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Stabilization of lipid bilayer vesicles by sucrose during freezing

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ABSTRACT **The freeze-induced fusion and leakage of small unilamellar vesicles (SUV) of natural and synthetic phosphatidylcholines and the suppression of these processes by sucrose was studied by electron microscopy, by high-resolution NMR, and by ESR techniques. During slow freezing of SUV suspensions in water, the lipid was compressed into a small interstitial volume and transformed into a multilamellar aggregate without vesicular structure. When frozen in sucrose solution, the lipid also was compressed between the ice crystals but remained in the form of vesicles. The fractional amount of lipid remaining as SUV after freezing was found to increase significantly only at sucrose/lipid molar ratios above 0.4. Eu^{3+} displaced sucrose from the lipid by competitive binding. During freezing in the absence of sucrose, the vesicles became transiently permeable to ions. ESR studies showed that fusion of vesicles in the absence of sucrose is far more extensive when they are frozen while above their phase-transition temperature (t_c) than when frozen while below their t_c. It is concluded that the extent of membrane disruption depends on the membrane mobility at the moment of freezing and that sucrose exerts its protective effect by binding to the membrane interface and/or by affecting the water structure.**

Zwitterionic phospholipids, such as phosphatidylcholine (PtdCho), which exhibit only limited swelling in excess water (1), form large (>100 nm) multilamellar vesicles (MLV) as the thermodynamically most-stable form at room temperature. These can be transformed into small unilamellar vesicles (SUV) (<80 nm) by sonication or other dispersion methods. Small vesicles are an ideal test system for the study of membrane stability because they undergo fusion and revert to MLV when subjected to certain processes, such as passage through the gel-to-liquid-crystal phase-transition temperature (t_c), dehydration, freezing/thawing, freeze-drying/rehydration, or, in the case of negatively charged vesicles, addition of divalent cations. The factor common to these diverse processes is the formation of two lipid phases, the liquid-crystalline and the gel state, whose coexistence leads to defects in molecular packing (2, 3). Membrane stability is also dependent on the degree of hydration in the sense that hydration can prevent interbilayer contact (4–6). By measuring the resistance of SUV to effects of phase transition or dehydration, the stabilizing or destabilizing effect of various additives can be tested. Known stabilizers include mono- and disaccharides (7) and certain ions such as tetraalkylammonium and perchlorate (8), whereas membranes become destabilized on addition of univalent metal ions above 0.5 M (9) or multivalent ones at 1–10 mM (10).

The stabilizing effect of sucrose during freezing of liposomes had been noted (11); systematic investigations of the stabilizing effect of various sugars on the lipid bilayers of cellular components were prompted by the observation that certain organisms that can survive dehydration are rich in trehalose, a nonreducing disaccharide consisting of two glucose units (12). Desiccation and rehydration at room temperature or freezing (which amounts to removal of liquid water) of cells or cell components was observed to cause disruption of membrane structure and function unless certain sugars were present (13).

We report here an investigation of the mechanism of membrane disruption during freezing of SUV made of various PtdChos and of the protective effect of sucrose.

MATERIALS AND METHODS

PtdCho from egg yolk and the corresponding dioleoyl and dipalmitoyl compounds (Ole_2-PtdCho and Pam_2-PtdCho) were purchased from Lipid Products (Surrey, UK). The purity of the lipids was checked by TLC. They were found to be pure by TLC standards. The spin labels CAT 16 and TEMPO were purchased from Molecular Probes (Junction City, OR). [TEMPO is 2,2,6,6-tetramethylpiperidine-*N*-oxyl and CAT 16 is 4-(*N*,*N*-dimethyl-*N*-hexadecyl)ammonium-2,2,6,6-tetramethylpiperidine-1-3 oxyl bromide.] All other chemicals were of reagent grade.

