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Conversion of a Lipoprotein from Egg Yolk into a Membranous Form in the Presence of Yolk Lipid

R. W. Burley, R. W. Sleigh, and Joan M. Bain

Division of Food Research, CSIRO, P.O. Box 52, North Ryde, N.S.W. 2113.

Abstract

Treatment of the major lipoprotein (density 0·95 g/ml) from hen's egg yolk with aqueous dispersions of yolk lipid resulted in a mixture that contained new large globular particles with membranous outer layers. These particles, referred to as 'membranous globules', had diameters in the range 50–200 nm when viewed in the electron microscope and in appearance, properties, and composition resembled the 'insoluble yolk globules' that are a natural part of whole yolk.

Usually about a quarter of the lipoprotein interacted, as estimated by ultracentrifugation or gel-filtration chromatography, but the extent of interaction was highly variable, for reasons that are not yet clear. With some exceptions there was more interaction under conditions favouring dissolution of the apoproteins. Most of the neutral lipid in the membranous globules came from the added lipid rather than from the lipoprotein, according to the distribution of yolk pigments. Evidently the lipids and proteins in yolk lipoprotein are sufficiently flexible in their arrangement to be able to take part in widely different lipid–protein structures when their proportions are different.

Introduction

Two lipid–protein structures of low density have been isolated from avian egg yolk: (1) the major lipoprotein, with particles 20–40 nm in diameter (Cook and Martin 1969), and (2) the 'insoluble yolk globules', which are much larger particles (diameters 125–250 nm) with membranous phospholipid–protein outer layers (Vadehra *et al.* 1977). Although these particles are different in size and also in properties they have the same constituents (mainly triacyl glycerols, phospholipids, and proteins) in approximately the same proportions. The only notable difference is in the proportion of the apoprotein of low molecular weight (apovitellenin I) which is present in smaller amount in the globules. This similarity in composition suggested to us a common origin for these different structures and that interconversion should be possible. We have now tested this possibility and we have found that by treating yolk lipoprotein with lipid under certain conditions the lipoprotein particles can be altered to give membranous structures similar in appearance and properties to those naturally present in yolk.

These results have two main implications, which we are following up. First, the ability of yolk lipoprotein to stabilize droplets of lipid helps to explain the emulsifying power of yolk, for which the lipoprotein is largely responsible (Davey *et al.* 1969). Second, there is a clear relationship between a soluble lipoprotein and a membranous structure. The interconversion *in vivo* of blood serum lipoproteins with widely differing sizes is well established (e.g. Nilsson-Ehle *et al.* 1980), but this does not usually involve membranous structures.

Materials and Methods

Yolk Low-density Lipoprotein

For most experiments yolk lipoprotein was from unfertilized eggs of Australorp hens that were kept on a commercial mash diet at the Division of Animal Production, CSIRO, North Ryde, N.S.W. For experiments in which highly pigmented yolks were needed, the eggs were from an Australorp hen allowed access to green plants. Eggs used in one experiment were from Peking ducks. The following procedure was used for isolating lipoprotein from the pooled yolks of five or six eggs: the yolk (70 g) at about 20°C was mixed gently with sodium chloride solution (0·16 M, 70 ml, 5°C). The mixture was centrifuged for 45 min in the SW 27 rotor of a Beckman preparative ultracentrifuge at 20000 rpm, (70000 *g*) and 10°C, to sediment the yolk granules which were discarded. Next the supernatant solution was mixed with an equal volume of 4·0 M sodium chloride and about 200 ml of the mixture centrifuged for 2 h under the same conditions, after which the upper floating layer of the 'insoluble yolk globules' (Vadehra *et al.* 1977) was removed. The remaining solution was then centrifuged for a further 20 h as before and the yellow floating lipoprotein layer removed, dispersed in water (about 50 ml), and dialysed for 16 h at 2°C into 2 litres of de-ionized water in a stoppered flask. When smaller quantities of yolk were available, e.g. pigmented yolks, a smaller rotor was used for centrifuging (SW 65) at higher speeds for a shorter time. In some experiments small amounts of antioxidant, butylated hydroxytoluene, were added to the yolk (0·1 mg/g) without affecting the interaction of lipoprotein and lipid. The lipoprotein was kept for up to 12 days as a concentrated solution (14–20%) in water at 2°C.

