Plant Physiol. (1984) 76, 759–762
0032-0889/84/76/0759/04/$01.00/0

# Association between Membrane Phase Properties and Dehydration Injury in Soybean Axes[1]

Received for publication March 21, 1984 and in revised form July 12, 1984

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## ABSTRACT

**Axes of soybean seeds are tolerant to dehydration at 6 hours of imbibition, but susceptible to dehydration injury if dried at 36 hours of imbibition. Smooth microsomal membranes were isolated from axes imbibed for 6 hours (dehydration tolerant state) and 36 hours (dehydration susceptible state) before and after dehydration treatment. The phase properties and the lipid composition of the membrane fraction were investigated. Wide angle x-ray diffraction patterns of microsomal membranes from axes imbibed for 6 or 36 hours indicated a liquid-crystalline to gel phase transition at approximately 7°C. Membranes from axes dehydrated at 6 or 36 hours of imbibition and rehydrated for 2 hours exhibited a phase transition at 7°C and 47°C, respectively. Changes in fatty acid saturation did not account for the changes in phase properties. However, the increased phase transition temperature of the membranes from dehydration injured axes was associated with an increase in free fatty acid:phospholipid molar ratio and a decrease in phospholipid:sterol ratio. These results suggests that dehydration prompted a deesterification of the linkage between glycerol and fatty acid side chains of the phospholipid molecules in the membrane. The resultant increase in free fatty acid content in the membrane is thought to alter the fluidity and phase properties of the membrane and contribute to dehydration injury.**

Injury to cellular membrane systems is a common phenomenon of environmental stresses (9, 11, 16, 25). Although increased leakage of cytoplasmic solutes after exposure to stress has been generally accepted as indicative of membrane lesions (11, 16, 22), the exact mechanism of membrane injury has not clearly been defined. Therefore, precise characterization of the nature of membrane injury is required to understand the mechanism of stress tolerance in plant tissues.

Seeds are generally tolerant to dehydration at maturity and during the early stages of germination but lose dehydration tolerance as germination proceeds (7, 22). Therefore, the germinating seed can be used as a system to study dehydration tolerance (7, 22). We have previously shown that soybean seeds can be imbibed for 6 h and dehydrated to 10% moisture without loss of viability and vigor. However, if soybean seeds were imbibed for 36 h, tolerance of this dehydration treatment is lost (22). Although the loss of dehydration tolerance coincides with cell elongation, cell enlargement does not appear to be related to the loss of dehydration tolerance (22). Furthermore, dehydration injury in soybean seeds occurred in the axis, not in the cotyledons, and only when dehydrated below 20% moisture (22).

Ultrastructural studies on dehydration-injured seeds (4, 5) and freeze fracture studies of the phycobiont *Trebouxia* after drying (20), demonstrated alterations in membrane structure induced by dehydration. Recent experimental evidence obtained using low angle x-ray diffraction (13) did not confirm Simons' (26) hypothesis that seed phospholipids change from a lamellar to a hexagonal phase at 20% moisture. However, kinetic analysis of solute efflux from dehydration-injured soybean axes is consistent with the proposal that a biophysical or biochemical reorganization of the membranes has occurred after dehydration stress rendering the lipid bilayer more permeable (23). The present study attempts to more clearly define the membrane injury associated with dehydration, by comparing the structural and compositional properties of the microsomal membranes from soybean axes in the dehydration tolerant (at 6 h of imbibition) and dehydration susceptible (at 36 h of imbibition) states, before and after dehydration stress.

## MATERIALS AND METHODS

Soybean (*Glycine max.* L. Merr. cv Maple Arrow) seeds were imbibed and dehydrated as previously described (22). Dehydrated axes were reimbibed for 2 h to fully rehydrate the tissue prior to membrane isolation.

