Bending Elasticities of Model Membranes: Influences of Temperature and Sterol Content

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ABSTRACT Giant liposomes obtained by electroformation and observed by phase-contrast video microscopy show spontaneous deformations originating from Brownian motion that are characterized, in the case of quasispherical vesicles, by two parameters only, the membrane tension $\bar{\sigma}$ and the bending elasticity k_c. For liposomes containing dimyristoyl phosphatidylcholine (DMPC) or a 10 mol% cholesterol/DMPC mixture, the mechanical property of the membrane, k_c, is shown to be temperature dependent on approaching the main (thermotropic) phase transition temperature T_m. In the case of DMPC/cholesterol bilayers, we also obtained evidence for a relation between the bending elasticity and the corresponding temperature/cholesterol molecular ratio phase diagram. Comparison of DMPC/cholesterol with DMPC/cholesterol sulfate bilayers at 30°C containing 30% sterol ratio shows that k_c is independent of the surface charge density of the bilayer. Finally, bending elasticities of red blood cell (RBC) total lipid extracts lead to a very low k_c at 37°C if we refer to DMPC/cholesterol bilayers. At 25°C, the very low bending elasticity of a cholesterol-free RBC lipid extract seems to be related to a phase coexistence, as it can be observed by solid-state ^{31}P-NMR. At the same temperature, the cholesterol-containing RBC lipid extract membrane shows an increase in the bending constant comparable to the one observed for a high cholesterol ratio in DMPC membranes.

INTRODUCTION

Biological membranes are highly dynamic systems. They are able to deform themselves following external stresses (erythrocytes in blood capillary) or cytoskeleton solicitations (mitosis). In a general way, such geometric transformations cannot be considered without involving at least some physical properties of the membrane such as lipid fluidity, shear, stretching, and bending elasticities (Sackmann et al., 1986; Bloom et al., 1991; Sackmann, 1994). Giant unilamellar vesicles were shown to deform similarly (Berndl et al., 1990; Käs and Sackmann, 1991), proving such behavior to be quite independent of the membrane nature.

Shape changes can be understood by using reduced parameters. This means that shape transition lines are found independently of liposome characteristics such as geometric (volume, area) or mechanical (elasticity) ones (Seifert et al., 1991; Heinrich et al., 1992). Nevertheless, the energy cost of any deformation can be evaluated according to effective parameters and compared to a reference state. To study the connection between membrane composition and deformability of sterol-containing bilayers, we can thus vary the former and measure the response of the system.

Sterols are widespread membrane molecules found in most of the plasma membranes. In mammals, cholesterol is known to act as a regulator of membrane fluidity and many other different bilayer properties. One point to notice is a modulation of the physical state of a sterol-containing membrane as a function of its sterol enrichment. This is the case for water permeability (Milon et al., 1986), chain order parameters of phospholipids (Davis, 1983; Léonard and Dufourc, 1991), polymorphism (Epand and Bottega, 1987; Vist and Davis, 1990; Tampé et al., 1991; McKay and Robert, 1994; Slotte, 1995), self-diffusion coefficients (Almeida et al., 1992), viscoelasticity (Chabanel et al., 1983; Elise Gabriel and Roberts, 1986), and membrane mechanical properties (Evans and Needham, 1986; Needham et al., 1988; Duwe et al., 1990; Needham and Nunn, 1990; Bloom et al., 1991). To reinvestigate the last point, we began a systematic study to compare bending elasticities as a function of sterol content and temperature, for model and natural membranes. We will briefly give some theoretical background for the understanding of the bending modulus measurement method using thermal fluctuations of giant quasispherical vesicles. The influences of temperature and sterol content were investigated for dimyristoyl phosphatidylcholine (DMPC)/cholesterol (Chol) bilayers. To check whether the sterol effect is modified by a change in its polar head region, cholesterol was substituted by an anionic derivative, the cholesterol sulfate (CholS), found mainly in the stratum corneum, a specialized outer layer of the skin. Finally, these complex lipid mixtures were related to membranes obtained from total lipid extracts of red blood cells (RBCs), in the presence and absence of physiological cho-

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lesterol concentration. The macroscopic behavior of the obtained liposomes using RBC lipid extracts is shown to be related to microscopic and macroscopic information obtained by solid-state ^{31}P-NMR measurements.

THEORETICAL BACKGROUND

Mechanical properties of membranes

In 1973, Helfrich introduced three elasticities characterizing the membrane mechanical responses to a stress (Helfrich, 1973), in the limit of a thin surface slightly deformed from its equilibrium shape. The first one, i.e., shear elasticity, has to play a role for biological membranes because of the cytoskeleton but seems to be ineffective for bilayers in their fluid state (Evans and Needham, 1987).

Stretching elasticity is important only if an expansion or a compression of the area is expected. The energy per unit area, f_s, required to increase a surface element S_0 by ΔS is equal to

$$f_s = \frac{k_s}{2}\left(\frac{\Delta S}{S_0}\right)^2$$

A typical value for k_s, the stretching modulus, is in the range 100–300 mJ/m^2 (Evans and Needham, 1987). Thus any deformation involving k_s needs a large amount of energy, comparable with that required when increasing an oil/water interface.

The third mechanical property of importance for membranes is bending elasticity. Considering S_0 deformed according to the two principal curvatures c_1 and c_2 at constant area, the bending energy per unit area f_c is

$$f_c = \frac{k_c}{2}(c_1 + c_2 - c_0)^2 + \bar{k}_c c_1 c_2 \qquad (1)$$

In this expression, k_c, the splay modulus, is always positive, a typical value for a lipid bilayer being 10^{-19} J (Faucon et al., 1989; Duwe et al., 1990; Evans and Rawicz, 1990; Niggemann et al., 1995). On the other side, $\bar{k}_c$, the saddle-splay modulus, can be either positive or negative, depending on the molecular characteristics of the membrane. Up to now there has been no experimentally determined value for the saddle-splay modulus; simulations give absolute values from k_BT (where k_B is the Boltzmann constant and T the temperature) to k_c, i.e., $\sim 25 \times k_BT$ (Szleifer et al., 1990). Moreover, when a closed object like a perfect or a slightly deformed sphere is considered, the integration of the Gaussian curvature over the total surface, $\oint c_1 c_2 \, dS$, is equal to 4π (Gauss-Bonnet theorem; Darboux, 1972). As a result, there is no contribution from the $\bar{k}_c$ term to the deformation free energy when we consider only continuous perturbations of a given shape.

Significant work has been done to invent experimental methods that make it possible to measure the bending modulus, k_c, of model membranes (Bo and Waugh, 1989; Evans and Rawicz, 1990; Kummrow and Helfrich, 1991). Historically, the first one was the analysis of thermal fluctuations of giant liposomes as seen by phase-contrast microscopy (Servuss et al., 1976; Schneider et al., 1984; Bivas et al., 1987; Faucon et al., 1989; Duwe et al., 1990; Niggemann et al., 1995); this is used herein to study k_c variation as a function of the model membrane composition. Consequently, a theoretical description of this phenomenon will be given first, before going into details of the experimental requirements.

Theoretical description of the thermal fluctuation phenomenon

In this description, only quasispherical liposomes will be considered, i.e., vesicles characterized, for a given inner volume $\mathcal{V}_0$, by a surface $\mathcal{S}_0$ in excess compared to the area of the sphere with the same volume. Defining R as the radius of the sphere:

$$\mathcal{V}_0 = \frac{4\pi R^3}{3} \qquad (2)$$

$$\mathcal{S}_0 = 4\pi R^2(1 + s) \qquad (3)$$

where $s \geq 0$ is the excess area. Looking at such a vesicle by a two-dimensional technique (Fig. 1), we observe the existence of membrane undulations originating from Brownian motion, as in the case of red blood cell flickering phenomena (Brochard and Lennon, 1975). These thermal fluctuations induce permanent oscillations of the vesicle shape around a spherical form. Then we can define $r(\theta, \varphi, t)$ as the function locating the bilayer center in the direction (θ, φ) (spherical coordinate system) at a time t:

$$r(\theta, \varphi, t) = R[1 + u(\theta, \varphi, t)] \qquad (4)$$

$u(\theta, \varphi, t)$ is the relative deformation assumed to be small compared to 1 ($|u| \ll 1$ and hence, $s \ll 1$). In that case, we can write all of the characteristic quantities of a liposome as a power series of $u(\theta, \varphi, t)$ (and its derivatives $u_\theta, u_\varphi, \ldots$), keeping only the terms up to the second order. This can be done for the volume, the surface, and the elastic energy functionals, respectively $\mathcal{V}\{u\}$, $\mathcal{S}\{u\}$, and $\mathcal{F}_u\{u\}$ (Mitov et al., 1992):

$$\mathcal{V}\{u\} = \oint V(u)\, d\theta\, d\varphi, \quad V(u) = R^3\left(\frac{1}{3} + u + u^2\right)\sin\theta \qquad (5)$$

$$\mathcal{S}\{u\} = \oint S(u, u_\theta, u_\varphi)\, d\theta\, d\varphi,$$
$$S(u, u_\theta, u_\varphi) = R^2\left(1 + 2u + u^2 + \frac{(\nabla u)^2}{2}\right)\sin\theta \qquad (6)$$

