AN ELECTROPHORETIC AND ULTRACENTRIFUGAL STUDY ON THE PROTEINS OF THE HIGH DENSITY FRACTION OF EGG YOLK*

G. BERNARDI** AND W. H. COOK

Division of Applied Biology, National Research Council, Ottawa, Ont. (Canada)

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SUMMARY

1. The high density fraction represents 31% of the non-dialysable egg yolk solids. Electrophoretic analyses show that it contains six proteins in the proportions indicated below in order of decreasing mobility: phosvitin 15%; α-livetin 7%; β-livetin 17%; α-lipovitellin 30%; β-lipovitellin 21%; and γ-livetin 10%. No evidence was obtained to indicate the presence of a comparable proportion of any other protein.

2. Two lipoproteins that can be resolved electrophoretically, termed α- and β-lipovitellin, were found in the high density fraction. As another lipoprotein is present in the low density fraction, there are three lipoproteins in whole egg yolk, and these were demonstrated electrophoretically by adding a small proportion of the low density fraction to the high density fraction solutions.

3. Both α- and β-lipovitellin sediment as a single boundary in 1 *M* NaCl, but at higher pH values they dissociate to yield a slower sedimenting component. β-lipovitellin dissociates in a lower pH range than α-lipovitellin, but both appear to behave as reversible association–dissociation systems.

INTRODUCTION

Egg yolk proteins can be separated, on the basis of their density, into a HDF and a LDF[1]. The latter, previously referred to as the floating or rising fraction[2,3], is formed by a low density lipoprotein. The high density, or sedimenting, fraction contains five proteins: phosvitin[4,5], α-, β-, and γ-livetin[6,7], and lipovitellin[1,8]. In the present work electrophoretic and ultracentrifugal analyses were undertaken on HDF with a variety of solvents and under various experimental conditions, in an effort to resolve, identify, and estimate the relative proportions of its components.

In this investigation it was found that lipovitellin contains two electrophoretic components. In accordance with usual practice these have been called α- and β-lipovitellin. Their separation and characterisation will be reported in a later paper[9].

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

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** Present address: Centre de Recherches sur les Macromolécules, Strasbourg (France).

METHODS

Concentration of solutions was determined primarily by sampling replicate aliquots of both solution and solvent after exhaustive dialysis, and drying these to a constant weight *in vacuo* at 60°. The specific refractive index increment (dn/dc) of the solute was then calculated from these gravimetric results, and routine concentration estimates made from the refractive index increments (Δn) of solutions as measured in a differential refractometer[10].

Sedimentation velocity measurements were made at 20° and 59,780 rev./min with conventional 12 or 30 mm cells in a Spinco Model E analytical ultracentrifuge equipped with temperature control. The boundary displacement was measured with a travelling microscope and sedimentation coefficients computed from plots of log x *vs.* t. These were corrected to standard conditions and extrapolated to zero concentration. When relative concentrations of different sedimenting components had to be estimated, double sector cells were used. Areas were measured on 9 × (linear) enlargements of the patterns with an Amsler mechanical integrator. No corrections were made for differences in the dn/dc of the components, for the JOHNSTON-OGSTON effect[11], or the dilution effect due to the sector shape of the cell.

Electrophoretic measurements were made in a Spinco Model H apparatus at 1°. Veronal buffer at pH 9.0 and 0.3 μ (containing 0.25 M sodium chloride) was the solvent used, unless otherwise stated. As the ionic strength necessary to keep the lipoprotein in solution was rather high, low voltage gradients were used to maintain a current of 16 mA, and most experiments extended for about 20 h. Boundary displacements were measured on the patterns of the descending limb with a travelling microscope and the mobilities computed from these values and the conductivity of the solvent at 0°. To estimate the relative proportions of the different components, area measurements were made with a mechanical integrator on enlargements (4 × linear) of the descending limb, where resolution of the components was more satisfactory. In a few instances electrophoretic components were isolated by means of a separation[12] or analytical cell.

