# Studies on Female-Specific Serum Proteins (Vitellogenin) and Egg Yolk Proteins in Teleosts: Immunochemical, Physicochemical and Structural Studies

Akihiko HARA

Department of Biochemistry, Hokkaido University
School of Medicine
Sapporo, Japan

CONTENTS

PAGE

PAGE

# 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 which they encounter. Since the initial investigations of electrophoretic patterns of serum and plasma proteins of carp *(Cyprinus carpio)* performed by Deutsch & Goodloe (1945) and Moore (1945) with Tiselius method, 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 & McShan (1949) studied serum protein fractions in various species of fishes using electrophoretic technique for the separation and quantitative analysis of proteins.

Irisawa & Irisawa (1961) working with elasmobranch sera concluded that the serum albumin fraction was missing in the skate *(Raja kenojei)* 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 of a variety of fishes obtained under almost the same condition. He ascertained the following fact that the specificity, in quantity as well as in quality, of the serum protein composition was more or less conspicuous corresponding to their taxisonomic 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 & 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 & 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 fish and those from diseased ones.

Solomon & 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 *(Sebastiscus 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 & 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 gairdnerii)* plasma protein separation using disc electrophoresis, gel filtration and salt solubility fractionation.

Yoneda & Ishihara (1974) reported disc electrophoretic patterns of serum proteins from chum salmon *(Oncorhynchus keta)* and masu salmon *(Oncorhynchus 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 & Yerger (1976) reported an electrophoretic investigation of subpopulations of the spotted sea trout *(Cynoscion nebulosus)* in the Gulf of Mexico and Atlantic coast of Florida. According to them, the serum protein patterns proved to be a more sensitive criterion for detecting population differences than lens proteins.

Studies of plasma or serum proteins of fish in regard to the sexual differences were also reported by many investigators. Vanstone & Ho (1961) reported a slowly migrating

fraction in the serum of maturing 2.5-year-old female coho salmon *(Oncorhynchus 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 *(Oncorhynchus nerka)* differed in their antigenic constitution when tested by the immunodiffusion method with immune antisera prepared in rabbits. 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 & Ridgway (1963) quoted that the above component, called Sm antigen by them, might be identical with the "serum vitellin" reported by Vanstone & Ho (1961). Fine & Drilhon (1963) identified a similar protein in Atlantic salmon *(Salmo salar)* by immunodiffusion. They explained that the existing evidence of serum vitellin might account for the following sequence of events in oviparous vertebrates: 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 there for the yolk formation.

Utter & Ridgway (1967) reported that a serologically detectable serum factor (HM factor) was associated with maturity in Engligh 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 *(Salmon gairdneri)*, and found sexual differences in 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 & Vanstone (1971) purified three egg yolk proteins of coho salmon *(Oncorhynchus kisutch)*. 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 *(Salmo*

*gairdnerii)* lipovitellin from eggs, serum and from several organ extracts.

Aida et al. (1973a) reported of ayu *(Plecoglossus altivelis)* a similar observations on the sexual difference in the composition of plasma proteins, its hormonal control and relationship between plasma protein and egg yolk protein, using electrophoretical and 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 result, he considered that vitellogenesis in the river lamprey was stimulated by estrodiol administration.

Emmersen & Petersen (1976) showed the presence of a specific lipophosphoprotein in female flounders *(Platichtys 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 estrodiol treatment in both sexes. They assume that the isolated lipophosphoprotein is identical to the yolk-precursor protein, vitellogenin. Furthermore, they found that the process of vitellogenesis was correlated closely with changes in the synthesis of RNA and DNA in the liver (Emmersen & 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.

Preceding various reports cited above clearly show the appearance of the specific protein in the blood during vitellogenesis of female as well as estrogen-treated fish. Recently, a 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 insect protein which specifically appears in female insects (Pan et al., 1969). It is purely functional and does not imply any definite characteristics of the proteins. Avian and amphibian vitellogenin, which is also 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 immediate precursor of these proteins in egg yolk (Wallace & Bergink, 1974; Christmann et al., 1977). However, such characterizations of the female specific proteins in fish (fish vitellogenin) has not 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 the better understanding of the reproduction and culture of fishes.

During a comparative study of serum protein profile in male and female fish (Hara, 1975), we found that a female-specific serum protein was capable of binding iron. 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 *(Salmo gairdneri)*, chum salmon *(Oncorhynchus keta)* 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, the paper includes the studies of female-specific serum proteins identified in serum of the medaka *(Oryzias latipes)* by immunological procedures. Finally, speculations of molecular structure of female-specific serum protein (vitellogenin) 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, *Salmo gairdneri*, chum salmon, *Oncorhynchus keta*, Japanese eel, *Anguilla japonica*, and medaka, *Oryzias latipes*.

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 of the years 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 Ichani River and the Shibetsu River, Nemuro, Hokkaido, which drain into the Pacific Ocean. Since the fish were in the 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 during the autumn in 1976. They were bred 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 at a week interval for 1-4 months (Yamamoto et al., 1974).

Medaka used in the present study were obtained from a pond at Yunokawa Hot Spring in the suburbs of Hakodate. They were then cultured in out-door ponds in the campus of the Faculty of Fisheries, Hokkaido University, until use.

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.

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.

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

Every blood sample was 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°C until use. No remarkable change in the antigenicity were observed during storage at

-20°C for several months.

3. Eggs

Ovulated eggs were collected from female rainbow trout, chum salmon and medaka during spawning season. Eggs of Japanese eel were stripped from the females which had attained their full maturation with pituitary treatments. These eggs obtained from the four fish species were at the tertiary yolk stage. They were kept frozen at -20°C until thawed before experiments.

Egg yolk proteins were extracted essentially as follows. Eggs were thoroughly washed with 0.9% NaCl and were then homogenized using a Waring blendor. The contents were centrifuged at 10,000 rev/min for 30 min at 4°C. The supernatant was collected and filtrated through a filter paper. The filtration was again centrifuged at 30,000 rev/min for 60-90 min at 4°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 a week interval. A test bleeding was taken from the ear vein

and the serum was immediately assayed by immunoelectrophoresis. When the immunoelectrophoresis pattern was judged satisfying, 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 as follows. The polyvalent antiserum mentioned above was absorbed 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 (Ouchterlony's) method and immunoelectrophoresis.

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.

The antisera used in the present study are listed in 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

Table 1. The antisera used for immunochemical characterization

Polyvarent Antiserum

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 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

Specific Antiserum

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

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 & Osborn (1969). Samples were incubated in a solution containing 1% SDS and 10 mM iodoacetamide at 37°C for 2 hours, 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 minutes. 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 portein 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).

Immunoelectrophoresis was performed by the method of Grabar & 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 procedure was as follows: the sample was subjected to a disc of 5% or 7.5% polyacrylamide gel and electrophoresed. After the finish of the electrophoresis, the disc was taken out from the glass tube, and implanted in plate of 1.2% agarose gel. Then the antiserum was placed in troughs made parallel to the implanted disc gel.

Crossed immunoelectrophoresis and tandem-crossed immuno-electrophoresis 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 finally 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

a 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% $NaN_3$ 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 µl of fish serum or egg yolk extracts, containing approx. 1 mg protein, were incubated for 1 hr at room temperature with 5 µl $^{59}FeCl_3$ corresponding

to 0.1 μCi. The sample was then submitted to cellulose acetate membrane electrophoresis, immunoelectrophoresis, crossed immunoelectrophoresis or tandem-crossed immuno-electrophoresis. 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. While, 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. Then the filter paper was stained with Amido 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 γ-counter.

H. Chemical analysis

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

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

Determination of $NH_2$-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 a 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 a female-specific serum protein of chum salmon.

Sedimentation coefficient, $S^0_{20,w}$ was evaluated by extrapolation to zero protein concentration and corrected to the viscosity and density of water at 20°C (Svedberg & Perderson, 1940).

Diffusion coefficient was measured in the 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. Values were calculated by the Fujita's equation modified by Kawahara (1969).

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

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

Extinction coefficient ($E^{1\%}_{280\ nm}$) was evaluated as follows: a weighed sample, after drying over $P_2O_5$ in a vacuum was

dissolved in 0.1 N NaOH and the optical density of the solution was determined at 280 nm by a Hitachi Perkin-Elmer Model 139 spectrophotometer.

The determination of the isoelectric point was performed 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 hr 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-17β experiment, a group of rainbow trout consisting of males or immature females weighing about 350 g, were injected with 0.5 mg of estradiol-17β in 50% ethanol. Blood samples were taken from the fish 2,4,6 and 8 days after the single injection of estrogen.

The other group of rainbow trout estimated to be about 100 g in body weight were chosen to ensure that they were sexually immature. One hundred μg of estradiol-17β in 50% ethanol (0.2 ml) was injected into the muscle behind the head. The blood sample was repeatedly taken every 2 days after the single estrogen injection from the dorsal aorta after anesthesia with MS222*. The fish were sampled for histological and immunofluorescent study of the liver.

*MS222: meta-aminobenzoic acid ethylester methanesulfonate

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

2. Medaka

In the present study of medaka, ethinylestradiol, estradiol-17β, 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 on pellets containing hormones at a dose of 100 μg/g diet during the experimental period (Nov. - Dec. 1977). The control group was 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.

*G.S.I.: Gonadosomatic index (ovarian weight×100/body weight)

## III. RESULTS

### A. Rainbow trout

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

Typical electrophoretic patterns of immature, maturing and ripe rainbow trout which were sampled in two trout farms are shown in Fig. 1. Differences between sexes were seen in the β-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.

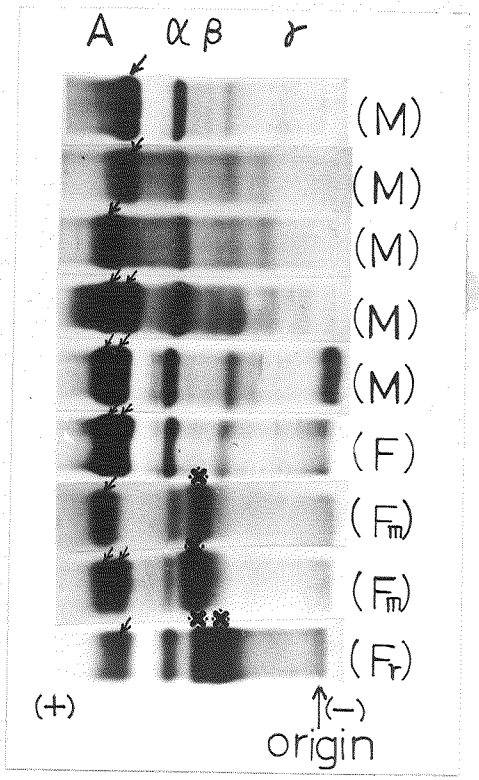


Fig. 1. Typical cellulose acetate membrane (Cellogel) electrophoretic patterns of serum of male (M) and female (F) rainbow trout. The electrophoregrams show one (↙) or two bands (↙↙) in the albumin fraction. All speciments of maturing (Fm) and ripe (Fr) females have one or two components (*) in the β-globulin 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 precipitin reaction with male serum proteins. However, the absorbed antiserum kept 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). Results of immunoelectrophoretic analysis of anti-trout FSSP with pooled ripe female serum and egg yolk extracts are shown in Fig. 2. As can be seen in Fig.2, 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 a minor faint line. The result indicates that there are antigens (female-specific serum proteins) specifically existing in female serum but not in male serum, and that similar antigens are also present in egg yolk extracts.

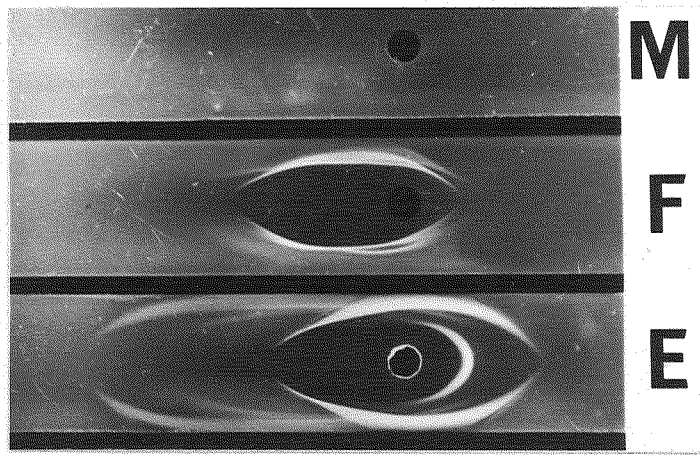


Fig. 2. Immunoelectrophoresis of serum of ripe male (M) and female (F) rainbow trout, and egg yolk extracts (E) with rabbit anti-trout FSSP.

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

Autoradiography of cellulose acetate membrane electrophoresis of the serum mixed with $^{59}FeCl_3$ clearly indicated the difference between female and male in the spawning condition. As shown in Fig. 3, the female serum showed two strong radioactive bands, while the male serum showed a single weak radioactive band. One of the two bands of female sera was the same mobility with the 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).

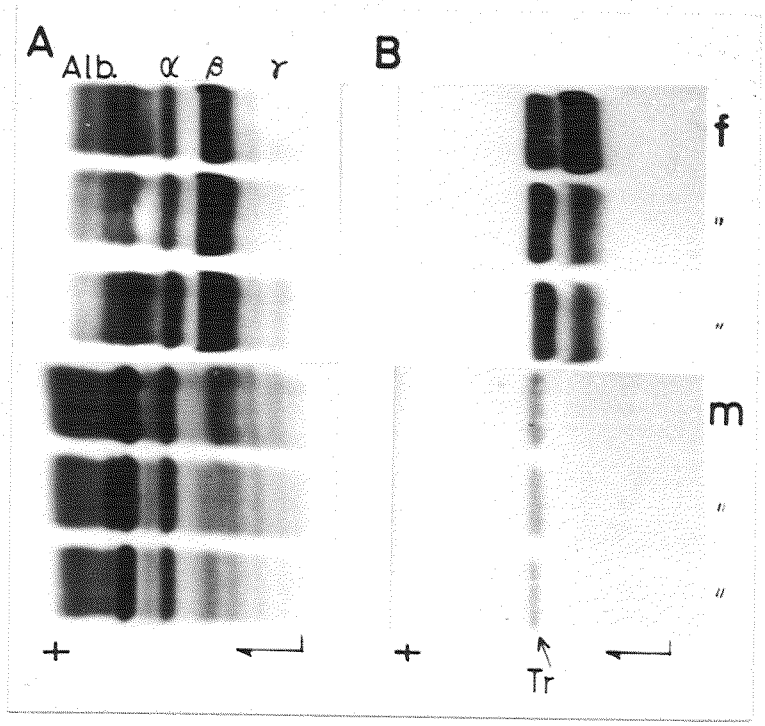


Fig. 3. Cellulose acetate membrane (Cellogel) electrophoresis of ripe rainbow trout sera (A) and its autoradiography with $^{59}Fe$ (B). f: female serum, m: male serum, Tr: transferrin. To 25 µl of the serum was added 10 µl of $^{59}FeCl_3$ solution with an activity of 0.1 µCi, corresponding to 0.003 µg Fe. After incubation the mixture was subjected to electrophoresis, and the gels were exposed to X-ray films for 2 weeks.

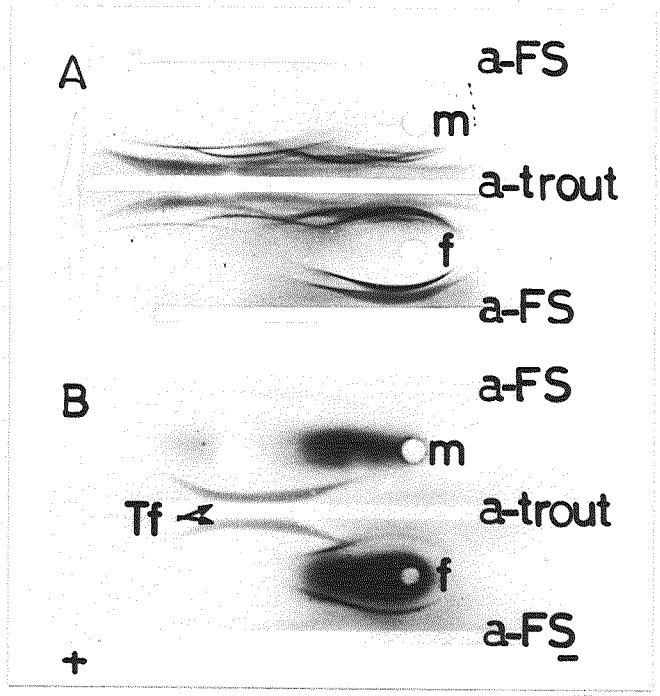


Fig. 4. Immunoelectrophoresis of ripe rainbow trout sera. A: protein staining, B: autoradiography with $^{59}Fe$, a-trout: anti-trout serum, a-FS: a-trout FSSP, m,f,: male or female serum.

The results of immunoelectrophoresis combined with autoradiography of pooled sera of ripe rainbow trout are shown in Fig. 4. The autoradiography of the immunoelectrophoresis with anti-trout serum indicated that the male sera yielded only one radioactive precipitin line at the α-globulin region which was commonly observed in female serum. This component is considered to be transferrin. On the other hand, the female sera showed another radioactive line which migrated to the β-globulin region. As indicated in Figs. 2 & 4, there were two precipitin lines at the β-region with anti-trout FSSP. However, only one line near the antigen well was capable of binding the radioactive iron as seen in Fig. 4.

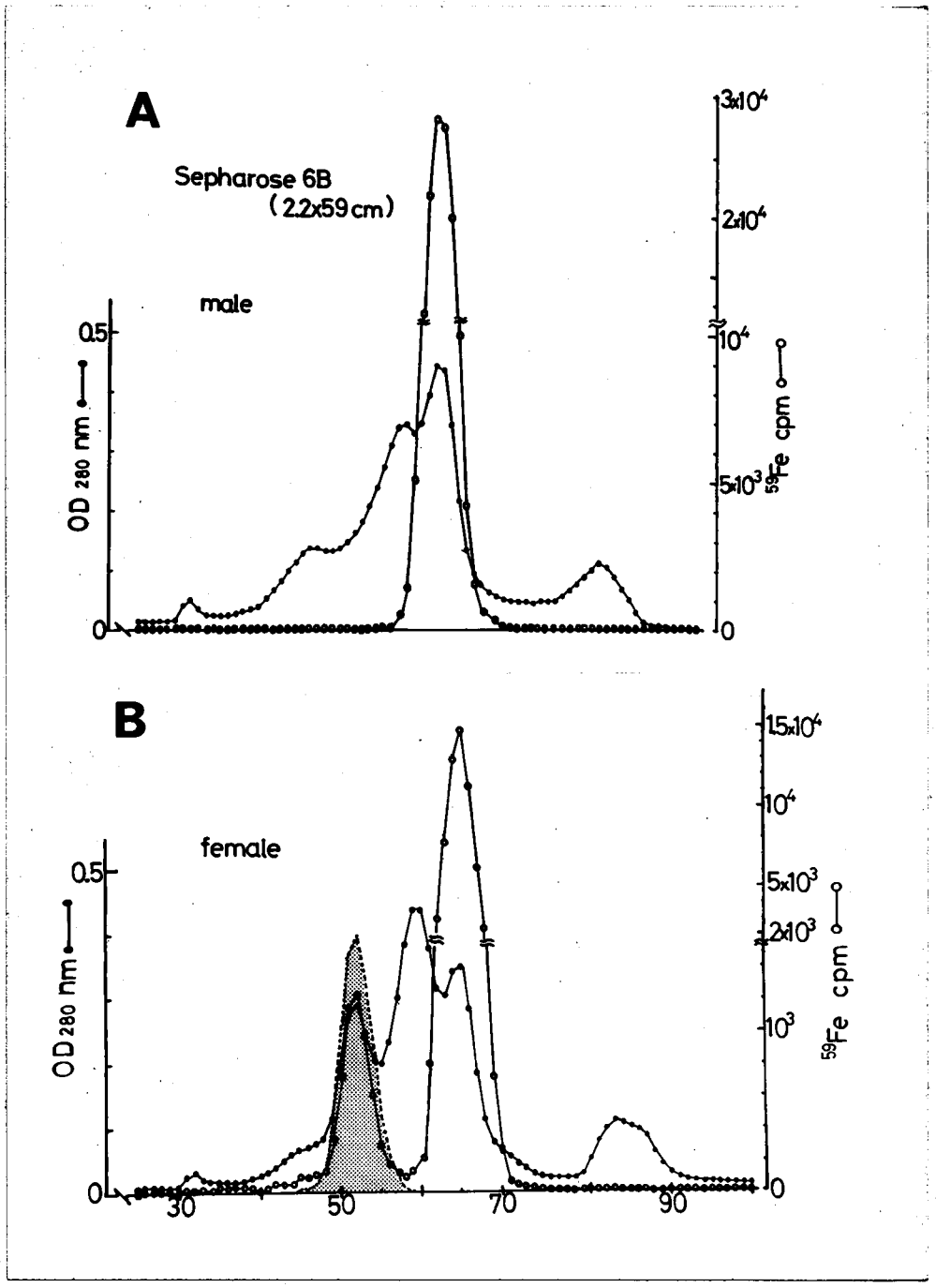


Fig. 5. Gel filtration on Sepharose 6B of sera of ripe male (A) and female (B) rainbow trout. The fractions in shadowed area of female contained female-specific serum protein. One radioactive protein (transferrin) was observed in male, while two radioactive proteins were obtained in female.

Gel filtration on Sepharose 6B of serum mixed with $^{59}FeCl_3$ was also performed in an attempt to separate the female-specific iron-binding protein from putative fish transferrin (Fig. 5). Radioactive protein observed in male was eluted as a fraction of relatively low molecular weight (approx. mol. wt. 80,000) and was concluded to be transferrin of the fish (Fig. 5A). In female trout, however, two distinct radioactive peaks were obtained (Fig. 5B). The second radioactive peak corresponded to the peak of the 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 $^{59}FeCl_3$ using Sephadex G-200 gel filtration. The elution pattern of egg yolk extracts is shown in Fig. 6. Only one radioactive peak

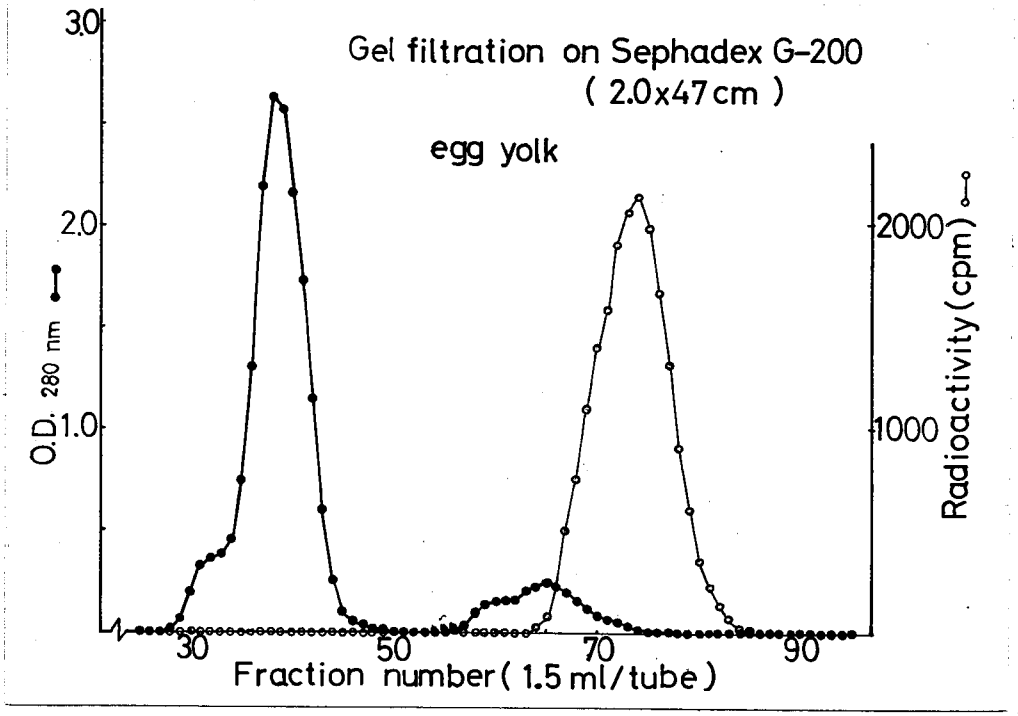


Fig. 6. Gel filtration on Sephadex G-200 of egg yolk extracts from rainbow trout. One radioactive peak was observed at the very small molecular weight fraction.

was observed in this case at the very small molecular weight fraction (mol. wt. around 20,000)(see Fig. 10).

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

It was reported that proteins specifically present in the female of chum salmon are precipitable by a dilution of the serum of maturing females with a low ionic strength (Yoneda & Ishihara, 1974). 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 dialyzed against distilled

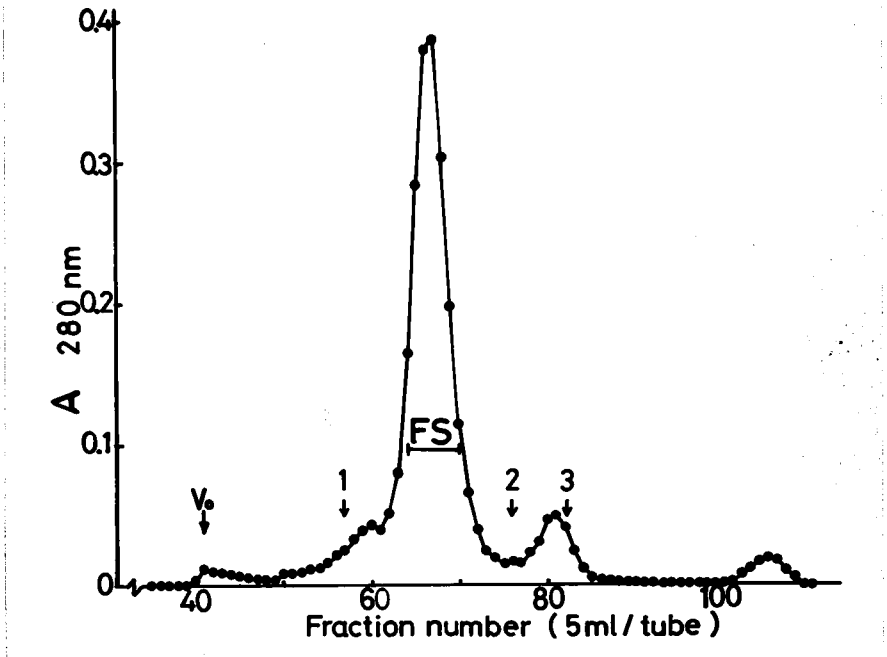


Fig. 7. Chromatography of rainbow trout serum on Sepharose 6B. Material: water-insoluble proteins isolated from pooled ripe female rainbow trout serum. Column: 2.6×90cm, Elution buffer: 0.02 M Tris-HCl, pH 8.0, containing 2% NaCl and 0.1% $NaN_3$. FS: fractions eluted in the position indicated by a horizontal bar where collected and designated as FS(see text). Vo: void volume. Marker proteins: (1) IgM, (2) IgG, (3) human serum albumin.

