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Isolation and Properties of a Low Molecular Weight Protein (Apovitellenin I) from the High-Lipid Lipoprotein of Emu Egg Yolk†

R. W. Burley

ABSTRACT: As part of a study of protein–lipid interactions in avian egg yolk, the high-lipid lipoprotein (density 0.96 g/ml) of the egg yolk of the emu (*Dromaeus novaehollandiae*) has been examined. The major fraction of this lipoprotein has a particle weight of 3×10^6 with 13% of apoprotein. This apoprotein is more soluble than the corresponding apoprotein from the egg yolk of the hen (*Gallus domesticus*). Consequently several proteins (the emu "apovitellenins") that range in molecular weight from 10^4 to more than 10^5 have been recognized in the emu lipoprotein. The protein of lowest molecular weight ("apovitellenin I"), which accounts for nearly half the total apoprotein, has been isolated by chromatography in urea solution. Purified apovitellenin I contains no histidine, cystine, sulfhydryl groups, or phosphate. It contains a small amount of amino sugar. Lysine is the N-terminal residue. In disaggregating solvents (6 M guanidine hydrochloride and 6 M urea) apovitellenin I is present as a randomly coiled monomer at low concentrations. In water, in methanol, and in aqueous methanol, the protein has a high viscosity and a large proportion of α-helical structure according to optical rotatory dispersion, the maximum (nearly 80% helix) being in 50% aqueous methanol. In aqueous solutions above about pH 4 large aggregates were present and the protein was precipitated by low concentrations of salt. From its physical properties it is suggested that apovitellenin I has a structural role in the lipoprotein.

The arrangement of lipid and protein molecules in the soluble high-lipid low-density lipoproteins of avian egg yolk is not known. Lipoproteins of domestic hen's eggs have been studied intensively, but little is known about their apoproteins mainly because they are extremely insoluble in the usual nondegradative solvents for proteins. Consequently, reliable methods for their isolation and purification have not been available (Martin, 1961; Steer *et al.*, 1968; Evans *et al.*, 1968; Cook and Martin, 1969). From measurements in formic acid Martin (1961) suggested that the apoproteins of hen's egg lipoprotein have a low monomeric molecular weight. Recently, by using a mild procedure, it has been possible to isolate from the hen's lipoprotein a low molecular weight apoprotein fraction that is soluble in a water–methanol–chloroform mixture but not in solely aqueous media (Burley, 1968; Burley and Sleigh, 1971). In an extension of this work, it has been found that the apoproteins from the high-lipid lipoprotein of emu's egg yolk are all more soluble in aqueous solutions and in other solvents than those of the hen, although the lipoproteins are similar. There is apparently a series of emu apoproteins for which the term "emu apovitellenins" is proposed. The emu apovitellenin of lowest molecular weight, referred to here as "apovitellenin I," has been studied in detail in order to try to explain its function in the lipoprotein. Experiments on the location of this protein in the yolk, and on its isolation, purification, composition, and properties are described here. Its effect on lipid will be reported later.

Materials and Methods

Egg Yolk. Eight emu's eggs were studied, six of them from birds fed on a commercial laying-hen diet. When necessary, the eggs were stored at 1° for up to 9 months. Results of experiments on an egg less than 1 day old suggested that storage had no effect on apovitellenin I. The lipid composition of the yolks varied slightly with diet, and there may have been slight variations in the proportions of the apovitellenins in different eggs. Most of the results given here were from four eggs from birds fed on hen's food. The emu's eggs contained 225–243 g of yolk (about 31% of the weight of the egg) of which 56% by weight was nonvolatile in fresh yolk.

Acidic 6 M Urea. This was prepared by adding 10 N hydro-

† From the Commonwealth Scientific and Industrial Research Organization, Division of Food Research, North Ryde, N.S.W., 2113, Australia. *Received November 17, 1972.*

chloric acid to 6 M urea that had been through a mixed-bed ion exchange resin column and a Millipore filter, the final concentration being 0.05 N acid and the pH 3.3. The yolk proteins showed no signs of degradation in this solvent for several weeks. A solution of apovitellenin I contained a small amount of lower molecular weight material after a month in solution at 22°.

Guanidine hydrochloride was from Mann Research Laboratories, New York, N. Y. It was used as a 6 or 8 M solution, pH 5.5, without addition of salt. For some experiments 2-mercaptoethanol (to 0.1 M) was added.

