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

I. Electrophoretic Analyses of Egg Protein

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(Figs. 1-5; Table 1-5)

Lipoprotein is the predominant component in the yolk constituency of all eggs. It is designated as the major energy source during the embryonic and larval development. Larger varieties of investigations have been carried out on the yolk protein in hen's eggs than on any other animals'. Consequently, not less than six proteins have been isolated from the hen's egg yolk before fertilization[1-3]. Two kinds of lipoproteins, one of phosphoproteins, and three of serum proteins. The qualitative and quantitative changes of these proteins, after the onset of embryogenesis[4,5], have been determined also.

The investigations of fish protein have been carried out by means of the histochemical and biochemical technics. The egg proteins of different species of fish, such as salmonoid, killifish, pond smelt, herring, *etc.* were examined by many workers[6-18]. However, very little is known in detail on the nature of specific fish eggs. Since the salmonoid egg possesses several advantages for research, the egg protein has been investigated by YOUNG *et al.*[6], FUJII[7], ITO *et al.*[8-10], SCHMIDT *et al.*[11], WALLACE *et al.*[12], MANO *et al.*[13,14], and JARED *et al.*[15] Three proteins were found in the yolk of the unfertilized salmonoid egg: lipoprotein, phosphoprotein, and one unidentified protein which was suggested as the serum protein by JARED and WALLACE[12].

Through the histochemical studies, it is well known that the salmonid egg contains two different types of lipid: the oil globule and the yolk globule. Although the lipids of egg have been studied by many workers[20-26], the lipid composition was never analyzed individually by the biochemical method. As in fact the lipids provide considerable energy for the developing embryo as well as the lipoproteins, it is of interest to investigate the relation between lipid and protein in the yolk during the development.

It is the purpose of the studies to characterize the chemical changes of the yolk proteins of the developing egg. In the present paper we described the electrophoretical studies of the yolk protein obtained from the immature egg of rainbow trout, in order to gain the basic information on the nature of egg yolk.

MATERIALS AND METHODS

Materials were the eggs obtained from a rainbow trout (*Salmo gairdnerii irideus*)

reared in the ZAOH RAINBOW TROUT BREEDING STATION, Miyagi-prefecture, in December of 1964. The fish was three years old female, 46 cm in body length and 1.43 kg in body weight. The ovary was about 330 g in weight and the eggs were ranged from 3.5 to 5.2 mm in diameter. It was noticed that the egg was still immature, because it was unable to ovulate freely from a ventral opening of the fish.

Extraction of protein: The ovary taken out from the fish was cleaned from its adherent membrane and washed with 1 per cent saline solution to remove blood. Then each egg was punctured with a needle, and the contents were drawn out with 5 per cent saline solution and centrifuged at 15,000G for a half an hour. The top layer was the oil globule, the middle the protein which was low in density and insoluble in saline solution (LDF), and the bottom was the protein fraction which was high in density and soluble in saline solution (HDF). Each fraction was collected and centrifuged again with a small amount of saline solution, respectively. The HDF, however, was further treated as follows; after dialyzing with distilled water in a cellophane tube at 5°C for 4 days, the precipitates formed were collected by centrifugation, dissolved in 5 per cent saline solution and dialized with distilled water. After repeating three times this treatment, the precipitates were collected by centrifugation, washed with distilled water, and dissolved in carbonate buffer solution (pH=9.8, i=0.1) to make sample solution.

Isolation of lipid: Lipid from the lipoprotein was prepared by the method of HILLYARD *et al.*[27] as follows; 100 m*l* of a mixture of methanol-ether-chloroform (6:2:1 v/v) was added to 5 m*l* of the protein solution with stirring and left standing at 60°C for 2 hours. After extracting the lipid, the residue was again heated with another 50 m*l* of the same solvent for 15 minutes, followed with 50 m*l* of a mixture of ethanol-ether (3:1 v/v), and then with 50 m*l* of ether. Each extract was mixed together and the solvent was removed off under reduced pressure. The resulted lipid portion was dissolved in ether and washed several times with water, then dried by passing through nitrogen gas under reduced pressure. There after the non-lipid moiety was filtrated, washed with a small amount of water, and then dried over anhydrous calcium chloride under vacuum to obtain a yellowish white powder.

