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Fragmentation into small vesicles of dioleoylphosphatidylcholine bilayers during freezing and thawing

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Abstract

Multilayered liposomes of some phosphatidylcholines progressively fragment into small vesicles when the electrolyte solution in which they are suspended is subjected to successive cycles of freezing and thawing. The fragmentation process, routinely monitored by absorbance measurements and verified by electron microscopy and dynamic light scattering, involves bilayer breakage and resealing. After 10 cycles of freezing and thawing in 0.1 M electrolyte solution, the result is a population of vesicles smaller than 200 nm diameter. Sucrose, a common cryoprotectant, completely inhibits fragmentation. Fragmentation is absolutely dependent upon the presence of an electrolyte. Those electrolytes most effective in promoting liposome fragmentation have large freezing point depressions and corresponding high solubilities at the freezing point. This, coupled with the observation that saturating concentrations of electrolyte are less effective than 0.1 M solutions indicates that an essential stage in the fragmentation process is osmotic extraction of water from the vesicles, i.e., ice formation in the external phase leads to a progressive increase in the electrolyte concentration of the residual external solution, which, in turn, dehydrates the vesicle. In addition, for maximal fragmentation, the minimum temperature must be at least as low as the solute eutectic temperature. Particular physical properties of the bilayer are also important, for dioleoyl and diphytanoyl derivatives are much more susceptible to fragmentation than are other phosphatidylcholines, and inclusion of 50 mol% cholesterol in dioleoylphosphatidylcholine completely inhibits membrane breakup. This system provides insight into mechanisms of freezing damage to membranes and may also offer a very simple and rapid assay for biological cryoprotectants.

Key words: Liposome; Phospholipid; Vesicle; Bilayer; Freezing; Thawing; Cryobiology

1. Introduction

Liposomes have become an essential element in many procedures of cell and membrane biology [1,2]. Not only are they used to study bilayers and model their response to environmental challenges, but they represent essentially the only mode of reconstituting purified membrane proteins in a form that enables appropriate testing of their function. A number of liposome preparation methods have a freezing and thawing step as an important part. Those leading to reconstitution usually follow the procedure pioneered by Kasahara and Hinkel, whose reconstitution of the erythrocyte glucose-transporter involved freezing and thawing of sonicated vesicles [3]. Subsequently, it was found that freeze-thaw-sonication produced liposomes that very efficiently encapsulate the aqueous phase [4] and that maximal encapsulation was reduced by high ionic strength, high lipid concentrations and the presence of sucrose. An osmotic inflation procedure that generates giant vesicles up to tens of microns in diameter also relies upon freezing thawing [5]. Recently, it has been emphasized that freezing and thawing of multilamellar vesicles (MLVs) is necessary to produce a uniform distribution of solute throughout the layers [6].

Freezing and thawing causes disruption of liposomal as well as cell membranes and liposomes have therefore found application as test systems to assess potential biological cryoprotective agents. Particular attention has been given to sugars as cryoprotectants, several of which were found to contribute to vesicle stabil-

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Abbreviations: MLV, multilamellar vesicles; DOPC, dioleoylphosphatidylcholine; Mops, 4-morpholinepropanesulfonic acid.

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ity during freeze-thawing [7–9]. The mechanism of cryoprotectant action remains to be elucidated, but commonly discussed possibilities involve the sugar binding directly to the lipid headgroups or influencing the process of vitrification of the freezing aqueous phase. Cholesterol can act as an 'internal' membrane cryoprotective agent, perhaps by maintaining the bilayer in a fluid-like state over a large range of temperatures [10,11].

We have examined the effects that the nature of the electrolyte has for the consequences of freezing and thawing suspensions of 1,2-dioleoylphosphatidylcholine vesicles. Remarkably, in the presence of certain salts, the freeze-thaw process leads to the complete fragmentation of the original large multilamellar liposomes into much smaller (200 nm) vesicles. The effect involves a complex interplay between the eutectic temperature of electrolyte and the lowest temperature reached during the freezing phase. Vesicle fragmentation was inhibited in the presence of sucrose in the aqueous phase and cholesterol in the membrane phase.

2. Materials and methods

Chemicals

Most lipids were obtained from Avanti Polar Lipids (Alabaster, AL), but some came from Sigma (St. Louis, MO). They were stored in chloroform (10 mg/ml) at −20°C. Unless otherwise stated, the lipid used in all experiments was 1,2-dioleoylphosphatidylcholine (DOPC). Calcein was obtained from Hach (Ames, IA). Its purity was verified by thin layer chromatography with 60:30 chloroform/methanol as eluting solvent. Water, from the deionized building supply, was passed through charcoal and ion exchange cartridges and finally through a Barnstead 'organopure' mixed bed charcoal-ion exchange cartridge. Buffers and salts were from conventional sources. Aqueous solutions were stored in borosilicate glassware.

