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Morphology and phase behavior of two types of unilamellar vesicles prepared from synthetic phosphatidylcholines studied by freeze-fracture electron microscopy and calorimetry

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Differential scanning calorimetry and freeze-fracture electron microscopy have been used to characterize the phase behavior and morphology of two types of unilamellar vesicles composed of synthetic phosphatidylcholines. The first type displayed an average diameter of roughly 100 nm and was formed by slow dilution and dialysis of octylglucoside-solubilized lipid. These large, unilamellar vesicles were termed dialyzed, octylglucoside vesicles and could be obtained as a fairly well defined and uniform population of vesicles. The second vesicle type was prepared by a unique procedure involving dialysis of deoxycholate-solubilized lipid at its pre-transition temperature. This procedure produced a much more heterogeneous distribution of vesicle sizes (500 to 4000 nm in diameter) and left some dilamellar and oligolamellar species which could not be conveniently separated from the giant, unilamellar vesicles constituting the major portion of the sample. Both populations of vesicles displayed phase behavior similar, but not identical to that of large, multilamellar vesicles (LMV). Fracture-face morphology of the gel phase was also observed to differ between the two unilamellar and the multilamellar species. LMV have previously been shown to have clear undulated or banded fracture-faces in the $P_{\beta'}$ phase, while octylglucoside vesicles are shown here to have facetted fracture-faces. Giant, unilamellar vesicles displayed a faint banded morphology similar to but less distinct than that of the LMV $P_{\beta'}$ phase. These results have demonstrated that bilayer apposition is not required to support the banded fracture-face morphology characteristic of the $P_{\beta'}$ phase but that a limiting curvature is necessary.

Introduction

The most widely used models for biological membranes have been large, multilamellar vesicles (LMV) and sonicated small, unilamellar vesicles (SUV). Unfortunately, the juxtaposed bilayers of LMV and their lack of a well defined surface area make them imperfect models for cell membranes.

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Abbreviations $DC_{15}PC$, 1,2-dipentadecanoyl-3-*sn*-phosphatidylcholine, DPPC, 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine, LUV, large, unilamellar vesicle(s), LMV, large, multilamellar vesicle(s), SUV, small, unilamellar vesicle(s), Tes, 2-([2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino)ethanesulfonic acid

Likewise, the high degree of bilayer curvature of SUV makes them best suited for modeling the highly curved regions of biological membranes The disadvantages of these popular vesicle types have led to several efforts to develop a better model system, namely, large, unilamellar vesicles (LUV) having a defined surface area and sufficiently large diameters to overcome extreme curvature effects.

Many LUV preparative procedures have been proposed. These include solvent injection methods [1,2], reverse phase evaporation [3], and detergent removal techniques [4–9], along with a variety of other methods [10–13]. Reported vesicle radii for different methods of preparation have ranged from 25 nm to 500 nm. We have chosen to define the term large, unilamellar vesicle (LUV) to encompass those vesicles of radii of greater than limiting size (25 nm) but less than 500 nm. Vesicles with radii on the order of 500 nm or greater are referred to here as giant, unilamellar vesicles.

To date, few efforts have been made to systematically study the lamellar phase behavior of these large, unilamellar structures. Recently, using three synthetic phosphatidylcholines, we have shown the calorimetrically-determined phase behaviors of large, unilamellar vesicles formed by dialysis of octylglucoside/lipid mixtures, reverse phase evaporation [3], and SUV fusion [14] to be similar to those of LMV prepared from the same lipids; at least with regard to displaying a 'pretransition' at temperatures just below the main order to disorder transition [9]. This implies that LUV may support a two-dimensional ordered phase similar to the $P_{\beta'}$ phase found in LMV. In this communication, we report a detailed electron microscopic investigation of bilayer fracture-face morphology in two types of vesicles (octylglucoside vesicles and giant, unilamellar vesicles) prepared from synthetic phosphatidylcholines. The results have revealed that bilayer apposition is not required to support the rivuletted fracture-face morphology characteristic of the $P_{\beta'}$ phase but that minimal curvature is necessary. For vesicles of greater curvature, a facetted fracture-face morphology was found to replace the rivuletted morphology.

