Short Communication

A Rapid Reverse Phase Evaporation Method for the Reconstitution of Uncharged Thylakoid Membrane Lipids That Resist Hydration[1]

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ABSTRACT

Comparison of several lipid reconstitution methods showed that they were not equally efficient at transferring the predominant thylakoid lipid, monogalactosyldiglyceride (MG), to the aqueous phase. We report a reverse phase evaporation method that employs Freon 11 as a lipid solvent and is capable of successfully hydrating MG in spinach (*Spinacia oleracea* L.) at room temperature within minutes. Using this method it is possible to force an equal weight mixture of MG and digalactosyldiglyceride into small bilayer vesicles without the formation of inverted micellar 'lipidic particles' in the membranes.

Our aim in this work was to establish a procedure by which bilayer vesicles of variable and known composition could be formed quickly and reproducibly from endogenous thylakoid lipids. Such vesicles will be used to lipid-enrich (9, 10, 16) thylakoid membranes so that we can (a) positively identify the protein complexes seen on the P-face of freeze-fractured thylakoids, and (b) investigate the physiological effects of increasing the lipid to protein ratio of these membranes.

Siegel et al. (16) have shown that lipid enrichment of thylakoid membranes using phospholipid vesicles causes extensive membrane fragmentation as well as particle aggregation. In contrast, lipid enrichment of inner mitochondrial membranes with phospholipids (9, 10) can be carried out without any structural degradation of the membranes. The important difference between the two membrane systems probably lies in the fact that phospholipids are the major lipid component of inner mitochondrial membranes and only a minor (10–13%, Ref. 3) component of thylakoid membranes. The predominant thylakoid lipids are the uncharged MG[2] and DG. They contribute approximately 51 and 26%, by weight, of the thylakoid lipids, respectively. Addition of large amounts of charged phospholipids to thylakoid membranes could be sufficient to cause the structural alterations observed by Siegel et al. (16). Thus, we have undertaken to form bilayer liposomes from endogenous thylakoid lipids.

[2] Abbreviations: MG, monogalactosyldiglyceride; DG, digalactosyldiglyceride; RPEV, reverse phase evaporation.

MATERIALS AND METHODS

Lipids were extracted from commercial spinach (*Spinacia oleracea* L.) thylakoids with chloroform:methanol and separated on a preparative silicic acid column (17). Thylakoid lipids (mixtures and individual classes) were transferred to an aqueous medium by (a) hydration in distilled H_2O, 0.1 M NaCl solution, or buffer (50 mM Na phosphate, 0.1 M KCl, pH 7.2); (b) solubilization in detergent; or (c) RPEV from Freon 11 (trichlorofluoromethane, bp 23°C; Matheson, East Rutherford, NJ).

Heating times in the simple hydration experiments were varied from 5 to 10 min at 30°C to 60 min at 80°C to maximize transfer of lipid from the glass to the solution. Sonication was either in a bath for 45 min (simple hydration experiments) or with a probe for two or three 30-s bursts (Triton experiments). Lipids were routinely used at 2, 10, and 20 mg/ml in the final aqueous volume and all treatments were carried out in a N_2 atmosphere. The RPEV method used here was modified from the work of Cafiso et al. (1) to accommodate larger volumes, rapid reconstitution, and lower temperatures. The schemes used for reconstitution are outlined in Figure 1. The ratio of Freon to water was 1:2 (v/v) in most of these experiments. Changing the ratio slightly did not affect the results as long as an emulsion could be maintained during warming. Continuous vortexing maintained an emulsion at all the Freon/water ratios tested. Slow warming (5–10 min) of the Freon/water emulsion was necessary to prevent boiling of the mixture. More rapid warming (approximately 2 min) produced such violent boiling that most of the lipid sample was spattered over the inside of the tube making recovery next to impossible.

The efficiency of transfer was assayed by TLC (chloroform:methanol:water [65:25:4, v/v] and/or chloroform:methanol:ammonium hydroxide [65:35:5, v/v]) of the material re-extracted from the glass vessel walls after removal of the hydrated sample.

For freeze-fracture electron microscopy, samples were either glycerinated to 35% (v/v) and dip-frozen in liquid nitrogen-cooled Freon 12 or jet frozen without glycerol. Pt-C replicas were made at −115°C in a Balzers 360M freeze-etch instrument equipped with electron guns.

