Hydrophobic chromatography of proteins in urea solutions

The separation of apoproteins from a lipoprotein of avian egg yolk

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(Received 22 June 1982/Accepted 13 September 1982)

A method is described for the chromatographic separation of mixtures of egg-yolk proteins of low solubility by using a hydrophobic column (phenyl-Sepharose) and eluting with increasing concentrations of aqueous urea at low pH. The resolving power of the method was established by tests on proteins and protein fragments of known sequence. The theoretical basis for the method remains, however, unclear. Factors such as the aggregation of the protein often appeared to be more important than its hydrophobicity in determining the urea concentration needed for elution. The method was applied to the mixture of apoproteins from the low-density lipoprotein (density about 0.95 g/ml) of avian egg yolk. For the previously studied apoproteins from egg yolk of the hen (*Gallus domesticus*), hydrophobic chromatography provided a new and convenient method for isolating the main apoproteins (hen apovitellenins I–VI). For the hitherto unexplored apoproteins from egg yolk of the duck (*Anas platyrhynchos*) the method has now been used to isolate three new proteins, two of which were not readily separated by methods based on molecular size. The elution pattern obtained with duck egg-yolk apoproteins is not the same as that of the hen egg-yolk apoproteins, although we suggest a relationship for the three new apoproteins based on their amino acid compositions and other properties. Possible roles for the apoproteins in avian egg yolk are described.

Hydrophobic chromatography, in which the stationary medium contains hydrophobic groups (Er-el *et al.*, 1972), has been successfully applied to the separation of soluble proteins (see, e.g., the review by Shaltiel, 1976). Theoretical aspects have been considered in detail by Srinivasan & Ruckenstein (1980). Usually the proteins are applied to the hydrophobic column in water or dilute aqueous solution, and eluted with salt solutions, detergents or mixed solvents. It has more recently been found that the range of hydrophobic chromatography can be extended to include highly insoluble proteins, including those associated with lipids, by using increasing concentrations of urea as eluent and thus avoiding the use of detergents and salts (Creamer & Matheson, 1981; Burley & Sleigh, 1981).

We have applied this method to the problem of the resolution of the complicated mixture of apoproteins isolated from the major, i.e. low-density, lipoprotein fraction (density about 0.95 g/ml) of avian egg yolk. The number, nature and purpose of these proteins is a problem in avian biochemistry. It

has been shown that one of them ('hen apovitellenin I') is transferred unchanged in sequence from the blood very-low-density lipoprotein to egg-yolk lipoprotein (Dugaiczky *et al.*, 1981), where it possibly has a structural role (Burley, 1973). None of the other apoproteins has been studied in such detail. Their study has been complicated by the problem of isolation, as most of them are insoluble in aqueous buffers. Methods used for their isolation have so far depended on molecular size. These methods include gel-filtration chromatography in various media such as detergents, dilute HCl and urea (Burley, 1975; Raju & Mahadevan, 1976; Bengtsson *et al.*, 1977; Burley & Sleigh, 1980), and also gel electrophoresis in detergent plus urea (Burley & Sleigh, 1980). Hydrophobic chromatography in urea solutions provides a useful alternative to these methods, and should enable large amounts of the yolk apoproteins to be isolated for further study. In the present paper we give examples of its use on known and unknown apoprotein mixtures. A brief account of some of our results has been reported (Burley & Sleigh, 1981).

Materials and methods

Urea solutions

Standard solutions of urea of analytical grade were passed through a mixed-bed ion-exchange resin column and a micropore filter (pore size $0.45\ \mu\text{m}$). Small volumes of concentrated acid, or occasionally buffer salt, were then added. Solutions that contained concentrations of urea higher than 3 M were acidified with HCl (final concn. 20 mM, pH 3.0–3.5). For lower concentrations of urea, HCl was replaced by formic acid or acetic acid to prevent the pH from falling below about 3, because of possible damage to the chromatographic gels.

Chromatographic materials

Sephadex, Sepharose and the hydrophobic materials phenyl- and octyl-Sepharose CL-4B were from Pharmacia, Sydney, N.S.W., Australia. For the results reported in the present paper one batch of phenyl-Sepharose (lot no. FM19078) was used. No change in its properties was observed over 10 months, during which it was in contact with acidic urea at 21°C. A batch used for experiments reported previously (lot no. 9705; Burley & Sleight, 1981) gave slightly different results in that the resolution of the less-retarded proteins was improved and they were eluted at lower urea concentrations. The order of elution was, however, unchanged. Treating this batch of Sepharose with the proteolytic enzyme Pronase (Kaken Chemical Co., Tokyo, Japan) in case its properties had been altered by the permanent adsorption of a small amount of protein did not affect its behaviour, which probably depended on the original agarose.

