Liquid Crystals
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713926090

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To cite this Article Tsvetkova, N. M. , Kapasi, M. F. and Quinn, P. J.(1993) 'A time-resolved X-ray diffraction study of the lamellar to non-lamellar phase transitions in 1,2-distearoylmonogalactosylglycerol', Liquid Crystals, 15: 1, 65 — 74
To link to this Article: DOI: 10.1080/02678299308027839
URL: http://dx.doi.org/10.1080/02678299308027839

LIQUID CRYSTALS, 1993, VOL. 15, NO. 1, 65–74

A time-resolved X-ray diffraction study of the lamellar to non-lamellar phase transitions in 1,2-distearoylmonogalactosylglycerol

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(Received 21 October 1992; accepted 1 March 1993)

Monogalactosyldiacylglycerol (MGDG) has been isolated from *Vicia faba* leaves and distearoyl derivatives have been prepared by catalytic hydrogenation of the unsaturated galactolipid. The structure and the phase behaviour of 1,2-distearoyl MGDG in an excess of water were studied using a time-resolved X-ray diffraction method. The crystalline to liquid crystalline ($Lc_1 \rightarrow \alpha$) transition observed calorimetrically at 86°C was found to correspond to a lamellar crystalline to non-lamellar inverted hexagonal (H_2) phase transformation. This transition appeared to be a two state process with no detectable intermediate states. In contrast cooling H_2 gives first $L\alpha$ and then $L\beta$ before reverting to Lc_1. The transitions from the metastable lamellar gel ($L\beta$) phase to the lamellar liquid crystalline ($L\alpha$) state and from $L\alpha$ to H_2 were reversible on cooling and reheating with a hysteresis of 4–5°C. A scheme consistent with the structural and calorimetric data obtained from MGDG is proposed. The similarities in the thermal and structural properties of MGDG and other non-bilayer forming lipids are discussed in terms of related functional roles of these lipid classes in biomembranes, in which they are found abundantly as natural constituents.

1. Introduction

Galactolipids are the principal polar lipids of photosynthetic plant membranes of a wide variety of species from algae to higher plants [1–3]. In higher plants, the dominant molecular species are lipids with polyenoic fatty acyl substituents and in some species, trienoic fatty acids, of which linolenic [18 : 3w3] predominates. Studies of the phase behaviour and structure of polyunsaturated monogalactosylglycerols (MGDG) have shown that, even when dispersed in an excess of water, an inverted hexagonal (H_2) phase is formed at 20°C [4–7]. On cooling, a phase transformation from H_2 to the lamellar gel, $L\beta$ state was observed [7]. Structural and thermal studies of saturated (1,2-distearoyl) MGDG and 1,2-dialkylgalactoglycerolipids performed using differential scanning calorimetry (DSC), freeze-fracture electron microscopy and X-ray diffraction methods have been reported [5, 8–11]. The lipid used in our laboratory [5, 6, 8, 9] has been prepared using semi-synthetic methods which involve first the isolation of the dilinolenoyl molecular species of MGDG from fresh leaves and then saturating the lipid by catalytic hydrogenation to form the distearoyl derivative. More recently, an homologous series of fully synthetic β-D-galactosyldiacylglycerols has been prepared and examined by thermal and static (constant temperature) X-ray diffraction methods [12].

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0267–8292/93 $10·00

At least three different phases of 1,2-distearoyl MGDG have been described depending on the temperature and the level of hydration. A low temperature crystalline Lc_2 phase is formed during storage at 20°C for two days and converts exothermally at 47°C to a higher temperature crystalline Lc_1 phase on heating.

The Lc_1 phase melts at 86°C to a liquid crystalline α state. This transition is not reversible. Cooling of the α state leads to the formation of a metastable gel $L\beta$ phase at 69°C, which transforms exothermally to a crystalline Lc_1 state during 3–4 hours of equilibration at 20°C [8, 9].

The structures of the two crystalline states, Lc_2 and Lc_1, and the gel $L\beta$ state of saturated MGDG/water mixtures, and the interconversions between them have been extensively characterized [8, 9]. In the present work we have applied a time-resolved X-ray diffraction method to examine the structural changes associated with transitions between the phases, namely, the crystalline to liquid crystalline ($Lc \rightarrow \alpha$) and gel to liquid crystalline ($L\beta \rightarrow \alpha$) phase transitions of 1,2-distearoyl MGDG in an excess of water.

