Effect of Sugars on Headgroup Mobility in Freeze-Dried Dipalmitoylphosphatidylcholine Bilayers: Solid-State ^{31}P NMR and FTIR Studies

Nelly M. Tsvetkova,* Brian L. Phillips,# Lois M. Crowe,* John H. Crowe,* and Subhash H. Risbud#
*Section of Molecular and Cellular Biology and #Department of Chemical Engineering and Materials Science, University of California at Davis, Davis, California 95616 USA

ABSTRACT The effect of the carbohydrates trehalose, glucose, and hydroxyethyl starch (HES) on the motional properties of the phosphate headgroup of freeze-dried dipalmitoylphosphatidylcholine (DPPC) liposomes was studied by means of ^{31}P NMR, Fourier transform infrared spectroscopy (FTIR), and differential scanning calorimetry (DSC). The results show that trehalose, which is a strong glass former (T_g = 115°C), elevates the onset of the lipid headgroup rotations and preserves some rotational mobility of the phosphate headgroups after cooling from the liquid-crystalline state. Glucose (T_g = 30°C), a very effective depressant of the phase transition temperature of freeze-dried DPPC, markedly elevates the initiation of the temperature of headgroup rotations. On the other hand, the monosaccharide does not preserve the headgroup disordering when cooled from the liquid-crystalline state. These effects are consistent with formation of hydrogen bonds between the OH groups of the sugar and the polar headgroups of DPPC. They show, however, that hydrogen bonding is not sufficient for preservation of the dynamic properties of freeze-dried DPPC. HES, although a very good glass former (T_g > 110°C), does not depress the phase transition temperature and affects only slightly the rotational properties of freeze-dried DPPC. This lack of effect of HES is associated with the absence of direct interactions with the lipid phosphates, as evidenced by the FTIR results. These data show that vitrification of the additive is not sufficient to affect the dynamic properties of dried DPPC.

INTRODUCTION

The ability of sugars to depress the gel-to-liquid-crystalline phase transition temperature (T_m) of dry phospholipid bilayers is a well known phenomenon (Crowe et al., 1984a,b; Tsvetkov et al., 1989). There is ample evidence that this depression of T_m is involved in the stabilization of liposomes (Crowe et al., 1985), intact membranes (Crowe et al., 1983), and whole cells (Hoekstra et al., 1992; Leslie et al., 1994) during drying. Lee et al. (1986, 1989) studied the dynamic properties of dipalmitoylphosphatidylcholine (DPPC)/trehalose mixtures by ^{31}P and ^{2}H NMR and found that the sugar restricts the rotational mobility of the lipid molecules below and above the thermal phase transition. They reported that the DPPC/trehalose mixtures at temperatures above their phase transition (T_m = 48°C, as determined by differential scanning calorimetry (DSC)), exist in a liquid-crystalline phase, with highly disordered acyl chains but rigid phosphate headgroups (Lee et al., 1986, 1989). Previous studies in our laboratory provided evidence that the depression of T_m involves direct interaction between the sugar and phospholipid headgroups, a proposal called the water replacement hypothesis (Crowe and Crowe, 1992).

Koster et al. (1994) suggest an alternative mechanism to direct interactions. They propose, based partly on previous work by Green and Angell (1989), that vitrification of the sugar is sufficient to reduce T_m of the dried lipid and provide successful preservation. Many sugars, including mono-, di-, and polysaccharides form glasses during dehydration. The glassy state is a thermodynamically unstable solid state characterized by very high viscosity and low molecular mobility (Slade and Levine, 1995; Franks, 1993). During heating, sugar glasses undergo a second-order transition to a rubber-like state that exhibits a lower viscosity and higher mobility than the glass.

In the present study, we investigated the effect of two saccharides and a carbohydrate polymer on the motional properties of phospholipids and the nature of the phase transitions of the phospholipid/sugar assemblies. We used ^{31}P NMR spectroscopy to examine the effect of sugars on the mobility of DPPC phosphate headgroups and FTIR to study spectral changes associated with hydrogen bonding between the lipid phosphates and the OH groups of the sugars. The disaccharide trehalose, which is a strong glass former (T_g = 115°C) (Ding et al., 1996; Crowe et al., 1996b), has been shown to depress the temperature of phase transition of dry DPPC (Crowe and Crowe, 1988; Crowe et al., 1988; Tsvetkova et al., 1988). We have previously proposed that trehalose forms hydrogen bonds with the polar lipid headgroups during drying (Crowe et al., 1984a), and we found this sugar to be very effective in stabilizing liposomes in the dry state (Crowe et al., 1985; Crowe et al., 1996b). Previous studies of the effect of the monosaccharide glucose show that it lowers the T_m of dry DPPC, forms hydrogen bonds with the phosphate headgroups as assessed by FTIR (Crowe et al., 1996a), has a T_g at ~30°C (Roos, 1993; Slade and Levine, 1995) but does not preserve liposomes during drying (Crowe et al., 1997). The carbohydrate

Received for publication 27 April 1998 and in final form 12 August 1998.
Address reprint requests to Dr. Nelly M. Tsvetkova, Section of Molecular and Cellular Biology, University of California at Davis, Davis, CA 95616. Tel.: 530-752-1094; Fax: 530-752-5305; E-mail: nmisvetk@ucdavis.edu.

0006-3495/98/12/2947/09 $2.00

polymer hydroxyethyl starch (HES), although a very good glass former ($T_g > 110$°C) (Crowe et al., 1997), shows no evidence of direct interactions with the DPPC and is ineffective in preserving liposomes. HES does not depress the temperature of the phase transition of freeze-dried DPPC (Crowe et al., 1996a, 1997).

Our results show that both trehalose and glucose raise the temperature of initiation of the lipid headgroup rotation, compared with pure freeze-dried DPPC. However, trehalose preserves some motional properties of the lipid headgroups upon cooling from the liquid-crystalline state, whereas glucose does not. These effects are consistent with direct interactions between the lipid headgroups and the OH groups of the sugar. By contrast, HES affects only slightly the rotational properties of DPPC, consistent with a lack of direct interactions with the lipid phosphates.

MATERIALS AND METHODS

Sample preparation

Trehalose dihydrate and glucose were purchased from Pfanstiehl (Waukegan, IL). HES was a gift from Dr. Barry Spargo and was dialyzed against water before use. Liposomes were prepared by drying aliquots of DPPC (Avanti Polar Lipids, Alabaster, AL) in chloroform under a stream of dry nitrogen followed by at least 2 h on a lyophilizer (VirTis Co., New York, NY) to remove the residual solvent. Aqueous solutions of trehalose or glucose were used to hydrate the lipid (sugar:lipid, 1:1, w/w). In the case of HES, dry polysaccharide was added to the lipid in the same weight ratio, and the mixture was then hydrated. The aqueous mixtures were heated to 55°C followed by vortexing. Unilamellar vesicles were formed by extruding the multilamellar vesicles through 200-nm polycarbonate filters (Poretics, Livermore, CA) in a hand-held extruder (Liposofast, Avestin, Ontario, Canada) that was heated to 60°C. The liposomes were frozen by immersing in liquid nitrogen, lyophilized, and kept under vacuum until used. The vials containing the dried lipids were placed in a dry box purged with dry air at 0–1% relative humidity. The humidity was monitored with an Omega RH 201 meter (Omega Engineering, Stamford, CT). The sample holders for the DSC and NMR experiments were loaded and sealed in the dry box. The samples for FTIR were loaded between CaF_2 windows in the dry box.

