Plant Physiol. (1997) 114: 705–713

Subcellular Localization of Secondary Lipid Metabolites Including Fragrance Volatiles in Carnation Petals[1]

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Pulse-chase labeling of carnation (*Dianthus caryophyllus* L. cv Improved White Sim) petals with [^{14}C]acetate has provided evidence for a hydrophobic subcompartment of lipid-protein particles within the cytosol that resemble oil bodies, are formed by blebbing from membranes, and are enriched in lipid metabolites (including fragrance volatiles) derived from membrane fatty acids. Fractionation of the petals during pulse-chase labeling revealed that radiolabeled fatty acids appear first in microsomal membranes and subsequently in cytosolic lipid-protein particles, indicating that the particles originate from membranes. This interpretation is supported by the finding that the cytosolic lipid-protein particles contain phospholipid as well as the same fatty acids found in microsomal membranes. Radiolabeled polar lipid metabolites (methanol/water-soluble) were detectable in both in situ lipid-protein particles isolated from the cytosol and those generated in vitro from isolated radiolabeled microsomal membranes. The lipid-protein particles were also enriched in hexanal, *trans*-2-hexenal, 1-hexanol, 3-hexen-1-ol, and 2-hexanol, volatiles of carnation flower fragrance that are derived from membrane fatty acids through the lipoxygenase pathway. Therefore, secondary lipid metabolites, including components of fragrance, appear to be formed within membranes of petal tissue and are subsequently released from the membrane bilayers into the cytosol by blebbing of lipid-protein particles.

Lipid-protein particles resembling oil bodies have been identified in the cytosol of carnation (*Dianthus caryophyllus* L. cv Improved White Sim) petals (Hudak and Thompson, 1996). Oil bodies are present in oil-bearing seeds and are formed by blebbing from the ER, the site of triacylglycerol synthesis (Yatsu and Jacks, 1972; Gurr, 1980; Huang, 1992). Indeed, oil bodies typically contain approximately 95% triacylglycerol by weight (Murphy, 1990). They also contain a major structural protein, oleosin, which is thought to prevent their coalescence, particularly during dehydration of the seed (Tzen and Huang, 1992). Oleosins range from 17 to 25 kD in size and have a hydrophobic midsection that loops into the hydrophobic interior of the oil body. The more polar carboxy and amino termini of the protein associate with the phospholipid headgroups on the outside surface of the oil body (Huang, 1992).

The finding that lipid-protein particles resembling oil bodies are present in carnation petals is of interest inasmuch as this is not an oil-bearing tissue. The cytosolic lipid-protein particles from carnation petals are similar to oil bodies in that they can be isolated by flotation centrifugation and contain phospholipid and triacylglycerol, but they are distinguishable from oil bodies in that they have less triacylglycerol and are smaller in size (Hudak and Thompson, 1996). Indeed, cytosolic particles range from 30 to 300 nm in diameter (Hudak and Thompson, 1996), whereas oil bodies are typically 0.2 nm to 2.5 μm in diameter (Huang, 1992). The cytosolic lipid-protein particles also contain a lower-molecular-weight protein (approximately 17 kD) that may be analogous to oil body oleosin (Hudak and Thompson, 1996). Lipid-protein particles similar to those in carnation petals have also been isolated from the cytosol of bean cotyledon tissue (Yao et al., 1991a; McKegney et al., 1995) and from the stroma of chloroplasts (Ghosh et al., 1994). Of particular interest is the finding that the cytosolic and organellar lipid-protein particles are enriched in membrane lipid and protein metabolites (Yao et al., 1991a; Ghosh et al., 1994; Hudak et al., 1995). This has prompted the proposal that their formation may be an integral feature of membrane turnover, allowing removal of metabolites that would otherwise destabilize the structure of the membrane bilayer (Yao et al., 1991a; Hudak et al., 1995). Lipid-protein particles with essentially similar properties can also be generated in vitro from isolated membranes under conditions in which membrane lipid metabolism has been activated (Yao et al., 1991a; Hudak and Thompson, 1996).

There is evidence that certain cell membranes serve as a platform for the synthesis of secondary lipid metabolites. In animal systems, for example, Ca^{2+} appears to mediate the association of lipoxygenase with membranes, which then acts on arachidonic acid to form fatty acid hydroperoxides that lead to the formation of prostaglandins (Axen et al., 1973). In plant cells there are both cytosolic and membranous forms of lipoxygenase (Fobel et al., 1987; Hildebrand et al., 1988; Vianello et al., 1995) that form fatty acid hydroperoxides from free linolenic and linoleic acids. Fatty acid hydroperoxides in turn serve as substrates for both hydroperoxide lyase and allene oxide synthase. Hy-

[1] This work was supported by the Natural Sciences and Engineering Research Council of Canada.

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Abbreviation: Epps, 4-(2-hydroxyethyl)-1-piperazine propane sulfonic acid.

droperoxide lyase is membrane-associated (Hatanaka et al., 1987; Gardner et al., 1991; Matsui et al., 1991) and converts fatty acid hydroperoxides to C6-aldehydes, which are components of flavor and fragrance and also exhibit antimicrobial and antifungal activity (Hamilton-Kemp et al., 1992; Croft et al., 1993; Mishra and Dubey, 1994). The product of the reaction of allene oxide synthase with fatty acid hydroperoxides is an intermediate in the synthesis of methyl jasmonate, which is an active element of plant response to injury (Andresen et al., 1992; Farmer et al., 1992; Yamagishi et al., 1993). Allene oxide synthase is known to be a Cyt P450 (Song and Brash, 1991) and is likely localized in plastids (Song et al., 1993). It is also the major protein of rubber particles (Pan et al., 1995), which have properties in common with cytosolic lipid-protein particles.

