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Differences in the susceptibility of plant membrane lipids to peroxidation

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Peroxidation of three membrane lipid preparations from plants was initiated using Fe-EDTA and ascorbate and quantified as the production of aldehydes and loss of esterified fatty acids. Using liposomes prepared from commercial soybean asolecithin, the degree of peroxidation was shown to be dependent on: the free radical dose, which was varied by the ascorbate concentration; the presence of tocopherol in the liposome; the configuration, of the liposome, multilamellar or unilamellar; and time after initiation. There were dramatic interactions among these factors which led to the conclusion that in comparing the susceptibility of different membrane preparations it is essential to examine the kinetics of the peroxidation reactions. The composition of the liposome was a major determinant of the degree of peroxidation and of the type of degradative reactions initiated by the oxygen free radicals. A fresh polar lipid extract from *Typha* pollen had very similar fatty acid composition to the soybean asolecithin, but was more resistant to peroxidation as shown by less aldehyde production and increased retention of unsaturated fatty acids after treatment. Similarly, microsomal membranes from the crowns of non-acclimated and cold acclimated winter wheat (*Triticum aestivum* L.) seedlings had a much higher linolenic acid content than soybean asolecithin but was much more resistant to peroxidation. In the winter wheat microsomes, the loss of esterified fatty acids was not selective for the unsaturated fatty acids; consequently, even with 40% degradation, the degree of unsaturation in the membrane did not decrease. These different reaction mechanisms which occur in plant membranes may explain why measurements of fatty acid unsaturation fail to detect peroxidative reactions during processes such as senescence, aging and environmental stress.

Introduction

After exposure of plants to freezing temperatures, desiccation, anoxia or several other stresses, a common symptom of injury is a disruption of membrane function and integrity as shown by leakage of cytoplasmic solutes and loss of osmotic responsiveness [1,2]. Several models have been proposed to explain the nature of this injury to cellular membranes [1–5]. We have recently advanced two models, which are not necessarily mutually exclusive [4,5]. The first proposes that at low temperatures or low water contents, the phospholipid bilayer, which is normally in a liquid-crystalline phase, forms domains of gel phase lipid which increase membrane permeability and irreversibly disrupt membrane function [4]. As predicted by this model, increased cytoplasmic sucrose levels and increased fatty acid unsaturation, which both tend to maintain the liquid-crystalline phase, have been correlated with increased tolerance of stress [4,6]. Our second model proposes that several environmental stresses promote the formation of oxygen free radicals which mediate the degradation of phospholipids, leading to the accumulation of free fatty acids and/or peroxidation products, the irreversible formation of gel phase domains and loss of membrane function [5]. Evidence supporting the latter model includes the observations: (1) that the changes in the lipid phase transition temperature and lipid composition seen in microsomal membranes isolated from lethally stressed plant tissue can be simulated in vitro

Abbreviations: DHPC, diheptadecanoylphosphatidylcholine; GLC, gas-liquid chromatography; MDA, malondialdehyde; PC, phosphatidylcholine; PA, phosphatidic acid; PG, phosphatidylglycerol; PI, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; TBA; thiobarbituric acid; TLC, thin-layer chromatography.

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by exposure of isolated membranes to oxygen free radicals from xanthine oxidase [7,8]; (2) that microsomal membranes from tolerant plant tissue are more tolerant of free radical treatment than those from susceptible tissues [7,8]; and (3) that stress increases the titer of free radicals produced by microsomal membranes [8]. The most commonly accepted mechanism of free radical attack on phospholipids involves peroxidation of unsaturated fatty acids [9], thereby predicting that fatty acid saturation should increase after exposure to stress. This is not observed even when 50% of the membrane lipid is degraded [10–13]. This model also predicts that increased tolerance of free radical attack, and therefore, stress tolerance, would be correlated with decreased unsaturation of the fatty acids (reduced levels of substrate) whereas just the opposite is observed [11].

There are, however, alternative mechanisms of free radical attack on membrane lipids. Superoxide has been reported to deesterify phospholipids by nucleophilic addition [14] and it has been proposed that hydroxyl radicals may abstract H from the tertiary carbon of the glycerol backbone of the phospholipid almost as easily as from the divinylmethane group of the unsaturated fatty acid [15].

