Lipid-Sugar Interactions[1]

RELEVANCE TO ANHYDROUS BIOLOGY

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ABSTRACT

The ability of seeds and other anhydrous plant forms to survive the withdrawal of water must involve a mechanism for protecting the integrity of cellular membranes. Evidence from animal systems implicates sugars as protective components, and we have tested the changes in mesomorphic phase state of phospholipid model membranes upon hydration and dehydration in the presence of sucrose and/or sucrose plus raffinose. X-ray diffraction studies of dry dimyristoylphosphatidylcholine (DMPC) indicate that the presence of sucrose lowers the chain order/disorder transition temperature to that of hydrated lipid; likewise, the lamellar repeat spacings showed the dry DMPC/sucrose mixture to be similar to that of the hydrated lipid. These results support the proposed potential of sugars to substitute for water in biomembranes. If sucrose is to serve as a protectant during desiccation of seeds, its tendency to crystallize would lessen its effectiveness. Raffinose is known to serve as an inhibitor of sucrose crystallization, and is abundant in seeds. The addition of raffinose to make DMPC/sucrose/raffinose mixtures (1/1/0.3 mass ratio) prevented sucrose crystallization, suggesting this as a possible in vivo role for raffinose.

The membrane seems to be the structural component of organisms which would be most vulnerable to damage upon withdrawal of water (10). The liquid crystal structure of membranes is dependent on the organizing forces of water (17). Recent evidence from desiccation tolerant animal systems such as tardigrades, nematodes, and brine shrimp indicate that trehalose serves to preserve the intactness of membranes upon drying, thus providing desiccation tolerance (6, 8). In angiospermous seeds, however, no trehalose has been found to occur (15); in fact, trehalose is toxic to many higher plants (23). Thus there must be some alternative manner of protection in seeds which survive desiccation without losing viability (16).

Sucrose is almost universally present in seeds (1); it can provide much of the same type of membrane protection as does trehalose in vitro (10, 19). Therefore, the possibility of its participating in the desiccation tolerance of seeds becomes an attractive possibility. The experiments reported here were undertaken to test this concept. We selected DMPC[3] as the phospholipid for model experiments, and we used x-ray diffraction as the method for measuring its interactions with sucrose (5).

A possible complication in the assignation of desiccation protection to sucrose is that, in nearly dry conditions, it has a strong tendency to crystallize (21), thereby limiting its availability. Accordingly, we measured the crystallization tendencies of sucrose in mixtures with DMPC, and the ability of raffinose, a very common component of desiccation-tolerant seeds (1) to suppress crystallization and to protect vesicle integrity upon desiccation.

METHODS AND MATERIALS

DMPC was obtained from Avanti Polar Lipids Inc. (Birmingham, AL), sucrose from Fisher Scientific (ACS Grade, Fairline, NJ), and raffinose pentahydrate from Sigma Chemical Co. For x-ray studies, the DMPC was dissolved in methanol (50 mg/ml) and mixed with methanolic solutions of the sugars (1 g/ml) to obtain the DMPC/sucrose mass ratios of 1/2, 1/1, 1/0.7, and 1/0.2. These represent mole ratios of 1/3.94, 1/1.98, 1/1.39, and 1/0.40, respectively, each sample containing 40 to 50 mg of lipid. Each sample was combined with 1.5 ml of benzene and vortex-stirred. The mixtures were frozen over dry ice/acetone and lyophilized (16 h). Dry samples which were to be hydrated were placed in jars maintained at 75% RH by a saturated NaCl solution. The lipid/sugar mixes (40–100 mg each) were held in the humid atmosphere for 4 d at 30°C. The wet and dry samples were loaded into glass capillaries (1 mm, Charles Supper Co., Natick, MA) and flame-sealed followed by an epoxy sealant. Transfer to the capillaries was carried out in a dry box in which air had been circulating over anhydrous $CaSO_4$ for 24 h. The high transition temperature observed with pure DMPC indicates that anhydrous conditions prevailed.

