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Shape Change and Physical Properties of Giant Phospholipid Vesicles Prepared in the Presence of an AC Electric Field

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ABSTRACT Giant unilamellar vesicles with diameters ranging from 10 to 60 μm were obtained by the swelling of phospholipid bilayers in water in the presence of an AC electric field. This technique leads to a homogeneous population of perfectly spherical and unilamellar vesicles, as revealed by phase-contrast optical microscopy and freeze-fracture electron microscopy. Freshly prepared vesicles had a high surface tension with no visible surface undulations. Undulations started spontaneously after several hours of incubation or were triggered by the application of a small osmotic pressure. Partially deflated giant vesicles could undergo further shape change if asymmetrical bilayers were formed by adding lyso compounds to the external leaflet or by imposing a transmembrane pH gradient that selectively accumulates on one leaflet phosphatidylglycerol. Fluorescence photobleaching with 7-nitrobenz-2-oxa-1,3-diazol-4-yl–labeled phospholipids or labeled dextran trapped within the vesicles enabled the measurement of the membrane continuity in the dumbbell-shaped vesicles. In all instances phospholipids diffused from one lobe to the other, but soluble dextran sometimes was unable to traverse the neck. This suggests that the diameter of the connecting neck may be variable.

INTRODUCTION

Liposomes are often used as models of biological cells. For that purpose, it is important to have a homogeneous population of unilamellar vesicles with a well-defined interior and a size comparable to the size of cells. Sonicated vesicles are unilamellar; however, they have a diameter limited to about 40 nm, whereas large unilamellar vesicles (LUVs) obtained by extrusion of multilamellar vesicles through filters have a maximum size of about 200 nm. Other techniques such as the mere swelling of lipids in buffer allow one to obtain larger vesicles but in a nonsystematic manner and with a low yield (Lasic, 1988; Winterhalter and Lasic, 1993). Recently Angelova and collaborators (Angelova and Dimitrov, 1986; Angelova et al., 1992) have shown that a homogeneous population of giant unilamellar vesicles (GUVs), with diameters in the range 30 to 60 μm, can be obtained by swelling in the presence of an AC electric field of lipids deposited on a glass plate. The vesicles are perfectly spherical and their unilamellar nature was demonstrated by measurement of the elastic modulus via a statistical analysis of the thermal undulations (Faucon et al., 1989; Méléard et al., 1990). The advantage of the AC electric field technique is the reproducibility of the generation of giant vesicles. In addition, the vesicles can be obtained with different types of phospholipids in the presence or absence of cholesterol. On the other hand, the spherical shape constitutes a disadvantage in the investigation of shape changes because spheres cannot undergo shape changes at constant volume. This can be overcome by deflating the vesicles with the help of a small osmotic pressure before using them for such studies.

The objective of the present article is to characterize GUVs obtained in the presence of AC electric field and to demonstrate that they can be used to investigate experimentally shape deformations of liposomes.

MATERIALS AND METHODS

Chemicals

Egg phosphatidylcholine (EPC) was prepared according to the method of Singleton et al. (1965). Egg phosphatidylglycerol (EPG), brain phosphatidylserine, and cholesterol were purchased from Sigma. The fluorescent phospholipids 1-acyl-2-[12-(NBD-amino)stearoyl]-sn-glycerol-3-phosphocholine (NBD-PC) and the corresponding 2-lyso derivatives NBD-LPC were synthesized in the laboratory (Colleau et al., 1991). Rhodamine-LPE (*N*-(lissamine rhodamine B sulfonyl)-*O*-(1-oleoyl-*sn*-glycero-3-phospho)-ethanolamine) was obtained by hydrolysis of rhodamine PE (*Avanti Polar Lipids*).

Formation of giant vesicles

The chamber for vesicle preparation consisted of a microscope slide and a cover slide, each coated with a thin transparent film of indium tin oxide (ITO), which made them electrically conductive. The voltage was applied through thin wires sealed on each glass plate. Phospholipids (0.5 mg) were first dissolved in 1 ml of chloroform-methanol (9/1). The following mixtures were used: pure EPC, EPC/EPG (99/1, mol ratios); EPC/EPG (90/10); EPC/EPG/cholesterol (76/1/23); EPC/EPG/NBD-PC (94/1/5); EPC/PS (85/15). For each sample 2.5 μg lipids in organic solvent was deposited on the microscope cover slide on the conductive face. The cover slide was then put under vacuum for 2 h to eliminate the solvent. It is noteworthy that for the success of liposome generation with an AC field, the film formed by evaporation had to be as uniform as possible. A teflon spacer of 0.5 mm was placed on the cover slide, and a microscope slide was put on top, with the conductive face

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oriented toward the cover slide. The system was held together with small clips and sealed with a sigillum paste. An aqueous solution was injected between the slide and cover slide with a syringe through a hole in the teflon spacer. The aqueous solution contained in general 50 mM sucrose and 0.02% azide (w/v). In some cases, fluorescent dextran (0.1 mM) was included in the sucrose solution. Two types of dextran were used: fluoresceine dextran (fluorescein isothiocyanate-dextran (FITC-dextran) from Sigma) and rhodamine dextran (rhodamine B isothiocyanate-dextran from Sigma). Vesicles were also formed in a glucose solution (50 mM) containing dextran (molecular weight 73,000) at a concentration of 15 mg/ml and 0.02% azide.

The chamber was placed under an inverted light microscope (Zeiss IM35), which allowed the monitoring of the vesicle formation. A power supply generated a low-frequency voltage (10 Hz) that was progressively increased from 0 to 1.2 V in 40 min. Giant vesicles were obtained in 3 to 5 h. The vesicles had a maximum diameter of 10 to 60 μm. They were detached from the cover slide by raising the voltage to 1.4 V while simultaneously decreasing the frequency to 4 Hz. The vesicles were then recovered by suction with a syringe and transferred to a medium containing 50 mM glucose. The difference in density between the sucrose inside the vesicles and the glucose outside facilitated their sedimentation and provided a better contrast in the light microscope.

Characterization of giant vesicles

Light microscopy

To determine the size distribution of a population of giant vesicles, several pictures of the same ensemble of sedimented liposomes were taken, focusing the microscope at different different levels. For each liposome the larger diameter measured was assumed to correspond to its true diameter. The estimated error was $\pm 2.5\%$.

Electron microscopy

The most appropriate methods for studying liposomes in solution by electron microscopy are based on cryofixation followed by direct observation of very thin frozen-hydrated specimens or by observation of the replicas of freeze-fracture preparations. Because of the very large dimensions of giant vesicles, the latter method is more appropriate, provided the sample is cryoprotected, to avoid morphological perturbations induced by the formation of ice crystals.

A small droplet of solution of giant vesicles containing dextran and glycerol (30% v/v) was deposited on a thin copper holder and then rapidly quenched in liquid propane. The frozen sample was fractured at -125°C, in a vacuum better than 10^{-6} Torr, with a liquid nitrogen-cooled knife in a Balzers 301 freeze etching unit. The replication was done using unidirectional shadowing, at an angle of 35°, with platinium carbon. The replicas were washed with organic solvents and distilled water, and were observed in a Philips 301 electron microscope.

