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Effect of chlorophyll *a* on the phase behavior of hydrated monogalactosyldiacylglycerols

Phoebe Dea [a,*], Laurence T. Pearson [a], Martin Caffrey [b] and Sunney I. Chan [a,**]

[a] *Arthur Amos Noyes Laboratory of Chemical Physics* ***, *California Institute of Technology, Pasadena, CA 91125* and [b] *Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, NY 14853 (U.S.A.)*

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We have studied the effect of chlorophyll *a* (chl *a*) on the X-ray diffraction patterns and the appearance of freeze-fracture electron micrographs of aqueous dispersions of monogalactosyldiacylglycerols (MGDG), the most abundant lipid in the thylakoid membrane. In MGDG systems containing 0–18 mol% of chl *a*, the diffraction patterns indicate the presence of a well-ordered reverse hexagonal phase. When 30 mol% of chl *a* was incorporated into the MGDG, the low-angle X-ray diffraction lines of the hexagonal lattice were slightly broadened and were accompanied by additional weak lines. With higher mol percents of chl *a*, the low-angle lines could no longer be indexed on a hexagonal or lamellar lattice. The freeze-fracture electron micrographs of similar samples showed that the patterns characteristic of the reverse hexagonal phase of an aqueous dispersion of pure MGDG were replaced by large liposomes, the fracture pattern of which is circular. We conclude that chl *a* in excess of 20 mol% destabilized the orderly reverse hexagonal phase of aqueous MGDG dispersions and disturbed the long-range order of the lipid array. These results are summarized in a temperature-composition isobaric phase diagram over a temperature range of −60°C to 60°C.

Introduction

Photosynthesis occurs at specialized chloroplast membranes which consist of approximately 50% protein, 40% lipid and 10% chlorophyll by weight [1]. The monogalactosyldiacylglycerols (MGDG) and digalactosyldiacylglycerols (DGDG) represent the main constituents of the lipid matrix in the thylakoid membranes, being nearly 40% and 20% of the lipid mass, respectively [2]. It is both tempting and logical to correlate this unique chemical composition with its unique biochemical functions.

X-ray diffraction and freeze-fracture electron microscopy studies by Shipley et al. [3] have shown that MGDG from *Pelargonium* leaves do not form bilayers in an aqueous dispersion but exist in a reverse hexagonal lipid-water phase (H-II) with the polar groups facing inwards towards the aqueous phase. Esterification with a second galactose unit to form DGDG completely alters the phase

* Permanent address: Department of Chemistry and Biochemistry, California State University at Los Angeles, Los Angeles, CA 90032, U.S.A.
** To whom reprint requests should be addressed.
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Abbreviations: chl *a*, chlorophyll *a*; H-II, reverse hexagonal phase; MGDG, monogalactosyldiacylglycerols; DGDG, digalactosyldiacylglycerols.
Correspondence address: Department of Chemistry and Biochemistry, California State University at Los Angeles, Los Angeles, CA 90032, U.S.A.

morphology, and a lamellar bilayer phase is formed. In the presence of excess water, DGDG forms dispersions of liposomes [4]. More recently, Williams and co-workers [5–8] have carried out rather extensive freeze-fracture studies on MGDG and DGDG/water systems from broad bean plants (*Vicia faba* L., var. Express). Their results indicate that when hydrated, MGDG forms an H-II phase, DGDG forms liposomes, while a mixture of these lipids in the molar ratio of 2 : 1 (MGDG/DGDG) forms bilayers in which there are either interbilayer lipid particles corresponding to an inverted micellar phase or arrays of particles between apposed bilayers.

Many studies of unsaturated phosphatidylethanolamines have shown that their phase states are modified by the presence of other components. For example, when phosphatidylcholine is mixed with phosphatidylethanolamine, the bilayer-forming phosphatidylcholine tends to stabilize the bilayer or lamellar phase over the reverse hexagonal phase [9]. Two components of different molecular shape can often form a new phase when mixed. In a dispersion of lysophosphatidylcholine/cholesterol (1 : 1), the lipids are found to be organized in extended bilayers [10]. Furthermore, monoolein and dioleoyl-phosphatidylcholine when mixed in equimolar amounts at 5% water form an inverted hexagonal phase. In isolation under the same conditions, they form the lamellar phase [11]. It is possible that a mixture of chl *a* and MGDG may form a phase other than that formed by the pure lipids. In particular, if the chlorophyll and galactolipids form stable complexes, the molecular shape of such complexes may be such as to permit stabilization of a bilayer phase.