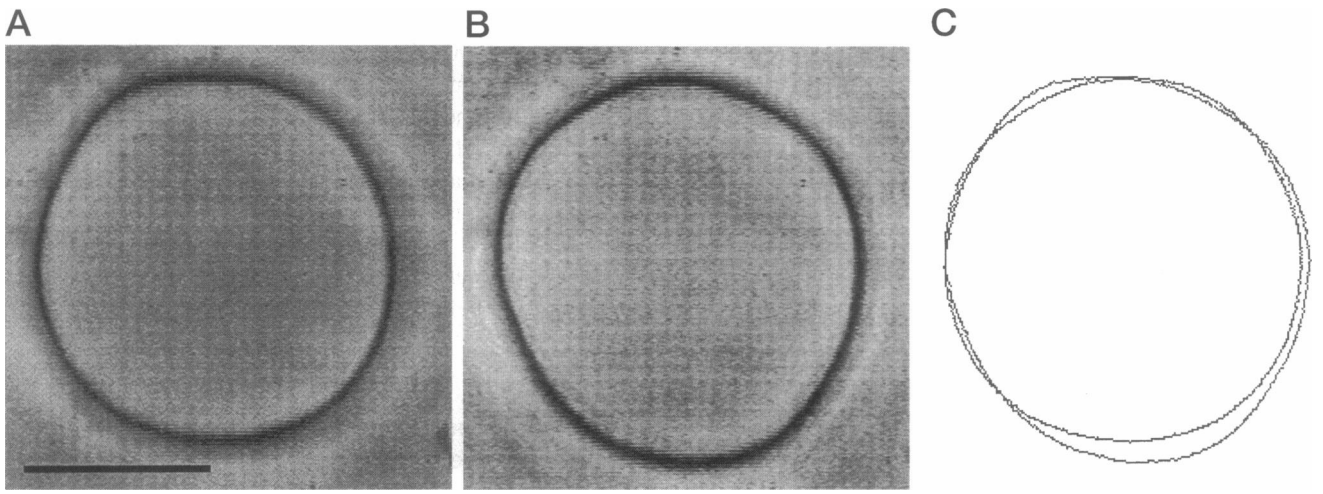

FIGURE 1 Two video images of a quasispherical vesicle, as seen through a phase-contrast microscope at different times (*A* and *B*). After image analysis, the contour fluctuations function, $\rho(\varphi,t)$, can be drawn and superimposed for the two video images (*C*) and related to the amplitudes of the three-dimensional shape through the autocorrelation function of the contour deformations. The bar at the lower corner in *A* corresponds to 10 μm.

$$\mathcal{F}_c\{u\} = \oint F_c(u, u_\theta, u_\varphi, u_{\theta\theta}, u_{\varphi\varphi})\, d\theta\, d\varphi,$$

$$F_c(u, u_\theta, u_\varphi, u_{\theta\theta}, u_{\varphi\varphi}) \tag{7}$$

$$= \frac{k_c}{2}[4 - 4\nabla^2 u + 4u\nabla^2 u + (\nabla^2 u)^2 + 2(\nabla u)^2]\sin\theta$$

with

$$(\nabla u)^2 = \left(u_\theta^2 + \frac{u_\varphi^2}{\sin^2\theta}\right) \quad \nabla_u^2 = \left(u_{\theta\theta} + \frac{\cos\theta}{\sin\theta}u_\theta + \frac{u_{\varphi\varphi}}{\sin^2\theta}\right)$$

where we can omit the Gaussian term in Eq. 7 as a constant when the sphere topology is maintained (Darboux, 1972).

We consider now the volume and the area of our liposome. We have shown previously that it is very expensive to increase the overall area of the vesicle. On the other hand, our experiences last no more than 10 min, the hydrostatic pressure difference between the interior and the exterior of the liposome being very small (otherwise, we cannot expect large thermal fluctuations). We can thus assume that both the area and the volume are constant during the observation of the thermal fluctuation phenomenon. Consequently we can write

$$\mathcal{V}\{u\} = \mathcal{V}_0 \tag{8}$$

$$\mathcal{S}\{u\} = \mathcal{S}_0 \tag{9}$$

where $\mathcal{V}_0$ and $\mathcal{S}_0$ are the constants representing, respectively, the total volume and area of the liposome.

The quasispherical vesicle, limited by a single bilayer and fully characterized by ($\mathcal{V}_0$, $\mathcal{S}_0$), is immersed in a thermal bath at a temperature T. The shapes minimizing the bending energy, Eq. 7, and keeping both the volume $\mathcal{V}\{u\}$ and area $\mathcal{S}\{u\}$ constant are obtained by solving a variational problem or, equivalently, by minimization of the functional (Arfken, 1985):

$$\mathcal{F}\{u\} = \mathcal{F}_c\{u\} + \sigma\mathcal{S}\{u\} - \Delta p \cdot \mathcal{V}\{u\} \tag{10}$$

σ and Δp are the Lagrange multipliers associated with the constant area and volume constraints (physically, σ can be considered as a membrane tension and Δp as the pressure difference between the inside and the outside of the membrane).

After calculation (Mitov et al., 1992), we obtain the expression for the fluctuation amplitudes $u(\theta, \varphi, t)$:

$$u(\theta, \varphi, t) = \frac{A_0^0}{\sqrt{4\pi}} + \sum_{n=2,|m|\le n}^{n_{max}} U_n^m(t) Y_n^m(\theta, \varphi) \tag{11}$$

Y_n^m being the spherical harmonics (Arfken, 1985). n_{max} is a cutoff introduced in the summation to limit the deformation to wavelengths larger than molecular size. Using the reduced membrane tension $\bar{\sigma} = \sigma R^2/k_c$ and the reduced pressure difference $\bar{p} = \Delta p R^3/k_c$, we write for the constant factor A_0^0 (the static deformation compared to the sphere with a radius R):

$$\frac{A_0^0}{\sqrt{4\pi}} = -\frac{\bar{p} - 2\bar{\sigma}}{2(\bar{p} - \bar{\sigma})} \sim 0 \Rightarrow \bar{p} \sim 2\bar{\sigma} \tag{12}$$

which is somehow an equivalent expression for the well-known Laplace relation (Mitov et al., 1992). The dynamic part itself, $U_n^m(t)$, is not directly related to a theoretical quantity, but the time average of its square modulus, $\langle|U_n^m(t)|^2\rangle$, can be obtained using the equipartition theorem:

$$n \ge 2, \qquad |m| \le n$$

$$\langle|U_n^m(t)|^2\rangle = \frac{k_B T}{k_c} \times \frac{1}{(n-1)(n+2)[\bar{\sigma} + n(n+1)]} \tag{13}$$

Measurable quantities

Equations 11 and 13 show that the dynamic part of the deformation (or equivalently the thermal fluctuations) is defined using spherical harmonics, $Y_n^m(\theta, \varphi)$. Unfortunately, we cannot observe the liposome in three-dimensional space (using, for example, a confocal microscope) at a sufficiently high resolution in time. As a result of the spherical symmetry of the static shape, one can demonstrate that a two-dimensional section of a quasispherical vesicle (Fig. 1) is enough to obtain the information we are interested in, i.e., the fluctuation amplitudes $\langle|U_n^m|^2\rangle$.

