Cryo-Transmission Electron Microscopy of a Superstructure of Fluid Dioleoylphosphatidylcholine (DOPC) Membranes

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ABSTRACT Using cryo-transmission electron microscopy, we have obtained abundant and reproducible evidence for a superstructure of dioleoylphosphatidylcholine (DOPC) bilayers. Dispersions of vesicles were prepared by gentle shaking of a 2% suspension of DOPC in water followed in part by extrusion through a porous technical membrane. Sampling and cryofixation took place at various times within 3 weeks after the preparation. From the micrographs we infer that the small fraction of vesicles enclosing one another develop passages (connections) between the bilayers. In contrast, the superstructure is basically a feature of disconnected membranes. Among its modifications are isolated membrane bends or folds and a grainy membrane texture with a minimal grain spacing of 4–6 nm. In the extruded dispersions the passages and the superstructure seem to be formed mostly within the first day. The fraction of smooth and unilamellar vesicles is large at all times and in all dispersions.

INTRODUCTION

Biological cells differ widely in their functions and activities. These differences are usually attributed to a similar diversity of the membrane proteins. However, the proteins reside in a fluid lipid bilayer the composition of which is likewise characteristic of a given kind of biological membrane. The multitude and greatly variable concentrations of the lipid molecules involved suggest that the lipid composition is another factor governing membrane properties. Apart from specific molecular interactions between lipids and proteins, the fluid lipid bilayer may affect the activity of embedded proteins by its continuum properties. Of particular interest in this respect is the stress profile of the bilayer, i.e., the negative pressure acting along a normal cut through the lipid bilayer as a function of the normal coordinate. The stress profile may have a direct effect on the conformation of membrane proteins. Moreover, it is intimately related to the bending elasticity of the bilayer that controls the shapes of vesicles and, to an unknown extent, of biological cells and intracellular membranes.

There is at least one example of a biological membrane on which the bilayer impresses a periodically curved superstructure. In a series of papers (Sternberg et al., 1986, 1987; Meyer et al., 1990), Meyer and co-workers established a square pattern in the membranes of L-form (i.e., wall-less) cells of *Streptomyces hygroscopicus*. It resembles an egg carton looking equal from both sides. The same superstructure reappeared in the bilayers of the extracted lipid and phospholipid fractions (which both contain cardiolipin), its period ranging from 75 to 15 nm (Meyer et al., 1990). As mentioned by Meyer et al., Verkleij and Wilschut found the egg carton texture in mixtures of a bacterial cardiolipin with egg yolk phosphatidylcholine (EYPC) in the presence of Ca^{2+}. In all of these cases, the method of detection was freeze-fracture electron microscopy.

In our laboratory, the properties of lipid membranes have been studied for many years, mostly by light microscopy of giant vesicles. In the course of time, various odd effects came to the surface that could not be explained by existing models for fluid membranes. They finally led us to postulate that the fluid bilayers of some of the most common lipids can assume a superstructure. The electrically neutral lipids of these studies include EYPC, several one-component phosphatidylcholines (PCs), a few one-component phosphatidylethanolamines (PEs), and natural digalactosyldiacylglycerols (DGDGs). The series of strange phenomena began with the observation of intermittent wiggles and knees in freely fluctuating bilayer tubes (Beblik et al., 1985) and ended with a study of the bilayer bending rigidity that shows its value to depend on the method of measuring it (Niggemann et al., 1995). In recent small angle x-ray scattering experiments we found PC multilayer systems to disintegrate while the monitored repeat distance remained fixed at the so-called equilibrium spacing. This was taken to suggest that the membranes peel off the stack when they acquire the superstructure (Hartung et al., 1994). However, the most compelling reason for postulating a superstructure is the need to explain an anomalous membrane roughness that can absorb as much area again as is visible under the optical microscope ($\Delta A/A \leq 1$). A variable anomalous roughness up to this magnitude, in addition to the roughness of a few percent ($\Delta A/A \leq 0.05$) produced by the usual undulations, was inferred from an analysis of induced adhesion, i.e., the mutual adhesion of fluid bilayers induced by lateral tension (Servuss and Helfrich, 1989). The experimental results and theoretical arguments suggesting a superstructure or an anomalous roughness have been compiled in a recent review (Helfrich, 1995).

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To detect the postulated superstructure we took up cryo-transmission electron microscopy (cryo-TEM) of very dilute vesicle dispersions (Klösgen and Helfrich, 1993), studying first EYPC bilayers. We found a small number of vesicles with grainy membranes, the irregular grain spacing being on the order of the membrane thickness of ~4 nm, a similar number of opaque vesicles, and several other vesicles with unusually bumpy or angular shapes. The graininess and the angularity were interpreted as evidence for the superstructure and the anomalous roughness, respectively, that we were looking for. The vast majority of vesicles, in fact, the totality in almost all the samples, displayed only the smooth membranes and regular shapes known in larger sizes from light microscopy. We attributed the extreme rarity of graininess and angularity as well as the lack of pictures showing both phenomena to a leveling of these membrane structures during the rapid cooling that precedes freezing. This seems possible because of the enormous thermal area expansivity of the lipid bilayers ($\sim 3 \times 10^{-3} K^{-1}$; Evans and Needham, 1987). The effect of lateral membrane contraction upon cooling is increased by the well known 6% dilation of water upon freezing (Heide and Zeitler, 1985).

The first pictures of grainy membranes allowed us to propose a preliminary model for the superstructure (Helfrich, 1989). It starts from the assumption that the superstructure is built from highly curved local saddles in the membrane. The saddles are thought to be due to non-Hookean terms in the bending energy, at least one of them being negative. As each saddle is surrounded by two highs and two lows in an otherwise flat membrane, the saddles are cooperative, which enables them to form patterns. Energy barriers have to be overcome when a saddle is created or destroyed, and lateral tension of sufficient strength should level the saddles and their patterns. Recent Monte Carlo simulations based on the simplest possible version of the model and plausible values for the bending elastic moduli have resulted in the periodic superstructure of the egg carton with a period of 6 nm (Goetz and Helfrich, 1996). Current simulations (Jud and Helfrich, in preparation) suggest that the same model reproduces also the graininess and, perhaps, other modifications of the disordered superstructure that we observe in dioleoylphosphatidylcholine (DOPC) membranes. The model will be briefly reviewed in the Discussion.

More recently, x-ray microscopy was used as a new and rapidly progressing technique to study vesicles of EYPC as well as of dimyristoylphosphatidylcholine (DMPC) and of palmitoyloleoylphosphatidylcholine (POPC) at room temperature and in the fluid membrane state (Thiene et al., 1993; Guttmann and Klösgen, 1993; Klösgen and Guttmann, 1996). Some of the micrographs also point to anomalies of the bilayer structure, which, however, are not fully resolved, the limit of resolution being ~25 nm at present (Schliebe et al., 1996).

The superstructure need not be restricted to single bilayers in water. In fact, an undulated bilayer structure in practically waterless stacks of polymer-complexed soy lecithin membranes was recently inferred from small-angle x-ray scattering (Antonietti et al., 1995).

Our former search by cryo-TEM for superstructure and anomalous roughness may be regarded as unsatisfactory in two respects. First, the yield of grainy or at least opaque membranes was extremely low and we had no method to improve it. Second, EYPC as a natural material is a mixture of several similar lipids, so that the graininess might result from their segregation upon cooling. The second deficiency can be corrected by using a one-component lipid. We rejected the typical saturated PCs such as DMPC because they have a rippled crystalline phase in a temperature range just below the main transition that could be confused with any superstructure of the fluid bilayer. In addition, they are rather rare in biological membranes. Cryo-TEM of POPC vesicle dispersions was discontinued, without finding a superstructure, when we learned that POPC tends to isomerize into OPPC by exchanging its hydrocarbon chains (Pfeiffer, 1995).

Finally, substantial fractions of vesicular bilayers exhibiting a superstructure were reliably obtained by cryo-TEM of dispersions of DOPC vesicles. Surprisingly, the superstructure appeared in several modifications. The simplest of them has the form of one or more furrows (or ridges), i.e., sharp bends or folds, in an otherwise smooth vesicle membrane. Some of the two-dimensional modifications are very similar to the dense graininess of EYPC membranes, including a 4- to 6-nm grain spacing. In others, the texture is less dense and a general tendency of the grains to arrange themselves in rows becomes more conspicuous. The texture of lowest density resembles a network of short lines, probably furrows and ridges, and seems to crumple the membrane. There are also unusually bumpy or angular vesicles of the type found previously with EYPC.