Inasmuch as lipoxygenase, hydroperoxide lyase, and probably allene oxide synthase are membrane-associated enzymes, it is likely that secondary metabolites of fatty acids are formed within membranes. These metabolites, like those formed during turnover of membrane lipids and proteins, are nonbilayer molecules that would destabilize membrane structure if allowed to accumulate in the bilayer. In the present study we obtained evidence indicating that cytosolic lipid-protein particles originate from membranes and that their formation may be a mechanism for releasing secondary lipid metabolites from membranes.

MATERIALS AND METHODS

Plant Material and Subcellular Fractionation

Rooted carnation (*Dianthus caryophyllus* L. cv Improved White Sim, California Florida Plant, Salinas, CA) cuttings were grown in a greenhouse in 26-cm-diameter pots containing Pro-Mix BX medium (Premier Horticulture, Riviere-du-Loup, Quebec, Canada) and fertilized on a continuous-feed schedule with 28-14-14 (Plant Products, Brumpton, Ontario, Canada). Lighting was supplemented from dusk to dawn with high-pressure sodium lamps (400 W, 210 μmol m^{-2} s^{-1}). Day and night temperatures were 25 and 18°C, respectively. The flowers were harvested prior to petal senescence, when they were fully open with yellow-tinted centers.

Fractionation of the petal tissue was carried out at 4°C. The petals were removed from freshly cut flowers and homogenized (25 g fresh weight 150 mL $^{-1}$) at 4°C in a homogenization buffer (50 mM Epps, 0.25 M sorbitol, pH 7.4, 10 mM EDTA, 2 mM EGTA, 1 mM PMSF, 1 mM benzamadine, 10 mM amino-*n*-caproic acid, and 4% polyvinylpolypyrrolidone) for 45 s in a mixer (Omnimixer, Sorvall) and for an additional 1 min in a homogenizer (Polytron, Brinkmann). All subsequent resuspensions were in a homogenization buffer without polyvinylpolypyrrolidone (isolation buffer). The homogenate was filtered through four layers of cheesecloth, and the filtrate was centrifuged at 10,000*g* for 20 min. The pellet was discarded, and the supernatant was centrifuged for 1 h at 250,000*g* to sediment microsomal membranes. The supernatant from this centrifugation (postmicrosomal supernatant) was retained for the isolation of cytosolic lipid-protein particles. The microsomal membranes were washed by resuspension in 5 volumes of isolation buffer and centrifugation at 250,000*g* for 1 h and resuspended in the isolation buffer (2 mg protein mL^{-1}). Protein was quantified according to the method of Bradford (1976) using BSA (Sigma) as a standard.

Lipid-protein particles were isolated from the postmicrosomal supernatant by ultrafiltration (Hudak and Thompson, 1996). The postmicrosomal supernatant was centrifuged at 305,000*g* for 12 h to sediment any residual membrane, and the resulting 140 mL of membrane-free cytosol was concentrated to 10 mL by filtration through a 1000-kD cutoff filter (Pharmacia). The particles remained in the retentate and were washed three times by dilution with 3 volumes of isolation buffer, pH 7.4, and subsequent concentration to 10 mL with the cutoff filter. In some experiments, lipid-protein particles were separated from membrane-free cytosol (305,000*g* 12-h supernatant) by Sephadex G-25 column chromatography (25 g, Pharmacia). For this purpose, 3 mL of membrane-free cytosol was loaded onto the column and eluted with 5 × 3 mL volumes of the isolation buffer.

In Vivo Labeling with [^{14}C]Acetate

To establish the origin of lipids in the cytosolic lipid-protein particles, intact carnation petals were labeled with [^{14}C]acetate, and the appearance of label in microsomal membranes and lipid-protein particles was followed over time. For this purpose, the petals were excised from the flower at the point at which the green pigmentation began and placed upright in small cups (2 cm in diameter) containing 4 mL of Epps-sorbitol buffer (50 mM Epps, 0.25 M sorbitol, pH 7.4) and 3.7 × 10^5 Bq in each cup of [1-^{14}C]acetate (1.85 × 10^9 Bq/mmol, NEN Dupont). All incubations were performed in the light at 23°C.

To establish the time course for loading of radioactivity, the petals were allowed to take up the [^{14}C]acetate over a 4-h period, and at 1-h intervals they were removed from the radiolabeled acetate and rinsed in Epps-sorbitol buffer. One-gram samples of the washed petal tissue were then suspended in 3 mL of scintillation fluid (EcoLite, ICN), and levels of radioactivity were determined using a liquid scintillation counter (model LS 101, Beckman).

For pulse-chase radiolabeling studies, the petals were allowed to take up the [^{14}C]acetate for 2 h and were then rinsed with Epps-sorbitol buffer and transferred to cups containing 4 mL of 3.75 mM unlabeled sodium acetate in Epps-sorbitol buffer. At various periods during the incubation in excess unlabeled acetate, portions of the petal sample were removed, rinsed in Epps-sorbitol buffer, weighed, and homogenized for isolation of microsomal membranes and cytosolic lipid-protein particles.

In Vitro Generation of Radiolabeled Lipid-Protein Particles

Radiolabeled microsomal membranes isolated from carnation petals that had been pulse-labeled with [^{14}C]acetate for 2 h were used for in vitro generation of lipid-protein particles (Hudak and Thompson, 1996). Resuspended

membranes were incubated in in vitro buffer (50 mM Epps-0.25 M sorbitol, 1 mM PMSF, 1 mM benzamadine, 10 mM amino-*n*-caproic acid, 40 μM $CaCl_2$, pH 8.3) at 10 mg protein 50 mL^{-1} at 23°C with gentle shaking. After 2 h the suspension was centrifuged at 250,000*g* for 1 h at 4°C to pellet the microsomal membranes.