X-ray diffraction measurements were made with wiggler-enhanced synchrotron radiation on the A1 line at the Cornell High Energy Synchrotron Source (CHESS). The experimental arrangement and precautions implemented to minimize x-radiation damage have been previously described (2, 3, 5).

Diffraction patterns were recorded on x-ray sensitive film (CEA Reflex 25, CEA America Corp.; DEF-5, Kodak) and temperature was controlled to $+/-1.5°C$ using a forced-air crystal heating/cooling apparatus (3). The air stream was coaxial with the sample capillary. Exposure times varied from 10 s to 3 min depending on the beam current, sample-to-film distance, sample composition, and mesomorphic phase type. X-ray wavelength (1.568 Å) was determined using a lead nitrate standard and a carefully measured sample-to-film distance.

Crystallization studies were carried out with about 50 mg DMPC

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[3] Abbreviation: DMPC, dimyristoylphosphatidylcholine.

in 5 ml vials. The lipid was solubilized in methanol, appropriate sugars added as aqueous solutions to produce the mass ratios desired, and the mixtures were stirred on a vortex stirrer, frozen over dry ice, and lyophilized (16 h). Triplicate dry samples were weighed, and then placed in a 75% RH atmosphere (saturated NaCl) in a 30°C oven. Changes in sample weight occurring during equilibration with the humid atmosphere were measured at intervals over an 8 d period. Crystallization is observed as an exclusion of water (lowering the sample weight) as crystals form (18).

Vesicle leakage experiments were carried out using mixtures of palmitoyloleoylphosphatidylcholine (18 mg) and bovine brain phosphatidylserine (2 mg) (Avanti Polar Lipids, Inc.). The lipids were combined in chloroform and the mixture was dried under nitrogen in a rotoevaporator (2 h, 25°C). The dry mixture was taken up in degassed buffer (10 mM TES, 10 μM EDTA, pH 7.4) containing 5 mg/L isocitric acid plus the amount of sugar required for the mass ratios specified. This suspension was made into vesicles by sonication (Braunsonic 1510, Melsungen A. G.) for 10 min at 50 W following the procedures of Mouradian et al. (19). The sonicated vesicles were filtered from the suspending medium through Sephadex G-50 in a 10 ml syringe barrel, taken up again in buffer, and dried in a rotoevaporator under nitrogen (45–55°C) until dry (2 h) and stored under nitrogen. The vesicles were again taken up in TES-EDTA buffer (1 ml) and tested for the amount of isocitrate that had leaked out of the vesicles upon drying. This involved addition of isocitrate dehydrogenase (5 mg in 100 μl TES-EDTA buffer, 6 mg NADP/ml, 20 mg MnCl$_2$/ml). Control samples of vesicles were disrupted with 10% w/w Triton X-100 and assayed for total isocitrate. The sample solution (0.5 ml) was added to 3.5 ml enzyme mixture, and the NADPH produced was measured at 340 nm when it reached a steady value (20). Leakage was expressed as a ratio of isocitrate that had leaked out of the vesicles to the amount inside the control (undried) vesicles.

RESULTS

The effects of sucrose on the mesomorphic phase behavior of DMPC were examined by x-ray diffraction. The resulting DMPC/sucrose temperature-composition 'phase diagram' is shown in Figure 1. In this and in subsequent experiments, comparisons were made between mixtures of DMPC and sucrose after lyophilization from organic solvent, with and without subsequent partial hydration by incubation for 4 d in a 75% RH atmosphere. We will refer to these as wet and dry samples, respectively. The results in Figure 1 indicate that the upper limit or liquidus boundary to the hydrocarbon chain order/disorder transition for DMPC initially in the lamellar phase was drastically lowered by the addition of sucrose. A mass ratio of DMPC to sucrose of 1/0.7 or lower caused the transition of dry DMPC to occur at about 55°C. Each of the wet samples showed transitions at about 55°C. Thus, sucrose can alter the mesomorphic phase properties of dry DMPC to resemble those of wet samples. We note that in a separate experiment with fully hydrated DMPC, the corresponding chain order/disorder transition was sharp, falling between 24 and 24.5°C.

