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REACTION CHARACTERISTICS AND MECHANISMS OF LIPID BILAYER VESICLE FUSION

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Small unilamellar lipid bilayer vesicles were prepared from brain phosphatidylserine, egg phosphatidylcholine, and synthetic dipalmitoylphosphatidylcholine, and were fused into larger structures by freezing and thawing, addition of calcium chloride, and passage through the lipid phase transition temperature. Fusion reactions were studied by electron microscopy, light scattering, and use of fluorescent probes. Fusion was accompanied by leakage of lipid vesicle constituents and of water-soluble solutes in the inner vesicle compartments, and by uptake of these types of components from the external solution. Such leakage was greater during fusion by freezing than by Ca^{2+}. Passage through the transition temperature produced a moderate degree of fusion, without loss of membrane components. It is concluded that each fusion method gives rise to a characteristic size or narrow range of sizes of fusion products. The fraction of small vesicles fused into larger structure depends on the method of vesicle preparation, composition of the lipid bilayer, and composition of the external solution. Fusion is induced by creation of a discontinuity in the bilayer or by removal of water associated with the bilayer. The amount of water removed controls the extent of fusion. This is maximized in bilayers when in the liquid-crystal phase, as against the gel phase, in vesicles made by ethanol injection, as against sonication, and in charged bilayers, as against neutral ones.

Introduction

The fusion of lipid bilayer vesicles (liposomes) has received much attention in recent years. Transformation of small unilamellar vesicles into larger uni- or multi-lamellar vesicles is important as a model reaction for the adhesion and fusion of cell and organelle membranes in processes such as secretion and endocytosis. It is of additional interest since fusion is accompanied by capture and release of vesicle components. Fusion of small unilamellar vesicles can be induced in several ways, including (a) passage through the phase transition temperature of the bilayer [1–3]; (b) addition of fusogenic agents such as lysophosphatidylcholine and other single-chain polar lipids [4]; (c) addition of millimolar concentrations of Ca^{2+} or other multivalent ions [5–7]; and (d) freezing and thawing [8–10].

The fused structures produced by these methods are found to be resistant to further growth under fusion-inducing conditions. The reverse reaction, the breakdown of large aggregates into small unilamellar vesicles occurs only when energy is supplied, in the form of sonication. The larger structures, therefore, are thermodynamically stable, whereas small vesicles represent a metastable

Abbreviations: Chl, chlorophyll *a*; DPPC, synthetic dipalmitoylphosphatidylcholine; DCY, dioctadecyloxacarbocyanine toluene sulfonate; EDTA, ethylenediamine tetraacetic acid, sodium salt; PC, phosphatidylcholine; PS, phosphatidylserine, T_c, lipid phase transition temperature; Tris, tris(hydroxymethyl)aminomethane.

form of higher free energy. Fusogenic agents thus represent a means of lowering the activation energy of the fusion reaction.

Bilayer vesicles exist due to a balance of stabilizing and destabilizing forces [11]. Included in the former are hydrophobic and ionic interactions, and hydration of the polar headgroups. Destabilizing forces include lateral pressure between adjacent lipids, expecially in the inner layer of small-diameter vesicles. Hydration of the headgroups creates a strong repulsive force, preventing the close approach of two vesicles [12].

The present objective was to understand the mechanism whereby various fusogenic procedures disturb this balance of forces. Our previous studies of freezing effects had shown that fusion proceeds to different extents under various reaction conditions [8]. It was of interest, therefore, to investigate the effects of the method of vesicle preparation, of the ionic charge density in the bilayer, and of the salt concentration on the course of fusion. Possible fusion mechanisms may be represented by: (a) Fusion of variable fractions of small vesicles to give essentially the same size and type of fused structure; or (b) Fusion of all small vesicles to give aggregates and fused structures of a range of different sizes and types. As we will show, (a) is the principal mechanism, at least in the initial stages of fusion. Removal of water associated with the bilayer is the principal destabilizing effect which triggers fusion.

Materials and Methods

Phosphatidylcholine (PC) (Type III E from egg yolk, as a 10% solution in hexane), phosphatidylserine (PS) (from bovine brain, as a 1% solution in chloroform/methanol), dipalmitoylphosphatidylcholine (DPPC) (synthetic), and chlorophyll *a* (Chl) (from spinach) were obtained from Sigma Chemical Co. *N,N'*-Dioctadecyloxacarbocyanine-*p*-toluene sulfonate was obtained from Eastman Organic Chemicals. Terbium chloride hexahydrate and dipicolinic acid were obtained from Aldrich Chemical Co. Sephadex G-75 gel filtration medium was obtained from Pharmacia, Inc.

All fluorescence and light scattering measurements were made with a Hitachi-Perkin Elmer fluorescence spectrometer, Model MPF-3, fitted with corrected spectra accessory.

