Low Amounts of Sucrose Are Sufficient to Depress the Phase Transition Temperature of Dry Phosphatidylcholine, but Not for Lyoprotection of Liposomes

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ABSTRACT Disaccharides such as sucrose and trehalose play an important role in stabilizing cellular structures during dehydration. In fact, most organisms that are able to survive desiccation accumulate high concentrations of sugars in their cells. The mechanisms involved in the stabilization of cellular membranes in the dry state have been investigated using model membranes, such as phosphatidylcholine liposomes. It has been proposed that the lyoprotection of liposomes depends on the depression of the gel to liquid-crystalline phase transition temperature (T_m) of the dry membranes below ambient and on the prevention of membrane fusion by sugar glass formation, because both lead to leakage of soluble content from the liposomes. Since fusion is prevented at lower sugar/lipid mass ratios than leakage, it has been assumed that more sugar is needed to depress T_m than to prevent fusion. Here, we show that this is not the case. In air-dried egg phosphatidylcholine liposomes, T_m is depressed by $>60°C$ at sucrose/lipid mass ratios 10-fold lower than those needed to depress fusion to below 20%. In fact, T_m is significantly reduced at mass ratios where no bulk sugar glass phase is detectable by Fourier transform infrared spectroscopy or differential scanning calorimetry. A detailed analysis of the interactions of sucrose with the P=O, C=O, and choline groups of the lipid and a comparison to published data on water binding to phospholipids suggests that T_m is reduced by sucrose through a "water replacement" mechanism. However, the sucrose/lipid mass ratios necessary to prevent leakage exceed those necessary to prevent both phase transitions and membrane fusion. We hypothesize that kinetic phenomena during dehydration and rehydration may be responsible for this discrepancy.

INTRODUCTION

It is well established that disaccharides such as Suc (sucrose) and Tre (trehalose) play an important role in the protection of biological membranes under stress conditions, particularly during drying. In fact, the ability of some organisms to survive in the dry state (anhydrobiosis) has been related to the accumulation of large amounts of disaccharides in their cells and tissues (1–3). It has, however, also been shown recently that some organisms can survive complete dehydration without the accumulation of sugars (4).

In addition to the biological interest in the molecular mechanisms of anhydrobiosis, recent applied efforts have been directed at the preservation of mammalian cells in the dry state. This involves blood components such as platelets (5,6) but also other cell types (7–9). In these cases, Tre has been accumulated in the cells, either by endocytosis or by genetically engineering the cells to take up or synthesize trehalose.

The mechanism by which disaccharides protect membranes in the dry state has been studied extensively (see 1–3, 10–16 for reviews). According to the water replacement hypothesis, sugars insert between the lipid headgroups in dry membranes, interacting with the lipids through H-bonds. These interactions between sugar and lipid result in maintenance of dry membranes in a physical state similar to that seen in the presence of water.

It has been proposed that the establishment of sugar-lipid interactions is responsible for the depression of the gel to liquid-crystalline phase transition temperature (T_m) observed in dry phospholipid bilayers (3). As a consequence, phase transitions are avoided during dehydration and rehydration, thus preventing the associated loss of internal solutes (1,3, 15). Additionally, it has been shown that the protection of membrane vesicles by sugars is associated with the ability of sugars to form a glassy state (vitrify) at ambient temperatures. The formation of a rigid and stable glassy matrix prevents vesicle membrane fusion (see Oliver et al. (1), Buitink and Leprince (10), and Crowe et al. (13) for reviews). This is important, not only because fusion increases vesicle size but also because it often results in leakage (10,13). It has been suggested that the prevention of both phase transitions and fusion is necessary and sufficient to stabilize both model and biological membranes in the dry state (1,2).

It has been shown that the prevention of leakage from liposomes after drying and rehydration depends on the mass ratio of sugar/lipid (17). Several studies, both on freeze-dried and air-dried vesicles, have shown that leakage can be completely, or almost completely, prevented at sufficiently high sugar/lipid ratios if the sugar is present both inside and outside of the vesicles. It has also been shown that a lower sugar/lipid mass ratio is necessary to prevent fusion than to

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prevent leakage (12,18–22). From this fact it was concluded that more sugar is needed to depress T_m than to prevent fusion (18,19).

In this contribution, we show that this is not the case. T_m of dry egg phosphatidylcholine (EPC) vesicles is effectively depressed at significantly lower Suc/EPC mass ratios than are necessary to prevent fusion and leakage. In fact, there is considerable depression of T_m before any bulk sugar glass becomes detectable by Fourier transform infrared spectroscopy (FTIR) or differential scanning calorimetry (DSC). This effective depression of T_m can be clearly related to the ability of Suc to H-bond to the P=O, C=O, and choline parts of the lipid. Comparison of our data to published data on water binding to phospholipids suggests that T_m of dry EPC in the presence of Suc is indeed depressed by a water replacement mechanism but that the prevention of phase transitions and fusion is still not sufficient to completely prevent leakage.

MATERIALS AND METHODS

Materials

EPC was purchased from Lipid Products (South Nutfield, Surrey, UK). Suc (purity 99.5%) was obtained from Sigma (St. Louis, MO).

Preparation of liposomes

EPC was dried from chloroform under a stream of N_2 and stored under vacuum overnight to remove traces of solvent. Liposomes were prepared from hydrated lipids using a hand-held extruder with two layers of polycarbonate membranes with 100-nm pores ((23); Avestin, Ottawa, Canada).

Leakage and fusion experiments

Liposomes for leakage and fusion studies were made as previously described (24). Liposomes were mixed with concentrated Suc solutions to give the appropriate sugar/lipid mass ratios, and samples were air dried in desiccators at 28°C and 0% relative humidity for 24 h in the dark (21).

FTIR spectroscopy

Spectra were obtained from samples containing EPC liposomes and Suc at different mass ratios in the range 0.0–2.0 (mg Suc/mg EPC). Liposomes were extruded in the presence of Suc, so that the solute was present on both sides of the membranes. Samples (50 μl) were spread on CaF_2 windows and dried as described above. A window was then fixed in a cuvette holder connected to a temperature control unit (Specac Eurotherm, Worthington, UK; compare Hincha et al. (21)). The cuvette holder was placed in a vacuum chamber with KBr windows, which was placed in the infrared beam. Temperature was controlled by a liquid N_2 reservoir and an electrical heater. The temperature was measured with a thermocouple attached to the cuvette holder next to the sample. The sample was first heated to 50°C for 20 min under vacuum to remove residual moisture the lipid had taken up during sample handling. The sample was then cooled to −50°C and, after 30 min equilibration, the temperature was increased at a constant rate of 1°C min^{-1} to 100°C.

To obtain lipid melting curves and determine T_m values, spectra with 4 cm^{-1} resolution and two coadded scans were recorded in a Perkin-Elmer (Foster City, CA) GX 2000 FTIR spectrometer every minute. After normalization of absorbance and baseline correction using the Spectrum 5.0.1 software, the wavenumber of the CH_2 symmetric stretching (νCH_2s) band around 2850 cm^{-1} was determined by the automatic peak identification routine. The T_m values were estimated as the midpoints of the lipid melting curves (25). Additionally, the T_m values were also obtained by fitting a sigmoidal/Boltzmann function to the experimental curve and determining the center of this curve (OriginPro 7.0 software). The obtained T_m values were similar (±1°C) in both cases.

