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Periodically curved bilayer structures observed in hyphal cells or stable L-form cells of a *Streptomyces* strain, and in liposomes formed by the extracted lipids

Helmut W. Meyer [1], Walter Richter [1] and Johannes Gumpert [2]

[1] *Department of Electron Microscopy, Medical School, Friedrich Schiller University Jena, Jena and* [2] *Academy of Sciences GDR, Central Institute of Microbiology and Experimental Therapy, Jena (G.D.R.)*

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Periodically curved bilayer structures showing a tetragonal pattern were revealed by freeze-fracture electron microscopy in hyphal cells, stable L-form cells, and liposomes prepared from extracted lipids of *Streptomyces hygroscopicus* NG 33-354. The pattern is formed by alternating convex and concave curvatures of the bilayer. It has been found with different repeat distances (multiples of about 15 nm) and with a different degree of expression (from just visible to very pronounced). An interpretation as infinite periodic minimal surface (IPMS) structures is more probable than an inducement of the pattern by underlying small vesicles. The occurrence of nonbilayer textures and the similarity of the tetragonal pattern with a 'bilayer sector' from a cubic phase structure (Andersson, S. et al. (1988) Chem. Rev. 88, 221–242) support such an interpretation.

Introduction

The planar bilayer is the characteristic arrangement of lipid molecules within biological membranes. Among the membrane lipids there are always some species which, after separation, prefer the nonbilayer structures of the inverted hexagonal phase (H_{II}) or of an inverted cubic phase [1–4]. Furthermore, a regulated balance between bilayer- and nonbilayer preferring lipids has been found in membranes of some microorganisms [2,5,6].

The inverted nonbilayer phase structures consist in arrangements of curved monolayers, and therefore a highly curved bilayer organization has been postulated for an intermediate between the planar bilayer and the inverted nonbilayer structures [7]. We have observed a membrane structure with periodic curvatures (wafer pattern) in hyphal cells and protoplast-type L-form cells of *Streptomyces hygroscopicus* [8–11]. The pattern is caused by the membrane lipids because the same structures could be observed in bilayers made from the extracted lipids. The lipids have been found to be composed of about 45% phospholipids, 35% neutral lipids and 20% glycolipids. Of importance is a high content of constituents with a tendency to adopt nonbilayer structures, e.g., cardiolipin, phosphatidylethanolamine and branched fatty acids. The phospholipid fraction alone is able to produce this periodically curved bilayer structure, but glycolipids and neutral lipids show an amplifying effect [9].

In a previous paper [10] the formation of the hexagonal and tetragonal pattern of the periodically curved bilayer structures was attributed to underlying small vesicles. Based on conflicting freeze-fracture observations, especially the occurrence of the pattern also in absence of small vesicles, an 'infinite periodic minimal surface (IPMS)'-structure was proposed recently as an alternative explanation by one of us [11]. This type of structure is already known from the cubic phases [12]. In this study, we focus on further details especially considering the tetragonal pattern.

Materials and Methods

The cultivation of *Streptomyces hygroscopicus* NG 33-354 or of the L-form of this strain and the preparation of membranes have been described previously [10,13]. Hyphal cells or L-form cells grown in liquid medium at 28°C were harvested after different periods of incubation and prepared for freeze-fracturing without chemical fixation and without cryoprotection.

Correspondence: H.W. Meyer, Abteilung für Elektronenmikroskopie des Bereichs Medizin, Friedrich Schiller Universität Jena, Ziegelmühlenweg 1, 6900 Jena, G.D.R.

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For the chloroform/methanol extraction of lipids the purified and lyophilized membranes of the L-form cells were used. Multilamellar liposomes have been commonly produced by shaking the lipids with phosphate-buffered saline (PBS), pH 7.2. For some investigations distilled water or citrate buffer (0.1 M, pH 3) has been used.

Freeze-fracturing was performed with a Balzers BAF 400 D device, and for quenching the sandwich-technique and liquified propane were used. The cleaned replicas were examined in a Jeol JEM 100 B or a Tesla BS 500 electron microscope.

Results

In freeze-fractured preparations hyphal cells cultivated as submerged culture at 28°C reveal remarkable alterations on their cytoplasmic membranes by formation of well demarcated areas without particles. These particle-free areas, consisting obviously in segregated

