Biochimica et Biophysica Acta, 777 (1984) 306–322
Elsevier

BBA 72276

EFFECTS OF RECONSTITUTION METHOD ON THE STRUCTURAL ORGANIZATION OF ISOLATED CHLOROPLAST MEMBRANE LIPIDS

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(Received April 9th, 1984)

Key words: Membrane reconstitution; Structural organization; Galactosylacylglycerol; Freeze-fracture electron microscopy; (Chloroplast)

Plant galactolipids were isolated from spinach thylakoids and reconstituted by (1) hydration in water or buffer, (2) solubilization in Triton X-100 and subsequent slow detergent removal, and (3) reverse phase evaporation using Freon 11 (trichlorofluoromethane, b.p. 23°C). Digalactosyldiacylglycerol (DG) formed bilayer liposomes when reconstituted by any of these methods. Monogalactosyldiacylglycerol (MG) was very difficult to transfer quantitatively to the aqueous phase. Reverse phase evaporation was the most successful method, and conventional hydration in water or buffer the least efficient, for reconstituting MG quantitatively. Freeze-fracture electron microscopy of pure MG showed arrays of hexagonal II tubes as well as packed inverted micelles (7–9 nm diameter in both cases) covered by a monolayer of lipid. Reconstitution of binary mixtures of MG and DG using the various methods produced the same structures. However, the concentration of MG at which various structural changes occurred depended on the method used for reconstitution. Some of the differences between buffer-hydrated and reverse phase evaporated-reconstituted DG/MG mixtures were traced to the conventional hydration method leaving MG selectively behind on the glass. Reverse phase evaporation allowed the most MG to be incorporated into vesicular structures (about 50% vs. about 30–40% by the detergent method). Irregularities in the bilayer vesicles, 'lipidic particles' and 'fusion pores', varied proportionally with the amount of MG in the mixture. The transition from vesicular structures to packed tubes and particles had occurred by approx. 66% MG using reverse phase evaporation and by approx. 50% MG using detergent solubilization. Aggregates of packed inverted micelles were present in several DG/MG mixtures. The diameters of the inverted micelles varied from 7–9 nm (pure MG) to 20–21 nm (60:40, DG/MG). A model is presented that relates this variation in diameter geometrically to the overall 'cone' shape of an MG molecule and the cylindrical shape of DG. In contrast to a previous report, glycerol had no effect on the type of structures observed in replicas of mixed DG/MG samples. However, all structures were more clearly defined in freeze-fracture replicate from glycerinated samples.

Introduction

Our aim in this work has been to establish a procedure by which we can quickly and reproducibly form bilayer vesicles of variable and known composition from endogenous thylakoid lipids. The majority of the thylakoid lipids, mono- and digalactosyldiacylglycerols (MG and DG), are uncharged and could be expected to behave differently in reconstituted systems than the commonly used, charged phospholipids. MG and DG constitute approximately 51% and 26%, respectively, of the total non-pigmented thylakoid lipids [1]. Additional lipids are sulfoquinovosyldiglyceride

(SL, approx. 7%), phosphatidylglycerol (PG, approx. 9%) and phosphatidylcholine (PC, approx. 3%).

The phase behavior of pure MG and DG in water has been characterized by Shipley et al. [2] using X-ray diffraction and freeze-fracture electron microscopy. They showed that MG formed an hexagonal phase encapsulating water cylinders in a lipid matrix, and that DG formed lamellar structures. The presence of excess water did not alter the lipid structure in either case.

More recently, Sen et al. [3–8] have confirmed these basic structures using freeze-fracture electron microscopy. They looked extensively at the structures formed by a 2 : 1 MG/DG mixture subjected to heating and/or incubation with ethylene glycol. Their results indicate that MG behaves like phosphatidylethanolamine in bilayers [9], i.e., that it forms 'lipidic particles' or inverted micellar structures within the lipid bilayer. The theoretical calculations of Murphy [10–12] provide an explanation for this behavior of MG: the two trienoic fatty acid tails occupy a much larger volume than does the galactose headgroup producing an overall cone shape for the MG molecule (headgroup at the apex), similar to the situation proposed for phosphatidylethanolamine [9]. The monolayer studies of Bishop et al. [13] further demonstrate the relative importance of the unsaturated fatty acids to the overall volume occupied by MG.

The relationship between lipidic particles, inverted micelles and membrane contact or fusion has been extensively debated [14–16]. Recently the role of such lipid structures in membrane fusion has been challenged using ultrarapid freezing of phospholipid vesicles following Ca^{2+}-induced fusion [17]. For reconstitution studies we would like to avoid vesicles containing lipidic particles because their presence would unnecessarily complicate identification of reconstituted protein particles even though the two types of particles can be distinguished in freeze-fracture replicas [15,16].

In repeating some of the experiments of Sen et al. [4,7], we have found that their conventional hydration method is relatively inefficient in reconstituting MG into aqueous solutions, and that under certain conditions, significant amounts of MG can remain stuck on the glass. To overcome these problems, we have tested the ability of several reconstitution methods to incorporate significant amounts of MG quantitatively into bilayer liposomes. In addition, we describe other structures formed when MG and DG are mixed in various ratios. Some of our preliminary studies have been reported [18].

Materials and Methods

Purification of lipid classes. Thylakoids were obtained from commercial spinach leaves after homogenization and differential centrifugation [18]. Thylakoids from 300 to 400 g of prepared leaves were resuspended in 30 ml of buffer (0.05 M sodium phosphate (pH 7.2)/0.1 M KCl/0.4 M sucrose), and extracted with cold chloroform/methanol essentially as described by Allen and Good [19].

The concentrated thylakoid extract (40–60 mg/ml) was separated by column chromatography on silicic acid according to a procedure modified from that of Rouser et al. [20]. For preparative work a 5.5 cm (o.d.) × 6–8 cm column, containing 60–70 g silicic acid, was used. Such columns could hold 0.8–0.9 g of extract (silicic acid/lipid $\geqslant$ 75, Ref. 20).

The 'total extract minus pigment' (see below) was obtained by eluting pigments with chloroform as above and the remaining lipids with chloroform/methanol (1 : 1, v/v). Similarly, the 'total extract minus pigment, minus MG' was obtained by elution of pigments and MG with chloroform and chloroform/acetone (1 : 1), respectively, and then collecting the remaining lipids with chloroform/methanol (1 : 1).

Analysis of lipids. Routine identification of lipids was carried out by thin-layer chromatography (TLC) on Silica gel G (Rediplates, Fisher Scientific) using the solvent systems described by Allen and Good [19]. Unpigmented lipids were identified with iodine vapor and standard spray reagents [21]. DG standard was obtained from PL Biochemicals (Milwaukee, WI). The presence of sulfur (SL), phosphorus (PL), and magnesium (chlorophyll) in column fractions was confirmed by X-ray energy spectrometry in a Kevex 5500 series spectrometer attached to a Cambridge S4 scanning electron microscope.

Preparation of lipid structures. Thylakoid lipids (mixtures and individual classes) were transferred to an aqueous medium by (1) hydration in distilled water, buffer (0.05 M sodium phosphate/0.1 M KCl (pH 7.2)), or 0.1 M NaCl solution, (2) solubilization in detergent, or (3) reverse phase evaporation [22] from Freon 11 (trichlorofluoromethane, b.p. 23°C, Matheson, East Rutherford, NJ) as outlined in Fig. 1.

Preparation of samples for electron microscopy. Samples were routinely dip-frozen in (1) liquid-nitrogen cooled Frèon 12 after glycerination to 35% (v/v) or (2) liquid-nitrogen cooled propane (non-glycerinated samples). In addition several samples were jet-frozen [23] with and without cryoprotectant. For jet-freezing of liquid samples, gold mesh grids (12 μm thick) were electrode discharge machined with a 1 mm brass rod to produce uniform holes to contain the sample. The machined grids were glued to gold supports with a small drop of 0.5% Formvar in 1,2-dichloroethane and air dried overnight before use.

