Biochimica et Biophysica Acta 858 (1986) 169–180
Elsevier

BBA 73105

The interaction of saccharides with lipid bilayer vesicles: stabilization during freeze-thawing and freeze-drying

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(Received December 6th, 1985)

Key words: Membrane fusion; Freeze-thawing; Freeze-drying; Cryoprotection; Saccharide-membrane interaction

The fusion of small unilamellar vesicles of phosphatidylcholines during freeze-thawing and freeze-drying/rehydration, and the suppression of fusion under these conditions by various saccharides, was investigated by gel filtration on Sepharose 4B, quasielastic light scattering, high-resolution ^{1}H-NMR, ESR spin labeling, and differential scanning calorimetry. Freeze-thawing and freeze-drying of aqueous small unilamellar vesicle suspensions in the presence of sufficient sucrose had no significant effect on the average size and size distribution of small unilamellar vesicles. In the presence of sucrose the structural integrity and the permeability properties of the phosphatidylcholine bilayers were retained during freeze-thawing and freeze-drying. A comparison of the stabilizing effect of sucrose with that of trehalose and glucose showed that the stabilization is not sugar-specific but is a general property of saccharides. The fraction of small unilamellar vesicles recovered after freeze-thawing depended on the saccharide/phosphatidylcholine molar ratio. The mechanism of the cryoprotective effect involves binding of the sugar to the phospholipid polar group, probably through hydrogen bonding.

Introduction

Aqueous suspensions of zwitterionic phosphatidylcholine can form various types of aggregates. Multilamellar vesicles, typically several micrometers in size, represent the thermodynamically most stable form for this type of phospholipid [1]. Multilamellar vesicles are formed when the lipid is gently shaken with water or an aqueous buffer. These are transformed into small unilamellar vesicles by various methods, including sonication [2], transient pH change in the presence of phosphatidic acid [3] or addition of limited amounts of single-chain amphiphiles [4]. The resulting small unilamellar vesicles represent a metastable state of higher free energy. They can, under certain conditions, persist for long periods of time (e.g., when stored at 4°C in the case of egg PC). They revert to the more stable form of multilamellar vesicles only when perturbed in certain ways, such as incubation at or below the gel–liquid-crystal phase-transition temperature (T_c), freeze-thawing, or freeze-drying/rehydration [5,6]. In each of these cases the small unilamellar vesicles aggregate and fuse, forming multilamellar vesicles. Simultaneously, the vesicular bilayer structure opens up, allowing the bidirectional exchange of entrapped compounds with the external aqueous phase.

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Abbreviations: CAT 16, 4-(*N*,*N*-dimethyl-*N*-hexadecyl)ammonium-2,2,6,6-tetramethylpiperidine-*N*-oxyl iodide; T_c, phase-transition temperature; TEMPO, 2,2,6,6-tetramethylpiperidine-*N*-oxyl; TEMPO-palmitate, 2,2,6,6-tetramethylpiperidine-*N*-oxyl-4-yl hexadecanoate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

It has been known for some time that various mono- and disaccharides can stabilize lipid membranes against disruption. For example, organisms rich in trehalose, such as nematodes, were found to survive dehydration [7]. The presence of various sugars during freeze-drying and rehydration of the sarcoplasmic reticulum, or freeze-thawing of microsomes, prevented loss of both structural and functional integrity of these subcellular compounds [7,8]. In previous studies, we found that sucrose and other saccharides prevented the fusion of small unilamellar vesicles made from egg PC during freeze-thawing [9]. Recently, we observed by freeze-fracture electron microscopy that freezing of small unilamellar vesicles made from egg PC caused the lipid to be squeezed out and compressed between ice crystals, both in the absence and presence of sucrose. Without sucrose, the lipid lost its vesicular structure and became a multilamellar aggregate; in the presence of sucrose, however, the small unilamellar vesicles were merely concentrated into a small volume, without loss of structure or significant change in size [10].

We here present results of studies designed to describe the cryoprotective effect of saccharides upon lipid vesicles in greater detail, using high resolution ^{1}H-NMR, ESR, gel filtration, light-scattering and differential scanning calorimetry techniques. As we will show, saccharides act by binding to lipid bilayers, thereby preventing or inhibiting dehydration of bilayers during freezing.

Materials and Methods

Egg PC, egg PA and DPPC were purchased from Lipid Products (Surrey, U.K.). The purity of the lipids was checked by TLC, and was found to be satisfactory by TLC standards. The spin labels TEMPO, CAT 16, TEMPO-palmitate, 5-doxyl- and 16-doxylstearic acid were purchased from Molecular Probes, Junction City, OR, U.S.A. All other chemicals were of reagent grade.

Lipid dispersions were prepared by rotary evaporation of the phospholipid, together with a spin probe when needed, from $CHCl_3/CH_3OH$ (2 : 1, v/v) solution, in a round flask. The resulting film was thoroughly freed of remaining solvent under high vacuum. The lipid was dispersed in 1–2 ml aqueous buffer by brief vortexing, and sonicated for 30–60 min until the dispersion was clear. A Branson probe-type sonicator with microtip was used. To prepare vesicles in the presence of saccharides, the phospholipid was dispersed and sonicated in aqueous sugar solutions. The resulting vesicles therefore had sugar solution on both the inside and outside of the bilayer. When such vesicles were gel-chromatographed, the elution buffer also contained sugar. For freeze-thawing, 1.0 ml samples of sonicated lipid dispersion were placed into 8-mm diameter glass test tubes which were immersed in a solid CO_2–methanol cooling bath at $-72°C$ for 20 min, then thawed in a water bath at room temperature, and kept at room temperature for a minimum of 1.5 h to allow for completion of the fusion process. For freeze-drying, 1.0 ml samples were placed in 100-ml round flasks and frozen in a coooling bath while being rotated to form a film of frozen solution. The flasks were placed under vacuum on a freeze dryer for 15 h. The samples were then reconstituted with 1 ml of water.

