Reconciliation of opposing views on membrane–sugar interactions

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It is well established that small sugars exert different types of stabilization of biomembranes both in vivo and in vitro. However, the essential question of whether sugars are bound to or expelled from membrane surfaces, i.e., the sign and size of the free energy of the interaction, remains unresolved, and this prevents a molecular understanding of the stabilizing mechanism. We have used small-angle neutron scattering and thermodynamic measurements to show that sugars may be either bound or expelled depending on the concentration of sugar. At low concentration, small sugars bind quite strongly to a lipid bilayer, and the accumulation of sugar at the interface makes the membrane thinner and laterally expanded. Above ∼0.2 M the sugars gradually become expelled from the membrane surface, and this repulsive mode of interaction counteracts membrane thinning. The dual nature of sugar–membrane interactions offers a reconciliation of conflicting views in earlier reports on sugar-induced modulations of membrane properties.

membrane interface | membrane structure | preferential binding | preferential exclusion | interaction free energy

Small sugars such as the disaccharides sucrose and trehalose are among the so-called osmolytes (1) or compensatory solutes (2), which are accumulated in response to environmental stress in virtually all taxa. Their function is to act as inert regulators of the osmotic pressure, but they also optimize the physical properties of the cytosol (3) and stabilize biomolecular conformations against cold, drought, and heat (4–7). The same small carbohydrates have also proven useful in vitro as protectants or excipients for biopreservation (8). Many reports have shown that membranous structures are particularly stabilized by small sugars (4, 6, 9), but the definition of stabilization covers a wide range of biological and physical parameters. Thus, studies on intact cells have documented improved survival following exposure to heat, cold, drought, or chemical stressors (6, 10, 11). Other works have analyzed stabilization on the basis of phenomenological properties of model membranes, for example, the leakage or intermixing of probes in liposomes (12, 13). Finally, stability has been discussed with respect to rigorous physical parameters such as the structure or mechanical properties of lipid bilayers (14, 15). The current work addresses membrane dimensions and the thermodynamics of interaction with the purpose of elucidating fundamental aspects of membrane–sugar interrelationships. The different observations of sugar stabilization have sparked a large number of studies on sugars and model membranes (usually phospholipid bilayers) over the past 30 y. Investigations of fully hydrated membranes show an interesting tendency to fall into two groups with mutually conflicting conclusions. Thus, many investigations have suggested direct (favorable) interaction of sugars and the phospholipid interface (16–23), and it is obvious that such interactions could be the origin of sugar effects, for example, through interlocking of several lipids molecules that simultaneously hydrogen bond to the same disaccharide molecule (24). Other stabilizing consequences of sugar binding have been put forward, and in this paper, we collectively refer to this interpretation as the "interaction hypothesis." In contrast to this, other works have concluded that the sugars are preferentially expelled from the hydration zone and that their effect on the membrane is exerted indirectly by the local osmotic imbalance and the concomitant increase in interfacial free energy (25–30). We call this the "exclusion hypothesis."

The most unambiguous support for the interaction hypothesis is probably the lateral expansion of phospholipid monolayers, which is observed when, e.g., sucrose or trehalose is added to the aqueous subphase in a Langmuir–Blodgett trough (16–18, 31). The obvious interpretation of this is that the sugar molecules increase the area through intercalation between the lipid head groups. Other experimental approaches have reached analogous conclusions in work on lipid bilayers (19, 20, 24, 32), and Viera et al. (33) found that trehalose that was bound to a bilayer during dehydration remained associated for hours upon rehydration in buffer without sugar. Most recently, the interaction hypothesis has been supported by many molecular dynamics simulations (see, e.g., refs. 21–23 and references therein), which consistently reported membrane–sugar attraction and an associated buildup of sugar at the membrane interface. Conversely, the exclusion hypothesis is supported by a multitude of studies on the phase behavior of fully hydrated phospholipids showing that the addition of sugars and other kosmotropic solutes consistently stabilize the phase with the smallest surface area (34, 35). For example, the gel phase is stabilized over the fluid phase (25, 27), hexagonal phases are favored over lamellar phases (34, 36), and the lateral expansion associated with interdigitation is strongly disfavored (37). This ubiquitous correlation is explained by a preferential expulsion or exclusion that increases the interfacial free energy and thus promotes the stability of lipid phases with low water accessible areas. The exclusion hypothesis has been supported by some spectroscopic evidence (28), and recently both small-angle scattering studies (36, 38) and vapor pressure measurements (39) have provided direct evidence for a partial depletion of sugar in the hydration zone of lipid bilayers. Several aspects of the exclusion hypothesis were recently discussed by Lenné et al. (40) who concluded that "sugars partition away from the phospholipid headgroups, rather than inserting between the headgroups."