In this paper, we report the effect of chl *a* on the phase behavior of aqueous dispersions of MGDG as revealed by freeze-fracture electron microscopy and X-ray diffraction.

Materials and Methods

Chlorophyll *a* was isolated from spinach extracts following the dioxane precipitation procedures of Iriyama et al. [12] and powdered sugar chromatographic purification [13,14]. Final purification was carried out in milligram quantities with high pressure liquid chromatography, using an Alltech 5 μm C-18 reverse phase column on a Waters High Pressure Liquid Chromatography apparatus with a UV detector at 254 nm. Complete resolution of chlorophyll *a* was achieved using a mobile phase consisting of 6% water in methanol at a column flow rate of 4 ml/min. Care was taken to minimize exposure of the chlorophyll *a* samples to room light and to keep the samples under nitrogen.

Monogalactosyldiacylglycerols (average M_r 773) from wheat flour were purchased from Sigma Chemical Co. The predominant fatty acid of monogalactosyldiacylglycerols is linoleic acid [15].

Galactolipids in benzene and chlorophyll *a* in chloroform were thoroughly mixed in a sample vial and the solvent evaporated under a stream of nitrogen. The residual solvent was removed by drying the sample under vacuum overnight. The dried film was then hydrated by adding excess nitrogen-saturated water and vortexing at room temperature. For X-ray diffraction measurements, samples were prepared similarly and transferred to thin-walled 1 mm diameter quartz capillaries (Blake Industries, Inc.) and flame sealed under vacuum.

Wide-angle and low-angle X-ray diffraction patterns were recorded on X-ray-sensitive film as a function of temperature over the range −60°C–60°C. Diffraction measurements were carried out using monochromatic (1.5 Å) wiggler-enhanced synchrotron radiation at the Cornell High Energy Synchrotron Source as described previously [16,17]. Radiation damage was minimized by implementing the precautions outlined in an earlier publicaiton [18]. Some of the measurements were made using the newly developed time-resolved X-ray diffraction method [19,20]. The samples contained in 1 mm capillaries were placed in a brass holder and clamped in a brass block through which water from a thermostated water bath was circulated. Sample temperatures were stable to ±0.5°C from 7°C to 55°C. Lower temperatures were achieved by passing over the sample capillary a stream of dry nitrogen gas that had been cooled in a methanol/solid CO_2 bath or an FTS crystal cooling device. Sample temperature was recorded by a copper-constantan thermocouple positioned in or next to the sample capillary to within 2 mm of the X-ray beam. The sample

assembly was mounted on a concentric goniometer head (Supper) attached to a Buerger precession camera (Supper) which acted as an optical bench in the alignment of collimator, sample, beam stop and film. Exposure times varied from 1 to 15 min depending on the electron current and sample concentration.

Freeze fracturing was carried out on a Balzer 360 M instrument using a 'Clamshell' type sample holder. Approximately 1.5 μl of sample was loaded into a sandwich consisting of 25 μm thick copper discs which had been cleaned by dipping in 50% nitric acid. The sample was quenched rapidly from 20°C by plunging the sandwich into liquid Freon 22. In some experiments, a 58% solution of ethylene glycol in water was used as cryoprotectant. The frozen sandwich was then mounted, fractured at −130°C and etched for 30 s using the surface of a knife at −190°C. The etched surface was shadowed with platinum from 30° to the horizontal, then coated with carbon. Replicas were freed from the copper discs by dissolving the discs in 50% nitric acid. The replicas were then washed in dimethylformamide, followed by water, then floated onto formvar coated grids for observation in a Philips 201C electron microscope at 80 kV.

