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

V. Further Studies on the Yolk Protein during Embryogenesis

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(Figs. 1–5, Tables 1–7)

There have been found at least three kinds of protein in the yolk throughout the embryogenesis of rainbow trout, by electrophoretic analysis[1]. In rainbow trout it is well known that the egg hatches out in 20–30 days after fertilization and the yolk content in the sac fry lasts for a further period of 20–30 days, then signs of starvation begin to appear. Therefore, it was suggested that the yolk sac content resulted in heavy loss at the starvation period. Changes of the yolk content at various stages of embryogenesis have been reported in previous paper[1]. However, no major variations in yolk reserve could be detected until hatching. The decrease of yolk content mainly takes place after hatching, and the low molecular substances produced are accumulated in the yolk sac at the later stage of the development. It appeared that the accumulation of low molecular substance in yolk was more rapid than the absorption ay alevin[1]. This paper describes further details of the properties of the yolk during embryogenesis.

MATERIALS AND METHODS

Rainbow trout egg and alevin were obtained from the Zaoh Rainbow Trout Breeding Station, Miyagi-prefecture in December of 1966. Experiments were carried out on the mature egg before fertilization, the 29 th-day alevin after fertilization (immediately after hatching), and the 42 nd-day alevin after fertilization in starvation period.

Treatment of yolk: The yolk sac of the fish egg or alevin was washed with a dilute saline solution, punctured with a needle, and the contents were rinsed out

with a 2 % saline solution. After oil globules and light materials (LDF*) were removed by centrifugation, the yolk globule which had dissolved into a saline solution was dialyzed to distilled water, then lyophilized (HDF*). The dominant portion, HDF, was used for analysis. For comparison with the hen's yolk protein it was dissolved in a 5 % saline solution and treated by the same procedure.

Solubility test: The following procedure was used for the salting-in curve. Exactly 1 m*l* of HDF solution was introduced into a series of test tubes containing 10 m*l* of 0–3 % sodium chloride solution. After the mixtures were throughly shaken with care they were conserved in a chill room at a temperature of 5°C, for an overnight. Then they were centrifuged and the optical density of the supernatants were measured at 275 nm with a Hitachi-Parkinelmer EPS-3 Spectrophotometer. These values were plotted against the protein content of the starting solution in order to determine the solubility.

Electrophoretic analysis: Tiselius electrophoretic analysis was done on HDF with a Hitachi HTB-2A instrument. A carbonate buffer at pH 9.8 (ion strength 0.1) was used. The current was 5 mA/cm^2 for minutes.

Gel filtration: About 50 mg of HDF were dissolved in a 2 % saline solution, and placed on a column of Sephadex G-200. The column used was 46 cm in length and 1.3 cm in diameter. The flow rate was 10 m*l* per an hour, and the fraction volume was 2 m*l*. The effluents were taken at intervals and their optical density was measured at 280 nm with a spectrophotometer. Each fraction was dialyzed against distilled water and used for chemical analysis.

Enzymatic digestion of HDF: The enzymes used were trypsin (Kanto-kagaku), papain (Katayama-kagaku), and pepsin (E. Merck). Five m*l* of substrate solution containing 20 mg of HDF, 10 mg of EDTA, and 0.02 *M* phosphate buffer solution were mixed at 37°C. In the case of papain digestion, 4 mg of cystein were added. After that 5 m*l* of 0.44 *M* trichloroacetic acid were added to the solution in order to precipitate the undigested protein. The extent of proteolysis was calculated comparing the amount of soluble low-molecular substance with the protein content of the starting HDF solution. For comparison, the casein according to Hammarsten was treated by the same conditions.