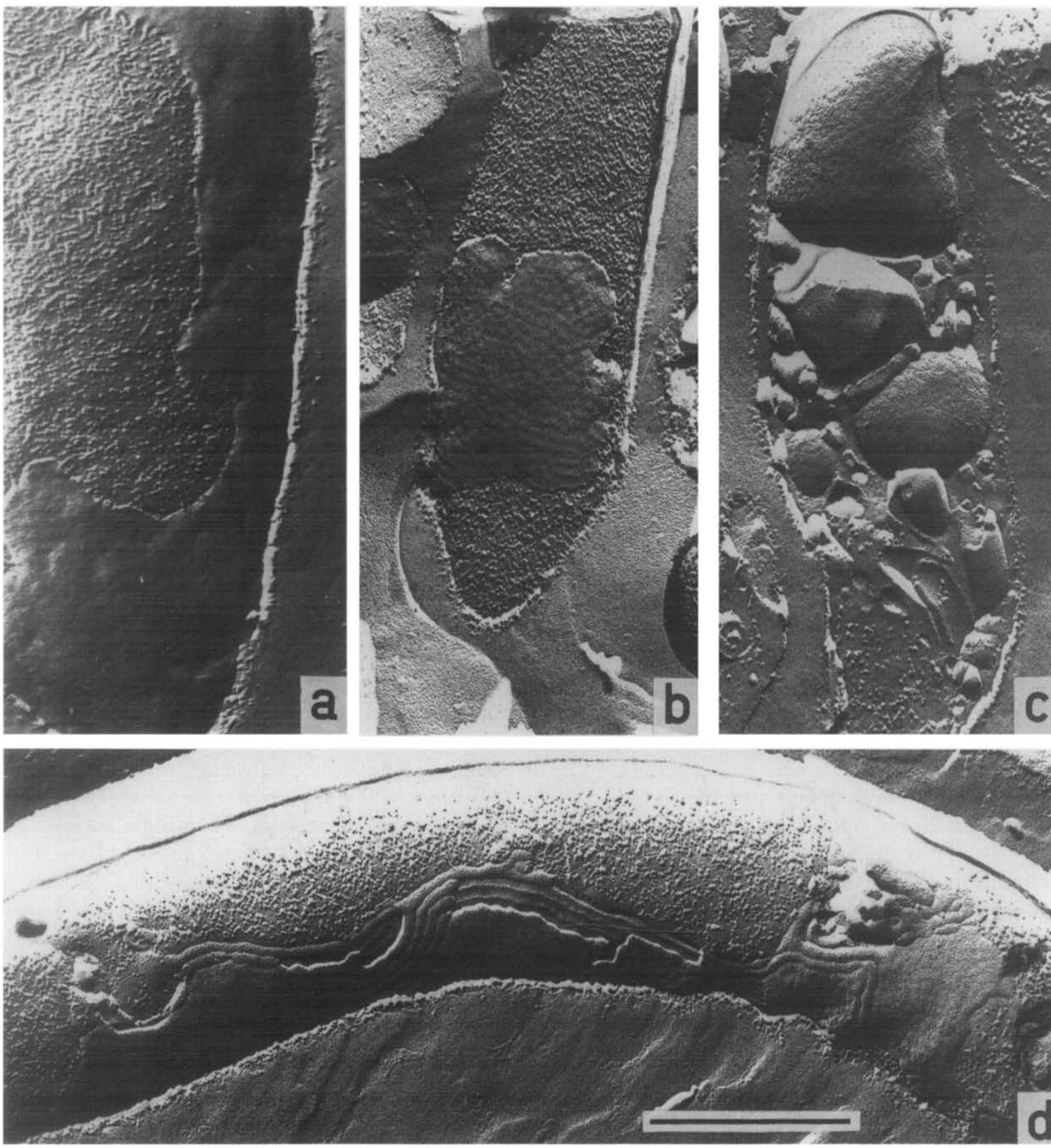

Fig. 1. Hyphal cells of *Streptomyces hygroscopicus* cultivated for one (a), or four days (b–d). Pattern formation by curvatures in well demarcated particle-free areas of the cytoplasmic membrane is weak (a), more distinct and then revealing a regular tetragonal pattern (b), or exceptionally in parts transformed into stripes (d). The pattern can also occur in the membrane of intracellular granules in disintegrated cells (c). Bar: 500 nm. All micrographs are arranged with shadow direction from bottom to top.

membrane lipids, have been found already after one day of cultivation (Fig. 1a), but they are more frequent after 3–5 days of cultivation (Figs. 1b and 1d). Interestingly these areas are often not planar but have a regular pattern by curvatures, an effect also observed on particle-free membranes of some granules in disintegrated old cells (Fig. 1c).

Commonly the expression of the pattern in the hyphal cells is only weak (Figs. 1a and 1c), but if the pattern is distinct enough a tetragonal arrangement can be seen (Fig. 1b). The center-to center spacing within this pattern is in the range of 25–30 nm. Exceptionally the texture differs, because partially one direction is more prominent resulting in worm-like stripes (Fig. 1d).