Differential scanning calorimetry

DSC was carried out on a Perkin-Elmer DSC 7 with a helium gas purge and a high-sensitivity DSC from Calorimetry Sciences Corp. (Provo, UT) with a nitrogen gas purge. Scanning rates were 10°C/min with the Perkin-Elmer DSC and 12°C/hour with the high-sensitivity DSC. Sample pan sizes were 20 μl for the Perkin-Elmer DSC and 1 ml for the Calorimetry Sciences DSC.

^{31}P NMR

The ^{31}P NMR spectra were taken with a Chemagnetics CMX-400 spectrometer, operating at 162 MHz. The samples were contained in epoxy-sealed glass tubes designed to fit into 7.5-mm (outer diameter) zirconia rotors (Wilmad Glass Co., Buena, NJ) and were loaded under flowing liquid nitrogen boil-off. Most of the ^{31}P spectra were measured by spin-echo detection with excitation provided either by ^{31}P{^{1}H} cross-polarization (CP; 1-ms contact time) or directly by a single-resonance (SR) spin-echo sequence (90x-τ-180y-τ-acquire). The 90° pulse width was 6 μs for both ^{1}H and ^{31}P. In general, the CP-echo works well at low temperature (below T_m), but magnetization transfer is very inefficient in the liquid-crystalline state. Typically, we used a 40-μs interpulse delay (τ) and a 2-s relaxation period for both CP-echo and SR-echo experiments. Between 20,000 and 40,000 acquisitions were required for adequate signal-to-noise ratio. Some additional spectra were taken with single-pulse excitation and acquisition to check the spin-echo results at temperatures near the initiation of headgroup motions. High-power ^{1}H decoupling was employed during acquisition of all spectra. The sample temperature was regulated by flowing heated air over the sample and controlled by the standard Chemagnetics MAS-probe heater assembly. We report the nominal (set) temperatures, although independent calibration with a chemical shift thermometer (van Gorkom et al., 1995) indicates that the mean sample temperature is ~3 K higher at 70°C. This correction is linear from 25°C to 90°C. Under slow MAS conditions at 70°C, approximately 75% of the sample experiences a temperature within ±1.5 K of the mean, but we expect the temperature gradient to be much smaller for the nonspinning experiments reported here.

Fourier transform infrared spectroscopy

Spectra were obtained with a Perkin-Elmer 1750 optical bench, assisted by a microcomputer equipped with IRDM software and a Peltier temperature controller (Crowe et al., 1989). Data processing consisted of baseline flattening of the CH_2 stretch region from 3000 to 2800 cm^{-1}, using the interactive flat routine, and normalization of absorbance, using the abex routine. The temperature was controlled by a Peltier device and monitored by a fine thermocouple on the FTIR window (Crowe et al., 1989). The frequencies of the CH_2 symmetric stretch were estimated by eye to the nearest 0.1 cm^{-1} at 80% of peak height. The frequencies of the phosphate asymmetric stretches were taken to the nearest 0.1 cm^{-1} from the peak absorbance of these bands, using the unflatted 1280–1100 cm^{-1} region.

RESULTS AND DISCUSSION

Differential scanning calorimetry

In the present experiments, DPPC freeze-dried from excess water shows a single thermal event at 72°C, which is fully reproducible during reheating and does not depend on the thermal history (Fig. 1, *a* and *b*). This T_m corresponds to water contents of ~5 wt % (Kodama et al., 1982), which is equivalent to DPPC-dihydrate.

Freeze-dried DPPC/trehalose liposomes show a phase transition from gel to liquid-crystalline state at 62°C, 10°C lower than T_m for freeze-dried DPPC (Fig. 1 *c*), which is in reasonable agreement with our previous studies (Crowe and Crowe, 1988; Crowe et al., 1988, 1996a). This is in contrast to the work of Lee et al. (1986) where the DPPC/trehalose sample showed a T_m of 48°C, using DSC. Those samples were prepared from a mixture of organic solvents and resulted in multilamellar structures with excess crystalline trehalose. Previous work from this laboratory (Crowe and Crowe, 1988; Crowe et al., 1988) has shown that unilamellar vesicles prepared from aqueous trehalose solution maintain their unilamellar structure after freeze-drying.

Upon subsequent reheating, only a broad, low-enthalpy peak centered at 24°C is observed, which is reproduced upon subsequent scans (Fig. 1, *d* and *e*). In accordance with our previous studies, these data show that rapid cooling of freeze-dried DPPC/trehalose from the liquid-crystalline state produces a metastable phase (Crowe and Crowe, 1988; Crowe et al., 1988). Lee et al. (1986) do not report this low-temperature transition on subsequent scans, possibly because their scans were started at higher temperatures. We

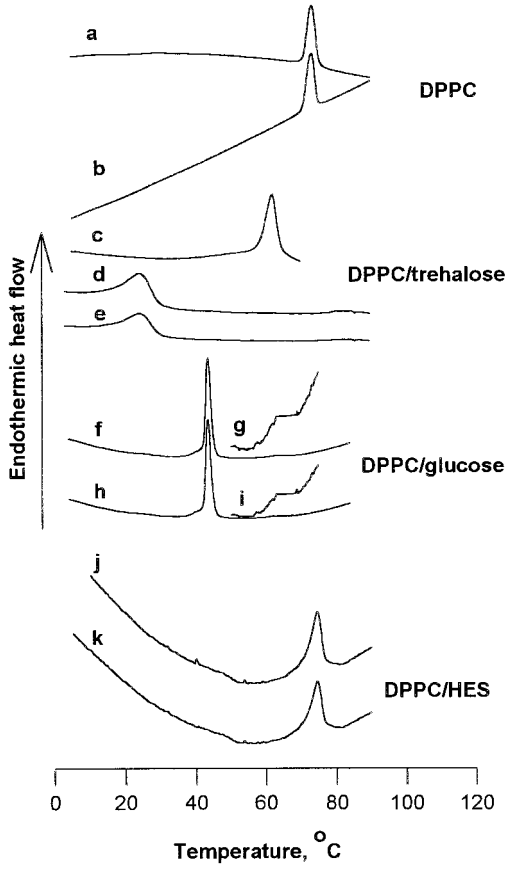

FIGURE 1 DSC traces of phase transitions of freeze-dried DPPC during first (*a*) and second (*b*) heating; freeze-dried DPPC/trehalose (1:1, w/w) liposomes during first (*c*), second (*d*), and third (*e*) heating; freeze-dried DPPC/glucose (1:1, w/w) liposomes during first (*f*) and second (*h*) heating; DPPC/glucose traces at higher sensitivity (*g* and *i*); freeze-dried DPPC/HES (1:1, w/w) liposomes during first (*j*) and second (*k*) heating. For each sample, second (and third) heating runs were started immediately upon completion of previous scan.

