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FORMATION OF INVERTED LIPID MICELLES IN AQUEOUS DISPERSIONS OF MIXED *sn*-3-GALACTOSYLDIACYLGLYCEROLS INDUCED BY HEAT AND ETHYLENE GLYCOL

ARINDAM SEN [a], ANTHONY P.R. BRAIN [c], PETER J. QUINN [a] and W. PATRICK WILLIAMS [b]

Departments of [a] Biochemistry, [b] Biophysics and [c] Electron Microscopy Unit, Chelsea College, University of London, Manresa Road, London SW3 6LX (U.K.)

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The formation of 'lipidic' particles corresponding to inverted lipid micelles in freeze-fracture replicas of aqueous dispersions of mono- and digalactosyldiacylglycerols can be greatly enhanced either by increasing the temperature from which the samples are thermally quenched or by the addition of cryoprotectants such as ethylene glycol. In the case of the heated samples, the lipids tend to form quasi-crystalline structures consisting of sheets of 8–9 nm diameter particles organised on an orthorhombic lattice. The orientation of alternate sheets varies giving rise to a characteristic herring-bone pattern. Ethylene glycol-treated samples, in contrast, form more regular structures consisting of 13–16 nm diameter particles. Lowering the temperature from which the samples are quenched and/or decreasing the concentration of ethylene glycol reduces the frequency of formation of such structures. A number of intermediate states associated with the re-incorporation of the lipid molecules of the inverted micelles into the lamella phase are also identified. The factors influencing particle formation are briefly discussed. It is concluded that the destabilisation of lipid-water interactions play a major role in this process.

Introduction

The occurrence of 'lipidic' particles in freeze-fracture replicas prepared from aqueous dispersions of certain phospholipid [1–6] and galactolipid [7–9] mixtures is well established. The basic requirement for the formation of such particles appears to be that at least one component of the mixture when dispersed alone in water should assume an hexagonal II phase; the other component(s) forming a lamella phase under these conditions.

Verkleij et al., [1,3] have demonstrated that the formation of such particles in phosphatidylcholine/cardiolipin mixtures is triggered by the addition of Ca^{2+}. De Kruijff et al. [2] and more recently Hui et al. [6] have shown that raising the temperature of suitable lipid dispersions has a rather similar effect. Massive increases in the number of such particles formed in galactolipid mixtures can, as we have recently reported [8], also be brought about by the addition of ethylene glycol.

Verkleij and his collaborators [1–4] have suggested that the particles they see in their preparations correspond to inverted lipid micelles sandwiched between the leaflets of a lipid bilayer. The particles they observe in replicas prepared from Ca^{2+}-treated phosphatidylcholine/cardiolipin mixtures are attributed to inverted lipid micelles formed at the fusion interface of adjacent bilayers [1,3]. The formation of such particles has been suggested as playing a major role in membrane

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fusion [10,11]. Our observation [8] that ethylene glycol, a well known membrane fusogen [12], promotes such particle formation is consistent with this view. The factors involved in the induction of inverted lipid micelle formation are thus clearly of great potential interest.

In a recent study [9], we showed that several different but related particle, or particle-like, structures could be recognised in replicas prepared from aqueous dispersions of mono- and digalactosyldiacylglycerols. The smallest of these particles, which we identified as corresponding to inverted lipid micelles, were about 10–12 nm in diameter and were often found to be organised in the form of planar arrays. The other larger structures were identified as local deformations in the fracture face arising in response to a temperature-dependent incorporation of these micelles into the lamellar structure.

In this paper we investigate the effects of temperature and of added ethylene glycol on the formation of inverted lipid micelles in dispersions of this type in more detail. We describe a number of different crystal-like structures observed in such preparations and discuss the processes involved in lamella-inverted micelle transformations in such systems.

Materials and Methods

Galactolipids were isolated from fresh leaf tissue of 4–5 weeks post-emergent broad bean plants (*Vicia faba* L., var. Express), purified and estimated as described previously [13]. The lipids were mixed in the desired molar ratio and the solvent removed under a stream of nitrogen. Dry lipid mixtures were stored under vacuum overnight to remove any residual solvent. The lipid mixtures were then dispersed in nitrogen-saturated water, or different concentrations of ethylene glycol in water, by ultrasonic irradiation under nitrogen. The dispersed lipids were equilibrated at the desired temperature for 15 min and immediately frozen using a slurry of nitrogen. The frozen samples were fractured at −115°C in a Polaron freeze-fracture device and shadowed by platinum-carbon immediately after fracture. The replicas were washed with chloroform/methanol (2:1, v/v) and examined in a Philips EM 301 electron microscope.

