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# Protection of large unilamellar vesicles by trehalose during dehydration: retention of vesicle contents

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**The ability of trehalose and other sugars to maintain the integrity of large unilamellar vesicles subjected to dehydration and rehydration has been investigated. It is shown, employing freeze-fracture techniques, that large unilamellar vesicles prepared in the presence of trehalose at 125 mM or higher concentration do not exhibit significant structural changes during the dehydration-rehydration cycle. Further, up to 90% of entrapped $^{22}Na$ or [$^{3}H$]inulin is retained during this process. Other sugars also exhibited similar protective effects where trehalose was most effective, followed by sucrose, maltose, glucose and lactose. It is demonstrated that proton or $Na^+/K^+$ electrochemical gradients can be maintained during the dehydration-rehydration process, which can subsequently be used to drive the uptake of lipophilic cationic drugs such as adriamycin. The implications for long-term storage of liposomal systems for use in drug-delivery protocols are discussed.**

## Introduction

The ability of certain organisms such as brine shrimp cysts and some species of nematodes to survive dehydration appears to be related to their production of the nonreducing disaccharide, trehalose [1,2]. Such anhydrobiotic organisms can remain viable for years in the dry state and upon rehydration resume their normal metabolic activities. It has been proposed that trehalose preseves membrane structure by hydrogen bonding to the phospholipid headgroup and effectively replacing the bound water [3]. Evidence in support of this hypothesis has been provided by differential scanning calorimetry and infrared spectroscopic studies. The thermotrophic transition between the gel and liquid-crystalline states occurs at about 41°C for fully hydrated dipalmitoylphosphatidylcholine while the anhydrous lipid has a transition at 68°C. In the presence of trehalose, however, the transition temperature of the anhydrous lipid is lowered in an analogous manner to that observed on addition of water [4]. In addition, infrared spectroscopic studies have demonstrated an interaction between the hydroxyl groups of trehalose and the lipid phosphate headgroup, probably indicative of hydrogen bonding [4].

The remarkable ability of trehalose to preserve membrane structure in the dry state is not restricted to the anhydrobiotic organisms. Microsomal vesicles isolated from the abdominal muscles of lobster when dried in the presence of this

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

disaccharide retained their structural and functional integrity upon rehydration [5].

We therefore decided to study the properties of large unilamellar vesicles dried in the presence of trehalose, particularly whether they maintain a permeability barrier in the anhydrous state. The amount of initially entrapped sodium, inulin or adriamycin retained by vesicles which had been dried and then rehydrated was examined as a function of trehalose concentration. In addition, vesicles across which a sodium/potassium ion gradient was established, and which were then dried in the presence of trehalose, were shown to retain their ability to establish a membrane potential upon rehydration (in the presence of the potassium ionophore, valinomycin). This potential could then be used to drive uptake of the lipophilic cation, adriamycin, into the vesicles.

## Materials and Methods

*Materials.* Egg phosphatidylcholine was isolated employing standard procedures and was more than 99% pure as determined by TLC. Trehalose, maltose, sucrose and glucose were obtained from Sigma while lactose and fructose were purchased from Fisher Scientific and Mallinckrodt, respectively. $^{22}Na^+$, [$^3$H]inulin, tetra[$^3$H]phenylphosphonium bromide and $^3$HHO were obtained from New England Nuclear. Adriamycin was a generous gift of Dr. Alan Eaves.

*Vesicle preparation.* Large unilamellar vesicles were prepared using the LUVET (large unilamellar vesicles by extrusion techniques) procedure [6]. Unless otherwise stated, 80 $\mu$mol egg phosphatidylcholine were hydrated with 2 ml of 150 mM NaCl/20 mM Hepes (pH 7.4) containing the indicated concentration of trehalose. $^{22}Na^+$ (5 $\mu$Ci) or [$^3$H]inulin (5 $\mu$Ci, spec. act. 409 mCi $\cdot$ g$^{-1}$) were added to the dry lipid prior to hydration. The mixture was dispersed by vortexing and then passed ten times through two stacked polycarbonate filters of 100 nm pore size (Nuclepore, Pleasanton, CA) under 250 psi. When the freeze-thaw procedure was utilized, the vesicles were sized as above, freeze-thawed (employing liquid nitrogen) and then resized.

