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(VITELLOGENIN) AND EGG YOLK PROTEINS IN TELEOSTS :  
IMMUNOCHEMICAL, PHYSICOCHEMICAL AND  
STRUCTURAL STUDIES\*

By

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## I. Introduction

Intraspecific variations in the blood serum proteins of animal species occur as a result of their genetic constitution and the influence of environmental and physiological factors. Deutsch and Goodloe (1945) and Moore (1945) performed the initial electrophoretic patterns of serum and plasma protein investigations of carp (*Cyprinus carpio*) using the Tiselius method. Since that time, a large number of studies have been concerned both with fundamental analyses of fish serum components and with differences in these due to migration, age and development, starvation, temperature, salinity, and diseases.

Deutsch and McShan (1949) studied serum protein fractions in various species of fishes using electrophoretic technique for the separation and quantitative analysis

of proteins.

Irisawa and Irisawa (1961), working with elasmobranch sera, concluded that the serum albumin fraction was missing in the skate (*Raja kenosjoi*) and shark (*Heterodontus japonicus*), and suggested that the lower vertebrates had a different plan of producing serum proteins than do the higher vertebrates.

Saito (1957) examined the electrophoretic patterns of serum proteins in a variety of fishes obtained under almost the same condition. He ascertained that the specificity, in quantity as well as in quality, of the serum protein composition was more or less conspicuous corresponding to taxonomic relationships or to the habitual environment.

Engle *et al.* (1958) found proteins which show electrophoretic characteristics of gamma globulins in the serum of elasmobranchs but not in the serum of teleosts. Their finding was based on the presence or absence of a fraction comparable to human gamma globulin in electrophoretic characteristics.

Mairs and Sindermann (1960) made a thorough study of Atlantic herring (*Clupea harengus*) serum by means of electrophoresis. They found a considerable variability and concluded that it was inadvisable to assume species specificity of electrophoretic patterns unless a considerable number of specimens were examined.

Fujiya (1961) showed a variation in the electrophoretic patterns of various fishes caused by starvation and by various concentrations of industrial wastes and chemicals in ambient water.

Rall *et al.* (1961) examined the plasma components of lamprey (*Petromyzon marinus dosatus*) under metamorphosis. They described that a new component in the mature fish, which was of major quantitative importance, was considered to be an alpha globulin rather than albumin.

Sulya *et al.* (1961) reported the electrophoretic studies on plasma proteins of 26 species of fishes from the Gulf of Mexico. They showed that analbuminemia was observed not only in elasmobranchs, but in gars and some clupeids, as well as in two species of higher fishes.

Fine *et al.* (1963) examined the number of serum proteins in the eel (*Anguilla anguilla*) by means of starch gel electrophoresis and immunological techniques. They observed twenty-one components in eel serum including a new component at the  $\gamma_2$ -globulin region after immunization of eel against human proteins.

Thomas and McCrimmon (1964) found that variations in lamprey (*Petromyzon marinus*) serum protein concentration, positions and sizes of the fractions were associated with growth stage, sex and disease.

Mulcahy (1967) examined the total amount of serum proteins and electrophoretic patterns of sera from diseased and from healthy Atlantic salmon (*Salmo salar*). He found marked differences between the sera from healthy and diseased fish.

Solomon and Allanson (1968) reported the effects of exposure to low temperatures on the serum protein of cichlid fish (*Tilapia mossambica*).

Rolan (1968) reported the species difference of catfish plasma comparing to that of human serum by means of polyacrylamide gel electrophoresis.

Yamashita (1969) observed seasonal changes in blood elements and in electrophoretic patterns of serum proteins in rockfish (*Sebastes marmoratus*). He showed that the components of serum proteins were separated clearly throughout the



year in the female, while sometimes they were not separated distinctly in the male.

Belcheva and Khristov (1972) studied on serum proteins of two species of salmonids and five species of cyprinids by means of electrophoresis and immunoelectrophoresis in agar gel.

Perrier *et al.* (1973) reported experiments concerning rainbow trout (*Salmo gairdneri*) plasma protein separation using disc electrophoresis, gel filtration and salt solubility fractionation.

Yoneda and Ishihara (1974) reported disc electrophoretic patterns of serum proteins from chum salmon (*Oncorhynchus keta*) and masu salmon (*O. masou*). They observed interspecies differences and individual variations in both species of teleosts.

Harris (1974), in an investigation of the protein components of dace (*Leuciscus leuciscus*), found intraspecific variations throughout the year. He also made a comparison between polyacrylamide gel electrophoretic characteristics of human and dace serum proteins.

Weinstein and Yerger (1976) reported an electrophoretic investigation of spotted sea trout (*Cynoscion nebulosus*) subpopulations in the Gulf of Mexico and Atlantic coast of Florida. According to that study, the serum protein patterns proved to be a more sensitive criterion for detecting population differences than lens proteins.

Studies of fish plasma or serum proteins in regard to sexual differences have also been reported by many investigators. Vanstone and Ho (1961) reported a slowly migrating fraction in the serum of maturing 2.5-year-old female coho salmon (*O. kisutch*) which, they believed, might be serum vitellin (an egg yolk protein). It was absent in the plasma of males, immature females, spawning females and spawned-out females.

Ridgway *et al.* (1962) reported that blood samples taken from individual red salmon (*O. nerka*) differed in antigenic constitution when tested by the immunodiffusion method with rabbit immune antisera. They found that maturing female salmon possessed a complex antigen in their sera which was not detectable in the sera of males and immature females. Subsequently, Krauel and Ridgway (1963) quoted that the above component, which they termed Sm antigen, might be identical with the "serum vitellin" reported by Vastone and Ho (1961). Fine and Drilhon (1963) identified a similar protein in Atlantic salmon by immunodiffusion. They explained that the existing evidence of serum vitellin might account for the sequence in oviparous vertebrates where, under the control of the pituitary, estrogen produced in the ovary stimulates the liver to produce proteins that are transported through the blood to the ovary and are utilized for yolk formation.

Utter and Ridgway (1967) reported that a serologically detectable serum factor (HM factor) was associated with maturity in the English sole (*Parophrys vetulus*) and Pacific halibut (*Hippoglossus stenolepis*). The factor was detected in the serum of some immature females of both species during the spawning season. Evidence associating the synthesis of this factor with the action of estrogenic hormones was obtained when the production of the factor was induced by the injection of estradiol into male English soles.

Thurston (1967) examined acrylamide gel electrophoretic patterns of blood

serum proteins from rainbow trout, and found sexual differences in the composition of plasma proteins in relation to gonadal maturation.

Plack *et al.* (1971) reported the occurrence of egg proteins in cod (*Gadus morhua*) serum. They noted that the major components in the egg extract were two similar lipoproteins with molecular weight of about 400,000. These lipoproteins were identified by immunochemical methods in the serum of female cod with developing ovaries, but not in the serum of males or of immature females.

Markert and Vanstone (1971) purified three egg yolk proteins of coho salmon. They showed by immunological techniques that these proteins were present in the plasma of sexually maturing female coho and of estrogenized immature coho of both sexes, but not of sexually maturing males or nonestrogenized immature females. They concluded that at least two of the three egg yolk proteins were corresponding to the lipovitellin and phosvitin of amphibians.

Amirante (1972) also reported the physicochemical and immunochemical characteristics of rainbow trout lipovitellin from eggs, serum, and from several organ extracts.

Aida *et al.* (1973a) reported of the ayu (*Plecoglossus altivelis*), similar observations on the sexual differences in plasma protein composition, its hormonal control and relationship between plasma protein and egg yolk protein, using electrophoretical immunological methods. They named newly formed proteins in maturing female ayu as Female Specific Plasma Protein (FSPP).

Pickering (1976) reported that the electrophoretic analysis of the serum from gonadectomized, estradiol-implanted, female lamprey (*Lampetra fluviatilis*) revealed a very dense protein band which migrated slowly towards the anode. From the results, he considered that vitellogenesis in the river lamprey was stimulated by estradiol administration.

Emmersen and Petersen (1976) showed the presence of a specific lipophosphoprotein in female flounders (*Platichthys flesus*) during vitellogenesis. They concluded that a lipophosphoprotein containing alkali-labile protein phosphorus was present in vitellogenic flounders, and that the synthesis of this protein could be induced by estradiol treatment in both sexes. They assume that the isolated lipophosphoprotein is identical to the yolk-precursor protein, vitellogenin. Furthermore, it was found that the process of vitellogenesis was correlated closely with changes in the synthesis of RNA and DNA in the liver (Emmersen and Emmersen, 1976), suggesting that this organ was the hormonal target as well as the site of synthesis of the yolk precursor protein in the flounder.

Craik (1978) demonstrated the occurrence of vitellogenin in an elasmobranch (*Scyliorhinus canicula*) for the first time. The author reported that in this group, as in other oviparous vertebrates, the yolk granule protein was synthesized in the liver and conveyed to the growing oocytes by the plasma.

The preceding cited reports clearly show the appearance of a specific protein in the blood during vitellogenesis of the female as well as estrogen-treated male fish. Recently, the term "vitellogenin" has become the generally accepted name for this female-specific protein, especially in teleosts, elasmobranchs and lampreys as well as other oviparous vertebrates. The name "vitellogenin" was first used for the protein which specifically appears in female insects (Pan *et al.*, 1969). It is purely func-

tional and does not imply any definite characteristics of the proteins. Avian and amphibian vitellogenin, which is also a sex-limited serum protein, has been well-characterized and is considered to be a complex of lipovitellin and phosvitin. It is now regarded as the immediated precursor of these proteins in egg yolk (Wallace and Bergink, 1974; Christmann *et al.*, 1977). However, such characterizations of the female specific proteins in fish (fish vitellogenin) has yet been done. Because of the very close relationship of the fish vitellogenin with egg yolk formation, it is easily concluded that the studies of such a protein can provide much information for the disclosure of the mechanism of egg development. In this respect, investigation of vitellogenin of fishes can surely contribute to a better understanding of the reproduction and culture of fishes.

During a comparative study of serum protein profile in male and female fish (Hara, 1975), a female-specific serum protein that is capable of binding iron was found. The present paper describes some comparative aspects between the female-specific serum proteins (vitellogenin) and their related egg yolk proteins which were purified from rainbow trout, chum salmon and Japanese eel (*Anguilla japonica*). Some chemical and immunological properties of both female-specific serum proteins and the egg yolk proteins from several other fishes are also presented. Furthermore, studies of female-specific serum proteins identified in the medaka (*Oryzias latipes*) by immunological procedures are included. Finally, speculations of female-specific serum protein (vitellogenin) molecular structures from several different fishes are also given.

## II. Materials and methods

### A. Fish and eggs

#### 1. Fish

Fish used in this study were rainbow trout, chum salmon, Japanese eel, and medaka.

Rainbow trout were collected from the Toya Rinko Biological Station at the Lake Toya, and "Akashi Rainbow Trout Culture Farm" at Kamikawa, Hokkaido, in various seasons from 1974 to 1978. Fish were classified as "immature" if their gonads were poorly developed and if their sex was difficult to be determined without microscopic examination. Fish were classified as "maturing" if their gonads were developed so that their sex was apparent without magnification. Fish were classified as "ripe" if the gonads were in spawning condition to the degree that eggs or sperm were readily released when the fish were handled.

Chum salmon were taken in autumn of 1974 to 1978 at the mouth of the Ichiani River and the Shibetsu River, Nemuro, Hokkaido, which drain into the Pacific Ocean. Since the fish were in spawning condition, their eggs and sperm were readily released by pressing the abdomen with fingers.

Silver migratory forms of Japanese eel were caught at rivers in the Aomori Prefecture, Honshu, during the autumn of 1976. They were held in laboratory tanks which contained circulating sea water without feeding. Female eels were injected intramuscularly with a saline suspension of 12-16 mg of acetone-dried pituitary glands from ripe female chum salmon 5-14 times weekly in 1-4 months

(Yamamoto *et al.*, 1974).

Medaka were obtained from a pond at Yunokawa Hot Spring in the suburbs of Hakodate, Hokkaido. They were then cultured in ponds on the campus of the Faculty of Fisheries, Hokkaido University.

## 2. Blood

Blood of rainbow trout and chum salmon was collected by cutting the tail or by cardiac puncture, immediately after the fish were killed by a blow on the head. In one experiment of rainbow trout in which repeated samplings of the same individual were required, blood was collected with a needle from the dorsal aorta after anesthesia.

Blood samples from maturing female Japanese eels treated with pituitary, and those from silver migratory forms of maturing male eel were collected following cutting of the tail after anesthesia.

Blood of medaka was collected by using micro-hematocrit tubes following cutting of the tail.

All blood samples were allowed to clot at room temperature followed by centrifugation at 3,000 rev/min for 20-30 min to collect the serum. Blood samples from medaka were centrifuged at 10,000 rev/min for 5 min to obtain a clear serum.

The serum was stored at  $-20^{\circ}\text{C}$  until use. No remarkable change in the antigenicity were observed during storage at  $-20^{\circ}\text{C}$  for several months.

## 3. Eggs

Ovulated eggs were collected from female rainbow trout, chum salmon and medaka during the spawning season. Eggs of the Japanese eel were stripped from females which had attained their full maturation with pituitary treatments. Eggs obtained from all species were at the tertiary yolk stage. Eggs were kept frozen at  $-20^{\circ}\text{C}$  and thawed before experiments.

Egg yolk proteins were extracted as follows: Eggs were thoroughly washed with 0.9% NaCl and were then homogenized using a Waring blender. The contents were centrifuged at 10,000 rev/min for 30 min at  $4^{\circ}\text{C}$ . The supernatant was collected and filtrated through filter paper. The filtration was again centrifuged at 30,000 rev/min for 60-90 min at  $4^{\circ}\text{C}$ . A clear intermediate layer was present between the top floating layer and a small amount of precipitate. This middle layer was collected as the egg yolk protein extracts.

## B. Antisera

All antisera were raised in rabbits. The injections consisted of a mixture, in equal volumes of the antigen solution at a volume ranging from 0.5 ml to 1.0 ml, and of Freund's complete adjuvant. These injections were made intradermally into the back of rabbits, four or five times at weekly intervals. A test bleeding was taken from the ear vein and the serum was immediately assayed by immunoelectrophoresis. When the immunoelectrophoresis pattern was judged satisfactory, the animal was exsanguinated by bleeding from the carotid artery.

Polyvalent antiserum against rainbow trout, chum salmon, or Japanese eel, serum proteins was prepared by immunizing rabbits with pooled maturing or ripe female serum of more than 20 fish.

The specific antiserum to the female-specific serum protein(s) of rainbow trout,

chum salmon, or Japanese eel, was prepared by first absorbing the polyvalent antiserum mentioned above with an equal volume of pooled male serum of more than 20 fish. The absorbed antiserum thus obtained react only with female serum but not with male serum when tested by the double immunodiffusion method and immunoelectrophoresis (mentioned below).

The polyvalent antiserum to egg yolk proteins of rainbow trout, chum salmon, Japanese eel, or medaka, was raised in rabbits by immunization with a 0.9% NaCl extract of eggs.

Seven polyvalent antisera and 3 specific antisera are used in the present study (Table 1).

### C. Electrophoresis

Cellulose acetate membrane electrophoresis was performed with Cellogel (Chemetron; Milano, Italy) in a 0.05 M barbital buffer, pH 8.6

Disc electrophoresis was carried out in 5% or 7.5% polyacrylamide gel by the method of Davis (1964). The proteins were stained with Amido black 10B. Lipids were visualized in the gel by staining them with Sudan black B (Prat *et al.*, 1969). Carbohydrates were stained with periodic acid-Schiff's reagent according to the method of Zacharius *et al.* (1969).

SDS (sodium dodecyl sulfate) polyacrylamide gel electrophoresis was carried out according to the procedure of Weber and Osborn (1969). Samples were incubated in a solution containing 1% SDS and 10 mM iodoacetamide at 37°C for 2 h, and then applied to the polyacrylamide gel electrophoresis. Reduction of the proteins was carried out by 1% 2-mercaptoethanol in 1% SDS at 100°C for 2 min. The marker proteins used in the SDS electrophoresis were secretory IgA (mol. wt. 380,000), human IgG (mol. wt. 150,000), human transferrin (mol. wt. 76,000), bovine serum albumin (mol. wt. 67,000), ovalbumin (mol. wt. 45,000), pepsin (mol. wt. 35,000), and horse heart cytochrome-C (mol. wt. 12,400). The relative proportion of stained protein bands was measured by densitometry at 610 nm with a densitometer (OZ-802 Asuka Mfg. Co., Ltd., Japan) equipped with a linear transporter and a recorder.

### D. Immunological procedures

Double immunodiffusion was performed in 1.2% agarose by the method of Ouchterlony (1953).

Single radial immunodiffusion in agarose gel was performed by the method of Mancini *et al.* (1965).

Immunolectrophoresis was performed by the method of Grabar and Williams (1953) with 1.2% agarose in a 0.05 M barbital buffer, pH 8.6.

Disc immunoelectrophoresis was carried out by combining the disc electrophoresis and immunodiffusion. The sample was subjected to a disc of 5% or 7.5% polyacrylamide gel and electrophoresed. Following electrophoresis, the disc was taken out from the glass tube and implanted in a plate of 1.2% agarose gel. The antiserum was then placed in troughs made parallel to the implanted disc gel.

Crossed immunoelectrophoresis and tandem-crossed immunoelectrophoresis were carried out with 1% agarose gel (Litex HSA, Denmark) containing Tris-

barbital buffer, pH 8.6, ionic strength 0.02 according to Weeke (1973) and Krøll (1973). After electrophoresis, the plates were washed, dried, and stained with Coomassie brilliant blue R-250.

### E. Immunofluorescent techniques

Immunofluorescence staining for anti-trout FSSP (see Table 1) was carried out by the method of Kawamura (1966). Smears of liver cells from rainbow trout were dried at room temperature for 60 min and then fixed in acetone-methanol (1:1) for 10 min. Rabbit anti-trout FSSP was placed on the smears for 50 min at 37°C. The slides were then washed thoroughly with phosphate buffer in saline, stained with FITC (fluorescein isothiocyanate)-conjugated anti-rabbit IgG at 37°C for 50 min, washed again and mounted in 20% buffered glycerol. All the stained preparations were examined under an Olympus fluorescence microscope.

### F. Chromatography

Thin-layer gel filtration of serum protein on Sephadex G-200, superfine, was performed in a 0.02 M Tris-HCl buffer, pH 8.0, containing 2% NaCl and 0.1% NaN<sub>3</sub> according to the method of Andrews (1964).

Column chromatography with Sephadex G-75, G-200 and Sepharose 6B (Pharmacia Fine Chemicals) was performed in the same Tris-HCl buffer as mentioned above.

Hydroxylapatite (BDH Chemicals Ltd.) chromatography was carried out by use of 0.2-1.2 M potassium phosphate buffer, pH 6.8 at 4°C.

The determination of molecular weight was done using a gel filtration column with horse heart cytochrome-C (mol. wt. 12,400), trypsin (mol. wt. 23,300), ovalbumin (mol. wt. 45,000), serum albumin (mol. wt. 67,000), alcohol dehydrogenase (mol. wt. 140,000), human IgG (mol. wt. 150,000), bovine liver catalase (mol. wt. 240,000), apoferritin (mol. wt. 480,000), bovine thyroglobulin (mol. wt. 670,000) and human IgM (mol. wt. 900,000) as marker proteins.

