Crustacean Lipovitellin. Isolation and Characterization of the Major High-Density Lipoprotein from the Eggs of Decapods*

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ABSTRACT: A simple procedure is described by which the principal protein component of the eggs and mature ovaries of six decapod crustaceans was isolated in relatively pure form and in large amounts. The component, in all cases, was a lipoprotein (30% lipid) which lacked protein-bound phosphorus and had an average molecular weight of 3.5×10^5. The relatively intense chromatic properties of the crustacean lipoproteins were due to the presence of a carotenoid noncovalently bonded to the lipid and/or protein. Those proteins displaying colors (purple, blue, and green) associated with shorter wavelengths of the visible spectrum undergo spectral changes dependent upon solvent conditions. Although these crustacean proteins may not be homologous to the vertebrate lipovitellins, they appear to serve in the same capacity, and generic use of the term "lipovitellin" is thus suggested for the major high-density lipoprotein found within animal eggs.

The crustacean lipovitellins appear to offer several advantages for studies of lipoproteins in general and lipoprotein synthesis in particular.

The eggs of many of the crustaceans have long been noted for their intense and distinctive coloration, which ranges from purple (*Pachygrapsus*), blue (*Scapholebris*), and green (*Daphnia*) to yellow (*Callinectes*), orange (*Emerita*), and red (*Polyonyx*). When preserved or "fixed" for cytological study, those eggs which display colors associated with the shorter wavelengths of the visible spectrum undergo a color change to orange or red. Similar color changes have been noted as a normal course during embryonic development (*e.g.*, Ball, 1944; Goodwin, 1951; Green, 1957). The cellular agent primarily responsible for this variable pigmentation has been identified as a carotenoprotein (see Fox (1953), p 117 ff), and the conjugated lobster protein has been briefly characterized with regard to molecular size (Wyckoff, 1937; Ceccaldi *et al.*, 1966), and given the name "ovoverdin" (Stern and Salomon, 1937). The carotenoid moiety of the pigments has been shown to be noncovalently bound to the protein since it can be extracted readily with polar lipid solvents, and in most cases it has been identified as astaxanthin or an astaxanthin ester (Goodwin, 1960; Cheesman and Prebble, 1966).

Ovarian maturation in many crustaceans, and hence the deposition of the above carotenoproteins in the vitellogenic oocyte, is apparently under neurosecretory control and can be initiated in mature, but "dormant," females by eyestalk ablation (Panouse, 1943; Brown and Jones, 1949; Ōtsu, 1963). The synthetic site for the crustacean egg carotenoproteins, in agreement with well-documented information available for insects (Telfer, 1965) and vertebrates (Urist and Schjeide, 1961; Heald and McLachlan, 1965), has generally been suggested as being external to the oocyte (Smith, 1911; Abeloos and Fischer, 1926; Green, 1957; Kerr, 1966). Beams and Kessel (1963), however, have demonstrated a well-established endoplasmic reticulum in the crayfish oocyte, and contend that "the precursors of proteinaceous yolk are formed largely within the rough surfaced, stacked cisternae (of the oocyte's endoplasmic reticulum) and become distributed throughout the ooplasm by moving along the largely smooth surfaced and branched cisternae, where they eventually aggregate and undergo chemical change resulting in definitive yolk bodies."

Because of the many interesting properties of the crustacean egg carotenoproteins, the ability to subject their production to experimental control, and the conflicting reports regarding the mechanism of their synthesis and accumulation, the present investigation was undertaken to define, in biochemical terms, the major component of crustacean egg yolk. This information should be of some help in further and perhaps less speculative studies regarding the synthesis and transport of this component. We shall describe a general method for the isolation and temporary preservation of six decapod yolk proteins and provide some information regarding their molecular and spectral characteristics.

Materials and Methods

Reagents. Pepsin, yeast alcohol dehydrogenase, bovine catalase, and bovine thyroglobulin were ob-

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tained from Sigma Chemical Co., bovine serum albumin from Armour Pharmaceutical Co., and human hemoglobin from Pentex, Inc. Agarose was purchased from Bausch and Lomb; Basophor EL was obtained from BASF Colors and Chemicals, Inc. (Charlotte, N. C.); and Blue Dextran 2000 was a product of Pharmacia Fine Chemicals, Inc. (New Market, N. J.). Dimethyldichlorosilane, as a 1% solution in benzene, was obtained from Bio-Rad Laboratories. Reagent grade ammonium sulfate was recrystallized from EDTA[1] and a 0° saturated solution prepared as described previously (Wallace, 1963b). All water was glass distilled.

Animals. *Pagurus pollicaris* (hermit crab), *Uca pugilator* (fiddler crab), *Sesarma reticulatum*, and *Libinia emarginata* (spider crab) were collected from the Woods Hole area in the latter half of May, and *Homarus americanus* (lobster) and *Cancer irroratus* (rock crab) were obtained from the same region during the first part of August. At the time of collection, the ovaries of all animals, with the exception of *Pagurus*, were fully ripe with mature or ovulating oocytes and were dissected out and used as a source material. Females of *Pagurus* were carrying eggs in very early stages of development, and clusters of such embryos were removed from the pleopods for processing.