Unencapsulated $^{22}Na^+$ or [$^3$H]inulin was removed by passing the vesicles through a column (1.4 $\times$ 10 cm) of either Sephadex G-50 (fine) for removal of $^{22}Na^+$ or Ultragel AcA 34 for removal of [$^3$H]inulin.

*Dehydration of vesicles.* Samples (1 ml) were dried in 10-ml Kimex tubes at room temperature under high vacuum (Vitro Freeze Drier) normally for 24 h. In some cases, the samples were frozen in liquid nitrogen prior to dehydration. In experiments where the amount of residual water present after drying was determined, $^3$HHO (tritiated water, 30 $\mu$Ci) was added to the initial sample.

Following dehydration, samples were rehydrated with distilled water (900 $\mu$l) and the vesicles were dispersed by gentle vortexing. The amount of initially entrapped $^{22}Na^+$, [$^3$H]inulin or adriamycin remaining within the vesicles was determined following passage of 100-$\mu$l aliquots of the vesicle suspension over columns (1 ml) of Sephadex G-50 (fine) or Ultragel AcA 34 [6] equilibrated with the same solution in which the vesicles were suspended.

*Membrane potential.* In order to produce a $Na^+/K^+$ chemical gradient to establish a membrane potential, LUVET's were prepared in a potassium glutamate buffer (169 mM potassium glutamate/250 mM trehalose/20 mM Hepes (pH 7.4). Subsequently, the external buffer was replaced by a NaCl buffer (150 mM NaCl/250 mM trehalose/20 mM Hepes (pH 7.4)) by passage through a Sephadex G-50 (fine) column (1.4 $\times$ 10 cm) which was preequilibrated with the NaCl solution [7]. Samples of vesicles were then either kept at 4°C for 24 h or dried under high vacuum for the same period and then rehydrated.

Where employed, valinomycin (Sigma, St. Louis) was added in ethanol to a concentration of 0.5 $\mu$g/$\mu$mol phospholipid. The membrane potential generated was measured by determining the distribution of the lipophilic cation tetra[$^3$H]phenylphosphonium bromide (New England Nuclear, Canada) as described previously [6].

Similarly, transmembrane pH gradients (interior acid) were formed using a trap buffer with low pH (150 mM KOH/135 mM glutamic acid/250 mM trehalose (pH 5.5)) which was then exchanged with a high-pH buffer (150 mM KOH/125 mM glutamic acid/30 mM NaCl/250 mM trehalose (pH 7.5)) on a Sephadex G-50 (fine) column. Where used, the proton ionophore CCCP was

added to a final concentration of 20 μM.

*Freeze-fracture electron microscopy.* Samples for freeze-fracture contained 25% glycerol and were fractured and replicated as described previously [8] using a Balzers freeze-fracture apparatus. Replicas were visualized on a Phillips 400 electron microscope.

*Quasi-elastic light scattering measurements.* Vesicles were sized by employment of a Nicomp 200 Laser Particle Sizer (Nicomp Instrument, Goleta, CA) operating at 632.8 nm and 5 mW.

*Assays.* Phospholipids were quantified by determination of inorganic phosphorus as described by Chen et al. [9]. Adriamycin uptake was measured following solubilization of vesicles in 0.5% Triton X-100 from its absorbance at 480 nm. [$^3$H]inulin, $^3$HHO and tetra[$^3$H]phenylphosphonium were counted in a Phillips PW 4700 liquid scintillation counter while $^{22}Na^+$ was quantified by gamma counting on a Beckman Gamma 800.