### G. Iron-binding property

Autoradiography was used for demonstration of iron binding capacity of proteins according to the method of Giblett *et al.* (1959). Twenty-five  $\mu$ l of fish serum or egg yolk extracts, containing approximately 1 mg protein, were incubated for 1 h at room temperature with 5  $\mu$ l <sup>59</sup>FeCl<sub>3</sub>, corresponding to 0.1  $\mu$ Ci. The sample was then submitted to cellulose acetate membrane electrophoresis, immunoelectrophoresis, crossed immunoelectrophoresis or tandem-crossed immunoelectrophoresis. After electrophoresis, the plates were stained and dried. Finally they were placed in close contact with X-ray films (Fuji 400). The autoradiogram was developed after standing for 2-3 weeks. Autoradiography of thin-layer gel filtration chromatography was performed essentially according to the above mentioned method. When the chromatography finished, the plates were covered with a filter paper, which was then stained with Amide black 10B, dried and exposed to an X-ray film.

For determination of the labeled protein in effluent fractions eluted from the gel filtration column, samples of 2.5 ml from the collected tubes were counted by

Dainabot  $\gamma$ -counter.

## H. Chemical analysis

The amount of phosphorus in purified proteins were estimated by the method of Fiske and Subbarow (1925).

Amino acid analyses were carried out by a Hitachi Model KLA-3 automatic amino acid analyser. Hydrolyses were performed in 6N HCl for 24 h at 110°C in evacuated, sealed tubes.

Determination of NH<sub>2</sub>-terminal amino acid was performed by the dansyl chloride method using a polyamide gel thin-layer chromatography (Gray, 1967).

Protein concentration was determined by the method of Lowry *et al.* (1951), using bovine serum albumin as standard.

## I. Physicochemical analysis

Analytical ultracentrifugations were carried out in a Spinco Model-E ultracentrifuge at 56,100 rev/min using protein concentrations varying from 13.0 mg/ml for the female-specific serum protein from rainbow trout, 14.9 mg/ml for one egg yolk protein (E1) of rainbow trout, 4.7 mg/ml for the other egg yolk protein (E2) of rainbow trout, and 8.9 mg/ml for the female-specific serum protein of chum salmon.

Sedimentation coefficient,  $S_{20,w}^0$  was evaluated by extrapolation to zero protein concentration and corrected to the viscosity and density of water at 20°C.

Diffusion coefficients were measured in a Spinco Model-E ultracentrifuge, using a synthetic boundary cell at 12,590 rev/min at protein concentrations of 4.6 mg/ml for a female-specific serum protein of chum salmon. Coefficient values were calculated by Fujita's equation modified by Kawahara (1969).

Molecular weights were calculated by using sedimentation and diffusion coefficients in the Svedberg equation (Svedberg 1937).

Partial specific volumes of rainbow trout and chum salmon female-specific serum proteins were calculated from the amino acid composition (McMeekin *et al.*, 1949).

Extinction coefficient ( $E_{280\text{nm}}^{1\%}$ ) was measured in 0.1 N NaOH with a Hitachi Perkin-Elmer Model 139 spectrophotometer. A weighed sample, after drying over P<sub>2</sub>O<sub>5</sub> in a vacuum was dissolved and the optical density of the solution was determined at 280 nm.

The isoelectric point was determined by an LKB 8100 electrofocusing column. A purified female-specific serum protein and two egg yolk proteins in rainbow trout were applied and run in a pH gradient from 3 to 10 for 72 h and collected in 1 ml fractions. The pH was measured with a Hitachi-Horiba Expandomatic pH meter at 0°C and each protein was determined with a single radial immunodiffusion and/or absorbance at 280 nm.

## J. Experiments on hormone-treated fish

### 1. Rainbow trout

For the estradiol-17 $\beta$  experiment, a group of rainbow trout consisting of males or immature females weighing about 350 g, were injected with 0.5 mg of estradiol-

17 $\beta$  in 50% ethanol. Blood samples were taken from the fish 2, 4, 6 and 8 days after the single injection of estrogen.

An additional group of immature (based on an average body weight of 100 g) rainbow trout were injected with 100  $\mu$ g of estradiol-17 $\beta$  in 50% ethanol (0.2 ml) into the muscle behind the head. Blood samples were taken every 2 days for 22 days after the single estrogen injection from the dorsal aorta after anesthesia with meta-aminobenzoic acid ethylester methanesulfonate (MS 222). The fish after 6 days were sampled for histological and immunofluorescent study of the liver.

For light microscopic observations, the liver was fixed with Bouin's fluid. Serial paraffin sections were cut at 5-6  $\mu$  in thickness, and stained with Delafield's hematoxylin and eosin.

## 2. Medaka

In the present study of medaka, ethinylestradiol, estradiol-17 $\beta$ , methyltestosterone and testosterone were employed for the four different steroid hormone treatments.

One hundred sexually inactive female fish (1.6-2.4% in G.S.I.\*) were divided into 5 groups at the start of the experiment and reared under the same conditions. Each group was fed pellets containing these hormones at a dose of 100  $\mu$ g/g diet during the experimental period (Nov.-Dec. 1977). A control group received the same pellets without hormones. Five fish of each group were sampled at one time to collect the blood at 5, 10, 20 and 30 days after the beginning of the hormonal treatment.

## III. Results

### A. Rainbow trout

#### 1. Sexual differences of serum proteins in cellulose acetate membrane electrophoretic patterns

Male and immature rainbow trout had similar electrophoretic patterns (Fig. 1). Differences between sexes were seen in the  $\beta$ -globulin region, in which maturing and ripe female trout showed strongly stained components, whereas no males and immature females showed such components in that region.

#### 2. Identification of female specific proteins in sera and egg yolk extracts by means of immunological procedures

Absorption of a polyvalent antiserum to female serum proteins with male serum produced the loss of a precipitin reaction with male serum proteins. However, the absorbed antiserum maintained reactivity with female serum as well as with egg yolk extracts. This kind of antiserum was designated as anti-trout FSSP (female-specific serum proteins) (see Table 1). Anti-trout FSSP failed to react with male serum, whereas it reacted with the female serum forming two parallel lines, and with egg yolk extracts developing two distinct lines together with minor faint lines (Fig. 2). The result indicated that there are antigens (female-specific serum proteins)

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\* G.S.I.: Gonadosomatic index (ovarian weight  $\times$  100/body weight)



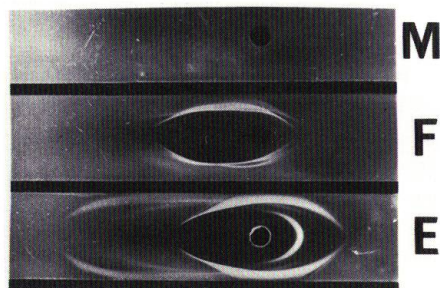
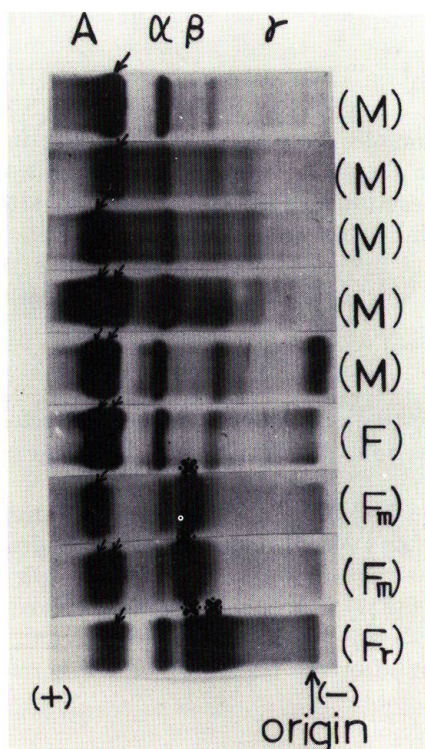


Fig. 2. Immunoelectrophoresis of ripe male (M) and female (F) rainbow trout serum, and egg yolk extracts (E) with rabbit anti-trout FSSP.

Fig. 1. Typical cellulose acetate membrane (Cellogel) electrophoretic patterns of male (M) and female (F) rainbow trout serum. The electrophoregrams show one (✓) or two bands (✓✓) in the albumin fraction. All specimens of maturing (Fm) and ripe (Fr) females have one or two components (\*) in the  $\beta$ -globulin region.

Table 1. The antisera used for immunochemical characterization

<i>Polyvalent Antiserum</i>	
1.	Anti-trout serum: Rabbit antiserum against pooled ripe female rainbow trout sera
2.	Anti-salmon serum: Rabbit antiserum against pooled ripe female chum salmon sera
3.	Anti-eel serum: Rabbit antiserum against pooled maturing female Japanese eel sera
4.	Anti-trout eggs: Rabbit antiserum against 0.9% NaCl egg yolk extracts of rainbow trout
5.	Anti-salmon eggs: Rabbit antiserum against 0.9% NaCl egg yolk extracts of chum salmon
6.	Anti-eel eggs: Rabbit antiserum against 0.9% NaCl egg yolk extracts of Japanese eel
7.	Anti-medaka eggs: Rabbit antiserum against 0.9% NaCl egg yolk extracts of medaka
<i>Specific Antiserum</i>	
1.	Anti-trout female-specific serum proteins (anti-trout FSSP): absorbed anti-trout serum with male rainbow trout sera
2.	Anti-salmon female-specific serum proteins (anti-salmon FSSP): absorbed anti-salmon serum with male chum salmon sera
3.	Anti-eel female-specific serum protein (anti-eel FSSP): absorbed anti-eel serum with male Japanese eel sera

existing specifically in female serum but not in male serum, and that similar antigens are also present in egg yolk extracts.

3. *Identification of the iron-binding property in female-specific serum and egg yolk proteins*

Autoradiography of cellulose acetate membrane electrophoresis of the serum mixed with  $^{59}\text{FeCl}_3$  clearly indicated the difference between females and males in the

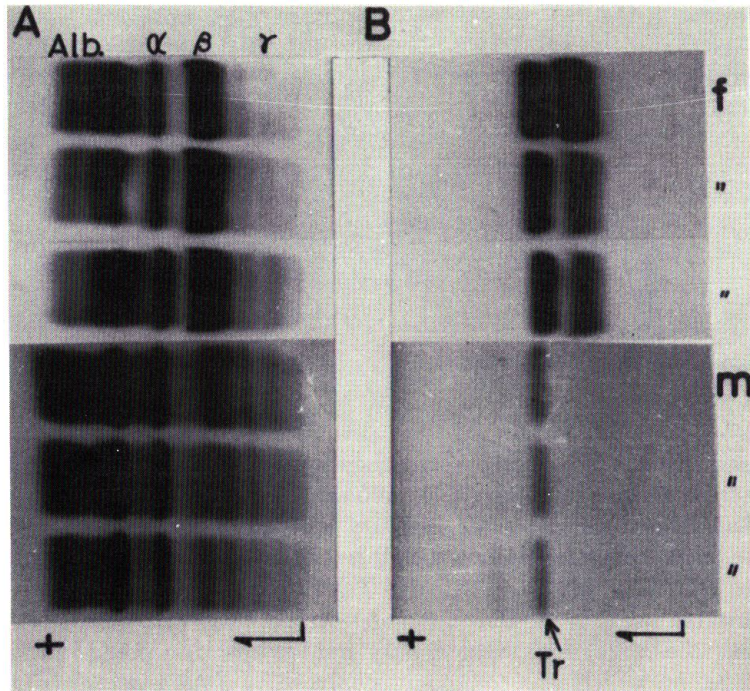


Fig. 3. Cellulose acetate membrane (Cellogel) electrophoresis of ripe rainbow trout sera (A) and its autoradiography with  $^{59}\text{Fe}$  (B). f: female serum, m: male serum, Tr: transferrin. To 25  $\mu\text{l}$  of the serum was added 10  $\mu\text{l}$  of  $^{59}\text{FeCl}_3$  solution with an activity of 0.1  $\mu\text{Ci}$ , corresponding to 0.003  $\mu\text{g}$  Fe. After incubation the mixture was subjected to electrophoresis, and the gels were exposed to X-ray films for 2 weeks.

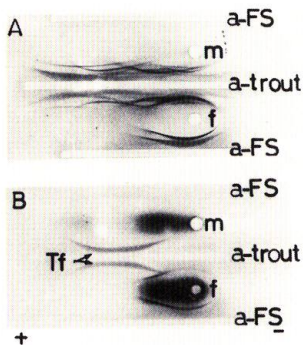


Fig. 4. Immunelectrophoresis of ripe rainbow trout sera. A: protein staining, B: autoradiography with  $^{59}\text{Fe}$ , a-trout: anti-trout serum, a-FS: a-trout FSSP, m, f: male or female serum.

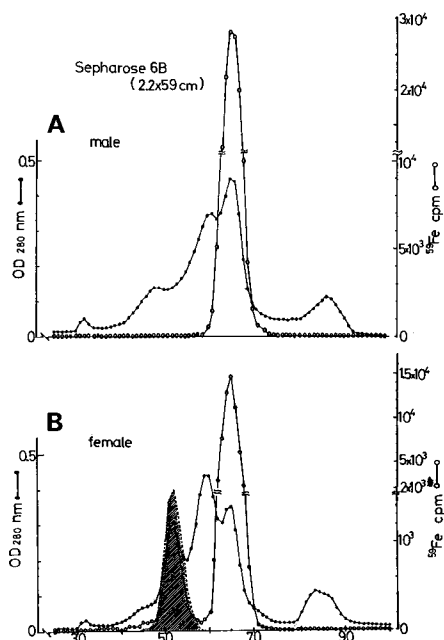


Fig. 5. Gel filtration on Sepharose 6B of ripe male (A) and female (B) rainbow trout sera. The fractions in shadowed areas of the female contained female-specific serum proteins. One radioactive protein (transferrin) was observed in males, while two radioactive proteins were obtained in females.

spawning condition. The female serum showed two strong radioactive bands, while the male serum showed a single weak radioactive band (Fig. 3). One of the two bands of female sera was the same mobility with band of male sera. This component is considered to be transferrin which has been previously identified in salmon by Utter *et al.* (1970), Møller (1970) and Payne *et al.* (1971).

The autoradiography of the immunoelectrophoresis with anti-trout serum indicated that the male sera yielded only one radioactive precipitin line at the  $\alpha$ -globulin region which was commonly observed in female serum (Fig. 4). This component is considered to be transferrin. On the other hand, the female sera showed another radioactive line which migrated to the  $\beta$ -globulin region. There were two precipitin lines at the  $\beta$ -region with anti-trout FSSP (Fig. 2 & 4). However, only one line near the antigen well was capable of binding the radioactive

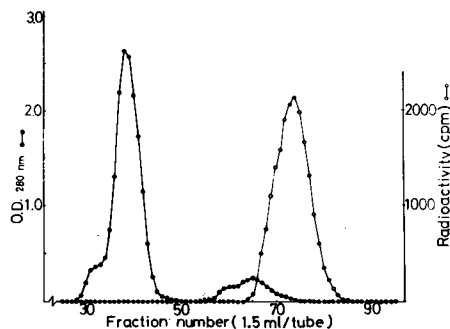


Fig. 6. Gel filtration on Sephadex G-200 of egg yolk extracts from rainbow trout. One radioactive peak was observed at a very small molecular weight fraction.

iron (Fig. 4).

Gel filtration on Sepharose 6B of serum mixed with  $^{59}\text{FeCl}_3$  was also performed to separate the female-specific iron-binding protein from putative fish transferrin (Fig. 5). Radioactive protein observed in males was eluted as a fraction of relatively low molecular weight (approximately mol. wt. 80,000) and was concluded to be transferrin (Fig. 5A). In female trout, however, two distinct radioactive peaks were obtained (Fig. 5B). The second radioactive peak corresponded to the peak of transferrin as indicated above. The first radioactive peak observed in female serum only was considered to be a female-specific serum protein with iron-binding activity. A similar experiment was made for egg yolk extracts with  $^{59}\text{FeCl}_3$  using Sephadex G-200 gel filtration. Only one radioactive peak was observed in the case of egg yolk extracts at a very small molecular weight fraction (mol. wt. around 20,000) (Fig. 6).

#### 4. Purification of female-specific iron-binding serum protein (FS)

Yoneda and Ishihara (1974) reported that proteins specifically present in the female of chum salmon are precipitative by a dilution of maturing female serum with a low ionic strength. Therefore, a similar procedure was used for the purification of the female-specific iron-binding serum protein (hereafter abbreviated to FS) of the trout: pooled ripe female serum was dialysed against distilled water, and the precipitate formed was centrifuged at 10,000 rev/min for 30 min at 4°C. The pellet was suspended in water, recentrifuged, and dissolved in 0.5 M NaCl. The solution was then applied to a gel filtration column of Sepharose 6B (2.6×96 cm) with 0.02 M Tris-HCl buffer, pH 8.0, containing 2% NaCl and 0.1%  $\text{NaN}_3$ . The main peak of elution pattern was found to consist of FS when assessed by means of a single radial immunodiffusion using the anti-trout FSSP (Fig. 7). Its elution position suggested a molecular weight of about 600,000 (see Fig. 9).

#### 5. Purification of egg yolk proteins (E1 and E2)

Egg yolk proteins were isolated according to the procedure of Markert and Vanstone (1971). Eggs were washed with 0.9% NaCl, and homogenized in a Waring blender. The contents were strained through a Buchner funnel, and centrifuged at 30,000 rev/min for 60 min at 4°C. A clear middle layer with a yellowish color was collected. This clear solution was dialysed against distilled water overnight in 4°C.

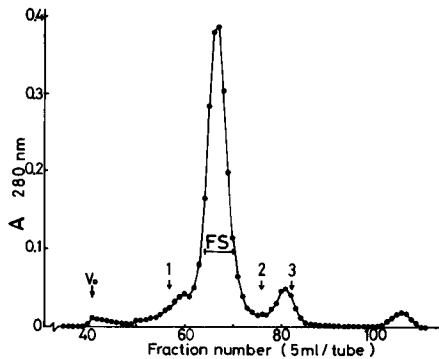


Fig. 7. Chromatography of rainbow trout serum on Sepharose 6B. Material: water-insoluble proteins isolated from pooled ripe female rainbow trout serum. Column: 2.6×96 cm, Elution buffer: 0.02 M Tris-HCl, pH 8.0, containing 2% NaCl and 0.1%  $\text{NaN}_3$ . FS: fractions eluted in the position indicated by a horizontal bar where collected and designated as FS.  $V_0$ : void volume. Marker proteins: (1) IgM, (2) IgG, (3) human serum albumin.

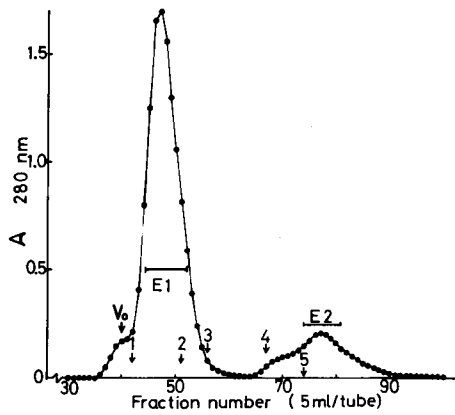


Fig. 8. Chromatography of rainbow trout egg yolk proteins on Sephadex G-200. Material: water-insoluble proteins isolated from rainbow trout egg yolk. Column:  $2.6 \times 95$  cm. Elution buffer: same as in Fig. 7. E1, E2: two major components were designated as E1 and E2. Vo: void volume. Marker proteins: (1) horse spleen apoferritin, (2) IgG, (3) yeast alcohol dehydrogenase, (4) bovine serum albumin, (5) ovalbumin.

