Shape Modification of Phospholipid Vesicles Induced by High Pressure: Influence of Bilayer Compressibility

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ABSTRACT Giant vesicles composed of pure egg yolk phosphatidylcholine (EYPC) or containing cholesterol (28 mol%) have been studied during a high hydrostatic pressure treatment to 285 MPa by microscopic observation. During pressure loading the vesicles remain spherical. A shape transition consisting of budding only occurs on the cholesterol-free vesicles during pressure release. The decrease in the volume delimited by the pure EYPC bilayer between 0.1 and 285 MPa was found to be 16% of its initial volume, whereas the bulk compression of water in this pressure range is only 10%. So the compression at 285 MPa induced a water exit from the pure EYPC vesicle. The shape transition of the EYPC vesicle during pressure release is attributed to an increase in its area-to-volume ratio caused by the loss of its water content during compression. Because bulk compression of the cholesterol-containing vesicle is close to that of water, no water transfer would be induced across the bilayer and the vesicle remains spherical during the pressure release.

INTRODUCTION

A change in area-to-volume ratio of large unilamellar vesicles induces their shape modification. These phenomena have been studied on lipid bilayers as models because biomembranes are subjected to complex morphological transitions that are involved in many basic cellular functions such as endocytosis, exocytosis, budding, or vesicle transport. Works accomplished by Dobereiner (Dobereiner et al., 1993) have demonstrated that the heating of vesicles, which results in a high expansion of the phospholipid bilayer while the volume of the vesicle water contents remains constant, causes budding of vesicles. The shrinkage of a vesicle generated by an osmotic gradient across the bilayer leads to the budding and fission of the vesicle (Kas and Sackmann, 1991). Shape transitions that occur during heating or shrinking are governed by the minimum bending energy constraint of closed shells, the area-to-volume ratio variation, and the area difference between the outer and the inner leaflets of the bilayer (Mui et al., 1995; Svetina and Zeks, 1989).

Shape changes of vesicles composed of a phospholipid mixture are influenced by additional factors, such as the preferential distribution of components between the two monolayers, the different spontaneous curvatures of the lipid domains, and the interfacial energy between the domains (Jülicher and Lipowsky, 1993; Seifert, 1993).

High hydrostatic pressures (from 100 to 1000 MPa) have been extensively applied to study changes in phospholipid bilayer structure as a consequence of increased acyl chain order, which could be implied in hydrostatic pressure injury of biomembranes. Many studies supported by different methods, including neutron diffraction, Raman scattering, fluorescence spectroscopy, and differential thermal analysis, have shown that high pressure decreases lateral motion and induces phase transition in phospholipid bilayers (Macdonald, 1984; Wong et al., 1988). In fact, the gel to liquid-crystalline phase transition temperature (Tm) rises linearly with hydrostatic pressure level in accordance with the Clausius-Clapeyron relationship, giving values of dTm/dP in the range of 0.15–0.3°C/MPa for phospholipids. However, most of these studies have been carried out with indirect measurement, which does not generally take into account vesicle shape or suppose it to be spherical. Moreover, the vesicles are usually made of phospholipids composed of a polar headgroup attached to two similar acyl chains, whereas phospholipids in biological membranes are essentially composed of two different acyl chains (New, 1990).

The aim of this study was to observe the volume variation of giant phospholipid vesicles during hydrostatic pressure application. The vesicles were composed of egg yolk phosphatidylcholine containing cholesterol or not, to measure their bulk compressibility and to estimate the influence of cholesterol on lipid packing. The second purpose was to observe the effects of a high-pressure treatment on vesicle shapes, considering that pressure influences phospholipids' physical state, bilayer area, and water volume.

We report here that egg yolk phosphatidylcholine vesicles remain spherical and that their volume is reduced by 16% during pressure loading to 285 MPa. A shape modification consisting of budding occurs on such vesicles during pressure release. Cholesterol-containing vesicles (28 mol%) remain spherical during the whole hydrostatic treatment; their volume decrease during pressurization to 285 MPa is 8%.

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MATERIALS AND METHODS

Materials

Egg yolk phosphatidylcholine (EYPC) and cholesterol of high purity (>99%) were purchased from Sigma Chemical Co. (St. Louis, MO).

