Osmotic and Curvature Stress Affect PEG-Induced Fusion of Lipid Vesicles but Not Mixing of Their Lipids

Vladimir S. Malinin,* Peter Frederik,† and Barry R. Lentz*
*Department of Biochemistry and Biophysics, CB#7260, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7260 USA, and †Pathology Department, University of Maastricht, Maastricht, the Netherlands

ABSTRACT Poly (ethylene glycol) (PEG) in the external environment of membrane vesicles creates osmotic imbalance that leads to mechanical stress in membranes and may induce local membrane curvature. To determine the relative importance of membrane stress and curvature in promoting fusion, we monitored contents mixing (CM) and lipid mixing (LM) between different sized vesicles under a variety of osmotic conditions. CM between highly curved vesicles (SUV, 26 nm diameter) was up to 10 times greater than between less curved vesicles (LUV, 120 nm diameter) after 5 min incubation at a low PEG concentration (<10 wt%), whereas LM was only ~30% higher. Cryo-electron microscopy showed that PEG at 10 wt% did not create high curvature contacts between membranes in LUV aggregates. A negative osmotic gradient (−300 mOs/kg, hypotonic inside) increased CM two- to threefold for both types of vesicles, but did not affect LM. A positive gradient (+220 mOs/kg, hypertonic inside) nearly eliminated CM and had no effect on LM. Hexadecane added to vesicles had no effect on LM but enhanced CM and reduced the inhibitory effect on CM of a positive osmotic gradient, but had little influence on results obtained under a negative osmotic gradient. We conclude that the ability of closely juxtaposed bilayers to form an initial intermediate ("stalk") as soon as they come into close contact was not influenced by osmotic stress or membrane curvature, although pore formation was critically dependent on these stresses. The results also suggest that hexadecane affects the same part of the fusion process as osmotic stress. We interpret this result to suggest that both a negative osmotic gradient and hexadecane reduce the unfavorable free energy of hydrophobic interstices associated with the intermediates of the fusion process.

INTRODUCTION

Membrane fusion is a key event in many essential cell processes, such as exocytosis, endocytosis, membrane recycling, protein sorting and transport, fertilization, and cell division. Membrane fusion is also crucial to the processes of viral infection and budding. It is not surprising, then, that the molecular mechanism of cell membrane fusion is currently under intensive investigation. Substantial evidence suggests that viral and cell membrane fusion, although mediated by proteins, is lipidic in its basic mechanism (Chernomordik et al., 1998; Lentz and Lee, 1999). Thus, studying model membrane fusion provides insight into the basic mechanism of cell membrane fusion. Poly(ethylene glycol) (PEG) has been widely used to induce lipid bilayer fusion. PEG is a hydrophilic polymer that causes aggregation and fusion of lipid vesicles and cells (Lentz, 1994). It is believed that depletion forces that induce aggregation arise from the exclusion of PEG from the vesicle surface layer (depletion layer, thickness on the order of 1 nm) and particularly from the contact region between the membranes (Arnold et al., 1990; Evans and Needham, 1988; Kuhl et al., 1996; Yamazaki et al., 1989). The imbalance of osmolality between the depletion layer and the bulk aqueous phase provides a thermodynamic potential that drives aggregate formation. In addition, PEG dehydrates the lipid bilayer, leading to formation of nonlamellar structures (Boni et al., 1981), raising the gel-to-fluid phase transition temperature (Tilcock and Fisher, 1979) and enhancing membrane permeability (Aldwinckle et al., 1982). Finally, PEG has a very high osmolality (1 Os/kg and up) (Michel, 1983) at concentrations of 20–40 wt%, causing osmotic stress of cells or vesicles when PEG added externally is not matched by an internal osmolyte.

The effect of osmotic stress on membrane fusion has been investigated in a number of different systems. In comparing studies, it is important to use a common nomenclature for osmotic stress. Here, we use the terms "positive" and "negative" osmotic gradients to express the difference of osmotic pressure in the trapped compartment relative to the external compartment ($\Pi_{in} - \Pi_{out}$). Thus, adding PEG to the external compartment, without compensating with an increase in osmolyte concentration in the trapped compartment, created hypertonic osmotic stress. According to some reports, positive osmotic stress ($\Pi_{in} > \Pi_{out}$) promoted Ca^{2+}-induced fusion of cells (Ahkong and Lucy, 1986) or fusion of lipid vesicles to planar bilayers (Chanturiya et al., 1997; Cohen et al., 1982). In contrast, another study reports that hydrostatic cell inflation (also positive stress) inhibited hemagglutinin (HA)-induced fusion (Markosyan et al., 1999). Still, a third study shows that 30-nm diameter liposomes fused better under a negative osmotic gradient ($\Pi_{in} < \Pi_{out}$), but, when these vesicles grew in size to more than 100 nm, positive stress was necessary to promote further fusion (Miller et al., 1976). From these disparate results, it seems that our understanding of the effects of osmotic stress on membrane fusion remains incomplete.

Submitted July 10, 2001, and accepted for publication December 20, 2001.

Address reprint requests to Dr. Barry R. Lentz, University of North Carolina, Department of Biochemistry, 418 MEJB, CB #7260, Chapel Hill, NC 27599-7260. Tel.: 919-966-5384; Fax: 919-966-2852; E-mail: uncbrl@med.unc.edu

In our previous studies, we have shown that large unilamellar vesicles (LUVs) fuse at high PEG concentrations (20–40 wt%), which can create strong negative osmotic stress and significant vesicle shrinkage (Burgess et al., 1992). We proposed that flattening of vesicles created highly curved edges that drove fusion of large vesicles. Numerous studies show that high membrane curvature promotes fusion (Lentz et al., 1992; Nir et al., 1982; Ohki, 1984; Talbot et al., 1997). However, model membrane vesicles can experience not only a shape change but also membrane mechanical stress in the presence of PEG and osmotic stress. In this paper, we examine the balance between osmotic stress and curvature effects in PEG-induced fusion of lipid vesicles. We were surprised to find that negative osmotic stress did not enhance fusion through dramatic changes in membrane curvature and that positive stress (vesicle swelling) actually inhibited fusion. Based on the parallels that we have found between the effects of osmotic gradients and hexadecane, we suggest that these unexpected effects of osmotic stress on PEG-mediated membrane fusion may result from the ability of osmotic gradients to impede or encourage movement of lipids in ways that accommodate nonlamellar structures created during the fusion process.

MATERIALS AND METHODS

1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dilinolenoyl-*sn*-glycero-3-phosphocholine ($DC_{18:3}PC$), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) were purchased from Avanti Polar Lipids (Birmingham, AL), and used without further purification (except cholesterol (Ch)). Ch (Avanti Polar Lipids) was purified via the dibromide form (Schwenk and Werthessen, 1952). Concentrations of phospholipid stocks in chloroform were determined by phosphate assay (Chen et al., 1956). 1-hexadecanoyl-2-(3-(diphenylhexatrienyl)propanoyl)-*sn*-glycero-3-phosphocholine (β-DPHpPC) was purchased from Molecular Probes (Eugene, OR). Dodecyl octaethylene glycol monoether ($C_{12}E_8$) was purchased from Calbiochem (La Jolla, CA). PEG of molecular weight 7000–9000 (PEG 8000) was purchased from Fisher Scientific (Fairlawn, NJ) and purified as described previously (Lentz et al., 1992). Polybead polystyrene microspheres were purchased from Polysciences (Warrington, PA). All other reagents were of the highest quality available.

