

## RETINALS AND RETINOLS INDUCED BY ESTROGEN IN THE BLOOD PLASMA OF *XENOPUS LAEVIS*

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### Summary

Injection of estrogen into male *Xenopus laevis* induced the appearance of retinals (retinal and 3-dehydroretinal) and a considerable increase in the amount of retinols (retinol and 3-dehydroretinol) in the blood plasma. These retinoids were mainly in the all-*trans* form. Without estrogen injection, retinols were normally found in the blood plasma of both males and females, but only trace amounts of retinals were detected and these were restricted to the plasma of females.

The proteins in the blood plasma of estrogen-injected males were separated into two fractions. One fraction included vitellogenin, the precursor of egg yolk proteins, and the other contained some plasma proteins other than vitellogenin. Retinals were detected in the former and retinols in the latter. It is suggested that retinals are bound to vitellogenin and are taken up into oocytes in the process of vitellogenesis.

### Introduction

Previous papers (Seki *et al.* 1987; Azuma *et al.* 1988, 1990) have described our studies on retinoids in the eggs of toads and frogs. All-*trans* retinals were present but other retinoids and carotinoids were hardly detectable. In *Xenopus laevis*, a decrease in the level of egg retinals (all-*trans* retinal and 3-dehydroretinal) during the period of development before the larvae began to feed was accompanied by the formation of retinols (all-*trans* retinol and 3-dehydroretinol), retinyl esters (all-*trans* retinyl ester and 3-dehydroretinyl ester) and 11-*cis* retinals (retinal and 3-dehydroretinal). These results suggest that egg retinals are the source of the vitamin A necessary for larval development in *Xenopus laevis*.

Recent experiments have confirmed that retinals in the egg are bound to a yolk protein, lipovitellin 1, through a Schiff base linkage (Irie *et al.* 1991). It is known that egg yolk proteins are derived from a maternal blood plasma protein, vitellogenin, and that vitellogenin is synthesized in the liver, secreted into the blood circulation and sequestered by oocytes (Wallace and Jared, 1968, 1969; Wallace and Bergink, 1974; Wallace, 1985). If retinals are bound to vitellogenin, then retinals should also be produced in the liver, bound to vitellogenin and taken up into oocytes. Vitellogenin synthesis is normally

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controlled in females, and can be initiated in males by exogenous estrogen (Follett *et al.* 1968; Munday *et al.* 1968; Wallace and Dumont, 1968; Wallace and Jared, 1968; Dolphin *et al.* 1971). The concentration of vitellogenin in the blood plasma of an estrogen-injected male is higher than that of a normal female (Wallace and Jared, 1968; Selman and Wallace, 1983; Wallace, 1985). This study shows that retinals can be detected in the blood plasma of females and estrogen-injected males and that the amount of retinals in the former is smaller than that in the latter. Furthermore, the quantity of blood plasma retinol is also increased in estrogen-injected males. The fractionation of blood plasma proteins shows that the vitellogenin fraction contains retinals but little retinol. The results suggest that retinals are bound to vitellogenin but that retinols are not.

## Materials and methods

### *Animals and blood plasma*

Adult male and female *Xenopus laevis* were purchased from Hamamatsu Seibutsu Kyozaï Co., Ltd (Japan). They were reared at approximately 23°C and fed twice weekly with toad food supplied by the same company. Animals were injected *via* the dorsal lymph sac with 1mg of estradiol-17 $\beta$  dissolved in 0.5ml of olive oil (Dolphin *et al.* 1971). Control animals received injections of the medium only. Several days or weeks after the injection, the toads were anesthetized by hypothermia and subjected to ventrotomy. Blood was collected from the heart using a syringe filled with 50% Dulbecco's Ca<sup>2+</sup>-free saline with 0.07mol l<sup>-1</sup> sodium citrate (Wiley *et al.* 1979). The blood sample was centrifuged (1400g, 10min) and the plasma was obtained as the supernatant.

