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GENERATION OF MULTILAMELLAR AND UNILAMELLAR PHOSPHOLIPID VESICLES

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Multilamellar and unilamellar vesicles can be generated by a variety of techniques which lead to systems with differing lamellarity, size, trapped volume and solute distribution. The straightforward hydration of lipid to produce multilamellar vesicles (MLVs) results in systems which exhibit low trapped volumes and where solutes contained in the aqueous buffer are partially excluded from the MLV interior. Large trapped volumes and equilibrium solute distributions can be achieved by freeze-thawing or by 'reverse phase' procedures where the lipid is hydrated after being solubilized in organic solvent. Unilamellar vesicles can be produced directly from MLVs by extrusion or sonication or, alternatively, can be obtained by reverse phase or detergent removal procedures. The advantages and limitations of these techniques are discussed.

Keywords: liposomes; multilamellar vesicles; large unilamellar vesicles; freeze-thaw; extrusion; reverse phase evaporation.

I. Introduction

Most diacyl phospholipids and mixtures thereof spontaneously adopt the bilayer organization when dispersed in excess water [1] to form closed vesicular structures ('liposomes'). In this work we consider the methods of dispersal, which can dictate whether these bilayer systems are multilamellar or unilamellar, large or small, and which can result in large variations in trapped volumes and trapping efficiency.

The simplest liposome to construct is obtained by the mechanical dispersion of dry lipid in water. The resulting structures are usually MLVs which consist of concentric bilayers separated by narrow aqueous channels. Since their initial characterization [2], MLVs have been employed extensively to determine details of bilayer structure. The regular arrays of bilayers in MLVs are ideally suited for X-ray studies, whereas their relatively large size ($\geq$400 nm diameter) makes structural and motional analysis employing nuclear magnetic resonance (NMR) more straightforward than in smaller systems (see Ref. 1 and references therein).

However, the size, heterogeneity and the presence of many internal compartments limit the use of MLVs in studies of bilayer properties such as permeability and fusion. Permeability studies are restricted due to the complications in interpret-

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ing ion flux across numerous internal bilayers. Similarly, the interactions between apposed bilayers during membrane fusion cannot be easily studied in MLV systems because of the size heterogeneity and the lack of a single well-defined internal compartment [3].

Methods have therefore been devised to produce single bilayer vesicles. These include disruption of preformed MLVs by sonication [4] and extrusion [5,6] or modifications of the phospholipid hydration procedure such that unilamellar systems form spontaneously. This is achieved in some solvent evaporation procedures [7,8] and detergent dialysis techniques [9,10].

The unilamellar vesicle is normally categorized as either a small unilamellar vesicle (SUV) or LUV [7] though a clear distinction between these two vesicle types is often difficult to make (see §IV). LUVs, which are usually more stable than SUVs and exhibit significantly larger trapped volumes, are the most popular model membrane system.

The purpose of this review is to summarize the large number of processes developed for generating liposomal systems and provide a rational guide to the reader who is looking for a particular model membrane to suit a specific purpose.

II. Characterization of Liposomal Preparations

Liposomal preparations can be adequately characterized by three main parameters. The first is lamellarity, which concerns the number of internal lamellae sequestered within MLVs and therefore not exposed to the external medium. Size is also an important parameter as is the distribution of sizes within a particular preparation. The third property of closed bilayer structures is the trapped volume. Here we indicate appropriate techniques to determine these parameters.

1. Lamellarity

MLV preparations are usually heterogeneous and can consist of single bilayer systems in addition to MLVs. However, an averaged indication of lamellarity is given by the proportion of lipid exposed to the external medium. This reflects the lipid located in the outer monolayer of the external bilayer. This will be a small proportion of the total lipid for highly multilamellar systems but will be approximately half of the total lipid in (large) unilamellar systems.

Labelling of amino-containing lipids by impermeable reagents [11] or binding of radiolabeled ions [12] has been used to determine outermonolayer lipid. However, one of the most accurate and straightforward procedures for determining the lamellarity of phospholipid dispersions is to use ^{31}P-NMR to monitor the phospholipid phosphorus signal intensity. In particular, adding an impermeable paramagnetic or broadening reagent [6] to the external medium will decrease the intensity of the initial ^{31}P-NMR signal by an amount proportional to the fraction of lipid exposed to the external medium.

Electron microscopy can provide a more qualitative indication of lamellarity.

