Swelling of a Lecithin Lamellar Phase Induced by Small Carbohydrate Solutes

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ABSTRACT In this paper, we consider the effect of adding small carbohydrate solutes (small sugars) to DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine) L_α dispersions and the consequences on the force balance at zero osmotic pressure (maximal swelling). We show the importance of long incubations required to obtain samples at thermodynamic equilibrium where molecular diffusion has been completed. The monotonic increase of maximal swelling versus sugar content occurs as a combined effect of the screening of the van der Waals contribution and fluctuations in the lamellar stacks. According to this new approach, it is shown that changes in dielectric properties result in a much less pronounced effect than entropic forces (undulations) generated by the softening of the membranes at high sugar content. However, this sugar-induced swelling cannot be explained quantitatively by adding an entropic contribution to molecular interactions. Quantitative disagreement between the proposed mechanism and our observations is due either to nonadditivity of molecular interactions with entropic forces or to the relation used to account for the entropic contribution.

INTRODUCTION

Quantitative determinations of intermolecular and surface forces are of primary importance in phenomena such as vesicle-vesicle interaction, adhesion, and membrane fusion. Because these processes occur mainly in biological solutions and in the presence of buffers and various types of solutes, it is important to determine bilayer interactions in the presence of model hydrosoluble solutes. Sugar units of molecular or macromolecular size are the major constituent of the soluble parts of glycolipids and glycoproteins and play a major role in the dynamical properties of the membrane and in recognition processes.

Over a wide range of concentrations and temperatures, model biological membranes made of the synthetic phospholipid DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine) form neutral lyotropic lamellar phases. The biologically relevant phase is the L_α, which has been the most studied, pure, in excess water, or in the presence of various aqueous solutes, polymers, or proteins.

The identification of the dominant interactions in biphasic samples involving a lamellar phase in coexistence with a reservoir requires the knowledge of the exact content of each of the two microphases in equilibrium. In most binary systems, the knowledge of the average composition combined with scattering experiments is sufficient to describe the samples in terms of structure and composition of the coexisting phases. In ternary systems, scattering experiments combined with an analysis of the third component's concentration to determine its osmotic pressure is the most suitable way to proceed. For example, when large polyelectrolytes coexist with concentrated clay dispersions, the complete separation of the two phases allows the thermodynamics to be understood (Morvan et al., 1994). However, such samples often have the appearance of gels that are very difficult to separate in two "pure" phases unless ultracentrifugation is used. Such samples are supposed to be studied at thermodynamic equilibrium (although in principle the pressure applied by centrifugation could be quantified as well). Alternative strategies rely on delicate determination of Bragg-reflection shapes (Salditt et al. 1998; Salditt, 1999), adsorption in a surface force apparatus, and pipette aspiration (Parsegian and Rand, 1995). However, bilayer adsorption quenches all modes of interaction related to fluctuations to create systems irrelevant to the equation of state in bulk.

Lyotropic lamellar phases prepared in an excess of water and in the presence of a host molecule represent a general case where the host can be either a soluble molecule or a confined polymer (Demé et al., 1996, 1997), a cosurfactant, a polymer confined in the membranes (Radlinska et al., 1995). In the case of soluble molecules, their osmotic pressure can be considered in the force balance. Assuming additivity of molecular and entropic forces (low fluctuations), the osmotic pressure of the excess solution Π_s can be written:

$$\Sigma\Pi_i(d_w) = \Pi_{hyd.}(d_w) + \Pi_{vdw}(d_w) + \Pi_{und.}(d_w) + \Pi'_s(d_w) = \Pi_s \quad (1)$$

in which $\Pi_{hyd.}$, Π_{vdw}, and $\Pi_{und.}$ refer to the distance dependant hydration, van der Waals, and entropic contributions to membrane interactions. Π'_s is the added osmotic pressure due to the presence of sugar confined between the membranes. At maximal swelling, the difference between the osmotic pressure of the coexisting phases is zero. If one of the phases is pure or almost pure water (solution of lipid at the CMC), then the total osmotic pressure in the lyotropic domains is zero as well,

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resulting from the balance between dispersion forces and short range hydration forces (Lis et al., 1982).

Finally, in the case of a coexistence between inner and external sugar solution in excess, and when equilibrium via molecular diffusion of the sugar has been completed, we have to consider the concentration in the midplane and the concentration in the reservoir. These two concentrations are likely to be equal (Demé et al. 2000). Then, the two osmotic terms related to the sugar compensate and Eq. 1 simplifies to:

$$\Sigma\Pi_i(d_w) = \Pi_{hyd.}(d_w) + \Pi_{vdw}(d_w) + \Pi_{und.}(d_w) = 0 \quad (2)$$

The well-known case of osmotic compression by polymer solutions corresponds to:

$$\Sigma\Pi_i(d_w) = \Pi_{hyd.}(d_w) = \Pi_{vdw}(d_w) + \Pi_{und.}(d_w) = \Pi_p \quad (3)$$

in which Π_p is the osmotic pressure of the external polymer solution. Exclusion of the polymer from the water layers results in a zero repulsive contribution in the force balance of the lamellar phase.

However, according to Diamant (submitted manuscript), in the case of multilamellar domains dispersed in an excess of solution, and if the sum of the pressures is low (of the order of 100–1000 Pa), the contact energy between the two macroscopic phases coexisting in the sample cannot be neglected. The repulsion between bilayers may be compensated by the macroscopic surface tension between the lamellar domains and the sugar solution in excess. Eq. 2 becomes:

$$\Sigma\Pi_i(d_w) = \gamma \frac{\Delta A}{\Delta V} \approx 2\gamma/R \quad (4)$$

in which γ is the macroscopic surface tension between lamellar domains and the reservoir solution. ΔA and ΔV are the variations associated to contact area and volume of the lamellar domains, approximated in the form of a Laplace relation in which R is the mean radius of the domains. Eq. 4 shows that a significant contribution can result from a nonzero surface tension combined to small lamellar domains (MLVs (multilamellar vesicles)). This tension stabilizes onions against unbinding (Diamant and Cates, 2001).

A founding paper of biological membrane physics (Le-Neveu et al., 1977) proposed a qualitative explanation of the observed maximal swelling of neutral phospholipid membranes in the presence of small carbohydrate molecules. From the measurement of the maximal swelling versus sugar concentration, it has been inferred that the primary effect of sugar addition was to shield the frequency-dependent contribution of the dispersion force by matching the permitivity of the layers, thus producing a minimum in the attractive dispersion force. The associated maximal swelling observed when sugar is added to zwitterionic lecithin/water dispersions has been explicitly calculated and has been shown to be consistent with the experimental observation made at that time: a swelling till 0.22 sugar weight fraction followed by a deswelling at higher concentration. The explanation of the unexpected nonmonotonic effect led to similar works (McDaniel et al., 1983; Stümpel et al., 1985) in which qualitatively analogous results were observed. In the latter reference a similar trend was reported, although much less pronounced and in the L_β' phase (5°C).

