BIOCELL
2002, 26(1): 111-118

ISSN 0327 -9545
PRINTED IN ARGENTINA

Workshop: "Biology of Ampullariidae"

Minireview

Lipoproteins from plasma and perivelline fluid of the apple snail *Pomacea canaliculata*

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Key words: Lipoproteins; lipids; proteins, snail embryogenesis

Introduction

Structural lipids, primarily phospholipids and sterols, as well as energy-storage lipids such as triacylglycerols are insoluble in water. In the aqueous fluids of animals, they are carried as lipoproteins, complexes of lipids bound to polypeptides. These water-soluble lipoproteins can be separated into different classes, depending upon their hydrated densities. These classes include very low density (VLDL), low density (LDL), high density (HDL) and very high-density (VHDL) lipoproteins. The two first ones predominate in the blood of vertebrates, while HDLs are the main lipoproteins in invertebrate hemolymph. Lipid circulation systems in invertebrates have been studied mainly in the phyla Arthropoda and Mollusca. In terrestrial invertebrates, the mechanisms of lipid circulation are well known in insects and comparatively less known in arachnids. The most studied group of aquatic invertebrates, respect to lipoproteins, is the Crustacea and, in contrast, the phyllum Mollusca has received little attention. In fact, the lipoproteins of only one species of the classes Bivalvia, Cephalopoda and Gastropoda were described up to date. In addition to the lipoproteins common to males and females, there are female-specific lipoproteins, which appear in the hemolymph plasma during vitellogenesis. They are similar to vitellins, the egg lipoproteins that provide energy and nutrient for the developing embryo. These egg storage molecules can also play a role in the growth and survival of larvae. Among aquatic invertebrates, egg lipoproteins have been described in crustaceans, sea urchins and molluscs (for a review, see Lee, 1991).

The present review deals with the lipoproteins present in hemolymph and perivitelline fluid of the snail *Pomacea canaliculata*, their lipid and protein composition, as well as their consumption during the embryo development. This is the only Gastropoda species whose plasma and egg lipoproteins were studied. Results are discussed comparatively with other invertebrates.

Interorgan transport of lipids

Hemolymph of aquatic invertebrates usually has a low lipid content. The lipid concentration in *P. Canaliculata* plasma varies between 127 and 210 μg/

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Received on November 29, 2000. Accepted on November 13, 2001.

ml and the values in hemocytes are within the range of 54 – 68 μg/ml hemolymph. The lipid content in hemolymph of *P. canaliculata* is very low when compared with that from other gastropods such as *Planorbis corneus* (Ottaviani, 1984) or *Biomphalaria glabrata* (Fried *et al*., 1989). Nevertheless, values are similar to those reported for plasma of bivalves living in the same habitat (Pollero *et al*., 1985), which varies from 80 to 260 μg/ml, and also for cephalopods (Heras and Pollero, 1989). Variations in blood lipid composition in aquatic invertebrates have been attributed to food intake and gonad maturity (Thompson, 1977). The scanty lipid concentration in hemolymph of these invertebrates makes it rather difficult to study the mechanism of lipid transport. This obstacle is overcome by using radioactive tracers, so that plasma lipoproteins can be detected and their relative importance in the transport of tracers or metabolites is determined. The low-lipid-content hemolymph of *P. canaliculata* was labeled by *in vivo* injections of liposomes containing 14C-cholesterol (Pollero et al., 1992) or salts of 14C-palmitic acid (Garin and Pollero, 1995), at different times of incubation, in order to investigate the possible occurrence of lipoproteins and their role and that of hemocytes in lipid transport.

