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Lipovitellin from the Crustacean, *Artemia salina*

BIOCHEMICAL ANALYSIS OF LIPOVITELLIN COMPLEX FROM THE YOLK GRANULES*

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Didier de Chaffoy de Courcelles‡ and Masatoshi Kondo§

From the Laboratory of Microbiology, Department of Cell Biology, University of Antwerp, 2610 Wilrijk, Belgium

The polypeptide, lipid, and carbohydrate components of the lipovitellin complex from anostracan crustacean, *Artemia salina*, have been investigated. The lipovitellin isolated from the yolk granules is a carotenoid•lipoglycoprotein complex, containing 3.3% carbohydrate and 8.6% lipid. About half of the carbohydrate fraction is mannose and the rest consists of galactosamine, galactose, and glucosamine. The lipid fraction contained 67% phospholipid and 33% neutral lipid. Phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine are three major phospholipid components and their relative ratio changes by developmental stages. The neutral lipid fraction is composed of mainly triacylglycerol and cholesterol.

Two polypeptide chains are identified as apoprotein components of the lipovitellin complex. Their molecular weights are estimated by sodium dodecyl sulfate gel electrophoresis to be 190,000 and 68,000, respectively. The third polypeptide with M_r = 85,000 is a very minor component and exists in nonstoichiometric levels in the lipovitellin complex. On the basis of an apparent molecular weight of the lipovitellin complex determined by gel filtration chromatography, the dimeric lipovitellin form is suggested, each monomer of which contains one set of M_r = 190,000 and one set of M_r = 68,000 apoproteins. Each dimeric lipovitellin complex contains approximately 70 associated lipid molecules, of which about 4 are attributed to canthaxantin, and 100 bound carbohydrate molecules.

Vitellogenesis is one of the key events occurring in the growing oocytes of both vertebrate and invertebrate animals. This process is usually under control of several hormones as has been documented in amphibians (1-3), reptiles (4, 5), birds (6-8), and insects (9-11). Thus, the vitellogenesis has been extensively studied as a model system for the hormonal control of gene regulation at the molecular level (12-15). By contrast, our present knowledge on vitellogenesis in crustaceans is extremely limited so that the site of biosynthesis of the vitellogenin molecule has not been established to date. There is only one indirect evidence for a possible control of vitellogin synthesis by ovarian hormone in the amphipod, *Orchestia gammarella* (16). The structure of apovitellogenin and/or apolipovitellin components in crustacean has not been reported except one study in the crayfish, *Procambarus*, where the stoichiometry of five possible peptides in the lipovitellin complex has not been determined (17).

Since accurate knowledge of the polypeptide chains involved in the formation of the lipovitellin complex, which derive from the yolk precursor protein, vitellogenin, is essential for the study of vitellogenin gene activation, the present study was carried out. The brine shrimp, *Artemia salina* (L.), belonging to the order anostraca, has a great advantage as an experimental object owing to its ease in the cultivation in a large scale under controlled conditions (18). Therefore, we have chosen *A. salina* for our study in order to obtain essential data on the lipovitellin complex structure. In this report, we describe the structure of apolipovitellin components and analysis of lipid and carbohydrate components in detail and compare these results with those from known invertebrate sources.

EXPERIMENTAL PROCEDURES

Materials—All chemicals used were of "Pro Analyse" quality and obtained from E. Merck, Darmstadt, Germany. Kieselgel 60 plates for thin layer chromatography were also from E. Merck. Tris was purchased from Sigma Chemical Co., St. Louis, Mo. *Staphylococcus aureus* V8 protease was obtained from Miles Laboratories, Elkart, Ind. DEAE-cellulose A-52 and all electrophoresis materials were obtained from Serva Feinbiochemica, Heidelberg, Germany. All Bio-Gel resins were from Bio-Rad Laboratories Richmond, Ca. Sepharose 6B and Sephadex resins were obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Molecular weight protein markers were purchased from Boehringer Mannheim, Mannheim, Germany. Amicon PM 10 was from Amicon, Oosterhout, The Netherlands. Encysted cryptobiotic gastrulae of *A. salina* were obtained from Metaframe, Newark, Ca. Authentic samples of carotenoids were kindly supplied by Drs. U. Gloor and F. Weber, Hoffmann-La Roche & Co., Basel, Switzerland.

Cultivation of Brine Shrimps—Nauplius larvae were hatched from cryptobiotic gastrulae and adults at various stages were cultured from freshly hatched nauplii in 80-liter aquaria at 28°C in a medium containing 0.51 M NaCl with an oxygen concentration of 0.17 to 0.21 mM (standard conditions) as described in Heip *et al.* (18).

Isolation of Lipovitellin Complex—Yolk granules from the oocytes, gastrulae, and nauplii were prepared as previously described (19). All operations were carried out at 0-4°C in quartz bidistilled water. The lipovitellin complex was solubilized at 0°C from purified yolk granules in 50 mM Tris-HCl (pH 9) containing 1 M NaCl for 1 to 2 h and the insoluble material was removed by centrifugation. The supernatant was filtered through Whatman No. 1 filter paper and then chromatographed on Sepharose 6B or Bio-Gel A-1.5m using the solubilization buffer. Further purification was achieved by chromatography on DEAE-cellulose. The sample was dialyzed or diluted with 50 mM Tris-HCl (pH 9) to 0.02 M NaCl, applied to columns, and eluted with 0.02 to 0.12 M NaCl gradient in the same buffer. The lipovitellin was eluted at 0.08 to 0.09 M NaCl from the column after a small peak at 0.03 to 0.04 M NaCl. The pool fraction containing the lipovitellin was concentrated by ultrafiltration on Amicon PM-10 membranes. Lipid globules from gastrulae were prepared as for yolk granules (19), except that the last centrifugation at 10,200 × *g* in a Beckman JS 13 rotor was for 10 min. The upper yellow, loosely packed lipid globule layer on top of the tightly packed yolk granules was removed by gently suspending on quartz bidistilled water. This

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§ To whom correspondence should be addressed.

fraction was further purified by repeated centrifugation as above. The last pellet was used for analysis.