PREPARATIONS

HDF

One volume of egg yolk, obtained by puncturing yolks from which any adhering white had been removed by rolling them on absorbent paper, was diluted to 3 volumes with 2 M NaCl and enough solid NaCl to bring the final concentration to 2 M NaCl. This suspension was stirred for 1 h at 4° and then centrifuged at 30,000 rev./min (No. 30 rotor) in a Spinco Model L preparative ultracentrifuge at 0° for 24 h. Four zones were then evident in the tubes (Fig. 1): A, a firm layer of yellow gel; B, a clear colorless solution; C, a viscous yellow solution grading to a firm pellet at the bottom; D, a fluffy yellow suspension. Layer A and the suspended material in layer D constitute LDF and the remainder HDF. Their separation was accomplished by breaking layer A and decanting layers B and D. The pellet C and adherent layer A were then separated by cutting the tube, and pellet C collected by washing with solution BD and then with the original solvent. When the pellet C had completely dissolved, the yellow suspension was centrifuged, as above, for 12 h and the small

amount of LDF, originally present in suspension D, was removed from the top by suction. The remainder represents all the sedimenting material, termed HDF.

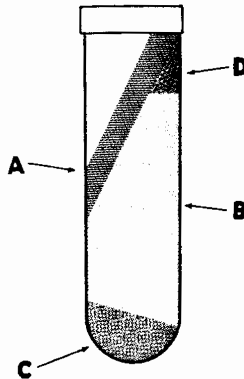

Fig. 1. Zones present in preparative ultracentrifuge tubes after high-speed centrifugation (24 h at 0° and 30,000 rev./min) of egg yolk diluted 1:3 with 2 *M* NaCl.

Fast sedimenting component (S_1)

The procedure was similar to that used to prepare HDF, except that both centrifugations were of 65-h duration. Only pellet C was retained after the first centrifugation. This was dissolved in the original solvent and centrifuged. Again only pellet C was retained after the second centrifugation.

Lipovitellin

Based on earlier observations[1,7,8], an extremely mild but effective procedure, outlined in Fig. 2, was developed for preparing lipovitellin, phosvitin, and the

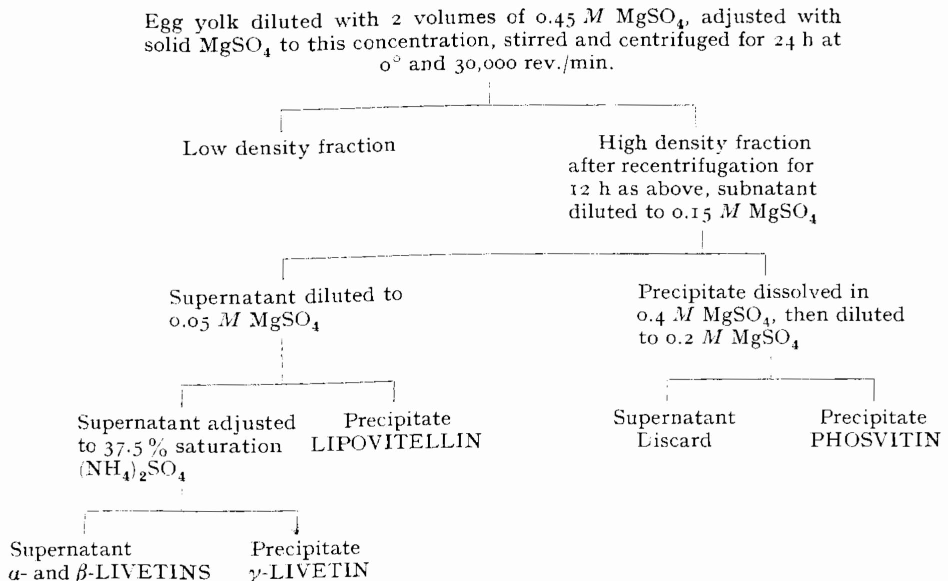

Fig. 2. Procedure for the fractionation of HDF.

livetins. The HDF was first prepared as indicated above, except that 0.45 *M* $MgSO_4$ was used instead of 2 *M* NaCl. Two volumes of water were then added to the HDF drop-wise (0.15 *M* $MgSO_4$) with mechanical stirring in a room at 4°. After standing overnight, a yellow gelatinous precipitate adhered to the bottom of the flask. Resolution and precipitation of this fraction yielded phosvitin. The supernatant was decanted, diluted with two volumes of water (0.05 *M* $MgSO_4$), and allowed to stand overnight at 4°. The white precipitate of lipovitellin was recovered by centrifugation, dissolved in 1 *M* NaCl, and stored at 0°. This material was then dialysed exhaustively against the experimental solvent.

