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Protection of liposomes during dehydration or freezing

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When liposomes are subjected to dehydration or freeze-thawing, vesicle fusion and/or leakage of vesicle contents can occur. The disaccharide, trehalose and the cryoprotectant, glycerol, are known to protect vesicle integrity during dehydration and freezing respectively. Here we examine their protective abilities as a function of vesicle size and lipid composition. It is shown that fatty acyl composition, cholesterol content and, with the exception of phosphatidylglycerol, acidic lipid content do not significantly alter the retention of aqueous contents by vesicles dehydrated and rehydrated in the presence of trehalose. The susceptibility to leakage induced by both dehydration and freezing is, however, critically dependent upon vesicle size with the smallest systems (70—100 nm diameter) being most stable. The mechanism whereby trehalose protects against vesicle fusion and leakage is also discussed.

Keywords: trehalose; liposomes; dehydration; cryoprotection; sugars; glycerol.

Introduction

The ability of trehalose to preserve the integrity of dehydrated membranes was first recognized in anhydrobiotic organisms such as brine shrimp cysts and certain nematodes [1]. Later it was shown to protect the Ca^{2+}-transport ability of muscle microsomes during dehydration/rehydration [2]. While a number of other sugars share this protective property, trehalose is said to be most effective [2]. Similarly to biological membranes, lipid vesicles dehydrated in the absence of a protective solute undergo massive fusion with concomitant release of entrapped material. We have shown previously that trehalose and certain other sugars prevent such fusion and greater than 80% of vesicle contents can be retained following rehydration [3]. Similar results were reported by Crowe et al. [4] for large unilamellar vesicles composed of a phosphatidylcholine/phosphatidylserine mixture. Recently, it has been demonstrated that this trehalose-dependent stabilization cannot simply be ascribed to the retention of residual water in the dehydrated sample [5].

Small unilamellar vesicles subjected to a freeze-thaw cycle also undergo fusion and again this can be prevented by the inclusion of cryoprotectants such as sugars or glycerol in the medium [6].

In the present study we have examined the protective abilities of trehalose and glycerol during dehydration or freezing as a function of vesicle size and composition. By entrapping millimolar concentrations of carboxyfluorescein

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DOPC, dioleoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; EPC, egg phosphatidylcholine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MTPP$^+$, methyl triphenylphosphonium ion; OGP, octyl β-D-glucopyranoside; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; QELS, quasi-elastic light scattering.

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during vesicle formation leakage can be quantified by relief of fluorescence quenching without the need to separate vesicles from released fluorophore. These studies also provide further insight into the mechanism whereby trehalose exerts its protective effect during dehydration.

Experimental procedures

Materials

5(6)-Carboxyfluorescein (Molecular Probes) was purified by ethanol recrystallization and Sephadex LH20 chromatography according to Weinstein et al. [7]. All phospholipids were obtained from Avanti Polar Lipids while cholesterol (standard for chromatography), octyl-B-D-glucopyranoside and trehalose were obtained from Sigma. [^{14}C]Glucose and [^{14}C]potassium thiocyanate were purchased from New England Nuclear. All salts and buffers were of analytical grade.

Vesicle preparation

Lipid mixtures were co-lyophilized from benzene/methanol (70:30, v/v) and the dry lipid stored at −20°C. Large unilamellar vesicles were prepared using an "Extruder" (Lipex Biomembranes, Inc.) as described previously [8,9]. In most of the experiments described egg phosphatidylcholine was used. Unless otherwise stated, 40 μmol of egg phosphatidylcholine were hydrated in 1 ml of 100 mM carboxyfluorescein, 250 mM trehalose, 150 mM NaCl, 20 mM HEPES (pH 7.4). The mixture was dispersed by vortexing, subjected to five freeze-thaw cycles in liquid nitrogen and then passed ten times through two stacked polycarbonate filters (Nuclepore, CA). Generally, filters of 100 nm pore size were used but where the influence of vesicle size was examined, vesicles were sized through either 15, 30, 50, 100, 200 or 400 nm pore size filters.

Multilamellar vesicles were prepared simply by dispersing the lipid in the same buffer as above with gentle vortexing.

Unencapsulated carboxyfluorescein was removed by passing the vesicle suspension over a column (1.4 × 15 cm) of Sephadex G-50 (fine) equilibrated with 250 mM trehalose, 150 mM NaCl, 20 mM HEPES (pH 7.4).

Dehydration and freezing

Samples (500 μl) in 13 × 100 mM test tubes were dehydrated under high vacuum (60 mTorr) (Virtis Freeze Drier) for 24 h at room temperature. The mobile powder obtained was rehydrated to its original volume at 30°C with distilled water equilibrated at this temperature and then dispersed with vortexing.