When microsomal membranes from the crown of winter wheat seedlings or from soybean seeds are exposed to free radicals generated by xanthine oxidase, changes in fatty acid unsaturation do not occur [7,8]. It may be that plant membranes react with oxygen free radicals in distinctly different ways than purified lipids, liposomes, or other artificial membrane systems because of the complex mixture of antioxidants found in plants [16]. Alternatively, different types of oxygen free radicals (i.e., superoxide vs. hydroxyl) may initiate different degradative reactions in plant membranes (i.e., deesterification vs. peroxidation). The objectives of this study were to develop a method of treating plant membrane lipids with oxygen free radicals which would initiate peroxidative reactions, to characterize some of the factors which might alter the degree of peroxidation, and finally to determine if different membrane preparations differed in their susceptibility to peroxidation. This would allow the nature of the free radical reactions in plant membranes to be characterized in more detail, and provide a better understanding of free radical and environmental stress tolerance in plants. Xanthine oxidase, the system used previously [7,8] was inadequate; it was not possible to vary the free radical dose because of inhibition of superoxide formation at high concentrations of xanthine [17]. Furthermore, since it seemed essential to have a non-protein free radical generating system if changes in membranes proteins were to be studied, the Fe-EDTA and ascorbate system [18,19] seemed preferable. In this study, we have used this free radical generating system to study the peroxidation reactions in three types of plant membrane systems: liposomes of commercially available soybean asolecithin, liposomes of polar lipid extract from desiccation tolerant *Typha* pollen, and microsomal membranes from wheat seedlings before and after cold acclimation.

Materials and Methods

Preparation of liposomes and membrane isolation

Soybean asolecithin (Fluka Chemical) was used as a source of mixed phospholipids for the preparation of liposomes. A stock solution of 100 mg/ml was prepared in chloroform and stored at 4°C until needed. The stock was purified immediately before use on a Sep-Pak silica cartridge (Waters Associates). Usually 30–60 mg of lipid from the stock asolecithin solution was applied to the column in chloroform and washed with a minimum of 10 ml chloroform, and 20 ml acetone and the polar lipid fraction was eluted with 20 ml methanol. In some treatments, tocopherol (Vitamin E, Fluka Chemical) dissolved in chloroform, was added to the methanol fraction. The eluted methanol fraction was dried under vacuum, and the dried phospholipids were resuspended by vortexing in 10 mM KH_2PO_4 (pH 6.8), at a final concentration of 2 mg lipid (as applied to the column) per ml. Vesicles from this preparation were considered to be multilamellar. In some treatments these multilamellar liposomes were sonicated in a circular bath sonicator (Laboratory Supplies Company, Hicksville, NY) until clarity to form small unilamellar vesicles.

A total lipid extract from *Typha* pollen was prepared as described previously [20]. The total lipid extract was separated into neutral and polar fractions on a Sep-Pak cartridge as described above. The polar (methanol) fraction was used to form multilamellar vesicles.

Microsomal membranes were isolated from the crowns of winter wheat (*Triticum aestivum* L.) cv. Fredrick using the method of Albro et al. [21], except that the final membrane pellet was resuspended in 50 mM $NaHCO_3$ (pH 7.0) instead of mannitol-Mops buffer. Membranes were isolated from 7-day-old seedlings (non-acclimated) or from seedlings acclimated by a subsequent 35 day growth period at 2°C as described previously [8,10].

Free radical treatment

The liposomes or isolated membranes were treated with free radicals generated using Fe-EDTA and ascorbate [18,19]. Solutions of 10 mM Fe-EDTA (ferric monosodium ethylenediaminetetraacetic acid, BDH Chemicals Ltd.) in water, and 20 mM ascorbic acid (Merck) in 10 mM KH_2PO_4 (pH 6.8) were made immediately before use. The reaction was started by the addition of 0.5 ml of Fe-EDTA to a mixture of 2.0 ml liposomes, 0.5 ml ascorbate, and 2.0 ml KH_2PO_4 buffer.

The reaction mixture was incubated at room temperature with shaking and sampled at designated times.

The rate of oxygen consumption was followed by transferring 2-ml samples of the reaction mixture to a Clark type oxygen electrode at designated times after the addition of Fe-EDTA.

Analysis of aldehyde and fatty acid saturation

The production of aldehydes in the reaction mixture was followed using TBA [22]. Acetone/chloroform (1 : 1, v/v) was added to clear the aqueous phase after heating. The absorbance was read at 532 nm, and a molar extinction coefficient of 156000 was used to calculate malondialdehyde equivalents formed. In all experiments, the values presented are the means of at least two separate determinations.

To determine the recovery and fatty acid saturation of the phospholipids in the reaction mixture, a 2 ml sample of the reaction mixture was added to 4 ml of 1 : 1 (v/v) mixture of chloroform/methanol containing 500 μg DHPC as an internal standard. The lower chloroform layer was dried on a column of anhydrous Na_2SO_4 and evaporated under nitrogen. The dried lipids were resuspended in 3 ml methanolic KOH and heated at 70°C for 15 min, which methylates the esterified fatty acids only [20]. To this mixture was added 1.5 ml of a saturated NaCl solution and 1.5 ml hexane, and the mixture centrifuged to separate two phases. The hexane layer was dried on anhydrous Na_2SO_4, reduced in volume and analyzed by GLC using a 30 m capillary column of DB225 (J&W Scientific).

