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STUDIES ON AMPHIBIAN YOLK

III. A RESOLUTION OF YOLK PLATELET COMPONENTS

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SUMMARY

Intact isolated yolk platelets have been analyzed for nucleic acids, lipid, hot trichloroacetic acid-extractable carbohydrate and water-extractable constituents. It is concluded that yolk platelets *in situ* contain no RNA, but the amount of DNA which they contain is still uncertain. Also, no evidence thus far has been obtained for a readily extractable polysaccharide. When dissolved in strong salt solutions under non-oxidative conditions, the main part of the yolk platelet (water-insoluble) resolves as two components in the ultracentrifuge. A method for separating these two components in quantitative yield is indicated.

INTRODUCTION

Recently we isolated yolk platelets from ovulated eggs of *Rana pipiens* in a state that resembles their condition *in vitro*, *i.e.*, with the superficial layer still present around the main body[1]. Electron microscopy of numerous platelet preparations showed no contamination from nuclear elements and a minimum contamination from cytoplasmic elements. This paper (a) presents results of tests on these preparations for several of the components ascribed to yolk platelets by various authors, (b) describes a method for resolving the components of these preparations, and (c) presents some analytical data on these components.

METHODS AND MATERIAL

Isolation of yolk fractions (F-1)

Yolk platelets from ovulated eggs of *Rana pipiens* were isolated by using a sucrose–PVP medium[1]. After draining, the wet pellets were rehomogenized in water (10 ml per pellet) and dialyzed against two changes of water (12 l each change) over a period of 24–48 h to remove the sucrose. To resolve the components further, the dialyzed suspension (WY + PVP) was centrifuged for 10 min at 1700 × *g* ($F_{av.}$) and the

Abbreviations: BSA, bovine serum albumin; PVP, polyvinylpyrrolidinone; WY, whole yolk.

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

slightly opalescent supernatant decanted from the well-packed pellets. The pellets were then gently rehomogenized in water and the suspension centrifuged again. The final pellet constituted the *Yi* fraction, whereas the supernatants were combined to provide the *Ys* + PVP fraction. The entire procedure was carried out in the cold (1–5°). All water was glass-distilled. The dialysis tubing used was size 18/32 (Visking Nojax Casings) which does not pass insulin or ribonuclease[2,3] and through which PVP (Matheson Company) was 98 % non-dialyzable as determined by dry weight recoveries.

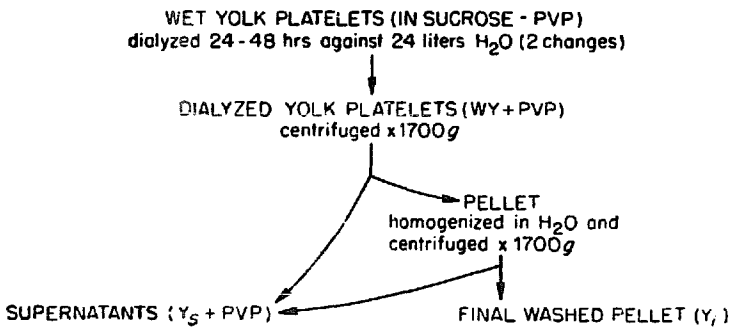


Fig. 1. Schematic diagram of the procedure used to isolate the various yolk fractions described in the text.

Analytical procedures

Dry weights of all samples were determined by incubating the samples in a vacuum oven at 100° until a constant weight was obtained. Solutions were slowly evaporated under an infrared lamp before dry-weight determinations were made.

Lipid was extracted by the procedure of FOLCH *et al.*[4]. Phospholipid content was alternately determined by multiplying the P content by 25 (see ref. 5) or by gravimetric recovery after silicic acid chromatography[6]. Nucleic acid was determined on lipid-extracted WY + PVP by the procedures of WEBB[7] and WEBB AND LEVY[8] for RNA and DNA respectively. Values relating to whole yolk (WY) were derived by correcting for the presence of PVP. Carbohydrate was determined as glucose equivalents using the anthrone procedure of SCOTT AND MELVIN[9]. Elemental analyses were performed by the Galbraith Laboratories Inc., Knoxville.

