Differential Effects of Alkali Metal Chlorides on Formation of Giant Liposomes by Freezing and Thawing and Dialysis†

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ABSTRACT: The formation of liposomes by hydration of egg phospholipids in solutions of the alkali metal chlorides has been investigated. A procedure requiring neither organic solvent nor detergent has been developed which yields much larger vesicles than any other hydration procedure by which vesicles have been generated from preformed bilayers in contact with aqueous phases. It involves a first step in which the lipids are completely hydrated by sonication in dilute buffer solution. The resultant small vesicles are subsequently enlarged by freezing and thawing in a very high concentration of the electrolyte of interest so that the large vesicles formed become equilibrated with that electrolyte solution. Finally, these vesicles are dialyzed against a lower concentration of electrolyte to cause the internal volume to expand through an osmotically driven influx of water. Vesicle fusion is also probable at this step. Liposome formation was monitored both by measuring the encapsulated volume and by determining the size distribution of vesicles visible under the light microscope. This investigation has led to the elucidation of several significant effects of the kind and concentration of electrolyte on the liposomes generated. (1) The resultant liposomes had large encapsulated volumes when the electrolyte concentration during the freeze–thaw step was either very high (>1 M) or very low (a few millimolar) but not when it was intermediate (physiological range). In spite of the significant trapped volume of liposomes generated in the low concentration case, practically none of these vesicles had diameters more than a few micrometers. (2) When high concentrations (>1 M) of some alkali metal ion chlorides were present during the freeze–thaw step, giant (diameters greater than 10 μm) vesicles, most of which are uni- or oligolamellar, became apparent after dialysis against a low concentration of electrolyte. For optimal results, both the freeze–thaw step and dialysis are essential; however, variable effects of the alkali metal ions were observed only in the former step. Giant vesicles could not be produced under any conditions in the presence of LiCl. Solutions of NaCl generated giant vesicles only when the lipid concentration was very high. In the presence of CsCl, only small numbers of large vesicles formed. In contrast, vesicles formed in the presence of KCl or RbCl during the freeze–thaw step were extremely large and numerous. Low concentrations of $CaCl_2$ slightly stimulated the formation of giant vesicles from RbCl or KCl solutions but synergized dramatically with CsCl to produce giant vesicles. (3) Interfacial properties of the liposomes, as reflected in electrophoretic mobility and turbidity in the presence of different alkali metal ions, differed significantly, although not enough to explain the differences in hydration of lipids in these solutions. The eutectic temperatures of frozen alkali metal chloride solutions decreased in the order K > Rb > Na > Cs > Li, roughly in parallel with the formation of large vesicles in those solutions. (4) The presence of sugar during the freeze–thaw step inhibited the subsequent formation of large liposomes, perhaps through a cryoprotectant action. Nevertheless, glycerol led to the formation of small numbers of vesicles up to 50 μm in diameter.

Liposomes continue to prove useful in a variety of ways—as models of cell membranes, as microcapsules for delivery of agents into cells, and as matrices for reconstitution of membrane proteins and their functions. Accordingly, a number of investigators have developed methods to generate vesicles that feature one or more characteristics useful for specific applications. These methods, recently reviewed by Szoka & Papahadjopoulos (1980), have generally involved re-formation of lipid bilayers in solutions from which a solubilizing agent—either organic solvents or detergents—has been removed. In contrast, there have been few systematic investigations of liposome formation by the method originally described by Bangham et al. (1965), namely, hydration of an anhydrous lipid phase. The characteristics of vesicles generated by that method could be influenced by a number of parameters which warrant attention, particularly since the Bangham method is still the way most liposomes are made.

For the purpose of studying membrane–membrane interactions, we have had special interest in vesicles which are clearly visible under the light microscope and stable in physiological electrolyte solutions. In the course of developing procedures for generating such large vesicles, we discovered a number of substantial influences of solutes on the characteristics of liposomes formed from solutions containing them. Here, we describe one method for preparing vesicles with diameters of tens of micrometers, the success of which is substantially affected by different alkali metal ions. Our results clearly show that liposome formation by the manipulation of hydrated bilayers offers considerably more promise for controlling liposome characteristics than has been generally appreciated and, perhaps more importantly, that there remains much to be learned about the interactions of lipid bilayers with themselves and with aqueous phases. One step in the procedure, freezing and thawing, was previously known to cause enlargement of small vesicles (Kasahara & Hinkle, 1977; Pick, 1981).

The procedure described here is based upon an initial hydration of lipid by sonication. Next, freezing and thawing in a concentrated solution of electrolyte causes the vesicles to become simultaneously enlarged and loaded with that electrolyte. Subsequently, the vesicles are dialyzed against a lower concentration of electrolyte to form giant vesicles. Surprisingly, large differences in water uptake were found when different alkali metal chlorides were used—Li^+, Na^+, and Cs^+ being far less effective than K^+ and Rb^+. In the latter cases, vesicles with diameters up to 50 μm were formed. A process(es) in addition to osmotically driven inflation of vesicles

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must be promoted by electrolytes, since the latter are not equally effective in generating large vesicles. Among the additional possibilities are fusion of vesicles, influence of electrolyte on bilayer properties, and effects of different solutions on van der Waals attractive forces and electrostatic repulsive forces between bilayers, which could be instrumental during or after the freeze–thaw step.

Three other procedures have been described that also lead to extremes of hydration, i.e., giant vesicles wherein the ratio of lipid to aqueous phase in the vesicle actually approaches that of the solution as a whole. Two of these require nonelectrolytes and probably rely upon the electrical repulsion that exists between even weakly charged bilayers in such solutions (Reeves & Dowben, 1969; Boroske et al., 1981). The third involves a double emulsion with organic solvent and also requires the presence of a minimum proportion of charged lipid (Kim & Martin, 1981). Effects of different electrolyte solutions were not apparent in either strategy. A number of X-ray diffraction studies have been done on lipid hydration. Such studies have been done with electrolyte solutions at concentrations far less than the several molar that we have used, with the exception of that of Gottlieb & Eanes (1972) which revealed only slight differences among the alkali chlorides.

Materials and Methods

Chemicals. Chemicals were obtained from the following companies: Sigma Chemical Co., St. Louis, MO (both crude and pure egg yolk phosphatidylcholine, dicetyl phosphate, stearylamine, and cholesterol); Hach Chemical Co., Ames, IA [calcein, 4′,5′-bis[*N*,*N*-bis(carboxymethyl)aminomethyl]-fluorescein]. All lipid preparations except crude egg yolk phosphatidylcholine were pure by silica gel thin-layer chromatography criteria. All lipid was dissolved in $CHCl_3$ and stored at −20 °C.