In earlier work (Demé et al., 1996), we reported monotonic swelling of the DMPC lamellar phase induced by added mono- or disaccharides. It was also shown that in samples containing oligosaccharides where molecular diffusion was not completed, compression due to residual excess of sugar in the reservoir (equivalent to a deswelling of the lamellar phase) could be observed as in the case of osmotic compression by polysaccharides. The oligosaccharides were from the series of glucose oligomers ranging from $n = 2$ (maltose) to $n = 5$ (maltopentaose). It was shown that for a given incubation time the osmotic stress was a function of the sugar size. Thus, the excess of sugar in the coexisting solution is higher when the molecular weight is increased until the extreme case of the polysaccharide (pullulan) where complete exclusion of the chains takes place. In this case, the applied osmotic pressure is known from separate measurements on the polymer solution.

DMPC binary phase diagrams are given in Janiak et al. (1976, 1979) and Smith et al. (1988) for DMPC. We reconsider here the case of DMPC in the presence of large amounts of added sugar (mono- or disaccharide) and propose a new interpretation of our recent results (Demé and Zemb, 2000). These contradict older work but confirm those of our previous studies (Demé, 1995; Demé et al., 1996; Ricoul, 1997).

MATERIALS AND METHODS

Materials

Chemicals

Glucose and fructose were obtained from Fluka (Buchs, Switzerland). DMPC was obtained from Avanti Polar Lipids (Alabaster, AL). Samples were prepared in Millipore water.

X-ray camera

Small-angle X-ray scattering (SAXS) patterns were recorded on a home-build Huxley-Holmes laboratory camera in pinhole geometry using the CuKα radiation selected by a curved monochromator and focused at the position of the two-dimensional detector (Le Flanchec et al., 1996).

Methods

Particular care was taken in sample preparation to ensure thermodynamic equilibrium. There are several possible strategies: either by relying on diffusion of solute in water and through defects in bilayers or by starting from nearly molecularly dispersed dry powder obtained by freeze drying very dilute ternary solutions. We have noticed in previous work that the material obtained by freeze drying from the dilute mixture allows rapid reswelling equivalent to what is obtained

after several weeks of molecular diffusion (Demé et al., 1996). This rapid reswelling is a critical step, particularly when the "solvent" is a polymer solution (Demé et al., 1997). In the partition equilibrium of a small solute, it is crucial that the samples are studied at equilibrium of the chemical potentials of all entities, because any difference between the coexisting phases may have dramatic effects. In the present case of high sugar concentrations, a residual osmotic stress can be strong and lead to important modifications of the equilibrium distance between membranes. However, diffusion coefficients of small sugars are such that the equilibrium time remains within the range of 1 to 2 weeks, i.e., reasonable time compared with the quasi infinite time required for polymers to diffuse in confined water layers and compatible with the chemical stability of the compounds at incubation temperature.

Samples were incubated for 2 weeks at 30°C with regular vortexing. SAXS experiments were performed at 30°C as well. This is well above the chain melting temperature of pure DMPC, corresponding to the P_{β}'-L_{α} transition (23°C) and still above the transition in the presence of sugar (Stümpel et al., 1985). SAXS experiments are performed directly on the biphasic mixtures. The ternary samples are microseparated with the lamellar phase at "maximal swelling" in equilibrium with excess sugar solution. Large multilayer vesicles are formed on a mesoscopic scale, i.e., they are too small to be easily separated from the pure coexisting solvent but large enough to produce sharp and perfectly isotropic Debye-Sherrer rings whose profile is not limited by the number of layers but by the interlayer fluctuations (Dubois and Zemb, 1991). Lamellar domains appear in the form of onions or MLVs producing Maltese crosses under polarizing microscope and hence contain several thousands of membranes and some macroscopic surface tension (Diamant, 2001), which may quench fluctuations (Seifert, 1995). Unfortunately, the value of the tension and how it changes in the presence of sugar are not known.

Force balance

The three major contributions we consider are the van der Waals, the hydration, and the entropic forces (Eq. 1). The membranes composing the lamellar stack of alternated water and lipid layers are described by a simple model consisting of an aliphatic core of melted chains surrounded by hydrated polar heads. The membrane thickness d_m, is defined by:

$$d_m = d_h + 2d_p \tag{5}$$

in which d_h is the thickness of the hydrocarbon core and d_p that of the polar head layer. The measured periodicity is the sum of the membrane and water layer thicknesses:

$$d = d_m + d_w \tag{6}$$

van der Waals forces

In the following calculations only the force between first neighbors is considered (Israelachvili, 1991). The thickness of the membrane in excess water is considered constant. It is known to be concentration dependent in the monophasic L_{α} domain and to vary at phase transitions where chain-packing rearrangements take place (Janiak et al., 1979). Regarding retardation effects that affect the frequency-dependent contribution of the Hamaker constant (Ninham and Parsegian, 1970), we have neglected them because of the membrane separations, which do not exceed 50 Å.

Double film model

The relation used to calculate the van der Waals pressure considers, in the case of the double film model, a thickness d_a that corresponds to the thickness of the aqueous region separating the hydrophobic layers. The contribution of the van der Waals attraction to the total pressure of the sample can be calculated according to Ninham and Parsegian (1970):

$$\Pi_{vdw}(d_w) = -\frac{A}{6\pi}\left[\frac{1}{d_a^3} - \frac{2}{(d_a + d_h)^3} + \frac{1}{(d_a + 2d_h)^3}\right] \tag{7}$$

in which A is the nonretarded Hamaker constant calculated for the symmetric case of two identical apolar phases interacting across water. It can be decomposed into a zero frequency contribution ($A_{v=0}$) and a frequency-dependent contribution ($A_{v>0}$) (Israelachvili, 1991):

$$A = A_{v=0} + A_{v>o} = \frac{3}{4}kT\left(\frac{\epsilon_1 - \epsilon_2}{\epsilon_1 + \epsilon_2}\right)^2 + \frac{3h\nu_e}{16\sqrt{2}}\frac{(n_1^2 - n_2^2)^2}{(n_1^2 - n_2^2)^{3/2}} \tag{8}$$

in which subscripts 1 and 2 refer to the apolar phase and to the water, respectively. h is the Planck constant = 6.63×10^{-34} J×s, ϵ_1 and ϵ_2 the dielectric constants, n_1 and n_2 the refractive indexes, and ν_e the absorption frequency. Taking $\epsilon_1 = 2$, $\epsilon_2 = 80$, $n_1 = 1.464$, $n_2 = 1.333$, and a single absorption frequency in the ultraviolet $\nu_e = 3 \times 10^{15}$ s^{-1} one obtains:

$$\begin{aligned} A &= A_{v=0} + A_{v>0} \\ &= 2.9 \times 10^{-21} + 2.2 \times 10^{-21} \\ &= 5.1 \times 10^{-21} J\,(1.2\,kT_{room}) \end{aligned} \tag{9}$$