Freeze fracture and replication of samples was carried out in a Balzers 360 M freeze-etch device equipped with electron guns. Standard fracture and replication was done at −115°C while etching was at −108°C. Replicas were cleaned in commercial bleach, distilled water and chloroform/methanol (1 : 1, v/v), Micrometer bars are included on the electron micrographs.

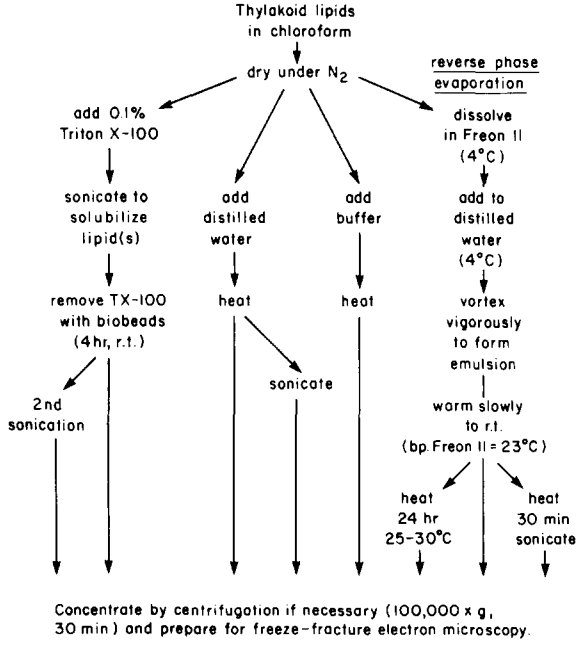


Fig. 1. Methods for formation of lipid structures.

Results

Individual lipid classes

Monogalactosyldiacylglycerol

MG proved very difficult to transfer quantitatively to the aqueous phase. The methods employed, their efficiencies and the structures observed by freeze-fracture electron microscopy are summarized in Table I. Simple hydration of MG in buffer or water with or without heating and/or sonication transferred only a small amount of the lipid to the aqueous phase. Most of the lipid remained attached to the walls of the glass containers. To overcome this problem, we tried solubilizing the lipid by sonication in 0.1% Triton X-100. Removal of the Triton-lipid solution from the tube and washing of the tube with chloroform showed that solubilization was complete; no lipid was detectable by TLC in the chloroform washes (not shown). However, during the subsequent detergent removal, clumps of lipid formed and attached firmly to the glass. Reverse phase evaporation forced a large portion of the MG to remain in the aqueous phase. Again a substantial amount of clumping of the lipid was observed, but most of the clumps remained in suspension instead of attaching to the glass. A slight turbidity was observed in the bulk water suggesting the presence of some smaller suspended clumps (these were confirmed by phase contrast light microscopy, 400 ×). Removal of the water/Freon emulsion from the test tube at 4°C and subsequent washing of the tube with chloroform showed that only a trace of MG remained associated with the glass.

Freeze-fracture electron microscopy of MG samples prepared by reverse phase evaporation showed the presence of both extensive particle arrays and tubular lattices (Fig. 2). In almost all cases, particles and tubes did not co-exist in a single aggregate. The particles and tubes had diameters of 7–9 nm. In some fractures a smooth lipid layer was revealed covering the tubes or

TABLE I

INDIVIDUAL LIPID CLASSES

Lipid	Method of transfer	Yield	Structures observed by freeze--fracture electron microscopy
MG	Hydration in water	Negligible but more than with buffer	Amorphous aggregates with occasional tubes
	Hydration in buffer	Negligible	None, as lipid remained stuck to glass vessel
	Solubilization in detergent	Low (lipid sticks to glass during detergent removal)	Amorphous aggregates with occasional tubes
	Reverse phase evaporation	Initially high (see text)	Tubular lattices, particle arrays, possibly monolayer lipid sheet covering lattices and arrays
DG	Hydration in water	high	Bilayer vesicles (diameter is inversely proportional to extent of sonication)
	Hydration in buffer	high	Bilayer vesicles
	Solubilization in TX-100	high	Bilayer vesicles (diameter is inversely proportional to extent of sonication after detergent removal)
	Reverse phase evaporation	high	Bilayer vesicles (small diameter due to extensive vortexing, diameter increased by freezing and thawing)

particles (Fig. 2a, b). This suggests that a monolayer of lipid covers the hydrophobic tails of the tubes or particles which are presumed to be in the hexagonal II configuration [2]. Examination of freeze-fractured material obtained by either of the other methods was more difficult due to the low efficiency of transfer of MG into the aqueous phase. In addition the aggregates that were observed generally lacked a well-defined substructure. Most of them had a slightly granular appearance which was occasionally interrupted by small tubular arrays.

Digalactosyldiacylglycerol

In contrast to MG, DG was very efficiently transferred to the aqueous phase by all the methods indicated in Table I. Freeze-fracture replicas showed that bilayer liposomes were formed by all methods (Fig. 3). The largest liposomes were produced by the Triton solubilization method due to the gentle agitation of the sample during detergent removal, the step at which vesicles formed in the procedure. There was, however, a large heterogeneity in the diameter of vesicles produced by this method. As expected, sonication or vigorous vortexing produced a smaller and more uniform size distribution. Freezing (at $-20\,^{\circ}C$ or $-196\,^{\circ}C$) and thawing of vesicle preparations increased the average diameter of the vesicles as well as the size heterogeneity of the population.

Lipid mixtures

All methods tested for reconstitution of total thylakoid lipids (including pigments) produced bi-

