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Factors affecting the stability of dry liposomes

John H. Crowe and Lois M. Crowe

Department of Zoology, University of California, Davis, CA (U.S.A.)

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Previous studies have shown that liposomes can be preserved in the dry state in the presence of certain sugars, of which trehalose is particularly effective. There have been some discrepancies in results obtained by the various laboratories in which this phenomenon has been studied, both with respect to the efficacy of the sugars tested and the degree to which the dry vesicles can be stabilized. We show here that several factors that affect the stability of the dry liposomes may be responsible for the discrepancies between measurements by different laboratories. These factors include: (1) Size: small, sonicated vesicles are comparatively very unstable, and retain no more than 70% of trapped solute after drying, even in extremely high concentrations of sugars. Very large vesicles are similarly unstable. (2) Charge: a small amount of negatively charged lipid in the bilayer significantly increases stability. (3) Stabilizing sugar: the comparative efficacy of the sugar used varies with the size of the vesicles. (4) Dry-mass ratio. It is the dry-mass ratio between the stabilizing sugar and lipid that is important in the preservation during freeze-drying, not the concentration of either lipid or sugar in bulk solution.

Introduction

It has often been suggested that liposomes in which water-soluble molecules are entrapped could be used for delivery of those molecules to cells in whole organisms, including humans [1–2], as well as for lipid-based assay systems [3]. These suggestions are becoming a reality, with the creation of several commercial products that are based on liposomes [4]. One of the difficulties in the practical application of these products has been the long-term stability of the liposomes. We [5–10] and others [11–13] have addressed this problem and have found that liposomes can be reduced to dry powders if they are dried in the presence of certain sugars. In the initial studies on this phenomenon, we reported that trehalose, a non-reducing disaccharide of glucose commonly found at high concentrations in organisms that naturally survive dehydration [14–18], is particularly effective in this regard [5–6]. When liposomes, about 50 nm in diameter and composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC) and PS (9 : 1), were freeze-dried in the presence of trehalose in a mass ratio of 2 g trehalose to 1 g lipid, upon rehydration the vesicles were seen to retain 100% of their original contents [5]. In subsequent studies we showed that trehalose is the most effective of the sugars tested [6] and provided a physical explanation for the mechanism for the preservation [6,10]. The best evidence available suggests that there is a direct interaction between the sugar and the polar head group of the phos-

Abbreviations: Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethal)ethyl]amino}ethanesulfonic acid; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine.

Correspondence: J.H. Crowe, Department of Zoology, University of California, Davis, CA 95616, U.S.A.

pholipid [10,12,18–22], the result of which is a depression of the transition temperature of the lipid and its maintenance in a fluid state even in the absence of water [5,10,13,18,20].

Other laboratories have confirmed these results, with some puzzling discrepancies. For example, Madden et al. [11] reported that much greater quantities of the stabilizing sugar were required to stabilize the liposomes and that retention of trapped solute was significantly less in their hands than we had reported. Strauss et al. [13] found that trehalose and sucrose have approximately similar stabilizing properties, in distinction from our own results. However, all three laboratories are using different methods to prepare the liposomes, different lipids, and different methods to record stability. In the present paper we show that these factors lead to differences in the stability of dry liposomes.

