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ARTICLE *in* DEVELOPMENTAL BIOLOGY · MARCH 1986

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Characterization of Yolk Platelets Isolated from Developing Embryos of *Arbacia punctulata*

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Received March 21, 1985; accepted in revised form August 8, 1985

Yolk platelets, a major organelle of sea urchin eggs and embryos, were isolated from *Arbacia punctulata* and biochemically characterized over the course of development to the pluteus stage. Fractionation by sucrose gradient centrifugation revealed yolk platelets in two major density classes. The low-density yolk platelet fraction could be obtained as a very homogeneous preparation and was highly enriched in acid phosphatase activity, while depleted of mitochondrial (cytochrome *c* oxidase) and plasma membrane (phosphodiesterase) marker enzymes. The chemical composition of low-density yolk platelets prepared from eggs and embryos at various stages of development remained unchanged in terms of phospholipid, triglyceride, hexose, sialic acid, RNA, and protein. However, analysis of the major yolk platelet glycoproteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed a number of stage-specific changes. These glycoproteins were found to be major glycoproteins of crude embryo lysates and were predominantly of the polymannose, N-linked type. The predominance of polymannose-type glycoproteins in yolk platelets was further demonstrated by their staining with concanavalin A-colloidal gold in Lowicryl-embedded sections of embryos. These studies represent the first systematic biochemical characterization of intact yolk platelets and the changes in them during early embryonic development.

INTRODUCTION

Although yolk platelets are a major organelle of sea urchin eggs and embryos, relatively little is known about their biochemical properties and function. Analysis of the lipoprotein fraction of yolk platelets by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) has revealed that this component contains the majority of protein constituents that stain positively for carbohydrate using the periodic acid-Schiff (PAS) method (Ii *et al.*, 1978). This observation is consistent with earlier histochemical and cytochemical studies that demonstrated that the yolk platelets are the major PAS-staining organelles of sea urchin eggs (Takashima, 1971; Immers, 1960). It has been reported that in several sea urchin species (*Strongylocentrotus purpuratus, Lytechnius pictus*, and *S. droebachiensis*) the yolk platelets contain a predominant glycoprotein (M_r 200,000) that is the major protein constituent of the egg (Harrington and Easton, 1982). In a brief report (Harrington and Easton, 1980), it was noted that this glycoprotein is present during early embryogenesis, but disappears after the late blastula stage. Similar studies concerning the presence of glycoproteins in the yolk platelets of sand dollar eggs have been reported (Ozaki, 1980).

In amphibians the major yolk proteins of eggs provide a reservoir of materials for utilization during embryogenesis (Karasaki, 1963). Because the general characteristics of the yolk platelets of sea urchin embryos are similar to those of other species (Williams, 1967), a similar function has been assumed. An alternative but related function for sea urchin yolk platelets as a site of catabolism is suggested by the presence of several acid hydrolases, including acid phosphatase (Schuel *et al.*, 1975). Such catabolic enzymes could be used to generate amino acids, fatty acids, or carbohydrates from their stored macromolecular forms.

To gain a better understanding of the role of yolk platelets during embryonic development, we developed a simple method for isolating intact yolk platelets from *Arbacia punctulata* eggs and embryos over the course of development to the pluteus stage. This procedure revealed the presence of two major density classes of yolk platelets, the less dense one being nearly homogeneous. Analysis of the low-density yolk platelets isolated throughout development revealed little or no change in the mass of a variety of constituents including protein, lipid, carbohydrate, and nucleic acid. Although the total protein content remained constant, SDS-PAGE profiles of nearly all glycoproteins from intact yolk platelets

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0012-1606/86 $3.00

were altered during development. This suggests that certain dynamic processes occur during development that affect the glycoproteins comprising all yolk platelet components. Characterization of the oligosaccharide chains of the yolk platelet glycoproteins indicates that they constitute the majority of the polymannose-type, N-linked oligosaccharide chains of the embryo.

MATERIALS AND METHODS

Embryo dissociation. Eggs of *A. punctulata* were collected and fertilized, and embryos were cultured at 23°C as previously detailed (Schneider and Lennarz, 1976). Embryo cultures (500–1000 ml of a 1% suspension) were harvested by hand centrifugation at various stages of development. The embryos (5–10 ml packed embryos) were washed in 100 ml of Ca^{2+}-, Mg^{2+}-free seawater containing 25 m*M* EGTA and 2.5 m*M* $NaHCO_3$, pH 8.0 (CMF-SW; Detering *et al.*, 1977). After resuspension in 20 ml CMF-SW, the embryos were incubated on ice for 15 min with occasional pipetting to effect dissociation into single cells. The cells were collected by centrifugation at 1000*g* for 2 min at 4°C and resuspended in 15–20 ml CMF-SW containing the following protease inhibitors (PI): 25 KIU/ml aprotinin, 1 μg/ml leupeptin, 2 μg/ml antipain, 10 μg/ml benzamidine, 1 μg/ml chymostatin, and 1 μg/ml pepstatin (CMF-SW/PI).

Sucrose gradient centrifugation. All steps outlined in this and the following section were performed at 4°C. The cells from dissociated embryos were homogenized in a French pressure cell at 900–1000 psi, diluted to 100 ml with CMF-SW/PI, and centrifuged at 27,000*g* for 25 min. The material in the pellet was then resuspended in 4 ml CMF-SW/PI and layered on top of a 30-ml discontinuous sucrose gradient. The sucrose gradient was composed of 5 ml of 35% (w/v) sucrose, 15 ml of 25% sucrose, and 10 ml of 10% sucrose, osmotically adjusted with CMF-SW/PI. The gradient was centrifuged at 100,000*g* (24,000) rpm in a Beckman SW 28 rotor for 16 hr and fractionated from the bottom of the tube. The fractions containing yolk platelets and other organelles were identified by light scattering (absorbance at 600 nm) using a Guilford spectrophotometer. After pooling the appropriate fractions, they were diluted 10-fold with CMF-SW/PI and centrifuged at 27,000*g* to collect the yolk platelets.

Metrizamide gradient centrifugation. Yolk platelets isolated by sucrose gradient centrifugation and collected at 27,000*g* were resuspended in 1.0 ml CMF-SW/PI containing 33% (w/v) metrizamide. The sample was placed in a 12-ml centrifuge tube and overlayed consecutively with 2 ml 25%, 2 ml 19%, 2 ml 10%, 2 ml 5%, and 1 ml 0% metrizamide in CMF-SW/PI. After centrifugation for 3 hr at 100,000*g* (28,000 rpm in a Beckman SW 41 rotor), the bottom of the centrifuge tube was punctured and fractions were collected. The fractions containing yolk platelets were identified, pooled, and concentrated as described above for sucrose gradient centrifugation.

Biochemical analysis. Total esterified fatty acids were determined by their corresponding hydroxamates as described by Stern and Shapiro (1953) using dipalmitoylphosphatidylcholine as standard. Phospholipid content was estimated by extraction with chloroform:methanol, 2:1 (v/v) as described by Folch *et al.* (1957) and subsequent determination of the phosphorous content of the organic extracts by the Bartlett procedure (1959) using inorganic phosphate as standard. To estimate the triglyceride content of samples, lipids were extracted as described above and the triglyceride fraction was separated from other lipids by preparative thin-layer chromatography on silica gel 60 developed with petroleum ether:diethylether:acetic acid, 80:20:1 (v/v/v). Lipids were eluted from the area of the plate containing triglycerides with chloroform:methanol, 2:1 (v/v) and the fatty acid content was determined following alkaline hydrolysis as described by Novak (1965) using tripalmitin as standard. Neutral hexose content was measured by the phenol-sulfuric acid method (Dubois *et al.*, 1956) using glucose as standard. Sialic acids were estimated by the thiobarbituric acid method (Warren, 1959) using *N*-acetylneuraminic acid as standard. Total nucleic acid content was estimated spectophotometrically (Layne, 1957). Ribonucleic acid content was estimated from the ribose content of samples by reaction with orcinol (Schneider, 1957) using ribose as standard. RNA determination by both methods gave essentially identical results. Protein content was determined by the procedure of Lowry *et al.* (1951) using bovine serum albumin as standard.