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 (2.6×96cm) with 0.02 M Tris-HCl buffer, pH 8.0, containing 2% NaCl and 0.1% $NaN_3$. The elution pattern is shown in Fig. 7. The main peak was found to consist of FS when assessed by means of a single radial immunodiffusion using the anti-trout FSSP. 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 & Vanstone (1971). Eggs were washed with 0.9% NaCl, and broken by a mixer. The contents were strained through a Buchner funnel to remove egg membranes, and centrifuged at 300,000 rev/min for 60 min at 4°C. A clear middle layer with a yellowish color was collected. This clear solution was dialyzed against distilled water overnight in cold. 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 (2.6×95cm), and eluted with Tris-HCl buffer as mentioned above. The elution pattern yielded two peaks, E1 and E2 as shown in Fig. 8. These two peaks (E1 & E2) collected as in Fig. 8 were tested for their reactivity to the anti-trout FSSP and were

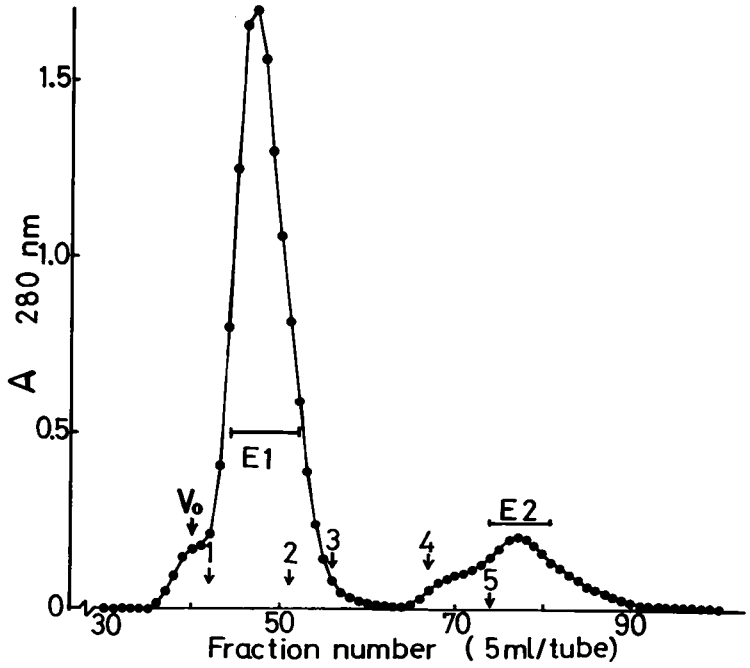


Fig. 8. Chromatography of rainbow trout egg yolk proteins on Sephadex G-200. Material: water-insoluble proteins isolated from rainbow trout egg yolk. Column: 2.6× 95cm. Elution buffer: same as in Fig. 7. E1, E2: two major components were designated as E1 and E2. Vo: void volume. Marker proteins: (1)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

found both 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 & 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

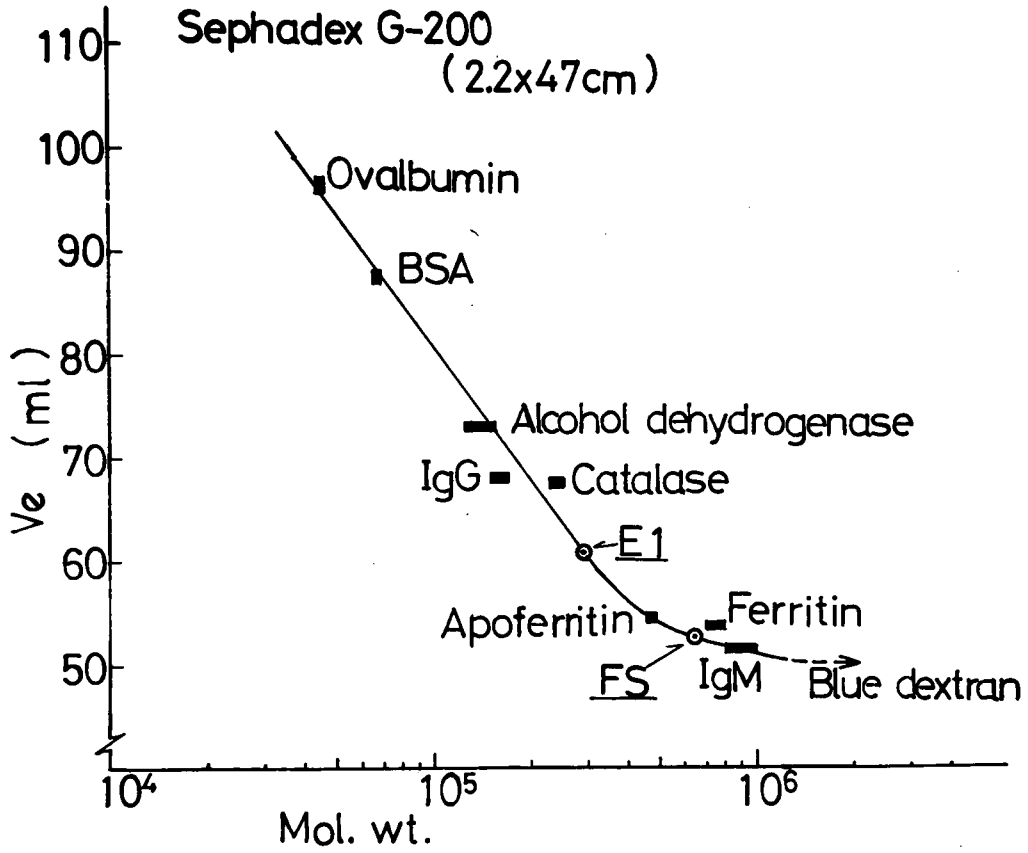


Fig. 9. Estimation of molecular weight from the exclusion volume on Sephadex G-200. Calibration curve of Sephadex G-200 (2.2×47cm). The samples used for calibration are indicated by dark bars. The open circles correspond to the elution volume of trout female-specific iron-binding serum protein (FS) and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

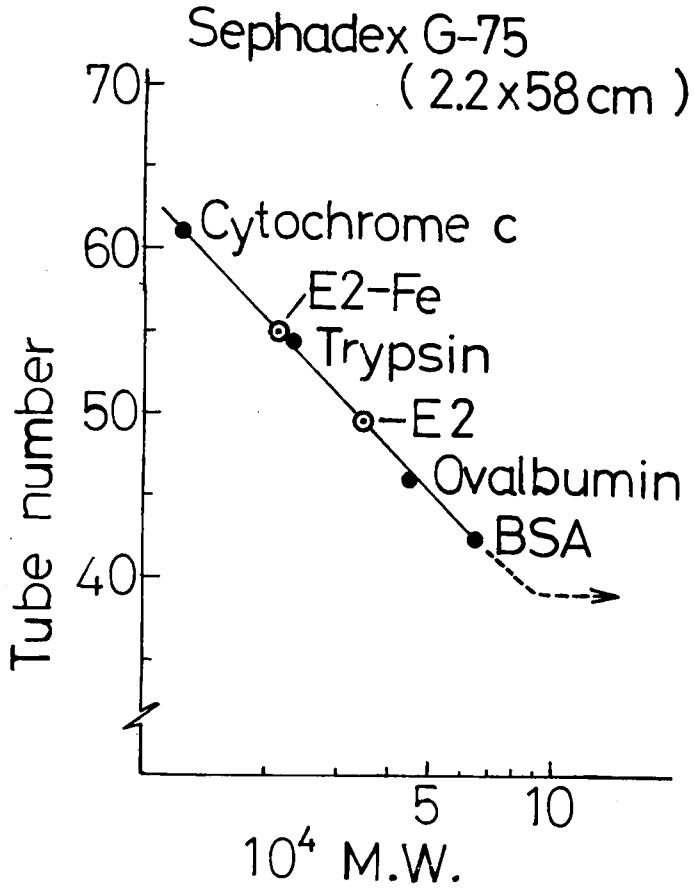


Fig. 10 Estimation of molecular weight from the exclusion volume on Sephadex G-75. Calibration curve of Sephadex G-75 (2.2×58cm). The samples used for calibration are indicated by dark circles. The open circles correspond to the elution volume of trout egg yolk proteins (E2) and radioactive peak of E2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

4.9 to 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).

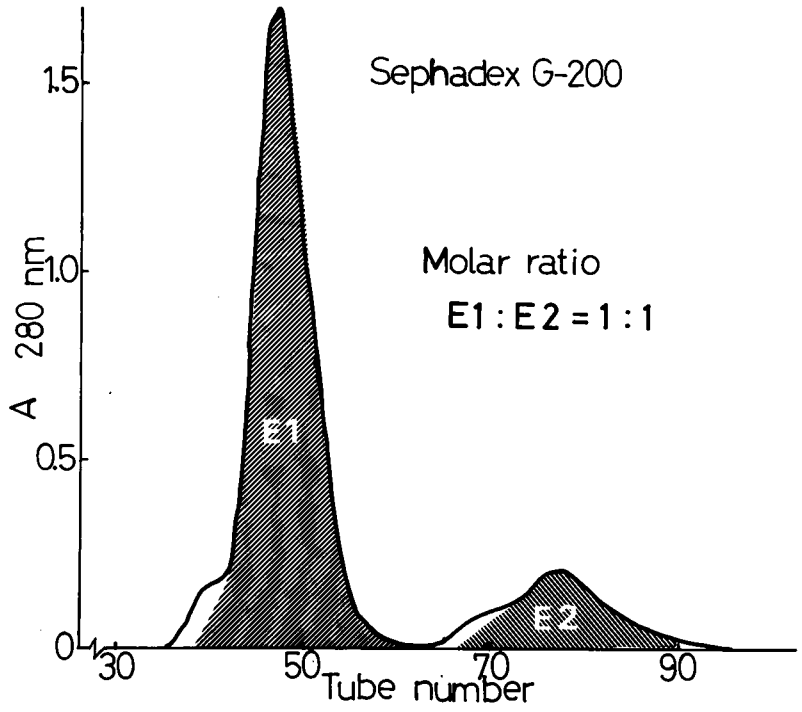


Fig. 11 Elution pattern on Sephadex G-200 of rainbow trout egg yolk proteins (see Fig. 8). Ratios of protein amount between E1 and E2 (shadowed area) were determined planimetrically from charts of gel filtration on Sephadex G-200 monitored by 280 nm absorption. A molar ratio of E1 and E2 can be roughly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

## 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 Ouchterlony technique. It showed a single homogeneous band in the disc electrophoresis when

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 as shown in Fig. 13.

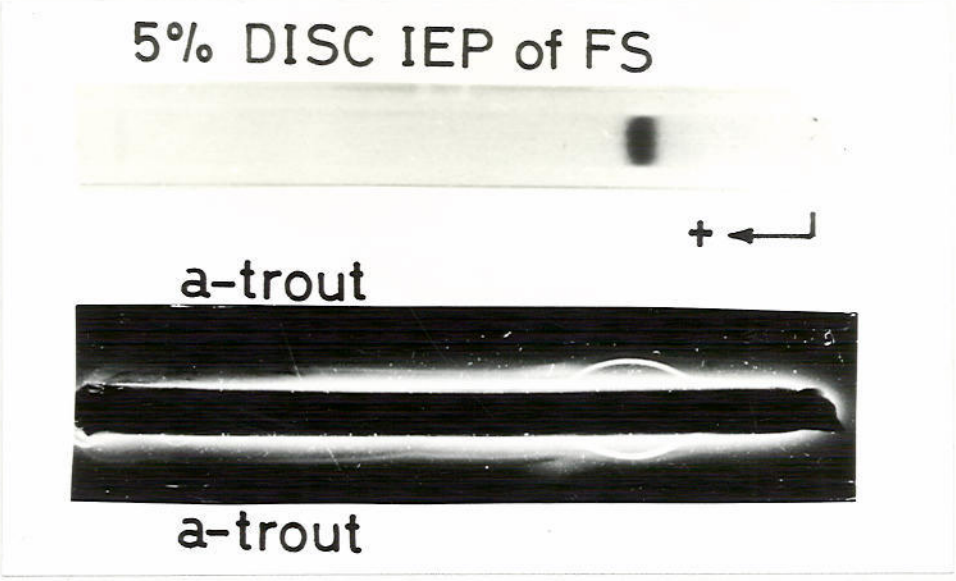


Fig. 12. Disc immunoelectrophoresis of purified FS from rainbow trout. The sample was subjected first to disc electrophoresis in 5% polyacrylamide gel, followed by immunodiffusion in 1.2% agarose gel. a-trout: anti-trout serum.

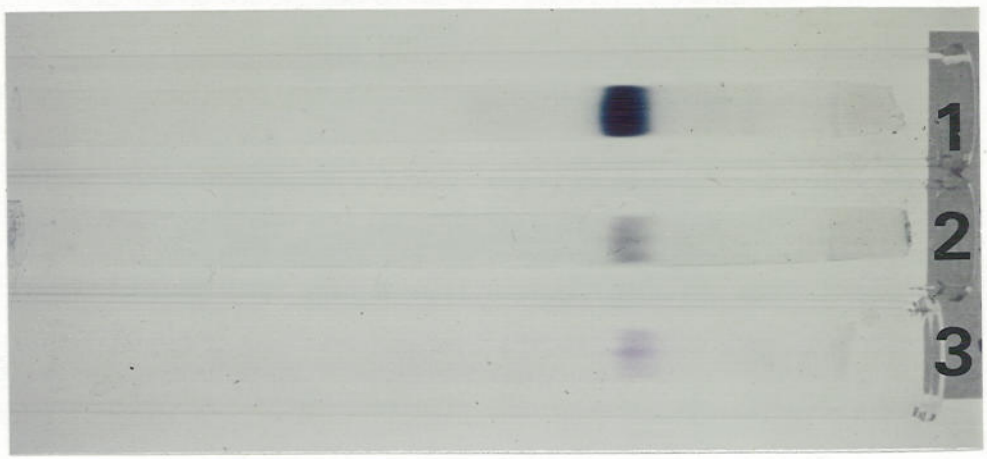


Fig. 13. Disc electrophoresis of purified FS from rainbow trout. 1: protein staining. 2: lipid staining. 3: carbohydrate staining.

Disc immunoelectrophoresis of E1 and E2 from egg extracts gave the pattern shown in Fig. 14. E1 showed a sharp single band in 5% polyacrylamide gel electrophoresis. 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). E2 was stained neither with the carbohydrate nor the lipid reagents.

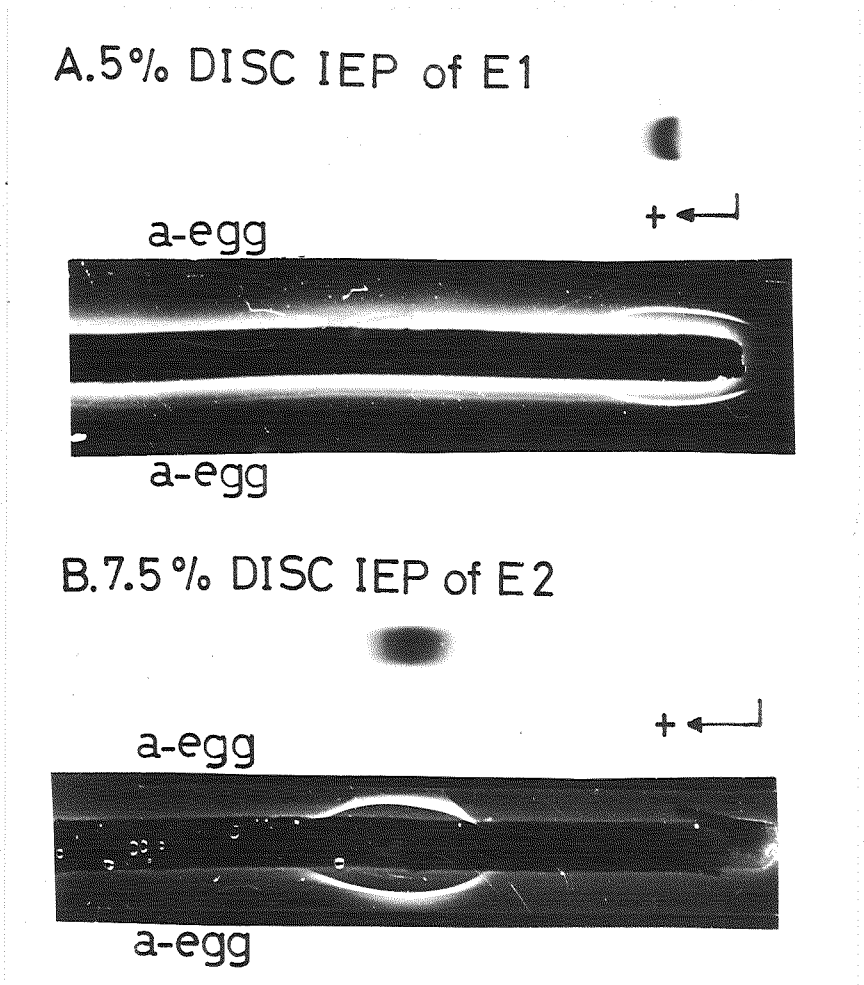


Fig. 14. Disc immunoelectrophoresis of purified E1 (A) and E2 (B) isolated from egg yolk proteins of rainbow trout. The samples were subjected first to disc electrophoresis in 5% or 7.5% polyacrylamide gel, followed by immunodiffusion in 1.2% agarose gel. a-egg: antiserum to trout eggs (anti-trout eggs).

7. Antigenic relation between FS, E1 and E2

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

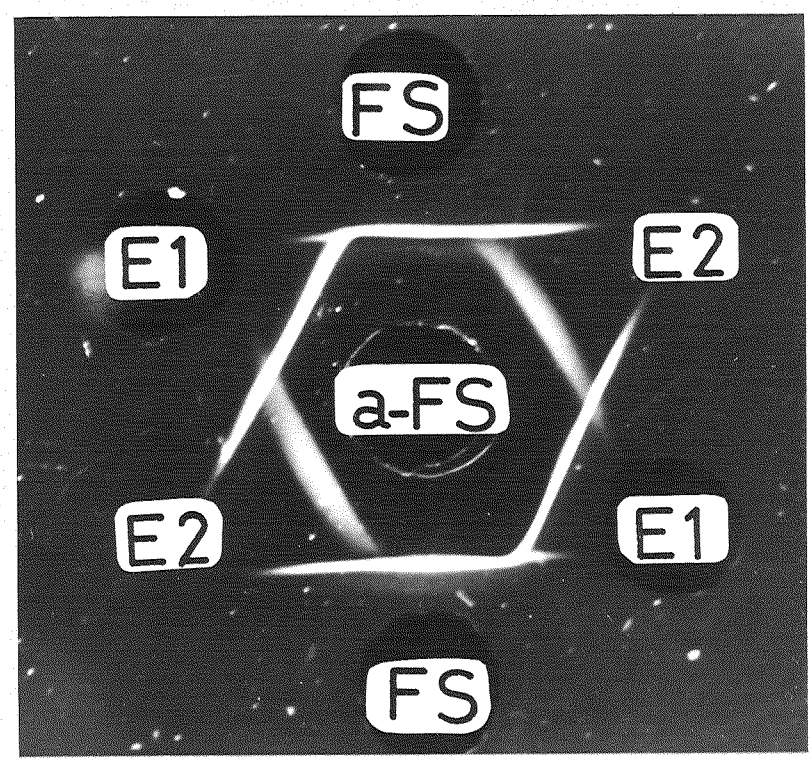


Fig. 15. Precipitin reaction of FS, E1 and E2 from rainbow trout. FS: female-specific iron-binding serum protein. E1 and E2: fractions of egg yolk proteins. a-FS: anti-trout FSSP.

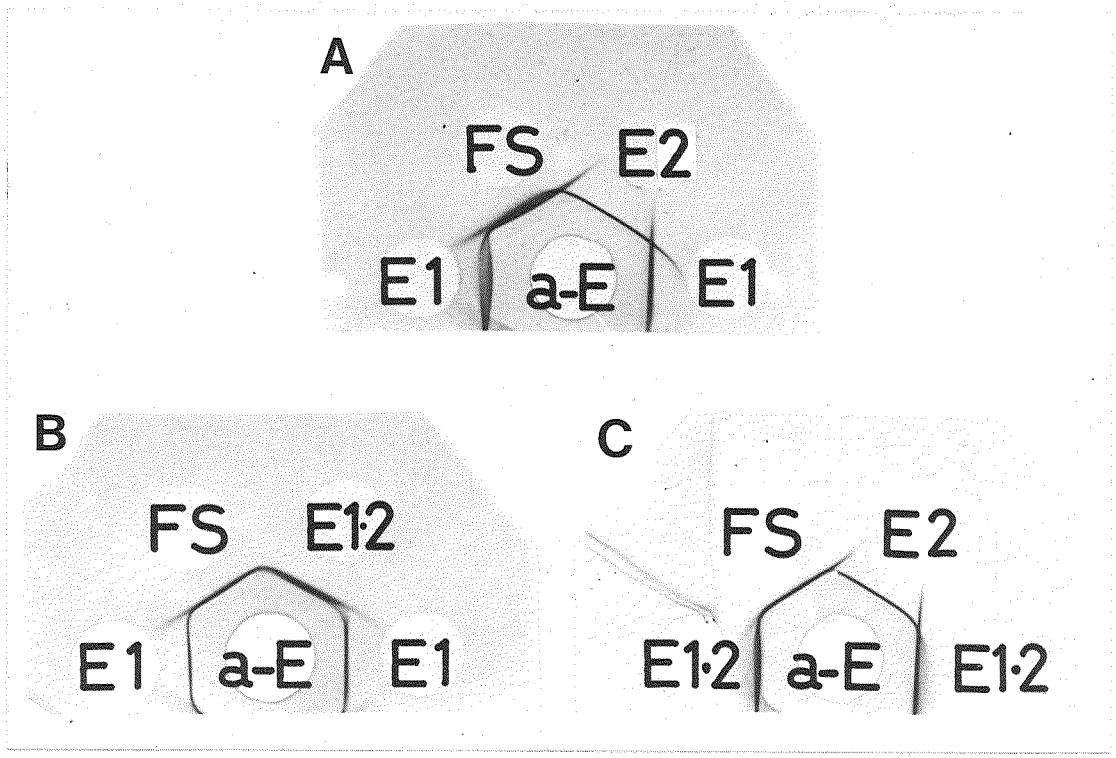


Fig. 16. Precipitin reaction of FS, E1 and E2 from rainbow trout. FS, E1 and E2: same as in Fig. 15. E1·2: E1 plus E2. a-E: anti-trout eggs. After removal of umprecipitated protein by washing and drying, the precipitin arcs were stained with Amido black 10B.

8. SDS polyacrylamide gel electrophoresis

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

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 same for the two subunits, the ratio of protein amount between the two subunits (mol. wt. 90,000 and 15,000) can be calculated to be 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 into a smaller molecular weight of 15,000.

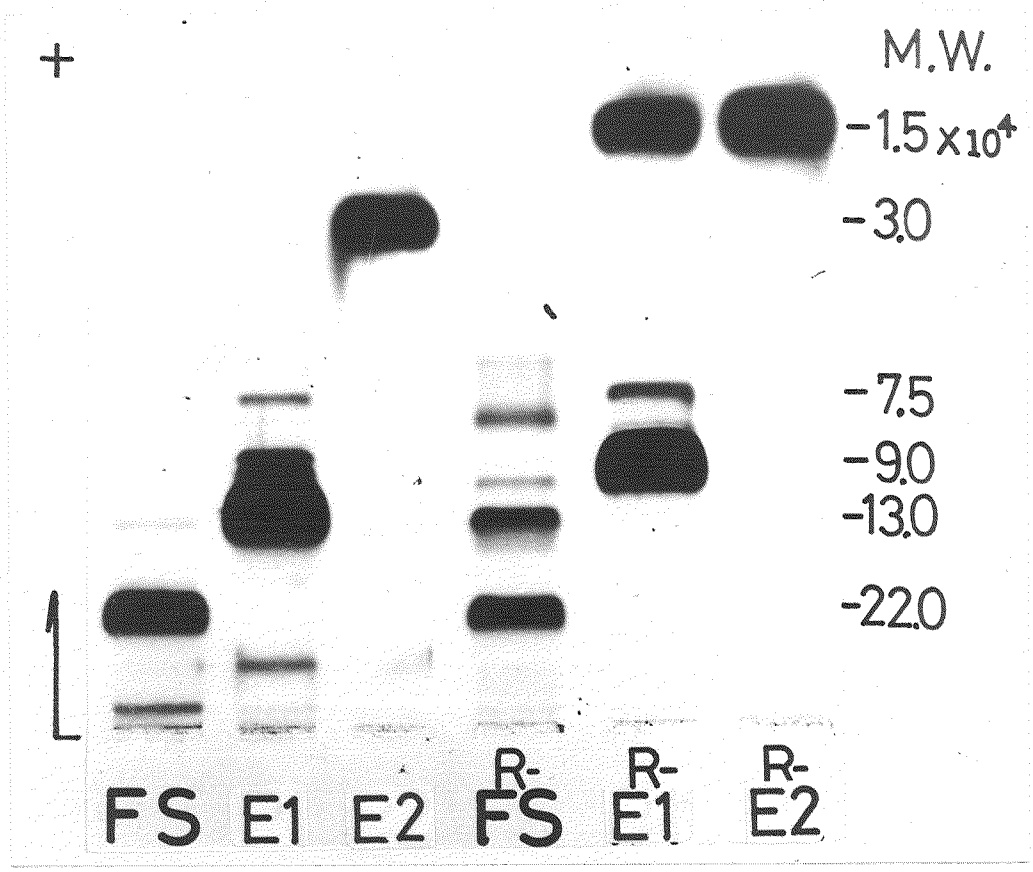


Fig. 17. SDS polyacrylamide gel electrophoresis. R-FS, R-E1 and R-E2: each protein reduced with 2-mercaptoethanol. After electrophoresis, proteins were stained with Coomassie brilliant blue R-250.

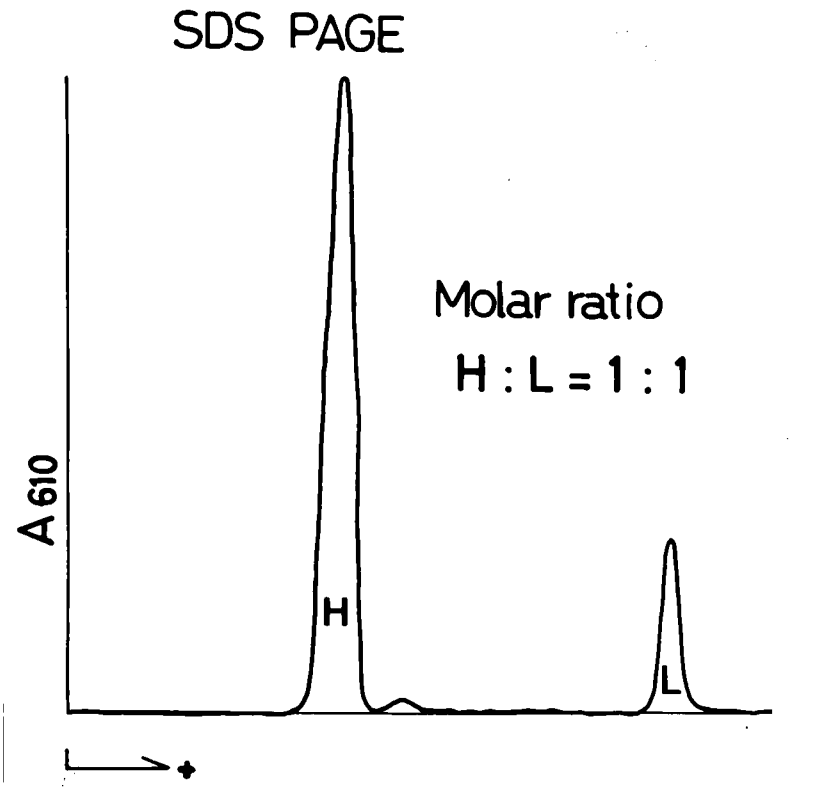


Fig. 18. Spectrophotometric scan of SDS polyacrylamide gel of reduced E1 (R-E1)(Fig. 17). The electrophoretically separated proteins were stained with Coomassie brilliant blue R-250. A molar ratio of 1:1 is obtained for the two subunits (mol. wt. 90,000(H) and 15,000(L)) of E1 of rainbow trout egg yolk.

9. Determination of phosphorus

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.

10. Amino acid composition

The amino acid compositions of FS, E1 and E2 were shown in Table 2. The values for 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.

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.

Table 2. Amino acid composition of FS, E1 and E2 of rainbow trout

| 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 |

The results are expressed as moles/100 moles of amino acid.

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 | 1913 | 271 | 2184 | 2201 |

11. Sedimentation coefficient

The analytical ultracentrifugal experiment of purified proteins from trout was shown in Figs. 19, 20 and 21. The infinite dilution value of the sedimentation coefficient, $s^0_{20,w}$, of FS and E1 was 10.5S and 9.7S, respectively. The value of $S^0_{20,w}$ of E2 could not be calculated because of its asymmetrical peak (Fig. 21).

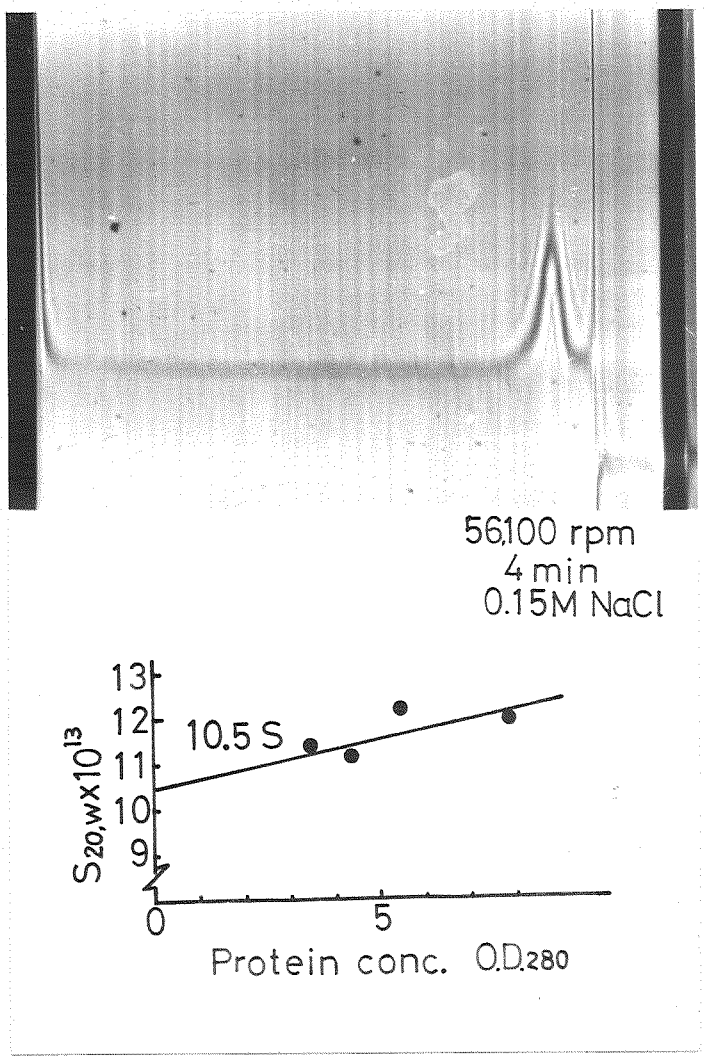


Fig. 19. Ultracentrifugal pattern of purified trout FS and concentration dependency of the sedimentation rate.