Tiselius electrophoresis: The electrophoresis of egg protein was carried out by using a Hitachi HT-D type Tiselius electrophoretic apparatus. Carbonate buffer solution was applied at pH 9.8, and ion strength 0.1. A field strength of 5 mA per cm^2 was employed. Areas of each peak in the electrophoretic pattern were measured at the ascending side.

Isolation of protein: The protein solution was applied to the starch-grain electrophoretic analysis. The potato starch (Maruishi Pharmaceutical Co.) was purified by washing 10 times with 5 volumes of water and twice with 2 volumes of acetone. Then it was packed in a migrating cell ($6 \times 1.5 \times 26$cm). The sample was loaded on the groove of the starch at 6–7cm from the cathode side and applied to electrophoresis. After 48 hours, each of the migrated protein was extracted with the buffer solution at pH 9.8, and the relative concentration was determined by measuring the optical density at 280 mμ with a Beckmann DU spectrophotometer.

Fingerprinting: The zone of the electrophoretic pattern of the proteins separated by the above procedure was reprinted on filter paper by "finger printing".

Color and precipitation tests: Bromophenol blue test for proteins, o-tolidine test[28] for peptides, Sudan black B test for lipids, acetonic silver nitrate[29] and aniline hydrogen phthalate test for carbohydrates, and ammonium molybdate test for phosphorus were applied to the finger printed filter paper. Each protein separated by electrophoresis was also examined by the following color reactions and precipitation tests.; Biuret, Xanthoproteic, Millon's, Sakaguchi, Hopkins-Cole, Ninhydrine, and Molisch tests were used for color reactions, and trichloroacetic acid (TCA), ethanol, tannic acid, ammonium sulphate, picric acid, metaphosphoric acid, sulphosalicylic acid, and phosphotungstic acid for precipitation tests. Heat coagulation test for protein was also done.

Analytical method: Nitrogen was estimated by the micro-Kjeldahl method and phosphorus by the method of ALLEN[30]. Phospholipid was given as lecithin from multiplying the quantity of phosphorus in lipid by 25.

RESULTS

The general compositions of egg content were shown in Table 1. The yolk globule was separated from the yolk content by centrifugation.

Table 1. General Compositions of yolk globule and water-insoluble matter of HDF.

	Mosisture %	Lipid* %	Nitrogen %	Protein %	TCA-insoluble matter %
Yolk globule	64.0	6.6	4.2	26.3	34.3
Water-insoluble matter	—	19.1	13.6	85.0	100.0

*extracted by the method of Hillyard *et al.*[27]

The Tiselius electrophoretic patterns of the total protein and water-insoluble matter in HDF of the fish egg were demonstrated in Fig. 1. There are three peaks in both Fig. 1-A and Fig. 1-B. Each of them corresponds to each other. The electrophoretic composition was calculated from the ascending patterns. They were 84.0, 11.6, and 4.4 per cent in Fig. 1-A, and 88.3, 9.4, and 2.3 per cent in Fig. 1-B, respectively.

Three components were also found in the starch grain zone electrophoretic pattern presented in Fig. 2. The component I and II extracted from this starch block showed a single peak by the Tiselius electrophoresis as demonstrated in Fig. 3, respectively.

On each component replaced on filter paper by fingerprinting the color reactions were examined (Table 2). The table shows that the component I contained lipid, phosphorus, and carbohydrate, and emitted a greenish fluorescence when irradiated with ultraviolet ray (253.7mμ). The component II was negative for phosphorus-

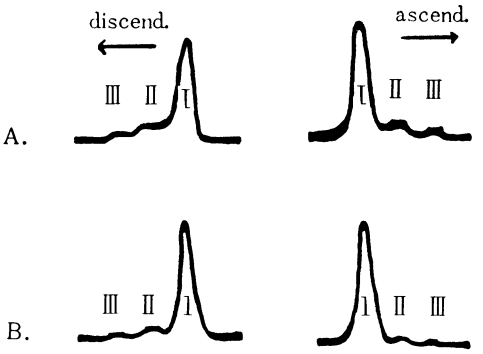

Fig. 1. Tiselius electrophoretic patterns of the egg protein of rainbow trout.
A; total egg protein
B; water-insoluble matter in HDF

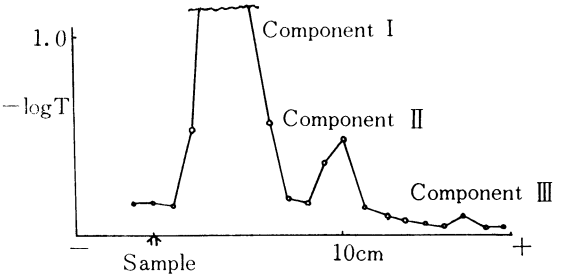

Fig. 2. Zone electrophoretic patterns of egg protein, pH 9. 8, 1.9mA/cm², 48 hours.