To address the obvious contrast between the interaction and exclusion hypotheses, we have used small-angle neutron scattering (SANS) and thermodynamic measurements and found that the interaction of membranes and small sugars indeed includes both an attractive and a repulsive component. The former leads to a buildup of sugar at the interface (in accord with the interaction hypothesis) at low sugar concentration, whereas the latter

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brings about an expulsion of sugar (as described in the exclusion hypothesis) in more concentrated samples. This unique mode of interaction is shown to make the membrane thinner and laterally expanded even if the interfacial free energy is increased. We suggest that this is the origin of earlier disagreement on sugar–membrane interactions in excess water and that this unusual interaction pattern should be considered in analyses of sugar-induced stabilization of fully hydrated membranes (whereas the issue of sugar effects on dry membranes is beyond the current scope).

Results

Data from SANS measurements were fitted with a molecular constrained analytical model for polydisperse bilayer liposomes. We used a three-shell model for the bilayer cross-section and modeled the bilayer in terms of a central hydrophobic core, containing the hydrocarbon chains, sandwiched between inner and outer hydrophilic layers, composed by the hydrophilic head groups and hydration water. This approach, including its use of molecular constraints, was pioneered by Luzatti and Husson (41), later refined (42, 43), and recently further modified and extensively applied to lipid bilayers by Kucerka et al. (44, 45). Our approach is similar in spirit to Kucerka's approach (see *SI Text*). However, as the single SANS bulk contrast does not contain sufficient information to use the detailed structural model of Kucerka et al., we have, for simplicity, used a step-function-based scattering length density profile instead of Gaussian-based function. The free parameters of the model are R, the average radius of the liposomes, σ_R/R, the relative standard deviation of the Gaussian describing the size distribution, N_{hyd}, the number of hydration water molecules per hydrophilic head group of the phospholipids, and $D_{bilayer}$, the total bilayer thickness. In addition to these model parameters, we also fitted an overall scaling factor, accounting for the product of the experimental uncertainties in the absolute scale calibration and sample concentration, respectively, and a small constant background. A more detailed description of the model is available in *SI Text*.

The central SANS parameter for the current analysis is the thickness, $D_{bilayer}$, which is plotted as a function of the sugar concentration in Fig. 1. It appears that the sugars consistently make the membranes thinner. The largest changes exceed 3 Å or about 8% of the total thickness, and the effect is quite similar for the two investigated lipids, DMPC (1,2-dimyristoyl-sn-glycero-3-phosphocholine) and DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine). Interestingly, the monosaccharide (glucose) induces only about half the thinning of the disaccharides (trehalose and sucrose) at the same weight fraction.