Phospholipid classes were separated by thin-layer chromatography and identified as previously described [6,20]. The individual phospholipid classes were eluted from the silica gel and fatty acids were methylated with methanolic KOH as described above. The recovery of total esterified fatty acids in each phospholipid class was used to calculate the relative recovery of the phospholipid after free radical treatment.

Results

Oxygen uptake

The rate of oxygen uptake in the reaction mixture of multilamellar asolecithin vesicles, Fe-EDTA, and ascorbate was dependent upon the concentration of ascorbate added. With no ascorbate added to recycle Fe from the ferric to the ferrous form, there was no measurable oxygen consumption, and presumably negligible formation of activated oxygen free radicals (Fig. 1). As the concentration of ascorbate was increased, the initial rate of oxygen consumption was higher. The rate of oxygen uptake declined with time as the ascorbate was oxidized. At low levels of ascorbate, the reaction was completed sooner than at higher levels, so that at higher levels of ascorbate the production of free radicals proceeded not only at a higher rate, but also for a longer period of time.

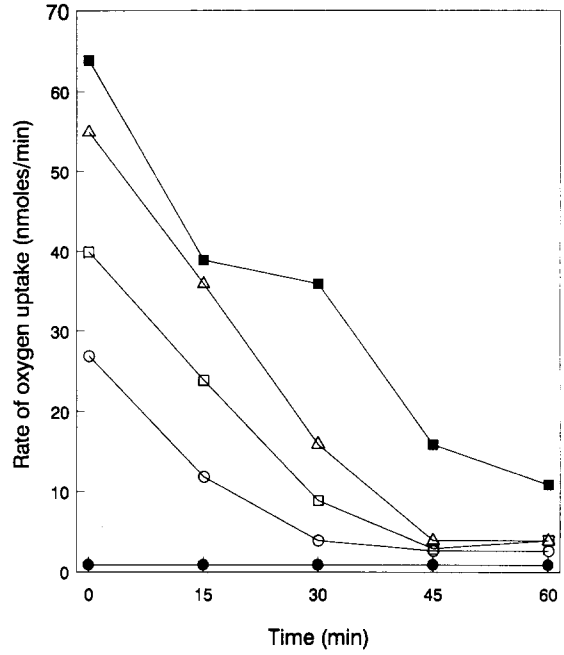

Fig. 1. Oxygen uptake as measured with a Clark-type oxygen electrode in a 2-ml sample of the reaction mixture containing multilamellar liposomes of 1 mg/ml phospholipid from soybean asolecithin, 1 mM Fe-EDTA, 10 mM phosphate buffer (pH 6.8) and ascorbate added to 0 (●), 0.42 (○), 0.83 (□), 1.67 (△), and 3.33 (■) mM. Oxygen consumption followed the relationship $y = 38 - 1.5t + 0.12t^2 + 7.8c$ ($r^2 = 0.92$), where t and c are time and ascorbate concentration, respectively.

Peroxidation of soybean asolecithin

The reaction of the oxygen free radicals with liposomes composed of soybean asolecithin was expected to produce a range of TBA-reactive products such as MDA, other aldehydes, and lipid hydroperoxides [23]. Consequently, this assay was used only to estimate the relative degree of peroxidation occurring under different treatment conditions, and not as an assay specifically for MDA. The results are expressed as MDA equivalents for comparative purposes.

The degree of peroxidation, measured either as production of MDA equivalents or as loss of esterified fatty acids, was dependent on several factors: the amount of ascorbate in the reaction mixture; the presence of tocopherol in the liposome; the configuration of the liposome, whether multi- or unilamellar; and the time at which the measurements were made. Furthermore, there were dramatic interactions among these factors.

As the amount of ascorbate was increased, the quantity of peroxidation products formed after 120 min incubation also increased (Fig. 2). Liposomes without tocopherol were severely damaged by the addition of 2 mM ascorbate, and increasing the ascorbate concentra-