The hemolymphatic transport of lipids in association with hematic cells had been demonstrated for triacylglycerol and cholesterol in a bivalve (Pollero *et al*., 1985; Pollero, 1987; Pollero and Heras, 1989) and, as a secondary way of lipid transport in an octopus (Heras and Pollero, 1990). Nevertheless, such role of hemocytes in lipid circulation does not seem to be important in *P. canaliculata,* as it was demonstrated in experiments conducted *in vivo*. When 14C-cholesterol–containing liposomes were injected into the snail foot and incubated, they were absorbed and incorporated into the hemolymph. The radioactivity was largely recovered in plasma and, in lesser amount, in the hemocytes. The low values of radioactivity found in hemocytes were attributed to a plasma-cell interchange and not to any special function of hematic cells in lipid transport (Pollero *et al*., 1992; Garin and Pollero, 1995). This distribution of radioactivity among the hemolymph components indicates that the interorgan transport of cholesterol is mostly performed by plasma and, perhaps to a very lesser extent, by hematic cells. A marked difference is shown between the cholesterol transport in this snail and that of the bivalve *Diplodon delodontus,* where more than 40% of hemolymphatic cholesterol circulates associated with hemocytes (Pollero, 1987). We can conclude that the lipid transport in *P. canaliculata* hemolymph is mostly performed via plasma lipoproteins.

Plasma lipoproteins

Labeled plasma of *P. canaliculata* was ultracentrifuged in density gradients, fractionated, and the total proteins, radioactivity and density were measured in isolated fractions. Fractions containing radioactivity and protein maxima, which indicated the occurrence of lipoproteins, were pooled and used to analyze lipid and protein compositions, using radiochromatographic and electrophoretic techniques, respectively.

On the basis of labeled cholesterol experiments, a cholesterol transporting HDL was detected, it was the first lipoprotein reported for the gastropods (Pollero *et al*., 1992). Table I collect the lipid classes composition of this *P.canaliculata* plasma lipoprotein. The observation on the labeled-cholesterol transport system implies that the HDL transports the largest amount of this sterol in the free form. Nevertheless, free sterol concentration, as well as free fatty acids and triacylglycerols, reach moderate mass values in this plasma fraction, while phospholipids account for the highest percentages of the total lipids. Other lipids including a significant percentage of hydrocarbons, and small amounts of sterol esters, complete the lipid classes spectrum of this lipoprotein.

When the HDL is analyzed by electrophoresis under dissociating conditions, it shows a unique apoprotein band with an apparent molecular weight of 70 kDa (Garin, 1996). It points out a difference respect to the HDL from *O. tehuelchus*, the other mollusc whose apolipoproteins were described. In octopus HDL three polipeptides of 129, 230 and 340 kDa were detected, corresponding the last one to the monomer of hemocyanin. This observation suggested, for the first time, the apoprotein role for this respiratory pigment (Heras and Pollero, 1992), function of hemocyanin then corroborated in arachnids (Cunningham and Pollero, 1996; Cunningham *et al*., 1999). Nevertheless, hemocyanin's role concerning the lipid transport in hemolymph of invertebrates cannot be generalised; upon the results obtained in the *P. canaliculata* HDL, the role of hemocyanin as an apolipoprotein may be discarded.

Protein/lipid ratio of *P. canaliculata* HDL, of about 1.9, falls into the range presented by other HDLs from different origins (Chapman, 1980). Concerning density and lipid composition, the HDL from *P. canaliculata* may be compared with those HDLs isolated from the bivalve *D. Delodontus* and from the cephalopod *O. Tehuelchus*, the only molluscs whose lipoproteins were characterized. Density of the snail HDL is higher than

that of the clam and it is coincident with octopus HDL. Each HDL differs in protein/lipid ratio, and perhaps this explains the density differences. However, lipid and protein compositions must also be considered. While cholesterol and phospholipids are both the main lipid classes in clam and octopus HDLs, triacylglycerols are significant components only in the clam. On the other hand, in the snail, phospholipids predominate, while triacylglycerols contribute to a lesser extent. Such differences in lipid composition confer particular characteristic to each lipoprotein and the same could be expected concerning the protein composition. The characterization of HDL apoproteins from other molluscs was done in detail in *O. tehuelchus* and only partialy in *D. Delodontus*. Both apoprotein compositions differ from that of the snail described above.