The X-ray data reported here show that the $Lc \rightarrow \alpha$ transition observed calorimetrically at 86°C corresponds to the lamellar crystalline to inverted hexagonal H_2 phase transformation and proceeds as a one step process with no detectable intermediate states. Transition from $L\beta$ to H_2 phase proceeds via an intermediate lamellar liquid crystalline $L\alpha$ phase between the gel and the non-lamellar H_2 phase, which appears over a temperature range of about 5°C during cooling and subsequent reheating with a hysteresis of 4–5°C.

2. Materials and methods

Lipid extraction and purification: The lipid was extracted according to the procedure described by Sen *et al.* [5].

Fresh leaves of 4–5 week-emergent broad bean plants [*Vicia faba* L., var., Express] were homogenized in 10 volumes of chloroform/methanol (2:1, v/v) in a Waring blender. The extract was allowed to stand for 2 h and then filtered through glass wool. Monogalactosylglycerol was isolated from this total lipid extract by chromatography on an acidified Florisil column and purified by thin layer chromatography (TLC) [13]. The plates were developed with chloroform:methanol: water (65:25:4, by volume). The lipid bands were resolved under ultraviolet light after spraying with aqueous Rhodamine 6G (0·01 per cent, w/v).

The purified MGDG band was recovered and separated into different molecular species by TLC on silica gel containing $AgNO_3$ (10 per cent, by weight of $AgNO_3$). The lipid was visualized as before and the band containing lipid with two 18:3 fatty acyl chains was scraped off the plate and extracted into chloroform:methanol (2:1, by volume).

Lipid preparation and analysis: The unsaturated galactolipid was hydrogenated using Adam's catalyst in benzene as described previously [14]. The final product was chromatographically pure and stored at -80°C under N_2 prior to examination. Total lipid content and fatty acid composition were estimated by gas chromatography of the methyl esters [15]. The fatty acid composition after hydrogenation of MGDG was 99·2 wt% stearoyl and 0·8 wt% palmitoyl.

Sample preparation: Freeze-dried MGDG was dispersed in distilled water and equilibrated at 20°C for 2 days. The lipid concentration was 25 g%.

X-ray measurements: Time-resolved X-ray diffraction measurements were performed using a monochromatic (0·150 nm) focused X-ray beam at Station 8·2 of the

Daresbury Synchrotron Laboratory as previously described [16]. The samples were mounted on a modified Linkam THM 600 temperature controlled microscope stage (Linkam Scientific Instruments, Surrey, U.K.) between mica sheets 1 mm apart. The temperature of the sample was monitored by a thermocouple placed adjacent to the sample. Diffraction patterns were recorded using a quadrant detector. The X-ray data were acquired in 255 consecutive time frames of 3 s each separated by a 10 μs wait-time. Static patterns were also recorded with an exposure time of 100 s. The X-ray scattering was plotted as a function of reciprocal spacing, $S = 2\sin\theta/\lambda$, using teflon (0·48 nm) as a calibration standard. The time-resolved X-ray measurements were made for both heating and cooling cycles at a rate of 5° min^{-1} in the range 30–95°C. The raw scattering data were subjected to a smoothing routine.

3. Results

Crystalline to liquid crystalline ($Lc_1 \rightarrow \alpha$) transition. Equilibration of aqueous dispersions of 1,2-distearoyl MGDG for 2 days at 20°C induces the formation of a lamellar crystalline phase that upon heating undergoes a single endotherm at 86°C. This transition is known to be associated with a disordering of the hydrocarbon chains, but the mechanism of mesophase transformation in the 1,2-distearoyl MGDG has not been characterized by the time-resolved X-ray diffraction method. Static X-ray diffraction patterns of fully hydrated 1,2-distearoyl MGDG in the crystalline state, in the liquid crystalline state and during the transition are shown in figure 1. Dynamic changes in the low and wide angle scattering intensities are shown in greater detail in figure 2.

