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LIPID MIXING DURING FREEZE-THAWING OF LIPOSOMAL MEMBRANES AS MONITORED BY FLUORESCENCE ENERGY TRANSFER

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A new pair of fluorescence-energy-transferring probes, dansylphosphatidylethanolamine and dioctadecylindocarbocyanine, were incorporated separately into phospholipid vesicles to monitor intervesicle lipid mixing under various conditions. The transfer efficiencies of mixtures of sonicated vesicles labeled with 2 wt% donor dansylphosphatidylethanolamine (DnsPE) or with 1 wt% acceptor dioctadecylindocarbocyanine (DiI-C_{18}) were negligible, but increased to about 25% after the vesicles had been frozen in a solid CO_2/ethanol bath, thawed and diluted. The freeze-thaw-induced mixing of lipids between vesicles, signified by energy transfer, was dependent on lipid concentration and was promoted by 0.5–1.5 M KCl, 0.5 M potassium trichloroacetate and 5 mM sodium acetate (pH 4) and inhibited by 0.5 M LiCl, 0.5 M glycerol, 0.5 M sucrose, 0.15 M KCl and 0.15–1.5 M NaCl. These results support and complement previously reported measurements of the trapped volumes, turbidities and population size distributions of similarly treated liposomes. Comparison of the responses of paucilamellar vesicles with those of multilamellar vesicles suggests that lipid mixing during freeze-thawing can occur either during interaction of the outermost bilayers of vesicles or during interaction of all bilayers, possibly as a result of breakdown and reformation of bilayer structure.

Introduction

Attempts to improve methods for the preparation of large (5 μm or greater diameter) unilamellar liposomes have led to an analysis of the effects of freeze-thawing on phospholipid suspensions under a variety of conditions [1–3]. A standard procedure followed in our laboratory involves freezing and thawing of small, sonicated liposomes in various solutes prior to dialysis against a low salt buffer. Large changes in the trapped volume, population size distribution (a parameter which reflects the frequency of different sizes in a given population of liposomes), turbidity and the light microscope image of liposome samples have been produced by freeze-thawing under certain conditions. Pick [1] has reported similar effects of freeze-thawing without subsequent dialysis on the trapped volumes of lipid vesicles.

To facilitate the interpretation of these results in molecular terms, we have exploited the principle of fluorescence energy transfer as the basis of a convenient means of quantitating the degree to which lipids undergo interliposome mixing. We have found the efficiency of energy transfer between a pair of fluorophores originally incorporated into separate vesicles to parallel the changes in the parameters originally measured after freeze-thawing under various conditions. In the course of this work, a new pair of energy-transfer-

Abbreviations: DiI-C_{18}, dioctadecylindocarbocyanine; DnsPE, dansyldistearoylphosphatidylethanolamine; Mops, 3-morpholinepropanesulphonic acid.

ring fluorophores – i.e., dansyl and indocarbocyanine – was found to be suitable for monitoring intervesicle mixing of lipids and may prove useful for other purposes.

Materials and Methods

Chemicals

Most of the liposomes were made of phosphatidylcholine, Type IX-E from dried egg yolk (Sigma Chemical Co., St. Louis, MO). Lysophosphatidylcholine was not detected by thin-layer chromatography and could not have been more than a few percent, if any. This preparation does contain a component which is present as 2.1 negative charges per 100 molecules of lipid by microelectrophoresis (Oku and MacDonald, unpublished data) and may be due to ganglioside, since it also contains about 1.8 mol% sialic acid, assayed by the method of Warren [4]. The other lipid used was diisostearoylphosphatidylcholine, synthesized by the method of Johnson et al. [5]. The fluorescent probes were dioctadecylindocarbocyanine (DiI-C_{18}, Molecular Probes, Junction City, OR) and dansyldistearoylphosphatidylethanolamine (DnsPE, Molecular Probes). All probes gave a single spot when applied in excess to and run on a silica gel thin-layer plate. The levels of all probes are given as weight percent, which approximate mole percent since their molecular weights are close to those of phospholipids – i.e., DiI-C_{18} = 825 and DnsPE = 1000. All lipids and probes were dissolved in chloroform/methanol, 2:1, and stored at −20°C. Dextran sulphate, 3-morpholinepropanesulphonic acid (Mops) and Triton X-100 were also from Sigma Chemical Co.

