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Biochimica et Biophysica Acta 1368 (1998) 150–160

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

The effects of chloroplast lipids on the stability of liposomes during freezing and drying

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Received 18 July 1997; accepted 14 August 1997

Abstract

Chloroplast thylakoids contain four classes of lipids, monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulfoquinovosyldiacylglycerol (SQDG), and phosphatidylglycerol (cpPG). We have investigated the effects of these lipids on the stability of large unilamellar vesicles made from egg phosphatidylcholine (EPC), by substitution of different fractions of EPC in the membranes by the various chloroplast lipids. Damage to liposomes after freezing to −18°C was measured as carboxyfluorescein leakage or fusion between vesicles. The presence of all chloroplast lipids increased leakage. However, the maximum amount of leakage and the concentration dependence were dramatically different between the different lipids. Only SQDG induced vesicle fusion, while the non-bilayer lipid MGDG did not. The presence of MGDG in the membranes led to more leakage than the presence of another non-bilayer lipid, egg phosphatidylethanolamine (EPE). In EPE-containing liposomes, leakage was strongly associated with fusion. Combinations of different chloroplast lipids had an additive effect on leakage induced by freezing. Most of the leakage from galactolipid-containing vesicles occurred during the first 15 min of freezing at −18°C. After a 3 h incubation period, most leakage occurred between 0°C and −10°C. Lowering the temperature to −22°C had only a small additional effect. Incubation of liposomes at −10°C in the presence of 2.5 M NaCl without ice crystallization, approximately the same concentration obtained by freezing to −10°C, resulted in very little leakage. Air drying of liposomes to low water contents resulted in massive leakage, both from pure EPC vesicles and from vesicles containing galactolipids. The latter vesicles showed more leakage at any given water content than EPC vesicles. © 1998 Elsevier Science B.V.

Keywords: Chloroplast lipid; Drying; Freezing; Galactolipid; Liposome

1. Introduction

For over 30 years, chloroplast thylakoid membranes have been used as a convenient and productive model system to study mechanisms of freeze-thaw damage and cryoprotection in higher plants (see [1], for a recent review). Because of the unique composition of these membranes, it is difficult to compare directly the results of these studies with data obtained

Abbreviations: CF, carboxyfluorescein; cpPG, chloroplast phosphatidylglycerol; DGDG, digalactosyldiacylglycerol; DG^+, chloroplast lipids after separation from MGDG; DW, dry weight; EPC, egg phosphatidylcholine; EPE, egg phosphatidylethanolamine; H_{II}, hexagonal II phase; MGDG, monogalactosyldiacylglycerol; POPG, 1-palmitoyl-2-oleoyl-*sn*-3-phosphatidylglycerol; PS, bovine brain phosphatidylserine; SQDG, sulfoquinovosyldiacylglycerol; TEN, Tes-EDTA–NaCl-buffer

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PII S0005-2736(97)00204-6

with other membrane systems. Thylakoids have a lipid composition that is reminiscent of the membranes of prokaryotic organisms, such as the well-studied *Acholeplasma laidlawii* [2–4]. The non-bilayer lipid MGDG accounts for approximately 50% of the thylakoid lipid content, while the bilayer lipids DGDG (≈25%), cpPG (≈15%), and SQDG (≈10%) make up the other half [5]. The surface of thylakoid membranes is, therefore, densely covered with the sugars galactose and glucose-6-sulfate, and there is evidence that this can lead to specific solute-membrane interactions that have important repercussions for the action of several cryoprotectants (summarized in [1]).

For instance, some galactose-specific lectins have been shown to bind to DGDG headgroups and reduce freeze-thaw damage to thylakoids [6]. Cryoprotection is mediated through a hydrophobic interaction between the lectins and thylakoid lipids, which leads to a reduction in lipid fluidity and membrane solute permeability [7]. It has also recently been possible to purify a cryoprotective protein (cryoprotectin) from cold acclimated cabbage leaves [8] by using a thylakoid cryoprotection assay [9].

Furthermore, it has been shown that different sugars protect thylakoids from freeze-thaw damage with very different efficiencies (reviewed in [1]). In the case of trehalose, it was shown that its exceptionally high cryoprotective efficiency [10] was related to the ability to reduce thylakoid membrane permeability for other solutes, such as glucose, both during freezing and in unfrozen solutions [11]. The available evidence suggests that this was achieved by hydrogen bonding of trehalose with DGDG headgroups.

In all these cases, investigations with model membranes of defined lipid composition would be important to define exactly the interactions between these solutes and specific membrane lipids and to clarify the physical mechanisms that lead to cryoprotection. Specific interactions between a peptide and thylakoid galactolipids through hydrogen bonding have recently been described in unfrozen solutions [12]. The vast majority of liposome studies into the cryoprotective effects of solutes have been conducted with pure phospholipid bilayers [13]. It should be obvious that these results are only of limited relevance for a membrane that contains only about 15% phospholipid.