Results

Temperature-dependent structural changes

The organisation of aqueous dispersions of mixtures on mono- and digalactosyldiacylglycerols as revealed by freeze-fracture electron microscopy is largely dependent on the temperature from which the mixtures are thermally quenched. Replicas from samples quenched from room temperature show mainly lamella structures which usually take the form of buckled sheets or liposomal aggregates. Such replicas are characterised by the presence of a number of easily recognisable particle, or particle-like, structures. These, as we have demonstrated elsewhere [8,9], can be attributed to the presence of inverted lipid micelles sandwiched within the lamellae or to local deformations of the fracture-face associated with the reincorporation of the lipids of such micelles into the lamella phase. If, however, the same samples are heated to about 50°C and then quenched directly from this temperature an entirely different organisation, involving several different structural forms, is observed. This is illustrated in Fig. 1 which shows an hexagonal II type structure in conjunction with a particulate quasi-crystalline phase. The hexagonal II structure appears to be similar to that observed in replicas prepared from pure unsaturated monogalactosyldiacylglycerol [14]. The possibility that it corresponds to a mixed phase of monogalactosyl- and digalactosyldiacylglycerols analogous to that recently reported for mixed phospholipids [15] cannot, however, be discounted.

Electron micrographs showing two of the most commonly observed crystal-like arrangements are shown in Figs. 2 and 3. The structure shown in Fig. 2 is made up of sheets of particles. Within each sheet, the particles are organised on an orthorhombic lattice. Measurements of the length of long rows of such particles and division of the length of such rows by the number of particles present in the rows yielded repeat distances along the principal axes of this quasi-crystalline arrangement of 8–9 nm. The arrangement of the particles is somewhat asymmetric giving rise to a distinct pattern of ridges and grooves in the individual sheets. The orientation of these ridges alternates with each sheet giving rise to a characteristic herring-bone pattern in the replica surface. The indi-

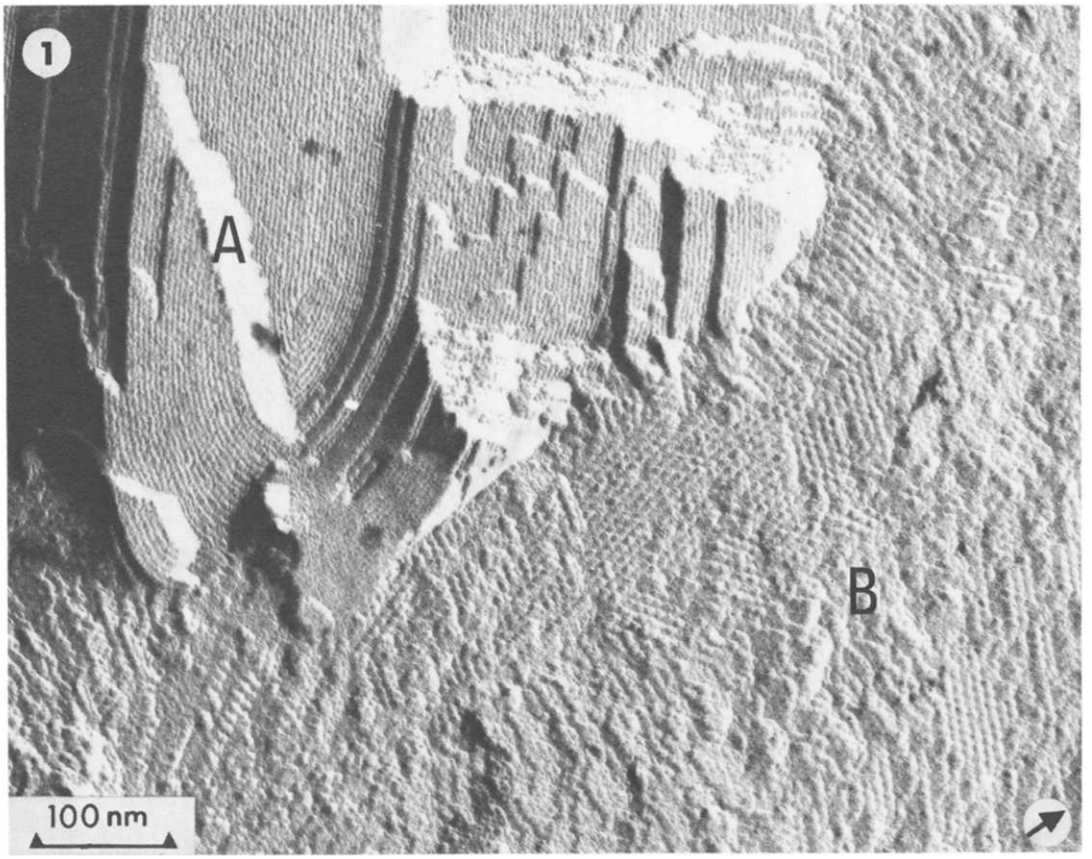


Fig. 1. Electron micrograph of a free-fracture replica prepared from a sonicated dispersion of 2 : 1 mole ratio mixture of mono- and digalactosyldiacylglycerols quenched from 50°C. In region A, the lipid appears to be organised in an hexagonal II phase whilst in region B it is organised into a quasi-crystalline particulate phase. The arrow indicates the shadow direction.

vidual particles are clearly spherical, or near-spherical. They are thus similar to, but significantly smaller than, the 10–12 nm particles corresponding to inverted lipid micelles observed in replicas prepared from samples quenched from room temperature [8]. An alternative, more uniform arrangement of these particles, is shown in Fig. 3. The repeat distance within the lattice is almost identical to that of the herring-bone structure and the possibility that the structure shown in Fig. 3 represents a cross-fracture of the structure shown in Fig. 2 cannot be excluded.