Amino acid composition: Each sample was defatted with methanol and chloroform, and hydrolyzed with 6 *N* hydrochloric acid at 105–110°C for 22 hours. Then the amino acid composition was determined by a Hitachi KLA-2 Amino

* Abbreviation HDF: High Density Fraction
LDF: Low Density Fraction

Acid Analyzer. The amount of tryptophane in the protein was determined according to the method of GOODWIN *et al.*[2]

Nitrogen was determined by the micro-Kjeldahl procedure, phosphorus by the method of ALLEN[3], and lipid extraction from the lyophilized sample by the method of FOLCH *et al.*[4]

RESULTS

The natures of the fish yolk at three different stages are shown in Table 1. In the course of the embryogenesis, protein and lipid progressibly diminished, and the corresponding decrease of the yolk weight and increase of non-proteic nitrogen was evident. Moreover the water-soluble protein occurred in the 42 nd-day yolk.

Table 1. Chemical analyses of yolk in three embryonic stages

		Unfertilized egg	29th-day	42nd-day
Yolk content (mg)		135.5	111.0	80.0
Yolk ratio (%)*		94.5	88.3	37.5
Oil globule (mg)		5.6	5.3	4.4
Lipid of lipoprotein (mg)		9.8	7.5	4.4
Nitrogen compound**	Protein (%)	99.7	94.7	86.1
	Non-protein (%)	0.3	5.3	13.9
Protein	Water insoluble (%)	100.0	100.0	71.5
	Water soluble (%)	0.0	0.0	28.5

* percentage of yolk weight to whole egg or alevin

** Proteic nitrogen was calculated by measuring that of the 5% trichloroacetic acid insoluble matter.

The solubility curves of HDF obtained from the various embryogenetic stages are shown in Figure 1. HDF from the immature egg is also illustrated. When HDF of the 42 nd-day was dialyzed against distilled water, a fairly high amount of water-soluble protein appeared within the cellophane tube. Hen's yolk protein was less soluble than that of the rainbow trout egg in a low concentration of saline solution.

Table 2 shows the results of the analyses of HDF in three periods of development. Nitrogen, phosphorus, and lipid contents of HDF are also indicated in Table 2. The nitrogen content of HDF stayed relatively constant, but the decrease of phosphorus and lipid was evident in the course of the embryogenesis. The slight decrease of nitrogen content in non-lipid moiety of HDF was observed at the

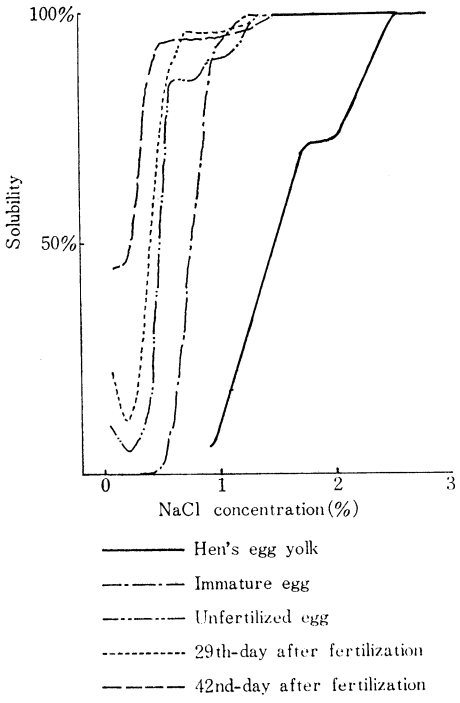

Fig. 1 Solubility curves of HDF obtained from various embryogenetic stages of rainbow trout.

Table 2. Chemical analyses of HDF

	Unfertilized egg	29th-day	42nd-day
Nitrogen (%)	13.7	13.7	13.8
Phosphorus (%)	1.0	0.9	0.7
N/P (gram atoms)	30.3	33.8	43.8
Lipid moiety (%)	23.6	19.6	17.8
Phosphorus (%)	2.6	2.5	2.0
Non-lipid moiety(%)	76.4	80.4	82.2
Nitrogen (%)	15.3	15.3	14.3
Phosphorus (%)	0.6	0.5	0.4

Table 3. Chemical analyses of HDF-P and HDF-S obtained from 42nd-day yolk.