Liposomes

Phospholipid vesicles were prepared by drying chloroform/methanol aliquots containing 10 mg phospholipid under nitrogen and oil-pump vacuum, prior to hydration in 1 ml 10 mM Mops (pH 7) for at least 30 min at 55°C. After suspension by vortexing, the multilamellar liposomes were usually probe-sonicated for 3 min in an ice/water bath. Samples which had been bath-sonicated under argon to clarity reacted to freeze-thawing in the same way as probe-sonicated samples. Suspensions to be stored at 4°C contained 0.02% NaN_3, which did not affect the response of the liposomes to freeze-thawing. Phospholipid was quantitated by measuring inorganic phosphorus by a modification of the procedure of Bartlett [6]. 10–100-μl samples of liposomes (half of them donor labeled and half of them nonfluorescent or acceptor-labeled plus additives, as appropriate) in borosilicate tubes were frozen by immersion in a solid CO_2/95% ethanol bath for 2 min. The samples were thawed by letting them stand at room temperature. 1 ml of 10 mM Mops, pH 7, was then added to each sample before measurement of fluorescence and/or light scattering.

Fluorescence measurements

After surveying a number of bilayer-compatible, amphiphilic fluorophores as possible energy-transferring donor-acceptor pairs, we chose to use DnsPE and DiI-C_{18}. DiI-C_{18} has a high quantum yield and, in addition, an excitation spectrum with a minimum in the region of the excitation maximum of DnsPE. The maxima for excitation and emission were 355 nm and 515 nm, respectively, for DnsPE and 540 nm and 575 nm, respectively, for DiI-C_{18}. Plots of the concentrations of DnsPE and DiI-C_{18} in egg phospholipid vesicles vs. their fluorescence intensities gave straight lines up to the concentrations of probes used.

Transfer efficiency was determined by exciting a sample containing both DiI-C_{18}- and DnsPE-labeled liposomes, as well as an identical sample containing DnsPE-labeled and nonfluorescent liposomes, in a quartz cuvette at the donor excitation maximum. Fluorescence emitted on excitation of the dansyl donor was measured in a Farrand spectrofluorimeter and recorded between 450 and 600 nm to obtain a value for the donor at 515 nm and the acceptor at 575 nm. The latter value was not used in the calculation of transfer efficiency but served to show that transfer to acceptor, as opposed to donor quenching alone, was occurring. In all cases, acceptor emission was inversely related to donor emission. 10-nm slits were used. Resonance energy transfer efficiency was calculated from the equation, $E = 1 - (F/F_0)$, where F is the donor fluorescence in the presence of acceptor and F_0 is the donor fluorescence in the absence of acceptor [7]. Initially, samples without DnsPE were also monitored, but the practice was discon-

tinued, as the background not contributed by emission from DnsPE was negligible. In addition, it was established that transfer was abolished when the donor and the acceptor were diluted into Triton micelles.

Results

The effects of various treatments on intervesicle mixing of phospholipid were determined by combining DnsPE-labeled vesicles with either nonfluorescent vesicles or vesicles labeled with DiI-C_{18}, exposing them to various treatments and measuring the transfer efficiency as described in Materials and Methods. To relate the efficiency of energy transfer to the degree of lipid mixing, liposomes containing both 1 wt% donor and 0.5 wt% acceptor, 2 wt% donor alone or 1 wt% acceptor alone were prepared and transfer efficiency was determined as a function of the percent liposomes of the mixed type – i.e., with both donor and acceptor. Data were obtained with either egg phosphatidylcholine or diisostearoylphosphatidylcholine, each at 10 mg/ml and either in sonicated or unsonicated form, and pooled to produce Fig. 1. Neither the type of lipid nor the liposome morphology affected the results. Fig. 1 shows that vesicles containing either 2 wt% DnsPE, 1 wt% DiI-C_{18}, or both 1 wt% donor and 0.5 wt% acceptor increase in transfer efficiency in a linear fashion as a function of mixed vesicle content. Throughout this work, therefore, results which are expressed as transfer efficiencies can be related to percent content of mixed vesicles, i.e., the degree of lipid mixing induced by a particular treatment, by referring to Fig. 1. Complete mixing of lipids between equal amounts of vesicles containing 1 wt% DiI-C_{18} and vesicles containing 2 wt% DnsPE would correspond to nearly 70% transfer efficiency.

