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Special features of phosphatidylcholine vesicles as seen in cryo-transmission electron microscopy

Beate Klösgen, Wolfgang Helfrich

Fachbereich Physik, Freie Universität Berlin, Arnimallee 14, D-14195 Berlin, Germany

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Abstract. Vesicles of egg yolk phosphatidylcholine (EYPC) were studied by cryo-transmission electron microscopy. The electron micrographs indicate that, despite the rapidity of cooling, membrane undulations are flattened and some vesicles change their shapes before the samples freeze. These artefacts are attributed to the action of the lateral tension that results from the membrane area contraction associated with the temperature drop. Other micrographs represent grainy membranes and angular vesicles. We regard them as the first direct evidence for the superstructure and optically invisible roughness which were recently postulated for these membranes.

Key words: Vesicle shapes – Membrane roughness – Cryo-transmission electron microscopy

Introduction

The bilayers of phosphatidylcholines (PC), phosphatidylethanolamines (PE) and digalactosyldiacylglycerols (DGDG) have been studied extensively, being typical and electrically neutral lipid membranes. Their mechanical properties are thought to be controlled by a stretching modulus and a bending rigidity, both of which have been measured for many of them. The measurements of the stretching modulus, which is generally near 200 mN/m, seem well reproducible with a normal scatter, at least at lateral tensions above 0.1 mN/m (Needham and Evans 1988; Evans and Rawicz 1990). There are many experimental studies of the bending rigidity of PC, PE and DGDG bilayers (see, e.g., Engelhardt et al. 1985; Faucon et al. 1989; Mutz and Helfrich 1990 and earlier references cited therein; Evans and Rawicz 1990; Kummrow and Helfrich 1991; Waugh et al. 1992). The measured values usually display a spread among vesicles which is larger than the experimental error for the single vesicle. The averages obtained by different methods vary even more widely, ranging in the case of egg yolk PC (EYPC) from $2.3 \cdot 10^{-19}$ J to $2.4 \cdot 10^{-20}$ J. The highest value was derived from the bending fluctuations of tubular vesicles (Servuss et al. 1976; Beblik et al. 1985), while the lowest was calculated from the elongation of spherical vesicles by electric fields, where at tensions below 10^{-2} mN/m the stretching consists essentially in a flattening of thermal undulations (Kummrow and Helfrich 1991). The differences are still unexplained and may be regarded as a sign of strange membrane behavior.

A suspicion that these lipid bilayers are more complex than expected and possess individual properties arose for the first time in a study of tube fluctuations when it was noted that the PC membranes tended to go intermittently and locally into a state of rapid wiggle-like motion (Beblik et al. 1985). In the meantime the impression of complexity was reinforced by other observations. Apart from the excessive variation of the bending rigidities, they include chaotic blebbing from vesicles (Evans and Rawics 1990), a dispersed phase of EYPC (Harbich and Helfrich 1990a), an occasional excessive stretchability of PC membranes (Kummrow and Helfrich 1991) and some peculiarities of budding from vesicles (Käs and Sackmann 1991) and extended membranes which were noted in a theoretical analysis (Wiese et al. 1992). The most impressive phenomenon indicating complexity is, perhaps, mutual membrane adhesion induced by lateral tension (Servuss and Helfrich 1989; Harbich and Helfrich 1990b; Mutz et al. 1990). While spontaneous adhesion and an unbinding transition were found in our laboratory only with DGDG in NaCl solution (Mutz and Helfrich 1989), induced adhesion was common to all the electrically neutral lipid membranes studied (Servuss and Helfrich 1989; Harbich and Helfrich 1990b; Mutz et al. 1990). As a rule, it is rare and accidental, but it can be obtained reproducibly in swollen multilayer systems by the simple trick of cooling (Harbich and Helfrich 1990b). Two membrane quantities can be measured at an adhesive contact: the contact angle and the rounding length. The latter was in general visible as the tensions were very low ($< 10^{-3}$ mN/m/; it permits the tension to be estimated if the bending rigidity is known. The contact angles were found to be independent of later-

Correspondence to: B. Klösgen

al tension over up to two orders of magnitude. The constancy seems to agree with the predictions of conventional undulation theory. However, a deeper analysis revealed that the experimental contact angles of 40° and more, depending on material and geometry, would require bending rigidities much lower than the minimum values measured. In an attempt to resolve the discrepancy, a new membrane roughness was postulated which is capable of storing much more membrane area than the few percent typical of undulations. The roughness would have to be at the same time very sensitive to lateral tension and of a scale below optical resolution. It would require for its support an unknown superstructure of the membranes.

A possibility of directly checking the hypothesis of a roughness associated with a superstructure is offered by cryo-transmission electron microscopy (cyro-TEM; for a comprehensive summary see Dubochet et al. 1988). This technique allows the study of vesicle suspensions prepared as thin water lamellae. At cooling rates faster than is common in freeze fracture electron microscopy, the specimens are frozen so rapidly that the water solidifies into the glassy state. In contrast to other techniques, there is no further treatment of the samples which are photographed in the frozen state. Cyro-TEM has already been used by several groups to study vesicle suspension, resulting in pictures which look very similar to those obtained by light microscopy (Lepault et al. 1985; Talmon 1986; Siegel et al. 1989; Vinson et al. 1989; Walter et al. 1990; Frederik et al. 1991; Edwards 1991; Chestnut et al. 1992). However, the question of whether the cooling, despite its rapidity, produces artefacts in the lipid membranes was not investigated. In the present work, we did cryo-TEM of PC vesicle suspensions with two purposes in mind. On the one hand, we searched for evidence of the postulated superstructure, including an extra roughness, of electrically neutral lipid membranes. On the other hand, we looked for artefacts, especially those which may be expected to arise from an enormous contraction of the membrane area before freezing. The two problems cannot be separated as a high lateral tension resulting from the contraction may destroy the superstructure. Accordingly, we will document apparent artefacts before we shown some of the rather rare pictures revealing grainy membranes or angular vesicles which seem to reveal the anticipated superstructure or roughness. Some of the immediate interpretations of our results will be substantiated by estimates in the discussion.

Preliminary accounts of the present work were given in conference proceedings (Helfrich and Klösgen 1990, 1991; Klösgen and Helfrich 1992). In addition, a model for the superstructure allowing for the postulated roughness has been proposed (Helfrich 1989). It is based on the conjecture of highly localized saddles of very large curvature which because of their cooperativity are capable of deforming the membrane on a larger scale.

Materials and methods

Egg yolk-L-α-phosphatidylcholine (EYPC) and L-α-dimyristoyl-phosphatidylcholine (DMPC) were used as purchased (Sigma Chemical Co., Munich; Avanti Polar Lipids, Alabama) without further purification. Control by thin layer chromatography only showed the spots characteristic of DMPC and EYPC, which is a mixture of molecules (Stahl 1967). Mostly 1% (wt/wt) vesicle suspensions were prepared by the following procedure similar to the standard method of Saunders et al. (1962) and others (Reeves and Dowben 1969; Kim and Martin 1981). 5 ml of lecithin solution in chloroform (Merck, spectroscopic grade) at a concentration of 10 mg/ml is placed in a 10 ml flask which is mounted onto a rotary evaporator (Büchi, Rotavapor). On slowly removing the solvent by gentle rotation of the flask under reduced pressure (water pump vacuum) at a temperature of about 318 K, a rather uniform layer of pure lipid is deposited onto the bottom of the flask. After addition of 5 ml of pure water from a Millipore water system or, alternatively, a 10 mM solution of NaCl in distilled water the flasks are sealed and stored for a day at 318 K. This temperature is above the main transition temperature of the lipid membranes which does not exceed $T_m = 253$ K (Lammers et al. 1978) for EYPC and which is about 294 K for DMPC (Szoka and Papahadjopoulos 1980). Gradual water uptake results in the formation of a milky cloud in the flask. Finally, the sample is transformed into a suspension of vesicles either with a sonifier (Branson, Cell Disrupter B15; typically for 5 min at 318 K in the interrupt mode), or with a laboratory vibrator (Heidolph; about 5 min at moderate modes), or by gentle mechanical rotation (ETI, orbital Mini-Shaker; at room temperature with 200 cycles/min for about 8 h), or by manual rotation as the gentlest method.