A 2,4-dinitrophenyl derivative of Sepharose CL-4B, referred to as 'dinitrophenyl-Sepharose' was prepared by coupling bis-(2,4-dinitrophenyl)lysine to Sepharose CL-4B by the procedure of Axen *et al.* (1967). The coupling was performed in a tetrahydrofuran at room temperature by using [1-cyclohexyl-3-(2-morpholinoethyl)carbodi-imide methotoluene-*p*-sulphonate] (from Aldrich Chemical Co., Milwaukee, WI, U.S.A.).

Proteins

Egg-yolk apoproteins. These were from the low-density lipoprotein of fresh egg yolk from Australorp hens (*Gallus domesticus*) or Pekin ducks (*Anas platyrhynchos*). The lipoproteins were obtained by centrifuging granules-free yolk in 2 M-NaCl as described previously (Burley, 1978). Apoproteins were isolated by removing the lipid with chloroform/methanol (1:1, v/v) at neutral pH (Burley, 1975) and dissolved in 3 M-urea (pH 3) to give a protein concentration of about 0.5%.

The low-molecular-weight apoprotein, duck's apovitellenin I, and its CNBr-cleavage fragments were isolated as described by Inglis & Burley (1977).

Hen's apovitellenin I was isolated by gel-filtration chromatography on Sephadex G-75 (Burley, 1975). The monomeric, i.e. reduced, form was obtained by treating a solution (0.4%) in 6 M-urea, pH 7, with 2-mercaptoethanol (final concn. 0.1 M) for about 4 h at 21°C and then dialysing it against acidic urea solution.

Other proteins. A mixture of α -, β -, and κ -caseins was obtained by acid precipitation from fresh bovine milk (Manson & Annan, 1971). Phosphate groups were removed with potato acid phosphatase (Bingham *et al.*, 1976).

Hydrophobic chromatography

A column (50 cm $\times$ 2.2 cm) containing 175 ml of phenyl-Sepharose CL-4B was used at 21°C with upward flow at a rate of 16–18 ml/h, during which three fractions were collected. Linear urea gradients were prepared by means of a gradient mixer (model GM-1 from Pharmacia). Usually acidic 3 M- and 9 M-urea in equal volumes of 200–280 ml were mixed. Proteins in the fractions were detected at 280 nm in 1 cm cells in a Unicam SP.3000 spectrophotometer (Pye-Unicam Instruments, Cambridge, U.K.). Proteins or fragments with low absorbance at 280 nm were detected by applying spots (25 μl) to filter paper and staining with Coomassie Blue (0.1%) in acetic acid/ethanol/water (1:4:5, by vol.).

Hydrophobicity index

As a measure of the hydrophobicity of proteins and peptides, the hydrophobicity index (Φ) of Bigelow (1967) was used. This index represents the average value of the free energy of transfer of their amino acid residues from ethanol to water, expressed as ΔF_T kJ/mol of amino acid. The corrected values given by Nozaki & Tanford (1971) were taken into account.

Electrophoresis

Yolk proteins and polypeptides were identified on tubes (8 cm $\times$ 0.7 cm) or slabs (8 cm $\times$ 8 cm $\times$ 0.2 cm) of polyacrylamide gel in minor modifications (Burley & Sleight, 1980) of the procedure of Weber & Osborn (1969). Better resolution was obtained with tubes, but slabs were more convenient for comparisons and for photoelectric scanning. For the gel patterns given in the present paper, slabs containing 6 M-urea/65 mM-Tris/borate buffer/1 mM-EDTA, pH 8.6, were used. Gels used for proteins (Figs. 3 and 5) contained 7.5% acrylamide. Those used for peptides (Fig. 6) contained 14% acrylamide. The gels were stained with a mixture of Coomassie Blue plus Amido Black [each 0.05% in acetic acid/water/ethanol (1:4:5, by vol.)] and scanned on a Shimadzu photoelectric densitometer (Schimadzu Corp., Kyoto, Japan). Finally, the scans were traced. Molecular weights were estimated by using a series

of standard proteins, as described previously (Burley & Sleigh, 1980).

Milk caseins were identified by electrophoresis in urea without detergent.

