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Protection by sugars against phase transition-induced leak in hydrated dimyristoylphosphatidylcholine liposomes

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The disaccharides trehalose and sucrose have small effects on temperature and enthalpy of the pre- and main phase transition in hydrated DMPC bilayers. In contrast, these sugars cause a considerable retention of carboxyfluorescein when large unilamellar vesicles of DMPC are heated through the main transition. This effect is sugar specific, as the monosaccharides glucose and fructose are less effective and ethyleneglycol has no effect at all.

Interest in sugar–membrane interactions has been greatly stimulated by studies on the role of trehalose in anhydrobiosis [1–3] and the discovery that sugars can stabilise the permeability barrier of membranes during drying and freezing [4–6]. Dehydration and cooling can cause phase changes in the lipid part of membranes, which affect cellular functioning [7,8]. These phase changes can be either from a liquid-crystalline to a gel state organisation or are of a polymorphic nature, i.e. bilayer → non-bilayer organisation. The general hypothesis is that sugars can interfere with these phase changes and thereby exert their membrane stabilising action. The hypothesis is largely based on studies on the influence of sugars on the phase properties of lipids in model membrane systems [5,9–12]. One particular aspect of the phase behaviour of lipids in model and biological membranes is the increased membrane permeability under conditions of co-existence of gel and liquid-crystalline domains [13–16]. Dehydration and cooling will favour such membrane conditions, and therefore it is conceivable that sugars exert their protective action (partially) by preventing such harmful permeability changes. To analyse this possibility, we studied the influence of different carbohydrates on the leak from hydrated phospholipid vesicles at the phase transition temperature.

1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) was either synthesised and purified according to standard procedures [17,18] or obtained from Avanti Polar-Lipids (Birmingham, AL, U.S.A.). The lipid of both sources was judged to be more than 99% pure by HPTLC and GLC and gave identical results for the transition temperatures and enthalpies as determined with DSC. The pre-transition of the Avanti DMPC was, however, twice as broad. Furthermore, both phospholipids showed a similar leak profile though vesicles prepared from Avanti DMPC were about 1.7-times less leaky above the transition temperature, for which we have no explanation. Trehalose dihydrate, sucrose and fructose were from Sigma (St. Louis, MO, U.S.A.). Glucose and ethyleneglycol (EG) were from Merck (Darmstadt, F.R.G.). All carbohydrates were of the highest quality available and were used without further purification. 6-Carboxyfluorescein (Eastman Kodak Co., Rochester, NY, U.S.A.) was purified by active carbon treatment, recrystallisation from water/ethanol (2:1, v/v) and Sephadex LH-20 (Pharmacia) column chromatography [19].

For differential scanning calorimetry, samples of 10 μmol DMPC in chloroform were dried and stored overnight under high vacuum. The samples were then hydrated at a temperature of 40 °C with 1 ml buffer, containing the desired concentration of either sucrose, trehalose or EG and 100 mM NaCl, 25 mM Pipes and

Abbreviations: CF, 6-carboxyfluorescein; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DSC, differential scanning calorimetry; EG, ethyleneglycol; MLV, multilamellar vesicle; LUVET, large unilamellar vesicle produced by extrusion techniques; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid.

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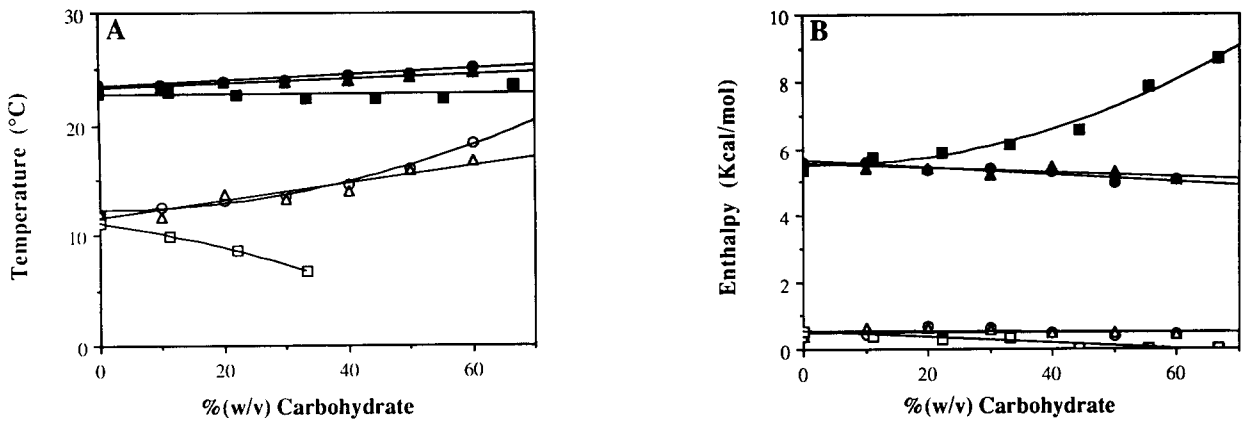

Fig. 1. Effect of sucrose (○, ●), trehalose (△, ▲) and ethyleneglycol (□, ■) on the onset transition temperatures (A) and the enthalpies (B) of the pre-transition (open symbols) and the gel to liquid-crystalline transition (closed symbols) of aqueous dispersions of DPMC.