Isolation of Yolk Proteins. Ovaries or eggs were washed and thoroughly dispersed with a tight-fitting Teflon homogenizer in three volumes of 0.5 M NaCl–5 mM EDTA (pH ~5). The mixture was centrifuged at 30,000 rpm for 30 min (Spinco Model L centrifuge) to remove debris and fatty material. The centrifuge tubes were then punctured and the clear subnatant was collected through a Whatman No. 1 filter. To the filtrate was added, slowly with stirring, two volumes of 100% saturated ammonium sulfate. The precipitate was collected by centrifugation at 30,000 rpm for 30 min and could be stored at 0–5° for about 1 month when overlaid with fresh 67% saturated ammonium sulfate.

To purify further the yolk proteins for analytical work, the precipitates from the above step were solubilized by the addition of several volumes of water and the mixture was subsequently dialyzed against at least 20 volumes of 45% saturated ammonium sulfate. Any insoluble material was removed centrifugally, the volume of the solution was noted, and four-sevenths volume of saturated ammonium sulfate slowly added to give a final concentration of 65% saturation. The precipitate was collected centrifugally and dissolved in and thoroughly dialyzed against three to four changes of 0.5 M NaCl–5 mM EDTA.[2] Any insoluble material which appeared after dialysis was also removed centrifugally. All of the above operations were performed in the cold and with a minimum exposure to light.

Determination of Diffusion Coefficients with a 6% Agarose Column. Agarose beads were prepared according to the method of Hjertén (1964). Each batch of emulsion mixture consisted of 300 ml of 6% agarose (6 g of agarose/100 ml of H_2O dissolved by autoclaving several times), 450 ml of toluene, 150 ml of carbon tetrachloride, and 10 ml of Basophor EL. The beads were freed of organic solvents, and, by a combination of wet sieving and decantation, a 120–200 mesh (75–125-μ diameter) fraction, representing about 20% of the original agarose, was collected for use.

A 2.5-cm diameter Sephadex K-25 column (Pharmacia Fine Chemicals, Inc.), whose inner surface had been rendered hydrophobic by treatment with a dimethyldichlorosilane solution, was poured to a height of 95 cm, essentially as described by Gelotte (1964). A Sephadex "sample applicator" was then positioned and maintained on top of the bed. The applicator, which did not measurably compress the agarose bed, routinely allowed an even application of sample without disturbing the top of the column. The column was kept at 2.5 ± 1.0°, and a constant flow rate maintained at 24.3 ± 0.2 ml/hr with a Milton-Roy Minipump. The effluent was monitored with an ultraviolet absorption meter (UV-280IF, Gilson Medical Electronics).

The void volume (V_0) of the column was periodically determined with solutions of Blue Dextran 2000,[3] and was consistently found to be 166.5 ml. The volume of solvent internal to the gel phase (V_i) was taken as the elution volume of tritiated water minus the void volume, and was found to be 303.0 ml. The distribution coefficient (K_D) for a given solute was then defined (Gelotte, 1964) as $K_D = (V_e - V_0)/V_i$, where V_e is the effluent peak position of the solute. The distribution coefficient can then be related to the effective Stokes radium (a) of the solute molecule by employing the relationship derived by Ackers (1964) for restricted diffusion processes in molecular sieve chromatography, whereby

$$K_D = \left(1 - \frac{a}{r}\right)^2\left[1 - 2.104\frac{a}{r} + 2.09\left(\frac{a}{r}\right)^3 - 0.95\left(\frac{a}{r}\right)^5\right] \quad (1)$$

provided that r, the effective pore radius within the gel and the only column parameter, is known. To determine r, 10-mg samples of proteins with a known diffusion coefficient were applied to the agarose column, and the K_D value for each protein was measured (Table I). The equivalent Stokes radii were calculated from the known diffusion coefficients (D^0) by the Stokes–Einstein equation, $a = kT/6\pi\eta D^0$, where k is the Boltz-

[1] Abbreviation used: EDTA, disodium ethylenediaminetetraacetic acid.

[2] A slight opalescence, as yet unidentified, frequently appeared in the last diffusates of the *Homarus* preparations.

[3] The weight-average molecular weight of this polydisperse conjugated polysaccharide is claimed to be 2×10^6 by the manufacturer. The initial peak coming off the column and comprising about two-thirds of the ultraviolet-absorbing material (see Figure 1) was judged to represent very high molecular weight material completely excluded from the agarose matrix. A similar peak position was noted for the floating fraction of hen's egg yolk, which has an average molecular weight of 5×10^6 (Martin *et al.*, 1959).

