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Studies on the Apoproteins of the Major Lipoprotein of the Yolk of Hen's Eggs I. Isolation and Properties of the Low-molecular-weight Apoproteins

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Abstract

In a continuing study of protein–lipid interactions in egg yolk, the total apoprotein mixture (i.e. the 'apovitellenins') from the high-lipid, low-density lipoprotein (density 0·97 g/ml) of the yolk from hen's eggs has been isolated in a soluble form. By gel-filtration chromatography in 6M urea the mixture has been separated into several fractions from which three new low-molecular-weight proteins (I, I*a*, and II), making up about 30% of the total, have been isolated. The most plentiful of these (I) consists of stable aggregates with several identical subunits each of molecular weight about 10000. This protein is analogous to the principal protein from the corresponding lipoprotein of emu's egg yolk, i.e. emu's apovitellenin I. Hen's apovitellenin I has a slightly different amino acid composition from that of the emu; notably it contains a sulphydryl group. The hen's protein also forms more stable aggregates that are dissociated by detergent and by guanidine hydrochloride but are stable in urea.

The molecular weight of I*a* is similar to that of I and the amino acid composition is the same, with the exception that I*a* has a higher proportion of amide groups. It aggregates less readily than I under the same conditions.

The third new protein (II, 'hen's apovitellenin II') has a molecular weight of about 20 000. It has no tyrosine or methionine residues, but contains glucosamine and has several disulphide groups. It has been isolated in very small amount only.

Introduction

The main constituent of the yolk of avian eggs other than water is a lipoprotein that has a high proportion of lipid and a low density. In egg yolk of the domestic hen, *Gallus domesticus*, this lipoprotein is about 60% of the dry weight of the yolk and contains about 16% of protein by weight, the rest being neutral lipid and phospholipid (for reviews of the earlier literature see Cook and Martin 1969, Burley 1971, Vadehra and Nath 1973). Studies on the protein (i.e. the apoprotein) have been hampered by its insolubility in the usual solvents. Martin (1961) was able to dissolve the total apoprotein in 88% formic acid and from hydrodynamic measurements concluded that it was heterogeneous and contained a monomer with low molecular weight (10 000). More recently a somewhat similar conclusion was reached by Hillyard *et al.* (1972) using apoprotein solubilized by chemical treatments. In 1968 it was reported that part of the apoprotein was soluble in lipid solvents (Burley 1968). It was later found that this protein had a low molecular weight but was heterogeneous (Burley and Sleigh 1971).

The work described here shows that the hen's apoproteins are not inherently insoluble—they are difficult to study largely because of their strong tendency to bind lipids and to form aggregates. This work followed from experiments with egg yolk

of other avian species, especially that of the emu. The principal protein from the high-lipid lipoprotein of emu's egg yolk has been isolated in a pure state (Burley 1973*a*). This protein, referred to as 'emu's apovitellenin I', has 84 amino acid residues in a single chain and a molecular weight of 9741 according to the sequence, which has recently been reported by Dopheide and Inglis (1974). During experiments on the emu's apoprotein (e.g. Burley 1973*b*), it was observed that extensive aggregation and insolubility were to be expected if the lipid and salt had not been completely removed from the protein during isolation. This observation was found to be applicable to apoproteins of other birds' eggs. Accordingly, when suitable precautions are taken during isolation, the total apoprotein mixture of hen's egg yolk is soluble in aqueous solutions at low ionic strength and in solvents such as 6M urea, and is amenable to the usual methods of protein chemistry. The isolation and some preliminary properties of the low-molecular-weight apoproteins from the hen's apoprotein mixture are reported here.

Materials and Methods

Eggs were obtained either from a flock of White Leghorn hens at the Division of Food Research, CSIRO, that had just started laying, or from hens at the Division of Animal Genetics, CSIRO. These eggs were freshly laid and were still warm when opened. Eggs of uncertain age that were obtained commercially were used for some preparations. The source of the egg yolk did not much affect the properties of the isolated lipoprotein, but removal of lipids and isolation of pure apoproteins were easier with fresh eggs.

The acid urea reagent was 6M urea, 0·025N HCl, pH 3·3. The *N*-(4-dimethyl-3,5-dinitrophenyl)maleimide reagent was from Aldrich Chemical Co. Inc., Wisconsin.

