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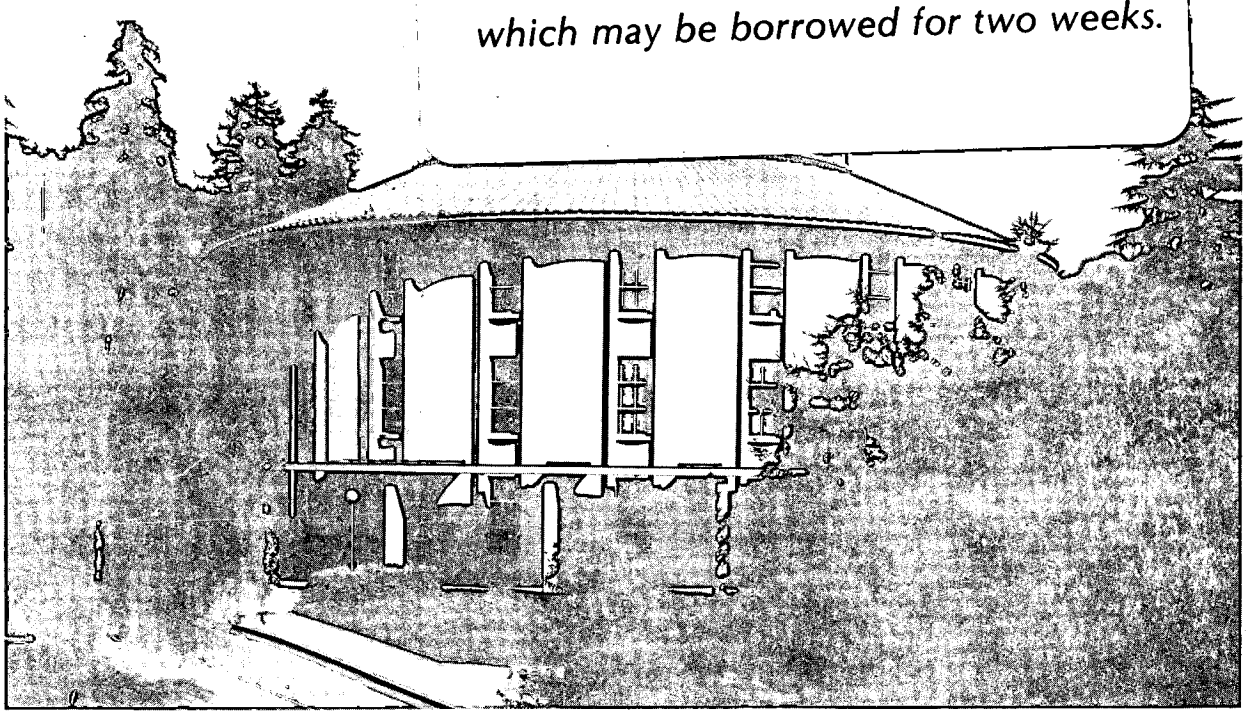

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Triterpenoid Biosynthesis in Euphorbia lathyris Latex Associated with a Vacuole.

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Abstract

Latex isolated from Euphorbia lathyris laticifer cells maintains its ability to synthesize triterpenols (and their esters) from acetate. When the latex is centrifugated at 5000 xg for 15 min., this biosynthetic activity can be subdivided into two separate fractions: the acetate to mevalonic acid activity remains in the supernatant, while the mevalonic acid to triterpenol activity is pelleted. Further purification of the pellet by isopycnic centrifugation on Percoll gradients yielded a particle responsible for the conversion of mevalonic acid to triterpenol. Electron microscopy of this particle and comparison with marker enzyme activity indicated that this organelle is a vacuole.

Additional key words: hydrocarbons, isoprenoids, latex particles

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Introduction

The laticifer cells of Euphorbia species allocate a significant amount of their carbon to the production of triterpenoids; triterpenols and their fatty acid esters constitute up to 50% of the dry weight of Euphorbia lathyris latex (Nemethy et al. 1983). These energy-rich compounds may provide a renewable alternative to petroleum for both fuel and chemical feedstocks. Before isoprenoid-producing plants can become an economically viable alternative to fossil fuels, their isoprenoid yields must be increased. This will require an understanding of the physiological processes that control carbon allocation to isoprenoid production. Since subcellular compartmentation is one form of biological control, we have attempted to isolate the various particles involved in the biosynthesis of these isoprenoids so that we may further study their role in the regulation of hydrocarbon production.

Many previous studies of the subcellular organization of the laticifer cell have been done with Hevea brasiliensis. Ultrastructural studies have shown that in addition to the normal cellular structures, including nuclei, mitochondria, endoplasmic reticulum and ribosomes, Hevea laticifers contain three unique particles (for review, see Archer 1980). The major unique component, called a lutoid, is a single-membrane-bounded structure with a diameter from 0.5 to 5 μm. A second, less abundant particle is the Frey-Wyssling complex, a double-membrane-bounded particle 4 to 6 μm in diameter. This organelle contains lipid globules and various membrane structures, and is thought to be a plastid. The third structures are minute polyisoprene (rubber) particles.

When Gomez (1975) tapped latex from H. brasiliensis plants he found lutoids, Frey-Wyssling complexes and polyisoprene particles, but few mitochondria or nuclei were observed. The lutoids were separated by centrifugation, and found to comprise about 20% of the total latex volume. The biochemistry of these organelles has been well studied. They have been found to contain acid hydrolases, peroxidase, lysozyme, and α-mannosidase, and accordingly are thought to be lysosomal vacuoles (D'Auzac et al. 1982).

Ultrastuctural studies of Euphorbia species have shown that their laticifers resemble those of H. brasiliensis. Although the major isoprenoid components of Euphorbia species are triterpenoids, not rubber, these hydrocarbons are still contained in small particles (Groeneveld

1976). Fineran (1982, 1983) examined the laticifers of both mature and developing tissue of Euphorbia pulcherrima.. Carefully fixed mature tissue had a wall-lining layer of cytoplasm bounded by the plasmalemma, and a large central vacuole with an intact tonoplast, indicating that the mature laticifer was a living cell. Nuclei were observed, but mitochondria were poorly differentiated and ribosomes were scarce. Plastids containing a single large starch grain were also observed. The triterpenoid particles were contained within the central vacuole. Laticifers in the developing tissue of the sub-apical region of the stem had many smaller vacuoles each containing triterpenoid particles, along with normal appearing mitochondria and higher levels of ribosomes.