Preparation of liposomes

Preparation of liposomes was as follows unless otherwise stated. 100 μl of a chloroform solution of phospholipid (10 mg/ml) was freed of bulk solvent under a stream of argon and kept under vacuum (oil pump) for 2 h. The lipid was then hydrated to a final volume of 1 ml with the appropriate aqueous phase. For most experiments, this was 0.1 M NaCl buffered with 10 mM sodium phosphate buffer (pH 7.2). Other salts were substituted for NaCl for comparative purposes, but samples containing 0.1 M NaCl were included in all assays as internal controls. Following vortex agitation to emulsify the mixture, the vesicle suspensions were subjected to one of the freeze-thaw protocols described below. Thawing was in a water bath at approximately 50°C. Freeze-thaw cycles were normally carried out 10 times. Turbidity of the samples was evaluated by measuring their absorbance at 420 nm with a Beckman DU-50 Spectrophotometer. Where indicated, some samples contained sucrose (25 mM–0.4 M) in the aqueous phase and others contained cholesterol (33 mol%–50 mol%) in the membrane phase.

Freezing procedures

Normal freezing. Samples contained in 13 × 100 mm disposable borosilicate culture tubes were frozen in a dry-ice ethanol bath (mean cooling rate ≈ 60C°/min) until they had cooled to approximately the bath temperature. Samples in solutions of LiCl and $CaCl_2$ were also immersed in liquid nitrogen following cooling in the dry ice bath. The eutectic temperatures of solutions of these two salts are quite low and supercooling without complete freezing appeared likely unless they were cooled below solid CO_2 temperature.

Rapid freezing. Samples were immersed in liquid nitrogen for 40 s. This produces a mean cooling rate ≈ 125C°/min from room temperature to −80°C.

Slow freezing. Samples in 10 × 60 mm test tubes were placed in a nested set of 3 larger test tubes, all of which were immersed in a solid CO_2-ethanol bath. The cooling rate between −20°C and −60°C was ≈ 5C°/min. This procedure was used for experiments involving sets of vesicles in NaCl, KCl, LiCl, NaI, and $MgCl_2$ solutions. After cooling to −60°C, the sample test tube was placed in a dry-ice ethanol bath to complete cooling to solid CO_2 temperature.

Very slow freezing. Samples were placed in a test tube rack in a −20°C freezer for 2 h. This procedure generates a mean cooling rate of a few tenths of a degree per minute.

Temperatures were monitored with a thermocouple immersed in one of the samples. Comparisons of cooling rates with and without a second, dummy thermocouple showed that heat conduction through the thermocouple wires was insignificant.

Eutectic temperatures and solute concentrations at the eutectic temperature were obtained from the literature [12–14].

Determination of liposome encapsulated volume

The volume encapsulated within the liposomes after various treatments was determined using a fluorometric method based on quenching of extra-vesicular calcein by cobalt ion [15]. Vesicles were hydrated with 10 mM 4-morpholinepropanesulfonic acid (Mops) buffer (pH 7.2) containing 0.1 M NaCl and 0.1 mM calcein at a lipid concentration of 10 mg/ml. A higher concentration of lipid was used in these experiments so that the internal volume was an appreciable fraction of the total volume, allowing greater accuracy in the determination of encapsulated volume. After vortexing, the

liposome suspension was frozen and thawed 0, 5, or 10 times. 20 μl of the corresponding samples were diluted in 480 μl of Mops (10 mM Mops (pH 7.2)) and 0.1 M NaCl. Fluorescence was measured before and after addition of 12 μl of 20 mM $CoCl_2$ and again after lysing the vesicles with 1/20 volume of 10% Triton X-100. The excitation wavelength was 480 nm and emission was monitored at 520 nm. After appropriately correcting for dilution and background, as described in [15], the ratio of fluorescence after $CoCl_2$ addition to that before its addition is the ratio of internal to external volume.

Determination of vesicle rupture

DOPC (10 mg) was hydrated in 1 ml of phosphate-buffered 0.1 M NaCl, vortexed and frozen and thawed 10 times. 5 μl of 10 mM calcein was added to the sample and vesicles were frozen and thawed an eleventh time. Post freeze-thaw treatment of samples was the same as for those of the previous section. Fluorescence measurements before and after cobaltic ion quenching of external calcein fluorescence enabled calculation of the internal volume that had equilibrated with the external phase during the eleventh freeze-thaw cycle.