The electron micrograph presented in Fig. 4 shows a region of a similar replica in which a crystalline area abutts directly onto a lamella stack. The presence of parallel strings of particles in the faces of the lamellae directly adjacent to the crystalline region strongly suggests that the two phases are very closely related and that they are probably interconvertible under appropriate conditions. The particles in the lamellae faces clearly correspond to the inverted lipid micelles of the type that we have reported previously [7–9] adding further support to the idea that the crystalline structures are made up of such micelles.

If instead of quenching the samples from higher temperatures, the samples are allowed to cool to room temperature before thermal quenching, the crystalline regions largely disappear. The only feature distinguishing replicas of such samples from those obtained from non-heated samples is that they still contain regions in which the lipid has

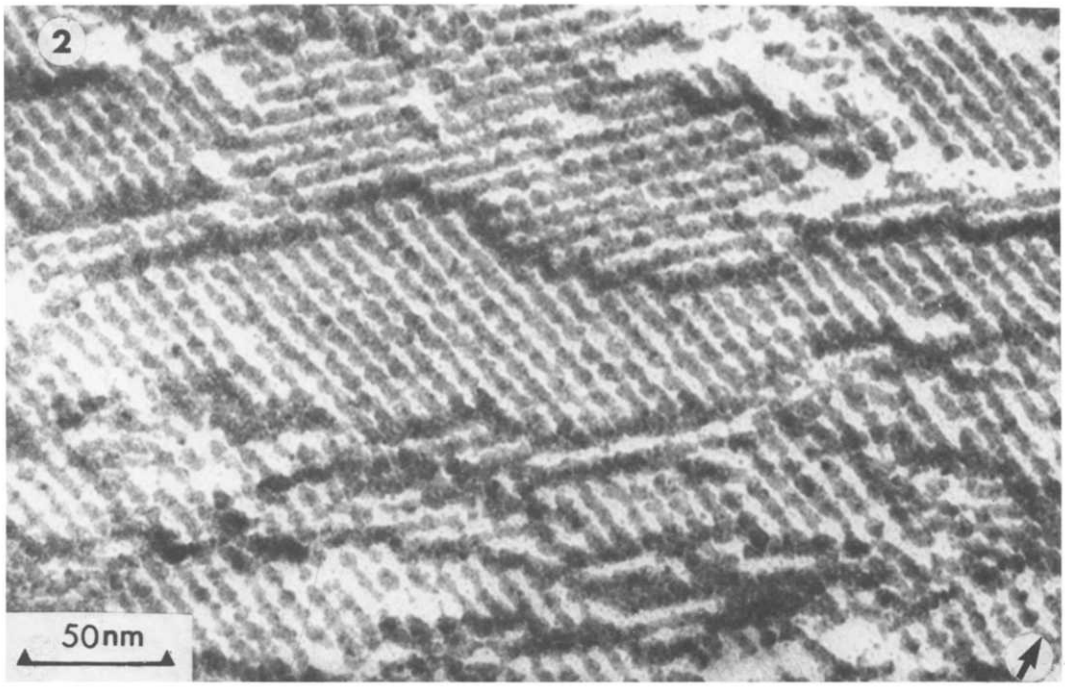


Fig. 2. Electron micrograph of a freeze-fracture replica prepared from 2:1 mole ratio mixture of mono- and digalactosyldiacylglycerols ultrasonically dispersed in water showing particles arranged in sheets. The change in the orientation of the particles in alternate sheets gives rise to a characteristic 'herring bone' pattern. The sample was quenched from 50°C. Shadow direction is indicated by the arrow.