The L-form cells have no cell wall structures, and they are spherical bodies different in size. After cultivation in liquid medium for several days on the membrane faces not always the normal random distribution of intramembranous particles has been found. Often the membranes have particle-free areas of different size and

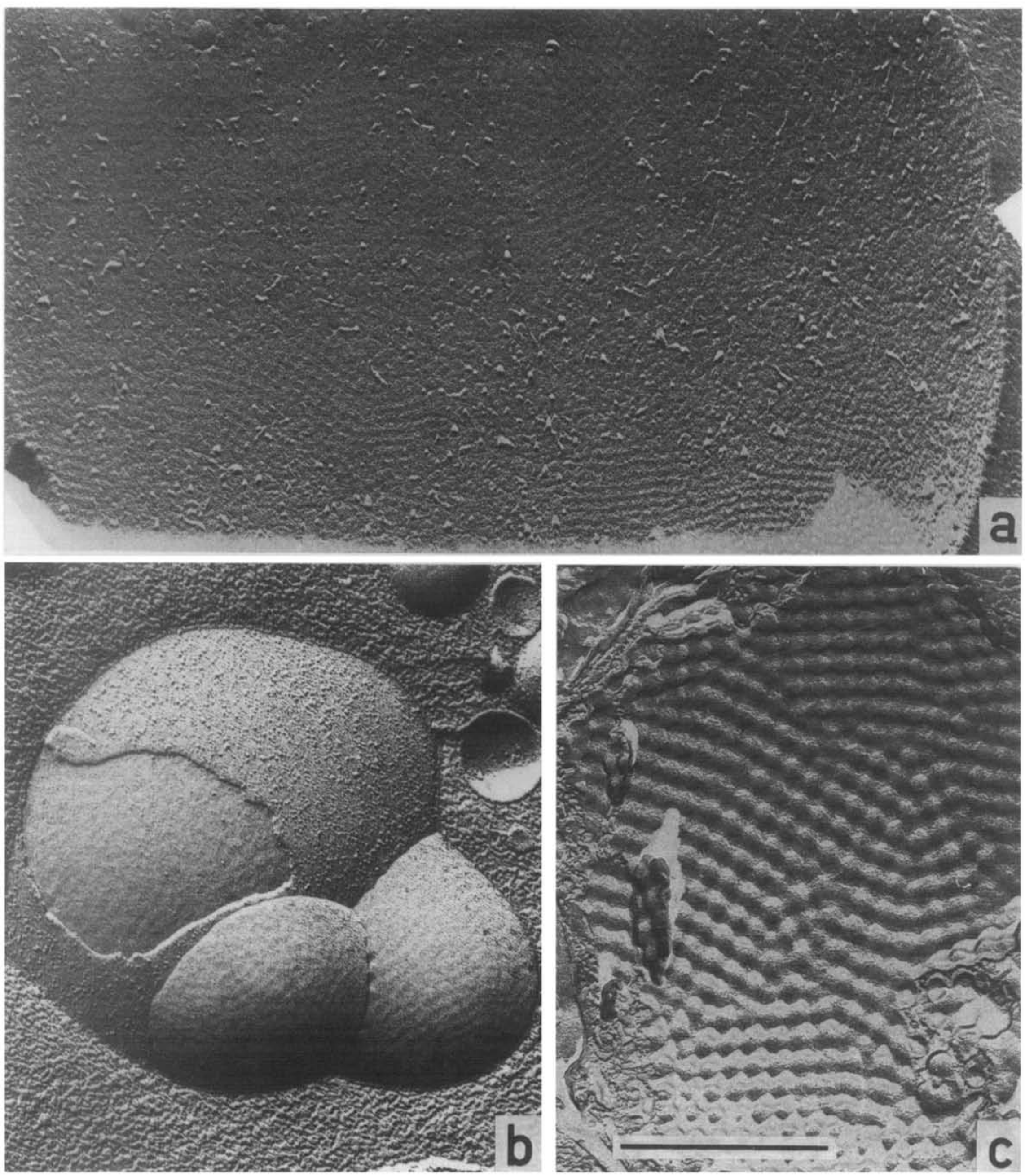

Fig. 2. Stable L-from cells of *Streptomyces hygroscopicus*. Regular tetragonal pattern with a repeat distance of 25–30 nm in particle-free areas of a cytoplasmic membrane (a), weak expression of the pattern in two attached lamellar bodies and in an underlying membrane area with only a few particles (b), and a very distinct regular tetragonal pattern with a repeat distance of about 60 nm on a particle-free membrane (c). Bar: 500 nm.

appearance. Additionally lamellar bodies without particles or with only a few particles are present within or separated from the cells. The tendency to form a pattern by periodic curvatures is high in particle-free areas of L-form membranes as well as in the lamellar bodies. The membrane in Fig. 2a shows a number of particle-free areas and all of them are characterized by a very regular tetragonal pattern with a repeat distance of 25–30 nm. The same dimension shows the pattern in Fig. 2b expressed on two lamellar bodies budded from or attached to a membrane with normal appearance and on the fracture-exposed part of an underlying membrane with only a few particles. In comparison to the weak expression of the pattern in Fig. 2b a very distinct regular tetragonal pattern can be seen in Fig. 2c. The pattern has been formed in a lipid layer without particles. The repeat distance is in the range of 60 nm.