Preparation of Liposomes. Sonicated phospholipid vesicles were prepared as follows: a phospholipid film, made by drying a chloroform solution of the lipid under nitrogen, was held under oil pump vacuum for 1 h and then hydrated with 10 mM 4-morpholinepropanesulfonic acid (Mops)[1] buffer, pH 7.2, containing 0.1 mM calcein. After suspension by vortexing, the resultant multilamellar liposomes were sonicated for about 1 min at maximum power in a probe-type apparatus (Biosonic IV). After addition of various solutes to the desired concentration, the lipid dispersion (0.3-mL final volume) was frozen in a dry ice–acetone bath, thawed at room temperature, and vortexed. The freeze–thaw procedure was repeated 3 times. Finally, the lipid dispersion was dialyzed for 2 days against 10 mM Mops buffer containing calcein (0.1 mM), EDTA (ethylenediaminetetraacetic acid) (0.01 mM), and NaN_3 (0.02%). After dialysis, the phosphorus content of aliquots of samples was assayed by using a modification of the Bartlett procedure (1959).

Determination of the Trapped Volume of Liposomes. The volume inside the liposomes was determined according to a method described elsewhere (Oku et al., 1982). In brief, 20 μL of sample was diluted with 480 μL of 10 mM Mops buffer, and the fluorescence intensity was measured before and after addition of 2 μL of 10 mM $CoCl_2$. Cobalt ion quenches the fluorescence of the calcein that is accessible to it, i.e., that which is outside the liposomes, so that the fraction of the fluorescence (corrected for background fluorescence) remaining after cobalt addition corresponds to the fraction of the total volume that is encapsulated. Background fluorescence (due to incomplete quenching) is a few tenths of a percent of the total and was determined for each sample by lysing the vesicles with 25 μL of 10% Triton X-100 after the cobalt addition.

Determination of the Population Size Distribution of Liposomes. A 16-μL portion of an appropriately diluted sample was applied to a microscope slide and covered with an 18 × 18 mm cover glass sealed with Vaseline. Under these conditions, the sample thickness is approximately 50 μm. Using a microscope equipped with a video camera and monitor, we selected 15 or more fields at random. The depth of focus was chosen to maximize the largest number of liposomes in focus so that their diameters could be easily measured. Liposome population size distribution was then calculated according to the following arbitrary formula:

$$\text{population size distribution} = \frac{x(5\text{–}10) + [x(10\text{–}15) \times 2] + [x(15\text{–}20) \times 3] + \ldots}{NP}$$

where $x(a\text{–}b)$ means the number of liposomes between a and b micrometers in diameter, N is the number of fields surveyed, and P is the concentration of the phospholipid phosphate in millimolar in the sample examined. This formula was chosen largely on the basis of simplicity and reproducibility. It contains an empirically chosen bias in favor of large vesicles, i.e., the weighing factors in the numerator; this bias was intended to counteract the bias introduced by comparing a sample containing a few very large liposomes with a sample containing medium-sized lipsomes. In essence, the parameter is the weighted number of vesicles per field for a standard lipid concentration of 1 mM. The most important application of this measurement was to allow us to quantify the formation of vesicles significantly larger than a few micrometers in diameter. A comparison of the trapped volume with the population size distribution provides an index of how skewed the distribution of sizes is: a large trapped volume but a low population size distribution will indicate significant numbers of vesicles below 5 μm in diameter, whereas a large trapped volume with a high population size distribution will indicate significant numbers of vesicles above 5 μm.[2]

Determination of the Turbidity Change of Liposomal Suspensions. Liposomes were prepared in water and briefly sonicated. Samples containing 0.2 mM liposomes, 5 mM EDTA, and 1.0 M each of various salts were mixed well and allowed to stand at room temperature for 30 min. Turbidity was measured at 400 nm. After three cycles of freezing and thawing, turbidity was measured again.

Electrophoresis of Liposomes. Liposomes were prepared in water. Samples contained 0.1 mM phospholipid, 1.0 mM Mops buffer (pH 7.2), 0.5 mM EDTA, and 0.1 M each of various electrolytes. The Rank Bros. (Cambridge, U.K.) apparatus was used. The procedure has been described previously (Bangham et al., 1958).

Determination of Eutectic Temperatures. A Perkin-Elmer differential scanning calorimeter (DSC 1B) was used to de-

[1] Abbreviation: Mops, 4-morpholinepropanesulfonic acid.

[2] Some appreciation for the meaning of the population size distribution parameter can be gotten from the recognition that, if all large vesicles were 10–15 μm in diameter and evenly distributed throughout the sampled volume, a population size distribution of 10 would mean that a dispersion of lipid at 1.0 mM would yield about 5 vesicles per field and that there would be about 100 000 000 such vesicles per μmol of lipid. If it is assumed that they are unilamellar, such vesicles would contribute about 4% of the total lipid, and then the trapped volume would be about 16 μL/μmol.