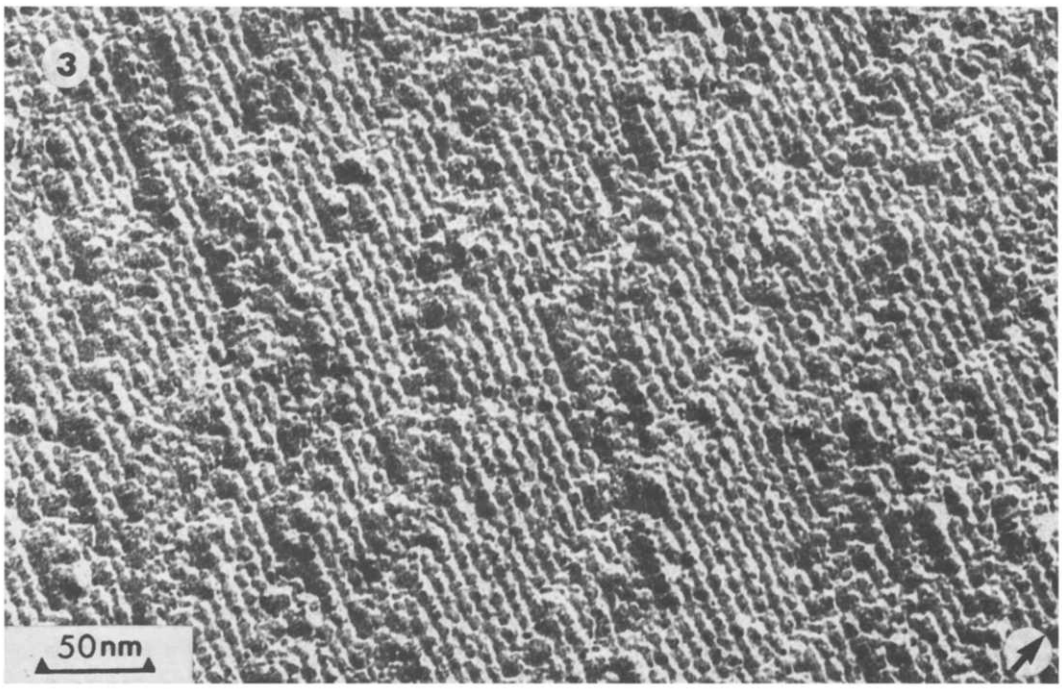


Fig. 3. Electron micrograph of a replica obtained from freeze-fractured sample of a sonicated dispersion of 2:1 mole ratio mixture of mono- and digalactosyldiacylglycerols quenched from 50°C. The particles are arranged in a uniform symmetrical lattice. The arrow indicates the shadow direction.

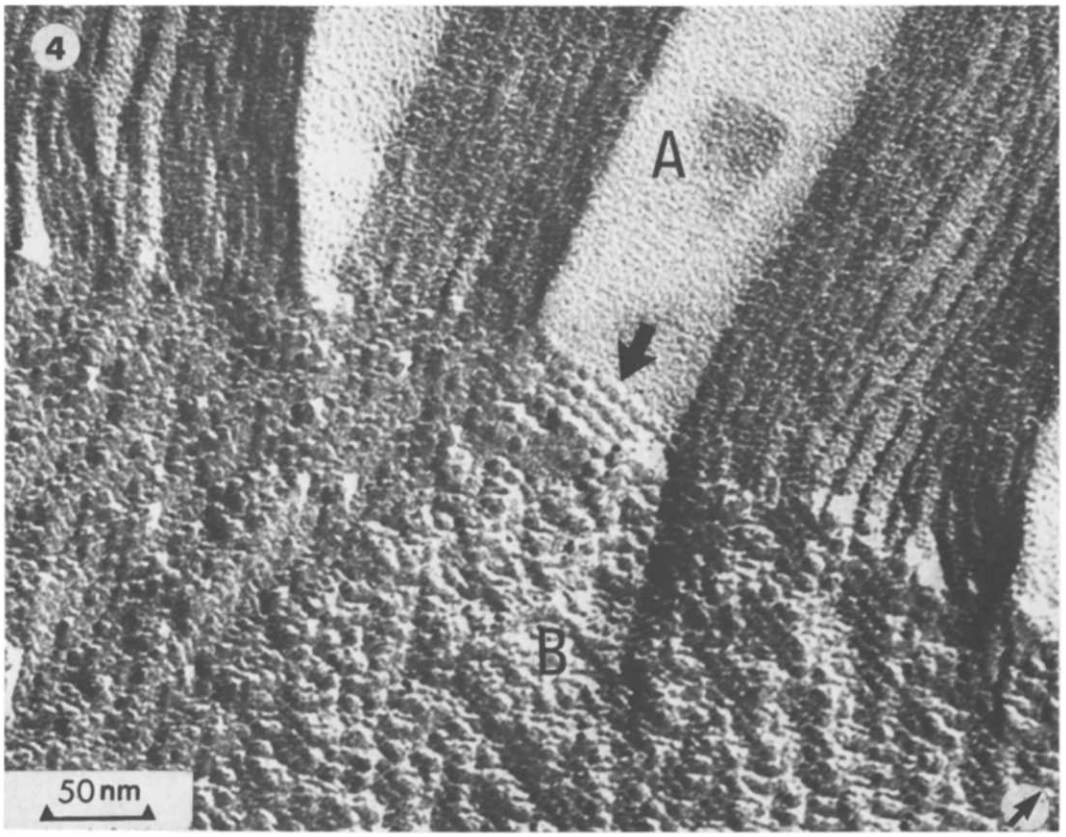


Fig. 4. Electron micrograph of a freeze-fracture replica prepared from sonicated dispersion of a 2 : 1 mole ratio mixture of mono- and digalactosyldiacylglycerols in water, quenched from 50°C. Area (A) shows bilayer structures which break down to form aggregates of particles in area (B). A linear array of particles (arrowed) is seen at the junction of the two phases. Shadow direction is shown by the smaller arrow at lower right corner.

taken up an hexagonal II structure. This presumably reflects the fact that pure phase-separated monogalactosyldiacylglycerol cannot be re-incorporated into the dispersion as a whole. No traces of the crystal-like phases described above were observed even for samples held at room temperature for as little as one minute indicating that these phases, in contrast, are readily re-incorporated.

A series of experiments were performed in which the samples were heated to a given temperature, held at this temperature for 15 min and then quenched directly to −115°C. Replicas prepared from samples heated to 24–55°C were effectively identical. As the pre-treatment temperature was reduced below 20°C, the replicas took an appearance increasingly similar to those quenched from room temperature (see Reference 9 for details of such samples). On lowering the temperature still further, fewer and fewer particulate structures were seen in the replicas. Samples quenched from 2°C or below showed few if any such structures. The temperature resolution of these quenching experiments is inadequate to allow an accurate determination of the temperature at which the quasi-crystalline structures disappear. Use of the jet-freezing technique, recently applied to studies of this type by Van Venetie and Verkleij [15] would, however, almost certainly improve this situation.