Materials and Methods

Liposomes

Egg phosphatidylcholine (PC) and crude bovine phosphatidylserine (PS) were purchased from Avanti Polar Lipids (Birmingham, AL) and used without further purification. Trehalose, sucrose, and lactose were purchased from Pfanstiehl Laboratories (Waukegan, IL). Highly purified carboxyfluorescein was obtained from Molecular Probes (Junction City, Oregon) and used without further purification. PC and PS at a mole ratio of 9 : 1 were mixed together in chloroform solution, dried under nitrogen, and placed under vacuum (10–25 mTorr) on a VirTis lyophilizer (The VirTis Co., Gardiner, NY) for at least 12 h. The lipids were rehydrated to a final lipid concentration of 20 mg/ml with double-distilled deionized water containing 20 mM Tes buffer (pH 7.5), 0.1 M carboxyfluorescein, and 0.25 M sugar (either lactose, sucrose or trehalose). The resulting multilamellar vesicles were frozen four times in liquid nitrogen and thawed in a 40 °C water bath after each freezing event. With this fluid mixture of lipids, simply freeze-thawing them reduces the original multilamellar vesicles to large unilamellar and oligolamellar vesicles (Fig. 1a). Smaller unilamellar vesicles were prepared from the freeze-thawed vesicles essentially as described by Mayer et al. [23]. Briefly, the vesicles were successively extruded through polycarbonate filters of 400, 200, 100, 50 and 30 nm pore diameter. In order to obtain unilamellar vesicles nominally 100 nm in diameter, for example, the multilamellar vesicles were extruded five times through 400 nm filters, followed by five passes through 200 nm filters, an then by five passes through 100 nm filters. Excess carboxyfluorescein and sugar not trapped in the unilamellar vesicles was eluted through a Sephadex G-50 fine column (15 × 180 mm) with Tes buffer as the mobile phase. The column chromatography under these conditions results in an unavoidable osmotic shock, leading to leakage of about one-third of the trapped solute during passage through the column. After elution from the column the vesicles are stable, however, showing no further leakage for at least 72 h while they are stored at 4 °C. Before using the stored vesicles in the freeze-drying experiments, aliquots were diluted to produce samples containing 10 mg lipid per ml. Various amounts of sugars were added to the final solutions to produce the desired sugar/phospholipid ratio before freeze-drying.

Retention of trapped carboxyfluorescein after freeze-drying

The amount of carboxyfluorescein inside and outside the vesicles was measured with a Perkin-Elmer LS-5 fluorometer as follows. Fluorescence of 10 μl aliquots of the sample dispersed in 2.5 ml Tes buffer (pH 7.5) was recorded with the excitation beam set at 460 nm and emission at 550 nm, after which the vesicles were lysed by addition of 20 μl of 2% (v/v) Triton X-100, and fluorescence was measured again. The fraction of carboxyfluorescein contained within the vesicles was calculated from the ratio of fluorescences recorded before and after the vesicles were lysed. 100-μl aliquots were frozen in liquid nitrogen and freeze-dried for at least 12 h. The dry samples were rehydrated to their original volumes with distilled water, and the concentration of carboxyfluorescein inside and outside the vesicles was recorded by measuring fluorescence before and after the vesicles were lysed. The percentage of carboxyfluorescein retained after freeze-drying was calcu-