Shape changes

The vesicles were visualized with the inverted Zeiss microscope, using Nomarski differential interference contrast or fluorescence detection modes; the objective lens was 63× or 100×. A Leitz phase-contrast microscope (Labovert) was also used. Both microscopes were equipped with a video camera, which allowed the change in vesicle shape to be recorded as a function of time.

The giant spherical vesicles were deflated by osmotic pressure. If the vesicles were tranferred directly with a micropipette to a medium of higher osmolarity, they usally collapsed. Therefore, a special chamber that allowed a very gradual increase of the osmotic pressure without turbulance was designed as follows. The chamber containing the vesicles in a medium of low osmolarity was made of a silicon frame (internal diameter 10 mm, thickness 2 mm) deposited on a cover slide. The top of the silicon frame was covered with a membrane filter (Cyclopore; Poly Labo, Strasbourg, France) with 0.2-μm holes and glued to a 4-mm microscope glass slide. The glass slide had a hole forming a well in the center in contact with the filter, through which the medium with higher osmolarity diffused.

Once vesicles were partially deflated, further shape transformation could be triggered by adding lysolipids injected through a 10-μm-diameter micropipette near a selected vesicle or by mixing lysolipids with the vesicle suspension. Shape change of discoid vesicles containing phosphatidylcholine and phosphatidyglycerol was triggered by the transmembrane reorientation of phosphatidylglycerol induced by a pH gradient as described by Hope et al. (1989a) and Redelmeier et al. (1990). This was achieved by transferring the vesicles with the help of a 30- to 60-μm-diameter micropipette and a micromanipulator system to a medium at pH 9. In a few experiments, the pH of the medium was modified directly without the transfer of vesicles.

Photobleaching experiments

A fraction of a selected vesicle was bleached with a brief and intense illumination from an argon laser beam (Coherent Innova 90-5) directed toward an area selected with a diaphragm. The recovery of the fluorescence was analyzed qualitatively.

RESULTS

Formation of giant vesicles

Fig. 1 shows the progressive increase in size of phosphatidylcholine vesicles that takes place during the application of an ac field. The small vesicles that are formed initially fuse to reach a maximum diameter of about 30 to 60 μm. If the solution is incubated for more than 5 h, the vesicles do not grow further. They may, in fact, collapse. Not all vesicles reach the maximum size. When the domain of exploration of the microscope is varied by changing the depth of field, smaller vesicles become apparent (see Fig. 1).

The vesicles shown in Fig. 1 appear to be in close contact but seem independent. Yet the following experiment suggests that they may be connected. In some experiments a small percentage of long-chain fluorescent phospholipids, NBD-PC, were mixed with the unlabeled phospholipids, rendering the vesicles fluorescent (Fig. 2 *A*). Bleaching a fraction of the vesicles with a laser beam extinguished a selected population of vesicles (Fig. 2, *B* and *C*). Although the long-chain phospholipids are unable to jump from one vesicle to another through the aquous phase, the fluorescence was recovered within 2 min in all vesicles (Fig. 2 *D*), suggesting that lipid can diffuse from vesicles to vesicles. The type of connection between the vesicles is not known. For example, they may be connected by small tethers, but the close packing does not allow the vizualization of such features. When the vesicles were sucked into a syringe and transferred to a different medium, the connections between the vesicles were probably ruptured mechanically, leaving independent vesicles. However, the stability of the optical contrast proved that the vesicles remained sealed after transfer. The same photobleaching experiment was performed

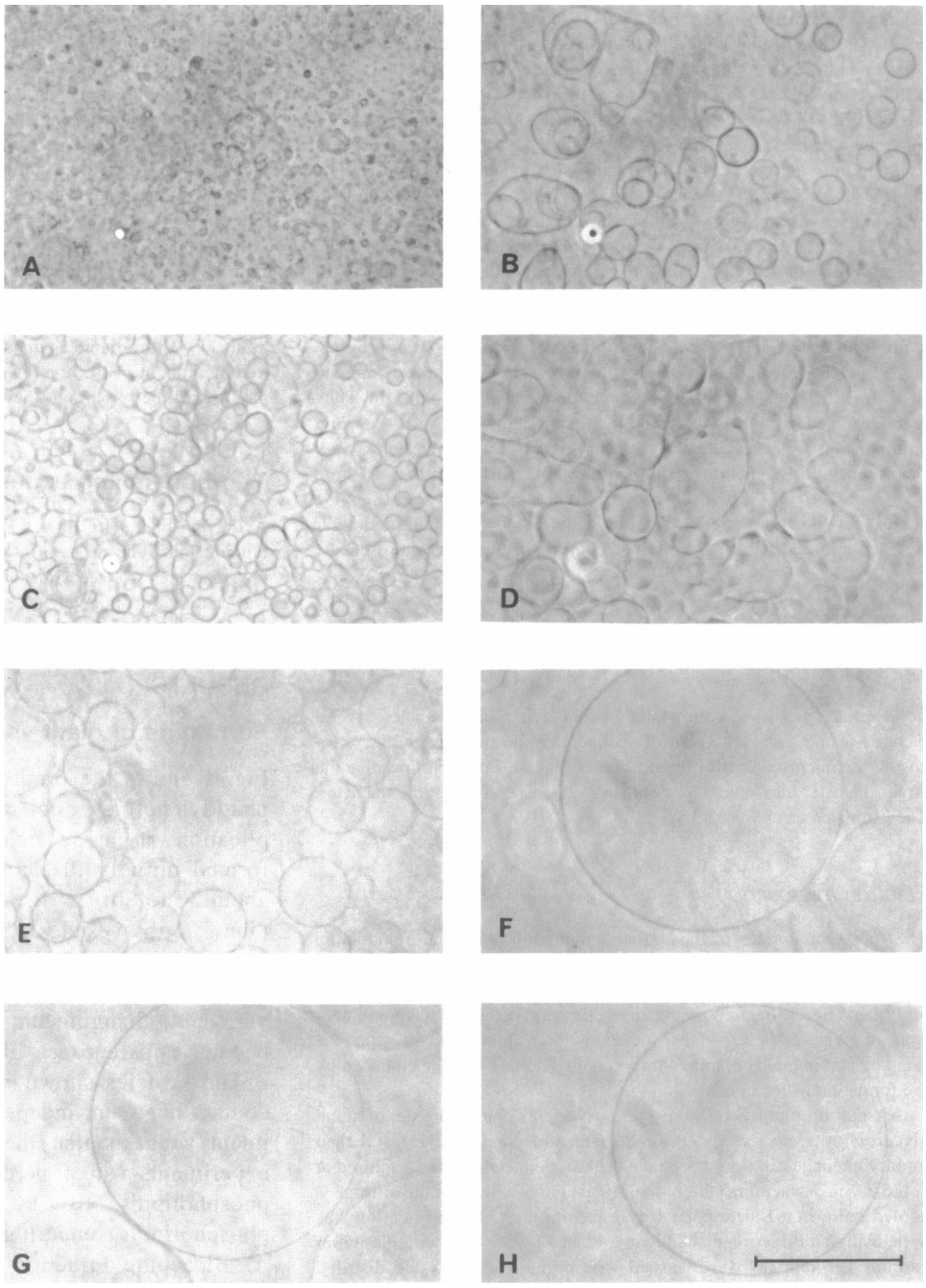

FIGURE 1 Swelling of EPC liposomes in 50 mM sucrose with an AC electic field. Vesicles are observed with an optical microscope in Nomarski configuration. (*A*) Lipid film before application of the electric field. (*B*) 20 min after application of 10 Hz AC electric field, which was gradully increased from 0 to 0.5 V. (*C*) 30 min after application of the field, which has reached 0.9 V. (*D*) Same area as in *C*, but the image is focused to an upper region of the suspension. (*E*) $t = 3$ h, voltage, 1.1 V. (*F*) same area as in *E* but with a different focus. (*G* and *H*) Two different views after 4-h incubation; the frequency is raised to 4 Hz and induces vesicle detachment from the supporting glass. The bar corresponds to 20 μm.

on a cluster of such transferred vesicles containing long-chain fluorescent phospholipids, but no recovery of fluorescence was observed (not shown).

