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Convex-concave curvatures in bilayers of dipalmitoylphosphatidylcholine and cholesterol induced by amphotericin B/deoxycholate after prolonged storage

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Abstract

Freeze-fracture investigations on the influence of amphotericin B/deoxycholate on multilamellar vesicles (MLV) of DPPC containing cholesterol have revealed a new phase structure. Alternating convex and concave curvatures are observed after storage of the vesicles at temperatures below 25°C for at least 4 weeks. Three types of these patterns occur, a small-dimensional (repeat distance ~ 100 nm), an intermediate-dimensional (repeat distance ~ 400 nm) and a large-dimensional (repeat distance ~ 700 nm). The types can be formed on the same bilayer side by side. Additionally, the types differ in the morphology of the tops. In the case of the small-dimensional type the shape of the top can be described as a circular flat plane or opening and in the other cases as a hemispherical cap. The large dimensional type differs from the others by involvement of bilayer stacks. The formation of this new phase after prolonged storage could be confirmed by DSC measurements. The new structure can be explained in the framework of bicontinuous cubic phases and periodically curved bilayer structures. From the electron micrographs a *lo* (liquid ordered) phase is suggested.

Key words: Bilayer curvature; Amphotericin B; Phosphatidylcholine; Cholesterol; Liquid ordered phase; Freeze-fracture; Electron microscopy

1. Introduction

Local curvatures are important shape transformations in biological membranes, especially in connexion with the budding of small vesicles (e.g., in endocytosis). However, there are also relatively stable deformations by local curvatures, often having the appearance of intermediate budding stages. Examples are the caveolae, small invaginations of the plasma membrane of endothel cells, smooth muscle cells etc. [1], the fenestrations and tubular networks of the Golgi-apparatus [2] and the invaginations in the plasma membrane of yeasts and other fungi [3].

The formation of local curvatures can be accompanied by special proteins. Most representative for peripheral proteins is clathrin in connection with the coated pits and coated vesicles [4]. Integral proteins can also be responsible for a local membrane curvature if their shape is cone-like [5], similar to the role of cone-shaped lipid molecules in the 'bilayer couple hypothesis' [6], where an asymmetrical distribution of this lipids between the two apposing monolayers in the membrane is assumed to induce a membrane curvature. On the other hand peripheral proteins can also act as a framework allowing a local curvature of the lipid bilayer only in large gaps. Such an example has been found in the vacuolar membrane of a secretory yeast mutant with circular and/or elongated concavely curved areas free of intramembrane particles [7]. A 'bilayer couple' effect is to assume in this case, because no plausible external reason for the concave curvature exists.

Complementary to the 'bilayer couple hypothesis' different reasons exist for pure lipidic bilayers to deform into protrusions or invaginations. On the basis of a mixture of lipids two mechanism can result in local

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curvatures. One is the formation of intramembrane domains by lateral phase separation [8], and the other concerns only fluid bilayers, where the stress by the contrary bending tendencies of both monolayers becomes reduced by periodic curvatures, three-dimensionally in the bicontinuous cubic phase structures [9] and two-dimensionally in periodically curved bilayers [10].

The occurrence of areas which are alternately convex and concave curved in a very regular pattern is extraordinary for biological membranes. We have found these 'periodically curved bilayers' in the membrane of a *Streptomyces* strain and in the liposomal bilayers made from the mixture of the extracted lipids [7,10]. For interpretation a deduction from the bicontinuous cubic phase structure Im 3 m (Q^{229}) was proposed. The arrangement of bilayers in the bicontinuous cubic phases is topologically equivalent to IPMS = infinite periodic minimal surfaces, and is an organization of frustrated *fluid* films [11]. Frustration is an internal stress caused by the conflict of the coupled monolayers of the bilayer in their tendency to curve. The phenomenological parameter 'monolayer intrinsic curvature' [12,13] represents a colligative property of the system and the value of intrinsic curvature in a monolayer summarizes the tendency to curve for all components. This tendency arises from an imbalance in the distribution of lateral forces within the monolayer [9], more easily imaginable considering geometric packing constraints [14] where differences in the cross section of the headgroup and the chain region result in a cone shape of the molecules. Presence of cone-shaped molecules (preferring nonbilayer structures when isolated) in a high proportion will result in an enlarged frustration of the bilayer and the periodic curvature is a possible mechanism for relaxation.

The responsibility of proteins or lipids for local curvatures is difficult to separate in the complex biological membranes, but the induction of such an effect in bilayers from the extracted lipids [10] or from lipids in a defined composition excludes the influence of the membrane proteins. In earlier investigations on the effect of amphotericin B and nystatin on the membrane of human erythrocytes the formation of local convex bulgings with a diameter in the range of 50 nm was observed in case of high concentrations of these polyene-antibiotics and low ionic strength [15,16]. These local deformations represent a kind of membrane damage and have been observed also in a fungal plasma membrane [17]. Additionally they are of interest as an indication on possible side-effects of these antibiotics on mammalian cell membranes. The induction of such deformations is not restricted to amphotericin B, the same effect could be induced, e.g., by higher concentrations of the lectin concanavalin A in the membrane of turkey erythrocytes [18].

The intramembrane particles are usually absent in these wart-like deformations. Therefore, a membrane deformation due to lipid–lipid interactions was suggested. However, we have not been able to support this interpretation with artificial membranes consisting of mixtures of different lipids and incubated with amphotericin B at room temperature or at 37°C. No structural changes of the bilayers could be observed. The quite regular deformations described in this paper have been observed only after a prolonged incubation at a temperature below 25°C.

There are similarities in the appearance between the convex-concave bilayer deformation described in this paper and the periodically curved bilayer suggesting the same relaxation mechanism. A contradiction seems to be the importance of fluidity for the latter process, whereas the formation of the convex-concave bilayer deformation occurs also at the low temperature of 4°C and after a long storage only. A plausible explanation comes from the special phase *lo* (liquid ordered), which is restricted to bilayers with at least two different components. Admixture of cholesterol as well as of amphotericin B has been found to be effective in formation of this phase. The *lo* phase finds especially interest in connection with cholesterol [19,20] since it may be a phase existing in biological membranes containing large amounts of cholesterol.