interpret the present effect in the following way. During drying, the phosphate headgroups in DPPC are tightly packed, thus limiting access of the trehalose to the polar headgroups. As a result, on the first scan T_m is partially depressed. When the acyl chains are melted, thus decreasing the headgroup packing, the sugar can access the headgroups and, as a result, depress the T_m on the subsequent scans. In subsequent scans, T_m always remains at 24°C, but if the sample is cooled to 4°C, equilibrated at this temperature, and rescanned, T_m returns to its original value of 62°C, indicating that the initial lipid conformation can be restored. Recovery of the initial T_m has been noted before (Crowe and Crowe, 1988; Crowe et al., 1996a). Lee et al. (1989) also observed metastability of DPPC/trehalose multilayers freeze-dried from organic solvents. Their x-ray diffraction data showed gel and liquid-crystalline phases typical for DPPC/trehalose multilayers freeze-dried from excess of water (Quinn et al., 1988). However, the recooled gel phase was similar but not identical in chain packing to the gel phase they observed before reheating.

DSC of freeze-dried DPPC/glucose liposomes shows a highly cooperative phase transition at 43°C and a small endotherm at 63°C (Fig. 1, *f* and *g*). The T_m at 43°C indicates that the monosaccharide strongly depresses the transition temperature of freeze-dried DPPC and is similar to that reported previously for dried DPPC/glucose measured both with DSC and FTIR (Crowe et al., 1996a). The appearance of the smaller endotherm at 63°C suggests a phase separation that is most likely due to inhomogeneous distribution of the glucose molecules in freeze-dried DPPC. Both transition temperatures are stable, persisting at the same value after repeated scanning and storage below T_m for at least a week. Unlike the results for DPPC/trehalose, the thermal history of the sample has no effect on T_m. By contrast, HES has no significant effect on the transition temperature of freeze-dried DPPC (Fig. 1, *j* and *k*). In agreement with previous data, repeating cooling and heating does not affect the lipid phase transition in the presence of HES, which remains similar to that of freeze-dried DPPC (Crowe et al., 1996a, 1997).

The samples used for the NMR experiments, contained in sealed glass tubes, were also examined by high-sensitivity DSC. The endotherms displayed during the first and second heating were very similar to those obtained for the parallel samples with the Perkin-Elmer DSC.

^{31}P NMR

The ^{31}P NMR spectra of freeze-dried DPPC, obtained during heating, are shown in Fig. 2. At 25°C an asymmetric chemical-shift powder pattern, with principal values of 79, 23, and −100(5) ppm, is observed. These values are similar to those reported previously for DPPC-monohydrate (81, 25, and −110) (Herzfeld et al., 1978), but with a slightly reduced overall width. This spectrum corresponds to the ^{31}P shielding tensor near the rigid-lattice limit and is attributed to stationary lipid phosphate headgroups with the O-P-O plane tilted with respect to the plane of the bilayer (Griffin et al., 1978).

Upon heating to above 40°C, the spectrum narrows into an axially symmetric pattern with shielding anisotropies of $\Delta\sigma = -57$ ppm in the temperature range 50–60°C, and $\Delta\sigma = -45$ ppm above 70°C. The reduction of the spectral width and appearance of axially symmetric powder patterns with increase in temperature indicate the onset of the lipid headgroup rotations, the rates of which exceed the width of the rigid-lattice spectrum observed at 25°C (~30 kHz). Similar changes have been observed by Griffin et al. (1978) for fully hydrated DPPC, but at much lower temperatures. Collapse of the rigid powder pattern for hydrated DPPC occurs at ~−10°C ($\Delta\sigma = -69$ ppm), which has been attributed to an onset of rotation of the lipid headgroups about the bilayer normal (Griffin et al., 1978). Further width reduction with an increase of temperature (to $\Delta\sigma = -47$ ppm at $T = 48$°C) has been attributed by these authors to additional rotation of the phosphate groups, most likely

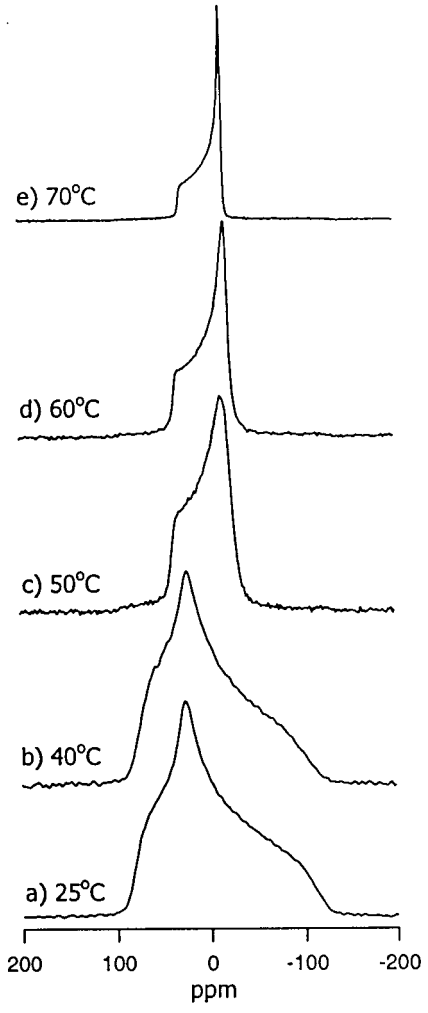

FIGURE 2 ^{31}P NMR spectra of freeze-dried DPPC, obtained with increasing temperature at (*a*) 25°C, (*b*) 40°C, (*c*) 50°C, (*d*) 60°C, and (*e*) 70°C. No change occurs with further increase of the temperature to 90°C. All spectra were obtained by spin-echo sequence with a 40-μs interpulse delay, 500-kHz sampling rate, 2-s relaxation delay, and 12,000–20,000 acquisitions. *a* and *b* were obtained with ^{31}P{^{1}H} cross-polarization (CP-echo) and 1-ms contact time; all others were from direct ^{31}P excitation.

about the C(2)-C(3) bond of the glycerol backbone (Griffin et al., 1978). Interestingly, the anisotropy we observe for freeze-dried DPPC at the highest temperatures (70°C and 80°C) is very close to that observed by Griffin et al. (1978) for fully hydrated DPPC, suggesting that the range of motions of the polar headgroups in the liquid-crystalline state is very similar in the dry and fully hydrated lipid.

It should be noted that from 25°C to 60°C, the spectra of freeze-dried DPPC show evidence of some sample inhomogeneity (Fig. 2). Smoothing of the low-frequency edge of the rigid powder pattern at 25°C suggests a variation in σ_{33} of $\sim\pm5$ ppm. At 50°C, the CP-echo experiment produces a complicated spectrum that appears to be the sum of an axially symmetric pattern and a rigid-lattice spectrum, whereas the spin-echo spectrum shows a broad baseline feature suggesting the presence of some stationary lipid phosphate groups. Such spectral features associated with the presence of stationary phosphates are observed up to 70°C. One possible explanation for these observations could be an inhomogeneous distribution of residual water molecules in freeze-dried DPPC. Both the breadth of the stationary powder pattern and the onset of headgroup rotations depend on water content (Griffin, 1976).