Analytical procedures

Protein concentrations were determined gravimetrically after the solution had been dialysed into water. Dialysis tubing with high retention (Spectrapor; Spectrum Medical Industries, Los Angeles, CA, U.S.A.) was used.

Amino acid compositions of proteins were determined on HCl hydrolysates, by using a Beckman model 120C analyser (Beckman Industries, Palo Alto, CA, U.S.A.).

Tryptophan was determined by the spectrophotometric method of Edelhoch (1967).

Results

Hydrophobic chromatography of egg-yolk apovitellenin I and other proteins of known sequence

Fig. 1 shows the separation of a synthetic mixture of two similar proteins of low molecular weight from egg yolk of different avian species, duck's apovitellenin I and the reduced form of hen's apovitellenin I. Table 1 gives the positions of elution and other data for these proteins, and shows that they were eluted in the order expected from their hydrophobicities, i.e. the more hydrophobic protein was eluted later. Subsequent tests suggested, however, that hydrophobicity was not always dominant. Thus the oxidized, dimeric, form of hen's apovitellenin I was eluted much later (Table 1) and was completely separated from the monomer; and tests on other mixtures of known proteins showed that, although separation was often complete, there was no consistent relationship between elution pattern

and hydrophobicity. For example, Table 1 also gives data for the elution of two polypeptide series: (i) three CNBr-cleavage fragments of duck's apovitellenin I, and (ii) three caseins from bovine milk before and after removal of phosphate groups. For each series the constituents were separated at least partly. Resolution could be improved by using a shallower gradient, but neither the composition (e.g. number of aromatic residues) nor the sequence (e.g.

Fig. 1. *Hydrophobic chromatography of apovitellenin I* A column of phenyl-Sepharose was used (see the Materials and methods section), and samples (each 10 mg) of duck apovitellenin I (peak A) and the reduced form of hen apovitellenin I (peak B) were applied in 8.0 ml of acidic 2M-urea. The fractions were each of 5.3 ml, and the points refer to optical absorbance at 280 nm. The straight line refers to the urea concentration gradient. Peaks were identified by the amino acid composition of the isolated proteins.

Table 1. *Hydrophobic chromatography of proteins and polypeptides*

Positions of elution of proteins and protein fragments during chromatography as in Fig. 1 are given, plus other data. For experimental details see the text.

Protein	Elution position ([urea], M*)	Hydrophobicity index† (kJ/mol)	No. of residues	Aromatic residues
Duck apovitellenin I	6.85 ± 0.10	5.15	82	9
Hen apovitellenin I (reduced)	6.64 ± 0.11	4.69	82	7
Hen apovitellenin I (oxidized)	8.0 ± 0.2	4.69	164	14
Duck apovitellenin I				
CNBr fragment 1	5.6	5.52	29	4
CNBr fragment 2	6.7	4.18	39	2
CNBr fragment 3	6.1	6.36	14	3
α_{s1} -Casein	7.0 (7.4‡)	4.48	199	19
β -Casein	4.9 (5.0‡)	6.82	209	13
κ -Casein	4.5 (4.7‡)	4.69	169	13

* Urea concentration at centre of peak. Standard errors are given where three or more values were available.

† See the Materials and methods section.

‡ Values in parentheses refer to the dephosphorylated protein.

Fig. 2. *Hydrophobic chromatography of proteins from hen's-egg yolk*

A mixture (172 mg) of the apoproteins from hen's-egg-yolk lipoprotein was applied within 3 h of dissolving in acidic 3 M-urea (45 ml); other conditions were as given in Fig. 1 legend, although the fractions were each 5.4 ml. The letters (A–H) denote the principal peaks and the horizontal bars the proteins identified by electrophoresis (Fig. 3), which are indicated by roman numerals. The fractions used for electrophoresis in Fig. 3 are indicated by arrow heads. The straight lines refer to the urea concentration gradient.

distribution of hydrophobic residues) was helpful in predicting the order of elution.

Before using phenyl-Sepharose for the tests in Table 1, we tried other materials. Octyl-Sepharose retained the yolk apoproteins more firmly than did phenyl-Sepharose, although the order of elution was the same. We also observed some difference between different batches of phenyl-Sepharose (see the Materials and methods section). A new material, dinitrophenyl-Sepharose, did not retain any of the yolk apoproteins.