### *Preparation of vitellogenin from blood plasma*

Vitellogenin was prepared from the blood plasma according to Wiley *et al.* (1979). Briefly, 5ml of the plasma sample was mixed with 20ml of 20mmol l<sup>-1</sup> EDTA solution and 1.6ml of 0.5mol l<sup>-1</sup> MgCl<sub>2</sub> was added. This was further mixed gently and centrifuged (13000g, 10min) to obtain a supernatant (sample A) and a precipitate. The precipitate was dissolved in 3ml of 1mol l<sup>-1</sup> NaCl, 50mmol l<sup>-1</sup> TrisCl buffer (pH7.5) and centrifuged (13000g, 10min). The supernatant obtained in this way was mixed with 25ml of distilled water, resulting in the precipitation of vitellogenin. The precipitate was redissolved in 3ml of 1mol l<sup>-1</sup> NaCl, 50mmol l<sup>-1</sup> TrisCl buffer (pH7.5) and dialyzed against 1mol l<sup>-1</sup> NaCl and then twice against 50mmol l<sup>-1</sup> TrisCl buffer (pH7.5). The sample obtained (sample B) included vitellogenin.

### *Detection of retinoids in the samples*

Retinoids in the blood plasma or fractionated samples (samples A and B) were extracted by the oxime method and analysed by high-performance liquid chromatography (HPLC) as reported previously (Azuma *et al.* 1988, 1990; Irie *et al.* 1991). An HPLC system (JASCO, Japan) equipped with a 4.6 mm $\times$ 250 mm column of YMC-Pack A-003-3 SIL (Yamamura Chemical Laboratories Co. Ltd, Japan) was used

and a mixture of *n*-hexane, diethylether and ethanol (90:10:0.1 v/v) was eluted at a flow rate of 1.3 mlmin<sup>-1</sup>. The absorbances of fractions at 350nm and 330nm were measured with JASCO 875UV detectors and peak areas were determined by integrating with a Chromatopac C-R4A (Shimadzu, Japan). Sometimes the fluorescence, excited at 330 nm, was monitored at 470nm using a fluorescence spectrophotometer F1000 (Hitachi, Japan). Quantities of several retinoid isomers were estimated from their absorption coefficients and the peak areas of known amounts of standard retinoids (Azuma *et al.* 1988, 1990; Irie *et al.* 1991).

#### *SDS-polyacrylamide gel electrophoresis*

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970) with 7.5% acrylamide gel as the separating gel. The quantification of proteins in the samples was carried out by the Lowry method (Lowry *et al.* 1951) and by a method measuring the fluorescence excited at 280nm using bovine serum albumin as the standard protein. The detailed procedure has been described previously (Irie *et al.* 1991).

## Results

### *Estrogen induction of retinals and retinols in the blood plasma*

Fig. 1 shows HPLC chromatograms of retinoids extracted from female plasma (Fig. 1B) and from estrogen-injected male plasma (Fig. 1C) together with a chromatogram of standard retinaloximes and retinols (Fig. 1A). The volume of blood plasma for the extraction of retinoids was 0.3ml. In the case of Fig. 1C, the blood was collected 10 days after estrogen injection. Retinals in the samples are extracted as retinaloximes, so that the peaks of retinaloximes in Fig. 1B,C correspond to retinals in the plasma of a female and of an estrogen-injected male. Small *syn*-peaks of all-*trans* retinaloximes (peaks 1 and 2) in Fig. 1B indicate that small amounts of all-*trans* retinal and 3-dehydroretinal (retinal<sub>1</sub> and retinal<sub>2</sub>) are present in female plasma. However, large peaks of all-*trans* retinaloximes are observed in Fig. 1C, indicating the presence of large amounts of all-*trans* retinal and 3-dehydroretinal. Although not shown in this figure, retinals were barely detectable in the plasma of males without estrogen injection. The peaks due to all-*trans* retinol and 3-dehydroretinol (retinol<sub>1</sub> and retinol<sub>2</sub>) (peaks 5 and 6) are also shown in Fig. 1B,C and are much larger in the latter. The unnumbered peaks have not been identified. The amounts of retinals and retinols in the blood plasma were calculated from such HPLC data.