Lipid dispersions were prepared by rotary evaporation of the phospholipid, together with CAT 16 when needed, from 1–2 ml of $CHCl_3/CH_3OH$, 2:1 (vol/vol). The resulting lipid film was thoroughly freed of solvent under high vacuum, dispersed by Vortex mixing in 1–2 ml of aqueous solution, and sonicated for 30–60 min with a Branson probe-type sonicator with microtip.

For electron microscopy, the samples were cryofixed by flash-freezing them in a propane jet as described by Müller *et al.* (14). They were freeze-fractured as described elsewhere (15).

^1H NMR spectra of vesicle suspensions in ^2H_2O were recorded on a Bruker Fourier-transform spectrometer at 360 MHz. Sodium acetate was included in the samples as an internal integration standard. A relaxation delay of 20 sec was used between pulses to avoid saturation of the acetate. The integrals of the $-N(CH_3)_3$ and of the hydrocarbon chain signals (defined as the sum of all ^1H signals upfield of the sodium 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 E 104-A X-band spectrometer fitted with variable temperature control. Samples of *ca.* 50 μl were sealed into 1-mm-diameter glass tubes. Temperatures were measured with a thin thermocouple inserted into the magnet cavity alongside the sample capillary and were accurate to within 0.5°C. For quenching experiments, the signal intensity was expressed by the normalized height h_0 of the $m = 0$ line, defined as

$$h_0 = \frac{\text{measured height (cm)}}{\text{receiver gain} \times \text{modulation amplitude} \times [\text{probe}].}$$

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Abbreviations: CAT 16, 4-(*N*,*N*-dimethyl-*N*-hexadecyl)ammonium-2,2,6,6-tetramethylpiperidine-1-3 oxyl bromide; PtdCho, phosphatidylcholine; Ole_2-PtdCho, dioleoyl phosphatidylcholine; Pam_2-PtdCho, dipalmitoyl phosphatidylcholine; MLV, multilamellar vesicle; SUV, small unilamellar vesicle; t_c, phase transition temperature; TEMPO, 2,2,6,6-tetramethylpiperidine-*N*-oxyl.

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RESULTS

Electron Microscopy. To study the effect of sucrose as a stabilizer of bilayers during freezing, SUV were formed by sonication of egg PtdCho dispersions (10 mg/ml = 13 mM) in water and in 10% sucrose solution, referred to as the aqueous and the sucrose sample, respectively. They were examined by freeze-fracture electron microscopy as described. Aliquots of the dispersions were subjected to the following treatments before being freeze-fractured.

(*i*) They were flash-frozen at a rate of *ca.* 10,000°C/sec in a propane jet. This is the generally accepted method for structural preservation. (*ii*) One-milliliter volumes of the sample, contained in small plastic tubes, were frozen slowly by placing them into a freezer cabinet at −100°C. (*iii*) The samples were frozen slowly, as in *ii*, then thawed by warming them to room temperature, and finally flash-frozen as in *i*.

Results are presented in Fig. 1. For the aqueous sample, flash-freezing showed that sonication had produced SUV of 20- to 50-nm diameter (Fig. 1*A*). During slow freezing, the lipid was squeezed out into the small interstitial spaces between the ice crystals (Fig. 1*B*). The lipid appeared to be compressed into multilamellar aggregates, with complete absence of closed vesicles. Thawing of the slowly frozen sample followed by flash-freezing gave large multilamellar vesicles of 0.7–20 μm, characteristic of unsonicated egg PtdCho suspensions (Fig. 1*C*).

For the sucrose sample, flash-freezing showed that, here too, SUV of 20–50 nm were initially present (Fig. 1*D*). As mentioned later, the SUV formed in sucrose were about 20% larger on average than in water, but this difference cannot be discerned by eye. Slow freezing again squeezed the lipid into a small interstitial volume between ice crystals. Here, however, in great contrast to the aqueous sample (Fig. 1*B*), the lipid retained its vesicular structure (Fig. 1*E*). Thawing and flash-freezing of the slowly frozen sample resulted in SUV, apparently unchanged in size from those originally present (Fig. 1*F*).