The transition temperatures detected in the wide-angle region of the x-ray diffraction pattern provide information on the packing of the lipid acyl chains. Over most of the temperature range examined, the DMPC/sucrose mixtures exist in the lamellar phase with either crystalline, gel, and/or liquid crystalline hydrocarbon chain packing. Complementary information in the form of lattice type, size, and symmetry is available from an analysis of the low-angle diffraction region. The calculated lamellar repeats or d-spacing values from representative wet and dry samples are compared in Figure 2. The data indicate that in wet samples sucrose has minimal effect on the lattice size, which decreases dramat-
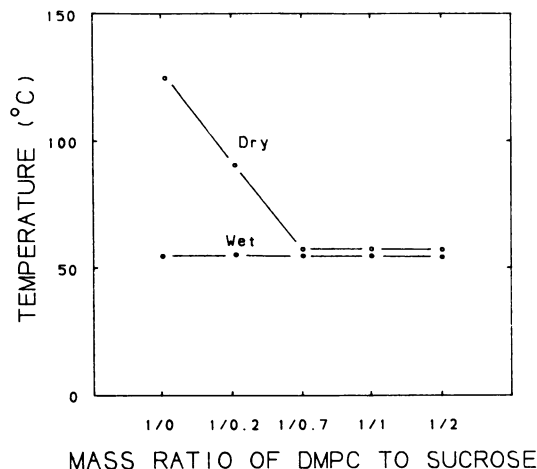

FIG. 1. Dependence of the chain order/disorder transition temperature of DMPC on the presence of sucrose at various mass ratios, comparing dry and partially hydrated samples. Transition temperatures were determined using wide-angle x-ray diffraction to distinguish between the lamellar gel or crystalline and the liquid-crystalline phases. Transition temperatures represent the liquidus boundary where the last trace of the gel or crystalline phase is seen upon first heating and are good to +/ −5°C. We note that for dry DMPC, the first indication of chain disordering as evidenced by the appearance of diffuse scatter in the vicinity of 4.5 Å was observed between 101 and 110°C and that disordering is complete by 129°C. Lattice type and symmetry of the liquid crystalline phase(s) has/have not been determined.
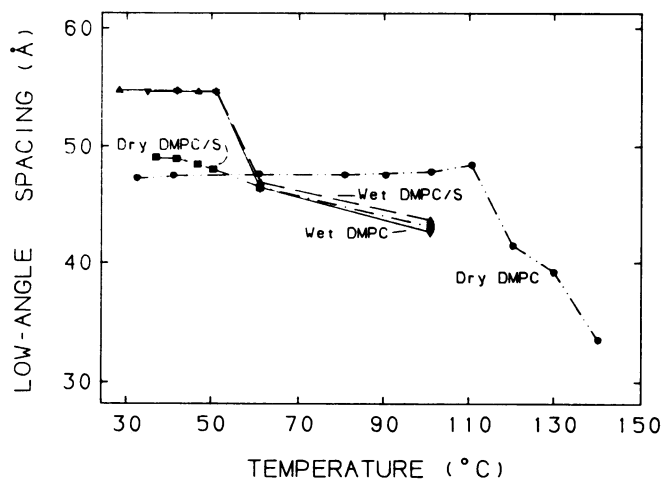

FIG. 2. Temperature dependence of the long-spacing of dry and partially hydrated DMPC with and without added sucrose. Determination of d-spacings is based on low-angle x-ray diffraction measurements. The lipid/sucrose mixture was at a mass ratio of 1/1. Up to 100°C, phase type is predominantly lamellar. At 110°C and above, other phase types may be present. Temperatures are reliable to ±2°C. Solid lines are wet samples, dashed lines are dry. Circles are DMPC alone, triangles are DMPC/sucrose at 1/1 by weight.

ically above the transition temperature. In dry samples, the behavior is quite different. For example, with pure DMPC, the lattice parameter has a small, positive thermal expansion coefficient which changes abruptly at the transition temperature. In contrast, the lattice size for DMPC/sucrose shows a sizeable negative expansion coefficient with little change in lattice parameter through the transition region. The lamellar repeat at and above the transition range is almost identical for wet DMPC and for the DMPC/sucrose mixture, regardless of its state of hydration.