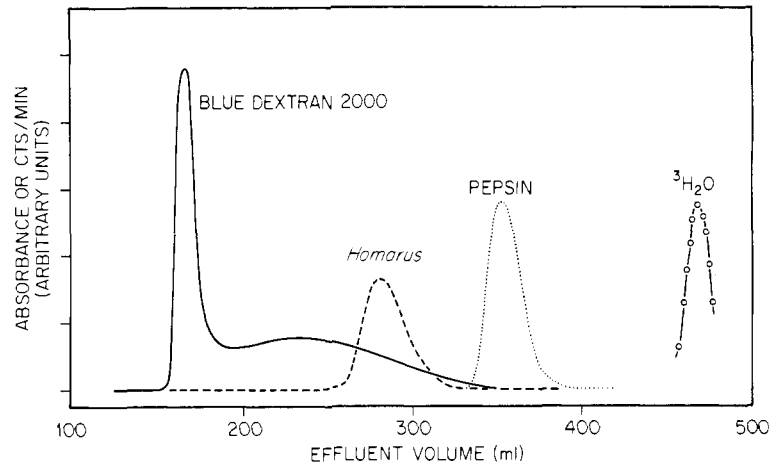

FIGURE 1: Representative elution patterns using a 6% agarose column (r = 25.8 mμ). The patterns for Blue Dextran 2000, *Homarus* egg lipoprotein, and pepsin were traced from the chart paper, whereas 2-ml fractions of the effluent were measured in a scintillation counter for ^3H_2O; solvent, 0.5 M NaCl–5 mM EDTA (pH 5).

mann constant, T the absolute temperature, and η is the viscosity of water (all values are corrected to water as a solvent at 20.0°). The effective pore radius for the 6% agarose column was then determined from K_D and a by using the computed relationship between K_D and a/r in eq 1 as provided by Ackers (1964) in his Table III. This value for r was found to be 25.8 mμ (Table I).

The column, once calibrated, could thus be used to determine the diffusion coefficient of unknown proteins by a similar procedure. For this purpose, 5–25-mg samples of each crustacean egg protein in a 1–2-ml volume were applied to the column, and the average K_D values determined from 6 to 10 samples were used for the calculations.

TABLE I: Determination of the Mean Effective Pore Radius (r) for a 6% Agarose Column.

Protein	K_D	$D^0_{20,w} \times 10^7$ (cm²/sec)	a (mμ)	r (mμ)
Thyroglobulin	0.186[a]	2.60[d]	8.24	26.1
Catalase	0.401[a]	4.46[e]	4.80	25.1
Alcohol dehydrogenase	0.429[a]	4.70[f]	4.56	25.6
Serum albumin	0.543[b]	5.90[g]	3.63	27.1
Hemoglobin	0.613[a,c]	6.90[h]	3.10	28.4
Pepsin	0.616[b]	8.71[i]	2.46	22.8
	Mean effective pore radius			25.8

[a] Solvent, 0.5 M NaCl–5 mM EDTA–0.1 M Tris-Cl (pH 8). [b] Solvent, 0.5 M NaCl–5 mM EDTA (pH 5). [c] Using 0.5 M NaCl as a solvent, K_D = 0.685 and, assuming a half-mer (Andrews, 1962; Ackers, 1964), a = 2.46 mμ and the value for r is 28.3 mμ. [d] Derrien *et al.* (1949). [e] Summer *et al.* (1940). [f] Hayes and Velick (1954). [g] Wagner and Scheraga (1956); value corrected for the presence of 3% dimer. [h] Lamm and Polson (1938); Field and O'Brien (1955). [i] Edelhoch (1957).

Other Physical and Chemical Determinations. Sedimentation studies were performed at 20.0° with a Spinco Model E ultracentrifuge, using 4° Kel-F standard and wedge cells at 56,100 and 59,780 rpm, schlieren optics, and a Wratten No. 77A filter for the mercury lamp. Photographs were taken on Kodak metallographic plates, and the boundary positions were measured with a Nikon comparator. The least-squares fit to a plot of log x *vs.* t was used to obtain s_{20}, which was then corrected to standard conditions.

Partial specific volumes were measured gravimetrically with a 10-ml pycnometer at 20.0° using 1–2% protein solutions. Lipid determinations were performed as previously described (Wallace, 1965), and nitrogen and phosphorus analyses of the delipidated samples were made by Galbraith Laboratories (Knoxville, Tenn.). Absorptivity measurements were made at 280 mμ on solutions of known concentration, as determined by dry weight analysis employing a solvent displacement correction (Cook and Wallace, 1965). Spectral analyses were performed with a Cary Model 15 recording spectrophotometer. In all cases, the solvent used in the above determinations was 0.5 M NaCl–5 mM EDTA (pH ~5).