Labeled palmitate experiments provide to the plasma, depending on the incubation times, either with radioactive free fatty acids or radioactive glycerides . On the basis of radioactive palmitate injections and incubations *in vivo*, a second lipoprotein containing labeled lipids was detected in *P. canaliculata* plasma (Garin and Pollero, 1995). Its buoyant density, between 1.03 and 1.05 gr/ml, corresponds to a low-density lipoprotein (LDL). Although LDLs are common vertebrate lipoproteins, they have been also reported in some invertebrates. Lipoproteins ranging within density intervals of 1.04 – 1.08 gr/ml, were isolated from the saturniid silkmoth *Hyalophora cecropia* (Thomas and Gilbert, 1968), the clam *D. Delodontus* (Pollero and Heras, 1989) and *O. tehuelchus* (Heras and Pollero, 1990). In the housefly *Musca domestica*, the occurrence of a lipoprotein tentatively identified as LDL was also reported (Dwivedy and Bridges, 1973). LDL also appears during the flight of migratory insects when diacylglicerol uptake by HDL lipophorin converts it to LDL lipophorin (Van der Horst *et al*., 1993). The presence of LDL has not been reported in other arthropods other than insects. It may be speculated that the LDLs occurrence among aquatic invertebrates is a characteristic of molluscs, since they have been found in the three classes examined up to now.

Lipids from *P. canaliculata* LDL, separated into their classes and quantified, gave the quali-quantitative composition shown in Table I. Free fatty acids, triacylglycerols and phospholipids represented quantitatively important lipid fractions whereas hydrocarbons were, comparatively, of minor significance. Concerning total lipids transported in plasma, the LDL of this snail has, similarly to its counterparts in clams, octopus

TABLE 1.

Composition of *P. canaliculata* plasma lipoproteins.

COMPONENT	HIGH DENSITY LIPOPROTEIN (HDL)		LOW DENSITY LIPOPROTEIN (LDL)	
	μg lipids/ml plasma	% wt /wt	μg lipids/ml plasma	% wt /wt
HYDROCARBONS	3.1	9.1	1.4	6.8
ESTERIFIED STEROLS	0.4	1.1	ND	
TRIACYLGLYCEROLS	4.8	14.4	6.1	28.6
FREE FATTY ACIDS	5.2	15.5	7.1	33.8
FREE STEROLS	4.6	13.8	ND	
PHOSPHOLIPIDS	15.5	46.1	6.5	30.8
TOTAL LIPIDS	33.6	34.4	21.1	44.5
TOTAL PROTEINS	64.1	65.6	26.3	55.5
HYDRATED DENSITY (gr/ml)	1.10 – 1.15		1.03 – 1.05	

Values are the mean of triplicate analyses. ND: not detectable.

and silkmoth, a minor quantitative importance when compared to those lipoproteins of superior density (HDL). Nevertheless, some similarities and differences are evident between this LDL and those of other invertebrates regarding their lipid composition. Incidentally, in clam and octopus, phospholipids are by far the main lipid in LDL., while the free fatty acids predominate over phospholipids in *P. canaliculata.* In relation to energetic lipids, LDL in P. canaliculata, as in clam, is the principal carrier of triacylglycerol (Pollero and Heras, 1989). In contrast, as expected in an insect, diacylglycerols are the main fuel in the LDL of silkmoth (Thomas and Gilbert, 1968).

Proteins from LDL, analyzed by dissociating electrophoresis, show two polypeptides with apparent molecular weights of 15.5 and 17 kDa, respectively. The high-molecular-weight protein, termed apo-B, is the main protein constituent of LDLs in vertebrate groups that are evolutionary distant, such as mammals, reptiles, amphibians and fish (Chapman, 1980; 1983). As regards aquatic invertebrates, the only previous report dealing with the LDL protein composition has been done on the cephalopod *O. tehuelchus* . This lipoprotein, termed LP-I by Heras and Pollero (1992), contains hemocyanin as its major protein and in a lesser amount, another polypeptide of minor molecular weight. In the snail *P. canaliculata*, the apoprotein composition seems to be rather simple since it is composed of only two small polypeptides and, as in the HDL, the apolipoprotein role of hemocyanin must be discarded in this species.