Particles were isolated from the resulting supernatant either by ultrafiltration following a 12-h centrifugation at 305,000*g* to sediment any residual membrane or by flotation. For flotation, the supernatant fraction was made 10% (w/v) with Suc, and 23 mL of supernatant was placed in centrifuge tubes (60Ti, Beckman) overlaid with 1.5 mL of isolation buffer and centrifuged at 305,000*g* for 12 h. The lipid-protein particles floated into the 1.5 mL of isolation buffer during centrifugation and were removed with a Pasteur pipette (Hudak and Thompson, 1996). Radiolabeled lipid-protein particles were also generated in vitro by incubating microsomal membranes in the presence of [^{14}C]linoleic acid. For this purpose, the membranes were suspended in in vitro buffer (10 mg 50 mL^{-1}) containing 9.25×10^5 Bq of [1-^{14}C]linoleic acid (1.96×10^9 Bq/mmol, NEN Dupont) and maintained at 23°C with gentle shaking. After 2 h lipid-protein particles were isolated by ultrafiltration or flotation.

Lipid Analysis

Total lipids were extracted from the radiolabeled microsomal membranes and cytosolic lipid-protein particles according to the method of Bligh and Dyer (1959). Phospholipids were quantified according to the method of Rouser et al. (1966). Levels of radioactivity in both the chloroform phase and methanol/water phase of the Bligh and Dyer extraction were determined by adding 100-μL aliquots to 3 mL of scintillation fluid and counting the samples in a liquid scintillation counter (LS 1701, Beckman). Levels of radiolabeled volatile compounds in the methanol/water phase were determined by taking the samples to dryness under a stream of N_2 prior to the addition of scintillation fluid. To determine the level of radiolabeled free fatty acids in the microsomal and cytosolic lipid-protein particle fractions, total lipid extracts were fractionated by TLC (Yao et al., 1991a). The separated fraction of free fatty acids was scraped into 3 mL of scintillation fluid for counting. Fatty acids were identified and quantified as methyl esters by GC (McKegney et al., 1995).

Volatile Analysis

C6-aldehydes and alcohols in microsomal membranes and cytosolic particle fractions were identified and quantified by GC/MS. Ten milliliters of the membrane and particle suspensions were preheated to 40°C for 5 min and purged with N_2 at a flow rate of 200 mL/min for 20 min. The purged volatiles were adsorbed onto multibed thermal desorption tubes (Carbotrap 300, Supelco, Bellefonte, PA) using a dynamic thermal stripper (Dynatherm model 1000, Supelco) with block, oven, and tube temperatures set at 110, 50, and 40°C, respectively. The tubes were transferred to a thermal desorption unit (Dynatherm model 890, Supelco) and desorbed for 3 min at 350°C. The desorbed volatiles were transferred directly to a gas chromatograph (5890 series II, Hewlett-Packard)/mass spectrometer (model 5970, Hewlett-Packard) through a transfer line maintained at 250°C. Fractionation was achieved using a capillary column (30 m × 0.25 mm i.d., 0.25-μm film thickness; Wax 10, Supelco). The oven temperature was maintained at 50°C for the first 5 min and increased at 6°C/min for 20 min to a final temperature of 250°C.

Electron Microscopy

Isolated lipid-protein particles were examined by electron microscopy after staining with uranyl acetate. To prepare the samples a droplet of freshly isolated particle suspension was placed on a Formvar-coated copper grid, the moisture was absorbed with a piece of filter paper, and the film was stained with 70% ethanol saturated with uranyl acetate for 30 s and allowed to air-dry for 30 min. The lipid-protein particles were examined with an electron microscope (model CM10, Philips, Eindhoven, The Netherlands) operating at 60 kV.

RESULTS

Lipids and lipid metabolites in membranes and cytosolic lipid-protein particles of carnation petals were radiolabeled during de novo synthesis by pulse-labeling with [^{14}C]acetate. Uptake of [^{14}C]acetate into the petals was rapid over a 2-h period (Fig. 1). Accordingly, petals were routinely pulse-labeled for 2 h and then transferred to buffer containing a 75-fold molar excess of unlabeled acetate.

At various periods throughout the pulse-chase labeling protocol, the petals were fractionated to obtain microsomal membranes and cytosolic lipid-protein particles. The cytosolic particles were spherical in nature and ranged from 30 to 300 nm in diameter (Fig. 2). The incorporation of radiolabeled acetate into microsomal membrane lipid reached a peak at 2 h and then declined as the petals incorporated unlabeled acetate into membrane lipids (Fig. 3). The appearance of radiolabeled lipid in cytosolic lipid-protein particles was delayed by comparison with microsomal

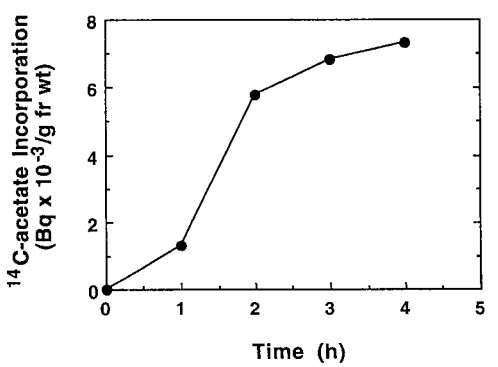

Figure 1. Time course for [^{14}C]acetate uptake into carnation petals. Data are expressed relative to petal fresh weight (fr wt).

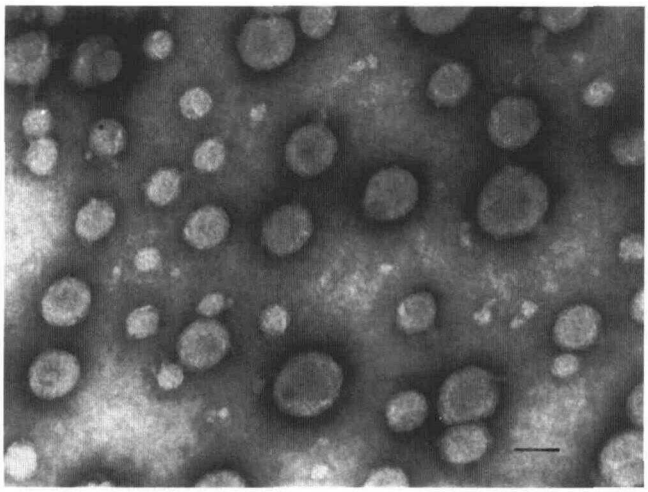

Figure 2. Electron micrograph of cytosolic lipid protein particles isolated from carnation petals by ultrafiltration and stained with uranyl acetate. Bar = 100 nm.

membranes and continued to increase during uptake of unlabeled acetate as the level of radiolabeled lipid in the microsomal membranes was declining (Fig. 3). A similar trend was observed when the lipid extracts were fractionated and the levels of radiolabeled free fatty acids in microsomal membranes and cytosolic lipid-protein particles were examined (data not shown). This temporal pattern of in situ radiolabeling supports the contention that cytosolic particles originate from membranes.

