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Simulation of Dehydration Injury to Membranes from Soybean Axes by Free Radicals[1]

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ABSTRACT

Smooth microsomal membranes were isolated from axes of soybean (*Glycine max* L. Merr.) seeds at the dehydration-tolerant (6 hours of imbibition) and dehydration-susceptible (36 hours of imbibition) stages of development and were exposed to free radicals *in vitro* using xanthine-xanthine oxidase as a free radical source. Wide angle x-ray diffraction studies indicated that the lipid phase transition temperature of the microsomal membranes from the dehydration-tolerant axes increased from 7 to 14°C after exposure to free radicals, whereas those from the dehydration-susceptible axes increased from 9 to 40°C by the same free radical dose. The increased phase transition temperature was associated with a decrease in the phospholipid:sterol ratio, and an increase in the free fatty acid:phospholipid ratio. There was no significant change in total fatty acid saturation, which indicated that free radical treatment induced deesterification of membrane phospholipid, and not a change in fatty acid saturation. Similar compositional and structural changes have been previously observed in dehydration-injured soybean axes suggesting that dehydration may induce free radical injury to cellular membranes. Further, these membranes differ in their susceptibility to free radical injury, presumably reflecting compositional differences in the membrane since these membranes were exposed to free radicals in the absence of cytosol.

The precise mechanism by which dehydration injures plant tissues has not been clearly defined, although considerable evidence suggests that cellular membranes may be at least one site of injury (8, 11, 17, 18). Our previous studies (17, 18) using germinating soybean seeds to elucidate possible mechanisms of dehydration injury, have shown that soybean seeds are tolerant of dehydration during the early stages of germination, up to 6 h after commencing inbibition, and become susceptible to injury during the progress of germination. Thus, dehydration at 36 h of imbibition leads to a complete loss of seed viability (17). Typical symptoms of dehydration injury, such as increased rates of solute leakage, occur only in the axes not in the cotyledons (17, 18). The transition from dehydration tolerance to susceptibility does not seem to involve cell enlargement or protein synthesis (17).

Further experimentation characterizing properties of membranes isolated from soybean axes in the dehydration-tolerant (6 h of imbibition) and dehydration-susceptible (36 h of imbibition) states indicated that dehydration induced a deesterification of membrane phospholipid with a resultant increase in the free fatty acid content of the membranes, and altered phase properties of the lipid bilayer (19). Phospholipid deesterification has been suggested to be the major consequence of superoxide (O_2) mediated lipid deterioration (15). Although lacking conclusive evidence, some authors have hypothesized a free radical mediated mechanism of injury during dehydration stress (1, 4).

Xanthine oxidase, an autooxidizing enzyme, produces superoxide as a byproduct during the conversion of xanthine to uric acid (7). In this study, the xanthine-xanthine oxidase system was used to produce free radicals *in vitro* in an attempt to simulate the symptoms of dehydration injury on the membranes from soybean axes.

MATERIALS AND METHODS

Soybean seeds (*Glycine max* L. Merr. cv Maple Arrow) were imbibed for 6 or 36 h as previously described (17).

Isolation of Membranes. Microsomal membranes from soybean axes imbibed for 6 or 36 h were isolated as described previously (9, 10, 19). For each preparation, about 10 g of the tissue were homogenized in 30 ml of 0.3 M sucrose-50 mM $NaHCO_3$ (pH 7.0). The homogenate was filtered through four layers of cheesecloth, made 15 mM with CsCl and centrifuged through 1.3 M sucrose-15 mM CsCl for 2 h. Each centrifuge tube contained 7 ml of supernatant overlaying 3 ml of 1.3 M sucrose-15 mM CsCl. Smooth microsomal membranes which were collected at the interface were removed with a pipet, diluted with three volumes of 50 mM $NaHCO_3$ and pelleted by centrifugation at 165,000*g* for 1 h. The pellet was washed with 50 mM $NaHCO_3$.