Egg-reserve lipoporteins

During yolk synthesis primary oocytes increase their size by accumulating vitellus. This vitellus is composed of proteins, lipids and carbohydrates and it represents the energy source for the developing embryo. The major egg-yolk proteins are either called vitellins or lipovitellins, being the latter high density lipoproteins (HDL). Such lipoproteins have been isolated from the eggs of two molluscs: the bivalve *Pecten maximus* and the cephalopod *Sepia oficialis* (Lee, 1991). In gastropods, Barre *et al*. (1991) reported the presence of vitellins in oocytes and gonads of *Helix aspersa*. Unlike this general mechanism, most gastropods have a perivitelline fluid, mainly synthesized by accessory glands of the female tract called albumen gland, that represents the major source of nutrients for the embryo. Therefore proteinaceous yolk granules found in embryos developing from egg cells provided with perivitelline fluid do not serve as nutrient storage, but they function as primary lysosomes in charge of perivitelline fluid digestion instead (Raven, 1972; Jong-Brink *et al*., 1983; Wourms, 1987; Bretting *et al*., 1991). *P. canaliculata* belongs to this latter invertebrate group where yolk is a minor source of nutrients to the embryo. In the pulmonate snail *Lymnaea stagnalis* the perivitelline fluid is composed of calcium, proteins and the carbohydrate galactogen, but no lipid was detected (Hortsman, 1956; Raven, 1972; Jong-Brink *et al*., 1983). A more detailed analysis of the perivitelline polysaccharides in *P. canaliculata* showed that they are composed of galactose and fucose units (Raven, 1972). On the other hand, Cheesman (1958) found a carotene-glycoprotein complex with no lipids associated in the eggs of this snail that he called ovorubin.

To characterize the lipid and protein composition of the perivitelline fluid of the eggs of *Pomacea canaliculata*, we performed a sequencial centrifugation of egg homogenates followed by a purification step using a combination of gradient ultracentrifugation and HPLC using size exclusion and ion exchange columns. Thus we were able to isolate three lipoprotein fractions whose lipids were analyzed by microchromatography, and the protein moiety studied by gel electrophoresis. Two lipoproteins (PV 1 and PV 2) and one lipopoprotein fraction (PV 3) were detected for the first time in gastropods (Garin *et al*., 1996). They represent 57.0, 7.5 and 35.5% of the egg total proteins, respectively. Compositions are shown in Table 2.

PV 1 is a glyco-carotene-protein complex with characteristics of a very high density lipoprotein (VHDL). It has 0.33% lipids, mainly free sterols and phospholipids. PV1 is a red lipoprotein particle of high molecular weight. This particle was first described by Cheesman (1958) in the same snail, and it was called ovorubin. More recent results (Garin *et al*., 1996) showed that this carotene-glycoprotein complex has also lipids attached (0.33%), which account for 6.7% of the total lipids of the perivitelline fluid. The presence of lipids has not been reported before for the ovorubin and it would allow the inclusion of PV 1 as a lipo-glyco-caroteneprotein, similar to other invertebrate lipovitellins (Zagalsky, 1985; Cheesman *et al*., 1967). Nevertheless, it can not be considered a true lipovitellin because it is not transported by hemolymph to the vitellogenic oocytes, but it would be incorporated to the fertilized oocyte as a secretion of the albumen gland instead. This distinction between ovorubin and true lipovitellins agrees with some of the differences reported by Zagalsky (1971) on the amino acid composition.

PV 1 would be taken up by the developing embryo by pinocytosis, as some authors have pointed out for

other components of the perivitelline fluid (Elbers and Bluemink, 1960; Rigby, 1979; Raven, 1972). The fact that the predominant lipid classes of ovorubin are biomembrane components (free sterols and phospholipids) would suggest that the major function of these lipids during embryogenesis would be structural, while galactogen and apoproteins would provide the energy and precursor molecules. The approximate molecular weight of 300 Kd agrees with the one found by Cheesman (1958) using another methodology. In his original work, Cheesman did not study the subunits of ovorubin. According to our results, the approx MW of the three subunits found was 35, 32, and 28 kDa (Garin *et al*., 1996).