These changes in mesomorphic phase behavior and of lamellar lattice size of DMPC/sucrose mixtures indicate that the sugar can substantially alter the properties of the phospholipid, and that in general, the presence of sugar induced physical properties in the dry phospholipid similar to those occurring under hydrated conditions. Below the transition temperature, the dry DMPC/sucrose showed a lamellar spacing which was intermediate between wet and dry DMPC.

Crowe and Crowe (10) have shown that sucrose is less effective than trehalose in protecting membranes from damage upon drying. We have reported preliminary evidence suggesting that a limitation of sucrose effectiveness might be related to its tendency to crystallize during the drying process (16). Examination of the x-ray films for sucrose crystal reflections indicated that indeed crystallization had occurred in numerous samples, especially in those incubated for 4 d at 75% RH. An arbitrary scoring (on a scale from 0–5) of the x-ray films was used to estimate the extent of sucrose crystallization in the wet and dry samples of DMPC and DMPC/sucrose mixtures. The results shown in Figure 3 are averages from four experiments, and they indicate that at DMPC/sucrose mass ratios below 1/0.7, some crystallization of sucrose occurred even in the dry samples. The incubation of samples under 75% RH for 4 d however, caused crystallization to occur at every sucrose content examined.

If sucrose provides an important contribution to desiccation tolerance in seeds, it would seem imperative to prevent its withdrawal through crystallization. Angiospermous seeds commonly contain large amounts of oligosaccharides, especially raffinose and stachyose (1). Since raffinose is known to serve as an effective inhibitor of sucrose crystallization (21), it would be reasonable to expect that the presence of raffinose would contribute to the desiccation tolerant characteristic in angiospermous seeds.

Evidence for the progress of crystallization in an amorphous sample of sucrose can be obtained by following sample weight changes during storage in an atmosphere of controlled relative humidity (21). Using this type of experimental procedure, we have followed the hydration of DMPC, sucrose, or mixes of the two (Fig. 4). As DMPC absorbs water from the humid atmosphere, its weight rises to a plateau corresponding to 2.7 mol of water/mol of lipid in about 10 h. The literature equilibrium value determined for DMPC at 25°C (12) was approximately 3.5 and at 22°C (14) it was 4.8 mol of water/mol of lipid. As sucrose takes up water its weight increases for about 20 h, after which water is released as would be expected to occur during crystallization. Mixtures of DMPC plus sucrose at mass ratios of 1/0.1

FIG. 4. The time course of water absorption and release upon sucrose crystallization for samples of DMPC and sucrose held in a moist atmosphere. Samples were held in 75% RH at 30°C, and weights determined at intervals. The DMPC/sucrose mixtures are indicated as mass ratios, and weight changes as a % of initial weight with an accuracy of +/−2%.

FIG. 5. The time course of water absorption and release upon sucrose crystallization for samples of DMPC, sucrose, and raffinose alone or in combination. Conditions of the test are described in the legend to Figure 4. The DMPC/sucrose/raffinose mixture was at a mass ratio of 1/1/0.3.

or 1/1 (Fig. 4) show gradual losses of wet weight suggestive of a slow sucrose crystallization in the mixture. If raffinose is present in the sample, as in Figure 5 (DMPC/sucrose/raffinose, mass ratio 1/1/0.3), this weight loss does not occur, indicating that crystallization of sucrose is prevented.

