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Fructans insert between the headgroups of phospholipids

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Abstract

Fructans are polysaccharides consisting of one glucose unit and two or more fructose units. It was hypothesized that fructans play a role in drought tolerance in plants by interacting directly with the membrane. In this paper we investigated this hypothesis by studying fructan-membrane interactions in hydrated mono- and bilayer systems. It was found that fructans inserted between the headgroups of different kinds of phospholipids with some preference for phosphatidylethanolamine. Insertion occurred even under conditions of very tight lipid packing. The presence of a surface associated layer of fructan was observed in both model systems. This layer was able to reduce the ability of a surface-active protein to interact with the lipids. Fructans showed a much stronger effect on the different lipid systems than other (poly)saccharides, which appears to be related to their hydrophobic properties. Fructans were able to stabilize the liquid-crystalline lamellar phase, which is consistent with a drought protecting role in plants.

Keywords: Bilayer; Phase transition; Drought tolerance; Monolayer; Phosphatidylcholine; Phosphatidylethanolamine

1. Introduction

Fructans are polysaccharides, consisting of a single glucose unit attached to two or more fructose units. Their polymerization grade (DP) varies from 3 to over 100 000, depending on the producing species. Both plants and bacteria produce fructans. Two different glycosidic linkages can be found in fructans: β(2-1) linkages (inulin type) and β(2-6) linkages (levan type). Both linkages can be found in the same molecule.

In plants, fructans function as carbohydrate storage. It has been suggested that they have an important additional function, namely to protect plants against cold and dry conditions [1–3]. This hypothesis is supported by several observations. First, fruc-

Abbreviations: PE, phosphatidylethanolamine; PC, phosphatidylcholine; DPPC, 1,2-dipalmitoyl-*sn*-glycerol-3-phosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycerol-3-phosphocholine; DMPE, 1,2-dimyristoyl-*sn*-glycerol-3-phosphoethanolamine; DOPC, 1,2-dioleoyl-*sn*-glycerol-3-phosphocholine; DOPE, 1,2-dioleoyl-*sn*-glycerol-3-phosphoethanolamine; DEPE, 1,2-dielaidoyl-*sn*-glycerol-3-phosphoethanolamine; DOPG, 1,2-dioleoyl-*sn*-glycerol-3-phosphoglycerol; CF, carboxyfluorescein; MC540, merocyanine 540; MSG, 1-[$^2H_{35}$]stearoyl-*rac*-glycerol; TLC, thin layer chromatography; FPLC, fast protein liquid chromatography; LUVETs, large unilamellar vesicles prepared by extrusion technique; NMR, nuclear magnetic resonance; DSC, differential scanning calorimetry

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tan accumulating plants appeared at the time that seasonal rainfall developed, around 30 000–35 000 years ago, requiring plants to survive dry periods [1]. Second, the natural distribution of these plants coincides with regions that have a temperate to subtropical climate with seasonal or more sporadic rainfall [1]. Third, drought induces fructan accumulation in species capable of synthesizing fructans [4]. More direct evidence is presented by Pilon-Smits et al. [5], who genetically engineered a fructan accumulating tobacco plant able to synthesize levan type fructan. This plant showed an improved performance under drought stress. The growth rate was significantly higher, as were fresh weight and dry weight yields. The performance under non-stress conditions was identical to that of the wild-type tobacco plant.

Although fructan accumulation appears to be related to drought resistance, the mechanism by which fructans protect the plant is not clear yet. An important cause of cell death under dry circumstances is the loss of the selective barrier function of membranes. In the last two decades it has become clear that disaccharides can act as drought protectants for plant membranes. Crowe and Crowe [6,7] showed that membrane phase behavior under dehydrated conditions was markedly influenced by sugars in vitro. The liquid-to-gel phase transition temperature (T_m) was shifted to lower temperatures, thereby preventing a phase transition under biological circumstances. In later reports they stated that especially sucrose and trehalose are capable of membrane preservation [8].

Demel et al. [9] put forward the hypothesis that fructans would fulfill a similar function. They showed for the first time that fructans penetrate into lipid membranes using monomolecular lipid layers. This interaction was orders of magnitude larger than the interaction between disaccharides and lipids [9,10], which points towards a different type of interaction.

In this study the interactions between fructans and membrane lipids in aqueous medium are studied with a range of biophysical techniques in order to answer the following questions. What is the lipid specificity of the fructan-lipid interactions and is this interaction dependent on the lipid packing? Do fructans interact with lipids in bilayer systems and what are the consequences of this interaction for the bilayer properties? To answer these questions levan type fructan produced by *Bacillus subtilis* levansucrase was studied, the same fructan as used in the drought stress experiments in transgenic plants [5] and for which interaction with lipid monolayers was shown [9]. Special attention was paid to obtain a very pure polysaccharide preparation.

Fructans were found to insert between the headgroups of many different lipids even at very high packing densities both in mono- and bilayers. Fructans had a stronger effect on the membrane surface properties than several other (poly)saccharides tested. Most importantly, fructans were able to stabilize the liquid-crystalline phase, which is consistent with a drought protecting role in plants.

2. Materials and methods

2.1. Materials

1,2-Dipalmitoyl-*sn*-glycerol-3-phosphocholine (DPPC), 1,2-dimyristoyl-*sn*-glycerol-3-phosphocholine (DMPC), 1,2-dioleoyl-*sn*-glycerol-3-phosphocholine (DOPC) 1,2-dimyristoyl-*sn*-glycerol-3-phosphoethanolamine (DMPE), dielaidoyl-*sn*-glycerol-3-phosphoethanolamine (DEPE), 1,2-dioleoyl-*sn*-glycerol-3-phosphoethanolamine (DOPE) and 1,2-dioleoyl-*sn*-glycerol-3-phosphoglycerol (DOPG) were obtained from Avanti Polar Lipids (Birmingham, AL). 1-Hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine (pyrene PC) was purchased from Molecular Probes (Eugene, OR). Carboxyfluorescein (CF) was obtained from Kodak and purified according to Ralston et al. [11]. Sucrose [fructose-1-^{3}H] in ethanol:water 7:3 (37 MBq/ml, specific activity 377.4 Gbq/mmol) was obtained from NEN Life Science Products (Boston, MA). Dextran (MW 76 900) and merocyanine 540 (MC540) were purchased from Sigma (Zwijndrecht). SecA was obtained as described in [12]. 1-[$^2H_{35}$]Stearoyl-*rac*-glycerol (MSG) was synthesized as described in [13].