Amino acids were determined by the method of SPACKMAN *et al.*[10], employing a Spinco Model-120 Analyzer. Thoroughly N_2-flushed and evacuated samples were hydrolyzed for 48 h in constant-boiling HCl at 110°. Results were corrected for hydrolytic destruction from the data of MAHOWALD *et al.*[11] on myokinase and BECKER[3] on ribonuclease and insulin.

Sedimentation analyses were performed at 20° with a Spinco Model-E Analytical Ultracentrifuge, using conventional 3- or 12-mm cells and a 12-mm wedge cell.

RESULTS

When yolk platelets were isolated and allowed to drain for 5 min as described previously[1], a 440 ± 30-mg "wet pellet" was obtained from 250 ± 20 eggs. An analysis of the components of this wet pellet and the fractions which were derived from it are given in Tables I, II and III. The wet pellet consists of whole yolk platelets in a sucrose–PVP medium; the sucrose and PVP may be separately removed by a

TABLE I

COMPONENTS OF THE ORIGINAL "WET PELLET" PREPARATION

Component	*Percentage of "wet pellet" weight*	*Average*
Dry weight	46, 47, 47, 49, 50	48
Dialyzed dry weight (WY + PVP)	28, 29, 31, 32	30
Sucrose	18, 18, 19	18
PVP*	2.7, 2.7, 2.9	3
Medium H_2O*	39, 39, 42	40
Platelet solids (WY)	27, 28, 29	28

* Based on sucrose analysis and a determination that the suspension medium contains sucrose, PVP and H_2O in the weight ratios of 30.3, 4.5 and 65.2, respectively.

TABLE II

COMPONENTS OF DIALYZED YOLK PLATELETS (WY + PVP)
All values are expressed on a dry weight basis.

Component	*Per cent of WY + PVP*	*Elemental analysis of components*		
		% N	*% P*	*P/N*
Dialyzed platelets (WY + PVP)	100	13.0	1.6	0.12
Dialyzed platelets after H_2O extraction (Yi)	81–84	14.1	1.8	0.13
Dialyzed platelets after lipid extraction	87	14.7	1.7	0.12
H_2O extract (Ys + PVP)	16–19	8.6	<0.1	—
Lipid extract	12.5	1.8	2.6	0.67*
PVP	9–10	12.6	<0.1	—
Whole yolk (WY, calculated)	90–91	13.1	1.8	0.14

* Atomic ratio.

dialysis and a water extraction, respectively. In addition, this treatment causes the superficial layer of the yolk platelet to disappear[1], so that the soluble components within the dialysis bag (Ys + PVP) assume a special importance.

An alternate scheme whereby the intact yolk platelets in sucrose–PVP medium are first extracted (rather than dialyzed) several times with water also causes the superficial layer to disappear. Thus it is conceivable that a dialysis of the water extract could result in a similar soluble fraction (Ys + PVP). The analyses reported in this paper, however, were all performed on fractions obtained by the procedure outlined in Fig. 1.

Yolk platelets after isolation in sucrose–PVP

Table I is an analysis of the wet pellet weight, 52 % of which is water. After the intact yolk platelets are isolated, the sucrose is removed by dialysis and the loss in dry weight after dialysis seems to be entirely caused by the loss of this sucrose. After dialysis the contents of the dialysis tubing is designated as WY + PVP, the PVP content of which is almost 10 % on a dry weight basis. Whole yolk in the theoretical absence of PVP is designated as WY and is about 28 % of the "wet pellet" weight.

The data in Table I can also be used to give an indication of the water content

TABLE III

AMINO ACID COMPOSITION OF YOLK FRACTIONS

All values are expressed as g residue/100 g total residue. Not included in the "total" tabulations are the values for NH_3 and tryptophan.

