BBA 4053

STUDIES ON AMPHIBIAN YOLK

IV. AN ANALYSIS OF THE MAIN-BODY COMPONENT OF YOLK PLATELETS

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(Received December 28th, 1962)

SUMMARY

A procedure for the separation and isolation of Components S and F from the insoluble main-body fraction of isolated yolk platelets is described. The principal features of this procedure involve an ammonium sulfate precipitation and a minimum contact with oxidizing agents. Chemical, physical, and amino acid analyses have been performed on the two components. It has been found that Component S is a phosphoprotein (phosphorus = 8.4%, molecular weight = 32000) similar to phosvitin and Component F is a lipoprotein (lipid = 17.5%, molecular weight = 420000) similar to lipovitellin. These two components have accordingly been designated as amphibian phosvitin and lipovitellin, respectively. It has been further determined that there are two molecules of phosvitin for every molecule of lipovitellin in the main-body crystal, and that the phosvitin molecules are arranged in a simple hexagonal lattice. With these considerations, a definition of the structural unit and the unit cell of the main-body crystal has been proposed, and a tentative model for the crystalline structure has been presented.

INTRODUCTION

Recent electron microscopic studies of amphibian yolk platelets have revealed that they are primarily composed of a main body which displays a crystalline periodicity[1,2]. The visible units of the main body are approximately 40–45 Å in diameter, have a center-to-center distance from one another of 81 Å, and seem to be arranged in a simple hexagonal lattice[2]. However the relationship between these observed units and the macromolecular elements which comprise the main body of yolk platelets is still largely unknown. For this reason a biochemical investigation of the components of the main body has been undertaken.

Intact yolk platelets have been isolated from ovulated eggs of *Rana pipiens* and

Abbreviations: PVP, polyvinylpyrrolidinone; *Yi*, main-body fraction of isolated yolk platelets.

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** Operated by Union Carbide Corporation for the United States Atomic Energy Commission.

the surrounding superficial layer has been removed by a water extraction[3]. The water-extracted yolk platelet was thus found to consist of the main body of the yolk platelet and was designated as the *Yi* fraction[3,4]. When dissolved in strong salt solutions, *Yi* was found in the ultracentrifuge to consist of three components indicated as S, F, and X in the order of their increasing sedimentation rate[4]. Component X, however, was demonstrated to be an oxidation product of F and did not appear when *Yi* was fractionated or analyzed under non-oxidative conditions. An ammonium sulfate fractionation procedure which was employed to separate Components S and F is further detailed in this paper, and some of the chemical and physical properties of the isolated components are provided.

METHODS

Preparation of the main-body fraction of yolk platelets

The main-body or *Yi* fraction of yolk platelets from *Rana pipiens* was prepared by centrifuging homogenates of ovulated eggs in a discontinuous sucrose–PVP gradient and extracting the isolated yolk platelets with water as previously described[4].

It was found that larger amounts of a preparation suitable for the biochemical analyses reported here could also be more conveniently prepared by using whole ovaries and a sucrose–EDTA solution as a suspending medium (the presence of EDTA helps to inhibit an undesirable aggregation within the homogenate[4,5]). In this procedure, ovaries from *Rana pipiens* were removed, washed in distilled water, and thoroughly homogenized as a 20% (w/v) suspension in 0.25 M sucrose–2 mM EDTA. The homogenate was quickly filtered through 8-ply gauze, 20 ml layered over 20 ml 1.0 M sucrose–2 mM EDTA in a 40-ml conical centrifuge tube and centrifuged in an International centrifuge at 1000 rev./min ($F_{av.} = 200 \times g$) for 10 min. The supernatant was discarded and the loosely-packed pellet rehomogenized, layered, and centrifuged twice more (all operations performed at 3–5°). The third pellet thus obtained was well-packed and bright yellow. This fresh pellet, when examined with the light microscope, was composed of "yolk platelets" which frequently showed signs of cracking. Under the electron microscope, the fixed preparations were seen to be composed only of the main-body components of yolk platelets (*i.e.*, no superficial layer) and these components lacked much of the normal crystalline periodicity. Although mitochondria and other cytoplasmic structures were not observed, contamination with nuclei was greater than that observed for sucrose–PVP preparations. However, it was never more and was frequently less than the contamination observed in the preparations obtained by the method of RINGLE AND GROSS[6]. Although the crystalline structure of the isolated main-body components appeared (in fixed and embedded material) to be rather damaged (perhaps caused by a partial removal from the main body of divalent cations by EDTA), it was found, nevertheless, that Components S and F obtained from such material were entirely analogous to S and F obtained from *Yi*. Also, Component X was absent from the sucrose–EDTA preparations, perhaps because the presence of EDTA inhibited a heavy-metal-catalyzed oxidation of Component F to form X.