Triple film model

It has been shown (Attard and Mitchell, 1987; Attard et al., 1988) that the shape of experimental pressure-distance curves was better reproduced when a more detailed triple film model was considered to describe the membrane as a well-defined medium composed of distinct hydrophobic and polar regions with distinct dielectric properties (Ninham and Parsegian, 1970; Evans and Needham, 1987). In this case, the triple film model describes the van der Waals pressure, according to the relation (Ninham and Parsegian, 1970):

$$\begin{aligned} \Pi_{vdw}(d_w) = &-\frac{A_1}{6\pi}\left[\frac{1}{d_w^3} - \frac{2}{(d_w + 2d_p + d_h)^3} + \frac{1}{(d_w + 4d_p + 2d_h)^3}\right] \\ &-\frac{A_2}{6\pi}\left[\frac{1}{(d_w + d_p)^3} - \frac{1}{(d_w + d_p + d_h)^3} - \frac{1}{(d_w + 3d_p + d_h)^3} + \frac{1}{(d_w + 3d_p + 2d_h)^3}\right] \\ &-\frac{A_3}{6\pi}\left[\frac{1}{(d_w + d_p)^3} - \frac{1}{(d_w + 2d_p + d)^3} + \frac{1}{(d_w + 3d_p + 2d_h)^3}\right] \end{aligned} \tag{10}$$

which accounts for a Hamaker constant related to correlations of differences in polarizability between adjacent layers, integrated over the electromagnetic spectrum. It is the sum of three terms related to polar heads

and water ($A_1(\ldots)$), aliphatic chains and head groups ($A_3(\ldots)$), and a cross-term ($A_2(\ldots)$). This latter term is one order of magnitude below the two others and is usually neglected (Evans and Needham, 1987).

Hydration forces

This exponential interaction dominating all other contributions at short distances (<25 Å) has been largely studied in a number of systems and has been reviewed (Rand and Parsegian, 1989). The hydration pressure is known for DMPC from previous osmotic stress measurements using pullulan solutions (Demé et al., 1996). It is characterized by an exponential decay length of 1.91 Å and an extrapolated pressure at zero separation of 4.5×10^9 N/m^2 yielding the distance-dependent contribution:

$$\Pi_{\text{hyd.}}(d_w) = 4.5 \times 10^9 e^{-d_w/1.91} \tag{11}$$

The decay length is close to the value of 1.93 Å reported for egg lecithin (LeNeveu et al., 1976) but slightly below the 2.2 Å reported for DMPC (Lis et al., 1982).

In the following, we will consider that confined sugar molecules do not interfere with the polar head layer and do not modify the hydration force. This assumption is supported by a recent small angle neutron scattering study where contrast variation was used to determine the partition of sugars between lamellar domains and excess solution (Demé et al., 2000). It was shown that sugar molecules are partially depleted from the confined region. The absence of interaction between glucose monomers and phosphocholine headgroups was also shown by neutron reflectivity on DMPC monolayers spread on glucose and pullulan solutions (Demé and Lee, 1997). Finally, in the range of periodicities observed in the presence of sugar it vanishes and can be considered negligible at high sugar concentration.

Entropic forces

The entropic contribution (Helfrich, 1978) that originates in excluded-volume interactions is usually neglected in the force balance of lipids forming stiff membrane stacks. Effectively, considering a membrane bending rigidity k_c of the order of 10 kT for pure DMPC in water (Sackmann, 1995) and introducing a repulsive term of entropic origin in the force balance increases the equilibrium distance of the membranes by less than 1 Å. The effect is minor in the binary system, but it has important consequences in the ternary system (when the lamellar phase is swollen and the van der Waals contribution in the balance weaker). We will take this contribution into account by using the relation (Helfrich, 1994):

$$\Pi_{\text{und.}}(d_w) = \frac{3\pi^2}{64}\frac{(kT)^2}{{d_m}^3.k_c}\left(\frac{\Phi}{1-\Phi}\right)^3 \tag{12}$$

in which Φ is the volume fraction of the lipid in the lamellar domains deduced from the measured lamellar spacing and from the known membrane thickness d_m (35.5 Å).

Forces associated with sugar exclusion from multilayer vesicles

If the multilayer vesicle, on the time scale considered, is permeable to the small solute, the activity of molecules inside and outside is the same and there is no osmotic term due to sugar exclusion from the MLVs. Another view of the same effect is to consider that the first hydration layer is not available for the solute (Lyle and Tiddy, 1986; Demé and Zemb, 2000). Thus, the concentration of solute in the mid-plane between bilayers is the same as in the bulk and hence, no compression due to osmotic stress induced via depletion has to be considered. The case of uncharged molecules has been recently considered in detail (Bonnet-Gonnet et al., 2001).

However, we have to consider the general mechanism proposed recently (Diamant, 2001) where the partial exclusion of solute from the bilayers allows the release of surface tension and thus minimizes the free energy. In the presence of this mechanism implying depletion of sugar, the Laplace Eq. 4 has to be corrected according to:

$$\Sigma\Pi_i(d_w) = p_o + 2\gamma/R_{\text{MLV}} \tag{13}$$

in which the osmotic stress is difficult to evaluate. This extra osmotic compression due to this new type of depletion cannot be evaluated quantitatively. There is only a higher bound for it, considering the concentration difference inside and outside. If the solution is equivalent to a perfect gas, this upper limit is given by:

$$p_o \leq (\Psi_s' - \Psi_s)kT \tag{14}$$

if Ψ_s is expressed as a number density (m^{-3}).

The mechanism proposed by Diamant is analogous to a depletion mechanism. However, it is not a depletion due to sterical incompatibility as in the case of polymers, but the concentration inside and outside are different because of the nonvanishing surface tension of the bilayers in a given crystallite of smectic phase, i.e., one multilayer vesicle (or onion) versus external medium. Thus, to minimize the bilayer free energy, depletion of the nonadsorbing solute can be associated to the undulations of the bilayer without intrinsic local softening of the bilayer.