Investigation of exuded Euphorbia latex has centered in large part on the unique rod-shaped starch grains. Mahlberg and his coworkers (Mahlberg 1973, Mahlberg et al. 1983) have used starch grain morphology along with latex triterpenoid composition to determine phylogenic relationships within the genus. Some work has been done on isoprenoid production in isolated latex. Ponsinet and Ourisson (1967, 1968), Bisboer and Mahlberg (1979), Groeneveld et al. (1982, 1987), and Nemethy et al. (1983) have all reported on various aspects of isoprenoid biosynthesis in isolated Euphorbia latex. Groeneveld et al. (1987) reported that triterpene biosynthesis occurred in two separate sites, the amyloplast and latex particle-containing vesicles.

We have combined centifugation techniques with biochemical assays and electron microscopy to determine the structure-function relationship of the various components of Euphorbia lathyris latex. In this paper we report on the assignment of a biochemical function to one of the latex subcellular particles.

abbreviations: MES, 2-N-morpholinoethane sulfonic acid; MVA, mevalonic acid; SEM, scanning electron microscopy; TEM, transmission electron microscopy; TP, triterpenols; TPE, triterpenol-fatty acid esters.

Materials and Methods

Plant Material. Euphorbia lathyris L. plants were propagated from seed collected in the wild

near Healdsburg, California. Plants were grown in a Controlled Environment PGW-36 growth chamber, under a light regime of 16 h day at 600 μmol quanta m^{-2} s^{-1}, followed by 8 h of darkness. Light was provided by a combination of General Electric warm-white fluorescent and General Electric 52 watt incandescent lamps. The temperature was set at 27 °C day/ 18 °C night. Latex was collected from shallow incisions made by razor blade at the base of the petioles.

Centrifugation. Latex was first fractionated by differential sedimentation. Two hundred microliters of latex was combined with an equal volume of centrifugation buffer (final concentration of 10 mM MES, pH 5.5, 0.25 M sorbitol, 2 mM $MgCl_2$) in a 1.2 ml microfuge tube and spun at 5000 xg for 10 min. in a Beckman 11 microfuge. This pellet was used for much of the biochemical and structural analysis.

Further fractionation was accomplished by isopycnic centrifugation of the 5000 xg pellet on Percoll gradients. Percoll (Pharmacia) was dialysed overnight against 10 mM MES, pH 5.5. A self-generated gradient (ρo=1.015-1.14) was formed by mixing 3.1 ml of the dialysed Percoll with 7.9 ml of centrifugation buffer (same final concentration as above) in a 15 ml tube, and centrifuging in a Beckman Ti50 rotor at 30,000 xg for 30 min. Density Marker Beads (Pharmacia) were used to monitor the formation of the gradient. The 5000 xg latex pellet was resuspended in I ml of the centrifugation buffer, layered on the top of the Percoll gradient, and centrifuged at 800 xg for 25 min. at 4 °C. The gradient was fractionated from the bottom of the tube into 1 ml aliquots.

Biosynthesis Assays. Triterpenol biosynthesis from acetate and MVA was monitored in whole latex, 5000 xg supernatant and pellet, and Percoll gradient fractions. Whole latex (200 μl) was combined with an equal volume of centrifugation buffer and added to a tube containing U-^{14}C-acetate (final concentration = 1.0 mM, 8.25 mcuries $mmol^{-1}$) and/or ^{3}H-MVA (final concentration = 0.75 mM, 33 mcuries $mmol^{-1}$). Samples were incubated for 2-4 h and then quenched by the addition of acetone. The triterpenols were extracted with hexane and the solvent was evaporated under a stream of nitrogen gas. The triterpenols and triterpenol esters were further purified by thin layer chromatography on silica gel G plates (Analtech), developed in ethyl ether: petroleum ether (3:1). The triterpenols and their esters were localized by comparison of a marker spot (visualized by spraying with H_2SO_4 (conc) followed by charring)

with known standards. Bands were cut corresponding to the triterpenols and triterpenol esters, and radiolabel content was determined by performing liquid scintillation counting on the material scraped from half of each band. The incorporation of radiolabel into sterols was confirmed by eluting the triterpenols from the other half of these bands and separating them further by HPLC. The eluted compounds were chromatographed on an Altex ODS column with 100% methanol, monitored at 214 nm, and retention times of the peaks were compared to those of known triterpenols. These fractions were collected and analyzed by liquid scintillation counting.

The 5000 xg supernatant was assayed as above, substituting the supernatant for the whole latex/buffer mix. The 5000 xg pellet was resuspended in 400 µl of centrifugation buffer and assayed. The reconstituted latex was assayed after recombining a 5000 xg pellet with 5000 xg supernatant. Two hundred fifty microliter aliquots of Percoll gradient fractions were added to directly to the ^{3}H-MVA and assayed.

Marker Enzyme Assays. The presence of subcellular organelles in the gradient fractions was monitored using specific marker enzymes. Endoplasmic reticulum were identified by a cytochrome reductase assay (Phillips and Langdon, 1962), mitochondria by a fumarase assay (Hill & Bradshaw 1969), peroxisomes by a catalase assay (Maehly & Chance 1954), and vacuoles by a α-mannosidase assay (Chrispeels & Boulter 1975). Protein content was determined using the procedure of Vincent and Nadeau (1983).

Electron Microscopy. All buffered solutions, unless noted otherwise, used 0.1 M Na cacodylate, pH 6.0 as the buffer.

The 5000 xg pellet was prepared for scanning electron microscopy (SEM) by dividing it up into small pieces and then adding 1 ml of fixative (2% glutaraldehyde, 1% OsO_4, in cacodylate buffer) and incubating for 1 h in the dark on ice. The sample was then centrifuged at 5000 xg for 15 min, and the supernatant removed. The pellet was washed 3x with cacodylate and incubated for 30 min at room temperature in I ml of 1% glutaraldehyde in cacodylate. Following the removal of the glutaraldehyde solution, the sample was washed 3x with buffer, postfixed by incubation with 1 ml of buffered 0.5% OsO_4 for 20 min, and then was washed 8x with buffer.

The sample, broken into small pieces, was dehydrated using an ethanol series and critical point dried from liquid CO_2. The dried, powdery sample was mounted on stubs by shaking onto drying graphite glue. The samples were sputter coated with a 15 nm Pt layer using a Polaron sputter coater equipped with a quartz crystal thickness monitor.