EYPC, which is a complex mixture of phosphatidylcholines, exhibits a gel to liquid-crystalline transition temperature ranging from −15°C to −7°C (New, 1990).

Vesicle preparation

Giant vesicles were obtained using a modification of the technique described by Reeves and Dowben (1969). The lipid was dissolved in chloroform:methanol (1:1 v/v) at a concentration of 1.2×10^{-3} M. The solution was drawn into glass capillary tubes (0.6 mm in diameter) and then expelled with a stream of nitrogen. After solvent evaporation for 24 h, capillary tubes containing a thin film of phospholipids were filled with distilled water saturated with nitrogen, sealed at one end, and then allowed to equilibrate for 8–12 h. Vesicles were collected in distilled water and were used immediately after preparation. Cholesterol-containing vesicles were prepared identically, except that cholesterol was added in chloroform: methanol at a molar ratio of 40 molecules for 100 molecules of phosphatidylcholine (28 mol of cholesterol for 100 mol of total lipids). The vesicles selected for microscopic observations had a large core free of internal vesicle and were not subjected to sedimentation.

Pressurization chamber, microscope, image analysis

The high-pressure visualization device was designed to observe on line vesicles or biological cells during a high-pressure treatment through a light microscope (Fig. 1). The high-pressure device is connected to a manually operated piston screw pump of 3 ml (Novaswiss, Cesson, Switzerland), which operates up to 700 MPa. This pump is especially designed for applications during which a small volume (typically 5 ml) is to be compressed.

The two sapphire windows and the spacer delimit the chamber (about 30 μl) where samples could be observed. Pressure and temperature were respectively controlled in this chamber with a high-pressure gauge (Kistler, Paris, France) and a thermocouple (Butec, Erie, PA). The temperature was not regulated but stayed almost constant (23 ± 0.5°C) during the whole experiment because of the small liquid amount in the circuit and the great exchange surface.

The system was filled with 5 ml of a vesicle suspension. The small volume of the high-pressure visualization chamber allows the observation of individual floating vesicles during the whole experiment. Because of the flux induced by compression, only one or two vesicle(s) could be observed during each high-pressure treatment. Fifteen EYPC and 15 cholesterol-containing vesicles have been observed at the time of 30 distinct experiments.

The hydrostatic pressure treatment in the chamber consisted of pressure loading to 285 MPa within 3 min, 2-min holding time, and a release to atmospheric pressure completed in about 3 min.

The high-pressure device could be disposed directly on the stage of an inverted phase-contrast light microscope (Leitz, Wetzlar, Germany), equipped with an objective lens (Fluotar GF 25/0.35; Leitz, Wetzlar, Germany). The magnification of the objective lens was limited by the long working distance (17 mm) required for the observation in the chamber.

Measurement of areas and volumes of vesicles

Microscopic observation could be recorded instantaneously via a 1/3′ CCD camera (I2S, Pessac, France) on a microcomputer (Hewlett Packard, Sunnyvale, CA) through an image-grabbing system (Matrox, Dorval, Canada). The images were analyzed with Visilog software (Noesis, Velizy, France). Each image consists of a 758 × 576 pixel matrix of 256 grey levels. Vesicles were individually analyzed to find their projected area. The vesicle surface and volume can then be calculated from the projection area data if their shape can be assumed to be spherical (discoid projection). The image analysis technique has been described previously (Berner and Gervais, 1994) and has been improved for this type of observation.

The sharpness of the whole measurement system has been tested with standardized latex beads (9.06 μm in diameter; Coulter Electronics, Luton, England). The effect of pressure on visualization has also been estimated with glass beads (diameter < 40 μm; Prolabo, Fontenay, France), on which hydrostatic pressure had no noticeable effect at the level used. These two experiments have given a deviation lower than 3% in the relative volume estimation. Moreover, analysis was only carried out on very large vesicles, which enhances the system accuracy.

