Novel Multilayered Lipid Vesicles: Comparison of Physical Characteristics of Multilamellar Liposomes and Stable Plurilamellar Vesicles†

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ABSTRACT: The preparation of a new kind of multilayered liposome, called a stable plurilamellar vesicle (SPLV), is described. Although SPLVs and classical multilamellar vesicles (MLVs) are made of the same materials and appear overtly similar in the electron microscope, the two types of vesicles differ as determined by stability, entrapment efficiency, electron spin resonance (ESR), NMR, X-ray diffraction, and biological effects. It is demonstrated that, contrary to what has been assumed, classical MLVs exclude solutes during their formation and, thus, are under a state of osmotic compression. By contrast, the SPLV process produces liposomes that are not compressed. The effects of osmotic compression are discussed. It is suggested that the state of osmotic stress is an important variable that distinguishes various types of liposomes and that has significant physical and biological consequences.

The idealized multilamellar vesicle (MLV),[1] or liposome, consists of a sequence of concentric lipid bilayer shells enclosing some central aqueous volume. For egg phosphatidylcholine (EPC) liposomes the bilayers are separated by aqueous layers of a well-defined width (typically 10–20 Å thick), which is a compromise between the repulsive and attractive forces between the bilayers (Rand, 1981). Because liposomes are often intended to be used in vivo as drug carriers, the aqueous fluid normally contains buffered physiological saline, as well as other dissolved solutes. The standard method for producing MLVs involves vacuum drying lipid dissolved in a solvent, such as chloroform, to a thin film onto the bottom of a round-bottom flask (Bangham et al., 1965). Liposomes are formed by adding the aqueous solution and shaking or vortexing until the dry film is removed from the wall of the flask.

An alternative procedure for producing multilamellar liposomes, which have been named stable plurilamellar vesicles (SPLVs), is reported below. MLVs and SPLVs exhibit markedly different physical properties. For example, the two types of liposomes differ in their stability, ESR, NMR, and X-ray diffraction signals, and encapsulation efficiency. Moreover, antibiotics encapsulated in SPLVs are effective in curing Brucella infections (Fountain et al., 1985). By contrast, MLV encapsulation of the same antibiotics are considerably less effective in curing Brucella infections (Dees et al., 1985; Fountain et al., 1985). These results were puzzling because both MLVs and SPLVs are made from the same materials and look overtly similar in the electron microscope. Consequently, we were led to investigate the source of the physical differences between the two types of liposomes.

The exact details whereby a dry lipid film is converted to MLVs is not well understood. Lipid bilayers are semipermeable membranes: water passes readily through them, but solutes, such as salts, are retained (Bangham et al., 1967). It has been generally assumed that the aqueous solute concentration is uniform throughout the MLV. In this paper we demonstrate that standard methods of producing MLVs result in vesicles with aqueous compartments that are depleted in solutes, i.e., that the MLV formation effects a separation of water from its dissolved solutes. This leads to an osmotic stress on the liposomes. By contrast, the SPLV process produces multilamellar liposomes with a uniform solute distribution and relatively little osmotic gradient on the liposomes. In other words, MLVs are really quite different than previously thought while SPLVs conform closely to the picture that was thought to characterize MLVs. The state of osmotic stress on the liposomes affects a broad spectrum of physical properties of the vesicles and has profound implications on the understanding and use of lipid bilayers.

Experimental Procedures

MLV Preparation. EPC (100 mg in chloroform, Sigma Chemical Co., type VIIe) was rotary evaporated (room temperature) to a thin dry film in a 50-mL round-bottom flask. Occasionally, as described in the text, other lipids and/or solvents were used. Two milliliters of an aqueous phase (typically, HEPES buffer consisting of 72.5 mM KCl, 72.5 mM NaCl, and 10 mM HEPES, pH 7.4) was added to the flask. If a particular solute was to be entrapped, it was also mixed into the aqueous phase added to the flask. The flask was vortexed until the lipid film coating the flask was completely suspended. The suspension was then set on the bench to equilibrate for 2 h after which it was washed 4 times. Each wash was done by mixing the suspension with buffer to a total volume of 20 mL, followed by centrifugation (10000*g*) to pellet the liposomes. The supernatant was removed, and the pellet

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[1] Abbreviations: DOPC, dioleoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; EPC, egg phosphatidylcholine; MLV, multilamellar vesicle; MPV, monophasic vesicle; LSS, long-spacing signature; SPLV, stable plurilamellar vesicle; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ESR, electron spin resonance.

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was then resuspended for further washes or to a designated final volume.

SPLV Preparation. EPC in chloroform (100 mg) or, occasionally, other lipids as described in this text were rotary evaporated to dryness in a 50-mL round-bottom flask. The lipid film was dissolved in 5 mL of ethyl ether. Then, 0.3 mL of the aqueous phase (typically HEPES buffer) was added to the ether–lipid solution. If a particular solute was to be entrapped, it was dissolved in the aqueous phase prior to adding it to the ether–lipid solution. The two-phase mixture (aqueous and ether) was emulsified in a bath sonicator (Laboratory Supplies Co., Model G1125P1G), during which time a gentle stream of nitrogen was passed over the mixture. This was continued for approximately 2 min until the ether was largely evaporated and ether could no longer be smelled. The resulting cake was resuspended in 10 mL of buffer by swirling the fluid in the flask. This liposome suspension was pelleted and washed, as described for MLVs.

MPV Preparation. An EPC film was prepared in a 50-mL round-bottom flask by rotary evaporation from chloroform, as for SPLVs. Five milliliters of 95% ethanol and 0.2 mL of HEPES buffer were added to the flask, and the flask was vortexed until the lipid film was dissolved. The result was a monophase solution of lipid, ethanol, and the buffer. The monophase was then rotary evaporated to dryness. The film contained the solutes, which were dissolved in the buffer. This dry film was suspended and washed as described under MLV Preparation.

Gas Chromatography. Ether contamination of the liposomes was measured on a Beckman GC72-E gas chromatograph equipped with a flame-ionization detector and a Spectra Physics "minigrator" electronic integrator. The column was a Waters Associates copper $6' \times {}^1/_4''$ poropak P. The carrier flow was 60 cm^3/min. The detection limit was 250 μM.

ESR. SPLVs and MLVs were made as previously described, except that 1 mol % of 5-doxylstearate (Molecular Probes, Junction City, OR) was added to 40 mg of EPC in chloroform prior to the initial rotary evaporation step. After the formation of liposomes, the preparations were washed, and ESR spectra of both samples were recorded. Following the addition of ascorbate (10 mM final concentration), spectra were recorded at regular intervals in order to follow reduction of signal as a function of time.

Electron Microscopy. Liposomes (0.025 mL of a 100-mg EPC liposome suspension in 1.0 mL of buffer containing 30% glycerol) were applied to a gold specimen support. This was immersed in liquid freon 22 maintained just above its freezing point (−168 °C) and then transferred to liquid nitrogen. The support was then transferred to a Balzars freeze–fracture apparatus while still cold. After evacuation, the samples were cleaved with the microtome arm, and the ice was sublimed for 3 min. A thin film of carbon–paladium was then evaporated on the freshly exposed surface. Replicas were removed and cleaned of lipid by placing them in liquid bleach overnight. The replicas were picked up on copper grids and examined on a Phillips EM300.

Problems inherent in determining vesicle-size histograms from freeze–fracture micrographs arise from (1) biased field selection and (2) the fact that a fracture plane through a frozen liposome does not necessarily reveal a given liposome's diameter. To obviate the former, micrograph fields were taken without consciously selecting objects other than ensuring that a given micrograph contained several liposomes. Fields were taken at eigher 3400× or 14000×. All distinct liposomes in the micrographs were measured with a magnifying loupe equipped with a reticle. For oblate spheroids, the largest reasonable diameter was selected. The sample sizes always exceeded 200 measurements.