Percoll gradient samples were prepared for SEM using the technique of Mazia et al (l975). Fractions from a gradient were applied to poly-L- lysine coated cover slips, which were allowed to sit for 20 min. on ice, and were then rinsed 4x with a buffered solution (50 mM MES, pH 6.1, 0.4 M sorbitol, 5 mM $MgCl_2$, 5 mM DTE). The samples were fixed for 1 h in 2% glutaraldehyde in cacodylate buffer, washed with buffer, and then postfixed in buffered 1% OsO_4. The samples were rinsed with distilled water and dehydrated as before with an ethanol series. The samples (in 100% ethanol) were dried from liquid CO_2, mounted on stubs and coated with 15 nm Pt. All SEM was performed with an ISI DS-130 SEM at 10 kv.

The 5000 xg pellet was prepared for transmission electron microscopy (TEM) by incubation with 5 ml of 1% glutaraldehyde and 0.5% OsO_4, in cacodylate buffer, in the dark for 1 h at 0-4 °C. The sample was centrifuged at 1000 xg for 5 min, and rinsed 3x with 0.1 M Na cacodylate, pH 7.0. The sample was then postfixed in 4 ml of 2% glutaraldehyde in 0.1M Na cacodylate, pH 7.0 for 30 min and rinsed 3x with Na cacodylate buffer. Samples were next incubated in 4 ml of 0.5% OsO_4 for 45 min, and rinsed 3x with water. The samples were dehydrated through an ethanol series to 70% ethanol, placed in a 1:1 mixture of 70% ethanol and LR White resin (Polysciences, Inc) for 30 min, and then infiltrated with three changes of LR White resin. The samples were placed in capsules, degassed for 1-2 h and hardened for 18-20 h in a vacuum oven at 60 °C. Thin sections were cut from the resin blocks using a glass knife, collected on formvar-coated 200 mesh copper grids, and poststained with 5% uranyl acetate in water for 30 min and lead citrate stain for 5 min (Reynolds 1963).

Fractions from the Percoll gradients were prepared for TEM by incubation with the fixative solution (final concentration 0.3% OsO_4 and 1% glutaraldehyde in in cacodylate buffer) for 1.5 h at 0-4 °C. The samples were then centrifuged at 5000 xg for 15 min, and the pellets washed 3x with cacodylate buffer, followed by two washes of distilled water. Samples were dehydrated

using an 8 step ethanol series to 70% ethanol, then infiltrated with a 1:1 solution of 70% ethanol and LR White resin for 45 min followed by incubation with 100% LR White resin overnight. The samples were then incubated with fresh LR White resin for 1.5 h and placed in capsules which were degassed and hardened as before. Sections were cut with a glass knife, collected on naked 300 mesh copper grids, and poststained with uranyl acetate and lead citrate. All TEM was performed with a Zeiss 109 TEM at 80 kv.

Results

As we have reported previously (Nemethy et al. 1983, Skrukrud et al 1987) isolated latex is capable of converting both acetate and MVA to the triterpenols and their esters (TP+TPE) (Figure 1,Table 1). When latex was fractionated by differential centrifugation the ability to use both of these substrates was altered. The 5000 xg supernatant exhibited little TP+TPE biosynthetic activity. The level of acetate incorporation was reduced to 21% of that of whole latex, and the incorporation of MVA into triterpenols was negligible at 2% of whole latex rates. The 5000 xg pellet showed no acetate to triterpenol activity, while the ability to convert MVA into triterpenols remained relatively high at 38% of control levels. Recombining the supernatant and the pellet restored acetate to triterpenol activity to 45% of whole latex values, and MVA incorporation was increased slightly above that of the pellet to 41%.

The ratio of incorporation of MVA into triterpenol esters and tritepenols also changed with centrifugation. Whole latex had a TPE/TP ratio of 6.7, while the 5000 xg pellet ratio dropped to 0.8. When the supernatant and pellet were reconstituted the ratio returned to 6.8. The incorporation of MVA in the supernatant was too low to generate an accurate TPE/TP ratio.

To further purify the organelles, the 5000 xg pellet was resuspended in buffer, applied to a Percoll density gradient and centrifuged. Fractions were collected from the gradient, and aliquots from these fractions were tested for the ability to convert ^{3}H-MVA into TPE's and TP's. In addition, marker enzyme assays were performed on aliquots of the fractions to identify the presence of specific organelles, and protein levels were determined. The particle responsible for the conversion of MVA to TP+TPE banded within a narrow region in fractions 3-5 (figure 2). This corresponded to a major peak of α-mannosidase activity, indicating the presence of vacuoles within this region. The major microsomal peak, as indicated by cytochrome c reductase

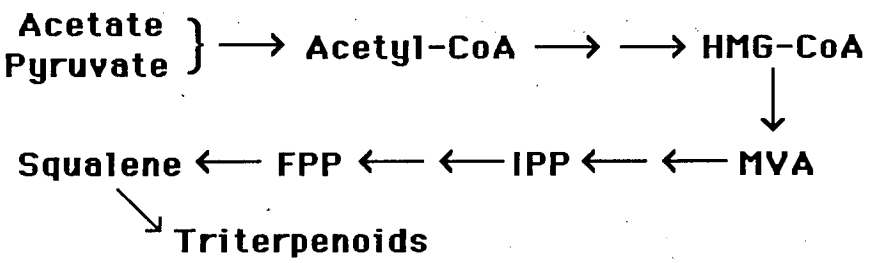

Figure 1) The triterpenoid biosynthetic pathway

Table 1

Incorporation into Triterpenols and Triterpenol Esters
(p mol substrate incorporated hr^{-1} ml $latex^{-1}$)

substrate:	Acetate	MVA	Triterpenol Esters: Triterpenols
Sample			
whole latex	24.2	530	6.7
5000 xg supernatant	5.0	9.0	1.7
5000 xg pellet	0	158	0.8
reconstituted (supernatant + pellet)	10.8	217	6.6

The triterpenol ester: triterpenol ratio was calculated from the assays using MVA as the substrate.