Results

Isolation and Stability of the Proteins. Preliminary experiments with *Homarus* and *Cancer* indicated that the majority of proteins extracted from the eggs and ovaries could be precipitated as the ammonium sulfate concentration was increased from 45 to 65%. This was also found to be true of embryos in very early stages and of fresh, mature ovaries of the other decapods

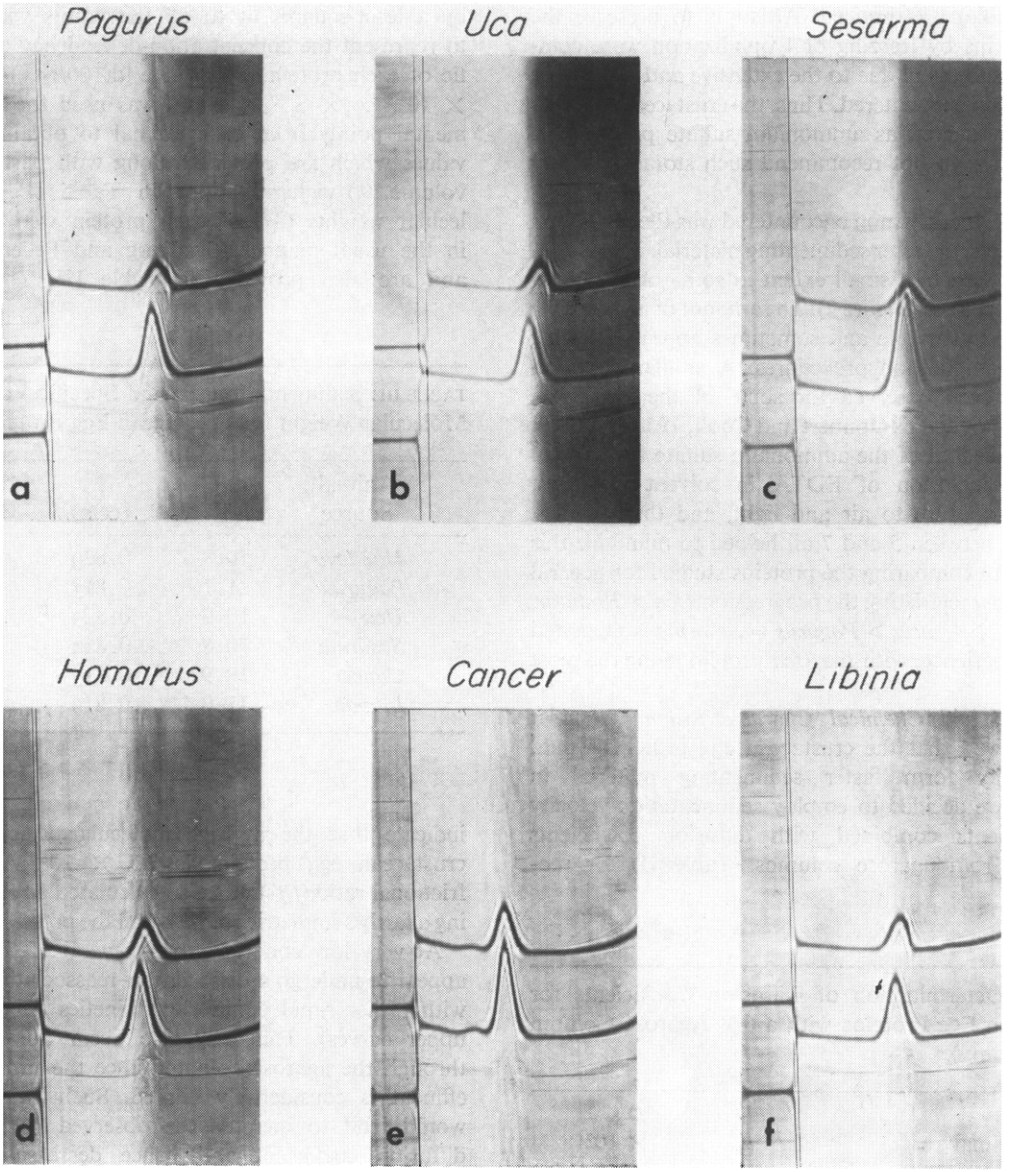

FIGURE 2: Sedimentation patterns for crustacean egg lipoproteins. (a) *Pagurus*, 1.76 (upper) and 4.40 (lower) mg; (b) *Uca*, 1.42 and 3.56 mg; (c) *Sesarma*, 1.96 and 6.88 mg; (d) *Homarus*, 2.34 and 4.68 mg; (e) *Cancer*, 3.22 and 6.11 mg; and (f) *Libinia*, 1.98 and 3.97 mg. Light-absorbing material can be seen to sediment simultaneously with light-refracting material, the absorbance being most pronounced for the purple proteins (a–c). A small amount of faster sedimenting material is present in a, b (upper), c (lower), and f (upper). Material sedimenting more slowly than the main peak is present in the *Cancer* preparation (e), but this was not a normal occurrence. Skewed peaks, possibly indicating dissociation of the main component at low concentrations, are seen in the upper curves of a, b, and f. Minutes after reaching speed and speed for each preparation, respectively: (a) 35 min at 59,780 rpm; (b) 34 min at 59,780 rpm; (c) 40 min at 56,100 rpm; (d) 41 min at 56,100 rpm; (e) 50 min at 59,780 rpm; and (f) 39 min at 56,100 rpm. Bar angle is 65° and solvent is 0.5 M NaCl–5 mM EDTA (pH 5) in all cases.

studied. This procedure was therefore used as a general method for purifying and eventually recovering at least 70% of the protein originally present as a relatively homogeneous fraction as judged by gel filtration (Figure 1) and ultracentrifugation (Figure 2).