X-ray Diffraction. X-rays were generated on a Rigaku RU-200 X-ray generator using a 0.2 × 2 mm focus cup and a loading of 50 kV, 60 mA. The beam was focused horizontally via single-mirror Franks optics and collimated vertically as described in Gruner (1977). X-rays were detected by using a quantum-limited two-dimensional slow-scan TV detector (Gruner, 1977; Reynolds et al., 1978), yielding the X-ray intensity in each of 240 × 240 adjacent areas or pixels. Typical X-ray exposure times were 5–30 s. The low-angle diffraction consisted of concentric rings of Bragg orders arising from the liposome multilayer repeat. X-ray patterns were real-time reduced to one-dimensional traces of intensity vs. scattering angle by radial integrations over 20–50° of the two-dimensional pattern. Multilayer repeat spacings were determined by a least-squares fit to the peak positions of the Bragg orders, where the peak positions were taken as the centers of parabola least-squares fit to the peak profiles.

Wide-angle X-ray patterns were acquired via the TV detector and reduced to one-dimensional traces via radial integration. As opposed to the low-angle patterns, the high-angle integrations at each radius were divided by the length of the arc integrated at that radius.

The X-ray specimen cell consisted of a 1 mm thick neoprene washer jacketed by two thin mylar or aluminum foil X-ray windows. Liposomes were concentrated by spinning at 10000*g* for 5 min. A few milligrams of the pellet were loaded into the well formed by the hole in the neoprene washer and the lower X-ray window. The washer–window sandwich was clamped between copper plates machined to slide snugly into a thermostated jacket (±1 °C; slew rate ~0.3 °C/s). Small holes in the thermostat and copper plates allowed passage of the X-rays through the specimen. A typical X-ray run consisted of diffraction patterns taken at several temperatures with 2-min equilibration times after temperature changes.

Nuclear Magnetic Resonance. We are indebted to Dr. Pieter Cullis of the University of British Columbia for seeing to the performance of the NMR. SPLVs and MLVs were prepared and suspended in HEPES buffer containing 2 mM $MnCl_2$. ^{31}P NMR spectra were collected at 20 °C by employing a Bruker WP 200 Fourier-transform NMR spectrometer operating at 81 MHz for ^{31}P. Accumulated free-induction decays (FIDs) from 5000 transients were obtained with a 20-kHz sweep width, a 7-μs 90° radio-frequency pulse, and a 1-s interpulse time in the presence of gated broad-band proton decoupling. An exponential multiplication corresponding to 50-Hz line broadening was applied to the FID prior to Fourier transformation.

MLV and SPLV signals were accumulated under identical conditions, and samples were run immediately after each other so that the relative signal intensities were accurate. The integrated ^{31}P NMR spectra were normalized by the SPLV and MLV concentrations (84.2 and 109.9 mg/mL, respectively) so as to be directly comparable.

Enzyme Digestion. MLVs and SPLVs were made containing a ^3H label in the membranes and a ^{14}C label in the aqueous spaces by mixing trace quantities of [^{3}H]DPPC with the EPC and by preparing the liposomes in buffer containing traces of [^{14}C]sucrose. The [^{3}H]DPPC had the label on the choline head groups. These double-labeled liposomes were washed 4 times and divided into nine 0.5-mL aliquots so that each aliquot contained 5.0 mg of lipid. Phospholipase C (0.2 unit) was added to each fraction, and timing was initiated.

Immediately, and every 15 min thereafter for 2 h, one fraction from each group was removed and spun at 10000*g*, 50 μL of the aqueous supernatant was recovered, and the counts per minute of each label was determined by liquid scintillation counting. Phospholipase C digests PC by cleaving the phosphocholine head groups that contain the ^3H label. Thus, the two reaction products are [^3H]phosphocholine and mixed diglycerides. Since neither of these compounds is capable of maintaining membrane integrity, the [^{14}C]sucrose entrapped within the aqueous space circumscribed by a bilayer will be released as the bilayer is digested. Upon centrifugation, three distinct layers are seen: a layer of diglycerides floating on the surface, a clear aqueous supernatant, and a pellet composed of undigested liposomes. Since both [^{14}C]sucrose and [^3H]-phosphocholine are water soluble, counting the supernatant layer will indicate the amount of membrane digested, as well as the amount of solute ([^{14}C]sucrose) released. These data are plotted as a ratio of [^3H]phosphocholine/[^{14}C]sucrose, which indicates the amount of membrane digested per amount of solute released.

Results

One measure that distinguishes between liposomes is the efficiency with which an aqueous solute may be entrapped. Entrapment is defined as the fraction of the initial solute (see Experimental Procedures) remaining with the liposomes after four washes. If radiolabeled inulin is dissolved in the water used to prepare the liposomes, MLVs typically entrapped 2% of the inulin whereas SPLVs entrapped 37%. Incubating the MLVs for 48 h in the labeled inulin buffer prior to washing only marginally increased the relative entrapment. If one assumes that 2% (2% × 2 mL = 40 μL) of the inulin solution is entrapped in MLVs and that 37% (37% × 0.3 mL = 111 μL) is entrapped in SPLVs, then the latter should contain 111/40 = 2.8 times as much fluid. In fact, upon centrifugation, SPLVs yielded pellets that were larger than MLVs, but only by about 30%. Consequently, the difference in entrapment efficiency cannot be simply due to the difference in the volume of the initial aqueous fluid. When the experiment was done with $^{22}NaCl$, the MLVs entrapped 2% and the SPLVs entrapped 27%.

The rheological properties of MLV and SPLV pellets differed. After centrifugation (10000*g*), and upon pouring off the supernatant, the MLV pellet remained intact in the bottom of the test tube. By contrast, the SPLV pellet was soft and tended to run out of the tube with the last of the supernatant. This difference was apparent even after the first preparative wash (see Experimental Procedures).

MLVs and SPLVs were also distinguished by the way they leaked entrapped markers. In general, SPLVs maintained their entrapped solutes dramatically longer than MLVs. This was demonstrated by mixing the calcium-sensitive dye Arsenazo III (3 mM) in the buffer used to prepare the liposomes. The liposomes were then washed to remove any unentrapped Arsenazo III, suspended in HEPES buffer with 500 mM $CaCl_2$, purged with N_2, sealed in screw-top vials, and stored at room temperature. Leakage of the Arsenazo III was readily detected by eye by the color change that occurred when the dye contacted Ca^{2+}. Whereas MLVs leaked within a few days, the SPLVs did not leak after 15 months. A number of antibiotics have also been tested (Table I), and the results also demonstrated the stability of the SPLV entrapment.

Another experiment that demonstrated that SPLVs and MLVs differed relied on the ability of ascorbate to reduce nitroxide spin probes. In aqueous solutions the reduction occurs rapidly with concomitant loss of ESR signal. If the spin probe is in a protected environment, such as within the hydrocarbon of a lipid bilayer, it may be reduced more slowly or not at all by the hydrophilic ascorbate ion. Thus, the rate of nitroxide reduction can be used to study the rate of penetration of the ascorbate ion into lipid bilayers. Figure 1 shows the percentage remaining spin signal vs. time for SPLVs and MLVs suspended in an ascorbate solution. At 90 min, the ascorbate has reduced 25% of the probe embedded in MLVs but 60% of the probe embedded in SPLVs. The simplest interpretation is that SPLVs allow for a dramatically greater penetrability of ascorbate than do MLVs.