2. Materials and methods

L(α)-Dipalmitoylphosphatidylcholine (DPPC) and cholesterol were purchased from SERVA (Heidelberg) and used without further purification. 1 mg Fungizone®, from Squibb, consists of 0.55 mg amphotericin B and 0.45 mg sodium deoxycholate. All other chemicals were of analytical grade and the water was double glass-distilled.

Multilamellar vesicles (MLV) were produced in the following way: DPPC and cholesterol were codissolved in chloroform, resulting in 2, 10 or 30 mol% cholesterol, applied to an amount of 14–21 mg lipids in 1–2 ml chloroform. A dry thin lipid film on the wall of a glass vessel was produced by evaporation under rotation and reduced pressure, followed by a subsequent storage under high vacuum for at least 6 h. For rehydration 0.5 ml solution of Fungizone® (1 mg Fungizone® in 10 ml physiological saline) was added and the solution was heated to 50°C. By shaking at this temperature formation of MLV becomes evident by a milky appearance.

Then, the carefully sealed vessels were stored below 25°C, commonly in a refrigerator at 4°C. For storage over a long time at higher temperatures the MLV in Fungizone®-saline suspension were incubated at 32°C for 3 weeks respectively at 45°C for 3 months. To test

the short-time influence of a lower temperature small amounts of the suspension were placed between the copper supports for freeze-fracturing and these sandwiches were incubated in a freezer at −15°C for 20 h.

For freeze-fracture experiments of the cold suspensions precooled copper supports for the sandwich as well as precooled instruments, which have been placed in the refrigerator for at least 30 minutes, have been used. The sandwiched samples were frozen from this temperature by plunging immediately into liquified propane, cooled by liquid nitrogen. Fracturing and shadowing was performed at −130°C in a BAF 400D (Balzers, Liechtenstein) equipped with electron guns. The replicas were cleaned with chloroform and than observed with a JEM 100B electron microscope. The micrographs in Figs. 1–10 are oriented with direction of shadowing from bottom to top.

Calorimetric studies were performed using a DSC-2 apparatus (Perkin Elmer, Norwalk, CT). The samples prepared as described above were stored at around 4°C for more than 3 months. 15 μl of this suspension were deposited into precooled aluminium pans and hermetically sealed. Heating and cooling runs were performed between the temperatures of −20°C and 60°C with a scanning rate of 2.5 K/min.

3. Results

In the presence of amphotericin B/deoxycholate after storage for 3–4 weeks or longer at 4°C, or at least at a temperature below 25°C, in the bilayers of MLVs consisting in DPPC and 2, 10 or 30 mol% cholesterol a new structure was induced by this polyene antibiotic. The new structure is revealed by bilayer deformations consisting of local convex and concave curvatures. This effect is found only in a fraction of the bilayers, even after an incubation for 9 months. Especially affected are the outer bilayers of multilamellar vesicles (Fig. 1) and separated single bilayers (Fig. 2).

A special feature is the occurrence of patterns with convex and concave curvatures in three types, mainly different in their dimensions (Figs. 2 and 8). In the small-dimensional type the projections have a volcano-like appearance. They have a sharp flat and circular edge, 40–50 nm in diameter, which often looks like an opening. In reality the 'volcanos' seem to be closed and the bilayer is not perforated at these sites (Fig. 3). The projections have a radius of curvature of about 20–30 nm and the convex respectively concave ones are separated by a distance of about 100 nm. The intermediate-dimensional type (Fig. 4) is characterized by a