Ethylene glycol-induced changes

Addition of ethylene glycol to dispersions of mixtures of mono- and digalactosyldiacylglycerols leads, as we have noted elsewhere [8], to the for-

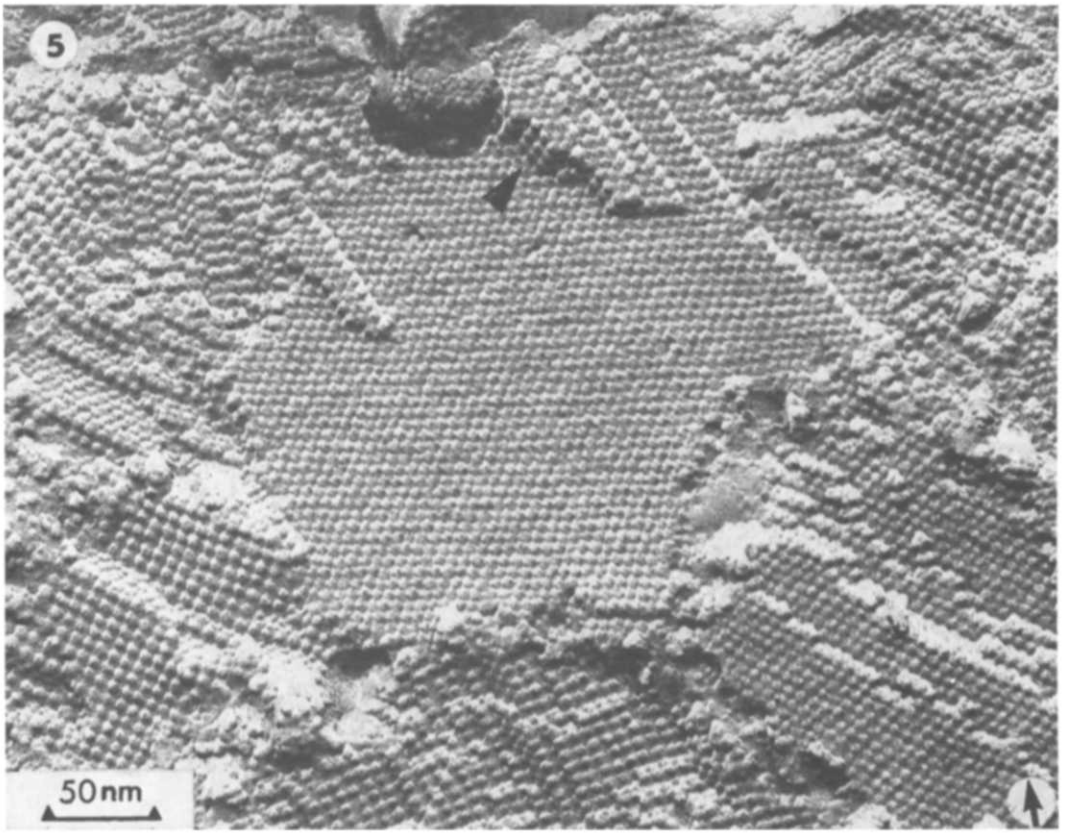


Fig. 5. Electron micrograph of a replica obtained from freeze-fractured sample of a sonicated dispersion of 2 : 1 mole ratio mixture of mono- and digalactosyldiacylglycerols in 58.4% ethylene glycol. Particles are seen organised in an orthorhombic array in a planar structure. Cross-fracture, indicated by an arrow-head, reveals the three dimensional nature of lattice. The arrow indicates the shadow direction.

mation of large numbers of inverted lipid micelles. These micelles are again often found to form large crystal-like structures. Typical examples of such structures are shown in Fig. 5. Their organisation is similar, but not identical, to those of the temperature-induced structures shown in Figs. 2 and 3. The most obvious difference lies in the particle size; the lattice repeat distance in the ethylene glycol-treated sample is about 13–16 nm. This compares to 8–9 nm for the heat-treated samples. As in Fig. 2, the structure shown in Fig. 5 shows an orthorhombic symmetry in the plane of the sheets. Viewed end-on, however, the sheets appear to show a square, or near-square, symmetry. In contrast to the temperature-induced structures, there appears to be no asymmetry in the packing within the individual sheets and herring-bone patterns are not observed in these replicas. Neither is there any indication of the presence of phase-separated monogalactosyldiacylglycerol.

In addition to these crystal like structures, the replicas contain many well-formed liposomes. The fracture faces of these liposomes are frequently studded with arrays of inverted lipid micelles. In many cases the crystal-like structures are, as illustrated in Fig. 6, found in close association with

Fig. 7. Electron micrograph of a replica from a freeze-fractured sample of 2 : 1 mole ratio mixture of mono- and digalactosyldiacylglycerols ultrasonically dispersed in 58.4% ethylene glycol. Particles are in parallel, linear arrays within a planar lamella (indicated by an arrow-head). The concave blister-like regions are essentially free of particles. Shadow direction is indicated by the arrow.