The precipitate thus formed was separated by centrifugation at 10,000 rev/min for 30 min. The pellet was dissolved in 0.5 M NaCl, and dialyzed against water. This procedure was repeated twice. The final clear yellowish solution in 0.5 M NaCl was applied to a gel filtration on Sephadex G-200 ( $2.6 \times 95$  cm), and eluted with Tris-HCl buffer as mentioned above. The elution pattern yielded two peaks, E1 and E2 (Fig.

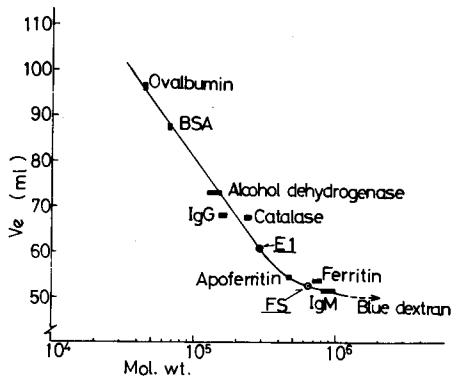


Fig. 9. Estimation of molecular weight from the exclusion volume on Sephadex G-200. Calibration curve of Sephadex G-200 ( $2.2 \times 47$  cm). The samples used for calibration are indicated by dark bars. The open circles correspond to the elution volume of trout female-specific iron-binding serum protein (FS) and egg protein (E1) passed through the Sephadex G-200 column.

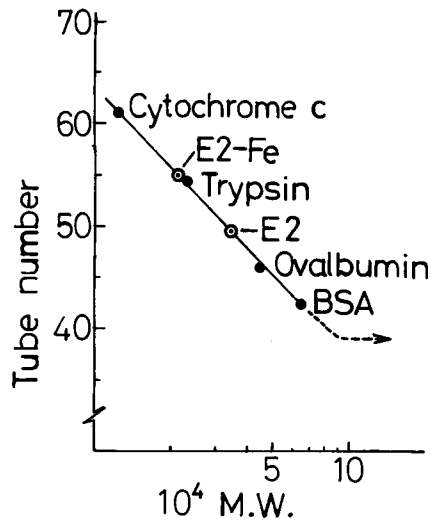


Fig. 10. Estimation of molecular weight from the exclusion volume on Sephadex G-75. Calibration curve of Sephadex G-75 ( $2.2 \times 58$  cm). The samples used for calibration are indicated by dark circles. The open circles correspond to the elution volume of trout egg yolk proteins (E2) and radioactive peak of E2 fraction (E2-Fe) (see Fig. 6).



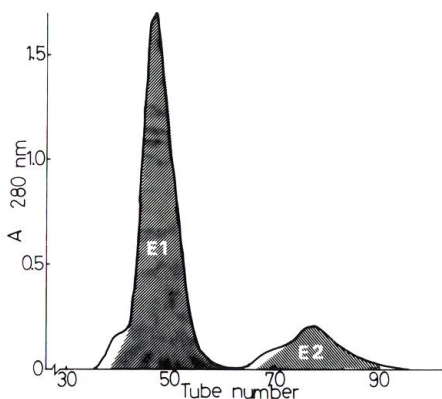


Fig. 11. Elution pattern on Sephadex G-200 of rainbow trout egg yolk proteins (see Fig. 8). Ratios of protein amount between E1 and E2 (shaded area) were determined planimetrically from charts of gel filtration on Sephadex G-200 monitored by 280 nm absorption. A molar ratio of E1 and E2 can be roughly calculated to be 1 to 1.

8). These peaks were tested for their reactivity to the anti-trout FSSP and both were found to be antigens immunologically related to the FS, showing the precipitative reaction with the anti-trout FSSP. The molecular weights estimated by gel filtration were about 300,000 for E1 with Sephadex G-200, and 35,000 for E2 with Sephadex G-75 (Figs. 9 and 10).

The ratio of protein amount between E1 and E2, which were precipitable together in a low ionic strength, was determined planimetrically from charts of gel filtration on Sephadex G-200 monitored by 280 nm absorption, and were found to be 4.9 and 1, respectively. Putting the ratio of protein amount, molecular weights of E1 and E2, and their extinction coefficient (see later) together, the molar ratio of E1 and E2 can be roughly calculated to be 1 to 1 (Fig. 11).

#### 6. Purity of FS, E1 and E2

The preparation of FS gave rise to only one precipitin line with the antiserum to trout serum as well as with the anti-trout FSSP by the double immunodiffusion method. It showed a single homogeneous band in the disc electrophoresis when

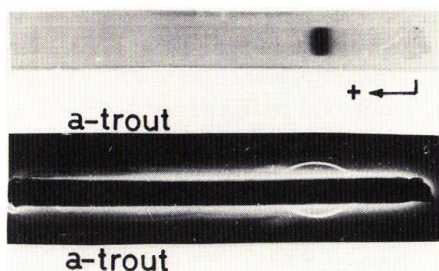


Fig. 12. Disc immunoelectrophoresis of purified FS from rainbow trout. The sample was subjected first to disc electrophoresis in 5% polyacrylamide gel, followed by immunodiffusion in 1.2% agarose gel. a-trout: anti-trout serum.

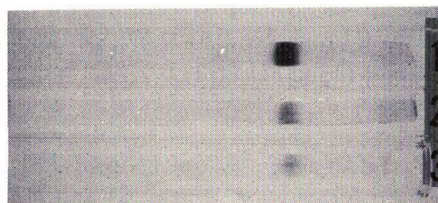


Fig. 13. Disc electrophoresis of purified FS from rainbow trout. 1: protein staining. 2: lipid staining. 3: carbohydrate staining.

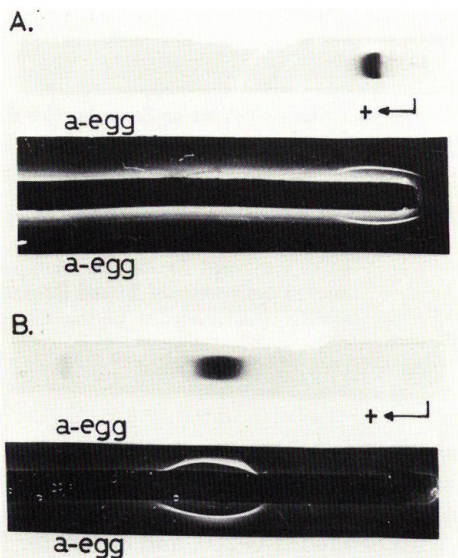


Fig. 14. Disc immunoelectrophoresis of purified E1 (A) and E2 (B) isolated from egg yolk proteins of rainbow trout. The samples were subjected first to disc electrophoresis in 5% or 7.5% polyacrylamide gel, followed by immunodiffusion in 1.2% agarose gel. a-egg: antiserum to trout eggs (anti-trout eggs).

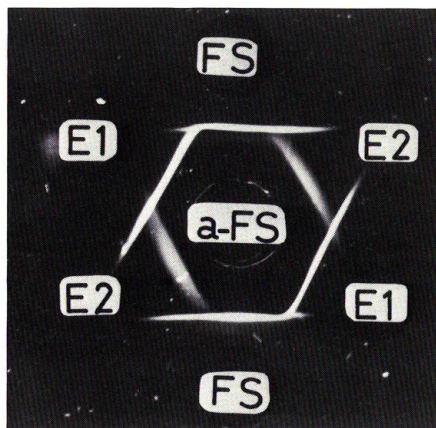


Fig. 15. Precipitin reaction of FS, E1 and E2 from rainbow trout. FS: female-specific iron-binding serum protein. E1 and E2: fractions of egg yolk proteins. a-FS: anti-trout FSSP.

stained by Amido black 10B. Disc immunoelectrophoresis of FS with the anti-trout serum gave a single precipitin arc at the position corresponding to the protein band (Fig. 12). This protein could be stained with Sudan black B and Schiff's reagent indicating the presence of lipid and carbohydrate in the molecule (Fig. 13).

E1 showed a sharp single band in 5% polyacrylamide gel electrophoresis and one precipitin line with the antiserum to egg proteins (a-egg) (Fig. 14A). This protein was positive to stainings for lipids and carbohydrates similar to FS. E2 gave rise to a broad band in 7.5% gel and a broad single arc with the antiserum to egg proteins (a-egg) (Fig. 14B). E2 was stained neither with carbohydrate nor the lipid reagents.

#### 7. Antigenic relation between FS, E1 and E2

FS formed a spur over E1 and E2, and the lines of E1 and E2 crossed with each other on double immunodiffusion in 1.2% agarose gel against the anti-trout FSSP (Fig. 15). Precipitin reactions of these proteins with anti-trout eggs (antiserum to egg proteins) are shown in Fig. 16. E1 and E2 cross each other, but FS spurs over E1 and E2 similar to the case with the anti-trout FSSP (Fig. 16A). However, the precipitin line of FS fuses with a mixture of E1 and E2 indicating that FS consists of E1 and E2 antigens (Figs. 16B and 16C).



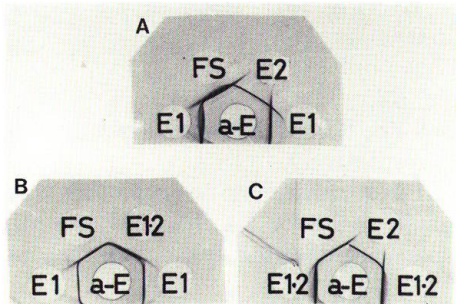


Fig. 16. Precipitin reaction of FS, E1 and E2 from rainbow trout. FS, E1 and E2: same in Fig. 15. E1·2: E1 plus E2. a-E: anti-trout eggs. After removal of unprecipitated protein by washing and drying, the precipitin arcs were stained with Amido black 10B.

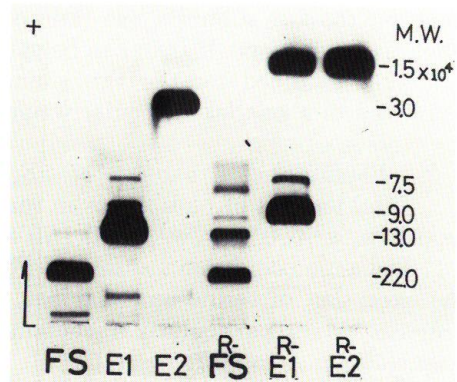


Fig. 17. SDS polyacrylamide gel electrophoresis. R-FS, R-E1 and R-E2: each protein reduced with 2-mercaptoethanol. After electrophoresis, proteins were stained with Coomassie brilliant blue R-250.

#### 8. SDS polyacrylamide gel electrophoresis

FS displayed a main band which migrated at a position corresponding to a molecular weight of 220,000–240,000 (Fig. 17). E1 displayed a major band corresponding to a molecular weight of about 130,000, and E2 revealed a single band corresponding to 30,000 (Fig. 17).

FS reduced with 2-mercaptoethanol (R-FS) showed two main bands which migrated at positions corresponding to a molecular weight of 220,000–240,000 and 130,000, respectively, and some minor bands which migrated faster than the two main bands. Reduced E1 (R-E1) showed two major bands, one of which corresponded to a molecular weight of 90,000 and the other corresponded to 15,000. Provided that the ratio of color yield with Coomassie brilliant blue R-250 is the

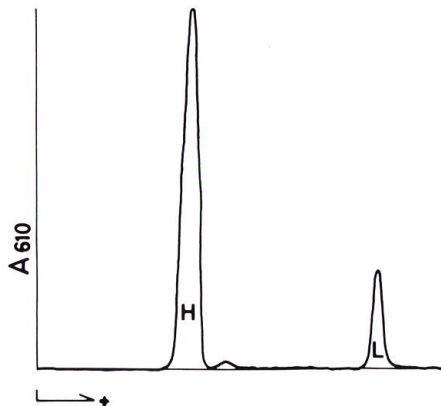


Fig. 18. Spectrophotometric scan of SDS polyacrylamide gel of reduced E1 (R-E1) (Fig. 17). The electrophoretically separated proteins were stained with Coomassie brilliant blue R-250. A molar ratio of 1:1 is obtained for the two subunits (mol. wt. 90,000 (H) and 15,000 (L)) of E1 of rainbow trout egg yolk.



same for the two subunits, the ratio of protein amount between the two subunits (mol. wt. 90,000 and 15,000) can be calculated as 1 : 5.25, and the molar ratio of 1 : 1 is thus obtained for the two subunits of E1 (Fig. 18). Reduced E2 (R-E2) converted to a smaller molecular weight of 15,000.

#### 9. Physicochemical analyses of FS, E1 and E2

Total amount of phosphorus in the purified FS, E1 and E2 were determined to be 0.68 w/w %, 0.95 w/w % and 1.70 w/w %, respectively.

The amino acid compositions of FS and E1 were quite close to each other with the exception of serine and alanine. Compared to FS or E1, E2 showed quite different values, particularly in contents of aspartic acid, serine, lysine, alanine, and phenylalanine (Table 2).

The number of amino acid residues per mole of FS, E1 and E2 was evaluated from the molecular weights (Table 3). The summation of the respective amino acids of E1 and E2 showed close similarity to that of a half molecule of FS except for serine and alanine.

The infinite dilution value of the sedimentation coefficient,  $S_{20,w}^0$ , of FS and E1 was 10.5 S and 9.7 S, respectively (Figs. 19 and 20). The value of  $S_{20,w}^0$  of E2 could not be calculated because of its asymmetrical peak (Fig. 21).

Table 2. Amino acid composition of FS, E1 and E2 of rainbow trout expressed as moles/100 moles of amino acids.

Amino acid	Residue		
	FS	E1	E2
Asp	8.40	7.60	15.69
Thr	4.95	5.29	3.99
Ser	7.52	4.77	10.86
Glu	11.51	11.51	9.97
Pro	5.21	5.32	3.17
Gly	4.21	4.02	5.72
Ala	11.68	14.13	3.51
Cys/2	1.18	0.80	2.74
Val	7.10	7.63	7.76
Met	2.55	2.54	3.03
Ile	5.46	5.87	5.00
Leu	9.47	10.33	6.99
Tyr	2.99	2.87	3.89
Phe	4.04	4.60	1.72
His	2.12	2.19	2.25
Lys	7.10	6.23	10.59
Arg	4.53	4.38	3.15
Total	100.02	100.08	100.03

Table 3. Number of amino acid residues per mole of FS, E1 and E2 from rainbow trout

Amino acid (mol. wt.)	E1 (210,000)	E2 (30,000)	E1+E2	FS/2 (240,000)
Asp	145	42	187	185
Thr	101	11	112	109
Ser	91	29	120	165
Glu	220	27	247	253
Pro	102	9	111	115
Gly	77	15	92	93
Ala	270	9	279	257
Cys/2	15	7	22	26
Val	146	21	167	156
Met	49	8	57	56
Ile	112	14	126	120
Leu	197	19	216	208
Tyr	55	11	66	66
Phe	88	5	93	89
His	42	6	48	47
Lys	119	29	148	156
Arg	84	9	93	100
Total	1,913	271	2,184	2,201

Extinction coefficient ( $E_{1\text{cm}, 280\text{nm}}^{1\%}$ ) were determined for the purified proteins in 0.1 N NaOH. The value for FS, E1 and E2 was 5.31, 5.51 and 7.76, respectively.

Patterns of FS purified protein isoelectrofocusing revealed somewhat heterogeneous isoelectric points, showing three peaks (Fig. 22A). The main peak of FS showed the pI of 6.4 and the other minor peaks corresponded to the pI of around 5.5 and 5.8. On the other hand, isoelectric focusing of the E1 and E2 displayed rather homogeneous peaks (Figs. 22B and 22C). PI of E1 and E2 were determined to be 10 and 7.2, respectively.

#### 10. Amounts of FS in fish at various ovarian stages

The amount of FS in female fish was determined by a single radial immunodiffusion technique on 1.2% agarose gel containing anti-trout FSSP (Fig. 23). The standard curve determined by a typical radial immunodiffusion is shown in Fig. 24.

The concentration of FS in the serum of female trout at various maturational stage was determined (Fig. 25). Five out of 15 immature females produced no ring in the radial immunodiffusion and the concentration of their FS was estimated to be less than 20  $\mu\text{g/ml}$ . Mean levels of concentration at various maturational stages were 147  $\mu\text{g/ml}$  for immature, 11,080  $\mu\text{g/ml}$  for maturing, 3,166  $\mu\text{g/ml}$  for ripe and 640  $\mu\text{g/ml}$  for spawned-out stages, respectively.

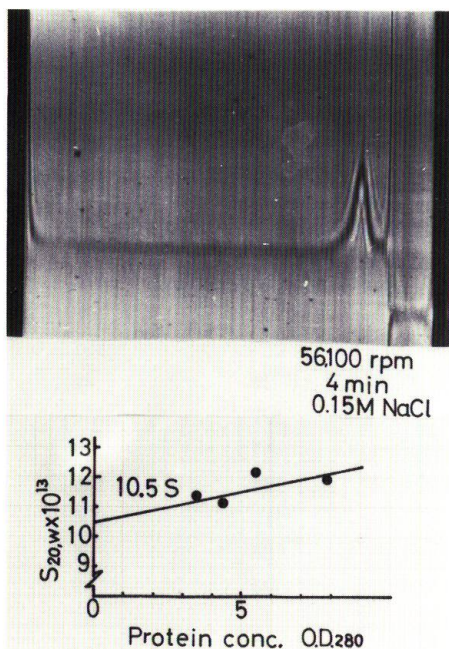


Fig. 19. Ultracentrifugal pattern of purified trout FS and concentration dependency of the sedimentation rate.

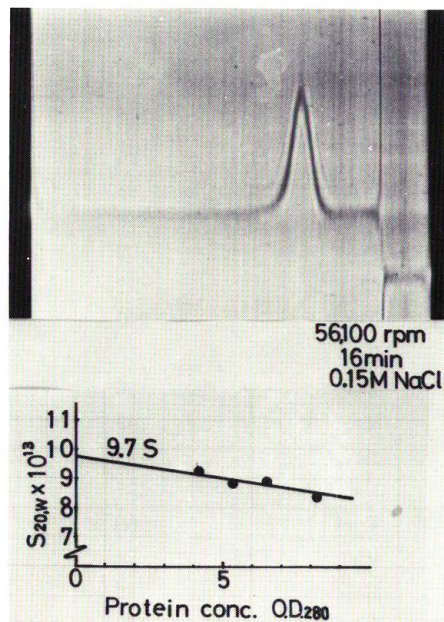


Fig. 20. Ultracentrifugal pattern of purified trout E1 from egg yolk proteins and concentration dependency of the sedimentation rate.

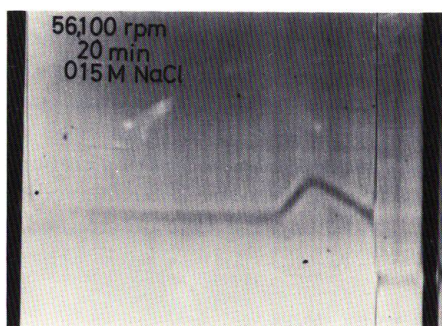


Fig. 21. Ultracentrifugal pattern of purified trout E2 from egg yolk proteins.

11. Production of FS in male and in immature trout by a treatment with estradiol-17 $\beta$

Serum was collected from fish 2, 4, 6 and 8 days after a single estrogen injection. Possible production of FS by the treatment with estrogen was assessed by means of double immunodiffusion with the anti-trout FSSP (Fig. 26). Two precipitin lines were developed with the maturing female fish as well as with the estrogen-treated male and immature females. One of the two lines fused completely with that of purified FS, indicating the production of FS in the estrogen-treated fish.