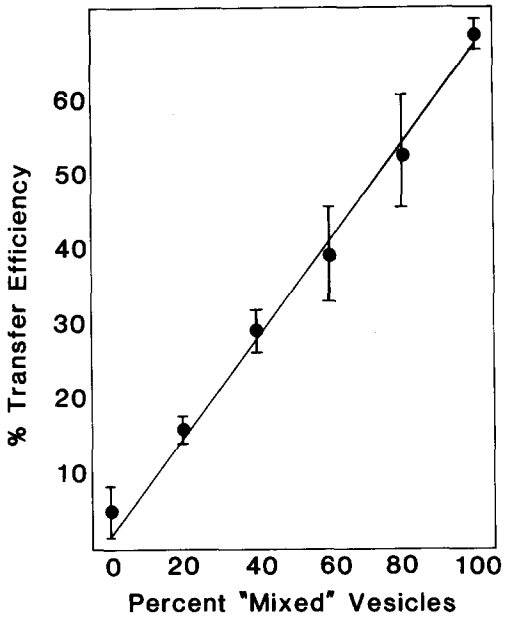

Fig. 1. Transfer efficiency as a function of content of mixed vesicles. The vesicles were composed of either egg phospholipid or diisostearoylphosphatidylcholine (10 mg/ml), either sonicated or unsonicated and labeled either with 2 wt% DnsPE alone, with 1 wt% DnsPE and 0.5 wt% DiI-C_{18}, or with 1 wt% DiI-C_{18} alone. 10 μl aliquots of liposomes containing various proportions of DnsPE + DiI-C_{18} vesicles were diluted with 1 ml 10 mM Mops (pH 7) and the transfer efficiencies ± S.D. determined as described under Materials and Methods.

Having related the degree of lipid mixing to percentage transfer efficiency, we next tested whether freeze-thawing of vesicles separately labeled with donor and acceptor and probe-sonicated to clarity would cause donor and acceptor to occupy the same vesicle, i.e., to display fluorescence energy transfer. Liposomes containing fixed concentrations of either donor (2–3 wt% DnsPE) or acceptor (2–2.5 wt% DiI-C_{18}) were combined with equal amounts of liposomes containing increasing concentrations of either acceptor or donor, respectively. The final concentration of egg phosphatidylcholine was 10 mg/ml in an aliquot of 10 μl. Tubes containing these samples were either left standing at room temperature or kept in solid CO_2/95% ethanol for 2 min. After the frozen samples had thawed at room temperature, 1 ml of 10 mM Mops (pH 7) was added to all samples and their fluorescence was determined.

Figs. 2A and B depict two important features of this system: (1) With one exception, samples not frozen and thawed exhibited minimal energy transfer and, therefore, contained few vesicles bearing both donor and acceptor. Indeed, even samples containing 1 wt% DiI-C_{18} and 2 wt% DnsPE and left covered at room temperature overnight exhibited minimal energy transfer. The exception – mixtures of vesicles containing 2–3 wt% DnsPE and vesicles containing 5 wt% DiI-C_{18} –