We think that our pictures unequivocally demonstrate the existence of a superstructure. However, the detailed interpretation of the images is difficult and has not yet been done. In addition, there is always the possibility that sample freezing obliterates part of the superstructure. A special problem, absent with EYPC, arises from the bilayer connections, or passages, which formed numerously in some vesicular objects after the dispersions had been prepared. In the micrographs, the contours of passages can be confused with a modification of the superstructure. Also called interlamellar attachment sites or ILAs (Siegel, 1986a,b), passages are known to develop very rapidly in dispersions of *N*-methylated dioleoylphosphatidylethanolamine (Me-DOPE) (Siegel et al., 1989, 1994).

In the following, we will first describe the new experiments on DOPC membranes and then report the results, showing pictures of passages and diverse modifications of the superstructure. In the subsequent discussion we will try to interpret the observations.

MATERIALS AND METHODS

In our earlier experiments with EYPC, grainy membranes were found only in shaken dispersions, which tend to contain large fractions of big non-

spherical vesicles, but not in sonicated ones, the vesicles of which typically are spheres. In the present work we added extrusion as a technique to produce small vesicles that in part are nonspherical. Angular vesicles, i.e., vesicles with anomalously bumpy shapes, were obtained previously by cooling shaken dispersions from room temperature to 8°C, keeping them there for 2 weeks, and reheating them to room temperature for 1 or 2 h before sampling. This time the dispersions were briefly quenched from 40°C to −20°C and then incubated at 40°C for at least 4 h. The common idea was to reduce the enclosed volume of initially spherical vesicles, either by water permeation or by rupturing the membrane at the lower temperature.

DOPC with a purity >99% (as indicated by the producer, Avanti Polar Lipids, Alabaster, AL) was used as delivered in chloroform solution (20 mg/ml) without further purification. The lipid solution had been stored uninterruptedly at low temperature (−20°C or lower) before it was used. Chemical purity and stability were checked by thin layer chromatography in some cases. No components other than DOPC were ever noticed at a sensitivity of approximately 1%, even at the end of a 3-week experiment. The preparation of lipid suspensions started with removing the chloroform from roughly 5 ml of solution held under reduced pressure at elevated temperature (~40°C) in a rotary evaporator until a thin film of dry lipid almost free of solvent formed on the wall of the preparation flask. Subsequent overnight storage at 40°C under reduced pressure (~1.5 kPa) presumably removed all chloroform remnants. From this deposit, a lipid suspension was obtained by slow addition of distilled water (Millipore, Seradest, Eschborn, Germany), the final lipid content being ~2% by weight. To promote swelling, the suspensions were gently shaken with a frequency of 20 cycles/min in an orbital shaker (ETI, MiniShaker, Zurich, Switzerland) at 40°C for 8 to 24 h.

The resulting shaken dispersions were the basis for the preparation of two additional kinds of vesicle dispersions. A sonicated dispersion was made by exposing the original dispersion to ultrasound (Branson, Cell Disruptor B15, Shelton, CT; 1:1 interrupt mode) for 5 min at 40°C. To obtain an extruded dispersion, the original dispersion was repeatedly pressed through a 400-nm porous technical membrane (polycarbonate) in a micro-extruder (Avestin, LiposoFast, Ottawa, Canada; $V_{int} \approx 500$ μl) by slowly pushing the pistons through 51 times.

In each series of experiments, the original and the extruded dispersions were investigated. In a few cases, the sonicated variety was also prepared and studied. Each of these vesicle dispersions was divided in two halves that were handled differently: one half was kept continuously at 40°C, while the other half was rapidly quenched to ~−20°C, left there for 10 min, and then reheated. All dispersions were incubated at 40°C for another 4 h before the first samples were taken.

As the temperature decrease of quenching from 40°C to −20°C is very large as compared with the previous cooling from 20°C to 8°C, we did not leave the samples at the lower temperature to allow equilibration but returned them to the higher temperature after only 10 min. As DOPC bilayers remain fluid down to −22°C (Ladbrooke and Chapman, 1969), we expected the area contraction associated with the quenching to result in transitory rupture and volume loss of vesicles that initially were spherical or nearly so. Back at room temperature, these vesicles should possess much more area than the surface of a sphere of equal volume. The excess of area should facilitate the formation of a superstructure.

Samples were taken and frozen after the 4-h waiting time after the preparation of the vesicle dispersions, 1 day later and 1 week later; occasional samplings in between and after 3 weeks complemented the experiments. At any time of sample preparation, at least four grids of each dispersion were frozen for observation in the electron microscope.

Cryofixation was performed according to standard procedures (Dubochet et al., 1988; Jäger, 1990; Klösgen and Helfrich, 1993). As a rule, a droplet of vesicle dispersion was put on a special microscope grid (400 meshes/inch and covered with a holey carbon foil) and blotted to obtain water lamellae of a thickness of <300 nm. The grid was mounted on a guillotine-type plunger device (Robards and Crosby, 1983). By releasing a spring, the grid was shot with a velocity of >2 ms^{-1} (Talmon et al., 1990) into liquid ethane kept at a few degrees above the temperature of liquid nitrogen (76 K), where it cooled with a rate of at least 10^4 Ks^{-1} (Bald, 1987). The vitrified sample had to remain at temperatures below 130 K, thought to be the devitrification temperature of water (Johari et al., 1992). All the steps of sample preparation were performed in a controlled vitrification chamber (Bellare et al., 1988a,b; Bailey et al., 1991) to guarantee a constant temperature (usually near 30°C) and a high humidity (>90%). The latter was needed to prevent evaporation of water and, thus, any osmotic shrinkage of the vesicles that might result from it. Without this precaution, water permeation through the membrane may produce a spontaneous curvature (Boroske et al., 1981) that, when strong enough to deform the vesicle, could give rise to a lateral tension leveling the superstructure. Osmotic effects on vesicle shape were studied in parallel experiments and shall be described elsewhere (Klösgen, in preparation).

The specimen was then cryo-transferred for direct imaging into a precooled microscope (DEEKO 250 (Jäger, 1985) or CM12, Philips). An underfocus of $\Delta z = -1.2$ μm was used to achieve sufficient phase contrast to make visible thin structures such as single bilayers (~4 nm) without losing too much resolution. Membranes are seen in cross section where they are parallel to the optical axis of the microscope. In addition, they slightly darken the whole area occupied by a vesicle by electron scattering and absorption. Unfortunately, we do not know the thickness of the lamella for all pictures taken. With the Deeko 250, we could measure the beam current and thus estimate the local lamella thickness. It ranged from 100 nm or even less up to ~300 nm. Thicker samples were hard to investigate as absorption and multiple scattering weaken the image beam. Both effects required longer exposure times (>1 s), which was not acceptable because of the increase of radiation damage. In addition, the amount of inelastically scattered electrons becomes more and more important in thick lamellae. This results in bad image contrast. In the course of our experiments, we could not successfully use another microscope (Zeiss, EM 902) with a built-in omega filter for energy selection and contrast enhancement. Images were recorded on film negatives (SO 163 from Kodak or Scientia from Agfa).

RESULTS

General Observations

Before presenting and discussing micrographs of the superstructure, we will report some general observations concerning the shapes of smooth vesicles in the various dispersions and their comparison, where possible, to those found previously with EYPC. We will also remark on the evolution of the superstructure with time, a problem that remains to be studied systematically.

The contours of sonicated vesicles, in general unilamellar, were always circular and mostly of diameters ≤400 nm, the average size decreasing with sonication time. As less water was evaporated from the lamellae than in the previous experiments, the vesicles did not crowd in any of the samples. Accordingly, we did not see the flattening of the contours by which adjacent vesicles keep a certain distance. From this effect, which occurred regularly in dense enough sonicated EYPC dispersions, we inferred the presence of thermal undulations and undulatory mutual repulsion of the membranes before freezing (Klösgen and Helfrich, 1993).

In freshly extruded samples, the fraction of vesicles enclosing others varied from preparation to preparation and was usually between 7 and 16%. Almost all of those objects were bilamellar, the spacing of the bilayers being usually less than twice the bilayer thickness. Only rarely was the inner vesicle much smaller than the outer one.

Most of the vesicles seen in extruded dispersions were spherical, the diameters ranging from ~400 nm to <100

nm, with an accumulation of sizes around 100 nm, in accordance with the observations of others (Hope et al., 1985; Ertel et al., 1993). In contrast to sonicated dispersions, however, there were also ellipsoidal and even tubular vesicles. Nonspherical shapes should permit the superstructure to develop, and vesicles with diameters less than the thickness of the water lamellae (≤300 nm) will experience as little membrane tension as possible during sample preparation. This combination may be expected to offer ideal conditions in a search for the superstructure.

