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Effects of sugar alcohols and disaccharides in inducing the hexagonal phase and altering membrane properties: implications for diabetes mellitus

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A number of sugars lowered the bilayer to hexagonal phase transition temperature of dielaidoylphosphatidylethanolamine. Disaccharides had the greatest effect followed by sugar alcohols. The monosaccharides, glucose and galactose had no effect on this phase transition temperature. The sugars promoted vesicle leakage only under conditions where the lipid was near its hexagonal phase transition temperature. Leakage from lipids in the bilayer state was inhibited by the sugars. Polyols, such as sorbitol, promote hexagonal phase formation and alter membrane permeability. These membrane effects may contribute to the damage caused by sorbitol accumulation in certain tissues of diabetic patients.

Introduction

It has been demonstrated that certain sugars protect phospholipid membranes against damage caused by freezing and dehydration [1–5]. This has stimulated a number of investigations on the effects of sugars on membrane properties. Trehalose causes a substantial lowering of the phase transition temperature of dry dipalmitoylphosphatidylcholine [6,7]. Trehalose also increases molecular motions of dry phospholipids above their phase transition temperature [8]. It has been shown that the preservation of intact liposomes in the dry state does not require the retention of residual water [7]. These effects have been interpreted in terms of the sugars binding to the lipid head groups. Models have been proposed in which hydroxyl groups of the sugars are hydrogen bonded to the oxygen of the lipid phosphates [9,10].

Most of the studies on carbohydrate-phospholipid interactions have been done using phosphatidylcholine or binary phosphatidylcholine/phosphatidylserine as lipids which tend to form bilayer phases. In addition to effects of sugars on lipids in the bilayer phase, sugars may also modulate the tendency of membranes to form non-bilayer structures. Lipid polymorphism is believed to play an important role in the function of biological membranes [11]. Phosphatidylethanolamines are a significant component of membrane lipids which have a propensity for forming non-bilayer phases [12]. The bilayer to hexagonal phase

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Abbreviations: DEPE, dielaidoylphosphatidylethanolamine; DOPE, dioleoylphosphatidylethanolamine; PC, egg phosphatidylcholine; TPE, phosphatidylethanolamine prepared by transesterification from egg phosphatidylcholine; DSC, differential scanning calorimetry.

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transition of phosphatidylethanolamines is particularly sensitive to the presence of small amounts of certain additives [13]. Relatively few studies have been done on the effect of carbohydrates on the behaviour of phosphatidylethanolamines. The bilayer to hexagonal phase transition of phosphatidylethanolamine was not observed by DSC in the presence of a 1:4 lipid to trehalose ratio [1,14].

As carbohydrates are very abundant molecules in living organisms and also some of them play a very important role in metabolic diseases such as diabetes mellitus, it was of interest to study further their influence on the properties of model membranes particularly with regard to their effect on the bilayer to hexagonal phase interconversion.

Materials and Methods

Materials. Dielaidoylphosphatidylethanolamine (DEPE), dioleoylphosphatidylethanolamine (DOPE), egg phosphatidylcholine (PC), and phosphatidylethanolamine prepared by transesterification from egg phosphatidylcholine (TPE) were obtained from Avanti Polar Lipids. Carboxyfluorescein was from Eastman Kodak and was used without further purification. Carbohydrates were purchased from Sigma (fructose, trehalose, maltose, lactose, *myo*-inositol); BDH Chemicals Limited (glucose, galactose, sucrose, sorbitol); glycerol from Fisher Scientific Company; and polyethylene glycol (mol. wt. 6000) from J.T. Baker Chemical Company. Sugars were of analytical grade and were used without further purification with the exception of sorbitol which was recrystallized from ethanol and the melting point was measured as a criterion of purity.

Sample preparation for DSC. The dry lipid film was suspended at a concentration of 7 mM in 20 mM Pipes, 1 mM EDTA, 150 mM NaCl and 0.02 mg/ml NaN_3 (pH 7.4) or in a sugar solution in the same buffer by warming the tube to 45 °C and vortexing vigorously for 30 s. The resulting suspensions and the buffer or sugar solutions were degassed under vacuum and loaded into an MC-2 scanning calorimeter (Microcal Company, Amherst, MA). The concentrations of sugars used varied within the range of 0.2–2.0 M. In some cases, the highest concentration was limited by the solubility of the sugar.