For the freezing experiments the vesicle suspension (300 μl) in a cryovial (Simport Plastics) was frozen in liquid nitrogen and then thawed in warm water (30—35°C).

Fluorimetry

In the present work 5(6)-carboxyfluorescein is used as an aqueous marker. Following entrapment at self-quenching concentrations, its release can be monitored as a relief of fluorescence quenching. This procedure is preferable to the use of column chromatography which can exhibit varying lipid recovery if massive fusion occurs during dehydration.

Fluorescence mesurements were performed using a SLM Aminco SPF 300 C ratio spectrofluorimeter at an excitation wavelength of 492 nm (bandpass 0.25 nm) and an emission wavelength of 520 (bandpass 10 nm).

Measurements were made at a lipid concentration of 15—30 μM in 250 mM trehalose, 150 mM NaCl, 20 mM HEPES (pH 7.4) with or without 25 mM octyl-β-D-glucopyranoside. These lipid and detergent concentrations have little effect on the fluorescence intensity of carboxyfluorescein.

Membrane potential determination and glucose permeability

The possibility of a membrane potential (interior positive) in vesicles containing carboxyfluorescein was probed using [^{14}C]-potassium thiocyanate essentially as described previously using $MTPP^+$ [8]. Control vesicles composed of egg phosphatidylcholine exhibiting a positive potential were prepared in 150 mM KSCN, 20 mM HEPES (pH 7.4) and external thiocyanate

removed by passage of the vesicles over a column (1.4 × 15 cm) of Sephadex G-50 (fine) equilibrated with 150 mM NaCl, 20 mM HEPES (pH 7.4). The vesicles are relatively impermeable to sodium or chloride ions but permeable to thiocyanate. This anion, therefore, diffuses out of the vesicles down its chemical gradient generating a membrane potential, interior positive. The potential was quantified from the distribution of [^{14}C]KSCN across the vesicle membrane with the interior concentration determined following centrifugation of aliquots (100 μl) of the suspension on Sephadex G-50 (medium) "minicolumns" [10].

Similarly, glucose permeability was determined following preparation of vesicles in 150 mM NaCl, 10 mM glucose (containing 4 μCi [^{14}C]glucose ml^{-1}) 20 mM HEPES (pH 7.4) with or without 100 mM carboxyfluorescein. External glucose was removed by column chromatography and subsequent leakage determined with 1 ml minicolumns as above.

Vesicle size determination

Vesicle size was determined by quasi-elastic light scattering (QELS) using a Nicomp 200 Laser Particle Sizer (Nicomp Instruments, Goleta, CA) operating at 632.8 nm and 5 mW. In addition, some samples were examined by freeze-fracture electron microscopy as described in Mayer et al. [9]. Replicas were visualized on a JEOL JEM-1200 EX microscope.

Results

Before carboxyfluorescein can be used as an aqueous marker to follow vesicle leakage, two criteria must be established. First, at the self-quenching concentrations employed it must not of itself affect membrane permeability and second that the probe should be passively entrapped within the vesicle as a result of the lipid permeability barrier and not merely retained due to electrostatic forces. To validate the first criterion, [^{14}C]glucose was trapped in egg phosphatidylcholine vesicles in the presence or absence of 100 mM carboxyfluorescein and its passive permeability monitored. As shown in

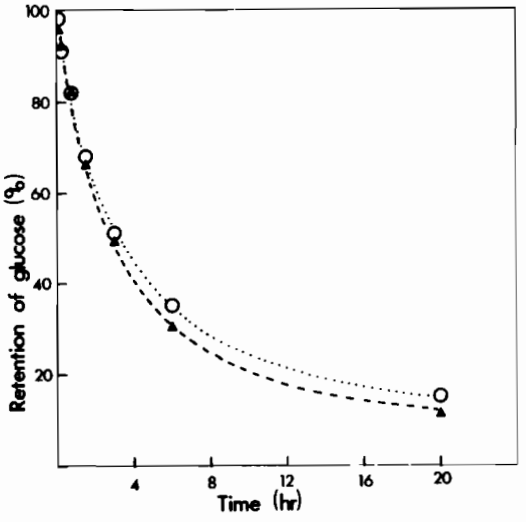

Fig. 1. Influence of carboxyfluorescein on membrane permeability. The permeation of [^{14}C]glucose from large unilamellar vesicles composed of egg phosphatidylcholine prepared in the presence, - - - - ▲ - - - - ; or absence, ... O . . . . , of 100 mM carboxyfluorescein was followed as described in Methods.