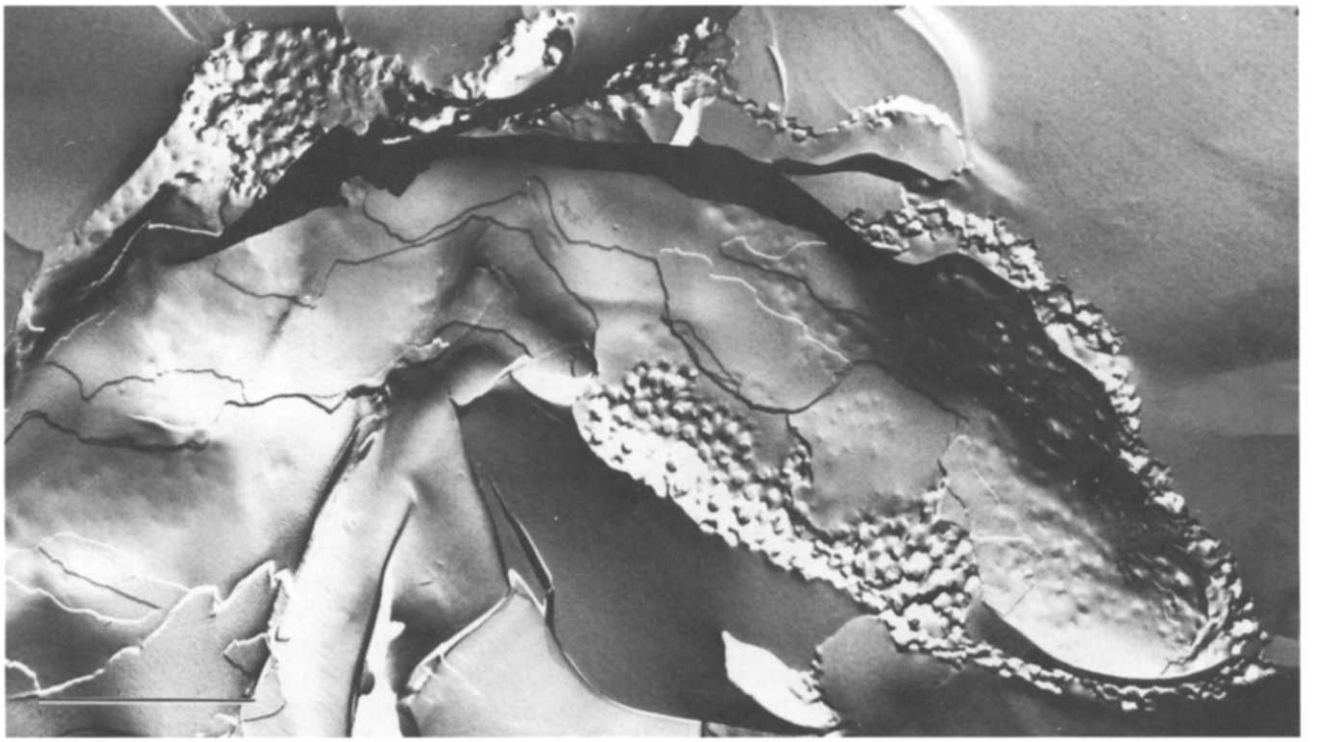

Fig. 1. Multilamellar vesicles of DPPC/cholesterol incubated with amphotericin B/deoxycholate, where the outer loosened bilayers are deformed by similar structures of the same size as in biological membranes. The inner tightly packed bilayers are smooth or with markings from adjacent deformed bilayers. DPPC with 2 mol% cholesterol incubated for 4 weeks with amphotericin B/deoxycholate at 4°C. The bar indicates 1 μm.

separation-distance of about 400 nm and hemispherical caps, 150–200 nm in diameter, instead of the volcano-like protrusions. Accordingly, the radius of curvature is enlarged to about 50–80 nm. Both types form an irregular pattern, as can be seen in cross-fractures (Fig. 5) as well as in fractures along the bilayer-face (Fig. 6). Most irregular is the large-dimensional type (Figs. 7 and 8), which has been found, together with both other types, hitherto only in case of bilayers containing 30 mol% cholesterol. This type has a separation distance in the range of 700 nm and a radius of curvature from 150 to 200 nm. A speciality of this large-dimensional type is the involvement of oligolamellar stacks of bilayers (arrows in Fig. 7).

The close similarity between the three types becomes evident not only by their occurrence in two neighbouring bilayers (Figs. 2 and 8) but also by their presence side by side in the same bilayer (Fig. 5). Nothing can be said about a transformation from one into the other type, but all can evolve from the planar bilayer directly. Often structural alterations are visible where a flat bilayer contacts the projections of a deformed bilayer. This can be striking in the case of the small-dimensional type, because circular corrugations (Fig. 2, arrowheads) seem to be an early stage in the formation of the protrusions. The origin of these structures from an underlying and already deformed bilayer is revealed in Fig. 9 (arrow). The degree of deformations of the planar bilayer is different, from nearly not visible to the circular corrugations. Intermediate deformations (Figs. 1 and 6) are the common picture, as it is also the case with the intermediate-dimensional type (Fig. 4).

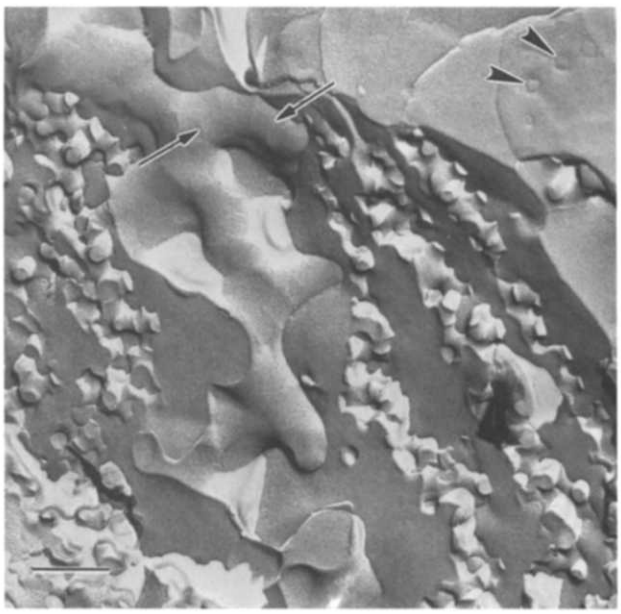

Fig. 2. Separated bilayers with convex-concave deformations, one (in the middle) in the intermediate-dimensional type, the others in the small-dimensional type. Where the bilayers are still tightly packed they are smooth (upper right corner of the picture). The circular corrugations (arrowheads) are impressions from an adjacent bilayer with small-dimensional deformations. The arrows indicate on rims separating 'domains' of the deformed bilayer. DPPC with 10 mol% cholesterol incubated for 4 weeks with amphotericin B/deoxycholate at 4°C. The bar indicates 200 nm.

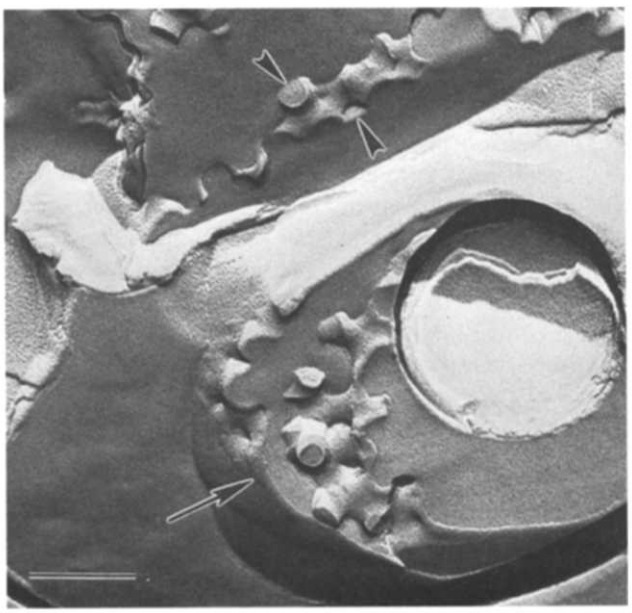

Fig. 3. Deformation into the small-dimensional type. The transition from the smooth separated bilayer (arrow) reveals no intermediate stages. The flat and circular closing of the convex and the concave protrusions is obviously not an opening, because their fracture faces (arrowheads) have the appearance of the bilayer fracture face and not of the sourrounding ice. Same preparation as in Fig. 2. The bar indicates 200 nm.