Microsomal membranes and cytosolic lipid-protein particles both contained radiolabeled polar lipid metabolites (methanol-water soluble), but the temporal patterns of lipid metabolite radiolabeling in the two fractions during the pulse-chase protocol were clearly distinguishable. Specifically, levels of radiolabeled lipid metabolites in the microsomal membranes remained low and essentially constant throughout the period of pulse-chase labeling, whereas levels of radiolabeled metabolites in the cytosolic lipid-protein particles increased to reach a peak at 2 h,

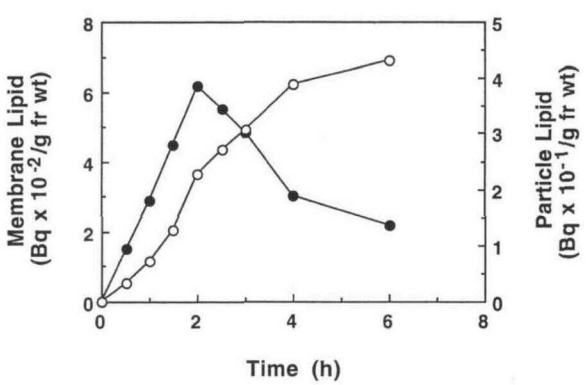

Figure 3. Time course for [^{14}C]acetate incorporation into lipids (chloroform-soluble) of subcellular fractions from pulse-chase-labeled carnation petals. ●, Microsomal membranes; ○, ultrafiltered cytosolic lipid-protein particles. Data are expressed relative to petal fresh weight (fr wt) and are from one of four separate experiments showing the same results.

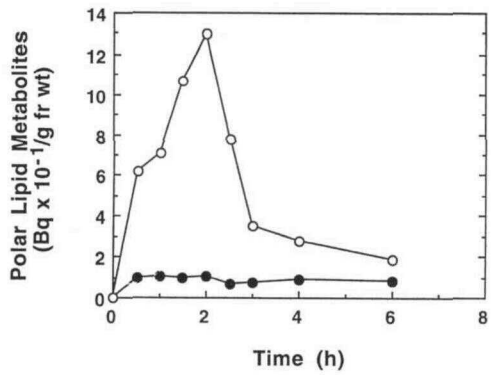

Figure 4. Time course for [^{14}C]acetate incorporation into polar lipid metabolites (methanol/water-soluble) of subcellular fractions from pulse-chase-labeled carnation petals. ●, Microsomal membranes; ○, ultrafiltered cytosolic lipid-protein particles. Data are expressed relative to petal fresh weight (fr wt) and are from one of four separate experiments showing the same results.

coincident with the addition of excess unlabeled acetate, and then declined (Fig. 4). Moreover, the lipid-protein particles were enriched by approximately 80-fold in polar lipid metabolites relative to lipid by comparison with microsomal membranes. After the petals were pulse-labeled for 2 h with [^{14}C]acetate, the ratio of radiolabeled polar lipid metabolites to radiolabeled lipid was 2.4 in the lipid-protein particles, whereas the corresponding ratio for microsomal membranes was only 0.03 (Table I). Also, a large proportion of the radiolabeled polar lipid metabolites in the cytosolic lipid-protein particles proved to be volatile. This was determined by measuring the proportion of the radiolabel in the methanol/water phase of lipid extracts that evaporated when samples were dried under N_2. For lipid-protein particles, 63.8 ± 3.7% (mean ± SE for n = 3) of the radiolabel in the methanol/water phase of lipid extracts evaporated under N_2, whereas only 34.7 ± 3.5% (mean ± SE for n = 3) of the radiolabel in the methanol/water phase of lipid extracts from corresponding microsomal membranes evaporated under identical conditions.

These labeling patterns suggest that polar lipid metabolites formed in membranes are released by blebbing of lipid-protein particles and do not accumulate in the membrane bilayers. The isolated microsomal membranes and cytosolic lipid-protein particles were thoroughly washed to remove any radiolabeled cytosolic contaminants. However, to confirm that the polar lipid metabolites were formed in

Table I. *Level of ^{14}C-radiolabeled lipids (chloroform-soluble) and polar metabolites (methanol/water-soluble) in microsomal membranes and lipid-protein particles isolated by ultrafiltration of cytosol from carnation petals pulse-labeled for 2 h with [^{14}C]acetate*

Values are means ± SE for n = 3.

Fraction	^{14}C Label		Metabolite/Lipid Ratio
	Lipids	Polar Lipid Metabolites	
	% total		
Microsomal membranes	97.2 ± 3.2	2.8 ± 3.2	0.03
Lipid-protein particles	29.3 ± 5.6	70.7 ± 5.6	2.4

membranes and released by blebbing, lipid-protein particles were generated from microsomal membranes labeled in vitro with [^{14}C]linoleic acid. The radiolabeled linoleic acid was administered to isolated microsomes, and the membranes were allowed to metabolize the radiolabeled fatty acid and generate lipid-protein particles under in vitro conditions for 2 h. At the end of this period, microsomal membranes and the in vitro generated lipid-protein particles both contained radiolabeled lipid (chloroform-soluble) and polar lipid metabolites (methanol/water-soluble), indicating that the lipid metabolites originated from the membranes (Table II). Moreover, like their in situ cytosolic counterparts, the in vitro generated lipid-protein particles were enriched (approximately 4-fold) in lipid metabolites relative to lipid by comparison with microsomal membranes (Table II).