## Results

Egg phosphatidylcholine LUVET's containing entrapped $^{22}Na^+$ were dried in the presence of varying concentrations of trehalose. Samples were either frozen in liquid nitrogen or dried without prior freezing. The amount of initially trapped $^{22}Na^+$ retained by the dried vesicles upon rehydration is dependent upon the trehalose concentration

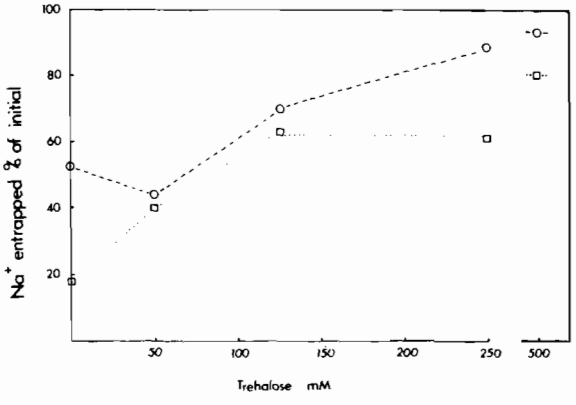


Fig. 1. Retention of $^{22}Na^+$ by dehydrated vesicles as a function of trehalose concentration. Large unilamellar vesicles were dried without prior freezing (○- - - - - -○), or after freezing in liquid nitrogen (□· · · · · ·□).

(Fig. 1) with up to 90% of the sodium retained at the highest concentration of trehalose tested. It can be seen that vesicles dried without prior freezing retain more of their contents than those first frozen in liquid nitrogen. When trehalose is omitted from the buffer, the vesicles upon rehydration give a milky suspension in contrast to the translucent appearance of the original sample. In addition, the recovery of the vesicles from the 1-ml Sephadex columns is low (< 10%), suggesting that they have fused to form larger structures. This was confirmed by freeze-fracture electron microscopy. In contrast to the small uniform size of the LUVET's prior to drying (Fig. 2a), vesicles dried in the absence of trehalose and then rehydrated are in general much larger as shown in Fig. 2b. Vesicles dried in the presence of 50 mM trehalose and then rehydrated are generally the same size as prior to dehydration, but a small fraction of larger structures are also observed (Fig. 2c). At trehalose concentrations of 125 mM or greater, there is no discernible structural difference between vesicles before and after dehydration and rehydration (Fig. 2d).

To verify that vesicles dehydrated in the presence of trehalose retain their contents and do not simply reencapsulate label upon rehydration, vesicles were prepared in 250 mM trehalose and $^{22}Na^+$ then added to the external medium. Following dehydration and rehydration, aliquots of the suspension were passed down 1-ml Sephadex columns as detailed in Materials and Methods. Of the available $^{22}Na^+$, less than 0.02% was sequestered by the rehydrated vesicles confirming that they do not encapsulate solute in the external medium upon rehydration.

The ability of LUVET's to retain inulin ($M_r$ 5000) is shown as a function of trehalose concentration in Fig. 3. As might be predicted from its greater molecular weight, more of the inulin is retained following drying than sodium. At the higher trehalose concentrations, however, this difference is quite small and suggests that the small amount of each label lost may be the result of vesicle rupture rather than permeability changes.

To determine whether trehalose needs to be present on both sides of the vesicle membrane to preserve its structure in the dry state, large unilamellar vesicles were prepared with the disacchar-