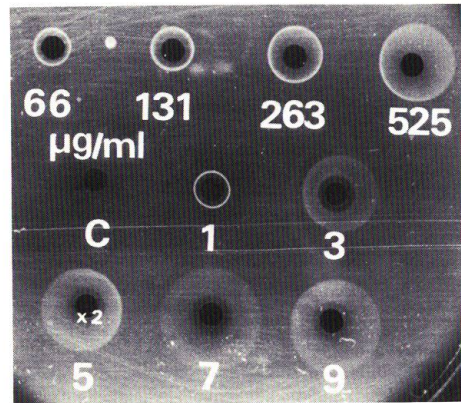
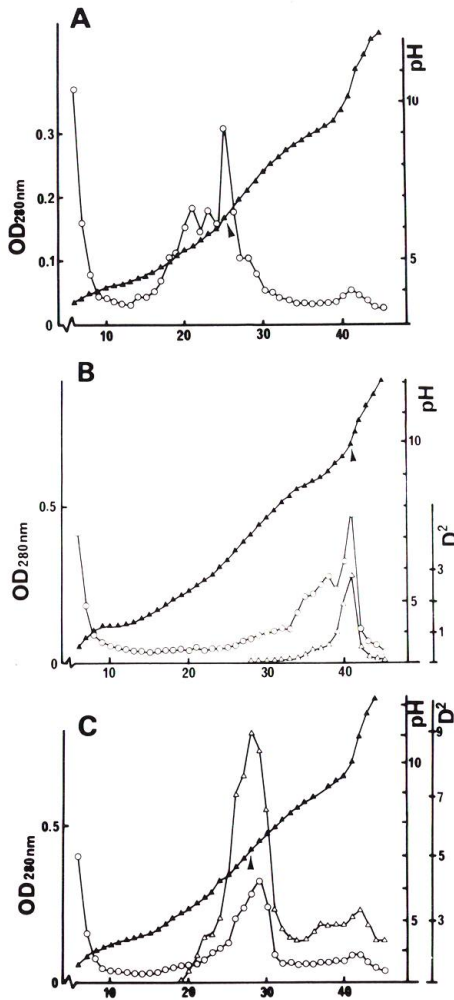


Fig. 23. Typical immunodiffusion plate (unstained). The agarose contained anti-trout FSSP. The antigen wells were filled with varying amounts of purified FS from rainbow trout used as standard and serum samples (C, 1, 3, 5 and 7).

Fig. 22. Isoelectrofocusing patterns of FS(A), E1(B) and E2(C) purified proteins isolated from rainbow trout. Ampholite: pH 3-10, voltage 700 V for 72 h. Sample: 1.0 ml. The pH ( $\blacktriangle$ ) was measured with a pH meter at 0°C and each protein was determined with a single radial immunodiffusion ( $\triangle$ ) and/or absorbance at 280 nm ( $\circ$ ). Single radial immunodiffusion in agarose gel was performed using anti-trout eggs.

The iron binding activity of the FS produced after the treatment of fish with the hormone was assessed by autoradiography of the immunoelectrophoresis with  $^{59}\text{FeCl}_3$  (Fig. 27), and by gel filtration on Sephadex G-200 of the serum admixed with  $^{59}\text{FeCl}_3$  (Fig. 28). Precipitin lines of hormone-treated fish developed by anti-trout FSSP show the radioactivity for  $^{59}\text{Fe}$ , whereas the control fish (male and immature female) do not show any radioactive precipitin line other than transferrin (Fig. 27). Gel filtration of the serum from the hormone-treated fish (Fig. 28) disclosed the appearance of a new radioactive peak at a higher molecular weight fraction besides the peak of transferrin (Fig. 5).

Results of radio immunoelectrophoresis surmised the increase of FS after a longer time-interval (Fig. 27). Therefore, more precise determination of FS after the hormone treatment was made using the single radial immunodiffusion with



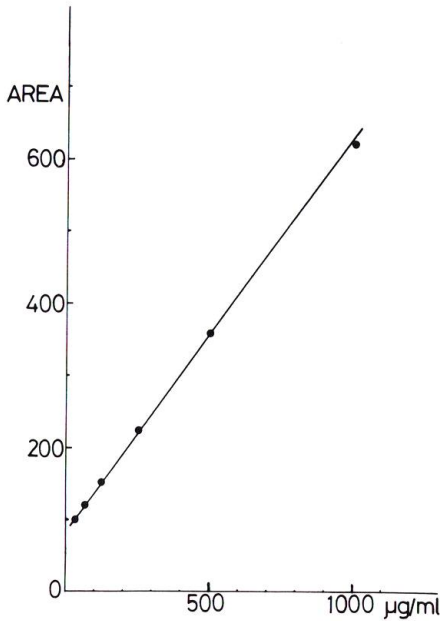


Fig. 24. The standard curve determined by a typical single radial immunodiffusion (see Fig. 23). Horizontal line indicates the concentration of trout FS.

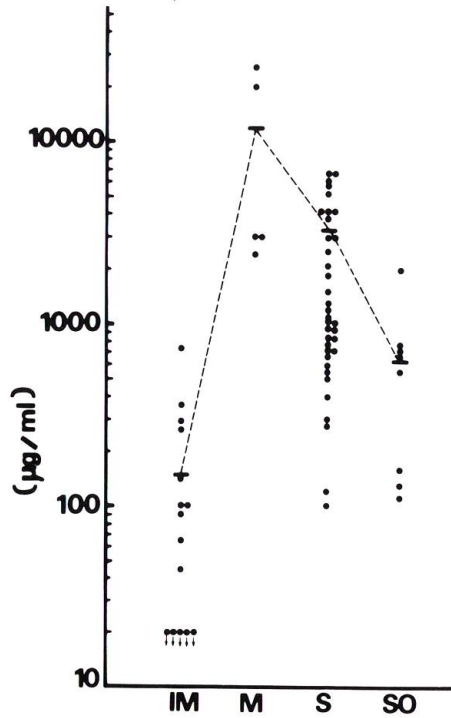


Fig. 25. The concentration of FS in the serum of female rainbow trout at various maturational stages. IM: immature, M: maturing, S: ripe, SO: spawned-out.

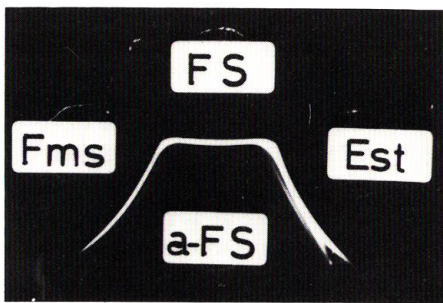


Fig. 26. Precipitin reaction of FS, sera of maturing female and estrogen-treated male rainbow trout. Fms: serum of maturing female fish. Est: serum of estrogen-treated fish (6 days after injection). FS: female-specific iron-binding serum protein of rainbow trout, a-FS: anti-trout FSSP.

anti-trout FSSP for the determination of FS. Four different doses of estradiol-17 $\beta$ , 40  $\mu$ g, 400  $\mu$ g, 1.4 mg, and 7 mg, per kg fish body weight, were administered once into muscle of 10 fish in four groups. Two fish in each group were sacrificed for collecting the serum several days after injection (Fig. 29). A gradual increase of FS

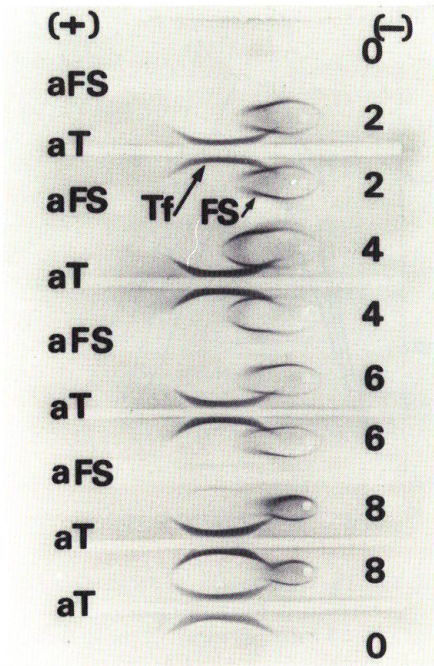


Fig. 27. Immunoelectrophoretic autoradiography of  $^{59}\text{Fe}$ -binding proteins in estrogen-treated rainbow trout sera. The antigen wells were filled with the serum collected from fish 0 (control), 2, 4, 6, and 8 days after administration of estrogen. Antisera used were anti-trout FSSP (aFS) and anti-trout serum (aT). Tf: transferrin, FS: female-specific iron-binding serum protein.

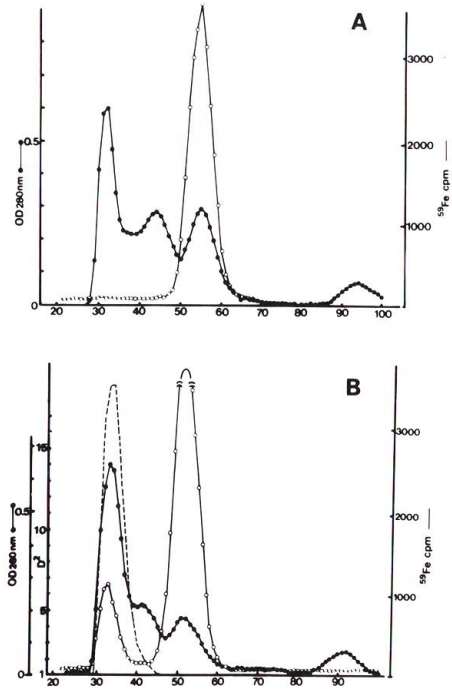


Fig. 28. Gel filtration on Sephadex G-200 of rainbow trout serum collected from non-estrogen treated (A) and estrogen-treated (B) fish. The presence of FS (dotted line) was assessed by the Mancini method using anti-trout FSSP. Column:  $2.0 \times 47$  cm, Elution buffer: 0.02 M Tris-HCl, pH 8.0, containing 2% NaCl and 0.1%  $\text{NaN}_3$ . Each serum was 0.5 ml and was mixed with  $10 \mu\text{l}$  of  $^{59}\text{Fe}$  ( $0.25 \mu\text{Ci}$ ).

after administration of estradiol- $17\beta$  was observed until the around day 7, after which, the concentration tended decrease (Fig. 29). Dose response of FS production was also found up to the 1.4 mg hormone per kg body weight.

The gradual decrease of FS after the highest concentration at around day 7 was further confirmed by surveying the change in concentration of FS in four individual fish by collecting serum repeatedly for up to 3 weeks, after being administered 1 mg of estradiol- $17\beta$  per kg body weight (Fig. 30). FS reached its highest concentration on day 7 to day 12, and disappeared from the serum within 10 days.

#### 12. Histological changes of the liver following estrogen administration

Liver cells of immature fish were histologically changed after the treatment with estradiol- $17\beta$ . In the estrogen-treated fish, the liver cells became somewhat enlarged and some round vacuoles appeared within the cytoplasm (Fig. 31). These

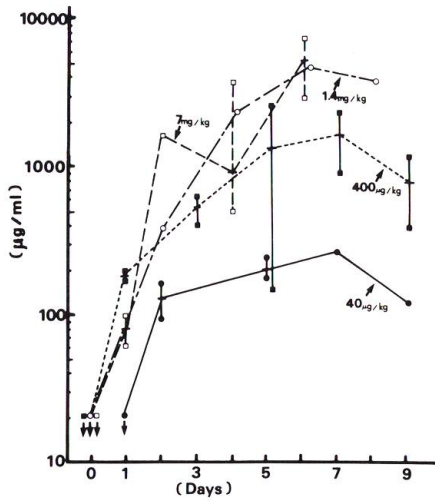


Fig. 29. The concentration of FS of rainbow trout (on a logarithmic scale) at different times after the injection of various doses of estradiol-17 $\beta$ .

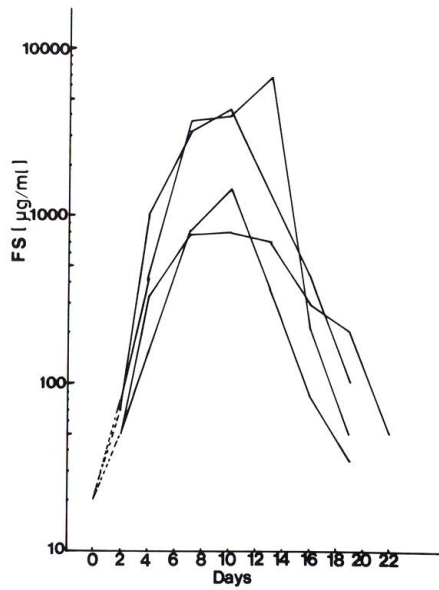


Fig. 30. The concentration of FS of individual rainbow trout after administration with 1 mg of estradiol-17 $\beta$  per kg body weight.

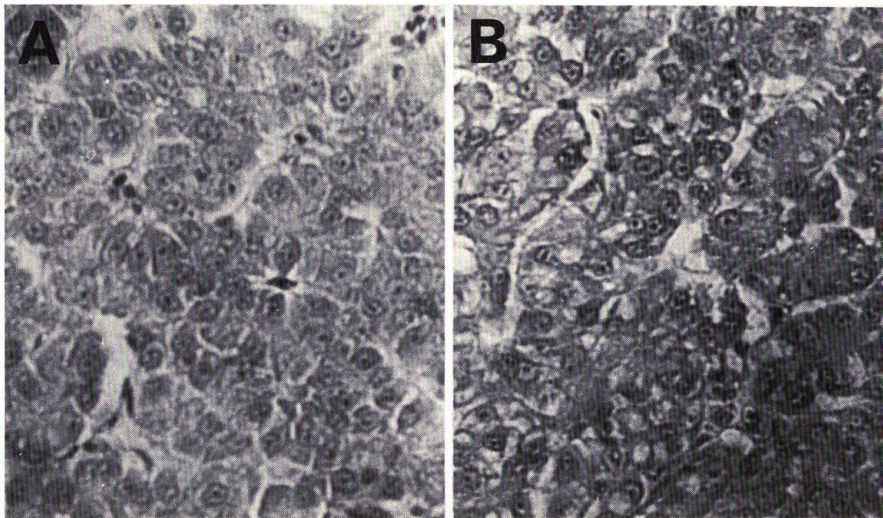


Fig. 31. Liver cells of immature rainbow trout (A) and of estradiol-17 $\beta$ -treated immature fish (B).



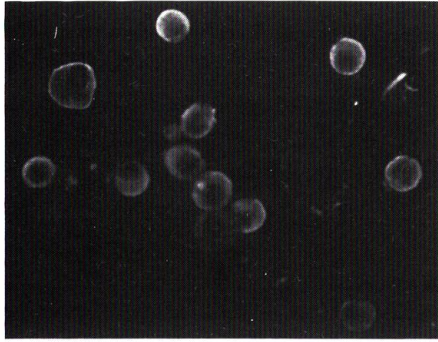


Fig. 32. Localization of female-specific serum protein(s) in fixed rainbow trout liver cells as revealed by immunofluorescence. The cytoplasm is homogeneously stained.

aspects of liver cells after estrogen treatment were quite similar to those of the cells of maturing females. Similar observation have been reported in landlocked red salmon (kokanee) by Ishii (1971) and in ayu by Aida *et al.* (1973b).

13. *Immunofluorescence study on estradiol-treated fish for the detection of female-specific serum proteins in liver cells*

Immunofluorescence staining showed that the cytoplasm of liver cells of estrogen-treated fish was consistently positive to female-specific serum protein(s), but that of normal immature female or male fish (control) was negative (Fig. 32). These results indicate that the liver cells are responsible for synthesis of female-specific protein(s) stimulated by estrogen.

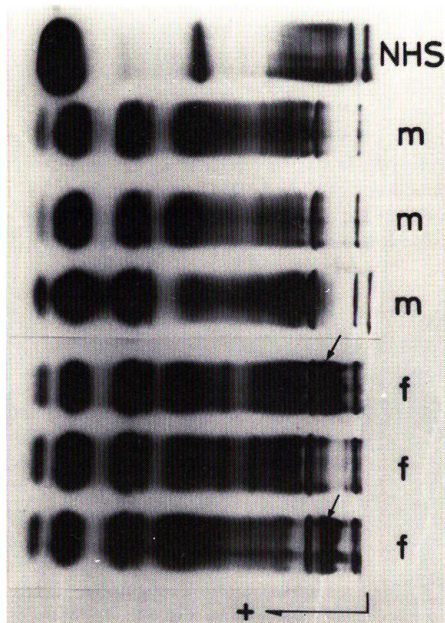


Fig. 33. 7.5% polyacrylamide disc electrophoresis of ripe chum salmon serum. The band indicated by the arrow was observed in some females but not in males. NHS: normal human serum, m: male serum, f: female serum.



**B. Chum salmon**

*1. Sexual differences in serum protein*

Patterns of 7.5% polyacrylamide gel disc electrophoresis of ripe chum salmon showed a band appearing near the origin in some females, but never in males (Fig. 33). This band was seen in 46 out of 65 individual samples from ripe females.

Although much differences between sexes of chum salmon were not seen on the electrophoretic pattern stained for protein, some female samples showed strongly

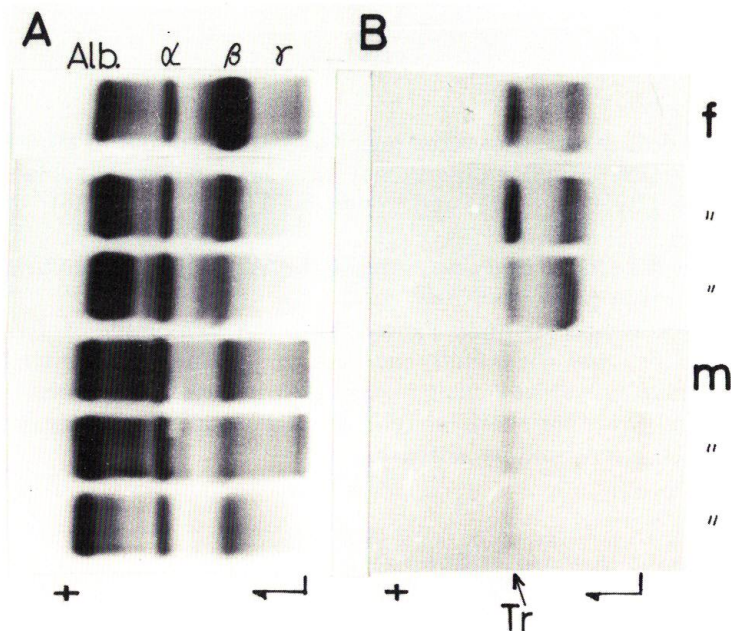


Fig. 34. Electrophoresis of chum salmon sera. A: cellulose acetate electrophoresis (Cellogel), B: autoradiography with  $^{59}\text{Fe}$ ; f: female sera, m: male sera, Tr: transferrin. Twenty five  $\mu\text{l}$  of the serum was added to 10  $\mu\text{l}$  of  $^{59}\text{FeCl}_3$  solution with an activity of 0.1  $\mu\text{Ci}$ , corresponding to 0.003  $\mu\text{g}$  Fe. After incubation, approximately 1  $\mu\text{l}$  of the mixture was subjected to electrophoresis, and the gels were exposed to X-ray films for 2 weeks.