Preparation of High-lipid Low-density Lipoprotein from Egg Yolk

The following method was used, all solutions being deoxygenated by boiling followed by cooling in oxygen-free nitrogen: To 100 ml of egg yolk, free of white, an equal volume of 0·16N NaCl containing EDTA (total concentration 10^{-3}M) was added. The mixture was stirred, nitrogen was bubbled through, and the yolk granules were sedimented by centrifuging for 30 min at 100 000 *g* at 2°C and then discarded (Burley and Cook 1961). After the supernatant liquid had been saturated with NaCl, the high-lipid lipoprotein was isolated as a floating, yellow, greasy or oily layer by centrifuging the solution for 5 h at 400 000 *g*, or for 15 h at 100 000 *g*, at 10°C. The crude lipoprotein was dispersed under nitrogen in 4N NaCl (total volume 100 ml) by use of a homogenizer, and then recentrifuged to remove livetins and other soluble impurities. Finally the lipoprotein in 1N NaCl (about 100 ml) was dialysed against water under nitrogen to reduce the concentration of NaCl to 10^{-3}N or less.

The lipoprotein prepared in this way had a wide range of particle sizes, according to gel-filtration chromatography (Burley 1970), although this would not be expected to affect the isolation of the apoproteins (Burley 1973*a*). The concentration of lipoprotein was usually 10–15% as determined from the dry weight, and the lipoprotein contained 15–16% by weight of protein.

Isolation of Total Apoproteins

Two methods were used. In both, the aqueous solutions were free of oxygen. The first method was carried out at 20–25°C and the second method at 2°C.

Method 1. To a solution of the above salt-free lipoprotein (100 ml, approximately 10% w/v) solid EDTA (1·7 g disodium salt) was added. The mixture was stirred, nitrogen was bubbled through it, and NaOH was added until the EDTA had dissolved and the pH was 7–7·2. About 2·2 ml of 1·0N NaOH was needed for the disodium salt of EDTA. The solution was then added to a 1 : 1 mixture of chloroform and methanol (400 ml) containing antioxidant (butylated hydroxytoluene, 2 mg about 0·02% of the weight of lipid). After standing for 20 min, the mixture was filtered through paper (Whatman 541) and the solid protein washed with about 300 ml of a 1 : 1 chloroform–methanol mixture. The protein was then transferred to glass centrifuge tubes and washed further by stirring with chloroform–methanol followed by centrifuging at low speed (2000 r.p.m., 15 min). EDTA was

removed from the precipitate by stirring it with water (50 ml) and recentrifuging. Finally the protein was again washed twice with chloroform–methanol and dried under suction. The dried protein was available about 60 h after breaking the eggs. It should contain less than 0·02% phosphorus and it should be used as soon as possible. It was usually dissolved in acid urea or, if necessary, stored at −20°C. For complete dissolution in acid urea, HCl was added to reduce the pH to 3·3. Aqueous solutions were best prepared by dialysing the urea solution into water.

Method 2. To a solution of salt-free high-lipid lipoprotein (100 ml, about 10% w/v), HCl (0·6 ml, 5N) was added and the mixture (pH 2·5) poured into a separating funnel containing methanol (210 ml), chloroform (125 ml), and butylated hydroxytoluene (2 mg). The small sedimented layer of neutral lipid in chloroform was removed as soon as possible and petrol (30 ml, b.p. 30–40°C) was added. Soon after settling, the lower yellow chloroform–petrol layer was removed. More petrol (30 ml) and chloroform (60 ml) were then added with a little antioxidant and the lower layer again removed. After this separation had been repeated the upper aqueous layer was clear or slightly opalescent. Solid urea (36 g) was then added to the aqueous layer which was allowed to warm to room temperature before extraction with chloroform. The mixture was centrifuged at low speed, if necessary, to separate the layers, and the organic solvents were sucked off from the aqueous solution on a rotary evaporator at less than 40°C, after which the pH was 3·3. The aqueous solution was then extracted twice more with chloroform, which was finally sucked off as before. After passing through a Millipore filter (RA) portions of this solution, which contained 1–2% w/v of protein in about 90 ml, were used for chromatography.

In both methods of preparation, insolubility of the final protein in 6M urea at pH 3·3 was a sign that it contained too high a concentration of lipid, salt, or chloroform. The yield of protein was variable. Almost 100% was sometimes recovered by the first method, but with the second method the yield was often 70% or less. More protein could be recovered if all the solvents used for extraction of lipid were combined, diluted with water, and allowed to stand for 24 h or longer at 20°C, when a precipitate of protein formed at the solvent interface. The precipitate was washed well to remove lipid and precipitated EDTA. It contained a high proportion of low-molecular-weight proteins.

Both methods have been successfully applied to the lipoproteins of the yolk of emu's eggs and of other birds' eggs. Probably because of the greater solubility of the emu's proteins in organic solvents, loss of protein during solvent extraction was greater than for hen's lipoprotein, so it was necessary to recover protein from the mixed solvents.

After the hen's apoproteins had been separated by chromatography in 6M acid urea, they were recovered either by dialysis against water followed by freeze-drying, or by precipitation with trichloroacetic acid.