The convex-concave bilayer deformations consist possibly of a patchwork of 'domains' as seen sometimes in replicas with a good resolution (Figs. 2 and 10). The 'domains' differ only a little in smoothness of the bilayer fracture-face and in degree of curvature. Rims often separate the 'domains' and these rims can be seen sometimes in the small-dimensional type also (Figs. 6 and 10).

Reflattening of the convex-concave bilayer deformations occurs after incubation at higher temperatures. The bilayer deformations are absent after heating to 45°C for only 1 min. No curvature-deformation had been induced by a storage at 32°C and 45°C, even after 3 months. A shorter storage for 24 h at the lower temperature of −15°C was also not effective. Induction of convex-concave bilayer deformations was further not possible without cholesterol in the MLV nor after an incubation without the antibiotic and after the incubation only with cholate, the additive in Fungizone®.

The results of the calorimetric studies (Fig. 11) include for comparison pure L-DPPC. Under normal conditions fully hydrated DPPC shows two phase tran-

sitions on heating. The phase sequence is $L_{\beta'} - P_{\beta'} - L_{\alpha}$. A prolonged incubation of the enantiomeric DPPC at temperatures around 4°C results in a very slow conversion of the L_{β}, phase to the so-called subgel phase L_c. Therefore heating of L-DPPC after a long incubation in the cold results in three phase transitions. Fig. 11a shows the first heating run of such a sample. The corresponding phase transition temperatures and enthalpies amount to 22.6°C; 17.9 kJ/mol (subtransition), 36.3°C; 7.2 kJ/mol (pretransition) and 41.3°C; 38.5 kJ/mol (main transition). In the second heating run (not shown) only the pre- and the main transition can be observed.

Fig. 11b shows the first heating run of the mixture of DPPC/cholesterol/Fungizone® prepared as desribed above. The main transition is shifted to a higher temperature (T_m = 42.8°C). A clear pronounced pretransition could not be observed, however, a deviation of the base line below the main transition peak occurs. A second transition centered at around 29°C appears only in the first heating run. The transition region is relatively broad, it starts at 25°C and finishes at 32°C. On cooling the sample (Fig. 11c) the main transition can be observed with a slight supercooling. In the second heating run (Fig. 11d) only the main transition appears. This behavior can be compared with the subtransition of pure L-DPPC. The formation of the low-temperature phase of the mixture has also a very slow kinetics.

4. Discussion

The result of our freeze-fracture studies is the finding of a new type of bilayer structures consisting of three different pattern of convex-concave deformations. The system, a mixture of DPPC and cholesterol influenced by amphotericin B/deoxycholate, is complex and includes therefore different aspects and problems.

4.1. The low temperature state

A first speciality is the formation of these structures only after a long-time storage at temperatures below 25°C, also at the refrigerator temperature of 4°C, indicating a connection with the subgel phase (L_c) found e.g., in bilayers of DPPC and other disaturated phosphatidylcholines after a low temperature storage for several days [21–24] or, as in the case of DPPG bilay-

