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FORMATION OF GIANT LIPOSOMES FROM LIPIDS IN CHAOTROPIC ION SOLUTIONS

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Liposomes with diameters in the range of 10–20 μm, evidently uni- and oligolamellar, were generated upon removal of sodium trichloroacetate by dialysis or dilution from a solution containing egg phospholipids and sodium trichloroacetate. At room temperature, giant liposomes were formed only from concentrations of sodium trichloroacetate which induced the transformation of phosphatidylcholine from the lamellar to the micellar phase. The yield of giant liposomes increases with increasing phospholipid concentration in the sodium trichloroacetate solution. Inclusion of a freeze-thaw step reduced the concentration of sodium trichloroacetate needed to generate giant liposomes to less than 0.1 M and substantially lowered the minimum lipid concentration. Apparently sodium trichloroacetate is concentrated during freezing to above the critical concentration which solubilizes phospholipids. The micelles, so generated, also become concentrated so that giant liposomes form upon thawing as the melting ice dilutes the trichloroacetic acid and the micellar phase reverts to the lamellar phase. Other chaotropic solutions, such as guanidine-HCl and urea, which did not solubilize lipids at room temperature, also generated giant liposomes when their solutions, containing dispersed lipids, were frozen, thawed and then dialyzed to remove the solutes. In the case of chaotropic anions such as thiocyanate and nitrate, potassium salts are more effective than sodium salts.

Introduction

Liposomes have been widely used as models for natural membranes [1], microcapsules for delivery of drugs into cells [2,3] and tools for microinjection [4]. Homogeneous, large unilamellar vesicles can be desirable for such purposes. A number of investigators have developed methods for preparing unilamellar vesicles as large as about a micron in diameter, using detergents or organic solvents [5], and several methods exist for preparing cell-sized, unilamellar vesicles [6–9] although there are some limitations to their application. In this paper, we describe a new method for generating giant, uni- and oligolamellar vesicles from lipid in solutions of strong chaotropic agents.

Chaotropic ions are well known to disturb water structure [10], and they may perturb lipid bilayers in a way different from detergents. Although there has been one report of guanidine thiocyanate solubilizing human erythrocyte ghost membranes [11], it has only recently been shown that lipids are actually solubilized in chaotrope solutions. We found that concentrated solutions of trichloroacetate and tribromoacetate dissolve choline containing phospholipids as micellar solutions [12]. Since chaotropes are highly water-soluble and have relatively low affinities for lipids, their removal

Abbreviations: Mops, 4-morpholinepropanesulfonic acid; Docs, *N*,*N*′-dioctadecyloxacarbocyanine-*p*-toluenesulfonate; calcein, 4′,5′-bis[*N*,*N*-bis(carboxymethyl)aminomethyl]fluorescein; egg PC, egg yolk phosphatidylcholine Type V-E; egg PL, egg yolk phosphatidylcholine Type IX-E.

should be easier than that of detergents.

We also describe here the application of freeze-thawing to generation of giant liposomes from chaotropic agent solutions. Freezing causes concentration of lipid and chaotropic agent by their exclusion from bulk ice, reducing substantially ($> 10\times$) the concentration of trichloroacetate required. The procedure is similar to that previously described involving KCl or RbCl [13].

Materials and Methods

Chemicals. Egg yolk phosphatidylcholine (Type V-E and IX-E), dicetyl phosphate, stearylamine and cholesterol were purchased from Sigma Chemical Company, St. Louis, MO. Bovine brain phosphatidylserine was purchased from Avanti Biochemicals Inc., Birmingham, AL. *N,N'*-Dioctadecyloxacarbocyanine-*p*-toluenesulfonate (DOCS) was from Eastman Organic Chemicals (Rochester, NY), and calcein was from Hach Chemical Company (Ames, IA). All lipid preparations except Type IX-E egg yolk phosphatidylcholine, which is about 60% phosphatidylcholine and about 30% phosphatidylethanolamine, gave a single spot on silica gel thin layer chromatography. Sodium or potassium trichloroacetate was prepared by neutralizing trichloroacetic acid with the appropriate hydroxide.