Force balance and additivity

A long standing problem is the validity of adding entropic forces to molecular interactions. This was reviewed recently (Lipowsky, 1995b). The general difficulty is that fluctuations enhance any exponentially decreasing interaction. Analytical solutions to these problems are not available, and treatments using renormalization theory only indicate trends. Because additivity of molecular forces with entropic contributions cannot be supposed a priori, we only indicate qualitative trends for the "effective stiffness" of the membrane, either directly induced by the presence of the solute or via the tension release suggested by Diamant.

Partition of sugars between lamellar phase and excess solution

Using small-angle neutron scattering and solvent contrast variation with deuterated sugars, we have determined the sugar partition coefficient in microseparated samples (Demé and Zemb, 2000). In the system presented here, detailed knowledge of the composition of the two coexisting phases allows a quantitative reinterpretation of the equation of state (pressure versus distance). At equilibrium, in samples prepared with a mean sugar volume fraction $<\Phi_s> = 0.115$ the sugar concentration in the interbilayer water layers is lower by ~one-third than in the excess solution (respectively $\Psi_s = 0.095$ and $\Psi_s' = 0.155$). This corresponds to a partial exclusion of 18% of sugar molecules from the water layers relative to the mean sample concentration $<\Phi_s>$, leading to $\Psi_s' > \Psi_s$. We take this effect into account by considering the exact sugar concentration between bilayers (Ψ_s) to calculate the extent of screening of the dispersion force.

Force balance in the presence of sugar

Addition of sugar to water increases the index of refraction of the aqueous layers and as a consequence decreases the difference in polarizabilities (Eq. 7). This reduces the contribution of visible frequencies to the total van der Waals interaction. At a sufficiently high interbilayer sugar concentration (Ψ_s), the aqueous polarizability begins to exceed that of the hydrocarbon and polar head layers and the total interaction increases with added sugar. Thus, the Hamaker constant is modified but not monotonically with a

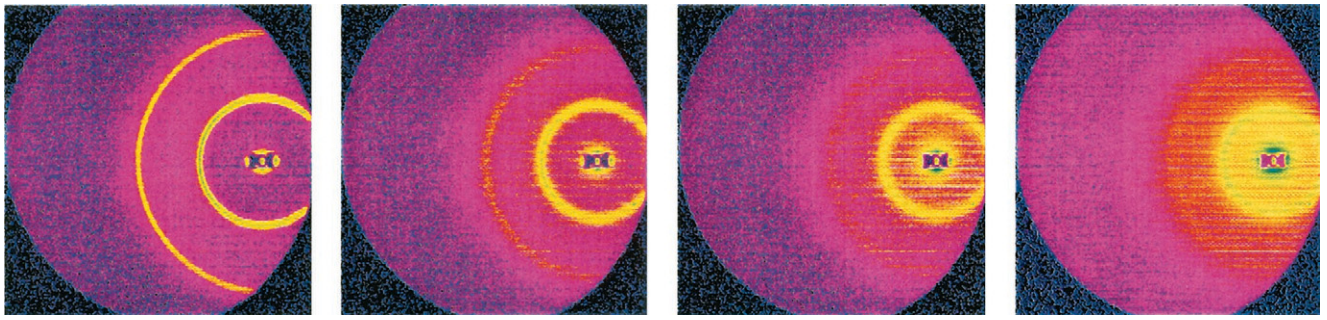

FIGURE 1 Selection of two-dimensional SAXS patterns of DMPC/glucose suspensions prepared with increasing concentrations of glucose (from left to right: Φ_s = 0, 0.05, 0.10, and 0.20). The volume fraction of the lipid $<\Phi_L>$ relative to the "water + sugar volume" is 0.20.

minimum at a sugar concentration Ψ_s = 0.22 and a maximum of the weakening of the van der Waals attraction at that concentration (LeNeveu et al., 1977). To account for the presence of the sugar in the aqueous layers of the lamellar phase, we have calculated Ψ_s for the different sugar concentrations Ψ_s considering the partition of the sugar between lamellar domains and the excess solution (Demé and Zemb, 2000). From Ψ_s = 0 to 0.22, the van der Waals attraction is progressively weakened, and the relative strength of repulsive contributions increases, leading to the observed swelling. Above Ψ_s = 0.22, van der Waals forces are reinforced leading to a stronger attractive contribution and an expected deswelling. In the presence sugar, we use the double film model, which gives a good approximation of the triple film model (see Fig. 4) and for which the dependence of the Hamaker constant is known (LeNeveu et al., 1977).

RESULTS

Scattering curves

Fig. 1 shows a selection of two-dimensional SAXS patterns illustrating the effect of adding glucose to the DMPC lamellar phase. Two sharp quasi-Bragg reflections characteristic of the lamellar structure are clearly visible. The effect of adding sugar is already visible at 5%: the reflections broaden and the second order disappears around $<\Phi_s>$ = 0.20 by weight of sugar in the aqueous phase. On radially averaged data (Fig. 2, in $I(q)\times q^2$ versus q representation),