Enzyme assays. Acid phosphatase was assayed at 25°C in 100 m*M* sodium acetate buffer, pH 5.0, containing 4 m*M* *p*-nitrophenylphosphate (Dore and Cousineau, 1967). Phosphodiesterase activity was similarly determined using a reaction mixture composed of 25 m*M* Tris-HCl, pH 9.0, and 2 m*M* *p*-nitrophenyl-TMP (Touster *et al.*, 1970). In both assays the reaction was terminated by the addition of 2 vol of 0.5 *M* Na_2HPO_4 adjusted to pH 12.0, and the absorbance of the sample was determined at 410 nm in a Gilford spectrophotometer. Cytochrome *c* oxidase activity was determined by monitoring the oxidation of ascorbate-reduced cytochrome *c* spectrophotometrically at 550 nm (Wharton and Tzagoloff, 1967). The substrate was prepared at a concentration of 1% in 10 m*M* phosphate, pH 7.0, and the assay performed at 25°C as described (Wharton and Tzagoloff, 1967).

Electrophoretic analyses. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed according to Laemmli (1970), and the gels were stained

either with Coomassie brilliant blue R or by the periodic acid-Schiff (PAS) method (Segrest and Jackson, 1972). High-mannose oligosaccharides were removed from N-linked glycoproteins by digestion with endoglucosaminidase H (Endo H, Miles Laboratories). Protein samples were brought to 0.05% SDS in 40 μl, boiled 1 min, and incubated at 30°C for 15 hr in 30 m*M* citrate buffer, pH 6.0, containing Endo H and the PI mixture cited above. The samples were then prepared in SDS sample buffer (Laemmli, 1970) with β-mercaptoethanol, boiled 3 min and analyzed by SDS-PAGE.

Concanavalin A (Con A) Sepharose chromatography. Con A-Sepharose CL-4B was pretreated with ovalbumin to reduce the nonspecific binding of proteins. After elution of specifically bound ovalbumin with 0.5 *M* α-methyl-D-mannoside, the resin was preequilibrated with a buffer containing 0.5 *M* NaCl, 0.2% Triton X 100, 1 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, 0.02% sodium azide, and 20 m*M* Hepes, pH 7.5, plus the PI mixture. Samples were incubated overnight with 1 ml con A-Sepharose, shaken gently at 4°C, and then poured into a small column and washed with 50 ml of buffer. The column was then brought to room temperature and eluted with buffer containing 0.5 *M* α-methyl-D-mannoside. Fractions of 1 ml were collected and those with the highest protein concentration were pooled for further analysis by SDS-PAGE.

Radioiodination of crude yolk platelets. Intact yolk platelets in the heterogeneous mixture isolated from 10^6 blastula stage embryos by centrifugation of homogenates at 27,000*g* were radioiodinated by the lactoperoxidase-glucose oxidase method of Hynes (1973). The 27,000*g* pellet was resuspended in 1 ml CMF-SW containing 5 m*M* glucose, lactoperoxidase (820 μg/ml), glucose oxidase (0.1 unit/ml), and carrier-free Na^{125} I (1 mCi/ml). The reaction was allowed to continue for 10 min at room temperature and was stopped by the addition of 10 μl of 1 mg/ml sodium metabisulfite followed by a 5-min incubation. The iodinated material containing yolk platelets was then washed several times by centrifugation for 1.5 min at 11,600*g* in a Beckman microfuge and resuspensed in CMF-SW containing 50 m*M* NaI. Approximately 6×10^5 cpm were incorporated into 0.2 mg of total protein.

Fixation for electron microscopy. Eggs or embryos were fixed for 1.5 hr in 3.0% (v/v) glutaraldehyde in seawater containing Ca^{2+} (Decker and Lennarz, 1979). Subsequently, the specimens were washed by hand centrifugation, suspended in 1.0% (w/v) OsO_4 and incubated on ice for 1 hr. Sediments prepared from the diluted yolk fractions (see above) were similarly fixed in CMF-SW. Occasionally, the embryos and isolated yolk platelet pellets were fixed in 1.0% (w/v) OsO_4 in artificial seawater or CMF-SW at pH 8.0 without previous exposure to glutaraldehyde. Dehydration and embedding were performed as previously reported (Decker and Lennarz, 1979). Lowicryl K4M embedments were prepared by the method of Altman *et al.* (1983), with the exception that 1.5% (w/v) paraformaldehyde and 1.0% (v/v) glutaraldehyde in isotonic phosphate-buffered saline were routinely used for fixation.