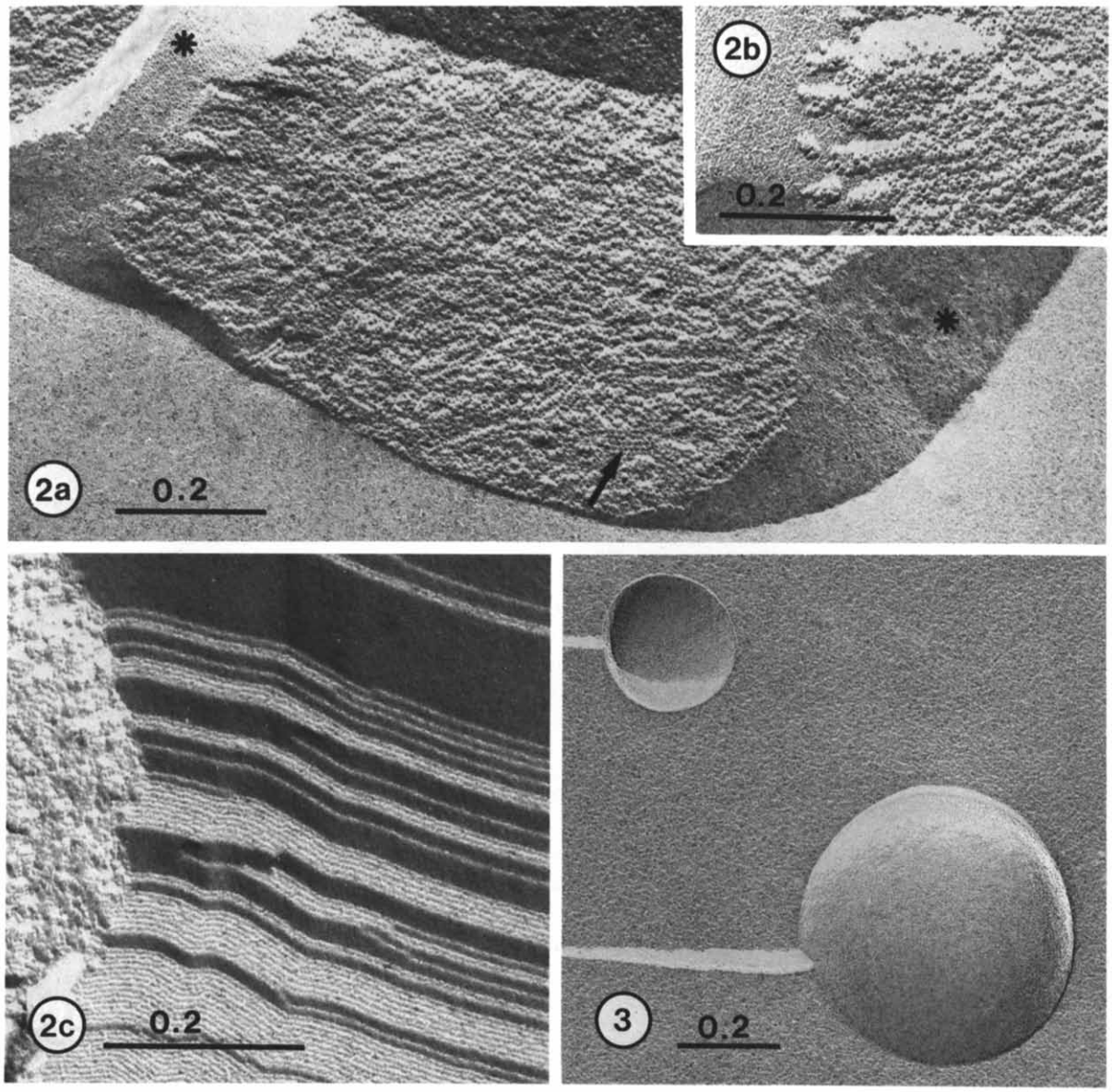


Fig. 2. Lipid aggregates formed when MG was reconstituted by reverse phase evaporation at 10 mg/ml. Packed lipid spheres (a) are shown in cross-fracture (arrow) and along the surface immediately underlying the smooth lipid layer (asterisks) covering the aggregate. The lipid layer (b) is clearly seen when the particles are removed during the fracturing process. Aggregates containing hexagonal II tubes (c) are also seen in reconstituted MG samples, in longitudinal and cross-fracture. Magnifications: (a) 107 500×; (b) 127 500×; (c) 142 500×.

Fig. 3. In marked contrast to MG, DG formed typical bilayer liposomes when reconstituted by reverse phase evaporation (shown here), hydration and detergent solubilization methods. Magnification: 74 100×.

layer vesicles with lipidic particles (Table II). Samples reconstituted by hydration (Fig. 1) also contained arrays of tubes and/or particles (Fig. 4a). The efficiency of transfer of lipid to the aqueous phase was much greater by reverse phase evaporation than by hydration. After hydration, signifi-

TABLE II

TOTAL THYLAKOID LIPIDS

Lipid extract	Method of transfer	Yield	Structures observed by freeze--fracture electron microscopy
Total thylakoid lipids (+pigments)	Hydration in buffer	Low	Some bilayer vesicles, many tubular arrays
	Hydration in water	Moderate	Predominantly bilayer, some tubular lattices and particle arrays, lipidic particles in bilayer
	Reverse phase evaporation	High	Bilayer vesicles with lipidic particles
(−pigments)	Reverse phase evaporation	High	Bilayer vesicles with lipidic particles
(−pigments, −MG)	Reverse phase evaporation	High	Bilayer vesicles with very few lipidic particles

cant amounts of the lipid remained in the thin film stuck to the glass.

Removing the pigments from the total lipid extract did not significantly change the number of lipidic particles visible in the bilayer vesicles after reconstitution by reverse phase evaporation (Fig. 4b). It did reduce the amount of clumping of the lipid during reconstitution. There was a small concentration effect with this lipid sample: liposomes that were reconstituted by reverse phase evaporation at 10 mg lipid/ml aqueous volume (Fig. 4b) contained more lipidic particles than those reconstituted at 2 mg/ml.

The molecular organization of lipidic particles is shown in Fig. 4c. The structure should give rise to particles and pits (see below) depending on the path of the fracture plane around the inverted micelle (see Ref. 24). In other instances, inverted micelles are observed where adjacent bilayer membranes are in the process of fusing (Figs. 4b, d). The sample shown in Fig. 4b exhibits, besides several completed fusion pores (arrowheads), lipid particles along lines of attachment of the adjacent membranes [14–16]. These latter lipidic particles are shared between the bilayers as depicted in Fig. 4d. Lipidic particles were essentially non-existent in samples reconstituted from thylakoid extracts from which MG as well as the pigments had been removed.

Reconstitution by hydration of a lipid mixture containing DG and SL (approx. 97 : 3) produced bilayer vesicles (Table III). Addition of approx. 24% MG to this mixture produced bilayer vesicles that contained only a few lipidic particles. However, not all the lipid was transferred to the aqueous phase in the latter case. Analysis of the lipid residue on the glass walls showed that it contained approx. 50% MG (not shown) instead of 24% as was present in the original mixture. Thus MG was disproportionately retained on the glass after hydration. Consequently the lipid composition of the bilayer vesicles was lower in MG than the original lipid mixture. This lower MG composition could account for the unexpected scarcity of lipidic particles in the liposomes produced from the 73 : 24 : 2 DG/MG/SL sample.

In another experiment the 2 : 1 MG/DG mixture of Sen et al. [7] was hydrated in 100 mM NaCl (33 : 66 DG/MG mixture in Table III). After 45 min of sonication at $< 10\,^{\circ}C$, about one third of the lipid was still stuck to the glass. The lipid in the aqueous phase and that on the glass were re-extracted and compared with the original mixture by TLC (not shown). MG was enriched in the

TABLE III

THYLAKOID LIPID MIXTURES

Lipid mixture	Method of transfer	Yield	Structures observed by freeze-fracture electron microscopy
DG/SL (97 : 3 wt%)	Hydration in water	High	Bilayer vesicles, some fused vesicles
DG/MG/SL (73 : 24 : 2)	Hydration in water	High	Predominantly bilayer vesicles, a few lipidic particles (MG selectively retained on glass)
DG/MG (wt%)			
90 : 10	Solubilization in Triton X-100	High (detergent to lipid = 1)	Bilayer vesicles
80 : 20	Reverse phase evaporation	High	Bilayer vesicles with some membrane 'fusion pores' and a few lipidic particles
75 : 25	Solubilization in Triton X-100	High (detergent to lipid = 1)	Extensive bilayer sheets interconnected through 'fusion pores', some lipidic particles
70 : 30	Solubilization	High (detergent to lipid = 2)	Bilayer vesicles, areas smaller than in 75 : 25 sample
60 : 40	Reverse phase evaporation	High	Primarily bilayer vesicles, some segregated regions with membrane 'fusion pores', lipidic particles and tubes
50 : 50	Solubilization in Triton X-100	High	Crystalline particle arrays, tubular lattices, lipidic particles in very limited bilayer areas
	Reverse phase evaporation	High	Small mixed bilayer and tubule-containing vesicles
40 : 60	Reverse phase evaporation	High	Small 'solid vesicles' some with tubular and particle cores
33 : 66	Hydration in 100 mM NaCl	Moderate (45 min bath sonic 4–10 °C)	Amorphous aggregates with particle arrays and tubular lattices
	Reverse phase evaporation	Moderate (clumping)	Extensive hexagonal II tubes, lipidic particles in very limited bilayer
30 : 70	Reverse phase evaporation	Moderate (clumping)	Extensive tubular arrays, covered with smooth lipid layers (angular and spherical surface layers)