The vesicles observed in the original, shaken-only dispersions of DOPC were similar in shapes and sizes to those obtained by the same method from EYPC. Besides spheres and ellipsoids we found many tubular vesicles of widely varying radii and lengths, mostly extending over more than one hole of the carbon foil. The temperature being higher this time than previously, 40°C instead of room temperature, we reduced the time of shaking from 1 day to usually 8 h in the present experiments. In fact, to speed up vesicle formation and equilibration was one of the reasons for raising the temperature of the dispersions.

As to the evolution of the superstructure, we never saw indications of it in sonicated dispersions, in agreement with our previous experiments. Even after several weeks of incubation, quenched and unquenched vesicles were always spherical (or oblate ellipsoids of rotational symmetry when they were larger than the lamella thickness), their contours being smooth and circular. Although the sonicated vesicles should have had some excess area at least in quenched samples, it was probably not enough to allow any superstructure to develop or survive the area contraction in the course of cryofixation.

Neither passages nor signs of a superstructure were visible in extruded dispersions in the fresh state, i.e., right after the 4-h waiting time. On the other hand, in samples taken after a day from the same dispersions, many vesicles displayed passages or one of the modifications of the superstructure. Part of the original, shaken dispersions exhibited passages and superstructure already in the fresh state. When superstructure was present, it occurred in general on at least 10% of the vesicles. Textures of intermediate or low density seemed to prevail over the densely packed graininess or angularity when the dispersions were 3 weeks old. No other systematic differences could be established between 1-day-old and older dispersions. There were many smooth vesicles, including some with passages, even in the oldest dispersions.

Surprisingly, quenching was found to have little or no effect on the incidence and evolution of superstructure. In particular, it appeared not to destroy preexisting superstructure as vesicles with a superstructure were also seen in fresh shaken dispersions that had been quenched just before the 4-h hour waiting time.

Micrographs

A typical ensemble of vesicles in an extruded dispersion is shown in Fig. 1. Freely floating objects of different shapes,

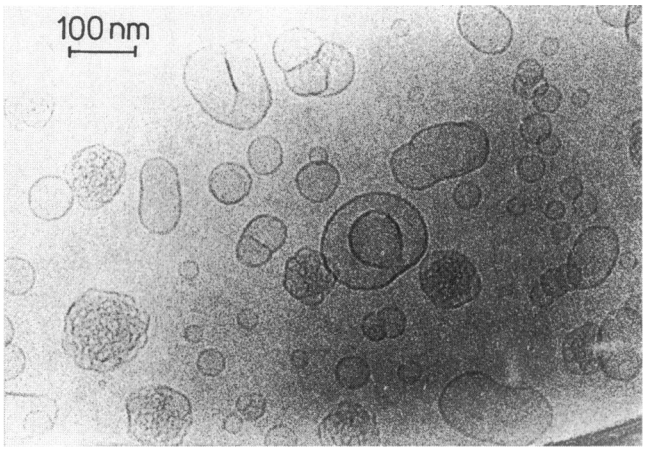

FIGURE 1 Vesicle dispersion prepared by extrusion through a 400-nm pore membrane, quenched to −20°C for 10 min, incubated for 1 day at 40°C before cryofixation. Note the great variety of vesicles in size, shape, and membrane features. The vesicle membranes are smooth, textured, or marked with lines (but otherwise smooth). The short heavy line on one of the vesicles at the top may be a membrane fold.

many of them spherical, exist side by side. Apart from their shapes, the vesicles differ by their membrane features. Most of the vesicles have smooth membranes, but a significant fraction of them exhibit a membrane texture. Other vesicles display lines, one of them a short and particularly dark line. Along this line the otherwise smooth membrane appears to be sharply bent, perhaps being folded upon itself. The bottle shape of one of the bigger vesicles may be due to the fusion of a trapped vesicle with the enclosing one by formation of a passage. Incidentally, the large width of the bottleneck might be a transient deformation due to rapid area contraction that was captured by fast freezing. Some vesicles seem to adhere in groups of two or three, but the contrast of the apparent contact areas seen from their sides is relatively low. Alternatively, such a line could be a sharp membrane bend or a single bilayer separating two adjoining vesicles in a state of semifusion (Kozlov et al., 1989).

A general problem in describing and analyzing two-dimensional textures results from the possibility of seeing a superposition of two pictures from the near and far sides of the vesicle. Although the effective depth of focus of the electron microscopes is always larger than the theoretical depth of 250 nm, there are indications that in many cases the contributions from the two halves differ appreciably in contrast. Another problem arises from the possibility that different membrane structures produce similar and, perhaps, indistinguishable images. In particular, there is a risk of mistaking arrays of passages for the crumpled texture of the superstructure (and vice versa).

To minimize ambiguities we show in the following five figures tubular and spheroidal vesicular objects displaying passages (ILAs). Passages had been noticed several years ago in light microscopy of highly swollen PC in water (Harbich et al., 1978).

Long tubular vesicles with often non-uniform diameters are typical of shaken dispersions. Fig. 2 gives an example.

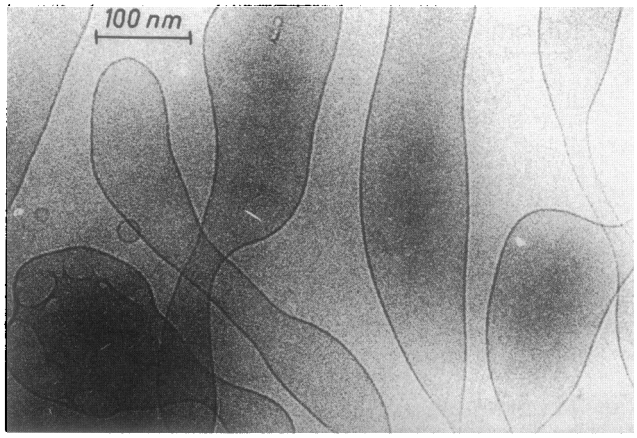

FIGURE 2 Shaken dispersion, unquenched and incubated for 1 day. The membranes of the irregular tubes are smooth. Note the garland enclosing a darkened region on the club-shaped vesicle (lower left) and the series of bright spots on one of the long tubes (top), all of which indicate passages. The vesicles touch a strip of carbon foil (not shown, beginning just at the bottom of the figure) and are probably connected to other vesicles across the strip.

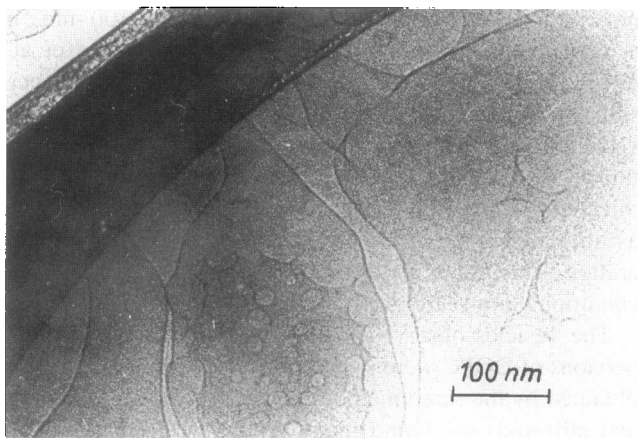

FIGURE 3 Same sample as in Fig. 2, with view of adjacent hole of carbon foil. In addition to the garlands in the center and upper left, note the pointed cushions on the right, which represent tube-like membrane overpasses (passages).

Of particular interest in this picture is the club-shaped vesicle on the lower left. It is marked by a line resembling a garland and enclosing a region slightly darker than the surroundings. The line forms tips at several places where it reverses its direction. Based on the interpretation of similar cryo-TEM micrographs with another phospholipid (Siegel et al., 1989, 1994) we believe that the darkening is due to a second vesicle inside the tube and that the garland is the contour of the enclosed vesicle and of several passages connecting the bilayers of the two vesicles (which thus lose their identities). Passages opening toward the contours are especially easy to recognize, looking like a pair of inverted parentheses. The circles around lightened regions within the dark region are most likely the contours of other passages (see next paragraph). The three bright spots with diameters of less than 10 nm, arranged in a row at the top of the tube crossing the club, may also be passages, this time between the near and far sides of the tubular vesicle.