Yolk Lipid

This was isolated from a solution of the above lipoprotein (5% w/v in 0·01 M EDTA, pH 6·5) by extraction with four volumes of a 1 : 1 chloroform: methanol mixture as in a previous method for the isolation of the apoproteins (Burley 1975). The lipid was stored in the chloroform phase after it had been concentrated to about 20% (w/v). This solution contained antioxidant added during the isolation of the lipoprotein. For most experiments the solvent was removed from the lipid under water-pump vacuum at 40°C: for some experiments an oil pump was used and evacuation continued for 16 h at 20°C to ensure the removal of solvents. The lipid mixture consisted largely of triacyl glycerols and phospholipids (e.g. Burley 1968). It is referred to here as 'yolk lipid' although it was not from the whole yolk.

Interaction of Lipoprotein and Lipid

The standard conditions used throughout this work were as follows: A sample of the above yolk lipid (0·15–0·4 g; within this range the quantity did not have a measurable effect) was homogenized for 20 s with 8 ml of 0·106 M acetic acid at 20°C in an Ultraturrax mixer (Janke and Kunke KG, Staufen, Germany) at 30% of maximum power. Lipoprotein (2·0 ml of a 14–18% w/v solution) was then added and the mixture (0·085 M acetic acid, pH 3·3) homogenized as before for 60 s. As mentioned later, homogenization helped greatly but was not essential. When different aqueous media were needed (e.g. salt solutions, buffers, urea) these were included in the 8 ml of aqueous solution added initially to the lipid. Where necessary sulfhydryl and other reagents were usually added to the lipoprotein before mixing. The samples were used as soon as possible after homogenization, although there was no evidence of further change on standing for several days.

Isolation of the 'Membranous Globules'

Two methods were used, corresponding to those used by Vadehra *et al.* (1977) for the 'insoluble yolk globules'. (1) The above mixture after homogenization was added to an equal volume of 4 M sodium chloride and centrifuged for 2 h (20000 rpm, SW 27 rotor, 20°C) to float the globules which were present in a waxy upper layer. This method was not used for analyses because unreacted lipid was not removed. (2) Alternatively, unreacted lipid and lipoprotein were removed by gel-filtration chromatography. The reaction mixture (10 ml) was applied to a column (2·2 by 120 cm) containing agarose (Bio-Gel A15m, 200–400 mesh, Bio-Rad Laboratories Inc., California) and eluted with 1·0 M sodium chloride, 0·025% (w/v) sodium azide, 20°C. Unreacted lipid and some protein remained at the top of the column as a white layer. When necessary, lipid was extracted from this layer with chloroform : methanol (1 : 1) v/v and the protein dissolved from the residual agarose by addition

of 4% (w/v) sodium dodecyl sulfate after the lipid and solvents had been removed. About 98% of the lipid applied to the column could be accounted for, either retained at the top or eluted. It is possible that the remainder adhered to the whole column because the resolving power decreased slowly with use.

Analytical Procedures

Lipid and protein were determined gravimetrically after separation by the one-phase method of Bligh and Dyer (1959). Phosphorus was determined by the method of Fiske and Subbarow (1925) after digestion in perchloric acid. Apoproteins were identified by gel electrophoresis in detergent and column chromatography in urea as described by Vadehra *et al.* (1977) for apoproteins from 'insoluble yolk globules'.

Centrifugation

A Spinco model E analytical ultracentrifuge (with double-sector cells) was used for sedimentation patterns, such as shown in Fig. 5. Preparative centrifugation was done on a Beckman L2-65 ultracentrifuge.

Electron Microscopy

Samples of lipid, lipoprotein, or reacted mixtures, were first isolated as floating pellets by centrifugation in 1 or 2 M sodium chloride for between 20 min and 4 h at 20000 rpm (SW 65 rotor, 10°C) depending on their density. Small pieces of the pellets were treated overnight in formaldehyde–glutaraldehyde fixative (Karnovsky 1965) at pH 7·2. This was followed by: (1) washing in phosphate buffer, pH 7·2; (2) post-fixation in 1% (w/v) phosphate-buffered osmium tetroxide, pH 7·2, for 2 h; (3) several washings in water; (4) staining in 2% (w/v) aqueous uranyl acetate for 1 h; (5) dehydration in an alcohol series; (6) embedding in an epoxy resin mixture (Spurr 1969); and (7) sectioning. Thin sections were stained on the grid with 2% (w/v) aqueous uranyl acetate and lead citrate (Reynolds 1963) and examined in a Siemens Elmiskop I electron microscope.

