Immunological identification of two female-specific proteins from the plasma of Indian freshwater murrel, *Channa punctatus* (Bloch)

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Vitellogenin, the egg yolk precursor, has been isolated from the blood of several fish species\(^1\). It is an estrogen-inducible female-specific multicomponent protein; present in the adult vitellogenic females but absent in males and in immature non-vitellogenic females\(^2\). In addition, cDNA sequence for vitellogenin has been cloned and analysed\(^3\). A characteristic feature of vitellogenin molecule is the presence of alkali-labile phosphorus that permits its in vivo labelling with radiolabelled phosphorus. This characteristic has been employed by most investigators for identification and estimation of vitellogenin in the blood of several fishes and have led to the belief that vitellogenin is the sole female-specific protein in the fish blood. Estrogen induces, not only the synthesis of vitellogenin, but also of lipoproteins, egg envelope proteins that lack phosphorus\(^4\)-\(^11\). Obviously, these sex-specific, estrogen inducible non-phosphorylated proteins cannot be detected by these conventional techniques.

Immunology, which offers analytical tools of exquisite precision, has been applied to develop immunoassays (RIA and ELISA) for measurement of plasma vitellogenin and egg-yolk proteins, and to localize vitellogenin in fish hepatocytes. Immunological techniques can also be employed for detection of female-specific plasma proteins that, unlike vitellogenin, lack phosphorus.

Present communication reports on immunological identification of a female-specific protein, in addition to vitellogenin, in the blood of the Indian freshwater murrel, *Channa punctatus*, by using specific antisera.

**Materials and Methods**

**Collection and care of fish**—Adult specimens of *C. punctatus* (body weight 100-150 g) were collected from the backwaters of river Yamuna around Delhi (Lat. 28°35' N, Long. 77°12' E) and maintained at 25±1°C and a photoperiod of 12L:12D for at least 7 days prior to use in experiments\(^12\). Fishes were fed *ad libitum* every evening with minced beef. Water in the aquaria was renewed daily with dechlorinated water adjusted to laboratory temperature.

**Chemicals**—Unless otherwise specified, all chemicals used were of analytical grade and were obtained from Sigma Chemical Co., St. Louis, U.S.A. and British Drug House Laboratory, Mumbai, India.

**Induction and in vivo labelling of female-specific plasma proteins**—Female murrels were collected during prespawning period and injected im every day with 10 \(\mu\)g estradiol-17\(\beta\) (E\(\_\)2)/100 g fish body weight for 7 days\(^13\). Twenty four hours prior to bleeding each fish was administered, ip, 0.25 mCi of \(^{32}\)P-NaH\(_2\)PO\(_4\) (sp. activity 104 mCi/mM; BARC, Mumbai). Fifteen minutes before collection of blood each fish was
injected with 4-5 TIU of aprotinin. Blood samples were collected, centrifuged and the pooled plasma was processed immediately to prevent possible aggregation of plasma proteins. Plasma was either subjected directly to gel filtration chromatography or after prior ultracentrifugation at 136,000 g for 1 hr to remove low-density lipoproteins.

Isolation of plasma proteins—Ultrogel AcA 34 (LKB) packed in a glass column (100×2.5 cm) was equilibrated with several volumes of 0.1M KCl, 0.1M Tris-Cl buffer at pH 7.5 containing 0.2% sodium azide. Plasma proteins were eluted at 4°C at a flow rate of 20 ml/hr. Fractions of 5 ml were collected and the absorbance recorded at 280 nm. One hundred μl from each fraction was applied to Whatman 3 MM filter paper disks and processed for radioactivity measurement.

Preparation of antibodies—New Zealand white rabbits were injected im with an emulsion of an equal volume of estrogenized murrel plasma and Freund’s Complete Adjuvant four times at weekly intervals. Rabbits showing a positive response were administered a booster injection and bled one week later. Blood was allowed to clot at 4°C overnight and the serum stored in small aliquots at -20°C after addition of sodium azide. This antiserum is referred to as poly-specific antiserum (PSAS). Two ml of this antiserum was absorbed with 1.5 ml of normal male serum. The resultant antiserum showed no cross-reaction with male serum when tested by double immunodiffusion and is referred to as female-specific antiserum (FSAS).