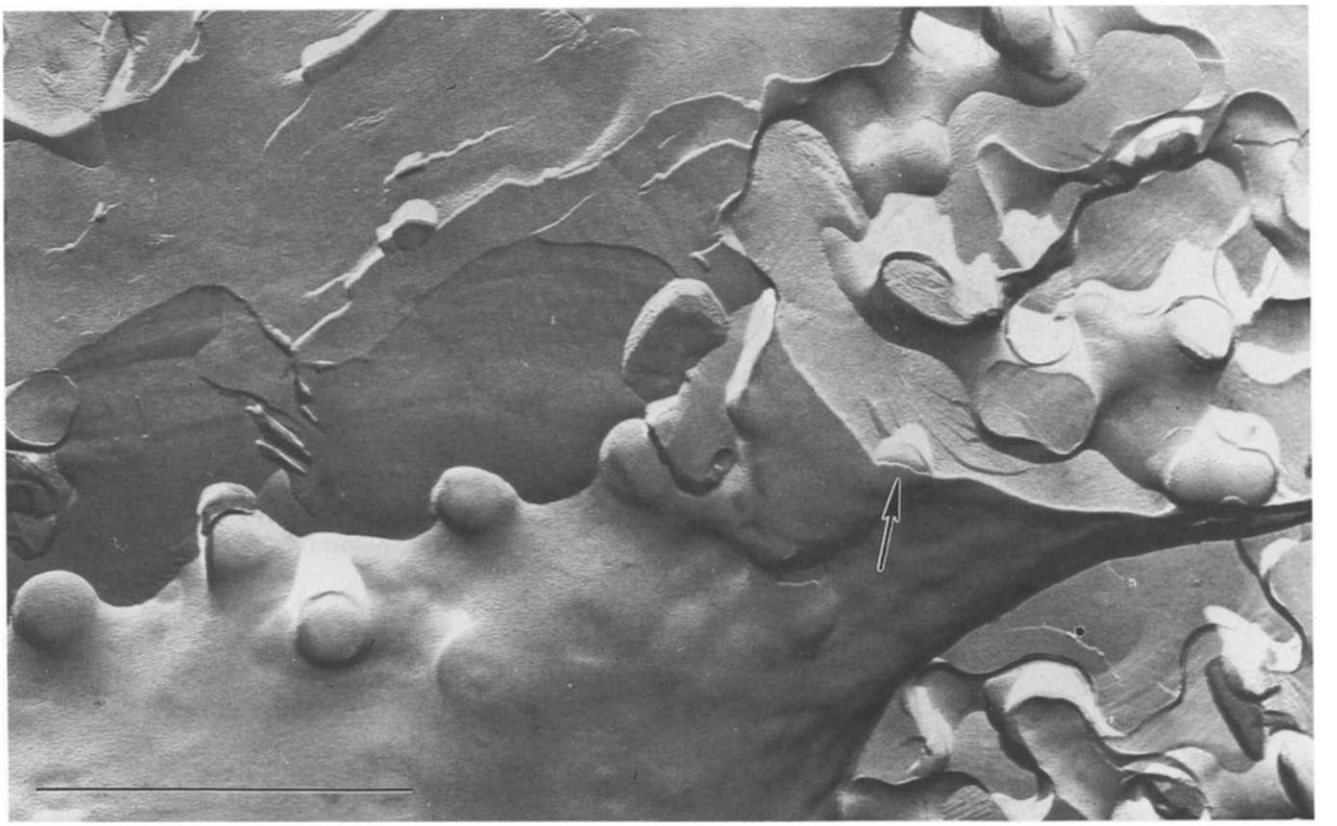

Fig. 4. Deformation into the intermediate-dimensional type. The markings on the smooth bilayer are produced by contact with already deformated bilayers (arrow). DPPC with 10 mol% cholesterol incubated for 9 months with amphotericin B/deoxycholate at 4°C. The bar indicates 1 μm.

ers [25], for months. This L_c phase is more ordered ('crystal state') than the L_β, phase. L_β, is a gel phase with chains packed in a disordered twodimensional rectangular lattice. The chains are tilted.

The L_c phase is a highly ordered, condensed phase with a hybrid subcell having specific chain-chain packing. The area per chain is about 19 $Å^2$. It is the most dense form. The head groups are relatively immobile and the transition into the L_c phase is connected with a partial dehydration of the hydrophilic region of the lipid bilayer. The freeze-fractured bilayer of the L_c phase appears flat or has some slightly inclined areas [22,26].

The subtransition has been found in L-DPPC also in the presence of 20 mol% cholesterol [27] and is therefore expected in our samples, too. The convex-concave deformations already from their appearance do not belong to the more crystalline L_c phase. More plausible for shape changes into curved structures is a liquid-like state. The measured temperature of the 'subtransition' is with 25–32°C higher than the 18–20°C for hydrated L-DPPC and L-DPPC/cholesterol [27], indicating a conversion from another lateral packing which is probably more complex by the additional components amphotericin B and deoxycholate.

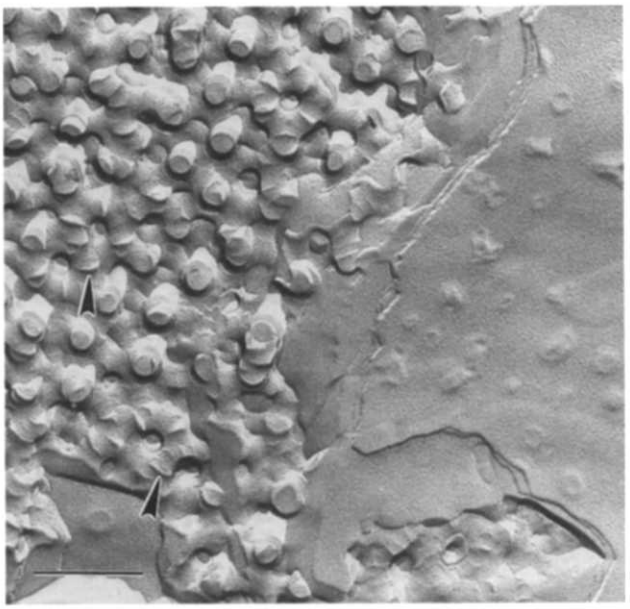

Fig. 6. Transition between flat areas and areas deformed into the small-dimensional type presented by fracturing along the bilayer face. The pattern formed by the convex-concave deformations appears only relatively regular. Rims (arrowheads) separating domains are not visible on the flat parts. At the flat parts markings from adjacent deformed bilayers are visible. Same preparation as in Fig. 2. The bar indicates 200 nm.

Fig. 5. Within a loose stack of bilayers deformed into the small-dimensional type two transitions into the intermediate-dimensional type are visible (arrows). Same preparation as in Fig. 1. The bar indicates 1 μm.

4.2. Cholesterol and the lo phase

Mixtures of phospholipids with cholesterol have been studied intensively. The detailed molecular organization is not fully understood, but the picture becomes more and more clarified. For the system DPPC/cholesterol below 20 mol% cholesterol the coexistence of a cholesterol-rich and a cholesterol-poor phase is accepted [28–31]. Above the main transition the cholesterol-poor phase is liquid-disordered (*ld*) and the cholesterol-rich phase is liquid-ordered (*lo*). Below the main transition the *lo* phase still exists, now together with the normal (gel state) solid-ordered (*so*) phase. An indication for the separation into cholesterol-rich and cholesterol-poor regions are parallel stripes in the freeze-fractured bilayers, representing the cholesterol-rich phase [32].

In the *lo*-phase (which was also termed β-phase [30]) the acyl chains show a high degree of conformational order. This phase exhibits zero shear restoring force also at temperatures well below the main transition of the pure lipid [33]. Complete breaking of the crystalline structure in the solid phase of DPPC by cholesterol needs a concentration of 20 mol% cholesterol and more. At lower concentrations lateral phase separation into *lo* and *so* domains occurs [30].

In our investigations the microdomain structure was stable in the solid state for months as demonstrated by

the presence of stripes in the controls without amphotericin B/deoxycholate. These structures could be found after such long times also in the samples containing the antibiotic, but restricted to the shielded inner layers of the multilamellar vesicles. However, smoothness of the bilayer with no indications of other existing microdomains seems to be the prerequisite for the development of the convex-concave bilayer deformations.

4.3. Amphotericin B and the lo phase

The stripes disappear due to the penetration and incorporation of amphotericin B/deoxy-cholate into the accessible outer bilayers. Amphotericin B/deoxyycholate acts obviously as a crystal breaker too, leading together with cholesterol to a transformation into a homogeneous *lo* phase. This homogenization into an overall liquid state is one precondition for the formation of a curvature pattern.