Particle diameters were measured on enlarged photographs by means of a magnifying scale.

Results

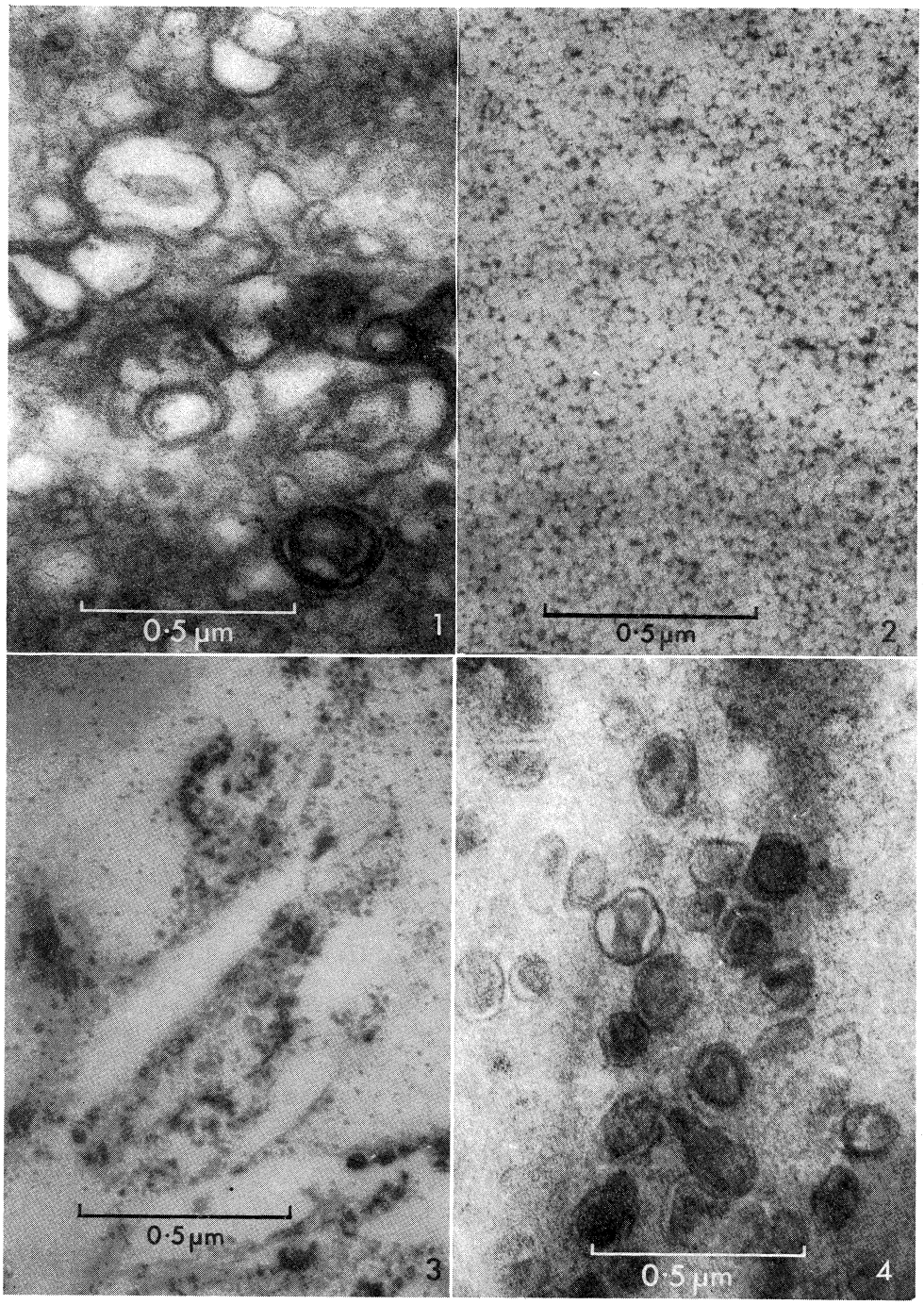
Evidence for interaction between yolk lipoprotein and lipid was obtained by electron microscopy, ultracentrifugation, and chromatography after mixtures had been homogenized in dilute acetic acid under the standard conditions given in Materials and Methods.

