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Lipid Peroxidation and Plant Tissue Disorders

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Lipid Peroxidation and Plant Tissue Disorders: Introduction to the Workshop

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Among the lipid peroxidation reactions, the addition of molecular O₂ to polyunsaturated fatty acids (PUFAs) is of particular interest in biological systems. The lipoxygenase pathway is the most-studied enzymatic pathway for oxidizing PUFAs, but nonenzymatic reactions have also been characterized. When one considers the significance of lipid peroxidation, it is surprising to discover the many diverse areas that are affected. In fact, whether one considers the process beneficial or detrimental depends on the circumstances. In situations where lipid peroxidation has been implicated in tissue damage, controversy exists over whether it is involved in the primary events causing injury or merely a by-product.

Lipid peroxidation is involved with normal developmental processes, including production of flavor and odor volatiles, formation of compounds with growth-regulator-like activities, and senescence. Characteristic flavors and aromas, such as those associated with cucumber (*Cucumis sativus* L.) and tomato (*Lycopersicon esculentum* Mill.), are due in part to the presence of various enzymes in the respective lipoxygenase pathways (Gardner, 1989). Although desirable characteristics may be imparted to foods by oxidized lipids, rancidity and other off-flavors can also develop (Perkins, 1989).

Induced defenses, including the hypersensitive response associated with resistance to pathogens and herbivore defense involving proteinase inhibitors, rely in part on oxidation of PUFAs (Farmer and Ryan, 1992). These authors proposed that methyl jasmonate, formed from linolenic acid, may serve as a secondary messenger in the lipid-based signaling pathway. A direct role of aldehydes formed from PUFAs in inhibiting fungal growth also has been established (Hamilton-Kemp et al., 1992).

Lipid peroxidation via free-radical-mediated reactions has been implicated in abiotic stresses. Evolution of ethane, a breakdown product of linolenic acid, was detected after temperature stresses (Harber and Fuchigami, 1986; Nanaiah and Anderson, 1992) and exposure to gaseous pollutants (Kimmerer and Kozlowski, 1982). Extensive peroxidation of membrane lipids may impair function and structure, possibly through the formation of gel-phase domains in the membrane (Pauls and Thompson, 1980). A similar decrease in membrane fluidity observed in senescing tissues was blocked by a lipoxygenase inhibitor (Fobel et al., 1987), but factors such as sterol content also may be important in determining membrane lipid viscosity (Duxbury et al., 1991).

Plant cells combat the formation of free radicals with antioxidants and free-radical scavengers. Leaf senescence mediated by free radicals was related to a decrease in superoxide dismutase and catalase activity (Dhindsa et al., 1981)—enzymes that detoxify superoxide and hydrogen peroxide, respectively. Purvis and Shewfelt (1993) proposed that an accumulation of stress-triggered oxidizing agents may overcome the defensive capabilities of the cell, leading to injury.

Lipid peroxidative reactions play key roles in plant responses to biotic and abiotic stresses, developmental processes, and food quality. The papers from this workshop cover many of these areas, beginning with an overview of the lipoxygenase pathway, focusing on the biochemistry and biological roles of the two branches of the lipoxygenase pathway. Presentations dealing with specific disorders are followed by a comprehensive model addressing the question of cause and effect.

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Biological Roles and Biochemistry of the Lipoxygenase Pathway

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Comparative overview of the plant and animal kingdoms. This workshop emphasized the “dark side” of uncontrolled lipid oxidation in plants, but another aspect of this oxidative process is thought to be a “normal” part of the physiological status of the plant and animal kingdoms. That is, the so-called lipoxygenase pathway is common to plants and animals (Fig. 1). Because the pathway in plants is activated by wounding and pathogen attack, the argument can be made that the pathway is normally quiescent. In animals, certain levels of prostaglandins and lipoxygenase products are known to be maintained, but only a few studies have addressed the endogenous levels of lipoxygenase metabolites in plants (e.g., see Vick and Zimmerman, 1982). More data are available concerning endogenous levels of the lipoxygenase pathway-generated jasmonic acid family of metabolites (see review in Hamberg and Gardner, 1992). Although some lipoxygenase isoenzymes can oxidize certain glyceride lipids, it is generally acknowledged that free polyunsaturated fatty acids are the preferred substrates. In plants, a little-known portion of the pathway is how the initial step is triggered, namely the lipolytic action on glycerides. Lipoxygenase oxidation of polyunsaturated fatty acids, and metabolism of the resulting fatty acid hydroperoxides, are more thoroughly characterized. However, the biosynthesis of prostaglandins via prostaglandin endoperoxide synthase has not yet been observed in plants. There are a few reports of prostaglandins isolated from plants, but few higher plants contain the arachidonic acid necessary for the action of prostaglandin endoperoxide synthase. Since prostaglandin-like fatty acids are formed simply by peroxidative reactions of hydroperoxides of either arachidonic acid or linolenic acid (O'Connor et al., 1984), it is unlikely that the “prostaglandins” found in plants resulted from true enzymic reactions of prostaglandin biosynthesis. Because higher plants largely metabolize their endogenous polyunsaturated fatty acids, linoleic and linolenic acids, and animals use mostly arachidonic acid in these reactions, they have been called the octadecanoid and eicosanoid pathways, respectively.

André and Hou first detected lipoxygenase in plants in 1932, but its significance was not understood until about 50 years later. Novel possible physiological roles for the lipoxygenase pathway in plants are now emerging rapidly. However, prostaglandin endoperoxide synthase

and lipoxygenase activities in animals were discovered much later (Hamberg and Samuelsson, 1974; Nugteren et al., 1966), and their physiological roles were defined quickly thereafter. Although plants and animals diverged in their evolution more than 2.5 billion years ago, there remain several common enzymic reactions in the pathway in addition to lipoxygenase. Allene oxide synthase (formerly called hydroperoxide isomerase, hydroperoxide cyclase, and hydroperoxide dehydrase) is found in plants and coral (Fig. 1), and hydroperoxide lyase has been identified in plants and fish (see review in Gardner, 1991). Interestingly, both the octadecanoid and eicosanoid pathways are often triggered by stress. Many of the physiological responses are amelioration or repair of the effects of stress. For many years, aspirin has been known to block prostaglandin formation by inhibiting prostaglandin endoperoxide synthase activity. Recently, Peña-Cortés et al. (1993) reported that aspirin in plants also inhibits allene oxide synthase, the enzyme that produces the plant equivalent of prostaglandins (12-oxo-phytodienoic acid).

An overview of the lipoxygenase or octadecanoid pathway of plants is shown in Fig. 2. After lipolytic action, the first enzyme in the series is lipoxygenase. Most plant lipoxygenase isoenzymes hydroperoxidize polyunsaturated fatty acids in a stereo-specific manner giving either 13(S)- or 9(S)-hydroperoxides, and some yield a mixture of both. Only a few isoenzymes give a more racemic mixture, similar to fatty acid autooxidation products. Thus, the hydroperoxides usually obtained from linoleic acid are 13(S)-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid (13S-HPOD) and 9(S)-hydroperoxy-*trans*-10,*cis*-12-octadecadienoic acid (9S-HPOD), and those obtained from linolenic acid are 13(S)-hydroperoxy-*cis*-9,*trans*-11,*cis*-15-octadecatrienoic acid (13S-HPOT) and 9(S)-hydroperoxy-*trans*-10,*cis*-12,*cis*-15-octadecatrienoic acid (9S-HPOT). By Fischer convention the 13(S)- and 9(S)-hydroperoxides are (*L*) and (*D*), respectively. After lipoxygenase action, there are several hydroperoxide-metabolizing branches of the pathway that impact the physiology of plants; these aspects are discussed in the sections below.

The jasmonic acid family. The key enzyme involved in the biosynthesis of the family of 7-iso-jasmonic acid, jasmonic acid, their esters, and numerous related metabolites was first observed in flaxseed (*Linum usitatissimum* L.) to transform 13S-HPOD into 12-oxo-13-hydroxy-*cis*-9-octadecenoic acid (α -ketol). Zimmerman (1966) named this enzyme hydroperoxide isomerase. Subsequently, hydroperoxide isomerase was found in the germ of corn seed (*Zea mays* L.), and,

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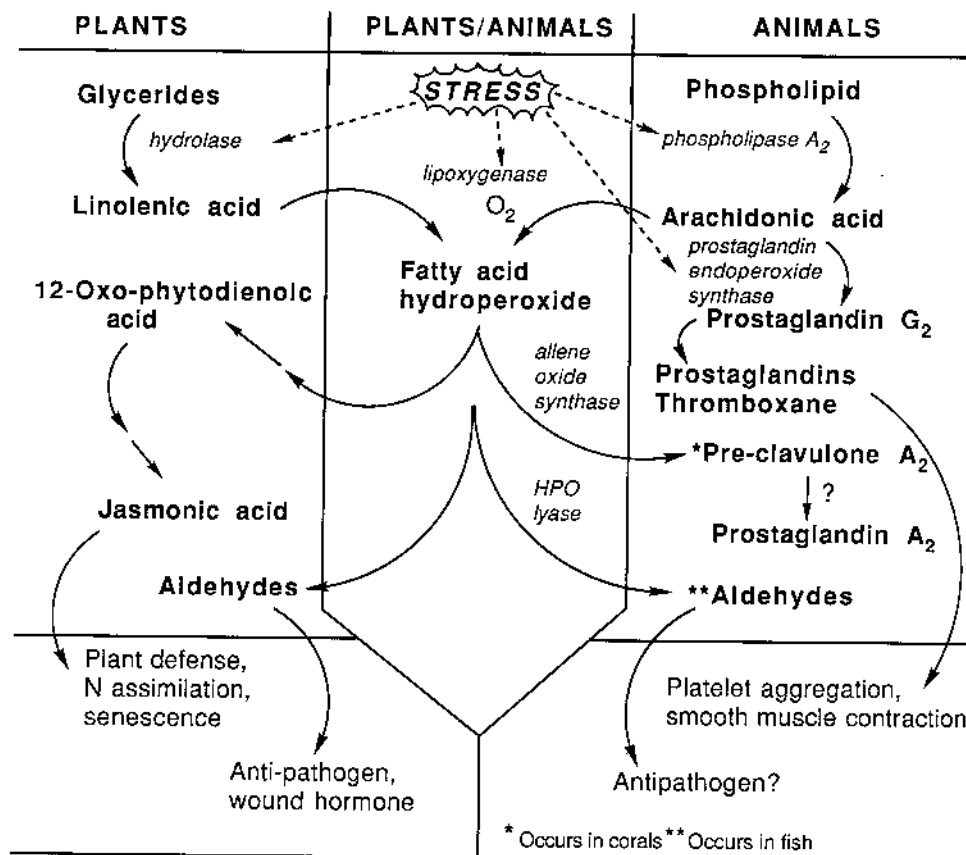


Fig. 1. Model of the stress-activated octadecanoid pathway of plants and eicosanoid pathway of animals showing reactions shared by both, as well as those unique to either the plant or animal kingdom.

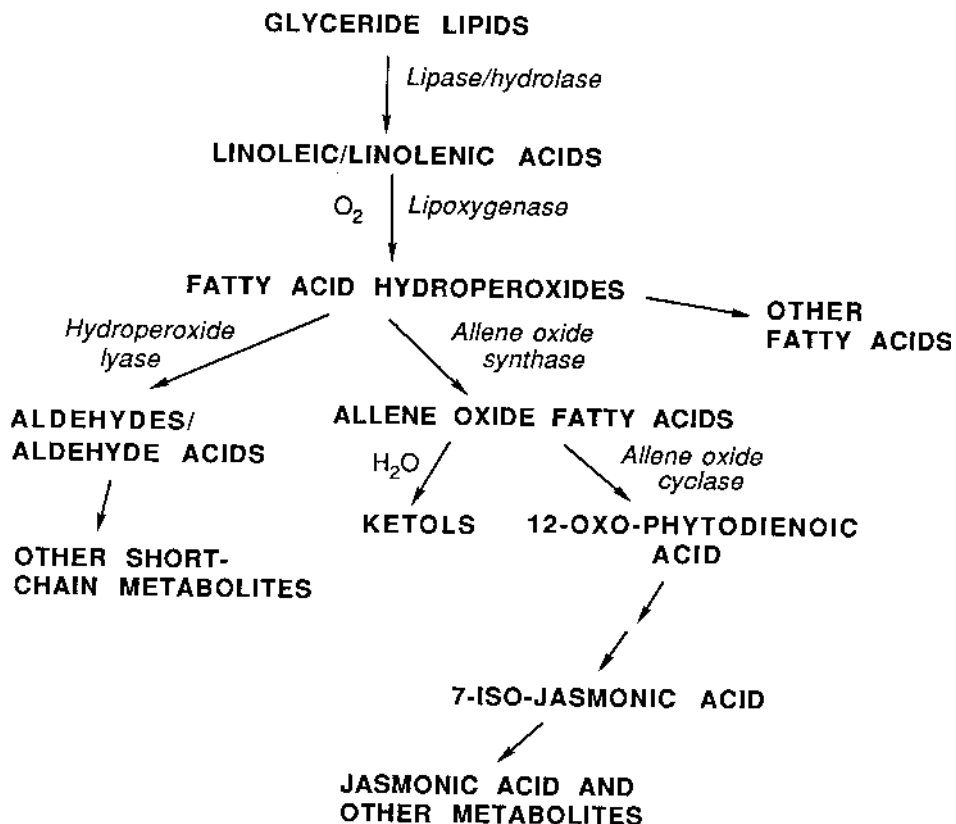


Fig. 2. A summary of the major metabolic branches in the plant octadecanoid pathway.