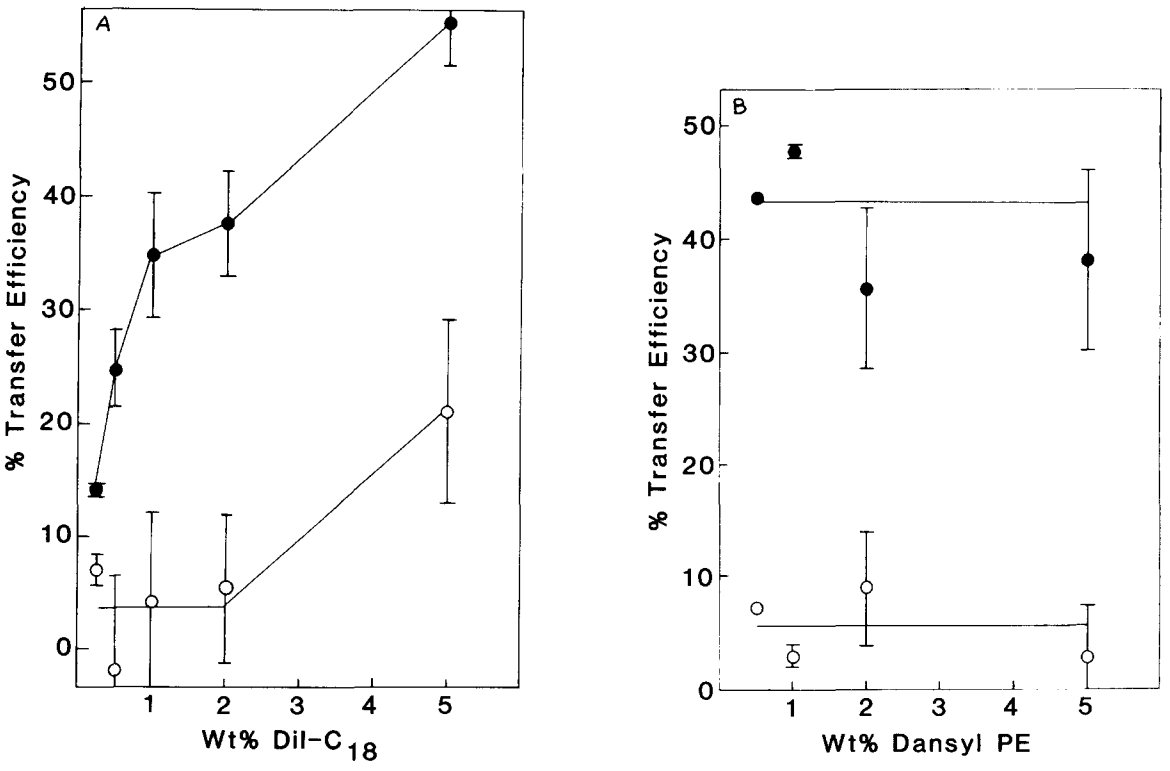

Fig. 2. (A) Effect of freeze-thawing on mean transfer efficiency (±S.D.) as a function of DiI-C_{18} or acceptor concentration. 5 μl of a suspension of liposomes containing 2–3 wt% DnsPE and 5 μl of a suspension of liposomes containing various concentrations of DiI-C_{18} were mixed, frozen and thawed, diluted with 1 ml of 10 mM Mops (pH 7) and the transfer efficiency ± S.D. determined as described under Materials and Methods. (B) Same as (A), except that DnsPE or donor concentration was varied and DiI-C_{18} held at 2–2.5 wt%. ●, frozen and thawed; ○, not frozen and thawed.

which displayed significant energy transfer, although unfrozen, indicates that concentrations of DiI-C_{18} greater than 2–2.5 wt% might be unsuitable for our purposes. (2) Increases in the diI-C_{18}or acceptor concentration (Fig. 2A) – but not increases in the DnsPE or donor concentration (Fig. 2B) – in the presence of fixed donor or acceptor concentrations, respectively, yielded increases in energy transfer. It is characteristic of fluorescence energy transfer in the static limit that transfer is dependent on acceptor, rather than donor, densi.ty or concentration [7]. Diisostearoylphosphatidylcholine, instead of egg phosphatidylcholine, vesicles were used in some experiments. The transfer efficiencies of 2 wt% DnsPE vesicles frozen and thawed with 1 wt% DiI-C_{18} vesicles were not affected by this lipid substitution – i.e., 25.4% ± 0.34% and 5.1% ± 0.36% for the synthetic lipid, frozen and unfrozen, respectively, and about 35% ± 5% for the natural lipid, frozen and unfrozen, respectively (see Fig. 2A). Extrapolating from Fig. 1, we estimate that about 35–50% of the lipids in sonicated vesicles mix with lipids from other vesicles after freeze-thawing.

The upper panel of Fig. 3 indicates that energy transfer induced by freeze-thawing – like increased turbidity [2], trapped volume [1,2], and population size distribution [2] – depends on lipid concentration. The samples containing different lipid concentrations were also checked for light scattering by setting both monochromators at 400 nm. The light scattering due to freeze-thawing (Fig. 3, lower panel) also increased with lipid vesicle concentration. The parallel between increased light scattering and increased energy transfer raised the unlikely possibility that both

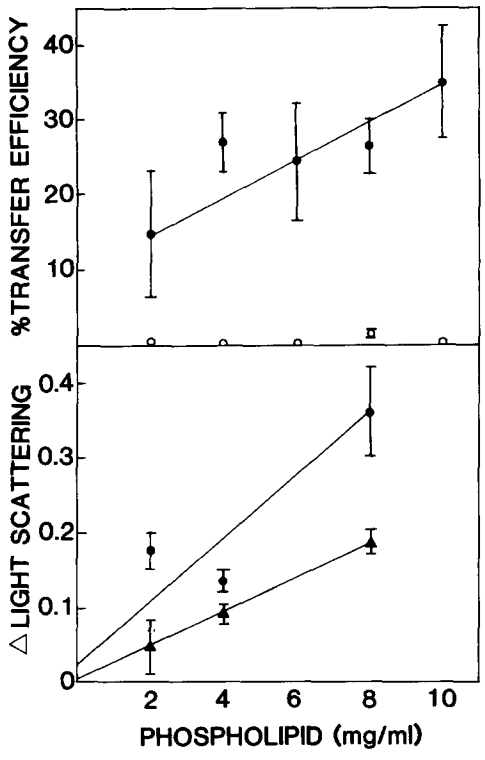

Fig. 3. Effect of freeze-thawing on mean transfer efficiency (±S.D.), upper panel, and on mean change in light scattering at 400 nm, lower panel, as a function of bulk egg phospholipid concentration during freeze-thawing. Net increase in light scattering varied between experiments 1 and 2 because different liposome preparations were used. Upper panel: ●, freeze-thawed; ○, not freeze-thawed. Lower panel: ●, experiment 1; ▲, experiment 2.