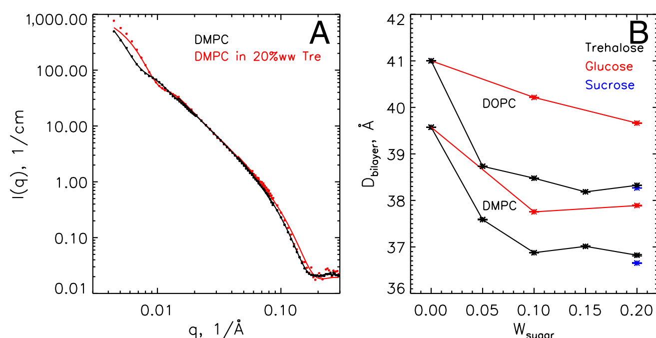

Fig. 1. Overview of the SANS results. (*A*) Examples of SANS data (points) and model fits (full lines). Black: DMPC in D_2O. Fit parameters: $R = 427$ Å, $\sigma_R/R = 28\%$, $N_{hyd} = 1$, $D_{bilayer} = 39.1$Å. Red: DMPC in D_2O with 20% trehalose. Fit parameters: $R = 295$ Å, $\sigma_R/R = 26\%$, $N_{hyd} = 1$, $D_{bilayer} = 35.7$ Å. Both samples are measured at 40 °C. The sugar-induced thinning is directly visible in the data around $q = 0.1$ Å^{-1}, where it makes the thinner bilayer (red) fall off toward zero at slightly higher q values. (*B*) The total bilayer thickness, $D_{bilayer}$, for, respectively, DOPC and DMPC as a function of the weight fraction of sugar.

The effect of sugar on the lipid volume was measured by densitometry. The specific volume, V_{spec}, of tertiary (water+sugar + lipid) samples were calculated from the resonant frequencies measured in the densitometer, and the apparent specific volume (46) of the lipid, V_{lip}, was determined (47) as $V_{lip} = \frac{[V_{spec}-(1-w_{lip})V_{solvent}]}{w_{lip}}$, where w_{lip} is the weight fraction of lipid in the tertiary samples and $V_{solvent}$ is the specific volume of the binary aqueous solvent (water + sugar, with exactly the same composition as the aqueous part of the tertiary sample). Values of V_{lip} are plotted as a function of the sugar concentration in Fig. 2.

The net affinity of sugars for the membranes (i.e., the free energy of membrane–sugar interaction) was calculated from the dialysis measurements and expressed by the so-called preferential binding parameter, Γ_3, as described in *SI Text* and Table S1. This parameter quantifies the binding (or expulsion) in mol sugar per mol lipid, and it may be positive or negative. Positive values of Γ_3 imply favorable interactions of sugar and membrane, and thus an accumulation of sugar at the membrane interface. Conversely, negative values of Γ_3 occur when water–membrane interactions are stronger than sugar–membrane interactions and it entails a partial (or "preferential") exclusion of the solute from the interfacial zone. The results in Fig. 3 show that both of these cases occur for the membrane–sugar systems investigated here. Thus, Γ_3 is positive in dilute sugar solutions but runs through a maximum and becomes negative at higher concentrations. We note that the abscissa in Fig. 3 is the free sugar concentration (measured outside the dialysis bag), whereas the abscissas in Figs. 1 and 2 are total (bound + free) concentrations. However, calculation of the bound fraction in Figs. 1 and 2 shows that this is small compared to the experimental errors, and it follows that the results in Figs. 1–3 may be compared directly and that the reported concentrations can be converted to osmolalities using activity coefficients for the binary aqueous sugar solutions.

Discussion

The understanding of sugar-induced stabilization of membranes will depend on the molecular description of sugar–membrane interactions. As illustrated in the Introduction, current views on this are divided on the question of whether sugars exert their effect through direct (attractive) interaction or indirect modulations, driven by preferential exclusion ($\Gamma_3 < 0$). The preferential binding data in Fig. 3 emphasize the importance of sugar concentration in these discussions. Thus, at low concentrations, the sugars accumulate at the membrane interface ($\Gamma_3 > 0$) in accord