Table 1 shows the amounts of retinoids in the blood plasma of adult females and males and estrogen-injected males. Blood samples were collected from estrogen-injected males 1, 2 and 3 weeks after injection. Only the amounts of all-*trans* retinals (retinal and 3-dehydroretinal) and all-*trans* retinols (retinol and 3-dehydroretinol) were calculated from HPLC data. As shown in Table 1, all-*trans* retinals were already present in the male plasma 1 week after estrogen injection and the levels increased with time after the injection. Maximum amounts of retinals were found 2–3 weeks after injection in this experiment. The percentage of 3-dehydroretinal in all-*trans* retinals (retinal and 3-

dehydroretinal) was about 50% in every case. In contrast, all-*trans* retinols (retinol and 3-dehydroretinol) were present in the plasma of females and males without estrogen injection (about 100pmolml<sup>-1</sup>). However, injection of estrogen increased the amount of all-*trans* retinols by a factor of 20–40 (Table 1). The percentage of 3-dehydroretinol in all-*trans* retinols (retinol and 3-dehydroretinol) is about 20% and is smaller than that of 3-dehydroretinol in all-*trans* retinals.

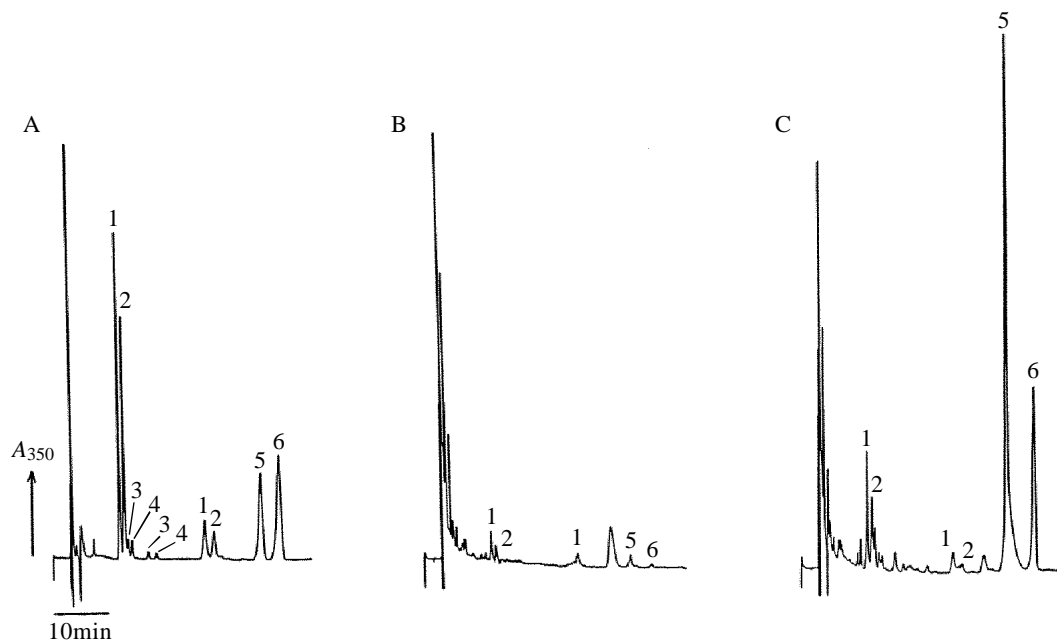


Fig. 1. HPLC chromatograms of standard retinaloximes and retinols (A) and of retinoids extracted from female plasma (B) and estrogen-injected male plasma (C). Male plasma was collected 10 days after estrogen injection. Peaks are labelled as follows: 1, 1', *syn* and *anti* all-*trans* retinaloximes; 2, 2', *syn* and *anti* all-*trans* 3-dehydroretinaloximes; 3, 3', *syn* and *anti* 13-*cis* retinaloximes; 4, 4', *syn* and *anti* 13-*cis* 3-dehydroretinaloximes; 5, all-*trans* retinol; 6, all-*trans* 3-dehydroretinol.