	HDF-P	HDF-S
Content (%)	71.5	28.5
Nitrogen (%)	13.9	13.6
Phosphorus (%)	0.8	0.6
N/P (gram atoms)	38.3	50.1
Lipid moiety (%)	17.7	17.9
Phosphorus (%)	2.1	1.8
Non-lipid moiety (%)	82.3	82.1
Nitrogen (%)	14.3	14.2
Phosphorus (%)	0.4	0.3

42 nd-day after fertilization. The gradual reduction of lipid and its phosphorus content might be due to the consumption of phospholipids.

HDF of the 42 nd-day was separated into two fractions according to their difference of solubility in water. The fraction insoluble in water was abbreviated HDF-P, and the soluble fraction HDF-S. The ratio of the former and the latter was respectively 71.5 and 28.5 per cents in weight, as seen in Table 1. Table 3 shows that the amounts of nitrogen and phosphorus in HDF-P were slightly higher than those in HDF-S, but reversely lower in the lipid content. When the protein composition was analysed with Tiselius electrophoresis, three components always appeared in all stages of the embryogenesis. In the case of HDF of mature egg before fertilization, three components corresponding to the electrophoretic diagram appeared on a gel filtration of Sephadex G-200. Moreover HDF at the 29 th and 42 nd-day were also clearly divided into three protein components by gel filtration (Fig. 2). It was proven that the three components appearing in the diagram were identified respectively as lipoprotein, glycoprotein, and phosphoprotein, in order of the eluted position. On the 42 nd-day the lipoprotein, however, was clearly resolved

into two components in gel filtration. The eluted lipoproteins were respectively collected and refined by the same procedure. The elution patterns of HDF-P and HDF-S are shown in Fig. 3.

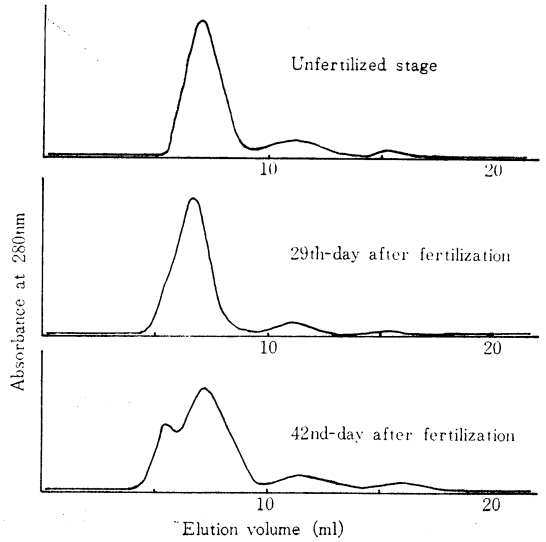

Fig. 2 Elution patterns on gel filtration in HDF of three embryogenetic stages.

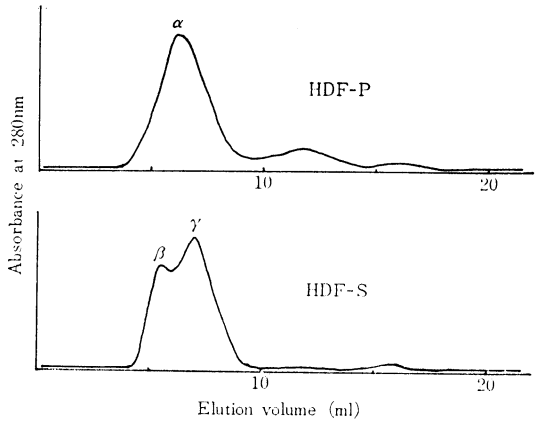

Fig. 3 Elution patterns on gel filtration in the water-insoluble (HDF-P) and soluble components (HDF-S) of HDF at 42nd-day.