The central tubular vesicle in Fig. 3 exhibits another garland revealing an enclosed vesicle and passages connecting the membranes of the two vesicles. In the darkened region inside the garland one sees numerous circles, the interiors of which lack the darkening. A few circles of the same types have already been noted in Fig. 2. They are interpreted as passages seen along their axis of rotational symmetry (Siegel et al, 1989, 1994). The passages vary considerably in size, the open diameters of the circles ranging from 25 nm to <10 nm. It cannot be ruled out that some of them connect the near and far sides of the enclosed vesicle. A diagram of a passage between parallel bilayers is given in Fig. 4 .

The "pointed cushions" visible on the large vesicle on the right of Fig. 3 seem to be a novel observation. Apparently, they are passages built into a single nearly flat membrane, a cushion being the image of a tube-like overpass crossing an identical underpass. (Topologically, passages are "handles", each increasing the genus of the membrane surface by unity.) Fig. 5 displays two diagrams of a passage residing in a single planar membrane and looking like a pointed cushion when seen from above or below the membrane. The passages may have been generated before sample preparation and swept to their positions when the dispersion was pipetted and spread on the grid. Pointed cushions of much larger size have in the meantime been observed in light microscopy of very dilute DOPC/water samples (Thimmel et al., in preparation).

The large vesicles of Figs. 2 and 3 are in contact with the carbon foil and extend over more than one hole of it. However, garlands enclosing darkened regions and circles with lightened interiors were also seen on an appreciable number of freely floating small vesicles in extrusion samples. Two of these structures resembling footballs are shown in Fig. 6. We suspect "footballs" to originate from initially bilamellar vesicles through membrane collisions producing

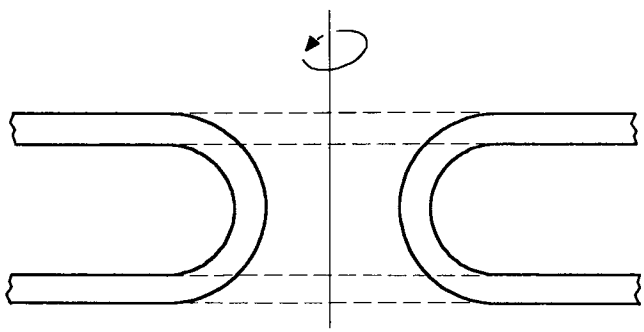

FIGURE 4 Schematic drawing of a passage connecting two parallel bilayers. The cross section contains the axis of rotation. Each line represents a monolayer.

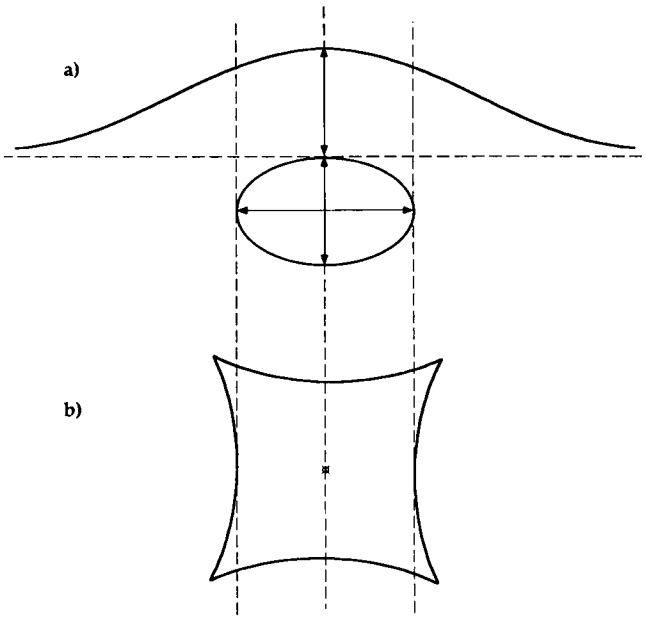

FIGURE 5 Schematic drawing of a pointed cushion, i.e., a single passage residing in a planar bilayer. (*a*) Cross section coinciding with a mirror plane. Each line represents the bilayer. (*b*) Corresponding membrane contours as seen from above or below the membrane. A contour line appears wherever the membrane is locally parallel to the direction of viewing.

passages. No other PC seems to be known that forms passages this easily.

Fig. 7 exhibits some simple vesicular objects containing one, two, or three passages. All of them were observed in extruded dispersions. The first two vesicles with one passage or handle (genus g = 1) resemble doughnuts. The completely smooth, nearly axisymmetric shape of Fig. 7 *a* is very close to the famous Clifford torus, a toroidal shape of minimal bending energy (Ou-Yang, 1990; Seifert, 1991). The vesicle in Fig. 7 *b* is so irregular in shape that it may be assigned to the class of angular vesicles. We assume the anomalous bumpiness to be a sign of the superstructure despite the fact that no furrows or texture can be seen. A button-shaped vesicle is shown in Fig. 7 *c*. Vesicles of this type and others of the same topology (g = 2) are well known from light microscopy (Fourcade et al., 1992; Michalet et al., 1994) and of special interest because of conformational diffusion among degenerate shapes (Jülicher et al., 1993). Finally, Fig. 7 *d* displays a vesicle with four passages (but only g = 3) lying in a plane. Unlike the typical football, it still looks like a bilamellar vesicle, with the two lamellae practically preserving their identities.

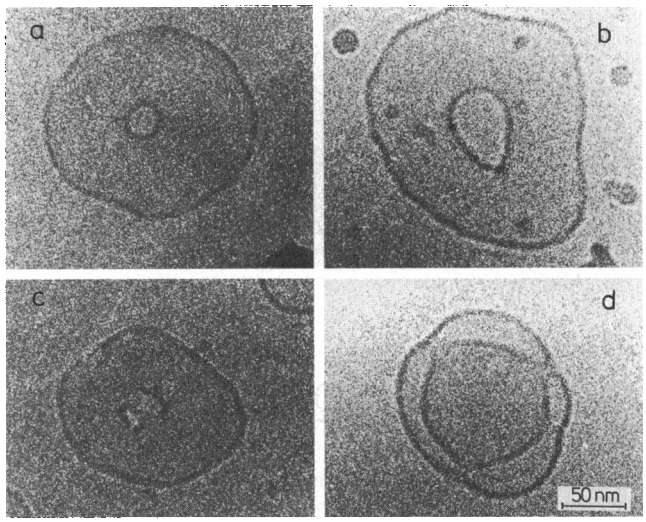

FIGURE 7 Simply shaped vesicles with passages from different suspensions of extruded vesicles. (*a* and *b*) Tori (genus g = 1) of different radii. The vesicle in b also is angular. (*c*) A button-shaped vesicle (g = 2). (*d*) Several passages aligned along the circumference of an originally bilamellar vesicle.

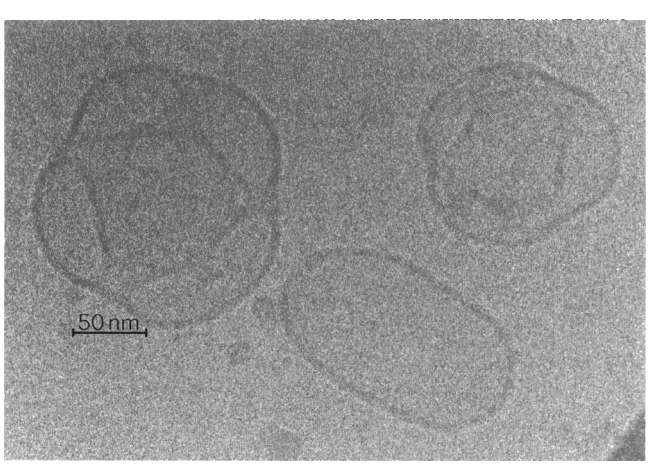

FIGURE 6 Extruded vesicle dispersion, unquenched and incubated for 7 days. Note the freely floating football-like vesicle with a garland and a circle on its membrane (upper left). A darkened membrane region is bounded by these lines. Another vesicle displays a poorly resolved garland (upper right).

The extended vesicular object in Fig. 8 consists of a pair of tubes, one of them enclosing the other. There seem to be many passages connecting the tubes as well as the lower and upper sides of each tube. Examples of the latter type are clearly visible in the lower part of the object, which is not occupied by the inner tube. Arrays of passages between extended membranes, one of them folded and enclosed by the other, have already been found and described (Siegel et al., 1989; Charitat and Fourcade, 1997). However, we cannot rule out that some of the lines in Fig. 8 represent the crumpled modification of the superstructure to be shown below.

Figs. 2–8 served to illustrate the tendency of DOPC bilayers toward fusion as manifested by passages. Bilayer collisions seem to be a precondition for fusion. The passages developing, e.g., in bilamellar vesicles will eventually hinder additional contacts. This may help to understand why our collection of pictures suggests that the footballs are relatively stable once they have formed.