SDS[1]-Polyacrylamide Gel Electrophoresis—Samples were denatured at 100°C for 5 min in 10 mM Tris, 0.33 M glycine (pH 7.5), 1% SDS, 1% 2-mercaptoethanol, 28% glycerol, and 0.01% bromphenol blue and applied to 5% gels (0.3 × 10 cm). Electrophoresis was performed at 2 mA/gel in an electrode buffer (10 mM Tris, 0.33 M glycine (pH 7.5), 0.1% SDS) essentially as described by Kamen *et al.* (20). The gels were stained with 0.25% Coomassie brilliant blue and after destaining with 35% methanol containing 5% acetic acid were scanned at 560 nm with a Gilford type 240 spectrophotometer equipped with a linear transport. Molecular weights of lipovitellin protein components were estimated by coelectrophoresis of reference proteins in 5% slab gels (0.2 × 11 × 11 cm) at a constant potential of 100 V.

For preparative runs, samples were first delipidated as described below (see lipid analysis) before denaturation in 62.5 mM Tris-HCl (pH 6.8), 2.3% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.001% bromphenol blue as above and applied to 5% slab gels (1 × 9.5 × 22 cm). Electrophoresis was carried out at 400 mA in an electrode buffer (25 mM Tris, 0.192 M glycine (pH 8.3), 0.1% SDS) essentially as described by Laemmli (21). The gels were stained briefly with Coomassie brilliant blue and the protein bands were cut out and homogenized by a VirTis homogenizer at 5000 rpm for 0.5 min. The protein was eluted from gel powders either by electrophoresis after polymerization in 7.5% cylindrical gels (1 × 1.2 cm) or by diffusion at 0°C in 3 volumes of 10 mM $(NH_4)_2CO_3$ for 48 h. The eluent was clarified by Whatman No. 1 filter, dialyzed against 10 mM $(NH_4)_2CO_3$ at 0°C, and finally lyophilized. The analysis of amino acid compositions and peptide mappings was performed on these preparations.

Two-dimensional Isoelectric Focusing/SDS-Polyacrylamide Gel Electrophoresis—The 4% polyacrylamide gels (0.3 × 10 cm) were polymerized chemically in the presence of 9.2 M urea, 2% Nonidet P-40, and 2% Ampholines. The sample buffer contained 9.5 M urea, 5% 2-mercaptoethanol, 2% Nonidet P-40, and 2% Ampholines. Electrophoresis was performed as described by O'Farrell (22). After isoelectric focusing, the gels were equilibrated in the loading buffer of Laemmli (21) (see above) for 1 h and then polymerized onto 5% slab gels (0.2 × 11 × 11 cm) with 1% agarose in the same buffer. SDS-gel electrophoresis in the second dimension was carried out as described by Laemmli (21).

One-dimensional Peptide Mapping by Limited Proteolysis—Gel slices containing appropriate proteins from analytical SDS gels were incubated at room temperature for 30 min in an enzyme buffer (0.125 M Tris-HCl (pH 6.8), 0.1% SDS, and 1 mM EDTA) (23), placed on a slab gel consisting of a 5% stacking gel (3.5 cm) and a 15% resolving gel (7 cm), and overlayed with the enzyme buffer containing 20% glycerol. After 5 μl of this buffer containing 10% glycerol and a given amount of V8 protease (2.5 to 5 μg) was placed on each slot, electrophoresis was carried out in the electrode buffer of Laemmli (21) containing 1 mM EDTA until the tracking dye reached the top of the resolving gel, when the current was turned off for 30 min. Electrophoresis was then continued until the dye front reached the bottom of the gel.

Amino Acid Analysis—The preparations of lyophilized individual apoprotein components which had previously been delipidated were hydrolyzed in 110°C in 6 N HCl and their amino acid compositions were determined by a JEOL J.L.C. 6 AH automatic amino acid analyzer as described by Moens and Kondo (24). Half-cystine was measured as cysteic acid after performic acid oxidation (25).

Molecular Weight Estimation by Gel Filtration—An apparent molecular weight of the native lipovitellin complex was estimated by a Bio-Gel A-1.5m column (1 × 55 cm) which had been equilibrated at 4°C with 50 mM Tris-HCl (pH 8) containing 1 M NaCl. Calibration of a column was performed by using thyroglobulin, apoferritin, bovine catalase, and yeast alcohol dehydrogenase. The column was developed at a flow rate of 4–8 ml/h.