Determination of vesicle size

The size distribution of liposomes was determined by photon correlation spectroscopy using Brookhaven Instruments equipment. The light source was the 488 nm line of an argon ion laser. In addition, manual size measurements were made of vesicles in photographs obtained by freeze-fracture electron microscopy. The rapid-freezing procedure has been described elsewhere [16]. The light microscope, with both phase and differential interference contrast optics, was used routinely to examine wet mounts of vesicle preparations.

3. Results

In the presence of salt, the absorbance of DOPC vesicles decreases with increasing numbers of freeze-thaw cycles

As shown in Fig. 1, the absorbance of suspensions of vesicles frozen and thawed in a 0.1 M saline solution decreased with each freeze-thaw cycle. After 10 cycles, the absorbance of the suspension has decreased approximately 0.8 absorbance units. In contrast to this marked reduction in absorbance when liposomes were frozen and thawed in NaCl solution, the absorbance of the samples actually increased by about 0.2 absorbance units when MLVs were frozen and thawed in water or in 10 mM sodium phosphate buffer solution. The effect of freezing and thawing in NaCl solutions as shown in Fig. 1 will be seen below to reflect a fragmentation of large multilamellar liposomes into smaller vesicles.

DOPC from two suppliers, Avanti and Sigma, exhibited indistinguishable behavior. Both of these phospholipids were pure by normal criteria of thin layer chromatography. Nevertheless, we tested the effect of lysophosphatidylcholine (oleoyl derivative) and found that inclusion of 4 mol% of this potential hydrolysis product of DOPC had no detectable effect on the fragmentation phenomenon, as least as detected by turbidity measurement.

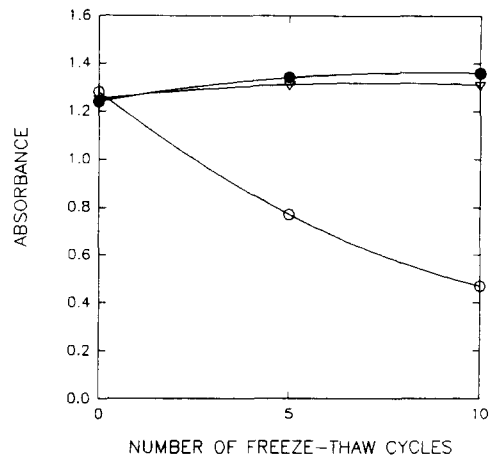

Fig. 1. Multilamellar liposomes fragment upon freezing and thawing in presence of 0.1 M NaCl, but not in water or in dilute phosphate buffer. 1 mg of DOPC was suspended in water (filled circles), in 10 mM sodium phosphate buffer (pH 7.4) (triangles) or in phosphate-buffered 0.1 M NaCl (open circles). The suspension was vortexed and then frozen in a solid CO_2-ethanol bath. Samples were thawed in warm water and the process repeated the number of times indicated in the Figure. Initially and after 5 and 10 freeze-thaw cycles, the absorbance of the samples at 420 nm was determined.

Measurements were also performed on samples hydrated with 10 mM potassium phosphate, sodium borate and Mops buffers (all at pH 7) instead of sodium phosphate buffer. These buffers neither measurably affected fragmentation in the presence of 0.1 M NaCl nor induced fragmentation in the absence of NaCl. Although phosphate buffer was routinely included in our measurements, its omission was without consequence.

After repeated freezing and thawing, vesicle diameters are reduced to a few tenths of a micron

Vesicles were examined by phase and differential interference contrast microscopy at 400 × magnification. Before freeze-thawing, vesicles were aggregated in large, bulky clusters, often interconnected by linear tethers. After 10 freeze-thaw cycles in 0.1 M NaCl as in the procedure of Fig. 1, virtually nothing in the sample was visible except for an occasional clump of lipid. Freeze-fracture electron micrographs of frozen and thawed DOPC vesicles, indeed, revealed large numbers of vesicles smaller than the limit of resolution of the light microscope. 75% of the vesicles were between 60 and 150 nm in diameter and none of those observed

were larger than 250 nm or smaller than 45 nm. Only unilamellar vesicles were seen, although cross fractures were not common. DOPC vesicles after freeze-thawing were also sized by photon correlation spectroscopy. According to this technique, the average particle diameter was approximately 145 nm; 80% of the particle diameters were between 200 and 500 nm, 4% were smaller than 90 nm and there were no detectable particles larger than 500 nm.

The aqueous volume encapsulated by liposomes increases with freeze-thawing

Since the size reduction that occurs upon freezing and thawing of DOPC vesicles in electrolyte solution must involve fragmentation of the bilayer of the source MLVs, each freeze-thaw cycle should produce a transient loss of barrier properties of the bilayer. This presumption was tested by including a fluorescent dye in the external phase and then determining how much of it became internalized during the freeze-thaw cycle. As shown in Fig. 2, the volume trapped by liposomes increased in proportion to the number of freeze-thaw cycles, as expected. For 0, 5, and 10 cycles the percentage of the external aqueous phase trapped by a 10 mg/ml suspension was 2.0, 11.7, and 17.4, respectively. The latter figure is approximately what would be expected from vesicles with 200 nm average radii, assuming complete equilibration with the external phase.