Preparation of liposomes. A lipid film, prepared by evaporation of a chloroform solution, was held in vacuo for more than 1 h, then hydrated with 10 mM Mops buffer, pH 7.2/0.1 mM calcein. After 1 min sonication of the lipid suspension at maximum power in a probe-type apparatus (Biosonic IV), the resultant suspension was mixed well with various amounts of sodium trichloroacetate, or other chaotropic agents, containing 10 mM Mops buffer/0.1 mM calcein. The lipid/chaotropic agent solution (0.3 ml final volume) was dialyzed for 2 days against 10 mM Mops buffer/0.1 mM calcein/0.01 mM EDTA/0.02% NaN_3. In some experiments the lipid/chaotropic agent solution was frozen in a solid CO_2-acetone bath, and thawed at room temperature for three cycles prior to dialysis.

Turbidity measurement of liposome suspension. Liposomes from egg yolk phospholipids were prepared in water and briefly sonicated in a probe-type sonicator. Samples containing liposomes (0.25, 0.5 or 1.0 mM as phospholipids) and various amounts of sodium trichloroacetate were mixed well, after which some were subjected to one cycle of freeze-thawing. Liposome turbidity was measured at 400 nm after the samples were incubated for 30 min at room temperature. Turbidity would be affected not only by the size of the scattering particles but also by the refractive index of the medium. Turbidity depends upon the square of the difference between the medium and particle refractive indices. In order to eliminate this influence we multiplied the measured turbidities by the factor of $(n_l - n_w)^2/(n_l - n_m)^2$, where n_l, the refractive index of lipid, was taken as 1.46 [14], n_w, that of water, is 1.333 and n_m, the medium refractive index, was taken from the table of the refractive index of trichloroacetic acid [15]. Although the last value is an approximation, comparison with our measured value for sodium trichloroacetate indicates a maximum error of $< 20\%$. To facilitate comparison of different suspensions we also normalized turbidities to 1.0 for the suspension in pure water.

Determination of marker trapped within liposomes. The encapsulation capacity of liposomes was determined as described elsewhere [16] using the fluorescent compound, calcein, as a marker of the internal aqueous volume. In brief, 20 μl of the sample solution, which contained 0.1 mM calcein, was diluted with 480 μl 10 mM Mops, pH 7.2, and the fluorescence intensity was measured before and after addition of 2 μl 10 mM $CoCl_2$, which quenched the fluorescence of calcein outside the liposomes. Background fluorescence was measured after lysing the liposomes with 25 μl 10% Triton X-100. Another 20 μl of sample solution was used for assay of phosphorus content by a modification of the Bartlett procedure [17].

Because of the possibility that marker may not be at the same concentration internally as externally after operations such as freeze-thawing or dialysis, and that marker may not equilibrate after 2 days dialysis, we express the results in terms of amount of calcein rather than volume of calcein solution. We choose the units of 10^{-10} mol/μmol phospholipid because this is numerically identical with the trapped volume in μl/μmol in the case when internal and external marker concentrations are actually equal.

Determination of population size distribution of liposomes. Population size distribution was determined according to a method described elsewhere [13]. In brief, a sample diluted to 1 mM as phospholipids was applied to a microscope slide and sealed under a cover glass with vaseline, such that the thickness of the sample was about 50 μm. Using a video monitor, large vesicles in random fields of 80 μm width were tabulated into classes of 5–10, 10–15, 15–20 and so forth, μm in diameter. We then calculated the sum of the number in the first class plus twice the number in the second class plus 3-times the number in the third class, and so forth. The average of this weighted sum of a minimum of 15 fields is the population size distribution. When a 1 mM solution contained too many vesicles to count conveniently, it was diluted and the vesicle sum was corrected accordingly. This parameter is approximately equal to the number of about 10 μm vesicles occurring in a $1 \cdot 10^{-4}$ μl volume of lipid at 1 mM.