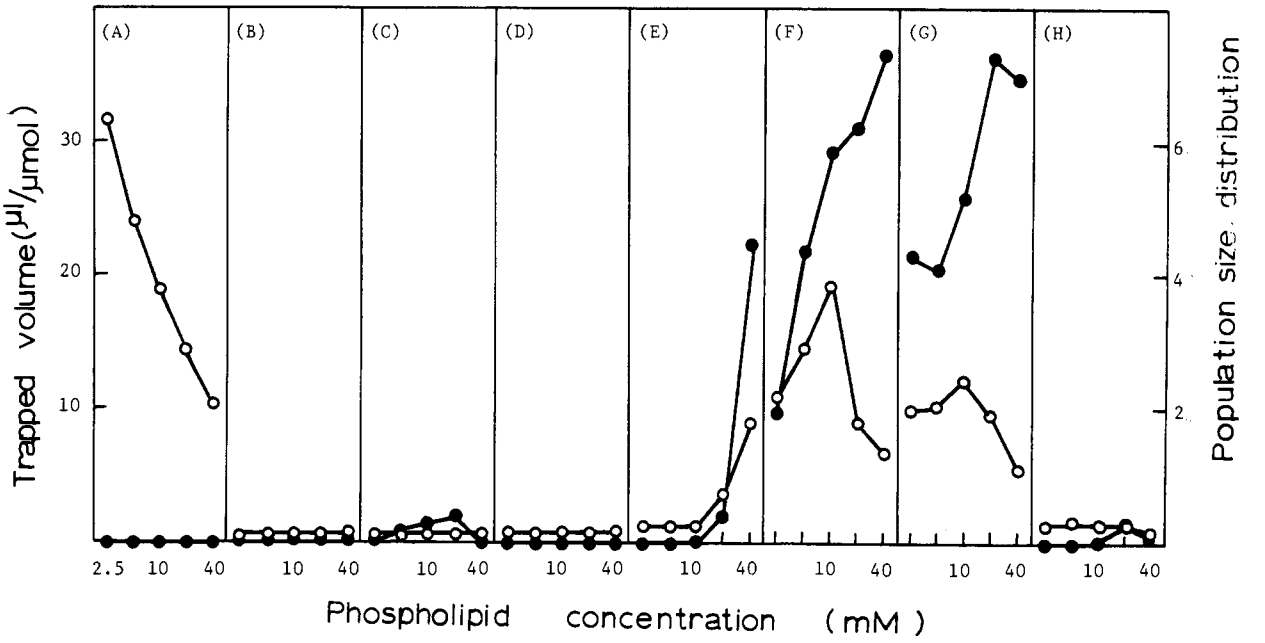

FIGURE 1: Effect on large liposome formation of lipid concentration and various solutes present during freezing and thawing. Liposomes prepared from egg yolk phospholipid at the concentrations indicated (scale is logarithmic) were frozen and thawed for three cycles in various solutes and dialyzed against 10 mM Mops buffer for 2 days. The trapped volume (O) and population size distribution (●) of large liposomes were determined after dialysis as described under Materials and Methods. (A) Without solutes except 10 mM Mops buffer; (B) 1.5 M glucose; (C) 25% glycerol; (D) 1.5 M LiCl; (E) 1.5 M NaCl; (F) 1.5 M KCl; (G) 1.5 M RbCl; (H) 1.5 M CsCl, present during freezing and thawing of the phospholipid dispersion.

termine eutectic temperatures of frozen solutions. Ten microliters of 3 M electrolyte solutions was used, and the heating rate was 2.5 °C/min. The reference pan was empty. The eutectic temperature was taken as the point of sharp departure of the endotherm from the base line. Samples were held a minimum of 10 °C below the expected eutectic temperature for at least 10 min to permit thermal equilibration. The instrument was calibrated with decane and water.

Results

The average degree of hydration of lipids exposed to the standard freeze–thaw–dialysis procedure was determined by measurement of the total trapped volume. Direct counting of large vesicles under the light microscope yielded a measure of what proportion of these were unusually large. The parameter used for comparison of different preparations is called the population size distribution and is described under Materials and Methods.

Figure 1 shows the trapped volume and population size distribution of large liposomes generated when water or various solutes were present at the freeze–thaw step. All solute concentrations were 1.5 M except glycerol which had a concentration of 25%. Concentrations of phospholipid in the liposomes were 40, 20, 10, 5, and 2.5 mM. All samples were dialyzed against 10 mM Mops buffer containing 0.1 mM calcein for 2 days. Figure 1A shows both parameters for liposomes which appeared after freezing and thawing of liposomes in the absence of added solutes. Before the freeze–thaw step, sonicated liposomes trapped about 0.5 μL/μmol, and there were virtually no liposomes observable under the microscope at 400× magnification. After three freeze–thaw cycles, the trapped volume of liposomes increased dramatically, especially at the lower concentration of liposomes, a trend previously described (Pick, 1981). Liposomes then became observable microscopically, but practically none exceeded 5 μm in diameter. Dialysis had no effect on the appearance of these vesicles, in contrast to those that were frozen and thawed in the presence of solute, as depicted in the remainder of the panels of Figure 1.

The presence of sugar during the freeze–thaw step significantly reduced the trapped volume. Under such conditions, the trapped volume was also independent of the lipid concentration, being similar to the value obtained prior to the freeze–thaw step. Data are shown for glucose, but the pattern was similar with methyl glucoside and sucrose. An additional obvious effect of the presence of a sugar was the absence of liposome aggregates even at lipid concentrations as high as 40 mM. Mutual adhesion was clearly much reduced under such conditions. Since production of large vesicles was also drastically reduced by sugars, vesicle–vesicle interactions may well be related to the formation of large vesicles. Only in the case of glycerol were a few giant vesicles observed following dialysis.

In contrast to the effect of the hydroxylic nonelectrolytes, the presence of electrolytes—particularly KCl or RbCl (and, under special circumstances, NaCl)—during the freeze–thaw step created conditions whereby the dialysis step became critical in the generation of very large vesicles. If LiCl was present during freezing and thawing, no aggregates were visible microscopically, and following dialysis, no large vesicles were apparent. The situation with NaCl and CsCl was intermediate; some aggregation of liposomes occurred during freezing and thawing, and some increase in the trapped volume occurred following dialysis. A high yield of large vesicles was obtained from NaCl solutions only at very high concentrations of lipid.

In addition to vesicle size changes, freezing and thawing in alkali chloride also caused agglutination, especially in the cases of KCl and RbCl. With this step, highly reticulated aggregates were generated, and subsequent dialysis yielded large encapsulated volumes as well as large numbers of giant vesicles. The striking differences among the alkali metal ion chlorides were investigated in some detail. In Figure 2 are shown population size distributions of liposomes generated by the standard procedure applied to lipid suspensions at 10 mM. In all cases,