Electron Microscopy of Lipoprotein–Lipid Mixtures

Fig. 1 shows that after homogenization such mixtures contained round particles with irregular membranous outer layers. These particles, which are referred to as 'membranous globules', were not observed in either the lipoprotein (Fig. 2) or the yolk lipid (Fig. 3) after the same treatment. For comparison, Fig. 4 shows 'insoluble yolk globules' isolated from the same yolk mixture as described in Materials and Methods.

Ultracentrifugation of Lipoprotein–Lipid Mixtures

A measure of the extent of interaction between lipoprotein and lipid was provided by ultracentrifugal schlieren patterns. Fig. 5*a*, lower curve (II), shows the large reduction (by about 30%) in the size of the inverse lipoprotein peak when compared with lipoprotein alone (upper curve, I). The ill-defined rapidly floating material (X) possibly corresponded to the globules seen in Fig. 1. As described in the next section, interaction was not detected with all mixtures. Fig. 5*b*, for example, shows that with one sample at higher pH and high salt concentration there was no evidence for interaction.



Figs 1–4. Electron micrographs of thin sections prepared as described in Materials and Methods

Fig. 1. Membranous globules prepared from a lipoprotein–lipid mixture in acetic acid (0·085 M, standard conditions given in Materials and Methods) and isolated by centrifugation.

Fig. 2. Yolk lipoprotein, homogenized in acetic acid (as in the standard method for treatment of lipoprotein–lipid mixtures but without lipid) then chromatographed on agarose as in Fig. 6*a*.

Chromatographic Isolation of Membranous Globules

Gel-filtration chromatography on agarose columns provided a more convenient method for estimating the extent of interaction of lipoprotein and lipid, and it also enabled the main product of interaction, membranous globules, to be isolated free from the reactants. Fig. 6*a*, curves I*a*, I*b*, showed that after homogenization not all the lipoprotein emerged in the usual position (i.e. peak B). Instead, a new peak, A, well ahead of B, was present, and there were indications of a smaller intermediate peak, C. Peaks A and C were absent when the lipoprotein was homogenized in acetic acid without lipid (e.g. curves II*a*, II*b*). Homogenization of lipid without lipoprotein produced a little material that eluted in position A, but most of the lipid was retained at the top of the column.

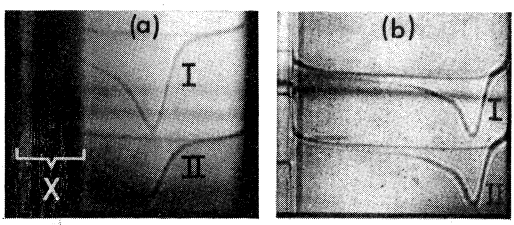


Fig. 5. Ultracentrifuge patterns of homogenized mixtures of lipoprotein and yolk lipid that had been dialysed into 1 M sodium chloride before centrifuging. Floatation from right to left. (*a*) Reaction in 0·085 M acetic acid (pH 3·3) as in Materials and Methods. Photograph taken 17 min after reaching 52 000 rpm, 20°C. Bar angle 60°. Curve, I control. Lipoprotein (3·7%) homogenized without added lipid. Curve II, lipoprotein (3·7%) homogenized with lipid (0·21 g) in acetic acid (0·085 M) as in standard method. The floating material 'X' refers to curve II only. (*b*) Reaction in 0·5 M sodium chloride, 0·05 M Tris buffer, pH 7·0. Photograph taken 20 min after reaching 42000 rpm. Bar angle 65°. Curve I, control, no lipid added, lipoprotein 2·0%. Curve II, lipoprotein 2·0% homogenized with lipid (0·15 g).

