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Effects of Fully and Partially Solubilized Amphiphiles on Bilayer Bending Stiffness and Temperature Dependence of the Effective Tension of Giant Vesicles

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Abstract. — We report the modification of the bending elastic modulus k_c of lipid bilayers (here DMPC) by small amounts ($c \leq 5$ mol %) of (i) small amphiphiles which exchange between the bilayer and the aqueous phase (*e.g.* the ion carrier valinomycin and the Ca^{++} carrier A23187) and (ii) amphiphiles solubilized in the membrane (cholic acid). Large reductions of the bending stiffness may be induced by a few percent of the solutes, *e.g.* 1 mol % of valinomycin reduce k_c by a factor of two. The effect is rationalised in terms of local thinning of the bilayer. The strong effect of solutes on k_c contrasts with its weak dependence on the lipid structure since the C18:0/C18:1-lipid stearyl-oleoyl-phosphatidyl-choline (SOPC) exhibits only a 15% higher value of k_c than DMPC. The effect of temperature on the flicker behaviour was analysed in order to establish correlations between the effective tension and the excess area of the quasi-spherical vesicles. The temperature dependence of the bilayer excess area for a DMPC vesicle leads to the thermal expansion coefficient, β , for which a value of $\beta = 10.4 \times 10^{-3} \text{ K}^{-1}$ is obtained. A much stronger tendency for budding on μm -scale (micro budding) during thermal area expansion of POPC and SOPC compared to DMPC was observed.

1. Introduction

The minimum bending energy concept of lipid bilayers and biological membranes (*e.g.* of erythrocytes) is one of the few examples demonstrating that universal physical laws may play a role for complex biological processes such as shape transitions and shape instabilities of cell membranes [1, 2].

Detailed studies of the elastic moduli of erythrocyte plasma membranes showed that the bending elasticity and the lateral compressibility are essentially determined by the lipid/protein bilayer [3–5] and for that reason erythrocyte plasma membranes are extremely flexible with

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respect to bending but are essentially incompressible with respect to lateral extensions. Studies of the amoeboid locomotion of cells of the slime mold *Dictyostelium discoideum* on substrates show that even in this case the lipid/protein bilayer elasticity and the constraint of finite area of the cell envelope imposed by the bilayer play an important role for the cell locomotion process [6].

Measurements of the bending elastic modulus, k_c , of erythrocytes based on the Fourier analysis of excitation of the membrane bending undulations as a function of the wavelength of excitation (1) yielded smaller k_c values for $0.25 \mu\text{m} \leq \lambda \leq 0.5 \mu\text{m}$ than for $\lambda > 1 \mu\text{m}$. Most surprisingly, the bending stiffness obtained from the analysis of the short wavelength excitations were considerably smaller than expected for bilayers containing also 50 mole % cholesterol such as erythrocytes [7].

Motivated by the question about the origin of this small bending stiffness of the natural lipid/protein bilayer, we studied the effect of small amphiphilic solutes on the bending stiffness of bilayers of DMPC. In order to compare the effect of the solutes on k_c with the dependence of the bending stiffness on the lipid chain structure we compared bilayers of DMPC, of SOPC and POPC.

Two types of solutes have been studied: (i) ion carriers (valinomycin and the Ca^{++} -ionophore A23187) which distribute between the aqueous phase and the bilayer and (ii) cholate which is supposed to generate transient pores within the bilayer.

To measure the effect of the apparent membrane tension originating in the constraint of fixed membrane area, measurements have been performed as a function of temperature in order to change the excess area systematically by thermal expansion.

2. Materials and Methods

2.1. MATERIALS. — The lipids DMPC, POPC and SOPC were purchased from Avanti Polar Lipids (Birmingham, AL) and the ion carriers Valinomycin and A23187 and the cholanic acid were products from SIGMA (St. Louis, MO). The $\text{SnO}_2/\text{InO}_2$ covered plates (ITO) were obtained from Balzers AG (Lichtenstein).

2.2. GIANT VESICLE PREPARATION. — The vesicles were prepared by the electric field supported swelling technique introduced by Dimitrov and Angelowa [8]. For that purpose a cell consisting of two glass plates ($30 \times 20 \times 0.5 \text{ mm}^3$) separated by a Teflon spacer was built. The inner side of the plates was covered by $\text{SnO}_2/\text{InO}_2$ electrodes. A thin layer of the lipid was deposited on the electrode covered surface of the cell by the solvent evaporation technique. In case of lipid mixtures a stock solution containing the lipid and the amphiphilic solute was used. After assembly of the cell it was deposited into a beaker containing 1.5 ml of the swelling buffer or Millipore water. A voltage of 1 V and 10 Hz was applied to the electrodes. Giant vesicles were formed after 1-2 hours which were carefully taken up by plastic pipettes. Many large thin walled vesicles with diameters of up to $100 \mu\text{m}$ were formed.

2.3. SAMPLE CELL. — For the present measurements the vesicles were transferred by micropipettes into a temperature controlled measuring chamber with inner dimension $40 \times 20 \times 0.25 \text{ mm}^3$. The chamber was surrounded by a copper block perforated by water from a thermostat. The temperature was measured within the chamber by a PT100 sensor that was coupled to a thermostat within an absolute accuracy of 0.1 K. The whole chamber could be deposited onto the stage of the inverted microscope (Fig. 1). Perturbations caused by thermal convection were very small because of the small inner height of the chamber (0.25 mm) and the small horizontal temperature gradients within the compartment.

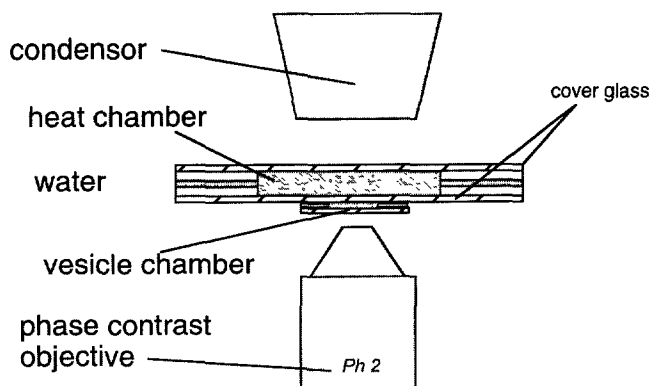


Fig. 1. — Schematic side view of observation chamber.

2.4. CONTOUR ANALYSIS. — The vesicles were observed by phase contrast microscopy using a Zeiss Axiovert 10 inverted microscope. An objective (Plan-Neofluar $40\times/0.75$ from Zeiss, Germany) with a working distance of 0.75 mm was used. Illumination occurred with a 200 W halogen lamp. Images of the vesicles were taken with a CCD camera (C3077 with the camera controller C2400 from Hamamatsu, Germany) and recorded with the SVHS-recorder AG7355 (Panasonic, Osaka, Japan). Digitalization of the movies was achieved with Pixelpipeline- and Pixelstore-cards from Perceptics (Perceptics, Knoxville, USA) using the same setup as described in [9]. The time between two image captures amounted to 40 ms.