Differential scanning calorimetry (DSC). Lipid suspension or appropriate reference solution were loaded into the sample or reference cell, respectively, filling a 1.42 ml fixed volume cell. A scan rate of 39 C°/h was employed. All scans were done in duplicate. Second heating scans on the same sample were essentially superimposable on the first scan. Thus, although the sugars would be expected to be impermeant to a lipid bilayer, they would readily equilibrate with both sides of a bilayer after reversibly passing through a hexagonal phase arrangement. The reproducibility of the DSC heating scans demonstrates that the effects of the sugars on the thermal transitions is not limited by the access of the sugar to all of the lipid, nor is the sample degraded by exposure to high temperature.

The calorimeter was interfaced with an IBM-PC through a Data Translation DT 2801 analog to digital I/O board. The calorimeter was controlled and data analyzed through the computer using software purchased from the Microcal Company. The bilayer to hexagonal phase transition was fitted to a single van't Hoff component in order to determine the temperature of the transition. Only one component was used for simplicity even though some transitions could be better fitted with multiple components (for example, see Fig. 1 below).

^{31}P-NMR. NMR spectra were recorded on a Bruker AM-500 spectrometer operating at 202.45 MHz. The probe temperature was maintained to within ±1°C by a Bruker B-VT 1000 variable temperature unit. Temperatures were checked by thermocouple measurements. A 10 mm broad band probe was used. A 30 kHz sweep width was employed with an acquisition time of 0.28 s and a relaxation delay of 0.3 s (16 K data points). The 90° pulse width was 16.6 μs with composite pulse proton decoupling. FID's were processed using exponential multiplication (line broadening 15.0 Hz). Chemical shifts are expressed in ppm from an external reference of 85% phosphoric acid in ^2H_2O.

Leakage from DOPE/PC vesicles. Small unilamellar DOPE-egg PC vesicles (DOPE/egg PC molar ratio of 1.19) containing entrapped carb-

oxyfluorescein were created by bath sonication above the transition temperature of a 10 mg/ml lipid suspension. The unencapsulated material was removed by passing the vesicle suspension through a G-50 (medium) Sephadex column (2 × 16 cm). To maintain the same osmotic pressure inside and outside of the vesicles, carboxyfluorescein (0.25 M) was dissolved in NaCl buffer (1 M NaCl, 10 mM Hepes, 0.002% NaN_3), whereas the external medium contained either 1.5 M monosaccharides or 1.2 M disaccharides in Hepes buffer (0.1 M NaCl, 10 mM Hepes, 0.002% NaN_3) or 1 M NaCl, 10 mM Hepes, 0.002% NaN_3 (absence of sugars). The Sephadex G-50 column was preequilibrated with the NaCl buffer.

The carboxyfluorescein was initially entrapped in the vesicles at high, self-quenching concentrations [15]. Its release was followed by the increase in fluorescence which occurs when the label is diluted in the external medium. The leakage was initiated by the addition of 5 μl of 1 mg/ml poly(L-arginine) to 2 ml of the sample and fluorescence measured with a Perkin-Elmer spectrofluorimeter (excitation 490 nm, emission 550 nm). A total release of the carboxyfluorescein was obtained by the addition of Triton X-100 (0.05% v/v).