Vesicle preparation

For most experiments, a volumetrically measured mixture of DOPC/DOPE/Ch (50/25/25 mol%) in chloroform was dried under a stream of nitrogen. In some experiments, vesicles prepared from DOPC/$DC_{18:3}PC$ (85/15 mol%) were used, as indicated. For vesicles used in lipid mixing (LM) measurements, an appropriate mole fraction of probe lipids was added to the chloroform solution before drying. The dried lipids were dissolved in cyclohexane with an aliquot of methanol (~5 vol%), frozen on dry ice, and lyophilized under high vacuum overnight. The lyophilized lipids were suspended with occasional agitation in an appropriate buffer for ~1 h at room temperature at a concentration of 10–20 mM. The fusion buffer contained 100 mM NaCl, 10 mM *N*-[tris(hydroxymethyl)methyl]2-2-aminoethane sulfonic acid, 1 mM EDTA, pH 7.4, and an appropriate concentration of sucrose where indicated. For the terbium/pyridine-2,6-dicarboxylic acid (DPA) assay, 1 mM $CaCl_2$ was added to the fusion buffer. LUVs were prepared by the extrusion method (Mayer et al., 1986). Lipid suspensions were extruded 10 times through a 0.1-μm polycarbonate filter (Nucleopore, Pleasanton, CA) at room temperature (above the phase transition) under a pressure of 80 psi of nitrogen (Lentz et al. 1992). LUVs had an average hydrodynamic diameter (volume weighted) of ~120 nm, with Gaussian distribution width of 45 nm, as determined by quasielastic light scattering (QELS). Small unilamellar vesicles (SUVs) were prepared as previously described (Lentz et al., 1987). To prepare SUVs, the lipid suspension was sonicated for 10 min using a Heat Systems Model 350 Sonicator (Plainview, NY) equipped with a titanium probe tip of 9.5-mm diameter. Vesicle preparations were fractionated by centrifugation at 70,000 rpm for 25 min at 4°C using a Beckman TL-100 ultracentrifuge in a TLA (Palo Alto, CA) (Barenholz et al., 1977). The average hydrodynamic diameter (volume weighted) of SUVs was 26 nm, with Gaussian distribution width of 13 nm. In one instance, DOPC/DOPE/Ch SUVs were sized on a Sepharose 4B (Pharmacia, Uppsala, Sweden) column and found to give a single peak corresponding to a mean diameter of ~25 nm in agreement with QELS data.

Contents mixing (CM) and leakage assays

Tb^{3+}/DPA CM and leakage assays (Talbot et al., 1997; Wilschut et al., 1980) were adapted to maintain necessary osmotic conditions. For the CM assay, two populations of vesicles were used, one encapsulating Tb^{3+} buffer (8 mM $TbCl_3$, 60 mM sodium citrate, 10 mM TES), and another DPA buffer (80 mM DPA, 10 mM TES, pH 7.4). In leakage experiments, vesicles prepared in Tb^{3+}/DPA buffer (4 mM $TbCl_3$, 30 mM sodium citrate, 40 mM DPA, 10 mM TES, pH 7.4) were used. Untrapped buffer was removed using a Sephadex G-75 column (Pharmacia Biotech AB, Uppsala, Sweden) equilibrated with buffer containing 100 mM NaCl, 10 mM TES, 1 mM EDTA, pH 7.4, which had the same osmolality as trapped buffer.

The CM assay was carried out by mixing equal amounts of vesicles (0.1 mM lipid) containing either $TbCl_3$ or DPA in a fusion buffer and an appropriate concentration of PEG. For experiments with controlled osmotic gradients, sucrose was added to the fusion buffer or the PEG to adjust osmolality. The resulting increase in fluorescence intensity attributable to formation of Tb/DPA complex was measured after 5 min of incubation using an SLM 48000 MHF spectrofluorometer (SLM Instruments, Rochester, NY) with an excitation wavelength of 278 nm (slit = 4 nm). A monochrometer set to a wavelength of 545 nm (slit = 32 nm) along with a cutoff filter OG500 (Schott Glass Technologies, Duryea, PA) were used to define the emitted light. The percent of CM at each particular PEG concentration was calculated based on the assumption that 100% CM corresponded to the fluorescence of Tb/DPA vesicles (representing fusion of one Tb^{3+} vesicle to one DPA vesicle). The calculated CM values will thus underestimate the actual extent of fusion to the extent that the ratio of Tb^{3+} to DPA vesicles present in a fusion aggregate is other than 1/1.

Percent leakage was calculated from the drop in fluorescence of vesicles containing both $TbCl_3$ and DPA upon addition of PEG, where 100% leakage was characterized as the fluorescence after addition of $C_{12}E_8$ detergent (octaethyleneglycol mono-*n*-dodecyl ether) at a concentration of 1 mM. The percentage of CM was always corrected for leakage as previously described (Talbot et al., 1997).

LM assay

The LM assay is based on the fact that the lifetime of the membrane-located probe 1-hexadecanoyl-2-(3-(diphenylhexatrienyl)propanoyl)-*sn*-phosphocholine (DPHpPC) is a sensitive function of its surface concentration in the membrane (Burgess and Lentz, 1993; Lentz and Burgess, 1989). Vesicles labeled with 5 mol% DPHpPC were mixed with probe-free vesicles at a 1:4 ratio and with an appropriate concentration of PEG (final lipid concentration of 0.2 mM). The average DPHpPC lifetime was ob-

tained from frequency-domain measurements analyzed using the SLM 48000 MHF software package assuming two lifetime components. A standard calibration curve for each system was obtained by measuring the lifetimes of DPHpPC in vesicles with different lipid:probe ratios. The percent of lipid mixing was calculated as documented previously (Burgess and Lentz, 1993), assuming that 100% LM corresponds to a fivefold dilution of the probe.

Fluorescence lifetime measurements were made on an SLM 48000 MHF spectrofluorometer using the laser UV multiline (351.1–363.8 nm) of a Coherent Inova 90 argon-ion laser (Coherent Auburn Group, Auburn, CA) for DPHpPC excitation and with the polarization of the laser beam oriented in the vertical direction. Emission was detected at an angle of 54.7° from the vertical through a 3-mm KV-418 filter (Schott Glass Technologies). Phase shifts and modulation ratios were measured at 30 frequencies (with a base frequency of 5 MHz) using a 5-s acquisition time, 300-acquisition average, and a glycogen solution as zero lifetime, using the acquisition software of the SLM 48000 spectrofluorometer.

Vesicle size determination

Average vesicle diameters were determined by QELS performed using a locally built multiangle instrument equipped with a Spectra-Physics Stabilite Model 120S helium-neon laser (632.8 nm) and a computer-controlled Nicomp 170 autocorrelator (Particle Sizing Systems, Santa Barbara, CA). Autocorrelation functions were analyzed by assuming a single Gaussian distribution of particle sizes in volume-weighted mode using software provided by Nicomp. For some preparations, we collected correlation data for ~45 min to run a multi-Gaussian analysis and never found evidence of more than a single peak.

Cryo-electron microscopy (EM)

Suspensions of vesicles and vesicles mixed with PEG solutions were applied to bare grids (700-mesh, hexagonal pattern) within an environmental chamber (relative humidity 100%, 24°C). The excess liquid was blotted away with filter paper using an automatic blotting device within the environmental chamber. The grid was subsequently dropped into melting ethane, using a guided drop principle and a shutter to direct the grid into the coolant placed just outside the environmental chamber (Frederik et al., 1991). The vitrified specimens were stored under liquid nitrogen and observed at −170°C (Gatan 626 cryoholder) in a Philips CM12 microscope. Micrographs were taken at 120 kV using low dose-conditions.

Osmolality measurements

Osmolality of sucrose and PEG solutions was measured using a Wescor Model 5100 Vapor Pressure Osmometer (Wescor, Logan, UT).

RESULTS

Fusion of LUVs is favored by but does not require negative osmotic stress

Fusion of egg phosphocholine LUVs requires high concentrations of PEG in the range of 30–35 wt% (Lentz et al., 1997; Talbot et al., 1997). The osmolality of such PEG solutions is as much as 0.9–1.4 Os/kg above the trapped buffer osmolality (Michel, 1983). Such an osmolality gradient might cause significant shrinkage of vesicles. Vesicles prepared with the highly unsaturated mixture of 85/15 DOPC/$DC_{18:3}$PC fuse more readily (Lentz et al., 1997).