Table I: 14-Week Stability of SPLV Antibiotic Entrapment[a]

entrapped drug	initial entrapment (%)	leakage into supernatant[b]	bioavailability of entrapped drug (%)
streptomycin sulfate	34.1	0	97
spectinomycin	37.2	0	84
chloramphenicol	35.2	0	89
oxytetracycline	18.8	0	91
sulfamerazine	6.3	0	93

[a] SPLVs were prepared with 127 μM egg phosphatidylcholine (EPC) and 25 μM drug. At the end of 4.5 months storage at 4 °C, the SPLVs were separated from storage buffer by centrifugation. Serial dilutions of the SPLV contents and the supernatant were applied to bacterial lawns in order to determine bioactivity as compared to standard dilutions of antibiotic. [b] 0 indicates below 1% of the amount of drug initially used.

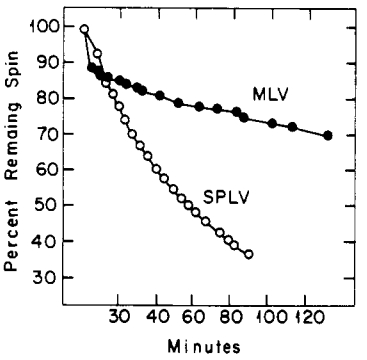

FIGURE 1: Ascorbate quenching of an ESR probe signal is shown for the two types of liposomes. The ESR probe resides in the membrane hydrocarbon.

Attempts to visualize differences between the two types of liposomes by optical microscopy were unsuccessful. Likewise, freeze–fractured vesicles appeared similar by electron microscopy. Size histograms of the freeze–fractured liposomes (Figure 2) were similar except for the appearance of a few very large MLVs.

X-ray Diffraction. X-ray diffraction was applied in an attempt to define signatures that could be used to distinguish MLVs from SPLVs. Three different X-ray signatures were found.

In the low-angle regime, both types of liposomes exhibit 3 lamellar orders of diffraction that arise from the radial stacking of membranes in the liposome. If the X-ray repeat spacing is graphed vs. temperature, the MLV and SPLV curves were seen to differ in characteristic ways (Figure 3): (a) MLVs exhibited a repeat spacing that fell linearly with increasing temperature. By contrast, the SPLV curve flattened out at higher temperatures. (b) The MLV curve fell below the SPLV curve. These two characteristics constitute the first of the diffraction signatures. We call it the long spacing signature, or LSS. The LSS is the most reliable and easily quantified of the three X-ray signatures to be discussed: for a given type of specimen the curves are repeatable to ±0.5 Å. Variation

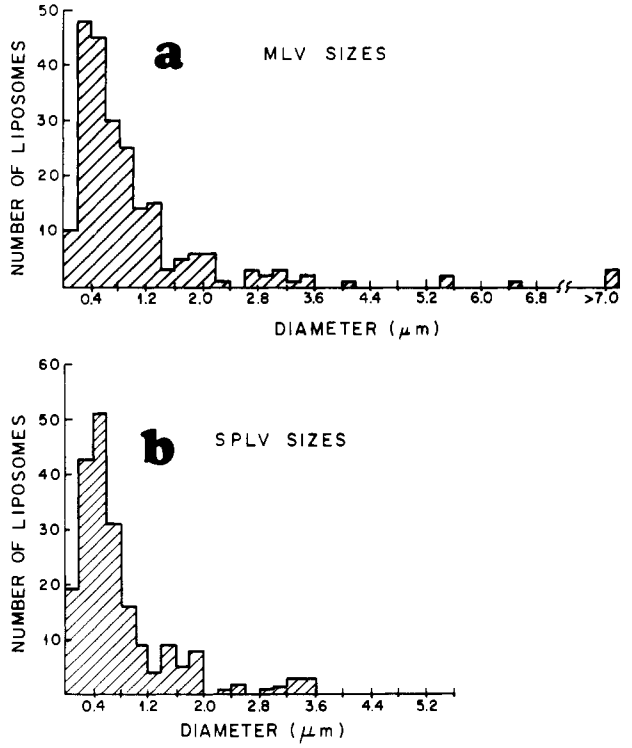

FIGURE 2: Size distribution histograms of a random sampling of MLVs (a) and SPLVs (b). The diameters of all liposomes in the electron micrograph field were included. The occasional occurrence of very large MLVs is the only substantial difference between the histograms. Although a more sophisticated sizing analysis is certainly possible, these histograms demonstrate that for most of the liposomes there are no obvious differences in the size distribution.

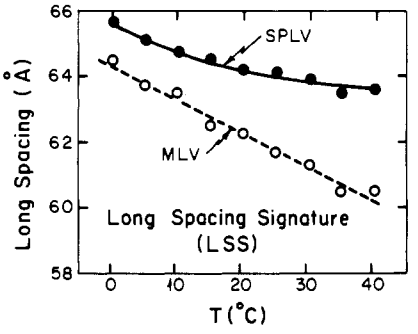

FIGURE 3: X-ray long spacing vs. temperature for MLVs and SPLVs. The characteristic forms of the MLV and SPLV curves constitute the long spacing signature, or LSS (see text).

of the liposomal parameters (e.g., lipid or buffer composition) may cause the absolute values of the curves to shift, but in so far as the liposomes are phenomenologically distinguishable as SPLVs and MLVs, the LSS appears to distinguish between the two types of liposomes (see below). The absolute values of the repeat spacings for MLVs exhibited more specimen to specimen variation and more variation over time than SPLVs. However, in all cases, even after 24 h of incubation in the preparation buffer, MLVs were clearly distinguishable from SPLVs by the LSS.

A second signature that distinguished MLVs from SPLVs was the width and asymmetry of the Bragg peaks (Figure 4a,b): MLVs exhibited peaks that were broader and often asymmetric. This may readily be interpreted as arising from a larger statistical variation of repeat spacings in the MLVs. The angle of diffraction, 2θ, for a given order, n, follows from the Bragg relation