2.2. Isolation and purification of fructans from *B. subtilis*

Fructans were isolated from the supernatant of *B. subtilis*, which synthesizes levan type fructans (β(2-6)

linkage). We observed that in the preparation used by Demel et al. [9] still some protein (1.5–4% w/w) was present, including levansucrase. Therefore, the purification protocol was improved. *B. subtilis* was grown as described by Demel et al. [9]. The cells were pelleted by centrifugation for 10 min at $4500 \times g$. Two volumes of cold ethanol (4°C) were added to the obtained supernatant and fructans were allowed to precipitate overnight at 4°C. The mixture was centrifuged for 10 min at $9000 \times g$ at 4°C and the pellet was subsequently dissolved in 5 mM Tris-HCl pH 8.0. The fructan solution was applied to a 1 ml resource Q column (Pharmacia) eluted with 5 mM Tris-HCl pH 8.0 or 5 mM phosphate buffer pH 8.0 on a Pharmacia FPLC system. The void volume peak was collected and freeze-dried. When removal of phosphate was needed, gel filtration was performed using a Sephadex G-10 column in the Pharmacia FPLC system eluted with 5 mM Tris-HCl pH 8.0. To reduce osmotic imbalances in the vesicle experiments all salts were removed by dialysis against MiliQ water using a SpectraPore dialysis membrane with a molecular cutoff of 1 kDa. TLC was performed to check the purity and to determine the concentrations of fructans [14]. The intensity of the spots was measured using an UltroScan XL (Pharmacia). The polydispersity of the sample was measured using gel filtration [15]. There was a very prominent peak at 25 kDa (± 2 kDa) and a small fraction of fructans was larger but less well defined. No fructans were found to be smaller than 22 kDa. The protein content of the fructan preparation was measured by Bradford analysis [16].

2.3. Synthesis of ^{3}H-labeled fructan

To synthesize labeled fructans, levansucrase was isolated according to Ebskamp et al. [17]. Levansucrase was desalted using a Sephadex G-10 column in a Pharmacia FPLC system eluted with 5 mM Tris-HCl pH 6.0 using detection at 280 nm. The fractions were stored at −20°C. The final protein concentration was 0.7 μg/μl. An aliquot of 50 μl ^{3}H-sucrose solution was dried under a stream of nitrogen to remove the ethanol. Subsequently, 3.3 μl 60% (w/w) sucrose in water, 10 μl levansucrase solution and 39 μl buffer (50 mM MES/KOH pH 6.0, 5 mM $CaCl_2$) were added to the ^{3}H-sucrose. This mixture was incubated for 48 h at 30°C. After the incubation, 5 μl 1 M Tris-HCl pH 8.0 was added. The mixture was loaded onto a 0.75 ml DEAE column and eluted with 5 mM Tris-HCl pH 8.0. Two column volumes were collected and lyophilized. This sample was hydrated and desalted using a Sephadex G-10 FPLC column as described in Section 2.2 to largely reduce the amount of sucrose. The purity of the samples was checked using TLC as described in Section 2.3. To ensure that the labeled fructan had the same interaction with lipids as the fructan isolated from *B. subtilis*, non-labeled fructan was produced using the same protocol in such an amount that the interaction with lipids could be tested in monolayer experiments. It was found that the fructan produced in this protocol behaved identically to the fructan isolated from *B. subtilis*.

2.4. Vesicle preparation

A lipid dispersion was obtained by drying a chloroform solution of lipids using rotational evaporation. After storage of the lipid film for at least 2 h under high vacuum, the lipids were dispersed in the appropriate buffer and warmed to above their gel to liquid-crystalline phase transition temperature under mechanical agitation.

Large unilamellar vesicles (LUVETs) were prepared by extrusion according to Hope et al. [18]. A dry lipid film was hydrated in the appropriate buffer. The sample was freeze-thawed ten times in a CO_2/ethanol bath and subsequently extruded ten times through two stacked 400 nm membrane filters.

The lipid-phosphate concentration in the resulting solutions was determined according to Rouser [19].

2.5. Monolayer experiments

2.5.1. Measurements at constant surface area

The surface tension was measured using the Wilhelmy plate method [9,20]. Experiments were performed in a 2.0 ml dish at room temperature. MiliQ water was used as subphase, on which a lipid layer was spread until a surface pressure between 20 and 35 mN/m was reached. Saccharides were added to the subphase through a small injection hole, while the subphase was continuously stirred. Within approx. 30 min, the pressure increase was stabilized.

The change in surface pressure at that time point was taken as a measure for the interaction of the saccharides with the lipid phase. In the absence of a lipid film the saccharides themselves showed a surface pressure increase less than 0.6 mN/m.

2.5.2. Pressure-area curve measurements

Pressure-area curves were measured, after spreading of 5 nmol lipid on a surface of 65 cm^2. As subphase either water or an 8 mg/ml fructan solution was used. After 10 min equilibration, the lipid layer was compressed till close to the collapse pressure with a rate of 15 cm^2/min and subsequently the lipid layer was expanded at the same rate. Expansion and compression were repeated several times and found to be reproducible.

2.5.3. Binding experiments

Fructans as obtained in Section 2.3 were mixed with the appropriate amount of radioactively labeled fructan. For this experiment a dish with a surface of 8.5 cm^2 (volume 5.5 ml) was used. The fructan mixture was added to the subphase via the small hole in the trough, while the subphase was continuously stirred. After reaching the maximum pressure increase, a 200 μl sample was taken from the subphase. Subsequently, the subphase was refreshed by flushing five times with water, while the surface pressure was monitored. The monomolecular layer was collected by suction into a counting vial. Simultaneously, the surface area was gradually reduced with a movable barrier to ensure that the monolayer was collected completely. After removal of the monolayer, a 200 μl sample was taken from the subphase to correct for the residual amount of radioactivity present in the subphase, which was collected together with the monolayer. To the different samples and the collected monolayer, 7.5 ml LSC cocktail (Packard, emulsifier-safe) was added and the amount of radioactivity was determined using the 1500 Tri-Carb liquid scintillation analyzer.

2.5.4. Accessibility assay

A DOPG film was spread to a surface pressure of 19 mN/m on a subphase of 10 mM Tris-HCl pH 7.5. An amount of fructan was injected into the subphase to yield a final concentration of 10 mg/ml. After equilibration for 30 min, 15 μg (8 μl) of the protein SecA was added and the pressure increase was monitored. As a control a DOPG film was spread which had the same surface pressure as the DOPG layer after fructan insertion. Next, 15 μg of SecA was injected and the surface pressure increase was monitored.