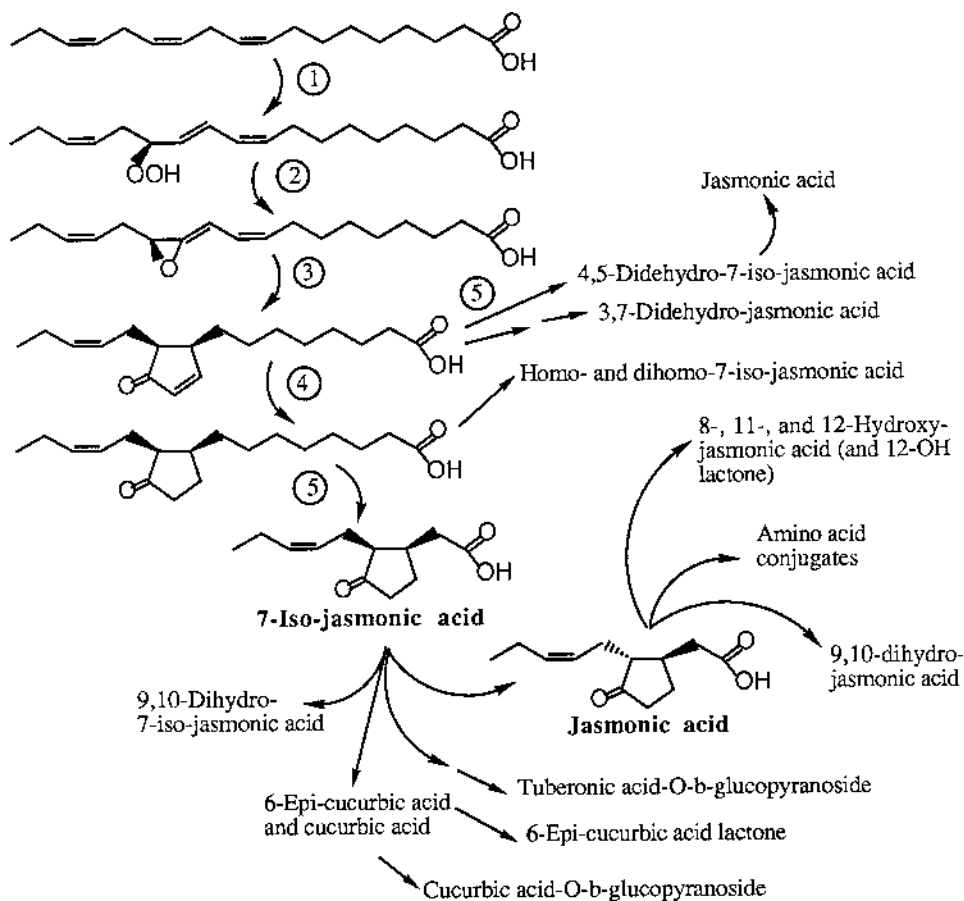


Fig. 3. The sequential conversion of linolenic acid into the jasmonic acid family of phytohormones. The enzymic reactions to 7-iso-jasmonic acid have been established as follows: 1) 13(*S*)-hydroperoxide-specific lipoxygenase, 2) allene oxide synthase, 3) allene oxide cyclase, 4) 12-oxo-phytodienoic acid reductase, 5) three β -oxidation steps. The other conversions shown have not been directly connected with specific enzymic reactions, but they are logical choices based on known biosynthetic reactions. Not shown are the esters of jasmonic and 7-iso-jasmonic acids; methyl and ethyl esters are known.

additionally, the γ -ketol, 12-oxo-9-hydroxy-*trans*-10-octadecenoic acid, was produced from 13*S*-HPOD (Gardner, 1970). The corn germ enzyme also converted 9*S*-HPOD into the corresponding α - and γ -ketols. Several years later, Zimmerman and Feng (1978) reported a "new" enzyme, hydroperoxide cyclase, that catalyzed the conversion of 13*S*-HPOT into a cyclic fatty acid, 12-oxo-phytodienoic acid. Subsequently, Hamberg (1987) showed that the α - and γ -ketols were derived from hydrolysis of the actual enzymic product, an unstable allene oxide fatty acid. He renamed the enzyme as hydroperoxide dehydrase, but this nomenclature was changed again to allene oxide synthase by Song and Brash (1991), who isolated the enzyme and characterized it as a cytochrome P450. The allene oxide synthase gene from flaxseed was recently cloned and sequenced (Song et al., 1993). Researchers soon recognized that the allene oxide fatty acid was the intermediate involved in cyclization of 13*S*-HPOT into 12-oxo-phytodienoic acid (Fig. 3) (Baertschi et al., 1988; Brash et al., 1988; Hamberg, 1988). Earlier, Corey et al. (1987) had suggested that an analogue of 12-oxo-phytodienoic acid, pre-clavulone A (Fig. 1), was formed in coral via a fatty acid allene oxide intermediate; a suggestion fully confirmed by Brash's group (Brash et al., 1987). In plants, only the 13*S*-HPOT was converted into the cyclic fatty acid; whereas allene oxides from the 9*S*-HPOT, 9*S*-HPOD, and 13*S*-HPOD exclusively underwent hydrolysis to the ketols. Reaction of 13*S*-HPOT catalyzed by allene oxide synthase results in the spontaneous cyclization of the intermediate allene oxide; however, hydrolysis to the ketols occurs to a larger extent. Also, spontaneous cyclization causes the formation of racemic 12-oxo-phytodienoic acid (9*S*, 13*S*, and 9*R*, 13*R*); but in the presence of another enzyme, allene oxide cyclase, mainly one isomer (98% 9*S*, 13*S*) was found in increased yield at the expense of ketol formation (Hamberg and Fahlstadius, 1990).

The next steps in the pathway to jasmonic acid involved the

reduction of the double bond of the cyclopentenone ring of 12-oxo-phytodienoic acid by a reductase (Vick and Zimmerman, 1986), followed by three successive β -oxidations (Vick and Zimmerman, 1983). As shown in Fig. 3, the immediate biosynthetic product is 7-iso-jasmonic acid, instead of jasmonic acid. Many derivatives related to jasmonic acid are known (see review in Hamberg and Gardner, 1992). Although the specific routes to these derivatives are unknown, the pathways shown in Fig. 3 were constructed by logical use of known biosynthetic reactions. The methyl ester of jasmonic acid, as well as 7-iso-jasmonic acid, are commonly occurring natural derivatives. Investigators have used synthetic methyl jasmonate extensively as a "volatile signal" in physiological research.

The biological activities of the jasmonic acid family are extensive (see recent reviews in Farmer and Ryan, 1992a; Hamberg and Gardner, 1992; Sembdner and Parthier, 1993; Staswick, 1992; van den Berg and Ewing, 1991; Weiler, 1993). Because these reviews are available, the literature from 1992 to present will be emphasized, with only a brief summary given of the earlier research (Table 1). Physiological functions of the jasmonic acid family have been largely researched with a synthetic racemate of methyl jasmonate or jasmonic acid. According to Nishida et al. (1985), synthetic methyl jasmonate tends to be composed of 47.5% methyl jasmonate (natural), 47.5% methyl *ent*-jasmonate (unnatural), 2.5% methyl 7-iso-jasmonate (natural), and 2.5% methyl *ent*-7-iso-jasmonate (unnatural); thus, it is reasonable to expect that the biological activity of the natural methyl esters may have a lower threshold than reported for synthetic racemates. As reported by Koda et al. (1992), methyl *ent*-7-iso-jasmonate and methyl *ent*-jasmonate had lower activity in some applications, but they equally inhibited the straight growth of oat (*Avena sativa* L.) coleoptiles, compared to the natural isomers. With some notable exceptions, such as the potato-tuberizing phytohormone, tuberonic acid-*O*- β -

Table 1. Biological activities of methyl jasmonate, jasmonic acid, and related metabolites.

Type of activity	Occurrence in plant and (tissue) ²	Investigators ³
Protein induction		
Vegetative storage proteins	Soybean (leaves)	Anderson; Franceschi & Grimes; Staswick et al.
Lipoxygenase	Soybean (leaves)	Bell & Mullet; Tranbarger et al.
Jasmonate-induced proteins	Barley (leaves, seedlings); 26 other species	Weidhase et al.; Mueller-Urri et al.; Hermann et al.
Proteinase inhibitors	Tomato, tobacco, alfalfa (plants)	Farmer & Ryan
Napin	Rape (embryo culture)	Wilen et al.
Cruciferin	Rape (embryo culture)	Wilen et al.
Oil-body proteins	Rape, flaxseed (embryo culture)	Wilen et al.
Phenylalanine lyase	Soybean (cell culture)	Gundlach et al.
Polyphenol oxidase	Apples (fruit), tulip (bulbs)	Czapski et al.; Saniewski & Czapski
Peroxidase	Barley (leaves), tulip (bulbs)	Weidhase et al.; Saniewski & Czapski
Protein degradation		
Rubisco	Barley (leaves)	Weidhase et al.; Popova & Vaklinova; Maslenkova et al.
Secondary metabolite induction		
Flavonoids, alkaloids, others	<i>Eschscholtzia</i> , others (cell culture)	Gundlach et al.
Anthocyanin	Soybean (seedlings)	Franceschi & Grimes
Tuberization induction		
	Potato (tubers)	Koda et al.; Yoshihara et al.
Senescence and growth inhibition		
Growth inhibition	Rice, wheat, oat, other (seedling)	Yamane et al.; Miersch et al.; Dathe et al.; others
Increased respiration	Barley (seedling)	Popova et al.; Satler & Thimann
Leaf abscission	<i>Ficus superba</i> Miq. (leaves)	Ueda et al.
Increased stomatal resistance	<i>Commelina benghalensis</i> L., other (leaves)	Raghavendra & Reddy; Satler & Thimann
Chlorophyll loss	Barley, oat, other (leaves)	Ueda & Kato; Miersch et al.; Ueda et al.; others
Other effects		
Ethylene stimulation	Tomato, apple (fruit)	Saniewski et al.; Czapski & Saniewski
Germination inhibition	Oat, wheat, other (seed), camellia (pollen)	Corbineau et al.; Yamane et al.; Wilen et al.; others
Germination stimulation	Seeds requiring cold stratification	Daletskaya & Sembdner; Berestetzky et al.
Tendrill coiling	<i>Bryonia dioica</i> Jacq. (tendrils)	Falkenstein et al.
Leaf opening inhibition	<i>Mimosa pudica</i> L. (leaves)	Tsurumi & Asahi
Volatile ester inhibition	Apple (fruit)	Olías et al.
Lycopene inhibition	Tomato (fruit)	Saniewski & Czapski

²Tobacco, *Nicotiana tabacum* L.; alfalfa, *Medicago sativa* L.; rape, *Brassica napus* L.; apple, *Malus* sp. and *Malus malus* cv. Golden Delicious; tulip, *Tulipa* sp.; *Eschscholtzia*, *Eschscholtzia californica* Cham.; wheat, *Triticum aestivum* L.; camellia, *Camellia sinensis* L.

³References are not available in Literature Cited. See Hamberg and Gardner (1992) for full citations.

glucopyranoside (Yoshihara et al., 1989), little is known of the biological activity of the various other metabolites of jasmonic acid and 7-iso-jasmonic acid. Another unknown is the fate or function of allene oxide hydrolysis products, the α - and γ -ketols.

Recent investigations have increasingly indicated that predation by herbivores (wounding), elicitation by fungi (hypersensitive response), or both are signaled by the lipoxygenase pathway, and the defense response to the signal occurs at the transcriptional level. This signal is theorized to be the jasmonic acid (or 7-iso-jasmonic acid) produced via the octadecanoid pathway. A reasonable model has been outlined by Farmer and Ryan (1992a, 1992b), which suggests that either a pathogen or a herbivore signals a receptor in the plasma membrane. According to the model, the receptors cause activation of lipase, and this in turn initiates the octadecanoid pathway to jasmonic acid and gene activation. Thus far, there is limited evidence for the activation of lipase by elicitation. Lipoxygenase's role in the hypersensitive response was demonstrated by a negative elicitor response using a lipoxygenase-null potato (*Solanum tuberosum* L.) callus culture, compared with a lipoxygenase-positive culture (Vaughn and Lulai, 1992). Mueller et al. (1993) found that adding fungal cell walls to a variety of plant cell cultures resulted in the release of free linolenic acid, and within 1 h, the transient formation of 7-iso-jasmonic acid, as well as 12-oxo-phytodienoic acid. In turn, jasmonic/7-iso-jasmonic acid produced as a result of elicitation induces mRNAs for the production of defensive proteins (Dittrich et al., 1992; Gundlach et al., 1992). These proteins, such as phenylalanine ammonia lyase, 4-coumarate:CoA ligase, and chalcone synthase, stimulated production of defensive secondary metabolites. Other recently reported defensive proteins induced by either jasmonic acid, methyl jasmonate, or their precursors (e.g., 12-oxo-phytodienoic acid) include a Kunitz-type proteinase inhibitor (Yamagishi et al., 1993), tobacco proteinase inhibitor (Rickauer et al., 1992), potato proteinase inhibitor II (Kim et al., 1992; Peña-Cortés et al., 1992), a precursor of leaf thionin (Andresen et al., 1992), and several proteinase inhibitors (Farmer and Ryan, 1992b; Farmer et al., 1992). However, it has been recently questioned if the proteinase inhibitors or other proteins play an important role in

protection against fungal attack. According to some workers (Cohen et al., 1993; Schweizer et al., 1993), other factors may be important. It has been amply demonstrated that lipoxygenase activity is induced by either wounding or fungal elicitors (e.g., Croft et al., 1990; Fournier et al., 1993; Hildebrand et al., 1989; Melan et al., 1993). Possibly, increased levels of certain lipoxygenase isozymes are a result of methyl jasmonate (or jasmonic acid) induction, such as observed for a lipoxygenase in soybean (*Glycine max* L.) (Grimes et al., 1992). The increased expression of lipoxygenase could function simply as a vegetative storage protein (Grimes et al., 1992) or could amplify the signal after pathogen attack.