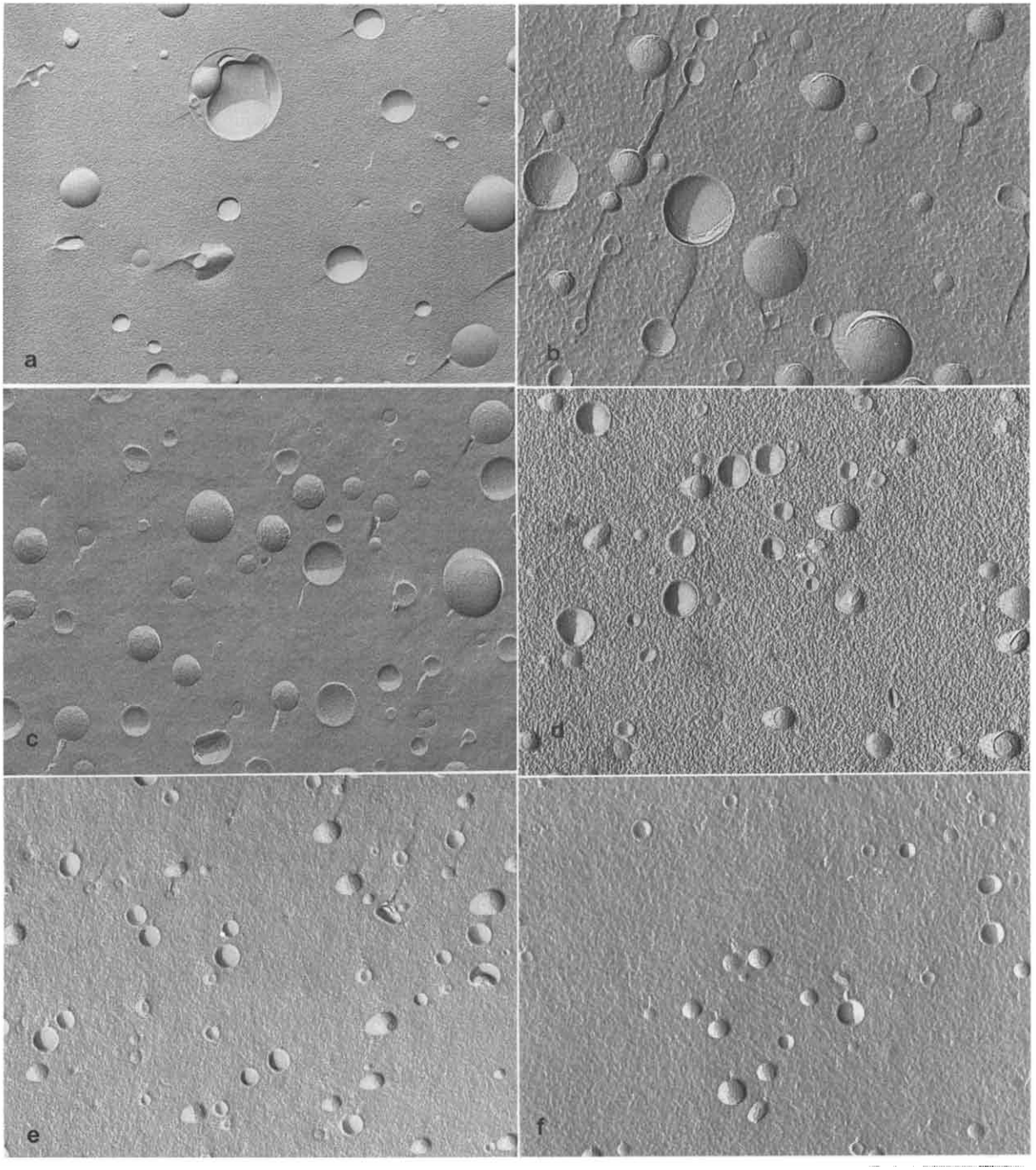

Fig. 1. Freeze-fracture images of vesicles composed of egg PC and PS (9:1) prepared by freeze-thaw of multilamellar vesicles. With five freeze-thaw cycles the multilamellar vesicles break down into unilamellar and oligolamellar vesicles (a), which are then reduced in size by extrusion through 400 (b), 200 (c), 100 (d), 50 (e) and 30 (f) nm pore-size polycarbonate filters.

lated according to the following formula:

$$\% \text{ retention} = \frac{F_a' - F_b'/F_a'}{F_a - F_b/F_a} \times 100$$

where F_b is the fluorescence before freeze-drying and before lysing; F_a the fluorescence before freeze-drying and after lysing; F_b' the fluorescence after freeze-drying and before lysing; and F_a' the fluorescence after freeze-drying and after lysing.

Freeze fracture

Samples were frozen in liquid freon, freeze-fractured on a Balzers BAF 400D freeze-fracture apparatus with platinum shadowing at a 45° angle. Replicas were examined in a Philips 410 transmission electron microscope. Diameters were measured from half-shadowed vesicles which had been fractured at the equator.

Results and discussion

Characterization of liposomes

Because we suspected at the outset that the size of the vesicles might be important in their stability, we have taken some care to characterize the vesicles extruded through polycarbonate filters, using freeze fracture. It was necessary to do these measurements even though Mayer et al. [23] have done similar studies on vesicles prepared from egg PC alone. The lipid mixtures used here behave considerably differently from PC alone, as we show below.