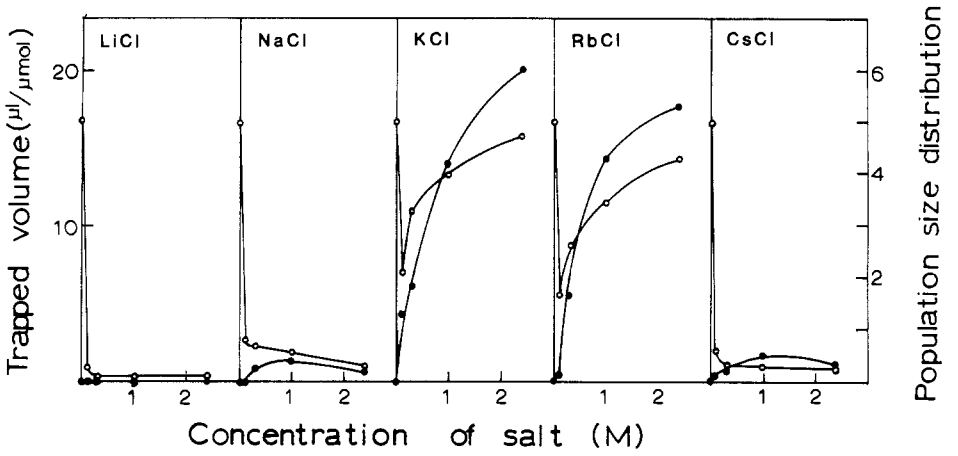

FIGURE 2: Effect of electrolyte concentration on liposome formation. The concentration of electrolytes during the freeze–thaw step was as given in the legend to Figure 1. Freezing and thawing were for three cycles. The trapped volume (○) and population size distribution of large liposomes (●) were determined after 2 days of dialysis against 10 mM Mops buffer.

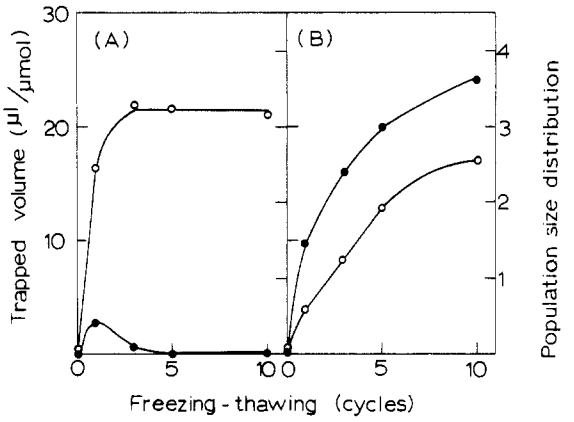

FIGURE 3: Effect on liposome formation of the number of freeze–thaw steps in water and in 1 M KCl. An egg yolk phospholipid dispersion (10 mM as phospholipid) was frozen and thawed in water (A) or in 1 M KCl solutions (B) for the number of times indicated. After dialysis against 10 mM Mops buffer, the trapped volume (○) and population size distribution (●) were determined.

Table I: Dialysis of Liposomes Preloaded with KCl[a]

lipid film hydrated	trapped volume (μL/μmol)	population size distribution
H_2O	0.42	0
1 M KCl	0.76	0.0037
2 M KCl	0.89	0.0031
3 M KCl	2.76	0.128
1 M KCl[b]	13.49	4.18

[a] Solvent was removed from 10 μmol of egg yolk phospholipid ($CHCl_3$ solution) under vacuum. The resultant lipid film was hydrated with 1 mL of either H_2O, 1 M KCl, 2 M KCl, or 3 M KCl, all containing 0.1 mM calcein. After brief sonication, samples were dialyzed for 2 days against 10 mM Mops buffer containing 0.1 mM calcein. Trapped volume and population size distribution were determined as described under Materials and Methods. [b] Liposomes containing 1 M KCl were frozen and thawed for three cycles before dialysis against 10 mM Mops buffer.

the presence of a low concentration of electrolyte during freezing and thawing drastically reduced trapped volumes (compare first point, which is zero concentration of added electrolyte, with subsequent points). In the absence of salt, the trapped volume was sizable, but no large vesicles were visible. In the cases of LiCl, NaCl, and CsCl, the only result of increasing concentrations was a decrease in the trapped volume. In the cases of KCl and RbCl, on the other hand, the initial reduction in trapped volume at a low concentration of electrolyte was followed by a very substantial increase as the solute concentration was increased. It is evident from the sharp rise in the population size distribution (right axis) that the presence of large and giant vesicles makes a substantial contribution to the rise in trapped volume. This increase in population size distribution with increasing solute concentration during freezing and thawing represents an increase in both average diameter and number of large vesicles.

Importance of Freezing and Thawing. The effects on trapped volume and large vesicle formation of the presence and absence of 1 M KCl during freezing and thawing are shown in Figure 3 as a function of the number of cycles of freezing and thawing. In the absence of solute, the trapped volume rose to its final value within three cycles. Few large vesicles were observed, and the number of these actually diminished after a few cycles. Similar behavior was reported previously (Oku et al., 1982). When 1 M KCl was present during freezing and thawing, the expected increase in trapped volume and number of giant vesicles was observed upon subsequent dialysis, but the effect of freezing and thawing became cumulative so that both parameters increased for at least 10 cycles. The consequences of freezing and thawing may thus be qualitatively different in water and electrolyte solutions (see Discussion).

One obvious consequence of freezing and thawing is to disrupt vesicles (Morris, 1981) so as to effect equilibration of the internal and external phases. The data in Table I show that freezing and thawing does more to generate large liposomes than simply loading sonicated liposomes with a high concentration of electrolyte prior to dialysis. Lipid films were swollen in solutions of differing KCl concentrations. The resultant liposomes were then sonicated to yield vesicles containing those KCl concentrations without freezing and thawing them. These vesicles were then dialyzed for 2 days against solutions of various compositions and osmolarity. The first four rows of the table reveal an increase in aqueous volume associated with vesicles as the concentration of entrapped KCl is increased. The effects are, however, smaller than those obtained when vesicles were subjected to freezing and thawing, as shown by the entry in the last row. It is clear from these results that vesicles loaded with solute by sonication, rather than by freezing and thawing, do become substantially larger upon dialysis in a manner which depends upon the internal concentration of solute. Vesicle fusion is one mechanism by which such growth could occur. The greater trapped volume and population size distribution of preparations subjected to

Table II: Production of Large Liposomes Requires an Osmolarity Decrease during Dialysis[a]

expt	medium during dialysis	medium during assay	trapped volume (μL/μmol)	presence of large liposomes
1		Mops buffer	3.7	no
2	0.3 M KCl	Mops buffer	4.8	no
3	0.3 M NaCl	Mops buffer	3.9	no
4	Mops buffer	Mops buffer	7.2	yes
5		0.3 M KCl	7.6	no
6	0.3 M KCl	0.3 M KCl	8.4	no

[a] Liposomal suspension (10 mM as phospholipid) containing 10 mM Mops buffer, 0.3 M KCl, and 0.1 mM calcein was frozen and thawed for three cycles. After freezing and thawing, the trapped volume and size were directly assayed with Mops buffer (experiment 1) or with 0.3 M KCl solution (experiment 5). The first sample was diluted 25-fold with Mops buffer. Other samples were dialyzed against the solution indicated for 2 days. All dialysis solutions contained 10 mM Mops buffer and 0.1 mM calcein.

freezing and thawing, however, may have to do with the fact that such vesicles are already larger than the sonicated liposomes as a consequence of the freeze–thaw step. Other influences considered under Discussion may also be important.