$$n\lambda = 2D \sin \theta \qquad (1)$$

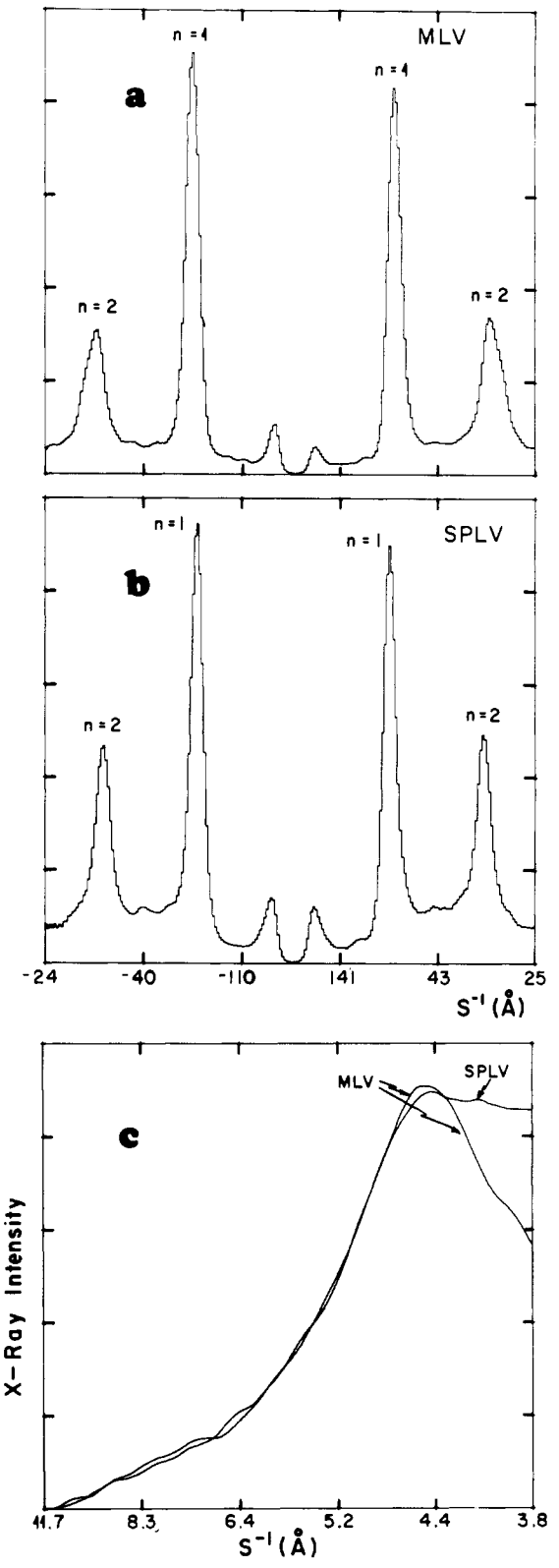

FIGURE 4: (a and b) Low-angle X-ray diffraction intensity (arbitrary units) vs. inverse reciprocal distance is shown for MLVs (a) and SPLVs (b). The first and second orders on either side of the beam stop shadow (dip near the center) are relatively sharp. However, the second-order MLV diffraction ($n = 2$) is wider than that for SPLVs and often asymmetric; this is the second X-ray signature that distinguishes MLVs from SPLVs. $T = 40$ °C. (c) Wide-angle X-ray diffraction of MLVs and SPLVs also differs, as shown. This X-ray signature is more variable than the LSS. The low-angle camera geometry is unsuitable for diffraction for $s^{-1} < 3.8$ Å. However, wide-angle X-ray film data (not shown) indicate that the SPLV diffraction falls rapidly for $s^{-1} < 3.7$ Å. $T = 10$ °C.

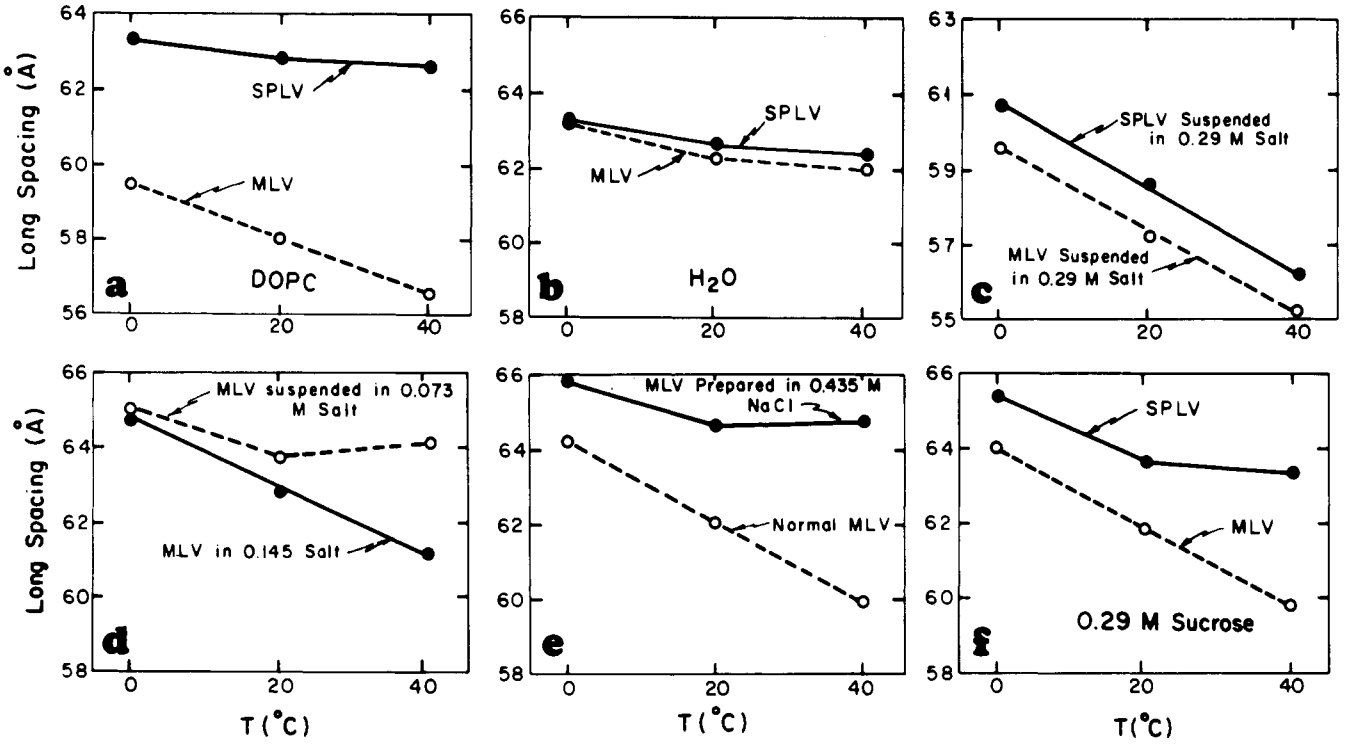

FIGURE 5: LSS for liposomes that have been prepared in different ways. In this figure, data points were acquired sequentially at 40, 20, and 0 °C. These data points are connected by straight lines to guide the eye. However, it is important to realize that the true shape of the bent curves is smooth, as seen in Figure 3. (a) DOPC liposomes; (b) liposomes prepared and suspended in distilled water; (c) liposomes prepared in 0.145 M salt but suspended in 0.29 M salt; (d) MLVs prepared in 0.145 M salt but suspended in 0.073 M salt; (e) MLVs prepared in 0.435 M salt but suspended in 0.145 M salt; (f) liposomes prepared and suspended in 0.29 M sucrose.

where λ = X-ray wavelength and D = repeat spacing of the lattice. Suppose the sample contains a distribution of repeat spacings characterized by a width ΔD. Then, for $\sin\theta \simeq \theta$, the spread in scattering angle is simply

$$|\Delta\theta| \simeq |d\theta/dD|\Delta D \simeq n\lambda\Delta D/D^2 \qquad (2)$$

Note that $|\Delta\theta|$ increases with the order number, n. This is why the peak asymmetry in Figure 4a is only apparent in the second order; the asymmetry of the first-order peak is hidden by the instrumental line width due to the X-ray camera. The use of this X-ray signature required careful measurement of the instrumental line width, which, in practice, was harder to measure than the repeat spacing. This signature also was more variable than the LSS. For these reasons, we chose to use the LSS for identification purposes.

The third X-ray signature was in the wide-angle regime ($\lambda/2 \sin\theta < 10$ Å). Melted-chain lipids exhibit a broad peak at about 4.4–4.6 Å due to correlations in the hydrocarbon region (Luzzati, 1968; Costello & Gulik-Krzywicki, 1976). As shown in Figure 4c, the MLVs yielded a well-defined peak at 4.4–4.6 Å but SPLVs exhibit diffraction that extends to much higher angles. This tells us that SPLVs have electron-density correlations that vary over a wider range of distances, in particular, over distances smaller that 4 Å. This signature required a rearrangement of the X-ray detector geometry, tended to vary, and was harder to quantify than the LSS.