Interactions between lipid and sugar and sugar-induced conformational changes were investigated by an analysis of the following vibrational modes arising from the lipid: phosphate asymmetric stretching (νP=Oas), carbonyl stretching (νC=O), asymmetric stretching of the choline methyl groups ($\nu CN(C\text{-}H_3)_3$as), and asymmetric stretching of the choline C-N bond ($\nu C\text{-}N(CH_3)_3$as). Also, the band attributed to the hydroxyl stretching vibration (νOH) of the sugar was investigated. Spectra at different temperatures between −50°C and 100°C were recorded with 2 cm^{-1} resolution and 16 coadded scans. For the νC=O band, peak deconvolution and curve fitting were performed using the peak-fitting module of OriginPro 7.0. A Pearson7 function was used for peak fitting, with only the number of peaks used as a set parameter. All other parameters were optimized by the software through an iterative process to find the best curve fit.

DSC

The thermal behavior of the samples was investigated by DSC. As for the FTIR experiments, DSC thermograms were obtained from samples containing sugar and EPC at Suc/EPC mass ratios between 0.0 and 2.0, with sugar present on both sides of the membranes. Either 25 or 50 μl of the liposome samples were transferred into preweighed aluminum pans and dried as described above. After drying, the pans were sealed under a N_2 atmosphere and reweighed before and after the DSC measurements. No differences in the weight of the dry samples were found. The amount of residual water in the samples was determined by puncturing the lids of the sample pans and drying the pans for 24 h at 80°C in a vacuum oven. In all cases, the amount of residual H_2O was ≤0.02 g H_2O/g dry weight.

DSC experiments were performed using a Netzsch (Selb, Germany) DSC 204. After cooling the samples from room temperature to −60°C at a rate of 20°C min^{-1}, they were scanned at 20°C min^{-1} while heating to 100°C. This cooling and heating cycle was performed four times. Lower heating rates were tested (1°C, 5°C, and 10°C min^{-1}), but a rate of 20°C min^{-1} was chosen to resolve clear transitions. Due to thermal disequilibrium in the calorimeter, a scan rate of 20°C min^{-1} overestimated the transition temperatures by ~2–3°C. Since the transition temperatures were all measured under the same conditions, the comparison between different samples is not affected by the scan rate. Duplicate samples were prepared and scanned for each Suc/EPC mass ratio.

The analysis of the thermograms and the determination of the phase transition temperatures were performed with the Netzsch software package. T_m was recorded as the temperature at the peak maximum. The glass transition temperature (T_g) was determined as the midpoint of the heat capacity change associated with the glass transition. All data used for the analysis were taken from the heating thermograms, but cooling scans were recorded to analyze reproducibility and hysteresis of the transitions.

RESULTS

Gel to liquid-crystalline phase transitions in lipids and Suc glass transitions

Fig. 1 shows the melting curves of air-dried EPC liposomes at different Suc/EPC mass ratios. In accordance with previous studies (21,22,25) the T_m of pure EPC liposomes was 40°C. Also, as previously shown (25), in the presence of Suc at a mass ratio of 2.0 (maximum ratio used in this study), the

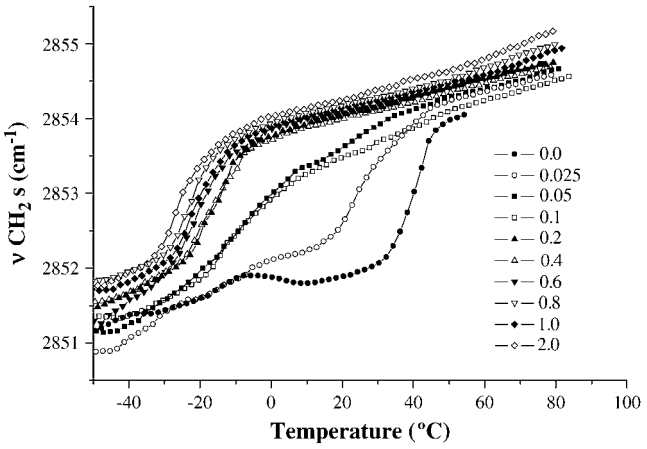

FIGURE 1 Lipid melting curves of dry EPC liposomes as determined by FTIR spectroscopy. The wavenumber of the symmetric CH_2 stretching band (νCH_2s) is plotted as a function of the sample temperature. The samples contained either only EPC liposomes or Suc and liposomes at the different mass ratios indicated.

lipid melting curve was shifted to lower temperatures by >60°C and T_m was ~−25°C.

Fig. 1 also indicates that the mass ratio can be reduced from 2.0 by a factor of 10 without a dramatic effect on T_m. For Suc/EPC mass ratios ≥0.2, the T_m values of dry EPC liposomes were all below −17°C, resulting in only a small change in T_m over this wide range of sugar contents ($\Delta T_{m\ (0.2-2.0)} \approx 7$°C). When the amount of Suc was reduced further, to give mass ratios of 0.1 or 0.05, the midpoint of the melting curves was found at about −8 to −9°C. However, the lipid melting curves were very broad, making T_m determination difficult, and it could not be distinguished whether one or more phase transitions were occurring. This was addressed further by the DSC measurements described below. Even at the lowest Suc/EPC mass ratio employed in our study (0.025), T_m was already shifted by ~15–25°C, indicating a high efficiency of Suc to depress T_m in dry membranes.

It is also clear from Fig. 1 that Suc had an effect on the mobility of the lipid acyl chains in both the gel and the liquid-crystalline phase. In both cases, νCH_2s increased by approximately one wavenumber with increasing Suc/EPC mass ratios from 0.0 to 2.0. This is most likely due to an increased spacing between the lipids in the presence of Suc, allowing for a higher number of *gauche* conformers in the fatty acyl chains. An intercalation of disaccharides into dry gel phase lipids has also been suggested from previous studies (26).

To confirm the phase transitions determined by FTIR and to get more information about the thermal behavior of the samples, DSC experiments were conducted. Fig. 2 shows the DSC thermograms from second heating scans for the Suc/EPC mass ratios also investigated by FTIR (Fig. 1). In agreement with the FTIR results, the thermogram of the pure dry lipid showed one endothermic peak centered at 40°C, which is attributed to the gel to liquid-crystalline phase transition.