Physical Methods of Analysis used on Isolated Apoproteins

In general these have been described previously (Burley 1973*a*). For molecular weight estimations the Yphantis (1960) short-column equilibrium method was used. Optical rotatory dispersion (O.R.D.) was measured on a Perkin–Elmer model 141 polarimeter in the visible region only. Apparent α-helical concentrations were calculated assuming a value of −630 for b_0. A Unicam SP 3000 spectrophotometer was used for optical absorbance measurements. Viscosity was measured using Ostwald microviscometers. For all measurements dilutions were made with dialysate.

Chemical Analyses of Isolated Apoprotein

For amino acid analyses a Beckman model 120C analyser was used. Corrections, based on hydrolyses in 6N HCl at 110°C for a series of times, were applied for losses during hydrolysis. Because of the small amount available a separate series was not done for fraction II. Amide groups were also determined from the ammonia liberated, measured on the amino acid analyser, during hydrolysis in 2N HCl for 1–6 h. Tryptophan was determined from ultraviolet absorption measurements (Edelhoch 1967).

Reactive sulphydryl groups were estimated using the coloured *N*-(4-dimethyl-3, 5-dinitrophenyl) maleimide reagent (Burley and Haylett 1959). This reagent is specific for sulphydryl groups in proteins and it will react under slightly acid conditions. It was used in large excess—about one-quarter of the weight of protein. For estimation of sulphydryl groups reactive during lipid removal, the reagent was dissolved in the chloroform–methanol mixture just before the lipoprotein was added. The protein was then isolated as before. For estimation of sulphydryl groups reactive in guanidine hydrochloride, the dry protein and reagent were added together to 6M guanidine hydrochloride

(20 ml for 0·4 g of protein) and the mixture stirred for 24 h at 25°C, alkali being added to maintain the pH between 7 and 8 (e.g. Fig. 3). Concentration of bound reagent was determined from the optical density at 440 nm.

Results

Experiments on the Total Apoprotein Mixture

Fig. 1*a* shows the chromatographic separation by gel filtration of the total apoprotein from the high-lipid lipoprotein of fresh hen's eggs. This protein had been isolated by the first procedure described in 'Methods', i.e. removal of lipids was done at pH 7. The chromatographic pattern has three well-defined regions (A, B, and C) corresponding to high-, intermediate-, and low-molecular-weight protein fractions. Isolation of the total apoprotein at low pH, i.e. by the second method, gave a similar pattern, although the relative height of region B was greater than that for the first method. In either case the proportion of B was variable; it was sometimes absent if the lipoprotein had been stored for more than a few days at 2°C or had been isolated from old eggs, and A was correspondingly larger. As shown in Fig. 2, B was also reduced in size by treatments that block sulphydryl groups.

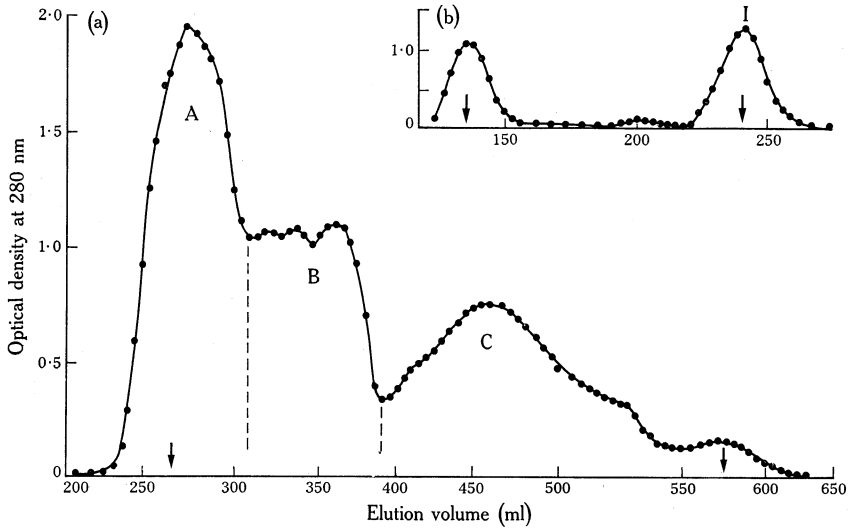
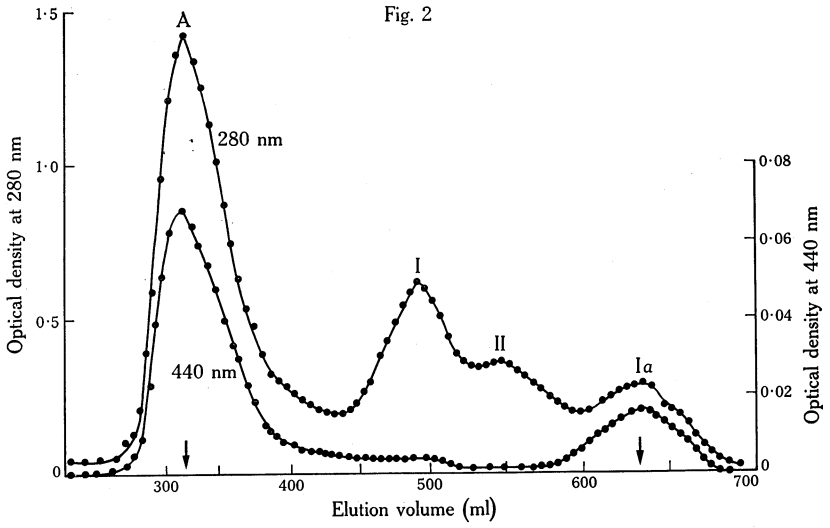