Considering its hydration density, PV 2 lipoprotein also falls into the VHDL range. It is a high molecular weight particle of 400 kDa. The major lipid classes of PV2 are free sterols and phospholipids and they also have significant quantities of energy-providing triacylglycerides and free fatty acids. This particle is composed of two subunits as revealed by SDS-PAGE, one of them 67 kDa and the other 31 kDa. The presence of the PV 2 particle in the perivitelline fluid was unexpected, since in previous descriptions of gastropod perivitellus proteins (Morril *et al*., 1964; Cheesman, 1958) there was no mention of a particle comparable to PV 2. It is the least concentrated of the three lipoprotein fractions of perivitellus (7.3%) and holds 3.75% lipids which corresponds to 10.1% of the total lipids for the egg. The lipid class composition of this particle suggested it may play an important role as both structural and energy source because it has significant amounts of triacylglycerides and free fatty acids as well as free sterols and phospholipids. A detailed study on the structure, aminoacid composition, and lipid binding properties of this lipoprotein would establish patterns and homologies with other proteins of great evolutive interest, and it is the subject of ongoing research in our laboratory.

Fraction PV 3 is composed of at least three lipoprotein particles. Hydration density of this fraction corresponds to the upper limit of HDLs. Analysis of this

TABLE 2.

Lipid class composition of lipoprotein fractions from the perivitelline fluid of *P. canaliculata.*

	μg lipid / g egg, wet wt (% w/w)			
LIPID CLASSES	PV 1	PV 2	PV 3h	PV 3p
HYDROCARBONS +	tr	3.4	tr	33.3
ESTERIFIED STEROLS	-	(8.5)	-	(28.1)
TRIACYLGLICERIDES	0.3	7.0	17.0	13.9
	(1.0)	(17.5)	(8.1)	(11.8)
FREE FATTY ACIDS	1.4	6.4	38.5	19.9
	(5.5)	(16.0)	(18.4)	(16.8)
FREE STEROLS	13.7	8.9	86.7	7.5
	(51.7)	(22.3)	(41.5)	(6.3)
PIGMENT	2.6	1.8	14.7	26.5
	(9.9)	(4.5)	(7.0)	(22.4)
PHOSPHOLIPIDS	8.4	12.5	52.8	17.2
	(32.0)	(31.2)	(25.2)	(14.6)
TOTAL	26.4	39.9	209.1	118.2
	(100)	(100)	(100)	(100)

Values are the mean of triplicate analyses. Values in parenthesis correspond to the percentage of each lipid class w/w; tr: traces; -:not determined.

fraction allowed us to study a separate particle "p" which is a carotenoprotein. This particle has an absorption maximum at 420 nm; it is responsible for the strong yellowish color of the fraction obtained from the ultracentrifuge gradient. This absorption maximum was already reported by Cheesman (1958) for the whole homogenate of the eggs during ovorubin purification. This author suggested that the chromoprotein responsible for that peak would be helicorubin, a chromoprotein found in the digestive tract of *H. pomatia.* Particle "p" is the most lipid-rich particle with 9.5% lipids that corresponds to 30% of the total perivitelline lipids. It is the fraction with the least concentration of proteins (8%) and it contains most of the carotenoid pigment as found by comparison of the absorption spectra at 420 nm of all fractions and also by the amount of the acetone-mobile peak in TLC-FID. The other fraction of PV 3 (fraction "h") is more heterogeneous, it represents 27% of the total protein and contains 5.16% lipids. The lipids account for 53.2 % of the total lipids of the perivitelline fluid. The major lipid classes are free sterols and phospholipids, as in the other two fractions, but in this case PV 3 also contains free fatty acids. The two subfractions, "h" and "p" have different lipid and apoprotein composition. Fraction "h" lipids are mainly free sterols, phospholipids and free fatty acids, and two particles of 100 and 64 kDa. Dissociating electrophoresis showed two subunits of 34 and 29 kDa. Fraction "p" is composed of a single particle of 26 kDa. It has high levels of a carotenoid pigment. Besides it contains free fatty acids, hydrocarbons, sterified sterols and triacylglycerides.

In general, the composition and amount of lipids suggested *P. canaliculata* egg lipoproteins would play a role in providing structural components and metabolic precursors for the developing embryo, and that they would not be considered only as energy sources for the embryo as lipovitellins are. In conclusion, all lipoprotein fractions of the perivitelline fluid of this freshwater prosobranch fall into the VHDL and HDL categories. This is basically the only feature in common with other invertebrate and vertebrate lipovitellins (Lee, 1991) because, as we already stated, it is not possible to consider homologies between this perivitelline lipoproteins and yolk lipovitellins based on their functional analogy.