Addition of ammonium sulphate to the supernatant to 37.5 % saturation precipitated γ-livetin, while α- and β-livetin remained in solution.

Each step of the fractionation procedure was examined by ultracentrifugation, electrophoresis and total and protein phosphorus analysis of the fractions obtained. On this basis the indicated purity of each fraction was higher than 95 %. Impurities could be removed by reprecipitating each fraction by the method indicated in Fig. 2.

ELECTROPHORETIC RESULTS

When HDF was examined in veronal buffer at pH 9.0 and 0.3 μ, seven components were evident in the descending, and five in the ascending, limb (Fig. 3). Since the three leading boundaries in the descending limb were found subsequently to be phosvitin, these have been marked 1a, b and c. At pH 10.5 (0.2 μ glycine buffer) and at pH 8.5 (0.3 μ veronal) the patterns were similar to those at pH 9.0.

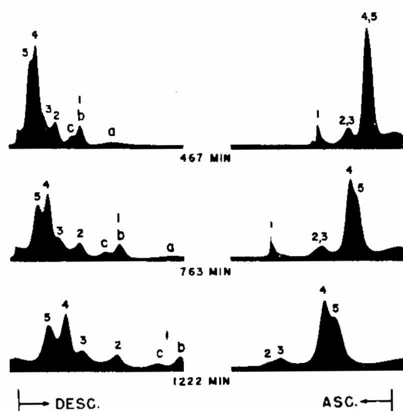

Fig. 3. Electrophoretic patterns of HDF in veronal pH 9.0, 0.3 μ.

In all buffers used, the shape of the patterns, the area and mobility of the components, differed in the ascending and descending limbs, a behavior that doubtless arises from the multiplicity of components of widely different charge density.

The electrophoretic components were identified by one or more of the following methods: (a) By adding isolated proteins to the HDF solution and comparing the patterns with the original material in parallel experiments; (b) by electrophoretic analyses of both the precipitates and supernatant solutions at each stage indicated in Fig. 2, supplemented by ultracentrifugal and chemical analyses; (c) by comparison

of the mobilities of the HDF components with those of the individual proteins. These mobilities are reported in Table I.

Component 1 was identified as phosvitin by all three criteria. This peak was enhanced when phosvitin was added (Fig. 4A). The crude phosvitin precipitate obtained at 0.15 *M* $MgSO_4$ shows a minor amount of lipovitellin, but the main component has the characteristic pattern and mobility of phosvitin (Fig. 4B), and these fast peaks were absent from the supernatant. Purified phosvitin at this ionic strength was found to give three peaks (Fig. 4C) in the descending limb and one in the ascending, all with similar mobilities (Table I) to the leading peaks observed in HDF solutions.

TABLE I

ELECTROPHORETIC MOBILITY OF THE COMPONENTS OF THE HIGH DENSITY FRACTION*

Mobility of HDF component		*Mobility of protein isolated from HDF*	
*Component***	$cm^2/sec/V \times 10^5$	*Protein*	$cm^2/sec/V \times 10^5$
1a	−10 to −12	Phosvitin	−10 to −12
1b	−8.5		−8.0
1c	−7.5		−7.4
2	−5.4	α-livetin	−5.1***
3	−3.7	β-livetin	−3.4***
4	−3.1	α-lipovitellin	−3.2 (see ref. 9)
5	−2.4	β-lipovitellin	−2.7 (see ref. 9)
		γ-livetin	−2.2

* All measurements in veronal buffer pH 9.0 and 0.3 μ.
** As indicated in Fig. 3.
*** Mobilities of α- and β-livetin were measured in a mixture of these two proteins.

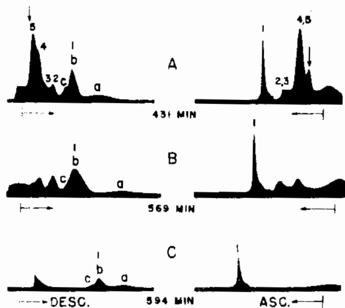

Fig. 4. Electrophoretic patterns in veronal pH 9.0, 0.3 μ of: A, HDF + phosvitin; B, precipitate obtained at 0.15 *M* $MgSO_4$ from HDF; C, phosvitin.