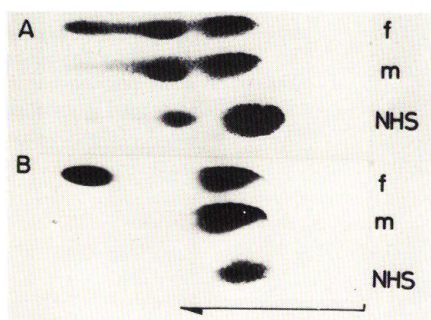


Fig. 35. Thin-layer gel filtration (A) and its autoradiography (B) with  $^{59}\text{Fe}$  on Sephadex G-200, superfine. Twenty five  $\mu\text{l}$  of the serum was added to 10  $\mu\text{l}$  of  $^{59}\text{FeCl}_3$  solution with an activity of 0.5  $\mu\text{Ci}$ . After being incubated for 1 h, 5  $\mu\text{l}$  of the mixture was subjected to thin-layer chromatography. NHS, m, f: same as in Fig. 33.

stained components in  $\beta$ -globulin region (Fig. 34). Autoradiography, however, displayed distinct differences between female and male. Female sera showed two strong radioactive bands, while male sera showed a single weak radioactive band (Fig. 34B). Female sera mobility in one of the two bands was the same as that of male sera. This component is considered to be transferrin.

Thin-layer gel filtration and its autoradiography of the pooled female serum as well as males with  $^{59}\text{Fe}$  with Sephadex G-200, superfine, showed that normal human serum was separated into three spots, M (macroglobulin fraction), G (IgG fraction) and A (albumin fraction) (Fig. 35). Salmon serum of both sexes was also separated into three spots. The spot corresponding to M fraction in the female stained stronger than in the male (Fig. 35A). Autoradiography disclosed that female serum showed two strong radioactive spots, while male serum showed one spot corresponding to the molecular weight of human transferrin (Fig. 35B). These results indicate that the female serum has an iron binding protein of a high molecular weight which is absent in male serum.

The results of immunoelectrophoresis and its autoradiography of salmon serum mixed with  $^{59}\text{FeCl}_3$  demonstrated that two precipitin lines were formed in female serum with anti-salmon FSSP (see Table 1), while male serum did not react with the antiserum (Fig. 36A). Autoradiography of the immunoelectrophoresis with polyvalent antiserum against salmon serum indicated that male serum yielded only one radioactive precipitin line at the  $\alpha$ -globulin region which was commonly observed in female serum (Fig. 36B). This component is considered to be transferrin. On the other hand, the female serum showed another radioactive line which migrated to the  $\beta$ -globulin region. Although two precipitin lines at the  $\beta$ -region were obtained in the immunoelectrophoresis with the anti-salmon FSSP, only one precipitin line formed near the antigen side was capable of binding the radioactive iron (Fig. 36B). This female-specific iron-binding protein was designated as chum salmon FS (see page 15).

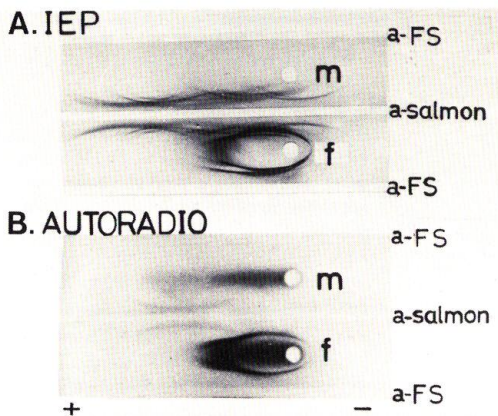


Fig. 36. Immunoelectrophoresis of chum salmon sera. A: protein staining, B: autoradiography with  $^{59}\text{Fe}$ , a-salmon: anti-salmon serum, a-FS: anti-salmon FSSP.

### 2. Purification of female-specific iron-binding serum protein (FS)

A pooled female salmon serum was dialysed against a 0.4 M potassium phosphate buffer, pH 6.8. This sample was then applied to a hydroxylapatite column ( $2.2 \times 23$  cm) which had been equilibrated with the same buffer. Proteins were eluted by stepwise elution using 0.4 M and 1.2 M potassium phosphate buffer, pH 6.8. The fraction eluted by 1.2 M potassium phosphate buffer contained the female-specific serum protein (Fig. 37). The fractions with 1.2 M potassium phosphate buffer was concentrated and then applied to a column of Sepharose 6B ( $2.2 \times 59$  cm) with 0.02 M Tris-HCl buffer, pH 8.0, containing 2% NaCl and 0.1%  $\text{NaN}_3$  (Fig. 38). One symmetric peak was obtained and was collected as the purified FS. Its elution position from the Sepharose 6B column suggested a molecular weight of about 600,000.

### 3. Purification of egg yolk proteins (E1 and E2)

Egg yolk proteins were isolated according to the procedure of Markert and Vanstone (1971) with modification. The eggs collected from ripe salmon were washed with 0.9% NaCl, and homogenized in a mixer. The resulting contents were strained through a Buchner funnel to remove egg membranes, and centrifuged at 30,000 rev/min for 90 min at 4°C. A clear middle layer of a reddish color was

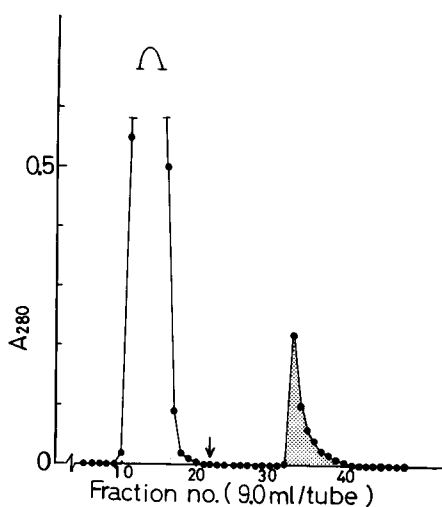


Fig. 37. Chromatography of female chum salmon serum on a hydroxylapatite column. The proteins were eluted with 0.4 M potassium phosphate buffer, pH 6.8 in the first step and in the second step (arrow) the protein was eluted with 1.2 M potassium phosphate buffer, pH 6.8. The fractions in shadowed areas were collected and concentrated for further analysis.  $A_{280}$ : absorbance at 280 nm.

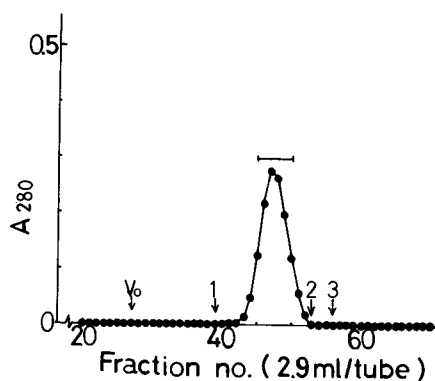


Fig. 38. Elution diagram of a partially purified preparation of chum salmon FS. The fraction containing FS isolated from the hydroxylapatite column was applied to a column of Sepharose 6B ( $2.2 \times 59$  cm) equilibrated with 0.02 M Tris-HCl buffer, pH 8.0 containing 2% NaCl and 0.1%  $\text{NaN}_3$ . The fractions marked with a bar which was eluted at a position of approximately 600,000 molecular weight were collected for further analysis. Marker proteins: 1. human IgM, 2. horse spleen apoferritin, 3. human albumin,  $V_0$ : void volume,  $A_{280}$ : absorbance at 280 nm.



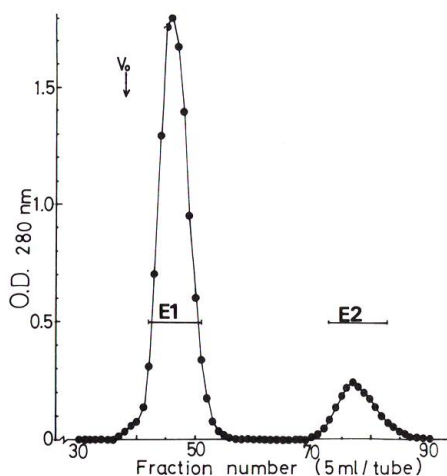


Fig. 39. Gel filtration of egg yolk proteins isolated from chum salmon egg. Column: Sephadex G-200 (2.6×95 cm). Elution buffer: 0.02 M Tris-HCl, pH 8.0, containing 2% NaCl and 0.1% NaN<sub>3</sub>. E1, E2: two major components were designated as E1 and E2 just same as in rainbow trout egg (see Fig. 8). Vo: void volume.

collected. This clear solution was dialysed against distilled water overnight. The precipitate thus formed was separated by centrifugation at 6,000 rev/min for 30 min. The pellet was then dissolved in 0.5 M NaCl, and again dialysed against water. This procedure was repeated twice. The final clear solution was applied to a gel filtration on Sephadex G-200 (2.6×95 cm), and eluted with the same buffer as in case of the Sepharose 6B. Two peaks were obtained from the gel filtration (Fig. 39) and were collected separately. They were tentatively denoted as E1 and E2 of salmon (see page 16).

#### 4. Purity of FS, E1 and E2

The purity of the above-mentioned preparations was assessed by means of cellulose acetate membrane electrophoresis, immunoelectrophoresis and disc electrophoresis. The preparation of FS gave a single band on cellulose acetate membrane electrophoresis (Fig. 40). The mobility of the purified material was faster than that

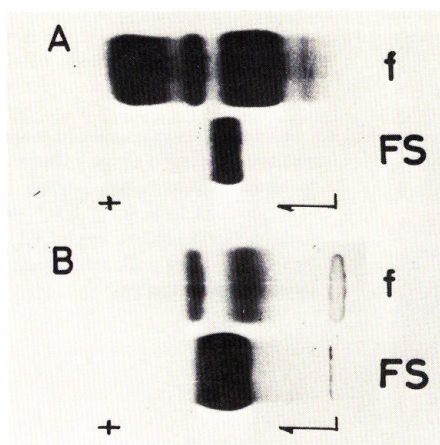


Fig. 40. Electrophoresis of purified chum salmon FS. A: Cellulose acetate membrane electrophoresis of female salmon serum (f) and the purified FS (FS), B: its autoradiography.

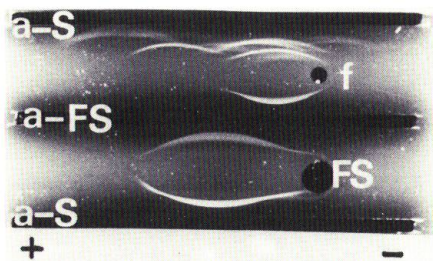


Fig. 41. Immunoelectrophoresis of purified chum salmon FS. a-S: anti-salmon serum, a-FS: anti-salmon FSSP, f: female serum, FS: purified chum salmon FS.

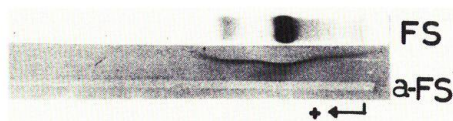


Fig. 42. Disc immunoelectrophoresis of purified chum salmon FS. The sample was subjected first to disc electrophoresis in 5% polyacrylamide gel which was subsequently implanted in 1.2% agarose gel, putting anti-salmon FSSP into the trough. The protein (FS) in polyacrylamide gel diffused into agarose gel and formed the precipitin line. FS: purified FS, a-FS: anti-salmon FSSP.

of the component in the original sera. This change in mobility of the purified material was confirmed by an immunoelectrophoresis with anti-salmon FSSP (Fig. 41). The purified preparation of FS retained the iron binding activity (Fig. 40B). Immunoelectrophoresis produced a single precipitin arc with polyvalent anti-salmon serum (Fig. 41). Disc electrophoresis of a preparation of FS displayed one major band with a minor band which migrated faster than the major band. The two bands, major and minor, showed the same antigenicity forming a bow formation of the precipitin line by disc immunodiffusion technique (Fig. 42). These bands were positively stained for both lipids and carbohydrates (not shown).

Each preparation of the egg yolk protein, E1 and E2, gave a sharp single band in cellulose acetate membrane electrophoresis (Fig. 43) and showed a single precipitin line in immunoelectrophoresis using polyvalent anti-salmon serum or anti-salmon eggs (Fig. 44). On disc electrophoresis, E1 gave a sharp single band in

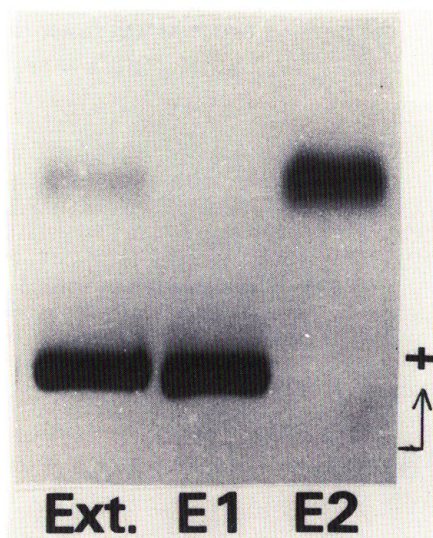


Fig. 43. Cellulose acetate membrane electrophoresis (Cello-gel) of egg yolk proteins in chum salmon. Ext.: water insoluble proteins from 0.9% NaCl extraction of chum salmon eggs. E1, E2: two purified egg yolk components isolated by gel filtration.



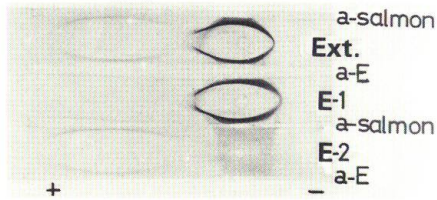


Fig. 44. Immunoelectrophoresis of 0.9% NaCl extracts (Ext.), E1 (E-1) and E2 (E-2) of chum salmon egg. The antisera used were anti-salmon serum (a-salmon) and anti-salmon eggs (a-E).

5% polyacrylamide gel and was stained positively for lipids and carbohydrates, whereas E2 gave a rather broad band in 7.5% gel (not shown). The broad band of E2, however, was negative for lipid and carbohydrate stainings.

##### 5. SDS polyacrylamide gel electrophoresis

The electrophoretic patterns of FS, E1 and E2 on SDS polyacrylamide gel were quite similar to those observed in FS, E1 and E2 of rainbow trout (Fig. 17). That is, FS displayed one main band of a molecular weight of approximately 220,000–250,000. E1 showed the main band corresponding to a molecular weight of about 130,000 and were split into two subunits (approximately mol. wt. 90,000 and 15,000) after reduction with 2-mercaptoethanol. E2 revealed a single band corresponding to 30,000 and this band was converted into 15,000 following the reduction.

##### 6. Antigenic relation between FS, E1 and E2

Figure 45 shows the pattern of the three purified proteins on double immunodiffusion in 1.2% agarose gel against anti-salmon FSSP. FS formed a spur over the two egg yolk proteins, E1 and E2, and the precipitin lines of each egg yolk protein crossed with each other. The results indicated that FS contains both E1 and E2 antigens in the molecules.

##### 7. Physicochemical analyses of FS

Ultracentrifugal analysis was performed for FS. The infinite dilution value of the sedimentation coefficient,  $S_{20,w}^0$ , was 12.5 (Fig. 46) and the diffusion coefficient was 1.87 for  $D_{20,w}$ . The partial specific volume was calculated from amino acid composition. The molecular weight calculated from S and D values in the Svedberg equation, employing a partial specific volume, 0.740, gave a value of 630,000 for FS.

The extinction coefficient ( $E_{280nm}^{1\%}$ ) determined in 0.15 M NaCl was 8.67.

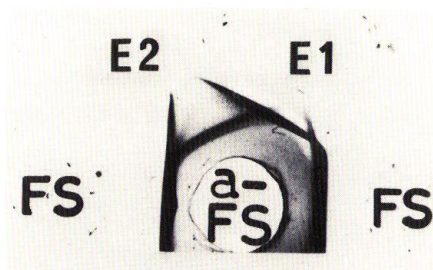


Fig. 45. Precipitin reaction of FS, E1 and E2 in chum salmon. FS: female-specific iron-binding serum protein, E1 and E2: fractions of egg yolk proteins. a-FS: anti-salmon FSSP.

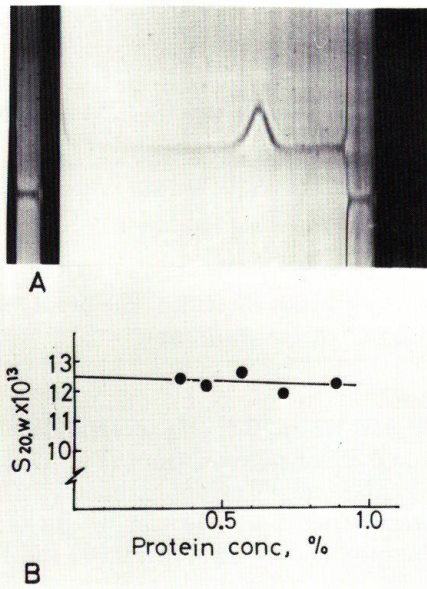


Fig. 46. Ultracentrifugation of purified chum salmon FS. (A) Schlieren pattern of the purified FS at a concentration of 0.9% in 0.15 M NaCl. Photograph was taken 24 min after reaching 56,100 rpm. (B) Concentration dependency of the sedimentation rate.

The isoelectric point (pI) of FS was 5.4, when determined by isoelectrofocusing. Several physicochemical data are summarized in Table 4.

The amino acid composition is shown in Table 5. The values for chum salmon FS and rainbow trout FS (Table 2) were quite close to each other.

### C. Japanese eel

#### 1. Sexual differences in serum proteins by cellulose acetate membrane electrophoresis

Typical electrophoretic patterns of silver migrating forms of male eels and of female eels treated with pituitary glands of salmon are shown in Fig. 47. Distinct differences between sexes were seen in the patterns, revealing that, in females, a strong protein band was concentrated in the  $\beta$ -region whereas, in males, the serum proteins were rather diffusely distributed from albumin to the  $\gamma$ -region.

Table 4. Physicochemical properties of purified chum salmon female-specific iron-binding serum protein (FS)

Sedimentation coefficient ( $S_{20,w}^0$ )	12.5S
Diffusion coefficient ( $D_{20,w}$ )	$1.87 \times 10^{-7} \text{ cm}^2 \cdot \text{S}^{-1}$
Partial specific volume (V)	0.740
Molecular weight ( $M_{s,D}$ )	630,000
(Gel filtration)	600,000
Extinction coefficient ( $E_{280\text{nm}}^{1\%}$ )	8.67
Isoelectric point (pI)	5.34

Table 5. Amino acid composition of chum salmon female-specific iron-binding serum protein (FS) expressed as moles/100 moles of amino acid.

Residue	Residue percent
	Salmon FS
Asp	8.70
Thr	5.08
Ser	7.16
Glu	11.08
Pro	6.04
Gly	4.50
Ala	10.66
Cys/2	1.46
Val	7.74
Met	2.39
Ile	5.65
Leu	8.74
Tyr	2.87
Phe	3.98
His	2.23
Lys	7.48
Arg	4.24
Total	100.00

## 2. Antigenic comparison between serum and egg yolk proteins

The antiserum to female serum protein absorbed with male serum (anti-eel FSSP) lost the reactivity male serum, but reacted with female serum, forming a single precipitin line as shown (Fig. 48). This immunodiffusion pattern indicated the presence of a female-specific serum protein which is not present in the male serum.

The anti-eel FSSP reacted also with egg yolk proteins extracted with 0.9% NaCl

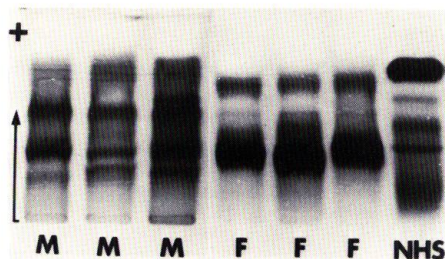


Fig. 47. Typical electrophoretic patterns of silver migrated forms of male Japanese eel (M) and of female Japanese eel (F) serum treated with salmon pituitary glands. NHS: normal human serum.