The prevention of sucrose crystallization by raffinose is further illustrated in Figure 6. The diffraction pattern obtained with DMPC alone is shown in Figure 6A. A mixture of DMPC/sucrose at a mass ratio of 1/1 reveals the superposition of additional reflections which derive from crystalline sucrose (Fig. 6B). A mixture of DMPC/sucrose/raffinose at a mass ratio of 1/1/0.3 shows no evidence of crystal formation (Fig. 6C). The appearance of a diffuse ring at about 4.5 Å indicates that at this temperature the lipid has undergone chain 'melting' in the presence of either sucrose or sucrose plus raffinose. In a separate experiment using dry DMPC/raffinose (1/0.3, by weight) we found that upon heating, lipid chain melting is completed somewhere between 50 and 68°C.

The effects of sucrose and raffinose in protecting membrane vesicles from damage during desiccation are illustrated by the data in Table I. In this case, vesicles constructed of palmitoyl-oleoyl-phosphatidylcholine plus bovine brain phosphatidylserine, with isocitrate inside, and sucrose or sucrose plus raffinose either on the inside or both inside and outside, were dried under
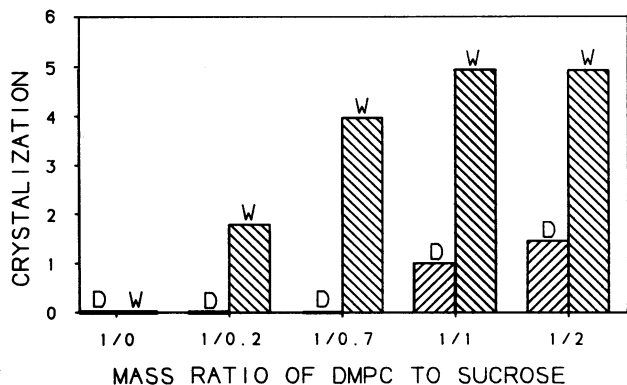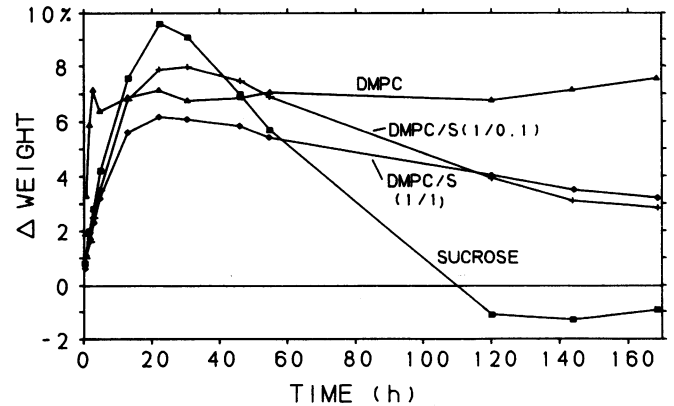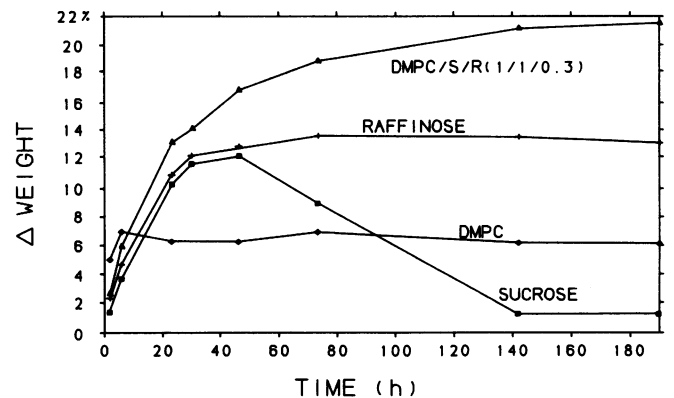