Ammonium sulfate fractionation of Yi

1 ml of the *Yi* pellet was made up to 40 ml with water, and 5 ml of 5.0 M NaCl were slowly added with stirring. The clarified suspension was then centrifuged to

remove "denatured" or extraneous material[4,6] and the yellow clear supernatant was filtered (to exclude pieces of the pellet which frequently break away). 1.5 vol. of water were slowly added to the filtrate, and the resulting flocculant precipitate centrifuged down. The supernatant was discarded and the precipitate was dissolved in about 20 ml of 3/7 0°-satd. ammonium sulfate* and briefly dialyzed against this solvent. The dialyzed solution was then centrifuged to remove a very small amount of turbidity, the volume of the clarified supernatant noted, and 3/7 vol. of 100% 0°-satd. ammonium sulfate was very slowly added. This provided a 60% 0°-satd. ammonium sulfate solution containing a copious precipitate of Component F. The mixture was then centrifuged at 50000 × g ($F_{av.}$) for 30 min to provide a well-packed yellow pellet and a water-clear supernatant containing Component S. Component F was reprecipitated from ammonium sulfate solutions twice more to free it from residual traces of Component S. Ammonium sulfate solutions of Components F and S were dialyzed overnight against 0.5 M NaCl and then for another 24 h against several changes of 0.1 M NaCl to free the proteins from ammonium sulfate. In 0.1 M NaCl, Component F was maximally insoluble and formed a copious precipitate, whereas Component S was completely soluble. Turbidity which appeared in the dialysis tubing containing Component S was removed by centrifugation, since this indicated that the ammonium sulfate precipitation of Component F was not complete and that the residual amounts were coprecipitating with Component S. At this stage, the precipitate of Component F was also centrifuged down and resuspended in fresh 0.1 M NaCl or an appropriate buffer. Component S was satisfactorily stored by further dialysis against distilled water and freeze-drying. Freeze-drying of Component F, however, resulted in the formation of Component X, so that physical determinations on Component F were performed within 24 h after dialysis against a solvent (in this case, 0.5 M NaCl–5 mM Tris·Cl, pH 8.0). All solutions mentioned in this paragraph, with the exception of those used for a suspension of Component S after its isolation, were previously heated to the boiling point, and then flushed with a fine stream of nitrogen (oxygen-free) while stirring until cooled to 3–5° in a cold room. They were then sealed and used within 48 h.

Determinations

Dry-weight and lipid determinations and elemental analyses were performed as previously described[4]. Phosphoprotein (alkali-labile) phosphorus was determined by incubating the protein in 1.0 N NaOH for 16 h at room temperature and analyzing the resulting solution for inorganic phosphorus according to MARTIN AND DOTY[7]. Protein-bound carbohydrate was analyzed by the orcinol procedure described by WEIMER AND MOSHIN[8], employing glucose as a standard.

Partial specific volumes were determined with a pycnometer containing 3–5% protein solutions. The viscosity of protein solutions at various concentrations was measured with an Ostwald viscometer and the intrinsic viscosity determined from a plot of $(1/c)\ln(\eta/\eta_0)$ *vs.* c. Spectral analyses were performed with a Cary Model-14 recording spectrophotometer.

* Crystalline reagent-grade ammonium sulfate was recrystallized from 0.1% EDTA. A saturated solution of the recrystallized product at 0° was neutralized (to pH 7) by the addition of approx. 1 ml/l concentrated ammonium hydroxide and designated as 100% 0°-satd. ammonium sulfate.

Sedimentation velocity measurements were made as previously described[4] and sedimentation coefficients computed from plots of log$x$ *vs.* t. These were corrected to zero concentration and standard conditions. Approximate values for molecular weight were subsequently calculated from sedimentation, partial specific volume and viscosity measurements from the formula provided by SCHACHMAN[9] (equation 147). Alternately, molecular weights were determined by ARCHIBALD's method[10,11], using 0.8–0.9 % protein solutions and speeds of 4790–6495 rev./min.

The amino acid composition of the proteins was determined by the method of SPACKMAN *et al.*[12], employing a Spinco Model-120 Analyzer. Constant-boiling HCl was added to the sample, the mixture alternately flushed with nitrogen and evacuated a dozen times and finally sealed under vacuum. If this procedure was carefully performed, cystine destruction was kept to a minimum and a reasonable quantitative estimate could be made from the chromatogram. Samples were hydrolyzed for 24, 48, and 96 h, vacuum-evaporated and stored at −20° under nitrogen until analysis. Analyses were performed alternately with standard determinations to correct for ninhydrin aging[13], and the results from the various samples plotted and extrapolated to zero time to correct for hydrolytic destruction and ammonia formation. Two long (150 cm) column runs were performed on each sample of Component S, using 2.0-ml and 0.5-ml aliquots of the hydrolyzed protein in sample buffer (1.5 mg/ml), respectively. The relatively high concentrations of hydrolyzed protein were necessary for a quantitative evaluation of those amino acids present in low concentrations, whereas the 0.5-ml aliquots were used primarily for serine determinations. The tryptophan content of Components S and F was estimated by incubating the proteins in 0.1 N NaOH for 30 min and 24 h respectively and recording the ultraviolet spectra according to BEAVEN AND HOLIDAY[14].