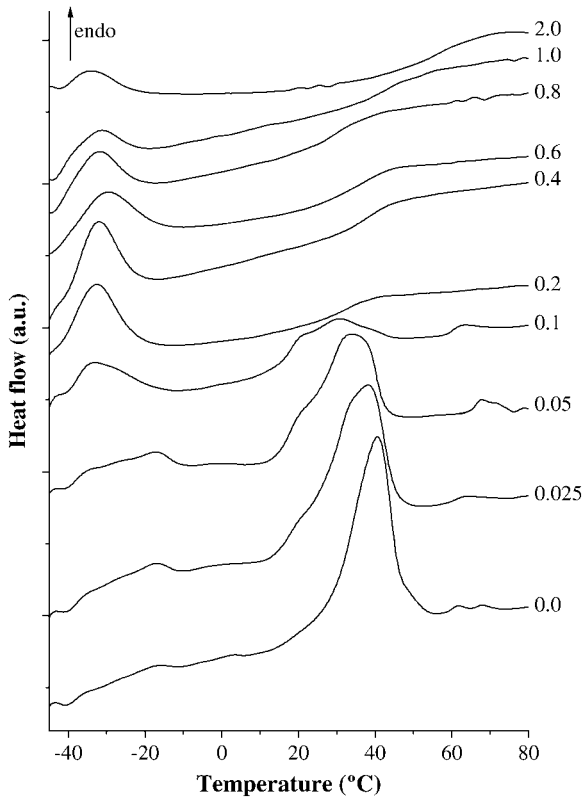

FIGURE 2 DSC heating thermograms of dry EPC liposomes in the presence of different amounts of Suc. The resulting Suc/EPC mass ratios are indicated to the right of each trace. Thermograms correspond to the second heating scan after cooling the samples from 100 to −60°C and subsequent equilibration at this temperature for 5 min.

For Suc/EPC mass ratios ≥0.2, the thermograms were all very similar. An endothermic peak was present at temperatures between −25 and −30°C, and the baseline showed a deflection at temperatures between 40 and 60°C. In accordance with previous DSC and x-ray diffraction studies on dry Suc and 1-palmitoyl-2-oleoylphosphatidylcholine at a mass ratio of 2.0 (27), the peaks localized at low temperatures are attributed to the lipid gel to liquid-crystalline phase transition, in good agreement with our FTIR data (Fig. 1). It is important to note that no differences were observed for this range of Suc/EPC mass ratios (≥0.2) between the first and the second heating scan (presented in Fig. 2) or any subsequent heating scans (data not shown).

Samples corresponding to Suc/EPC mass ratios between 0.025 and 0.1 had a more complex thermal behavior. In comparison to the pure lipid, the endothermic peak was slightly shifted to lower temperatures with increasing amounts of sugar. The peak also became broader and developed a shoulder on the low temperature side. These observations indicate that more than one transition may be associated with this peak. It has been noted previously (28,29) that the presence of sugars in liposome preparations can lead to broad, complex, and low enthalpic lipid phase transitions. The transition enthalpy

of the high temperature peak decreased with increasing Suc/EPC mass ratios. Concommittantly, a second peak at lower temperatures appeared, corresponding to the T_m of samples with Suc/EPC mass ratios $\geq$0.2. The enthalpy of this transition increased with increasing Suc/EPC mass ratios.

In contrast to what we observed for Suc/EPC mass ratios $\geq$0.2, for mass ratios between 0.025 and 0.1 the first and second heating scans differed from each other. In Fig. 3, the first and second heating scans of a sample with a Suc/EPC mass ratio of 0.1 are shown. From the first to the second scan there was an increase in the enthalpy of the low-temperature phase transition, whereas the enthalpy of the high-temperature phase transition decreased. Additionally, in the first heating scan the region of the high temperature phase transition showed several overlapping peaks that may be due to heterogeneity in the sample. In the second scan, usually only one broad peak with a shoulder was present. In some cases, the transition enthalpy of the low temperature transition still increased and the enthalpy of the high temperature phase transition decreased slightly from the second to the third heating scan (data not shown). In all cases, no difference was detected between the third and fourth heating scans.

The deflection in the baseline of the DSC themograms at higher temperatures (Fig. 2) corresponds to the glass transition of Suc. In fact, for a Suc/EPC mass ratio of 2.0, the glass transition temperature (T_g), determined as the midpoint of the deflection in the baseline, had a value of 58°C, in accordance with the T_g of 60°C for dry Suc at a water content of ~0.02 g H_20/g dry weight (30). It should be noted that a glass transition was only visible at mass ratios of 0.2 or above. At lower sugar concentrations, a possible glass transition was masked by the endotherm from the lipid phase transition.

However, FTIR (Fig. 4) also showed no evidence for the presence of a bulk glass phase at low Suc/EPC mass ratios. It has been shown that in general the formation of an H-bond X-H···Y leads to a greater length of the covalent bond X-H of the proton donor. Consequently, its force constant and the frequency of νXH decrease. Therefore, the establishment of H-bonds between Suc and lipid may affect νOH of the sugar. The νOH is a very broad band that is localized in the infrared spectral region of 3700–3000 cm^{-1}. This broad profile indicates the presence of a wide variety of OH conformers with different orientations, resulting from H-bonds of different length and strength. In addition to the lipid···HO interactions, the sugar molecules establish different intra- and intermolecular OH···OH bonds.

It has been shown (31) that the νOH band of pure amorphous Suc at 50°C is centered at $\approx$3370 cm^{-1}. In our samples, this wavenumber was only obtained at the highest Suc/EPC mass ratio investigated. With decreasing mass ratio, the peak wavenumber of νOH progressively declined (Fig. 4). This shift to lower wavenumbers indicates that the average strength of the interactions of the OH groups was greater in the presence of the lipid than for the pure sugar glass. Such a decrease in νOH of amorphous sugars after the

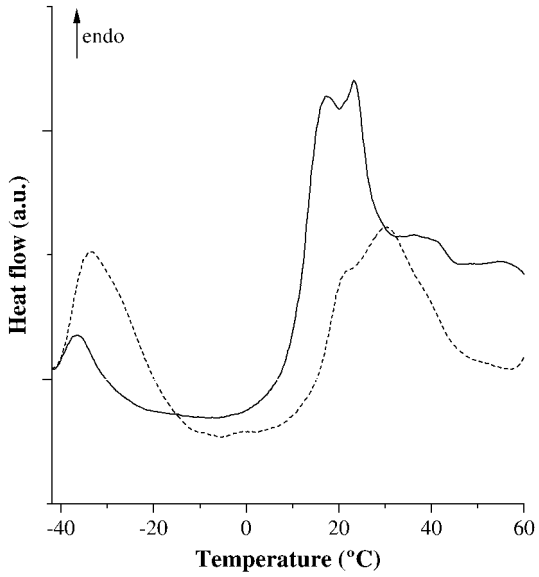

FIGURE 3 DSC heating thermograms of dry EPC liposomes in the presence of Suc at a Suc/EPC mass ratio of 0.1 in the temperature range −40–60°C. Solid line, first heating scan; dashed line, second heating scan. See Fig. 2 for experimental details.