TABLE I

LONG-SPACINGS FOR FULLY HYDRATED MGDG/CHL *a* AGGREGATES

Lipid	Temp. (°C)	Long-spacing (nm)
MGDG	20	(5.31 vs [a], 3.03 s, 2.66 s) [b]
	−40	3.78 vs
18 mol% chl *a* in MGDG	18	(4.97 vs, 2.90 s, 2.51 s)
	−63	4.0 vs
30 mol% chl *a* in MGDG	20	(5.19 s, 3.03 m, 2.64 w), 2.23 vw, 0.75 vw
	−40	4.16 s, 2.36 vw, 2.03 vw, 0.75 vw
46 mol% chl *a* in MGDG	20	5.13 s, 3.00 m, 2.79 vw, 2.28 m, 2.01 vw, 0.75 vw
	−40	[1.00 vs, 1.85 w, 2.07 vw, 5.61 vw] [c]
chl *a*	20	2.8 vw

[a] Relative intensities are denoted vs, very strong; s, strong; m, medium; w, weak; vw, very weak.

[b] Bracketed reflections index according to a hexagonal lattice.

[c] Because of uncertainties in sample-to-film distance absolute long-spacing determinations could not be made. The values reported correspond to radial position relative to that of the lowest angle reflection (see Fig. 3).

Results

We have carried out both static and time-resolved X-ray diffraction mesurements over the temperature range −60°C–60°C on a series of fully hydrated samples ranging from pure MGDG to pure chl *a*. The long-spacing data on representative samples are presented in Table I. In every condition examined but one (vide infra), the pure lipid and lipid/chl *a* mixtures were in the fluid or liquid-crystalline phase as evidenced by diffuse scatter in the vicinity of $(0.46\ \text{nm})^{-1}$.

The diffraction patterns of fully hydrated MGDG at 20°C and −40°C are shown in Fig. 1. The low-angle region consists of a series of extremely sharp reflections indexing on a hexagonal

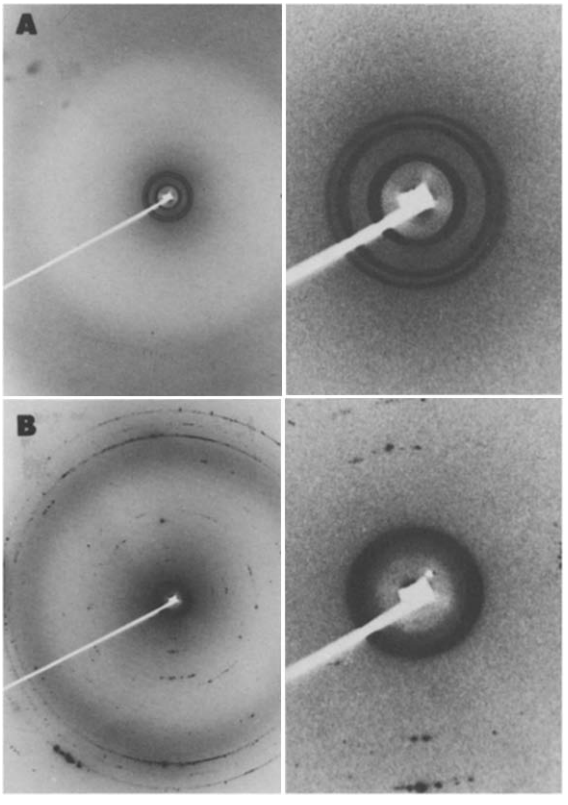

Fig. 1. X-ray diffraction patterns obtained for an aqueous dispersion of MGDG at 20°C (A), and at −40°C (B).

lattice with line spacings in the ratio $1/1 : 1/\sqrt{3} : 1/\sqrt{4}$. The hexagonal pattern persists upon cooling down to approximately $-15°C$ in the presence of supercooled water. However, once ice crystal formation occurs the low-angle diffraction pattern changes dramatically and exhibits a single strong reflection at $(3.78\ nm)^{-1}$ down to $-60°C$. A single reflection such as this precludes lattice type and symmetry determination and thus phase identification. As the sample is heated from $-60°C$ to $-3°C$, the diffraction pattern changes little, if at all. However, between $-3°C$ and $0°C$, diffraction lines corresponding to a hexagonal phase emerge and the dimensions of the hexagonal lattice increase, perhaps due to water uptake, with increasing temperature up to $0°C$. Thereafter, and as is observed with other systems [20], lattice size decreases with tempeature. At $20°C$, the MGDG d_{11} reflection at $(3.03\ nm)^{-1}$ (Table I) corresponds to a distance between cylinder axis in the hexagonal phase of 6.06 nm. This may be compared with the results of Shipley et al. [3] who observed a value of 6.05 nm at $20°C$ for fully hydrated MGDG prepared from *Pelargonium* leaves.