"Aged" material, whether from embryos in later stages of development or from ovaries which had not been immediately extracted or from preparations which had been stored as an ammonium sulfate precipitate for more than 1 month, tended to salt out at lower ammonium sulfate concentrations. The color of the ammonium sulfate pellets also tended to fade slightly with time (especially in the case of *Pagurus*, but also with *Sesarma*, *Cancer*, and *Homarus*), and a concomitant increase in material insoluble in solutions of low ionic strength (in which fresh preparations are normally

quite soluble) was noticed. Attempts to preserve the yolk proteins by freezing or lyophilization were completely unsuccessful due to the extensive and immediate denaturation encountered. Thus, the crustacean proteins are best preserved as ammonium sulfate precipitates, although we do not recommend such storage for any lengthy period.

Another phenomenon encountered was the formation of aggregates, or faster sedimenting material, in solution, as can be seen to a small extent in some of the ultracentrifuge studies (Figure 2). The amount of aggregating material was variable and sometimes appeared during normal manipulation procedures. A similar phenomenon has been observed for some of the vertebrate egg yolk lipovitellins (Joubert and Cook, 1958; Wallace, 1963a). Freedom of the ammonium sulfate from metal ions, the inclusion of EDTA in solvent solutions, minimal exposure to air and light, and the use of a pH range between 5 and 7 all helped to minimize this problem. In comparing the proteins studied for general stability characteristics, the progression *Uca* > *Homarus* = *Libinia* ≫ *Cancer* > *Pagurus* = *Sesarma* is suggested by our experience, with the *Uca* protein being the most stable.

Physical and Chemical Characterization. When it was observed that the crustacean egg proteins would occasionally form faster sedimenting material in solution, we decided to employ sedimentation-velocity measurements combined with diffusion coefficients obtained from agarose columns (Table II) for molecular weight determinations, since such procedures are influenced to a minimal extent by irreversible aggregate formation.

TABLE II: Determination of Diffusion Coefficients for Crustacean Egg Proteins with a 6% Agarose Column (r = 25.8 mμ).[a]

Animal Source	K_D	a (mμ)	$D^0_{20,w} \times 10^7$ (cm²/sec)
Homarus	0.374	5.3	4.1
Pagurus	0.358	5.4	3.9
Uca	0.408	4.9	4.4
Sesarma	0.362	5.4	4.0
Cancer	0.399	5.0	4.3
Libinia	0.363	5.4	4.0

[a] The solvent in all cases was 0.5 M NaCl–5 mM EDTA (pH 5).

The corrected $s_{20,w}$ values from all the sedimentation runs on the different proteins were plotted as the reciprocal against concentration (Figure 3). Because the various proteins seemed to display the same concentration dependence, the slope of the line obtained by a least-squares fit to all the points was assumed to represent the concentration-dependency characteristic of each protein. This value [$d(100/s_{20,w})/dc$ = 6.32 × 10^{-2} cc × S^{-1} × mg^{-1}] was used for the experimental points from each animal to obtain the $s^0_{20,w}$ values which are provided along with partial specific volume ($\bar{v}$) determinations in Table III. The molecular weights (M) of each protein were calculated in the usual manner (Svedberg and Pedersen, 1940) and are also provided in Table III. These values indicate that the average molecular weight for the crustacean egg proteins is 3.5 × 10^5. An average frictional ratio (f/f_0) was also calculated as 1.1, suggesting a fairly symmetric shape for all the proteins.[4]

TABLE III: Sedimentation, Partial Specific Volume, and Molecular Weight for Crustacean Egg Proteins.

Animal Source	$s^0_{20,w}$ (S)	$\bar{v}$ (cc/g)	$M \times 10^{-5}$
Homarus	10.8	0.820	3.6
Pagurus	11.1	0.815	3.7
Uca	10.9	0.823	3.4
Sesarma	10.8	0.814	3.5
Cancer	10.9	0.815	3.3
Libinia	11.0	0.819	3.7

At very low concentrations, several of the proteins appear to undergo a dissociation–reassociation reaction with rather rapid equilibrium kinetics (Figure 2a,b,f, upper curves). This may also occur during passage through the agarose column, since the protein in the effluent is considerably diluted. Such an occurrence would tend to increase the observed K_D value and diffusion coefficient and hence decrease the value derived for the molecular weight. The extent to which dissociation of the crustacean proteins may have occurred during passage through the agarose column was not thoroughly assessed, although no difference in elution position was noticed using samples with a concentration range of 5–25 mg/ml.