Preparation of High-Lipid Lipoproteins from Emu Yolk. The following method was used for most preparations. To 225 g of yolk, free of white, an equal volume of 0.16 M sodium chloride was added and the stirred mixture centrifuged for 30 min at 100,000*g*, 4°, to sediment the yolk granules, which were discarded (Burley and Cook, 1961). The supernatant liquid was then saturated with sodium chloride by adding the solid salt, and the lipoprotein was floated off by centrifuging for 4.5 hr at 360,000*g* in an angle rotor at 5°. The lipoprotein (59 g dry weight) was dispersed in 1.0 M sodium chloride, total volume 500 ml, and fractionated by chromatography in batches on columns (4.3 × 80 cm) of agarose (Bio-Gel A15 from Bio-Rad Laboratories) in 1.0 M sodium chloride at 4°, volumes of 80–100 ml being applied to each column. Figure 1 shows a typical chromatographic pattern although it refers to a smaller column. When needed for comparison, hen's egg-yolk lipoprotein was prepared in the same way. An alternative procedure that could not be used with hen's egg yolk avoided both high-speed centrifuging and column chromatography by taking advantage of the insolubility of the emu lipoprotein at very low ionic strength. After the removal of the yolk granules the supernatant mixture was passed through a Millipore filter, RA, and dialyzed into water at 1° for at least 24 hr. The precipitated lipoprotein was then filtered through high-porosity sintered glass, washed with water to remove soluble proteins, and dissolved in 1.0 M sodium chloride or 6 M acidic urea.

Preparation and Purification of Apovitellenin I. Two methods were used. The first was more rapid and convenient and could be used with large samples but the yield was much lower.

METHOD I. To a solution (750 ml, 4% w/v) of the purified high-lipid lipoprotein (usually corresponding to fraction D in Figure 1) in 1.0 M sodium chloride at 20°, a mixture of chloroform (890 ml) and methanol (1870 ml) was added. After standing for 1 hr or more at 20–25° the precipitated protein (consisting of the high molecular weight apovitellenins plus some aggregated apovitellenin I) was filtered off on Whatman No. 1 paper and more chloroform (900 ml) and water (850 ml) were added to the filtrate. This mixture was allowed to stand overnight at 20–25°. Precipitated protein was collected by filtering through sintered glass. The protein (about 1 g dry) was then dissolved in acidic 6 M urea (100 ml) and centrifuged for 30 min at 100,000*g* to sediment some gelatinous protein (apparently consisting of a complex of apovitellenin I and other proteins) and the supernatant solution chromatographed on columns (4.3 × 100 cm) of Sephadex G-100 in acidic 6 M urea at 20°. The pattern was similar to that in Figure 3a, although the first peak (IA) was smaller. The central part of the second large peak (IIB) was diluted with an equal volume of water and 15% by volume of 12% trichloroacetic acid was added. After the precipitated protein had been collected by centrifuging (20 min at 2000 rpm), it was dissolved in acidic 6 M urea to give a solution of concentration less than 1%. At higher concentrations gels formed, for reasons that are not clear. The protein was purified by chromatography on a column of Sephadex G-50 also in acidic 6 M urea (*e.g.*, Figure 3b). It was recovered as before, redissolved in acidic urea, and then exhaustively dialyzed into water at 4° and freeze-dried. The dry protein consisted of irregular birefringent microfilaments. About 500 mg was isolated from the lipoprotein in fraction D, Figure 1, of one yolk by this procedure. The same protein could be isolated from fractions A, B, C, and E and from the unfractionated lipoprotein. It could also be isolated at 2° although the yield was lower, possibly because aggregation in urea at this temperature led to greater losses.

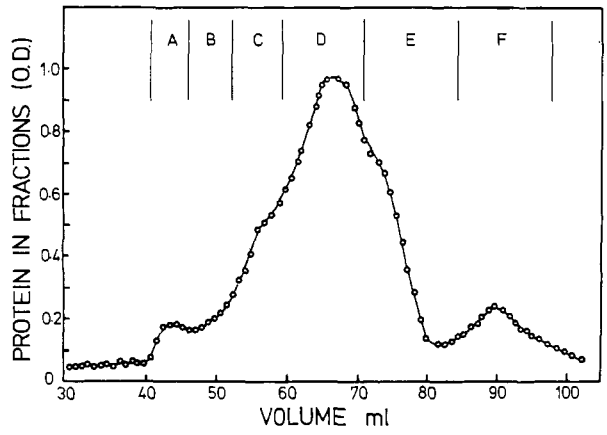

FIGURE 1: Chromatography of high-lipid lipoprotein (3.0 ml, 12% w/v) from emu yolk on a column (84 × 1.5 cm) of agarose (Bio-Gel A-15) at 4° eluting with 1.0 M sodium chloride at 5 ml/hr. Ordinates give total protein determined by Folin-biuret analysis on 100-μl samples, the optical density (OD) being measured at 720 nm. The lipoprotein in fractions A–E contained 8.0, 9.5, 11.0, 13.0, and 13.7% of protein, respectively, the rest being lipid. Fraction F, which emerged at the bed volume, contained the soluble yolk livetins with very little lipid.