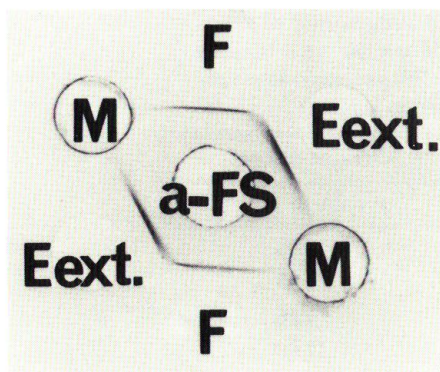


Fig. 48. Precipitin reaction of serum and egg yolk proteins of Japanese eel against anti-eel FSSP. M: male serum, F: female serum, Eext.: egg yolk proteins extracted with 0.9% NaCl, a-FS: anti-eel FSSP (see Table 1).

(Fig. 48). The latter precipitin line fused completely with the line formed with the female serum. This demonstrated the presence of an egg yolk protein which is immunologically identical to the female-specific serum protein. A reverse experiment using an antiserum to egg yolk proteins (anti-eel eggs) also demonstrated the complete identity between the female-specific serum protein and the egg yolk protein.

### 3. Iron-binding property of serum and egg yolk proteins

Radioactive iron ( $^{59}\text{Fe}$ ) was added to male and female sera and to a solution of egg extract, and the precipitin reaction was examined in agarose gel. The precipitin lines formed were examined by autoradiography. A rabbit antiserum to whole serum to female eel (anti-eel serum) formed one radioactive precipitin line with male serum, whereas female serum showed two lines (Fig. 49). The fused line, which shows the presence of iron-binding protein in sera of both sexes, was considered to be of serum transferrin. The female serum formed another line with anti-eel serum and this line completely fused with a line which appeared between female serum and anti-eel FSSP, and also fused completely with the line formed between anti-eel FSSP and egg yolk proteins. These results indicated that the female-specific serum protein and a protein in egg yolk shared antigenic identities which have an iron binding capacity. This female-specific iron-binding protein in serum was designated as FS of Japanese eel (see page 15 and 29).

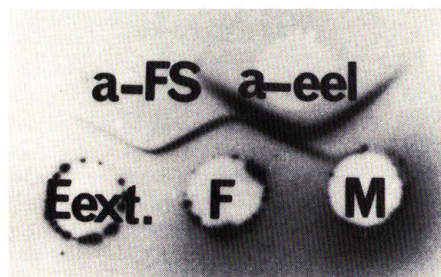


Fig. 49. Autoradiography of a gel diffusion reaction between the Fe-binding proteins in serum or egg yolk proteins of the Japanese eel, and rabbit antisera. a-eel: anti-eel serum. M, F, Eext., a-FS: same as in Fig. 48.

#### 4. Purification of female-specific iron-binding serum protein (FS)

Pooled female serum was dialysed against a 0.2 M potassium phosphate buffer, pH 6.8. This sample was then applied to a hydroxylapatite column which had been equilibrated with the same buffer. Proteins were eluted by stepwise elution using 0.2 M, 0.4 M and 1.2 M potassium phosphate buffer, pH 6.8. The eluate following washing of the column with 1.2 M potassium phosphate buffer, contained FS. FS was collected, concentrated and then applied to Sephadex G-200 gel filtration. The main peak which was eluted at the position near the void volume was collected. Further purification of FS was accomplished using Sepharose 6B gel filtration in Tris-HCl buffer. The second peak was collected as the purified FS. The presence of FS during these purifications was assessed by the Mancini method using the absorbed antiserum (anti-eel FSSP).

#### 5. Purification of egg yolk protein

An extract of egg yolk protein was first applied on a Sepharose 6B column. The main peak was close to the position where FS came out. It was collected and was further gel-filtrated through Sephadex G-200. Two peaks, a small peak at void volume and a large peak immediately after the small peak, were obtained. The second large peak was collected as a purified preparation of egg yolk protein which was related to FS.

#### 6. Purity of preparation of FS and its related egg yolk protein

The purity of both preparations was assessed by means of immunoelectrophoresis, disc electrophoresis and SDS polyacrylamide gel electrophoresis. Both preparations showed a single precipitin arc at  $\beta$ -region with polyvalent antisera raised to appropriate starting materials (Fig. 50). Disc electrophoresis of both the preparations displayed a single homogeneous band at the similar position (Fig. 51). SDS polyacrylamide (7.5%) gel electrophoresis of purified FS revealed one band (Fig. 52). However, preparation from eel yolk proteins disclosed a major band corresponding to the band of FS and two minor bands which migrated faster than the

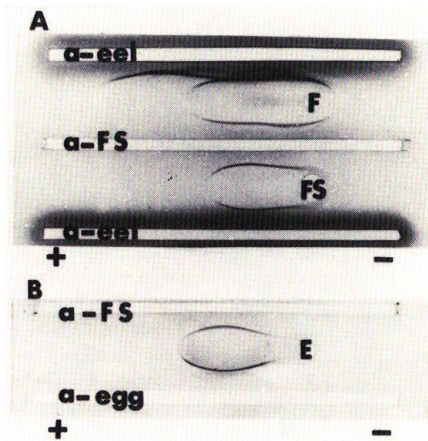


Fig. 50. Immunoelectrophoresis of purified FS (A) and egg yolk protein (B) of Japanese eel. FS: female-specific iron-binding serum protein, E: FS related egg yolk protein, a-egg: rabbit antiserum against egg yolk proteins extracted with 0.9% NaCl (anti-eel eggs), F, a-eel, a-FS: same as in Fig. 49.



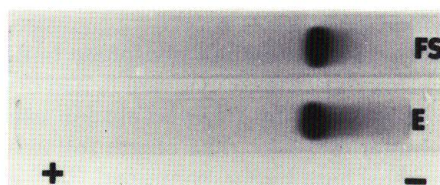


Fig. 51. Disc electrophoresis in 5% polyacrylamide gel of purified FS and its related egg yolk protein of Japanese eel. FS, E: same as in Fig. 50.

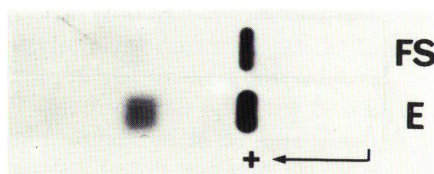


Fig. 52. SDS polyacrylamide gel electrophoresis of purified FS and its related egg yolk protein of Japanese eel. FS, E: same as Fig. 50.

major band. These patterns in SDS polyacrylamide gel electrophoresis were not altered by the reduction with 2-mercaptoethanol.

### 7. Molecular weight

The molecular weights estimated by gel filtration of Sephadex G-200 were about 350,000 for both FS and its related egg yolk protein (Fig. 53).

The molecular weight estimated with SDS polyacrylamide gel electrophoresis was 85,000 for FS as well as for the major band of its related egg yolk protein. Molecular weights of the two minor bands were assessed by 10% polyacrylamide gel with SDS and were estimated to be about 31,000 and 25,000. The protein ratio of these three components, which were found on the SDS gel electrophoresis of the purified egg yolk protein (Fig. 52), was determined by scanning the gel to be 6 : 1 : 1 from the major band to the minor two bands. When the molecular weights of the components were taken into account, a molar ratio of 2 : 1 : 1 was estimated for the three components.

### 8. Physicochemical analyses of FS and egg yolk protein

Purified preparations of FS and egg yolk protein were clearly stained for lipids and carbohydrates on disc electrophoresis. The results indicated that these proteins were glycolipoproteins.

Total amounts of phosphorus in the purified FS and egg yolk protein were determined to be 0.71 w/w % and 0.63 w/w %, respectively.

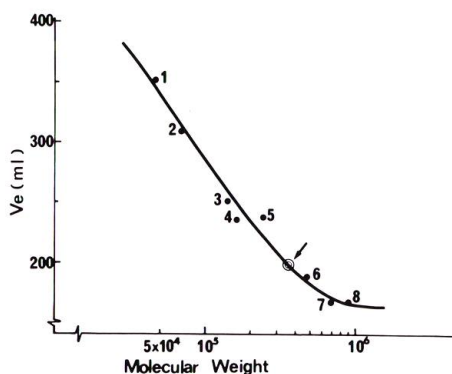


Fig. 53. Estimation of molecular weight by Sephadex G-200 gel filtration. Both FS and its related egg yolk protein of Japanese eel are eluted at the position of the open circle (arrow). The samples used for calibration are indicated by dark circles. 1: ovalbumin, 2: bovine serum albumin, 3: alcohol dehydrogenase, 4: human IgG, 5: bovine liver catalase, 6: apoferritin, 7: bovine thyroglobulin, 8: human IgM, Ve: elution volume.

Table 6. Amino acid composition of female-specific iron-binding serum protein (FS) and its related egg yolk protein (E) of Japanese eel expressed as moles/100 moles of amino acid.

Amino acid	Residue	
	FS	E
Asp	7.33	6.94
Thr	5.32	5.52
Ser	5.78	4.08
Glu	11.75	12.13
Pro	4.73	5.39
Gly	5.59	5.08
Ala	18.00	18.56
Cys/2	0.64	0.44
Val	6.08	5.93
Met	2.78	3.00
Ile	4.91	5.61
Leu	7.84	8.74
Tyr	2.79	2.89
Phe	3.79	3.75
His	1.97	1.83
Lys	5.92	5.91
Arg	4.79	4.17
Total	100.01	99.97

Table 6 shows the amino acid composition of these two purified proteins. The values of amino acids of FS and the egg yolk protein were quite close to each other except for only serine.

An alanine spot was identified as the  $\text{NH}_2$ -terminal amino acid for both purified FS and its related egg yolk protein on thin-layer chromatography of the dansylated samples.

Extinction coefficients ( $E_{1\text{cm}, 280\text{nm}}^{1\%}$ ) were determined for the purified proteins in 0.1 N NaOH. The values for FS and its related egg yolk protein were 9.04 and 7.60, respectively.

#### D. Medaka

##### 1. Properties of antiserum against egg yolk extracts

The polyvalent antiserum to egg yolk proteins (designated as anti-medaka eggs) was prepared by immunizing rabbits with 0.9% NaCl extracts of ovulated eggs.

In immunoelectrophoresis, egg yolk proteins revealed three or four precipitin lines which appeared very close with each other. The crossed immunoelectrophoresis, however, clearly demonstrated the presence of four components reacting with

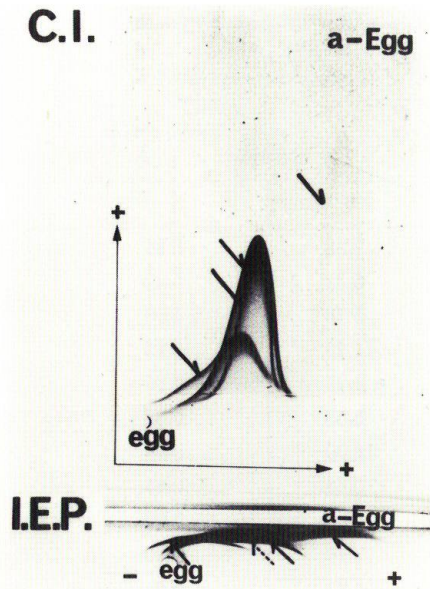


Fig. 54. Immunoelectrophoresis (I.E.P.) and crossed immunoelectrophoresis (C.I.) of egg yolk proteins of medaka. Antigen: 4  $\mu$ l of 0.9% NaCl extracts of ovulated medaka eggs. The antiserum used was the polyvalent antiserum to medaka egg yolk extracts (a-Egg) in both electrophoresis. First dimension (C.I.): 90 min, 10 V per cm. Anode to the right. Antibodies: 0.4 ml antiserum plus 6 ml agarose. Second dimension: overnight (20 h), 3 V per cm, anode at the top. Staining: Coomassie brilliant blue R-250.

the anti-medaka eggs (Fig. 54).

Autoradiography of crossed immunoelectrophoresis with a polyvalent anti-medaka eggs, indicated that egg yolk proteins yielded one clear radioactive precipitin line and another faint radioactive line. This means that one or two iron-binding proteins exist in egg yolk (Fig. 55).

The anti-medaka eggs were applied to the reaction with medaka serum. The antiserum reacted with the female serum forming more than two lines, whereas it did not react with the male serum (Fig. 56). The same tests were performed by means of crossed immunoelectrophoresis. The male serum did not show any reaction with the anti-medaka eggs whereas the female serum reacted with the antiserum forming three distinct precipitin lines (Fig. 57A). These results indicate that the anti-medaka eggs have similar characteristics to the antiserum of the female-specific

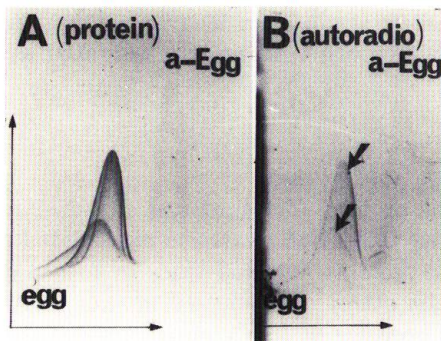


Fig. 55. Crossed immunoelectrophoresis (A) and its autoradiography (B). Antigen: egg yolk proteins of medaka mixed with  $^{59}\text{FeCl}_3$ . Antibody: anti-medaka eggs. The arrows show the radioactive precipitin lines.



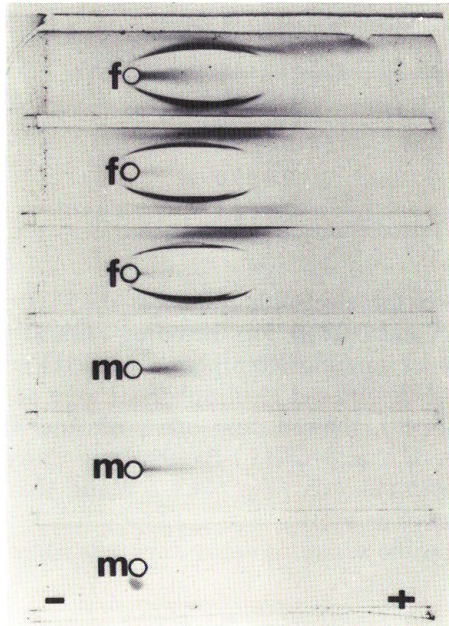


Fig. 56. Immunoelectrophoresis of female and male medaka sera in the breeding season. Each trough was filled with anti-medaka eggs. f: female, m: male.

serum proteins as described for trout, salmon and eel.

The female serum from the breeding season yield two radioactive precipitin lines in the autoradiography combined with crossed immunoelectrophoresis (Fig. 57B). This indicates that female fish in the breeding season contain two female-specific iron-binding proteins in blood serum.

## 2. Studies on female-specific serum proteins during maturation

Fishes used in these experiments were classified into three groups, i.e., "immature", "breeding", and "sexually inactive" (Table 7). Immature fish were selected by histological examinations of the ovary: fish which had not spawned before and where the oocytes were in the yolk vesicle stage were classified as "immature".

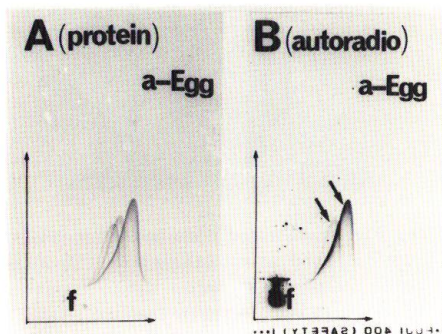


Fig. 57. Crossed immunoelectrophoresis (A) and its autoradiography (B). Antigen: female serum of medaka in breeding season mixed with  $^{59}\text{FeCl}_3$ . Antibody: anti-medaka eggs. After staining and dried, the plate was exposed to X-ray films.

Table 7. The gonadosomatic index (G.S.I.) of three groups of medaka.

Group	G.S.I. (%)
“immature”	0.5- 2.1
“breeding”	9.5-12.0
“sexually inactive”	1.8- 2.4

The amount of serum samples used for electrophoresis was 0.4  $\mu$ l for “immature”, 0.08  $\mu$ l for “sexually inactive” and 0.02  $\mu$ l for “breeding” fish (Fig. 58). Concentration of antiserum used for crossed immunoelectrophoresis was 0.1 ml per 6 ml of agarose. Two different patterns of the crossed electrophoresis were obtained for the immature serum. One immature fish showed only one peak and another immature fish formed two peaks (Figs. 58A and 58B). Serum from “breeding” formed three peaks on the crossed electrophoresis (Fig. 58C). While that from “sexually inactive” developed two peaks (Fig. 58D).

Two proteins out of three female-specific serum proteins were demonstrated to

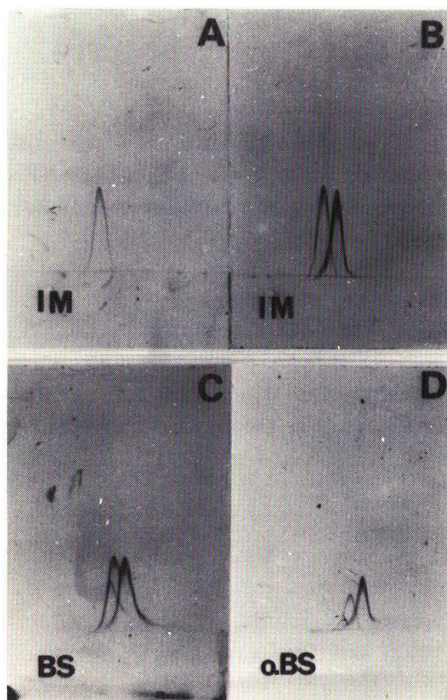


Fig. 58. Crossed immunoelectrophoresis of female medaka serum of various maturations. IM: “immature” (A, B), BS: “breeding”, o.BS: “sexually inactive”.

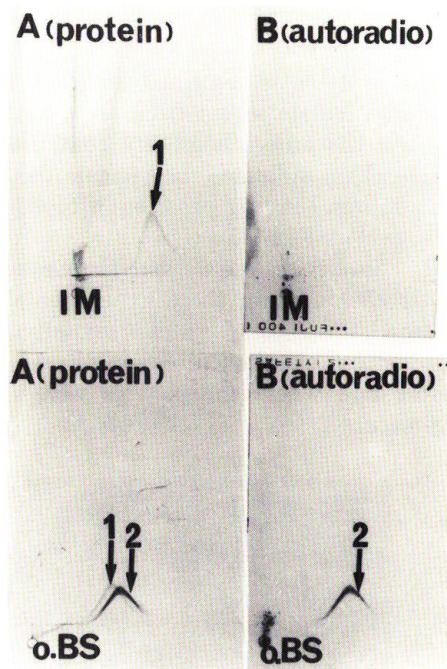


Fig. 59. Crossed immunoelectrophoresis (A) and its autoradiography (B) of female medaka sera. IM: “immature”, o.BS: “sexually inactive”.