The addition of chl *a* up to 18 mol% has no observable effect on the phase properties of MGDG. The hexagonal pattern was still present and it underwent similar changes upon ice formation as was observed with MGDG alone. The lattice parameters did undergo slight changes as shown in Table I. At 30 mol% chl *a*, the diffraction pattern continues to show reflections indexing on a hexagonal lattice (Table I, Fig. 2) and, as noted with pure MGDG, the lattice dimensions have a negative temperature coefficient above $0°C$. Additional reflections which do not index on a hexagonal lattice are seen in this sample (Table I)

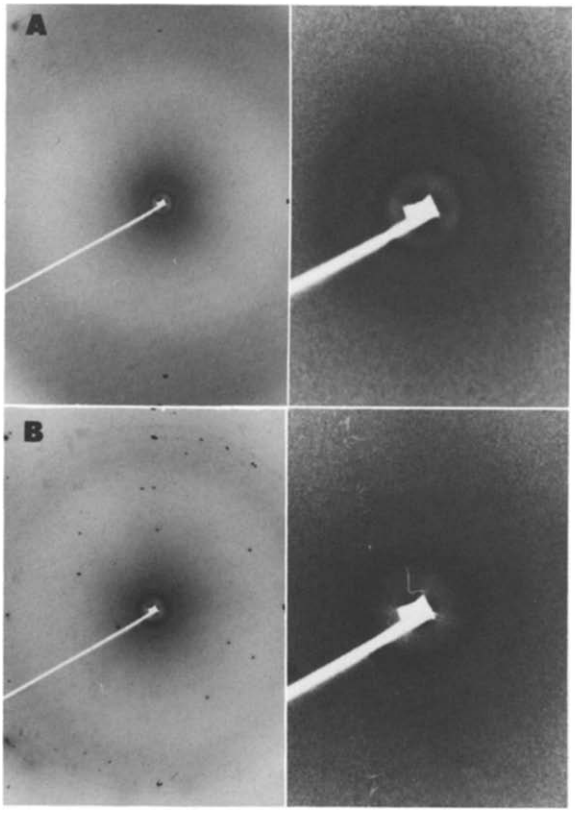

Fig. 2. X-ray diffraction patterns of a fully hydrated MGDG sample containing 30 mol% of chl *a* at $20°C$ (A), and at $-40°C$ (B).

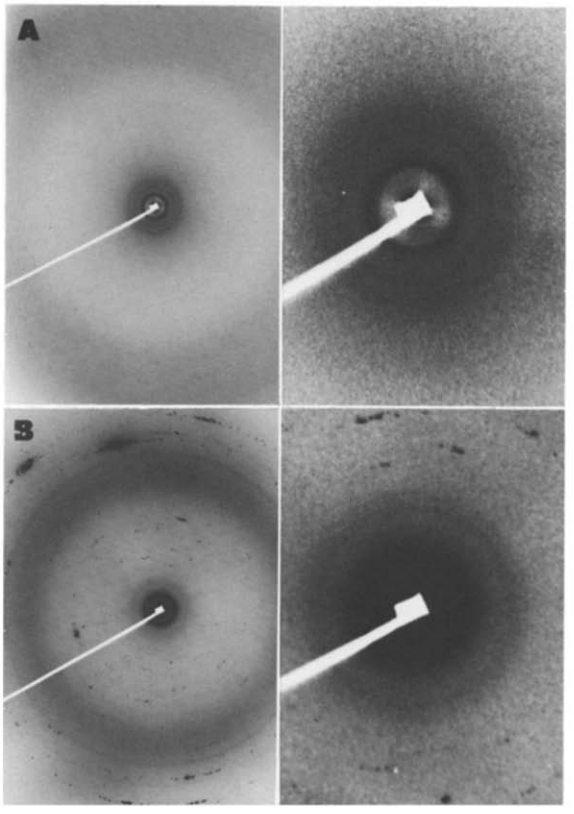

Fig. 3. X-ray diffraction patterns of a fully hydrated sample containing 46 mol% of chl *a* in MGDG at $20°C$ (A), and at $-40°C$ (B).