Component	*WY + PVP**	*Yi**	*Ys + PVP***
Lysine	8.8	9.1	8.6
Histidine	4.2	4.3	4.0
NH_3	1.8	1.8	2.7
Arginine	7.4	7.7	5.7
Aspartic acid	9.3	9.0	13.0
Threonine	4.3	4.3	5.7
Serine	9.2	9.1	9.1
Glutamic acid	12.6	12.4	14.0
Proline	3.5	3.6	3.6
Glycine	2.6	2.7	6.0
Alanine	4.5	4.6	3.2
Cystine/2	1.2	0.8	2.5
Valine	6.0	6.0	5.2
Methionine	3.0	2.9	2.1
Isoleucine	7.0	6.9	4.9
Leucine	8.0	8.2	4.2
Tyrosine	4.0	4.1	4.0
Phenylalanine	4.4	4.3	4.0
Total g residue per 100 g sample	69.5	83.1	4.7

* Values reported are ± 5%.
** Values reported are ± 10%.

of intact yolk platelets after isolation. Since 12% of the "wet pellet" weight is water not derived from the suspension medium, the intact platelet may be shown to contain only 30% water.

Yolk platelets after dialysis (WY + PVP)

From dialyzed yolk platelets (WY + PVP), two fractions can be obtained by a water extraction. These are designated (a) Ys + PVP, a soluble fraction which contains PVP and possibly the components of the superficial layer, and (b) *Yi*, which represents 81–84% of the dry weight of WY + PVP (Table II). An electron microscopic analysis of the latter fraction[1] has shown that it corresponds to the main body component of yolk platelets and hence is analogous to platelet fractions obtained and analyzed by other workers[12–16].

The components of intact yolk platelets (WY)

Dialyzed and lyophilized yolk platelets (WY + PVP) were analyzed for protein (by an amino acid analysis, see Table III), carbohydrate (extractable with hot 5% trichloroacetic acid), nucleic acids, and lipid. The results were corrected for the PVP and residual water present and are reported as components of whole yolk platelets (WY) in Table IV. Since the amount of RNA and DNA measured was very small relative to the amount of material extracted, a control determination was made on crystalline BSA to estimate non-specific color. The percentage RNA and DNA in BSA was 0.04 and 0.03 respectively. If these values are accepted as a true control, then WY can be estimated to contain 0.06% RNA and 0.08% DNA.

Further analysis of the water-soluble fraction (Ys + PVP)

Table II shows that there is about 55 % PVP by weight in the Ys + PVP fraction. If Ys + PVP is lyophilized, dissolved in 1.0 M NaCl and centrifuged in the analytical ultracentrifuge, a single component can be seen which sediments at a slower rate than the components present in Yi (Figs. 2 and 3). A comparison with PVP dissolved in 1.0 M NaCl (Fig. 4) suggests that the sedimenting component in Ys + PVP is primarily PVP. Based on the degree of refraction indicated by the schlieren patterns and assuming that the sedimenting component in Ys + PVP is entirely PVP, it can be calculated that Ys + PVP contains as much as 65 % PVP by weight.

TABLE IV

CHEMICAL COMPONENTS OF INTACT YOLK PLATELETS (WY)

Component	*Per cent of dry weight*
Protein	78*
Carbohydrate (extractable)	2.0
Lipid	14
RNA	0.10**
DNA	0.11**

* This value is based on amino acid analysis and hence does not include phosphate bound to the protein. A preliminary analysis indicates that this "alkali-labile" phosphate comprises at least 4 % of WY dry weight.