Leakage from TPE vesicles. The pure TPE, carboxyfluorescein-containing unilamellar vesicles were prepared by sonication of 10.5 mg TPE/ml with 0.25 M carboxyfluorescein in a glycine buffer (1 M NaCl, 0.1 mM EDTA, 10 mM glycine) at pH 9.5. The vesicles were separated from unencapsulated material on a Sephadex G-75 column, preequilibrated with the glycine buffer. The encapsulated solutions were isoosmotic to the buffers used for column chromatography and in leakage experiments. Leakage from vesicles was induced by raising the temperature and/or decreasing pH. Multilamellar vesicles with 0.25 M carboxyfluorescein were prepared in either 1.2 M sucrose or in 1 M NaCl, 20 mM Pipes, 1 mM EDTA, 0.02 mg/ml NaN_3. The pH of the solutions were adjusted to 7.4 or 9.5. The vesicle suspensions, containing 7 mg/ml lipid, were freed of extravesicular carboxyfluorescein by washing the vesicles with buffer after centrifugation. The lipid was pelleted in an Eppendorf centrifuge and resuspended in 0.5 ml fresh buffer without carboxyfluorescein. This process was repeated several times until no visible color was detected in the supernate. The pellet was resuspended in 0.5 ml buffer and 25 μl was used in 2.5 ml buffer for the leakage studies. Leakage was induced by raising the temperature or altering the pH outside of the vesicles.

Results

The effect of solutions of various carbohydrates on the bilayer to hexagonal phase transition temperature of DEPE was studied by DSC. The transition temperature for pure DEPE in Pipes buffer at pH 7.4 was 65.1°C. In the presence of sugar alcohols and disaccharides, but not glucose or galactose, the bilayer to hexagonal phase transition temperature was shifted to lower temperatures in a concentration-dependent manner, thus showing their destabilizing effect on the bilayer phase. Table I summarizes results for all carbohydrates studied, giving the linear regression slopes of the hexagonal phase transition temperature vs. the total concentration of sugar. A negative slope indicates bilayer destabilization. However, in contrast to hydrocarbon or amphiphilic additives

TABLE I

EFFECT OF POLYOLS AND SACCHARIDES ON THE BILAYER TO HEXAGONAL PHASE TRANSITION TEMPERATURE OF DEPE

Additive	Slope [a] (C°/mol fraction)
Monosaccharides	
Glucose	−0.2±0.6
Galactose	−0.6±0.7
Fructose	−2.5±0.1
Sugar alcohols	
Sorbitol	−5.4±0.2
myo-Inositol	−5.5±0.3
Other Polyols	
Glycerol	−2.76±0.03
PEG	+0.23±0.02
Disaccharides	
Sucrose	−12.6±0.3
Trehalose	−10.8±0.2
Lactose	−10.0±1.0
Maltose	−9.0±0.4

[a] Slope of a plot of the bilayer to hexagonal phase transition temperature vs. mol fraction of additive. Negative slopes indicate bilayer destabilization.

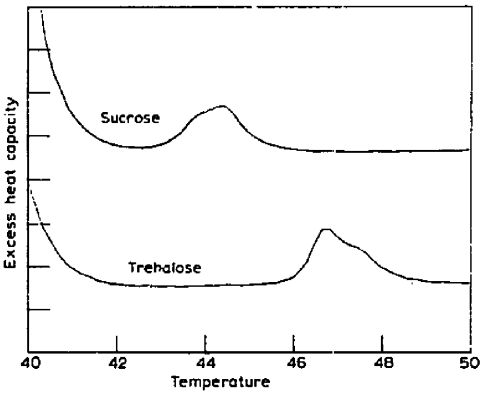

Fig. 1. DSC heating scans of the bilayer to hexagonal phase transition of DEPE in the presence of 1.5 M trehalose or 2 M sucrose. Each vertical tick mark represents 100 cal/mol per K. The high temperature shoulder of the main transition is visible in the temperature range 40–41°C.

which partition into the membrane and broaden the bilayer to hexagonal phase transition [13], this transition in the presence of sugars remains sharp for the entire range of sugar concentrations. Representative DSC curves for DEPE in the presence of trehalose or sucrose are shown in Fig. 1. An example of the DSC scan of pure DEPE can be found in Ref. 13.

