Biochimica et Biophysica Acta, 685 (1982) 297–306
Elsevier Biomedical Press

BBA 71071

BILAYER AND NON-BILAYER TRANSFORMATIONS IN AQUEOUS DISPERSIONS OF MIXED *sn*-3-GALACTOSYLDIACYLGLYCEROLS ISOLATED FROM CHLOROPLASTS

A FREEZE-FRACTURE STUDY

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(Received July 14th, 1981)

Key words: Non-bilayer structure; Model membrane; Galactosyldiacylglycerol; Freeze-fracture; (Chloroplast)

A number of different particle and 'particle-like' structures are observed in freeze-fracture replicas prepared from aqueous dispersions of mixtures of mono- and digalactosyldiacylglycerol. The smallest of these structures (10–12 nm in diameter) corresponding to inverted lipid micelles sandwiched within lipid bilayers are often organised into extensive planar arrays. A number of larger 'particle-like' features are also observed in replicas of this type. An analysis of the relationship between these structures suggests that they reflect responses to stresses associated with a temperature-dependent incorporation of the lipids of the inverted micelles into the lamellar structure.

Introduction

The existence of 'lipidic' particles in freeze-fracture replicas prepared from aqueous dispersions of cardiolipin/phosphatidylcholine mixtures containing Ca^{2+}, and in cardiolipin dispersions treated with Ca^{2+} or Mg^{2+}, were reported first by Verkleij et al. [1] and Vail and Stollery [2]. Subsequently, a number of studies have shown 'particle-like' structures in replicas prepared from dispersions of different phospholipid[3–8] and galactolipid mixtures [9,10]. In general these structures are observed only in lipid mixtures containing at least one component which normally assumes an hexagonal-II configuration when dispersed in water; the other component(s) forming a lamellar phase under such conditions. The molecular configuration associated with particle formation, however, is still a matter of controversy [8,12].

De Kruijff and Verkleij and their collaborators [3] have, on the basis of evidence obtained from freeze-fracture and ^{31}P-NMR studies, proposed a number of possible molecular arrangements to account for the existence of such particles. Of these they favour most an organisation involving the formation of inverted micelles sandwiched between the leaflets of a lipid bilayer. The particles seen in replicas prepared from Ca^{2+}/cardiolipin/phosphatidylcholine mixtures are interpreted as reflections of inverted micelles formed at the fusion interface between adjacent bilayers [1,4]. This model, which recently has been extended by Cullis et al. [11], envisages the particles as reflections of inverted micelles, or rows of micelles, formed at the nexus of two interconnecting bilayers. Miller [6] and, more recently, Hui and Stewart [12] have proposed an alternative model, sometimes referred to as the intermembrane attachment site model, in which the particles are thought to represent conical attachment points between two adjacent bilayers.

In an earlier paper [10], we have shown that the

10–12-nm particles seen in freeze-fracture replicas prepared from sonicated dispersions of mono- and digalactosyl diacylglycrols correspond to inverted micelle structures of the type suggested by Verkleij and his co-workers. These two lipids when dispersed alone in water form hexagonal II and lamellar structures, respectively [13,14] and thus fit into the general pattern previously observed for particle-containing phospholipid mixtures. We have now examined this galactolipid mixture in more detail and find that several other 'particle-like' features occur in such mixtures. It is concluded that these latter features do not necessarily represent structures that exist under equilibrium conditions but reflect responses to the stresses associated with a temperature-dependent incorporation of the lipids forming the inverted micelles into the lamellar structure.

Materials and methods

Monogalactosyldiacylglycerol and digalactosyldiacylglycerol were isolated from fresh leaf tissue of 4–5 week post-emergent bean plants (*Vicia faba*, L., var. Express) as described previously [13]. Mixtures of galactolipids were prepared in solvent which subsequently was removed; the preparation was stored under vacuum dessication to remove any traces of solvent. The dry lipid mixture was then dispersed in oxygen-free water or salt solution by ultrasonic irradiation. No cryoprotectants were added to the samples. The dispersed lipids were quenched thermally from about 20°C in a slurry of nitrogen and the frozen specimens fractured at −115°C in a Polaron freeze-fracture device. Platinum-carbon replicas were prepared, washed with solvent, and examined in a Philips EM 301 electron microscope.