Amphotericin B is indeed able to interact with phospholipids in the same manner as cholesterol. A 1:1 complex is also formed with DMPC, ^{2}H-NMR studies revealed an ordering effect of amphotericin B on the acyl chains of DMPC [34]. Especially informative in connection with our investigations are the results of Janoff et al. [35] on the effects of amphotericin B on DMPC/DMPG (molar ratio 7:3) using freeze-fracturing and other methods. The hydrated lipid system shows a main transition at 23–24°C and a pretransition at 13°C. Frozen from 20°C the bilayers without amphotericin B exhibited the normal ripples, but in contrast the bilayers containing amphotericin B are not spherical vesicles and the ripples are absent (partly at 5 mol% and generally at 25 and 50 mol%). The structural alteration with a more soft appearance consists in an irregular bending of loosely packed 'ribbon-like' structures. Main- and pretransition disappear as seen by calorimetry, but ^{31}P-NMR indicates progressively immobilized lipids with increase of the amphotericin B concentration. Wide-angle X-ray diffraction confirms this effect. In mixtures with amphotericin B a well ordered side-by-side packing of the acyl chains could be observed above the main transition temperature of the pure lipid mixture.

The assumption of a persistence of the gel-phase is in discrepancy with the morphological appearance after freeze-fracturing, but the results are in accordance with the formation of a *lo* phase by amphotericin B. Similar to cholesterol the amphotericin B molecule is stiff and smooth in its hydrophobic part (the rigid heptaenic macrolactone ring) and is therefore very

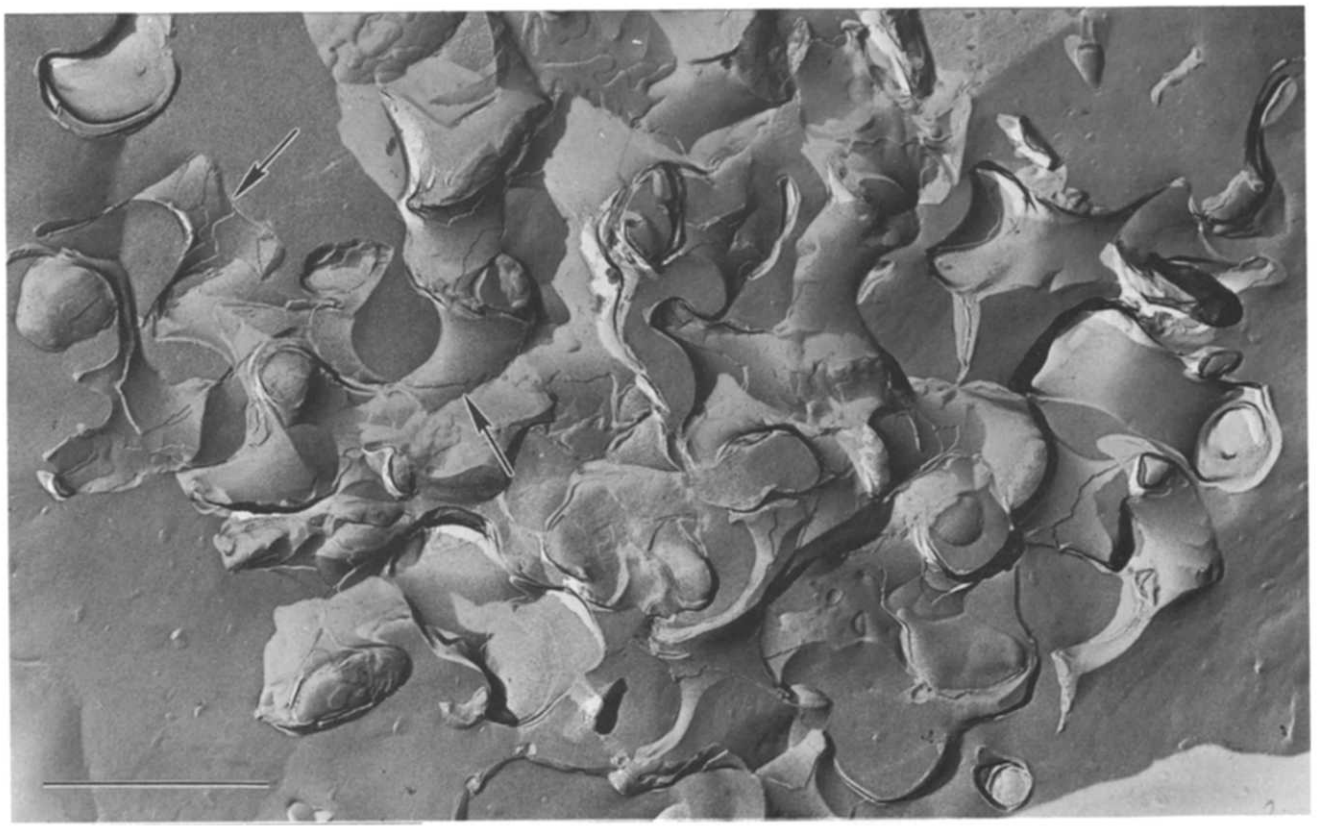

Fig. 7. Deformation into the large-dimensional type. Only in case of this type an oligolamellar stack of bilayers is involved (arrows). DPPC with 30 mol% cholesterol incubated for 10 weeks at 20°C with amphotericin B/deoxycholate. The bar indicates 1 μm.

Fig. 8. Occurrence of the large-dimensional type together with the intermediate-dimensional type and (inset) the small-dimensional type in the same sample. Same preparation as in Fig. 7. The bar indicates 1 μm.

