Phase Behavior of Large Unilamellar Vesicles Composed of Synthetic Phospholipids†

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ABSTRACT: Large unilamellar vesicles (LUV) have been prepared by three procedures from several synthetic and natural phosphatidylcholines. Reverse-phase evaporation vesicles (REV) and fusion vesicles were prepared by established procedures. A published procedure for the preparation of dialyzed octyl glucoside vesicles (DOV) was modified to allow its use with synthetic phospholipids. Negative-staining and freeze–fracture electron microscopy was used to determine the vesicle size distribution (mean diameters 800–1000 Å) and extent of oligolamellar contamination in DOV preparations. Trapping of 6-carboxyfluorescein yielded measurements of the internal volume (2.6 ± 0.3 μL/μmol of P_i) consistent with the size distributions determined by electron microscopy. An upper limit of less than 3 mol % oligolamellar vesicle contamination was indicated by calorimetric heat capacity profiles. The phase behaviors of large multilamellar vesicles and all three types of LUV were compared by using high-sensitivity differential scanning calorimetry and fluorescence depolarization of the membrane probe diphenylhexatriene. The most remarkable feature was the increased breadth of the main transition of DOV and of REV relative to the multilamellar species and to fusion vesicles. Both the main transition and the pretransition occurred at nearly the same temperatures in unilamellar and multilamellar species, but the unilamellar pretransition involved less than half the enthalpy observed in the multilamellar transition. Additional experiments indicated that the broadened main phase transition associated with DOV and REV reflected bilayer impurities resulting from preparation. It is concluded that LUV prepared by procedures that avoid impurities undergo a highly cooperative phase transition, as demonstrated here for fusion vesicles.

Despite the well-documented preparation of small unilamellar vesicles (SUV)[1] (Huang, 1969) and the ease of formation of large multilamellar vesicles (LMV) (Bangham, 1968), several considerations have pointed to the need for unilamellar vesicles of larger than limiting size but having a defined surface area. First, the high degree of bilayer curvature (Sheetz & Chan, 1972; Huang & Mason, 1978) and distinct phase behavior (Suurkuusk et al., 1976; Lentz et al., 1976a,b) of SUV as compared to LMV have led to the notion that SUV contained strained and unstable bilayers that are good models only of highly curved regions of biological membranes. Large unilamellar vesicles (LUV) would be better models for the more common uncurved regions. Second, purified integral membrane proteins have been reconstituted into unilamellar vesicles of 800–2000-Å diameter (Petri et al., 1980; Moore et al., 1981). Protein-free LUV of similar size are needed for comparison in order to establish the effect of protein on bilayer properties. Finally, a well-defined LUV population is needed to ascertain the extent to which interbilayer interactions influence the phase behavior of multilamellar vesicles. Much of our understanding of the lateral mixing properties of different lipid species comes from studies of the phase behavior in multibilayer structures. If interbilayer interactions significantly affect phase behavior in LMV, conclusions drawn about the structure of bilayer regions of biological membranes from such studies would be questionable.

A variety of preparatory methods for LUV have been proposed, including organic solvent evaporation techniques (Kremer et al., 1977; Deamer & Bangham, 1976; Schieren et al., 1978; Szoka et al., 1980), detergent removal methods (Milsmann et al., 1978; Enoch & Strittmatter, 1979; Zumbuehl & Weder, 1981; Petri et al., 1980; Mimms et al., 1981), and other procedures (Reeves & Dowben, 1969; Barenholz et al., 1979; Lichtenberg et al., 1981; Dufour et al., 1981). While advantageous for some applications, these preparations suffer from several shortcomings. These include small size typical of cholate-removal and ethanol-injection vesicles (Zumbuehl & Weder, 1981; Kremer et al., 1977), size heterogeneity (Reeves & Dowben, 1969), intricate and irreproducibile preparation techniques (Deamer & Bangham, 1976), applicability only to certain lipids (Lichtenberg et al., 1981), and contamination by multilamellar vesicles (Deamer & Bangham, 1976; Reeves & Dowben, 1969; Szoka et al., 1980).

A procedure that seemed likely to provide fairly homogeneous unilamellar vesicles with diameters of about 1000–2000 Å was the removal of octyl glucoside from a solubilized lipid sample (Zumbuehl & Weder, 1981; Mimms et al., 1981). However, when we attempted to apply a published procedure (Mimms et al., 1981) to *synthetic* phospholipid preparations, we encountered difficulties undocumented in the original description of the method as applied to a natural (egg yolk) phosphatidylcholine preparation. We report here detailed modification of octyl glucoside dialysis procedures that yield reproducible LUV preparations with a variety of synthetic and natural phospholipids. In addition, the phase behavior of LUV prepared by this procedure, by reverse-phase evaporation, and by SUV fusion is described and contrasted with the phase

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[1] Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; LUV, large unilamellar vesicle(s); LMV, large multilamellar vesicle(s); SUV, small unilamellar vesicle(s); REV, reverse-phase evaporation vesicle(s); DOV, dialyzed octyl glucoside vesicle(s); egg PC, egg phosphatidylcholine; DMPC, 1,2-dimyristoyl-3-*sn*-phosphatidylcholine; $DC_{15}PC$, 1,2-dipentadecanoyl-3-*sn*-phosphatidylcholine; DPPC, 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; DSC, differential scanning calorimetry; TLC, thin-layer chromatography.

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behavior of LMV prepared from the same lipids. The results have demonstrated that impurities associated with solvents and detergents used in LUV preparations are partly responsible for observed differences between LMV and LUV phase behavior. The results show that the phase behavior of LUV approached that of LMV when sufficient efforts were made to avoid contamination.

Materials and Methods

Materials. Chloroform solutions of recrystallized (Lentz et al., 1976a,b) lipids [1,2-dimyristoyl-3-*sn*-phosphatidylcholine (DMPC), 1,2-dipentadecanoyl-3-*sn*-phosphatidylcholine ($DC_{15}PC$), and 1,2-diaplmitoyl-3-*sn*-phosphatidylcholine (DPPC)] were obtained from Avanti Biochemical (Birmingham, AL) and determined to contain less than 1 mol % polar lipid impurity by thin-layer chromatography (Lentz et al., 1976a,b). Egg phosphatidylcholine (egg PC) was isolated from fresh hen egg yolks (Newman & Huang, 1975). Octyl β-D-glucopyranoside was purchased from Calbiochem (La Jolla, CA; lot 110012), and [^{14}C]octyl β-D-glucopyranoside was purchased from New England Nuclear (Boston, MA; lot 1141-235); ultrapure potassium chloride was supplied by Heico, Inc. (Delaware Water Gap, PA; lot 2179). 6-Carboxyfluorescein was obtained from Molecular Probes, Inc. (Junction City, OR). Deionized water for preparation of vesicles was distilled twice (initially from basic permanganate) by using an all-glass apparatus and argon bubbled before use. Isopropyl ether (Fisher certified, lot 73108-4) and diethyl ether (Baker reagent, lot 43649) were passed over an aluminum oxide column before use. In addition, it was found necessary to distill these commercial ethers from $LiAlH_4$ in order to minimize impurities in the REV preparations. All other materials were of the best quality available.