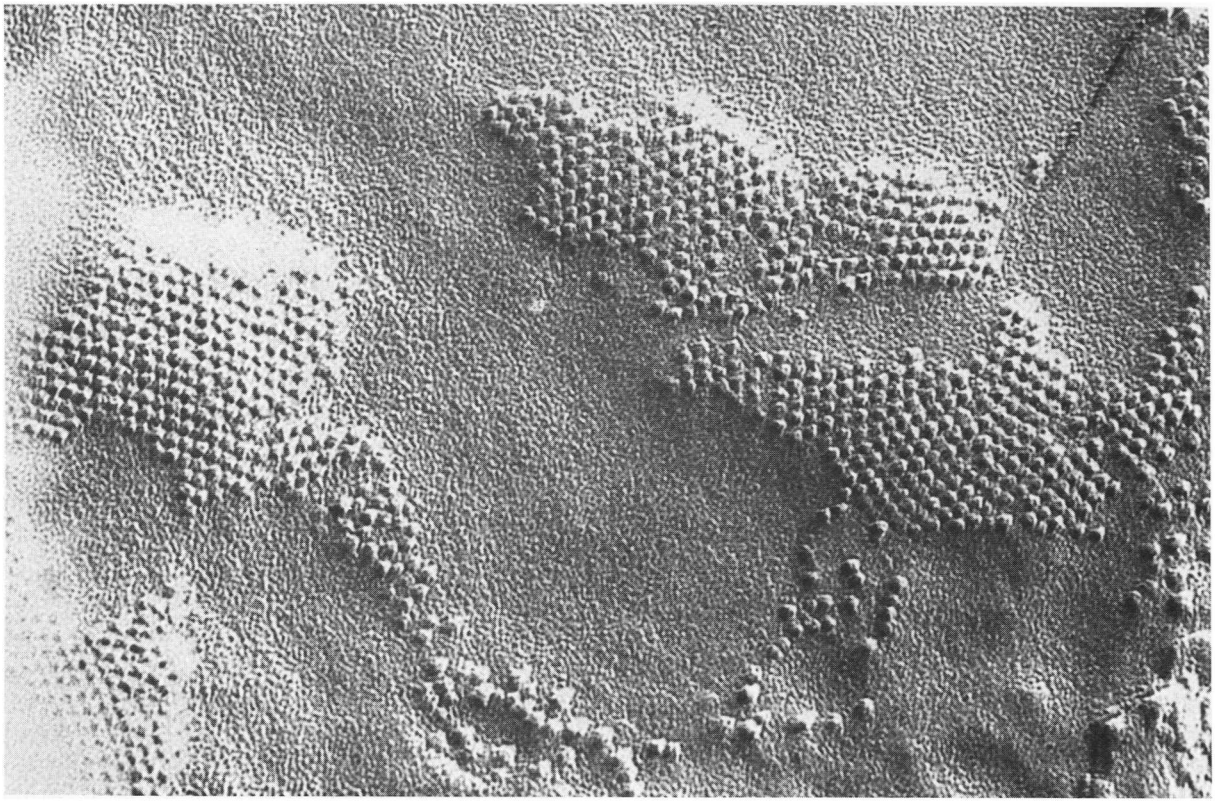

Fig. 1. Electronmicrograph of a freeze-fracture replica prepared from a 2:1 mole ratio mixture of mono- and digalactosyldiacylglycerols ultrasonically dispersed in 10 mM $CaCl_2$, showing extensive arrays of the smaller diameter particles (×212500).

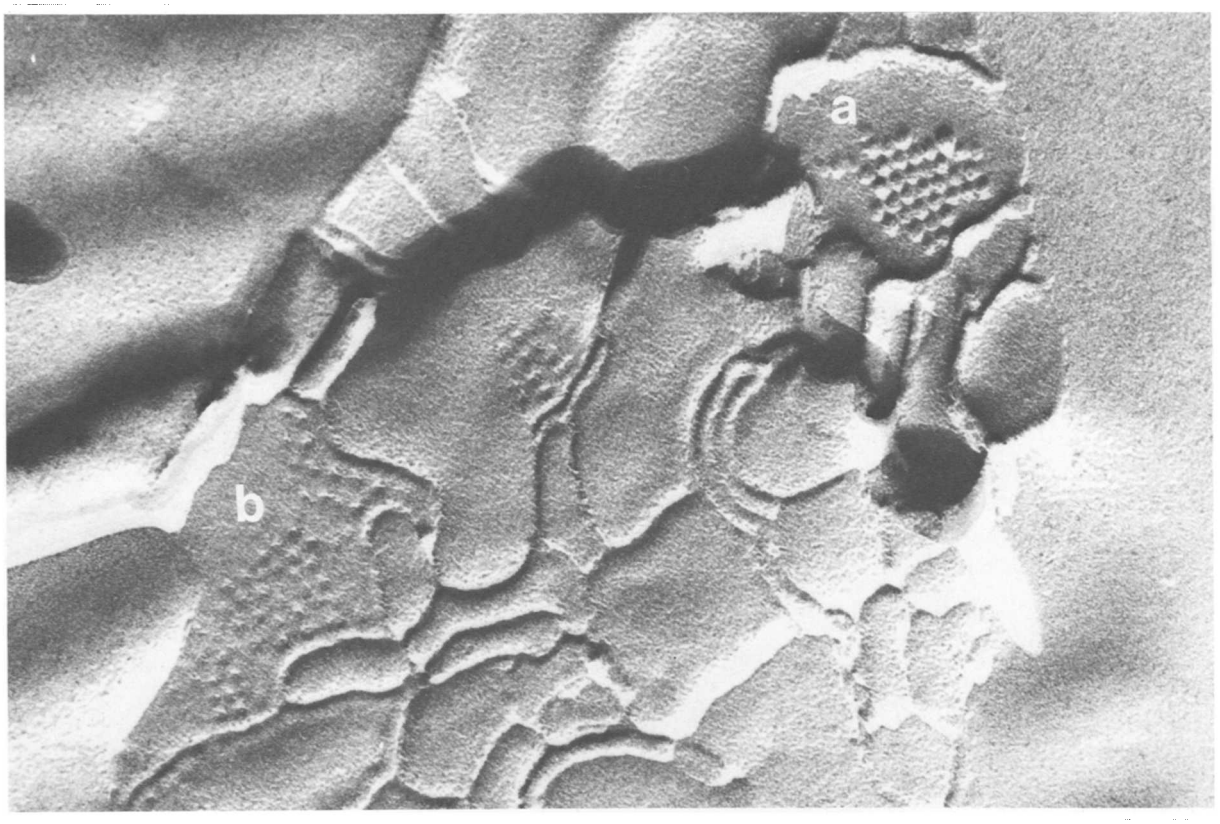

Fig. 2. Electronmicrograph of a replica obtained from a freeze-fractured sample of a sonicated aqueous dispersion of a 2 : 1 mole ratio mixture of mono- and digalactosyldiacylglycerols showing arrays of particles (a) and arrays of pits (b) within a lamellar structure. A system of ridges dividing the fracture face into small smooth areas can also be observed (×212500).