The radiolabeled lipid metabolite to lipid ratios in microsomal membranes and lipid-protein particles for the in vitro labeling experiments with [^{14}C]linoleic acid were not the same as those for the in situ labeling experiments with [^{14}C]acetate (Tables I and II). This presumably reflects the fact that only specific metabolites were radiolabeled with [^{14}C]linoleic acid, whereas all lipid species and their metabolites were labeled with [^{14}C]acetate. Lipid-protein particles were also generated in vitro from radiolabeled microsomal membranes isolated from petals that had been pulse-labeled in situ for 2 h with [^{14}C]acetate and again were enriched (approximately 31-fold) in polar lipid metabolites relative to lipid by comparison with microsomal membranes (Table III).

The finding that metabolites of [^{14}C]linoleic acid were released from microsomal membranes by blebbing of lipid-protein particles suggested the involvement of lipoxygenase in this metabolism. This enzyme appears to be associated with microsomal membranes of carnation petal tissue (Fobel et al., 1987). To test this hypothesis the effect of *n*-propyl gallate on the release of polar lipid metabolites from microsomal membranes was determined. Lipid-protein particles were generated over a 2-h period in the presence and absence of 2 mM *n*-propyl gallate from microsomal membranes isolated from petals that had been prelabeled in situ with [^{14}C]acetate. In the presence of *n*-propyl gallate, a general antioxidant that also inhibits lipoxygenase (Ho et al., 1995), levels of radiolabeled lipid and polar lipid metabolites in the in vitro-generated lipid-protein particles were both reduced by approximately 40% (Table IV). *n*-Propyl gallate at a concentration of 2 mM inhibits carnation petal microsomal lipoxygenase by >90% (Fobel et al., 1987). One possible interpretation of the finding that the appearance of radiolabeled polar lipid metabolites in the lipid-protein particles was only reduced by approximately 40% is that preformed radiolabeled metabolites are released under these conditions.

The observation that levels of microsomal lipids and lipid-metabolites in the lipid-protein particles were reduced in parallel by *n*-propyl gallate is consistent with the contention that lipoxygenase-derived lipid metabolites are removed from membrane bilayers by blebbing of lipid-protein particles. This possibility is supported by the finding that volatile C6-aldehydes and alcohols, metabolites of the lipoxygenase pathway, were present in both microsomal membranes and cytosolic lipid-protein particles. Microsomal membranes and membrane-free cytosol (305,000*g* 12-h supernatant) were isolated from carnation petals that had not been pulse-chase labeled. Several volatile components of carnation flower fragrance were identified in these fractions, including hexanal, *trans*-2-hexenal, 1-hexanol, 3-hexen-1-ol, nonanal, 2-hexanol, and 3-hexen-1-yl benzoate, which are all metabolites of the lipoxygenase pathway (Gardner, 1995), as well as caryophyllene, benzyl alcohol, hexyl benzoate, and benzyl benzoate (Fig. 5). Of these,

Table II. *Level of ^{14}C-radiolabeled lipids (chloroform-soluble) and polar lipid metabolites (methanol/water-soluble) in microsomal membranes and ultrafiltered lipid-protein particles generated in vitro from microsomal membranes*

Microsomes were isolated from carnation petals and incubated for 2 h in the presence of [^{14}C]linoleic acid to generate lipid-protein particles. The particles were isolated by ultrafiltration, and levels of radiolabeled lipids and polar lipid metabolites in the membranes and the particles were determined after the 2-h incubation. Values are means ± SE for $n = 3$.

Fraction	^{14}C Label		Metabolite/ Lipid Ratio
	Lipids	Polar Lipid Metabolites	
	% total		
Microsomal membranes	91.5 ± 1.8	8.6 ± 1.8	0.09
Lipid-protein particles	73.8 ± 3.2	26.2 ± 3.2	0.35

Table III. *Level of ^{14}C-radiolabeled lipids (chloroform-soluble) and polar metabolites (methanol/water-soluble) in microsomal membranes and in ultrafiltered lipid-protein particles generated in vitro from microsomal membranes isolated from carnation petals pulse-labeled for 2 h with [^{14}C]acetate*

Values are means ± SE for $n = 3$.

Fraction	^{14}C Label		Metabolite/ Lipid Ratio
	Lipids	Polar Lipid Metabolites	
	% total		
Microsomal membranes	96.8 ± 0.5	3.2 ± 0.5	0.03
Lipid-protein particles	52.2 ± 3.9	47.8 ± 3.9	0.92

Table IV. *Reduction of microsomal-derived radiolabeled lipids (chloroform-soluble) and polar lipid metabolites (methanol/water-soluble) in lipid-protein particles generated in vitro in the presence of n-propyl gallate*

Lipid-protein particles were generated in vitro in the presence and absence of 2 mM *n*-propyl gallate from microsomal membranes isolated from petals that had been pulse-labeled for 2 h with [^{14}C]acetate. The particles were isolated by flotation. Values are means ± SE for $n = 3$ and are based on measurements of becquerels per milligram of microsomal membrane protein.

Fraction	Reduction in the Presence of *n*-Propyl Gallate
	%
Lipids	37.4 ± 4.2
Polar lipid metabolites	40.9 ± 4.7

hexanal, *trans*-2-hexenal, 1-hexanol, 3-hexen-1-ol, 3-hexen-1-yl benzoate, benzyl alcohol, and benzyl benzoate were detectable in both microsomal membranes and membrane-free cytosol; nonanal, caryophyllene, and hexyl benzoate were detectable only in the microsomal membranes, and 2-hexanol was detectable only in the membrane-free cytosol (Fig. 5).