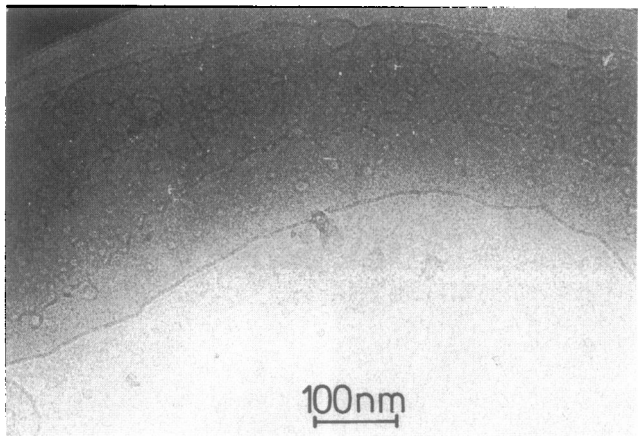

FIGURE 8 Big tubular vesicle from a shaken dispersion, quenched and incubated for 7 days. Passages intersperse the whole object. They connect the near and the far sides of an outer tube (lower part) and adjacent membranes where an inner tube is enclosed (upper part).

We will now deal with the evidence for a superstructure of DOPC bilayers in its various modifications, considering first vesicles that are smooth except for pronounced membrane furrows or ridges. Attention has already been drawn to a very conspicuous bend, possibly a fold, with well defined ends, that is seen on one of the vesicles in Fig. 1. Two other furrows, apparently closed to form rings, are visible on the branched tube of Fig. 9. Either of them marks the position of a sharp bend, constricting the stem and one of its two branches. A diagram of a bilayer tube exhibiting a constricting furrow is given in Fig. 10. Note that the membrane assumes saddle curvature in the centers of the sharp bends. Similar lines in other pictures (not shown) suggest that this is a common feature of furrows (or ridges). This may relate to our conjecture that a preference of the membrane for strong saddle curvature is the common cause of all modifications of the superstructure (see below).

A few of the prolate vesicles in the extruded samples are similar to the elongated vesicles with a notch (heart-shaped

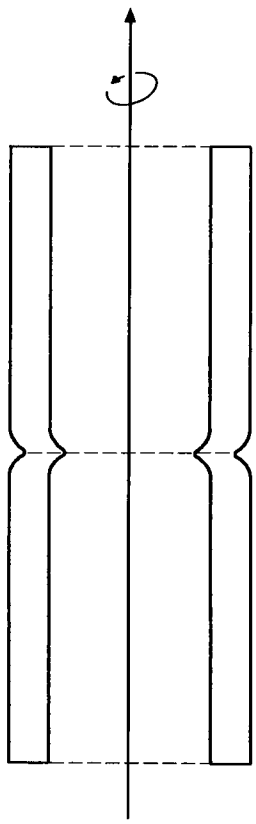

FIGURE 10 Schematic drawing of a bilayer tube with a constricting furrow. The cross section contains the axis of rotation. Each line represents a monolayer.

vesicles) found earlier with DMPC (Weiss, 1989) and EYPC (Klösgen and Helfrich, 1993). An example seen at an oblique viewing angle is shown in Fig. 11. No such shapes were ever observed in optical microscopy. The notch may be due to a single saddle, a row of saddles, or a short furrow, but our pictures do not reveal any disruption of membrane smoothness. It seems that the curvatures in the notch were

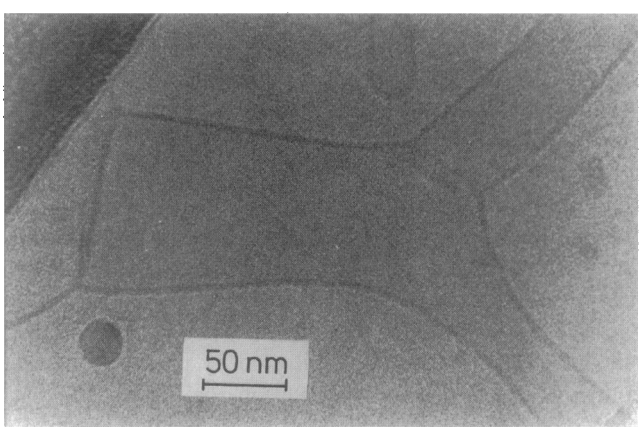

FIGURE 9 Branched tube in a shaken dispersion, unquenched and incubated for 7 days. Note the constricting lines that circle the stem and one of the branches.

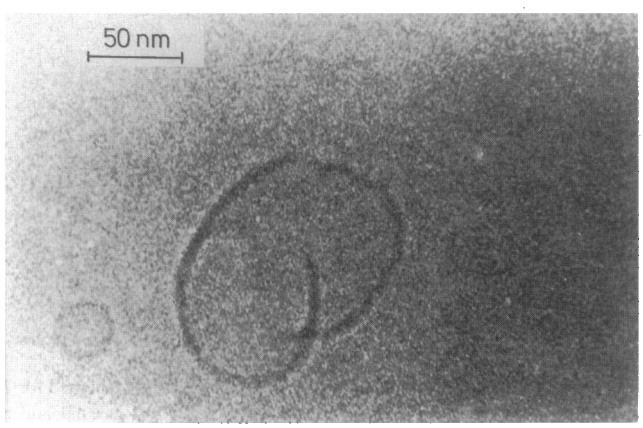

FIGURE 11 Vesicle with a notch in an extruded dispersion, quenched and incubated for 1 day.

not particularly sharp, or a sharp bend was rounded off by the area contraction during sample freezing.

A small overhang in a very extended membrane seems to occur, among other features, in Fig. 12. The overhang is inferred from the slightly darkened field between two lines continuing on both sides as a single line. One of the continuations is ~250 nm long and appears to split again in the middle into two closely spaced lines. It is not clear whether the lines represent the contours of the warped membrane where it happens to be parallel to the direction of viewing or, less likely, slightly irregular membrane ridges. In other words, the overhang illustrates either membrane angularity without a visible superstructure, such as in Figs. 7b and 11, or a pair of lines of very sharp and directly visible bend. Near the overhang there are three or four pointed cushions. Because of their proximity one may speculate that in an overhang the membrane can collide with itself and occasionally create a passage.

The rest of the figures present evidence for modifications of the superstructure that are two-dimensional rather than linear. In Fig. 13, one sees vesicle surfaces covered with a dense and uniform grainy texture. The nearby edge of the carbon foil protects even fairly large vesicles from being squeezed in the water lamella. Squeezing by the water surfaces can in principle produce in the vesicle membrane very large lateral tensions that may be expected to destroy any superstructure. The graininess appears to be the property of single membranes of unilamellar vesicles. An interpretation of the pattern in terms of an array of passages (see for comparison: Basáñez et al., 1997) is unlikely for the following reasons. First, a spherical sponge, i.e., a multiply self-connected membrane in the form of a sphere, would appear much darker because of the increased electron beam scattering and absorption. This can be concluded from the occasional observation of onion-like structures that look uniformly black inside the eighth skin. Moreover, we would expect a sponge or a lattice of passages to manifest itself in small-angle x-ray diffraction experiments. Examining a few samples of swollen DOPC, we never saw appropriate signals, not even with synchrotron radiation at very low angles and high beam intensity. Second, if the sponge occupies only a spherical shell of a certain thickness, one would expect a darkened rim along the circumference of the sphere, but there is none. Third, if the number of lamellae in the shell were very small, we should see some of the clearly drawn circles typical of passages parallel to the viewing direction as we did in Figs. 2, 3, and 8, but they are absent. Fourth, if the grainy texture results in any way from passages, they should be as small as the grains, which seems hardly possible with a bilayer thickness of 4 nm.

Fig. 14 shows a very large vesicle with a graininess of the maximal density observed. In both Figs. 13 and 14 we estimate the typical spacing of these grains to be 4–6 nm, which agrees well with what we found previously with EYPC (Klösgen and Helfrich, 1993).