Preparation and labeling of vesicles

Both the sonication [13] and the ethanol injection method [14,15] were used to prepare vesicles, to incorporate membrane-bound probes, and to trap water-soluble probes in the inner vesicle compartments. To monitor fusion by energy transfer between DCY and Chl and to monitor simultaneously the amalgamation of the inner compartments by the reaction of Tb^{3+} with dipicolinic acid, vesicle populations labeled in several ways were prepared, using either of the two methods. Vesicles 'separately labeled ' (I) with DCY in the membrane and $TbCl_3$ in the internal volume, and (II) with Chl in the membrane and dipicolinic acid in the core, were prepared. 'Doubly labeled' vesicles (III) carrying all four of these probes were also made.

When using the sonication method, procedures were as follows: To prepare I, 1.5 mg (approx. $2 \cdot 10^{-3}$ mmol) of phospholipid, as a solution in organic solvent, and 0.12 ml of a $3.4 \cdot 10^{-2}$ mM solution of DCY in ethanol ($4.1 \cdot 10^{-6}$ mmol) were evaporated to dryness in a rotary evaporator in a round-bottom flask, redissolved in ether, and redried. The resulting film of lipids was briefly dispersed on a Vortex mixer in 1.0 ml of a aqueous solution 20 mM in $TbCl_3$ ($2 \cdot 10^{-2}$ mmol), 200 mM in sodium citrate, and 2 mM in Tris buffer, pH 7.5, and then sonicated for 15 min with a Branson W 140 probe-type sonicator with microtip. The almost-clear solution was passed through a 1×15 cm Sephadex G-75 chromatographic column, and eluted with a solution 5 mM in NaCl, 0.1 mM in EDTA, and 2 mM in Tris. The vesicles eluted immediately after the void volume. An eluate volume of 2.5 ml was collected which contained essentially all the phospholipid. This was diluted with the same buffer as used for elution to a volume of 8 ml.

To prepare II, 1.5 mg of phospholipid and 0.23 ml of a $3.9 \cdot 10^{-2}$ mM solution of Chl in ethanol ($8.8 \cdot 10^{-6}$ mmol) were evaporated to dryness as described for I, then dispersed and sonicated with 1.0 ml of a solution 200 mM in dipicolinic acid and 2 mM in Tris, pH 7.5 and chromatographed. Again, 2.5 ml were collected and diluted to 8 ml.

Doubly labeled vesicles III were prepared by evaporating 1.5 mg phospholipid with 0.06 ml of the ethanolic DCY solution and 0.112 ml of the

ethanolic Chl solution, then sonicated with 1.0 ml of a 1 : 1 mixture of the above $TbCl_3$ and dipicolinic acid solutions, and chromatographed.

The resulting final concentrations in a 1 : 1 mixture of preparations I and II, or in preparation III, were: Phospholipid, 0.188 mg/ml or 0.25 mM; DCY, $2.6 \cdot 10^{-4}$ mM; Chl, $5.5 \cdot 10^{-4}$ mM. The concentrations of Tb^{3+} and dipicolinic acid in the trapped internal volumes were 1.25 mM and 12.5 mM, respectively, and approx. 500-times lower when calculated with respect to the total aqueous volume of the suspensions.

To prepare vesicles by the injection method, ethanolic solutions of phospholipid and the appropriate membrane labels were injected through a Hamilton syringe fitted with a 22-gauge needle, 10 cm long, into a 20 to 25-fold volume of an aqueous buffer during vigorous agitation on a Vortex mixer. Gel chromatography of negatively charged vesicles made by injection met with difficulties, since these became bound to the column packing and could be eluted only incompletely, if at all. Injected PS vesicles therefore were not labeled with Tb^{3+} and dipicolinic acid. Quantities used were as follows:

I: 2.25 mg phospholipid in 0.15 ml ethanol together with 0.12 ml of a $3.46 \cdot 10^{-2}$ mM solution of DCY in ethanol was injected into 7.5 ml of a solution 0.11 M in KCl and 0.05 M in Tris (pH 7.5).

II: As I, except that 0.20 ml of $3.9 \cdot 10^{-2}$ mM Chl in ethanol was used in place of the DCY solution.

III: As above, but using 0.06 ml of the DCY solution and 0.10 ml of the Chl solution. The resulting final concentrations in a 1 : 1 mixture of I and II, or in III, were: Phospholipid, 0.30 mg/ml or 0.40 mM; DCY, $2.75 \cdot 10^{-4}$ mM; Chl, $5.2 \cdot 10^{-4}$ mM.

Determination of the extent of vesicle fusion

Transfer of electronic excitation energy between donor and acceptor probes bound to the membranes in two vesicle populations was used to monitor proximity, and thus vesicle aggregation and/or fusion. The energy donor was DCY, and the acceptor was Chl. Both these probes were chosen because they remained firmly attached to intact membranes due to their long hydrocarbon chains. Another reason for their selection was that their wavelengths of absorption and emission did not interfere with the assay for mixing of the inner vesicle compartments (see below). Following a technique described previously [16], dipole-dipole energy transfer was measured by exciting the donor at 486 nm and observing sensitized fluorescence in the acceptor at 676 nm. Since energy transfer declines with the sixth power of the intermolecular distance, one may expect near-full energy transfer when donor and acceptor are on the same vesicle, or when differently labeled vesicles come in contact or fuse. Essentially zero energy transfer occurs between donor and acceptor on two different vesicles which are not in contact.