Some very interesting results, however, have been obtained in studies that were conducted with a different rationale. It has been argued that, since sugars in solution are cryoprotective for phospholipid vesicles, the immobilization of sugars at the membrane surface, due to the inclusion of synthetic glycolipids in the membranes, should lead to a superior stabilization [14]. This was indeed found in some cases [15–17]. Also, evidence has been presented, that less free sugar is needed to cryopreserve vesicles containing such synthetic glycolipids than is needed for pure phospholipid liposomes [16,17]. This was interpreted as indirect evidence for interactions between membrane-bound and free sugars. There are, however, to the best of our knowledge, no published reports on the effects of naturally occurring glycolipids on the freeze-thaw stability of liposomes and on the interactions of such lipids with cryoprotectants.

We have therefore, investigated the effects of the different classes of thylakoid lipids on the freeze-thaw stability of EPC liposomes by substitution of increasing fractions of EPC by thylakoid lipids. In addition, we have compared the effects of MGDG with those of another non-bilayer lipid, EPE. In order to gain information about possible mechanisms of freeze-thaw damage, we have also measured the stability of liposomes under osmotic stress and after drying to different water contents.

2. Materials and methods

2.1. Lipids

Phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL). Galactolipids were purified as described in [12,18,19], from fresh spinach (*Spinacia oleracea* L.) leaves obtained from a local market in Davis, CA. Briefly, thylakoids were isolated from 400 g of spinach leaves and washed twice by centrifugation and resuspension in fresh buffer to remove contaminations from other cellular membranes. Lipids were extracted in chloroform:methanol (1:2, v/v) and aqueous 100 mM NaCl was added to facilitate the partitioning of the lipids into the organic phase. This phase was concentrated by rotary evaporation and applied to a column filled with 80 g of silicic acid (SIL-LC, Sigma) pretreated with

petroleum ether and equilibrated in chloroform. Pigments were eluted with chloroform and MGDG with chloroform:acetone (1:1, v/v). The remaining lipids (DG^+) were eluted from the column with chloroform:methanol (1:1, v/v), dried under vacuum and redissolved in chloroform. This fraction was applied to a smaller column (30 g) of the same material and DGDG and SQDG were eluted with acetone. Then cpPG was eluted with methanol. The purity of the lipids was periodically checked by TLC and was found to be greater than 95% in all cases.

2.2. Preparation of liposomes

All liposomes were prepared from hydrated lipids using a hand-held extruder ([20]; Avestin, Ottawa, Canada) with two layers of polycarbonate membranes (Poretics, Livermore, CA) with 100 nm pores. The different lipids were mixed in chloroform, dried under a stream of N_2 and stored under vacuum over night to remove traces of solvent. Mixtures of different lipids were made by weight and are expressed as % (w/w).

2.3. Freezing and air-drying experiments

Liposomes (20 μl) were mixed with an equal volume of 10 mM TES, 0.1 mM EDTA, 50 mM NaCl (TEN buffer, pH 7.4) in 0.5 ml microcentrifuge tubes (final lipid concentration 5 mg ml^{-1}). The tubes were placed in a bath containing ethylene glycol cooled to −18°C. After 5 min the samples were crystallized by touching the outside of the tubes with a spatula cooled in liquid nitrogen. The samples were kept frozen for 3 h and thawed in a water bath at room temperature. Control samples were incubated at 0°C for 3 h. The figures show the means ± S.D. from three parallel samples, unless indicated otherwise. Where no error bars are visible, S.D. was smaller than the symbols.

For air-drying, small aluminum pans (Perkin–Elmer, Norwalk, CT) were tared using a Cahn-33 electrobalance (Cahn, Cerritos, CA). Extruded vesicles (lipid concentration 10 mg ml^{-1}) with encapsulated CF were placed in the pans in 20 μl aliquots, and the pans were transferred to a sealed desiccation chamber over anhydrous calcium sulfate (Drierite, Xenia, OH). At time intervals of 5–15 min, a single pan was removed, weighed on the electrobalance, and the sample was immediately tested for CF leakage as described below. The pecentage of water remaining in the samples was calculated from their weight. Approximately, 2.5 h were required to remove 99% of the water from the liposome samples.

2.4. Leakage and fusion measurements

For leakage experiments, 10 mg of lipid were hydrated in 0.5 ml of 100 mM CF (Molecular Probes, Eugene, OR; purified according to [21]), 10 mM TES, 0.1 mM EDTA (pH 7.4). After extrusion, the vesicles were passed through a column (0.5 × 10 cm) of Sephadex G-50 (Pharmacia) equilibrated in TEN to remove the CF not entrapped by the vesicles. The eluted samples had a lipid concentration of approximately 10 mg ml^{-1}. For leakage measurements, 5 μl of sample were diluted in a cuvette in 3 ml of TEN. Measurements were made in a Perkin–Elmer LS-5 fluorometer at an excitation wavelenght of 460 nm and an emission wavelenght of 550 nm. Fluorescence of CF is strongly quenched at the high concentration inside the vesicles and is increased when CF is released into the medium. The total CF content of the vesicles (100% leakage value) was determined after lysis of the membranes with 50 μl of 1% Triton X-100.