Table 1. Amounts of retinoids in the blood plasma of male *Xenopus* with and without estrogen injection and of female *Xenopus*

	All- <i>trans</i> retinals (pmolml <sup>-1</sup> )	All- <i>trans</i> retinols (pmolml <sup>-1</sup> )
Female (-)	Approx. 10	94±33
Male (-)	<10	96±17
Male (+) 1w	115.4±35.1	2292±1065
Male (+) 2w	222.9±107.9	2349±1919
Male (+) 3w	285.7±74.4	3776±905

Values are mean ± S.D., N=3.

(-), without estrogen injection; (+), with estrogen injection; 1w, 1 week after injection; 2w, 2 weeks after injection; 3w, 3 weeks after injection.

*Retinals and retinols in the fractions of blood plasma*

Blood plasma was prepared from estrogen-injected males 2 weeks after injection. After adding  $MgCl_2$  and EDTA to the plasma, the supernatant (sample A) and the precipitate (sample B) were obtained as described in Materials and methods. Sample A, sample B and the original blood plasma were assayed for retinoids by HPLC and for proteins by SDS-PAGE.

Fig. 2 shows HPLC chromatograms of retinoids extracted from sample A (Fig. 2A), sample B (Fig. 2B) and the blood plasma (Fig. 2C). Each sample was prepared from an aliquot of the same plasma solution. The elution peaks were identified using standard retinaloximes and retinols, as in Fig. 1. Fig. 2C shows the presence of both retinals and retinols in the blood plasma. In this case, the peaks due to all-*trans* retinal (1 and 1'), all-*trans* 3-dehydroretinal (2 and 2'), 13-*cis* retinal (3 and 3') and 13-*cis* 3-dehydroretinal (4 and 4') are clear. The two small peaks eluting just before peak 1 and two further peaks between peaks 4' and 1' are not numbered in this figure but are assumed to be due to 11-*cis* retinal and 3-dehydroretinal (M. Azuma, unpublished observation). Large peaks (5 and 6) due to all-*trans* retinols (retinol and 3-dehydroretinol) are also shown in Fig. 2C. As indicated in Fig. 2A, sample A has mostly all-*trans* retinol and 3-dehydroretinol (peaks 5 and 6), in amounts similar to those found in the blood plasma (Fig. 2C), and low levels of retinals, the peaks of which are not numbered. In contrast, sample B, shown in Fig. 2B, has

