Asymmetrical Membranes and Surface Tension

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ABSTRACT The $^{31}$P-nuclear magnetic resonance chemical shift of phosphatic acid in a membrane is sensitive to the lipid head group packing and can report qualitatively on membrane lateral compression near the aqueous interface. We have used high-resolution $^{31}$P-nuclear magnetic resonance to evaluate the lateral compression on each side of asymmetrical lipid vesicles. When monooleoylphosphatidylcholine was added to the external monolayer of sonicated vesicles containing dioleoylphosphatidylcholine and dioleoylphosphatic acid, the variation of $^{31}$P chemical shift of phosphatic acid indicated a lateral compression in the external monolayer. Simultaneously, a slight dilation was observed in the inner monolayer. In large unilamellar vesicles on the other hand the lateral pressure increased in both monolayers after asymmetrical insertion of dioleoylphosphatidylcholine. This can be explained by assuming that when monooleoylphosphatidylcholine is added to unilamellar vesicles the membrane bends until the strain is the same in both monolayers. In the case of sonicated vesicles, a change of curvature is not possible, and therefore differential packing in the two layers remains. We infer that a variation of lipid asymmetry by generating a lateral strain in the membrane can be a physiological way of modulating the conformation of membrane proteins.

INTRODUCTION

Lipid asymmetry in biomembranes is often viewed solely as a difference in chemical composition between the two monolayers. For example, it is well known that the plasma membrane of eukaryotic cells contains essentially phosphatidylcholine and sphingomyelin in the outer monolayer, whereas aminophospholipids are predominantly located in the inner monolayer (Devaux, 1991). However, lipid asymmetry can also mean a difference in surface density resulting from a difference in the number of lipids between the two coupled monolayers. Such a difference in lipid surface density can be achieved artificially by the addition of lysophosphatidylcholine (LPC) to the external surface of a lipid vesicle or of a biological membrane. Because of the very slow flip-flop of lipids, the asymmetrical surface density is generally stable. However, the mismatch causes a difference in lateral pressure or tension between the two leaflets that often, but not always, leads to vesicle shape change. In biological membranes, the lateral pressure formed by an asymmetrical lipid distribution could affect the structure and function of intrinsic membrane proteins.

We have used nuclear magnetic resonance (NMR) to probe the local variations of the lateral pressure in various types of unilamellar vesicles. High-resolution $^{31}$P-NMR spectra of phospholipid vesicles containing a mixture of dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatic acid (DOPA) were recorded and the chemical shift of DOPA was monitored. The potentiality of $^{31}$P-NMR spectroscopy for the assessment of lateral compressibility in a membrane was brought to light by Swairjo et al. (1994a). They showed that the $^{31}$P chemical shift of DOPA is different in the inner and outer monolayers of sonicated unilamellar vesicles (SUVs). They inferred that the difference in chemical shifts was due to the difference in local curvature of the two monolayers, which is certainly true in sonicated vesicles. In fact, via its sensitivity to the local pK_a, the chemical shift effectively reports on the packing of the lipid head groups and can be considered as a qualitative indicator of any change of surface tension.

NMR spectroscopy can be used to monitor qualitatively the surface tension of vesicles of various sizes as long as they contain phosphatic acid and that high-resolution NMR measurements are possible. We have investigated successively asymmetrical SUVs, LUVs (large unilamellar vesicle) and FTVs (freeze-thaw vesicles). FTVs are unilamellar vesicles obtained by repetitive cycles of freeze thawing, a process that generates unilamellar vesicles with a distribution of sizes but with a significant contribution of vesicles with a diameter above 200 nm (Traïkia et al., 2000). With giant unilamellar vesicles (GUVs) the lipid concentration would be too low to allow NMR experiments to be performed. In the case of LUVs and FTVs magic angle spinning was necessary to achieve high resolution. Shape changes were visualized by cryoelectron microscopy.