Synthetic Vesicles. (*A*) *Dialyzed octyl glucoside vesicles* (DOV) were made by using a slow dilution–dialysis method, starting with a detergent to lipid molar ratio of 10:1. In a typical preparation, 30 μmol of lipid dissolved in chloroform was mixed with 300 μmol of octyl glucoside dissolved in 2:1 chloroform:methanol, dried under a stream of argon as a thin film onto the bottom of a 10-mL Reacti-Flask (Pierce Chemical Co., Rockford, IL), and placed under high vacuum for 8–12 h to remove residual solvent. One milliliter of an aqueous solution containing 25 mM octyl glucoside and 50 mM KCl was added to the dried lipid–detergent sample with vigorous vortexing. The detergent in the aqueous medium was present to maintain the ratio of detergent to lipid in the mixed micelles at 10:1, taking into account the high critical micelle concentration of octyl glucoside (22 mM; Jackson et al., 1982). The clear lipid–detergent suspension was diluted slowly to a final volume of 10 mL with 50 mM KCl. The buffer was pumped into the magnetically stirred detergent–lipid solution by means of an LKB peristaltic pump set to a speed of 0.3 mL/h. Both the lipid–detergent suspension and the diluting buffer were kept at a temperature above the phase transition temperature of the lipid being used. Typically, the dilution required 30 h to complete, and the lipid reached a final concentration of approximately 3 mM. The lipid–detergent suspension was then dialyzed against 800 mL of 50 mM KCl. The initially clear dialysates became turbid at 0.5 h into the dialysis and remained cloudy throughout. DMPC and $DC_{15}PC$ preparations had a tendency to flocculate during dialysis. This was overcome by gently sonicating these samples in the dialysis bags for 30 s by using a Heat Systems sonicator equipped with a Heat Systems cup horn to break up aggregates. After this treatment, the lipid remained suspended for the duration of the dialysis. Dialysis proceeded for 3 days, above the main phase transition temperature of the lipid, with buffer changes every 8–12 h. After dialysis, the sample was divided among 1.5-mL tubes and spun for 20 s at maximum speed (13 000 rpm) in a Beckman Microfuge B. The supernatant was collected and filtered through a 0.4-μm Nucleopore filter (Nuclepore Corp., Pleasanton, CA). To concentrate the sample and remove small unilamellar vesicles, the filtrate was centrifuged for 60 min at 48 000 rpm in a Ti-75 rotor. The pellet was resuspended in 2.5 mL of 50 mM KCl for subsequent use.

(*B*) *Large multilamellar vesicles* (LMV) were prepared in 50 mM KCl at a lipid concentration of 5 mM as previously described (Lentz et al., 1976 a,b).

(*C*) *Fusion vesicles* were prepared from DMPC, $DC_{15}PC$, and DPPC in 50 mM KCl–0.02% NaN_3 by cold storage (4 °C) of SUV over 3–4 weeks, according to Wong et al. (1982). Starting lipid concentrations were 100 mM. At the end of storage, extracted lipid was found free of hydrolysis products by TLC on Analtech silica gel GHL plates eluted with 65:25:4 $CHCl_3$:CH_3OH:H_2O (v/v) (Lentz et al., 1976a,b). Vesicles were diluted to approximately 5 mM with 50 mM KCl before use.

(*D*) *Reverse-phase evaporation vesicles* (REV) were prepared in 50 mM KCl as described by Szoka et al. (1980) and filtered through 0.4- and 0.2-μm Nuclepore filters. Diethyl ether, or isopropyl ether when necessary (Düzgünes et al., 1983), was removed above the phase transition of the lipid under study.

Electron Microscopy. Negative-staining electron microscopy was employed to guage the range of vesicle sizes in DOV preparations (Valentine et al., 1968). A carbon-coated mica sheet was dipped into the vesicle suspension, and the carbon layer was subsequently floated onto 2% ammonium molybdate stain. The carbon layer adhered to a 400-mesh copper grid (Polysciences Inc., Warrington, PA), which was removed to dry on filter paper. Preparations were examined within 3 h. Vesicle size distributions were obtained by measuring vesicle diameters on electron micrographs from several preparations. Vesicle diameters obtained from negatively-stained preparations were corrected for shape changes associated with dehydration and adhesion to the substrata (Szoka et al., 1980) by dividing by a factor of $2^{1/2}$. Diameters obtained from freeze–fracture preparations were not corrected, although vesicles whose fracture faces only slightly protruded from the ice substrate (judged by the degree of platinum shadowing) were avoided. Average mass-weighted vesicle diameter distributions were calculated from the measured size distribution by assuming a bilayer thickness of 47 Å [on the basis of the X-ray diffraction data of Janaik et al. (1976)] and a lipid cross-sectional area of 60 Å (Jain, 1972).

Samples for freeze–fracture were preincubated 8–10 h at the quenching temperature. A propane jet freezing device was used at very rapidly freeze the samples, which were sandwiched between two thin copper sheets (Lentz et al., 1981). After being fractured, samples were shadowed with Pt from 45°.

JEOL 100 CX or JEOL 100 B microscopes operated at 80 kV were used to view freeze–fracture replicas or negatively stained samples.

Fluorescence. Fluorescence depolarization measurements (Lentz et al., 1978) were performed on an SLM 4800 spectrofluorometer using 1,6-diphenyl-1,3,5-hexatriene (DPH) as a membrane probe. Lipid samples were diluted to a concentration of 0.2 mM in 50 mM KCl. A small amount (0.8 μL) of a 2 mM stock solution of DPH in tetrahydrofuran was injected into the sample to give a final dye to lipid ratio of 1:2000 and allowed to equilibrate with the vesicles for 8–12

h in a rotary incubator at 48 °C. Measurements were taken every 0.1 or 0.2 °C at a scan rate of ±18 °C/h. Details of the calculation of the "microviscosity" and "microviscosity activation energy" parameters are given in the literature (Lentz et al., 1978, 1980).

Calorimetry. Heating and subsequent cooling scans were made on vesicle samples by using a Tronac 750 (Provo, UT) high-sensitivity, differential scanning calorimeter constructed for us by Roger Hart (Hart Scientific, Orem, UT). This instrument is an improvement of the basic heat flow design of Suurkuusk et al. (1976), having both increased sensitivity and the ability to cool as well as to heat. Data collection is under microprocessor control. Temperature accuracy is better than 0.05 °C, and base-line noise is less than 0.04 μcal/s. To obtain excess heat capacities corrected for the dynamic response of the calorimeter, computer calculations based on the assumption of Newtonian heat flow have been performed. The computational algorithms also subtract inherent base-line temperature dependence due to the inability to perfectly match the thermal behavior of sample and reference calorimeter cells. Their validity has been checked by using samples of known phase behavior (DPPC LMV and high-purity gallium; B. R. Lentz, Clubb, Hart, and Jensen, unpublished results). Experiments were performed at a scan rate of ±15 °C/h and data recorded at temperature intervals of 0.05 °C. Samples were composed of 3–5 mg of lipid in 1.5 mL of 50 mM KCl and were sealed in stainless-steel (316) ampules.

Trapped Volume Determination. The size of internal vesicle compartments was determined by the absorbance of trapped 6-carboxyfluorescein using the method of Wong et al. (1982). Vesicle samples (0.42 mL containing 4–5 μmol of lipid in 50 mM KCl) were adjusted to 10 mM in 6-carboxyfluorescein by adding a small aliquot of 0.2 M 6-carboxyfluorescein. Vesicle suspensions were equilibrated at 48 °C in a rotary oven for 18 h at pH 7.4. A 50-μL aliquot of 0.45% (w/v) KOH was added to the sample to bring the pH to ~8.5–9.0 and thereby decrease bilayer permeability to carboxyfluorescein (Wong et al., 1982). The sample was equilibrated for 1.5 h at 48 °C. Vesicles were separated from external carboxyfluorescein by chromatography on a Sephadex G-50 column (1.2 × 12 cm) equilibrated with 50 mM KCl–20 mM TES, pH 8.5. Vesicle-containing fractions were pooled and aliquots removed for total phosphate (P_i) determination (Chen et al., 1956). The amount of marker present in the pooled sample was assayed by measuring the absorbance at 490 nm in a 1-cm microcuvette on a Hitachi Model 100-20 spectrophotometer using 0.3% Triton X-100 to release trapped carboxyfluorescein. It was necessary to record measurements 2 h after Triton X-100 addition to ensure complete release of the internal marker and eliminate problems due to scattering.