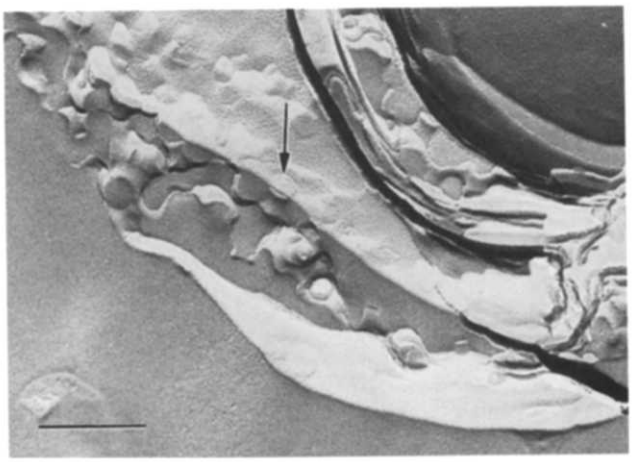

Fig. 9. Course of the fracture partially along a flat bilayer and then crossing the underlying ice containing deformed bilayers in the small-dimensional type clearly reveals the origin of the circular corrugations from contact with the flat circular top of the protrusions (arrow). Same preparation as in Fig. 2. The bar indicates 200 nm.

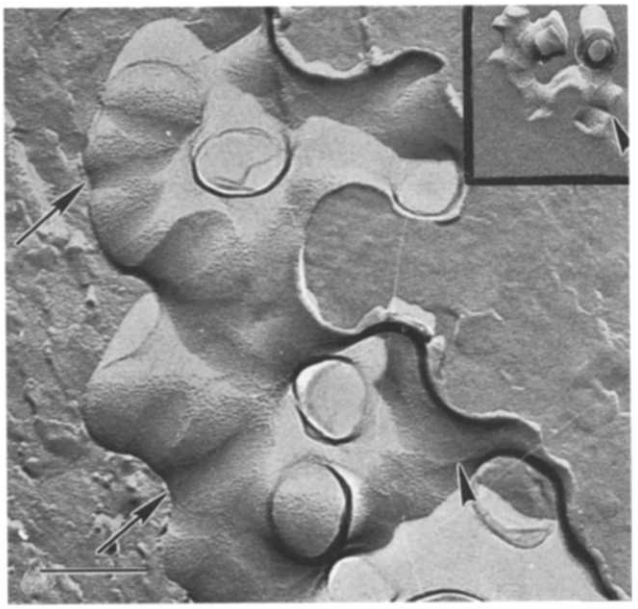

Fig. 10. The fracture face of the deformed bilayer in the intermediate-dimensional type reveals 'domains' of a more smooth appearance and a somewhat altered curvature (arrows). Rims between the 'domains' (arrowheads, see also Fig. 2) can be observed also in the small-dimensional type (inset, see also Fig. 6). DPPC with 10 mol% cholesterol incubated with amphotericin B/deoxycholate for 6 months at 4°C and than for 30 min at 16°C. The bar indicates 200 nm.

likely to prefer the interaction with acyl chains in the extended conformation. Both molecules are amphiphils with a small polar head and like to form an equimolar complex with lipids.

To this group belongs further the antibiotic filipin, which is structural similar to amphotericin B. Interaction with DMPC also results in formation of 'gel-like' lipids in the fluid-phase region above the main transition and in a disordering effect below the main transition [36]. The characteristic small membrane deformations induced by filipin are in the common interpretation filipin-sterol complexes (for review see [37]), however the possibility of filipin-lipid complexes was not excluded [36]. Because of this deformations (also termed lesions) filipin is of special interest concerning the convex-concave deformations induced by amphotericin B/deoxycholate. As seen in freeze fractured membranes (e.g., [38]) the filipin-induced deformations are small (diameter 20–25 nm) circular convex and concave structures.

4.4. Formation of curved bilayer structures

The convex-concave bilayer deformations are caused by such forces as intrinsic bending and packing. The intrinsic tendency for bending results mainly from the kind of the involved molecules. In case of asymmetrical distribution between the both monolayers the 'bilayer

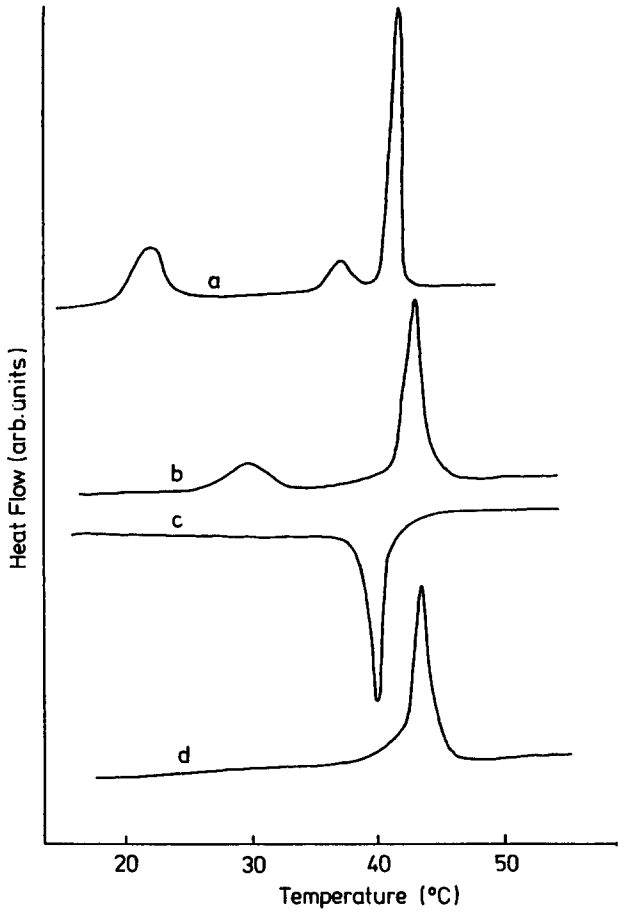

Fig. 11. Calorimetric transition curves for multilamellar vesicles of pure L-DPPC (plot a, heating scan after prolonged storage at 4°C) and of L-DPPC/cholesterol/Fungizone® (plot b, heating after a storage for 3 months at 4°C; plot c, cooling scan; plot d, second heating scan performed successively).

couple hypothesis' [6] explains the bending. However, symmetry of composition in both monolayers does not prevent bilayer curvature. The parameters *spontaneous curvature of the monolayer* and *packing stress* [12,13] have been used successfully to analyse lipid polymorphism, especially in the hexagonal and the cubic phases [9,39]. The *frustration* (internal stress) of the bilayer due to the two oppositely oriented monolayers can be reduced by periodic curvatures. The result is a three-dimensional network of curved bilayers in the case of bicontinuous cubic phases [40–42] and the so-called L_3 (= sponge) phase [43–45], and two-dimensional the periodically curved bilayer [10]. All these structures are more or less regular; however, it should be noted that their state is neither crystalline nor solid, but fluid (liquid).