For resonance energy transfer measurements [22], two liposome samples were prepared in TEN, one of which contained 0.5 mol% each of *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-phosphatidylethanolamine (NBD-PE) and *N*-(lissamine Rhodamine B sulfonyl)-dioleoylphosphatidylethanolamine (Rh-PE), while the other contained only unlabeled lipids. Both probes were purchased from Molecular Probes, Eugene, OR. After extrusion, liposomes were combined at a ratio of 1:9 (labeled:unlabeled), resulting in a lipid concentration of 10 mg ml^{-1}. Membrane fusion was measured by resonance energy transfer [22]. In a cuvette, 20 μl of sample were diluted with 2 ml of TEN. Fluorescence was measured in a Hitachi F-2000 fluorescence spectrometer at an excitation wavelength of 450 nm and an emission wavelength of 530 nm before (F) and after (F_0) the addition of 40 μl of a 1% Triton X-100 solution. The energy transfer efficiency (E) between the two fluorophores was calculated as $E = 1 - F/F_0$. E was calculated

for both control samples stored at 0°C (E_0) and for the frozen-thawed samples (E_s). The degree of vesicle fusion was calculated as % fusion = $100 - \{(E_s/E_0) \times 100\}$. Thus, fusion is detected as the decrease in energy transfer between the two fluorophores, as fused vesicles constitute a dilution of the probes by unlabeled lipid molecules.

3. Results

We have used two different methods to quantitate freeze-thaw damage to liposomes after freezing for 3 h at −18°C, namely the loss of a soluble marker from the interior of the vesicles (CF leakage), and fusion of the vesicle bilayers. In all cases, control samples of the same lipid composition were held for the same time at 0°C. In no case was leakage or fusion at 0°C increased during incubation, due to the presence of the various thylakoid lipids at the concentrations used in the freeze-thaw experiments (data not shown).

Fig. 1 shows that both non-bilayer lipids investigated in this study destabilized EPC vesicles. However, the amount of MGDG necessary to induce leakage during freezing was much lower than the amount of EPE required to achieve a similar effect.

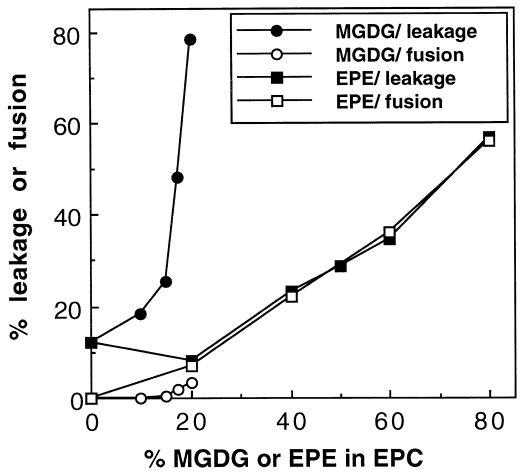

Fig. 1. Freeze-thaw damage to liposomes as a function of the lipid composition of the membranes (Damage was either measured as carboxyfluorescein (CF) leakage or as liposome fusion. The membranes were composed of EPC and varying fractions of either MGDG or EPE. All samples were frozen for 3 h at −18°C).

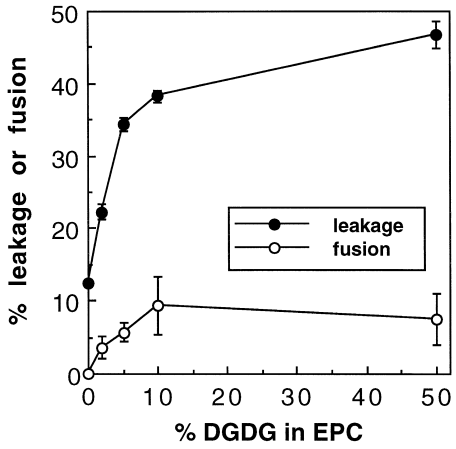

Fig. 2. Freeze-thaw damage to EPC liposomes containing different fractions of DGDG (see legend to Fig. 1, for details).

This correlates well with the fact that liposomes containing EPE in their bilayers only showed leakage at 0°C when 90% or more of the lipid was EPE, while the presence of only 30% MGDG in EPC bilayers resulted in markedly increased leakage already in the absence of freezing (data not shown).

In addition to the quantitative differences between the two non-bilayer lipids, revealed by the leakage measurements, fusion measurements showed that the mechanism by which the two lipids destabilize the membranes during freezing was also different. While MGDG induced no significant amount of fusion (4.1% fusion and 78.3% leakage with 20% MGDG/80% EPC), fusion in the presence of EPE increased in parallel with leakage (Fig. 1). Therefore, the leakage induced during freezing by EPE can be completely explained on the basis of increased bilayer fusion, while the much more drastic increase in leakage in MGDG-containing liposomes must be the result of a different mechanism.

DGDG is a bilayer forming lipid and can therefore be incorporated into membranes in any desired concentration without any detrimental effects on stability in the absence of freezing. Nevertheless, there was a marked increase in leakage after freezing when the vesicle membranes contained even a small portion of DGDG. Addition of as little as 2% DGDG led to a 10% increase in leakage, compared to the 100% EPC membranes (Fig. 2). However, leakage leveled off at more than 10% DGDG and only increased by 7% from 10% to 50% DGDG. This shows that the two

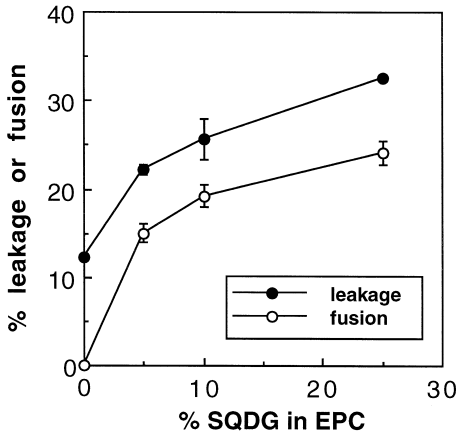

Fig. 3. Freeze-thaw damage to EPC liposomes containing different fractions of SQDG (see legend to Fig. 1, for details).