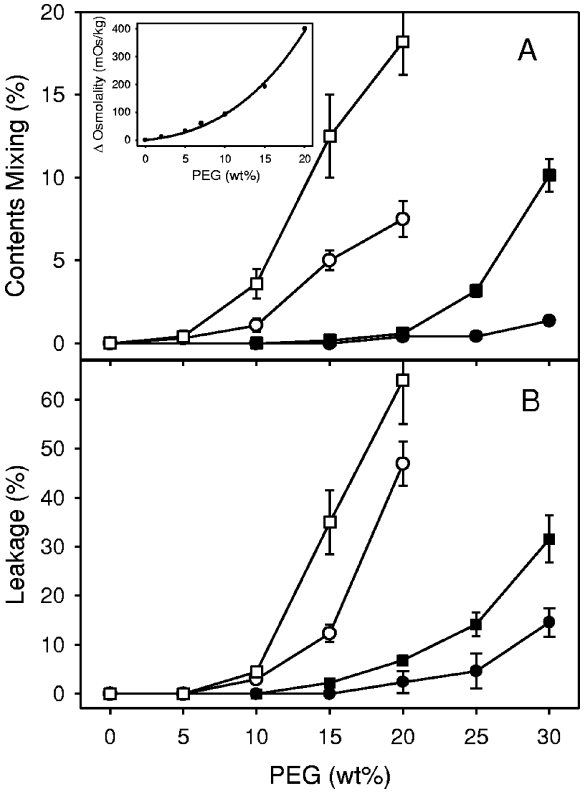

FIGURE 1 Fusion of LUVs is favored by but does not require negative osmotic stress. PEG-induced fusion of LUVs of different composition (DOPC/DOPE/Ch 50/25/25 mol%, ○ and □, and DOPC/$DC_{18:3}$PC 85/15 mol%, ● and ■) is shown in terms of CM (*A*) and leakage (*B*) of osmotically balanced (○ and ●) and unbalanced (□ and ■) vesicles as a function of PEG concentration. Measurements were performed after 5 min incubation with PEG of indicated concentrations at 23°C. Osmotically unbalanced vesicles were prepared in Tb^{3+} and/or DPA buffer of the same osmolality as the buffer in which PEG was dissolved. Osmotically balanced vesicles were prepared in the presence of sucrose at a concentration appropriate to match the osmolality of the inside and outside vesicle compartments after vesicles were added to buffer containing PEG. Vesicles exposed to PEG without balancing sucrose on the interior of the vesicle experienced the osmotic gradients (measured directly under our ionic conditions) shown in the *inset* in *A*. For reference, an osmotic gradient of 200 mOs/Kg corresponds to a pressure gradient of ~5 atm.

Still, 25 wt% PEG was necessary to detect CM of LUVs prepared with this lipid mixture (Fig. 1). In the presence of these high concentrations of PEG, the osmotic gradient experienced across vesicle membranes reaches significant levels (Fig. 1 *A*, *inset*). To avoid the large osmotic gradients associated with high PEG concentrations, we prepared vesicles in buffer containing appropriate concentrations of sucrose so as to balance the osmolality of each PEG concentration used. These osmotically balanced vesicles showed almost no CM and very little contents leakage at PEG concentrations up to 30 wt%. One might conclude that large vesicles with low membrane curvature do not fuse in the absence of high negative osmotic stress. Because an osmotic gradient is expected to cause membrane shrinkage and thus

induce curvature, this might suggest that high membrane curvature is necessary for PEG-mediated fusion.

To examine this possibility, we turned to vesicles with a different lipid composition. LUVs prepared with a more fusogenic lipid mixture, DOPC/DOPE/Ch (2/1/1), fused at a much lower PEG concentration than did DOPC/$DC_{18:3}$PC vesicles (Fig. 1, □ and ○). This is expected for two reasons. First, DOPE molecules have negative intrinsic curvature and reduce the energy barrier of creating a stalk, the commonly recognized initial fusion intermediate (Kozlov et al., 1989). In addition, vesicles containing phosphatidylethanolamine display closer interbilayer approach at a given PEG concentration (Burgess et al., 1992). Osmotic balancing between the trapped and external compartments also reduced CM and leakage in these LUVs, but only to 40–50% of that in the presence of an osmotic gradient. We conclude that vesicles having intrinsic curvature stress can fuse even in the absence of osmotic stress that may induce mechanical curvature stress. It seems that osmotically induced mechanical stress and curvature stress both play a role in promoting PEG-mediated fusion.

High curvature enhances fusion but not LM

In an attempt to isolate the roles of membrane curvature and osmotic stress, we examined osmotically balanced SUVs of limiting small size, which should remain approximately spherical in the presence of PEG (see Appendix). The data in Fig. 2 report different features of PEG-mediated fusion of these DOPC/DOPE/Ch vesicles: (*A*) CM, (*B*) contents leakage, and (*C*) LM. As one can see by comparing Figs. 2 and 1, the high membrane curvature of SUVs (○ and ●) compared with LUVs (□ and ■) strongly enhanced CM and leakage but had little influence on LM. This was reflected both in slightly lower threshold PEG concentrations for fusion and in considerably larger increments in CM and leakage with PEG concentration for SUVs (○ and ●) as compared with LUVs (□ and ■). Indeed, the extent of CM for SUVs at 10 wt% PEG was almost seven times larger than that for LUVs at this PEG concentration. By contrast, LM increased by only ~30% in SUVs versus LUVs at 5 to 10% PEG, a range for which SUVs fuse and LUVs show little fusion. This difference is approximately what we expect for inner leaflet mixing between fusing vesicles, whereas nonfusing vesicles are limited to LM of contacting leaflets. Fig. 2, *A* and *C*, insets show the time courses of CM and LM for DOPC/DOPE/Ch SUVs. These time courses are multiexponential, as documented elsewhere (Evans and Lentz, 2001; Lee and Lentz, 1997). The endpoint assays we use here sample comparable portions of the times courses and reflect a combination of the slow and fast components of both processes.

Another interesting observation is that considerable LM (nearly one-third of the maximum value observed) occurred for both LUVs and SUVs prepared from this fusogenic lipid

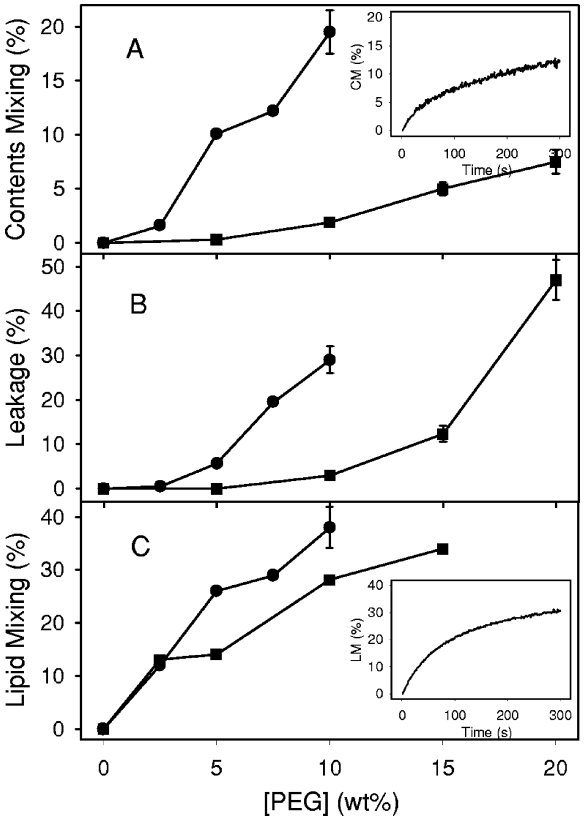

FIGURE 2 High curvature enhances fusion but not LM. CM (*A*), leakage (*B*), and LM (*C*) were measured at different PEG concentrations. Small (SUVs, ●) and large (LUVs, ■) DOPC/DOPE/Ch vesicles were prepared in the presence of appropriate sucrose concentrations to eliminate an osmotic gradient after addition of PEG. The average volume-weighted size of the vesicles was measured by QELS as 26 nm for SUVs and 120 nm for LUVs. The Tb/DPA assay was used for CM and leakage measurements, and the DPHpPC lifetime assay was used to measure mixing of membrane lipids. All measurements were performed after 5 min incubation with PEG at 23°C. The *insets* to *A* and *C* show representative times courses for fusion and LM, respectively, between SUVs in the presence of 10 wt% PEG.

mixture at a PEG concentration of only 2.5 wt%, which is close to the threshold value for vesicle aggregation. At the same concentration, CM and leakage were not detected for LUVs, whereas CM was ~2% for SUVs. This indicates that vesicle outer leaflets merge readily as soon as vesicles come into contact, independent of curvature. It is not discernible from our data whether this reflects formation of the first intermediate of the fusion process (the stalk) or whether it simply reflects an increase in the rate of intervesicle exchange when vesicles come into close contact. Based on two observations, we favor the explanation that the stalk intermediate forms even at prefusion PEG concentrations. First, our results (Fig. 2 *C*) show that LM varied in a smooth fashion from nonfusogenic to fusogenic PEG concentrations. Second, the activation energy of LM was the same at very low and subfusion PEG concentrations in SUVs (Evans and Lentz, 2001). Thus, we suspect that there is no clear demarcation between the process leading to LM just

below fusion conditions and that leading to LM just above the fusion threshold PEG concentration. These observations suggest that stalk formation occurs as soon as vesicles are brought into close contact, independent of bilayer curvature. Fusion pore creation, by contrast, seems to be enhanced by high curvature.