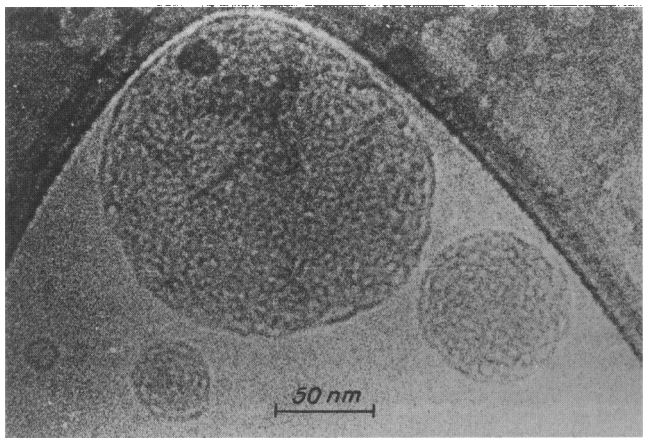

FIGURE 13 Vesicles from a preparation similar to that of Fig. 1. The surfaces of the three vesicles appear uniformly covered with graininess of high density.

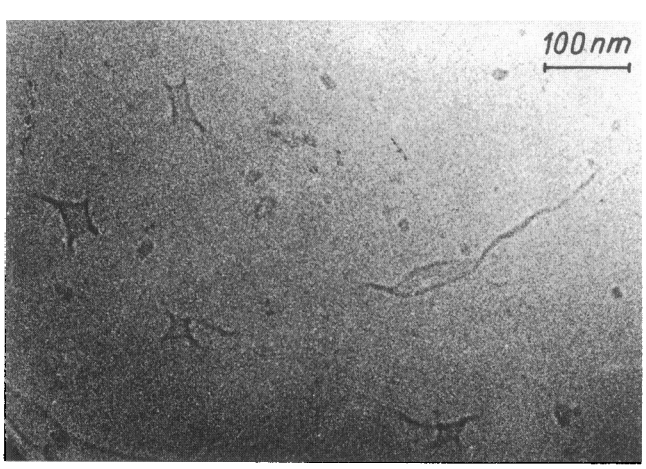

FIGURE 12 Section of a very large tubular vesicle, from a shaken dispersion, unquenched and incubated for 7 days. Note the pointed cushions indicating membrane overpasses (passages) and an elongated object apparently representing a membrane overhang.

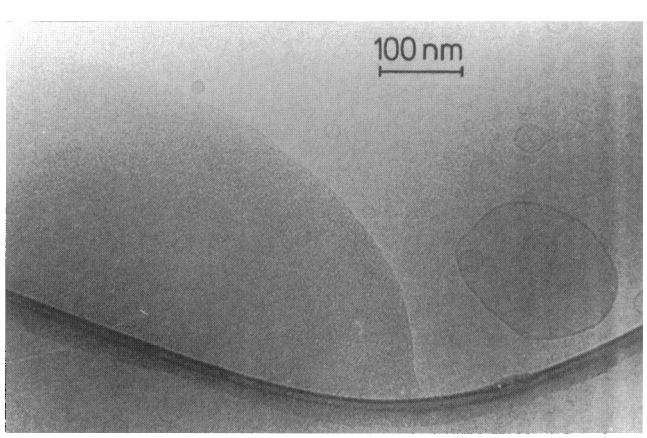

FIGURE 14 Vesicles in a dispersion shaken for one day, quenched, and incubated for 4 hours. Although the dispersion is fresh by our definition, the membrane of the largest vesicle exhibits graininess of maximal density.

It is difficult to differentiate the dense graininess seen in the last two figures from that of EYPC vesicles. The bright spots may be an exception, being more brilliant than the surroundings of the vesicles only in the case of EYPC. In the membranes of both EYPC and DOPC they look rounder (or more clearly separated) than their dark counterparts. Because of their brilliance we identified the bright spots with the grains in our study of EYPC. In the case of DOPC it seems more natural to regard the dark spots as the grains. They appear to form short parallel strings on the vesicles of both materials as a short-range order, a tendency that also exists but is less pronounced for the bright spots.

Two-dimensional modifications of the superstructure of looser texture than the graininess were seen for the first time in the present study. As the density decreases, the impression of continuous lines rather than sequences of dark spots becomes stronger, and at the lowest densities the texture is reminiscent of an irregular network of lines. The spacing of the lines varies from 6 to ~20 nm. Textures of low density may be seen on the membranes of several vesicles in Fig. 1. The remaining micrographs will give additional examples of low and intermediate densities. Loose textures cannot be unambiguously discriminated from arrays of passages. In some cases the superstructure may well coexist with passages.

The largest object in Fig. 15 is a vesicle with a membrane texture of intermediate density. Along its contour the superstructure gives place to smooth membrane domains that in this particular case are comparatively small and difficult to discern. Vesicles of this type are relatively frequent in extruded samples. The smooth domains often form bulges the size and prominence of which vary from sample to sample (see also Fig. 1). The smoothness suggests that the two-dimensional superstructure tends to avoid the membrane regions of maximal cylindrical curvature. This conclusion is based on the assumption that the vesicles in question are oblate and unable to rotate freely in the water lamella.

Among the smooth vesicles of Fig. 15 there is an object that resembles a pair of adhering vesicles. However, no dark contour of a contact area is discernible. The weak contrast of the constricting line rather suggests we see a closed membrane furrow like those in Fig. 9.

A separation into textured and smooth membrane regions depending on curvature is particularly evident in some of the samples made from shaken-only dispersions. An example is shown in Fig. 16. The membrane looks rather smooth where it turns around at the edge of what is probably a very large vesicle. The rest of the vesicle membrane seems to be approximately parallel to the water surface and uniformly covered with low-density superstructure. On the upper right, the vesicle continues as a tube running along the carbon border. Note the completely smooth surface of the tube, which is probably much thinner than the water lamella.

A similar separation into textured and smooth regions in the same membranes is seen in Fig. 17. Two large vesicles are mostly covered with low-density superstructure, but their contours display rather smooth domains. A long tube with a completely smooth membrane emanates from the lower vesicle. The other ends of the tubes of Figs. 16 and 17 are probably attached to the carbon foil, like the vesicles from which they originate. In both figures a few of the lines along the vesicle edges seem to indicate passages. Also, there may be some circular cross sections of passages in the flat parts of the vesicles.

Sharp boundaries between smooth and textured membrane regions differing little in curvature are seen in Fig. 18. The bounding lines constrict the vesicle, which suggests line tension. In contrast to the preceding three figures, this picture may be taken to indicate a general preference for phase separation, even in the absence of curvature. However, as we found large-scale separation only on vesicles extending over more than one hole in the carbon foil, we

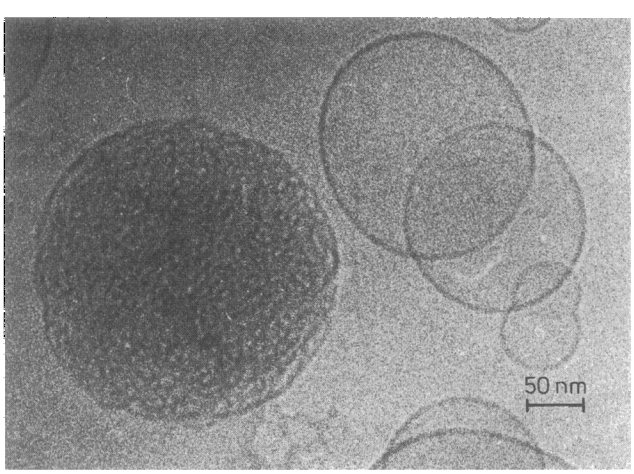

FIGURE 15 Vesicles in an extruded and quenched dispersion, incubated for 10 days. The membrane texture seen on one of the vesicles is of intermediate density. Note the smooth domains, some of them slightly bulgy, along its contour. Because of its size, this vesicle is probably oblate and unable to rotate freely in the water lamella.

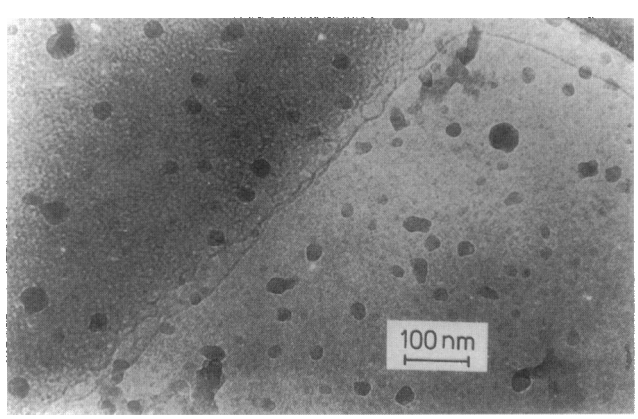

FIGURE 16 Grainy vesicle in a shaken dispersion, quenched and incubated for 4 hours. There are smooth bulges along the vesicle contour and an entirely smooth tubular appendix (upper right). The dark spots are residues of the coolant sticking to the top or bottom of the ice lamella.