RESULTS

Shape transitions

The morphological behavior of an EYPC vesicle without cholesterol during a high-pressure treatment is proposed through successive microscopic digitalized pictures in Fig. 2. During pressure loading (Fig. 2 *A*) and holding time at 285 MPa (Fig. 2 *B*), the vesicle remains spherical. A shape modification occurs during pressure release. A slight dissymmetry appears between 260 and 250 MPa and grows

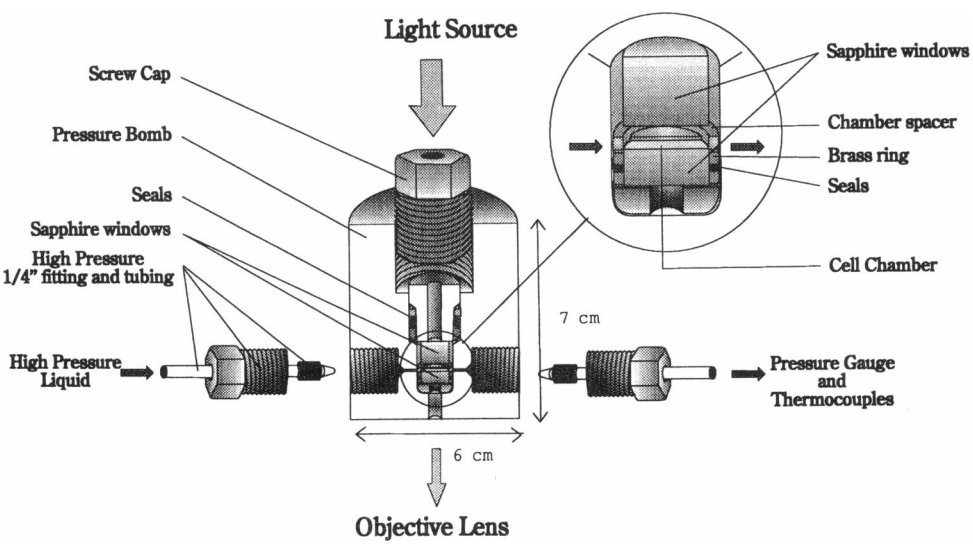

FIGURE 1 Schematic diagram of the device allowing microscopic observations during pressure application. The optical chamber (about 30 μl), which appears at the upper left, consists of two sapphire windows and a chamber spacer. To prevent leakage of pressurization medium, toric seals, brass rings, and gold films are fitted together. The water is externally pressurized and connected to the chamber through lateral inlet using high-pressure fittings and tubing. Pressure and temperature are always measured with a suitable pressure gauge and thermocouple.