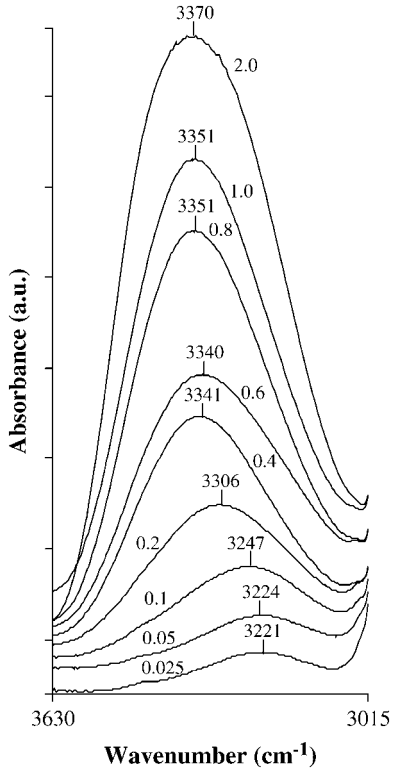

FIGURE 4 Infrared spectra in the Suc OH stretching vibration (νOH) region of dry samples containing Suc and EPC liposomes at the indicated mass ratios. The peak wavenumbers for each spectrum are indicated above the respective curves.

introduction of liposomes has been reported previously (32,33). Our data suggest that at very low mass ratios, the sugar is essentially completely bound to membrane lipids and as the mass ratio increases, an increasing fraction of the sugar is forming a bulk glass phase. In accordance with the DSC data (Fig. 2), bulk glass phase formation becomes evident at mass ratios of 0.2 and above in the FTIR spectra (Fig. 4).

Participation of the P=O groups in sugar-lipid interactions

It has been suggested that the reduction in the T_m of dry lipids in the presence of sugars is due to direct H-bonding interactions between the sugars and the lipid headgroups (see, e.g., Oliver et al. (1) and Oliver et al. (2) for reviews). FTIR spectroscopy has been frequently used to investigate H-bonding to the P=O moiety of various phosphatidylcholines, because the massive downshift of the νP=Oas band of phospholipids due to H-bonding allows the detailed analysis of such interactions.

In dry EPC liposomes, νP=Oas was localized at ~1260 cm^{-1} (Fig. 5). In the presence of Suc, νP=Oas was shifted to lower wavenumbers, indicating the expected participation of the P=O groups in H-bonds with the sugar molecules. With increasing amounts of sugar, the wavenumber of νP=Oas decreased. This decrease was steepest for mass ratios up to 0.2 and reached a minimum value of 1234 cm^{-1} at a mass ratio of 0.6. This indicates that there is a progressive engagement of the phosphate groups in interactions with sugar molecules.

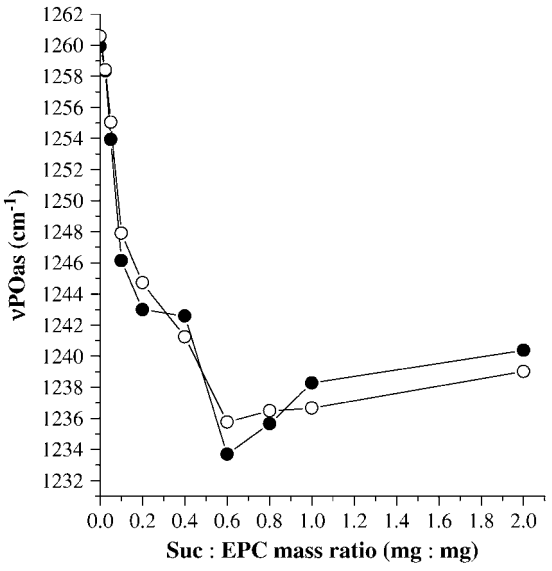

FIGURE 5 Wavenumber of the asymmetric P=O stretching vibrational mode (νP=Oas) as a function of the Suc/EPC mass ratio at 50°C (*solid symbols*) and 100°C (*open symbols*).

For mass ratios between 0.6 and 1.0, the H-bonding became slightly weaker (Fig. 5; at 50°C: $\nu P{=}Oas_{(Suc/EPC\,=\,0.6)} = 1234\ cm^{-1}$ and $\nu P{=}Oas_{(Suc/EPC\,=\,1.0)} = 1238\ cm^{-1}$). This effect was found at all investigated temperatures and in independent experiments. The apparent reduction of the P=O(lipid)···HO(sugar) interactions resulted most probably from the competition with HO(sugar)···HO(sugar) H-bonding. This coincides with the concentration range where a bulk sugar glass phase was clearly established, as determined by FTIR (Fig. 4) and DSC (Fig. 2) measurements.

For higher Suc/EPC mass ratios (≥1.0), increased ratios had no significant influence on the strength of the P=O···HO interactions (Fig. 5). This indicates that at higher mass ratios, the additional sugar molecules only contributed to the bulk sugar glass phase (Figs. 2 and 4) but not to interactions with the phosphate groups. Temperature did not have a significant effect on νP=Oas at any Suc/EPC mass ratio (data not shown).

Participation of C=O groups of the lipids in sugar-lipid interactions

In addition to the P=O moiety, the C=O groups in diacyl lipids are potential partners for H-bonding interactions with water or sugars (34–42). In the presence of water, the νC=O peak is shifted downfield and broadened on the downfield side, compared to the dry membranes (38,39,42), which is due to C=O···HO H-bonding interactions. In the presence of sugars, however, there is no apparent shift in the νC=O band in dry membranes (20,35), although evidence for H-bonding of disaccharides to the C=O groups has been obtained for hydrated systems both experimentally (37) and from molecular dynamics simulations (40,41).

There is evidence in the literature that the main νC=O peak can be decomposed into at least two components, contributing the νC=O vibrational modes of the H-bonded and nonbonded (free) conformers of the C=O group (34,43). The higher wavenumber band component (1740–1742 cm^{-1}) has been assigned to the νC=O of free C=O groups ($\nu C{=}O_{free}$), whereas the lower wavenumber component (≈1728 cm^{-1}) has been attributed to the νC=O vibration of H-bonded conformers ($\nu C{=}O_{bond}$). In addition, conformational changes in the headgroups may contribute to the observed differences in the C=O spectra.

To investigate H-bonding interactions between the disaccharide and the C=O lipid groups, deconvolution of the main νC=O band (located at ≈1739 cm^{-1}) into two components and curve fitting were performed. Fig. 6 shows the result for pure air-dried EPC (Fig. 6 *A*) and for a sample containing Suc and EPC at a mass ratio of 2.0 (Fig. 6 *B*). The two band components $\nu C{=}O_{free}$ (1740 cm^{-1}) and $\nu C{=}O_{bond}$ (1728 cm^{-1}) changed their relative area and intensity in the presence of the sugar. Assuming that the relative area of a band component is proportional to the respective conformer population, it can be concluded that the populations of