During the initial heating, freeze-dried DPPC/trehalose liposomes give rigid ^{31}P powder patterns up to $\sim$50°C that are nearly identical to those for pure DPPC (principal values 79, 23, and $-103(5)$ ppm, Fig. 3 *a*). Between 50°C and 60°C the spectrum collapses into a narrow, axially symmetric pattern that exhibits an anisotropy $\Delta\sigma = -42$ ppm similar to that of the corresponding high temperature spectra of freeze-dried DPPC. With further increase of temperature, the spectrum narrows slightly, to $\Delta\sigma = -38(1)$ ppm at 80°C. In the present study, no significant change in the spectral features is observed during heating through the thermal phase transition from gel to liquid-crystalline state (T_m = 62°C). Similar results were obtained by Lee et al. (1986) for freeze-dried DPPC/trehalose mixtures in the temperature range 21–50°C. They did not observe any change in ^{31}P spectra below and above the lipid phase transition, which was registered at 48°C in their DSC data, and did not observe the onset of headgroup rotation that occurs at higher temperature.

The present results show that in the presence of trehalose, the temperature at which the lipid headgroups begin to rotate is considerably elevated and is close to the T_m, in contrast to pure freeze-dried DPPC, which shows rotation at least 20°C below the T_m. We observed the same effect with DPPC/trehalose samples freeze-dried to monohydrate, but in this case the headgroups begin to rotate at higher temperature, between 80°C and 90°C.

Cooling the sample from the liquid-crystalline state to 10°C, which produces a metastable phase (based on DSC data), results in a featureless spectrum that is significantly narrower than that obtained at 25°C during the initial heating cycle (Fig. 3 *e*). With each subsequent increase in temperature, the spectrum narrows and sharpens until the axially symmetric pattern is fully regained at $\sim$50°C ($\Delta\sigma = -52$ ppm). The spectrum continues to narrow with further increase in temperature, and at 60°C the width ($\Delta\sigma = -41(1)$ ppm) is the same within experimental uncertainty to that observed at 60°C during the initial heating cycle. The narrow, featureless spectrum obtained at 10–25°C after cooling from the liquid-crystalline state most likely results from retention of at least one rotational mode (e.g., about the bilayer normal) combined with disorder in rotational state or angle of the other motions (e.g., a distribution of C(2)-C(3) order parameters). This interpretation is consistent with the observation that the full width of the low-temperature spectra during the second heating is similar to that of pure freeze-dried DPPC between 50°C and 60°C. We think it unlikely that the narrow width at 10°C could result from changes in the static shift anisotropy, considering that the full width of the rigid ^{31}P spectrum decreases by only 40

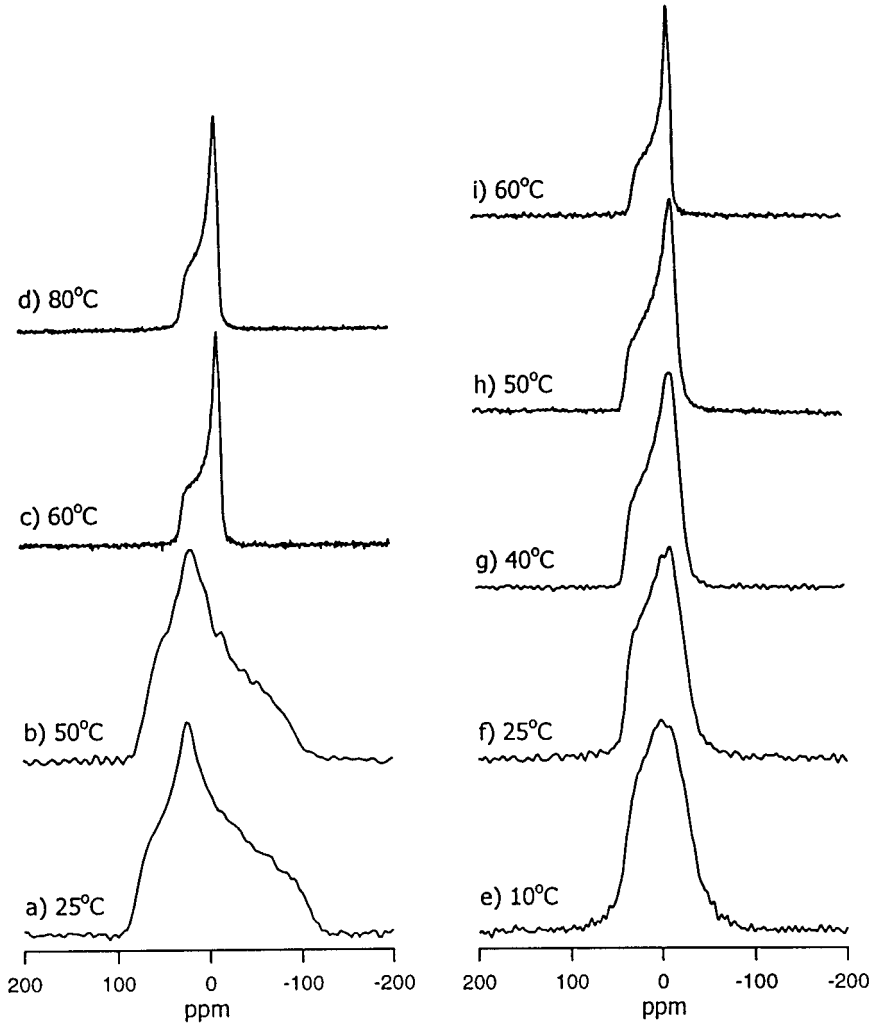

FIGURE 3 ^{31}P NMR spectra of freeze-dried DPPC/trehalose (1:1, w/w) liposomes obtained with increasing temperature during initial (*a–d*) and second (*e–i*) heating cycles. Immediately after acquisition of 80°C (*d*), sample temperature was decreased to 10°C in less than 2 min to begin second heating cycle. All spectra were obtained with direct-excitation spin-echo as described for Fig. 2, except *a* and *b* were obtained via CP-echo and *c* and *d* were from single-pulse excitation and acquisition with 100-kHz spectral window and 20,000 and 40,000 acquisitions, respectively.

ppm from the anhydrous to fully hydrated DPPC. These data show that the disaccharide continues to affect the motional properties of the phosphate headgroups after cooling from the liquid-crystalline state.

