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**Comparative biochemical and physiological studies of retinoid
storage in the eggs of oviparous chordates**

卵生脊索動物の卵におけるレチノイド貯蔵に関する
比較生理生化学的研究

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Comparative biochemical and physiological studies of retinoid storage in the eggs of oviparous chordates

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I. Abstract

The egg of *Xenopus laevis* contains both retinal (RAL1) and 3-didehydroretinal (RAL2). The sum of the two retinals (RALs) and the ratio of the RAL1 to RAL2 were in the ranges 40-60 pmol/egg and 0.67-1.35, respectively. RALs were shown to be bound directly to lipovitellin (LV), an egg yolk protein. The gel chromatography of LV after the treatment with sodium borohydride and SDS revealed that RALs were bound to lipovitellin 1 (LV1), the major component of LV. These results indicate that the RALs are bound to the protein moiety of LV1 *via* a Schiff base linkage.

Injection of estrogen into male *Xenopus laevis* induced the appearance of RALs and a considerable increase in the amount of retinols (retinol and 3,4-didehydroretinol) in the blood plasma. RALs in the blood plasma of estrogen-injected males were always detected in the vitellogenin, the precursor of egg yolk proteins. It is suggested that retinals are bound to vitellogenin and are taken up into oocytes in the process of vitellogenesis.

RAL1 or both RAL1 and RAL2 were major or exclusive retinoids in the eggs of four teleosts, chum salmon (*Oncorhynchus keta*), black porgy (*Acanthopagrus schlegeli*), marbled flounder (*Pleuronectes yokohamae*), and stingfish (*Inimicus japonicus*). In *O. keta* eggs, both RAL1 and RAL2 were present at the ratio of about 3:4, whereas RAL1 was the only retinal in the eggs of the other three marine species. RAL1 was the exclusive retinoid in the eggs of *P. yokohamae* and *I. japonicus*, whose eggs lack lipid bodies. In the eggs of *O. keta* and *A. Schlegeli*, which have lipid bodies, retinylesters were also detected. In *O. keta* eggs, retinals were present mostly in the aqueous part and were bound to a protein homologous to LV 1, and retinylesters were located in lipids. These results indicate that retinals are the essential mode of retinoid storage in eggs of teleosts and retinylesters are additional retinoids that accompany lipid accumulation.

RAL1 was the almost exclusive retinoid (>> 99%) in the eggs of the solitary ascidian, *Halocynthia roretzi*, and the concentration of RAL1 was 25.9 to 40.1 (30.6

on average) ng/mg of protein. The egg retinal consisted of four isomers: all-*trans* (50.9%), 9-*cis* (6.8%), 11-*cis* (20.4%) and 13-*cis* (21.9%). The presence of retinal in the eggs of this ascidian is a characteristic shared with the wide range of oviparous vertebrates, although the isomer composition differs between ascidian eggs and vertebrate eggs; in vertebrate eggs, almost all the retinal is in the all-*trans* form. The egg retinal was bound to a protein complex *via* a Schiff base linkage. The electrophoretic characteristics of the protein complex were similar to that of egg yolk proteins of oviparous vertebrates. The results presented in this study strongly suggest that, as is found with oviparous vertebrates, retinal in the ascidian eggs is the essential mode of retinoid storage.

It is strongly suggested that the accumulation of the retinal-protein complex into eggs is a common characteristic in the animals of every class of oviparous vertebrate and urochordate. Whereas, the eggs of mammals would not accumulate RALs. It is assumed that the ascidian does not metabolize retinol, and that echinoderms do not synthesize retinoids from carotenoids. The possible scheme on the retinoid storage and metabolism is presented.

II. General Introduction

Vitamin A and its derivatives, retinoids, are known to be involved in two different functions: retinal (RAL1) and 3,4-didehydroretinal (RAL2) for the photoreception, and retinoic acid (RA) for gene regulation. As retinoids play important roles for the visual pigment formation and embryonic morphogenesis during development, it is expected that some retinoid, which is the precursor of the functional retinoids, stored in the eggs.

The occurrence of RAL1 or both RAL1 and RAL2 (RALs), determined by Carr-Price colorimetric reaction, was shown to be distributed in the eggs or ovaries of a wide range of oviparous vertebrates (Plack et al., 1957, 1959, 1961; Plack, 1960; Plack, and Kon 1961). Seki et al. (1987) reported that RALs were almost exclusive components of retinoids in the eggs of some amphibians by using high performance liquid chromatography. Thus, the occurrence of RALs in vertebrate eggs have already been evident. The egg RALs were suggested to be bound to proteins, because vitamin A activity of herring eggs was precipitated by heating or by trichloroacetic acid (Plack et al., 1959) and RALs were not extracted with organic solvent after the treatment with sodium borohydride (Seki et al. 1987). However, the protein that binds the egg RALs has not been characterized. In the section III (Retinal and 3,4-didehydroretinal in the eggs of *Xenopus laevis*), therefore, the egg homogenate of *X. laevis* was fractionated by several methods, and evidence is presented that the RALs are bound to lipovitellin 1, a yolk protein, by a Schiff base linkage.

The egg yolk proteins are derived from the female-specific serum protein, vitellogenin, and vitellogenin is synthesized in the liver, secreted into the blood circulation and sequestered by oocytes (Wallace and Jared, 1968, 1969; Wallace and Bergink, 1975; Wallace, 1985). The vitellogenin synthesis is initiated by estrogen, even in males by exogenous estrogen administration (Follett et al., 1968; Munday et al., 1968; Wallace and Dumont, 1968; Dolphin et al., 1971). In the section IV (Effect of estrogen administration in the amount and composition of retinoids in the blood

plasma of *Xenopus laevis*), the retinoids in the blood plasma of male *X. laevis* after the injection of estrogen was investigated. The HPLC analyses revealed that RALs were present in the blood plasma of estrogen-injected males. The fractionation of blood plasma proteins showed that the vitellogenin fraction contained retinals, suggesting that the blood RALs are bound to vitellogenin. The egg RALs appears to be derived from retinyl ester stored in the maternal liver.

In the section III, it is evident that retinoids occur only as RALs in *X. laevis* eggs, in which no lipid bodies have been found. In contrast to the eggs of *X. laevis*, not only RALs but also retinol and/or retinyl ester have been shown to be present in the eggs or ovaries of several teleosts (Plack et al., 1959; Plack and Kon, 1961; Plack et al., 1964; Costaridis et al., 1996). It is generally known that many teleost eggs are composed of an aqueous part, which contains yolk proteins, and lipid bodies. Much variation occurs in lipid accumulation among fish species (Selman and Wallace, 1989). In the section V (Retinoid composition and retinal localization in the eggs of teleost fishes), therefore, intracellular localization of retinoids in the eggs of four teleosts was examined. The experimental results showed that retinyl esters were present in lipid bodies, apart from RALs, which was localized in a protein-bound form in the aqueous part. Only a trace amount of retinols were detected in the eggs of three teleosts, and no retinols were found in the other species.

In the former sections (III, IV and V), the egg retinoids of anamniote vertebrates were investigated. Other than in vertebrates, retinoic acid was shown to be involved in the embryonic morphogenesis of the closest relatives of vertebrates, the ascidians (subphylum Urochordata) and amphioxus (subphylum Cephalochordata), in several studies (Katsuyama et al., 1995; Holland and Holland, 1996; Katsuyama and Saiga, 1998; Hinman and Degnan, 1998, 2000; Escriva et al., 2002). However, the endogenous retinoids in eggs and embryos of nonvertebrate chordates have not been examined. Therefore, in the section VI (Storage of retinal in the eggs of the ascidian, *Halocynthia lorentzi*), the retinoids in the eggs of the solitary ascidian, *H. roretzi*, was

analyzed to clarify the mode of retinoid storage. The experimental results revealed that RAL1 in the ascidian eggs is practically only storage of retinoid.

In above sections (III to VI), the retinoids in the eggs of anamniote vertebrates and an urochrdate were surveyed. The experimental results provide evidence that RALs are the common and major retinoid storage in the eggs of every species examined. However, hepatic retinyl esters are known to be prevalent mode of retinoid storage in adult animals of every vertebrate class. In mammals, it is considered that RALs are not stored as retinoic acid precursors at any time in the life cycle. The storage and the metabolism of retinoids would have changed during chordate evolution. The evolutionary background of the retinoid storage and metabolism is discussed in the section VI (General discussion), on the basis of the results of my research including unpublished data.

III. Retinal and 3,4-didehydroretinal in the eggs of *Xenopus laevis*

Abstract

The egg of *Xenopus laevis* contains both retinal (RAL1) and 3-didehydroretinal (RAL2). The sum of the two retinals and the ratio of the RAL1 to RAL2 in one egg were not so diverse among the eggs in a brood, but the values between different broods varied in the ranges 40-60 pmol/egg and 0.67-1.35, respectively.

Both retinals in the egg homogenate were precipitated in 20 mM Tris-HCl buffer, redissolved in NaCl solution at concentrations above 400 mM, and precipitated at 50-65% saturation with ammonium sulfate. The proteins containing retinals corresponded to lipovitellin (LV) on SDS-PAGE. The LV was chromatographed by gel filtration in the presence of SDS into two components, LV1 and LV2, and retinals bound covalently to the binding site by the treatment with sodium borohydride (NaBH_4) were cochromatographed with LV1. The protein with retinals was converted to a fluorescent product by the NaBH_4 treatment, following which both retinals could no longer be extracted with organic solvents. The fluorescence was observed even after delipidation of the NaBH_4 -treated LV. These results indicate that the retinals are bound to the protein moiety of LV1 via a Schiff base linkage. The molar ratio of LV1 to one molecule of RAL1 or RAL2 was in the range of 12-23. We discuss evidence that the retinals are bound to the lipovitellin precursor, vitellogenin, prior to the uptake of the vitellogenin into the oocyte.

1. Introduction

Extraocular retinal was found for the first time in the eggs of herring by Plack et al. (1957). In subsequent papers (Plack, 1960; Plack and Kon, 1961; Plack et al., 1959), the occurrence of retinal, or both retinal and 3-didehydroretinal, in the megalecithal eggs of vertebrates was shown to be common, while no retinals were found in the eggs or ovaries of the invertebrates investigated (Plack and Kon, 1961). Plack et al. (Plack et al., 1961) also reported important observations that retinal is

found in cod ovaries only when they are maturing and contain large ripening eggs, and that its first appearance coincides with the onset of vitellogenesis. Plack et al. (1964) also found retinal in the blood of egg-laying hens, but only traces in the blood of cockerels and none at all in the blood of pullets. Using the fowl, an intimate relation between sexual maturation and the appearance of retinal in the plasma was shown, and the hormonal control of retinal concentration in the blood plasma was substantiated (Plack, 1964). These findings indicate a connection between egg retinal and vitellogenesis, but neither the origin of the egg retinal nor its physiological significance are yet known. For further our understanding, it is important to make clear the state in which retinal exists in the egg.

Plack reported in his early papers (Plack et al., 1957, 1959) that the retinal in herring eggs is bound to a lipid and, either directly or indirectly, to protein, for the retinal was extracted with a fat solvent only after denaturation of the proteins with ethanol. Recently, Seki et al. (1987) found that *Xenopus laevis* eggs contain both retinal and 3-didehydroretinal in nearly equal amounts, and it was suggested that the retinals are present in a protein-bound form, but the protein that binds the retinals has not been characterized. Therefore, in this study, *X. laevis* egg homogenate was fractionated by several methods to inspect the protein that binds the retinals. Evidence is presented that the retinals are bound to a yolk protein, lipovitellin 1 (LV1), and the origin of the egg retinals is discussed in terms of this and previous (Plack, 1964; Plack et al., 1961, 1964) results concerning egg retinals.

2. Materials and methods

2.1. Animals and eggs

X. laevis were supplied by Dr. H. Takasaki or purchased from Hamamatsu Seibutsu Kyozaï Co., Ltd. (Shizuoka, Japan). They were reared at 25°C and fed twice weekly with a feed for the toads supplied by the same company. The eggs were obtained by injection to the animals with chorionic gonadotrophic hormone (Nieuwkoop and Faber, 1956), Puberogen (for veterinary use; Sankyo Co., Tokyo) at

200-300 IU. Pre-gastrula eggs were used in this experiment. The jelly layers of the eggs were removed (Sakai and Kubota, 1981) by shaking the eggs gently in 0.6% NaCl solution containing 1.5% sodium thioglycolate at pH 9-10, then the eggs were rinsed with 0.6% NaCl solution in 20mM Tris-HCl buffer at pH 7.4.

2.2. Fractionation of egg homogenate

The rinsed eggs were homogenized in 20 mM Tris-HCl buffer at pH 7.4 (TB), using a glass homogenizer with a motor-driven teflon pestle or a high-speed homogenizer (Physoctron NS-50; Nichion Irikakikai Seisakusho Co. Ltd., Chiba, Japan). The homogenate was centrifuged at 5°C with a centrifuge (Kubota KR-180B, Tokyo) at $13,000 \times g$ for 20 min. The precipitate was resuspended in 400 mM NaCl in TB (TBS) and centrifuged again. To collect the TBS soluble substances, the material solved in TBS was reprecipitated by dilution with more than three times the volume of TB. After centrifugation, the collected material was redissolved into TBS and then fractionated further by ammonium sulfate (AS) precipitation. Till 50% saturation, no precipitation occurred. At 65% saturation, the turbid suspension was kept on ice for a few hours, or overnight in a refrigerator, and centrifuged to obtain the AS soluble (AS-sup) and precipitate (AS-ppt) fractions. The AS-ppt, which was insoluble in TB, was washed with sufficient TB by centrifugation to remove the AS.

The AS-ppt freed from AS was redissolved in TBS and fractionated by gel filtration on a 3×38 cm column of Sephacryl S-200, equilibrated with TBS containing 0.1% sodium dodecylsulfate (SDS). Prior to gel filtration, the AS-ppt in TBS was treated with sodium borohydride (NaBH_4), to bind the retinals covalently to the binding site (Bownds and Wald, 1965; Seki, 1984). To minimize the cleavage of the protein by NaBH_4 , the treatment was performed on ice for 30 min, which was long enough to complete the reduction at the binding site as monitored by the increase in fluorescence (see below). The reaction was terminated by addition of more than three times the volume of TB and centrifuged to wash out NaBH_4 . In the supernatant, no fluorescence due to retinol or retinyl product was observed. The precipitate was

dissolved in TBS, mixed with SDS to a final concentration of 2% and applied to the column for gel filtration. Five ml fractions were collected and their absorption spectra were measured from 220 to 400 nm with a spectrophotometer (Hitachi 200-20, Hitachi Corp., Tokyo, Japan). Fluorescence of the retinyl product could not be used to monitor the fractions because the fluorescence was quenched by SDS.

All the procedures of preparation of the proteins described above and the following retinoid analyses were carried out under dim red light.

2.3. Detection of retinals in the fractions

Fluorospectrometry and high performance liquid chromatography (HPLC) were used to detect the retinals. Although retinal itself is not fluorescent in most solvents, retinol and N-retinyl product (the reduced products of retinal and the retinylidene Schiff base, respectively) are fluorescent (cf. Bownds and Wald, 1965; Seki, 1984). The conversion of the material with retinals into the fluorescent product, on treatment with NaBH_4 , was monitored by the increase of fluorescence with an emission λ_{max} around 475 nm, induced by excitation at 330 nm using a fluoro photometer (Hitachi 650-10M, Hitachi Corp., Tokyo, Japan).

For HPLC analysis, retinals in the egg(s) or fractionated samples were extracted by the oxime method (Suzuki and Makino-Tasaka, 1983) following our routine procedures Seki et al., 1989). The eluent was 8% diethylether-0.08% ethanol in n-hexane (Seki et al., 1987), or 5% tert-butylmethylether-0.04% ethanol-25% benzene in n-hexane: both of which give good separation of the syn oximes of retinal (RAL1) and 3-didehydroretinal (RAL2). A HPLC-system (Hitachi 655, Hitachi Corp, Tokyo, Japan) was equipped with a 6×150 mm column of $3 \mu\text{m}$ silica gel (YMC-PACK-A-012-3 S-3 SIL; Yamamura Chemical Laboratories Co. Ltd., Kyoto), with a 4×50 mm precolumn, and was used at a flow rate of 2 ml/min. Absorption at 350 nm was monitored with a UV-detector (Hitachi 638-41, Hitachi, Japan). Quantities of RAL1 oxime and RAL2 oxime were estimated from the peak areas of the

syn isomers, using equimolar mixtures of standard oximes (Makino-Tasaka and Suzuki, 1986) supplied by Dr. T. Suzuki.

2.4. Quantification of the lipovitellin 1

Two methods, the Lowry's method (Lowry et al., 1951) and the fluorescence excited by 280 nm, were used to determine the amount of the protein that binds the retinals. The standard protein was bovine serum albumin. The amount of lipovitellin 1 (LV1) in the AS-ppt was calculated using the stoichiometry 1:1 (Bergink and Wallace, 1974) for LV1 and lipovitellin 2 (LV2), and the molecular weight 150,000 dalton (Bergink and Wallace, 1974; Willy and Wallace, 1981) for the sum of the molecular weights of the two proteins.

2.5. SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970) with a 7.5-15% acrylamide linear gradient gel as the separating gel. The gels were stained overnight with 0.05% Coomassie Brilliant Blue (CBB) R-250 in 20 mM AlCl₃, 25% isopropyl alcohol and 10% acetic acid (Willy and Wallace, 1981) to visualize phosphoprotein bands (Hegenauer et al., 1977). The following proteins were employed as molecular weight markers; Myosin (200KD), β -galactosidase (116KD), phosphorylase b (97.4KD), bovine serum albumin (66.2KD), ovalbumin (43KD), trypsinogen (24KD), β -lactoglobulin (18.4KD) and lysozyme (14.3KD).

3. Results

3.1. Amounts and the ratio of retinal and 3-didehydroretinal in an egg

Both ret₁ and ret₂ are found in the *X. laevis* egg. Table 1 shows the amounts of each retinal, along with their sum and ratios in the eggs produced by four different pairs of adults (A-D). The sum of the two retinals (pmol/egg) was nearly constant among eggs from one brood (A), though varied from brood to brood in the range of 40

and 60 pmol/egg. Similarly, the ratio of RAL1 to RAL2 was not so diverse within each brood, but varied from 0.67 to 1.35 in the four broods examined.

3.2. Survey of retinals in fractions of the egg homogenate

In the first step, eggs were homogenized in TB and separated into a TB-soluble (TB-sup) and a TB-precipitate (TB-ppt) fraction by centrifugation. They were assayed for retinals by HPLC as shown in Figure 1; almost all the retinals was found in the TB-ppt (Fig. 1, B), with little detected in the TB-sup (Fig. 1, A). It was found that the TB-ppt is soluble in 10% NaCl. On centrifugation, a small quantity of black sediment was precipitated but contained no retinal detectable by HPLC: all the retinals were detected in the clear supernatant. The concentration of NaCl was then altered, and the amount of retinal in the supernatant after centrifugation was examined by HPLC. About 97% of the retinals were detected in 400 mM NaCl solution, 31% in 200 mM NaCl and none in 100 mM NaCl. Subsequent solubilization was therefore routinely performed using the 400 mM NaCl in TB (TBS).

The retinal-containing material solubilized in the TBS (TBS-sup) was then fractionated by the AS precipitation. Almost all of the retinals was detected in the AS-ppt fraction. Table 2 shows an example of the quantitative representation of the retinals in the fractions mentioned so far. The ratio of RAL1 to RAL2 did not change markedly during the procedures. The behavior of the retinal-containing material described above is similar to that reported for lipovitellin (LV) in the yolk platelet of toad eggs (Wallace, 1963a, 1963b). The SDS-PAGE patterns (Fig. 2) of the egg proteins further suggests that the protein in the AS-ppt fraction is LV (Bergink and Wallace, 1974; Willy and Wallace, 1981). The AS-sup (lane 4) contained phosvitin, which was not stained by CBB without AlCl_3 (Willy and Wallace, 1981).

Another attempt to solubilize the TB-ppt without NaCl was performed. The TB-ppt suspended in TB was divided into aliquots and mixed with detergents (Triton X-100, Lauryl-sucrose ester or SDS) to a final concentration of 2%. In SDS solution, the turbid suspension was completely dissolved, but a heavier turbidity occurred in the

other two detergents. The TB-ppt could not be solubilized in the mild detergents, indicating that the TB-ppt is not a membrane substance.

3.3. Binding of the retinals with LV via the Schiff base

Figure 3 shows that the retinal in the lipovitellin fraction (AS-ppt solved in TBS) is reduced by NaBH_4 to a fluorescent product with an excitation maximum around 335 nm (A) and the emission maximum around 475 nm (B). After the addition of NaBH_4 , neither RAL1 nor RAL2 could be extracted by the organic solvent for HPLC analysis. These results indicate that the both retinals are bound to LV by the Schiff base linkage (Seki, 1984).

