Biochimica et Biophysica Acta 898 (1987) 223–230
Elsevier

BBA 73508

Electron microscopic and biophysical studies of liposome membrane structures to characterize similar features of the membranes of *Streptomyces hygroscopicus*

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(Received 10 June 1986)
(Revised manuscript received 4 November 1986)

Key words: Membrane structure; Liposome; Multilamellar liposome; Freeze-fracture electron microscopy; NMR, ^{31}P; Differential scanning calorimetry; (*S. hygroscopicus*)

To characterize the novel non-planar plasma membrane structure of bacteria (wafer structure), liposome membranes from the bacterial lipid mixture and individual lipid fractions were prepared and investigated by freeze-fracture electron microscopy, microcalorimetry and ^{31}P-NMR spectroscopy. The phospholipid content of the membranes is essential for the formation of the non-planar membrane structure and there is no indication that the formation of the structure is connected with temperature-induced lipid phase transition processes. An exaggerated form of the wafer structure (raspberry structure) is also visible and additionally, in both cases, many small spherical vesicles are observed. We suggest that both membrane features of the liposomal and bacterial membranes are induced by these vesicles, forming a hexagonal or cubic organization of vesicles on the cytoplasmic surface of the biological membrane, and in between the multilamellae in the artificial membranes.

Introduction

Many Gram-positive bacteria can be induced to form protoplasts and protoplast type L-forms devoid of cell wall structure. Protoplasts are usually unable to propagate and their cytoplasmic membrane corresponds to a great extent with the cytoplasmic membrane of normal vegetative cells. Stable protoplast type L-form cells, however, can grow and multiply, but the structural and physiological properties of their cytoplasmic membrane are altered.

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In *Streptomyces hygroscopicus,* a producer of the macrolid antibiotic Turimycin, the cytoplasmic membrane contains glycolipids (20%), neutral lipids (38%) and phospholipids (42%). Cardiolipin (40–45%) and phosphatidylethanolamine (35–40%) are the main components of the phospholipid fraction. The L-form membranes show similar composition with slightly higher phospholipid content (48%). For both membrane types the acyl chains of these lipids are branched (69–78%) and mainly anteiso $C_{15:0}$ and $C_{17:0}$ [1].

In view of the rather stable lipid bilayer of the L-form cells of *S. hygroscopicus,* and the predominance of phospholipids which are known to form polymorphic structures other than bilayers [2], it was of interest to study the membranes of the

normal bacteria and the protoplast type L-form in some detail.

Initial evidence that the cell membrane of such an organism display unusual structures has come from freeze-etch electron microscopy of the L-form membranes on which globular units of 75–80 nm were observed [1]. Uneven features, normally wave-like, have also been observed in membranes of other microorganisms in a few cases only, e.g. *Fusarium oxysporum* [3], *Candida parapsilosis* [4], *Streptomyces coelicolor* [5] and *Bacillus subtilis* [6].

Here we report the coexistence and close packing of vesicles together with a distorted extended lamellar bilayer, in both normal *S. hygroscopicus* and in their L-form cells. Two structural complexes are observed and termed 'wafer' and 'raspberry', depending upon the degree of lamellar distortion by spherical particles, which are either 30–40 nm or 55–65 nm in diameter. Since similar structures are observed in aqueous dispersions of total lipid extract from the normal cell- and L-form membranes they are thought to be induced by the particular lipid composition of these bilayers [7].

Materials and Methods

Cultivation and growth characteristics of *Streptomyces hygroscopicus* NG 33-354, and preparation of stable L-form protoplasts has been described previously [7,8]. The purified and lyophilized membranes were extracted with chloroform/methanol (2 : 1, v/v) to obtain the total lipids, which were fractionated into the neutral, glycolipid and phospholipid fractions by Silica gel 40 (Merck) column chromatography.

Multilamellar liposomes were prepared from extracted lipids as described previously [7]. These liposomes were quenched for freeze-fracture electron microscopy after incubation for 30 min at the required temperature.