Disaccharides are particularly potent bilayer destabilizers (Fig. 1). For example, a 1.5 M solution of trehalose or sucrose depresses the hexagonal phase transition temperature of DEPE from 65° to 47°C. Less effective seemed to be the sugar alcohols, sorbitol and *myo*-inositol. Among monosaccharides, two aldoses glucose and galactose had no effect on the bilayer-hexagonal phase transition temperature whereas a ketose, fructose, shifted this temperature about 5 C° for 2 M sugar concentration. From all of the compounds studied, only polyethylene glycol had a stabilizing effect on the bilayer phase. This may result from the size of polyethylene glycol which prevents the juxtaposition of bilayers which is required to form the hexagonal phase. Although polyethylene glycol would not permeate rapidly through bilayers, equilibration would take place after passing through the bilayer-hexagonal phase transition. At a concentration of 50% polyethylene glycol, the hexagonal phase transition temperature of DEPE is shifted to 73.4°C, and the hexagonal phase transition is significantly broadened. At the same time, the gel to liquid-crystalline phase transition temperature was raised by 50% polyethylene glycol about 3 C°. With hydrocarbon or amphiphilic additives, the bilayer to hexagonal phase transition temperature is generally lowered regardless of the effect on the hexagonal phase transition. In contrast sugars as well as polyethylene glycol, raise the gel to liquid crystalline phase transition temperature. Two molar solutions of disaccharide raise the temperature about 3 C°, 2 M sugar alcohols about 1.5 C°, 2 M fructose about 0.5 C° and glucose or galactose not at all. Sucrose also raises the transition temperature of dipalmitoylphosphatidylcholine [16].

Assignment of the high and low temperature phases in the presence of trehalose or sorbitol is confirmed by ^{31}P-NMR spectroscopy (Fig. 2). The conversion of the bilayer powder pattern to one representing the hexagonal phase occurs at a much lower temperature in the presence of these sugars than was found for pure DEPE using ^{31}P-NMR [13].

A DSC study of the effects of sugars on the thermotropic phase transitions of TPE was also undertaken. The results corroborated those obtained with DEPE showing the destabilizing effect of sugars on the bilayer phase of phosphatidylethanolamines (Table II). The gel to liquid-crystalline transition temperature for TPE in Pipes buffer (pH 7.4) was 18.3°C, whereas a bilayer to hexagonal phase transition appeared at 61.4°C. For pH 9.5, only the main transition ws present at 15.4°C, although the scan was run up to 90°C. Both transitions were moved when TPE was suspended in 1.2 M sucrose solution. Sucrose caused a dramatic shift of the bilayer to hexagonal phase transition which appeared at 66.8°C at pH 9.5 and at 43.8°C at pH 7.4. The effect of sucrose on the main transition was less profound. It was shifted towards higher temperatures by about 2.3 C° for pH 9.5 and 1.4 C° for pH 7.4. Thus, 1.2 M sucrose had almost the same effect on the bilayer phase of TPE as a change of pH from 9.5 to 7.4.

Carboxyfluorescein leakage experiments for mixed DOPE/PC vesicles showed that carbohydrates protect these vesicles against leakage (Fig. 3). The only partial exception was trehalose which

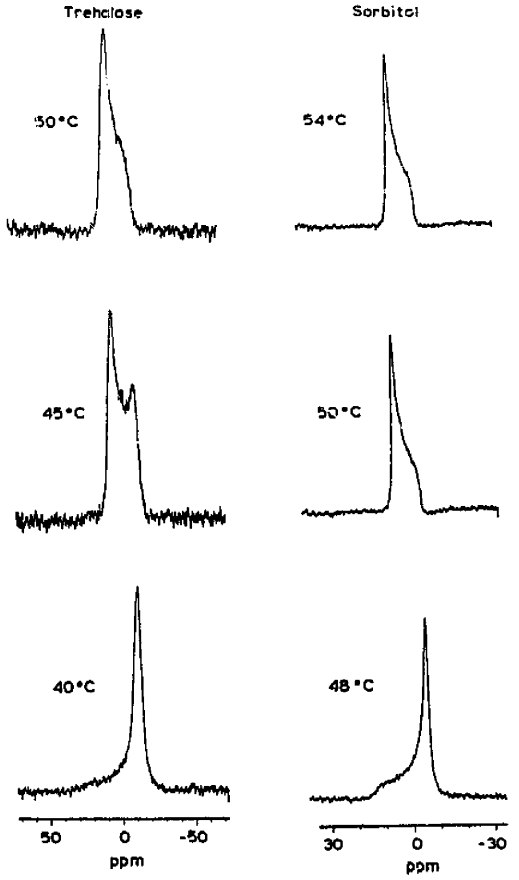

Fig. 2. ^{31}P-NMR spectra of DEPE in the presence of 1.5 M trehalose (left) or 2 M sorbitol (right) as a function of temperature.

enhanced the extent of leakage, although it lowered the rate of leakage.