Freeze-fracture images show that, while the vesicles extruded through filters of larger pore size do appear larger than those extruded through finer filters, both populations are heterogeneous (Fig. 1). When measurements of the diameters of half-shadowed vesicles similar to those shown in Fig. 1 were made, we found that the average diameters are surprisingly similar, regardless of the pore size in the filters used (Table I). However, since we are interested in comparing the stability of these vesicles with respect to retention of trapped solutes, it is not the diameter of the vesicles per se that is important, but rather their trapped volume. Because volume increases as the cube of the radius while surface area increases as its square, measurement of the diameter can provide a deceptive estimate of the trapped volume. This point is illustrated in Fig. 2. Based on the measurements of the diameters of the vesicles, the average size would appear to be about 90 nm for this sample. But since a small number of vesicles contain a large volume, the volume distribution is clearly skewed towards the larger sizes. Similar measurements for the other classes of extruded vesicles show that those extruded through the smaller pore sizes are reasonably uniform, whereas those made with the larger pore sizes are much more heterogeneous. Nevertheless, clear trends can be seen in the volume distributions from large to small vesicles. For convenience, we will refer to vesicles extruded through, for example, filters with 30 nm pores as '30 nm vesicles' even though they depart significantly from their nominal size.

TABLE I

AVERAGE DIAMETERS OF EGG PC-PS (9:1) VESICLES EXTRUDED THROUGH POLYCARBONATE FILTERS WITH THE INDICATED PORE SIZES

Pore size	Diameter of vesicles (±S.E.)
400	118±9
200	96±4
100	89±6
50	69±2
30	61±2

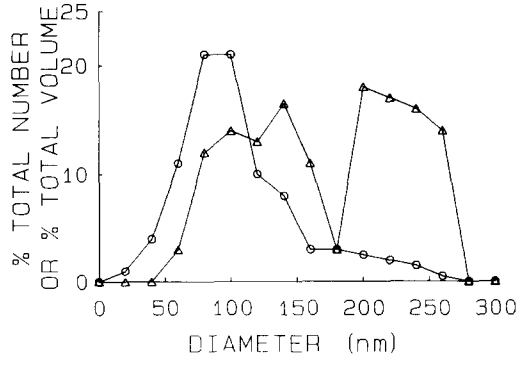

Fig. 2. Diameter and trapped volumes for vesicles extruded through 200 nm pore-size filters. Based on the diameters, the average size of the vesicles appears to be about 90 nm. However, most of the trapped volume is included in larger vesicles. ○, number of vesicles; △, volume.

Effect of size on retention of trapped solute

Size of the vesicles profoundly affects their stability during freeze-drying. As the data in Fig. 3 show, the optimal size for preservation is in the range of 50–100 nm. Such vesicles can be efficiently preserved by trehalose, achieving retention of 100% of the trapped carboxyfluorescein at mass ratios of trehalose/lipid of less than 4. By contrast, small, sonicated vesicles (approx. 25 nm in diameter) retain only about 40% of trapped solute at the same trehalose concentration. Similarly, very large vesicles are also comparatively unstable, retaining less than 50% of the trapped carboxyfluorescein following freeze-drying with 4 g trehalose per g lipid. The relative instability of the sonicated vesicles is intuitively easy to understand; such vesicles are thought to be under considerable strain due to their high radius of curvature and have been shown to fuse readily [24]. The relative instability of the larger ones is more difficult to explain, but it could be due to a lack of the structural rigidity that is required for stability.

Effects of different sugars

Different sugars have markedly dissimilar effects on stability, depending on the size of the vesicles. For example, Fig. 4 shows effects of two sugars, trehalose and sucrose, on retention of trapped solute by freeze-dried PC-PS vesicles of two different sizes. With vesicles about 100 nm in