Separation of the apoprotein mixture from hen's-egg-yolk lipoprotein

Table 1 suggested that chromatography on phenyl-Sepharose in acidic urea solution could profitably be applied to mixtures of similar hydro-

Fig. 3. *Photometric scans of gel-electrophoretic patterns of proteins from hen's-egg yolk*

Polyacrylamide slabs containing detergent were used, as described in the Materials and methods section. Gel T, total apoprotein mixture from the lipoprotein of hen's egg yolk; roman numerals refer to apovitellenins identified previously. Gels A–G, selected chromatographic fractions denoted by arrow heads in Fig. 2. The approximate molecular-weight scale derived from standards is indicated at the bottom of the Figure.

phobic proteins. Fig. 2 refers to the separation of a freshly prepared mixture of apoproteins from hen's egg-yolk lipoproteins. Seven peaks (A–G) were partly or completely resolved. The proteins in the peaks were identified by gel electrophoresis in detergent by comparison with the original mixture (Fig. 3, gel T), which had been elucidated previously (Yamauchi *et al.*, 1976; Burley & Sleight, 1980). Electrophoretic patterns of fractions within the peaks A–G are shown in Fig. 3. Table 2 summarizes

data from Figs. 2 and 3 and gives the hydrophobicities of the principal apoproteins. Table 2 also indicates how these proteins could be resolved or purified further by gel-filtration chromatography, so that it was possible to isolate the seven previously described apoproteins, apovitellenins I–VI (Burley, 1975; Burley & Sleight, 1980), starting with hydrophobic chromatography.

From a comparison of Table 2 and Table 1 it is apparent that apovitellenin I emerged at a slightly

Table 2. *Hydrophobic chromatography of a protein mixture from hen's-egg yolk*

Data for the separation of the mixture of lipoprotein apoproteins were as given in Fig. 2 legend. Identification of the principal proteins was by electrophoresis (Fig. 3) and gel-filtration chromatography (Burley & Sleight, 1980). For experimental details see the text.

Peak (Fig. 2)	[Urea] at centre of peak (M)	Principal proteins (apovitellenins)*	Minor proteins (apovitellenins)	Isolation of principal proteins†
A	3	III (4.52), V (4.27)	IV, VI	Not attempted
B	4.75	II (3.64), VI (4.14)	III	II and IV separated on CL-6B or G-100
C	5.3	III (4.52)	Ia, IV	Ia removed on G-75 and IV on CL-6B
D	6.2	Ia (4.69)	IV, III	Ia separated on G-75
E	6.8	VI (4.10)	Va and others unidentified	VI isolated on CL-6B
F	7.6	I (4.69)	Va, VI	I isolated on G-100 or G-75
G	9	V (4.27)	III, IV, VI	V isolated on CL-6B

* The hydrophobicity index (kJ/mol) is given in parentheses.

† Gel-filtration chromatography was used with columns (80 cm × 2 cm) of Sepharose CL-6B, Sephadex G-100, or Sephadex G-75 (Burley, 1975; Burley & Sleight, 1980) in acid 6 M-urea.

Fig. 4. *Hydrophobic chromatography of a protein mixture from duck's-egg yolk*

A sample (136 mg in 25 ml of acidic 3 M-urea) was applied to the same column of phenyl-Sepharose used for Fig. 2 and eluted in the same way. The letters a, b and c refer to proteins that were isolated and subsequently purified. Their electrophoretic patterns are given in Fig. 5. I refers to duck apovitellenin I, the principal constituent. This peak was cut off because of its size. The straight lines refer to the urea concentration gradient.

Table 3. *Amino acid compositions and other data for duck's-egg-yolk apoproteins*

The apoproteins a, b, and c are those indicated in Fig. 4. For comparison, compositions of the corresponding proteins from the hen are also given, i.e. hen's-egg-yolk apovitellenins IV, III and VI (Burley & Sleight, 1980). The standard errors (S.E.M.) refer to three or more estimations. For experimental details see the text.