Vitrified specimens to be examined in the cryo-transmission electron microscope were prepared from these vesicle suspensions by the bare grid method (Dubochet et al. 1988; Bellare et al. 1988; Jäger 1990). A pipette (Eppendorf) is used to apply a 10 µl droplet of the suspension on a copper grid (400 mesh/inch) mounted on a plunger device (Talmon 1986; Bellare et al. 1988). Quickly and by minutely controlling the formation of the lamella in the meshes through a microscope eyepiece the excess suspension is removed from the grid with blotting paper. When the lamella has sufficiently thinned, after at least 20 s, the grid is rapidly transferred into a small liquid nitrogen (76 K) cooled vessel containing liquid propane (Bellare et al. 1988; Jäger 1990; Ryan et al. 1990) which provides for very good heat conductance as no boiling bubbles hinder thermal contact. The cooling rate was measured by means of a thermocouple to be $3 \cdot 10^4$ K s^{-1} (Groll 1986), which may be regarded as a lower limit (Bald 1987; Lücken et al. 1992). By preventing crystallization, the rapid cooling transforms the water into low density ($\varrho \approx 0.94$ g cm^{-3}) vitreous ice (Brüggeler and Mayer 1980; Dubochet and McDowell 1981; Adrian et al. 1984; Heide and Zeitler 1985; Angell et al. 1986). This amorphous phase of ice is thermally stable between 76 K and the devitrification temperature at about 130 K (Heide 1974; Lepault et al. 1985; Johari et al. 1992) above which crystallization into cubic ice occurs. To preserve the vitreous state the sample grid was mounted under liquid nitrogen (76 K) in a cryo-specimen holder which was then transferred into the precooled ($\sim$ 76 K) vacuum chamber

of the electron microscope (DEEKO 250 (Jäger and Zeitler 1985)). The microscope was operated at 100 kV ($\lambda = 3.8$ pm) in the conventional transmission mode with a resolution of up to 0.7 nm. The phase contrast needed to enhance the contrast of fine structures such as the lipid bilayers (Adrian et al. 1984; Dubochet et al. 1987) was achieved by underfocussing (Hawkes 1972; Adrian et al. 1984; Stewart and Vigers 1988; Vogel et al. 1986) the objective lens to an underfocus of $\Delta f \approx -1$ μm. The sample thickness was crudely estimated from the diminution of the electron beam in a spot near the final exposure area. Another procedure of sample preparation that has the advantage of being faster was chosen in several cases. It consists in using a carbon foil containing a lot of holes instead of the copper grid with its regular meshes. In general, the holes are much smaller than the meshes. Therefore, the water lamellae are rather stable so that the excess suspension can be removed from the foil within a few seconds. This is achieved by putting a piece of blotting paper directly onto the carbon grid from behind to suck the suspension through the holes. After removing the blotting paper, one is left with thin lamellae of vesicle suspension spanning the smaller holes. The specimen is then rapidly transferred into the coolant. A vitrified probe can thus be obtained within approximately 5 s after coating the carbon grid.

Results

The size and the shape of the vesicles observed depended very much on the mechanical method of preparation. The use of 10 mM NaCl solution instead of pure water had no effect on the appearance of the samples. The most homogeneous vesicle suspensions were obtained by the sonication method. Here, the vesicles were rather small and more or less spherical. The size distribution became sharper and was shifted towards smaller vesicles if the sonication time was longer. The suspensions obtained by the mechanical or manual rotation method were quite heterogeneous as to both size and shape of the vesicles. They turned out to be the most interesting samples, revealing deformations due to area contraction as well as grainy membranes and angular vesicles. Micrographs of all types of preparations will be presented and evaluated.

The sample shown in Fig. 1 was taken from a 1% (wt/wt) egg yolk PC suspension in pure water. The sample thickness as estimated from the diminution of the electron current is about 80 nm. Apparently, a size separation of the vesicles has happened during film formation when the liquid lamella was thinning. The larger particles have been expelled from the center of the mesh and collected in the thick region near a copper bar. This process was reported to occur within less than a second (Dubochet et al. 1988). All the vesicles exhibit rather smooth surfaces as compared to flaccid giant vesicles depicted in the same size. Since vesicle shape fluctuations are easily visible in light microscopy and predicted to be scale invariant, we may infer from their absence that they have been largely supressed by lateral tension. The degree of suppression can in principle be used to estimate the lateral tension (see below). The size distribution ranges from many very small vesicles of radii as small as 5 nm to a few big ones, the largest diameters exceeding the thickness of the frozen water lamella by about a factor of three. Two observations indicate that the large vesicles did not project from the lamellae. First, if the deformed vesicles would stick out, the total sample thickness would be locally enlarged. This should result in an increased shading of the vesicle centers which was never seen in the micrographs. Second, tilt exposures done with several samples (Lücken and Jäger 1992) showed the contour shape of the largest vesicles to depend on the tilt angle. We suspect that the large vesicles have been squeezed flat during the thinning of the water lamella. This may be attributed to the enormous forces exerted by the surface tension of water on bulges in the air-water interface. The ensuing excess pressure inside the vesicle and the lateral tension of its membrane should be large enough to enforce rapid water permeation or produce transient holes, thus enabling the vesicle to reduce its volume (see also below).

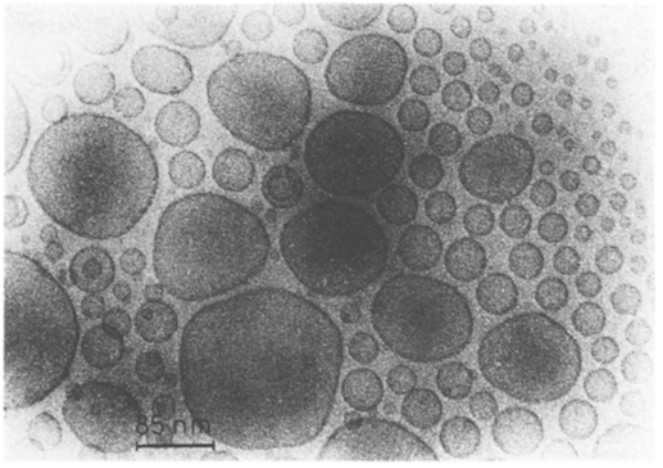

Fig. 1. EYPC vesicles in pure water, prepared by sonication. Some vesicles are larger than the measured thickness of the water lamella of $d \approx 80$ nm, but do not stick out (see text). Note the concentration of small vesicles in a thinner region near the middle of the mesh in a copper grid. Defocus: $\Delta f \approx -1$ μm [1]