The wide-angle scattering profile of the crystalline phase, which arises mainly from the hydrocarbon chain packing, is characterized by two intense peaks located at spacings corresponding to 0·379 nm and 0·414 nm and a weaker intensity band centred at 0·463 nm (see figure 1 (*a*)). Upon heating through the transition region, there is a progressive decrease in the intensity of these peaks (see figure 2 (*b*)) which are replaced by a broad scattering band centred at 0·464 nm and characteristic of disordered lipid acyl chains. There is no apparent shift in the position of these bands during the transition or evidence of any structural intermediates between the crystalline and the liquid crystalline phase. This behaviour indicates a two step pathway for the $Lc_1 \rightarrow \alpha$ phase transition.

The low angle diffraction patterns of fully hydrated 1,2-distearoyl MGDG equilibrated at 20°C show three prominent orders of reflection in the ratio 1 : 1/2 : 1/3 characteristic of a lamellar structure (see figures 1 (*a*) and 2 (*a*)) [17]. At 47°C, a slight increase in the lamellar spacing from 5·34 nm to 5·42 nm is observed (see figure 3). At this temperature an exotherm has been detected calorimetrically, corresponding to an $Lc_2 \rightarrow Lc_1$ transformation [8]. However, we were unable to detect any changes in the corresponding wide angle diffraction region over the range 40–50°C which would have indicated restructuring of the existing crystalline state. The increase in the lamellar spacing could be attributed to an increase in the inter-bilayer separation, due to an uptake of water. Heating through the $Lc_1 \rightarrow \alpha$ transition leads to the gradual appearance of two reflections in the low angle region corresponding to spacings of 3·39 nm and 3·08 nm superimposed on reflections characteristic of the crystalline phase (see figures 1 (*b*) and 2 (*a*)). This pattern of reflections was seen only over a range of 1°C (from 84° to 85°C) which is in agreement with the thermal characteristics of the phase transition recorded calorimetrically [8] (see figure 3). At

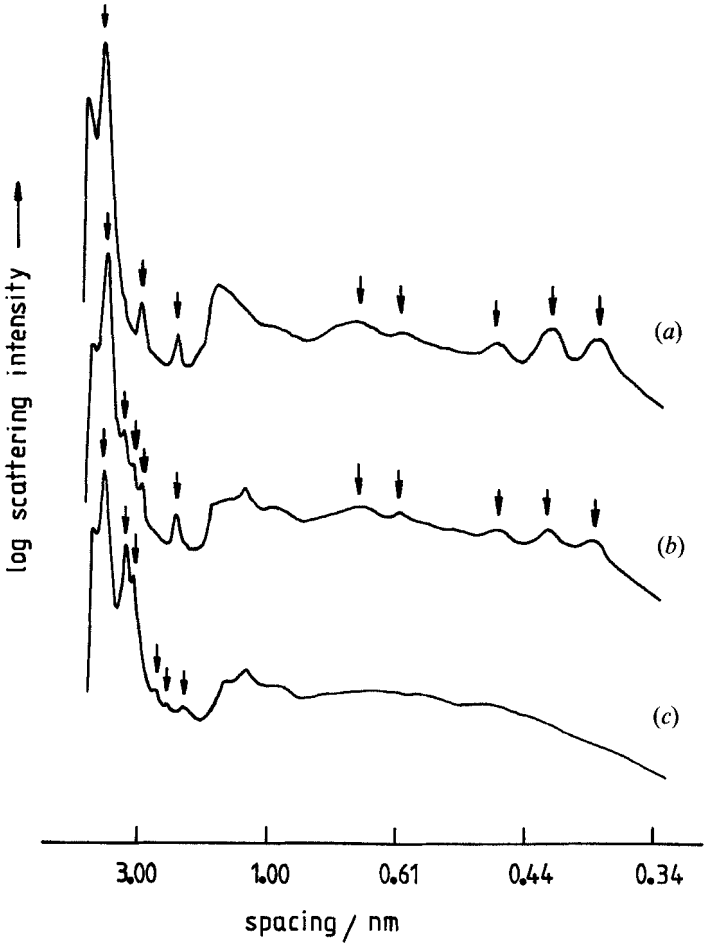

Figure 1. X-ray diffraction patterns showing the relationship between log scattering intensity and *d*-spacings of fully hydrated 1,2-distearoyl MGDG (*a*) in the crystalline state recorded at 43°C, (*b*) in the region of the transition from crystalline to inverted hexagonal phase recorded at 84°C, (*c*) in the inverted hexagonal phase recorded at 87°C.