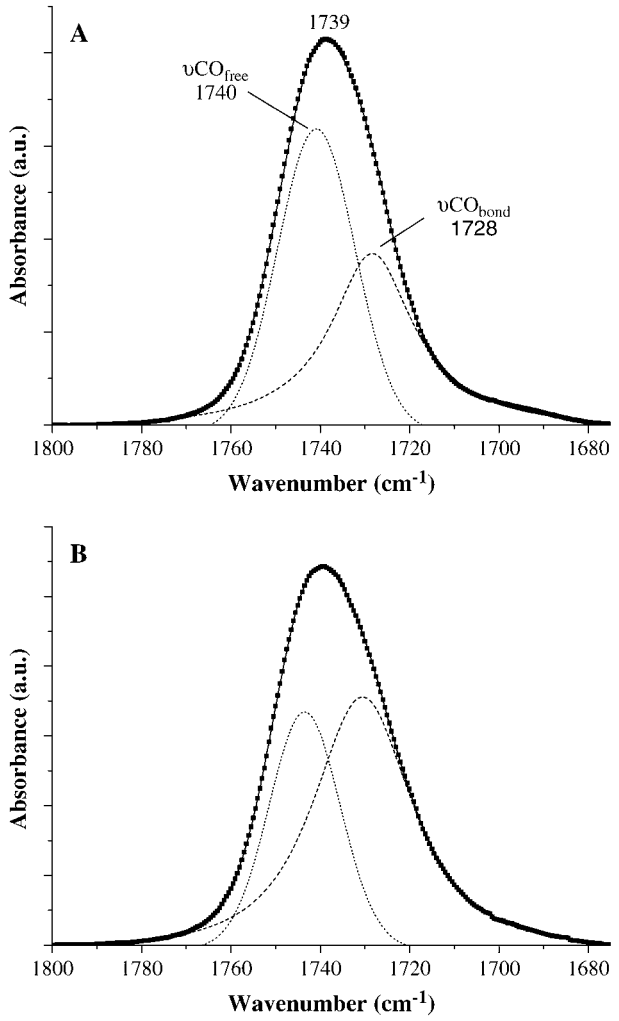

FIGURE 6 Infrared spectra in the carbonyl stretching region of (*A*) pure dry EPC liposomes and (*B*) dry EPC liposomes and Suc at a Suc/EPC mass ratio of 2.0. The peaks were deconvoluted and fitted into two band components corresponding to $\nu C{=}O_{free}$ (*short dashes*, upfield peak, no H-bonding) and $\nu C{=}O_{bond}$ (*long dashes*, lowfield peak, H-bonded). The solid curve comprises both the measured and the fitted absorbance curves. The correlation coefficients for the fitted curves were always higher than 0.999. Peak wavenumbers are indicated in panel *A*.

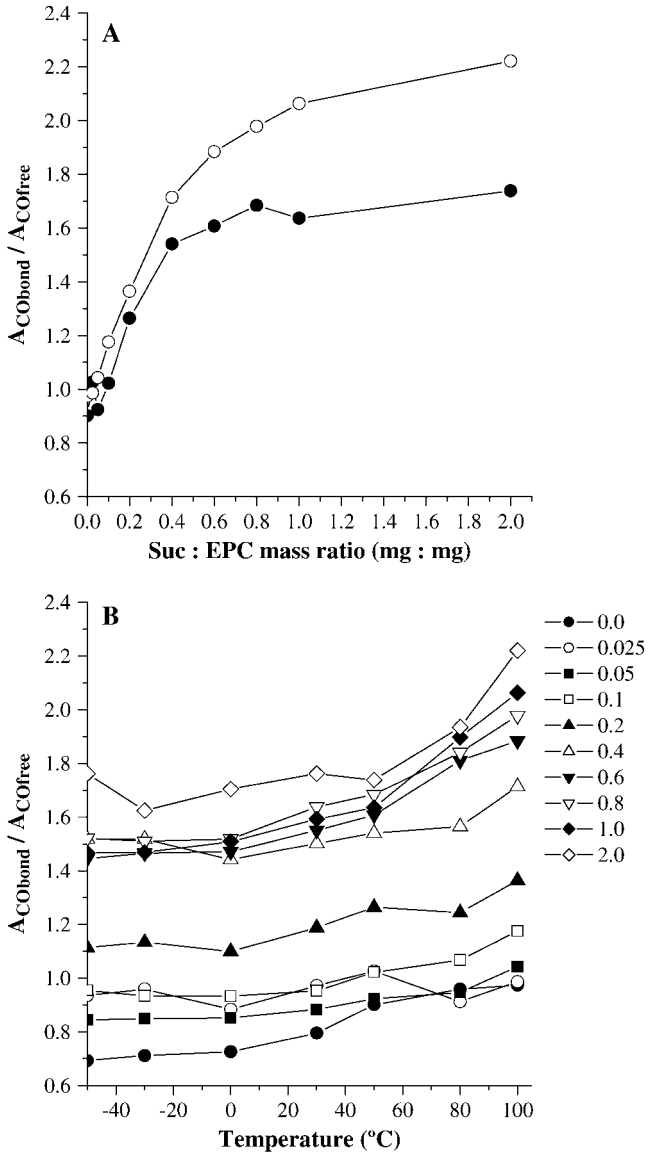

FIGURE 7 Ratio $A_{C=Obond}/A_{C=Ofree}$ (fitted peak areas of the two $\nu C{=}O$ band components identified in Fig. 6), (*A*) as a function of the Suc/EPC mass ratio of dry samples at 50°C (*solid symbols*) and 100°C (*open symbols*), or (*B*) as a function of temperature for the different Suc/EPC mass ratios indicated.

$C{=}O_{free}$ and $C{=}O_{bond}$ conformers change upon addition of Suc. The ratio $A_{C=Obond}/A_{C=Ofree}$, with $A_{C=Obond}$ and $A_{C=Ofree}$ the respective fitted peak areas, was 0.9 and 1.7 for pure EPC and Suc/EPC at a mass ratio of 2.0, respectively.

Fig. 7 *A* shows the ratio of $A_{C=Obond}/A_{C=Ofree}$ as a function of the Suc/EPC mass ratio at 50 and 100°C. At higher mass ratios $A_{C=Obond}/A_{C=Ofree}$ increased, indicating that the relative size of the population of $C{=}O_{bond}$ conformers increased. This was particularly pronounced at mass ratios between 0.1 and 0.4. At Suc/EPC mass ratios $\geq$1.0, $A_{C=Obond}/A_{C=Ofree}$ changed very little. As discussed above for the P=O···HO interaction, the higher mass ratios contribute only to the bulk sugar glass phase but not to lipid-sugar interactions.

There was an increase in $A_{C=Obond}/A_{C=Ofree}$ with temperature, especially at high Suc/EPC mass ratios (Fig. 7 *B*), indicating an increase in the $C{=}O_{bond}$ conformer population with temperature. This, however, was not correlated with T_m, although the accompanying lamellar extension might be expected to facilitate the access of sugar molecules to the interfacial region of the bilayer. The steepest increase in $A_{C=Obond}/A_{C=Ofree}$ was observed above ~60°C at Suc/EPC ratios above 0.4. This may indicate that the melting of the bulk sugar glass present in these samples (compare Figs. 2 and 4) allows additional lamellar extension of the already

fluid bilayers, thus allowing deeper penetration of Suc into the interfacial region of the membrane.

Participation of choline groups of EPC in sugar-lipid interacions

Fig. 8 shows the region of the $\nu CN(C\text{-}H_3)_3as$ vibration between 3000 and 3060 cm^{-1} of dry EPC liposomes at different Suc/EPC mass ratios. It is a weak band and its clear resolution is difficult because of the overlapping νCH_2 bands immediately downfield. Despite this, the $\nu CN(C\text{-}H_3)_3$ as band, with the peak maximum at 3011 cm^{-1}, is clearly visible. It has a shoulder on the highfield side at ~3031 cm^{-1}. With increasing mass ratios this shoulder moved gradually to higher wavenumbers, and at a Suc/EPC mass ratio of 0.6 it appeared as a separate peak at 3043 cm^{-1}. At higher mass ratios this peak was no longer resolved, due to spectral overlap with the νOH from the sugar (compare Fig. 4).