The thickness of the liquid lamella is hard to control. Only rarely did we succeed in obtaining a thick lamella without crystalline ice. An example is given in Fig. 2 which shows vesicles in a vitreous water lamella about 220 nm thick. The preparation was exactly the same as in the case of Fig. 1. Obviously, the vesicle concentration was too high to prevent overlap of vesicle contours. The increase of the gray value with the number of membranes passed by the electron beam is easily visible. Note that there is only one vesicle in Fig. 2 with a diameter larger than the lamella thickness. Two kinds of deviations from the spherical shape can be seen in Fig. 2. The first, also existing in Fig. 1, is a bumpiness of the larger vesicles associated with a tendency of the membranes not to touch each other. We noticed the avoidance of contact whenever

[1] All values given for d and Δf in any picture are estimations based on local measurements close to the site of taking the micrograph

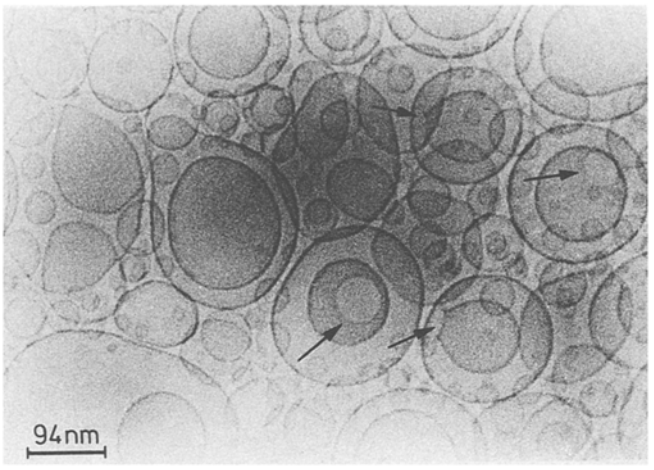

Fig. 2. EYPC vesicles in pure water, prepared by sonication. Note the bumpiness of some of the vesicles often associated with a tendency of the membranes to avoid each other. A few vesicles are bottled-shaped (marked by arrows). Sample thickness: $d \approx 220$ nm; $\Delta f \approx -1$ μm

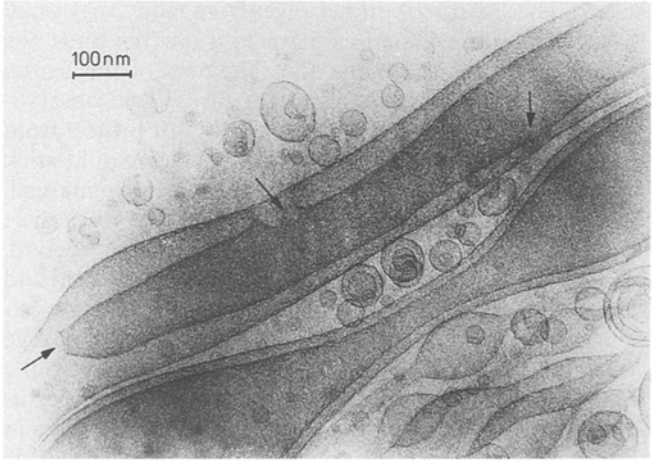

Fig. 3. EYPC vesicles in 10 mM NaCl solution, prepared by manual rotation. Note the spindle shapes of two big cylinders, one enclosing the other, and of some small ones. They probably indicate high lateral tensions, as do the rupture sites on another big cylinder (marked by arrows). $d \approx 260$ nm; $\Delta f \approx -2.8$ μm

the membranes were frozen in the fluid state. It indicates a repulsive interaction which we think is brought about by thermal undulations. Apparently, the latter have been smoothed out by the area contraction of the membranes during cooling so that they are absent in the pictures. Electrostatic repulsion as an alternative appears unlikely especially since the addition of salt made no difference. Moreover, in the course of our experiments we noted that DMPC vesicles frozen in the flat crystalline phase ($L_{\beta'}$) tend to display large areas of very close contact even in pure water (Klösgen and Lücken, unpublished observations). This rules out an important electrostatic repulsion, thus confirming the role of undulations in the fluid phase (L_α). Bottle shapes represent the second kind of nonspherical vesicles in Fig. 2. Most of them can easily be distinguished from large vesicles enclosing smaller ones. Often, they exhibit a typical rim (see small arrows) where the inner membrane connects to the outer one. In other cases, one sees an enclosed object which cannot be a vesicle as it looks brighter than its immediate surroundings (see large arrow). The origin of the bottles is unclear. A rather natural explanation would be the fusion of a vesicle with an enveloping larger one. It should be noted that the mutual avoidance of EYPC membranes has been documented but not discussed in a paper by Talmon (1986). Bottle shapes have also been found previously by other authors and interpreted in a different manner (Dubochet et al. 1988; Vinson et al. 1989).

A rich variety of vesicle shapes besides spheres is obtained if the swollen PC suspension is gently rotated by hand in a comparatively large flask. Resulting in many tubular structures of variable size, this treatment is evidently much less disruptive than sonication. Figure 3 shows a vesicle suspension thus prepared, containing 1% (wt/wt) of EYPC swollen at 318 K in 10 mM NaCl solution. The most prominent objects in this very thick sample are two pairs of wide tubes crossing the whole picture. In each pair, one tube is fitted into the other. There are also more or less spherical vesicles, single-walled or of more complex structure. One pair of the wide tubes as well as other, narrow tubes display a pronounced modulation of their diameters. It is also remarkable that the distance between the membranes of the pair is practically constant over the whole contour length. This is a further indication of the repulsive interaction discussed above. We believe the modulation of the tubes to be an artefact that results from the rapid area contraction of the still fluid membrane when the sample enters the coolant. The spindle-like shapes which we see are far from the shapes of constant mean curvature balancing a spontaneous curvature. The latter have been calculated; they range from the straight cylinder over beaded rods to the pearl string (Deuling and Helfrich 1970). The spindles can hardly be explained as preexistent equilibrium shapes arising from a lateral tension. In the absence of spontaneous curvature lateral tension lengthens and narrows a given tube, concentrating the fixed enclosed volume in a single inflated section. Vesicles with a tether are a familiar example (Waugh and Hochmuth 1987). The situation can be more complicated in the presence of both lateral tension and spontaneous curvature. However, the previously calculated shapes (Deuling and Helfrich 1976 a, b) are easily seen to remain solutions of the shape equation (Wiese and Helfrich, unpublished results 1992) if there is lateral tension. All this suggests as another explanation that the modulated shapes of Fig. 3 were produced during the cooling. Apparently, the lateral tension produced by area contraction destabilizes the original shape, probably a straight cylinder, thus initiating a separation into narrower and wider sections. The most direct evidence for lateral tension is provided in Fig. 3 by the inner tube of the other pair of wide tubes. Its membrane appears ruptured at some places (see small arrows) and completely broken in a narrow section outside the figure. Permitting water to escape from the tube, the holes could be the reason why there is so little modulation in this case.