An evaluation of the size distribution of giant vesicles was made. Fig. 3 shows a histogram obtained by analyzing a population of vesicles made with EPC/LPC (99/1). The average size was slightly larger with pure EPC.

Freeze-fracture electron microscopy was carried out on a population of vesicles containing dextran and glycerol (30%) and sedimented in glycerol (30%). Careful observation of a large number of freeze-fractured samples indicated that more than 90% of giant vesicles were cross-fractured, allowing the visualization of their interior, as illustrated in Fig. 4. All cross-fractured giant vesicles were unilamellar. Most of them contained a few small vesicles and only very few contained one smaller giant vesicle.

The same type of giant spherical vesicles were obtained under very similar conditions and with approximately the same efficiency with mixtures of EPC and EPG, or EPC mixed with 15% phosphatidylserine, or EPC mixed with 23% cholesterol. Phospholipids containing a 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) group on one acyl chain or a fluorescent cholesterol analog could

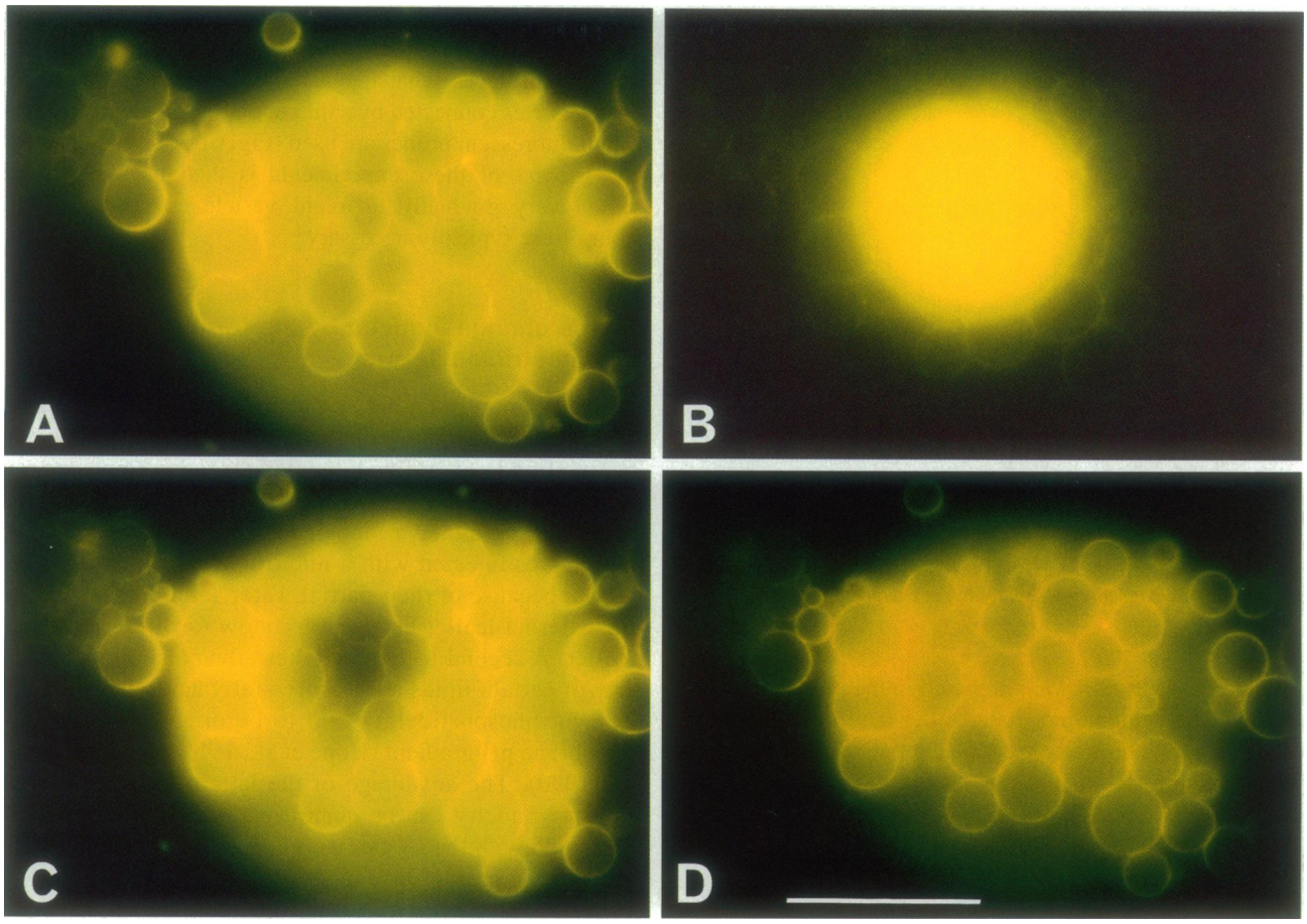

FIGURE 2 EPC liposomes containing 5% of fluorescent phospholipids (NBD-PC). (*A*) End of the swelling period. (*B*) A selected area corresponding to a circular domain of 30 μm diameter is illuminated with a laser beam for 1 s. (*C*) Immediately after illumination, a bleached domain is visible. (*D*) Two minutes after the bleach, the fluorescence appears to be homogeneously distributed, indicating a diffusion of the fluorescent phospholipids from vesicles to vesicles. The bar corresponds to 30 μm.

be introduced without decreasing the yield. Saturated phospholipids (dimyristoyl phosphatidylcholine, DMPC) gave similar results.

Increasing the buffer concentration decreased the efficiency of vesicle formation. In practice, 10 mM Bis-Tris or 2-[morpholino]ethanesulfonic acid was found to be the maximum concentration of buffer tolerable. Above that concentration, very few vesicles are formed.

Thermal undulations and vesicle aggregation

Freshly prepared vesicles, transferred to a glucose medium, were perfectly spherical with no visible thermal undulations. The application of a small osmotic pressure appeared to be sufficient to trigger large-scale surface undulations. Undulations in vesicles containing 50 mM sucrose were typically triggered by an external concentration of 70 mM glucose without complete modification of the overall vesicle shape. The progressive evaporation of water in unsealed samples achieved a similar result. Thermal undulations became spontaneously visible after several hours (20 h) of incubation at 40°C or several days (1 week) at 4°C. It is very likely that during that period some lipid degradation took place. Long incubations were carried out with DMPC vesicles, i.e., with saturated lipids. Undulations were produced similarly, indicating that the lipid degradation was not due to oxidation of the acyl chains but more likely the formation of lyso-lipids. We have attempted to analyze by thin-layer chromatography the lipids extracted from a large population of vesicles incubated under similar conditions. Lyso compounds could barely be detected. They corresponded to a maximum of 1% of the total lipids present.