The fraction of vesicles that had undergone aggregation or fusion following a given treatment was determined by comparing the energy transfer in a mixture of vesicles separately labeled with donor and acceptor with that in vesicles doubly labeled with both probes. The latter were subjected to the same fusion treatment as the test mixture to cancel out incidental changes in energy transfer not due to fusion.

The sensitized acceptor fluorescence, F_{DA}, was corrected by a blank value, $(F_{DA})_0$, representing zero energy transfer. The latter arises from the small absorbance of the acceptor (Chl) at the donor excitation wavelength of 486 nm, which results in direct excitation of Chl in addition to the induced fluorescence. In order to obtain self-consistent results, advantage was taken of the inertness of vesicles to further aggregation or fusion after an initial fusion reaction [17,18]. Separately labeled vesicles, containing only DCY or only Chl, were caused to fuse by freezing or by Ca^{2+}, and were subsequently mixed. This mixture, which contained the same label concentrations as the test mixture, was used to determine $(F_{DA})_0$. Blank values so obtained closely agreed with the mean of the blanks for the DCY and the Chl vesicles.

For internal standardization, the fluorescence of Chl resulting from direct excitation at 420 nm (where DCY has only negligible absorbance) was also measured, again using the inert mixture of separately labeled and separately fused vesicles to determine an intrinsic value in absence of energy transfer. This value is proportional to ϕ_A, the fluorescence quantum yield of the acceptor.

The observed fluorescence intensities (excita-

tion at donor absorption peak; emission measured at acceptor emission peak) are given by

$$F_{DA} = K(A_D\phi_{ET}\phi_A + A_A\phi_A) \quad (1)$$

and

$$(F_{DA})_0 = KA_A\phi_A \quad (2)$$

where ϕ_{ET} is the energy transfer efficiency, K is a proportionality constant, ϕ_A is the fluorescence quantum yield of the acceptor, and A_D and A_A are the absorbance of donor and acceptor, respectively, at the donor absorbance maximum (486 nm for the present system). The absorbances, provided they are small, are proportional to I_a, the intensity of light absorbed. It follows that

$$\phi_{ET} = \frac{F_{DA} - (F_{DA})_0}{K \cdot A_D \cdot \phi_A} \quad (3)$$

Both A_D and ϕ_A were changed somewhat by addition of Ca^{2+} or freezing, possibly due to changes in rigidity or polarity attending fusion. However, they were the same in separately labeled and doubly labeled vesicles after a given treatment, and thus cancelled out in comparative measurements. The fraction of vesicles fused thus is given by

$$\text{fraction fused} = \frac{[F_{DA} - (F_{DA})_0] \text{ sep. labeled}}{[F_{DA} - (F_{DA})_0] \text{ doubly labeled}} \quad (4)$$

Determination of mixing of aqueous vesicle cores and/or leakage

Following the method of Wilschut and Papahadjopoulos [19], the amalgamation of the inner aqueous volumes during fusion was detected and quantitated by trapping nonfluorescent Tb^{3+} (as a weak complex with citrate) and nonfluorescent dipicolinic acid in separate vesicle populations. When these were mixed and induced to fuse (e.g. by Ca^{2+}), a highly fluorescent complex of terbium with dipicolinic acid formed. In order to demonstrate that the complex did not form outside the vesicles as a result of leakage, EDTA was added to the external solution. This destroyed the fluorescent complex by competitively complexing with Tb^{3+}. This system was also used to determine the extent of leakage of vesicle core constituents during fusion: Vesicles which initially contained the terbium complex were fused in presence of EDTA in the outer solution. The decrease in fluorescence indicated the amount of leakage.

The fluorescence was measured at 545 nm, with excitation at 276 nm. Since the latter wavelength is close to one-half the emission wavelength, there was a possibility of getting second harmonic transmission by the emission monochromator. A cut-off filter was used to prevent this. A non-zero blank reading was observed even when only Tb^{3+} or only dipicolinic acid was present, apparantly due to the other labels present. Inert mixtures of separately fused vesicles were used to establish consistent base line readings.

Light scattering measurements

Vesicle sizes can be monitored by steady-state turbidity or light scattering determinations. In homogeneous suspensions of particles smaller than about 1/20 the wavelength used, the specific Rayleigh scattering intensity (i.e. intensity/concentration) is proportional to the volume of the particle, or to the cube of the diameter. For larger particle sizes, up to twice the wavelength, the scatter intensity roughly varies with the square of the particle diameter, as discussed by Kerker [20].