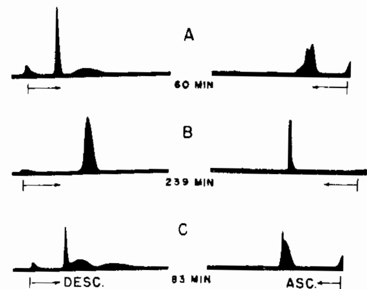

Fig. 5. Electrophoretic patterns of: A, phosvitin in veronal pH 9.0, 0.1 μ; B, phosvitin in veronal pH 9.0, 0.1 μ, + 0.01 *M* $MgSO_4$; C, phosvitin in veronal pH 9.0, 0.1 μ, + 0.005 *M* Na versenate.

This anomalous electrophoretic behavior of phosvitin was studied by determinations on purified material in veronal buffer at pH 9.0 and 0.1 μ (Fig. 5A) and in this buffer plus: 0.01 *M* $MgSO_4$ (Fig. 5B); 0.01 *M* $CaCl_2$ or 0.005 *M* sodium Versenate (Fig. 5C). At this lower ionic strength only two components were detectable in purified phosvitin; both magnesium and calcium complexes[5] were electrophoretically homogeneous; and the Versene-treated material had three components in the descending

and two in the ascending, limb. Evidently the electrophoretic behavior of phosvitin depends on the ionic strength and bivalent cation content of the solution.

The identification of the livetins in the HDF pattern was undertaken by adding a 2:5 mixture of α- and β-livetin (containing a minor amount of γ-livetin). This enhanced peak 2 and, to a greater extent, peak 3 (Fig. 6A). The lipovitellin precipitate obtained at 0.05 *M* $MgSO_4$ (Fig. 2) was free of these components (Fig. 6B), while the peaks in the supernatant had the same mobilities as α-, β- and γ-livetin. This evidence indicated that components 2 and 3 (Fig. 3) are α- and β-livetin.

Components 4 and 5 must contain γ-livetin and lipovitellin. The precipitate at 0.05 *M* $MgSO_4$ (Fig. 6B), containing little or no γ-livetin, had two components of similar mobility and proportions to those in HDF (Table I). Evidently lipovitellin has two electrophoretic components, one of which is indistinguishable from γ-livetin at pH 9.0. Addition of γ-livetin enhanced component 5 (lowest mobility) in this buffer and at pH 10.6 (0.2 μ glycine buffer) it was possible to resolve added γ-livetin from the two lipovitellin boundaries (Fig. 6C).

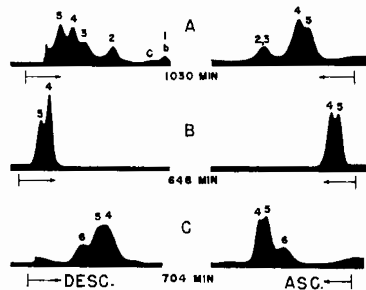

Fig. 6. Electrophoretic patterns in veronal pH 9.0, 0.3 μ of: A, HDF + α- and β-livetin; B, precipitate obtained at 0.05 *M* $MgSO_4$ from HDF; C, lipovitellin + γ-livetin in glycine pH 10.6, 0.2 μ.

The relative amounts and wide density differences of LDF and HDF made it impossible to maintain stable boundaries for electrophoretic analysis of whole egg yolk that had not been ether extracted. A small quantity of LDF could, however, be tolerated in the HDF veronal pH 9.0 solution. Upon electrophoresis LDF appeared as a component of still lower mobility than γ-livetin (Fig. 4A arrows) and was resolved (in the ascending limb) at an early stage.

SEDIMENTATION RESULTS

Ultracentrifugation in 1 *M* NaCl resolved HDF into two components termed S_1 and S_2[1], having $s^{\circ}_{20,w}$ values of 9.9 S (see ref. 9) and 3.4 S, (see ref. 6) respectively. All available evidence indicates that S_2 is formed by α- and β-livetin[6], so that S_1 must represent phosvitin, γ-livetin and lipovitellin, although its schlieren pattern (Fig. 7A1) suggests a monodisperse material.

When lipovitellin is prepared according to Fig 2, it also sediments in 1 *M* NaCl as a single component (Fig. 7A2) with $s^{\circ}_{20,w} = 10.5$ S (see ref. 9). This component is termed S_L to distinguish it from S_1 above.

