Pharmaceutical Research, Vol. 3, No. 1, 1986

Research Article

Formation of Multilayered Vesicles from Water/Organic-Solvent (W/O) Emulsions: Theory and Practice

Charles Pidgeon,[1,2] Ann H. Hunt,[1] and Katherine Dittrich[1]

Received January 15, 1985; accepted July 9, 1985

Multilayered liposome (MLV) formation from water/organic-solvent (W/O) emulsions was studied. A fundamental liposome population parameter, the E^{ratio}, was defined and used to estimate the bilayer number and water spacing in MLV liposomes. MLVs prepared from W/O emulsions have optimum drug entrapment at an emulsion-lipid/emulsion-water ratio of ~0.33. Drug entrapment is typically 50 to 65% under these optimal conditions.

KEY WORDS: liposome; membrane; drug delivery; lipid; multilayered vesicle.

INTRODUCTION

Liposomes have been used as drug carriers for many years, and it is generally accepted that multilayered vesicles (MLV) have low drug-trapping characteristics.[3] Low drug trapping is caused by the process typically used to make multilayered liposomes. Multilayered vesicles are made by adding an aqueous solution to a film of lipid dried on the bottom of a flask. The aqueous solution penetrates the dried lipid film and hydrates the phospholipids. For thermodynamic reasons, hydrated phospholipids spontaneously aggregate to form vesicles. MLV formation is probably mediated not through individual hydrated phospholipids but, rather, through pieces or sheets of phospholipids that have become hydrated. Low drug trapping is characteristic of this process because the surface area between the lipid phase and the aqueous solution is small; drug dissolved in the aqueous phase is not in intimate contact with lipids when they form the liposome membrane.

The REV technique circumvented the problem of low drug entrapment by increasing the surface area between the lipid and the aqueous phase during membrane formation (1). This was accomplished by forming liposomes from water droplets emulsified in an organic phase (W/O emulsion). Phospholipids in the W/O emulsion surround the water droplets, and removal of the organic solvent forced the water droplets (containing the drug) to coalesce into liposomes. The intimate contact established between the lipids and the aqueous phase during membrane formation ensured high drug entrapment. REV vesicles are usually single layered but recently a technique has been described that produces multilayered vesicles from W/O emulsions (2). Contrary to conventional MLVs, MLVs prepared from W/O emulsions have high drug entrapment.

This report describes liposome drug-trapping experiments using MLVs prepared fromn W/O emulsions. A model is presented to predict the type of liposomes generated from W/O emulsions, i.e., single layered or multilayered. Theory suggests that the most important parameter for MLV formation from W/O emulsions is the ratio of lipid to water in the emulsion. For MLV formation the amount of lipid in the W/O emulsion should exceed the amount of lipid needed to cover completely the emulsified water drops. Thus lipid in excess of that required to form inverted micelles forms the first or initial bilayer structure around the micelles. Lipid in excess of one complete bilayer forms additional bilayers around the first lamella; the number of bilayers depends on the amount of excess lipid.

MATERIALS AND METHODS

Chemicals and Solutions

Egg L-α-phosphatidylcholine (PC) was purchased from Avanti Polar Lipids, Inc., Birmingham, Ala. DL-α-Dipalmitoylphosphatidic acid (PA) and cholesterol (CH) were purchased from Sigma Chemical Co., St. Louis, Mo. Phospholipid purity was periodically evaluated by thin-layer chromatography (~2 μmol lipid was chromatographed on silica-gel plates and eluted with $CHCl_3CH_3OH/H_2O$:65/25/4). Phospray (Supelco, Bellefonte, Pa.) was used to detect phospholipid phosphate. Deuterium oxide, 99.8% (D_2O), was obtained from MSD Isotopes, Merk Frost Canada, Inc., Montreal, Canada. L-α-Dipalmitoyl [choline-me-^{3}H] (^{3}H-DPPC), 51 Ci/mmol, Lot 1782-187, and inulin [carboxyl-^{14}C] (^{14}C-inulin), 2.4 mCi/g, Lot 1950-040, were purchased from NEN,

[1] Eli Lilly Research Laboratories, Indianapolis, Indiana 46285.
[2] To whom correspondence should be addressed. Present address: Charles Pidgeon, Department of Industrial and Physical Pharmacy, School of Pharmacy and Pharmacal Sciences, Purdue University, West Lafayette, Indiana 47907.

[3] This work addresses aqueous soluble drugs, i.e., drugs that do not adhere to the membrane surface or dissolve in the hydrocarbon part of the bilayer. High entrapment of lipophilic drugs in MLVs is not addressed in this report.

0724-8741/86/0200-0023$05.00/0 © 1986 Plenum Publishing Corporation

Boston. Sucrose (U-^{14}C), 360 mCi/mmol, Lot 977801, was purchased from ICN, Irvington, Calif. Calcium- and magnesium-free Dulbecco's phosphate-buffered saline 10 × (Grand Island Biological Co.) was diluted with sterile water for injection to make isotonic PBS. PBS-D_2O was prepared by lyophilizing 1 ml PBS 10 × and reconstituting with 1 ml D_2O, followed by lyophilization and reconstitution with 10 ml D_2O. The second lyophilization from D_2O was necessary to exchange ^1H with ^2H. All other chemicals and solvents were of the highest purity available and used as received.

Preparation of Liposomes

All liposomes in this report were prepared from W/O emulsions using ether as the organic solvent. Other organic solvents may be useful. Single-layered REV vesicles were prepared in a 25 × 175-mm ground-glass test tube as described (1), and unentrapped aqueous space markers removed by floating the vesicles on a Ficoll gradient (3).

Stable plurilamellar vesicles (SPLVs) were prepared as described (2) in a 100-ml round-bottom flask except 10 ml instead of 5 ml ether was used to prepare the emulsion. The SPLV procedure requires (i) forming a W/O emulsion by sonicating 0.3 ml PBS with 10 ml ether containing 100 mg PC, followed by (ii) a nitrogen purge while sonicating. Aqueous soluble drugs are added to the PBS and organic soluble drugs added to the ether. Step (ii) removes the organic solvent and also generates a mist blown into the air. The mist contaminates the immediate environment when radiolabels are encapsulated. Because of this we always prepare SPLVs in a hood under a plexiglass shield.

Multilayered REV vesicles (MLV-REV) were prepared by modifying the REV procedure that generates single-layered vesicles as follows. Lipid (usually 100 mg) dissolved in 10 ml ether was emulsified with PBS (usually 0.3 ml) by 2 min of sonication under argon at 25°C (Branson bath sonicator, 100-ml round-bottom flask). The volume of water and amount of lipid were varied to test for MLV formation from W/O emulsions. Ether in the emulsion was removed at ~400 mm Hg by vacuum suction. Low vacuum is needed to prevent ether from flashing. After 5 to 30 min of vacuum, a gel forms. A higher vacuum can be used to break the gel, but increasing the vacuum is not necessary and frequently causes the system to bubble excessively.

MLV liposomes (SPLV and MLV-REV) were separated from unentrapped aqueous space markers by 10 min of centrifugation (10,000 rpm); longer (20-min) centrifugation times were required for negatively charged liposomes. Vesicles were resuspended in PBS, pelleted by centrifugation again, then diluted to the desired volume with PBS. ^{14}C-Sucrose or ^{14}C-inulin was used to measure trapping efficiencies, and ^{3}H-DPPC was used to measure lipid recoveries.

Measurement of "Gel-Suspension" Water

Removing the organic solvent in the SPLV and MLV-REV process results in water loss. The water content in the MLV-REV and SPLV gel suspension was measured by ^{1}H-NMR. The gel-suspension stage refers to the W/O emulsion after most of the organic solvent is removed but before excess buffer is added to resuspend the liposomes. Before each experiment the NMR spectrometer was calibrated with H_2O/D_2O standards by plotting the peak height vs the microliters of H_2O per milliliter. The H_2O/D_2O standards were prepared in NMR tubes and sealed by flame. Assay of H_2O by NMR required both samples and H_2O/D_2O standards to be measured in identical-size NMR tubes. We used 5-mm tubes (No. 506, Norell, Inc., Candisville, N.J.) for all measurements. Samples were examined using either a Bruker WH360 or a Bruker WM270 NMR spectrometer, operating in the Fourier transform mode. Spectra were recorded using a single scan and a 45° pulse width. Exponential multiplication using LB = 1.0 was applied to the free induction decay before transformation. Temperature equilibrium of the tubes within the probe was verified by observation of a constant HOD peak height on repeated scans. The linearity of both instruments was from 0 to 120 μl H_2O/ml, and all samples were kept in this range. Validation of the assay using known concentrations of H_2O/D_2O gave a coefficient of variation of 8%. Duplicate determinations of unknown gel-water samples gave a precision of ±8% (N = 15).

