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SEPARATION AND CHARACTERIZATION OF THE TWO HIGH DENSITY LIPOPROTEINS OF EGG YOLK, α- AND β-LIPOVITELLIN*

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SUMMARY

1. The electrophoretic components of lipovitellin (α- and β-) have been separated from each other, and from the livetins and phosvitin in the high density fraction of egg yolk, by chromatography on hydroxyapatite columns.

2. Both α- and β-lipovitellin have the same lipid content, nitrogen content, amino acid composition, and molecular weight ($4.0 \cdot 10^5$). They differ in their protein phosphorus content, electrophoretic mobility, absorption on hydroxyapatite, ultracentrifugal behaviour in alkaline media, and in solubility.

INTRODUCTION

It was shown in a recent paper[1] that the HDF of egg yolk contains two lipoproteins, α- and β-lipovitellin, differing in electrophoretic mobility. In addition, their sedimentation behaviour shows that both dissociate into smaller entities, β-lipovitellin being dissociated at pH 9.0 and α-lipovitellin at higher pH values. As the separation of

Abbreviation: HDF, high density fraction; LDF, low density fraction.

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these lipovitellins by preparative electrophoresis[1] was considered inadequate for further work, their separation was undertaken by column chromatography. The separation of α- and β-lipovitellin and their characterization by chemical and physical means are described in this paper.

METHODS

Total nitrogen and sulphur were determined by micro-Kjeldahl and micro-Carius, respectively.

Phosphorus was estimated either by ALLEN's method[2] or the method of BERENBLUM AND CHAIN[3], depending on the phosphorus content of the sample.

Total lipids were extracted from 1–1.5 g amounts of material as approx. 10% solutions in 1 *M* sodium chloride by 20 volumes of ethanol–ether (3:1)[4] at room temperature. The protein precipitate was removed by centrifugation, washed twice with ethanol–ether, and the combined extracts concentrated to a small volume on a rotary evaporator under reduced pressure. A large volume of ethyl ether was then added to precipitate the contaminating sodium chloride. Ethyl ether was used instead of petroleum ether because the latter is known to dissolve sodium chloride in the presence of phospholipids[5]. The sodium chloride was removed by centrifugation, washed twice with ethyl ether, centrifuged again, and the supernatant and washings concentrated, as above. The extract was then transferred quantitatively into weighing flasks, evaporated under a nitrogen stream and finally dried to a constant weight *in vacuo* at 40°.

Phospholipids were separated from the non-phospholipids by chromatography of the total lipids on silicic acid columns[6] using the method of LEA, RHODES AND STOLL[7]. The first fraction (non-phospholipids) was eluted with chloroform containing 2% methanol, and the second (phospholipids) with methanol. Both were estimated gravimetrically. The recovery from the column was quantitative. The phosphorus content of the total lipids, multiplied by a factor of 25, was also used to estimate the amount of phospholipids[8].

Chromatographic separations of the proteins of the HDF of egg yolk were made at 4° on columns of hydroxyapatite by the method of TISELIUS, HJERTÉN AND LEVIN[9], using a time-operated fraction collector. A few experiments were done with 1 × 25 cm columns using small amounts of HDF to obtain an elution curve. The O.D. of these fractions were measured in a Beckman DU spectrophotometer at 280 mμ using 1 cm silica cells. When the O.D. was greater than 1.0, silica plungers were used to reduce the depth of solution. The large-scale separations were performed on 2.5 × 90 cm columns at a flow rate of 40 ml/h. About 3 g proteins were generally applied to the column as about 5% solutions in 0.2 *M* potassium phosphate buffer pH 6.8, which was the lowest concentration that would maintain the lipovitellin in solution. Three elution steps were generally used, namely, 0.2 *M*, 0.6 *M*, and 2 *M* potassium phosphate. The high protein concentration of the eluates made further concentration unnecessary. The eluates were examined by electrophoretic and ultracentrifugal methods.