Fig. 1. (*a*) Chromatographic separation on a column (4·3 by 70 cm) of Sephadex G100 of the total apoprotein mixture (0·6 g in 40 ml) from the high-lipid lipoprotein of fresh hen's egg yolk. The solvent was 6M urea at pH 3·3 and 20°C. The fractions were 3–6 ml; larger fractions were collected between 350 and 500 ml. A, B, and C represent protein fractions of high, intermediate, and low molecular weight. (*b*) Separation of the corresponding emu's protein on another column (2·5 by 65 cm) using the same solvent. I, emu's apovitellenin I. In Figs 1–3 arrows represent void and bed volumes. These varied slightly because of differences in packing.

Chromatographic patterns for the hen's apoprotein were in marked contrast to those for the corresponding apoprotein isolated from emu's egg yolk by either method of preparation (e.g. Fig. 1*b*). The second major peak of the emu's chromatogram (labelled 'I' in Fig. 1*b*) consisted of the low-molecular-weight protein, emu's apovitellenin I. By the new methods of isolation, in which salt, divalent metals, lipid, and oxygen were excluded as far as possible, the yield of emu's apovitellenin I was increased from about 45% to about 65% of the total apoprotein.

Fig. 2 shows the effect on the chromatographic pattern of the hen's total apoprotein of the presence of a sulphydryl-blocking reagent, *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide, during lipid removal at pH 7. A similar pattern was found for lipid removal at pH 2·5. Evidently the chromatographic pattern was simplified by



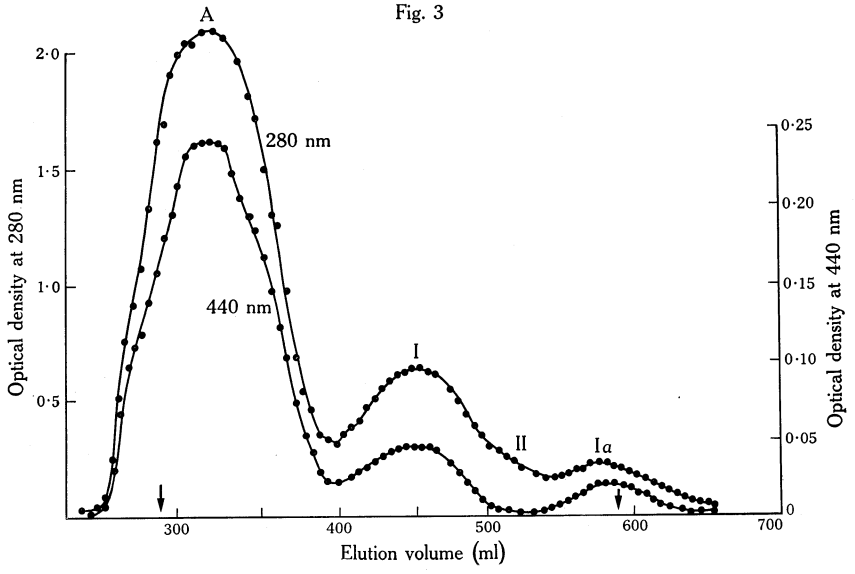


Fig. 2. Chromatographic separation on a G100 Sephadex column (4·3 by 70 cm) of hen's total apoprotein mixture treated with the sulphydryl reagent *N*-(4-dimethylamino-3,5-dinitrophenyl) maleimide during lipid removal at pH 7·0. Protein (0·16 g) was applied in 30 ml of 6M urea at pH 3·3 and 20°C. Fractions were measured at 280 and 440 nm to give protein and bound-reagent concentrations, the former being corrected for absorption of the reagent at 280 nm. Unreacted reagent was highly retarded on this column.