The ^{31}P NMR spectra of freeze-dried DPPC/glucose liposomes (Fig. 4) indicate that the lipid headgroup rotation begins only above 70°C, which is much higher than the temperature of the gel-to-liquid-crystalline phase transition, observed by DSC at 43°C (Fig. 1, *f* and *h*). At low temperatures, the powder pattern of DPPC/glucose is very similar to that of pure DPPC and DPPC/trehalose, giving principal values 79, 23, and −105 ppm at 25°C (Fig. 4). During the thermotropic phase transition at 43°C, there is no evidence of rotational motions of the polar headgroups, and only a decrease in the signal obtained from ^{31}P {^{1}H} cross-polarization is observed, due to acyl-chain disorder. Between 40°C and 60°C, the spectrum smooths and narrows somewhat, which could be due to either limited headgroup reorientation or a distribution of shielding tensors resulting from interaction with the sugar. However, the ^{31}P spin-lattice relaxation remains slow, indicating absence of rapid rotations. The rotation of the phosphate headgroups begins at ~70°C (Fig. 4), and the width of the axially symmetric pattern at 75°C ($\Delta\sigma = -39 \pm 1$ ppm) is similar to those of pure freeze-dried DPPC and DPPC/trehalose at high temperatures. Unlike DPPC/trehalose, however, the rigid powder pattern of DPPC/glucose observed at 25°C is restored immediately upon cooling the sample to 25°C from the disordered liquid-crystalline phase. This result indicates that glucose does not preserve the motional properties of the lipid phosphates upon cooling from the liquid-crystalline state. On the other hand, glucose, similarly to trehalose, markedly elevates the commencement of the lipid headgroup rotations, which appears to occur above 70°C (~25°C higher than the T_m). One possible reason for this result is a direct interaction between the sugar and the phosphate groups, most likely via hydrogen bonding, a possibility that we discuss further with FTIR results.

The changes in the ^{31}P spectra of freeze-dried DPPC/HES liposomes with increasing temperature (Fig. 5) resemble those of pure freeze-dried DPPC, with a slightly higher temperature for the onset of the lipid headgroup rotations. At 25°C, the rigid asymmetric powder pattern is slightly narrower than that of DPPC (principal values 75, 22, and

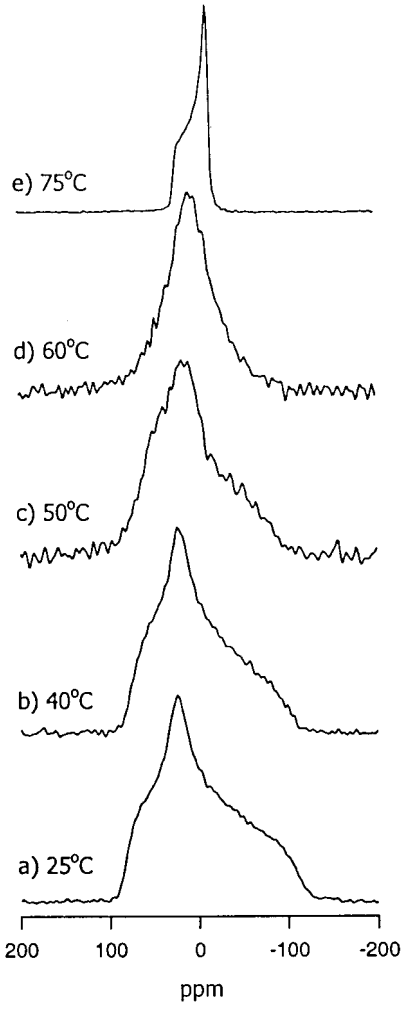

FIGURE 4 Spin-echo ^{31}P NMR spectra of freeze-dried DPPC/glucose (1:1, w/w) liposomes obtained with increasing temperature at (*a*) 25°C, (*b*) 40°C, (*c*) 50°C, (*d*) 60°C, and (*e*) 75°C. The spectrum at 25°C after the first heating cycle (not shown) is indistinguishable from *a*. All data were collected as described for Fig. 2, but with 40,000 acquisitions; *a* and *b* were obtained with ^{31}P {^{1}H} cross-polarization; and *c*–*e* were with direct ^{31}P excitation.

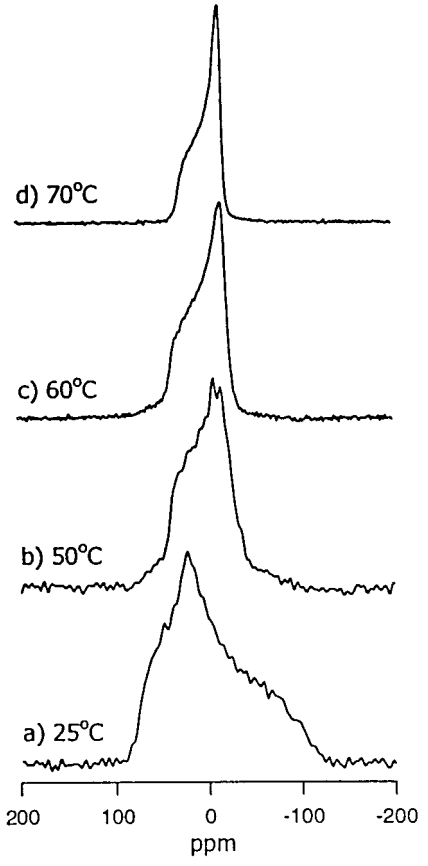

FIGURE 5 ^{31}P NMR spectra of freeze-dried DPPC/HES (1:1, w/w) liposomes, obtained with increasing temperature at (*a*) 25°C, (*b*) 50°C, (*c*) 60°C, and (*d*) 70°C. The spectrum at 40°C (not shown) is similar to that taken at 25°C. All data were collected as described in Fig. 2 with direct ^{31}P excitation except *a*, which was obtained with ^{31}P {^{1}H} cross-polarization.

-97 ± 3 ppm). Although the thermal phase transition of freeze-dried DPPC/HES is at 73°C (Fig. 1, *j* and *k*), axially symmetric patterns are observed above 50°C, and their width decreases with increasing temperature. During heating, the complicated spectra observed at 50°C and 60°C (Fig. 5) comprise a sum of the rigid powder pattern with an axially symmetric one ($\Delta\sigma = -57$ ppm), the relative intensity of which increases with temperature. At 70°C and above, only the axially symmetric pattern is present, which decreases slightly in width from $\Delta\sigma = -45$ ppm at 70°C to $\Delta\sigma = -42$ ppm at 80°C. Spectra taken at 25°C upon cooling from high temperature do not differ significantly from those observed initially. Subsequent heating of the same sample produces spectral data very similar to the ones from the first run.

These results clearly indicate that HES has only a slight effect on the motional properties of the DPPC headgroups in comparison to trehalose and glucose. The polymer induces a small increase of the temperature of onset of the lipid headgroup rotation and in this respect the properties of freeze-dried DPPC/HES are very similar to those of pure freeze-dried DPPC. The main difference appears to be the presence of two spectral components between 50°C and 60°C, indicating that the initiation of headgroup rotations varies through the sample, possibly due to sample inhomogeneity or to a distribution of lipid headgroup-HES interactions arising from the large size of HES molecules. In addition, at each temperature the axially symmetric patterns are slightly narrower for DPPC/HES than for pure DPPC, which could be due to the smaller rigid width of DPPC/HES and/or to a small effect of HES on the rotational modes of the polar headgroups.