Results

Aqueous dispersions of 2 : 1 mole ratio mixtures of mono- and digalactosyldiacylglycerols have been examined by freeze-fracture electron microscopy and several well-defined structures have been observed. These structures differ in their size, shape and organisation. By careful examination of a large number of replicas prepared under similar conditions, we have attempted to explain the origin of these features and to suggest likely molecular arrangements that may explain their existence.

A consistent feature in these replicas is the presence of small particles sandwiched within bilayers of a lamellar phase. The diameter of these particles appears to depend, to some extent at least, on the ionic strength of the suspending medium. Preparations dispersed in water contain particles which range in diameter between 10 and 12 nm but if the lipids are suspended in electrolyte solutions (100 mM monovalent or 10 mM divalent cations) the average particle diameter reduces to 8–10 nm. Fig. 1 shows a region of a replica prepared from a dispersion of the galactolipids in 10 mM $CaCl_2$ in which a large number of the small-diameter particles can be seen ordered into close-packed arrays in the lamellar structure. Arrays of particles such as these are often observed but pits corresponding to the presence of particles on the complementary fracture face are seen only rarely. Fig. 2, however, shows a region of a replica in which an array of particles is present together with arrays of pits which presumably arise from such particles. The pits are much more difficult to distinguish than the particles, suggesting that they are very shallow. Their apparent rarity probably

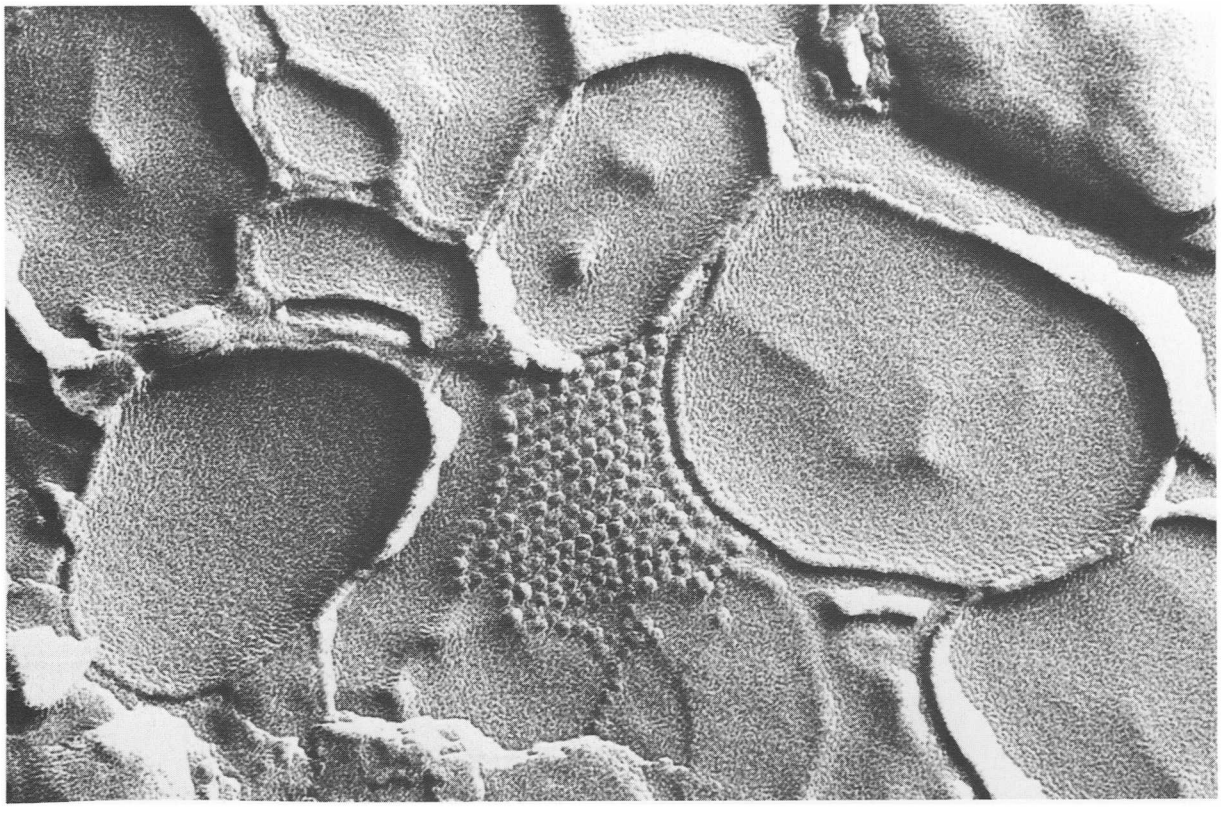

Fig. 3. Electronmicrograph of a replica obtained from a freeze-fractured sample of a sonicated aqueous dispersion of a 2 : 1 mole ratio mixture of mono- and digalactosyldiacylglycerols showing typical examples of planar arrays of the smaller diameter particles and of the tubular structures often found in association with such particles. Conical deformations in the underlying lipid can be seen in the centre of some regions bounded by the tubular ridges (×212500).

reflects the fact that they are difficult to replicate. We have reported previously the existence of particles of this type in aqueous dispersions of mixtures of galactolipids [9,10] and have shown that they correspond to inverted micelles sandwiched within the bilayer [10].