Fig. 3. Chromatographic separation of hen's total apoprotein under same conditions as Fig. 2 with the difference that the protein (0·29 g) was first treated with the sulphydryl reagent in 6M guanidine hydrochloride (20 ml) (see text), then applied to the column and eluted with 6M urea at pH 3·3.

blocking sulphydryl groups. Fig. 2 also reveals that reactive sulphydryl groups were present only in the fractions of highest and lowest molecular weight (i.e. A and I*a*). Fig. 3 shows that if the total apoprotein, isolated at pH 7, was dissolved in 6M guanidine hydrochloride in the presence of the above maleimide reagent before chromatography in 6M acid urea, more sulphydryl groups reacted. It is also clear that this treatment did not greatly alter the chromatographic pattern, although the proportion of low-molecular-weight proteins was smaller and one fraction, which did not contain a reactive sulphydryl group (II in Figs 2 and 3), was present in smaller amount after treatment, for reasons that are not known. Possibly the protein of this fraction formed large aggregates at some stage during treatment.

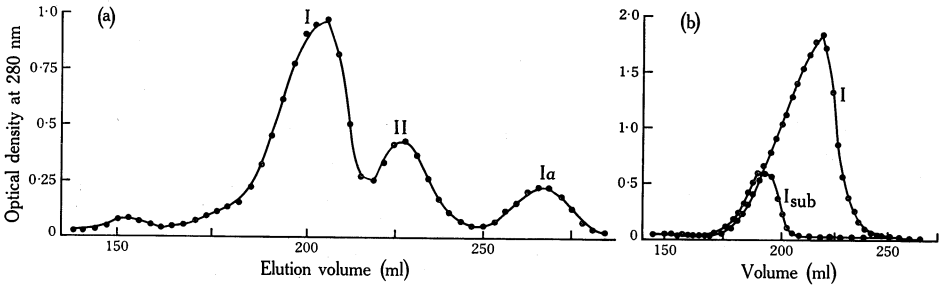


Fig. 4. (*a*) Chromatographic separation of hen's low-molecular-weight apoproteins (fraction C, Fig. 1*a*) on a column (2·5 by 80 cm) of Sephadex G75 in 6M urea at pH 3·3 and 20°C. About 0·15 g of protein was applied in 15 ml. (*b*) Rechromatography of isolated protein fractions on Sephadex G75 column used in (*a*). I, pooled fractions I as obtained in (*a*). I_{sub}, high-molecular-weight protein subfraction from I.

Properties of the Isolated Low-molecular-weight Apoproteins

The low-molecular-weight apoproteins (C, Fig. 1) were about 30% of the total apoprotein according to the weights isolated. Their chromatographic resolution on another column is shown in Fig. 4*a*. Three proteins were isolated from the major peaks in Fig. 4*a* (I, II, and I*a*). On rechromatography I gave an asymmetrical peak (e.g. I, Fig. 4*b*). Two further protein fractions—referred to as I and subfraction I (I_{sub})—were isolated by rechromatography of the main peak and the higher-molecular-weight leading edge. No indications of interconversion were found. In Fig. 4*b*, for example, curve I_{sub} shows the rechromatography of the subfraction.

Gel-electrophoretic patterns in the presence of detergent for four of the isolated proteins are shown in Fig. 5, according to which fraction I and its subfraction and also fraction I*a* each consisted essentially of a single protein. In tests on mixtures, these proteins could not be distinguished from each other or from emu's apovitellenin I, although under slightly different conditions (pH 7·5, 0·03M tris buffer) the hen's proteins ran ahead of emu's apovitellenin I. Fraction II gave a rather diffuse band with a lower mobility than the others. Addition of a disulphide reducing agent (dithiothreitol) to the samples before electrophoresis did not alter the mobility, although fraction II then gave a sharper single band. Evidently intermolecular disulphide groups were not present in these proteins.

In Table 1 some physical data, measured in various solvents, for fractions I, I_{sub}, I*a* and II are compared; and data for emu's apovitellenin I are also given (Burley 1973*a*). The results summarized in this table show that in the highly disaggregating

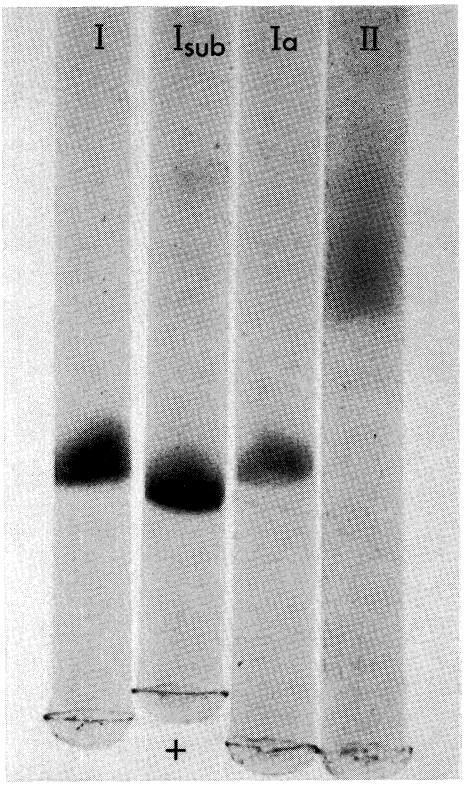


Fig. 5. Gel-electrophoretic separation of isolated hen's low-molecular-weight apoproteins using polyacrylamide gel (8%) containing sodium dodecyl sulphate (0·1% w/v) in 0·05M phosphate buffer at pH 7·0. Protein bands were stained with Coomassie Blue.
I, fraction I, main peak (Fig. 4*b*);
I_{sub}, high-molecular-weight subfraction (Fig. 4*b*);
I*a*, fraction I*a* (Fig. 4*a*);
II, fraction II (Fig. 4*a*).