The area detector that was used (see Experimental Procedures) acquired the needed low-angle X-ray exposures in 5–30 s. Moverover, the thermal kinetics of the LSS were at least as fast as our ability to slew over temperature (~0.3 °C/s) and acquire the data. Consequently, the LSS was an experimentally convenient signature in that it could be acquired in a few minutes. In what follows, we shall use the LSS to examine the liposomes that resulted when the normal MLV and SPLV preparation procedures were altered. As shall be seen, the availability of a fast, easy to measure signature of "SPLV-ness" or "MLV-ness" was essential in evaluating various hypotheses of what distinguished the two types of liposomes.

It was first hypothesized that the differences between MLVs and SPLVs may have resulted from contamination by the solvents used in the liposome preparations. When the liposomes were made, both chloroform and ethyl ether were used. These solvents, like many anesthetics, alter membrane properties (Janoff & Miller, 1982). To address whether residual ether contamination was important, SPLV ether was measured via gas chromatography. After one wash, 13 mol % ether remained; after the second wash, 0.5 mol % was left; after the third wash, the remaining ether was below the detectable limit of 0.1 mol %. Even so, ether was added to the chloroform–lipid stock solution used to prepare MLVs to see if this affected the MLVs. The resulting liposomes were indistinguishable from MLVs made without ether as indicated by the LSS (data not shown). Consequently, it was concluded that ether cannot account for the differences between MLVs and SPLVs.

Even though both MLVs and SPLVs were prepared from a lipid film dried from chloroform, it is possible that SPLVs would not contain the same concentration of residual chloroform because residual chloroform may have evaporated with the ether. To test if residual chloroform was involved, the lipid films used to produce MLVs and SPLVs were vacuum pumped for 72 h and then washed 8 times in the normal volume of wash buffer. The resulting LSS were indistinguishable from that of normal MLVs and SPLVs. (The resulting curves are, in fact, shown in Figure 3.) We conclude that chloroform cannot be responsible for the differences between the liposomes.

Another hypothesis was that the SPLV process produced bilayers that differed in acyl chain composition in the inner and outer leaflets. This was conceivable because EPC is a mixture of lipids and contains a spectrum of acyl chains. To

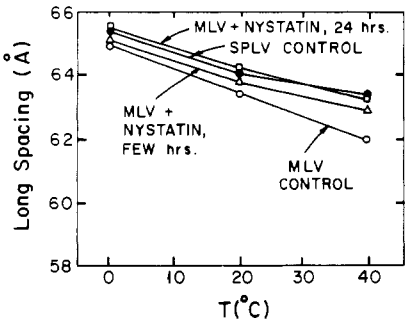

FIGURE 6: Nystatin in the presence of cholesterol is known to be a nonspecific ionophore. MLVs were prepared with EPC, 6.6 mol % cholesterol, and 1 mol % nystatin. The MLV LSS is shown after a few hours and after 24 h. Note that the MLV LSS relaxes to the LSS of the SPLV control. The MLV and SPLV controls contained cholesterol but no nystatin. The LSS of these controls did not change over the 24-h period. As seen in the MLV control, cholesterol affects the LSS; however, a detailed description of the effects is beyond the scope of this paper. At low concentrations, the effects are small.

test this hypothesis, the liposomes were made out of pure DOPC (Avanti Polar Lipids, Birmingham, AL), which contained only oleoyl chains. The LSS (Figure 5a) demonstrated DOPC MLVs and SPLVs to be distinct, thereby ruling out membrane asymmetry.

Besides lipids and water, the remaining major constituents of liposomes were the salts dissolved in the buffers used to prepare the vesicles. The fact that salts are important may be seen in Figure 5b, which shows the LSS of liposomes prepared and suspended in distilled water. Note that both MLVs and SPLVs yielded SPLV-like curves. Further experiments demonstrated that the salt concentration gradient across the liposomes, as opposed to the amount of the entrapped salt, was important. For example, Figure 5c shows that if SPLVs were prepared as normal, in 0.145 M salt, but suspended in 0.290 M salt for a few hours prior to X-ray diffraction, the resulting LSS resembles that of MLVs. Thus it appeared that a MLV-like signature resulted if an osmotic gradient was imposed on SPLVs by increasing the salt concentration outside the liposomes. The LSS typical of SPLVs could similarly be achieved by suspending MLVs in a solution of low salt concentration. Figure 5d demonstrates, indeed, that MLVs prepared with 0.145 M salt but suspended in 0.073 M salt exhibit a SPLV-like LSS. The fact that the salt *gradient* is important is also seen in Figure 5e, which shows the LSS of MLVs prepared with 0.435 M salt and then suspended in 0.145 M salt. The LSS is again seen to be typical of SPLVs.

Figure 5b–e established that a salt concentration gradient (less salt inside than outside) was sufficient to cause SPLV liposomes to reveal a MLV-like LSS and that relaxing or reversing this gradient yielded a SPLV-like LSS. A further test of this hypothesis was to prepare liposomes with a nonspecific ionophore, such as nystatin (Andreoli & Monohan, 1968; Holz & Finkelstein, 1970), incorporated into the bilayers. In the presence of cholesterol, this ionophore allows passage of salt ions and would gradually relax a salt gradient. As would be predicted, the LSS of MLVs gradually converted to that of SPLVs as the salts leaked through the nystatin pores (Figure 6).

The hypothesis that MLVs are solute depleted was also tested directly via NMR. It is known that the ^{31}P NMR signal that is associated with phospholipid is quenched by Mn^{2+}. If MLVs and SPLVs are prepared with and suspended in Mn^{2+} in the buffer, one expects the integrated ^{31}P NMR signal to be less (per unit of lipid) for SPLVs then for MLVs. The reason for this is that the SPLVs should have Mn^{2+} entrapped between the bilayers and, thus, accessible to all the ^{31}P; consequently, the ^{31}P NMR signal should be strongly quenched. By contrast, if MLVs are solute and Mn^{2+} depleted, less of the lipid signal should be quenched. When this experiment was performed (see Experimental Procedures), the integrated SPLV signal intensity was found to be 72% that of MLVs.

We next investigated if the differences between the liposomes were due to an osmotic gradient or an ionic salt gradient. To do this, liposomes were prepared in 0.290 M sucrose, which is a nonionic solute and a first approximation to the osmolarity of the 0.145 M salts that are normally used. Figure 5f shows the LSS differences between MLVs and SPLVs are preserved, suggesting that an osmotic gradient is sufficient.

The data thus far presented suggest that SPLVs have solute distributed throughout the aqueous compartments of the liposomes but that MLVs are solute depleted. It is likely that during the hydration of the dry lipid film used to make MLVs (see Discussion) water gained access to the space between the bilayers but most of the solutes were excluded. If it were possible to deposit a dry lipid film with solutes already between the layers, then the resultant liposomes might be expected to behave like SPLVs even though the dry lipid film is hydrated by the MLV process. A solute-containing lipid film can be prepared by solubilizing lipid in a solvent (e.g., ethanol) that is also miscible with water, drying the mixture under vacuum, and then rehydrating the resulting film in buffer. This process was devised by Dr. Michael Fountain of The Liposome Co. (Fountain, 1984) and produces liposomes called monophasic vesicles or MPVs (see Experimental Procedures). Note that this final hydration step is identical with that used to prepare MLVs; only the lipid film with which one starts is different. The LSS of MPVs was similar to that of SPLVs, as were the other two X-ray signatures (Figure 7). This, again, suggested that MLVs are solute depleted.