Materials and Methods

Materials. 1,2-Dipentadecanoyl-3-*sn*-phosphatidylcholine ($DC_{15}PC$) and 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine (DPPC) were obtained from Avanti Polar Lipids, Inc. (Birmingham, AL) and determined to be greater than 99% pure by thin-layer chromatography [15]. Lipids were stored in spectral grade chloroform. Deoxycholic acid was purchased from MCB (Cincinnati, OH). It was dissolved in ethanol, decolorized with Norit®-activated charcoal and recrystallized from a 50% aqueous ethanol solution before use. Octyl β-D-glucopyranoside was supplied by Calbiochem (La Jolla, CA; Lot No. 210131). [^{14}C]Octyl β-D-glucopyranoside (Lot No. 1141-235) and [^{3}H]deoxycholic acid (Lot No. 1319-014) were purchased from New England Nuclear (Boston, MA). All solvents were spectral or HPLC grade. Water was doubly distilled, with the first distillation being from basic $KMnO_4$. Utrapure KCl was from Heico, Inc. (Delaware Water Gap, PA; Lot No. 2179). Cellulose dialysis tubing (MW 12000, cutoff, 5/8 inch inflated diameter) was obtained from A.H. Thomas Co. (Philadelphia, PA). Prior to use, tubing was boiled in 10 mM Tris acetate/2.5 mM EDTA/0.1 M KCl for 3 h, rinsed well with deionized water and allowed to soak in 10 mM Tris acetate/2.5 mM EDTA at 4°C for 8–12 h. Other chemicals were reagent grade or better.

Vesicle preparation. Dialyzed, octylglucoside vesicles were formed by a detergent-dilution-dialysis method starting from an initial 10:1 octylglucoside to lipid molar ratio [9]. Lipids and octylglucoside were mixed in chloroform and dried under a stream of N_2 onto the wall of a small round bottom flask. Residual solvent was removed under high vacuum for 8–12 h. The lipid was solubilized in 1 ml 50 mM KCl/25 mM octylglucoside prior to dilution. Aqueous medium (50 mM KCl) was slowly pumped into the stirred sample, which was maintained above the lipid phase transition temperature. After dilution, detergent was removed by exhaustive dialysis above the phase transition temperature. Using [^{14}C]octylglucoside, the final dialysate was determined to contain less than 0.03 mol% contamination from detergent after 72 h. The sample (10 ml) was removed and spun in a Beckman microfuge® B for

20 s at 13000 rpm to remove large, multilamellar vesicle contamination and vesicle aggregates. the supernatant was collected and concentrated by ultracentrifugation in a Beckman Ti 75 rotor at 48000 rpm for one hour. The octylglucoside vesicle pellet was resuspended in 2.5 ml of 50 mM KCl and filtered through a 0.4 μm Nuclepore® filter. This population of vesicles has been characterized by negative staining electron microscopy and found to be homogeneous with diameters between 80–100 nm [9].

Giant, unilamellar vesicles were prepared by solubilizing a film of dried lipid (9 μmol) with 7.5% sodium deoxycholate (0.2 ml) in a buffer containing 20 weight % glycerol/0.1 mM $CaCl_2$/0.1 M KCl/0.01 M Tes (pH 7.5). The detergent was removed by dialysis against the same buffer at the pre-transition temperature of the lipid, analogous to a procedure developed for the reconstitution of the Mg^{2+}-dependent, Ca^{2+}-stimulated adenosine triphosphatase of sarcoplasmic reticulum [16]. Using [^{3}H]deoxycholate, detergent contamination was determined to be less than 0.8–1 mol% after 48 h of dialysis. Following the initial dialysis, the sample was further dialysed against 50 mM KCl for 72 h to remove glycerol. This transferred the giant, unilamellar vesicle sample to the standard aqueous medium in which we have performed previous studies of phosphatidylcholine phase behavior [17].

Freeze-fracture. Samples were incubated between two thin copper sheets, frozen in a propane jet freezing device, and fractured by procedures similar to those previously reported [18,19]. The preparation of octylglucoside vesicle samples was modified in an attempt to reduce the possibility of vesicle fusion during incubation in the copper sandwiches. First, Sephadex gel particles suspended in silicon grease were no longer used as a spacer between the apposing copper sheets. Instead, a piece of platinum wire (10 μm diameter) served as a spacer. The wire was removed after the sample had been introduced between the plates, but prior to freezing. Second, following nitric acid etching of the two copper surfaces, these were washed with acetone and a thin coat of Formvar® (0.25% in CH_2Cl_2) was applied. This helped reduce but still did not totally eliminate severe aggregation of octylglucoside vesicles on the copper sheets. Finally, we determined that only elimination of a long equilibration below the main lipid phase transition would alleviate the problem of vesicle fusion. For this reason, octylglucoside vesicle samples were kept above their phase transition temperature and cooled to the desired temperature only five minutes before freezing. Prolonged equilibration of highly concentrated (i.e. pelleted) octylglucoside vesicle samples at low temperatures appeared to induce spontaneous fusion of these vesicles. On the other hand, giant, unilamellar vesicle samples could be stored below their phase transition and equilibrated for 12 h at the specified temperature prior to jet freezing. Sample concentrations (octylglucoside vesicles, 2–3 mM; giant, unilamellar vesicles, 5–6 mM) were kept low to avoid aggregation artifacts. Samples were fractured and shadowed with Pt at a 45° angle using a Balzers freeze-fracture device. Replicas were viewed on a JEOL 100 CX microscope operated at 80 kV. In the case of octylglucoside vesicle preparations, roughly 4000 vesicles were examined. However, because of the very large size of giant, unilamellar vesicles, only about 100 such vesicles could be practically examined at reasonable magnifications (25000–50000 ×).