Importance of the Dialysis Step for Generation of Large Liposomes. The data of Figure 2 show that large liposomes form even when the concentration of KCl present during freezing and thawing is as low as 0.3 M. To gain additional information (see Table II) on the importance of the dialysis step, as well as the external solute concentration, we dialyzed liposomes that had been frozen and thawed in 0.3 M KCl against various solutions. Liposomes subjected to such conditions of freezing and thawing were observed by light microscopy to be extensively aggregated. A 25-fold dilution with 10 mM Mops buffer dispersed these aggregates, but the same dilution with 0.3 M NaCl or KCl was without effect. In none of these instances did simple dilution generate large vesicles. The morphology and trapped volume of these aggregates were, furthermore, unchanged upon dialysis against either alkali metal chloride at 0.3 M. On the other hand, when the aggregates were dialyzed against 10 mM Mops buffer for 2 days, many large vesicles were observed, and the trapped volume increased substantially. Since simple dilution into this solution generates no large vesicles, it may be that the slower dialysis process precludes osmotic fragmentation of the liposomes.

The data of Table II also indicate that it is not the solute gradient per se that is important but the presence of an osmotic gradient; dialysis of liposomes in 0.3 M KCl against the same concentration of NaCl was without effect on vesicle morphology. The difference between NaCl and KCl (see, e.g., Figure 2) is therefore ascribed to the freeze–thaw step.

Row 5 of Table II gives the encapsulated volume of liposomes after freezing and thawing, while row 6 gives the corresponding datum for vesicles that were, in addition, dialyzed against 0.3 M KCl. Since calcein trapped within the vesicles was assayed in the presence of 0.3 M KCl in both cases, there was no osmotic stress during the assay. In both cases, the encapsulated volume was rather large, so freezing and thawing increases lipid hydration without giving rise to vesicles that we would classify as large.

The dramatic size change in vesicles subjected to the procedure described is illustrated in the photographs of Figure 4. Most of the vesicles in the original sonicated suspension are not visible. As may be seen from Figure 4a, vesicles that have been frozen and thawed in KCl solution, and then dialyzed, are mostly large, and few multilayered vesicles are apparent. Vesicles subjected to the same treatment in the absence of KCl are small, as is shown by Figure 4b. Many of the vesicles present are too small to photograph, and those that are visible are quite dark under phase contrast, indicating a dense, presumably multilayered structure.

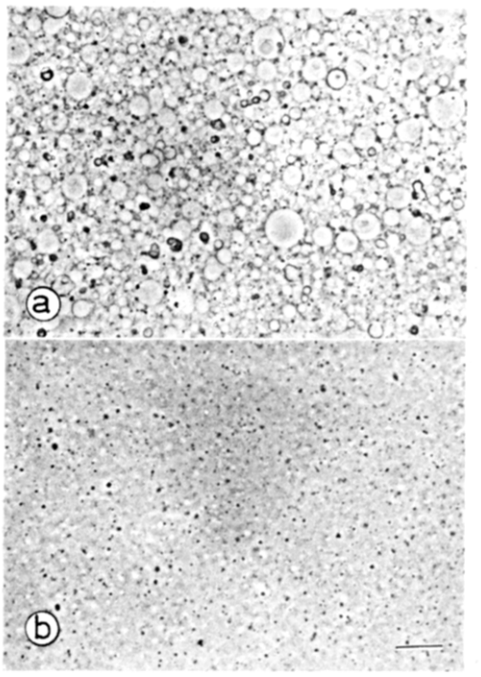

FIGURE 4: Large liposomes prepared with or without KCl. Egg yolk phospholipid dispersion (10 mM as phospholipid) was frozen and thawed for three cycles in the solution with (a) or without (b) 3 M KCl and dialyzed against 10 mM Mops buffer. Bar, 20 μm.

Table III: Effect of Calcium on the Formation of Large Liposomes[a]

salt	presence of Ca^{2+}: trapped volume (μL/μmol)	presence of Ca^{2+}: population size distribution	absence of Ca^{2+}: trapped volume (μL/μmol)	absence of Ca^{2+}: population size distribution
	10.5	0.05	14.4	0.02
LiCl	0.2	0	0.2	0.02
NaCl	2.6	0.10	2.2	0.20
KCl	12.5	5.88	10.4	3.97
RbCl	11.5	6.39	9.6	4.71
CsCl	8.0	3.52	1.0	0.19

[a] Liposome suspensions (10 mM as phospholipid) were frozen and thawed for three cycles with 1 M each of various salts, 10 mM Mops buffer, 0.1 mM calcein, and with or without 5 mM $CaCl_2$. In the absence of $CaCl_2$, 5 mM EDTA was added for protection against adventitious contamination by divalent cations. The trapped volume and population size distribution of large liposomes were determined after dialysis of the sample solutions against 10 mM Mops buffer containing 0.1 mM calcein, 0.02% NaN_3, and 0.01 mM EDTA.

Effect of Calcium Ion on the Hydration of Lipid in Alkali Metal Chloride Solutions. It has been found that phosphatidylserine bilayer membranes fuse upon treatment, first, with Ca^{2+} ions and, second, with EDTA (Düzgüneş et al., 1981), a process that may be related to natural membrane fusion (Wilschut et al., 1980). Since there were some indications that fusion might be involved in the generation of large vesicles under the conditions described here, the possibility of a synergistic effect of calcium ion was considered. Table III shows

Table IV: Effect of Electrolytes on the ζ Potential of Liposomes of Egg Phospholipids[a]

salt	ζ potential (mV)
	−43.1 ± 2.5
LiCl	−0.9 ± 0.5
NaCl	−5.5 ± 0.6
KCl	−7.3 ± 0.5
RbCl	−7.2 ± 0.7
CsCl	−7.7 ± 0.7

[a] Electrophoresis of liposomes (0.1 mM as phospholipid) was in solutions containing 1.0 mM Mops buffer (pH 7.2), 0.5 mM EDTA, and 0.1 M each of the indicated salts.

the effect of Ca^{2+} on the production of large vesicles. When present during the freeze–thaw step, this ion did indeed influence the population size distribution and trapped volume of liposomes generated by dialysis of lipid suspensions that had been frozen and thawed in 1.0 M solutions of the alkali metal chlorides. Although Ca^{2+} increased both the trapped volume and the population size distribution of liposomes formed from RbCl and KCl solutions, most noteworthy was its striking stimulation of both parameters in the case of vesicles formed from CsCl solution. Since the divalent cation was not present in the dialysis buffer, its effects must have been exerted during freezing and thawing or at the beginning of dialysis.