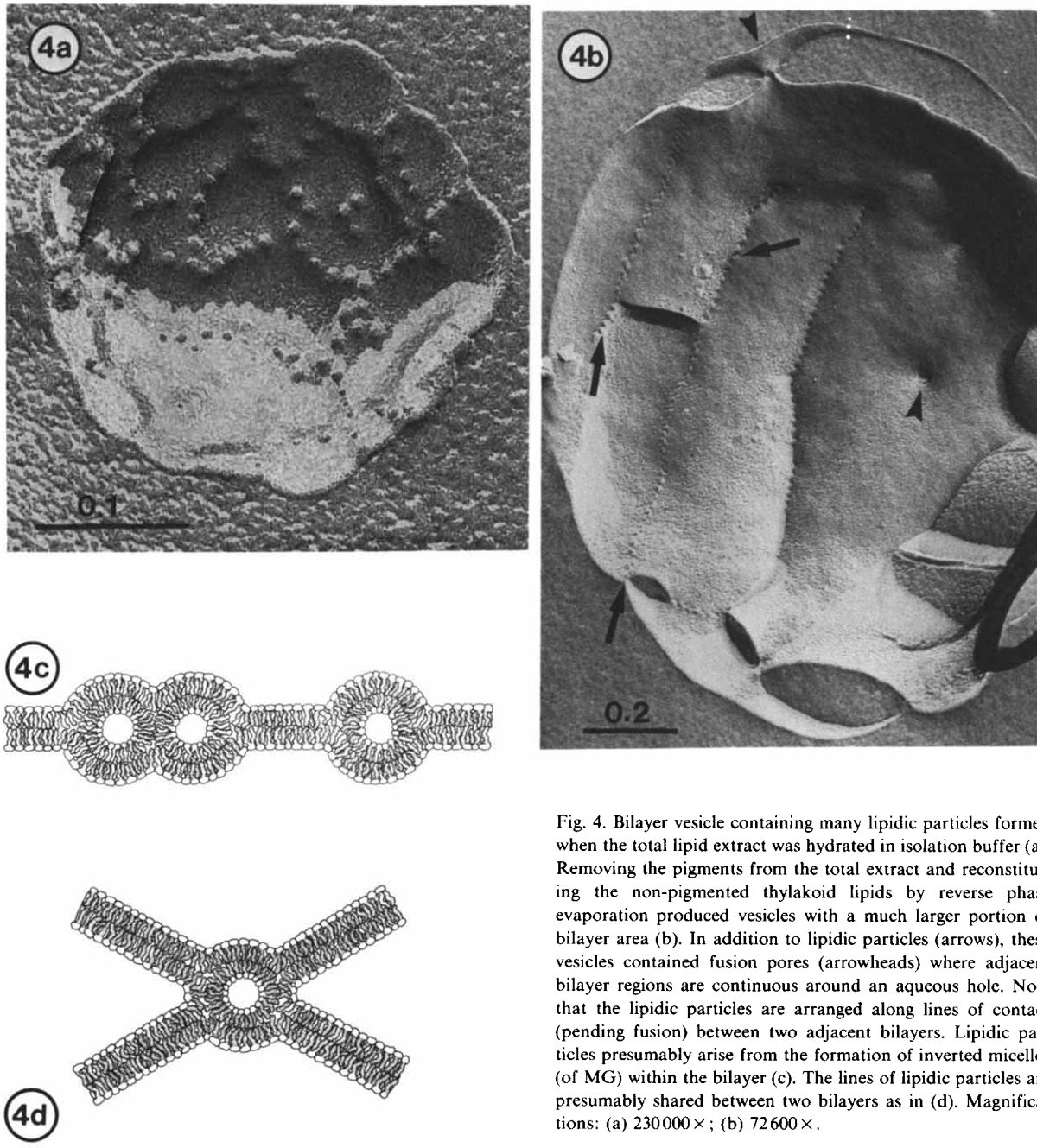


Fig. 4. Bilayer vesicle containing many lipidic particles formed when the total lipid extract was hydrated in isolation buffer (a). Removing the pigments from the total extract and reconstituting the non-pigmented thylakoid lipids by reverse phase evaporation produced vesicles with a much larger portion of bilayer area (b). In addition to lipidic particles (arrows), these vesicles contained fusion pores (arrowheads) where adjacent bilayer regions are continuous around an aqueous hole. Note that the lipidic particles are arranged along lines of contact (pending fusion) between two adjacent bilayers. Lipidic particles presumably arise from the formation of inverted micelles (of MG) within the bilayer (c). The lines of lipidic particles are presumably shared between two bilayers as in (d). Magnifications: (a) 230000×; (b) 72600×.

residue stuck on the glass, relative to both the starting mixture and the material in the aqueous phase. Most of the lipid in the aqueous phase was present as visible clumps. Some of these clumps were frozen (from 10 °C) without glycerol for freeze fracture analysis. Another aliquot of the sample was warmed to 50 °C for 30 min and then frozen, again without cryoprotectant, for microscopy.

Freeze-fracture electron microscopy of the 33 : 66 sample quenched from approx. 10 °C showed lipid aggregates containing 7–9 nm diameter tubes (Fig. 5), presumably of the hexagonal II-type, and indistinct, square-packed clumps of 14–16 nm particles (Fig. 5). The heated sample exhibited these same structures as well as extensive, distinct square (cubic) and hexagonal (rhombohedral lattice) arrays of 10–13 nm particles (Fig. 6). The particles resembled those seen in glycerinated samples of similar mixtures prepared by detergent solubilization and reverse phase evaporation at room temperature (see below). Along the edges of the aggregates in the hydrated and heated sample we also observed bilayer areas with some lipidic particles suggesting that at 50 °C some DG might be leaching out of the lipid aggregate. Reconstitution of the same mixture by reverse phase evaporation showed all of the same structures. Limited bilayer areas laced with lipidic particles were present in the interior of the aggregates as well as on the periphery.

Triton X-100 (0.1%) coupled with sonication readily removed all traces of lipid from the glass vessel at detergent to lipid ratios of 1 or 2. Lipid structures formed as the Triton was removed from the solution during the incubation with biobeads.

Using the detergent solubilization method allowed incorporation of 10–20% (by wt.) MG into DG liposomes without disruption of bilayer structure (detergent/lipid = 1 during solubilization). When MG was increased to 25% of a DG/MG mixture, extensive bilayer membrane networks with surface irregularities were seen (Fig. 7). Most of these irregularities appeared to be contact points between adjacent bilayer areas similar to those described for phospholipids [14–16], suggesting that fusion of bilayer liposomes had occurred as the detergent was removed. A few of these irregularities were much smaller (8–10 nm), resembling lipidic particles or inverted micellar structures within the bilayer. Mixtures containing equal weights of MG and DG (Fig. 8) produced mixtures of hexagonally-packed 8–9 nm tubes, presumably of the hexagonal II-type, crystalline arrays of 10–12 nm particles, and traces of bilayer areas rich in lipidic particles. The structural relationship between the tubes and particles is illustrated in

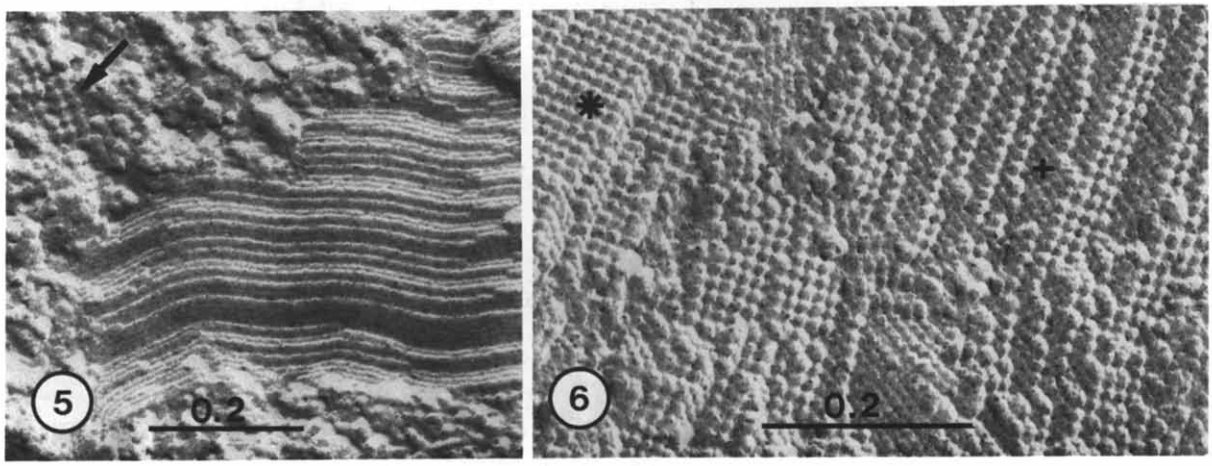


Fig. 5. Lattice of hexagonal II-type tubes seen in replicas of a 33 : 66 DG/MG sample formed at less than 10 °C by sonication in 100 mM NaCl. The sample was maintained at 10 °C and frozen without warming or glycerination. Some ordered particle arrays were also seen in these samples (arrow). Magnification: 100000×.