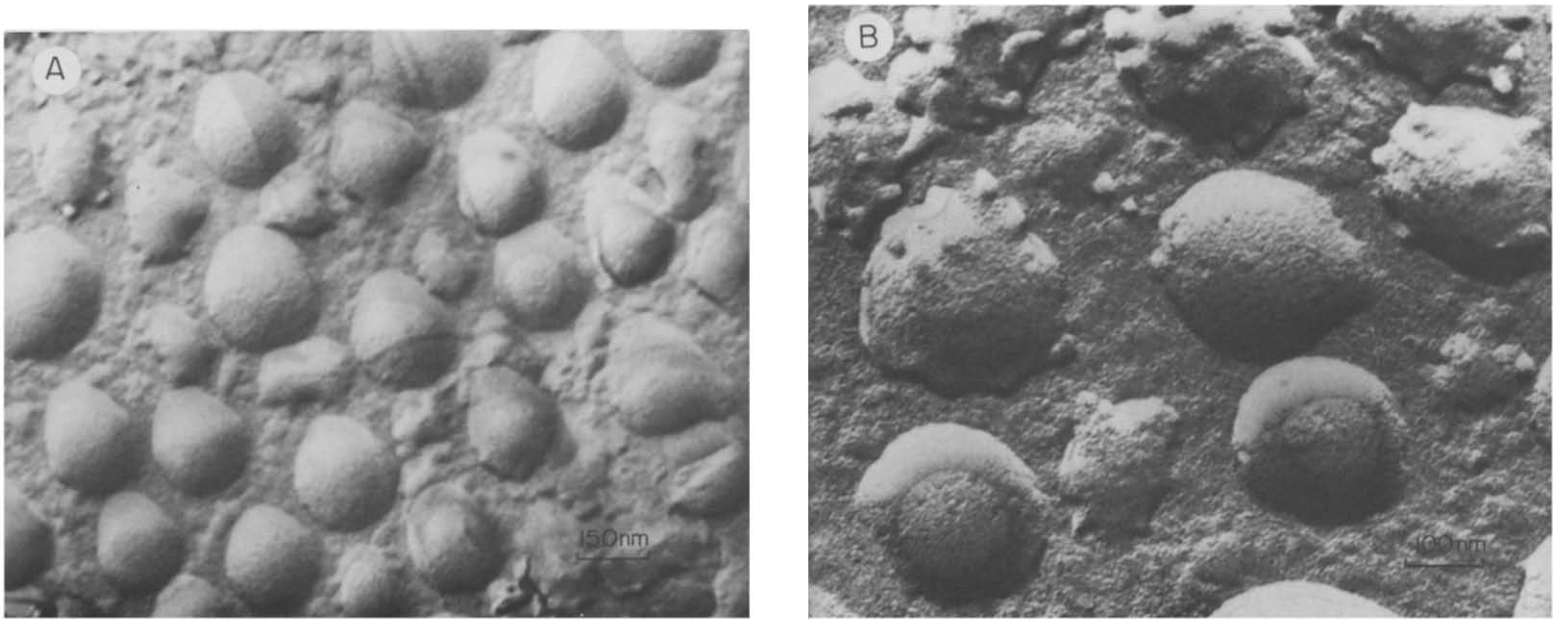

Fig. 4. Freeze-fracture electron micrograph of a fully hydrated sample containing 46 mol% of chl *a* in MGDG, with 58% ethylene glycol added as cryoprotectant. A, a representative micrograph; B, a magnified region showing the presence of blisters.

and we note also that the hexagonal lines are considerably broader than observed at the lower chl concentrations. This suggests that at 30 mol% chl *a*, the sample is probably in a mixed phase region and that the orderly hexagonal arrangement of lipid aggregates is being disrupted.