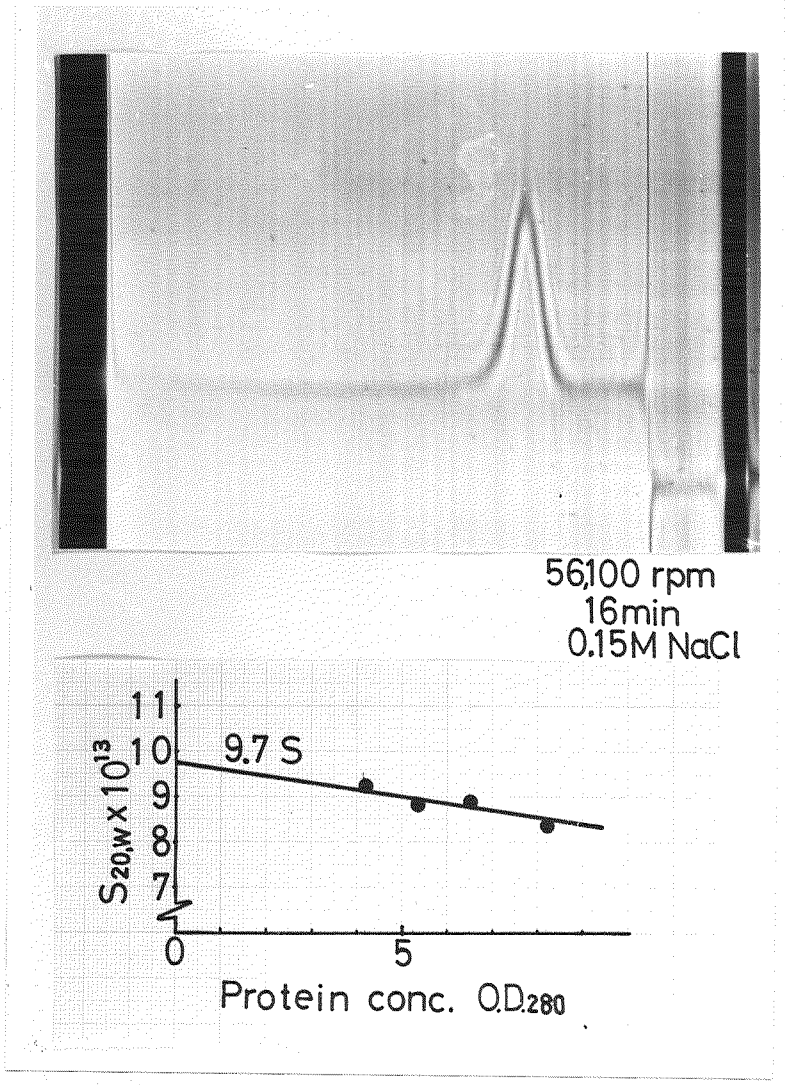


Fig. 20. Ultracentrifugal pattern of purified trout E1 from egg yolk proteins and concentration dependency of the sedimentation rate.

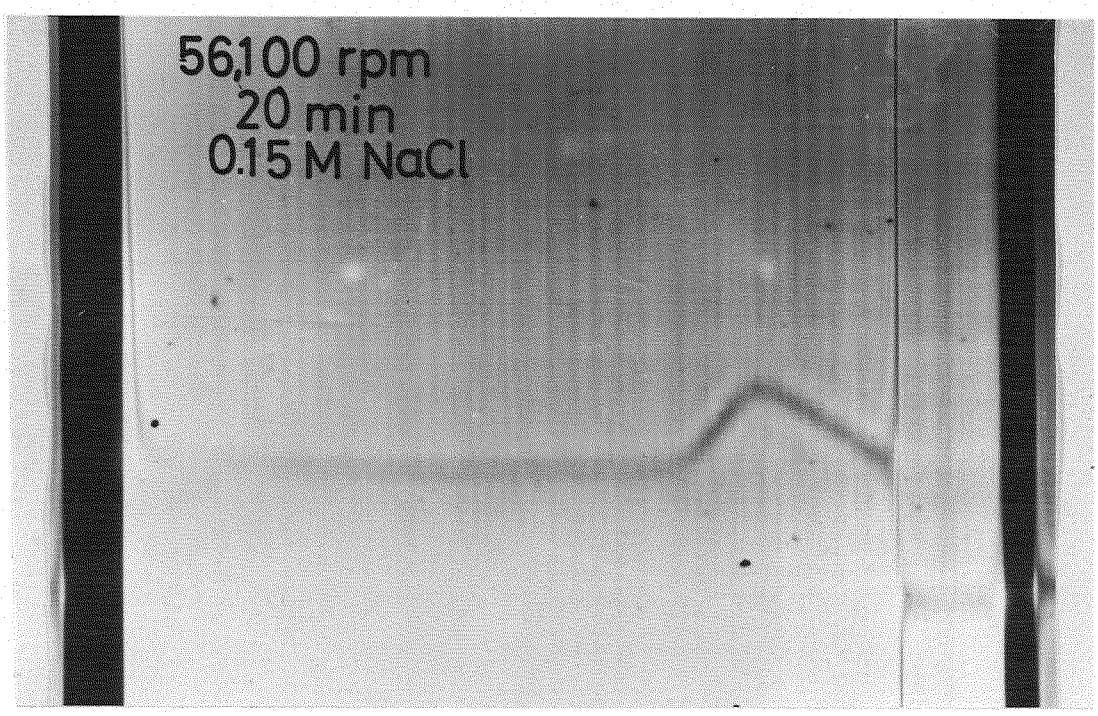


Fig. 21. Ultracentrifugal pattern of puried trout E2 from egg yolk proteins.

12. Extinction coefficient

Extinction coefficient ($E^{1\%}_{1cm,\ 280nm}$) 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.

13. Isoelectric point

Patterns of isoelectrofocussing of purified proteins of FS, E1 and E2 were shown in Fig. 22. FS revealed somewhat heterogeneous isoelectric points, showing three peaks as shown in 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 focussing of the E1 and E2 displayed rather homogeneous peak as seen in Fig. 22B and 22C. PI of E1 and E2 were determined to be 10 and 7.2, respectively.

14. Amounts of FS in fish at various ovarian stages

Amount of FS in female fish was determined by a single radial immunodiffusion techniques on 1.2% agarose gel containing anti-trout FSSP. Figure 23 shows the reactions of individual samples and varying amounts of purified protein used as standard. 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 stages is summarized in 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 µg/ml. Mean levels of concentration at various maturational stages were 147 µg/ml for immature, 11,080 µg/ml for maturing, 3,166 µg/ml for ripe and 640 µg/ml for spawned-out stages, respectively.

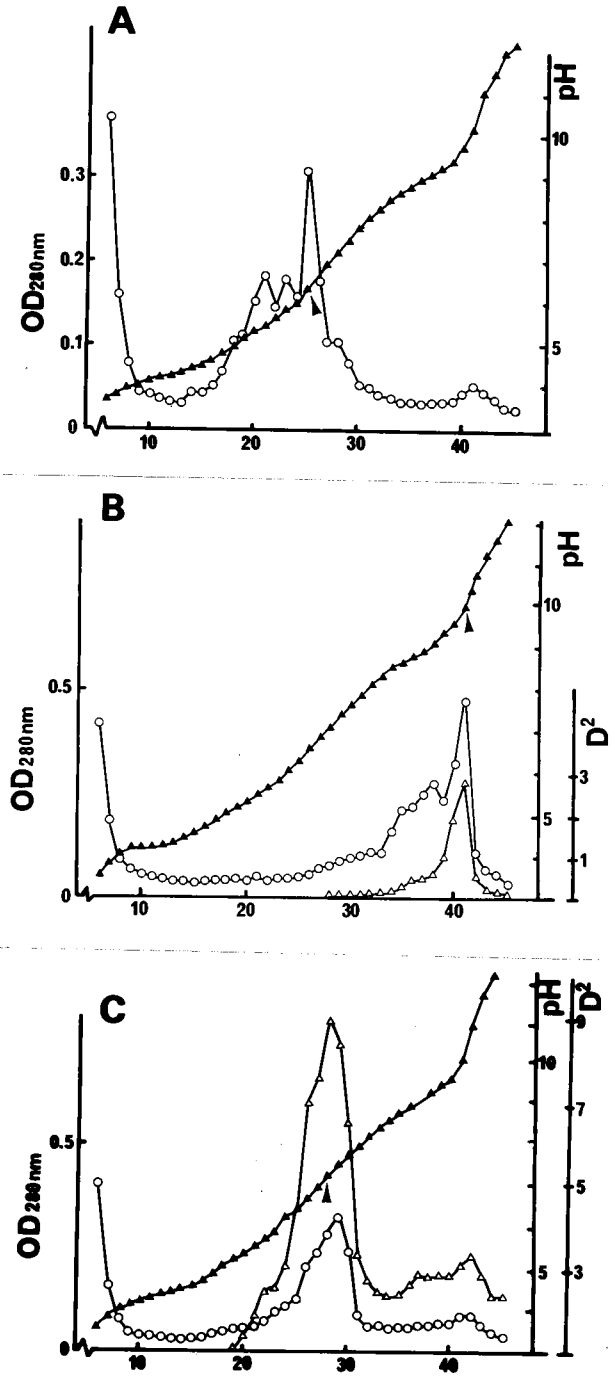


Fig. 22. Patterns of isoelectrofocussing of purified proteins of FS(A), E1(B) and E2(C) isolated from rainbow trout. Ampholite: pH 3-10, voltage 700 V for 72 hr. Sample: 1.0 ml. The pH (▲) was measured with pH meter at 0°C and each protein was determined with a single radial immunodiffusion (△) and/or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

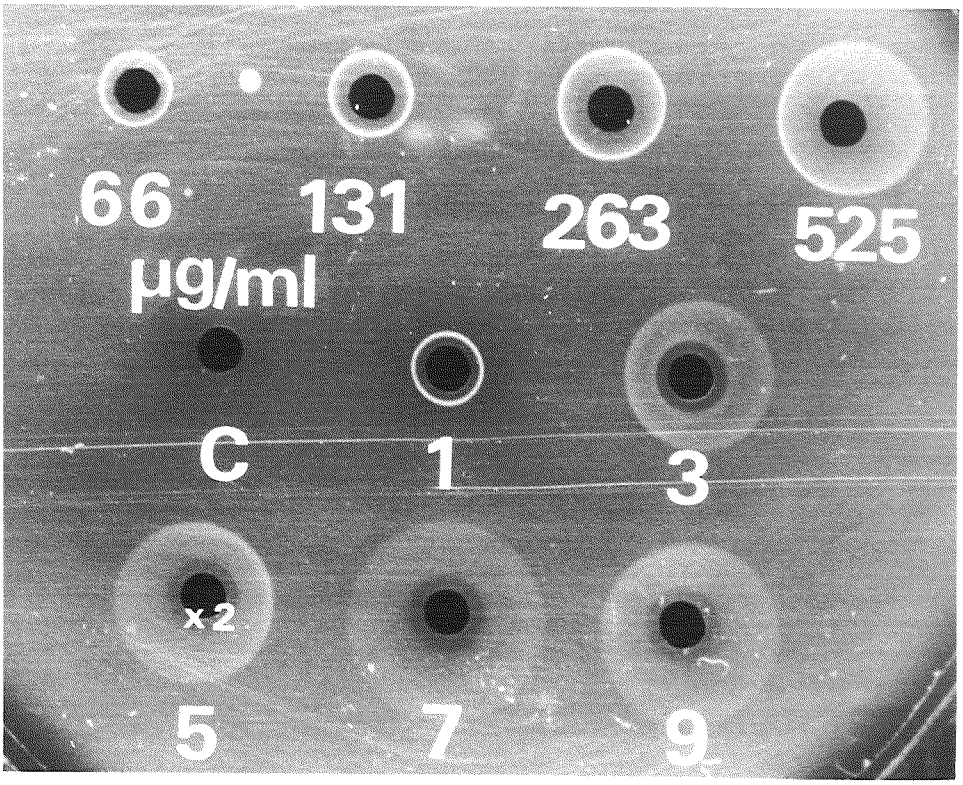


Fig. 23. Typical immunodiffusion plate (unstained). The agarose contained anti-trout FSSP. The antigen wells were filled with varying amounts of purified FS from rainbow trout used as standard and serum samples (C,1,3,5 and 7).

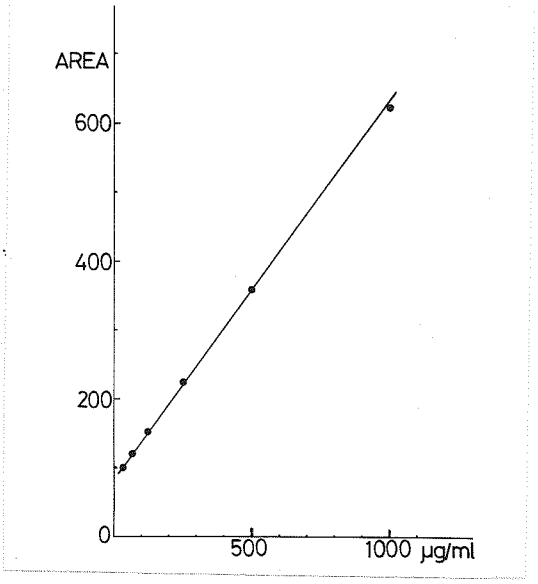


Fig. 24. The standard curve determined by a typical single radial immunodiffusion (see Fig. 23). Horizontal line indicates the concentration of trout FS.

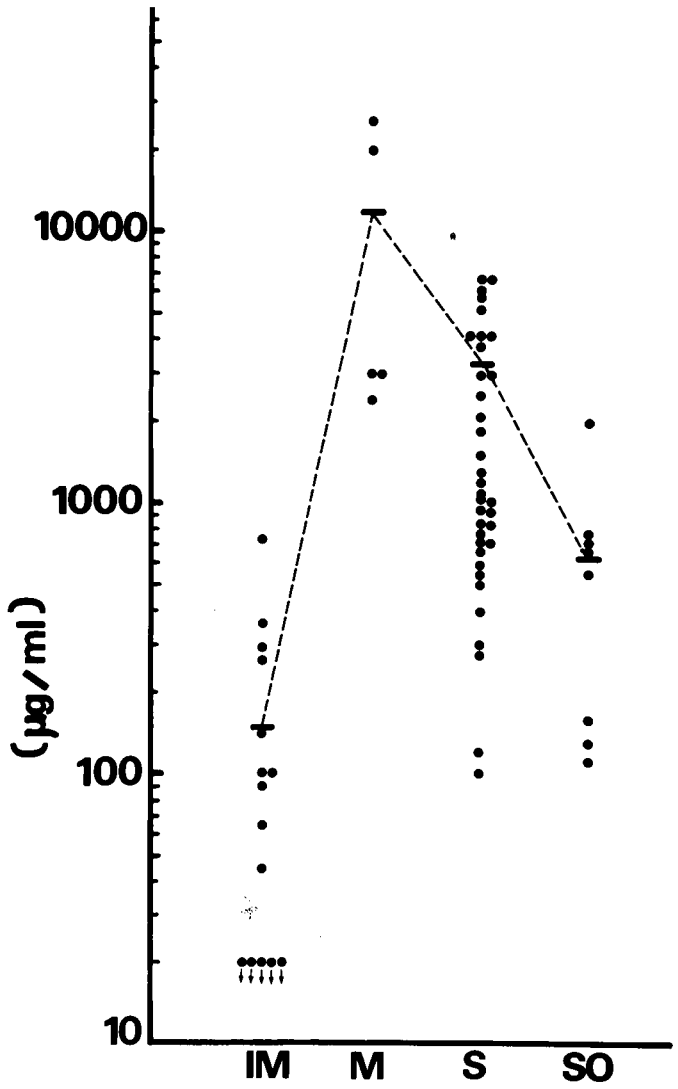


Fig. 25. The concentration of FS in the serum of female rainbow trout at various maturational stages. IM: immature, M: maturing, S: ripe, SO: spawned-out.

15. Production of FS in male and in immature trout by a treatment with estradiol-17β

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. A typical immunodiffusion pattern is shown in Fig. 26. Two precipitin lines were developed with the maturing female fish as well as with estrogen-treated male or immature female. One of the two lines fused completely with the line of purified FS, indicating the production of FS in the estrogen-treated fish.

The iron binding activity of the FS produced after the treatment of fish with the hormone was assessed by autoradiography of the immunoelectrophoresis with $^{59}FeCl_3$ (Fig. 27),

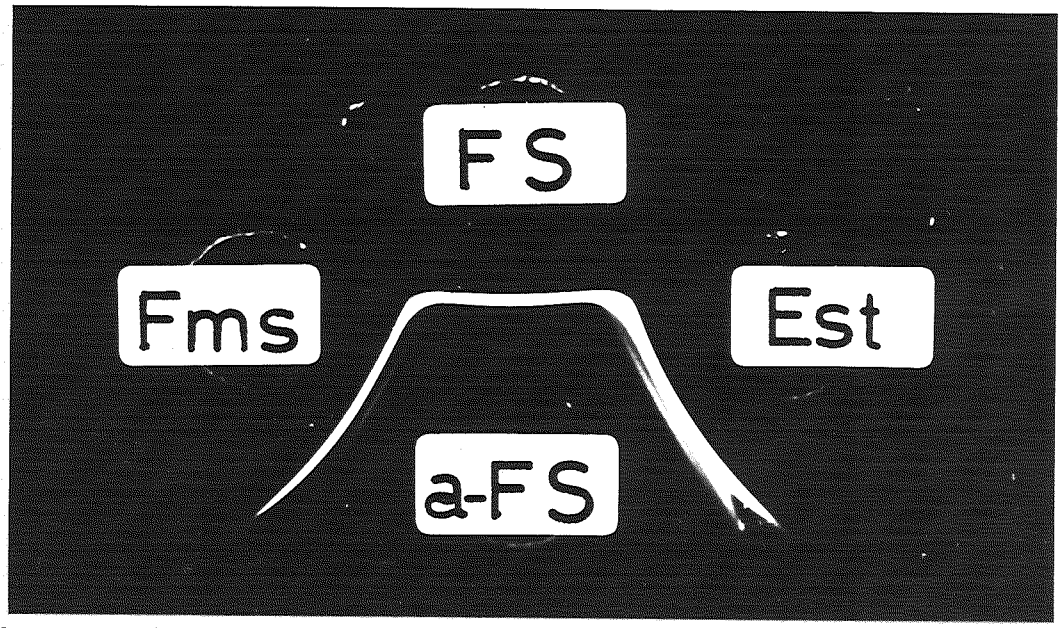


Fig. 26. Precipitin reaction of FS, sera of maturing female and estrogen-treated male rainbow trout. Fms: serum of maturing female fish. Est: serum of estrogen-treated fish (6 days after injection). FS: female-specific iron-binding serum protein of rainbow trout, a-FS: anti-trout FSSP.

and by gel filtration on Sephadex G-200 of the serum admixed with $^{59}FeCl_3$ (Fig. 28). As shown in Fig. 27, precipitin lines of hormone-treated fish developed by anti-trout FSSP show the radioactivity for $^{59}Fe$, whereas the control fish (male and immature female) do not show any radioactive precipitin line other than transferrin. Gel filtration of the serum from the hormone-treated fish (Fig. 28) disclosed the appearance of a new radioactive peak at the higher molecular weight fraction besides the peak of transferrin as similar to Fig. 5.

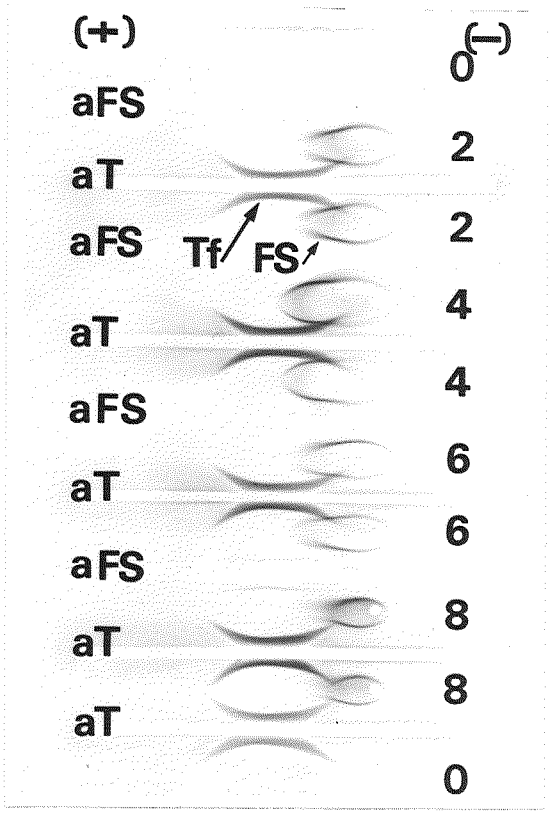


Fig. 27. Autoradiography of immunoelectrophoresis of $^{59}Fe$-binding proteins in estrogen-treated rainbow trout sera. The antigen wells were filled with the serum collected from fish 0(control), 2,4,6 and 8 days after administration of estrogen. Antisera used were anti-trout FSSP (aFS) and anti-trout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

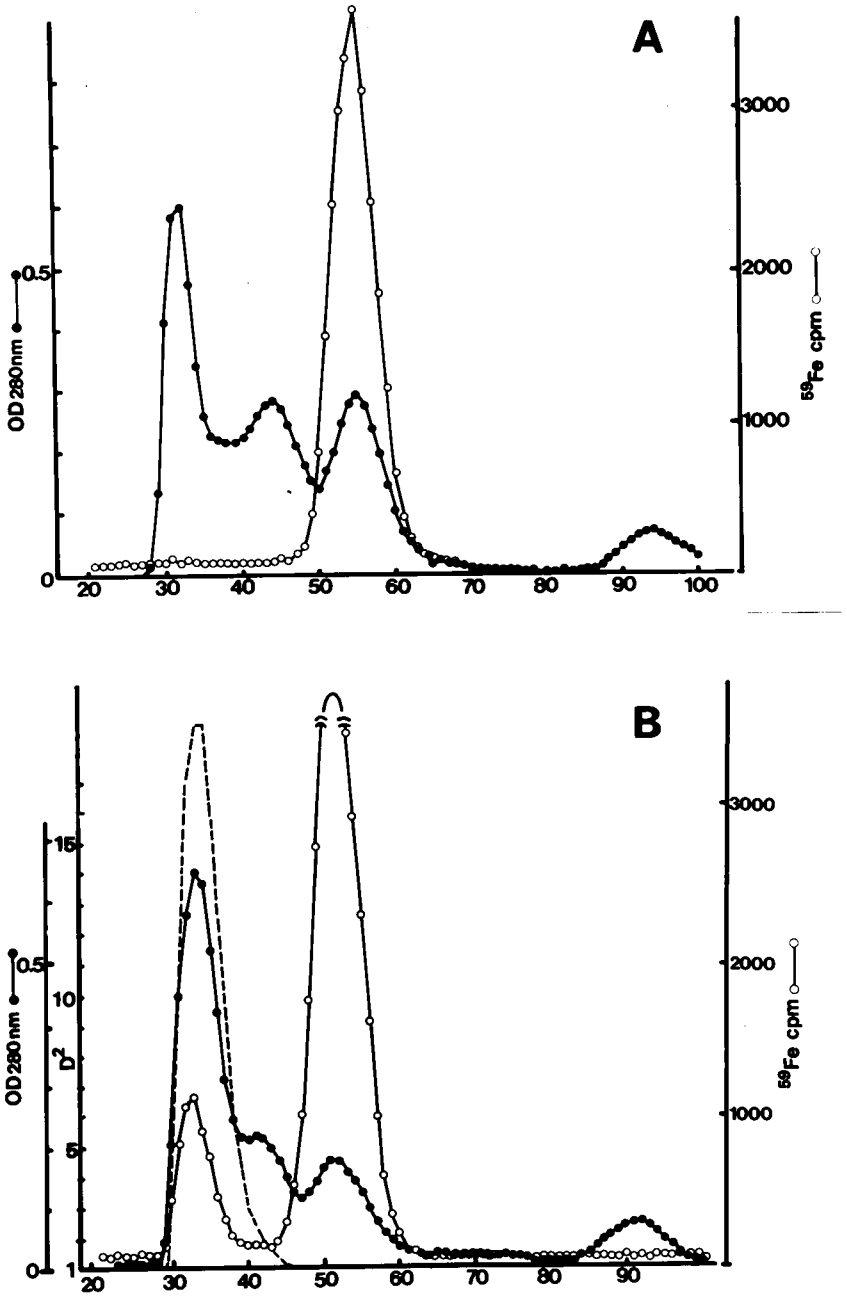


Fig. 28. Gel filtration on Sephadex G-200 of rainbow trout serum collected from non-estrogen treated (A) and estrogen-treated (B) fish. The presence of FS (dotted line) was assessed by the Mancini method using anti-trout FSSP. Column: 2.0×47cm, Elution buffer: 0.02M Tris-HCl, pH 8.0, containing 2% NaCl and 0.1% $NaN_3$. Each serum was 0.5 ml and was mixed with 10 µl of $^{59}Fe$ (0.25 µCi).

Results of radio immunoelectrophoresis as shown in Fig. 27 surmised the increase of FS after longer time-interval. Therefore, more precise determination of FS after the hormone treatment was made using the single radial immunodiffusion with anti-trout FSSP for the determination of FS. Four different doses of estradiol-17β, 40 μg, 400 μg, 1.4 mg and 7 mg per kg of fish body weight, were administered once into muscle of each 10 fish in four groups. Two fish in each group were sacrificed for collecting the serum at days after injection as indicated in Fig. 29. As can be seen in Fig. 29, the gradual increase of FS after administration of

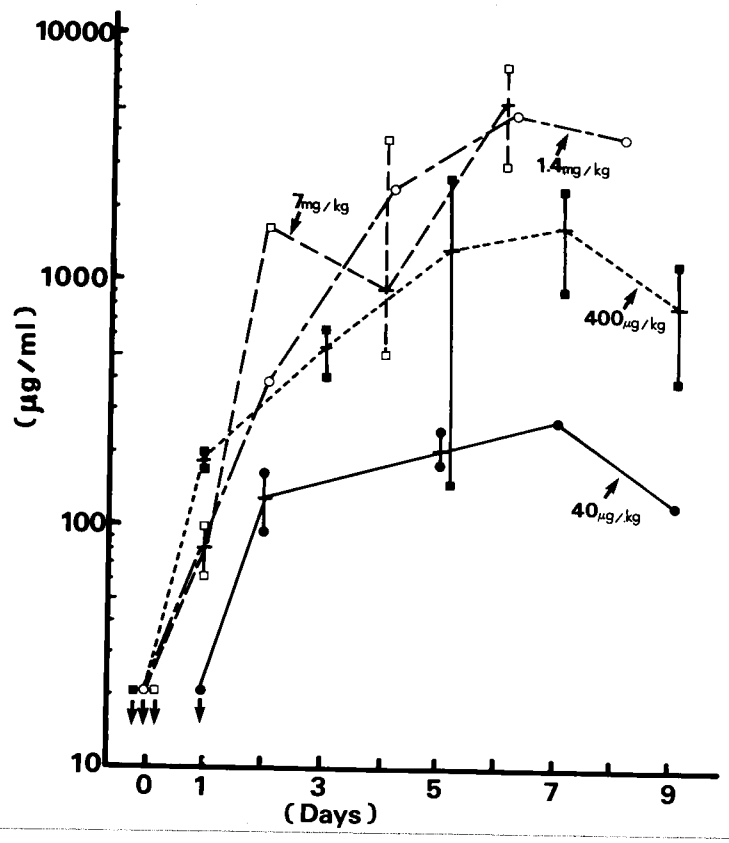


Fig. 29. The concentration of FS of rainbow trout (on a lagarithmic scale) at different times after the injection of various doses of estradiol-17β.