Calorimetry. A specially designed, high-sensitivity differential scanning calorimeter [20] with the ability to heat and cool was used to detect membrane phase transitions. Experiments were performed at scan rates of ±15 deg. C/h and data were recorded at temperature intervals of 0.05 deg. C under microprocessor control. Data were corrected for calorimeter response and thermal lag using procedures tested in this laboratory with known standards (unpublished data). Samples contained 3–5 mg of lipid in 1.5 ml 50 mM KCl.

Results

Phase behavior

The phase behavior of LMV of synthetic phosphatidylcholines has been characterized using differential scanning calorimetry [17] and will be referred to for comparison with the work reported here for octylglucoside vesicles and giant, unilamellar vesicles. The excess heat capacity as a function of temperature is plotted in Figs. 1 and 2 for octylglucoside vesicle and giant, unilamellar

vesicle preparations, respectively, made from the synthetic phosphatidylcholines, $DC_{15}PC$ and DPPC. Octylglucoside vesicle preparations retain the basic features of LMV phase behavior; namely, the presence of a main transition preceeded by a less-enthalpic pre-transition peak, a few degrees below the main transition temperature. The main peak transition temperatures of octylglucoside vesicles and LMV are comparable (see Table I).

Giant, unilamellar vesicles also exhibited a main transition peak at a temperature comparable to octylglucoside vesicles and LMV (see Table I). The transition was still broadened by comparison to multilamellar vesicles although it was quite similar to the transition observed for octylglucoside vesicles of the same lipid composition.

The main difference in octylglucoside vesicle and LMV phase behavior, namely broadened peak widths at half height for the main transition of octylglucoside vesicles (see Table I), has been investigated in detail [9] and found to result from apparent impurities in commercially available octylglucoside.

Pre-transition temperatures were slightly lower for octylglucoside vesicles and giant, unilamellar vesicles than for LMV. As seen in the insets of Figs. 1 and 2, there was a substantial hysteresis between the heating and cooling scans in this pre-transition region, while the main transition peaks were nearly superimposible. This effect has been noted previously in studies of LMV phase behavior using diphenylhexatriene as a fluorescent probe [17].

Diluted (1–2 mM) octylglucoside vesicle samples can be stored at low temperature (4°C) for at least a month before changes in their phase behavior can be noted by differential scanning calorimetry [9]. If scanned one week after being stored in this manner, DPPC octylglucoside vesicles exhibited a sub-transition (see Fig. 1) about 10°C below the pre-transition (see Table I). Once the sample was heated above its main transition temperature, the sub-transition was no longer visible upon subsequent cooling. These results are consistent with those of Chen et al. [21], who noted a sub-transition in DPPC LMV.

TABLE I

SUMMARY OF THE PHASE BEHAVIOR OF DIFFERENT VESICLE PREPARATIONS

Transition temperatures (T_m), enthalpies (ΔH), and peak widths at half height ($\Delta T_{1/2}$) are given for heating (first entry for each lipid) and cooling (second entry for each lipid) scans In one scan ($DC_{15}PC$ giant, unilamellar vesicles, cooling scan) the pre-transition cannot be accurately resolved from the baseline (n r)

Lipid	Main transition			Pre-transition	
	T_m (±0 1°C)	ΔH (kcal/mol)	$\Delta T_{1/2}$ (±0 15°C)	T_m (±0 1°C)	ΔH (kcal/mol)
Large, multilamellar vesicles					
$DC_{15}PC$	33.7	8.3±0 1	0.23	24 8	1 4±0 2
	33.8	8.7±0 1	0.23	20 9	1 0±0 2
DPPC	41 2	8 7±0.2	0.28	34 9	1 5±0 2
	41.3	8 7±0.1	0.33	31 2	0 9±0 2
Octylglucoside vesicles					
$DC_{15}PC$	33 6	7.7±0.5	0 6	21 9	0 5±0 2
	33 8	7.8±0.5	0 5	20.3	0.3±0.2
DPPC	41 0	10.5±0 5	1.4	32 1	1 2±0 2
	41 1	11.2±0.5	1.4	31.1	0 8±0 2
Giant, unilamellar vesicles					
$DC_{15}PC$	33.3	7 1±0.5	0.66	15 1	0 2±0 2
	33.3	7 1±0 5	0 69	n r	n r
DPPC	41.4	8 7±0.3	1 1	32 7	0 5±0 2
	41 3	8.8±0 3	1 0	32 3	0 2±0 2

