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Release of Fluorescent Peroxidized Lipids from Membranes in Senescing Tissue by Blebbing of Lipid-Protein Particles

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Several lines of evidence indicate that peroxidation of membrane lipids is an inherent feature of ripening and senescence of plant tissues. Dhindsa and Dhindsa (1981), for example, demonstrated a correlation between lipid peroxidation and increased membrane permeability in senescing leaves. Levels of H_2O_2 and lipid hydroperoxides also increase in ripening fruit, and Frenkel (1978) has reported that ripening can be delayed by inhibiting the formation of H_2O_2 . Pear (*Pyrus communis* L. var. Bartlett) and banana (*Musa cavendishii* L. var. Valery) fruit contain fluorescent products of lipid peroxidation that increase during natural or ethylene-induced ripening (Maguire and Haard, 1975). Senescing leaves and cotyledons also accumulate fluorescent peroxidation products (Wilhelm and Wilhelms, 1981), and evidence shows that they are localized in membrane lipid bilayers (Pauls and Thompson, 1984).

Fluorescent peroxidized lipids are a family of compounds with a characteristic Schiff base structure ($-N=C-C=C-N$) that is formed by the reaction of aldehydes from peroxidized lipids with free amino groups (Tappel, 1975). Schiff bases formed from malondialdehyde and amino compounds have characteristic fluorescence spectra that feature an excitation maximum at ≈ 360 nm and an emission maximum at ≈ 430 nm (Fig. 1), and are thus similar to the lipofuscins known to accumulate in animal tissues during aging (Tappel, 1975). Fluorescent peroxidized lipids appear to be formed in membrane bilayers (Pauls and Thompson, 1984), and if they were to accumulate therein, they could be expected to disrupt membrane structural integrity. Indeed, the accumulation of peroxidized lipids in senescing membranes may contribute to loss of membrane function as tissues age (Thompson et al., 1987).

In this report, we describe evidence indicating that fluorescent peroxidized lipids are released from membranes by blebbing (pinching of surface protrusions) of lipid-protein particles from the membrane surface. The data presented have been obtained from studies with senescing bean (*Phaseolus vulgaris* L.) cotyledons grown under etiolation conditions and senescing carnation (*Dianthus caryophyllus* L.) flower petals. These lipid-protein particles have been partially characterized previously (Yao and Thompson, 1993; Yao et al., 1991a, 1991b, 1993a), are present in plant and animal tissue (Yao et al., 1991a, 1993b), and have been tentatively termed deteriosomes to denote their putative role in removing lipid and protein catabolites from membranes (Yao et al., 1991a). Lipid and protein catabolism is a normal feature of membrane turnover, and any significant accumulation of these catabolites in the bilayer could have a disruptive effect on membrane structure. Recent evidence suggests that these catabolites

are selectively removed by blebbing of lipid-protein particles from the membrane surface (Yao and Thompson, 1993; Yao et al., 1991a, 1991b, 1993a). Impairment of this blebbing process with advancing senescence appears to result in an accumulation of lipid catabolites in senescing membranes that causes bilayer destabilization and loss of membrane function (Yao et al., 1991b).

Peroxidized lipid accumulation in senescing membranes

Fluorescence spectra of lipid extracts from microsomal membranes of cotyledons at various stages of senescence all show excitation maxima at 370 nm and emission maxima at 430 nm (Fig. 1). The

Fig. 1. Excitation (A) and emission (B) spectra for lipid extracts of microsomal membranes isolated from senescing bean cotyledons (age in days). (●) young, 2 days old; (▲) 4; (■) 7; (solid hexagon) 9. At 4 days, the cotyledons are beginning to show visible manifestations of senescence, and by 9 days, they are extensively senescent (data are from Pauls and Thompson, 1984). Inset: corresponding excitation spectra for lipid extracts of microsomal membranes from the petals of carnation flowers. (●) Young, stage 2 flowers (petals fully expanded and flowers fully open with yellow-tinted centers); (○) senescing stage 4 flowers (petals showing pronounced inrolling).

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intensity of fluorescence in the membrane lipid extracts expressed on a per micromole phospholipid basis increases about 10-fold between day 2, when the tissue is young, and day 9, when the tissue is extensively senescent (Fig. 1). Similar data have been obtained for microsomal membranes isolated from the petals of senescing carnation flowers (inset of Fig. 1). Fluorescent compounds of this type have also increased in ripening fruit (Maguire and Haard, 1975) and in the chloroplasts of senescing leaves (Wilhelm and Wilhelms, 1981).