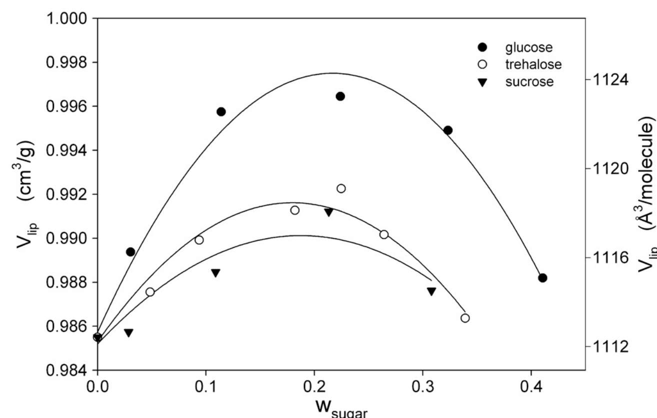

Fig. 2. Apparent volume of DMPC at 40 °C as obtained from the densitometry measurements and plotted against the weight fraction of sugar. Volumes are given in both cm^3/g (left-hand ordinate) and Å^3/molecule (right-hand ordinate).

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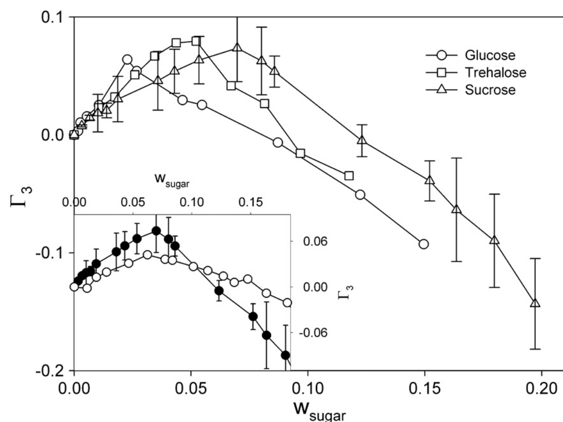

Fig. 3. Net affinity of sugars for unilamellar DMPC membranes expressed as the preferential binding parameter, Γ_3, and plotted as a function of the weight fraction of sugar. The experimental temperature was 30 °C for data in the main panel. (*Inset*) Data for sucrose at 30 °C (closed symbols) where DMPC is in the fluid phase and 18 °C (open symbols) where DMPC is in the gel phase. Error bars are ±SEM. In Table S2, results from this figure are expressed as partitioning coefficients and compared with literature data.

with the binding hypothesis. This effect reaches a maximum at about 3% (wt/wt) for glucose and about twice this concentration for the disaccharides (that is about 0.2 M in all cases). For the disaccharides the amount of bound sugar at the maximum is 0.07 mol/mol lipid; i.e., there is 1 sugar for each 14 lipids in the membrane. At higher sugar concentrations, Γ_3 decreases linearly to negative values thus signifying preferential exclusion of sugar from the membrane interface in accord with earlier reports (38, 39). These data are compiled and compared with the current results in Table S2. We interpret this nonmonotonic course of Γ_3 as the result of two independent modes of interaction. One mode contributes positively to Γ_3 (binding), whereas the other mode contributes negatively (exclusion). The positive values of Γ_3 at low sugar show that the binding mode dominates under these conditions, and this is likely to reflect sugar–head group hydrogen bonding, as identified in several computational studies (see, e.g., refs. 21–23). Recently, computational analysis of systems at low water contents (48) has suggested that this binding couples to an enlarged lateral area as also seen in this work. At higher sugar concentration, the binding mode appears to saturate and to be exceeded by the exclusion mode, which drives Γ_3 to negative values. This is in accord with the general kosmotropic nature of small sugars, which tend to exclude them from aqueous interfaces (49) and thereby affecting lipid phase behavior (25, 27, 29, 34, 37, 50). It is important to note that the two suggested modes overlap and that the (thermodynamic) function Γ_3 will quantify the sum of these contributions (and possibly other, as of yet unidentified effects). In a 5% trehalose solution, for example, where the binding mode is most pronounced, there will also be a numerically smaller negative contribution to Γ_3 from the exclusion mode. This overlapping also pertains to higher sugar concentrations where the negative Γ_3-values will be composed of a positive contribution from the bound sugars, which is exceeded by the exclusion, so that the average composition of the aqueous solvent in the hydration zone is poorer in sugar than the bulk.