Enzyme Digestion. Information about the distribution of solute within liposomes (i.e., the solute concentration profile) was obtained via enzymatic digestion of the lipid. Liposomes were prepared with a radioactive marker incorporated in the lipid head groups and a different isotopic marker incorporated in the aqueous solution. These liposomes were slowly degraded by exposure to phospholipase C, an enzyme that cleaves the lipid head group from the glycerol backbone. The amount of head-group marker released by the enzyme was a measure of the number of lipid molecules digested, which should also be proportional to the area of the lipid bilayer involved. As a layer of the liposome broke down, the aqueous marker entrapped beneath that layer was released. Consequently, the ratio of the amount of head group to aqueous marker released measured the ratio of bilayer area to aqueous marker entrapped beneath that layer. The sharp X-ray diffraction peaks indicated that, even for MLVs, the aqueous space between the bilayers was constant to within a small fraction of an angstrom. Because all the aqueous layers were essentially of fixed width, the amount of aqueous marker released per unit area of membrane was proportional to the aqueous marker solute concentration in that layer. Mathematically, if dN_H/dt and dN_A/dt were the rates of release of the head group and aqueous markers, respectively, then

$$(dN_A/dt)/(dN_H/dt) = dN_A/dN_H = \alpha c \qquad (3)$$

where c = concentration of aqueous marker and α = a proportionality constant. If the aqueous marker concentration varied with depth in the liposome, then the ratio of marker released (the left-hand side of eq 3) would vary over time as the enzyme degraded deeper and deeper layers of the liposomes. If, for example, the aqueous marker concentration

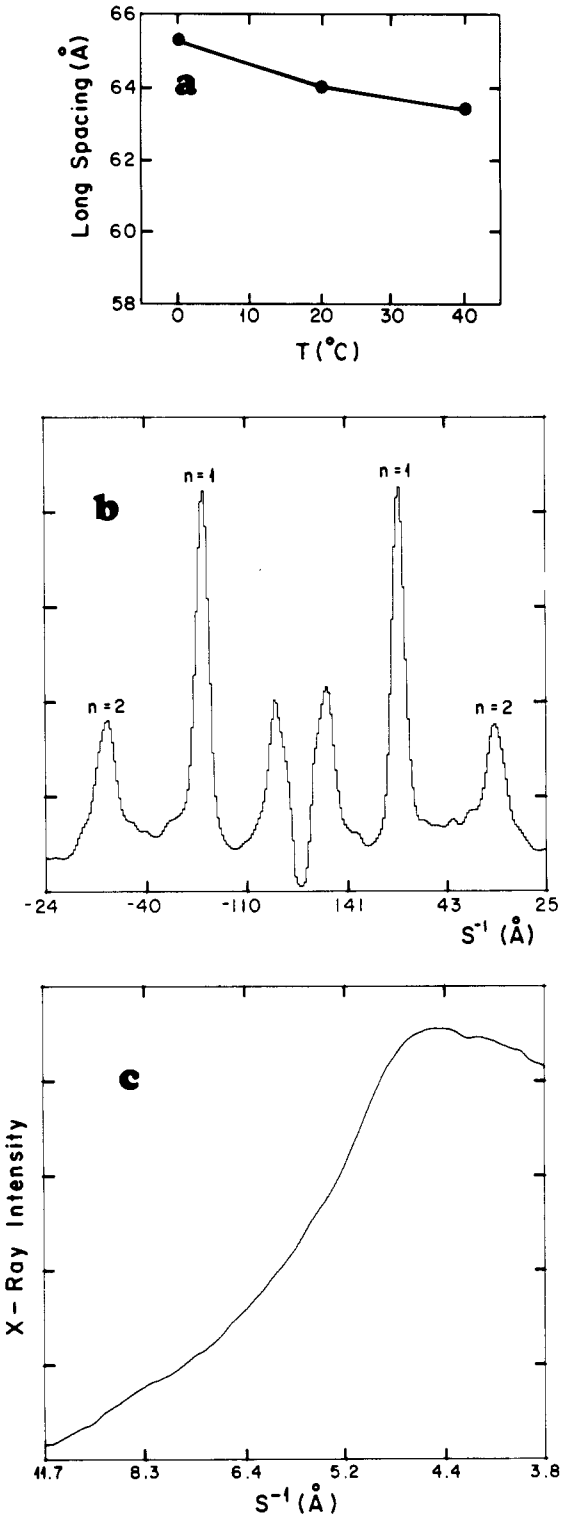

FIGURE 7: (a) MPV LSS. (b) Low-angle diffraction from MPVs illustrates the second X-ray signature. The peaks are sharp and relatively symmetric, like that of SPLVs (Figure 4a,b), even though the dry MPV lipid film is hydrated just like an MLV film. $T = 40$ °C. (c) Wide-angle diffraction from MPVs. Compare this to Figure 4c. $T = 10$ °C.

between the outer bilayers was of lower concentration than between the deeper bilayers, then one expects the ratio N_A/N_H to initially rise and then level out as the liposomes degrade.

This experiment was performed by incorporating a small amount of [^{3}H]DPPC as a head-group marker and entrapping [^{14}C]sucrose as an aqueous marker (see Experimental Procedures). The liposome suspensions were divided into nine aliquots and phospholipase C was added. Periodically over

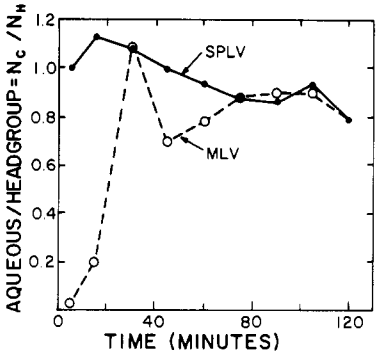

FIGURE 8: Ratio of release of ^{14}C aqueous marker counts to ^{3}H membrane head-group counts is shown as a function of time as the liposomes are enzymatically digested. The steep rise of the MLV curve suggests that the outer aqueous compartments of the MLVs have a relatively low concentration of the aqueous marker solute molecules. At 120 min, the liposomes had released about 30% of the total head-group counts. The MLV curve has been scaled by the factor $(N_C/N_H)_{SPLV\ tot}(N_H/N_C)_{MLV\ tot}$ to allow a direct comparison of the two curves.

the course of the enzyme digestion, aliquots were centrifuged to settle the liposomes, and the supernatant was scintillation counted to determine the amount of released head-group and aqueous marker counts. The results are shown in Figure 8. Note that the relative initial aqueous marker concentration of the MLVs is very low and then rises slowly while the SPLVs concentration is much more nearly constant. The simplest interpretation of Figure 8 is that MLVs are especially solute depleted in the outer layers while SPLVs are of essentially uniform concentration throughout. Note that this interpretation is dependent on assumptions of sequential degradation of the liposome layers and is also complicated by heterogeneity (size, number of layers, etc.) in the liposomes. For these reasons, we consider these experiments suggestive, but not compelling. We note in passing that both MLVs and SPLVs were digested at roughly comparable rates.

Discussion

Composition of MLVs. Liposomes are generally classified as multilamellar (MLV) or small or large unilamellar vesicles (SUV, LUV) [see Szoka & Papahadjopoulos (1980)]. Numerous reviews have been written that describe the preparation, properties, and uses of liposomes [e.g., Bangham (1972), Bangham et al. (1974), Szoka & Papahadjopoulos (1980, 1981), and Deamer & Uster (1983)]. The procedure used to prepare MLVs for this paper is similar to the original methods used by Bangham et al. (1965). A number of previously published procedures used lipids dispersed in mixtures of hydrophobic and hydrophilic solvents [Schneider, 1980; see also Deamer & Uster (1983)]. Of these, the reverse-phase evaporation (REV) (Szoka & Papahadjopoulos, 1978) method is the most commonly used process. However, these procedures produce largely unilamellar vesicles and, therefore, will not be discussed further.