Liposomes were prepared by the desired technique except the gel suspension was reconstituted with 2.5 ml D_2O buffer (PBS-D_2O). H_2O from the gel suspension carried over to the final D_2O/liposome population. The suspension was centrifuged (13 × 100-mm test tube, 2500 rpm, 1 min) to float the liposomes ($\rho \cong 0.99$) in heavy water (D_2O buffer $\rho \cong 1.1$). The subphase was sampled to measure the microliters of H_2O per milliliter of water (μl H_2O/ml). This technique is feasible because water equilibrates across bilayers instantaneously (4, 5). For some experiments liposomes reconstituted with PBS-D_2O were treated as any other liposome population and the encapsulation efficiency was calculated from radiolabels. It was therefore necessary (i) to assay the D_2O/liposome dispersion for radioactivity before vesicle flotation (see above) and (ii) to separate unentrapped aqueous space markers from the vesicles after flotation. To accomplish (ii), a 0.3-ml aliquot of floated vesicles was diluted to 1.5 ml with PBS/PBS-D_2O (10:1) and centrifuged in a microfuge for ~8 min. Floated liposomes were collected, diluted again to 1.5 ml with PBS/PBS-D_2O (10:1), and floated by centrifugation (microfuge). The collected vesicles were diluted and counted for radioactivity. Increasing the solvent density by using PBS-D_2O instead of PBS/PBS-D_2O (10:1) reduces microfuge times (3 min instead of 8 min).

Computer Generations and Theoretical Calculations

Computer-generated populations of liposomes describing E^{ratio} vs diameter were abstracted from the computer simulations generated by Pidgeon and Hunt (6).

The total surface area of emulsified water drops having a radius r was calculated from the area and volume of spheres with radius r after (a) finding the number of drops with radius r that 0.3 ml water will generate and then (b) multiplying the number of drops by the area per drop. From this, the drop diameter vs the total surface area was plotted. The amount of phospholipid needed to form a monolayer around water drops of radius r was estimated using the area per molecule for egg PC found in bilayers, 70 Å^2 (7). However, the area per molecule at oil/water interfaces may be relevant; lecithin monolayers at oil/water interfaces have an area per molecule of between 45 and 100 Å^2 (23.4°C) (8). We chose 70 Å^2 because the O/W interface is transient during

the process of liposome formation and the area per molecule requirements of bilayers must be met with the available phospholipid molecules. Nevertheless, changing the area per molecule to 45 or 100 Å^2, or to a distribution of values, will not alter the conclusions in the report.

Statistical Analysis

Statistical analysis correlating gel-suspension water to E^{ratio} was done using the statistical software package (SAS Institute, Inc.; Cary, N.C.). Least-squares calculations used the GLM (generalized linear model) routine.

RESULTS AND DISCUSSION

Gel and Liposome Formation: Gel-Suspension Phase

W/O emulsions containing lipid must invert from a W/O system to an O/W system for liposomes to form. Immediately preceding inversion, a gel phase forms and the W/O to O/W transition is actually a gel inversion. Gel inversion to a liposome suspension can be followed by the striking difference in the physical appearance of the mixture during the transition. Liposome suspensions are brilliant white and viscous, but the physical appearance of the gel varies depending on the type of liposome being formed.[4] Gels that invert to single-layered PC liposomes are cloudy opaque and viscous (resembles frosted glass), whereas gels that invert to multilayered PC liposomes are clear and viscous. For convenience, we define a "gel-suspension" stage as the W/O emulsion after most of the organic solvent is removed but before excess buffer is added to resuspend the vesicles. Gel inversion may or may not have occurred at the gel-suspension phase, and a gel or liposome suspension can be obtained as described above. Gel inversion at the gel-suspension stage depends on the amount of gel water in the lipid/water mixture. When the gel water is less than that needed to hydrate the lipids (~23 μl H_2O/100 mg PC), bulk water is not available to establish a continuous aqueous phase. Under these conditions, gel inversion cannot occur and liposomes are formed only after the gel suspension is reconstituted with excess buffer. However, gel inversion to liposome suspensions is facile when the gel water ≧50% by weight, e.g., 100 μl H_2O/100 mg PC.

The SPLV process inverts from a W/O to an O/W system rapidly, and gel inversion is difficult to see. Continuous sonication while removing the organic solvent facilitates a constant equilibration of water, solutes, lipid, and organic solvent. Sonication causes a rapid gel inversion, and instead of gels, white liposome suspensions are obtained in 1–2 min during the SPLV process. Long nitrogen purge times, however, can remove enough water to precipitate the lipid on the walls of the flask; the SPLV process thus should be terminated at ~1 to 2 min of nitrogen purge.

Liposomes formed without continuous sonication, REV and MLV-REV vesicles, are accompanied by bubble formation during gel inversion. Bubbling requires bulk water and therefore no bubbling occurs when <20% by weight water (i.e., PC hydration water) exists at the gel-suspension phase. In the gel suspension (particularly in the gel phase), ether is not the continuous phase and it can boil inside the water–lipid mixture. As ether boils, a bubble forms and lipid molecules form a monolayer or bilayer at the interface and stabilize the bubble. As ether continues to boil, more ether molecules enter the stabilized bubble and raise the internal bubble pressure. The pressure inside bubbles is greater than outside bubbles. As the ether is removed from the lipid/water mixture, less ether is available to initiate bubble formation and raise the internal pressure of existing bubbles. Thus bubbles are maintained longer. If the vacuum is increased to break the gel, the pressure outside the bubble (or gel suspension) decreases significantly. This causes ether to boil rapidly and small bubbles are formed. Thus as gel inversion proceeds, the rate and extent of bubble formation will depend on the amount of bulk water, external pressure (or vacuum), and residual ether. Increasing the bulk water increases the available volume of water that can form bubbles. Thus when the gel water is ~50% by weight water, gel inversion occurs smoothly, but increasing vacuum at the gel-suspension stage causes rapid bubbling.

Drug Trapping, Encapsulation Efficiency (E), and Encapsulation Ratio (E^{ratio})

Drug entrapment is typically calculated as the amount of added drug associated with the final liposome population. This method for calculating drug entrapment does not give information about the physical or structural properties of the liposome population. For this reason, liposome encapsulation efficiencies (E) are measured. E is calculated as the microliters of entrapped water per micromole of lipid; E measures how efficiently a micromole of lipid sequesters water. Usually E is measured using water-soluble tracers (e.g., ^{14}C-sucrose) under the assumption that the fraction of marker associated with the final liposome population reflects the fraction of water entrapped. Thus if 1 ml of buffer containing ^{14}C-sucrose was used to prepare liposomes and 10% of the added ^{14}C-sucrose was liposome associated, then 100 μl of water is inside the vesicles. Intraliposomal water is in equilibrium with bulk water, and the microliters of entrapped water actually reflects the internal vesicle volume (excluding lipid volume of multiple bilayers). In a mathematical treatment of liposome populations, another term, E^{ratio}, was used to characterize the ability of liposomes to entrap water and drugs (6). The E^{ratio} was defined as the microliters of water per microliter of lipid in the final liposome population. The E^{ratio} is a more fundamental liposome population parameter than E and a hypothetical experiment demonstrates this. Liposomes can be prepared from photoactive lipids that cross-link during irradiation. Light-induced phospholipid cross-linking can extend to only a few molecules or to every lipid molecule in the bilayer. Calculating the liposome trapped volume per micromole of lipid (E) will vary depending on the extent of polymerization, whereas the E^{ratio} will not vary. This is because polymerization affects the micromoles, not the volume, of lipid making up the membranes.

Converting E to E^{ratio} requires lipid molecular weights and the approximation that lipids have a density near unity [ρ lipid ≅ 1 g/ml, e.g., 100 mg lipid ≅ 100 μl of lipid (6)].

[4] Changing the lipid composition can change the appearance of the gel (manuscript in preparation).

Thus, calculating the microliters of H_2O trapped per microliter of lipid in the liposome population is as follows.

$$E^{ratio} = \frac{\mu l\ H_2O}{\mu mol\ lipid} \times \frac{\rho\ lipid}{MW\ lipid} \times 1000 \quad (1)$$

where μl H_2O/μmol lipid is the population encapsulation efficiency. For egg PC the encapsulation efficiency (E) is 78% of the encapsulation ratio E^{ratio}.

Calculating the E^{ratio} is useful because it gives structural information about the liposome population under study. For instance, in multiple bilayers, the E^{ratio} eauals the volume ratio of water to lipid in the repeating bilayer. The water-to-lipid volume ratio, however, equals the ratio of water thickness (*w*) to bilayer thickness (*l*) in the repeating bilayer (*w*/*l*). This condition, i.e., $E^{ratio} = w/l$, occurs only when most of the entrapped water resides in the repeating bilayers and not inside the cores of the multilayered vesicles. These relationships are demonstrated in Fig. 1 for homogeneous liposome populations (6). Each data point in Fig. 1 is a homogeneous population of liposomes generated from 1 mmol PC. Each curve in Fig. 1A was generated by adding repeating layers of water and lipid to single-layered vesicles.