Amino acid analyses were carried out using the technique of MOORE, SPACKMAN AND STEIN[10]. The effluent was analysed according to MOORE AND STEIN[11]. Lipoprotein samples, previously defatted by alcohol–ether[5], were hydrolysed by refluxing with

redistilled 6 *N* HCl for 22 h. The determination of cysteine–cystine as cysteic acid was done on samples oxidized with performic acid[12], dried *in vacuo*, and then hydrolysed, as above. The molar ratio of tryptophan and tyrosine was determined in 0.1 *N* NaOH with a Carey Model 11 recording spectrophotometer[13]. This ratio and the tyrosine content obtained by column chromatography were used to estimate the tryptophan content.

The protein concentration of the solutions, sedimentation velocity and electrophoretic mobility were measured by the methods previously described[1]. Partial specific volumes ($\bar{v}$) were determined at 20° in a magnetic float equipment[14], and here the concentrations were measured as weight fractions.

Molecular weight estimates were made by ARCHIBALD's method[15,16] at speeds between 4133 and 8225 rev./min, depending on the material investigated. Measurements were made only at the meniscus, since the use of either carbon tetrachloride or Dow Corning No. 515 silicone fluid to locate the base of the cell[17] caused a progressive broadening of the air-solution meniscus, possibly due to a piling-up of lipids set free from the lipoprotein. Protein concentrations of 0.8–1.0 % were generally used for these determinations, and the enlarged patterns (8 × linear) were measured with a mechanical integrator and computed according to SMITH, WOOD AND CHARLWOOD[18].

Diffusion coefficients were measured at 20° by either the Rayleigh fringe or the schlieren cylindrical lens method. The interference patterns were analysed according to LONGSWORTH's method[19]. The diffusion coefficients by the schlieren method were measured either in a Spinco Model H apparatus (avoiding any movement of the cell turret) or in a synthetic boundary cell (valve or capillary type) in the ultracentrifuge operated at 4133 or 8225 rev./min, and computed by the height–area method.

The HDF, and its major sedimenting component (S_1), were prepared by the sedimentation method, and lipovitellin by fractional precipitation from magnesium sulphate solutions, as previously described[1].

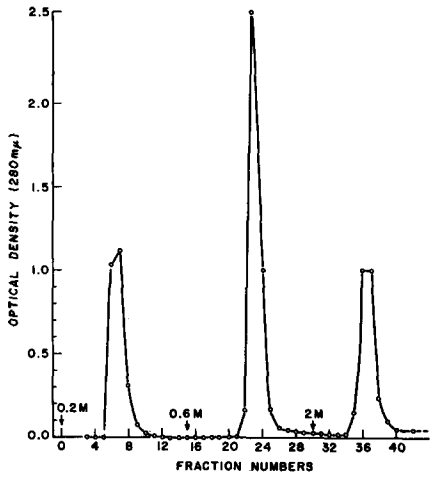
Chromatographic separation

Preliminary chromatographic experiments showed that no separation of the egg yolk proteins was obtained on brushite[9], but hydroxyapatite columns, prepared according to TISELIUS *et al.*[9], effectively fractionated HDF. Hydroxyapatite may also be useful for fractionating the LDF, since fractions were obtained from it at 0.3 and 0.7 *M* phosphate buffer pH 6.8 in initial trials, but the possibility of "false" components[20] was not investigated with LDF.

The elution curve obtained when HDF was chromatographed on a column of hydroxyapatite is shown in Fig. 1. The first fraction left the column without being absorbed at the buffer molarity required to dissolve the material (0.2 *M*). Ultracentrifugal examination of this fraction in veronal buffer pH 9.0 and 0.3 μ showed only the S_2 component (α- and β-livetin) and γ-livetin[1]. Electrophoretic analysis (Fig. 2A) also showed that it was made up of the three livetins in the proportions $\alpha:\beta:\gamma = 2:5:3$. When this first fraction is rechromatographed, it again leaves the column at 0.2 *M* potassium phosphate buffer. Preliminary experiments indicate that the three livetins can be fractionated on hydroxyapatite columns at phosphate buffer concentrations between 0.05 and 0.15 *M*.