3.4. Retinals bind to the protein moiety of LV1

Lipovitellin is composed of two major components, LV1 and LV2, as shown in lane 5 in Figure 2. They could be separated by gel filtration in the presence of SDS, as has been shown by Wiley & Wallace (1981), but our preliminary test indicated that the retinals also separated from the native binding site on addition of the SDS. This was prevented by adding NaBH_4 to the LV solution (in TBS) prior to the addition of SDS, and then gel filtration was performed. Figure 4A shows the resulting chromatogram detected by absorptions of the protein, A_{280} , and the retinyl product, A_{330} . The proteins were separated into two peak fractions but the retinyl product eluted out in a single peak which coincided precisely with the first peak of the protein. The SDS-PAGE results in Figure 4B confirm that the first peak is LV1 and the second one LV2 (Willy and Wallace, 1981). From these results it is concluded that the retinals are bound to LV1 with the Schiff base linkage.

Lipovitellin is a lipoprotein (Wallace, 1963b), and the retinal can form the Schiff base with an amino group of either the protein or such a lipid as phosphatidylethanolamine. The results shown above have suggested that the retinals are bound to the protein, but definite evidence that the retinals are bound to the protein moiety of the LV is lacking. This point was clarified as follows. A LV sample was

divided into two aliquots, to one of which NaBH₄ was added. The lipid was extracted from both samples by mixing with 3 volumes of an organic solvent (methanol:dichloromethane = 1:2), re-mixing with n-hexane and centrifuging at 1,400 × g for 10 min. The upper organic solvent layer was removed and the extraction with dichloro-methane/n-hexane was repeated again. In the organic solvent, the fluorescence with an emission maximum around 420 nm was observed at 330 nm excitation, but the intensity was not different between the two samples. The lower water layer was then recentrifuged at 13,000 × g for 20 min to precipitate the delipidated proteins. The precipitate was suspended with the TBS but it was not redissolved. SDS was added at the final concentration of 5% to make the suspension clear. Figure 5 shows the fluorescence spectra immediately after the addition of SDS. The concentration of the delipidated protein in each sample was almost the same (A), but the fluorescence with the emission maximum around 470 nm (B) was observed only in the NaBH₄-treated sample. It is therefore concluded that NaBH₄ treatment resulted in a retinyl-protein product.

3.5. The molar ratio of the retinals to LV1

The sum of the two retinals in a LV sample was determined with HPLC, and the amount of the LV1 in the same volume of the sample was determined. Table 3 shows the results obtained by the three experiments. The amount of LV1 in a sample differed according to method used to estimate the proteins: about 1.5 times larger by fluorometry than by the Lowry method. In spite of the ambiguity of the protein determination, the result was clear: only 4-8% of the LV1 molecules bind one molecule of RAL1 or RAL2.

4. Discussion

The present experiments revealed, first of all, that the egg retinals are not bound to a water soluble protein, since no retinal was detected in the TB-sup fraction (Fig. 1, Table 2). Secondly, the protein bound to retinals is not a membrane protein,

since it was not redissolved in mild detergent. Finally, a procedure to extract lipoproteins using NaCl solution as used for insect hemolymph (Chino et al., 1969; Chino, 1980) was successful, indicating that the retinal containing material is a lipoprotein that behaves like LV (Wallace, 1963a, 1963b). Further purification of the material by gel filtration and protein analysis by SDS-PAGE indicate definitively that the retinals are bound to LV1 (Fig. 4).

LV1 is a protein constituent of the yolk platelet, arising from a yolk protein precursor, vitellogenin (Willy and Wallace, 1981). The sequence of vitellogenesis, as summarized by Selman and Wallace (1989), includes (i) the hepatic synthesis and secretion of vitellogenin in response to circulating estrogen, (ii) the delivery of vitellogenin, via the maternal circulation, to the surface of the growing oocyte, (iii) selective uptake of vitellogenin by receptor-mediated endocytosis, and (iv) cytoplasmic translocation of vitellogenin to form yolk bodies, and concomitant proteolytic cleavage of vitellogenin into the polypeptide subunits of the yolk proteins, lipovitellin and phosvitin.

Seki et al. (1987) suggested that retinal is produced in the toad ovary, on the basis of finding of carotenoids and retinylester in the vary. However, the findings of the present study suggest instead that retinal binding to LV1 might have occurred at the hepatic vitellogenin formation. Moreover, the retinal occurs in the blood of egg-laying hens, but not of pullets or cockerels (Plack et al., 1964), and the appearance of retinal in the blood is influenced by hormonal control (Plack, 1964). These facts are further confirmation that retinals are bound to the vitellogenin upstream from its appearance in the ovary.

In insects, vitellogenin is found as a hemolymph lipoprotein II (Pan and Wallace, 1974; Chino et al., 1976), which contains carotenoids (Chino et al., 1969, 1976). The hemolymph lipoprotein II of the silkworm was shown to be identical with the egg lipoprotein II (Chino et al., 1976), and we have detected carotenoids but not retinoids in the eggs of a moth (unpublished observation) and a dragonfly (Seki et al., 1988). Also, Plack & Kon (1961) have shown that carotenoids (but no retinal) are

present in locust eggs. There seems, therefore, to be a common strategy for the delivery of egg retinals in the toad (and probably in other egg-laying vertebrates) and of egg carotenoids in insects.

The stoichiometry of one retinal per only several % of LV1 (Table 3) was unexpected. One possible explanation is the heterogeneity of *X. laevis* LV1 (α , β , γ ; Bergink and Wallace, 1974), which are produced from three (Willy and Wallace, 1978) or four (Wahli et al., 1979) different vitellogenin species. More precise determination of the egg retinals and the protein is a project for further examination.

The egg retinals were characterized to have bound to the protein moiety of the LV1 via the Schiff base linkage (Figs. 3, 4, 5). The fluorescence spectrum shown by Figure 5 has its emission maximum around 475 nm (for 330 nm excitation), due to the N-retinyl protein. The quantum efficiency, however, of the fluorescence of RAL2 is known to be extremely low, and so the Schiff base linkage of RAL2 is not deducible from the fluorescence data. However, the following two results indicate that binding states of RAL1 and RAL2 are the same: (i) the unity in the ratio of RAL1 to RAL2 during the purification steps of LV (Table 2), and (ii) both retinals become unextractable after treatment of LV with NaBH₄.

The N-retinylidene Schiff base has its absorption maximum around 360 nm, at alkaline pH, but at acid pH it is protonated and the maximum is around 440 nm (Pitt et al., 1955). It is well known that the chromophore retinals of visual pigments are bound to the protein moiety, opsin, via a protonated Schiff base linkage. It is therefore of interest to know whether or not the Schiff bases of the egg retinals are protonated. To answer this question, the absorption spectrum of the LV solution in TBS was measured, but the results were somewhat complex. The spectrum showed a broad peak in the visible range with a maximum at ca. 370 nm and shoulders around 350, 400 and 480 nm. In addition to retinals, *X. laevis* yolk protein has been reported to bind biliverdin, which has absorption bands around 360 and 660 nm (Redshaw et al., 1971). Furthermore, during this experiment, the characteristic excitation spectrum of the fluorescence due to a riboflavin was observed in the LV sample: the maxima were

at 375 nm and around 460 nm, with characteristic fine structure (unpublished observation). At least four substances (RAL1, RAL2, biliverdin, riboflavin) contribute to the absorption spectrum. Moreover, the addition of NaBH₄ reduced not only the retinylidene Schiff base but also the biliverdin, forming bilirubin with a λ_{max} around 440 nm. So the difference spectrum before and after the addition of the NaBH₄ was not appropriate to detect the absorption maximum of Schiff base. Addition of alkali or acid to the LV solution caused heavy precipitation rendering absorption measurement impossible. When SDS was added to the LV solution at neutral pH, however, a large red-shift of the absorption band was observed. The shifted spectrum had a maximum at 415 nm, with the shoulders around 390 and 450 nm. This change of the absorption spectrum is highly suggestive of inducing the protonation of Schiff base, since the pK_a of the Schiff base has been reported to rise in the anionic detergent, SDS (Kito and Nashima, 1980).

RAL1 and RAL2 are the only retinoids found in *X. laevis* eggs (Seki et al., 1987). The egg retinals are presumably used as the chromophore retinals of the visual pigments, for more than half of the egg retinals appear in the eyes of tadpoles, at stage 45, as RAL1 and RAL2 esters (Azuma et al., 1988). During the present experiment, the presence of retinoic acid in the egg was also inspected, using HPLC, but was not detected. The metabolic path of egg retinals, bound to LV1, into such substances having vitamin A activities is a problem for further investigation.

Acknowledgments

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Table 1. Content of all-*trans* retinal (RAL1) and 3-didehydroretinal (RAL2) in the egg from different pairs of adults

Pair	n	pmol/egg			RAL1/RAL2
		RAL1	RAL2	sum	
A	1	22.9	27.8	50.7	0.82
A	1	24.3	30.6	54.9	0.79
A	1	23.3	27.2	50.5	0.86
A	5	26.0	29.3	55.3	0.89
B	1	29.6	22.0	51.6	1.35
B	1	28.6	22.0	50.6	1.30
C	1	16.8	25.2	42.0	0.67
C	1	16.8	23.0	39.8	0.73
D	2	33.9	25.2	59.1	1.35

n: number of eggs analyzed

Table 2. Amounts of all-*trans* retinal (RAL1) and 3-didehydroretinal (RAL2) in different fractions of *X. laevis* egg homogenate

Fraction	nmol			Recovery	RAL1/RAL2
	RAL1	RAL2	sum		
TB-sup	0.39	0.44	0.83	0.01	0.9
TB-ppt	39.2	46.8	86	0.99	0.84
TBS-sup	38.3	44.2	82.5	0.96	0.87
AS-ppt	37	43	80	0.97	0.86

TB: 20mM Tris-HCl buffer, pH 7.4

TBS: 400mM NaCl in TB

AS: 65% saturation of ammonium sulfate

The egg homogenate in TB was centrifuged at 12,000 rpm for 20 min, producing a supernatant (TB-sup) and a precipitate (TB-ppt). The TB-ppt was resuspended in TBS and centrifuged to produce a second supernatant fraction (TBS-sup). The TBS-sup was then precipitated by AS to obtain the precipitate fraction (AS-ppt). The amounts and ratio of the retinals in these fractions were quantified by HPLC.

Table 3. The molecular ratio of lipovitellin (LV) to retinals (retinal (RAL1) and 3-didehydroretinal (RAL2))

Exp.	LV/ret _{1 or 2}		RAL1/RAL2
	Lowry	Fluor.	
A	16.4	20.7	0.87
B	12.7	18.8	1.2
C	13.9	22.8	0.72

The amount of protein in a LV sample was quantified by the Lowry's method and by fluorometry, and amount of the LV1 was calculated as shown in Materials and Methods. The amounts and the ratio of all-*trans* RAL1 and RAL2 in the same sample were determined by using HPLC.

Captions to Figures

Fig. 1. HPLC elution profiles of extracts from the supernatant and precipitate fractions of *X. laevis* egg homogenate in 20 mM Tris-HCl buffer at pH 7.4 (TB). Retinoids were extracted by the oxime method and analyzed by HPLC as described in Materials and Methods. The eluent was 5% tert-butylmethylether-0.04% ethanol-25 % benzene in n-hexane. Large peaks of *syn* and *anti* all-*trans* retinaloximes (1,1') and all-*trans* 3-dehydroretinaloximes (2,2') are observed only in the precipitate fraction (B).

A: 7.8×10^{-3} portion of the supernatant fraction (TB-sup).

B: 8.3×10^{-4} portion of the precipitate fraction (TB-ppt) resuspended in 400 mM NaCl in TB.

Fig. 2. SDS-PAGE of the proteins in the fractionated samples of *X. laevis* egg homogenate. The gel was stained with CBB in the presence of AlCl₃ [18].

1, precipitate in 20 mM Tris-HCl buffer at pH 7.4 (TB); 2, supernatant in TB; 3, supernatant in 400 mM NaCl in TB; 4, supernatant in the 65% saturated ammonium sulfate (AS); 5, precipitate in AS; and 6, marker proteins. The phosvitin band (PV; [18]), shown clearly in lane 4, is observed faintly in lane 3 but not at all in lane 5, although lipovitellin bands (LV1 and LV2; [18]) are much denser than in lane 3.

Fig. 3. Fluorescence spectra of the 65% saturated ammonium sulfate (AS)-soluble and -precipitate fractions before and after addition of NaBH₄. The AS-soluble (1,1') and -precipitate (2,2') fractions were measured before (1,2) and after (1',2') addition of NaBH₄. Note that the detection range is the same in all the samples except 2' in B, where the range is reduced to one third as can be seen from the relative size of the Raman scattering peak at 375 nm. The fluorescent product, with an excitation maximum around 330 nm and emission maximum at 475 nm, is produced in the AS-precipitate fraction on addition of NaBH₄, indicating the presence of retinal.

A: Excitation spectra obtained from emission at 520 nm.

B: Emission spectra measured during excitation at 330 nm.

Fig. 4. Gel filtration profile, in the presence of 0.1% SDS, of the ammonium sulfate precipitated material after treatment with NaBH_4 . The proteins were chromatographed into two fractions: lipovitellin 1 (LV1; Fr.11) and lipovitellin 2 (LV2; Fr.15) [18]. The retinyl-product detected by the absorbance at 330 nm cochromatographed with LV1. A, The elution profiles shown by the absorbances at 280 nm (\bigcirc) and at 330 nm magnified ten-fold (\bullet); B, SDS-PAGE of the proteins in peak fractions 11 and 15 in A, and marker proteins.

Fig. 5. Fluorescence spectra of the delipidated lipovitellin. An ammonium sulfate precipitate fraction was divided into two aliquots, NaBH_4 added to one of them, and both samples were delipidated as described in the text. The delipidated proteins were resolved in 5% SDS in Tris-HCl buffer at pH 7.4 containing 400 mM NaCl. The fluorescence due to the retinyl-product is observed even after delipidation of the lipovitellin sample treated with NaBH_4 .

A: The fluorescence excited at 280 nm.

B: The fluorescence excited at 330 nm.