Localization of fluorescent peroxidized lipids in the cytosol

Fluorescent peroxidized lipids also are present in the cytosol (postmicrosomal supernatant) of senescing tissues (Table 1). Indeed, for 2-day-old bean cotyledon tissue, $\approx 59\%$ of the total (microsomal plus cytosol) fluorescence-reflecting peroxidized lipids is in the cytosol, and $\approx 41\%$ is associated with the microsomal membranes. As the tissue ages, however, there is a pronounced accumulation of peroxidized lipids in membranes such that $\approx 72\%$ of the total (microsomal plus cytosol) fluorescence is associated with the microsomal fraction and only $\approx 28\%$ with the cytosol (Table 1). In absolute terms, this represents about a 2-fold increase with advancing senescence in levels of fluorescent peroxidized lipids in microsomal membranes and a 52% decline in cytosolic levels of fluorescent peroxidized lipids. Thin-layer chromatography has indicated that the major fluorescing products in the membranes are peroxidized phospholipids, whereas the cytosol contains peroxidized phospholipids and peroxidized free fatty acids (Yao et al., 1993a).

Further fractionation of the cytosol revealed that, for young and senescing tissue, $\approx 50\%$ of the cytosolic fluorescent peroxidized lipids are associated with nonsedimentable lipid-protein particles (Table 1, Fig. 2). These lipid-protein particles appear to be formed by blebbing from membranes and are isolated by centrifuging the cytosol (postmicrosomal supernatant) at $250,000\times g$ for 12 h to sediment residual membrane, and then filtering the resulting supernatant through a 300,000-Da cutoff filter to yield nonsedimentable lipid-protein particles in the retentate and particle-free cytosol in the filtrate (Yao et al., 1991a, 1991b). When these particles are further purified by gel filtration, the lipid and protein co-elute, which indicates that they are associated in a macromolecular complex (Yao and Thompson, 1993; Yao et al., 1991a). The particles are spherical (Yao et al., 1991a) (Fig. 3A), have a propensity to bleb into smaller particles (Fig. 3A), and range from 50 to 250 nm in diameter (Fig. 3B). Their protein composition is complex and clearly distinguishable from those of corresponding microsomal membranes and particle-free cytosol (Fig. 4). These particles contain phospholipids but are enriched in phospholipid catabolites, in particular free fatty acids and gel-phase-forming lipids (Yao et al., 1991b). Indeed, the free : esterified fatty acid ratio is typically 6- to 10-fold higher in nonsedimentable lipid-protein particles than in corresponding membranes.

These observations have prompted the proposal that these lipid-protein particles serve as a vehicle for removing destabilizing molecular catabolites from membranes, and the particles have been tentatively termed "deteriosomes" to connote this putative function (Yao and Thompson, 1993; Yao et al., 1991a, 1991b). This proposal is reinforced by the finding that the lipid-protein particles contain fluorescent peroxidized lipids as well (Fig. 2). Indeed, the level of

Table 1. Fluorescence of lipid extracts from microsomal membranes, cytosol, lipid-protein particles, and particle-free cytosol isolated from young 2-day-old and senescing 7-day-old bean cotyledons.

Fractions	Percentage of total fluorescence ^a	
	2-day-old cotyledons	7-day-old cotyledons
Microsomal membranes	41.6 $\pm$ 4.6	71.9 $\pm$ 10.9
Cytosol	58.4 $\pm$ 6.4	28.1 $\pm$ 4.2
Lipid-protein particles	26.4 $\pm$ 1.2	14.3 $\pm$ 2.3
Particle-free cytosol	32.0 $\pm$ 1.7	13.8 $\pm$ 4.7

^aTotal fluorescence is the sum of microsomal membrane plus cytosolic fluorescence and is based on 10 g of cotyledon tissue. Fluorescence emission was recorded at 430 nm (excitation 360 nm). Standard errors of the means are indicated; n = 3. Data are from Yao et al. (1993a).

Fig. 2. Excitation and emission spectra for lipid extracts of nonsedimentable lipid-protein particles. (○) Lipid-protein particles from young, 2-day-old bean cotyledon tissue; (●) lipid-protein particles from petals of young, stage 2 carnation flowers. The cotyledon data are from Yao et al. (1993a).

peroxidized lipids relative to phospholipid is about 15-fold higher in the lipid-protein particles than in microsomal membranes. The peroxidized lipids are also detectable in the particle-free cytosol of young and senescing tissue (Table 1), which may reflect either breakdown of the lipid-protein particles upon release into the cytosol, or partitioning of the fluorescent peroxidized lipids from the lipid-protein particles into the cytosol.