The image analysis of Fig. 1 *B* leads to the instantaneous contour of the equatorial section of the liposome, $\rho(\varphi, t) = R[1 + u(\pi/2, \varphi, t)]$ (Fig. 1 *C*). Two different methods have been proposed in the literature to link this experimental information to the bending modulus k_c. One was introduced by Engelhardt et al. (1985). Calling $V_q(t)$ the q mode amplitude obtained using a Fourier analysis of the relative contour deformation $u(\pi/2, \varphi, t) = (\rho(\varphi, t) - R)/R$ at t, we can write

$$q \geq 2, \quad V_q(t) = \frac{1}{\sqrt{2\pi}} \oint_{\varphi=0}^{2\pi} u(\pi/2, \varphi, t)\exp(\mathrm{i}q\varphi)\, \mathrm{d}\varphi$$

$$= \sum_{n=|q|}^{n_{max}} U_n^q(t)\cdot\Theta_n^q(\pi/2) \quad (14)$$

$$\Rightarrow \langle|V_q(t)|^2\rangle = \sum_{n=\max(2,|q|)}^{n_{max}} \langle|U_n^m(t)^2\rangle[\Theta_n^q(\pi/2)]^2 \quad (15)$$

where $\Theta_n^m(\theta)$ is defined through the Legendre polynomials, P_n^m, using the relation (Arfken, 1985)

$$\Theta_n^m(\theta) = (-1)^m\sqrt{\frac{2n+1}{2}\cdot\frac{(n-m)!}{(n+m)!}} \times P_n^m(\cos\theta)$$

The second method was introduced by Bivas et al. (1987). It is based on the analysis of the autocorrelation function of the contour fluctuations, $\xi(\gamma, t)$:

$$\xi(\gamma, t) = \frac{1}{R^2}\left[\frac{1}{2\pi}\oint_0^{2\pi} \rho(\varphi + \gamma, t)\overline{\rho(\varphi, t)}\, \mathrm{d}\varphi - \rho^2(t)\right] \quad (16)$$

where γ is the correlation angle, $\bar{\rho}$ is the ρ complex conjugate function, and $\rho(t)$ is the angle average of the contour radius function $\rho(\varphi, t)$:

$$\rho(t) = \frac{1}{2\pi}\oint 4\rho(\varphi, t)\, \mathrm{d}\varphi$$

Using spherical harmonics decomposition, the time average of the autocorrelation function, $\xi(\gamma)$, can be written equivalently (Faucon et al., 1989):

$$\xi(\gamma) = \sum_{n=2}^{n_{max}} \langle B_n(t)\rangle P_n^0(\cos\gamma) - \frac{k_BT}{k_c} P_0^0(\cos\gamma) \sum_{n=2}^{n_{max}} \frac{[\Theta_n^0(\pi/2)]^2}{(n+2)(n-1)[\bar{\sigma} + n(n+1)]} \quad (17)$$

$$\langle B_n(t)\rangle = \frac{k_BT}{4\pi k_c} \times \frac{(2n+1)}{(n+2)(n-1)[\bar{\sigma} + n(n+1)]} = \frac{(2n+1)}{4\pi}\langle|U_n^m(t)|^2\rangle \quad (18)$$

from which k_c and $\bar{\sigma}$ can be determined using a least-squares method (Mitov et al., 1992).

It is important to note that the autocorrelation function, Eq. 16, and its decomposition into Legendre polynomials, Eq. 17, give directly a proportionality relation, Eq. 18, between the theoretical amplitudes of the spherical harmonics, $\langle|U_n^m(t)|^2\rangle$, and the experimentally measurable quantities, $\langle B_n(t)\rangle$. Comparing to Eq. 15, one observes that $\langle|V_q(t)|^2\rangle$ is unfortunately a complex sum over many $\langle|U_n^q(t)|^2\rangle$, depending on k_c and $\bar{\sigma}$. This complicates the parameter-fitting procedure and makes this approach very tedious at least, if not unsuitable.

MATERIALS AND METHODS

Lipids were obtained from Fluka (Buchs, Switzerland). Cholesterol sulfate was kindly provided by Christian Dior Laboratory (St Jean de Braye, France). Red blood cell membrane lipids were extracted by hexane/isopropanol according to the method of Radin (1981). In the case of red blood cells this solvent mixture is preferable to commonly used chloroform/methanol mixtures, as it dissolves almost no pigments (Radin, 1981). Briefly, with constant stirring, 20 ml of packed cells are added dropwise to 500 ml of hexane/isopropanol (3:2, v:v), followed by filtering. The solvent is evaporated under reduced pressure. The resulting lipidic film is immediately dispersed in cyclohexane, lyophilized, and stored under vacuum at −20°C. For further purification of RBC lipids from nonlipid contaminants, Sephadex (G-25, coarse, beaded; Pharmacia Fine Chemicals) column chromatography is performed as described by Rouser et al. (1967) and Kates (1972). The integrity of the lipid extract was qualitatively checked by two-dimensional thin-layer chromatography (elution solvents: 1) chloroform/methanol/water (65:25:4 v:v:v); 2) butanol/acetic acid/water (6:2:2 v:v:v)). Only traces of lysolipids were detected. The relative amounts of the main phospholipid classes in the lipid extract were determined by high-resolution ^{31}P-NMR and found to be in accordance with known ratios (34% phosphatidylcholine, 27% phosphatidylethanolamine, 24% sphingomyelin, and 15% phosphatidylserine). The sterol content of the RBC lipids was determined with a pathology laboratory system (BM/Hitachi 911; Hitachi, Tokyo, Japan) by a standard kit (Boehringer Mannheim Meylan, France SA) using cholesterol esterase and cholesterol oxydase reactions. Sterol extraction from the total RBC lipids was achieved by the so-called acetone precipation (Kates, 1972). Separation was verified by thin-layer chromatography. The chromatography of the acetone-insoluble fraction shows all phospholipids initially present, as well as the absence of free fatty acids, cholesterol, and diacylglycerides.

Solid-state ^{31}P-NMR measurements on lipid dispersions in excess water were carried out on a Bruker ARX300 implemented for high-power solid-state spectroscopy and operating at 121.49 MHz. A phase-cycled Hahn-echo pulse sequence (Rance and Byrd, 1983) with quadrature detection and gated proton decoupling was used. Samples were allowed to equilibrate for at least 30 min at a given temperature before the NMR signal was acquired; the temperature was regulated to ±1°C. Typical acquisition parameters were a spectral window of 50 kHz, a $\pi/2$ pulsewidth of 8 μs, delay between the two pulses forming the echo equal to 40 μs, a recycle delay of 6 s (50 s for high-resolution spectra), and 2200 scans. Spectral simulations were performed on an Alpha computer (Dec 4000, Digital, Nashua, NH) as described by Pott and Dufourc (1995), assuming either a spherical or a slightly ellipsoidal distribution of the bilayer normal. The amount of isotropic lines superimposed on a powder pattern was determined by simulation of a Gaussian or Lorentzian line and subsequent subtraction from the experimental spectrum. Percentages are expressed relative to the total spectral area.

The samples used for NMR studies were observed under a phase-contrast microscope for direct visualization of the consequences of a temperature change for liposome dispersions. Deionized water was added to obtain a lipid concentration close to 0.2 mg/ml. The vesicles were injected in a cell similar to the one described by Faucon et al. (1989). Then the cell was sealed and maintained at different temperatures (room temperature, 37°C and 50°C) for 1 day to allow equilibrium. The evolution of the liposome shape was followed from their equilibrium temperature (37°C and 50°C) to room temperature.

Electroformation of giant quasispherical liposomes

According to the model introduced in the previous paragraph, bending elasticity of membranes can be measured using quasispherical and unilamellar liposomes. GUVs (giant unilamellar vesicles) with a typical radius of 10 μm are used to obtain an accurate measurement of the thermal fluctuation deformations by video microscopy (see below). They must also be isolated and have no (visible) surface defects.

Such vesicles are prepared using electric fields to increase their formation rate and yield (Angelova et al., 1992b). Lipids are first solubilized into an organic solvent (chloroform/methanol 9:1, lipid concentration ~0.2 mg/ml). Two different experimental chambers are prepared, depending on the working distance of the microscope objective. The first one is used with an oil immersion setup when a temperature control is needed (this thermal regulation device is presented by Fernandez-Puente et al., 1994, and detailed by Fernandez-Puente, 1994). It is built from two Indium Tin Oxyde (ITO) covered glass slides separated by a 0.3-mm-thick silicon spacer (Fig. 2 *A*) (inner volume ~100 μl). The second chamber is used for room temperature observation with a water immersion objective (Angelova et al., 1992b). It is made of a 1-mm-thick glass cell (similar to a fluorescence cell) where two parallel platinum wires of 0.8 mm diameter have been introduced (Fig. 2 *B*) (inner volume ~300 μl).

The lipid solution is deposited on the electroformation cell conductor (~2 μl of the chloroform/methanol lipid solution in a single spot for ITO slides or in four to six different locations along the platinum wires). Organic solvents are removed by evaporation under vacuum for 1 h, and deionized water (Millipore mQ, Bedford, MA) is added slowly to prevent lipid dispersion. Then the electric field is applied at 10 Hz from low voltage (30 mV/mm) to "high" voltage (400 mV/mm) in 10 min. After a typical period of 2 h, quasispherical vesicles are formed. Usually liposomes stick (probably through filaments) to the conductor. Dispersion is then promoted by a low-frequency (4-Hz) field, sometimes followed by "a few taps" on the cell bottom. During the whole process, electroformation can be controlled by microscopy.