To investigate the effect of pH on the dispersions, samples were made and examined at pH 1.3, 7.2 and 10, using different buffered media (citrate, borate, and phosphate-buffered saline). Electron microscopic examination (see Results) of all lipid dispersions gave similar results after more than one week at 4 °C indicative of rather stable structures.

The samples were quenched using the sandwich technique, using liquid propane. The specimens were fractured and shadowed in a Balzers BAF 400 D freeze-fracture device at −150 °C. The cleaned replicas were examined in a Tesla BS 500 or Jeol JEM 100 B electron microscope.

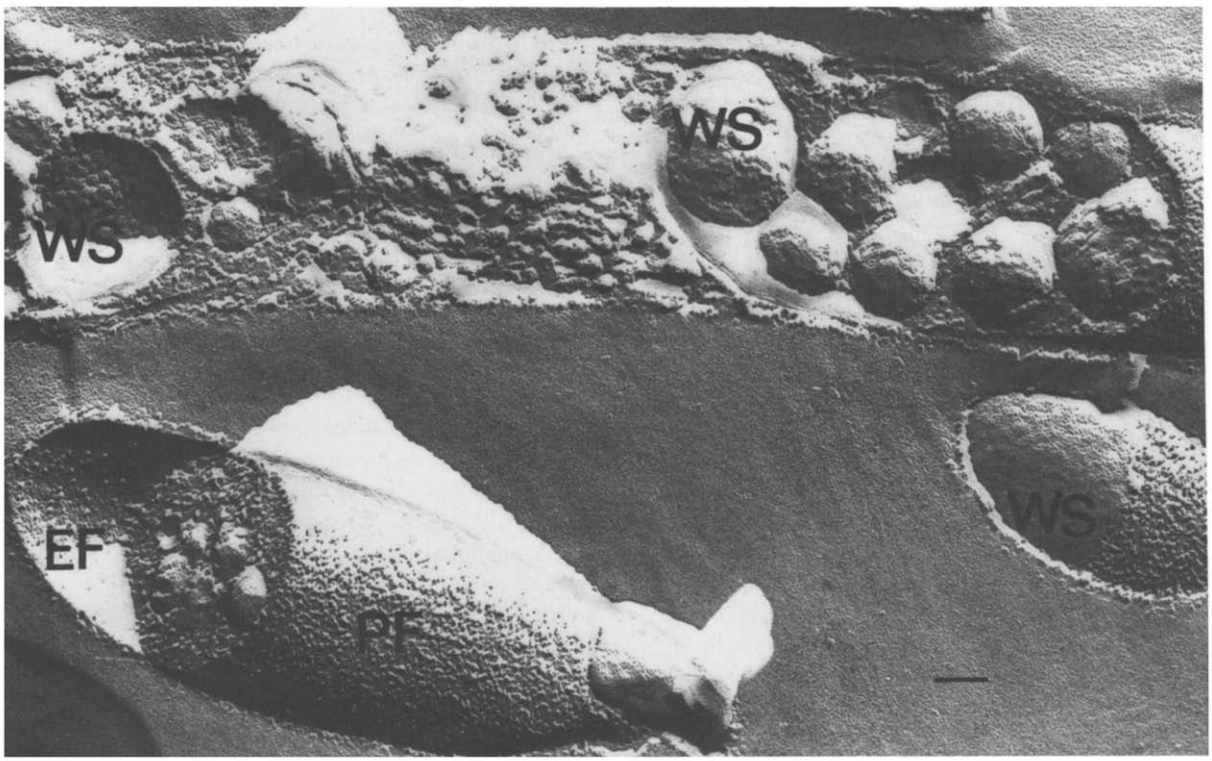
Multilamellar liposomes of the phospholipid fraction (52.2 mg lipid/ml; citrate buffer; pH 1.3; prepared with deuterated solutes) were investigated by DSC using a Perkin-Elmer DSC-2 calorimeter (scan rate 2 Cdeg/min, at maximum sensitivity of 0.1 mcal/s; sample volume approx. 25 μl) and by ^{31}P-NMR spectroscopy using a Bruker HX-90 spectrometer equipped for strong broad-band proton decoupling.