Earlier studies have indicated that the formation of nonsedimentable lipid-protein particles is a two-step process involving phospholipid catabolism, which is enzymatically mediated and gives rise to bilayer-destabilizing lipid catabolites, followed by blebbing from the membrane surface (Yao et al., 1991a, 1991b). This contention is based on the findings that nonsedimentable lipid-protein particles with essentially similar properties can be formed *in vitro* from isolated membranes under conditions in which phospholipid catabolism is activated, and that their formation can be prevented by heat denaturation of the membranes (Yao et al., 1991a). In particular, nonsedimentable lipid-protein particles formed from isolated membranes under *in vitro* conditions are also enriched in phospholipid catabolites, specifi-

Fig. 3. Morphology and size of nonsedimentable lipid-protein particles. (A) Electron micrographs of lipid-protein particles isolated from the petals of young, stage 2 carnation flowers and stained with uranyl acetate according to Yao et al. (1991a); bars = 0.1 μ m. (B) Size distribution of lipid-protein particles isolated from the petals of young, stage 2 carnation flowers obtained by dynamic light scattering using a helium-neon laser (Hallett et al., 1989).

Fig. 4. SDS-PAGE (10% to 20% linear gradient) of subcellular fractions isolated from the petals of carnation flowers. Each lane was loaded with 1 μ g of protein, and the gels were stained with silver (Wray et al., 1981). (A) Microsomal membranes; (B) nonsedimentable lipid-protein particles; (C) particle-free cytosol. Fractions isolated: lane 1, from the petals of stage 1 flowers (partially closed flower buds); lane 2, from the petals of stage 2 flowers (petals fully expanded and flowers fully open with yellow-tinted centers); lane 3, from stage 3 flowers (fully open flowers without yellow-tinted centers); lane 4, from petals of stage 4 flowers (senescing flowers showing pronounced petal inrolling).

cally free fatty acids and lipids that induce phase separations in membrane bilayers (Yao et al., 1991a, 1991b).

The contention that peroxidized lipids formed in membranes are released by blebbing from the membrane surface is supported by the finding that nonsedimentable lipid-protein particles formed in vitro from isolated membranes also contain fluorescent peroxidized lipids. For example, fractionation of the supernatant resulting from treatment of microsomal membranes from bean cotyledons with Ca^{2+} to activate phospholipid catabolism revealed that $\approx 65\%$ of the fluorescent peroxidized lipids released are associated with nonsedimentable lipid-protein particles, and the remaining 35% is in the particle-free supernatant (Table 2). Microsomal membranes from carnation petals and from bean cotyledons both exhibit lipoxygenase activity that is inhibited by *n*-propyl gallate (Lynch and Thompson, 1984; Lynch et al., 1985). *n*-Propyl gallate also inhibits the formation of fluorescent peroxidized lipids in microsomal membranes as well as blebbing of nonsedimentable lipid-protein particles (Table 3). These observations collectively indicate that the lipoxygenase reaction facilitates the formation of nonsedimentable lipid-protein particles, and that peroxidized lipids formed by the lipoxygenase reaction may be among the promptor molecules that engender blebbing.

Impairment of blebbing may account for accumulation of peroxidized lipids in senescing membranes

Several indirect lines of evidence suggest that the capability of membranes to release molecular catabolites by blebbing of lipid-protein particles is impaired with advancing senescence. Specifically, free fatty acids accumulate in membranes as tissues senesce (Fobel et al., 1987), and gel-phase-forming phospholipid catabolites also accumulate in senescing membranes (Barber and Thompson, 1983; Legge et al., 1982; McKersie and Thompson, 1979; Platt-Aloia and Thomson, 1985). Both of these compounds appear to be removed in young tissue by blebbing, thus leading to the formation of nonsedimentable lipid-

Table 2. Fluorescence of lipid extracts from fractions obtained by treating microsomal membranes from 2-day-old bean cotyledons with Ca^{2+} to activate phospholipid catabolism and release lipid-protein (L-P) particles by blebbing.^z

Fractions	Percentage of total fluorescence ^y
Supernatant containing nonsedimentable L-P particles	100
Nonsedimentable L-P particles	64.4 $\pm$ 9.2
Filtrate	35.6 $\pm$ 5.5

^zNonsedimentable L-P particles were generated from 10 mg protein equivalents of microsomal membranes. The supernatant containing nonsedimentable L-P particles was obtained by centrifuging the reaction mixture after treatment with Ca^{2+} , and the L-P particles were isolated from the supernatant by ultrafiltration as described (Yao et al., 1993a).

