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HAL Id: jpa-00210129
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Submitted on 1 Jan 1985

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Bilayer bending rigidity of some synthetic lecithins

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(Reçu le 21 février 1985, révisé le 22 mai, accepté le 31 mai 1985)

Résumé. — Nous avons mesuré le module d'élasticité de courbure des membranes fluides de DMPC, DPPC et DSPC dans l'eau. De la distribution d'équilibre des fluctuations thermiques de flexion de vésicules tubulaires, nous avons obtenu les valeurs de 2,4, 2,0 et 1,8 × 10⁻¹² erg avec une erreur d'environ 20 %. Nous avons également observé quelques déformations des tubules fluctuantes telles que des « coudes » bien marqués et des undulations prononcées de courtes longueurs d'onde dont l'énergie élastique fictive nous a paru trop grande pour être excités thermiquement.

Abstract. — The curvature elastic modulus of fluid membranes of DMPC, DPPC, and DSPC in water was measured. From the equilibrium distribution of the thermal bending fluctuations of tubular vesicles we obtained values of 2.4, 2.0, and 1.8 × 10⁻¹² erg, respectively, with errors of about 20 %. We also observed some deformations of the fluctuating tubes such as sharp knee-like bends and pronounced short-waved wriggles which apparent elastic energies seem too high for thermal excitation.

1. Introduction.

Amphiphilic molecules, such as lecithin, in excess water can organize spontaneously in a bimolecular array. Within the limits of continuum theory the resulting double layer with a thickness of approximately 4 nm may be treated as an infinitely thin elastic membrane. Nonspherical vesicles and flaccid spheres are practically free of lateral tension, so the only elasticity controlling their shape is that of curvature. Its free energy density $g_c$ per unit area may be written as [1]

$$g_c = \frac{1}{2} k_\varepsilon (c_1 + c_2 - c_0)^2 + k_c c_1 c_2$$  \hspace{1cm} (1)$$

where $c_1$ and $c_2$ are the principal curvatures, $c_0$ the spontaneous curvature which allows for an asymmetry of the membrane, and $k_\varepsilon$ and $k_c$ are elastic moduli. The modulus $k_\varepsilon$ is also called bending rigidity. As long as a vesicle is closed the integral over the second term gives a constant contribution, thus having no influence on the shape of the vesicle.

The bending rigidity of lecithin membranes is low enough to permit vesicular shape fluctuations of giant vesicles that are visible under a microscope. The first measurement, resulting in $k_\varepsilon = (2.3 \pm 0.3) \times 10^{-12}$ erg, was done with egg lecithin and employed the bending fluctuations of long tubular vesicles [2].

Recently Schneider et al. measured the bending rigidity of the egg-lecithin bilayer by analysing the statics and dynamics of the fluctuations of tubular [3] and spherical [4] vesicles. They obtained $k_\varepsilon = (1-2) \times 10^{-12}$ erg. Sakurai and Kawamura [5] deduced a smaller value of $4 \times 10^{-13}$ erg from the bending of myelin figures by magnetic fields. However, the vesicular objects were not well defined since they were attached to a lump of lecithin. Also, the spacing of the membranes in the rods which may have influenced the bending rigidity was not exactly known.

Theoretical estimates of the curvature elastic modulus are of the order of $10^{-12}$ erg [6]. A review on fluid-membrane elasticity is given by Petrov and Bivas [7].

The first measurement of the bending rigidity of a natural membrane, the human red blood cell membrane, was reported in an early paper by Brochard and Lennon [8]. They found from the analysis of the «flicker-phenomenon» $k_\varepsilon = (1.3 - 3) \times 10^{-13}$ erg. This is a surprisingly low value since the RBC wall is believed to be stabilized by a spectrin network.

Recently Evans [9] derived the bending modulus from buckling instabilities in the RBC membrane which were induced by micropipette aspiration, finding $k_\varepsilon = 1.8 \times 10^{-12}$ erg.

A low bending rigidity permits strong out-of-plane fluctuations of the membrane. The resulting undulation forces were predicted to be in close competition
with van der Waals attraction in the case of egg lecithin [10]. This may explain why lecithin vesicles form spontaneously in excess water and do not stick to each other [11, 12]. Recently, it was demonstrated that well-ordered multilayer systems of lecithin take up water without apparent limit (membrane spacings up to 200 Å were observed) [13]. Even if they do not dominate, undulation forces must always be taken into account in dealing with intermembrane interaction [14]. It is therefore desirable to measure the rigidities of some additional representative materials.