Osmotic gradients do not create high curvature contacts in LUV aggregates

Direct cryo-EM observation of PEG-aggregated vesicles was used to test whether LUVs might form toroidal stacks under the influence of a negative osmotic gradient, as previously suggested (Lentz et al., 1992). DOPC vesicles aggregate well at 10 wt% PEG but do not fuse (at least within the time scale of sample preparation for cryo-EM). For this experiment, we chose vesicles that do not fuse because we wanted to examine the effects of PEG on vesicle shape without the complication of fusion. Our observations were limited to 10 wt% PEG, because the electron density of a PEG solution at higher concentration becomes comparable with the density of vesicle membranes. This limits contrast and the quality of cryo-EM images.

As one can see from Fig. 3, LUVs are not absolutely spherical even in buffer without PEG (*A*), probably because the extrusion process used to prepare vesicles distorts them from a spherical shape (Jin et al., 1999). Addition of PEG failed to deform them to an appreciable extent in the case of both osmotically balanced (*B*) and unbalanced (*C*) LUVs compared with untreated vesicles in isotonic buffer (*A*). Moreover, vesicles in aggregates induced by this low PEG concentration had no preferential orientation to one another (i.e., they did not stack as we have previously speculated for higher PEG concentrations (Lentz et al. 1992)). We observed some flattened vesicles in the center of aggregates, and more rounded vesicles on the periphery. This could be because attractive forces directed from the periphery to the center create maximum tension at the center of an aggregate. But even these deformed LUVs contact one another mainly at regions of moderate curvature. We conclude that the number of intermembrane contacts at sites of high curvature is not substantially increased under conditions of negative osmotic gradient. Thus, an osmotic gradient probably influences fusion by creating mechanical tension in vesicle bilayers rather than by inducing contacting regions of high curvature.

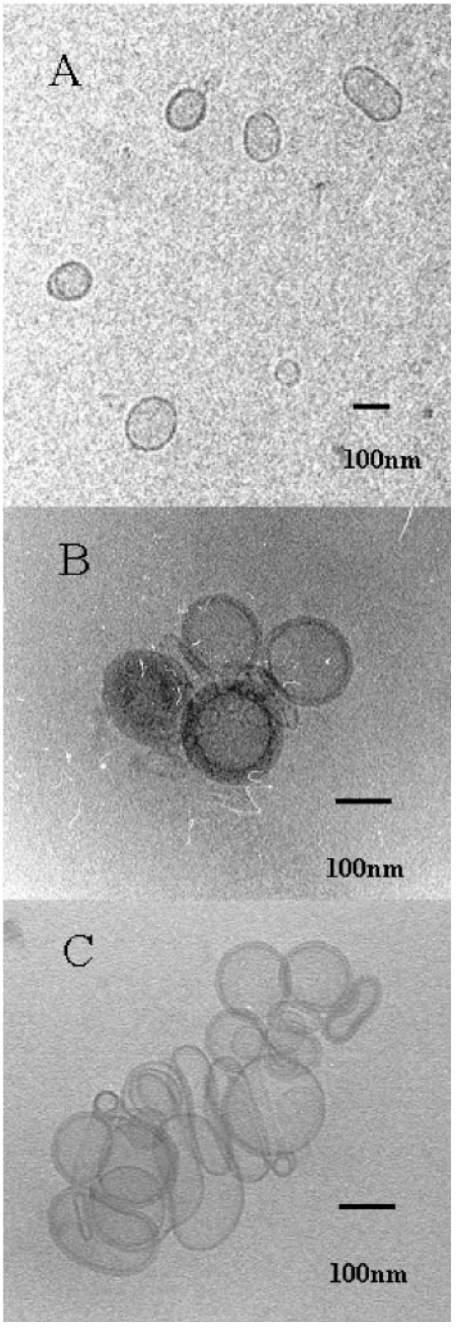

FIGURE 3 PEG does not create high curvature contacts in LUV aggregates. Cryo-EM of DOPC LUVs under different buffer and osmotic conditions: in isotonic buffer (*A*) or in 10 wt% PEG osmotically balanced (*B*) and unbalanced (*C*). *Bars*, 100 nm. DOPC vesicles aggregate well at this PEG concentration but do not fuse. LUVs are not absolutely spherical even in buffer because the extrusion process used to prepare vesicles distorts them from a spherical state.

Osmotic stress significantly affects CM and leakage but not LM

To examine the role of osmotic stress alone without changing membrane curvature, we applied different osmotic gradients to SUVs by adding appropriate concentrations of sucrose inside or outside the vesicles. We define the osmotic gradient as the difference in osmotic pressure inside and outside. To create a positive osmotic gradient, sucrose was added to the lipid sample before vesicle preparation.

It was assumed that small vesicles with diameters of ~26 nm are rigid enough to maintain a spherical shape even at a

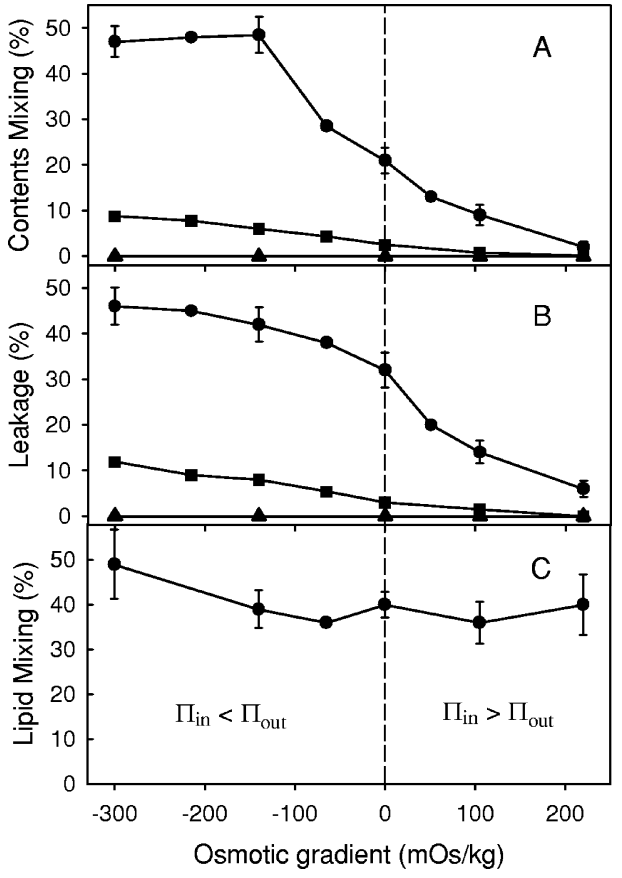

FIGURE 4 Osmotic stress significantly affects CM and leakage but not LM. The effects of osmotic gradient ($\Pi_{in} - \Pi_{out}$) on PEG-induced fusion of DOPC/DOPE/Ch SUVs (●) and LUVs (■). (*A*), CM; (*B*), leakage; and (*C*), LM at 10 wt% PEG. Osmotic gradients were created by adding appropriate amounts of sucrose inside or outside the vesicles. ▲ represent the results of control experiments without PEG.

high negative osmotic gradient. Our assumption is based on our experimental observations of only a slight deformation of LUVs at 10% PEG (Fig. 3 *C*), which corresponds to osmotic gradient of ~200 mOs/kg, and some theoretical considerations (Appendix). We show in the Appendix that vesicle resistance to an externally applied osmotic pressure is approximately proportional to R^{-3}. If we consider that SUVs are approximately six times smaller than LUVs, this gives a factor of ~200 increase in SUV resistance to osmotic deformation. Because the shape of LUVs changes very little under the influence of 10 wt% PEG (Fig. 3 *C*), one may conclude that SUV deformation by up to 10 wt% PEG would be negligible.

As one can see from Fig. 4, a negative osmotic gradient enhanced both CM and leakage in SUV preparations, whereas a positive gradient inhibited them almost completely (●). In control measurements, osmotic gradients in the range used did not induce leakage or CM for SUVs in the absence of PEG (▲). In contrast to the effect of osmotic gradients on CM, there was no significant effect of osmotic gradients on LM (Fig. 4 *C*). We have shown previously that lipid exchange occurs between contacting outer leaflets independent of whether fusion occurs (Burgess et al., 1991; Evans and Lentz, 2001; Wu and Lentz, 1991).