Data treatment

Fluctuating vesicles are observed with a phase-contrast microscope (Axiovert 135 with a water immersion ×40/0.75 objective or IM35 with an oil immersion ×100/1.25 objective; Zeiss, Oberkochen, Germany) and a CCD video camera (C2400–77; Hamamatsu, Hamamatsu City, Japan). Video images are recorded on a U-matic recorder (VO 7630; Sony, Tokyo, Japan), digitized on a Pericolor 1500 (Matra, Paris, France), and analyzed on an Alpha Computer (Dec 4000; Digital) (Mitov et al., 1992).

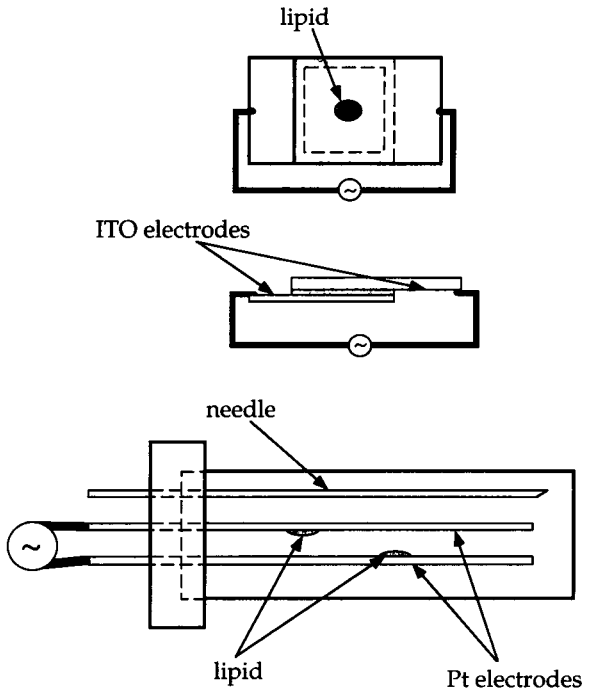

FIGURE 2 Sketches of the electroformation cells used (*A*) when temperature regulation is needed and (*B*) when observations are made at room temperature.

The contour extraction procedure (Fig. 1, *B* and *C*) is very simple when a phase-contrast microscope is used. The pictures in Fig. 1, *A* and *B*, were obtained after a background substraction. The equatorial cross section of the vesicle is a slightly deformed black circle. Looking at a defined direction φ from the center O, the intensity resembles that of an inverted Gaussian curve, which is close to the mean image intensity $\bar{I}$ everywhere, except in the contour region, where it decreases abruptly (within 5–10 pixels) through a minimum I_m, the value (but not the position) of which usually depends on the precise position of the objective with respect to the liposome bilayer. Fitting this intensity profile with a Gaussian function leads to the location of that minimum, assumed to be the membrane position in the φ direction. Using that procedure once for every φ direction, we obtain between 500 and 900 points for each contour, depending on the liposome size (Fig. 1 *C*).

A detailed description of the experimental limitations inherent in such a method can be found in Mitov et al. (1992). To summarize, the noise introduced by the video system (essentially the video recorder) and the digitization procedure leads to a white noise in the Fourier spectrum (an unknown constant value added to all $\langle|V_q(t)|^2\rangle$) and a Dirac δ-function added to the autocorrelation function $\xi(\gamma)$. Because of a nice mathematical property of the δ-function and the Legendre polynomials, this noise has no influence on the experimental $\langle B_n(t)\rangle$, thus leading to a higher precision than the Fourier method (Engelhardt et al., 1985). Using a large number of analyzed images ($N \approx 400$) leads to a precision of the mean value $\langle B_n(t)\rangle \approx N^{-1/2}$ due to the intrinsically stochastic nature of the thermal fluctuations. Moreover, it is known that video images are essentially the result of light signal accumulation on the camera target, each pixel of the CCD plate being illuminated for 40 ms. Each movement characterized by a speed greater than one pixel (to be exact, the distance separating two pixels) per 40 ms is blurred. Another important improvement in our treatment is the introduction of a correction factor to take into account this integration

effect of the video camera (Faucon et al., 1989). We also note that the integration effect becomes negligible when a stroboscopic lamp replaces the usual incandescent light as a microscope source (Méléard et al., 1992) or if a high-speed camera is used instead of an ordinary TV CCD camera.

The experimental autocorrelation functions can then be calculated and their Legendre polynomial amplitudes estimated. From Eqs. 17 and 18, we deduce these amplitudes to be independent quantities that can be directly corrected for the integration effect of the video camera (Faucon et al., 1989). This is the main advantage of the approach using the autocorrelation function compared to a more direct Fourier analysis of the contour deformation (Engelhardt et al., 1985). With these corrected data and for a set of p different experimental amplitudes B_n^{exp}, $2 \leq n \leq p + 1$, we must minimize the following sum using an iterative procedure:

$$\sum_{n=2}^{p+1}\left(\frac{B_n^{exp} - \langle B_n(t)\rangle}{D_n^{exp}}\right)^2$$

In this expression, B_n^{exp} and D_n^{exp} are, respectively, the n-order experimentaly estimated average amplitude and the corresponding dispersion of the P_n^0 Legendre polynomial amplitudes $\langle B_n(t)\rangle$ being the theoretical expression 18. A least-squares method leads to the estimation of $(k_c, \bar{\sigma})$ for a given liposome (Fig. 3). It can be noted that for low enough membrane tension, k_c is obtained independently from $\bar{\sigma}$ when n^2 is large compared to $\bar{\sigma}$ (Faucon et al., 1989).

Finally, this procedure is repeated on different liposomes to reach a k_c dispersion equal to a few percent (typically, a set of 10 different vesicles is required to obtain a dispersion for k_c of ~5%). It should be mentioned that such an average estimation for k_c requires about a month's work for each particular experimental condition, including a preliminary period for the electroformation procedure adjustment.

RESULTS

DMPC bilayers

The general requirements already introduced were taken into consideration for both the choice of fluctuating vesicles inside the electroformation cell and the experimental data that can be obtained from their analysis. In the case of DMPC in excess water (we are close to the infinite dilution condition, the lipid to water mass ratio being smaller than 0.1%), we know the phase diagram to be characterized by a thermotropic behavior. Whereas we already know that the vesicle reacts to a temperature change by a modification of its membrane tension (Käs and Sackmann, 1991), it has been reported recently that the bending rigidity also depends on the temperature (Fernandez-Puente et al., 1994; Hönger et al., 1994; Niggemann et al., 1995).

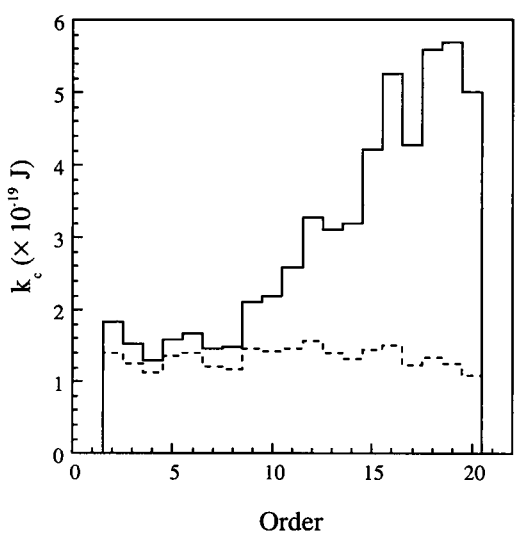

FIGURE 3 Results of the thermal fluctuations analysis of the liposome shown in Fig. 1. ——, Result of the fitting procedure obtained without introducing the correction factor (see text). – – –, Fitting procedure where the thermal fluctuation amplitudes are corrected from the video integration effect.

The results for k_c measurements obtained with pure DMPC liposomes are reported in Table 1. We should recall that for all of the results shown (as otherwise indicated), the cell temperature was held constant at the required temperature during the electroformation swelling and the thermal fluctuation video recording procedures. For a temperature range from 15°C to 50°C, the cell temperature is controlled with a precision of about ±0.25°C. The error bars in Table 1 are the usual standard deviations for k_c values only. It is possible to detect three different regions for k_c dependence as a function of the temperature. For $T > T_m + 6$°C (T_m = 23.9°C is the gel-to-liquid phase transition temperature for DMPC; Lewis et al., 1987), k_c seems to be independent of the temperature, the increase being too small to be meaningful compared to the error bars. This behavior does not seem to be general for unsaturated lipids far from their phase transition temperature (Niggemann et al., 1995), whereas it has also been found for other saturated phosphatidylcholine bilayers (Fernandez-Puente et al., 1994). Furthermore, k_c increases slowly when T is decreasing from $T_m + 6$°C to $T_m + 3$°C. This effect is not very important (~20%) but is statistically significant. This behavior is followed by a relatively sharp drop in the range $T_m + 3$°C to $T_m + 1$°C. Unfortunately, we were unable to measure k_c very close to T_m for technical reasons.