Results

Cytoplasmic membranes of the bacterial strain *Streptomyces hygroscopicus* display a nonplanar membrane structure in their normal (Fig. 1) and more noticeably in their viable and stable L-form cells (Fig. 2). These latter contain somewhat more phospholipid compared to the membranes of the normal form. We suggest that this membrane feature is a 'wafer'-like structure from its regular pattern of bulges with diameters of 30–40 nm. In some cases, in both normal and L-form cells, this structure is visible in an exaggerated form, called 'raspberry'-like structure, with bulges of 55–65 nm (Fig. 3a (RS)) (see Refs. 1 and 7).

Multilamellar liposomes were prepared in three different ways (i) from the total lipid extract, (ii) from the individual lipid fractions and (iii) from the reconstituted total lipid extract (isolated subfractions being combined in the same proportions as in the biological membranes) of the purified membranes of the L-form cells to simulate the unusual structures observed in the membranes of the normal bacteria and of their L-form cells. Among the different lipid fractions only phospholipids are able to form multilamellar liposomes. The glycolipid and neutral lipid fractions form only some small vesicles with smooth fracture faces.

The wafer structure is now observed if the dispersions contain the phospholipid fraction with (Fig. 3a) or without (Fig. 3b,c,d) other lipids, indicating that wafer structure formation is prim-



Figs. 1–3. All freeze-fracture electron micrographs are oriented with shadow direction from bottom to top. The bar always represents 100 nm.

Fig. 1. Normal cells of *Streptomyces hygroscopicus*, cultivated for 2 days at 28° C [8]. Incubation temperature before quenching, 23° C. Freeze-fracture representation with wafer structure (WS) on an area of the cytoplasmic membrane and on some membranes of intracellular granules. Protoplasmic fracture face (PF) of the cytoplasmic membrane with high density of integral protein particles and exoplasmic fracture face (EF) with less protein particles.

arily connected with the phospholipid content of the membranes [7].

In Fig. 3b the wafer-like structure (WS) of the planar membrane appears to enclose many vesicles which form the indentations of this planar membrane as shown more clearly in the cross-fracture boundary of Fig. 3c. Beneath each indentation is observed an associated vesicle marked by an arrow (Fig. 3b,c). In some regions, however, no vesicles are observed under the bulges, which may result from fracture between rows of vesicles.

In multilayered dispersions, impressions of vesicles can be seen as craters on the outer surface of an opposing inner bilayer (Fig. 3d), which are marked by a circle. Such impressions are observed inbetween the bulges from vesicles on the under side of the bilayer resulting in close regular packing in this system (see Discussion).

The phospholipid liposomes reveal the wafer structure at physiological pH (phosphate-buffered saline; pH 7.2) but it is not so pronounced as in the case of the total extractable lipids or the combined lipid fractions [7]. Investigations of the pH dependence of the structure formation in the model phospholipid membranes revealed an exaggerating effect of acid (Fig. 3b,c,d) or alkaline (not shown) conditions. Similarly, in the acid or alkaline buffered media, the structures are more pronounced than in the liposomal membranes of the total lipid mixtures at physiological pH.

In coexistence with the wafer structure, raspberry-like structures are visible in both the liposomal membranes (phospholipid, combined bacterial lipid fractions and total extract) and the L-

Fig. 2. Freeze-fracture electromicrograph of a *Streptomyces hygroscopicus* L-form cell membrane with wafer structure (WS). Incubation temperature before quenching, 23° C. Bacteria were cultivated for 9 days at 28° C [8].

form membranes [7]. Both structures are often observed together in the same sample, or even in the same liposome (Fig. 3a) or L-form cell [1]. It appears that there is a similarity between both structures, which, however, are quite distinct. However, it is important to note that no intermediate structures were observed in any system studied.

Higher incubation temperatures (between 50 and 70° C) of the lipid dispersion before quenching favour the raspberry structure formation, which dislays less regular packing than the wafer structure [7].