RESULTS AND DISCUSSION

Chemical and physical measurements

Chemical analyses on Components S and F are provided in Table I. Component S is apparently a highly phosphorylated phosphoprotein and almost all of its phosphorus is released as inorganic phosphorus in 1.0 N NaOH. Component F, on the other hand, is a high-density lipoprotein. Since the lipid in Component F seems to represent all the lipid previously found in *Yi* (see ref. 4), it may be concluded that it is about two-thirds phospholipid.

Some physical measurements performed on Components S and F are provided in Table II. From these data, mol. wts. of 32000 and 420000 have been calculated for Components S and F respectively. The molecular weight for the Component F isolated from yolk platelets of *Rana esculenta* has been indicated by FUJII[15] to be 390000*, in good agreement with the value reported here for the Component F of *Rana pipiens*. The most uncertain determination reported in Table II is the partial specific volume of Component S. This value (0.61 ml/g) is the result of only two determinations, since the supply of Component S was limited, and appears abnormally low even for a highly phosphorylated protein. JOUBERT AND COOK[16], however, have

* The molecular weight provided by FUJII is $4.5 \cdot 10^5$. This value was apparently derived by using ρ = the solvent density in his molecular weight calculation. A recalculation using his $s^0_{20,w}$ value and a $\rho = H_2O$ density at 20° gives a molecular weight of $3.9 \cdot 10^5$.

reported a value of 0.545 ml/g for the partial specific volume of the phosphoprotein (phosvitin) isolated from hen's egg yolk. They attribute their result in part to the presence of gegenions, and this may also apply to Component S.

TABLE I

SUMMARY OF CHEMICAL MEASUREMENTS

Values given are expressed as per cent of component dry weight.

Constituent	*Component S*	*Component F*
Nitrogen	15.2	14.4
Phosphorus		
(total)	8.4	0.90
(alkali-labile)	7.3	0.28
(lipid)	< 0.1	0.45
Sulfur	< 0.1	0.4
Lipid	< 0.1	17.5
Carbohydrate	0.5	< 0.1
Protein	68.5	81.5

TABLE II

SUMMARY OF PHYSICAL MEASUREMENTS

Property	*Component S*	*Component F*
$s^{\circ}_{20,w}$ (S)*	3.1	11.8
$[\eta]$ (ml/g)*	—	5.5
$\bar{v}$ (ml/g)*	0.61	0.77
Mol. wt. $\times 10^{-4}$		
from $s^{\circ}_{20,w}$ and $[\eta]$	—	41
from ARCHIBALD*	3.2	43
Volume (ml $\times 10^{21}$)	32.4	537
Spherical diameter (Å)	40	101
$E^{1\%}_{1\,cm}$ (280 mμ)**	1.96	7.59

* Solvent: 0.5 M NaCl–5 mM Tris–Cl (pH 8.0).
** Solvent: H_2O.

The rather low $E^{1\%}_{1\,cm}$ value reported for Component S in Table II has already been inferred by its low contribution to the total absorption of Yi at 280 mμ, as indicated by an ammonium sulfate precipitation curve[4]. The absorption spectra of both Components S and F are further detailed in Fig. 1. In 0.1 N NaOH, the spectrum of Component S shows a single and symmetrical tyrosine peak at 290 mμ, indicating that tryptophan is either absent or present in very low amounts ($moles_{try}/moles_{tyr} = 0.08$).

The spectrum of Component F in H_2O shows inflections at 260 and 290 mμ, indicating the presence of phenylalanine and tryptophan respectively. The inflection at 260 mμ previously reported by GROSS AND GILBERT[17] for the spectrum of dissolved yolk platelets is thus not necessarily considered to be evidence for the presence of nucleic acid, as suggested by these authors. The existence of a carotenoid as a constituent of Component F is indicated by the visible absorption spectrum, with peaks at 456 and 484 mμ. RINGLE AND GROSS[18] have previously isolated "yolk xanthophylls"

from *Rana pipiens* yolk platelets, and found that the main absorption peak for their material was about 440 mμ in light petroleum. The carotenoid pigment of Component F was readily extracted with methyl alcohol and the decidedly hypophasic solubility properties previously noticed by RINGLE AND GROSS[18] were corroborated. The pigment in light petroleum (b.p. 30–60°) was subsequently placed on a neutral alumina (6% H_2O) column and eluted by a light petroleum–acetone gradient.