Results

DOV Preparation. The slow dilution–dialysis procedure described here reproducibly yielded fairly homogeneous large unilamellar vesicles as judged by electron microscopic examination of negatively stained preparations (Figure 1A). A sufficiently slow initial dilution step was necessary to achieve this goal. Mimms et al. (1981) have reported that reproducible egg PC LUV preparations could be obtained only at detergent to lipid molar ratios of 10:1 or greater. Initial attempts to prepare synthetic phosphatidylcholine LUV by using either the dialysis procedure of Mimms et al. (1981) or the dilution procedure used by us at dilution rates of 2.2×10^{-3} mL μmol^{-1} h^{-1} led to inconsistent results regardless of the degergent to lipid molar ratio. Some of these preparations contained nonvesicular, "wormlike" structures (see Figure 1B). These

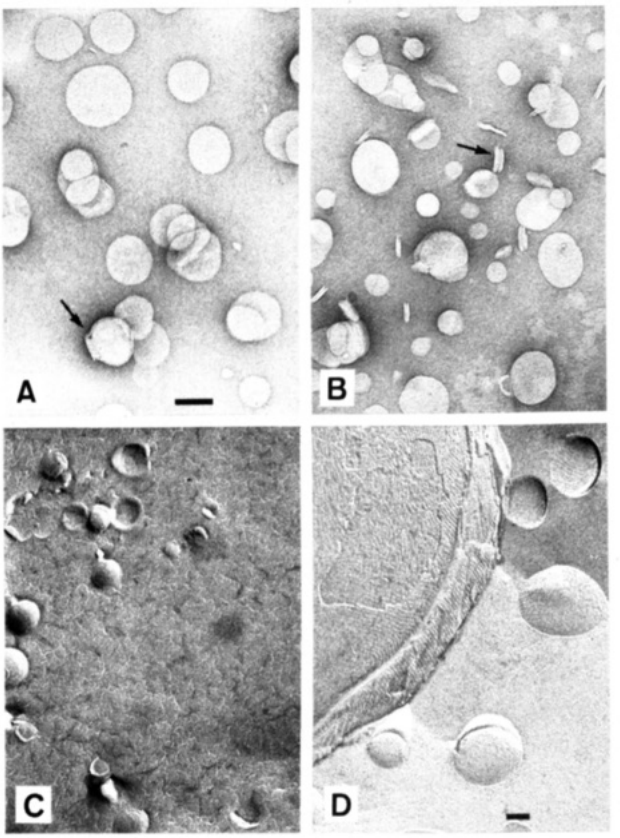

FIGURE 1: Electron micrographs of vesicle preparations. (A, B) Negative-staining electron micrographs (2% ammonium molybdate, see text) of (A) a representative DPPC preparation of DOV illustrating an oligolamellar contaminant (see arrow) and of (B) nonvesicular wormlike structures (see arrow) obtained from preparations diluted at 2.2×10^{-3} mL μmol^{-1} h^{-1}. Magnification 50000×. Scale marker = 1000 Å. (C, D) Freeze–fracture electron micrographs of (C) the same sample shown in (B) and of (D) the resuspended pellet from the initial Microfuge spin following dialysis (see text). Magnification 31520×. Scale marker = 1000 Å. Micrographs are shown with shadowing from below.

wormlike structures were not clearly visible above the ice matrix using freeze–fracture techniques (Figure 1C), indicating that this technique is not as sensitive to these structures. By contrast, at a slower dilution rate (0.9×10^{-3} mL μmol^{-1} h^{-1}), all preparations were unilamellar when a 10:1 detergent:lipid ratio was used.

Vesicle size distributions did not vary in any systematic fashion with dialysis temperature despite the fact that the rate of octyl glucoside removal was quite sensitive to temperature. We observed first-order time constants (τ) for initial rates of detergent removal by dialysis of 1.4 h at room temperature (~21 °C) with an egg PC preparation, of 0.9 h at 36 °C with a DMPC preparation, and of 0.5 h at 45–50 °C with a DPPC preparation. Using a radioactive tracer of [^{14}C]octyl glucoside, we found that dialysis at any of these temperatures for 72 h was sufficient to reduce octyl glucoside levels to less than 0.03 mol % of the total lipid.

After removing octyl glucoside, it was necessary to fractionate the preparations by centrifugation and filtration in order to obtain reasonably homogeneous populations of unilamellar vesicles. The initial Beckman Microfuge spin was performed to remove any lipid aggregates and eliminate the possibility of LMV contamination. Examination of the pellet from this centrifugation step by freeze–fracture electron microscopy revealed both aggregates of DOV and some LMV (see Figure 1D). Filtration through 0.4-μm Nuclepore polycarbonate filters was to assure an upper limit on vesicle size

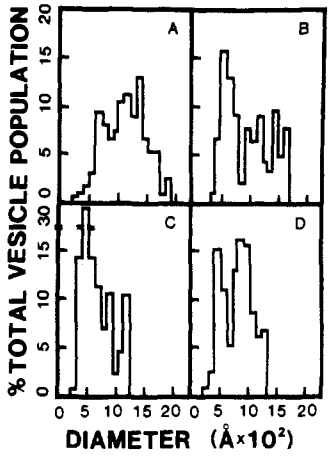

FIGURE 2: Vesicle size distributions determined by measuring 500–1500 vesicles from negative-stain electron micrographs. These histograms give mass-weighted vesicle diameter distributions calculated from observed, number-weighted distributions by using a bilayer thickness of 47 Å and a lipid cross-sectional area of 60 Å^2. (A) Egg PC LUV (mean diameter = 1120 ± 500 Å); (B) DMPC DOV (mean diameter = 980 ± 400 Å); (C) $DC_{15}PC$ DOV (mean diameter = 580 ± 200 Å); (D) DPPC DOV (mean diameter = 940 ± 400 Å).

and to remove remaining aggregates. Filters with other cutoff sizes (0.1 and 0.2 μm) were also tried. The 0.2-μm filter gave visibly reduced yields while the 0.1-μm filter did not allow any of the material to pass. A final ultracentrifugation at 48 000 rpm served two purposes. First, DOV will pellet under these conditions, while SUV (~250 Å in diameter) will not (Barenholz et al., 1977). This freed the sample from contamination by limiting-size small unilamellar vesicles and any nonvesicular material (see above), if present. Second, centrifugation provided a convenient means of concentrating the sample for further studies.

After fractionation, overall yields of phospholipid incorporated into DOV were roughly 45% for DPPC and 20% for both DMPC and $DC_{15}PC$. The largest loss (50% for DPPC and 75% for $DC_{15}PC$ and DMPC) occurred during the first microfuge spin, where extremely large aggregates were eliminated. Subsequent filtration was carried out in nearly quantitative yield, and the final ultracentrifugation step resulted in >90% recovery. The observation of aggregated DOV in the pelleted material after the the Microfuge spin suggests that somewhat better yields might be obtained by a brief sonication of the dialysate before centrifugation. In the interest of maintaining a reproducvible preparation for the studies of phase behavior reported here, this possibility was not explored further.

