

Studies on the Apoproteins of the Major Lipoprotein of the Yolk of Hen's Eggs

III.* Influence of Salt Concentration during Isolation on the Amount and Composition of the Apoproteins

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Abstract

When the major lipoprotein of hen's eggs was prepared by centrifuging yolk in salt solutions, the ionic strength affected the apoprotein mixture. At high salt concentrations (ionic strength above about 2) more protein was present in the lipoprotein than at lower ionic strength. The extra protein was not removed by subsequently lowering the ionic strength. This extra protein consisted largely of proteins of high molecular weight, including, according to electrophoresis, γ -livetins from the aqueous phase of yolk.

Introduction

As a result of studies in this and other laboratories, information is now available about the low-molecular-weight apoproteins from the low-density high-lipid lipoprotein of the yolk of hen's eggs (Burley 1975; Burley and Davies 1976; Raju and Mahadevan 1976; Bengtsson *et al.* 1977). The apoproteins of high molecular weight have, however, so far proved more difficult to study. One possible complicating factor is discussed here, namely, when the lipoprotein is isolated by centrifuging at very high salt concentrations the proportion of apoprotein, particularly the high-molecular-weight apoprotein, is larger than when low ionic strength is used. The reasons for this behaviour are not certain, but it is clear that the use of very high ionic strengths during preparations leads to an unnecessary complication in the study of the yolk apoproteins.

Materials and Methods

Yolk Lipoprotein

Pooled yolk from 6-12 freshly laid eggs of Australorp hens kept at the Division of Animal Production, CSIRO, North Ryde, N.S.W., was used. The lipoprotein was isolated by the following method: the yolk was diluted with an equal volume of 0.16 M NaCl and centrifuged for approximately 40 min at 80 000 *g* at 10°C. Usually an antioxidant (butylated hydroxytoluene, 0.1 mg/g of yolk) was added before centrifuging; alternatively O₂-free solutions were used and preparations were done under N₂ as far as possible. During centrifuging the yolk granules sedimented and were later discarded. The supernatant solution was mixed with an equal volume of salt solution of twice the desired final concentration, taking into account the sodium chloride added already but not the salt present naturally, and the lipoprotein was isolated by centrifuging at approximately 2×10^6 h $\times$ g, at 10°C, using either the SW 41 rotor or the SW 27 rotor of a Beckmann L2-65 preparative ultracentrifuge. The lipoprotein was removed from the top of the centrifuge tubes and resuspended in 10 times the volume of salt solution, of the same concentration used for the first prolonged centrifugation, and recentrifuged. The lipoprotein was finally dialysed against 1.0 M NaCl for physical

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measurements, or against water for isolation of the apoprotein. According to gel-filtration chromatography on columns of agarose (Burley 1970) the isolated lipoprotein did not contain free protein.

For certain of the more concentrated solutions it was necessary to add solid salt before centrifuging. This did not, however, appear to affect the results. For solutions of low ionic strength (<1) glycerol was included to increase the density and so prevent fractionation of the lipoprotein during centrifuging (Martin *et al.* 1964). In a few experiments the insoluble yolk globules—a very small proportion of the total yolk—were removed by centrifuging for 2 h at 40 000 *g* (Vadehra *et al.* 1977) without altering the results.

Analysis of Protein and Lipid

The amount of protein in the lipoprotein was determined gravimetrically after extraction of the lipid by the method of Bligh and Dyer (1959) modified as follows for small samples. To 5.0 ml of the aqueous solution of lipoprotein (about 3% w/v) in 1 M NaCl was added 6.25 ml of chloroform plus 12.5 ml of methanol. After 16 h at 20°C more chloroform (7 ml) and water (7 ml) were added, the protein was filtered off, washed with chloroform and water, and dried and weighed. The lipid in the chloroform layer was determined gravimetrically. Total recoveries of lipid and protein were usually between 95 and 98% of the initial weight of lipoprotein. It was found that recoveries were lower if the lipoprotein concentration was greater than about 5%, for reasons that have not been determined.

Phospholipid was estimated from the amount of phosphorus in the extracted lipid.

Isolation and Fractionation of the Apoproteins

Either of the methods described previously (Burley 1975) were used to remove lipid and dissolve the apoproteins. These were then chromatographed on a column of Sephadex G100 in 6 M urea, pH 3.3, as already described although the initial apoprotein concentration was 0.7% or less. Two fractions were collected: (1) the 'low-molecular-weight' fraction described previously, containing apovitellenins I, Ia, and II; the (2) the 'high-molecular-weight' fraction containing the remaining apoproteins. The amounts of these fractions were determined gravimetrically after dialysis against water.

Physical Measurements

Partial specific volumes were determined by extrapolation to infinite dilution after measuring solution densities at a range of lipoprotein concentrations on an Anton Paar density meter model DMA 02C. The solutions were first well dialysed against 1.0 M NaCl and the dialysate used for dilutions.

For ultracentrifuge measurements a Spinco model E ultracentrifuge was used. Because of their low density and refractive index, the yolk lipoproteins floated in aqueous solutions and gave inverse Schlieren patterns (see for example Faucher and Koleske 1965).