As observed by electron microscopy (see Results), our vesicles initially were fairly homogeneous and 300 to 500 Å in diameter. For these, specific scattering, i_{90}/c (where i_{90} is the scattering intensity at a scattering angle of 90°, and c is the lipid concentration). was used to estimate mean diameters on a relative scale which compared well with the electron micrographs. After fusion by freezing, the electron micrographs showed two distinct populations: Small vesicles of the size originally present, and larger vesicles, but few intermediate sizes. For such bimodal size distributions the fraction f of the vesicles transformed from monomers into aggregates can be calculated on a relative scale as follows:

For a sample originally containing n_M monomeric vesicles per cm^3 the specific scattering intensities i^0 and i before and after fusion are given by

$$i^0 = n_M \cdot m \quad (5)$$

and

$$i = (1-f)n_M m + \frac{f \cdot n_M}{p} \cdot a \quad (6)$$

where m and a are the scattering intensities per monomeric and per aggregate particle, respectively, p is the degree of aggregation, and $f n_M/p$ is the number of aggregates per cm^3. It follows that the net scattering ratio, R, is given by

$$R = \frac{i}{i^0} - 1 = f\left(\frac{a}{mp} - 1\right) \quad (7)$$

According to this model, the ratio of R/f is a measure of the average size of the aggregates. For a given size of aggregates, R is a measure of the fraction of vesicles that have fused. The value of R was found to be reproducible for a given fusion procedure, even though the values of i and i^0 varied for different preparations of a given vesicle type. Results are reported in terms of R, as a relative measure of the fraction of vesicles that have fused.

Measurements were made by setting both excitation and emission wavelengths of the fluorescence spectrophotometer to 350 nm. The instrument was set to read zero when the wavelengths differed. By scanning the emission wavelength, a sharp net scattering peak was obtained when the wavelengths coincided.

Results

Electron microscopy

To characterize the products of fusion, PS and PC vesicles were examined by electron microscopy as prepared by either sonication or ethanol injection, and after being fused by freezing or addition of Ca^{2+}. Negative staining with ammonium molybdate was used [17]. Vesicles prepared by either method were found to be unilamellar and fairly homogeneous in size. Those made by injection were estimated to average 300 Å in diameter; sonicated vesicles averaged 500 Å. After freezing-thawing, the vesicles consisted of two distinct populations: A group of larger vesicles, most of them unilamellar, but some having several lamellae, which ranged from 2000 to 2500 Å and from 1400 to 1800 Å for PS and PC vesicles, respectively; and a group of small unilamellar vesicles of the same size as seen before fusion. Only few vesicles had intermediate sizes.

These results show that small unilamellar vesicles upon freezing give a narrow size range of larger fused vesicles, corresponding to case (a) of the two mechanisms suggested earlier. The number of small vesicles reacting to form fused vesicles may be estimated from the diameters: If we assume that the surface area per phospholipid remains constant, that small unilamellar vesicles have 2/3 of their lipid in the outer layer, and that the fused vesicles are unilamellar with 50% of the lipid on the outside, then the observed size increases correspond to a fusion of about 25 PS vesicles and about 15 PC vesicles.

PS vesicles treated with 2.4 mM Ca^{2+} showed a completely different behavior: Large irregular, non-vesicular structures were observed. The average of the size range was estimated at 4000–15 000 Å. Some of these structures were seen to be multilamellar, and evidently represent the cochleate structures described by Papahadjopoulos and coworkers [21]. A few vesicles of the original size were also present.

Degree of completion of fusion reaction

The fusion of sonicated PS vesicles by freezing and by Ca^{2+} was monitored by determining, in the same reaction mixture, (a) energy transfer between membrane bound labels (DCY and Chl) in different vesicles, (b) release of these labels into the external solution, (c) formation of a fluorescent complex between Tb^{3+} and dipicolinic acid by amalgamation of the inner compartments of vesicles labeled with these probes, and (d) release of the terbium complex from the inner compartments into the external solution.

The vesicles carried DCY or Chl, or both, in their membranes as well as Tb^{3+} or dipicolinic acid or both, in aqueous solution trapped in the inner compartments. Such multiple labeling was found necessary when correlating membrane fusion with the amalgamation or retention of trapped interior solutes, since the presence of the latter affected the fusion behavior of the vesicles. To carry out a typical fusion experiment by freeze-thawing, 2.5 ml of a 1 : 1 mixture of separately labeled populations I (DCY and Tb^{3+}) and II (Chl

TABLE I

EFFECT OF FREEZE-THAW TREATMENT AND OF 2.4 mM $CaCl_2$ ON PS VESICLES

Vesicles made by sonication in 0.15 M salt solution, pH 7.5. Fluorescence energy transfer, ϕ_{ET}, and fluorescence of the terbium-dipicolinic acid complex, ϕ_{Tb}, given in arbitrary units. Fluorescence data were corrected by a blank (see text) appropriate for the treatment used. All values are the mean of two or three experiments.

Sample		Untreated	Freeze-thaw	Ca^{2+}
I and II mixed, then fused	ϕ_{ET}	0	0.084	0.15
	ϕ_{Tb}	0	0	0.12
III (doubly labeled)	ϕ_{ET}	0.40	0.33	0.35
	ϕ_{Tb}	0.73	0.14	0.51
Fraction of vesicles fused	$= \frac{\phi_{ET}(I+II)}{\phi_{ET}(III)}$	0	0.25	0.43
Fraction of membrane labels retained	$= \frac{\phi_{ET}(III, fused)}{\phi_{ET}(III, untreated)}$	1.0	0.83	0.88
Fraction of inner volumes amalgamated	$= \frac{\phi_{Tb}(I+II)}{\phi_{Tb}(III)}$	0	0	0.23
Fraction of terbium complex retained	$= \frac{\phi_{Tb}(III, fused)}{\phi_{Tb}(III, untreated)}$	1.0	0.19	0.70

and dipicolinic acid), contained in a 10×90 mm test tube, was immersed in a dry ice-acetone freezing bath at $-78°C$ for 15 min, then allowed to thaw in a 25°C water bath. A doubly labeled reference sample, population III (carrying all four labels) was also prepared and frozen.