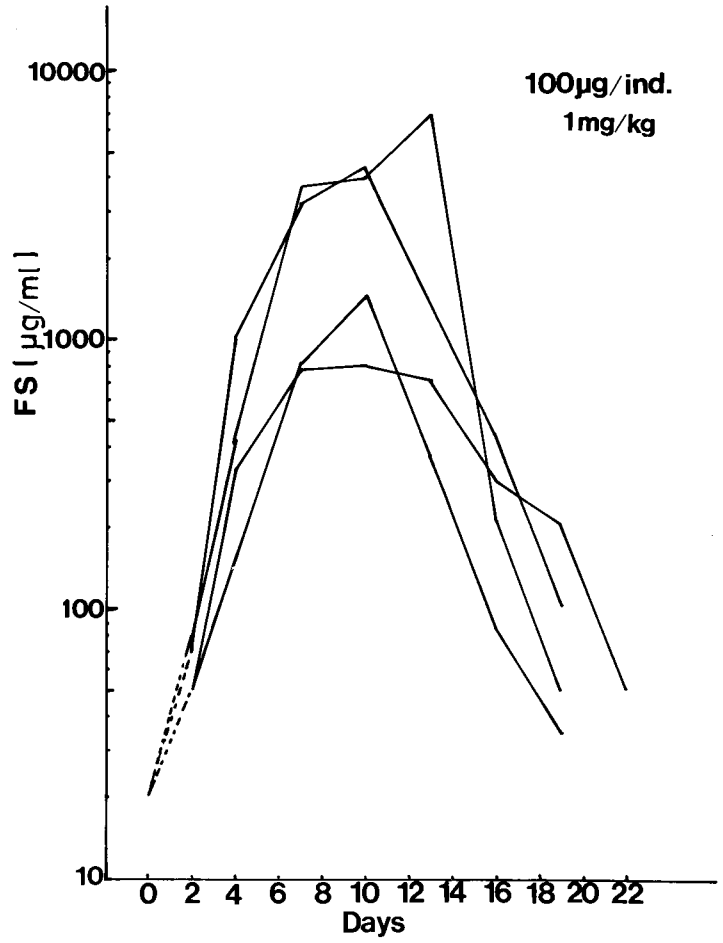


Fig. 30. The concentration of FS of individual rainbow trout after administration with 1 mg of estradiol-17β per Kg body weight.

estradiol-17β was observed until the day around 7. After that, the concentration of FS tended to decrease. Dose response of FS production was also found up to the 1.4 mg hormone per Kg body weight.

Preceding experiments shown in Fig. 29, indicated a gradual decrease of FS after the highest concentration at around day 7. Such a phenomenon was further confirmed by surveying the change in concentration of FS of the same

individual fish collecting the serum repeatedly for a long period up to 3 weeks. Figure 30 shows the changes of the FS concentrations for four individual fish administered with 1 mg of estradiol-17β per Kg body weight. FS reached its highest concentration on day 7 to day 12, and then disappeared from the serum within 10 days.

16. Histological changes of the liver following estrogen administration

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

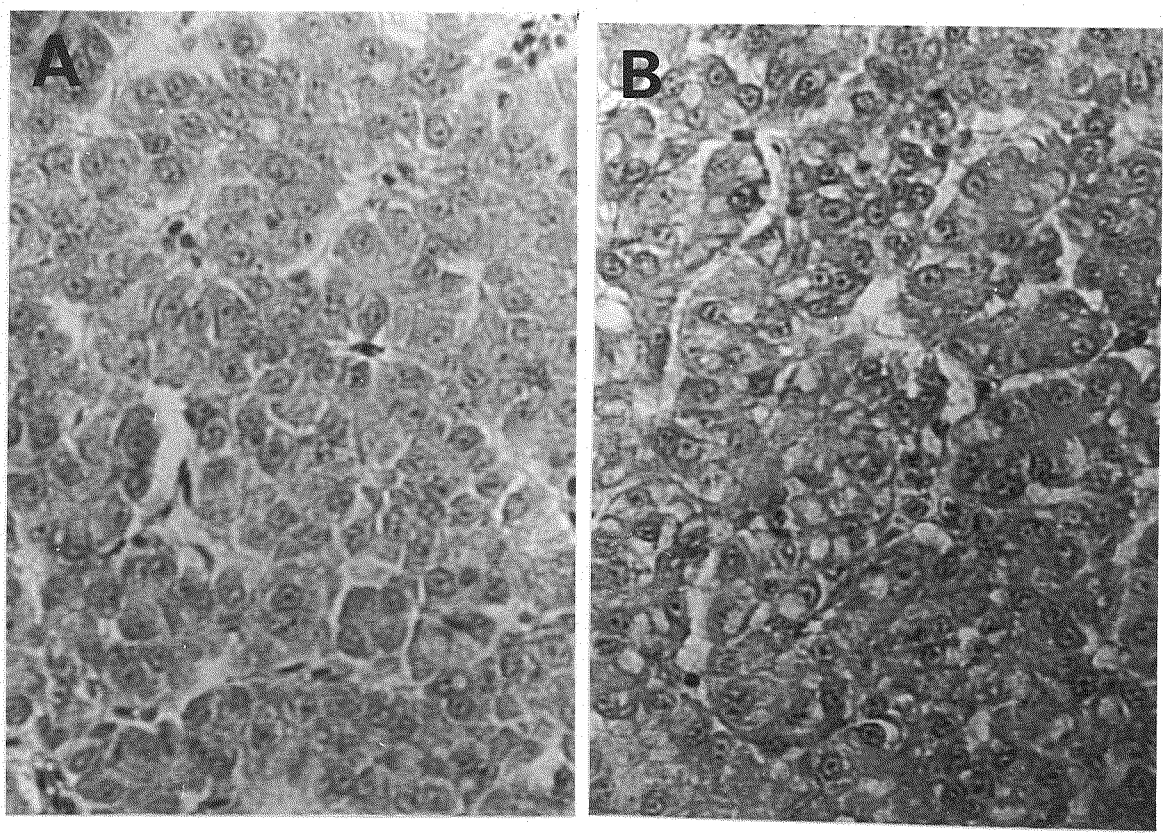


Fig. 31. Liver cells of immature rainbow trout (A) and of estradiol-17β-treated immature fish (B).

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

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

Representative results obtained in several immunofluorescence experiments with use of the anti-trout FSSP are shown in Fig. 32. 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. The results indicate that the liver cells are responsible for the synthesis of female-specific protein(s) stimulated by estrogen.

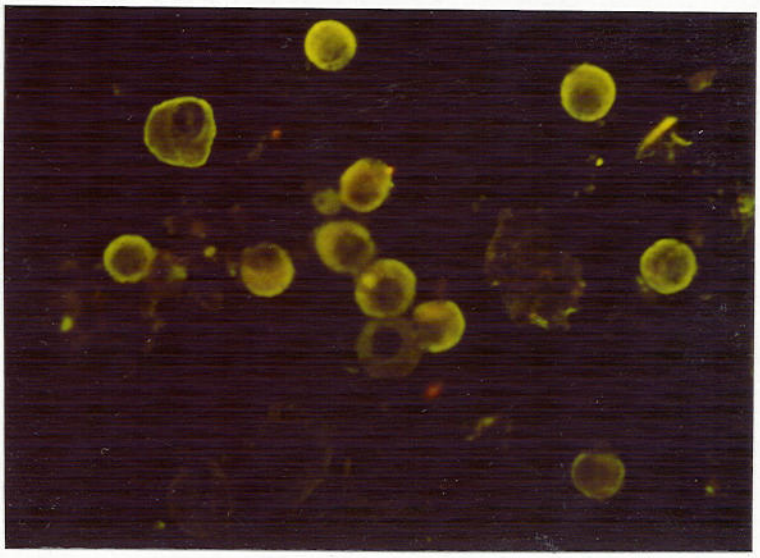

Fig. 32. Localization of female-specific serum protein(s) in fixed rainbow trout liver cells as revealed by immunofluorescence. The cytoplasm is homogeneously stained.

B. Chum salmon

1. Sexual differences in serum protein

Typical patterns of 7.5% polyacrylamide gel disc electrophoresis of chum salmon in the spawning season are shown in Fig. 33. A band appearing near origin, observed in some females was never seen in males. This band was seen in 46 out of 65 individual samples from ripe females.

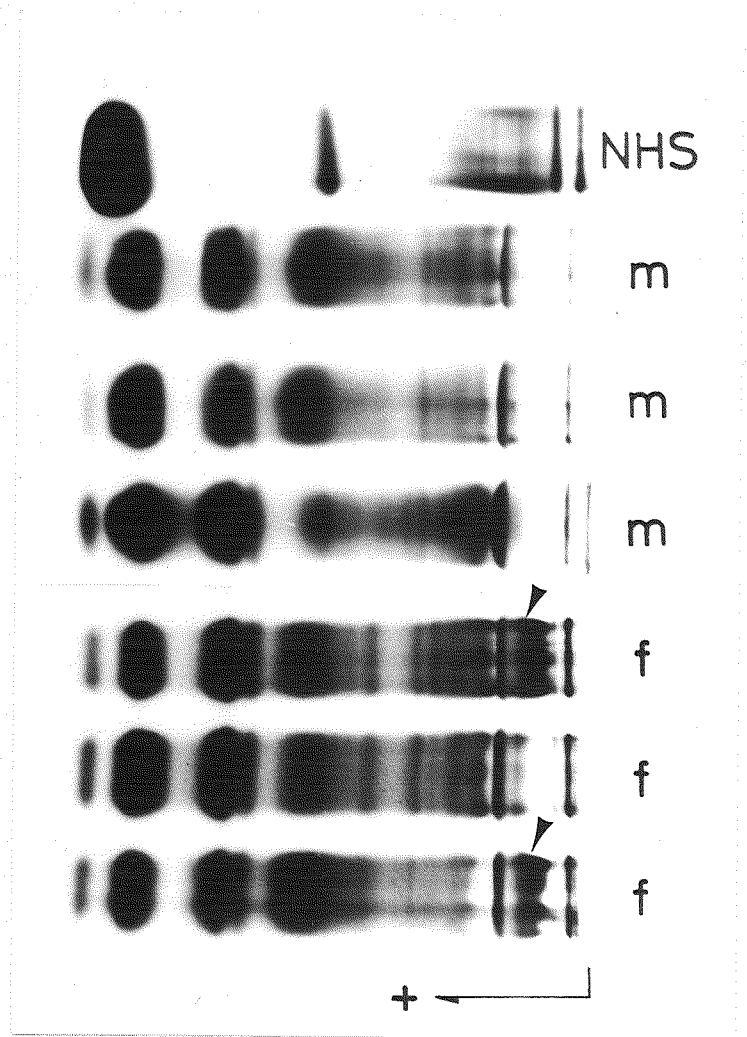


Fig. 33. 7.5% polyacrylamide disc electrophoresis of ripe chum salmon serum. The band indicated by arrow was observed in some females but not in males. NHS: normal human serum, m: male serum, f: female serum.

Electrophoretic patterns and autoradiography on cellulose acetate membrane in chum salmon are shown in Fig. 34. Although much differences between sexes were not seen on the electrophoretic pattern stained for protein, some female samples showed strongly stained components in β-globulin region. Autoradiography, however, displayed distinct differences between female and male. As shown in Fig. 34B, female sera showed two strong radioactive bands, while male sera showed a single weak radioactive band. The mobility of one of the two bands of female sera was the same as that of the band of male sera. This component is considered to be transferrin.

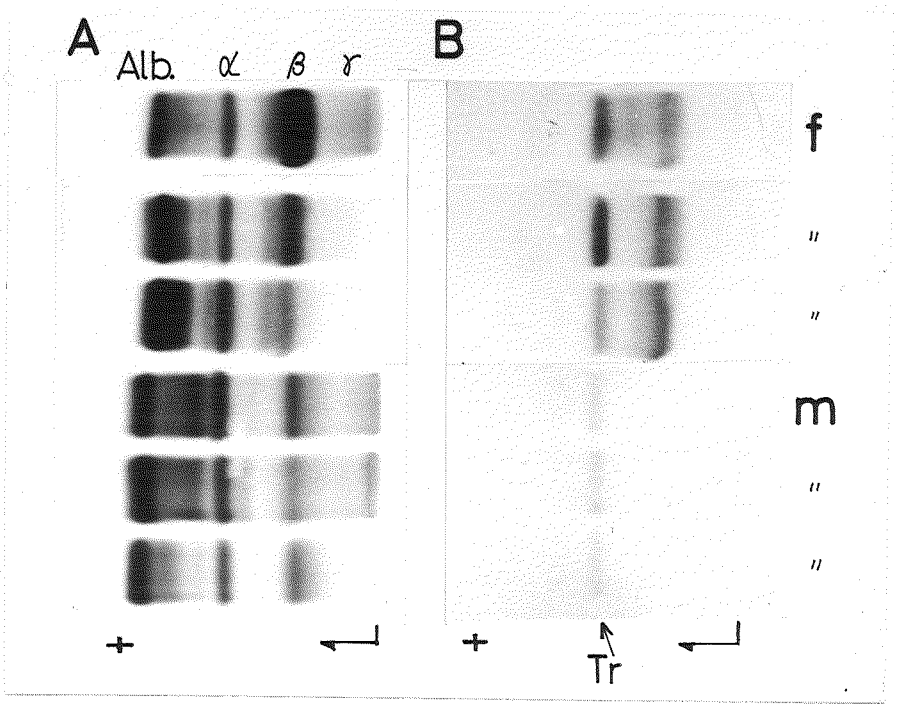


Fig. 34. Electrophoresis of chum salmon sera. A: cellulose acetate electrophoresis (Cellogel), B: autoradiography with $^{59}Fe$; f: female sera, m: male sera, Tr: transferrin. To 25 µl of the serum was added 10 µl of $^{59}FeCl_3$ solution with an activity of 0.1 µCi, corresponding to 0.003 µg Fe. After incubation approximately 1 µl of the mixture was subjected to electrophoresis, and the gels were exposed to X-ray films for 2 weeks.

Thin-layer gel filtration and its autoradiography of the pooled serum of females as well as males with $^{59}Fe$ with Sephadex G-200, superfine, are shown in Fig. 35. Normal human serum was separated into three spots, M (macroglobulin fraction), G(IgG fraction) and A(albumin fraction). Salmon serum of both sexes was also separated into three spots. The spot corresponding to M fraction in the female was 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 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.

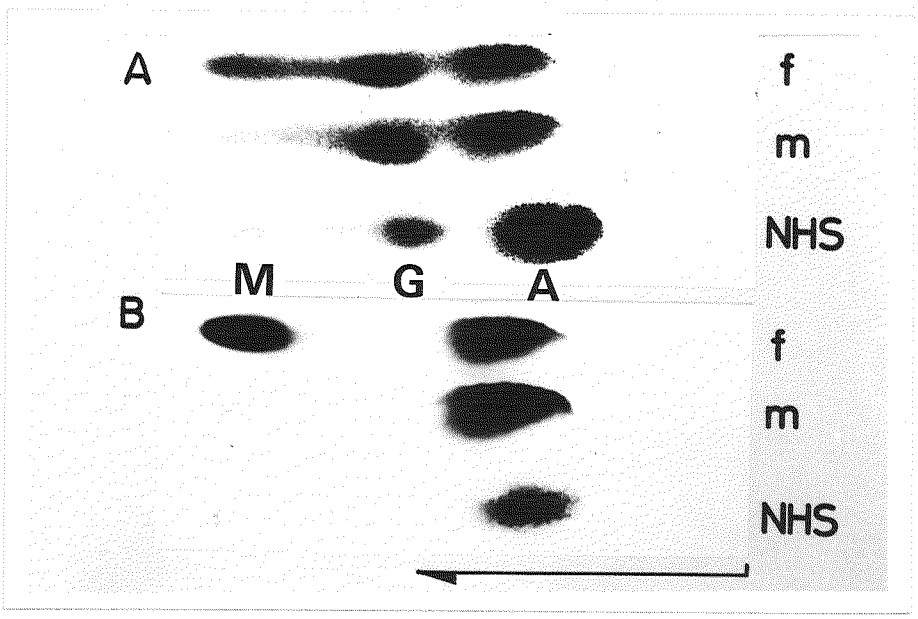


Fig. 35. Thin-layer gel filtration (A) and its autoradiography (B) with $^{59}Fe$ on Sephadex G-200, superfine. To 25 µl of the serum was added 10 µl of $^{59}FeCl_3$ solution with an activity of 0.5 µCi. After being incubated for 1 h, 5 µl of the mixture was subjected to thin-layer chromatography. NHS, m, f: same as in Fig. 33.

The results of immunoelectrophoresis and its autoradiography of salmon serum mixed with $^{59}FeCl_3$ are shown in Fig. 36. 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). As shown in Fig. 36B, autoradiography of the immunoelectrophoresis with polyvalent antiserum against salmon serum indicated that male serum yielded only one radioactive precipitin line at the α-globulin region which was commonly observed in female serum. This component is considered to be transferrin. On the other hand, the female serum showed another radioactive line which migrated to the β-globulin region. Although two precipitin lines at the β-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 radio-

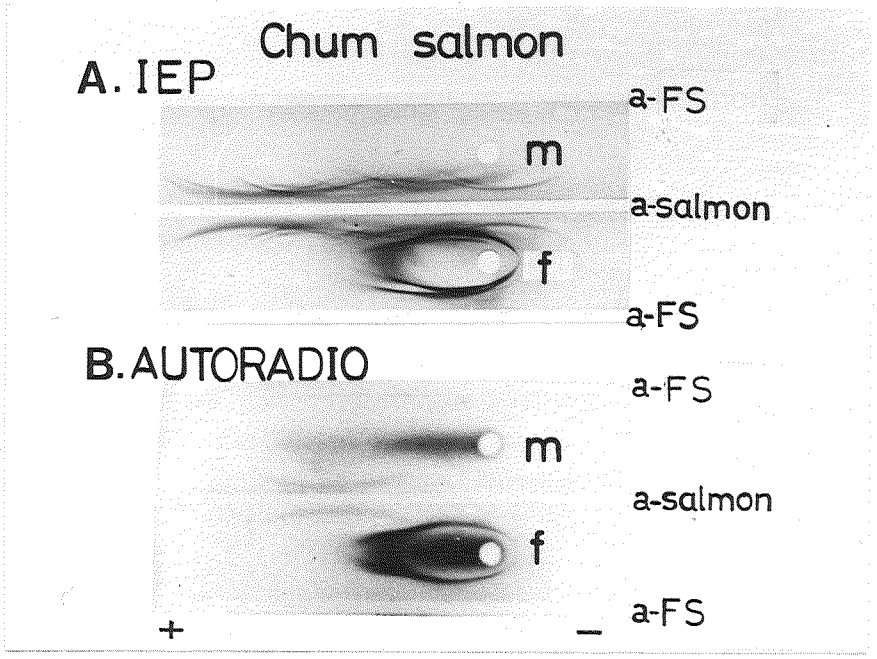


Fig. 36. Immunoelectrophoresis of chum salmon sera. A: protein staining, B: autoradiography with $^{59}Fe$, a-salmon: anti-salmon serum, a-FS: anti-salmon FSSP.

active iron (Fig. 36B). This female-specific iron-binding protein was designated as FS of chum salmon (see page 33 ).

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 (2.2×23cm) which had been equilibrated with the same buffer. Proteins were eluted

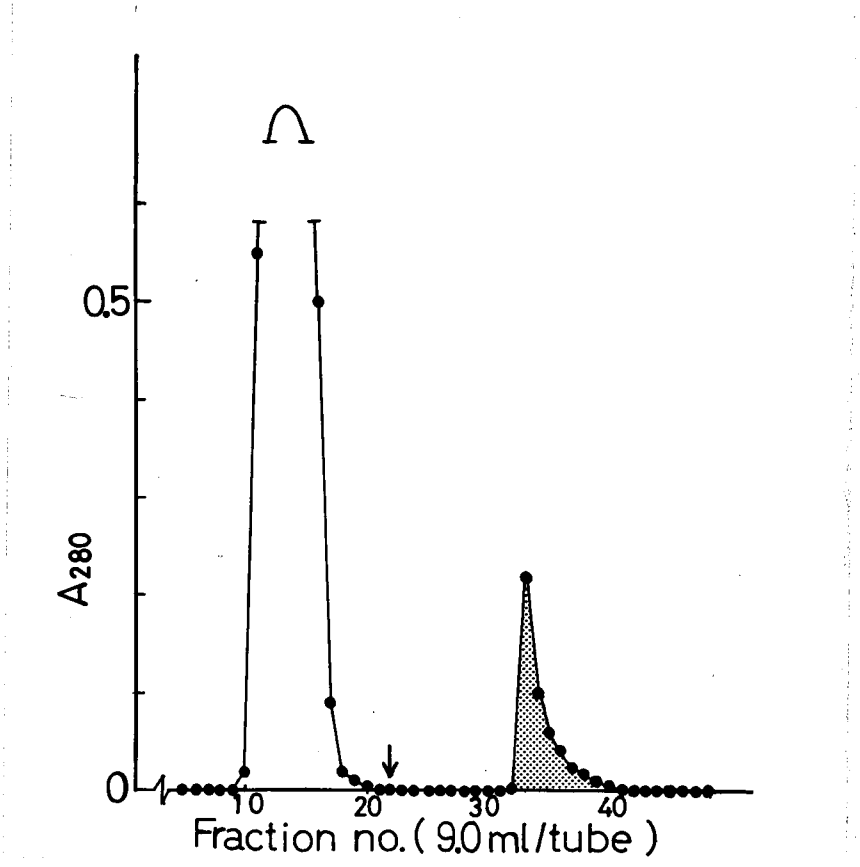


Fig. 37. Chromatography of female chum salmon serum on a hydroxylapatite column. The proteins were eluted with 0.4 M potassium phosphate buffer, pH 6.8 in the first step and in the second step (arrow) the protein was eluted with 1.2 M potassium phosphate buffer, pH 6.8. The fractions in shadowed area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

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. The elution pattern of hydroxylapatite column is shown in Fig. 37. The fractions with 1.2 M potassium phosphate buffer was concentrated and then applied to a column of Sepharose 6B (2.2×59cm) with 0.02 M Tris-HCl buffer, pH 8.0, containing 2% NaCl and 0.1% $NaN_3$. The elution pattern is shown in 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.

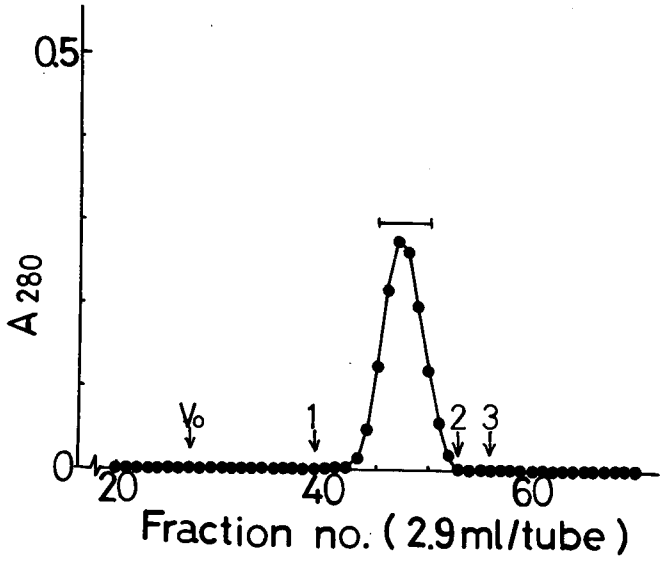


Fig. 38. Elution diagram of a partially purified preparation of chum salmon FS. The fraction containing FS isolated from hydroxylapatite column was applied to a column of Sepharose 6B (2.2×59) equilibrated with 0.02 M Tris-HCl buffer, pH 8.0 containing 2% NaCl. The fractions marked with a bar which was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

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

Egg yolk proteins were isolated essentially according to the procedure of Markert & Vanstone (1971). The eggs collected from ripe salmon were washed with 0.9% NaCl, and broken by a mixer. The 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 collected. This clear solution was dialysed against distilled water overnight in cold. 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

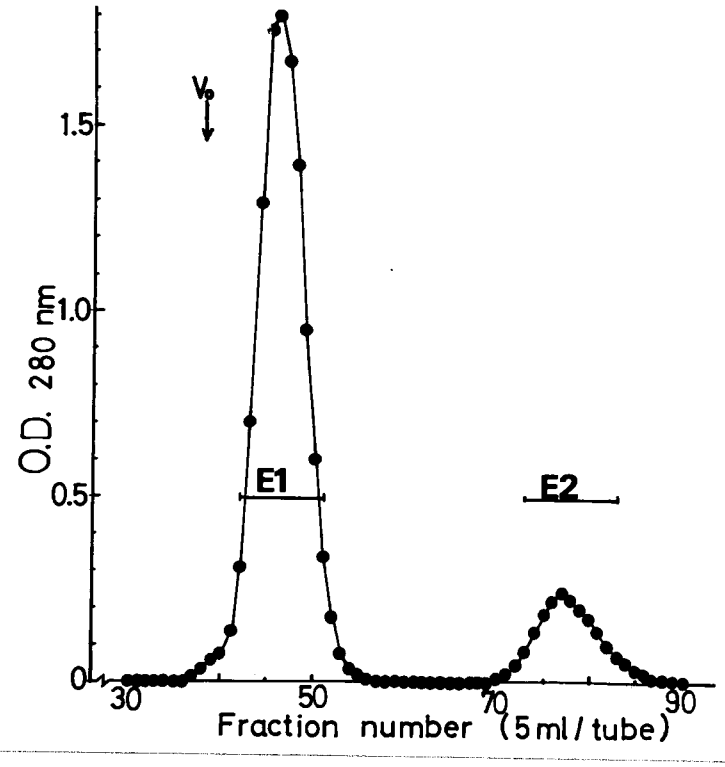


Fig. 39. Gel filtration of egg yolk proteins isolated from chum salmon egg. Column: Sephadex G-200 (2.6×95cm). Elution buffer: 0.02 M Tris-HCl, pH 8.0, containing 2% NaCl and 0.1% $NaN_3$. E1, E2: two major components were disignated as E1 and E2 just same as in rainbow trout egg (see Fig. 8). Vo: void volume.

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 (2.6×95cm), and eluted with the same buffer as in case of the Sepharose 6B. Two peaks were obtained from the gel filtration as indicated in Fig. 39 and were collected separately. They were tentatively denoted as E1 and E2 of salmon (see page 34).

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 as shown in Fig. 40. The mobility of the purified material was faster than that of the component in the original sera. This change in mobility of the purified material was confirmed by a immunoelectrophoresis with anti-salmon FSSP (Fig. 41). The purified preparation of FS retained the iron binding activity as shown in 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 antigenecity 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).

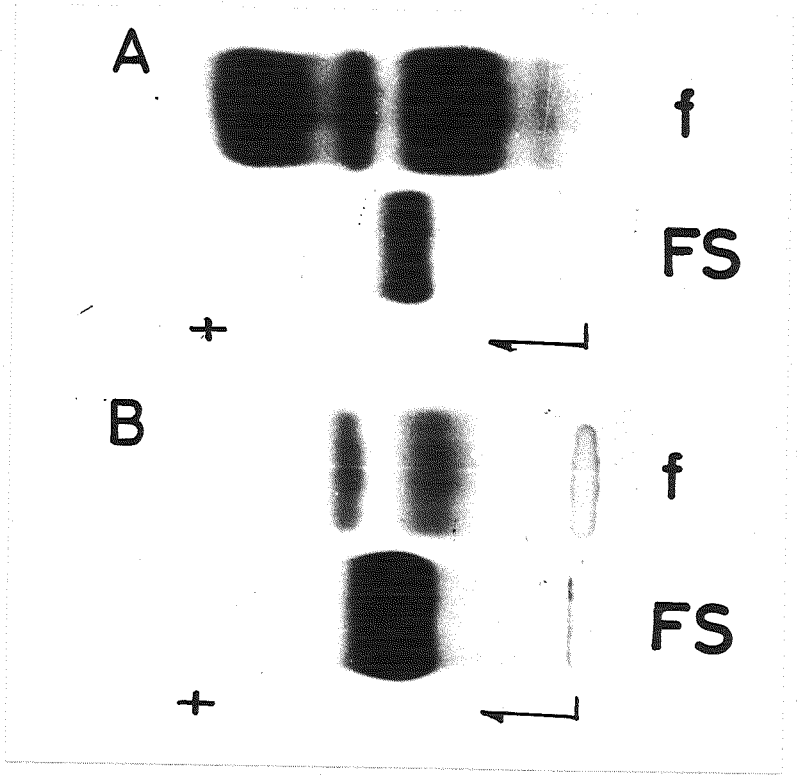


Fig. 40. Electrophoresis of purified FS of chum salmon. A: Cellulose acetate membrane electrophoresis of female salmon serum (f) and the purified FS(FS), B: its autoradiography.