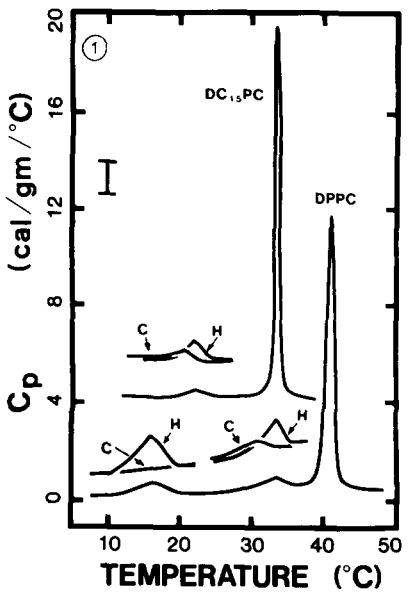

Fig. 1. Excess heat capacity (C_p) as a function of temperature for octylglucoside vesicles. Data was recorded at 0.05 deg C intervals at a scan rate of approx. 15 deg. C/h. Heating scans are shown here through the main transition region Subsequent cooling scans were virtually identical. Pre-transition regions for both heating (H) and cooling (C) scans have been enlarged in the insets (I, scale marker = 0.5 cal/g per deg. C). Note the 'subtransition' observed at about 15°C in DPPC heating but not cooling scans

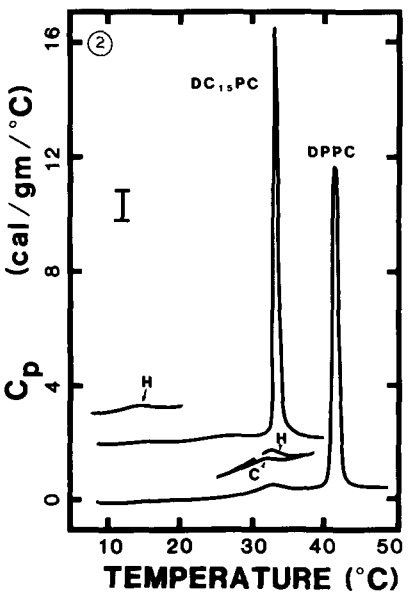

Fig 2 Excess heat capacity (C_p) as a function of temperature for giant, unilamellar vesicles. Calorimeter conditions were the same as for Fig. 1. The cooling pre-transition for $DC_{15}PC$ could not be accurately resolved from the baseline and therefore is not plotted here. I, scale marker for pre-transition regions = 0.5 cal/g per deg. C

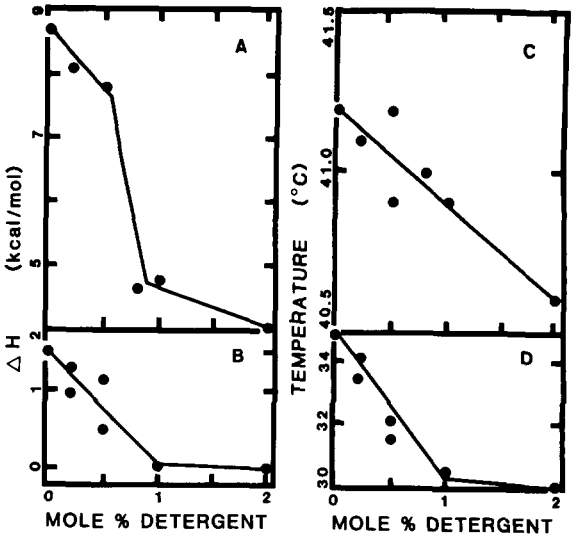

Fig 3 Enthalpy and transition temperature vs. mole fraction deoxycholate for DPPC multilamellar vesicles prepared in the presence of varying amounts of deoxycholate in 50 mM KCl Panels A and B display the enthalpy for the main and pre-transitions, respectively Panels C and D present the transition temperatures for the main and pre-transitions, respectively. Data are presented from heating differential scanning calorimetric scans only, although cooling scans were found to behave in an analogous manner.

Effects of detergent contamination on giant, unilamellar vesicle phase behavior

In light of the dramatic effect that impurities can have on the calorimetric phase behavior [9] of large, unilamellar vesicles, we carefully determined the amount of residual deoxycholate in giant, unilamellar vesicle preparations. Using [^{3}H]deoxycholate as a tracer, we found less than 0.8 mol% detergent present after 48 h. (This represented the limits of detection).