parameters could reflect vesicle aggregation, i.e., energy transfer from donor to acceptor in different vesicles, as opposed to a growth in vesicle size, i.e., energy transfer from donor to acceptor in the same vesicle. To determine whether intervesicle energy transfer could occur from DnsPE to DiI-C_{18}, we performed an experiment similar to that of Struck et al. [8]. Liposomes unlabeled or labeled with donor or acceptor were exposed to 150000 M_r dextran sulfate, 5 mg/ml, and 1.25 mM $MgSO_4$ for 30 min at room temperature, then diluted in 1 ml 10 mM Mops (pH 7). These reagents were suggested by the work of Kim and Nishida [9]. Both light scattering and energy transfer increased, the latter presumably resulting from a small amount of intervesicle fusion. When EDTA was added, light scattering decreased to that given by samples untreated with dextran sulphate-$MgSO_4$ but the energy transfer due to fusion caused by dextran sulphate-$MgSO_4$ remained at pre-EDTA but post-dextran sulphate-$MgSO_4$ levels. Hence, the vesicle aggregation promoted by dextran sulphate-$MgSO_4$ at least did not facilitate energy transfer.

The process by which freeze-thawing and dialysis causes small, sonicated vesicles to grow is inhibited by sugars [1,2], either inhibited [1,2] or promoted [2] by alkali metal cations and promoted by chaotropic reagents [3]. Particularly because the differential effects of alkali metal cations on liposome formation were not anticipated but are potentially of biological significance, we attempted to corroborate these findings by means of fluorescence energy transfer. The data in Fig. 4 affirm a differential effect of KCl and NaCl on freeze-thawing-dependent energy transfer as a function of salt concentration. It can be seen that NaCl tends to suppress energy transfer at concentrations between 0.05 to 1.5 M and especially at 0.15 M, whereas KCl first inhibits at 0.05 M but stimulates from 0.5 to 1.5 M. The difference between 43% energy transfer given by 1.5 M KCl and 28%

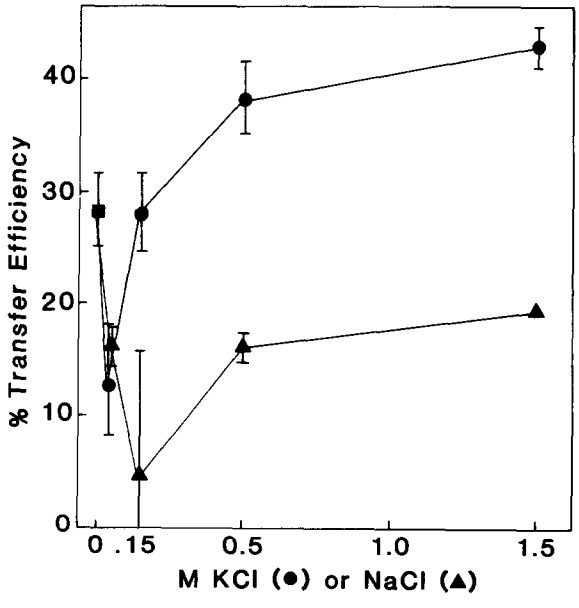

Fig. 4. Effect of various concentrations of KCl (●), NaCl (▲) or no salt (■) on the mean transfer efficiency (±S.D.) of frozen and thawed egg phospholipid vesicles.

energy transfer given in the presence of buffer only represent a KCl-dependent increase in lipid mixing of about 50%, according to Fig. 1. Neither salt at any concentration (not shown, except for KCl and NaCl at 0.5 M in Table I) affected energy transfer in the absence of freeze-thawing.

Other solutes which suppressed trapped volume increases induced by freeze-thawing [1,2] were tested, in addition to KCl and NaCl for comparison (Table I). LiCl/glycerol/sucrose at 0.5 M drastically inhibited energy transfer, as did 0.15 M NaCl (Fig. 4). Periodically, 1–2 mM EDTA (pH 7) was present during freeze-thawing, but did not affect the results. Thus, divalent cation contamination of the solutes used at high concentrations was not responsible for any of the observed effects.