Characterization of Lipid Components—Total lipids were extracted from the lipovitellin complex by the method of Bligh and Dyer (26) and the lipid content was determined gravimetrically with a Cahn balance according to Rouser *et al.* (27). The separation between neutral lipid and phospholipid was achieved by thin layer chromatography on Kieselgel 60 plates (20 × 20 cm) which had been activated by heating at 110°C overnight (28). To separate neutral lipids, the following solvent systems were used: (*a*) hexane:diethyl ether:acetic acid (70:30:1), (*b*) benzene:diethyl ether:ethanol:acetic acid (50:40:2:0.2), (*c*) hexane:diethyl ether (94:6). To separate phospholipids, the solvent system of chloroform:methanol:acetic acid:water (25:15:1.8:1.2) was employed. Each component was quantitated densitometrically after staining the plates with either iodine (29) or potassium dichromate-sulfuric acid (30). The quantitation of individual lipid components was also carried out by gas-liquid chromatography of methyl esters (31) with a Hewlett Packard 7620 gas chromatograph using a SE-30 chromasorb AWP 100 to 120 mesh column and a EGSS-X column. Individual samples were recovered from preparative thin layer plates which were then processed for gas-lipid chromatography together with an internal marker triacylglycerol containing C17 fatty acyl chains. To detect unsaturated fatty acids, gas-liquid chromatography was performed using a EGSS-X column before and after reduction of the samples with H_2 gas.

Cholesterol was recovered from thin layer plates, processed according to Skipski (32), and quantitated colorimetrically by the method of Hanel (33). Carotenoid was also recovered from thin layer plates, eluted with chloroform, and quantitated spectrophotometrically at 470 nm. Identification of carotenoid components was achieved by thin layer chromatography using a solvent system of benzene:diethyl ether:methanol (17:2:1) together with the authentic samples.

Analysis of Carbohydrate Components—The total hexose content of the delipidated lipovitellin was determined by the anthrone reaction with D-mannose as standard by the method of Roe (34). The identification of hexose and hexosamine components was carried out by gas chromatography of trimethylsilyated derivates of methylglycosides as described above. The delipidated lipovitellin was methanolyzed with 0.41 N HCl in methanol at 80°C overnight and dried under a stream of N_2. Deacetylated *N*-acetylhexosamines were reacetylated in methanol:acetic anhydride:pyridine (2:3:2) at room temperature for 30 min. After drying, the samples were trimethylsilyated by the method of Vance and Sweeley (35).

RESULTS

Carbohydrate Components of Lipovitellin Complex—The presence of hexoses and hexosamines in the *A. salina* lipovitellin complex was demonstrated by means of thin layer and gas chromatographies. The total carbohydrate content in this complex was 3.3%, about half of which was mannose (Table I). Mannose was also detected as a major or sole carbohydrate component in other invertebrate lipovitellins or vitellogenins, whose total carbohydrate content was found in the range of

TABLE I

Carbohydrate components of lipovitellin complex from A. salina gastrulae

The amount of hexose was directly determined and the relative quantity of individual hexoses and hexosamines was estimated by gas-liquid chromatography.

Carbohydrate	%, w/w[a]	% of total carbohydrate
Total	3.6[b]	100
Hexose	2.2 ± 0.1 (8)[c]	
Mannose	1.8[b]	50
Galactose	0.4[b]	11.1
Hexosamine	1.4 ± 0.1[b] (8)	
Galactosamine	1.2[b]	33.3
Glucosamine	0.2[b]	5.6

[a] Values expressed with respect to the weight of apoprotein.
[b] Calculated values on the basis of gas chromatographic data.
[c] Values in parentheses were the number of determinations.

TABLE II

Lipid components of lipovitellin complex from A. salina gastrulae

Lipid	%, w/w[a]	% of total lipid
Total lipid	8.8 ± 0.2 (5)[b]	100
Polar lipids	5.9 ± 0.3 (5)	67.0
Neutral lipids	2.9 ± 0.1 (5)	33.0

[a] Values expressed with respect to the weight of apoprotein.
[b] Values in parentheses were the number of determinations.

[1] The abbreviations used are: SDS, sodium docedyl sulfate; LV-α, LV-β, LV-γ, LV-δ, and LV-ε, polypeptide species of the lipovitellin complex of decreasing molecular weight as judged by SDS gel electrophoresis.

TABLE III

Lipid components of phospholipid and neutral lipid of lipovitellin complex and lipid globules in A. salina

Lipid component	%, w/w of total lipid		
	LVg[a]	LVn	LG
Phospholipid			
Phosphatidylcholine	40.5 ± 0.9[b] (4)[c]	36.0 ± 0.6[b] (4)	29.1 ± 1.2[b] (4)
Phosphatidylethanolamine	11.9 ± 0.4[b] (4)	16.6 ± 2.1[d] (10)	23.3 ± 0.5[b] (4)
Phosphatidylserine	8.7 ± 0.3[b] (5)	9.6 ± 0.8[b] (5)	4.4 ± 0.6[b] (5)
Unknown	3.0 ± 1.1[d] (10)	3.6 ± 1.2[d] (10)	3[e]
Neutral lipid			
Triacylglycerol	13.0 ± 0.3[b] (4)	18.6 ± 0.1[b] (4)	23.2 ± 0.1[b] (4)
Diacylglycerol	5.5 ± 0.2[b] (5)	1.6 ± 0.1[b] (5)	4.5 ± 0.2[b] (5)
Monoacylglycerol	2.7 ± 0.1[b] (5)	1.2 ± 0.7[b] (5)	1.9 ± 0.2[b] (5)
Free fatty acid	5.5 ± 0.1[b] (5)	2.7 ± 0.1[b] (5)	3.2 ± 0.1[b] (5)
Cholesterol	6.1 ± 0.4[d] (10)	4.5 ± 0.7[d] (10)	4[e]
Cholesterol ester	<0.5[e]	<0.5[e]	5[e]
Canthaxantin	2.6 ± 0.2[d] (11)	3.3 ± 0.3[d] (11)	0.1 ± 0.01[d] (11)
Unknown	<0.5[e]	<0.5[e]	<0.5[e]

[a] The abbreviations used here and in Tables IV, V, and VI are: LVg and LVn, lipovitellin complex derived from gastrulae and nauplii, respectively; LG, lipid globule.