Electron microscopic examination of lipoprotein particles was carried out following negative staining according to the procedure of Nichols *et al.* (1969). Particle diameters were measured on enlarged photographs.

Ionic strengths were calculated from the molarity of the salt solutions.

Gel Electrophoresis

The procedure of Weber and Osborn (1969) was used with columns (0.5 by 7 cm) of polyacrylamide gel (7.5% with 5% cross-linking), the electrode buffer being sodium dodecyl sulfate (0.1% w/v), tris chloride (0.03 M), pH 7.6. The protein samples, after chromatography, were isolated by precipitation with trichloroacetic acid, washed with water, and a small part (about 0.5 mg dry weight) dissolved in 4% (w/v) sodium dodecyl sulfate (100 μ l). After addition of marker dye (bromophenol blue), and glycerol, samples of 10 μ l were applied to the gels through which a current of about 7 mA at 100 V was passed for 1 h.

Results

The relationship between the percentage of protein in the yolk lipoprotein and the ionic strength at which it was isolated is shown in Fig. 1. Although sodium

chloride was used for most experiments sodium bromide and ammonium sulfate were also used. The results show signs of considerable scatter, especially at high ionic strengths. The source of this variation has not been identified. It could have been related to variations amongst different preparations of lipoprotein because

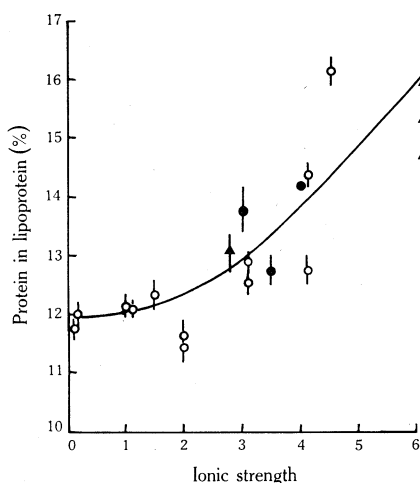


Fig. 1. Percentage of protein in yolk lipoprotein as a function of ionic strength during centrifuging. Each point refers to a separate isolation of lipoprotein on which between three and six analyses were done.
 ○ Sodium chloride.
 ● Sodium bromide.
 ▲ Ammonium sulfate.
 Standard errors are shown by vertical bars.

results from each preparation were more consistent, but the possibility that some factor other than the salt concentration was exerting an influence cannot be excluded. In spite of the variability, it is clear from Fig. 1 that the proportion of protein in the lipoprotein increased as the ionic strength was increased above about 2.

Table 1 summarizes some properties of lipoproteins from one sample of pooled yolk isolated at two ionic strengths. As expected from Fig. 1 the partial specific volume was lower when a higher salt concentration was used for isolation. This

Table 1. Properties of lipoproteins from one sample of hen's egg yolk after isolation at low and high ionic strengths of sodium chloride
 The standard errors refer to four or more determinations

	Ionic strength	
	1.0	4.1
Protein (g/100 g)	12.2±0.2	14.3±0.4
Lipid (g/100 g)	87.1±0.3	84.4±0.3
Phospholipid (% total lipid)	27.2±0.1	27.1±0.1
Partial specific volume (ml/g)	1.039	1.020
Flotation coefficient, $s_{20}^{f,M}$ NaCl(S)	21.8	19.5
Low-molecular-weight apoprotein fraction (% total apoprotein)	34±2	29±2

difference was due to the difference in protein as the proportion of phospholipid in the lipid did not change. The flotation patterns of the lipoproteins described in Table 1 are compared in Fig. 2. The general shapes of the curves in Fig. 2 are very similar. There was, however, a slight displacement of the whole curve. This suggests that the distribution of the extra protein was approximately uniform in the upper sample and was not confined to one lipoprotein fraction. Because these samples had

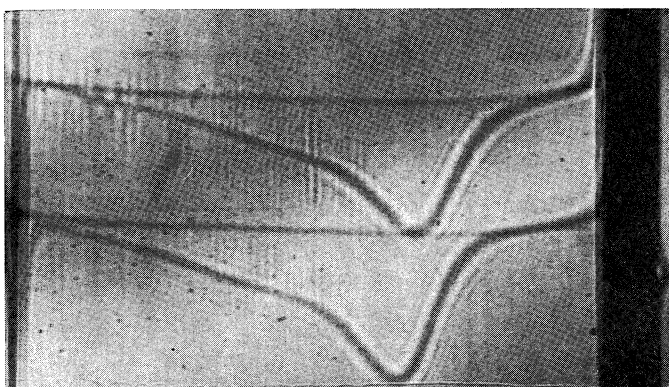


Fig. 2. Ultracentrifuge pattern of yolk lipoprotein isolated at ionic strength 4.1 (upper curve, lipoprotein concentration 1.8% w/v) and at ionic strength 1.0 (lower curve, lipoprotein concentration 2.0% w/v). The solvent was 1.0 M NaCl, and each sample was dialysed for 24 h. The plate was taken after 27 min at 42 000 rev/min, 20°C, bar angle 70°.