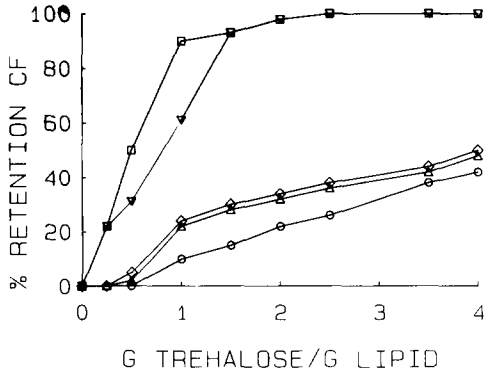

Fig. 3. Retention of trapped carboxyfluorescein (CF) by vesicles extruded through the indicated pore-sized filters, freeze-dried in the presence of trehalose at the indicated concentrations, and then rehydrated. ○, 25 nm; ▽, 50 nm; □, 100 nm; △, 200 nm; ◇, 400 nm.

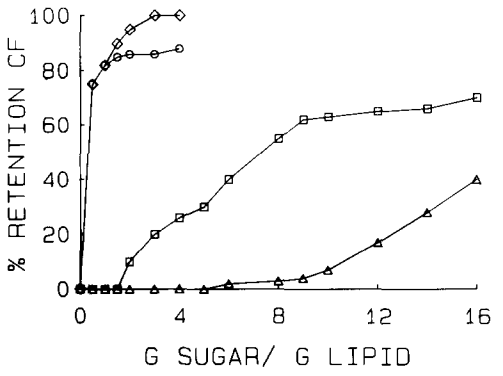

Fig. 4. Comparison between effects of sucrose and trehalose on retention of trapped carboxyfluorescein (CF) following freeze-drying of sonicated (25 nm; ▽, sucrose and □, trehalose) vesicles and vesicles prepared by extrusion through 100 nm filters (○, sucrose and ◇, trehalose).

diameter, trehalose and sucrose provide similar levels of stabilization, except that retention rises to 100% with trehalose and reaches maximal value to about 90% with sucrose. This is a small, but repeatable difference between these two sugars (cf. Ref. 6). With sonicated 25 nm vesicles, by contrast, the two sugars behave quite differently. Much more sugar is required for te preservation in both cases, but trehalose provides markedly more effective preservation that does sucrose, even at the highest mass ratios of sugars tested. With both sucrose and trehalose, the maximal retention seen is less than 70%. The apparent difference between the ability of these two sugars to preserve dry liposomes may be related to fundamental differences in their mode of interaction with the bilayer. Alternatively, Leopold and Vertucci [25] have suggested that sucrose may not be as effective as trehalose because the former molecule tends to crystallize more readily than the latter. Based on that proposition, they have recently [26] shown that the introduction of raffinose, which they suggest inhibits the crystallization of sucrose, improves the efficacy of sucrose both in depressing the transition temperature of dry phospholipids and in retention of trapped solutes following drying.

Effects of sugar on both sides of the bilayer

We [5,6] and others [11] previously reported that sugars are required on both sides of the

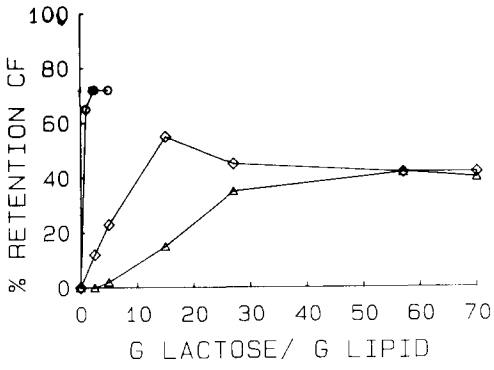

Fig. 5. Effects of providing lactose both inside and outside sonicated vesicles and on the outside only on retention of carboxyfluorescein (CF) following freeze-drying of sonicated vesicles. For comparison, retention by 50 nm vesicles with lactose on both sides is shown also. ○, 50 nm, inside and outside; ◇, 25 nm, inside and outside; and △, 25 nm, outside only.