Small unilamellar TPE vesicles were stable at 24°C and pH 9.5, showing the same residual leakage in buffer and in sugar solutions. Lowering of the pH to 7.4 did not change the stability of the liposomes. When the temperature was raised to 69°C at pH 9.5, TPE vesicles in buffer showed an increase in leakage of about 5%, whereas vesicles placed in glucose and sorbitol solutions leaked almost 50% of its contents (Table III). Maltose and sucrose completely protected the vesicles against disruption. When, in addition to enhanced temperature, the pH of the external solution was lowered to 7.4, vesicles in buffer leaked almost all

TABLE II

DSC RESULTS FOR MULTILAMELLAR TPE IN DIFFERENT SOLUTIONS

Solution	T_m [a] (°C)	$T_{H_{II}}$ [b] (°C)
Glycine buffer [c] (pH 9.5)	15.4	–
1.2 M sucrose [d] (pH 9.5)	17.7	66.8
Pipes buffer [e] (pH 7.4)	18.3	61.4
1.2 M sucrose [f] (pH 7.4)	19.7	43.8
Pipes buffer [g] with 1 M NaCl (pH 7.4)	18.9	54.7

[a] T_m is the temperature of the gel to liquid-crystalline phase transition.

[b] $T_{H_{II}}$ is the temperature of the bilayer to hexagonal phase transition.

[c] 10 mM glycine, 0.1 M NaCl, 0.1 mM EDTA, (pH 9.5).

[d] 1.2 M sucrose, 0.1 M NaCl, 0.1 mM EDTA, 10 mM glycine (pH 9.5).

[e] 20 mM Pipes, 1 mM EDTA, 0.02 mg/ml NaN_3, 0.1 M NaCl (pH 7.4).

[f] 1.2 M sucrose in 20 mM Pipes, 1 mM EDTA, 0.02 mg/ml NaN_3, 0.1 M NaCl (pH 7.4).

[g] 1 M NaCl, 20 mM Pipes, 1 mM EDTA, 0.02 mg/ml NaN_3 (pH 7.4).

of their contents, while in presence of sugars the leakage varied from 44 to 62% (Table III).

Large multilamellar TPE vesicles prepared at pH 7.4 were less stable at low temperatures (25°C) as compared to the vesicles obtained at pH 9.5, showing a high initial leakage when placed in various solutions. When sucrose or sorbitol was present, both inside and outside of the vesicles,

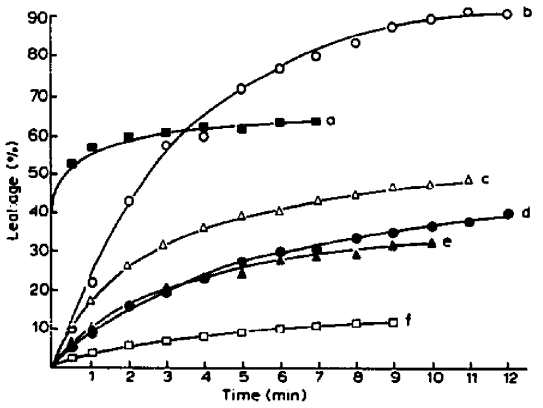

Fig. 3. Leakage of the carboxyfluorescein from DOPE/PC sonicated vesicles in the presence of 1 M NaCl (■, a), 1.2 M trehalose (○, b), 1.5 M sorbitol (△, c) 1.5 M glucose (●, d), 1.2 M sucrose ((▲, e), and 1.2 M maltose (□, f).