The rate of enzymatic digestion of native and defatted HDF were compared. HDF of the mature egg was defatted by the method of FOLCH *et al.*[4], and dispersed in incubation mixture. The original HDF was easy to be decomposed more than the defatted HDF in papain digestion, but in tryptic digestion it was in the

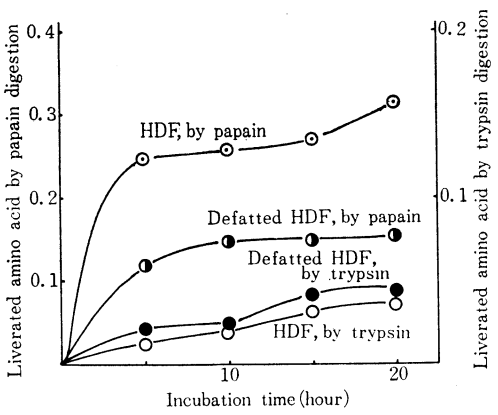

Fig. 4 Time course of enzymatic digestion of HDF obtained from unfertilized egg. The liverated amino acid from HDF by the enzyme was determined the absorbance of 0.2 *M* trichloroacetic acid-soluble matter at 275 nm.

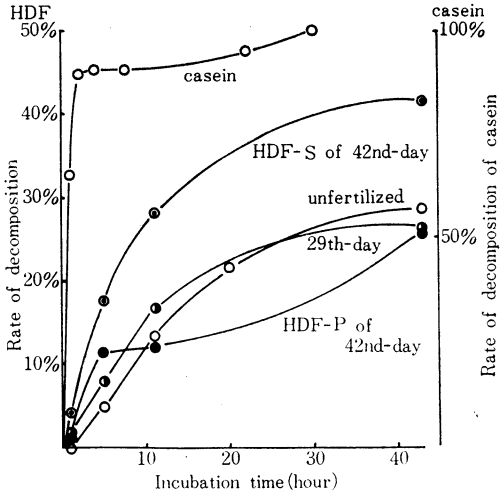

Fig. 5 Susceptivility of HDF of various stages for papain digestion.

opposite way (Fig. 4).

Fig. 5 illustrates the papain digestion of HDF in the various stages of the embryogenesis. The digestibity of HDF was less than 50 per cent even at the end of the reaction time. On the other hand the digestion of casein attained 90 per cent during the first 3 hours of the incubation. HDF-S of the 42 nd-day showed lower stability to papain digestion than the other HDF.

The results of proteolysis of various HDF by three enzymes are summerized in Table 4. HDF showed marked stability to trypsin in all stages of the embryo-

Table 4. Extent of proteolysis of HDF of three embryogentic stages at 37°C after 20 hours.

	Trypsin	Papain	Pepsin
Unfertilized egg (%)	2.6	21.1	57.1
29th-day yolk (%)	2.5	11.0	58.4
42nd-day yolk (%)	2.8	21.4	84.6
Casein (%)	70.0	87.5	64.6

Table 5. Analyses of yolk proteins obtained from various developmental stages.

	Lipoprotein						Glycoprotein		
	Immature	Unfertilized	29th-day	42nd-day α	42nd-day β	42nd-day γ	Immature	Unfertilized	42nd-day
Nitrogen (%)	11.9	11.8	12.4	12.3	12.1	12.6	12.7	12.6	12.4
Phosphorus (%)	0.5	0.5	0.5	0.3	0.4	0.5	0.0	0.7	0.8
N/P (gram atoms)	52.5	52.1	54.8	90.7	67.0	55.6		39.8	34.2
Tyr/Tryp*		2.8	3.5	2.3	4.6	3.6		3.3	3.6

* Molar ratio of tyrosine to tryptophan

genesis, but pepsin digested more than 50 per cent of HDF. The stability of HDF for pepsin digestion decreased with the proceeding of the embryogenesis.

Table 5 lists some analytical data of the isolated proteins. The lipoproteins of the 42 nd-day were designated as α, β, and γ, as shown in Fig. 3. The lipoproteins of the mature and immature eggs were almost similar to each other in content of nitrogen and phosphorus. However, it was seen that the phosphorus content of the glycoprotein apparently increased during maturation.