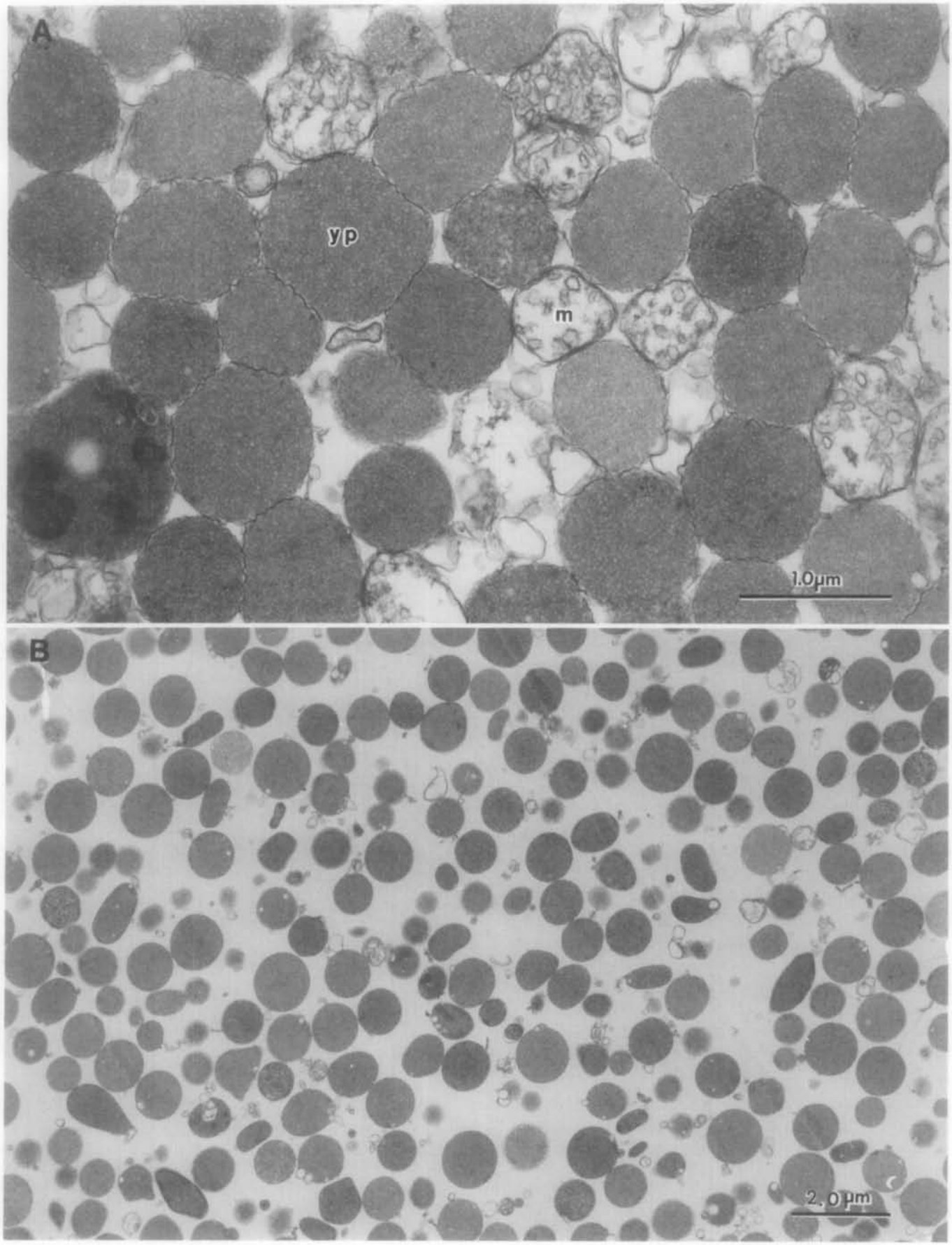
Preparation of con A-gold marker. Colloidal gold (16 nm average diameter) was prepared by the method of Frens (1973). Con A was electrostatically adsorbed to the colloid by dropwise addition of 2 mg of salt-free Con A (Sigma) in 1 ml of 0.15 *M* sodium phosphate, pH 7.4 to a 20-ml suspension of colloidal gold. Five milliliters of 1% polyethylene glycol was then added dropwise to the stirred suspension, followed by the addition of 1.5 ml of 1 *M* NaCl and 2.5 ml of 0.15 *M* sodium phosphate, pH 7.4. The samples were then centrifuged in siliconized ultracentrifuge tubes for 30 min at 25,000 rpm in a Beckman TY65 rotor. The colloidal gold-Con A pellet was then resuspended by Vortex mixing and recentrifuged to remove any unbound Con A from the preparation. The final colloidal gold-Con A pellet was then resuspended in 0.5 ml of sodium phosphate buffer containing 20% glycerol by Vortex mixing. The sample was stored at −20°C prior to use. Control experiments to establish the specificity of lectin binding were performed using hapten sugar competition by the method of Roth (1983). Thin sections of Lowicryl K4M embedded embryos were mounted on nickel grids and incubated in 20 μl of colloidal gold-Con A diluted 1:1 (v/v) with 0.15 *M* sodium phosphate or 0.5 *M* α-methyl-D-mannoside in 0.15 *M* sodium phsophate (pH 7.4). After incubation for 30 min at 23°C in a humid chamber, the specimens were serially washed in sodium phosphate buffer and distilled water. Lowicryl-embedded specimens were then stained for 5–10 min in 2.0% (w/v) aqueous uranyl acetate, washed in distilled water, air dried, and viewed in a Phillips 410 electron microscope operated at 60 kV.

RESULTS AND DISCUSSION

Isolation of Yolk Platelets

We have developed a simple procedure for isolating yolk platelets from eggs and embryos of *A. punctulata.* Sedimentation at 27,000*g* of homogenates of gastrula stage embryos resulted in a pellet that was primarily composed of yolk platelets and mitochondria (Fig. 1A).

FIG. 1. Fractionation of gastrula-stage yolk platelets. (A) Initial yolk platelet-enriched pellet isolated at 27,000*g* containing both yolk platelets (yp) and mitochondria (m); (B) purified low-density yolk platelets recovered after sucrose gradient centrifugation. Samples were fixed in glutaraldehyde, in presence of trace amounts of EGTA followed by OsO_4.


A
yp
m
1.0μm
B
2.0μm

Although it was clear from ultrastructural examination of yolk platelets *in situ* that the majority of these organelles exhibited similar staining properties and substructure (see below), yolk platelets varied sharply in their apparent buoyant density. Subsequent sucrose gradient centrifugation of the 27,000*g* pellet resulted in the separation of two major bands as determined by light scattering (Fig. 2A). The low-density band proved to be highly enriched in yolk platelets (Fig. 1B), while the high-density band was composed of yolk platelets heavily contaminated with mitochondria (not shown). Similar results were obtained by sucrose-gradient fractionation of the pellet isolated at 27,000*g* from eggs and from the cells of dissociated embryos at other developmental stages, including blastula, prism, and pluteus.

To further assess the purity of the low-density yolk platelet fraction, we utilized the observation that several hydrolytic enzymes, including acid phosphatase, are localized in the yolk platelets (Schuel *et al.*, 1975). Indeed, the specific activity of acid phosphatase was found to increase nearly sevenfold over that of the crude lysate upon sucrose gradient fractionation (Table 1). Since the yolk platelets comprise a large percentage of the cytoplasm, the purification obtained appears to be near the maximum possible. As shown in Table 1, the activity of phosphodiesterase, an enzyme marker of the plasma membrane, was absent from the yolk platelet preparation, and the mitochondrial marker enzyme, cytochrome *c* oxidase, was reduced nearly 15-fold in specific activity.

Metrizamide gradient centrifugation has been used very successfully for separating mitochondria and lysosomes (Wattiaux *et al.*, 1978; Wong *et al.*, 1982). When this procedure was included as an additional purification step, only a single peak was obtained as determined by both light scattering and acid phosphatase activity (Fig. 2B). Measurement of acid phosphatase in the peak fraction of the metrizamide gradient indicated that acid phosphatase specific activity was similar to that observed after sucrose gradient fractionation (Table 1). Therefore, based upon electron microscopic observation, the absence of phosphodiesterase and cytochrome *c* oxidase activities, and the approach to constant specific activity of acid phosphatase, we conclude that the low-density yolk platelet fraction obtained by sucrose gradient centrifugation was essentially homogeneous.

The biochemical quantification of yolk platelets and their components over the course of development (see

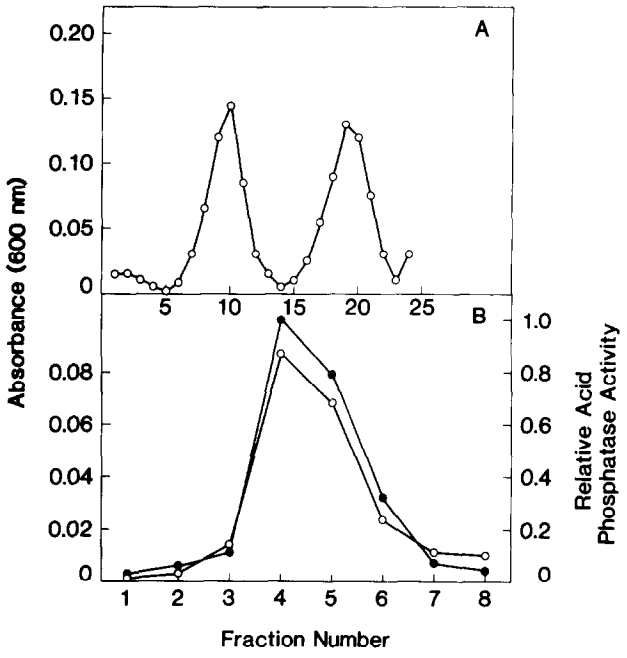


FIG. 2. Gradient centrifugation of yolk platelets. In (A), the 27,000*g* pellet was applied to a sucrose gradient and centrifuged as described under Materials and Methods. Fractions of 1 ml were collected from the bottom of the tube and their absorbance at 600 nm was determined. In (B), the low-density yolk isolated by sucrose gradient centrifugation was applied to a metrizamide gradient and centrifuged as described under Materials and Methods. Fractions of 1 ml were collected from the bottom of the tube, and their absorbance at 600 nm was determined (open circles). Each fraction was assayed for acid phosphatase activity as described under Materials and Methods (closed circles).