The small effect of HES is most likely due to the absence of a strong interaction with the lipid phosphate groups. Indeed, HES does not cause a shift in the phosphate vibration indicative of hydrogen bonding in dried egg phosphatidylcholine (Crowe et al., 1996a, 1997), in sharp contrast

with the samples dried with trehalose or glucose. Contrary to the hypothesis of Koster et al. (1994), which would predict that HES should be particularly effective in reducing T_m in dry phospholipid, we find that HES has no effect on the T_m and only slightly affects the motional properties of the lipid phosphates. These data indicate that vitrification is not sufficient in itself for stabilization of biomaterials. In fact, glucose is also inefficient in preserving biomaterials (Crowe et al., 1996a, 1997). However, when both HES and glucose are combined, a successful preservation can be achieved (Crowe et al., 1996a, 1997).

Fourier transform infrared spectroscopy

Discrete frequency ranges in the FTIR spectrum can be assigned to different parts of the lipid molecule, which allows detailed information about the molecular interactions to be obtained. Furthermore, the thermal phase transition can be monitored by following changes in the wavenumber of the CH_2 symmetric and asymmetric stretches that undergo an increase in frequency as the acyl chains melt and the number of *gauche* conformers increases. In this work, we observe changes in the vibrational frequency of the bands corresponding to the methylene symmetric stretching mode around 2850 cm^{-1} and the phosphate asymmetric stretching mode at 1240 cm^{-1} (Cameron and Mantsch, 1978; Casal and Mantsch, 1984; Wong and Mantsch, 1988) in freeze-dried DPPC/trehalose, DPPC/glucose, DPPC/HES, and DPPC liposomes.

In freeze-dried DPPC/trehalose liposomes, changes in the vibrational frequency of the CH_2 band around 2850 cm^{-1} show the gel-to-liquid-crystalline phase transition to be at 67°C (Fig. 6 *A*). The band corresponding to the PO_2 asymmetric stretch of DPPC in the presence of trehalose shifts from 1242 cm^{-1} to 1228 cm^{-1} during the first heating (Fig. 6 *A*). This shift to lower vibrational frequency is consistent with hydrogen bonding between the phosphate headgroups and the OH groups of the sugar and is in agreement with previous data (Crowe et al., 1984a,b, 1996a). During the second heating, performed immediately after the first one, the frequency of the CH_2 band increases (Fig. 6 *B*) indicating a phase transition at 24°C, in accordance with the DSC data (Fig. 1 *d*). However, no significant change in the frequency of the phosphate band is observed, and it remains at 1228 cm^{-1} (Fig. 6 *B*). The lack of spectral changes of the lipid phosphates during the second heating suggests that no change in the molecular environment at the polar headgroups occurs after cooling and upon subsequent reheating. These spectral data also suggest that the increase of the temperature of initiation of the headgroup rotations observed by the ^{31}P NMR is most likely associated with direct interactions between the lipid phosphates and the sugar. The ability of trehalose to preserve the motional properties of the lipid phosphates after cooling from the liquid-crystalline state may be due to increased headgroup spacing related to increased interaction via hydrogen bonding between the

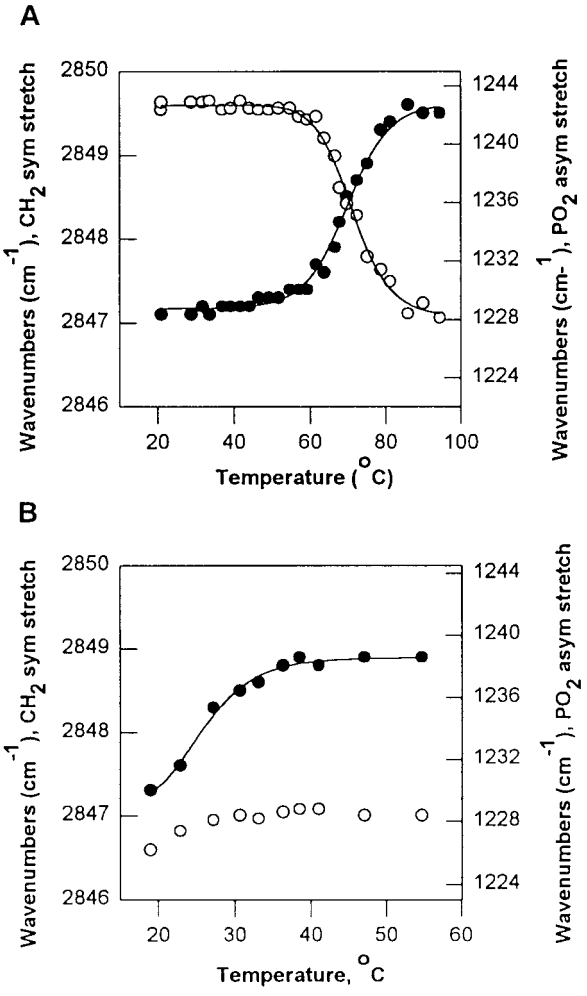

FIGURE 6 Changes in the vibrational frequency of the CH_2 symmetric stretch (●) and the PO_2 asymmetric stretch (○) in freeze-dried DPPC/trehalose (1:1, w/w) liposomes as a function of the temperature during the first (*A*) and second (*B*) heating.

lipid phosphates and the OH groups of the sugar after the initial temperature scan. Fig. 6 *A* shows that chain melting and the shift in phosphate frequency (increased hydrogen bonding) occur at the same temperature.

Changes in the vibrational frequencies of the CH_2 band at about 2850 cm^{-1} and the PO_2 band at 1242 cm^{-1} of freeze-dried DPPC/glucose liposomes are shown in Fig. 7. During the first heating, the CH_2 band shifts correspond to the gel-to-liquid-crystalline phase transition at 43°C. However, during further heating the frequency of this band continues to increase. This additional temperature-induced change in frequency is consistent with the appearance of the small endotherm at 63°C (Fig. 1 *g*), suggesting a phase separation most likely due to inhomogeneous distribution of the monosaccharide molecules in freeze-dried DPPC. These spectral changes in CH_2 are fully reproducible during the second heating (data not shown), in accordance with the DSC data (Fig. 1, *h* and *i*). During the first heating, the PO_2 band shifts from 1242 cm^{-1} to 1225 cm^{-1} which is indicative of increased hydrogen bonding between the lipid phos-