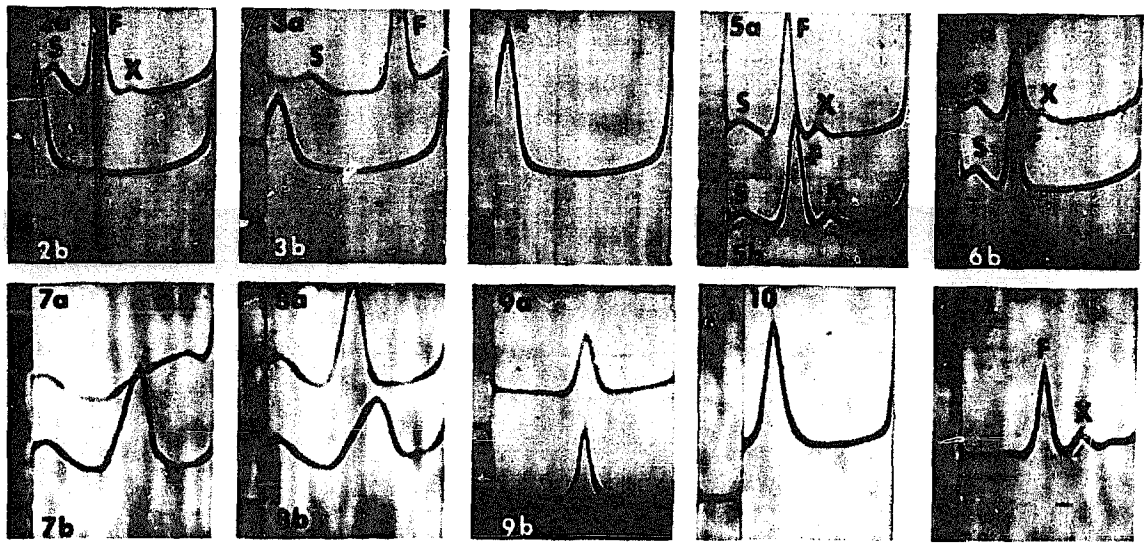
** Uncorrected values. See text.

About 5 % of the Ys + PVP dry weight is protein (Table III) and about 11 % is carbohydrate because the hot 5 % trichloroacetic acid-extractable sugar reported for WY (Table IV) can also be quantitatively (99 % recovery) extracted by water. A Pflüger test[17] on WY + PVP for glycogen was negative. In addition, 80 % methyl alcohol quantitatively (96 % recovery) extracts the carbohydrate from WY + PVP. This indicates that the sugar extracted into the Ys + PVP fraction has a low molecular weight[18]. Finally, if a lyophilized preparation of Ys + PVP is placed on a Sephadex G-50 column (180 × 1 cm, eluant = 0.25 M NaCl, $t = 25°$), all the carbohydrate comes off the column after, and distinctly separate from, the PVP and protein. Sucrose, placed on the same column under the same conditions, comes off the column in exactly the same position as the Ys + PVP carbohydrate.

Further analysis of the water-insoluble fraction (Yi)

Fraction Yi contains essentially all of the lipid and phosphorus of intact yolk platelets (WY) and about 98 % of the protein. The lipid content of Yi is thus about 15 %. Based on P content (Table II), the lipid is 65 % phospholipid, whereas chromatography on silicic acid indicates that the phospholipid content is 74 %. The latter value, however, includes a highly xanthophyllic carotenoid pigment which comes off the column in the phospholipid fraction.

When Yi was lyophilized, subsequently dissolved in 1.0 M NaCl and centrifuged in the analytical ultracentrifuge, three components were generally seen (Figs. 2a and 3a), here designated as S, F and X. When Yi was first dissolved in a strong salt



Figs. 2–11. Sedimentation patterns of yolk platelet components. The technical details for each figure are provided in the chart below. For further information, see text.