The influence of sugar molecules on the choline moiety was further supported by the analysis of the $\nu C\text{-}N(CH_3)_3as$ band at ~968 cm^{-1}. Fig. 9 *A* shows the $\nu C\text{-}N(CH_3)_3$ as peak position as a function of the Suc/EPC mass ratio at 50°C and

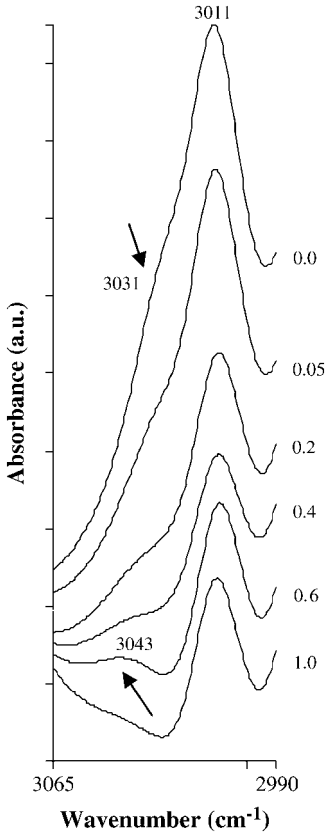

FIGURE 8 Infrared spectra in the asymmetric stretching region of the choline methyl group ($\nu CN(C\text{-}H_3)_3as$) of dry EPC liposomes in presence of sucrose at different Suc/EPC mass ratios. All spectra were recorded at 50°C.

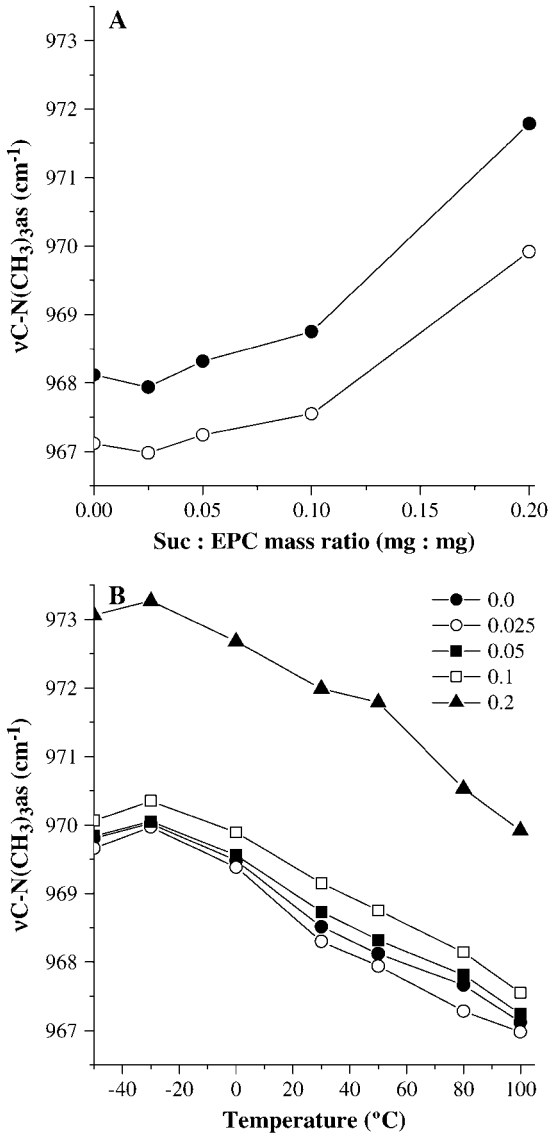

FIGURE 9 Wavenumber of the asymmetric stretching of the choline C-N bond vibrational mode ($\nu C\text{-}N(CH_3)_3as$) (*A*) as a function of the Suc/EPC mass ratio of dry samples at 50°C (*solid symbols*) and 100°C (*open symbols*); (*B*) as a function of temperature for the different Suc/EPC mass ratios indicated.

100°C. Due to overlap with bands from the sugar, the analysis of the $\nu C\text{-}N(CH_3)_3as$ band was only possible for mass ratios ≤0.2. With increasing mass ratios an upfield shift of ~4 cm^{-1} in the $\nu C\text{-}N(CH_3)_3as$ band was observed.

At all Suc/EPC mass ratios, $\nu C\text{-}N(CH_3)_3as$ was shifted to lower wavenumbers with increasing temperature (Fig. 9 *B*). From −50°C to 100°C the band position was shifted downfield by ~3 cm^{-1}, indicating that the CH···OH interactions became weaker. In fact, a downshift with increasing temperature was also apparent for the higher wavenumber component band of the $\nu CN(C\text{-}H_3)_3as$ vibration described above (data not shown).

DISCUSSION

Comparison of sugar-lipid and water-lipid interactions

It is clear from the results presented above that Suc interacts with the polar P=O and C=O groups of the lipid and also with the methyl groups from the choline moiety. Qualitatively similar interactions have also been detected between water and lipids (36,38,42,44–46). This section of the discussion will explore the similarities and differences between sugar-lipid and water-lipid interactions in more detail. The following sections will then focus on the functional significance of these interactions for the depression of T_m and membrane stabilization.

Two vibrational modes of the choline group were analyzed in this study, νCN(C-H_3)$_3$as and νC-N(CH_3)$_3$as, and both showed an upshift in the presence of sugar. νCN(C-H_3)$_3$as showed an upshift of the upfield component of its vibration of up to 12 cm^{-1} with increasing Suc/EPC mass ratios from 0.0 to 0.6. An analysis of higher mass ratios was not possible, due to an overlap of this band component with the νOH band from Suc. However, a comparison with the effect of water (36,44,45) shows that the behavior of the band is almost identical in both cases.

The maximum response of the νC-N(CH_3)$_3$as band in the presence of Suc was only ~4 cm^{-1}, and this is equal to the shift observed in EPC liposomes after rehydration (38). This indicates that the response obtained with Suc and EPC at a mass ratio of 0.2 may correspond to the maximum effect that can be induced in this vibration. Again, an analysis of higher mass ratios was not possible, due to spectral overlap with a band from Suc.

These results are in agreement with previous FTIR and Raman studies on phosphatidylcholines at different levels of hydration (36,44,45), or in the presence of different sugars at a sugar/lipid mass ratio of 2.0 (35). After deconvolution and fitting of the νCN(C-H_3)$_3$as band, an upshift of the upfield component was observed under both conditions (35,36). Also, a strong upshift of the νCH vibration band was found in the presence of water for both methylphosphocholine, a chain-depleted model compound, and headgroup-deuterated dimyristoylphosphatidylcholine (44).