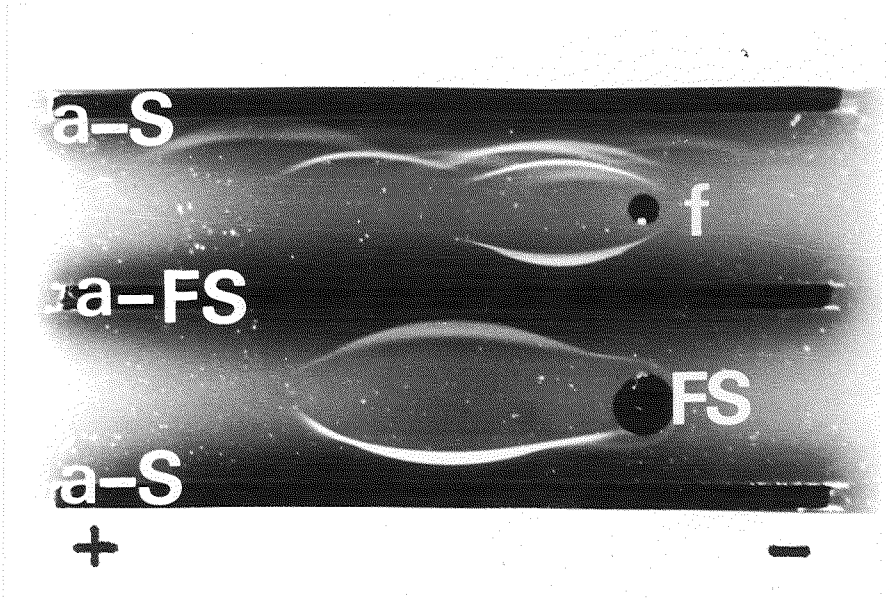


Fig. 41. Immunoelectrophoresis of purified FS of chum salmon. a-S: anti-salmon serum, a-FS: anti-salmon FSSP, f: female serum, FS: purified FS of chum salmon.

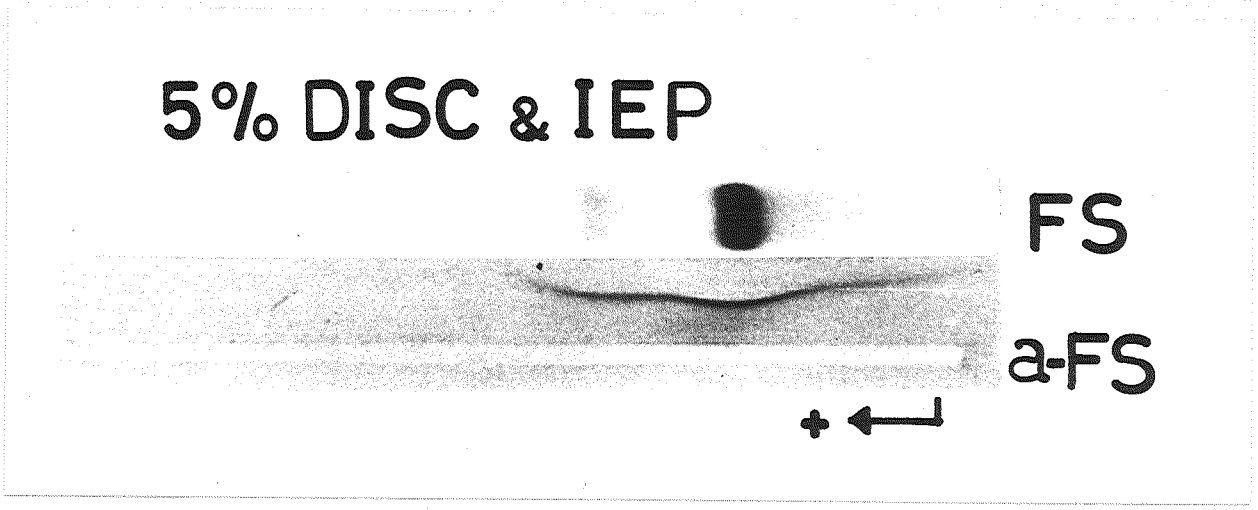


Fig. 42. Disc immunoelectrophoresis of purified chum salmon FS. The sample was subjected first to disc electrophoresis in 5% polyacrylamide gel which was subsequently implanted in 1.2% agarose gel, putting anti-salmon FSSP into the trough. The protein (FS) in polyacrylamide gel diffused into agarose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

Figure 43 shows the pattern of cellulose acetate membrane electrophoresis of purified egg yolk protein, E1 and E2. Each preparation of the egg yolk protein gave a sharp single band, showing a reactivity with polyvalent anti-salmon serum or anti-salmon eggs (Fig. 44), and developing a single precipitin line. On disc electrophoresis, E1 gave a sharp single band in 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.

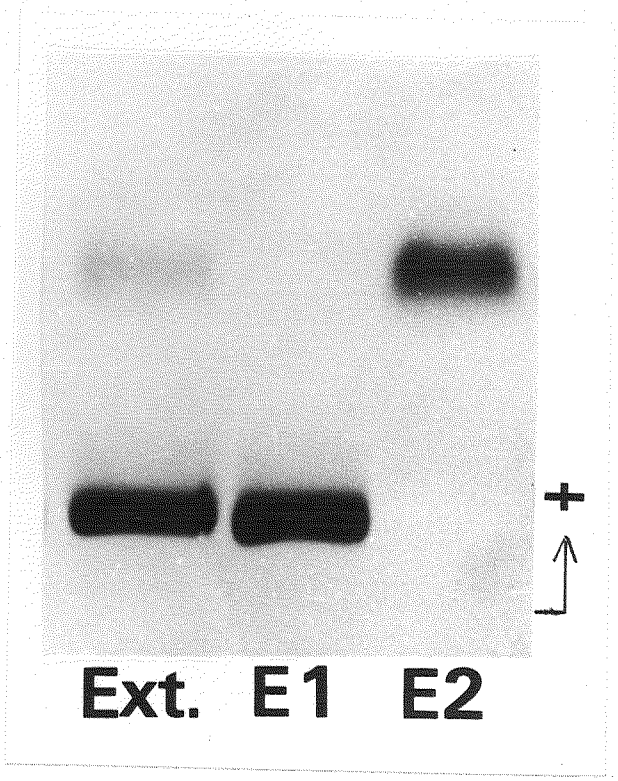


Fig. 43. Cellulose acetate membrane electrophoresis (Cellogel) of egg yolk proteins in chum salmon. Ext.: water insoluble proteins from 0.9% NaCl extraction of chum salmon eggs. E1, E2: two purified egg yolk components isolated by gel filtration.

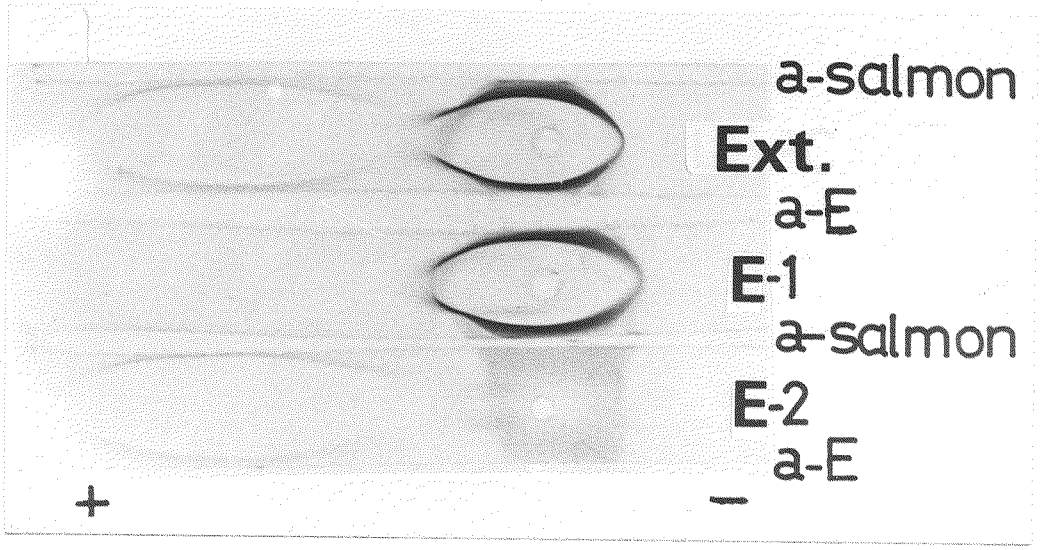


Fig. 44. Immunoelectrophoresis of 0.9% NaCl extracts (Ext.), E1 (E-1) and E2 (E-2) of chum salmon egg. The antisera used were anti-salmon serum (a-salmon) and anti-salmon eggs (a-E).

5. 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 around 220,000-250,000. E1 showed the main band corresponding to a molecular weight of about 130,000 and were split into two subunits (approx mol. wt. 90,000 and 15,000) after reduction with 2-mercaptoethanol. E2 revealed a single band correspond to 30,000, and this band was converted into 15,000 following the reduction.

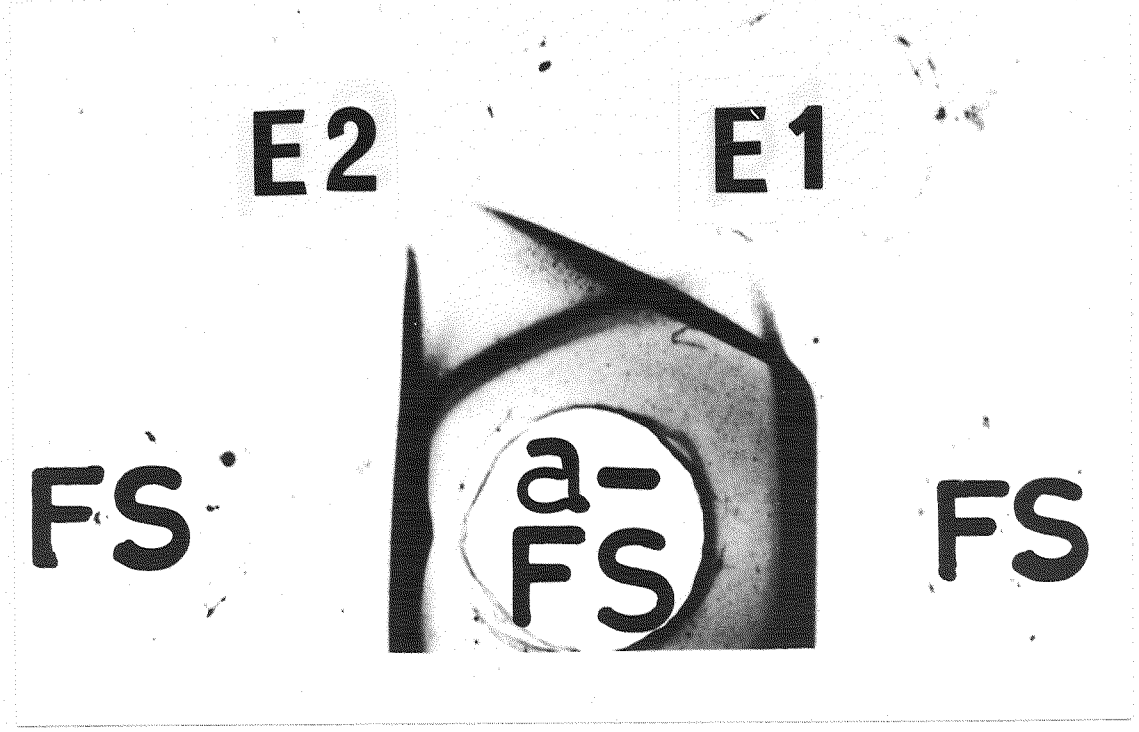


Fig. 45. Precipitin reaction of FS, E1 and E2 in chum salmon. FS: female-specific iron-binding serum protein, E1 and E2: fractions of egg yolk proteins. a-FS: anti-salmon FSSP.

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.

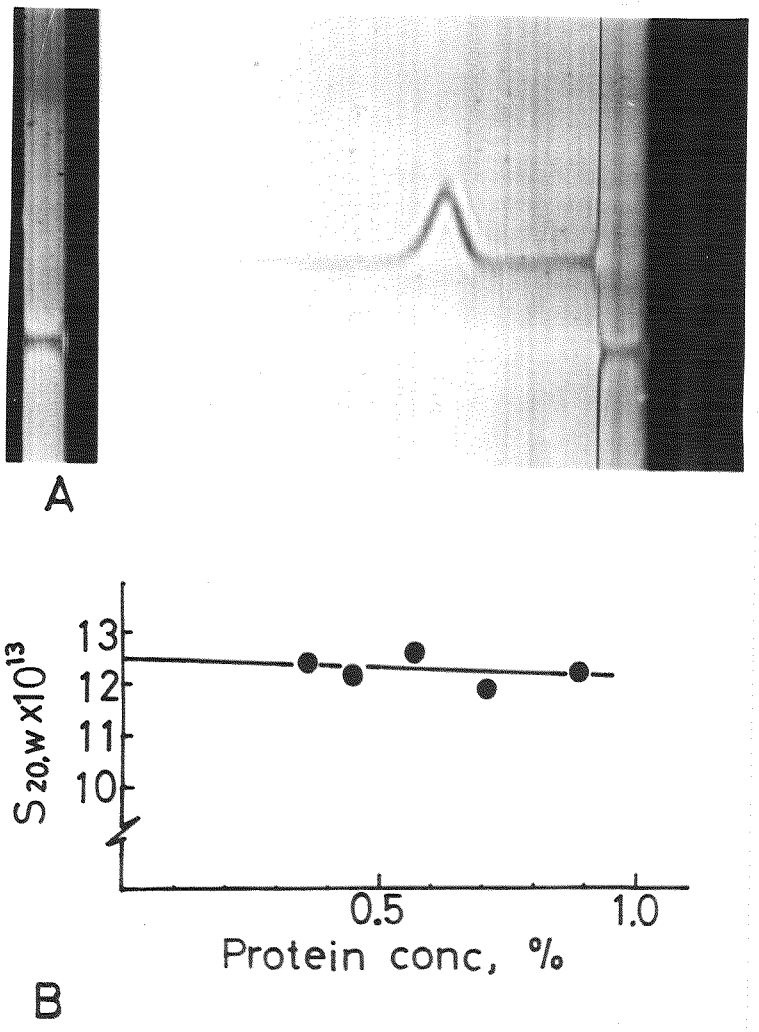


Fig. 46. Ultracentrifugation of purified FS of chum salmon. (A) Schlieren pattern of the purified FS at concentration of 0.9% in 0.15 M NaCl. Photograph was taken 24 min after reaching to 56,100 rpm. (B) Concentration dependency of the sedimentation rate.

## 7. Physicochemical analyses of FS

Ultracentrifugal analysis was made for FS. The infinite dilution value of the sedimentation coefficient, $S^0_{20,w}$, was 12.5 (Fig. 46) and the diffusion coefficient gave a value of 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 Svedverg equation, employing a partial specific volume, 0.740, gave a value of 630,000 for FS.

Extinction coefficient ($E^{1\%}_{280\ nm}$) determined in 0.15 M NaCl was 8.67.

Isoelectric point (pI) of FS was 5.4, when determined isoelectrofocusing.

Several physicochemical data are summarized in Table 4.

The amino acid composition is shown in Table 5.

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

| | |
|---|---|
| Sedimentation coefficient ($S^o_{20,w}$) | 12.5S |
| Diffusion coefficient ($D_{20,w}$) | $1.87\times10^{-7}cm^2\cdot S^{-1}$ |
| Partial specific volume (V) | 0.740 |
| Molecular weight ($Ms,_D$) | 630,000 |
| (Gel filtration) | 600,000 |
| Extinction coefficient ($E^{1\%}_{280\ nm}$) | 8.67 |
| Isoelectric point (pI) | 5.34 |

Table 5. Amino acid composition of chum salmon female-specific iron-binding serum protein (FS)

| Residue | Residue percent<br>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 |

The results are expressed as moles/100 moles of amino acid.

C. Japanese eel

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

Typical electrophoretic patterns of silver migrated 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 β-region whereas, in males, the serum proteins were rather diffusedly distributed from albumin to γ-region.

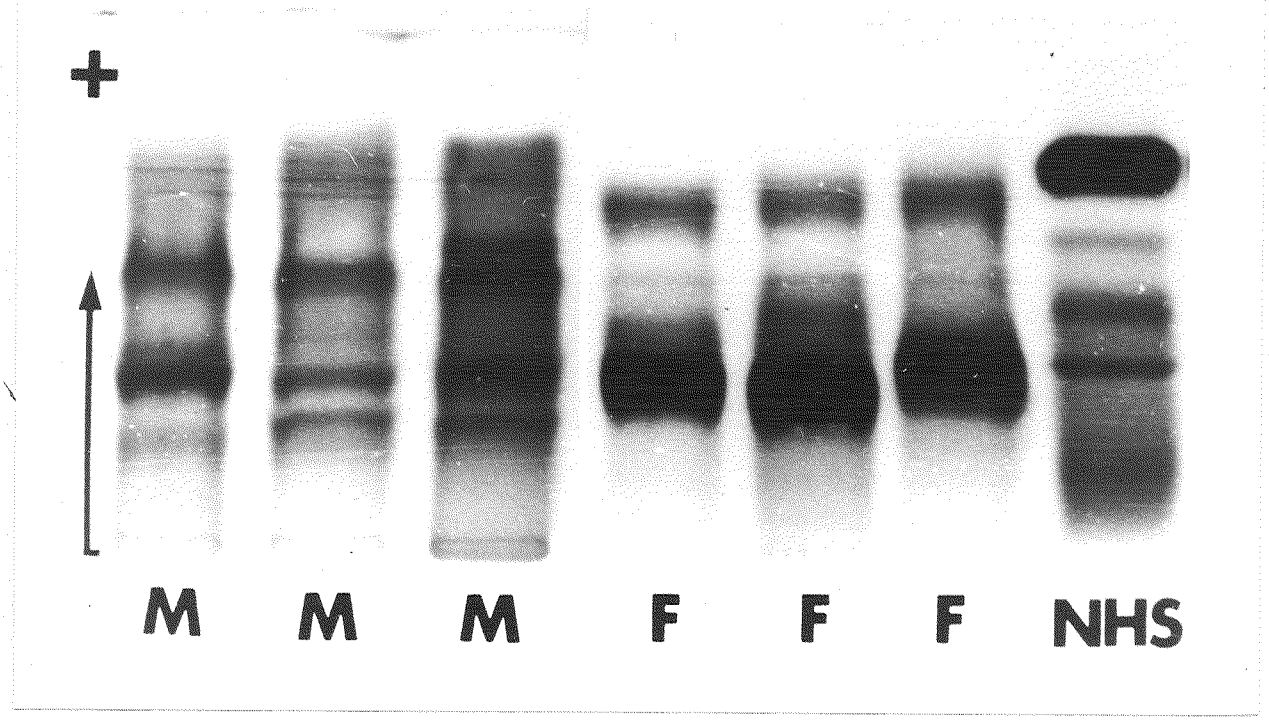


Fig. 47. Typical electrophoretic patterns of serum of silver migrated forms of male Japanese eel (M) and of female Japanese eel (F) treated with salmon pituitary glands. NHS: normal human serum.

## 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 with male serum, but reacted with female serum, forming a single precipitin line as shown in 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. 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.

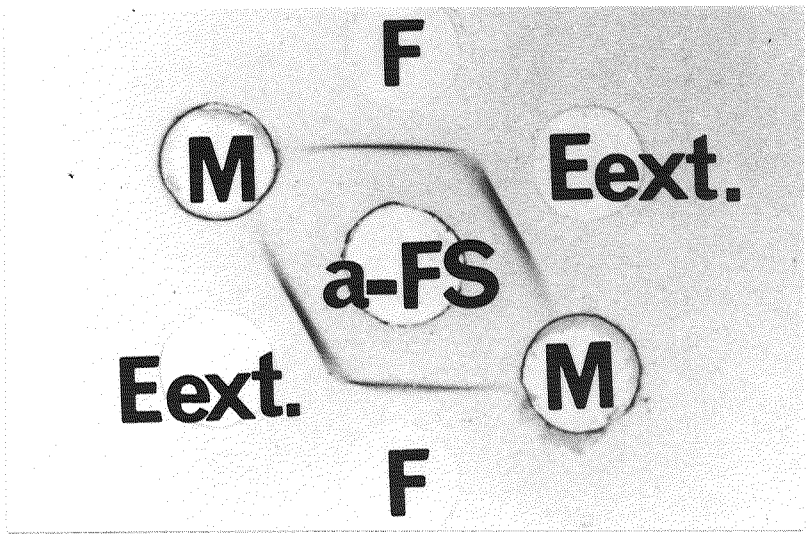


Fig. 48. Precipitin reaction of serum and egg yolk proteins of Japanese eel against anti-eel FSSP. M: male serum, F: female serum, Eext.: egg yolk proteins extracted with 0.9% NaCl, a-FS: anti-eel FSSP (see Table 1).

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

Radioactive iron ($^{59}Fe$) 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. As shown in Fig. 49, a rabbit antiserum to whole serum of female eel (anti-eel serum) formed one radioactive precipitin line with male serum, whereas female serum showed two lines. The fused line which shows 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 33 & 65).

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

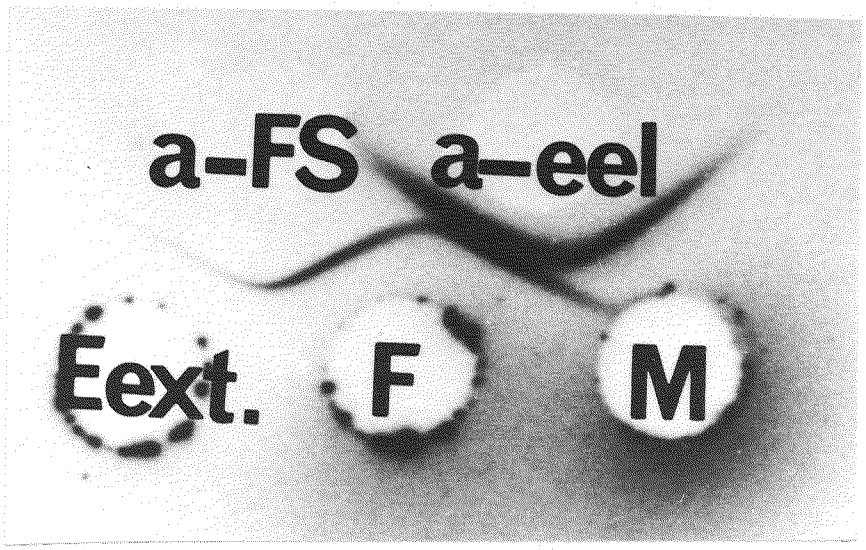


Fig. 49. Autoradiography of a gel diffusion reaction between the $^{59}$Fe-binding proteins in serum or egg yolk proteins of Japanese eel, and rabbit antisera. a-eel: anti-eel serum. M, F, Eext., a-FS: same as in Fig. 48.

1.2 M potassium phosphate buffer, contained FS. It 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 purification 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 β-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 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

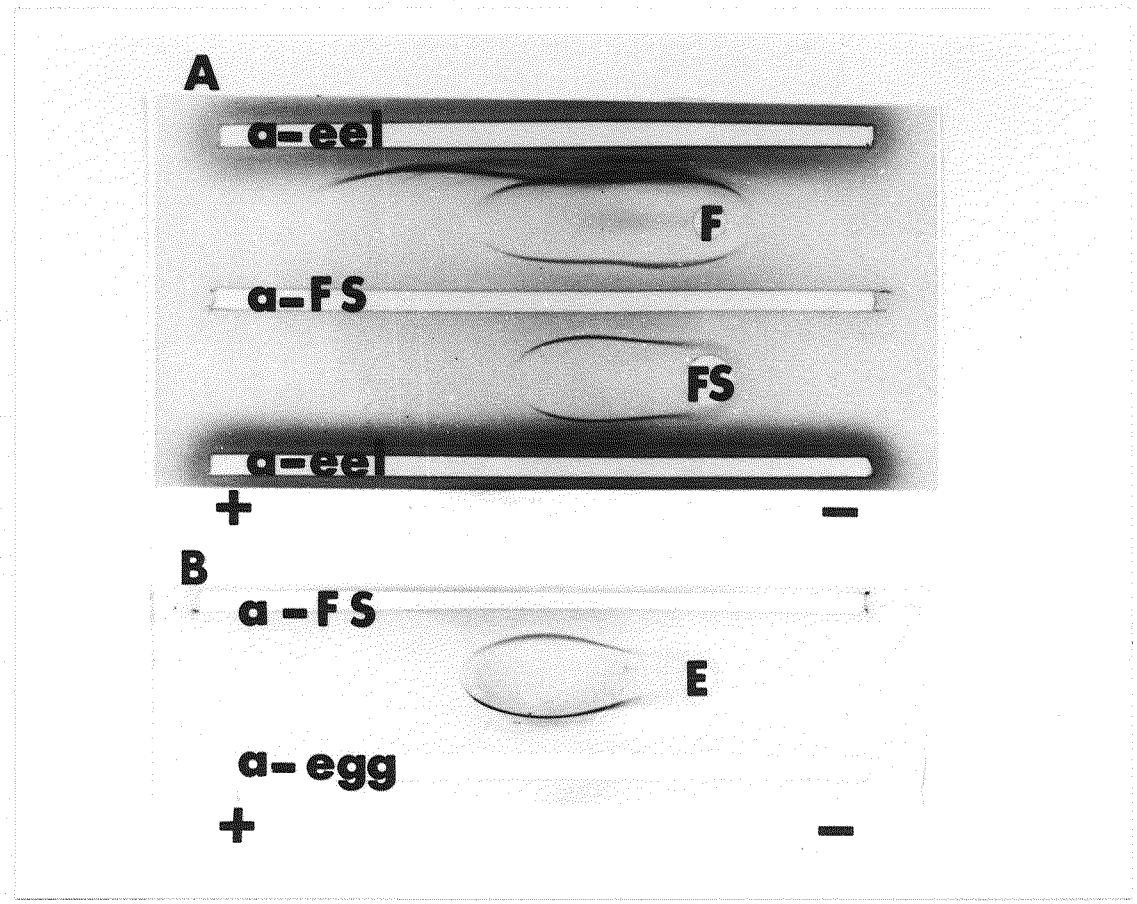


Fig. 50. Immunoelectrophoresis of purified FS (A) and egg yolk protein (B) of Japanese eel. FS: female-specific iron-binding serum protein, E: FS related egg yolk protein, a-egg: rabbit antiserum against egg yolk proteins extracted with 0.9% NaCl (anti-eel eggs), F, a-eel, a-FS: same as in Fig. 49.

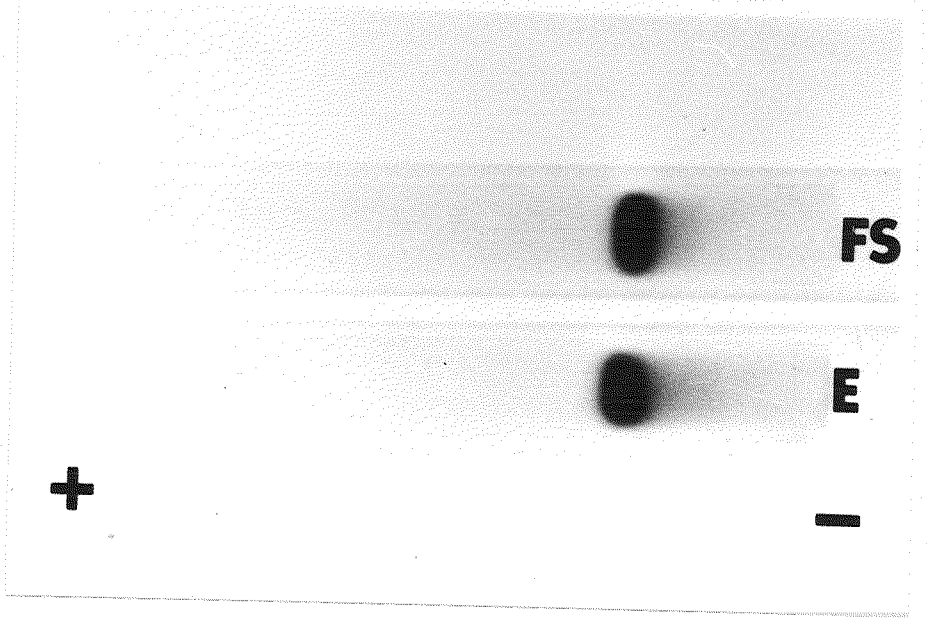


Fig. 51. Disc electrophoresis in 5% polyacrylamide gel of purified FS and its related egg yolk protein of Japanese eel. FS, E: same as in Fig. 50.