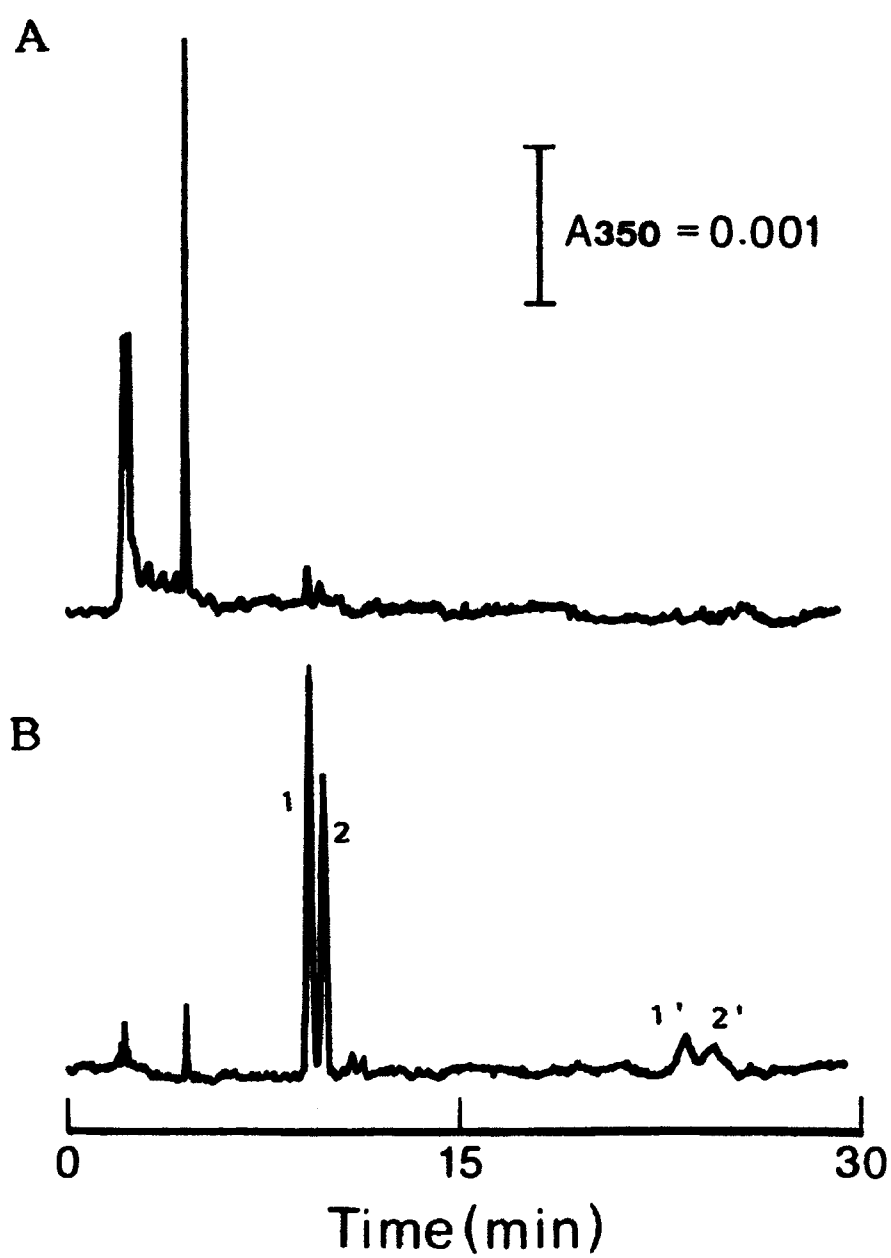


Fig. 1

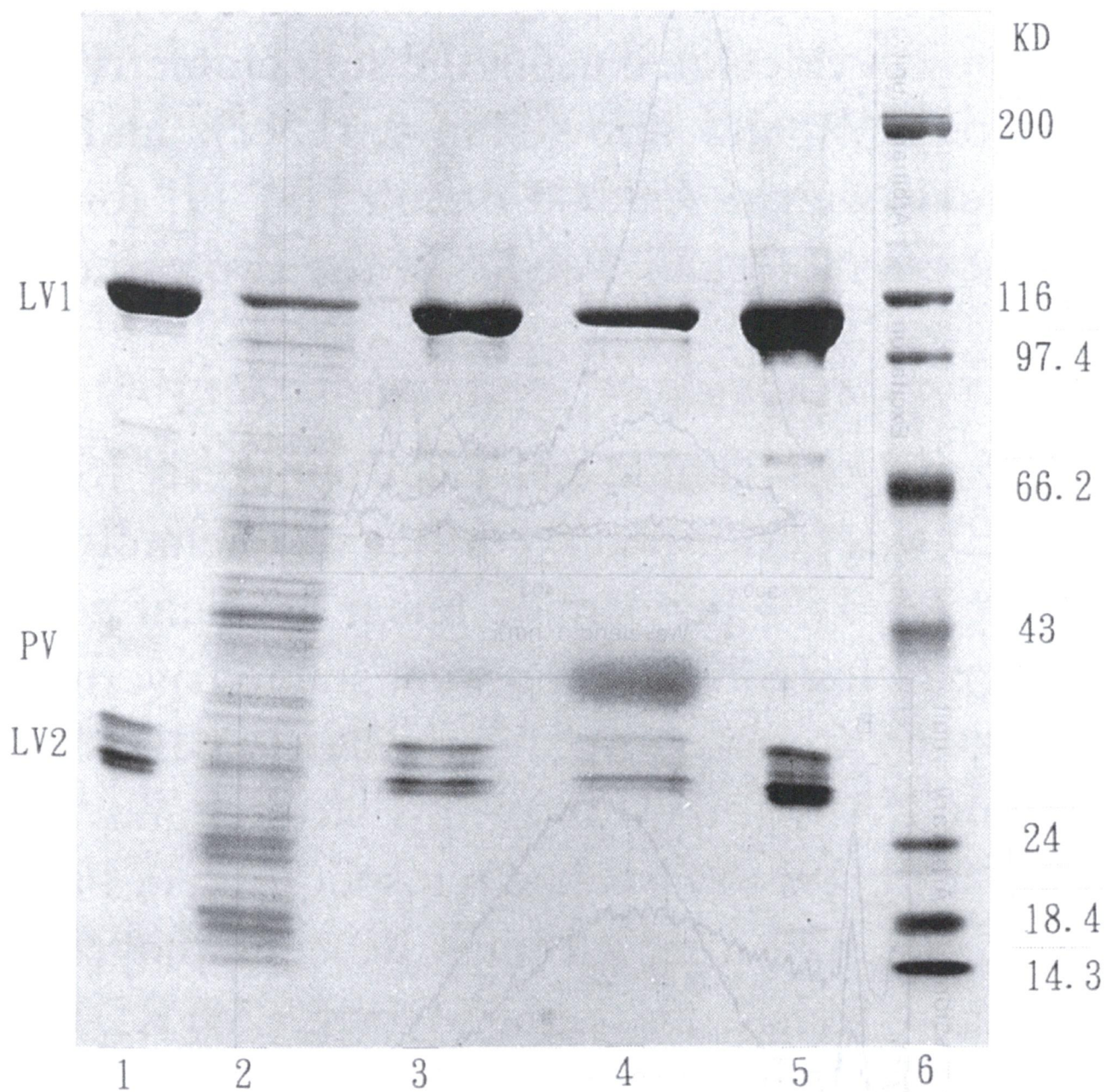


Fig. 2

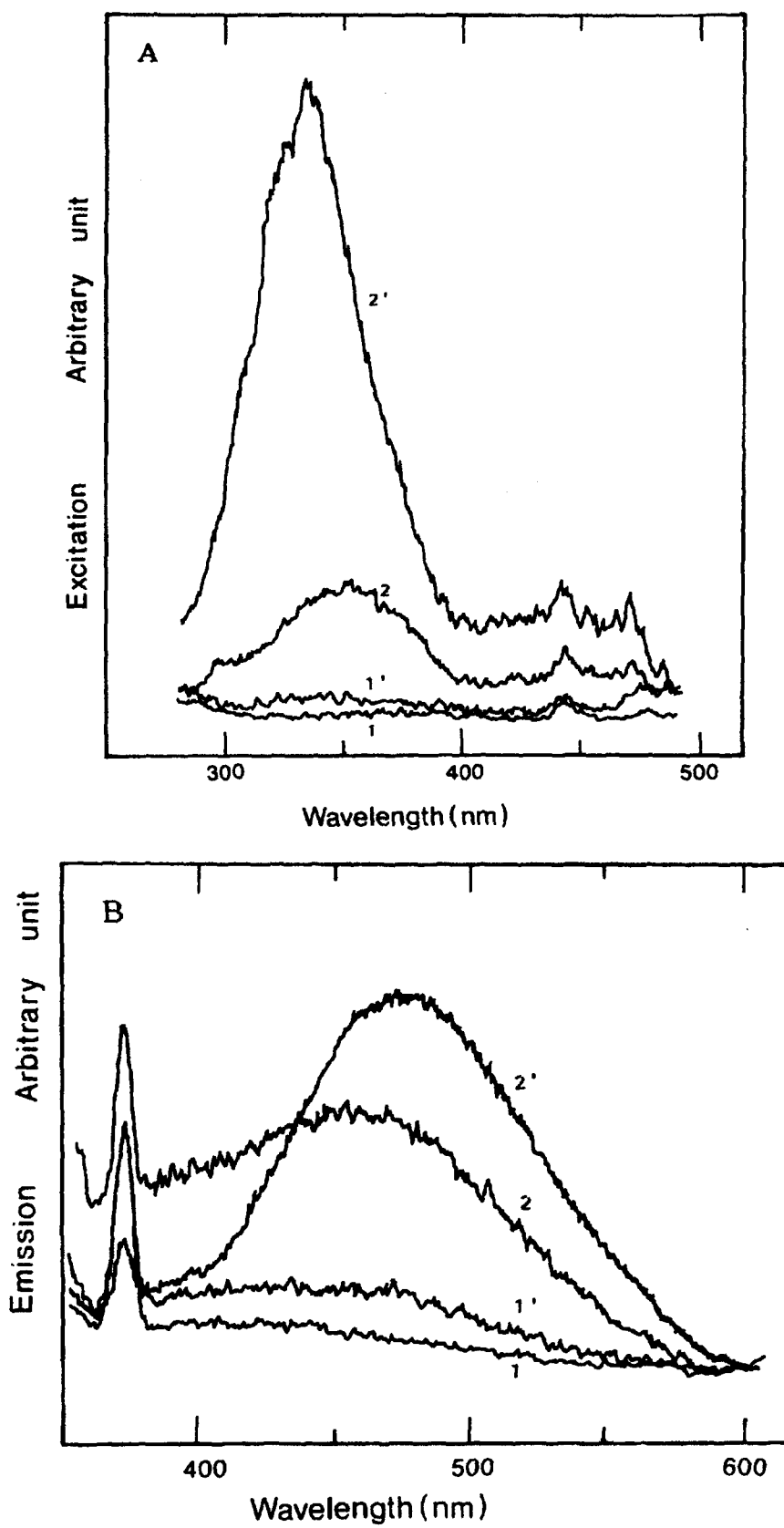


Fig. 3

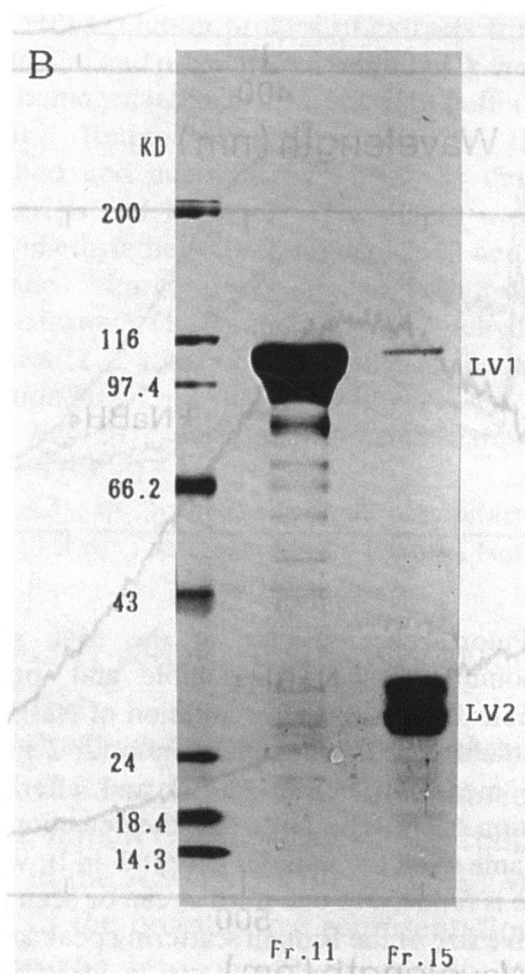
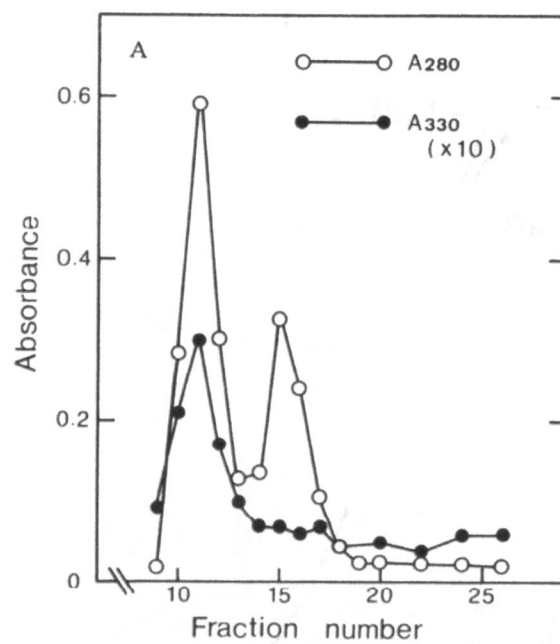


Fig. 4

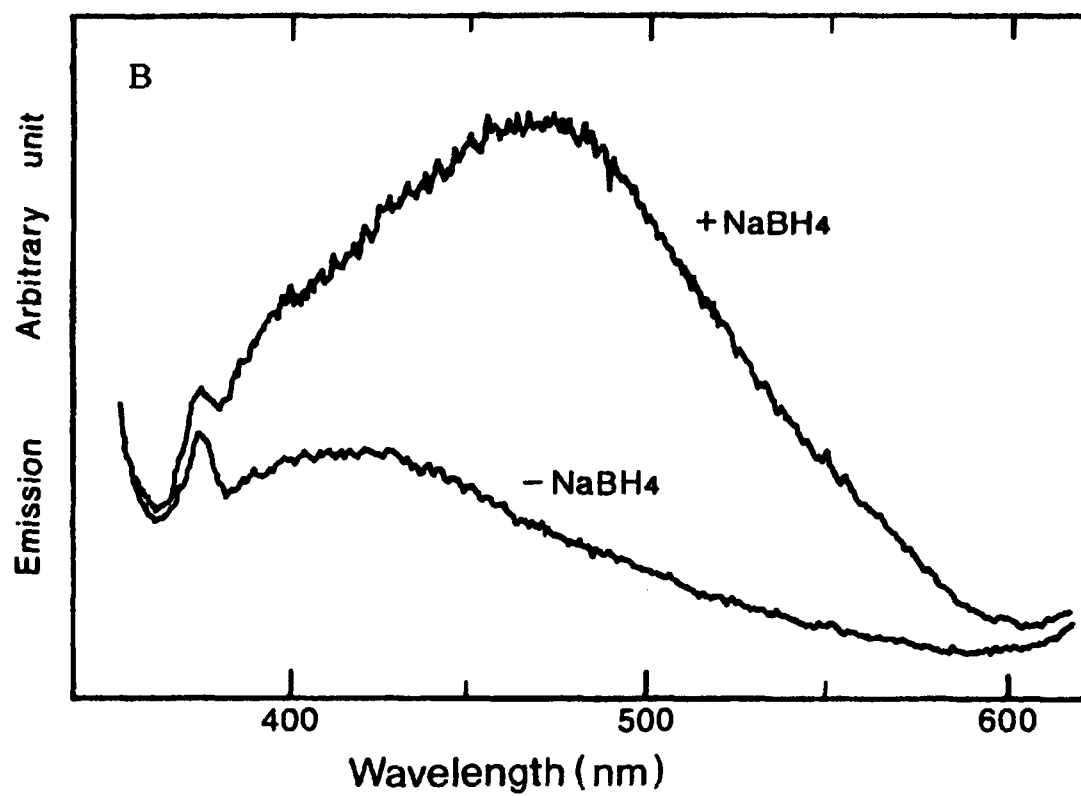
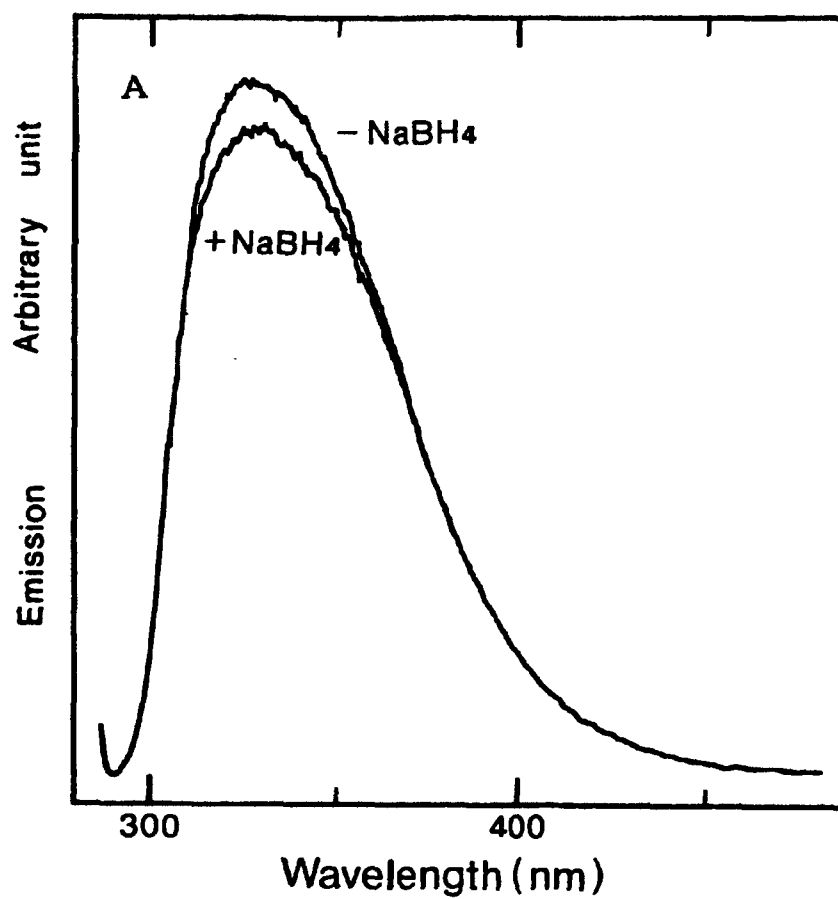


Fig. 5

IV. Effect of estrogen administration in the amount and composition of retinoids in the blood plasma of *Xenopus laevis*

Abstract

Injection of estrogen into male *Xenopus laevis* induced the appearance of retinals (retinal and 3,4-didehydroretinal) and a considerable increase in the amount of retinols (retinol and 3,4-didehydroretinol) 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 male 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.

1. 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 carotenoids were hardly detectable. In *Xenopus laevis*, a decrease in the level of egg retinals (retinal (RAL1) and 3,4-didehydroretinal (RAL2)) during the period of development before the larvae began to feed was accompanied by the formation of retinols (retinol (ROL1) and 3,4-didehydroretinol (ROL2)), retinylesters (retinylester (RE1) and 3,4-didehydroretinylester (RE2)). These results suggest that egg retinals are the source of the vitamin A necessary for larval development in *X. laevis*.

Recent experiments have confirmed that retinals in the eggs are bound to a yolk protein lipovitellin 1, through a Schiff base linkage (Irie et al., 1991). It is

2.2. Preparation of vitellogenin from blood plasma

Vitellogenin was prepared from the blood plasma according to Willy et al. (1979). Briefely, 5 ml of the plasma sample was mixed with 20 ml of 20 mM EDTA solution and 1.6 ml of 0.5 M MgCl_2 was added. This was further mixed gently and centrifuged at $13\,000 \times g$ for 10 min to a supernatant (sample A) and precipitate. The precipitate was dissolved in 3 ml of 1 M NaCl, 50 mM Tris-HCl buffer (pH 7.5) and centrifuged ($13\,000 \times g$). The supernatant obtained in this way was mixed with 25 ml of distilled water, resulting in the precipitation of vitellogenin. The precipitate was redissolved in 3 ml of 1 M NaCl, 50 mM Tris-HCl buffer (pH 7.5) and dialyzed against 1 M NaCl and then twice against 50 mM Tris-HCl buffer (pH 7.5). The sample obtained (sample B) included vitellogenin.

2.3. 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 analyzed 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 4.6×250 mm column of YMC-Pack A-003-3 SIL (Yamamura Chemical Laboratories Co. Ltd., Kyoto, 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 ml/min. The absorbances of fractions at 350 and 330 nm were measured with JASCO 875UV detectors and peak areas were determined by integrating with a Chromatopack C-R4K (Shimadzu Co. Ltd., Kyoto, Japan). Sometimes the fluorescence, excited at 330nm, was monitored at 470 nm using a fluorescence spectrophotometer F1000 (Hitachi Corp., Tokyo, 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).

2.4. 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's method (Lowry et al., 1951) and by a method measuring the fluorescence excited at 280 nm using bovine serum albumin as the standard protein. The detailed procedure has been described previously (Irie et al., 1991).

3. Results

3.1. 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 0.3 ml. 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 and 1C correspond to retinals in the plasma of a female and of an estrogen-injected male. Small *syn*-peaks of all-*trans* retinaloximes (peak 1 and 2) in Fig. 1B indicate that small amounts of all-*trans* RAL1 and RAL2 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* RAL1 and RAL2. Although not shown in this figure, retinals were barely detectable in the plasma of males without estrogen injection. The peaks due to a ROL1 and ROL2 (peaks 5 and 6) are also shown in Fig. 1B and 1C 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 and all-*trans* retinols 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 RAL1 in *all-trans* retinals was about 50% in every case. In contrast, *all-trans* retinols were present in the plasma of females and males without estrogen injection (about 100 pmol/ml). However, injection of estrogen increased the amount of *all-trans* retinols is about 20% and is smaller than that of RAL2 in *all-trans* retinals.

Retinals and retinols in the fractions of blood plasma

Blood plasma was prepared from estrogen-injected males 2 weeks after injection. After adding MgCl₂ 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* 3,4-didehydroretinal (2 and 2'), 13-*cis* retinal (3 and 3') and 13-*cis* 3,4-didehydroretinal (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 3,4-didehydroretinal (M. Azuma, unpublished observation). Large peaks (5 and 6) due to *all-trans* retinols (retinol and 3,4-didehydroretinol) are also shown in Fig. 2C. As indicated in Fig. 2A, sample A has mostly *all-trans* retinol and 3,4-didehydroretinol (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 large amounts of *all-trans* retinal (peaks 1 and 1') and *all-trans* 3,4-didehydroretinal (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 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 on sample B (Fig. 3B) and in the blood plasma (Fig. 3C). The pattern of 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.

4. Discussion

This study shows that an estrogen injection induces the appearance of retinals (mainly all-*trans* retinals) and vitellogenin in the blood plasma of male *X. laevis*. A method of purifying vitellogenin (Willy et al., 1979) produced the fraction that precipitated in the presence of MgCl₂ 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 (NaBH₄) 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 NaBH₄ 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 Schiff base linkage. Small amounts of retinals were detected in the blood plasma of female *X. 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 in the blood plasma. These retinols were barely detectable in the vitellogenin fraction (sample B) but were found mostly in the fraction that was soluble in the presence of MgCl_2 and EDTA (sample A). Sample A did not include vitellogenin but contained other proteins whose relative molecular masses were lower than those of vitellogenin polypeptides (approximately 200 kDa). Estrogen induction of serum retinol-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 (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 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 of the vitellogenin-retinal complex and its conversion to the lipovitellin-retinal complex.

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

	All-trans retinals (pmol/ml)	All-trans retinols (pmol/ml)
Female (–)	Approx. 10	94 ± 33
Male (–)	< 10	96 ± 17
Male (+) 1w	115 ± 35	2292 ± 1065
Male (+) 2w	223 ± 108	2349 ± 1919
Male (+) 3w	286 ± 74	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.

Captions to Figures

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 labeled as follows: 1 and 1', *syn* and *anti* all-*trans* retinaloxime; 2 and 2', *syn* and *anti* all-*trans* 3,4-didehydroretinaloxime; 3 and 3', *syn* and *anti* 13-*cis* retinaloxime; 4 and 4', *syn* and *anti* 13-*cis* 3,4-didehydroretinaloxime; 5, all-*trans* retinol; 6, all-*trans* 3,4-didehydroretinol.

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 MgCl₂ (sample A, see text); B, The fraction precipitated in EDTA and MgCl₂ (sample B, see text). The volume of the blood plasma (C) was 0.4 ml and sample A and B were derived from an approximately similar volume of blood plasma. Peaks are labeled as follows: 1 and 1', *syn* and *anti* all-*trans* retinaloxime; 2 and 2', *syn* and *anti* all-*trans* 3,4-didehydroretinaloxime; 3 and 3', *syn* and *anti* 13-*cis* retinaloxime; 4 and 4', *syn* and *anti* 13-*cis* 3,4-didehydroretinaloxime; 5, all-*trans* retinol; 6, all-*trans* 3,4-didehydroretinol.

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 samples are the same as for Fig. 2. The arrow indicates the position of vitellogenin.

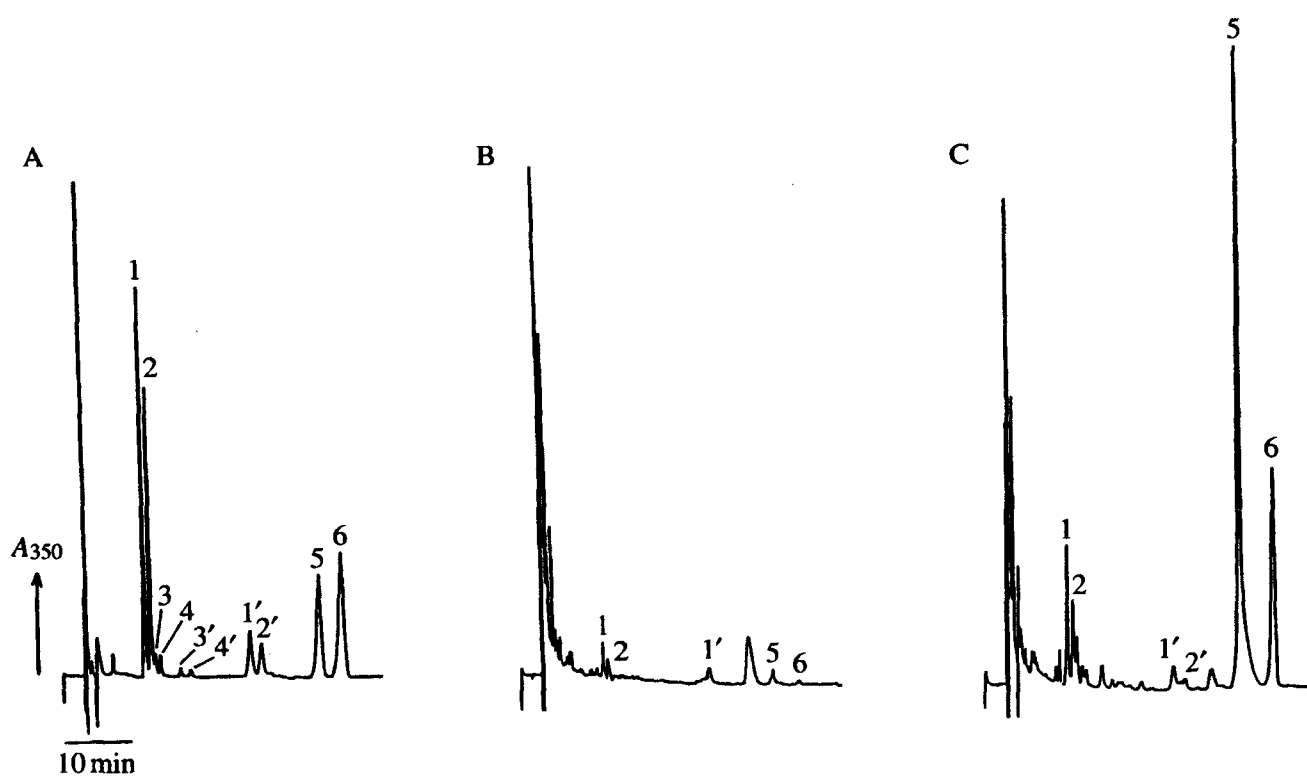


Fig. 1.