The membrane-free cytosol was further fractionated by Sephadex G-25 chromatography to distinguish between volatiles associated with lipid-protein particles (void volume) and those that were in the soluble phase of the cytosol (delayed volume). Five volatile C6-aldehydes and alcohols derived from the lipoxygenase pathway, specifically hexanal, *trans*-2-hexenal, 1-hexanol, 3-hexen-1-ol, and 2-hexanol, were detectable in both the void volume (lipid-protein particles) and the delayed volume (Fig. 6A). However, when the concentrations of volatiles in the void volume were expressed per unit volume of lipid-protein particles and compared with the concentration of volatiles per corresponding unit of delayed volume, it was apparent that there was a 24- to 216-fold higher concentration of volatiles in the hydrophobic lipid-protein-particle subcompartment of the cytosol than in the cytosol itself (Fig. 6B). There was also an approximately 5-fold enrichment of volatiles relative to phospholipid in the lipid-protein particles by comparison with corresponding microsomal membranes. Combined levels of hexanal, *trans*-2-hexenal,

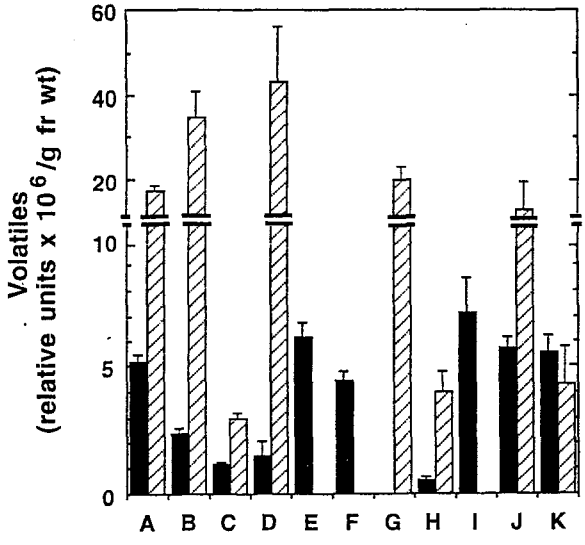

Figure 5. Volatile signatures of subcellular fractions isolated from carnation petals. Solid bars, Microsomal membranes; hatched bars, membrane-free cytosol (305,000*g* 12-h supernatant) containing lipid-protein particles. Values are means ± SE for $n = 3$ and are expressed as relative units (total ion counts obtained by GC-MS) relative to petal fresh weight (fr wt). A, Hexanal; B, *trans*-2-hexenal; C, 1-hexanol; D, 3-hexen-1-ol; E, nonanal; F, caryophyllene; G, 2-hexanol; H, benzyl alcohol; I, hexyl benzoate; J, 3-hexen-1-yl benzoate; and K, benzyl benzoate. Actual values (μg/g fresh weight) for volatiles for which authentic standards were available are 0.05, 0.03, 0.02, and 0.03 for hexanal, *trans*-2-hexenal, 1-hexanol, and 3-hexen-1-ol, respectively, in microsomal membranes and 0.16, 0.39, 0.04, and 1.48, respectively, for the same volatiles in the 12-h supernatant.

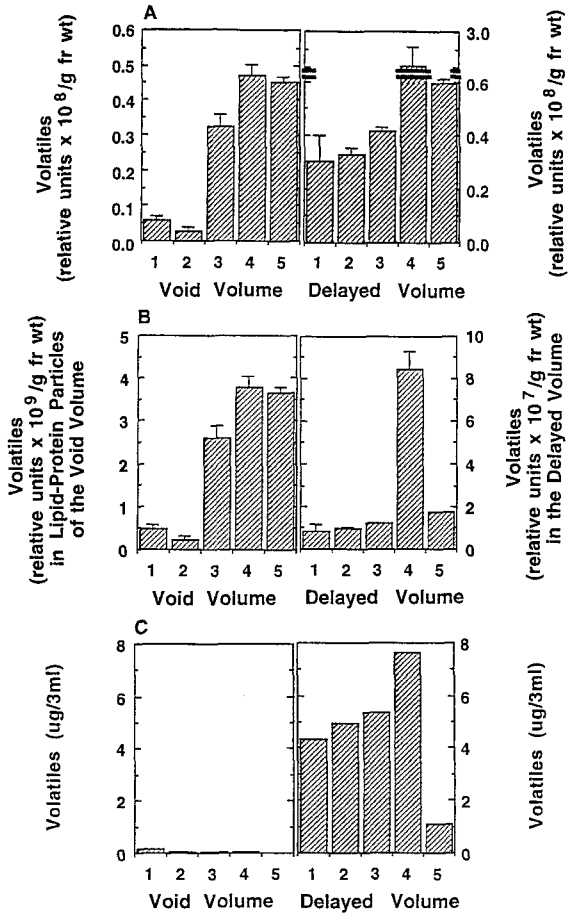

Figure 6. Levels of lipoxygenase pathway-derived volatiles in cytosolic lipid-protein particles from carnation petals. Membrane-free cytosol (305,000*g* 12- h supernatant) was fractionated by Sephadex G-25 column chromatography to obtain lipid-protein particles (void volume) and particle-free cytosol (delayed volume). The numbers 1 to 5 correspond to hexanal, *trans*-2-hexenal, 1-hexanol, 3-hexen-1-ol, and 2-hexanol, respectively. A, Levels of volatiles (means ± SE for $n = 3$) expressed as relative units (total ion counts obtained by GC-MS) relative to petal fresh weight (fr wt) in the void volume and delayed volume. Actual values (μg/g fresh weight) for volatiles for which authentic standards were available are 0.07, 0.03, 0.37, and 0.55 for hexanal, *trans*-2-hexenal, 1-hexanol, and 3-hexen-1-ol, respectively, in the void volume and 0.30, 0.33, 0.42, and 2.94, respectively, for the same volatiles in the delayed volume. B, Levels of volatiles (means ± SE for $n = 3$) per milliliter of lipid-protein particles in the void volume (the content of particles was determined by measuring phospholipid, and their volume was calculated assuming an average particle diameter of 120 nm [Hudak and Thompson, 1996] and that each particle is circumscribed by a monolayer of phospholipid) and per milliliter of delayed volume. Values are expressed as relative units (total ion counts obtained by GC-MS) relative to petal fresh weight. Actual values (μg/g fresh weight) for volatiles for which authentic standards were available are 2.41, 1.13, 12.96, and 18.92 for hexanal, *trans*-2-hexenal, 1-hexanol, and 3-hexen-1-ol, respectively, in the void volume and 0.04, 0.05, 0.06, and 0.42 for the same volatiles in the delayed volume. C, Levels of volatile standards (results of a single experiment) in the void volume and delayed volume of fractionated particle-free cytosol. Authentic volatile standards were dissolved in particle-free cytosol (filtrate obtained by ultrafiltration of membrane-free cytosol from which endogenous volatiles had been purged by thermal stripping with N_2) prior to fractionation.