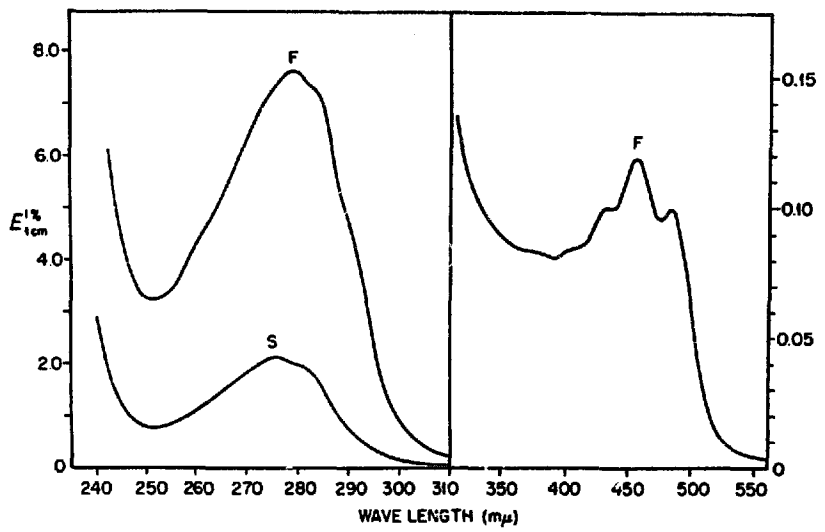

Fig. 1. Absorption spectra of 1% solutions of Components S and F. Solvent: H_2O.

A monitor of the eluant (at 443 mμ) indicated that a single peak (coming off at about 15% acetone concentration) contained more than 90% of the material, the rest being distributed in two or three minor peaks. The major absorption maxima for the main carotenoid component were 442 and 464 mμ in light petroleum, 443 and 470 mμ in methanol, 456 and 483.5 mμ in chloroform, and 475 and 504 mμ in carbon disulfide. No further identification of the pigment has been made. Assuming an $E^{1\%}_{1\,cm}$ value of 2000 (at the point of maximum absorption) for the carotenoid and a molecular weight of around 550, it has been calculated from the absorption spectrum of Component F that there is only about one carotenoid molecule for every 20 molecules of lipoprotein. A carotenoid pigment seems to be a component of all amphibian yolk platelets and is responsible for their light yellow color, but the association of the carotenoid molecules with the protein component of the platelet is thus perhaps not a specific one.

The solubility of Component F as a function of sodium chloride concentration is indicated in Fig. 2. Apparently, Component F has the peculiar property of being insoluble in low salt concentrations, but soluble in distilled water and high salt concentrations in a manner similar to certain nucleoproteins[19]. When Component F in distilled water is dialyzed against 0.5 M NaCl, a voluminous precipitate first appears within the dialysis tubing, and this precipitate is later solubilized. Preliminary investigations on the influence of pH on the salt-solubility properties of Component F indicate that Fig. 2 represents a curve for approximately neutral solutions. Below pH 4, the solubility in low-ionic-strength solutions increases, but marked salting out occurs at high ionic strengths ($I > 0.2$). High pH values (> 9.0) promote greater solubility regardless of ionic strength, as does aging of the preparations. Although

some of these properties have been previously noted for the high-density lipoproteins of hen's egg yolk[20-22], the moderate solubility in distilled water as opposed to solutions of low salt content has not yet been observed and remains rather difficult to explain without further experimentation.

The amino acid composition of Components S and F are provided in Table III. The recovered nitrogen agrees well with the values reported in Table I, yet only 91 %

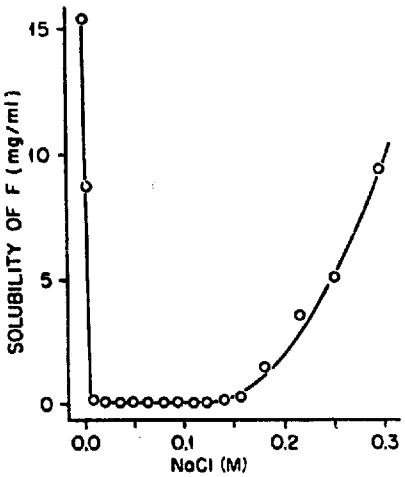

Fig. 2. Solubility of Component F as a function of NaCl concentration. A saturated solution of Component F exhaustively dialyzed against H_2O and a second solution of Component F dialyzed against 0.5 M NaCl (and subsequently adjusted to the same protein concentration as the H_2O solution) were mixed with each other in varying proportions (at 25°) to provide the NaCl molarities indicated. The solutions were slowly cooled to 1° and centrifuged at 425 × g ($F_{av.}$) for 20 h at this temperature. The amounts of protein remaining in solution were determined from the absorbancy of the supernatants.

of Component S is accounted for on a dry-weight basis. This is probably due to the presence of sodium in association with the phosphoprotein (see TABORSKY AND ALLENDE[23]). Component S is a highly unusual protein, about 45 % of its amino acids being represented by serine, and the low values for cystine, methionine, phenylalanine, and tryptophan indicate that these amino acids are most likely completely absent. Obviously, the protein is not completely pure and its major contaminant is probably Component F. The phosphate present is more than sufficient to account for a complete monoesterification of the serine residues, so that other types of phosphate–protein associations may occur.