Similar to induction by elicitors, wounding causes expression of a variety of proteins in a manner similar to the induction of these proteins by either methyl jasmonate, jasmonic acid, or their precursors (Farmer and Ryan, 1992b; Farmer et al., 1992; Hildmann et al., 1992). Wounding caused the transient formation of 7-iso-jasmonic acid, which peaked within 30 min to 2 h (Albrecht et al., 1993). Creelman et al. (1992) also observed jasmonic acid and methyl jasmonate accumulation after wounding soybean stems. Stimulation of *Bryonia dioica* Jacq. tendrils by streaking with a wooden stick was sufficient to increase the level of jasmonic acid (Weiler et al., 1993). Although wounding causes transient production of the jasmonic acid family of compounds and they induce proteins at the transcriptional level, surprisingly, gene deletion analysis showed that the induction mechanism of potato proteinase inhibitor II by wounding is different from induction by methyl jasmonate (Kim et al., 1992).

In addition, proteins that have no obvious plant defense function are expressed in the presence of either methyl jasmonate or jasmonic acid. Some serve as vegetative storage proteins, like those found earlier in soybean (*Glycine max* L.) leaves. Soybean storage proteins act as N sinks within the leaf before their mobilization during seed development. Recent reports include determination of the sequence of the jasmonate-inducible 23-kD (Andresen et al., 1992) and 60-kD (Becker and Apel, 1992) proteins of barley (*Hordeum vulgare* L.) and detection of an inducible vegetative storage protein in *Arabidopsis thaliana* L. (Staswick et al., 1992). In contrast, methyl jasmonate

caused the decline of certain photosynthetic enzymes. Methyl jasmonate impaired translation of transcripts for the large and small subunits of rubisco as well as several light-harvesting chlorophyll-protein complex apoproteins (Reinbothe et al., 1993a, 1993b). It is tempting to hypothesize that plants use the jasmonic acid phytohormones as a signal to switch normal photosynthetic function of the plant to a defensive mode through N storage and production of defensive substances. Possibly, they also may control N assimilate partitioning between vegetative and reproductive tissue.

Other biological effects of methyl jasmonate/jasmonic acid studied recently include ethylene biosynthesis (Chou and Kao, 1992a; Sanz et al., 1993), potato (*Solanum tuberosum* L.) tuberization (Matsuki et al., 1992), tendrils coiling (Weiler et al., 1993), seed germination (Ranjan and Lewak, 1992), leaf senescence (Chou and Kao, 1992b), and onion (*Allium cepa* L.) bulbing (Nojiri et al., 1992). Gene expression induced by methyl jasmonate has been compared with similar promoters, such as abscisic acid, desiccation, wounding, and sucrose (Lorbeth et al., 1992; Mason et al., 1992; Reinbothe et al., 1992).

The aldehyde pathway. Hydroperoxide lyase is an enzyme that cleaves fatty acid hydroperoxides into two fragments at the position between the hydroperoxide-bearing carbon and the double bond. This cleavage results in aldehydic functions on both sides of the site of cleavage, producing both an alkanal/alkenal and an oxo-acid. Generally, there are hydroperoxide lyases specific for either 9-hydroperoxides of linoleic/linolenic acids or 13-hydroperoxides of linoleic/linolenic acids. Although various hydroperoxide lyase enzymes have not been investigated completely in regard to their stereo-selectivity for hydroperoxide substrates, a few have been identified as being specific for hydroperoxides with the *S*-configuration, but thus far, none has been identified as *R*-specific. Recent reviews outline these pathways in more detail (Gardner, 1989, 1991; Hatanaka et al., 1987).

Hydroperoxide-lyase-catalyzed reactions are commonly observed in plants, particularly in wounded tissues, and they often result in characteristic plant odors. 13-Hydroperoxides of linoleic and linolenic acid cleave into hexanal and *cis*-3-hexenal, respectively, as well as 12-oxo-*cis*-9-dodecenoic acid (Fig. 4). A hydroperoxide lyase specific for 13-hydroperoxides was recently isolated from tea [*Camellia sinensis*

(L.) Ktze.] leaves (Matsui et al., 1991). The *cis*-3-hexenal and 12-oxo-*cis*-9-dodecenoic acid are often isomerized by an alkenal isomerase (Phillips et al., 1979) into *trans*-2-hexenal and 12-oxo-*trans*-10-dodecenoic acid, respectively. Zimmerman and Coudron (1979) showed that the latter oxo-acid is a wound hormone, "traumatol," and this compound is undoubtedly the precursor of "traumatic acid," which English et al. described in 1939. Hexanal has a rancid green odor, whereas *cis*-3-hexenal and *trans*-2-hexenal have intense grassy and spicy-green odors, respectively. Alcohol dehydrogenases reduce the aldehydes into the corresponding alcohols (Fig. 4) (Matoba et al., 1989), which lead to similar, more subdued odors. Recently, the pathway has been expanded to include the conversion of *cis*-3-hexenal into 4-hydroxy-*trans*-2-hexenal (Gardner and Hamberg, 1993). 4-Hydroxy-*trans*-2-hexenal, as well as the more intensively studied 4-hydroxy-*trans*-2-nonenal, are genotoxic and cytotoxic (reviewed by Esterbauer et al., 1991).

Cleavage of 9-hydroperoxides proceeds by the process described above, except the products are either *cis*-3-nonenal or *cis*-3,*cis*-6-nonadienal from the hydroperoxides of linoleic or linolenic acids, respectively, as well as 9-oxononanoic acid (Fig. 5). The 9-hydroperoxide-specific hydroperoxide lyase has been separated from the 13-hydroperoxide-specific lyase, indicating that they are individual isoenzymes (Matsui et al., 1989). After formation of the C-9 aldehydes, other transformations occur similar to those discussed above for the aldehydes derived from 13-hydroperoxides (Fig. 5). The odors produced by cleavage of 9-hydroperoxides are principally due to the intense cucumber (*Cucumis sativus* L.)-like odor of *cis*-3-nonenal and *cis*-3,*cis*-6-nonadienal with a more subdued and altered odor contributed by their corresponding alcohols or *trans*-2-alkenals.

Another type of hydroperoxide lyase, affording different cleavage fragments from those discussed above, has been reported in fungi, alga, and a species of grass. For example, this hydroperoxide lyase converts 13*S*-HPOD into either pentanol or pentane, as well as 13-oxo-*trans*-11,*cis*-9-octadecadienoic acid (see review in Gardner, 1991). A particularly interesting reaction of this type is found in mushroom (*Psalliota bispora* Lange). Apparently, a lipoxygenase may exist that is specific for oxidizing the 10*S*-position of fatty acid; that is, mush-

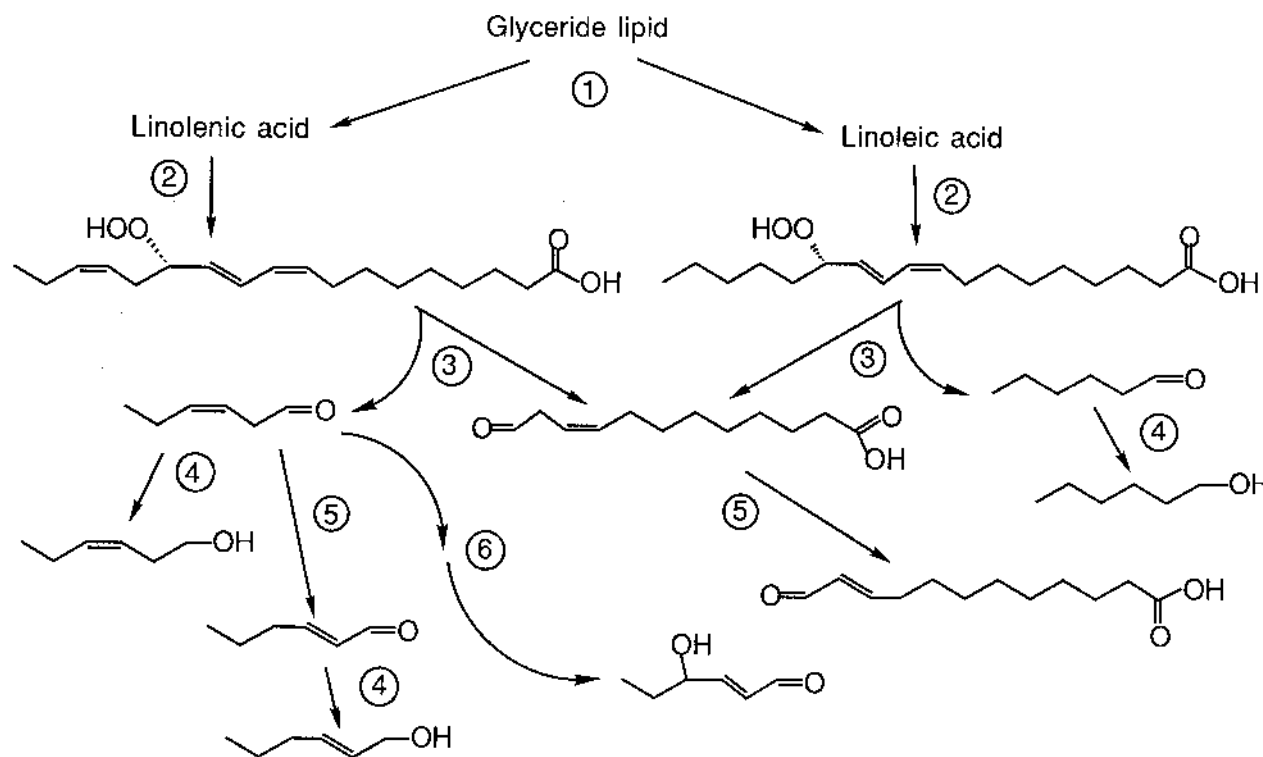


Fig. 4. The C₆-C₁₂ cleavage pathway to aldehydes and alcohols via a 13(*S*)-hydroperoxide-specific lipoxygenase. The specific enzymes involved are 1) a family of lipolytic enzymes, 2) 13(*S*)-hydroperoxide-specific lipoxygenase, 3) hydroperoxide lyase, 4) alcohol dehydrogenase, 5) *cis*-3(9):*trans*-2(10)-enal isomerase, 6) *cis*-3-alkenal oxygenase and hydroperoxide epoxygenase.

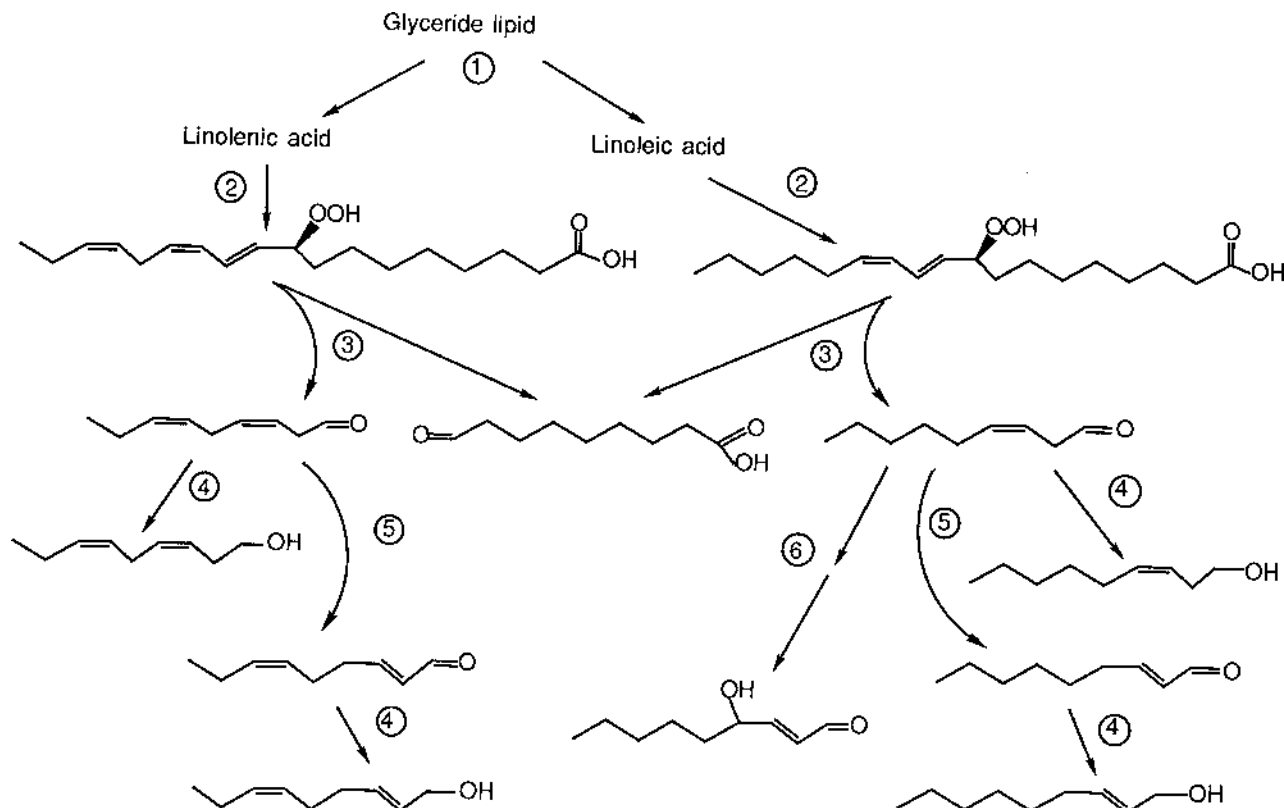


Fig. 5. The C₉-C₉ cleavage pathway to aldehydes via a 9(*S*)-hydroperoxide-specific lipoxygenase. The specific enzymes are 1) a family of lipolytic enzymes, 2) 9(*S*)-hydroperoxide-specific lipoxygenase, 3) hydroperoxide lyase, 4) alcohol dehydrogenase, 5) *cis*-3:*trans*-2-enal isomerase, 6) *cis*-3-alkenal oxygenase and hydroperoxide epoxidase.

room hydroperoxide lyase cleaved the 10*S*-hydroperoxide of linoleic acid into 1-octen-3-ol and 10-oxo-*trans*-8-decenoic acid (Wurzenberger and Grosch, 1984). 1-Octen-3-ol is an important flavor component of mushrooms.