Both S_1 and S_L have been shown to split into two components at pH 9.0, and, from its similar sedimentation properties, the minor component was taken to be

γ-livetin[8]. As this view was not consistent with information obtained in the present study, further investigations were undertaken. To avoid spurious effects of fractionation, the S_1 component was studied in the presence of S_2, as it occurs in HDF, and lipovitellin was prepared according to Fig. 2.

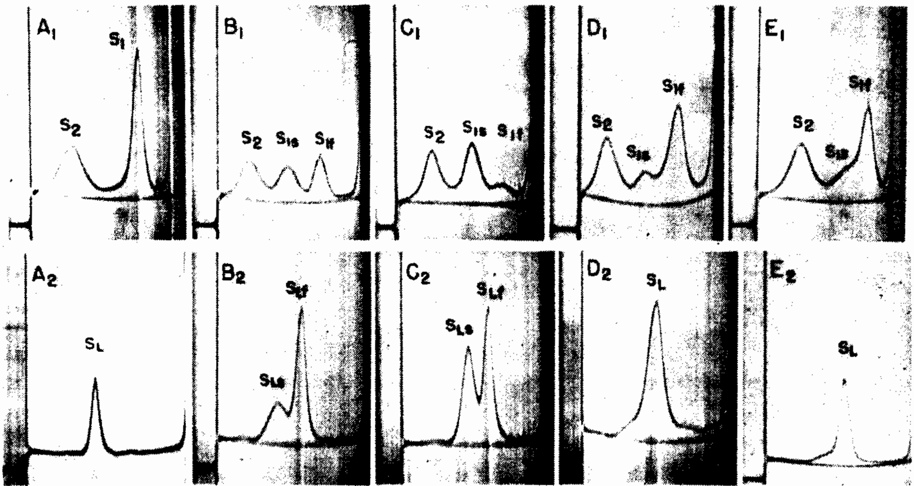

Fig. 7. Sedimentation patterns of HDF (upper row) and lipovitellin (lower row) in: A, 1 M NaCl; B, veronal pH 9.0, 0.3 μ; C_1, glycine pH 10.9, 0.2 μ; C_2, glycine pH 10.6, 0.2 μ; D, phosphate pH 6.8, 0.4 μ; E, veronal pH 9.0, 0.3 $\mu \longrightarrow$ 1 M NaCl.

The splitting that occurs when S_1 and S_L were examined in veronal at pH 9.0 is shown in Fig. 7B1 and 7 B2, respectively. The proportion of the slow components S_{1s} and S_{Ls} increased with increasing pH (Fig. 7C1 and C2), while S_2 remained constant in the HDF solutions. This confirms and extends an earlier observation[8] indicating that the major component, S_1, dissociates to an increasing extent as the pH is increased. In phosphate buffers pH 6.8 (0.4 and 0.8 μ) S_1 splits into two components (Fig. 7D1), but S_L does not (Fig. 7 D2). When solutions in veronal are dialysed into 1 M NaCl, S_{1s} remains in reduced amount and S_{Ls} practically disappears (Fig. 7 E). The proportions of S_{1s} and S_{Ls} components observed in these and other solvents are summarized in Table II.

The results in Table II show that there is about 20% more of a slow moving component in S_1 than in S_L. Presumably both S_1 and S_L are principally lipovitellin, which can dissociate into two components under certain conditions, especially at high pH. However, S_1 also contains approximately 20% of another slow moving component, which is either γ-livetin or an interaction product of γ-livetin and phosvitin.

Additional experiments were undertaken to obtain further information on the S_1 component. When this component, containing phosvitin, γ-livetin and lipovitellin, was prepared from HDF by centrifugation to remove S_2, and examined at pH 9.0, S_{1f} and S_{1s} were present, but phosvitin (3.5 S) was never evident. By diluting HDF directly from 0.45 M to 0.05 M $MgSO_4$ the precipitate contains lipovitellin and phosvitin, but no γ-livetin. This fraction sedimented as a single peak in 1 M NaCl,