Little is known about the mechanism by which freeze-thawing causes small, sonicated vesicles to become larger. To begin to address this subject, we tested whether unsonicated, mostly multilamellar liposomes would give the same results as sonicated, paucilamellar liposomes. Fig. 5 shows that unsonicated liposomes display significantly less fluorescence energy transfer than sonicated liposomes, even after three cycles of freeze-thawing. In addition to their having a higher energy transfer efficiency than unsonicated vesicles, the sonicated vesicles show markedly increased energy transfer between one and two cycles of freeze-thawing, after which complete mixing of donor and acceptor has occurred. These data suggest that only the outermost bilayer of a vesicle participates in whatever event leads to vesicle growth during intervesicle interaction. (It is assumed that the unsonicated preparation contains paucilamellar, as well as multilamellar, liposomes, since the initial cycle of freeze-thawing the unsonicated preparation results in about 33% lipid mixing.)

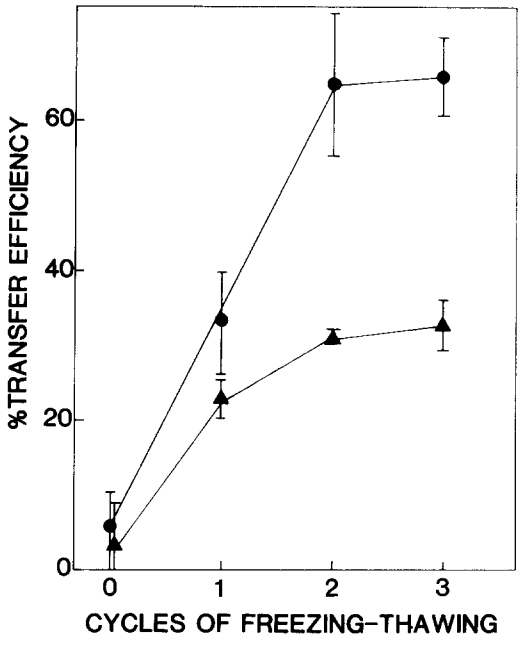

Fig. 5. Effect of the number of cycles of freeze-thawing on mean transfer efficiency (±S.D.) of sonicated (●) or unsonicated (▲) egg phospholipid vesicles.

The alternative possibility that small liposomes are endowed with a greater tendency to grow than large liposomes, as signified by their more extensive lipid mixing, is unlikely for two reasons. First, the freeze-thawing of small, sonicated vesicles causes them to grow significantly larger, as seen with the light microscope and indicated by an increase in turbidity (Fig. 3). Nevertheless, these much larger vesicles undergo as much lipid mixing during a second freeze-thaw (Fig. 5), unlike the once-frozen and thawed, largely multilamellar vesicles. Second, sonicated vesicles labeled with donor and sonicated vesicles labeled with acceptor, which have been frozen and thawed separately and become significantly larger in the process, still display increased energy transfer when subse-

TABLE I

EFFECTS OF 0.5 M ALKALI METAL CATIONS OF SUGARS ON FLUORESCENCE ENERGY TRANSFER EFFICIENCY IN SONICATED LIPOSOMES

Vesicles were composed of egg phospholipid, either unlabeled or labeled either with 2–3 wt% DnsPE or with 2–3 wt% DnsPE and 1 wt% DiI-C_{18}. 5 μl of DnsPE vesicles and 5 μl of one of the other two types of vesicle were mixed with 10 μl of the indicated additions at 1.0 M to give 0.5 M solute prior to freeze-thawing. Data are given as mean % transfer efficiency ± S.D.

Addition	Freeze-thawed	Not freeze-thawed
KCl	34.9 ± 5.5	13.0 ± 11.3
NaCl	18.0 ± 8.5	1.5 ± 2.8
LiCl	0.4 ± 6.2	0.8 ± 9.6
Glycerol	0.8 ± 7.0	8.3 ± 3.6
Sucrose	1.1 ± 6.4	5.4 ± 7.6

TABLE II

COMPARISON OF THE EFFECTS OF PROMOTERS OF FREEZE-THAW-DEPENDENT FLUORESCENCE ENERGY TRANSFER ON SONICATED AND UNSONICATED LIPOSOMES

Vesicles were composed of egg phospholipid, either unlabeled or labeled either with 2–3 wt% DnsPE or with 2–3 wt% DnsPE and 1 wt% DiI-C_{18}. 5 μl of DnsPE vesicles and 5 μl of one of the other two types of vesicle were mixed with 10 μl of each addition so that their final concentrations were half that shown. Data are given as mean % transfer efficiency ± S.D. KTCA, potassium trichloroacetate.