Fig. 1 glucose permeation is not altered by the presence of the fluorophore indicating no major perturbation of the membrane by carboxyfluorescein.

Bramhall [11] has reported that small unilamellar vesicles composed of dipalmitoylphosphatidylcholine (DPPC) are permeable to carboxyfluorescein. He suggests that when this compound is entrapped within such vesicles migration of the negatively charged species across the bilayer generates a membrane potential (interior positive) which limits further efflux of the fluorophore to the rate of influx of counterions such as protons or Na^+. To investigate this possibility, the existence of a membrane potential in carboxyfluorescein-loaded vesicles was directly probed using the lipophilic anion [^{14}C]thiocyanate. Control vesicles in which a positive membrane potential was generated using a potassium thiocyanate gradient (150 mM) rapidly accumulate [^{14}C]thiocyanate indicating a potential of greater than + 100 mV after 90 min. Similar vesicles loaded with carboxyfluorescein, however, show no measurable potential over the same time period. Secondly, if the efflux of carboxyflu-

orescein were limited by the influx of counterions, then increasing membrane permeability to such ions should cause rapid release of the fluorophore. The influence of various ionophores on carboxyfluorescein leakage was therefore examined. Neither gramicidin (5 μM), valinomycin (5 μM in presence of 10 mM KCl) nor CCCP (20 μM), which allow the rapid equilibration of Na^+, K^+ and protons respectively, affected the fluorescence of carboxyfluorescein-loaded vesicles (results not shown). If, however, the vesicles were lysed using octyl-β-D-glucopyranoside, release of the fluorophore with consequent relief of quenching was observed. These results indicate that electrostatic forces are not preventing carboxyfluorescein efflux in this system.

Following removal of external carboxyfluorescein by passage of egg phosphatidylcholine vesicles over a gel filtration column 94% fluorescence self-quenching is observed. The background fluorescence is not reduced by further column chromatography and is stable for several days. This may be attributed to a fraction of the fluorophore partitioning into the bilayer. Given that the butanol/water partition coefficient of carboxyfluorescein at pH 7.4 is 8.0×10^{-4} [7], a rough estimate of probe concentration in the lipid phase is of the order 10^{-5} M. At this concentration in aqueous solution self-quenching is nearly fully relieved. Consistent with carboxyfluorescein in the membrane itself being responsible for background fluorescence this level is decreased by about 50% for vesicles composed of dipalmitoylphosphatidylcholine. At room temperature this phospholipid is in the gel state and reduced partitioning of the probe into the bilayer would be expected.

The ability of trehalose or glycerol to protect vesicles from dehydration or freezing is compared in Figs. 2 and 3. If large unilamellar vesicles (mean diameter 100 nm) composed of egg phosphatidylcholine are dehydrated in the absence of trehalose they fuse to form structures considerably larger than 1 μm in diameter. As would be expected, greater than 95% of the carboxyfluorescein is released. Similar vesicles prepared in the presence of trehalose and then dried, however, maintain their size and retain more than 70% of their contents in agreement with previous results [3]. Also, as shown pre-

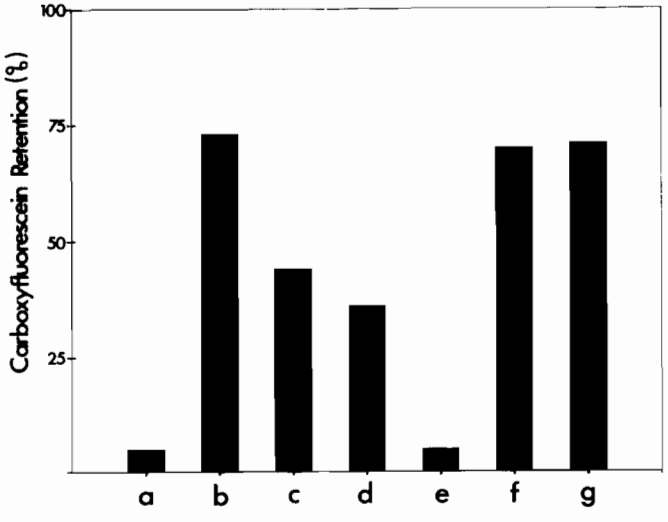

Fig. 2. Protection of egg phosphatidylcholine vesicles by trehalose during dehydration or freezing. Large unilamellar vesicles (100 nm mean diameter) containing carboxyfluorescein were dried or freeze-thawed and fluorophore retention determined (see Methods). Vesicles were dehydrated (a) in the absence of trehalose, (b) with trehalose (250 mM), (c) with trehalose (250 mM) and glycerol (10%, w/w), (d) with trehalose (250 nm) outside only and (e) with trehalose (250 mM) inside only. Similar vesicles were frozen and thawed, (f) in the absence of trehalose, (g) with 250 mM trehalose.