In agreement with a former theoretical article (Farge et al., 1990), we have found that the addition of lysophosphatidylcholine to one side of LUVs or FTVs triggers an overall change of curvature that is accompanied by an increased lateral compression in both leaflets and not only on the external one. However, with sonicated vesicles, which are spheres with the highest curvature that can withstand a
phospholipid bilayer, there is no possibility of changing the curvature and the surface pressure increases solely in the outer monolayer.

MATERIALS AND METHODS

Chemicals

Lipids (DOPC, DOPA, and monooleoylphosphatidylcholine (MOPC)) and chemicals (HEPES, KCl, and EDTA) were purchased from Sigma Chemical Co. (Saint Quentin Fallavier, France) and were used without further purification. The purity of all lipids was verified by thin layer chromatography and by high resolution NMR in chloroform.

Vesicles preparation

All samples were prepared as an initial dry lipidic film of DOPC/DOPA (80:20 mol%) and the buffer was: 0.1 M HEPES, 0.1 M KCl, and 0.005 M EDTA at pH 8.0. SUVs (30 mg/mL) were prepared by sonication of a lipid dispersion in buffer under a stream of argon using a probe type sonicator (model VC50, Bioblock Scientific, Paris, France) at 40 W in an ice bath until a clear solution was obtained. The sample was afterwards centrifuged at 11,000 × g for 15 min to ensure the removal of metallic particles. Unless otherwise mentioned, LUVs were prepared by the reverse phase evaporation technique followed by filtration of the preparation (50 mg/mL) through a polycarbonate membrane, successively at 1, 0.4, and at 0.2 μm (Szoka et al., 1980; Traikia et al., 2000). The LUVs were concentrated to 100 mg/mL by ultra centrifugation at 395,000 × g for at least 2 h (Beckman, TLC100). FTVs were prepared at 100 mg/mL. Characterization of their lamellarity and size distribution was described in Traikia et al. (2000). Stock solution of MOPC was prepared at 10 mg/mL in the buffer. Because of the large amount of lipids necessary (several hundred microliters of 100 mM phospholipids in one sample), a rather accurate determination of the ratio of DOPC/DOPA or DOPC/DOPA/MOPC could be achieved from the weight of the dry lipids and was always controlled by integration of the high resolution NMR spectra recorded with magic angle spinning (MAS). In a few instances, for SUVs or LUVs, the ratio of DOPC/DOPA was also checked by NMR in organic solvent but not for all samples.

NMR

NMR experiments were performed and processed on a Bruker AVANCE DMX400-WB NMR spectrometer (1H resonance at 400 MHz, 31P resonance at 162 MHz) using a Bruker 4-mm MAS probe with an external lock for the LUVs, FTVs, and MLVs (multilamellar vesicles). A few experiments were carried out with an insert-containing rotor, which allows better field homogeneity but because of the low volume contained in such rotor longer accumulations were necessary. The spinning speed of the 4 mm ZrO2 MAS rotor was controlled to within 5 Hz at 8 kHz and the temperature (fixed at 8°C unless otherwise specified) was calibrated as described in Traikia et al. (1997). A 10-mm broadband liquid state NMR probe was used for the SUVs experiments. The 31P-90° pulse lengths were 4.6 and 16 μs for the MAS and the liquid state probes, respectively. The recycle delay was 2 s and typical 31P spectral width was 20 ppm (3.24 kHz). 1H decoupling was not applied during acquisition of the 31P magnetization. In all experiments, 4096 complex points were acquired. Before Fourier transformation, the data were zero filled to 8192 points, exponentially multiplied with 10 Hz line broadening and treated with automatic baseline correction. The theoretical frequency reference, 0 ppm, corresponds to phosphoric acid. As reported by Swairjo et al. (1994a), we found that DOPC resonance at 8°C consist of a single peak at ~0.64 ppm that can be used as an internal reference. For broadband spectra, 0 ppm corresponds to the isotropic peak of DOPC micelles. Other conditions for broadband 31P-NMR spectroscopy were those of Traikia et al. (2000).