TABLE III

EFFECT OF SUGARS ON CARBOXYFLUORESCENE LEAKAGE FROM SMALL UNILAMELLAR TPE VESICLES

External medium [a]	% Release [b]	
	$t = 69°C$ (pH 9.5)	$t = 69°C$ (pH 7.4)
1 M NaCl	4.9	86
1.5 M glucose	54.0	62
1.5 M sorbitol	41.0	50
1.2 M maltose	2.0	44
1.2 M sucrose	1.0	44

[a] Medium external to the liposomes also contains 0.1 M NaCl, 10 mM Hepes, 0.002% NaN_3 and sufficient additive to make it isosmotic with the entrapped media containing 1 M NaCl, 10 mM Hepes, 0.002% NaN_3.

[b] % of total carboxyfluorescein released during the first three minutes (similar to Fig. 3). After this initial, relatively rapid release of carboxyfluorescein, the rate of carboxyfluorescein release decreases several fold.

TABLE IV

EFFECT OF SUGARS ON CARBOXYFLUORESCENCE LEAKAGE FROM MULTILAMELLAR TPE VESICLES

Buffers adjusted to pH 7.4 or 9.5 were made as follows. Internal medium: 0.25 M carboxyfluorescein, 20 mM Pipes, 1 mM EDTA, 0.02 mg/ml NaN_3 containing either 1 M NaCl or 1.2 M sucrose. External medium: 20 mM Pipes, 1 mM EDTA, 0.02 mg/ml NaN_3 containing either 1 M NaCl or 1.2 M sucrose or 1.5 M sorbitol.

	Release (%)			
Internal medium	Carboxyfluorescein in buffer (pH 7.4)		Carboxyfluorescein in 1.2 M sucrose solution (pH 7.4)	
External medium	$t = 25°C$ (pH 7.4)	$t = 44°C$ (pH 7.4)	$t = 25°C$ (pH 7.4)	$t = 44°C$ (pH 7.4)
Buffer	73.8	76.8	50.6	71.7
Sucrose	68.9	93.8	56.3	83.0
Sorbitol	68.2	93.8	56.2	79.2
Internal medium	Carboxyfluorescein in buffer (pH 9.5)		Carboxyfluorescein in 1.2 M sucrose solution (pH 7.4)	
External medium	$t = 25°C$ (pH 9.5)	$t = 25°C$ (pH 7.4)	$t = 25°C$ (pH 9.5)	$t = 25°C$ (pH 7.4)
Buffer	51.3	75.7	45.9	78.1
Sucrose	50.0	93.7	36.5	88.2
Sorbitol	46.5	88.1	35.3	78.5

they were less leaky at temperatures far below the hexagonal phase transition temperature (25°C) than vesicles with buffer both inside and outside of the vesicles. However, when sugars were present at that temperature only outside of the vesicles, they had little, if any, protective effect on the vesicles. When the temperature was raised to 44°C, which is the hexagonal phase transition temperature for TPE in sucrose, vesicles containing the buffer inside and placed into sucrose or sorbitol, leaked almost completely (≈ 90%), whereas vesicles containing entrapped sucrose and placed into a sugar solution were a little bit more stable (≈ 83% leakage), but leaked more rapidly than vesicles surrounded by buffer. This is quite understandable as the hexagonal phase transition temperature for TPE in the Pipes buffer (1 M NaCl) at pH 7.4 is 55°C. At 54°C, both populations of vesicles leaked 100% of their contents no matter what the external solution was. The results are summarized in Table IV.

Discussion

Crowe et al. [14] have demonstrated that the bilayer to hexagonal phase transition of DOPE is not observed in the presence of high concentrations of trehalose. They assumed that the transition was not observed because trehalose was stabilizing the bilayer phase. Small shifts in the transition temperature observed at low mol fractions of trehalose could be caused by kinetic phenomena since it is known that the bilayer to hexagonal phase transition of DOPE is very susceptible to these effects [17]. In contrast, the bilayer to hexagonal phase transition of DEPE occurs at a temperature 50 C° higher and is much more rapid. Hence, the DSC of this lipid is less susceptible to kinetic effects. Our systematic study using a range of concentrations of several sugar alcohols and disaccharides (Table I) clearly shows that these substances promote hexagonal phase formation in DEPE. This is confirmed by the ^{31}P-NMR spectra (Fig. 2).