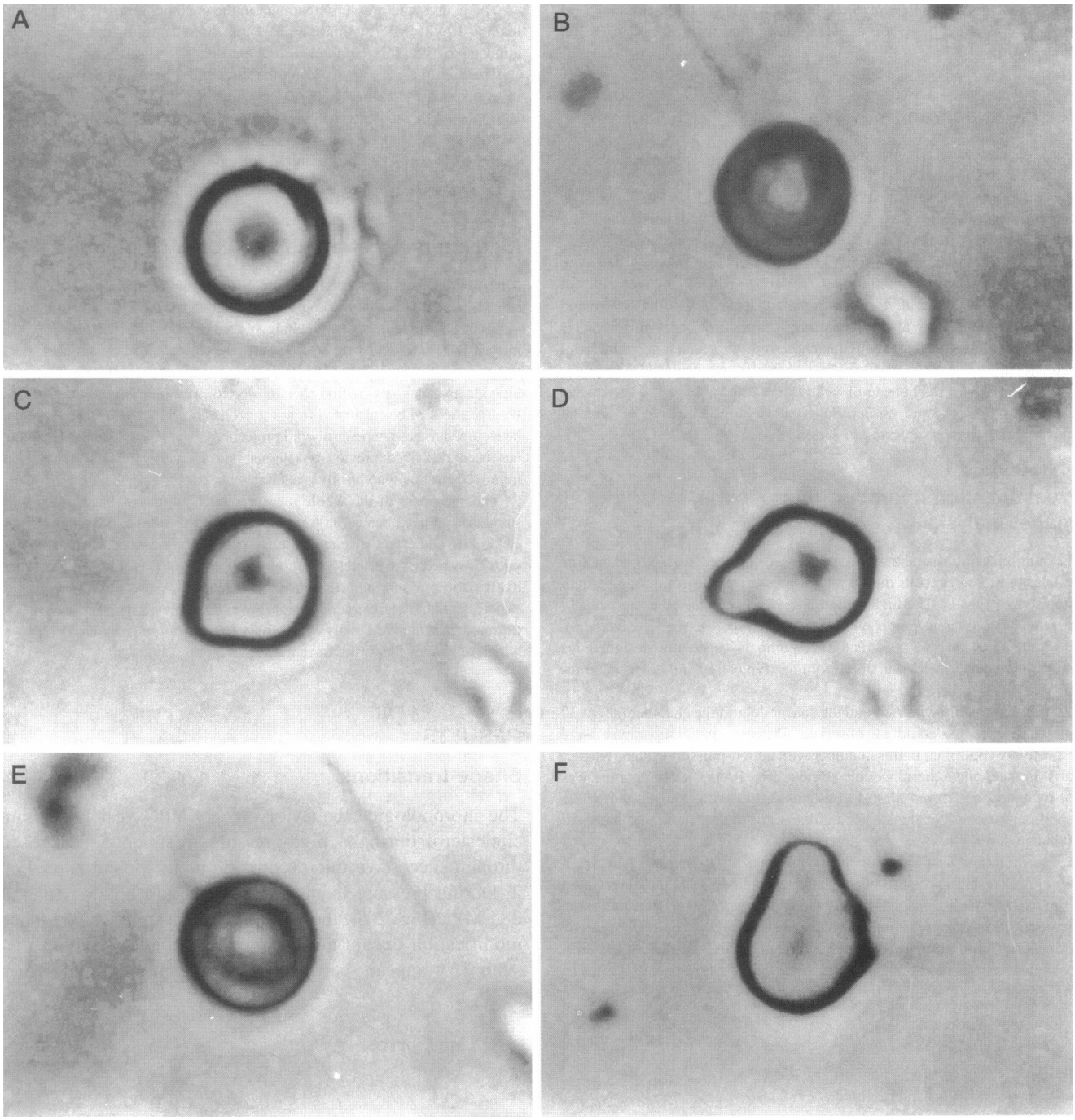

FIGURE 2 Example of a pure EYPC vesicle observation during two consecutive high-pressure cycles (approximate scale, ×1500). First pressurization cycle: (*A*) Pressure (p) = 0.1 MPa, time (t) = 0 min, volume measured (V) = 1875 μm^3. (*B*) p = 285 MPa, t = 3 min, V = 1569 μm^3. (*C*) p = 200 MPa, t = 6 min. (*D*) p = 0.1 MPa, t = 8 min. Second pressurization cycle (begining 9 min after the end of the first one): (*E*) p = 285 MPa, t = 20 min, V = 1580 μm^3. (*F*) p = 0.1 MPa, t = 25 min.

continuously (Fig. 2 *C*), with pressure release leading to bud formation. The vesicle definitively keeps this new shape (Fig. 2 *D*) at atmospheric pressure. This phenomenon has only been observed when the pressure level of the treatment was higher than 200 MPa. When a budded vesicle is compressed again, the bud progressively retracts during pressure loading and the vesicle recovers its spherical shape (Fig. 2 *E*) when the pressure level reaches 250 MPa. A consecutive pressure release produces a shape transition (Fig. 2 *F*) similar to the one described for the first pressurization cycle.

Successive pictures of a cholesterol-containing EYPC vesicle during a high-pressure treatment are shown in Fig. 3. In this case, the vesicle remains spherical during the whole treatment, including pressure release. The application of a