Interaction of Bilayer Membranes with Alkali Metal Ion Chlorides. It is apparent that liposome volume increases upon freezing and thawing and that large vesicles may appear upon subsequent dialysis. Since vesicle or membrane fusion may occur in one or both steps, membrane–membrane interactions are potentially of great importance in determining the extent of hydration in different electrolytes. These interactions were probed in several ways, the first being electrophoresis.

Table IV shows the ζ potentials of egg phospholipid vesicles in different electrolytes at 0.1 M. Small proportions of acidic lipids endow these liposomes with a small negative surface charge indicated by a significant negative potential in distilled water. As predicted from the double-layer theory (McLaughlin, 1977), the potential is drastically reduced in absolute magnitude by low concentrations of electrolyte. In contrast to that theory which predicts no differences among electrolytes of the same type, however, there is a striking variation, particularly in the case of LiCl. The potential of vesicles in the presence of this salt is so depressed that significant binding of Li^+ is indicated. If the potential in the presence of KCl is assumed to be the true value for a noninteracting electrolyte, then according to the Gouy-Chapman equation, 2% of the lipid molecules bound a Li^+ ion. (The lipid contains 2.1% negatively charged lipids, according to the ζ potential in KCl.) Eisenberg et al. (1979) have recently found that Li^+ binds tightly to phosphatidylserine membranes, and it may well be that Li^+ is attached predominantly to the negatively charged components of egg phospholipids. Na^+ also appears to bind to egg lipids; about 5 Na^+ ions are bound per 1000 lipid molecules at 0.1 M NaCl.

The lowest temperature to which a solution may be cooled before solvent and solute crystallize out is the eutectic temperature. Although there is some ambiguity in the literature about the eutectic temperature for LiCl solutions, at least one reference indicates that it may be as low as −80 °C (Seidell, 1965). This suggested that a reason for the lack of effectiveness of LiCl solutions in supporting formation of large vesicles was that these solutions did not become completely frozen in our dry ice bath. Since it was possible that there is another minimum at the other side of the series, at Cs^+, which might similarly account for the ineffectiveness of CsCl,

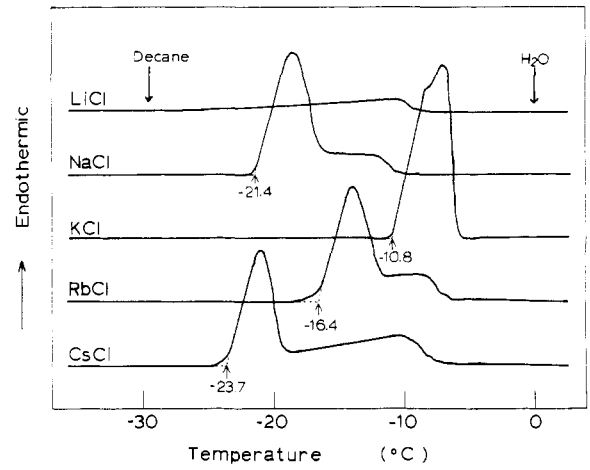

FIGURE 5: Differential scanning calorimetry of various electrolyte solutions. As indicated, the eutectic temperature is at the onset of the first endothermic event. The concentrations of the solutions were 3 M.

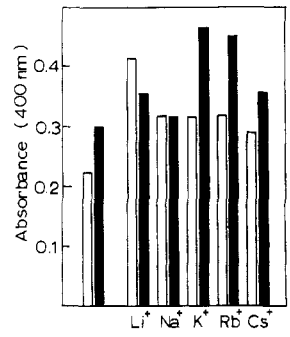

FIGURE 6: Turbidity of liposomes with or without electrolytes and effect of freezing and thawing. The turbidity of egg yolk phospholipid dispersions (0.2 mM as phospholipid) was measured with or without 1.0 M addition of various salts (as indicated) at 400 nm (open bars). The solid bars indicate the optical density after three cycles of freezing and thawing.

we determined its eutectic temperature as well as those of the solutions of the chlorides of Na, K, and Rb. Thermograms of these solutions are shown in Figure 5. The eutectic temperature is that of the beginning of the absorption of heat, i.e., the upward deflection. As may be seen, the eutectic temperatures of the two effective solutions, KCl and RbCl, are indeed above those of NaCl and CsCl, but the latter are by no means low enough that there would be unfrozen solution at dry ice temperatures. Our values for KCl and NaCl solutions agree with the literature values (−10.7 and −21.2 °C, respectively; D'Ans et al., 1962). We were unable to find literature values for the other two solutions. The eutectic temperature for LiCl does indeed seem to be very low, for we were unable to detect a transition in such solutions down to −50 °C.

For another indication of the influence of different electrolytes on membrane–membrane interactions, we measured turbidities of lipid suspensions. The data are presented in Figure 6. Turbidity was lowest in suspensions in water, an indication of the small average size of the vesicles present. Turbidity rose upon freezing and thawing, in agreement with an increase in average vesicle size found by trapped volume measurements. Initially, turbidity was highest in LiCl, probably because electrostatic repulsion is not sufficient to prevent formation of some aggregates, but curiously, it decreased upon freezing and thawing. Little change was seen in NaCl solution, but turbidity increased upon freezing and thawing the lipid suspensions in KCl, RbCl, and CsCl. The

largest change was seen with the former two electrolytes, those from which large vesicles formed upon dialysis. Some contribution to the turbidity increase must come from an increase in average vesicle size, at least in the case of KCl and RbCl (see below), but as revealed by direct observation under the microscope, freezing and thawing generates aggregates which must also contribute to an increase in turbidity.