Electron microscope. Liposomes were placed on a copper grid previously coated with carbon film. The grid negatively stained with 2% phosphotungstate was examined under an electron microscope (JEOL 100-CX).

Results

Solubilization of lipids by trichloroacetate and giant liposome formation

Trichloroacetate is a potent chaotropic agent, which at high concentrations of neutral sodium and potassium salts solubilizes some phospholipids as micellar solutions [12]. Optically clear solutions of phospholipids are obtained by adding 3 M sodium trichloroacetate to either dry lipid or liposome suspensions. Fig. 1a and b show photomicrographs of phospholipids consisting of egg PL plus a fluorescent marker, DOCS, at a 100 : 4 molar ratio in 3 M sodium trichloroacetate. Under phase optics, almost nothing is visible in such solutions (Fig. 1a). Fig. 1b is the fluorescence microscopic image of the same field as shown in Fig. 1a. The sample exhibited uniform fluorescence.

Other photomicrographs in Fig. 1 show preparations like those of a and b after dialysis for 2 days against 300 mM sodium trichloroacetate (c, d) or 10 mM Mops buffer (e, f). After dialysis

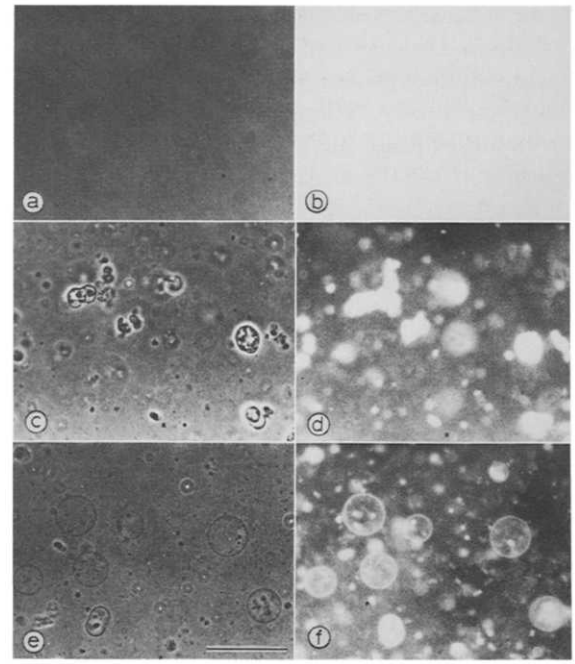

Fig. 1. Photomicrographs of giant liposomes generated from 3 M sodium trichloroacetate. Egg PL (20 μmol) and a fluorophore, DOCS, (0.8 μmol) were dissolved in 1 ml of 3 M sodium trichloroacetate, and dialyzed against 10 mM Mops, pH 7.2, buffered 300 mM sodium trichloroacetate solution (c, d) or 10 mM Mops buffer, pH 7.2, (e, f) for 2 days. Each sample was diluted 5-fold with 3 M sodium trichloroacetate (a, b) or dialysis buffer and observed under the phase microscope (a, c, e) or fluorescence microscope (b, d, f). Bar: 50 μm.

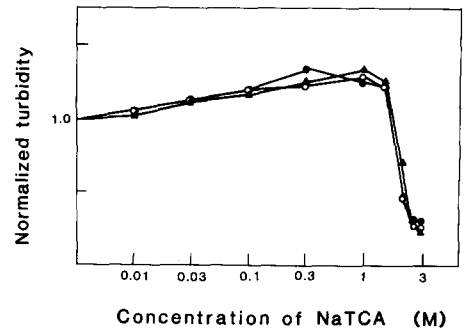

Fig. 2. Turbidity of phospholipids in trichloroacetate solutions of different concentrations. Egg PL liposomes were sonicated briefly and diluted with 10 mM Mops buffer, pH 7.2/0.5 mM EDTA and the concentrations of sodium trichloroacetate indicated in the Figure. The final concentrations of phospholipids were 0.1 mM (●), 0.25 mM (○) and 0.5 mM (▲). Turbidity measured at 400 nm was normalized to compensate for the effect of the refractive index of the sodium trichloroacetate solution as described in Materials and Methods. Turbidities at zero sodium trichloroacetate concentrations were 0.23 (0.1 mM), 0.57 (0.25 mM) and 1.00 (0.5 mM).