Gel filtration chromatography was carried out as described previously [11] on two Sepharose 4B columns (46 × 0.9 cm), with a flow rate of 5 ml/h. One column was run with an elution buffer containing 0.12 M NaCl, 0.01 M Hepes (pH 7.0) and 0.05% NaN_3. The other column was operated with an elution buffer containing 0.15 M sucrose, 0.04 M NaCl, 0.01 M Hepes (pH 7.0) and 0.05% NaN_3. The samples, which typically contained 10 mg lipid per ml, were centrifuged for 5 min at 12000 × *g*. This treatment pellets over 97% of any multilamellar vesicles present in a sample, for example due to insufficient sonication or due to aggregation during freezing. A 0.5 ml volume of the supernatant was then applied to the appropriate column, depending on whether or not the sample contained sucrose. Fractions, typically of 0.3 ml, were collected in scintillation vials on an automatic fraction collector with ultraviolet-detector. Elution profiles were generated: (a) by recording absorbance (turbidity) of the vesicles on the ultraviolet-detector; (b) by trapping the fluorescent dye pyrene trisulfonate in the vesicles, and analyzing the fractions on a fluorescence spectrometer; and (c) by adding [^{3}H]DPPC to the vesicles as a marker and analyzing the fractions on an automatic scintillation counter. In order to obtain

reproducible results, the Sepharose 4B columns were pre-saturated with egg PC by chromatographing sonicated egg PC dispersions repeatedly until reproducible elution profiles were obtained.

Turbidity measurements were made on an absorption spectrophotometer at 400 nm. Microcells with a pathlength of 10 mm and a compartment width of 3 mm were used. Apparent molar extinction coefficients, ε, defined by ε = absorbance/(molarity of the lipid), were recorded. Where necessary, highly turbid samples were serially diluted, and ε was found from the limiting slope of a plot of absorbance vs. lipid molarity. The fraction of lipid aggregated, i.e., present as multilamellar vesicles, was calculated on a relative basis by comparing its apparent extinction coefficient ε with a reference value ε_0 for a lipid dispersion that had been freeze-thawed without addition of a saccharide as cryoprotectant, and which was assumed to be completely in the form of multilamellar vesicles. The value of ε of unfrozen sonicated lipid dispersions, assumed to consist entirely of small unilamellar vesicles, was only about 0.5% of that of fully aggregated samples, and could be neglected. The fraction of small unilamellar vesicles in a sample therefore was given by $1 - (\varepsilon/\varepsilon_0)$.

^{1}H-NMR spectra of lipid dispersions in ^2H_2O were recorded on a Bruker Fourier-transform spectrometer at 360 MHz. Sodium acetate was included as an internal integration standard. A relaxation delay of 20 s was used between pulses to avoid saturation of the acetate. The spectra were integrated and the integrals of the -$N(CH_3)_3$ and of the hydrocarbon chain signals, defined as the sum of all ^{1}H signals upfield of the acetate standard, were recorded as a percentage of the theoretical amount expected for the total lipid present.

ESR spectra were recorded at 9.2 GHz on a Varian Model E104-A X-band spectrometer fitted with a variable temperature control. Samples of about 50 μl were sealed into 1-mm capillaries. Temperatures were measrued with a thermocouple inserted into the cavity alongside the sample tube, and were accurate to within 0.5°C. Spectra were recorded over a range of 100 or 200 G.

Differential scanning calorimetry was carried out in a Perkin-Elmer (Norwalk, CT, U.S.A.) DSC-2 instrument attached to a Perkin-Elmer model 3600 data station. Samples were heated and cooled repeatedly, usually at a rate of 5 Cdeg/min. To prepare unsonicated dispersions, the phospholipid was weighed into the DSC pan, 50 μl of the sucrose solution were added with a Hamilton syringe, and the pan was immediately sealed and transferred to the calorimeter.

Dynamic light-scattering measurements were carried out as described previously [12]. For experimental details, instrumentation, and data evaluation see Refs. 12–15.

Results

Gel filtration on Sepharose 4B and dynamic light scattering

The elution profile of sonicated egg PC dispersions in H_2O consisted of two peaks, a minor one eluted at the column void volume, V_0, and a major, broad peak at $V_e = 22 \pm 1$ ml corresponding to a Stokes radius of about 10 nm (Fig. 1A). Subjecting sonicated egg PC dispersions in H_2O to freeze-thawing as described under Materials and Methods caused aggregation and/or fusion of the unilamellar vesicles. Before freeze-thawing, less than 5% of sonicated egg PC dispersions were pelleted by centrifugation at $12\,000 \times g$ for 5 min, whereas after freeze-thawing 95% were spun down. When the phospholipid remaining in the supernatant was applied to Sepharose 4B, most of it was eluted in the void volume (Fig. 1A). Sonicated egg PC dispersions in buffer containing 0.15 M (about 5%) sucrose gave similar elution profiles (Fig. 1B). However, the two peaks were less well separated because the major one was eluted at a somewhat smaller elution volume, $V_e = 19 \pm 1$ ml. Using the calibration curve published previously [12], this V_e value corresponds to a Stokes radius of 11.5 ± 1.2 nm. The effect of freeze-thawing on sonicated egg PC dispersions in the presence of sucrose was drastically different from that observed in buffer without sucrose. This is evident from a comparison of the gel filtration patterns before (Fig. 1B) and after freeze-thawing (Fig. 1C). The two elution profiles are almost superimposable, as is demonstrated in Fig. 1D, which gives the difference between the two elution patterns; the points appear to be randomly scattered