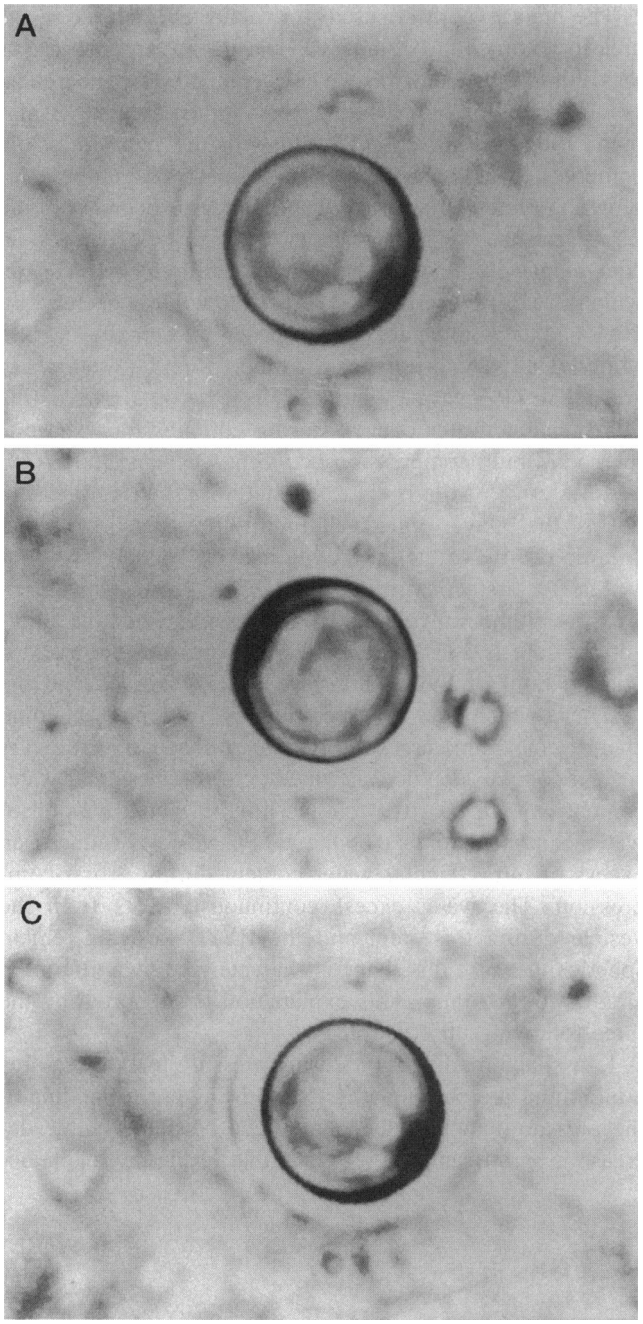

FIGURE 3 Example of EYPC vesicle containing cholesterol (28 mol%) during a high-pressure treatment (approximate scale, ×1500). (*A*) Pressure (p) = 0.1 MPa, time (t) = 0 min, volume measured (V) = 2600 μm^3. (*B*) p = 285 MPa, t = 3 min, V = 2400 μm^3. (*C*) p = 285 MPa, t = 8 min, V = 2620 μm^3.

second pressurization cycle to this vesicle does not induce any shape modification.

The morphological behavior of the vesicles during a complete high-pressure treatment was observed 15 times for each kind of vesicle. As the vesicles are subjected to rotation and displacement in the chamber, their apparent shapes are related to the observation angle, so the images of the pure EYPC vesicle that are proposed in Fig. 2 correspond to the most representative shape transition that was observed. Furthermore, pressurized vesicle samples, containing approximately 20 vesicles, were systematically observed after each treatment, and no large spherical vesicles remained in the EYPC vesicle samples, whereas all vesicles were spherical in the cholesterol-containing vesicle samples.

Bilayer lateral compressibilities

Areas of the two types of vesicles were calculated from image analysis data acquired during compression to 285 MPa while vesicles were spherical. The mean area of 15 individual vesicles for each type is presented in Fig. 4. Assuming that pressure-induced modification of the bilayer thickness is negligible in relation to vesicle size, the area variation is attributed to the isotropic lateral compression of the bilayer.

The area decrease of the pure EYPC vesicles during pressure loading to 285 MPa is 11% of the initial area. The main compression occurs between 125 and 225 MPa.

This increase in compressibility could be related to a structure change of phospholipids located in the bilayer. Indeed, EYPC are, at atmospheric pressure, in the liquid crystalline state because their transition temperature (*Tm*) ranges from −15°C to −7°C. As *Tm* increases with pressure level at a rate of about 0.15–0.3°C/MPa, the region of liquid-to-gel transition change is probably located around 150 MPa at 23°C. Lipids are more compressible in the liquid-crystalline than in the gel phase (Böttner et al., 1994). These properties agree well with the measured area variation of the vesicles.