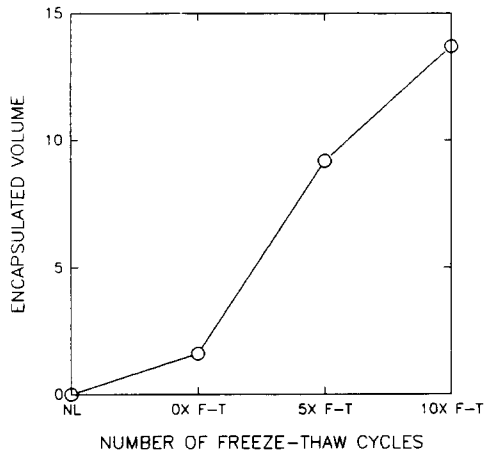

Fig. 2. Liposome fragmentation leads to increased encapsulation of the aqueous phase. Multilamellar liposome suspensions were prepared like the NaCl sample of Fig. 1 except that the fluorescent dye, calcein, was included in each sample. One sample was frozen and thawed 10 times (10X F-T), another 5 times (5X F-T). A third sample was not frozen at all (0X F-T) and a fourth lacked lipid (NL). The fluorescence of the samples was then determined after addition of cobalt chloride solution and again after addition of Triton X-100 detergent. Cobalt ion quenches external but not internal calcein, so the change in fluorescence allows determination of the percent of the calcein entrapped. These percentages were converted into microliters internal volume per micromole of lipid phosphorus and plotted as shown.

Small freeze-thaw vesicles also rupture when frozen and thawed

The entrapment of the aqueous phase demonstrated in the experiments of the previous paragraph showed that freezing and thawing MLV suspensions leads to MLV breakage. To determine whether the small vesicles produced by freezing and thawing would themselves rupture during additional freeze-thaw cycles, calcein was added to a sample after, rather than before, 10 freeze-thaw cycles. The fraction of the external calcein was then determined as described above. Before the eleventh freeze-thaw cycle, the amount of calcein trapped was essentially zero within experimental error (0.6%), whereas after the eleventh cycle, the percentage trapped increased to 22%. Given the several percent variation between experiments, this is not significantly different from the 17.4% found in the case in which vesicles were frozen and thawed 10 times in the presence of calcein (previous paragraph). From these results, we concluded that liposomes rupture completely even after they have been reduced to a small and presumably uni- or paucilamellar state.

The nature of the electrolyte affects the extent of fragmentation

A variety of electrolyte solutions in addition to NaCl were examined. Some were found to have an influence comparable to that of NaCl whereas others were noted to be significantly less effective in inducing freeze-thaw fragmentation of DOPC liposomes. The data in Fig. 3 show the change in absorbance over 10 freeze-thaw cycles in relation to the eutectic concentration of the electrolyte (positive vertical axis) as well as to the eutectic temperature (negative vertical axis). There is generally a good correlation between the maximal concentration of electrolyte reached as the solution freezes (eutectic concentration) and the change in absorbance after the standard series of freeze-thaw cycles. There is a similar parallel between maximum freezing point depression (eutectic temperature) and absorbance reduction.

The damage to phosphatidylcholine multilamellar vesicles by freezing in different electrolyte solutions was also examined by Brearley et al. [17], whose test was leakage of a marker from the internal compartment. By this measure, the dependence of extent of damage on the type of electrolyte was quite different from that shown here.

The concentration of electrolyte affects the extent of fragmentation

The results described above indicated that the concentration of solute remaining when ice crystallizes out of an electrolyte solution may be critical for vesicle fragmentation. To distinguish between the importance of this, an osmotic effect, and that of freezing and thawing per se, we examined the response of liposomes

to freezing in solutions close to the eutectic concentrations. Under such conditions, the solution effectively only solidifies when the temperature is lowered; there is no significant solute concentration change. The absorbance changes upon freezing and thawing DOPC suspensions were determined for solutions of LiCl at 7.0 M, NaCl at 5.1 M and KCl at 3.4 M. In Fig. 4, these absorbance changes are compared with those in experiments with 0.1 M solutions of the same salts. The first bar for each electrolyte represents the 0.1 M concentration sample and the second bar, the higher concentration sample. Fragmentation is much reduced when the electrolyte concentration is such that the osmotic effect of freezing and thawing is effectively eliminated.