Adding water–lipid layers onto single-layered liposomes can cause the population E^{ratio} to increase or decrease depending on the size of the initial single-layered liposomes. For instance, 1 mmol PC can form 3.6×10^{14} liposomes 0.44 μm in diameter with an E^{ratio} of 19.1. Using this liposome population to form vesicles with two bilayers from 1 mmol PC yields 1.7×10^{14} liposomes 0.46 μm in diameter, but the E^{ratio} decreases to 10.1. Some of the highly efficient single-layered liposomes in the first population were sacrificed so

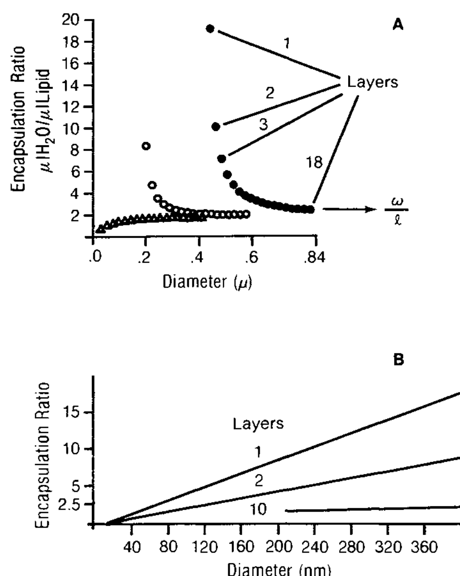

Fig. 1. Relationship between encapsulation ratio and liposome diameter. Data were generated using 1 mmol phosphatidylcholine, a 74-Å layer of water (*w*), and a 37-Å bilayer thickness (*l*). E values were obtained after adding successive water–lipid layers to single-layered liposomes with a diameter of 300 Å (△), 2000 Å (○), and 4400 Å (●). E^{ratio} always approaches the ratio *w*/*l* in MLVs. The curves generated in B were obtained by connecting the data points in A for 1-layered, 2-layered, and 10-layered vesicles.

the lipid could be used to add second layers to other single-layered liposomes. Thus the E^{ratio} and the number of liposomes per millimole of PC decreases as more bilayers are added. If one uses a 300-Å single-layered liposome, the trend is reversed because very small liposomes are themselves not efficient. Adding bilayers increases the efficiency because the volume of water in each bilayer is greater than the core volume.

From numerous computer-generated populations of liposomes we have found the E^{ratio} to approach the value *w*/*l* as the bilayer number increases. Figure 1A is an example of this. The first data point of each curve (i.e., the diameter of the first layer in the MLV population) varied from 300 to 4400 Å, but the E^{ratio} approached 2.0 (*w*/*l* = 74 Å/37 Å) after approximately 5 to 10 bilayers, depending on the core volume. Figure 1B was obtained from Fig. 1A and shows that multiple-layered vesicles have low encapsulation ratios compared to single-layered vesicles. The key findings from the computer-generated data (Fig. 1) are that (i) irrespective of the diameter of the innermost lamella, the E^{ratio} approaches *w*/*l*; (ii) the $E^{ratio} = w/l$ if most of the entrapped water is in the repeating bilayers and not the liposome core; and (iii) the E^{ratio}, number of layers, and vesicle diameter are dependent variables. These general conclusions are independent of the *w* (=74 Å) and *l* (=37 Å) values used to generate the data. The value of *l* in real liposome populations is not a fixed value and varies depending on the type of lipids making the membrane (9) and the temperature (10–12). The value *l* = 37 Å was obtained from hydrodynamic studies on small unilamellar vesicles (13). Estimating the bilayer number from Fig. 1 is, at best, a first approximation. However, Fig. 1 can easily distinguish 1 or a few bilayers (e.g., E^{ratio} = 5–10, liposome diameter of 0.5 μm) from ≧10 bilayers (e.g., $E^{ratio} < 2$, liposome diameter > 0.6 μm). We adhere to these limitations in estimating the bilayer number throughout this report. It is worth noting that the data in Fig. 1 were generated from geometry constraints of circular lipid and water layers. The water layer does not distinguish between hydration or bound water (10 to 15 H_2O molecules/phospholipid molecule) and free water.

As with the bilayer number, predicting *w* from the E^{ratio} is also an approximation. Table I shows the accuracy of the E^{ratio} in predicting *w* on populations of liposomes generated with water spacings between 20 and 74 Å. For liposomes containing 25 bilayers, the E^{ratio} predicts *w* well (except for *w* = 20 Å) even though core diameters varied 0.03 to 0.4400 μm. However, for liposome populations containing 15 bilayers, predictions of *w* are sensitive to the inside core diameter. When the core diameter is large and, therefore, contains a large volume of water, the E^{ratio} can overestimate *w* twofold (e.g., 0.44-μm core diameter, 15 layers, *w* = 30 Å; Table I). More than 15 bilayers are required to minimize the contribution of core water relative to bilayer water. In other words, if a large fraction of the entrapped water is in the core, one is not on the asymptotic curve in Fig. 1A, and the E^{ratio} overestimates *w*. While real liposome populations can have a distribution of core diameters, bilayer numbers, and liposome diameters, any given population of vesicles contains relatively constant values for *w* and *l*. Thus because Fig. 1 is asymptotic, the use of the E^{ratio} to predict *w* and bilayer number is justified for multilayered liposome populations as shown by Fig. 1.

Table I. Accuracy of (l) E^{ratio} in Predicting Water Spacing w in Multilamellar Liposomes[c]

Core diameter (μm)	w_i (Å)	(l) $E^{ratio} = w$ (Å)	
		NL = 15	NL = 25
0.03	20	18	19
0.20	20	29	23
0.44	20	53	35
0.03	30	27	28
0.20	30	37	32
0.44	30	60	43
0.03	40	36	38
0.20	40	44	41
0.44	40	67	52
0.03	50	46	47
0.20	50	54	50
0.44	50	75	60
0.03	60	54	56
0.20	60	62	60
0.44	60	80	69
0.03	74	67[a]	—[b]
0.20	74	74[a]	—[b]
0.44	74	93[a]	—[b]

[a] l = 37 Å for these data; l = 35 Å for all other data.
[b] Calculation was not done.
[c] Populations of liposomes were generated from input water spacing values (w_i) between 20 and 74 Å. The bilayer water thickness (w) was calculated by (l) E^{ratio} for liposomes containing 15 and 25 bilayers.

Drug entrapment measurements for REV, MLV-REV, and SPLV liposomes are given in Tables II and III and Fig. 2. Estimating the bilayer number for SPLVs and MLV-REVs from Fig. 1 requires liposome particle diameters and E^{ratio} measurements. We have estimated liposome diameters to be approximately 0.6 μm for SPLV and ≧0.7 μm for MLV-REV by electron micrograph and laser light scattering.[5] Encapsulation studies to obtain the E^{ratio} are described below.

Table II shows that REV vesicles have very high E^{ratio} values (21.8 ± 5.5). REV vesicles are approximately 0.4 to 0.5 μm in diameter (1, 18), and an E^{ratio} of 21.8 suggests single-layered vesicles (Fig. 1). Increasing the amount of lipid from 20 to 100 mg decreased the E^{ratio} values about fourfold (E^{ratio} 21.8 to E^{ratio} 4.75). Based on Fig. 1, this decrease in the E^{ratio} means vesicles with more than one bilayer were generated by the high amount of lipid. Forming single-layered vesicles by the REV process thus depends on the amount of lipid in the preparation. Comparing the MLV populations, MLV-REV is superior to SPLV in drug entrapment, E, and E^{ratio} values (Table II). For MLV-REV (>0.7 μm in diameter) an E^{ratio} of 1.5 to 2 implies more than 10 bilayers. Table III shows the same trends for negatively charged vesicles as found for neutral vesicles (Table II): (i) REV has optimum E values compared to MLV-REV and SPLVs; and (ii) MLV-REVs have higher E values and percentage entrapment than SPLVs. In addition, decreasing the amount of lipid in the sample increased the E^{ratio} for SPLV, MLV-REV, and REV vesicles. Thus, the REV process is more efficient with small amounts of lipids.

Neutral MLV-REV vesicles have very high drug entrapment when emulsion water is 300 μl and emulsion lipid is 100 mg (Table II). If emulsion water is increased from 300 to 500 μl or decreased from 300 to 125 μl, drug entrapment significantly decreases (Fig. 2A). Thus optimum drug entrapment for PC MLV-REV vesicles occurs at an emulsion-lipid/emulsion-water ratio of 0.33 when 100 mg PC is used to prepare the liposome. Doubling the lipid to 200 mg PC causes optimum drug entrapment to occur at approximately the same emulsion-lipid/emulsion-water ratio (Fig. 2B).

