Lipid-Protein Globules of Avian Egg Yolk

ISOLATION AND PROPERTIES OF GLOBULES STABLE IN CONCENTRATED SODIUM CHLORIDE SOLUTION

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A new type of globular particle, the 'insoluble yolk globule', was isolated from the egg yolk of three avian species (hen, duck and emu) by centrifugation or gel-filtration chromatography. These globules are stable in NaCl and urea solutions at concentrations that dissolve or disrupt other constituents of yolk. The isolated globules are about 1% of the dry yolk of hen's and duck's eggs but about 8% of emu's-egg yolk. Most of these globules are less than $2\mu\text{m}$ in diameter. Electron micrographs of sections show a preponderance of globules in the range $0.125\text{--}0.25\mu\text{m}$, each with a thick shell surrounding a featureless interior. Globules with the same appearance were seen in sections of unfractionated yolk. Two kinds of larger particle were also observed: (i) particles with a distinct outer membrane and a vesiculated interior; (ii) featureless spheres, possibly of lipid. The insoluble yolk globules comprise protein (8–11% by dry wt.), phospholipid (31–35% total lipid), triacylglycerols (49–53%), cholesterol (8%) and cholesteryl esters (2–3%); the variations being among species. The phospholipid is accessible to phospholipase C. The isolated protein is heterogeneous and resembles the apoprotein from the yolk low-density lipoprotein.

Avian egg yolk contains approx. 50% by weight of water and 35% of lipid, the rest being mostly protein. Most of the lipid is present in a soluble low-density lipoprotein that has 12–15% of protein and is about 30% of the yolk. The rest of the lipid is present in a variety of particles suspended in the aqueous medium (Bellairs, 1961; Cook & Martin, 1969; Bain & Hall, 1970; Bellairs *et al.*, 1972; Vadehra & Nath, 1973). The most plentiful of these particles are the 'yolk granules', which are 12% of the yolk and contain 30% lipid (Burley & Cook, 1961). We report here the isolation and analysis of another type of globular yolk particle, which we refer to as 'insoluble yolk globules' because they are unaffected by concentrated NaCl or urea solutions in which the granules and other particles dissolve. Although these globules are a small proportion of yolk, they are of interest because of their composition and properties. They do not resemble the granules but they have the same lipids and probably the same proteins as the low-density lipoprotein, thus suggesting a common metabolic origin. Their structure, however, appears to be quite different; nor do they resemble other large lipid-containing particles (e.g. chylomicrons) as they have a higher proportion of protein and a relatively high density.

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Materials and Methods

Materials

Hen's eggs were from White Leghorn or Australorp breeds (*Gallus domesticus*) kept at CSIRO Division of Animal Production, Sydney. No differences were observed between breeds in the insoluble yolk globules from the eggs. Eggs from Peking ducks (*Anas platyrhynchos*) were from a commercial farm at Annangrove, N.S.W. and eggs from emus (*Dromaeus novaehollandiae*) were from Kalkari Park, Ku-ring-gai Chase National Park, N.S.W. For each species, at least one preparation of insoluble globules was made from eggs opened within a day of laying. Similar preparations were obtained from eggs stored at 2°C for up to 3 months.

Preparative methods

Isolation of insoluble yolk globules. Three methods were used. Method (i). Fresh egg yolk (180 g) free of egg white was mixed rapidly with an equal volume of 0.16M-NaCl at 20°C, and centrifuged for 30 min at 40000g (r_{av} , 8 cm) at 10°C in the no. 30 rotor of a Beckman L2-65 preparative ultracentrifuge. During centrifugation the yolk granules sedimented as a pellet (Burley & Cook, 1961). The supernatant liquid containing insoluble yolk globules was made up to approx. 2M with solid NaCl added in small portions, each being dissolved before the next was added. This

mixture was then centrifuged for 1 h as above. The globules were apparent as a white layer floating in the upper 2–4 cm and were removed with a spatula and a pipette. Alternatively the supernatant solution was removed with a syringe. The crude preparation of globules was then diluted with 5 vol. of 2M-NaCl and centrifuged as above. This process was repeated five to six times to remove the low-density lipoprotein and other soluble yolk constituents.