40 μM EDTA (pH 7.4). Subsequently, the samples were frozen and thawed five times to get a better encapsulation of the carbohydrates [20] and the MLV were concentrated by centrifugation for 20 minutes at 4°C and 18000 × *g*. Calorimetric scans were run on a Perkin-Elmer DSC 4 differential scanning calorimeter at a scanning rate of 2 C°/min. Data were collected and handled on the Perkin-Elmer 3600 data Station, using Perkin-Elmer software.

For the permeability assay, MLV of DMPC were prepared by hydration with 0.8 ml buffer in the same way as for the DSC experiments. The buffer contained, apart from the desired concentration carbohydrate, 25 mM NaCl, 10 mM Hepes, 1 mM EDTA, 0.2 mM NaN_3 and 50 mM CF at pH 7.4. From these MLV, large unilamellar vesicles were prepared at room temperature by extrusion [21] through two (stacked) polycarbonate filters (Nuclepore; 0.4 μm pore size). The resulting $LUVET_{400}$ were stored overnight at 4°C and non-enclosed CF was removed at the same temperature by gel filtration on a Sephadex G-75 column (0.7 × 20 cm; Pharmacia). The column buffer was the same as the buffer used for hydration except for the replacement of the 50 mM CF by an additional 75 mM NaCl. For further experiments, the vesicles were diluted to 2.5 ml, divided into aliquots of 150 μl and stored on ice. From here, two different protocols were used. In the first method, the samples were incubated for 5 minutes at temperatures ranging from 0 to 60°C. For all incubations, the final temperature was reached in about 100 s and the presence of carbohydrates had no effect on the time dependence of the sample temperature. After incubation, 25 μl was as quickly as possible injected into the fluorescence cuvette with pipet tips that were kept at the same incubation temperature. The cuvette contained 2 ml buffer that was the same as the column buffer in case of the experiments with EG and an iso-osmotic concentration of NaCl in case of the sugars. The temperature of the cuvette was 10 or 40°C. After injecting, the initial fluorescence was measured on a SPF 500C spectrofluorometer (SLM instruments Inc., Urbana, IL, U.S.A.). The second protocol was used to evaluate the effect of increased permeability above T_c. The 5 min incubation period was replaced by direct injection of samples from ice into the cuvette with the sugar solution of interest and the fluorescence was followed for longer times. The difference in fluorescence, immediately after injecting beneath and above the phase transition temperature (i.e., 10 and 40°C), should reflect the leakage due to the phase transition and the subsequent gradual increase at 40°C is caused by the passive permeability above the phase transition. Excitation was at 430 nm and the increased CF fluorescence due to a dequenching of the released CF [22] was measured at an emission wavelength of 513 nm. The total amount of enclosed CF was determined by lysing the vesicles with 20 μl 10% (v/v) Triton X-100. The CF release is simply expressed as F/F_{Triton}. No correction was made for the low level (< 6% of intensity after lysis) of fluorescence immediately after gel filtration.

To prevent osmotic stress on the vesicles, the osmolality of all buffers was verified with a cryoscopic osmometer (Gonotec, osmomat 030). For some experiments, the effect of the medium viscosity was tested and relative viscosities were determined with a type 200000 Bohlin VOR rheometer (Bohlin Reologi AB, Sweden) at 20°C. Phospholipids were quantified after perchloric acid destruction using the method of Fiske-SubbaRow [23].

As test lipid, DMPC was chosen because model membranes made of this lipid show a well characterised leak in the phase transition [13,14,24]. To characterise the DMPC system with respect to the membrane action of sugars, we first studied the influence of the disaccharides sucrose and trehalose on the onset temperature and transition enthalpy of both the pre-transition and gel to liquid-crystalline transition of MLV in buffer. Trehalose and sucrose were chosen because both sugars

are considered natural cryoprotectants [5,25,26] and EG was included because it is often used as an artificial cryoprotectant [27,28].