Some further examples concerning the diversity of pattern structures observed in L-form preparations are presented in Fig. 3. In addition to the repeat distances of 25–30 nm and about 60 nm a 40–45 nm distance has also been found occasionally (Fig. 3a). The pattern is only weakly expressed in Fig. 3a, and simultaneously the number of particles is reduced but still relatively high. On the other hand the lamellar body in Fig. 3b without any particle shows a very prominent pattern. This pattern is arranged more hexagonally, probably as a result of dislocations by the small radius of curvature of the vesicular body. Quite another type with a hexagonal pattern is represented by the membrane deformations in particle-free areas seen in Fig. 3c. The units are tightly packed resulting in a hexagonal outline of the single units, and often they have less regular dimensions than in the tetragonal pattern. Another type of periodic curvatures is presented in Fig. 3e, where the single curved units are separated by particles in a net-like distribution. Sometimes also single particle-free areas can form bulges (Fig. 3d).

Liposome produced from extracted lipids of L-from membranes express the same pattern formation as seen in the particle-free areas of the cytoplasmic membranes or on the lamellar bodies. However, the sequence of repeat distances in the pattern has been found completed by a further dimension of about 15 nm (Fig. 4a). This small repeat distance was observed very seldom, and the arrangement within the pattern is not well defined. Examples demonstrating the tetragonal arrangement clearly are presented in Figs. 4b–4d for the

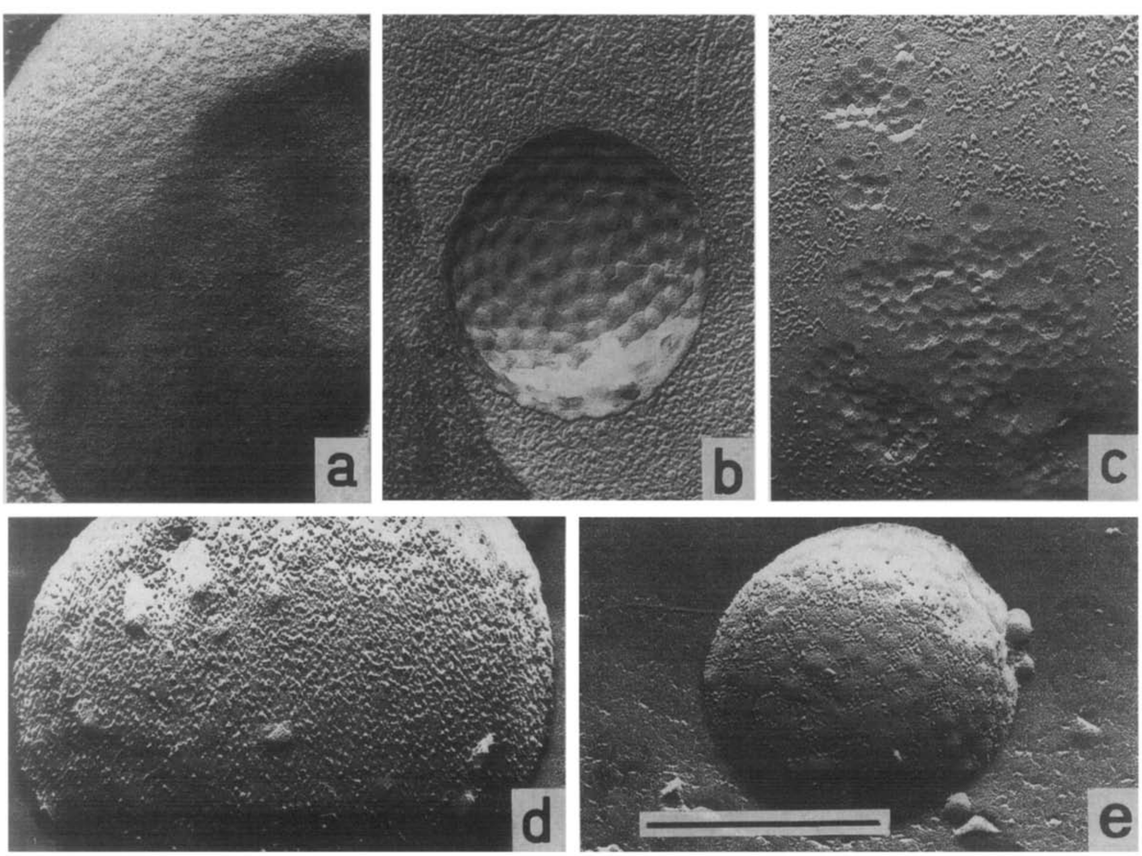

Fig. 3. Stable L-form cells of *Steptomyces hygroscopicus*. Weak expression of a regular curvature-pattern on a cytoplasmic membrane with a reduced number of particles (a), but distinct expression on a particle-free lamellar body (b). The pattern in (b) is distorted into a more hexagonal arrangement. A different type of hexagonal arrangement (c) is composed of 'impressions', tightly packed and less regular in size. Rarely single particle-free areas are bulged (d), and also regularly arranged bulges within a net-like distribution of particles occur (e). Bar: 500 nm.

repeat distances of about 30 nm, 45 nm and 60–75 nm, respectively. The pattern has an identical appearance looking at the concave (Fig. 4c) or at the convex (Fig. 4d) side of a lipid bilayer. Pattern formation was also observed in multilamellar liposomes with tightly stacked lamellae (Fig. 4e). No intercalated vesicles were visible in cross-fractured areas.