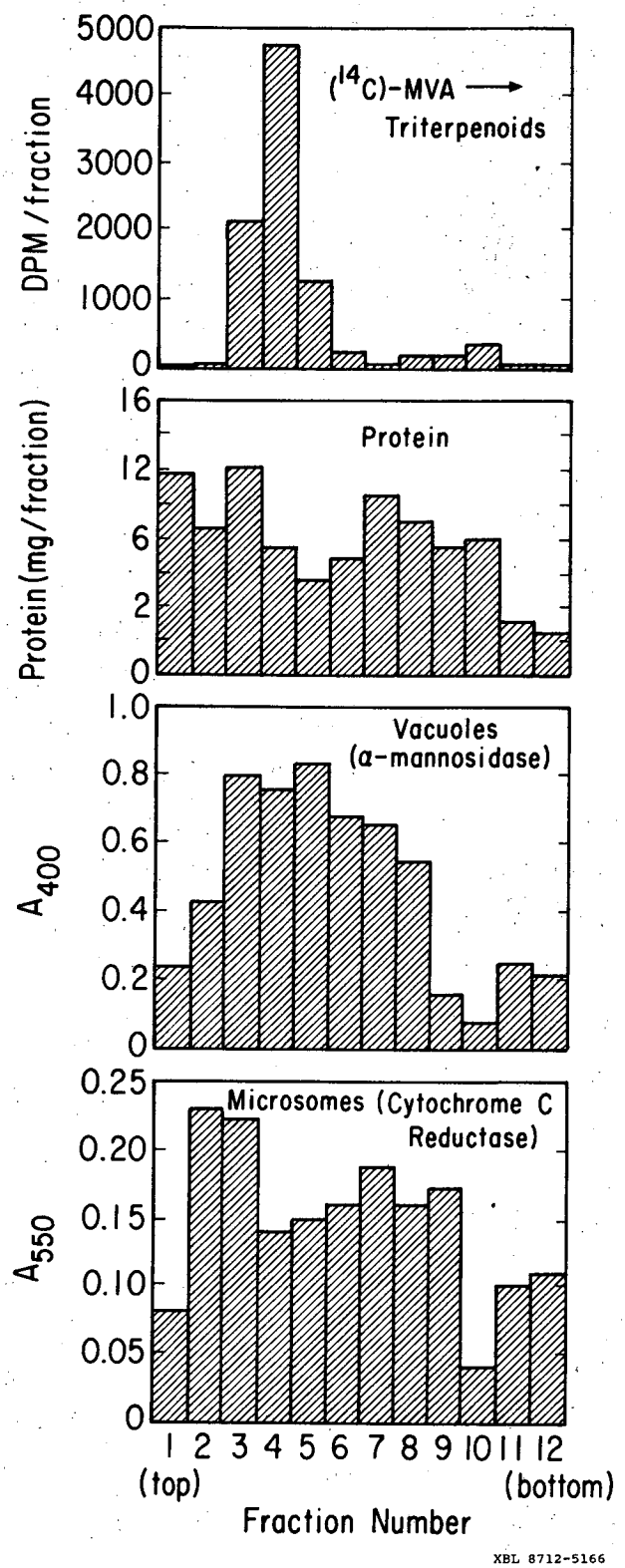

Figure 2) Activity of fractions collected from a Percoll gradient. The 5000 xg pellet was applied to the gradient and centrifuged as described in the text. Fractions were collected and assayed for triterpenoid biosynthetic activity A, protein content, α-mannosidase activity, and cytochrome C reductase activity. Assays for fumarase activity (for mitochondria) and catalase activity (for microbodies) were also performed; no activity for either was detected. activity, occurred above the band of TP+TPE synthesis. No fumarase nor catalase activity was detected, indicating that there were no intact mitochondria or microbodies in the gradient.

Scanning electron micrographs of the 5000 xg pellet were dominated by the presence of rod-shaped starch grains with lengths from 8 to 34 μm (figure 3). Other regular ovoid structures with lengths of 4 to 12 μm were also seen in this fraction (figure 4). Examination of thin sections of the 5000 xg pellet by TEM showed the presence of numerous ovoid structures with diameters from 0.5 to 4 μm (figure 5). Some of these structures had internal osmophilic structures, while others had a more dispersed and diffuse substructure. In addition, many osmophilic particles with diameters of 0.125 to 0.3 μm were found outside of the ovoid particles.

Fractions from Percoll gradients were prepared for SEM by first adhering any particles to poly-lysine coated glass coverslips and washing; this method captures negatively charged particles and allows the removal of Percoll particles. Only fractions 1 and 2, above the band of TP+TPE biosynthetic activity, exhibited structure in these SEM's. The structures, regular spheres of about 0.2 μm in diameter (figure 6), are not Percoll; Percoll particles range in size from 15 to 30 nm in diameter. No other structures were observed on the coverslips. Only starch grains were observed in the Percoll pellet (figure not shown);the fraction directly above the pellet (fraction 11) also contained a few starch grains. A structure was found in fractions from the biosynthetically active region of the gradient (fractions 3-5) when observed by TEM (figures 7 and 8) . These structures were found only in this region. They ranged in size from 2 to 9 μm and were bound by a single membrane (figure 8 insert).

Discussion

The results presented in Table 1 show that the conversion of acetate to the isoprenoids can be physically separated into two processes: the conversion of acetate into MVA, and the conversion of that MVA into triterpenoids. The 5000 xg pellet had no measurable acetate to TP+TPE activity but retained a significant proportion of its MVA to TP+TPE activity. When the latex was reconstituted after centrifugation, half of the acetate to TP+TPE activity was restored, showing that this activity was not lost by physical damage from centrifugation, but was actually separated from the pelleted activity.