The amino acid composition is listed in Table 6. For comparison the results of vitellin were cited[5]. When the amino acid composition was expressed as the molar ratio per aspartic acid, the four lipoproteins concerned were similar to each other with the exception of lysine, arginine, serine, methionine, and tyrosine. The amino acid content was the highest in the mature egg before fertilization, when represented as the defatted residue. A comparison of yolk lipoprotein of the rainbow trout with hen's yolk vitellin indicated that about an half of the amino acid composition was in good agreement. The fish egg lipoprotein was higher in ratio

Table 6. Amino acid compositions of lipoproteins

	Immature		Unfertilized		42nd-day β		42nd-day γ		Vitellin
	μ mol per mg	per asp. a.	μ mol per mg	per asp. a.	μ mol per mg	per asp. a.	μ mol per mg	per asp. a.	per asp. a.
Lysine	0.581	0.88	0.973	1.03	0.438	0.92	0.489	0.84	0.80
Histidine	0.187	0.28	0.297	0.31	0.153	0.32	0.170	0.29	0.27
Arginine	0.359	0.54	0.505	0.54	0.254	0.53	0.243	0.42	0.78
Cystine/2	0.018	0.03	0.020	0.02	0.012	0.03	0.024	0.04	0.20
Aspartic acid	0.661	1.00	0.943	1.00	0.479	1.00	0.582	1.00	1.00
Threonine	0.348	0.53	0.579	0.61	0.302	0.63	0.343	0.59	0.56
Serine	0.229	0.35	0.381	0.40	0.185	0.39	0.197	0.34	0.83
Glutamic acid	0.623	0.94	1.178	1.25	0.633	1.32	0.669	1.15	1.32
Proline	0.407	0.62	0.672	0.71	0.376	0.79	0.392	0.67	0.68
Glycine	0.319	0.48	0.476	0.50	0.261	0.55	0.297	0.51	0.60
Alanine	1.172	1.77	1.828	1.94	0.938	1.96	1.066	1.83	0.98
Valine	0.713	1.08	1.123	1.19	0.577	1.21	0.661	1.14	0.92
Methionine	0.151	0.23	0.209	0.22	0.152	0.32	0.023	0.04	0.31
Isoleucine	0.490	0.74	0.763	0.81	0.402	0.84	0.455	0.78	0.74
Leucine	0.807	1.22	1.288	1.36	0.680	1.42	0.751	1.29	1.13
Tyrosine	0.148	0.22	0.197	0.21	0.113	0.24	0.076	0.13	0.38
Phenylalanine	0.370	0.56	0.618	0.66	0.342	0.71	0.367	0.63	0.41
Tryptophan**			0.070	0.07	0.049	0.10	0.021	0.04	0.20
NH_3	1.082	1.64	1.499	1.59	0.911	1.90	1.031	1.77	

* Cited from WALLACE[5].

** Calculated from molar ratio of tyrosine to tryptophan by GOODWIN *et al.*[2]

of alanine and phenylalanine per aspartic acid than in vitellin, whereas the ratio of arginine, cystine, serine, tyrosine, and tryptophane was always smaller in fish than in hen.

Table 7. Amino acid compositions (μ mol/mg) of glycoproteins.

	Unfertilized	42nd-day
Lysine	0.650	0.669
Histidine	0.135	0.149
Arginine	0.115	0.126
Cystine/2	0.034	0.045
Aspartic acid	0.946	0.908
Threonine	0.191	0.195
Serine	0.346	0.409
Glutamic acid	0.493	0.515
Proline	0.171	0.173
Glycine	0.362	0.376
Alanine	0.239	0.263
Valine	0.528	0.516
Methionine	0.023	0.037
Isoleucine	0.317	0.320
Leucine	0.439	0.455
Tyrosine	0.092	0.107
Phenylalanine	0.122	0.124
Tryptophan*	0.028	0.030
NH_3	1.299	1.294

* Calculated from molar ratio of tyrosine to tryptophan by GOODWIN *et al.*[2]

Both glycoproteins of unfertilized eggs and the 42 nd-day yolk were almost similar in the amino acid composition as seen in Table 7. This shows that their glycoproteins were analogous.