An important finding in Table II, Table III, and Fig. 2 is that multilayered vesicles (SPLV and MLV-REV) prepared from W/O emulsions have high drug entrapment. In other words, the amount of liposome-associated ^{14}C-sucrose and ^{14}C-inulin is a large fraction of the amount added. Optimum drug entrapment is critical for the pharmaceutical development of liposomes as a drug delivery system.

Gel-Suspension Water and Water Thickness in Repeating Bilayers

Liposome formation by the SPLV process involves a 1- to 2-min nitrogen purge while sonicating an ether/H_2O mixture. Figure 3 shows the time course of H_2O removal during the process. The SPLV procedure uses 10 ml of ether, which solubilizes 90 μl of H_2O. Solubilized water is lost as an ether/water azeotrope when the ether is removed to form the gel-suspension phase. The total water loss, however, depends on the sonication time, nitrogen purge time, and emulsion water. Emulsions containing 300 μl PBS lose water at a rate of 21 μl/min, and emulsions containing 1000 μl PBS lose water at a rate of 40.9 μl/min (Fig. 3 slopes). This water loss is by evaporation because sonication has stopped. Sonication while purging with nitrogen causes water loss by both evaporation and mist. This is evident from Fig. 3, which shows the water loss after a short interval of 1–2 min of sonicating and purging. During this 1- to 2-min interval, the water loss is ~150 and 400 μl for ether/PBS 10/0.3 and ether/PBS 10/1 emulsions, respectively. Sonication generates a mist, and purging with nitrogen expels mist droplets into the immediate environment. In addition to water loss, this causes radiolabel losses of 7.5 ± 2.9% (N = 7) for ^{14}C-sucrose and 9 ± 3.0% (N = 4) for ^{14}H-DPPC. Radiolabel loss must occur from the mist, because ^{14}C-sucrose and ^{3}H-DPPC are nonvolatile. For the MLV-REV process, no mist is created (visual observation), and water loss is exclusively by evaporation. ^{14}C-Sucrose loss was <0.1%, and ^{3}H-DPPC loss was <1% for the MLV-REV process (N = 5). Because radiolabels are not extensively lost during the SPLV and REV processes, they can be used for estimating E and E^{ratio}.

The E^{ratio} was measured as a function of gel-suspension water for SPLV and MLV-REV vesicles prepared from ether/PBS 10/0.3 and 10/1 (v/v). All experiments were designed to study E^{ratio} vs gel-suspension water after >99% of the ether (measured by NMR) was removed, which occurs at ~210 and 900 μl gel water for the 10/0.3 and 10/1 (v/v) W/O emulsions, respectively.

Water loss during gel formation may cause membrane

[5] Pidgeon, unpublished results.

Table II. Encapsulation Studies of Neutral Liposomes Composed of Egg PC[g]

		Inulin encapsulation[a]						
		Liposome-associated inulin[c] (%)	W/O emulsion Lipid (mg)	W/O emulsion PBS[f] (μl)	Lipid recovery (%)	E (μl H_2O/μmol lipid)[d]	E^{ratio} (μl H_2O/μl lipid)[d]	Bilayer number
1	SPLV	36 ± 4	100	300	86 ± 6.5	1 ± 0.1	1.27 ± 0.14	≧15
2	MLV-REV	65 ± 15	100	300	97 ± 3.5	1.50 ± 0.33	2.04 ± 0.42	≧10
3	REV	28 ± 11	20	1000	65 ± 17[e]	17.01 ± 4.31	21.8 ± 5.5	1
		Sucrose encapsulation[b]						
		Liposome-associated sucrose[c] (%)	W/O emulsion Lipid (mg)	W/O emulsion PBS[f] (μl)	Lipid recovery (%)	E (μl H_2O/μmol lipid)[d]	E^{ratio} (μl H_2O/μl lipid)[d]	Bilayer number
4	SPLV	32.2 ± 4.5	100	300	92.6	0.84 ± 0.13	1.08 ± 0.17	≧15
5	MLV-REV	52.2 ± 14	100	300	92.4	1.14 ± 0.14	1.46 ± 0.18	≧10
6	REV	33.8 ± 11	100	1000	70.72[e]	3.71 ± 0.94	4.75 ± 1.21	2–5

[a] Values for inulin encapsulation are the means ± SD from three determinations.
[b] Values for sucrose encapsulation are the means ± SD from five determinations.
[c] These values depend on lipid recovery.
[d] These values are normalized for lipid recovery.
[e] Ficoll density gradient used to purify vesicles causes lipid losses.
[f] PBS, phosphate-buffered saline.
[g] All liposomes were prepared from W/O emulsions. REV liposomes contained 3 ml ether, whereas SPLV and MLV-REV vesicles contained 10 ml ether as the organic solvent for the emulsion. ^{14}C-Inulin or ^{14}C-sucrose was used to quantitate liposome-associated inulin and sucrose. ^{3}H-DPPC was used to quantitate lipid recovery after vesicle purification. Methods for calculating E, E^{ratio}, and bilayer number are described in Materials and Methods.

Table III. Encapsulation Studies Using Negatively Charged Liposomes[e]

		PC/PA (9:1) liposomes						
		Liposome-associated inulin[c] (%)	W/O emulsion Lipid (mg)	W/O emulsion PBS[d] (μl)	Lipid recovery (%)	E (μl H_2O/μmol lipid)	E^{ratio} (μl H_2O/μl lipid)	Bilayer number
1	SPLV	15	100	300	61	0.580	0.74	≧15
2	MLV-REV	26	100	300	82	0.748	0.95	≧15
3	REV	29 ± 7.5	100	1000	86 ± 12	2.65	3.45	2–5
		PC/PA/CH (5:1:4)[c]						
		Liposome-associated sucrose[a] (%)	W/O emulsion Lipid (mg)	W/O emulsion PBS[d] (μl)	Lipid recovery (%)	E (μl H_2O/μmol lipid)[b]	E^{ratio} (μl H_2O/μl lipid)[b]	Bilayer number
4	SPLV	19 ± 0.6	100	300	85 ± 1.0	0.418 ± 0.02	0.67 ± 0.02	≧15
5	MLV-REV	25 ± 2	100	300	87 ± 5.3	0.522 ± 0.036	0.86 ± 0.07	≧15
6	REV	62 ± 12	100	1000	93 ± 3.6	4.08 ± 0.9	6.66 ± 1.20	2–5
7	SPLV	9 ± 2.6	20	300	83.67 ± 1.5	0.987 ± 0.28	1.61 ± 0.46	>10
8	MLV-REV	12 ± 5.6	20	300	91.0 ± 3	1.22 ± 0.6	1.98 ± 0.98	>10
9	REV	24.3 ± 1.15	20	1000	82 ± 7.5	9.12 ± 0.53	14.88 ± 0.865	1

[a] These values depend on lipid recovery.
[b] These values are normalized for lipid recovery.
[c] The reported values are the mean ± SD from three determinations.
[d] PBS, phosphate-buffered saline.
[e] ^{14}C-Inulin or ^{14}C-sucrose was used to quantitate liposome-associated inulin and sucrose. ^{3}H-DPPC was used to quantitate lipid recovery after vesicle purification. Calculation of E, E^{ratio}, and bilayer number is discussed in the text.

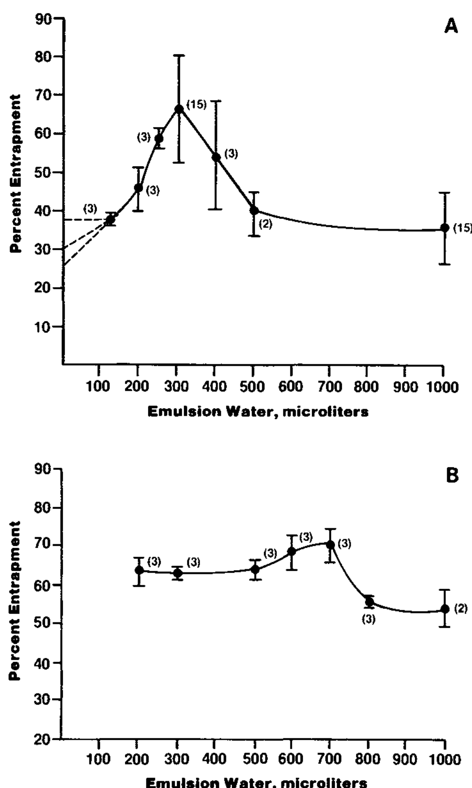

Fig. 2. Drug entrapment vs emulsion water. Egg PC, 100 mg (A) or 200 mg (B), was used to prepare the vesicles. Emulsion water (as PBS) varied from ~125 to 1000 μl. Values represent the mean ± SD. The number of separate liposome samples at different emulsion water values is given in parenthesis. ^{14}C-Sucrose was used to estimate percentage entrapment.