The area compression of the cholesterol-containing vesicles is found to be a quasilinear function of the pressure level. The cholesterol-containing vesicles are less com-

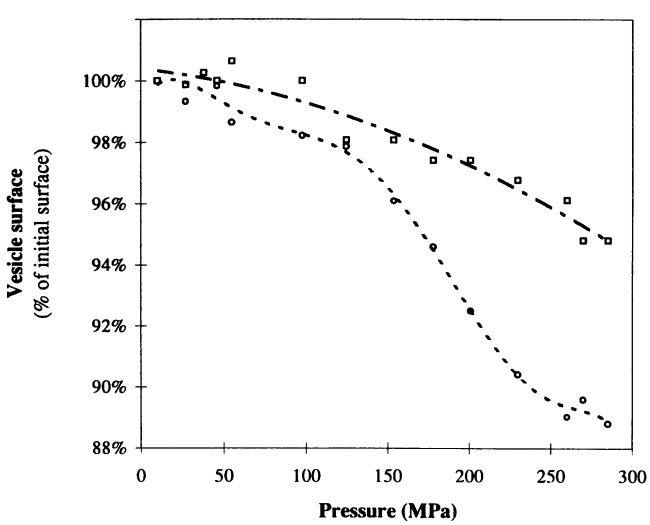

FIGURE 4 Relative surfaces of EYPC vesicle bilayer during pressure loading up to 285 MPa, as measured by image analysis: pure EYPC (○) and with cholesterol (28 mol%) (□).

pressible than the pure EYPC vesicles because their area decrease between 0.1 and 285 MPa is 5%.

The relevance of these compressibility values is confirmed by the good agreement with those determined by neutron diffraction, which give values of 6–14% for the lateral compressibility of dimyristoyl-phosphatidylcholine bilayers between 0.1 and 190 MPa (Braganza and Worcester, 1986). Concerning the influence of cholesterol on phospholipid lateral compressibility, it is of interest to note that cholesterol induces a drastic decrease in the pressure dependence of the dipalmitoylphosphatidylcholine (DPPC) phase transition temperature d*Tm*/d*P*. This value, typically 0.15–0.3°C/MPa, is 40% reduced in a 23 mol% cholesterol-containing DPPC vesicle (Muller and Galla, 1983). However, a much smaller decrease (5–10%) in the pressure-temperature dependence of dioleoylphosphatidylcholine (DOPC) bilayers caused by the addition of 35–40 mol of cholesterol for 100 mol of total lipids was reported by Chong and Cossins (1984). The nature of the acyl chains influences the effects of cholesterol on the bilayer compressibility. These differing results indicate that cholesterol reduces the compressibility of bilayers containing saturated acyl chains (DPPC) by its packing effect and had a less pronounced influence on the compressibility of unsaturated phosphlipid bilayers (DOPC). In fact, the presence of double bonds is thought to be a main cause of area compressibility of DOPC bilayers. Our results suggest that cholesterol has a condensing effect on EYPC bilayers, which, contrary to DOPC, are composed of a wild mixture of unsaturated and saturated acyl chains. Moreover, the addition of cholesterol (28 mol%) probably suppresses the phase transition of the EYPC bilayer because the cholesterol-containing vesicles do not exhibit a main area decrease around 150 MPa, unlike pure EYPC vesicles. It should be remarked that EYPC vesicles have undergone a phase transition between 125 and 225 MPa and that the coexistence of liquid and gel-crystalline domains in this pressure range has no influence on vesicle shape during compression.

DISCUSSION

The difference between the morphological behaviors of the two types of vesicles during pressure release shows that an area-to-volume ratio corresponding to a spherical shape was kept during the whole hydrostatic treatment for the cholesterol-containing vesicle. In fact, the shape modification of the EYPC vesicles implies an increase in its area-to-volume ratio during pressure release. After a second compression the EYPC vesicles recover a spherical shape and an area equal to the one measured after a first compression to 285 MPa. From these observations it can be deduced that the EYPC bilayers have not been subjected to fusion with other internal bilayers, which could have induced an increase in vesicle area, during the two compression cycles. Moreover, the oscillation between a nonspherical shape at 0.1 MPa and a spherical shape at 285 MPa was observed through 15 distinct experiments.