DISCUSSION

This study concerned itself with the changes in the yolk protein of the rainbow trout egg during the embryogenesis. It was observed that the changes of the protein constituent chiefly occured after hatching. Although the yolk protein composition was invariable in electrophoretic determination throughout embryogenesis, several changes occured in the lipoprotein composition by gel filtration on Sephadex G-200. A new component was eluted at the front of the usual lipoprotein position, when HDF of the 42 nd-day passed through the column. Chemical

changes in yolk protein were found to take place mainly after hatching out. From the result of Table 1, a relatively high storage of ash within HDF was suggested.

The lipoprotein of the rainbow trout egg was insoluble in low salt concentration during the begining stage of the embryogenesis. Since HDF at the unfertilized stage was apparently insoluble in sodium chloride solution of less than 0.5 %, its solubility gradually increased during embryogenesis. Besides phosphoprotein, lipoprotein and glycoprotein obtained from immature eggs were also insoluble in distilled water as previously published[6]. The glycoprotein in this present report, corresponding to the salmon livetin of YOUNG & PHINNEY[7] and β-component of JARED & WALLACE[8], was insoluble in water, whereas it was reported that the protein remained soluble after extensive dilution of a saline extract in case of the trout egg[8]. In this work, it was found that the solubility of HDF into a diluted saline solution gradually increased with the progress of the embryogenesis, 28.5 per cent of protein consequently became water-soluble from the 42 nd-day after fertilization. The solubility of lipoprotein was proven to be affected with a lipid composition[28)29)]. In a lipid of lipoprotein a certain alteration took place during the embryogenesis[30)]. HDF-S of the 42 nd-day was found to be composed of two distinct lipoproteins which mainly differed in molecular size. Therefore the lipoprotein in HDF of the 42 nd-day consisted of, at least, three components with different physical and chemical properties. They were homogeneous in electrophoretic mobility, but heterogeneous in solubility and elution behavior in gel filtration. Moreover, lipid content, N/P ratio, and their amino acid composition were somewhat different from each other. The appearance of the large molecular lipoprotein on the 42 nd-day showed that the lipoprotein had undergone reconstruction during the embryogenesis. In the studies on the hen's yolk protein during embryogenesis, it was reported by SAITO *et al.*[9)10)] that ovalbumin accompanied by a disappearance of α-livetin was observed in the yolk constituent. Electrophoretical changes were found to take place in the protein composition of the medake fish (*Oryzias latipes*) egg following embryogenesis[11)12)13)]. Moreover, in other fish eggs such changes were observed by electrophoretic determination[14)15)]. However, there was hardly a difference in protein components during the early stage of embryogenesis of the rainbow trout.

The chemical composition of the glycoproteins in two embryonic stages seemed to be homologous. However, the protein of several species of *Oncorhynchus* presented by MARKERT & VANSTON[16)] proved to be composed of several distinct components. The glycoprotein isolated from the immature egg obtained in a

previous research[6] appeared to be similar to that of the mature egg in electrophoretic behavior, although the phosphorus content was somewhat lower. The glycoprotein of the mature egg did not diminish its amount of phosphorus by purification procedures of starch-grain zone electrophoresis and gel filtration. YOUNG & PHINNEY[7] found out that salmon egg livetin contained 0.1 per cent phosphorus. The absence of phosphorus in the protein of unfertilized eggs of rainbow trout was observed also qualitatively[17)18)]. JARED & WALLACE[8], who presented the protein as a serum protein, reported that there occured a small amount of phosphorus, less than 0.02 per cent, in the protein of the rainbow trout egg. Consequently, the great increase of phosphorus content during egg maturation has to be examined and made clear in the future.