Table 1. Molecular weights and results of other physical measurements for isolated hen's low-molecular-weight apoprotein fractions and a comparison with emu's apovitellenin I

For fractions I and I_{sub}, see Fig. 4*b*; for fractions I*a* and II, see Fig. 4*a*. Emu I is emu's apovitellenin I (Burley 1973*a*)

Measurement	Solvent	Temp. (°C)	Protein fraction I	I_{sub}	Ia	II	Emu I
Molecular weight							
Sequence studies[A]							9741
Sedimentation equilibrium[B]	6M guanidine. HCl–0·05M C_2H_5SH, pH 7	25	9400	9400	9400	20 400	
Gel electrophoresis	2% SDS, pH 7		10 000	10 000	10 000	20 000	
Amino acid analysis[C]			9114	9114	9117	20 470	
O.R.D. (helix %)	6M urea, pH 3·3	20	17				0
	3M urea, pH 7	20	47				
	1:1 methanol–water, pH 3	20	81				78
	6M guanidine. HCl	20	0				0
Viscosity (ml/g)	3M urea, pH 7	20	6·5				
Extinction coefficient $\left(E^{1\%}_{1cm}\right)$	6M guanidine. HCl	20	13·3		13·3	20·5	17·0[D]
Partial specific volume[E] (ml/g)			0·752	0·752	0·753	0·712	

[A]Dopheide and Inglis (1974).
[B]Yphantis cell. Results for I and Ia at various concentrations fitted the same regression line; standard error for eight determinations = 140. Results for I_{sub} were more erratic but fitted the same line.
[C]Calculated as minimum values from Table 3.
[D]The published value (Burley 1973a) is in error and refers to a 2-mm cell.
[E]Calculated values from Table 3.

solvents, guanidine hydrochloride and sodium dodecyl sulphate (SDS), fractions I, I_{sub}, and I*a* consist of proteins with the same low molecular weight. By contrast, gel-filtration chromatography in 6M urea (Figs 4*a* and 4*b*) clearly indicates that I and I_{sub} are much larger than I*a*, presumably as a result of aggregation. Sedimentation measurements, details of which will be reported later, have confirmed that I and I_{sub} consist of aggregates in 6M urea.

Table 2. Amino acid analyses for low-molecular-weight hen's apoproteins

Mean value are in moles/10^4g. c.v., coefficient of variation

Amino acid	Fraction I[A]			I_{sub}[B]	Fraction I*a*[C]			Fraction II[D]		
	Mean	c.v. (%)	S.E.	Mean	Mean	c.v. (%)	S.E.	Mean	c.v. (%)	S.E.
Lys	5·94	7·1	0·15	5·13	6·07	6·3	0·17	1·31	6·9	0·17
His	0			0	0			0·54	11·0	0·03
Arg	5·80	8·2	0·22	5·70	5·68	6·8	0·17	6·67	7·8	0·26
Asp	7·99	5·6	0·14	8·09	7·92	2·7	0·09	9·48	9·5	0·36
Thr	4·92	11·8	0·21	4·62	4·78	4·7	0·10	5·58	5·4	0·15
Ser	4·13	6·3	0·35	4·09	3·89	8·7	0·15	6·52	6·1	0·20
Glu	8·24	12·7	0·41	8·97	8·42	5·3	0·20	10·26	6·6	0·34
Pro	2·04	10·9	0·08	2·02	2·06	5·9	0·05	5·32	2·6	0·07
Gly	3·10	13·5	0·16	3·01	3·07	12·8	0·18	12·87	4·7	0·30
Ala	8·13	7·1	0·21	8·38	7·87	2·8	0·10	4·72	5·1	0·12
½Cys	1·13	15·3	0·10	0·99	1·02	12·0	0·05	4·49	9·6	0·22
Val	6·81	6·9	0·19	7·89	6·66	5·7	0·16	6·07	5·8	0·18
Met	0·80	18·8	0·08	0·90	0·85	11·7	0·04	0		
Ile	6·06	7·9	0·20	5·99	5·73	8·1	0·21	2·32	5·6	0·07
Leu	9·25	8·1	0·26	9·45	9·41	7·9	0·34	7·81	11·7	0·46
Tyr	3·08	14·0	0·15	3·44	2·97	9·5	0·13	0		
Phe	2·02	7·0	0·07	2·17	1·96	11·5	0·10	3·67	10·1	0·19
Try	1·04	11·7	0·05	1·08	0·94	10·0	0·04	3·59[E]		
Glucos-amine				0	0			0·88	17·4	0·11
Amide	6·72	19·9	0·54	6·90	10·24	4·1	0·24	9·23	27·9	2·26

[A]From eight estimations. [B]From two estimations. [C]From five estimations.
[D]From four estimations. [E]Single estimation.