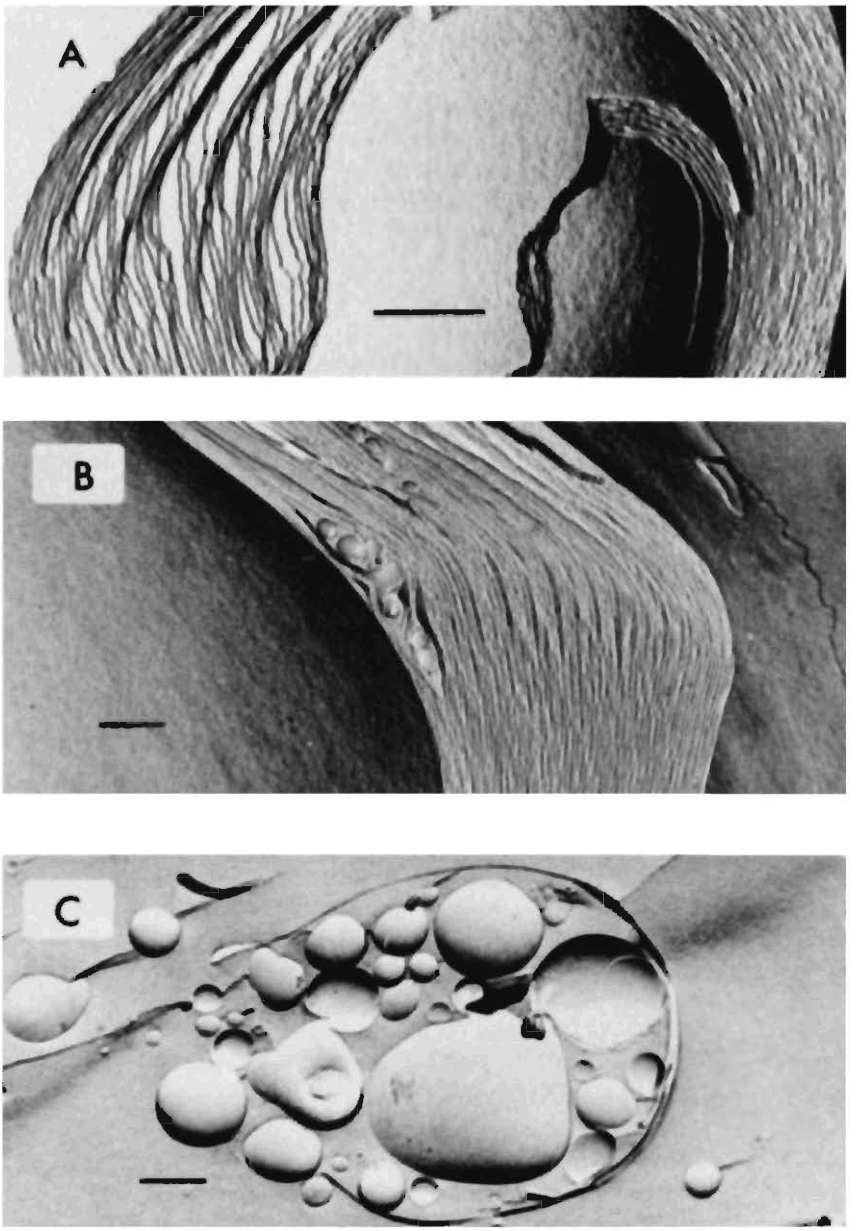

Fig. 1. Freeze fracture micrographs of egg-PC at 50 mg/ml concentration. (A) MLVs prepared in 150 mM NaCl, 20 mM HEPES at pH 7.4. (B) MLVs in the same buffer containing 25% (v/v) glycerol. MLVs were subjected to 5 freeze-thaw cycles as described in the text. (C) FATMLVs, prepared by subjecting MLVs to 5 freeze-thaw cycles in the absence of glycerol. Bar = 200 nm.

Both negative staining [13] and freeze-fracture [14] techniques are used regularly. However, negative staining is subject to artefacts that arise as vesicle dispersions are dried on grids. The resolution is often poor and the collapse of vesicles on top of vesicles to give rise to apparently multilayered systems complicates interpretation. Freeze-fracture, on the other hand, provides a unique view of internal lamellae (see Fig. 1) when cross-fracturing occurs. However, cross-fractures are usually relatively rare events and, as a result, freeze-fracture only provides a rough indication of lamellarity.

2. Size

The size distribution of liposomal systems is often a difficult parameter to measure. This is particularly true for MLVs which usually exhibit broad size distributions (centred around diameters of a micron or more) where a single technique may not be able to monitor all sizes present. Techniques such as employed by the Coulter channelizer [15] can give some estimate but pitfalls exist. More unimodal MLV size distributions can be achieved by extrusion of the dispersions through filters with relatively large pore sizes (e.g. 1 μm).

A variety of techniques can be employed for MLV and LUV systems with mean diameters <1 μm, including electron microscopy and light scattering procedures. Freeze-fracture electron microscopy procedures [16,17] assume spherical vesicles. If the angle of deposition of the shadowing material is 45° to the fracture plane then vesicle fracture faces that are 50% shadowed are cleaved equatorially and so reflect the true vesicle diameter [17].

Light scattering techniques, particularly laser-based quasi-elastic light scattering protocols, are becoming increasingly popular. These techniques are based on the fact that the time-dependent coherence of light scattered by a vesicle is sensitive to vesicle diffusion which is dependent on the viscosity of the aqueous medium and vesicle size. This technique can be applied to advantage for unimodal systems with mean diameters <1 μm, obtaining size distributions in good agreement with freeze-fracture techniques [18]. The advantage is that such information can be obtained in minutes, but misleading results are easy to obtain for heterogeneous systems exhibiting bimodal or more complex size distributions.

3. Trapped volume

The aqueous trapped volume of a particular vesicle preparation is normally expressed as the volume entrapped per lipid, and varies from 0.5 μl/μmol for some MLV and SUV preparations to 30 μl/μmol for some large unilamellar systems. In combination with information on size, trapped volume can be used as an indicator of vesicle lamellarity. For example, unilamellar vesicles with a mean diameter of 400 nm should exhibit a trapped volume of $\sim$13 μl/μmol phospholipid assuming an area per molecule of 0.7 nm^2 and a bilayer thickness of 4 nm (Fig. 2). If the size is known but the measured trap is substantially less than the theoretical value for LUVs of this size, it can be assumed that an appreciable proportion of the vesicles

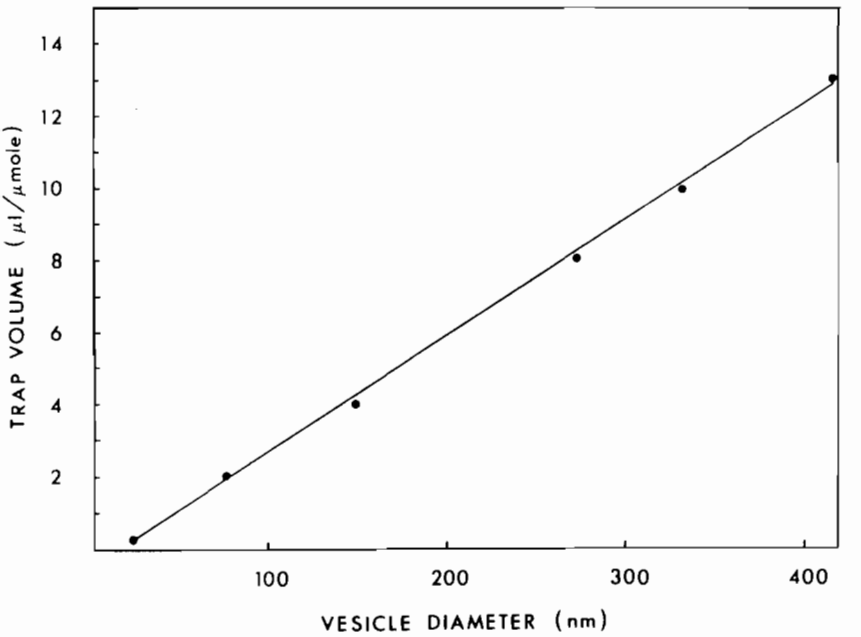

Fig. 2. A plot of calculated trapped volumes (μl/μmol of phospholipid) against the mean diameter of a ULV population. A Gaussian size distribution is assumed with a Gaussian spread of 40 nm. The area per phospholipid molecule was taken to be 0.7 nm^2 and the bilayer thickness 4 nm.

are multilamellar. Trapped volume is also a good indication of the extent of swelling or hydration in an MLV preparation (see §III).