Addition of sugars made the membranes thinner by as much as 8–9% for the highest concentrations (Fig. 1). The thinning produced by the disaccharides was similar both for DMPC and DOPC, whereas the monosaccharide, glucose, generated a much smaller reduction in D_{bilayer} for both lipids. In spite of the decreasing thickness, the molecular volume of DMPC exhibited a weak increase with increasing sugar concentration up to 20% sugar (Fig. 2). A detailed analysis of the resulting lateral expansion awaits comprehensive scattering studies of the sugar content in the membrane interface, but if the area per phospholipid is simply estimated as the ratio of the volume and thickness ($A = V_{\mathrm{lip}}/D_{\mathrm{bilayer}}$), we find a maximal increase in phospholipid lateral area of 5–6 Å^2 or 9% for the disaccharides (see Fig. S1). This number is estimated from the apparent volume of the lipid molecules and hydration water only. If the partitioning of sugar into the hydrophilic part of the membrane phase is included in the calculations, even higher degrees of expansion will be obtained. A more quantitative thermodynamic analysis of this can be carried out by means of the lateral equation of state that separates the intrabilayer free energy into three terms describing, respectively, the interfacial free energy, the head-group repulsion, and the repulsion resulting from hydrocarbon chain entropy (51).

It is interesting to compare the changes in membrane dimensions with the binding data in Fig. 3. Thus, at low sugar concentration (<5%), it is intuitive to conclude that the binding of sugar involves some degree of intercalation, which separates the lipid head groups and thus increases the lateral area. This, in turn, allows increased disordering of the lipid chains (more gauche conformers) and hence a thinning of the membrane, which is more pronounced for the disaccharides than for the smaller glucose (Fig. 1). This general behavior is typical for the interfacial binding of small molecules to membranes (52, 53), and we conclude that the structural and thermodynamic results at low sugar concentration are mutually supportive. At higher sugar concentrations we see a different and unorthodox picture. Thus, the negative values of Γ_3 and the associated increased interfacial free energy would be expected to compress the membrane laterally. Nevertheless, DMPC membranes in, e.g., 15% trehalose are thinner and laterally expanded compared to membranes in pure water. We suggest that this can be explained by the two modes of interaction discussed above. Thus, at 15%, the sugar molecules already bound at lower sugar concentrations are still in the membrane and these bound sugars still dominate the changes in membrane dimensions. At still higher sugar concentration, we find a compression of the lipids (negative slope of V_{lip} in Fig. 2), which suggests that the increased interfacial free energy associated with the exclusion eventually offsets the structural changes of the bound sugars. This conclusion is also in line with the occurrence of minima in the thickness data in Fig. 1 as the lateral expansion of the bound sugars is gradually counteracted by increasing interfacial free energy (unfortunately SANS measurements could not be extended above 20% sugar with the current methods).

Sugar–membrane interactions have been extensively discussed on the basis of lipid phase transition data, and many reports have concluded that addition of sugar favors the phase with the smallest interfacial area because of a preferential exclusion, which is common to all kosmotropes. We note that while Γ_3 for the fluid membrane [Γ_3(fluid)] may be a useful parameter, changes in, e.g., the main (gel-to-fluid) transition temperature, T_m, is not governed by Γ_3(fluid) itself, but by the difference, $\Delta\Gamma_3 = \Gamma_3(\mathrm{fluid}) - \Gamma_3(\mathrm{gel})$. If, for example, $\Delta\Gamma_3$ is negative, the solute interacts more favorably with the gel phase and consequently stabilizes this phase (i.e., raises the temperature where it melts). Conversely, solutes with $\Delta\Gamma_3 > 0$ will lower T_m. The behavior of Γ_3(fluid) and Γ_3(gel) for the sucrose-DMPC system is illustrated in Fig. 3, *Inset*. Obviously, it is not possible to study the two different phases under the exact same conditions, so the experimental temperature was 18 °C and 30 °C, respectively, for studies of gel- and fluid-phase DMPC. It appears that the two curves are qualitatively similar with a maximum at the same location and hence that the two modes of interaction pertain to both phases. The binding to the gel phase is somewhat weaker, but the difference is not large. The most conspicuous difference is the negative slope after the maximum, which is larger by a factor of three for the fluid phase compared to the gel phase (Fig. 3, *Inset*). This difference in slopes gives rise to substantial negative values of