Addition	Freeze-thawed		Not freeze-thawed	
	Sonicated	Unsonicated	Sonicated	Unsonicated
10 mM Mops (pH 7)	26.5 ± 1.5	15.8 ± 0.0	8.7 ± 3.8	−4.1 ± 0.0
10 mM sodium acetate (pH 4)	59.1	56.1 ± 1.4	1.0	1.9
1 M KTCA	69.2 ± 2.5	59.0 ± 2.6	6.2 ± 6.1	4.6 ± 0.0
3 M KCl	45.5 ± 4.8	7.8 ± 0.0	7.9 ± 4.8	−2.0 ± 2.9

quently mixed and frozen and thawed (not shown).

Furthermore, we found that lipid mixing can occur by different mechanisms, depending on the promoters of vesicle growth, for example. Table II shows that 5 mM sodium acetate (pH 4) and 0.5 M potassium trichloroacetate increase the transfer efficiencies of unsonicated and sonicated liposomes to the same extent, which indicates an interaction of all bilayers, not only the outermost. These promoters may have caused the disintegration of bilayer structure so as to facilitate intervesicle mixing of lipid. 1.5 M KCl, on the other hand, which stimulates energy transfer of sonicated – but not unsonicated – liposomes, seems to facilitate the type of interaction of outermost bilayers appearing to occur on freeze-thawing in 10 mM Mops (pH 7) (see Fig. 5).

It should be noted that dilution of the frozen and thawed suspension per se is necessary for the measurement of trapped volume, turbidity and population size distribution, as well as fluorescence energy transfer, even when the effect of only freeze-thawing on these parameters is of interest. Energy transfer, like the other parameters measured, is not affected by the osmolarity of the buffer used for prompt dilution; in one experiment, for example, transfer efficiencies of liposomes frozen and thawed in 1.5 M KCl and diluted with 10 mM Mops (pH 7) or with 3 M KCl differed by 1.8%. It is recalled that one of the standard procedures in our laboratory for forming large liposomes includes gradual dilution accomplished by dialysis against low-salt buffer subsequent to freeze-thawing. In the case of the slow dilution by dialysis, as opposed to prompt dilution, hypoosmotic buffer is necessary for the formation of large liposomes [2].

Discussion

Application of the fluorescence energy transfer technique has helped to clarify two ambiguities posed by the trapped volume and population size distribution measurements of liposomes frozen and thawed under different conditions [2].

(1) Whereas liposomes with diameters of 5 μm or greater seemed to form only from small liposomes frozen and thawed in high concentrations of KCl or RbCl and dialyzed against low-salt buffer, the trapped volumes of samples frozen and thawed in the absence of any alkali metal cation and dialyzed against low salt buffer were generally higher than the former. It was not clear whether the trapped volume measurement was influenced by the conditions of freeze-thawing and subsequent dialysis or whether large liposomes formed in the low-salt samples but fragmented prior to measurement of the population size distribution. Turbidities of both high- and low-salt samples increased on freeze-thawing but could not, of course, enable distinction between an increase in size and an increase in degree of aggregation. The fluorescence energy transfer measurements help to resolve this question by showing that lipid mixing, which is not reversed or altered by events subsequent to freeze-thawing, does occur to a significant

degree (i.e., about 25%) in the low-salt samples which is, nevertheless, less (i.e., about 66% as much) lipid mixing than in the high salt samples. Therefore, the trapped volume measurements, which are generally taken to indicate the relative proportions of unilamellar as opposed to oligo- or multilamellar liposomes [10] or the degree of hydration [11] provide an underestimate in this case of the hydrating effect of K^+, relative to no K^+. The population size distribution may provide an overestimate of the vesicle enlargement occurring in high – relative to low – salt, at least with respect to vesicles larger than 5 μm in diameter.