At an even higher chl *a* level of 46 mol%, indexing of the low-angle diffraction lines was not possible at any of the temperatures examined (Table I, Fig. 3). It appears that at this level, chl *a* and MGDG do not accommodate each other in either the hexagonal packing arrangement or indeed any other unique geometry recognizable by X-ray diffraction. This is the only sample in which additional sharp lines to the wide-angle side of the $(0.46\ nm)^{-1}$ peak were observed at $-40°C$. However, the phase state of the acyl chains cannot be established unequivocally under these conditions because of interference in this region by ice reflections (Fig. 3).

Diffraction patterns were also recorded on pure chl samples dispersed in water. Measurements were made at five temperatures in the range $-39°C$–$50°C$. At low angles, the diffraction patterns are featureless except for a single diffuse, ill-defined line centered at approximately $(2.81\ nm)^{-1}$ possibly indicating the presence of micellar aggregates. The wide-angle region shows a diffuse peak centered at $(0.43\ nm)^{-1}$ in the presence of ice. Above 0°C, the broad wide-angle peak due to water overlaps with that of chlorophyll and a well-defined pigment peak is no longer visible.

In the mixed MGDG/chl *a* systems containing in excess of 18 mol% chl *a*, a relatively sharp reflection at $(0.75\ nm)^{-1}$ was consistently observed in the diffraction patterns at all temperatures examined. The origin of this reflection is not known, and given the observations with pure chl *a*, it is unlikely to represent phase-separated chl *a*. However, it was absent in the 18 mol% chl *a* sample, the lowest concentration of chl *a* in MGDG examined in this study.

In conjunction with X-ray diffraction, freeze-fracture electron microscopy was carried out. Fig. 4A shows a characteristic region of a replica prepared from 46 mol% of chl *a* in MGDG. The dominant structure has the appearance of large (250 nm diameter) bilayer liposomes. The fracture pattern of the structures is circular, with the 'halo' that is characteristic of single bilayer vesicles which have been fractured and from which ice has then been removed from the outside surface by sublimation during etching. In addition, there appear to be blisters on or around the liposomes (Fig. 4B). The blisters may be due to areas of concentration of chl *a*. In none of the micrographs examined was there any evidence of the H-II phase normally associated with MGDG.

Discussion

Pure MGDG in the fully hydrated state adopts the H-II phase over the entire temperature range examined when water is part of the system. Ice formation drives the MGDG into an as yet unidentified phase with fluid acyl chains. Indeed, over the entire phase diagram, liquid-crystalline hydrocarbon packing prevails with only one possible exception at $-40°C$ for the 46 mol% chl *a* where evidence for the coexistence of gel and liquid-crystalline phase was obtained.

The addition of 18 mol% chl *a* to MGDG perturbs the lipid system very little. The MGDG H-II phase accommodates this level of chl *a* with slight changes in the dimensions of the hexagonal lattice. Beyond 18 mol%, the H-II phase begins to disorder and additional phase(s), as yet unidentified, begin to emerge. 30 mol% appears to be a transition region. It is here that a well-defined, albeit somewhat disordered, reverse hexagonal phase is seen in the presence of small amounts of these other phases.

Worthy of note in this context is the result concerning the interaction of chl *a* with certain phosphatidylcholines. Several groups [21–23] have demonstrated that chlorophyll can be integrally incorporated into the bilayer, with the porphyrin headgroup in the polar region of the membrane and the nonpolar phytol chain inserted into the hydrophobic core of membrane along the fatty acyl chains. Eigenberg et al. [24,25] have obtained evidence for compound formation between chl *a* and distearoylphosphatidylcholine involving interaction of the lipid phosphate with the magnesium ion in chlorophyll. More recently, Dea and Chan [26] have mapped the phase diagram of chl *a* in a bilayer membrane of dimyristoylphosphatidylcholine and showed that chlorophyll-rich domains are

formed in this lipid system. The monolayer solubility limit of chl *a* in both gel and liquid-crystalline dimyristoylphosphatidylcholine have also been reported by Heithier and Möhwald [27] to be in excess of 20 mol%. While the lipid phase and dispersion type differ considerably between these systems, a similar conclusion is drawn, namely that the two lipid systems accommodate chl *a* to the extent of at least 20 mol%.