Cryoelectron microscopy

A few microliters of the lipid mixtures were applied to a holey carbon film on a copper grid that was held by tweezers mounted on a shaft above a liquid ethane bath cooled by liquid nitrogen (Leica EM CPC). The sample was blotted with filter paper and immediately plunged into the liquid ethane (Dubochet et al., 1988). The vitreous specimens were transferred under liquid nitrogen to the cryo-transmission electron microscope cold stage (model 626 Gatan), which was inserted into the Philips CM 120 electron microscope and maintained at ~170°C throughout specimen observation. Specimens were imaged at 120 kV by low dose technique, and micrographs were recorded on Kodak SO163 films with a magnification of 45,000× and a 1-μm defocus.

RESULTS

Sonicated unilamellar vesicles

Fig. 1 shows 31P-NMR spectra of DOPC/DOPA (80:20 mol%) SUVs with and without MOPC added externally. The top spectra in Fig. 1 were recorded at 45°C. At this temperature DOPC gives rise to two resonance peaks partially overlapping. When at 45°C MOPC is added, the lines slightly broaden and the two phosphatidylcholine peaks collapse in agreement with the results of Kumar et al. (1989), who found that 31P-T2 of POPC decreases upon addition of LPC to SUVs. As reported previously by Swairjo et al. (1994a), DOPA in SUVs gives rise to a doublet with two well-separated peaks, the low field line corresponds to the phosphorus on the external side of the vesicles and the high field line to the inner leaflet molecules. We have carried out NMR experiments (not shown) in the presence of 10 mM Pr3+ ions, which selectively shifted the low field peak of DOPA and allowed us to confirm the conclusion of Swairjo et al. (1994a). Integration of DOPA peaks indicated roughly a ratio of 2 to 1, which was consistent with the proportion of outer to inner surfaces of SUVs.

The two DOPA peaks are still well separated in the presence of MOPC. The chemical shift of DOPA has been used to investigate the influence of MOPC on the surface pressure of both leaflets. Because the position and splitting between the two DOPA peaks are sensitive not only to the presence of MOPC but also to the temperature, the pH and the ratio of DOPA to DOPC (Swairjo et al., 1994a), it was important to maintain the latter parameters fixed in all further experiments. A relatively low temperature (8°C) was adopted to allow comparison with experiments involving SUVs that required a low temperature for reasons that will be explained below.

Fig. 2 shows the effect on the 31P-NMR spectra of increasing concentration of MOPC at 8°C. The two chemical shifts associated with DOPA molecules are modified: δPA(ext) moves up-field whereas δPA(int) moves slightly...
down-field. At this temperature there is no obvious change in the line width of the DOPC peak and no obvious redistribution between the spectral intensities of the two DOPA peaks. This suggests that addition of MOPC neither induced SUVs fusion nor caused their solubilization. Changes in δ<sub>PA</sub> would simply reveal changes in lateral pressure of each monolayer without important overall shape changes.

**Large unilamellar vesicles**

By cryoelectron microscopy, we found that the presence of an important percentage of DOPA (20 mol%) in the vesicles made it difficult to obtain an homogeneous population of LUVs when prepared by direct lipid extrusion under pressure according to the protocol designed by Cullis and collaborators (Hope et al., 1985). Fig. 3 shows some examples of extruded DOPC/DOPA vesicles with odd shapes. Furthermore, we found that the ratio of DOPA/DOPC was generally modified with a loss of DOPA. On the other hand, when LUVs were prepared by the phase reverse technique (Szoka et al., 1980), we obtained more homogeneous populations of unilamellar vesicles with many spherical vesicles and almost no tubular ones (Fig. 4). Besides, the proportion of DOPC/DOPA was not modified. Addition of MOPC to the latter vesicles provoked the formation of more elongated vesicles (Fig. 5, A and B). Budding was observed but required the addition of 5% to 10% of MOPC.

Because glycerol was used in some NMR experiments described below, we have attempted to determine LUVs shapes with and without lysophosphatidylcholine addition in the presence of glycerol. Unfortunately when glycerol is present in samples examined by cryoelectron microscopy, the contrast is weak. Nevertheless, we found that all vesicles

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**FIGURE 1** 31P-NMR spectra (162 MHz) of SUVs containing a mixture of DOPC/DOPA in 4:1 molar ratio prepared by sonication in buffer at pH 8. The dotted spectra were obtained with MOPC added to in the outer monolayer: (a) 45°C; (---) no MOPC; ( ) 30% MOPC; (b) 8°C; (---) no MOPC; ( ) 15% MOPC.