Another feature in Fig. 2 is the presence of large numbers of interconnecting ridges delineating small areas of smooth fracture face. This arrangement is illustrated again in Fig. 3 in which the juxtaposition of ridges and particles suggests that the ridges are probably tubular micelles formed by the lateral fusion of strings of 10–12-nm particles. Another feature that appears to be associated with the disappearance of these particles is the formation of conical deformations in the lamellar phase. Examples of these can also be seen in Fig. 3 where a number of such deformations are visible in the centres of the smooth areas of fracture face bordered by the tubular structures.

Other areas of the replicas show regions where these latter deformations appear to have migrated to form steeply contoured ridges involving many thicknesses of bilayer. Under these conditions, the tubular structures appear to break down to form linear arrays of particle-like structures following the crests of the ridges. The relationship between these various structures is clearly illustrated in Fig. 4 which shows examples of the small 10–12-nm particles, the conical deformations, the tubular structures in various stages of disruption and the larger particles associated with the ridges in the fracture face.

An example of a more advanced stage in this process is shown in Fig. 5. All traces of the small 10–12-nm diameter particles have disappeared and

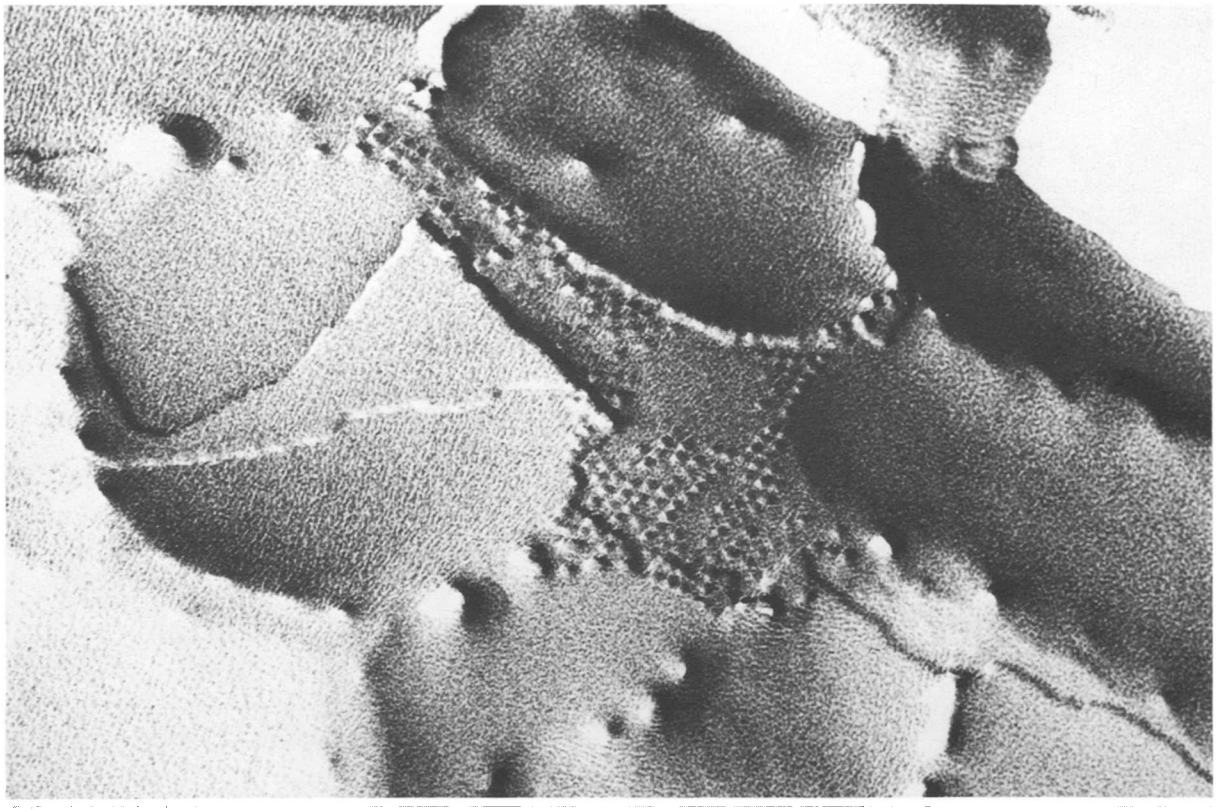

Fig. 4. Electronmicrograph of a freeze-fracture replica prepared from a sonicated dispersion of a 2 : 1 mole ratio mixture of mono- and digalactosyldiacylglycerols suspended in 0.1 M NaCl. Examples of arrays of the small 10–12-nm diameter particles, the tubular micelles formed from the fusion of such particles and the conical deformations are shown in the central and upper parts of the electron micrograph. In other regions the deformations appear to have migrated to form much more pronounced ridges which are capped by the remnants of disrupted micelles (×212500).

the remains of the tubular structures have been replaced by rows of 10–20-nm diameter particle-like structures. A complementary view of this organisation, taken from a different region of the same replica, is shown in Fig. 6 in which pits, corresponding to the particle-like structures seen in Fig. 5, are seen to follow apparent depressions in the fracture face. In our experience, particles of this type are always found to be associated with ridges, and pits with depressions, in the fracture face. In most cases, any one group of such features contains only particles or only pits. It is comparatively rare to find particles and pits in close association with each other. An example of such an association is shown in Fig. 7. It originates from a 1 : 1 mole ratio mixture rather than the 2 : 1 ratio employed in most of our experiments and the lipid organisation is clearly different to that shown in Figs. 5 and 6. The fracture face is extremely buckled, as evidenced by the long shadows associated with the particles lining the ridges. However, despite these differences it is quite clear that the general rule that particles are associated with ridges and pits with depressions still seems to apply.