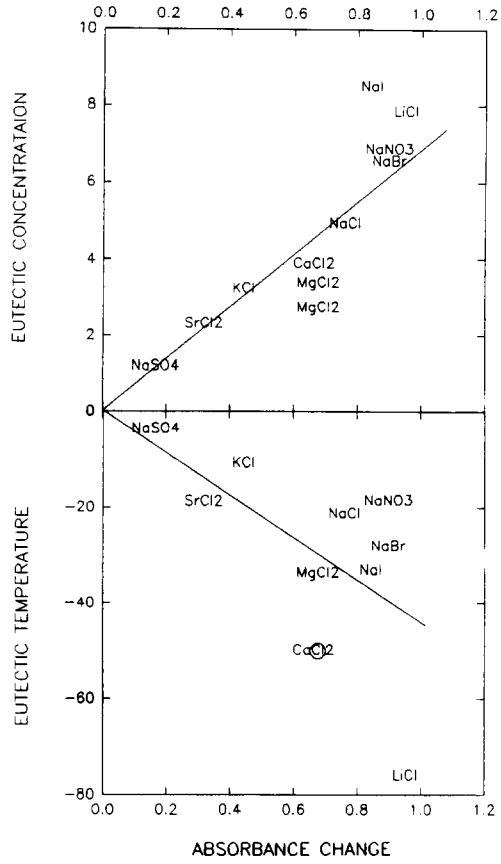

Fig. 3. Extent of liposome fragmentation depends upon the electrolyte; the more soluble the electrolyte, the more effective in promoting fragmentation. Samples were prepared as described for the NaCl sample of Fig. 1, except that the electrolytes were substituted according to the formulas in the Figure. The absorbances were determined before and after 10 freeze-thaw cycles and the change in absorbance for the samples in each electrolyte were plotted against either the eutectic temperature (lowest temperature attained before the salt crystallizes out) or the eutectic concentration (molal concentration of electrolyte in the solution just before solidification). The point corresponding to each solution is in the center of each formula. Two values are given for $MgCl_2$; the smaller absolute values in both halves of the Figure correspond to $MgCl_2 \cdot 12H_2O$. The larger value in the upper half of the Figure is for $MgCl_2 \cdot 8H_2O$ as is the circle near the middle of $CaCl_2$ in the lower half.

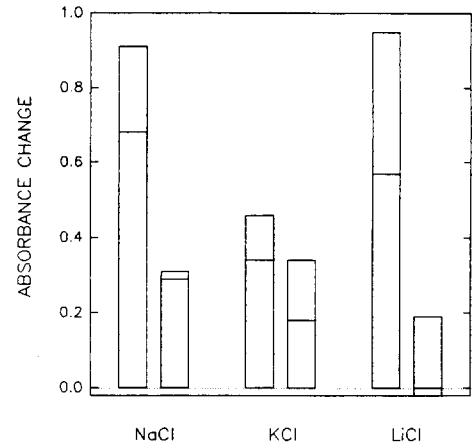

Fig. 4. Fragmentation is greatly reduced when the concentration of electrolyte is near saturation. Phospholipid was suspended in solutions of NaCl, KCl or LiCl at either 0.1 M or at a concentration close to saturation (5.1, 3.4, and 7.0 M, respectively). Absorbances were determined before and after freezing and thawing and the absorbance change is plotted for each sample, the first bar representing the sample at 0.1 M and the second bar, that the higher concentration. The top of each bar and the line within the bar represent absorbance changes for 10 and 5, respectively, freeze-thaw cycles. The absorbance reduction that occurs after freezing and thawing is much reduced when the concentration of electrolyte is such that little concentration change occurs during freezing and thawing.

It is also apparent that the magnitude of the reduction in fragmentation is greater, the higher the salt solubility.

Within wide limits, the freezing and thawing rate has little effect on vesicle fragmentation

Vesicles frozen in a dry-ice ethanol bath cool at a rate of about 60C°/min. When the cooling rate was increased to ≈ 125C°/min by immersion in liquid nitrogen or decreased to ≈ 5C°/min by insulating the tubes, the extent of vesicle fragmentation in 0.1 M NaCl (phosphate buffered) did not markedly change.

Vesicles were normally thawed in 50°C tap water, which produced a rate of warming of ≈ 80C°/min. When vesicles in 0.1 M NaCl (phosphate buffered) were thawed slowly at room temperature, the extent of fragmentation was unchanged, as assessed by the standard absorbance measurement.