^yFluorescence emission was recorded at 430 nm (360 nm excitation). Standard errors of the means are indicated; *n* = 3. Data are from Yao et al. (1993a).

Table 3. Inhibitory effect of *n*-propyl gallate on the formation of fluorescent peroxidized lipids and on blebbing of nonsedimentable lipid-protein (NS L-P) particles during treatment of microsomal membranes with Ca^{2+} to activate phospholipid catabolism.^z

Action	Percent inhibition
Formation of fluorescent peroxidized lipids in microsomal membranes ^y	47.5 $\pm$ 7.5
Blebbing of NS L-P particles from microsomal membranes ^x	70.9 $\pm$ 7.0

^zMicrosomal membranes (10 mg protein equivalent) from 2-day-old bean cotyledons were treated with Ca^{2+} in the presence and absence of 2 mM *n*-propyl gallate as described (Yao et al., 1993a) to initiate lipid peroxidation and blebbing of NS L-P particles.

^yFluorescence emission was recorded at 430 nm (excitation 360 nm). The standard error of the mean is indicated; *n* = 3.

^xFormation of lipid-protein particles was quantified as the release of lipid phosphate. The standard error of the mean is indicated; *n* = 3.

Fig. 5. Thin-layer chromatogram of total lipid extracts from nonsedimentable lipid-protein particles formed *in vitro* from microsomal membranes of bean cotyledons. The chromatogram was developed as described in Yao et al. (1991a). Lane 1, lipid-protein particles from microsomal membranes (10 mg protein equivalents) of young, 2-day-old cotyledons; lane 2, lipid-protein particles from microsomal membranes (10 mg protein equivalents) of senescing 7-day-old cotyledons. DG_{1,2} = 1,2-diacylglycerol; DG_{1,3} = 1,3-diacylglycerol; FFA = free fatty acids; U = unknown; SE = sterol esters; PA = phosphatidic acid.

Fig. 6. Changes in the abundance of nonsedimentable lipid-protein particles during senescence of carnation flower petals. Abundance was measured in terms of (A) free fatty acid levels, (B) phospholipid levels, and (C) protein levels in the particle fraction. Corresponding levels of (D) particle-free cytosol protein also are indicated. Means $\pm$ SE for $n = 3$ are shown for stages 2 through 4 of flower development as defined in the legend for Fig. 4.

protein particles (Yao et al., 1991a, 1991b). Fluorescent peroxidized lipids also accumulate in senescing membranes, and in young tissue, they appear to be removed by blebbing (Figs. 1 and 2).

More direct evidence that advancing senescence impairs the capability of membranes to remove phospholipid catabolites by blebbing has been obtained by quantifying the *in vitro* formation of lipid-protein particles from isolated microsomal membranes of young and senescing tissue. It is clear, for example, from thin-layer chromatography (Fig. 5) that the release of lipid catabolites by blebbing of lipid-protein particles from microsomal membranes of senescing 7-day-old cotyledon tissue is notably less than the release from microsomal membranes of 2-day-old cotyledon tissue. There also are changes in the quantity of nonsedimentable lipid-protein particles in senescing tissue that are consistent with the contention that blebbing from membranes becomes impaired as membranes age. In senescing carnation petals, for example, levels of particle phospholipid and free fatty acids expressed on a per gram of tissue dry weight basis decline by $\approx 57\%$ as the tissue ages (Fig. 6 A and B), and particle protein also declines by 46% over the same period (Fig. 6C). By contrast, levels of protein in the particle-free cytosol remain essentially unchanged as the petals senesce (Fig. 6D). The finding that the protein, phospholipid and free fatty acids of nonsedimentable lipid-protein particles decline essentially in parallel suggests that the decreases reflect a change in abundance of the particles rather than a change in their chemical composition. This contention is further supported by the finding that there is no significant change in the polypeptide profile of the lipid-protein particles during senescence despite the pronounced decline in total particle protein (Fig. 4).

In summary, these observations collectively indicate that the ability of membranes to release destabilizing molecular catabolites, including fluorescent peroxidized lipids, by blebbing decreases with advancing senescence. This decrease may be due in part to the decrease in bulk lipid fluidity that is characteristic of membrane senescence (Borochov et al., 1976; Fobel et al., 1987; Leshem et al., 1984; McKersie et al., 1978; Thompson et al., 1982). Impairment of this putative housekeeping mechanism with age could be expected to contribute to bilayer destabilization and loss of membrane function.