On close examination of the particles and pits shown in Figs. 5–7, it is clear that the 10–20-nm particles are not true particles of the inverted micelle type but local deformations of the lamellar structure involving a number of bilayer thicknesses. Examples of cross-fractured bilayers passing through and joining rows of particles and pits are arrowed in Figs. 6 and 7.

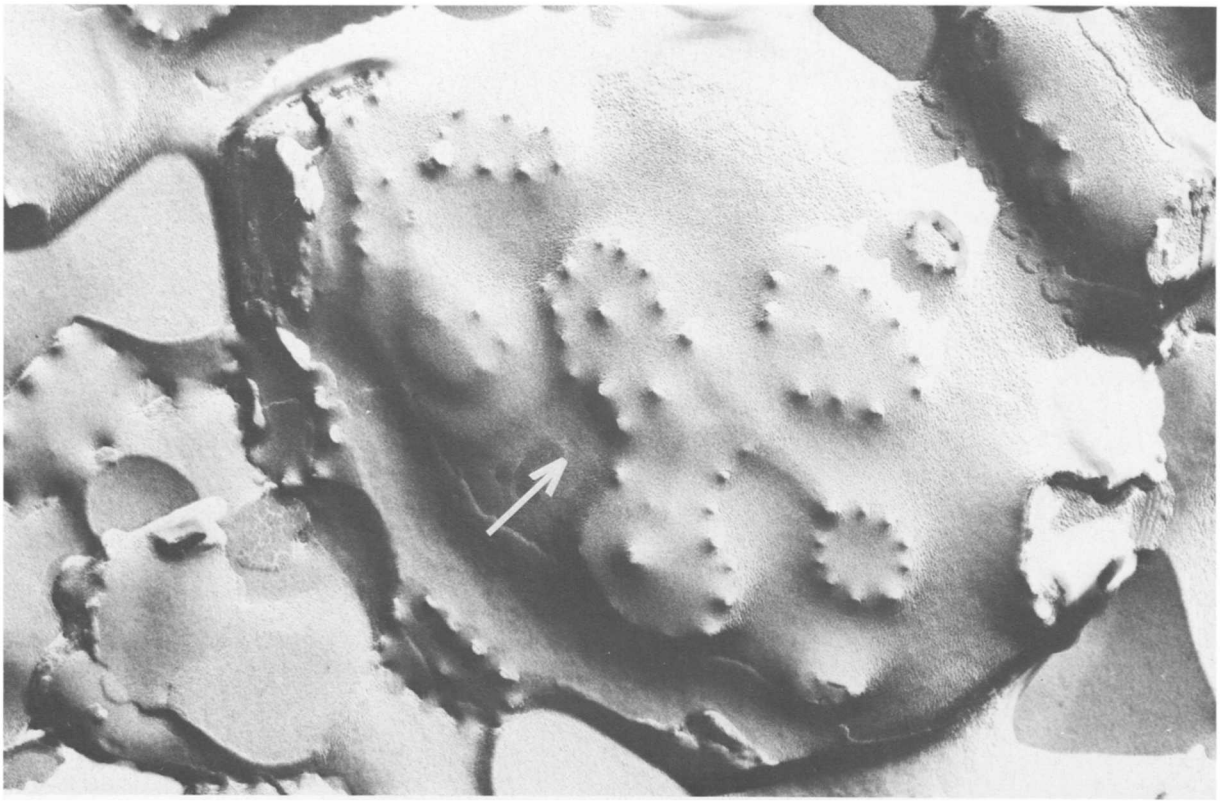

Fig. 5. Electronmicrograph of a freeze-fracture replica prepared from a 2:1 mole ratio mixture of mono- and digalactosyldiacylglycerols suspended in distilled water showing examples of the 10–20-nm particle-like structures associated with ridges in the underlying lipid. The shadowing direction is indicated by the large arrow (× 106250).

Most of the structures described above are associated with bilayers organised in what appear to be stacked planar sheets. These sheets, particularly in the case of structures of the type seen in Figs. 5–7, are often in a very distorted shape and it is extremely difficult to decide whether the sample is made up of buckled lamellae or of liposomal aggregates. the samples are difficult to suspend and precipitate readily on standing. If, however, the ratio of monogalactosyldiacylglycerol to digalactosyldiacylglycerol is decreased, the samples become much easier to disperse and freeze-fracture reveals many more discrete liposomes. At the same time, the particles we have described above are replaced increasingly by even larger particle-like structures which are typically 50–100 nm across and are associated with gross deformations of the underlying lipid. These deformations often span many hundreds of nanometres and involve many lamellae thicknesses. This is illustrated particularly well in Fig. 8 in which the particles appear to form a ring encircling a large spherical liposome. The distortion of the outline of the liposome in the region of the particle ring and the relationship between the individual particles and different lipid bilayers is quite clear. It is tempting to suggest that these larges particles and deformations are formed by a coalescing of features of the type shown in Figs. 5–7.