Trapped volume is normally determined by dispersing lipid in an aqueous medium that contains a radioactive solute, such as ^{22}Na or $^{3}H/^{14}C$ inulin, which does not readily permeate across bilayers. The proportion of solute trapped is ascertained after isolating the MLVs from external radioactivity by centrifugation, dialysis, or gel filtration, for example, and the aqueous trapped volume can then be calculated [6].

III. MLVs

1. Hydration and solute distribution

The primary event in the preparation of model membrane systems from dry lipids is hydration. This is the most important step in producing MLVs as the degree of hydration is most sensitive to the technique employed. Here we examine the process in detail.

The most common laboratory procedure for MLV formation was originally described by Bangham et al. [2] and involves the drying of lipid dissolved in organic

TABLE I

COMMON PROCEDURES FOR THE GENERATION OF MLVs AND UNILAMELLAR VESICLES

Category	Technique	Type	Trapped volume[a] (μl/μmol lipid)	Advantages	Disadvantages	Refs.
Direct hydration	Aqueous medium added to dry lipid	MLV	~0.5	Fast procedure	Low trapped volume; low trapping efficiency; unequal distribution of solute	2
	Plus freeze-thaw	FATMLV	5–10	Fast procedure; high trapped volumes; high trapping efficiency	Solute dependent	14, 18
	MLVs extruded through 0.1 μm polycarbonate filter	LUV	1–2	High trapping efficiency for extrusion techniques;	Trapped volumes relatively low unless freeze-thaw protocol is employed	6, 18
	Plus freeze-thaw	LUV	1–10	no detergents		18
	Sonication/french press	SUV	0.2–0.5	or solvents used; fast procedures		5, 16, 31
	Plus freeze-thaw	LUV	1–10			33
Hydration from organic solvent	SPLV technique	MLV	1–10	High trapping efficiency	Technically complex; limited by lipid solubility in organic phase; residual organic solvent.	20
	MPV technique	MLV	1–10			20
	Reverse phase evaporation	LUV	~10			16
	Ether evaporation	LUV	10–20			38
Detergent removal	Cholate (deoxylcholate) dialysis/gel filtration	LUV	0.5–5	Reconstitution of proteins possible; high trapped volumes	Detergents difficult to remove completely; procedures lengthy; generally low trapping efficiency; limited when using lipid mixtures.	10, 42
	Octylglucoside	LUV	~10			9

[a]Trapped volumes dependent on lipid concentration and lipid type, see references.

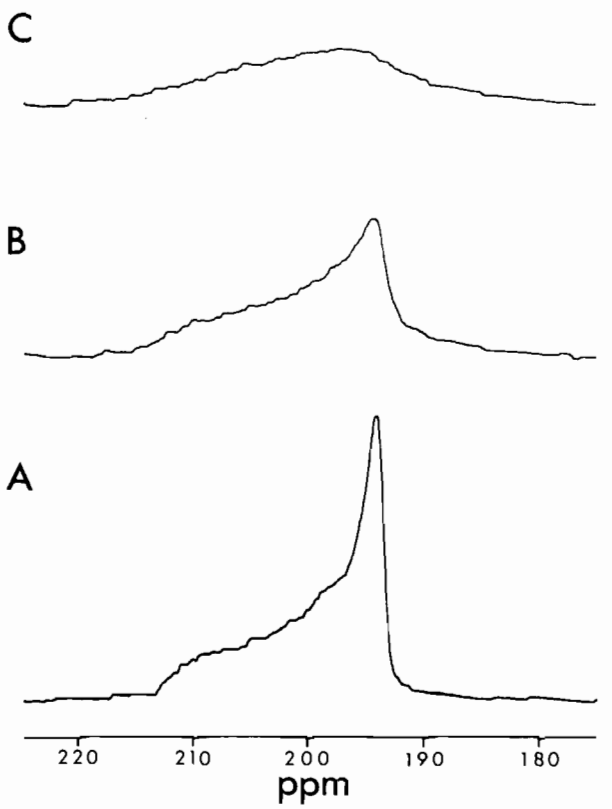

Fig. 3. 81.0 MHz ^{31}P-NMR spectra of egg-PC MLVs dispersed (A) in the absence of Mn^{2+} and (B) in the presence of 0.5 mM Mn^{2+}. The spectrum of part (C) was obtained from the MLVs dispersed in the presence of 0.5 mM Mn^{2+} which were subsequently subjected to five freeze-thaw cycles. The ^{31}P-NMR spectra were collected at 20°C employing a Bruker WP200 spectrometer employing a 20 KHz sweep width, 2 s interpulse delay and broad band proton decoupling. Phospholipid concentration was 100 mg/ml.

solvent (by vacuum evaporation or convection) to form a dry lipid film on the walls of a flask or test tube. An aqueous buffer is then added to hydrate the lipid film and MLVs are formed upon gentle agitaton of the mixture.

By way of example, egg phosphatidylcholine (egg-PC) MLVs produced at a concentration of 50 μmol/ml in this manner result in systems where less than 10% of the total lipid is present in the outer monolayer and thus exposed to the external medium [6,14]. The MLVs are heterogeneous in size, varying 0.05–10 μm in diameter. Moreover, the ratio of internal trapped volume to lipid is low, on the order of 0.5 μl/μmol of lipid [14] (see Table I). Characteristic freeze-fracture planes are observed in which cross-fractures graphically reveal the stacked inner-lamellae (Fig. 1A).