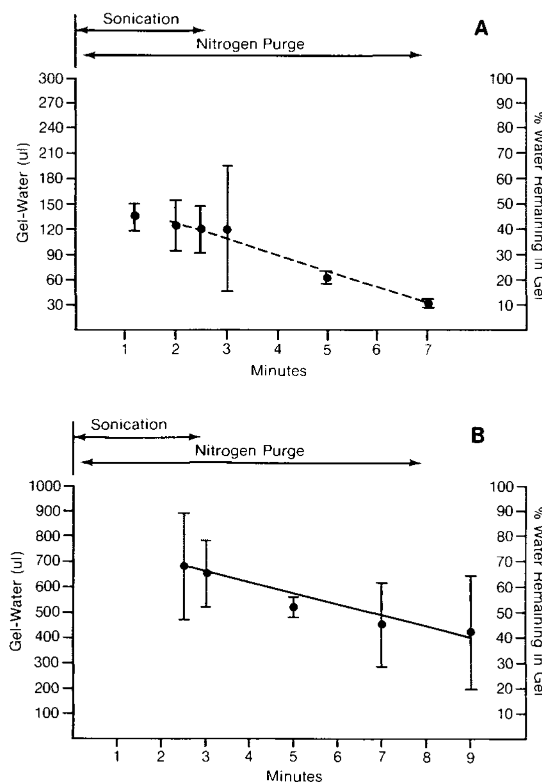

Fig. 3. Water loss during SPLV formation. SPLV liposomes were prepared by sonicating and purging an emulsion of phosphate-buffered saline and ether containing 100 mg egg PC. Liposomes were formed from either a 10/0.3 (v/v) (A) or a 10/1 (v/v) (B) ratio of ether/PBS. Water was measured by NMR (see text). Linear regression for the line in A yielded r = 0.931, m = 21.1, and an intercept of 180 μl. Linear regression for the line in B yielded r = 0.967, m = 40.7, and an intercept of 763 μl. Data in A reflect 16 populations of liposomes and the data in B reflect 18 populations of liposomes. Conditions used to prepare SPLV vesicles, shown at the top of A and B, show a 20-sec sonication interval before purging with nitrogen; sonication was from 0 to 2.5 min.

damage, particularly if insufficient water is left to hydrate the phospholipid molecules. Membrane damage is expected to affect the final amount of drug associated with liposomes. We make the assumption that the hypertonic gel suspension of SPLVs and MLV-REV vesicles can be diluted with excess isotonic buffer to generate vesicles that osmotically swell depending on the internal salt concentration.

Figure 4 shows that the E^{ratio} of the final liposome population depends on the amount of water remaining in the gel-suspension stage. The trends, however, are different for SPLVs and MLV-REV. For SPLVs, decreasing water in the gel suspension decreases the E^{ratio} until about 100 μl gel water, and then the E^{ratio} increases. For MLV-REV vesicles the E^{ratio} continues to decrease as gel-suspension water is lost. The best-fit line for MLV-REV (Fig. 4B) gave $\hat{y}$ = 1.054 + 0.0066x (P > 0.001).

The relationship between E^{ratio} and gel-suspension water for SPLVs is complicated. For SPLVs prepared from ether/H_2O 10/0.3 (v/v), the E^{ratio} decreases until ~90 μl of water remains in the gel; the E^{ratio} then increases as the remaining 90 μl of water is lost. Removing all but residual water required ~8 to 9 min of nitrogen purging. Statistical analysis of the SPLV curve (Fig. 4) using a linear combined model (i.e., simultaneously fitting two linear equations to minimize residual) showed the ascending and descending lines to be statistically significant. The best-fit lines gave $\hat{y}_1$ = 1.895 − 0.0126x (P < 0.005) and $\hat{y}_2$ = − 0.936 + 0.0149x (P < 0.02). The total mean square error was 0.0275, and the combined r^2 was 0.680. The estimated crossing point of the ascending and descending lines was 103 μl of gel-suspension water. The trends in Fig. 4 can be explained from the known hydration behavior of phospholipid molecules in water (10, 14–19) and the computer-generated data (Table I, Fig. 1).

Phosphilipids immersed in excess water (not buffered salt solutions) form lamellar phases of alternating layers of water and lipid (10). In excess water (>45 wt% water; Ref. 10) an equilibrium bilayer separation of $w \cong 29$ Å is found for PC bilayers. Dispersing PC in physiological saline or sucrose solution does not change the 29-Å intralamellar water spacing (23). These accurate measurements of w are derived from X-ray diffraction and have been performed on liposomes prepared from many different lipid compositions. X-ray diffraction measurements of the repeat spacing in SPLVs is 64–66 Å (23), which corresponds to a w of ~29 Å if one assumes that the bilayer thickness l is 37 Å. In these studies the SPLV repeat spacing varied less than 2 Å when the vesicles were prepared in water or 0.29 M salt, which is twofold the physiological osmolality. The population parameter E^{ratio} in conjunction with X-ray diffraction measure-

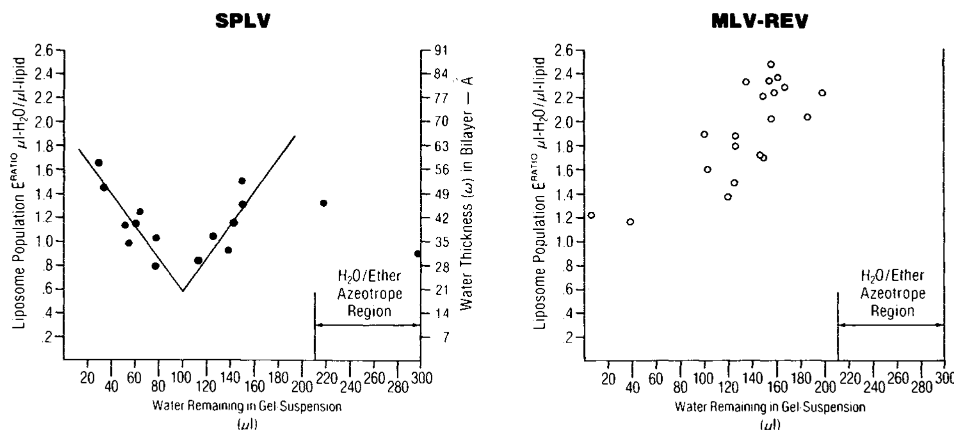

Fig. 4. The encapsulation ratio correlates with the amount of water remaining in the gel suspension. MLV-REV (○) and SPLV (●) liposomes were prepared from ether/H_2O 10/0.3 (v/v) emulsions. Encapsulation ratio (E^{ratio}) was calculated from radiolabels, and gel suspension was measured by ^{1}H-NMR. The water-layer thickness (w) in the repeating bilayers was calculated from $E^{ratio} = w/l$ and a bilayer thickness (l) of 35 Å (see Results).

ments of w must be compared to evaluate the vesicles. Figure 4 shows w for SPLVs to be near 30 Å for many of the measurements. In addition, Table II shows neutral SPLVs to have an E^{ratio} of 1.27 ± 0.14 or 1.08 ± 0.17 when inulin and sucrose were used to prepare the liposomes. The estimated w (from E^{ratio}) is 44 and 30 Å, respectively. Table III shows negatively charged SPLVs to have an E^{ratio} of 0.74 and 0.67 ± 0.02 using inulin and sucrose as aqueous space markers. These E^{ratio} values correspond to w = 26 and 23.5 Å, respectively. The data in Table II, Table III, and Fig. 4 show that SPLVs fit the computer-simulated model shown in Fig. 1. SPLVs are multilayered, $E^{ratio} \cong w/l$, and most of the trapped water is in the repeating bilayer. Stated differently, the SPLV core is not of sufficient volume to contain most of the trapped aqueous volume. The core can be collapsed by dehydration as described below.

SPLV vesicles prepared from 100 mg PC requires ~100 µl of water for the PC/water mixture to have 50 wt% water. Since w remains near 30 Å for >45 wt% water, the decrease in the E^{ratio} observed upon dehydrating the SPLV gel suspension to 100 µl gel water does not reflect a decrease in the interlamellar water spacing (Fig. 4). Dehydrating the SPLV PC/water mixture from 210 to 100 µl water reflects collapse of the SPLV core. From linear regression analysis, the crossing point of the ascending and descending line for the SPLV curve (Fig. 4) is 103 µl; 103 µl water corresponds to ~50 wt% water in the system. Fortuitously, data points were not obtained at 50 wt% water. In Fig. 4, the E^{ratio}, however, predicts ~19-Å water spacing at 50 wt% water, and Small (10) has measured the water thickness between the phosphorylcholine headgroups of sequential bilayers to be 19 Å. In Small's model the 13 water molecules of hydration per phospholipid molecule are not included in the 19-Å distance between PC headgroups. X-ray diffraction measurements, however, include the headgroup region as part of the aqueous compartment. The 19-Å SPLV water spacing at 50 wt% water estimated from the E^{ratio} therefore suggests that the SPLV process merely generated a PC/water mixture similar to equilibrating water with PC as was done in Small's work (10).