Hydration of Different Lipids in Alkali Metal Chloride Solutions. The procedure described above for hydrating lipids can produce very large and, in most cases, presumably unilamellar lipid vesicles without the addition of other agents such as organic solvent or detergent. It would appear that all that is required of the lipid is that it can be sonicated to yield a vesicle suspension. Since the potential applications of such large vesicles are numerous and important, it was of interest to determine whether the same procedure generated large vesicles from lipids other than egg phospholipids. A variety of other compositions were tested, all being frozen and thawed in 1 M KCl 3 times and dialyzed against the standard buffer. The compositions were egg phospholipid–cholesterol (1:1), egg phosphatidylcholine, phosphatidylcholine–cholesterol (1:1), phosphatidylcholine–dicetyl phosphate (10:1), phosphatidylcholine–cholesterol–dicetyl phosphate (10:10:1), phosphatidylcholine–stearylamine (10:1), and phosphatidylcholine–cholesterol–stearylamine (10:10:1). The phosphatidylcholine was chromatographically pure, and the numbers in parentheses are mole ratios. Trapped volumes were between about 5 and 10 μL/μmol for the uncharged liposomes and between about 15 and 25 μL/μmol for the charged liposomes, excluding stearylamine-containing compositions for which the calcein-based volume assay is unsuitable. Giant vesicles were obvious in all of the preparations of charged liposomes although population size distributions were not determined. Such vesicles were also present as very large aggregates, and it was difficult to assess the proportion of large vesicles in comparison with the other compositions. It appears from this survey that there is no limitation on the composition of the lipid for the generation of large vesicles by the procedure described here, although it is quite possible that different compositions may not exhibit the same dependence of hydration on the kind of electrolyte as do egg phospholipids.

Although most of the experiments described here involved dialysis against dilute buffer (plus, of course, calcein), this was largely for convenience and is not necessary. Many applications of liposomes require physiological concentrations of electrolyte; the hydration procedure described is effective in generating large liposomes under those conditions as well.

Discussion

In general, when anhydrous lipids are presented with an aqueous phase and allowed to hydrate to equilibrium, the amount of aqueous phase imbibed is far less than that presented unless there is a net repulsive force between bilayers. If there is little or no bilayer repulsion, an attractive force, due to van der Waals interactions, will hold the bilayers at an equilibrium separation dictated by the overall balance of forces and their dependence upon separation (Rand, 1981). In such cases, the result of dispersing lipid in an aqueous phase is the familiar multilayered liposome. We have explored alternative methods of hydrating vesicles that have the potential of overcoming attractive forces and giving rise to large unilamellar vesicles. The procedure we find most effective for generating vesicles with a large ratio of volume to area involves sonication in water, addition of electrolyte, freezing and thawing, and dialyzing against a hypoosmotic solution. These four steps will be considered in that order.

The first step, sonication, constitutes the initial hydration step. As is well-known, sonication produces small, unilamellar vesicles in which hydration is complete; all membranes are separated by at least several hundred angstroms of aqueous phase (Huang, 1969). The dispersion should not revert to multilayered liposomes unless the membranes are brought close together under conditions in which van der Waals forces become important and in which membranes break and can reform into multiple concentric layers.

The second step—addition of electrolyte—is an essential prelude to the third and fourth steps. It seems unlikely that any important changes occur at this point because it only involves adding sufficient electrolyte to bring the concentration to 1 M or higher. Water would be osmotically extracted from the vesicles, and the edges of the resultant flattened disks might become unstable because of small radii of curvature; however, it appears unlikely that anything more than an increase in vesicle permeability would occur.

The third step, freezing and thawing, on the other hand, is by no means a simple process. A number of influences come into play, and it is not yet clear which of these are most important for the large difference in response of vesicles to different electrolytes. In general, most substances are largely excluded from ice, so that as ice crystals form, the remaining solution becomes more and more concentrated (Meryman, 1966). In the case of the liposome suspensions we have studied most, vesicles will be forced together in an electrolyte solution that eventually becomes saturated. The interior of the vesicles will be hypoosmotic and will thus continuously lose water so that the vesicles could become flattened enough to rupture. Such an effect would, of course, be smaller, the higher the concentration of electrolyte initially.

The electrolyte solutions studied here all form eutectics; as the temperature is lowered, an increasingly concentrated liquid phase forms which, at a particular composition, crystallizes out as ice and an electrolyte phase, the latter being either hydrated or not hydrated (anhydrous). LiCl and NaCl form cryohydrates with between five and two water molecules associated with each equivalent of electrolyte. The remaining alkali metal ions have radii close to that of the water molecule; they do not strongly perturb ice structure, and their chlorides crystallize out without water of hydration (Meryman, 1966). Since the eutectic temperature of LiCl is close to that of dry ice, it is quite possible that such solutions have not come to equilibrium in our freezing regimen. If complete solidification is required for the subsequent dialysis step to be effective in production of large vesicles, then this may explain the absence of such vesicles in LiCl solutions; however, such an explanation cannot account for the differences among the other electrolytes. On the other hand, lack of complete freezing is a likely explanation of the inhibitory influence of sugars. Glycerol, for example, is well-known as a cryoprotectant; 30% of a 25% solution such as was used in the experiment of Figure 1 does not freeze at any temperature (Luyet, 1970). Why a few large vesicles are generated in such solutions remains unexplained, however.

The increasing concentration of electrolytes caused by water crystallization will tend to dehydrate the membrane itself as well as extract water from the internal compartment. In the case of the synthetic saturated phosphatidylcholines, which have been well studied, the effect of dehydration is to raise the gel to liquid-crystal phase transition temperature and lower the enthalpy of that transition (Chapman et al., 1967). Actual measurements in moderately concentrated alkali chloride solutions reveal little change in either of these thermodynamic

parameters (Simon et al., 1975; Chapman et al., 1977). In neither case is it obvious how the electrolyte, through an effect on water activity, could influence the hydration of lipids in such a way that it would account for our observations.