**FIGURE 2** Effect of MOPC addition to SUVs on the chemical shifts of the two DOPA resonance in DOPC/DOPA (4:1) SUVs. Temperature is 8°C. (a) 31P-NMR spectra (162 MHz) of SUVs with an increasing concentration of MOPC added externally. Percentages indicated on the right hand side of a are the molar percentages of MOPC added in relation to the total lipids present. (b) Dependence of the 31P-DOPA chemical shifts in the outer (○) and inner (△) leaflets, respectively. The uncertainty of the mol% of MOPC was estimated from lipid analysis in organic solvent.
seemed to be more spherical in the presence of glycerol than without and that the addition of MOPC tended on the contrary to give homogeneous populations of perfectly spherical vesicles instead of generating elongated or eight-shapes vesicles (see above). Fig. 5 C was obtained with 25% of glycerol and 10% MOPC added externally. With 50% glycerol the vesicles were very difficult to see but seemed to be perfect spheres (data not shown).

When $^{31}$P-NMR spectra of LUVs or MLVs are recorded without sample spinning, the resolution is poor and does not allow one to separate the various phospholipid head groups, even if the spectrum is recorded at 45°C. Consequently, we have performed all further $^{31}$P-NMR experiments with 8 kHZ sample spinning at the magic angle (MAS). The $^{31}$P-NMR spectrum of MLVs containing DOPA/DOPC exhibits a single narrow peak for DOPA at 2.2 ppm (Fig. 6 a). This is consistent with the lipid surface density being the same in both leaflets for lipid vesicles with large diameters. In the case of LUVs with a diameter of ~200 nm, vesicle tumbling and phospholipid lateral diffusion on the surface of LUVs create a nonnegligible incoherent averaging that, in practice, diminishes the efficiency of magic angle spinning (Traikia et al., 1997). In that case, it is preferable to reduce the averaging due to thermal motion and thus, paradoxically, to reduce the temperature to obtain narrow lines (Fig. 6 b). In fact, the best $^{31}$P-NMR spectra with LUVs were obtained at 8°C after addition of 50% glycerol to minimize vesicle tumbling (Fig. 6 c). However, besides the resolution enhancement reported above, glycerol can modify the interface between phospholipids and the aqueous environment due to the well known dehydration effect of glycerol (Fenske and Cullis, 1993). This may explain why, for example in the presence of 50% glycerol, $\delta_{PA}$ is moved up field by ~0.5 ppm in the absence of any MOPC. For that reason, we have recorded spectra of DOPA/DOPC-LUVs with and without glycerol despite the fact that in the latter case the resolution was poorer and required the use of an insert-
containing rotor. In all instances, we obtained a single line associated with DOPA (Fig. 6, b and c). When MOPC was added, the DOPA peak was shifted up field as shown by the dotted line spectra in Fig. 6, b and c.

Freeze-thawed vesicles

Cycles of freeze-thawing on lipid dispersions generate a population of unilamellar vesicles (MacDonald et al., 1994; Traïkia et al., 2000). Although these FTVs are not homogeneous in size, the contribution of the larger vesicles (200–500 nm) is dominant in the NMR spectrum (Traïkia et al., 2000). We have recorded $^{31}$P-MAS-NMR spectra of FTVs obtained after 10 freeze-thaw cycles of a DOPC/DOPA mixture (80:20 mol%) with or without MOPC. In the absence of MOPC a single line was assigned to DOPA (dotted curve in Fig. 6 a), indicating that the packing of the outer and inner monolayers were very similar, thereby confirming that the contribution of very
small vesicles (with a diameter analogous to that of sonicated vesicles) was not dominant in the spectra. When MOPC was added externally to preformed FTVs, the phosphorus peak of DOPA moved up field and there was still no indication of a line splitting, which would correspond to DOPA located, respectively, in the inner and the outer leaflets (Fig. 7). Thus, as in the case of 200-nm LUVs, the chemical shift of DOPA present in the inner leaflet of FTVs is modified by addition of MOPC in the outer leaflet.