The frustration can result from the discrepancy in cross-sectional area between the headgroup and the acyl chain region of the involved molecules [14]. In our investigated system the hydrated DPPC is not cone-shaped, but cholesterol has only a small 'headgroup'. Therefore cholesterol can promote the formation of H_{II} and cubic phase structures [46]. The combination of cholesterol with the phospholipid DPPC in a 1:1 complex results in a cone shape, but this is not sufficient to induce a bilayer destabilization. Only the stripes seem to represent a bulging of the cholesterol-rich domains in comparison to the planar cholesterol-poor part of the bilayer [32].

4.5. The interaction between DPPC, cholesterol and amphotericin B

The combination between DPPC, cholesterol and amphotericin B has two aspects, the interaction of the antibiotic with sterols as the generally accepted damaging action of amphotericin B [47] and the complexation between the three components. Concerning amphotericin B and sterols an increased permeability of ions is attributed to their interaction. The greater ability to induce this effect in the ergosterol-containing fungal cell membranes than in the cholesterol-containing mammalian cell membranes is the assumed basis for the fungicidal action. Whereas interactions by hydrogen bonds are equivalent for both ergosterol and cholesterol, a difference has been found in case of the non-specific van der Waal's forces, which depend on the tight fitting of the molecules. In the molecule of amphotericin B a rigid chain of seven conjugated double bonds is predestined for this kind of interaction with the sterols. Because the ergosterol molecule has a more flat shape than the cholesterol molecule a better intermolecular contact between ergosterol and amphotericin B is to expect [48].

In the same manner a dependence in the interaction between amphotericin B and the molecular shape of phospholipids exists. Amphotericin B has a greater affinity for phospholipids with disaturated chains than for such containing unsaturated acyl chains [49] and for lipids in the ordered gel state than for those in the disordered liquid-crystalline state [47].

The affinity between cholesterol and DPPC is also only partially attributed to a specific association by a single hydrogen bond. Van der Waals forces are involved too as demonstrated, e.g., by space-filling molecular models [50] and as responsible for additionally and only loosely associated phospholipid molecules [51].

The interaction between the three components together, disaturated phosphatidylcholine, cholesterol and amphotericin B, has been investigated by ^{2}H-NMR [52]. The results are in accordance with an exchange of associates between the three components ('dynamic complexation'). From the aspect of shape and spontaneous monolayer curvature the small polar heads of cholesterol as well as amphotericin B will result also in case of such a dynamic complexation in an altogether

smaller space for the headgroup region than for the hydrophobic region.

4.6. The formation of the convex-concave bilayer curvatures

The development of bilayer curvatures at low temperatures may be explained by the predominance of the weak van der Waals interactions between the different components, easily disturbed by thermal molecular motions. It can be anticipated that the bilayer deformations by alternate convex and concave curvatures without a strong periodicity are mainly based on the same forces as the periodically curved bilayer structures [10]. Both are manifestations of a relaxation, where a conflict in the spontaneous curvature between the two monolayers results in a frustration, which can be relaxed by configurations with alternating curvatures. This type of relaxation is realized with a strong periodicity three-dimensionally in the bicontinuous cubic phase structures and two-dimensionally in the periodically curved bilayer. The bilayer networks of the cubic phases have been found to belong to the infinite periodical minimal surface (IPMS) structures and for the periodically curved bilayer the similarity with a selected plane of a cubic phase indicates a relation to the IPMS structures. Minimal surfaces are defined mathematically by a mean curvature of zero at every point. There are only two possibilities (in our Euclidian space), the flat plane and the saddle [53]. In case of the periodically curved bilayer the hemispherical caps closing the holes in a selected plane of the cubic phase are therefore places of 'non minimal surface' interrupting the periodic saddle surface.

Such deviations from the IPMS structures exist also in the present system of convex-concave bilayer curvatures, but there are also connections. The surface exhibits saddles but the periodicity is weakened. The closing of the holes is completed with hemispherical caps in case of the intermediate- and large-dimensional type, but surprisingly by a flat plane in the small-dimensional type (openings instead of the flat plan additionally can not be excluded). May be, this structure represents a combination of the two types of minimal surfaces saddle and flat plane as a special kind of approach to relaxation. On the other hand the sometimes observed subdivision of the bilayer into a patchwork of 'domains' somewhat different in curvature (Fig. 10) indicates a more complex situation at least in the small- and intermediate-dimensional types.

An interesting yet not understood effect is the formation of two or three different types occurring together not only in the same sample and in directly neighbouring bilayers, but also on the same bilayer side by side (but all three types only in case of 30 mol% cholesterol). However, a parallelity exists to the periodically curved bilayer with four versions in periodicity. In comparison to the related cubic phases with lattice constants of at most 20 nm [53] the periods in the twodimensional regular pattern are in the range of 30, 45, 60 and 90 nm (Ref. 10 and unpublished data). The mean distance in the convex-concave bilayer deformations amounts to 100 up to 700 nm and more, and this is remarkably greater; probably it exceeds the border for the formation of a stable periodicity. The comparable three-dimensional structure is the L_3 or sponge phase, which is topological related to the bicontinuous cubic phases but less regular and also of a greater dimension (up to several hundreds nanometers [44]).

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6. References

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