Glucose, the principle monosaccharide found in higher organisms, has no effect on the bilayer to hexagonal phase transition temperature of phosphatidylethanolamine (Table I). In contrast, the sugar alcohols, sorbitol and *myo*-inositol, de-

stabilize the bilayer phase. Sorbitol is found at particularly high concentrations in cells of individuals with diabetes mellitus [18–20]. For example, sorbitol can reach concentrations as high as 20 μmol/g in the diabetic lens [18]. The accumulation of sorbitol in tissues of diabetic individuals is believed to be associated with degenerative processes leading to the secondary complications of diabetes [21–24]. The ability of sorbitol to destabilize membrane bilayers and promote hexagonal phase formation will lead to alterations in cell membrane properties which may be related to the destructive effects of sorbitol. The concentrations of sugars required to shift the bilayer to hexagonal phase transition temperature are about 100-fold higher than in the case of hydrocarbons or amphiphiles [13]. This is because, in contrast to more hydrophobic or amphiphilic substances, the sugars largely partition into the aqueous phase. Therefore, higher concentrations of sugars are required for them to effect membrane properties. Higher concentrations of sugars occur in biological systems in certain circumstances while this is not the case for hydrocarbons and amphiphiles. The failure of the sugars to partition extensively into the membrane is indicated by the fact that the cooperativity of the transition is not greatly diminished by these substances. Sorbitol is not highly toxic to cells and the secondary complications of diabetes are acquired over a prolonged period at highly elevated sorbitol concentrations. Therefore, the relatively weak bilayer destabilizing effect of sorbitol may be highly significant in explaining some of the degenerative processes occurring in diabetes mellitus, particularly in view of the fact that sorbitol is synthesized from glucose which has no effect on the bilayer to hexagonal phase transition.

We also tested the effect of sugars on the permeability of vesicles of TPE. It has recently been noted that the rate of carboxyfluorescein leakage from vesicles is often limited by counterion flux [25]. When bilayer lipid is transformed into the hexagonal phase, it can no longer retain its entrapped contents. Above the bilayer to hexagonal phase transition temperature of multilamellar TPE, there is almost quantitative release of carboxyfluorescein. At pH 7.4, the bilayer to hexagonal phase transition temperature of TPE is 55°C in 1 M NaCl but is lowered to 44°C in the presence of 1.2 M sucrose (Table II). At this pH and temperature, there is over 90% leakage from multilamellar TPE in the presence of sucrose or sorbitol but considerably less in the absence of sugars (Table IV). However, it is interesting that the sugars have an opposite effect, stabilizing vesicles against leakage, under conditions where hexagonal phase formation is suppressed. Thus, unilamellar DOPE/PC vesicles (Fig. 3), unilamellar TPE vesicles (Table III) or multilamellar TPE vesicles at pH 9.5 (comparing vesicles with pH 9.5 NaCl buffer on both sides of the membrane vs. vesicles with 1.2 M sucrose (Table IV)) are not likely to form the hexagonal phase either because of lack of bilayer-bilayer contact (unilamellar vesicles), the presence of PC or the high pH. In all of these cases sugars stabilized the vesicles against leakage. This dual effect of sugars, i.e. promoting leakage near hexagonal phase-forming conditions and inhibiting leakage of bilayer lipid, can be understood in terms of the solvating ability of sugars vs. water. Water can more effectively form hydrogen bonds with the lipid head groups. Thus, interaction of the lipid surface with sugars is like dehydration, resulting in a less permeable bilayer but also one more prone to hexagonal phase formation. Once the hexagonal phase is formed, all vesicle contents are lost. Thus, although certain sugars such as trehalose can profoundly stabilize liposomes, even against desiccation, these sugars also can induce hexagonal phase formation resulting in the loss of vesicle contents under certain conditions.

Acknowledgements

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