Figure	*Solute*	*Per cent of solute concentration*	*Solvent*	*Minutes after reaching speed*	*Rev./min*	*Bar angle*
2a	*Yi*	1.3	1.0 M NaCl	32	59 780	57.5°
2b	*Ys* + PVP	1.1	1.0 M NaCl	32	59 780	57.5°
3a	*Yi*	1.3	1.0 M NaCl	66	59 780	57.5°
3b	*Ys* + PVP	1.1	1.0 M NaCl	66	59 780	57.5°
4	PVP	1.2	1.0 M NaCl	69	59 780	57.5°
5a	*Yi*, reprecipitated	1.0	0.8 M NaCl	37	52 640	55°
5b	*Yi*, reprecipitated	0.9	0.6 M NaCl	37	52 640	55°
6a	*Yi*, fresh	0.7	1.0 M NaCl–0.15% H_2O_2	26	59 780	60°
6b	*Yi*, fresh	0.7	1.0 M NaCl	26	59 780	60°
7a	WY + PVP	1.2	0.40 M NaCl	46	52 640	60°
7b	WY + PVP	1.2	0.50 MNaCl	46	52 640	60°
8a	WY + PVP	1.2	0.55 M NaCl	43	52 640	60°
8b	WY + PVP	1.2	0.45 M NaCl	43	52 640	60°
9a	F	0.5	0.5 M NaCl, pH 8.0	51	52 640	60°
9b	F	1.7	0.5 M NaCl, pH 8.0	51	52 640	60°
10	S	1.0	0.2 M Tris, pH 8.8	69	59 780	55°
11	F + X	0.9	0.6 M NaCl	45	52 640	55°

solution, reprecipitated by dialysis against, or dilution with, water, then lyophilized and analyzed as above, the amount of X component was frequently larger (Fig. 5) than when *Yi* was simply lyophilized. It was subsequently noticed that the relative amount of X component was highly variable and seemed to depend upon the amount of handling and storage to which *Yi* was subjected. When freshly prepared *Yi* was dissolved into N_2-flushed 1.0 M NaCl without lengthy standing or preliminary lyophilization, the X component was absent (Fig. 6b). Finally, the addition of a small amount of H_2O_2 to the above "X-free" solution resulted in the appearance of the X component in addition to other heavy components (Fig. 6a). Thus it would seem that the presence of X is entirely due to an oxidation phenomenon, presumably involving the F component since the amount of F decreases in proportion to the amount of X present. Attempts to eliminate the X component by the addition of

reducing agents were unsuccessful, so that the oxidation appears irreversible and probably involves the lipid moiety of F (see ref. 19).

Yi is essentially insoluble in low salt concentrations and does not dissolve appreciably in NaCl solutions less than 0.35 M. The ultracentrifugal patterns of fresh *Yi* in 0.40 M to 0.55 M NaCl is given in Figs. 7 and 8. A reduction of heterogeneous heavy components into components S and F as the salt concentration is increased can clearly been seen. In 1.0 M NaCl, the s^0_{20} values for S and F are 2.7 and 9.1 respectively (Fig. 12). The heavy X component, if present at this salt concentration, has an s^0_{20} value of approx. 14, which may indicate that it is a dimer of component F.

Separation of components S and F

Since components S and F seemed to be somewhat analogous to the phosvitin and lipovitellin isolated from chicken yolk by a $MgSO_4$ fractionation[20], a similar procedure was first used to separate S and F. When a 0.25 M $MgSO_4$ solution containing dissolved *Yi* was gradually diluted to approx. 0.13 M, a precipitate was obtained which contained primarily S, so that a good separation of components seemed to be achieved. The major difficulty with this procedure, however, concerned the relatively large amounts of permanently insoluble material recovered from the F fraction after dialysis into solutions other than $MgSO_4$.

FUJII[21] apparently has been able to separate components F and X from S by a NaCl fractionation procedure, but gives no indication of the relative recovery of his fractions. Attempts by the author to program a similar NaCl fractionation procedure were considered unsatisfactory because of the narrow salt-concentration range required, the large amounts of material which were discarded relative to the recovery of F, and an inability to obtain S in a pure form.