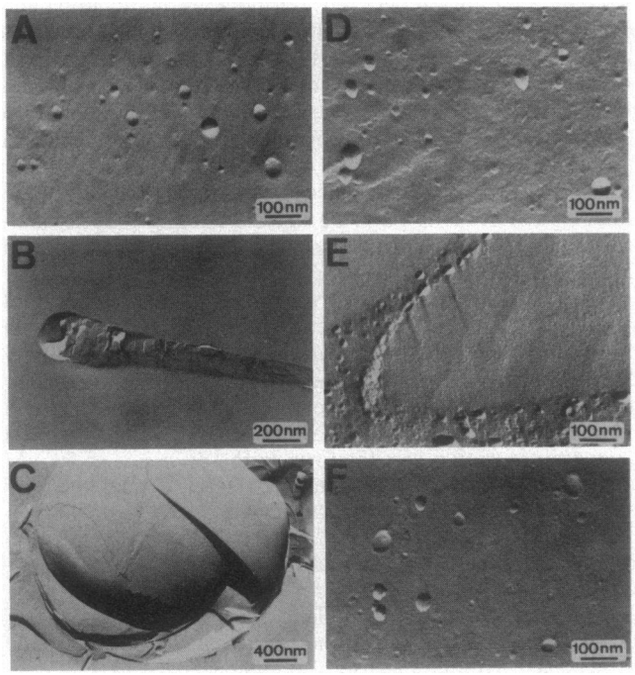

FIG. 1. Electron micrographs of egg PtdCho dispersions (10 mg/ml = 13.3 mM) in water (*A–C*) and in 10% sucrose solution (*D–F*). (*A* and *D*) Flash-frozen and freeze-fractured. (*B* and *E*) Frozen slowly at −100°C and freeze-fractured. (*C* and *F*) Frozen slowly at −100°C, rewarmed to room temperature, flash-frozen, and freeze-fractured.

NMR Studies. The fraction of lipid remaining as SUV after freezing was determined by ^{1}H high-resolution NMR spectroscopy as described. A standard freeze/thaw procedure was adopted for all samples: 1-ml aliquots, contained in 8-mm-diameter glass test tubes, were immersed for 20 min in a dry-ice/methanol bath at −72°C in a Dewar flask and then thawed in a water bath at ambient temperature. The NMR assay is based on the fact that only vesicles smaller than about 80–100 nm, or micelles, contribute to the high-resolution NMR spectrum (16–19). Comparisons with electron microscopy, gel filtration, dynamic light scattering (20), and ultracentrifugation have demonstrated that the NMR signal intensity of the hydrocarbon chain protons is a good measure of the concentration of SUV present (21). Fig. 2 shows the effect of the freeze/thaw procedure on NMR spectra of egg PtdCho dispersions in the absence and presence of sucrose. The high-resolution spectrum obtained in the original dispersion (Fig. 2, spectrum A) was almost completely lost after freezing/thawing in the absence of sucrose (Fig. 2, spectrum B). In contrast, the high-resolution spectrum was fully retained in the presence of 5% sucrose (Fig. 2, spectrum C).

A plot of the ^{1}H signal intensity as a function of sucrose concentration is shown in Fig. 3*A* for a series of samples, all containing 10 mg (13.3 mM) of egg PtdCho per ml and increasing concentrations of sucrose up to 30 mM. Below a sucrose/lipid molar ratio of 0.4, essentially no SUV remained