LM occurs at both stages of the fusion process, when outer leaflets merge to form the stalk intermediate and when inner leaflets merge to form the fusion pore (Lee and Lentz, 1997). Thus, inner leaflet mixing associated with substantial SUV fusion at highly negative osmotic gradients explains the slight increase in LM observed under these conditions (Fig. 4 *A*, ●). If additional LM should occur when a pore forms, how is it that a negative osmotic gradient can have such a large effect on pore formation but so small an effect on LM? This could be attributable to transbilayer redistribution of lipids in the early stages of the fusion process. This is not likely, as we have shown that transbilayer lipid redistribution occurs at a rate somewhat slower than pore formation (Evans and Lentz, 2001). The reason for the very small increase in LM with gradient, then, is likely the nature of the LM assay used in these studies. DPHpPC has the DPH group attached via propionic acid as the acyl chain in the 2 position of a phosphatidylcholine molecule. This positions DPH well into the hydrophobic portion of the bilayer and allows some energy transfer between probes from opposite monolayers, meaning that excited-state DPH dimers (Lentz and Burgess, 1989) remaining in unfused inner leaflets can transfer energy to DPH monomers in the fused outer leaflets in the hemifused state. This makes the DPHpPC lipid-mixing assay much less sensitive to inner-leaflet LM during pore formation than to outer-leaflet LM during the initial step of the fusion process, as we have previously reported (Lee and Lentz, 1997). LM during this stage of fusion can be measured with headgroup-labeled *N*-[7-nitrobenz-2oxa-1,3-diazole-4-yl]-dioleoylphosphatidylethanolamine–phosphatidylserine located exclusively in the inner leaflet, an assay that produces exactly the same time course as the CM assay for pore formation (Lee and Lentz, 1997).

For comparison, we summarize in Fig. 4 the effect of an osmotic gradient on LUV fusion (■). Although the effect was not as impressive as for SUVs because of low absolute levels of CM and leakage, in relative terms, CM changes were even greater for LUVs than for SUVs. Thus, osmotic swelling reduced CM to 9% of its original value (in the absence of a gradient) for SUVs and to 6% for LUV. Similarly, compression increased CM by up to 224% for SUVs and by 435% for LUVs. This is actually not surprising, as larger vesicles experience higher membrane lateral compression and membrane stress under the influence of an osmotic gradient (Appendix). Both because LUVs exhibit so little fusion when aggregated by PEG and to avoid these ancillary effects of an osmotic gradient, we focused our efforts to define the effects of an osmotic gradient on SUVs and did not pursue in depth the effects of osmotic gradients on LUVs.

We have examined several possible reasons for these unexpected effects of osmotic gradients. First, membrane tension could provide an additional energy, which plays the role of the driving force for fusion. Because of geometric restrictions, the outer leaflet compresses at some point during the fusion process whereas the inner leaflet expands. Under osmotic stress, the changes in elastic energy of the two leaflets have opposite signs, but these may not completely compensate for one another. The resulting elastic energy change could promote fusion under negative stress and inhibit under positive stress. Unfortunately, because there is no way to predict exactly how these effects might compensate, there is no way to check this hypothesis experimentally. Another reason could be that membrane stress somehow changes the energy barrier for formation of the two intermediate structures demonstrated in PEG-mediated fusion (Lee and Lentz, 1997) (presumably the stalk and septum). For example, a negative osmotic gradient could lower the free energy of hydrophobic interstices associated with fusion intermediates (Siegel, 1993) by pressing outer leaflet lipids into these regions where the presumed occurrence of nonlamellar structures causes hydrophobic mismatch (HMM) between lamellar leaflets (Siegel, 1993). To test for this latter possibility, we examined the influence of hexadecane on the different effects of positive and negative osmotic gradients on fusion.

N-hexadecane reduces the inhibitory effect of a positive osmotic gradient

Hexadecane and other hydrocarbons are proposed to lower the unfavorable free energy of hydrophobic interstices associated with fusion intermediates and thereby to promote fusion of cells and lipid vesicles (Basanez et al., 1998; Chen and Rand, 1998; Walter et al., 1994). It might be that the effect of a positive osmotic stress is to oppose lipid movement into these regions of HMM. If so, this would inhibit the ability of normal lipid fluctuations to reduce the free energy of these regions. In this case, adding hexadecane should reduce or even abolish the inhibition of fusion by a positive osmotic gradient. For lipid vesicles in the presence of 5 mol% hexadecane, we observed an increase in the extent of CM (Fig. 5 *A*, □ and ○) as compared with vesicles prepared without hexadecane (■ and ●) with both 5 wt% and 10 wt% PEG (□ and ○, respectively). This increase varied with osmotic conditions, with the maximum effect observed at the largest positive osmotic gradient. Contents leakage (not shown) also increased in the presence of hexadecane. Similar to CM, hexadecane enhanced contents leakage mainly under conditions of a positive osmotic gradient. Although conditions and membrane compositions can be found for which fusion occurs without leakage (Lentz et al., 1997; Massenburg and Lentz, 1993), it is more common to find that contents leakage increases as fusion increases (Massenburg and Lentz, 1993), as it does in DOPC/DOPE/Ch SUVs. LM was virtually unaffected by the presence of hexadecane.

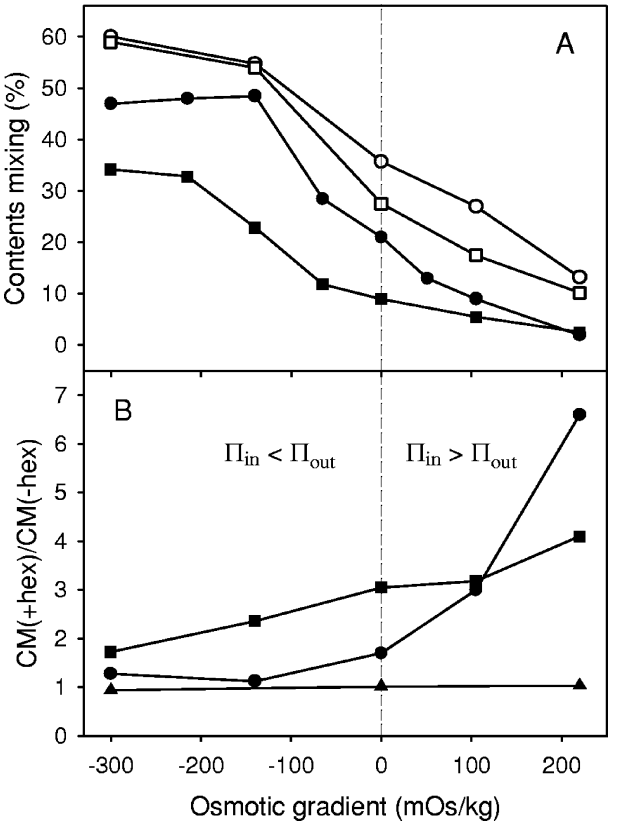

FIGURE 5 N-hexadecane reduces the inhibitory effect of a positive osmotic gradient. (*A*) CM of SUVs with (○ and □) or without (● and ■) hexadecane. Hexadecane at 5 mol% of lipid concentration was added to the lipid stock solution before it was dried for vesicle preparation. Osmotic gradients were created by adding appropriate amounts of sucrose inside or outside the vesicles. PEG concentrations were 5 wt% (□ and ■) or 10 wt% (○ and ●). (B) The fusion-enhancing effect of hexadecane is presented as the ratio of CM data with and without hexadecane as a function of the osmotic gradient for 5 wt% (■) and 10 wt% (●) PEG. Also shown is the ratio of the extents of LM data with and without hexadecane at 10 wt% (▲) PEG.

The interplay between the effects of hexadecane and osmotic stress is summarized in Fig. 5 *B*. In this figure, the influence of hexadecane on the extent of CM (● and ■) is shown as ratios of results in the presence of hexadecane to those in the absence of hexadecane, which are plotted as a function of osmotic gradient. Consistent with the hypothesis that a negative osmotic gradient enhances fusion through lowering the free energy of HMM, hexadecane had less influence on fusion under conditions of a negative gradient but substantially enhanced fusion under conditions of a positive osmotic gradient. The ratio of extents of LM in the presence of hexadecane to LM in its absence is also shown in Fig. 5 *B* (▲). This is seen to be essentially one (0.97 to 1.06), independent of osmotic stress. This shows that hexadecane, like osmotic stress and curvature, does not influence formation of the initial fusion intermediate.