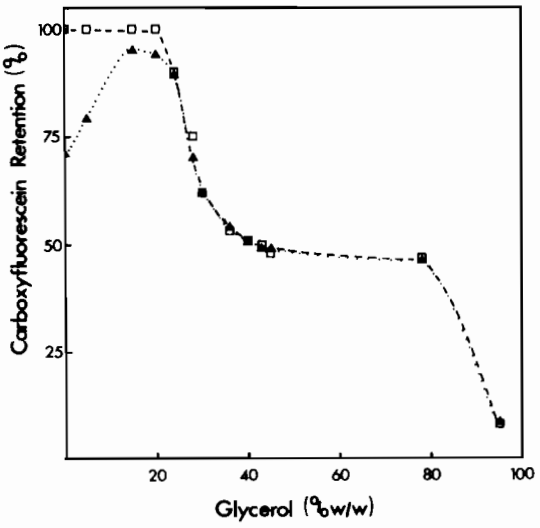

Fig. 3. The influence of glycerol concentration on carboxyfluorescein leakage from frozen and thawed egg phosphatidylcholine vesicle. Control vesicles were maintained at 25 °C for 30 min in the presence of varying glycerol concentrations, — — □ — —, while similar vesicles were frozen and thawed as described in Methods, ... ▲ . . . .

viously [3], trehalose must be present on both sides of the vesicle membrane for optimal protection (Fig. 2). If the sugar is present only on the outside of the vesicles, 70% of the entrapped carboxyfluorescein is released following dehydration and rehydration. However, it is interesting that the vesicles retain their original size distribution as determined by QELS, which we take to indicate that no fusion has occurred (before dehydration mean vesicle diameter, 105 nm S.D., 26 nm; following dehydration mean vesicle diameter, 103 nm, S.D. 28 nm). If trehalose is present only inside the vesicles, it has no protective effect against either fusion or leakage.

The large increase in size observed when small unilamellar vesicles are subjected to a freeze-thaw cycle is not observed in the presence of trehalose [6]. Surprisingly, however, large unilamellar vesicles used in the present study showed no increase in mean vesicle size by QELS and retained about 70% of the encapsulated carboxyfluorescein even when frozen and thawed in the absence of trehalose (Fig. 2). Further freeze-thaw cycles (up to ten cycles) caused only a small incremental release of fluorophore with no evidence of fusion. It should be noted that small unilamellar vesicles prepared, for example, by sonication have a mean diameter in the range of 20 nm and lipid packing problems may render them inherently unstable. In contrast, the smallest vesicles prepared by extrusion have a mean diameter of 72 nm which likely accounts for their much greater stability. The presence of trehalose on both sides of the vesicle membrane had no protective effect with regard to leakage during freeze-thawing (Fig. 2). If, however, glycerol is included in the sample buffer a concentration-dependent reduction in carboxyfluorescein leakage is observed (Fig. 3). The high bilayer permeability of glycerol will result in its equilibration on both sides of the vesicle membrane following addition to the external buffer. In Fig. 3 the influence of glycerol concentration on fluorophore leakage is shown for control vesicles maintained at 25 °C for 30 min and for similar vesicles following a freeze-thaw cycle. It can be seen that as the glycerol concentration is increased up to about 15% (w/w) significantly increased protection against freeze-thaw-induced leakage is observed. At higher concentrations of the cryoprotectant the level of carboxyfluorescein release increases for both the freeze-thawed and control samples. This is presumably the result of membrane destabilization at these high glycerol concentrations.

TABLE I

The influence of cholesterol content and fatty acyl composition on carboxyfluorescein retention during dehydration and rehydration.

Sample	Carboxyfluorescein retention (%) ± S.E.M.
EPC	73 ± 4
EPC/CHOL 9:1	76 ± 3
EPC/CHOL 8:2	74 ± 5
EPC/CHOL 7:3	75 ± 2
EPC/CHOL 6:4	75 ± 4
DOPC	82 ± 2
POPC	69 ± 6
DPPC	88 ± 2

The influence of phospholipid acyl chain saturation on the ability of trehalose to protect against dehydration is examined in Table I. As the degree of unsaturation of the chains increases (i.e. DPPC< POPC< EPC< DOPC) there is little change in the retention of carboxyfluorescein by trehalose-protected vesicles despite a large (approximately 60°C) difference in the gel to liquid-crystalline phase transition temperature of the different phospholipids. Vesicles of dipalmitoylphosphatidylcholine, which is in the gel state at room temperature both when fully hydrated and when dehydrated in the presence of trehalose [12] show the highest level of retention. A second mechanism whereby the hydrocarbon order of phospholipid vesicles can be manipulated is through the incorporation of cholesterol into the bilayer. As with the change in fatty acyl composition, however, no significant effect on carboxyfluorescein leakage is observed as the cholesterol content is increased from 0 to 40 mole% (Table I). These results will be discussed later in the context of the mechanism of trehalose protection during dehydration.