Free Radical Treatment. The membrane pellet was resuspended in 5 ml of 0.02 mM xanthine-50 mM $NaHCO_3$ in a centrifuge tube. Continuous mixing of the suspension was achieved using a stirring magnet, and 20 μl containing 0.5 units (1 unit converts 1 μmol of xanthine to uric acid/min at 25°C) of xanthine oxidase (Sigma) was added to the membrane suspension. Another 5 ml of substrate was gradually added to the reaction mixture during a 30-min period, then centrifuged for 1 h at 165,000*g* to reisolate the smooth microsomal membranes. An identical membrane sample was resuspended in 50 mM $NaHCO_3$ without xanthine but with the added enzyme as a control.

X-Ray Diffraction. The packed membrane pellet was prepared for x-ray diffraction as previously described (10, 19). Wide angle x-ray diffraction patterns were recorded for 6 to 8 h at various temperatures using a fine focused beam of collimated Cu Kα radiation. The beam was monochromated using an appropriate nickel filter. The lipid phase transition temperature of the isolated membranes was determined to within 1°C, as the highest temperature at which gel phase lipid could still be detected by x-ray diffraction.

Lipid Analysis. Total lipids were extracted from membrane samples according to the procedure of Nichols (14). Phospholipid content of the lipid extract was determined as Pi after $HClO_4$

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digestion (5). Methyl esters were prepared using BF_3 in methanol (13) and methyl esters were extracted with 2 ml pentane:water (2:1 v/v) and quantified by GC (19). Heptadecanoic acid (17:0) was used as internal standard. Free fatty acids and sterols were separated by TLC and quantified as previously described (19).

RESULTS

Wide angle x-ray diffraction patterns of the microsomal membranes isolated from soybean axes imbibed for 6 h displayed only a broad diffuse reflection centered at a Bragg spacing of 4.6 Å when recorded at room temperature, irrespective of the free radical treatment (Fig. 1a, b). This reflection derives from phospholipid packed in a liquid crystalline phase and is common to biological membranes (3, 9, 10). A similar 4.6 Å band was featured from the microsomal membranes isolated from 36-h imbibed axes with no free radical treatment (Fig. 1c). In contrast, membranes isolated from 36-h imbibed axes which were previously exposed to free radicals displayed an additional sharp ring at 4.15 Å Bragg spacing (Fig. 1d), representing gel phase lipid (3, 9, 10).