DISCUSSION

Our results support the following conclusions: 1) a negative osmotic gradient enhances fusion, but not through increasing curvature stress; 2) osmotic swelling inhibits fusion through effects on steps in the fusion process after formation of the initial and second intermediates; 3) osmotic compression and hexadecane seem to have complimentary effects on fusion through their effects on HMM; and 4) curvature stress in small vesicles promotes fusion, but, surprisingly, not through the formation of the initial intermediate, rather through its effect on a later step in the process. These conclusions will be discussed in order.

A negative osmotic gradient does not promote fusion through high curvature contacts between compressed or flattened vesicles at high PEG concentration

This work was initiated to evaluate the balance between osmotic stress and curvature effects in PEG-induced fusion of lipid vesicles. Before proceeding, it is worth noting that the effects we report reflect general osmotic stress rather than specific effects of PEG, as we varied the osmotic gradient using sucrose at constant PEG concentration. We proposed previously (Burgess et al., 1992; Lentz, 1994) that osmotic shrinkage of vesicles treated with 25–30 wt% PEG creates highly curved edges that drive fusion of large vesicles at high PEG concentration. From the results presented here, we now know this mechanism can not hold at PEG concentrations at or below 10 wt%. First, LUVs in 10 wt% PEG did not deform noticeably compared with untreated vesicles in isotonic buffer at this PEG concentration (Fig. 3). Second, our data show that a negative osmotic gradient increases fusion of both large and small vesicles regardless of the fact that SUVs are much more rigid and should not undergo significant deformation. Finally, osmotic balancing of LUVs in 10 wt% PEG also did not noticeably change vesicle shape (Fig. 3), but reduced CM by more than a factor of 2 (Fig. 1), making it clear that osmotic stress modulates fusion through something other than changes in curvature.

Osmotic stress affects steps in the vesicle fusion process after formation of the stalk but before the septum intermediate converts to a pore

Osmotically or hydrostatically applied membrane tension, which is always accompanied by a change in monolayer areas, has been used many times as a tool for studying the fusion process. Contradictory results have been reported for the effects of osmotic gradients or hydrostatic pressure on membrane fusion. Two reports, in disagreement with our results, state that a positive osmotic gradient (swelling) promotes fusion (Chanturiya et al., 1997; Cohen et al., 1982), leading to a proposal of an "osmotic mechanism" of exocytosis (Finkelstein et al., 1986). Another set of results reports that a positive hydrostatic pressure (which also creates membrane tension, as does a positive osmotic gradient in our experiments) inhibits fusion (Markosyan et al., 1999; Solsona et al., 1998). A third set of results reports that the effect of osmotic stress depends on the stage of fusion being examined. In this view, a negative osmotic gradient (compression) promotes the initial step of the fusion process, whereas a positive osmotic gradient promotes the late steps of the process (Ahkong and Lucy, 1986; Miller et al., 1976).

The apparent contradiction among these three groups of results can be resolved in terms of the types of fusing model membranes used in different experiments. Thus, all experiments in the first group involved lipid vesicles fusing to a planar bilayer membrane, whereas, in the second group, both fusing membranes enclosed trapped compartments (cells or exocytotic granules). Planar bilayer membranes are the simplest model of biological membranes but can not be considered a complete analog, mainly because they generally contain at least a trace of hydrocarbon solvent, and they have an edge that inevitably contains a reservoir of that solvent. Our results are consistent with results obtained with membranes without a solvent-rich edge (Markosyan et al., 1999; Solsona et al., 1998). Markosyan et al. (1999) proposed that cell inflation inhibits influenza virus HA-induced fusion between cells by preventing the formation of HA-mediated dimples that constitute the point of fusion. Although this result agrees with our observation that a positive osmotic gradient inhibited fusion, the interpretation disagrees with ours. First, in our studies of SUV fusion, we do not need a dimple to initiate fusion because SUVs already have a highly curved membrane. In addition, a positive osmotic gradient did not inhibit LM, so it could not have inhibited the initial step (stalk formation) of PEG-induced fusion of SUVs. Instead, it inhibited CM, which reflects later steps in the process. Thus, osmotic gradients (positive or negative) alter the fusion process through their effects on steps in the process after the formation of the initial intermediate. Finally, inhibition of CM by osmotic swelling suggests that the step that is altered is not the final one (i.e., opening of the fusion pore), because membrane expansion should promote opening and enlargement of the pore in the septum (Chizmadzhev et al., 2000). So, our results locate the osmotically sensitive step to conversion of intermediate 1 (stalk) to intermediate 2 (septum) (Lee and Lentz, 1997).

Another interesting observation was that leakage did not increase, but even decreased, under positive (swelling) osmotic stress. One might expect that leakage would increase as membrane tension was increased because of osmotic swelling pressure. The membrane tension achieved under the maximum osmotic pressure used (220 mOs/kg) is $\sim$3 mN/m for SUV. Such a tension is at least five times less than the critical tension required for membrane rupture (Needham and Nunn, 1990). One can imagine that even a

subcritical pressure could promote formation of very small transient pores. Our results (Fig. 4), as well as experimental data of other authors (Johnson and Buttress, 1973), show that LUVs are not leaky under moderate osmotic stress. It is also interesting that a compressive (negative) osmotic stress increased leakage much as it increased fusion (Fig. 4, *A* and *B*). It seems that leakage and fusion both varied with the applied osmotic gradient in a counter-intuitive fashion and in parallel to one another. We suggest that leakage might reflect the same physical processes that allow a compressive osmotic gradient to promote fusion. However, because leakage often accompanies but is not required for fusion (Massenburg and Lentz, 1993), leakage seems to be a consequence of these processes rather than being necessary for them.

Osmotic compression favors fusion probably by favoring movement of lipids into hydrophobic interstices

The commonly accepted model for membrane fusion has some nonlamellar or highly distorted lamellar structure at the point of contact between fusing membranes. One way to picture this structure is to imagine highly curved lamellar leaflets surrounding interstices in the hydrophobic interior of the structure (Lee and Lentz, 1997). Siegel (1993) first described the importance of hydrophobic interstices to the free energy of fusion intermediates by reference to the stability of inverted hexagonal phases. By assuming that the approximately lamellar portions of these structures have a constant thickness and a finite curvature, we have described the free energy associated with hydrophobic interstices in terms of an unfavorable free energy per unit interstice volume (Malinin and Lentz, in preparation). According to this simple geometric model, the volume of interstice becomes substantial at the intermediate step between stalk and septum, i.e., at the same stage of the fusion process that is osmotically sensitive in our experiments. To stabilize the hydrophobic interstice experimentally, we used N-hexadecane, which is thought to decrease the free energy of hexagonal phases by filling interstitial spaces (Rand et al., 1990), and by this means promote fusion (Basanez et al., 1998; Walter et al., 1994). Hexadecane is quite soluble in a bilayer and does not form separate phases (at least in the concentration range up to 15 wt%). It also does not change the intrinsic curvature and bending modulus of a DOPE monolayer (Chen and Rand, 1998). In lamellar lipid bilayers, hexadecane seems to distribute to the more ordered hydrophobic environment of a monolayer with its long axis primarily parallel to the acyl chains (Walter et al., 1994). However, at concentrations used in our experiments (5 mol% that corresponds to 1.7 wt%), hexadecane most likely does not change significantly the physical properties of a lipid monolayer because it had no effect on LM at any osmotic pressure gradient (Fig. 5 *B*). This observation must also mean that hexadecane affects steps in the fusion process after the initial step when structures other than lipid monolayers are present. Because hexadecane partitions easily to the interstitial region in a hexagonal phase (Chen and Rand, 1998), it is most likely that it acts by partitioning into and lowering the free energy associated with hydrophobic interstices in one or both of the fusion intermediates. If the interstitial energy were affected somehow by osmotic stress, then adding hydrocarbons or other nonpolar lipids to lower the free energy of the interstices should alter the effects of osmotic stress on fusion. Our results confirmed this expectation, with the largest effect of hexadecane occurring at positive osmotic gradients (Fig. 5). The complimentary effects of osmotic stress and hexadecane suggest that osmotic stress affects PEG-induced fusion by changing the energy of interstice formation, the same point in the process affected by hexadecane. If so, how might osmotic stress affect the free energy associated with hydrophobic interstices?