2.6. Centrifugation experiments

LUVETs were prepared of DMPC and 0.5% pyrene PC for detection purposes as described in Section 2.4. Vesicles (0.5 mM lipid) were incubated with or without the indicated amount of fructan at 30°C for 30 min in a total volume of 300 μl. After incubation, the solutions were centrifuged for 15 min at $20\,000 \times g$ at 30°C. Since the pellet was not very stable, only 80% of the volume was collected as supernatant. The remaining 20% was mixed together with the pellet and is referred to as pellet fraction. The amount of lipid in the fractions was determined in the presence of 0.5% Triton X-100 by fluorescence spectroscopy using a SPF-500 SLM Aminco fluorimeter. Excitation was performed at 345 nm, and the amount of fluorescence was measured at 378 nm.

2.7. Carboxyfluorescein leakage experiments

Carboxyfluorescein (CF) leakage experiments were performed according to Breukink et al. [21]. LUVETs were prepared in the presence of 50 mM CF, 50 mM K_2SO_4, 50 mM MES/KOH pH 6.5. After extrusion (see Section 2.4), the LUVETs were separated from the remaining CF by size exclusion chromatography with a Sephadex G-50 column using 50 mM MES/KOH, 100 mM K_2SO_4 as elution buffer. For the fluorescence experiments, vesicles (25 nmol lipid) were diluted in 1.2 ml elution buffer and fructan solutions were added under continuous stirring. CF leakage was measured by determining the fluorescence at 514 nm with the excitation wavelength at 492 nm. Total permeabilization was obtained by addition of 50 μl 10% Triton X-100.

2.8. MC540 partitioning

The surface properties of a vesicle can be accessed by the probe molecule MC540 [22]. The partitioning of the probe between hydrophobic and hydrophilic

environment depends on the packing of the headgroup region. The ratio between the absorption at 530 nm and 570 nm is a measure for this partitioning [23]. LUVETs were made as described in Section 2.4 using 10 mM Tris-HCl pH 7.5 buffer. In the sample 0.5 mM lipid was incubated at the indicated temperature with the appropriate amount of saccharide in a final volume of 1 ml. After 30 min, 2 μl of an ethanol/water (1:2 v/v) solution of the dye was added resulting in a final dye concentration of 6.6 μM. The mixture was incubated for another 5 min. Next, the mixture was transferred to a 1 ml cuvette and the absorption of MC540 was measured in a Perkin Elmer Lambda 18 UV/VIS spectrometer.

2.9. DSC measurements

Lipid dispersions of DEPE and DPPC were formed in 10 mM Tris-HCl pH 7.4 buffer in the absence or presence of the indicated amount of fructan. DPPC phase transitions were also measured using LUVETs prepared in the same buffer. Thermograms were recorded on a Microcall DSC differential scanning calorimeter. Samples were repetitively scanned at a rate of 20°C/h. DEPE was measured in the range of 30–70°C and DPPC was measured from 20°C to 55°C. The scans were processed using Microcall software.

2.10. ^{31}P-NMR

Lipid dispersions of DEPE were prepared in 10 mM Tris-HCl pH 7.4 buffer in the absence or presence of the indicated amount of fructan. Samples were spun down at 5000×*g* for 5 min at room temperature to concentrate the lipid dispersion. ^{31}P spectra were recorded on a Bruker MSL 300 spectrometer. A Bruker B-VT1000 temperature controller regulated the sample temperature. Proton decoupling was carried out at 121.5 MHz, with a 15 μs 90° pulse, a 1.3 s interpulse time, and gated proton-noise decoupling. At each temperature approx. 1000 scans were acquired. A sweep width of 50 kHz, 4096 data points, and a 100 Hz line broadening was used.

2.11. ^{2}H-NMR

An amount of 20–25 mg dry MSG was hydrated in 3 mM Tris-HCl pH 7.4 buffer in deuterium depleted water with or without fructan. The samples were heated for 15 min at 65°C and subsequently cooled to room temperature. The samples were stored at 4°C till the ^{2}H-NMR measurements. The measurements were carried out as described in [13].

3. Results

3.1. Monolayer studies

3.1.1. Fructan-PC interaction

In the paper of Demel et al. [9] a large pressure increase was measured upon injection of fructans under a PC monolayer. We noticed that the purification protocol used in their paper resulted in a sample which still contained a small amount of protein that could potentially contribute to the measured effects. Therefore, we improved the purification method as described in Section 2. This resulted in a fructan preparation in which no protein could be detected. Given the detection limit of the method, a maximum protein concentration of 0.1% w/w can be estimated, which is at least a 20-fold reduction as compared to the previous preparation. To get insight into the lipid-interacting properties of this preparation we measured the pressure-area curve of DPPC in the absence and presence of fructans as shown in Fig. 1A. The pure DPPC showed a phase transition from liquid expanded to liquid condensed state at 5 mN/m as reported in the literature [9]. Upon the addition of fructans the curve was shifted to larger areas both in the liquid expanded and the liquid condensed state. This demonstrates penetration of fructan into the monolayer under both lipid packing conditions. The phase transition was observed in the presence of fructans in contrast to the study of Demel et al. [9] in which the transition was broadened beyond detection.

The interaction of this highly purified fructan fraction with the liquid expanded state of lipids was further investigated using DMPC as shown in Fig. 1B,C. Fructans penetrated into the liquid expanded monolayer of this shorter chain lipid as demonstrated by the maximal surface pressure increase of 7.5 mN/m observed at approximately 10 mg/ml fructan (1B). To gain further insight into the fructan-