Finally we have carried out experiments with FTVs for which MOPC was mixed with DOPC/DOPA before vesicle formation. In that case, MOPC is likely to be present in both leaflets. These “symmetrical” vesicles, as expected, give rise to a single DOPA peak (not shown). The position of this peak moved slightly up field when MOPC concentration was increased. However, the variation of DOPA peak position with MOPC concentration in symmetrical vesicles was much smaller than in asymmetrical vesicles as shown in Fig. 8. The obvious difference between the influence of MOPC on the chemical shift of DOPA in asymmetrical versus symmetrical vesicles confirms that MOPC, when added externally, does not equilibrate rapidly between both leaflets. Nevertheless, the change in chemical shift observed at high MOPC concentration in symmetrical vesicles is indicative of a lateral strain in both leaflets. This requires some comments that will be given in the Discussion section.

**Broadband spectra of LUVs in the presence of MOPC**

The quantitative interpretation of the data summarized in Fig. 8 would require a precise knowledge of the fraction of MOPC added that effectively is localized within the outer monolayer of each type of vesicles. Because of the limited expansitivity of a lipid monolayer, the addition of MOPC to only one monolayer in a lipid vesicle is likely to be limited. To determine what proportion of MOPC added actually resides in the membrane, we have examined broadband $^{31}$P-NMR spectra of DOPC/DOPA-LUVs in the presence of increasing concentrations of MOPC (Fig. 9). If a fraction of MOPC remains in micelles, it can be identified in broad-
band NMR spectroscopy by a narrow peak in the central part of the spectrum. Ideally, such experiments should be done with unilamellar vesicles with a diameter of several hundred nanometers to avoid narrowing of the NMR spectrum by vesicle tumbling. Because the $^{31}$P-NMR spectra of LUVs with a diameter in the 100- to 200-nm range is partially narrowed (Traikia et al., 2000), we have used for these experiments LUVs prepared by phase reversion and filtered with 400 nm pores. Fig. 9, a to e, are broadband $^{31}$P-NMR spectra of 400-nm DOPC/DOPA-LUVs recorded in the presence of increasing concentrations of MOPC added externally. Fig. 9 f is the spectrum of MOPC in buffer recorded under the same conditions and corresponds to micelles of MOPC. Fig. 9 a was recorded in the absence of MOPC. It is not the typical spectrum of a homogeneous population of very large liposomes. Indeed, the central part of the spectrum reveals the existence of a distribution of sizes (Traikia et al., 2000). This confirms that the extrusion of liposomes with pore sizes of 400 nm does not lead to a homogeneous population of unilamellar vesicles. In fact, these vesicles probably contain bilamellar vesicles as reported by Mayer et al. (1986). Nevertheless, such vesicles permit an easier evaluation of the micellar contribution in a composite NMR spectrum than 200-nm LUVs do.

Fig. 9 (b to e) shows that the addition of MOPC gives rise to a central narrow peak, the intensity of which grows with the proportion of MOPC added. At or above ~5% MOPC, the contribution of this peak is nonnegligible, which suggests that a significant fraction of MOPC does not incorporate in the membrane. Fig. 9, b, c, d, and e, cannot be simulated by a linear combination of spectra a (spectrum of liposomes) and f (spectrum of micelles) because the peak at 0 ppm is too broad (for example, see Fig. 9 e). Most likely, the broadening is due to rapid exchange of MOPC between micelles and membranes. By subtracting a fraction of spectrum a from spectra b, c, d, and e respectively, we have estimated the contribution of MOPC nonincorporated into the vesicles and deduced the percentage of MOPC, which was effectively in the membrane. For sake of simplicity the hypothesis used to draw the solid curve in Fig. 9 B was that all vesicles were unilamellar. If these “LUVs” actually contain a nonnegligible proportion of bilamellar vesicles (Mayer et al., 1986), then the proportion of MOPC per phospholipid in the external monolayer is higher than indicated in Fig. 9 B. Hence, the curve corresponds to a lower limit of MOPC percentage that is effectively incorporated into unilamellar vesicles.