An algorithm was developed to determine the contour of the stored images to an accuracy of at least one pixel. This corresponds to an absolute accuracy smaller than 250 nm measuring an ideal picture with the optical properties mentioned above. The position of the contour is defined by the minimum of the brightness of the image, whereby one can find each point along the circumference of the contour by determining the minimum of the intensity in a direction normal to the position to the contourline. The major problem to determine the position of each point with an accuracy better than one pixel was solved by the following algorithm: The intensity of one arbitrary point in the neighbourhood of the contour is represented by the following equation:

$$I(x, y) \propto \sum_{i,j=0}^{n_i, n_j} I_{i,j} e^{-\frac{(x-i)^2 + (y-j)^2}{N^2}}, \quad (1)$$

where $I_{i,j}$ is the intensity of the i -th and j -th pixel in the x and y direction of the image, respectively. The exponentials serve the Gaussian smoothening of the intensities for each pixel and the degree of smoothening can be determined by the mean square width N^2 . This procedure generates another intensity distribution of the form shown in Figure 2c. It has the great advantage of being nearly independent of the discontinuities caused by digitalisation with a CCD-camera and thus enabling subpixel resolution. The above procedure was performed for 1024 angle positions along the contour. The contour points determined in this way for a contour at time t are then used as starting point for the determination of the contour at time $t + \Delta t$. The procedure described above was implemented into NIH-image (version 1.49 developed by Wayne Rasband, National Institutes of Health, Washington D.C.) using think pascal (version 4.02 by Symantec Corporation, USA) on a Macintosh Quadra 950 computer.

The so-obtained contours are then treated like in [10] to achieve the mean square amplitudes as a function of the mode number q . The further treatment of the mean square amplitudes is done with Igor Pro (WaveMetrics, Oregon).

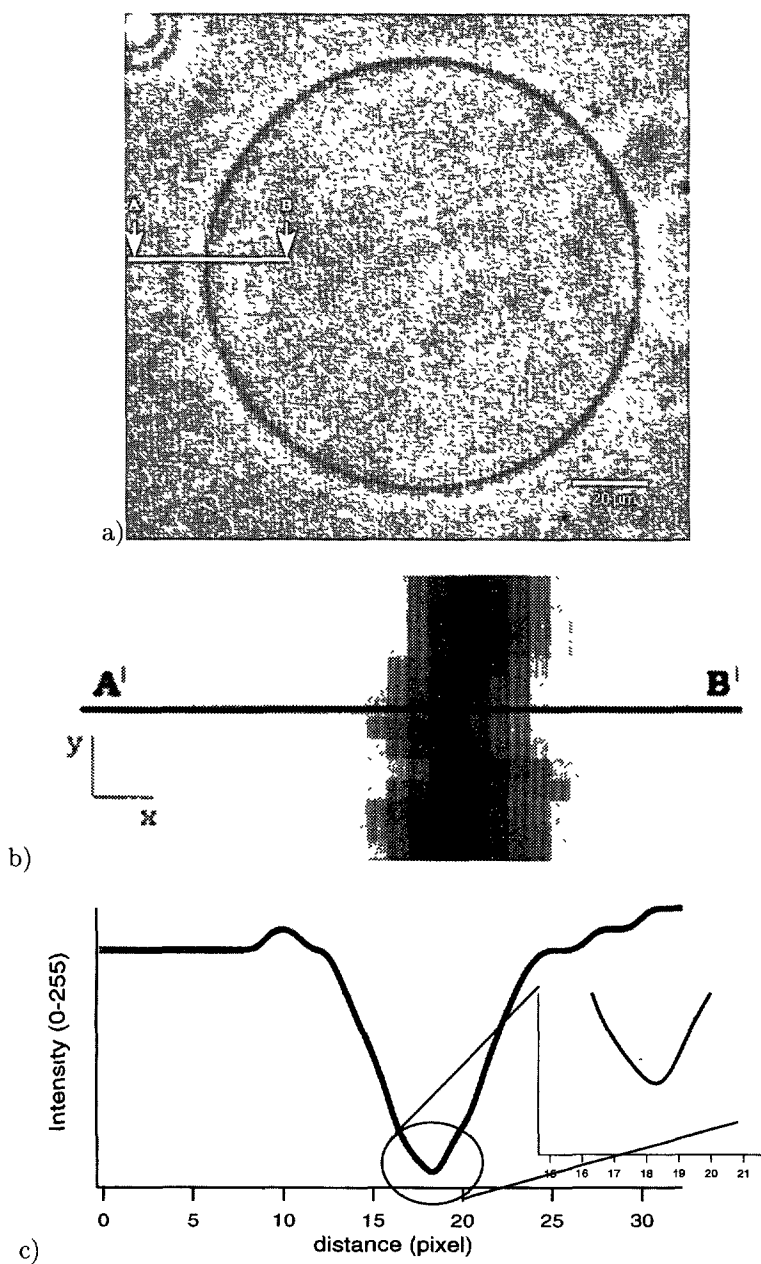


Fig. 2. — a) DMPC giant vesicle observed by phase contrast microscopy. b) Section of trace of one vesicle. Each quadratic spot defines one pixel. c) Intensity plot along the black line $\overline{A'B'}$ of Figure 2a. The solid line shows the intensity plot obtained with the Gaussian smoothing algorithm in a direction normal to the contour of the vesicle. The grey bars show the intensity plot one would obtain by the conventional procedure.

The testing of the algorithm was done in two ways: (i) Artificial drawn circles of known diameter that are treated twice with the “smooth”-function provided by NIH-Image were measured with the above described procedure and the measured circumference was compared to the theoretical. (ii) The algorithm was used to measure a spherical (non flickering) vesicle and the data were transferred into spherical coordinates. The radius data for each angle could then be easily compared. One obtains that the minimum static resolution for the algorithm is about 0.7 pixel. The later described measurement precision is a function of this static resolution of the algorithm. But the measurement precision for the flicker amplitudes depends strongly on the optical resolution of all the optical units, too, so that one cannot compute the dependence between this static resolution of the trace finding algorithm and the later on described measurement precision. Moreover the optical resolution for one frame of a non spherical shape of a quasi-spherical vesicle is worse than a spherical shape because of the physical properties of phase contrast microscopy. So the dynamic resolution of the algorithm strongly depends on the shape of the vesicle measured.