We suppose that dehydrating the SPLV gel suspension beyond 50 wt% water initiates lipid precipitation onto the walls of the flask. The increased E^{ratio} for rehydrating SPLV gel suspensions that have previously been dehydrated to <50 wt% water (as in Fig. 4) would thus be similar to the preparation of conventional MLVs. To prepare conventional MLVs, lipids are dried or precipitated on the floor of the container. Rehydrating dried lipid with excess buffer (e.g., 10 ml) generates conventional MLVs, and conventional MLVs have E^{ratio} values greater than SPLVs prepared from 100 mg lipid and 300 µl PBS. All the dehydrated gel suspensions in Fig. 4 were reconstituted with 10 ml of D_2O buffer to measure H_2O and E^{ratio}. Thus the more one dehydrates beyond 50 wt% gel water, the more lipid precipitates and rehydration generates more vesicles with a large core volume. It should be emphasized that decreasing the gel water from 90 to 0 µl forces intimate contact between the drug and the lipid so that rehydration of the gel results in increased drug trapping.

Figure 4 demonstrates that MLV-REV vesicles are different from SPLV vesicles with regard to the distribution of water throughout the particles. The E^{ratio} for MLV-REV vesicles ranges between 2.0 and 2.4 when only azeotropic water is lost. This corresponds to a water thickness between 70 and 84 Å based on the E^{ratio}. As described above, neutral PC bilayers are separated by ~30 Å of water (10, 13, 22, 23) and thus the E^{ratio} overestimated w. Based on Fig. 1 and Table II, the E^{ratio} overestimates w when the core contains a large fraction of the entrapped water.[6] Thus the formation of MLV-REV liposomes from 100 mg PC and 300 µl PBS does not generate sufficient bilayers per particle for MLV-REV liposomes to fit the computer-generated data in Fig. 1 and

[6] It is interesting to compare conventional MLVs to MLV-REVs. Conventional PC MLVs typically entrap 2 µl H_2O/µmol lipid, corresponding to an E^{ratio} of ~2.5. The E^{ratio} predicted w is 87.5 Å, which is much greater than the measured values near 30 Å (10, 13, 22, 23). Conventional PC MLVs must also have a large core where a large fraction of the entrapped volume resides.

Table I. Removing water in excess of azeotropic water results in a continued decrease in the E^{ratio}. Thus, in spite of the E^{ratio} overestimating w, Fig. 5 applies to MLV-REV vesicles. The E^{ratio} decreases as gel-suspension water is lost (Fig. 4) and the amount of trapped solutes and trapped water decreases. Figure 5 describes the dehydration of neutral MLV-REV vesicles, but the majority of water loss is from the MLV-REV core instead of the repeating bilayers. All MLV-REV vesicles with gel-suspension water greater than 40 μl were brilliant white at the gel-suspension stage. This indicates that liposomes were formed at this point in the process.

The correlation between E^{ratio} and gel water depends on the amount of emulsion water. No trends are established when emulsion water is increased from 300 to 1000 μl (Fig. 6). The average E^{ratio} was 3.66 ± 0.86 (N = 15) for REVs and 3.67 ± 0.63 (N = 15) for SPLVs. These E^{ratio} values suggest that the liposomes prepared by both processes are multilayered and have between two and five layers. Because these vesicles contain so few bilayers, the use of the E^{ratio} to estimate w is prohibited. It is difficult to remove gel-suspension water by vacuum (750 mm Hg), and the gel water ranged from 600 to 900 μl for the REV process. However, nitrogen purging removes water rapidly by evaporation (~40 μl/min; Fig. 3B) and gel-suspension water ranged from 250 to 900 μl for the SPLV process (Fig. 4). We intentionally removed significant amounts of water from the gel suspension to elucidate the mechanism of liposome formation from W/O emulsions.

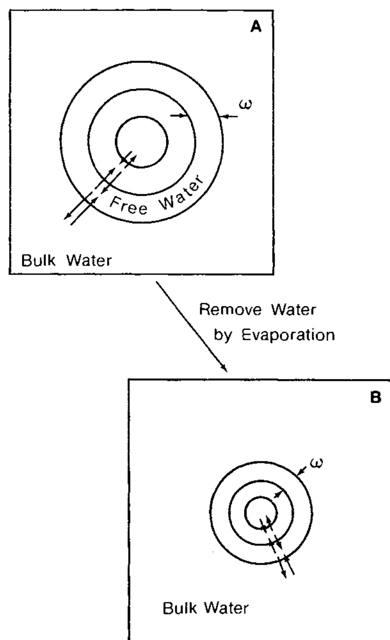

Fig. 5. Dehydration of phospholipid water systems for ≧45% weight water. Liposomes formed from W/O emulsions have water in three states, bulk or extraliposomal, free, and bound. Bound water is not shown in the diagram. Because water equilibrates across the membranes, rapidly removing water from the "gel-suspension" stage may cause core collapse and/or the water thickness (w) to decrease in the liposome.

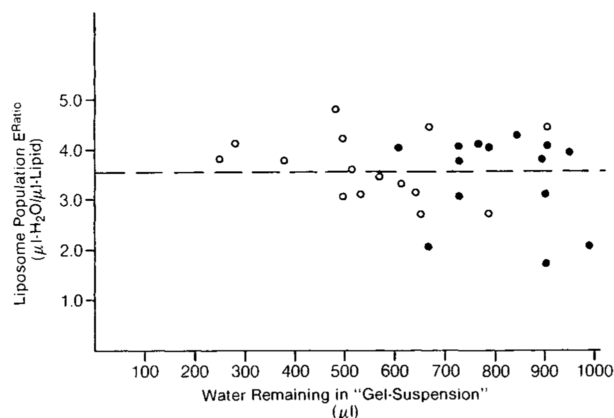

Fig. 6. E^{ratio} vs gel-suspension water. The REV (●) and SPLV (○) processes were followed using 100 mg egg PC and an ether/PBS 10/1 (v/v) emulsion. The mean E^{ratio} (dashed line) was 3.66 ± 0.86 (N = 15) for REVs and 3.67 ± 0.63 (N = 15) for SPLVs. Gel-suspension water was measured by NMR and E^{ratio} was calculated from ^{14}C-sucrose and ^{3}H-DPPC recoveries in the final liposome population.

Model

A model to predict multilayered vesicles from the REV process must explain the experimental drug-trapping data; the bilayer number depends on both the lipid concentration and the volume of emulsified water.

A model can be developed to explain these observations if one considers the process used to make multilayered vesicles from W/O emulsion. In a broad sense the REV procedure forms liposomes from water droplets emulsified in an organic phase. Phospholipids in the emulsion form "inverted micelles"[7] and surround the water droplets; removal of the organic solvent makes the inverted micelles coalesce into liposomes (1). Multilayered vesicles are formed when the emulsified aqueous phase is small yet the lipid content is high. The theoretical amount of lipid (egg PC) needed to form a lipid monolayer around emulsified water droplets is shown in Fig. 7. Water droplets were calculated from 300 μl of water emulsified in excess ether. For this constant volume of water, increasing the water drop diameter above ~0.5 μm does not significantly alter the combined surface area of all the drops in the emulsion. Thus the asymptotic curve (Fig. 7) shows very little change in the amount of lipid needed to cover 300 μl of emulsified water droplets between 0.5 and 0.9 μm (e.g., ~6 mg lipid for 0.5-μm droplets vs 3.7 mg lipid for 0.9-μm droplets). One can see how a heterogeneous population of emulsion water drops (300 μl total water) between 0.5 and 1 μm in diameter can generate a heterogeneous distribution of liposomes. Albeit heterogeneous in diameter, such liposomes would be predominantly single or multilayered (with an average number of bilayers) depending on the ratio emulsion lipid/emulsion water. Figure 7 can be

[7] In a thermodynamic sense emulsion water drops coated with lipid are not inverted micelles, because inverted micelles do not have a large aqueous core. The term "inverted micelle" is used as a functional definition for the process.

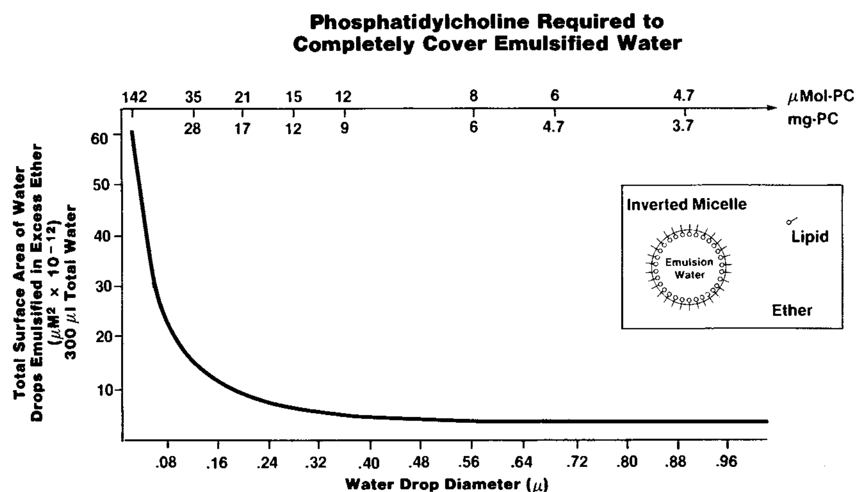

Fig. 7. Phosphatidylcholine (μ*M*) required to form a monolayer around water drops emulsified in excess ether. An area per molecule of 70 A^2 for PC was used to estimate the amount of lipid needed to cover the surface area generated by 300 μl of water emulsified in excess ether.

scaled up or down depending on the volume of emulsion water. For instance, 600 μl of emulsion water would require two times the lipid (in Fig. 7) to form inverted micelles around all emulsion drops of defined size.