The second chromatographic component, obtained with 0.6 *M* phosphate buffer,

was evidently β-lipovitellin, since it was largely dissociated when examined ultracentrifugally at pH 9.0 (see ref. 1). Electrophoretic analysis showed that it was contaminated with a minor amount of α-lipovitellin, which was removed on rechromatographing (Fig. 2B).



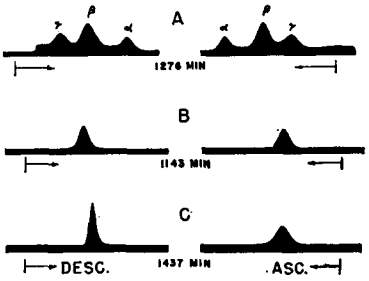


Fig. 1. Chromatography of HDF on hydroxyapatite.

Fig. 2. Electrophoretic patterns in veronal buffer pH 9.0, 0.3 μ of HDF fractions obtained by column chromatography: A, total livetin fraction; B, α-lipovitellin; C, β-lipovitellin.

The third chromatographic fraction obtained with 2 *M* phosphate buffer was evidently α-lipovitellin contaminated with a small amount of β-lipovitellin, which was removed on rechromatographing (Fig. 2C). Phosvitin was retained on the column and did not appear in any of the eluates, as indicated by electrophoretic and other analyses. This behaviour is in accordance with the reported affinity of polyphosphates for hydroxyapatite[9].

Results of physical measurements

The results of the physical measurements on the S_1 component of HDF, prepared by the sedimentation procedure already described[1], lipovitellin and its α- and β-components, are summarized in Table I. The electrophoretic mobilities of isolated α- and β-lipovitellin in veronal buffer (pH 9.0, 0.3 μ) are in good agreement with their individual mobilities in the mixture obtained by preparing lipovitellin by precipitation.

Most of the measurements were made in 1 *M* NaCl to avoid the dissociation of lipovitellin in alkaline solvents of lower ionic strength. Small but consistent differences in the sedimentation coefficients of α- and β-lipovitellin suggest minor differences in volume or shape. The molecular weights obtained from the sedimentation and diffusion coefficients and the transient state method[15], however, are the same for α- and β-lipovitellin and their parent mixture within experimental error. As expected, the molecular weight of lipovitellin is somewhat higher than that previously observed* in dissociating solvents. The molecular weight of the S_1 component will be discussed later.

* Through a computation error involving the magnification factor, the diffusion coefficient reported earlier for lipovitellin[21] in veronal buffer (pH 9.0, 0.3 μ) was too high and M too low. The correct values are: $D^{\circ}_{20,w} = 3.26 \cdot 10^{-7}$ and $M = 3.7 \cdot 10^{5}$. Likewise, for S_1 in 1 *M* NaCl[21], $D = 2.68 \cdot 10^{7}$, $M = 4.1 \cdot 10^{5}$; and for phosvitin in acetate buffer (pH 4.0, 0.1 μ)[22], $D = 4.60 \cdot 10^{7}$ and $M = 3.6 \cdot 10^{4}$.