[b] Determined by gas-liquid chromatography using an appropriate internal standard after separation of each component by thin layer chromatography.

[c] Values in parentheses were the number of determinations.

[d] Determined colorimetrically after separation of individually components by thin layer chromatography. Phospholipids, cholesterol, and canthaxanthin are according to the methods of Rouser *et al.* (27), Hanel (33), and de Chaffoy *et al.* (19), respectively.

[e] Determined by densitometric quantitation of thin layer chromatograms.

TABLE IV

Distribution of fatty acyl chains in individual lipid components from lipovitellin complex and lipid globules of A. salina

Fatty acyl chain	% w/w within individual lipid component																				
	Free fatty acid			Triacylglycerol			Diacylglycerol			Monoacylglycerol			Phosphatidylcholine			Phosphatidylethanolamine			Phosphatidylserine		
	LVg	LVn	LG	LVg	LVn	LG	LVg	LVn	LG	LVg	LVn	LG	LVg	LVn	LG	LVg	LVn	LG	LVg	LVn	LG
14:0	2.1	4.3	2.2	5.1	5.4	2.4	2.8	2.5	2.0	0.9	12.3	2.8	1.3	0.9	1.0	6.9	< 0.5	< 0.5	6.7	t	1.2
16:0	16.8	21.5	17.0	16.6	13.7	15.8	11.7	16.7	12.0	9.9	9.0	10.6	24.5	18.0	17.5	13.4	10.1	7.3	14.1	7.2	8.9
16:1	11.5	12.8	17.0	14.7	23.0	18.6	8.7	19.7	15.9	6.9	2.3	1.8	11.5	15.0	10.1	4.6	5.9	3.3	2.7	2.4	t
16:2	< 0.5	t[a]	t	1.2	0.6	< 0.5	t	t	t	t	t	t	t	t	t	4.0	t	t	9.2	t	t
16:n1	0.6	1.6	0.7	< 0.5	0.6	< 0.5	< 0.5	< 0.5	< 0.5	t	t	t	< 0.5	t	t	< 0.5	< 0.5	< 0.5	–	–	–
16:n2	2.2	2.4	1.4	6.3	3.7	1.6	1.7	2.6	< 0.5	–	–	–	1.3	< 0.5	< 0.5	1.5	< 0.5	< 0.5	1.0	–	–
18:0	5.2	6.3	4.2	2.6	1.7	2.7	6.7	3.3	3.7	10.7	23.5	5.1	5.5	3.6	6.8	6.9	4.8	7.0	14.6	9.1	23.5
18:1	42.7	26.2	45.0	40.3	31.1	47.2	46.6	35.6	52.7	41.8	8.5	7.3	48.7	44.7	42.5	52.9	53.1	47.3	30.0	15.0	3.0
18:2	2.6	1.3	2.0	1.8	1.7	1.2	1.8	1.3	1.8	1.1	t	t	1.3	1.8	1.5	t	1.0	1.7	2.1	–	–
18:n	2.3	1.6	1.6	3.3	1.8	2.0	2.1	1.9	1.5	1.8	t	t	2.7	1.4	1.2	2.1	1.8	1.1	t	t	t
20:0	< 0.5	t	0.8	< 0.5	t	0.7	t	t	t	1.3	2.1	1.9	< 0.5	< 0.5	< 0.5	1.9	< 0.5	< 0.5	2.5	t	t
20:1	0.8	–	1.0	0.6	t	t	1.2	t	t	1.3	–	–	–	–	–	1.8	0.7	0.9	–	–	–
20:2	–[b]	–	–	0.7	< 0.5	0.6	1.2	t	t	–	–	–	2.1	0.8	< 0.5	< 0.5	< 0.5	t	–	–	–
20:n	3.8	9.1	4.2	2.5	3.2	5.1	2.3	1.7	6.6	1.7	3.9	12.4	1.5	5.9	7.8	1.8	4.0	7.5	3.5	6.6	5.4
22:0	t	t	t	< 0.5	t	0.5	0.7	t	< 0.5	9.3	33.2	20.6	< 0.5	< 0.5	< 0.5	–	1.1	1.5	2.5	10.9	5.5
22:n1	4.1	9.5	1.2	1.8	1.0	0.8	t	t	t	1.3	6.3	28.9	0.7	3.2	6.3	t	11.4	15.5	–	33.4	48.7
22:n2	13.0	3.4	–	1.5	0.5	< 0.5	0.5	t	1.1	–	–	8.7	0.9	1.0	2.1	1.9	2.6	3.4	11.8	6.8	3.4

[a] Trace amounts.

[b] Not detected.

2.5 to 13.6% (36–41). In contrast, the lipovitellin·phosvitin complex from *Xenopus laevis* contains only trace amounts of carbohydrate, if any (42).

Characterization of Lipid Components—Purified lipovitellin complex from *A. salina* gastrulae which had not been treated with DEAE-cellulose was analyzed for lipid components by the method as described under "Experimental Procedures." The total lipid content in the complex was determined to be 8.6%, of which approximately 67% was phospholipids (Table II). The phospholipid content of 5.9% in the *A. salina* lipovitellin complex is significantly lower than the value of 15 to 17% found in the corresponding complex from another anostraca crustacean, *Branchipus stagnalis* (36). Moreover, the total lipid content of 8.6% in *A. salina* is distinctly lower than the value of about 30% identified in lipovitellins from several decapod crustaceans [43]. On the other hand, its lipid content rather resembles the values (8 to 16%) reported for certain insect vitellogenins or lipovitellins (37–40, 44, 45).