However, we notice that it is possible to pass through the phase transition region without destroying the vesicle dispersion (results not shown). Independently of the liposome considered when reducing T to T_m, we observe first a rapid disappearance of the thermal fluctuations at the microscope resolution. This phenomenon is not related to a very precise temperature, because other liposomes still fluctuate. At a temperature closer to T_m, one can distinguish between two

TABLE 1 Bending moduli for DMPC/Chol membranes

T (°C)	$k_c^{100/0}$ ($\times 10^{-19}$ J)	$k_c^{90/10}$ ($\times 10^{-19}$ J)	$k_c^{70/30}$ ($\times 10^{-19}$ J)	$k_c^{50/50}$ ($\times 10^{-19}$ J)
20			6.1 ± 0.2	
24.8		0.9 ± 0.1		
25	0.8 ± 0.13			
25.2		1.45 ± 0.06		
26	1.1 ± 0.1	1.63 ± 0.07		
27	1.52 ± 0.06	2.23 ± 0.07		
30	1.30 ± 0.08	2.00 ± 0.1	4.1 ± 0.25	6.1 ± 0.3
40	1.27 ± 0.09	1.84 ± 0.09	3.07 ± 0.13	3.7 ± 0.3

T is the cell temperature. The superscript on the bending modulus k_c indicates the DMPC/Chol molar ratio.

different behaviors. Depending on the liposome followed during the cooling process, either an "explosion" process is observed (the liposome being destroyed), or the liposome assumes a spherical shape that stays stable at $T < T_m$. The latter restores its fluctuating shape after increasing the temperature to $T > T_m$. All of the liposomes remaining now show very large thermal fluctuation amplitudes, and the mean shape resembles an ellipsoid.

Sterol-containing membranes

An important feature of the addition of cholesterol to a pure saturated phospholipid seems to be the appearance of diphasic equilibria (coexistence of two lamellar phases within a bilayer) as a function of the temperature and the cholesterol content (Vist and Davis, 1990; Almeida et al., 1992; McMullen and McElhaney, 1995). With reference to the above, we suspect that such phase transitions influence the bilayer mechanical properties. Thus we first measured k_c as a function of the temperature for DMPC/cholesterol mixtures with variable mole fractions of sterol.

According to the phase diagram published by Almeida et al. (1992), we choose three different cholesterol molar ratios: 10%, 30%, and 50%. A 10% cholesterol-containing membrane should be a bilayer in which phase separation occurs at $T < 30\%$. Thirty percent cholesterol membranes are close to the limit of the liquid order phase in the temperature range studied here. A 50% cholesterol ratio is roughly the upper limit content for mammalian lipid systems. It is also important for a comparison with the measurements for RBC lipids (see below).

The bending elasticity obtained as a function of temperature and cholesterol ratio for DMPC/cholesterol bilayers is summarized in Table 1. We notice that k_c changes versus temperature are qualitatively identical for both pure DMPC and DMPC systems containing 10% cholesterol. However, this is not the case for larger sterol contents, at 30% and 50%, where k_c increases continuously with a decrease in the temperature T. At a given high temperature (30°C and 40°C), k_c also increases as a function of the cholesterol fraction.

Qualitatively, a 10% cholesterol ratio in DMPC leads to a liposome behavior very similar to the one of pure DMPC bilayers when T is lowered to T_m. All of the fluctuating liposomes change their shape from a Brownian spheroid to a nonfluctuating sphere. Some of them disappear at a temperature close to T_m, whereas others cross T_m without any apparent damage. At higher cholesterol content, it is always possible to cross T_m (defined for pure PC) without dramatic changes in the stability and morphology of the GUVs, leading to k_c measurements at temperatures below T_m for 30% cholesterol molar ratio bilayers.

We also measured the bending rigidity of other cholesterol-containing model systems, to compare their behaviors to that of DMPC/cholesterol bilayers. This was the case for DMPC/CholS bilayers or for a total lipid extract from red blood cells. The data are reported in Table 2. DMPC/Chol and DMPC/CholS systems containing both 30% sterol contents at 30°C are characterized by the same k_c (within the experimental error).

Using the RBC lipid extraction procedure (see Materials and Methods), we also succeeded in measuring bending rigidity of natural protein-free membranes. The analysis of the RBC lipid extract yield a cholesterol to lipid mass ratio of 18.4%, as expected in the case of human red blood cells (Devaux and Seigneuret, 1985), leading to a molar ratio close to 40%. The measurements of k_c for the lipid extract give very interesting results (Fig. 4). At room temperature (taken to be 25°C), bending elasticity of RBC lipid membranes is better represented by three distinct values in the range $(2.68-4.7) \times 10^{-19}$ J, whereas the analysis of sterol-free RBC lipid membranes leads to a single k_c estimation of $\sim 1.0 \times 10^{-19}$ J. The electroformation method was also used with lipid extracts to produce giant vesicles at physiological temperature. This procedure did not create any technical problems, except for sterol-free RBC lipid extracts. In that case, all of the vesicles obtained were spherical (no thermal fluctuations), explaining the absence of k_c values at 37°C. To improve the yield of fluctuating vesicles and in that case only, the liposomes were swollen at room temperature first, then T was slowly increased to 37°C. Unfortunately, no improvement was noticeable. However, comparing the results presented in Tables 1 and 2, we see that the bending rigidity for cholesterol containing RBC lipid extract at 37°C is quite identical to that of DMPC membranes at high temperature.

To understand the results obtained for k_c measurements on lipid extracts by microscopic observations and, particularly, the strange behavior of cholesterol-free RBC lipid extract liposomes, samples can be studied by solid-state ^{31}P-NMR experiments (Bloom et al., 1991). Thermal variations have been accomplished by first heating the samples from 25° to 60°C, followed by a decrease in temperature to 25°C (temperature steps: 5°). Lipid extracts with and without cholesterol are both characterized by a superposition of an isotropic line on a powder pattern (data not shown). The phospholipid fraction characterized by a fast isotropic reorientation was found to vary strongly, depending on the method used for the dispersion preparation. However, for

TABLE 2 Bending moduli for DMPC/CholS and RBC lipid extract bilayers

	DMPC/CholS $T = 30$°C	RBC extract $T = 25$°C	RBC extract $T = 37$°C	Sterol-free RBC extract $T = 25$°C
k_c ($\times 10^{-19}$ J)	3.8 ± 0.2	2.68 ± 0.19 3.56 ± 0.14 4.7 ± 0.2	1.46 ± 0.16	1.0 ± 0.15

T is the temperature and k_c is the bending rigidity. The concentration of cholesterol sulfate (CholS) in DMPC bilayers is 30 mol%. In the case of red blood cell lipid extracts, two kinds of bilayers were studied: with a natural cholesterol amount (RBC extract) and after the elimination of uncharged lipids (sterol-free RBC extract).

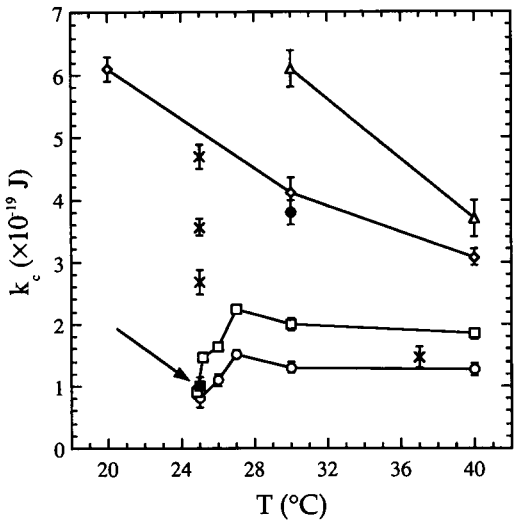

FIGURE 4 k_c dependence on temperature, as a function of the membrane composition. For DMPC/Chol mixtures: ○, pure DMPC; □, 10 mol% Chol; ◇, 30 mol% Chol; △, 50 mol% Chol. ●, Bending rigidity of 30 mol% cholesterol sulfate/DMPC bilayers at 30°C only. The behavior of red blood cell lipid extract is shown with (×) and without sterol (■) for 25°C and 37°C. Lines are used to help the reader; the arrow points to the bending modulus of sterol-free RBC lipid extracts.