Two parallel very thin spindle-like tubes of EYPC in pure water are shown in Fig. 4. Note the clear resolution

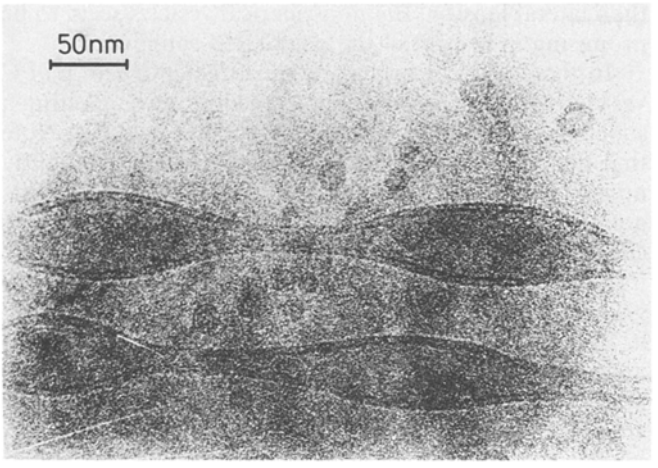

Fig. 4. A pair of tubular EYPC vesicles in pure water, prepared by manual rotation. As in Fig. 3, the spindle shapes presumably are the result of a destabilization of straight cylinders by high lateral tension. $d \approx 50$ nm; $\Delta f \approx -1.0$ μm

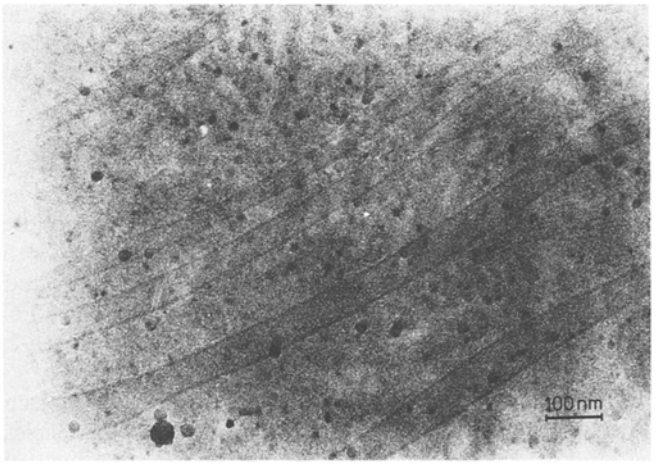

Fig. 5. Set of parallel thin tubular vesicles of EYPC in pure water, prepared by manual rotation. $d \approx 70$ nm; $\Delta f \approx -1$ μm

of the monolayers in each of the bilayer contours. For the same reason as before, the modulation is presumably an artefact due to membrane contraction during cooling. Figure 4 is part of a larger picture comprising an area of 2.0 μm by 1.73 μm. The tubes run across the whole picture, their ends being probably fixed somewhere on the grid. In contrast to the wide tubes which were usually modulated, many of the narrow tubes had rather constant radii. An example of five such tubes, again of EYPC in pure water, is given in Fig. 5. Being straight and parallel, the tubes were probably fastened to the grid. Similar sets of this parallel tubes occured several times in our samples. The alignment of the tubes is probably due to the hydrodynamic effects connected with sample preparation (pipetting, blotting and water lamella formation). As a rule, the tubes are not completely straight, deviating from their overall direction by up to 10°. Rupture sites of very thin tubes have not been seen in any of our samples. Most of these features agree with earlier findings of Miller et al. (1987) who also documented, without discussion, a wide

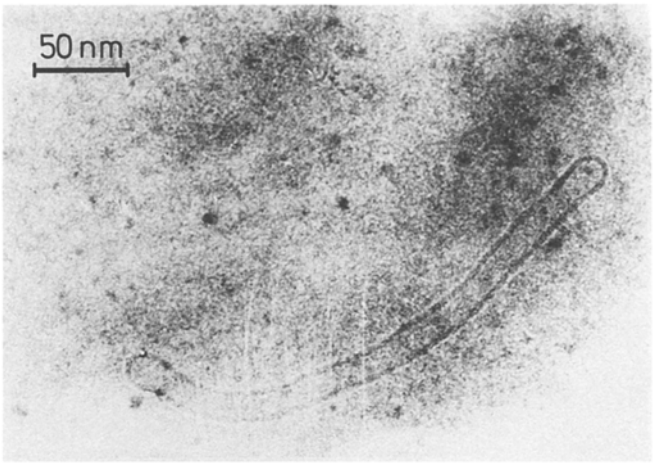

Fig. 6. Thin tubular EYPC vesicle with free ends, frozen in 10 mM NaCl solution, prepared by mechanical rotation. Note the bending fluctuation of the tube and also the well-developed small-scale fluctuations of the tube membrane. $d \approx 60$ nm; $\Delta f \approx -1.4$ μm

variety of vesicle shapes including spindles and the mutual avoidance of neighboring membranes in systems of synthetic ionic surfactants (SHBS). However, it is not clear whether the mutual repulsion of these membranes is due to the undulations or to surface charges.

A freely floating tubular EYPC vesicle is seen as a whole in Fig. 6. Apart from the visible membrane thickness, it looks like a fluctuating giant tube in light microscopy (Beblik et al. 1985). First, there is a pronounced bending fluctuation of the whole tube, the angle between the tube ends having a typical value. Second, remnants of the short wavelength undulations along the contour of the tube are easily visible. The apparent scale invariance strongly suggests that small cylindrical vesicles can adjust to area contraction without developing significant lateral tensions. Such an adjustment requires the vesicle to change its length and diameter while conserving its overall shape. In contrast to the small tube, short-wavelength undulations seem to be smoothed out by lateral tension on the extended tubes of the preceeding figures.

The principal aim or our work was to find evidence for a superstructure and a small-scale roughness of the membranes. Their absence in the pictures shown so far does not necessarily mean that they did not exist prior to freezing. The high lateral tensions (see below for an estimate) brought about by the contraction of the membrane area may have destroyed the suspected cooperative saddles and any roughness due to them. The shear flow associated with filling and emptying the transfer pipette as well as the blotting of the suspension droplet from the grid also produce lateral tensions which could be damaging. A destruction would be particularly relevant if the buildup of the superstructure takes time as was previously speculated (Helfrich 1989). Furthermore, the postulated membrane roughness arising from the superstructure could be incompatible with the small dimensions of the vesicles as its scale should be of the same order of magnitude. We may infer that the chances of finding to local saddles should be best with vesicles so small and nonspherical that area contraction results in a change of shape rather

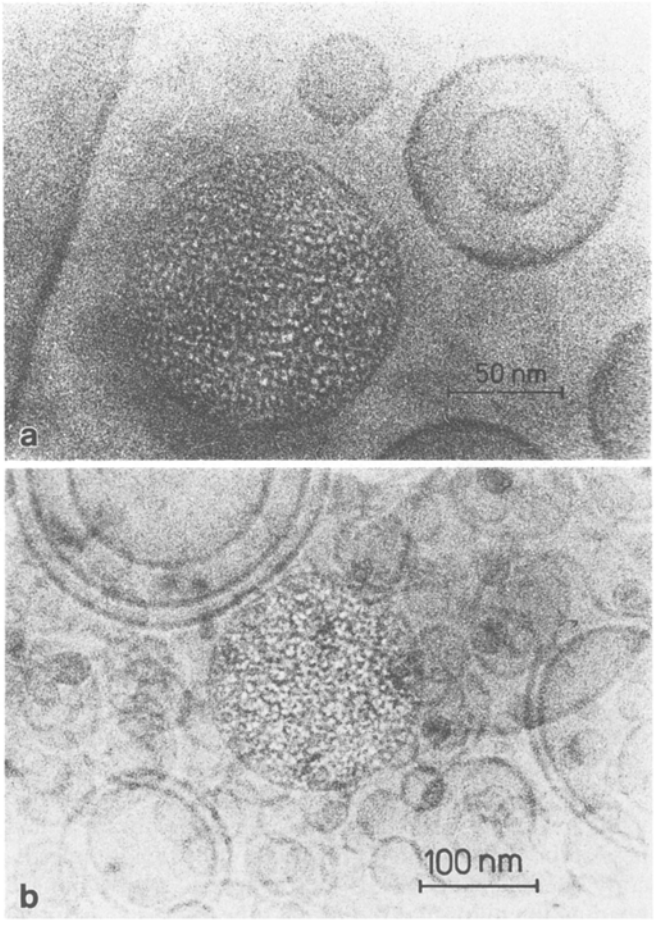

Fig. 7 a, b. EYPC vesicles with a grainy membrane among others with smooth membranes, prepared in pure water with a laboratory vibrator (Fig. 7 a) or by a rotation shaker (Fig. 7 b). No visible traces of radiation damage can be observed all over both pictures. In the very crowded sample shown in Fig. 7 b lots of "normal" vesicles are grouped around the grainy one. The graininess is thought to reveal a superstructure of the membrane. **a** $d \approx 200$ nm; $\Delta f \approx -0.8$ µm. **b** $d \approx 50$ nm; $\Delta f \approx -1$ µm