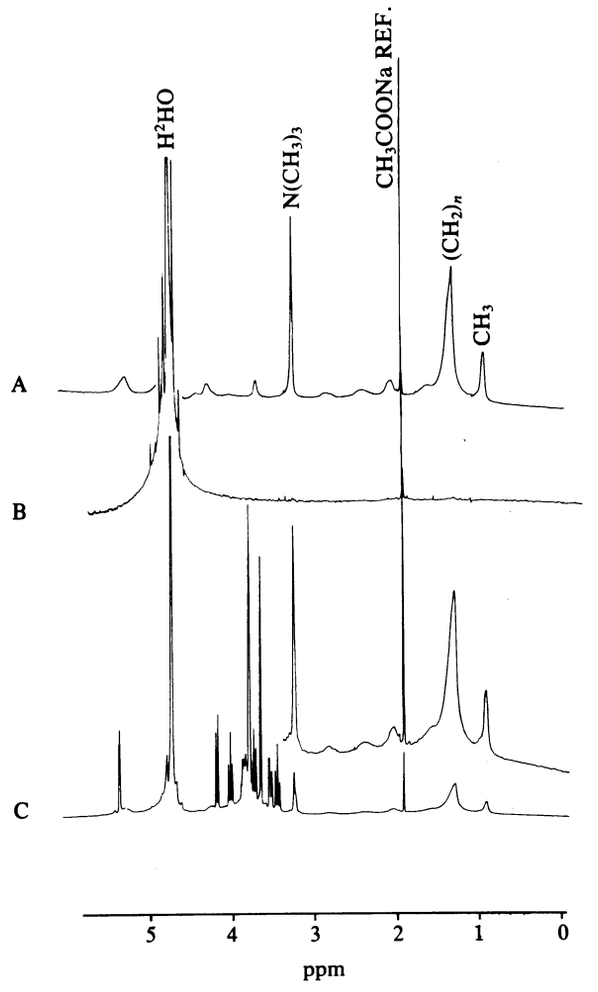

FIG. 2. ^{1}H NMR spectra (360 MHz) of sonicated egg PtdCho dispersions (10 mg/ml = 13.3 mM) in ^2H_2O. Spectra: A, in water at room temperature; B, in water after freezing/thawing; C, in 5% sucrose, after freezing/thawing. A vertically expanded version of spectrum C with amplification as in spectrum A is also shown. REF., reference.