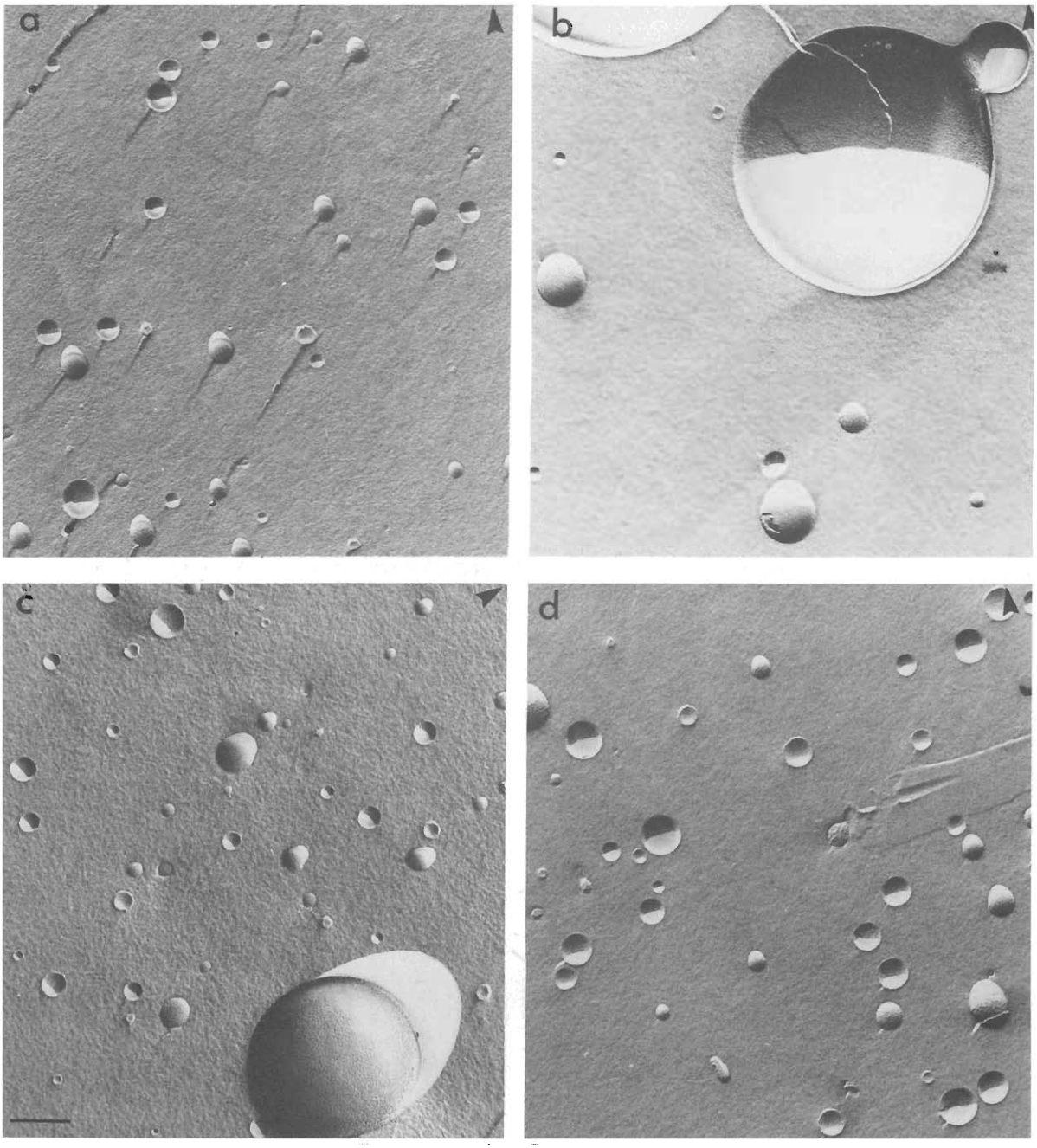


Fig. 2. Freeze-fracture electron micrographs of egg phosphatidylcholine vesicles before and after dehydration and rehydration. Egg phosphatidylcholine vesicles prepared by extruding large multilamellar vesicles through a 100 nm polycarbonate filter are shown in (a). Following dehydration in the absence of trehalose, much larger structures are obtained (b). Vesicles dried in the presence of 50 mM trehalose (c), or 125 mM trehalose (d), are also shown. The bar represents 200 nm and the arrows indicate the direction of shadowing.

ide on both sides of the membrane or only on the outside. The vesicles were dried and at different times up to 72 h samples were rehydrated and the level of $^{22}Na^{+}$ retained was determined. These results are shown in Table I together with the values of the amount of residual water present in