(2) Given the potential ambiguity of the trapped volume, population size distribution and turbidity measurements and the already mentioned importance of the differential effects of the alkali metal cations on these parameters, it was necessary to corroborate these findings by means of the fluorescence energy transfer technique. We had to explore the possibility that a 'vesicle growth process' does occur during freeze-thawing of vesicles in high concentrations of Na^+, Li^+ and Cs^+, as well as K^+ and Rb^+, but that vesicles frozen and thawed in the former leaked and fragmented prior to the measurement of the parameters previously investigated. Since energy transfer was nil in the case of 0.5 M Li^+ (see Table II), reduced to one half that in Mops alone in the case of Na^+ (see Fig. 4), and increased 10% above that in Mops alone in the case of 0.5 M K^+ (see Fig. 4), the 'vesicle growth process' stimulated during freeze-thawing was indeed inhibited by Na^+ and Li^+ and promoted by K^+. Like Li^+, glycerol and sucrose inhibited this phenomenon to an even greater extent than did Na^+. Again, however, whether a given treatment causes giant liposomes to form is not wholly a function of its promoting lipid mixing, since about 20 and 40% of the vesicles underwent lipid mixing when frozen and thawed in the presence of 0.5 M NaCl and in the presence of 10 mM Mops (pH 7), respectively, neither of which produce giant liposomes. 55% of the vesicles underwent lipid mixing when frozen and thawed in 0.5 M KCl, which produces some giant liposomes. Finally, the influence of alkali metal cations on giant liposome formation cannot be attributed entirely to their influence on the chemical activity of water, as suggested by Pick [1], in view of the now well-substantiated differential effects of specific cations and of the same cation – i.e., K^+ – at different concentrations (Ref. 2 and Fig. 4).

It is not entirely clear how the freeze-thaw technique causes small vesicles to grow, although freeze-thawing of membranes, biological [12,13] as well as artificial [14], is not new and although freeze-thawing has proven particularly useful for the reconstitution of certain membrane proteins [15,16] into lipid vesicles (see reviews in Refs. 17, 18). In addition to aiding the interpretation of previously obtained data, results reported here shed some light on the mechanism by which freeze-thawing promotes the growth of lipid vesicles. There has been little evidence for the commonly stated assertion that freeze-thawing causes vesicle fusion, especially fusion of the type characteristic of biological systems. In fact, there is evidence against freeze-thawing-dependent vesicle growth resembling biological fusion, since vesicles so treated lose their integrity [14,19].

The transient loss of integrity implied by freeze-thaw-associated leakiness is not so extensive that bilayer structure disintegrates, however, except possibly in the presence of potassium trichloroacetate or 5 mM sodium acetate (pH 4) (Table II). This inference is based on the greater energy transfer efficiency displayed by small, sonicated vesicles (Fig. 5, once frozen) and by larger, oligo- and unilamellar vesicles (Fig. 5, twice or thrice frozen) than by multilamellar vesicles (Fig. 5) frozen and thawed in 10 mM Mops (pH 7). The transfer efficiency of sonicated vesicles is also greater than that of unsonicated vesicles after freeze-thawing in 0.5 M KCl (Table II). If lipid mixing occurred among all bilayers and not only between outermost bilayers, or if bilayer structure were lost during freeze-thawing, little difference would be expected between the oligo- and unilamellar versus the multilamellar vesicles.

In contrast to vesicles frozen and thawed in 10 mM Mops or high KCl, sonicated vesicles undergo the same amount of lipid mixing as unsonicated vesicles when freeze-thawing is performed in the presence of 5 mM sodium acetate (pH 4) or 0.5 M potassium trichloroacetate. Either all bilayers interact or bilayer structure breaks down during freeze-thawing in those solutes. At high concentrations, trichloroacetic acid does dissolve lipid bi-

layers into micelles without freeze-thawing [3]. (Incidentally, the effect of 5 mM sodium acetate (pH 4) appears to be due to acetic acid and not to protons (MacDonald, R.I., unpublished data).) In summary, vesicle growth induced by freeze-thawing can occur either during interaction of vesicles at their outermost bilayers (e.g., in 10 mM Mops (pH 7) or in high concentrations of KCl) or during interaction among all bilayers – possibly as a result of the collapse and reformation of bilayer structure (e.g., in potassium trichloroacetate or 5 mM sodium acetate, pH 4). Both mechanisms can lead to extensive intervesicle mixing of phospholipids.

A final novel aspect of this work is the introduction of a new donor-acceptor pair of resonance-energy-transferring fluorophores, i.e., dansyl and indocarbocyanine. The amply demonstrated usefulness of fluorescence energy transfer for monitoring vesicle-vesicle and vesicle-cell interactions [8,20–25] should be served by the availability of an alternative to the donor-acceptor pairs already in use, especially one, the acceptor of which is as stable to illumination and characterized by as high an extinction coefficient and quantum yield as indocarbocyanine [26]. It does have the disadvantage of possessing a positive charge, but because it can be used at low concentrations, this characteristic is unlikely to prove troublesome. Since one of the drawbacks to fluorescence spectroscopy of extrinsic fluorophores is the possibility that the fluorophores themselves will perturb the system, the high extinction coefficient could be a particular asset. It should be possible to perform experiments with this pair at even less than the 1–2 wt% employed in the present study by operating the spectrofluorimeter at higher sensitivity and/or increasing the slit widths.

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