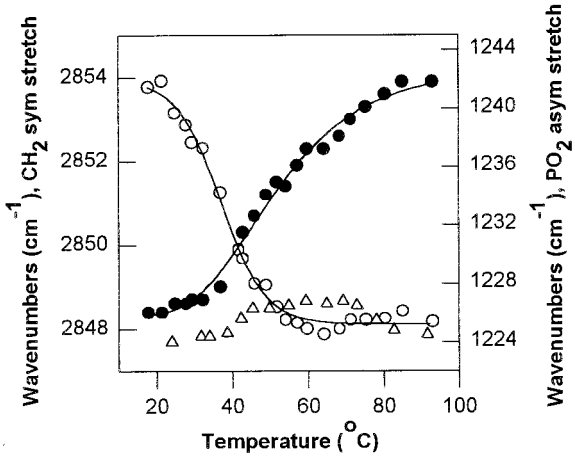

FIGURE 7 Changes in the vibrational frequency of the CH_2 symmetric stretch (●) and the PO_2 asymmetric stretch (○) in freeze-dried DPPC/glucose (1:1, w:w) liposomes as a function of the temperature during the first heating. △, PO_2 asymmetric stretch during immediate reheating.

phates and the glucose. During the second heating, the vibrational frequency of the PO_2 band changes only slightly with temperature (Fig. 7), suggesting that the molecular interactions (most likely hydrogen bonding) remain through subsequent cooling and reheating. These spectral changes in the PO_2 band are similar for both DPPC/glucose and DPPC/trehalose and are consistent with previous data (Crowe et al., 1984a,b, 1996a). Although both sugars induce a shift to lower vibrational frequency of the lipid phosphates, they differ in their effects on headgroup motional properties. Glucose does not preserve some lipid headgroup rotations after cooling from the liquid-crystalline state, whereas trehalose does, as evidenced by ^{31}P NMR.

For DPPC/HES liposomes, changes in the vibrational frequency of the CH_2 band show a phase transition at ~75°C (Fig. 8 *A*). The frequency of the PO_2 band changes only slightly with the temperature (Fig. 8 *A*), indicating that there is no hydrogen bonding between the lipid phosphates and the polymer. Subsequent reheating reproduces essentially the same spectral changes for the CH_2 (data not shown) and PO_2 stretching bands (Fig. 8 *A*). These spectral changes in CH_2 and PO_2 stretching bands of DPPC/HES are similar to the ones observed in freeze-dried DPPC (Fig. 8 *B*). The shift in the vibrational frequency of the CH_2 band in DPPC shows a phase transition at ~72°C. At the same time, the frequency of the PO_2 band changes insignificantly with temperature (Fig. 8 *B*). The same spectral changes of the CH_2 (data not shown) and PO_2 stretching bands of freeze-dried DPPC are observed during immediate reheating (Fig. 8 *B*). In accordance with the ^{31}P NMR data, HES has only a slight effect on the properties of DPPC phosphates in comparison with trehalose and glucose. The polymer induces only a small shift in the frequency of the PO_2 stretching band, and in this respect the properties of freeze-dried DPPC/HES are similar to those of pure freeze-dried DPPC.

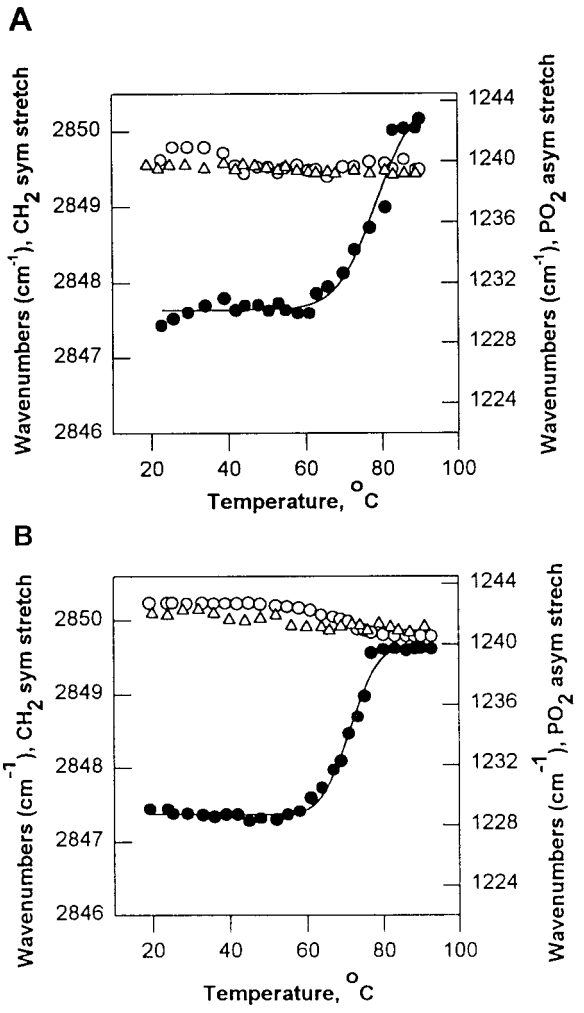

FIGURE 8 Changes in the vibrational frequency of the CH_2 symmetric stretch (●) and the PO_2 asymmetric stretch (○) in freeze-dried DPPC/HES (1:1, w:w) liposomes (*A*) and freeze-dried DPPC (*B*) as a function of the temperature during the first heating. △, PO_2 asymmetric stretch during immediate reheating.

SUMMARY AND CONCLUSIONS

The ^{31}P NMR spectra of DPPC/trehalose show that the disaccharide elevates considerably the onset of the lipid phosphate headgroup rotations and preserves some motional properties of the lipid phosphate groups upon cooling from the liquid-crystalline state (Fig. 3). These effects appear to be consistent with formation of hydrogen bonds between the OH groups of the disaccharide and the polar groups near the thermal phase transition. By contrast, glucose elevates considerably the onset of the lipid phosphate headgroup rotations but does not preserve the rotational motions of DPPC headgroups during cooling from the liquid-crystalline state. As shown by the ^{31}P NMR spectra, although glucose elevates markedly the onset of the headgroup rotations, to above 70°C (Fig. 4), the lipid phosphate groups are stationary immediately upon cooling. Elevation of the onset of headgroup rotations and changes in the PO_2 stretching fre-

quency are consistent with hydrogen bonding between the lipid phosphates and the sugar. Therefore, hydrogen bonding between the sugar and lipid is not sufficient for preservation of headgroup rotations upon cooling from the liquid-crystalline state.

Although a very good glass former, HES does not depress the T_m of the dried lipid. It induces only a slight increase in the temperature of onset of the headgroup disordering and small changes in the rotation angles. This lack of effect of HES is associated with the absence of direct interactions with the DPPC phosphates as shown by the FTIR data, which could be attributed to the large molecular weight of this additive. The ^{31}P NMR data indicate that vitrification of the additive is not sufficient to affect the dynamical properties of dried DPPC.

This work was supported by grant IBN93–08581 from the National Science Foundation, and the NMR equipment was provided by the W. M. Keck Foundation.

REFERENCES

Cameron, D. G., and H. H. Mantsch. 1978. The phase transition of 1,2-dipalmitoyl-sn-phosphatidylcholine as seen by Fourier transform infrared difference spectroscopy. *Biochem. Biophys. Res. Comm*un. 83: 886–892.

Casal, H., and H. H. Mantsch. 1984. Polymorphic phase behavior of phospholipid membranes studies by infrared spectroscopy. *Biochim. Biophys. Acta*. 779:381–401.