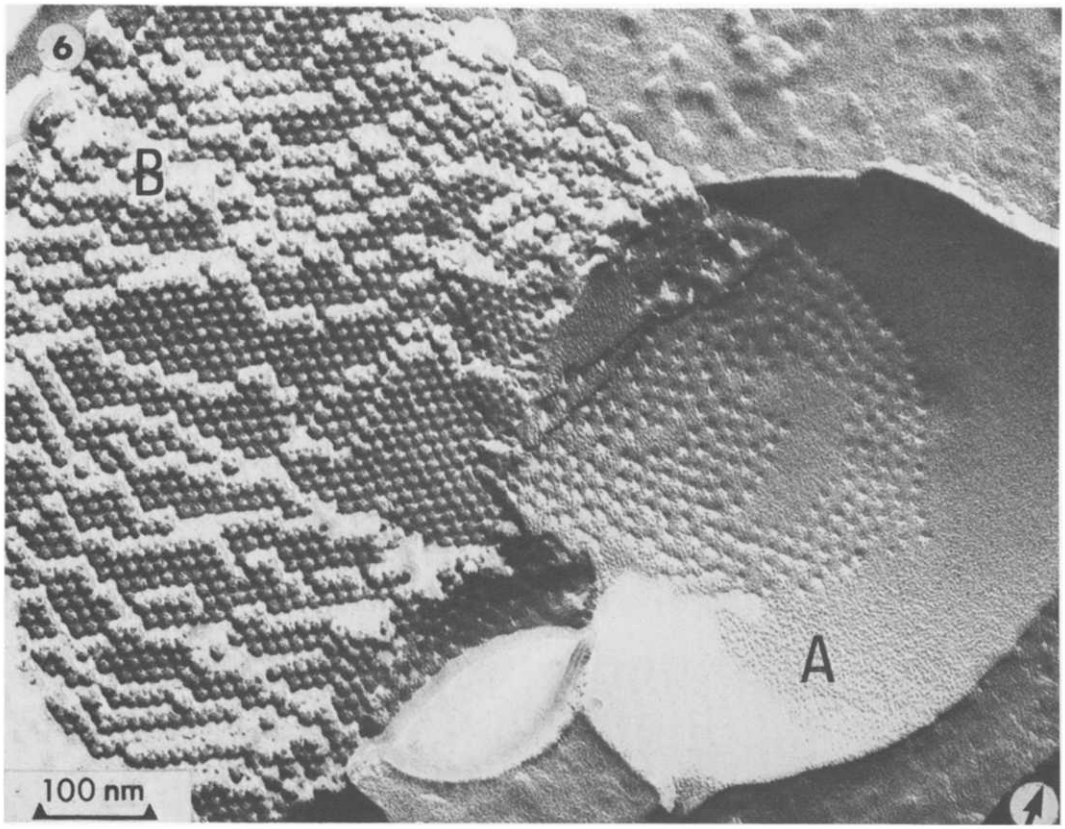
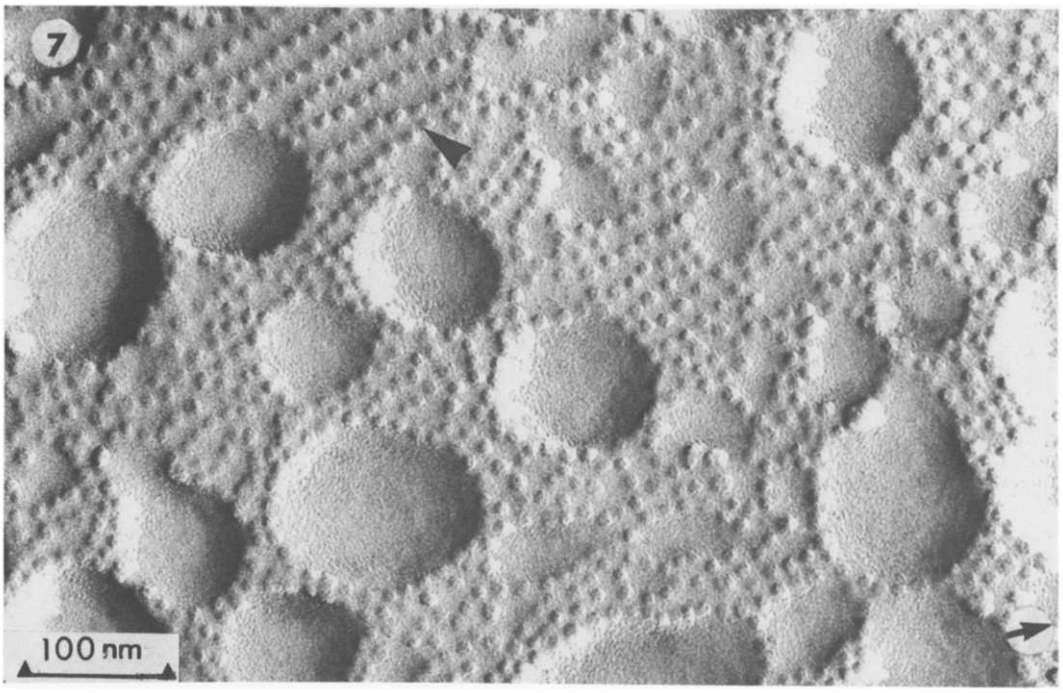