$\Delta\Gamma_3$ in accord with the increased T_m reported at high sucrose (25, 50). Sucrose binding in dilute solutions is quite similar for fluid- and gel-phase DMPC ($\Delta\Gamma_3$ is small), and hence this binding is of minor importance for the phase equilibrium. The important parameter for sugar effects on T_m is the unfavorable interaction of fluid membranes and sucrose at moderate and high concentration. This interpretation is in line with the exclusion theory (34, 54), and we conclude that although this approach neglects the binding mode, exclusion theories may provide reasonable results on phase behavior because the binding mode has little effect on $\Delta\Gamma_3$. This limited effect of the binding mode on phase behavior is in strong contrast to the structural changes that couple to the binding.

The main conclusions of this work are reiterated in the cartoon in Fig. 4. The data show that membrane–sugar interactions may be described by the overlapping action of an attractive and a repulsive component. The former is likely to reflect hydrogen bonding and to saturate at intermediate sugar concentrations. The latter is driven by the kosmotropic or "water-structure-making" effect (55), which causes a general depletion of sugars from aqueous interfaces (34, 54). The kosmotropic contribution dominates except at low sugar concentrations, where the attractive contribution is stronger. The binding triggers significant changes in the dimensions of the bilayer (Fig. 1), which are increasingly counteracted by the exclusion, and reverted at very high sugar concentration (Fig. 2). It appears that this dual mode of interaction may be the cause of some controversy in earlier studies on sugar–membrane interactions, which is discussed in the Introduction. Thus, experimental approaches that are sensitive to the membrane dimensions (e.g., monolayer methods) will primarily detect the consequences of the binding mode, whereas macroscopic approaches (e.g., phase behavior and thermodynamics of interaction) will detect exclusion except at quite low sugar concentration, where the effects are difficult to measure. This interpretation provides a reconciliation of the binding and exclusion theories discussed in the Introduction. It is also inclusive to other observations on sugar–membrane interactions such as increased order parameters of the acyl chains at very high (67%) trehalose (56) where the compression exerted by the exclusion mode exceeds the binding-induced expansion. It appears to be of interest to study if this unique mode of interaction is important for the different types of membrane stabilization provided by small carbohydrates.

Methods and Materials

Chemicals. All lipids (99%) were purchased as powders (Avanti Polar Lipids) and used as supplied. Glucose [>99.5% D-(+)-glucose] and sucrose (>99.5% α-D-Glucopyranosyl β-D-fructofuranoside) was from Sigma-Aldrich, and trehalose (>99% α-D-glucopyranosyl α-D-glucopyranoside) was from AppliChem. Normal water was freshly made in a MilliQ equipment (Millipore) and D_2O (99.9 atom % D) was from Sigma-Aldrich.

Small-Angle Neutron Scattering. Dry DMPC or DOPC was hydrated on the day of use by solutions of sugars in D_2O. The lipid concentration was 6.2 mg/mL. The lipid suspension was extruded to unilamellar liposomes through two stacked polycarbonate filters with 100-nm pore size in a Lipex extrusion device (Northern Lipids). SANS experiments were performed at the SANS-II instrument at the Swiss Spallation Neutron Source (SINQ) at the Paul Scherrer Institute. Using three different combinations of neutron wavelengths and sample-to-detector distances, a q range from 0.005 to 0.3 Å^{-1} was covered. The SANS data were azimuthally averaged and background subtracted by the standard software used at the facility and normalized to absolute units of differential scattering cross-section per unit volume (cm^2/cm^3) by division by the scattering spectrum of H_2O (57). The wavelength spread $\Delta\lambda/\lambda$ was 10.5% FWHM. The resolution effects arising from wavelength spread and finite collimation were taken into account in the data analysis by convolution of the model with the appropriate resolution function at each setting (58). This is done automatically in the programs used for the data analysis. All samples were measured in 5 mm Hellma Quartz cells and at a temperature of 40 °C.