TABLE I

SUMMARY OF PHYSICAL MEASUREMENTS

Property	*Solvent**	*S_1 component*	*Lipovitellin*	*α-lipovitellin*	*β-lipovitellin*
Electrophoretic mobility (10^{-5} cm²/sec/V)	B		—3.2 (α) —2.8 (β)	—3.2	—2.7
dn/dc (concentration in g/ml) at 5780 Å, 25°	A	0.179	0.181	0.180	0.180
v (ml/g) at 20°	A	0.765**	0.778	(0.777)***	0.777
$S°_{20,w}$ (Svedbergs)	A	9.9	10.5	10.9	10.4
	B		11.9§	12.0	
ds/dc (concentration in g %)	A	—0.75	—0.35	—0.50	—0.40
	B		—1.80§	—1.02	
$D_{20\,w}$ (10^{-7} cm²/sec) at concentration given					
Rayleigh	A	2.5 (0.28 %)			
Schlieren					
Electrophoresis cell			2.9 (0.33 %)		
Ultracentrifuge				3.0 (0.47 %)	2.8 (0.75 %)
Molecular weight ($\times 10^5$)					
from s and D	A	4.1	4.0	4.0	4.0
from ARCHIBALD's method§§	A	4.0 (0.93 %)	4.0 (0.94 %)	3.8 (0.47 %)	3.8 (1.00 %)

* Solvent A was 1 *M* NaCl; solvent B veronal buffer pH 9.0, 0.3 μ (0.25 *M* NaCl).

** At 25°.

*** Assumed value based on lipovitellin and β-lipovitellin.

§ In solvent B values given are for major component, minor component had $S°_{20,w}$ = 7.4 S and ds/dc = —0.30.

§§ Not corrected for concentration dependence. Correction would have increased reported value by 5 % or less.

The ultracentrifugal behaviour of α- and β-lipovitellin in neutral and alkaline solvents, together with comparable studies on the S_1 component and lipovitellin, supplementing earlier results[1], are shown in Fig. 3. In 1 *M* NaCl the S_1 component (Fig. 3A1), lipovitellin (Fig. 3A2), α-lipovitellin (Fig. 3B1) and β-lipovitellin (Fig. 3B2) all form single, sharp and symmetrical boundaries. In veronal buffer (pH 9.0, 0.3 μ) the slower sedimenting component is barely evident in α-lipovitellin (Fig. 3C1), whereas in β-lipovitellin (Fig. 3C2) this slow component represents about 80 % of the material. In glycine buffer (pH 10.9, 0.2 μ) α-lipovitellin (Fig. 3D1) dissociates to about the same extent as β-lipovitellin (Fig. 3D2), the degree of dissociation of the latter being about the same as at pH 9.0.

RESULTS OF CHEMICAL ANALYSES

The results of the chemical analyses made on lipovitellin and its α- and β-components are given in Table II. Lipovitellin and its separated components all contain about 20 % lipid, made up of 12 % phospholipid in terms of the total lipoprotein, and the remainder as triglycerides, cholesterol etc. The differences found between the reported lipid content and composition of the different lipovitellins are of doubtful significance.

Analysis of the two vitellins (*i.e.*, defatted lipovitellins) indicates that they have the same nitrogen content and tyrosine/tryptophan ratio. They differ markedly in phosphorus content and to a lesser extent in sulphur content. The latter difference