TABLE 1
MARKER ENZYME ACTIVITIES DURING FRACTIONATION OF THE LOW-DENSITY YOLK PLATELETS

Fraction	Specific activity		
	Phospho-diesterase[a]	Acid phosphatase[a]	Cytochrome *c* oxidase[b]
Crude lysate	1.41	14.8 (1.0)[c]	0.35 (1.0)[c]
Sucrose gradient peak	ND[d]	98.7 (6.7)[c]	0.02 (0.06)[c]
Metrizamide gradient peak	ND[d]	133 (9.0)[c]	ND[d]

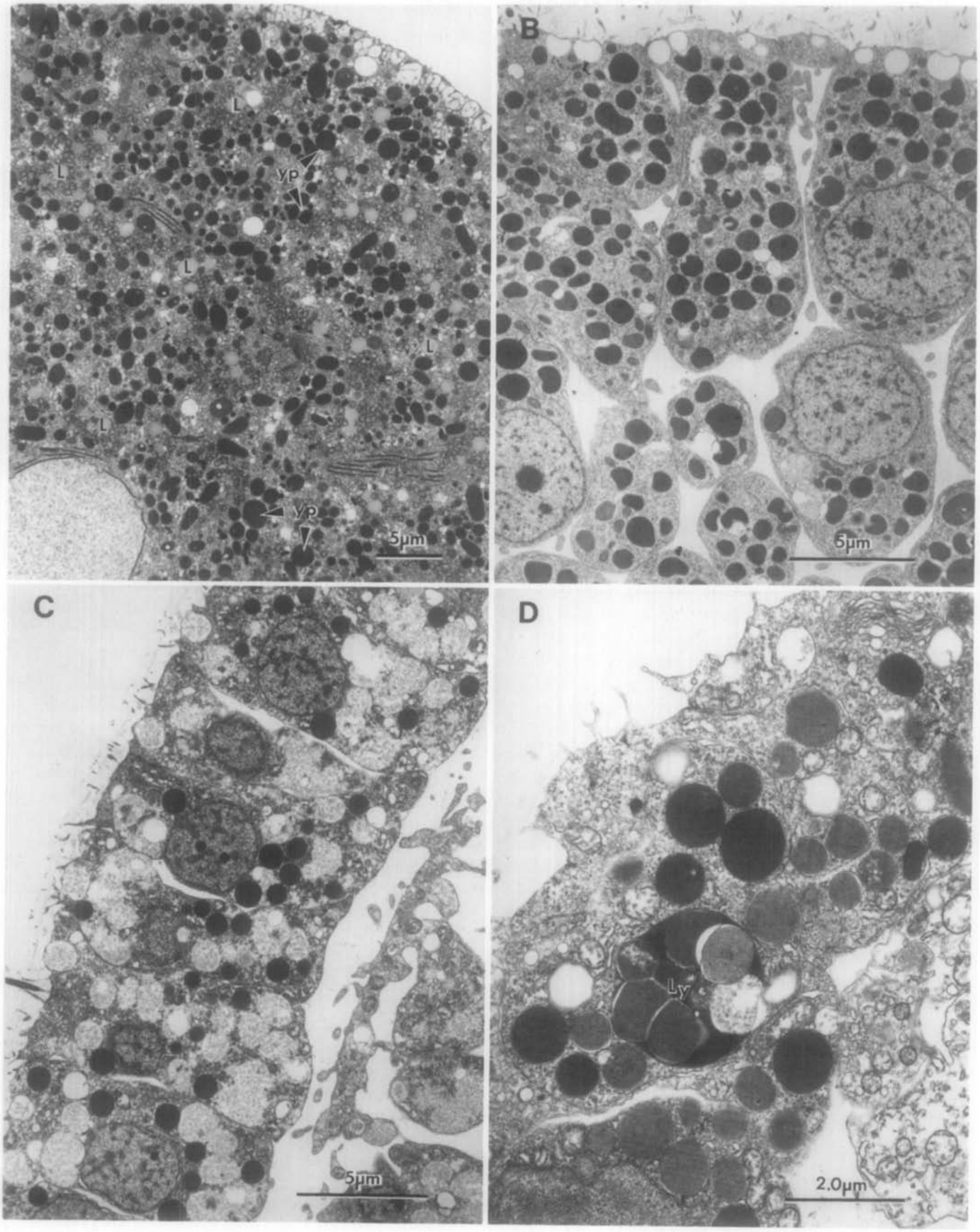
[a] Specific activity expressed as nmole of product formed per min per mg protein.

[b] Specific activity expressed as arbitrary units per mg protein.

[c] The specific activity relative to the crude lysate is provided in parentheses.

[d] Not detectable.

FIG. 3. Morphology of yolk platelets *in vivo*. Yolk platelets (yp) are prevalent in *A. punctulata* eggs and embryos up to the pluteus stage. (A) Unfertilized egg; (B) hatched blastula; (C) gastrula; (D) pluteus. In Figs. 3A, B, and D, samples were fixed in OsO_4 without prior exposure to glutaraldehyde. In Fig. 3C, poor preservation of the yolk platelets is observed in preparations fixed in the presence of Ca^{2+} with glutaraldehyde followed by OsO_4 (see Materials and Methods). Lipid droplets (L) are commonly seen in the unfertilized egg (A), but are rarely found at subsequent stages. Lysosome-like structures (Ly) occasionally appear at later developmental stages (D).


L
L
YP
L
L
L
YP
5μm
B
5μm
C
5μm
D
Ly
2.0μm

below) could be complicated by differential recovery during isolation at the various stages. To test this possibility, the crude yolk platelets contained in the 27,000*g* pellet of blastula homogenates were radioiodinated as described under Materials and Methods and 120,000 cpm were combined with crude cell lysates of embryos at the 2-cell, blastula, gastrula, and prism stages. The low-density yolk platelets were then isolated from each of these stages by sucrose gradient centrifugation and the recovery of exogenously added radiolabeled yolk platelets was determined. Although this is not a perfect control, the finding that approximately equal amounts of radioactivity were recovered in each yolk platelet preparation, suggests that the yolk platelets are not differentially degraded by components of the various cell lysates prepared at these four stages of development.