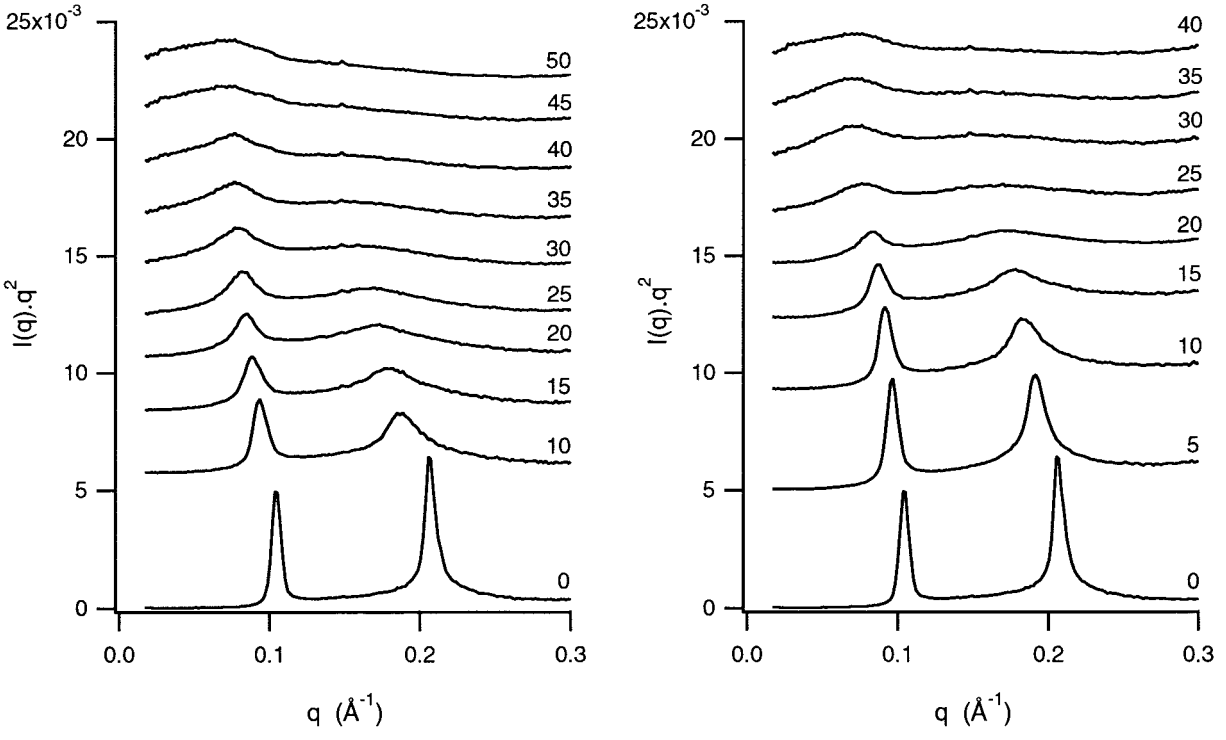

FIGURE 2 Radially averaged SAXS curves ($I(q).q^2$ versus q plots) showing the swelling and the softening of the lamellar phase upon addition of glucose or fructose to DMPC suspensions. (*Left*) DMPC ($<\Phi_L>$ = 0.20) + glucose from Φ_s = 0 to 0.50 by weight in water. (*Right*) DMPC ($<\Phi_L>$ = 0.20) + fructose from Φ_s = 0 to 0.40 by weight in water.

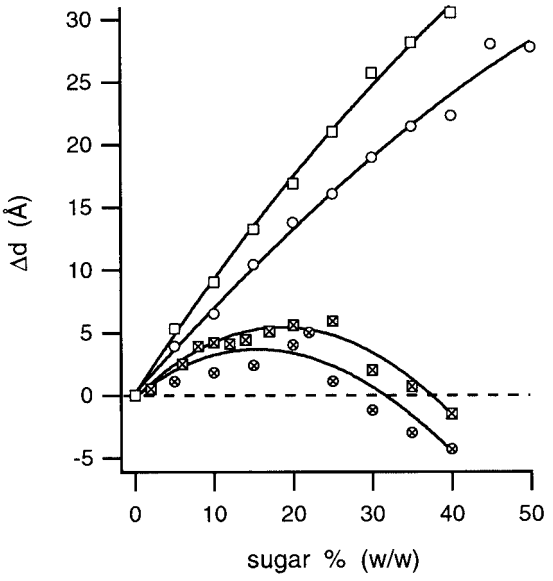

FIGURE 3 Change of the periodicity of the lamellar domains versus sugar concentration in the aqueous phase as measured by SAXS. Our results obtained with DMPC + glucose (○) and fructose (□) are compared with those of LeNeveu et al. (1977) obtained with egg lecithin + glucose (⊗) and saccharose (⊠). Plotting Δd instead of d accounts for the membrane thickness difference between the two phospholipids. Data obtained with DMPC are calculated from curves of Figs. 1 and 2. The error on d is of the order of ± 0.5 Å (± 1 Å on Δd). The solid lines are guides to the eye.

one can verify that despite the important disorder, the samples are still lamellar at high sugar concentration. In both cases (glucose and saccharose), the monotonic swelling and the simultaneous softening are evidenced by a shift of the peaks toward low angles and by the progressive broadening of the quasi-Bragg reflections. Because the indexing is kept for any sugar content explored, the shift of the peak to small angles corresponds to a monotonic increase of the interlamellar spacing. In binary suspensions (DMPC + water), the membrane thickness is known to vary only in the monophasic L_α domain where no excess of water is present (Janiak et al., 1976) and at the L_β-P_β' and P_β'-L_α transitions due to different chain tilt angles or melting of the chains. In ternary mixtures with saccharose (Stümpel et al., 1985) small deviations can be attributed to an untilting of the chains but never lead to a swelling increase of several tens of angstroms as observed here. The change in periodicity of the lamellar phase is due to an increase of the water layer thickness.

Swelling versus sugar concentration

The effect of mono- and disaccharides on the periodicity of the lamellar phase is shown on Fig. 3. Data obtained with egg lecithin (two lower curves) are the results from LeNeveu et al. (1977) obtained with glucose and saccharose, whereas data obtained with DMPC (two upper curves) correspond to the spacings calculated from the scattering curves shown in Fig. 2 with glucose and fructose. Here, we have plotted the periodicity change Δd instead of the periodicity d to account for the difference in periodicity between DMPC (60.4 Å) and egg lecithin (63 Å) in the absence of sugar. The difference is due to the chain composition of DMPC (two C_{14} chains) and egg lecithin (mixture of various chain lengths). DMPC and egg-lecithin have bending rigidities of the order of ~ 10 kT (Sackmann, 1995). Small differences in thickness due to different chain lengths may affect the van der Waals contribution (Eqs. 7 and 10) but not significantly in regard to the deviations of several tens of Angstroms observed here.

Fig. 3 shows the monotonic swelling observed upon addition of sugar in the range of studied concentrations (solid lines are guides to the eyes). It emphasizes the global effect of adding small hydrosoluble molecules such as glucose or fructose: favoring repulsive interactions leading to a monotonic swelling. The same trend is observed for the mono- and the disaccharide with a more pronounced swelling excess with the disaccharide, as previously reported with other disaccharides like lactose (Demé et al., 1996) or saccharose (Ricoul et al., 1997). There is a large difference between our data and older results (LeNeveu et al., 1977; Stümpel et al., 1985) and an opposite trend at high sugar concentration. In a previous study (Demé, 1995; Demé et al., 1996), we already observed a swelling-deswelling sequence induced by the addition of oligosaccharides. But this was observed when samples were equilibrated only a few days. In such a case, nonequilibrium of the sample results in an excess of sugar in the reservoir leading to an osmotic compression of the lamellar phase analogous to the one observed with polysaccharides. Osmotic pressures of concentrated sugar solutions can be that high that an uncompleted diffusion can result in opposite effects to those observed at equilibrium.