Crowe, L. M,., and J. H. Crowe. 1988. Trehalose and dry dipalmitoylphosphatidylcholine revisited. *Biochim. Biophys. Acta*. 946:193–201.

Crowe, J. H., and L. M. Crowe. 1992. Preservation of liposomes by freeze-drying. *In* Liposome Technology. G. Gregoriadis, editor. CRC Press, Boca Raton, FL. 229–252.

Crowe, J. H., L. M. Crowe, J. F. Carpenter, A. S. Rudolph, C. Aurell Wistrom, B. J. Spargo, and T. J. Anchordoguy. 1988. Interactions of sugars with membranes. *Biochim. Biophys. Acta*. 947:367–384.

Crowe, J. H., L. M. Crowe, and D. Chapman. 1984a. Preservation of membranes in anhydrobiotic organisms: the role of trehalose. *Science*. 223:701–703.

Crowe, J. H., L. M. Crowe, and D. Chapman. 1984b. Infrared spectroscopic studies on interactions of water and carbohydrates with biological membranes. *Arch. Biochem. Biophys*. 232:400–407.

Crowe, J. H., L. M. Crowe, and S. A. Jackson. 1983. Preservation of structure and functional activity in lyophilized sarcoplasmic reticulum. *Arch. Biochem. Biophys*. 220:477–484.

Crowe, L. M., J. H. Crowe, A. Rudolph, C. Womersley, and L. Appel. 1985. Preservation of freeze-dried liposomes by trehalose. *Arch. Biochem. Biophys*. 242:240–247.

Crowe, J. H., F. A. Hoekstra, L. M. Crowe, T. J. Anchordoguy, and E. Drobnis. 1989. Lipid phase transitions measured in intact cells with Fourier transform infrared spectroscopy. *Cryobiology*. 26:76–84.

Crowe, J. H., F. A. Hoekstra, K. H. N. Nguyen, and L. M. Crowe. 1996a. Is vitrification involved in depression of the phase transition temperature in dry phospholipids? *Biochim. Biophys. Acta*. 1280:187–196.

Crowe, J. H., A. E. Oliver, F. A. Hoekstra, and L. M. Crowe. 1997. Stabilization of dry membranes by mixtures of hydroxyethyl starch and glucose: the role of vitrification. *Cryobiology*. 35:20–30.

Crowe, L. M., D. S. Reid, and J. H. Crowe. 1996b. Is trehalose special for preserving dry biomaterials? *Biophys. J*. 71:2087–2093.

Ding, S.-P., J. Fan, J. L. Green, Q. Lu, E. Sanchez, and C. A. Angell. 1996. Vitrification of trehalose by water loss from its crystalline dihydrate. *J. Thermal Analysis*. 47:1391–1405.

Franks, F. 1993. Solid aqueous solutions. *Pure Appl. Chem*. 65:2527–2537.

Green, J. L., and C. A. Angell. 1989. Phase relations and vitrification in saccharide-water solutions and the trehalose anomaly. *J. Phys. Chem*. 93:2880–2882.

Griffin, R. G. 1976. Observation of the effect of water on the ^{31}P nuclear magnetic resonance spectra of dipalmitoyllecithin. *J. Am. Chem. Soc*. 98:851–853.

Griffin, R. G., L. Powers, and P. S. Pershan. 1978. Head-group conformation in phospholipids: a phosphorus-31 nuclear magnetic resonance study of oriented monodomain dipalmitoylphosphatidylcholine bilayers. *Biochemistry*. 17:2718–2722.

Herzfeld, J., R. G. Griffin, and R. A. Haberkorn. 1978. Phosphorus-31 chemical shift tensors in barium diethyl phosphate and urea-phosphoric acid: model compounds for phospholipid head- group studies. *Biochemistry*. 17:2711–2718.

Hoekstra, F. A., J. H. Crowe, L. M. Crowe, T. Van Roekel, and E. Vermeer. 1992. Do phospholipids and sucrose determine membrane phase transitions in dehydrating pollen species? *Plant Cell Environ*. 15:601–606.

Kodama, M., M. Kuwabara, and S. Seki. 1982. Successive phase-transition phenomena and phase diagram of the phosphatidylcholine-water system as revealed by differential scanning calorimetry. *Biochim. Biophys. Acta*. 689:567–570.

Koster, K., M. S. Webb, G. Bryant, and D. V. Lynch. 1994. Interactions between soluble sugars and POPC during dehydration: vitrification of sugars alters the phase behavior of the phospholipid. *Biochim. Biophys. Acta*. 1193:143–150.

Lee, C. W. B., S. K. Das Gupta, J. Mattai, G. G. Shipley, O. H. Abdel-Mageed, A. Makriyannis, and R. G. Griffin. 1989. Characterization of the L_λ phase in trehalose-stabilized dry membranes by solid-state NMR and X-ray diffraction. *Biochemistry*. 28:5000–5009.

Lee, C. W. B., J. S. Waugh, and R. G. Griffin. 1986. Solid-state NMR study of trehalose/1,2-dipalmitoyl-sn-phosphatidylcholine interactions. *Biochemistry*. 25:3737–3742.

Leslie, S. B., S. A. Teter, L. M. Crowe, and J. H. Crowe. 1994. Trehalose suppresses phase transitions in dry yeasts. *Biochim. Biophys. Acta*. 1192:7–13.

Quinn, P. J., R. D. Koynova, L. J. Lis, and B. G. Tenchov. 1988. Lamellar gel-lamellar liquid crystal phase transition of dipalmitoylphosphatidylcholine multilayers freeze-dried from aqueous trehalose solutions: a real-time x-ray diffraction study. *Biochim. Biophys. Acta*. 942:315–323.

Roos, Y. 1993. Melting and glass transitions of low molecular weight carbohydrates. *Carbohydrate Res*. 238:39–48.

Slade, L., and H. Levine. 1995. Glass transitions and water-food structure interactions. *In* Advances in Food and Nutrition Research. G. E. Kinsella, editor. Academic Press, San Diego. 103–269.

Tsvetkova, N., B. Tenchov, L. Tsonev, and T. Tsvetkov. 1988. Dependence of trehalose protective action on the initial phase state of dipalmitoylphosphatidylcholine bilayers. *Cryobiology*. 25:256–263.

Tsvetkov, T. D., L. I. Tsonev, N. M. Tsvetkova, R. D. Koynova, and B. G. Tenchov. 1989. Effect of trehalose on the phase properties of hydrated, and lyophilized dipalmitoylphosphatidylcholine multilayers. *Cryobiology*. 26:162–169.

van Gorkom, L. C. M., J. M. Hook, M. B. Logan, J. U. Hanna, and R. E. Wasylishen 1995. Solid-state lead-207 NMR of lead (II) nitrate: localized heating effects at high magic angle spinning speeds. *Magn. Res. Chem*. 33:791–795.

Wong, P. T. T., and H. H. Mantsch. 1988. High-pressure infrared spectroscopic evidence of water binding sites in 1,2-diacyl phospholipids. *Chem. Phys. Lipids*. 46:213–224.