A role for the aldehydes languished in obscurity for many years after the first reports of physiological activity. Major et al. (1960) and Schildknecht and Rauch (1961) found that *trans*-2-hexenal was fungitoxic, and Nandi and Fries (1976) found antifungal activity with other hydroperoxide-lyase-derived aldehydes. Subsequently, Zimmerman and Coudron (1979) reported a wound-healing action of 12-oxo-*trans*-10-dodecenoic acid. More recently, there have been numerous investigations of antifungal and antimicrobial activity of the hydroperoxide-lyase-generated aldehydes, including activity against certain pathogenic strains of these organisms (Bradow, 1991; Croft et al., 1993; Deng et al., 1993; Doehlert et al., 1993; Gardner et al., 1990; Gueldner et al., 1985; Hamilton-Kemp et al., 1992; Urbasch, 1987; Vaughn and Gardner, 1993; Zeringue and McCormick, 1989, 1990). In one particularly interesting study (Croft et al., 1993), *Phaseolus vulgaris* L. leaves inoculated with an avirulent strain of *Pseudomonas syringae* pv *phaseolicola* Burkholder caused a burst of hexenals and hexenols after 15 to 24 h, which corresponds to the time of the hypersensitive response. One of the hexenals, *trans*-2-hexenal, was particularly inhibitory to the growth of *P. syringae* pv *phaseolicola*. Because the hexenal/hexenol burst occurred before phytoalexin accumulation, these compounds may be early volatile phytoalexins important to early plant responses. However, a virulent, compatible strain of *P. syringae* pv *phaseolicola* elicited hexenals/hexenols only at levels slightly above the controls. In addition to antifungal and antimicrobial activity, anti-insect activity has been demonstrated for some of the aldehydes (Chamberlain et al., 1991; Hildebrand et al., personal communication; Mohri et al., 1990).

The fungitoxicity of 4-hydroxy-*trans*-2-nonenal is comparatively high (Vaughn and Gardner, 1993). In addition to antifungal activity, the 4-hydroxy-alkenals, including 4-hydroxy-*trans*-2-hexenal, may cause other physiological effects. According to Esterbauer (1992), 4-hydroxy-*trans*-2-nonenal may modulate gene expression. Since 4-

hydroxy-*trans*-2-nonenal and 4-hydroxy-*trans*-2-hexenal activate phospholipase D, these 4-hydroxy-alkenals also appear to have some control over the generation of second messengers, such as phosphatidic acid and diacylglycerol (Natarajan et al., 1993). At least one enzyme, glucose-6-phosphate dehydrogenase, has been inactivated by exposure to 4-hydroxy-*trans*-2-nonenal (Szweda et al., 1993).

Other hydroperoxide-decomposing enzymes. Another major pathway of linoleic/linolenic acid hydroperoxide metabolism involves the formation of hydroxy-diene/triene, epoxy-ene/diene, epoxyhydroxy-ene/diene, and trihydroxy-ene/diene fatty acids. There has been some debate about the relative importance of two possible pathways of formation of these fatty acids. As ascertained in the laboratories of Blée (Blée and Schuber, 1990a, 1990b; Blée et al., 1993) and Hamberg (Hamberg and Fahlstadius, 1992; Hamberg and Hamberg, 1990), one pathway, catalyzed by hydroperoxide-dependent peroxygenase (or epoxygenase), involves the epoxidation of double bonds with peroxide oxygen, resulting in the simultaneous reduction of hydroperoxide to hydroxide. Primary products are epoxy-, epoxyhydroxy-ene/diene, and hydroxy-diene/triene fatty acids. Trihydroxy-ene/diene fatty acids are hydrolysis products of epoxyhydroxy-ene/diene fatty acids. An alternative pathway involves formation of an alkoxy radical from hydroperoxide and subsequent alkoxy rearrangement into epoxyhydroxy-ene/diene fatty acids (for a discussion of these two possibilities, see review by Gardner, 1991).

The physiological importance of the various oxygenated fatty acids discussed in the preceding paragraph has been attributed to their antifungal action against rice blast disease (Kato et al., 1983, 1984, 1985, 1986a, 1986b, 1991; Ohta et al., 1990; Shimura et al., 1983). Both lipoxygenase and lipid hydroperoxide-decomposing activities are activated after infection of rice (*Oryza sativa* L.) with rice blast fungus, especially with an incompatible strain of the fungus (Ohta et al., 1991). In addition, two products of the lipoxygenase and lipid hydroperoxide-decomposing system elicited phytoalexins in *Oryza sativa* L. (Li et al., 1991). Similarly, a trihydroxy-ene fatty acid is produced by the root of *Colocasia esculenta* var. *antiquorum* L. in defense against *Ceratocystis fimbriata*, the black rot fungus (Masui

and Kojima, 1990; Masui et al., 1989). And, according to Blée and Schuber (1993), the various fatty acids produced by the hydroperoxide-dependent peroxygenase reaction are cutin monomers and their precursors. The production of a cutin barrier may be yet another important defensive function of the lipoxygenase pathway in plants.

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Peroxidative Activity of Apple Peel in Relation to Development of Poststorage Disorders

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Lipid peroxidation is a factor in the development of plant tissue senescence (Thompson et al., 1991), and in responses to physiological stresses such as chilling (Parkin and Kuo, 1989). Apple (*Malus domestica* Borkh.) fruit are commonly stored for long periods at low temperature, and during this time a variety of disorders can develop, only some of which appear to be uniquely associated with senescence. Many apple cultivars are chilling sensitive, although several months near 0C are required for symptoms to develop (Bramlage and Meir, 1990). During this time, both senescence and chilling injury can occur, and an interaction between these conditions could contribute to the development of certain disorders.

Superficial scald develops on the surface of certain apple cultivars following long-term storage near 0C. The scald results from peroxidation of the sesquiterpene α -farnesene in fruit peel, producing conjugated trienes that presumably perturb membrane lipids under certain conditions, causing disruption, discoloration, and death of surface cells (Anet, 1974; Huelin and Coggiola, 1970a). The disorder is usually controlled by applying the antioxidant diphenylamine (DPA) to fruit before placing them in storage. DPA inhibits the oxidation of α -farnesene (Huelin and Coggiola, 1970b).

We recently presented evidence that only a portion of the conjugated trienes are positively correlated with scald development, while another portion is negatively correlated with it (Du and Bramlage, 1993), and that ethylene is fundamentally involved in development of conditions that help determine scald development (Du and Bramlage, 1994). Ethylene production is mediated by free radicals (Gardner and Newton, 1987), and ethylene regulates many aspects of fruit ripening and senescence (Abeles et al., 1992). Also, DPA treatment affects ethylene production, respiration, and enzyme activity (Baker, 1963; Lurie et al., 1989), as well as reducing α -farnesene oxidation in apple peel. Since lipid peroxidation is a prominent factor in apple senescence (Feys, 1985; Feys et al., 1980), we proposed that lipid peroxidation products may contribute to conditions that determine scald development, and possibly to symptom expression itself. To investigate this hypothesis, we sampled apple fruit under a variety of conditions related to scald development, and assayed them for some general peroxidation products, and for activities of enzymes that are important in controlling the accumulation of peroxidation products in plant tissues. We now detail this work as part of this workshop.

Plant materials

Investigations occurred over 3 years using fruit grown under normal orchard conditions at the Univ. of Massachusetts Horticultural Research Center, Belchertown.

On 26 Sept. and 10 Oct. 1990, \approx 200 'Cortland' and 'Delicious' apples, respectively, were harvested from each of three single-tree replications at commercial maturity. Before storage, half of the harvested fruit of each cultivar was dipped in fungicide [227 g of 50% methyl 1-(butyl carbamoyl)-2-benzimidazocarbamate (benlate) and 454 g of 3a,4,7,7a-tetrahydro-2-[(trichloromethyl)thio]-1-*H*-isoindole-1,3(2*H*)-dione (captan) per 400 liters of water] as control, and the other half was dipped in 2 g DPA/liter plus fungicide. All fruit then were stored at 0C in air. Ten-fruit samples were taken from each replicate at harvest and at every 4 weeks during storage, up to a maximum of 16 weeks. Sampled fruit were peeled, and the peel was cut into small pieces, mixed, frozen immediately in dry ice, and stored at -25°C for subsequent analyses. Scald incidence on the remaining fruit was evaluated visually after 16 weeks at 0C plus 7 days at 20C. No scald had developed on 'Delicious' at this time, so fruit were returned to storage for an additional 8 weeks plus 7 days at 20C for a second scald evaluation.

On 30 Sept. 1991, \approx 400 'Empire' fruit were harvested from each of four single-tree replications and stored at 0C in air for 24 weeks. Ten-fruit samples were taken at 4-week intervals, peeled, and frozen for analyses as described above. After 24 weeks at 0C, senescent breakdown was present in some fruit. Ten fruit with the disorder and 10 fruit free of it were selected from each replication. Peel was taken from disorder-free fruit as above, but diseased fruit peel was taken only from areas with symptoms. Peel was frozen for assay as above.

On 9 Sept. 1992, \approx 200 'Cortland' fruit were harvested from each of four single-tree replications and stored at 0C for 20 weeks. Stored fruit then were transferred to 20C for 7 days and evaluated for scald development. Fruit within each replication then were sorted into three groups according to their scald intensity, i.e., no scald; 1% to 33% of fruit surface; and $>33\%$ of fruit surface affected with scald. Ten fruit without senescent breakdown were selected randomly from each group, peeled, and frozen for analyses.

Assays of lipid peroxidation products

Extraction. Ten grams of frozen peel were homogenized in a blender with 50 ml of cold acetone for 3 min and filtered through Whatman no. 4 paper. The filtrate was brought to 75 ml with water and kept at 4C under refrigeration for measurements.

Measurements. Two groups of products of lipid peroxidation were measured in the present study. The thiobarbituric acid-reactive substances (TBARS) were measured using the thiobarbituric acid assay modified to adjust for the high sugar content of fruit [expressed as nmoles of malondialdehyde (MDA) per gram of fresh weight; Du and Bramlage (1992)]. Total peroxides were determined according to Brennan and Frenkel (1977), modified as follows: 1 ml of properly diluted extract and 4 ml of water were added to 1 ml of titanium reagent (20% titanous tetrachloride in concentrated HCl, v/v). After mixing and

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standing for 5 min, the absorbance was measured at 415 nm against a blank that contained all components except the extract. A standard curve was made from commercial hydrogen peroxide (H₂O₂), and it gave good linearity for concentrations <2 mM. The total peroxides were expressed as nmoles of H₂O₂ per gram of fresh weight.

Assays of enzyme activities

Extraction. Two grams of frozen tissue was ground with a mortar and pestle in 10 ml of 50 mM phosphate buffer (containing 0.2% insoluble polyvinylpyrrolidone, 0.1 mM EDTA, and 3 mM MgCl₂, pH 7.0), and a small amount of washed sand. The homogenate was centrifuged at 15,000×g for 15 min, and the supernatant obtained was used for assays of enzyme activities. Extract preparation was carried out at 0 to 4°C. Enzyme activities were expressed as units per gram of fresh weight.

Assay of catalase. Catalase activity was assayed by the ability to decompose H₂O₂. Four milliliters of assay mixture (50 mM Tris buffer, pH 6.8, containing 5 mM H₂O₂) was added to 0.5 ml of extract. After 10 min at 20°C, the reaction was stopped by adding 0.5 ml of titanium reagent, as described above. The resulting solution was appropriately diluted and the residual H₂O₂ was determined as described above for total peroxides. A control, in which the enzyme activity was stopped at "zero" time, was run at the same time. One unit of catalase activity is defined as the amount of enzyme that decomposed 0.01 μmol of H₂O₂ per minute under the assay conditions.

Assays of peroxidase (POD) and polyphenoloxidase (PPO). POD and PPO activities were assayed essentially according to Kar and Mishra (1976). One unit of activity of POD or PPO is defined as the amount of the enzyme that caused an increase of 0.1 in the absorbance at 420 nm by the purpurogallin formed in the assay.