METHOD II. This method did not involve the precipitation of protein. A solution (about 6% w/v) of the emu high-lipid lipoprotein in 1.0 M sodium chloride was dialyzed into several changes of acidic 6 M urea at 20° to remove the sodium chloride almost completely. Alternatively, salt was first removed by dialysis into water at 1°. To the urea solution (17 ml) a mixture of chloroform (20 ml) and methanol (40 ml) was added and the one-phase solution was allowed to stand for 30 min at 20–25°. More chloroform (20 ml) was then added. After settling, the lower chloroform layer was discarded and the upper solution treated with another 10 ml of chloroform. If necessary the mixture was centrifuged. Chloroform was then sucked off and the solution left for 24 hr at 20°. A small precipitate of protein that had formed during extraction dissolved on standing. The solution was then dialyzed into 6 M urea and chromatographed on a column of Sephadex G-100 (Figure 3a). The protein in peak IIB was collected and purified as in the first method.

Isolation of Total Apovitellenins for Gel Electrophoresis. Two methods were used with similar results. In the first, the solution of apoprotein in acidic 6 M urea (see Method II above) was diluted with an equal volume of water and 12% trichloroacetic acid was added to precipitate the protein, which was collected by centrifuging (15 min at 2000 rpm). To the sediment a little solid sodium bicarbonate was added to neutralize the acid and the protein was dissolved in a solution containing sodium dodecyl sulfate (1%) and mercaptoethanol (1%) in Tris buffer, pH 8. The solution was then di-

TABLE 1: Physical Properties and Composition of Emu Yolk High-Lipid Lipoprotein (Fraction D, Figure 1) Compared with the Lipoprotein of Hen's Egg Yolk.[a]

	Emu	Hen
Partial sp vol (ml/g)	1.045	1.029
Sedimentation coeff $s^0_{20,w\,NaCl}$ (S)	−18.9	−14.0
ds/dc (S per g per 100 ml)	1.2	1.8
Intrinsic viscosity (ml/g)	3.0	2.8
Particle wt (daltons)		3.3[b]
(i) Scheraga and Mandelkern (1953) method	2.6×10^6	
(ii) Electron microscopy[c]	3.2×10^6	
Protein (% by wt)	13.0	15.6
Phospholipid (% of total lipid)	22.7	27.2
Triglycerides[d,e] (% of total lipid)	74.3	69.0
Fatty acid residue[e] (% of total)		
C-16:0	24.1	31.8
C-16:1	2.8	8.2
C-18:0	7.0	7.6
C-18:1	57.9	44.6
C-18:2	5.6	7.2
C-20:4	0.6	0.0
Saturated fatty acid residues (% total)	31.7	35.0

[a] All physical measurements were in 1.0 M sodium chloride at 20.0°. [b] From Martin *et al.* (1964). [c] Using an average particle diameter of 214 Å as measured in the electron microscope. [d] The remaining lipid was largely cholesterol and esters. [e] Analyses by Mrs. J. A. Pearson using thin layer and gas chromatography.

alyzed at 20° into the same mixture before electrophoresis as in the next section. In the second method, to the lipoprotein solution (1.0 ml, 5% w/v) in 1.0 M sodium chloride, methanol (2.5 ml), chloroform (2.5 ml), and 12% trichloroacetic acid (1 ml) were added. After 30 min at 20° the mixture was centrifuged at 2000 rpm for 20 min and the mat of protein at the liquid–liquid interface was removed, washed in ether, and dispersed in 1 ml of the detergent solution used in the previous method, before electrophoresis.

Gel Electrophoresis. The method was that of Weber and Osborn (1969) as modified by Greaser and Gergely (1971). The protein solution (5–10 μl containing about 10 μg of each protein) in 1% sodium dodecyl sulfate was applied to 4% cross-linked polyacrylamide gel disks (8 × 0.3 cm). A current of 7 mA/tube was used, the electrode buffer being 0.1% sodium dodecyl sulfate–0.05 M phosphate, pH 7.0. As molecular weight standards the proteins used were: myosin, bovine serum albumin, ovalbumin, actin, hemoglobin, and lysozyme.