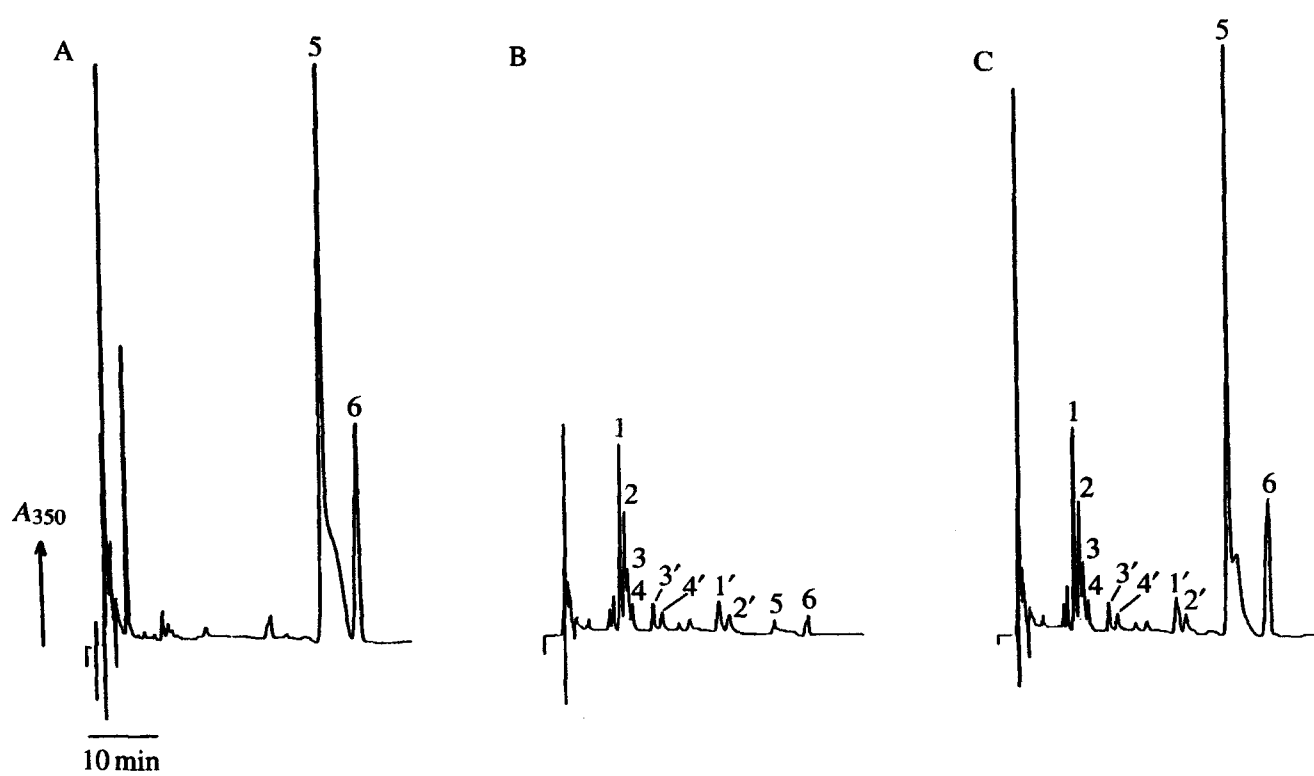


Fig. 2.

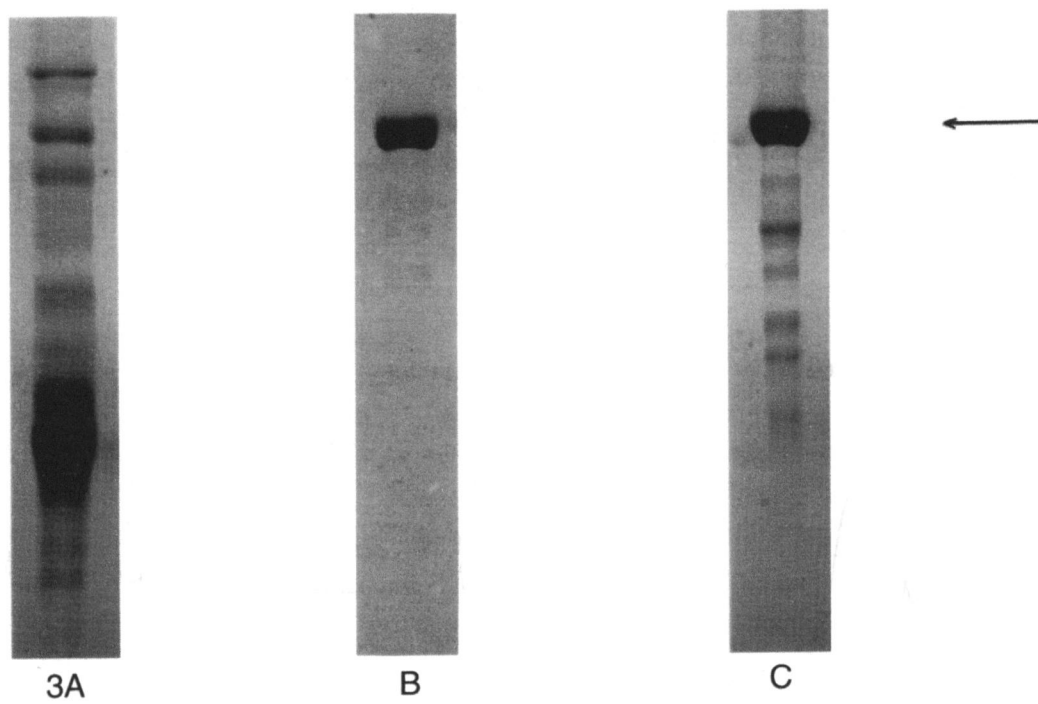


Fig. 3

V. Retinoid composition and retinal localization in the eggs of teleost fishes

Abstract

Retinoids in the eggs of four teleosts, chum salmon (*Oncorhynchus keta*), black porgy (*Acanthopagrus schlegeli*), marbled flounder (*Pleuronectes yokohamae*), and stingfish (*Inimicus japonicus*), were analyzed by high performance liquid chromatography. Retinal (RAL1) or both RAL1 and 3,4-didehydroretinal (RAL2) were major or exclusive retinoids in the eggs of every species examined. In *O. keta* eggs, both RAL1 and RAL2 were present at the ratio of about 3:4, whereas RAL1 was the only retinal in the eggs of the other three marine species. RAL1 was the exclusive retinoid in the eggs of *P. yokohamae* and *I. japonicus*, whose eggs lack lipid bodies. In the eggs of *O. keta* and *A. Schlegeli*, which have lipid bodies, retinylesters were also detected, and retinals composed 69% and 93%, respectively, of total retinoids. In *O. keta* eggs, retinals were present mostly in the aqueous part and were bound to a protein homologous to lipovitellin 1, an amphibian yolk protein, and retinylesters were located in lipids. These results indicate that retinals are the essential mode of retinoid storage in eggs of teleosts and they are the precursors of functional retinoids, such as retinoic acid and visual pigment chromophores. Retinylesters are additional retinoids that accompany lipid accumulation.

1. Introduction

The occurrence of retinal in tissues other than the eye was shown for the first time in the eggs of several fishes (Plack et al., 1957). In subsequent studies, Plack and colleagues used the Carr-Price reaction, as improved by them, to show the distribution of retinal (RAL1), or of both RAL1 and 3,4-didehydroretinal (RAL2) in the eggs or ovaries of a wide range of oviparous vertebrates: lampreys, elasmobranchs, teleosts, amphibians, reptiles, and birds, (Plack et al., 1959; Plack, 1960; Plack and Kon, 1961; Plack et al., 1961). Their bioassay examinations also showed that the egg retinal was

vitamin A active, and they solved the problem that vitamin A activity in herring eggs, measured biologically, was 7–10 times greater than the activity of retinol determined by the Carr-Price reaction (Plack et al., 1959; Plack et al., 1961). The supernatant fraction of herring egg homogenate was always vitamin A active, and the activity was precipitated by heating or by trichloroacetic acid, suggesting the association of vitamin A activity with protein (Plack et al. 1959).

In 1987, Seki et al. reported that retinoids detected by high performance liquid chromatography (HPLC) in the eggs of *Xenopus laevis* and several other amphibians were exclusively retinals (RAL1 and RAL2). Later, we presented evidence that the retinals in *X. laevis* eggs are bound to the protein moiety of lipovitellin 1, a yolk lipoprotein, by a Schiff base linkage (Irie et al., 1991).

In contrast to the eggs of *X. laevis*, not only retinal but also retinol and/or retinylester have been shown to be present in the eggs or ovaries of several teleosts (Plack et al., 1959; Plack and Kon, 1961; Plack et al., 1964; Costaridis et al., 1996). It is generally known that many teleost eggs are composed of an aqueous part, which contains yolk proteins, and lipid bodies. Much variation occurs in lipid accumulation among fish species, ranging from eggs devoid of lipids to those in which 37% of the weight is a wax ester (Selman and Wallace, 1989). No lipid bodies have been found by morphological observations in *X. laevis* eggs, in which retinoids occur only as retinals. On the basis of these results, we hypothesized that retinols and/or retinylesters are present in lipid bodies, apart from retinals, which might be localized in a protein-bound form in the aqueous part. However, intracellular localization of retinoids in teleost egg cells has not been verified. In this study, therefore, we examined retinoid composition and localization in the ovulated eggs of four teleost species with and without lipid bodies in their eggs, and we obtained evidence of the state of retinals in teleost eggs.

In *X. laevis* embryos, egg retinals have been suggested to be the source of functional retinoids such as visual pigment chromophores in the eyes (Azuma et al., 1988, 1990) and retinoic acid (Durstun et al., 1989; Kraft et al., 1994), although retinals

are not stored as retinoic acid precursors in mammals at any time in the life cycle. On the basis of the present results, we discuss the storage and metabolism of retinoids in vertebrate eggs and adults.

2. Materials and methods

2.1. Animals and eggs

Unfertilized eggs, which were ovulated into the coelum of matured chum salmon (*Oncorhynchus keta*), were purchased from a fisher who harvested the fish from the Chitose River, Hokkaido, Japan. Fertilized eggs in early developmental stages (pregastrula) of black porgy (*Acanthopagrus schlegeli*), and unfertilized eggs of marbled flounder (*Pleuronectes yokohamae*) and stingfish (*Inimicus japonicus*) were supplied from the Osaka Prefectural Foundation for Fishery Promotion, Sea Farming Center, Osaka, Japan. *O. keta* eggs were rinsed with 0.65% NaCl and frozen individually with liquid nitrogen. The eggs of other marine species were rinsed with a 3% NaCl solution. The eggs of every species were stored in a deep freezer at -80°C until use.

2.2. Preparation of samples

The eggs were thawed and an approximately 5 times volume of cold 20 mM Tris-HCl buffer (pH 7.4) for marine species or a buffer containing 0.2 M NaCl (saline buffer) for *O. keta* was added. Then the eggs were homogenized with a high-speed cutting homogenizer (Physoctron NS-50, Nichion Irikakikai Seisakusho Co., Ltd., Chiba, Japan). The homogenate was filtered through a layer of laboratory tissue paper to remove egg membrane fragments. The filtrate was used for whole retinoid analyses by HPLC.

To examine the localization of the retinoids, *O. keta* eggs were divided into aqueous and lipid fractions as follows. Several eggs were put in a beaker with an appropriate volume of saline buffer. The egg membrane was cut into pieces with small scissors, and the contents were mixed gently with a glass rod. The mixture was then

filtered with a small piece of filter paper (No. 2, Toyo Roshi Kaisha Ltd., Tokyo, Japan); the fragments of egg membrane and lipid substance were trapped on the paper, and the lipid was collected with a Pasteur pipette. The aqueous filtrate containing yolk proteins was diluted 5 times with a Tris-HCl buffer to precipitate the yolk proteins. The resulting precipitate was collected by centrifugation at $13\,000 \times g$ for 20 min. The pellet obtained was dissolved and made up to initial volume with the saline buffer.

The yolk protein complex (lipovitellin-phosvitin) of the salmonid teleosts has been shown to be soluble in 0.5 M NaCl solution, and to precipitate by dialysis against distilled water (Markert and Vanstone, 1968). In our preliminary tests, however, the yolk proteins of *O. keta* eggs were soluble in 0.2 M NaCl solution, and precipitated by a 5-times dilution with 20 mM Tris-HCl buffer. Therefore, the pellet obtained is the yolk protein complex fraction.

2.3. Extraction and analysis of retinoids and quantification of protein

The retinoids were extracted with organic solvents by the oxime method (Suzuki and Makino-Tasaka, 1983) following our routine procedure (Seki et al., 1989); briefly, 0.1 ml of 2 M hydroxylamine hydrochloride (freshly neutralized) and 1 ml of methanol were added to 1 ml of each sample in that order, and then retinoids were extracted with dichloromethane and hexane. The HPLC-system (Hitachi 655, Hitachi Ltd., Tokyo, Japan) was equipped with a 6×150 mm column of 3 μ m silica gel (YMC-PACK-A-012 S-3 SIL, Yamamura Chemical Laboratories Co., Ltd., Kyoto, Japan), with a 4×50 mm precolumn, and was used at a flow rate of 2 ml/min. Absorbances of dual wavelengths (330 and 350 nm) were monitored with UV-Visible detectors (Jasco 875-UV and UV-970, JASCO Corp., Tokyo, Japan) and recorded with an integrator (Labchart 80, System Instrument Co. Ltd., Tokyo, Japan). The eluent was 5% *tert*-butylmethyl ether–0.04% ethanol–25% benzene in *n*-hexane. Because retinylesters elute out in the void fraction under this eluent condition, the fraction (retention time for 1.5–3 min) was collected, saponified with ethanolic KOH (Bridges and Alvarez, 1982), extracted with organic solvents, and rechromatographed

under the same conditions as described above. Retinylesters were detected as retinols, the products of the saponification, on the second HPLC. The retinoids were identified based on the retention time and the ratio of absorbance at 330 and 350 nm in comparison with the standard retinal isomers and retinols. The extraction and the analyses of retinoids were performed under dim red light.

The protein content of the homogenate was measured by using the method of Lowry et al. (1951), with bovine serum albumin as the standard protein.

2.5. Treatment with sodium borohydride

Sodium borohydride (NaBH_4) was added to the filtered homogenate to reduce the retinals bound to yolk proteins by a Schiff base linkage into n-retinyl products (Bownds and Wald, 1965; Irie et al., 1991). In contrast to retinylidene Schiff bases in *X. laevis* eggs, those in the fish eggs were not reduced readily, so a protein denaturant, solid urea or 10% sodium dodecylsulfate (SDS), was added immediately after the addition of NaBH_4 . The final concentrations of urea and SDS were 8 M and 2%, respectively. Preceding the extraction of retinoids by the oxime method described above, the mixtures were allowed to stand for 30 min on ice to complete the reaction.

2.6. Gel chromatography and SDS-polyacrylamide gel electrophoresis

The yolk protein complex of *O. keta* was treated with NaBH_4 and SDS as described above, dialyzed against the 20 mM Tris-HCl buffer containing 0.1% SDS and 0.02% dithiothreitol, and applied to a Sephacryl S-300 HR column (2.5×86 cm) equilibrated with the same solution. The eluent was collected in 6-ml fractions. After the gel chromatography, absorbances at 280 nm (absorption of protein) and 330 nm (absorption of retinyl product) of each fraction were measured.

The SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed principally according to the procedure described by Laemmli (1970) using 1-mm-thick 7.5% slab gels. The yolk protein complex or gel chromatography fractions were treated with the sample buffer (at final concentrations of 0.125 M Tris-phosphate

buffer at pH 6.9, 2% SDS, 5% 2-mercaptoethanol, and 0.01% bromophenol blue). The electrophoresis was performed under a constant current of 12.5 mA per gel for about 2 h until the bromophenol-blue marker reached about 1 cm from the end of the gel. The molecular weight marker used was a protein mixture including the following proteins: β -galactosidase (116 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). After the electrophoresis, the gels were stained with 0.1% Coomassie Brilliant Blue R-250 in 45% methanol and 10% acetic acid, and then destained with 20% methanol and 10% acetic acid.

3. Results

3.1. Retinoid composition in the eggs

Figure 1 shows HPLC chromatograms of whole retinoids in the egg homogenate of the four teleost species. Retinal was observed to be the major retinoid in the eggs of every species examined. Only RAL1 was detected in the eggs of the three marine species (Fig. 1, B₁, C₁, and D₁), but both RAL1 and 3,4-didehydroretinal (RAL2) were found in *O. keta* eggs (Fig. 1, A₁); the proportion of RAL1 to the sum of the retinals was 40%–45% (42% on average). Most retinal(s) in the eggs of every species detected were the all-*trans* isomer, and a small amount of the 13-*cis* form was present.

Other than retinals, small peaks of all-*trans* retinol (ROL1) and 3,4-didehydroretinol (ROL2) were found in *O. keta* eggs (Fig. 1, A₁), and a small peak of all-*trans* ROL1 was detected in *P. yokohamae* and *I. japonicus* eggs (Fig. 1, C₁ and D₁). The free retinol content was less than 1% of the total retinoids (Table 1). Retinols were scarcely detected in *A. schlegeli* eggs.

To analyze the retinylesters, the solvent front fraction was collected and rechromatographed after saponification (Fig. 1, A₂, B₂, C₂, and D₂). Considerable amounts of all-*trans* ROL1 and ROL2 were detected in *O. keta* eggs (Fig. 1, A₂), and a little all-*trans* ROL1 in *A. schlegeli* eggs (Fig. 1, B₂), indicating the presence of

corresponding retinylesters in these eggs. The sum of the retinylesters in *O. keta* eggs was 21%–42% (31% on average) of the total retinoids, and the ratio of retinylester to 3,4-didehydroretinylester was about 9:1. The retinylester content in *A. schlegeli* eggs was 4.9%–8.5% (6.7% on average) of the total retinoids (Table 1). No retinylesters were found in *P. yokohamae* or *I. japonicus* eggs (Fig. 1, C₂ and D₂).

The retinal concentration was 16.0 to 76.5 ng per mg of protein, and the content was 0.65 to 729 ng per egg, depending on the teleost species (Table 1).

3.2. Localization of retinals and retinylesters in *O. keta* eggs

Lipid bodies were observed in the eggs that contained retinylesters (*O. keta* and *A. schlegeli*), but no lipid bodies were observed in the eggs that did not contain detectable retinylesters (*P. yokohamae* and *I. japonicus*) (Table 1). A large lipid body seen by microscopic observation accounts for the large amount in *O. keta* eggs, and several small lipid bodies account for the small amount in *A. schlegeli* eggs. *O. keta* eggs were divided into aqueous and lipid fractions, and the aqueous fraction was subdivided into supernatant and precipitate fractions (see Materials and Methods). Figure 2 shows the retinoid analyses in each of the three fractions. Most retinals were observed in the precipitate of the aqueous fraction, whereas retinylesters were observed mostly in the lipid fraction. A small amount of free retinols was detected mainly in the precipitate fraction. As the egg yolk protein complex was confirmed to be localized in the precipitate of the aqueous fraction by SDS-PAGE (see Fig. 4, lane C), RAL1 and RAL2 were apparently bound to the egg yolk protein.

3.3. Effect of sodium borohydride

To confirm the Schiff base linkage of retinals to the egg yolk protein, NaBH₄ was applied to convert the retinylidene Schiff base into retinyl protein, which is unextractable with organic solvents. After the reduction of egg homogenates with NaBH₄, the amount of retinaloximes extracted decreased, but considerable quantities

of retinaloximes were still detected in organic solvents (Table 2). The effect of NaBH₄ on the eggs with lipids was considerably less than on the eggs without lipid bodies.

Thus, a protein denaturant, urea (8 M) or SDS (2%), was added immediately after the addition of NaBH₄ to facilitate the reaction of NaBH₄. The protein denaturants markedly accelerated the effect of NaBH₄; the amount of extracted retinaloximes decreased. In the presence of urea, the effect of NaBH₄ on the Schiff base in the eggs with lipid bodies was considerably less than on the eggs without lipid bodies. In the presence of 2% SDS, no retinaloximes were extracted from any egg homogenate with the organic solvent after treatment with NaBH₄.

An increase in retinol was not observed after these treatments either with or without the protein denaturants, suggesting the absence of free retinals, which would have been reduced to retinols by NaBH₄.

3.4. Identification of retinal binding protein in *O. keta* eggs

After the yolk proteins of *O. keta* eggs were treated with NaBH₄ and SDS, the sample was chromatographed with the Sephacryl S-300 HR column (Fig. 3). The proteins were separated into two major peaks (absorbance at 280 nm), whereas the retinyl product (absorbance at 330 nm) eluted out in a single peak, which coincided with the first protein peak.