Fig. 6. Replicas of warmed (non-glycerinated) samples (33 : 66 DG/MG as in Fig. 5) show all the features seen in the samples frozen from 10 °C but more crisply. In addition, the warmed sample contained extensive arrays of ordered particles, some of which were in rhombohedral or trigonal (hexagonal close-) packing (+) and many of which were in the less energetically favorable square-square (cubic) packing configuration (*). Magnification: 140000×.

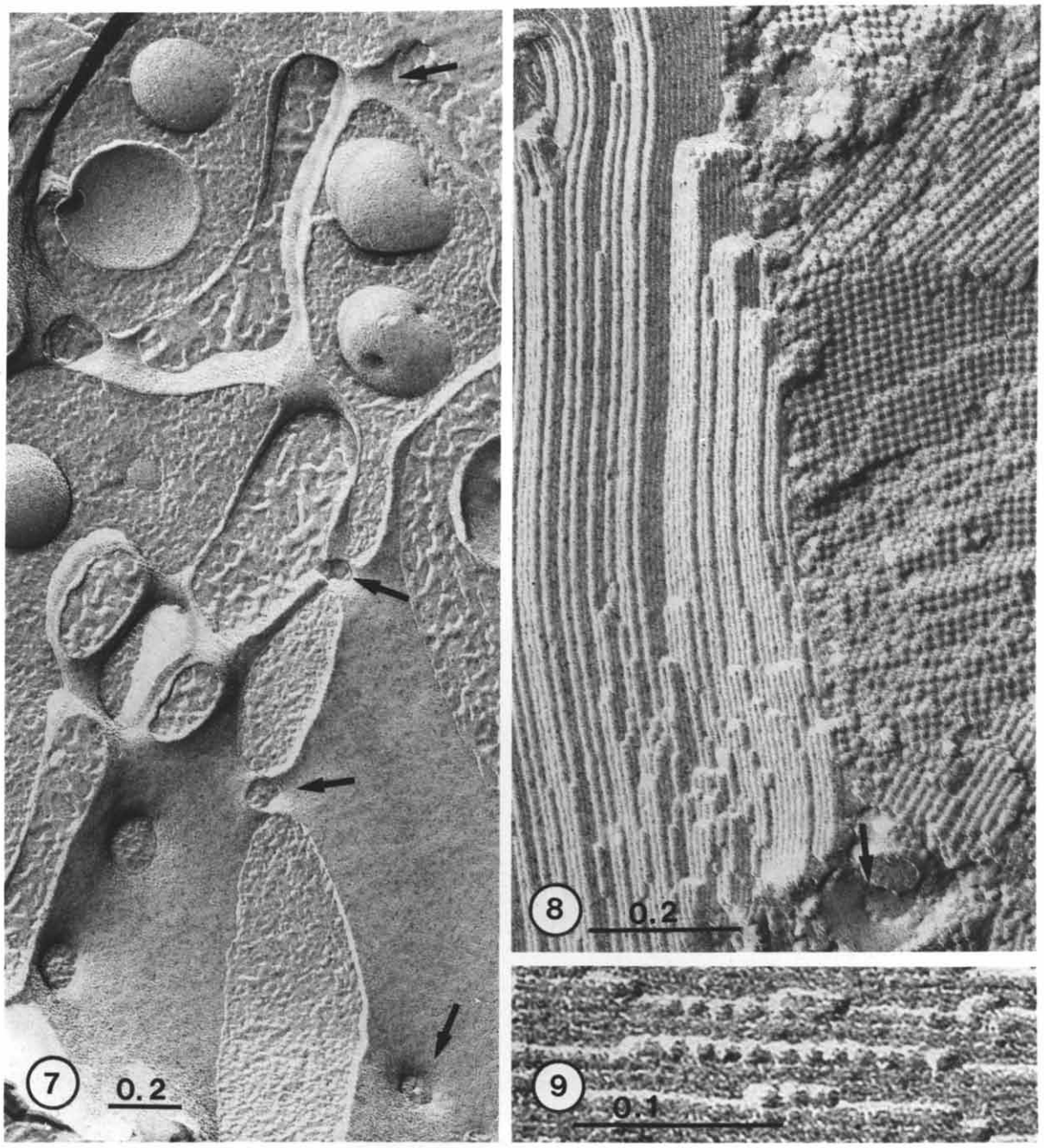


Fig. 7. Samples containing 25% MG and prepared by detergent solubilization exhibited large bilayer areas with fusion pores (arrows). The aqueous areas are easily distinguished from the membranes due to the slight etching of the sample before replication. Magnification: 56000×.

Fig. 8. Increasing the MG content to 50% changed the structures observed from primarily bilayer to non-bilayer organization when the detergent solubilization method was used for reconstitution. Extensive hexagonal II-type tubular lattices and packed inverted micellar spheres predominated. Occasionally a small bilayer area with lipidic particles was observed (arrow). Magnification: 113850×.

Fig. 9. This micrograph shows the interrelatedness of the tubes and spheres, suggesting a dynamic equilibrium between these two structures. Magnification: 320000×.

Fig. 9 where the tubular array is interrupted by rows of spheres, suggesting that the two structures may be interconvertible (see models in Ref. 25 and 26). Most of the particle arrays appeared to be in the form of a rhombohedral, or trigonal, lattice (Fig. 10) exhibiting adjacent square (a) and hexagonally (b) packed areas depending on the fracture plane (compare Figs. 6, 8 and 19). In a rhombohedral lattice, hexagonally packed layers of spheres are 'nestled' in the closest packing arrangement where centers of spheres in one layer are over spaces between spheres in the adjacent layer.

By increasing the detergent to lipid ratio to two, 30% MG could be incorporated into bilayer structures with only a very limited number of lipidic particles. In addition, vesicles formed with the higher detergent ratio (two) were much smaller than those of similar mixtures formed with the lower detergent ratio (one). These two observations suggested to us that detergent removal was incomplete (or at least not reproducible). Detergent molecules remaining incorporated into the liposomes could easily be complexed with MG in a fashion that would suppress lipidic particle formation. Alternatively, smaller vesicles with a greater radius of curvature could result, thereby allowing proportionately more MG to be associated with the inner leaflet. Regardless of the molecular mechanism, the presence of any detergent in the liposomes was unacceptable for any future experi-