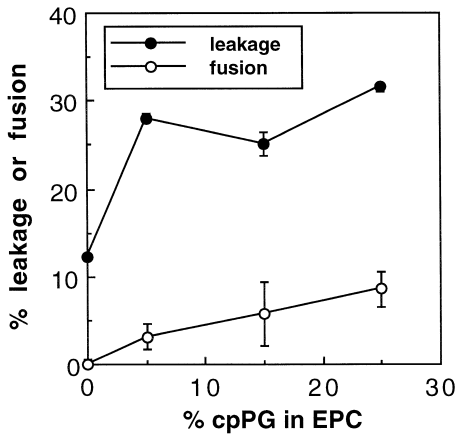

Fig. 4. Freeze-thaw damage to EPC liposomes containing different fractions of cpPG (see legend to Fig. 1, for details).

galactolipids had different effects on bilayer stability during freezing. MGDG was much more effective in inducing leakage during freezing and the effect was not saturated at higher concentrations (Fig. 1), as was the case with DGDG (Fig. 2). DGDG also led to slightly increased fusion, but this was not enough to quantitatively account for the observed leakage (7.4% fusion and 46.7% leakage with 50% DGDG/50% EPC).

A different effect was seen in the third class of thylakoid glycolipids. SQDG induced a moderate increase in freeze-thaw damage in EPC liposomes, which was strictly correlated ($r = 0.98$ in a linear regression analysis) with increased bilayer fusion (Fig. 3). Quite surprisingly, also the PG fraction isolated from thylakoids led to a small increase in leakage and fusion (Fig. 4). Control experiments with liposomes made of either 25% POPG/75% EPC or 25% PS/75% EPC showed that this increase in leakage during freezing was not a general effect of phosphatidylglycerols or negatively charged lipids, as there was no difference in leakage between these vesicles and those composed of pure EPC (data not shown).

Since natural membranes always contain a combination of several different lipids, it was of interest to investigate the effects of different combinations of chloroplast lipids on freeze-thaw damage. Fig. 5 shows that the destabilizing effects of the single lipids in EPC membranes (Figs. 1–4) are also observed in combinations. When the two galactolipids were combined in a single membrane, the liposomes showed more leakage after freezing than vesicles containing EPC and either MGDG or DGDG (Fig. 5, Table 1). However, when 25% cpPG or POPG were added to the mixture containing 50% DGDG and 20% MGDG, there was no significant change in

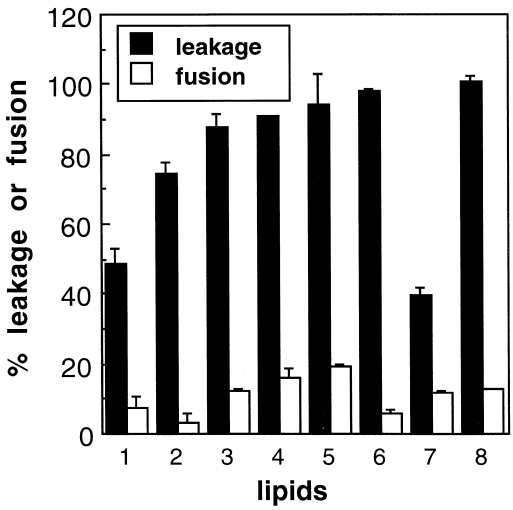

Fig. 5. Effect of varying lipid composition on freeze-thaw damage to liposomes (1: 50% DGDG/50% EPC ($n = 4$); 2: 20% MGDG/80% EPC ($n = 4$); 3: 50% DGDG/20% MGDG/30% EPC ($n = 4$); 4: 50% DGDG/20% MGDG/25% cpPG/5% EPC ($n = 1$); 5: 50% DGDG/20% MGDG/25% POPG/5% EPC ($n = 3$); 6: 50% DGDG/20% MGDG/25% SQDG/5% EPC ($n = 2$); 7: 80% DG^+/20% EPC ($n = 3$); 8: 80%DG^+/20% MGDG ($n = 3$). The values in paranthesis show the number of independent experiments (with three parallel samples each) performed with the different lipid mixtures).

Table 1
Statistical analysis of the freeze-thaw induced CF leakage from liposomes of different lipid composition as shown in Fig. 5

Lipids [a]	P [b]
1 vs. 2	0.0005
2 vs. 3	0.0005
3 vs. 4	n.s.
3 vs. 5	n.s.
3 vs. 6	0.0005
7 vs. 8	0.0005
1 vs. 7	0.0005
3 vs. 8	0.0005
4 vs. 8	0.0005
6 vs. 8	0.005

[a] The numbers are the same as in Fig. 5, indicating the lipid compositions listed in the legend to Fig. 5.
[b] Level of significance of the differences in CF leakage from liposomes of different composition, as determined by an unpaired Student's t-test (n.s. = not significant, $P > 0.05$).

leakage (Table 1). On the other hand, the addition of 25% SQDG, instead of the phospholipids, increased leakage significantly (Table 1).