**X-Ray Diffraction.** Microsomal membranes from dehydrated and nondehydrated soybean axes were prepared for x-ray diffraction as described by McKersie and Thompson (14, 15). For each preparation, about 10 g of 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 and centrifuged at 10,000*g* for 20 min. The resulting supernatant was made 15 mM with CsCl and centrifuged through 3 ml of 1.3 M sucrose-15 mM CsCl at 165,000*g* for 2 h. Smooth microsomal membranes which collected at the interface were removed with a syringe, diluted with 3 volumes of 50 mM $NaHCO_3$, and pelleted by centrifugation at 165,000*g* for 1.5 h. The packed membrane pellet was mounted between the jaws of a brass holder and equilibrated under $N_2$ to remove water around and between membranes (15). The holder bearing the membrane sample was placed in a temperature-controlled chamber and wide angle diffraction patterns were recorded for 6 to 8 h at various temperatures. A beam of collimated Cu K $\alpha$x-rays from a fine focused sealed source operated at 40 kv and 20 mamps was used. An appropriate nickel filter was used to monochromate the beam. Lipid phase transition temperature was determined to within 1°C as the highest temperature at which gel phase lipid could be detected.

**Lipid Analysis.** Total lipids were extracted from the isolated membranes according to the procedure of Nichols (18). Phospholipid content of the lipid extract was determined as Pi after $HClO_4$ digestion (6). Fatty acid methyl esters were prepared from the total lipid extract using $BF_3$ in methanol (17). Methyl esters were extracted with 2 ml pentane:water (2:1 by volume) and

[1] Supported by the Natural Sciences and Engineering Research Council of Canada.

quantified by GC using a stainless steel column (GP 10% SP 2330 on 100/120 chromosorb W) maintained at 180°C. Heptadecanoic acid (17:0) was used as an internal standard.

Free fatty acids and sterols were separated by TLC in petroleum ether:diethyl ether:acetic acid (70:30:2 by volume) and identified using authentic standards. Free fatty acids were extracted from the silica gel using developing solvent. Fatty acids were methylated by heating with $BF_3$ in methanol for 3 min (17), and quantified as described above. Sterols were eluted from TLC plate with petroleum ether and derivatized in equal volume of acetonitrile and BSTFA (Chromatographic Specialties) for 1 h at room temperature (15). The sterol derivatives were then analyzed by GC using a glass column (3% OV-101 on 100/120 chromsorb W HP). Experiments were replicated three times.

## RESULTS

At room temperature, wide angle x-ray diffraction patterns from smooth microsomal membranes of soybean axes which were (a) imbibed for 6 h, (b) imbibed for 6 h and dehydrated, or