3. Theoretical Background of the Method

3.1. IN GENERAL. — The vesicle is considered as a quasi-spherical shell with fixed volume V and area A . The area A exhibits an excess with respect to a spherical shell of the same volume which allows to define an equivalent radius according to

$$V = \frac{4\pi}{3} R_0^3, \quad A = R_0^2(4\pi + \Delta) \quad (2)$$

where Δ is the dimensionless excess area. The thermal excitations may then be described in terms of the normalised spherical harmonic expansion ($Y_{lm}(\vartheta, \varphi)$) of the surface according to [11,12]

$$r(\vartheta, \varphi, t) = R_0 \left(1 + \sum_{l,m} a_{lm}(t) Y_{lm}(\vartheta, \varphi) \right) \quad (3)$$

where ϑ and φ are the polar and azimuthal angles. For a general shape the elastic energy associated with the bending excitations will be different in either the (i) spontaneous curvature model [12], (ii) the bilayer coupling model [13] or (iii) the area-difference-elasticity model, which is a combination of both models [14]. For a quasi-spherical shape, the dimensionless mean square amplitudes of the spherical harmonic modes are independent of the model [15] and given by

$$\langle |a_{lm}|^2 \rangle = \frac{k_b T}{k_c(l+2)(l-1)[l(l+1) + \sigma(\Delta)]} \quad (4)$$

where $\sigma(\Delta)$ is an effective tension. The next step is to express the mean square amplitudes $\langle |v_q|^2 \rangle$ of the two-dimensional contour as a functional of the mean square amplitude of the spherical harmonics $\langle |a_{lm}|^2 \rangle$. If the contour is Fourier transformed according to

$$v(\varphi, t) = R_0 \cdot \sum_{q=0}^{q_{\max}} v_q(t) \exp\{+iq\varphi\} \quad (5)$$

where φ is the longitudinal angle, the mean square amplitudes in the equatorial plane $\langle |v_q|^2 \rangle$ may be expressed as [16–18]

$$\langle |v_q|^2 \rangle = \sum_{l=q}^{l_{\max}} \langle |a_{lq}|^2 \rangle (\cos \pi/2) \cdot N_{l,q} \quad (6)$$

where $P_{l,q}(\cos(\pi/2))$ are the values of the associated Legendre polynomials in the equatorial plane. The factor $N_{l,q}$ is given by $N_{l,q} \equiv \frac{2l+1}{4\pi} \cdot \frac{(l-q)!}{(l+q)!}$. The key point is that the summation starts at $l = q$ and the sum is thus a function of the (dimensionless) wave number of the contour. This approach may be applied if

- (i) the vesicle is quasi spherical so that the image plane goes through its centre and if;
- (ii) the time over which the fluctuations are observed is much longer than either the recurrence or the relaxation times of the excitation, which may require half a minute observation time for larger vesicles.

The bending stiffness k_c is then given by

$$k_c = \frac{k_b T}{\langle |v_q|^2 \rangle} \cdot S(q) \quad (7)$$

with

$$S(q) = \sum_{l=q}^{l_{\max}} \frac{N_{l,q} \cdot [P_{l,q}(\cos \pi/2)]^2}{(l-1)(l+2)[l(l+1) + \sigma(\Delta)]}. \quad (8)$$

The sum on the right side of this equation is rapidly converging with $l_{\max}$ for moderate values of $\sigma(\Delta)$.

So far, fluctuation spectra of quasi-spherical vesicles have been analysed by using σ as a fitparameter [10, 17–19]. This tension, however, is determined by the constraint of fixed area of the vesicle in terms of the excess area parameter Δ alone by the implicit equation

$$\sum_{l \geq 2}^{l_{\max}} \frac{2l+1}{l^2 + l + \sigma(\Delta)} = \frac{2\Delta k_c}{k_b T} \quad (9)$$

where $l_{\max}$ is the cut-off quantum number characterizing the bending mode of the shortest possible wavelength. It is of the order of the bilayer thickness and therefore

$$l_{\max} \propto \frac{2\pi R}{d_m} \approx 10^4. \quad (10)$$

Since in equations (4) and (9) neither the spontaneous curvature nor the area-difference elasticity show up, analysis of the quasi-spherical flickering will neither depend on or even reveal any information about these quantities.

3.2. EVALUATION OF THE BENDING STIFFNESS k_c . — In order to assess the relevance of a non-vanishing effective tension for the determination of the bending elastic constant, in Figure 3a, $S(q)$ is plotted as a function of q for the situation $\sigma(\Delta) = 0$ and for $\sigma(\Delta) = 5$. For $\sigma(\Delta) = 0$, $S(q)$ obeys a simple scaling law $S(q) \propto q^{-3}$ practically over the whole range of q values, while for moderate values of $\sigma(\Delta)$ this law still holds for a rather large regime of q -values ($q > 8$ in Fig. 3b). For such situations, the procedure for the determination of k_c can be still simplified drastically by plotting the reduced mean square amplitudes $\langle |v_q|^2 \rangle \cdot q^3$ as a function of q . For q values for which $\sigma(\Delta)$ is negligible a plateau is observed from which k_c can be directly determined without fitting the correct sum of equation (8) to the data. As will be shown below, a plateau regime can always be found if (i) the excess area of the vesicle is made large enough (*e.g.* by thermal expansion of the bilayer) and (ii) video integration time effects do not influence the measurement of the flicker amplitudes [17].