Further controls to determine the effects of residual deoxycholate were performed. DPPC LMV were made in the presence of small amounts of deoxycholate ranging from 0.2 to 2 mol%. The phase behavior of these samples was then determined calorimetrically. With increasing amounts of added deoxycholate, both the main and pre-transition temperatures and the main and pre-transition enthalpies decreased (Fig. 3). This trend was evident in both heating and cooling scans though only data from heating scans are presented. If one assumes the phase behavior of

unilamellar and multilamellar vesicles to be similar in the absence of any impurities (supported by SUV fusion vesicle [14] data [9]), these controls would indicate a maximum of only 0.5–1 mol% contamination in giant, unilamellar vesicle preparations, in agreement with the estimate obtained using [^{3}H]deoxycholate.

Freeze-fracture

Based on our calorimetric determination of octylglucoside vesicle and giant, unilamellar vesicle phase behavior, three temperatures were chosen to study the fracture-face morphology in these two vesicle types: (1) below the pre-transition, in what would be the $L_{\beta'}$ phase * in LMV [22]; (2) between the pre-transition and main transition corresponding to the $P_{\beta'}$ region of LMV; and (3) above the main transition in what would be the L_α phase in LMV. $DC_{15}PC$ and DPPC octylglucoside vesicles have been well characterized with respect to size by negative staining electron microscopy [9]. These vesicles are 80–100 nm in diameter and contain less than 3% oligolamellar contamination [9]. $DC_{15}PC$ and DPPC giant, unilamellar vesicles are more heterogeneous in size than octyl-glucoside vesicles since these were not subjected to similar exhaustive fractionation steps during their preparation. From freeze-fracture micrographs, giant, unilamellar vesicles were found to be 500 to 4000 nm in diameter. An estimate of the oligolamellar contamination of these vesicles was made by examining vesicles frozen from 0°C, where cross fractures were common (45% frequency). Only 10–20% of these cross fractures were to other lamellae (indicating oligolamellar structures) while the majority were to the ice substratum. Both $DC_{15}PC$ (Fig. 4b, d, f) and DPPC giant, unilamellar vesicles (not shown) were studied by freeze-fracture and found to show similar morphological changes associated with their phase transitions. Micrographs of DPPC (Fig. 4a, c, e) and $DC_{15}PC$ octylglucoside vesicles (not shown) also demonstrated morphological changes through their phase transitions although these were distinct from those demonstrated by giant, unilamellar vesicle preparations.

It is instructive to compare the fracture-face morphologies of our octylglucoside vesicle and giant, unilamellar vesicle structures with those of LMV in corresponding temperature ranges. Fig. 4a shows octylglucoside vesicles below their pre-transition. These vesicles had a unique facetted structure that could be seen in both concave and convex fracture planes. Arrows in Fig. 4a indicate vesicles which best exemplify this facetted morphology. Giant, unilamellar vesicles at a temperature well below their pre-transition are shown in Fig. 4b. A fracture through to the ice matrix of this convex surface can be seen and demonstrates the unilamellar nature of this vesicle. The faint irregular banding pattern seen in giant, unilamellar vesicles at low temperatures likely represents kinetically trapped remnants of the $P_{\beta'}$ structure which exists at higher temperatures. Similar irregular bands have been noted in DPPC LMV frozen from comparable temperatures [23].

In comparing octylglucoside vesicles and giant, unilamellar vesicles, the most prominent differences occurred at temperatures between the pre- and main phase transition peaks. In this temperature range, LMV fracture-faces were characterized by a distinctive parallel banding pattern typical of the $P_{\beta'}$ phase [23,24]. Octylglucoside vesicles in this temperature range exhibited a facetted fracture-face morphology even more distinctive than that seen at lower temperatures. This is indicated by the arrows in Fig. 4c. Electron micrographs of giant, unilamellar vesicles in this temperature range (Fig. 4d) revealed a weakly banded fracture pattern reminisent of, but much less distinct than, that observed with LMV. A banded pattern seen in a vesicle containing a cross fracture to the ice matrix would unambiguously demonstrate that a $P_{\beta'}$-like phase can exist in a unilamellar vesicle. For unknown reasons, cross fractures to the ice were very rare at higher temperatures in our preparations. At 28°C, only one $DC_{15}PC$ vesicle demonstrated a cross fracture to the ice substratum, verifying that it was unilamellar. The vesicle fracture-face still clearly showed the faint banded pattern typical of all giant vesicles frozen from the $P_{\beta'}$ region and illustrated in Fig. 4d.