Assays of superoxide dismutase (SOD). SOD activity was measured according to Beauchamp and Fridovich (1971) and Dhindsa et al. (1981) by measuring the ability of SOD to inhibit the photochemical reduction of nitro blue tetrazolium (NBT). The assay mixture contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 μM NBT, 2 μM riboflavin, 0.1 mM EDTA, and 100 μl of enzyme extract. Three milliliters of the assay mixture in uniform, transparent tubes was shaken and placed 50 cm below a lightbank consisting of eight 15-W fluorescent lamps. The reaction was started by switching on the lights and was allowed to proceed for 10 min. The reaction then was stopped by switching off the lights, and the absorbance by the assay mixture at 560 nm was read. The diffuse room light had no measurable effect. The nonirradiated assay mixture did not develop color and served as control, and the assay mixture lacking enzyme extract developed maximum color, which decreased with increasing amount of extract added. One unit of SOD activity is defined as the amount of enzyme that inhibited the NBT photoreduction by 50% under the assay conditions. However, the percent inhibition was not linear with SOD concentration. Thus, units of SOD activity were calculated from transformed data as described by Asada et al. (1974) and Giannopolitis and Ries (1977). SOD units per milliliter = (V₀/V - 1) × (dilution factor), where V₀ is the slope of the change in absorbance in the absence, and V in the presence, of enzyme extract. The transformation resulted in a linear correlation between the SOD activity and the amount of the extract used in this study [V₀/V = 0.989 + 0.48 (μl), R = 0.951***].

Data were subjected to analyses of variance. Least significant difference or Duncan's new multiple range test was used to separate means when applicable.

Findings

Peroxidative characteristics in fruit peel during storage at 0°C were compared among three apple cultivars with different scald susceptibilities (Table 1). 'Cortland' and 'Delicious' are scald susceptible, while 'Empire' is scald resistant. 'Empire' fruit peel accumulated significantly more TBARS and peroxides during storage than did either 'Cortland' or 'Delicious' fruit peel, and 'Delicious' accumulated somewhat higher concentrations than did 'Cortland'. 'Empire' peel exhibited lower catalase, POD, and PPO activities than that of the

Table 1. Comparisons among cultivars of lipid peroxidation products and enzyme activities in apple peel and development of superficial scald after storage.^z

Measurement ^y	Cultivar		
	Cortland	Delicious	Empire
TBARS (nmol·g ⁻¹ fresh wt)	55 a ^x	79 b	102 c
Peroxides (nmol·g ⁻¹ fresh wt)	20 a	24 b	31 c
Catalase (unit/g fresh wt)	58 b	58 b	56 a
POD (unit/g fresh wt)	53 c	25 b	15 a
Total SOD (unit/g fresh wt)	248 c	51 a	157 b
PPO (unit/g fresh wt)	18 b	21 b	10 a
Scald (%)	23 b	20 b ^w	0 a

^z'Cortland' and 'Delicious' were harvested on 26 Sept. and 10 Oct. 1990, respectively, and stored for 16 weeks at 0°C. 'Empire' was harvested on 27 Sept. 1991 and stored for 24 weeks at 0°C. Each cultivar was sampled at 4-week intervals and data are means of all sample times.

^yTBARS = thiobarbituric acid-reactive substances; POD = peroxidase; SOD = superoxide dismutase; PPO = polyphenoloxidase.

^xMean separation within rows by Duncan's new multiple range test, P ≤ 0.05.

^w'Delicious' did not scald following 16 weeks at 0°C and was returned to storage for an additional 6 weeks. Scald developed during subsequent 7 days at 20°C.

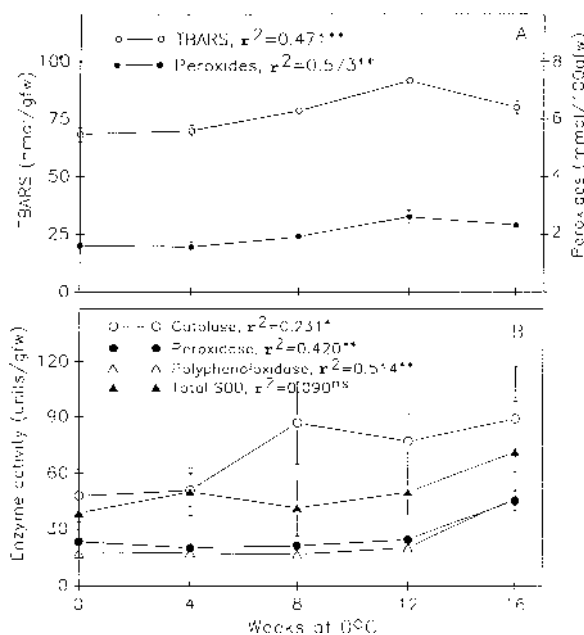


Fig. 1. Changes in (A) concentrations of peroxidation products and in (B) activities of certain enzymes in 'Delicious' apple peel during storage at 0°C. ns, **, ***Nonsignificant or significant at P ≤ 0.05 or 0.01, respectively, for linear regressions over time.

Table 2. Effects of diphenylamine (DPA) applied at 2 g-liter⁻¹ before storage on accumulation of peroxidation products, enzyme activity, and superficial scald development on apples after storage.^z

Measurement ^y	Cortland		Delicious	
	-DPA	+DPA	-DPA	+DPA
TBARS (nmol·g ⁻¹ fresh wt)	55 NS	54	78 NS	80
Peroxides (nmol·g ⁻¹ fresh wt)	22 **	17	24 *	20
Catalase (unit/g fresh wt)	83 **	39	70 **	45
POD (unit/g fresh wt)	55 NS	32	27 *	22
Total SOD (unit/g fresh wt)	248 NS	267	51 NS	45
PPO (unit/g fresh wt)	18 NS	18	24 *	18
Scald (%)	20 **	1	20 **	5

^z'Cortland' and 'Delicious' were harvested on 26 Sept. and 10 Oct., respectively, and stored for 16 weeks at 0°C. Fruit were sampled every 4 weeks, and data are the means of all sample times.

^yTBARS = thiobarbituric acid-reactive substances; POD = peroxidase; SOD = superoxide dismutase; PPO = polyphenoloxidase.

^xScald did not develop on 'Delicious' after 16 weeks at 0°C. Fruit were returned to 0°C for an additional 6 weeks, and scald developed during the subsequent 7 days at 20°C.

ns, **, ***Nonsignificant or significant at P ≤ 0.05 or 0.01, respectively. Significance of DPA treatment effect for a cultivar and measurement.

other cultivars, and 'Empire' and 'Delicious' peel had lower SOD activity than did that of 'Cortland'. Despite the indications of higher peroxidative activity in 'Empire' peel during storage, these fruit developed no scald after storage, while the disorder occurred on the other two cultivars.

During storage of 'Delicious' at 0C, slow but significant linear increases in TBARS and peroxides occurred over time (Fig. 1). Catalase, POD, and PPO activities also increased significantly over time, but SOD activity did not change significantly (Fig. 1). Similar results were recorded for 'Cortland', except that SOD activity declined 75% during 16 weeks at 0C (data not shown). In 'Empire', only TBARS and catalase increased significantly during storage, while SOD activity decreased slowly (data not shown).

DPA applied before storage slightly reduced peroxide concentrations in peel of both 'Cortland' and 'Delicious' apples during storage but had no effect on TBARS concentrations (Table 2). DPA also markedly reduced catalase activity in both cultivars, slightly reduced POD and PPO activities in 'Delicious', but had no effect on SOD activity in either cultivar. Treatment reduced scald development in both cultivars.

'Cortland' apples at 20C developed no scald, but after 24 weeks of storage at 0C, about one-third of the fruit had developed the disorder (Table 3). At 20C, TBARS and peroxides were higher after 1 week than at harvest, but after 2 weeks, they were at the same levels as at harvest. This transient increase is associated with the onset of ripening (Du, 1993), but it was not accompanied by significant changes in activity of any of the measured enzymes. At 0C, no significant change in either TBARS or peroxides was evident after 12 or 24 weeks, while catalase and POD activities increased during storage. Overall, temperature had no significant effect on peroxidative characteristics other than inducing higher POD activity at 0C than at 20C.

Peel from 'Cortland' apples with different amounts of superficial scald on their surfaces had similar peroxidative activity after storage for 20 weeks at 0C (Table 4). There was no significant difference in peroxidative activity of peel between scald-free and scalded fruit.

During storage of 'Empire' apples, some fruit developed symptoms of senescent breakdown: soft, brown areas with dark vascular strands developing just beneath the peel, primarily near the calyx end

of the fruit. When peel from above these areas ("senescent") was compared with peel from disorder-free fruit ("healthy"), marked differences in peroxidative activity were evident (Fig. 2). Senescent tissue contained much higher concentrations of TBARS and peroxides, and higher activities of catalase, POD, and SOD, but there was no difference in PPO activity.

Implications

During storage of 'Delicious' apples at 0C, concentrations of TBARS and peroxides gradually increased (Fig. 1), and peroxidation products also increased in 'Cortland' and 'Empire' (data not shown). Since temperature had no significant effect on TBARS or peroxides (Table 3), the increases in apples during storage apparently reflected effects of fruit senescence and were not the result of chilling damage. The suggestion that lipid breakdown results from some combination of chilling and senescence (Parkin et al., 1989) also appears unlikely.

Since plant tissues possess complex systems that control buildup of oxidation products (Winston, 1990), these assays gave limited insight into cellular processes. Catalase, POD, and SOD activities were assayed to see if substantial changes occurred in protecting tissues from peroxide accumulation. Catalase activity increased substantially in fruit peel during storage (Table 3, Fig. 1); the only indications of a marked loss of protection were a 75% reduction in SOD activity in 'Cortland' peel during 16 weeks at 0C (data not shown), and a possible reduction of SOD activity in 'Empire' (Table 3). Nevertheless, the TBARS and peroxide data suggest that control systems in apple peel largely kept peroxidative activities in check during prolonged storage of the fruit, and that overall changes were remarkably slow.

Superficial scald is induced soon after harvest, but its symptoms are not expressed until fruit have remained in storage for 3 or more months (Bramlage and Meir, 1990). During this extended storage time, conjugated trienes usually increase in fruit peel, and scald development is positively correlated with maximum conjugated triene concentration (Huelin and Coggiola, 1968). Our hypothesis was that lipid peroxidation products in tissue increase over time, to a point where they contribute to development of scald symptoms. No general relationship existed between scald susceptibility and concentration of peroxidation prod-

Table 3. Effects of temperature on accumulation of lipid peroxidation products, enzyme activity, and superficial scald development on 'Cortland' apples.

Time of measurement	TBARS ² (nmol·g ⁻¹ fresh wt)	Peroxides (nmol·g ⁻¹ fresh wt)	Catalase (units/g fresh wt)	POD ² (units/g fresh wt)	Total SOD ² (units/g fresh wt)	PPO ² (units/g fresh wt)	Scald (%)
At harvest	53	19	29	41	155	25	---
20C, 1 week	75	27	51	37	108	23	---
20C, 2 weeks	51	19	65	40	100	22	2 ^y
0C, 12 weeks	52	20	58	54	98	23	---
0C, 24 weeks	52	17	77	60	68	27	34
LSD _{0.05}	20	8	48	14	89	9	16
0C vs. 20C	NS ^z	NS	NS	*	NS	NS	***

²TBARS = thiobarbituric acid-reactive substances; POD = peroxidase; SOD = superoxide dismutase; PPO = polyphenoloxidase.

^ySlight scald-like symptoms were present on a few fruit at harvest, but did not become more prevalent during the postharvest period.

^zThis comparison indicates only the main effects of temperature.

NS, *, *** Nonsignificant or significant at $P \leq 0.05$ or 0.001, respectively.

Table 4. Concentrations of lipid peroxidation products and activities of certain enzymes in 'Cortland' apple peel affected with different intensities of superficial scald.²

Surface affected with scald (%)	TBARS ^y (nmol·g ⁻¹ fresh wt)	Peroxides (nmol·g ⁻¹ fresh wt)	Catalase (units/g fresh wt)	POD ^y (units/g fresh wt)	Total SOD ^y (units/g fresh wt)	PPO ^y (units/g fresh wt)
None	62	22	35	27	36	11
1 to 33	57	22	21	24	40	12
34 to 100	55	22	21	26	35	12
Significance	NS	NS	NS	NS	NS	NS

²Fruit were harvested on 9 Sept. 1992, and stored at 0C for 20 weeks, plus 1 week at 20C.

^yTBARS = thiobarbituric acid-reactive substances; POD = peroxidase; SOD = superoxide dismutase; PPO = polyphenoloxidase.

^zNonsignificant.

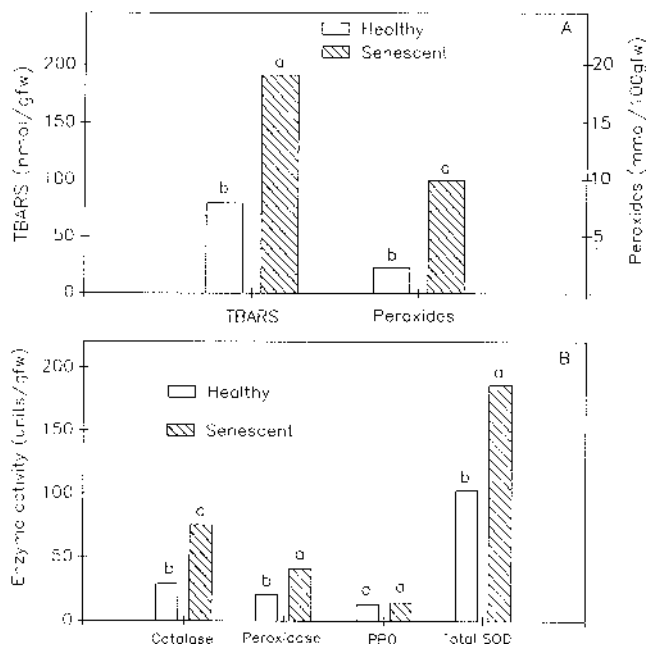


Fig. 2. Comparisons of concentrations of lipid peroxidation products and enzyme activities between peel of 'Empire' apples above areas with senescent breakdown ("senescent") and peel from fruit free of senescent breakdown ("healthy") after storage at 0C for 24 weeks. Mean separation within pairs at $P \leq 0.05$.

ucts. Scald-resistant 'Empire' apples contained higher TBARS and peroxide concentrations than scald-susceptible 'Cortland' and 'Delicious' (Table 1). In addition, there was no difference in TBARS or peroxide concentrations in 'Cortland' at scald-inducing and noninducing temperatures (Table 3), and no sudden increase occurred as 'Cortland' apples became liable to scald development at removal from storage (data not shown). However, the increase in peroxidation products (Fig. 1) may have been associated with the increase of conjugated trienes (Huelin and Coggiola, 1968), or with changes in cellular conditions that result in symptom development.