The result of the SDS-PAGE analysis (Fig. 4) shows that the first peak from the gel chromatography (Fr. 42) contained mostly a single protein, with a molecular weight of about 90 kDa (Fig. 4, lane A). Several proteins of lower molecular weight were present in the second peak (Fr. 55; Fig. 4, lane B). These results show that the retinals in *O. keta* eggs were bound to the protein in the first peak by Schiff base linkage.

4. Discussion

Both RAL1 and RAL2 were found in the eggs of *O. keta*, an anadromous fish, and the proportion of RAL1 to total retinals (42%) was higher than that of Atlantic salmon (*Salmo salar*) eggs (28%; Plack and Kon, 1961). In contrast to salmonid

teleosts, RAL2 was scarcely detected in the eggs of the marine teleost species we examined (Fig. 1). However, the presence of RAL2 (4% – 18% of total retinals) has been reported in the eggs of several marine teleosts by Plack and Kon (1961). This inconsistency may be accounted for by the different teleost species examined and the analytical method: Carr-Price colorimetric reaction vs. HPLC analysis. Plack and Kon reported the presence of a considerable amount of RAL2 (34% – 97% of total retinals) in the eggs or ovaries of freshwater teleosts, whose visual pigment chromophores are predominantly RAL2 (Bridges, 1972); we did not use HPLC to confirm this. Geometric isomers of retinal detected in this experiment were mostly the all-*trans* form (Fig. 1), similar to previous results on eggs of some teleosts (Plack et al., 1957), zebrafish (Costaridis et al., 1996), and amphibians (Seki et al., 1987; Irie et al., 1991).

In the present experiment, RAL1 or both RAL1 and RAL2 were the major or exclusive components of retinoids in the eggs of every species we examined. Similar results have been obtained for the eggs of the Atlantic cod (*Gadus morhua*; Plack et al., 1961), zebrafish (Costaridis et al., 1996), some amphibians (Seki et al., 1987), and *X. laevis* (Irie et al., 1991). Besides retinals, retinylesters were detected in the eggs of *O. keta* and *A. schlegeli* (with lipid bodies), but not in those of *P. yokohamae* or *I. Japonicus* (without lipid bodies). As shown in Table 1, the amount of retinylester correlated with the developmental degree of the lipid bodies. According to these results, it is presumed that retinylester was not present in the aqueous part. To obtain further confirmation of the retinoid localization, the contents of *O. keta* eggs, which included a well-developed lipid body of reddish-orange color, were divided into aqueous and lipid fractions, and then the aqueous fraction was subdivided into the yolk protein fraction and the non-yolk protein fraction. Retinoid analysis of each fraction (Fig. 2) showed that retinals were present in the yolk protein fraction and that retinylesters were mostly in the lipid fraction. Teleost eggs are enveloped by a hard egg membrane. Although *O. keta* eggs are large enough (about 7 mm in diameter) to manually incise the egg membrane using a pair of scissors, *A. schlegeli* eggs (0.8 to 1.1 mm) are too small to rupture the membrane gently. Vigorous homogenization of *A.*

schlegeli eggs created a homogenate emulsion, and the attempt to separate the homogenate into aqueous and lipid fractions was not successful. In spite of this failure, our results presented above strongly suggest that retinals and retinylesters in teleost eggs are localized in the aqueous and lipid parts, respectively.

The presence of a considerable amount of retinylesters has been shown in the ovaries of several fish species (Plack and Kon, 1961; Plack et al., 1961). The ovary of *I. japonicus* included a small but clearly detectable amount of retinylester (data not shown), although retinylester was not found in the ovulated eggs of this species, as shown in Figure 1 (D₂) and Table 1. The retinylester detected in the ovaries of fishes that lay eggs without lipid bodies is considered to be present in follicle cells or in ovary tissue other than the eggs.

To examine the form of the retinals in teleost eggs, NaBH₄ was added to the egg homogenate with SDS prior to the extraction of retinoids with organic solvents by the oxime method. After the treatment, retinaloximes were not detected in the extract. Although retinals with an unprotected aldehyde are reduced to retinols by NaBH₄, retinols were not increased by the NaBH₄ treatment. The results shown by Table 2 indicate that all retinals in the eggs of the four teleosts were bound via a Schiff base linkage to proteins. The retinal binding protein in the eggs of *O. keta* was shown by gel chromatography (Fig. 3) and SDS-PAGE (Fig. 4) to be a single polypeptide with a molecular weight of about 90 kDa. The electrophoretic pattern of egg yolk proteins in *O. keta* eggs was similar to those in amphibian eggs (Wallace, 1963a, 1963b), rainbow trout eggs (*Oncorhynchus mykiss*; Hara and Hirai, 1978; Tyler 1993), and coho salmon eggs (*Oncorhynchus kisutch*; Hara et al., 1993), and the retinal binding protein in *O. keta* eggs corresponded to lipovitellin 1 in amphibian eggs. We have already shown that the retinals in *X. laevis* eggs are bound to lipovitellin 1 by a Schiff base linkage (Irie et al., 1991). It is suggested that retinals in the eggs of amphibians and teleosts are commonly bound to lipovitellin 1 or its homologous protein by Schiff base linkage.

The ratio of retinal to total egg protein measured by Lowry's method varied among the teleost species (Table 1). As yolk proteins contribute up to 90% of the dry weight of eggs in most lower vertebrates, including teleosts (Selman and Wallace 1989), the molecular ratio of retinal to its binding protein may also vary among the teleost species. Except for *O. keta* eggs (about 7 mm in diameter), the eggs of the 3 marine teleosts were similar in size (0.8 to 1.2 mm), and the retinal content in one egg was also similar among the 3 marine species.

The NaBH₄ reactivity with or without the presence of urea (Table 2) correlated inversely with the developmental degree of lipid bodies in the eggs. The different efficiency of NaBH₄ may reflect the difference in hydrophobicity of the retinal binding site of the binding protein. It would appear that the hydrophobic submolecular environment interrupts access of NaBH₄ to the retinal binding site. As the NaBH₄ reacts easily with *X. laevis* egg yolk protein without any protein denaturants (Irie et al., 1991), the retinal binding site of the teleost yolk proteins may be much more hydrophobic than that of *X. laevis*.

The precursor of egg yolk proteins in oviparous vertebrates, vitellogenin, is known to be a female-specific serum protein, synthesized in the liver by the effect of estrogen. The appearance of retinal in cod ovaries occurred when there was histological evidence for the onset of vitellogenesis (Plack et al., 1961). Retinals have been detected in the blood and liver of laying hens, but they are not measurable in the blood of immature pullets and only trace amounts are found in the blood of cockerels (Plack, 1964; Plack et al., 1964). In the blood of estrogen-injected male *X. laevis*, considerable amounts of retinals, as well as of vitellogenin, have been detected, and the retinals were found in the vitellogenin fraction (Azuma et al., 1993). These findings suggest that, in common with oviparous vertebrates, the retinals are synthesized in the liver and transported in a vitellogenin-bound form in the blood to the eggs, where they accumulate.

During the development of *X. laevis* embryos, the egg retinals have been shown to be the precursors of visual pigment chromophores (Azuma et al., 1988,

1990). Another role of egg retinals is as the precursors of endogenous retinoic acids, which have important physiological functions for axial patterning of vertebrates during development (Means and Gudas, 1995). Egg retinals must be the precursors of endogenous retinoic acids in the embryos of zebrafish (Costaridis et al., 1996) and *X. laevis* (Ang and Duester, 1999). Therefore, it is evident that retinals are the common and essential mode of retinoid storage in eggs of teleosts and amphibians, and they are the precursors of the functional retinoids during development. Moreover, retinylesters are additional retinoids that accompany the accumulation of lipid substances.

Although retinals are commonly found in the eggs and embryos of the anamniote vertebrates, retinals are undetectable in mouse embryos (Horton and Maden, 1995), chick limb buds (Thaller and Eichel, 1987), and chick embryos (Maden et al., 1998). Costaridis et al. (1996) reported that retinoid metabolism appears to be fundamentally different in the embryos of lower vertebrates compared with the embryos of higher vertebrates. In other studies, however, endogenous retinal has been found in quail embryos (Dong and Zile, 1995) and in mouse embryos at the egg cylinder stage (Ulven et al., 2000). This discrepancy with respect to embryonic retinal in mammals and birds is probably due to the difficulty of retinal detection. It is suggested that the amount of retinal in embryos of mammals and birds is very small, because the metabolic rate of retinol to retinoic acid via retinal is very high. This assumption is supported by the results of several studies. Two classes of enzymes, alcohol- and aldehyde dehydrogenases, are involved in the biotransformation of retinol to retinoic acid (Duester, 1996). The rate-limiting step for retinoic acid synthesis is the oxidation of retinol to retinal in mammals (Chen et al., 1995) and in avian embryos (Dong and Zile, 1995), as opposed to the conversion of preexisting retinal to retinoic acid in *X. laevis* embryos (Ang and Duester, 1999).

Although retinals are the stored form in the eggs of oviparous vertebrates, retinylester in the liver is the prevalent mode of retinoid storage in adults of both oviparous and viviparous vertebrates. The retinol derived from the hepatic retinylester is considered to be supplied to mammalian embryos during development, and the

retinol is converted to retinoic acid via retinal, with retinal dehydrogenase type 2 as the key regulator in retinoid signaling (Haselbeck et al., 1999; Niederreither et al., 1999). Obviously, the retinoid metabolism is fundamentally identical among the classes of vertebrates, as has been described by the recent review (Duester, 2000). The only difference is the stored form in the metabolic pathway of retinoids. It is a very important finding that the stored form of retinoids, which play an essential role as the precursors of functional retinoids, is different between eggs and adults of oviparous vertebrates. The biological basis of the difference of the mode of retinoid storage is a problem for further investigation.

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Table 1.

Retinoid composition (%) and retinal concentration in the eggs of the four teleost species.

Species	Lipid body amount	Retinoid composition (%)			Retinal concentration	Retinal content
		RAL	RE	ROL	ng / mg protein*	ng / egg
<i>O. keta</i>	++	68.9	31.1	trace	16.1	729
<i>A. schlegeli</i>	+	93.3	6.7	n.d.	76.5	0.65
<i>P. yokohamae</i>	—	~100	n.d.	trace	40.5	1.10
<i>I. japonicus</i>	—	~100	n.d.	trace	16.0	0.69

RAL, retinal; RE, retinylester; ROL, retinol; n.d., not detected; * measured by Lowry's method

Table 2.

Effect of sodium borohydride (NaBH_4) to yolk proteins with or without protein denaturant.

Species	Lipid body amount	Retinals extracted by oxime method (%)			
		control – NaBH_4	+ NaBH_4	+ NaBH_4 +urea	+ NaBH_4 +SDS
<i>O. keta</i>	++	100	98	43	0
<i>A. schlegeli</i>	+	100	85	33	0
<i>P. yokohamae</i>	–	100	44	2	0
<i>I. japonicus</i>	–	100	35	0	0

The egg homogenates were extracted with organic solvents by the oxime method. Except for the control, the samples were treated with NaBH_4 with or without a protein denaturant before the extraction. The amount of retinals extracted from the control (without NaBH_4 treatment) was set at 100%. After NaBH_4 was added in the presence of SDS, no retinals were detected in the extract.

Captions to figures

Fig. 1. HPLC analyses of egg extract of the four teleost species. The egg homogenate of each species was extracted by the oxime method and analyzed (A₁, B₁, C₁, D₁). The solvent front of each chromatograph (1.5–3 min) was collected, saponified, and then rechromatographed under the same conditions (A₂, B₂, C₂, D₂). Retinylesters were detected as retinols in the second HPLC. The scale bars show the absorbances at 350 nm (A₁, B₁, C₁, D₁) or 330 nm (A₂, B₂, C₂, D₂).

A, Chum salmon (*Oncorhynchus keta*); B, Black porgy (*Acanthopagrus schlegeli*); C, Marbled flounder (*Pleuronectes yokohamae*); D, Stingfish (*Inimicus japonicus*)

a and a', *syn* and *anti* all-*trans* retinaloxime; b and b', *syn* and *anti* all-*trans* 3,4-didehydroretinaloxime; c and c', *syn* and *anti* 13-*cis* retinaloxime; d and d', *syn* and *anti* 13-*cis* 3,4-didehydroretinaloxime; e, all-*trans* retinol; f, all-*trans* 3,4-didehydroretinol

Fig. 2. Retinoid localization in the eggs of chum salmon (*Oncorhynchus keta*). The egg substances were divided into two fractions (aqueous and lipid), and then the aqueous fraction was subdivided into the precipitate and supernatant parts after the yolk proteins were precipitated by a 5-times dilution with 20 mM Tris-HCl buffer, pH 7.4 (see Materials and Methods). Each sample was extracted by the oxime method and analyzed by HPLC (A₁, B₁, C₁). After the first HPLC, each solvent front (1.5–3 min) was saponified and rechromatographed under the same conditions (A₂, B₂, C₂). Retinylesters were detected as retinols in the second HPLC. The scale bars show the absorbances at 350 nm (A₁, B₁, C₁) or 330 nm (A₂, B₂, C₂).

A, Precipitate of the aqueous fraction; B, Supernatant of the aqueous fraction; C, Lipid fraction

a and a', *syn* and *anti* all-*trans* retinaloxime; b and b', *syn* and *anti* all-*trans* 3,4-didehydroretinaloxime; c and c', *syn* and *anti* 13-*cis* retinaloxime; d and d', *syn* and

anti 13-*cis* 3,4-didehydroretinaloxime; e, all-*trans* retinol; f, all-*trans* 3,4-didehydroretinol

Fig. 3. Gel chromatography of chum salmon (*Oncorhynchus keta*) egg yolk protein treated with NaBH₄ and SDS, using a Sephacryl S-300 HR column (2.5 × 86 cm) equilibrated with the 20 mM Tris-HCl buffer containing 0.1% SDS and 0.02% dithiothreitol. Absorbances at 280 nm (protein, ○) and 330 nm (retinyl product, ●, 5-times enlargement) of each fraction were measured after the chromatography.

Fig. 4. SDS-polyacrylamide gel electrophoresis of the fractions (Fr. 42, lane A; Fr. 55, lane B) of the gel chromatography (see Fig. 3) and yolk proteins (lane C) of chum salmon (*Oncorhynchus keta*) eggs. D, Molecular weight markers.

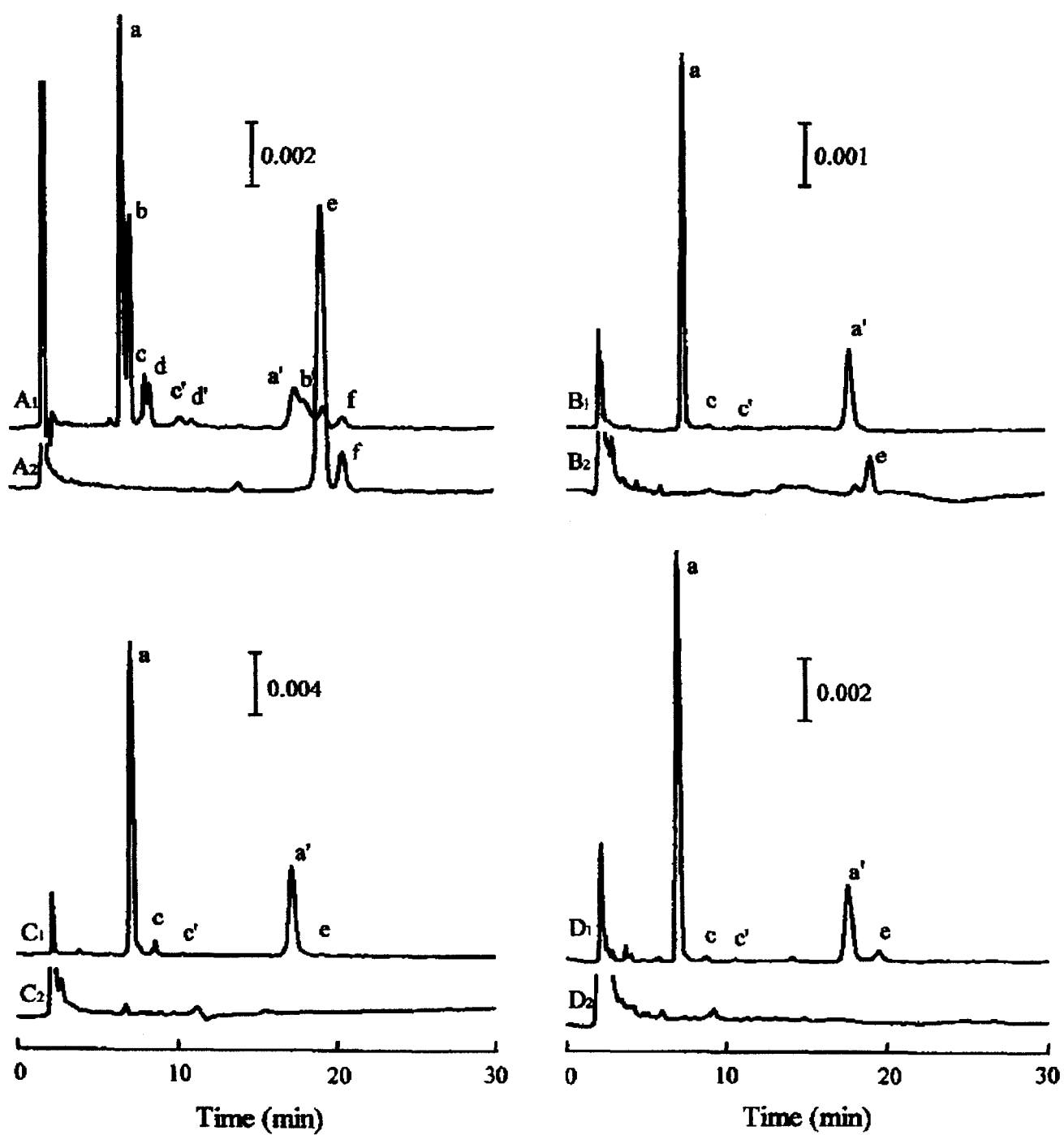


Fig. 1.

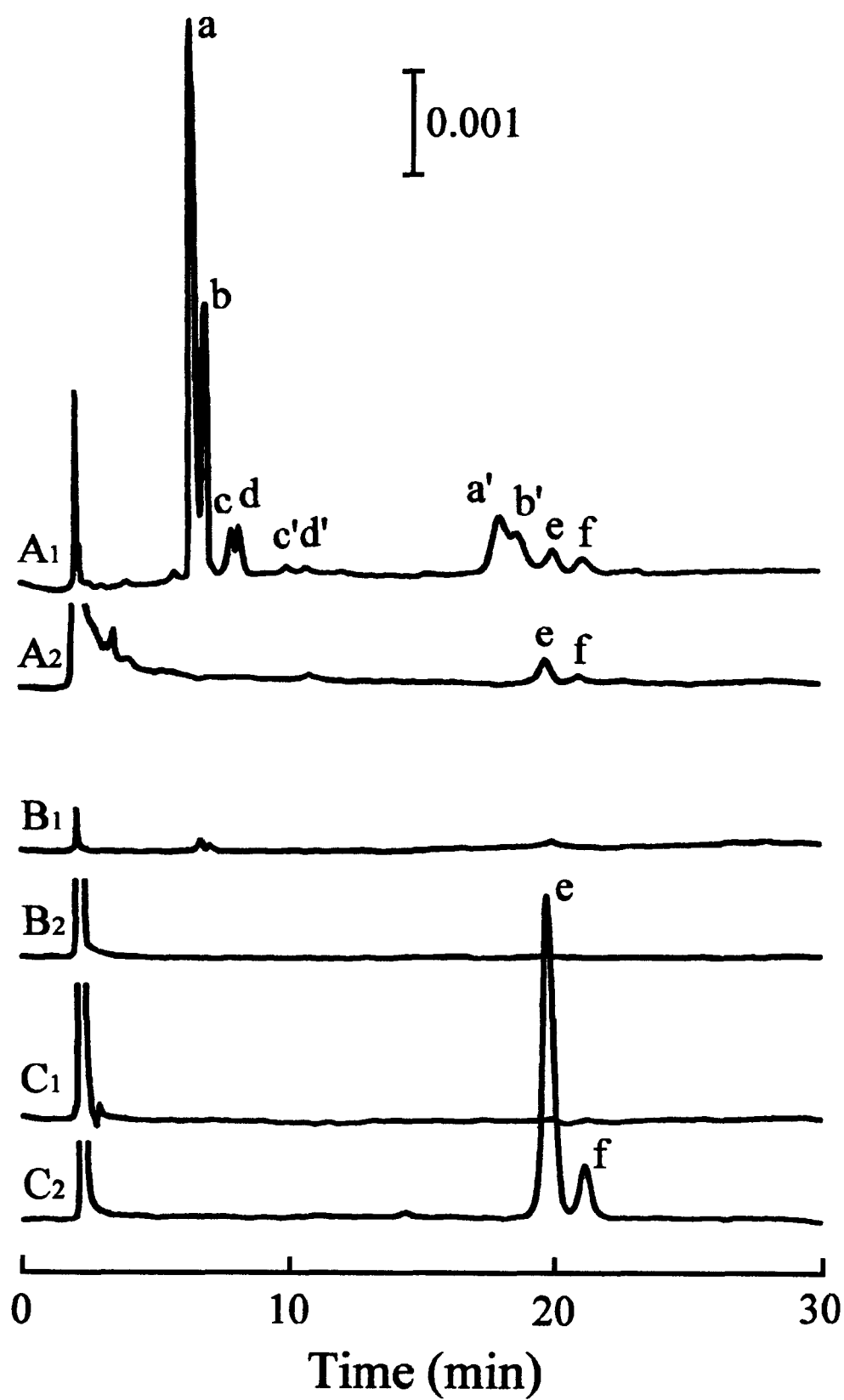


Fig. 2.