Extensive vesicle fragmentation requires that the temperature of the vesicle suspension fall below the eutectic temperature of the electrolyte

Since neither the rate of freezing nor thawing had a significant effect on MLV fragmentation, it was of obvious interest to determine whether the lowest temperature reached by the frozen suspension was a critical factor. Thus, DOPC vesicles, suspended in several different electrolytes, were cooled to temperatures intermediate between the ice point and the temperature of solid CO_2. To do so, the temperature of the suspen-

sions was monitored with a thermocouple, and the sample was removed from the solid CO_2 bath when its temperature had dropped to the desired value.

Fig. 5 illustrates the relationship between the absorbance measured after 10 freeze-thaw cycles and the final minimum temperatures of samples containing either NaCl, NaI, or $MgCl_2$. The eutectic temperatures for NaCl and NaI are −21°C and −33°C, respectively [13]. The eutectic temperature for $MgCl_2$ dodecahydrate is −34°C, but there is also a metastable octahydrate which has a eutectic temperature of −50°C [14] and we cannot be sure which applies in our situation. Nevertheless, the $MgCl_2$ eutectic temperature is certainly lower than that of the other electrolytes included in the Figure. It is evident from Fig. 5 that, the lower the eutectic temperature of the electrolyte, the lower the minimum temperature of the frozen suspension necessary for fragmentation. Although the correlation is not perfect, the absorbance begins to decrease near the eutectic temperature of the salt and most of the change is complete when the sample has cooled 15–20°C below the eutectic temperature. It should be noted that eutectic temperatures are normally determined upon heating, since when temperatures fall, supercooling effects lead to variation among measurements. It is expected that supercooling of vesicle contents would be especially common because of the small volume and thus unlikely presence of heterogeneous nucleation substances.

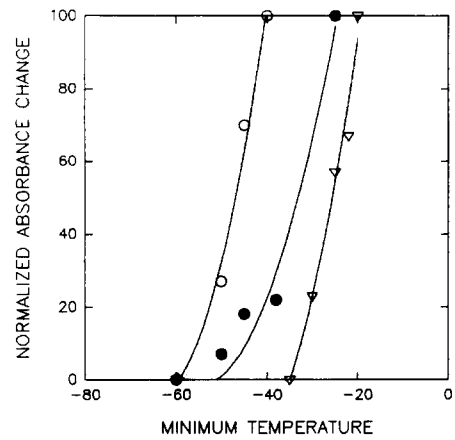

Fig. 5. Liposomes do not fragment upon freezing and thawing unless the sample temperature falls below a minimum temperature which is characteristic of each solute. Sets of multilamellar liposome suspensions in NaCl (triangles), NaI (filled circles) or $MgCl_2$ (open circles) were frozen and thawed 10 times. Each symbol in the Figure corresponds to a sample that had been cooled to the temperature given by the value of the point on the abscissa and then rewarmed. The absorbance change for each sample in each electrolyte was then plotted on scale normalized for each electrolyte (100 = maximum change in A for each electrolyte). As is evident, the minimum temperature for a series of freeze-thaw cycles must fall to a value that is characteristic of each electrolyte, and that these temperatures have the same relationship within this group as do their eutectic temperatures.

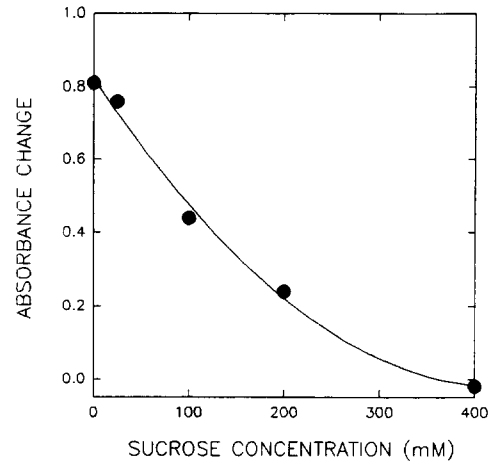

Fig. 6. Sucrose inhibits fragmentation during freezing and thawing. Dioleoylphosphatidylcholine suspensions in phosphate-buffered 0.1 M NaCl and containing sucrose at the concentrations shown in the Figure were frozen to solid CO_2 temperature and thawed 10 times. Absorbance changes for the series of freeze-thaw cycles are plotted against the concentration of sucrose in the sample.

Sucrose inhibits fragmentation of liposomes

It has been reported that sugars, particularly arabinose and sucrose, stabilize lipid bilayers to freeze-thaw damage [18,19]. The phenomenon is thought to be due either to binding of the sugar to the lipid or by promoting vitrification. Given the unusual fragmentation of DOPC vesicles we have observed during freezing and thawing, it was of interest to determine whether sucrose would also inhibit this process. We found that sucrose can completely prevent fragmentation of MLVs during freezing and thawing. As shown in Fig. 6, the extent of inhibition of fragmentation is related to the molar concentration of sucrose and is maximal at about 0.4 M sucrose. The results shown are for a solution of 0.1 M NaCl, but a similar diminution in absorbance change was seen with the same concentrations of KCl and LiCl.