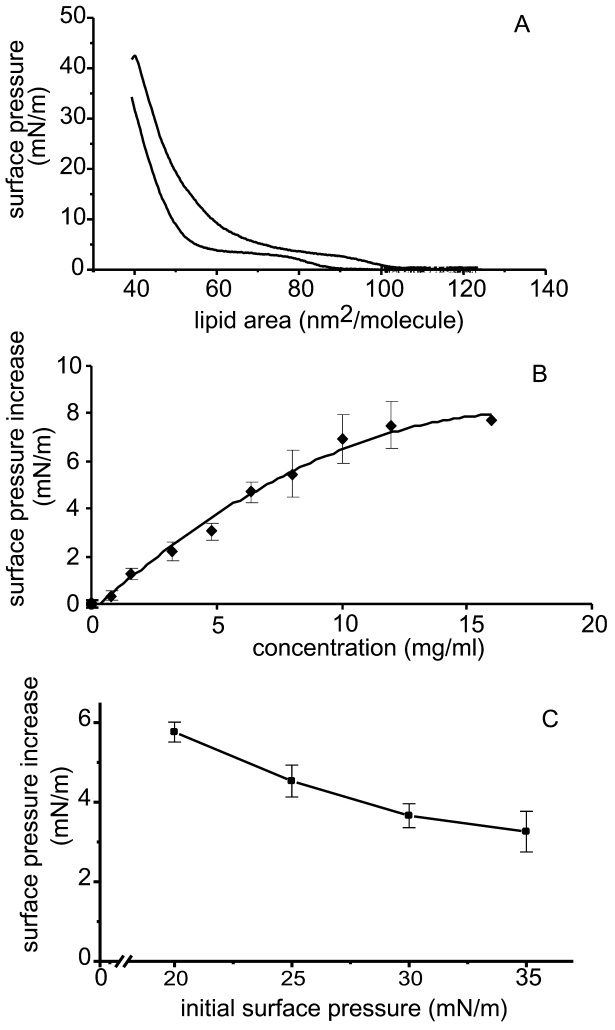

Fig. 1. The effect of fructan on the surface pressure of PC monolayers. (A) Pressure-area curve of DPPC monolayer in the absence (lower curve) and presence (upper curve) of 8 mg/ml fructan. (B) Surface pressure increase induced by different fructan concentrations in a DMPC monolayer spread at 20 mN/m. (C) Surface pressure increase induced by 8 mg/ml fructan as a function of the initial surface pressure of a DMPC monolayer.

DMPC interaction, the surface pressure increase was measured as a function of the initial surface pressure using a constant amount of fructan as shown in Fig. 1C. It was shown that fructans also inserted into monolayers at very high initial surface pressures, even exceeding pressures corresponding to packing in lipid bilayer systems [24].

The binding of fructan to DMPC monolayers was studied using radioactively labeled fructan. To wash away the radiolabel, the subphase was flushed several times (see experimental section). It was observed that during this procedure the surface pressure of the monolayer was hardly affected demonstrating the stability of the fructan-DMPC interaction. Fructan binding was determined as the amount of fructose residues bound per DMPC molecule. For a DMPC layer spread at an initial surface pressure of 20 mN/m and a subphase concentration of 8 mg/ml fructan, the amount of fructose per DMPC molecule was found to be 5:1 (± 2 fructose units, mean $\pm$ S.D. obtained from five separate experiments). The relatively large error in the measurements was most likely due to the fact that only 0.1% of the added radioactively labeled fructan adhered to the lipid monolayer. This ratio cannot be interpreted as insertion of 5 fructose units per lipid molecule, since part of the long fructan molecules is likely to be in the subphase and not in direct interaction with the monolayer.

The polysaccharide-lipid interaction showed specificity towards the polysaccharide chain, since the

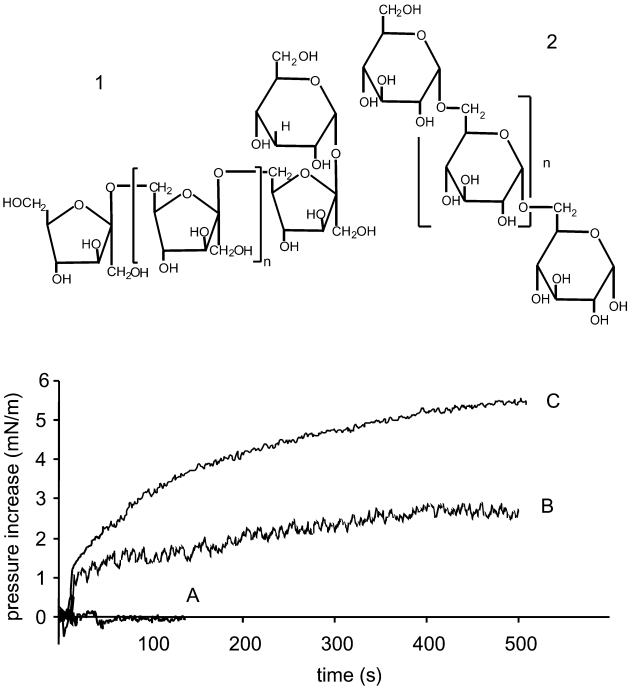

Fig. 2. Effect of different sugars on the surface pressure of DMPC monolayers. A monolayer was spread at an initial surface pressure of 20 mN/m. At time point zero a sugar solution (sucrose, A; dextran, B; fructan, C) was injected underneath the monolayer resulting in a final concentration of 8 mg/ml. The structures of the polysaccharides are displayed in the top of the figure: 1, fructan (levan type); 2, dextran.

surface pressure increase in a DMPC monolayer was much larger for fructan than for dextran (Fig. 2). The chemical structures of the polysaccharides are included in the figure, demonstrating that the repeating unit of a dextran is a six-membered ring and of a fructan is a five-membered ring. Small saccharides as sucrose (Fig. 2) and trehalose (data not shown) hardly affected the surface pressure of the monolayer as reported earlier [9,10].

3.1.2. Fructan-PE interaction

To get insight in the headgroup specificity of fructan insertion into monolayers, PE, which is another abundant zwitterionic phospholipid in plants, was tested. DMPE was chosen, because it has a well documented phase transition [9], which is similar to DPPC monolayers as shown by the pressure-area curve (compare Fig. 3A and 1A). The presence of 8 mg/ml fructan caused a large area increase both below and above the phase transition, indicating insertion into the monolayer in both phases. The observed effects were even more pronounced when compared to DPPC, showing that the insertion of fructans into lipid layers is not restricted to PC, but is a more general phenomenon. The phase transition of DMPE was still observed in the presence of fructans, but shifted to larger areas due to the penetration of fructans into the monolayer.

In the liquid condensed state of DMPE (initial surface pressure 20 mN/m) a comparable fructan concentration dependence for the surface pressure increase was found as for DMPC in the liquid expanded state. The saturation concentration for the DMPE-fructan interaction was approx. 15 mg/ml. However, the maximum surface pressure increase obtained in this system was larger than for DMPC. This was most likely not due to a larger extent of penetration, but reflects the steeper pressure-area curve of DMPE in the liquid condensed state. Since DMPC and DMPE can not be easily compared due to the different state of the monolayer, we also measured the interaction of 8 mg/ml fructans with a monolayer of DOPC and DOPE spread at 20 mN/m. Fructans interacted more favorably with PE than PC, since the surface pressure increase for DOPE was 7.7 ± 0.4 mN/m and for DOPC 4.0 ± 1.0 mN/m at 8 mg/ml (data not shown).