According to electron microscopy (Fig. 7), peak A of Fig. 6 contained globules similar to those in the original mixture, although they were better-defined, probably because unreacted lipid and products such as partly formed globules did not pass down the column. The weights of solids and the proportions of protein and lipid in peaks A and B (Fig. 6) were determined. Material in peak C was included with peak A. Peak C apparently represented an unstable intermediate because on rechromatographing it emerged in position A. The decrease in peak B after homogenization was used as a measure of the extent of interaction. Approximately half the lipoprotein apoprotein lost was found in the globules (peak A). The rest remained at the top of the column.

Considerable variability in the extent of interaction was found under the standard conditions (i.e. low pH, low ionic strength). Thus for 13 determinations on different preparations, the average decrease in peak B (Fig. 6) compared with the control, was 26·6% (s.d. 12·3), the range being from 9·0 to 37·8. This variation is well outside the error of measurement, but the reasons for it have not been found. The following treatments did not decrease the amount of lipoprotein in peak B or increase the amount of globules: adding mercaptoethanol (to 0·025 M) or EDTA (to 1 mM) to the lipoprotein before mixing, adding chloroform (40 mg/g) or sodium dodecyl sulfate (8 mg/g) to the lipid, drying the lipid under oil-pump vacuum, omitting

Fig. 3. Yolk lipid homogenized in 0·085 M acetic acid without lipoprotein, then separated by centrifuging.

Fig. 4. Natural 'insoluble yolk globules' isolated by the centrifugal procedure of Vadehra *et al.* (1977).

antioxidant from the mixture. An experiment with lipoprotein from duck's eggs gave a much higher interaction (41%) when homogenized with hen's lipid than that usually found with hen's lipoprotein.

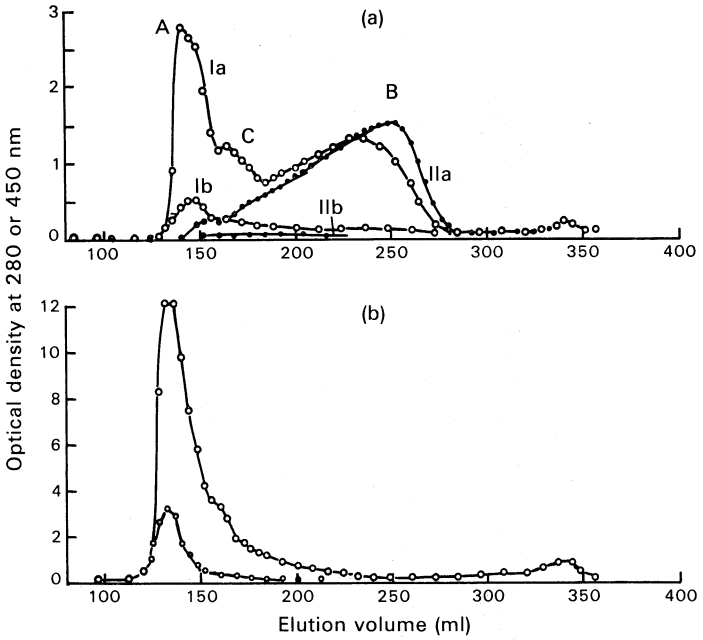


Fig. 6. Chromatography of lipoprotein–lipid mixtures and controls on an agarose column as described in Materials and Methods. (*a*) Curves I*a* and I*b*: homogenized mixture (10·0 ml) containing lipoprotein (0·194 g) and yolk lipid (0·20 g) in 0·085 M acetic acid. I*a*, optical absorbance at 280 mn; I*b* optical absorbance at 450 nm, i.e. turbidity. Curves II*a* (280 nm) and II*b* (450 nm): control, lipoprotein (0·194 g) homogenized in 0·085 M acetic acid without lipid. Peaks A and C were opaque, the unchanged lipoprotein, peak B, was almost clear. (*b*) Mixture of lipoprotein (0·194 g) and lipid (0·20 g) homogenized in 4·8 M urea, 0·85 M acetic acid. Upper curve, absorption at 280 nm. Lower curve, absorption at 450 nm.