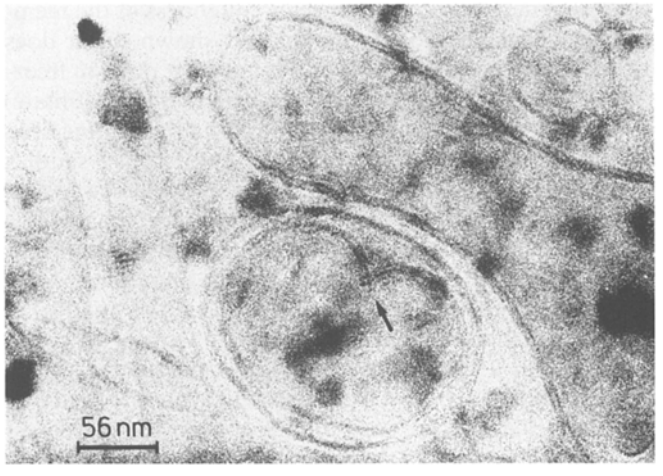

Fig. 8. EYPC vesicles of various shapes in pure water, prepared by manual rotation. Note the deep notch (see arrow) in one of the vesicles resulting in a heart shape. $d \approx 170$ nm; $\Delta f \approx -1.0$ µm

than lateral tension. Big nonspherical vesicles seem to be promising in respect of the postulated roughness.

In the course of our work we detected a few EYPC vesicles with an inhomogeneous membrane suggesting a superstructure. Examples are given in Fig. 7 a, b. Note that the contours of these particular vesicles are slightly nonspherical. The "graininess" in the membranes differs greatly from the appearance of the vast majority of vesicles, including those surrounding them. It is also distinct from the effect of radiation damage which was nowhere visible in the larger pictures, of which sections are shown in Fig. 7 a, b. The contours of the vesicles look pale and appear a bit fuzzy in comparison with their "normal" neighbors. However, the unevenness accompanying the graininess does not seem to store enough area to represent the postulated membrane roughness. The bright spots of the grainy pattern are brighter than the background outside the vesicles. We may assign the bright (and dark) spots to the imaging action of the deformed membrane which varies in thickness and inclination. The spacing of the bright spots is on the order of the membrane thickness of approximately 4 nm. This would agree with a dense array of highly localized saddles. It cannot be ruled out that the pattern of dots is a superposition of two images from the two sides of the vesicle.

Altogether, we found five vesicles with a distinct graininess and several others that look opaque, possibly suggesting indadequate focusing. Graininess as well as opaqueness always extended over the whole picture of the vesicle. The radii of all those vesicles ranged from 12 to 62 nm. Recently, grain membranes have also been found with vesicles prepared from a mixture of soy-bean-PC and cholesterol or DMPC and cholesterol (Lücken 1992, private communication).

A roughly elliptic EYPC vesicle contour with a deep notch (see arrow) is shown in Fig. 8. Very similar vesicles of about the same size have also been noticed and called heart-shaped in a cryo-TEM study of DMPC vesicle preparations by the Sackmann group (Weiss 1989). The indented vesicle of Fig. 8 happens to be enclosed by another, elliptic vesicle. With the possible exception of these two vesicles, undulations seem to be strongly suppressed in the other membranes seen in the figure. The notch need not be as sharp in reality as it appears since the indented contour may not be properly aligned. However, the indentation can hardly be understood in terms of bending elasticity, and a notch has never been observed with giant vesicles in light microscopy. At the center of the indentation could be a local saddle or a row of such saddles, in accordance with the proposed model of a superstructure (Helfrich 1989).

In order to increase the chances of our search for the postulated roughness we modified the gentle method of EYPC vesicle preparation. After manual rotation the dispersions were put in a refrigerator and kept at 281 K for two weeks. The flasks were brought back to room temperature, ca. 295 K, one or two hours before the contents were processed. Reheating after a period of incubation sufficiently long to establish a new equilibrium at the lower temperature was expected to result in generally nonspherical vesicles. At the same time, we hoped to ben-

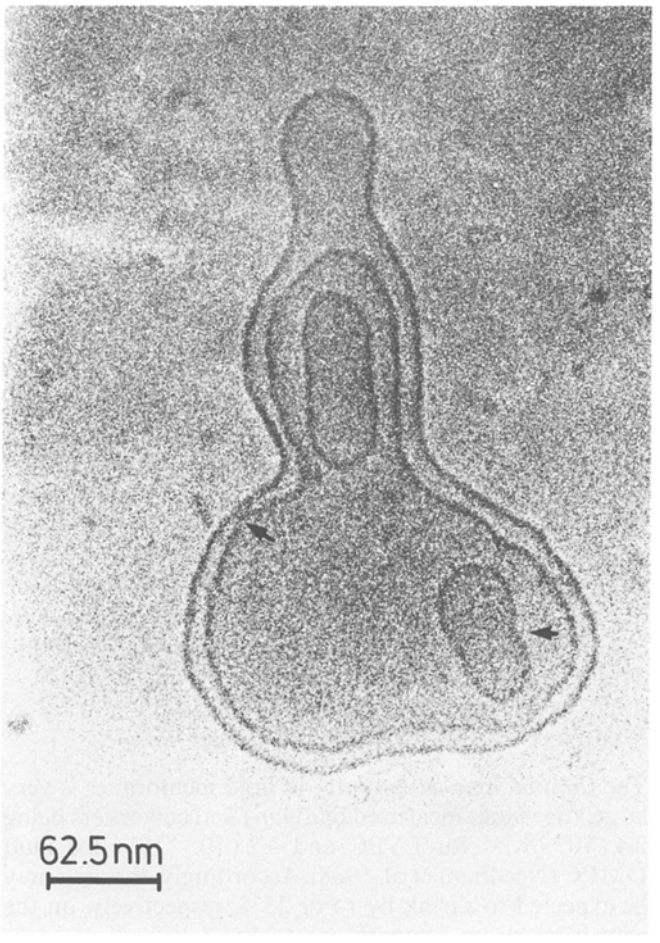

Fig. 9. Large EYPC vesicles in 10 mM NaCl solution, prepared by manual rotation and subsequent incubation at 218 K for 12 d. Note the strange shapes and the extraordinary bumpiness of the two outer-most membranes, thought to indicate a novel membrane roughness. $d \approx 170$ nm; $\Delta f \approx -2.8$ μm