FIG. 3. Crystallization of sucrose in dry and hydrated DMPC, comparing various mass ratios of DMPC to sucrose. The degree of crystallization is rated on an arbitrary scale from 0 to 5 based on x-ray diffraction patterns. A rating of 0 indicates absence of the spotty reflections characteristic of sucrose crystals; a rating of 5 corresponds to the degree of spottiness seen with a pure sucrose powder (see also Fig. 6).
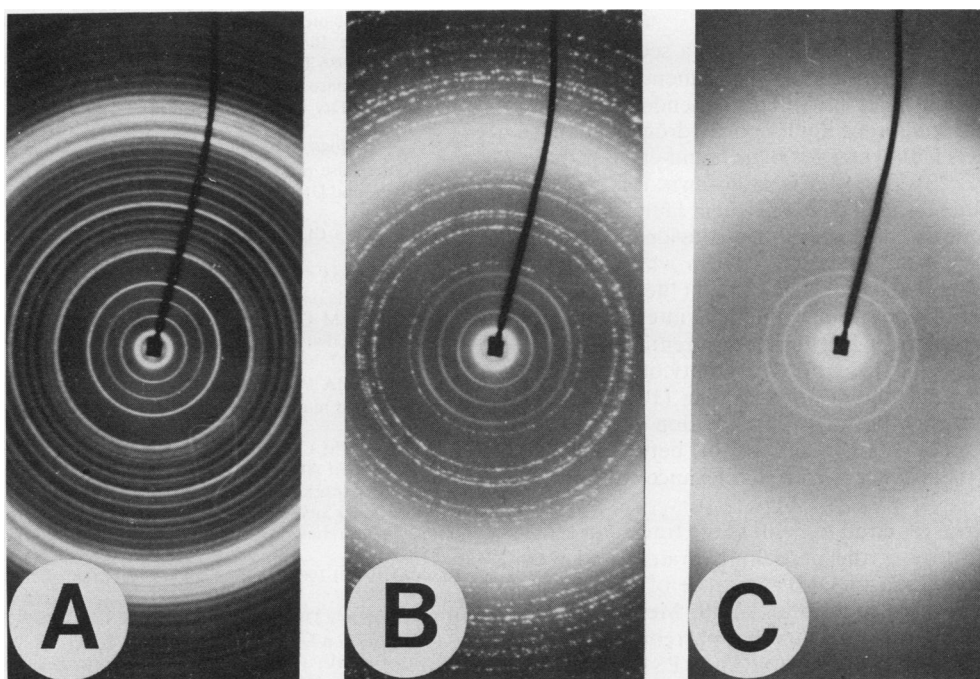
FIG. 6. Representative diffraction patterns recorded at 50°C of (A) DMPC, (B) DMPC/sucrose at a mass ratio of 1/1, and (C) DMPC/sucrose/raffinose at a mass ratio of 1/1/0.3. Samples A and B were exposed to 75% RH for 4 d, sample C is dry. In (B) the spotty reflections derive from crystalline sucrose. Experimental conditions: sample-to-film distance, 8.42 cm; wavelength, 1.568 Å; collimator diameter, 0.3 mm; incident flux, 10^{10} photons/s; electron beam current, 20 to 30 mA; machine energy, 5.2 GeV; exposure times, 15 s. The d-spacing values of the various reflections or scattering peaks in these photographs are as follows: (A) 56.2, 27.26, 18.39, 13.75, 9.33, 8.74, 8.44, 8.14, 7.98, 7.44, 6.94, 6.74, 6.40, 6.14, 5.89, 5.44, 5.34, 5.17, 5.04, 4.90, 4.78, 4.66, 4.52, 4.40, 4.35, 4.18, 4.11, 4.00, 3.91, 3.87, 3.82, 3.72, 3.59, 3.48, 3.35, 3.30, 3.20, 3.11, 3.05, and 2.93 Å; (B) 48.92, 24.49, 16.46, 12.19, (10.61, 7.60, 6.96, 6.76, 5.73, 5.45, 5.30, 4.89, 4.73, 4.54, 4.50, 4.37, 4.36, 4.28, 4.04, 3.96, 3.80, 3.74, 3.61, 3.55, 3.45, 3.38, 3.25, 3.13, 2.90, 2.81, 2.75, and 2.69 Å); and (C) 48.92, 24.26, 16.16, 12.08, and 4.50 Å. The bracketed reflections in (B) co-index with crystalline sucrose (13).