In order to check, whether our approach of first separating the different lipids by chromatography and then reconstituting them in different fractions into the bilayers resulted in a freeze-thaw behavior of the vesicles that came reasonably close to the stability of the original lipid mixture, we also conducted experiments with a DG^+ lipid fraction. This fraction is obtained by separating first the pigments and then MGDG from the rest of the thylakoid lipids on a silicic acid column as described in Section 2. The DG^+ fraction, therefore, contains DGDG, cpPG, and SQDG in approximately the same relative amounts as the intact thylakoid membrane.

Membranes made from 80% DG^+ and 20% EPC were slightly more stable than membranes containing only 50% DGDG and 50% EPC (Fig. 5, 1 vs. 7). When the 20% EPC in the DG^+ containing membranes were substituted by MGDG, leakage after freezing increased to almost 100%. This was significantly more than in samples containing 50% DGDG, 20% MGDG, and 30% EPC, but only marginally more than in samples containing 50% DGDG, 20% MGDG, and 25% SQDG (Table 1).

While leakage varied dramatically in liposomes of different lipid composition, the degree of fusion was always low (Fig. 5). This emphasizes that fusion plays only a minor role in freeze-thaw induced leakage of vesicles containing chloroplast lipids. The fusogenic effect of SQDG in combination with EPC (Fig. 3) was no longer apparent in the more complex lipid mixture containing also MGDG and DGDG (Fig. 5).

These results show that the main contribution to membrane destabilization during freezing is made by the galactolipids, which together accounted for about 90% CF leakage (Fig. 5). SQDG and cpPG, on the other hand, had only a small additional influence. We have therefore limited the remainder of our analysis to liposomes containing MGDG, DGDG, or both, in combination with EPC.

Fig. 6 shows the dependence of CF leakage from liposomes on the incubation time at −18°C. For all three lipid combinations, most of the freezing damage happened within the first 15 min. Leakage increased with longer freezing times in samples containing 20% MGDG and 80% EPC. After 4.5 h, CF leakage had increased by 21.9% (from 54.7% to 76.6%) compared to samples frozen for 15 min. In contrast, liposomes containing 50% DGDG and 50% EPC showed no further leakage after the initial loss (48.4%). Vesicles made of both galactolipids showed an intermedi-

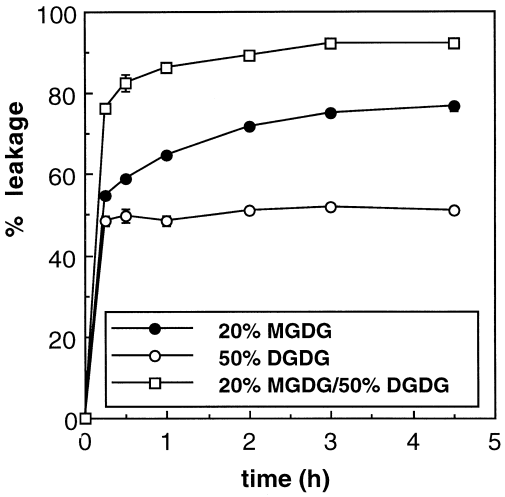

Fig. 6. Time dependence of CF leakage from liposomes (Samples were frozen to −18°C for the indicated times and leakage was measured after thawing. The membranes contained the indicated lipids in EPC).

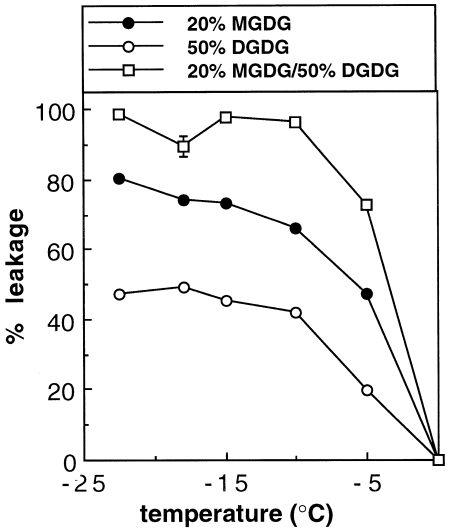

Fig. 7. Temperature dependence of CF leakage from liposomes (Samples were frozen for 3 h to the indicated temperatures or stored for the same time at 0°C).

ate behavior. Since these vesicles had already lost about 90% CF after 2 h, this may have limited further leakage. Nevertheless, it is clear that the presence of MGDG in the membranes led to a slow, time dependent component of leakage in addition to the major rapid component.

The freezing temperature had a very similar effect on CF leakage for all three types of liposomes (Fig. 7). Damage increased strongly with decreased temperature between 0°C and −10°C. Lowering the temperature further to −22°C had only a comparatively small effect.

There are several physical changes during a freeze-thaw cycle that could lead to the observed leakage from liposomes. The crystallization of ice leads to an increase in the solute concentration in the solution surrounding the vesicles, and could also bring the vesicles into close and potentially damaging contact with ice crystals and with each other. In addition, the lowering of the temperature and the increased osmolality could result in lipid phase transitions, or phase separations (see Section 4, for details).