**DISCUSSION**

Asymmetrical insertion of lysophosphatidylcholine

MOPC was used here to create asymmetrical membranes. Long chain lysophosphatidylcholine molecules have a critical micellar concentration in water in the micromolar range (Kumar and Baumann, 1991; Needham et al., 1997). Adding lysophosphatidylcholine to a very concentrated suspension of unilamellar vesicles (several millimolar of phospholipids) allows one to rapidly intercalate new molecules in the outer surface. NMR experiments shown in Fig. 9 indicate nevertheless that a fraction of MOPC is rapidly exchanging between micelles and membranes, when the percentage of MOPC compared with total phospholipids present is above 2% to 3%. At least 50% of MOPC added penetrate the vesicle surface. There was no saturation under our experimental conditions (Fig. 9 B).

NMR data (Fig. 9 A) and electron microscope micrographs (Fig. 5) obtained with LUVs show that LUVs are not destroyed, neither do they form smaller vesicles when 5%
are values for the area compressibility modulus of fluid bilayers

**Vesicles shape and surface tension**

The shapes of giant unilamellar vesicles with an asymmetrical lipid distribution has been documented in previous articles (Farge and Devaux, 1992; Mui et al., 1995; Mathivet et al., 1996). Complete phase diagrams of vesicles morphologies have been calculated by bending energy minimization as proposed originally by Helfrich (1973; Svetina and Zeks, 1989; Berndl et al., 1990; Seifert et al., 1991). A more recent and elaborate model takes into account the lateral elasticity of each lipid monolayers (Miao et al., 1993). In other words, an asymmetrical lipid distribution can generate an important surface tension in LUVs. In those vesicles, tension builds up before shape change takes place.

DOPA as a lateral pressure sensor

A basic assumption for the interpretation of the NMR experiments is that the phosphorus chemical shift of phospha-
phosphatidic acid allows one to detect a lateral compression of the lipids near the aqueous interface and not only membrane bending. A water/lipid interface represents an interphase between a bulk aqueous phase of high dielectric constant ($\epsilon \approx 80$) and the hydrophobic phase of a membrane of very low dielectric constant. One consequence of the lower dielectric constant is that the adsorption of cations and anions to the ionizable groups of lipids is facilitated (Tocanne and Teissié, 1990). However, the actual ionization state of phosphatidic acid (PA) at the aqueous interface is also strongly dependent upon the phosphate accessibility to the buffer. Close packing by reducing the area available for the head-group phosphate allows the monoanionic form of PA to be more favorable than the dianionic form. Titration curves at various pH with sonicated dispersions of phosphatidylcholine/PA mixtures allowed Swairjo et al. (1994a) to show that the difference in chemical shift of PA in the two monolayers of SUVs can be explained by a difference in the apparent pK$_a$ of the phosphate moiety on the two sides of SUVs. They calculated a pK$_a$ value around 12 in the inner leaflet instead of 7 for a free phosphate. The pK$_a$ shift in the inner leaflet would arise from the tight packing associated with the inner leaflet curvature. On the other hand, the PA head-group in the outer-leaflet would be in a more relaxed geometry that allows the phosphorus to sense the buffer pH.