Discussion

All the features illustrated in Figs. 1–8 were observed in mixtures of mono- and digalactosyldiacylglycerols thermally quenched from about 20°C. The sequence in which the various features

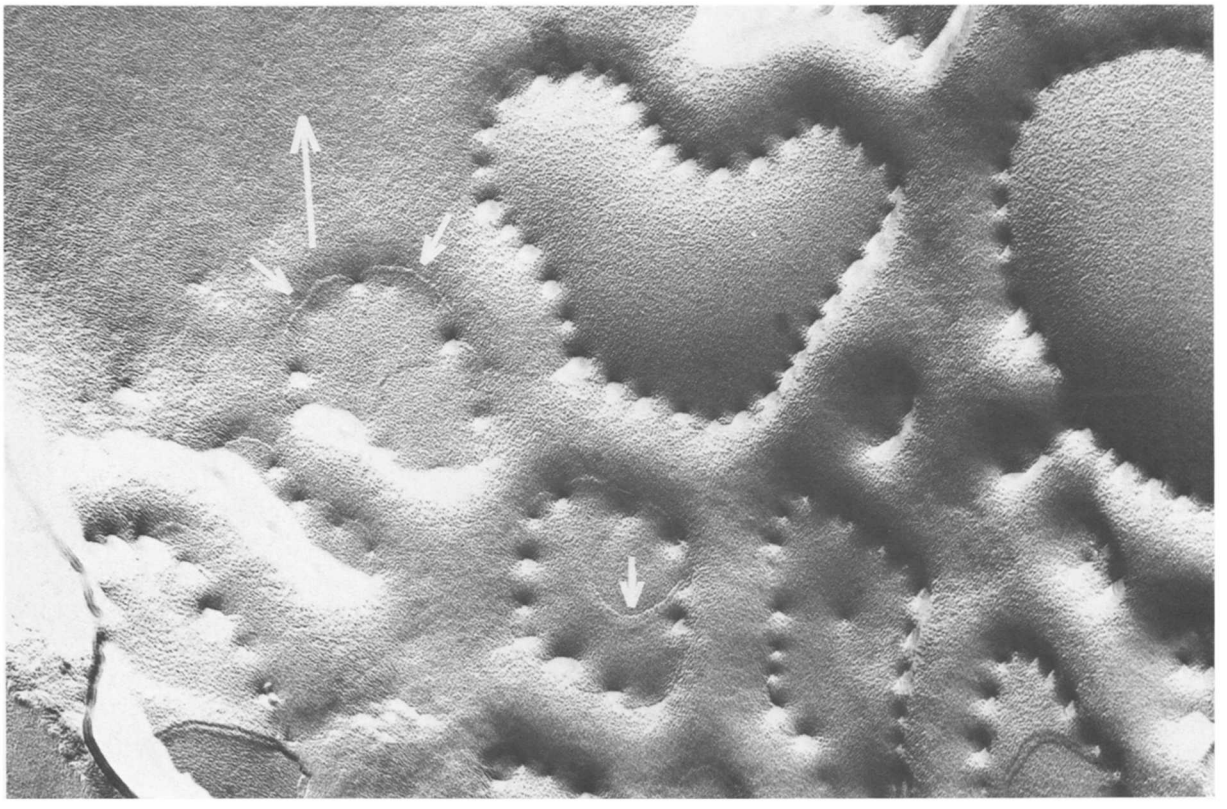

Fig. 6. An electronmicrograph of a freeze-fracture replica prepared from a 2:1 mole ratio mixture of mono- and digalactosyldiacylglycerols showing an arrangement of pits and depressions complementary to the particle-ridge organisation shown in Fig. 5. Example of cross-fractured bilayers passing through, and joining, individual particles are indicated by the small arrows. The large arrow indicates the shadowing direction (×106250).

have been presented corresponds, we believe, to a series of transitionary states between a phase-separated state in which the monogalactosyldiacylglycerol is localised predominantly in inverted micelles to one in which the two lipids are mixed more intimately in a uniform lamellar phase. The simultaneous presence of these states is explained by the fact that at room temperature the lipids exist in a heterogenous state mid-way between these two extremes. We have observed that an increase of only a few degrees in the temperature from which the samples are thermally quenched is sufficient to shift the balance towards the former, and a similar temperature decrease towards the latter state (unpublished data).

Our interpretation of the structures formed when the mixtures are trapped in this intermediate state is based on a consideration of the stresses likely to be generated when the lamellar phase undergoes a rapid lateral expansion associated with the incorporation of the inverted micelles into the lamellar phase. In principle the inverted micelles can disappear in either of two ways. They either can fuse with each other within the plane of the membrane to form larger structures or they can be incorporated directly into the bilayer, releasing their contents into the aqueous compartment between adjacent bilayers (see Fig. 9). These processes, in their initial stages, give rise to the tubular structures and conical deformations that we have identified in Figs. 2 and 3. The relative proportions of inverted micelles involved in the two processes will be determined by the restraining forces existing within a given bilayer and between bilayers.