Immunological analysis and autoradiography—³²P-labelled murrel plasma as well as fractions obtained after gel filtration were tested against PSAS and FSAS. Ouchterlony immunodiffusion and immunoelectrophoresis were performed following routine procedures. After development of precipitin lines, the plates were washed free of excess reactants and dried. They were coated with a thin film of photographic emulsion (Kodak NTB 3) and after seven days developed in Kodak D-19b developer.

SDS PAGE and peptide mapping—Samples were subjected to polyacrylamide gel electrophoresis under denaturing conditions (SDS PAGE) on 5% gel. Peptide mapping of proteolytic digests was performed according to the procedure of Cleveland et al. Samples were digested with either papain or chymotrypsin at 37°C for 30 min and proteolysis stopped by heating the mixture to 100°C for 2 min. The digests were electrophoresed at 200 V and stained with Coomassie Brilliant Blue.

Fig. 1.—Gel filtration profile of ³²P labelled plasma from estradiol-treated murrel C. punctatus. Almost the entire ³²P activity is associated with vitellogenin (peak III).
Results

Elution profile and immunological identification of plasma proteins in murrel—Figure 1 depicts the elution pattern of $^{32}$P-labelled plasma of female murrel on Ultrogel AcA 34 after 7 injections of $E_2$. Plasma resolved into five absorbance peaks. The first peak,
which eluted within void volume, was opalescent due to presence of low density lipoproteins with some \(^{32}\)P activity. Almost the entire \(^{32}\)P activity was associated with third peak (fractions 23-33). This protein was characterised as vitellogenin on the basis of \(^{32}\)P activity and its elution position (Ve/Vo 1.42). Interestingly, an additional \(^{32}\)P-containing component was observed at 1.8 elution position in the fractions (38-44) of the fourth peak.

Alternate fractions from the entire gel filtration profile were tested for immunoreactivity against PSAS and FSAS. The results presented in Fig. 2 show that with the exception of low density lipoprotein fraction (11), all other fractions crossreacted with PSAS. With FSAS, the precipitin reaction was confined only to fractions of third and ascending part of the fourth absorbancy peaks (see Fig. 2). A perusal of Fig. 2 clearly shows that the antigen in the IIIrd peak (fractions 23-33) was immunologically distinct from that in the ascending part of IVth peak (fractions 35-41). Descending fractions (43, 45) of the IVth peak showed no reaction with FSAS. When fraction 27 (III peak) and fraction 39 (from IV peak) were placed in the adjacent wells, the precipitin arcs formed against FSAS exhibited a cross over reaction of non-identity (Fig. 2). Subsequently in the text, these two immunologically discrete female-specific plasma proteins are referred to as FS I and FS II respectively.

One step gel filtration chromatography on Ultrogel AcA 34 is sufficient to resolve murrel plasma proteins\(^{15}\). The present investigation showed that prior ultracentrifugation of \(^{32}\)P-labelled plasma at 136,000 g for 1 hr that removes low density lipoproteins, results in a better resolution of FS I and FS II. The gel filtration profile of the clear subnatant is shown in Fig. 3. Almost the entire \(^{32}\)P was confined to FS I fractions. In contrast, FS II has no \(^{32}\)P activity. A small peak of radioactivity was associated with a protein, which...
eluted after FS II. These two proteins (FS I and FS II) isolated by gel filtration chromatography of ultracentrifuged plasma of estrogenized murrel were employed for a more detailed immunological characterization.

Results of radial immunodiffusion experiments show that FS I and FS II are immunologically distinct and both proteins are present in the unfraccionated E2-treated plasma. When Ouchterlony plates were subjected to autoradiography, the radioactivity was found to be associated only with FS I; radioactive precipitin line corresponding to FS II was completely absent both in the native plasma as well as in the isolated protein (Fig. 4). When plasma was subjected to immunoelectrophoresis, two precipitin arcs with different electrophoretic mobility were formed against FSAS; of these, one corresponded to purified FS I and the other to purified FS II (Fig. 5).

