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The Retinal and 3-Dehydroretinal in *Xenopus laevis* Eggs are Bound to Lipovitellin 1 by a Schiff Base Linkage

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ABSTRACT—The egg of *Xenopus laevis* contains both retinal (ret_1) and 3-dehydroretinal (ret_2). The sum of the two retinals and the ratio of the ret_1 to ret_2 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 ret_1 or ret_2 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.

INTRODUCTION

Extraoccular retinal was found for the first time in the eggs of herring by Plack *et al.* [1]. In subsequent papers [2–4], the occurrence of retinal, or both retinal and 3-dehydroretinal, in the megalecithal eggs of vertebrates was shown to be common, while no retinal was found in the eggs or ovaries of the invertebrates investigated [4]. Plack *et al.* [5] 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.* [6] 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 [7]. 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 [1, 2] that the retinal in herreing 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.* [8] found that *Xenopus laevis* eggs contain both retinal and 3-dehydroretinal 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),

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and the origin of the egg retinals is discussed in terms of this and previous [5–7] results concerning egg retinals.

A preliminary account was presented at the 60th Annual Meeting of the Zoological Society of Japan in 1989 [9].

MATERIALS AND METHODS

Animals and eggs

Xenopus laevis were supplied by Dr. H. Takasaki or purchased from Hamamatsu Seibutsu Kyozai 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 [10], 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 [11] 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 20 mM Tris-HCl buffer at pH 7.4.

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 (Physcotron NS-50; Nichion Irikakikai Seisakusyo Co. Ltd., Chiba, Japan). The homogenate was centrifuged at 5°C with a centrifuge (Kubota KR-180B, Tokyo) at 13,000×*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 dodecyl sulfate (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 [12, 13]. 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, 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.

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. 12, 13]. 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 fluorophotometer (Hitachi 650–10 M, Hitachi, Japan).

For HPLC analysis, retinals in the egg(s) or fractionated samples were extracted by the oxime method [14] following our routine procedures [15]. The eluent was 8% diethylether-0.08% ethanol in

n-hexane [8], or 5% *tert*-butylmethylether-0.04% ethanol-25% benzene in *n*-hexane: both of which give good separation of the *syn* oximes of retinal (ret_1) and 3-dehydroretinal (ret_2). A HPLC-system (Hitachi 655, Hitachi, Japan) was equipped with a 6×150 mm column of 3 μm 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. Absorbance at 350 nm was monitored with a UV-detector (Hitachi 638–41, Hitachi, Japan). Quantities of ret_1 oxime and ret_2 oxime were estimated from the peak areas of the *syn* isomers, using equimolar mixtures of standard oximes [16] supplied by Dr. T. Suzuki.

Quantification of the lipovitellin 1

Two methods, the Lowry method [17] 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 [18] for LV1 and lipovitellin 2 (LV2), and the molecular weight 150,000 dalton [18, 19] for the sum of the molecular weights of the two proteins.

SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli [20] 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 $AlCl_3$, 25% isopropyl alcohol and 10% acetic acid [19] to visualize phosphoprotein bands [21]. The following proteins were employed as molecular weight markers; Myosin (200 KD), β-galactosidase (116 KD), phosphorylase b (97.4 KD), bovine serum albumin (66.2 KD), ovalbumin (43 KD), trypsinogen (24 KD), β-lactoglobulin (18.4 KD) and lysozyme (14.3 KD).

RESULTS

Amounts and the ratio of retinal and 3-dehydroretinal in an egg

Both ret_1 and ret_2 are found in the *X. laevis* egg.

TABLE 1. Content of all-*trans* retinal (ret_1) and 3-dehydroretinal (ret_2) in the egg from different pairs of adults

Pair	n	pmol/egg			ret_1/ret_2
		ret_1	ret_2	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 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 ret_1 to ret_2 was not so diverse within each brood, but varied from 0.67 to 1.35 in the four broods examined.

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) fractions by centrifugation. They were assayed for retinals by HPLC as shown in Figure 1; almost all the retinals were 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 there-

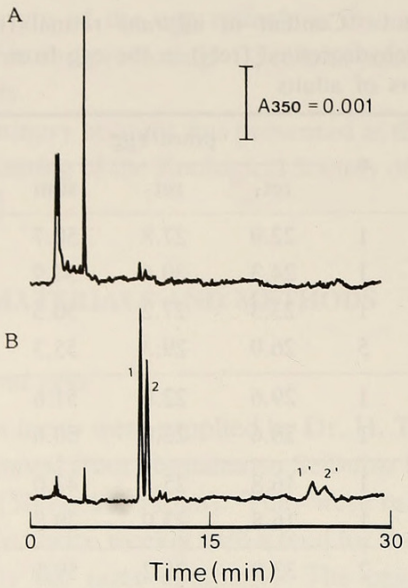


FIG. 1. HPLC elution profiles of extracts from the supernatant and precipitate fractions of *Xenopus laevis* egg homogenate in 20 mM Tris-HCl buffer at pH 7.4 (TB). Retinoids were extracted by the oxime method and analyzed by HPLC as described in Materials and Methods. The eluent was 5% *tert*-butylmethylether-0.04% 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.