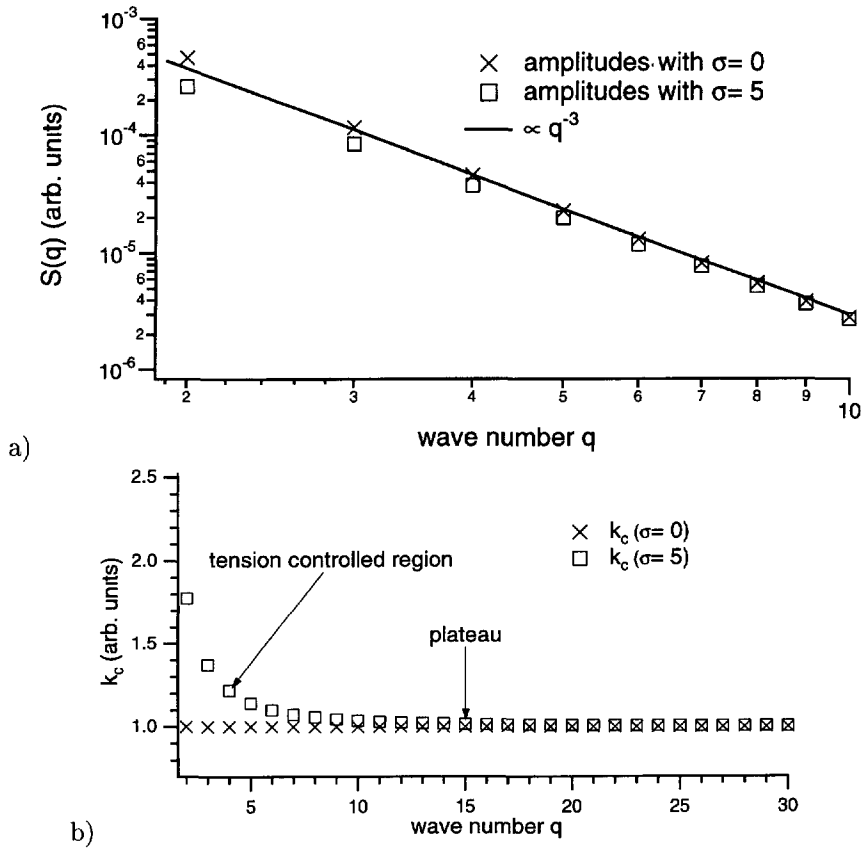


Fig. 3. — a) Plot of the sum of right side of equation (8) in arbitrary units for zero effective tension $\sigma(\Delta) = 0$ (datapoints $\times$) and for non-zero effective tension $\sigma(\Delta) = 5$ (datapoints $\square$). The straight line corresponds to the scaling law $S(q) \propto q^{-3}$. b) Plot of the normalized bending rigidity k_c one would get with the mean square amplitudes of Figure 3a assuming that the effective tension is zero. For non zero effective tension ($\sigma > 0$) too large k_c -values are obtained for low wave numbers ($q < 15$).

In Figure 4, we illustrate the accuracy of the k_c measurements for a moderately large membrane tension. In Figure 4a the mean square amplitudes for a DMPC vesicle of $55 \mu\text{m}$ diameter are presented. Three regimes are clearly visible: (i) a central regime in which the mean square amplitudes scale as q^{-3} , (ii) a regime at $q < 8$ where the tension becomes essential and the mean square amplitudes lie below the q^{-3} line and (iii) a regime at $q > 35$ where the amplitudes are determined by random noise. In Figures 4b and 4c the q -dependence of the bending elastic modulus obtained by evaluation of the mean square amplitudes by equations (7) and (8) with $\sigma(\Delta) = 0$ are shown. The values of k_c converge rapidly from the tension dominated regime to the plateau regime where k_c is practically independent of the wave number. At $q > 35$ the flicker amplitudes are dominated by noise and thus the k_c -value in this regime is too low. The values in the plateau yield an average value $k_c = 1.2 \times 10^{-19} \text{ J}$.

3.3. EVALUATION OF THE ACCESS AREA Δ AS A FUNCTION OF TEMPERATURE. — On the other side, the parameter $\sigma(\Delta)$ and therefore the excess area Δ may be determined by fitting equation (7) to the observed $\langle |v_q|^2 \rangle$ — versus — q plots. It is of course clear that one has

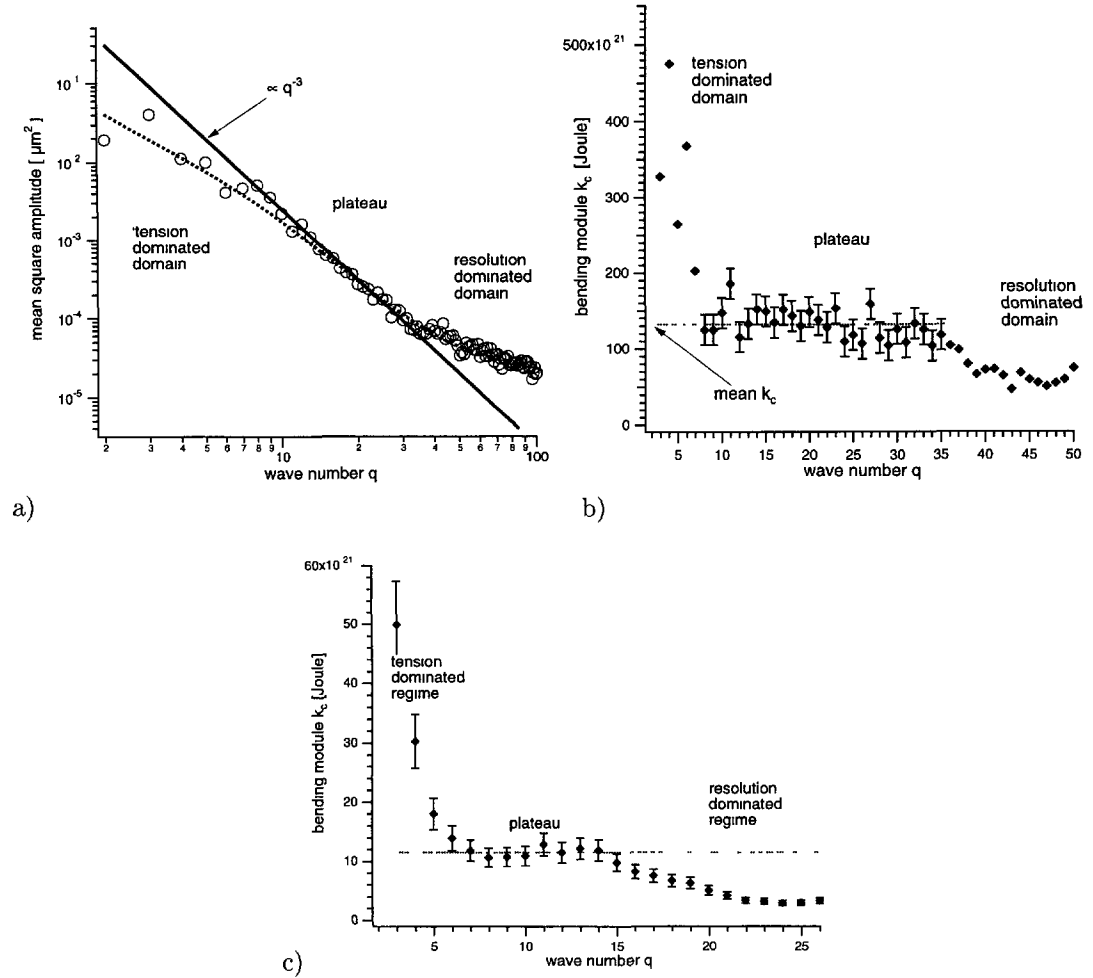


Fig. 4. — a) Mean square amplitudes for DMPC-vesicle with a diameter of $55 \mu\text{m}$ measured at a temperature of 25.7°C . The drawn line obeys the scaling law $S(q) \propto q^{-3}$ and the dotted line corresponds to a fit using equations (7) and (8) with the parameters $k_c = 1.3 \times 10^{-19} \text{ J}$ and $\sigma = 63$. b) Bending elastic modulus of DMPC vesicle plotted as function of the excitation wave vector q by analysis of data of Figure 4a using equations (7) and (8) for $\sigma = 0$. c) Bending elastic modulus of DMPC containing 0.01 mM cholic acid vesicle plotted as function of the excitation wave vector q by analysis of data of using equations (7) and (8) for $\sigma = 0$.

carefully to check if corrections of the measured flicker amplitudes caused by video integration effects of the camera are necessary [17]. This becomes most important if one chooses the two parameter fit. In the present work we avoided the correction by choosing large vesicles (correlation times $\tau \propto R_0^3$) and checking the necessity of corrections with the procedure given in [17] in order not to run into numerical unpleasantness. The bending stiffness is known as temperature dependent [19,20]. We could not detect a temperature dependence of the bending stiffness within the narrow temperature region (0.5 K) so we treated k_c as constant in order to have a better statistics for $\sigma(\Delta)$.