After suspending the extracted lipids in citrate buffer (pH 3) the pattern formation was promoted (Fig. 4c) and also textures indicating the occurrence of nonbilayer structures could be observed. After heating to 80 °C and freezing from this high temperature the H_{II}-phase texture in Fig. 5a as well as the regularly arranged 'impressions' on the outermost layer of a lipid body in Fig. 5b (obviously produced by a tight-fitting H_{II} or cubic phase structure) have been found. The repeat distance within the regular pattern in Fig. 5b is about 12 nm and the repeat distance of the striation of the H_{II} texture in Fig. 5a about 5 nm. A distance of 5 nm is already near to the region of limited lateral resolution of the replica. A very fine striation with a repeat distance lower than 5 nm, detected in the sediment after mixing of the dried lipid extract with distilled water (Fig. 5c), probably belongs to an H_{II} texture, too, but is problematic because of this limited resolution. Another structure possibly representing an H_{II} texture was found under neutral pH conditions in PBS, exactly in the sediment formed after some days of storage at 4 °C (Fig. 5d). The repeat distance of about 10 nm is more in accordance with the 12 nm pattern in Fig. 5b, but in such sediments also other and more complex textures with repeat distances varying between 5 nm and 20 nm have been found (not shown).

Discussion

The membrane lipids are responsible for the formation of the unusual 'wafer pattern' in membranes of *Streptomyces hygroscopicus* because the same structures were found in bilayers made from the extracted lipids [8–10]. Furthermore, these periodically curved bilayers preferentially occur in membrane areas without intra-