In the absence of hydrocarbons, lipids themselves probably adjust to the shape of the fusion intermediates. It is believed that the energy of hydrophobic interstices is determined mainly by stretching of acyl chains of the lipids in the lamellar regions surrounding the interstices (Kirk et al., 1984). This lowers the free energy of hydrophobic interstices, but at a free energy cost. Filling the added interstice volume without addition of any lipid to the fusing membranes leads to an overall decrease in lipid packing density, which is equivalent to membrane expansion. Membrane expansion at constant bilayer thickness leads to an increase in surface area in the lamellar regions of the fusing vesicles, a process that exposes water to hydrocarbon and is thus quite unfavorable. A compressive osmotic force will oppose this expansion and should favor the movement of hydrocarbon mass into the interstice regions. For example, filling 4 nm^3 of mismatched hydrophobic region (we calculate a volume ranging from 8 to 40 nm^3 at different stages of the fusion process) would require removing this volume from lamellar regions of fusing vesicles and thus reduce membrane area by $\sim$1 nm^2. Under a compressive pressure of 3 mN/m, reducing the membrane area by this amount would be favorable by $\sim$0.7 kT. Thus, osmotic compression makes it energetically favorable to reduce the volume of lamellar regions of fusing vesicles. Adding cyclohexane provides material to fill the interstices without the need for membrane expansion.

Another possible mechanism for filling interstices is translocation of an entire lipid molecule from a monolayer to the interstice. We note that, during the fusion of SUVs, lipids must move from the compressed outer leaflet of vesicles toward the expanding inner leaflet (Lentz et al., 1997). During this transbilayer lipid movement, the mismatch space would be filled, thus lowering the free energy barrier to formation of the septum and fusion pore. Phospholipids could move across the bilayer in this

way, although Ch might be a better candidate for this transbilayer lipid movement. Ch is a fairly hydrophobic molecule, and one might expect that the free energy of Ch translocation from a lamellar region to a hydrophobic interstice would be less than the free energy of stretching phospholipid acyl chains to fill the interstice. Vesicle compression would crowd lamellar regions and favor Ch movement into regions of HMM; conversely, membrane expansion should inhibit it. It might also be that fusion peptide or transmembrane regions of fusion proteins could also help fill hydrophobic interstices during protein-mediated fusion in the cell.

High membrane curvature promotes fusion but through later steps in the process rather than through the formation of the initial intermediate

Our results comparing SUVs to LUVs (Fig. 2) show that membrane curvature did not alter the extent of LM significantly, and thus must not affect significantly formation of the initial intermediate. However, increased membrane curvature did enhance CM, and thus must affect later steps in the fusion process. We might have expected to see the greatest effects of curvature on LM, because the high positive curvature of SUV outer leaflets should destabilize them and favor stalk formation. This result suggests that there is some other aspect of the structure of a highly curved membrane that favors the later stages of the fusion process. Efforts to define better the effects of curvature on this stage of the fusion process are under way in terms of calculations of the free energies of the structures present during the late stages of fusion.

APPENDIX

Let us consider an extreme situation of a nonexpansible membrane (large expansion modulus), when all the osmotic energy transfers to bending energy (membrane area is fixed). Assume also that deviations from spherical shape are small ($\delta R/R \ll 1$). The change in the osmotic energy can be estimated as:

$$\Delta G_{OS} \approx -\Delta\Pi\Delta V \sim -\Delta\Pi\int_A \delta R dA \sim -4\pi\Delta\Pi R^3\frac{\langle R\rangle}{R} \quad (1)$$

where $\Delta\Pi = \Pi_{in} - \Pi_{out}$ is osmotic gradient (negative value), R is vesicle radius, and $\langle\delta R\rangle$ is the average vesicle deformation over the surface area A (also negative, because vesicle volume decreases). The change in the bending energy becomes:

$$\Delta G_b \sim \frac{1}{2}K_b\Delta\int_A\left(\left(\frac{2}{r} - C_0\right)^2 + \left(\frac{2}{r}C_0\right)^2\right)dA$$

$$\sim -\frac{1}{2}K_b\int_A\frac{16}{r^3}\delta r dA \sim -32\pi K_b\frac{\langle\delta r\rangle}{R} \quad (2)$$

where K_b is the bending modulus and C_o is intrinsic curvature of a monolayer, and r is the radius of membrane curvature. For small deformations $r \sim R$, but $\langle\delta r\rangle \neq \langle\delta R\rangle$. Membrane thickness is assumed to be negligible compared with R, so the inner and outer leaflets have the same radius. If vesicles of different size have a similar deformation (i.e., $\langle\delta R\rangle/R$ and $\langle\delta r\rangle/R$ are invariant), then they would have the same change of bending energy, but the change of osmotic energy would be proportional to R^3. In other words, vesicle resistivity to a negative osmotic stress is approximately proportional to R^{-3}. For really small vesicles such as SUVs, our assumption about large expansion modulus of a membrane is no longer valid, but this makes vesicle resistivity even stronger, because part of the osmotic energy converts to the expansion energy. Our estimation is also consistent with more accurate calculations of the stability of a spherical interface under a negative pressure drop (Kozlov and Markin, 1990), which has the same dependence of critical pressure ($\sim R^{-3}$) necessary to loose its spherical shape.

We thank Drs. D. Siegel, F. Cohen, L. Chernomordik, and K. Gawrisch for useful advice and discussions.

This work was supported by USPHS grant GM32707 to B.R.L.

REFERENCES

Ahkong, Q. F., and J. A. Lucy. 1986. Osmotic forces in artificially induced cell fusion. *Biochim. Biophys. Acta.* 858:206–216.

Aldwinckle, T. J., Q. F. Ahkong, A. D. Bangham, D. Fisher, and J. A. Lucy. 1982. Effects of poly(ethylene glycol) on liposomes and erythrocytes. Permeability changes and membrane fusion. *Biochim. Biophys. Acta.* 689:548–560.

Arnold, K., O. Zschoernig, D. Barthel, and W. Herold. 1990. Exclusion of poly(ethylene glycol) from liposome surfaces. *Biochim. Biophys. Acta.* 1022:303–310.

Barenholz, Y., D. Gibbes, B. J. Litman, J. Goll, T. E. Thompson, and R. D. Carlson. 1977. A simple method for the preparation of homogeneous phospholipid vesicles. *Biochemistry.* 16:2806–2810.

Basanez, G., F. M. Goni, and A. Alonso. 1998. Effect of single chain lipids on phospholipase C-promoted vesicle fusion. A test for the stalk hypothesis of membrane fusion. *Biochemistry.* 37:3901–3908.

Boni, L. T., T. P. Stewart, J. L. Alderfer, and S. W. Hui. 1981. Lipid-polyethylene glycol interactions: II. Formation of defects in bilayers. *J. Membr. Biol.* 62:71–77.

Burgess, S. W., and B. R. Lentz. 1993. Fluorescence lifetime measurements to monitor membrane lipid mixing. *Methods Enzymol.* 220:42–50.

Burgess, S. W., D. Massenburg, J. Yates, and B. R. Lentz. 1991. Poly(ethylene glycol)-induced lipid mixing but not fusion between synthetic phosphatidylcholine large unilamellar vesicles. *Biochemistry.* 30: 4193–4200.

Burgess, S. W., T. J. McIntosh, and B. R. Lentz. 1992. Modulation of poly(ethylene glycol)-induced fusion by membrane hydration: importance of interbilayer separation. *Biochemistry.* 31:2653–2661.

Chanturiya, A., L. V. Chernomordik, and J. Zimmerberg. 1997. Flickering fusion pores comparable with initial exocytotic pores occur in protein-free phospholipid bilayers. *Proc. Natl. Acad. Sci. U.S.A.* 94: 14423–14428.

Chen, P. J., Jr., T. Y. Torribara, and H. Warner. 1956. Microdetermination of phosphorus. *Anal. Chem.* 28:1756–1758.

Chen, Z., and R. P. Rand. 1998. Comparative study of the effects of several n-alkanes on phospholipid hexagonal phases. *Biophys. J.* 74:944–952.

Chernomordik, L. V., V. A. Frolov, E. Leikina, P. Bronk, and J. Zimmerberg. 1998. The pathway of membrane fusion catalyzed by influenza hemagglutinin: restriction of lipids, hemifusion, and lipidic fusion pore formation. *J. Cell. Biol.* 140:1369–1382.