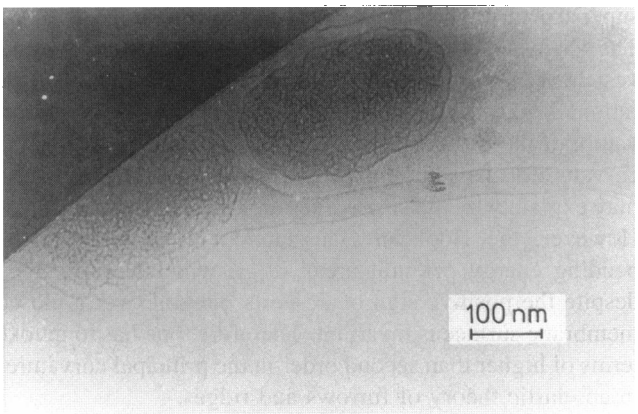

FIGURE 17 Shaken vesicle dispersion, unquenched and incubated for 7 days. Note another example of a smooth tubule emanating from a rough vesicle.

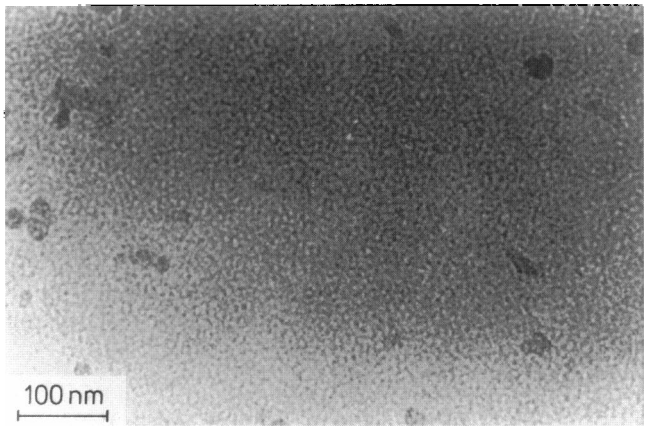

FIGURE 19 Very extended vesicle or single membrane in a shaken dispersion, unquenched and incubated for 7 days. The graininess is of intermediate density and covers the whole film negative, i.e., an area of ~3 μm^2.

cannot rule out that it resulted from special effects such as strong lateral tensions during sample preparation.

Vesicles in samples prepared from shaken dispersions can be extremely large. Sometimes they span several holes in the carbon foil, and it is not always clear whether one sees a vesicle or, perhaps, a single membrane. A grainy membrane of this type is shown in Fig. 19. No border of the vesicle (or single membrane) is visible in the whole micrograph.

DISCUSSION

The following discussion of the superstructure is based on the inspection of 103 micrographic negatives displaying more than 2000 vesicles. Only micrographs of satisfactory quality are included in these numbers. After dealing with passages and their appearances in the pictures we consider the various modifications of the bilayer superstructure and their suitability to produce anomalous membrane roughness. In addition, we speculate on the evolution of the superstructure and the problem of spontaneous mutual membrane adhesion.

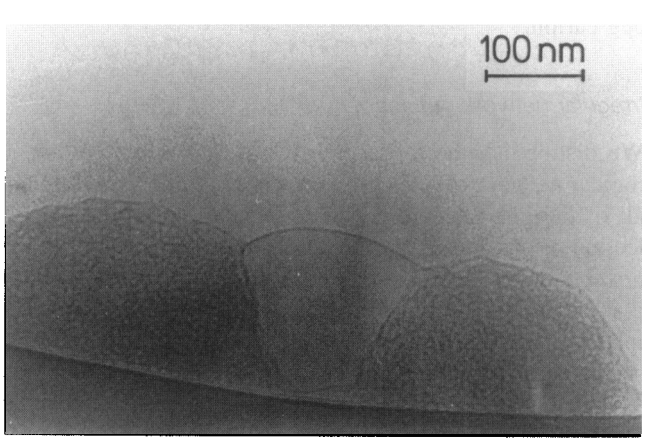

FIGURE 18 Big vesicle in a shaken dispersion, unquenched and incubated for 7 days. Note the constrictions associated with the two boundary lines between smooth and rough regions.

Passages

Let us begin with the passages (or ILAs) that we observe in a small fraction of the vesicular objects. They do not represent the superstructure and, in fact, complicate the search for it. Their frequent appearance was quite confusing because they were absent in our previous pictures of EYPC vesicles (Klösgen and Helfrich, 1993). The passages seem to arise almost exclusively in spheroidal and tubular vesicles that enclose one another. Among the unilamellar exceptions are vesicles in the shape of tori and buttons (Fig. 7). Very likely, in all of these cases the bilayer fusion leading to passage formation was preceded by numerous bilayer collisions. The collision rate may be expected to approach zero as the vesicular structure becomes saturated with passages, their final number depending on the water volume enclosed between the originally separate lamellae. This scenario of passage formation is supported by the fact that no objects with passages, but a proportionate fraction of bilamellar vesicles, have been found in fresh extruded dispersions.

The minimal energy needed to create a passage between parallel bilayers is $-4\pi\bar{\kappa}$, where $\bar{\kappa}$ is the bending elastic modulus of Gaussian curvature $\bar{\kappa} = c_1 c_2$, with c_1 and c_2 being the principal curvatures (Harbich et al., 1978). Accordingly, one can infer $\bar{\kappa} > 0$ from the tendency of passage formation. A positive value of $\bar{\kappa}$ is indeed anticipated for bilayers of electrically neutral two-chain phospholipids. However, other PCs seem to be nearly inert with respect to fusion, probably because of high-energy barriers for changes of topology, which imply cutting and resealing of the bilayer.

Modifications of the superstructure

The superstructure of DOPC bilayers was frequently and reliably observed in the present experiments, in contrast to our previous study of EYPC membranes, and may be divided into four different modifications. The division is governed by practical reasons, and there may be intermediate states and gradual transitions between all four of them.

As the anomalous membrane shapes will be interpreted in the spirit of the curvature elastic model mentioned in the Introduction, let us briefly review the model. It starts from the following formula for the energy per unit area:

$$g = \frac{1}{2}\kappa(c_1 + c_2)^2 + \bar{\kappa}_2(c_1c_2)^2 + \bar{\kappa}_4(c_1c_2)^4$$

Here a (weak) gradient term, $[\nabla(c_1 + c_2)]^2$, and the term of the last paragraph, $\bar{\kappa}c_1c_2$, have been omitted. The integral of the latter is known to be a topological invariant depending only on the genus of a closed surface (Gauss-Bonnet theorem). The first of the elastic moduli, κ, is the ordinary bending rigidity of fluid membranes, which is generally on the order of 1×10^{-19} J for PC bilayers. The second is negative, $\bar{\kappa}_2 < 0$, and thus tends to warp the membrane. Its value, $\sim -1 \times 10^{-36}$ Jm^2, including the exceptional negative sign, was derived from a simplified bilayer stress profile. The third modulus is assumed to be positive, thus limiting the warping. Its magnitude, $\sim 1 \times 10^{-71}$ Jm^8, was estimated from the same stress profile by dimensional analysis. The model is of course a gross simplification, disregarding a large number of other moduli of fourth, sixth, and eighth order in the principal curvatures and the gradient terms of equivalent orders.

Whenever the flat membrane is destabilized by terms of higher than quadratic order in the principal curvatures, a first-order transition is to be expected between the flat and warped states. This implies nucleation energies for all modifications of the superstructure and could explain a phase separation into smooth and textured regions on a flat membrane. However, the avoidance of cylindrical membrane curvature by the loosely textured superstructure is presumably a special effect of higher-order bending elasticity.

The model was shown to produce in Monte Carlo simulation a periodic superstructure, the so-called egg carton, with an optimal period of 6 nm (Goetz and Helfrich, 1996). In the meantime, it was possible to obtain also disordered modifications (Jud and Helfrich, in preparation). The preliminary results suggest that at least the graininess can be reproduced by this simple model with very little variation of the elastic moduli from the values giving the egg carton. Even though the simulations may have influenced the following discussion, it would be premature to refer to them in detail.

Furrows and ridges

The dark lines representing sharp bends of the membrane, called furrows or ridges, are the most direct evidence of a superstructure. They are a novel and relatively rare feature. The furrows end somewhere on the membrane or encircle a vesicle. The lines of this new modification can in no way be rationalized as the contour lines of passages. The line curvature of the furrows seems to be generally opposite to the curvature of the sharp bend. The resulting saddle curvature may explain why the single lines are furrows and not ridges. However, the Hookean Gaussian curvature term of the bending energy per unit area, $\bar{\kappa}c_1c_2$, will have no effect, despite the positive sign of $\bar{\kappa}$, as its integral over a closed membrane surface is invariant. Therefore, one has to invoke terms of higher than second order in the principal curvatures in an elastic theory of furrows and ridges.