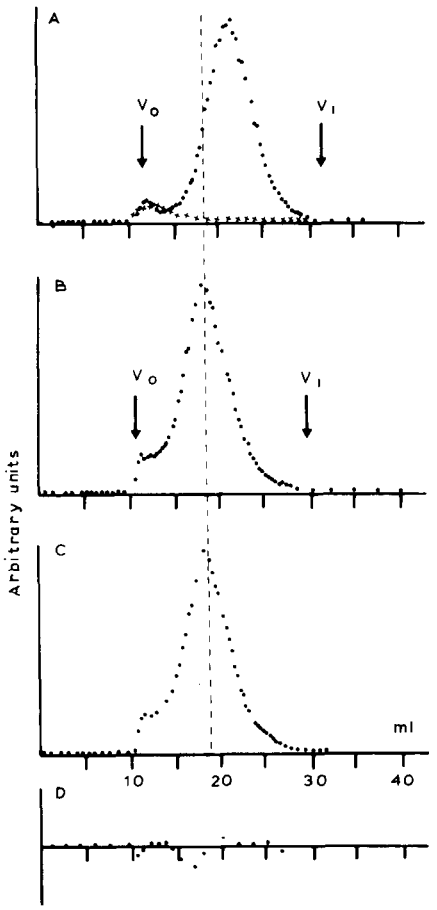

Fig. 1. Gel filtration patterns on Sepharose 4B. (A) A 0.5 ml sample of a sonicated egg PC dispersion at 10 mg/ml = 0.013 M in buffer (0.12 M NaCl/0.01 M Hepes (pH 7.0)/0.05% NaN_3) was applied to the column (46×0.9 cm) preequilibrated with the same buffer; the column was eluted with buffer at a flow rate of about 5 ml/h. In each of the 0.3 ml fractions collected, the phospholipid concentration was determined by radioactivity measurement (●). The same sonicated egg PC dispersion as above was subjected to freeze-thawing. After centrifugation at 12000×*g* for 5 min, 0.5 ml of the supernatant was applied to the same column (×). (B–D) A second Sepharose 4B column of similar dimensions as in (A) was equilibrated with buffer (0.04 M NaCl/0.01 M Hepes (pH 7.0)/0.05% NaN_3/0.15 M sucrose). A sonicated egg PC dispersion in the same buffer (0.5 ml at 10 mg/ml = 0.013 M) was chromatographed on this column before (B) and after freeze-thawing (C). The difference between the gel filtration patterns (B) and (C) is given in (D). Both columns were presaturated with egg PC as described before [12] and handled in the same way. Phospholipid recovery was not less than 85%, usually 90–95%. The void volume, V_0, and the total volume, V_t, are indicated by arrows. The dashed line indicates the elution volume, V_e, of the main peak of sonicated egg PC in the presence of sucrose.

about the base line. The results obtained by gel filtration are supported by dynamic light-scattering data summarized in Table I. Freeze-thawing in the absence of sucrose produced aggregation and/or fusion of small unilamellar vesicles, as shown by the considerable increase in hydrodynamic radii obtained either by a cumulant analysis or by a two-exponential analysis of the intensity autocorrelation function. In contrast, freeze-thawing in the presence of sucrose produced no significant changes in these values.

NMR studies

The fraction of lipid remaining in vesicular form after a freeze-thaw cycle or a freeze-drying/rehydration cycle was measured by high-resolution ^{1}H-NMR spectroscopy, as described under Materials and Methods. The determination of the small unilamellar vesicle content by NMR is made possible by the fact that only vesicles smaller than 80–100 nm, or micelles, give a high-resolution NMR spectrum [16–19]. The NMR assay method for small unilamellar vesicles has been shown to give good agreement with the results of electron microscopy, gel filtration, and dynamic light scattering [3,12]. Fig. 2 shows ^{1}H-NMR spectra of sonicated egg PC dispersions at room temperature (Fig. 2A) and after freeze/thawing (Fig. 2B). In the latter spectrum the lines due to the $-N(CH_3)_3$ and hydrocarbon chain protons were completely lost in the baseline. Fig. 2C shows the spectrum of an egg PC dispersion in the presence of 10% sucrose, after freeze/thawing. Here, the high-resolution spectrum is seen to be fully retained. It is almost the same as in Fig. 2A, except that the polar group region (above about 3 ppm) is dominated by sugar resonances. Comparison of the integrals of the spectra in Fig. 2A and C showed complete recovery of small unilamellar vesicles after freeze/thawing. Comparative NMR measurements made on sonicated egg PC/egg PA (2:1) dispersions in 10% sucrose showed that freeze-drying/rehydration was somewhat more damaging than freeze-thawing. In the former case only about 80% of the high-resolution signal was retained, compared to almost 100% for the latter.

Effect of saccharide and lipid concentrations

The NMR assay discussed above was used to

TABLE I

DYNAMIC LIGHT SCATTERING OF SONICATED EGG PC DISPERSIONS IN H_2O IN THE PRESENCE AND ABSENCE OF SUCROSE

The plus sign in the Freeze-thawing column indicates that the sonicated dispersion was subjected to the freeze-thaw treatment described in Materials and Methods. r_H is the mean hydrodynamic radius as derived from a cumulant analysis of the intensity autocorrelation function. r_1 and r_2 are the hydrodynamic radii derived from the analysis of the intensity autocorrelation function in terms of two exponentials.