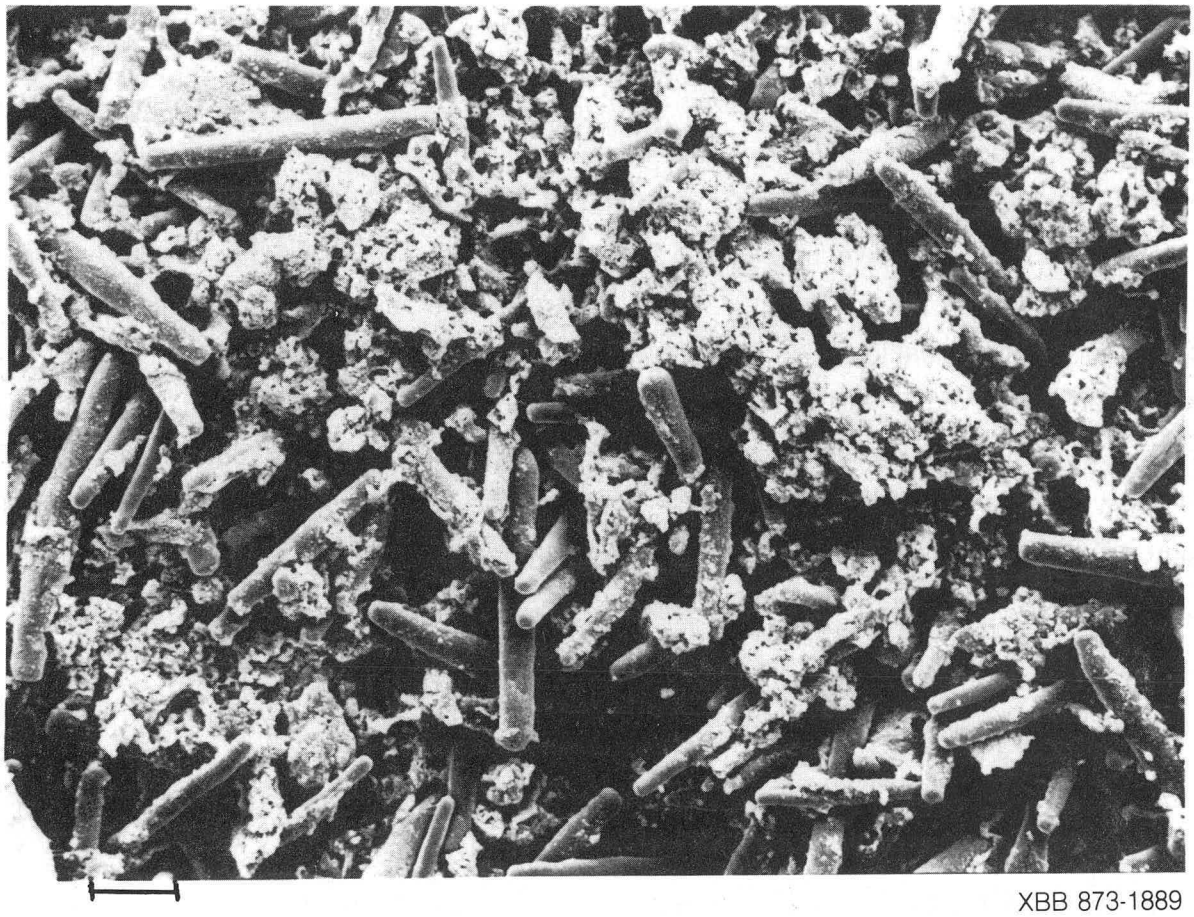

Figure 3) Scanning electron micrograph of dried 5000 xg pellet of *E. lathyris* latex. Bar equals 10 μm.

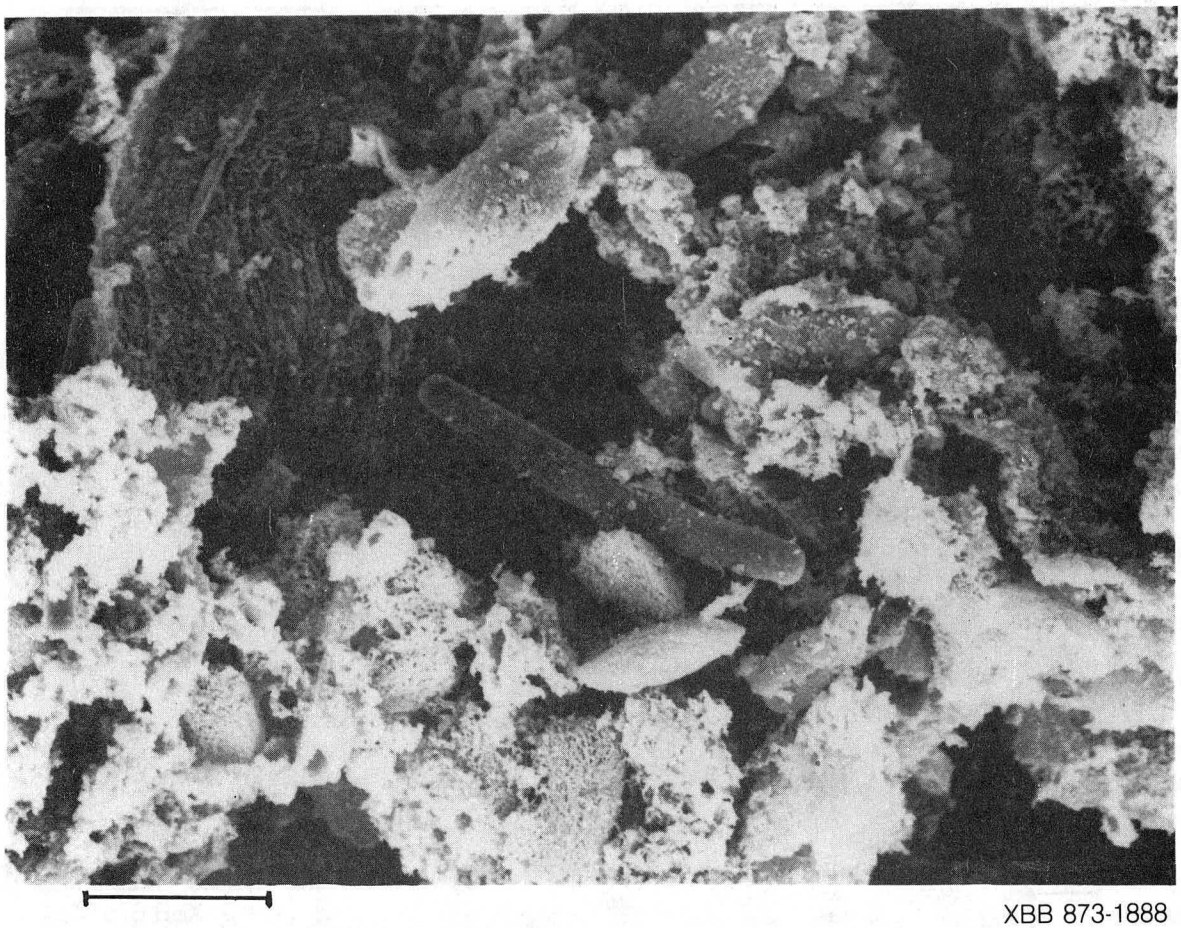

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Figure 4) Scanning electron micrograph of dried 5000 xg pellet of *E. lathyris* latex. Bar equals 10 μm.