While fatty acyl chain composition may not greatly influence trehalose protection, the phospholipid headgroup is of considerable importance. In Fig. 4 the retention of carboxyfluorescein by trehalose-protected vesicles containing various ratios of egg phosphatidylcholine and egg phosphatidylglycerol is shown. Clearly, the presence of the anionic phospholipid destabilizes the dried vesicles. Control samples of the different lipid mixtures not subjected to dehydration and incubated at 4°C showed no leakage over several days. Only the dehydrated sample of 100% egg phosphatidylglycerol showed an increased mean vesicle size following rehydration. This leakage of carboxyfluorescein is not, however, a general feature of anionic phospholipids, since phosphatidylserine, phosphatidylinositol and phosphatidic acid vesicles dehydrated in the presence of trehalose show similar retention levels following rehydration to those composed of phosphatidylcholine (Fig. 4). Why phosphatidylglycerol should behave in a different manner to other anionic lipids is not known. Recently, however, we have shown that multilamellar vesicles containing dimyristoylphosphatidylglycerol will spontaneously vesiculate under appropriate conditions while similar systems containing

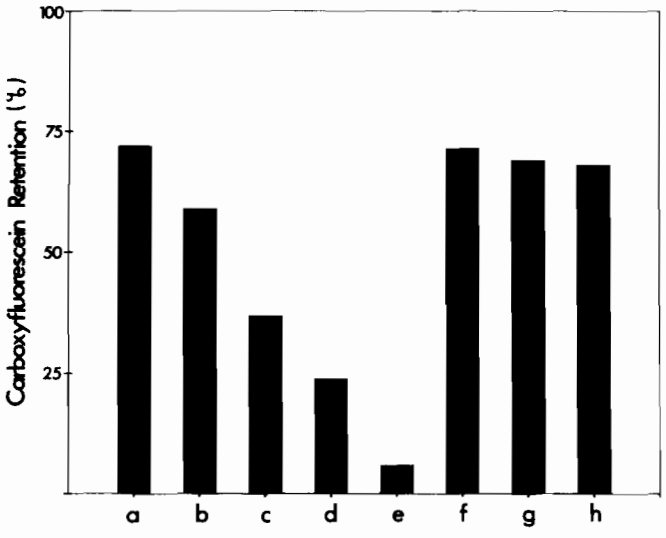

Fig. 4. Protective effect of trehalose as a function of the lipid headgroup composition. The retention of carboxyfluorescein by large unilamellar vesicles of varying compositions dehydrated in the presence of 250 mM trehalose is shown. Samples are (a) egg phosphatidylcholine (EPC); (b) EPC/egg phosphatidylglycerol (EPG) (3 : 1); (c) EPC/EPG (1 : 1); (d) EPC/EPG (1 : 3); (e) EPG; (f) egg phosphatidylserine; (g) egg phosphatidic acid; (h) liver phosphatidylinositol.

dimyristoylphosphatidylserine are structurally stable [13].

In an earlier study the influence of vesicle size on stability to dehydration in the presence of trehalose was examined [3]. Leakage of entrapped $^{22}Na^+$ was determined following passage of the vesicles over a 1 ml "minicolumn" of Sephadex G-50. This experimental protocol could, however, underestimate leakage, particularly for larger systems, if fused or aggregated vesicles are retained on the column support while smaller ones elute through. Using the present carboxyfluorescein technique, therefore, we prepared vesicle systems of varying mean diameter in the presence of trehalose using filters ranging in pore size from 15 to 400 nm [9]. As the filter pore size is reduced below 100 nm the resulting vesicles have smaller mean diameters but these do not correspond to the pore diameter indicating some elastic deformation of the vesicles during extrusion (see Fig. 5). Vesicles extruded through 30, 50 and 100 nm pore size filters show comparable retention of carboxyfluorescein following dehydration and rehydration, but as the mean diameter is further increased, their ability to withstand dehydration is reduced with large multilamellar vesicles leaking almost 80% of their contents. As predicted, when compared to the earlier data, these results indicate an underestimation of leakage for the larger systems using $^{22}Na^+$. Recently, it has been reported that limit size small unilamellar vesicles prepared by sonication (average vesicle diameter 25 nm) leak much of their contents during dehydration even when trehalose is present [14]. This is perhaps not surprising given that the lipid packing constraints in such small systems render them susceptible to fusion and leakage even when fully hydrated [15]. A similar size dependence to the dehydration data shown above is seen when stability to freezing is examined (Fig. 6) with the smallest extruded vesicles (mean diameter 72 nm) retaining 100% of their contents while MLVs release more than half of the entrapped fluorophore. Limit size vesicles prepared by sonication were found to be inherently leaky as reported by others [15] and the influence of freeze-thaw was not examined. It