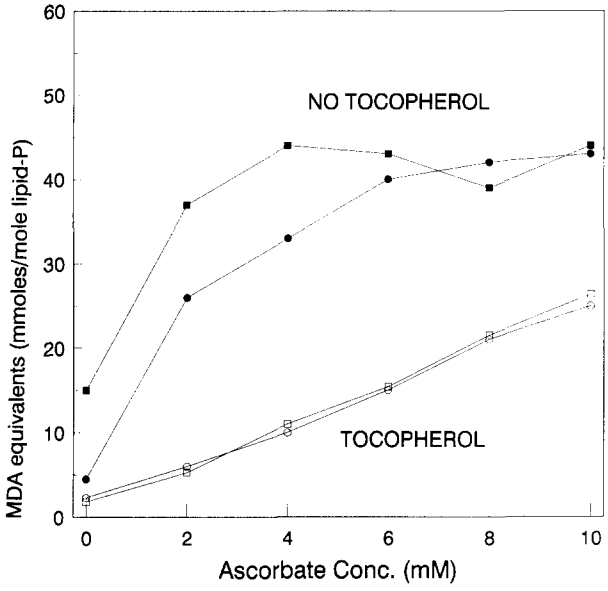

Fig. 2. Effect of ascorbate concentration on the formation of TBA-reactive products by oxygen free radicals generated from Fe-EDTA and ascorbate in multilamellar (○,●) and unilamellar (□,■) asolecithin liposomes. Liposomes were formed from the polar lipids of soybean asolecithin (10 mg), with or without the addition of 0.5 mol% tocopherol, and were incubated in the reaction mixture for 120 min.

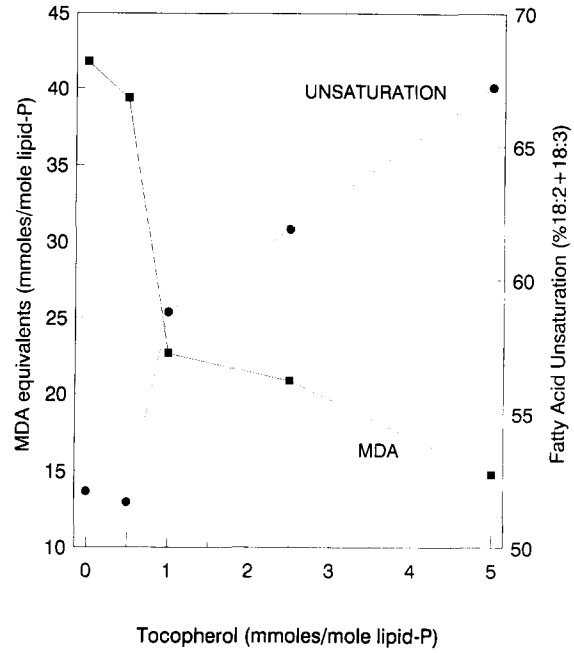

Fig. 3. Effect of tocopherol concentration in multilamellar soybean asolecithin liposomes on the formation of TBA-reactive products by oxygen free radicals generated from Fe-EDTA and ascorbate. Liposomes were formed from polar lipids in soybean asolecithin, and incubated in the reaction mixture for 60 min. Values are the mean of duplicate experiments.

tion 5-fold only marginally increased the degree of peroxidation at 120 min. Unilamellar liposomes without tocopherol were more sensitive to peroxidation at low concentrations of ascorbate. Both multilamellar and unilamellar liposomes containing 0.5 mol% tocopherol, were substantially protected at low levels of free radical exposure, but the degree of damage increased linearly with the concentration of ascorbate.

A threshold of 0.1 mol% (1 mmole tocopherol/mole lipid-P) tocopherol was required to give any degree of protection, and increasing the tocopherol above this threshold reduced peroxidation linearly (Fig. 3). The substrates for these peroxidative reactions producing the MDA equivalents are primarily the unsaturated fatty acids, linoleic (18 : 2) and linolenic (18 : 3), which are selectively degraded in the absence of tocopherol (Table I). The recovery of these two fatty acids was inversely proportional to the amount of MDA equivalents formed (Fig. 3). As the amount of tocopherol in the liposome increased, the recovery of 18 : 2 and 18 : 3 increased, again indicating that tocopherol had inhibited the peroxidative reactions.

The kinetics of the reaction indicated that TBA-reactive products were formed in the liposomes without tocopherol throughout the entire 120 min incubation, although only minimal differences were observed between 2 and 10 mM ascorbate (Fig. 4). In the tocopherol containing liposomes, the reaction stopped at specific times depending on the amount of ascorbate added. Therefore, it seems that tocopherol was terminating the lipid peroxidation chain reaction, and that once the production of oxygen free radicals had ceased (Fig. 1), there was no further lipid peroxidation.

As previously observed, the sonicated unilamellar

TABLE I

Recovery of esterified fatty acids (nmoles per sample) from soybean asolecithin liposomes after treatment with oxygen free radicals

Liposomes were formed with or without the addition of 0.5 mol% tocopherol to the asolecithin, and resuspended by vortexing in phosphate buffer (pH 6.8). A sample was taken as control and the remainder was treated with Fe-EDTA and ascorbate for 120 min. The recovery of esterified fatty acids was quantitatively determined in each sample using diheptadecanoylphosphatidylcholine as the external standard. The experiment was repeated three times with similar trends. Values are from a single representative experiment. Values in parentheses represent % recovery relative to control.