Ultrastructure of the Low-Density Yolk Platelets

As shown in Figs. 3A–D, numerous yolk platelets of *A. punctulata* were found within the cytoplasm of the unfertilized egg and in embryos throughout early development to the pluteus stage. In the presence of artificial seawater containing Ca^{2+}, the use of OsO_4 as the sole fixative resulted in superior preservation of yolk platelets *in situ* as compared to primary fixation in glutaraldehyde with subsequent exposure to OsO_4 (compare Figs. 3A, B, and D with Fig. 3C). It can be seen in Fig. 3C that yolk platelets often appeared to fuse with the plasma membrane when fixed in glutaraldehyde in the presence of Ca^{2+}. Addition of the Ca^{2+} chelator, EGTA, to the primary fixative, glutaraldehyde, resulted in yolk platelet preservation similar to that obtained when OsO_4 was used alone (data not shown). In the unfertilized egg (Fig. 3A), presumptive lipid droplets were relatively well preserved by OsO_4 fixation. However, at the blastula stage (Fig. 3B), the lipid inclusions were rarely observed, although yolk platelets remained abundant. In contrast to the unfertilized egg (Fig. 3A), lysosome-like structures containing yolk platelets were occasionally seen at the later stages (Fig. 3D), suggesting that some of these organelles may undergo degradation during development.

Electron microscopic examination of yolk platelets from *Hemicentrotus pulcherrimus* has revealed a substructure consisting of coarse granules or micelles 20–30 nm in diameter, packed within a limiting membrane (Takashima, 1971). Lipoprotein particles isolated from yolk platelets of *H. pulcherrimus* vary between 25 and 50 nm in diameter and may represent the granular components of this organelle observed *in situ* (Ii *et al.*, 1978).

As shown in Fig. 4A, the low-density yolk platelets of *A. punctulata* were approximately 1 μm in diameter and were limited by a membrane approximately 9 nm thick. The major identifiable substructures consisted of small particles, some of which resembled membrane vesicles of about 60 nm diam (Fig. 4A, arrowheads). In Fig. 4B, aggregates of the yolk subparticles are shown; they were visualized using negative staining after removal of the limiting membrane by osmotic lysis. In accordance with the findings of Ii *et al.* (1978), yolk platelet subparticles revealed by negative staining vary in diameter from about 10 to 50 nm (Fig. 4B). These vesicular yolk subparticles were commonly found throughout development upon examining thin sections of whole embryos. Note the obvious differences in the amount of vesicular subparticles (arrowheads) within the two yolk platelets shown in Fig. 4C. This variability in the content of matrix subparticles was observed at all developmental stages, suggesting the accumulation and/or depletion of these constituents within the organelle. Furthermore, at no point in development was there any indication of a change in the relative proportion of the two morphologically distinguishable yolk platelet forms. The presence of both morphological forms in the low-density yolk platelet fraction (see Fig. 1B) indicates that they are not separated by sucrose gradient centrifugation.

Biochemical Characterization of the Low-Density Yolk Platelets

The low-density yolk platelets isolated by sucrose gradient centrifugation were analyzed biochemically to determine possible compositional changes over the course of development. It can be seen from Table 2 that the yolk platelets isolated at three stages showed no marked, consistent change with respect to their content of lipid, carbohydrate, nucleic acid, and protein. These results corroborate our morphological observations and further suggest that this yolk platelet fraction is not utilized as a store of readily catabolizable components during the first 48 hr of embryonic development.

It is possible that fatty acids, which are plentiful in the yolk platelets (Ii *et al.*, 1978), are utilized during development, but are not depleted to an extent that is detected in our analysis. However, there are other sources of lipid in the embryo, most notably the lipid droplets (Fig. 3), which could provide a reservoir of catabolizable organic precursors. Indeed, when the lipid content of yolk platelets was compared with that of crude lysates (Fig. 5), it was apparent that the yolk platelet fraction

FIG. 4. Ultrastructure of gastrula-stage yolk platelets. (A) Yolk platelets are limited by a membrane approximately 9 nm thick and contain particulate substructures that may form membrane vesicles (arrowhead). (B) Hypotonic lysis and negative staining with phosphotungstate result in removal of the limiting membrane and reveal an aggregate of particles thought to be lipoproteins (Ii *et al.*, 1978). (C) In some cases yolk platelets visualized *in situ* appear depleted of matrix contents (arrowhead), suggesting the occurrence of dynamic changes during development (fixed in OsO_4 only).

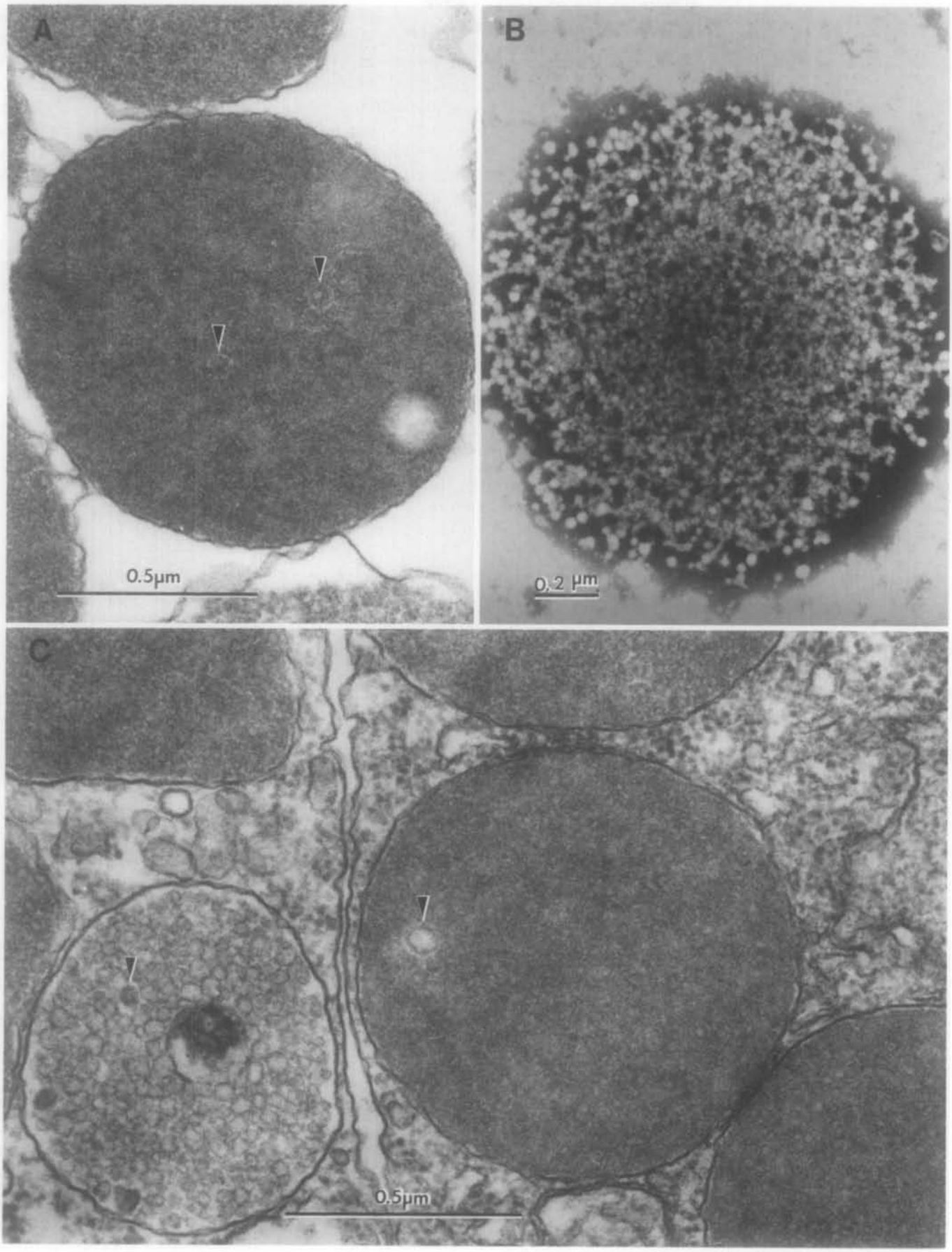

A
0.5μm
B
0.2 μm
C
0.5μm

TABLE 2
CHEMICAL ANALYSES OF YOLK PLATELETS ISOLATED AT THREE DEVELOPMENTAL STAGES[a]