The hydrocarbon chains of galactolipids are characterized by a high degree of unsaturation resulting in extremely low liquid-crystalline-to-gel phase transition temperatures. This may enable the phytol chain to insert easily into the hydrophobic region along the acyl chains. While some long-range order is maintained in the MGDG/chl *a* mixtures, the X-ray diffraction results showed that the order is different from that of pure MGDG when the chlorophyll mol% is 30% or greater. This is confirmed by the large liposomes observed with freeze-fracture electron microscopy and the absence of any H-II fracture patterns. Thus, when chl *a* is incorporated into MGDG, it destabilizes the orderly H-II phase structure and disturbs the long-range order of the lipid array.

The phases adopted by MGDG and chl *a* are consistent with the idea that the phase behavior of amphiphiles is strongly dependent on the relative cross-sectional area of headgroup and hydrocarbon regions of each molecule [28,29]. In this sense, chl *a* and MGDG represent opposite extremes, the former having a large headgroup (the porphyrin ring) relative to a single hydrocarbon chain, and the latter a small headgroup (a galactose residue) and two unsaturated hydrocarbon chains giving it a wedge shape. Consistent with these dimensions, chl *a* forms normal micelles [30] when hydrated whereas MGDG forms a reverse hexagonal phase.

A schematic representation of the H-II phase is shown in Fig. 5A. Upon incorporation of the chl *a* molecules, the disruption which arises from large differences in molecular shape between chl *a* and MGDG may allow the H-II cylinders to open up to form pseudobilayers, as schematically exemplified in Fig. 5B. The same may also be responsible for interrupting the long-range order of the lipid as reflected in the X-ray diffraction patterns. These models are consistent with those that have been proposed as possible intermediates in the lamellar-to-H-II phase transition [20,31] and in the process of membrane fusion such as alamethicin-mediated fusion of lecithin vesicles [32].

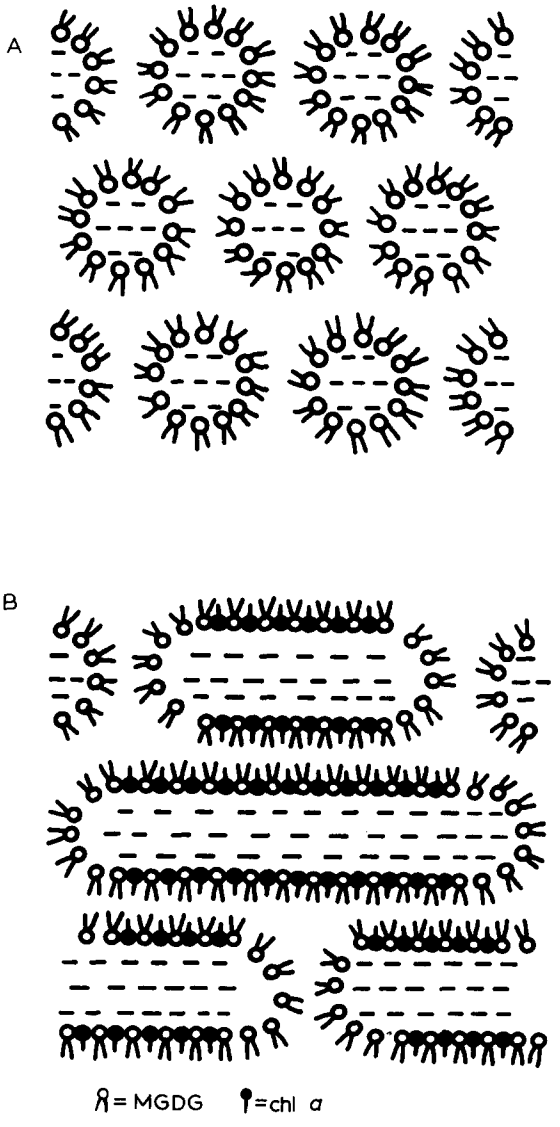

Fig. 5. Diagrammatic representation of (A) the H-II structure of pure MGDG, and (B) the structures formed upon incorporation of chl *a* into MGDG.