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Toward a Comprehensive Model for Lipid Peroxidation in Plant Tissue Disorders

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Lipid peroxidation has been observed during the development of metabolic disorders in plant (Shewfelt and Erickson, 1991; Winston, 1990) and animal tissues (Kehrer, 1993; Pacifici and Davies, 1991). A partial list of recent reports linking lipid peroxidation to plant disorders, senescence, and aging is shown in Table 1. It is not clear whether peroxidation represents a primary cause or merely a secondary effect of these disorders. Lipid peroxidation and associated defense mechanisms in the cell occur via an exceedingly complex process. While certain steps appear to be nonenzymic chemical reactions governed strictly by kinetics (Buettner, 1993), others are clearly under genetic control (Kane et al., 1993). Statements to proclaim or dismiss lipid peroxidation as the primary cause of these disorders have been based on oversimplification of the processes involved. The case against a causative role is based on the variability of cell and tissue response to various types of stress and the frequent lack of evidence of peroxidative product accumulation before onset of the disorder (see Table 2 for recent examples). The objective of this review is not to catalog lipid oxidation in plant stresses, but to provide a framework for evaluating a cause-and-effect relationship in these disorders. This article presents an overview of peroxidative processes in plant tissues, a comprehensive model to explain how peroxidation of membrane lipids could function to cause disorders, specific explanations for problematical observations of affected tissue, and implications of the model if the theory is correct. The proposed model is an amplification and extension of one described earlier (Purvis and Shewfelt, 1993; Shewfelt and Erickson, 1991).

PEROXIDATION IN PLANT TISSUE

Lipid peroxidation is a natural consequence of metabolic processes in the cell, occurring via initiation, propagation, and termination reactions (Schaich, 1992; Winston, 1990). Active oxygen species such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) are by-products of

electron transport in chloroplasts, mitochondria, and plasma membranes (Winston, 1990). Interaction of superoxide and hydrogen peroxide with iron species (Fe^{2+} , Fe^{3+}) in membranes leads to formation of lipid-free radicals, presumably via formation of the hydroxyl radical ($HO\cdot$) (Borg and Schaich, 1988) or by an iron intermediate that is most active as the $Fe^{2+} : Fe^{3+}$ ratio approaches 1.0 (Minotti and Aust, 1992). Singlet oxygen (1O_2) is formed in chloroplasts during photosynthesis (Chakraborty and Tripathy, 1992) and is highly reactive with lipids (Bradley and Min, 1992).

Lipid hydroperoxides (LOOH) are also formed in plant tissue by lipoxygenase (LOX) (Gardner, 1991). Unlike the free radical processes described above, lipoxygenase, acting under aerobic conditions, does not produce lipid-free radicals. In addition, lipoxygenases in plant tissue either require or preferentially attack free fatty acids (Hildebrand, 1989), usually acting in concert with hydrolytic enzymes (Leshem, 1992).

For each source of lipid peroxidation within the cell there is a defense mechanism. Thus, superoxide can be degraded to hydrogen peroxide by superoxide dismutase (SOD) and hydrogen peroxide can be further degraded to water and oxygen by catalase. Alpha-tocopherol, β -carotene, and ascorbic acid will scavenge free radicals, including the hydroxyl radical (Buettner, 1993), while carotenoids (DiMascio et al., 1989) and ubiquinone (Cabrini et al., 1986) quench singlet oxygen. Lipoxygenase activity is controlled by compartmentation (Elstner, 1991) and by maintaining the fatty acids in the esterified form (Hildebrand, 1989).

Despite the presence of defense systems, membrane lipids are peroxidized, probably via generation of the hydroxyl radical in the membrane in close proximity to a polyunsaturated fatty acid (PUFA). The resultant lipid peroxyl radical will be quickly converted to a lipid hydroperoxide by interaction with α -tocopherol or other hydrogen donors (Fig. 1) (Buettner, 1993). One α -tocopherol molecule is estimated to protect from 220 (Cogrel et al., 1993) to 1000 (Buettner, 1993) phospholipid molecules within a membrane. Rapid mobility of α -tocopherol in the bilayer is achieved by the association of its polar head group with the polar head group of phosphatidylcholine (PC) (Urano et al., 1993). The tocopheroxyl radical is more stable than a

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