Calorimetry studies (traces not shown) on the liposome suspensions made of the total extractable lipids, or of the phospholipid fraction of the L-form membranes, show no detectable enthalpy changes in either the heating or the cooling experiments between 4 and 70° C.

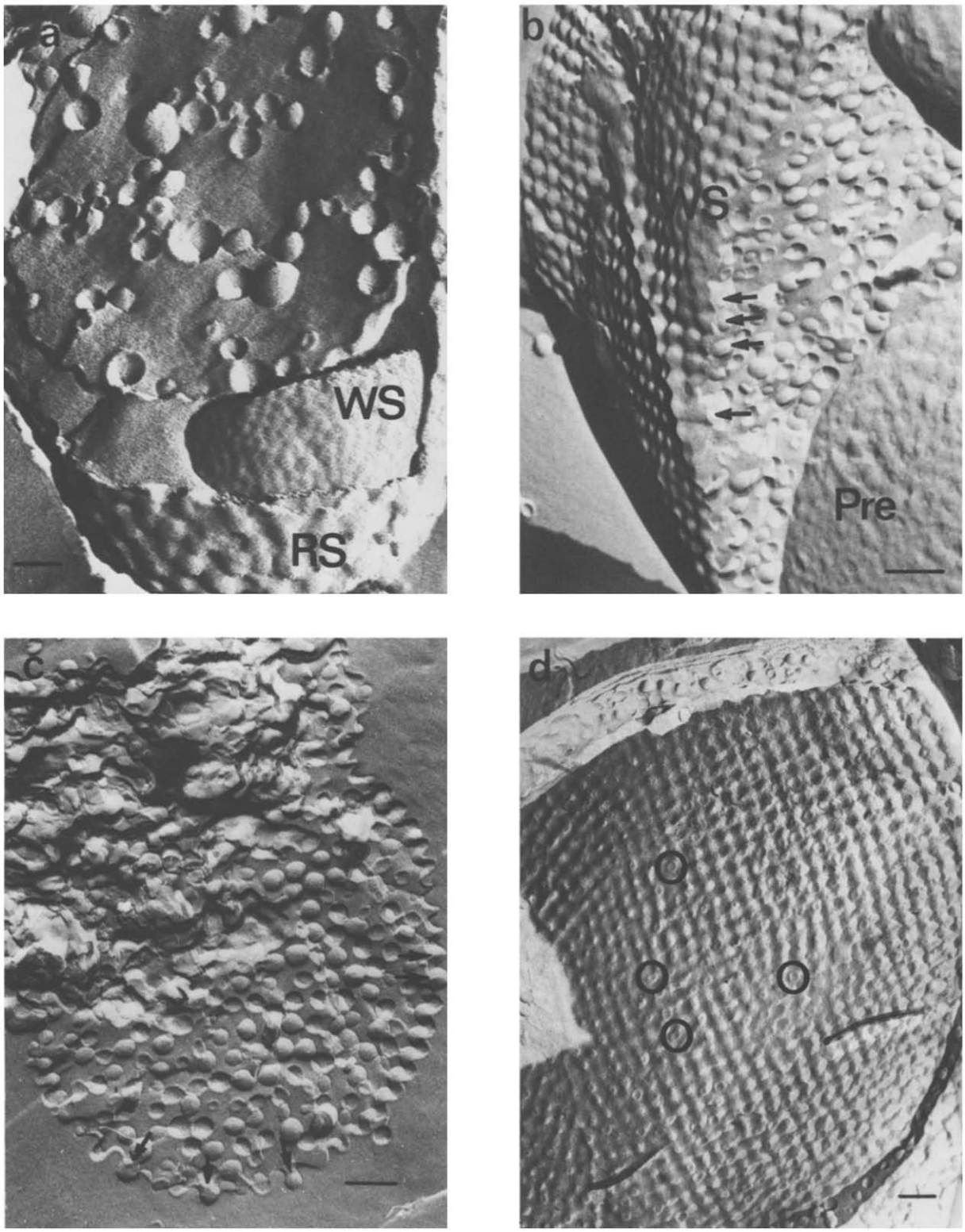
^{31}P-NMR examination of liposome suspensions, prepared from the phospholipid fraction of the L-form membranes, showed a spectrum with a single, relatively narrow and near symmetrical peak (Fig. 4), which is typical for relatively small, isotropically tumbling structures, and not for large extended multilayered liposome dispersions [9,10].