In Fig. 3C it is shown that fructans had the ability

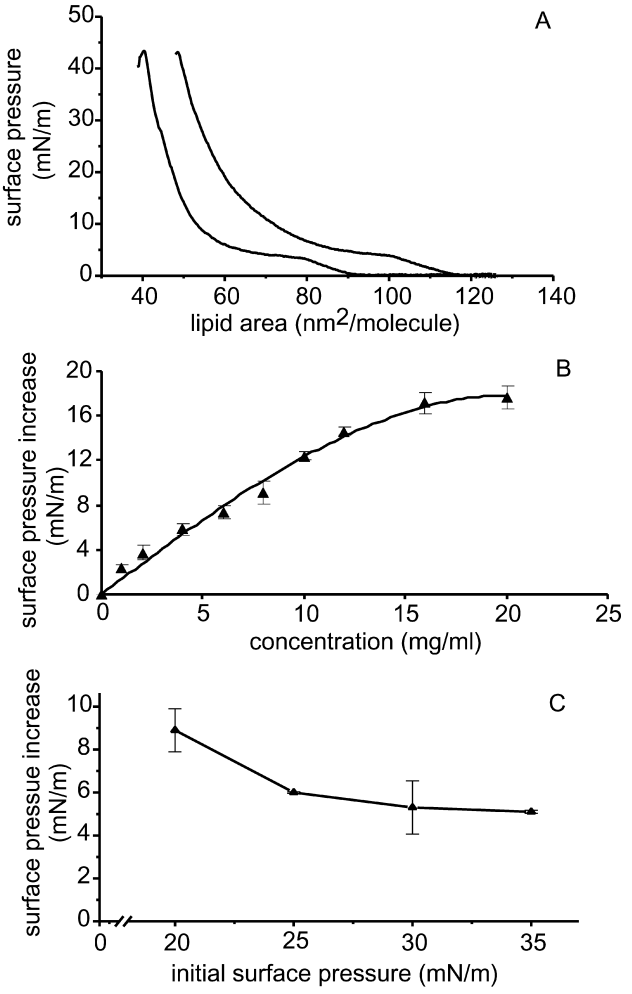

Fig. 3. The effect of fructan on the surface pressure of DMPE monolayers. (A) Pressure-area curve of DMPE in the absence (lower curve) and presence (upper curve) of 8 mg/ml fructan. (B) Surface pressure increase induced by different fructan concentrations in a DMPE monolayer spread at 20 mN/m. (C) Surface pressure increase induced by 8 mg/ml fructan as a function of the initial surface pressure of the DMPE monolayer.

to insert into condensed PE lipid layers even at very high initial surface pressures. Interestingly, the penetration of fructan into the DMPE monolayer is reversible, since washing of the subphase after stabilization of the fructan-DMPE interaction resulted in a surface pressure decrease of at least 65%. Consequently, no significant amount of radioactivity could be detected in the monolayer (data not shown). To investigate whether this effect was due to the lipid phase or the lipid headgroup we tested DOPE, which is in the liquid expanded state at 20 mN/m. The sur-

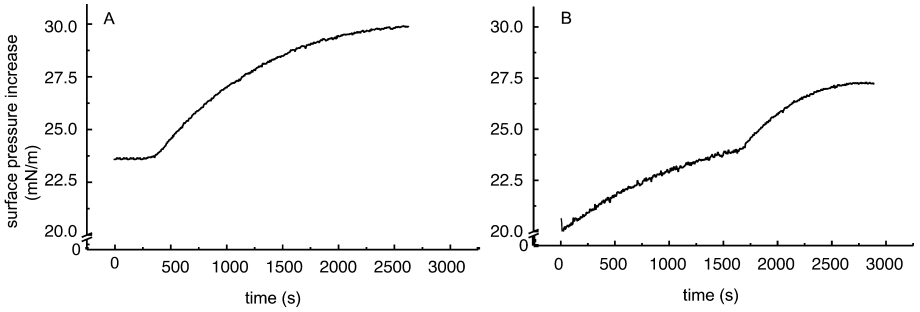

Fig. 4. The effect of fructan on the accessibility of SecA to a DOPG monolayer. Panel A shows the surface pressure increase induced by 15 μg SecA into a DOPG monolayer with an initial surface pressure of 20 mN/m. Panel B shows the surface pressure increase upon injection of 10 mg/ml fructan and subsequent injection of 15 μg SecA underneath a DOPG monolayer with an initial surface pressure of 15 mN/m.

face pressure during washing remained stable, suggesting that a tight interaction between PE and fructan is possible under liquid expanded conditions. Therefore it was concluded that fructans bind less tightly to the lipids which are in the liquid condensed state.

3.1.3. Accessibility of the monolayer in the presence of fructan

One important consequence of the fructan-lipid interaction could be that other membrane interacting molecules such as proteins are hindered in their interaction with lipids in the presence of fructans. We tested this possibility using SecA as a model protein. This water soluble dimeric protein (100 kDa per monomer) is an important component of the preprotein translocase in bacteria and due to its amphipathic properties can readily insert into monolayers containing anionic lipid [25]. This is demonstrated in Fig. 4A where injection of SecA causes a large increase in surface pressure as previously shown by Breukink et al. [25]. Fructans also interact with monolayers of this unsaturated anionic phospholipid as indicated by the pressure increase of 4.1 ± 0.5 mN/m shown in Fig. 4B. In this experiment the initial surface pressure of the monolayers was chosen such that after injection of fructans the surface pressure increase resulted in a value similar to the initial pressure of the experiment shown in Fig. 4A. Subsequent injection of an equivalent amount of SecA underneath this monolayer caused a $30 \pm 10\%$ reduction in surface pressure increase as compared to the control. It was verified that this reduction was not due to complexation of SecA to fructan in the solution, since a subsequent addition of SecA did not increase the surface pressure any further. This result shows that fructans associated with a DOPG monolayer can partially prevent the SecA-DOPG interaction, possibly by shielding the lipid headgroup region.

3.2. Studies on lipid vesicles and dispersions

3.2.1. Fructan association to vesicles

Our monolayer results suggested that fructans should be able to interact with phospholipid bilayers. To investigate this possibility we studied the association of fructans with LUVETs of DMPC in the liquid-crystalline state. Unfortunately, direct binding experiments using centrifugation methods could not be performed because the largest fructan molecules precipitate readily even at low speeds. Therefore, an alternative method was applied, in which the ability of fructans to pellet DMPC LUVETs was measured

Table 1
Fructans associate with DMPC LUVETs[a]

Fructan concentration (mg/ml)	% lipid in pellet fraction
0	45 ± 2
1	52 ± 2
2.5	53 ± 2.5
5	57 ± 2.5
10	57 ± 5

[a]LUVETs were composed of DMPC and 0.5% pyrene lipid and incubated at 30°C with the indicated amounts of fructan and subsequently centrifuged for 15 min at $20\,000 \times g$ at 27°C. The amount of lipid in the pellet fraction (pellet fraction was defined as the bottom 20% of the total volume of the sample) was determined using fluorescence.