Table I. — *Summary of bending stiffnesses of single walled vesicles of DMPC, SOPC and POPC.*

	DMPC	SOPC	POPC
bending stiffness k_c [10^{-19} J]	1.16	1.44	1.43
accuracy Δk_c [10^{-19} J]	0.17	0.35	0.22

4. Data Representation

In principle the measured k_c -values k_c^i of an ensemble of vesicles (enumerated by i) could be presented in terms of a histogram. In the present work a different procedure is chosen.

The distribution of k_c -values k_c^i is represented in terms of the Gaussian-distribution

$$F(k_c) = \frac{1}{N} \sum_{i=1}^N \exp \left\{ -\frac{(k_c - k_c^i)^2}{b_i^2} \right\} \quad (11)$$

where N is the number of vesicles measured and b_i^2 is the mean square deviation of each k_c -measurement. The advantage of this different representation compared to conventional histogram bars is that every k_c -value is weighted by its own error in contrary to histogram bars where there is no weighting at all except of the estimated thickness of the bar. So this representation carries more information and the judgment of the measurement error is more realistic.

5. Results

5.1. EFFECT OF SOLUTES ON BENDING STIFFNESS. — Table I summarizes the observed variation of the bending stiffness of pure lecithin vesicles with the hydrocarbon chain structure. It is found that k_c varies only slightly (by about 15%) with the structure of the hydrocarbon chain of the lipids used in the present work.

Figure 5 shows distributions of k_c -values of pure DMPC vesicles and vesicles of this lipid with a lipid to valinomycin molar ratio of 1%. To a first approach a bimodal distribution is clearly found in each case and the k_c -values of the maxima of the two bands differ by a factor of nearly two. The two distributions are therefore attributed to single and double walled vesicles. It should be stated that the band at high k_c could contain some three walled vesicles in particular considering the two outermost points. However, we only consider the k_c -values obtained from the maximum of the low k_c band in our further discussion and therefore no attempt was made to analyse the high k_c band more extensively. Comparison of Figures 6a and b shows that the bending stiffness is drastically reduced by the small ion carriers.

Figure 6 summarizes the variation of the bending stiffness of DMPC-vesicles with increasing concentrations for the ion carriers valinomycin and A23187, respectively. A remarkable difference is that the effect of valinomycin of decreasing k_c is much more dramatic whereas for A23187 k_c is essentially constant (within experimental error) up to 2.5 mole % and decreases only essentially above this limit.

5.2. EVALUATION OF THE EXCESS AREA. — As noted above a sufficient large excess area of the membrane is required in order to obtain plateaus in the $\langle |v_q|^2 \rangle \cdot q^3$ versus q plots. The simplest way to increase the excess area consists in heating [21]. In Figure 7 the variation of the mean square amplitudes of a quasi-spherical DMPC vesicle with increasing temperature is

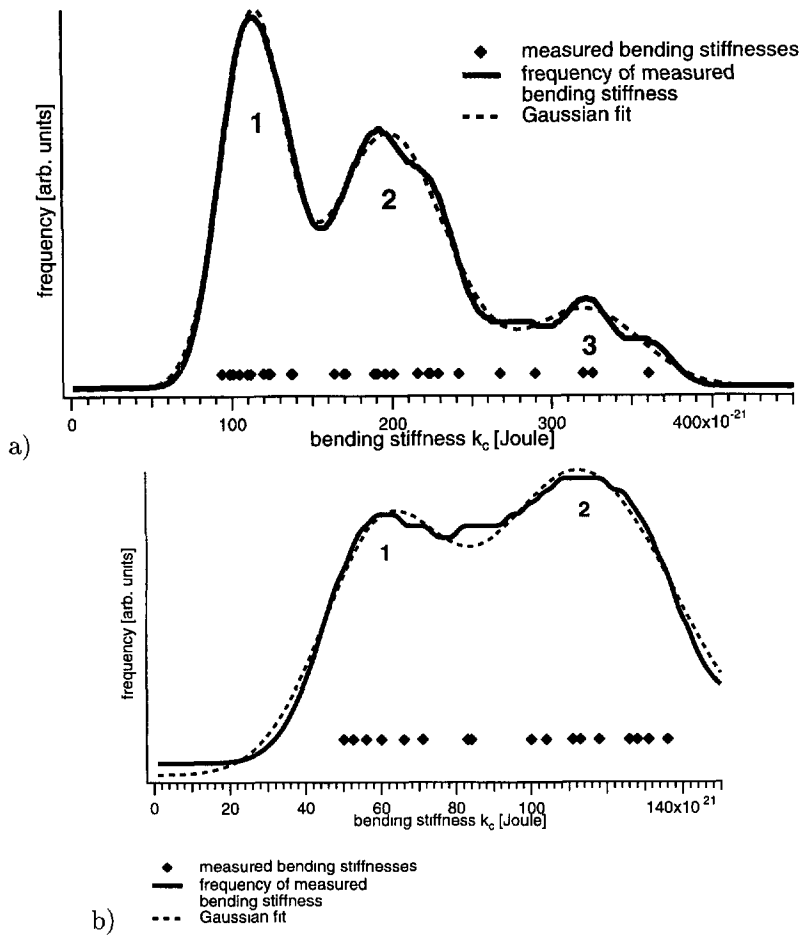


Fig. 5. — Distribution of measured stiffness k_c for a) vesicles of pure DMPC (58 vesicles analysed) and b) vesicles of DMPC containing 1 mole% of valinomycin (23 vesicles analysed). The points denote the measured values for k_c , the drawn line gives the distribution of k_c -values determined according equation (11) and the dotted line is a Gaussian fit to the first band. Both figures clearly show a bimodal distribution maxima differing by about a factor of two which is attributed to distributions of single walled and double walled vesicles

shown for excitation wave numbers $q = 2-11$. Most amplitudes increase about linearly with temperature. As expected, the increase of the mean square amplitudes with the temperature is the smaller the higher the mode number q . A notable exception is found for $q = 2$ for which $\langle |v_q|^2 \rangle$ increases up to $T = 26.4^\circ\text{C}$ but decreases again upon further heating. This is attributed to a change of the vesicle from a quasi-spherical to an ellipsoidal shape. As already noted previously [10] the apparent bending stiffness of elongated vesicles obtained by flicker analysis is increased. The strange behaviour of the $q = 6$ mode is attributed to an artefact. The amplitudes of the high order modes $q \geq 10$ (Fig. 7c) do not increase remarkably by increasing T by 0.3°C showing that they are fully excited already at 26.2°C . The method is not accurate enough to observe the small expected increase of $\langle |v_q|^2 \rangle$ for those modes.