It is interesting to consider the progression of the hydration process during formation of these MLV systems. The major point to note is that the lipid on the surface of the dry film will first adopt bilayer structure in response to the aqueous buffer. Water can permeate readily through such bilayers [19,20], but ions and other solutes permeate much more slowly. It may therefore be expected that the outermost bilayers act as a molecular sieve, where water permeates through to achieve equilibrium hydration of interior bulk lipid (~30–40 molecules of water per phospholipid [19]) but where solutes are excluded. This would suggest that the interbilayer solute concentrations should be lower than exterior concentrations for MLV systems, and effects corresponding to such non-equilibrium solute distributions have been observed [14,20]. A particularly graphic example of this is shown in Fig. 3B, where it is shown that dispersion of PC in a buffer containing Mn^{2+} still gives rise to a 'bilayer' ^{31}P-NMR signal even though sufficient Mn^{2+} is present to broaden the resonance appreciably. As discussed in §IV, freeze-thawing the dispersion (or solubilizing by addition of detergent) allows all phospholipids to experience the Mn^{2+} to the same extent, resulting in a much broader ^{31}P-NMR spectrum (Fig. 3C). These results are consistent with a non-equilibrium distribution of solute in the initial MLV preparation.

The bilayer separation in MLVs is determined by an equilibrium between attractive van der Waals forces and repulsion due to electrostatic and hydration forces [19]. For phosphatidylcholines this equilibrium distance is approx. 2.5 nm, equivalent to about 35 molecules of water per phospholipid [19]. In order to improve the aqueous trap and ensure an equal distribution of solute between internal aqueous compartments, methods of improving hydration resulting in increased separation are necessary. In the following sections, we review various methods of achieving this.

2. Lipid composition

The internal trap of neutral phospholipid MLVs can be increased by incorporating charged lipids into the membrane [19,21]. The effect of the resulting surface charge is to increase the electrostatic repulsion between bilayers thus inducing swelling. X-ray analysis demonstrates inter-bilayer separations in excess of 10–20 nm for pure charged lipid systems as well as PC MLVs containing small proportions of charged lipid when hydrated in water [12,19,21]. Even in 150 mM salt, swelling is reflected in the increased trap volume of egg-PC when egg phosphatidylserine (egg-PS) is incorporated into the membrane. This is demonstrated in Figs. 4A and 4B. Figure 4A shows the trapped volume increase that occurs for egg-PC MLVs when they are subjected to freezing and thawing (see §III.3). The maximum trap obtained under these conditions was 9 μl/μmol lipid. However, Fig. 4B demonstrates that frozen and thawed MLVs that contain <2 mol% PS exhibit trap volumes of 15 μl/μmol. A positive surface charge is as effective as negative surface charge at inducing swelling [12].

3. Freeze-thaw

Freezing and thawing MLV preparations can offer a convenient procedure for

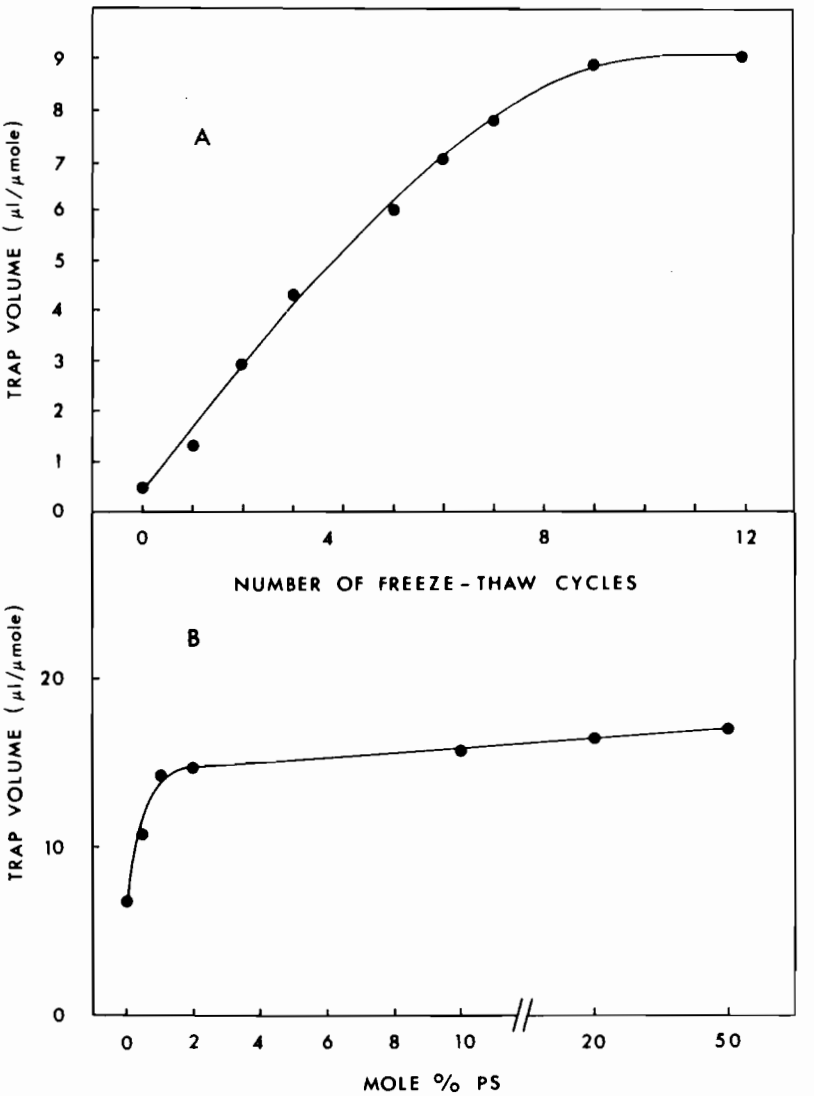

Fig. 4. (A) The trapped volumes (μl/μmol of lipid) of egg-PC MLVs that have been subjected to freeze-thaw cycles employing liquid N_2. Phospholipid concentration was 50 mg/ml in 150 mM NaCl, 20 mM HEPES at pH 7.4. (B) The trapped volumes of egg-PC MLVs containing various amounts of egg-PC. Each sample was subjected to five freeze-thaw cycles at a lipid concentration of 50 mg/ml in 150 mM NaCl, 20 mM HEPES at pH 7.4. All trapped volumes were determined employing [^{14}C] inulin.