To induce fusion by Ca^{2+}, 30 μl of a 0.2 M $CaCl_2$ solution was added with stirring to 2.5 ml of a 1 : 1 mixture of I and II, giving 2.4 mM Ca^{2+}. The reference population III was treated similarly.

The fraction of vesicles that had fused was found by calculating the fluorescent energy transfer in the mixture of I and II after fusion and comparing it with the energy transfer in III, also after fusion. This eliminated the effect of losses of membrane labels during fusion, assumed to be the same in (I + II) and in III.

The loss of membrane labels from the vesicles by migration into the external solution was obtained from the same set of energy transfer data by comparing III after fusion with III in the initial state. Data are collected in Table I. Ca^{2+} caused a larger fraction of the vesicles to fuse than did freezing. The loss of membrane labels, however, was slightly greater during fusion by freezing.

The statistics of formation of fused vesicles from small unilamellar ones carrying identical or different labels must be considered when interpreting these results: For random aggregation of differently labeled monomers, the relative number of aggregates of fixed size n having d donor-labeled and $(n-d)$ acceptor-labeled monomers is given by the binomial coefficient $C_d^n = n!/d!(n-d)!$. For aggregates of 15 or more monomers, inferred from electron microscopy, the binomial distribution function shows a sharp maximum for aggregates of equal numbers of donor-labeled and acceptor-labeled monomers. The function is symmetric about this maximum, so that the average composition is also one of equal numbers of donors and acceptors. Moreover, only about 4% of the aggregate (of size 15) contain less than 3 or more than 12 donor-labeled monomers. Aggregates formed by fusion of monomers from populations I and II therefore have, on average, a 1 : 1 ratio of donors and acceptors, the same as the doubly labeled population III. The ratio of ϕ_{ET} of (I + II) relative to that of III thus will be practically equal to the fraction of vesicles that have fused.

The extent of amalgamation of the inner compartments was found by comparing the fluorescence of the terbium complex formed during

fusion of (I + II) with that of III, also after fusion. As seen in Table I, a certain amount of amalgamation occurred after Ca^{2+} addition, but this was much less than the fraction of membrane labels that had mixed. No mixing of inner compartments could be detected after freezing.

Comparison of the fluorescence of the terbium complex in III before and after fusion gave the percentage of complex retained. This was slightly lower than the retention of membrane labels for Ca^{2+} addition, and very much lower after freezing. Because of the time needed for freezing and thawing, measurements were made 20 min after freezing began, but within 5 min after addition of Ca^{2+}. The lower retention figures after freezing are thus likely to be due, at least in part, to a slow leakage during this time delay. However, the great discrepancy between retention of inner probes and membrane probes during freezing, and the absence of any detectable formation of terbium-dipicolinic acid complex during fusion, indicates that vesicle fusion by freezing is inherently more leaky than Ca^{2+} fusion.

Incorporation of probes from external solution

The reaction opposite to loss of vesicle components, i.e. the incorporation of constituents from external solution, was investigated with PS and PC vesicles made by injection. To 3 ml of a vesicle suspension, 10 μl of an ethanolic solution of DCY or Chl was added. Essentially no fluorescence was observed at this point since these probes form non-fluorescent aggregates in water. When the PS vesicles were then fused by freezing or Ca^{2+}, the fluorescence of the added probes increased substantially, showing that they had become incorporated into the vesicles. PC vesicles, in contrast, showed only marginal uptake during fusion. Addition of 1.0 M NaCl at room temperature, without freezing, also caused considerable uptake of DCY and Chl by PS vesicles. In all cases the fraction of probe taken up was calculated by comparison with another sample in which the same amount of probe had been incorporated by co-injection during formation of the vesicles in the usual manner.

Results are shown in Table II. The uptake of DCY was similar during freezing and Ca^{2+} fusion, whereas the incorporation of Chl was greater during freezing than after Ca^{2+}. This is consistent with a greater loss of membrane labels during freezing, compared to Ca^{2+}.

Effects of method of vesicle preparation

Comparative data for fusion of PS vesicles made by injection and by sonication are shown in Table III. Injected vesicles fused to a much greater extent than sonicated ones, both after Ca^{2+} addition and after freezing. Consistent with this greater reactivity was a lower percentage of membrane-bound labels retained by injected vesicles, i.e. greater leakiness. When passed through a Sephadex G-75 chromatographic column, injected PS vesicles were extensively retained whereas sonicated ones eluted freely, as is also shown in Table III.