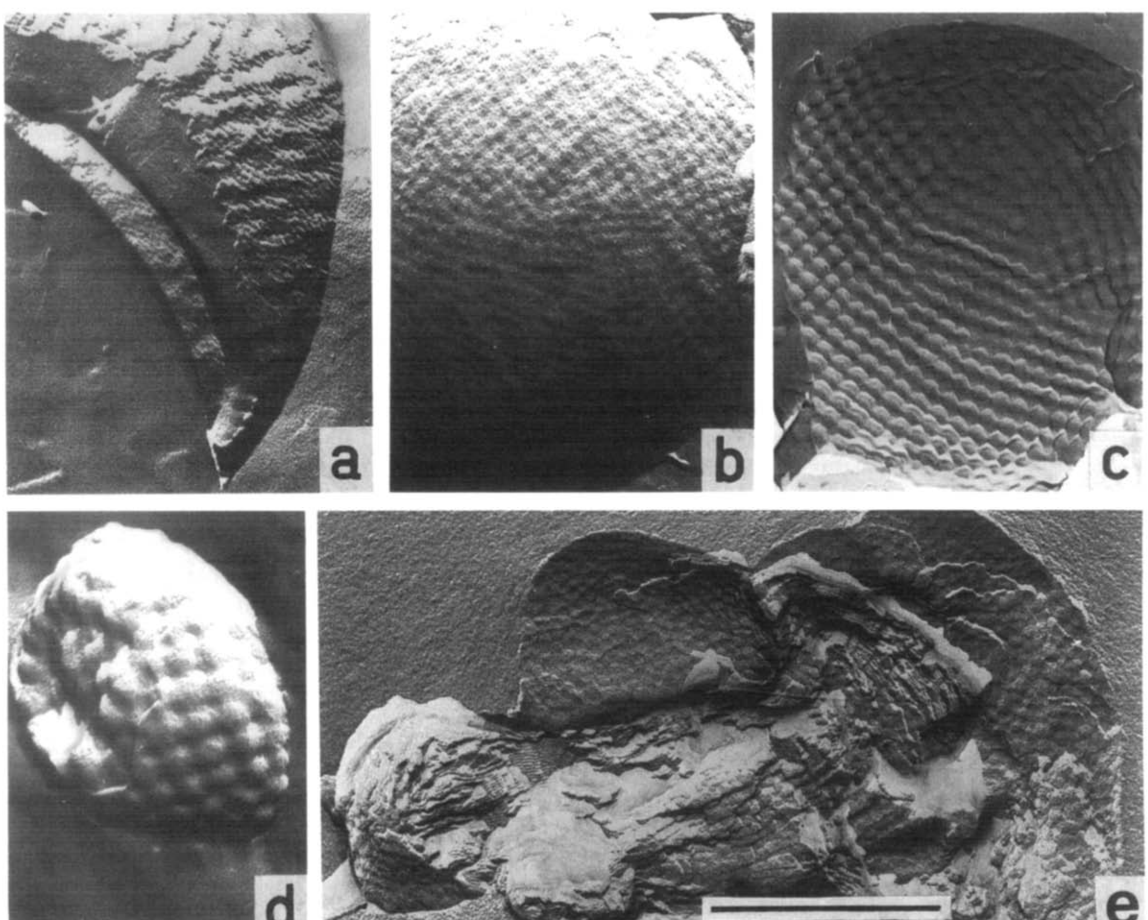

Fig. 4. Liposomes produced from the total lipid extract (a, b, e) or from lipid fractions containing the phospholipids (c, d) of *Streptomyces hygroscopicus*. The pattern can exist in repeat distances of about 15 nm (a), 30 nm (b), 45 nm (c) and 60–75 nm (d). The pattern has the same appearance on concave (c) and on convex (d) faces. In multilamellar bodies (e) the pattern is expressed without intercalated small vesicles.
Bar: 500 nm.

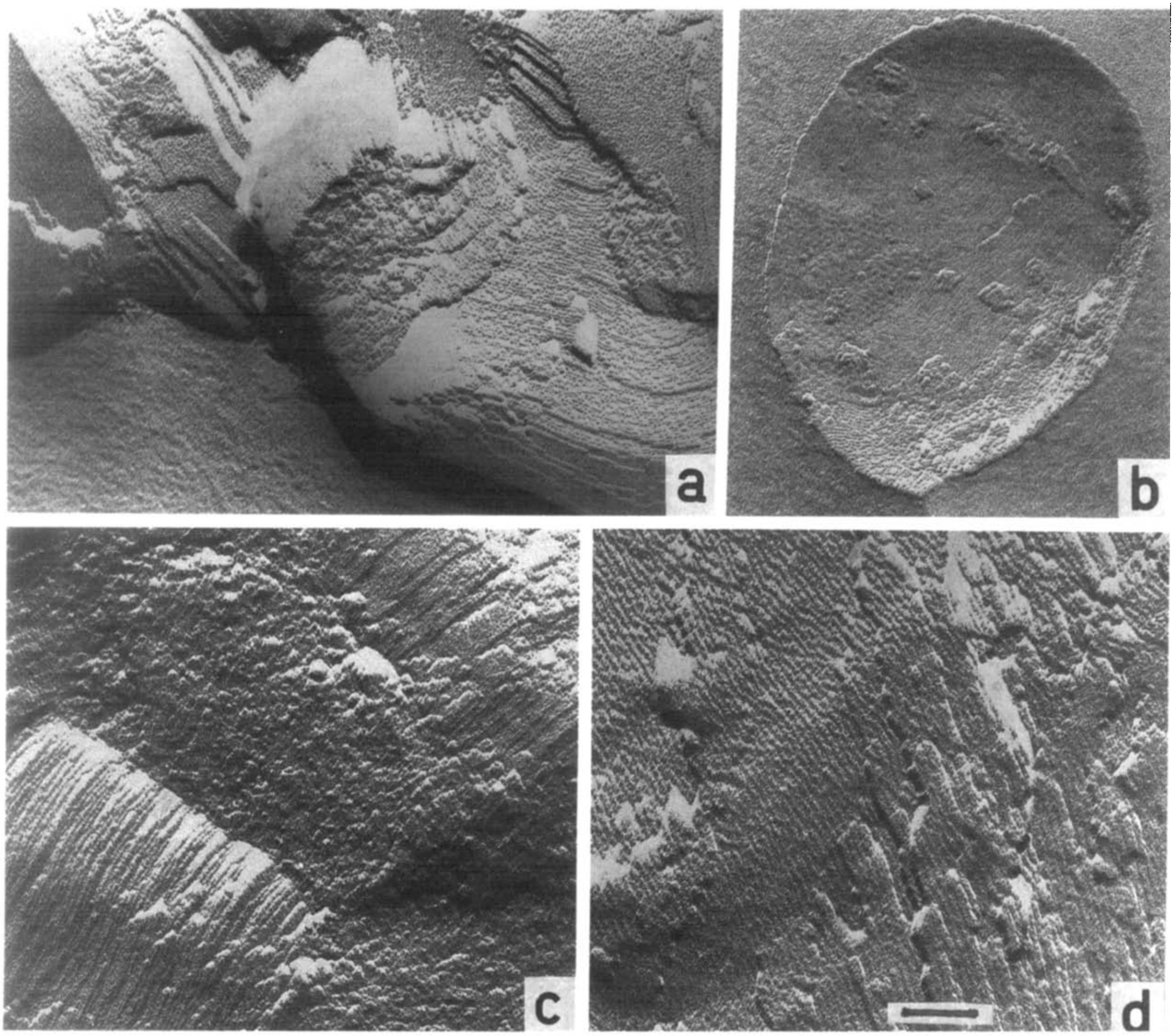

Fig. 5. Nonbilayer textures in lipid bodies formed by the total lipid extract of *Streptomyces hygroscopicus* in citrate buffer pH 3 (a, b), in distilled water (c) and in PBS stored for some days at 4° C (d). The structures obviously representing H_{II}-structures have repeat distances of about 5 nm (a), lower than 5 nm (c) and about 10 nm (d). The regular pattern with a repeat distance of about 12 nm in (b) is seen on the outermost layer of a lipid body and may be caused by a tightly attached nonbilayer structure. Bar: 100 nm.