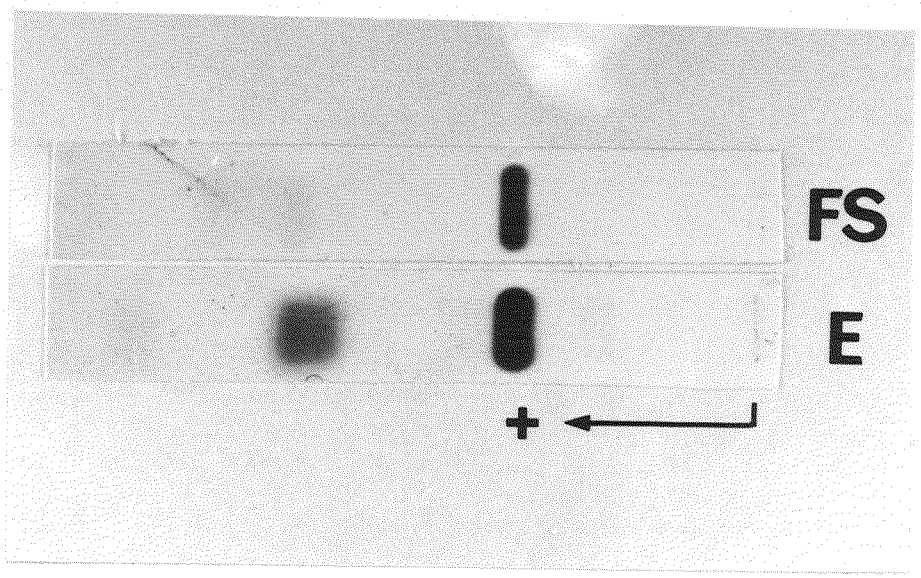


Fig. 52. SDS polyacrylamide gel electrophoresis of purified FS and its related egg yolk protein of Japanese eel. FS, E: same as Fig. 50.

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.

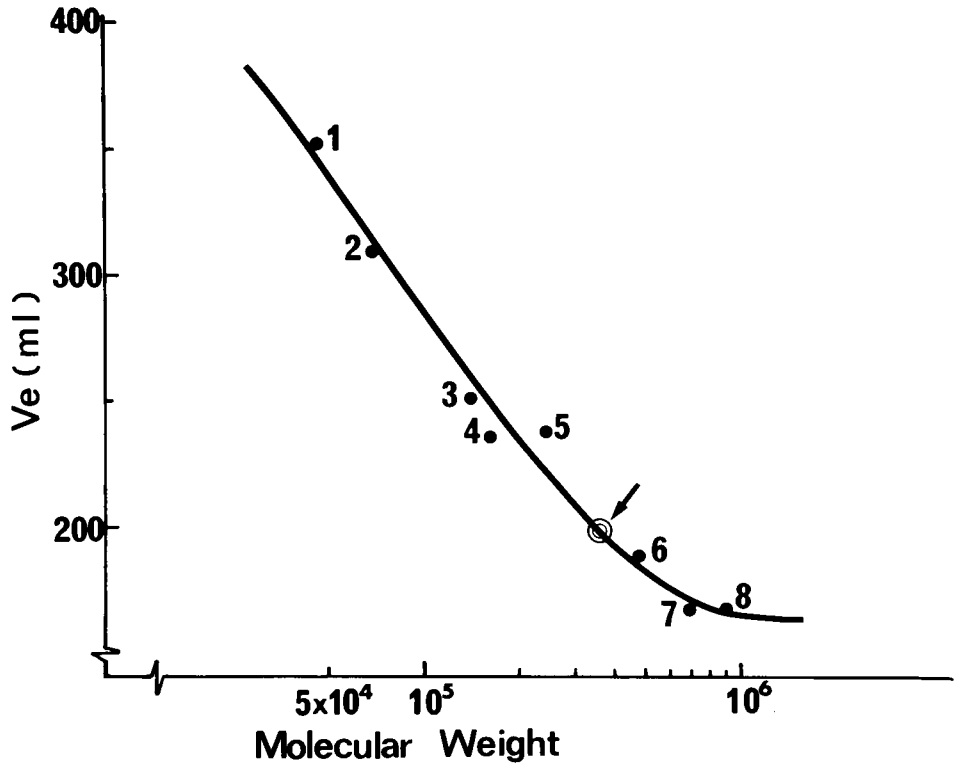


Fig. 53. Estimation of molecular weight by Sephadex G-200 gel filtration. Both FS and its related egg yolk protein of Japanese eel are eluted at the position of open circle (arrow). The samples used for calibration are indicated by dark circles. 1: ovalbumin, 2: bovine serum albumin, 3: alcohol dehydrogenase,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

8. Presence of lipid and carbohydrate in each purified 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.

9. Determination of phosphorus

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.

10. Amino acid composition

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.

11. $NH_2$-terminal amino acid

An alanine spot was identified for both purified FS and its related egg yolk protein on thin-layer chromatography of the dansylated samples.

12. Extinction coefficient

Extinction coefficients ($E^{1\%}_{1\ cm,\ 280\ nm}$) 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.

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

| 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 |

The results are expressed as moles/ 100 moles of amino acid

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.

Figure 54 shows immunoelectrophoresis and crossed immunoelectrophoresis of egg yolk proteins with the anti-medaka 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 the anti-medaka eggs.

The results of crossed immunoelectrophoresis combined with autoradiography of egg extracts mixed with $^{59}FeCl_3$ are shown in Fig. 55. As can be seen in Fig. 55, 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.

The anti-medaka eggs was, then, applied to the reaction with medaka serum. The results of immunoelectrophoresis of sera of females and males in the breeding season are shown in Fig. 56. The antiserum reacted with the female serum forming more than two lines, whereas it did not react with the male serum. The same tests were performed by means of crossed immunoelectrophoresis. The male serum did not show any

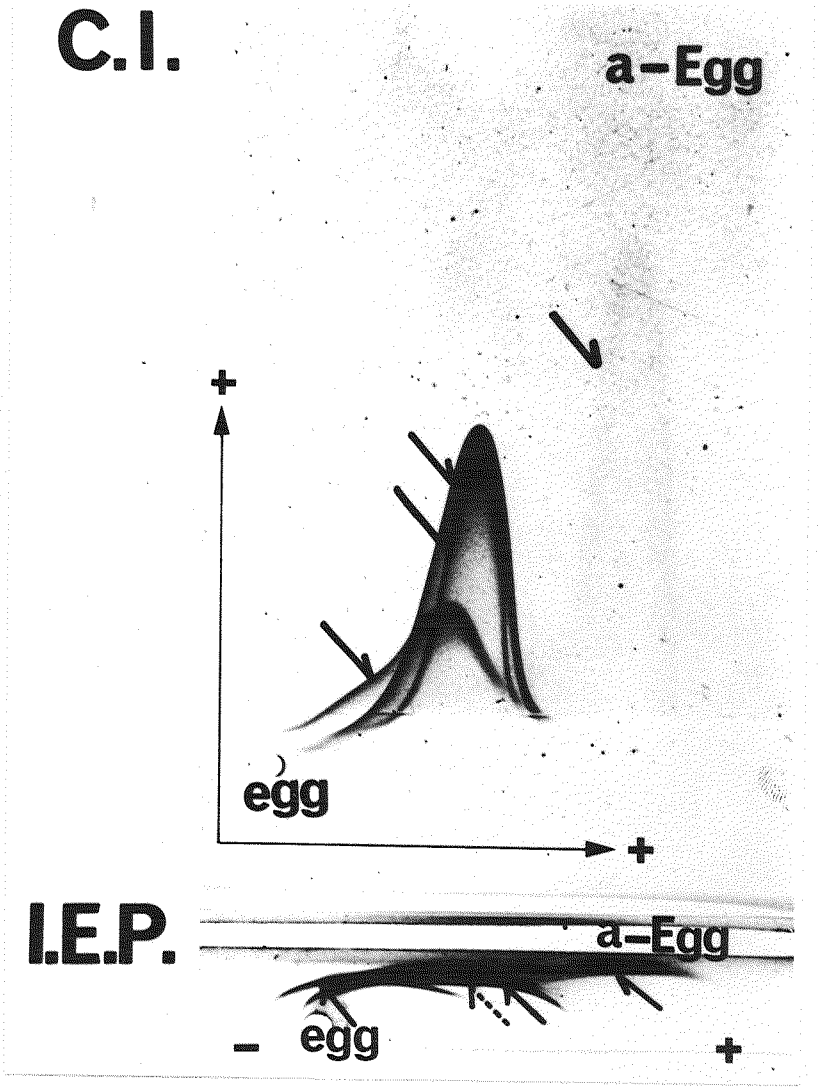


Fig. 54. Immunoelectrophoresis (I.E.P.) and crossed immunoelectrophoresis (C.I.) of egg yolk proteins of medaka. Antigen: 4 µl of 0.9% NaCl extracts of ovulated eggs of medaka. The antiserum used was the polyvalent antiserum to medaka egg yolk extracts (a-Egg) in both electrophoresis. First dimension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

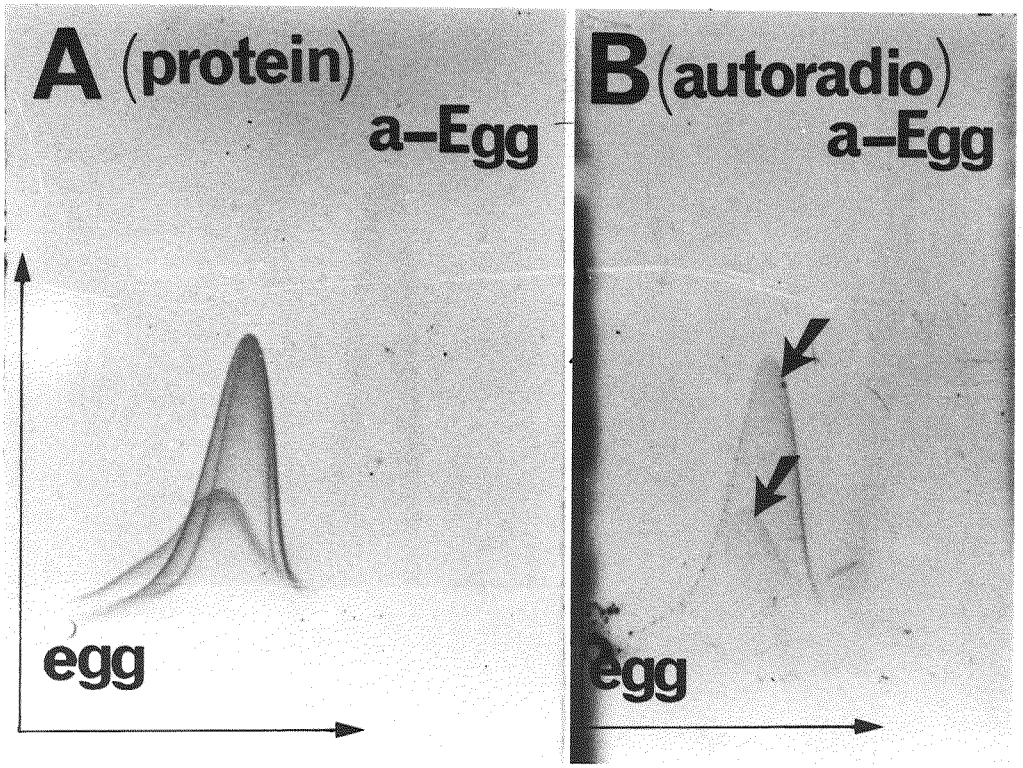


Fig. 55. Crossed immunoelectrophoresis (A) and its autoradiography (B). Antigen: egg yolk proteins of medaka mixed with $^{59}FeCl_3$. Antibody: anti-medaka eggs. The arrows show the radioactive precipitin lines.

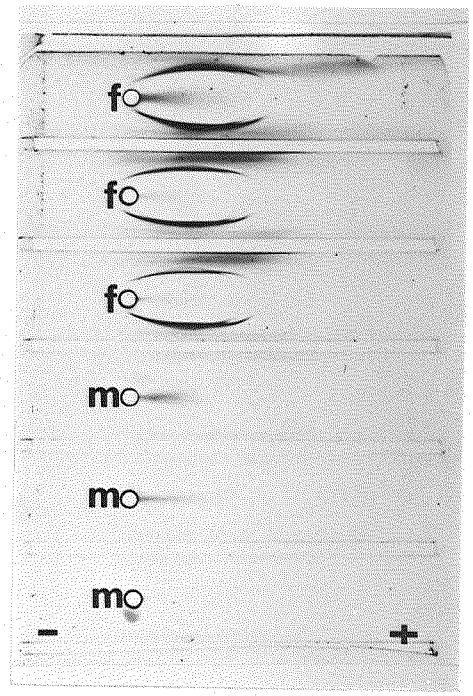


Fig. 56. Immunoelectrophoresis of female and male sera of medaka in breeding season. Each trough was filled with anti-medaka eggs. f: female, m: male

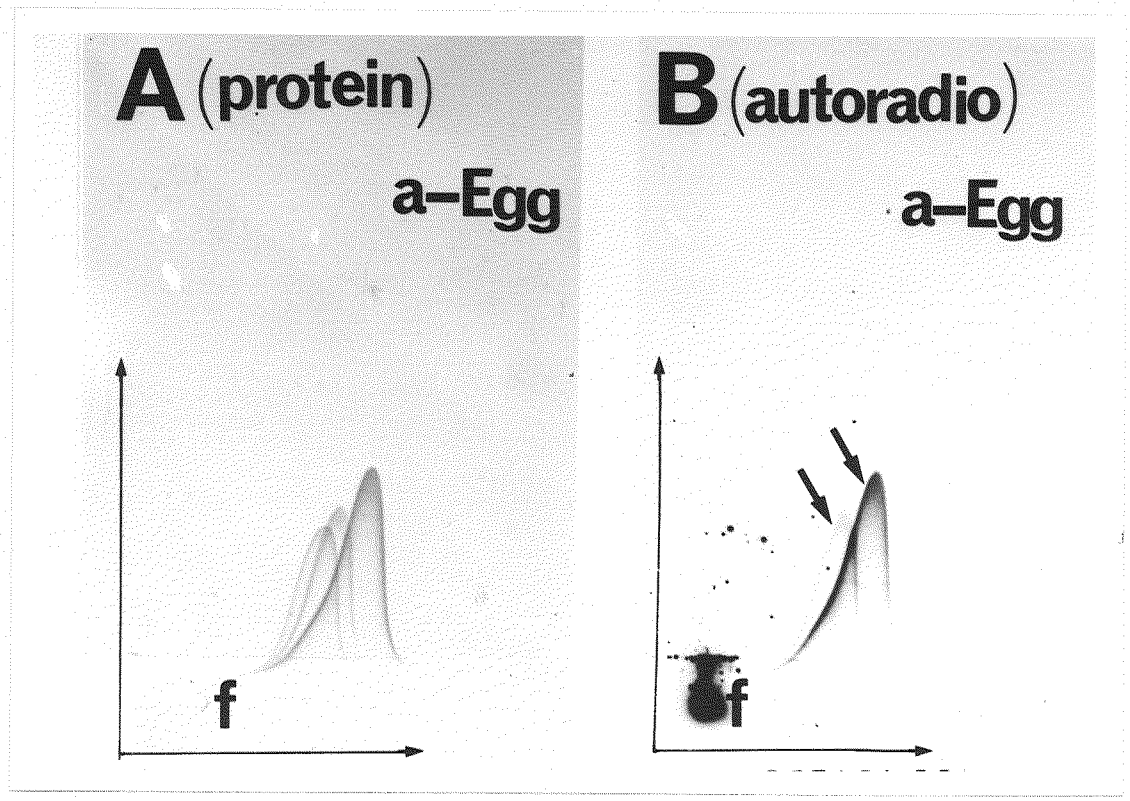


Fig. 57. Crossed immunoelectrophoresis (A) and its autoradiography (B). Antigen: female serum of medaka in breeding season mixed with $^{59}FeCl_3$. Antibody: anti-medaka eggs. After staining and dried, the plate was exposed to X-ray films.

reaction with the anti-medaka eggs whereas the female serum reacted with the antiserum forming three distinct precipitin lines as shown in Fig. 57A. These results indicated that the anti-medaka eggs has similar characteristics to the antiserum to the female-specific serum proteins as described for trout, salmon and eel.

Figure 57B shows the autoradiography combined with crossed immunoelectrophoresis of the female medaka in the breeding season. The female serum yielded two radioactive precipitin lines. This indicated that female fish in the

breeding season contains two female-specific iron-binding proteins in serum.

2. Studies on female-specific serum proteins during maturation

Fishes used in this experiments were classified into three groups, i.e., "immature", "breeding" and "sexually inactive". Immature fish were selected by histological examinations of the ovary: fish that had never spawned before and their ovarian oocytes that were in the yolk vesicle stage were classified as "immature". The gonadosomatic index (G.S.I.) of the three groups used are summarized in Table 7.

Figure 58 demonstrates the results of crossed immunoelectrophoresis of female serum at the three maturational stages. Amount of serum samples used for electrophoresis was 0.4 µl for "immature", 0.08 µl for "sexually inactive" and 0.02 µl for "breeding". 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. As shown in Figs. 58A & 58B,

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 |

one immature fish disclosed only one peak and another immature fish formed two peaks. Serum from "sexually inactive" developed two peaks (Fig. 58D), whereas that from "breeding" formed three peaks on the crossed electrophoresis (Fig. 58C).

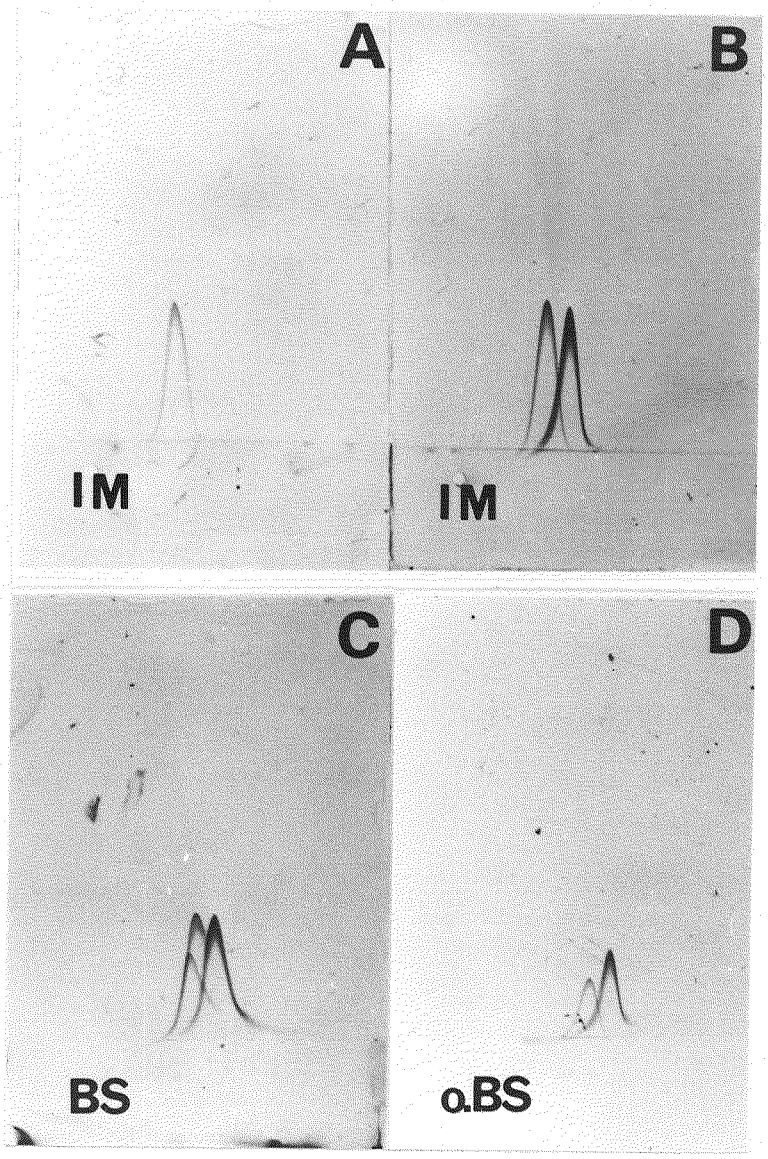


Fig. 58. Crossed immunoelectrophoresis of female medaka serum of various maturations. IM: "immature"(A.B), BS: "breeding", o.BS: "sexually inactive". Details in test.

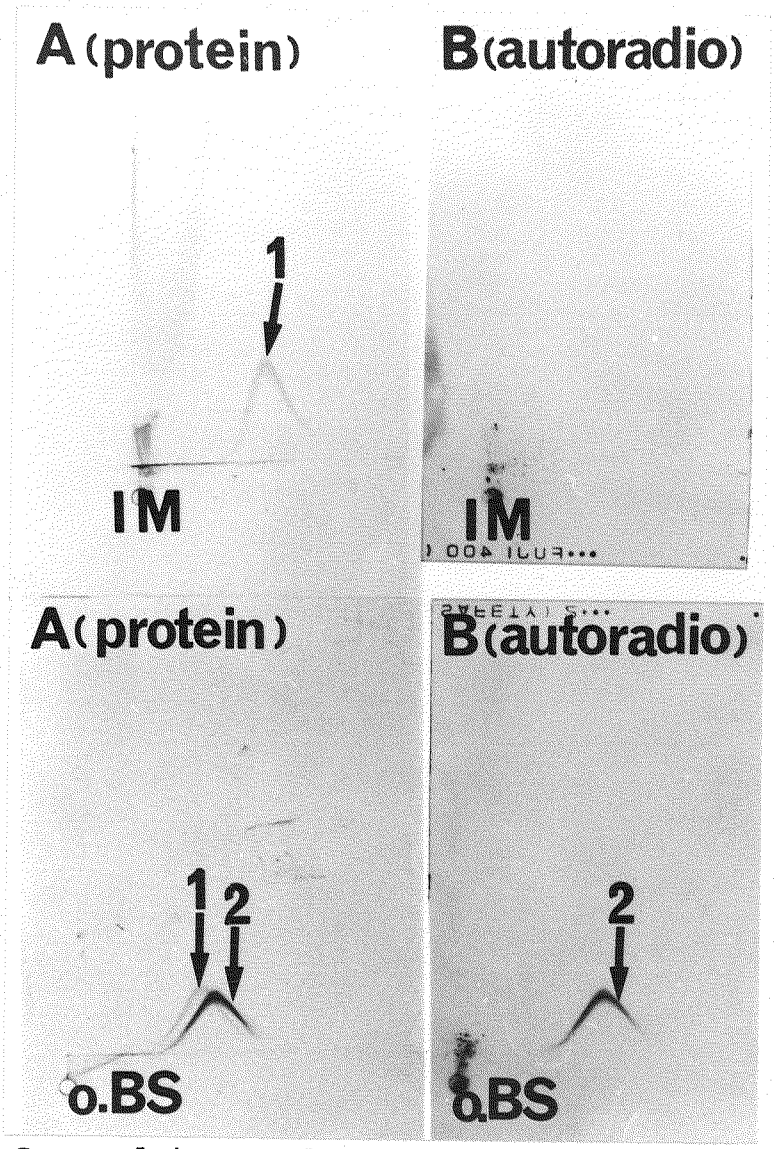


Fig. 59. Crossed immunoelectrophoresis (A) and its autoradiography (B) of female medaka sera. IM: "immature", o.BS: "sexually inactive".

Two proteins out of three female-specific serum proteins were demonstrated to have iron-binding activity (Fig. 57). Presence of such an activity was investigated for the female-specific protein of "immature" and "sexually inactive". As shown in Fig. 59, 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 know 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. 60-1). 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. 60-2). In the fish at the breeding season, oocytes were at the tertiary yolk stage (Fig. 60-3). Oocytes of sexually inactive females were found to be in the late phase of yolk vesicle

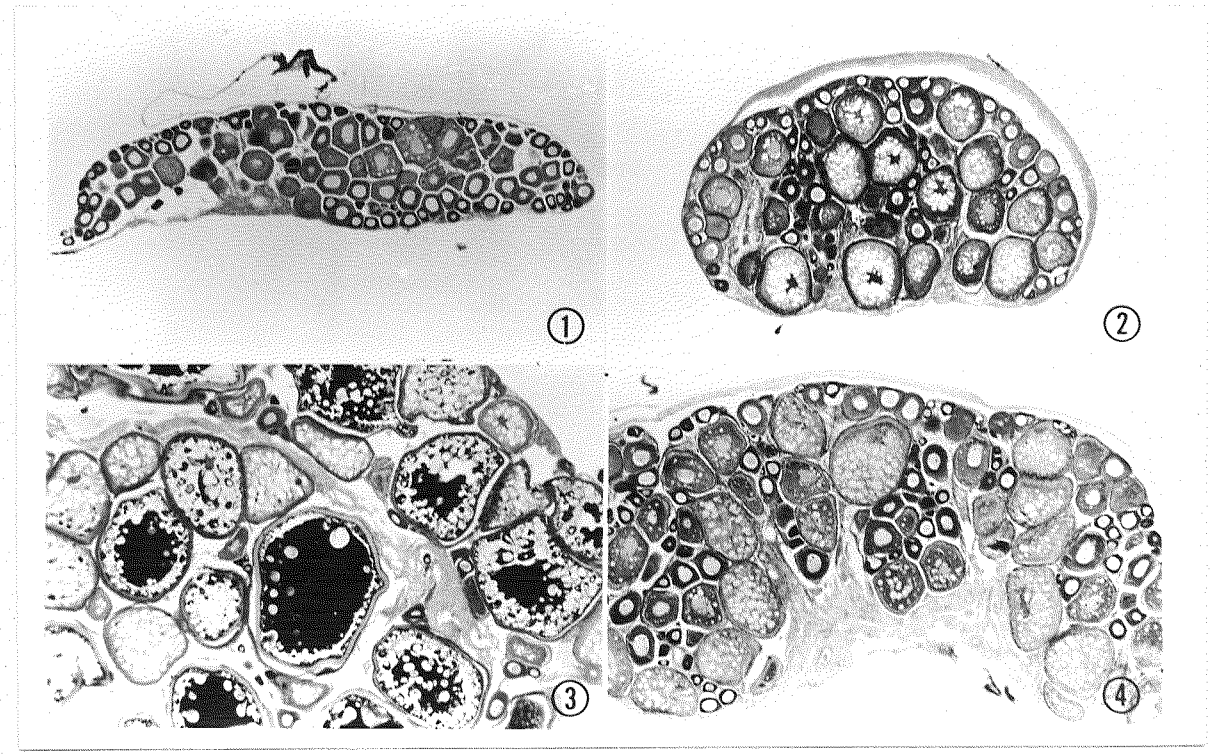


Fig. 60. Histology of the ovary at various stages in medaka. 1,2: "immature", 3: "breeding", 4: "sexually inactive". X 28.

stage, similar to the immature fish with two female-specific serum proteins (Fig. 60-4). The results of histological study indicated that the occurrence of female-specific serum proteins corresponded to the stage of ovarial maturation.

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

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

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 in 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 precipitin 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 (Fig. 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 sera of breeding females and sexually inactive females. Two female-specific serum

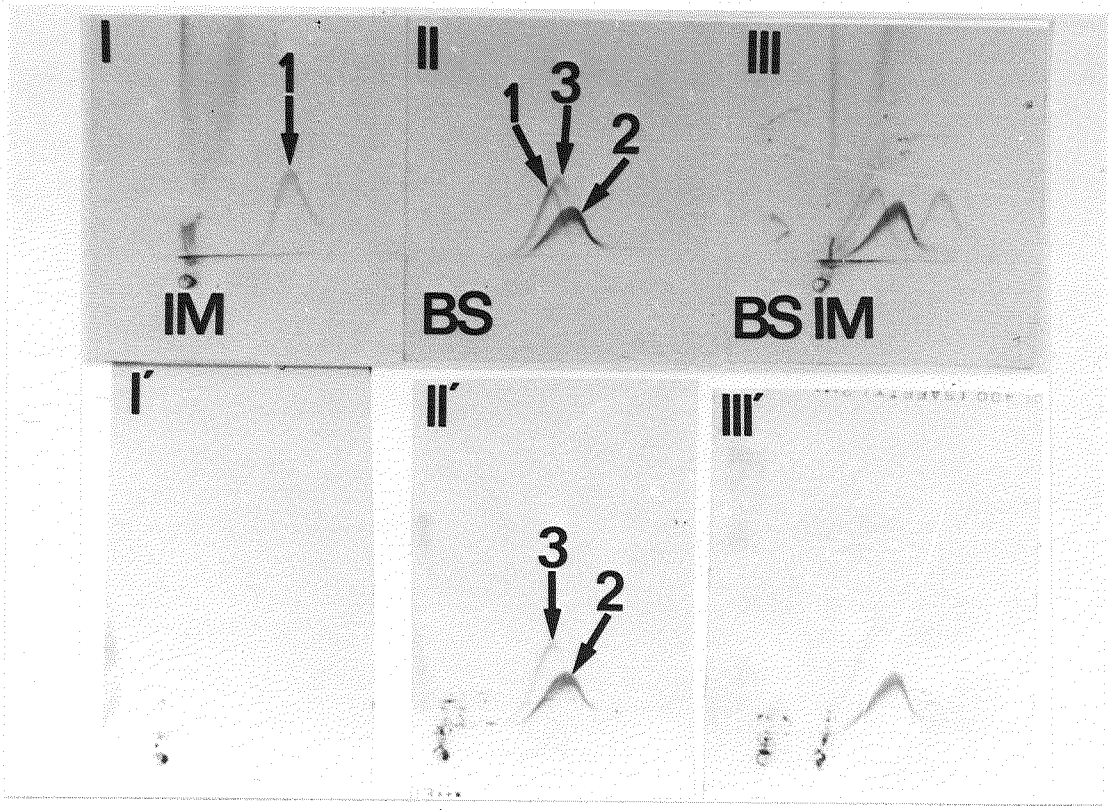


Fig. 61. Tandem-crossed immunoelectrophoresis (I, II, III) and their autoradiography (I', II', III') of female medaka serum. Antigen: immature (IM) and breeding (BS) female medaka serum mixed with $^{59}FeCl_3$ (III). Serum sample and 0.9% NaCl (I and II). First dimension: The first dimensional run was done at 10 V/cm for 90 minutes. Anode to the right. Antibodies: anti-medaka eggs. Second dimension: The immunoelectrophoretic run was done at 3 V/cm for 20 hours (anode at top). Details in text.