Knowledge of the internal composition of MLVs has been hampered by a lack of detailed information about how a dry lipid film reacts when exposed to solute-containing water. The prevailing view point has been summarized by Bangham et al. (1974), who have written in a review about liposomes that "their usefulness as a model system derived from the fact that, as the dry phospho- and/or other lipids of biological origin undergo their sequence of molecular rearrangements, there is an opportunity for *unrestricted* (emphasis added) entry of solutes, e.g., isotopically labelled salts and proteins, between the planes of hydrophilic headgroups before an unfavorable entropy situation of an oil-water interface intervenes." They

go on to say that there is a subsequent sealing of membranes into concentric, closed compartments, sequestering water and solutes that can thereafter only diffuse across compartments by crossing the bilayer walls. It was realized shortly after the development of the MLV procedure by Bangham and colleagues (1965) that lipid bilayers are highly permeable to water and relatively impermeable to alkali metal salts and a host of other solutes (Bangham et al., 1967). This being the case, one may ask if the sealing of the outermost bilayer wall, upon hydration of a dry film, occurs prior to or after the enclosed lipid has been fully hydrated. If the sealing were to occur before full hydration, then water will continue to enter, via diffusion, because lipid head groups have a high natural affinity for water (Parsegian et al., 1979). However, salts and other such solutes may be excluded because of the relative impermeability of the outermost bilayer wall to these substances. The result would be a liposome in which the internal concentration of solute in the aqueous layers would be less than in the external bulk water. However, we know of no place in the literature where the question of the last four sentences is posed and discussed. We also know of no study that has examined the distribution of solute within liposomes formed by the MLV procedure. In the absence of such discussion it has generally been implicitly assumed that the aqueous solute concentration inside the liposome is the same as the concentration of the fluid in which the liposomes are formed [e.g., see Bangham et al. (1967)].

In attempting to understand the differences between SPLVs and MLVs, one is forced to question if the internal MLV solute concentration is the same as that of the fluid used to prepare the liposomes. The high solute entrapment efficiency to SPLVs relative to MLVs raised the question of where the entrapped solute was being held within the liposome. In the past, the low entrapment efficiency of MLVs seemed reasonable in view of an inadequate knowledge of specific adsorption by the bilayers and the wide heterogeneity in the number of bilayers and sizes of the central aqueous volumes of the liposomes. However, when MLVs and SPLVs were compared, these reasons were rapidly seen to be inadequate to explain the differences in entrapment efficiency. Electron microscopy revealed similar size distributions (Figure 2). Specific adsorption could not account for the differences because both types of liposomes were made of the same lipid. X-ray diffraction (Figure 3) revealed the repeat spaces to differ by only about 2 Å (25 °C). Using an established bilayer thickness of roughly 40 Å (Small, 1967; Worcester, 1976), this implied the aqueous thickness between the bilayers differed by only 10%–15%. Large differences in the central aqueous volume or in the number of layers could not be reconciled with the fact that the SPLV and MLV pellet volumes per unit mass of lipid were comparable to within 30%. It appeared that MLVs were somehow diluting the solutes and SPLVs were concentrating them.

The X-ray signatures (Figures 3 and 4) provided a rapid means of evaluating hypotheses as to the differences between the two types of liposomes. It was seen that a signature characteristic of MLVs could be obtained from SPLVs by increasing the external salt concentration (Figure 5c) and vice versa for MLVs by decreasing the external salt concentration relative to the concentration of the solution used to prepare the liposomes (Figure 5d,e). This suggested that the X-ray signatures were sensitive to a concentration gradient. The LSS was reproduced with sucrose (Figure 5f), suggesting that the X-ray signatures were primarily due to osmotic, as opposed to ionic, effects. [Although changing the salt composition (data not shown) has strong effects on the LSS, MLVs and SPLVs still always differ.] When liposomes were prepared in distilled water (Figure 5b), to remove any concentration gradients, both MLVs and SPLVs yielded a SPLV-like LSS. The ionophore (Figure 6), MPV (Figure 7), enzyme digestion (Figure 8), and NMR experiments all supported the picture of an MLV that is under osmotic compression.

Importance of Osmotic Stress. Much of the liposome literature is concerned with the differences between multilamellar and unilamellar liposomes. Literally speaking, both SPLVs and MLVs are multilamellar. Relatively little attention has been paid to comparisons of different types of multilamellar liposomes. One of the reasons given for using unilamellar liposomes is that they can have a high entrapment efficiency [e.g., Szoka & Papahadjopoulos (1978)]. While this is true, in light of the results of this paper, this is not a compelling argument against the use of multilamellar vesicles, since SPLVs also have a high entrapment efficiency. Another highly discussed variable is the size of the liposomes. Again, this is important but does not differentiate MLVs from SPLVs. The lipid composition of liposomes is the concern of an enormous literature. But both MLVs and SPLVs are made of EPC. Neither the buffering system nor the specific ionic composition is of paramount importance. The most important variable appears to be the sign and magnitude of the osmotic stresses exerted upon the liposomes. Although numerous studies have used the osmotic properties of liposomes, this stress has not been generally recognized as a variable with which to differentiate certain liposomal types.

When multilamellar liposomes are subjected to an osmotic stress, there is a rearrangement of the solute concentrations in the various aqueous compartments. Kwok & Evans (1981) have determined that the elastic limit in large unilamellar EPC vesicles is 3–4 dyn/cm after an expansion in bilayer area of 2%–3%. In the unilamellar case, this membrane tension would be reached when an osmotic gradient of roughly 20 mosmols (depending on the size of the vesicle) is imposed on the vesicle. However, with multilayered vesicles the stress is distributed over many concentric layers. It is unclear as to how solutes repartition in this case. The fact that the curves of Figure 5c–e are not all identical after the imposition of a large change in the osmotic strength of the suspending buffer suggests that the bilayers do not simply lyse and result in a liposome in which all compartments have a solute concentration equal to that of the suspending buffer.

When an osmotic gradient is imposed on a liposome, the bilayers must dilate or shrink to accomodate the stress. This has the effect of changing the area per molecule in the bilayer. Small changes in the area per molecule may be highly significant. In the statistic mechanical models that have been proposed for bilayers, the area per molecule is a fundamentally important parameter. This has been emphasized, for example, by Nagle (1980), by Israelachvili et al. (1980), by Pink (1982), by Kirk et al. (1984), and by Gruner et al. (1985). As the area per molecule changes, the hydrocarbon order parameter must also change. Several studies [see Israelachvili et al. (1980)] have examined the hydrocarbon order parameter (state of inclination from the membrane normal) vs. carbon number down the chain. We know of no studies that have done this for stressed bilayers. The wide-angle X-ray signature (Figure 4c) suggests this order parameter profile may change under stress. In fact, in most statistical mechanical models, the area per molecule is introduced as a fundamental constraint or as a parameter derived from an independent calculation of the head-group interactions. The change in the area per molecule

is likely to affect the hydrogen bonding and steric interactions between head groups. It must also affect the magnitude of the interaction with water: it is known that the thickness of a bilayer is coupled to the hydration of the multilamellar lattice. The area per molecule decreases as water is withdrawn from the lattice (Luzzati, 1968; Rand, 1981). We suggest the coupling may be reversible, i.e., that expanding or shinking the area per molecule may affect the interaction with water (we think this is an important area for future experimental investigation). In sum, the entire molecular basis of the bilayer is affected by the osmotic stress.