The facetted nature of 80–100 nm diameter

* Nomenclature of smectic phases is after Tardieu et al [22] L_α, lamellar, chains disordered; $P_{\beta'}$, two dimensional, pleated, chains ordered; $L_{\beta'}$, lamellar, chains ordered

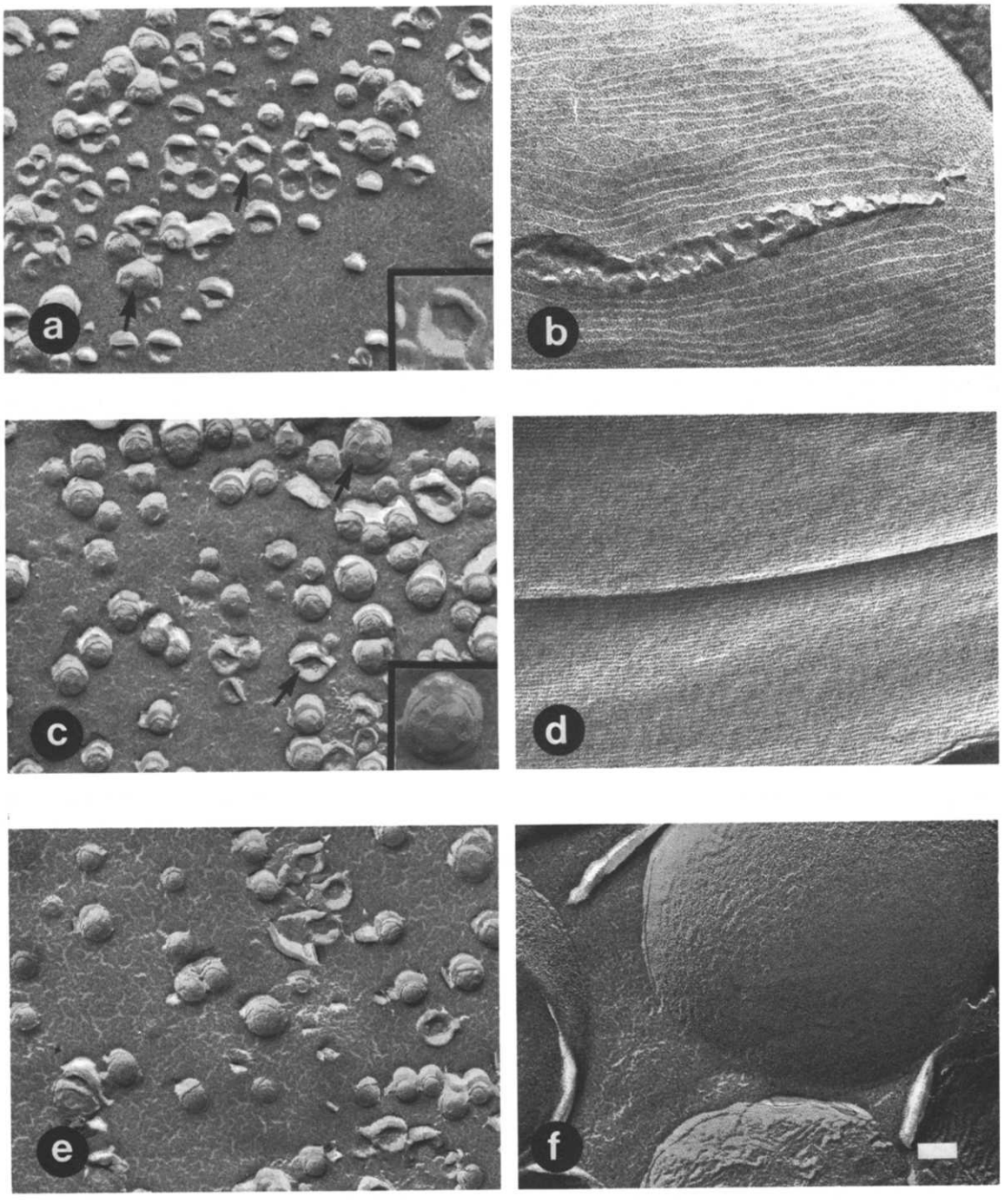

Fig. 4. Freeze-fracture electron micrographs of vesicle samples prepared by 'jet freezing' Platinum shadowing is from below Magnification 62500×. Bar indicates 100 nm. (a) DPPC octylglucoside vesicles frozen from 10°C Arrows indicate facetted morphology (b) $DC_{15}PC$ giant, unilamellar vesicles frozen from 0°C (c) DPPC octylglucoside vesicles frozen from 35.2°C. Arrows indicate facetted fracture-faces. (d) $DC_{15}PC$ giant, unilamellar vesicles frozen from 28°C (e) DPPC octylglucoside vesicles frozen from 55°C. (f) $DC_{15}PC$ giant, unilamellar vesicles frozen from 55°C Insets in the lower right of frames (a) and (c) are enlargements (magnification 100000×) of individual vesicles showing facetted fracture-faces.