86°C, six reflections were observed in the low angle scattering region corresponding to long spacings in the ratio $1:1/\sqrt{3}:1/\sqrt{4}:1/\sqrt{7}:1/\sqrt{9}:1/\sqrt{12}$ (see figures 1(*c*) and 2(*a*)) characteristic of an hexagonal lattice and consistent with an inverted hexagonal arrangement of lipid molecules [17]. The repeat spacing increases from 5·44 nm for the lamellar structure to 6·05 nm for the H_2 phase (at 5°C above the transition) (see figures 2(*a*) and 3). The lattice spacing (distance between axes of water cylinders) is $a=6{\cdot}99$ nm ($a=2d/\sqrt{3}$, where $d=6{\cdot}05$ nm).

Examination of the individual frames indicates that the wide angle patterns characteristic for the crystalline Lc_1 phase, although decreasing in intensity, persist for 12 s after the temperature passes the phase transition temperature at 85°C (see figure 2(*b*)). At the same time, the first order reflection in the low angle region retains its position at 5·40 nm during the 12 s period, where the reflections of the Lc_1 phase are still present in the wide angle diffraction region. The first order then shifts to 5·98 nm, when the reflections of the Lc_1 phase in the wide angle region disappear.

Gel to liquid crystalline (Lβ—>α) transition. The lamellar gel to liquid crystalline transition was investigated by cooling dispersions from the non-lamellar, H_2 phase and subsequently reheating them from the gel, Lβ, phase. A cooling experiment is illustrated in figure 4. This shows a three-dimensional projection of diffraction intensity recorded in the low angle scattering region during a temperature scan in the