Chemical constituent	Yolk platelets from Egg	Blastula	Pluteus
Esterified fatty acids (total)[b]	0.250	0.450	0.350
Esterified fatty acids (phospholipid)[b]	0.201	0.242	0.208
Esterified fatty acids (triglycerides)[b]	0.06	0.06	0.03
Hexose[b]	0.174	0.250	0.167
Sialic acids[b]	0.020	0.045	0.029
RNA[c]	54	47	70
Protein[c]	212	280	219

[a] The low-density yolk platelet fraction was isolated as described under Materials and Methods by sucrose gradient centrifugation.
[b] Expressed as μmole/10^6 embryos.
[c] Expressed as μg/10^6 embryos.

did not contribute significantly to the total lipid content of the embryo. Furthermore, it is evident from the results in Fig. 5 that, whereas the lipid content of yolk platelets remained constant during the course of development (see Table 2), the other larger store of lipid present in the crude lysate fraction was depleted. This was the case if we measured either triglyceride content (Fig. 5A) or total esterified fatty acids (Fig. 5B).

The vesicular structure of the yolk platelets (Fig. 3) suggested the possibility that these organelles functioned in either lipogenesis or membrane biogenesis. Therefore, the following enzymes were assayed by Dr. Robert Bell, Department of Biochemistry, Duke University, using a lysed yolk platelet preparation derived from gastrula stage embryos: fatty acyl coenzyme A lyase, glycerol phosphate acyl transferase, choline phosphotransferase, and ethanolamine phosphotransferase (data not shown). Because enzyme activity was in no case detectable, it seems unlikely that the yolk platelet was a site of lipid synthesis.

Also of note was the high content of RNA in the yolk platelets (about 12% by weight, see Table 2). Previous studies (Dubois *et al.*, 1971) identified unique classes of 9 S and 12 S RNA in the yolk platelets of *S. purpuratus*. To determine whether any of this RNA represents translatable mRNA, the total RNA isolated from purified yolk was used to program an *in vitro* translation system. However, no translation products were detected (data not shown).

Proteins of the Low-Density Yolk Platelets

Although the total protein content of the yolk platelet fraction does not change during embryogenesis, SDS-PAGE analysis revealed changes in individual proteins. In Fig. 6 is shown the SDS-PAGE profile of the proteins of a crude cell lysate and the purified low-density yolk platelet fraction at several developmental stages. The gels were stained with either Coomassie blue to visualize protein (Figs. 6A and C) or PAS reagent to visualize glycoproteins (Figs. 6B and D). It is apparent that the major proteins associated with the yolk platelets were also major protein components of the unfractionated cell extract. All of these proteins stained with PAS reagent, indicating that they were glycoproteins. They ranged in molecular weight from about 35,000 to over 300,000. A comparison of the yolk-associated glycoproteins from embryos at different stages of development revealed changes in their relative amounts (Fig. 6D). The major yolk-associated glycoprotein (designated d), as well as glycoprotein a, decreased in amount over the course of development, whereas several other proteins (designated c, g, h, k, and l) increased. Other yolk-associated glycoproteins (designated b, e, f, i, and j) remained relatively constant over the course of early development.

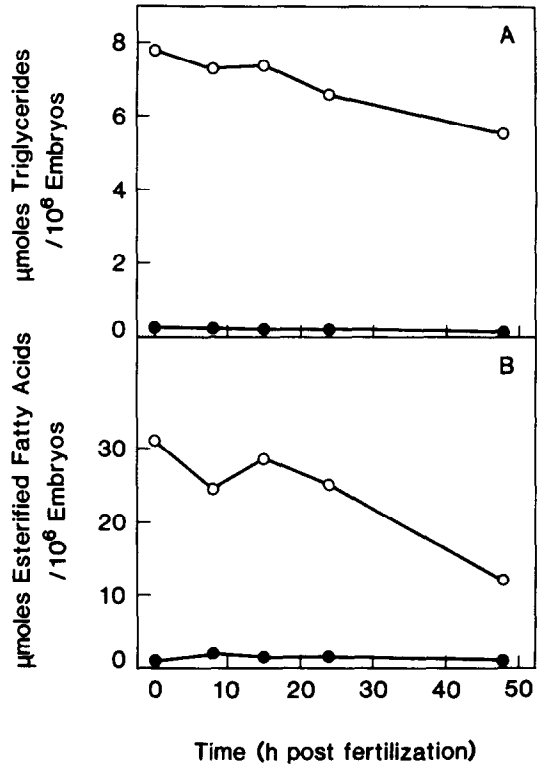


FIG. 5. Lipid composition of the low-density yolk platelet fraction. Triglycerides (A) and total esterified fatty acids (B) were measured as described under Materials and Methods in crude lysates (open circles) or in the purified yolk platelet fraction (closed circles) of unfertilized eggs (0 hr) and embryos at the hatched blastula (8 hr), mesenchyme blastula (15 hr), gastrula (24 hr), and pluteus (48 hr) stages of development.

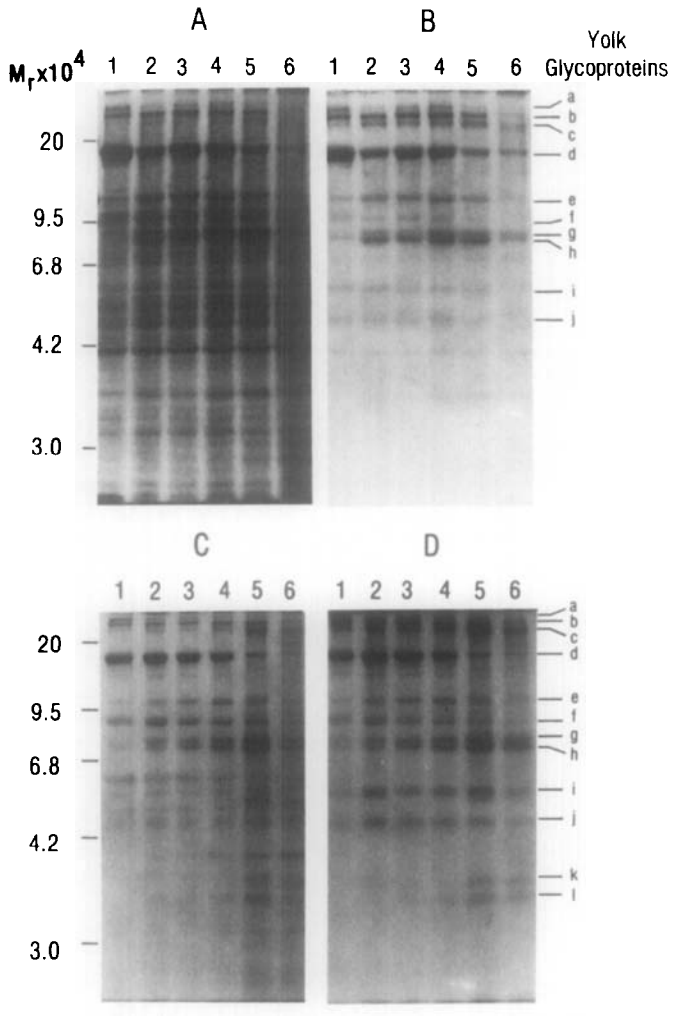


FIG. 6. SDS-PAGE analysis of yolk platelet proteins. Crude lysates (A, B) or purified yolk platelets (C, D) were analyzed by SDS-PAGE in 8.75% polyacrylamide. Gels A and C were stained with Coomassie brilliant blue R and gels B and D were stained by the PAS method for carbohydrate. The lane numbers represent samples containing 50 μg protein taken from unfertilized eggs (1), early blastula (2), mesenchyme blastula (3), gastrula (4), prism (5), and pluteus (6) stage embryos. The migration of molecular weight standards are indicated to the left and the major glycoproteins of the yolk platelet are identified by letters to the right.