RESULTS AND DISCUSSION

MG, the predominant thylakoid lipid (3), is very difficult to transfer quantitatively to the aqueous phase by hydration and sonication. MG can be completely solubilized with 0.1% (v/v) Triton X-100 but does not stay in solution during removal of
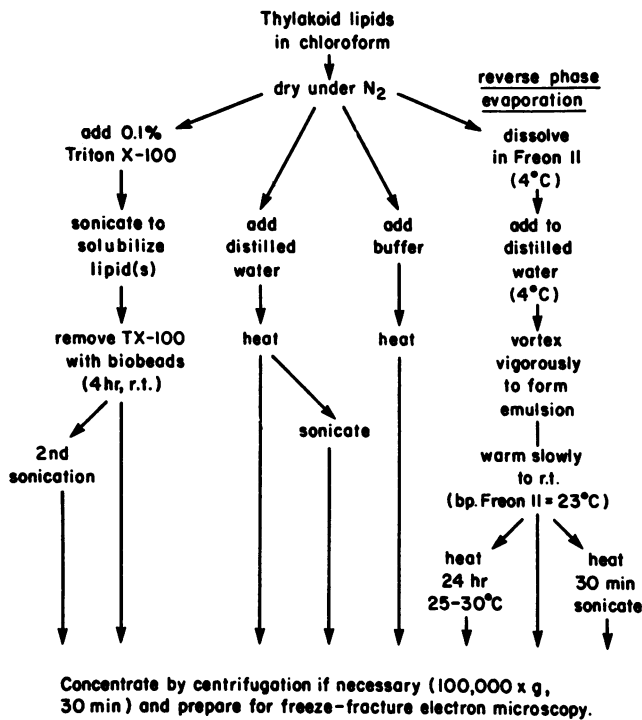
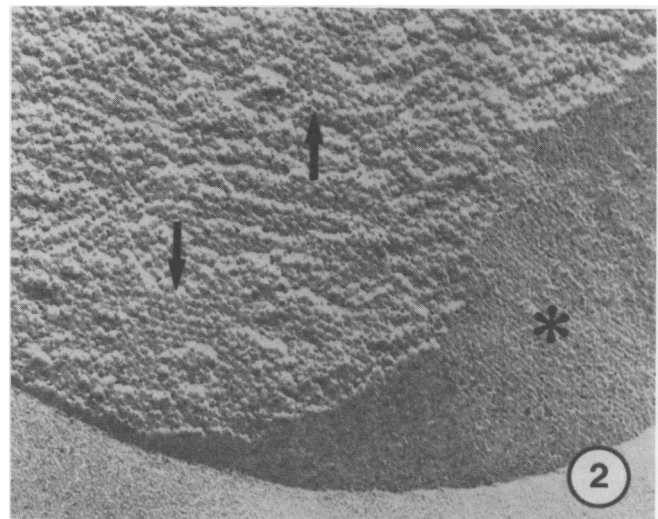FIG. 1. Methods for formation of lipid structures.

FIG. 2. Packed lipid spheres produced when pure MG was reconstituted by RPEV at 10 mg/ml. The spheres are shown in cross-fracture (arrows) and along the surface immediately underlying the smooth lipid layer covering the aggregate (asterisk). Magnification: × 158,000.

the detergent with Biobeads (BioRad, Richmond, CA). Instead, the lipid forms large clumps that stick to the glass. Reverse phase evaporation is more efficient in rehydrating MG: the lipid forms clumps but these clumps do not stick to the glass. The clumping is most apparent at the highest concentration of MG that we tested (20 mg/ml). Lowering the concentration of MG to 10 or 2 mg/ml decreases the amount and size of the clumps. MG is readily soluble in Freon 11 at 40 to 50 mg/ml, the highest concentration we tested.

Freeze-fracture electron microscopy shows that MG transferred by RPEV is organized into extensive arrays of hexagonal II-type tubes (periodicity: 7–9 nm) as previously reported (15) or into large aggregates of small spheres. Both the tubes and the spheres are covered by a smooth lipid monolayer (Fig. 2). We favor the interpretation of the spheres as inverted lipid micelles, analogous to the hexagonal II-type tubes, rather than as small bilayer vesicles because of (a) their small size (approximately 7–9 nm) and (b) the existence of the surface monolayer of lipids protecting the hydrophobic lipid tails of the inverted micelles from the aqueous surroundings.

In contrast, DG, the second most prevalent thylakoid lipid (3), readily disperses into aqueous phases as bilayer liposomes. All of the methods that we used successfully transfer all of the DG into the aqueous phase. As expected, the diameter of the liposomes decreases with sonication and increases after freezing and thawing. DG is also readily soluble in Freon 11 at 50 mg/ml.

The efficiency of transfer of binary mixtures of thylakoid galactolipids to the aqueous phase varies significantly with the method employed. The primary problem in all of the simple hydration experiments was that MG is selectively retained on the glass walls of the vessel. Use of Triton to solubilize the lipid overcomes this problem completely but creates another: Biobeads do not appear to remove all the detergent. The presence of any detergent is unacceptable since we plan to use these vesicles for lipid-enriching native membranes; thus, we developed a simple and rapid RPEV method (Fig. 1) to transfer all the lipid to the aqueous phase efficiently and reproducibly.