The initial size of vesicles made by injection, as inferred from light scattering data, could be varied by adjusting the lipid concentration in the alcoholic solution injected into aqueous buffer. Higher lipid concentrations gave larger vesicles, as previously reported by Kremer at al. [15]. Data in Table IV show that the final light scattering values attained after freezing of PS vesicles were almost the same for different initial scattering values.

TABLE II

INCORPORATION OF MEMBRANE-BOUND PROBES INTO PS AND PC VESICLES DURING FUSION, AS PERCENTAGE OF PROBES INCORPORATED DURING VESICLE PREPARATION

Lipid	Probe	Room temp., 0.005 M NaCl	Room temp., 1.0 M NaCl	After freezing	After Ca^{2+}
PS	DCY	5.3	35.3	94	100
	Chl	7.3	20.0	98	80
PC	DCY	4.7		7.0	6.7
	Chl	8.0		29	12.7

TABLE III

COMPARATIVE REACTIVITY OF PS VESICLES MADE BY ETHANOL INJECTION AND SONICATION

		Injected	Sonicated
Fraction of vesicles fused by:	Freezing	0.75	0.26
	Ca^{2+}	0.86	0.42
Fraction of membrane probes retained after:	Freezing	0.40	0.60
	Ca^{2+}	0.60	0.89
Fraction of vesicles eluted from Sephadex G-75 column		0.37	0.88

Thus, the smallest vesicles showed the greatest incremental factor in size. This shows that, for a given lipid type and given preparative method, the equilibrium state attained after fusion is the same for different starting conditions.

Effect of lipid composition of vesicles

To study the effect of the type of headgroup and net charge of the lipid, vesicles were made by ethanol injection from anionic PS, zwitterionic PC and mixtures of these. They were fused by freeze-thawing, and by Ca^{2+} addition. Results are plotted in Fig. 1 which records the extent of fusion in terms of energy transfer, ϕ_{ET}, and also in terms of the net ratio R of light scattering increment. As discussed under Methods, R is a measure of the weighted ratio of fused to unfused vesicles.

Pure PC was unreactive towards Ca^{2+}; PS/PC mixtures responded only when the PS content was over 50%. The response to freezing also increased with increasing PS content, but not as steeply as the response to Ca^{2+}. The enhanced reactivity of PS and PS-rich mixtures towards freezing appears to arise from the greater amount of water associated with such vesicles, compared to PC. This point is discussed further in a later section.

TABLE IV

EXTENT OF FUSION OF PS VESICLES MADE BY ETHANOL INJECTION AS FUNCTION OF INITIAL SIZE

PS in ethanolic injection solution (mg/ml)	Reduced light scattering (i_{90}/c)		Net scattering increment (R)
	Initially	After freezing	
10	25	215	7.6
20	43	258	5.0
30	72	252	2.5

Inhibition of fusion during freezing in presence of electrolytes

To clarify further the role of ionic interactions [22,23] PS and PC vesicles were prepared by injection in 0.005 M salt, and frozen in presence of salts at various concentrations up to 3.5 M. Light scattering measurements were made before and after fusion. As shown in Table V, the net scattering ratio R declined with increasing NaCl concentration, both for PS and PC. Because both

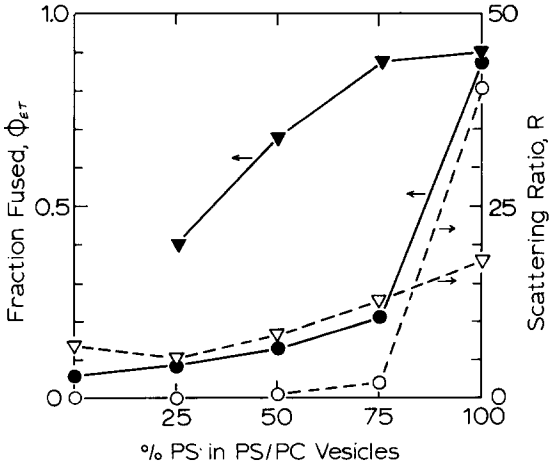

Fig. 1. Effect of PS content in PS/PC vesicles made by injection. Fraction fused, determined by energy transfer: ▼, on freezing; ●, on Ca^{2+} addition. Net scattering increment, R: ▽, on freezing; ○, on Ca^{2+} addition.

TABLE V

INHIBITION OF FUSION OF INJECTED PS AND PC VESICLES BY ELECTROLYTES IN EXTERNAL SOLUTION

Net scattering increments, R are given.