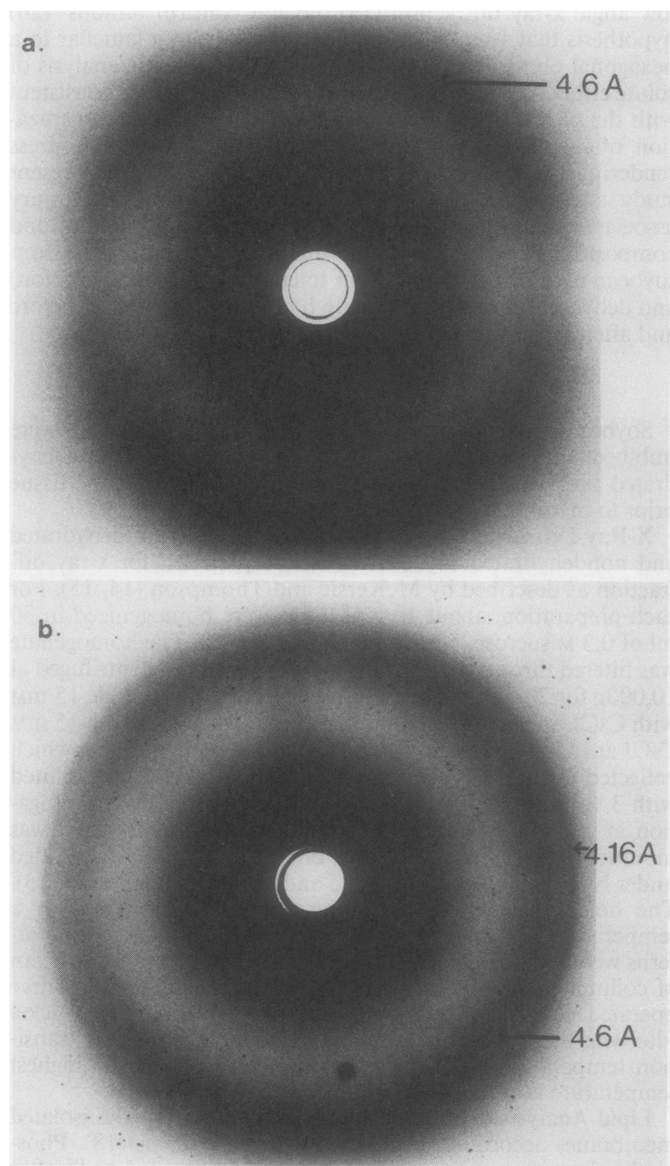


FIG. 1. Wide angle x-ray diffraction patterns of smooth microsomal membranes from soybean axis. (a), Imbibed for 36 h; (b) imbibed for 36 h and then dehydrated and rehydrated for 2 h.

(c) imbibed for 36 h, featured a broad diffuse lipid reflection centered at a Bragg spacing of 4.6 Å (Fig. 1a). This is believed to be derived from phospholipid packed in a liquid crystalline phase and is typical of those routinely obtained from biological membranes (3, 14, 15). By contrast, corresponding diffraction patterns for smooth microsomal membranes isolated from axes dehydrated at 36 h of imbibition contained, in addition to the 4.6 Å liquid-crystalline reflection, a sharp ring at 4.15 Å indicating the presence of phospholipid packed in a gel phase (Fig. 1b).

The phase transition temperature measured by x-ray diffraction represents the highest temperature at which the gel phase could be detected (12, 14). In the membranes isolated from axes imbibed for 6 and 36 h but not dehydrated, the liquid-crystalline to gel phase transition temperatures were 7°C and 9°C, respectively (Table I). There was no change in the phase transition temperature if the axes were dehydrated at 6 h of imbibition. The membranes from axes dehydrated at 36 h of imbibition exhibited a much higher phase transition temperature of 47°C.

The formation of gel phase lipid domains in the microsomal membranes of axes imbibed for 36 h before dehydration was not associated with major changes in fatty acid unsaturation (Table II). The proportion of unsaturated to saturated fatty acids was not changed significantly after the dehydration treatment at either 6 or 36 h of imbibition. There was slight, nonsignificant ($P \leq 0.05$) decrease in linoleic acid (18:2) and a quantitatively similar increase in oleic acid (18:1) and stearic acid (18:0) in the membranes dehydrated at 36 h of imbibition. These small changes in saturation, however, were not sufficient to account for the changes in phase transition temperature. The unsaturated:saturated fatty acid ratio decreased during the progress of germination from 3.6 at 6 h of imbibition to 2.5 at 36 h of imbibition. This was mainly due to the decreased proportion of linoleic acid (18:2) with a corresponding increase in the proportion of palmitic acid (16:0).

Stigmasterol and $\beta$-sitosterol collectively comprise more than 80% of the total sterols. Cholesterol and campesterol were the other detectable sterols in these microsomal membranes (Table III). Dehydration at 6 or 36 h of imbibition did not induce major changes in the proportion of these sterols. There was a small decrease in $\beta$-sitosterol (6%) and a slight increase in stigmasterol (4%) and cholesterol (2%) when axes were dehydrated at 36 h of imbibition.

More striking changes occurred in the phospholipid:sterol ratio of the microsomal membranes. Microsomal membranes from axes imbibed for 36 h but not dehydrated had a molar phospholipid:sterol ratio of 38. Dehydration at 36 h decreased the ratio to 16 (Table I). There was no significant change in the ratio if the axes were dehydrated at 6 h. This reduction in the phospholipid:sterol ratio may indicate either a breakdown or loss of phospholipid, or a synthesis of sterol. The former is more likely since dehydration injury was accompanied by a large increase in the free fatty acid content of the microsomal membrane fraction. The total free fatty acids in the smooth microsomal membrane fraction averaged 42 and 34 mmol $mol^{-1}$ phospholipid at 6 and 36 h of imbibition, respectively (Table I). This ratio did not increase when the axes were dehydrated at 6 h, but when dehydrated at 36 h, the free fatty acid content increased approximately 10-fold to 343 mmol $mol^{-1}$ phospholipid, implying that there was an extensive degradation of phospholipids induced by dehydration treatment of axes at 36 h of imbibition.