Fatty acid	nmoles fatty acid recovered			
	no tocopherol		0.5% tocopherol	
	control	treated	control	treated
16:0	257	209 (81)	306	283 (93)
18:0	50	41 (82)	59	51 (87)
18:1	125	88 (70)	141	120 (85)
18:2	933	333 (36)	1056	882 (84)
18:3	103	23 (22)	113	92 (81)
Total	1468	694 (47)	1676	1428 (85)
Unsaturation [a]	70.6	51.2	69.8	68.3

[a] Unsaturation: proportion (%) of (18 : 2 + 18 : 3) relative to all esterified fatty acids.

liposomes were much more extensively and rapidly damaged than multilamellar ones, if they did not contain tocopherol (Fig. 5). The accumulation of peroxidation products from unilamellar liposomes exposed to 2 mM ascorbate reached a maximum at 30 min incubation; afterwards the MDA equivalents declined towards the levels seen in the multilamellar liposomes. As a result, at 120 min, the differences between multilamellar and unilamellar liposomes were minimal. Presumably, the Fe-EDTA and ascorbate, which were added to the vesicles after formation, produced oxygen radicals which had access only to the outer layer of the multilamellar liposome and peroxidation reactions slowly worked along successive layers of membrane. In the sonicated, unilamellar liposomes, the free radicals generated in the outer aqueous phase had access to the total surface of the membrane from the beginning of the reaction. Because of the increased surface area exposed to the aqueous phase, this effectively increased the availability of lipid substrate for the peroxidation reactions.

In the soybean asolecithin liposomes, the oxygen free radicals initiated other types of reactions than those

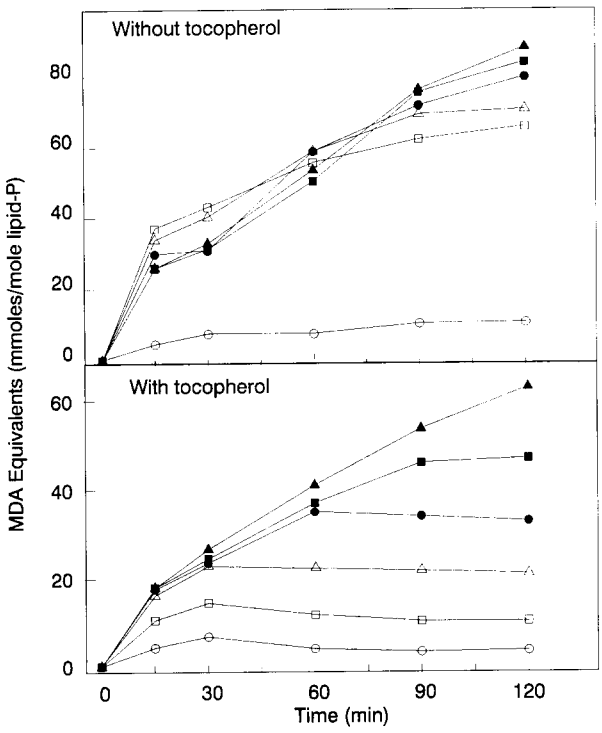

Fig. 4. Time course of the formation of TBA-reactive products by oxygen free radicals generated from Fe-EDTA in multilamellar soybean asolecithin liposomes. Ascorbate was included in the reaction at concentrations of 0 (○), 2 (□), 4 (△), 6 (●), 8 (■), and 10 (▲) mM. Liposomes were formed from polar lipids in soybean asolecithin, without or with the addition of 0.5 mol% tocopherol. Without tocopherol, MDA production in the presence of all ascorbate concentrations (excluding 0 mM) followed the relationship $y = 7.2t$ ($r^2 = 0.96$), where t is time (min); the concentration effect was not significant. With tocopherol, MDA increased as $y = 1.77t^{1/2} + 2.14c$, where c is ascorbate concentration, including 0 mM ($r^2 = 0.66$).