Force balance in pure water

We have calculated the van der Waals force according to three different models: the double film model with the head groups either in or out of the aqueous layers and the triple film model with Hamaker constants for the chains/heads interface, the head/water interface, and the cross-term. We used the following Hamaker constants: $A_1 = 3 \times 10^{-21}$ J, $A_2 = 10^{-22}$ J, and $A_3 = 10^{-21}$ J (Ricoul, 1997). The three pressure-distance curves are shown in Fig. 4 *A*. We have calculated the total pressure-distance curve in the three cases considered here (Fig. 4 *B*). The two series of curves show that the triple film model (solid line) is best approximated by the double film model when the head group layer is included in the membrane (dashes) rather than in the aqueous phase (dots). This is due to the fact that dielectric properties of the layer of hydrated headgroups are closer to

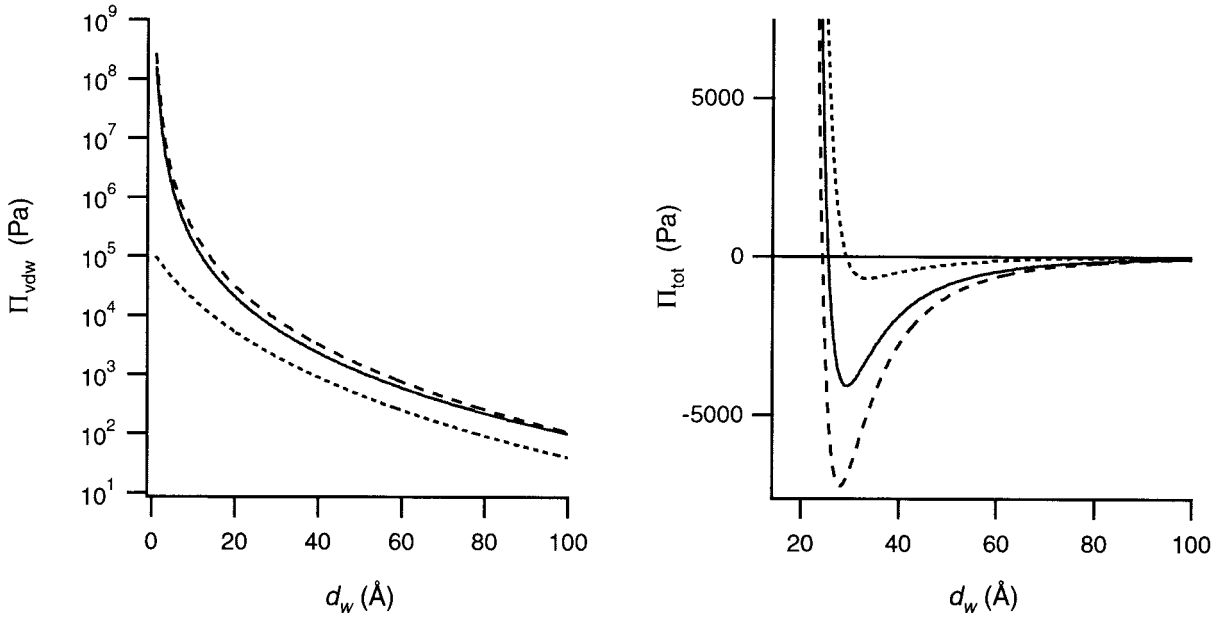

FIGURE 4 van der Waals pressure as calculated according to the three models described in text (*left*) and total pressure-distance curves resulting from the sum of van der Waals, hydration, and entropic contributions in the frame of the additivity approximation (*right*). The zero on the *x* axis corresponds to the head-solvent interface. The zero on the *y* axis corresponds to the equilibrium pressure (lamellar domains in equilibrium with excess water or sugar solution). The osmotic pressure of the sugar solution is equilibrated by the sugar confined between the membranes and producing the same pressure (equilibrium condition). (*Dotted lines*) Double film model with $d_a = d_w + 2d_p$; (*dashed lines*) double film model with $d_a = d_w$; (*full lines*) triple film model. Calculated equilibrium distances corresponding to the three models are: 30.2 Å (double film model, $d_a = d_w + 2d_p$), 25.7 Å (double film model, $d_a = d_w$), and 24.5 Å (triple film model). When the polar head layer is incorporated in the aqueous phase ($d_a = d_w + 2d_p$) divergence occurs for $d_w = -d_p$ instead of $d_w = 0$ for the two other models.

those of the hydrophobic chain layer than those of pure water. As expected when headgroups are considered as part of the aqueous layer, the van der Waals pressure diverges at the chain-head interface ($d_w = -d_p$), whereas divergence occurs at the head-solvent interface ($d_w = 0$) for the two other models. Note that periodicities calculated by adding the water and membrane thicknesses ($d_w + d_b$) differ by only 1.2 Å between the two models: 60 Å ($d_w = 24.5$ Å) for the triple film model and 61.2 Å ($d_w = 25.7$ Å) for the double film model with $d_a = d_w$. In regard to these small differences between the measured periodicity (60.4 Å by SAXS) and calculated ones, the zero of the van der Waals attraction can be adjusted to fit exactly the experimental equilibrium distance. It yields $\Delta(\text{vdw}) = 0.4$ Å for the triple film model and $\Delta(\text{vdw}) = -0.8$ Å for the double film model. This value defines the position where the contribution diverges and is used in the following calculation in the presence of sugar.

DISCUSSION

Force balance versus sugar concentration

Our goal was to investigate in detail the swelling of DMPC lamellar dispersions in equilibrium with excess solutions of glucose and fructose. We have proposed to reconsider the force balance of the ternary system by taking into account the full balance of forces including the modification of the dielectric properties in the aqueous layers and entropic interactions induced by the softening of the membranes upon addition of sugar molecules. To determine the key role of the confined solute in the force balance, we had developed a neutron contrast variation method to determine the exact amount of sugar in the lamellar domains and in the coexisting excess solution (Demé and Zemb, 2000). This method is the most suited when macroscopic separation of the lamellar domains from the excess aqueous solution is not possible by means that do not modify the equilibrium between the phases in coexistence. We applied it using deuterated sugars so that the delicate step of macroscopic separation of the two coexisting phases was avoided.