2. Theory.

We consider the bending fluctuations of a tubular vesicle restricted to the \(xy\) plane which may be thought of as the object plane of a microscope. (Out-of-plane bending fluctuations do not interfere when they are weak enough; see also below.) The elastic modulus \(K_T\) of tube bending was calculated on the assumption of constant circular cross section of the tube [2]

\[ K_T = \pi k_c r, \]  

(2)

where \(r\) denotes the tube radius. The modulus \(K_T\) is independent of spontaneous curvature [2]. Bending can be described in terms of curvatures, angles, or displacements. Figure 1 sketches the geometry of a bent tube. If \(s\) is the length measured along the tube's axis of total length \(L\), the curvature \(C(s)\) may be expressed by the Fourier expansion

\[ C(s) = \sum_{n=1}^{\infty} C_n \sin \left( n \frac{\pi}{L} s \right), \quad 0 < s < L. \]  

(3)

According to the equipartition theorem the mean square amplitudes of the modes obey

\[ \langle C_n^2 \rangle = \frac{2 k_B T}{K_T L} \]  

(4)

where \(k_B\) is Boltzmann's constant and \(T\) absolute temperature. Integrating equation (3), we obtain the angle difference

\[ \varphi(s) - \varphi(0) = \sum_{n=1}^{\infty} \frac{L}{2\pi n} C_n \left( 1 - \cos \left( n \frac{\pi}{L} s \right) \right). \]  

(5)

Only the odd modes contribute to the angle \(\Delta \varphi = \varphi(L) - \varphi(0)\) made by the two ends of the tube. Use of equations (2) and (4) and of \(\sum_{n \text{ odd}} n^{-2} = \pi^2/8\) leads to the mean square angle

\[ \langle (\Delta \varphi)^2 \rangle = \frac{k_B T L}{\pi k_c r}. \]  

(6)

The formula was derived previously in a slightly different way [2]. It is exact even for large \(\Delta \varphi\), provided the tube remains in the plane. Displacements are given by integrals of the type \(\int \sin \varphi(s) \, ds\). A simple, approximate formula can be obtained for weak bending fluctuations (\(|\varphi| \ll 1\) which permit substitution of \(\varphi\) for \(\sin \varphi\). The displacement \(y(s)\) with respect to the straight line between the tube's ends obtained by integration of equation (5) is then

\[ y(s) = -\sum_{n=1}^{\infty} \left( \frac{L}{2\pi n} \right)^2 C_n \sin \left( n \frac{\pi}{L} s \right). \]  

(7)

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(7)

Schneider et al. [3] retained only the contribution of the first mode

\[ \langle (\Delta y_j)^2 \rangle = \frac{2 k_B T L^3}{\pi k_c r} \]  

(9)

which because of the fast convergence of \(n^{-4}\) reproduces (8) to better than 5 %. In thermal equilibrium the statistical distributions of \(\Delta \varphi\) as well as \(\Delta y\) may be expected to be Gaussian and centred at zero. The membrane bending rigidity can be calculated with (6) and (8) from the respective second moments and the tube's length and radius. However, the validity of the former approach is more general than that of the latter.

3. Materials and methods

The saturated lecithins 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-palmitoyl-sn-glycero-3-phosphocholine (DPPC), and 1,2-stearoyl-sn-glycero-3-phosphocholine (DSPC) were obtained from Sigma (Munich), and for comparative measurements sometimes from Serva (Heidelberg) or Fluka (Neu-Ulm). The purity claimed by the distributor was 97-99 %. Purified egg lecithin (EYPC) was obtained from Serva. We made sure that there were no ionic impurities that influenced the experiment by repeated washing of a chloroform solution of the lecithin in water (see, e.g. [15]). We checked the purity of the samples by thin-
layer gel chromatography on Sephadex 60 plates with a mixture of methanol/chloroform/water (65 : 65 : 2,5) and obtained always a single spot, which indicates that impurities were less than 1 % Water was ion-depleted by an ion exchanger (Seradex) and had a pH between 5 and 6 and a conductivity of less than 0.1 µS/cm.

The sample chamber consisted of a stainless steel support glued to an object slide and a lid containing the cover glass which was screwed to the lower part of the chamber. The inner volume was about 200 µm high and the base area 12 mm in diameter. The chamber was well sealed against evaporation and allowed observation of the probe for several hours and even days.