Method (ii). The yolk granules were removed from fresh yolk as in Method (i). The supernatant liquid was then made approx. 2M with solid urea and 0.4M with solid sucrose, the solids being added slowly. The mixture was then centrifuged and the globules were recovered as above. The globules were washed by centrifuging five to six times with 2M-urea/0.44M-sucrose/0.16M-NaCl.

Method (iii). Fresh yolk was first slowly mixed with an equal volume of 4M-NaCl and the mixture centrifuged for 1 h at 40000g to float off the insoluble globules. These were then washed five to six times with 2M-NaCl as in method (i). This method was used mainly to give a measure of the total weight of insoluble globules in yolk because some globules sedimented with the yolk granules and were missed by the other methods. It had the disadvantage that, because the yolk granules dissolved, a small amount of the phosphoprotein phosvitin was able to interact with other yolk constituents and contaminate the protein.

Preparation of soluble lipid-free protein from yolk globules. A method that removed all lipid from the low-density lipoprotein of hen's-egg yolk and gave soluble apoprotein (Burley, 1975) was adapted as follows, all operations being at 2°C. A suspension of the globules (>3%, w/v) was dialysed overnight against water, O₂ being excluded by bubbling N₂ through the mixture. To 10 ml of the aqueous suspension (0.3–0.4 g of globules) 10M-HCl (25 µl) was added and the acidic suspension poured into 12.5 ml of chloroform plus 21 ml of methanol in a separating funnel. This mixture was then agitated gently; vigorous shaking gave a stable emulsion in which, according to observations in the light microscope, the insoluble protein tightly surrounded droplets of chloroform plus lipid. After the yellow chloroform/lipid layer had settled and had been removed, light petroleum (b.p. 30–40°C, 3 ml) was added with gentle agitation. The lower chloroform/lipid layer was again removed and more petroleum (3 ml) plus chloroform (6 ml) added. Finally, the cloudy upper aqueous layer, containing undissolved protein, was added to urea (3.6 g) at 20°C, and the solvents were sucked off with warming to 40°C. The protein dissolved in the urea solution (6M) that remained after removal of the solvents. The amount of protein isolated was determined gravimetrically after dialysis against water. The protein remained soluble in water and contained very little phosphorus.

Analytical methods

Lipid and protein in globules. The procedure of Bligh & Dyer (1959) was used to separate lipid and protein which were determined gravimetrically. Values for protein were in good agreement with those from protein solubilized in acid urea. Protein was also determined colorimetrically by the Folin–biuret method (Lowry *et al.*, 1951), with approximately the same results.

Lipid analyses. Lipid classes were estimated by the chromatographic procedure of Carroll (1961, 1963) on Florisil (Floridin Co., Tallahassee, FL, U.S.A.). The sterol was at least 96% cholesterol according to g.l.c. on a column of 3% SE30 on Gas Chrom Q (Applied Science Laboratories, State College, PA, U.S.A.) at 250°C at flow rate 30–35 ml/min. Phosphorus in the phospholipids was determined by the method of Fiske & SubbaRow (1925) after digestion in HClO₄. Other lipid classes were estimated gravimetrically. Methyl esters of the total lipid were prepared by the method of Glass & Christopherson (1969) as modified by Hood *et al.* (1972) and the fatty acid compositions estimated by g.l.c. on a column of 10% SP 222-PS at 155°C.