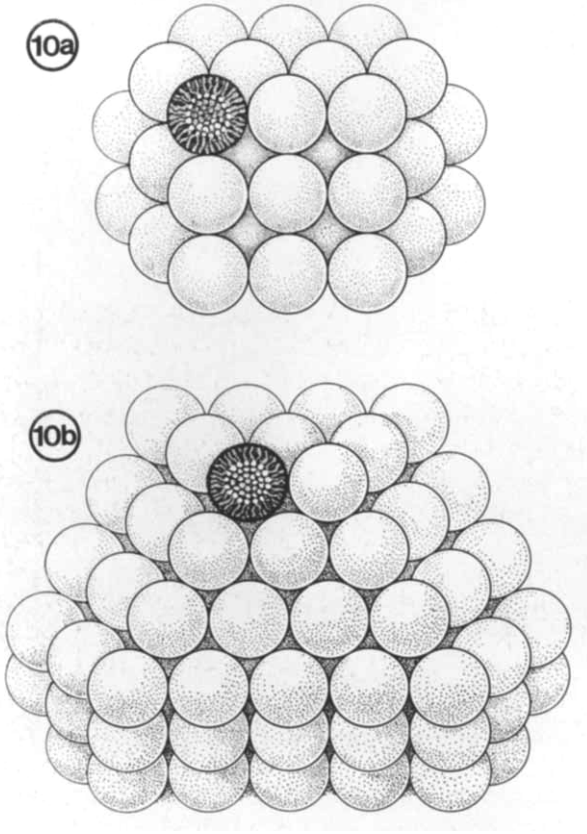
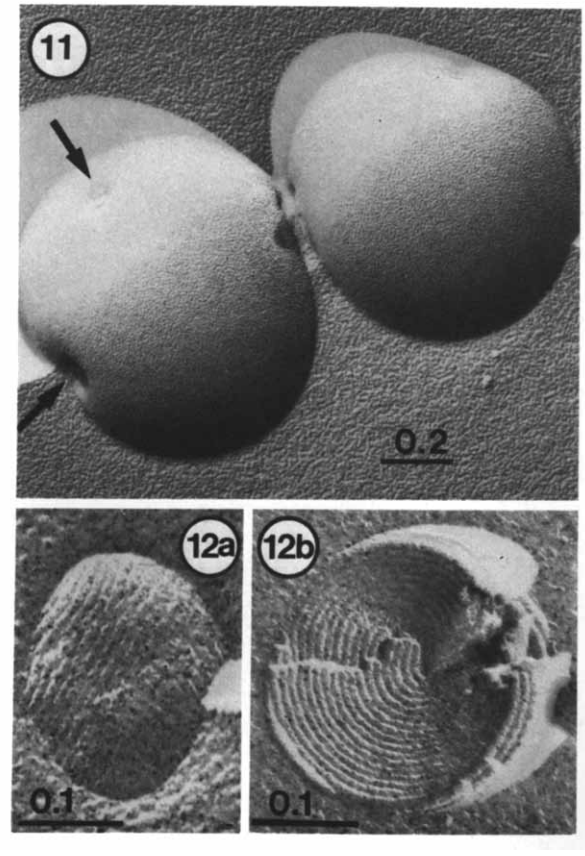
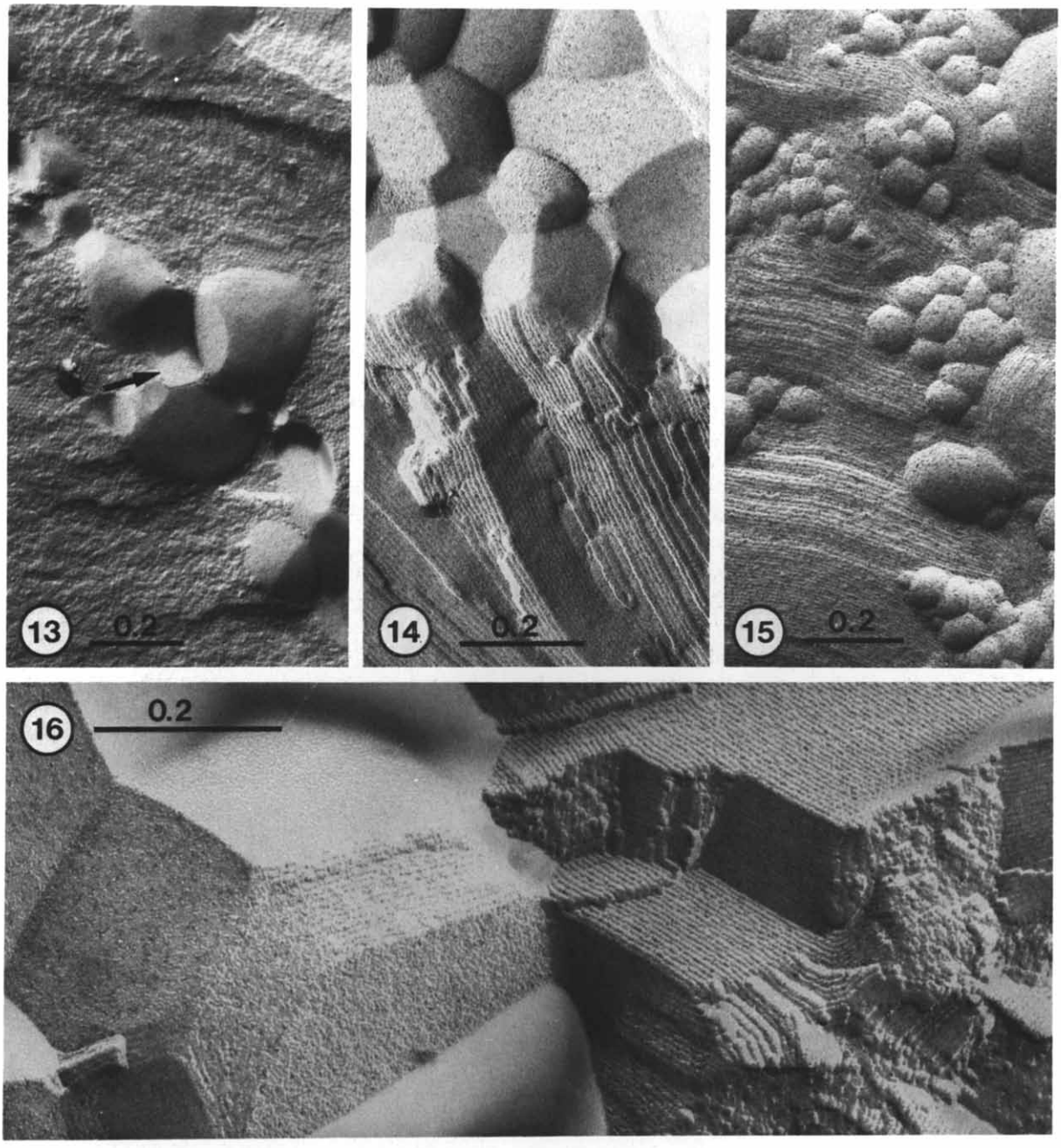


Fig. 10. The packed spheres observed in the 50:50 DG/MG (detergent solubilization) and the 30:70 DG/MG reverse phase evaporation mixtures appear most often to be in a rhombohedral or trigonal lattice. The fracture plane can thus pass through the lattice in many directions giving rise to either square (a) or hexagonally (b) packed particle arrays.



Figs. 11 and 12. Using reverse phase evaporation for reconstitution of a 50:50 DG/MG mixture produced bilayer vesicles (Fig. 11) with fusion pores (arrows) as well as some small tubular vesicles (Fig. 12). Surface (a) and deeper cross (b) fractures are shown. Magnifications: Fig. 11, 45500×; Fig. 12 (a) and (b) 140000×.



Figs. 13–16. Transition regions in MG-rich lipid aggregates showing the relationship between packed particles or tubes and the smooth lipid layers. Fig. 13 shows smooth layers which presumably formed around water pockets within an extensive particle aggregate of 100% MG (reverse phase evaporation). Note the rounded surfaces of the pocket lining adjacent to the particles and the smooth surfaces (arrow) where they abut (resembling aggregated soap bubbles). The transition from tubular arrays to the angular water-interface layers is shown in Fig. 14, in a 30:70 DG/MG aggregate formed by reverse phase evaporation. On the surface of an aggregate, the smooth surfaces tend to take on a more spherical shape (Fig. 15) suggesting that DG is leaching out of the aggregate into the aqueous surroundings. A higher magnification view of smooth layers covering bundles of aggregated tubes in a 30:70 DG/MG sample is shown in Fig. 16. Magnifications: Fig. 13, 70000×; Fig. 14, 92400×; Fig. 15, 72000×; Fig. 16, 140000×.

ments involving, e.g., reconstitution of biological samples. Thus even though the detergent solubilization method allowed incorporation of a significant amount of MG into bilayer vesicles, the method appeared inadequate for providing liposomes suitable for use in biological experiments.