Chemical data, together with absorptivity values, are provided in Table IV, and indicate that the crustacean egg proteins are lipoproteins with an average lipid content of 30% and are free of protein-bound

[4] An approximate frictional ratio (f/f_0) was calculated from the relationship a/a_0, where $a_0 = (3M\bar{v}/4\pi N)^{1/3}$ and represents the radius of the smallest sphere compatible with the molecular weight. As a test of this calculation, the relatively asymmetric bovine fibrinogen molecule was found to have a K_D value of 0.118 on the agarose column employed, thus providing a value for a of 9.81 mμ and indicating that f/f_0 = 2.19, in fair agreement with the literature value of 2.34 (Shulman, 1953). Alternatively, if the fibrinogen data were included in the column calibration (Table I), the mean effective pore radius would be 26.1 mμ.

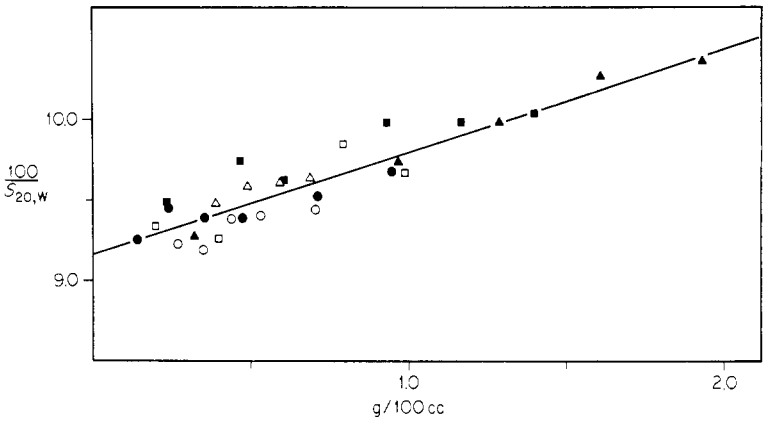

FIGURE 3: Sedimentation coefficient of crustacean egg lipoproteins as a function of concentration: (○) *Pagurus*, (●) *Uca*, (△) *Sesarma*, (▲) *Cancer*, (□) *Libinia*, and (■) *Homarus*. Solvent: 0.5 M NaCl–5 mM EDTA (pH 5). The line represents the least-squares fit to all the points.

phosphorus. The lipid extract contained the chromatic agent and was always orange or orange-red regardless of the color of the original lipoprotein, whereas the delipidated protein was colorless.

Spectral Characteristics. Solutions of some of the crustacean egg lipoproteins change color dependent upon the solvent conditions. This is more a property of purple, blue, and green proteins than of orange and red. As examples, characteristic spectra in the solvent used for these studies, 0.5 M NaCl–5 mM EDTA (pH 5), are provided in Figure 4 together with spectra for the same proteins under alkaline conditions. At pH 5, the green lipoprotein from *Homarus* displays a large peak with a maximum at 463 mμ and a smaller broad peak at about 645 mμ. At pH 10.5, the latter peak is completely gone and the major peak has shifted to 477 mμ, providing an orange solution. The purple lipoprotein from *Uca* has a single broad peak at pH 5 with a maximum around 513 mμ. At pH 9, the peak is considerably sharpened and shifted toward shorter wavelengths (maximum at 488 mμ), again providing an orange solution. A spectral shift displayed by the *Sesarma* lipoprotein is essentially the same as observed for *Uca*, except that it is complete at pH 7.5. At both pH 5 and 9, however, the visible absorption maxima for the orange lipoprotein from *Cancer* remain the same, with a doublet peak at 467 and 492 mμ.

As represented in Figure 4, the values for solutions at pH 5 are absorptivity values, whereas those spectra recorded under alkaline conditions were equated with the pH 5 spectra at 280 mμ and subsequently plotted. An isosbestic point has been noted for proteins at or very near 280 mμ (Shugar, 1952), so that whatever small difference in absorptivity that may exist at this wavelength at pH 5 and under alkaline conditions is most likely insufficient to account for the pronounced hyperchromicity observed in the visible spectrum with alkaline solvents. A hyperchromatic effect during the transformation of the *Homarus* protein from green to red under conditions whereby the protein concentration was kept strictly constant was also noted by Stern and Salomon (1938).

TABLE IV: Absorptivity Values and Chemical Data for Crustacean Egg Proteins.

Animal Source	Absorptivity at 280 mμ (l./g × cm)	Lipid (%)	Protein N (%)	Protein P (%)
Homarus	0.85	29	15.3	<0.01
Pagurus	1.18	33	13.6	<0.01
Uca	1.25	32	14.6	<0.01
Sesarma	1.39	29	15.0	<0.01
Cancer	1.11	28	14.8	<0.01
Libinia	0.76	30	13.8	<0.01

Discussion

A single lipoprotein from the mature ovaries and early embryos of six different decapods has been recovered in each case as the primary (although perhaps not exclusive) yolk protein of these crustaceans. Although a considerable difference exists among the lipoproteins with respect to spectral characteristics, physical and chemical characterization indicates that within an experimental error of ±5% they are very similar components. Lipoproteins which previously have been isolated in reasonably pure form from crustacean ovaries or embryos have included the green protein from the lobster (*Homarus* sp.; Stern and Salomon, 1938; Kuhn and Sörensen, 1938; Ceccaldi *et al.*, 1966), the blue protein from the goose barnacle (*Lepas* sp.; Ball, 1944), the purple protein from the hermit crab