Lipid	Electrolyte	Molarity			
		0.005	0.1	0.5	3.5
PS	NaCl	9.0	5.5	3.9	3.8
	KI	7.0		2.0	1.9
	KNO_3	5.8	1.0	0.3	0
PC	NaCl	3.7		1.2	0.9
	KI	4.0		4.0	0.3
	KNO_3	2.6		2.6	2.6

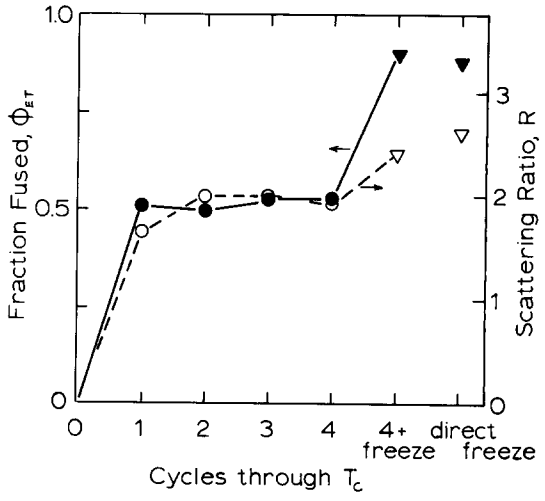

Fig. 2. Extent of fusion of injected DPPC vesicles by cycling through the transition temperature, and freezing. Fraction fused: ●, during T_c cycling; ▼, after freezing. Net scattering increment: ○, during T_c cycling; ▽, after freezing.

lipids were affected, and charge apparently was not a factor, it was considered possible that NaCl, with a eutectic point of −22°C, might lower the local freezing point in the vicinity of the bilayer below the t_c of the lipids which are +5 to +1°C for PS, and −7 to −15°C for PC.

To check this, another salt, KNO_3, with a eutectic point of only −3°C, was tested. This salt inhibited fusion of PS but not of PC, as seen in the table, evidently because freezing had occurred below the T_c of PS, thus inhibiting its fusion, but above the T_c of PC so that its fusion was not diminished. To allow for a possible effect due to weaker binding of K^+, compared to Na^+ [24], measurements were also made with a third salt, KI, with a eutectic point of −22°C. As seen in the table, KI inhibited the fusion both of PS and PC, as predicted by this hypothesis, although only when raised to 3.5 M for the latter lipid. The inhibitory effect seen at different salt concentration can be understood qualitatively by considering that the local freezing point will decrease with increasing local ionic concentration, to a limit represented by the eutectic point.

A lowering of the local freezing point below the T_c of the bilayer therefore is seen to be an important factor controlling the extent of fusion during freezing.

Fusion by passage through the transition temperature

Passage of lipid bilayers through their liquid crystal - gel phase transition temperature, T_c, or incubation slightly above T_c, is known to cause rapid fusion. Since the natural lipids used in the preceding experiments have t_c values between +5 and −15°C, freezing them at −78°C necessarily includes passage through T_c. To observe the effect of passage through T_c separately from freezing effects, vesicles made from DPPC, with a t_c of 41°C, were used. They were separately labeled with DCY and Chl. After being formed by injection at 60°C, the two populations were mixed, slowly cooled to 1°C, and slowly rewarmed to 60°C. This cycle was repeated four times. Light scattering and energy transfer were measured initially, and after each cooling/warming step.

As shown in Fig. 2, energy transfer, ϕ_{ET}, and the light scattering ratio R increased sharply after one cooling/warming cycle, and leveled off during successive cycles. The vesicles were then frozen, thawed, and reheated above T_c. This resulted in further sharp increases in these values. The final values were essentially the same as those found in a sample which had been frozen and thawed after preparation at 60°C, i.e. after one passage through T_c.

Another DPPC sample was doubly labeled with DCY and Chl. Energy transfer measurements showed that these membrane labels were fully

retained during four cycles through T_c, and during subsequent freeze-thawing.

These results show that different equilibrium states are reached by passage through T_c, and by a freeze-thaw treatment. As measured by energy transfer and light scattering, these states represent fusion of different fractions of the small vesicles originally present. The retention of membrane labels by DPPC vesicles during fusion, in contrast to PS and PC, may be a consequence of the saturation of this lipid.

Correlation of light scattering and energy transfer increments

Experimental data from the preceding sections were used to examine the correlation of light scattering increments resulting from fusion with the corresponding increase in energy transfer a measure of the degree of completion of the fusion reaction. Plots of the scattering ratio R as a function of f, the fraction of vesicles fused (obtained from energy transfer, ϕ_{ET}) are shown in Fig. 3. The points fell onto three distinct curves for fusion by passage through T_c, by freezing, and by Ca^{2+} addition. The scattering ratio increased most steeply with energy transfer for Ca^{2+} fusion and less steeply for freezing, in agreement with the much larger fused structures seen in electron micrographs after Ca^{2+}. Fusion by T_c passage gave the smallest rise in R, implying the smallest fused structures. According to Eqn. 7, the linear proportionality of the data, seen for fusion by passage through T_c and at low extents of fusion by freezing, implies that in these cases the average size of the aggregates was constant within each fusion method. Upward curvatures indicate that the aggregate size increased when fusion by freezing neared completion, and that it increased throughout the range with increasing extent of fusion by Ca^{2+}.

It is of interest to note that points for sonicated and injected PS vesicles fell onto the same curves, within each fusion method. Points for mixtures of highly reactive PS with less reactive PC also fell onto the same curves as those for pure PS.