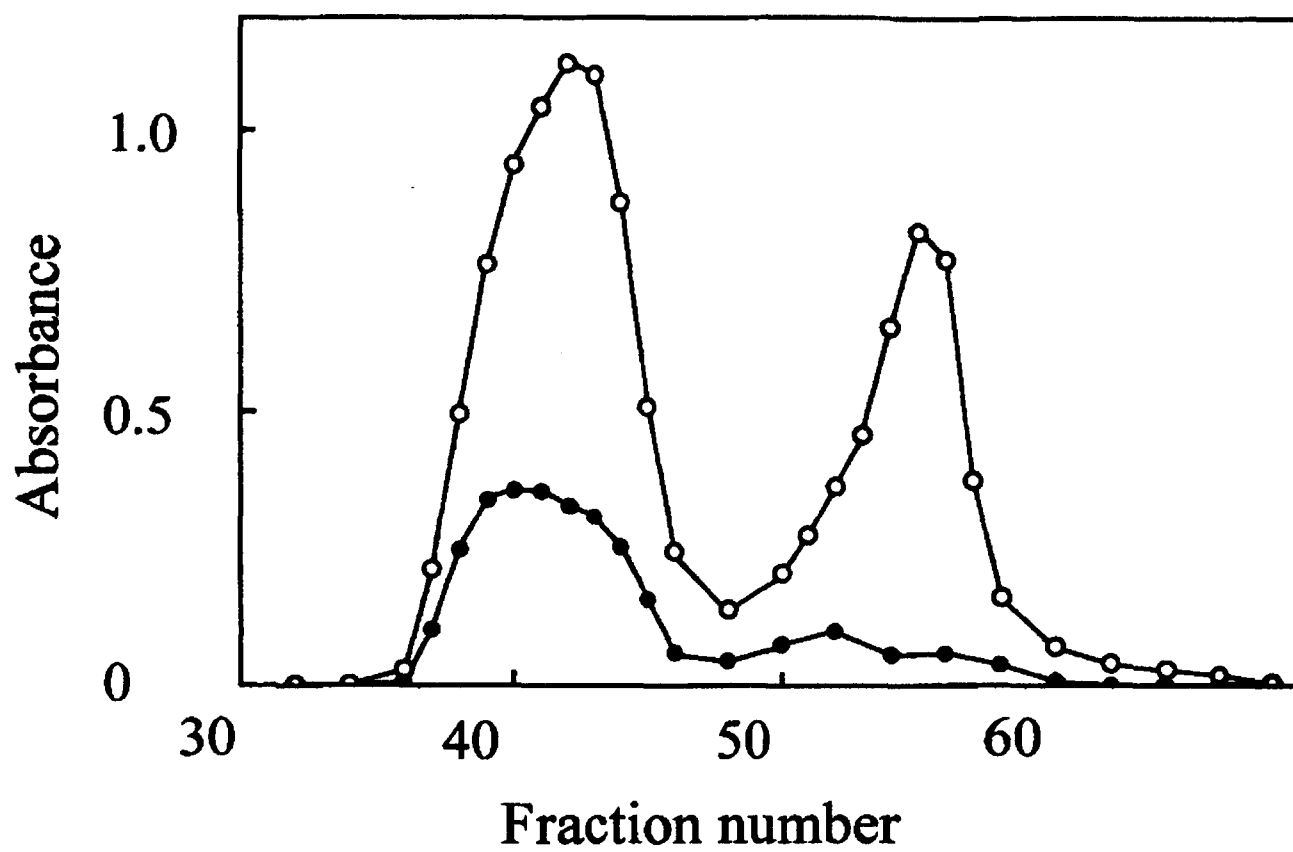


Fig. 3.

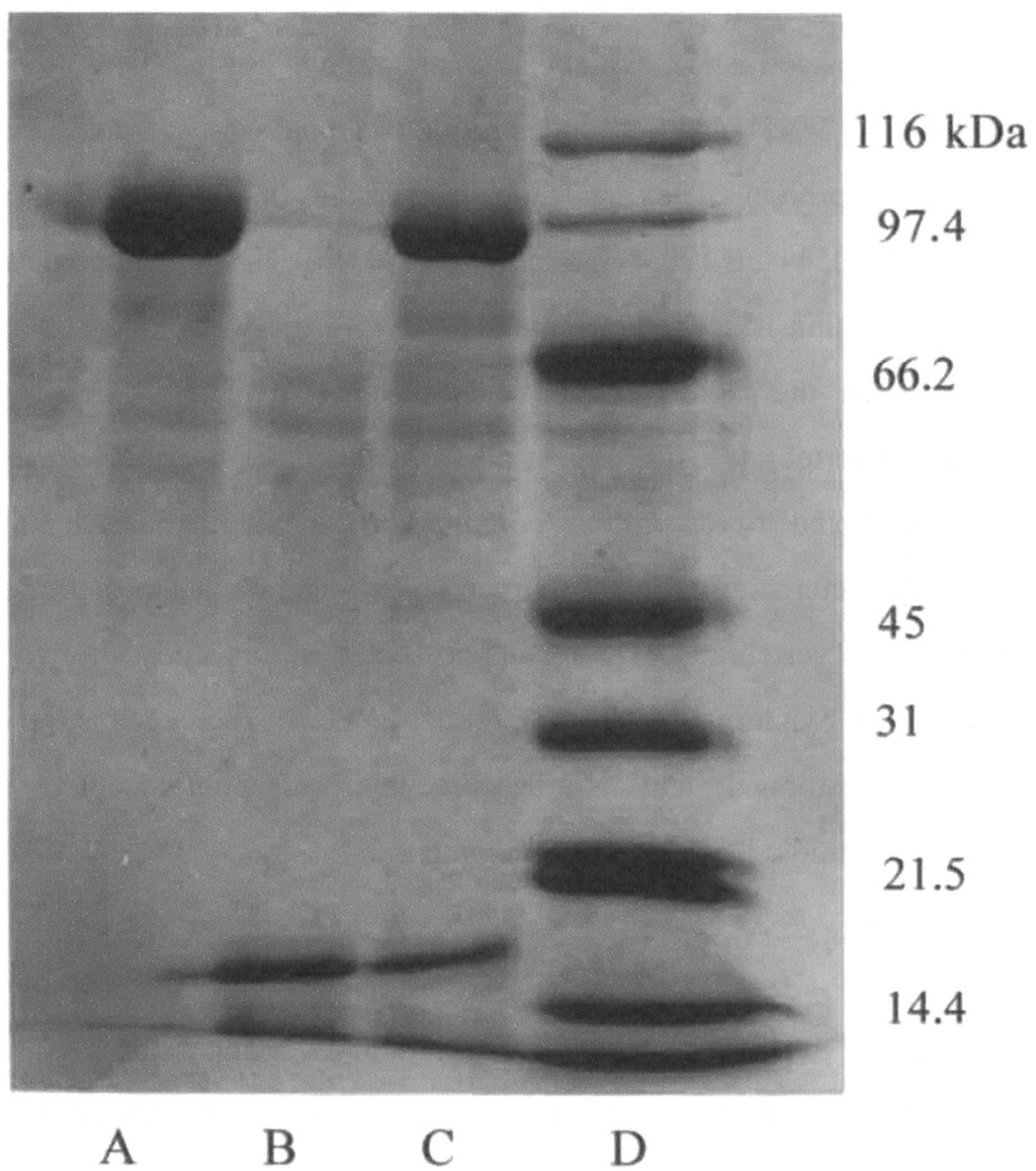


Fig. 4.

VI. Storage of retinal in the eggs of the ascidian, *Halocynthia roretzi*

Abstract

Retinoids in the eggs of the solitary ascidian, *Halocynthia roretzi*, were analyzed by high performance liquid chromatography. Retinal was the almost exclusive retinoid (>> 99%), and the concentration of retinal was 25.9 to 40.1 (30.6 on average) ng/mg of protein. The egg retinal consisted of four isomers: all-*trans* (50.9%), 9-*cis* (6.8%), 11-*cis* (20.4%) and 13-*cis* (21.9%). The presence of retinal in the eggs of this ascidian is a characteristic shared with the wide range of oviparous vertebrates, although the isomer composition differs between ascidian eggs and vertebrate eggs; in vertebrate eggs, almost all the retinal is in the all-*trans* form. The egg retinal was bound to a protein complex *via* a Schiff base linkage. The electrophoretic characteristics of the protein complex were similar to that of egg yolk proteins of oviparous vertebrates. The results presented in this study strongly suggest that, as is found with oviparous vertebrates, retinal in the ascidian eggs is the essential mode of retinoid storage, and is the precursor of photoreceptive pigment chromophores and retinoic acid during development.

1. Introduction

Retinoids are well known to be involved in two quite different functions: retinal (RAL1) and 3,4-didehydroretinal (RAL2) play a role in photoreception, and retinoic acid (RA) plays a role in gene regulation. These functions appear to have different evolutionary backgrounds: the photoreceptive function is evolutionarily ancient while the RA function is specific to chordates (Duester, 2000).

The 11-*cis* isomers of the retinals (RAL1 and RAL2) have been well characterized as the visual pigment chromophores in the retinas of both vertebrate and invertebrate animals. The pineals and deep brains of lower vertebrates are extraocular photoreceptors involved in maintaining biological rhythms, and the retinals present in these organs are suggested to be photoreceptive pigment chromophores (Sun et al.,

1991; Masuda et al., 1994; Yoshikawa and Oishi, 1998). Okano et al. (2002) detected 11-*cis* RAL2, which appears to be a photoreceptive pigment chromophore in dermal melanophores, in the larval tail fins of *Xenopus laevis*. Kajiwara et al. (1990) proposed that the ganglion of the solitary ascidian, *Halocynthia roretzi*, was a photoreceptor that regulated gamete release, and suggested that RAL1 found in the ganglion was the chromophore of the photoreceptive pigment.

In addition to the photoreceptive function of retinals, RA is involved in pattern formation during vertebrate development. Other than in vertebrates, endogenous RAL1 and a homolog of retinoid X receptors have also been found in colonial (budding) ascidians; the RAL1 is suggested to be the precursor of RA, which plays a role regulating morphogenesis during bud growth (Kawamura et al., 1993; Kamimura et al., 2000). Katsuyama et al. (1995) reported that the exposing embryos to RA affected the embryonic development of *H. roretzi*.

RAL1 or both RAL1 and RAL2 are commonly present in the eggs of a variety of oviparous vertebrates (Plack et al., 1959; Plack and Kon, 1961). These egg retinals are suggested to be the precursors of functional retinoids, such as visual pigment chromophores (Azuma et al., 1988, 1990) and RA (Costaridis et al., 1996; Ang and Duester, 1999). Retinals in the eggs of amphibians and a teleost were shown to be bound to lipovitellin 1, a yolk protein, or its homologous proteins by a Schiff base linkage (Irie et al., 1991; Irie and Seki, 2002; Irie et al., in press), and were suggested to be the essential mode of retinoid storage (Irie and Seki, 2002).

However, the endogenous retinoids in eggs and embryos of chordate animals other than vertebrates have not been well characterized, although we made a preliminary examination of retinoids in ascidian eggs (Irie et al., in press). Therefore, to clarify the mode of retinoid storage in eggs of non-vertebrate chordates, we analyzed the retinoids in eggs laid by the solitary ascidian, *H. roretzi* (subphylum Urochordata). In the present study, we provide evidence that RAL1 in the ascidian eggs is practically only storage of retinoid and that the composition of isomers is different from that in vertebrate eggs.

2. Materials and Methods

2.1. *Animals and eggs*

We purchased adults of the solitary ascidian *Halocynthia roretzi*, which had been raised in Mutsu Bay (Aomori, Japan), and kept them in an aquarium at the Marine Biological Station of Tohoku University (Aomori, Japan). The eggs were laid in the station under the controlled light and temperature conditions described by Numakunai and Hoshino (1980).

Because ascidian eggs are covered by a chorion and two types of accessory cells (follicle cells and test cells) (Satoh, 1994), we dechorionated the unfertilized eggs by immersing them in filtered natural seawater containing 0.1% actinase E (Tokyo Chem. Co., Tokyo, Japan) and 1.5% sodium thioglycolate, adjusted to about pH 11 by dropwise addition of 1 N NaOH (Mita-Miyazawa et al., 1985; Mita-Miyazawa and Satoh, 1986), and gently shaking them for 15 to 20 min until the chorion was completely removed. The eggs were then rinsed several times with filtered natural seawater to decant off the dissociated accessory cells. The naked egg cells obtained were collected by manual centrifugation and stored in a deep freezer ((80 (C) until use.

2.2. *Extraction of retinoids*

The eggs were thawed, homogenized, and then diluted with approximately ten-times volume of 20 mM Tris-HCl buffer (pH 7.4) containing 0.1 M NaCl. The homogenate obtained was extracted with organic solvents by the formaldehyde method (Makino-Tasaka and Suzuki, 1986) or the oxime method (Suzuki and Makino-Tasaka, 1983) following our routine procedures (Seki et al., 1989). Briefly, 2 ml of 35% formaldehyde and 1 ml of methanol (formaldehyde method), or 0.1 ml of 2 M hydroxylamine hydrochloride (NH₂OH-HCl, freshly neutralized) and 1 ml of methanol (oxime method), were added to 1 ml of each sample. Samples were then extracted with dichloromethane and hexane. To confirm the Schiff base linkage, the homogenate

was extracted by the oxime method after treatment with sodium borohydride (NaBH_4) (Bownds and Wald, 1965).

To create RAL1 isomers in a photo-equilibrated state, an appropriate amount of standard all-*trans* RAL1 (a kind gift from Dr Y. Kito) was dissolved in ethanol and irradiated for 10 min under a fluorescent light. Then the sample was extracted by the oxime method for the following analysis.

2.3. Analysis of retinoids and quantification of protein

High performance liquid chromatography (HPLC) was performed principally by the method described in a previous report (Irie and Seki, 2002). The HPLC system (Hitachi 655, Hitachi Ltd., Tokyo, Japan) was equipped with a 6 (150 mm column of 3((m silica gel (YMC-PACK-A-012 S-3 SIL, Yamamura Chemical laboratories Co. Ltd., Japan), with a 4 (50 mm precolumn, and was used at a flow rate of 2 ml/min. Absorbances of dual wavelengths (330 and 350 nm, or 330 and 360 nm) were monitored with UV/Visible detectors (Jasco 875-UV and UV-970; Jasco Corp., Tokyo, Japan) and recorded with an integrator (Labchart 80; System Instrument Co. Ltd., Tokyo, Japan). The eluent was 5% *tert*-butylmethyl ether, 0.04% ethanol and 25% benzene in *n*-hexane. Because retinylester (RE) isomers elute out in the void fraction under this eluent condition, the fraction (retention time for 1.5-3 min) was collected, saponified with ethanolic KOH (Bridges and Alvarez, 1982), and rechromatographed under the same conditions as described above. RE isomers are detected as retinol (ROL) isomers, the products of the saponification, on the second HPLC.

RAL1 isomers elute out in a retention time of 3-6 min under the HPLC conditions. To confirm the presence of RAL1 isomers in the formaldehyde method extract, the 3-6 min fraction was collected, treated with $\text{NH}_2\text{OH}\cdot\text{HCl}$ to convert the RAL1 to RAL1-oxime, or with NaBH_4 to reduce the RAL1 to ROL, and rechromatographed under the same conditions. The RAL1 isomers and the derivatives were identified on the basis of retention time and the ratio of absorbance at 330 and

360 nm (RAL1), or 330 and 350 nm (RAL1-oxime and ROL) in comparison with standard retinoids. The extraction and analyses of retinoids were performed under dim red light.

The protein content of the homogenate was measured by Lowry's method (Lowry et al., 1951) with bovine serum albumin as the standard protein.

2.4. Gel chromatography and polyacrylamide gel electrophoresis

The egg homogenate was centrifuged at 13 000 (g for 20 min, and the supernatant obtained was applied to a Sephacryl S-300 HR column (2.5 (86 cm) equilibrated with 20 mM Tris-HCl buffer containing 0.1 M NaCl. The eluent was collected in 5-ml fractions. After the gel chromatography, absorbances at 280 and 440 nm of each fraction were measured. The peak fractions at 280 nm absorption were extracted by using the oxime method and analyzed by HPLC, and the proteins in the fractions were analyzed by electrophoresis.

Sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE) was performed principally according to the procedure described by Laemmli (1970) using 1-mm thick 10% slab gels. The molecular weight marker used for SDS-PAGE was a protein mixture including the following proteins: phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa) and trypsin and inhibitor (21.5 kDa). After the electrophoresis, the gels were stained with 0.1% Coomassie Brilliant Blue R-250 (CBB) in 45% methanol and 10% acetic acid, and then destained with 20% methanol and 10% acetic acid.

3. Results

3.1. Confirmation of the presence of RAL1 isomers

In the HPLC chromatogram of the extract (formaldehyde method) from the ascidian egg homogenate (Fig. 1A₁), five peaks were detected in the retention time of 3 × 6 min. Of these five peaks, four corresponded to those of the isomers (9-*cis*, 11-*cis*, 13-*cis* and all-*trans*) of the standard RAL1 (Fig. 1A₂). The 3-6 min fraction

was collected, treated with $\text{NH}_2\text{OH}\cdot\text{HCl}$ to convert the RAL1 to retinaloxime, and rechromatographed under the same conditions (Fig. 1B₁). The peaks on the second chromatogram corresponded to the peaks of the RAL1-oxime isomers derived from the standard RAL1 isomers treated by the same procedure (Fig. 1B₂). When the 3-6 min fraction was treated with NaBH_4 to reduce RAL1 to ROL and rechromatographed (Fig. 1C₁), the peaks corresponding to standard ROL isomers derived from the standard RAL1 isomers after the same treatment (Fig. 1C₂) were detected. These results are indubitable evidence of the presence of the RAL1 isomers in the ascidian eggs.

3.2. Concentration and isomer composition of retinal

In the HPLC chromatogram of the extract (oxime method) of standard all-*trans* RAL1 after photo-equilibration in ethanol (Fig. 2A), several *cis* isomers created by photoisomerization were observed. In the HPLC analysis of the extract (oxime method) from the egg homogenate (Fig. 2B), the four RAL1-oxime isomers were clearly detected. These two chromatograms were similar to each other, although the ratio of the isomers was somewhat different. Table 1 summarizes the concentrations and the isomer compositions of RAL1 in the eggs from 6 different animals. Quantitative analyses revealed that the concentration of the sum of the RAL1 isomers ranged from 25.9 to 40.1 ng/mg of protein. The RAL1 isomers detected were all-*trans* (50.9%), 9-*cis* (6.8%), 11-*cis* (20.4%) and 13-*cis* (21.9%).

3.3. Retinoid composition and the state in which RAL1 exists

The four isomers of RAL1-oxime were detected, whereas no RAL2-oxime isomers were found (Figs. 1 and 2B). A small peak with the retention time close to that of all-*trans* ROL (around 19 min) was observed (Fig. 2B). To analyze the RE, the solvent front fraction (1.5-3 min) was collected and rechromatographed after saponification, (Fig 2C). A small peak around 19 min was also found on the second chromatogram. However, the peaks were too small to identify as all-*trans* ROL. Even

if the small peaks were quantified as all-*trans* ROL, RAL1 was the major retinoid component (>> 99%), and the amounts of ROL and RE were less than 0.1% and 0.2%, respectively.

The egg homogenate was extracted by the oxime method after treatment with NaBH₄ and then analyzed (Fig. 2D). None of the RAL1 isomers were detected in the extract, and no increase in the amount of ROL was observed after this treatment. This suggests the absence of RAL1 with unprotected aldehyde, which would have been reduced to ROL by the NaBH₄ treatment (Bownds and Wald, 1965).

3.4. Identification of retinal binding protein

The egg homogenate was centrifuged and the supernatant obtained was applied to a Sephacryl S-300 HR column (Fig. 3). The substances with absorption at 280 nm were separated into several peaks. The fractions of the first peak were turbid. The first and second peaks (I and II in Fig. 3) assumed a yellow color, which was represented by the absorption at 450 nm, and showed a visible absorption spectrum similar to carotenoids (spectral data not shown). The fraction of each peak (I to VI) was extracted by the oxime method and analyzed by HPLC (Fig. 4). Retinaloxime isomers were detected in the first and second peaks, but not in any other peak. These findings indicate that the fractions of peaks I and II contained the retinal binding protein.

On the SDS-PAGE (Fig. 5), several bands were present in the first and second peaks of the gel chromatography (peaks I and II in Fig. 3). The electrophoretic patterns of the two peaks were almost identical, although the protein concentrations were different. No bands were observed after the staining with Coomassie Brilliant Blue R250 in the other peaks of the gel chromatography (peaks III to VI in Fig. 3).