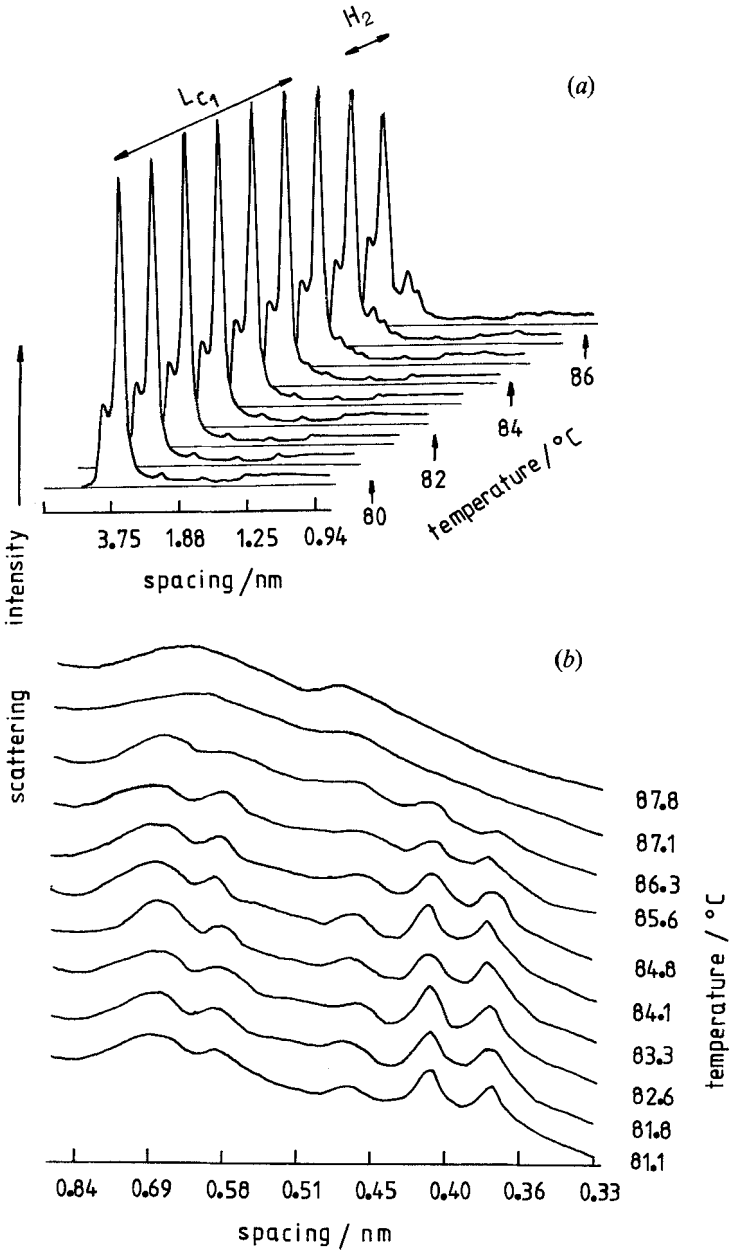

Figure 2. X-ray scattering intensity versus *d*-spacing as a function of temperature of fully hydrated 1,2-distearoyl MGDG heated from 35°C to 95°C at 5°C min^{-1}, showing selected patterns recorded through the phase transition. (*a*) Low angle scattering region; (*b*) wide angle scattering region. Diffraction patterns accumulated over 9 s.

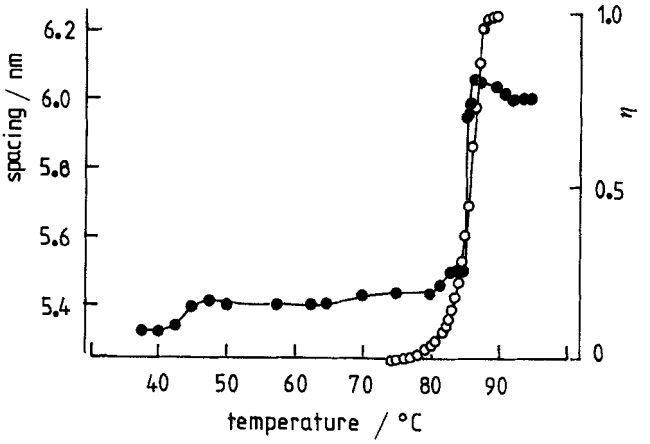

Figure 3. Low angle diffraction *d*-spacings of fully hydrated 1,2-distearoyl MGDG versus temperature recorded during heating from 35°C to 95°C at 5° min^{-1} (●); integral of specific heat, $\eta = \int^{T}_{T_0} Cp\ dT/\Delta H cal$, $T_0 = 76°C$, obtained from the thermogram in [8] (○).

range of the non-lamellar to lamellar transition and two-dimensional plots of scattering intensity in the wide angle region over the corresponding temperature range (see figures 4 (*a*) and (*b*)). Cooling of the H_2 phase leads to the formation of the lamellar liquid crystalline state commencing at 72°C. In the low angle region, there is evidence for a coexistence of the non-lamellar, H_2 phase and the lamellar liquid crystalline, Lα phase, but only in individual diffraction patterns and not throughout the whole transition region. Further cooling leads to the appearance of a sharp symmetric peak at a spacing corresponding to 0·411 nm and a weaker reflection at 0·445 nm in the wide angle region, characteristic of hexagonal packing of the acyl chains in a lamellar gel, Lβ phase (see figure 4 (*b*)). Subsequent reheating restores the lamellar liquid crystalline, Lα phase in fully hydrated MGDG at about 71°C and a transition to the inverted hexagonal structure appears at about 76°C.

A detailed observation of the individual diffraction patterns during the Lβ→Lα transition showed that the changes in the lamellar period take place simultaneously with the changes in the wide angle diffraction pattern. Compared with the two state $Lc_1 \rightarrow H_2$ transition, this sequence of structural events suggests a somewhat different transition mechanism.

In summary, the X-ray information obtained for 1,2-distearoyl MGDG in an excess of water provides the following description of the structural changes involved during heating of annealed specimens and upon subsequent cooling and reheating. After equilibration for two days at 20°C, 1,2-distearoyl MGDG forms a lamellar crystalline state with a lamellar repeat spacing of 5·34 nm and wide angle peaks at spacings of 0·379 nm, 0·411 nm and 0·463 nm (see figure 1 (*a*)). At 47°C, an increase in the lamellar spacing from 5·34 nm to 5·42 nm was observed (see figure 3) without changes in the corresponding wide angle diffraction pattern and was associated with an increase in the interlamellar spacing between adjacent bilayers. The high temperature transition at 86°C, observed by differential scanning calorimetry, can be assigned to a direct lamellar crystalline to inverted hexagonal structural change with corresponding increase in the long spacing from 5·44 nm to