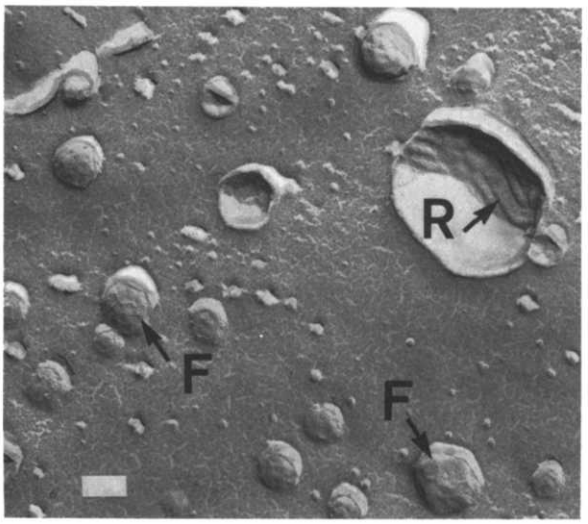

Fig. 5 Freeze-fracture electron micrograph of DPPC octylglucoside vesicles 'jet frozen' from 35.2°C after several hours of incubation at this temperature Vesicles with diameters less than 200 nm exhibit the facetted morphology (F) while vesicles larger than this begin to exhibit a rivuletted pattern (R). Platinum shadowing is from below Magnification 55000× Bar indicates 100 nm

octylglucoside vesicles in the temperature range of the $P_{\beta'}$ phase is further illustrated in Fig. 5. The sample shown in this micrograph contained DPPC octylglucoside vesicles stored at high concentration (i.e. 10–20 mM) for 12 h at 35.2°C before being jet frozen from this temperature. In our experience, these conditions (storage at high concentration below the phase transition) have reproducibly resulted in fusion to yield a heterogeneous population of vesicles (see Methods). This heterogeneous vesicle population allows us to emphasize the importance of vesicle size in determining fracture-face morphology. In such micrographs, normal, large, unilamellar vesicles (i.e., 80–100 nm diameter) clearly show a facetted fracture-face morphology (see 'F' in Fig. 5) while larger, fused vesicles demonstrated a rivuletted morphology (see 'R' in Fig. 5) reminiscent of, but not identical to, that characteristic of LMV. From consideration of several micrographs of this type, we estimate the cut-off between these two types of morphologies to occur for vesicles of roughly 200 nm diameter.

Above the main transition temperature, micrographs of octylglucoside vesicles showed a smoother, more rounded vesicle shape (Fig. 4e) as compared to the facetted structures (Figs. 4a and 4c). These micrographs were somewhat etched in preparation and the resulting ring around each vesicle reflects the juncture between an internal fracture-face and exposed membrane surface. These should not be misinterpreted as indicative of multilamellar structures or of the facetted fracture-faces referred to above. At high temperatures, giant, unilamellar vesicles were found to have a somewhat mottled surface similar to that noted previously for pure phosphatidylcholine, phosphatidylglycerol and sphingomyelin LMV [18,19,23] (Fig. 4f). Worm-like structures, as previously reported by Lentz et al. [23] were also seen in some of these vesicles above their phase transition. These probably represent fluid phase regions that have experienced a slightly slower freezing rate.

Discussion

While interest has increased considerably in the use of large, unilamellar vesicles as biomembrane models and as drug entrapment devices, very little serious effort has been made to determine the phase behavior of these vesicles. Thus, only a handful of reports exist on this subject, and they disagree even as to the existence of a pre-transition. For example, both Petri et al. (octylglucoside dialysis [7]) and, more recently, Wong et al. (fusion vesicles [14]) failed to detect a pre-transition using differential scanning calorimetry. A study by Düzgüneş et al. [28] using reverse phase evaporation vesicles also reported no pre-transition by differential scanning calorimetry, although, on close inspection, a faint pre-transition may be visible above their instrumental baseline. A fourth calorimetric study [12] claimed to have detected a pre-transition in deoxycholate-dialyzed vesicles, although it was, in our opinion, quite difficult to distinguish from a curved baseline. Freeze-fracture electron microscopy performed on ether-injection vesicles [26] and giant, unilamellar vesicles gently peeled from the wall of a flask [27] lends evidence to the existence of an intermediate gel phase. Düzgüneş and coworkers reported facetted vesicle fracture-faces when reverse phase evaporation vesicles were frozen from temperatures just above the main phase transition. In response to the dis-

parity of published opinion on this issue, we have performed high sensitivity differential scanning calorimetric measurements on unilamellar vesicles prepared by a variety of procedures [9]. We report here and elsewhere [9] that all unilamellar vesicles display a pre-transition, although this can be obscured by the presence of solvent- or detergent-derived impurities (Fig. 3B).