Discussion

It is evident from Figures 1 and 2 that RAL1 is the almost exclusive component of retinoid in the ascidian eggs. RAL1 or both RAL1 and RAL2 are commonly present in the eggs of oviparous vertebrates (Plack et al., 1959; Plack and Kon, 1961; Seki et

al., 1987; Irie et al., 1991; Costaridis et al., 1996; Rønnestad et al., 1998; Irie and Seki, 2002). In teleost eggs, retinals and retinylesters were localized in the aqueous and lipid parts, respectively, and RAL1 was the only retinoid found in teleost eggs lacking lipid bodies (Irie and Seki, 2002). The egg retinal of *Oncorhynchus keta* (chum salmon), an anadromous fish, was shown to be composed of both RAL1 and RAL2, whereas RAL1 was the only retinal in the eggs of marine teleosts (Irie and Seki, 2002). The retinoid composition in the ascidian eggs, which do not contain lipid bodies, closely resembled that in marine teleost eggs without lipid bodies.

The amount of RAL1 in the eggs as shown in Table 1 (30.6 ng/mg of protein on average) is different from the result of our previous analysis (17.0 ng/mg of protein; Irie et al., in press). The difference is evidently due to whether the eggs were dechorionated; only dechorionated eggs were used in this experiment, whereas dechoriation was not performed in our previous analysis. On the basis of this difference, RAL1 appears to be localized in egg cells, rather than in accessory cells, although we did not analyze the retinoids in the accessory cells, because the large amount of contamination from egg cell fragments could not be removed from the accessory cell fraction.

Four isomers of RAL1 were detected in the extracts of the ascidian eggs (Table 1) with the percentages of RAL1 isomers being quite different from those found in vertebrate eggs; in vertebrate eggs, retinal is present mostly in the all-*trans* form (Plack et al., 1959; Plack and Kon, 1961; Seki et al., 1987; Irie et al., 1991; Rønnestad et al., 1998; Irie and Seki, 2002). It is known that 11-*cis* retinals play a role in the chromophores of photoreceptive pigments involved in both ocular and extraocular photoreception in a variety of animals. In the ascidians, the cerebral ganglia in *H. roretzi* (Kajiwara et al., 1990; Ohkuma et al., 2000) and *Ciona savignyi* (Tsutsui and Oka, 2000) were suggested to be the photoreceptors, and RAL1 in the ganglion of *H. roretzi* appears to be the photoreceptive pigment chromophore (Kajiwara et al., 1990; Ohkuma and Tsuda, 2000). In the larval ocellus of *H. roretzi*, the retinal protein appears to play a role in photoreception (Nakagawa et al., 1999). However, the

presence of 11-*cis* RAL1 is not evidence that RAL1 is involved in a photoreceptive function in the eggs. The isomer composition in the eggs was similar to that of the standard retinal dissolved in ethanol after photo-equilibration (Figs. 2A, 2B). The unique isomer composition, including the presence of 11-*cis* RAL1, might be due to photo-equilibration occurring in the ascidian eggs. To the best of our knowledge, no evidence has been presented showing that the egg retinal is itself involved in any physiologically dynamic function.

On the gel chromatography, the egg substances with absorption at 280 nm were separated into several peaks (Fig. 3). Because the first peak was turbid and the SDS-PAGE pattern was identical to that of the second peak, the protein in the first peak appears to be the protein in the second peak in an aggregated state. The yellow pigments that bind to the protein in these peaks appear to be xanthophylls, because of the absorption spectrum and the fact that the pigments did not elute out under the eluent conditions (for small polar molecules) of the HPLC analysis.

RAL1 was shown to be bound to the protein in the first and second peaks of the gel chromatography (Fig. 4). The SDS-PAGE (Fig. 5) revealed that the retinal binding protein consisted of several polypeptide chains. The SDS-PAGE pattern of the protein complex was similar to that of egg yolk proteins of amphibians (Selman and Wallace, 1989; Irie et al., 1991; Irie et al., in press) and teleosts (Hara and Hirai, 1978; Tyler, 1993; Hara et al., 1993; Irie and Seki, 2002; Irie et al., in press), and the four bands with molecular weight around 90 000 (Fig. 5) corresponded to amphibian lipovitellin 1. We previously reported that the egg retinal is bound to a single polypeptide, lipovitellin 1 or its homologous protein, in the eggs of *X. laevis* (clawed toad; Irie et al., 1991), *O. keta* (chum salmon; Irie and Seki, 2002) and *Bufo japonicus* (Japanese toad; Irie et al., in press). In *X. laevis*, egg retinals are evidently derived from hepatic RE, because retinals were bound to vitellogenin, the precursor of egg yolk protein synthesized in the liver, in the blood of estrogen-treated male toads (Azuma et al., 1993). In the ascidians, however, the origin of the egg retinal and yolk

proteins has not been clarified, although vesicles derived from the Golgi complex in oocytes appear to play a role in egg yolk formation (Kessel, 1966).

No isomer of RAL1 in the ascidian eggs was extracted with organic solvents after the NaBH₄ treatment (Fig. 2D). This result indicates that RAL1 is bound *via* a Schiff base linkage. We previously reported that egg retinals in amphibians and teleosts are bound to yolk proteins *via* a Schiff base linkage (Irie et al., 1991; Irie and Seki, 2002). Our results presented above suggest that the retinal-protein complex accumulates in eggs by a mechanism common to all oviparous chordates. As we have pointed out in a previous paper, the effect of the Schiff base linkage, which protects the reactive aldehyde group of retinals, would negate the toxicity of the aldehyde, and would enable the accumulation of a large amount of retinals in eggs (Irie et al., in press).

The presence of SDS was necessary for a complete reaction of NaBH₄ with teleost egg yolk, because the hydrophobicity of the retinal binding site appears to affect the reactivity of NaBH₄; the hydrophobic submolecular environment appears to interrupt access of NaBH₄ to the binding site without the presence of SDS (Irie and Seki, 2002). However, the environment of the retinal binding site in ascidian eggs appears to be more hydrophilic than that in teleost eggs, because the NaBH₄ was effective without the presence of any protein denaturants.

RA has an important physiological function in axial patterning of vertebrates during development (Means and Gudas, 1995). The biological significance of RA in the closest relatives of vertebrates, the ascidians (subphylum Urochordata) and amphioxus (subphylum Cephalochordata), has been examined in several studies. In the colonial ascidian, *Polyandrocarpa misakiensis*, treatment with RA induced a secondary axis in developing buds (Hara et al., 1992) and RA derived from the endogenous RAL1 was suggested to trigger morphallactic development of buds (Kawamura et al., 1993). In *P. misakiensis*, an RA receptor (RAR) homolog (Hisata et al., 1998) and retinoid X receptors (RXR) (Kamimura et al., 2000) were detected, suggesting that RA plays a role in gene regulation in the budding process. Treatment

with RA perturbed morphogenesis (Hinman and Degnan, 1998; Katsuyama and Saiga, 1998) and HOX gene expression (Katsuyama et al., 1995; Katsuyama and Saiga, 1998; Hinman and Degnan, 2000) during embryonic and postembryonic development of the ascidians, *Herdmania curvata* and *Halocynthia roretzi*. RA also affected the HOX gene expression of the amphioxus, *Branchiostoma floridae* (Holland and Holland, 1996). The involvement of RA in the formation of the anterior-posterior axis and in HOX gene expression is evidently a characteristic shared by the subphyla of the phylum Chordata (Holland and Holland, 1996; Shimeld, 1996). These studies indicate that RA functions in the non-vertebrate chordates during the developmental process. Thus, it is not unexpected that some retinoid, which appears to be the precursor of RA, is stored in the eggs until the animals begin to feed.

The present research demonstrates that RAL1 is practically the only form of retinoid stored in ascidian eggs; the amount of the other retinoids is negligible. The storage of retinal in the ascidian eggs would be useful to provide biosynthesis of RA, as well as the photoreceptive pigment in the ocellus, during development. We have previously reported that retinals are the major and essential mode of retinoid storage in the eggs of amphibians and teleosts (Irie and Seki, 2002). Evidently, the mode of retinoid storage in the eggs of the urochordate is the same as that of the anamniote vertebrates, agreeing with our preliminary assumption (Irie et al., in press). The stored RAL in the eggs is certainly converted to RA by the metabolic enzyme, RAL dehydrogenase, which would be synthesized during development. The enzyme was shown to be first expressed at the neurula stage of *X. laevis* embryos (Ang and Duester, 1999) and at the primitive streak stage of mouse embryos (Ang and Duester, 1997). Our observations strongly suggest that the accumulation of retinals as retinoids stored in eggs originated before the divergence into subphyla during chordate evolution, although the expansion of the family of enzymes that catalyze the first step of the retinoid signaling pathways probably occurred after the cephalochordate-vertebrate split (Dalfó et al., 2001; Cañestro et al., 2002).

Acknowledgments

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Table 1

Retinal concentration and isomer composition in *Halocynthia roretzi* eggs collected from different individuals, extracted by the oxime method.

Animal	Concentration (ng/mgP)	Isomer composition (%)			
		<i>all-trans</i>	<i>9-cis</i>	<i>11-cis</i>	<i>13-cis</i>
A	Not measured	56.1	6.9	20.8	16.2
B	31.8	50.9	6.5	18.4	24.1
C	28.0	49.5	7.4	20.0	23.1
D	40.1	48.4	6.3	19.1	26.2
E	27.9	48.5	7.0	22.6	21.9
F	25.9	52.0	6.5	21.5	20.0
Average	30.6	50.9	6.8	20.4	21.9

mgP, mg of protein measured by Lowry's method.

Captions to Figures

Fig. 1. Confirmation of the occurrence of retinal isomers in the eggs of the ascidian *Halocynthia roretzi*. The egg homogenate was extracted with organic solvents by the formaldehyde method (see text). The egg extract (A_1) and a mixture of standard retinal isomers (A_2) were analyzed by HPLC. The 3 - 6 min fraction of each HPLC was collected (between the broken lines), treated with hydroxylamine-hydrochloride to convert retinal isomers to retinaloxime isomers and rechromatographed under the same conditions (B_1 and B_2). Then, each of the first HPLCs was performed again (A_1 and A_2), the 3 - 6 min fraction was reduced with sodium borohydride to convert retinal isomers to retinol isomers and rechromatographed (C_1 and C_2). The scale bar shows the absorbances at 360 nm (A), 350 nm (B) or 330 nm (C).

1, 13-*cis* retinal; 2, 11-*cis* retinal; 3, 9-*cis* retinal; 4, all-*trans* retinal; a and a', *syn* and *anti* 11-*cis* retinaloxime; b and b', *syn* and *anti* all-*trans* retinaloxime; c and c', *syn* and *anti* 9-*cis* retinaloxime; d and d', *syn* and *anti* 13-*cis* retinaloxime; e, 11-*cis* and 13-*cis* retinol; f, 9-*cis* retinol; g, all-*trans* retinol; *, unidentified substance.

Fig. 2. HPLC analyses of the standard retinaloxime isomers and the egg extract. The standard retinal (initial form was all-*trans*) solubilized in ethanol after light equilibration (A) and the ascidian egg homogenate (B) were extracted by the oxime method. As retinylesters elute out in the void fraction under the eluent conditions, this fraction (1.5-3 min retention time) of analysis B was collected, saponified to convert retinylesters to retinols and then rechromatographed under the same conditions (C). Chromatogram D shows the analysis of the egg extract after the egg homogenate was treated with sodium borohydride. The scale bars show the absorbances at 350 nm (A, B and D) or 330 nm (C).

a and a', *syn* and *anti* all-*trans* retinaloxime; b and b', *syn* and *anti* 11-*cis* retinaloxime; c and c', *syn* and *anti* 9-*cis* retinaloxime; d and d', *syn* and *anti* 13-*cis* retinaloxime.

Fig. 3. Gel chromatography of the supernatant of the egg homogenate, using a Sephacryl S-300 HR column (2.5 × 86 cm) equilibrated with 20 mM Tris-HCl buffer containing 0.1 M NaCl. Absorbances at 280 nm (○) and 440 nm (●) of each fraction were measured after the chromatography. The 440-nm absorption represents yellow pigments, which we presumed to be xanthophylls. The Roman numerals designate the peaks of the 280-nm absorption.

Fig. 4. HPLC analyses of the extracts (oxime method) from the peak fractions of the gel chromatography (Fig. 3). A to F represent peaks I to VI (fractions 45, 51, 67, 75, 97 and 107) of Fig. 3.

a and a', *syn* and *anti* 11-*cis* retinaloxime; b and b', *syn* and *anti* all-*trans* retinaloxime; c and c', *syn* and *anti* 9-*cis* retinaloxime; d and d', *syn* and *anti* 13-*cis* retinaloxime.

Fig. 5. SDS-PAGE of the fractions of the gel chromatography (Fig. 3).

Lane A, peak I (fraction 45); Lane B, peak II (fraction 51); C, Molecular weight markers.