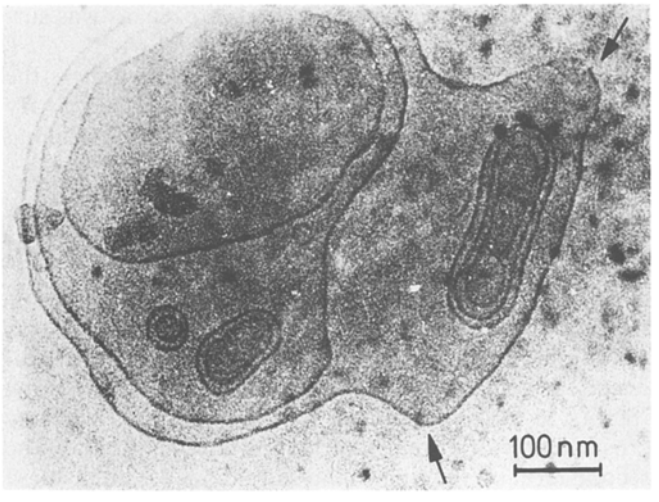

Fig. 10. EYPC vesicles, prepared as those of Fig. 9. Note the strange shapes of the two outermost membranes. $d \approx 170$ nm; $\Delta f \approx -1.4$ μm

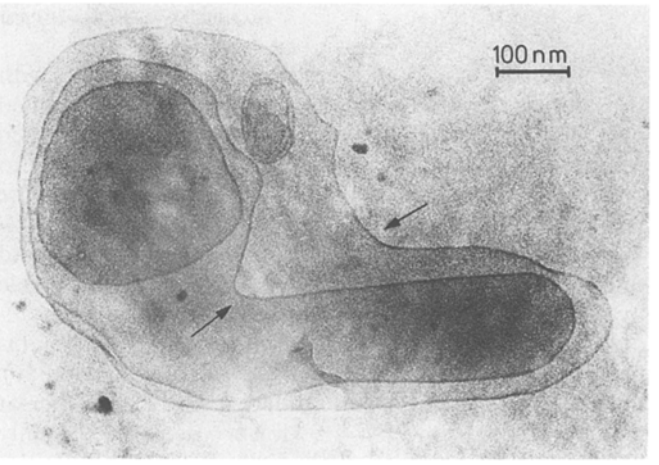

Fig. 11. EYPC vesicles, prepared as those of Figs. 9 and 10. Note the strange pistol shape of one of the vesicles and the sharp corners (see arrows) in the contours of the larger vesicles. $d \approx 180$ nm; $\Delta f \approx -1.8$ μm

efit from a tendency of EYPC membranes to disintegrate at temperatures below 288 K into an optically invisible dispersion (Harbich and Helfrich 1990 a). Any mechanism of membrane breakup is likely to involve holes. They would permit the vesicles to lose volume as is required for strong deformations. Both effects would increase the relative area contraction up to which the vesicles remain nonspherical. The electron micrographs obtained from the incubated dispersions looked indeed different from the rest. While there were no more grainy membranes to be seen, most of the vesicles, including the smaller ones, were unusually bumpy. Some of the big vesicles displayed, in addition, fairly sharp membrane bends giving them an angular appearance. Examples of angular vesicles are shown in Figs. 9 to 11 where the largest vesicle always encapsulates other ones. In all three figures the larger contours are dramatically irregular. Having no counterparts among the giant vesicles studied by light microscopy, the angular shapes can arise neither from undulations nor from spontaneous curvature. We believe them to be a manifestation of the new roughness which was postulated to rationalize a considerable storage of membrane area. As a check, we also investigated osmotically shrunken EYPC vesicles. The electron micrographs displayed reduced volumes but no angular shapes.

Discussion

The electron micrographs show that the effects of lateral tension are ubiquitous in rapidly frozen PC membranes. Most membranes look smoother than their counterparts in optical microscopy. Nevertheless, a few vesicles have grainy membranes and others angular contours. Their membranes seem to reveal the conjectured superstructure and extra roughness, respectively. Before discussing these special phenomena, we have to consider the frozen state in general as well as the lateral tension generated by cooling and its consequences.

a) The frozen state

A preliminary remark may be in place before we deal with the samples themselves. Some of our pictures were spoiled by a superficial artefact, namely blobs of propane frozen onto the surface of the water lamella. They are easy to distinguish from lipid structures because they evaporate when the sample is exposed to the electron beam.

Ideally, the freezing of the samples should be so rapid that there are no changes whatever on the scale resolvable by electron microscopy. Water seems to satisfy this requirement as it converts into vitreous ice if the temperature is lowered quickly enough (Angell and Choi 1986). However, this vitreous state differs from liquid water at room temperature by its lower density of only $0.94\,g\,cm^{-3}$ (Dubochet et al. 1982; Heide and Zeitler 1985; Franks 1986). The increase in volume of the water reinforces, in effect, the contraction in area of the membranes, as will be discussed below. If the freezing is not fast enough, liquid water may also transform into cubic or hexagonal crystalline ice. In fact, the striations typical of hexagonal ice are present in some of our micrographs. Traces of cubic ice were detected in part of the samples when checked in the standard way by electron diffraction. Sometimes hexagonal ice is seen only on one side of a membrane. None of our pictures suggests that the incipient crystallization affected vesicle contours. The micrographs shown here are all supposed to be taken from regions in the samples that contain lipid vesicles suspended in vitrified water.

Phosphatidylcholine molecules are larger and more complex than water molecules. EYPC membranes solidify at c. 253 K (Lammers et al. 1978), i.e., much below the crystallization temperature of water. Therefore, we expected these membranes to vitrify if the cooling is rapid enough for the vitrification of water and to do so below 230 K, the approximate onset temperature for the vitrification of water (Dubochet et al. 1982; Franks 1986; Johari et al. 1992). The smoothness of the vesicle contours even in high resolution pictures indicates the absence of breaks of the frozen membranes, thus suggesting that the frozen EYPC membranes are glassy rather than crystalline. The slowness of molecular rearrangements as measured by the temperature jump method (Genz and Holzwarth 1985; Holzwarth 1986) is a further argument for glassiness. Fluid membranes in frozen water could continue to contract, thus smoothing out locally while the larger features of their shapes are preserved, provided a hydration shell is still fluid. Such a mechanism, if it exists, could help to explain why the membranes of narrow tubes are smooth although the tubes are not quite straight. A more direct explanation of small scale smoothing may be based on size effects (see below).