Fig. 6. Electron micrograph of a freeze-fracture replica from sonicated dispersion of a 2:1 mole ratio mixture of mono- and digalactosyldiacylglycerols in 30% ethylene glycol. Area (A) shows the presence of pits on a liposome and area (B) shows particles in a crystal-like structure. The close juxtaposition of the two structures suggests an interconversion of the two phases. Shadow direction is indicated by the arrow.



such liposomes. Another common feature of such replicas is the presence of planar lamellae containing large numbers of inverted lipid micelles. These lamellae often show small convex or concave blister-like features that are themselves usually particle-free. Examples of such structures are shown in Fig. 7. In this particular case, shadows cast by surrounding particles indicate that the particle-free areas are in fact depressions in the fracture face. In our experience, within any given fracture face these features are always either all convex or all concave suggesting that they represent complementary views of the same type of structure. It is noteworthy that the particles surrounding these features tend to be organised in parallel rows similar to those seen at the border of lamella and crystal regions in the heat-treated samples (Fig. 4). This suggests that the lamellae are probably formed from individual sheets of the crystal-like structures shown in Figs. 5 and 6 and that the blister-like features correspond to deformations in the lamellae resulting from the re-incorporation of inverted micelles directly into the lamella phase.

The frequency of occurrence of all these features is strongly dependent both on the concentration of ethylene glycol present in the dispersions and the temperature from which the dispersions are quenched. Samples containing 58.4% ethylene glycol, the highest concentration employed, when quenched from room temperature show very extensive crystalline regions. No significant difference is observed on decreasing the concentration of ethylene glycol to 30%, but when the concentration of ethylene glycol is decreased fur-

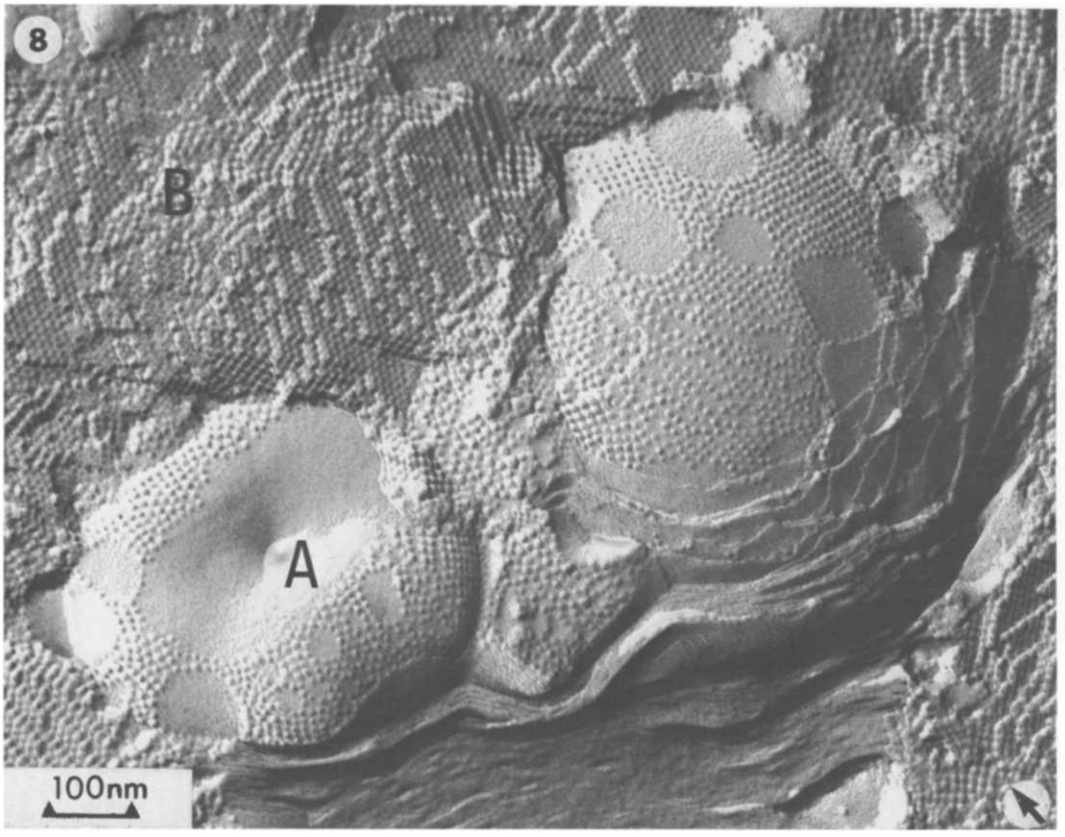


Fig. 8. Electron micrograph of a replica from freeze-fractured dispersion of a 2 : 1 mole ratio mixture of mono- and digalactosyldiacylglycerols in 50% dimethyl sulphoxide. Multi-lamellar liposomes with particles within the lamellae (A) are seen together with crystal-like particle aggregates (B). The close proximity of the two phases suggests an interconversion between the two structures. The arrow indicates the shadow direction.

ther, these features become rarer until at about 5% ethylene glycol the replicas are indistinguishable from ethylene glycol-free dispersions. Similarly if the temperature from which the samples are quenched is reduced the frequency of occurrence of crystalline regions decreases.