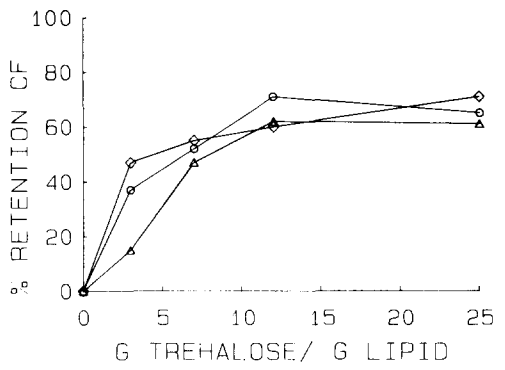

Fig. 6. Effects of preparing sonicated vesicles in the presence of the indicated concentrations of trehalose on retention of carboxyfluorescein (CF) following freeze-drying. Although the amount inside the vesicles varies widely, retention of CF does not differ significantly between the three treatments. ○, 0.06 M; △, 0.125 M; and ◇, 0.5 M.

bilayer to achieve maximal stabilization, and we confirm and extend that observation here. Lactose is commonly used in the pharmaceutical industry for preservation of drugs and other proteins of pharmaceutical interest. This sugar is not particularly effective at stabilizing either larger vesicles or small, sonicated ones, achieving only about 70% retention with larger ones and at most 60% with small sonicated ones, even with the sugar on both sides of the bilayer (Fig. 5). When lactose was provided only on the outside of the vesicles, markedly inferior preservation was seen; with 15 g lactose per g lipid, less than 20% retention was achieved, while the vesicles retained about 50% of the trapped carboxyfluorescein with the sugar on both sides of the bilayer (Fig. 5). It is possible that this effect is simply due to the fact that having the lactose on both sides of the bilayer is additive, i.e., that the effective concentration of sugar affecting the bilayer is the arithmetic total of the amount inside and outside. To test this possibility, we prepared sonicated vesicles in the presence of various concentrations of trehalose, thus trapping sundry amounts inside. If the effect of the trehalose inside is simply additive to that outside, these vesicles should have been stabilized by differing amounts. Fig. 6 shows clearly that this is not the case. Only a small amount of trehalose is required inside, and adding more inside has no apparent effect on stability.

Concentration of sugar required to stabilize dry liposomes

In previous studies we have shown that a remarkably small amount of sugar is required to stabilize vesicles of POPC-PS about 50 nm in diameter – only about 2 g trehalose per g lipid [5]. Further, we found that maximal effects of this sugar on the physical properties of the dry lipids are achieved by the time this mass ratio of trehalose/lipid is added. The question can now be asked: which is important for the stabilization, the mass ratio between sugar and lipid in the dry state, or the bulk concentration of the sugar before the drying is begun? To distinguish between these possibilities, the following experiment was conducted. Vesicles of PC-PS were prepared by extrusion through 50 nm filters and sufficient trehalose was added to produce the desired mass ratio of trehalose/lipid. The samples were then sequentially diluted to produce lipid-trehalose mixtures with widely varying concentrations in the bulk medium, but with the same lipid/trehalose mass ratios. The results (Fig. 7) show that the dilution of the samples before freeze-drying has no effect on the preservation of the liposomes, indicating that it is the mass ratio between sugar and lipid that is important for preservation. The trehalose concentration required in bulk solution is, in fact, very low, in the millimolar range. By contrast, Madden et al. [11] uniformly used 250

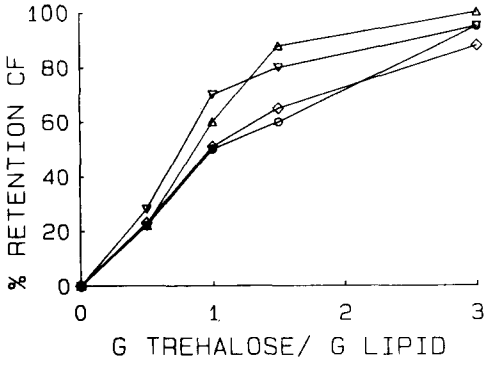

Fig. 7. Effects of diluting sugar-vesicle preparations by the indicated amounts on retention of trapped carboxyfluorescein (CF) following freeze-drying. Although the bulk concentrations of sugar and lipid vary widely, retention of trapped solute is not significantly different between thre treatments. This result indicates that retention varies with the mass ratio between sugar and lipid and not the concentration of the sugar in the bulk phase. △, no dilution; ◇, 1:100; ▽, 1:10; and ○, 1:1.