TABLE II

PROPORTIONS OF SLOW SEDIMENTING COMPONENTS IN S_1 AND S_L, IN VARIOUS SOLVENTS

Solvent	*Ionic strength*	*pH*	S_{1s}*	S_{Ls}*
NaCl	1.0		0	0
Phosphate buffer	0.4	6.8	20	0
Phosphate buffer	0.8	6.8	18	0
Phosphate buffer	0.3	7.5	—	25
Veronal buffer	0.3	9.0	52	31
As above, dialysed back to NaCl	1.0		28	6
Veronal buffer	1.0	9.0	—	28
Glycine buffer	0.2	10.0	59	—
Glycine buffer	0.2	10.6	66	47
As above, dialysed back to NaCl	1.0		25	< 5
Glycine buffer	0.2	10.9	80	—

* Values are expressed as % of total S_1 or S_L.

but at pH 9.0 the 3.5 S boundary (phosvitin) was evident, in addition to the other two, and this 3.5 S boundary persisted when the preparation was dialysed back into 1 *M* NaCl. Evidently the removal of γ-livetin affects the sedimentation behavior of phosvitin.

The anomalous sedimentation behavior of γ-livetin, already reported[7], was again observed in veronal at pH 9.0 and 1 *M* NaCl solution. In sodium chloride solutions the *s vs. c* plot was positive, indicating aggregation with increasing concentration, although extrapolation yielded the reported[7] value of $s^{\circ}_{20,w} = 7.5$ S. In veronal at pH 9.0 the *s vs. c* plot gave the same intercept but was slightly negative. At $c = 0.5$ % the sedimentation coefficient in veronal was about 2 S units lower than in 1 *M* NaCl, and this behavior was reversible between these solvents. This indicates that failure to resolve γ-livetin from the S_1 component at finite concentration in 1 *M* NaCl may arise from its similar sedimentation rate rather than by interaction.

Composition of high density fraction

In order to express the proportions of the several components observed electrophoretically in HDF in terms of yolk solids, the proportion of HDF in yolk solids had to be determined. Since the components are dialysed before examination, the whole yolk solids were dialysed to permit their proportions to be expressed on a comparable basis. Evaporation and other factors affect the percentage solids found in egg yolk, but the loss on dialysis was about 2.5 %, and the non-dialysable solids were about 46.5 % of the total yolk contents. The separation of HDF (Fig. 2) was then performed quantitatively by using 2 *M* NaCl as solvent and somewhat more dilute solutions of yolk, washing the LDF, and adding these washings to the HDF. The amount of the starting material and HDF were determined by dry weight after exhaustive dialysis against sodium chloride solutions.

The results of replicated experiments, summarized in Table III, show that 31 ± 1 % of the non-dialysable yolk solids was HDF, and the LDF by difference about 69 %. As the electrophoretic separation of γ-livetin from the lipovitellin components in HDF was inadequate for measurement, the proportion of this protein was obtained from the content and patterns of the total livetins, separated from the other constituents on hydroxyapatite columns, to be described later[9].

TABLE III

PROPORTION OF PROTEINS IN HIGH DENSITY FRACTION BY ELECTROPHORETIC ANALYSIS

Component	As HDF %		As non-dialysable egg yolk solids %
HDF	100		31
Phosvitin	15		4.6
α-livetin	7		2.2
β-livetin	17		5.3
α-lipovitellin	30		9.3
β-lipovitellin	31	21	6.5
γ-livetin		10*	3.1

* As γ-livetin was not resolved in HDF, it was estimated from its electrophoretic proportion in the total livetins (α:β:γ = 2:5:3) obtained by chromatographic separation[9].

DISCUSSION

Electrophoretic analysis of HDF solutions, conducted over long periods at low current densities in suitable solvents, revealed a maximum of seven boundaries in the descending limb. These were identified by electrophoretic, ultracentrifugal, and chemical analyses. It was found that the three boundaries of highest mobility were all given by phosvitin. The next two boundaries were given α- and β-livetin with mobilities in general agreement with previous reports[6]. These were followed by lipovitellin, which was found to have two electrophoretic components, termed α-lipovitellin and β-lipovitellin in order of decreasing mobility, as is the usual practice. Although γ-livetin was not resolved in HDF, it was found, on increasing its proportion by addition, that it had a lower mobility than either of the lipovitellins. This finding requires an earlier interpretation[6] of an electrophoretic pattern of whole egg yolk to be revised. With the recognition of α- and β-lipovitellin, all the electrophoretic components can be identified as proteins and lipoproteins previously isolated from HDF, and no evidence was obtained for the existence of other proteins in sufficient proportion to be detectable by the methods employed.