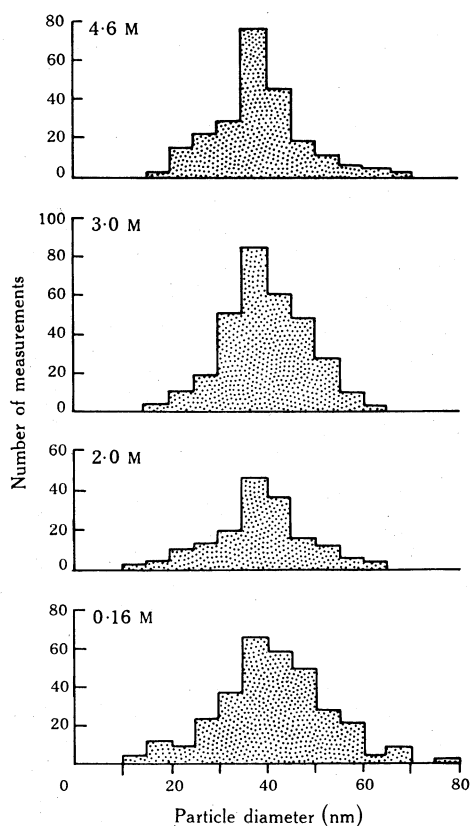


Fig. 3. Distribution of the diameters of lipoprotein particles in preparations isolated at four concentrations of NaCl (4.6, 3.0, 2.0 and 0.16 M). The preparations in 4.6 and 2.0 M NaCl were from one sample of yolk and the others from another sample measured at a different time.

been dialysed against sodium chloride of ionic strength 1.0, the ultracentrifuge experiments also showed that protein was not lost from the high-protein sample when the ionic strength was lowered. A more sensitive test was provided by the gel-filtration chromatography of this lipoprotein in 1.0 M NaCl (see Materials and Methods) when little free protein was detected.

Electron microscopic examination revealed little difference in the appearance of lipoprotein particles isolated at different ionic strengths, and there was no difference in size distribution (Fig. 3).

The apoproteins were fractionated by gel-filtration chromatography as described in Materials and Methods. Those from lipoprotein isolated at high ionic strength had a higher proportion of high-molecular-weight protein and a lower proportion of low-molecular-weight protein than those isolated at low ionic strength (Table 1).

The nature of the high-molecular-weight proteins from both lipoprotein preparations was explored by gel electrophoresis. Fig. 4 shows that both patterns are complex and are similar except for two distinct bands (arrows) that are almost absent in the low-ionic-strength preparation. In its position, the darker band resembles that of γ -livetins (Martin and Cook 1958) which is the only high-molecular-weight soluble protein found so far in yolk.

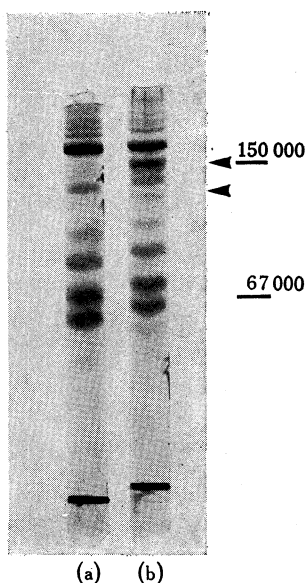


Fig. 4. Gel electrophoresis in detergent (sodium dodecyl sulfate) of the high-molecular-weight apoprotein fraction from yolk lipoproteins isolated in sodium chloride at (a) ionic strength 1.0 and (b) ionic strength 4.1. The arrow heads indicate the main differences and the horizontal lines refer to the positions of standard proteins: hen's γ -livetins (molecular weight 150 000) and bovine serum albumin (67 000). These markers refer to gel (b) because of the slight difference in the alignment of the two gels. Electrophoresis was from top to bottom. The lower bars on the gels represent the marker dye. Gels were stained with Coomassie blue.

Discussion

Centrifugation in concentrated solutions of inorganic salts has long been a common procedure in the isolation of soluble lipoproteins (e.g. Lindgren and Nichols 1960). The above experiments show that for one system, hen's egg yolk, the use of very high salt concentrations leads to lipoprotein with a higher proportion of protein than when lower salt concentrations are used. Apparently no similar effect has been reported for other systems. Thus although increased binding of protein to lipid would be expected at high salt concentrations, the effect appears to be specific rather than general. Two suggestions can be offered to explain this effect of ionic strength:

(1) at high ionic strength some of the soluble proteins, i.e. the livetins, combine with the lipoprotein particles; alternatively, (2) in intact yolk there is a close association between livetins and lipoproteins that is stabilized by a high ionic strength. Neither of these, however, really explains the fact that the extra protein is not readily removed. It should be mentioned that attempts at combining isolated lipoprotein with livetins have not been successful which is evidence against suggestion (1). It is therefore likely that the extra protein enters into the lipoprotein structure in an irreversible manner that is at present not understood.

Acknowledgments

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