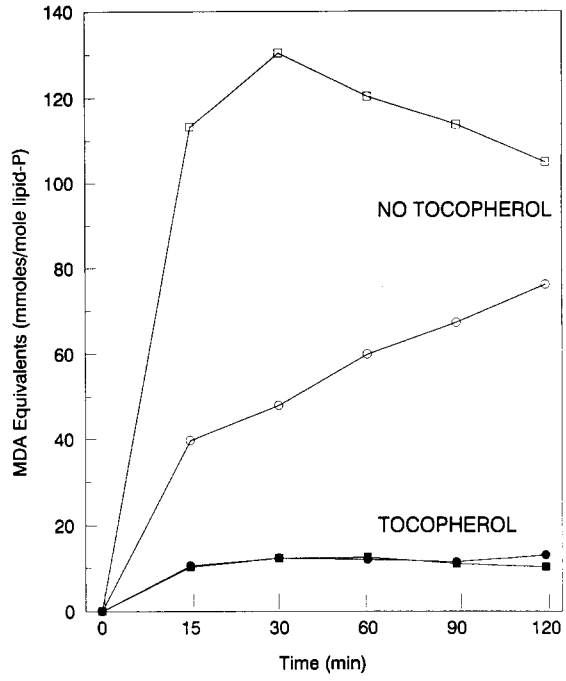

Fig. 5. Effect of sonication on the time course of the formation of TBA-reactive products by oxygen free radicals generated from 1 mM Fe-EDTA and 2 mM ascorbate. Liposomes were formed from polar lipids in soybean asolecithin, with or without the addition of 0.5 mol% tocopherol, and were subsequently used as multilamellar vesicles (○,●) or sonicated to form unilamellar vesicles (□,■). Values are the means of duplicate determinations.

leading to MDA production. For example, in liposomes which contained tocopherol, there was consistently a 10 to 15% reduction in the recovery of all esterified fatty acids (Table I), only slight MDA production (Fig. 5), and a slight change in unsaturation (Table I).

Peroxidation of plant membranes

Peroxidation of the polar lipids from soybean asolecithin was compared to peroxidation of freshly extracted polar lipids from *Typha* pollen. The fatty acid composition of the polar lipids from the two preparations was very similar; both preparations contained approx. 61% linoleic acid and 6–7% linolenic acid. The *Typha* pollen contained negligible amounts of stearic and oleic acids, and about 30% palmitic, whereas the soybean extract contained 20% palmitic and 5% of the others. In spite of this similarity, the rate and extent of fatty acid degradation were quite different in the two samples when exposed to 2 mM ascorbate and Fe-EDTA (Fig. 6). The *Typha* pollen was slower to form TBA-reactive products, and even after 240 min the proportion of unsaturated fatty acids was only reduced from 70 to 59%, whereas in the asolecithin the proportion was reduced from 70 to 35%.

TLC separation of the phospholipids indicated that the primary phospholipids in both samples were PC and PE (Table II). The asolecithin contained twice as much

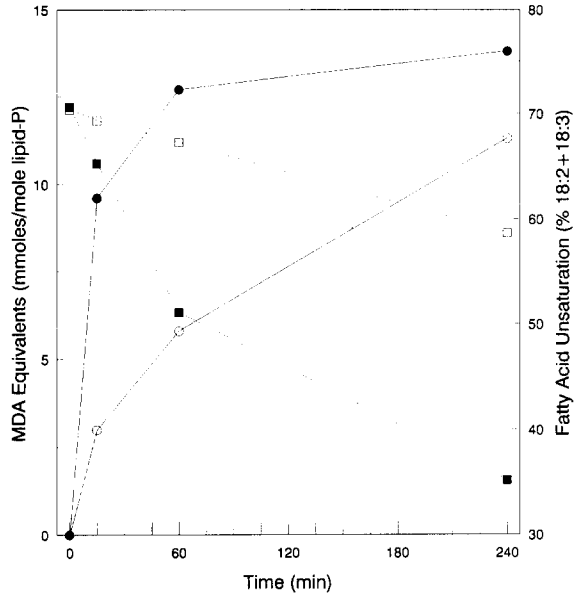

Fig. 6. Formation of TBA-reactive products (○,●) and degradation of polyunsaturated fatty acids (□,■) in multilamellar vesicles of soybean asolecithin (closed symbols) and *Typha* pollen polar lipids (open symbols) during exposure to oxygen free radicals generated by 1 mM Fe-EDTA and 2 mM ascorbate. Values are the means of duplicate determinations.

PE as the *Typha* preparation and correspondingly less PC. Both fractions had relatively minor quantities of other phospholipids. The degradation of the phospholipids in both preparations was apparently random with no obvious selectivity since the relative proportion of PC/PE/PI was not changed. There was a substantial accumulation of phospholipid at the origin of the treated samples which presumably represents phospholipids which were crosslinked by the peroxidative reactions. This fraction had a higher proportion of palmitic acid, and relatively lower proportion of linoleic acid than the total esterified fraction (data not shown), which further supports this assumption. Free radical treatment decreased the proportion of the unsaturated fatty acids in all phospholipid classes (data not shown). There was no detectable lyso PC, and no major increase in PA observed in either preparation.