The important aspect of Table III is the comparison of the amino acid ratios of Components S and F with two known proteins isolated from hen's egg yolk, *i.e.*, phosvitin and lipovitellin. The values reported for Component F and lipovitellin are in good agreement, with the exception of arginine and possibly proline. A comparison of Component S with phosvitin also indicates that these two proteins are analogous, although the agreement is perhaps not as striking. In particular, phenylalanine is present in phosvitin and is apparently lacking in Component S, whereas Component S contains several amino acids (glutamic acid, glycine, arginine) in much greater proportion than phosvitin. Recent chemical measurements on phosvitin have indicated nitrogen values of 11.9–12.9 % (see refs. 16, 25 and 26). The higher value reported here for Component S (15.2 %) may thus be partly due to the much higher arginine value, although to what extent ammonium ions (from ammonium sulfate) may

contaminate the preparation has not been established. Sulfate contamination is negligible, according to the sulfur determinations.

Thus, from the amino acid analyses and from a comparison of the physical and chemical properties of Components S and F with those of phosvitin and lipovitellin[27], it may be concluded that they are analogous proteins*. Components S and F will therefore be referred to as amphibian phosvitin and lipovitellin respectively. Since avian phosvitin and lipovitellin are found in the "granule" fraction of hen's egg

TABLE III

AMINO ACID COMPOSITION OF COMPONENTS S AND F

Constituent	Component S			Phosvitin*	Component F			Vitellin**
	g residue × 10^{-2} per mole	Residues per mole	Residues per aspartic	Residues per aspartic	g residue × 10^{-2} per mole	Residues per mole	Residues per aspartic	Residues per aspartic
Aspartic acid	14.5	12.6	1.00	1.00	307	267	1.00	1.00
Threonine	4.2	4.2	0.33	0.33	145	144	0.54	0.56
Serine	81.5	93.7	7.43	10.33	222	257	0.96	0.83
Glutamic acid	25.4	19.7	1.56	0.89	423	329	1.23	1.32
Proline	3.5	3.6	0.29	0.25	145	149	0.56	0.68
Glycine	6.7	11.8	0.94	0.44	90	158	0.59	0.60
Alanine	3.1	4.8	0.38	0.56	178	251	0.94	0.98
Cystine/2	0.4	0.4	0.03	0.00	30	29	0.11	0.20
Valine	1.3	1.3	0.23	0.17	229	231	0.87	0.92
Methionine	0.3	0.2	0.02	0.00	105	80	0.30	0.31
Isoleucine	2.0	1.8	0.14	0.11	250	221	0.83	0.74
Leucine	2.3	2.0	0.16	0.19	304	269	1.01	1.13
Tyrosine	4.8	2.9	0.23	0.19	139	85	0.32	0.38
Phenylalanine	0.6	0.4	0.03	0.19	155	105	0.39	0.41
Tryptophan	0.4	0.2	0.02	0.08	63	34	0.13	0.20
Lysine	20.0	15.6	1.24	1.25	283	221	0.83	0.80
Histidine	14.5	10.7	0.85	0.83	125	91	0.34	0.27
Arginine	33.4	12.4	1.70	0.86	231	148	0.55	0.78
NH_3	6.0	37.5	2.98		60	375	1.45	
Total***								
residues	(68.5%)§	206.9			(81.5%)	3069		
H_2PO_3	(22.0%)	114.1			(1.2%)	62		
lipid	(0.0%)				(17.5%)			
carbohydrate	(0.5%)				(0.0%)			
Total recovered	(91.0%)				(100.2%)			
Nitrogen								
residues	(15.1%)	346.2			(14.3%)	4291		
lipid	(0.0%)				(0.3%)	80		
total	(15.1%)	346.2			(14.6%)	4371		

* Values calculated from the data of TABORSKY AND ALLENDE[23].

** Average values for $\alpha + \beta$-vitellin (lipid-free $\alpha + \beta$-lipovitellin) from COOK *et al.*[24].

*** NH_3 values not included in total tabulation, assuming that they replace OH in the protein.

§ Values in parentheses are per cent of the gram molecular weight.

* SCHJEIDE AND URIST[28] have indicated that their "X_2" component isolated from the granule fraction of chicken yolk is a lipoglycoprotein containing a polysaccharide, and SUGANO[29] has found carbohydrate present in his "α-lipovitellin". These proteins are apparently analogous to the combined lipovitellins described by COOK and his coworkers[30]. Component F (lipovitellin) of the amphibian yolk platelet does not contain a polysaccharide, however, and, as indicated in Table I, if there is any protein-bound carbohydrate in the platelet, it is more likely to be associated with Component S (phosvitin).

yolk[26,28], it would seem that these granules are homologous with the crystalline main-body component of the amphibian yolk platelet. According to COLVIN AND COOK[31] the granules correspond to what has been described by BELLAIRS[32] in an electron-microscopic study of hen's egg yolk as "lipid drops" (see her Fig. 9). At present, it is interesting to note that, although these granules are comprised of small dense particles, they appear to have lost their crystalline nature.