Action of phospholipase C on globules. The method was essentially that used previously for other yolk constituents (Burley & Kushner, 1963). Suspensions of globules in 0.16M-NaCl/0.01M-NaHCO₃, pH 7.8 (0.12 g of globules in 1.0 ml) containing the enzyme (0.1 mg; EC 3.1.4.3 from Sigma Chemical Co., St. Louis, MO, U.S.A.) were dialysed against 10.0 ml of the same solution at 20°C with Visking cellulose casing. During dialysis, about 24 h, portions (0.2–0.5 ml) of the outer solutions were withdrawn for analysis of phosphate (Fiske & SubbaRow, 1925). The results were calculated as diffusible phosphorus per cent of total phosphorus.

Physical methods

Gel electrophoresis. Protein fractions, about 2 µg in 10 µl of 1% sodium dodecyl sulphate, were separated in tubes (0.5 cm × 7 cm) of polyacrylamide gel (7.5% w/v, 5% cross-linking) by a procedure similar to that of Weber & Osborn (1969), the buffer being 0.03M-Tris containing 0.1% sodium dodecyl sulphate, pH 7.7.

Electron microscopy. Small centrifuged pellets (50–100 mg) of insoluble yolk globules were fixed in 6% glutaraldehyde in 0.1M-sodium phosphate buffer (pH 7.2) plus 7% (w/v) sucrose (1 h or overnight), post-fixed in 1% phosphate-buffered OsO₄ (1–2 h), washed, stained in 2% aq. uranyl acetate (1 h), washed again, and then dehydrated in an ethanol series, before being embedded in Epon 812. The embedded samples were sectioned, stained on the grid with 2% aq. uranyl acetate and lead citrate (Reynolds, 1963),

and examined in a Siemens Elmiskop 1. The effect of fixation on the structure of the hen's and emu's globules was observed by placing additional samples overnight in the formaldehyde/glutaraldehyde fixative of Karnovsky (1965) with post-fixation in OsO_4 (1–2 h) or in OsO_4 alone for 1–2 h, the rest of the embedding procedure being the same. Small blobs of yolk (about 100 mg) from freshly opened emu's eggs were treated by the three procedures in the same way as the pellets of insoluble globules.

Results and Discussion

In the light microscope the insoluble yolk globules appeared small and approximately circular, although a few very large globules were seen. Fig. 1(a) shows the distribution of particle sizes in a typical light micrograph of emu's-egg-yolk globules. Most of the globules could not, however, be measured in the light microscope, so Fig. 1(b) refers to thin sections viewed in the electron microscope (e.g. Plate 1). Globules from hen's- and duck's-egg yolk gave a similar distribution and their cross-sections were similar (Plates 1a–1d). The shape of the globules was similar after initial glutaraldehyde or formaldehyde/glutaraldehyde fixation followed by OsO_4 (Plates 1b and 1e), but there was a difference when OsO_4 alone was used for fixation (Plate 1f). Globules similar to those in Plates 1(a)–1(e) were

Fig. 1. Histograms showing the distribution of the diameters of insoluble yolk globules

The globules were isolated from emu's-egg yolk by method (i). The other methods gave similar histograms. (a) From light-micrographs of globules in 1% agar measured by Nomarski interference contrast optics in a Zeiss WL microscope. Particles of less than 0.5 μm were not measured. (b) From electron micrograph of a thin section (see Plate 1c).

Fig. 2. Chromatography of insoluble yolk globules. A sample (10 ml; 3%, w/v) of globules was passed through a Millipore filter (5 μm), applied to a column (120 cm × 2.5 cm) of Bio-Gel A-15m (Bio-Rad Laboratories, Richmond, CA, U.S.A.) and eluted with 1 M-NaCl/0.02% sodium azide at 20°C. The turbidity was estimated from the A_{650} (●), and protein was estimated on 100 μl portions by the method of Lowry *et al.* (1951) at A_{720} (○). The void volume was 100 ml and the bed volume 290 ml. (a) Globules that had been centrifuged once. Peak A contained only small globules, peak B low-density lipoprotein, and peak C yolk livetins. (b) Globules purified by centrifuging five times, as given in the Materials and Methods section. The large peak contained only small globules.

seen in unfractionated yolk (Plate 1g), thus decreasing the possibility that they were artifacts produced during isolation. In unfractionated yolk the globules were usually near large densely staining particles, possibly yolk granules, one of which is shown in part in Plate 1(g), although no attachment was visible. The isolated globules were also seen in the electron microscope after negative staining. They tended, however, to clump together so that diameter measurements were not feasible.