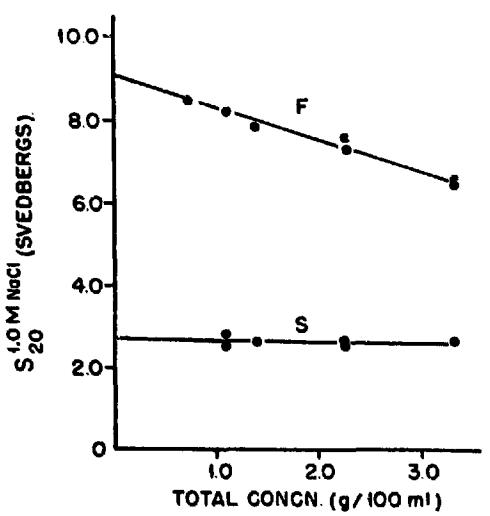


Fig. 12. Sedimentation–concentration diagram for the two sedimenting components of *Yi* in 1.0 M NaCl.

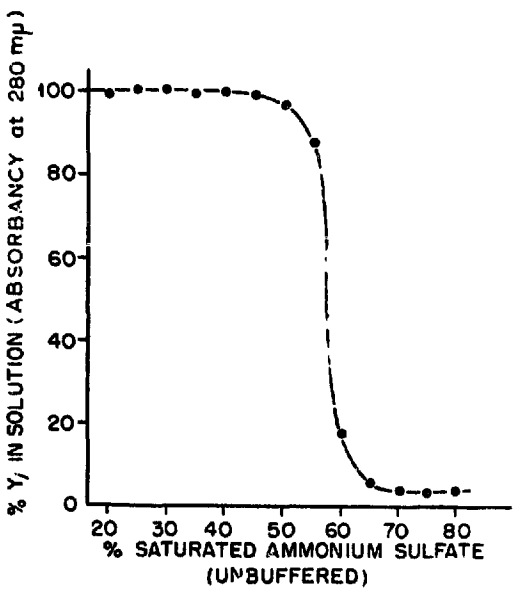


Fig. 13. Ammonium sulfate precipitation curve for *Yi*. Equal aliquots of dissolved *Yi* (in 20% saturated ammonium sulfate) were thoroughly mixed with solutions of ammonium sulfate to provide the concentrations indicated. The mixtures were allowed to stand several hours and then centrifuged at 425 × *g* ($F_{av.}$) for 36 h. The absorbancy of the resulting supernatants was read at 280 mμ. Temp., 1–2°.

In a third attempt to separate S from F, a solubility curve of *Yi* in unbuffered ammonium sulfate was plotted (Fig. 13) and seemed to indicate that practically all of *Yi* was precipitated by 65% saturation. In buffered ammonium sulfate (to pH 7 with NH_4OH), the same degree of precipitation was achieved with a 60% saturated solution. However, ultracentrifugal analysis of the washed and reprecipitated protein indicated that it was entirely composed of F (Fig. 9), and that S could be recovered from the 65% saturated ammonium sulfate supernatant (Fig. 10) so that component S apparently has a weak absorbancy at 280 mμ. Therefore it was concluded that an ammonium sulfate precipitation provides the quickest and simplest way of obtaining apparently pure fractions of S and F in almost quantitative yield.

When using *Yi* samples which contained various amounts of X component, an ammonium sulfate fractionation failed to separate F from X, the two always coprecipitating (Fig. 11). Attempts to separate these two components by CM-cellulose chromatography have also been unsuccessful. This seems to indicate, then, that components F and X are chemically quite similar except for molecular size. To obtain component F completely free from X, it was necessary to use fresh *Yi*, N_2-saturated solutions and ammonium sulfate which has been recrystallized in the presence of EDTA.

DISCUSSION

The availability of intact and relatively uncontaminated yolk platelets[1] makes possible a more accurate investigation of the biochemistry of these structures and their role in embryological processes. A recurrent theme in many papers concerned with the amphibian yolk platelet is that it may act as a reservoir of nucleic acid or other specific substances which assume a prominent role during various differentiative processes[22-28].