against dilute sodium trichloroacetate some fluorescent objects of unknown structure appeared (c, d). As can be seen in e and f, further removal of sodium trichloroacetate generates numerous uni- or oligolamellar liposomes with diameters of 10–20 μm, although many small vesicles are also present. Under an electron microscope, we observed extended sheets with one or a few layers. We presume that these sheets were parts of giant liposomes, but because of their large size and apparent ease of distortion during drying on the grid, they do not seem to be very satisfactory objects for electron microscopy.

Fig. 2 shows the turbidity of liposome dispersions in the presence of various concentrations of sodium trichloroacetate. Such briefly-sonicated liposomal suspensions became optically clear at once when high concentratoins of sodium trichloroacetate were added. Absorbance, determined after 30 min incubation at room temperature, remained constant for at least a day. When a liposome solution (0.75 mM as phospholipid) containing 3 M sodium trichloroacetate was diluted 3-fold

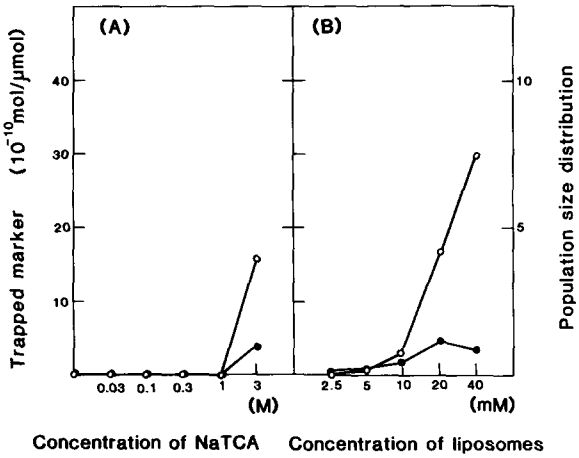

Fig. 3. Formation of giant liposomes from 0–3 M sodium trichloroacetate solutions. A. Liposomes prepared from egg PL were mixed with sodium trichloroacetate solutions of the indicated concentrations and dialyzed against 10 mM Mops buffer, pH 7.2/0.1 mM calcein/0.01 mM EDTA/0.02% NaN_3 for 2 days (final concentration of phospholipids was 20 mM). Trapped marker (●) and population size distribution (○) of the resultant liposomes were then determined according to the methods described in Materials and Methods. B. As indicated in A except that the sodium trichloroacetate concentration was held constant at 3 M and the lipid concentration was varied as shown.

with buffer, the absorbance of the resulting solution was much lower than that of a phospholipid solution (0.25 mM as phospholipid) originally made to contain 1 M sodium trichloroacetate. The absorbance of the latter sample remained low for at least 1 day (data not shown). On the other hand, once phospholipid was solubilized by 3 M sodium trichloroacetate, heating at 70°C for 1 h caused the solution to become turbid which is perhaps due to the increased strength of hydrophobic interactions with increased temperature. After a 5 h incubation at room temperature, such solutions clarified. No large liposomes were generated upon dialysis of these turbid solutions unless they were incubated at room temperature until they clarified. The dependence of absorbance of lipid solutions on the concentration of sodium trichloroacetate was independent of phospholipid concentration, at least from 0.05 to 0.5 mM. The concentrations of sodium trichloroacetate which clarify the lipid solutions appear to be critical to the generation of giant liposomes (see Discussion).

Giant liposome formation following dialysis of phospholipids and sodium trichloroacetate for 2 days is evaluated on the basis of these two parameters in Fig. 3A. Giant liposomes were generated by dialysis of solutions containing > 1 M sodium trichloroacetate. This concentration corresponds to that which induced the sharp drop in turbidity of liposome suspensions shown in Fig. 2. Fig. 3B shows the dependence of giant liposome formation on lipid concentration at 3 M sodium trichloroacetate. Large numbers of giant liposomes were generated only above about 10 mM lipid. The amount of marker trapped by liposomes formed at high concentrations of phospholipids is small relative to the population size distribution. This may be due to a limitation of the dialysis bag volume (liposomes formed at high concentration are indeed close-packed) and/or dilution of entrapped calcein during formation of giant liposomes (their calcein content is seen under the fluorescence microscope to be quite variable).