(Table 1). The centrifugation rate was chosen such that in the absence of fructan 45% of the vesicles were present in the pellet fraction. Increasing fructan concentrations caused an increase in the amount of vesicles, which were recovered in the pellet fraction up to 57% at 10 mg/ml. It should be realized that the increase in density of the solution by fructans will counteract pelletation of the vesicles resulting in an underestimation of the effects shown.

The most straightforward interpretation of the data is that fructans associate with the lipid vesicles. Further support for this interpretation came from experiments using the lipid bilayer probe MC540. This amphipathic dye partitions between the membrane and the water phase depending on the interfacial packing density [22]. Partitioning is reflected by the absorbance ratio A_{570}/A_{530}; upon entering a hydrophobic environment the absorbance at 570 nm increases, whereas the absorption maximum at 530 nm is slightly reduced [23]. Fig. 5A illustrates the sensitivity of the method using DMPC LUVETs in the gel (L_β) and liquid-crystalline phase (L_α). Addition of vesicles increased the A_{570}/A_{530} ratio due to entry of the dye into the vesicle bilayer, a process that was saturated at 0.5 mM lipid. The much larger ratio in the L_α phase as compared to the L_β phase reflects the much tighter packing of the lipids in the gel state.

In Fig. 5B the effect of polysaccharides on the partitioning of MC540 into DMPC vesicles is shown. Dextrans did not hinder the partitioning of the probe into the lipid phase. In contrast, fructans decreased the partitioning into DMPC vesicles both in the gel phase and in the liquid-crystalline phase of lipids. In the liquid-crystalline phase the partitioning for MC540 was reduced even further than in the gel phase. This experiment indicated that the packing density at the surface of the lipid vesicle was higher in the presence of fructans.

To investigate whether the effects observed in the experiment were due to dye-polysaccharide interactions, the effect of carbohydrates on MC540 was studied in the absence of lipid vesicles (Fig. 5C). Dextran hardly changed the absorbance ratio of the dye. Fructans, however, showed a large increase in the ratio with increasing concentrations, which is opposite to the effect observed in the presence of the vesicles. These results show that the effect measured

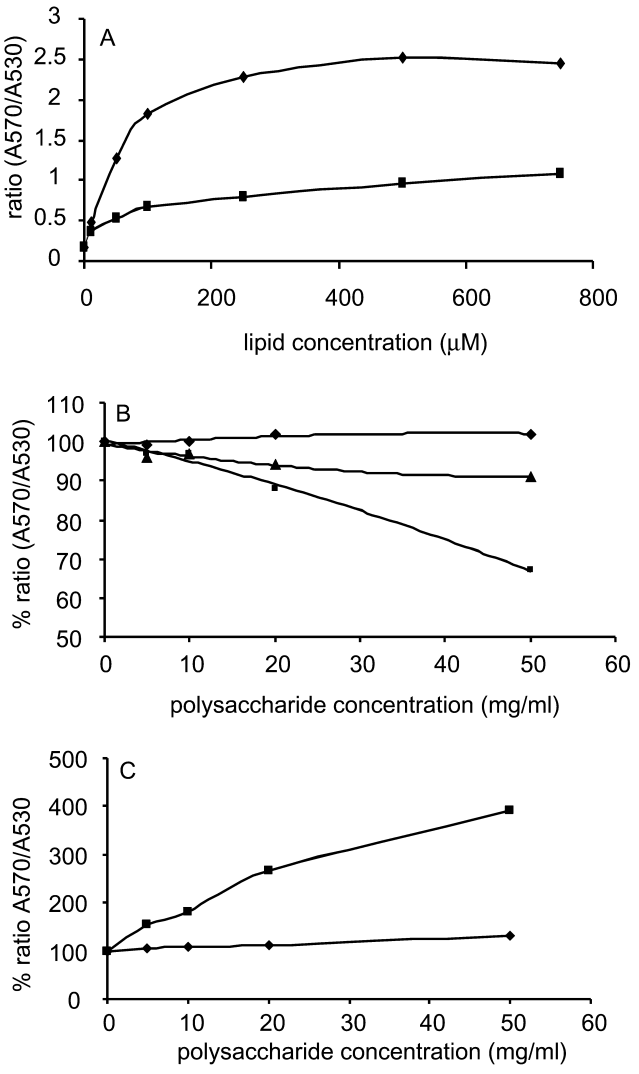

Fig. 5. The effect of fructans on the partitioning of MC540 between water and DMPC vesicles. (A) The effect of incubation temperature and DMPC vesicle concentration on the A_{570}/A_{530} ratio of a constant amount of MC540. The samples were incubated for 30 min at 30°C (♦) and 15°C (■), respectively. (B) The effect of fructan and dextran on the partitioning of MC540. 0.5 mM of DMPC LUVETs was mixed with the indicated amount of polysaccharide and the A_{570}/A_{530} ratio was measured. The ratio at the given temperature in the absence of any polysaccharide was set as 100%. ♦, incubation with dextran at 30°C; ▲, incubation with fructan at 15°C; ■, incubation with fructan at 30°C. (C) The effect of an increasing polysaccharide concentration (■, fructan; ♦, dextran) on the A_{570}/A_{530} ratio of MC540. In the absence of polysaccharide the ratio was set to 100%.