increasing trapped volume and promoting equilibrium solute distributions. The MLV morphology is dramatically altered by this procedure, as shown in Fig. 1. Figure 1C shows a freeze-fracture micrograph of an egg-PC MLV sample (50 mg/ml) that has undergone five cycles of freezing in liquid nitrogen followed by thawing in

warm water [14]. The resulting frozen and thawed MLV (FATMLV) gives rise to cross-fracture characteristics not observed in the precursor MLV samples (Fig. 1A). Vesicles within vesicles and vesicles between lamellae are commonly observed. Cross-fractures revealing closely stacked lamellae are rarely seen and the improved swelling is reflected by a large increase in trapped volumes. Most importantly, if FATMLVs are formed in the presence of manganese, the ^{31}P-NMR signal is identical to that obtained for the same mixture solubilized in detergent (Fig. 3C). In other words, the freeze-thaw procedure results in equilibrium distribution of solute between lamellae in the FATMLV system.

The mechanism by which freezing and thawing improves the swelling of MLVs and induces solute equilibration is not clear. However, it likely reflects a physical disruption of lamellar structure, possibly by ice crystals formed during the freezing process. This is supported by the observation that addition of cryoprotectants prior to freeze-thawing increases the number of cross-fractures typical of normal MLVs (Fig. 1A) whilst reducing the type of fracture typical of FATMLVs as shown in Fig. 1C. It is interesting to note that small vesicles can be observed forming within the stacked bilayer sheets as shown in Fig. 1B. The generation of small systems during freeze-thaw cycles is also observed by ^{31}P-NMR. An isotropic component appears in the ^{31}P-NMR spectrum of egg-PC following 10 freeze-thaw cycles (Fig. 5C). Even after two cycles a small isotropic component can be observed, as shown in Fig. 5B. The isotropic signal arises from the rapid diffusion of phospholipid around bilayer systems with diameters $\leqslant$200 nm [1].

4. Reverse phase

The term 'reverse phase' is used here as a general term for the hydration of lipids directly from an organic solvent. When dissolved in an organic phase, lipid molecules are dispersed in monomer form. Several procedures have been developed whereby hydration occurs either as the organic phase is diluted, or as solvent evaporates. In both instances lipids aggregate into bilayer structures as the hydrophilic nature of their environment increases.

Reverse phase procedures have been employed to directly produce LUVs [7,8] as well as MLVs [20], and this type of hydration can circumvent the disadvantages associated with direct addition of aqueous medium to dry lipid, described in §III.1. Unfortunately, a number of other restrictions are imposed; for instance, a major limitation is the solubility of lipids in the solvent being used and subsequent removal of solvent from the final preparation.

Many organic solvents have been employed in reverse phase procedures, but ether and ethanol are perhaps the most common. MLVs with high trapping efficiencies can be produced by adding aqueous buffer containing the agent to be trapped to an ethanolic solution of lipid, which is then dried under vacuum. This results in a dry lipid film within which solute is trapped. Subsequent hydration results in an MLV preparation that efficiently traps the solute initially present within the film [20,23]. Similarly, stable plurilamellar vesicles (SPLVs) produced from lipid dissolved in ether are also multilamellar preparations but with unique properties of

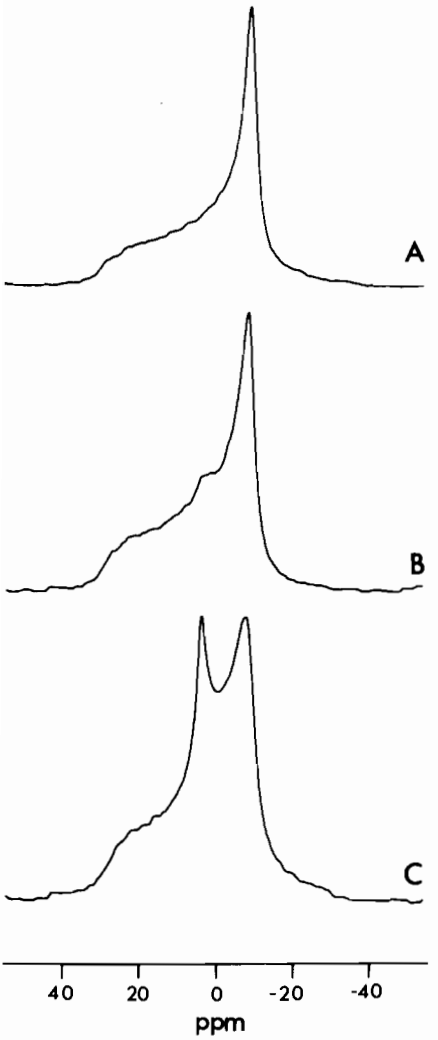

Fig. 5. 81.0 MHz ^{31}P-NMR spectra of egg-PC MLVs dispersed at 100 mg/ml in 150 mM NaCl, 20 mM HEPES at pH 7.4. (A) MLVs prior to freezing and thawing, (B) MLVs subjected to two cycles of freezing and thawing, (C) MLVs subjected to ten cycles of freezing and thawing. Freezing and thawing was performed using liquid N_2 and warm water. The ^{31}P-NMR spectra were collected at 20°C employing a Bruker WP200 spectrometer employing a 20 KHz sweep width, 0.8 s inter-pulse delay and broad band decoupling.

stability [20]. In this process, a small volume of buffer is added to an ether-lipid solution and the mixture is then bath sonicated while ether is evaporated. As the ether phase is depleted, multi-bilayer systems form in the residual aqueous phase. These MLV preparations exhibit an equilibrium solute distribution [20].

A related technique is known as reverse phase evaporation (REV) and produces vesicles which generally exhibit fewer lamellae than the MLV systems described so far [7,16]. REVs are formed from an emulsion of ether (containing dissolved lipid) and buffer. The ether is gradually removed under reduced pressure to form a gel thought to consist of a continuous bilayer network enclosing aqueous pockets [7]. Vortexing and continued rotary evaporation under vacuum collapses the gel into a mixture of oligolamellar and unilamellar vesicles, which exhibit relatively high trapped volumes (see Table I).