If after this long period of incubation, fatty acid free bovine serum albumin (BSA) was added to the medium containing the spherical vesicles, the giant vesicles were observed to aggregate and form domains, sometimes with a honeycomb morphology. See Fig. 5 *A–D*. With fresh vesicles, on the other hand, addition of BSA did not trigger aggregation (not shown).

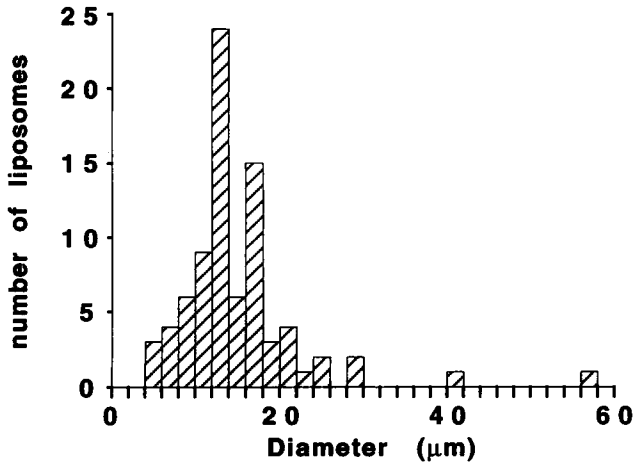

FIGURE 3 Size distribution of a population of giant liposomes obtained in one experiment by swelling in an AC electric field of a lipid mixture containing EPC/LPC (95/5). Liposomes with a diameter below 1 μm are not counted. Pure EPC gives a slightly larger average diameter.

When giant vesicles were prepared in the presence of 1% LPC and BSA added immediately after the formation of the vesicles, aggregation also took place, but without the honeycomb pattern.

Shape changes induced by osmotic pressure

The shape of a spherical vesicule cannot be modified without a change in the volume-to-area ratio. This can be achieved by imposing an osmotic pressure, which pumps out water. Spherical vesicles are transformed into flaccid vesicles with discoid (or obloid) shapes and then into series of apparently connected spheres of smaller size. Note that, in some instances, what appears to be separate vesicles is, in fact, vesicles connected by a thin tether that is clearly visible when fluorescent probes are used (Fig. 6). A prerequisite for the success of these experiments is that the pressure be raised very gradually to avoid vesicle collapse. Several hours were typically necessary.

Shape change induced by the formation of asymmetrical bilayers

As shown in a previous publication (Farge and Devaux, 1992), the shape of a giant discoid or obloid vesicle can be modified, at a fixed temperature, by imposing an asymmetrical lipid composition. The simplest technique of generating a bilayer unbalance consists of adding lyso derivatives injected with a micropipette. The lyso derivatives, because of their partial water solubility, penetrate the external leaflet but are very slow to flip to the inner leaflet. A second technique takes advantage of the property of certain lipids such as free fatty acid, phosphatidic acid, or phosphatidylglycerol to be oriented by a transmembrane pH gradient (Hope et al., 1989a; Redelmeier et al., 1990). The advantage of the latter technique is that lipid asymmetry can be quantified, which is more difficult to do with the former technique. Both techniques are used in the present investigation.

In all instances, excess lipid on the external side was accompanied by shape deformation, as illustrated in Figs. 7–9. Obloid giant vesicles are transformed into dummbell-shaped (or 8-shaped) vesicles made of two closely associ-

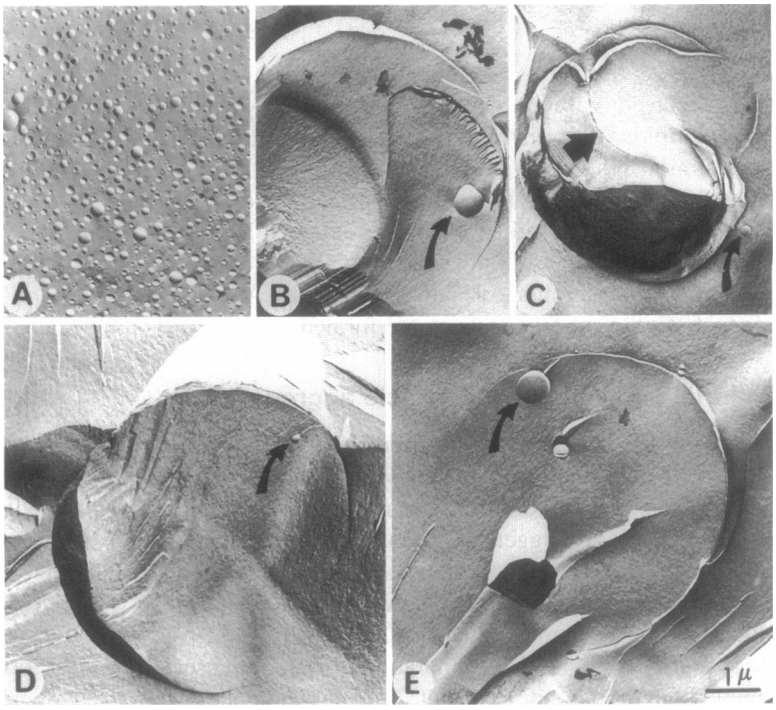

FIGURE 4 Freeze fracture electron micrographs of large unilamellar vesicles (LUVs) (*A*) and of giant unilamellar vesicles (GUVs) (*B*, *C*, and *D*). The curved arrows in *B*, *C*, *D*, and *E* point to vesicles of the size of LUVs, and the large arrow in *C* points to a GUV enclosed in a larger one. The magnification indicated in *E* is the same for all micrographs.