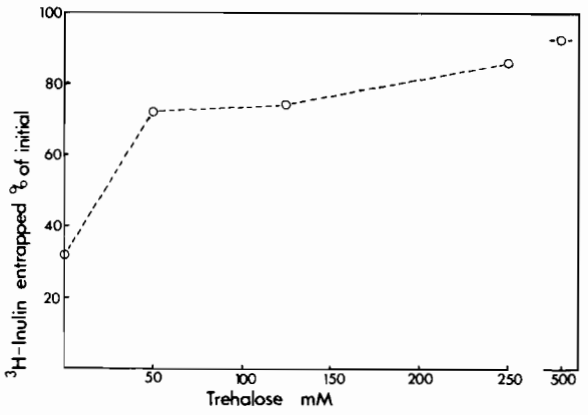


Fig. 3. Retention of [$^3$H]inulin as a function of trehalose concentration. Large unilamellar vesicles containing entrapped [$^3$H]inulin were dried under high vacuum without prior freezing. For details, see Materials and Methods.

the samples following drying. It is clear that trehalose must be present on both membrane surfaces to fully protect the vesicles, and further the amount of residual water present in samples with trehalose on the external membrane surface only is critical in determining the amount of structural damage occurring.

To further characterize the factors influencing membrane stability, the salt concentration was varied for a fixed trehalose concentration and the percentage of $^{22}Na^+$ retained by dried vesicles was determined. In addition, the size of the vesicles

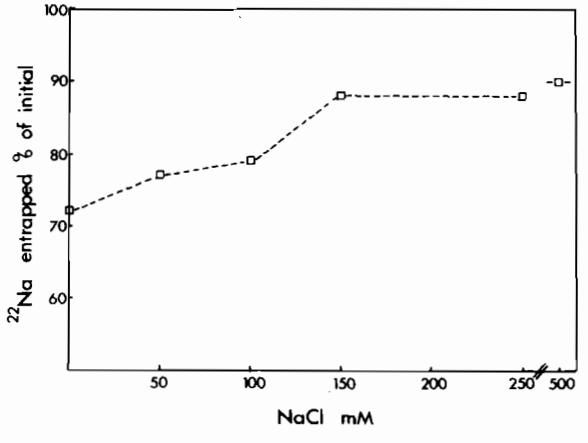


Fig. 4. Influence of sodium chloride concentration on the amount of $^{22}Na^+$ retained by dehydrated vesicles. Vesicles prepared in 250 mM trehalose were dried under high vacuum for 24 h as detailed under Materials and Methods.

TABLE I

EFFECT OF TREHALOSE ON PRESERVATION OF THE VESICLE MEMBRANE STRUCTURE IN THE DRY STATE

| Sample | Drying time (h) | $^{22}Na^+$ retained (%) | Residual water (%) |
|---|---|---|---|
| Trehalose on both sides of membrane (250 mM) | 24 | 94 | 5.6 |
| | 48 | 84 | 5.4 |
| | 72 | 84 | 5.2 |
| Trehalose on outside of membrane (250 mM) | 24 | 68 | 5.4 |
| | 48 | 49 | 5.0 |
| | 72 | 17 | 4.2 |

was modified by passing them through polycarbonate filters with pore sizes ranging from 800 to 50 nm. A freeze-thaw cycle was also included to enhance the trapped volume of the resultant vesicles [6].

As shown in Fig. 4, there is a small but significant increase in the amount of $^{22}Na^+$ retained by the vesicles dried at higher salt concentrations (up to 500 mM). In Table II, the influence of vesicle size on stability to drying is shown. It is interesting that vesicles prepared by passing multilamellar

TABLE II

INFLUENCE OF VESICLE SIZE ON ABILITY TO RETAIN $^{22}Na^+$ ON DEHYDRATION AND REHYDRATION

Vesicles were prepared with varying mean diameters by extruding multilamellar vesicles through polycarbonate filters of appropriate pore size (see Materials and Methods). The samples all contained 250 mM trehalose and were dehydrated for 24 h. Vesicles prepared by extrusion through 200 or 400 nm pore size filters showed two size populations by quasi-elastic light scattering. The means of each are given below. In addition, vesicles extruded through the 800-nm filters showed a small population ( $<10\%$) with a mean diameter of 130 nm.