The lipovitellin of hen's egg yolk can be electrophoretically and chromatographically resolved into two components, designated as α- and β-lipovitellin[33,34]. Little difference has been found in the amino acid composition of these two proteins[24]; but the protein phosphorus contents are 0.50 and 0.27 % respectively[26], and higher pH values are required for the dissociation of α-lipovitellin into subunits[21]. It has yet to be established whether amphibian lipovitellin also resolves into two components, although the preliminary evidence is negative. The protein phosphorus content of amphibian lipovitellin (0.45 %) and its existence as a single component in the ultracentrifuge at pH 8.0 would indicate that it corresponds to α-lipovitellin, although freedom from contaminating phosvitin and its chromatographic behavior on a hydroxyapatite column must be established before this conclusion is warranted.

A model for the crystalline structure of the main body

It is apparent that the crystalline main body of the amphibian yolk platelet is comprised of not one, but at least two proteins herein designated as amphibian phosvitin and lipovitellin. As a first consideration of the crystalline structure, it is necessary to determine the molecular ratio of these two proteins within the main body (Y*i*). One of the analytical results used for such a determination is provided in Fig. 3, which represents a $MgSO_4$ precipitation curve for Y*i* dissolved in (originally) 0.25 M

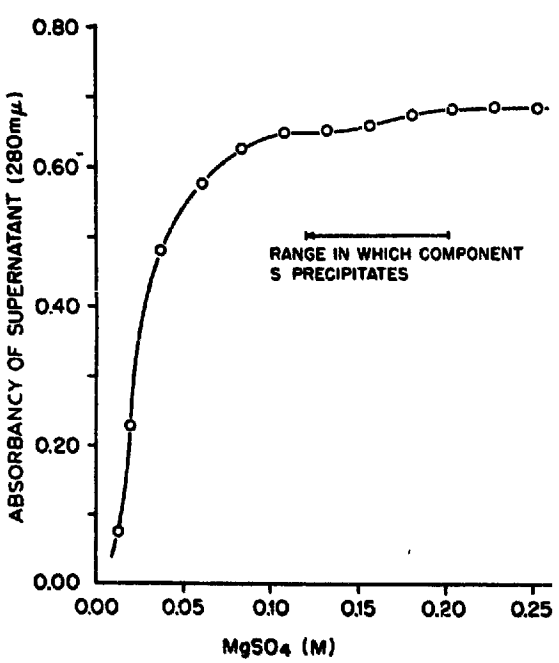

Fig. 3. Solubility of Y*i* as a function of $MgSO_4$ concentration. 500 μl of an approx. 2 % solution of Y*i* in 0.25 M $MgSO_4$ was quantitatively added to 10.0-ml solutions of $MgSO_4$ at 20° to provide the molarities indicated. After thorough mixing the solutions were placed in a cold room for 30 min, centrifuged at 425 × *g* ($F_{av.}$) overnight (16 h at 2–3°), and the absorbancy of the resulting supernatants was read at 280 mμ.

$MgSO_4$. As the concentration is lowered to 0.125 M $MgSO_4$, phosvitin precipitates and lipovitellin remains in solution, a phenomenon originally described by JOUBERT AND COOK[16]. Lipovitellin can be precipitated by a further dilution. This method cannot be used to isolate amphibian phosvitin and lipovitellin for reasons previously indicated[4], but the curve can give some indication of the relative amounts of the two proteins*. The molecular ratio of phosvitin and lipovitellin computed from such a curve and from other analytical determinations in which there is a significant difference between the two proteins is provided in Table IV. From this table, it may be tentatively concluded that one lipovitellin molecule is associated with two phosvitin molecules in the crystalline structure of the main body. These three molecules thus make up what shall be defined as the structural unit of the main-body crystal.

TABLE IV

THE RELATIVE AMOUNTS OF PHOSVITIN AND LIPOVITELLIN IN *Yi*

Method of analysis	*Percentage by weight*		*moles phosvitin per moles lipovitellin*
	Phosvitin	*Lipovitellin*	
Lipid determination*	14.3	85.7	2.2
Phosphorus determination*	12.0	88.0	1.8
Serine determination*	11.4	88.6	1.7
Ultraviolet absorption ($MgSO_4$ curve)**	15.1	84.9	2.3
Schlieren refraction (ultracentrifuge)***	10.6	89.4	1.6

* The weight percentage of each protein was derived from simultaneous equations, using the analytical values for phosvitin and lipovitellin reported here and for *Yi* reported previously[4].

** The percentage of the total absorbancy contributed by each protein was divided by its $E^{1\%}_{1\,cm}$ value.

*** The relative areas under the curves for phosvitin and lipovitellin were computed by weighing the tracings from runs on a variety of protein concentrations. Approximate corrections for the Johnston–Ogston effect were made by an extrapolation of the values for the relative areas to infinite dilution[9]. The corrected values for the relative areas were then divided by the refractive-index constant of each protein as determined with a synthetic boundary cell.