To prepare the vesicles a small amount (some µg) of the lecithin, in the case of DMPC preferably slightly hydrated in a water-saturated atmosphere at temperatures below the main transition [16], was gently spread on the object slide and the chamber then filled with water.

The chamber was mounted on the heatable object stage of a Leitz microscope. Temperature was controlled by an external thermostat (Haake) to better than 1 °C. The sample was observed with phase-contrast microscopy. The lamellarity of a vesicle, i.e. the number of double-layers in the wall of the vesicle, could be determined with a photometer (Zeiss) adapted to the microscope. Details of the apparatus have been given elsewhere [17, 18]. In the present experiments a second beam-splitter allowed the simultaneous mounting of a video camera (Grundig) connected to a video recorder (Sony).

To obtain an equilibrium distribution of the states of a fluctuating tubular vesicle we analysed video frames at intervals which were larger than the correlation time of the fluctuations. Analysis was done with the help of a digitizer tableau and a Z80-based semi-automatic image analyser (MOP-Videoplan, Kontron).

The tangents to the axis of the tube at s = 0 and s = L were drawn and their angle measured on the tableau. The displacement of the tube's centre from the straight line connecting the ends was obtained by means of the image analyser upon recording the course of the tube's axis.

4. Observations and results.

Below the main transition temperature \( T_m \) there was almost no change of the lecithin crystals visible in the light microscope. When the temperature was raised just above \( T_m \) fluid membranes started to grow at the edge of the crystals. After one hour or more of swelling the sample usually contained a lot of freely moving tubular, spherical and other vesicles. When the temperature was lowered again, at \( T_m \) most of the membranes ceased to fluctuate, i.e. became rigid, and sometimes crumpled as observed earlier [19]. Some vesicles, however, remained fluid even at 10 °C below \( T_m \). On re-heating the sample these vesicles did not become fluid below \( T_m \), so that the effect is not likely to be caused by impurities in the membrane. This subcooling phenomenon confirms the prevailing view that the phase transition at \( T_m \) is of first order.

Most of the vesicles studied were unilamellar. They were selected by looking for contours of lowest optical contrast. In several cases the lamellarity was determined photometrically [18]. In general there was agreement between the visual judgement based on experience, the photometric result, and the lamellarity inferred from the value of the elastic modulus obtained later. Tubes were only studied if their lengths were at least 15 times larger than their radii.

The tubes usually remained in the object plane, which could be checked with an accuracy of ± 2 µm. Rotations and bending fluctuations out of this plane were rare. If they occurred the experiment had to be interrupted until the tube regained its usual horizontal position at the same or a similar height. The reason for this preference is not known; perhaps it was due to a small temperature gradient in the chamber. Only tubes at least 1 µm away from each slide were used so that interaction with the glasses was unlikely. Gravitational settling was not observed within one day.

We selected vesicles which appeared to be neither connected to other objects nor hindered by them in their fluctuations. In particular, we checked that the ends of each tube were moving freely. The motion of vesicles fulfilling these criteria was video recorded for 1 to 3 hours. Translational and rotational motion of the vesicle as a whole was usually very slow, thus not disturbing the observations.

The lengths \( L \) of the tubes were defined by omitting sections of the order of the tube diameter at the ends of the tubes. The resulting lengths ranged from 16 µm to 110 µm and in general fluctuated less than 5 % in the course of an experiment. The tube radii \( r \) ranged from 0.8 µm to 3.1 µm, the error being about 0.2 µm. The number of states analysed for a tube varied from 31 to 109 and was typically about 60. The time intervals between subsequent states were usually 60 s, thus being sufficiently longer than the correlation time of 10 to 20 s, as e.g. obtained for a tube of DPPC with \( L = 30 \) µm and \( r = 1.6 \) µm. The necessary autocorrelation functions were computed from sufficiently rapid sequences of \( \Delta \phi \). The angle \( \Delta \phi \) made by the tube's ends was typically less than 25°, but sometimes reached 60°. The local angle of the tube's axis with the line connecting its ends usually was less than 13°.