DOPC and diphytanoylphosphatidylcholine are particularly sensitive to fragmentation during freezing and thawing

Once several of the relevant parameters of the process had been established for DOPC, it was of interest to determine whether this behavior is shared by phosphatidylcholines having other acyl chain compositions. The following exhibited less than a 10% absorbance change upon 10 freeze-thaw cycles in phosphate-buffered 0.1 M NaCl: dilinoleoyl, dilinolenoyl, palmitoylarachidonoyl, stearoylarachidonoyl, myristoylarachidonoyl, dimyristoyl and palmitoyloleoyl. Egg-yolk PC vesicles were also relatively resistant to fragmentation after 10 freeze-thaw cycles. It should be emphasized that in most of these cases there was a decline in the absorbance of the suspensions of these lipids of

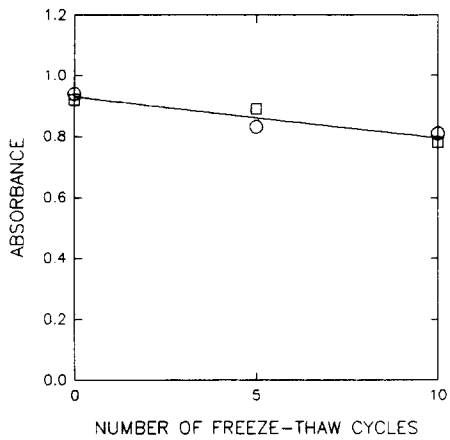

Fig. 7. Cholesterol in the bilayer reduces fragmentation induced by freezing and thawing. The standard freeze-thaw protocol (0.1 M NaCl, 10 freeze-thaw cycles) was applied to lipid samples consisting of 50 mol% dioleoylphosphatidylcholine and 50 mol% cholesterol. Results for duplicate samples are given. Although the light scattered by cholesterol-containing bilayers is somewhat less than that scattered by bilayers lacking cholesterol, the change in absorbance upon freezing and thawing cholesterol-containing membranes is much smaller than that for bilayers lacking cholesterol.

about 0.01 A per freeze-thaw cycle. We did not extend our experiments beyond 10 cycles, but it does appear likely that with a large number of cycles, say 50 or more, the absorbance of most of these suspensions would approach that of DOPC which has been subjected to 10 cycles. Diphytanoylphosphatidylcholine was the one other phospholipid among those tested which fragmented essentially to the same extent as did DOPC.

Cholesterol inhibits DOPC vesicle fragmentation

The finding that freezing-induced fragmentation was a property of few phosphatidylcholines indicates that the phenomenon depends upon some rather subtle properties of the lipid bilayer, perhaps involving characteristics such as the phase transition temperature or a mechanical property. Most characteristics of phospholipid bilayers are modified in some way by cholesterol, so we examined the effect of incorporation of this sterol in DOPC vesicle membranes. Indeed, the presence of either 33 mol% or 50 mol% cholesterol dramatically inhibited fragmentation during freeze-thawing, as shown for the latter case in Fig. 7. Although the absorbance of 50 mol% cholesterol-containing membranes is initially lower than that of DOPC membranes, the former scarcely change upon freezing and thawing, in stark contrast to the latter.

4. Discussion

The remarkably extensive fragmentation of DOPC multilayers upon freezing and thawing in a variety of common electrolytes is one of the most dramatic examples of freeze-thaw damage to membranes that has been observed. We have documented the formation of a population of vesicles of less than 200 nm diameter after 10 freeze-thaw cycles. The absorbance change has not levelled off at 10 cycles, so it is evident that more freezing and thawing would cause additional fragmentation to even smaller vesicles. Others have observed loss of barrier properties of vesicles during freezing and thawing [5,17,20–28], but massive disintegration as observed here has, as far as we are aware, not been described elsewhere.

Since the phenomenon is dependent upon the presence of an electrolyte with a high solubility in water at low temperatures, freezing- and/or thawing-dependent osmotic effects evidently play a critical role in vesicle fragmentation. Freezing must invariably begin in the external phase before it does within vesicles, since many more nuclei (e.g., dust) are available in the external phase to initiate crystallization. As ice forms, the solute becomes more and more concentrated and induces an osmotic flow of water out of the vesicles. The volume reduction of vesicles will hence be greater, the higher the saturating concentration of solute at the point of solidification of the remaining liquid phase. This, the eutectic concentration, will be approximately related to the freezing point depression of the given solute. (It would be a function of the molal concentration of solute and independent of the nature of the solute except for the fact that electrolyte solutions are seldom ideal and freezing point depression is hence not uniformly related to solubility at the freezing point.) As is consistent with an osmotic effect, the more effective electrolytes are those whose solutions generally have the lowest freezing points. If one considers NaCl under our standard conditions, the concentration change during freezing is from 0.1 to about 4.9 osmolal, 0.1 being the concentration of the vesicle suspension at room temperature and 4.9 being the eutectic concentration. Shrinkage of the vesicle, if it did not rupture before internal freezing began, would hence correspond to a volume reduction factor of nearly 50. The potential for very significant dehydration is clearly present during freezing of vesicles in solutions of moderate salt concentration.