5. Dehydration-rehydration

The majority of model membrane research is carried out on model systems prepared by direct hydration or reverse phase technology. However, a method of producing MLVs with high trapping efficiency specifically designed for pharmacological applications is the dehydration-rehydration procedure [24,25]. The technique involves the drying of either MLVs or unilamellar vesicles in aqueous medium in the presence of solute followed by controlled rehydration. Both freeze-drying (lyophilization) and direct drying by vacuum or convection are effective. During the drying process, lipid vesicles are concentrated concomitantly with solute, and at some point, fuse into large aggregates containing the material to be trapped. If rehydration is performed gradually, MLVs are produced as described in §III.1. However, solute is already present in many of the interbilayer spaces, and so efficient trapping occurs.

In summary, MLVs produced by hydration of a dry lipid film result in systems exhibiting low trapped volumes and non-equilibrium solute distributions. Larger trapped volumes and equilibrium solute distributions can be achieved by a variety of procedures including freeze-thaw, dehydration-rehydration, and reverse phase (see Table I). Remaining differences between these MLV systems likely arise due to the proportion of closely stacked lamellae remaining, the size of the systems, lipid composition and salt concentrations. The choice of a particular procedure is likely to be dictated to some extent by the nature of the agents to be entrapped in the MLV systems [26]. For example, entrapment of a hydrophobic drug may well be optimized in a reverse phase procedure where the drug is cosolubilized (with the lipid) in the organic phase. Alternatively, for water soluble molecules freeze-thaw and hydration techniques are more suitable.

IV. Unilamellar Vesicles

Unilamellar vesicles are the most popular model membrane systems. The well-defined aqueous compartment enclosed by a single bilayer enables their use in a wide range of applications. It is common practice [7,16] to characterize unilamellar vesicle systems as small (sonicated) SUVs and LUVs. For the purpose of this review, we will consider SUVs as 'limit size' vesicles. A bilayer vesicle composed of diacyl-

phospholipids has a limit size, below which the radius of curvature at the inner-monolayer becomes so acute that lipid packing restraints prevent smaller diameters being achieved. For (egg) PC this diameter is approx. 25 nm [4], and vesicles of this diameter exhibit a 2:1 excess of lipid in the outer monolayer compared with the inner monolayer and an aqueous trapped volume on the order of 0.2 μl/μmol phospholipid. Characteristics of these small systems include greater lipid disorder and vesicle instability [26,28,29]. As vesicle diameters exceed 40–50 nm, packing constraints decrease markedly [26]. Consequently, we will refer to vesicles above 50 nm in diameter as LUVs and those below as SUVs.

The techniques available to produce SUVs or LUVs can be divided into three major categories. These are techniques which produce unilamellar systems directly from MLVs, techniques which involve initial solubilization of the lipid in organic solvent (reverse phase) and techniques which utilize detergents in the initial solubilization of lipid.

1. Unilamellar vesicles produced from MLVs

Three procedures can be employed to produce unilamellar vesicles directly from MLV preparations. Until recently, the most common procedure has been to sonicate MLVs to form limit size vesicles [4]. Such SUVs can also be produced by French press techniques [5,30,31]. These small systems offer certain advantages for drug delivery applications [16,30,31]; however, use of limit size vesicles is restricted due to their instability and low trapped volumes [28]. The size distribution of a particular preparation is dependent on the lipid composition. For example, inclusion of equimolar cholesterol results in an increase in the limit size from 25–30 nm to 30–50 nm [31,32], presumably because cholesterol limits the curvature of the bilayer.

The inherent instability of SUVs offers a means by which they can sometimes be enlarged to form LUVs. For example, incubation of sonicated vesicles at 4°C for 3–4 weeks [28] or subjecting them to a freeze-thaw cycle [33] induces fusion. This results in LUVs on the order of 80 nm diameter [29,33]. Practical problems with these techniques include a dependence upon the composition and ionic strength of the aqueous medium, as well as lipid composition. Moreover, frozen and thawed vesicle systems are often heterogeneous and the presence of high concentrations of solute (when trapping drugs, for example) sometimes reduces the effectiveness of freeze-thaw protocols.

2. Medium pressure extrusion

Sequential extrusion of MLVs at low pressures (< 80 lb/in^2) through filters of decreasing pore size has been shown to produce LUV systems [22]. It has recently been shown that the repeated extrusion of MLVs under moderate pressures (800 lb/in^2) through two stacked polycarbonate filters of 0.1 μm pore size results in a relatively homogeneous population of LUVs with a mean diameter of