When the lipid-soluble antioxidant DPA was applied to apples at harvest, it reduced the peroxide concentration in apple peel during storage, despite reducing catalase and peroxidase activities (Table 2). This might have resulted from significantly reduced ethylene production (Du and Bramlage, 1994), but it also may indicate that DPA significantly supplemented the endogenous antioxidant concentrations in the peel. Barden and Bramlage (1994b) found that water-soluble antioxidants in apple peel decreased during fruit storage, and that among different lots of fruit, differences in lipid-soluble antioxidants at harvest usually persisted as levels increased during storage. These at-harvest differences among fruit lots were negatively correlated with scald development (Barden and Bramlage, 1994a). Applied DPA may reduce scald by contributing directly, as a lipid-soluble antioxidant, to cellular resistance to initiation of scald symptoms, perhaps by inhibiting fruit senescence (Lurie et al., 1989) or ω -farnesene oxidation to conjugated trienes (Huelin and Coggiola, 1970a).

Our results provide no insight into events during scald symptom development. Tissue with different levels of symptom expression contained TBARS and peroxide concentrations equal to those in scald-free tissue, and equal enzyme activities (Table 4). PPO activity was equal in peel regardless of symptom development (Table 4), and no difference existed in PPO activity among cultivars (Table 1) or between temperatures (Table 3). Lurie et al. (1989) suggested that DPA may reduce scald symptom development by maintaining low PPO activity. We found only slight increases of PPO activity during fruit senescence (Fig. 1), and slight reduction of PPO by DPA (Table 2).

Peel over tissue with senescent breakdown had markedly different peroxidative characteristics than that from breakdown-free fruit

(Fig. 2). These results also suggest that development of scald symptoms is distinct from general cell breakdown and discoloration.

In summary, our work provides no evidence that marked changes in peroxidative activity of apple peel are related to scald development in apples. However, it does not preclude the possibility that lipid peroxidation is related to scald development in more subtle ways, especially in the susceptibility of cell membranes to perturbation by conjugated trienes, which may produce cellular disorganization and symptom expression.

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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 H₂O₂ and lipid hydroperoxides also increase in ripening fruit, and Frenkel (1978) has reported that ripening can be delayed by inhibiting the formation of H₂O₂. 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

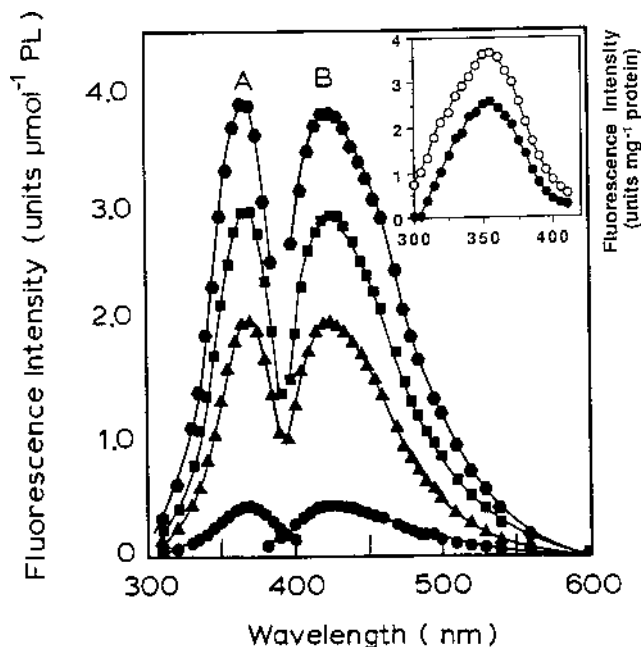


Fig. 1. Excitation (A) and emission (B) spectra for lipid extracts of microsomal membranes isolated from senescing bean cotyledons (age in days). (●) young, 2 days old; (▲) 4; (■) 7; (solid hexagon) 9. At 4 days, the cotyledons are beginning to show visible manifestations of senescence, and by 9 days, 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).

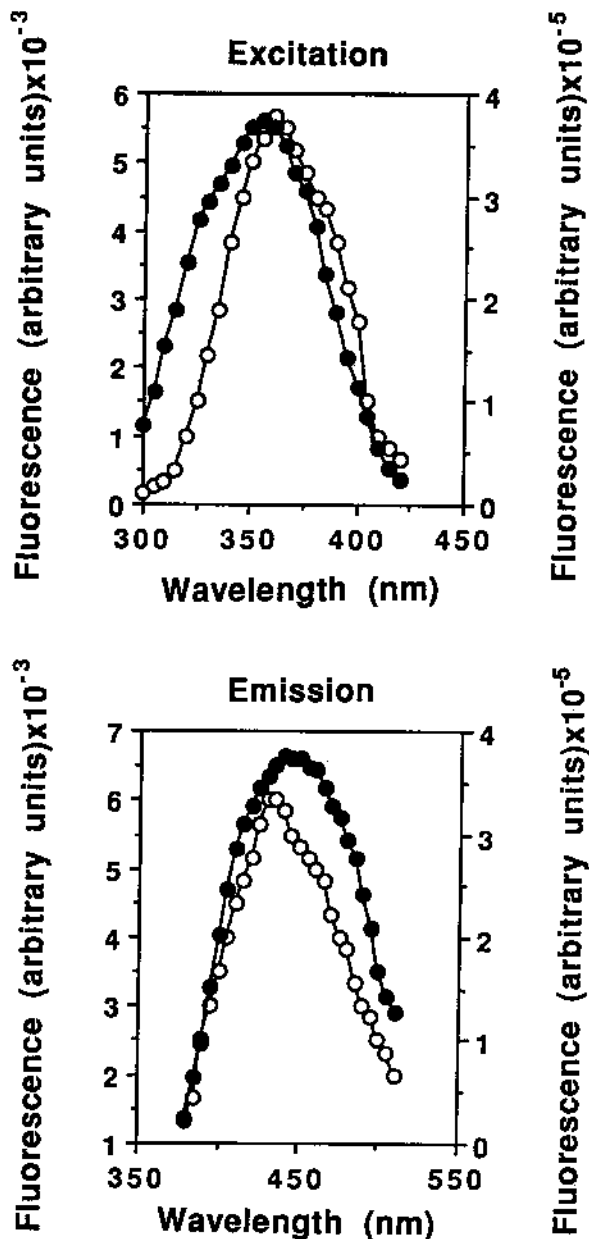


Fig. 2. Excitation and emission spectra for lipid extracts of nonsedimentable lipid-protein particles. (O) Lipid-protein particles from young, 2-day-old bean cotyledon tissue; (●) lipid-protein particles from petals of young, stage 2 carnation flowers. The cotyledon data are from Yao et al. (1993a).

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-

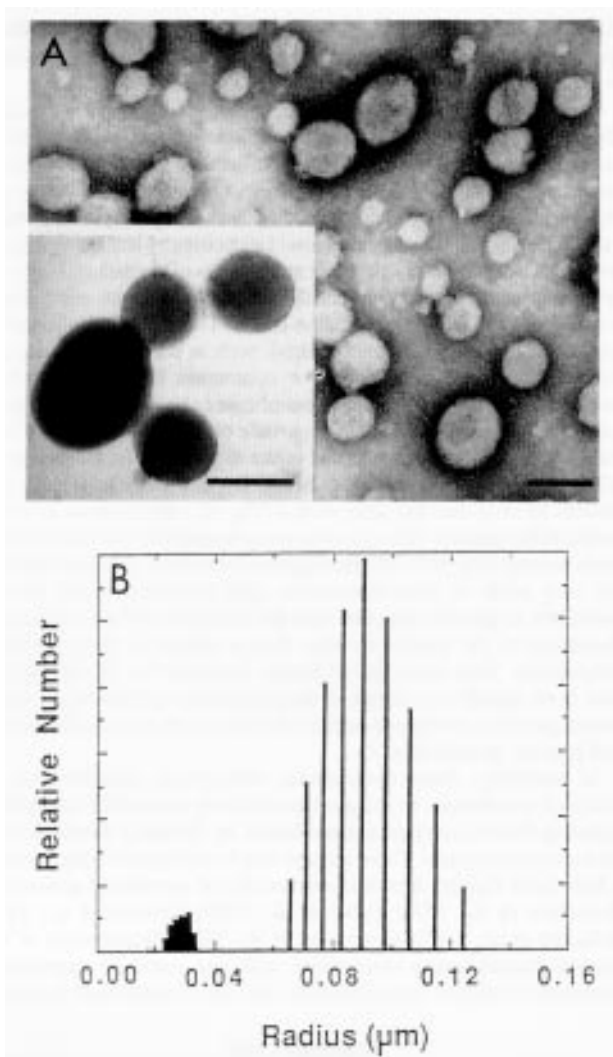


Fig. 3. Morphology and size of nonsedimentable lipid-protein particles. (A) Electron micrographs of lipid-protein particles isolated from the petals of young, stage 2 carnation flowers and stained with uranyl acetate according to Yao et al. (1991a); bars = 0.1 μm . (B) Size distribution of lipid-protein 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).

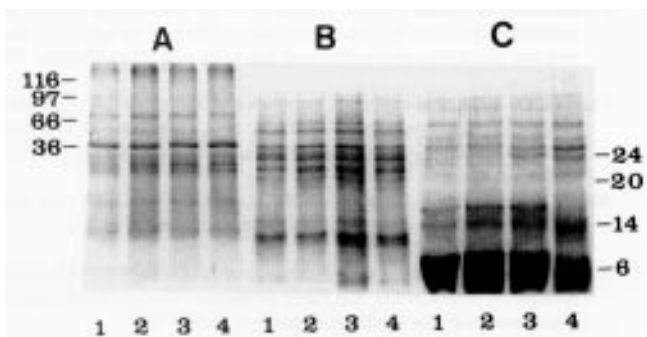


Fig. 4. SDS-PAGE (10% to 20% linear gradient) of subcellular fractions isolated from the petals of carnation flowers. Each lane was loaded with 1 μg of protein, and the gels were stained with silver (Wray et al., 1981). (A) Microsomal membranes; (B) nonsedimentable lipid-protein particles; (C) particle-free 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; $n = 3$.

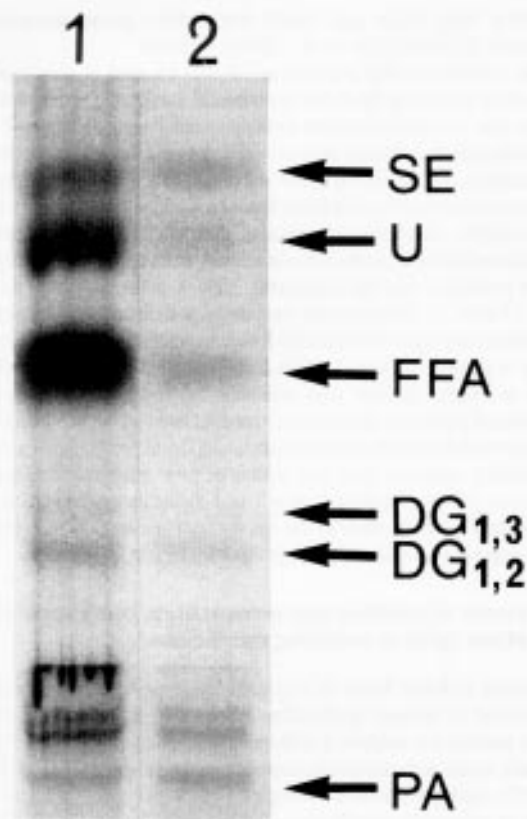


Fig. 5. Thin-layer chromatogram of total lipid extracts from nonsedimentable lipid-protein particles formed *in vitro* from microsomal membranes of bean cotyledons. The chromatogram was developed as described in Yao et al. (1991a). Lane 1, lipid-protein particles from microsomal membranes (10 mg protein 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.