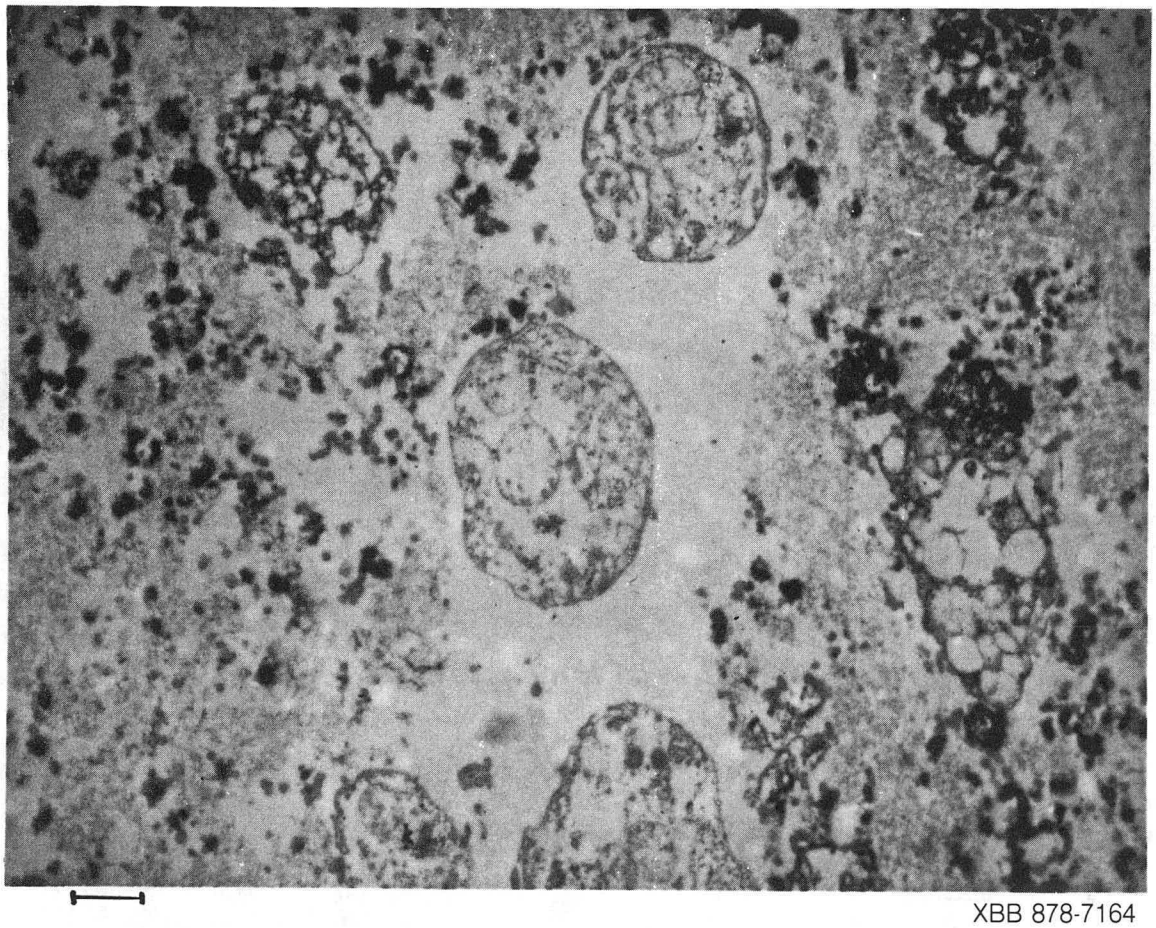

Figure 5) Transmission electron micrograph of resin-embedded 5000 xg pellet of *E. lathyris* latex. Bar equals 1 μm.

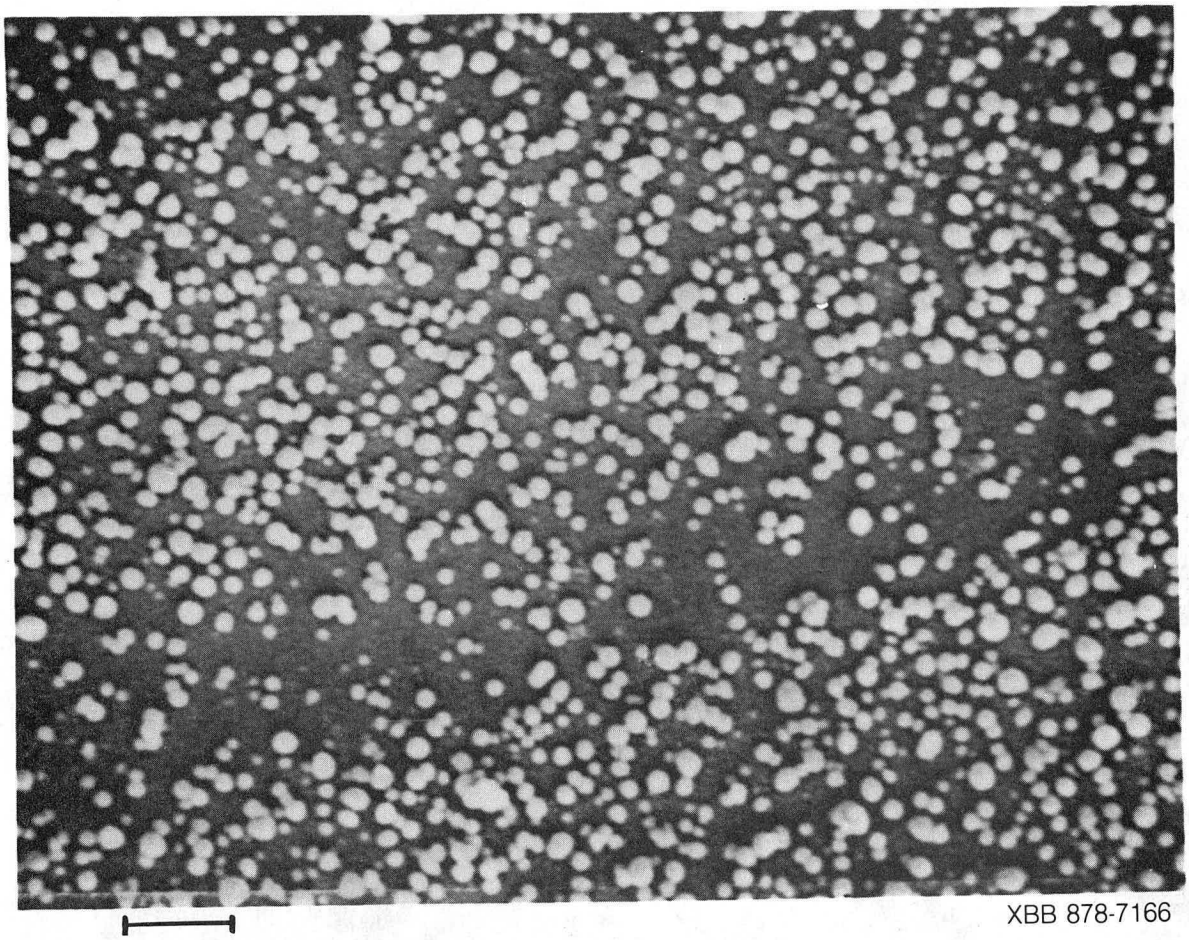

Figure 6) Scanning electron micrograph of latex particles from *E. lathyris* latex, isolated on a Percoll gradient. Bar equals 1 μm.