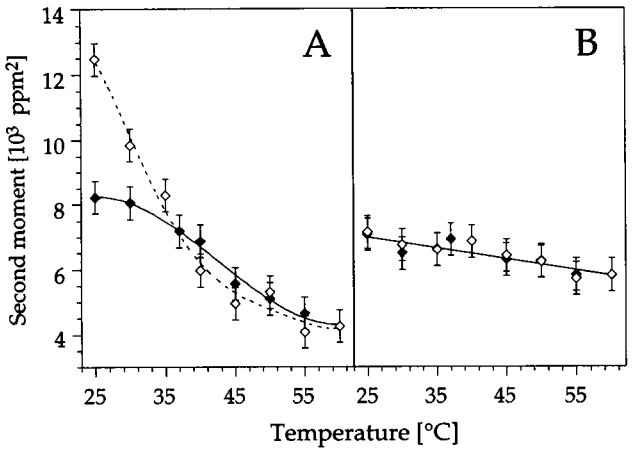

FIGURE 5 Second moments of ^{31}P-NMR spectra of RBC lipid extracts without (*A*) and with (*B*) cholesterol, as a function of temperature. Filled symbols correspond to an increase in the temperature and open symbols to a decrease. Lines are drawn to help the reader.

both systems (with and without cholesterol) and whatever the sample preparation, the amount of isotropic line increases with increasing temperature. In the case of the cholesterol-free RBC extract, the amount of isotropic line increases significantly for $T \geq 40$°C. This behavior is irreversible, i.e., a decrease in temperature leads to unchanged percentages of the isotropic line. In the case of the cholesterol-containing RBC lipids, the amount of isotropic line rises quasicontinuously with increasing temperature, but in contrast to the sterol-free RBC lipids, this behavior is reversible. Furthermore, one remarks an important difference between the two bilayer systems in the evolution of the second spectral moment, M_2, with temperature (Fig. 5). The polar RBC lipids exhibit a substantial hysteresis in the thermal variation. The first increase in temperature leads to an important decrease in M_2 centered on 40°C. Once 60°C is reached, cooling results in a remarkable increase in M_2, leading to a value of $\sim 12.5 \times 10^3$ ppm^2 at 25°C, in contrast to $\sim 8 \times 10^3$ ppm^2 observed before the thermal treatment (Fig. 5 *A*). In contrast, in the case of the cholesterol-containing RBC lipids, M_2 decreases only slightly and almost linearly with increasing temperature, and reversibility is observed (Fig. 5 *B*), a typical behavior for lipid dispersions containing high amounts of cholesterol (≥30 mol%). Yet the thermal evolution of M_2 for the dispersion without cholesterol is quite surprising; the high values of M_2 at 25°C in particular are remarkable. In this context it is interesting to further analyze the shape of the ^{31}P-NMR spectra. Actually, the unexpectedly high M_2 value obtained after thermal treatment is reflected by the presence of a very large spectral contribution, with the limit of the very broad powder pattern lineshape on the left side at ~50 ppm (see magnification in Fig. 6 *A*). By spectral simulation it was estimated that ~10% of the phospholipids contribute to this subspectrum, with a chemical shift anisotropy, $\Delta\sigma$, of ~90 ppm. In the case of the cholesterol-containing lipid extract, spectra acquired under the same conditions are characterized by a single axially symmetrical powder pattern component with a $\Delta\sigma$ of ~42 ppm (Fig. 6 *B*).

Finally, one can correlate NMR and k_c measurements in a qualitative manner by following the behavior of giant vesicles when changing the temperature using direct microscope visualization of NMR sample preparations. Qualitatively, RBC lipid extract GUVs (with cholesterol) never show fluctuations when maintained for a long time at room temperature. Some liposomes stick to others, indicating a lowering of long-range repulsions. Raising the temperature to ~50°C leads to the appearance of fluctuating vesicles. While observing such liposomes during the lowering of T to room temperature, a rapid decrease in the thermal fluctuation amplitudes is detected. Finally, their shapes transform into spherical objects. The cholesterol-free lipid extract behaves differently when GUVs are observed at 25°C after the incubation at 50°C. In the first seconds after the begin-

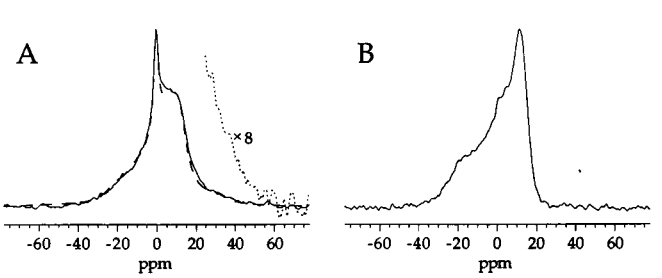

FIGURE 6 Selected ^{31}P-NMR spectra of RBC lipid extracts without (*A*) and with (*B*) cholesterol, at 25°C. In *A* a magnification (*dotted line*) and a simulation (*dashed line*) are shown. The corresponding parameters were 10% for the isotropic line and 10% $\Delta\sigma \approx 20$ ppm, 10% $\Delta\sigma \approx 90$ ppm, and 70% $\Delta\sigma \approx 40$ ppm for the powder patterns.

ning of the observation, the larger liposomes resemble quasispherical or ellipsoidal objects with large thermal fluctuations (not shown). After a short time (~1 min) as T decreases, most of these vesicles change their shape to unusual polyhedral forms (Fig. 7). It can be noted that flat membrane areas are quite rigid at microscope resolution, whereas the high curvature points fluctuate and seem to be responsible for the overall vesicle shape changes. This behavior is maintained when the cell stays at room temperature for a few days.

DISCUSSION

Since the pioneering work of Brochard and Lennon in 1975 clearly explaining the origin of the RBC flickering phenomenon, bending elasticities have been measured by using different experimental approaches. The thermal fluctuation analysis of giant vesicles is one of them. It is based on optical observations and image analysis that does not imply physical contacts with a solid material such as glass pipettes (Evans and Rawicz, 1990; Waugh et al., 1992). However, the application of this technique is clearly restricted to sparse objects: we are speaking about giant, isolated, fluctuating, unilamellar vesicles, a large number of qualities for a simple mesoscopic-scale structure. However, from the practical point of view, their formation rate and yield were improved by using electroformation to obtain significant populations of such objects.

As already mentioned, giant fluctuating liposomes are fully characterized by two distinct parameters, the reduced membrane tension $\bar{\sigma}$ and the bending elasticity k_c (Faucon et al., 1989). The first one is related to a geometric property of a particular vesicle (surface/volume ratio), and the second, k_c, is a characteristic of the lipid bilayer itself. This is checked for given experimental conditions (temperature, membrane composition) by recording the thermal fluctuation of ~10 liposomes. This leads to a mean k_c and an associated error bar (standard deviation) generally smaller than $k_c/10$ that can be used to analyze the relationships between the mechanical modulus and any other biophysical parameter. We demonstrate here that k_c is tightly related to the thermodynamic state characterizing the membrane, depending on the phase diagram (temperature and/or composition).

Model membranes

For pure DMPC bilayers, k_c remains constant within the experimental error for large temperature changes far above T_m. Then it increases to T_m + 3°C (Fig. 4). To explain the observed behavior, we return to simple microscopic models relating bending modulus to the most characteristic molecular parameters in the case of one-component membranes, the molecular area A and the bilayer thickness b (Petrov and Bivas, 1984; Szleifer et al., 1990):

$$k_c \sim K \frac{b^n}{A^m} \qquad n, m \geq 1 \tag{19}$$

It is known from x-ray data from DMPC bilayers that a temperature decrease induces a larger increase in the membrane thickness b in the range 25–30°C than in the range 30–40°C (Kirchner and Cevc, 1993), and the thermal area expansivity obtained by micromanipulation remains larger at 29°C than at 35°C (Needham et al., 1988). Considering our results, it appears that any changes in b or A are not large enough to influence k_c behavior for temperatures greater than 30°C. This is no longer the case in the range 27–30°C, where a small but significant increase is observed