We also looked for giant liposomes following simple dilution of sodium trichloroacetate/lipid solution. A solution of 20 mM egg PL in 3 M sodium trichloroacetate was gently infused into 10 ml of 10 mM Mops or Mops-buffered saline at the rate of a few μl/min. The sodium trichloro-

acetate/lipid solution sank slowly in the buffer and giant liposomes were recovered at the bottom of the vessel.

Effect of freeze-thawing on generation of giant liposomes by sodium trichloroacetate

Freezing a solution concentrates solute(s) by eliminating water as ice and thus might be expected to reduce the concentration of sodium trichloroacetate necessary to solubilize lipids. That this is true was revealed by measurements of turbidity using low concentrations of lipids. Fig. 4 shows the absorbance change of liposome suspensions after freezing in solid CO_2/acetone and thawing at room temperature. Compared with samples not subjected to freeze-thawing, liposomes in relatively low concentrations of sodium trichloroacetate displayed significantly less turbidity. In contrast to samples treated with sodium trichloroacetate at room temperature, the reduction of turbidity by freeze-thawing was greater, the lower the concentration of liposomes. This may reflect the formation of smaller liposomes, perhaps as a consequence of smaller amounts of lipid being trapped together in interstices of the ice. For the experiments of Fig. 4, suspensions were subjected to freeze-thawing for one cycle; additional cycles produced little additional change.

Fig. 5 shows that large liposomes are produced by freeze-thawing in relatively dilute sodium trichloroacetate solutions; after three cycles of freeze-thawing and dialysis against 10 mM Mops, giant liposomes were generated even in 30 mM sodium trichloroacetate. Both trapped marker and population size distribution indicate the presence of abundant giant liposomes. The dependence of giant liposome frequency on lipid concentration under such conditions is much less than when a high sodium trichloroacetate concentration is used without freezing.

Next, vesicle formation from dilute sodium trichloroacetate solutions was characterized with respect to duration of dialysis. When the sample solution was simply diluted with buffer after freeze-thawing (zero time), numerous giant liposomes were generated, but the amount of trapped marker was rather low, since the dilution buffer did not contain calcein. Thus, giant liposomes can

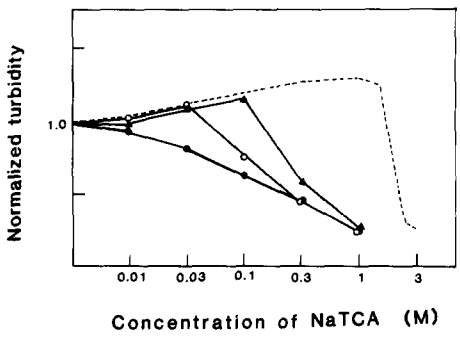

Fig. 4. Effect of freeze-thawing on the turbidity of lipid suspensions in the presence of sodium trichloroacetate. Egg PL suspensions were prepared by brief sonication and diluted with 10 mM Mops buffer, pH 7.2/0.5 mM EDTA and various amounts of sodium trichloroacetate. The final concentrations of phospholipids were 0.1 mM (●), 0.25 mM (○) and 0.5 mM (▲). The turbidity of freeze-thawed lipid suspensions was measured at 400 nm after 30 min at room temperature. Turbidities were normalized to constant refractive index and lipid concentration as described in Materials and Methods. Turbidities at zero sodium trichloroacetate concentrations were 0.27 (0.1 mM), 0.61 (0.25 mM) and 1.06 (0.5 mM), respectively. The dotted line gives the expected behavior of all concentrations if not freeze-thawed (average values from Fig. 2).