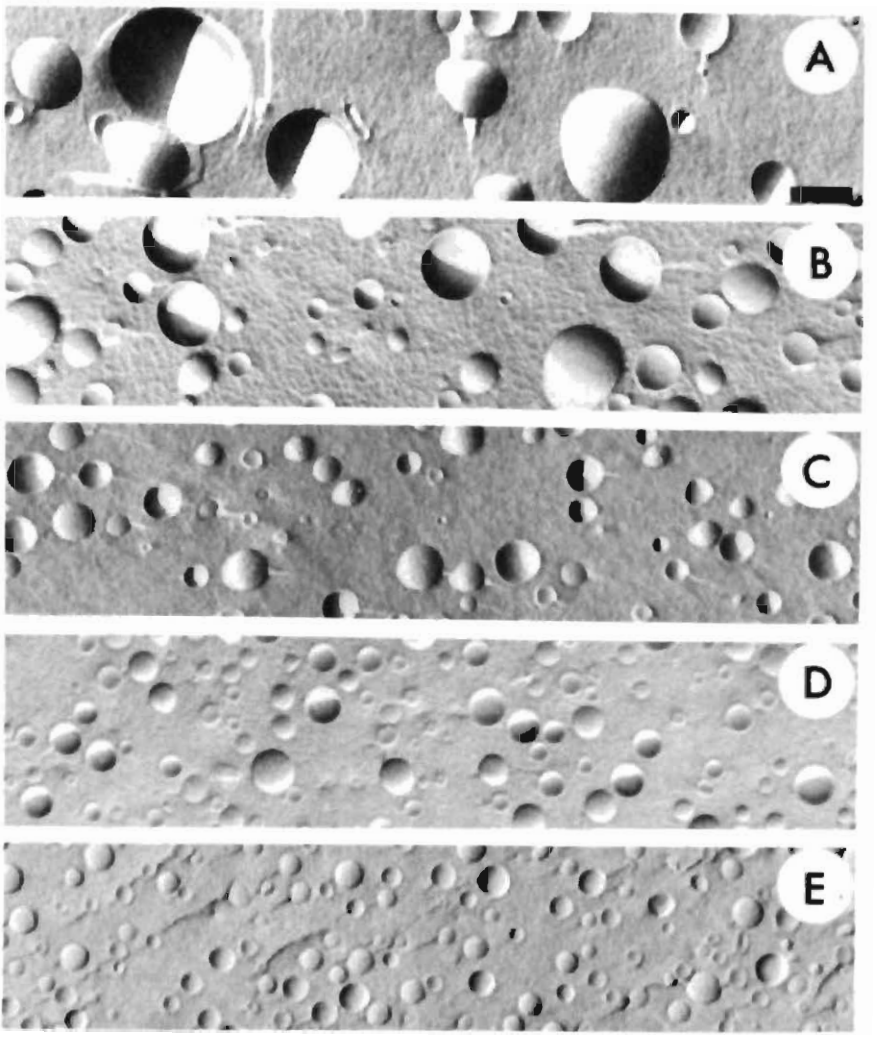

Fig. 6. Freeze-fracture electron micrographs of FATMLVs passed 20 times through filters of various pore sizes. Vesicles were prepared from egg-PC at 100 mg/ml. The pore sizes of the filters employed were (A) 400 nm, (B) 200 nm, (C) 100 nm, (D) 50 nm and (E) 30 nm. The bar in panel A represents 150 nm and all panels exhibit the same magnification.

approx. 90 nm [6]. The considerable advantage of this technique is that it is rapid (preparation time on the order of 10 min), works directly from MLVs and on a wide variety of lipid species and mixtures. In combination with freeze-thaw protocols, it is possible to produce homogeneous populations of unilamellar vesicles in the range 40–150 nm [6,18] by using filters of different pore size. Freeze-fracture micrographs of vesicles by extrusion techniques (VETs) produced by extrusion of

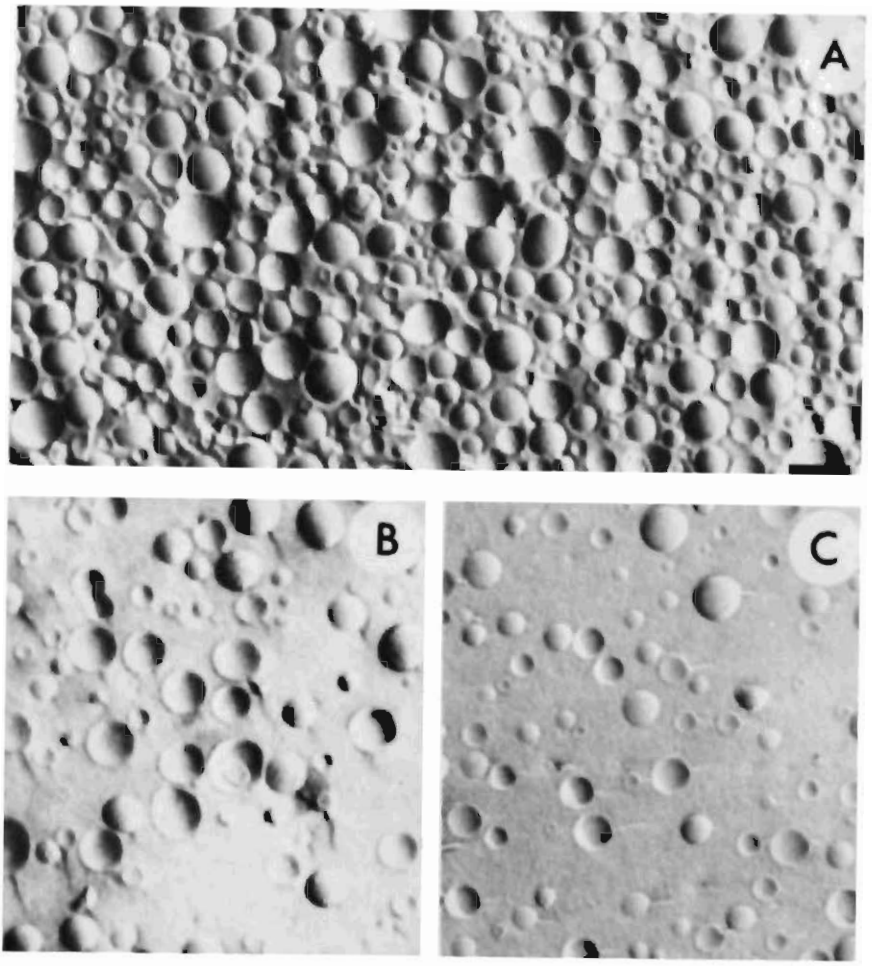

Fig. 7. Freeze-fracture electron micrographs of FATMLVs passed through 100 nm pore size filters. (A) Vesicles extruded at 400 mg egg-PC ml of aqueous buffer; (B) vesicles from (A) diluted to 100 mg lipid/ml; (C) vesicles extruded at 100 mg lipid/ml. All micrographs are at the same magnification and the bar = 150 nm.

FATMLVs through a variety of filter pore sizes are shown in Fig. 6. Approximately 90% of the vesicles passed through a filter with a pore size of 200 nm (Fig. 6B) are unilamellar [18]. A major feature of the extrusion procedure is the fact that concentrations as high as 400 mg/ml can be employed, enabling high aqueous trapping efficiencies of 60% for VET_{200} systems. Figure 7 shows the freeze-fracture plane of egg-PC LUVs prepared at 400 mg/ml (diluted 25% with glycerol).