Egg lipoprotein dynamics during embryogenesis

To determine the lipoprotein dynamics during embryogenesis, we followed each of them in perivitelline fluid and inside the embryos from the moment of fertilization until hatching (Heras *et al.*, 1998). In that study, egg development was divided into 5 stages according to major developing events from morulla until 3-day juveniles measuring for the first time in molluscs the utilization of the different lipid classes, proteins and carbohydrates in the embryo and the purified perivitelline fluid separated from each other. Embryo, egg shell and perivitellus were separated using a Percoll® discontinuous density gradient. Samples were loaded onto the gradient and then centrifuged at low speed. Thus, a shell-containing pellet was formed while the embryos were located in the interphase of the two Percoll solutions, and the perivitelline fluid floated on top of the gradient. Stage V embryos were manually isolated from perivitellus under a stereoscopic microscope. Stage VI juvenile snails were collected manually on their first 3 days of free life.

During development, total protein levels undergo significant changes, decreasing in the perivitelline fluid while increasing in embryos. Morrill *et al.* (1964) also found that the perivitelline fluid proteins were used for the growth of *Lymnaea* embryos. Embryos of *P. canaliculata* took up 90% of perivitelline fluid proteins with a sharp decrease in perivitellus proteins between stages IV and V.

Interestingly, there was no net increase in embryo protein content until hatching, suggesting that most proteins incorporated at stages IV and V must have been either consumed as an energy source or converted into other components. A trend to increase the protein level between stages III and IV was also observed. Some of these findings were clarified when the dynamics of individual protein behavior during development was studied by electrophoresis (Fig. 1). Perivitelline fluid from stage I to V (Fig. 1A) shows the lipoprotein bands corresponding to the perivitellins PV1 (300 kDa), PV2 (400 kDa) and PV3 (100, 64 and 26 kDa) (Garin *et al.*, 1996). PV1 is the most abundant lipoprotein, followed by PV2. PV3, which is a minor component better identified in Fig. 1B by its 21 kDa apoprotein band. Perivitelline fluid at stage V is very poor in proteins and it seems to conserve mostly the stored perivitellin PV1 which is a thermostable protein. Its presence throughout development would serve as an osmotic regulator, and the pigment that PV1 contains would protect the embryo from sunlight as suggested by Cheesman (1958).

Embryos from stage II to IV absorb PV1 from perivitellus (Fig. 1A) and to a lesser extent, PV2 (67 kDa band of Fig. 1B). New proteins are synthesized in the embryo, such as one of 560 kDa which becomes quantitatively more important at the end of development. Although no net increase in the embryo protein content between stages IV and V was observed, new protein

bands appeared corresponding to PV2, PV3 perivitellins and a 560 kDa protein, suggesting that the uptake systems along with the catabolic pathways of embryos are more active at these stages. Embryos at stage V displayed a pattern characterized by a higher concentration of PV2 and PV3 (Figs. 1A and 1B, respectively). After hatching, only PV1 and the one of 560 kDa were found to be the major proteins. On the other hand, PV2 (Fig. 1A) and PV3 were almost absent, probably used as an endogenous source of energy and structural molecules during this transition to free life (Fig. 1B).

It is interesting to note that egg perivitellins are temporarily stored unaltered by the embryos. After hatching (stage VI), the perivitellins are rapidly depleted in juveniles. Hatchlings show an important increase in their total protein content which is difficult to explain unless there is an external carbon source. Therefore, protein conversion efficiency (calculated as % of perivitellus energy transformed into embryonic tissues) was calculated between stages I and V, and the low value observed 29.0% suggests they are not only used for embryo structure but also as an energy source.