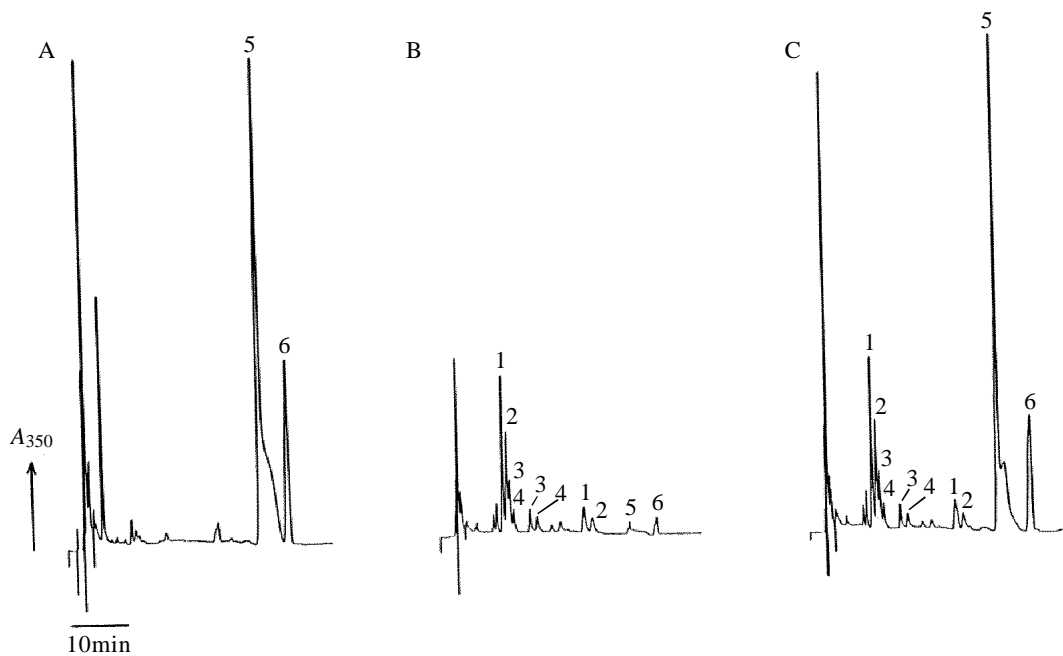


Fig. 2. HPLC chromatograms of retinoids in the fractionated samples of estrogen-injected male plasma (A and B) and in the original plasma (C). Male plasma was collected 2 weeks after estrogen injection. (A) The fraction soluble in EDTA and  $MgCl_2$  (sample A, see text). (B) The fraction precipitated in EDTA and  $MgCl_2$  (sample B, see text). The volume of the blood plasma (C) was 0.4ml and samples A and B were derived from an approximately similar volume of blood plasma. Other details as for Fig. 1.

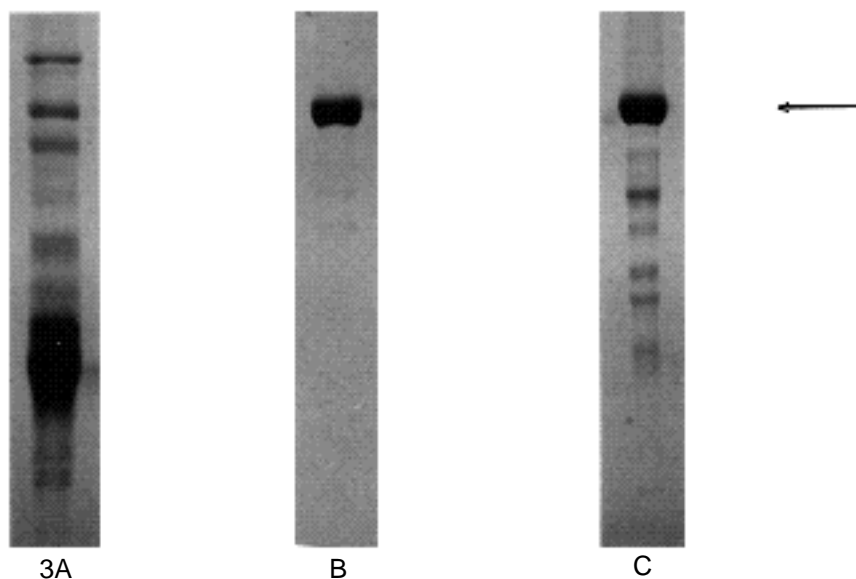


Fig. 3. SDS-PAGE of the proteins in the fractionated samples of estrogen-injected male plasma (A and B) and in the original plasma (C). Details of sample A (A), sample B (B) and of the plasma are the same as for Fig. 2, but the amounts of protein in these samples are different; 35  $\mu\text{g}$  (A), 5.1  $\mu\text{g}$  (B) and 8.3  $\mu\text{g}$  (C). The arrow indicates the position of vitellogenin.

large amounts of all-*trans* retinal (peaks 1 and 1') and all-*trans* 3-dehydroretinal (peaks 2 and 2') but small amounts of retinols (peaks 5 and 6). The amount of retinals in sample B seems to be equal to that in the original blood plasma shown in Fig. 2C.