Dialysis Measurements. Stock suspensions of 2–4% (wt/wt) DMPC in MilliQ water were extruded to unilamellar liposomes. About 500 μL of the extruded solution was transferred to dialysis bags (Spectrum Laboratories Inc., molecular weight cutoff = 12–14 kDa), and placed in a closed beaker with 250 mL of sugar solution of the desired concentration. The beaker was placed on a shaking table in a thermostatted box (air bath) and slowly temperature cycled between 20 and 30 °C for 12 h. Slow passage through the main transition (at about 24 °C for DMPC) is associated with a massive transient leakage that provides transbilayer equilibration of small polar molecules (59). After the T cycling, the samples were held at 30 °C (±0.2 °C) for an additional 12 h. It is implicit for the subsequent data analysis that this treatment equilibrates the sugar across the membrane, and hence that the local sugar concentration in the hydration zone is the same for the two membrane leaflets. Samples from inside and outside the dialysis bag were retrieved and diluted 1∶50 (or 1∶100 for the highest sugar concentrations) in 50% 1-propanol, so that the liposomes dissolved and released their contents. The amounts of sugar and lipid were measured in a Varian 9012 HPLC equipped with a Sedex 85 evaporative light scattering detector and converted into molal concentration units as described in *SI Text*. Samples from the dialysis experiments were analyzed in five separate HPLC runs, and the standard error of mean of repeated measurements was less than 0.5%.

Densitometry. Vacuum dried DMPC aliquots were hydrated with 2 mL of either pure water or previously prepared sugar solution (5–30% wt/wt). All steps were quantified gravimetrically to within 0.01 mg. The samples were temperature cycled, shaken, and intermittently treated with light ultrasound prior to the densitometry. The density of both tertiary (water–sugar–lipid) and binary (water–sugar) samples was measured in a DMA 601 vibrating tube densitometer (Anton Parr) at 40 ± 0.01 °C. Prior to the measurements, the samples were degassed by stirring under vacuum

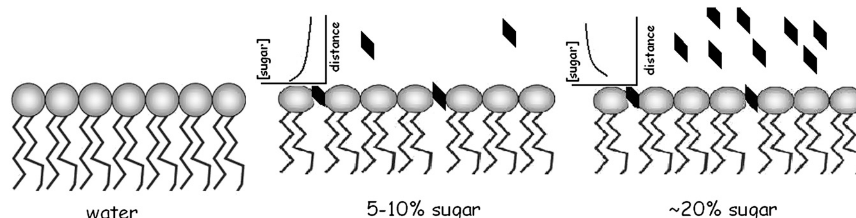

Fig. 4. Simplified illustration of the hypothesis for sugar–membrane interactions. Intercalation of the bound sugar molecules at low sugar concentration (5–10%) makes the membrane thinner and laterally expanded. Under these conditions, the sugar:water molar ratio is increased in the vicinity of the membrane as indicated by the small graph (*Middle*). At about 20% sugar the concentration gradient near the interface has changed sign ($\Gamma_3 < 0$), as indicated in the small graph (*Right*), but the membrane is still laterally expanded by the bound sugar.

for ~45 s. This removal of microbubbles proved to be necessary for a satisfactory precision, and the associated loss of water through evaporation (~1 mg) was quantified gravimetrically and corrected for in the data analysis. The instrument was regularly calibrated against pure water and air. The experimental repeatability was 3×10^{-6} cm^3/g.

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