Electron micrographic studies[1,2] of yolk platelets in oocytes and embryos of *Rana pipiens* have indicated that the main body is composed of electron-dense particles, sometimes arranged in a regular hexagonal array. The diameter of the particles has been estimated to be 35–40 Å by WARD[1] and 45 ± 5 Å by KARASAKI[2]. A second consideration thus involves the relation of these particles to phosvitin or lipovitellin. WARD[1] has demonstrated that the particles are "naturally" electron dense, *i.e.*, they can be visualized with the electron microscope in formalin-fixed and unstained preparations, even when uranium is not included in the embedding medium[35]. He has also obtained negative evidence that iron may be responsible for the electron density. KARASAKI[2] has further noticed that the density was considerably increased by uranium staining, a procedure which preferentially stains such high phosphorus-containing substances as nucleic acids for electron microscopy[36]. These observations seem to implicate phosvitin as the observed electron-dense particle. Its spherical diameter has been calculated to be 40 Å, and its density is rather high (Table II). *In situ*, phosvitin may be associated with considerable amounts of cal-

* The relative amounts indicated in Fig. 3 for the phosvitin and lipovitellin are approximately the same as those indicated by the ammonium sulfate curve previously published[4].

cium[28,37], which would increase its density even further. Also, the phosphorus content of phosvitin (Table I) is similar to that found in nucleic acids, so that the phosvitin molecule is the most likely substance present in the main body which would bind added uranium. Thus it is concluded that individual phosvitin molecules represent those electron-dense particles which are sometimes observed by electron microscopy to be arranged in a regular hexagonal array within the main-body crystal.

More frequently, however, individual particles were not observed by electron microscopy, but rather a system of alternating light and dark bands with an average spacing between dark bands of 71 Å. The appearance of particles or bands seemed to depend entirely upon the angle of sectioning and observation, and if a 200–500-Å-thick section exhibiting a hexagonal dot pattern was slightly tilted in the microscope, a band pattern was subsequently observed[2]. Considering the frequency of the observed types of band patterns and the frequency of the appearance of a regular hexagonal packing of elements, KARASAKI[2] deduced that the dense particles could only be organized into either a simple hexagonal or a face-centered cubic lattice. The latter

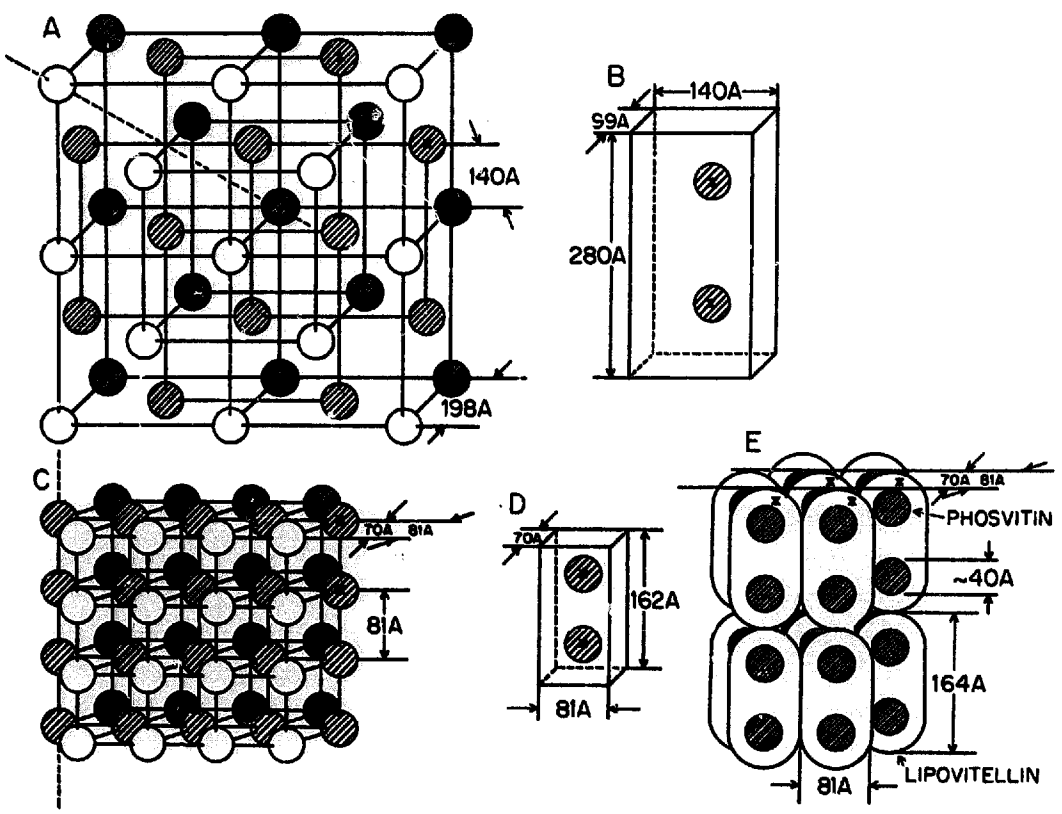

Fig. 4. Models for the possible arrang nents of yolk proteins within the main-body crystal, all drawn to the same scale. A, an arrangement of phosvitin molecules in a face-centered cubic lattice; the more darkly shaded molecules are in the background; the solid lines connect molecules which are 198 Å apart; when viewed from the orientation indicated by the dashed line, a regular hexagonal array of particles would appear to have an 81-Å center-to-center distance from one another; B, a structural unit from A (containing the two phosvitin molecules indicated by an asterisk and an unindicated lipovitellin molecule); C, an arrangement of phosvitin molecules in a simple hexagonal lattice; the solid lines connect molecules which are 81 Å apart; when viewed from the orientation indicated by the dashed line, a hexagonal array of particles similar to the case in A would be observed; D, a structural unit from C; E, proposed model for the arrangement of structural units in the main-body crystal of the amphibian yolk platelet; a rhombohedral unit cell would be composed of four structural units (indicated by an asterisk); the phosvitin molecules are arranged in a simple hexagonal lattice.