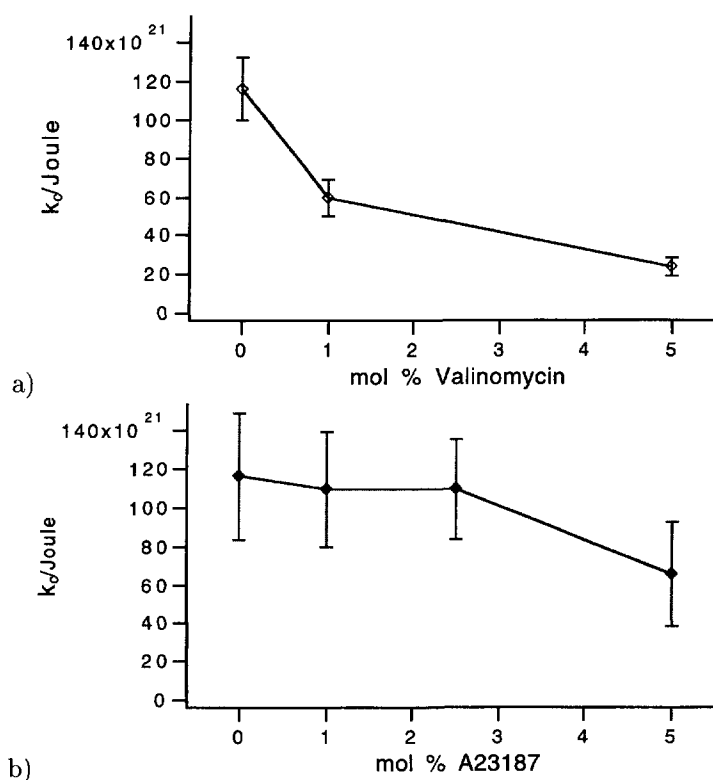


Fig. 6. — Variation of bending stiffness of single walled DMPC vesicles with increasing amount of the monovalent ion carrier valinomycin (a) and the Ca^{++} -carrier A23187 (b).

By analysing the temperature dependent mean square amplitudes in terms of the effective tension $\sigma(\Delta)$, one can even obtain the thermal expansion coefficient β as follows. The effective tension for the data of Figure 7 is given in Figure 8a. From $\sigma(\Delta)$, the temperature dependence of the excess area Δ , defined in equation (2), has been determined with the help of the implicit equation (9) and the result is represented in Figure 8b. As expected, Δ increases approximately linearly with temperature. From the temperature dependence of the excess area the thermal area expansion coefficient β is obtained according to

$$\Delta = 4\pi\beta(T - T_0) \quad (12)$$

where T_0 is the temperature at which the vesicle formally has no excess area. The temperature T_0 of course has to be smaller than an estimated temperature T_0^{est} where the flickering is not detectable any more. A value of $\beta = 10.4 \times 10^{-3} \text{ K}^{-1}$ measured at a temperature of 24°C was obtained for DMPC which agrees reasonably well with the values $\beta = 6 \times 10^{-3} \text{ K}^{-1}$ obtained for DMPC by neutron scattering experiments [22] or the value $\beta = (4-6) \times 10^{-3} \text{ K}^{-1}$ for DMPC measured by the micropipette technique [3,4].

5.3. BUDDING ON μM SCALE-MICROBUDDING. — An interesting behaviour was found for vesicles of SOPC and POPC. Heating did not lead to an increase of the area-reservoir available for flickering that is to an increase in the vesicle flaccidity. As demonstrated in Figure 9 this can be explained in terms of the formation of microbuds. Figures 9a-c show that the vesicle

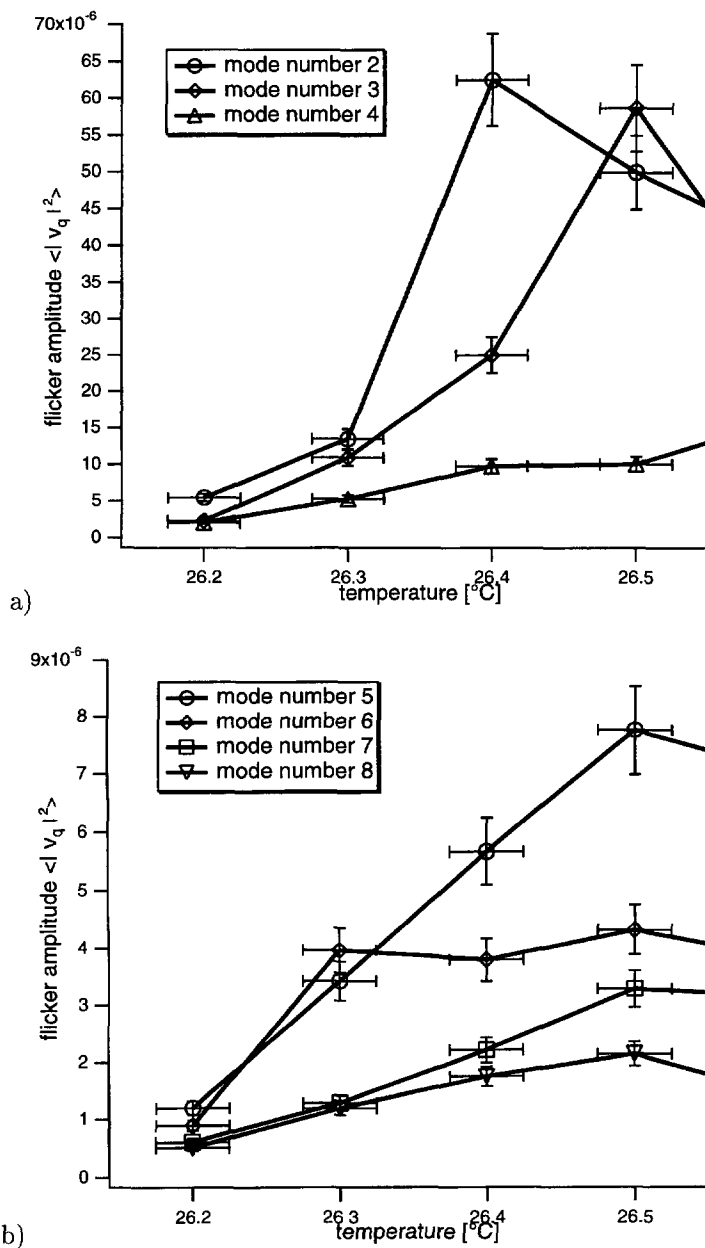


Fig. 7. — Variation of the mean square flicker amplitudes $\langle |v_q|^2 \rangle$ of the contour of a DMPC-vesicle with temperature for different groups of excitation mode numbers q . Note the different scales of the ordinate.

remains essentially spherical even after heating by more than 7 °C. A close inspection of the membrane at higher magnification (*cf.* Fig. 9d) shows that the inside exhibits many small budded vesicle which eventually merge into large tethers [21].