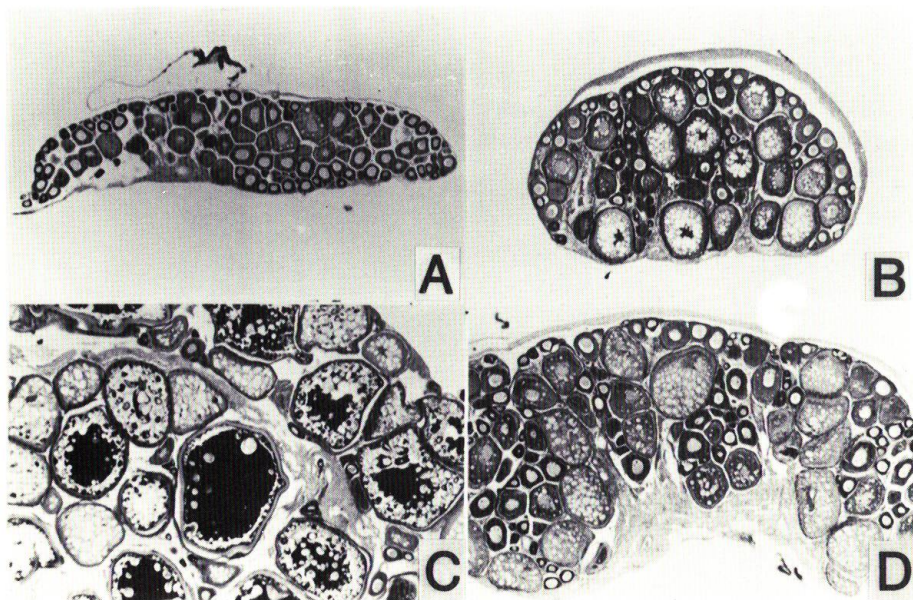


Fig. 60. Histology of the ovary at various stages in medaka. A, B: "immature", C: "breeding", D: "sexually inactive".

have iron-binding activity (Fig. 57). Presence of such an activity was investigated for the female-specific protein of "immature" and "sexually" inactive" fish. The female-specific protein which appeared in immature fish as a single peak had no such an activity, while one of the female-specific serum proteins from sexually inactive fish showed the activity (Fig. 59).

The histological examination of the ovary was made to find whether or not the changes in pattern of the crossed immunoelectrophoresis correlated to the change in histology of the ovary. Oocytes of immature fish, in which only one female-specific serum protein was observed, were found to be in the early phase of yolk vesicle stage (Fig. 60A). On the other hand, oocytes of another immature fish, in which two female-specific serum proteins were seen, were found to be in the middle or late phase of yolk vesicle stage (Fig. 60B). In the fish at the breeding season, oocytes were at the tertiary yolk stage (Fig. 60C). Oocytes of sexually inactive females were found to be in the late phase of yolk vesicle stage, similar to the immature fish with two female-specific serum proteins (Fig. 60D). The results of histological study indicated that the occurrence of female-specific serum proteins corresponded to the stage of ovarian maturation.

### 3. *Antigenic comparison female-specific serum proteins and egg yolk proteins*

In order to compare the antigenicity between female-specific serum proteins and egg yolk proteins, the tandem-crossed immunoelectrophoresis technique was employed.



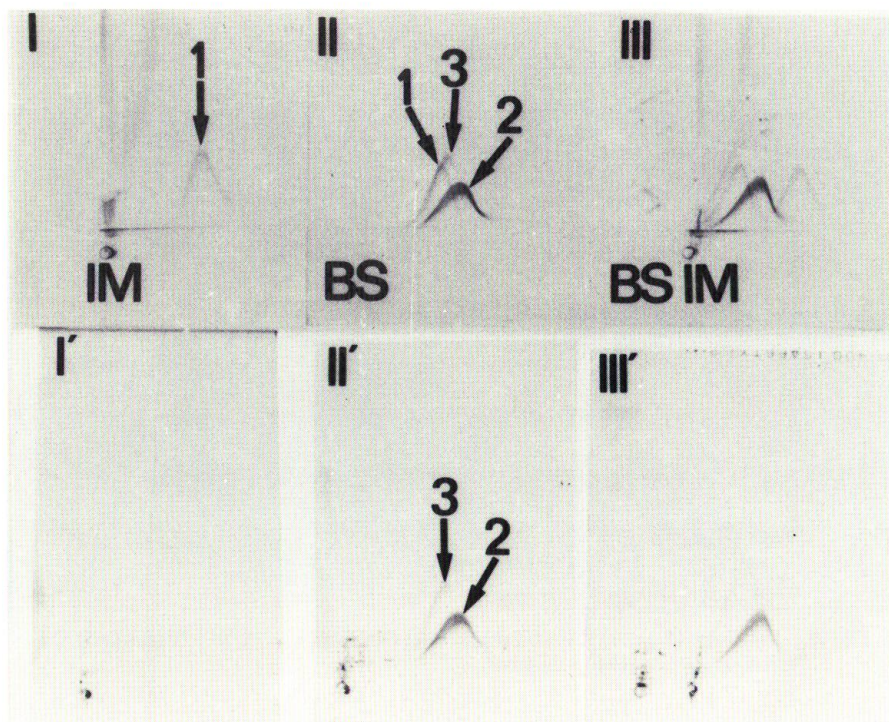


Fig. 61. Tandem-crossed immunoelectrophoresis (I, II, III) and their autoradiography (I', II', III') of female medaka serum. Antigen: immature (IM) and breeding (BS) female medaka serum mixed with  $^{59}\text{FeCl}_3$  (III). Serum sample and 0.9% NaCl (I and II). First dimension: The first dimensional run was done at 10 V/cm for 90 min. Anode to the right. Antibodies: anti-medaka eggs. Second dimension: The immunoelectrophoretic run was done at 3 V/cm for 20 h (anode at top).

The patterns of tandem-crossed immunoelectrophoresis for the comparison between immature females with only one female-specific serum protein and breeding females with three female-specific serum proteins, and autoradiography of the same plate are shown (Fig. 61). Three female-specific serum proteins observed in breeding females are tentatively named FS1, FS2 and FS3, respectively (Fig. 61-II). FS1 did not produce the radioactive precipitine line, but FS2 and FS3 developed the radioactive precipitine lines (Fig. 61-II'). One precipitine line in immature females at the early phase of yolk vesicle stage fused completely with the line of FS1 of breeding females (61-III). These results indicated that one female-specific serum protein in immature fish shares antigenic identities with FS1.

Figure 62 shows the tandem-crossed immunoelectrophoresis combined with autoradiography of breeding female sera and sexually inactive females. Two female-specific serum proteins, one of which bound iron, were seen in the sexually inactive females (Fig. 62-I, I'). This iron-binding female-specific serum protein from sexually inactive females completely fused with the line of FS2 observed in breeding females (Fig. 62-III'). The other line, which had no iron-binding prop-

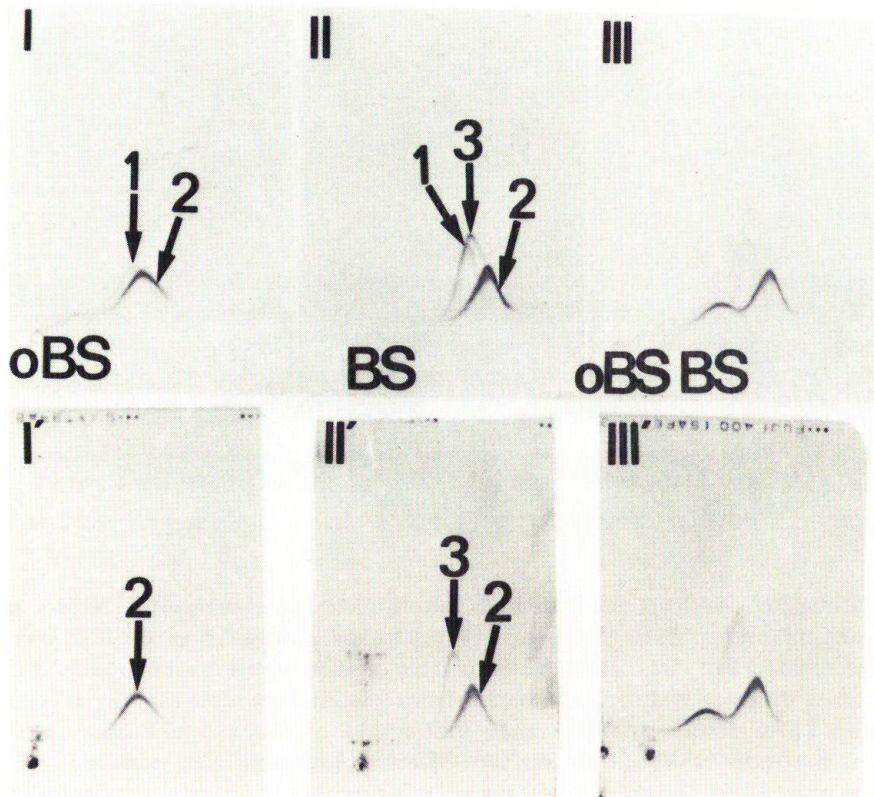


Fig. 62. Tandem-crossed immunoelectrophoresis (I, II, III) and their autoradiography (I', II', III') of female medaka serum. Antigen: female medaka sera, of "sexually inactive" (o.BS) and "breeding" (BS).

erty, was identical with FS1 (Fig. 62-III). The two lines observed in the immature fish at the middle or late phase of yolk vesicle stage (Figs. 58B and 60B), were identified by this procedure to correspond to FS1 and FS2 (not shown).

Figure 63 shows the tandem-crossed immunoelectrophoresis of female serum in the breeding season and egg yolk proteins with anti-medaka eggs. Three female-specific serum proteins in the breeding season named FS1, FS2 and FS3 were identified with egg yolk proteins.

#### 4. Female-specific serum proteins in steroid hormone-treated fish

Fish were treated with four different steroid hormones (methyltestosterone, testosterone, ethinylestradiol, and estradiol-17 $\beta$ ) and serum samples were collected at 5, 10, 20 and 30 days after the initial oral administration of each hormone. The serum from the control group developed two precipitin peaks with anti-medaka eggs (Fig. 64). The serum from androgen (methyltestosterone and testosterone)-treated fish also produced two precipitin peaks which were similar to those found in the control group (Figs. 65 and 66). On the other hand, the serum collected from both



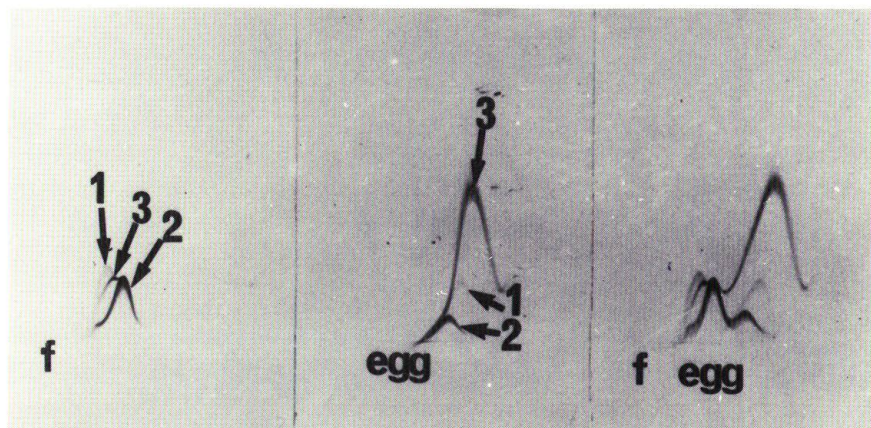


Fig. 63. Tandem-crossed immunoelectrophoretic comparison of serum and egg in medaka. Antigen : female medaka serum in breeding season (f), egg yolk extracts of medaka (egg).

ethinylestradiol- and estradiol-treated fish formed three precipitin peaks, all of which being higher than the peaks of the other groups (Figs. 67 and 68). In estrogen-treated fish, the height of the female-specific serum protein peaks did not show much differences during the period from 5 to 30 days after the initial administration. These results indicate that production of three female-specific serum proteins are induced by administration of ethinylestradiol and estradiol-17 $\beta$  and that the rate of synthesis of the specific proteins reaches its maximum within 5 days.

Production of the three female-specific serum proteins was also induced in male fish after administration of estrogen (Fig. 69). Two out of the three female-specific serum proteins induced by the hormone treatment showed the iron binding activity (Fig. 70).

Table 8 summarizes the occurrence of the female-specific serum proteins in egg yolk extracts and in serum of medaka at various maturational stages.

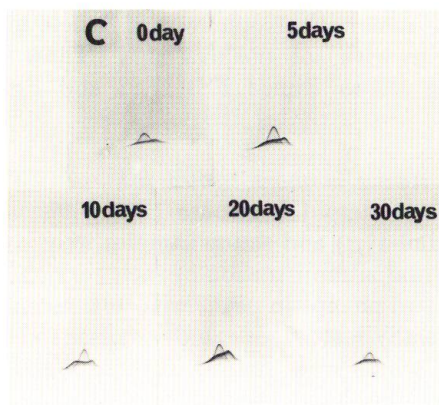


Fig. 64. Crossed immunoelectrophoresis of sera of female medaka of the control group.

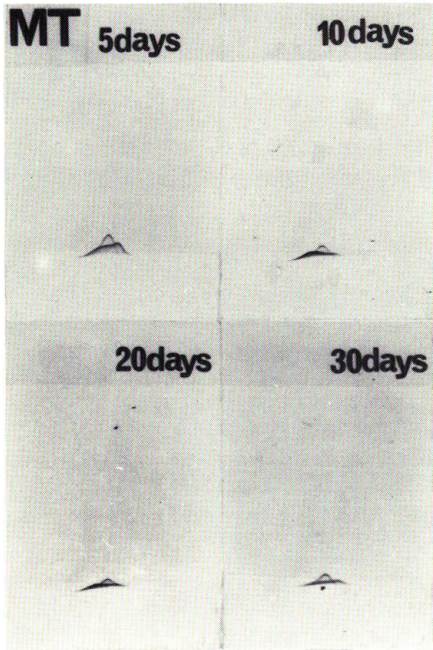


Fig. 65. Crossed immunoelectrophoresis of sera from methyltestosterone-treated medaka.

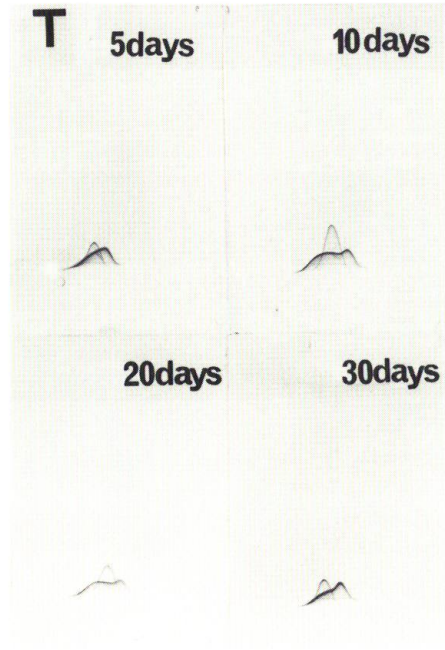


Fig. 66. Crossed immunoelectrophoresis of sera from testosterone-treated medaka.

Table 8. Occurrence of the female-specific serum proteins in egg yolk proteins and in serum of medaka at various maturational stages.

	Female-Specific Serum Proteins		
	FS1	FS2 (iron-binding)	FS3 (iron-binding)
“immature” type 1*	+	—	—
“immature” type 2*	+	+	—
“sexually inactive”	+	+	—
“breeding”	+	+	+
estrogen-treated fish	+	+	+
male fish	—	—	—
egg yolk proteins	+	+	+**

\* Oocytes of type 1 “immature” were found to be in the early phase of yolk vesicle stage and those of type 2 were found to be in the middle or late phase of yolk vesicle stage (see page 43).

\*\* uncertain iron-binding activity.



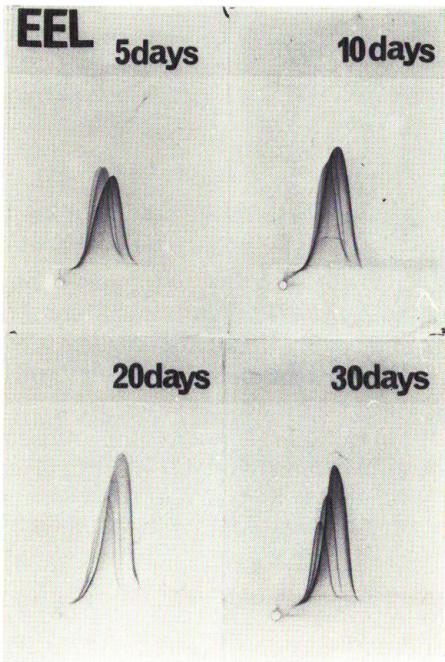


Fig. 67. Crossed immunoelectrophoresis of sera from ethinylestradiol-treated medaka.

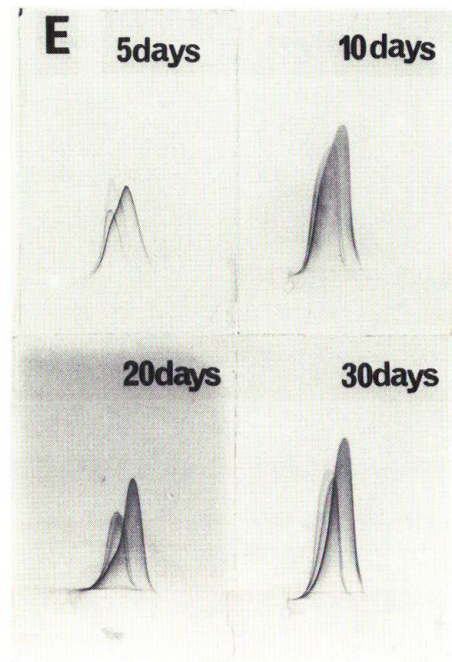


Fig. 68. Crossed immunoelectrophoresis of sera from estradiol-17β-treated medaka.

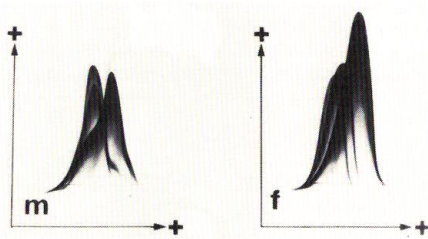


Fig. 69. Crossed immunoelectrophoresis of sera from male (m) and female (f) medaka after administration of ethinylestradiol. Samples were collected at 10 days after hormone treatment.

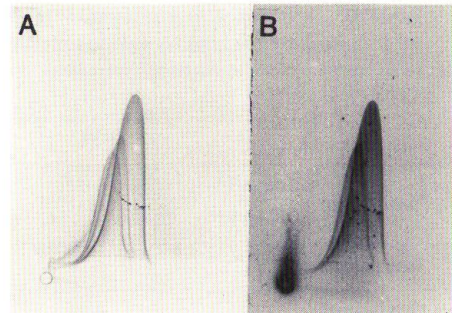


Fig. 70. Crossed immunoelectrophoresis (A) and its autoradiography (B). Antigen: male medaka serum after administration of estradiol-17β mixed with <sup>59</sup>FeCl<sub>3</sub>.

## VI. Discussion

Previous studies on the protein vitellogenin in oviparous vertebrates have revealed some characteristic properties which include the following: (1) protein vitellogenin appears in serum of the female during vitellogenesis (Drilhon and Fine, 1963; Thurston, 1967; Crim and Idler, 1978) and, (2) can be induced in male and immature female sera by the administration of estrogen and/or a pituitary extract (Follett and Redshaw, 1968; Campbell, 1978). (3) It also has a characteristic capability of binding calcium as a glycolipophosphoprotein complex (Wallace, 1970; Redshaw and Follett, 1971; Ansari *et al.*, 1971), (4) it may be a possible precursor of egg yolk proteins, phosvitin and lipovitellin (Wallace and Jared, 1968, 1969; Bergink and Wallace, 1974; Deeley *et al.*, 1975; Christmann *et al.*, 1977), and (5) it has a molecular weight of 450,000-500,000 and consists of two identical polypeptide chains, each of which has a molecular weight of about 200,000-240,000 (Bergink and Wallace, 1974; Deeley *et al.*, 1975; Christman *et al.*, 1977; Gordon *et al.*, 1977; Penning *et al.*, 1977).