fore 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 ret_1 to ret_2 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 [22, 23]. The SDS-PAGE patterns (Fig. 2) of the egg proteins further suggest that the protein in the AS-ppt fraction is LV [18, 19]. The AS-sup (lane 4) contains phosvitin, which is not stained by CBB without $AlCl_3$ [19].

Another attempt to solubilize the TB-ppt with-

TABLE 2. Amounts of all-*trans* retinal (ret_1) and 3-dehydroretinal (ret_2) in different fractions of *Xenopus laevis* egg homogenate

Fraction	nmol			recovery	ret_1/ret_2
	ret_1	ret_2	sum		
TB-sup	0.39	0.44	0.83	0.01	0.90
TB-ppt	39.2	46.8	86.0	0.99	0.84
TBS-sup	38.3	44.2	82.5	0.96	0.87
AS-ppt	37.0	43.0	80.0	0.97	0.86

TB: 20 mM Tris-HCl buffer, pH 7.4
TBS: 400 mM NaCl in TB
AS: 65% saturation of ammonium sulfate
The egg homogenate in TB was centrifuged at $13,000\times g$ 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.

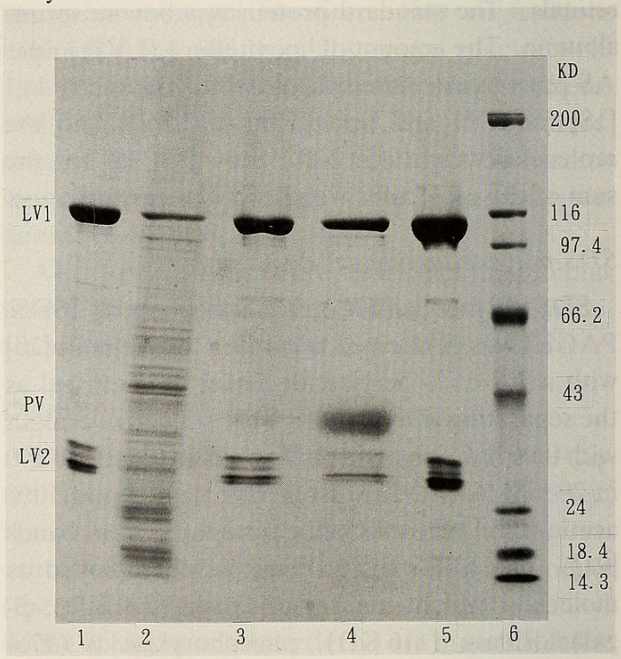


FIG. 2. SDS-PAGE of the proteins in the fractionated samples of *Xenopus laevis* egg homogenate. The gel was stained with CBB in the presence of $AlCl_3$ [18]. 1, precipitate in 20 mM Tris-HCl buffer at pH 7.4 (TB); 2, supernatant in TB; 3, supernatant in 400 mM NaCl in TB; 4, supernatant in the 65% 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.

out 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 dissoved, 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.

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 ret_1 nor ret_2 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 [13].

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 [19], but our preliminary test indidated 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 [19]. From these results it is concluded that the retinals are bound to LV1 with the Schiff base linkage.

Lipovitellin is a lipoprotein [23], 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