Any sudden increase in area of a given bilayer

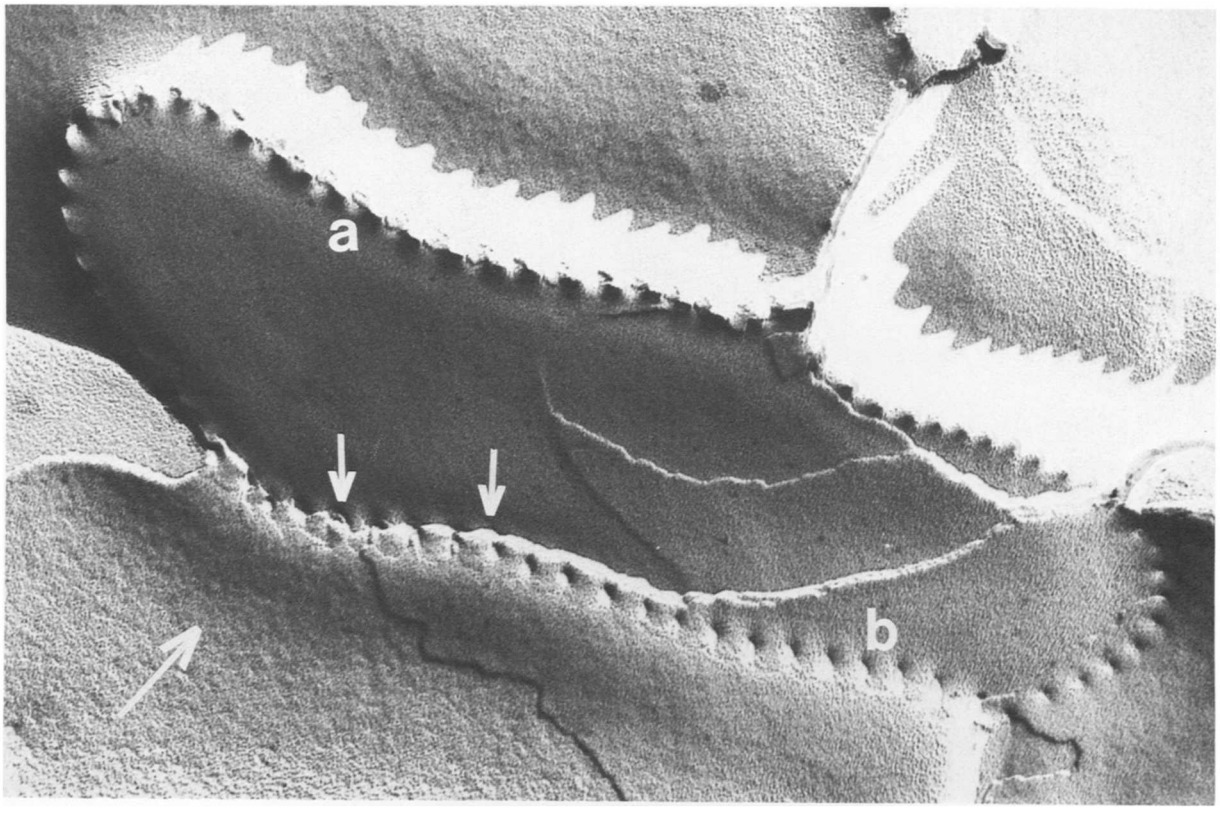

Fig. 7. Electronmicrograph of a freeze-fracture replica prepared from an aqueous dispersion of a 1:1 mixture of mono- and digalactosyldiacylglycerols showing examples of the 10–20-nm particle-like structures arranged in linear arrays along the crests of ridges in the lipids. The particle array (a) appears to be continuous with the row of pits (b) associated with depressions in the feature face. Examples of cross-fractured bilayers passing through these pits and particles are indicated by the small arrows. The large arrow indicates the direction of shadowing (×106250).

will necessarily give rise to stress in the surrounding bilayers. This stress can be relieved most readily by the migration of the small local deformations existing in the individual bilayers to form larger deformations which can be propagated through many bilayer thicknesses. Assuming the bilayers are part of a closed system, which may or may not be of the conventional liposome type, the increase in surface area will not be accompanied by an equivalent increase in volume. This probably explains why the deformations are propagated as ridges of the types seen in Figs. 4 and 5. The system of depressions seen in Fig. 6 is, in our opinion, simply another aspect of the same structures viewed in a complementary fracture face. The much more steeply contoured fracture face shown in Fig. 7 presumably reflects a region where the lamellae have buckled in both directions to form a system of parallel ridges and depressions.

Some at least of the structures reported here appear to have direct counterparts in mixtures of hexagonal and bilayer-forming phospholipids. The small 10–12-nm diameter particles that make up the planar arrays seen in Figs. 1–4 are very similar to the particles reported by Verkleij et al. [4] as occurring in replicas prepared from dispersions of phosphatidylcholine/phosphatidylethanolamine/cholesterol and phosphatidylcholine/monoglucosyldiglyceride mixtures preheated to 60°C prior to freeze-fracture. The fact that these latter particles are often distributed in parallel strings suggests that they could be formed by the breakdown of tubular micelles of the type we find in galactolipid mixtures. We have observed similar parallel par-