mM sugar in their experiments, which is far more than needed to preserve the liposomes. We hasten to add that freeze-drying is different from freeze-thawing in this regard. In order to stabilize vesicles during freeze-thawing, much higher concentrations of the sugars are needed, and, in this case, it appears that the bulk concentration of the sugar in solution is the important factor [7,12,13,27,28], an effect that is possibly related to the effect of the sugars on recrystallization of ice during thawing.

Effect of charged lipids included in the bilayer

All the results shown above were obtained with mixtures of PC and PS. Vesicles of similar size can be prepared with PC alone, but with the following differences. During the freezing of PC-PS mixtures before they are extruded through the polycarbonate filters, the multilamellar vesicles are reorganized into oligolamellar vesicles. With PC alone, by contrast, the multilamellar vesicles persist following frezing, as previously reported [23]. After extrusion, the PC vesicles do not differ significantly from those made from PC-PS, in either average diameter or trapped volume.

Madden et al. [11] have shown that vesicles of PC alone can be preserved by freeze-drying with sugars, but the maximal level of stabilization they were able to achieve was about 80–90% retention. We have conducted similar studies with different sizes of vesicles made of PC alone, with the results shown in Fig. 8. Even with the optimal size of vesicles (50–100 nm), not much more than 70% retention was achieved even at the highest ratios of sugars to phospholipids (Fig. 8). Two factors might account for the apparent disagreement between these data and those previously published by Madden et al. [11]. (a) Those authors expressed their data in molar quantities for the sugars used, so it is not clear just what the final sugar/lipid ratio was in their samples, but it is probably considerably more than the amounts shown in Fig. 8. It is possible that at these higher sugar contents, the retention of trapped solute increases. (b) An alternative explanation is that there is an experimental artifact introduced into the data of Madden et al. for the following reasons. To measure retention of trapped solute they passed the vesicles through an ion-exclusion column after they were dried and rehydrated, thus removing the leaked solute from the outside of the liposomes. They then measured the concentration of lipid recovered from the column and expressed the retention data in terms of the recovered lipid. In our hands, a significant fraction of the lipid is not recovered from such columns with the liposome fraction following freeze-drying, probably because fusion during the freeze-drying leads to formation of multilamellar vesicles that do not comigrate with the unilamellar ones [5]. Thus, Madden et al. were measuring retention of trapped solute by a

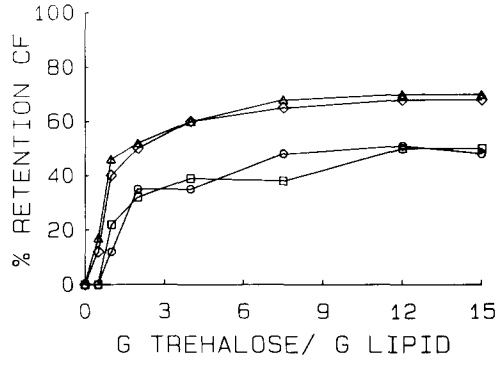

Fig. 8. Retention of trapped carboxyfluorescein (CF) by vesicles of egg PC alone. Retention is much less than in vesicles of similar size prepared by the addition of PS. ◇, 50 nm; △, 100 nm; ○, 200 nm; and □, 400 nm.

selected fraction of the original population. We believe that this is the most likely factor that accounts for the higher level of apparent stabilization they were able to achieve with PC alone compared with the results reported here. In keeping with this suggestion is the fact that they reported retention values of 50% or more after their vesicles were freeze-dried without addition of sugar. With the techniques used here and those described previously we have seen no significant retention of trapped solute without adding sugar before freeze-drying.