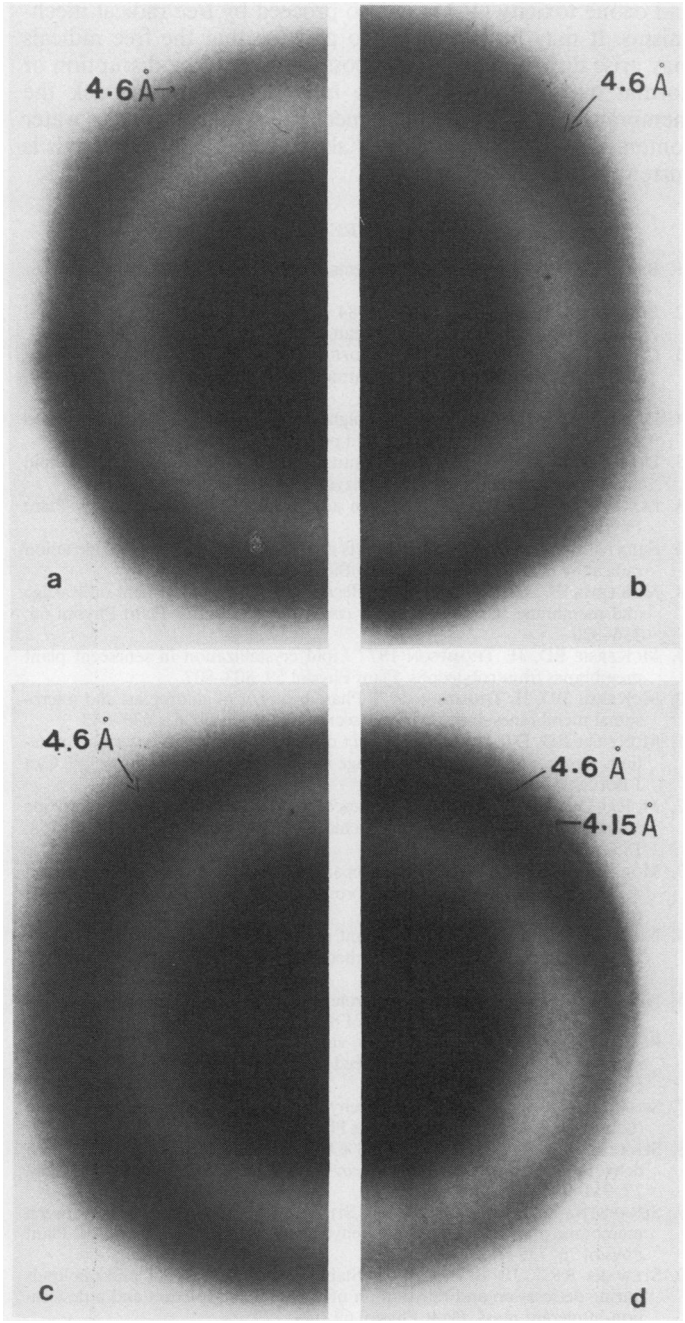

FIG. 1. Wide angle x-ray diffraction patterns of smooth microsomal membranes from soybean axes recorded at 25°C: (a), imbibed for 6 h; (b), imbibed for 6 h—exposed to free radicals; (c), imbibed 36 h; (d), imbibed 36 h—exposed to free radicals.

The lipid phase transition temperatures indicate this trend more quantitatively (Table I). *In vitro* free radical treatment of microsomal membranes isolated from soybean axes imbibed for 6 h increased the lipid phase transition temperature from 7 to 14°C. In contrast, an identical free radical treatment of microsomal membranes isolated from axes imbibed for 36 h increased the lipid phase transition temperature from 9 to 40°C.

In vitro free radical treatment did not induce significant changes in fatty acid composition or in sterol composition in

Table I. *Effect of Free Radicals on Phase Transition Temperature, Phospholipid:Sterol Ratio, and Free Fatty Acid:Phospholipid Ratio of Microsomal Membranes from Soybean Axes*

Imbibition Period	Free Radical Treatment	Phase Transition Temperature	Phospholipid:sterol Ratio	Free Fatty Acid Phospholipid
h		*°C*	*mol·mol⁻¹*	*mol:mol*
6	Control	7	57	0.052
	Treated	14*[a]	51*	0.088NS
36	Control	9	52	0.043
	Treated	40*	30*	0.212*

[a] NS, * indicate that control and treated samples were not significantly different or significantly different at $P \leq 0.05$, respectively, according to LSD test.

Table II. *Effect of Free Radical Treatment on the Fatty Acid Composition of the Total Lipid Extract from Microsomal Membranes from Soybean Axes*

All differences between control and treated samples were not significantly different at $P \leq 0.05$ according to LSD test. Statistical analysis was performed after square root transformation.

Imbibition Period	Free Radical Treatment	Mol % of Total Fatty Acid					Unsaturated: saturated Ratio
		16:0	18:0	18:1	18:2	18:3	
h							
6	Control	18.5	4.4	4.9	51.6	20.6	3.3
	Treated	20.2	4.2	4.8	50.6	20.2	3.1
36	Control	26.0	4.2	3.9	46.0	19.8	2.3
	Treated	26.6	4.5	4.0	45.1	19.1	2.2

Table III. *Effect of Free Radical Treatment on the Sterol Composition of Microsomal Membranes from Soybean Axes*

All differences between control and treated samples were not significantly different at $P \leq 0.05$ according to LSD test. Statistical analysis was performed after square root transformation.

Imbibition Period	Free Radical Treatment	Mol % Sterol			
		Cholesterol	Campesterol	Stigmasterol	β-Sitosterol
h					
6	Control	1.6	11.7	11.3	75.3
	Treated	2.0	11.9	9.8	76.1
36	Control	1.5	12.0	11.1	76.9
	Treated	2.0	10.6	11.6	75.7

either membrane isolation (Tables II and III). For both membranes the unsaturated:saturated fatty acid ratio remained unchanged after free radical treatment (Table II).