Despite the careful selection of the tubes free fluctuations seemed to be hindered in some cases, as signaled by large deviations from a Gaussian distribution. We discarded all experiments where the mean of \( \Delta \phi \) differed by more than 10° from zero or was larger than one third of the r.m.s. of \( \Delta \phi \). We
further calculated $M_i = \left( \sum (\Delta \varphi_n)^i \right)/N$, $i = 1, 2, 3, 4,$ and omitted all experiments which showed a skewness $a_3 = (M_3/M_2^2)$ of more than $\pm 1.0$ or where the curtosis $a_4 = (M_4/M_2^2)$ deviated by more than $\pm 1.0$ from its Gaussian value 3. Figure 2 shows the distribution of angles $\Delta \varphi$ for two representative experiments, one with DMPC and the other with DSPC, respectively. An apparent elastic modulus $k'_e$ was calculated from $\langle (\Delta \varphi)^2 \rangle$ and equation (6). The error of $\langle (\Delta \varphi)^2 \rangle$ was estimated to be 10-20% mainly by comparing the experimental cumulative distribution to the Gaussian distribution with the same second moment. The typical error of a single measurement of $k'_e$ was about 20% if allowance was made for the uncertainties of the quantities other than $\langle (\Delta \varphi)^2 \rangle$ which enter equation (6).

In addition to the study of the three saturated lecithins we re-examined the bending rigidity of egg lecithin. The original experiments [2] were done with crude egg yolk lecithin showing a 30% content of phosphatidylethanolamine in thin layer chromatography. The material used in this work was pure with respect to the head group. Within experimental error, however, we recovered the former value of $k'_e$.

Figure 3 shows the distribution of the measured values for $k'_e$ of the four substances. The gap between the majority group of values and higher ones marks the transition from uni- to bilamellar vesicles. It was assumed as earlier [2] that for low lamellarity $l$ the effective elastic modulus $k'_e$ changes linearly with $l$. The few values of $k'_e$ that were near multiples of the mean of the unilamellar group were reduced to the unilamellar value. The results for all substances as calculated from the unweighted single values are summarized in Table I. The standard deviations of the individual values of $k'_e$ from their mean values are about equal to the experimental errors of the single measurements. The uncertainty assigned to the final values in the table is representative of the single measurements. The reason for this caution is that we cannot entirely exclude systematic errors, e.g. an occasional wrong lamellarity. It is also hard to estimate the influence of a possible correlation between the observed $\Delta \varphi$ and fluctuations of the length $L$ (see above) which were not taken into account in our calculation.

For comparison we calculated the moduli $k_e$ for some tubes also from the distribution of center displacements and equation (8). The error bars of the two values, one obtained from the angles and the other from the displacements, usually overlapped. There seemed to be a tendency for the latter to be smaller than the former. This is not understood since the opposite is expected on the basis of the approximations used in deriving equation (8). More measurements would be needed to find out if there is a systematic trend.

In the course of our measurements we noticed a
novel and rather strange phenomenon. Approximately 30% of the pictures taken for the Δρ analysis showed pronounced wriggles, i.e. more or less coherent bending modes with wavelengths less than two times the tube's diameter. In general, these fluctuations did not look like peristaltic modes. Closer analysis showed that the wriggles had lifetimes of 1/4 to 1 s. Between the disappearance of a wriggling and its reappearance, usually at a different position on the tube's axis, intervals of up to 2 s elapsed in which the tube appeared to be smooth within microscopic resolution. Sometimes wriggling extended just over a single wavelength which resulted, e.g. in a dislocation or a buckle of the tube's axis. In very few cases the wriggles covered almost the whole length of the tube; figure 4 shows such an example with a tube of DSPC. The wriggling is much more impressive when the dynamics are watched on the video monitor. Comparing the four lecithins, we found that with DSPC the wriggles were much stronger and more frequent than with the others. For DSPC the amplitudes were up to 20% of the radius while e.g. for DMPC they just reached 10%. A few checks with the higher homologues containing 20 and 22 carbons in the fatty acid chain did not reveal effects stronger than those seen with DSPC.

Another curious phenomenon were knee-like deformations which were observed with all substances but were again the most pronounced with DSPC. We speak of a "knee", if the angle Φ(s) changes by more than 25° over a length not larger than the tube's diameter. Figure 5 shows such a knee in a tube of EYPC. Sometimes the occurrence of a knee was accompanied by a comparatively long relaxation time of Δρ. Occasionally there was a regeneration of the knee from a more continuous bend. The typical lifetime of a knee was 1/4 to 4 seconds. Some knees migrated with a velocity of up to 2.5 μm/s along the tube's axis. Both phenomena, the wriggles as well as the knees, are remarkable for the high curvature elastic energies which seem necessary for their formation. This will be discussed below.