The main hypothesis used to explain the increase in the area-to-volume ratio of the EYPC vesicles is an intravesicular volume decrease. Thus we have compared the theoretical volume variation of the intravesicular water content (Bridgman, 1964) during compression with the measured bulk compression of the two types of vesicles. The curves presented in Fig. 5 demonstrate that the volume decrease of the EYPC vesicles is greater than the one expected for an equivalent volume of water. The bilayer area compression, which is 11%, corresponds to a vesicle volume decrease of 16%, and the volume decrease of water between 0.1 and 285 MPa is 10%. The difference in compression between water and the EYPC vesicles induces the exit of a part of the intravesicular water content during compression. The difference in bulk compression between water and the EYPC vesicles at 285 MPa is approximately 6%. During pressure release the bilayer regains its initial area, and the internal volume is 94% of the initial volume.

After a first compression the EYPC vesicles have an area-to-volume ratio characterizing spheres when the ambient pressure is 285 MPa. At a lower pressure the area-to-volume ratio of the EYPC vesicles is increased and the vesicles, which are governed by the minimum bending energy constraint, are subjected to a shape transition. A second compression does not induce further vesicular water content exit. In fact, the relative low bulk compression of water compared to that of the EYPC vesicles induces an excess of intravesicular water content during a first compression. The water excess continuously exits from the vesicle during this step, and the EYPC vesicles remain spherical because this shape is characterized by a minimum area-to-volume ratio. This explanation is supported by the three following observations:

1. A shape modification during pressure release requires a minimum pressure loading to 200 MPa, corresponding to the pressure at which the pure EYPC vesicle volume decrease is significantly higher than that of the internal aque-

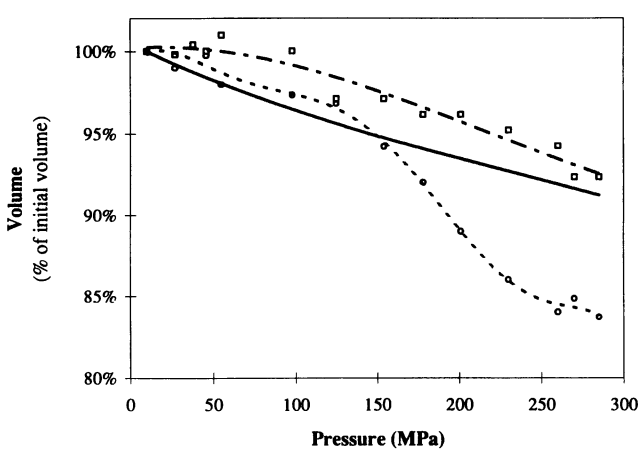

FIGURE 5 Relative volumes of EYPC vesicle during pressure loading up to 285 MPa, as measured by image analysis: pure EYPC (○) and with cholesterol (28 mol%) (□) compared to pure water theoretical compression (——).

ous content. The change in the physical state of the EYPC bilayer around this pressure level is probably the main cause of the high bulk compressibility difference between the vesicle and its water content.

2. After the first compression the EYPC vesicle shape is spherical at 285 MPa and nonspherical at ambient pressure.

3. The pear shape that characterizes the EYPC vesicles after pressure release is a shape of low bending energy, theoretically determined by Seifert et al. (1991), for a vesicle of constant area and subjected to an internal volume decrease of 5–10%.

Fig. 5 shows that the measured volume of the cholesterol-containing vesicles during compression is always equal to, or slightly higher than, the theoretical volume of the vesicle water contents. The low lateral compression of the cholesterol-containing bilayer does not imply any intravesicular water content loss during compression and explains the constant spherical shape of the cholesterol-containing vesicle during the entire high-pressure treatment.

This work demonstrates with direct visualization that EYPC vesicles, whether or not they contain cholesterol, are not subjected to shape transition during hydrostatic compression. The shape change of pure EYPC vesicles observed through pressure release is a direct result of the higher bulk compression of these vesicles compared to that of water. This difference in bulk compressions induces a greater vesicle volume decrease than their water content volume decrease and causes a water exit from the vesicle during pressure loading. This study confirms the fact that cholesterol significantly reduces phospholipid bilayer compressibility. The incorporation of cholesterol (28 mol%) into EYPC bilayers prevents the loss of water from the vesicles during an entire high-pressure treatment to 285 MPa. These results confirm the hypothesis that cholesterol acts in membranes by limiting the consequences of the changes in environmental physical parameters (Reis et al., 1996).

The most important biological implication of this phenomenon is that cholesterol, which is already known to reduce phase separation of phospholipids, could prevent the loss of intracellular content during high-pressure treatment by its condensing effect on lipid bilayers.

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