Physical Measurements. Most procedures and apparatus were those described previously (Burley, 1968, 1970). For sedimentation equilibrium measurements, either short columns (Yphantis, 1960) or conventional 3-mm columns were used, with schlieren optics. For the latter, apparent molecular weights were determined by Lamm's method, with extrapolation to zero protein concentration using the procedure of Marler *et al.* (1964). Optical rotatory dispersion (ORD) was measured on a Perkin-Elmer Model 141 polarimeter with filters for between 365 and 589 nm. Optical absorption was measured on a Unicam SP3000 automatic spectrophotometer.

Amino Acid Analyses. A Beckman Model 120C amino acid analyzer was used. Protein samples (2–4 mg) were first hydrolyzed for 24 hr at 110° in 5.7 N hydrochloric acid (3–5 ml) in the absence of air. Corrections based on six hydrolyses, from 4 to 72 hr, were applied to the results if necessary. For the estimation of amino sugars, the protein was first hydrolyzed for 4 hr at 100° in 4 N hydrochloric acid. Tryptophan was estimated from ultraviolet measurements in 6 M guanidine hydrochloride as in the method of Edelhoch (1967). No evidence for cystine, cysteine, or their decomposition products was found in the hydrolysates of apovitellenin I, nor could SH groups be detected in the intact protein by the nitroprusside or other tests.

N-Terminal Residue Estimation on Apovitellenin I. To the protein (6 mg) dispersed in ethanol (5 ml) was added fluorodinitrobenzene (12 mg) followed by sodium bicarbonate (12 mg) in water (3 ml) as in the method of Sanger. The mixture was shaken gently at 20° for 72 hr or longer. The dinitrophenylated protein was then precipitated by adding trichloroacetic acid (2 ml, 12%) and water (5 ml), and centrifuged down. After washing in water and ether it was hydrolyzed in 5.7 N hydrochloric acid for 16 hr at 110°. The ether-soluble dinitrophenylated amino acid was isolated by chromatography on a column of pH 6.5 buffered Celite and identified by paper chromatography as usual, the concentration being measured at 360 nm in 0.2 M sodium bicarbonate. No acid-soluble dinitrophenyl amino acids could be detected by paper electrophoresis at pH 9.

Tryptic Digestion. The freeze-dried apovitellenin I (20 mg) was allowed to stand in water (2 ml) at 40° for 30 min. Trypsin (2 mg) was then added in 0.5 ml of water. The mixture was either kept at pH 8.0, 40°, in a pH-Stat, or the pH was maintained by additions of 0.4 M Tris–maleate buffer. The protein had largely dissolved in 3 hr. According to the pH-Stat about 24 hr was needed for complete digestion.

Results

High-Lipid Lipoproteins of Emu's Egg Yolk and Resolution of Their Apoproteins. Gel-filtration chromatography of the lipoproteins isolated from emu's egg yolk by centrifuging (see Materials and Methods) is shown in Figure 1, which suggests a wide distribution of particle sizes with one fraction (fraction D) predominating. Fraction A, which may have contained a small proportion of free lipid, was distinguished by an opaque white appearance in 1.0 M sodium chloride even when concentrated, whereas fractions B–E were clear yellow. There were considerable differences in the proportions of protein in the lipoprotein from different regions of the chromatogram. Properties of fraction D are summarized in Table I, which also gives values obtained under the same conditions for the major fraction of normal hen's egg-yolk lipoprotein. The properties of the two lipoproteins are very similar. Values for the hen's lipoprotein are closely comparable to those in the literature (*e.g.*, Martin *et al.*, 1964). The electron microscope appearance of the lipoprotein particles of fraction D was also similar to that of the lipoprotein of hen's eggs (Nichols *et al.*, 1969; Burley, 1971).

After isolation of the total apoprotein from the emu high-lipid lipoprotein, several protein fractions (the apovitellenins) were separated by gel electrophoresis in the presence of detergent. The apovitellenins from fraction D, Figure 1, are shown in the center gel in Figure 2, which also gives some approximate molecular weights. The electrophoretic patterns of the apoproteins from fractions A, B, and E in Figure 1