The origin of excess swelling (from 0 to ~30 Å) results from an increase of the water layer thickness. A swelling of 30 Å cannot result from a change of membrane thickness due to a rearrangement of the chains and/or of the heads, although a small contribution to the observed swelling cannot be rejected (Stümpel et al., 1985). Appearance of fluctuations is also supported by SAXS data (Figs. 1 and 2). The shape of the first reflection is shown Fig. 5 for several sugar concentrations. It is

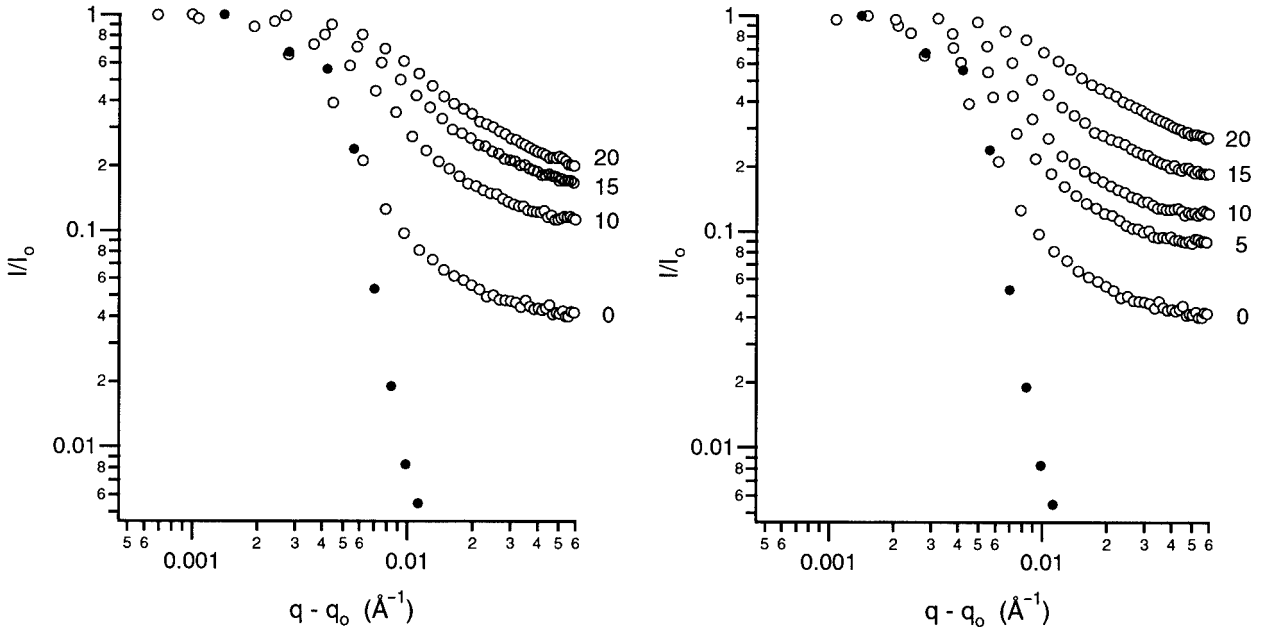

FIGURE 5 Peak shapes (○) in normalized representation (I/I_o versus $q-q_o$), and experimental instrument resolution function of the SAXS camera (●), showing the broadening of quasi-Bragg reflections upon addition of glucose and fructose to the lamellar suspensions. Results are shown for sugar concentrations ranging from 0 to 0.20 w/w of glucose (*left*) and fructose (*right*). The FWHM of the resolution function is 8.8×10^{-3} Å^{-1}.

compared with the resolution function of the camera determined experimentally with an attenuated transmitted beam (FWHM = 8.8×10^{-3} Å^{-1}). This comparison shows that the effect of softening increases with the amount of sugar and that it is strong enough to be observed with a setup not particularly optimized for high-resolution experiments.

It is known that added compounds can drastically modify the bending rigidity of phospholipid membranes, either in the direction of a stiffening or in that of a softening (Sackmann, 1995). However, we are not aware of any evidence of a membrane softening by nonlipophilic or nonamphiphilic molecules. The general underlying mechanism of membrane softening by small carbohydrate molecules is not understood, although the data clearly show the combination of a swelling and a softening. McDaniel et al. (1983) have proposed a mechanism in the case of a softening induced by another small carbohydrate (glycerol). Surface tension measurements on water-glycerol mixtures show that glycerol reduces the surface tension of water. Using Gibbs' equation and for 50% glycerol, 90% of the surface is occupied by glycerol. Thus, lateral repulsions and the area per molecule could be increased and this could favor a softening of the bilayer by thinning of the apolar layer. But there is no direct evidence of such mechanism in the ternary system. The reason why disaccharides induce more swelling than monosaccharides is also not completely clear, although a relation with the solute size has already been reported (Demé et al., 1996) and may seem straightforward.

The change of dielectric properties of the water layers induces deviations of the order of a few Angstroms. The Hamaker constant is calculated for every interbilayer sugar concentration (ψ_s) and by taking into account the known optical properties of the solution. Without sugar $A = 1.24\ kT$, at the optical match point $A = A_{v=0} = 0.71\ kT$, and for the largest concentration investigated $A = 0.84\ kT$.

On Fig. 6 we show a few characteristic pressure-distance curves from a series of simulations where hydration, van der Waals, and entropic contributions have been added and where the only variable is the bending rigidity constant of the membranes k_c. The calculation was done in the absence of sugar (6A) and at the match point of the frequency-dependent van der Waals contribution where $A_{v>0} = 0$ ($\psi_s = 0.22$). In this approach, we do not introduce tension release but incorporate all softening effects into an effective bending constant and extract the equilibrium distance versus k_c. For any value of k_c it is given by the intercept of the pressure-distance curve with the x axis, where attractive and repulsive terms counterbalance. Fig. 7 shows the full set of equilibrium distances versus k_c resulting from the simulation compared with experimental equilibrium distances obtained without sugar, with glucose or fructose. As shown on Fig. 6, *A* and *B*, below a certain value of k_c the curves do not go through an attractive regime anymore, and the pressure-distance curve becomes purely repulsive. This predicts an unbinding transition and yields both the equi-