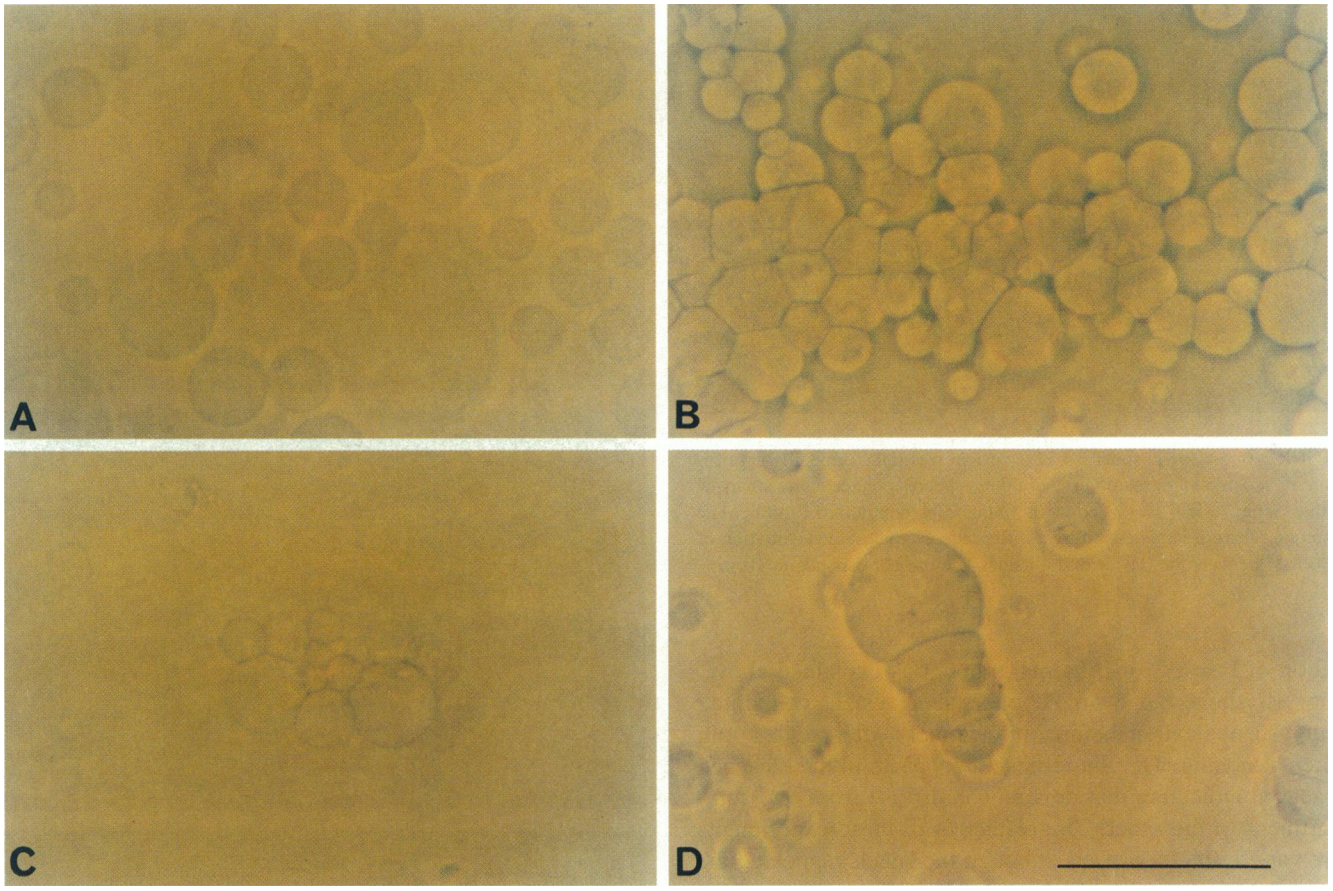

FIGURE 5 Vesicle aggregation. (*A*) Control showing a population of EPC vesicles after swelling, transfer with a syringe, and storage (30 days at 4°C). (*B*) Stored vesicles 2 mn after adition of 1% BSA. (*C*) Freshly prepared EPC vesicles containing initially 1% LPC and incubated with BSA. (*D*) Another example of aggregation triggered by the addition of BSA to DMPC vesicles stored 7 days. The bar corresponds to 30 μm.

ated spheres or into vesicles made of a series of spheres (Figs. 7 and 8). Fig. 9 should be considered a curiosity that is rarely found. In this particular case, the giant vesicle seemed to possess initially a very unusual topology. The transfer of 1% EPG from the inner leaflet to the outer leaflet resulted in the shape transformation indicated in Fig. 8. If 10% EPG was used instead of 1%, the shape transformation was very rapid and the intermediate shapes shown in Fig. 8, *B* and *C*, were not visible. In most cases the final shapes were series of spheres.

The use of fluorescent probes enabled us to determine whether the spheres were still connected or if fission (i.e., is physical separation) had taken place. As in the case of the shape transformation triggered by an osmotic pressure, we found that vesicles that may appear as separated entities by phase contrast microscopy were in fact sometimes linked by a lipid tether whose physical structure is still unclear.

The same type of shape transformation was achieved with vesicles containing EPC and cholesterol (23% mol/mol). No significant difference in the time scale of transformation or in the propensity of the vesicles to separate was observed (not shown).

Shape transformation was also obtained by the addition of fluorescent lyso-phospholipids to liposomes containing unlabeled EPC. A lyso-PC with a NBD moiety on the acyl chain was first used (NBD-LPC); a second analog had a fluorescent rhodamine probe on the headgroup of lyso-PE. The objective of these experiments was to see if the cone-shaped molecules formed by the lyso-compounds had any tendency to accumulate in the regions of high curvature, namely at the interface between the two vesicles formed. However, we were not able to detect any lipid segregation with these probes.

Photobleaching experiments

Optical microscopy does not allow one to give detailed information on the structure of the neck between two spherical vesicles that are still connected. To determine whether the bilayer was continuous, we performed photobleaching experiments wherein one part of the vesicle was bleached by a short pulse from a laser beam while the rest was protected by a diaphragm. For these experiments, giant spherical vesicles containing either long-chain fluorescent phospholipids or soluble FITC-dextran (molecular weight 73,000) were made by the AC field technique. Figure 8-shaped vesicles (or dumbbells) were induced by the ad-

FIGURE 6 A single spherical EPC vesicle was transformed by application of an osmotic pressure into these three spheres connected by a narrow tether. The vesicle is loaded with fluorescent dextran (0.1 mM). The osmotic pressure is generated by a difference in glucose concentration of 100 mM between the inside and outside. The bar corresponds to 10 μm.

dition of lyso-PC or by a pH gradient as explained above. Fig. 10 shows an example of such a vesicle containing fluorescent dextran before, immediately after, and 4 min after bleaching. The fluorescence reappears in the bleached fraction, indicating that dextran can diffuse from one compartment to the other. The results from these experiments are varied. In other cases, although the "neck" appeared the same when viewed with the optical microscope, the fluorescence intensity could not be recovered in the bleached fraction. A possible explanation is that the "hole" between the two spheres derived from a single giant vesicle may have a different size. In contrast, when such experiments were performed with long-chain fluorescent phospholipids, the bleached compartment always regained intensity, indicating free diffusion of the phospholipids between the two parts.

The order of magnitude of time necessary to homogenize the probes (1 mn) was consistent with what can be estimated from the lateral diffusion coefficient of phospholipids. Note that the long-chain phospholipids have no solubility in water and cannot hop from one vesicle to the other.

DISCUSSION

GUVs, with a diameter of 10 μm or more, have the size of biological cells and constitute useful model systems. They present the advantage of being visible with an optical microscope. Their shape and shape modifications can be easily followed, in contrast to small unilamellar vesicles (SUVs) and LUVs, which can only be observed by electron microscopy. GUVs can be obtained by the careful swelling of lipids in pure water in the absence of agitation. However, this technique has a low yield and gives rise to a mixture of vesicles, only a small fraction of which are unilamellar; furthermore, the efficiency depends very much upon the lipid composition (Farge and Devaux, 1992).