| Polycarbonate filter pore size (nm) | Mean vesicle diameter (nm) | % $^{22}Na^+$ retained following dehydration and rehydration |
|---|---|---|
| 800 | 500 | 80 |
| 400 | 220, 500 | 84 |
| 200 | 180, 375 | 87 |
| 100 | 170 | 92 |
| 50 | 112 | 88 |

TABLE III

A COMPARISON OF DIFFERENT SUGARS AND THEIR ABILITY TO STABILIZE VESICLES IN THE ANHYDROUS STATE

Large unilamellar vesicles were prepared in the presence of 500 mM of each sugar, dehydrated for 24 h and the amount of initially trapped $^{22}Na^+$ retained upon rehydration was determined as described under Materials and Methods.

| Sugar tested (500 mM) | % $^{22}Na^+$ retained following dehydration and rehydration |
|---|---|
| Trehalose | 88 |
| Glucose | 73 |
| Sucrose | 86 |
| Maltose | 76 |
| Lactose | 66 |

systems through filters of 50 and 100 nm pore size have a mean diameter considerably in excess of the pore size. In contrast, in the absence of trehalose the mean diameter (determined by freeze fracture) is slightly smaller than the pore size [6]. When vesicles are prepared by extrusion of lipid

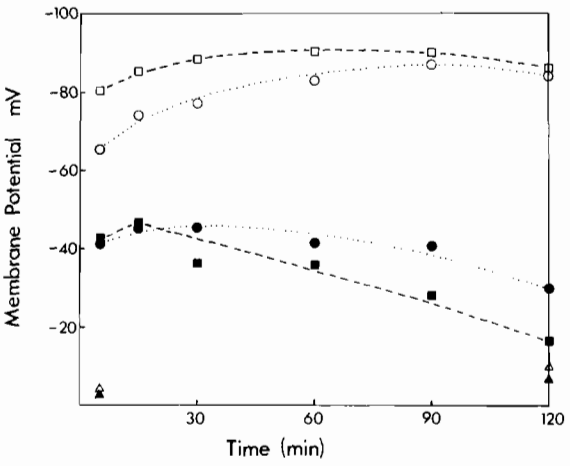


Fig. 5. Membrane potential generated by a proton gradient for control and dehydrated vesicles. Vesicles with a preexisting proton gradient (see Materials and Methods for details) were maintained at 4°C for 24 h, control, or dehydrated under high vacuum for the same time. Both samples contained 250 mM trehalose. The potential generated by the dried vesicles upon rehydration was determined in the absence (○·····○), or presence (●·····●) of 20 μM CCCP. The control without CCCP (□- - - - -□) and with CCCP (20 μM) (■- - - - -■) is shown. In addition, control vesicles were prepared with no proton gradient across the membrane (pH 7.5 buffer inside and outside). The measured membrane potentials for these vesicles are shown as: dehydrated, ▲; and control maintained at 4°C, △.