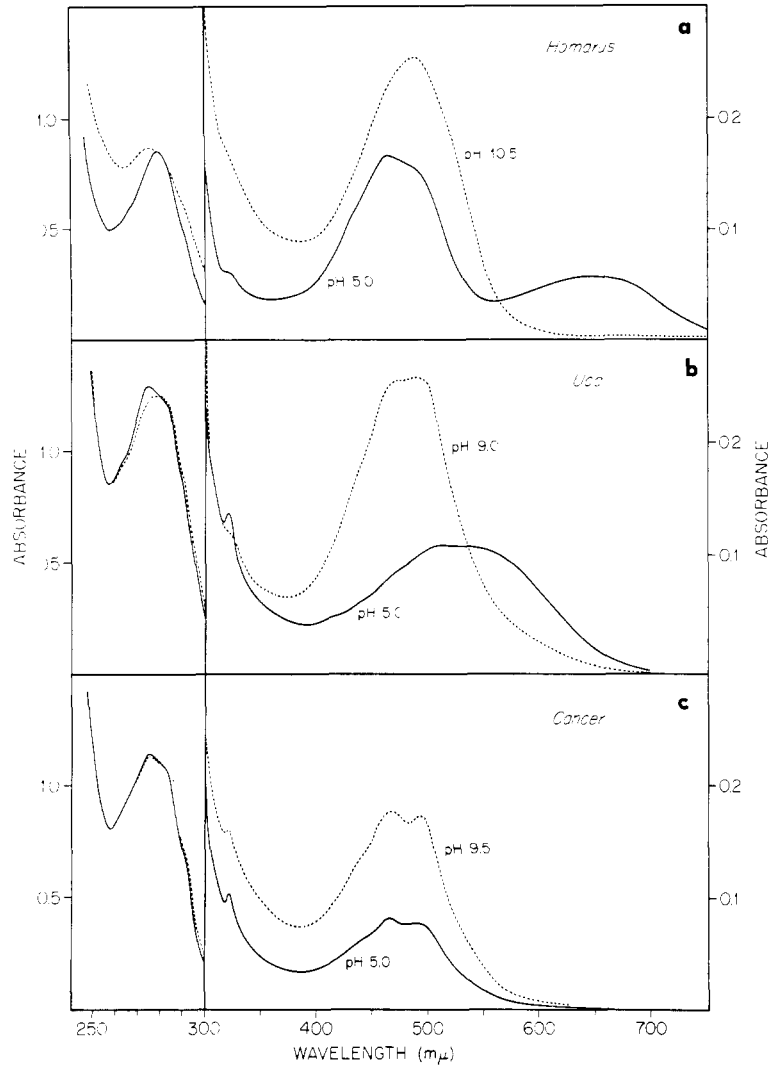

FIGURE 4: Ultraviolet and visible absorption spectra for *Homarus* (a), *Uca* (b), and *Cancer* (c) egg lipoproteins in 0.5 M NaCl–5 mM EDTA at pH 5 (solid line) and pH 9–10.5 (dashed line). The latter condition was achieved by thorough dialysis against 0.5 M NaCl–5 mM EDTA adjusted with NaOH. The spectra at pH 5 are true absorptivity values (solute concentration = 1 mg/cc), whereas the spectra for alkaline solvents were equated at 280 mμ in each case with the curve for pH 5.

(*Eupagurus* [*Pagurus*] *bernhardus;* Cheesman and Prebble, 1966), and the yellow protein from the blue crab (*Callinectes sapidis;* Kerr, 1966). Most of these investigators were interested in either the spectral characteristics of the carotenoproteins under a variety of conditions or the identification of the prosthetic group, whereas little information exists regarding other properties.

Wyckoff (1937), on the basis of several runs in the ultracentrifuge, suggested a molecular weight of 3.0 $\times$ 10^5 for "ovoverdin," the carotenoprotein isolated from *Homarus* eggs. His corrected sedimentation constant (10.8) and that found here for the same protein are identical. The inclusion of the diffusion coefficient and particularly the partial specific volume into the molecular weight calculation, however, provide a larger value of 3.6 $\times$ 10^5 (Table III). More recently, Ceccaldi *et al.* (1966) have estimated the molecular weight for ovoverdin as 3.8 $\times$ 10^5. This estimation is based entirely on the gel filtration method of Andrews (1964), which relates log M to V_e. Such a procedure, however, would tend to give too large a value since lipoproteins have a larger effective Stokes radius per unit molecular weight than other proteins with a greater density but similar configuration.