Discussion

Formation of wafer and raspberry structures in the limiting bilayer of normal *Streptomyces hygroscopicus* cells, their L-form cells and in dispersions of their extracted lipids, appear to be determined by the phospholipid content of the bilayer, which on average is branched chain cardiolipin and phosphatidylethanolamine, which are 40–45% and 35–40% of total phospholipid respectively. In the model membranes, the structures are present in only phospholipid containing dispersions. In addition, the structures are more frequent and exaggerated in L-form membranes than in the membranes of normal cells, the former having a higher phospholipid content (48%) than the later (42%) [1].

The absence of any detectable thermal transitions from the calorimetric studies, as well as the

Fig. 3. (a) Freeze-fracture representation of liposomes, made of the reconstituted total lipid extract of the *Streptomyces hygroscopicus* L-form membranes. Raspberry structure (RS, outer membrane) and wafer structure (WS, inner membrane) in coexistence together and with many vesicles in the same multilamellar liposome. Incubation temperature, 33° C (pH 7.2). (b) Freeze-fracture micrograph of a multilamellar liposome prepared from the phospholipid fraction of the purified *S. hygroscopicus* L-form membranes under acid conditions. Incubation temperature, 4° C. Cross fracture of a part of this liposome with wafer structure (WS) displays a bilayer membrane area and many vesicles, some of them (marked by an arrow) are situated directly under the bulges. Preforms (Pre) of the wafer structure have less vesicles at the inner liposome membrane. (c) Cross fracture of a liposome prepared as for (b) showing many vesicles bounded by the bulged liposome membrane. Some of the vesicles (marked by an arrow) are situated directly under the bulges. (d) Liposome membranes, formed from the phospholipid fraction of the bacterial L-form membranes as for (b). Wafer structure and vesicle impressions in the gaps between the pattern rows at the inner liposome membrane can be seen. Some of the vesicle impressions are marked by a circle.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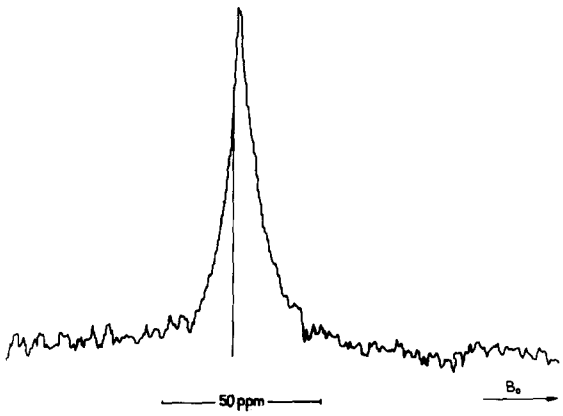

Fig. 4. 36.4 MHz ^{31}P-NMR spectrum from an aqueous dispersion of liposomes prepared from the phospholipid fraction of the *Streptomyces hygroscopicus* L-form membranes (52.2 mg lipid/ml; deuterated citrate buffer; acid pH). The spectrum was obtained at 26 °C from 25000 transients in the presence of high power proton noise decoupling, using a 60°-pulse and 2 K data points. The mark indicates the position of the reference ^{31}P-NMR signal of H_3PO_4 (85% v/v).

similarity of the structures observed in micrographs from samples quenched from various temperatures, strongly indicates that the structures are well defined and coexist as stable entities. No indication of non-lamellar phases were observed under any conditions described or of lipid lateral phase separation. This not preclude, however, the possibility that the different structural components, bilayer vesicles and extended bilayers, are composed of the same lipid composition or their relative amounts.