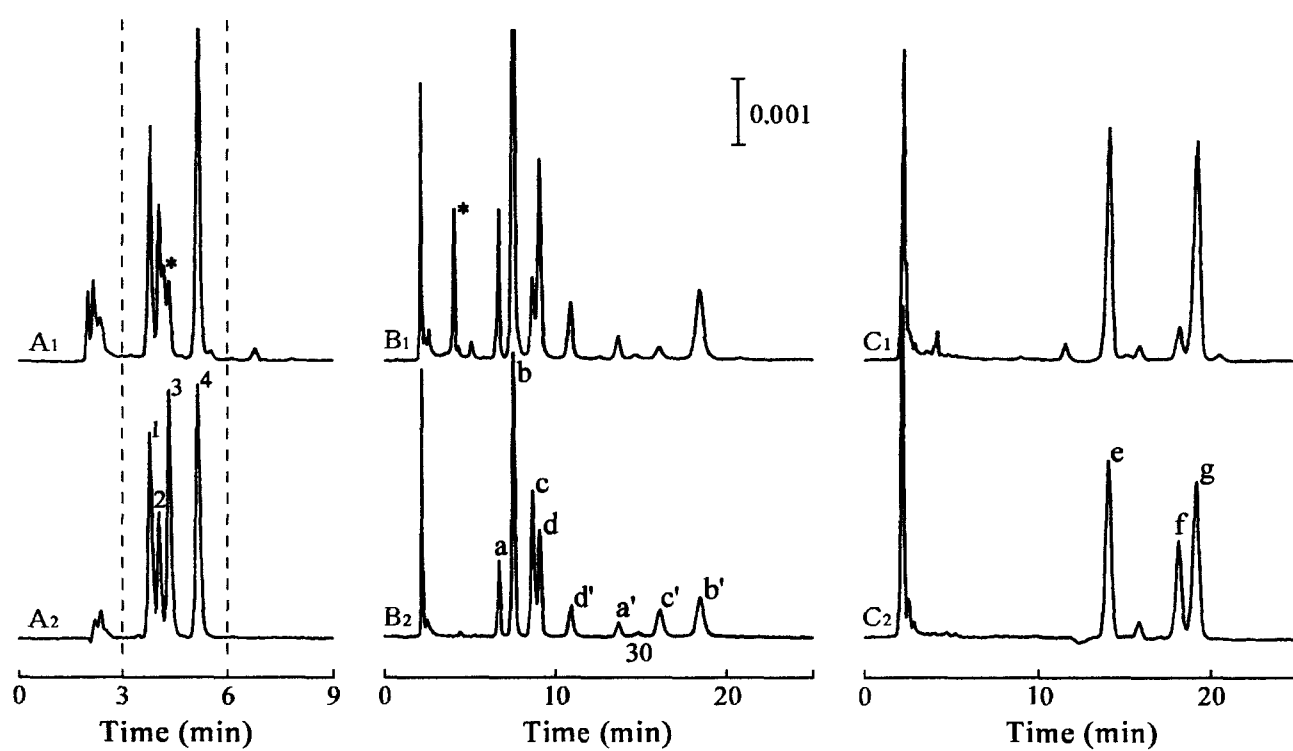


Fig. 1

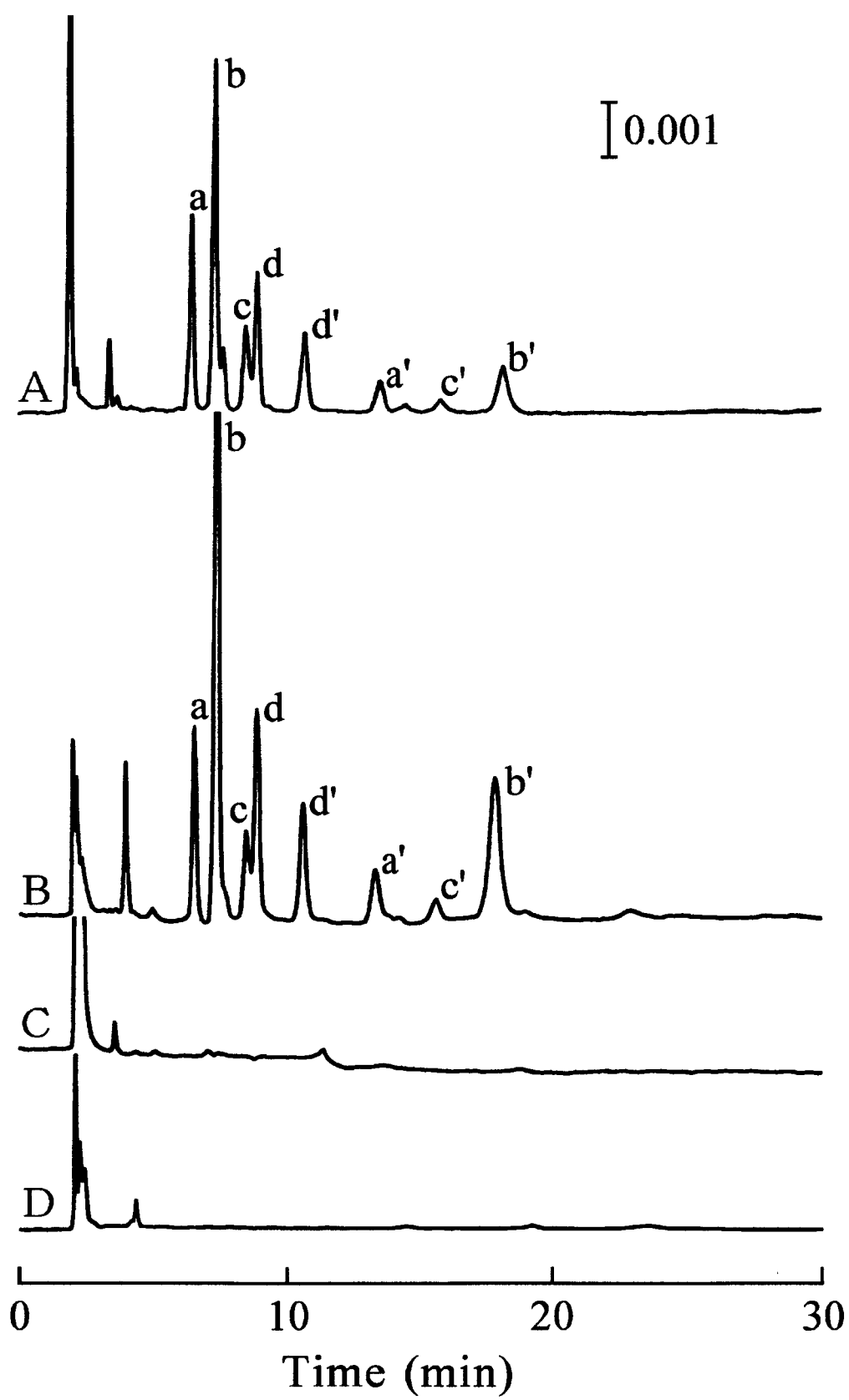


Fig. 2

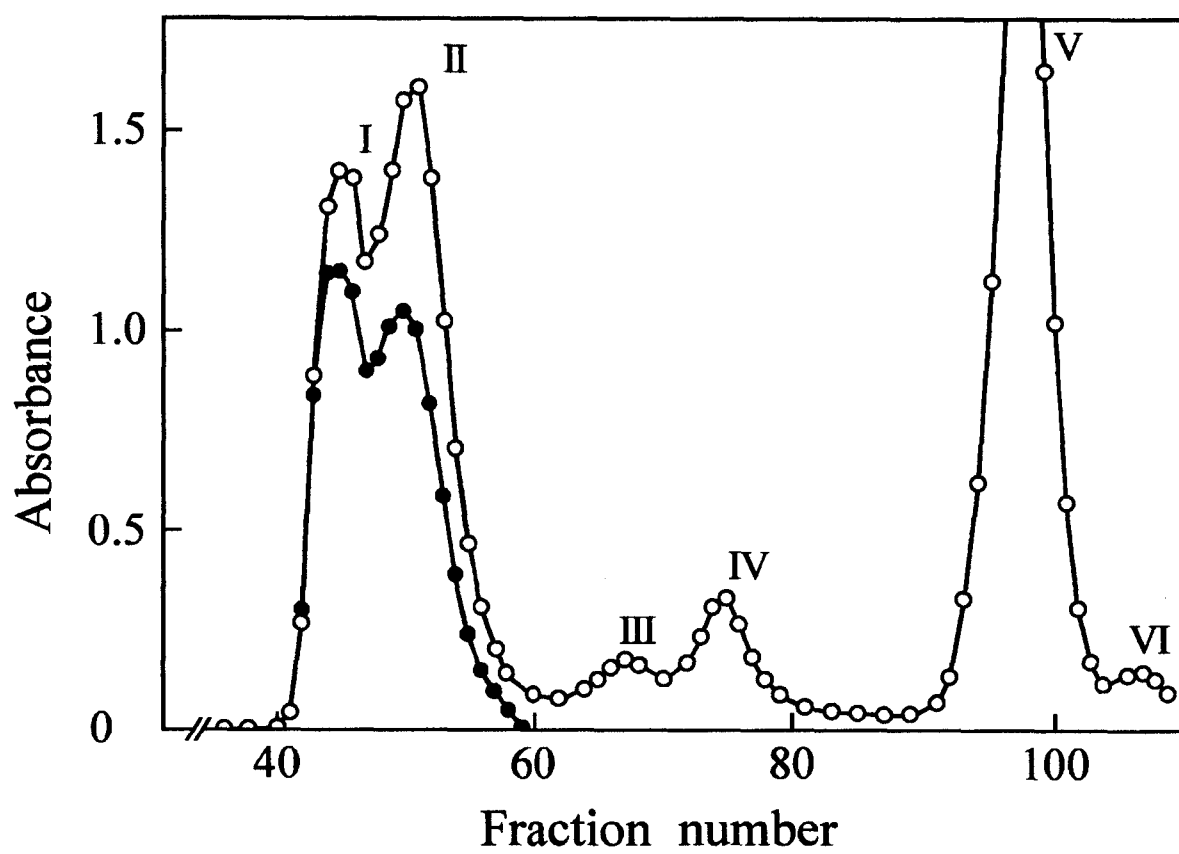


Fig. 3

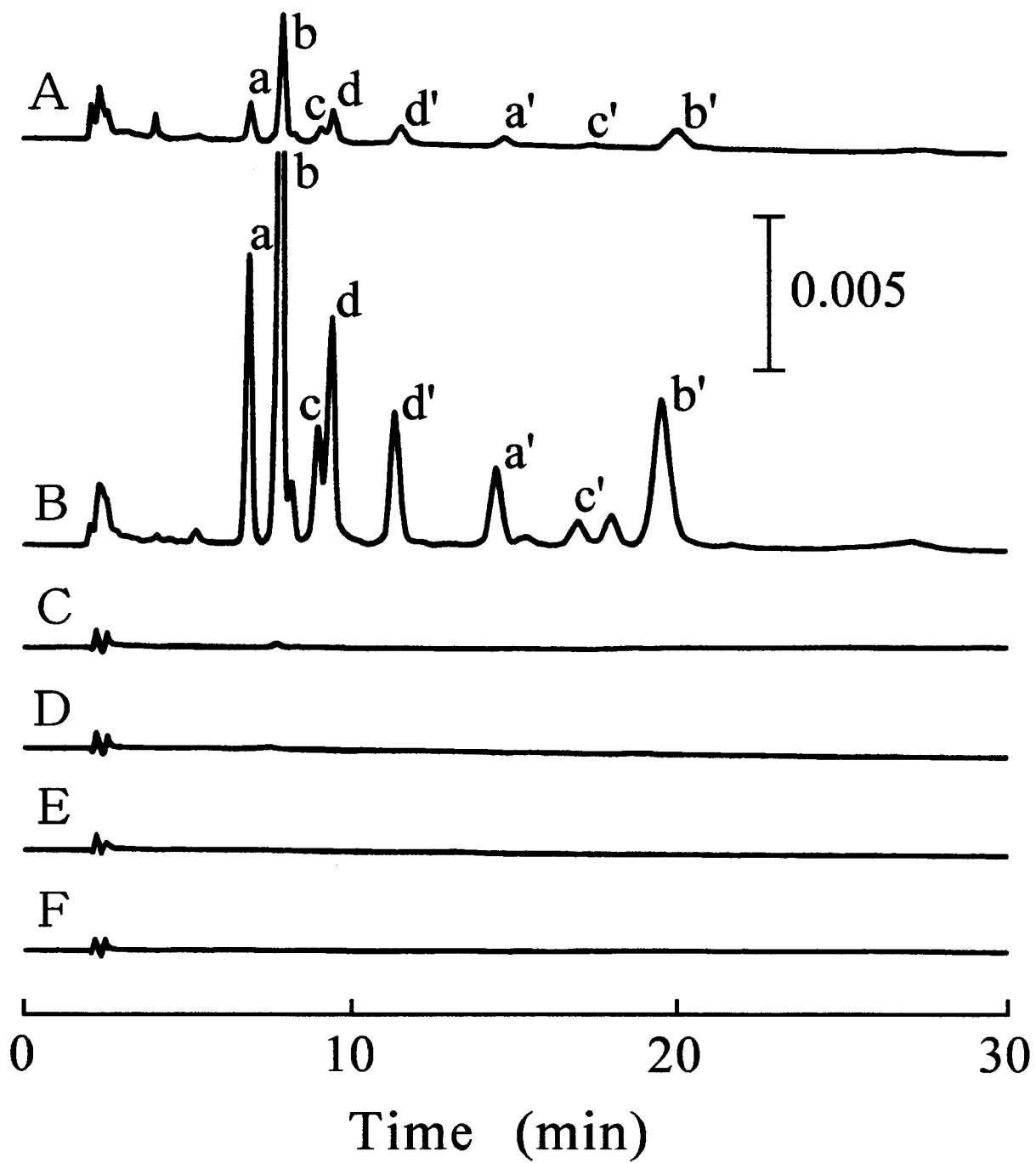


Fig. 4

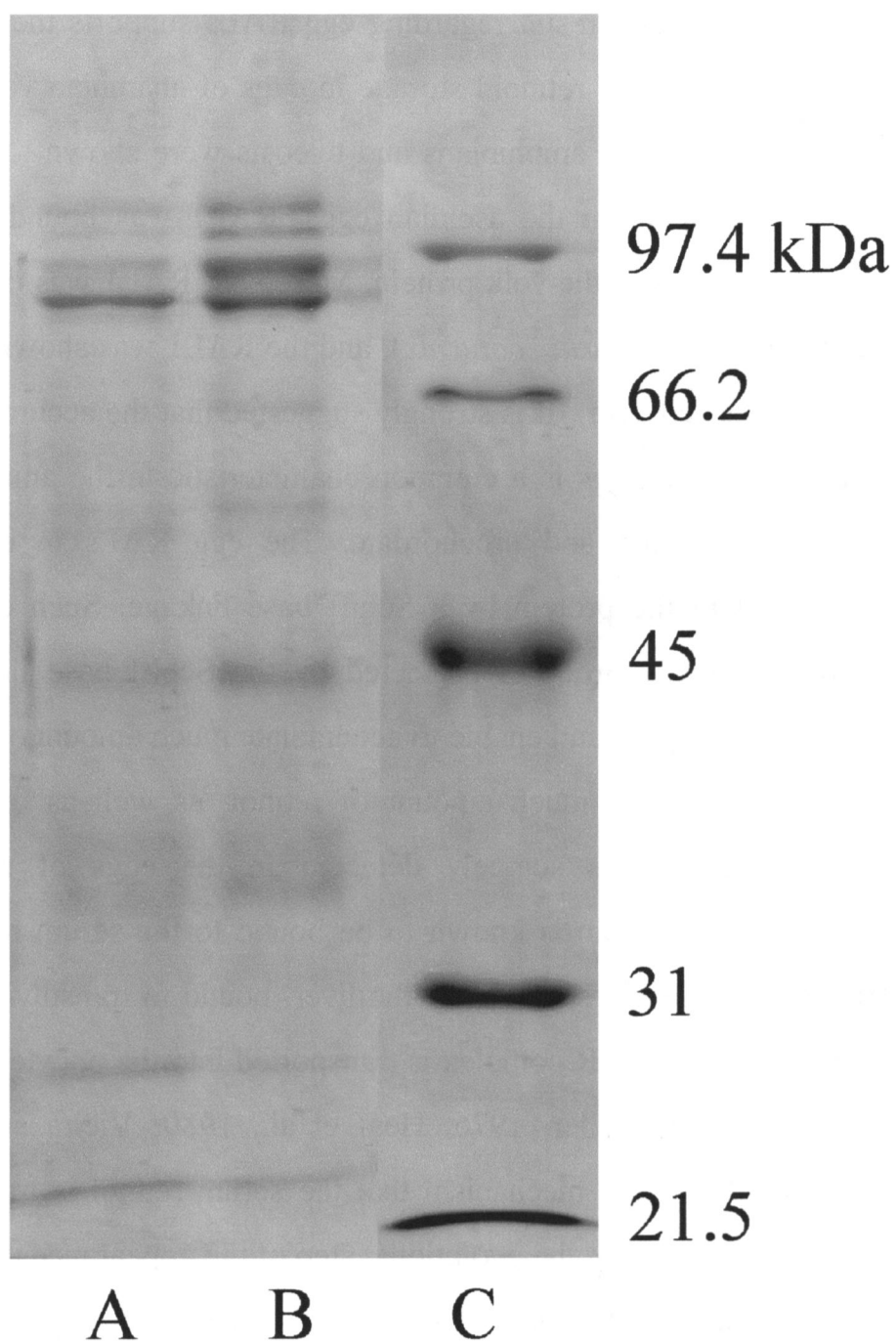


Fig. 5

VII. General Discussion

The every experimental result regarding egg RALs supports the idea that the RALs are the essential mode of retinoid storage in eggs of anamniotic vertebrates and urochordates. The egg RALs in amphibians and teleosts were shown to be bound to lipovitellin 1, a yolk protein. In the ascidian eggs, RAL1 was bound to a protein complex, which appeared to be the yolk protein. Moreover, RAL1 was detected in the eggs of the Japanese quail (*Coturnix coturnix*), and the RAL1 was shown to be bound to lipovitellin 1 (unpublished data). It is strongly suggested that the accumulation of the retinal-protein complex into eggs is a common characteristic in the animals of every class of oviparous vertebrate and urochordate. The egg RALs in every species examined were bound to the protein by a Schiff base linkage. Such existing state, which the reactive aldehyde group is protected by the Schiff base linkage, would negate the toxicity of aldehyde, and enable to accumulate much amount of RALs.

In hen and quail eggs, much amount of retinol, as well as of RAL1, was detected, although retinol was scarcely detected in the eggs of the anamniotic vertebrates. Retinol in the serum is known to be bound to the serum retinol binding protein (RBP). The holoRBP is secreted by the liver, bound to transthyretin (TTR) in the serum, and the holoRBP-TTR complex is transported into the oocytes mediated by the specific TTR receptor (Heller, 1976; Heaf et al., 1980; Vieira and Schneider, 1993; Vieira et al., 1995). The mechanism that the serum retinol is transported into oocytes would have originated at the evolutionary step of the appearance of amniote.

In the blood plasma of male *X. laevis*, RALs were induced by the estrogen administration, and the RALs were always detected in the fraction of vitellogenin, the precursor of egg yolk proteins, which is known to be synthesized in the liver. As described above, the egg RALs are bound to yolk proteins. The egg RALs are probably derived from retinyl esters in the maternal liver, and transported in vitellogenin-bound form to the eggs. In mammals, it is known that the liver does not

synthesize vitellogenin even in the presence of estrogen. Presumably the eggs of mammals do not accumulate RALs.

The storage mode of retinoids would change during embryonic development in oviparous vertebrates, because it is known that, in general, hepatic retinyl ester is the stored retinoid in adult. In the adult ascidian, however, RAL1 was the major retinoid, and the RAL1 was localized mostly in the gonads and the body wall muscle (Irie et al., in preparation). Retinol and retinyl ester were scarcely detected in every organ examined (gonads, body wall muscle, gill, hemolymph cells and plasma proteins). RAL1 is certainly the storage mode of retinoid throughout the life cycle of the ascidian. On the basis of these results, it is assumed that the ascidian does not metabolize retinol. This assumption is supported by the results of several studies. In the ascidian (*Ciona intestinalis*) and amphioxus (*Blanchiostoma floridae* and *B. lanceolatum*; cephalochordate), no medium-chain dehydrogenase/reductases-alcohol dehydrogenase (ADH) other than ADH class 3 (glutathione-dependent formaldehyde dehydrogenase) were found in the amphioxus, indicating that no retinol metabolizing enzymes are present in the urochordate and cephalochordate (Cañestro et al., 2000, 2002; Dalfó et al., 2001). In the recent paper, however, the same group also reported that amphioxus contained retinol as well as retinal, and that developmental effects of retinol treatment were comparable to those reported for retinoic acid (Dalfó et al., 2002). These results suggest that amphioxus metabolizes endogenous retinol to retinoic acid by the common oxidative pathway in chordates. This discrepancy in the retinoid metabolism of cephalochordate has not yet been solved.

Animals are, in general, unable to synthesize retinoids *de novo*. Retinoids are derived from the oxidative cleavage of carotenoids. β -caroten 15,15'-dioxygenase catalyzes the conversion of β -caroten into two molecules of retinal. In vertebrates, retinal, the first product of the carotenoid cleavage, is converted to retinol at the next enzymatic step. Retinol or its ester form are most abundant as the storage retinoid. In the retinoid signaling pathways, retinol is converted to retinoic acid *via* retinal. The metabolic pathway of retinoids is summarized in Fig. A.

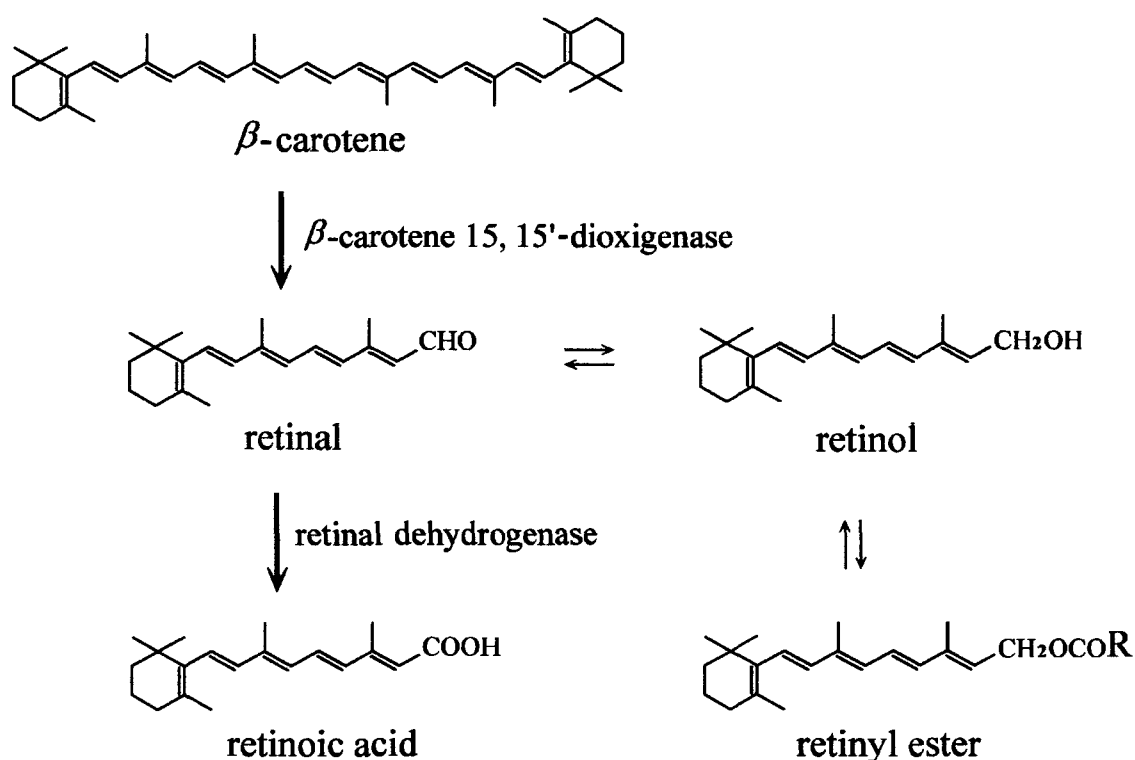


Fig. A. The main steps in retinoid metabolism of animals.

Other than chordate animals, it is reported that treatment of retinoic acid did not affect the anterior/posterior axis formation in sea urchin embryos, although the treatment of exogenous retinoic acid caused a developmental delay and appearance of some dismorphic embryos (Sciarrino and Matranga, 1995; Sconzo et al., 1996; Kuno et al., 1999). It is not evident that endogenous retinoids are involved in any physiological function in echinoderms. On HPLC analyses, any of the retinoid were not detectable in the eggs of the starfish (unpublished data) and the sea urchin (Tsushima, personal communication), but much amount of carotenoids were present, assuming that echinoderms do not synthesize retinoids. It is likely that the β -caroten 15,15'-dioxygenase was acquired at the evolutionary step of appearance of the chordate during the evolution of Deuterostomia. In the ascidian, as described above, RAL1 was the practically only retinoid not only in eggs but also in adult organs. The mechanism that retinal, the first product of carotenoid cleavage, is stored as the protein bound

form appears to be the beginning of the retinoid storage in the chordate evolution. On the basis of the above argument, the probable assumption of the evolutionary process of retinoid storage and metabolism is summarized in Fig. B.

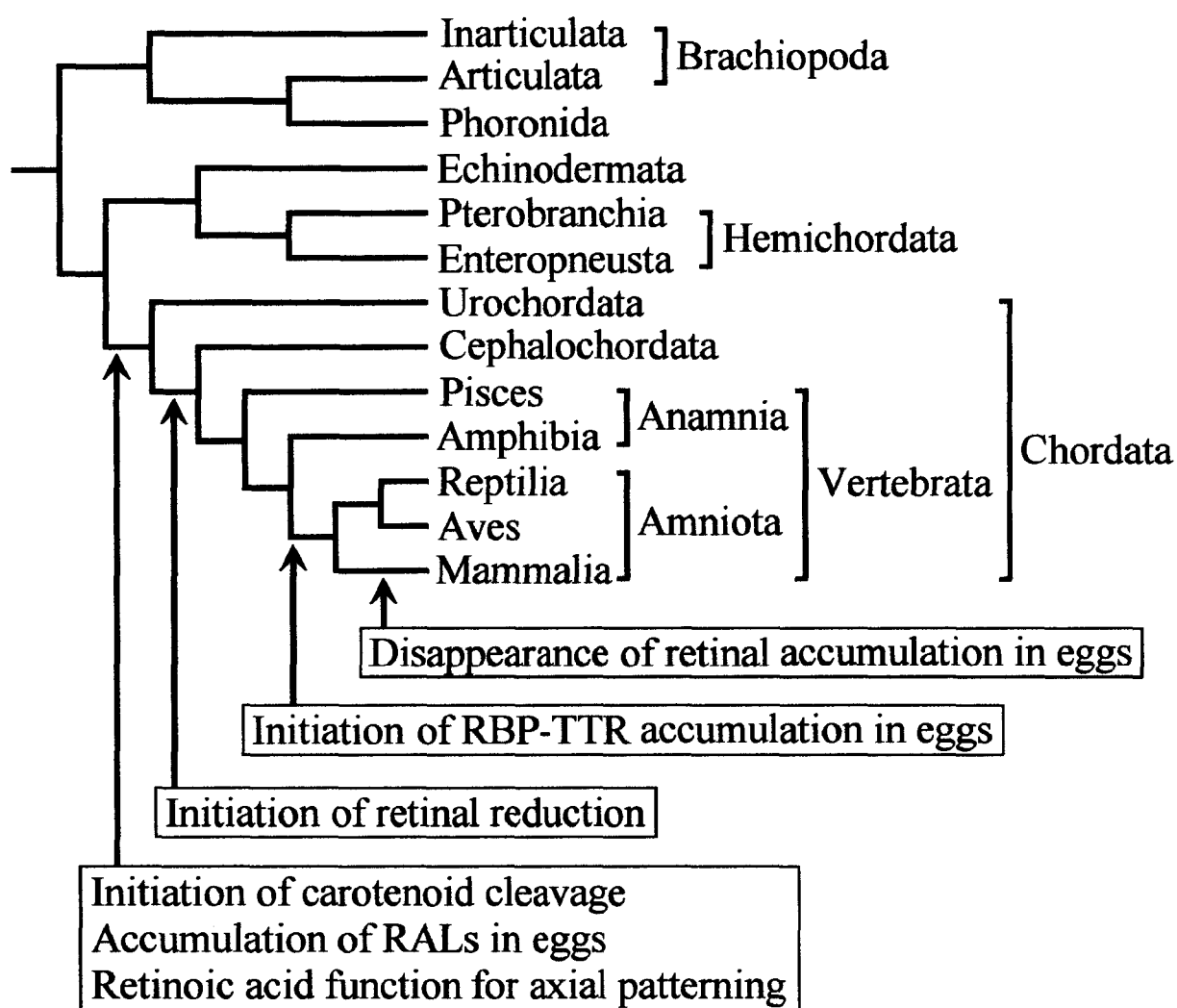


Fig. B. Probable scheme of the evolutionary process of retinoid storage and metabolism in Deuterostomia. (Original diagram: Zrzavý et al., 1998; modified)

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X. List of publications

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