[Egg PC] (mM)	[Sucrose] (mM)	Freeze-thawing	Mean hydrodynamic radii (nm)		
			r_H	r_1	r_2
1.3	0	–	43	14	68
13.3	146 (5%)	–	54		
1.3	14.6 (0.5%)	–		15	72
1.3	146 (5%)	–		17	95
1.3	14.6	+		14	71
1.3	146	+		15	75
1.3	0	+	245		
1.3	0	+		54	187

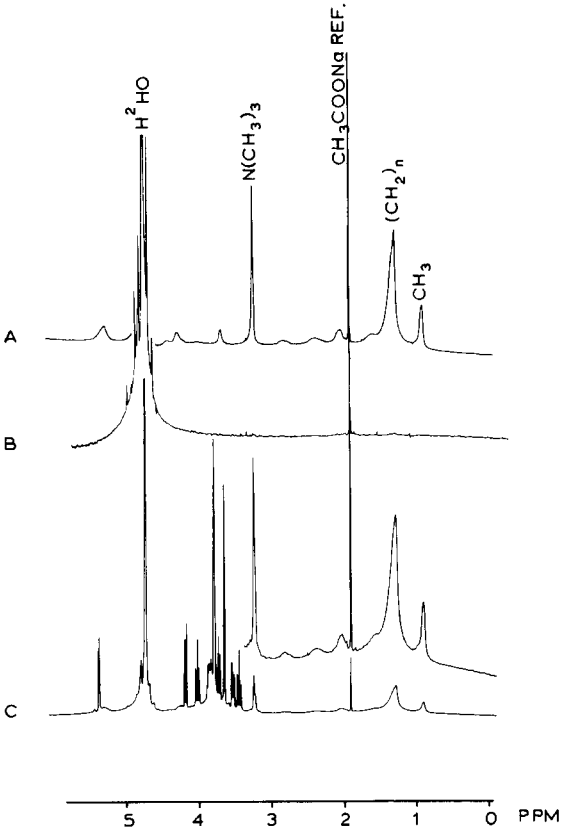

Fig. 2. ^{1}H-NMR spectra of sonicated egg PC dispersions (10 mg/ml = 0.013 M) in ^2H_2O recorded at 360 MHz at room temperature. A, control; B, the same sample as in (A) after freeze-thawing; C, egg PC (10 mg/ml) in ^2H_2O containing 10% sucrose, after freeze-thawing. The sensitivity of the vertically expanded spectrum in (C) is very similar to that of (A) and (B).

study the recovery of SUVs after freeze-thawing as a function of sucrose and lipid concentrations. When the egg PC concentration was varied between 1 and 10 mg/ml (1.33–13.3 mM) while holding the sucrose concentration constant at 0.1% (2.9 mM), the ^{1}H signal intensity after freeze-thawing passed through a maximum near a sucrose : lipid molar ratio of 1.0 (results not shown). This suggested that the cryoprotective effect is determined by the saccharide : lipid molar ratio, and not by the absolute sugar concentration. Fig. 3, curve A, shows the percentage of small unilamellar vesicles recovered after freeze-thawing as a function of the sucrose : egg PC molar ratio, at a constant egg PC concentration of 10 mg/ml (13.3 mM). A steep increase in the fraction of small unilamellar vesicles occurred near a molar ratio of 0.5. Almost 100% small unilamellar vesicle recovery resulted with molar ratios of 2 or more. Curve A also shows results of small unilamellar vesicle recovery, as calculated by turbidity measurements, as described under Materials and Methods. The agreement between the two techniques is seen to be good. The relative cryoprotective effects of sucrose, trehalose and glucose are compared in curves B and C of Fig. 3, which show data obtained by turbidity measurements. Trehalose and sucrose (curve B) were found to be equally effective, within experimental error. The monosaccharide glucose (curve C) was less effective, espe-

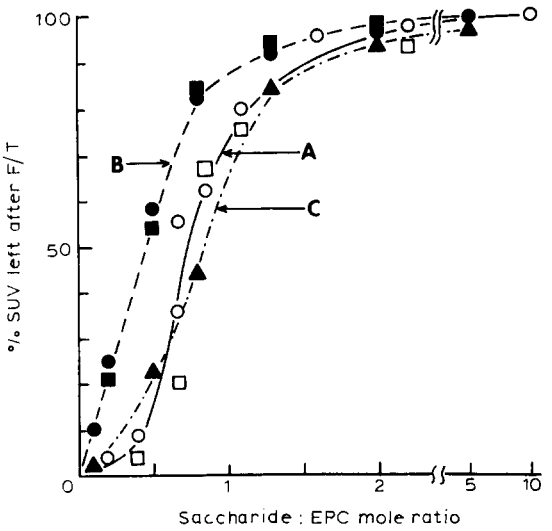

Fig. 3. Percentages of the lipid in sonicated dispersions that remained in the form of small unilamellar vesicles (SUV) after freeze-thawing (F/T), as a function of the saccharide : lipid molar ratio and of the type of saccharide. (A) Egg PC (EPC) (10 mg/ml = 13.3 mM) plus sucrose. % SUV determined by ^{1}H-NMR signal intensity (□) and by turbidity (○). (B) and (C) EPC (5 mg/ml = 6.7 mM) plus sucrose (■), trehalose (●) or glucose (▲).