The proportion of the individual free fatty acids was similar to that observed for the total fatty acid composition of these membranes (*cf.* Tables II and IV). The proportion of each fatty acid was not altered by the dehydration treatment, but a slight nonsignificant ($P \leq 0.05$) decrease in linoleic acid and an increase in palmitic acid were observed between 6 and 36 h of imbibition.

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

| Imbibition Period | Treatment | Phase Transition Temperature[a] | Phospholipid:Sterol Ratio | Free Fatty Acid:Phospholipid |
|---|---|---|---|---|
| *h* | | *°C* | *mol·mol⁻¹* | *mmol·mol⁻¹* |
| 6 | Nondehydrated | 7 b[b] | 46 a | 42 b |
| | Dehydrated | 7 b | 38 a | 38 b |
| 36 | Nondehydrated | 9 b | 38 a | 34 b |
| | Dehydrated | 47 a | 16 b | 343 a |

[a] Phase transition temperature is the highest temperature at which gel phase lipid can be detected.
[b] Values in a column followed by the same letter are not significantly different at $P \leq 0.05$ according to LSD test.

Table II. *Effect of Dehydration on Fatty Acid Composition of Smooth Microsomal Membrane Lipids from Imbibed Soybean Axes*

| Imbibition Period | Treatment | Mol % of Total Fatty Acid | | | | | Unsaturated:Saturated Ratio |
|---|---|---|---|---|---|---|---|
| | | 16:0 | 18:0 | 18:1 | 18:2 | 18:3 | |
| *h* | | | | | | | |
| 6 | Nondehydrated | 17.9 b[a] | 3.6 b | 5.7 b | 54.0 a | 18.6 a | 3.6 a |
| | Dehydrated | 18.2 b | 3.6 b | 6.6 b | 53.4 a | 18.4 a | 3.6 a |
| 36 | Nondehydrated | 23.5 a | 4.4 b | 5.0 b | 46.6 b | 20.4 a | 2.5 b |
| | Dehydrated | 24.7 a | 6.2 a | 8.4 a | 42.4 b | 18.2 a | 2.2 b |

[a] Values in a column followed by the same letter are not significantly different at $P < 0.05$ according to LSD test. Statistical analysis was performed after square root transformation.

Table III. *Effect of Dehydration on Sterol Composition of Smooth Microsomal Membranes from Imbibed Soybean Axes*

| Imbibition Period | Treatment | Mol % Sterol | | | |
|---|---|---|---|---|---|
| | | Cholesterol | Campesterol | Stigmasterol | β-Sitosterol |
| *h* | | | | | |
| 6 | Nondehydrated | 2.7 ab[a] | 12.3 a | 11.7 b | 73.4 a |
| | Dehydrated | 1.3 b | 12.4 a | 11.0 b | 75.3 a |
| 36 | Nondehydrated | 1.2 b | 11.3 a | 11.8 b | 75.6 a |
| | Dehydrated | 3.9 a | 12.4 a | 15.6 a | 68.2 b |

[a] Values in a column followed by the same letter are not significantly different at $P \leq 0.05$ according to LSD test. Statistical analysis was performed after square root transformation.

Table IV. *Effect of Dehydration on Free Fatty Acid Contents of Smooth Microsomal Membranes from Imbibed Soybean Axes*

| Imbibition Period | Treatment | Mol % of Free Fatty Acid | | | | |
|---|---|---|---|---|---|---|
| | | 16:0 | 18:0 | 18:1 | 18:2 | 18:3 |
| *h* | | | | | | |
| 6 | Nondehydrated | 24.2 a[a] | 3.5 a | 4.2 a | 52.7 a | 15.3 b |
| | Dehydrated | 22.9 a | 4.6 a | 4.5 a | 53.3 a | 15.3 b |
| 36 | Nondehydrated | 26.1 a | 5.0 a | 6.1 a | 44.3 ab | 18.2 a |
| | Dehydrated | 28.3 a | 5.3 a | 5.8 a | 41.9 b | 18.4 a |

[a] Values in a column followed by the same letter are not significantly different at $P \leq 0.05$ according to LSD test. Statistical analysis was performed after square root transformation.