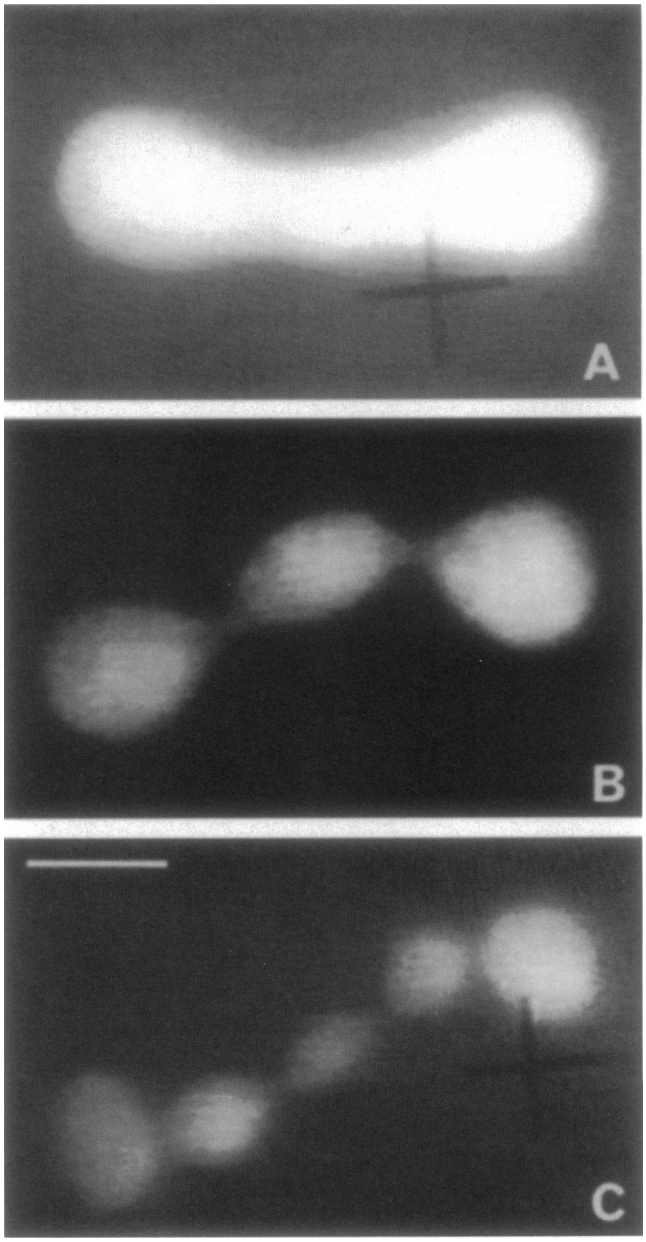

FIGURE 7 Shape transformation of a single giant EPC vesicle containing fluorescent dextran (0.1 mM). (*A*) Discoid shape obtained by a small osmotic pressure applied to a spherical vesicle. (*B*) Four minutes after addition of LPC in the proximity of the vesicle. (*C*) Seven minutes after addition of LPC. The bar corresponds to 10 μm.

The technique invented by Angelova and Dimitrov (1986) to form GUVs is based on the swelling of phospholipids in the presence of an AC electric field. It is a simple and reproducible technique that appears to be efficient for many types of different lipid mixtures, as long as the salt concentration is maintained at a low level (<10 mM). The vesicles, which are perfectly spherical, have a large internal volume and are stable for many days, which makes them attractive for biotechnical applications. The unilamellar nature of such vesicles was determined first by Faucon et al. (1989) by analysis of the thermal undulations and is con-

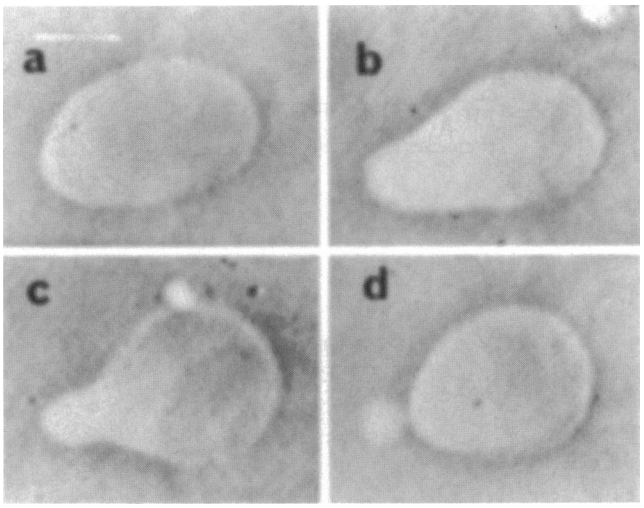

FIGURE 8 Shape transformation of a giant vesicle containing EPC:EPG with a ratio of 99:1. The shape change is triggered by raising the external pH from 6 to 9. The time scale for the shape transformation is approximately 10 s. The bar corresponds to 10 μm.

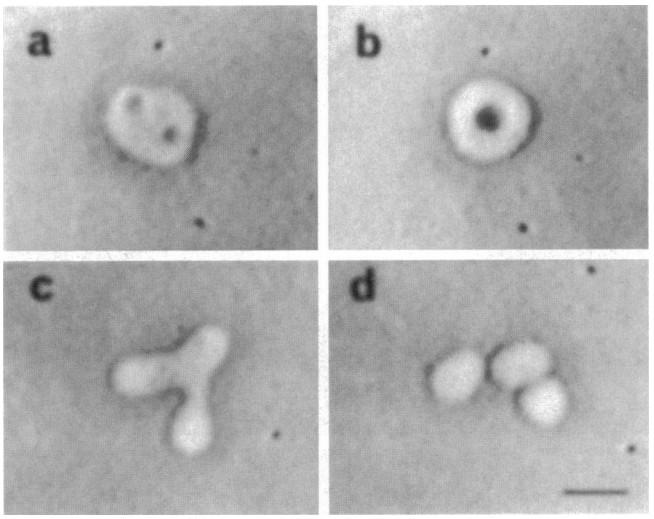

FIGURE 9 Shape transformation of a giant vesicle containing EPC:EPG with a molar ratio of 99:1, as in Fig. 8. This type of transformation is very rare. Same scale as in Fig. 8.

firmed in the present study by freeze-fracture electron microscopy. Freeze fracture can be ambiguous in the case of small vesicles or even LUVs, where this technique can only be used qualitatively to assess lamellarity (Hope et al., 1989b). However, there is a high probability of cross-fractures in giant liposomes, and thus the lamellarity can be ascertain by this method.

The mechanism of formation of the vesicles is still unclear. Angelova and collaborators have noticed that the vesicles, while they are growing, vibrate at the same frequency as the applied field. They suggested that one effect of the electric field would be to create a gentle mechanical agitation, which would help vesicles to form, fuse, and detach from the support. It is not certain, however, that the vesicles are detached from the support before transfer or micromanipulation. The photobleaching experiment described in Fig. 2 strongly suggests a connectivity between vesicles during the swelling period. Indeed, the spontaneous transfer of long-chain phospholipids between large-sized vesicles through the aqueous phase, or via vesicle collisions, is a very unlikely event that requires a long period of incubation, on the order of several hours (Bayerl et al., 1988; Geldwerth et al., 1991). The control experiment, which indicated that, after vesicle transfer, bleached vesicles do not recover any fluorescence even if the vesicles are in close contact, confirms that long-chain fluorescent lipids do not hop from one vesicle to another.

The absence of visible thermal undulations suggests a surface tension that is probably responsible for the sphericity of the vesicles. Whereas Angelova et al. have reported that the vesicles relaxed and fluctuated after a few hours, we found that vesicles stored at low temperature, under argon, remained spherical and did not fluctuate for days and even weeks. Temperature seems to be the most important parameter. Partial lipid degradation may take place at high temperatures and, as already noted by Faucon and collaborators (1989), may help to relax the vesicle tension. If the vesicles are stored at high temperatures in a nonsealed environment, water evaporation is substantial. Because the external medium contains glucose, the osmotic pressure increases progressively, and we have shown that this causes vesicle deformation and, hence, allows undulation to take place.