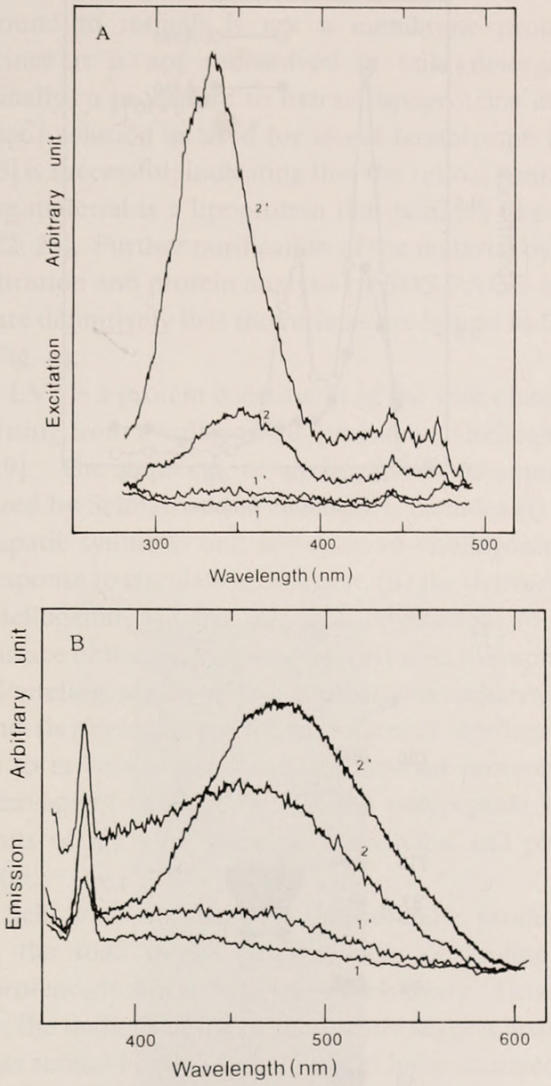
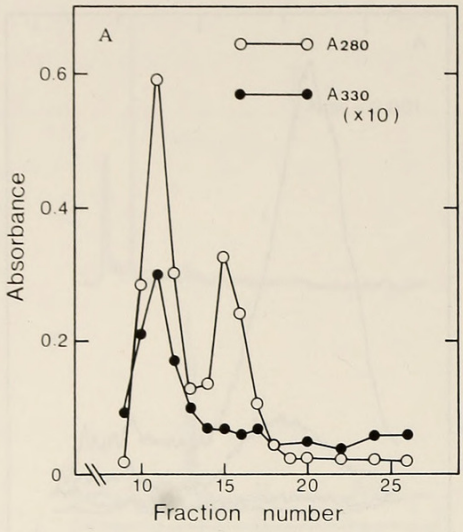


FIG. 3. Fluorescence spectra of the 65% saturated ammonium sulfate (AS)-soluble and -precipitate fractions before and after addition of $NaBH_4$. The AS-soluble (1, 1′) and -precipitate (2, 2′) fractions were measured before (1, 2) and after (1′, 2′) addition of $NaBH_4$. Note that the detection range 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 $NaBH_4$, indicating the presence of retinal.

A: Excitation spectra obtained from emission at 520 nm.

B: Emission spectra measured during excitation at 330 nm.



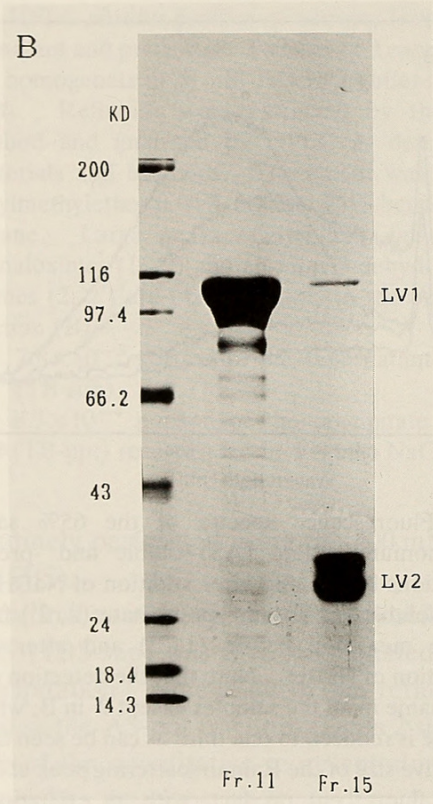


FIG. 4. Gel filtration profile, in the presence of 0.1% SDS, of the ammonium sulfate precipitated material after treatment with $NaBH_4$. The proteins were chromatographed into two fractions: lipovitellin 1 (LV1; Fr. 11) and lipovitellin 2 (LV2; Fr. 15) [18]. The retinyl-product detected by the absorbance at 330 nm cochromatographed with LV1.

A: The elution profiles shown by the absorbances at 280 nm (○) and at 330 nm magnified ten-fold (●).

B: SDS-PAGE of the proteins in peak fractions 11 and 15 in A, and marker proteins.

was divided into two aliquots, to one of which $NaBH_4$ 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 dichloromethane/*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