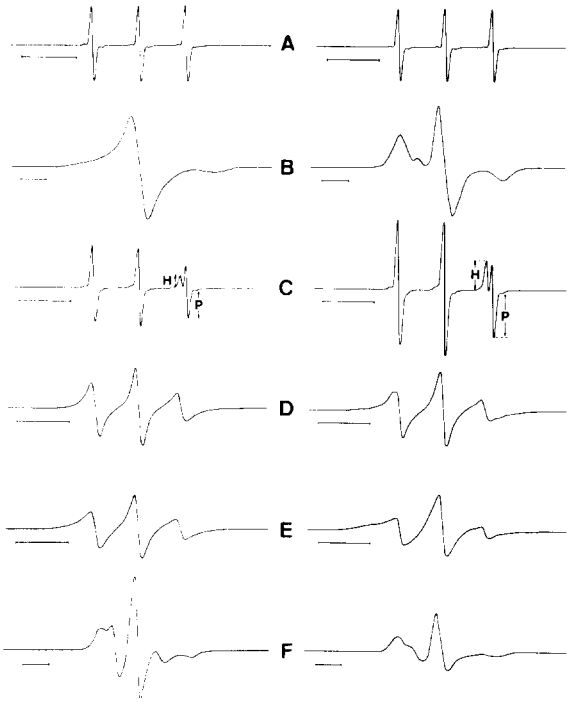

Fig. 4. ESR spectra of TEMPO (0.1 mM) in different systems and at different temperatures. Left-hand side: samples in H_2O. Right-hand side: samples in sucrose solutions varying between 10% and 37%. A, TEMPO in H_2O and 10% sucrose solution at room temperature; B, TEMPO in H_2O and 10% sucrose solution at −50°C; C, TEMPO in the presence of sonicated egg PC in H_2O and 37% sucrose solution at 28°C; D, TEMPO in sonicated egg PC in H_2O and 30% sucrose solution at −15°C; E, TEMPO in sonicated egg PC in H_2O and 30% sucrose solution at −25°C; F, TEMPO in sonicated egg PC in H_2O and 37% sucrose solution at −50°C. The concentration of the sonicated egg PC dispersions was 10 mg/ml = 0.013 M. *H* and *P* are the half-heights of the TEMPO signal arising from the probe being present in the egg PC bilayer and in H_2O, respectively.

cially at low molar ratios, than the two disaccharides tested.

The difference in the shapes of the plot of small unilamellar vesicle recovery vs. molar ratio for egg PC at 10 mg/ml plus sucrose (curve A) and the plot for egg PC at 5 mg/ml plus sucrose (curve B) can be accounted for by equilibrium considerations: the extent of complexation of small unilamellar vesicles with a saccharide to form a complex that remains in the form of small unilamellar vesicles after freezing depends on the first power of the lipid concentrations, whereas the extent of fusion of small unilamellar vesicles into multilamellar vesicles during freezing depends on a higher power of the lipid concentration. Therefore, the saccharide : lipid ratio needed in order to obtain a given small unilamellar vesicle : multilamellar vesicle ratio after freezing will increase with increasing initial lipid concentration, as was actually observed.

ESR studies

To elucidate the mechanism by which sucrose suppresses the fusion and leakage of vesicles during freezing [10], ESR spectra of the amphiphilic spin probe TEMPO were examined as a function of temperature in the presence and absence of egg PC vesicles and of sucrose. The temperature dependence of the ESR spectra of TEMPO in different environments is illustrated in Fig. 4. At room temperature the spectra were the same in water and in sucrose solution (row A). When these solutions were cooled to −50°C, however, very different spectra resulted (row B). In ice (left), TEMPO gave essentially a spin-exchange spectrum, indicating that upon formation of ice crystals the label was squeezed out and concentrated between ice

crystals. In 10% sucrose at −50°C the spectrum of TEMPO resembled a glass spectrum (row B, right), indicating that the spin label was practically immobilized but still accommodated within the sucrose-H_2O glass. The splitting between the low and high field extrema was 79 ± 1 G. Rows C to F in Fig. 4 show spectra of TEMPO in the presence of egg PC suspensions in the presence (right-hand side) and absence (left-hand side) of sucrose. At room temperature (row C) the $m = -1$ line was split, indicating that the probe was distributed between the phospholipid bilayer and the aqueous phase. The TEMPO distribution can be expressed by the spectral parameter f, defined as $H/(H + P)$ [20], where H and P are signal half-heights for the label present in the bilayer and the aqueous phase, respectively. At 20°C, f was 0.22 in the absence, and 0.34 in the presence of sucrose. This suggests that sucrose makes the bilayer more expanded. At −15 and −25°C (rows D and E), when the extravesicular bulk solution was frozen, the spectra indicated reduced mobility of the spin probe, with less mobility of the probe in the presence of sucrose. At −50°C (row F) the TEMPO spectrum in the egg PC dispersion without sucrose indicated that there was still some mobile component present compared to the corresponding TEMPO spectrum in the presence of sucrose, which showed the probe to be immobilized.

Other ESR spectra taken between +15°C and the freezing point of the solvent of about −13°C (recognized, independently of any lipid phase transition, by a sudden change in the microwave absorption of the sample) indicated that f decreased with temperature and became near zero as the T_c temperature of the lipid was approached (Fig. 5).

These results demonstrate that TEMPO is expelled from the lipid phase of egg PC dispersions below T_c (−15 to −20°C). As mentioned above, the spin-exchange spectrum of TEMPO in water at −50°C (Fig. 4B, left) indicates that TEMPO in the absence of lipid is squeezed out from ice. This, together with the evidence in Fig. 5, suggests that the mobile spectra of TEMPO seen at −15°C to −50°C (Fig. 4D–F, left column) arise from the presence of the label in the unfreezable water. Fig. 6A shows the maximum hyperfine splitting $2A_{\parallel}$ of the ESR spectra for TEMPO in water and in 30%

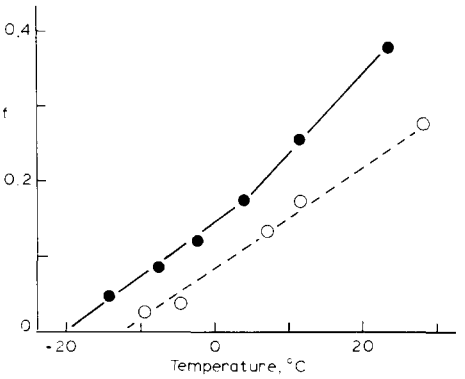

Fig. 5. The TEMPO distribution parameter f in sonicated egg PC dispersions (10 mg/ml = 0.013 M) in H_2O (○) and in 30% sucrose solution (●). $f = H/(H + P)$ where H and P are as defined in Fig. 4.

sucrose solution (in the absence of egg PC) as a function of temperature. In 30% sucrose, $2A_{\parallel}$ increased steeply between 0 and −20°C. Rigid glass-like spectra were obtained at temperatures below −30°C. In water the maximum hyperfine splitting increased less steeply as the solution was cooled. A value for $2A_{\parallel}$ of 47 G was measured at −35°C, indicating that the label still had considerable mobility even though the temperature was below the freezing point of about −13°C. Upon further cooling, this mobile spectrum transformed into a spin-exchange spectrum as demonstrated in Fig. 4 (row B, left).