Wheat crowns which are acclimated at low temperatures are more tolerant of freezing and other environmental stresses associated with winter [8,10,12]. The microsomal membranes from these crowns had a higher proportion of linolenic acid than those from nonacclimated crowns (Table III), which is typical of cold acclimated plants [1,8,11]. The microsomal membranes from both nonacclimated and acclimated crowns had much higher proportion of 18:3 than the soybean asolecithin or the *Typha* pollen (Table III) and as such may have been assumed to be more susceptible to peroxidative reactions. The opposite response was observed. After 120 min exposure to 2 mM ascorbate and Fe-EDTA, there was no major change in the distribution of fatty acids recovered from these membranes, whereas a substantial change was observed in the soybean asolecithin liposomes included in the same experiment for comparative purposes.

TABLE II

Phospholipid composition of soybean asolecithin and Typha pollen polar lipid fractions after treatment with 1 mM Fe-EDTA and 2 mM ascorbate for 0, 60 and 240 min

Phospholipids were separated by TLC and quantified by GLC analysis of esterified fatty acids. PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidyl ethanolamine; PG, phosphatidylglycerol; PA, phosphatidic acid.

Lipid fraction	% of total PL					
	asolecithin			*Typha*		
	0 min	60 min	240 min	0 min	60 min	240 min
Origin	2	8	25	1	3	23
PC	47	45	38	64	63	53
PI/PS	13	13	9	9	9	6
PE	35	29	22	18	17	10
PG	3	4	4	7	7	7
PA	1	1	3	2	1	1

TABLE III

Distribution of esterified fatty acids (% of total) of soybean asolecithin liposomes and wheat microsomal membranes from nonacclimated and acclimated crowns before and after exposure to Fe-EDTA and 2 mM ascorbate for 120 min

Values are the mean of duplicate determinations.

Fatty acid	Asolecithin		Nonacclimated		Acclimated	
	0	120 min	0	120 min	0	120 min
16:0	21	34	33	34	29	30
18:0	3	5	2	2	1	1
18:1	7	10	11	11	6	7
18:2	61	47	36	36	28	29
18:3	8	4	18	18	37	35

TABLE IV

Recovery of esterified fatty acids from the microsomal membranes of nonacclimated and cold acclimated winter wheat crowns after exposure to oxygen free radicals from Fe-EDTA and 2 mM ascorbate for 120 min

Values represent the moles of each fatty acid recovered after treatment relative to an untreated control sample (100%). Values are from the same experiments as Table III.

Fatty acid	% of time = 0		
	asolecithin	non-acclimated	acclimated
16:0	68	63	67
18:0	64	52	67
18:1	64	62	67
18:2	32	60	65
18:3	23	63	60
Total	42	62	64

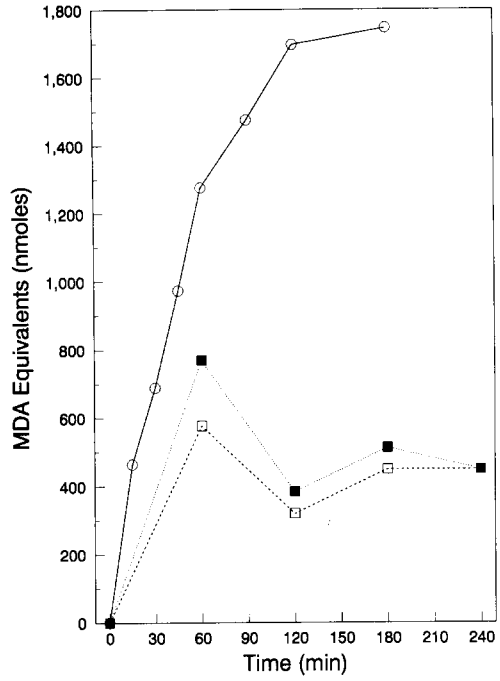

Fig. 7. Formation of TBA-reactive products in soybean asolecithin liposomes (○) and in the microsomal membranes from non-acclimated (□) and cold acclimated (■) winter wheat crowns during exposure to oxygen free radicals generated by 1 mM Fe-EDTA and 2 mM ascorbate. The asolecithin sample contained 0.5 mg lipid, whereas the wheat microsomal membranes contained a fraction equivalent to that extracted from 0.8 g crown tissue. Values are the means of two or three determinations. Peroxidations of the asolecithin (nmoles of MDA) followed the relationship $y = 144t^{1/2}$ ($r^2 = 0.96$) but peroxidation of the wheat microsomes followed the relationship $y = 111t^{1/2} - 5.6t$ ($r^2 = 0.75$).