Fig. 2(a) shows the gel-filtration chromatography of a sample of emu's-egg-yolk globules that had been isolated by centrifuging but had not been washed. The globules emerged with the front peak (peak A) and were estimated by their high turbidity. They were well separated from the low-density lipoprotein (peak B), and the low-molecular-weight constituents of yolk, i.e. the livetins, peak C. In preparations of washed globules, peak C was absent and peak B was very small, e.g. Fig. 2(b). Globules from hen's- and duck's-egg yolk gave the same chromatographic

Table 1. *Physical and analytical data for insoluble yolk globules from three species*

Where four or more values, from separate preparations, were available, means $\pm$ S.E. are given. The other values are means of two or three values. No differences were found between methods (i) and (ii) so their results were combined. Values for fatty acids are percentages of fatty acid residues in lipid extracted from globules. These values are means of duplicates or triplicates. In an experiment on ten known samples the average standard error was 0.1–0.2.

Weight isolated (g/100 g of dry yolk)*	Hen 0.7	Duck 1.0	Emu 8.2
Protein (g/100 g of dry globules)	10.3 $\pm$ 0.5†	11.3 $\pm$ 0.6	8.05 $\pm$ 0.3
Lipid (g/100 g of dry globules)	89.9 $\pm$ 0.8†	88.8 $\pm$ 0.8	91.7 $\pm$ 0.4
Particle density (g/ml)‡			1.02
Lipids (% by wt. of total lipid)			
Phospholipid	35.1 $\pm$ 6.7†	33.1 $\pm$ 7.0	30.6 $\pm$ 5.1
Triacylglycerols	48.5 $\pm$ 0.5†	52	53
Cholesterol	7.6 $\pm$ 0.7†	7.5	7.8
Sterol esters	2.2 $\pm$ 0.2†	2.8	2.3
Fatty acids			
C _{14:0}	0.1	0.3	0.2
C _{16:0}	26.6	25.0	28.1
C _{16:1}	3.1	3.4	2.7
C _{17:0}	0.2	0.4	0.2
C _{17:1}	0.1	0.3	Trace
C _{18:0}	11.3	5.8	8.2
C _{18:1}	45.9	54.1	58.8
C _{18:2}	9.7	6.2	6.5
C _{18:3}	0.4	0.4	0.2
C _{20:1}	0.2	0.2	Trace
C _{20:3}	0.1	0.4	Trace
C _{20:4}	2.1	3.3	1.1

* Isolated by method (iii).

† These values refer to eight or ten estimations, half from yolk of White Leghorn hens and the rest from yolk of Australorp hens. No differences were found.

‡ In 0.5M-NaCl (density 1.02 g/ml) 80% by wt. of the globules remained suspended after centrifuging for 16 h at 40000g, 20°C.

patterns. The electron-microscopic appearance of sections of globules from peak A was similar to that of globules before fractionation although they tended to stick together (Plate 2b). No large globules were observed; presumably they were retained on the filter or the column.

Table 1 gives data for insoluble yolk globules from eggs of three avian species from different genera. It shows that, apart from the higher proportion of globules in emu's-egg yolk, there is not much difference between the species. The chemical composition of globules prepared by chromatography (Fig. 2b) was indistinguishable from that of the others.