As soon as the vesicles undulate, the addition of fatty acid-free BSA, which selectively depletes the outer monolayers of all lyso derivatives, provokes vesicle aggregation (see the preliminary report by Devaux et al., 1993). This phenomenon can be interpreted as being due to three consecutive events: i) generation of a surface tension caused by the mismatch between inner and outer monolayers; ii) inhibition of the thermal undulations by surface tension; and iii) attraction of the vesicles by van der Waals forces. Tension-induced adhesion of vesicles has been discussed theoretically and observed experimentally by previous workers (Servuss and Helfrich, 1989; Lipowsky, 1995; Rädler et al., 1995; Netz and Lipowsky, 1995). In most cases, tension is caused by osmotic pressure. However, experimental and theoretical work has shown that the addition of lyso compounds on one monolayer can generate surface tension (Farge and Devaux, 1992, 1993). Here the mismatch is obtained by depleting selectively the outer monolayer. Removing lyso-phospholipids that form spontaneously upon storage or exist initially as trace compounds is equivalent to the selective addition of the lyso-lipid to one monolayer. In both instances, an unbalance between the two monolayers is created and is the cause of surface tension.

In the present article the vesicle shape was modified by change of the volume-to-surface ratio via osmotic pressure, or by modification of the transmembrane phospholipid distribution. The final shapes were similar to those obtained in other laboratories by changing the temperature (Käs and

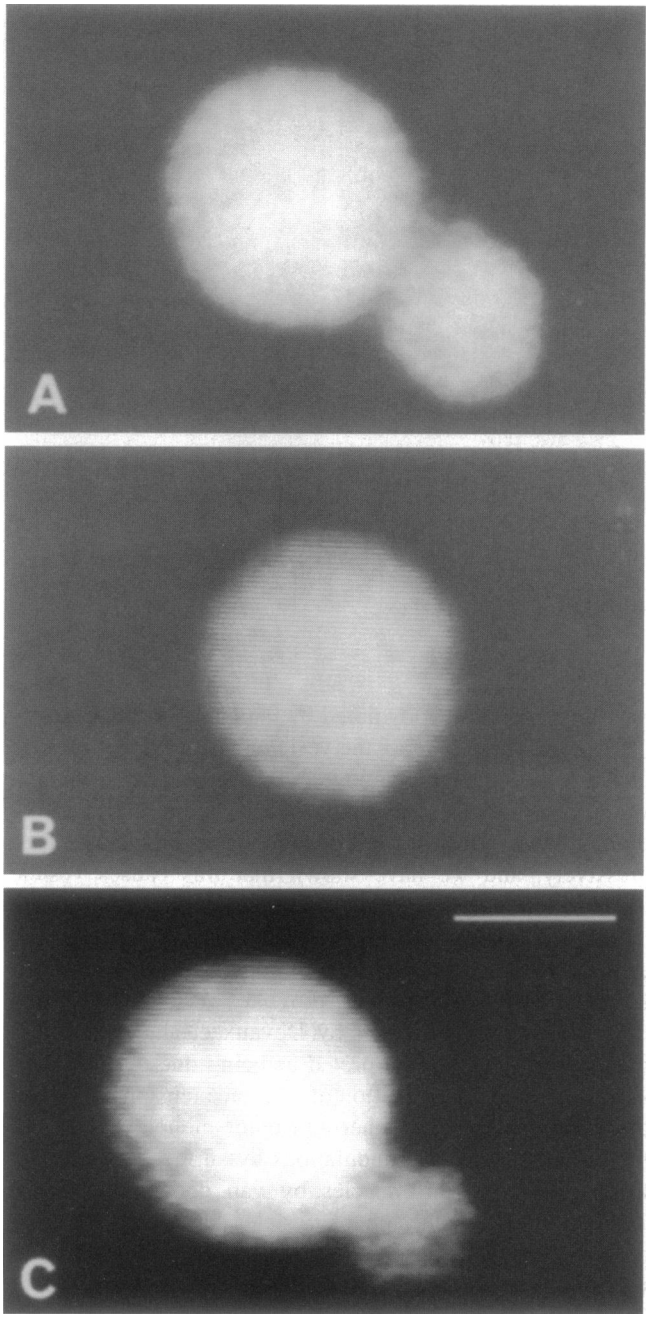

FIGURE 10 Recovery of fluorescence after photobleaching. (*A*) This giant liposome of EPC:EPG (99:1), which looks like two adhering vesicles, was obtained by imposing a ΔpH of 3 pH units through the membrane of a single discocyte liposome. The liposome contains FITC dextran (0.1 mM). (*A*) Before photobleaching. (*B*) Immediately after bleaching of the smaller vesicle. (*C*) Three minutes after bleaching, the fluorescence reappears in the bleached region, indicating that dextran can freely diffuse between the two vesicles.

Sackmann, 1991; Nazil et al., 1992; Döbereiner et al., 1993). The most frequently observed morphology was two closely connected spheres or series of connected spheres. These shapes correspond to states of minimum elastic energy predicted by theoreticians (Deuling and Helfrich, 1976; Svetina and Zeks, 1989; Seifert et al., 1991; Lipowsky, 1991; Gompper and Kroll, 1995). When a lipid redistribution is imposed by a pH gradient to a membrane containing EPG, an upper limit to the percentage of lipid redistribution can be given by the percentage of EPG present. In agreement with a previous investigation (Farge and Devaux, 1992), we found that the redistribution of less than 1% of the total phospholipid population was sufficient to provoke shape changes in giant vesicles. In a very recent article, Mui and collaborators showed by cryoelectron microscopy that LUVs can undergo shape change when dioleoylphosphatidylcholine is translocated from one leaflet to the other by a pH gradient (Mui et al., 1995). In agreement with our prediction (Farge and Devaux, 1993), the shape change of LUVs required a redistribution of 10% of the lipids, whereas with GUVs less than 1% was sufficient. If 10% EPG was present in GUVs and the vesicles submited to a pH gradient, the sequence of shape transformation was accelerated and sometimes caused the vesicle to collapse. The addition of cholesterol, in our experiments, did not modify significantly the results, and in particular, contrary to the results of Döbereiner et al. (1993), we did not observe vesicle fission when cholesterol was present.

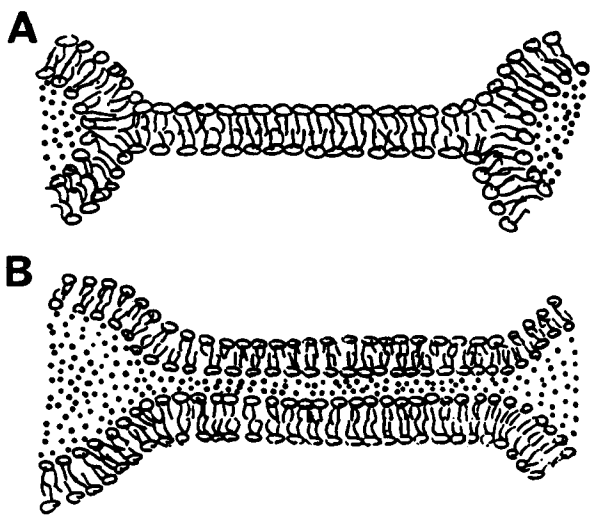

FIGURE 11 Two models suggesting how phospholipids could be organized in the narrow tube (or tether) connecting two vesicles.