The molar phospholipid:sterol (PL:S) ratio of the membranes isolated from axes imbibed for 6 h was decreased only slightly, from 57 to 51, by free radical treatment (Table I). On the other hand, the ratio was decreased from 52 to 30 when membranes from axes imbibed for 36 h were exposed to free radicals (Table I).

The observed decrease in PL:S ratio after exposure to free radicals was associated with an increase in the free fatty acid content of these membranes. When membranes isolated from 6-h imbibed axes were exposed to free radicals, the molar free fatty acid:PL ratio increased slightly from 0.052 to 0.088 (Table I), but was not significantly different at $P \leq 0.05$ according to the LSD test. Similar treatment of membranes from 36-h imbibed axes, however, induced a 5-fold increase in free fatty acid levels and the molar free fatty acid:PL ratio rose from 0.043 to 0.211 (Table I).

DISCUSSION

Free radical treatment of intact microsomal membranes *in vitro* caused significant changes in the lipid phase transition temperature as detected by wide angle x-ray diffraction, the PL:S ratio and the free fatty acid:PL ratio, but did not significantly alter the degree of fatty acid saturation of the total lipid extract from these membranes. Lipid peroxidation, as manifested by changes in fatty acid saturation or malondialdehyde production, has been assumed to be the primary mechanism of free radical injury to plant membranes (3, 4, 16). As a consequence, some studies have been conducted which failed to detect changes in fatty acid saturation after dehydration (20) and, therefore, an involvement of free radicals in mediating dehydration injury was not investigated. The structural and compositional analysis of the microsomal membranes from soybean axes would suggest that free radical damage to cellular membranes in aqueous solutions proceeds by means of the deesterification of membrane phospholipids possibly as a result of the nucleophilic attack by superoxide, as originally proposed by Niehaus (15). The resultant accumulation of high levels of free fatty acids in the membrane would be expected to contribute to membrane dysfunction (19).

Cellular membranes can apparently differ in their susceptibility to free radical damage. The microsomal membranes from soybean axes which were susceptible to dehydration injury (at 36 h of imbibition) were also very susceptible to free radical injury, whereas a similar membrane fraction from dehydration-tolerant axes (at 6 h of imbibition) exposed to similar free radical dose exhibited quantitatively much less molecular damage. The molecular mechanism imparting this relatively greater tolerance is unknown, but it does involve membrane components and not cytosol components such as superoxide dismutase, because in these experiments the membranes were exposed to free radicals after isolation when most of the cytosol had been removed.

There is a marked similarity between the effects of dehydration (19) and *in vitro* free radical treatment on the structural and compositional properties of the microsomal membranes from soybean axes. Damaged membranes (those from 36-h imbibed axes) exhibited increased lipid phase transition temperatures, decreased PL:S ratios, increased free fatty acid:PL ratios, and no significant change in fatty acid saturation, after free radical or dehydration treatment. Microsomal membranes from axes imbibed for 6 h did not exhibit any significant structural or compositional change after dehydration stress (19) and exhibited only relatively minor changes after free radical treatment. This similarity in the injury symptoms and the close temporal relationship between increased susceptibility to dehydration injury and increased susceptibility to free radical injury suggests that dehydration stress may induce a free radical-mediated deesterification of membrane phospholipid and, thus, may promote the accumulation of free fatty acids in the membrane. The source of these free radicals remains obscure, although the occurrence of free radicals in plant systems is well documented (6). Many enzymatic reactions generate free radicals (6). Natural and accelerated aging of soybean seeds can induce the accumulation of high levels of free radicals in the axis (2). The herbicide paraquat is thought to cause cellular death as a result of its catalysis of the production of superoxide and other free radicals (3). Natural senescence (12) and ozone toxicity (16) may also proceed by free radical mechanisms. It may be reasonable to propose that the free radicals may arise during dehydration, possibly due to the disruption or normal metabolism, and these free radicals may attack the membrane phospholipid. The mechanism by which low water contents or high osmotic potentials can generate free radicals is currently under investigation.

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