Factors that *did* influence the extent of conversion were pH and ionic strength, although here a further complication was observed. For most lipoprotein preparations the extent of conversion decreased by about 30% from pH 3·3 to pH 8 at low ionic strength, and there was a similar decrease at pH 3·3 on raising the ionic strength from 0·1 to 1·0 by addition of sodium chloride; but for about 10% of the preparations conversion was greatest at high ionic strength and was not affected much by pH. This difference was not associated with any change in the method of preparation or subsequent treatment.

The presence of urea also had a large effect on the lipoprotein–lipid interaction. Fig. 6*b* shows the effect of 4·8 M urea. More concentrated urea (6 M) had a similar

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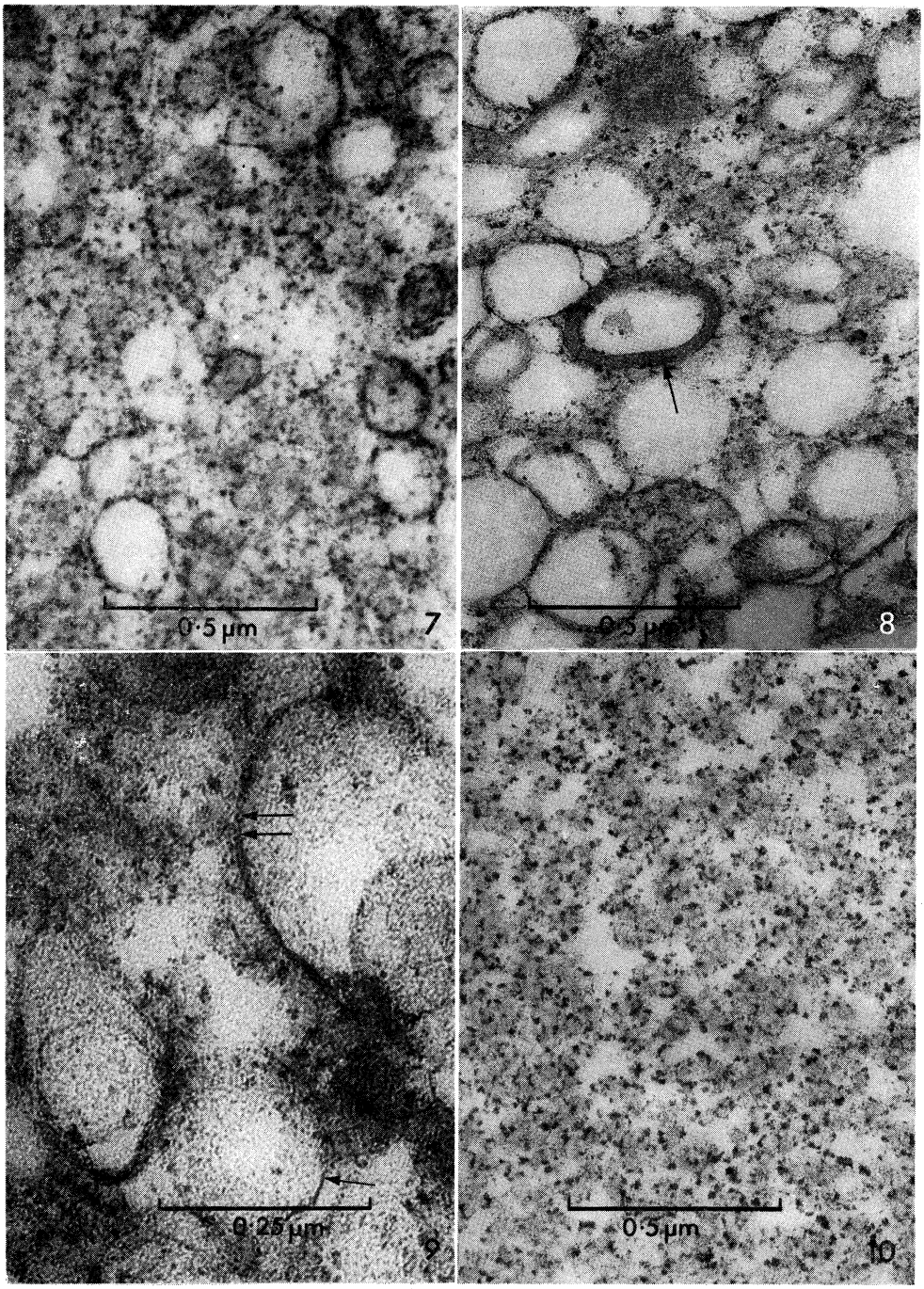
Fig. 9. Membranous globules prepared as in Fig. 8 but showing the single (↑) and double layered (↑↑) membranes at greater magnification

Fig. 10. Lipoprotein homogenized in 4·8 M urea (pH 3·3) then chromatographed (Fig. 6*b*), as for Figs 8 and 9 but without lipid.