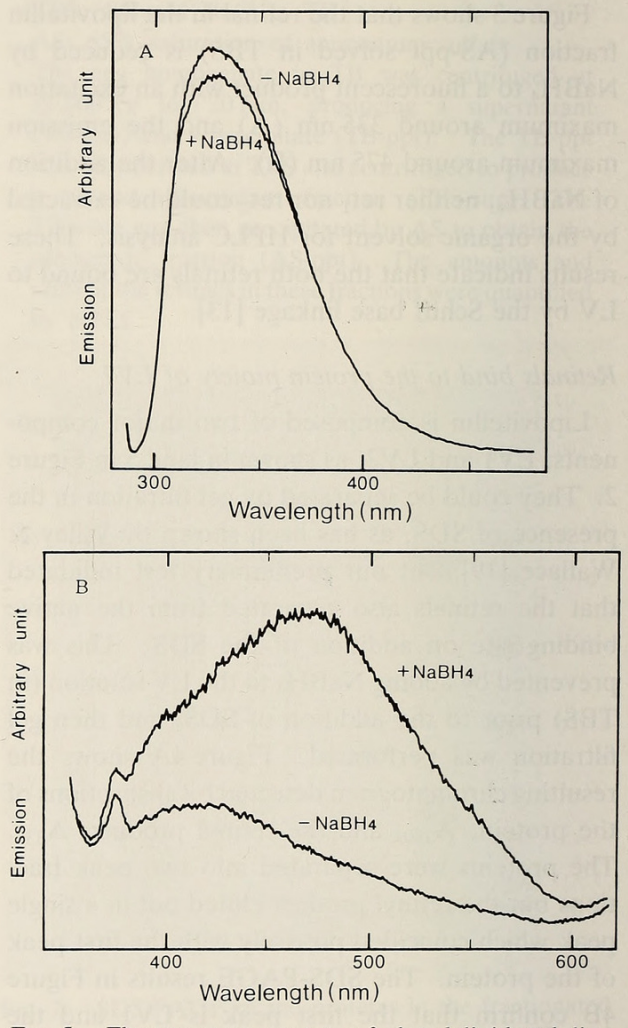


FIG. 5. Fluorescence spectra of the delipidated lipovitellin. An ammonium sulfate precipitate fraction was divided into two aliquots, $NaBH_4$ added to one of them, and both samples were delipidated as described in the text. The delipidated proteins were resolved in 5% SDS in Tris-HCl buffer at pH 7.4 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.

between the two samples. The lower water layer was then recentrifuged at 13,000×*g* for 20 min to preceipitate 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 $NaBH_4$-treated sample. It is therefore concluded that $NaBH_4$ treatment resulted in a retinylprotein product.

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 ret_1 or ret_2.

TABLE 3. The molecular ratio of lipovitellin (LV) to retinal (ret_1) or 3-dehydroretinal (ret_2)

Exp.	$LV/ret_{1\ or\ 2}$		ret_1/ret_2
	Lowry	Fluor.	
A	16.4	20.7	0.87
B	12.7	18.8	1.20
C	13.9	22.8	0.72

The amount of protein in a LV sample was quantified by the Lowry method [17] and by fluorometry, and amount of the LV1 was calculated as shown in Materials and Methods. The amounts and the ratio of all-*trans* ret_1 and ret_2 in the same sample were determined by using HPLC.

DISCUSSION

The present experiments reveal, first of all, that the egg retinals are not bound to a water soluble protein, since no retinal is detected in the TB-sup fraction (Fig. 1, Table 2). Secondly, the protein bound to retinals is not a membrane protein, scince it is not redissolved in mild detergent. Finally, a procedure to extract lipoproteins using NaCl solution as used for insect hemolymph [24, 25] is successful, indicating that the retinal containing material is a lipoprotein that behaves like LV [22, 23]. 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 [19]. The sequence of vitellogenesis, as summarized by Selman and Wallace [26], 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.* suggested [8] that retinal is produced in the toad ovary, on the basis of finding of carotenoids and retinylester in the ovary. 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 [6], and the appearance of retinal in the blood is influenced by hormonal control [7]. 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 [27, 28], which contains carotenoids [24, 27]. The hemolymph lipoprotein II of the silkworm was shown to be identical with the egg lipoprotein II [28], and we have detected carotenoids but not retinoids in the eggs of a moth (unpublished observation) and a dragonfly [29]. Also, Plack & Kon [4] 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; [18]), which are produced from three [30] or four [31] 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 ret_2 is known to be extremely low, and so the Schiff base linkage of ret_2 is not deducible from the fluorescence data. However, the following two results indicate that binding states of ret_1 and ret_2 are the same: (i) the unity in the ratio of ret_1 to ret_2 during the purification steps of LV (Table 2), and (ii) both retinals become unextractable after treatment of LV with $NaBH_4$.

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 [32]. 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 abosrption 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 [33]. 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 (ret_1, ret_2, biliverdin, riboflavin) contribute to the absorption spectrum. Moreover, the addition of $NaBH_4$ 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 $NaBH_4$ 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 [34].

Ret_1 and ret_2 are the only retinoids found in *X. laevis* eggs [8]. 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 ret_1 and ret_2 esters [35]. During the present experiment, the presence of retinoic acid in the egg was also inspected, 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.

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