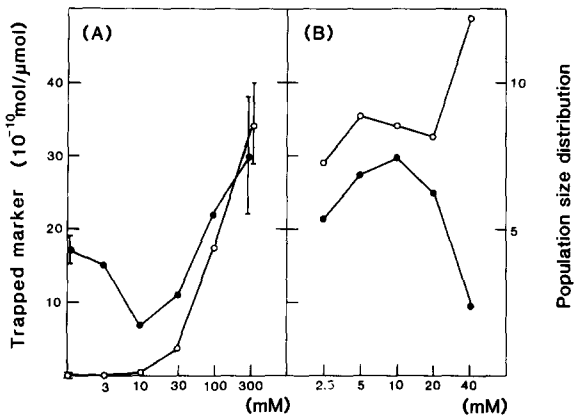

Fig. 5. Formation of giant liposomes from 0–300 mM sodium trichloroacetate solutions by inclusion of freeze-thawing. A. Liposomes (10 mM as egg PL) were suspended in sodium trichloroacetate solutions of the indicated concentrations, freeze-thawed for three cycles and dialyzed against 10 mM Mops buffer, pH 7.2/0.1 mM calcein/0.01 mM EDTA/0.02% NaN_3 for 2 days. Trapped marker (●) and population size distribution (○) of the resultant liposomes were then determined. The standard errors of five experiments are marked at 0 and 300 mM. B. The procedures were as in A except that the sodium trichloroacetate concentration was held constant at 300 mM and the lipid concentration was varied as indicated.

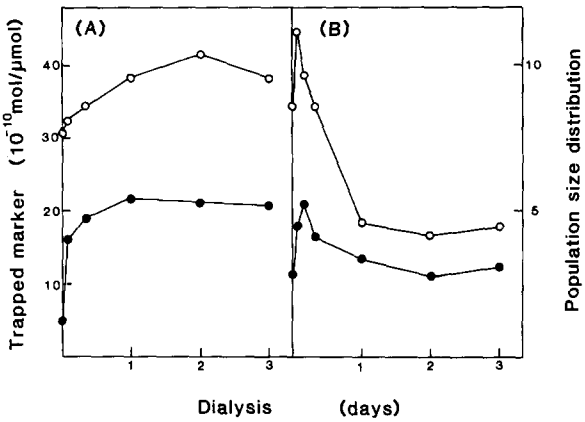

Fig. 6. Dependence of giant liposome formation upon duration of dialysis. A. Egg PL liposomes, final concentration 10 mM, were mixed well with 300 mM sodium trichloroacetate/0.1 mM calcein, and freeze-thawed three times. The samples were then dialyzed against 10 mM Mops buffer, pH 7.2/0.1 mM calcein/0.01 mM EDTA/0.02% NaN_3 for the times indicated. Samples were diluted 25-fold with 10 mM Mops buffer, pH 7.2, for measurement of trapped marker (●) and population size distribution (○). Samples at zero time were only diluted with buffer before measurement of both parameters. B. As A, except that dialysis and dilution buffers contained 0.15 M NaCl.

TABLE I

GENERATION OF GIANT LIPOSOMES OF VARIOUS LIPID COMPOSITIONS

Liposomes composed of various lipids were prepared in 10 mM Mops buffer, pH 7.2/0.1 mM calcein/0.01 mM EDTA, and sonicated briefly. After addition of sodium trichloroacetate (final concentration of sodium trichloroacetate was 300 mM and final concentration of lipid was 10 mM as phosphatidylcholine or phosphatidylserine), 0.3 ml of each sample was freeze-thawed three times and dialyzed against the same buffer + 0.02% NaN_3 for 2 days. Trapped marker and population size distribution were measured as described in Materials and Methods. The calcein assay is not compatible with positively-charged liposomes.

Lipid composition	Trapped marker (10^{-10} mol/μmol)	Population size distribution
Egg PC	11	4.0
Egg PC/cholesterol (1 : 1)	13	4.2
Egg PC/dicetyl phosphate (10 : 1)	15	4.7
Egg PC/stearylamine (10 : 1)		6.8
Bovine brain phosphatidylserine	21	9.2

be produced without dialysis, however, it should be noted that the vesicles are subjected to hypo-osmolarity both during thawing and dilution, so, there was still opportunity for osmotic inflation to occur.