Thermodynamically equilibrated DMPC membranes in excess water are in the fluid phase (L_α) above their main transition at 296 K. Between this temperature and 289 K they have been observed to form the rippled crystalline phase ($P_{\beta'}$) and at lower temperatures the planar crystalline phase ($L_{\beta'}$) (Rüppel and Sackmann 1983; Zasadzinski and Schneider 1987). In our experiments, DMPC membranes froze from the fluid (L_α) state to give the same smooth contours as EYPC membranes if the temperature prior to cooling was a few degrees (3 K or more) above the main transition. In disagreement with Lepault et al. (1985) our sonication vesicles were in general spheres (except those which are deformed for the reasons discussed in the interpretation of Figs. 1, 2) and never had faceted or broken contours at that. The smoothness suggests that the DMPC membranes are in the glassy state. The effects of area contraction did not differ from those seen with EYPC bilayers. No grainy membranes, angular vesicles, or other possible indications of a superstructure have been noticed in the relatively few DMPC samples frozen in the fluid state, all of which were prepared by the sonication method. Starting from temperatures closer to the phase transition resulted in membrane contours full of fine cracks. If the initial temperature was below the main transition, the pictures showed the respective crystalline phases, the membranes being either rippled ($P_{\beta'}$) or flat ($L_{\beta'}$) between the edges of polygonal vesicles. In order to avoid ambiguities we have shown here only EYPC membranes. Pictures of the crystalline phases of DMPC will be presented elsewhere (Klösgen and Lücken, to be published).

b) Area contraction and lateral tension

The thermal area expansivity of lipid membranes is very large, the values measured by Evans and coworkers being $2.4 \cdot 10^{-3}\,K^{-1}$ for EYPC and $4.2 \cdot 10^{-3}\,K^{-1}$ for fluid DMPC (Needham et al. 1988). Accordingly, the area may be expected to shrink by 14 or 25%, respectively, on the way from room temperature to the vitrification onset temperature of water at about 230 K, i.e. over a temperature span of c. 60 K. The estimates presuppose that the membranes remain fluid and, specifically, that the area is a linear function of temperature. The shrunken area decreases, in effect, by another 44% as the volume of the vitrified water is 6% higher than that of the liquid water (Heide and Zeitler 1985). This adds up to apparent decreases of 17% and 28% for EYPC and DMPC, respectively. A further decrease of area is conceivable if the membrane continues shrinking in a liquid hydration layer while the bulk of the water is already frozen, as was surmised above.

Area contraction produces lateral tension unless the shape of the vesicle can adjust to it. The tension necessary to compensate thermal shrinking by elastic stretching may be computed from (Helfrich and Servuss 1984)

$$\frac{\Delta A}{A_0} = \frac{\sigma}{\lambda} + \frac{k_B T}{8\pi k_c} \cdot \left[\ln\left(\frac{A}{a^2}\right) - \ln\left(\frac{\pi^2/a^2 + \sigma/k_c}{\pi^2/A + \sigma/k_c}\right)\right] \quad (1)$$

if the initial tension is taken to be zero. Here $\Delta A/A_0$ is the relative area increase required to neutralize the shrinkage, σ is the lateral tension, λ the membrane stretching modulus, k_B Boltzmann's constant, k_c the membrane bending rigidity, A the membrane area, and a^2 an area near the cross section of a lipid molecule in the membrane. The second term on the right-hand side stands for an increase not of real but of base area. It is due to the fact that lateral tension reduces the thermal undulations superimposed on a given (equilibrium) vesicle shape. The maximum

contribution of the second term, reached for $\sigma = k_c \cdot \pi^2/a^2$, is

$$\mathrm{Max}\left(\frac{\Delta A}{A}\right)_{\mathrm{und}} = \frac{k_B T}{8\pi k_c} \ln\left(\frac{A}{a^2}\right).$$

If is interesting to estimate this upper limit. Inserting $k_B T = 4 \cdot 10^{-21}$ J (at room temperature), $k_c = 2.4 \cdot 10^{-20}$ J (the smallest bending rigidity measured for PC's (Kummrow and Helfrich 1991)), $A = 4\pi \cdot 10^4$ mm^2 (for a spherical vesicle of radius 100 nm) and $a^2 \cong 1$ nm^2, we find $(\Delta A/A)_{\mathrm{und}} = 6\%$. The lateral tension needed to obtain half the maximum contribution, i.e. 3%, is computed from Eq. (1) to be 0.8 mN/m if one uses the same numbers and neglects the σ/λ term. The estimates indicate that the area stored in the undulations is not enough to compensate the lateral shrinking of the membrane brought about by cooling. The remaining deficits of area are 11% for EYPC and 22% for DMPC according to our crude calculations. With a stretching modulus $\lambda =$ 140 mN/m as measured for EYPC and DMPC (Evans and Needham 1987) the lateral tension needed for a compensation by Hookean stretching is 16 mN/m and 32 mN/m, respectively.

In discussing possible artefacts arising from area contraction and lateral tension we begin with vesicles of round shape. Exact spheres of fixed volume cannot adjust their shape to a reduction of surface area and remain unchanged. A vesicle that before cooling was a freely fluctuating, flaccid sphere with vanishing lateral tension should become a very tight sphere before it freezes. The cutoff wave vector q_c up to wich the undulations are largely suppressed is given by (Helfrich and Servuss 1984)

$$q_c^2 = \frac{\sigma}{k_c}. \tag{2}$$

Adopting from above $k_c = 2.4 \cdot 10^{-20}$ J and $\sigma \approx 15$ mN/m, we have $q_c \approx 7 \cdot 10^8$ m^{-1}. The associated limiting wavelength $\lambda_c = 2\pi/q_c \approx 10$ nm is so short that no undulations should be visible in the micrographs. There are in fact many tight vesicles with perfectly circular contours in our pictures. However, the tensions expected to develop before freezing are an order of magnitude above the tension of rupture which was measured to be 2 to 3 mN/m for the two PC's (Evans and Needham 1987). The limiting tension may have been higher in our experiments because of shorter waiting times and lower temperatures. The actual tension of the membranes before freezing is difficult to estimate from the pictures since 1 mN/m or less may suffice to convey the impression of perfect smoothness within the resolution of the micrographs.

The time needed to go from room temperature to the vitrification temperature of water is about 2 ms at the minimum cooling rate of $3 \cdot 10^{-4}$ K s^{-1}. This period is in general not long enough to allow significant permeation of water through the membranes. For a proof we use the following formula for the bulk speed, v, of water permeation (Katchalsky and Curran 1965)

$$v = \frac{\alpha P}{N_A k_B T} \cdot \Delta p$$

where $\alpha = 18 \cdot 10^{-6}$ m^3 mol^{-1} is the molar volume of water, $P = 4 \cdot 10^{-5}$ ms^{-1} the permeability of EYPC membranes for water (Boroske et al. 1981), N_A Avogadro's number and $\Delta p = 2\sigma/r$ the Laplace pressure. Assuming a time averaged tension $\sigma = 15$ mN/m and a spherical vesicle of radius $r = 10$ nm, one calculates at room temperature $v \approx 0.9 \cdot 10^{-6}$ ms^{-1}. Accordingly, the water proceeds ≈ 1.8 nm in 2 ms. The actual advance of bulk water is probably much smaller during the time of cooling because the permeability is likely to decrease rapidly with decreasing temperature. Accordingly, the permeation of water should be negligible as a mechanism to relax lateral tension excepts, perhaps, for the very smallest spheres. In any event, the estimated area contraction is more than sufficient to explain the smoothing of undulations.