Figs 7–10. Electron micrographs of thin sections prepared as described in Materials and Methods.

Fig. 7. Membranous globules prepared from a lipoprotein–lipid mixture as in Fig. 1 but isolated by chromatography as in Fig. 6*a*.

Fig. 8. Membranous globules prepared from a lipoprotein–lipid mixture in 4·8 M urea (pH 3·3) and isolated by chromatography as in Fig. 6*b*. The arrow indicates a frequently occurring multilayered membranous globule.

effect; namely, almost complete removal of lipoprotein and a much enhanced peak A. Electron microscopy (Fig. 8) showed that this peak contained membranous globules similar to those observed in dilute acetic acid although the membranes were more distinct. Multilayered membranes were visible, in addition to those with single and double membranes. The single and double membranes are shown at greater magnification in Fig. 9. By contrast with the behaviour in the absence of urea, homogenization without lipid in 4·8 M urea led to a loss of lipoprotein. Some of the product emerged in peak A where electron microscopy (Fig. 10) indicated ill-defined material, probably degraded lipoprotein, with no sign of membranous globules (cf. Fig. 2). The small, densely stained particles seen in Figs 7–10 have not been identified. They did not appear to be related to the treatment with urea or to the staining.

Table 1. Composition of membranous globules and a comparison with other yolk constituents

Values are percentages ± standard errors for three to six estimations

Preparation	Protein content (%)	Lipid content (%)	Phospholipid[A] (as % of total lipid)
Membranous globules			
Prepared in acetic acid	7·5±2·7	93·8±1·6	34·6±1·7
Prepared in 4·8 M urea	9·7±2·6	90·2±1·1	n.d.[B]
Insoluble yolk globules (see Vadehra *et al.* 1977)	10·3±0·5	89·9±0·8	35·1±6·7
Low-density lipoprotein (see Burley 1978)	12·2±0·2	87·1±0·3	27·2±0·1

[A] Lipid phosphorus × 25. [B] Not determined.

In the above experiments the interaction of lipoprotein and lipid was studied after homogenization at low speed. Homogenization was not, however, essential although it induced a higher percentage of conversion. Thus, if a mixture was shaken gently for a few minutes, instead of homogenizing it for 60 s, there was loss of lipoprotein and formation of globules—about 75% of that resulting from the other treatment of the same sample.

Composition and Size of Isolated Membranous Globules

Table 1 refers to globules prepared in acetic acid by the standard procedure and by homogenization in urea. For comparison, results for natural insoluble yolk globules and for the yolk lipoprotein are included. The membranous globules were evidently closer to the natural globules than to the original lipoprotein. The amount of protein in the membranous globules was variable, and part of it was insoluble in urea or detergent solutions plus mercaptoethanol. Nevertheless, the apoprotein pattern resembled that of the natural globules, i.e. there was a lower proportion of the apoprotein of low molecular weight, apovitellenin I, compared with the original lipoprotein. The protein retained at the top of the column after chromatography, as in Fig. 6*a*, had a relatively high proportion of apovitellenin I presumably because it contained protein lost from the lipoprotein but not included in the membranous globules.

In Fig. 11 the size distribution of the insoluble yolk globules is compared with the membranous globules produced by interaction of lipoprotein and lipid as in Materials and Methods. The sizes are evidently similar, and they are close to those for insoluble yolk globules reported previously by Vadehra *et al.* (1977) when account is taken of the finer subdivisions in Fig. 11.