Fig. 1 shows that both sugars cause a slight increase of the gel to liquid-crystalline transition temperature of less than 2C° for sugar concentrations up to 60% (w/v). The effect on the onset temperature of the pre-transition is larger and results in a shift of about 5–6 C° to higher temperatures. The influence of EG is clearly different from that of the sugars. The onset temperature of the pre-transition decreases about 4 C° in the presence of 30% (v/v) EG and at 40% (v/v) EG the pre-transition has completely disappeared. The transition temperature of the main transition is not affected at all by EG. Concerning the transition enthalpies, there is also a distinct difference between the effect of EG and the sugars. Trehalose and sucrose show at high concentrations only a small decrease for the gel to liquid-crystalline phase transition whereas addition of EG results in a significant increase from 5.6 to about 8.5 kcal/mol at a concentration of 60% (v/v). None of the carbohydrates caused obvious changes in the peak width of the main transition up to concentrations of 50% (w/v). These results are in agreement with preliminary observations made by Dr. E.A. Disalvo when visiting our laboratory in 1982. For sucrose, the same effects were reported for DMPC [29] and DPPC (dipalmitoylphosphatidylcholine) [25]. An increase of the main transition enthalpy, in the presence of 50% (v/v) EG, was also reported for dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatidic acid (DOPA) although for these lipids an effect on the transition temperature was noticed [30]. DMPC was therefore considered to be a good representative for studying sugar-lipid interaction.

Fig. 2 confirms that DMPC model membranes show a pronounced loss of barrier function at the main transition temperature. In a narrow temperature range, corresponding to the main transition, complete release of CF occurs in 5 min. At higher temperatures almost complete CF release (≈ 88%) occurs which could in principle be due to two phenomena: (i) leak due to transient passage through the phase transition and (ii) isothermal leak at the incubation temperature above T_c. The relative importance of each phenomenon was evaluated by following the fluorescence increase in time according to the second permeability protocol. After injection, only 7–9% of the closed CF was released in less than 5 s in which the phase transition is passed very rapidly. An additional passive leak of 10–12% was measured during incubation for another 5 min. Therefore, the major part of the large CF release above T_c as presented in Fig. 2 can be ascribed to transient passage through the phase transition.

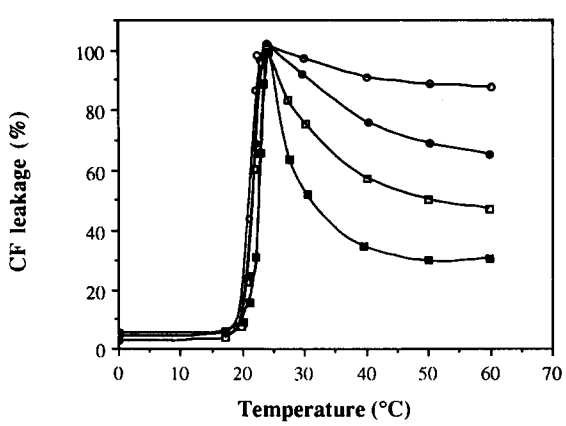

Fig. 2. Leakage of trapped carboxyfluorescein (CF) from DMPC $LUVET_{400}$ after incubation of the samples for 5 min at the indicated temperatures in the presence of 0 M (○), 0.25 M (●), 0.5 M (□) and 1.0 M (■) trehalose. The temperature of the cuvette in which the samples were injected was 10 °C.

The effect of increasing concentrations trehalose on the CF leakage is represented in Fig. 2 as a function of the incubation temperature. Two effects can be noticed. Firstly, in accordance with the DSC data, there is a small shift of the phase transition towards higher temperatures upon increasing the trehalose concentration. Secondly, and more interestingly, the presence of trehalose causes an effective protection against phase transition induced leakage.

The $LUVET_{400}$ that were incubated above T_c and subsequently measured at 10 °C passed the phase transition twice. To determine the effect of the second passage the experiments were repeated by injecting the 25 μl samples at a cuvette temperature of 40 °C. The differences in leak at both temperatures were less than 5%, demonstrating that the main leak is caused by passage of the phase transition during warming up of the 150 μl samples.

The leakage assay was repeated for 1.0 M solutions of sucrose, fructose, glucose and EG. All carbohydrates showed similar shaped leak curves though the extent of leakage above the transition temperature differed for the various carbohydrates. Fig. 3 compares the CF leakage at an incubation temperature of 50 °C. Sucrose is equally effective as trehalose in reducing the phase transition induced CF release. The monosaccharides are less capable whereas EG is virtually inactive. CF leak is at most 3.5% increased when the samples with sugars are incubated at 50 °C and measured below T_c. The samples with EG or without carbohydrate show, however, a 4–5% smaller leakage when the phase transition is passed a second time, for which we have no explanation.