In order to elucidate the role of osmotic stress and temperature in the absence of freezing, we incubated liposomes made of 20% MGDG and 80% EPC in NaCl solutions of different concentrations. After 3 h at either 22°C, 0°C, or −10°C, the vesicles were diluted in TEN and CF leakage was measured (Fig. 8). Samples at −10°C were not crystallized and remained supercooled during the entire incubation time. The solute concentration obtained in the unfrozen phase of an NaCl solution frozen to −10°C is approximately 2.5 M [23]. Therefore, liposomes in the samples incubated at −10°C in the presence of 2.5 M NaCl experienced the same conditions as samples frozen to this temperature, except for any effects due to the presence of ice crystals. It is obvious from the data shown in Fig. 8 that neither the low temperature nor the high solute concentration resulted in a degree of leakage, comparable to that measured after freezing vesicles of this lipid composition to −10°C (compare Fig. 7). In fact, the highest leakage was only 14.5% and was seen in samples incubated at 22°C.

We then conducted air drying experiments with liposomes of different lipid compositions to see whether the removal of water by evaporation would result in leakage similar to that seen when water was removed by ice crystallization. It can be seen that the gradual removal of water from the samples resulted in massive CF leakage from liposomes made of pure EPC, or of mixtures of EPC and chloroplast galactolipids (Fig. 9). The degree of leakage at any given water content was strongly dependent on the lipid

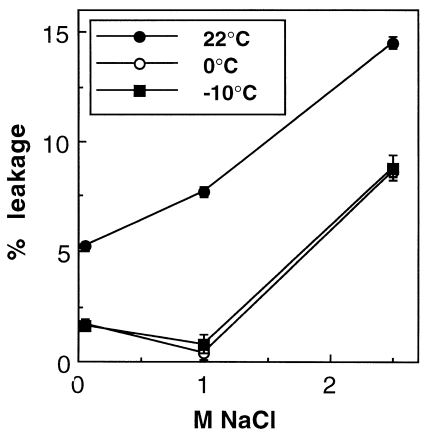

Fig. 8. Effect of osmotic stress on CF leakage from liposomes containing 20% MGDG and 80% EPC (Liposomes were suspended in TEN buffer containing 0.05 M, 1 M or 2.5 M NaCl and were incubated at the indicated temperatures for 3 h. Samples incubated at −10°C were not frozen but remained supercooled during the incubation time).

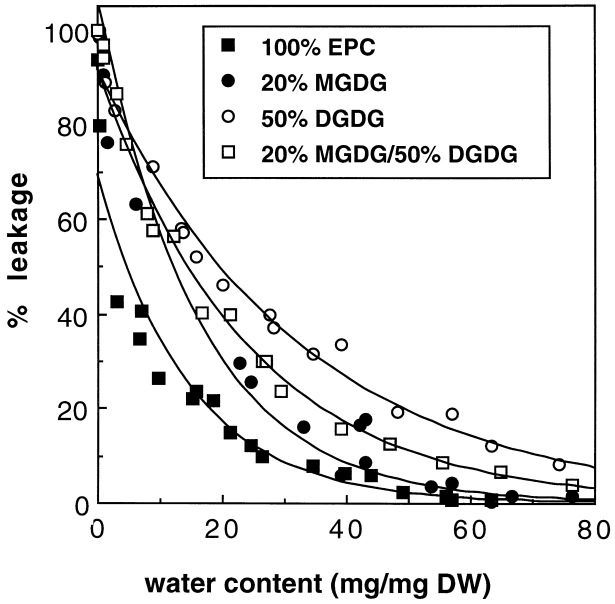

Fig. 9. Leakage of CF from air-dried liposomes after rehydration (Samples were dried at 0% relative humidity at room temperature and rehydrated in TEN buffer. After different drying times, the water content of the samples was determined. It is expressed as mg water/ mg dry weight (DW). The membranes contained the indicated lipids in EPC. The exponential curves were fitted to the data by regression analysis (100% EPC: $r = 0.98$; 20% MGDG/80% EPC: $r = 0.92$; 50% DGDG/50% EPC: $r = 0.99$; 20% MGDG/50% DGDG/30% EPC: $r = 0.99$). This regression analysis (Sigma Plot, Jandel Scientific, San Rafael, CA) also showed that the curve obtained from 100% EPC samples is significantly different from all other curves at a 95% confidence level. The 50% DGDG/50% EPC curve is significantly different from all other curves at a 99% confidence level. The 20% MGDG/80% EPC and the 20% MGDG/50% DGDG/30% EPC curves are not significantly different from each other).

composition of the membranes. Analogous to the situation during freezing, EPC vesicles were destabilized by the presence of both MGDG or DGDG. In contrast to the situation during freezing, however, DGDG was more destabilizing during drying than MGDG. Also, in combination, the effects of the galactolipids were not additive, as shown for freeze-thaw damage (Fig. 5). Instead, leakage in these samples was intermediate between those containing either only MGDG or only DGDG in combination with EPC (Fig. 9).