A priori, the expansion (or contraction) of each monolayer is associated with a stress profile through the membrane (Gruner and Shyamsunder, 1991; Cantor, 1997). Lipids labeled with $^{13}$C or $^2$H could be investigated by NMR the entire stress profile (Chiu and Wu, 1990; Koenig et al., 1997). $^{31}$P, on the other hand, is a natural probe that enables one to explore easily at least the two interfaces between phospholipids and water. Although it appears difficult to relate theoretically in a quantitative and rigorous manner the variation of DOPA chemical shift ($\delta_{PA}$) to the lateral pressure or surface tension, the modification of $\delta_{PA}$ is a convenient indicator of lateral stress modifications. Providing the two membrane leaflets have the same lipid composition and are facing the same buffer, identical values of $\delta_{PA}$ for the inner and outer leaflets reveal identical stress. The actual modification of the stress profile in a given experiment depends on what causes the membrane to bend. An external constraint, for example the aspiration of the membrane in a micropipette or actin polymerization pushing the membrane are processes that are likely to stretch one monolayer and compress the other monolayer (Marsh, 1996). But when a new lipid distribution, caused by the insertion of drugs or by a flippase activity, is sole responsible for the bending, the constraint is internal, i.e., the deformation must reach a final equilibrium with zero torque as long as the membrane remains elastic.

Our NMR experiments and those of Kumar et al. (1989) indicate that the addition of LPC to SUVs has practically no influence on the line width, hence on vesicles size and finally on the average curvature. Yet, $\delta_{PA}$ varied consider-ably in the outer leaflet upon addition of MOPC, whereas it remained almost constant in the inner one. Because it is hard to imagine how the curvature could change on one side of a membrane and remain constant on the opposite side, we suggest that a change of $\delta_{PA}$ should not be associated systematically with a change of membrane curvature but rather to compression/dilation near each interface accessible to the buffer.

**Interpretation of data obtained with sonicated unilamellar vesicles**

Compression of SUVs outer monolayer after the addition of MOPC is indicated by the up-field shift of $\delta_{PA}$(ext). This is not surprising; inserting MOPC in the outer monolayer should allow less area per lipid (Fig. 10 A). Perhaps more surprising is the slight downfield shift of $\delta_{PA}$(int), which suggests membrane dilation, hence a small increase of area per head group in the inner monolayer. However, because SUVs are spherical, there is no alternative to a slight expansion of the outer surface upon insertion of new molecules (elastic response). Because the inner monolayer adheres to the outer monolayer, it can expand slightly and release some pressure in the head-group region, which is probed by the phosphate chemical shift.

A similar observation was made by Swairjo et al. (1994b). The authors reported that the addition of annexin V to SUVs resulted in the same modification as the one reported here. They proposed a protein-induced change in vesicle morphology that corresponds to reduced curvature. Their data could be explained as well by implying a slight expansion of the outer leaflet due to the interaction of annexin V with the lipid head groups, involving perhaps a partial protein intercalation within the outer monolayer. Simultaneously the inner leaflet would be slightly relaxed as indicated by the sign of the change of $\delta_{PA}$(int) reported by these authors. Kumar et al. (1989) concluded from $^{31}$P-NMR-T$_2$ measurements that the addition of LPC to sonicated POPC vesicles tightens phospholipid packing but only in the outer monolayer. In the present study, we see a small variation of $\delta_{PA}$(int), it may simply tell that T$_2$ and $\delta_{PA}$, which are measurements of two different magnetic resonance properties, are not simply correlated and may have different sensitivity domains.

**Large unilamellar vesicles and freeze-thawed vesicles**

Comparison between Figs. 4 and 5 reveal that the addition of MOPC to the outer leaflet of LUVs, triggers an overall shape change. Although we have not seen the formation of protrusions and pseudopods as reported by Mui et al. (1995), we saw the formation of elongated vesicles and even eight shaped vesicles, at least in the absence of glycerol.
The vesicles shapes shown in Fig. 5, A and B resemble giant vesicles with an asymmetrical lipid distribution (Farge and Devaux, 1992), it should be stressed that it was necessary to add 5% LPC to achieve these transformations, whereas less than 1% mismatch suffice to obtain these shapes with GUVs. These observations are consistent with the scaling factor discussed above.