This method of preparation was also found to work well with dipentadecanoylphosphatidylglycerol–dimyristoylphosphatidylcholine mixtures. Vesicles prepared from these lipids did not flocculate during dialysis and gave yields in the same range as DPPC. One would expect that other lipid mixtures could be used equally well to make unilamellar vesicles by this technique.

DOV Morphology. Size distributions for several preparations of vesicles were obtained by measuring vesicle diameters in negative-stain (Figure 1A) and freeze–fracture (Figure 1C) electron micrographs. The measured diameters of 500–1500 vesicles were used to obtain the histograms shown in Figure 2 and to determine the mean, mass-weighted diameter of each vesicle population. As can be seen from Figure 2, our dilution–dialysis procedure resulted in distributions of vesicle sizes with mean diameters uniformly in the range of 800–1000 Å and with standard deviations in the diameters of 200–500 Å.

Figure 1A was also chosen to illustrate that vesicles fractionated by the double centrifugation–filtration procedures were nearly but not completely free of contamination by multibilayer species (one is indicated by an arrow in the figure). Extensive searching for oligolameller species revealed that they constituted between 0.7 and not more than 1.5 no. % of all vesicles in the final fractionated samples. Similarly, freeze–fracture electron micrographs of DPPC DOV preparations revealed less than 1 no. % of vesicles showing two or more fracture faces (data not shown).

Trapped Volume Measurements. A crucial requirement of any procedure designed to reconstitute lipids into model membrane vesicles is the formation of a permeability barrier resulting in a trapped compartment. Mimms et al. (1981) have previously shown that egg phosphatidylcholine vesicles prepared by octyl glucoside dialysis contained a trapped space. We have used 6-carboxyfluorescein to measure a trapped compartment (2.6 ± 0.3 μL/μmol of P_i) in DOV produced from DPPC (see Materials and Methods). This trapped volume would be predicted for a spherical, unilamellar vesicle of 1045-Å diameter, assuming a bilayer thickness of 47 Å (Janiak et al., 1976) and a phosphatidylcholine cross-sectional area of 60 Å^2 (Jain, 1972). By contrast, egg phosphatidylcholine SUV trap 0.17 μL/μmol of P_i as measured by the incorporation of [^{14}C]glucose and potassium ferricyanide (Roseman et al., 1978). By carboxyfluorescein trapping, we obtained a value of 0.17 ± 0.03 μL/μmol of P_i. The good agreement with the previous report serves to validate the carboxyfluorescein method.

DOV Stability. DOV prepared by our method were stable under various storage conditions. Vesicles stored with continuous agitation above (48 °C) or below (4 °C) their phase transitions were examined by negative-staining electron microscopy for up to 1 month after preparation. Vesicles sized as described above had a mean diameter of 940 ± 400 Å directly after preparation which remained essentially unchanged after storage for 3 weeks at 48 °C. However, some changes became apparent in the fourth week. At 4 °C, vesicles were stable for 4 weeks if samples were agitated and stored at low (1–2 mM) lipid concentration. However, without agitation, vesicles stored at low temperatures settled out of solution. Larger vesicles and LMV formed in a short time (7–10 days) under these conditions. In addition, cold storage of DPPC DOV at high concentrations (10–20 mM) led to vesicle fusion, in a matter of hours as judged from freeze–fracture electron micrographs (R. A. Parente, M. Hoechli, and B. R. Lentz, unpublished results).

DOV Phase Behavior. The phase behavior of DMPC, $DC_{15}PC$, and DPPC DOV preparations has been investigated by fluorescence depolarization of the membrane probe DPH and by high-sensitivity differential scanning calorimetry. As shown previously (Lentz et al., 1978), lipid phase transitions are conveniently detected fluorometrically in plots of the microviscosity activation energy vs. temperature, as depicted in Figure 3.

The fluorometric phase transition data agreed well with calorimetric data obtained on the same preparations. Excess heat capacity profiles obtained during heating and subsequent cooling calorimetric scans are plotted in Figure 4 for DOV and in Figure 5 for LMV, and important transition parameters are summarized in Table I. Transition temperatures for DOV and LMV prepared from identical lipid stocks were comparable. As noted in a previous study of LMV phase behavior (Lentz et al., 1978), fluorometrically measured peak temperatures were slightly lower and peaks were somewhat

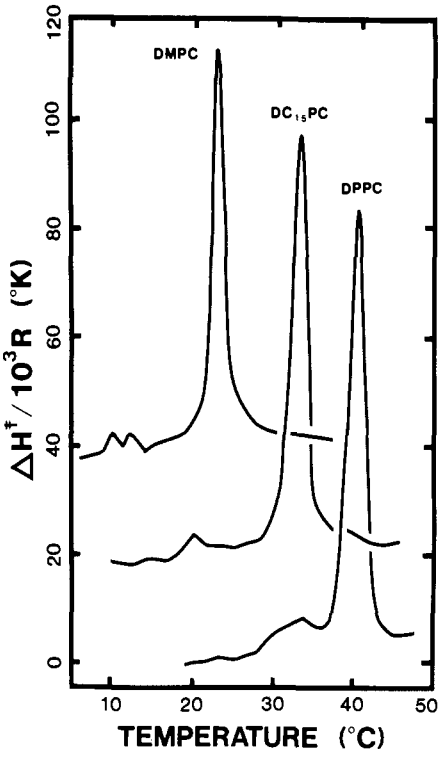

FIGURE 3: Temperature dependence of the microviscosity activation energy derived from DPH fluorescence depolarization for DMPC, $DC_{15}PC$, and DPPC DOV. Samples containing DPH were cooled and subsequently heated at a scan rate of 18 °C/h. The heating scans shown here were virtually identical with cooling scans (not shown) except in the pretransition region (see Table I).

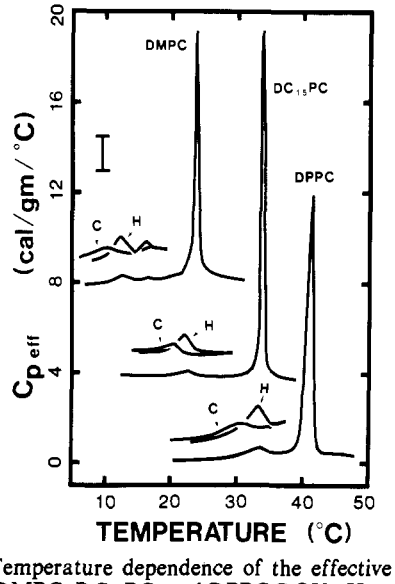

FIGURE 4: Temperature dependence of the effective excess heat capacity for DMPC, $DC_{15}PC$, and DPPC DOV. Heating and subsequent cooling scans were made at a scan rate of approximately 15 °C/h. In the main transition region, cooling scans (not shown) were essentially identical with the heating scans shown here. Pretransition regions of both heating (H) and cooling (C) scans have been enlarged in the insets (scale marker = 0.5 cal g^{-1} $°C^{-1}$).

broader than observed calorimetrically for DOV as well as LMV. The added DPH probably accounts for the lower phase transition temperature (Lentz et al., 1978), while the greater sensitivity of DPH to local, noncooperative changes likely accounts for the broader transition (Lentz et al., 1981).