Amino acid	Protein a		Hen apovitellenin IV	Protein b		Hen apovitellenin III	Protein c		Hen apovitellenin VI
	Mean	S.E.M.		Mean	S.E.M.		Mean	S.E.M.	
Asp	11.62	0.14	17.16	12.81	0.32	12.27	9.42	0.18	10.74
Thr	8.42	0.18	6.75	7.90	0.36	5.70	6.98	0.01	6.85
Ser	11.90	0.06	9.07	8.48	0.12	6.27	8.27	0.11	9.43
Glu	9.86	0.06	10.07	13.28	0.31	12.34	14.19	0.39	10.50
Pro	4.19	0.13	3.85	3.97	0.20	2.86	4.53	0.10	3.74
Gly	6.98	0.07	6.34	6.30	0.09	4.64	5.77	0.19	5.71
Ala	6.56	0.11	5.51	5.86	0.10	7.26	6.78	0.26	6.79
CyS	0.26	0.06	0.31	0		0.31	0.93	0.03	0.71
Val	5.05	0.06	5.35	6.37	0.18	5.46	5.86	0.19	6.31
Met	2.09	0.01	1.79	1.84	0.06	2.17	2.45	0.04	2.04
Ile	5.10	0.18	5.35	4.44	0.10	8.57	4.69	0.04	4.93
Leu	9.93	0.13	10.07	9.43	0.29	9.93	10.09	0.18	10.42
Tyr	3.13	0.25	3.17	3.45	0.26	3.04	3.49	0.10	3.28
Phe	5.25	0.20	5.34	5.97	0.17	4.77	4.08	0.07	4.30
Try	3.06	0.25	1.05	3.26	0.25	1.04	2.79	0.08	0.48
Lys	7.41	0.41	7.17	7.12	0.27	9.36	8.80	0.69	7.85
His	1.51	0.06	2.17	2.42	0.09	1.26	1.71	0.04	1.72
Arg	2.87	0.14	3.84	4.40	0.25	4.28	3.76	0.06	4.20
$10^{-4} \times \text{Mol.wt.}^*$	7.5		7.5	6.5		6.5	12.0		17.0
$\Phi^\dagger$	4.44		4.14	4.48		4.52	4.48		4.10
$\Delta^\ddagger$	6.1			11.8			6.7		

* Estimated by gel electrophoresis in detergent; see the Materials and methods section.

† Hydrophobicity index (kJ/mol); see the Materials and methods section.

‡ Amino acid difference index, referred to the neighbouring hen's-egg-yolk apoprotein.

lower urea concentration when part of the whole mixture. Moreover, the chromatographic pattern in Fig. 2 would not be expected from the hydrophobicities. Peak A, which was not retarded and was also eluted with 2M-urea, was clearly anomalous. It contained two proteins (apovitellenins III and V) that were also present in two other peaks (peaks C and G). It is possible that peak A contained a complex of these proteins, because we have previously found evidence (Burley & Sleight, 1980) that they are present as an unstable complex when the apoprotein mixture is first dissolved in urea. Peak A decreased in size after the mixture had been left at 2°C in acidic urea solution, as expected for an unstable complex. We also observed that apovitellenin III (peak C) changed on standing in urea solution after separation, so that on rechromatography it was eluted in the position of peak B, possibly a result of the formation of an aggregate. A more definite example of the effect of aggregation of a single protein was provided by apovitellenin VI (peak E), which is known to aggregate slowly in urea (Burley & Sleight, 1980). In old solutions this protein emerged earlier, between positions D and E in Fig. 2.

The hen's egg-yolk apoproteins were more

difficult to elute from phenyl-Sepharose if the pH of the urea solution was 4 or 7 instead of 3.

Separation of the apoproteins from the lipoprotein of duck's-egg yolk

Fig. 4 shows the hydrophobic chromatography of the total apoprotein mixture. The corresponding electrophoretic pattern is shown in Fig. 5 (gel T). Four of the proteins were isolated. The others have not been studied. As expected from its large size, peak I contained duck's egg-yolk apovitellenin I, identified by its amino acid composition (Inglis & Burley, 1977). Peaks a, b, and c contained single proteins with some overlapping, according to gel electrophoresis (Fig. 5). They were purified by rechromatography on Sepharose CL-6B, although the proteins in peaks a and b could not be separated from each other in this way because their molecular weights were too close: 65 000 and 75 000 according to gel electrophoresis in detergent (Table 3). Hen's-egg-yolk apoproteins with the same molecular weights as these were separated by gel-filtration chromatography because they behaved abnormally on Sepharose CL-6B (Burley & Sleight, 1980). The proteins in peaks a and b were well separated on

Fig. 5. Photometric scans of gel-electrophoretic patterns of proteins from duck's-egg yolk

Polyacrylamide slabs were used as indicated in Fig. 3 legend. Gel T represents the total apoprotein mixture. Most of the proteins have not been identified. The large peak corresponded to duck apovitellenin I. Gels a, b and c refer to proteins isolated from three of the peaks in Fig. 4.

phenyl-Sepharose by using a shallower gradient than that shown in Fig. 4.