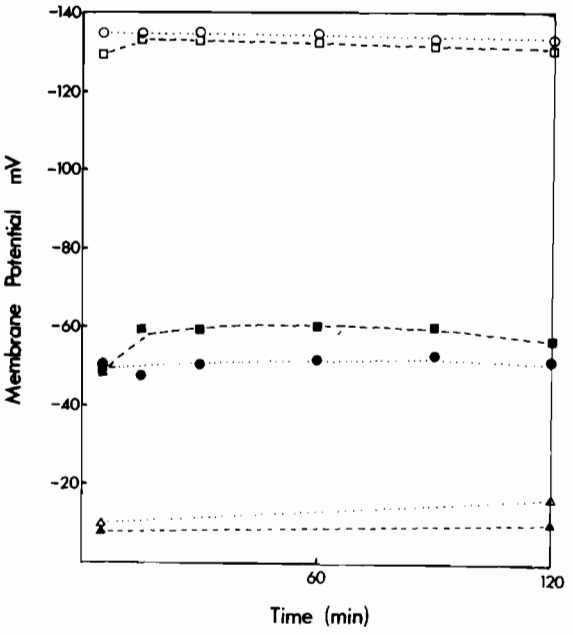


Fig. 6. Membrane potential generated by a $Na^+/K^+$ chemical gradient for dehydrated vesicles. Vesicles with a preexisting $Na^+/K^+$ gradient were dehydrated and rehydrated for 24 h in the presence of 250 mM trehalose. Following rehydration, the potential in the presence (○·····○), or absence (●·····●) of valinomycin (0.5 μg/μmol phospholipid) was measured using tetra[$^3$H]phenylphosphonium bromide. The potential generated by control vesicles maintained at 4°C for the same period was also measured in the presence (□- - - - -□), or absence (■- - - - -■) of valinomycin. Control vesicles with potassium glutamate on both sides of the membrane were also prepared. The potentials exhibited by the vesicles in the presence of valinomycin are also shown: ▲- - - - -▲, dehydrated; △·····△, kept at 4°C.

through filters of 200 or 400 nm pore size, two size populations are observed and the mean diameters of each are given in Table II. While the most stable vesicles appear to be those with a mean diameter of about 170 nm, the larger vesicle systems contain some multilamellar structures which make a rigorous comparison difficult.

The ability of trehalose to preserve the permeability barrier in dried vesicles was compared to a number of other sugars. Vesicles were prepared in (500 mM) trehalose, maltose, lactose, sucrose, and glucose and the amount of $^{22}Na^+$ retained by the vesicles following drying and rehydration was determined (Table III). It would appear to be a general property of sugars that they can stabilize vesicles in the anhydrous state. In this regard,

however, trehalose and sucrose are superior to maltose, glucose and lactose.

A potentially useful application for large unilamellar vesicles is as carriers for drugs or other biologically relevant molecules. Such an application, however, requires that the membrane-encapsulated drug be stored in a stable form. We have therefore examined the ability of vesicles to retain the antitumor drug adriamycin when dried in the presence of trehalose. In Table IV, the level of adriamycin trapped in large unilamellar vesicles before and after drying is shown as is the subsequent rate of drug leakage following rehydration. As with $^{22}Na^+$ and [$^3$H]inulin, more than 90% of the drug is retained following drying and rehydration. The rate of leakage of adriamycin from the rehydrated vesicles is comparable to the rate with normal vesicles [7].

We have shown previously that a large number of drugs have the property of being both lipophilic and cationic and respond to a membrane potential (negative inside) by accumulating to high concentrations in the vesicle interior [7]. We have therefore examined the possibility that vesicles can be prepared with a preexisting membrane potential, dried in the presence of trehalose and rehydrated with retention of the membrane potential. Vesicles with a proton gradient or a $Na^+/K^+$ chemical gradient were therefore prepared in 250 mM trehalose (see Materials and Methods for details). Following drying and rehydration, the potentials exhibited by these vesicles were compared to controls which had been maintained at 4°C for the corresponding time. As shown in Figs. 5 and 6, the potentials exhibited by vesicles dried and rehydrated are essentially the same as for the controls. In the case of the proton-gradient-driven potential, however, this is developed somewhat more slowly for the dehydrated and rehydrated vesicles.