The spectral shifts observed by various investigators for the egg lipoproteins of *Homarus* (Stern and Salomon, 1938), *Lepas* sp. (Ball, 1944), and *Pagurus* (Cheesman and Prebble, 1966), under a variety of conditions, have also been noted here for *Homarus* and *Uca*, but not for *Cancer*. Presumably, the carotenoid present in *Cancer* is either of a different type, or else is already in a state which is achieved in the other forms only after

a configurational change. The precise cause of the spectral shifts will remain an unresolved problem until a better understanding is achieved concerning the relationship of the carotenoid to its lipid and protein environment. It is known, at least, that the carotenoid is not covalently bound to the protein, since it can be extracted with lipid solvents, especially those of the polar type which most effectively "denature" or unfold the structure of the protein. The carotenoid may thus reside in hydrophobic "pockets" of the native protein and may not necessarily be associated with the protein through ionic linkages as suggested by Kuhn and Sörensen (1938; see also Cheesman and Prebble, 1966). In addition, the amount of carotenoid relative to the protein may not be stoichiometric since this relationship would depend upon the availability of the carotenoid to the protein at the time of the protein's synthesis and transport. As a possible relevant example, the eggs of *Daphnia* when reared under normal conditions are dark green due to the presence of a carotenoprotein, but are colorless if the appropriate carotenoid is missing from the diet (Teissier, 1932) or pale green if the cultures are kept in the dark (Green, 1957). Thus, it would seem that debate concerning the number of astaxanthin molecules in "ovoverdin" (Stern and Salomon, 1938; Kuhn and Sörensen, 1938; Ceccaldi *et al.*, 1966) is meaningless if based on the assumption of a stoichiometric relationship.

The major high-density lipoprotein present in hen's egg yolk has received considerable attention and has been given the name "lipovitellin" (Joubert and Cook, 1958). A similar (Fujii, 1960) and apparently homologous (Wallace, 1963b, and unpublished data) lipoprotein has been identified from the egg yolk of other vertebrate species. Vertebrate lipovitellin has a molecular weight of $4.2–4.5 \times 10^5$ (Wallace, 1963b; Cook and Wallace, 1965) and a lipid content ranging from 16 to 22% (Fujii, 1960; Burley and Cook, 1961; Wallace, 1965), and undergoes dissociation in alkaline solvents (Burley and Cook, 1962; Cook and Wallace, 1965; Wallace, 1965). Furthermore, they are phosphoproteins (Fujii, 1960; Burley and Cook, 1961; Wallace, 1963b), with the most recent protein phosphorus values ranging from 0.27 to 0.73% (Radomsky and Cook, 1964; Wallace, 1965). The crustacean lipoproteins studied here have a smaller average molecular weight (3.5×10^5) and a larger lipid content (30%) than the vertebrate lipovitellins. Also, they are not phosphoproteins, are considerably more soluble in solutions of low ionic strength than the vertebrate lipovitellins, and, from indications of preliminary evidence (R. A. Wallace, unpublished data), do not undergo dissociation in alkaline solvents at relatively high protein concentrations. Although a further difference between the crustacean egg lipoproteins and the vertebrate lipovitellins concerns the relatively intense and variable absorptivity of the former in the visible spectrum, this factor may be greatly influenced by diet (Teissier, 1932; DuPraw, 1958; Ferrando *et al.*, 1966) or environmental conditions (Green, 1957). Thus, the crustacean egg lipoproteins and the vertebrate lipovitellins would appear to be analogous but not homologous proteins, although a final conclusion on homology must await peptide and amino acid sequence studies.

The lipoprotein from lobster eggs has been named "ovoverdin" by Stern and Salomon (1937), thus indicating its green color. However, in view of the fact that the crustacean carotenoproteins display a variety of colors, we feel that the designation of these proteins according to color evokes a historical concern of presently diminished significance and if used would arbitrarily assign, for example, all purple proteins under one name and orange proteins under another, although they may be closely related and even have the same prosthetic group. Furthermore, an adjustment of solvent conditions could very well produce similarly colored solutions for proteins of different nomenclature. We therefore propose the use of the term "lipovitellin" in a generic sense to designate the major high-density lipoprotein found within the mature egg, and would call the six proteins of the present report "crustacean lipovitellin." Such terminology would appear to be more in keeping with the guidelines of protein nomenclature, under which nonhomologous (in terms of protein structure or amino acid sequence) proteins may be given the same name, depending upon their disposition, function, or particular prosthetic group. Such a situation, for instance, exists for enzymes in general (Dixon and Webb, 1964) and for various blood proteins such as hemoglobin (Keilin and Hartree, 1951). With the definition provided, the lipoprotein isolated by Fujii (1960) from squid eggs would be a lipovitellin; but the protein described by Malkin *et al.* (1965) from sea urchin eggs must await lipid analysis before classification, and the snail carotenoprotein "ovorubin" described by Cheesman (1958) would not be a lipovitellin because it is a component of the egg jelly and hence is external to the egg.

The crustacean lipovitellins discussed in the present report can be isolated in abundant amounts as apparently homogeneous lipoproteins. As lipoproteins, they appear to be relatively stable and display interesting spectral phenomena dependent upon environmental conditions. They would thus appear to serve as excellent models for the study of lipoproteins in general, both in terms of their structural and chemical characteristics and in reference to the manner of their synthesis, assembly, and transport within the cell.

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