membranous particles (integral membrane proteins). Obviously a high content of lipid components preferring nonbilayer structures is important for this pattern formation [10]. This conclusion is supported by the observation of nonbilayer textures after appropriate incubation conditions of the lipid mixture (Fig. 5), but other factors, especially the occurrence of branched-chain lipids, should not be neglected.

An accompanying effect is the formation of small vesicles relatively homogeneous in size. This has been one reason to interpret these structures as deformations of the planar membrane caused by underlying vesicles [10]. A more extended analysis of the freeze-fracture observations, however, cannot support this interpretation, particularly in case of the tetragonal pattern. Arguments are the absence or a too low number of underlying vesicles in cases where there is a prominent pattern [11], the worm-like stripes in Fig. 1d formed by the preference of one direction within the pattern and clearly not composed by impressions of underlying vesicles, and the multilamellar structures with an expression of the pattern but without inserted vesicles between the lamellae (Fig. 4e). The occurrence of the tetragonal pattern in different repeat distances is particularly striking. Besides those of about 60 nm, which is in the range of the vesicle size, others with 40–45 nm, 25–30 nm and about 15 nm could be found. This variation requires an interpretation of the periodically curved bilayer pattern without the aid of underlying vesicles.

For such an interpretation aspects of lipid polymorphism and lipid segregation, commonly by phase separation, are of importance, but also an exact image of the pattern is helpful. Because the tetragonal pattern

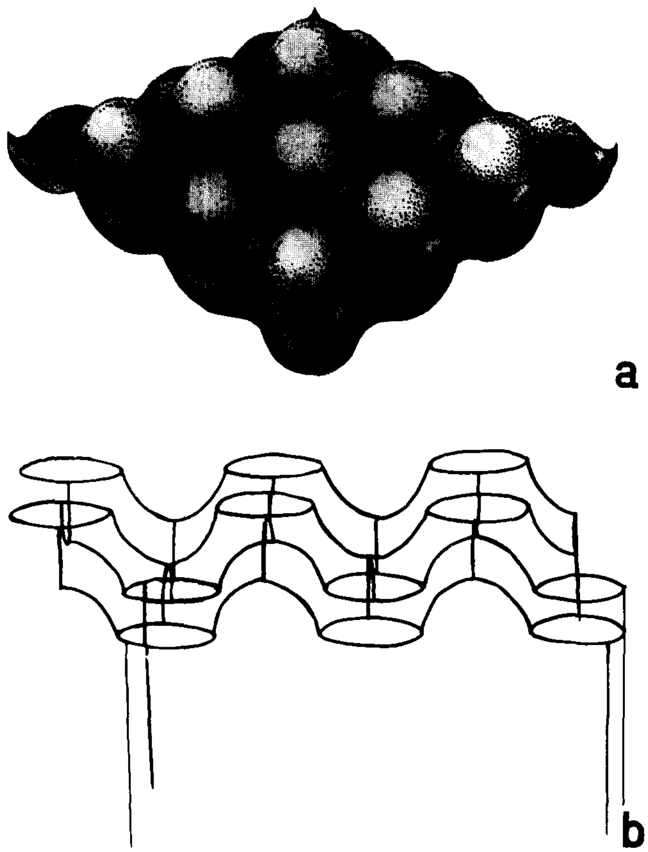

Fig. 6. Model of the observed tetragonal pattern of a periodically curved bilayer (a) and model of a selected plane from a cubic phase structure representing a two-dimensional minimal surface with zero average curvature at all points (b). The model (b) was taken from Andersson et al. (1988) [12].

reveals the same appearance independent of the view on a convex or a concave face (e.g., Figs. 4c and 4d) the regular bilayer deformation results in a structure with alternating convex and concave curvatures (Fig. 6a). A pattern formed only by curvatures in one direction, in this paper presented with a hexagonal arrangement in Fig. 3c, can be caused with more probability by underlying vesicles. However, such a 'one-sided' pattern has also been found in other systems without small vesicles [11].