When LUVs were formed in the presence of 25% glycerol, their shapes before addition of MOPC were rather spherical. Upon addition of MOPC, there was no significant elongation. On the contrary they formed homogeneous populations of perfect spheres (Fig. 5 C). Such shape change implies an average change of volume: perhaps glycerol that has a disordering effect on lipid acyl chains (Fenske and Cullis, 1993) makes the bilayer more permeable to water. In any case, when MOPC is added to large vesicles, it can be inferred from the high resolution $^{31}$P-NMR spectra that there is an increased surface pressure not only in the outer leaflet but also in the inner leaflet because the signal corresponding to DOPA remains a single peak and moves up-field. The high spectral resolution in Fig. 6 excludes the possibility that $\delta_{\text{P}_A}(\text{ext})$ could move up-field, whereas $\delta_{\text{P}_A}(\text{int})$ would move down-field or even remain constant. Thus, for LUVs (and FTVs) the insertion of MOPC provokes bending, which stops when both leaflets experience the same lateral pressure, hence the momentum is zero. (C) Symmetrical FTVs. Both monolayers have a positive spontaneous curvature due to the presence of wedge shape LPC. The adhesion of the two monolayers is only possible with formation of some surface tension (lateral pressure) but no overall membrane curvature is generated by the presence of LPC.

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### Symmetrical membranes containing MOPC

Fig. 8 shows that if MOPC is included in both monolayers of FTVs, the variation of DOPA resonance position is very small at least as long as the percentage of MOPC included in each monolayer is less than 10%. Thus, symmetrical and asymmetrical distributions of MOPC have very different effects. Clearly $\delta_{\text{P}_A}$ variation after addition of MOPC to the external monolayer cannot be explained by the dilution of the DOPA head group. For symmetrical membranes, there is no curvature expected from MOPC addition. On the other hand, adhesion of the two monolayers, which have opposite spontaneous curvatures, generates a “frustrated” flat bilayer with a constrained packing near the phospholipid head groups of both leaflets (Fig. 10 C) (Charvolin and Sadoc, 1988; Marsh, 1996).
Biological relevance

We have shown that an excess of lipids as small as 2% to 3% on one side of a vesicles can generates surface tension. Such situation can be triggered in a biological membrane by the synthesis and release of new lipids on one side of a membrane or by the activity of a phospholipid translocase. For small organelles, with a diameter ~200 nm, a surface tension should appear even before a significant shape change (budding) takes place.

The shape change caused by the aminophospholipid translocase activity in human erythrocytes (Seigneur and Devaux, 1984) has led to propose that the accumulation of phospholipids on the inner leaflet of the plasma membrane of eukaryotic cells by the aminophospholipid translocase could be involved at the early stage of endocytosis (Devaux, 1991; Müller et al., 1994; Farge et al., 1999; Devaux, 2000; Rauch and Farge, 2000). The hypothesis that modulation of lipid asymmetry could also modulate surface pressure and, by this process, conformation of membrane protein has also been suggested (Bogdanov et al., 1993). There are experimental observations actually demonstrating the role of surface pressure on the modulation of membrane protein function. Martinaud et al. (1990) have shown that the addition of amphiphiles such as lysolecithin or chlorpromazine to giant Escherichia coli spheroplasts resulted in the opening of mechanosensitive ion channels. The work of Martinac et al. (1990) shows that compounds intercalated in the outer or in the inner leaflet have the same physiological effect. This is a strong indication that the mechanism involved is not the local curvature per se but rather the lateral compression. The influence of lateral pressure on phospholipase activity has been illustrated in monolayers (Bougulavsky et al., 1994) and in large unilamellar vesicles (Lehtonen and Kinnunen, 1995). The lytic activity of melittin is also strongly influenced by the tension of vesicles under osmotic stress (Benachir and Laffleur, 1996). Using osmotic stress, Goulian et al. (1998) have shown that the tension of giant vesicles can modulate the dimerization of gramicidin and hence the opening of the channel.

Finally, we might speculate that the influence of the flipside family on the lateral pressure of a membrane could be a way to regulate the activity of transmembrane proteins and not only of mechanosensitive channels. The fact that in reconstituted vesicles with a diameter of ~200 nm the P-glycoprotein activity was found quasi null (Rothnie et al., 2001) or very small (Romsicki and Sharom, 2001) is perhaps the consequence of the lateral pressure generated by the unidirectional transport of lipids.

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