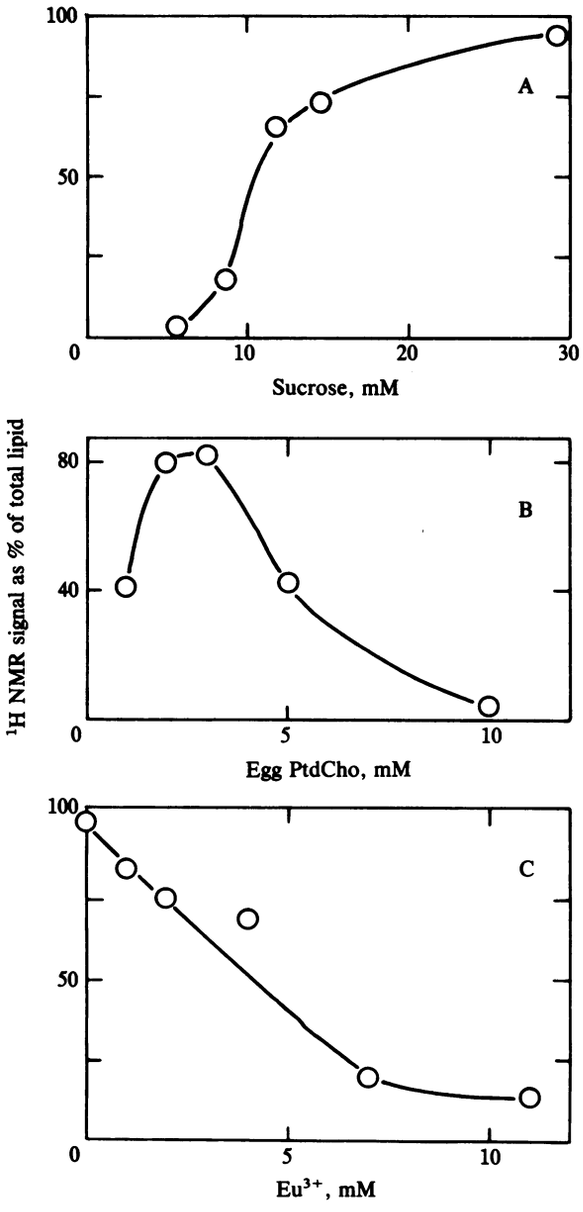

FIG. 3. ^{1}H NMR signal intensity of hydrocarbon protons in frozen and thawed egg PtdCho vesicles as a function of lipid and sucrose concentrations. (*A*) Effect of various sucrose concentrations from 0 to 29 mM at a constant egg PtdCho concentration of 13.3 mM. (*B*) Effect of various egg PtdCho concentrations from 1.33 to 13.3 mM at a constant sucrose concentration of 2.67 mM. (*C*) Suppression of the protective effect of sucrose by $Eu(NO_3)_3$ during the freeze/thaw procedure with egg PtdCho at 13.3 mM, sucrose at 66.7 mM, and Eu^{3+} varying from 0 to 11 mM.

after freezing/thawing. Above this critical ratio, the ^{1}H signal increased sharply. Nearly 100% retention of SUV after freezing resulted at sucrose/lipid ratios of 2–3 or higher (data not shown). To establish whether the effect is a function of the bulk concentration of sucrose or of the sugar/lipid ratio, the lipid concentration was varied, in another set of samples, from 1 to 10 mg/ml (1.33–13.3 mM) while keeping the sucrose concentration constant at 0.1% (2.9 mM). Fig. 3*B* shows that the ^{1}H signal intensity passed through a maximum near a sucrose/lipid ratio of 1. This shows that it is the molar ratio rather than the bulk concentration of sucrose that determines the protective effect. In a further series of samples, the concentrations of both lipid and sucrose were varied while keeping their ratio constant. The ^{1}H signal intensity was found to decrease as the concentrations were lowered (data not shown). This result is consistent with a reversible binding equilibrium.

To investigate the type of binding of sucrose to the vesicles, the protective effect of sucrose was measured in the presence of europium ions. The ^{1}H signal intensity was found to decrease with increasing concentrations of Eu^{3+} (Fig. 3*C*), producing a 50% loss in protection at ≈5 mM Eu^{3+}. The results suggest that sucrose binds to the same site as Eu^{3+}—namely, the phosphate group of the lipid (22).

As found previously (23), the vesicle structure opens up during freeze-induced fusion, causing release of solutes from the interior aqueous phase. The reverse movement of solutes from the external solution into the vesicles also occurs. To check whether sucrose suppresses the transient opening of the vesicles during freezing, 100 mM $K_3Fe(CN)_6$ was added as a paramagnetic shift reagent to sonicated egg PtdCho dispersions. As a result, part of the ^{1}H signals from the phospholipid polar groups [—$N(CH_3)_3$ and —CH_2N—] were shifted upfield as shown in Fig. 4, spectrum A. The split signal intensities were in a ratio of about 2:1, corresponding to the outer and inner polar groups. The presence of 30% sucrose affected neither the shift nor the relative intensity of the signals from the outer and inner polar groups (Fig. 4, spectra B and C). Upon freezing/thawing in the absence of sugar, the high-resolution spectrum was lost. A broad-line spectrum resulted in which the proton signal from the —$N(CH_3)_3$ group was just detectable; it was at the same upfield position as the shifted signal in the original spectrum, indicating complete penetration of the shift reagent. In the presence of sucrose and $K_3Fe(CN)_6$, the splitting of the —$N(CH_3)_3$ signal was retained after the freeze–thaw procedure (Fig. 4, spectrum D). A difference spectrum of the samples before and after freezing/thawing was practically a flat baseline (Fig. 4, spectrum E), proving that the vesicles had stayed intact during the freeze–thaw procedure in the presence of sucrose.