At this point, one may wonder whether the lines, although looking continuous, are actually sequences of highly localized saddles smoothed out, perhaps, by the membrane area contraction before freezing. Such a modulation would locally increase the saddle curvature $-c_1c_2$ by raising the relatively weak line curvature, which in the center of the furrow is one of the principal curvatures of the membrane. In a model of superstructure involving non-Hookean bending elasticity, the modulation could thus lower the overall energy of the line.

Dense graininess

Another modification of membrane superstructure is the dense grainy texture with grain spacings of 4–6 nm. It is not clear whether it is essentially the image of one side of the vesicle or (less likely) a superposition of two images of equal intensity. The reason we think that this pattern cannot be attributed to an irregular array of passages have been listed below Fig. 13. The main arguments are the smallness of the grains as compared with the bilayer thickness and the absence of the clearly drawn dark circles characteristic of passages viewed along their axes of symmetry. We add here as a further argument that the same graininess but no passages were found in the previous study of EYPC bilayers (except for the single "passage" of bottle-shaped vesicles). It seems natural to view the dense grainy texture as a melted egg carton.

Irregular network of lines

We distinguish the loose grainy texture resembling an irregular network of lines from the dense graininess, although all of the intermediate densities seem to occur in our collection of pictures. A particular problem with this third and novel modification of the superstructure is the possibility that part of the apparent lines are contours of passages. For instance, in micrographs such as Fig. 16 one may imagine seeing at some places the dark circles that are absent on vesicle faces bearing the dense graininess. On the other hand, there are hardly any side views of passages along the vesicle contours in Fig. 16 and the similar Fig. 17. An exception may be the pair of inverted parentheses at the bottom of the lower vesicle in Fig. 17. After inspecting

many examples of loosely textured vesicle surfaces we tend to believe that they represent essentially a superstructure of the disconnected bilayer. The obvious difference in structure is a second reason to distinguish two modifications. Only the dense graininess can be associated with a two-dimensional disordered array of saddles (although this remains to be proved, e.g., by image analysis) whereas the network of lines seems to consist of furrows and ridges that could be rows of smoothed out saddles, as argued above. Its apparently crumpled nature, to be seen along the vesicle circumference, makes this texture a new candidate for the anomalous membrane roughness that we have been looking for.

Angularity

We regard the unusual bumpiness or angularity of a small part of the membranes as a fourth modification of the superstructure. In the earlier study of EYPC it was the only modification available to explain the huge anomalous membrane roughness of PC and other phospholipid membranes (with $\Delta A/A$ up to unity, see above) that we had set out to discover. The fact that angularity is not frequent in the previous and present micrographs could be due to its suppression in small vesicles and narrow tubes. In large membranes, the anomalous roughness may have been leveled by lateral tensions arising during the preparation of the samples.

In our previous discussion of EYPC vesicles, we suspected that the membranes of angular vesicles, like all other membranes, are grainy before they are frozen. However, the conjecture that a two-dimensional texture is generally present and in suitable cases produces bumpiness is in conflict with some of the new observations. For instance, the survival of superstructure in quenched membranes indicates that the graininess is more robust than expected, at least in the case of DOPC. Moreover, the occasional membrane overpasses and overhangs (see Fig. 12), being easily deformable, should provide extra protection from the lateral tensions associated with the area contraction before freezing.

As there are not even islands of surviving membrane texture in the micrographs, we now believe that membranes looking smooth in cryo-TEM had also been smooth before they were frozen. Angularity and bumpiness could then result from furrows or isolated saddles that are not pronounced enough (perhaps because of area contraction during cooling) to be visible by themselves in electron microscopy.

Evolution of the superstructure

Our pictures tell us very little about the evolution of the superstructure on a vesicle membrane. Its final or energetically most favorable state seems to be the two-dimensional texture of low or intermediate density, as may be inferred from its relative frequency in old samples. An additional clue is the fact that we did not see any superstructure on sonicated vesicles, whether quenched or not. It implies that a large excess of area is needed for nucleation, more than the few percent associated with the usual bending fluctuations. This, in turn, suggests that the first step of nucleation is the creation of a furrow that strongly deforms the vesicle and thus requires much more excess area than a small patch of graininess. Additional furrows or ridges would then produce angularity and, from there or directly, the irregular network of lines or loose graininess. According to this scheme, the dense graininess would not be the state of minimal free energy.

Mutual membrane adhesion

In none of the micrographs did we notice clear signs of mutual membrane adhesion. If the membranes are indeed smooth before freezing, this would contradict our previous assumption that the membranes adhere to each other spontaneously when they are smooth and have to acquire a superstructure before they can separate. A new argument against spontaneous adhesion is provided by extruded vesicles, studied for the first time in the present work. Extrusion may be expected to destroy any superstructure. However, even in fresh extruded samples we never saw adhesion. In the case of separate vesicles this can be a consequence of the minuteness of the contact areas and the smallness of the adhesion energy, $\sim 1.5 \times 10^{-5}$ Jm^{-2}, as measured with aspirated PC vesicles (Evans and Needham, 1987). At the same time, these limitations should not prevent adhesion within bilamellar vesicles of small membrane spacing, but it did not occur.

CONCLUSION

Despite the progress made in establishing the existence of a bilayer superstructure, at least for a particular lipid, two of the specific questions we intended to solve in our cryo-TEM studies remain open. In the first place, it is not clear which type of anomalous roughness dominates in extended membranes. We believe it is the newly discovered loose network of lines that appears to be a strongly crumpled membrane texture but cannot rule out entirely membrane bumpiness and angularity found with both EYPC and DOPC. Mutual adhesion induced by lateral tension, the analysis of which made us postulate an anomalous roughness and a superstructure, seems to support the new concept because one may expect more contact or closeness between crumpled than angular membranes.

The other unresolved problem concerns spontaneous adhesion. The so-called equilibrium spacing of aqueous multilayer systems of electrically neutral lipids, among them PCs, is a well established phenomenon (Rand and Parsegian, 1989). Repeat distances have been measured in many laboratories and found to be reproducible. The theory of membrane unbinding, worked out in great detail (Cook-Röder and Lipowsky, 1992; Lipowsky, 1994), does not offer a ready explanation why in other experiments bilayers

of the same lipids separate from the stack to form giant vesicles. In a recent study mentioned in the Introduction we found in addition that the stack keeps its spacing while it disintegrates. To resolve these inconsistencies we proposed that the membranes acquire a superstructure as a precondition for separation. The results of the present work appear to disprove the idea, indicating instead that even smooth membranes do separate. In our cryo-TEM study of EYPC, spontaneous adhesion was absent also when the same experiments were done in NaCl solution (up to 5 mM). This seems to apply as well to DOPC (at the low salinity of up to 0.5 mM; Klösgen, in preparation). An alternative mechanism to explain how a multilayer system disintegrates by membrane peeling will be proposed elsewhere (Helfrich, in preparation). It employs a slight reduction of the bilayer bending rigidity that is due to the negative $(c_1c_2)^2$ term in Eq. 1 and effective only at large enough membrane spacings. The hat model of fluid membrane fluctuations (Helfrich and Kozlov, 1994) allows a convenient estimate of this effect.

At last, we remark on the term superstructure, which sometimes elicits protests from solid-state physicists, especially those interested in crystal surfaces. We chose this designation as it is short and obviously not incorrect. (Incidentally, theoretical mechanics also used to know only solid membranes.) Mesoscopic structure or mesostructure would be other possible names, but we feel that at least the dense grainy texture with a grain spacing on the order of the bilayer thickness is too fine to be called mesoscopic.

We are grateful to E. Zeitler for his generous permission to do electron microscopy at the Fritz-Haber-Institut in Berlin, Germany. B. K. thanks J. Jäger and C. Zecha for engaged and excellent technical assistance, M. Swoboda for being on her side in hopeless situations, and K. Zickfeld for photographic work. In addition, we benefitted from the electron microscope (CM12) provided by the Deutsche Forschungsgemeinschaft for Sfb 312. Both of us are also grateful to G. Klose for advising us of the isomerization of POPC and to Y. Talmon and D. Siegel for several critical and helpful discussions. It was a pleasure to compare together with A. Jud the superstructure and its Monte Carlo simulation.

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