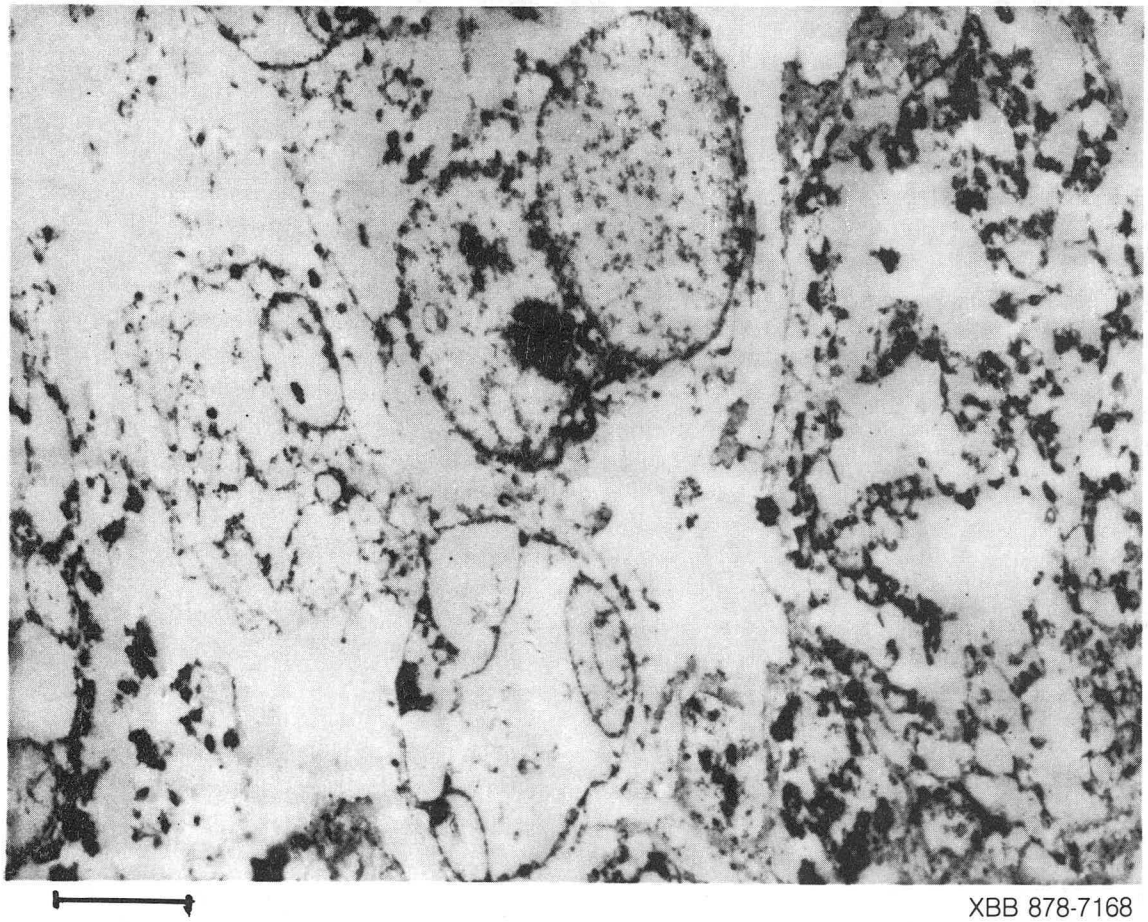

Figure 7) Transmission electron micrograph of triterpenoid-synthesizing fraction of *E. lathyris* latex, isolated on a Percoll gradient. Bar equals 1 μm.