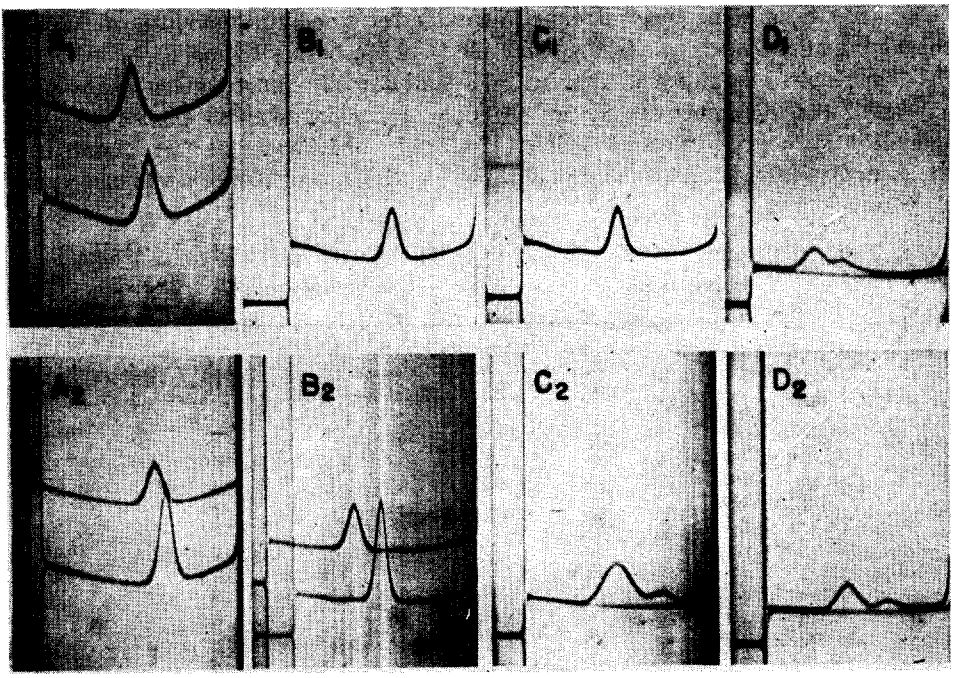


Fig. 3. Sedimentation patterns of: A_1, S_1 component in 1 *M* NaCl; A_2, lipovitellin in 1 *M* NaCl; B_1, α-lipovitellin in 1 *M* NaCl; B_2, β-lipovitellin in 1 *M* NaCl; C_1, α-lipovitellin in veronal buffer pH 9.0, 0.3 μ; C_2, β-lipovitellin in veronal buffer pH 9.0, 0.3 μ; D_1, α-lipovitellin in glycine buffer pH 10.9, 0.2 μ; D_2, β-lipovitellin in glycine buffer pH 10.9, 0.2 μ.

TABLE II

SUMMARY OF CHEMICAL ANALYSES*

Constituent		*Lipovitellin*	*α-lipovitellin*	*β-lipovitellin*
Total lipids	%	19	(20)**	20
Phospholipids				
(lipid P × 25)	%	12.5	11.0	13.5
(chromatography)	%	11.6		12.4
Non-phospholipids				
(chromatography)	%	7.4		7.6
Total phosphorus	%	1.05	1.40	0.95
Lipid phosphorus	%	0.50	0.44	0.54
As % of lipid	%	2.63	2.20	2.70
Protein phosphorus	%	0.50	0.96	0.36
As % of vitellin		0.62	1.20	0.45
Protein nitrogen	%		13.76	13.72
As % of vitellin	%		17.20	17.15
Protein sulphur	%		1.15	0.96
As % of vitellin	%		1.44	1.20
Tyrosine/tryptophan				
molar ratio		3.54	3.84	3.85

* Reported values are expressed as percent of lipoprotein, except as otherwise indicated.
** Assumed value.

must arise from some over-all experimental error since the amounts of sulphur-containing amino acids (see below) were the same for both vitellins.

The results of amino acid analyses on α- and β-lipovitellin are given in Table III, expressed as the number of residues per mole of vitellin taken as $3.2 \cdot 10^5$, *i.e.*, lipovitellin of $M = 4.0 \cdot 10^5$ less 20% lipid. The results of LEWIS *et al.*[23] are reported on the same basis for comparison, although these authors recognized that their preparations were impure.

TABLE III

AMINO ACID COMPOSITION OF α- AND β-LIPOVITELLIN

Expressed as residues/320,000 g of lipid-free vitellin.