Fig. 3(a) shows the gel-filtration chromatography of lipid-free proteins from the globules of duck's-egg yolk. For comparison Fig. 3(b) refers to the apoproteins isolated by the same procedure from the low-density lipoprotein of the same eggs. The chromatographic patterns are qualitatively similar although the dry weight of protein in the second peak was 38% of the total, compared with 73% for the second peak of the lipoprotein. The second peak was not smaller for globules that had been through an agarose

column. For the proteins of emu's-egg-yolk globules and low-density lipoprotein the chromatographic patterns were similar to Figs. 3(a) and 3(b), with the difference that peak B was a lower proportion of the total in both the globules (25%) and the lipoprotein (60%). From previous work it is known (Burley, 1973; Inglis & Burley, 1977) that peak B contains a single protein (emu's or duck's apovitellenin I) with a characteristic amino acid composition from which histidine is absent. The protein isolated from peak B (Fig. 3a) contained only a trace of histidine and the composition was that of apovitellenin I of the respective species. The gel-electrophoretic pattern of this protein in detergent was not distinguishable from that of apovitellenin I [Plate 2a (i)]. By contrast, the first peaks (A in Figs. 3a and 3b) contained a complicated mixture [Plates 2a (ii) and 2a (iii)], with the same bands but with obvious differences in their proportions. None of these proteins has yet been isolated, so a reliable comparison is not possible. The lipid-free proteins from the hen's-egg-yolk globules gave a different chromatographic pattern to those of the other species (Fig. 3c). This pattern does, how-

EXPLANATION OF PLATE I

Electron micrographs of thin sections of insoluble yolk globules and unfractionated yolk

(a) and (b) Hen's-egg yolk globules shown at two magnifications fixed with glutaraldehyde and OsO₄. (c) Emu's- and (d) duck's-egg-yolk globules fixed with glutaraldehyde and OsO₄. (e) Hen's-egg-yolk globules fixed with formaldehyde/glutaraldehyde and OsO₄. (f) Hen's-egg-yolk globules fixed with OsO₄. (g) Unfractionated emu's-egg yolk fixed as for (c) showing insoluble yolk globules (►). Part of a possible yolk granule (GR) is shown. Bars represent 0.25 μm.

EXPLANATION OF PLATE 2

Gel-electrophoretic patterns of lipid-free proteins and electron micrographs of thin sections of large yolk globules
 (a) Electrophoresis in polyacrylamide gels with sodium dodecyl sulphate. The bands moved from right to left. On gels (ii) and (iii) the thin dark lines at the left give the position of the marker dye. (i) Low-molecular-weight protein from globules of emu's-egg yolk isolated by chromatography (cf. peak B, Fig. 3a). (ii) High-molecular-weight-protein fraction from the same globules (cf. peak A, Fig. 3a). (iii) High-molecular-weight-protein fraction from emu's-egg-yolk low-density lipoprotein (cf. peak A, Fig. 3b). (b)–(e) Thin sections fixed with glutaraldehyde and OsO_4 . Bars represent $0.25\ \mu\text{m}$. (b) Insoluble yolk globules from emu's-egg yolk chromatographed on an agarose column (from peak A, Fig. 2a). (c) and (d) large globules isolated from hen's- and duck's-egg yolk respectively. (e) Part of a larger globule, possibly a lipid droplet, from hen's-egg yolk.

Fig. 3. Chromatography of lipid-free proteins from insoluble yolk globules and low-density lipoprotein

Samples (approx. 0.2 g of protein in 4 ml) were applied to a column (50 cm $\times$ 2.6 cm) of Sephadex G-150 (superfine grade), eluted with 6 M-urea/25 mM-HCl, pH 3.3. Protein was estimated from the A_{280} measurements (ϵ 17000 litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$) in 6 M-urea. The void and bed volumes were 48 and 110 ml respectively. (a) Protein from duck's-egg-yolk globules. (b) Protein from duck's-egg-yolk low-density lipoprotein prepared as for that of hen's-egg yolk (Burley, 1975). (c) Protein from hen's-egg-yolk globules. Peak A contained a mixture of proteins of relatively high molecular weight; peak B contained the low-molecular-weight protein, ducks' apovitellenin I, and peak X contained hen's apovitellenin I dimer.

ever, correspond approximately to that of the apo-proteins from hen's-egg-yolk low-density lipoprotein (Burley, 1975). Peak X contained only hen's apovitellenin I. Presumably this occurs as a dimer in the globules as it does in the low-density lipoprotein (Burley & Davies, 1976).