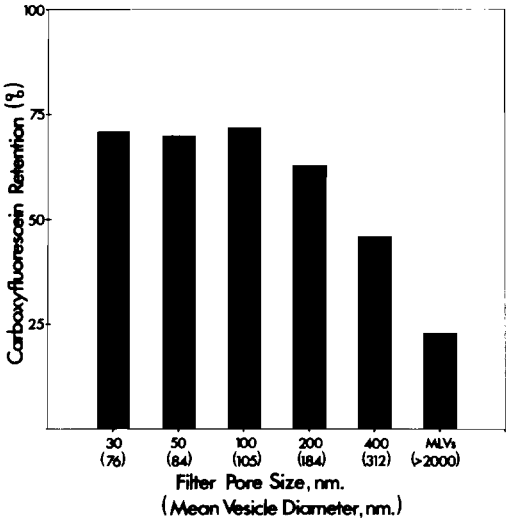

Fig. 5. Influence of vesicle size on ability to retain carboxyfluorescein following dehydration. Egg phosphatidylcholine vesicles were prepared by extrusion through filters of varying pore size as described in Methods. The mean vesicle diameter as determined by QELS is indicated in brackets after the filter pore size. Dehydration in the presence of 250 mM trehalose was as described in Methods.

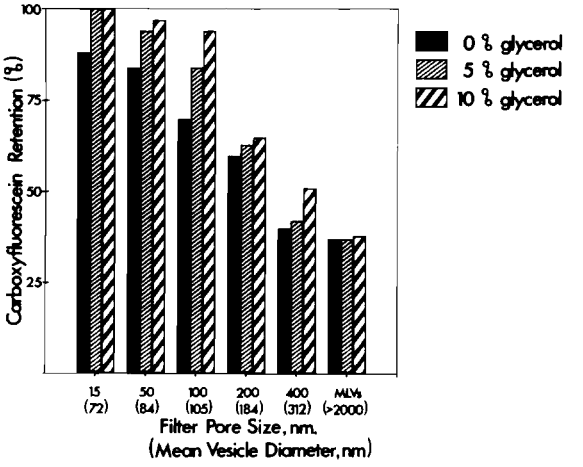

Fig. 6. Influence of vesicle size on ability to retain carboxyfluorescein following a freeze-thaw cycle. Egg phosphatidylcholine vesicles were prepared by extrusion through filters of varying pore size as described in Methods. The mean vesicle diameter as determined by QELS is indicated in brackets after the filter pore size. Samples were incubated at 4 °C for 24 h with either 5 or 10% w/w glycerol before freeze-thawing.

should be noted that when vesicles are extruded through filters of pore size greater than 100 nm some multilamellar systems will be present [9].

Discussion

The results presented here have implications in two main areas: the design of a liposomal system to maximize stability under appropriate storage conditions and the mechanism whereby trehalose and similar sugars protect dehydrated membranes. These two topics will be discussed in turn.

While the concept of using liposomes as a "magic bullet" to target encapsulated drugs to a specific site has yet to be realized [16], in several areas, simple liposome-drug complexes have immediate therapeutic potential (see Ref. 17 for review). The use of liposome encapsulation to buffer drug toxicity, for example, has proved viable for several drug classes. These include the anthracycline, doxorubicin, which is active against a range of cancers [18] and amphotericin B, which is widely used in the treatment of systemic fungal infections [19]. Other classes of liposomally-encapsulated drugs reported to show superior therapeutic indices include antibacterials [20], antivirals [21] and certain antiparasitics [22]. In addition, immunomodulating agents entrapped in liposomes show therapeutic promise for potentiating macrophage-mediated destruction of cancer metastases [23]. Clearly, in the development of pharmaceutically useful liposomal systems problems relating to storage and stability will need to be addressed. In particular, aqueous suspensions of lipid vesicles would be expected to exhibit drug leakage and possibly lipid degradation over the time scale required between preparation and their administration. For this reason, more stable dehydrated or frozen lipsomes are attractive possibilities if conditions can be found which maintain vesicle integrity and prevent loss of entrapped material.