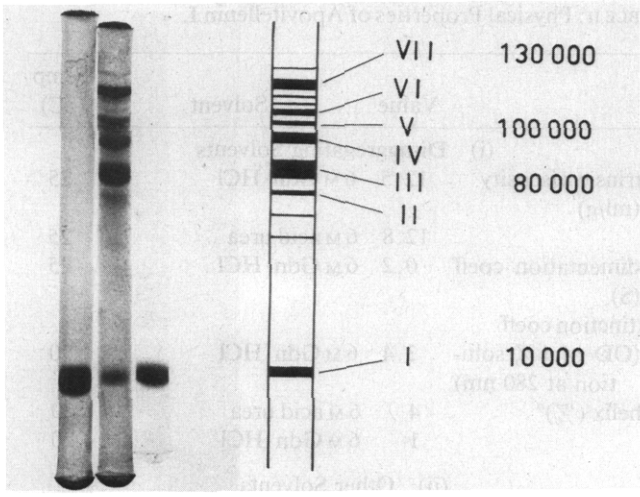

FIGURE 2: Gel electrophoretic separation of emu apovitellenins in the presence of detergent: center gel, mixed apovitellenins from fraction D, Figure 1; outer gels, isolated apovitellenin I, overloaded to show absence of other proteins. The right-hand gel was stained with Amido Black and the others with Coomassie Blue. The figures are approximate molecular weights according to standards.

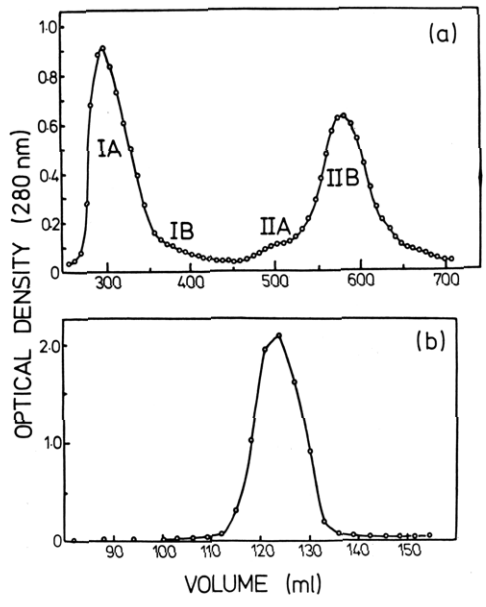

FIGURE 3: (a) Chromatography of total apovitellenins from emu egg-yolk lipoprotein (fraction D, Figure 1) on a column of Sephadex G-100 (100 × 4.3 cm) in 6 M urea–0.05 N HCl, at 20°, 20 ml/hr. (b) Rechromatography of fraction IIB on a column (150 × 2.5 cm) of Sephadex G-50 in 6 M urea–0.05 N HCl, 20°. Ordinates give protein concentrations expressed as optical density at 280 nm for a 1-cm path length.

were similar to this, although the relative intensities of some of the bands were different, *e.g.*, for fraction E the bands between I and II were more intense. Apovitellenin I (band I) was not detected in fraction F, which contained some of the yolk livetins, nor in any other part of the yolk tested.

The emu apovitellenins were readily divided into two groups by their solubility in a methanol–chloroform mixture, the soluble fraction being about 35% of the total apoprotein. Separation by solubility, however, was incomplete. The soluble fraction was largely apovitellenin I, according to gel electrophoresis, but it also contained higher apovitellenins, especially those designated II and VI in Figure 2, and the insoluble residue contained some apovitellenin I, presumably part of a complex or aggregate, in addition to the rest of the apovitellenins. By means of gel filtration chromatography in acidic urea, apovitellenin I could be isolated from the soluble fraction. Better separation was achieved if the lipoprotein was first dialyzed into acidic urea, in which it remained soluble, before extracting the lipid, so that the protein never precipitated. Figure 3a shows the usual chromatographic separation. Peaks IIA and IIB contained apovitellenin I, according to gel electrophoresis. Presumably IIA represented an aggregate. The other apovitellenins were in IA and IB, also according to gel electrophoresis. From the weights of protein recovered after chromatography, the percentage of apovitellenin I was calculated at about 45% of the total protein. Band intensities in gel electrophoresis suggested a lower proportion of apovitellenin I (Figure 2) for reasons that are not yet clear. Possibly there was loss of the low molecular weight constituent.

Rechromatography of the protein in the central part of peak IIB, Figure 3a, on a column of Sephadex G-50 gave a single peak, Figure 3b, that contained one protein according to gel electrophoresis (Figure 2, outer gels). Attempts at fractionating the protein on columns of DEAE Sephadex (in 6 M urea) and on Sephadex LH 20 (in methanol–chloroform–water) were not successful. The latter experiment gave no signs of residual lipid in the purified apovitellenin I.