The tetragonal pattern observed in our investigations can vary in the degree of curvature from nearly not discernible to very pronounced and in the center-to-center spacing in multiples of about 15 nm, but within every pattern the degree of curvature and the repeat distance are constant. Primarily such a regularity seems to reflect more an order of a crystalline structure than of a bilayer in the fluid state. However, the lipids in the nonbilayer phases are also in the fluid state, and these are structures with a high regularity, too. The concerning structures are the inverted hexagonal phase (H_{II}) formed by tightly packed cylinders and the more complicated cubic phases [14]. These inverted phases consist in monolayers with a high curvature where in segments also a bilayer arrangement exists.

Andersson et al. [12] have presented a selected segment of a cubic phase structure with a tetragonal arrangement (Fig. 6b) showing a great similarity with the periodically curved bilayer structure in Fig. 6a. Schematically the curvatures and the tetragonal arrangement of circular holes of the cubic phase segment can be produced from the structure in Fig. 6a by cutting off the caps of the protrusions at both sides. This similarity is of great importance because the selected plane from a cubic phase structure represents a part of a structure belonging to 'infinite periodic minimal surface (IPMS)' structures. An IPMS is an intersection-free surface periodic in three dimensions with an average curvature that is everywhere zero. The selected plane in Fig. 6b as a two-dimensional minimal surface with zero average curvature at all points has been proposed for modelling of membranes [12], and on this basis IPMS structures for the biological membranes have been discussed [4,15–17]. Complementing these assumptions, but on another niveau, the periodically curved bilayer structure observed in our investigations is with high probability such an IPMS structure or closely related to it.

The planar lipid bilayer can be inserted in the IPMS structures as the extreme case of two-dimensional IPMS with infinite curvature everywhere [12]. A phase transition between the planar bilayer and the periodically curved bilayer is energetically inexpensive and will be controlled by specific structural parameters only, and not primarily by the temperature or the water content. The lipid composition, exactly a change which disturbs the equilibrium within the membrane, is obviously the factor responsible for the transition into the periodically curved bilayer.

A phenomenological parameter in lipid polymorphism is the 'monolayer intrinsic curvature' [18,19]. A small radius of monolayer curvature drives to nonbilayer structures (H_{II}, cubic), and a large radius is in accordance with the bilayer arrangement. In mixtures of different lipids the monolayer intrinsic curvature is a colligative property of the system. In biological membranes the lipid composition is adjusted to such a value of intrinsic curvature which is compatible with the functional integrity of the bilayer arrangement [19].

The observed lipid segregation at growth temperature in the plasma membrane of *Streptomyces hygroscopicus* is very probably the result of a discrepancy between the amounts of bilayer and nonbilayer preferring lipids in the membrane. This is supported by the increase of the effect in older cells. The high content of nonbilayer lipids was suggested by the formation of nonbilayer textures in the hydrated extracted lipids by a low pH and/or high temperature. Both factors shift lipids to go from the fluid bilayer phase into a nonbilayer phase

[20]. A too high content in nonbilayer preferring lipids by unbalanced lipid metabolism or by liberation from their interaction with membrane proteins seems to result in a phase transition into the periodically curved bilayer accompanied by a phase separation. A restrictive effect of membrane proteins on membrane curvatures is indicated by both, the occurrence of the curvature pattern solely in areas without or with less particles and by a more weak expression if still some particles are present (e.g., Fig. 3a).

It should be emphasized that the periodically curved bilayer is not a transition stage to the structures of the nonbilayer phases. On the one hand, a mixture of lipids involving such of bacterial origin seems to be a prerequisite, and on the other hand, this structure occurs also at low temperatures where the formation of nonbilayer phases should be suppressed. In our investigations a formation of this pattern was detected not only at or above the growth temperature but also at 4 °C [9,10]. Verkleij, A. and Wilschut, J. (personal communication) have observed a well developed tetragonal pattern at 0 °C using a mixture of egg phosphatidylcholine and bacterial cardiolipin in the presence of Ca^{2+}, and in an earlier investigation such a pattern was seen in case of mixed bacterial lipids even at −10 °C [21]. With calorimetry for the *Streptomyces hygroscopicus* lipids no indication for a phase transition between 4 °C and 70 °C could be detected [10] and the same lack has been reported for the bacterial lipid mixture showing the pattern at −10 °C [21]. Therefore no definite coordination with a fluid or a solid phase state is possible. The proposed mechanism for the formation of regular convex and concave curvatures by a molecular rearrangement into a IPMS structure is a transition of second order without a direct dependence from the temperature. A more detailed analysis of the involved factors, however, is a possible field for further studies.

The formation of the periodically curved bilayer structure is a possible mechanism or driving force also behind other curvature formations in biological and artificial membranes [11]. It might be that also single local curvatures, e.g. in small particle-free areas like in Fig. 3d, can be caused by a similar mechanism. The formation of the small vesicles of uniform size, existing parallel to the periodically curved bilayers [9,10] seems to be a final result of the mechanism responsible for periodically curved structures. Freeze-fracture observations supporting this conclusion will be presented in a separate paper.

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