In spite of the lack of change in fatty acid unsaturation, there was a substantial loss of esterified fatty acids in all three samples (Table IV). The difference being that the polyunsaturated fatty acids were selectively degraded in the soybean asolecithin liposomes, whereas the degradation was evenly distributed across all fatty acids in both types of wheat microsomes. Thus, there seems to be very different types of free radical reactions in the two preparations, presumably determined by the nature of the lipid substrate.

The kinetics of the formation of TBA-reactive products was similar to that previously observed for soybean asolecithin liposomes which contained tocopherol (compare Fig. 4 with Fig. 7). A similar degree of degradation was noted in the first 60 min between the wheat microsomes and the asolecithin liposomes, but there was no subsequent increase in aldehyde formation in the wheat microsomes suggesting that the chain reactions had been quenched.

Discussion

Exposure of liposomes prepared from soybean asolecithin to oxygen free radicals resulted in peroxidative reactions leading to the production of aldehydes, and to the selective degradation of the unsaturated fatty acids. The amount of peroxidation detected was dependent on complex interactions among the concentration of ascorbate, the presence of antioxidants such as tocopherol, the configuration of the liposome, and the length of reaction time.

As the ascorbate concentration was increased in the reaction mixture, the rate and duration of oxygen consumption also increased suggesting increased superoxide and hydroxyl radical production. For the liposomes without tocopherol, this increased free radical dose had negligible effects on peroxidation. In other words, it did not matter how many free radicals started the chain reactions; once started, the rate of peroxidation was dependent on the propagation of these reactions, not on the production of more oxygen radicals. With tocopherol present in the liposome, peroxidation was dependent linearly on the concentration of ascorbate or more precisely the dose of oxygen free radicals to which it was exposed. In this system, tocopherol seemed to terminate the chain reaction, and thus more primary free radicals were required to continually initiate the peroxidation reactions.

Unilamellar liposomes were much more rapidly and extensively degraded than multilamellar ones. In the unilamellar preparation, aldehyde production was maximal at 30 min and the subsequent decline reflects the reaction of these aldehydes to produce non-TBA-reactive products. In contrast, reactions subsequent to 30 min in the multilamellar liposomes lead to increased aldehyde formation. Lipid hydroperoxides decompose in the presence of Fe^{2+} to form alkoxy and peroxy radicals [9,23] and this type of reaction would be expected to contribute to these continued peroxidative reactions. With an antioxidant in the liposomes, liposome configuration and time had negligible effects, indicating that extreme caution must be used in comparing peroxidation in different membrane preparations, and that kinetic studies of MDA formation are essential and more informative than quantitative measurements.

The Fe-EDTA/ascorbate system was used to study the peroxidative reactions in phospholipids from two well characterized plant systems — *Typha* pollen and winter wheat crowns. Both systems behaved in a markedly different manner than the asolecithin liposomes. The polar lipids from *Typha* pollen reacted more slowly than asolecithin even though fatty acid unsaturation was nearly identical. Differences in phospholipid composition have been shown to influence the susceptibility of different liposomes to peroxidation with phospholipid charge, lateral distribution and density being important considerations [19,24,25]. The presence of PE in membranes has also been shown to suppress formation of TBA-reactive compounds [26]. Since the *Typha* pollen had lower PE content than the soybean

asolecithin (Table II), these observations fail to explain the difference in susceptibility between the two samples.

The wheat microsomal membranes are another interesting system because in spite of the fact that they contained more linolenic acid than the asolecithin, they were much less susceptible to peroxidation. The kinetics of aldehyde formation (Fig. 7) indicate the presence of a chain reaction terminating antioxidant, such as tocopherol, but the nature of this antioxidant(s) has not been established. The Fe-EDTA/ascorbate system for inducing peroxidation in soybean asolecithin would seem to be an excellent system to characterize these antioxidants found in plant membranes. After treating the wheat membranes with oxygen free radicals, there was a loss of esterified fatty acids which was slightly less than in soybean asolecithin, 40 vs. 60%, respectively. However, the loss was random or non-selective, whereas in the soybean asolecithin, the loss was preferential for the unsaturated fatty acids. This qualitative difference in the degradative reactions initiated by oxygen free radicals cannot be explained by any of the parameters examined in this study but presumably reflects differences in composition of the two preparations.

Many degenerative processes in plants are thought by some to be mediated by oxygen free radicals, including leaf senescence, seed aging, and response to environmental stress [5,8,15,27] but the hypothesis has remained controversial primarily because changes in fatty acid unsaturation are observed only infrequently (see, for example, Ref. 28). These observations indicate that oxygen free radicals can promote degradative reactions causing loss of membrane phospholipid in the absence of changes in fatty acid unsaturation.

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