To interpret the ESR spectra observed in sucrose solutions, the phase behavior of sugar solutions as a function of temperature must be considered: when cooled, sugar solutions of less than approximately 50% concentration (w/w) are known to form a two-phase system consisting of ice crystals and a sugar water matrix that increases in viscosity with decreasing temperature, ultimately becoming a glass. More concentrated solutions form a single-phase sugar-water matrix [21]. The increase in the maximum hyperfine splitting with decreasing temperature observed in sucrose solutions of TEMPO, without lipid (Fig. 6A) and with sonicated egg PC (Fig. 6B), is consistent with this phase behavior, and suggests that the probe was dispersed in a sucrose-water matrix in both the absence and the presence of lipid.

Figs. 6B and 6C show the temperature depen-

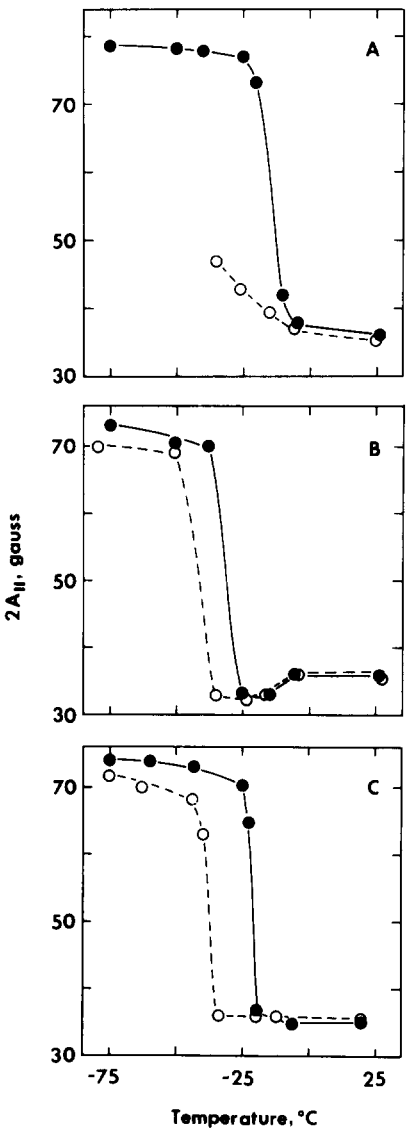

Fig. 6. Temperature dependence of the maximum hyperfine splitting, $2A_{||}$ (gauss). (A) TEMPO dissolved in H_2O (○) and in 30% sucrose solution (●). No phospholipid was present. (B) TEMPO in sonicated egg PC dispersions. (C) TEMPO in sonicated DPPC dispersions. The phospholipids (10 mg/ml = 0.013 M) were dispersed either in H_2O (○) or in 30% sucrose (●).

dence of the maximum hyperfine splitting $2A_{||}$ for TEMPO in egg PC and DPPC dispersions, in the presence (closed circles) and absence of sucrose (open circles). Consistent with the spectral shapes shown in Fig. 4D and E, the apparent $2A_{||}$ values for egg PC in the presence of sucrose increased steeply at about $-30°C$, while in the absence of sucrose this steep increase was shifted to about $-40°C$. A similar temperature dependence was observed for DPPC, except that the steep increase in $2A_{||}$ occurred at $-22°C$ and $-35°C$, respectively. These abrupt increases in $A_{||}$ occurred at temperatures when both egg PC and DPPC were below their respective T_c values of -15 and $41°C$, and thus seem not to be due to lipid phase transitions.

The question of whether or not the motion of the phospholipid was affected by sucrose was addressed by probing different bilayer regions. For this purpose CAT 16, TEMPO-palmitate, 5-doxylstearic acid and 16-doxylstearic acid were incorporated into sonicated egg PC dispersions. The temperature dependence of the maximum hyperfine splitting $2A_{||}$ derived from the ESR spectra of these probes is shown in Fig. 7. The presence of sucrose did not significantly affect the $2A_{||}$-temperature profile recorded for CAT 16 (Fig. 7A), indicating that the motion of phospholipid polar group is not greatly changed in the presence of sucrose. The sigmoidal curve obtained for TEMPO-palmitate (Fig. 7B) probably reflects the phase transition of egg PC. The $2A_{||}$ values, both above and below T_c, in the presence of sucrose agreed with those in the absence of sucrose, within experimental error. There were, however, significant differences between the two labels (Fig. 7A and B): the $2A_{||}$ values for CAT 16 were consistently higher than those for TEMPO-palmitate. Furthermore, the temperature profile of CAT 16 does not reflect the phase transition of egg PC: the $2A_{||}$ values appear to increase linearly with decreasing temperature, indicating a gradual loss in the motion of the spin probe. CAT 16 with a positively charged quaternary ammonium group probably interacts electrostatically with the phospholipid phosphate group; its motion possibly reflects that of the latter group. The temperature profiles of both 5-doxyl- and 16-doxylstearic acid again show little difference in the presence and absence of sucrose (Fig. 7C and D). It is interesting to note that the temperature dependence of 5-doxylstearic acid resembled that of CAT 16, and that the profiles of 16-doxylstearic acid and of TEMPO-palmitate were similar.