DOV also exhibited main-transition peak widths that were about 2 (DMPC and $DC_{15}PC$) or 4–5 (DPPC) times broader than those of multilamellar vesicles. This broadening was not a result of residual octyl glucoside contamination since DPPC

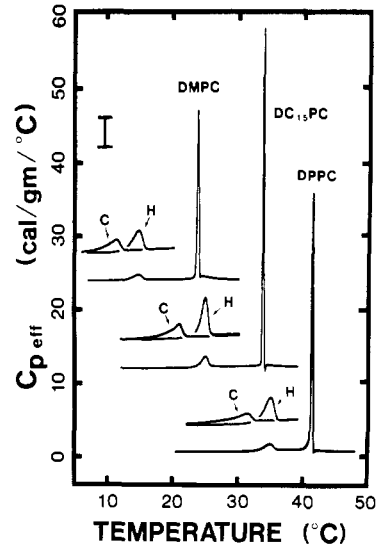

FIGURE 5: Temperature dependence of the effective excess heat capacity for DMPC, $DC_{15}PC$, and DPPC LMV. Conditions, insets, and inset scale markers are as in Figure 4. The slight drop below the base line seen just above the major transition is an artifact of our calorimetric correction algorithm (see Methods and Methods). This is due to the inability of a function of finite curvature to fit a heat flow pulse that approaches a square wave shape.

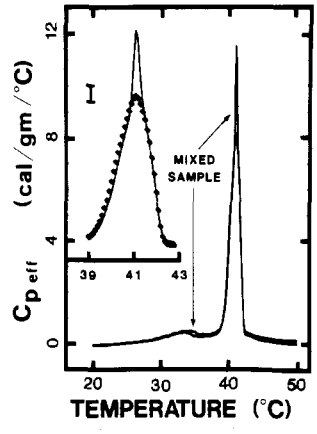

FIGURE 6: Temperature dependence of the effective excess heat capacity for DPPC DOV with 10 mol % added DPPC LMV contamination. Heating scans are plotted. These were virtually superimposable except where indicated by arrows pointing to the larger pretransition and sharp "cap" on the main transition of the DOV–LMV mixture. The inset contrasts clearly the sharp cap seen in the main transition of the mixed samples (solid curve) relative to the smooth, broad transition seen in the pure DOV sample (♦). The scale marker refers to the inset and corresponds to 1.0 cal g^{-1} $°C^{-1}$.

multilamellar vesicles made in the presence of 0.1 mol % octyl glucoside demonstrated the same transition temperature (41.2 °C) and $\Delta T_{1/2}$ (0.24 °C) as reported in Table I for noncontaminated LMV.

Calorimetrically determined main transition enthalpies were comparable in DOV and LMV except for DPPC, which displayed anomalously large enthalpies when incorporated into DOV (see Table I).

The pretransition region of DOV was also different from that of LMV. First, the pretransition heat capacity peak in DOV consistently occurred at lower temperatures (about 2 °C) in heating scans than in LMV. Second, pretransition enthalpies were always reduced in DOV relative to LMV. However, the hysteresis previously reported from fluorometric studies of LMV (Lentz et al., 1978) was seen here in both calorimetric and fluorometric data collected for both DOV and LMV samples. An unexplained but reproducible feature

Table I: Phase Transition Parameters[a] for Synthetic Phosphatidylcholine LMV, LUV, and Fusion Vesicles

		main transition			pretransition		
lipid	method[b]	T_m[c] (°C)	ΔH[d] (kcal/mol)	$\Delta T_{1/2}$[e] (°C)	T_m[c] (°C)	ΔH[d] (kcal/mol)	ref
				Large Multilamellar Vesicles			
DMPC	DSC-H	23.5	6.5 ± 0.4	0.26	13.9	1.1 ± 0.2	this work
	DSC-C	23.9	6.5 ± 0.2	0.26	10.9	0.5 ± 2.0	this work
	DSC-H	23.6	5.73	0.085	15.5	0.83	Dufour et al. (1981)
	DSC-H	23.9	5.0 ± 0.2	0.2	14.1	1.0 ± 0.5	Lentz et al. (1978)
	DSC-H	23.9	5.4	0.3	14.2	1.0	Mabrey & Sturtevant (1976)
	DSC-H	23.59	5.03	0.13	14.4	0.83	Chen & Sturtevant (1981)
	fluor-H	23.8 ± 0.25		1.6	12.4		Lentz et al. (1978)
$DC_{15}PC$	DSC-H	33.7	8.3 ± 0.1	0.23	24.8	1.4 ± 0.2	this work
	DSC-C	33.8	8.7 ± 0.1	0.23	20.9	1.0 ± 0.2	this work
	DSC-H	33.5	NA	NA	23.5	NA	Chen et al. (1980)
	fluor-H	33.9		2.0	23.6		this work
DPPC	DSC-H	41.2	8.7 ± 0.2	0.28	34.9	1.5 ± 0.2	this work
	DSC-C	41.3	8.7 ± 0.1	0.33	31.2	0.9 ± 0.1	this work
	DCS-H	41.3	8.6 ± 0.2	0.4	35.2	1.5 ± 0.5	Lentz et al. (1978)
	DCS-H	41.4	8.7	0.3	35.3	1.8	Mabrey & Sturtevant (1976)
	DSC-H	41.55	8.5	0.067	NA		Albon & Sturtevant (1978)
	DSC-H	41.05	6.43	0.14	34.8	0.92	Chen & Sturtevant (1981)
	DSC-H	41.6	8.7	0.3	NA		Wong et al. (1982)
	fluor-H	40.6 ± 0.25		1.6	31.9		Lentz et al. (1978)
				Dialyzed Octyl Glucoside Vesicles			
DMPC	DSC-H	23.5	6.3 ± 0.5	0.6	12.3	0.5 ± 0.2	this work
	DSC-C	23.8	6.2 ± 0.5	0.6	10.5	0.3 ± 0.2	this work
	DSC-H	24.4	6.8	0.6	15	2.17	Dufour et al. (1981)
	fluor-H	23.2		1.7	12.6 ± 0.4		this work
	fluor-C	23.5		1.5			this work
$DC_{15}PC$	DSC-H	33.6	7.7 ± 0.5	0.6	21.9	0.5 ± 0.2	this work
	DSC-C	33.8	7.8 ± 0.5	0.5	20.3	0.3 ± 0.2	this work
	fluor-H	33.6 ± 0.2		2.2	20.3 ± 0.2		this work
	fluor-C	33.7 ± 0.2		2.4	20.3 ± 0.6		this work
DPPC	DSC-H	41.0	10.5 ± 0.5	1.4	32.1	1.2 ± 0.2	this work
	DSC-C	41.1	11.2 ± 0.5	1.4	31.1	0.8 ± 0.2	this work
	DSC-H	41.0	8.0 ± 1.6		NO	NO	Petri et al. (1980)
	fluor-H	40.4 ± 0.2		2.2	33.9 ± 0.6		this work
	fluor-C	40.9 ± 0.2		2.2	30.9 ± 1.0		this work
				Fusion Vesicles			
DMPC	DSC-H	23.5	4.6 ± 0.3	0.3	13.4	0.4 ± 0.2	this work
	DSC-C	23.9	4.9 ± 0.3	0.3	11.1	0.2 ± 0.1	this work
$DC_{15}PC$	DSC-H	34.0	5.9 ± 0.2	0.7	NO	NO	this work
	DSC-C	33.9	6.4 ± 0.2	0.7	NO	NO	this work
DPPC	DSC-H	41.2	9.7 ± 0.2	0.3	34.6	0.8 ± 0.2	this work
	DSC-C	41.2	8.8 ± 0.2	0.3	31.4	0.4 ± 0.2	this work
	DSC-H[f]	41.6	6.9	0.6	NO	NO	Wong et al. (1982)
				Reverse-Phase Evaporation Vesicles			
$DC_{15}PC$	DSC-H	33.3	7.9 ± 0.2	0.45	22.0	1.3 ± 0.2	this work
	DSC-C	33.7	8.0 ± 0.2	0.45	19.9	0.5 ± 0.2	this work
DPPC	DSC-H	40.8	9.0 ± 0.2	0.96	31.9	1.3 ± 0.2	this work
	DSC-C	41.1	9.3 ± 0.2	0.96	26.6	0.5 ± 0.2	this work
	DSC-H	40.8	8.9 ± 0.2	1.3	NO	NO	Düzgünes et al. (1982)