From the results presented in Table III, it is evident that phosphatidylcholine is the major phospholipid component in the *A. salina* lipovitellin complex from gastrulae and nauplii. However, the ratio of phosphatidylcholine to phosphatidylethanolamine, the second abundant phospholipid component, apparently decreases from 3.3 to 2.1 as yolk degradation process proceeds during embryonic development. This tendency is more pronounced in isolated lipid globules, in which this ratio becomes about 1.3 (Table III). It has been shown that approximately 50% of yolk granules present in the gastrulae embryo is degraded at the time when the nauplius larva is hatched (46). The relative quantity of lipid globules in the embryo increases during development[2] and they are presum-

[2] D. de Chaffoy de Courcelles, G. De Maeyer-Criel, and M. Kondo, unpublished observation.

ably derived from the unutilized lipid released from the degrading yolk granules. The ratio of 3.3 found in *A. salina* gastrulae is higher than the value of 1.7 determined in silkworm lipovitellin (37), but it is lower than that of 4.7 reported in *X. laevis* one (42).

The third component is phosphatidylserine (Table III), whose amount occupies 14 to 15% of the total phospholipid and whose presence appears very significant, because only small amount (2%) was detected in the lipovitellin complex from *X. laevis* (42) or none was identified in the silkworm lipovitellin (37). In addition, sphingomyelin seems to be a major component (50–70%) in the unknown fraction of the phospholipids. Considering the amounts present in the *A. salina* lipovitellin complex (Table III), three major phospholipid components seems likely to be bound to apoproteins at stoichiometric levels as suggested by Ohlendorf *et al.* (42).

The neutral lipid constitutes about one-third of the total lipid (Table II). Triacylglycerol is the major component (37 to 38%) as is found in *X. laevis* lipovitellin complex (42) and its relative quantity in the neutral lipid seems to increase during embryonic development (Table III). By contrast, more diacylglycerol and cholesterol are present in the silkworm lipovitellin than is triacylglycerol (37). Approximately equal amounts (15 to 17%) of diacylglycerol, free fatty acid, and cholesterol are present in the gastrula lipovitellin, but diacylglycerol and free fatty acid components exist in significantly reduced amounts in the nauplius one (Table III). These changes in the relative amount among lipid components are likely to reflect the situation of lipid metabolisms at certain developmental stages.

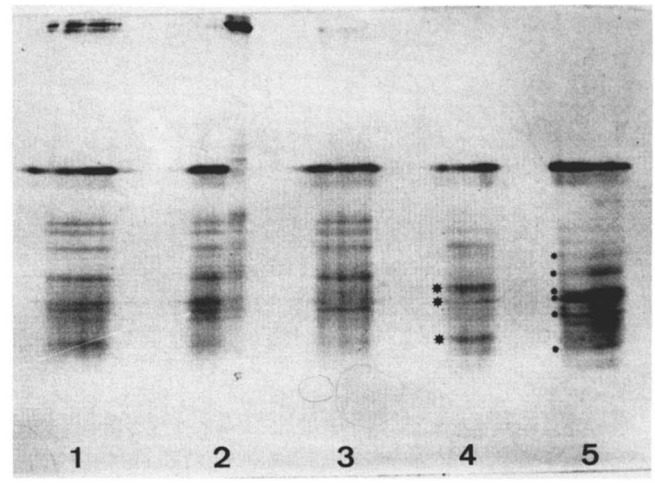

FIG. 2. **One-dimensional peptide mapping by limited proteolysis of isolated apolipovitellins with *S. aureus* protease V8.** The pattern represensts the hydrolysis performed at the enzyme/substrate ratio of 1:3 for LV-α1 (*lane 1*), intermediate LV-α (*2*), LV-α10 (*3*), LV-δ (*4*), and LV-ε (*5*). The peptides marked by *stars* are unique components of LV-δ and LV-ε. See "Experimental Procedures" for details.