Addition of glycerol or dimethyl sulphoxide leads to effects similar to those observed for ethylene glycol. A typical electron micrograph obtained from a suspension containing 50% dimethylsulphoxide is shown in Fig. 8. The large particle-free regions seen in the fracture-faces of the liposomes are probably related to the blister-like structures seen in Fig. 7.

Discussion

The 8–9 nm diameter particles forming the crystal-like structures seen in heated aqueous dispersions of mono- and digalactosyldiacylglycerol and the corresponding 13–16 nm diameter particles in ethylene glycol-water dispersions both appear to reflect the presence of inverted lipid micelles. We have previously reported the occurrence of planar arrays of such micelles in replicas prepared from aqueous dispersions of these lipids quenched from room temperature [7–9]. We have also noted the presence of crystal-like structures in such dispersions under negative staining conditions [8]. Our present observation that such structures can be induced by raising the temperature from which the dispersions are quenched is consistent with earlier observations of De Kruijff et al. [2] and Hui et al. [6] working with heat-treated dispersions of phospholipid mixtures. Verkleij and his collaborators [3] have also reported crystal-like structures, resembling those we find in ethylene glycol-water dispersions, in phosphatidylcholine/cardiolipin mixtures treated with >10 mM Ca^{2+}.

It is extremely difficult on the basis of freeze-fracture evidence alone to be certain whether the crystal-like structures we observe consist of inverted micelles sandwiched within individual bilayers that are stacked on top of each other or whether the micelles are contained in a continuous matrix of lipid as in the conventional hexagonal II structure. The fact that the fracture-face tends to jump frequently between different planes tends to suggest that the structure is continuous. X-ray diffraction studies indicate that aqueous dispersions of mixed galactolipids [16] and pure monoacylgycerols [17,18] can, under appropriate conditions, form optically isotropic cubic phases in which water is trapped within a three-dimensional structure related to the more familiar hexagonal II phase. The relationship of such phases to the quasi-crystalline structures we observe using freeze-fracture electron microscopy remains to be established.

The transition between crystalline and bilayer structure appears to involve an initial separation of the crystal planes into discrete bilayers followed by an incorporation of the lipids of the inverted micelles sandwiched within these bilayers directly into the lamella phase. Such an incorporation, as detailed elsewhere [9], of necessity results in large localised increases in the surface area of the bilayers. This accounts for the formation first of the blister-like features seen in Fig. 7 and finally the development of the liposomal structures of the type seen in Figs. 6 and 8. The relatively small energies associated with interconversions of this type is reflected in the small enthalpy changes associated with lamella-hexagonal II phase transitions in pure lipids. Cullis and De Kruijff [19], for example, have shown that the enthalpy changes associated with transition between the lamella and hexagonal II phases of egg yolk phosphatidylethanolamine is only of the order of 10–15% of that associated with the corresponding transition between gel and liquid crystalline phases. This again emphasises the importance of introducing rapid freezing techniques such as those employed by Van Venetie and Verkleij [15] and Gulik-Krzywicki and Costello [20] into studies of this type.

The organisation adopted in aqueous dispersion by any given lipid, or lipid mixture, is determined by the balance of forces existing between the molecules of the system. These can be divided into those arising from (1) lipid-lipid, (2) lipid-water and (3) water-water interactions. (1) Lipid-lipid interactions involve Van der Waal's forces between hydrocarbon chains, electrostatic interactions between the charges (or dipoles) of the headgroups and possible hydrogen bonding interactions between neighbouring lipid headgroups.

(2) Lipid-water interactions mainly involve lipid hydrogen bonding between the lipid headgroups and water and (3) water-water interactions, hydrogen bonding in ordered water associated with these headgroups. In addition to these forces, entropic factors and limitations imposed by steric hinderance have also to be taken into consideration.

A detailed analysis of these factors is clearly beyond the scope of this paper. The fact that the addition of cryoprotectants such as ethylene glycol leads to marked increases in inverted micelle formation in galactolipid mixtures suggests, however, that lipid-water and water-water interactions are likely to be particularly important in this system. These agents exert their cryoprotectant effect by disrupting hydrogen-bond formation in water. Their role in inducing the formation of inverted lipid micelles may involve their ability to interfere with hydrogen bonding between the sugars of the lipid headgroups and water and the formation of ordered water structures around these groups.

Wieslander et al. [21] have suggested that the increased tendency of certain lipids to take up hexagonal II phases at higher temperatures might be associated with increased bulkiness of their hydrophobic acyl chains arising from their greater mobility. Our observations would suggest that whilst the increased kinetic energy of these residues may well be a contributory factor both in lamella-hexagonal II transitions in pure lipids and inverted micelles formation in lipid mixtures, the effect of increased temperature on the destabilisation of interactions between the lipid and aqueous phases may be at least, if not more, important. In our view, attempts to predict molecular arrangements of this type in the absence of adequate thermodynamic and structural data are unlikely to be successful and rationalisations of known arrangements on the basis of ideas of molecular shape alone prove misleading.

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