The present results would indicate that with regard to fatty acyl composition, cholesterol content and, with the exception of phosphatidylglycerol, acidic lipid content considerable flexibility is possible in the design of liposomal systems suitable for storage in a dehydrated or frozen form. In the case of size, however, smaller vesicle systems (70—100 nm) are clearly more ameanable to both freezing and dehydration than larger ones.

The mechanism whereby trehalose protects dehydrated membranes has been the focus of considerable attention. It has been suggested that the sugar hydrogen bonds to the phospholipid headgroup effectively replacing bound water [24]. Infrared spectroscopic studies have provided evidence for such an interaction and in addition the gel to liquid-crystalline phase transition temperature of anhydrous dipalmitoylphosphatidylcholine is lowered by trehalose in a similar fashion to water [24]. It has been suggested that trehalose protects membranes by preventing the anhydrous lipid from undergoing a transition to the gel state with subsequent lateral phase separation and membrane destabilization [25]. In support of this hypothesis, Lee et al. [12] showed by deuterium NMR that for a trehalose/dipalmitoylphosphatidylcholine mixture above 46 °C the lipid adopts what is termed the lamda-phase where the fatty acyl chains are highly disordered. While this is similar to the liquid-crystalline state of the fully hydrated lipid, the lamda-phase differs in that the headgroups are immobilized due to binding to trehalose. Below this temperature the lipid slowly reverts to the gel phase. It is suggested that the lamda-phase is important in maintaining the integrity of dehydrated biological membranes. Some of the results presented here, however, are not consistent with these hypotheses. In

TABLE II

The influence of rehydration conditions on carboxyfluorescein retention by dehydrated dipalmitoylphosphatidylcholine vesicles.

Rehydration protocol	Carboxyfluorescein retention (%)
Rehydration at 30 °C	88
Rehydration at 50 °C	86
Dry powder heated to 50 °C, cooled to 4 °C for 48 h, rehydrated at 50 °C	88