Besides the budding of a small vesicle from a giant vesicle, we have also observed a rarer shape with a threefold symmetry (see Fig. 9 *C*). The latter shape was predicted by Mui et al. (1995) and would result from a transformation of a discocyte by breaking of the rotational symmetry.

One feature that becomes particularly visible when fluorescent lipids are used is the connection between separated vesicles by narrow lipid tubes or tethers (Fig. 6). The structure of this elongated lipid protuberance is unknown. Bar-Ziv and Moses (1994) have generated narrow tubes anchored at both ends to massive lipid globules. The tubes were shown to correspond to spheres connected like pearls of a necklace. Here, the resolution is not sufficient to rule out such a structure. Fig. 11 proposes two models. In Fig. 11

A there is no aqueous volume inside the tube, whereas in Fig. 11 *B* water-soluble molecules can penetrate. The very fact that the tether is visible with fluorescent dextran (Fig. 7) rules out model A. Such tubules are not only difficult to visualize; they are also fragile. One might speculate that if they exist in biological systems, they could have been overlooked. It is always postulated that an intense traffic of vesicles takes place within organelles and that proteins are shuttled from organelle to organelle by random diffusion of vesicles, for example, in the Golgi system. An alternative model could involve connections between organelles by small lipid tubules or vesicles still attached by such protrusions.

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REFERENCES

Angelova, M. I., and D. S. Dimitrov. 1986. Liposome electroformation. *Faraday Discuss. Chem. Soc.* 81:303–312.

Angelova, M. I., S. Soleau, P. Méléard, J.-F. Faucon, and P. Bothorel. 1992. Preparation of giant vesicles by external ac electric fields: kinetics and applications. *Prog. Colloid Polym. Sci.* 89:127–131.

Bar-Ziv, R., and E. Moses. 1994. Instability and "pearling" states produced in tubular membranes by competition of curvature and tension. *Phys. Rev. Lett.* 73:1392–1395.

Bayerl, T. M., C. F. Schmidt, and E. Sackmann. 1988. Kinetics of symmetric and asymmetric phospholipid transfer between small sonicated vesicles studied by high-sensitivity differential scanning calorimetry, NMR, electron microscopy, and dynamic light scattering. *Biochemistry.* 27:6078–6085.

Colleau, M., P. Hervé, P. Fellmann, and P. F. Devaux. 1991. Transmembrane diffusion of fluorescent phospholipids in human erythrocytes. *Chem. Phys. Lipids.* 57:29–37.

Deuling, H. J., and W. Helfrich. 1976. The curvature elasticity of fluid membranes: a catalogue of vesicle shapes. *J. Physique France.* 37: 1335–1345.

Devaux, P. F., L. Mathivet, S. Cribier, and E. Farge. 1993. How lipid asymmetry can make vesicles fusion-competent by inhibition of the thermal undulations. *Biochem. Soc. Trans.* 21:276–280.

Döbereiner, H. G., J. Käs, D. Noppl, I. Sprenger, and E. Sackmann. 1993. Budding and fission of vesicles. *Biophys. J.* 65:1396–1403.

Farge, E., and P. F. Devaux. 1992. Shape changes of giant liposomes induced by an asymmetric transmembrane distribution of phospholipids. *Biophys. J.* 61:347–357.

Farge, E., and P. F. Devaux. 1993. Size-dependant response of liposomes to phospholipid transmembrane redistribution: from shape change to induced tension. *J. Phys. Chem.* 97:2958–2961.

Faucon, J. F., M. D. Mitov, P. Méléard, I. Bivas, and P. Bothorel. 1989. Bending elasticity and thermal fluctuations of lipid membranes. Theoretical and experimental requirements. *J. Physique.* 50:2389–2414.

Geldwerth, D., A. de Kermel, A. Zachowski, F. Guerbette, J.-C. Kader, J.-P. Henry, and P. F. Devaux. 1991. Use of spin-label and fluorescent lipids to study the activity of the phospholipid transfer protein from maize seedlings. *Biochim. Biophys. Acta.* 1082:255–264.

Gompper, G., and D. M. Kroll. 1995. Phase diagram and scaling behavior of fluid vesicles. *Phys. Rev. E.* 51:514–525.

Hope, M. J., T. E. Redelmeier, K. F. Wong, W. Rodrigueza, and P. R. Cullis. 1989a. Phospholipid asymmetry in large unilamellar vesicles induced by transmembrane pH gradients. *Biochemistry.* 28:4181–4187.

Hope, M. J., K. F. Wong, and P. R. Cullis. 1989b. Freeze fracture of lipids and model membrane systems. *J. Electron Microsc. Tech.* 13:277–287.

Käs, J., and E. Sackmann. 1991. Shape transitions and shape stability of giant phospholipid vesicles in pure water by area-to-volume changes. *Biophys. J.* 60:825–844.

Lasic, D. D. 1988. The mechanism of vesicle formation. *Biochem. J* 256:1–11.

Lipowsky, R. 1991. The conformation of membranes. *Nature.* 349: 475–481.

Lipowsky, R. 1995. The morphology of lipid membranes. *Curr. Opin. Struct. Biol.* 5:531–540.

Méléard, P., M. D. Mitov, J.-F. Faucon, and P. Bothorel. 1990. Dynamics of fluctuating vesicles. *Europhys. Lett.* 11:355–360.

Mui, B. L.-S., H.-G. Dobereiner, T. D. Madden, and P. R. Cullis. 1995. Influence of transbilayer area asymmetry on the morphology of large unilamellar veiscles. *Biophys. J.* 69:930–941.

Nazil, A. N., S. Bayerl, and M. Bloom. 1992. Temperature-reversible eruptions of vesicles in model membranes studied by NMR. *Biophys. J.* 61:1413–1426.

Netz, R. R., and R. Lipowsky. 1995. Stacks of fluid membranes under pressure and tension. *Europhys. Lett.* 29:345–350.

Rädler, J. O., T. J. Feder, H. H. Strey, and E. Sackmann. 1995. Fluctuation analysis of tension-controlled undulation forces between giant vesicles and solid substrates. *Phys. Rev. E.* 51:4526–4536.

Redelmeier, T. E., M. J. Hope, and P. R. Cullis. 1990. On the mechanism of transbilayer transport of phosphatidylglycerol in response to transmembrane pH gradients. *Biochemistry.* 29:3046–3053.

Seifert, U., K. Berndl, and R. Lipowsky. 1991. Shape transformations of vesicles: phase diagram for spontaneous curvature and bilayer coupling model. *Phys. Rev. A.* 44:1182–1202.

Servuss, R. M., and W. Helfrich. 1989. Mutual adhesion of lecithin membranes at ultralow tensions. *J. Phys. France.* 50:809–827.

Singleton, W. S., M. S. Gray, M. L. Brown, and J. L. White. 1965. Chromatographically homogeneous lecithin from egg phospholipids. *J. Am. Oil Chem. Soc.* 42:53–56.

Svetina, S., and B. Zeks. 1989. Membrane bending energy and shape determination of phospholipid vesicles and red blood cells. *Eur. Biophys. J.* 17:101–111.

Winterhalter, M., and D. D. Lasic. 1993. Liposome stability and formation: experimental parameters and theories on the size distribution. *Chem. Phys. Lipids.* 64:35–43.