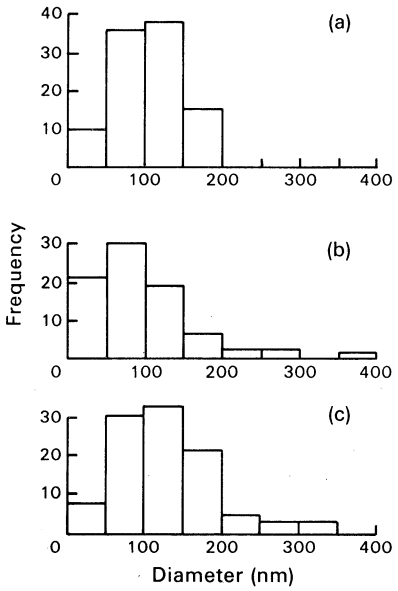


Fig. 11. Histograms showing distribution of diameters of globular particles seen in thin sections. (*a*) Insoluble yolk globules, Fig. 4; (*b*) Membranous globules prepared in 4·8 M urea, Fig. 8; (*c*) Membranous globules prepared by standard procedure, Fig. 7.

Redistribution of Neutral Lipid during Lipoprotein–Lipid Interaction

In an attempt at determining the source of the neutral lipids in the membranous globules, the following experiment was performed. Brightly coloured lipoprotein from highly pigmented yolk was homogenized under the standard conditions with lightly coloured lipid from less-pigmented yolk. After isolation by chromatography, as in Fig. 6*a*, lipid was extracted from fraction A plus C and from B with chloroform–methanol (1 : 1 v/v) and the optical absorption of the organic phase measured at 450 nm. Absorption at this wavelength corresponds to the concentration of carotenoid pigments. The optical extinctions ($E^{1\%}_{450\ nm}$) for fraction A plus C (Fig. 6), fraction B (Fig. 6), and for the lightly coloured lipid added before homogenization were 0·055±0·026, 0·174±0·069, and 0·028±0·001, respectively. The errors in this experiment were rather large; nevertheless it is apparent that the material in peaks A plus C did not acquire much pigment. On the assumption that the movement of the carotenoid pigments is a measure of that of other neutral lipids, it is likely that most of the neutral lipid in the globules was from the free lipid rather than from the lipoprotein.

Discussion

It is clear from our results that the major low-density lipoprotein of avian egg yolk is not entirely stable in the presence of aqueous dispersions of yolk lipid. In fact, under some conditions, the dispositions of much of the lipoprotein apoprotein and phospholipid can be altered to give membranes surrounding large globules of lipid. The globules formed in this way are similar in appearance under the electron microscope (Figs 1 and 7), in size distribution (Fig. 11), and in composition (Table 1), to the natural particles in yolk referred to previously as 'insoluble yolk globules' (Vadehra *et al.* 1977). The latter are notable for their resistance to urea and strong salt solutions. These are also properties of the new particles.

We have not quite eliminated the possibility that the conversion into membranous globules involved an enzymic or microbial process; but conversion also took place under conditions that do not favour such processes; for example, in concentrated acidic urea (Figs 8 and 9). Interchange of non-covalent bonds between lipids and apoproteins is therefore implicated in the conversion.

Understanding the mechanism of conversion presents considerable difficulty. An important problem is why, according to one type of experiment, neutral lipid from the lipoprotein took little part in the globules. From the composition, it should be possible to convert the lipoprotein into larger particles with elimination of some protein and the rearrangement of the remaining protein and the lipid. In practice even when the lipoprotein structure was disrupted, e.g. by homogenization in urea (Fig. 10), there was no evidence for formation of membranous globules. The presence of excess lipid was evidently necessary to produce these structures, possibly because a preformed lipid surface is essential. Other problems concern the variability of the results with different preparations using the same procedure, and the fact that a few preparations gave high conversions under most conditions whereas most needed a low ionic strength and low pH. We have not yet solved these problems. The factors we have considered, such as multivalent metals, detergents, oxidation, do not appear to be responsible. The difference between hen's and duck's egg-yolk lipoprotein implies that subtle differences in lipid–protein organization are important.

The general resemblance of the 'membranous globules' to the natural 'insoluble yolk globules' suggests that the latter could be produced *in vivo* from lipoprotein and lipid. Possibly the natural particles are formed when there is an excess of lipid in yolk, as a means of ensuring the stability of lipid–protein particles with a lower proportion of protein than the lipoprotein.

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