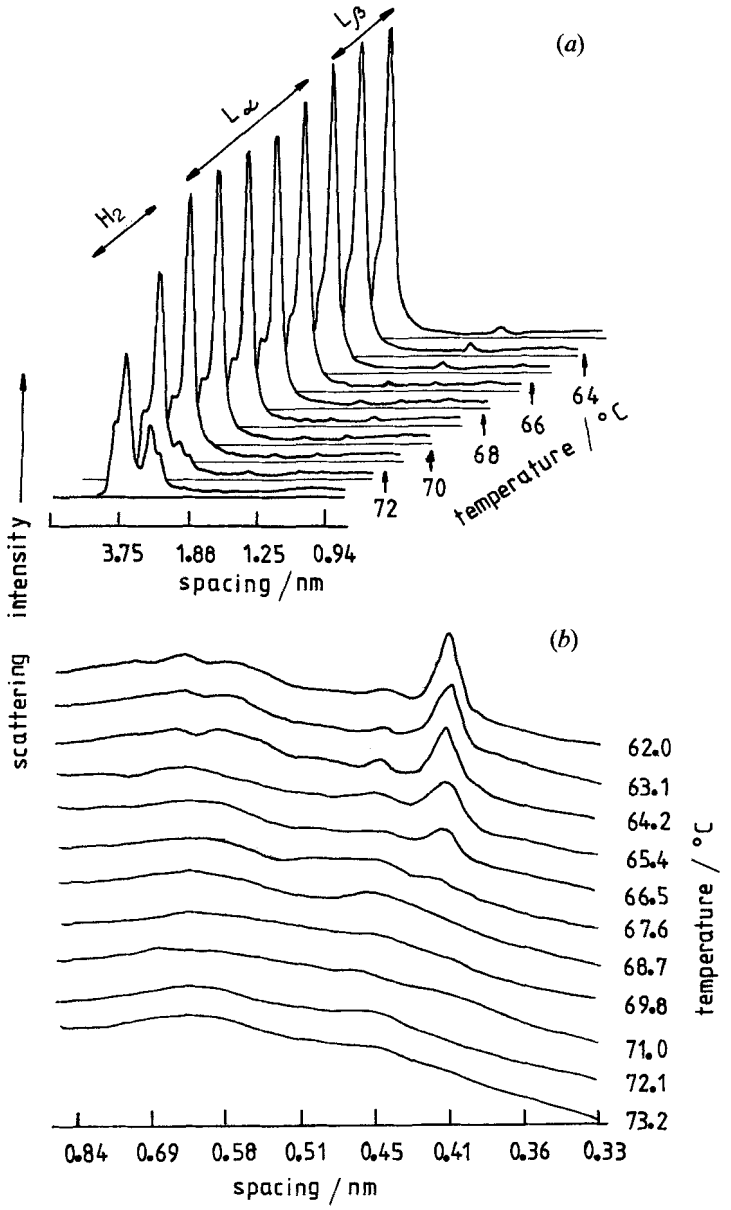

Figure 4. X-ray scattering intensity versus *d*-spacings as a function of temperature of fully hydrated 1,2-distearoyl MGDG cooled from 90°C to 31°C at 5° min^{-1} showing selected patterns recorded through the phase transition region. (*a*) Low angle scattering region; (*b*) wide angle scattering region. The diffraction patterns were accumulated over 3 s and every fourth frame is plotted.

6·05 nm (see figures 1, 2, and 3). On cooling, the low angle pattern indicates formation of a mesophase with a repeat spacing of 5·47 nm, that arranges to form a lamellar liquid crystalline, Lα structure (see figure 4 (*a*)). The Lα phase exists from 71°C to 66°C and transforms to a lamellar gel, Lβ phase with a *d*-spacing of 6·24 nm and a prominent wide angle peak at 0·411 nm (see figure 4 (*b*)). On reheating, a

sequence of structural transitions, from Lβ to Lα at 69°C and from Lα to inverted hexagonal H_2 phase, at 76°C was observed.

4. Discussion

MGDG isolated from broad bean leaves and hydrogenated to give the distearoyl derivative has been studied by DSC, freeze-fracture electron microscopy and X-ray diffraction methods [5, 8, 9]. Three mesophases have been identified and characterized: two crystalline phases, Lc_2 and Lc_1, and a metastable gel, Lβ phase.