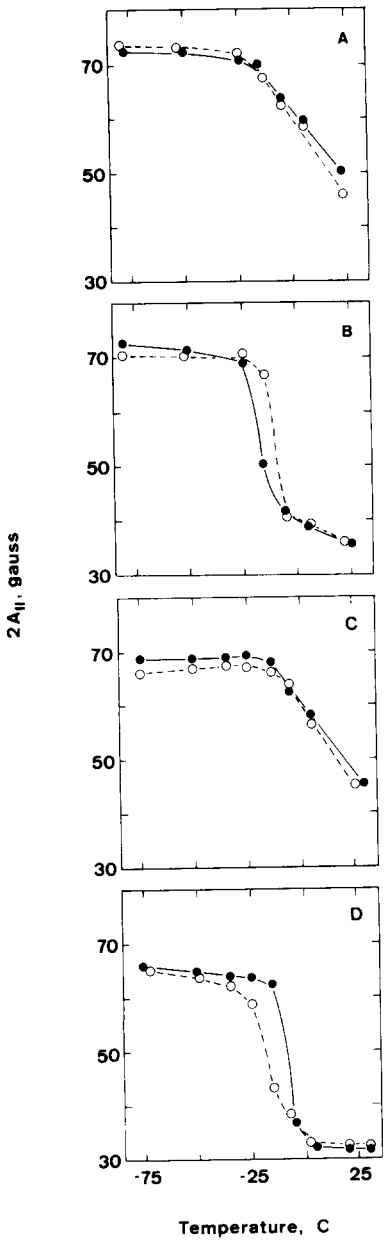

Fig. 7. Temperature dependence of the maximum hyperfine splitting, $2A_{||}$ (gauss), for different spin labels present in bilayers of sonicated egg PC dispersions. A, CAT 16; B, TEMPO-palmitate; C, 5-doxylstearic acid; D, 16-doxylstearic acid. The egg PC (10 mg/ml = 0.013 M) was dispersed either in H_2O (○) or in 30% sucrose solution (●).

Differential scanning calorimetry (DSC)

Fig. 8 shows DSC results obtained with dry DPPC and aqueous DPPC dispersions in the presence and absence of sucrose. The effect of sucrose on the crystal-to-liquid-crystal phase transition temperature of dry DPPC is shown in Fig. 8A. DPPC was dried and was present either as the monohydrate or the dihydrate [22]. It gave a transition at 69°C (curve 1, Fig. 8A) with an enthalpy change $\Delta H = 30.8$ J/g. When a DPPC dispersion in 70% sucrose was taken to dryness and the residue was thoroughly dried in vacuo, the heating curve in Fig. 8A (curve 4) was obtained. The main crystal-to-liquid-crystal transition of DPPC was reduced by about 20 Cdeg to 47°C and there was a high-temperature shoulder. The heating curve is similar to that obtained with DPPC dispersed in 70% sucrose (see curve 4, Fig. 8B). The effect of sucrose on the thermal behavior of aqueous DPPC dispersions is depicted in Fig. 8B. Up to about 10% sucrose (0.3 M), no effect on the heating and cooling curves was observed. The heating curve of

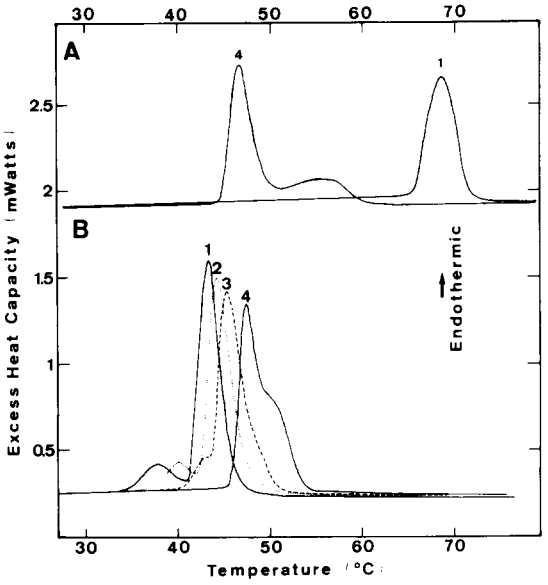

Fig. 8. (A) Differential scanning calorimetry heating curves of dry DPPC (about 1 mg) present probably as the mono- or dihydrate [22] (curve 1), and of dry DPPC (about 1 mg) in the presence of sucrose (curve 4). The latter sample was obtained by the thorough drying in vacuo of a DPPC dispersion originally made up in 70% sucrose solution (cf. curve 4, B). (B) Differential scanning calorimetry heating curves of unsonicated DPPC dispersions (20 mg/ml = 0.027 M) in sucrose solutions. DPPC dispersed in 10% (0.29 M) sucrose (solid curve 1), in 30% (0.87 M) sucrose (dotted curve 2), in 50% (1.95 M) sucrose (dashed curve 3), and in 70% (2.03 M) sucrose (solid curve 4). All heating curves were recorded at 5 Cdeg/min.

DPPC in 10% sucrose (Fig. 8B, curve 1) was indistinguishable from that of an aqueous DPPC dispersion consisting typically of a small pretransition at about 36.5°C and a main transition at 42°C with a total enthalpy chang, ΔH, of 58.4 J/g (sum of pre- and main transition). With increasing sucrose concentration, both pre- and main transition shifted to higher temperatures (curve 2); at the same time the ΔH of the phase transition decreased slightly. At 50% sucrose the pretransition appeared as a low-temperature shoulder of the main transition (curve 3). At 70% sucrose the main transition was increased to about 46°C with a conspicuous shoulder at higher temperatures; ΔH was decreased by about 10% to 52 J/g (curve 4).