1-hexanol, 3-hexen-1-ol, and 2-hexanol relative to phospholipid proved to be 37 ± 4 μg/mg in the lipid-protein particles compared with a corresponding value of only 7 ± 0.1 μg/mg for microsomal membranes. The data in Figure 6C are from control experiments in which authentic volatile standards dissolved in particle-free cytosol were fractionated by Sephadex G-25 chromatography. In the absence of lipid-protein particles, >98% of the volatiles eluted in the delayed volume (Fig. 6C). This indicates that the presence of volatiles in the void volume obtained by Sephadex G-25 chromatography of membrane-free cytosol (Fig. 6, A and B) does not reflect adsorption of volatiles to cytosolic protein, which co-eluted with the lipid-protein particles in the void volume and accounts for >90% of the total protein in the cytosol.

DISCUSSION

Cytosolic lipid-protein particles resembling oil bodies have been identified in cotyledon tissue and in flower petals (Yao et al., 1991a; Hudak and Thompson, 1996). Several lines of evidence suggest that these particles originate from membranes. In particular, they contain phospholipid and the same fatty acids that are found in microsomal membranes of corresponding tissue, and they are enriched in free fatty acids and steryl and wax esters of membrane origin (McKegney et al., 1995; Hudak and Thompson, 1996). Notwithstanding these common elements, the lipid composition of lipid-protein particles is clearly distinguishable from that of membranes (McKegney et al., 1995; Hudak and Thompson, 1996). For example, phospholipid fatty acids make up only approximately 10% of the total fatty acids in lipid-protein particles of carnation petals (Hudak and Thompson, 1996). The lipid-protein particles are also morphologically distinguishable from membrane vesicles (McKegney et al., 1995; Hudak and Thompson, 1996).

The presence of phospholipid suggests that the particles originate from phospholipid-bearing membranes. Galactolipid was not detected in the full complement of lipid-protein particles isolated from petal tissue (Hudak and Thompson, 1996), but this does not preclude the possibility that a subpopulation of particles originates from galactolipid-bearing membranes; indeed, this is known to be the case for leaf tissue (Ghosh et al., 1994). Isolated lipid-protein particles range from 30 to 300 nm in diameter and are uniformly osmiophilic in thin sections. Osmiophilic particles of the same size and shape as those isolated from the cell cytosol are discernible in the cytoplasm of intact cells (McKegney et al., 1995; Hudak and Thompson, 1996). Also, lipid-protein particles with properties similar to those isolated from the cell cytosol can be generated in vitro from isolated microsomal membranes under conditions in which phospholipid catabolism has been activated by the addition of Ca^{2+} (Yao et al., 1991a; Hudak and Thompson, 1996). Analogous lipid-protein particles originating from thylakoids have been identified in the stroma of chloroplasts. The chloroplast particles are enriched in free fatty acids as well as in catabolites of thylakoid proteins, contain galactolipid rather than phospholipid, and can also be generated in vitro by illumination of isolated thylakoids (Ghosh et al., 1994).

These observations have prompted the proposal that blebbing from membrane surfaces of lipid-protein particles enriched in lipid metabolites is an integral feature of membrane turnover, allowing removal of molecules such as free fatty acids and steryl and wax esters that would otherwise phase-separate and destabilize membrane bilayers (Yao et al., 1991b; Hudak et al., 1995). Indeed, the accumulation of these metabolites in membranes and ensuing lipid-phase separations are well-established manifestations of senescence for a number of plant tissues (Barber and Thompson, 1983; Platt-Aloia and Thomson, 1985; Faragher et al., 1987) and have been shown to correlate temporally with impairment of blebbing (Yao et al., 1991b; Hudak et al., 1995). Particles of similar size and shape to those isolated from the cytosol are evident in the cytoplasm of intact cells in electron micrographs of thin-sectioned carnation petal tissue (Hudak and Thompson, 1996), which supports the contention that they are in situ elements of the cytosol. Also, the finding in the present study that the pulse-chase labeling patterns of microsomal membranes and lipid-protein particles are temporally separated provides further evidence that the particles originate from membranes in situ and participate in membrane lipid turnover.

The finding that radiolabeled lipid synthesized in situ from [^{14}C]acetate appeared first in microsomal membranes and subsequently in cytosolic particles coincident with declining levels in membranes indicates that the lipid of the particles is derived from membranes. Radiolabeled free fatty acids also increased in microsomal membranes during pulse-labeling of the petals with [^{14}C]acetate and then declined during the chase with unlabeled acetate coincident with a corresponding increase in labeled free fatty acids in cytosolic lipid-protein particles. Phospholipid catabolism results in the formation of free fatty acids in membrane bilayers, and this temporal pattern of free fatty acid labeling in microsomal membranes and cytosolic lipid-protein particles is consistent with the contention that phospholipid catabolites formed during membrane turnover are released from the membrane bilayer by blebbing of lipid-protein particles into the cytosol. Indeed, cytosolic lipid-protein particles in carnation petals are enriched by approximately 10-fold in free fatty acids relative to phospholipid fatty acids compared with microsomal membranes (Hudak and Thompson, 1996).