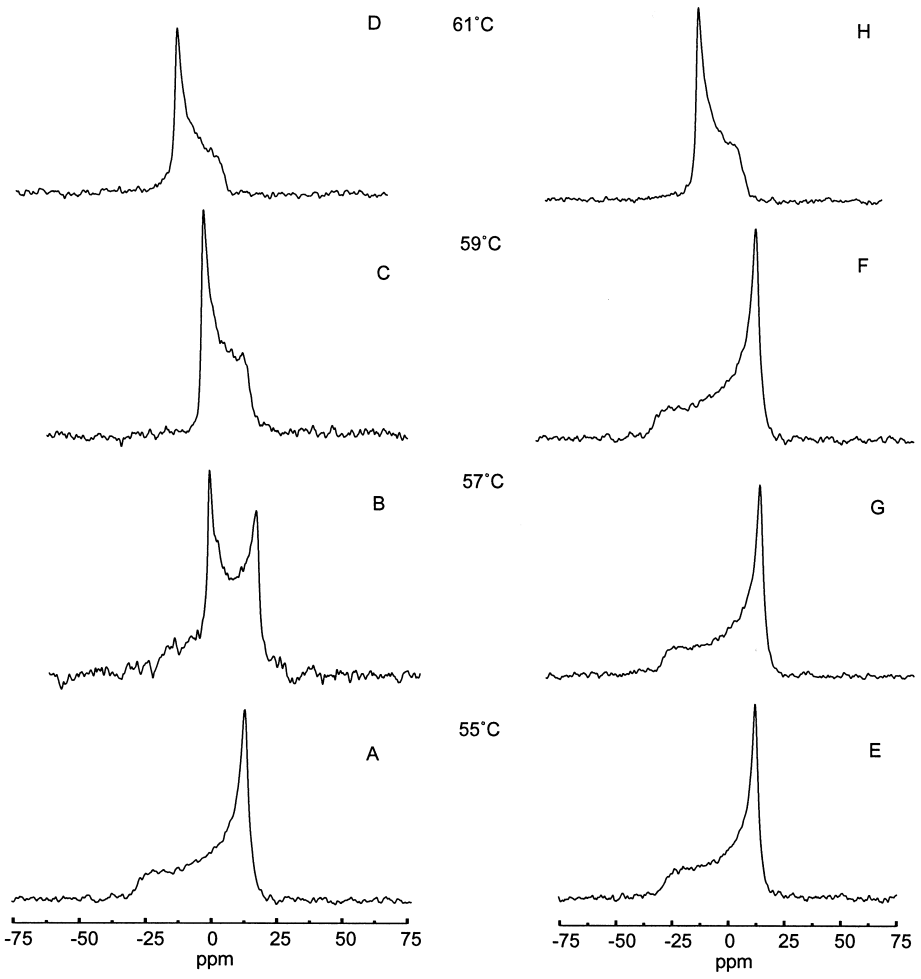

Fig. 6. The effect of fructans on the phase transition of DEPE as measured by ^{31}P-NMR. In the left panel pure DEPE is depicted. In the right panel the DEPE mixed with 10 mg/ml fructan is displayed.

in the presence of vesicles originates from the fructan-bilayer interaction and that the interaction might be larger due to the counteracting spectral changes caused by the fructan-dye interaction. It also indicates that fructans probably possess hydrophobic sites to which the dye can bind.

Because fructans associate with lipid vesicles, they could compromise the membrane integrity. This was tested by CF leakage experiments using DMPC vesicles at 30°C. Addition of maximally 20 mg/ml fructan did not give any CF efflux demonstrating the integrity of the DMPC bilayer in the presence of the sugar (data not shown).

3.2.2. *The influence of fructans on lipid polymorphism*

To test whether the fructan-lipid interaction affected the gel-to-liquid phase transition we analyzed lipid dispersions of DPPC and DEPE in the absence or presence of fructans using DSC. No significant influence of fructans on the L_β-L_α transition was measured for both lipids (effects on the phase transition temperature were smaller than 0.2°C and 0.5°C, respectively). However, using DEPE the transition temperature of L_α to the inverted hexagonal phase (H_{II}) was shifted slightly upward (data not shown). To investigate the latter transition in more detail ^{31}P-NMR was performed. This technique gives information on the different phases adopted by dispersions of DEPE [26]. In Fig. 6 the spectra of DEPE are shown, both in the presence (right) and absence of fructans (left). The spectrum in Fig. 6A, recorded at 55°C, had a low field shoulder and a high

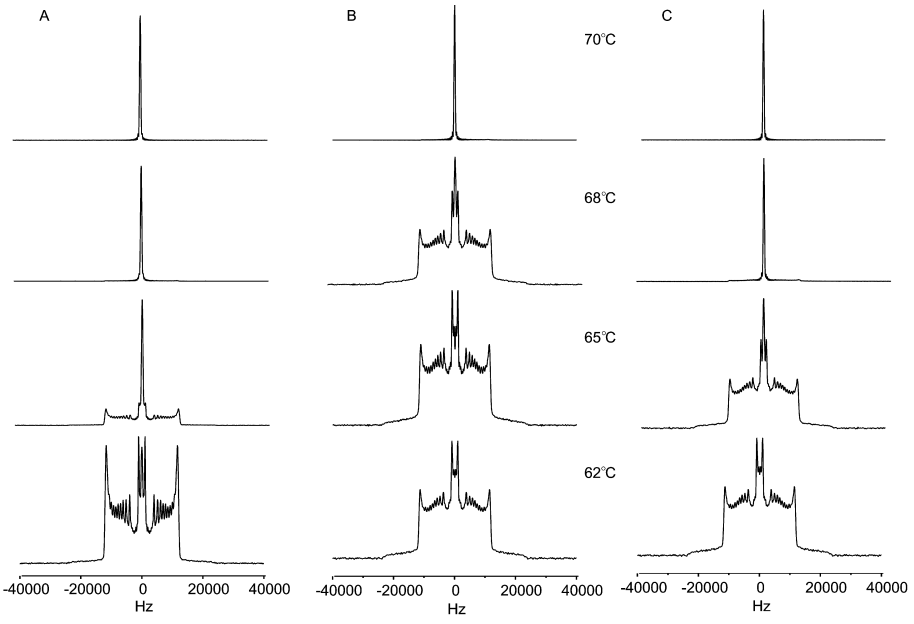

Fig. 7. The effect of fructans and dextrans on the phase transition of MSG as measured by ^{2}H-NMR. In the left panel pure MSG is depicted. In the middle panel MSG is mixed with 200 mg/ml fructan and in the right panel with 200 mg/ml dextran.

field peak, typical for a liquid-crystalline phospholipid bilayer organization. When the temperature was increased, the typical spectral characteristics of the H_{II} phase appeared. These spectra now have a 2-fold reduced chemical shift anisotropy of inverted sign as compared to the spectrum of the L_α phase (Fig. 6C,D). The spectra recorded in the presence of 10 mg/ml fructan showed that the L_α-H_{II} phase transition temperature was increased by 4°C. This result indicates a stabilization of the L_α phase in the presence of fructans. In addition, the spectral characteristics were unaffected in both phases, indicating that the fructans hardly affect the motional properties of the lipid headgroups at the NMR time scale.