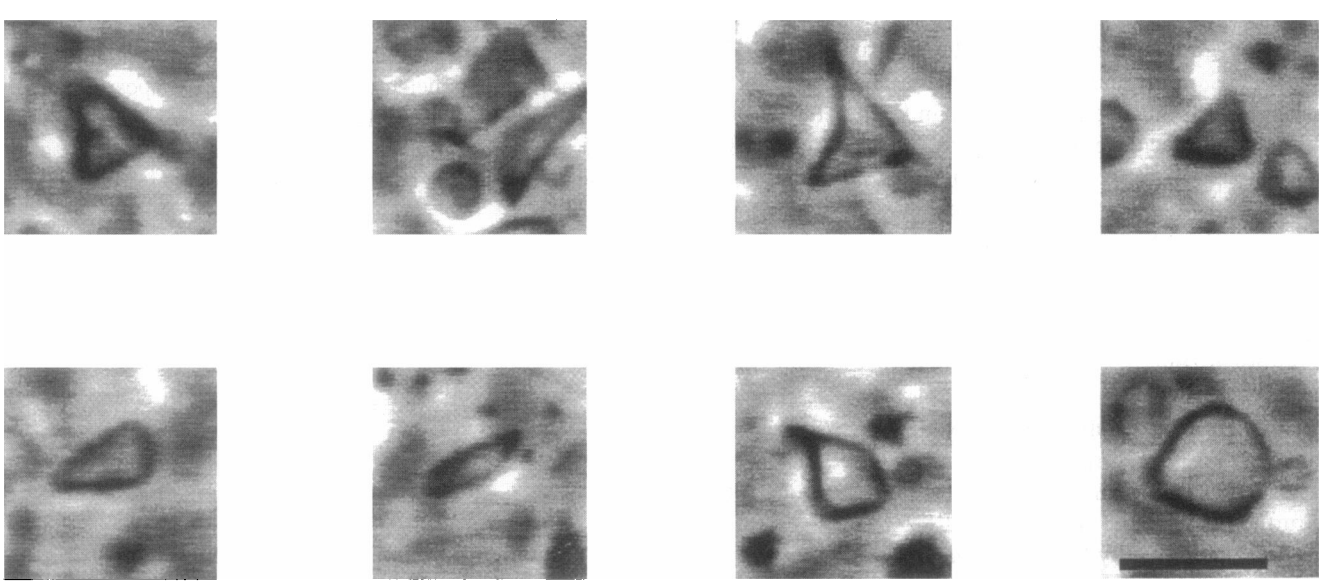

FIGURE 7 Video images of liposome suspensions from sterol-free RBC lipid extract used for NMR studies at room temperature. The bar in the lower right image corresponds to 10 μm.

(around 20%). However, it has been reported that a larger temperature dependence is obtained with unsaturated lipids (Niggemann et al., 1995).

The dependence of k_c on the molecular parameters was also demonstrated by following the behavior of saturated phosphatidylcholines as a function of their chain length (Fernandez-Puente et al., 1994). It has been shown experimentally that the bending modulus increases with the square of the hydrophobic thickness (Fernandez-Puente et al., 1994), meaning that the n-exponent in relation 19 is equal to 2 (Petrov and Bivas, 1984) for three saturated phosphatidylcholines (dilauroylPC, dimyristoylPC, and dipalmitoylPC).

To understand the thermal dependence for k_c close to T_m, one must consider a local deformation. For one-component bilayers, a curvature change induces a compression for the hydrophobic part of the outer monolayer (located in the opposite side compared to the curvature center) and an expansion of the inner monolayer. This is the result of the elastic interactions in the monolayer, which are concentrated mainly in the proximity of the hydrophilic region. Very close to T_m, a small increase in the chain density (related to the lateral tension in the hydrophobic part) after a curvature change would initiate a pressure-induced phase transition toward a pseudophase characterized by a more condensed chain region. As a result, a coexistence of fluid-like and gel-like hydrophobic domains will take place. Changing the curvature will lead to a change of domain relative proportion only, without any elastic response of the chain region. Thus the vanishing stretching modulus of the chain region strongly facilitates the curvature deformation, explaining the decrease in the apparent bending modulus of the bilayer in the vicinity of the phase transition temperature (Fernandez-Puente et al., 1994).

With regard to Fig. 4, we conclude that the variation of k_c with temperature for a 10 mol% cholesterol bilayer is similar to that of pure DMPC membranes. This is probably due to the low amount of cholesterol used. However, Table 1 shows a k_c decrease ($\sim$10%) when T is changed from 30°C to 40°C, whereas the corresponding bilayer thickness variations in the same temperature range are not significantly different (Léonard, 1993). This indicates that the microscopic model presented above cannot simply be extended to other binary membrane systems. The molecular area that must be used is not defined, for example, as well as the leading factor K relating k_c to b^n/A^m (Eq. 19). K is modified when intramolecular forces change within a bilayer. This is the case in the range 25–27°C when one observes a strong decrease in the bending stiffness for pure DMPC membranes (Fernandez-Puente et al., 1994; Hönger et al., 1994), and is especially evident in the present study when both the temperature and the composition are modified. For DMPC/Chol membranes, one must take into account the fact that reinforcement of the lipid interactions in the hydrophobic region is usually involved when the sterol amount is increased, and a possible phase segregation into cholesterol poor and rich regions (Almeida et al., 1992; McMullen and McElhaney, 1995). This phase separation process may be important for mechanical properties, as they are known to be related to molecular parameters (Needham and Evans, 1988; Needham et al., 1988; Needham and Nunn, 1990; Fernandez-Puente et al., 1994). It may induce large variations as the different phases change in size, shape, and relative amount. Such behavior can explain the lowering of k_c close to T_m at 10 mol% in cholesterol, but we did not detect any anomalies for k_c as a function of T for higher sterol ratios.

Nevertheless, it is clear from our data that an increase in cholesterol in a DMPC membrane induces a large increase in k_c for any given temperature. This effect is so important for 50 mol% compared to DMPC bilayers at 30°C that it was impossible to measure k_c at 20°C. At 30 mol% cholesterol content (T = 30°C), our k_c value agrees with those published, despite the fact that such values were characterized by larger error bars (Duwe et al., 1990). Surprisingly, their data for 20 mol% cholesterol at T = 30°C seem to be very close to our k_c value for a 10 mol% cholesterol bilayer at the same temperature. Other results gave systematic k_c increase with cholesterol content, using PC lipids (Evans and Rawicz, 1990; Song and Waugh, 1993), with noticeable differences compared to our measurements. Such differences may be due to their different technical approaches, as already mentioned in Materials and Methods. This should be tested using the different approaches, at a given place, similar to the work of Niggemann et al. (1995); this point is under study in our laboratory.

For the same 30 mol% sterol fraction at 30°C, one may be surprised to find an identical k_c for DMPC/Chol and DMPC/CholS membranes. This behavior was unexpected, because NMR and fluorescence polarization results showed that the ordering properties of cholesterol sulfate are less important than that of cholesterol at the same molecular ratio (Le Grimellec et al., 1984; Faure et al., 1996). Concerning the influence of the charge density on k_c, theoretical predictions are contradictory. The basic idea involves the formation of a double layer in front of charged membranes (Russel et al., 1989). For a rigid interface, the double-layer ion distribution is well defined and results from an equilibrium between repulsive and attractive electric forces and Brownian motion for solvated ions. Owing to the thermal fluctuation phenomenon of the bilayer, such an ideal equilibrium can never be reached. The double-layer ion distribution must accommodate the removal of local osmotic unbalances induced by local curvature changes. On one hand, a k_c increase was foreseen because of increases in the area density charge or the Debye length (Winterhalter and Helfrich, 1992), whereas Lekkerkerker (1989) predicted a negligible effect. If the addition of 30 mol% cholesterol sulfate to a membrane results in a superposition of charge and sterol effects, we conclude by comparison to DMPC/Chol results that the sterol effect is the dominant one.

Lipid extracts

The lipid fraction of plasma membranes is generally a complex mixture of molecules that differ in their hydrophobic and hydrophilic regions. Moreover, these lipids are organized asymmetrically with respect to the inner and outer monolayers, and within a given monolayer, domains do exist with different physical states. Although the precise role of such domains is not known, they are commonly accepted to be important to biological functions.

In the case of human erythrocytes, the membrane phospholipid fraction is essentially composed of sphingomyelin (SM) and phosphatidylcholine (PC) in the outer leaflet, whereas phosphatidylethanolamine and phosphatidylserine are mainly localized on the inner side (Barenholz and Thompson, 1980). In an excess of water, the gel-to-liquid phase transition temperature of a natural SM bilayer is in the range 30–50°C, the heat capacity peak being characteristic of a large biphasic region (Döbereiner et al., 1993). When mixed with other phospholipids, the gel-to-fluid phase transition is supposed to be governed by SM, because the other phospholipids remain in the fluid state down to −10–0°C. Thus the thermotropic behavior of the RBC lipid extract in the absence of cholesterol must be induced primarily by SM in the temperature range we are interested in, i.e., from 20°C to 40°C. ^{31}P-NMR is obviously well suited to showing such phase transitions at a microscopic scale (Bloom et al., 1991; Nezil et al., 1992) (Figs. 5 and 6), the same mixture is observed by phase-contrast microscopy (Fig. 7).