The work reported here focuses on the lipid morphological changes in unilamellar vesicles analogous to those occurring in large, multilamellar vesicles at the pre-transition. The pre-transition occurs in phosphatidylcholine multilayers between an ordered phase with chains tilted from the bilayer normal ($L_{\beta'}$) and an ordered phase at higher temperature with the bilayer plane undulated in a regular fashion ($P_{\beta'}$) [24,25]. For two reasons, we were uncertain whether such a transition could occur in small, or moderately-sized unilamellar vesicles. First, the periodicity of the repeating, pleated structure has been reported to be a sensitive function of hydration and, therefore, interbilayer distance [25]. This implies an interaction between bilayers stabilizing the $P_{\beta'}$ structure. Second, the extended, pleated structure would seem topologically inconsistent with all but fairly large vesicles, since it would, after very few repeats, close back on and interface with itself. In vesicles of appreciable curvature, there would be additional problems in forming stable structures at vesicle poles.

Consistent with the arguments outlined above, the fracture-face morphology of octylglucoside vesicles was observed to be facetted rather than rivuletted at temperatures just below the main phase transition (Fig. 4c). In the corresponding temperature range, giant, unilamellar vesicle fracture-faces showed a faint banded morphology with a band periodicity of 10.9 nm. This is considerably shorter than the band periodicity which we have observed for pure DPPC LMV in either the 'rivuletted' (14.5 nm) or 'banded' morphology (16.0 nm) [20]. It is also shorter than the limiting periodicity calculated from X-ray scattering data for DPPC (14.0 nm) or DMPC (12.0 nm) at high water content [25]. This implies that the structure of the intermediate gel phase in giant, unilamellar vesicles is somewhat different from the structure of the $P_{\beta'}$ phase in LMV. These results indicate that even isolated bilayers experience forces favoring formation of the pleated gel phase but that the normal expression of this phase in terms of a rivuletted or banded fracture-face morphology is constrained in vesicles of approx. 100 nm or smaller diameter. Instead, a facetted fracture-face morphology reflects the local lipid packing tendencies of vesicles in this size range (see Fig. 4 for 100 nm vesicles and the work of Blaurock and Gamble [29] for 20 nm vesicles). It seems that facetting may be the spontaneous response of phospholipid molecules to their inability to pack in a $P_{\beta'}$-like structure and meet the topological constraints of a small or intermediate-sized vesicle. We suspect that it is favorable inter-vesicle interactions involving apposed facetted surfaces of small, unilamellar vesicles and our larger octylglucoside vesicles that account for their tendency to aggregate when concentrated below their main phase transition for many days (see Results), ultimately leading to vesicle fusion [14]. On the other hand, it may be that vesicle contact and adhesion is the cause of the facetting we observe rather than the result of it. Three observations argue against this possibility. First, at the low concentrations (2–3 mM) used in our studies, vesicles aggregation was not substantial (see Fig. 4a, c, e). Second, facets were observed even in apparently isolated vesicles (Fig. 4a, c). Third, no facets were observed above the main phase transition temperature (Fig. 4e) in samples of the same concentration as those showing facets below the phase transition. Therefore, we conclude that the facetted morphology likely reflects lipid phase behavior rather than vesicle aggregation.

At temperatures below the pre-transition temperature, a banded morphology remained in the giant, unilamellar vesicles (Fig. 4b), but the regular periodicity was lost, probably indicative of the long equilibration times necessary to anneal out the $P_{\beta'}$ structure [23]. In the same manner, less distinct facets were observed in octylglucoside vesicle preparations at low temperatures (Fig. 4a).

Our conclusions hold despite any ambiguity resulting from slight di- and trilamellar contamination of the giant, unilamellar vesicle samples. The vesicles shown in Fig. 4b, d, and f were verified as being greater than 80–90% unilamellar by the presence of thru-fractures to the

ice lattice when frozen from low temperature (e.g., see Fig. 4b). In the temperature range corresponding to the $P_{\beta'}$ phase, faint banding was observed in all giant, unilamellar vesicle fracture-faces, making it highly unlikely that banding was due to oligolamellar structures. In addition, one clearly unilamellar vesicle (as evidenced by cross fracture) showed the same faint banding pattern in the $P_{\beta'}$ temperature range as did all other vesicles frozen from this temperature range. We conclude that unilamellar vesicles display a morphologically distinct phase intermediate between the fluid, high-temperature phase and the low-temperature solid-like phase. However, the morphology of this phase is banded or rivuletted only in vesicles of sufficiently low curvature and is facetted in smaller vesicles.

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