[a] Unreported values were either not available (NA) or not observed (NO). [b] DSC-H(C) indicates a heating (cooling) calorimetric span; fluor-H(C) indicates a heating (cooling) scan by fluorescence depolarization of DPH. [c] Errors in peak temperatures for our data were ±0.1 °C unless otherwise indicated. We have made no attempt to represent error ranges for data from our laboratories. T_m for the pretransition is scan-rate dependent (Lentz et al., 1978). [d] In general, errors for ΔH measurements represent variations in analysis using different intervals for integration. For lareg unilamellar vesicles, errors were further determined by the range of ΔH values from different preparations (two for DMPC, two for $DC_{15}PC$, and six for DPPC). [e] Peak widths at half-height for our data ($\Delta T_{1/2}$) were measured by hand, and errors are ±0.15 °C. Values from the literature are those reported in the tables or the text, except for the value of 0.6 measured from Figure 5 of Wong et al. (1982). [f] The scan reported by Wong et al. (1982) yielded anomalous results. More recent experiments have yielded $\Delta T_{1/2}$ = 0.3–0.4 °C (M. Wong and T. E. Thompson, unpublished results).

of DMPC DOV phase behavior was the dual-peak pretransition observed in heating calorimetric and fluorometric scans (see Figures 3 and 4).

In order to obtain another measure of multilamellar or oligolamellar contamination in DOV, we have examined the excess heat capacity profile of a DOV sample to which 10% LMV contamination was added. In Figure 6, the excess heat capacity profile for this mixed DOV–LMV sample is clearly seen to be intermediate between those observed for pure DOV and for pure LMV (Figures 4 and 5, respectively). Since the maximum excess heat capacity for LMV was consistently observed to be 3 times that for DOV, a 10% LMV contamination in DOV would be expected to produce a peak heat capacity approximately 1.3 times greater than that observed in pure DOV. The observed ratio was 1.5. In addition, the excess heat capacity peak for the mixture appeared as the superposition of a small, narrow LMV peak plus a large, broader peak characteristic of the pure DOV (see inset to

Table II: Tests for Bilayer-Associated Impurities

phospholipid	vesicle type	protocol[b]	T_m (°C)	$\Delta T_{1/2}$ (°C)	$\Delta T_{1/2}/\Delta T_{1/2}$ (LMV)[a]
DPPC	DOV	lyophilized total sample	41.4	2.0	7.0
DPPC	DOV	sonicated, diluted, centrifuged vesicles; pellet resuspended and lyophilized	41.1	0.8	2.9
DPPC	LMV	lyophilized total sample	41.2	0.4	1.4
$DC_{15}PC$	REV	commercial ether, lyophilized total sample	33.1	1.3	5.8
$DC_{15}PC$	REV	purified ether, lyophilized total sample	33.4	0.7	3.0
$DC_{15}PC$	LMV	lyophilized total sample	33.2	0.4	1.7

[a] $\Delta T_{1/2}$(LMV) is the full peak width at half-height of LMV prepared in the normal fashion from fresh lipid stocks. [b] All vesicle samples were freeze-dried, and the resultant lipid was suspended in doubly distilled water to form LMV, which were then analyzed by DSC.

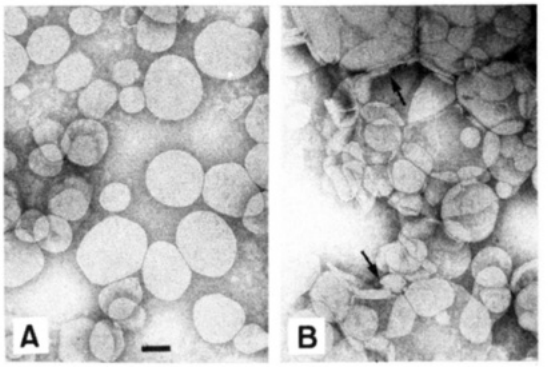

FIGURE 7: Negative-staining electron micrographs of (A) a DOV preparation and (B) the same sample with added 10% LMV contamination. Arrows indicate LMV which are easily visible in the DOV population in (B). Magnification 37500×.

Figure 6). Given the obvious compound nature of the mixed profile, it would be possible to detect as little as 3 wt % multilamellar contamination.

The mixed DOV–LMV sample described above was also characterized by negative-staining electron microscopy. The results (Figure 7) clearly demonstrated the sensitivity of the negative-staining technique in detecting multilamellar contamination and established a correlation with the calorimetric measure of contamination (Figure 6).

Phase Behavior and Characterization of Fusion Vesicles and REV. By way of comparison to our DOV preparations, we have also studied the phase behavior of two other well-characterized large unilamellar vesicle preparations: the fusion vesicles reported by Wong et al. (1982) and the reverse-phase evaporation vesicles (REV) of Szoka et al. (1980). Figure 8 shows the excess heat capacity profiles obtained during heating and subsequent cooling scans performed on a sample prepared by the low-temperature fusion procedure of Wong et al. (1982). Transition properties of these preparations are summarized in Table I. In the case of DMPC and $DC_{15}PC$ fusion vesicles, however, this summary can be somewhat misleading, since the heat capacity profiles were broad at the base and clearly showed multiple peaks (see Figure 8), suggestive of substantial sample heterogeneity. Only for DPPC fusion vesicles did we obtain a heat capacity profile with a single, sharp main-transition peak. For this reason, storage of sonicated vesicles at 4 °C seems to be a viable method of forming large unilamellar vesicles only in the case of DPPC. In our hands, DPPC fusion vesicles displayed a sharper transition with a larger enthalpy than reported by Wong et al. (1982). In this regard, the scan reported by Wong et al. (1982) appears to have been an anomalous result, since subsequent experiments on fusion vesicles in that laboratory gave a sharp heat capacity peak similar to our results for these vesicles (M. Wong and T. E. Thompson, unpublished results). Thus, the phase

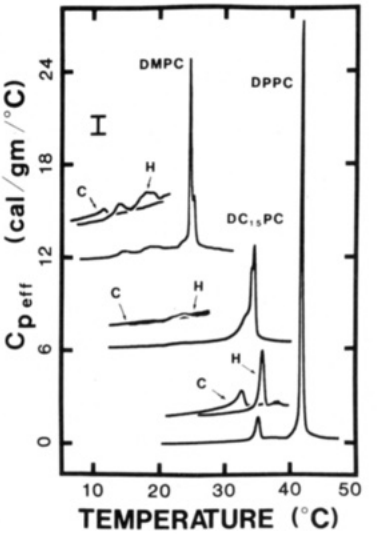

FIGURE 8: Temperature dependence of the effective excess heat capacity for DMPC, $DC_{15}PC$, and DPPC fusion vesicles prepared according to Wong et al. (1982). Conditions, insets, and inset scale markers are as in Figure 4.

behavior of DPPC fusion vesicles was quite similar to that of DPPC LMV, except for a somewhat reduced pretransition enthalpy (see Table I). The trapped compartment of DPPC fusion vesicles has also been characterized by the 6-carboxyfluorescein trapping method. We obtained a value of 2.7 ± 0.1 μL/μmol of P_i, which is comparable to the value obtained by Wong et al. (1982) of 2.5 ± 0.3 μL/μmol of P_i.