ESR Studies. In order to determine the temperature at which vesicle fusion occurred during a gradual cooling and warming cycle, the amphipathic spin label CAT 16 was incorporated into the PtdCho bilayers. The free radical is at the polar end of this probe and, hence, lies at the outer and inner interfaces of the vesicle membrane. The ESR signal from the outer label was quenched by addition of sodium ascorbate, a reducing agent. Loss of the remaining inner signal (which in intact membranes was about one-third of the total signal) at some point during the cooling process indicated just when the membrane had become permeable to ascorbate.

Vesicle suspensions were prepared containing 67 mM lipid and 1 mM CAT 16. ESR spectra were recorded, and signal heights h_0 were determined as described at progressively lower temperatures, starting at 2°C. For each lipid, a temperature profile was run without ascorbate at first, giving a signal representative of the total spin label present. A parallel temperature profile was then run in the presence of 50 mM ascorbate. At each temperature the signal from the latter sample was recorded as a fraction of the total signal height. The ascorbate was added at 2°C, since even intact membranes are somewhat permeable to this reagent at higher temperatures. Fig. 5*A* shows results for egg PtdCho. In the presence of 30% sucrose, no loss of signal occurred during cooling to −33°C and rewarming, showing complete absence of leakage. Without sucrose, the inner signal was suddenly and almost completely quenched at the freezing point and not recovered on rewarming. The freezing point was recognized quite independently of any lipid transition by a sudden change in the microwave absorption by the sample (which necessitated a retuning of the cavity). The freezing point was consistently observed at about −13°C, probably because of

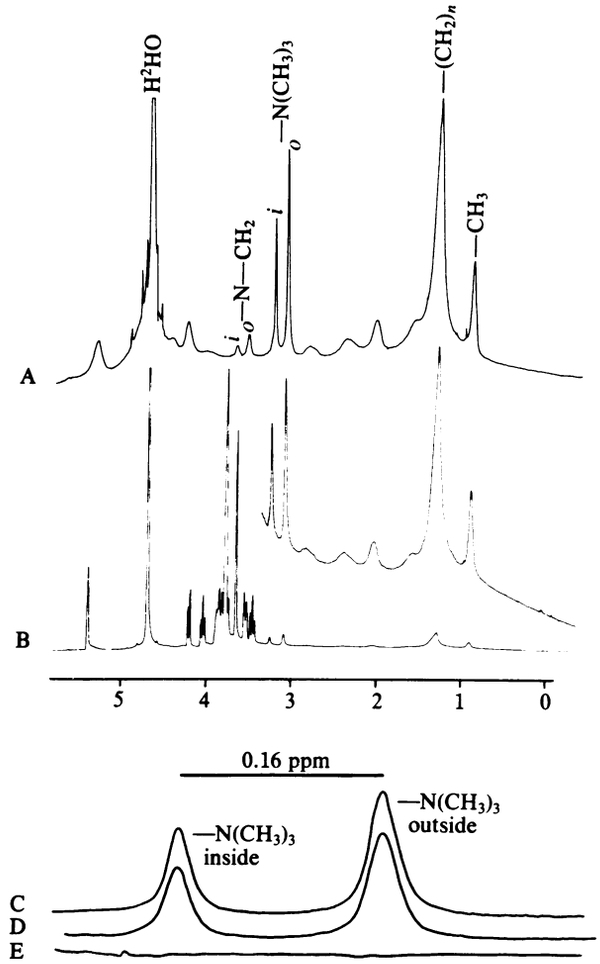

FIG. 4. ^1H NMR spectra (360 MHz) of sonicated egg PtdCho dispersions (10 mg/ml = 13.3 mM) in ^2H_2O to which $K_3Fe(CN)_6$ (final concentration, 100 mM) had been added after sonication. Spectra: A, In ^2H_2O at room temperature. Outside (shifted) and inside (nonshifted) signals of the polar groups are labeled *o* and *i*, respectively; B, As in spectrum A, but in 30% sucrose solution (a vertically expanded version of this spectrum, with amplification as in A, is also shown); C, horizontally expanded section of spectrum B, showing outside and inside $—N(CH_3)_3$ signals; D, As in spectrum C, but after the freeze–thaw procedure; E, difference spectrum of spectra C and D.