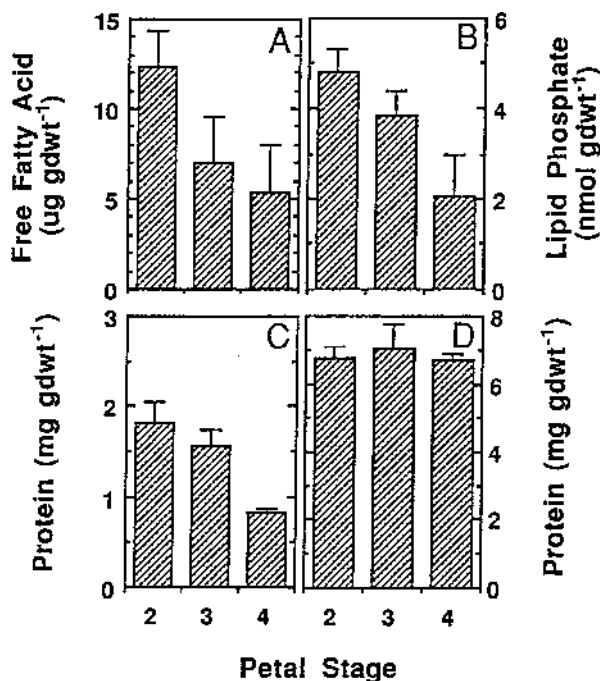


Fig. 6. Changes in the abundance of nonsedimentable lipid-protein particles during senescence of carnation flower petals. Abundance was measured in terms of (A) free fatty acid levels, (B) phospholipid levels, and (C) protein levels in the particle fraction. Corresponding levels of (D) particle-free 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 (Borochoy 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 (Cabrin 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 peroxy 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 tocopheroxy radical is more stable than a

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Table 1. Recent references supporting a link between lipid peroxidation and plant tissue disorders or senescence and aging.

Inducer	Description	Reference
Calcium deficiency	Correlation between necrosis and catalytic activity of superoxide dismutase in <i>Solanum tuberosum</i> L. tubers	Monk-Talbot et al., 1991
Chilling	Suggests resistant <i>Lycopersicon hirsutum</i> Humb. & Bonpl. decreases production of active oxygen species at low temperatures	Walker and McKersie, 1993
Heat and light	Decreased electron transport in <i>Triticum aestivum</i> L. leaves linked to peroxidation of thylakoid lipids	Mishra and Singhal, 1992
Microbial attack	Formation of free radicals in <i>Solanum tuberosum</i> L. tubers that are infected with <i>Erwinia carotovora</i> Oxidative response is one of earliest signals in hypersensitive response in <i>Glycine max</i> Merr. cells	Deighton et al., 1992 Legendre et al., 1993
Paraquat ²	Increased activity of antioxidant enzymes in drought-resistant <i>Zea mays</i> L. strain	Pastori and Trippi, 1993
Pollutants	In vitro demonstration of ozone-induced damage to <i>Populus maximowizii</i> A. Henry rubisco via membrane lipids	Landry and Pell, 1993
Water deficit	Resistance capacity of lipid extracts to peroxidation are affected by drought in <i>Phaseolus vulgaris</i> L. leaves	Ferrari-Iliou et al., 1993
Aging	Suggests gradual accumulation of free-radical products in <i>Solanum tuberosum</i> L. tubers but not consistent with lipoxygenase	Kumar and Knowles, 1993
Senescence	Increased lipid peroxidation products detected during senescence of <i>Petroselinum crispum</i> Mill. leaves	Meir et al., 1992

²Gramoxone(1,1'-dimethyl-4-4'-bypridinium ion) (paraquat).

Table 2. Recent references that do not support a link between lipid peroxidation and plant tissue disorders.

Inducer	Description	Reference
Bisulfite	Evidence of protein damage to photosystem II occurs before evidence of lipid peroxidation in <i>Phaseolus vulgaris</i> L. leaves	Covello et al., 1989
Chilling	Differences in sensitivity of <i>Cucumis sativus</i> L. leaves not explained on the basis of vulnerability to superoxide or presence of SOD	Hodgson and Raison, 1991
Dehydration	Degree of fatty acid unsaturation does not decrease and production of superoxide did not increase in <i>Helianthus annuus</i> L. seedling thylakoids	Sgherri et al., 1993
Light or methylviologen	Elevated cystolic glutathione reductase in leaves of transgenic <i>Nicotiana tabacum</i> L. plants did not affect oxidative balance	Foyer et al., 1991
Microbial attack	Oxidative burst in <i>Trifolium repens</i> L. and <i>Nicotiana tabacum</i> L. is independent of phytoalexin production and hypersensitive response Free radical scavenger tests indicate that •OH and LOX are not involved in stress ethylene production of <i>Glycine max</i> Merr. cuttings	Devlin and Gustine, 1992 Pennazio and Roggero, 1992
Paraquat ²	Higher levels of chloroplast SOD in transgenic <i>Nicotiana tabacum</i> L. plants did not prevent O ₂ toxicity	Tepperman and Dunsmuir, 1990
Aging	No measurable loss of PUFA observed in <i>Typha latifolia</i> L. pollen Absence of LOX isozymes in transgenic <i>Glycine max</i> Merr. seeds did not increase resistance to accelerated aging	van Bilsen and Hoekstra, 1993 Wang et al., 1990
Senescence	No free-radical accumulation associated with loss of viability of <i>Triticum aestivum</i> L. seeds Tocopherol levels in <i>Petroselinum sativum</i> Mill. and <i>Apium graveolens</i> L. leaves do not decrease predictably	Girard and LeMeste, 1992 Rise et al., 1989

²Gramoxone(1,1'-dimethyl-4-4'-bypridinium ion) (paraquat).

phospholipid free radical and can be regenerated by the oxidation of ascorbic acid to dehydroascorbic acid (Buettner, 1993), which in turn can be regenerated by the oxidation of glutathione and additional oxidation-reduction reactions in the cell (Meister, 1992). In addition, glutathione protects protein thiols in the membrane in the presence and absence of α-tocopherol (Palamanda and Kehrer, 1993). Note that since lipoxygenase produces lipid hydroperoxides directly without the free-radical intermediate (Gardner, 1991), lipoxygenase-catalyzed peroxidation does not lead directly to tocopheroxyl-radical formation. The action of lipoxygenase on lipids is also specific to the position of the double bond.

Lipid hydroperoxides increase the hydrophilic character of the internal bilayer (Frenkel, 1991). Relative to the active oxygen species and lipid radicals, lipid hydroperoxides are stable (Leshem, 1992), but they will degrade to undesirable free radicals (Schaich, 1992). Membrane repair systems have been identified that remove hydroperoxides by selective hydrolysis (Pacifci and Davies, 1991) and detoxify them by reactions coupled to glutathione reductase and nonspecific peroxidases (Salin, 1988).

In the absence of the necessary defense and repair systems, free-radical chain propagation of lipid peroxidation occurs within the membrane. As lipid hydroperoxides degrade, the lipid free radicals come into contact with other polyunsaturated fatty acids such that 10 to 100 additional lipid hydroperoxides are formed before the chain is broken (Niki et al., 1991). The rate of propagation is a factor of the interaction of lipid peroxy radicals and polyunsaturated fatty acids in

the membrane. This interaction is affected by both the concentration of available polyunsaturated fatty acids and the fluidity of the membrane. Although fluidity is frequently described in terms of chain-ordering, as affected by degree of unsaturation of fatty acids, rotational and lateral diffusion are more important factors in fluidity of membrane lipids (Carruthers and Melchior, 1988; Leshem, 1992; Webb and Green, 1991).

A COMPREHENSIVE MODEL

In the context of this view of lipid peroxidation, a model of peroxidative degradation of plant tissue can be constructed to explain the similarities and differences observed in plant tissue disorders. The model (Fig. 2) is offered to provide a basis for systematically testing the role of lipid peroxidation in the etiology of plant tissue disorders. It is consistent with most of the data collected to date, but has not been conclusively demonstrated.

Under normal conditions in the cell, active oxygen species are produced. Most of these species are scavenged or quenched by primary defense mechanisms, and little peroxidation of membrane lipids and proteins occurs. Any peroxidized lipids and proteins are quickly repaired. Upon exposure of plant tissue to stress, however, the level of active oxygen species tends to increase (Walker and McKersie, 1993). Stress may also induce the synthesis of defense molecules such as the antioxidant enzymes—superoxide dismutase, catalase, ascorbate peroxidase, and glutathione peroxidase (Mishra et al., 1993). Upon

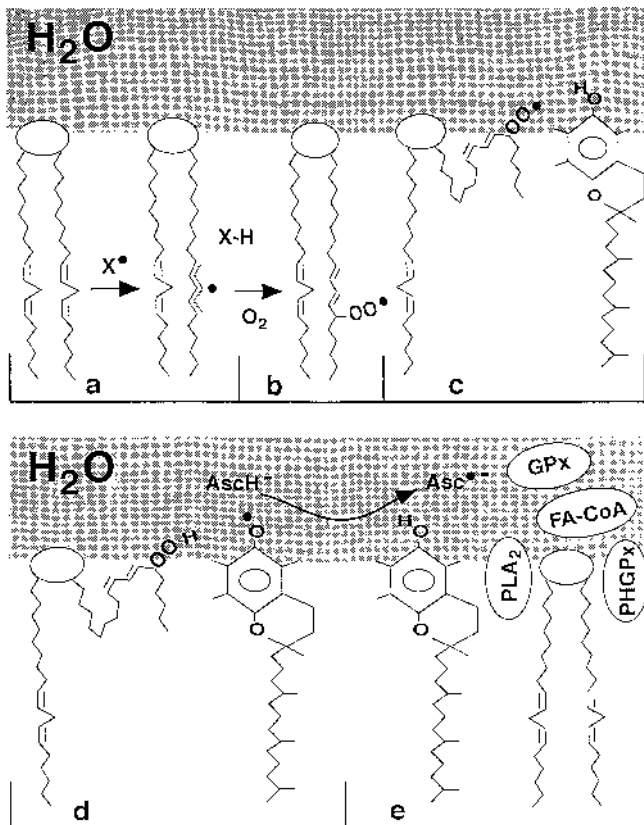


Fig. 1. Membrane lipid peroxidation. Only one leaflet of the bilayer is represented. (a) Initiation of the peroxidation process by an oxidizing radical, X^\bullet , by abstraction of a bis-allylic hydrogen, thereby forming a pentadienyl radical. (b) Oxygenation to form a peroxy radical and a conjugated diene. (c) Peroxy radical moiety partitions to the water-membrane interface where it is poised for repair by tocopherol. (d) Peroxy radical is converted to a lipid hydroperoxide, and the resulting tocopherol radical can be repaired by ascorbate. (e) Tocopherol has been recycled by ascorbate; the resulting ascorbate radical can be recycled by enzyme systems. The enzymes phospholipase A_2 (PLA_2), phospholipid hydroperoxide glutathione peroxidase (PH-GPx), glutathione peroxidase (GPx), and fatty acyl-coenzyme A (FA-CoA) cooperate to detoxify and repair the oxidized fatty acid chain of the phospholipid. This cartoon cannot show the dynamic aspects of this process. Tocopherol (TOH) in the membrane will undoubtedly be bobbing "up and down" so that the position of the "OH" is variable. In addition, TOH and TO^\bullet may have somewhat differing positions at the interface. Reprinted from *Archives of Biochemistry and Biophysics* [Buettner (1993) 300:535-543] with permission.

prolonged exposure, however, the level of prooxidants can exceed the defense capacity of the cell, or a specific membrane in the cell, against peroxidative attack. The tipping of this balance from net defense to net peroxidation could result from a continued increase in prooxidant formation, a decrease in one or more of the primary defense mechanisms, or a combination of these events (Purvis and Shewfelt, 1993). Although lipids and proteins are subject to free-radical attack, lipid peroxidation is kinetically favored (Murphy et al., 1992). Thus, if the process is regulated strictly by kinetics, peroxidation of polyunsaturated fatty acids may serve a protective function to prevent direct attack on proteins. Evidence in some animal systems suggests that protein damage is incurred by direct attack (Pacifici and Davies, 1991).

As lipid free radicals are converted to lipid hydroperoxides, α -tocopherol concentrations decrease, particularly when ascorbic acid, glutathione, and other cell reductants in contact with the affected membrane(s) are depleted. When the tocopherol concentration in the membrane is too low to protect the lipids, free-radical chain propagation occurs much faster than the repair mechanisms can function. Modification of the physical properties of the membrane (Bruch and Thayer, 1983) and direct peroxidation of proteins by lipid free radicals (Murphy et al., 1992) lead to decreased protein activity (Thomas et al.,

a. NORMAL CONDITIONS			b. INITIATION		c. CHAIN-BLOCKING	
O_2^-	SOD	LL	LL	LL	LL	LL
		LL	HO^\bullet	LL	LL	LL
H_2O_2	Catalase	LL	$\bullet OOLL$	HOOLL	$\bullet EL$	HOOLL
		LL	HO^\bullet	LL	LL	LL
Fe^{3+}		EL	EL	EL	LL	LL
	Chelator	LL	LL	LL	LL	LL
Fe^{2+}		[P]	[P]	[P]	[P]	[P]
		LL	LL	LL	LL	LL
1O_2	Carotenoids	LL	LL	LL	LL	LL
		LL	LL	LL	LL	LL
LOX		LL	LL	LL	LL	LL
		LL	LL	LL	LL	LL

PROOXIDANTS DEFENSE MEMBRANE

d. REPAIR		e. PROPAGATION		f. DAMAGE	
LL	LL	LL	LL	LL	LL
PLA_2	LL	LL	LL	LL	LL
$FA-CoA$	LL	HOOLL	HOOLL	HOOLL	HOOLL
C^\bullet	EL	dHC	$\bullet EL$	$\bullet EL$	$\bullet EL$
GSH	LL	GSSG	LL	LL	LL
LL	LL	LL	LL	LL	LL
[P]	[P]	[P]	[P]	[P]	[P]
LL	LL	$\bullet OOLL$	HOOLL	HOOLL	HOOLL
LL	LL	HOOLL	HOOLL	HOOLL	HOOLL
LL	LL	$\bullet OOLL$	$\bullet OOLL$	$\bullet OOLL$	$\bullet OOLL$
LL	LL	LL	LL	LL	LL
LL	LL	LL	LL	LL	LL

Fig. 2. A comprehensive model depicting free-radical attack of membrane lipids, initiation, chain-blocking, repair, propagation, and protein damage, where O_2^- represents superoxide; H_2O_2 , hydrogen peroxide; Fe^{3+} , ferric iron; Fe^{2+} , ferrous iron; 1O_2 , singlet oxygen; LOX, lipoxygenase; SOD, superoxide dismutase; L, phospholipid molecule with two fatty acids; E, α -tocopherol; [P], membrane-bound protein; HO^\bullet , hydroxyl radical; $\bullet OOLL$, lipid peroxy radical; HOOLL, lipid hydroperoxide; $\bullet E$, tocopherol radical; PLA_2 , phospholipase A_2 ; $FA-CoA$, fatty acyl coenzyme A; C^\bullet , ascorbic acid radical; GSH, glutathione; dHC, dehydroascorbic acid; GSSG, oxidized glutathione; $\bullet OL$, lipid alkoxy radical; and [P], a protein with a disulfide bond at an active site implying damage. Under normal conditions, defense mechanisms protect the membrane from peroxidation. Initiation occurs when oxygen radicals, such as $\bullet OH$, overcome the defense system to form $\bullet OOL$ (see Fig. 1 a and b). Chain-blocking is achieved by α -tocopherol forming HOOLL and $\bullet E$ (Fig. 1 c and d). Repair occurs by cleavage of the radical to C^\bullet (Fig. 1 e). Propagation results upon depletion of enough E, C, and GSH such that $\bullet OOLL$ and HOOLL are formed more rapidly than repair mechanisms can be effective. Damage to proteins is incurred through interaction with an adjacent $\bullet OOLL$.