In an attempt to eliminate microbial degradation and minimize the possibility of enzyme action during the preparation of apovitellenin I, in one experiment a sample of whole yolk (4.8 g) was cooled to −10° and 1.0 M sodium chloride (8 ml) at the same temperature was added. To the stirred mixture, which was stringy, chloroform–methanol, also at −10°, was added to give one liquid phase. The precipitated protein was then filtered off while warming to room temperature. The filtrate contained apovitellenin I according to gel electrophoresis in detergent and chromatography in urea, the amount isolated being slightly greater than that in an equivalent experiment at 20°.

Physical Properties of Apovitellenin I. Table II gives some approximate physical data for apovitellenin I in several solvents. Sedimentation equilibrium measurements in disaggregating solvents (*e.g.*, Figure 4) at different speeds at concentrations of protein up to about 1% suggested a homogeneous protein of low molecular weight (Table III), although at high concentrations there were occasionally indications of aggregation (*e.g.*, Figure 4b). Measurements of optical rotatory dispersion showed virtual absence of the α helix in these solvents. According to the equation used by Tanford *et al.* (1967), the viscosity in 6 M guanidine hydrochloride indicated a molecular weight that agreed with those derived from other methods (Table III), thus suggesting that in this solvent apovitellenin I was present as a random coil. Neither the intrinsic viscosity nor the sedimentation characteristics were altered by the inclusion of mercaptoethanol, indicating the absence of disulfide bonds.

Apovitellenin I was readily soluble in 98% formic acid and in formamide. It was difficult to dissolve in water, but aqueous solutions could be prepared by prolonged dialysis of precipitates from trichloroacetic acid, or by dialyzing solutions in 6 M urea. Solubility in water decreased with pH from 0.5% at pH 3 to 0.06% at pH 6 at 20°. In the range pH 4–9 addition of salt greatly decreased the solubility. The protein was again soluble above pH 10, but no physical measurements

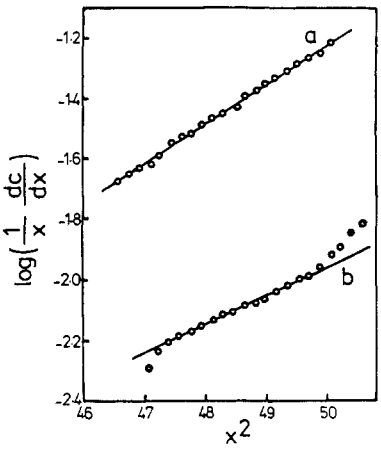

FIGURE 4: Ultracentrifuge sedimentation–equilibrium experiments on apovitellenin I using 3-mm columns, 25°, 30 hr. (a) Solvent, 6 M guanidine hydrochloride, pH 5.5; protein concentration, 0.84%; speed 29,500 rpm. (b) Solvent, 8 M guanidine hydrochloride; protein concentration, 1.1%; speed, 33,600 rpm. x is the distance from the center of rotation and c the concentration expressed as centimeters.

were attempted above pH 7. According to sedimentation measurements, in water above pH 4 the protein existed largely as a highly aggregated species. The aggregates could not be dissociated by prolonged dialysis at pH 3. Apovitellenin I was more readily soluble in methanol than in water. Precipitates from trichloroacetic acid dissolved rapidly to give solutions that remained clear on dialysis into pure methanol provided the concentration did not exceed about 0.4%. The dry protein was not, however, immediately soluble in methanol. In water and in methanol apovitellenin I contained a large proportion of α-helical structure according to the optical rotatory dispersion, the maximum being about 80% in aqueous methanol. The variations in helical content were reversible as long as the pH did not exceed about 5. All solutions of apovitellenin I had high viscosities, as would be expected if the protein always had a high axial ratio.

Apovitellenin I was precipitated as a gel from solution in 100% methanol by addition of ethanol, tetrahydrofuran, pyridine, or chloroform. If the methanol contained sufficient water, however, chloroform could be added to give one phase without precipitation.

Chemical Properties of Apovitellenin I. The amino acid composition and other analytical data for purified apovitellenin I are given in Table IV. The amino acid analyses fit a monomeric molecular weight of 10,200 best, although they would also be consistent with 6600 or 13,300. No trace of histidine, cysteine, or cystine could be detected, and the amount of phosphorus was less than 0.02%. The total nitrogen found agreed with the theoretical value and the total weight of amino acid residues and other known constituents was within 2% of the total weight. It is therefore unlikely that large amounts of fatty acid residues or other lipids were present. No significant variations in amino acid composition were found among different preparations. All preparations did, however, contain small amounts of amino sugar, usually galactosamine with a little glucosamine—always much less than 1 mol/10^4 g—and it has not been found possible to remove this. Possibly sugar residues are attached to a few amino acid residues of the protein; alternatively, a small amount of a contaminant rich in amino sugar may have been present. The

TABLE II: Physical Properties of Apovitellenin I.