We conclude from these results that drying was only partially equivalent to freezing under our experimental conditions and that most likely the dehydration induced by the formation of ice crystals has somewhat different effects on bilayer stability than evaporative dehydration at room temperature.

4. Discussion

Natural membranes are composed of a wide variety of lipids. Only in a few cases has it been possible to ascribe a specific function to any class of lipids in a given membrane. It can be assumed that membranes with vastly different lipid compositions also have different properties in many regards. In terms of the stress resistence of cellular membranes, both the direct contribution of the different lipids to the stability of the membrane, and possible stabilizing interactions with protective solutes are of interest.

The vast majority of studies on the stability and protection of model membrane systems during freezing and drying have been conducted on pure phospholipid bilayers (see [13,24], for a review). Consequently, very little is known about the contribution of glycolipids to membrane stress resistence. The present contribution is a first step in an ongoing project to characterize the interactions between soluble cryoprotectants and chloroplast glycolipids.

The most abundant lipid in thylakoid membranes is MGDG. It is a non-bilayer forming lipid that forms a H_{II} phase in excess water at temperatures above -15°C [25]. It can, however, be included in bilayers formed by other lipids, such as EPC or DGDG. The relative amount of MGDG that can be included without compromising the permeability barrier properties of the bilayer structure differs somewhat depending on the method of liposome preparation (see [5], for a review). Under our experimental conditions, CF leakage at 0°C increased measurably when the fraction of MGDG was raised from 20% to 30% in EPC liposomes (data not shown). It has been shown that in the native thylakoid membrane, a large fraction of the MGDG is tightly associated with intrinsic membrane proteins [26], which make up almost 50 wt% of the total membrane. Therefore, the amount of motionally free MGDG in thylakoid membranes, that could participate readily in H_{II} formation, is much less than the total amount of MGDG present in the membrane. For this reason, we feel that liposomes containing up

to 20% MGDG would still be a valid model system to study the contributions of different thylakoid lipids to freeze-thaw damage and cryoprotection of the native membrane.

Our data (Fig. 1) show that the presence of MGDG severely destabilized liposomes during a freeze-thaw cycle. Low concentrations (up to 10% MGDG/90% EPC) were tolerated, but at concentrations above 15%, leakage increased dramatically. As a comparison, we conducted a similar series of experiments with liposomes containing increasing fractions of EPE in EPC. EPE is, like MGDG, a H_{II} forming lipid. It was therefore, not possible to prepare liposomes with more than 80% EPE. At 90% EPE/10% EPC, the resulting vesicles did not encapsulate any CF at room temperature (data not shown). However, even liposomes containing 80% EPE showed less freeze-induced leakage than vesicles containing only 20% MGDG (Fig. 1). It is therefore clear, that MGDG is a much more destabilizing lipid than EPE.

In addition, our data indicate that the observed leakage was the result of different physical events in the presence of these non-bilayer lipids. In the case of EPE, leakage was closely associated with extensive bilayer fusion. It can be assumed that fusion was the result of H_{II} formation during freeze-induced dehydration. It has been shown before, that in mixtures of PEs and PCs, the PE can undergo a lyotropic phase transition during dehydration (see [27], for a review).

Fusion is not a conclusive indicator for H_{II} formation, as bilayer fusion can also occur in the absence of H_{II} formation. On the other hand, H_{II} formation is always an interbilayer event that, by necessity, leads to the mixing of membrane components [28], which would be detectable by a resonance energy transfer assay. Therefore, the absence of fusion in vesicles containing MGDG (Fig. 1), is strong evidence that in this case no H_{II} formation occurred during the freeze-thaw cycle.

We conclude from these data that at the concentrations employed in our study, MGDG formed no extensive H_{II} phase during freezing, although it did induce massive leakage. Therefore, its propensity for lyotropic H_{II} formation seems to be weaker than that of EPE. This is, at least qualitatively, in good agreement with the finding that in protoplasts isolated from the leaves of *Arabidopsis thaliana*, H_{II} phase lipid was observed during freezing only in the plasmamembrane, chloroplast envelopes, and endoplasmic reticulum, but not in thylakoid membranes [29].

Although MGDG and DGDG are, respectively, non-bilayer and bilayer forming lipids, their effects on membrane stability during freezing were in some respects surprisingly similar (Figs. 1 and 2). Like MGDG, DGDG strongly increased leakage during a freeze-thaw cycle, without inducing a corresponding increase in vesicle fusion. One physical characteristic that both lipids have in common is that they are very poorly hydrated. MGDG and DGDG, similar to the corresponding glucolipids from *A. laidlawii*, bind only 8–12 mol H_2O per mol lipid [4,30,31]. EPC is much more hydrated and has been shown to bind 23 mol H_2O per mol lipid, while EPE is also more poorly hydrated with 12 mol H_2O per mol lipid (reviewed in [32]). The poor hydration of the glycolipids may seem surprising, given the large number of hydroxyl groups that could potentially participate in hydrogen bonds with water molecules. It has, however, been shown that the sugar headgroups are oriented almost parallel to the membrane plane [33]. It is therefore, likely that they hydrogen bond preferentially with other glycolipid headgroups instead of water, leading to a very low degree of hydration.