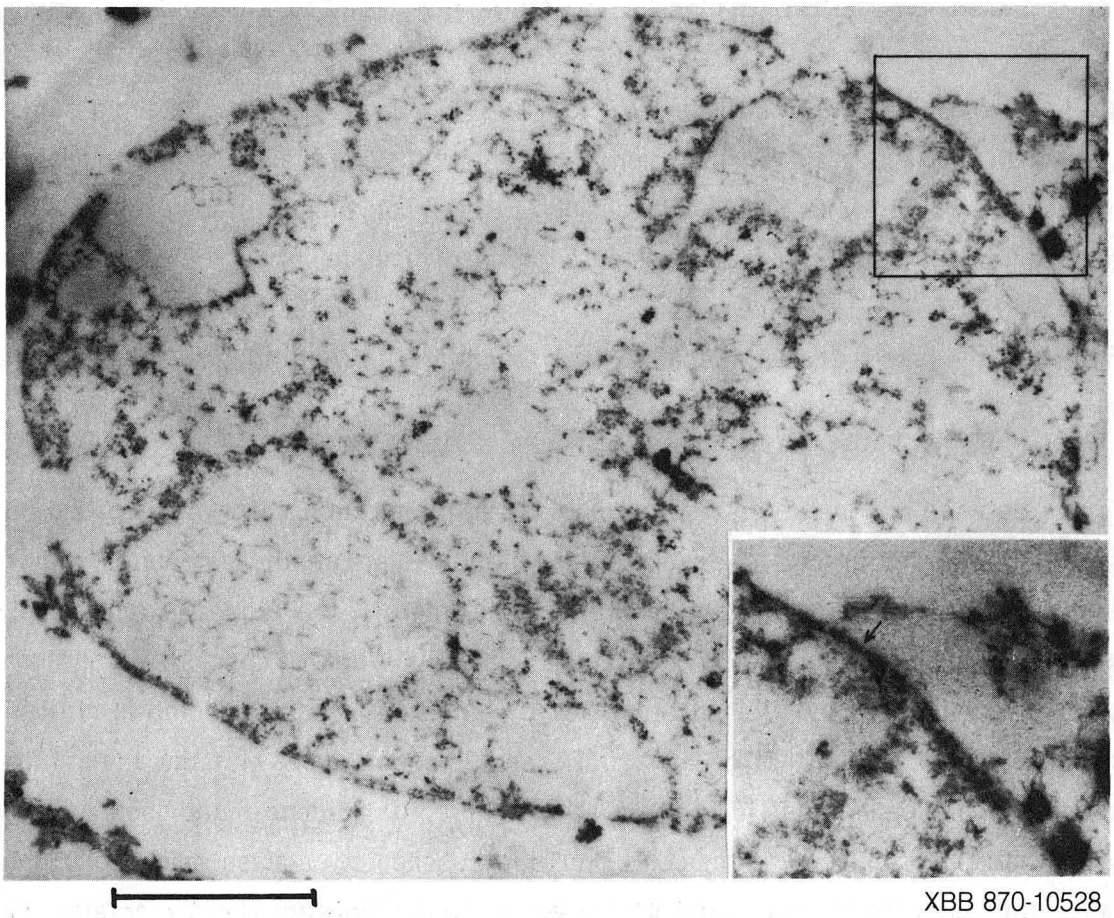

Figure 8) Transmission electron micrograph of triterpenoid-synthesizing structure. Bar equals 1 µm. Insert is an enlargement of bounding-membrane, showing single bilayer structure (arrow).

The reconstituted latex also exhibited a small increase in MVA to TP+TPE activity over the pellet, suggesting a synergistic relationship between the supernatant and the pelleted material. One possible explanation for this observation has been reported previously (Nemethy et al. 1983): the synthesis of the TPE's requires a phospholipid as the acyl donor and the phospholipid pool may be present in the supernatant. This is further supported by a comparison of the ratio of MVA incorporation into TPE and TP. The whole latex had a TPE/TP ratio of 6.7, while the pellet had a ratio of only 0.8, due to a large decrease in TPE biosynthesis. Recombining the pellet with the supernatant restored TPE biosynthesis, and the ratio returned to whole latex levels (6.8).

The 5000 xg supernatant had little MVA to TP+TPE activity (2%), but retained some of its ability (21%) to utilize acetate. At first glance this is confusing, since it should be impossible to utilize acetate without MVA to TP+TPE activity. But the conversion of acetate to MVA is rate limiting (Skrukrud et al. 1987), and the incorporation of acetate into TP+TPE occurs at only 5% of the MVA to TP+TPE rate. Thus the MVA to TP+TPE activity that remains in the supernatant, while low, is still capable of converting any MVA produced from acetate into triterpenoids.

We were able to observe three different particles in the electron micrographs of the 5000 xg pellets and the Percoll gradient fractions: membrane-limited organelles, starch grains, and triterpenoid or latex particles. The structure of most interest, the organelle (figures 5,7 and 8), was collected from the region of MVA to TP+TPE activity. We have concluded that this vesicle is a vacuole. It is limited by a single membrane, with some organized internal structure. Its physical characteristics are that of a vacuole, and it co-migrates with the marker enzyme α-mannosidase, indicating that it is vacuolar in nature. Its structure and lack of fumarase activity both indicated that it is not mitochondrial, the absence of catalase activity eliminates the microbody, and both its structure and its having a greater bouyant density greater than the major peak of cytochrome c reductase activity rule out it being microsomal. It is not a plastid because it is limited by a single membrane. In addition, vacuoles are extremely osmosensitive, which would explain the absence of the organelles on the polylysine-coated coverslips used for SEM of the Percoll gradient fractions.

The observation that the MVA to TP+TPE activity is associated with this structure agrees with

Fineran's (1983) ultrastructural determinations that indicated that E. pulcherrima latex particles are synthesized in tubular vacuoles found in the cytoplasm. Though the size of these vacuoles (2-9μm) is greater than that reported for the vacuolar lutoids of Hevea brasiliensis (1-5 μm; D'Auzac et al. 1982), we believe that this organelle is similar in both structure and function. Groeneveld et al. (1987) also reported that a vesicle was the site of triterpenol biosynthesis, but found that this vesicle contained large amounts of latex particles. In contrast to these data, we were able to physically separate the latex particles from the organelle responsible for triterpenol biosynthesis, which indicating that the site of storage and the site of synthesis differ.

The second structures we observed were starch grains (figures 3 and 4). The elongated shapes observed were characteristic of non-articulated Euphorbia laticifers (Mahlberg 1973). Mahlberg et al. (1983) ascribed the wrinkled surface of these grains in SEM to the membrane of the plastid. Since the site of starch synthesis in higher plants is the plastid, this is a logical conclusion, though the extrachloroplastic production of starch in latex must be ruled out before this structure can be positively identified. We also observed an irregular surface on the starch grains, and these structures did adhere to the polylysine-coated coverslips (figure not shown), indicating a negative charge on the surface. We could not determine if this is due to the outer envelope of an amyloplast or to adhesion of smaller particles to the starch grain's surface. Unlike Groeneveld et al. (1987) we were unable to detect any MVA to TP+TPE biosynthetic activity in fractions containing starch grains. If these structures are amyloplasts, they may have lost their structural integrity during centrifugation, with a resulting loss of biosynthetic capability. Plastids are a likely site of isoprenoid biosynthesis; in whole plant extracts of E. lathyris a major portion of the ß-Hydroxymethyl glutaryl-CoA Reductase activity (the enzyme responsible for the synthesis of MVA) is associated with the plastids (Skrukrud, unpublished results).

The final structures observed were the latex (triterpenoid) particles (figures 5 and 6). Most of these particles were found in the 5000 xg supernatant (data not shown), but some pelleted, probably adhering to more dense particles. The lipid nature of these structures is indicated by their heavy staining by osmium and low buoyant density. They also adhered to the polylysine-coated coverslips, indicating a negative surface charge. The question of whether latex particles are encapsulated within some limiting material has been studied by many groups.

Fineran (1982) observed boundary layer on some latex particles of E. pulcherrima, and Groeneveld (1976) reported a membrane-like film surrounding latex particles of Hoya australis (Asclepiadaceae), but found no membrane around the latex particles of E. milii. Our data suggest that E. lathyris latex particles may indeed be membrane-bounded.

Conclusion

The results indicate that the conversion of acetate to TP and TPE in latex is compartmentalized into two separate subcellular regions. The early steps, the conversion of acetate into MVA, remain in the supernatant when latex is centrifuged at 5000 xg, while the later steps, the utilization of MVA to make isoprenoids, occurr within an organelle that pellets at 5000 xg. The co-migration of this pelleted organelle with the marker enzyme α-mannosidase, and electron micrographs showing that a single membrane encloses this organelle, indicate that this structure is a vacuole.

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