To determine if the appearance of new yolk-associated glycoproteins during development was the result of *de novo* synthesis, embryos were labeled with [^{35}S]-methionine and the yolk platelets were isolated and analyzed by SDS-PAGE followed by autoradiography. No major yolk-associated glycoproteins were labeled, indicating that they were not synthesized *de novo* during embryogenesis (data not shown). The nature of the changes observed in some of the yolk-associated glycoproteins remains unclear. It seems unlikely that these changes reflected nonspecific proteolysis in the isolated yolk platelets since extensive precautions were taken to inhibit proteolysis during yolk platelet isolation. However, these changes may have resulted from specific proteolytic processing of the larger glycoproteins, i.e., glycoproteins a and d, leading to the formation of the smaller glycoproteins.

Studies on the 27 S component of sea urchin yolk platelets are relevant to our findings (Malkin *et al.*, 1965; Kari and Rottmann, 1985). In addition to the lipoproteins, yolk platelets contain a 27 S particulate component having a cylindrical shape of 12.5 × 18 × 0.9 nm (Malkin *et al.*, 1965). This component is rich in both protein and carbohydrate and upon analysis by SDS-PAGE followed by PAS staining was shown to have a glycoprotein profile different from that of the lipoprotein component (Ii *et al.*, 1978). In a paper that appeared while this manuscript was in preparation, it was reported that in *S. purpuratus* and *L. pictus* the mass of the 27 S component remains stable during development, although the glycoprotein profile changes significantly (Kari and Rottmann, 1985). During development the total protein content of the 27 S component remains constant and radioactive amino acids are not incorporated (Malkin *et al.*, 1965). However, the 27 S glycoproteins do undergo changes which, in most cases, appear to result from proteolytic processing (Kari and Rottmann, 1985). Unfortunately, detailed comparisons of these results and ours cannot be made because (a) we have studied the intact yolk platelet and (b) our studies were carried out in *A. punctulata*, a species not examined by Kari and Rottmann (1985).

As shown in Fig. 7, the oligosaccharide chains of the

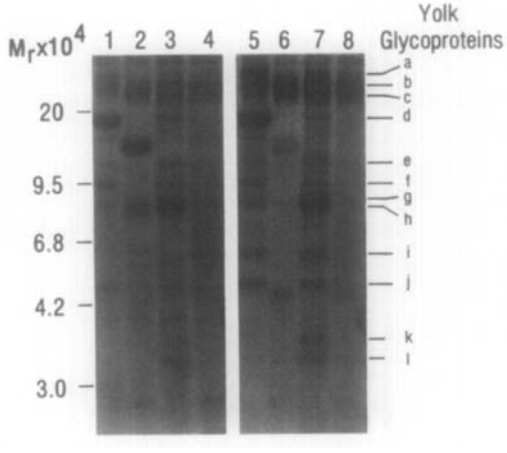
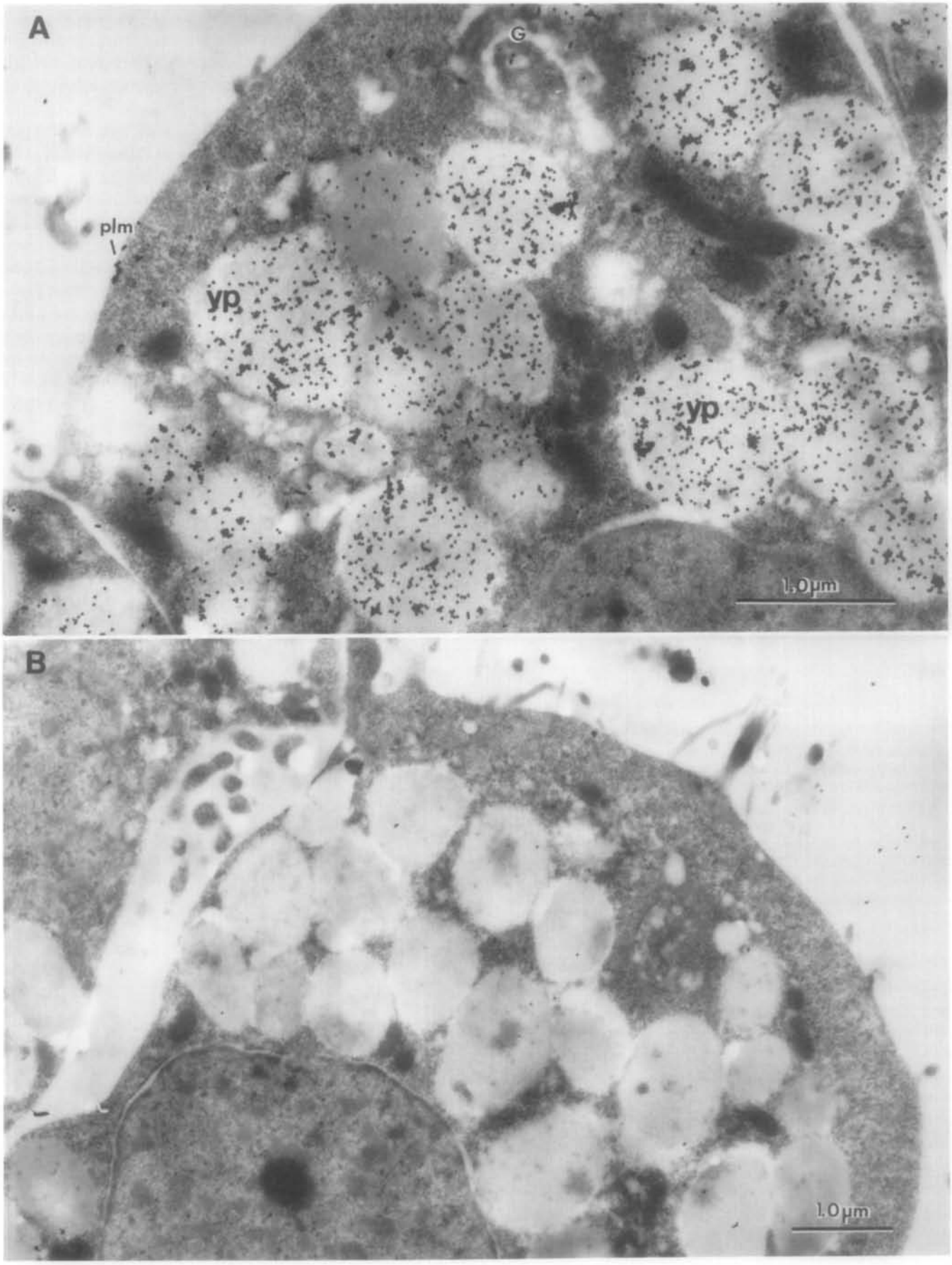


FIG. 7. Endo H sensitivity of yolk platelet glycoproteins. Purified yolk platelet proteins were incubated at 30°C for 15 hr in 30 m*M* citrate buffer, pH 6.0, in the absence (lanes 1, 3, 5, and 7) or presence of Endo H (lanes 2, 4, 6, and 8) as described under Materials and Methods. The samples, which contained 50 μg protein, were then analyzed by SDS-PAGE in 8.75% polyacrylamide and stained with either Coomassie blue (lanes 1-4) or PAS reagent (lanes 5-8). Yolk platelet samples were isolated from embryos at both the early blastula (lanes 1, 2, 5, and 6) and prism (lanes 3, 4, 7, and 8) stages. The yolk platelet bands and molecular weight markers are as indicated as in Fig. 6.