Immunoelectrophoresis followed by autoradiography also confirmed the absence of 32P radioactivity in FS II. Plasma from normal untreated male murrel showed no reaction with FSAS (Fig. 6A), whereas the

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**Fig. 6**—Ouchterlony double diffusion in agarose gel. The central well contained 50 μl female-specific antiserum (FSAS). Wells A and D contained purified FS II; wells B and E contained purified FS I in Figs 6A & 6B; wells C and F contained plasma from normal male murrel (Fig. 6A) or plasma from normal vitellogenic female murrel (Fig. 6B). In Fig. 6C wells A and D contained purified FS II and FS I respectively, wells B, C, E and F contained crude homogenate (10 μl) of ovary from gravid murrel. Note the absence of precipitin line against male plasma (Fig. 6A) and presence of two precipitin lines against vitellogenic female plasma (Fig. 6B) as well as against ovarian homogenate (Fig. 6C). Also note the cross-over (reaction of non-identity) between precipitin lines FS I and FS II (Figs 6A & 6B). Confluence of either line (reaction of identity) with precipitin lines against vitellogenic female plasma (Fig. 6B) and against ovarian homogenate (Fig. 6C).

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**Fig. 7**—Electrophoretic pattern on SDS PAGE (Fig. 7A) and Peptide maps (Fig. 7B) of FS I (lane a) and FS II (lane b). Marker proteins (lane c in Fig. 7A) electrophoresed were A: myosin (205 kD), B: β-galactosidase (116 kD), C: phosphorylase (97 kD), D: bovine serum albumin (66 kD), E: chicken egg albumin (45 kD), F: carbonic anhydrase (29 kD). Samples were loaded on 5% resolving gel. For peptide maps (Fig. 7B) either protein at a final concentration of 0.5 mg/ml in sample buffer, was incubated at 37°C for 30 min with 3 μg/ml of chymotrypsin (substrate: enzyme::100:1). 25 μg of substrate was loaded per well of a 10% resolving gel.
native plasma from normal seasonally vitellogenic murrel formed two precipitin lines with FSAS which exhibited reactions of complete identity with the respective precipitin lines of FS I and FS II (Fig. 6B). Further, these two female-specific proteins were also present in ovarian homogenate of gravid murrel, since of the two precipitin lines formed between FSAS and the ovarian homogenate, one precipitin line was confluent with purified FS I and the other with purified FS II (Fig. 6C).

**SDS PAGE and peptide mapping**—On SDS PAGE two proteins, FS I and FS II, resolved into one peptide (Fig. 7A). The molecular weight of FS I peptide was 175kD whereas that of FS II was 110 kD. Papain and chymotrypsin were used for digestion. Papain did not generate fragments of sufficiently wide range of molecular weights. However, a satisfactory pattern was observed with chymotrypsin. The peptide maps of the two proteins, FS I and FS II, were completely different, with each protein containing numerous peptides of the mobility not represented in the other (Fig. 7B).

**Discussion**

The present study clearly demonstrates the presence of two distinct female-specific plasma proteins in the plasma of female murrel, *C. punctatus*, following exogenous administration of estradiol-17β. Both the proteins are absent in the plasma of male murrel. Out of the two, only one (FS I) contains alkali-labile phosphorus and can incorporate radioactive phosphorus, thereby indicating that this protein is vitellogenin. In murrel, FS II is a unique non-phosphorylated plasma protein whose chromatographic and electrophoretic behaviour differs from FS I (vitellogenin).

The elution pattern of E2 plasma on Ultrogel AcA 34 shows that FS II is sometimes insufficiently separated from other plasma components. Prior removal of lipoproteins by ultracentrifugation greatly improves the resolution of FS II from vitellogenin and from other plasmatic proteins. This protein lacks radio-labelled phosphorus.

A minor phosphoprotein (elution position 1.8) is observed in the plasma of normal untreated male murrel. Similar protein is reported in the plasma of E2-treated male and that of vitellogenic female murrel. Neither the concentration nor the 32P activity of this component changes significantly after E2 treatment. Presence of this protein in either sex at any time of the year suggests that it is not a sex-specific phosphoprotein.