configuration was considered unlikely since it is isotropic and thus would not account for the birefringence and lamellar breakdown patterns frequently observed for yolk platelets. However, the structural unit for the main body has been here defined as two phosvitin molecules (or dense particles) in association with one lipovitellin molecule. From this consideration it can be demonstrated that a main body with a face-centered cubic packing of phosvitin molecules could nevertheless display a slight birefringence promoted by the lamellar arrangement of lipovitellin molecules. Thus, the face-centered cubic configuration of phosvitin molecules has not been ruled out, but this model may be eliminated by other considerations.

Figs. 4A and 4C represent models for a face-centered cubic packing and simple hexagonal packing of phosvitin molecules respectively. They are drawn to the same scale and both would provide a hexagonal array of dense particles with a center-to-center distance of 81 Å when observed from the appropriate orientation. A structural unit (or the space associated with two phosvitin molecules) from the face-centered cubic model (Fig. 4B) would have a volume of $3881 \cdot 10^{-21}$ ml, whereas a structural unit from the simple hexagonal model (Fig. 4D) would have a volume of $919 \cdot 10^{-21}$ ml. The volume of one lipovitellin and two phosvitin molecules is $602 \cdot 10^{-21}$ ml, so that the face-centered cubic model may be ruled out as providing a structural unit which is clearly too large.

For these reasons, the simple hexagonal lattice is presently adopted as a model for the arrangement of phosvitin molecules within the main body*. A structural unit provided by such a model, however, has only 66% of its space accounted for by the measured volumes of the component proteins. Part of the remaining 34% may be attributed to water. HONJIN AND NAKAMURA[39] have recently published an abstract concerning some X-ray diffraction measurements they have made on amphibian yolk-platelet homogenates. They found a primary spacing of 164 Å in fresh or formalin-fixed material (and in good agreement with a unit cell comprising the structural units proposed here). Furthermore, they noticed that the periodicity diminished to 151 Å in frozen-dried material. This would indicate a volume reduction through loss of water of 22% and seems to be in reasonable agreement with the calculated value (30% by weight) for the water content of whole yolk platelets[4]. A more accurate indication of the water content of *Yi* is needed, however, before the exact amount of water in each structural unit can be specified. Finally, SCHJEIDE AND URIST[28,37] have indicated that the granule fraction of hen's egg yolk also contains considerable amounts of calcium bound to phosvitin as a normal constituent. Some of their more recent results[40] would indicate that the calcium content of amphibian yolk is much lower, but even if calcium is similarly a normal constituent of the amphibian main body, it would probably become lost during the isolation procedures employed here for amphibian phosvitin and lipovitellin[37]. A careful determination of the calcium content of the intact main body will thus also have to be performed before a further elaboration of the structural unit can be made.

In collaboration with S. KARASAKI and in consideration of the available data

* Other highly suggestive evidence has been presented by WARD[1,38], who describes the morphology of the small yolk bodies in the young oocyte of *Rana pipiens* as octahedral plates appearing within the mitochondria. During maturation the plate-like bodies grow in size while retaining their regular hexagonal outline, thus possibly indicating a deposition of yolk protein along the planes of a simple hexagonal lattice.

from electron microscopy, biochemical studies, and X-ray diffraction, a tentative model for the crystalline structure of the main body is thus proposed in Fig. 4E. The features of the model which are presented here with some degree of certainty concern the simple hexagonal packing of phosvitin molecules and the 2:1 ratio of phosvitin to lipovitellin. The positioning of the lipovitellin molecules within the hexagonal system of phosvitin molecules, however, was based upon an entirely arbitrary decision. A unit cell provided by the molecule as drawn would consist of four structural units (marked with an asterisk) arranged in the form of a rhombohedron. It is hoped that such a model will serve to visualize the molecules comprising the main-body crystal and to indicate those relationships which require a more exact specification. In particular, the juxtaposition of the lipovitellin molecules and the manner of their association with the phosvitin molecules need to be examined further.

ACKNOWLEDGEMENTS

I wish to thank Dr. N. G. ANDERSON for his encouragement and for making available the facilities of his laboratory during the course of this work. I am also grateful to Miss E. LYBARGER for technical assistance with the amino acid analyzer, to Drs. R. R. BECKER, S. KARASAKI and L. M. KRAUSZ for many helpful discussions, and to Drs. W. H. COOK and R. B. SETLOW for a critical reading of the manuscript.

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