	Value	Solvent	Temp (°C)
(i) Disaggregating Solvents			
Intrinsic viscosity (ml/g)	12.5	6 M Gdn·HCl	25
	12.8	6 M acid urea	25
Sedimentation coeff (S)	0.2	6 M Gdn·HCl	25
Extinction coeff (OD of 1% solution at 280 nm)	3.4	6 M Gdn·HCl	20
α helix (%)[a]	4	6 M acid urea	20
	1	6 M Gdn·HCl	20
(ii) Other Solvents			
Intrinsic viscosity (ml/g)	10	Water, pH 5.5	20
	23	0.02 N formic acid, pH 2.9	20
	16	50% methanol (pH 3)[b]	20
Sedimentation coeff (S)	2	Water, pH 5.5	20
	0.2	0.02 N formic acid, pH 2.9	20
α helix (%)[a]	59	Water, pH 5.5	20
	42	0.02 N formic acid, pH 2.9	20
	78	50% methanol (pH 3)[b]	20
	80	50% methanol (pH 4)[b]	20

[a] From measurements of optical rotatory dispersion assuming $b_0 = -630$. [b] The pH refers to the aqueous constituent which was either 0.02 N formic acid (pH 3) or 0.02 N acetic acid adjusted to pH 4 with ammonium hydroxide.

presence of such a constituent is implied by the results of Augustyniak and Martin (1968) on the hen's egg apoprotein.

The only N-terminal residue found by Sanger's method was lysine. Reaction with fluorodinitrobenzene was very slow; even after 90 hr of reaction at 20° the equivalent weight (about 17,000, not corrected for decomposition during hydrolysis) was greater than expected from physical measurements (Table III).

Tryptic digestion of apovitellenin I was relatively slow. According to gel filtration chromatography on polyacrylamide gel (Bio-Gel P4) the tryptic peptides were small. None had more than about 14 residues, so presumably the distribution of basic residues (arginine plus lysine) was more or less uniform.

Discussion

The above results help to establish the physical and chemical properties of a new egg-yolk protein, provisionally termed emu apovitellenin I. This protein is the major apoprotein of the high-lipid low-density lipoprotein of emu yolk. It has been isolated by methods that appear to preclude the possibility of covalent bond splitting, although isolation without the prior removal of lipid has not been achieved. By all the tests applied so far, including gel electrophoresis (Figure 2), chromatography (Figure 3), ultracentrifugation (Figure 4), and end group analysis, the protein isolated was homogeneous, although none of these would detect small amounts of

TABLE III: Molecular Weight of Apovitellenin I.[a]

Method	Solvent	Temp (°C)	Mol Wt
Sedimentation equil, column (mm)			
3	6 M Gdn·HCl	25	10,600
1	6 M Gdn·HCl	25	10,200
3	8 M Gdn·HCl	25	8,900
Viscosity[b]	6 M Gdn·HCl	25	8,500
Gel filtration chromatography[c]	6 M acid urea	20	9,500
Gel electrophoresis	1% sodium dodecyl sulfate	20	10,000
Amino acid analysis[d]			10,200

[a] The partial specific volume used was 0.753 ml/g. [b] Assuming a random coil, average residue weight 114; Tanford *et al.*, 1967. [c] Sephadex G-50 column, 120 cm. [d] Best minimum value from data in Table IV.

TABLE IV: Amino Acid Composition of Apovitellenin I.[a]

Amino Acid	Mol/10^4 g Mean	CV	SE	Residues per 10,200 g
Lys	7.28	7.8	0.15	7
Arg	5.09	3.6	0.05	5
Asp	7.90	2.9	0.06	8
Thr	4.83	2.7	0.03	5
Ser	2.90	3.6	0.03	3
Glu	8.84	3.1	0.07	9
Pro	2.93	6.3	0.05	3
Gly	3.03	5.4	0.04	3
Ala	7.15	2.4	0.05	7
Val	9.47	3.5	0.09	10
Met	2.97	8.1	0.06	3
Ile	5.57	4.9	0.07	6
Leu	7.13	2.0	0.04	7
Tyr	3.78	4.3	0.04	4
Phe	4.05	5.5	0.06	4
Trp	2.71	4.2	0.03	3
Gal	0.2			
Amide N	7.2	6.4	0.39	7
Total N[b] (%)	14.9			
Total S[b] (%)	1.3			
Ash[b] (sulfated) (%)	0.5			

[a] The coefficients of variation (CV) and standard errors (SE) refer to measurements on 14 samples from four yolks, except for amide N, which was measured on five samples. Galactosamine (Gal) was measured on two samples from one yolk. [b] Duplicate analyses by Australian Microanalytical Laboratory, Melbourne.

other constituents. The molecular weight is undoubtedly very low, in the range 8000–11,000 (Table III), but there is doubt about the precise value because the partial specific volume has not been determined experimentally. A value of 0.753 ml/g was calculated from the amino acid composition (Table IV) and was used without correction for the solvent. Such a correction may have given a lower value (*e.g.*, see Tanford *et al.*, 1967). A value of 0.742 ml/g was determined by Martin *et al.* (1959) for the total apoprotein of hen's egg lipoprotein measured in formic acid.