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References

1 Stryer, L. (1981) Biochemistry, pp. 434–435, Freeman, New York
2 Hashimoto, H. and Murakami, S. (1975) Plant Cell Physiol. 16, 895–902
3 Shipley, G.G., Green, J.P. and Nichols, B.W. (1973) Biochim. Biophys. Acta 311, 531–544
4 Larsson, J. and Puang-Ngern, S. (1979) in Advances in the Biochemistry and Physiology of Plant Lipids (Appelqvist, L.A. and Liljenberg, C., eds.), pp. 27–33, Elsevier, New York
5 Sen, A., Williams, W.P., Brain, A.P.R., Dickens, M.J. and Quinn, P.J. (1981) Nature 293, 488–490
6 Sen, A., Williams, W.P., Brain, A.P.R. and Quinn, A.J. (1982) Biochim. Biophys. Acta 685, 297–306
7 Sen, A., Brain, A.P.R., Quinn, A.J. and Williams, W.P. (1982) Biochim. Biophys. Acata 686, 215–224
8 Gounaris, K., Sen, A., Brain, A.P.R., Quinn, P.J. and Williams, W.P. (1983) Biochim. Biophys. Acta 728, 129–139
9 Boni, L.T. and Hui, S.W. (1983) Biochim. Biophys. Acta 731, 177–185
10 Van Echteld, C.J.A., De Kruijff, B., Mandersloot, J.G. and DeGier, J. (1981) Biochim. Biophys. Acta 649, 211–220
11 Rilfors, L., Lindblom, G., Wieslander, A. and Christianson, A. (1984) Biomembranes 12, 205–245
12 Iriyama, K., Ogura, N. and Takamiya, A. (1974) J. Biochem. 76, 901–904
13 Strain, H.H., Thomas, M.R., Crespi, H.L., Blake, M.I. and Katz, J.J. (1960) Ann. N.Y. Acad. Sci. 84, 617–666
14 Strain, H.H., Thomas, M.R. and Katz, J.J. (1963) Biochim. Biophys. Acta 75, 306–311
15 Carter, H.E., Ohno, K., Nojima, S., Tipton, C.L. and Stanacev, N.Z. (1961) J. Lipid Res. 2, 215–222
16 Caffrey, M. and Bilderback, D.H. (1984) Biophys. J. 45, 627–631
17 Caffrey, M. and Feigenson, G.W. (1984) Biochemistry 23, 323–331
18 Caffrey, M. (1984) Nucl. Instrum. Methods Phys. Res. 222, 329–338
19 Caffrey, M. and Bilderback, D.H. (1983) Nucl. Instrum. Methods Phys. Res. 208, 495–510
20 Caffrey, M. (1985) Biochemistry 24, 4826–4844
21 Lee, A.G. (1975) Biochemistry 14, 4397–4402
22 Oettmeier, W., Norris, J.R. and Katz, J.J. (1976) Biochem. Biophys. Res. Commun. 71, 445–451
23 Podo, F., Cain, J.E. and Blasie, J.K. (1976) Biochim. Biophys. Acta 419, 19–41
24 Eigenberg, K.E., Croasmun, W.R. and Chan, S.I. (1982) Biochim. Biophys. Acta 679, 353–360
25 Eigenberg, K.E., Croasmun, W.R. and Chan, S.I. (1982) Biochim. Biophys. Act 679, 361–368
26 Dea, P. and Chan, S.I. (1986) Biochim. Biophys. Acta 854, 1–8
27 Heithier, H. and Möhwald, H. (1983) Z. Naturforsch. 38c, 1003–1010
28 Israelachvilli, J.N., Mitchell, D.J. and Ninham, B.W. (1977) Biochim. Biophys. Acta 470, 185–201
29 Mitchell, D.J. and Ninham, B.W. (1981) J. Chem. Soc. Faraday Trans. II 77, 601–629
30 Flaumenhaft, E. and Katz, J.J. (1973) J. Inorg. Nucl. Chem. 35, 1719–1725
31 Siegel, D.P. (1984) Biophys. J. 45, 399–420
32 Lau, A.L.Y. and Chan, S.I. (1975) Proc. Natl. Acad. Sci. USA 72, 2170–2174