The ether evaporation procedure of Szoka et al. (1980) created vesicles (REV) of similar size to the DOV described in this paper (about 1260-Å diameter on the basis of our measurements of 6-carboxyfluorescein trapping; see Trapped Volume Measurements under Results) but contained approximately 5% oligolamellar contamination (determined by negative-staining and freeze–fracture electron microscopy). The phase behavior of REV prepared from $DC_{15}PC$ and DPPC is reported in Table I. Main transition enthalpies and temperatures were in reasonable agreement with LMV and DOV data. Peak widths were intermediate between those of LMV and DOV. Heat capacity scans were very similar in appearance to those of DOV (Figure 4) and, therefore, have not been reproduced here.

Evidence for Impurities Associated with DOV and REV Bilayers. The broadened main transition enthalpy and diminished pretransition enthalpy observed in DOV and REV could be characteristics of unilamellar vesicle phase behavior or they could reflect bilayer-associated impurities derived from the detergent or solvent used in vesicle preparation. Analysis of extracted phospholipids by thin-layer chromatography (Lentz et al., 1976a,b) was routinely carried out following DOV preparation and revealed no indication of contaminants.

In addition, radioactive tracer analysis demonstrated very low contamination by residual octyl glucoside. In response to the suggestion of a reviewer, however, we performed even more sensitive tests for bilayer contamination, as outlined below and summarized in Table II.

A portion of a freshly prepared DPPC DOV sample was evaporated to dryness and lyophilized from benzene. LMV were prepared in purified water from the dried lipids and analyzed by DSC. Broad phase transitions were observed in such samples (Table II). These results imply that a bilayer-perturbing contaminant is present in normal DOV preparations. Presumably, the contaminant was somehow tightly associated with the vesicles, since it was not removed by extensive dialysis followed by centrifugation and resuspension of the pellet. In addition, not all the contaminant seemed to be associated with the bilayer until water was removed by lyophilization, since the main transition of the rehydrated sample was even broader than that of the DOV sample.

Another fraction of the original (DOV) preparation was sonified for 10 min, diluted, and centrifuged for 1 h at 48 000 rpm in a Ti-75 rotor. The pellet, containing 45% of the lipid, was resuspended in 0.2 mL of 50 mM KCl and lyophilized overnight. LMV were prepared in purified water and analyzed by DSC. The phase transition was still broadened relative to the transition in an LMV preparation, but much less so than in lipid obtained from an unsonicated DOV preparation (see Table II). These results indicate that a contaminant may become trapped within the vesicles during dialysis and be only partially released upon soniciation.

As a control, LMV were prepared, analyzed by DSC, and lyophilized as described above. The lyophilized lipid was then re-formed into LMV and subsequently analyzed by DSC. The resultant peak widths, while only slightly broadened, were still 1.3–1.5 times the original LMV widths, indicating that even the slightest contaminant in the water is detectable by this calorimetric analysis.

Since the source of contamination in DOV was likely the detergent used in vesicle preparation, octyl glucoside was recrystallized 3 times from petroleum ether after dissolution in acetone (Baron & Thompson, 1975). The odor of octanol was evident in the dried supernatant from the first and second, but not the third, recrystallizations. The trace quantities of octanol present in the commercial octyl glucoside were not detectable by thin-layer chromatography on Analtech GHL plates (9:4:1 ethyl acetate:2-propanol:water; iodine staining). The purified octyl glucoside chromatographed like the impure commercial product, showing only one spot on a thin-layer chromatogram. Quite surprisingly, DOV made with this recrystallized octyl glucoside contained hydrolyzed lipid by the time dialysis was completed, while the original octyl glucoside did not have this effect. We were unable to identify the reason for this hydrolytic activity.

We have also checked whether the phase behavior of REV might be sensitive to the purity of the ether used in the preparation. DSC analysis of rehydrated, lyophilized $DC_{15}PC$ REV samples, prepared by using commercial diethyl ether, showed a main phase transition (Table II) broader than the original REV phase transition ($\Delta T_{1/2} = 1.2$ °C). This suggests substantial contamination, as was the case for DOV. When freshly distilled diethyl ether was used in the $DC_{15}PC$ REV preparation, the breadth of the REV phase transition was reduced (0.45 °C, see Table I), and the transition breadth of the rehydrated, lyophilized sample was also reduced by a factor of 2 but was still greater than that of lyophilized LMV (Table II). Comparable results were obtained in comparing commercial and freshly distilled isopropyl ether in the preparation of DPPC REV (results not shown). Therefore, purification of the ether did reduce the level of impurity in REV but did not remove all impurities contaminating REV samples.

Discussion

Our major goal has been to obtain a well-defined preparation of large unilamellar vesicles to use in establishing the phase behavior of individual, synthetic phosphatidylcholine bilayers. For this purpose, it was essential to have a population of vesicles with diameters greater than those for which curvature effects should be important [i.e., greater than or roughtly equal to 800–1000 Å (Sheetz & Chan, 1972)]. A relatively homogeneous LUV population has been prepared by very slow dilution of mixed lipid–detergent dispersions, with subsequent removal of detergent by dialysis, followed by simple fractionation procedures. The nonionic detergent octyl glucoside was chosen over cholate or deoxycholate since it had been shown to be useful in forming 1000–2000-Å diameter egg PC LUV (Mimms et al., 1981) and has a high critical micelle concentration (Jackson et al., 1982), making it easier to remove by dialysis. Cholate dialysis vesicles are smaller (250–800-Å diameter) (Rhoden & Goldin, 1979; Milsmann et al., 1978; Zumbuehl & Weder, 1981) and contain high residual detergent levels (0.5–1.5 mol %) (Zumbuehl & Weder, 1981).

In our experience, the following two factors principally influenced the success of our dialysis procedure for preparing LUV: (1) the detergent:lipid molar ratio, and (2) the rate of detergent dilution. Mimms et al. (1981) have stressed the importance of the detergent:lipid ratio in forming egg PC vesicles and reported that a ratio greater than 5:1 was required even for egg PC solubilization by octyl glucoside. However, Mimms et al. removed octyl glucoside by rapid dialysis and gel filtration. We have found that the rate of octyl glucoside removal is a parameter of at least equal importance in determining the success and reproducibility of LUV preparation. For example, Mimms et al. reported that only 50% of the initially solubilized lipid was found in vesicular form when a detergent:lipid ratio of 5:1 was used in vesicle preparation. By contrast, we had had no trouble making egg PC vesicles at 80–90% yielded with a detergent to lipid ratio was low as 3:1 when a very slow dilution preceded the removal of octyl glucoside by dialysis.