Note, incidentally, in Figures 5c–e and 6 that it is the direction and magnitude of the osmotic *gradient* which seem most important. Similar findings pertain to osmotically induced fusion (Cohen et al., 1980, 1982; Zimmerberg et al., 1980).

We propose that MLVs are solute depleted relative to the buffer in which they are made. The direction of the osmotic stress is so as to make the liposome collapse onto itself. However, as Parsegian, Rand, and co-workers have shown [see Rand (1981) for a review], very strong repulsive hydration forces keep the bilayers apart. Parsegian et al. (1979) have measured the repeat spacing for EPC in pure water at 25 °C; they obtain a value of 62.5 Å. Reference to Figure 3 shows the MLV value to be 61.7 Å, indicating that the MLV layers are collapsed against the "hard wall" of the hydration force. (Note, incidentally, that the repeat spacing of either MLVs or SPLVs in pure water, as shown in Figure 5b, is about 62.5 Å at 25 °C.) The bilayers, in fact, are under a stress that has hitherto been rarely considered in liposomes: the bilayers are under *compression*. It is suggested that the effects of expanding and compressing bilayers are important and very much in need of investigation.

Solute depletion of the layers of MLVs has many implications. For example, one of the solutes most commonly involved is NaCl. The Nernst equations states that an imbalance of the concentrations, C_1 and C_2, of an ionic species across a membrane gives rise to an electrical potential [see, for example, Schwartz (1971)]:

$$\Delta\phi = -RT/(zF) \ln (C_2/C_1) \quad (4)$$

where R = the gas constant, T = temperature, z = ionic charge, $\Delta\phi$ = electrical potential, and F = Faraday's number. Now, the bilayer permeability coefficient for Na^+ (10^{-12} cm/s) is almost 2 orders of magnitude smaller than that of Cl^- (Stryer, 1975). This means that the Na^+ gradient cannot relax in weeks; the Cl^- gradient relaxes almost 100 times faster, leading to a Nernst potential across the bilayer. The biological implications of this may be significant. The potential may also be responsible for the rheological differences between MLV and SPLV pellets. Other implications arise from the many studies that have used MLVs. Many of these studies [e.g., Bangham et al. (1967)] are predicted on the assumptions that the solute concentration profile is uniform throughout the MLV and/or across the outer bilayer walls. The results of some of these studies may now have to be reconsidered.

Meaning of the X-ray Signals. The three X-ray signatures each probe different, although related, parts of the liposome structure. In principle, it is possible to stress bilayers in different ways so as to affect some parts of the structure more than others. The result is that a change in one of the X-ray signatures need not necessarily be reflected by simple changes in the others. To understand this, let us consider the microscopic sources of each of the signatures.

Of the three signatures, the width and asymmetry of the diffracted orders are most easily understood. For liposomes consisting of many layers, the shape of the diffracted orders (Figure 4a,b) is a direct indication of the width and asymmetry of the distribution of membrane repeat spacings. MLVs exhibit relatively wide, asymmetric peaks, indicative of a repeat spacing whose distribution mode and mean differ. This is consistent with the data of Figure 8, since a nonuniform distribution of solutes would lead to nonuniform osmotic forces between the layers and result in a slight variation in the repeat spacings.

The LSS (Figure 3) is relatively insensitive to the repeat spacing distribution; rather, by definition, it maps out the variation in the mean repeat spacing as the temperature is varied. The repeat spacing is the sum of the thicknesses of the bilayer and of the interbilayer aqueous space; both these component widths may be expected to be thermally sensitive. As the temperature is raised, more gauche rotamers are excited in the bilayer hydrocarbon, resulting in a thinner membrane and an increase in the area per molecule (Reiss-Husson, 1967). In the presence of excess water, the thickness of the fluid space is set by a complicated balance of van der Waals, hydration (Rand, 1981), membrane tension, and osmotic forces. In particular, the strong hydration force is likely to be coupled to the area per molecule, since it is known that the area per molecule changes as membranes are rehydrated (Small, 1967). In addition, although there has been little investigation of the thermal variation in the hydration force at a fixed area per lipid molecule, it would be surprising if the force coefficients were thermally insensitive. Thus, there is a complicated interaction between the thicknesses of the water and lipid layers such that forces which act laterally in the lipid plane also affect both the lipid and water thicknesses. Our understanding of the statistical mechanics of the hydrocarcon, of the interactions in the lipid polar region, and of the hydration force is not yet sufficiently sophisticated to completely predict the LSS.

It would be of interest to measure the component bilayer and aqueous thicknesses. Unfortunately, the two X-ray techniques that are used to determine these thicknesses cannot be simply applied to liposomes. The classic method of allocation of mass [see Luzzati (1968)] fails in the presence of excess water. Direct profile determinations (Stamatoff & Krimm, 1973) often use the assumption that the membrane profile remains constant as the repeat spacing is varied, usually by changing the osmotic strength of the suspending medium. The validity of this assumption is suspect for osmotically intact liposomes; besides, in light of this paper, variation of the imposed osmotic stress is a poor way to investigate multilamellar liposomes. Even so, there are many direct means of membrane profile determination, and these should be explored. Neutron diffraction (Worcester, 1976) is another possible means of determining the thicknesses.

The wide-angle signature (Figure 4c) is also ill understood. Broad, diffuse diffraction in the 4.6–3.5 Å range arises primarily from density correlations in the lipid hydrocarbon, although we cannot exclude the possibility that the wide-angle SPLV signal arises, in part, from water associated with the bilayers. The similarity of this peak to diffraction from paraffin oil is the source of the assertion that membrane hydrocarbon is "liquid-like" above a cooperative chain melting temperature [see Luzzati (1968)]. However, NMR has shown that the hydrocarbon is not at all like an isotropic liquid [see Israelachvili (1980) for a review]. Further, it is commonly observed in our laboratory that different lipid preparations exhibit melted-chain peaks that differ reproducibly in the exact peak position, shape, and width. Relatively little systematic experimental (Costello & Gulik-Krzywicki, 1976) or theo-

retical investigation has been done on this X-ray peak. It should be straightforward to compute the diffraction expected from a detailed statistical description of the carbon atom positions in the melted lipid hydrocarbon. We do not know of any study that has done this. We would guess that the diffraction would change as the area per molecule is changed.

The wide-angle signature may be expected to be less sensitive to changes in the aqueous region than the LSS. A systematic comparison of the LSS and the wide-angle signatures under various manipulations of the bilayers is warranted. In principle, a single bilayer sheet may be used to probe the wide-angle diffraction since this feature does not arise from a multilayer repeat. A single bilayer would, of course, have no LSS.

Summary and Conclusions

MLVs and SPLVs are made of the same chemicals but are formed by different procedures. The result is that MLVs appear to be under osmotic compression whereas SPLVs are not. The fact that the two types of liposomes differ with respect to stability, ESR, NMR, X-ray diffraction, and biological effects suggests that this physical distinction is important. Ultimately, the use of liposomes depends on their material properties, as well as their chemical composition. In other disciplines, such as metallurgy or polymer science, it is well accepted that the way materials are formed and worked is as important as their overall composition. By and large, the multilamellar liposome literature has emphasized distinctions between liposomes based on the chemical composition with relatively little emphasis on the procedure whereby the liposomes are made. The interactions of liposomes with other bilayers, chemical agents, and with living cells depend, in the end, on the molecular forces at the liposome surface. We have suggested reasons to believe that these forces are dependent on the stress imposed on the liposome bilayers and that these stresses result from the way the liposomes are made. Further work on the properties of stressed liposomes is called for.

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