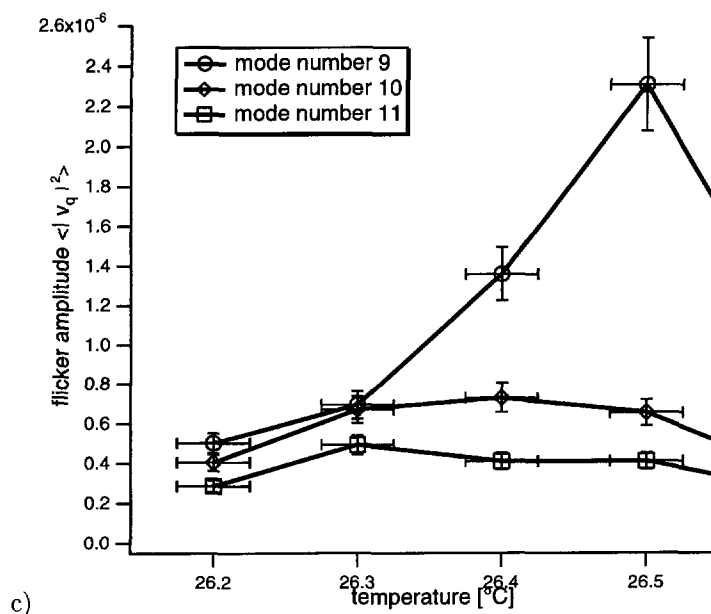


Fig. 7. — (Continued).

6. Discussion

The present experiments have shown that the spectral analysis of contour fluctuations provides a reliable and fast method for measurements of bending elastic moduli of lipid bilayers in order to study effects of solutes on the bilayer rigidity. It is, however, necessary to evaluate about 10 or more vesicles of the same type in order to distinguish single shelled from double shelled vesicles or to eliminate other artefacts *e.g.* caused by microbud formation or the adsorption of particles. Other sources of error are unprecise measurements of the vesicle radius or non-spherical shapes of the vesicles. For the latter, the fluctuation can no longer be described by an expansion around a sphere. For vesicles of DMPC sufficient excess area may be generated by thermal expansion in order to obtain a broad regime of wavevectors in which the membrane excitations are dominated by the bending energy, which facilitates the evaluation procedure. In other cases, k_c can still be measured by application of the more general equations (7) and (8). Both the bending stiffness k_c and the generalised tension σ can be determined by fitting these equations to the experimental plots of $\langle |v_q|^2 \rangle$ versus q . Following [15], the excess area Δ may be determined from the value of the generalised tension $\sigma(\Delta)$.

The bending elastic moduli of bilayers composed of one lipid component depend only weakly on the lipid structure of the lipid. Thus for the lipids studied in this work the values of DMPC and SOPC differ by only 15%. k_c increases slightly with increasing chain length in agreement with calculations of the chain length dependence of k_c by molecular statistical theories [23]. It has also been predicted by the same model that for mixtures of lipids of different chain lengths k_c may decrease in a non-linear way with increasing molar fractions of the short chain component. The correlation between k_c and the bilayer thickness has been experimentally well-established for mixtures of four long chain surfactants and pentanol [24]. However the effects of valinomycin and cholanic acid appears to be much more dramatic and are reminiscent of the previously found strong reduction of k_c by bola lipids [10].

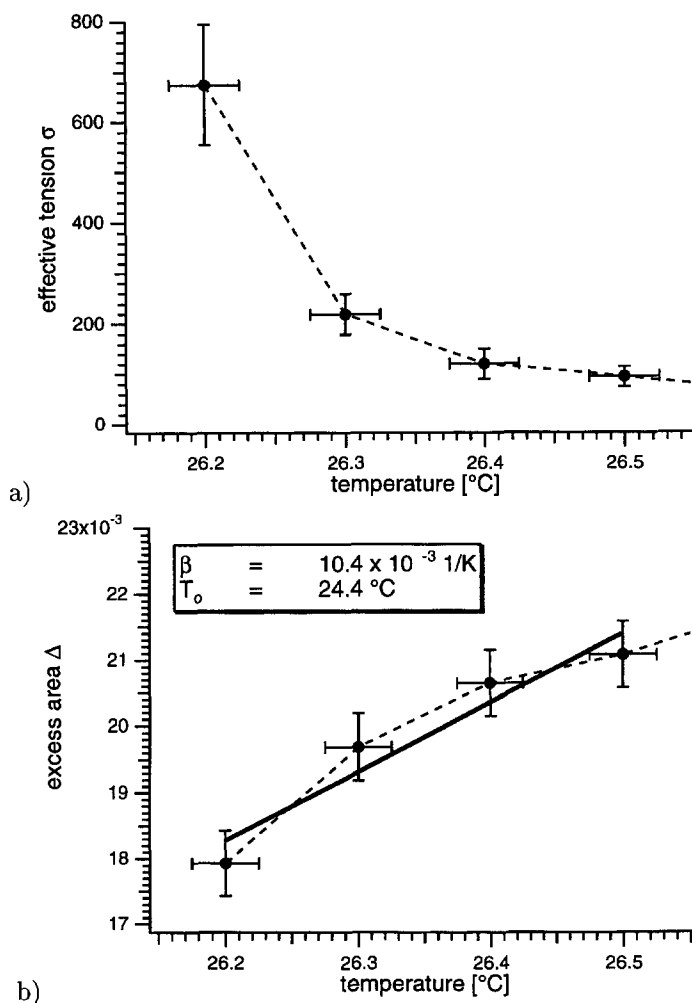


Fig. 8. — a) Temperature dependence of effective tension of DMPC-vesicle calculated from the temperature dependence of the mean square amplitudes exhibited in Figure 7. b) Temperature dependence of excess area Δ obtained from the T -dependence of the effective tension by solving the implicit equation (9).