The separation of proteins in the fractionated samples (sample A and sample B) and in the blood plasma by SDS-PAGE is shown in Fig. 3. The amount of protein in sample A (Fig. 3A) was much greater than the amounts in sample B (Fig. 3B) and in the blood plasma (Fig. 3C). The pattern of sample B is very similar to that reported for vitellogenin in the blood plasma of *Xenopus laevis* (Wiley *et al.* 1979; Wiley and Wallace, 1981), indicating that the main protein in sample B is vitellogenin (arrowed in Fig. 3). The pattern of sample A shows that the main proteins have relative molecular masses smaller than that of vitellogenin. The results shown in Figs 2 and 3 indicate that the fraction including vitellogenin (sample B) has retinals but little retinol while the other fraction (sample A) contains retinols and some proteins other than vitellogenin

### Discussion

This study shows that an estrogen injection induces the appearance of retinals (mainly all-*trans* retinal and 3-dehydroretinal) and vitellogenin in the blood plasma of male *Xenopus laevis*. A method of purifying vitellogenin (Wiley *et al.* 1979) produced the fraction that precipitated in the presence of  $\text{MgCl}_2$  and EDTA and included vitellogenin and retinals (sample B). To examine the possibility that retinals in the blood plasma were

bound to vitellogenin by the Schiff base linkage, as in the egg (Irie *et al.* 1991), the effects of sodium borohydride (NaBH<sub>4</sub>) on the retinals in the blood plasma and in the fractionated sample (sample *B*) were tested (results not shown). Hardly any retinals could be extracted from either the blood plasma or sample *B* after the addition of NaBH<sub>4</sub> but retinols could be extracted from the blood plasma. This is very similar to results obtained with egg retinals (Seki *et al.* 1987; Irie *et al.* 1991), and supports the idea that the retinals in the blood plasma are bound to vitellogenin by the Schiff base linkage. Small amounts of retinals were detected in the blood plasma of female *Xenopus laevis* without estrogen injection. These retinals also seemed to be bound to vitellogenin. The vitellogenin–retinal complex in the maternal blood plasma may be taken up into oocytes and converted into a lipovitellin–retinal complex during the process of vitellogenesis.

An estrogen injection induces not only the appearance of retinals but also an increase in the amount of retinols (all-*trans* retinol and 3-dehydroretinol) in the blood plasma. These retinols were barely detectable in the vitellogenin fraction (sample *B*) but were found mostly in the fraction (sample *A*) that was soluble in the presence of MgCl<sub>2</sub> and EDTA. Sample *A* did not include vitellogenin but contained other proteins whose relative molecular masses were lower than those of vitellogenin polypeptides ( $M_r$  approximately 200000). Estrogen induction of serum retinal-binding protein (RBP) mRNA has been reported in several papers (McKearin *et al.* 1987; McKearin and Shapiro, 1988; Whitman *et al.* 1990), suggesting that sample *A* obtained in this study probably contains large amounts of RBPs binding retinols. Retinols were barely detectable in oocytes (M. Azuma, unpublished observation) or eggs (Seki *et al.* 1987; Azuma *et al.* 1990; Irie *et al.* 1991). If oocytes have vitellogenin receptors but not RBP receptors, retinols would not be taken up into oocytes. These retinols may function in the same way as vitamin A in cells other than oocytes.

Small amounts of 11-*cis* retinals (retinal and 3-dehydroretinal) were detected in the blood plasma of estrogen-injected males (Fig. 2C) but were practically absent from the egg (Seki *et al.* 1987; Azuma *et al.* 1990; Irie *et al.* 1991). Retinals present in the egg occur mainly in the all-*trans* form together with small amounts of the 13-*cis* form (about 10%) (Seki *et al.* 1987). These results are inconsistent with the idea that retinals binding lipovitellin in the egg are derived only from retinals binding vitellogenin in the blood plasma. Experiments to investigate binding sites for retinal on both vitellogenin and lipovitellin, and to examine a possible stoichiometry between retinal and each of these proteins, are now in progress. The results will offer more reliable information on the vitellogenin–retinal complex and its conversion to the lipovitellin–retinal complex.

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