Chizmadzhev, Y. A., P. I. Kuzmin, D. A. Kumenko, J. Zimmerberg, and F. S. Cohen. 2000. Dynamics of fusion pores connecting membranes of different tensions. *Biophys. J.* 78:2241–2256.

Cohen, F. S., M. H. Akabas, and A. Finkelstein. 1982. Osmotic swelling of phospholipid vesicles causes them to fuse with a planar phospholipid bilayer membrane. *Science.* 217:458–460.

Evans, E., and D. Needham. 1988. Attraction between lipid bilayer membranes in concentrated solutions of nonabsorbing polymers: comparison of membrane-field theory. *Macromolecules.* 21:1822–1831.

Evans, K. O., and B. R. Lentz. 2001. Kinetics of lipid rearrangements during poly(ethylene glycol)-mediated fusion of highly curved unilamellar vesicles. *Biochemistry.* 41:1241–1249.

Finkelstein, A., J. Zimmerberg, and F. S. Cohen. 1986. Osmotic swelling of vesicles: its role in the fusion of vesicles with planar phospholipid bilayer membranes and its possible role in exocytosis. *Ann. Rev. Physiol.* 48:163–174.

Frederik, P. M., M. C. Stuart, P. H. Bomans, W. M. Busing, K. N. Burger, and A. J. Verkleij. 1991. Perspective and limitations of cryo-electron microscopy. From model systems to biological specimens. *J. Microsc.* 161:253–262.

Jin, A. J., D. Huster, K. Gawrisch, and R. Nossal. 1999. Light scattering characterization of extruded lipid vesicles. *Eur. Biophys. J.* 28:187–199.

Johnson, S. M., and N. Buttress. 1973. The osmotic insensitivity of sonicated liposomes and the density of phospholipid-cholesterol mixtures. *Biochim. Biophys. Acta.* 307:20–26.

Kirk, G. L., S. M. Gruner, and D. L. Stein. 1984. A thermodynamic model of the lamellar to inverse hexagonal phase-transition of lipid-membrane water-systems. *Biochemistry.* 23:1093–1102.

Kozlov, M. M., S. L. Leikin, L. V. Chernomordik, V. S. Markin, and Y. A. Chizmadzhev. 1989. Stalk mechanism of vesicle fusion. Intermixing of aqueous contents. *Eur. Biophys. J.* 17:121–129.

Kozlov, M. M., and V. S. Markin. 1990. Mechanics of interfaces: the stability of a spherical shape. *J. Phys. France.* 51:559–574.

Kuhl, T., Y. Q. Guo, J. L. Alderfer, A. D. Berman, D. Leckband, J. Israelachvili, and S. W. Hui. 1996. Direct measurement of polyethylene glycol induced depletion attraction between lipid bilayers. *Langmuir.* 12:3003–3014.

Lee, J., and B. R. Lentz. 1997. Evolution of lipidic structures during model membrane fusion and the relation of this process to cell membrane fusion. *Biochemistry.* 36:6251–6259.

Lentz, B. R. 1994. Polymer-induced membrane fusion: potential mechanism and relation to cell fusion events. *Chem. Phys. Lipids.* 73:91–106.

Lentz, B. R., and S. W. Burgess. 1989. A dimerization model for the concentration dependent photophysical properties of diphenylhexatriene and its phospholipid derivatives. DPHpPC and DPHpPA. *Biophys. J.* 56:723–733.

Lentz, B. R., T. J. Carpenter, and D. R. Alford. 1987. Spontaneous fusion of phosphatidylcholine small unilamellar vesicles in the fluid phase. *Biochemistry.* 26:5389–5397.

Lentz, B. R., and J. K. Lee. 1999. Poly(ethylene glycol) (PEG)-mediated fusion between pure lipid bilayers: a mechanism in common with viral fusion and secretory vesicle release? *Mol. Membr. Biol.* 16:279–296.

Lentz, B. R., G. F. McIntyre, D. J. Parks, J. C. Yates, and D. Massenburg. 1992. Bilayer curvature and certain amphipaths promote poly(ethylene glycol)-induced fusion of dipalmitoylphosphatidylcholine unilamellar vesicles. *Biochemistry.* 31:2643–2653.

Lentz, B. R., W. Talbot, J. Lee, and L. X. Zheng. 1997. Transbilayer lipid redistribution accompanies poly(ethylene glycol) treatment of model membranes but is not induced by fusion. *Biochemistry.* 36:2076–2083.

Markosyan, R. M., G. B. Melikyan, and F. S. Cohen. 1999. Tension of membranes expressing the hemagglutinin of influenza virus inhibits fusion. *Biophys. J.* 77:943–952.

Massenburg, D., and B. R. Lentz. 1993. Poly(ethylene glycol)-induced fusion and rupture of dipalmitoylphosphatidylcholine large, unilamellar extruded vesicles. *Biochemistry.* 32:9172–9180.

Mayer, L. D., M. J. Hope, and P. R. Cullis. 1986. Vesicles of variable sizes produced by a rapid extrusion procedure. *Biochim. Biophys. Acta.* 858: 161–168.

Michel, B. E. 1983. Evaluation of the water potential of solutions of polyethylene glycol 8000 both in the absence and presence of other solutes. *Plant Physiol.* 72:66–70.

Miller, C., P. Arvan, J. N. Telford, and E. Racker. 1976. Ca^{2+}-induced fusion of proteoliposomes: dependence on transmembrane osmotic gradient. *J. Membr. Biol.* 30:271–282.

Needham, D., and R. S. Nunn. 1990. Elastic deformation and failure of lipid bilayer membranes containing cholesterol. *Biophys. J.* 58: 997–1009.

Nir, S., J. Wilschut, and J. Bentz. 1982. The rate of fusion of phospholipid vesicles and the role of bilayer curvature. *Biochim. Biophys. Acta.* 688:275–278.

Ohki, S. 1984. Effects of divalent cations, temperature, osmotic pressure gradient, and vesicle curvature on phosphatidylserine vesicle fusion. *J. Membr. Biol.* 77:265–275.

Rand, R. P., N. L. Fuller, S. M. Gruner, and V. A. Parsegian. 1990. Membrane curvature, lipid segregation, and structural transitions for phospholipids under dual-solvent stress. *Biochemistry.* 29:76–87.

Schwenk, E., and N. T. Werthessen. 1952. Studies on the biosynthesis of cholesterol. III. Purification of C14-cholesterol from perfusion of livers and other organs. *Arch. Biochem. Biophys.* 40:334–341.

Siegel, D. P. 1993. Energetics of intermediates in membrane fusion: comparison of stalk and inverted micellar intermediate mechanisms. *Biophys. J.* 65:2124–2140.

Solsona, C., B. Innocenti, and J. M. Fernandez. 1998. Regulation of exocytotic fusion by cell inflation. *Biophys. J.* 74:1061–1073.

Talbot, W. A., L. X. Zheng, and B. R. Lentz. 1997. Acyl chain unsaturation and vesicle curvature alter outer leaflet packing and promote poly(ethylene glycol)-mediated membrane fusion. *Biochemistry.* 36:5827–5836.

Tilcock, C. P., and D. Fisher. 1979. Interaction of phospholipid membranes with poly(ethylene glycol)s. *Biochim. Biophys. Acta.* 557:53–61.

Walter, A., P. L. Yeagle, and D. P. Siegel. 1994. Diacylglycerol and hexadecane increase divalent cation-induced lipid mixing rates between phosphatidylserine large unilamellar vesicles. *Biophys. J.* 66:366–376.

Wilschut, J., N. Duzgunes, R. Fraley, and D. Papahadjopoulos. 1980. Studies on the mechanism of membrane fusion: kinetics of calcium ion induced fusion of phosphatidylserine vesicles followed by a new assay for mixing of aqueous vesicle contents. *Biochemistry.* 19:6011–6021.

Wu, J. R., and B. R. Lentz. 1991. Mechanism of poly(ethylene glycol)-induced lipid transfer between phosphatidylcholine large unilamellar vesicles: a fluorescent probe study. *Biochemistry.* 30:6780–6787.

Yamazaki, M., S. Ohnishi, and T. Ito. 1989. Osmoelastic coupling in biological structures: decrease in membrane fluidity and osmophobic association of phospholipid vesicles in response to osmotic stress. *Biochemistry.* 28:3710–3715.