At least three explanations for this effect are possible:

- (i) one possibility is that the small amphiphilic solutes exchange rapidly between the bilayer and the aqueous phase. They could reduce the local density fluctuations by accumulating in convexly curved areas and escaping from concavely curved areas. This is expected to result in an amplification of the undulations;
- (ii) a second explanation is that the solutes create local “defects” within the bilayer. Such defects could be pores or local thinnings. It is intuitively clear that such local discontinuities should decrease the flexural rigidity of the bilayer. In fact the formation of pores by cholates is well-established [25];

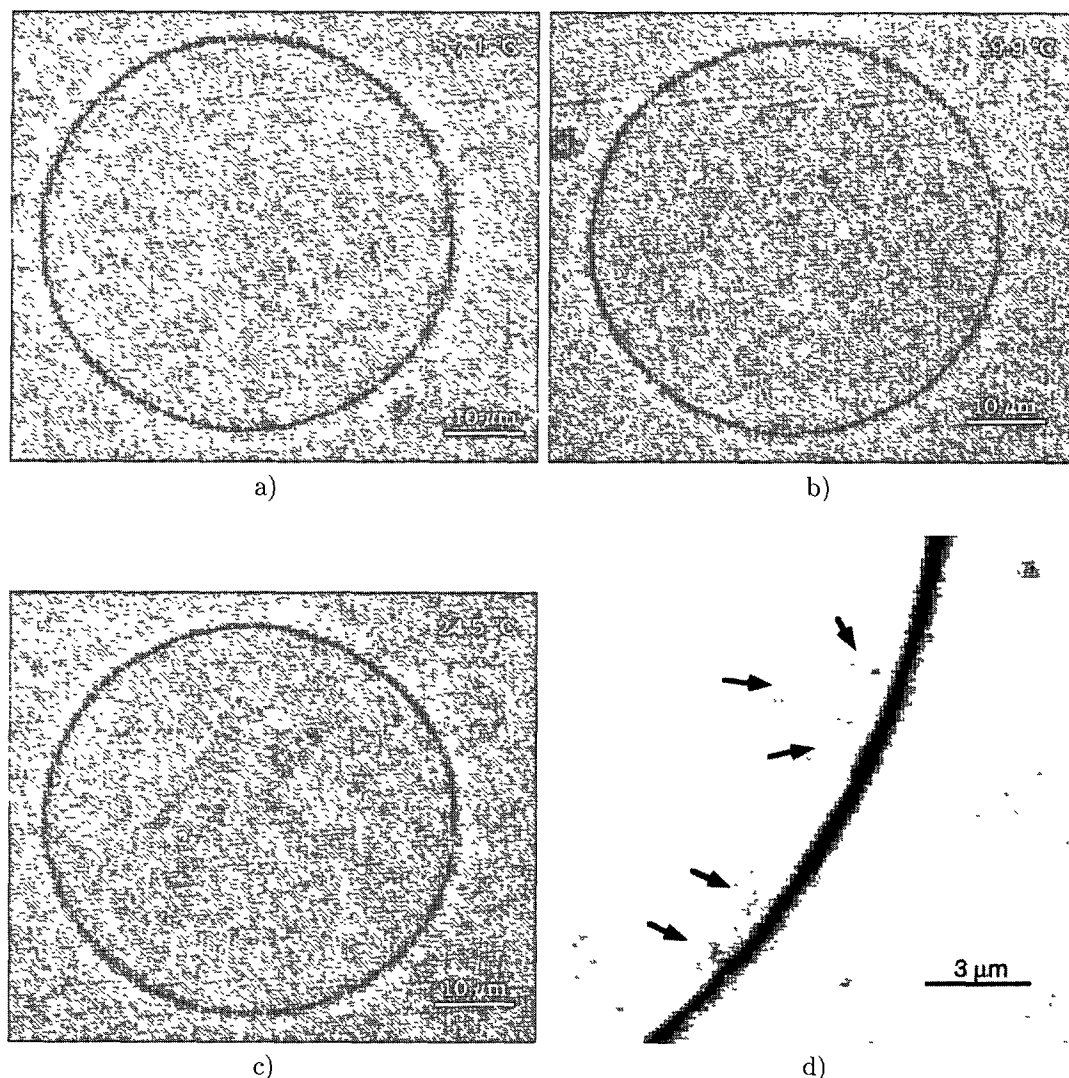


Fig. 9. — Demonstration of maintenance of the spherical shape of a POPC-vesicle during heating caused by microbudding. Images a) to c) show the vesicle at three different temperatures 17.1 °C, 19.9 °C and 24.5 °C. d) shows the enlargement of the vesicle contour at 24.5 °C with clearly visible microbuds protruding to the inside (arrows).

- (iii) mobile impurities or solutes can decrease the effective bending rigidity if they create a local spontaneous curvature. This has been shown *theoretically* both for planar membranes [26] and for closed vesicles [27]. In the latter case, this effect can lead to an enrichment of solutes in the bud if budding occurs.

Another surprising result of the present work is the continuous removal of excess area during heating by the formation of microbuds for POPC and SOPC vesicles which contrasts with the behaviour of DMPC. It has already been observed previously that POPC vesicles exhibit only a very narrow range of excess areas where oblate ellipsoids or stomatocytes are stable [21].

The non-equilibrium distribution of the number of lipid molecules in the inner and outer monolayer provides a driving force for a flip-flop which may play a role for the different behaviour of the lipids DMPC and POPC or SOPC. It was found that the transmembrane lipid transfer is much faster for DMPC than for DPPC [28]. Suggesting that the non-equilibrated number difference ΔN equilibrates much faster for DMPC than for POPC or SOPC therefore giant vesicles of the latter lipid may well have a stronger tendency for budding than DMPC-vesicles under the experimental conditions of the present work.

7. Conclusions

The conventional analysis of the fluctuation spectrum of quasi-spherical vesicles can be refined by replacing the effective tension in favour of the excess area. Studying the temperature dependence yields satisfying agreement with independent measurements of the thermal expansion coefficient.

Surprisingly large reductions of the bending stiffness k_c of bilayers can be induced by a few mol percent of amphiphilic solutes. The particular large effects induced by cholanic acid and valinomycin together with previous observation of a dramatically strong reduction of k_c by bipolar lipids which are short compared to bilayer thickness suggest that such strong reduction are caused by local thinning of the bilayer, by pore formation or by an induced inhomogeneous spontaneous curvature.

The strong softening effect of valinomycin strongly suggests that these amphiphiles cluster transiently within the bilayer and the question arises whether this clustering is not also important for the mechanism of ion translocation. Similar strong effects on the bending stiffness could also be exerted by slightly amphiphatic but water soluble proteins. Possible examples are spectrin (the basic building unit of the cytoskeleton of erythrocytes) or hisactophilin, a protein involved in the coupling of actin to membranes [29]. These proteins bind strongly to membranes in the presence of negatively charged lipids but can simultaneously penetrate partially into the bilayer [30]. This effect could provide an explanation for the surprising small bending elasticity of the erythrocyte membrane as revealed by flicker spectroscopic analysis of the short wavelengths bending excitations [5, 7]. In fact the present study was motivated by this question and studies of the effect of adsorbed proteins are in progress.

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Abbreviations

DMPC: dimyristoylphosphatidylcholine;
POPC: palmitoyloleoylphosphatidylcholine;
SOPC: stearoyloleoylphosphatidylcholine.

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