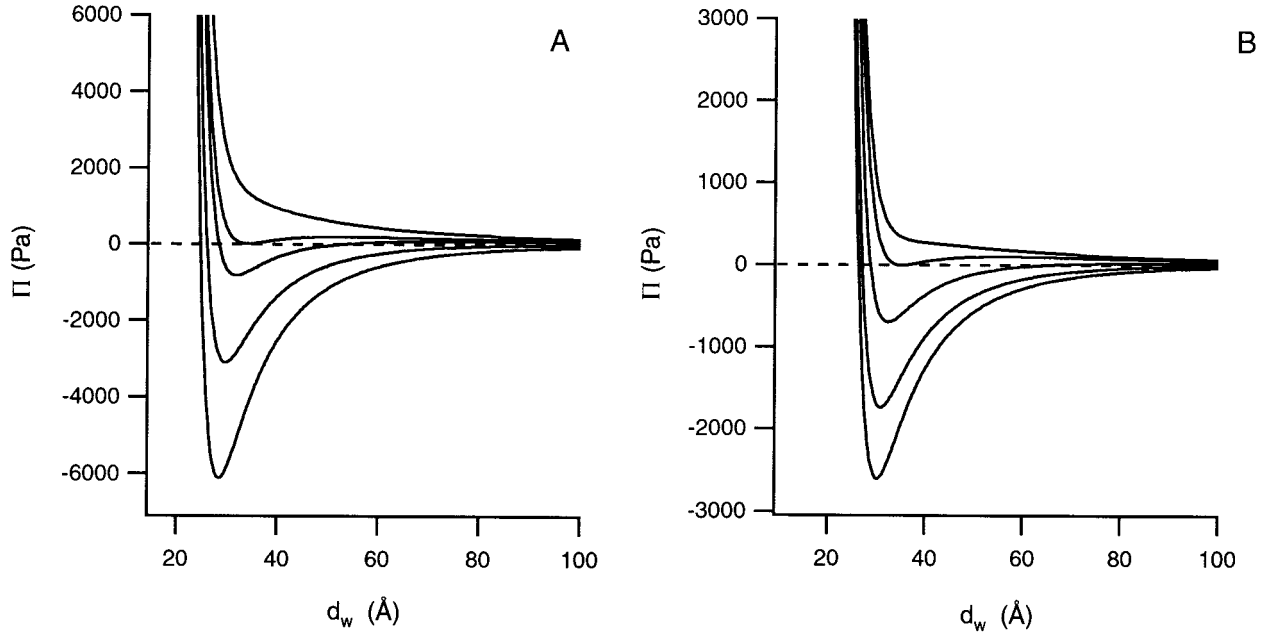

FIGURE 6 Simulated pressure-distance curves versus k_c obtained by summing intermolecular forces and an entropic contribution according to Eq. 11. (*A*) In the absence of sugar and (*B*) at a sugar concentration $\Psi_s = 0.22$ corresponding to the match point of the frequency-dependant contribution of the van der Waals attraction ($A_{v>0} = 0$). (*A*) From bottom to top $k_c = 20, 5, 3, 2.55$ (unbinding), and 2 *kT*. (*B*) From bottom to top $k_c = 20, 10, 6, 4.55$ (unbinding), and 4 *kT*.

librium distance of the membranes in lamellar domains before unbinding and the value of k_c at which unbinding should occur. As shown on Fig. 6 *B* in the presence of sugar, the prediction is not in agreement with the experimental result, unbinding being predicted for distances below those observed experimentally. In the absence of sugar the entropic term is less pronounced and the value of k_c that yields the experimental distance is 20 ± 5 *kT*.

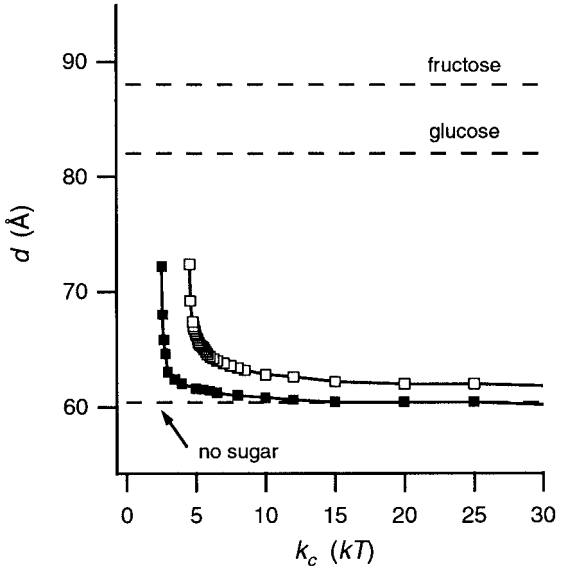

FIGURE 7 Comparison of simulated periodicities to those observed experimentally. Simulated values of *d* are extracted from pressure-distance curves as those shown in Fig. 6 in absence of added sugar (■) and in the presence of sugar at $\Psi_s = 0.22$ (□). Experimental values are represented as dotted lines without sugar (61.4 Å) and at the point of maximal screening of the van der Waals contribution in the case of glucose and fructose (respectively $d_{max} = 82$ and 88 Å). Both series of simulations suggest an unbinding transition for a value of *d* well below the experimental values.

In the presence of sugar, we observe experimentally an increase of 22 Å (glucose) and 28 Å (fructose), which would be due to fluctuation of membranes directly or indirectly associated to bilayer softening or tension release. Note that within this simple additive model, ignoring the effect of surface tension release, an unbinding at 72 Å for a $k_c = 4.5$ *kT*, associated to the Helfrich force dominating van der Waals, is predicted. If we consider the increase in maximal periodicity observed here without unbinding, we have to conclude that repulsion forces are increased monotonically in the presence of sugar. Depletion due to undulation also adds an attractive potential that prevents unbinding. Because we have the combination of two effects and nonlinearity of the interactions, numerical simulations of pressure-distance curves are done using an effective bending constant. In principle, one could simulate these curves and extract the equilibrium periodicity by keeping k_c constant and varying γ, according to Seifert's expression (Seifert, 1995).

The presence of fluctuations due to membrane softening and/or release of surface tension is consistent with the observed evolution of the shape of the quasi-Bragg peaks (Fig. 5). Without added sugar, the central part of the peak is dominated by the resolution of the Germanium monochro-

mator (full circles). Large wings are easily detected but with a significant diffuse scattering, which disables a quantitative determination of the bilayer stiffness using a Caillé-type approach. The Caillé parameter can only be fitted on one peak and not on several orders using the same parameter (Salditt et al., 1998) and the approach is only consistent using three detectable orders when one is far from the phase boundary, i.e., far from maximal swelling, unlike here. Moreover, broadening of the quasi-Bragg peaks can be induced either by a strengthening of the Helfrich force or by partial exclusion due to tension release mediated by the mechanism proposed by Diamant. The two mechanisms may coexist and be of similar magnitude. This coexistence disables the possibility of full calculation of the effect until the complete pressure-distance relation is measured for several sugar concentrations. The full relation (experimental equation of state) of lecithin bilayers in the presence of small solutes is not known and will be considered in the near future by using the osmotic stress induced by polymers in the presence of sugar. The case of sugar addition discussed here, where partial exclusion of sugar occurs together with excess swelling, generalizes the concept of Donnan exclusion, long known in the case of added salt, to nonpolar solutes.

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