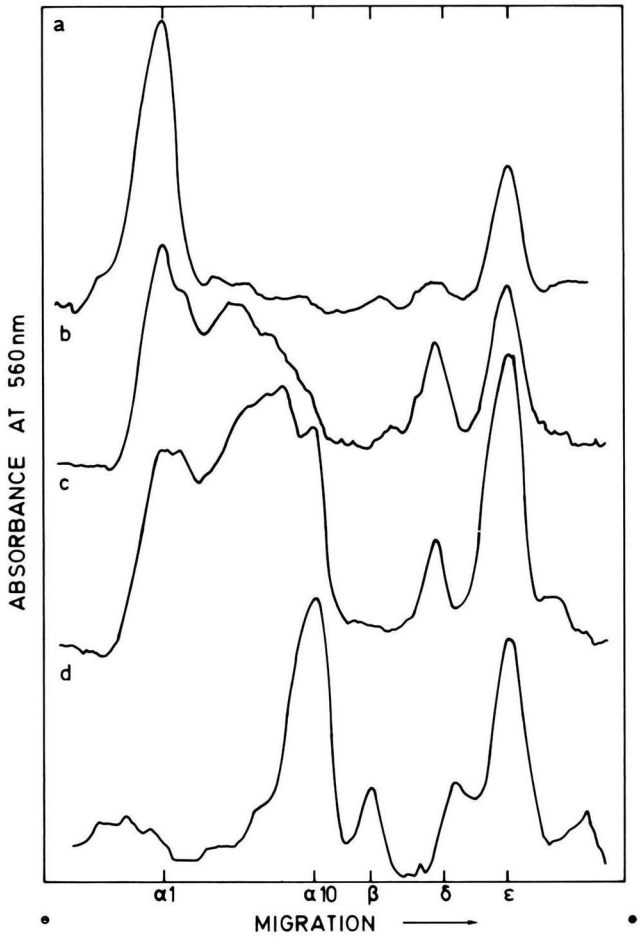

FIG. 1. **Densitometric scans of the apoprotein components of the lipovitellin.** The lipovitellin prepared from the oocytes (*a*), gastrulae (*b* and *c*), and nauplii (*d*) were separated by SDS disc gels (5% acrylamide and 0.1% SDS) and stained with Coomassie brilliant blue. α1, α10, β, δ, and ε indicate the positions for the corresponding apovitellins.

Lipovitellin complexes of the crustacean are generally characterized by their specific association with carotenoids (47–50). It has also been demonstrated that the lipovitellin from *A. salina* contains canthaxanthin (4,4′-diketo-β-carotene) as its prosthetic group (19, 51–54). As shown in Table III, canthaxanthin is present in the amount of about 3% of the total lipid. The presence of low amounts could make our estimate less accurate, as compared with those for other major lipid components. Nevertheless, we suggest the presence of 3 to 4 molecules of canthaxanthin in the native lipovitellin complex from *A. salina* (Table VI). Similar estimate was reported for another lipovitellin complex also containing canthaxanthin from the fresh water shrimp (*B. stagnalis*) (36). In addition, an extremely minor carotenoid component, echinenone (4-keto-β-carotene), was identified in *A. salina* lipovitellin by thin layer chromatography (19). Echinenone is a possible precursor to canthaxanthin in *A. salina* (52). No carotenoids are detected in the lipid globule (Table III). It is known that the carotenoid becomes concentrated in the nauplius eye and chromatophores in the crustacean (55).

Distribution of Fatty Acyl Chains—Table IV summarizes the results obtained for the distribution of fatty acyl chains in individual lipid components present in the lipovitellin complex and the lipid globule. It is clear that 18:1 is the most prominent fatty acyl chain in all lipid components except in monoacylglycerol and phosphatidylserine from the nauplius lipovitellin and the lipid globule. In the latter complexes, 22:n1 is the major fatty acyl chain, except that 22:0 is in monoacylglycerol of the nauplius complex. Both 22:n1 and 22:0 are only minor species in all other lipid components (Table IV).

Identification of Apoprotein Components—The protein component of the lipovitellin complex was analyzed by SDS-polyacrylamide gel electrophoresis. Fig. 1 illustrates the densitometric tracings of SDS gels analyzing the apoprotein components from *A. salina* at different developmental stages. It is quite evident that the oocyte lipovitellin contains principally only two apoprotein components (Fig. 1*a*), whose molecular weights are estimated 190,000 (LV-α1) and 68,000 (LV-ε), respectively. The molar ratio of LV-α1 to LV-ε was calculated

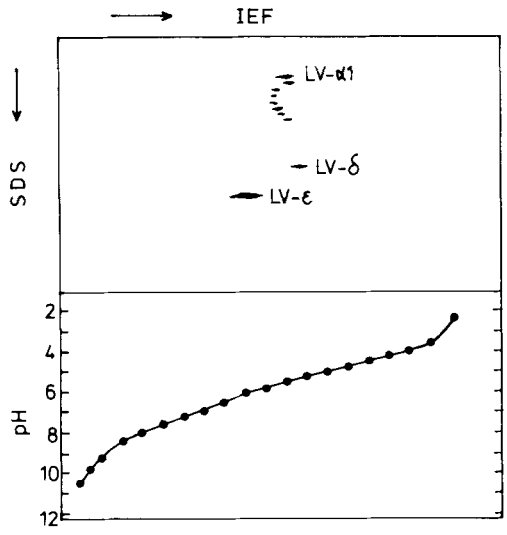

FIG. 3. **Two-dimensional polyacrylamide gel analysis of the apolipovitellin components of the gastrulae.** The *upper panel* represents a schematic presentation of the electrophoretogram separating LV-α1 to LV-α8, LV-δ and LV-ε, and the *lower panel* indicates pH-gradient of the first dimensional isoelectric focusing. (*IEF*). See "Experimental Procedures" for details.

by measuring several densitograms of the oocyte lipovitellin and was found to be 1.0:1.0 to 1.3. We, thus, propose that the apolipovitellins, LV-α1 and LV-ε, are present in an equimolar ratio in the oocyte lipovitellin complex.

When the lipovitellin of gastrula embryos was examined in an identical manner, we found the reduction in the relative amount of LV-α1 with respect to LV-ε, and also the concomitant presence of multiple components in varied amounts between the two apoprotein components. Figs. 1b and 1c, represents the densitometric tracings of SDS gels resolving apolipovitellins prepared from two different gastrula samples. It is quite clear that the decrease in the LV-α1 parallels with the simultaneous increase in the polypeptides with intermediate molecular weights (Fig. 1). When the lipovitellin is isolated from nauplii which have not been fed for 3 or 4 days after hatching and analyzed by SDS gels, it is found that the LV-α1 is completely degraded to LV-α10 (M_r = 120,000) whereas the LV-ε is not (Fig. 1*d*). In addition, the minor components, LV-β (M_r = 100,000) and LV-δ (M_r = 85,000), are evident in the lipovitellins from gastrulae and nauplii (Fig. 1), and will be discussed below. We suggest that (*a*) the lipovitellin from *A. salina* consists of LV-α1 and LV-ε in an equimolar quantity, amounting about 88% of its total weight (Table VI), (*b*) LV-α1 undergoes the stepwise proteolysis, giving rise to LV-α10, and (*c*) LV-ε, on the other hand, is resistant to this proteolytic attack.