Although leakage during freezing is not related to fusion, the data shown in Fig. 8 suggest that it may nonetheless depend on bilayer–bilayer interactions. Osmotic stress at temperatures from 22°C to −10°C in the absence of ice formation induced only very little leakage in vesicles containing 20% MGDG and 80% EPC. In this experiment, liposomes incubated in the presence of 2.5 M NaCl at −10°C experienced the same osmotic stress as samples frozen to −10°C [23]. However, during freezing not only low molecular weight solutes are concentrated around the vesicles, but the vesicles themselves are concentrated in the aqueous channels between the ice crystals. Therefore, freezing will bring the membranes into close physical contact with each other.

This close approach of adjacent bilayers, together with osmotic dehydration, can lead to a demixing of differently hydrated lipids [34]. It has been shown for liposomes containing dioleoyl-PC and dioleoyl-PE that during dehydration the lipids demix and form two seperate fluid phases in the membranes, because the less hydrated PE headgroups allow a closer ap-

proach of bilayers at a given degree of dehydration [35,36].

In the case of PE, this is a prelude to H_{II} formation [35]. For MGDG and DGDG we propose that during freezing a similar demixing from EPC occurs, due to the difference in hydration. In contrast to EPE (Fig. 1), the galactolipids induce no H_{II} or bilayer fusion (Figs. 1 and 2). This is due to specific interactions between adjacent bilayers that stabilize the membranes during close approach. It has been shown before that liposomes formed from pure DGDG or from DGDG/MGDG mixtures aggregate and form stacks of flattened vesicles in the presence of divalent or monovalent cations. This close physical contact of the bilayers was completely reversible when the cations were removed from the solution and resulted in no measurable degree of fusion [37,38]. Other glycolipids, such as galactosylceramide and cerebroside sulfate, have been shown to have similar properties [39].

The leakage resulting from this demixing of glycolipids and EPC during freezing could follow a mechanism similar to the lipid phase separation damage originally proposed by Quinn [40] for lipids with different liquid–crystalline to gel phase transition temperatures. This would imply that lipid demixing and the interaction of part of the lipid in one monolayer with lipid in another monolayer from a different membrane would lead to packing defects in the membrane. These defects could result in increased solute permeability of the membranes [41] that would lead to CF leakage and/or uptake of highly concentrated solutes from the unfrozen aqueous phase. This solute loading during freezing would lead to osmotic swelling and rupture of the vesicles during thawing, which would also result in CF leakage from extruded vesicles [42]. If solute loading occurs, equilibration across the membrane must be rapid, as most of the leakage occurs in the first 15 min of freezing (Fig. 6).

Whether a liquid–crystalline to gel phase transition is involved in CF leakage during freezing can not be unequivocally decided on the basis of our present data. However, we find it unlikely for two reasons. First, the temperature dependence of leakage is independent of the membrane lipid composition (MGDG, DGDG, or both in combination with EPC, Fig. 7). Second, the phase transition temperature of DGDG has been reported at -50°C [30], far below the lowest freezing temperature used in our experiments.

Interestingly, drying had somewhat different effects on leakage than freezing. MGDG was more effective in increasing leakage during freezing than DGDG, and the two lipids acted additively in combination (Fig. 5, Table 1). During drying, however, DGDG had a more destabilizing influence than MGDG, and the combination showed an intermediate behavior (Fig. 9). Also, at water contents above 40 mg mg^{-1} DW, where leakage from EPC vesicles after drying was approximately the same as from EPC vesicles frozen to -10°C, leakage from galactolipid-containing vesicles was much lower after drying than after a corresponding freeze-thaw cycle. Conversely, at a water content after drying, where leakage from vesicles containing 20% MGDG/80% EPC was approximately 80%, the same degree of leakage as observed in such vesicles after freezing to -18°C for 3 h, EPC vesicles had lost about 40% CF after drying, while leakage was only 12% after freezing. These two examples illustrate, that dehydration during freezing and drying affected liposomes differently, and that the effects were also strongly and differentially dependent on the membrane lipid composition in each case.

These differences could be the result of the different treatment temperatures (room temperature (approximately 25°C) during drying, -18°C during freezing), or the different rates of dehydration. During air drying the samples lose water slowly (approximately 2.5 h until samples had reached 1% water content), while the small samples (40 μl) were cooled to the final temperature within a few minutes. The differences in leakage seen in liposomes of different composition, however, were not a consequence of a different rate of water loss from the differentially hydrated lipids. The gravimetrically determined kinetics were the same for all lipid compositions (data not shown). It has been shown that the presence of ice can have direct effects on the phase behavior of lipids [43]. It could therefore also have an effect on membrane stability during freezing.

Clearly, further studies will be necessary to elucidate the mechanisms of membrane destabilization by thylakoid galactolipids in detail. Also, an understanding of the much smaller contributions of SQDG and cpPG to membrane destabilization (Figs. 3–5), and

possible interactions of these lipids with MGDG and DGDG will require further experiments. Nevertheless, the data in this paper provide a basis for future investigations into the lipid-specific cryoprotective or cryotoxic behavior of different solutes.

Acknowledgements

DKH gratefully acknowledges financial support through a Heisenberg stipend from the Deutsche Forschungsgemeinschaft. This work was also supported by grant IBN93-08581 from the National Science Foundation (USA) to JHC.

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