The α- and β-lipovitellins mentioned here are not the same as those described by SUGANO[13], which were prepared from ether extracted whole egg yolk and identified as lipovitellin and lipovitellenin, respectively. It was found here that the addition of LDF (not ether extracted) containing lipovitellenin to the HDF solutions gave a third lipoprotein boundary, the added LDF having the lowest mobility. SUGANO's lipovitellins differ both qualitatively and quantitatively from ours but, as he has found both his preparations to be heterogeneous mixtures ultracentrifugally[14], useful comparisons are impossible.

The several components of HDF cannot be separated quantitatively. Ultracentrifugation resolves only two boundaries, both containing two or more components. In consequence the best quantitative estimate of the concentration of the components was obtained from the electrophoretic patterns of HDF. As γ-livetin was not adequately resolved for satisfactory measurements, the proportion of the three livetins was estimated after their chromatographic separation[9] and found to be α:β:γ = 2:5:3. Phosvitin represents about 15 % and the two lipovitellins about half of HDF. Since HDF was found to represent 31 % of the nondialysable yolk solids, this factor can

be used to estimate their content in terms of yolk solids. As the protein content of the yolk solids is approx. 35 %, and HDF is approx. 31 %, the proportions of the proteins in HDF approximate, within experimental error, their proportions in the total lipid-free protein, if the lipid content of the lipovitellins is subtracted.

When HDF is dissolved in 1 *M* NaCl solutions, the lipovitellin, phosvitin and γ-livetin sediment as a single boundary, S_1 (see ref. 1, 8). At pH 9.0 S_1 splits and the slower minor boundary (termed S_{1s}) has the sedimentation properties of γ-livetin[7]. Lipovitellin behaves similarly, having a single boundary, S_L, in 1 *M* NaCl, and a slower component, S_{Ls}, at pH 9.0, originally regarded as a γ-livetin impurity[8]. Since lipovitellin, prepared according to Fig. 2, was free of phosvitin and γ-livetin by all criteria available, the origin of this second component was re-investigated. Comparison of HDF and lipovitellin solutions showed that S_{1s} was proportionately greater than S_{Ls} in a number of solvents, that both increased in quantity with increasing pH, and that only S_{Ls} disappeared completely when returned to 1 *M* NaCl. Evidently lipovitellin can dissociate reversibly into smaller units (S_{Ls}) under mildly alkaline conditions. The greater proportion of S_{1s}, and its incomplete reversibility on returning to 1 *M* NaCl, indicate that it contains two proteins that cannot be resolved ultracentrifugally. One of these is dissociated lipovitellin (S_{Ls}) and the other is γ-livetin or its interaction product with phosvitin. Recent measurements of the partial specific volume of S_{Ls} by an ultracentrifugal method[15] shows that it resembles lipovitellin in this property.

When the second component in S_1 at pH 9.0 was assumed as its γ-livetin content, the observed molecular weight of S_1 in sodium chloride solutions could not be harmonized with the molecular weight and apparent quantity of its components[8]. Since much of the second component is a dissociation product of lipovitellin that does not exist in sodium chloride solution, this inconsistency disappears. However, molecular weight estimates reported[8] for lipovitellin at pH 9.0 are likely to be somewhat low due to the presence of this dissociation product.

The relation between α- and β-lipovitellin and the association–dissociation products of lipovitellin observed ultracentrifugally was examined by recovering the separated components from the electrophoretic cell for sedimentation analyses. At pH 9.0 α-lipovitellin sedimented as a single S_L boundary, whereas β-lipovitellin sedimented as a mixture of approx. 80 % S_{Ls} and 20 % S_{Lf}. Since most of the total lipovitellin dissociates at pH 10.9, it is evident that α-lipovitellin also dissociates under more alkaline conditions.

In earlier studies[8] it was found that the content of the S_{Ls} component at pH 9.0 could be reduced following certain precipitation procedures. In the present study repeated precipitation at 0.1 *M* $MgSO_4$ yielded a sample that contained only 5 % S_{Ls}. Electrophoretic examination also showed that the precipitate obtained on dilution to 0.1 *M* $MgSO_4$ contained less β-lipovitellin than the precipitate obtained at 0.05 *M* $MgSO_4$. Repeated precipitation could therefore fractionate the two components.

The separation of the two electrophoretic components[9] and quantitative measurements on these and their dissociation products will be reported later.

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