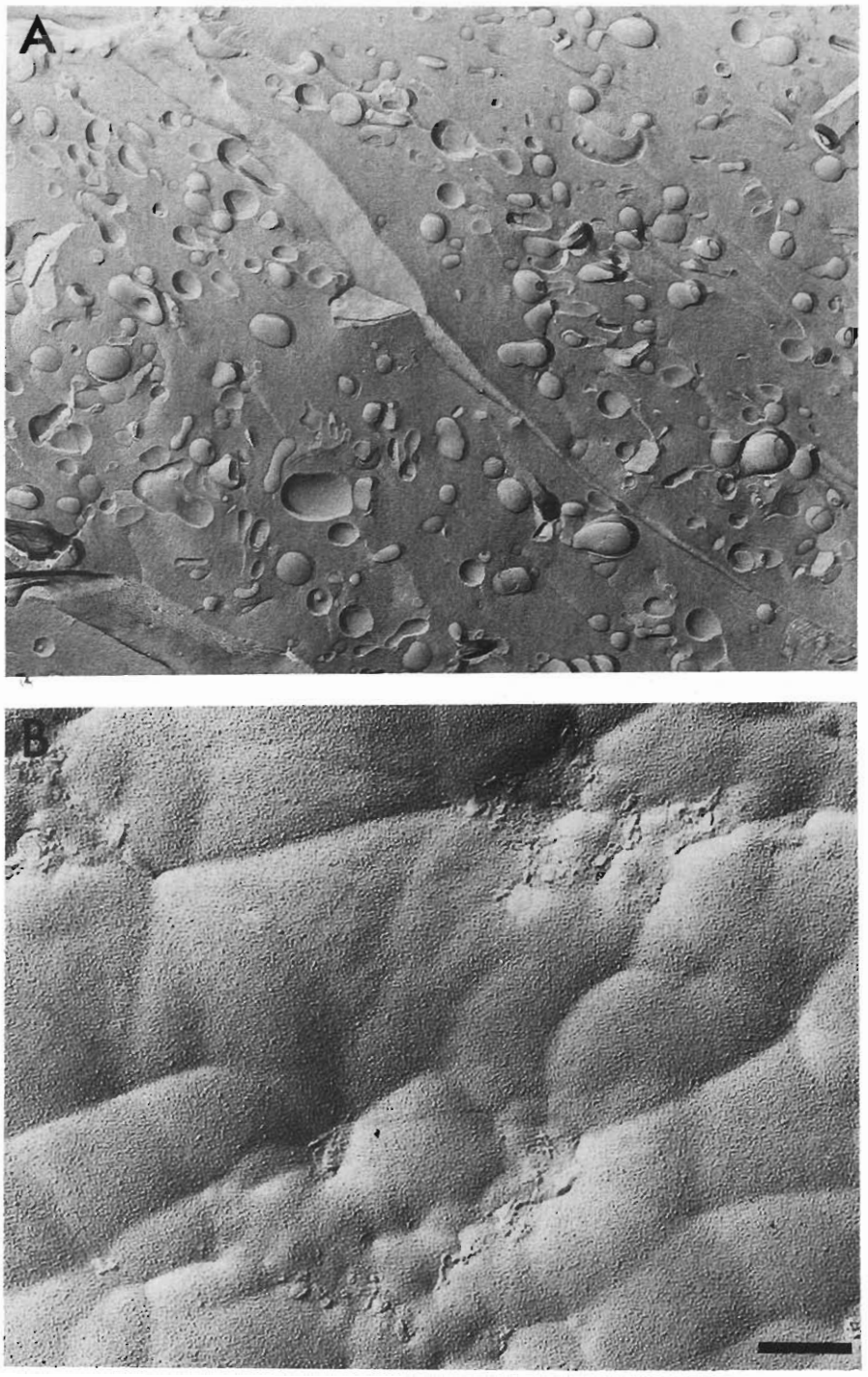

Fig. 7. Freeze-fracture electron micrographs of dehydrated vesicles. Large unilamellar vesicles (100 nm) of egg phosphatidylcholine were dried with (A) or without (B) trehalose (250 mM) and freeze-fracture replicas prepared as described in Methods. The bar represents 200 nm.

particular, one would expect the fatty acyl composition of the vesicles to markedly affect their stability to dehydration. As the degree of unsaturation was increased and the gel to liquid-crystalline transition temperature lowered vesicle stability should be enhanced. The results presented in Table I, however, show that little difference in carboxyfluorescein leakage is observed for vesicles of varying fatty acyl compositions despite a 60°C difference in gel to liquid-crystalline phase transition temperature. Further, vesicles of dipalmitoylphosphatidylcholine dehydrated at room temperature should be in the gel state in both the hydrated and dehydrated (trehalose-complexed) state (see Ref. 12) and yet these show the highest retention of fluorophore. It might be suggested that membrane destabilization and vesicle leakage occur as the lipids undergo a transition from the gel to lambda phase or vice versa but we observe no difference in carboxyfluorescein retention when the dry trehalose-dipalmitoylphosphatidylcholine vesicles are warmed to 50°C and then reequilibrated for 48 h at 4°C prior to rehydration or rehydrated with distilled water equilibrated at 50°C (Table II).

One of the prerequisites to membrane fusion is close apposition of two bilayers. At least part of the protective effect of trehalose and other sugars may result from an ability to act as a spacing matrix between vesicles preventing apposition. Such a role is illustrated by electron micrographs of samples dehydrated in the presence of or absence of trehalose (Fig. 7). When trehalose is present during drying vesicle-like structures can be observed in the fracture plane through the solid dehydrated sample (Fig. 7a). In contrast freeze-fracture replicas of dry samples dehydrated in the absence of sugar show no recognizable structures but instead irregular, amorphous aggregates are observed (Fig. 7b). It should be emphasized that these micrographs do not constitute evidence as such that sugars protect by preventing apposition but simply illustrate that they can provide a rigid matrix in which vesicle structure is retained. At the molecular level a strong interaction between the phospholipid and sugar would be important in preventing contact between approaching bilayers, particularly at lower sugar concentrations. As suggested previously [2], therefore, the protective ability of a particular sugar will be influenced by the number of hydroxyls available to hydrogen bond to the phospholipid headgroup. Within this "spacer" concept the greater effectiveness of disaccharides over monosaccharides (at the same molar concentration) [2] would be partly due to their larger size. Such a hypothesis is supported by the observation that the amount of sugar bound to dry membranes appears to be related to its protective ability [5]. In this context the results obtained when trehalose is present on only one side of the vesicle membrane are interesting. If the sugar is present only outside then considerable vesicle leakage is observed but no fusion, consistent with a "spacer" role. This result also demonstrates, however, that membrane integrity is compromised despite the absence of fusion indicating a further role for trehalose in maintaining the membrane permeability barrier. This could be attributed, as suggested previously [26], to trehalose effectively substituting for water. When trehalose is present only inside the vesicle, then both fusion and leakage are observed. In this situation no effective spacer molecules would be present between the vesicles, allowing apposition and subsequent fusion.

Acknowledgements

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