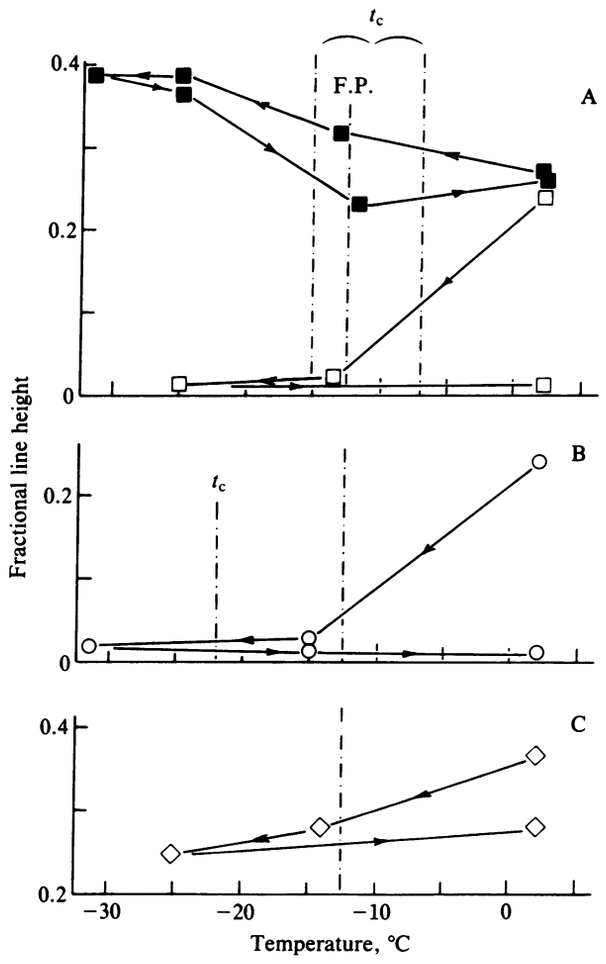

FIG. 5. Detection of the onset of membrane fusion. Internal egg PtdCho signal of sonicated PtdCho vesicles containing CAT 16 after addition of 50 mM sodium ascorbate to the external solution, as a function of temperature. Each internal signal is expressed as fraction of the total ESR signal (as measured in the absence of ascorbate) at the same temperature. In all cases the lipid was 67 mM, CAT 16 was 1 mM, and sucrose (where used) was 30%. Transition temperatures, t_c, and the freezing point, F.P., are shown. The connecting lines show the sequence of measurements only and do not represent linear changes. (A) Egg PtdCho with (■) and without (□) sucrose. (B) Ole_2-PtdCho without sucrose. (C) Pam_2-PtdCho without sucrose.

supercooling and/or eutectic effects. A differentiation between the effects of freezing and of lipid-phase transition was not possible with egg PtdCho because the t_c range of this lipid, −7° to −15°C (24), and the freezing point coincide. Results for Ole_2-PtdCho, which has a phase-transition temperature of −22°C, are shown in Fig. 5*B*. A sudden drop of the interior ESR signal from 24% to 2.5% (relative to the ascorbate-free sample) occurred at the freezing point. Subsequent cooling to −32°C and rewarming to −12.5°C, which involved two passes through t_c, caused a further slight drop in signal height to 0.8%. Pam_2-PtdCho, with a t_c of 41°C, was examined for comparison. The vesicles were formed by sonication at 50°C and then quickly cooled to 2°C. This caused only a minor degree of fusion, since the sample had passed quickly through the transition point. Data for the temperature profile with ascorbate are shown in Fig. 5*C*. A partial loss of the inner signal from 38% to 28% of the ascorbate-free reference occurred when the sample was frozen and rewarmed to 2°C. It may be noted in passing that the higher initial value of 38% for the inner Pam_2-PtdCho signal before freezing, compared to 24% for egg PtdCho and Ole_2-PtdCho, reflects a larger vesicle size, which may have resulted from passage through t_c. With Pam_2-PtdCho the extent of vesicle leakage due to phase transition could not be determined by the ascorbate permeation method because it is applicable only at low temperatures. The above experiments show clearly that membrane disruption is far more extensive for lipids that are still in the liquid-crystalline phase when the freezing point of the aqueous suspension is reached than for those lipids that are already in the gel phase at the freezing point (compare *B* and *C* of Fig. 5).