Figure 7 can help explain many of the results. Single-layered REV liposomes are typically >0.5 μm in diameter (1, 20) and are prepared from 1 ml water and 20 mg lipid (Tables II and III). From Fig. 7 it can be shown that 1 ml of water droplets requires 30 μmol of PC (~23 mg) to form a lipid monolayer around every water drop. Thus liposome formation, i.e., bilayer formation, requires some water drops to transfer lipids from their monolayers to other inverted micelles so bilayers can be formed. Single-layered vesicles are formed under these limiting conditions. Increasing the lipid content to 127 μmol PC (100 mg) allows enough lipid to form approximately two bilayers (127 μmol ÷ 60 μmol/bilayer), in agreement with Fig. 6 and Table II (line 3 vs line 6). Decreasing the emulsified water from 1.0 to 0.3 ml significantly decreases the amount of lipid needed to form monolayers around all emulsified water drops. Only 8 μmol of PC is needed to cover the total surface area of 0.3-ml water drops 0.5 μm in diameter (Fig. 7). Water loss during the SPLV and MLV-REV process is at least 100 μl (90 μl of H_2O is removed with 10 ml ether; see Fig. 2), and instead of ~8 μmol only ~5 μmol is required to cover the surface area. MLV-REV vesicles prepared from 127 μmol PC have enough excess phospholipid to form ~12 bilayers (Tables II and III).

Thus the simple model in Fig. 8 explains single- and multilayered liposome formation by the REV process. Single-layered vesicles are formed from W/O emulsions containing minimal amounts of lipid. Minimal lipid is defined as the amount needed to form less than or equal to one/half bilayer (inverted micelle) around emulsified water drops. Multiple bilayer formation from inverted micelles occurs when lipid excess exists in the W/O emulsion. Successive lamellae are established as a function of time during removal of the organic solvent in the emulsion. As the ether is removed, the concentration of lipid increases and the lipids begin to coat the inverted micelles. The coating forms a bilayer structure and single-layered liposomes are generated. Removing more ether forces additional lipid to form bilayer structures on top of existing liposomes. The number of lamellae depends on the amount of excess phospholipid and the surface area of the water drops. Lamellae are the most favorable orientation for phospholipids in water/phospholipid mixtures from 9 to 50% (w/w) (10). Bilayer structures are formed on top of existing liposomes, because the total surface area of the emulsion particles (Fig. 7) is 10^4 times the surface area of the vessel used to prepare the liposomes; phospholipid molecules must stack and assemble on top of each other. Phospholipid self-assembly requires the polar headgroup to align and the nonpolar tails to align regardless of the solvent.

As successive bilayers are laid down, ether remaining in the gel suspension allows solutes to equilibrate across bilayers. Bubbling and gel inversion (MLV-REV process) will also facilitate solvent and solute distribution across bilayers. However, sequentially formed bilayers will have an unequal distribution of solutes if the above mechanisms are insufficient to force solutes to equilibrate. Solute equilibration may not exist in the outermost lamella of MLV-REV vesicles. In contrast, SPLV liposomes were formed during sonication and one can expect an even distribution of solute among bilayers regardless of gel inversion and residual ether. In addition, sonication will cause the PC molecules to be continually dispersed and then reassembled. This will generate structually different liposomes. Consider a hypothetical MLV-REV gel suspension containing 100 mg lipid, 200 μl water, a 30-Å water spacing between the lamellae, and a 0.5-μm diameter of the innermost lamella (i.e., core diameter). Sonicating this water/lipid mixture will cause collapse of the core and release of entrapped solutes to bulk water. The SPLV process is similar to this example. If one sonicates throughout the REV process, one can expect structurally different MLV-REV vesicles (i.e., SPLV vesicles) compared to performing the process without sonication. Sonication causes the phospholipids to be continually dispersed throughout the W/O emulsion and subsequent gel-suspension phase where a lipid–water mixture exists. Thus compared to MLV-REV vesicles, sonicating throughout the REV process (SPLV vesicles) causes a smaller liposome di-

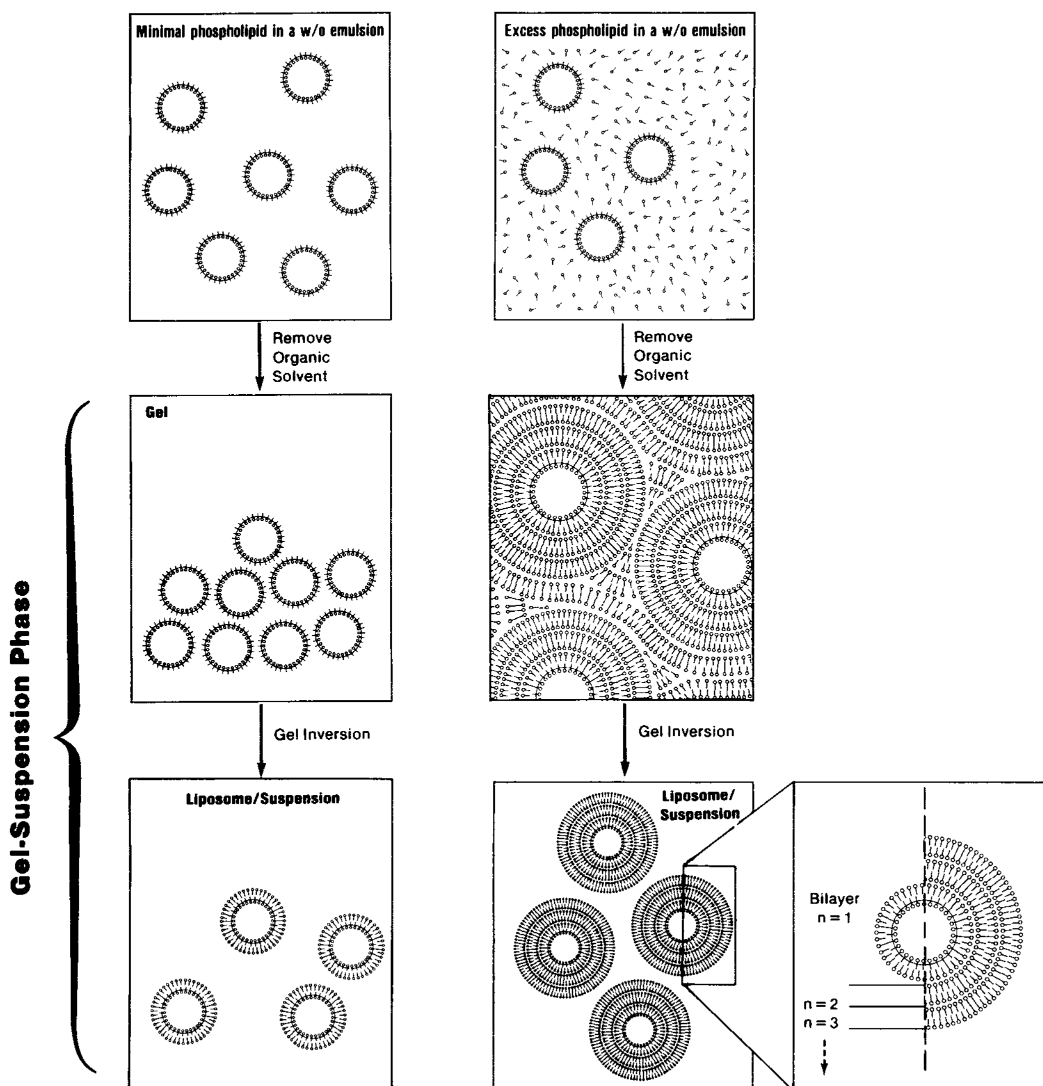

Fig. 8. Emulsion, gel-suspension, and liposome formation during the REV process in the presence of minimal and excess phospholipid molecules. Phospholipid excess exits when the amount of phospholipid in the W/O emulsion exceeds the amount of lipid needed to form a monolayer around the emulsified water drops. Phospholipid molecules are designated as lollipops. At the emulsion stage, inverted micelles composed of phosphatidylcholine surround the water drops, and the excess phospholipid is dissolved in the organic solvent. A gel forms after most of the organic solvent is removed. Under conditions of minimal phospholipid, a gel is formed from a collection of inverted micells. Bilayer formation under these conditions require some inverted micelles to transfer lipids to other micelles; some micelles need to be sacrificed so other micelles can become liposomes. Under conditions of excess phospholipid, lamella phases are established around the inverted micelles when the organic solvent is removed from the W/O emulsion. The number of lamellae depends on the excess phospholipids.

ameter, a greater number of bilayers, a smaller inside core diameter, and a lower drug entrapment (Tables II and III, Fig. 4). The internal core of the SPLV vesicles will decrease for the same reason the diameter of large single-layered REV vesicles would decrease to that of small unilamellar vesicles by continuous sonication.