Revere phase evaporation also transferred all of the lipid to the aqueous phase. DG liposomes prepared by reverse phase evaporation could accomodate 30–40% MG without disruptions of the bilayer structure. Sonication of DG liposomes (45 min, 40 °C) prepared by reverse phase evaporation forced slightly more MG into vesicles with the bilayer configuration, 40–50% MG. Comparison of the samples prepared from DG/MG mixtures by reverse phase evaporation and detergent solubilization methods showed no major differences in the type of structures formed. There was, however, a difference in the concentration of MG at which the transition from 'primarily bilayer' to 'crystalline aggregate of particles and tubes' occurred. With the Triton solubilization method the transition was clearly complete in a 50 : 50 mixture of DG/MG (see Table III and Fig. 8). When this same 50 : 50 mixture was used for reverse phase evaporation, we observed mostly small bilayer vesicles as well as some tubule-containing vesicles (Figs. 11 and 12). Only when the mixture contained 66–70% MG did a sample prepared by reverse phase evaporation produce a crystalline lipid aggregate. Additionally, clumping of lipids in the aqueous phase did not occur until the mixture was 66–70% MG. In contrast to the 100 % MG samples which always exhibited smooth lipid layers, generally over particles (Figs. 2 and 13) and occasionally over tubes (Fig. 13, arrow); the 30 : 70 (DG/MG) mixture contained smooth lipid layers covering extensive tubular arrays (Fig. 14–16). The smooth layers over clusters of packed tubes produced very angular structures in some areas (Figs. 14 and 16) while in others it appeared more spherical as in soap bubbles (Fig. 15). The latter were observed mostly along the external surfaces of the tubes, suggesting the blebbling of vesicular structures out of the aggregate into the surrounding aqueous phase (see 60 : 40 sample below). As mentioned above, the smooth fracture faces illustrated in Figs. 13–16 represent lipid/water interface regions, either on the surface of the aggregates (hexagonal II-type tubes or inverted micelles) or surrounding aqueous pockets within the lipid masses. The diagram in Fig. 17 shows our interpretation of how lipids in a monolayer configuration cover the hydrophobic surfaces of the aggregates at these interfaces. The relationship between rounded (Figs. 13 and 15) and angular (Figs. 14 and 16) interfacial monolayers can be discerned in Fig. 13 (arrow). Here a rounded configuration of the monolayer surfaces is visible adjacent to the inverted micellar aggregates, while flat, angular surfaces are seen where adjacent monolayers abut (Fig. 13, arrow), much like the associations seen in aggregates of soap bubbles. While most flat angular surfaces appear to arise in this manner, others may reflect on the angular nature of the packed hexagonal II-type tubes. At the extreme surfaces of lipid aggregates, rounded monolayer structures may arise where surface blistering is occurring.

The 60 : 40 (DG/MG) mixture consisted mainly of many small bilayer liposomes (Fig. 18) with a few interesting lipid aggregates (Figs. 19 and 20) as well as some bilayer vesicles rich in lipidic particles (Fig. 21). The lipid aggregates (Fig. 19) were remarkable because they contained: (1) typical hexagonal II-type tubes (8–9 nm); (2) larger than usual particles (20–21 nm) in a square-packed (cubic) array; (3) bilayer vesicles apparently for-

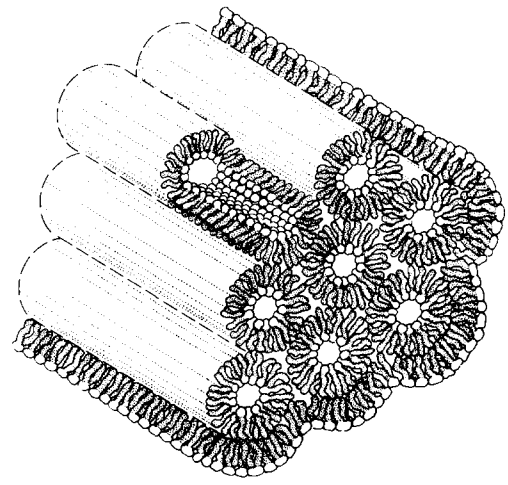


Fig. 17. Model showing the monolayer arrangement of lipids at the interface between the water phase and tightly packed hexagonal II-type tubes that could give rise to the images seen in Figs. 13–16.

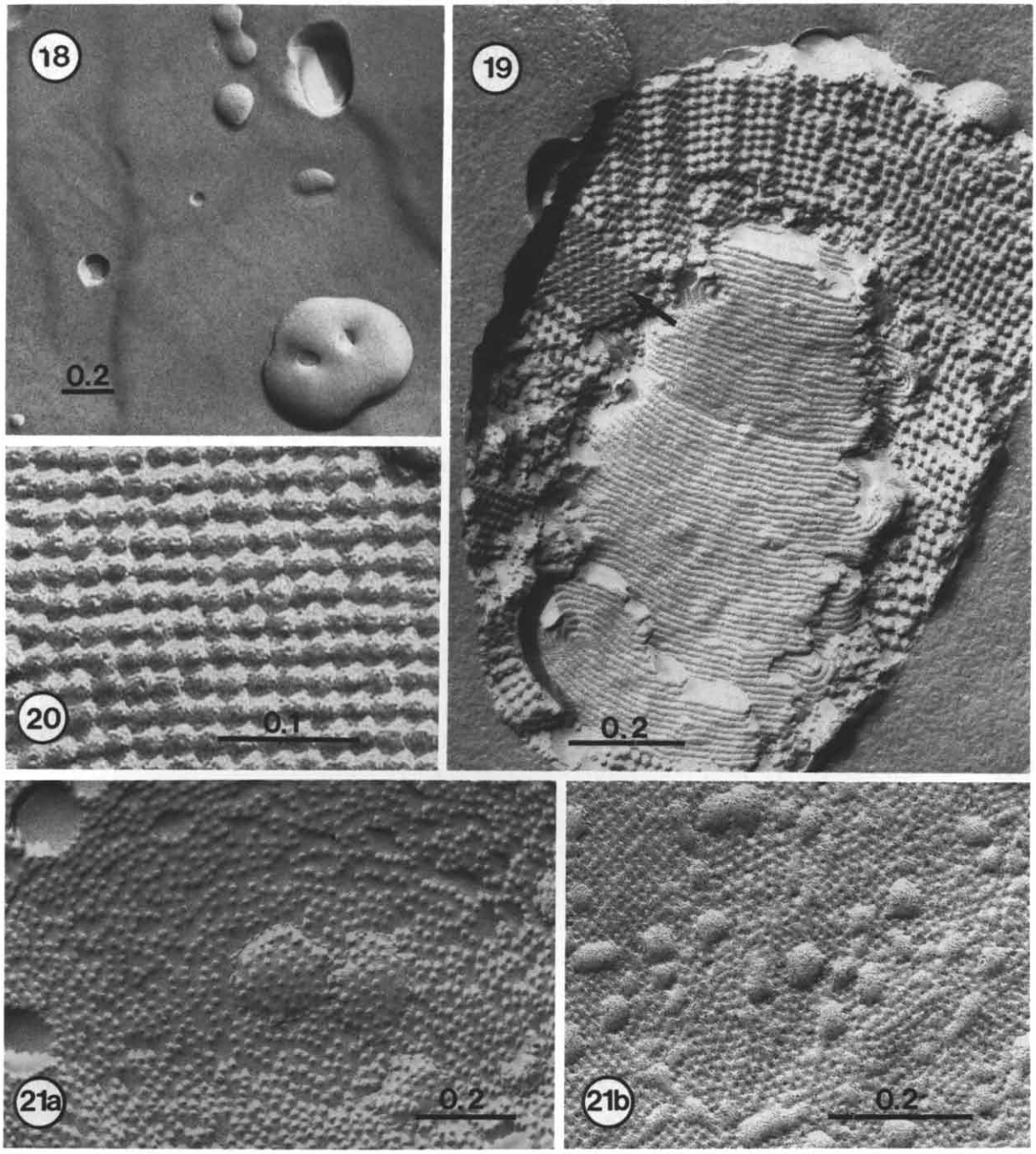


Fig. 18. Representative field of liposomes (including fusion pores) from 60:40 DG/MG mixture prepared by reverse phase evaporation. Magnification: 40300×.

Fig. 19. Lipid aggregate from the 60:40 DG/MG sample. Note the hexagonal (arrow) and square-packed regions of inverted micelles; the surface blebbling of bilayer regions (presumably DG); and the holes in the square-packed spheres. Magnification: 90000×.

Fig. 20. Higher magnification of a portion of the lipid aggregate showing the holes present in many of the square-packed spheres. Magnification: 218000×.