The data in Table 1 and Fig. 3 show that in composition the insoluble yolk globules resemble the low-density lipoprotein more than any other known

constituent of yolk. The yolk granules, for example, are larger and have more protein and less cholesterol (Burley & Cook, 1961; Cook & Martin, 1969). Table 2 compares data for the globules and lipoprotein from yolk of one species. As far as can be judged from these results the constituents of the two particles are the same but their proportions differ. The sizes of the

Table 2. A comparison of the composition of the insoluble globules and the low-density lipoprotein from the yolk of emu's eggs

Data for the lipoprotein are from Burley (1973).

	Globules	Lipo-protein
Mean particle diameter (nm)	170	21
Density (g/ml)	1.02	0.96
Protein (% of dry wt.)	8	13
Neutral lipid* (% of dry wt.)	58	67
Phospholipid (% of dry wt.)	28	20
Apovitellenin I (% of total protein)	25	60
Fatty acid residues (% of total)		
C _{16:0}	28.1	24.1
C _{18:0}	8.2	7.0
C _{18:1}	58.8	57.9
C _{18:2}	6.5	5.6

* Including cholesterol.

Fig. 4. Action of phospholipase C on insoluble yolk globules and yolk low-density lipoprotein

Small volumes (1.0 ml) containing the enzyme and substrate were dialysed against buffer solution (10.0 ml, pH 7.8) and the phosphorus released into the outer solution was measured at intervals. A. $\circ$, Action of enzyme on insoluble yolk globules from emu's egg. $\bullet$, Action of enzyme on low-density lipoprotein from the same egg. B. Control, insoluble yolk globules as used for A, but without the enzyme. C. Control, rate of dialysis of phosphocholine (in 1.0 ml of buffer) under the same conditions.

particles are also quite different, so the arrangement of the constituents must be different. The lipoprotein does not contain enough protein to cover the surface of an average particle. From the data in Table 2 plus the value of 1.33 g/ml for the density of the protein, we calculated that the protein in the lipoprotein could form a layer only about 0.3 nm thick at the surface. By contrast, the globules, because of their larger diameter, have enough protein to form a layer 1.7 nm thick. Phospholipid and cholesterol would also be expected to be at the surface (Schneider & Tatttrie, 1968). Electron-microscopic examination of sections (Plate 1) does in fact show that the globules are surrounded by a densely-staining layer 10–20 nm thick, and we suggest that this is a membrane containing protein, phospholipid and cholesterol. The presence of myelin figures after fixation with OsO_4 (Plate 1f) implies that phospholipid is associated with this membrane. Other evidence comes from the action of phospholipase C.

Fig. 4 shows that phospholipase C releases phosphorus, presumably as phosphocholine, almost completely from the globules and the low-density lipoprotein and that the rate of release is the same. From measurements on pure phosphocholine (curve C) it is clear that the rate of dialysis in this experiment was fast compared with the rate of enzyme action.

Among the isolated insoluble globules was a very small proportion of much larger particles (Fig. 1a). These could be concentrated on the surface by centrifuging in water. Some of them had well-defined outer membranes with small vesicles inside (Plates 2c and 2d); others had no structure and resembled lipid droplets (Plate 2e). We also observed in the light microscope that yolk contains other relatively large globules less resistant to the action of NaCl or urea. On the addition of 4M-NaCl or 2M-urea to fresh yolk these globules either disintegrated or shrivelled up, becoming first pear-shaped and finally rod-shaped. The rods remained insoluble, but the contents mixed with the aqueous phase.

The function of the various globules in yolk is not known. It should be noted that the insoluble globules are most numerous in emu's-egg yolk (Table 1) which

differs from the other species studied in that the eggs may have to survive for up to 4 months before hatching

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