The pulse-labeling studies with [^{14}C]acetate have also indicated that polar lipid metabolites formed within membranes are released from the membrane bilayer by blebbing of lipid-protein particles. Unlike free fatty acids, the radiolabeled polar lipid metabolites did not accumulate in microsomal membranes, even during the initial pulse of labeling with [^{14}C]acetate. Rather, they increased in the cytosolic lipid-protein particles, reaching an 80-fold higher concentration relative to chloroform-soluble lipid in the particles by comparison with microsomes, and then decreased during the chase with unlabeled acetate. This decline during the period when the petals were taking up unlabeled acetate presumably reflects partitioning of the

radiolabeled lipid metabolites out of the lipid-protein particles into the cytosol in accordance with partitioning coefficients. Lipid-protein particles generated in vitro from microsomal membranes, which had been labeled in situ with [^{14}C]acetate or in vitro with [^{14}C]linoleic acid, were also enriched in radiolabeled polar lipid metabolites compared with their membranes of origin. These observations collectively indicate that at least some of the polar lipid metabolites detectable in the lipid-protein particles were formed by membranous lipoxygenase, which utilizes free linoleic and linolenic acids as substrates (Fobel et al., 1987). Moreover, >60% of the [^{14}C]acetate-derived polar lipid metabolites in lipid-protein particles proved to be, and several metabolites of the lipoxygenase pathway are known to be, volatile (Hildebrand, 1989; Gardner, 1995). These include C6-aldehydes and alcohols, which are components of flavor and fragrance (Buttery et al., 1988; Raghavan et al., 1993) and also exhibit antifungal and antimicrobial activity (Gueldner et al., 1985; Croft et al., 1993; Mishra and Dubey, 1994).

Lipoxygenase and hydroperoxide lyase, which form fatty acid hydroperoxides and C6-aldehydes, respectively, are known to be membrane-associated (Fobel et al., 1987; Hatanaka et al., 1987; Matsui et al., 1991; Vianello et al., 1995). This, together with the finding in the present study that lipoxygenase pathway-derived C6-aldehydes and alcohols are present in microsomal membranes, suggests that volatile lipid metabolites originate from membranes. Other volatiles, specifically caryophyllene, hexyl benzoate, and benzyl benzoate, which are elements of flower fragrance (Knudsen et al., 1993) but not metabolites of the lipoxygenase pathway, were also detectable in microsomal membranes. These volatile lipid metabolites are nonbilayer molecules with a propensity to destabilize membrane bilayers, and several lines of evidence suggest that they are removed by blebbing of lipid-protein particles from the membrane surface into the cytosol. First, there is an enrichment of C6-aldehydes and alcohols in the lipid-protein particles by comparison with microsomal membranes. Second, levels of radiolabeled polar lipid metabolites formed in situ during pulse-chase labeling of intact petals with [^{14}C]acetate, approximately 60% of which are volatile, are approximately 80-fold higher in the lipid-protein particles than in corresponding microsomal membranes. Third, these radiolabeled polar lipid metabolites can be formed in microsomal membranes but do not accumulate therein. Fourth, the lipid-protein particles are also enriched in C6-aldehydes and alcohols compared with particle-free cytosol. Under steady-state conditions these volatiles would be expected to equilibrate between the hydrophobic lipid-protein particle subcompartment of the cytosol and the hydrophilic particle-free cytosol in accordance with partitioning coefficients.

The octanol/water partitioning coefficient for hexanal is 60 (Kalmet et al., 1988), and yet the concentration of the C6-aldehydes and alcohols is up to 216-fold higher in the lipid-protein particles than in the particle-free cytosol. The hydrophobicity for octanol is not likely to be identical to that for the interior of lipid-protein particles. Judging from the chemical composition of the particles, however, their interior is likely to be largely composed of fatty acid side chains (Hudak and Thompson, 1996), and therefore it seems reasonable to infer from this comparison that the concentration of volatile alcohols and aldehydes in the particles exceeds that which would be achieved by partitioning from the cytosol into the particles. The possibility that these volatile lipid metabolites are formed within the lipid-protein particles rather than originating from membranes seems unlikely in light of the fact that the lipid-protein particles do not contain measurable levels of lipoxygenase or hydroperoxide lyase (G. McKegney and J.F. Thompson, unpublished data). Also, the possibility that aliphatic alcohols and aldehydes stick to hydrophobic regions of proteins in the lipid-protein particles during tissue homogenization seems unlikely by analogy with the oleosin of oil bodies (Huang, 1992), because hydrophobic portions of the proteins could be expected to be imbedded in the hydrophobic interior of the particles, whereas those portions of the proteins exposed on the surfaces of the particles would be hydrophilic.

Cytosolic lipid-protein particles have properties in common with oil bodies found in oil-bearing seeds. They both contain phospholipid and triacylglycerol, although the ratio of triacylglycerol to phospholipid is higher in oil bodies than in cytosolic lipid-protein particles from carnation petals (Hudak and Thompson, 1996). In addition, oil bodies and cytosolic lipid-protein particles both appear to be formed by blebbing from membranes (Huang, 1992; Hudak and Thompson, 1996). The mechanism of blebbing is not understood. It is likely, however, that nonbilayer lipid metabolites including triacylglycerol, free fatty acids, steryl and wax esters, and C6-aldehydes and alcohols phase-separate within the bilayer. This would engender discrete domains enriched in these metabolites with a propensity to break away from the bilayer because of packing imperfections at the phase boundaries (Dobereiner et al., 1993; Sackmann and Feder, 1995). This concept is consistent with the fact that oil bodies and cytosolic lipid-protein particles are both enriched in one or more of these metabolites (Styme and Stobart, 1987; Yao et al., 1991b; McKegney et al., 1995).

ACKNOWLEDGMENT

The authors gratefully acknowledge the expert technical assistance of Ms. Lynn Hoyles in cultivation of the carnation plants.

Received November 25, 1996; accepted March 13, 1997.

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