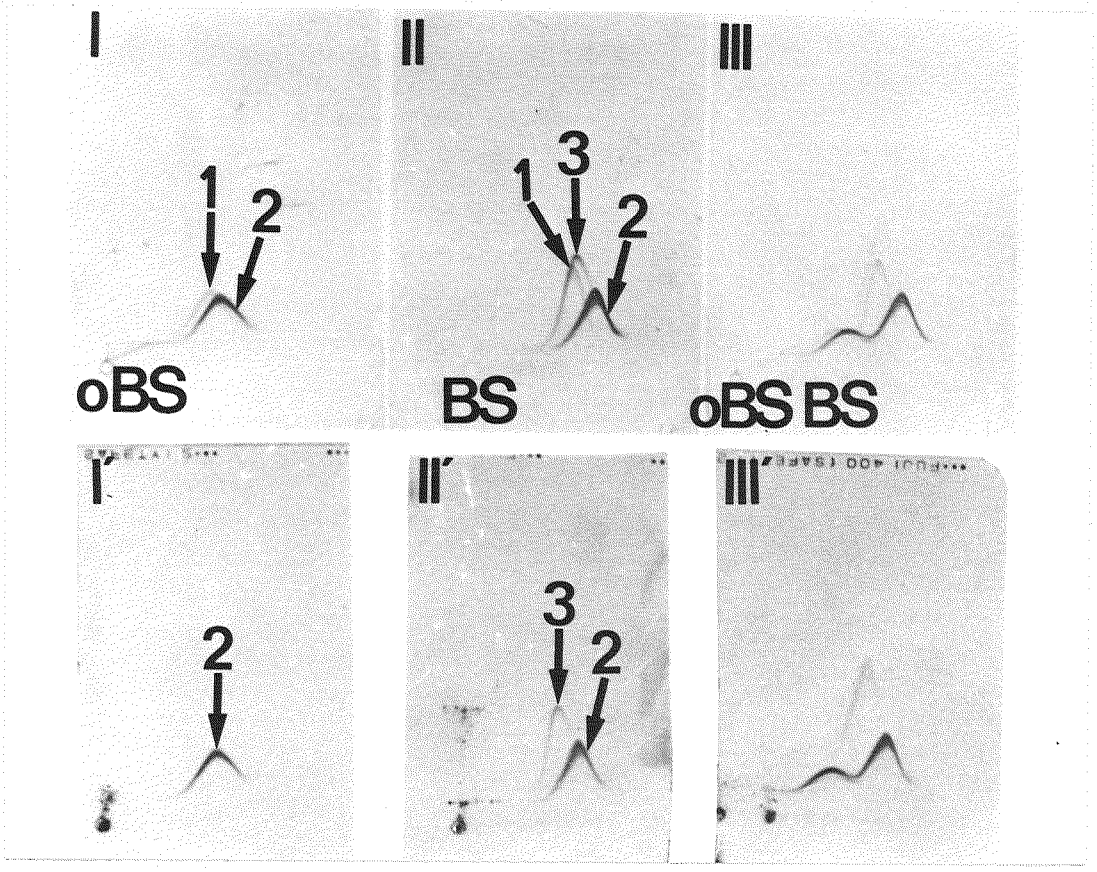


Fig. 62. Tandem-crossed immunoelectrophoresis (I, II, III) and their autoradiography (I', II', III') of female medaka serum. Antigen: female medaka sera, of "sexual inactive" (o.BS) and "breeding" (BS).

proteins, one of which bound iron, were seen in the sexually inactive females as shown in 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 property, 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 (Fig. 58B & 60-2), were identified by this procedure to correspond to FS1 and FS2 (not shown).

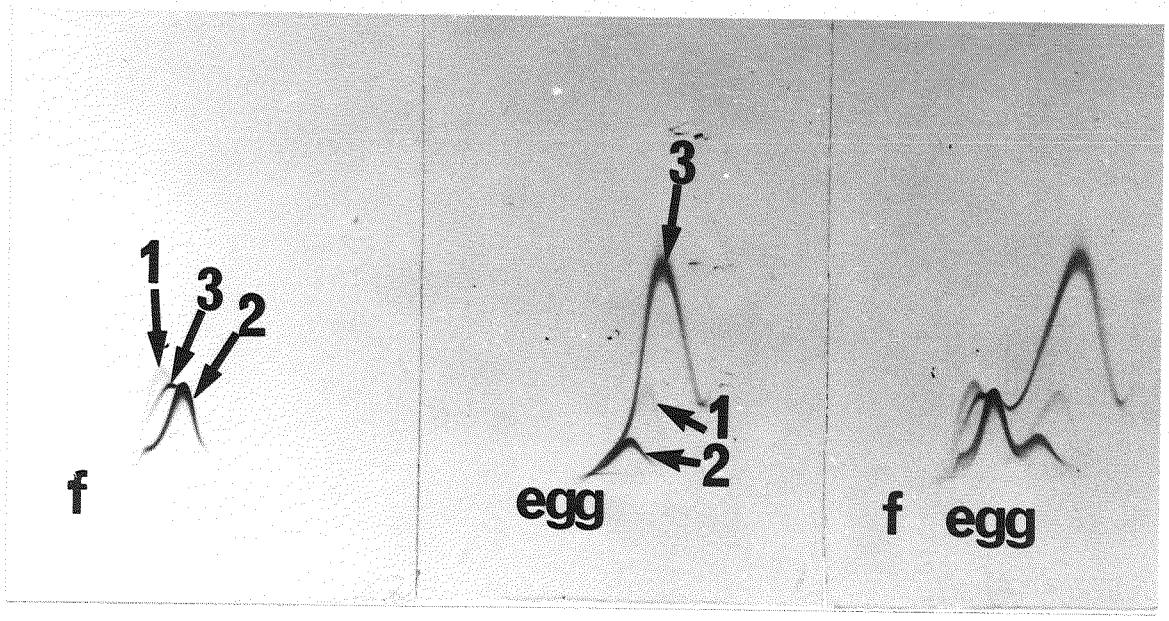


Fig. 63. Tandem-crossed immunoelectrophoretic comparison of serum and egg in medaka. Antigen: female medaka serum in breeding season (f), egg yolk extracts of medaka (egg).

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-17β) and serum samples were collected at 5, 10, 20 and 30 days after the initial oral administration of each hormone. Typical crossed immunoelectrophoretic patterns of serum from hormone-treated and control fish were shown in Figs. 64-68. The serum from control group developed two precipitin peaks with anti-medaka eggs as shown in 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 & 66). On the other hand, the serum collected from both ethinyl-estradiol- and estradiol-treated fish formed three precipitin peaks, all of which being higher than the peaks of the other groups, as can be seen in Figs. 67 & 68. In estrogen-treated fish, the height of the peaks of female-specific serum proteins 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

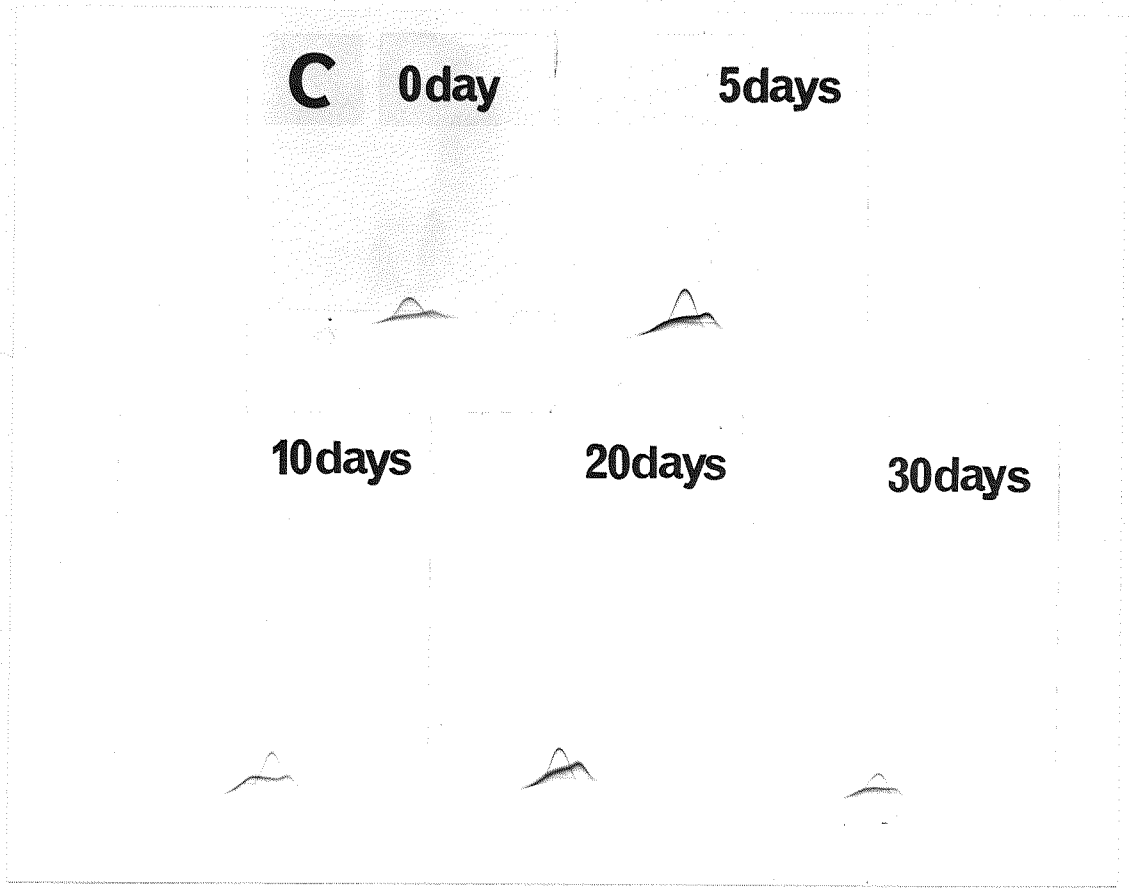


Fig. 64. Crossed immunoelectrophoresis of sera of female medaka of the control group.

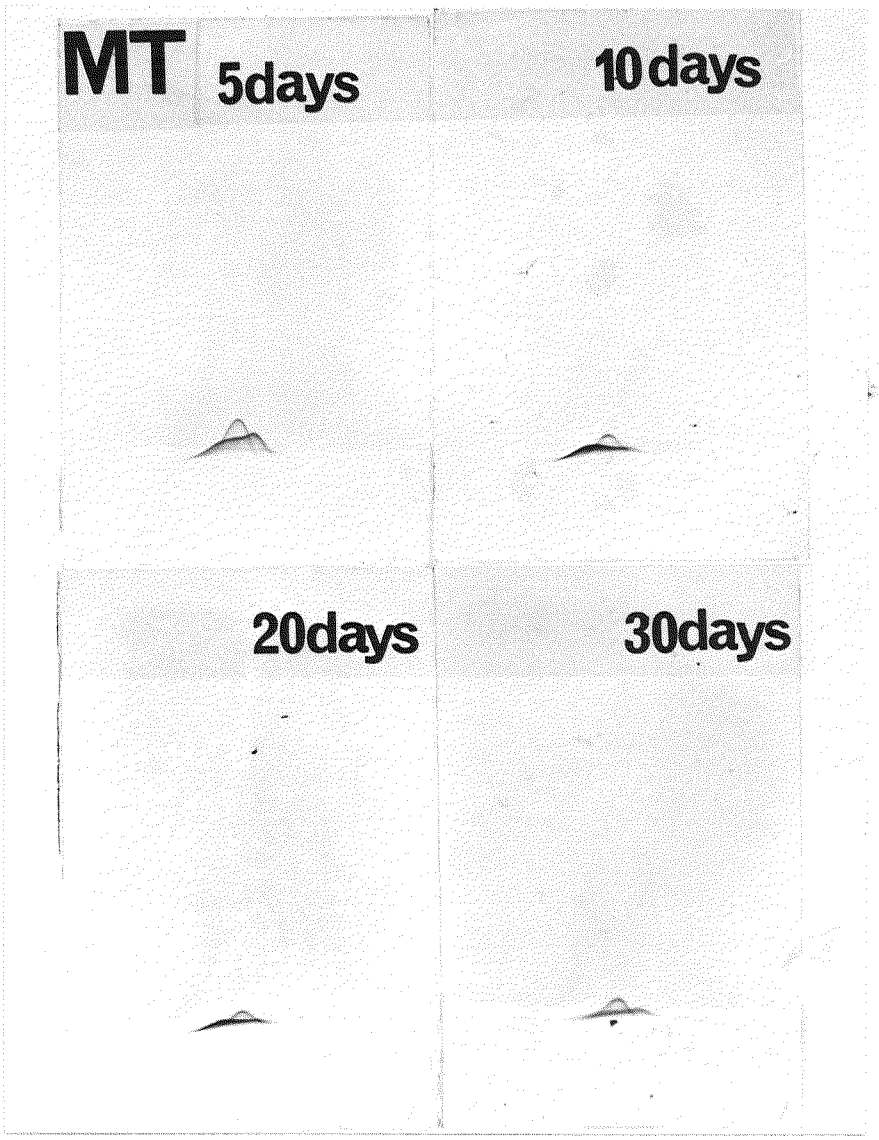


Fig. 65. Crossed immunoelectrophoresis of sera from methyltestosterone-treated medaka.

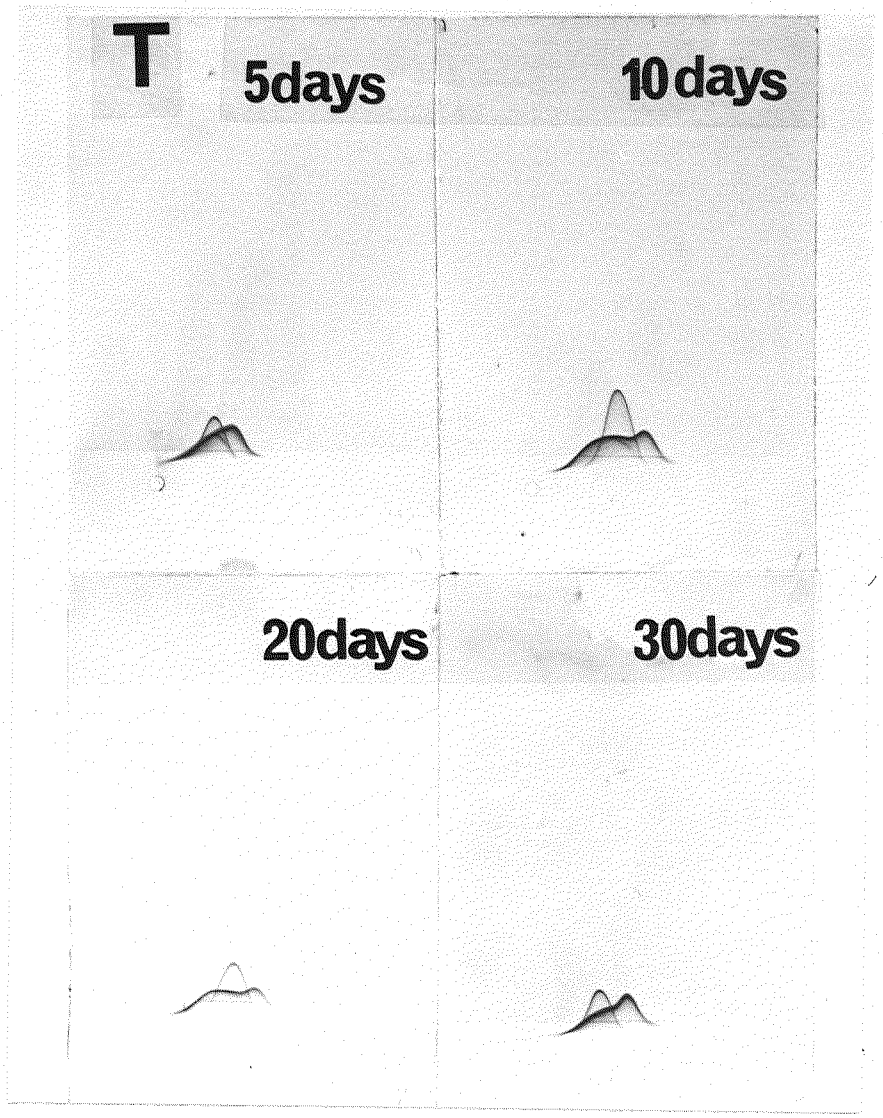


Fig. 66. Crossed immunoelectrophoresis of sera from testosterone-treated medaka.

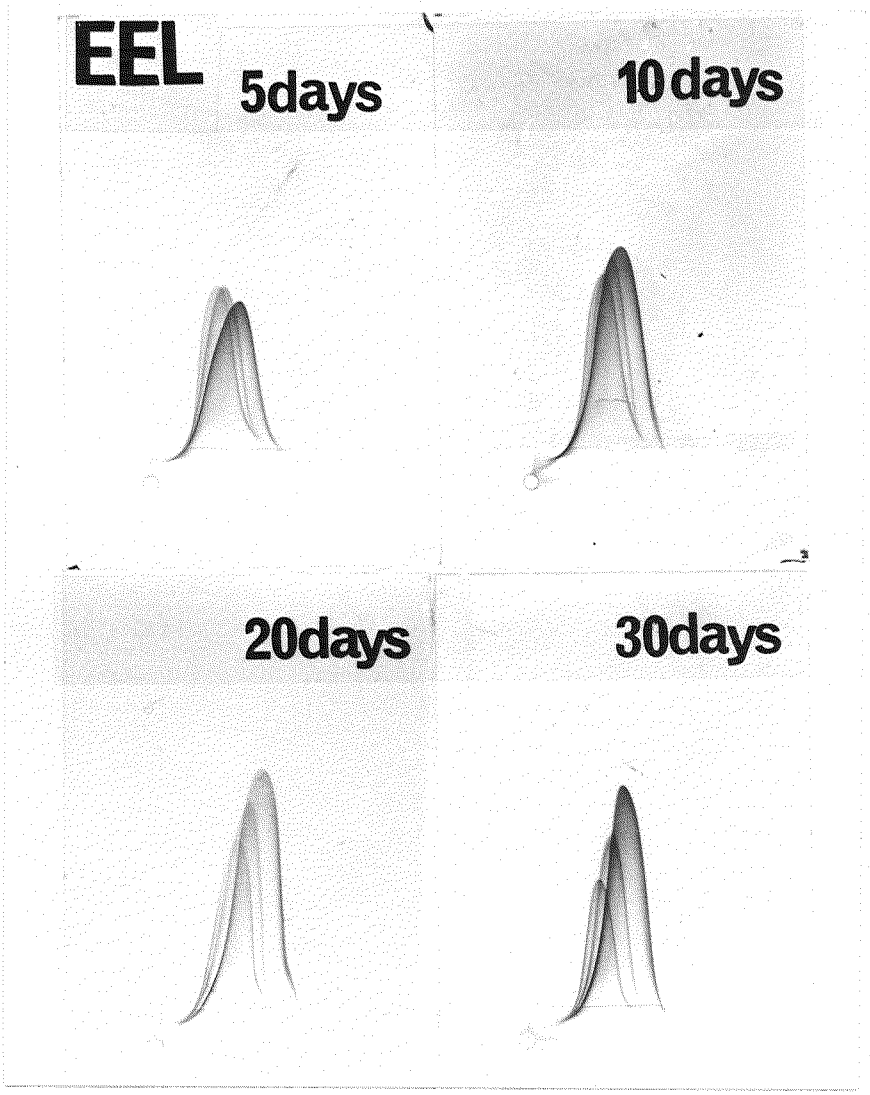


Fig. 67. Crossed immunoelectrophoresis of sera from ethinyl-estradiol-treated medaka.

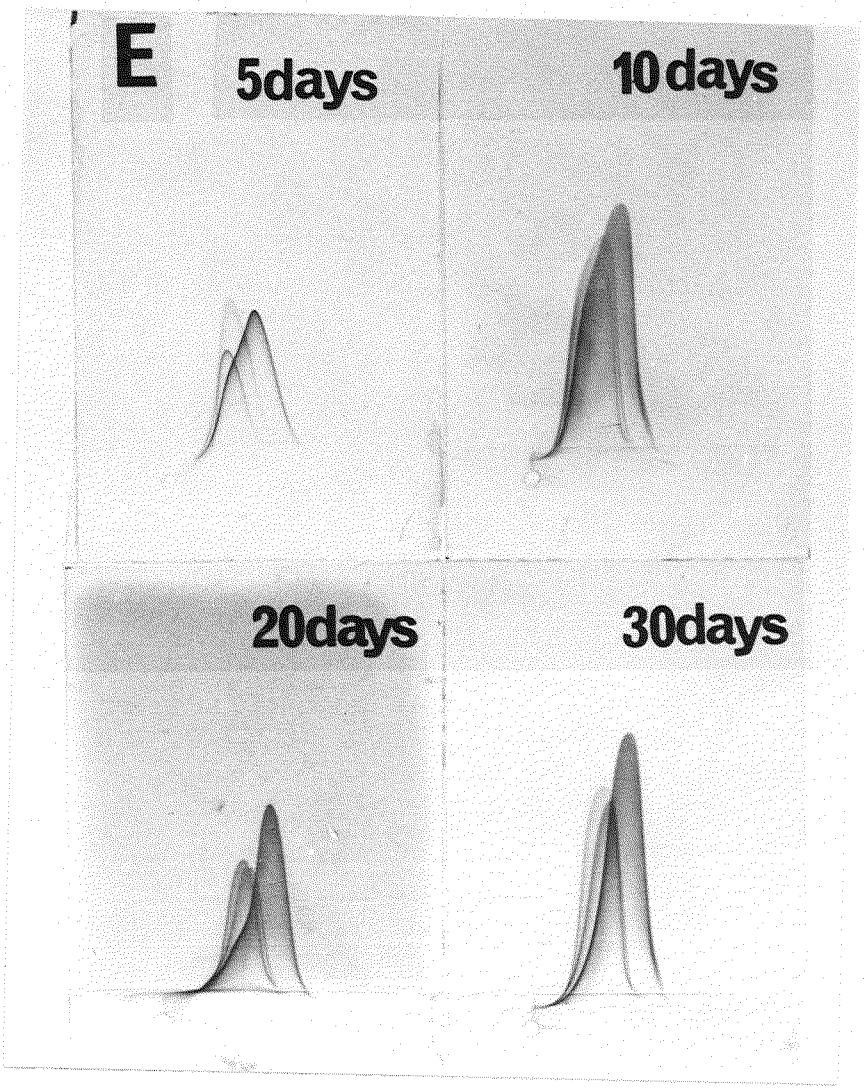


Fig. 68. Crossed immunoelectrophoresis of sera from estradiol-17β-treated medaka.

proteins are induced by administration of ethinylestradiol and estradiol-17β 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 as shown in Fig. 69. Two out of the three female-specific serum proteins induced by the hormone treatment showed the iron binding activity as shown in 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.

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 "immature" of type 1 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 93).

** uncertain iron-binding activity

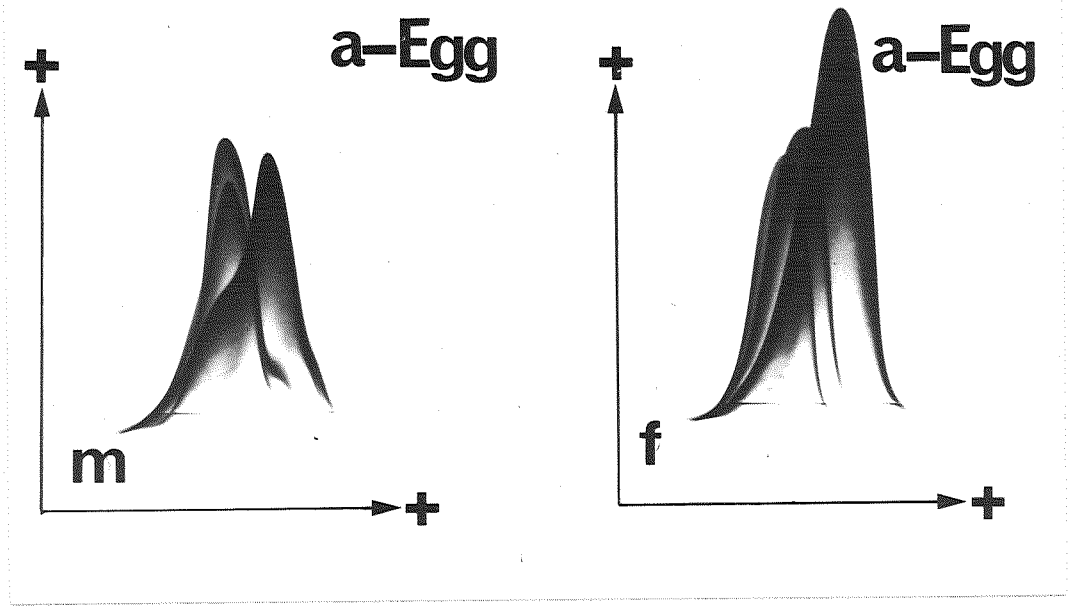


Fig. 69. Crossed immunoelectrophoresis of sera from male and female medaka after administration of ethinylestradiol. Samples were collected at 10 days after hormone treatment.

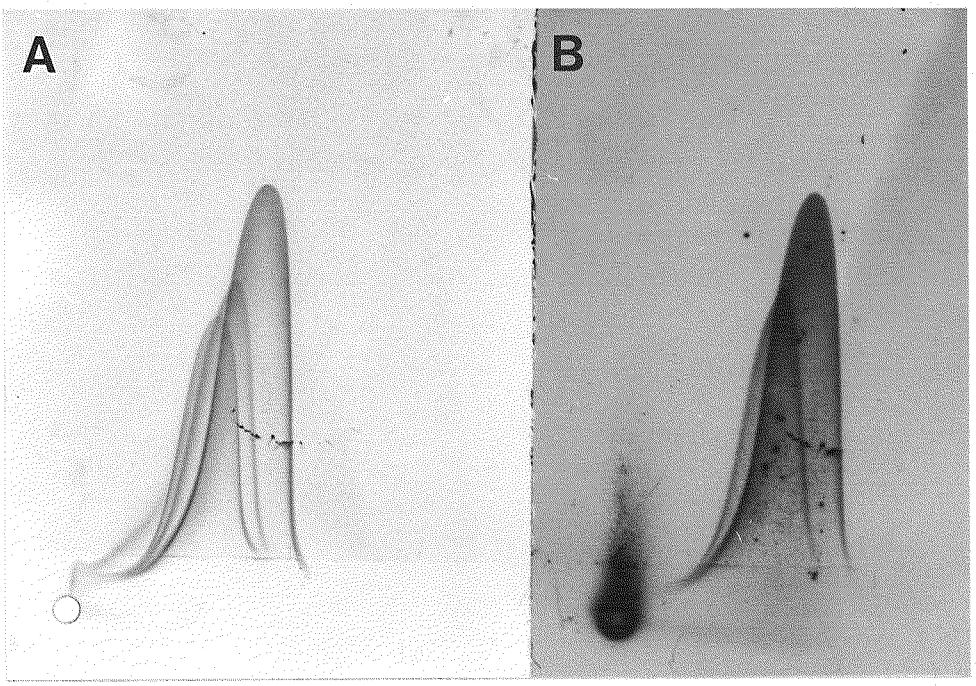


Fig. 70. Crossed immunoelectrophoresis (A) and its autoradiography (B). Antigen: male medaka serum after administration of estradiol-17β mixed with $^{59}FeCl_3$.