In the present study, the antigen which was isolated from female serum in rainbow trout, chum salmon and Japanese eel, satisfied several of the above-cited properties of vitellogenin; (1) it is a female specific protein, which is (2) induced in the serum of males and immature females by the administration of estrogen (trout) or a pituitary extract (eel). It (3) has carbohydrates, lipids and phosphorus in the molecule (glycolipophosphoprotein) (trout and eel), (4) the same or similar antigenic components is found in egg yolk and, (5) it is a macromolecular protein, having a subunit structure. It is therefore highly likely that the antigen in trout, salmon and eel serum corresponds to vitellogenin and that the antigen found in egg yolk may be the equivalent of the so-called phosvitin-lipovitellin complex.

Some physicochemical properties of fish, amphibian and avian vitellogenins, which were drawn from the results of this and other previous works, have been summarized in for comparison (Table 9).

One of the female-specific serum proteins (vitellogenin) which was seen in female trout, salmon and eel possessed iron-binding capacity (Figs. 4, 36 and 49). Wallace (1970) noted that vitellogenin of South African clawed toad (*Xenopus laevis*) can strongly bind circulating iron. Possibly, this iron-binding capacity is an important characteristic of vitellogenin. Normally, iron transport is carried out by transferrin, but, when the female fish is carrying eggs which are presumed to require a large amount of iron for vitellogenesis, vitellogenin may play an important role in iron transportation. It was observed in the domestic fowl during egg laying and after estrogen administration (Morgan, 1975) that plasma iron bound to transferrin might be taken up by the liver, incorporated into vitellogenin which was in turn secreted into the plasma to transfer iron to the ova. It is quite likely that the same mechanism is involved in the transport of iron in fish.

It has been proposed by Bergink and Wallace (1974) that amphibian vitellogenin with a molecular weight of 450,000 consists of two identical polypeptides, about 200,000 in molecular weight, that can be cleaved by protolysis to yield phosphorylated polypeptides of approximately the same size as phosvitin and lipovitellin. Although vitellogenin of avian (White leghorn chicken) has been

Table 9. Some physicochemical properties of fish, amphibian and avian vitellogenins

	Rainbow trout	Chum salmon	Japanese eel	Amphibian <sup>a)</sup>	Avian <sup>b)</sup>
S <sub>20,w</sub> <sup>0</sup>	10.2S	12.5S	n.d.		
Molecular weight (Gel-filtration)	600,000	600,000	350,000	470,000-540,000 <sup>c)</sup> 450,000 <sup>d)</sup>	450,000-500,000 <sup>e)</sup>
Phosphorus (w/w%)	0.68	n.d.	0.71	1.4	3.4
Carbohydrate (staining)	+	+	+	0.3 w/w%	
Lipid (staining)	+	+	+	12 w/w%	
Fe-binding	+	+	+	+	
Subunit	22-240,000	22-250,000	85,000	200,000	235,000-240,000
Amino acid					
Asp	8.40	8.70	7.33	9.23	8.67
Thr	4.95	5.08	5.32	5.48	4.82
Ser	7.52	7.16	5.78	11.50	18.30
Glu	11.51	11.08	11.75	13.70	9.92
Pro	5.21	6.04	4.73	4.87	4.72
Gly	4.21	4.50	5.59	5.01	4.72
Ala	11.68	10.66	18.00	8.29	7.13
Cys/2	1.18	1.46	0.64	0.81	
Val	7.10	7.74	6.08	4.55	6.07
Met	2.55	2.39	2.78	1.48	2.12
Ile	5.46	5.65	4.91	3.70	5.11
Leu	9.47	8.74	7.84	7.79	7.71
Tyr	2.99	2.87	2.79	2.66	2.89
Phe	4.04	3.98	3.79	3.48	2.99
His	2.12	2.23	1.97	3.20	3.08
Lys	7.10	7.48	5.92	8.30	6.45
Arg	4.53	4.24	4.79	5.88	5.30
Total	100.02	100.00	100.01	99.93	100.00

<sup>a)</sup> Wallace (1970), Redshaw and Follett (1971) and Bergink and Wallace (1974). <sup>b)</sup> Deeley et al. (1975) and Christmann et al. (1977). <sup>c)</sup> calculated from D<sub>20,w</sub><sup>0</sup> and S<sub>20,w</sub><sup>0</sup>. <sup>d)</sup> by sedimentation equilibrium. <sup>e)</sup> by electrophoresis in gradient polyacrylamide gels.

purified by Deeley *et al.* (1975), detailed knowledge of the molecular structure is limited. Native avian vitellogenin has been shown to be a dimer (mol. wt 480,000) composed of two identical subunits, each of a molecular weight of 240,000. From a combination of phosphate and amino acid analyses, and molecular weight determinations of purified egg yolk phosphoproteins, each vitellogenin monomer appeared to contain one lipovitellin and two phosvitin polypeptides (Deeley *et al.*, 1975; Christmann *et al.*, 1977).

Phosvitin and lipovitellin have been much more intensively studied than has vitellogenin. Phosvitin is unique in that half of its constituent amino acids are serine residues (Clark, 1970; Redshaw and Follett, 1971). Wallace and Jared (1969) reported that amphibian phosvitin has a molecular weight of 35,000. Clark (1973) has isolated two phosvitins from avian egg yolk. One had a molecular weight of 34,000 and the other had a molecular weight of 28,000.

The lipovitellin monomer, purified from amphibian eggs, consists of two different polypeptides, a heavy and light chain with molecular weights of 120,000 and 31,000, respectively, in a molar ratio of 1 to 1, and is present in the egg as a dimeric form with molecular weight of 400,000 (Bergink and Wallace, 1974; Redshaw and Follett, 1971). Similarly, the lipovitellin ( $\beta$ -lipovitellin) monomer, isolated from avian eggs, is composed of two fractions with molecular weights of 30,000 (light chain) and 110,000-140,000 (heavy chain) (Franzen *et al.*, 1968), and it is present in eggs as a dimeric form with molecular weight of 400,000 (Cook and Wallace, 1965). Bernardi and Cook (1960) isolated two avian lipovitellins,  $\alpha$  and  $\beta$ , from the egg yolk and demonstrated that there was no significant difference between the two in amino acid composition and the molecular weight (400,000).

In the present study, the precipitin reactions of vitellogenin (FS) in trout and salmon (Figs. 15, 16 and 45) suggested that it is a complex of two egg yolk proteins, E1 and E2. Gel filtration suggested that the molecular weights of trout vitellogenin, E1 and E2 were approximately 600,000, 300,000 and 35,000, respectively (Figs. 9 and 10). SDS polyacrylamide gel electrophoresis devoid of 2-mercaptoethanol has a molecular weight of 220,000-240,000 for trout vitellogenin, 130,000 for E1 and 30,000 for E2 (Fig. 17). Although trout vitellogenin yielded a small fraction with molecular weight of 130,000 by reduction with 2-mercaptoethanol, the main fraction remained at 220,000-240,000 dalton. On the other hand, the 130,000 component of E1 further split into two different polypeptides with a molecular weight of 90,000 (heavy chain) and 150,000 (light chain) in a molar ratio of 1 to 1, and E2 split into a component with a molecular weight of 15,000. From the observations mentioned above, it may be concluded that native vitellogenin in trout is a non-covalently bonded dimer of protein with molecular weight of 220,000-240,000, which is bound in the serum with some lipids, carbohydrates and, phosphorus, giving a molecular weight of about 600,000. Probably, E1 has a non-covalently bonded dimeric structure with two identical 130,000 components each of which consists of two different small subunits (mol. wt. 90,000 and 15,000) associated by disulfide bond, giving a molecular weight of 300,000 in egg yolk. E1 behaved quite similarly to amphibian and avian lipovitellin.

On the other hand, the results suggested that E2 was a disulfide linked dimer of a polypeptide with a molecular weight of 15,000. Behavior of trout egg yolk E2



in gel filtration appears to be similar to that of the phosvitin fraction of salmon eggs demonstrated by Markert and Vanstone (1971). They indicated that the phosvitin fraction from gel filtration consisted of two different polypeptides with almost the same molecular weight, phosvitin and  $\beta'$ -component. In the present study, however, trout E2 was found to consist of only one antigenic component. Fish phosvitin has been reported by several workers (Ito *et al.*, 1963; Barman *et al.*, 1964; Schmidt *et al.*, 1965; Mano, 1970) to contain high amounts of phosphorus and serine. However, the serine content of trout E2 shown in the present study was only 11 moles per 100 moles of amino acids (Table 2). The double immunodiffusion analysis indicated that the antigenicity of E1 completely differs from that of E2 (Figs. 15 and 16). Therefore, the component of 15,000 daltons in reduced E1 must be a different polypeptide from reduced E2 (mol. wt. 15,000). Although trout vitellogenin may be assumed to be a complex of E1 and E2, the reduction of vitellogenin with 2-mercaptoethanol in the presence of SDS did not give clear bands corresponding to E1 and E2 (Fig. 17). This means that the binding of the two components (E1 and E2) in vitellogenin is other than disulfide bond. As previously described, it was presumed that vitellogenin in amphibian sera split into lipovitellin and phosvitin in eggs probably enzymatically, because chemical degradation of the protein with SDS or guanidine hydrochloride did not demonstrate this splitting (Bergink and Wallace, 1974). This may probably be the case in fish. Structural concepts of the trout vitellogenin, E1 and E2 obtained from the molecular weight data are summarized in Table 10. This concept can be confirmed by the analyses of amino acid compositions (Table 3).

The eel vitellogenin and the related protein in egg yolk had a similar molecular weight of 85,000 daltons when determined by SDS polyacrylamide gel electrophoresis, though two minor components (mol. wt. 31,000 and 25,000) were found in the related egg protein. Gel filtration of the vitellogenin and the related egg protein disclosed the same molecular weight of 350,000 daltons. The results indicate that both the macromolecular components (mol. wt. 350,000) from serum and from egg yolk consist of four identical subunits with the molecular weight of 85,000 (e.g.  $85,000 \times 4 = 340,000$ ). Amino acid compositions of the components from both the

Table 10. Structural concepts of the rainbow trout vitellogenin, E1 and E2 obtained from molecular weight data.

	E1	E2	FS
Mol. wt. ( $\times 10^3$ ) (Gel filtration)	300	35	600
Molar ratio	1	: 1	FS=2(E1+E2)
Mol. wt. ( $\times 10^3$ ) (SDS PAGE)	90 , 15 (H) (L)	15 (E2/2)	220-240 FS/2
Molar ratio	1 : 1		
Composition	E1=2(H+L)	2 $\times$ E2/2	FS/2=2(H+L)+2 $\times$ E2/2 = E1 + E2

serum and the egg were quite similar except for serine (Table 6) and the NH<sub>2</sub>-terminal residue was alanine for both proteins. Although SDS polyacrylamide gel electrophoresis of egg protein indicated the presence of minor components, it may be considered that eel vitellogenin and the same antigen in egg yolk are almost the same protein, having very similar structures. However, it is interesting that the molecular weights of the minor components in the eel egg protein (mol. wt. 31,000 and 25,000) are rather similar to that of phosvitin in avian egg. In this respect, it would be quite intriguing to know whether or not the minor components of eel egg have properties of phosvitin.

Vitellogenin may be split into three small polypeptide chains (lipovitellin subunits, mol. wt. 31,000 and 120,000, and phosvitin, mol. wt. 35,000) in amphibian or four small polypeptide chains (lipovitellin subunits, mol. wt. 30,000 and 135,000, and two phosvitin, mol. wt. 34,000 and mol. wt. 28,000) in avians after taken up by the oocyte. A similar mechanism may operate in the case of rainbow trout, for the structural relationship between vitellogenin and egg yolk proteins from rainbow trout resembled that of amphibians (Bergink and Wallace, 1974) and avians (Deeley

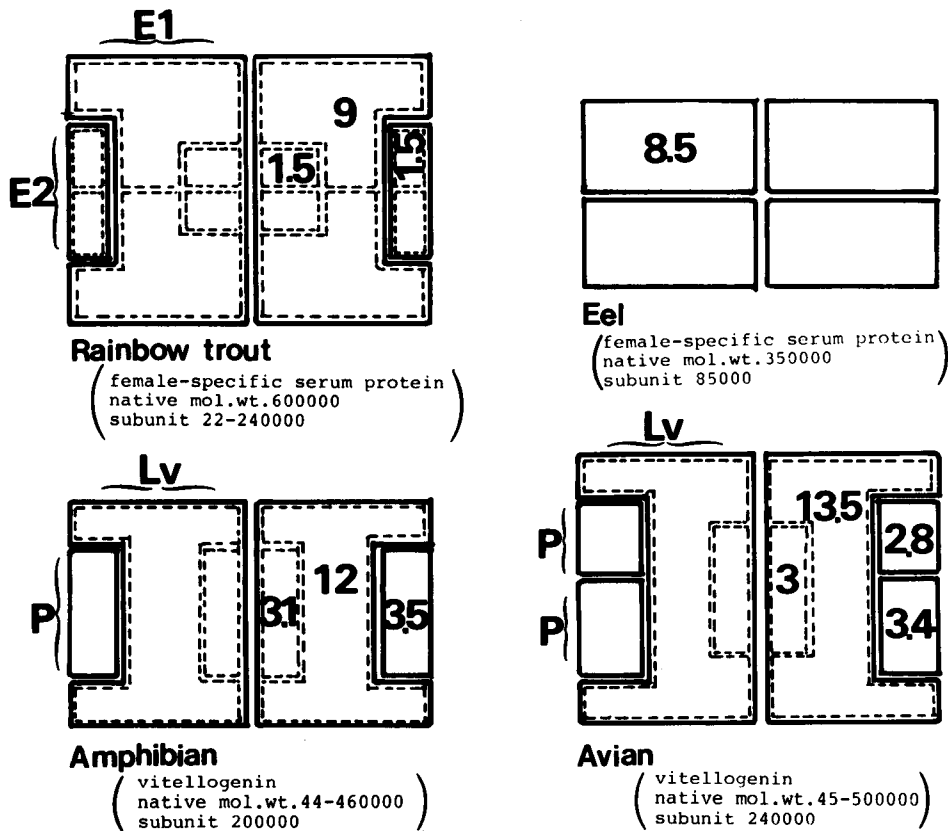


Fig. 71. Schematic models of various vitellogenin molecules. Lv: lipovitellin, P: phosvitin.

*et al.*, 1975). The relationship between vitellogenin and its egg protein in Japanese eel is quite different from that of rainbow trout. From the results mentioned above and previous reports of amphibians and avians, schematic models of various vitellogenin molecules may be possible (Fig. 71).

Differences in serum proteins among fish species have frequently been observed (Gemeroy, 1943; Hara, 1975). The structural differences between vitellogenins of eel and other animals may also be due to interspecific differences. Probably, eel vitellogenin, which has the same molecular structure as that of egg protein, may readily go into egg yolk without any structural conversion.

In medaka, it was shown that three female-specific serum proteins, which are antigenically related to egg yolk proteins, are present in breeding females and they can be induced in males by injections of estrogen (Figs. 57 and 69). These three female-specific serum proteins showed similar characteristic properties to vitellogenin in the following aspects: (1) they were specific proteins which appear during gonadal maturation, (2) they were induced in the male by the administration of estrogen and, (3) the same antigenic components were found in egg yolk extracts. Therefore, it may be concluded that the three female-specific serum proteins in medaka correspond to vitellogenin. It was shown in the present study that trout, salmon and eel vitellogenins have iron binding properties. Among the three female-specific serum proteins of the medaka, FS3 binds radioactive iron and appears only in breeding females (Figs. 61 and 62). This indicates that FS3 in medaka may correspond to the fish vitellogenin as defined in the present paper.

The analysis of medaka serum by means of electrophoresis combined with histological examinations of the ovaries clearly disclosed that the occurrence of three female-specific serum proteins are closely related to the development of ovaries (Figs. 58 and 60). Ridgway *et al.* (1962), Fine and Drilhon (1963) and Utter and Ridgway (1967) detected, by immunological methods, the presence of egg proteins in the serum of female fish with developing ovaries. In these respects, analyses of vitellogenin or its related protein in serum can provide information for the assessment of ovarian development. Among several analytical methods of such antigens which are available, the immunological procedure, such as the crossed immunoelectrophoresis, is a useful method for the analysis of serum from a tiny individual or very small samples.

The occurrence of three female-specific components as serum proteins in medaka was somewhat different from vitellogenins in other fish. This may be due to characteristic seasonal changes in the maturity factor of this fish (Yamamoto and Yoshioka, 1964). It is also possible that the mechanism of vitellogenesis differs from that in other fishes. Another possibility could be sought in the different analytical method, for crossed immunoelectrophoresis used for the medaka is a more sensitive technique than conventional immunoelectrophoresis and immunodiffusion (Figs. 56 and 57).

So far, four different species of teleosts have been investigated for their female-specific serum proteins. The vitellogenin of rainbow trout and chum salmon, which belong to the family Salmonidae, had a similar molecular structure. However, the vitellogenin of Japanese eel showed a rather different structure compared to that of the above two fish species. Furthermore, the proteins of medaka seemed to have

another molecular form, revealing three egg yolk proteins in the serum. Presumably, more different molecular forms or occurrence of the protein(s) might be present in other fish species, especially, in elasmobranchs and lampreys that are phylogenetically far from teleosts.

It has been reported that the presence of fish vitellogenin can be estimated indirectly by measuring calcium levels (Bailey, 1957; Oguri and Takada, 1967; Woodhead, 1969), total lipid (Plack and Woodhead, 1966), phospholipid (Plack and Pritchard, 1968), alkali-labile protein phosphorus (Emmersen and Petersen, 1976), phosphoprotein (Craik, 1978) and total protein (Booke, 1964) in plasma or serum of female and/or estrogen-treated fish. Plack *et al.* (1971) measured directly the concentration of egg proteins in the serum of immature male and female cod treated with estrogen, using the double immunodiffusion technique.

In the present study, the concentration of vitellogenin in trout serum was determined by the single radial immunodiffusion technique using a purified preparation as standard. Therefore, the concentrations of vitellogenin obtained in the present paper are considered the most reliable value when compared to those in the previous reports. The concentration of vitellogenin increased rapidly in 10 days after injection and then decreased sharply within the subsequent 10 days (Fig. 30). The half-life of trout vitellogenin was roughly calculated as 1.5 days from this profile (Fig. 30). The rapid synthesis of vitellogenin can operate in the liver under the influence of estrogen as demonstrated by histological and immunological observations of estrogen-treated fish (Figs. 31 and 32). The vitellogenin synthesized by the liver will be released into blood, conveyed to the oocyte and utilized for vitellogenesis.

The precise mechanism of how the vitellogenin can be taken into oocytes via the associated follicle layers is not known. In order to elucidate the mechanism, further studies on some special proteins or enzymes which can act as a receptor or a transfer of the vitellogenin are necessary. Otherwise, it is quite difficult to explain why such amounts of vitellogenin (or its related egg yolk protein) can accumulate specifically in the oocyte.

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