A further advantage of our RPEV method is demonstrated by

freeze-fracture electron microscopy. The Biobeads incubation for detergent removal is slow and allows formation of large lipid clumps when the percentage of MG is high (>40% by weight), RPEV is rapid and sufficiently vigorous that large scale aggregation of lipid does not occur until the mixture contains more than about 66% (by weight) MG. A 50:50 (w/w) mixture of MG and DG prepared by detergent solubilization and removal shows extensive arrays of hexagonal II-type tubes and packed spheres (Fig. 3). In contrast, a mixture of the same composition (Fig. 4) prepared by RPEV shows primarily bilayer liposomes with fusion pores and a few small vesicles filled with aggregates of coiled hexagonal II-type structures. TLC of extracts of the hydrated material and of the glass vessel shows that in fact no lipid remains associated with the glass after either Triton solubilization or RPEV of 50:50 (w/w) mixtures.

In summary, we find that the proportion of lipid in non-bilayer configurations in binary mixtures of plant galactolipids increases with the amount of MG in the mixture as expected from the work of Sen et al. (11–14), Shipley et al. (15), and the theoretical calculations of Murphy (6, 7). The first non-bilayer structures to appear are 'lipidic particles' (2, 4) and fusion points (5, 8) between adjacent bilayer vesicles. The MG concentration at which we observe the transition from bilayer liposomes to mostly non-bilayer structures (hexagonal II-type tubes and packed inverted micellar spheres) depends on the method used for transfer of the lipids to the aqueous phase. The commonly used simple hydration method produces inconsistent results due to the incomplete removal of MG from the glass. For the detergent solubilization method, the transition to non-bilayer structures is clearly complete in the 50:50 (w/w) mixture. For the RPEV method, the transition only becomes significant at an MG concentration of about 66%. This composition is of interest because the ratio of MG to DG in the native membrane is 2:1 (w/w). Thus, the RPEV method is the only one we have found that permits the preparation of bilayer vesicles from mixtures of the two major chloroplast lipids at concentrations that approximate the native composition. Addition of the other thylakoid lipids (sulfolipid and small amounts of phosphatidylglycerol and phosphatidylcholine) to 2:1 (w/w) mixtures of MG and DG further promotes the formation of bilayer liposomes using the RPEV technique.
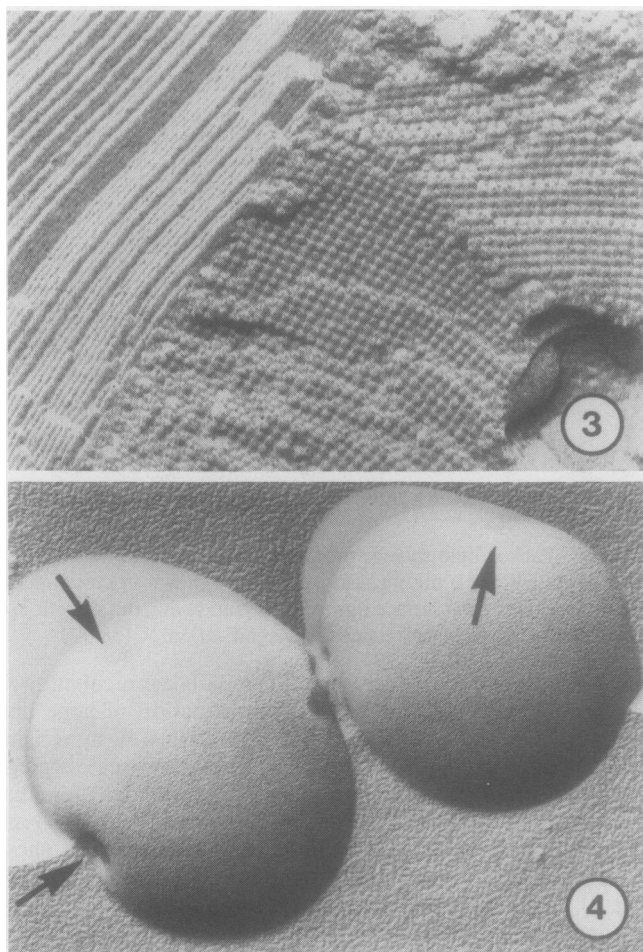
FIGS. 3 and 4. Organization of lipids observed in replicas of equal weight mixtures of DG and MG. Samples prepared by the detergent solubilization method (Fig. 3) contained hexagonal-II type tubes and packed inverted micellar spheres. In contrast, samples prepared by RPEV (Fig. 4) contained bilayer vesicles with fusion pores (arrows). Magnifications: Figure 3, × 104,000; Figure 4, × 49,000.

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