It is difficult to understand why the contours of adjacent vesicles usually are not quite circular but keep a fairly uniform distance from each other at the expense of forming pronounced bumps (Figs. 1, 2). We have interpreted these deformations as a vestige of undulatory repulsion between parallel membranes. The frozen bumpiness in otherwise very smooth membranes suggests in addition that there was not enough time for long wavelength undulations to relax. The relaxation time of the deformation modes of a sphere, represented by spherical harmonics, is given by (Milner and Safran 1987; Faucon et al. 1989)

$$\tau = \frac{\eta R^3}{k_c} \cdot \left(2 - \frac{1}{n(n+1)}\right) \cdot \left(\frac{2n+1}{(n-1)(n+2)[\bar{\sigma} + n(n+1)]}\right), \quad \text{for } n \geq 2, \tag{3}$$

where R is the radius of the sphere, n the "principal quantum number" of the mode, η the viscosity of water and $\bar{\sigma} = \sigma \cdot R^2/k_c$. The upper limit of the shape relaxation time of a shrinking deformed sphere as obtained for $\sigma = 0$ and $n = 2$ is approximately

$$\tau_{\max} = 0.38 \cdot \frac{\eta}{k_c} R^3.$$

For $R = 100$ nm and the room temperature values $\eta = 1 \cdot 10^{-3}$ Pa s and $k_c = 2.4 \cdot 10^{-20}$ J one obtains $\tau_{\max} \approx 1.6 \cdot 10^{-5}$ s. Since this is a very short period, the survival of bumps may indicate that the viscosity of the supercooled water increased dramatically as the temperature dropped which is in accordance with other findings (Franks 1986; Johari et al. 1992). The rapid dcrease of τ with increasing n predicted by Eq. (3) might explain why the contours are perfectly smooth apart from the round bumps which be regarded as long-wavelength deformations (small n). Another conceivable explanation, continued small-scale smoothing in a still fluid hydration layer, was suggested above. Also, the bulk viscosity of the membrane itself, being at room temperature a hundred times above that of water (Gaub et al. 1984; Lentz 1989), and the mutual friction of the monolayers (Evans et al. 1992) may matter in the shape changes.

Most of the tubular vesicles seen in our micrographs seem to have frozen in a state of rather high lateral tension since their short-wavelength undulations are weak or absent. The uniform spacing of the contours of tubes

fitted into each other indicates as in the case of bumpy spheres that before cooling the membranes undulated and thus repelled each other. Only the membrane of the short and thin tube of Fig. 6 appears to have escaped stretching. This vesicle may have adjusted its shape to area contraction rapidly enough because of its small dimensions.

The very conspicuous spindle-like deformation of many tubes has already been interpreted as an artefact of freezing. It is thought to be generated by the lateral tension arising from area contraction. To estimate the time τ_{sp} needed for the buildup of the spindles we use the crude formula

$$\tau_{sp} \approx \eta \cdot \frac{r}{\sigma},$$

r being the radius of the cylinder. It is based on dimensional analysis and neglects the problems of mode selection and fluctuation amplitudes. Insertion of $\eta = 10^{-3}$ Pa s, $r = 100$ nm and $\sigma = 1$ mN/m leads to $\tau_{sp} = 10^{-7}$ s which is much shorter than the 10^{-3} s at best available (Groll 1986). Accordingly, the formation of spindles seems possible even for the thickest tubes in our samples.

Many of the tubes seen in our pictures were thin ($r < 20$ nm) and belonged to sets of parallel tubes. Being rather straight, they were probably attached to the supporting grid. The force aligning them was small enough to permit local deviations from the average direction. We may try to estimate this force f by means of the relationship

$$\tfrac{1}{2} \cdot \langle \varphi^2 \rangle \cdot L \cdot f = kT \qquad (4)$$

where $\langle \varphi^2 \rangle$ is the mean square deviation of a tube section of length L. Putting $(\langle \varphi^2 \rangle)^{1/2} \approx 10°$ and $L = 200$ nm as suggested by the pictures, we find $f \cong 10^{-12}$ N at room temperature. It is interesting to compare this result to the force $2\varkappa k_c/r$ required to pull a tether of radius r from a vesicle whose membrane has zero spontaneous curvature (Waugh and Hochmuth 1987). With $k_c = 3 \cdot 10^{-20}$ J and $r = 20$ nm the latter force comes out to be c. $5 \cdot 10^{-11}$ N. The discrepancy by more than an order of magnitude may be due to the crudeness of our estimate. Alternatively, it might hint at a stabilization of very thin tubes by membrane superstructure existent prior to cooling.

c) Superstructure and angular vesicles

The previously proposed model of a superstructure of electrically neutral membranes (Helfrich 1989) starts from the assumption of strongly curved, highly localized saddles. In an otherwise flat membrane, a saddle has to be surrounded by two highs and two lows which require regular bending energy, while the saddle itself is thought to lower the free energy through higher order bending elasticity, thus stabilizing the whole structure. The saddles should be cooperative because when two of them come together they can share a high or a low. The cooperativity is expected to produce membrane shape deformations, thus giving rise to a new membrane roughness. The large area contraction estimated and the high lateral tension derived from it may be expected to destroy any local saddles and to flatten at least part of the postulated extra roughness. Especially, the local saddles are likely to disappear as short wavelength fluctuations seem more systematically suppressed than large-scale deformations. Also, simple estimates employing a plausible value for the extra area stored by a saddle suggest that the conjectured superstructure may not withstand tensions above 10^{-2} mN/m (Helfrich and Klösgen 1990). It is therefore surprising that some EYPC membranes display a graininess which could reflect an array of local saddles. The vesicles formed by the grainy membranes are round but their contours seem to be not exactly circular (see Fig. 7). They may have been ellipsoids that froze just before they became spheres, thus avoiding the buildup of large lateral tensions. However, this alone cannot explain why graininess was so rare since there are many vesicles in our pictures which are not far from the spherical shape. Recently, grainy membranes were relatively often observed in small vesicles prepared of DMPC and cholesterol (Lücken 1992, private communication). This may indicate a stabilization of local saddles through admixed cholesterol, a molecule with hardly a polar head.

The very bumpy vesicles called angular raise no such problems as their extra roughness is of a larger scale than the graininess. The fact that no graininess is visible on their membranes may be attributed to the high lateral tension arising just before freezing and leveling all small-scale features. The samples displaying angular vesicles had be reheated to room temperature after incubation at 291 K. The thermal expansion of the membrane area in going from a probably equilibrated state at 281 K to ca. 293 K does not seem enough to explain the strongly nonspherical shapes which we found. Rather, we suspect that their generation is related to the tendency of EYPC membranes to break up into a dispersed phase at the temperature of incubation (Harbich and Helfrich 1990a).

Conclusion

This work began as a search for a postulated membrane roughness which should be on a scale below optical resolution and absorb many times more area than can be stored by the familiar thermal undulations. It soon become clear that the enourmous area contraction and the resulting lateral tensions of the cooling membranes were serious obstacles to this enterprise. A cooling rate of $3 \cdot 10^4$ K s^{-1} is not nearly high enough to prevent the flattening of thermal undulations and other waviness. It also permits major shape changes of the vesicles before the water transforms into amorphous ice after ca. 2 ms of cooling. These artefacts in the cryo-transmission electron microscopy of lipid membranes have as yet not been noticed.

A small number of our vesicles displayed a graininess of the membrane which had never been seen before. When discovered in the course of the present work these patterns led to the formulation of a particular model for the suspected superstructure (Helfrich 1989). Its basic elements are highly localized saddle deformations in the

membrane. Because of their cooperativity they should tend to be grouped and from patterns. The extreme rarity of the graininess is ascribed to the leveling effect of the lateral tension which arises during cooling. Other vesicles, in samples prepared in a special way, showed an extraordinary bumpiness. These angular vesicles may be examples of the roughness which we have been seeking.

Additional experiments are needed to corroborate the present observations and conclusions. Particularly desirable would be the production and TEM study of angular vesicles of a synthetic phosphatidylcholine. Grainy membranes, unless containing cholesterol and other admixtures, may be much more elusive than angular vesicles if our ideas about them are correct. As a technical improvement a "controlled envirement chamber" (Bellare et al. 1988) should be used in order to control humidity and prevent water evaporation from the sample before freezing (Trinick and Cooper 1990; Frederik et al. 1991). At the same time, the investigations could be extended to a wider range of temperatures. Also of interest would be a theoretical treatment of the spindle-like deformations of tubes, including their dynamics.

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