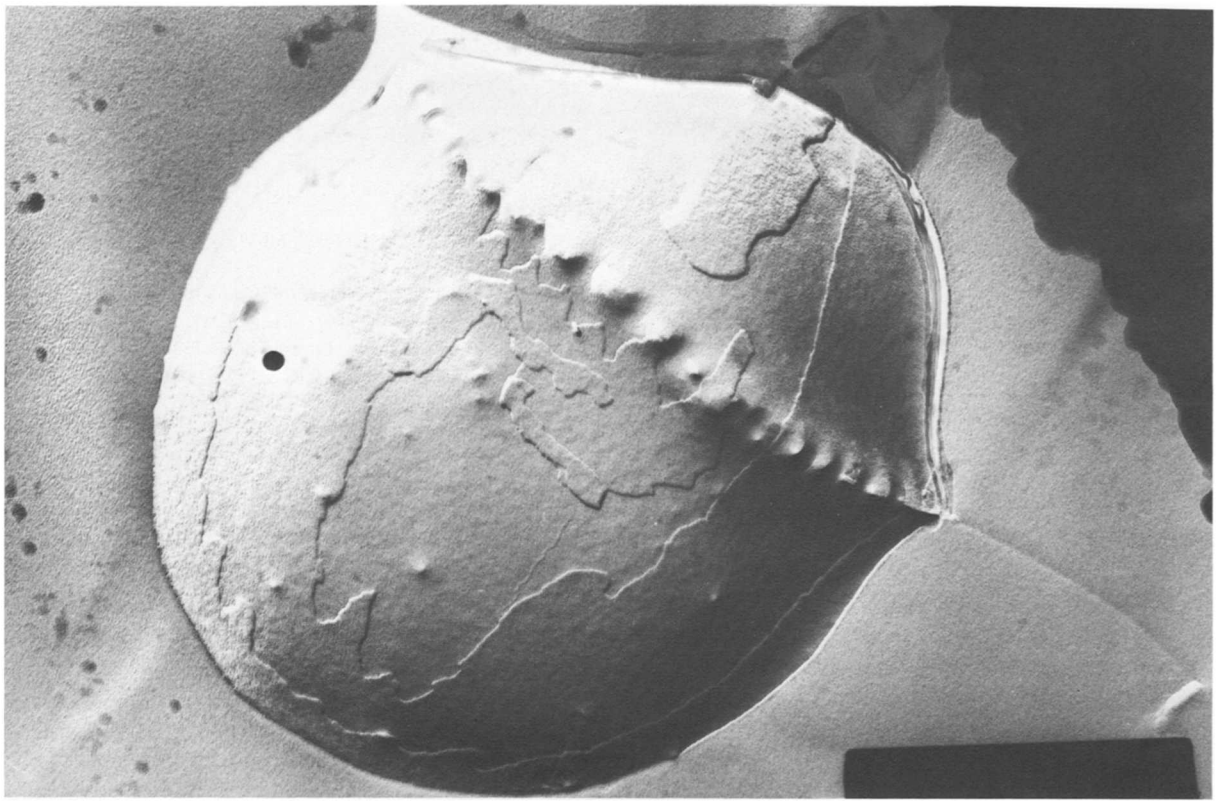

Fig. 8. Electronmicrograph of a freeze-fracture replica prepared from an aqueous dispersion of a 1:2 mixture of mono- and digalactosyldiacylglycerols showing examples of the large particle-like structures thought to be associated with shear deformations of the underlying lipid (×77350).

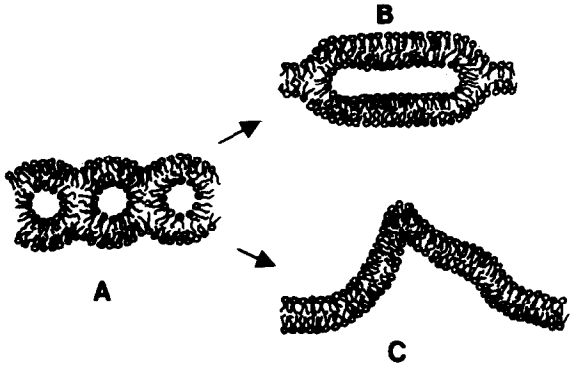

Fig. 9. Diagrammatic representation of a group of inverted micelles sandwiched within a lipid bilayer (A) and the relative effects of fusion of these micelles within the bilayer (B) and of the direct incorporation of the molecules making up the micelles into the bilayer (C). Note the very large increase in surface area, and consequent deformation, of the bilayer associated with (C).

ticle strings, together with other more complex arrangements, in replicas prepared from galactolipid mixtures quenched from 50°C (unpublished data) and from mixtures dispersed in aqueous ethylene glycol [10]. Verkleij et al. [4], and more recently Hui et al. [7], have also noted that such heat-treated dispersions often take on a ridged appearance that bears a strong resemblance to the organisation seen in Fig. 5.

Verkleij and his collaborators [3–4] and Miller [6] have reported the presence of particle structures similar to those shown in Figs. 5 and 6 in Ca^{2+}-treated dispersons of cardiolipin/phosphatidylcholine mixtures. These latter structures appear, however, to be associated with the fusion of pre-existing liposomes rather than a relaxation of a pre-formed phase-separated state. Whilst the two processes are clearly related, the details of the

intermediate stages are not necessarily identical and the interpretations offered by these authors to explain the apparance of their electronmicrographs are not, in our opinion, relevant to the present situation.

All of the structures illustrated by freeze-fracture in this work were obtained from preparations thermally quenched from physiological temperatures. this suggests that a balance exists between the bilayer and non-bilayer structures in the temperature range appropriate to the functioning chloroplast. Whether or not such lipid structures represent an important feature of the chloroplast thylakoid membrane remains to be established.

Acknowledgements

Financial suport was provided by grants from the Agricultural Research Council and the Royal Society.

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