There are some aspects which suggest that the wafer and the raspberry structures are derivative of the same mechanism for their formation. The two structures are seen in the same phospholipid dispersion and the same liposomal membrane (Fig. 3a). The particular lipid composition of the membranes is primarily of two phospholipids, which are wedge-shaped (branched chains and small head-groups) in their molecular geometry [2]. Marked molecular asymmetry of the bilayers can therefore occur [11] favouring both vesicle and indentation formation (Fig. 5). Only the average lipid composition is known for these membranes and not for the individual components. It is therefore not possible to deduce precise details of this molecular organization. However, such a high degree of molecular asymmetry in the vesicles would drive a corresponding asymmetry in the overlying extended bilayer thereby stabilizing the whole macromolecular complex (Fig. 5).

The gross packing of vesicles is seen to be rectangular for multilayered model membranes (Fig. 3a,b,d) and hexagonal for vesicles on a single extended biological bilayer, such as the viable bacterial (Fig. 1) and L-form cells (Fig. 2). The reason for this is related to the way vesicles can pack in a hexagonal fashion (Fig. 6a) when unre-

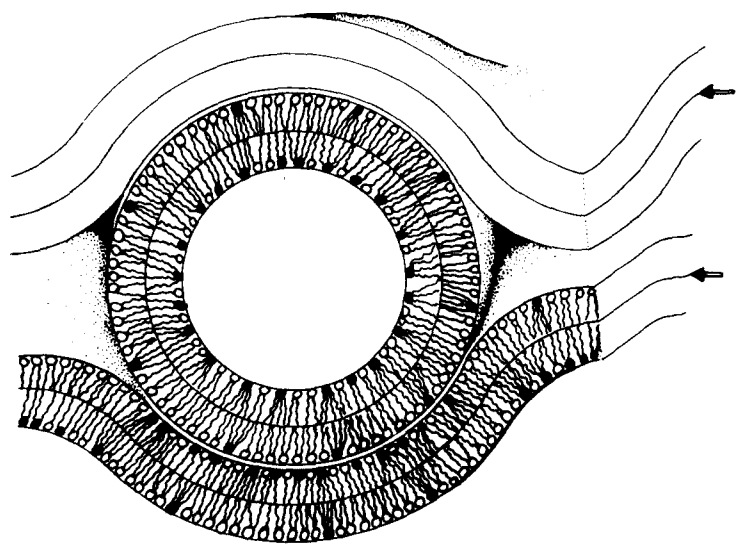

Fig. 5. Schematic representation of the vesicle structure and the formation of the bulges. This vesicle-membrane arrangement is energetically stabilized by accumulation of wedge-shaped phospholipid molecules as cardiolipin and phosphatidylethanolamine with branched acyl acids (represented by phospholipid symbols with full circles) in highly curved inner shells of vesicles and bulges (arrows: fracture faces in the membranes of the bulges, but the fracture face may also run along between both halfs of the vesicle membranes).

strained on a single bilayer (Fig. 6b), but when restrained between two bilayers (Fig. 6d) in which case they become cubic in packing (Fig. 6c). The cubic packing is clearly seen in micrographs of multibilayered lipid dispersions (Fig. 3b,d). It therefore appears that hexagonal packing is not possible in multilayered systems.

The proton decoupled ^{31}P-NMR spectrum of liposomes made from the phospholipid-fraction of the L-form membranes (Fig. 4), provides a strong indication that most of the phospholipid molecules (80%) are localized in curved membranes. As shown previously [9,10] small bilayer vesicles give rise to a narrow, symmetrical spectrum due to averaging through isotropic motion of the ^{31}P chemical shift anisotropy. The width of the line is related to the particle sizes which in the example shown here, have diameters of less than 100 nm [10]. Thus, both bulges and vesicles are likely to be of rather similar average diameter of curvature giving rise to the experimental observed spectrum due to fast lateral diffusion of molecules around both such curved structures [9].