DISCUSSION

The results reported here show that freezing of a suspension of small egg PtdCho vesicles in water results in loss of vesicular structure at the moment of freezing, with conversion of the lipid to multilamellar aggregates. This supports the model of Crowe and Crowe (12) for the disruption of vesicles during freezing and accounts for the observed mixing of the external and internal aqueous phases of the vesicles. In sucrose solutions up to about 30% (0.88 M), the vesicles do

not coalesce despite the fact that here, too, large ice crystals form on freezing, which expel the lipid and confine it to a small volume. The question arises whether this protective action of sucrose is due only to its attachment to the membrane, which our results have shown to occur, or whether there is a second effect due to the influence of sucrose on water structure.

From low-temperature ESR experiments with TEMPO (unpublished results), we have found that, upon freezing of an aqueous TEMPO solution, the spin label is squeezed out between ice crystals, giving rise to a spin-exchange spectrum. However, when TEMPO is present in sucrose-free egg PtdCho suspensions, it exists in a mobile phase even down to −50°C. Since TEMPO also has been shown to be squeezed out from lipid bilayers below t_c, it must be considered to be associated with unfreezable bound water at the membrane interface at temperatures below t_c. In the presence of sucrose, no such mobile phase was seen: at −50°C the probe was immobilized, probably in a glassy sucrose–water matrix. Such matrices are known to form in solutions of sucrose or other sugars (25) because of supercooling or supersaturation, without crystallization of the solute. A rigid glass could by enveloping the vesicles prevent their deformation and collapse. Thus, sucrose could act as a stabilizer in two ways, (*i*) by binding to the bilayer and preventing direct interbilayer contact and (*ii*) by embedding the vesicles in a rigid external phase, thus immobilizing them. Our results, particularly the effect of Eu^{3+} in abolishing the protective effect of sucrose, suggest that the second effect alone, without the first, is insufficient for cryoprotection.

The ESR experiments on ascorbate permeation clearly establish that the degree of vesicle disruption during freezing depends on the bilayer fluidity: disruption is high for lipid bilayers that are in the liquid-crystalline phase at the time of freezing of the lipid suspension, less so for lipid suspensions that are frozen with the phospholipid in the gel phase, and absent for lipids with sufficient sucrose bound to them. This is in agreement with the observed dependence of the fusion rate on membrane fluidity (26) and with the resistance to disruption during freezing of distearoyl PtdCho vesicles (27). If the greater resistance to disruption of a gel-phase bilayer, compared to a liquid-crystalline bilayer, is due to less water of hydration and greater rigidity, then the even greater resistance of bilayers having sucrose bound to them supports the theory that sugars can replace membrane-associated water (28). Our results show that sucrose binds to vesicles, most likely at the phosphate group (22), but not necessarily only to that site because nonspecific hydrogen bonds must be involved. The presence of a critical minimum sugar/lipid molar ratio suggests that sucrose inserts itself between the polar groups, since this will facilitate the binding of further sucrose by generally increasing the average distance between groups. Such expansion by sugars has been reported for phospholipid monolayers (29). We have observed that sucrose increases the average size of SUV formed by sonication by about 20%. At this stage, little can be said about the presence of water at an interface containing bound sucrose. The water that forms hydrogen-bonded bridges between lipids (30) may be replaced, or it may remain at least partially and aid in the bonding of sucrose. There also could exist an extensive sucrose–water network projecting some distance from the membrane interface.

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