1989), metabolic imbalances, cellular dysfunction, and ultimately tissue disorders.

Although no comprehensive study of peroxidative degradation of a specific membrane has been conducted with respect to a specific disorder in a plant tissue, a wide range of studies supports specific steps in the proposed model. In addition to the evidence presented in Table 1, increased superoxide generation has been noted in hypersensitive response (Adam et al., 1989; Keppler and Novacky, 1987). Formation of the hydroxyl radical in microsomes isolated from seedlings has been documented (Simontacchi and Puntarulo, 1992). Initial increases in the levels of antioxidant enzymes have been reported during winter hardening of red spruce (*Picea rubens* Sarg.) (Doullis et al., 1993), of herbicide-treated leaves (Schmidt and Kunert, 1986), and in manganese-deficient plants exposed to high-intensity light conditions (Cakmak and Marschner, 1992). Decreases in levels of antioxidant enzymes have been reported during chilling of susceptible seedlings (Jahnke et al., 1991) and dehydration of germinating seeds (Leprince et al., 1990). Superoxide dismutase activity decreases in mitochondria of senescing petals while increasing in the peroxisomes of the same petals (Droillard and Paulin, 1990). Depletion of cellular, water-soluble antioxidants

has been observed during hydration of seed axes (Senaratna et al., 1985) and chilling of susceptible fruit (Hariyadi and Parkin, 1991). Alpha-tocopherol levels decrease in thylakoids from chilled fruit before evidence of injury (Hariyadi and Parkin, 1991). Measurable levels of peroxidative products have been observed before symptom development of hypersensitive reaction (Adam et al., 1989) and loss of desiccation tolerance (Leprince et al., 1990). Evidence of lipid-protein peroxidative complexes detected by fluorescent pigment methodology has been described in senescing leaves (Meir et al., 1992) and chilled fruit (Hariyadi and Parkin, 1991).

EXPLANATIONS

A comprehensive model for lipid peroxidation as the primary cause of plant tissue disorders must explain contrary evidence, the wide variability of symptoms observed for a diverse set of stimuli, the delay between stress induction and evidence of peroxidation, cross protection observed between stress responses, localized damage as noted in hypersensitive reaction and chilling injury (CI), and the enhancement of CI observed upon rewarming.

Evidence against lipid peroxidation as a causal agent in plant tissue disorders can be categorized by failure to observe an increase in a specific oxidative species (Sgherri et al., 1993), inability to relate sensitivity to stress on the basis of specific antioxidants or scavenging enzymes (Foyer et al., 1991; Hodgson and Raison, 1991; Rise et al., 1989; Tepperman and Dunsmuir, 1990), the lack of a relationship between lipoxygenase activity and the disorder (Pennazio and Roggero, 1992; Wang et al., 1990), and the lack of selective decreases in polyunsaturated fatty acids (Sgherri et al., 1993; van Bilsen and Hoekstra, 1993). The proposed model suggests that, rather than focusing on a single event (e.g., increase in an active oxygen species or decrease in activity of a specific enzyme), the overall balance between prooxidants and defense reactions must be studied. Much of the research has focused on superoxide production and superoxide dismutase defenses, but if superoxide dismutase is present and catalase is deficient then hydrogen peroxide could accumulate to toxic levels. In addition, compartmentation of enzymes and substrates in the cell, ignored by most of the studies cited in Tables 1 and 2, would be expected to affect the oxidative balance at a particular membrane site. For example, if superoxide is produced and is able to migrate to a membrane site outside compartments containing superoxide dismutase, the superoxide could be converted to the hydroxyl radical via the Haber-Weiss reaction, with damage incurred at cellular superoxide dismutase levels that appear to be sufficient for protection. Exposure of susceptible and resistant leaves to chilling conditions resulted in increases in production of active oxygen species and antioxidant enzyme activity in both species (Walker and McKersie, 1993). Decreased susceptibility was attributed to a more rapid decline in subsequent production of active oxygen species in the resistant species, supporting the suggestion that an understanding of the balance of prooxidants and defense mechanisms is the critical area of study in lipid peroxidation research.

The proposed model is based on the premise that damage to a single membrane system (e.g., plasma membrane, thylakoid, inner mitochondrial membrane) in the cell is sufficient to induce metabolic disorders and tissue injury. Thus, measurement of total cellular polyunsaturated fatty acids may not reveal significant losses of polyunsaturated fatty acids in the affected membrane. The model is based on initiation and propagation of lipid free radicals in the membrane and considers formation of the hydroxyl radical in the presence of Fe^{2+} , Fe^{3+} , and singlet oxygen to be primary initiators of peroxidative degradation in plant membranes. Lipoxygenase is probably not a major factor in causing these disorders. Transgenic soybean seeds with decreased lipoxygenase were not more resistant to accelerated aging (Wang et al., 1990). More likely, however, hydrolytic enzymes and lipoxygenase are part of a programmed turnover or retailoring mechanism rather than being responsible for membrane degradation and resultant tissue disorders.

In this model, the wide variability in response and symptom development in diverse types of plant tissue to the same stress or similar types of tissue to various stresses is attributed to differences in

loss of protein function (e.g., ion transport, enzyme activity) in the membrane that is the site of attack. Studies on CI have shown differences in susceptibility of organs as influenced by genotype (Bodner and Larcher, 1978) as well as differences in susceptibility of specific membranes in a cell (Cheng and Shewfelt, 1988; Marangoni et al., 1989). The development of symptoms would result from the specific metabolic imbalances incurred by disruption of specific enzymes in the affected membrane or loss of membrane permeability. While the membrane site of attack would vary from tissue to tissue and potentially from stress to stress, the membrane of greatest susceptibility to a specific stress within a particular organ should always be the same, and response, including symptom development, should be predictable based on the function of that particular membrane in that cell in that particular organ.

The delay between stress induction and evidence of peroxidation can be explained by a greater understanding of the peroxidation process. Comparing the rate of reaction of active oxygen species occurring within nanoseconds or microseconds (Buettner, 1993) and the development of symptoms measured in hours (Adam et al., 1989), days (Cakmak and Marschner, 1992; Jahnke et al., 1991; Leprince et al., 1990), or weeks (Cheng and Shewfelt, 1988) suggests that the process is slow and incremental. Most of the peroxidation products measured as thiobarbituric acid-reactive substances (TBA-RS) and fluorescent pigments would not be expected to accumulate until late in the response process, probably during the propagation stage (Winston, 1990) and then only in the affected membrane. Thus, it should not be surprising that, in some tissues, evidence of these types of peroxidation could coincide with evidence of injury. Earlier measures of peroxidation, such as the presence of conjugated dienes, changes in electron spin resonance (ESR), and depletion of α -tocopherol or β -carotene in the affected membrane, should precede evidence of symptoms.

Mitigating treatments, such as cold acclimation (Doulis et al., 1993; Shewfelt, 1992), temperature conditioning (Hatton, 1990), and cross protection from one stress to another (Lurie et al., 1991; Shaaltiel et al., 1988), are attributed in this model to induction of defense mechanisms in the susceptible membrane at sublethal levels of stress exposure. Likewise, localized responses observed in hypersensitive reaction (Keppler and Novacky, 1987) and pitting, a symptom of CI in many fruits (Hatton, 1990; Jackman et al., 1988), may represent signaling from cells that are directly affected, permitting adjacent cells to synthesize new antioxidants to prevent injury. Thus, those cells exposed to direct microbial attack or earlier exposure to low temperatures within microclimates would induce defense mechanisms in adjacent cells, enabling them to survive.

One of the striking features of CI in harvested products is that symptoms not evident during chilling exposure develop rapidly upon rewarming (Jackman et al., 1988). If chilling is prolonged, however, symptoms usually will appear. A potential explanation for this phenomenon is that lipid hydroperoxides formed are stable enough at low temperatures that defense and repair processes present are sufficient to prevent propagation. As the temperature is raised, however, the breakdown of preformed lipid hydroperoxides in the affected membrane could occur more rapidly than the recovery of defense and repair mechanisms.

IMPLICATIONS OF THE MODEL

If the proposed model provides an accurate view of changes in the cell in response to the conditions described, we must change our view of stress physiology in plant tissue. The model as proposed suggests that 1) chemical processes in the membrane play a more direct role in development of disorders than biochemical (enzymic) processes; 2) the common link between many, if not all, membrane-associated disorders is free-radical propagation in the most susceptible membrane; 3) the critical oxidative process and susceptible membrane are nonspecific in plant response to stress but specific and predictable within a particular organ in response to a specific stress; and 4) headgroup mobility is as important as degree of fatty acid unsaturation in the development of these disorders.

One difficulty in conducting lipid peroxidation research is the lack of specificity of key antioxidants. Ascorbate, β -carotene, glutathione,

and α -tocopherol are all multifunctional (Winston, 1990). Thus, the effects on peroxidation of adding any of these compounds cannot be attributed to a single function. This lack of specificity of these molecules implies that the reaction kinetics in nonenzymic processes are at least as important as their role in enzyme-catalyzed reactions (Reed, 1990). Many of the primary defense reactions are enzymic in nature, such as those catalyzed by superoxide dismutase and catalase, as well as some repair reactions catalyzed by ascorbate peroxidase, glutathione reductase, and hydrolytic enzymes. These enzymes represent the ability of a cell to respond to increased levels of peroxidation. The disorder develops, however, as chemical reactions in a cell occur more rapidly than one or more of the critical enzymes is able to respond at the site. Thus, the breaching of the defense and repair mechanisms at the most susceptible membrane could be the result of a deficiency in a limiting enzyme, and that critical enzyme could vary from tissue to tissue. Genetic manipulation to control these disorders will require identifying the particular critical defense enzyme in the tissue studied. Overexpression of a critical enzyme might not alleviate the disorder if more than one enzyme is limiting. Also, much emphasis in membrane-associated disorders has been placed on membrane fluidity, usually with respect to chain ordering as determined by fatty acid unsaturation (Shewfelt, 1992; Webb and Green, 1991). If free-radical propagation is the critical step in the process as proposed in the model, then physical interactions of lipid free radicals with other polyunsaturated fatty acids would be as important as the concentration of the polyunsaturated fatty acids. Lateral and rotational diffusion, as controlled by the mobility of the lipid headgroup, plays a major role in fatty acid interaction within the membrane (Carruthers and Melchior, 1988; Leshem, 1992).

The importance of compartmentation within the cell cannot be overemphasized (Elstner, 1991; Schaich, 1992). The model suggests that a single membrane within the cell might be attacked, leading to dysfunction and even cell death. Thus, localized defense and repair or lack of defense and repair could be critical in the prevention or development of a disorder. Zhu and Scandalios (1992) demonstrated increased resistance to oxidative stress by increasing Mn superoxide dismutase expression, confined to mitochondria, in a deficient yeast strain.

This model provides a framework for systematically testing the role of membrane lipid peroxidation in the etiology of plant tissue disorders. It implies that future work must be directed at the specific membrane that is being attacked rather than at cellular membranes as a whole. Modifying genes either to limit production of active oxygen species or to increase synthesis of an antioxidant enzyme should not be attempted without prior or concurrent identification of the primary site of attack and the factor(s) causing the imbalance leading to net peroxidation. The causal factor(s) should be the target of the gene-modification studies. In addition to measuring products of lipid peroxidation, studies should also follow disappearance of antioxidants, such as α -tocopherol and carotenoids, associated with the membrane of interest as well as cellular reductants, such as ascorbic acid and glutathione, to determine whether depletion precedes evidence of peroxidation. Furthermore, developing better means of assessing the progress of peroxidation *in vivo* is essential. Future studies expected to contribute to our understanding of peroxidative degradation of membranes include those that 1) can successfully partition healthy and unhealthy domains of cells within tissue that has been exposed to a specific stress and 2) provide an evolution of peroxidative degradation by histochemistry.

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