In some electron micrographs, however, no vesicles are observed under the bulges (Fig. 3b,c), which may result from fracture between rows of vesicles. It may also, however, be taken as an indication for another explanation of the wafer and raspberry structures, namely that the formation of bulges is a cooperative membrane process, induced by the distinct distribution of certain lipid components within the membrane. The molecular basis of such a highly periodic and regular undulation remains, however, unknown. There is no reason for structure formation in such a highly fluid multicomponent bacterial or liposomal membrane where no phase transition processes of lipids occurs. In this type of membranes with a lateral diffusion of lipid molecules of $D \approx 10^{-12}$ m^2/s, a spontaneous aggregation of molecules (for instance in a bulge) would decay immediately.

The observed unusual membrane structures must have their origin in profound changes in the lipid organization of the bacterial and liposomal membranes. It is possible that the specific structures reported here are similar to those formed during vesicle formation processes in some bio-

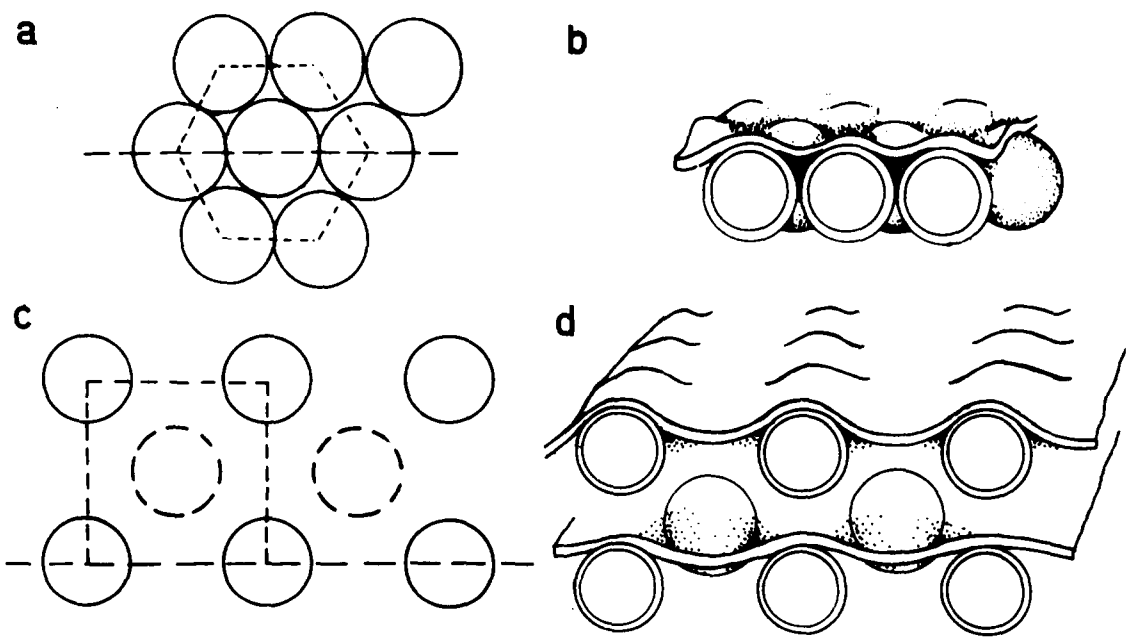

Fig. 6. (a and c) Pattern arrangement of the wafer and raspberry structures in a single bilayered biological (a) and multilayered artificial (c) membranes in top view; small hatching lines show the hexagonal (a) and the cubic (c) pattern arrangement; larger hatching lines represent the cut planes for the front views of (b and d). (b and d) Schematic illustration of the vesicle packing arrangement under the unilamellar biological membranes (b) and within multilamellar liposomes (d) in front view.

logical systems, for instance, the formation of secretory granules, lysosomes or synaptosomes.

Acknowledgements

We wish to thank Dr. H.W. Meyer for the freeze-fracture micrograph of Fig. 2 and for many critical discussions and Dr. H.-J. Flammersheim for performing the calorimetric measurements. We thank Dr. A. Watts for reading the manuscript.

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