Constituent	*α-vitellin*	*β-vitellin*	*"Vitellin" of* LEWIS *et al.*[23]
Aspartic acid	273	267	194
Threonine*	149	160	126
Serine*	255	260	341
Glutamic acid	326	334	239
Proline	156	155	122
Glycine	140	132	119
Alanine	226	217	144
Valine	178	188	169
Methionine	73	76	60
Isoleucine	162	175	129
Leucine	263	257	210
Tyrosine	95	88	67
Tryptophan**	25	23	17
Phenylalanine	91	92	77
Lysine	161	169	151
Histidine	61	58	62
Arginine	153	162	154
Cystine/2***	58	56	40
Total amino acid N	3612	3663	3175
Total N §	3929 (17.20%)	3917 (17.15%)	3587 (15.7%)
Amide N §§	(317)	(254)	295
P §	124 (1.20%)	46 (0.45%)	227 (2.2%)
S §	144 (1.44%)	120 (1.20%)	99 (0.99%)

* Corrected for hydrolysis by extrapolating values after 24 and 72 h hydrolysis to zero time.
** From tyrosine–tryptophan ratio (by u.v. spectroscopy) and tyrosine content.
*** As cysteic acid after performic acid oxidation, corrected by assuming 82% recovery[24].
§ Computed from values in Table II and LEWIS *et al.*[23].
§§ Values in parentheses are difference between total protein and amino acid nitrogen.

Most of the amino acids in α- and β-vitellin do not differ in amount by more than 4% and none by more than 8%. In some hydrolysates of both vitellins, the aspartic and glutamic acid contents were about 10% lower than those reported. A satisfactory explanation for this behaviour is lacking but, tentatively, it is suggested that the presence of any residual lipid may have caused some destruction of these acidic amino acids, and the higher values are taken to be more reliable.

The reported values for half-cystine have been corrected for 82% recovery[24]. The moles of sulphur-containing amino acids found in both vitellins are in good agreement and suggest a sulphur content between those (Table II) found for α- and β-vitellin, respectively. from elementary analysis.

The serine and threonine values were corrected for losses during hydrolysis by

extrapolating the figures obtained after 24 and 72 h hydrolysis back to zero time. These corrections indicated about 20% destruction of serine in both vitellins during the 22-h hydrolysis period used. It is noteworthy that the moles of serine exceed the moles of phosphorus present in both fractions.

When the amino acid composition of α- and β-vitellin is compared with that reported for impure vitellin[23], it is evident that phosvitin was the main impurity. Since about 50% of phosvitin consists of phosphoserine residues[23], its presence as a contaminant would increase the phosphorus and serine content of vitellin, and decrease the amount of nitrogen and of most of the other amino acids. This effect is indicated by the results in Table III. The difference in the serine content of LEWIS's vitellin, and ours, may actually be greater than the reported values, since the former estimated the destruction of serine during hydrolysis to be 10%, a value which is probably too low.

A further similarity between α- and β-lipovitellin is suggested by a similarity of their N-terminal amino acids[25]. Lysine and an as yet unidentified amino acid are present in comparable proportions in both vitellins.

DISCUSSION

The physical measurements reported here and in an earlier paper[1] give a consistent picture of α- and β-lipovitellin and the parent mixture of these two components. The two electrophoretic components have been separated chromatographically and found to have the same molecular weight in 1 *M* NaCl. Small but consistent differences in the sedimentation coefficients suggest a slight difference in volume or shape. Both α- and β-lipovitellin dissociate into smaller sub-units when dissolved in alkaline solvents and reassociate when returned to 1 *M* NaCl. β-lipovitellin dissociates at lower pH values than α-lipovitellin, but neither is completely dissociated at pH 10.9. No attempt was made to obtain quantitative estimates of the sizes of the sub-units from the measurements made on these paucidisperse systems at pH 10.9, but estimates based on the frictional ratios, and other known properties of α- and β-lipovitellin, and the sedimentation coefficients of the sub-units suggest that the latter are half the size of their parent molecules. This has been confirmed in other solvents (4 *M* urea) that cause complete dissociation, and will be reported later[26].

Obviously, the sub-units from α-lipovitellin cannot both be identical with those of β-lipovitellin, since these two lipoproteins differ in phosphorus content, but the available evidence is too limited to determine whether the sub-units of each lipovitellin are identical or different. Similarity of the sub-units of β-lipovitellin is suggested by its electrophoretic homogeneity in veronal buffer at pH 9.0 and 0.3 μ, a solvent in which 80% of this lipoprotein is dissociated. The presence of at least two N-terminal amino acids in β-vitellin, however, indicates that either the sub-units are different or that each contains more than one polypeptide chain.