The data reported here help provide a preliminary evaluation of such considerations, the first of which concerns nucleic acid content. Analysis of whole yolk (WY) for RNA and DNA reveals that nucleic acids may be present in amounts as low as 0.06% and 0.08% respectively. A comparison of these results with those of other workers[16,23,29] must include a consideration of possible contamination of the yolk fraction by non-yolk material. Nuclear material derived from follicle cells would certainly contaminate yolk platelets obtained by a low-speed centrifugation of ovarian homogenates, and nuclei have been observed in the preparations of PANIJEL and RINGLE AND GROSS[1]. Furthermore, when yolk platelets are isolated in simple salt or sucrose solutions, the superficial layer is lost and the residual main body frequently becomes invested by denatured material[1,15]. That this material (or "ghost" material[15]) may trap or actually constitute extraneous cytoplasmic material is indicated by the studies of NASS[30] and RINGLE AND GROSS[15,16], and the latter authors are careful to mention this fact when interpreting their RNA-P values. NASS[30] has also suggested that the greater phosphoprotein phosphatase activity observed by PANIJEL[12] for small platelets (as opposed to large) is largely a reflection of their greater surface/volume ratio and hence greater degree of contamination. This argument may be extended to include also the greater amount of RNA-P found by PANIJEL[12] in small platelets and the smaller amounts of RNA found by various authors for yolk platelets in general. Therefore it is concluded, in agreement with these histochemical results of OHNO *et al.*[31], that yolk platelets most likely contain no RNA *in situ*. The chemical

resolution of nucleic acids reported here is not sensitive enough to derive a similar conclusion for DNA content, however, and at this time serves only to substantiate the suggestion that yolk platelets may harbor most, if not all, of the cytoplasmic DNA[16,32,33].

Besides nucleic acids, other components which have been associated with yolk platelets include polysaccharide substances[28,31,34,35], histone-like proteins[26] and lysozyme[36]. More specifically, these components have generally been associated with the periphery or superficial layer of yolk platelets. Since a water extraction of yolk platelets seems to effect a disappearance of the superficial layer[1], a careful analysis of the water extract (Ys + PVP) may help to indicate the presence or absence of the various substances mentioned above.

In the preliminary work reported here, it has been found that about 8% of the yolk-platelet dry weight is water-soluble (= Ys) and 92% is insoluble (= Yi). Thus far, Ys has been found to consist of about 12% protein and 25% carbohydrate. This carbohydrate represents all of the hot-trichloroacetic acid-extractable carbohydrate from whole yolk (WY). Evidence has been presented, however, which indicates that the carbohydrate is probably sucrose which was derived from the original isolation medium and which apparently was trapped by the platelets and not removed by extensive dialysis. Although this low-molecular-weight carbohydrate has not been definitely identified, a gel filtration of Ys + PVP and the methanol solubility of the carbohydrate was definitely shown that the hot-trichloroacetic acid-extractable sugar is not a polysaccharide. The possibility still exists, however, that a polysaccharide which is covalently bound to hot-trichloroacetic acid-insoluble material may be present in yolk platelets. A precedent for such a possibility is provided by the demonstration of SCHJEIDE AND URIST[37] that the lipoprotein from the chicken yolk granule fraction contains a polysaccharide.

The Yi fraction in the ultracentrifuge consists of two components designated as S and F. A third heavy component observed by other workers[13,14,21] has now been suggested to be an irreversibly dimerized oxidation product of F. Corrected sedimentation values for 1.0 M NaCl solutions are 2.7 for S and 9.1 for F. These values, when corrected for the suspending medium, agree with the published values of GROSS AND GILBERT[13] and FUJII[21], but not with those of SCHJEIDE *et al.*[14]. Specifically, it is difficult to reconcile the difference between the value reported by SCHJEIDE *et al.* for the slow component ($s = 6$) and the value reported here ($s = 2.7$). The next paper in this series[19], however, will be concerned with a more accurate determination of the physical and chemical properties of components S and F, and will relate them to the electron-microscopic images of the main body of yolk platelets as observed by WARD[38] and KARASAKI[39].

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