The patterns of amino acid composition of lipoproteins were similar to each other but differed from those of the later stage in their lower rate of lysine, arginine, serine, methionine, and tyrosine. The loss of these amino acids might be due to the absorption by the embryo. Thus, considering the changes of amino acid composition, molecular size, and solubility, the authors may conclude that a partial reconstruction of the lipoprotein took place before absorption by embryo. However, this hypothesis remains to be checked by further experimentation. ITO *et al.*[19] indicated that the peptides from the egg yolk lipoproteins of hen, rainbow trout, and cuttle fish had a similar amino acid composition, and these lipoproteins of the hen's egg and the amphibian egg were apparently analogous from the point of view of the physical and chemical properties[5)20)]. A comparison of the lipoprotein and the lipovitellin indicated that thes proteins consisted of a somewhat analogous constituent. The higher value of alanine reported here was characteristic for the lipoprotein of the rainbow trout egg. Methionine and tyrosine, of which the disappearance took place at a later stage of the embryogenesis, might be presumed as one of the growth factors for developing in the embryo.

As for non-proteic nitrogen in yolk, an increase throughout embryogenesis was reported[1]. The study on free amino acid in the salmonoid egg during development has been carried out by many scientists[21)~25)]. HOLLETT & HAYES[26] reported that the increase of non-proteic nitrogen meant either that excretory products were appearing in a faster rate than they done away, or free amino acids could be appearing. The free amino acids in yolk were suggested to originate from yolk protein by the action of proteolytic enzyme. According to VAN DER GHINST[27], yolk breakdown apparently took place chiefly in the yolk sac. The enzyme activity of the yolk was confined to the yolk sac, and closely linked with yolk utilization.

The free amino acids originated from degradation of yolk protein and were gradually accumulated in the yolk sac at a later stage of the embryogenesis. However, the relation between the free amino acid and the protein in yolk has not yet been clarified. Therefore, further investigations on this problem are needed. OGINO & SUYAMA[23] reported that the amino acid composition of the total protein of the rainbow trout egg did not vary up to hatching. Comparing the stability of HDF in several proteolytic enzymes, it was clear that their lipoproteins were somewhat heterogeneous. The data reported here revealed that the yolk protein became gradually unstable to the enzymatic hydrolysis with the progress of the embryogenesis.

Several observations provided evidence that each lipoprotein molecule was digested by proteolytic enzymes of embryo or alevin. Moreover, it appeared that unabsorbed lipoprotein resulted in the increase of the molecular size, in decrease of the prosthetic group, and in the release of specific amino acids. It was suggested that the increase of molecular size was due to polymelization after the release of amino acids or of the prosthetic group.

SUMMARY

The yolk proteins obtained from various stages of embryogenesis of rainbow trout were analyzed in order to determine their chemical and physical properties.

1. The yolk protein gradually changed its physical and chemical properties with the progressing of the embryogenesis. The increase of molecular size of the lipoprotein was observed in the gel filtration. It was suggested that the lipoprotein molecule was rearranged in the yolk sac after a partial degradation by embryo.

2. The amino acid composition of the rainbow trout egg lipoprotein was somewhat similar to that of lipovitellin, although the high value of alanine and the low content of serine, cystine, and tryptophane were characteristic for the former.

3. A decrease in the ratio of several amino acids in the lipoprotein was found from the 42 nd-day after fertilization.

4. The amino acid composition of glycoprotein in both the unfertilized egg and 42nd-day yolk were similar to each other. Therefore the glycoprotein catabolized without consumption of specific amino acid.

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虹鱒卵の生化学的研究

V 胚発生過程における卵黄蛋白質の変化

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未成熟卵，未受精卵，29日目および42日目の卵黄蛋白質の性状を分析し，以下の結果を得た．

1．卵黄蛋白質は胚発生が進むに従って物理的，化学的性状が変化する．特に受精後42日目に分子量の大きいリポ蛋白質の生ずることを認めた．これはリポ蛋白質が部分的に分解された後再構成される為と考える．

2．虹鱒卵のリポ蛋白質のアミノ酸組成はアラニン含量が高く，セリン，シスチン，トリプトファンが低いことを除けば大体リポビテリンと似た組成を示した．

3．受精後42日目のリポ蛋白質のアミノ酸のうちいくつかのものの比率が減少することを認めた．これは特異なアミノ酸が選択的に胚発生に利用されていると考える．

4．卵黄の糖蛋白質のアミノ酸組成は未受精卵と受精後42日目のものでは殆ど変化は認められなかった．