Discussion

Recently, unilamellar lipid vesicles have become important as potential drug-delivery systems. The reason for this is that drugs and pharmacologically active compounds encapsulated within unilamellar lipid vesicles have been shown to have several advantages over conventional drug applications. The most significant of these is the possibility of selective drug delivery or drug targeting. When considering this kind of application, the stability of the drug-phospholipid system is important. Related to stability is the question of storage. It is clear that drugs encapsulated within lipid vesicles cannot be stored as aqueous lipid dispersions because such dispersions are neither chemically nor physically stable. They have been shown to undergo chemical degradation upon storage; furthermore, they usually are also thermodynamically unstable, particularly small unilamellar vesicles, which are known to undergo aggregation and/or fusion during storage at room temperature. The work presented here is relevant to the question of stability and long-term storage of drug-delivery systems that are based on small unilamellar vesicles.

The main conclusions that we can draw from the data presented here are that the average size and size distribution of small unilamellar vesicles made from egg PC are practically unaffected by freeze-thawing or dehydration/rehydration, provided that the dispersion medium contains sufficient sugar. Evidence for this conclusion is derived from a number of independent techniques: gel filtration on Sepharose 4B, dynamic light scattering, ^{1}H-NMR signal intensity measurements, and freeze-fracture electron microscopy. In addition, we were able to show that the structural integrity of the phospholipid bilayer is maintained during freeze-thawing or freeze-drying in the presence of sufficient quantities of sucrose. Evidence for this conclusion is based on the observation that the phospholipid bilayer in the presence of sucrose maintains its sealing properties and does not become permeable to ions. The ability of sucrose to preserve small unilamellar vesicles and their permeability barrier during freezing and dehydration was compared to that of other sugars. From the limited evidence presented it would appear that the protective and stabilizing effect is a general property of sugars. The disaccharides tested, sucrose and trehalose, were more effective than the monosaccharide glucose when compared on a molar basis (see Fig. 3B and C). Even when compared on the basis of the number of OH-groups per sugar molecule, the disaccharides were more effective than the monosaccharide, at least at low sugar/lipid molar ratios.

The mechanism of the cryoprotection of small unilamellar vesicles by sucrose seems to be related to the ability of the sugar to replace water molecules of hydration. Bound water has been shown to be hydrogen-bonded to the phospholipid polar group, particularly to the phosphate group [22]. The observation that the presence of 10 mM Eu^{3+} almost completely abolished the cryoprotective effect of sucrose [10] strongly suggests that the sugar is hydrogen-bonded to the same binding site, probably replacing water of hydration. Further evidence that sucrose is bonded to the phospholipid polar group is provided by DSC experiments with DPPC. In the presence of 70% sucrose the gel-to-liquid-crystal transition temperature of DPPC was raised by about 5 Cdeg. This is consistent with published data [23]. Furthermore, adding sucrose to dry DPPC had qualitatively the same effect as adding H_2O; it lowered the transition temperature T_c by about 20 Cdeg from 68 to 48°C (Fig. 8). The observed increase in T_c of aqueous DPPC dispersions containing sucrose could be due to sucrose being hydrogen-bonded

both to the phospholipid polar group and to neighboring sucrose molecules. In this way a hydrogen bonded network at the lipid-solvent interface would be produced that could cause an increase in T_c. At the same time, the interaction of sucrose with the phospholipid seems to produce an expansion in the packing of the phosphatidylcholine polar groups. This is indicated by a significant increase in the TEMPO distribution parameter f of sonicated egg PC suspensions upon the addition of sucrose (Fig. 5). An expansion in the packing of the lipid molecules in the presence of trehalose, sucrose, glucose, raffinose and inositol was also observed by Crowe et al. [24]. These authors showed by surface pressure studies that monolayers of DMPC and DPPC were expanded in the presence of the above saccharides. However, the molecular and segmental motion of the phospholipid does not appear to be significantly affected by the interaction of the phospholipid polar group with the sugar, as shown in Fig. 7. This is true at least when different regions of the egg PC bilayer are probed with spin labels having the paramagnetic group in different positions along the bilayer normal.

Our conclusions regarding the binding of sugar to the phosphatidylcholine polar group are consistent with the data of Crowe et al. [25]: from DSC and infrared spectroscopic studies using aqueous DPPC dispersions as a model system, these authors concluded that trehalose interacts with the phospholipid polar group, probably through hydrogen bonding of the sugar hydroxyl groups with the phosphate group of the lipid.

We have shown that sugar bound to the phospholipid polar group, probably through hydrogen bonding, stabilizes small unilamellar vesicles and suppresses vesicle aggregation and fusion induced by freeze-thawing or freeze-drying. Undoubtedly the sugar hydroxyl groups play an important role in this stabilization. A question of considerable interest is whether sugar OH-groups covalently attached to lipid molecules have a similar protective effect. To test this possibility, sonicated aqueous dispersions of phosphatidylinositol (from yeast, at 10 mg/ml) were subjected to freeze-thawing and freeze-drying in the absence of added sugars. Preliminary ^{1}H-NMR signal intensity measurements show that small unilamellar vesicles made of phosphatidylinositol, which contains a *myo*-inositol group covalently linked to the phosphate group, survive either of these treatments, to an extent of 80–100%.

The ability to freeze or dehydrate phospholipid small unilamellar vesicles in the presence of sugars with retention of their structural and permeability properties is important when considering the exploitation of small unilamellar vesicles as drug-delivery systems. It offers the possibility of long-term storage of such liposomes in the frozen or anhydrous state.

Acknowledgements

This work was supported by the Swiss National Science Foundation (Grant no. 3511-0.83) and by the Busch Memorial Fund (Rutgers University).

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