## DISCUSSION

Soybean seeds retain their viability if they are dehydrated to 10% moisture after 6 h of imbibition, but they lose viability and are unable to germinate if they are dehydrated to a similar extent at 36 h of imbibition (22). The loss in viability is accompanied by an increase in solute efflux which has been interpreted as indicating an increase in membrane permeability (23). The wide angle x-ray diffraction patterns obtained in the present study indicate that the smooth microsomal membranes from soybean axes imbibed for 36 h and exposed to a lethal dehydration treatment contain regions of gel phase lipid, in addition to regions of liquid-crystalline lipid, at physiological temperatures. In contrast, a similar membrane fraction isolated from axes imbibed for 6 h, which tolerated the same dehydration treatment and remained viable, contained only liquid-crystalline lipid at physiological temperatures. Similarly, membranes isolated from both types of soybean axes prior to the dehydration treatment contained only a liquid-crystalline phase. Thus, gel phase lipid was detected only in those axes which have been previously shown to have reduced viability and increased solute efflux during rehydration (22, 23).

The physical state of the membrane lipid bilayer influences both the structural and the functional properties of biological membranes. For example, the association between membrane lipid and protein (24), enzyme activity (21), transport (1), and permeability (2) are affected by the viscosity and phase properties of membrane lipids. The formation of gel phase lipid, which is an indication of a lateral phase separation of phospholipids

within the plane of the membrane, would thus be expected to contribute to the loss of viability of the axes after dehydration. Similar phase separations have been observed during leaf and cotyledon senescence (14, 15) after treatment of plants with the herbicide paraquat (3), and after exposure of isolated membranes to ozone (19).

The formation of gel phase lipid in these membranes occurred in the absence of major changes in fatty acid saturation, which is a consistent observation in the plant systems shown to contain gel phase lipid (3, 14, 15). Similarly, dehydration injury in the susceptible moss *Cratoneuron filicinum* has not been correlated with changes in fatty acid saturation (27). Thus, lipid peroxidation as manifested by changes in fatty acid saturation is not a contributing factor to dehydration injury nor to the formation of gel phase lipid domains in these membranes.

The decrease in the phospholipid:sterol ratio in membranes isolated from dehydration-injured axes (imbibed for 36 h) was associated with the increased proportion of gel phase and increased phase transition temperatures. This may reflect either a synthesis of sterol during dehydration or a degradation of phospholipid. Since extensive synthesis is unlikely in the dehydrated state, these data probably reflect the latter possibility, namely that dehydration induced a selective degradation of the phospholipid. The increased proportion of free fatty acid within the membrane fractions which had elevated phase transition temperatures also supports this conclusion. Collectively, the lipid analyses suggest that dehydration of axes at 36 h of imbibition promoted a hydrolysis of the ester linkages between the glycerol and fatty acid tails of phospholipids. The head group and glycerol moieties which are hydrophilic are thus lost from the membrane as indicated by the reduced phospholipid:sterol ratio. It is also interesting to point out that the efflux of inorganic P during the rehydration period is greatly enhanced from axes dehydrated at 36 h of imbibition (23). The fatty acid tails which are hydrophobic apparently remain in the membrane after hydrolysis and accumulate to a proportion of approximately 34 free fatty acids:100 phospholipids or approximately 15% of the total fatty acid chains in the membrane. The presence of free fatty acids in artificial membranes increases the phase transition temperature (8, 10, 28) and it is possible that the accumulation of free fatty acid in these membranes contributes to the lateral phase separation and formation of gel phase domains, and ultimately to the loss of viability.

*Acknowledgments*—The facilities provided by the Ontario Ministry of Agriculture and Food are gratefully acknowledged. The authors wish to thank Margot Loosley-Millman for her assistance in the x-ray diffraction laboratory, and Petar Gostovic for supplying seeds.

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