The only chemical difference found between α- and β-lipovitellin lies in their protein phosphorus content. Contamination arising from several sources may affect their apparent phosphorus content. The affinity of phosvitin for hydroxyapatite and the electrophoretic homogeneity of the separated α- and β-lipovitellin give assurance that the difference in protein phosphorus content is not due to the presence of phosvitin. Another possibility is that the lipovitellin fractions retain some phos-

phate from the eluent buffer. All samples were dialysed exhaustively against 1 *M* NaCl on removal from the column and the only analyses reported in Table II are those in which the phosphorus content of the diffusate (control) was zero. Some earlier analyses, in which the control sample was positive, showed a smaller differential in the phosphorus content of α- and β-vitellin, after subtracting the control value. In all instances, however, the minimum value for α-vitellin always exceeded the maximum value for β-vitellin. Therefore, even if the quantities reported are subject to revision on further study, there is little doubt that the two vitellins differ in phosphorus content. Their electrophoretic and chromatographic behaviour support this conclusion.

The observed molecular weight of S_1 ($4.1 \cdot 10^5$) in 1 *M* NaCl substantiates earlier conclusions on the nature of this component. In this solvent S_1 is a mixture of: a predominating amount of undissociated α- and β-lipovitellins, an associated form of γ-livetin and phosvitin. While γ-livetin, in its associated form, may have a molecular weight lower than the lipovitellins, its effect on the average molecular weight is offset by the interaction of phosvitin with one or more of the other proteins. The net result is a mixture having the physical constants of an entity with a molecular weight that is the same as that of the major lipovitellin components.

From previous electrophoretic studies[1] it was indicated that the two lipovitellins are present in the proportions $\alpha:\beta = 3:2$. When lipovitellin is prepared by the precipitation procedure, it is evidently fractionated to some extent, and the proportions present after successive precipitations indicate that β-lipovitellin is more soluble than α-lipovitellin. Consequently, the proportions of the two lipovitellins in preparations obtained by precipitation may not represent their ratios in HDF. This unavoidable fractionation of precipitated lipovitellin, and some uncertainty about the precise phosphorus contents of α- and β-vitellin, also preclude a valid estimate of their proportions in HDF based on their protein phosphorus content. In principle, an independent estimate of their proportions could be obtained from the chromatographic elution curve. Integration of the O.D. of the individual fractions obtained on elution indicated a 1:1 ratio. The sharp separation observed in the small-scale experiments, however, may be only apparent, since the material had to be rechromatographed to effect complete separation in the large-scale work. This possibility, and the variation in the fraction volumes with different density eluents, make the electrophoretic evaluation of the α-:β-lipovitellin ratio in HDF the most reliable.

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MOLECULAR WEIGHT AND BEHAVIOR OF LIPOVITELLIN IN UREA SOLUTIONS*

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SUMMARY

Lipovitellin in 4 *M* urea has a molecular weight of $2.0 \pm 0.2 \cdot 10^5$, which is half that observed in 1 *M* NaCl. The two sub-units present in urea are undistinguishable on the basis of their ultracentrifugal properties. β-Lipovitellin dissociates at lower urea concentrations than α-lipovitellin. Although lipovitellin is denatured by urea and suffers a loss of lipid in this solvent, its dissociation is partly reversible on reducing the concentration of urea.

INTRODUCTION

Recent studies[1,2] have shown that lipovitellin and its two electrophoretic components (α- and β-lipovitellin) all sediment as a single component in 1 *M* NaCl, and have a molecular weight of $4.0 \cdot 10^5$ in this solvent. In alkaline solvents, however, lipovitellin partially dissociates to yield a second slower sedimenting component[1]. A monodisperse solution of these sub-units is required to estimate their molecular weight but, since

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