Cubic phases occur in a phase diagram in between the lamellar and inverted hexagonal phases [27]. They are suggested to play an important role in several biological processes [27,28] as for instance membrane fusion. To analyze the effects of fructans on the formation of a cubic phase we selected monostearoyl glycerol (MSG) as test lipid. The phase behavior of hydrated dispersions of this lipid is well known [13] and this lipid forms a stable cubic phase. We used chain deuterated MSG, because this gave us the possibility to determine both the phase behavior and the chain order of the lipid. In Fig. 7 the ^{2}H-NMR spectra of a dispersion of MSG in water are shown. The spectrum at 62°C is typical of the L_α phase and consisted of a series of overlapping spectra originating from the various quadrupolar splittings of the deuterons along the hydrocarbon chain. The peaks corresponding to carbons 9–18 are well resolved. The terminal methyl group gives the narrow doublets in the center of the spectrum. At 68°C and higher temperature the ^{2}H-NMR spectra showed one isotropic peak typical for the cubic phase where rapid isotropic motion capability averages the quadrupolar interaction. The L_α-I transition occurred around 68°C. In the presence of 200 mg/ml fructans the L_α-I transition temperature was increased by 4°C showing the stabilization of the L_α phase (Fig. 7B). This is a specific effect because addition of a similar amount of dextrans hardly affects the L_α-I transition (Fig. 7C). The quadrupolar splittings of MSG in the L_α phases were not affected by the carbohydrate demonstrating that chain order/mobility was not changed.

4. Discussion

In this study we have investigated the interactions of fructans with lipid monolayers and we have shown that fructans also interact with hydrated bilayer sys-

tems. The lipid specificity of the interaction was analyzed and a comparison between different (poly)saccharides was made. Insight into the nature of the interaction was obtained using the MC540 dye, and ^{31}P-, ^{2}H-NMR experiments. The results lead to a model for the interaction of fructans with hydrated bilayers and give suggestions on the cryoprotective action of the polymer.

Using the monolayer technique we observed that fructans insert into monolayers composed of lipids with a PC headgroup (DMPC, DPPC and DOPC) and also into monolayers of DMPE, DOPE and DOPG. This suggests that the fructan-phospholipid interaction is of a general nature with a limited lipid specificity. However, some lipid preference can be observed. The surface pressure increase for DOPE was twice as large as for DOPC or DOPG. The latter two showed a similar surface pressure increase. The increased insertion of fructans into PE monolayers may be due to the relatively small headgroup, which probably leaves more room for insertion. The capability of the PE headgroup to form hydrogen bonds may also play a role [29].

Fructan can insert in both the liquid expanded and the liquid condensed state (comparable to the liquid-crystalline and the gel state of bilayers, respectively). Moreover, in both phases the fructan can insert even at very high initial surface pressures. This demonstrates the membrane penetration power of fructans. Interestingly, a large difference in the stability of the interaction between the liquid expanded and liquid condensed state was observed. In the latter phase most of the bound fructan can be readily washed away, as for the liquid expanded state the fructan-lipid interaction is stable. This suggests that the interaction is sensitive to the packing density and that the fructan-lipid interaction is less tight when the lipids are closely packed.

In this study we observed that fructans interact with bilayer lipid systems. The first observation was that in the presence of fructans more DMPC lipid vesicles were pelleted after a centrifugation step. Whether this effect is due to aggregation of the vesicles or simply due to binding of fructans to the vesicles was not further investigated. However, confirmation of the notion that fructans bind to vesicles was obtained by studying the absorption ratio A_{570}/A_{530} of MC540. This ratio reflects the partitioning of the dye between the water and the membrane phase [23] and is dependent on the surface pressure density [22]. When MC540 was added to a LUVET/fructan mixture the ratio was smaller than in the absence of fructans. This suggests that the partitioning of the probe into the membrane is reduced, indicating that the surface packing density is higher in the presence of fructans. These data strongly indicate that fructans are bound to the lipid vesicles. Moreover, these experiments confirmed that fructans display an interaction with both the gel and the liquid-crystalline phase, since in both phases the A_{570}/A_{530} ratio was decreased.

These results provide insight into the molecular basis of the fructan-lipid interaction. Most likely, fructans bind in the lipid headgroup region. Electrostatic interactions do not play a significant role, since fructans showed a similar surface pressure increase for both the anionic DOPG and the zwitterionic DOPC. Instead, hydrophobicity is probably important in the interaction as shown in the experiments using MC540. When MC540 was added to fructans, the absorption ratio was increased which indicates that the fructans contain hydrophobic sites. In contrast, when MC540 was added to a dextran solution, the absorption ratio was not increased. Dextrans did not interact with the lipid vesicles under these conditions and had a minimal effect on the surface pressure. Fructans contain an additional CH_2 group in comparison to dextrans (Fig. 2). This feature will increase the hydrophobicity of fructans, which could be responsible for the increased interaction with phospholipid model membranes. Taking into account the polar nature of the fructans with their large amount of hydroxyl groups it is logical to assume that fructans do not insert deeply into the membrane.

Superficial interactions are not expected to have a large effect on the acyl chain packing, which is consistent with the observations that fructans hardly affect the gel-to-liquid phase transition in bilayers and the order parameters of the acyl chains of the lamellar phase of MSG. The L_α-H_{II} transition of DEPE and the L_α-I transition of MSG were shifted upwards in temperature demonstrating a bilayer stabilizing action of the polysaccharide. In contrast, small disaccharides lower the L_α-H_{II} transition temperature [30]. The stabilization by fructans can be ex-

plained by the insertion of the polysaccharides between the lipid headgroups, thereby reducing the negative curvature stress induced by the rather small headgroup of these lipids.

What are the consequences of these findings for the biological function of fructans under hydrated conditions? First, the fructans are expected to stabilize the L_α phase, which is relevant, because many membranes contain large amounts of non-bilayer lipids as PE. In addition, we also measured a very large amount of fructan subunits associated per lipid molecule in the in the monolayer (5:1, respectively). It is improbable that this reflects the amount of inserted fructose units per lipid, but it most likely reflects the presence of an extended layer of carbohydrate adhered to the lipids and partially sticking into the aqueous phase. Most likely, the membranes are coated by fructans. In a biological system, this may have many implications, for example a reduction in the accessibility of the membrane surface for proteins. Consistent with this hypothesis we observed that insertion of SecA was reduced in the presence of fructans.

In a recent study Hincha et al. [31] observed that fructans stabilized the liquid-crystalline bilayer of vesicles under dehydrated conditions. This suggests that the fructan-lipid interactions reported here are also present under conditions of low amounts of water, supporting the hypothesis that fructan can protect membranes by interacting with the membrane lipids.

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