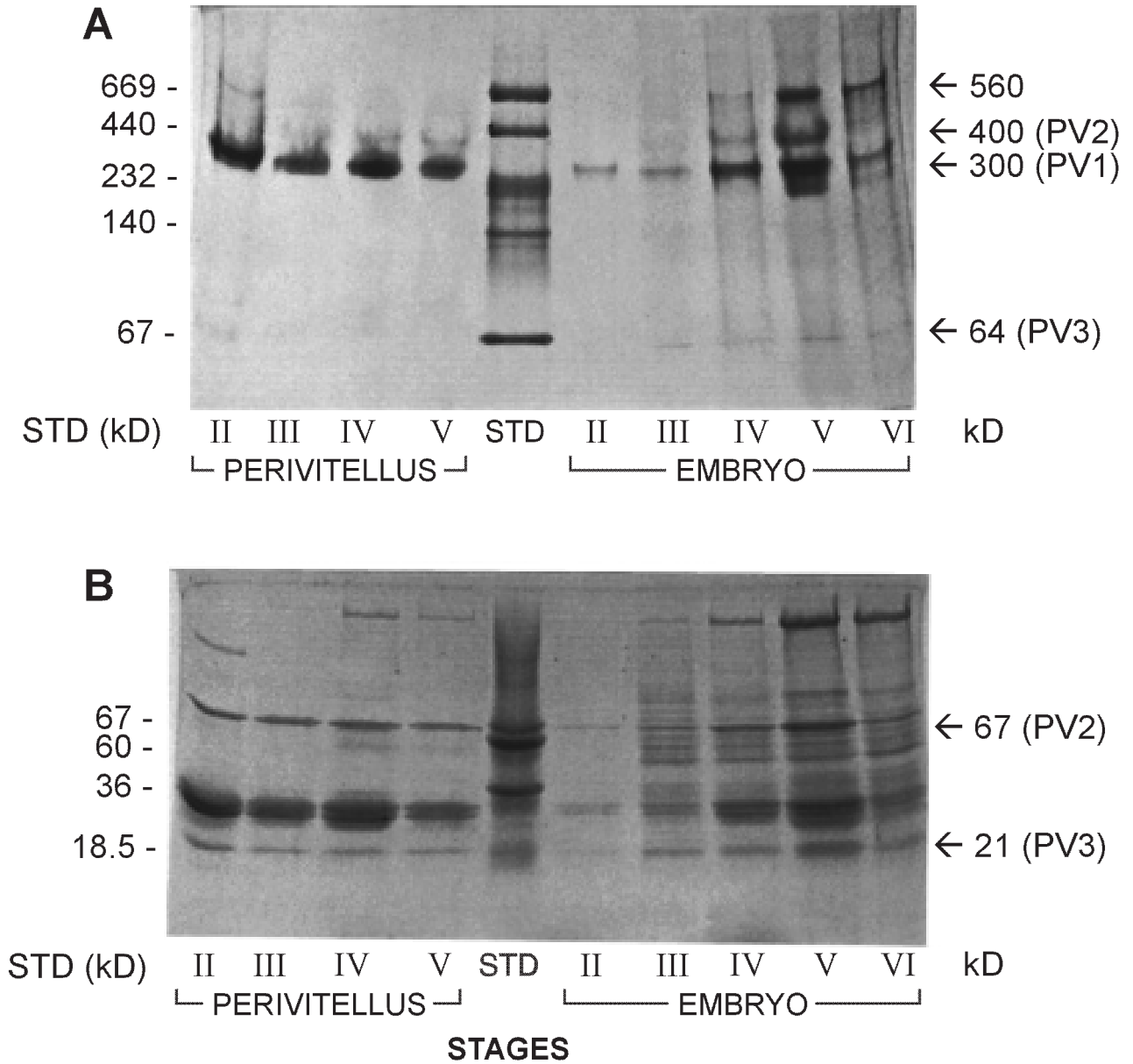

FIGURE 1. Native (A) and dissociating gel electrophoresis (B) of embryo and perivitelline fluid samples from each developing stage. (Taken from Heras *et al.*, 1998, with author permission)

We could, therefore, suggest that the absorption of perivitelins from the perivitelline fluid is divided into two phases. The first one shows a mild uptake up to stage IV followed by a second phase of very active uptake of all nutrients and selective consumption of proteins from stage IV to V where they would be utilized for organogenesis. After snail hatching, there would be a specific consumption of PV2 lipoprotein and its associated triacylglycerols.

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