A
G
plm
yp
yp
1.0μm
B
1.0μm

yolk platelet glycoproteins were partially characterized by digestion with Endo H followed by SDS-PAGE. When yolk platelet proteins from blastula and prism stages were treated with the Endo H, all except glycoproteins b and c from prism showed an increased mobility on SDS gels. As shown, staining with Coomassie blue revealed discrete bands (left panel), indicating that the shift observed upon treatment with Endo H was not merely the result of proteolysis by proteases contaminating the Endo H preparation. PAS staining revealed that nearly all of the oligosaccharide chains of these glycoproteins were subject to removal by Endo H (right panel) and were, therefore, of the N-linked, polymannose type. The carbohydrate remaining on some Endo H treated glycoproteins (e.g., the apparent products of a, b, c, d, and j) may reflect incomplete digestion, complex type N-linked chains or O-linked oligosaccharide chains. Consistent with these findings, all of the yolk-associated, Endo H-sensitive glycoproteins were bound to Con A-Sepharose and could be specifically eluted with α-methyl-D-mannoside (data not shown). Although it was difficult to match most of the yolk glycoproteins with their Endo H-digested products, the Endo H-treated major yolk glycoprotein (M_r 170,000) appeared to migrate with a M_r of about 135,000 (compare lanes 1 and 2). This molecular weight shift of M_r 35,000 indicates that the major yolk glycoprotein is heavily glycosylated with multiple polymannose-type oligosaccharides, perhaps containing as many as 15 such chains.

Ultrastrucutral Localization of the Yolk Glycoconjugates

To verify the finding that purified yolk platelets contained the majority of polymannose-type, N-linked glycoproteins found in the embryonic cells and to demonstrate that they indeed are components of the yolk platelets, we used colloidal gold conjugated to Con A to stain Lowicryl-embedded sections of embryonic cells. The results, shown in Fig. 8A, clearly demonstrate that the yolk platelet is the predominant organelle in the embryo to which the probe binds. Other subcellular sites which were specifically labeled with Con A-gold included the plasma membrane and the Golgi apparatus. The specificity of this binding was demonstrated in control preparations incubated with α-methyl-D-mannoside (Fig. 8B). Virtually no gold particles were evident in the control preparations.

CONCLUSIONS

This study has provided detailed biochemical information on the composition of intact yolk platelets from sea urchin embryos. No biochemical evidence of yolk platelet depletion was detected over the course of embryonic development up to the pluteus stage. In a very early study, Kavanau (1954) measured the synthesis and degradation of protein in acid-precipitable material from extracts of *S. purpuratus* embryos, assuming this fraction to represent yolk platelets. The results of that study, which were thought to demonstrate the utilization of this protein fraction during development, were later shown to have been the result of autolytic artifacts (Kavanau, 1958). The only change that we observed was in the profile of yolk platelet-associated glycoproteins analyzed by SDS-PAGE. Since during embryogenesis the major yolk platelet-associated glycoproteins are not metabolically labeled with amino acid precursors, it seems likely that these glycoproteins were synthesized during oogenesis and underwent molecular weight changes, perhaps owing to proteolytic processing during embryonic development. However, despite the changes in molecular weight of these proteins, no decrease in the concentration of total yolk platelet protein was detected over the course of development. Furthermore, the same constancy for content was observed for lipids, RNA, hexose, and sialic acid. Our observations regarding yolk platelet glycoproteins confirm the report of Kari and Rottmann (1985) and further provide information on the other biochemical components of the yolk platelet fraction during development. In addition, we have shown that, at least in *A. punctulata*, yolk platelets can be separated into two buoyant density classes; one of these classes cofractionates with mitochondria upon sucrose gradient centrifugation and was not examined in detail. The other class of yolk platelets, of lower density, was shown by marker enzyme and microscopic analyses to be essentially homogeneous.

Although yolk platelets in certain other embryonic systems are known to provide a store of nutrients and energy (Williams, 1967), their function in sea urchin embryos remains unclear. Schuel *et al.* (1975) suggested that the yolk platelets may be lysosome-like storage particles based on their content of lysosomal hydrolases. Although no study has provided convincing evidence that yolk platelet constituents are catabolized as embryogenesis proceeds, it is possible that they are present in a large excess and are depleted to an extent not readily detectable. Alternatively, yolk platelets could serve as a reserve to be used in the event that a food source is not immediately available when feeding begins at the late pluteus stage. Yolk platelet utilization in amphibians occurs late in development (Karasaki, 1963) and is

FIG. 8. Subcellular localization of Con A bindable glycoconjugates in blastula-stage cells. (A) Con A-gold label was highly concentrated over yolk platelets. The nuclei, mitochondria, and the cytoplasm were nearly devoid of gold particles, and only low levels of binding to Golgi (G), and plasma membrane (plm) were observed. (B) The specificity of binding of Con A-gold is demonstrated by the observation that the presence of 0.5 *M* α-methyl mannoside prevented the lectin-gold complex from binding to the sections. Label was essentially absent over all subcellular compartments.

initially regionalized in *Xenopus laevis* (Selman and Pawsey, 1965). Sea urchin embryos, which are normally cultured in sea water devoid of organic nutrients, initiate protein synthesis shortly after fertilization (Epel, 1967). Since this process occurs in the absence of yolk utilization, it may be that the carbon backbone for the amino acids required for this early translational activity are provided by the products of glycogen degradation that occurs following fertilization (Orstrom and Lindberg, 1940). In any case, it is clear that the function of the membrane- and glycoprotein-rich yolk platelets remains to be elucidated. In this context, monoclonal antibodies should prove to be useful in clarifying the function of this organelle in the sea urchin since they would enable one to elucidate the structural relationship between the various glycoproteins and their possible presence in other subcellular compartments.

The authors thank Dr. Robert Bell, Department of Biochemistry, Duke University, for performing assays of lipid synthesizing enzymes and Dr. Joseph Lau, Department of Biological Chemistry, The Johns Hopkins University School of Medicine, for carrying out *in vitro* translation assays. We are also indebted to Dr. Winston W. Chen, Department of Neurology, The John F. Kennedy Institute, for helpful suggestions and discussions. The excellent technical assistance of Ms. Betty J. Earles and Ms. Helen Park and the expertise of Ms. Diana Welch and Dr. Maria Valdizan in preparing the manuscript are greatly appreciated. This work was supported by a National Institutes of Health Grant (HD-18600) to W.J.L. and a National Research Service Award (GM-07965) from the NIGMS to D.R.A. Dr. William J. Lennarz, who is a Robert A. Welch Professor of Chemistry, acknowledges with gratitude the Robert A. Welch Foundation.

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