Since liposomes in physiological solutions are desirable for many applications, we examined the formation of giant liposomes during dialysis against saline for up to 3 days. As shown in Fig. 6B, during the first several hours, many giant liposomes were generated, however, after 1 day of dialysis, the population size distribution decreased by half. The remaining giant liposomes were rather stable for at least 3 days in salt solutions.

We also examined whether giant liposomes would be produced by this procedure even from phospholipids which are not dissolved in 3 M sodium trichloroacetate. Table I shows the results of varying the lipid composition. Liposomes of egg PC and cholesterol (1 : 1 as molar ratio) which are

TABLE II

FORMATION OF GIANT VESICLES BY FREEZE THAWING IN THE PRESENCE OF CHAOTROPES AND ALKALI CHLORIDES

Liposomes, 10 mM egg PL, were sonicated briefly and mixed with 2.4 M of the various solutes indicated except for the trichloroacetates and two alkali chloride samples which were used at 100 mM. After three cycles of freeze-thawing, samples were dialyzed against 10 mM Mops buffer/0.1 mM calcein/0.01 mM EDTA/0.02% NaN_3 for 2 days. Trapped marker and population size distribution were determined as described in Materials and Methods.

Solutes	Concentration (M)	Trapped marker (10^{-10} mol/μmol)	Population size distribution
None		17	0
Potassium trichloroacetate	0.1	22	5.2
Sodium trichloroacetate	0.1	15	4.4
KCl	0.1	7	1.3
NaCl	0.1	3	0
Urea	2.4	21	2.3
GuCl	2.4	17	6.2
KNO_3	2.4	15	3.4
$NaNO_3$	2.4	5	1.2
KSCN	2.4	—	6.0
NaSCN	2.4	—	0
KCl	2.4	11	5.5
NaCl	2.4	4	1.2

not dissolved in 3 M sodium trichloroacetate [12] became large after freeze-thawing in 300 mM sodium trichloroacetate followed by dialysis. The size and marker trappings were almost the same as those of liposomes composed of egg PC alone. Giant liposome formation was even more extensive with phosphatidylserine, which is also insoluble in 3 M sodium trichloroacetate. Thus, solubility in sodium trichloroacetate is not a requirement for giant liposome formation by the freeze-thaw dialysis procedure, although the mechanism may well differ from that which operates when complete lipid dissolution occurs.

Generation of giant liposomes by other chaotropic agents

Among a number of common chaotropic agent solutions examined, only tribromoacetate and trichloroacetate dissolved phosphatidylcholine [12]. It was thus of interest to compare formation of giant liposomes from solutions of chaotropes of different potency as well as from solutions of other electrolytes. The data obtained are shown in Table II. The freeze-thawing step was included in all cases. All chaotropes, except NaSCN, supported formation of giant liposomes. (Thiocyanate quenches calcein fluorescence so encapsulated marker measurements could not be done in such solutions.) It is of interest that there is a large difference between potassium and sodium thiocyanate. A similar but smaller effect was seen with the trichloroacetate and nitrate salts, and is consistent with a difference between the alkali metal chlorides reported previously [13].

Discussion

We have described several procedures for the preparation of liposomes which are extremely large and evidently uni- and oligolamellar. One procedure takes advantage of the fact that some phospholipids, such as phosphatidylcholine and sphingomyelin, dissolve as micelles in solutions of high concentrations of certain chaotropic ions, i.e., trichloroacetate and tribromoacetate. When the chaotropic agent is removed from such solutions – e.g., by dialysis — the lipids reform into bilayers as giant vesicles. Since reformation of bilayers during the removal of sodium trichloroacetate should depend upon the collision of lipid micelles, the size of generated liposomes should depend on lipid concentration, as shown in Fig. 3B. During dialysis of the sodium trichloroacetate there is a large reduction in osmolarity and it seems likely that liposomes formed under such conditions would continuously imbibe water, a factor that may well be critical for formation of giant vesicles.