Amino acid compositions and other data for the purified proteins from peaks a, b and c (Fig. 4) are given in Table 3. This Table also gives data for the hen's-egg-yolk apoproteins that were judged to be equivalent by the amino acid 'difference index' (Metzger *et al.*, 1968). According to this test proteins a and c were closest to hen's-egg-yolk apovitellenins IV and VI respectively. Protein b resembled apovitellenin III, although the difference was larger than for the others. As already noted, proteins a and b corresponded to apovitellenin IV and III in molecular weight and in chromatographic behaviour on phenyl-Sepharose, but protein c differed by about 30% in molecular weight from apovitellenin VI and behaved differently on phenyl-Sepharose. CNBr digests of the proteins of lower molecular weight (i.e. proteins a and b) gave different electrophoretic patterns (Fig. 6). Neither

Fig. 6. Photometric scans of gel-electrophoretic patterns of CNBr digests of duck's-egg-yolk proteins

Duck's-egg-yolk apoproteins a and b (see Fig. 4) were digested as described in the Materials and methods section and applied to a polyacrylamide-gel slab with a high proportion of acrylamide (14%). Other conditions were as indicated in Fig. 5 legend.

agreed completely with the closest hen's egg-yolk proteins (apovitellenins IV and III; Burley & Sleight, 1980), but in general they are similar. From all these data it seems appropriate to name proteins a, b and c duck's-egg-yolk apovitellenins IV, III and VI respectively.

Discussion

Our results show that elution of proteins from a hydrophobic column is sensitive to small changes in urea concentration. It is likely that proteins are attached to such a column by hydrophobic groups, i.e. phenyl groups on phenyl-Sepharose, and not by other groups, because the hen's-egg-yolk apoproteins were not retained on a column of dinitrophenyl-Sepharose. Urea interacts with protein side chains, especially with aromatic groups (Nozaki & Tanford, 1963; Prakash *et al.*, 1981), so it would be expected to weaken the binding of proteins to phenyl-Sepharose. Only for very similar proteins, however, did the elution position depend on the hydrophobicity alone (Fig. 1). Co-operative binding would explain the large difference between the monomeric (reduced) and dimeric forms of hen's-egg-yolk apovitellenin I (Table 1) and possibly some of the difference between duck's-egg-yolk apovitellenin I and its CNBr-cleavage peptides (Table 1). We suggest that an important additional factor is the effect of urea on the protein structure. In general, proteins that are unfolded in 3M-urea would be expected to expose more binding sites and so be more firmly attached to the column; thus compact aggregates should be more easily eluted than

extended monomers. Data on the shapes in 3 M-urea of the proteins we have tested and on the effect of increasing concentrations of urea are not yet available, so that the validity of this suggestion cannot be assessed. It is not, however, surprising that there is no consistent relationship between elution pattern and hydrophobicity, as also noted by Creamer & Matheson (1981) for elution of milk proteins by a combination of aqueous buffers and a gradient of neutral urea.

Although the theoretical basis for hydrophobic chromatography in urea solutions is not clear, we have found it useful for isolating the apoproteins from avian lipoproteins, especially in conjunction with other methods. As a result, four proteins are now available for comparison between two species, and it is already apparent that there are surprisingly large differences (Table 3) that will have to be taken into account in explaining the function of these proteins. As mentioned above, it is likely that apovitellenin I, the main constituent that has also been isolated from a blood lipoprotein (Dugaiczky *et al.*, 1981), is a determinant of lipoprotein structure. For the other apoproteins there are several possibilities. They may also be structural proteins without stringent requirements as to composition or size. They could be highly specific and essential for the functions of the lipoprotein as it exists in yolk. They need not be related to the lipoprotein, but could be driven into the lipoprotein fraction during isolation; a related example of such behaviour has been described (Burley, 1978). Information that would distinguish among these possibilities is not available. As a guide to further experiments we emphasize that in yolk, with 50% solids, the environment of the lipoprotein is different from that in blood, with about 7% solids, so that the requirements for lipid-protein interactions may be different. Thus the differences that we have observed may be an indication of important functional differences between the yolks of different species. Such functions might include the stabilization of the lipoprotein before incubation and control of its accessibility during incubation. Minor apoproteins that are not present in sufficient amounts to take part in all the lipoprotein particles possibly represent other lipid-protein structures in yolk.

We thank Dr. M. Howden for a supply of duck's eggs.

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