Table I. *Effects of Sucrose and Sucrose plus Raffinose in Protecting Membrane Vesicles from Disruption by Drying*

Vesicles were constructed of POPC and bovine brain PS (9/1 by weight), containing isocitrate; the amount of isocitrate leaking out of the vesicles after drying was measured as the ratio of isocitrate in the ambient solution to the amount in control vesicles determined after Triton X-100 treatment. Sucrose was provided inside or both inside and outside the vesicles (lipid/sucrose, 1/1 by weight), and sucrose plus raffinose (lipid/sucrose/raffinose, 1/1/0.3 by weight) was likewise provided inside or both inside and outside. The percent protection represents the percentage of the isocitrate retained in the vesicles after drying and rehydration.

Treatment	Sugars Inside		Sugars Inside and Outside	
	Leakage ratio	Protection	Leakage ratio	Protection
		%		%
Phospholipid only	1.0		1.0	
Sucrose	0.083	61	0.118	87
Sucrose/raffinose	0.062	81	0.072	94

vacuum. The data indicate that in the absence of sugars, the dried vesicles were unable to retain isocitrate. When the sugars were present only on the inside of the vesicles, sucrose allowed the retention of 61% of the isocitrate, and sucrose plus raffinose provided an additional 20% improvement in retention of isocitrate by the vesicles. When sugars were present on both the outside and the inside, sucrose allowed the retention of 87% of the isocitrate and sucrose plus raffinose gave a 7% improvement over the value for sucrose alone.

DISCUSSION

The experiments reported here provide for two conclusions relevant to the physiology of desiccation tolerance. A sugar such as sucrose can markedly alter the physical characteristics of a membrane phospholipid, causing it to retain characteristics of hydrated lipid even when water is absent. This is analogous to the protection of membrane vesicles or liposomes by trehalose as described by Crowe et al. (9, 10). The retention of wet characteristics by the dry phospholipid in the presence of sucrose may represent a central feature of desiccation tolerance. We also conclude that a sugar such as sucrose may be withheld from exerting protective effects because of its tendency to crystallize, especially at low moisture contents. The presence of an oligosaccharide, such as raffinose, can assist in the protection of membranes from desiccation damage by restricting or preventing the

crystallization of sucrose.

As water is withdrawn from a tissue such as a seed embryo, one of the most vulnerable sites for the development of damage will be the cellular membranes (10). The dependence of the bilayer conformation of membrane lipids upon hydrogen bonding and hydrophobic effects with water for their liquid-crystal structure is well known (17, 22). As water is withdrawn, it is reasonable to expect that membranes will lose their lamellar liquid crystalline structure unless some protection is provided. A 'water replacement theory' has been suggested (24) in which a sugar such as trehalose can form a substitute for water at the membrane surface. Evidence for a trehalose-phospholipid interaction has been provided by Crowe *et al.* (7, 9) using differential scanning calorimetry and infrared spectroscopy. The ability of sucrose to provide protection to a membrane upon drying (10), and the enhancement of this effect with the further addition of raffinose (Table I), suggest a similar *in vivo* interaction between phospholipids and sugars that may have direct relevance to the viability of dry seeds.

The amount of sucrose required to depress the transition temperature of DMPC to that of the partially hydrated lipid was approximately at a mass ratio of DMPC/sucrose of 1/0.7 (Fig. 1). This corresponds to a molar ratio of 1/1.39. Measurements by Crowe *et al.* (11) indicate that the amount of trehalose which fully occupied the hydrophilic sites of POPC and PS occurred at mass ratio of 1/0.67. This represents a molar ratio of 1/1.49. The corresponding values for sucrose in this same system were 0.5 g/g lipid and 1.11 mol/mol lipid.

The high concentrations of sucrose found in angiospermous seeds make this sugar an attractive candidate for the protection of membrane integrity. Because of its tendencies to crystallize, sucrose alone may not provide good membrane protection. The interaction of oligosaccharides such as raffinose, which are also widespread in orthodox seeds, may restrict the crystallization of sucrose, thus enabling maximal desiccation tolerance.

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