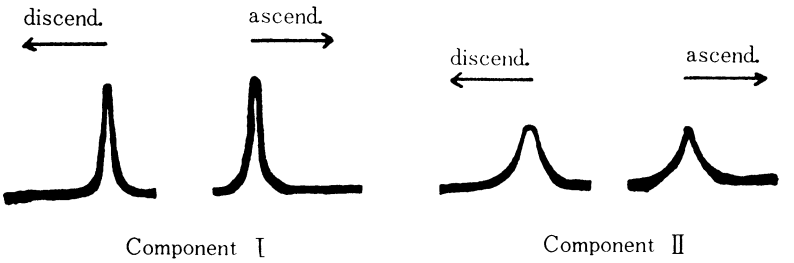

Fig. 3. Tiselius electrophoretic patterns of isolated components, pH 9. 8, 60 minutes.

Table 2. Color reaction of isolated component on filter paper.

	Component		
	I	II	III
Bromophenol blue	+	+	±
O-tolidine	+	+	+
Sudan back B	+	−	−
Ammonium molybdate	+	−	+
Acetonic silver nitrate	+	−	−
Aniline hydrogen phthalate	+	−	−

test. The results of spectral analyses for three components are seen in Fig. 4. The

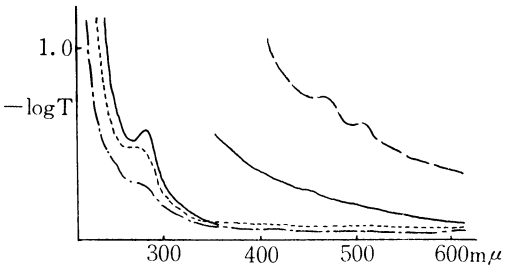

Fig. 4. Absorption spectra of isolated proteins.
———component I, ····· component II,
– – – – component III, — — — lipovitellin.

ultraviolet absorption spectra of the trout egg proteins were almost similar to those of hen's lipovitellin. However, in the visible region, there could be found distinctly two absorption maxima only in lipovitellin while none in fish egg protein. The lipid of lipovitellin gave also three maxima due to carotenoid pigment in the lipid of component I as seen in Fig. 5.

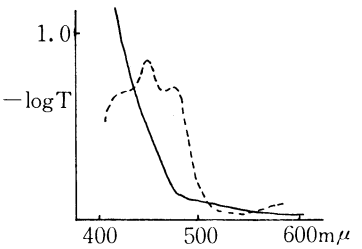

Fig. 5. Absorption spectra of lipid isolated from lipoprotein.
The lipids were dissolved in n-hexane.
———— component I, ····· lipovitellin.

Table 3. Color test for isolated component in solution.

	Component		
	I	II	III
Biulet	+	+	+
Xanthoproteic	+	+	−
Millon	+	+	+
Sakaguchi	+	+	+
Hopkins-Cole	+	±	±
Molisch	+	±	±
Ninhydrine	+	+	+

Table 4. Precipitation test of isolated component.

	Component		
	I	II	III
5% trichloroacetic acid	+	+	+
Ethanol	+	+	−
2% tannic acid	+	+	+
Sat. ammonium sulphate	+	+	+
Sat. picric acid	+	+	−
25% metaphosphoric acid	+	+	+
2% phosphotungstic acid	+	+	+
Lead acetate	+	−	+
20% sulphosalicylic acid	+	+	−
Heat coagulation	+	+	−

Chemical analyses of the components were summerized in Table 5.

Table 5. Chemical composition of proteins isolated from immature trout egg.

	Component		
	I	II	III
Ratio of content (%)	84.0	11.6	4.4
Total nitrogen (%)	11.9	12.7	14.3
Total phosphorus (%)	0.5	<0.1	8.2
Lipid moiety (%)	22.8		
Phosphorus of lipid moiety (%)	2.0		
Non-lipid moiety (%)	77.2		
Nitrogen of non-lipid moiety (%)	14.6		
Phosphorus of non-lipid moiety (%)	0.01		

DISCUSSION

It seems probable that the yolk globule consisted entirely of the insoluble matter in TCA solution. And the water-insoluble matter of HDF contained 19.1% of lipid and was also insoluble in TCA solution.