Characterization of Apoprotein Components—One of the minor components, LV-β, cannot be detected in the oocyte lipovitellin and is also almost absent in the gastrula complex. It becomes more evident in the lipovitellin complex isolated from embryos at the advanced stages when more extensive proteolysis of the lipovitellin takes place (for example, Fig. 1*d*). The amount of LV-β increases slowly and accumulates to a certain extent during an *in vitro* proteolysis by the isolated protease of the yolk granules.[3] It is therefore suggested that although further hydrolysis of LV-α10 seems a rate-limiting step, in accummulating large amounts of this polypeptide, a small part of the LV-α10 is rendered to further proteolysis and is converted to the LV-β.

[3] D. de Chaffoy de Courcelles and M. Kondo, unpublished observation.

The second minor component, LV-δ, exists in a very small amount in the oocyte lipovitellin, but is present in distinct quantities in the gastrula and nauplius lipovitellins (Fig. 1). Moreover, in contrast to LV-β, the relative amount of LV-δ appears independent of the degree of hydrolysis taken place in the LV-α polypeptides. The presence of LV-δ in the oocyte lipovitellin can be demonstrated when the solubilization process is prolonged to 4 h or more. Since the LV-δ is found only in small quantities as compared with LV-α1 and LV-ε, when the total protein from the yolk granules of oocyte is directly solubilized by SDS without the prior salt extraction,[3] it seems that LV-δ becomes concentrated in the solubilized oocyte lipovitellin fraction only after the prolonged solubilization of these yolk granules with 1 M NaCl, whereas LV-δ can readily be extracted from those granules of the gastrulae and nauplii. This indicates the possibility that the structural organization incorporating the LV-δ polypeptides within the yolk granule undergoes certain alterations during embryonic development.

These indirect observations suggest that LV-δ is not a simple proteolytic degradation product of LV-α1. To clarify this point one-dimensional peptide mapping by limited proteolysis using *S. aureus* V8 protease was carried out on iso-

TABLE V

Amino acid composition of polypeptides of A. salina lipovitellin complex

The amino acid compositions were determined using 24-h digests and were not corrected for amino acid destruction during hydrolysis. The molecular weights used for the compositions are: LV-α1, 190,000; LV-δ, 85,000; LV-ε, 68,000.

Amino acid	LV-α1		LV-δ		LV-ε	
	residues/mol	*mol %*	*residues/mol*	*mol %*	*residues/mol*	*mol%*
Lysine	115.7	7.5	46.5	6.6	34.4	6.1
Histidine	31.8	2.1	9.3	1.3	8.3	1.5
Arginine	60.0	3.9	20.5	2.9	18.7	3.3
Asparatic acid	139.9	9.1	49.3	7.0	44.4	7.9
Threonine	79.8	5.2	45.7	6.5	31.4	5.6
Serine	157.3	10.2	82.5	11.8	66.6	11.8
Glutamic acid	167.9	10.9	78.6	11.2	60.6	10.8
Proline	94.1	6.1	26.6	3.8	23.6	4.2
Glycine	172.0	11.2	87.2	12.5	70.6	12.6
Alanine	134.3	8.7	63.0	9.0	54.9	9.8
Valine	116.7	7.6	58.0	8.3	43.5	7.7
Methionine	24.2	1.6	7.4	1.0	4.6	0.8
Isoleucine	73.9	4.8	27.9	4.0	25.9	4.6
Leucine	97.0	6.3	57.0	8.1	44.1	7.8
Tyrosine	34.6	2.2	16.9	2.4	11.6	2.1
Phenylalanine	41.4	2.7	23.6	3.4	13.6	2.4
Half-cysteine[a]	0	0	0	0	5.6	1.0
Tryptophan	ND[b]		ND		ND	

[a] Determined as cysteic acid according to Hirs (25).
[b] ND, not determined.

TABLE VI

Summary on the components of lipovitellin complex from A. salina

Component	Number of molecules	Molecular weight of individual component
Apoprotein LV-α1	2	190,000
LV-ε	2	68,000
Apoprotein total	(4)	516,000
Carbohydrate	105	180[a]
Carbohydrate total	(105)	19,000
Lipid		
Phospholipid	42	780[a]
Neutral lipid	26	580[a]
Canthaxanthin	4	550
Lipid total	(72)	50,000
Total	181	585,000

[a] Weight-average molecular weight based upon the data in Tables I and III.

lated LV-α polypeptides, and LV-δ and LV-ε components. As shown in Fig. 2, whereas all LV-α polypeptides display mostly common fragments, LV-δ and LV-ε yield significantly different peptide patterns from those of the LV-α polypeptides. It is also evident that LV-δ and LV-ε are distinct components to each other. Fig. 3 demonstrates two-dimensional gel analysis of the apoprotein components of the gastrula lipovitellin using isoelectric focusing in the first dimension and SDS gel electrophoresis in the second according to the method of O'Farrell (22). All the LV-α polypeptides present in this preparation are well separated within the pI range of 5.24 to 5.35. LV-δ is found slightly more acidic (pI = 5.17) than LV-α1 (pI = 5.25), whereas LV-ε is more basic (pI = 6.04) than the latter. Furthermore, the amino acid composition of LV-δ is different from that of LV-α1, but is very similar to that of LV-ε. However, LV-ε contains 5 to 6 residues of cysteine, whereas LV-δ (and also LV-α1) lacks this residue (Table V). On the basis of these data, we conclude that LV-δ is an independent minor component in the lipovitellin complex of the *A. salina*.