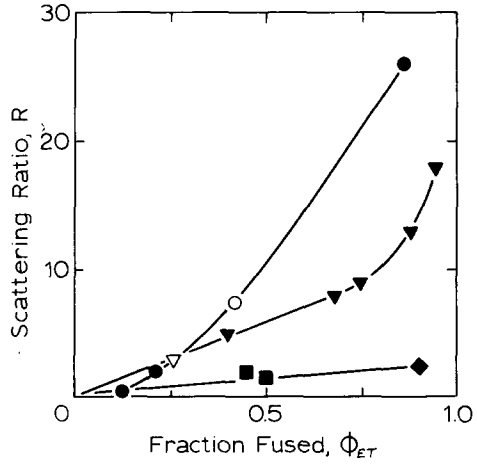

Fig. 3. Correlation of net scattering increment $R = (i/i^0) - 1$, with fraction of vesicles fused as determined by energy transfer, ϕ_{ET}. Sonicated PS vesicles: ▽, after freezing; ○, after Ca^{2+}. Injected PS vesicles: ▼, after freezing; ●, after Ca^{2+}. Injected DPPC vesicles: ■, after T_c cycling; ◆, after freezing.

Discussion

The correlation of the light scattering ratio R with f, the fraction fused (from energy transfer), supplemented by qualitative observations from electron microscopy, clearly establishes that fusion of small unilamellar vesicles results in fused aggregates having a narrow range of sizes which is characteristic for each fusion method. At low extents of fusion, the reaction may thus be written (with M standing for monomer vesicles)

$$n\,\mathrm{M} \overset{K}{\rightleftharpoons} \mathrm{M}_n$$

where the equilibrium constant K depends on fusion conditions, as discussed below, and n is approximately constant. An alternative course of fusion in which essentially all monomers fuse, but form a range of differently-sized aggregates, must be ruled out as the principal mechanism. A secondary reaction, represented by

$$m\,\mathrm{M}_n \rightarrow \mathrm{M}_{mn}$$

may come into play when the fusion nears completion and the concentration of free monomers is depleted.

The losses of liposome constituents and their uptake from solution during fusion show that a transient loosening of the bilayer structure occurs, increasing the permeability. The much greater

losses during freezing than on Ca^{2+} addition may be a consequence of the lateral condensation and tightening of the bilayer by Ca^{2+}.

The greater reactivity of vesicles made by the ethanol injection method, compared to sonicated vesicles, seems to arise from a looser packing and possible exposure of projecting lipid molecules. From the standpoint of reaction mechanisms, the different behavior of these vesicle types shows that distinct, non-interconvertible types of small vesicles exist. Injected vesicles thus represent a metastable state of higher energy than sonicated ones. Both of these can fuse into larger vesicles of basically the same size and same lower energy, but the equilibrium constant is larger for the fusion of injected vesicles. A similar observation can be made for injected vesicles of different initial sizes (formed by varying the lipid concentration in the ethanol solution): All fuse to give about the same final size.

The various means of inducing liposome fusion can now be seen as procedures which trigger a reorganization of metastable small vesicles into larger structures. All experimental results indicate that removal of water associated with the bilayer is one effect common to all methods of initiating fusion: During freezing, water is removed as ice; addition of Ca^{2+} causes displacement of water by competitive binding to polar sites; and transition from liquid crystal to gel phase also removes water since the gel is compact and binds less water. Creation of a discontinuity, as occurs during phase transition or on binding of Ca^{2+} [25], may be regarded as another basic effect initiating fusion, but actually this too is a way of removing water.

The different extents of fusion, that is, fractions of small vesicles reacted, can be shown to correlate with the amount of water removed in each instance:

(a) More extensive fusion and solute incorporation during freezing of anionic PS vesicles than of neutral PC vesicles can be related to the higher hydration of PS, inferred by Finer and Darke [26] from NMR linewidths. Different degrees of hydration can also be expected on the basis of the number of charged groups (three in PS, two in PC), even though one cannot distinguish between bound water and bulk water, as demonstrated by Lis et al. [27].

(b) Greater reactivity of vesicles made by injection, compared to sonicated vesicles, is consistent with a more open structure and, presumably, more water surrounding the exterior portions of the lipids.

(c) The inhibitory effects of different salts during fusion by freezing support the concept that fusion is more extensive when freezing occurs while the bilayer is above its T_c than in cases where the salt has lowered the freezing point below T_c. In the latter case, the bilayer is in the gel phase which is compact, less hydrated and therefore relatively immune to the disruptive effect of water removal. Addition of Ca^{2+} to a bilayer below its T_c (such as DPPS at room temperature) also causes much less fusion than above T_c, for the same reason.

(d) In addition to effects due to a lowering of the freezing point below T_c by certain salts, there may also exist specific effects connected with the structure-breaking ability of certain anions which can interfere with the hydrogen-bonded water structure around polar groups, as discussed by Chapman et al. [28].

Water surrounding the bilayer exerts a strong repulsive pressure which prevents close approach of vesicles and their fusion [12,27]. The fact that small vesicles are stable for prolonged periods, despite their high energy due to curvature and packing strain, means that removal of associated water is prevented by an activation energy barrier. This water effectively locks in the metastable small-vesicles structure, or several types of such structures. Triggering by one of the fusogenic methods provides the energy needed to surmount the energy barrier.

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