The major advantage of this technique over other procedures designed to produce

LUVs is that vesicles are prepared directly from MLVs, eliminating the problems associated with removal of organic solvents or detergents from final preparations. This technique, however, is limited to producing LUVs in the diameter range 40–150 nm. On the other hand, the most commonly used LUV systems fall within this size range; for instance, 100 nm LUVs appear to be the optimum model membrane size for drug delivery to cells in vitro [34]. Such vesicles also readily pass down gel filtration columns without clogging, enabling the rapid exchange of external buffers. This is useful for permeability studies [6,33,35].

3. Unilamellar vesicles from organic solvents

Hydration of lipids from an organic phase (§III.4) can result in the spontaneous formation of largely unilamellar vesicles. For example, two common procedures produce LUVs upon injection of lipid dissolved in ethanol [36] or ether [8,36,38] into buffer. The difference between the two techniques is that for lipid solutions in ethanol, hydration occurs upon solvent dilution whereas in the ether procedure hydration occurs as solvent evaporates.

Ethanol dilution is performed rapidly and vesicle formation presumably occurs locally near the point of injection. However, when lipid dissolved in ether is injected into an aqueous phase maintained at 60°C, LUVs are thought to form at the air/water interface as bubbles of ether surrounded by monolayers or multilayers of lipid leave the solution [38]. As shown in Table 1, the trapped volumes produced by the ether injection procedure are higher than for most other LUV procedures.

These procedures suffer from a fundamental problem faced by all reverse phase techniques, namely, the solubility of lipid and lipid mixtures in the organic phase. Diethyl ether [38], petroleum ether [37], or pentane [38] can be used in this technique, and methanol is sometimes added to increase the solubility of saturated lipids in these solvents [38].

Since its inception the reverse phase evaporation (REV) protocol has become a widely accepted method for LUV production [7,16]. This is despite the inherent problems associated with hydration of lipid from organic phases and the need for column chromatography or dialysis to remove traces of solvent from vesicle preparations. The main attractions of this protocol are the high trapping efficiencies and the ability to produce vesicle systems to high lipid concentrations. The REV procedure requires that lipid be solubilized in organic solvents such as diethyl ether, isopropylether or mixtures such as isopropylether and chloroform (1 : 1) depending upon the solubility of the lipids being used. Solvent and buffer are combined at specific volume ratios depending upon the organic phase chosen (volume ratios and lipid concentration are critical factors in the protocol). An emulsion is formed by sonicating mixtures for 15–30 min followed by the careful removal of solvent at 400 mmHg pressure until a thick 'stable' [7] gel has been formed. Formation of the gel is also a critical part of the process (discussed in §III.4) and is dependent upon the ratio of surface area/volume of the preparation during the evaporation procedure [16]. The gel is collapsed by vortexing or mild sonication and evaporation con-

tinued under vacuum (as achieved by a water aspirator) for 30 min. The resulting dispersion is a heterogeneous mixture of oligolamellar and unilamellar vesicles which requires extrusion through polycarbonate filters to achieve relatively homogeneous preparations of unilamellar vesicles [16,39]. Consequently, as a method for producing LUVs with diameters in the range of 100 nm, it is now superceded by procedures that are faster, more flexible and do not require organic solvents [6,18]. On the other hand, reverse phase hydration of some lipids and lipid mixtures from ether is most effective at producing, spontaneously, a largely unilamellar preparation with high trapped volumes (see Table I) and trapping efficiencies, and may trap some solutes more effectively than other techniques [16].

4. Unilamellar vesicles from detergents

Detergent removal represents the third main category of procedures commonly employed to produce LUVs. These techniques have arisen from methods first devised to reconstitute integral membrane proteins into lipid bilayers [40,41].

Dry lipid or pre-formed vesicles are first solubilized in the desired (detergent containing) buffer to form mixed micelles. As detergent is then removed by dialysis, the micelles coalesce and the phospholipid adopts the bilayer configuration resulting in sealed vesicles. The type of detergent employed as well as the rate and method of detergent removal determine the type of LUV preparation obtained [9,10,42,43]. For example, if egg-PC and deoxycholate (2:1) are mixed by sonication and the bile salt is subsequently removed by gel filtration, unilamellar vesicles are produced with an average diameter of 100 nm [10]. Similar procedures employing octylglucoside as detergent results in relatively large unilamellar vesicles on the order of 250 nm diameter [9]. All detergent removal techniques are dependent upon the type of lipid or lipid mixture being used. For instance, the detergent/lipid ratios and rates of detergent removal (slow dialysis, gel filtration) may have to be modified in order to get reproducible LUV dispersions from different lipid mixtures. This was found to be the case for octylglucoside preparative procedures applied to synthetic phospholipids [29] rather than natural egg-PC [9].

Elimination of contaminating detergent is the primary problem in this technique. Consequently, the majority of procedures employ cholate (deoxycholate) or octylglucoside as these detergents can be removed most rapidly and completely [16,42]. LUVs are produced in the 50–200 nm diameter size range and must be subjected to a variety of treatments designed to remove residual detergent from the bilayer. These include exhaustive dialysis over several days, rapid dialysis employing large surface areas of exchange (as in hollow fibre techniques), gel filtration and dialysis against adsorbent resins that lower detergent monomer concentrations [42]. Despite these treatments, residual detergent is often present at concentrations on the order of 1 mol/100 mol phospholipid [42], levels that might be expected to influence lipid disorder and bilayer permeability properties. However, LUVs produced by octylglucoside dialysis exhibit permeability coefficients for sodium that are similar to those obtained using LUVs produced by other techniques [6,9]. Generally,

problems associated with detergent removal which include low trapping efficiency and length of preparation limit the use of detergent-based techniques in producing protein-free LUVs. On the other hand these procedures are often the only available method whereby integral membrane proteins can be incorporated into unilamellar vesicle systems [40,41; also Ref 44 and references therein].

VII. Concluding

It should be clear from this review that techniques for the rapid and efficient generation of well characterized multilamellar and unilamellar lipid systems are now generally available. In our view a particularly important advance concerns the observation that standard MLV systems may not exhibit equilibrium transbilayer solute distributions. The subsequent advent of techniques (such as freeze-thaw) to achieve equilibrium solute distributions without involving organic solvents and the ability to rapidly produce unilamellar systems from multilamellar precursors represent additional important progress.

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