Dissociated fraction I in 6M guanidine hydrochloride reaggregated when the solvent was replaced by 6M acid urea by gel filtration or by dialysis. The re-formed aggregate had the same chromatographic behaviour as the original, thus suggesting that aggregation is reversible. Fraction I*a* showed no tendency to aggregate in 6M urea. Fractions I and I*a* formed covalent aggregates in 6M guanidine hydrochloride unless precautions were taken to prevent disulphide bond formation.

Fractions I and I*a* dissolved in the same aqueous and non-aqueous solvents as emu's apovitellenin I (Burley 1973*a*) although their solubility was slightly less. Thus fraction I was soluble in acidified aqueous methanol, but not very soluble in pure methanol. Fraction II was more soluble than the others. It was soluble in 0·16N NaCl and in methanol–water mixtures but not in pure methanol.

Table 2 gives amino acid analyses for the hen's low-molecular-weight apoproteins. The 'half-cystine' found after hydrolysis of fractions I and I*a* probably arose from cysteine in the protein; the presence of a sulphydryl group was shown by treating the protein with *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide in 6M guanidine

hydrochloride, after which a coloured protein derivative with between 0·88 and 0·95 moles of maleimide per 10^4 g of protein was isolated by chromatography in 6M urea. Sulphydryl groups could not be detected in fraction II by this procedure.

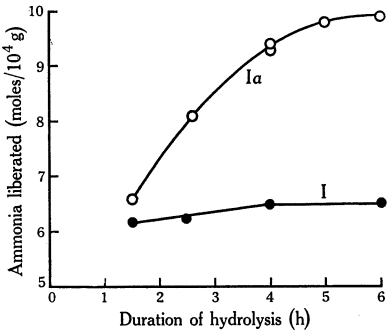


Fig. 6. Rate of liberation of ammonia from protein fractions I and I*a* in 2N HCl at 100°C.

For fractions I and I*a* the rate of liberation of ammonia during hydrolysis in 2N hydrochloric acid is compared in Fig. 6. The higher values for I*a* are consistent with the presence of additional amide groups that hydrolyse more slowly.

Table 3. Probable amino acid compositions of low-molecular-weight apoproteins from hen's lipoprotein, from Table 2, compared with those of emu's apovitellenin I

Values are given in moles per monomer

Amino acid	Hen's I	Hen's I*a*	Hen's II	Emu's I[A]
Lys	6	6	2	7
His	0	0	1	0
Arg	6	6	14	5
Asp	8	8	20	8
Thr	5	5	12	5
Ser	4	4	13	3
Glu	8	8	20	8
Pro	2	2	10	3
Gly	3	3	26	3
Ala	8	8	10	7
½Cys	1	1	9	0
Val	7	7	12	9
Met	1	1	0	3
Ile	6	6	4	6
Leu	9	9	17	7
Tyr	3	3	0	4
Phe	2	2	8	4
Try	1	1	7	2
Glucos-amine	0	0	2	0
Amide	7	10	18	7
Residues	80	80	187	84

[A]From Dopheide and Inglis (1974).

Probable amino acid compositions of the hen's apoproteins, calculated from the results in Table 2 to give minimum molecular weights, are given in Table 3. These

molecular weights agree reasonably well with those from physical measurements (Table 1). The similarity between I, I_{sub}, and I*a* suggests that the aggregates of I and I_{sub} consist of identical subunits, and that these are very similar to I*a*. The data in Tables 2 and 3 also suggest the possibility that fraction II has a sulphydryl group that was not detected with the maleimide reagent.

Discussion

The above results show that by mild, simple procedures it is possible to isolate the proteins in the high-lipid lipoprotein of the yolk of hen's eggs in a soluble form to which the usual methods of protein chemistry may be applied. For complete solubility of the protein, several conditions appear to be necessary, including removal of all phospholipid, prevention of oxidation of lipids and sulphydryl groups, and the avoidance of a high concentration of ions, especially multivalent cations. The relative importance of these conditions has not, however, been determined. There is a large difference in the ease with which the apoproteins of the hen's and emu's lipoproteins may be solubilized; but even with the emu's lipoprotein, much larger yields of low-molecular-weight protein were isolated than reported previously if precautions were taken to exclude salt, phospholipid and oxygen. It now seems likely that the low-molecular-weight protein (emu's apovitellenin I) is as much as 65% of the total apoprotein.