MLV formation by sonicating concentrated lipid/water mixtures as in the SPLV process is distinct from forming small unilamellar vesicles (SUVs) by sonication. SUVs are made by sonicating conventional MLVs suspensions containing small amounts of lipid (e.g., 20 mg) and large amounts of water (e.g., 1 ml). SUVs are generated because individual phospholipid molecules or membrane fragments can be temporarily dispersed in bulk water. The self-assembly of these isolated lipid molecules or membrane fragments in bulk solution generates small liposomes with low drug entrapment. Low drug entrapment occurs because the internal SUV aqueous compartment is a small fraction of the total aqueous phase in which the parent MLVs were sonicated. However, water is minimal in the SPLV process and sonication cannot produce isolated lipid molecules in bulk solution. High drug entrapment occurs from the SPLV process because these MLV vesicles entrap a large fraction of the water containing drug. From these considerations, it is evident that Fig. 7 does not apply to the SPLV process because continuous sonication causes the emulsified water drops to change in size. Bilayer numbers will be greater than predicted by Fig. 7 for SPLV vesicles. The amounts of emul-

sion water, gel-suspension water, sonication time, and sonication power, however, are all important and can influence the type of liposome formed under a particular set of experimental conditions. For instance, 1 ml emulsion water generated vesicles with similar E^{ratio} values ($E^{ratio} \sim 3.6$) for both the SPLV and the MLV-REV process (Fig. 6). An E_{ratio} of 3.6 suggests vesicles with two to five bilayers; not enough excess emulsion lipid is available to form multilayers.

Figure 8 depicts excess phospholipid as only monomers; however, phospholipid dimers, trimers, *n*-mers, and lamellae are also possible. For example, Aarts *et al.* (21) reports that ethanolic lipid solutions contain lipid aggregates when the ethanol is evaporated.

SUMMARY

This report introduced the concept of the E^{ratio} and then used the E^{ratio} to elucidate some of the mechanisms associated with liposome formation from W/O emulsions. The E^{ratio} was described as a fundamental liposome population parameter that can be used to estimate the number of bilayers and water thickness layer in multilayered vesicles (Fig. 1).

We have studied MLV formation from W/O emulsions whereby the organic solvent is removed during continuous sonication or after the cessation of sonication. Without sonication, water loss during the REV process is due to a water–organic solvent azeotrope. In our experiments, ether was used to form the W/O emulsion and 90–100 μl water was lost by azeotrope. Other organic solvents will remove different amounts of water depending on their azeotropic ratio. Removing the organic solvent during sonication creates a mist that also causes water loss from W/O emulsions. Thus removing the organic solvent with a nitrogen purge while sonicating the W/O emulsion causes water loss by both azeotrope and mist. This water loss is rapid and substantial (Fig. 3) and depends on the amount of water in the W/O emulsion.

We have defined the gel-suspension stage of the REV process to refer to the W/O emulsion after most of the organic solvent is removed but before excess buffer is added to resuspend the liposomes. The amount of water remaining in the gel suspension affects percentage of the drug entrapped and E^{ratio} (and perhaps w) in the final liposome population (Fig. 4). Gel-suspension water also determines the physical appearance of the PC/water mixture and determines whether the PC/water mixture does not invert, inverts slowly, or inverts rapidly from a gel to a liposome suspension.

NOTATION

REV	Reverse-phase evaporation; a process used to make liposomes from W/O emulsions
SPLV	Stable plurilamellar vesicle(s)
MLV-REV	REV liposomes with multiple bilayers
MLV	Multilamellar vesicle
E	Encapsulation efficiency (μl H_2O/μmol lipid)
E^{ratio}	Encapsulation ratio (μl H_2O/μl lipid)
PC	Phosphatidylcholine
CH	Cholesterol
PA	Phosphatidic acid
PBS	Phosphate-buffered saline
PBS-D_2O	Deuterated phosphate-buffered saline
w	Water-thickness layer in MLVs
l	Bilayer thickness
W/O	Water/organic solvent
^{3}H-DPPC	^{3}H-Dipalmitoylphosphatidylcholine
Gel water	Water content in the gel formed by the REV process
Gel lipid	Lipid content in the gel formed by the REV process
Gel-suspension phase	A lipid–water mixture obtained from W/O emulsions after most of the organic solvent is removed

ACKNOWLEDGMENTS

The authors wish to thank Angela Simpson for assistance in preparing the manuscript. The authors would also like to thank M. C. Smith for helpful discussions and J. Q. Oeswein for SAS computer simulations. We are grateful to S. Gruner and M. Ostro for sharing unpublished X-ray diffraction data on SPLV liposomes with us.

REFERENCES

1. F. Szoka and D. Papahadjopoulos. *Proc. Natl. Acad. Sci. USA* 75:4194–4198 (1978).
2. European Patent Office EP-92453-A, South Africa Patent ZA8302197-A, and Japan Patent JS9500952-W.
3. R. Fraley, S. Subramani, P. Berg, and D. Papahadjopoulos. *J. Biol. Chem.* 255:10431–10435 (1980).
4. N. Haran and M. Shporer. *Biochim. Biophys. Acta* 426:638–646 (1976).
5. J. Andrasko and S. Forsen. *Biochem. Biophys. Res. Comm.* 60:813–819 (1974).
6. C. Pidgeon and C. A. Hunt. *J. Pharm. Sci.* 70:173–176 (1981).
7. S. M. Johnson. *Biochim. Biophys. Acta* 307:27–41 (1973).
8. B. A. Pethica, J. Mingins, and J. A. G. Taylor. *J. Colloid Interfac. Sci.* 55:2–8 (1975).
9. H. Hauser, I. Pascher, R. H. Pearson, and S. Sundell. *Biochim. Biophys. Acta* 650:21–51 (1981).
10. D. Small, *J. Lipid Res.* 8:551–557 (1967).
11. D. L. Melchior and H. J. Morowitz. *Biochemistry* 11:4558–4562 (1972).
12. D. Marsh. *Biochim. Biophys. Acta* 363:373–386 (1974).
13. J. T. Mason and C. Huang. *Ann. N.Y. Acad. Sci.* 308:29–49 (1978).
14. D. A. Wilkinson, H. J. Morowitz, and J. H. Prestegard. *Biophys. J.* 20:169–179 (1977).
15. D. M. LeNeveu, R. P. Rand, D. Gingell, and V. A. Parsegian. *Science* 191:399–400 (1976).
16. D. M. LeNeveu, R. P. Rand, V. A. Parsegian, and D. Gingell. *Biophys. J.* 18:209–230 (1977).
17. D. Chapman. In G. Gregoriadis (ed.), *Liposome Technology, Vol. I. Preparation of Liposomes,* CRC Press, Boca Raton, Fla., 1983, pp. 1–18.
18. R. M. Williams and D. Chapman. *Phospholipids Liquid Crystals and Cell Membranes, Progress with Chemistry of Fats and Lipids,* Pergamon Press, Oxford, 1970.
19. D. A. Wilkinson and J. Nagle. In C. G. Knight (ed.), *Thermodynamics of Lipid Bilayers, Liposomes: From Physical Structure to Therapeutic Applications,* 1981.
20. F. Szoka, F. Olson, T. Heath, W. Vail, E. Mayhew, and D. Papahadjopoulos. *Biochim. Biophys. Acta* 601:559–571 (1980).
21. P. A. M. M. Aarts, O. L. J. Gijzeman, J. M. H. Kremer, and P. H. Wiersema. *Chem. Phys. Lipids* 19:267–274 (1977).
22. L. J. Lis, M. McAlister, N. Fuller, R. P. Rand, and V. A. Parsegian. *Biophys. J.* 37:657–666 (1982).
23. S. M. Gruner, R. P. Lenk, A. J. Janoff, and M. J. Ostro. *Biochemistry* 24:2833–2842 (1985).
24. R. P. Rand. *Annu. Rev. Biophys. Bioeng.* 10:277–314 (1981).
25. V. A. Parsegian, N. Fuller, and R. P. Rand. *Proc. Natl. Acad. Sci. USA* 76:2750–2754 (1979).