Apovitellenin I does not appear to contain covalently or noncovalently bound lipid or fatty acid residues. It differs from other egg proteins in the complete absence of histidine. Its amino acid composition is also notable for a low proportion of residues that would be expected to disrupt the α helix, *i.e.*, proline, glycine, serine, and asparagine (Lewis and Scheraga, 1971). Residues that prefer or are compatible with α-helical structure are therefore at least 80% of the total. Physical measurements, which are open to some uncertainty, suggest that the protein has a large proportion of α helix in water and a larger proportion in aqueous methanol (Table II).

Emu apovitellenin I is likely to have an essential role in the high-lipid lipoprotein. It is almost half the total protein, but is not present in any other part of the egg yolk. Hen's egg-yolk lipoprotein contains an analogous protein, with a lower solubility and a higher molecular weight (about 16,000), that possibly contains sulfhydryl groups (Burley and Sleigh, 1971). It has not so far been found possible to purify this protein, but the hen and emu lipoproteins are similar (Table I), so the emu apovitellenin must possess the essential properties needed for interactions with large amounts of lipid in avian high-lipid lipoproteins. What these properties are is unclear. It does seem certain though that some chemical groups, such as sulfhydryl and phosphate, are not essential, nor is a large preponderance of hydrophobic residues (Table IV). In fact 47% of the residues of apovitellenin I are polar, although the "hydrophobicity" is also relatively high ($H\phi_{av}$ = 1333 cal/residue (Bigelow, 1967). From the small amount present carbohydrate does not appear to be essential.

The relevant physical properties are even more difficult to assess because they may not be manifest in the absence of lipid. There is also the possibility of irreversible structural change to the protein during isolation, although for a small protein with no disulfide groups this possibility should be minimal. The most conspicuous physical properties of isolated apovitellenin I are firstly, a high proportion of α helix in water, and especially in aqueous methanol, which may be closer to its natural environment, and, secondly, a tendency to aggregate. The full extent of its aggregation has not yet been explored, but aggregates were formed very readily so it would not be surprising if they are also present in the lipoprotein.

The work of Steer *et al.* (1968) on the susceptibility of the apoprotein of hen's egg high-lipid lipoprotein to proteolytic enzymes suggests that a protein fraction relatively low in histidine is more readily digested and is therefore presumably at the lipoprotein surface rather than in the interior. This evidence, together with its high proportion of hydrophilic residues, implies that apovitellenin I occurs in the surface layer of lipid. Further evidence should be available from the amino acid sequence, at present being elucidated. The above preliminary results for the tryptic digestion of apovitellenin I suggest a relatively uniform distribution of basic residues along the polypeptide chain. Such a result would be consistent with an elongated rather than a globular structure for the protein at the lipid surface. The high viscosity of the aqueous

solutions would also be expected if the isolated protein had an elongated structure.

In conclusion it is suggested that apovitellenin I aids the stability of the lipoprotein by taking part in a highly aggregated protein network at the surface of the particles. An average emu lipoprotein particle with 13% of protein (Table I) should contain about 18 apovitellenin I molecules. Each of these has 87 amino acid residues and could have an extended length somewhere between a completely α-helical and a β-configuration, *i.e.*, between about 130 and 350 Å. Thus, as an average particle has a circumference of about 670 Å, there should be enough apovitellenin I to form a wide-mesh net at the surface even without the participation of the rest of the apoprotein. Such a structure should permit the interchange of lipid among the lipoprotein particles (Burley, 1971) but would prevent them from coalescing. The formation of a gel at low temperatures by certain yolk lipoproteins that have an abnormally high proportion of saturated lipid (Burley, 1970) may be interpreted, on this view, as an example of the interparticle aggregation of part of the apovitellenin I that has not been adequately accommodated at the lipid surface. The proposed structure assumes that apovitellenin I has an extended configuration at the lipid surface and is highly aggregated. As yet there is no evidence for either of these assumptions.

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