Fig. 21. Lipidic particles (a) and pits (b) in aggregates formed by reverse phase evaporation of a 60:40 DG/MG mixture demonstrating the complementary fracture planes around inverted micelles within a bilayer. Magnifications: (a) 77000×; (b) 110000×.

ming from the surface; and (4) small central holes (Fig. 20) on the exposed surface of some of the 20 nm particles, suggesting continuity with another square-packed layer removed during fracturing. Views of both particle and pit fields (Fig. 21) were obtained from this sample, demonstrating the complementary fracture faces associated with inverted micelles within bilayer membranes [24].

Discussion

Bilayer liposomes of DG can be readily formed by a variety of lipid hydration techniques (Fig. 1). In contrast, quantitative transfer of MG to an aqueous medium is not as easily achieved. While Triton X-100 solubilization of MG from the glass vessel is very efficient, subsequent removal of the detergent with biobeads appeared neither complete nor reproducible. Since the presence of any detergent in liposomes is unacceptable if such liposomes are to be used for biological studies, we have abandoned the use of detergent-solubilization methods in favor of the reverse phase evaporation method for transfer of lipids or lipid mixtures to the aqueous phase. This method has proven to be not only simple, but also quantitative and reproducible in our laboratory.

Our results suggest two problems associated with characterization of the lipid structures associated with a given lipid mixture after conventional hydration. The first is that the lipid in 'solution' or in a lipid clump may not accurately reflect the initial mixture if the lipid is hydrated simply by heating and/or sonication (MG sticks to the glass). Long incubation/sonication times may partially overcome this problem but reproducibility is not assured. The second problem is that the structures formed immediately (in the absence of detergent) may not be stable during lengthy incubations at or above room temperature (leeching of DG from DG/MG clumps).

In the context of the first problem, incomplete transfer of MG in a mixture of MG and DG would produce an overestimation of the concentration of MG necessary to create non-bilayer structures. This could account for our observation that all the structural configurations seen by Sen et al. [4,6] in a 33 : 66 DG/MG mixture were already present in our 50 : 50 DG/MG mixture. Replication of some of their experiments in our laboratory suggests that it was impossible for them to obtain a complete transfer of MG into the aqueous phase. This incomplete transfer of MG would also account for our observation of both hexagonal II-type tubes and some particles in a cold-produced and quenched DG/MG 33 : 66 sample (Fig. 5) where Sen et al. [5] observed primarily bilayers (DG) with a relatively low density of lipidic particles (MG).

The effects of heating and ethylene glycol [4] on the structures obtained from DG/MG mixtures appear, in our hands, to be ones of degree rather than of kind. We see much the same structures in the absence of glycerol or heat (Fig. 5) as we do in glycerinated samples. The glycerinated or heated samples appear structurally to be much more well defined than those without glycerol, suggesting a stabilizing effect of glycerol possibly in favoring specific forms in an equilibrium or by reducing plastic deformation during fracturing [27].

One of the most intriguing features of the DG/MG samples containing between 30 and 70% MG is the presence of extensive particle arrays in which square and hexagonal packings of particles co-exist in close association with each other and with hexagonal II-type tubular structures. As shown in the diagram Fig. 10 the two types of particle arrays can be produced by fracturing a rhombohedral, or trigonal, lattice along different planes. The lattice depicted in Fig. 10b reveals, besides several planes in which the particles are hexagonally packed, three square-packed planes (one on each side and one at the bottom of the structure). Another puzzling aspect of the particle arrays is the variability of the particle sizes in samples containing different ratios of MG and DG (7–9 nm in Fig. 2, 10–15 nm in Figs. 4 and 21, and 20–21 nm in Figs. 19 and 20). In Fig. 22 we provide a model that could account for the observed variations in the particle diameters. Assuming different overall molecular shapes for MG and DG [15,10–12] one can estimate the radii of inverted micelles containing varying ratios of MG and DG molecules. The estimated diameters for the inverted micellar structures fall within the measured values. Nevertheless, the tendency of DG to form bilayers may alter the DG/MG ratio in a given particle aggregate with time such that

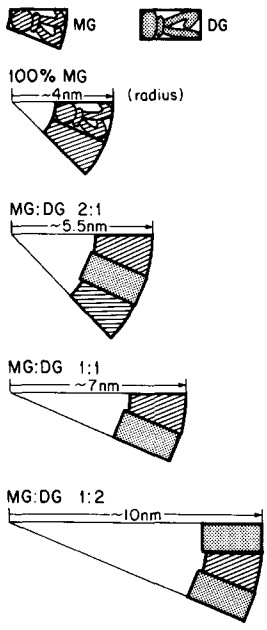


Fig. 22. Model suggesting how different ratios of MG and DG could give rise to inverted micelles with different diameters. Assuming an overall cone shape for MG and an overall cylindrical shape for DG allows for an increase in the radius of curvature as the DG : MG ratio increases.

the diameters would not depend absolutely on the compositions of the starting mixture. Our interpretation of the smooth surfaces seen in the 100% MG and 30 : 70 DG/MG samples (Fig. 17), where a monolayer covers the hexagonal-II type tubes at the interface with the aqueous phase, can be attributed to either MG or DG, depending on the sample, if one assumes that the interaction between the hydrophobic fatty acid tails and water is so unfavorable that even MG will form a relatively smooth or planar structure under adverse conditions.

The most puzzling structures observed in our samples are the small lipid aggregates that contain, besides hexagonal II-type tubes, large spheres (20–21 nm) with holes (Figs. 19 and 20). The large size of the spheres could arise from packing DG/MG in a ratio as in Fig. 22, but the holes remain perplexing. The spheres with holes may reflect an intermediate state in a dynamic equilibrium between closed spheres and tubes, a budding off process for example, as proposed for several phospholipid systems [25,26]. However, such structures should be extremely short-lived and therefore very hard to capture during freezing. Additionally, the tubes in all of our samples are much smaller (8–9 nm) and hexagonally packed whereas the particles are obviously square-packed in the viewing plane and with respect to the removed plane, indicative of a pure cubic packing. The holes, presumably related to the regions of contact between particles of adjacent planes, appear centered in the particles. Similar structures have been reported for phospholipid samples, and have been implicated as transition structures between lamellar, hexagonal II and cubic phases [28–30]. An alternative explanation for the origin of the holes is that the spheres are actually bilayer vesicles joined together as in a prolamellar body [31] but that they are more tightly packed (see model in Ref. 30). The minimum size for a bilayer vesicle of MG and DG is about 18 nm assuming that the inner leaflet is 100% MG and given that the holes are 6–7 nm. Addition of any DG to the inner leaflet would increase the radius of curvature and hence the size of the sphere. Again this kind of tightly packed and fused structure would be reasonably unstable and the likelihood of capturing it during freezing is not great. It seems more likely that the large (20–21 nm) particles are pure inverted micelles and not tiny bilayer vesicles, because the lipid aggregates containing the large particles are always covered by what appears to be a monolayer of lipids as in the 100% MG samples, suggesting a hydrophobic exterior surface. In contrast to the structures seen by Borovjagin et al. [28] and Sen et al. [7], we do not believe that any of our tubular arrays are bilayer tubes, partily due to their small diameter but also because of the tight packing of the tubes.

In conclusion, the reverse phase evaporation of lipids in Freon appears to provide the best method for rapidly and reproducibly forming galactolipid liposomes of a known composition, efficiently transferring both MG and DG to the aqueous phase. Although we still have not been able to produce bilayer structures with a native ratio of MG to DG of 2 : 1, we can incorporate more MG (40–50%) into DG bilayer liposomes without forming lipidic particles by using this method than by using either of the others described above (Fig. 1). Preliminary work suggests that sonicating or heat-

ing the samples, or adding other thylakoid lipids (SL, PG or PC) promotes bilayer formation from DG/MG mixtures containing high concentrations of MG.

Acknowledgements

We would like to thank Dr. David McKay for assistance in describing crystal structures, and Ms. Jan Logan for her excellent art work. This work was supported by the National Science Foundation, Grant PCM 8118627.

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