Also consistent with an osmotic origin of fragmentation is the finding that when the lipid is suspended in a solution containing electrolyte at a concentration close to the eutectic concentration, freeze-thaw induced fragmentation is greatly reduced. In these cases, the solution will simply crystallize out as hydrated salt. Since ice does not form, the residual liquid does not become more concentrated in solute and water will not be osmotically extracted from the vesicles.

Since our measurement of freeze-thaw effects requires an entire freezing-thawing cycle, we could not

determine whether the essential part of the cycle is freezing, thawing or both. Similarly, although it is quite clear that osmotic events during freezing or thawing are critical for liposome fragmentation, it remains indeterminate whether it is the vesicle interior or the membrane *per se* that is involved. That is, water will be extracted both from the liposome interior and from its membranes (water associated with the head groups) during freezing. During thawing, water will return to the membrane and, after the vesicle has resealed, to the vesicle interior as well. The membrane and the vesicle as a whole are thus both subjected to dehydration-hydration during the freeze-thaw cycle.

Water can be extracted from multilamellar vesicles in seconds or less by osmotic gradients imposed in rapid mixing devices [29], so it is not surprising that freezing rate did not have a large effect on fragmentation given that at our fastest rates, 10s of seconds were required for the sample to freeze fully.

Although a large osmotic gradient appears to be important for vesicle fragmentation, it is clearly not sufficient, since unless the lowest temperature reached by the sample is in the vicinity of the eutectic temperature of the solute, little fragmentation is observed (Fig. 5). For substantial fragmentation to occur, the temperature of the sample must actually fall to below the eutectic temperature, but since eutectic supercooling of 10–20°C is normal, this probably means that fragmentation (or an essential step thereto) occurs when the internal phase of the liposomes solidifies.

Freeze-thaw induced fragmentation of lipid vesicles has been described prior to the present report, although it evidently occurred to a much smaller extent. By light microscopy, Morris and McGrath [27] observed disruption and extrusion of material from multilamellar vesicles. This was visible during the thawing phase and it has been suggested to be due to freezing-induced dehydration of the membrane [26,27]. Similarly, a critical dependence of liposome damage upon eutectic freezing has also been previously observed [28]. In this study, which involved vesicles in 0.9% NaCl, internal solidification occurred at about −40°C although crystallization of external water began at ~ −12°C. There is thus considerable supercooling relative to the eutectic temperature, as suggested by Fig. 5.

It remains puzzling that some, but not more phosphatidylcholines exhibit such a conspicuous response to freezing and thawing. The phase transition temperature of DOPC is ~ −23°C [30]. Other lipids tested had phase transition temperatures both above and below that of DOPC, yet did not fragment to an extent comparable to DOPC. Diphytanoylphosphatidylcholine, the other lipid which fragmented to a similar extent exhibits no sharp phase transition [31]. It thus appears that the lipid phase transition plays no role in damage to these membranes caused by freezing and thawing. Alternative explanations for the apparent uniqueness of these two lipids are not obvious. Phosphatidylcholines interact weakly with a number of ions [32–34] and, given the high concentrations the lipid is exposed to just before eutectic solidification, the surface density of ions might be enough to affect membrane curvature so as to promote vesiculation. There is no apparent reason, however, why the two susceptible lipids should bind electrolytes in a unique way.

Sucrose inhibited fragmentation upon freezing-thawing, a result consistent with other studies of freeze-thaw damage in liposomes [18,20,22,35]. Since eutectic crystallization seems to be the factor with the greatest effect on fragmentation, the mechanism of action of sucrose in this case is apparently promotion of glass formation and concomitant inhibition of ice crystallization [19]. Some effect of sucrose bound to lipid [18] would, however, be difficult to rule out.

The incorporation of cholesterol into the DOPC bilayer inhibits its fragmentation. Although it is well known that cholesterol suppresses the lipid phase transition and maintains a fluidlike state, given that diphytanoylphosphatidylcholine also fragments and it has no sharp phase transition in the absence of cholesterol, it appears that the effect of cholesterol is on the mechanical rather than the thermodynamic properties of the bilayer [11].

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