These observations have been interpreted as reflecting the occurrence of conformational changes in choline, because of intercalation of water or sugar molecules between the choline and phosphate groups due to H-bonding to P=O or because of conformational transitions due to hydrocarbon chain melting (35,36,45). Ab initio calculations (44,47), however, indicated the existence of water binding to choline methyl groups. In fact, despite the apolar nature of these groups, they can act as donor sites in H-bonding, since they are substantially acidified by the electron-withdrawing influence of N^+. In contrast to classical H-bonds, the establishment of this C-H···OH-bonding interaction leads to a shortening of the C-H bonds, resulting in an increase in the force constant and in an upshift of the νCN(C-H_3)$_3$as vibration. H-bonds of the type C-H···O are known to be weak and have been designated as improper, blue-shifting H-bonds (44,48). Of course, contributions from conformational changes to the effects observed in the FTIR spectra cannot be excluded.

Collectively, however, these studies demonstrate the existence of direct water and sugar binding to lipid headgroups via CH···OH H-bonding. Our data indicate that the H-bonding interactions between the choline moiety and the disaccharide are of similar strength as the corresponding interactions of the lipid with water.

The presence of Suc did not lead to a shift of the νC=O band in the dry lipid, in agreement with previous observations (20). However, through deconvolution of this band (Fig. 6) we were able to quantify the contributions of H-bonded and free C=O groups in the membranes to this vibrational band. For the pure lipid, the population of free C=O groups was larger than the population of C=O groups involved in interactions. These interactions probably involve residual water molecules and also choline groups of other lipid molecules, which may interact through charge-pair interactions (49). In the presence of Suc up to a mass ratio of 1.0, the C=O···HO interactions increased, as indicated by an increase in the ratio of C=O_{bond} to C=O_{free} conformers (Fig. 7). An influence of conformational changes in the headgroups may of course also contribute to the observed differences in the C=O spectra. However, major conformational changes would be expected to take place in the concentration range where binding of Suc to the P=O groups is increasing most. From the fact that changes in νC=O mainly occurred at higher Suc/EPC mass ratios (see below), we conclude that at least the majority of these changes are due to H-bonding interactions between C=O groups and sugar molecules.

Interestingly, the ratio of C=O_{bond} to C=O_{free} could be further increased by heating the samples through the T_g of Suc, indicating that the bulk glass phase limits the expansion of the fluid bilayer (16) and thereby the penetration of the sugar into the membrane. During hydration of dry phosphatidylcholine films, νC=O was shifted downfield by as much as 5 cm^{-1} (38,39,50). Based on this, it is obvious that the effect of Suc on the C=O groups is much less pronounced than the effect of water. The data indicate that the larger size of the sugar molecules, compared with water, limits the penetration of sugar into the lipid bilayer and the interaction with the C=O groups.

H-bonding of Suc and water to the P=O groups can be quantitatively compared by comparing the effects of different Suc/EPC mass ratios on νP=Oas (Fig. 5) with the effects of different H_2O/1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) mass ratios extracted from published data (35,36). Fig. 10 shows that for mass ratios <0.2, νP=Oas decreases at a similar rate with increasing mass ratios of both additives. However, at mass ratios >0.2, νP=Oas continues to decrease steeply for the partially hydrated membranes, reaching a value of 1223 cm^{-1} for a mass ratio of 0.75 (fully

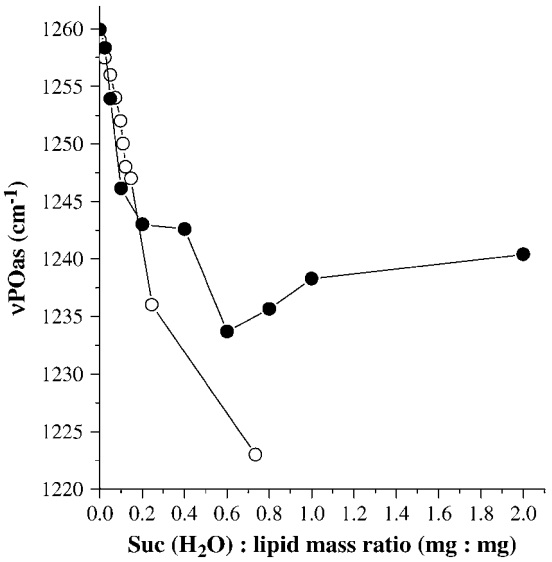

FIGURE 10 Wavenumber of the asymmetric P=O stretching vibration as a function of the Suc/EPC mass ratio for dry EPC liposomes (*solid symbols*) or as function of the H_20/DPPC mass ratio for DPPC liposomes (*open symbols*). Published data (35,36) for the hydrated DPPC samples were used.

hydrated), whereas for dry Suc/EPC, νP=Oas reaches a minimum value of 1234 cm^{-1} at a mass ratio of 0.6.

This shows that the extent of H-bonding between Suc and the P=O groups is much smaller than that achieved by H_2O. We assume that due to the larger size of the disaccharide, steric effects are limiting the strength of the interactions. This cannot be overcome by the presence of additional sugar, which only contributes to the bulk sugar glass phase. However, it should be mentioned that the hydration effect on νP=Oas may not be exclusively due to direct water binding to the phosphate. A mass ratio of H_2O/lipid of 0.75 corresponds to ~30 molecules of H_2O per molecule of DPPC, whereas the first solvation shell around the phosphate contains only approximately four molecules of water (40). Cooperative effects, conformational changes of the headgroups, and alterations in molecular packing (35) could also contribute to the increasing strength of P=O···HO(water) at high hydration levels.

In conclusion, our data show that although the H-bonding interactions between Suc and lipid are qualitatively very similar to the interactions between water and lipid, the sugar establishes weaker interactions with the C=O and P=O groups than water. Only at the level of the choline groups is the average strength of the interactions similar.

The Suc/EPC mass ratio required to induce the maximum increase in the strength of P=O···HO interactions was 0.6. This was lower than the ratio required to maximize the interaction of Suc with the C=O groups (1.0). Also, the largest variation of νP=Oas was observed for a mass ratio range 0.0–0.1, whereas it was 0.1–0.4 for νC=O and 0.1–0.2 for νC-N$(CH_3)_3$as. We therefore expect the sugar to bind to the lipid groups in the following order: phosphate, methyl choline, and finally carbonyl. This sequence is in accordance with ab initio calculated energies of interaction for one water molecule binding to the charged phosphate oxygen (15.67 kcal mol^{-1}), to tetramethylammonium (9.82 kcal mol^{-1}), and to the ester carbonyl (4.48 kcal mol^{-1}) (36,47).

Effects of H-bonding interactions on T_m

Fig. 11 shows the dependence of T_m on νP=Oas for dry membranes in the presence of different Suc/EPC mass ratios. The T_m values were determined by FTIR (Fig. 1). Since we found by DSC (Fig. 2) that at least two lipid domains with different T_m values were present in samples corresponding to mass ratios of 0.05 and 0.1, these data were not included in the graph. It is clear from Fig. 11 that increased H-bonding of Suc to the lipid P=O (lower νP=Oas) leads to lower T_m values. However, this relation is far from linearity. In fact, an exponential curve could be fitted to the data with a high correlation coefficient. This indicates that the first sugar molecules that bind to the lipid have the strongest effect on T_m. T_m reaches a plateau at ~−23°C, although the maximum strength of the H-bonding is not reached yet. The further increase in H-bonding at higher Suc/EPC mass ratios, however, has no additional influence on T_m.