Acknowledgements

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References

1 Poste, G. (1983) Biol. Cell 47, 19–38.
2 Ostro, M.J. (1987) Sci. Am. 256, 102–111.
3 Janoff, A.S., Carpenter-Brown, S., Weiner, A.J., Siebold, J., Weismann, G. and Ostro, M.J. (1983) Clin. Chem. 29, 1587–1592.
4 Gregoriadis, G. (1984) Liposome Technology, CRC Press, Boca Raton, FL.
5 Crowe, L.M., Crowe, J.H., Womersley, C. and Appel, L. (1985) Arch. Biochem. Biophys. 242, 240–247.
6 Crowe, L.M., Womersley, C., Crowe, J.H., Reid, D., Appel, L. and Rudolph, A.S. (1986) Biochim. Biophys. Acta 861, 131–140.
7 Womersley, C., Uster, P.S., Rudolph, A.S. and Crowe, J.H. (1986) Cryobiology 23, 245–255.
8 Carpenter, J.F., Hand, S.C., Crowe, L.M. and Crowe, J.H. (1986) Arch. Biochem. Biophys. 250, 505–512.
9 Carpenter, J.F., Crowe, L.M. and Crowe, J.H. (1987) Biochim. Biophys. Acta 923, 109–115.
10 Crowe, J.H., Crowe, L.M., Carpenter, J.F. and Aurell Wistrom, C. (1987) Biochem. J. 242, 1–10.
11 Madden, T.D., Bally, M.B., Hope, M.J., Cullis, P.R., Schieren, H.P. and Janoff, A.S. (1985) Biochim. Biophys. Acta 817, 67–74.
12 Strauss, G. and Hauser, H. (1986) Proc. Natl. Acad. Sci., USA 83, 2422–2426.
13 Strauss, G., Schurtenberger, P. and Hauser, H. (1986) Biochim. Biophys. Acta 858, 169–180.
14 Madin, K.A.C. and Crowe, J.H. (1975) J. Exp. Zool. 193, 335–342.
15 Panek, A.C., Barnardes, A. and Panek, A.D. (1987) in Membranes, Macromolecules, and Stability in the Dry State (Leopold, C., ed.), pp. 123–142, Cornell University Press, Ithaca, NY.
16 Martin, C.M., Diaz, L.A., Manzanal, M.B. and Hardisson, C. (1986) FEMS Microbiol. Lett. 35, 49–54.
17 Van Laere, A.J. and Slegers, L.K. (1987) FEMS Microbiol. Lett. 41, 247–252.
18 Crowe, J.H., Crowe, L.M. and Chapman, D. (1984) Science 223, 701–703.
19 Crowe, J.H., Spargo, B.J. and Crowe, L.M. (1987) Proc. Natl. Acad. Sci., USA 84, 1537–1540.
20 Lee, C.W.B., Waugh, J.S. and Griffin, R.G. (1986) Biochemistry 25, 3737–3742.
21 Anchordoguy, T.J., Rudolph, A.S., Carpenter, J.F. and Crowe, J.H. (1987) Cryobiology 24, 324–331.
22 Gaber, B.P., Chandrasekhar, I. and Pattabiraman, N. (1986) in Membranes, Macromolecules, and Stability in the Dry State (Leopold, C., ed.), pp. 231–241, Cornell University Press, Ithaca, NY.
23 Mayer, L.D., Hope, M.J. and Cullis, P.R. (1986) Biochim. Biophys. Acta 858, 161–168.
24 Nir, S., Wilschut, J. and Bentz, J. (1982) Biochim. Biophys. Acta 688, 275–278.
25 Leopold, A.C. and Vertucci, C.W. (1987) in Membranes, Macromolecules, and Stability in the Dry State (Leopold, C., ed.), pp. 22–34, Cornell University Press, Ithaca, NY.
26 Caffrey, M., Fonseca, V. and Leopold, A.C. (1987) Plant Physiol., in press.
27 Rudolph, A.S. and Crowe, J.H. (1985) Cryobiology 22, 367–377.
28 Rudolph, A.S., Crowe, J.H. and Crowe, L.M. (1986) Arch. Biochem. Biophys. 245, 134–143.