Composition of Lipovitellin Complex—The results from gel filtration experiments using a column of Bio-Gel A-1.5m, where a linear relationship of the logarithm of molecular weight for the reference proteins against partition coefficient is utilized to estimate an apparent molecular weight of *A. salina* lipovitellin complex. An apparent molecular weight of about 600,000 was determined, which might reasonably agree with a sedimentation coefficient of 14–15 S estimated by linear sucrose density gradients (19). Due to the lack of other biophysical data, we consider our estimate for the molecular weight of *A. salina* lipovitellin still tentative. Nevertheless, we could calculate the number of molecules of each components constituting the lipovitellin complex on the basis of the data presented in this study. Since only a small amount of LV-δ is detected in the oocyte lipovitellin, this component is not considered as a constant constituent of every lipovitellin molecule and therefore is omitted from the calculation. The calculated molecular weight is 585,000 (Table VI) and this value represents the complex containing two sets of the apolipovitellin pair (LV-α1 and LV-ε). It seems likely that although a monomeric form is also possible, *A. salina* lipovitellin in the native state exists in a dimeric form. In fact, the dimeric form of lipovitellin complex has been found in the silkworm (37) as well as in the african toad (42, 56).

DISCUSSION

The results presented in this study demonstrate that *A. salina* lipovitellin complex consists of approximately 3.3% carbohydrate, 8.6% lipid, and 88% apoprotein. The M_r = 190,000 LV-α1 and the M_r = 68,000- Mr LV-ε in an equimolar ratio constitute the apoprotein fraction. During embryonic development, LV-α1, but not LV-ε, undergoes a preferential proteolysis *in vivo* giving rise to multiple components of intermediate molecular weights. The examples of the apovitellin component containing two subunits, as in the case of *A. salina*, have been reported for a few insects and amphibian lipovitellins (37, 57, 58). The mosquito (*Culex pipiens fatigans* (58) as well as the silkworm (37) contain a set of two polypeptide chains of M_r = 160,000 and M_r = 82,000, and of M_r = 230,000 and M_r = 55,000, respectively, in their lipovitellins. Earlier it was also proposed that the toad lipovitellin consisted of two polypeptide chains of M_r = 120,000 and M_r = 31,000 (58). However, recent studies showed that the lower molecular weight component of the two toad apolipovitellins was heterogeneous, containing two polypeptides of M_r = 35,000 and M_r = 32,000 in an approximately equimolar ratio, and possibly also the third component of M_r = 31,000 in a lesser amount (42). Moreover, by employing a high resolution SDS gel electrophoresis system, Wiley and Wallace (59) were able to separate in the toad serum three vitellogenin molecules of similar molecular weights (M_r = 197,000, 188,000, and 182,000, respectively). The vitellogenin is the precursor molecule which gives rise to apolipovitellin and phosvitin by the specific proteolysis. Therefore, the situation in the toad lipovitellin complex appears more complex and remains to be clarified.

Very few reports have appeared to date dealing with the apolipovitellin structure in the crustacean. The fresh water shrimp (*B. stagnalis*) contains five major components of apparent molecular weights between 45,000 and 85,000, and several minor ones (36). In this case, considering the fact that *B. stagnalis* belongs to the same order anostraca as does *A. salina*, it might be possible that the lipovitellin had already undergone a partial proteolysis before SDS gel analysis as observed in *A. salina*. However, the crayfish lipovitellin consists of five components, two larger ones (M_r = 83,000 and 73,000) of which are sufficiently distinct to each other on the basis of amino acid compositions, excluding a possibility of the latter being a degradation product of the former (17, 50). In the case of insects, the fruit fly (*Drosophila melanogaster*) lipovitellin is found to contain three distinct but similar sized polypeptides (M_r = 46,000, 45,000, and 44,000) (60, 61), whereas the house cricket (*Acheta domesticus*) lipovitellin consists of four different sized polypeptides with the molecular weights between 130,000 and 47,000 (62). The locust lipovitellin, on the other hand, displays a more complex structure having at least eight polypeptides of molecular weights ranging from 140,000 to 52,000 (39). It thus appears from the limited data available to date that no particular subunit pattern of the lipovitellin complex can be ascribed to any specific classes or orders in the animal kingdom.

The molecular weight of the native lipovitellin from several decapod crustaceans was determined by the biophysical method and was found to be around 350,000 (43). The molecular weight of the crayfish (50) and the prawn (*Plesionika edwardsi*) lipovitellins was estimated to be about 500,000 (41). In either case, these lipovitellins contained relatively high lipid content of around 30% (41, 43, 50), in comparison with those from *A. salina* and several insects, in which only about 10% lipid was detected (37–40, 45). The estimated molecular weight of the lipovitellin from the anostraca crustacean, *A. salina* and *B. stagnalis* (36), has turned out to be even higher in the range of 600,000 to 650,000. This, however, compares rather well with the value (M_r = 500,000 to 660,000) found in insect lipovitellins with the low lipid content (37–40). No statement can be made at present on a possible structural similarity in the higher order between these two types of lipovitellins.

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