DSC measurements (Fig. 2) showed that a Suc/EPC mass ratio of at least 0.2 was necessary to completely depress T_m in dry membranes. At lower mass ratios, two phase transitions were observed, one occurring at a temperature characteristic of samples at high mass ratios, whereas the other occurred at a temperature similar to T_m in the absence of sugar. This suggests that at Suc/EPC mass ratios between 0.025 and 0.1 (molar ratios between 0.05 and 0.2), a sig-

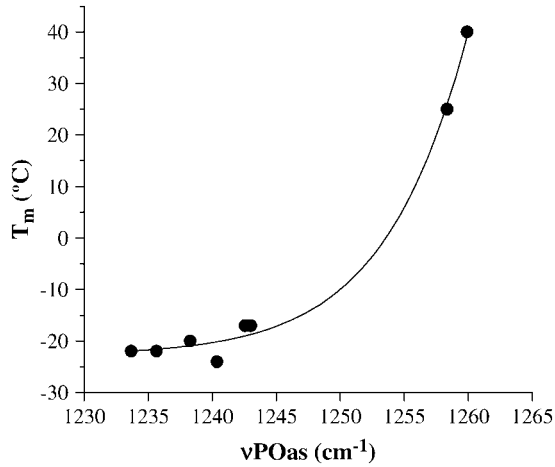

FIGURE 11 Correlation between the position of the νP=Oas peak and T_m for dry samples with different Suc/EPC mass ratios. T_m was determined from the FTIR lipid melting curves (Fig. 1). The wavenumber of νP=Oas was determined from spectra recorded at 50°C. An exponential curve was fitted to the data with a correlation coefficient $r = 0.9946$.

nificant fraction of EPC molecules had no interaction with the sugar. It is interesting to note that low amounts of Suc did not result in a gradual decrease in T_m for all lipids, but rather in the formation of membrane domains with either low or high T_m. These domains persist even after heating the samples through T_m and T_g, although the available sugar molecules maximized their interactions with the phospholipid headgroups in the fluid phase (13,51), increasing the relative amount of the lipid domains with a lower T_m. The absence of a detectable bulk sugar glass phase at these low mass ratios also supports our conclusion that the Suc molecules are quantitatively bound to the lipids until saturation of the binding sites is reached.

Although water shows stronger H-bonding to the lipid P=O groups than sugars (Fig. 10), sugars are able to depress the T_m of phospholipids considerably below the T_m at full hydration (our data and (27,29,30,51)). Obviously, there is a lack of correlation between H-bonding and T_m. We advance the following hypothesis to account for this discrepancy. Each Suc molecule has a theoretical capacity for eight H-bonds, whereas H_2O has the capacity for only two, when they are acting as proton donors. Molecular dynamics simulations showed that the most probable number of H-bonds that a Tre molecule can establish with a membrane is about six or seven (41). Also, simulations showed that the number of lipid molecules interacting with a common Suc or Tre molecule is often three or higher (40,52). This capability of a disaccharide to bridge several lipid molecules was shown to increase under conditions of high temperature stress and could be a key factor for membrane stabilization (52). In addition, the simulations show that the lifetime of H-bonds between sugar and lipid is considerably longer than the lifetime of water-lipid bonds (41). These facts lead to the conclusion that Suc is more stably bound to a membrane than water. Consequently, a sugar molecule will be harder to expel from a membrane during cooling and it will more readily enter a membrane during warming than water, resulting in a lower T_m. Therefore, the degree of H-bonding is only one factor that determines T_m. Others, such as steric factors and specific binding energies, will also contribute to the ability of a specific solute to depress T_m in a dry membrane.

The role of H-bonding interactions in membrane preservation during drying

The protective effect of sugars for dry membranes, which is usually determined as the retention of a soluble dye in membrane vesicles after drying and rehydration, is generally attributed to the ability of sugars to depress T_m and the fusion of membrane vesicles (1,2). In fact, fusion can lead to a transient leakiness of the participating vesicles (53,54) and a linear correlation between solute leakage and membrane fusion has been observed in drying experiments with liposomes in the presence of various solutes (20–22). It has, however, been shown that the prevention of fusion is not

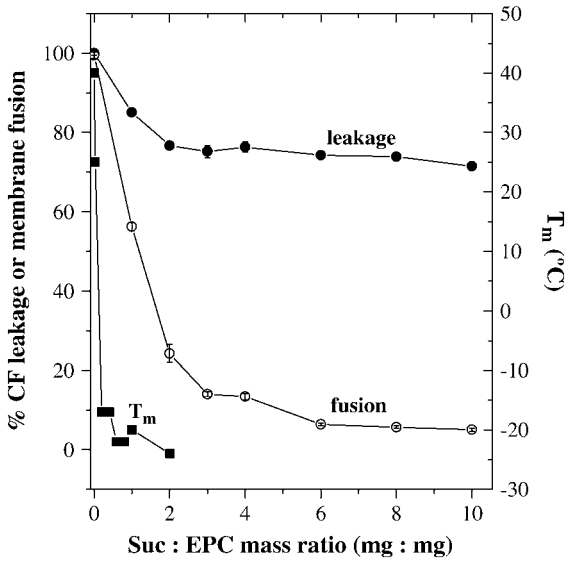

FIGURE 12 Protection of large unilamellar EPC liposomes from damage during drying by different Suc/EPC mass ratios. Leakage of CF from the vesicles and membrane fusion were determined after air drying and rehydration. T_m was determined from the FTIR lipid melting curves (Fig. 1).

sufficient to prevent leakage, since under all investigated experimental conditions a much higher sugar/lipid mass ratio was necessary to prevent leakage than to prevent fusion (18–22). From this fact, it has been concluded that the depression of T_m requires a higher sugar/lipid mass ratio than the depression of fusion (18,19).

In Fig. 12 the percentage of membrane leakage, measured by the amount of the soluble marker carboxyfluorescein (CF) released from the vesicles after rehydration, the percentage of membrane fusion, and membrane T_m, are presented as a function of the Suc/EPC mass ratio. The leakage and fusion data are virtually indistinguishable from previously published data (20,22), indicating the high reproducibility of the observed effects. It is obvious from Fig. 12 that the depression of T_m in dry lipids by ~60°C only required an ~10-fold lower Suc/EPC mass ratio than the depression of fusion to below 20%. Leakage was only reduced by ~25% in this experiment. The degree of protection against CF leakage can be increased by loading the liposomes with sugar before drying and also by drying the liposomes at a faster rate or by freeze drying. Nevertheless, it will always require more sugar to reduce leakage than to reduce fusion. We would like to forward the following hypothesis to explain this unexpected observation. The high degree of leakage could be due to a kinetic phenomenon. During drying, samples are transiently exposed to a decrease in water content and a concomitant increase in solute concentration. During this time, conditions may exist that are highly destabilizing and that are not sufficiently mirrored by our measurements of the dry endpoint. Careful measurements of T_m at different stages of the drying process and of the dependence of leakage on

drying velocity will be necessary to test this hypothesis. The knowledge gained from such experiments might help in devising experimental protocols for lyopreservation that might require much less sugar for complete liposome stabilization.

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