The difference between emu's and hen's lipoprotein may indicate tighter binding between proteins and lipids in the hen's lipoprotein. An alternative explanation is that the hen's apoproteins have a greater tendency to bind to lipid during isolation. Such a possibility is made plausible by the work of Folch-Pi (1972) on the lipophilic protein (proteolipid protein) of myelin. This protein, which resembles apovitellenin I in many of its physical properties, binds irreversibly to sphingomyelin under some conditions, which are likely to occur during isolation unless precautions are taken. In the membrane it is apparently not bound to this lipid. Thus some proteins may have an unused lipid-binding capacity. A possible role for divalent cations in binding phospholipid to the apoproteins, which is indicated by the beneficial effect of EDTA in removing lipid, would be consistent with the recent observations of Bulkin and Hauser (1973) on phospholipid binding by small peptides in the presence of calcium.

In addition to an apparent difference in lipid binding, the hen's apoprotein differs from that of the emu in its protein pattern as indicated by gel-filtration chromatography in urea solution (Figs 1*a*, 1*b*). Whereas for the emu there is a clear separation of the low-molecular-weight apovitellenin I from the proteins of high molecular weight, for the hen a variable mixture was found. The reasons for these variations have not yet been established but they appear to be related, in a way that is obscure, to the presence of sulphydryl groups (Fig. 2). Three new proteins of low molecular weight have been isolated from the hen's apoprotein mixture (Fig. 4). One of these was predominant (fraction I), the amount isolated being at least 25% of the total apoprotein. From its amino acid composition (Table 3) and properties (Table 1) this protein resembles emu's apovitellenin I. Thus it contains no histidine (a property shared by no other egg protein), it has a low proportion of proline, serine, and glycine, and the distribution of other residues is similar. In its molecular weight and ability to acquire a high proportion of α-helical structure in certain solvents (Table 1) it is also similar to the emu protein and unlike other egg proteins. It therefore seems reasonable to

assume that the two proteins are homologous and to name the hen's protein 'hen's apovitellenin I'. Presumably they have undergone considerable mutational divergence, which should be revealed when work on the sequence of hen's apovitellenin I, at present being undertaken by Dr T. A. A. Dopheide, is complete.

The most conspicuous chemical difference between hen's and emu's apovitellenin I is that the hen's protein has a sulphydryl group. A notable physical difference is the greater tendency of the hen's protein to aggregate. Emu's apovitellenin I also forms aggregates in aqueous buffers and, if the ionic strength is above about 0·05, in 6M urea (Burley 1973*a*); but the hen's protein is aggregated in 6M urea in the absence of salt. The 'resistant aggregates' of emu's apovitellenin I that were not disrupted by detergent or guanidine hydrochloride (Burley 1973*b*) have now been found to contain lipid.

A useful indication of the nature of the aggregation of hen's apovitellenin I is provided by another protein isolated from the total apoprotein mixture: viz. fraction I*a* (Fig. 4*a*). Within experimental accuracy this protein was indistinguishable in properties (Table 1) and composition (Table 2) from hen's apovitellenin I, with two exceptions: it did not aggregate in urea solution (Fig. 4*a*), and a larger proportion of ammonia was given off during acid hydrolysis (Fig. 6). The most likely explanation for the latter difference is that I*a* contains more amide groups (Table 3). It is suggested that the extra amide groups are responsible for inhibiting aggregation. It is further suggested that aggregation involves interaction at specific sites. Possibly at such sites one or more carboxyl groups interact with positively charged groups; alternatively, replacement of carboxyl groups with acid amides might alter a specific structure that is necessary for interaction. In either case it is possible that amide groups are important biologically for preventing aggregation of the protein until required for interaction with lipid. It has previously been suggested that apovitellenin I has a structural role on the lipoprotein surface by forming an aggregated network (Burley 1973*a*).

The third new low-molecular-weight protein isolated from the hen's apoprotein (II, Fig. 4*a*), provisionally termed 'hen's apovitellenin II', has an entirely different amino acid composition from that of the other two (Table 3) and from those of most other proteins. It has a high proportion of internal disulphide groups, with possibly an unreactive sulphydryl, but no methionine or tyrosine. It contains glucosamine and so is one of the yolk's proteins containing amino-sugar. It was present in very small amount, less than 1% of the total apoprotein being isolated as this protein, and not enough has been obtained for a complete study. Proteins equivalent to hen's apovitellenins II and I*a* may be present in the emu's lipoprotein, but they would be difficult to detect by chromatography in urea because of the large monomeric apovitellenin I peak (Fig. 1*b*).

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