The osmolarity of polyalcohol solutions is markedly different from the molarity due to strong hydration. Therefore, it was verified whether the difference in protection for various carbohydrates was a consequence of differences in the osmolarity by performing experiments with glucose, fructose and EG with the same

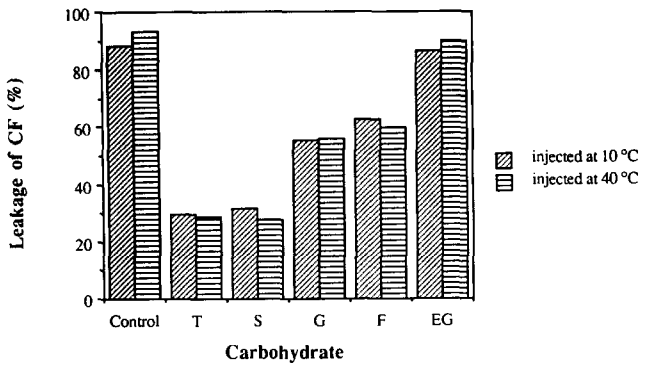

Fig. 3. Total leakage of trapped carboxyfluorescein (CF) from DMPC $LUVET_{400}$ after incubation of the samples for 5 min at 50 °C in the presence of 1 M trehalose (T), sucrose (S), fructose (F), glucose (G) and ethyleneglycol (EG). Samples were both injected at a cuvette temperature of 10 °C (left bars) and 40 °C (right bars).

osmolarity as the 1 M disaccharide solutions (i.e., 1.5 M monosaccharides; 1.66 M EG). At iso-osmotic concentrations, the monosaccharides were still less effective (60–70%) than the disaccharide solutions and EG had again no effect (data not shown). Furthermore, it was verified whether differences in the medium viscosity of the various polyalcohol solutions could explain the differences in CF release. To test this possibility, EG was chosen because of the relatively low viscosity of solutions of this polyalcohol. When an EG solution, with the same viscosity as 1 M disaccharide (i.e., 48% (v/v) EG), was incubated above T_c there was no retention of CF at all. Therefore, it seems obvious that the retention of CF is not a result of the viscosity of the solution but a specific property of the sugars. In conclusion, sugars protect the permeability barrier at the phase transition in contrast to EG which is currently used as a cryoprotectant.

Both the DSC and leak measurements show that trehalose and sucrose cause a small increase of the main transition temperature of hydrated DMPC. This effect is different from the effect of sugars on dehydrated lipid bilayers for which large decreases of T_c were observed [5,31–34]. Several possible mechanisms should be considered. Firstly, it is possible that sugar–lipid interactions, as is the case in dehydrated systems, are responsible for the small shift of T_c, for instance by replacing part of the lipid bound water molecules. Secondly, such a small increase is not necessarily caused by molecular interactions but can also be a consequence of osmotic dehydration due to a preferential exclusion of the sugars from the surface as has been shown for proteins [35]. The larger shift of the pre-transition probably indicates more direct sugar–lipid interactions at the lipid/water interphase. Sugar–lipid interactions are also reflected in ^{2}H-NMR experiments which show that changes in the orientation of the polar headgroups, in the presence of trehalose, are accompanied by an increase of the order parameter of the acyl chains of hydrated lipids [36]. Change in the nature of the phase transition is probably also the cause of the decrease in leakage in the presence of sugars.

As an explanation for the enhanced permeability during phase separation two mechanisms have been proposed: (i) mismatch in molecular packing at the interphases of the fluid and ordered domains [24,37] and (ii) altered lateral compressibility near the phase transition [13,38,39]. It is plausible that the effects of these mechanisms become less important when the difference in order in the paraffin chains below and above the phase transition decreases, for instance as a result of a more ordered liquid-crystalline state. Such a suggestion is consistent with the finding that the ion permeability at the phase transition in DPPC liposomes is much smaller than for the shorter chained DMPC [13]. This might explain the striking difference between the effects of the sugars and EG, especially because it also has been shown that EG causes an acyl chain disordering [36,40].

Crowe and co-workers suggested that the mechanism of protection by trehalose during dehydration is based on lowering the main transition temperature of the dry membranes, thus preventing a gel to liquid-crystalline phase transition upon rehydration by keeping the lipids in a fluid state [5]. This mechanism, however, is not applicable to hydrated DMPC bilayers and since prevention of passing the phase transition is no prerequisite for the preservation of the membrane permeability barrier, this mechanism may not hold for dry membranes as well.

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