## IV. DISCUSSION

Previous studies on the protein vitellogenin in oviparous vertebrates have revealed some characteristic properties which include the followings: (1) it appears in serum of the female during vitellogenesis (Drilhon & Fine, 1963; Thurston, 1967; Crim & Idler, 1978), (2) it can be induced in male and immature female sera by the administration of estrogen and/or a pituitary extract (Follett & Redshaw, 1968; Campbell, 1978), (3) it has a characteristic capability of binding calcium as a glycolipophosphoprotein complex (Wallace, 1970; Redshaw & Follett, 1971; Ansari et al., 1971), (4) it may be a possible precursor of egg yolk proteins, phosvitin and lipovitellin (Wallace & Jared, 1968, 1969; Bergink & Wallace, 1974; Deeley et al., 1975; Christmann et al., 1977), and (5) it has a molecular weight of 450,000-500,000 and it consists of two identical polypeptide chains, each of which has a molecular weight of about 200,000-240,000 (Bergink & Wallace, 1974; Deely 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 three different species, viz. rainbow trout, chum salmon and Japanese eel, satisfied several of the above-cited properties of vitellogenin; (1) it was a female specific protein, (2) it was induced in the serum of males and immature females by the administration of estrogen (trout) or a pituitary extract (eel), (3) it had carbohydrates, lipids

and phosphorus in the molecule (glycolipophosphoprotein) (trout and eel), (4) the same or similar antigenic components were found in egg yolk and (5) it was 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 to 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, are summarized in Table 9, for the comparison of their similarity.

As shown in Figs. 4, 36 and 49, one of the female-specific serum proteins (vitellogenin) which was seen in female trout, salmon and eel possessed iron-binding capacity. 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 be transfered iron to

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

The results for amphibian (South African clawed toad) vitellogenin are from Wallace (1970), Redshaw & Follett (1971) and Bergink & Wallace (1974).
The results for avian (white leghorn chicken) vitellogenin are from Deeley et al. (1975) and Christmann et al. (1977).

| | Rainbow trout | Chum salmon | Japanese eel | Amphibian | Avian |
|---|---|---|---|---|---|
| $S^{o}_{20,w}$ | 10.2S | 12.5S | n.d. | | |
| Molecular weight (Gel-filtration) | 600,000 | 600,000 | 350,000 | 470,000-540,000[a] 450,000[b] | 450,000-500,000[c] |
| 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 |

a) calculated from $D^{o}_{20,w}$ and $S^{o}_{20,w}$, b) by sedimentation equilibrium, c) by electrophoresis in gradient polyacrylamide gels.

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 molecular weight of 450,000 consists of two identical polypeptides, about 200,000 in molecular weight, that can be cleaved by proteolysis to yield phosphorylated polypeptides of approximately the same size as phosvitin and lipovitellin. Although vitellogenin of avian (White leghorn chicken) has been 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 & Follett, 1971). Wallace & 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, 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 & Wallace, 1974; Redshaw & Follett, 1971). Similarly, the lipovitellin (β-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 & Wallace, 1965). Bernardi & Cook (1960) isolated two avian lipovitellines, α and β, from the egg yolk and demonstrated that there was no significant difference between the two in the 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. The 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 & 10). SDS polyacrylamide gel electrophoresis devoid of 2-mercaptoethanol gave a molecular weight of 220,000-240,000 for trout vitellogenin, 130,000 for E1 and 30,000 for E2, as shown in Fig. 17. Although trout vitellogenin yielded a small fraction with molecular weight of 130,000 by reduction with 2-mercaptoethanol, the main fraction remained to be 220,000-

240,000 dalton. On the other hand, the 130,000 component of E1 further split into two different polypeptide with a molecular weight of 90,000 (heavy chain) and 15,000 (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 molecular weight of 15,000. Behavior of E2 of trout egg yolk in gel filtration appears to be similar to that of the phosvitin fraction of salmon eggs demonstrated by Markert & Vanstone (1971). They indicated that the phosvitin fraction from gel filtration consisted of two different polypeptides with almost the same molecular weight, phosvitin and β'-component. In the present study, however, trout E2 was disclosed 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 amino acids (Table 2). The double immunodiffusion analysis indicated that the antigenecity of E1 completely differs from that of E2 (Figs. 15 & 16). Therefore, the component of 15,000 daltons in reduced E1 must be 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 as shown in 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 & Wallace, 1974). This may probably be the case in fish. Structural concepts of the trout vitellogen, 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 as shown in 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

Table 10. Structural concepts of the rainbow trout vitellogenin, E1 and E2 obtained from the 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×E2/2 | FS/2=2(H+L)+2×E2/2 =E1+E2 |

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. 85,000×4=340,000). Amino acid compositions of the components from both the serum and the egg were quite similar except for serine (Table 6) and the $NH_2$-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, interesting is 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 avian 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 amphibian (Bergink & Wallace, 1974) and avian (Deely et al., 1975). The relationship between the vitellogenin and its egg protein of Japanese eel is quite different from that of rainbow trout. From the results mentioned above and previous reports of amphibian and avian, possible schematic models of various vitellogenin molecules may be those shown in 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, in the eel, the 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

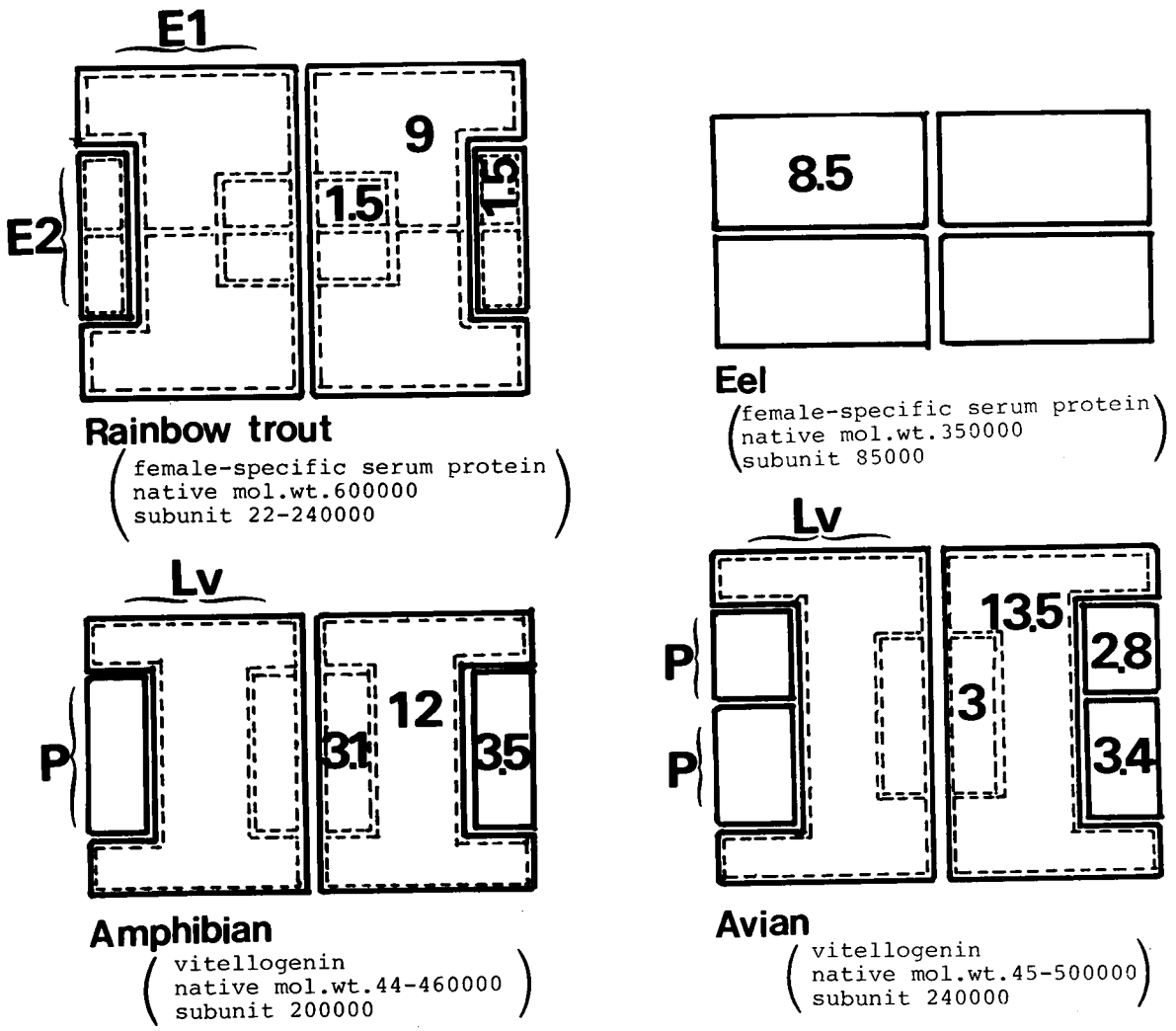


Fig. 71. Schematic models of various vitellogenin molecules. Lv: lipovitellin, P: phosvitin. Details in text.

induced in males by injections of estrogen (Figs. 57 & 69). These three female-specific serum proteins showed similar characteristic properties to vitellogenin in 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 property. As shown in Figs. 61 & 62, among the three female-specific serum proteins of the medaka FS3 binds radioactive iron and appears only in breeding females. 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 is closely related to the development of ovaries (Figs. 58 & 60). Ridgway et al. (1962), Fine & Drilhon (1963) and Utter & 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 the ovarial development. Among several analytical methods of such antigens are available, the immunological procedure, such as the

crossed immunoelectrophoresis is a quite 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. It may be due to characteristic seasonal changes in the maturity factor in this fish (Yamamoto & 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 as shown in Figs, 56 and 57.

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

(Baliley, 1957; Oguri & Takada, 1967; Woodhead, 1969), total lipid (Plack & Woodhead, 1966), phospholipid (Plack & Pritchard, 1968), alkai-labile protein phosphorus (Emmersen & 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 to be most reliable value when compared to those in the previous reports. A precise survey of the vitellogenin concentration after injection of estrogen was shown in Fig. 30. The concentration of vitellogenin increased rapidly in 10 days after injection and then decreased sharply within the subsequent 10 days. The half-life of trout vitellogenin could be roughly calculated to be 1.5 day from the profile shown in Fig. 30. The rapid synthesis of the vitellogenin can operate in the liver under the influence of estrogen as demonstrated by histological and immunological observations of the liver of estrogen-treated fish (Figs. 31 & 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 oocyte via its 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.

## Acknowledgements

During the course of my training many people have influenced my activities. First I wish to express my gratitude to Professor H. Hirai, Department of Biochemistry, Hokkaido University School of Medicine, for his invaluable guidance throughout this study.

Thanks are also due to Professor H. Takahashi, Faculty of Fisheries, Hokkaido University, for his critical reading of the manuscript.

I am deeply indebted to Professor J. Yamada, Professor I. Tsujino and Associate Professor K. Takano, Faculty of Fisheries, Hokkaido University, for their kind advice and reading of the present manuscript.

I also thank to Drs. Y. Tsukada, S. Nishi, K. Kobayashi, T. Konno and N. Hibi in our laboratory, Dr. H. Watabe, Higashi-Nippon-Gakuen University, Faculty of Pharmaceutical Sciences, and Dr. T. Matsuo, Department of Virology, Cancer

Institute, Hokkaido University School of Medicine for their valuable suggestions.

I wish to thank Drs. K. Yamauchi, T. Yoneda and Mr. Y. Takashima, Faculty of Fisheries, Hokkaido University, for their cooperation.

Appreciates are further extended to Dr. N. Kurohagi and Mr. H. Haruna, Toya Rinko Biological Station, at Lake Toya, and to Messrs. A. Haga, I. Takizawa and T. Oshuga, Salmon Hatchery in Shibetsu and Ichani, at Nemuro, for their kind supply of the samples.

I thank Miss K. Yoneta for her technical assistance and Miss M. Takada for her competent editorial assistance.

## REFERENCES


Aida K., Ngan P.V. & Hibiya T. (1973a) Sexual difference in composition of plasma protein of ayu in relation to gonadal maturation. Bull. Jap. Soc. Scient. Fish. 39, 1091-1106.

Aida K., Hirose K. & Yokote M. (1973b) Histological changes in the liver cells of ayu following gonadal maturation and oestrogen administration. Bull. Jap. Soc. Scient. Fish 39, 1107-1115.

Amirante G.A. (1972) Immunochemical studies on rainbow lipovitellin. Acta Embryol. exp. Suppl. 373-383.

Andrews P. (1964) Estimation of the molecular weights of proteins by Sephadex gel-filtration. Biochem. J. 91, 222-233.

Ansari A.Q., Dolphin P.J., Lazier C.B., Munday K.A. & Akhtar M. (1971) Chemical composition of an oestrogen-induced calcium-binding glycolipophosphoprotein in *Xenopus leavis*. Biochem. J. 122, 107-113.

Bailey R.E. (1957) The effect of estradiol on serum calcium, phosphorus, and protein of goldfish. J. exp. Zool. 136, 455-469.

Barman T.E., Nguyen-Kim Bai & Nguyen-Van Thoai (1964) Studies on a herring-egg phosphoprotein. Biochem. J. 90, 555-558.

Belcheva R.G. & Khristov D.I. (1972) A comparative electrophoretic and immunoelectrophoretic study of serum proteins in teleostean fishes. Зоологический Журнал 51, 682-687.

Bergink E.W. & Wallace R.A. (1974) Precurosor-product relationship between amphibian vitellogenin and the yolk proteins, lipovitellin and phosvitin. J. Biol. Chem. 249, 2897-2903.

Bernardi G. & Cook W.H. (1960) Separation and characterization of the two high density lipoproteins of egg yolk, α- and β-lipovitellin. Biochim. Biophys. Acta 44, 96-105.

Booke H.E. (1964) Blood serum protein and calcium levels in yearling brook trout. Progressive Fish-Culturist 26, 107-110.

Campbell C.M. (1978) In vitro stimulation of vitellogenin incorporation into trout oocytes by salmon pituitary extracts. Ann. Biol. anim. Bioch. Biophys. 18, 1013-1018.

Christmann J.L., Grayson M.J. & Huang R.C.C. (1977) Comparative study of hen yolk phosvitin and plasma vitellogenin. Biochemistry 16, 3250-3256.

Clark R.C. (1970) The isolationand composition of two phosphoproteins from hen's egg. Biochem. J. 118, 537-542.

Clark R.C. (1973) Amino acid sequence of a cyanogen bromide cleavage peptide from hens egg phosvitin. Biochem. Biophys. Acta 310, 174-187.

Cook W.H. & Wallace R.A. (1965) Dissociation and subunit size of β-lipovitellin. Canadian J. Biochem. 43, 661-669.

Craik J.C.A. (1978) Plasma levels of vitellogenin in the elasmobranch *Scyliorhinus canicula* L. (Lesser spotted dogfish). Comp. Biochem. Physiol. 60B, 9-18.

Crim L.W. & Idler D.R. (1978) Plasma gonadotrophin, estradiol, and vitellogenin and gonad phosvitin levels in relation to the seasonal reproductive cycles of female brown trout. Ann. Biol. anim. Bioch. Biophys. 18, 1001-1005.

Davis B. (1964) Disc electrophoresis-II. Method and application to human serum proteins. Ann. N.Y. Acad. Sci. 121, 404-427.

Deeley R.G., Mullinix K.P., Wetekam W., Kronenberg H.M., Meyers M., Eldridge J.D. & Goldberger R.F. (1975) Vitellogenin synthesis in the ovian liver. J. Biol. Chem. 250, 9060-9066.

Deutsch H.F. & Goodloe M.B. (1945) An electrophoretic survey of various animal plasmas. J. Biol. Chem. 161, 1-20.

Deutsch H.F. & McShan W.H. (1949) Biophysical studies of blood plasma proteins of some lower animals. J. Biol. Chem. 180, 219-234.

Drilhon A. & Fine J.M. (1963) Dimorophisme sexuel dans les protéins sériques de *Salmo salar*: etude électrophorétique. C. r. Soc. Biol. 157, 1897-1900.

Emmersen B.K. & Emmersen J. (1976) Protein, RNA and DNA metabolism in relation to ovarian vitellogenic growth in the flounder *Platichthys flesus* (L.). Comp. Biochem. Physiol. 55B, 315-321.

Emmersen B.K. & Petersen I.M. (1976) Natural occurrence, and experimental induction by estradiol-17-β, of a lipophosphoprotein (vitellogenin) in flounder *(Platichthys flesus*, L.*)* Comp. Biochem. Physiol. 54B, 443-446.

Engle R.L., Woods K.R., Paulsen E.C. & Pert J.H. (1958) Plasma cells and serum proteins in marine fish. Proc. Soc. Exp. Biol. Med. N.Y. 98, 905-909.

Fine J.M. & Drilhon A. (1963) Etude immunologique des protéins du sérum de *Salmo salar*: étude par immunodiffusion. C. r. Soc. Biol. 157, 1937-1940.

Fine J.M., Boffa G.A. & Drilhon A. (1963) The proteins of eel serum: starch gel electrophoresis and immunological studies. Proc. Soc. Exptl. Biol. Med. 114, 651-654.

Fiske C.E. & Subbarow Y. (1925) The colorimetric determination of phosphorus. J. Biol. Chem. 66, 375-400.

Follett B.K. & Redshaw M.R. (1968) The effects of oestrogen and gonadotrophins on lipid and protein metabolism in *Xenopus laevis* Daudin. J. Endocr. 40, 439-456.

Franzen J.S., Bobik C.M., Stuchell R.N. & Lee L. (1968) Polypeptide subunits of lipovitellin. Arch. Biochem. Biophys. 123, 127-132.

Fujiya M. (1961) Use of electrophoretic serum separation in fish studies. J. Water Pollution Control Federation 33, 250-257.

Gemeroy G.G. (1943) On the relationship of some common fishes as determined by the precipitin reaction. Zoologica 28, 109-123.

Giblett E.R., Hickman C.G. & Smithies O. (1959) Serum transferrins. Nature 183, 1589.

Gordon J.I., Deeley R.G., Burns A.T.H., Paterson B.M., Christmann, J.L. & Goldberger R.F. (1977) In vitro translation of avian vitellogenin messenger RNA. J. Biol. Chem. 252, 8320-8327.

Grabor P. & Williams C.A. (1953) Méthode permettant l'etude conjuguée des propriétes électrophorétiques et immunochimiques d'un mélange de protéines. Application au sérum sanguin. Biochim. Biophys. Acta 10, 193-194.

Gray W.R. (1972) End-group analysis using dansyl chloride. Methods Enzymol. 25, 121-138.

Hara A. (1975) Electrophoretical and immunological studies of fish serum proteins. Bull. Jap. Soc. Sci. Fish. 41, 105-113.

Harris J.E. (1974) Electrophoretic patterns of blood serum proteins of the cyprinid fish, *Leuciscus leuciscus (L)*. Comp. Biochem. Physiol. 48B, 389-399.

Irisawa H. & Irisawa A.F. (1954) Blood serum protein of the marine elasmobranchii. Science 120, 849-851.

Ishii K. (1971) Morphological changes in the liver of the kokanee, *Oncorhynchus nerka*, accompanied with sexual maturation. Bull. Fac. Fish. Hokkaido Univ. 22, 215-220.

Ito Y., Fujii T. & Yoshioka R. (1963) On a phosphoprotein isolated from trout egg. J. Biochem. 53, 242-243.

Kawamura A. (1966) Fluorescent antibody technique. Protein, nucleic and enzyme 11, 1621-1634.

Kawahara K. (1969) Evaluation of diffusion coefficients of proteins from sedimentation boundary curves. Biochemistry 8, 2551-2557.

Krauel K.K. & Ridgway G.J. (1963) Immunoelectrophoretic studies of red salmon *(Oncorhynchus nerka)* serum. Int. Archs. Allergy. appl. Immun. 23, 246-253.

Krøll J. (1973) Tandem-crossed immunoelectrophoresis. Scand. J. Immunol. 2, Suppl. 1, 57-59.

Lowry O.H., Rosebrough N.J., Farr A.L. & Randall R.J. (1951) Protein measurement with the Folin phenol regent. J. Biol. Chem. 193, 265-272.

Mairs D.F. & Sindermann C.J. (1960) Intraspecies variability in electrophoretic patterns of fish serum. Anat. Record 137, 377-378.

Mancini G., Carbonara A.O. & Heremans J.F. (1965) Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry 2, 235-254.

Mano Y. (1970) Mechanism of phosphorylation of phosphoprotein during oogenesis in trout in vivo. Biochem. Biophys. Acta 201, 284-294.

Markert J.P. & Vanstone W.E. (1971) Egg proteins of coho salmon *(Oncorhynchus kisutch)*. J. Fish. Res. Bd. Can. 28, 1853-1856.

Moore H.D. (1945) Species differences in serum protein patterns. J. Biol. Chem. 161, 21-32.

Møller D. (1970) Transferrin polymorphism in Atlantic salmon *(Salmo salar)*. J. Fish. Res. Bd. Can. 27, 1617-1625.

Morgan E.H. (1975) Plasma iron transport during egg laying and after oestrogen administration in the domestic fowl *(Gallus domesticus)*. Quarterly J. Exp. Physiol. 60, 233-247.

Mulcahy M.F. (1967) Serum protein changes in diseased Atlantic salmon. Nature 215, 143-144.

McMeekin T.L., Groves M.L. & Hipp N.J. (1949) Apparent specific volume of α-casein and β-casein and the relationship of specific volume to amino acid composition. J. Am. Chem. Soc. 71, 3928-3300.

Oguri M. & Takada N. (1967) Serum calcium and magnesium levels of goldfish with special reference to gonadal maturation. Bull. Jap. Soc. Scient. Fish. 33, 161-166.

Ouchterlony Ö. (1953) Antigen-antibody reactions in gels. IV. Types of reactions in coordinated systems of diffusion. Acta Path. Microbiol. Scand. 32, 231-240.

Pan M.J., Bell W.J. & Telfer W.H. (1969) Vitellogenic blood protein synthesis by insect fat body. Science 165, 393.

Payne R.H., Child A.R. & Forrest A. (1971) Geographical variation in the Atlantic salmon. Nature 231, 250-252.

Penning T.M., Merry A.H., Munday K.A. & Akhtar M. (1977) Studies on the biosynthesis, assembly and secretion of vitellogenin, an oestrogen-induced multicomponent protein. Biochem. J. 162, 157-170.

Perrier H., Delcroix J.P., Perrier C. & Gras J. (1973) Disc electrophoresis of plasma proteins of fish. Physical and chemical characters; localization of fibrinogen, transferrin and ceruloplasmin in the plasma of the rainbow trout *(Salmo gairdnerii* Richardson). Comp. Biochem. Physiol. 48B, 679-685.

Pickering A.D. (1976) Effects of gonadectomy, oestradiol and testosterone on the migrating river lamprey, *Lampetra fluviatilis* (L). Gen. Comp. Endocr. 28, 473-480.

Plack P.A. & Woodhead P.M.J. (1966) Vitamin A compounds and lipids in the blood of the cod, *Gadus morhua*, from the arctic in relation to gonadal maturation. J. mar. biol. Ass. U.K. 46, 547-559.

Plack P.A. & Pritchard D.J. (1968) Effect of oestradiol 3-benzoate on the concentrations of retinal and lipids in cod plasma. Biochem. J. 106, 257-262.

Plack P.A., Pritchard D.J. & Fraser N.W. (1971) Egg proteins in cod serum. Biochem. J. 121, 847-856.

Prat J.P., Lamy J.N. & Weill J.D. (1969) Coloration des lipoprotéins après électrophorèse en gel de polyacrylamid.

Rall D.P., Schwab P. & Zubrod C.G. (1961) Alteration of plasma proteins at metamorphosis in the lamprey *(Petromyzon marinus dosatus)*. Science 133, 279-280.

Redshaw M.R. & Folhett B.K. (1971) The crystallin yolk-platelet proteins and their soluble plasma precursor in an amphibian, *Xenopus laevis*. Biochem. J. 124, 759-766.

Ridgway G.J., Klontz G.W. & Matsumoto C. (1962) Intraspecific differences in serum antigens of red salmon demonstrated by immunochemical methods. Bull. Int. North Pac. Fish. Comm. 8, 1-13.

Rolan R.G. (1968) Electrophoretic comparison of human and catfish plasma proteins. Trans. Am. Fish. Soc. 97, 455-459.

Saito K. (1957) Biochemical studies on the fish blood-VIII on the electrophoretic specificity of serum proteins. Bull Jap. Soc. Sci. Fish. 22, 752-759.

Schmidt G., Bartsch G., Kitagawa T., Fujisawa K., Knolle J., Joseph J., De Marco P., Liss M. & Haschemeyer R. (1965) Isolation of a phosphoprotein of high phosphorus content from the eggs of brown brook trout. Biochem. Biophys. Res. Commun. 18, 60-65.

Solomon K. & Allanson B.R. (1968) The effects of exposure to low temperatures on the serum protein of *Tilapia mossambica*. Comp. Biochem. Physiol. 25, 485-492.

Sulya L.L., Box B.E. & Gunter G. (1961) Plasma proteins in the blood of fishes from the Gulf of Mexico. Am. J. Physiol. 200, 152-154.

Svedberg T. & Perderson & K.O. (1940) "The Ultracentrifuge", Oxford Univ. Press, London, p.6.

Thomas M.L.H. & McCrimmon H.R. (1964) Variability in paper electrophoretic patterns of the serum of landlocked sea lamprey, *Petromyzon marinus* Linnaeus. J. Fish. Res. Bd. Can. 21, 239-246.

Thurston R.V. (1967) Electrophoretic patterns of blood serum proteins from rainbow trout *(Salmo gairdneri)*. J. Fish. Res. Bd. Can. 24, 2169-2188.

Utter F.M. & Ridgway G.J. (1967) A serologically detected serum factor associated with maturity in English sole, *Parophrys vetulus*, and Pacific halibut, *Hippoglossus stenolepis*. Fishery Bull. Fish Wildl. Serv. U.S. 66, 47-58.

Utter F.M., Ames W.E. & Hodgins H.O. (1970) Transferrin polymorphism in coho salmon *(Oncorhynchus kisutch)*. J. Fish. Res. Bd. Can. 27, 2371-2373.

Vanstone W.E. & Ho C.W. (1961) Plasma proteins of coho salmon, *Oncorhynchus kisutch*, as separated by zone electrophoresis. J. Fish. Res. Bd. Can. 18, 393-399.

Wallace R.A. & Jared D.W. (1968) Studies on amphibian yolk. VII. Serum phosphoprotein synthesis by vitellogenic females and estrogen-treated males of *Xenopus laevis*. Can. J. Biochem. Physiol. 46, 953-959.

Wallace R.A. & Jared D.W. (1969) Studies on amphibian yolk. VIII. The estrogen-induced hepatic synthesis of a serum lipophosphoprotein and to selective uptake by the ovary and transformation into yolk platelet proteins in *Xenopus laevis*. Devl. Biol. 19, 498-526.

Wallace R.A. (1970) Studies on amphibian yolk. IX. *Xenopus* vitellogenin. Biochem. Biophys. Acta 215, 176-183.

Wallace R.A. & Bergink E.W. (1974) Amphibian vitellogenin: Properties, hormonal regulation of hepatic synthesis and ovarian uptake, and conversion to yolk proteins. Am. Zool. 14, 1159-1175.

Weber K. & Osborn M. (1969) The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244, 4406-4412.

Weeke B. (1973) Crossed immunoelectrophoresis. Scand. J. Immunol. 2, Suppl. 1, 47-56.

Weinstein M.P. & Yerger R.W. (1976) Electrophoretic investigation of subpopulation of the spotted seatrout, *Cynoscion nebulosus* (Cuvier), in the Gulf of Mexico and Atlantic coast of Florida. Comp. Biochem. Physiol. 54B, 97-102.

Woodhead R.M.J. (1969) Influence of oestradiol 3-benzoate upon the plasma content of calcium and vitamin A aldehydes in the cod, *Gadus morhua*. J. mar. biol. Ass. U.K. 49, 939-944.

Yamamoto K. & Yoshioka H. (1964) Rhythm of development in the oocyte of the medaka, *Oryzias latipes*. Bull. Fac. Fish. Hokkaido Univ. 15, 5-19.

Yamamoto K., Morioka T., Hiroi O. & Omori M. (1974) Artificial maturation of female Japanese eels by the injection of salmonid pituitary. Bull. Jap. Soc. Sci. Fish. 40, 1-7.

Yamashita H. (1969) Haematological study of a species of rockfish, *Sebastiscus marmoratus* - V. Seasonal changes of blood elements, electrophoretic pattern of serum proteins and their percentage fractions. Bull. Jap. Soc. Sci. Fish. 35, 370-385.

Yoneda T. & Ishihara Y. (1974) Disc electrophoretic patterns of blood serum proteins from chum salmon, *Oncorhynchus keta* and cherry salmon, *Oncorhynchus masou*. Bull. Fac. Fish. Hokkaido Univ. 24, 76-89.

Zacharius R.M., Zell T.E., Morrison J.H. & Woddlock J.J. (1969) Glycoprotein staining following electrophoresis on acrylamide gels. Anal. Biochem. 30, 148-152.