5. Discussion.

The values of $k_s$ for the three synthetic lecithins differed by hardly more than the experimental error and are in fairly good agreement with the values reported for EYPC [2-4].

We did not investigate if the bending rigidity displays a pretransitional effect just above $T_m$, the temperature of the main transition. Experiments done in this laboratory several years ago yielded $T_m = 22.6 \, ^\circ C$, 40.2 °C and 54.3 °C with giant unilamellar vesicles of DMPC, DPPC and DSPC [19]. More recently Evans and Kwok reported $T_m = 24.2 \, ^\circ C$ for such vesicles of DMPC [20]. The higher transition temperature is, perhaps, due to better purity. The lecithins used in the present work seemed to be very pure, as evidenced by the novel observation of strongly supercooled fluid membranes. Supercooling by 10 °C indicates a clear first-order character of the phase transition, so we expect pretransitional effects not to be very important.

Since, in addition, we have performed our measurements always a few degrees above $T_m$, we think that the variation of $k_s$ in the series of synthetic lecithins has little to do with any critical behaviour. An increase of hydrocarbon chain flexibility with temperature is probably more relevant. A crude elastic model suggests that the bending rigidity varies as $d^3$, $d$ being the membrane thickness, and is proportional to a bulk elastic modulus of compression (see e.g. [21]). On the basis of this model and X-ray data [22] for membrane thicknesses, DSPC should be more rigid by about 20% than DMPC. However, as the area per molecule in the membrane increases more rapidly than the membrane thickness with increasing chain length, the increase of $d$ may be more than compensated by a decrease of the bulk elastic modulus.
The short-waved wriggles of DSPC tubes were conspicuous for their large amplitudes together with their coherence. Typical values are an amplitude of $y_0 = 0.2 \, \text{r}$, a wavelength of $\lambda = 4 \, \text{r}$, and a coherence over just this length.

The elastic energy $G$ of such a sinusoidal wriggle may then be estimated by means of

$$ G = \frac{1}{2} K_T \left\langle C^2 \right\rangle \lambda $$

(10)

where the mean quadratic curvature $\left\langle C^2 \right\rangle$ is approximated by

$$ \left\langle C^2 \right\rangle = \frac{1}{2} y_0^2 \left(2 \frac{\pi}{\lambda} \right)^4 .$$

With the elastic modulus of tube bending $K_T$ as measured an astonishingly high excitation energy of about $1 \, \text{eV}$ is obtained. In certain cases, see figure 4, this energy is even higher because of the greater coherence of the wriggles. Considering the associated Boltzmann factors this appears to be far above thermally attainable energies.

A similar situation holds for the knee-like bends. The extra elastic energies of knees as estimated on the basis of equation (1), or more simply, by using the elastic modulus of tube bending (Eq. (2)), were very high, reaching 0.5 eV to 1 eV and sometimes even 1.5 eV. The occurrence of knees in some tubes was, however, rare enough not to influence the equilibrium distribution of angles $\Delta \phi$ and thus affect $k_c$.

We cannot yet explain the novel phenomena of knees and wriggles and would not like to draw any firm conclusions on the basis of the available evidence. Both phenomena seem to be characterized by regions of strong curvature. The formation of such regions, unexpected on the basis of equation (1) which is quadratic in the curvatures, may be energetically favoured by higher-order terms of the elastic energy density [23]. This might explain the easy formation of saddles and, as their counterparts, caps. However, microscopically resolvable curvatures do not seem strong enough, on a molecular scale, to give rise to higher-order effects.

6. Conclusion.

We have used an established method to determine the curvature elastic moduli of the three best known synthetic lecithins DMPC, DPPC and DSPC in the fluid phase from the bending fluctuations of tubular vesicles. Short-waved wriggles and kneelike deformation of the tubes, most prominent with DSPC, do not seem explicable by ordinary bending elasticity. They may point to a new type of membrane « roughness » which, if confirmed, should have many interesting implications.

Note added in revised version. — After submitting this manuscript we obtained a preprint by Engelhardt et al., now published [24], who derived the bending rigidity from a Fourier analysis of surface undulations of flaccid vesicles. For DMPC at 26°C they found $k_c = (3.5 - 6.5) \times 10^{-13} \, \text{erg}$ which is significantly less than our value.

References