Although alkali metal cations have not been noted for strong binding to phospholipids, such a direct influence is possible and could become substantial at the high concentrations that occur during freezing. Indeed, microelectrophoresis indicated substantial binding to egg phospholipids of Li^+ at a concentration of 0.1 M, and it is possible that at concentrations of several molar, enough ions bind to give the membranes a positive charge. Microelectrophoresis measurements are considered unreliable at high electrolyte concentrations because of heating effects, so this possibility could not be tested directly. If bilayer vesicles in LiCl actually acquire a positive charge, electrostatic repulsion might prevent their interaction and enlargement during the freezing step. It will be recalled that the turbidity of vesicle suspensions in LiCl actually decreased upon freezing and thawing, indicating that the scattering unit had become smaller. Na^+ also binds to egg phospholipid bilayers, but less strongly than Li^+, and turbidities remained unchanged upon freezing and thawing in NaCl. Turbidities of vesicles rose upon freezing and thawing in KCl, RbCl, and CsCl solutions, and electrophoresis indicated similar interactions (or lack thereof) of these salts with phospholipids. Nevertheless, large vesicles could be obtained from CsCl solutions only if $CaCl_2$ was included. The latter would absorb at least weakly, reduce the negative surface potential, and favor vesicle aggregation. It is perhaps noteworthy in this connection that aggregates were formed following freezing and thawing in KCl solutions but not in LiCl solutions. If fusion during the dialysis step is necessary for the generation of large vesicles, adhesion (reflected in the formation of aggregates) would certainly be a prerequisite. The important question of why aggregation is so extensive in KCl and RbCl solutions following freezing is, however, still unanswered.

Whatever the differential effects of the alkali metal chlorides on vesicles that have been frozen and thawed, it is clear that freezing will cause vesicle disruption by ice crystals and, with the possible exception of LiCl, by salt crystals as well. Vesicle flattening because of extraction of water may also be significant. The latter should generate broken edges of flattened vesicles which may anneal into larger vesicles as the solution thaws again. In any case, the vesicle interior should, at some point, acquire electrolyte from the external phase. As more ice melts, the external phase would become dilute and the resultant reduction of its osmolarity should cause some "osmotic" inflation of vesicles even prior to the dialysis step, where such a stress is deliberately imposed. The general effects of freezing and thawing are thus readily interpreted, but distinguishing among the specific influences of different electrolytes appears to require additional information on the properties of lipids in solutions of high electrolyte concentration.

The final step in the procedure we have investigated for full hydration of lipid is dialysis against an electrolyte at a lower concentration than that present in the prior step. The data of Table II indicated that this step does not depend upon the kind of electrolyte present outside the dialysis bag, so that it is likely that an osmotic flux of water into vesicles, independent of the nature of the species creating the osmotic gradient, is important for the appearance of large vesicles during this step. Some of the vesicles observed are so large that it is unlikely that correspondingly large flattened vesicles could have been generated by freezing and thawing. The conclusion thus seems inescapable that vesicle fusion occurs during dialysis. The mechanism is presumably very simple: adherent or tightly packed vesicles swell and rupture into one another. Since the yield of large vesicles is far from 100%, the small vesicles that remain may be those that did not find a partner to reseal with at the time of rupture. It should be recognized, however, that we have no direct evidence for vesicle fusion.

If there is significant fusion during dialysis, then a substantial influence of the aqueous phase would be expected, independent of the solution outide the bag. Vesicles must come into contact to fuse, and any electrolyte that facilitated contact would promote formation of large vesicles by such a mechanism. Two interactions between bilayers may be considered, electrostatic repulsion and van der Waals attraction (Parsegian, 1975). The lipids we have used contain only small amounts of acidic components, but this is sufficient to generate a ζ potential of nearly −50 mV in water. Repulsion between such vesicles will be substantial. Even if such vesicles were swelling osmotically, they would be very unlikely to come into close enough contact to fuse. The presence of an inert electrolyte during dialysis has two effects on intermembrane interactions; both the electrostatic repulsive force and the van der Waals attractive forces are reduced. The extent of reduction of both forces is expected to differ among the alkali metal ion salts. Electrostatic forces are reduced the same amount by ions of the same valence type, but differences in binding of the alkali cations are significant and will thus give rise to net differences in electrostatic interaction. We suspect that Li^+ binds strongly enough to egg phospholipid membranes that, at several molar concentration, the membranes could acquire a positive charge. The resultant potential would, of course, be small at such electrolyte concentrations but may still be sufficient to prevent membrane contact. The situation is less clear in the case of the other ions. Binding of Na^+ ions is relatively slight and that of Cs^+ appears negligible, yet the yield of large vesicles from these two solutions upon dialysis of vesicles suspended in NaCl and CsCl is very low.

van der Waals interactions of transient dipole-induced dipole (London dispersion) type will also influence the degree of contact of membranes. These forces depend upon the polarizability of the membrane and diminish as the polarizability of the intervening aqueous phases is increased. The latter is related to the refractive index (Nir, 1977) which, with the exception of similar values for Na^+ and K^+, increases with atomic weight from Li^+ to Cs^+ (Weast, 1980). Thus, membranes should experience strongest van der Waals attraction in solutions of LiCl and weakest interactions in solutions of CsCl. Furthermore, the polarizability of KCl and NaCl solutions is practically identical, and van der Waals forces should be very similar in these solutions. It would therefore appear that if contact between membranes and, consequently, vesicle fusion depend upon dispersion forces, we would find the largest vesicles formed in lipid dispersions that were dialyzed from LiCl and the smallest in those dialyzed from CsCl solutions. This was not found. On the other hand, electrostatic repulsive forces decreased in the same direction, so it is possible that a subtle balance of interactions occurs such that ions in the middle of the alkali metal ions series, namely, K^+ and Rb^+, constitute solutions wherein overall membrane–membrane attractive forces are, in fact, strongest. An accurate evaluation of the forces between phospholipid bilayer membranes in solutions of the alkali metal chlorides is obviously difficult, but the fact remains that vesicles that have been frozen and thawed in solutions of KCl and RbCl are highly aggregated. The empirical evidence thus favors the assumption that the balance

of forces is such that membrane–membrane contact is most favorable in these solutions. Since osmotic swelling of vesicles in close contact could well lead to their fusion, the finding of giant vesicles upon dialysis of a suspension of lipid in KCl and RbCl solutions is not surprising. The extent of vesicle aggregation in such solutions remains to be rationalized.

We have found significant differences in the interactions among H_2O, phospholipid, and alkali metal chlorides, and some of these can give rise to substantial differences in the formation of liposomes. We hope that this will lead to even better procedures for the formation of large vesicles, as well as to a more complete understanding of the basis of lipid hydration and liposome formation.

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Registry No. LiCl, 7447-41-8; NaCl, 7647-14-5; KCl, 7447-40-7; RbCl, 7791-11-9; CsCl, 7647-17-8; Ca, 7440-70-2; glycerol, 56-81-5; cholesterol, 57-88-5; dicetyl phosphate, 2197-63-9; stearylamine, 124-30-1.

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