An additional factor that may be critical in minimizing the proportion of multilayered vesicles in these preparations is that trichloroacetate binds to (with an affinity constant of about 1 M^{-1}) and confers a substantial negative charge upon phosphatidylcholine bilayers. The surface potential of phosphatidylcholine liposomes (as measured by microelectrophoresis [12]) is nearly −20 mV in 0.1 M sodium trichloroacetate, so that electrostatic repulsion may neutralize van der Waals forces that otherwise would lead to the formation of multilayered liposomes. In essence, then, the procedure transiently generates an anionic lipid.

Although we have not attempted to characterize completely the distribution of sizes of vesicles produced under the conditions described, very large numbers of vesicles with diameters in excess of 10 μm were generated. Both light and electron microscopic observations suggest that these liposomes are uni- and oligolamellar.

The proportion of the lipid that forms very large vesicles is difficult to evaluate from trapped volume measurements (see below). An alternative way to estimate the proportion of lipid in large vesicles is from the population size distribution. If we assume that the vesicles counted are unilamellar, then we calculate that a population size distribution of 10 would account for about 2% of the lipid present. Most of the lipid is therefore found in vesicles that cannot be accurately sized by light microscopy. These are visible as vesicles of a micron or so in diameter and although small in comparison with the giant vesicles present, would be described as large in typical liposome preparations.

Giant liposomes also formed on freeze-thawing and dialysis of lipid suspended in low concentration of sodium trichloroacetate. In this procedure, sodium trichloroacetate as well as lipids should become concentrated during freezing, leading to solubilization of the latter. At the thawing step,

the concentrated sodium trichloroacetate becomes diluted again from melted ice, and concentrated micelles would revert back to bilayers.

Freeze-thawing also enables the generation of giant liposomes from lipids which do not dissolve in sodium trichloroacetate, for example, phosphatidylserine, although the mechanism appears not to be the same as that in the case in which the lipid dissolves. We hoped to elucidate this issue by examining the extent to which other chaotropes support giant liposome formation by the freeze-thawing technique. It appeared reasonable that the disruption that occurs upon freeze-thawing of vesicles [18] would be accompanied by exposure of hydrophobic edges of membranes. In the presence of chaotropic agents, such broken edges should be stabilized, increasing the probability of the annealing of fragments into larger structures [19] which, upon dialysis against hypotonic buffer would be osmotically inflated into giant liposomes. Thus, the difference between lipids that dissolve and those that do not may just be in the extent of disruption prior to the annealing stage. Although the pattern of activity shown in Table II generally favors this hypothesis, there are clear exceptions. KCl, for example, is much more effective than $NaNO_3$, although the latter is chaotropic while the former is not. This and the difference between sodium and potassium salts indicate that factors other than chaotropic activity are important.

The formation of giant liposomes was characterized by two parameters, one equal to the amount of trapped marker and another related to the number of large vesicles/unit volume. These parameters provide a useful guide for evaluating the efficiency of different conditions with respect to the production of giant liposomes. Nevertheless, trapped calcein measurements of frozen and thawed vesicles, even after dialysis for 2 days, should be viewed with caution. We now know that calcein is very impermeant and we suspect that calcein concentrations, which may change during freeze-thawing [16] and hypotonic dialysis, do not equilibrate even after 2 days dialysis. For example, calcein is far less permeant than glucose (Oku, unpublished data). Hence, we have avoided referring to this measurement as a trapped volume measurement, a restraint which should have been, but was not, exercised previously in reporting differential effects of alkali metal cations [13]. Such effects on the population size distribution are genuine; however, as effects on trapped volume determined by calcein assay, they are not as readily interpretable as we originally thought. In particular, if one uses high concentrations of lipids, many of the giant vesicles generated by present procedures contain rather low concentrations of calcein as observed under the fluorescence microscope. In these cases expression of encapsulated marker in terms of trapped volume actually underestimates the volume of the vesicles.

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