Our observation of substantially increased rates of detergent removal by dialysis at higher temperatures led us to try a slow dilution step to enhance vesicle formation prior to dialysis. Since ratios of (3–5):1 octyl glucoside:lipid had been reported to result in apparent solubilization (Mimms et al., 1981; Jackson et al., 1982), we reasoned that the rate at which octyl glucoside was diluted below this level would be crucial to maintaining equilibrium between nascent vesicular structures and detergent–lipid micelles. While we had no information on the exact concentration range in which this cricial equilibrium might occur, we reasoned that it would occur between 0.022 M (octyl glucoside critical micelle concentration) and 0.022 M plus 3–5 times the lipid concentration, the concentration at which total solubilization appeared to occur. This line of reasoning led us to the preparative protocol described in this paper. The success of this protocol convinces us of the importance of the rate of octyl glucoside dilution and removal.

The vesicles prepared by the slow dilution and dialysis method appear to be unilamellar. Four lines of evidence support this conclusion: negative-staining electron microscopy, freeze–fracture electron microscopy, the sensitivity of excess

heat capacity profiles to multilamellar contamination, and trapped volume determination. While none of these criteria alone could be viewed as conclusive, their combined evidence is reasonably compelling. Both negative-staining and freeze–fracture techniques were capable of revealing oligolamellar vesicles (less than 1 no. %), but these techniques can be criticized as potentially disclosing selective subpopulations of vesicles. However, our calorimetric results clearly reflect the entire vesicle population and set an upper limit of 2–3 wt % (approximately 0.2–0.3 no. %) on the level of multilamellar contamination possible in our DOV preparations. Finally, the vesicle diameter of 1045 Å calculated from trapped volume measurements on a DPPC preparation agrees well with the mass-weighted average diameter of 940 ± 400 Å obtained by measuring electron micrographs of DPPC DOV (see Figure 2).

Since the start of this project, five reports have appeared on the phase behavior of large unilamellar vesicles, and their results are summarized in Table I. The first study reported that octyl glucoside dialyzed vesicles had a broadened main phase transition but otherwise nearly the same thermal properties as LMV (Petri et al., 1980). A second study reported that DMPC vesicles prepared by sodium deoxycholate partial solubilization and removal (Enoch & Strittmatter, 1979) had a main transition with a larger enthalpy, an increased half-width, and a higher phase transition temperature than found in the main transition of LMV (Dufour et al., 1981). A third report (Wong et al., 1982) stated that DPPC vesicles prepared by fusion of extremely concentrated SUV at low temperatures resulted in no change in the main transition temperature and essentially no change in peak width when compared to LMV (see Results and Table I). A fourth study reported a broadened transition and higher transition temperatures in REV relative to LMV using Raman spectroscopy to follow the main phase transition in DMPC SUV, LMV, and REV (van Dael et al., 1982). The fifth and most recent examination of this issue described the main phase transition in DPPC REV as slightly broadened and shifted by about 1 °C to lower temperature relative to DPPC LMV (Düzgünes et al., 1983). In all but the report of Dufour et al., a pretransition could not be clearly detected.

There are several possible reasons for the disagreements between results reported in the literature, including (a) differences in instrumental response, (b) differences in synthetic lipids, (c) differences in lipid handling and methods of preparation, and (d) real and significant differences in the phase behavior of various types of large unilamellar vesicles. In our studies, we have used the same instrument and corrected for the instrumental response. Furthermore, the same synthetic lipid stocks have been used to prepare all three types of unilamellar vesicles studied. In our hands, all three vesicle preparations displayed a pretransition, but with slightly lower heat content than found in LMV preparations. All unilamellar vesicles had a main phase transition peak at nearly the same (or slightly lower) temperature as that of LMV, and with nearly the same transition enthalpy (with the exception of DPPC LUV, within 1 kcal/mol) as LMV. However, fusion vesicles did not show the main phase transition broadening, relative to LMV, observed in DOV and REV preparations.

A simple hypothesis will account for the disagreements as to the phase behavior of large unilamellar vesicles. It appears that LUV prepared by either detergent removal or solvent evaporation contain bilayer-perturbing contaminants that broaden the main transition and, in some cases, eliminate the pretransition. The broadening probably reflects the large molar excess of detergent or solvent used in unilamellar vesicles preparation. As a result, even minor contaminants in the detergent or solvent can become significantly concentrated in the lipid bilayer. The presence of such contaminants in DOV and REV preparations is clear from the broad phase transitions seen in rehydrated, lyophilized samples (Table II). When measures were taken to remove contaminants, either from DOV or from ether used to prepare REV, the extent of phase transition broadening in lyophilized samples was reduced (Table II).

Table III: Bilayer Contamination[a] due to Main Transition Broadening

vesicle type[b]	mol % contamination[c]	mol % contamination[d]
DOV	2	4
REV		3
fusion vesicles	0.5	

[a] Estimates were derived by methods suggested by Albon & Sturtevant (1978) and Sturtevant (1982), assuming that broadening of the main transition is an additive property of solute broadening and van't Hoff broadening terms. The equation obtained is

$$\frac{T}{T_0} = 1 - RT_0\left[\frac{1}{\Delta H_{VH}}\ln\left(\frac{1-\alpha}{\alpha}\right) + \frac{X_2}{\Delta H_{cal}}\frac{1}{k/(1-k)+c}\right]$$

where R is the gas constant, ΔH_{VH} is the van't Hoff enthalpy, ΔH_{cal} is the calorimetrically determined main transition enthalpy of the "pure" main component, X_2 is the mole fraction of contaminant, k is the equilibrium constant for partitioning of the contaminant from fluid to gel phases, α is the fraction of the main component melted at a given temperature (T), and T_0 is the transition temperature for the pure main component. All impurity levels are relative to our LMV as pure lipid, using T_0 = 314.2 K, ΔH_{cal} = 8.7 kcal/mol, and effective ΔH_{VH} = 348 kcal/mol (obtained by fitting the van't Hoff expression to the entire observed LMV enthalpy profile). [b] All vesicles were prepared from the same DPPC stock. [c] Data fit on both sides of T_0 by using this procedure. [d] Data fit only on the low-temperature side of T_0 by using this procedure.

The arguments above suggest that only DPPC fusion vesicles should have a phase behavior that truly reflects the lipid phase behavior of a unilamellar vesicles, since these were prepared in the absence of solvents or detergents. We have found DPPC fusion vesicles to have a phase behavior nearly identical with that of DPPC LMV, except that the heat content of the pretransition was slighltly enhanced. This would be consistent with stabilization of the $P_{\beta'}$ phase of LMV through interbilayer interactions. Such interactions are also implied by analysis of careful X-ray diffraction studies on LMV (Janiak et al., 1976).

In summary, we conclude that noncontaminated LUV preparations and LMV should have similar phase behavior. The widely reported decrease in main phase transition cooperativity in LUV (Petri et al., 1980; Dufour et al., 1981; van Dael et al., 1982) appears to reflect impurities picked up from the detergent or solvent used in vesicle formation. We have used the methods suggested by Albon & Sturtevant (1978) and Sturtevant (1982) to estimate the extent of bilayer contamination in REV, DOV, and fusion vesicle preparations from the degree of main transition broadening. These estimates are presented in Table III. Despite the presence of low levels of contaminants, DOV and REV preparations have distinct advantages for many applications requiring unilamellar vesicles. For instance, DOV and REV can be made with lipids other than DPPC, a distinct advantage over fusion vesicles. Thus, the choice of a large unilamellar vesicle preparation will depend on the particular application for which they are intended.

Acknowledgments

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Registry No. DMPC, 18194-24-6; $DC_{15}PC$, 3355-27-9; DPPC, 63-89-8; DPH, 1720-32-7; octyl β-D-glucopyranoside, 29836-26-8; dipentadecanoylphosphatidylglycerol, 73731-64-3; 6-carboxyfluorescein, 3301-79-9.

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