It has generally been recognized that hen's egg yolk protein consisted mostly of three protein fractions which were lipoprotein (α-, β-lipovitellin), phosphoprotein (phosvitin), and serum protein (α-, β-, γ-livetin), respectively. Recently it was found that phosvitin and livetins correlated with those of hen's serum proteins by the immuno-electrophoretic and chromatographic methods[31)32)33)].

In the present work electrophoretic analyses showed that the immature egg of rainbow trout contains three protein components in the proportion of 84.0, 11.6, and 4.4 per cent. However, it was unsuccessful in the fractionation of the three protein components by salting-out or salting-in procedures as well as in the case of hen's protein.

The component I, which was a main fraction in the fish egg protein, contained lipid, carbohydrate, and phosphorus. The lipid content amounted to about 22.8 per cent, of which some 50 per cent were phospholipid. The component I showed fluorescence by irradiation of ultraviolet ray. This readily suggested that there exists a fat soluble vitamin in it. In 1958 SUGANO[34] reported that there exists carotenoid pigment in lipovitellin of hen's eggs, but we did not observe any inflection of absorption spectrum of trout egg lipoprotein in the range of 400mμ to 500mμ (Fig. 5).

ITO *et al.*[8] reported the presence of the phosphorus compound in the defatted residues of trout egg lipoprotein. In this case, however, it was disregarded. It might have been due to the reduction of phosphorus by the repeated procedures of delipidation. There was no difference of nitrogen content in the defatted lipoproteins of the immature egg and the mature one. This protein moiety became insoluble in acid or alkaline solution. The color reactions for lipid and carbohydrate were negative in the component II, although JARED *et al.*[15] found a small amount of lipid. The component II was supposed to be livetin-like substance.

The component III was a kind of phosphoprotein which was high in phosphorus content and easily soluble in water. SCHMIDT *et al.*[11] reported that phosphoprotein isolated from brown brook trout (*S. trutta*) was not precipitated by the addition of TCA. On the other hand, this component III became slightly turbid by the same procedure. Since the phosvitin fraction is generally small in quantity, but distributed widely in various vertebrate eggs[7,12,13,14], it is suggested that the component III was a substance analogous to phosvitin.

SUMMARY

The nature of protein in the immature egg yolk of rainbow trout was investigated. There exist three components in the proportion of 84.0, 11.6, and 4.4 per cent in the yolk.

Among these three components the principal one, component I, was a kind of lipoprotein. It contained 11.9 per cent of nitrogen, 0.5 per cent of phosphorus, a little carbohydrate, and 22.8 per cent of lipid of which some 50 per cent was phospholipid. It is noteworthy that this lipoprotein-like substance was deficient in carotenoid pigment.

The component II was an unidentified protein composed of 12.7 per cent of nitrogen and less than 0.1 per cent of phosphorus.

The component III was a phosphoprotein soluble in water. It contained 8.2 per cent of phosphorus and 14.3 per cent of nitrogen.

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虹鱒卵の生化学的研究

I. 蛋白質の電気泳動的研究

中川平介・土屋靖彦

虹鱒卵の卵黄形成後期の未成熟卵の内容物の分析を行った結果，ほとんどが蛋白質であることを明らかにした．蛋白質の主なる成分は電気泳動的に三つあり，それぞれは 84.0%．11.6%，4.4%の割で存在する．

三成分の蛋白質のうち 84.0% を占める成分は窒素含量 11.9%，リン含量 0.5%，脂質含量 22.8% および少量の炭水化物を含むリポ蛋白質である．脂質のうちの50%はリン脂質である．この成分は鶏卵のリポビテリンと同様の蛋白質と考えるが，カロチノイドを含まない点でそれとは異っている．11.6% を占める蛋白質は窒素含量 12.7%，リン含量 0.1% 以下で，リン含量の少ないのが特徴的である．

三成分の蛋白質のうち最も少ない成分は窒素 14.3%，リン 8.2% を含み，リン含有量の多いことと水溶性蛋白質であることで前二者の蛋白質とは異なる．この蛋白質は鶏卵のフォスビチン様のリン蛋白質と考える．