TABLE IV

ABILITY OF DEHYDRATED VESICLES TO RETAIN ADRIAMYCIN ON REHYDRATION

Adriamycin was entrapped in large unilamellar vesicles and the samples were then dehydrated in 250 mM trehalose for 24 h. The adriamycin content of the initial sample and the rehydrated vesicles was determined as described under Materials and Methods.

| | | Adriamycin content (nmol/μmol lipid) |
|---|---|---|
| Adriamycin trapped | before drying | 197 |
| | after drying | 185 |
| | 1 h after drying | 158 |
| | 2 h after drying | 145 |

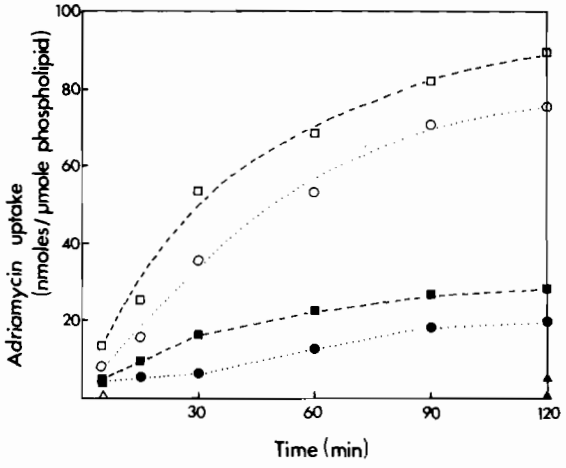


Fig. 7. Uptake of adriamycin driven by a membrane potential into previously dehydrated vesicles. Vesicles with a preexisting $Na^+/K^+$ gradient were dehydrated in the presence of 250 mM trehalose for 24 h. Following rehydration, the ability of the vesicles to accumulate adriamycin in the presence (○·····○), or absence (●·····●) of valinomycin was measured. Control vesicles maintained at 4°C for the same period were also tested in the presence (□-----□), or absence (■-----■) of valinomycin. In addition, control vesicles with potassium glutamate on both sides of the membrane were assayed in the presence of valinomycin as shown: △, dehydrated; or ▲, maintained at 4°C.

The ability of vesicles dried with a preexisting $Na^+/K^+$ chemical potential to accumulate adriamycin upon rehydration was then compared to control vesicles. Little difference in the rate or extent of uptake was observed (Fig. 7).

## Discussion

The preservation of membrane structure in the anhydrous state by trehalose appears to be related to its ability to replace the water molecules normally hydrogen-bonded to the lipid polar headgroups. In this regard, it has been shown to be superior to other sugars. Crowe and co-workers [5,10] examined a number of carbohydrates and their effectiveness at maintaining the structural

and functional properties of microsomal membranes at low water activities. Trehalose was the most effective while the alcohol sugars such as sorbitol and *myo*-inositol were least effective and appeared to be fusogenic [5]. The results we have obtained here support those earlier observations although, at higher concentrations, some other sugars, notably sucrose, appear almost equally effective.

At the present time, considerable interest is centered on the use of liposomal systems in the delivery of drugs and other biologically active molecules (for review, see Ref. 11). The ability to encapsulate a drug within a large unilamellar vesicle offers a number of advantages over conventional delivery procedures and offers the possibility of selective drug delivery. Before such an aim can be commercially realized, however, the liposomally entrapped drug must be obtained in a form that can be conveniently stored in a stable condition. In this regard, storage of the vesicles in aqueous suspension would not be suitable due to leakage of the drug. The observation, therefore, that large unilamellar vesicles loaded with adriamycin can be dried in the presence of trehalose and then rehydrated with minimal leakage of the drug offers exciting possibilities for storage of liposomal delivery systems in the anhydrous state.

We have shown previously that a membrane potential can be used to drive the accumulation of a number of drugs and biologically active molecules which share the property of being lipophilic cations [7,12,13]. The observation that vesicles dried with a preexisting proton or $Na^+/K^+$ chemical gradient can generate a membrane potential upon rehydration and that adriamycin is accumulated in response to this potential offers further exciting possibilities for drug entrapment and delivery. Liposomal delivery systems could be stored for long time periods in the anhydrous state and then loaded with the drug molecule of interest following rehydration using this intrinsic membrane potential.

The ability to dehydrate large unilamellar vesicles with retention of their structural and permeability properties clearly offers a number of important advantages for the long-term storage of liposomal systems.

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