The analysis of ^{31}P-NMR spectra as a superposition of several powder patterns has already been described for the case of phospholipid mixtures (Marassi and MacDonald, 1991; Shin et al., 1995). These spectral features are generally interpreted as being due to partial mixing (domain formation of different phospholipids) or slow exchange (different states for the same lipid, i.e., gel/fluid coexistence), resulting in distinct powder patterns for each phospholipid species.

For cholesterol-free RBC lipid extracts at 25°C (Fig. 5 *A*), we observe a subspectrum with a very large $\Delta\sigma$ that is likely to correspond to a phospholipid subpopulation with strongly restricted dynamics, i.e., in a gel-like organization. As already mentioned in the temperature range studied (20–60°C) (Döbereiner et al., 1993), SM is known to be the only lipid that can produce such a behavior. At a mesoscopic scale, this temperature decrease behavior can be correlated with the appearance of polyhedral vesicles (Fig. 7), which was seen previously for DMPC GUVs at $T < T_m$ (see, for example, Sackmann, 1994). Despite the fact that such vesicles were not suitable for the analysis of thermal fluctuations, their excess area was large enough to allow the development of gel domains on very extended flat regions. Quasispherical vesicles obtained directly by electroformation are used to measure k_c independently. The bending rigidity of sterol-free RBC lipid extracts is close to 10^{-19} J, which is just above that of egg yolk phosphatidylcholine ($k_c \approx 0.7 \times 10^{-19}$ J; Angelova et al., 1992a). This is quite strange, if we consider the area covered by gel-like domain, as can be seen in Fig. 7. Because the gel domain cannot bend, the fluid region (which is obviously the continuous phase) dominates the mechanical behavior of the membrane.

At room temperature, cholesterol-containing RBC lipid extracts were characterized by a second moment M_2 comparable in its thermal evolution to those of PC/Chol systems (Fig. 5 *B*). This can be attributed to the high cholesterol amount (~40%) known to abolish any cooperative phase transition. Bending elasticity measurements in the same system lead to dispersed results. We note, however, that the highest k_c at 25°C is almost twice as large as the lowest one. This might be due to the scarcity of double-walled vesicles. Clearly, the remaining two bending rigidities (2.7×10^{-19} J and 3.6×10^{-19} J) are values that are easily distinguished from the error bars. They are obtained in distinct cells prepared at different times. Thus a possible temperature shift between the two cells may be responsible for the large bending elasticity changes if the temperature dependence of k_c is strong enough.

At physiological temperatures, we did not succeed in the formation of fluctuating liposomes with cholesterol-free RBC lipid extracts. Such a behavior can be explained if we refer to Eq. 13 and assume a high reduced membrane tension $\bar{\sigma}$ at 37°C (remember that in that case only, the liposomes were previously formed at 25°C, and then the cell temperature was increased to 37°C). In comparison with fluctuating vesicles at room temperature, an increase in $\bar{\sigma}$ can only be due to a smaller liposome excess area, an increase in the inner volume being physically unacceptable. This is not a common behavior for lipid vesicles, inasmuch as the molecular area of the lipids usually increases as the temperature rises. As a reduction of the total area is not reasonable, we have to assume the formation of vesicles (budding) that are too small to be observed. This interpretation is supported by the appearance of an increasing fraction of isotropic signal in the powder pattern of ^{31}P-NMR spectra with increasing T. Such an isotropic peak in solid NMR is generally attributed to the existence of very small objects (<0.2 μm, a size smaller than the optical resolution), showing fast isotropic reorientation on the NMR time scale (Burnell et al., 1980). This budding process was seen previously in different model systems (Nezil et al., 1992) and also with pure SM bilayers as the temperature increased (Döbereiner et al., 1993). Contrary to the systems studied by Nezil et al. (1992) (mixtures of 1-palmitoyl-2-oleoylphosphatidylcholine:1-palmitoyl-2-oleoylphosphatidylserine with or without cholesterol), we observe an irreversible budding process for cholesterol-free lipid extracts, the irreversbility being attributed, in that case, to vesicularization. With regard to the study of Döbereiner et al. (1993), one may propose this phenomenon to be due to the presence of SM within the membrane. In this context it is noteworthy that temperature-dependent budding and vesicularization phenomena were also observed in the case of human erythrocytes (Lelkes and Fodor, 1991), making our physical observation biologically relevant.

The very low bending modulus obtained for that system compared to other model systems containing large amount of cholesterol at high temperatures was very surprising for us. Such membranes are characterized by a bending modulus ($k_c \approx 1.5 \times 10^{-19}$ J) comparable to that of DMPC bilayers far above T_m ($k_c \approx 1.3 \times 10^{-19}$ J). This can be due to the existence of a transition of low cooperativity, previously seen for erythrocytes in which lipid phase transitions or separations were reported for the range of 11–41°C (Galla and Luisetti, 1980).

Strictly speaking, we cannot directly compare the mechanical effect of cholesterol at physiological concentrations and temperatures with the corresponding cholesterol-free natural mixture at 25°. Actually, the bending modulus is not very different from that found with the cholesterol-free DMPC model system. Close to T_m, we saw the effect of cholesterol to be related to the so-called condensing effect, which can be simply interpreted by a molecular density increase in the hydrophobic region. We must also note that a cholesterol-free RBC lipid extract is shown here to be very temperature sensitive, probably because of the existence of SM phase separation within the bilayer. Thus the main influence of cholesterol in natural mixtures could be presented as a solvent for such gel-like domains that regulates this thermotropic behavior. This is obvious in Fig. 5 *B*, where we do not detect any dramatic variation of the second moment with temperature in the range 20–60°C. This finding might be of biological relevance, knowing that the SM and cholesterol contents in various membranes are often coupled (Barenholz and Thompson, 1980).

CONCLUSION

The precise role of cholesterol in overall membrane stability is not yet totally understood, but an important set of data now seems to be converging. For example, Sankaram and Thompson (1990a,b), Almeida et al. (1992), and McMullen and McElhaney (1995) describe the consequences of a variable amount of cholesterol added to different model membranes on the phase diagram, in relation to natural membrane functioning (protein partitioning related to the lipid state) or to other mechanical properties (Needham and Nunn, 1990; Bloom et al., 1991). Depending on the technique used, the information obtained is generally different but complementary. The universal belief about natural or model membranes is that they can be represented as composite materials: different phases are spread within the bilayer structure. The precise shape, size, or connectivity of the coexisting phases are not known, but they clearly change with the temperature, the cholesterol ratio, and the molecular characteristics of the bilayer-forming lipid. In this paper we document that the bending elasticity at $T > T_m$ is related to the ability of the sterol core to induce an increase in the ordering within the fluid chain region while maintaining the fluid-state behavior of the phospholipid (Almeida et al., 1992). This point is well demonstrated here, at least for a high cholesterol content (≥30%), where the synthetic or natural bilayers fluctuate in all temperature ranges studied (from 20°C to 40°C).

Phase transitions can also induce large k_c changes for pure bilayers that had been presented as originating from density fluctuations (Hönger et al., 1994) or a mechanical failure of one monolayer close to T_m (Fernandez-Puente et al., 1994). However, the precise relationship between any mechanical property and the molecular arrangement within the bilayer of binary mixture is not totally understood (Milner and Witten, 1988; Szleifer et al., 1990; Kozlov and Helfrich, 1992). Experimentally, we detect a k_c variation with temperature similar to that of pure DMPC bilayers for a 10 mol% cholesterol/DMPC mixture only. At a higher cholesterol content (≥30%), a monotoneous nonlinear decrease in k_c occurs when T is decreased. The interdigitized model introduced by Sankaram and Thompson (1990b) should lead to interesting behavior because of the restriction of the slipping motion for the two monolayers (Yeung and Evans, 1995). Whereas phase connectivity may play a role in biological functioning, it is clear that reaching the percolation threshold in a membrane mixture can induce a very large change in mechanical properties. Finally, the physical budding phenomenon already seen with other model systems (Nezil et al., 1992; Döbereiner et al., 1993; Jülicher and Lipowsky, 1993) and, in our case, with cholesterol-free RBC lipid extracts is supposed to be related to the differential area increase between the inner and outer layers of the vesicle. This can occur when T is increased or when submicronic vesicular structures fuse with a cytoplasmic membrane and may thus be related to biological processes such as endocytosis.

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