Unfractionated (native) plasma from normal seasonally vitellogenic female murrel as well as from E2-treated murrel yields two distinct precipitin lines against FSAS; one of these is confluent with the precipitin line formed against purified vitellogenin (FS I) and the other with that formed by FS II. This shows that FS II is not an artifact but a natural plasma protein of vitellogenic murrel. Further, two precipitin lines are formed against FSAS by a crude homogenate of yolky oocytes, of which one shows reaction of identity with vitellogenin and the other with FS II. The immunological similarity between vitellogenin and egg-yolk proteins is only to be expected, since the former is an established precursor of the latter. That vitellogenin shares antigenic sites with egg-yolk proteins has often been demonstrated.

The present results further show that the two female-specific plasma proteins of murrel are immunologically different from each other. This is evidenced by the fact that these two proteins exhibit precipitin reactions of complete non-identity with FSAS. Further, the two proteins have entirely different electrophoretic mobilities, FS I (vitellogenin) being more negatively charged than FS II. The molecular weight of FS I peptide is more than that of FS II peptide. Further, autoradiography shows that unlike vitellogenin, FS II lacks phosphorus. Peptide mapping is a stringent test of protein identity and that if two peptide profiles are different then the original proteins are not closely related. The highly divergent peptide profiles of FS I (vitellogenin) and FS II observed in the present study should, therefore, be regarded as sufficient proof that the two proteins are dissimilar and unrelated.

Estrogen stimulates not only the synthesis of vitellogenin but of a variety of other components which, like vitellogenin, are eventually deposited in the oocytes. For example, in many avian and fish species estradiol promotes, in addition to vitellogenin synthesis, a pronounced accumulation of low and very low density lipoproteins which are sequestered by the growing oocytes. Estrogens also induce the synthesis of several vitamin-carrying proteins, specially those concerned with the binding and transport of riboflavin and thiamine which are considered essential for embryonic development. Polymorphic forms of estrogen-dependent proteins have been reported in the plasma and egg-yolk of quail, *Coturnix coturnix*. In *Xenopus*, Holland and Wangh have reported an additional protein which is responsive to estradiol treatment but is absent in normal males. Yet another protein (EP 20) is present in both sexes of...
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Xenopus but its levels increase four-fold following estradiol treatment.31

In majority of fishes vitellogenin has been reported to be the only female-specific plasma protein. Nonetheless, in plasma of few fishes, precursor for egg envelope proteins, a female-specific protein other than vitellogenin, has been reported10,14,22-28. In coho salmon, Oncorhynchus kisutch, three egg-yolk components are present in the plasma of sexually maturing and estrogenized female fish.25 Two egg-yolk components, E1 and E2, of the rainbow trout exhibit an immunological reaction of non-identity when tested against a female specific antiserum; however, vitellogenin spurs over either protein suggesting that these two egg-yolk proteins are derived from vitellogenin.27 A significant finding reports on the isolation from the ascites fluid of medaka, Oryzias latipes, of spawning female-specific protein other than vitellogenin which cross reacts with an antichorion glycoprotein antibody.25,26 Hyllner et al.25 and Hyllner and Haux30 have detected plasma proteins, immunologically related to vitelline envelope proteins, which can be induced by estradiol administration in both sexes in rainbow trout (Oncorhynchus mykiss), brown trout (Salmo trutta) and turbot (Scophthalamus maximus). Similar observation has been reported by Larson et al.27. Molecular biological analysis of egg envelope has suggested that vitelline envelope protein genes are expressed in the liver only, implying an extra-ovarian origin of these proteins.28,31 These results suggest that some major components of egg envelope be synthesized in the liver under the influence of estrogen.

Egg yolk provides nutrition whereas egg envelope protects the developing embryo. Vitellogenin and chorionigen, precursor proteins for egg yolk and egg envelope respectively, are synthesized in the liver under the influence of estradiol-17β. The present study shows that two unrelated proteins, FS I (vitellogenin) and FS II, are synthesized by the hepatocyte under the influence of estradiol-17β. Both the proteins are transported via blood to the ovaries where they are incorporated in the oocytes.

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