The time-resolved X-ray diffraction data presented here reveal the liquid crystalline polymorphism of 1,2-distearoyl MGDG in an excess of water. The $Lc_1 \rightarrow \alpha$ transition observed calorimetrically at 86°C corresponds to a lamellar crystalline to inverted hexagonal H_2 phase transformation and appears to be a two state process characterized by coexistence of the initial Lc_1 and the final H_2 states with an absence of any detectable intermediate states. During this transition, the reflections in the wide angle region of the Lc_1 phase decreased in intensity without shift in position, while the broad band at 0·464 nm indexing the chain packing of the H_2 phase appeared (see figure 2(*b*)). The absence of any detectable intermediate states leads to the conclusion that, at least at the resolution of our experiment (3 s in the temperature scans), the $Lc_1 \rightarrow H_2$ transition of 1,2-stearoyl MGDG in an excess of water proceeds as a two state phase transition, characterized by a coexistence between the initial and final states during the transition and direct conversion of the former to the latter.

Cooling from temperatures where the H_2 phase exists produced a lamellar liquid crystalline, Lα phase which was present over the temperature range 72°C–66°C and has not been identified in previous studies. The phase sequence $L\beta \rightarrow L\alpha \rightarrow H_2$ is reversible on cooling and subsequent reheating with hysteresis of 4–5°C.

The mechanism of the $L\beta \rightarrow L\alpha$ phase transition does not appear to conform to either a two state or a continuous phase transition [18]. The changes in the lamellar spacing occur simultaneously with the changes in the wide angle diffraction pattern. The precise mechanism of this transition can be revealed, however, using quasi-static heating conditions.

Although the reflections in the wide angle region between 0·40 nm and 0·46 nm can be assigned to the packing of the hydrocarbon chains, the remaining reflections at 0·69 nm and 0·59 nm (see figures 1(*a*),(*b*) and 2(*b*)) have not yet been definitively assigned. It has been suggested [9] that these reflections originate from the galactose head groups, by comparison with crystallographic studies of galactocerebrosides [19] and n-alkyl glucosides [20, 21]. In our study, these two reflections exist in the crystalline, Lc_1 and gel, Lβ phases and are replaced by a broad band at 0·603 nm in the Lα and H_2 phases (see figures 1, 2(*b*)) and 4(*b*)). They were found to disappear simultaneously with the reflections of the crystalline, Lc_1 phase in the wide angle region and, on cooling, to reappear at 0·65 nm and 0·57 nm simultaneously with the sharp reflection centred at 0·411 nm of the Lβ phase (see figures 2(*b*) and 4(*b*)). However, in the absence of a definitive crystallographic study of glycoglycerolipids, this assignment remains tentative.

A scheme consistent with the structural and calorimetric evidence obtained from this and previous studies on semi-synthetic MGDG is presented in figure 5. The transition temperatures reported in this study are in agreement with those reported by other investigators and the two liquid crystalline states (Lα and H_2) identified and characterized by the present time-resolved X-ray diffraction study have been

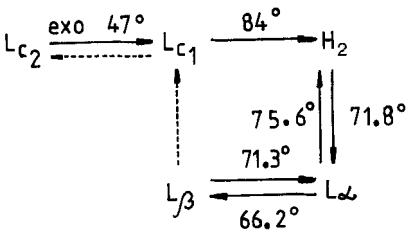

Figure 5. Block phase diagram of fully hydrated 1,2-distearoyl MGDG showing the relationship between the lamellar and non-lamellar phases in fully hydrated MGDG. The temperatures shown correspond to the *beginning* of the phase transitions. Information relating to the formation of the lamellar crystalline phases has been obtained from published data [8, 9].

included. We show that 1,2-distearoyl MGDG in an excess of water equilibrated at low temperature exhibits a two state Lc_1 to H_2 phase transition during first heating. Cooling from H_2 results in formation of the metastable $L\beta$ phase via an intermediate $L\alpha$ state; this phase has not hitherto been identified.

The phase sequence observed for semi-synthetic 1,2-distearoyl MGDG/water mixtures is in agreement with that reported for the pure synthetic analogue [12]. In this case, the phase transformation from $L\beta$ to H_2 takes place with an intermediate liquid crystalline, $L\alpha$ phase observed over a short temperature range. It is noteworthy that $L\alpha$ was not detected for synthetic 1,2-dialkylgalactosylglycerol with the same number of carbon atoms [10, 11]. Cooling of the inverted hexagonal H_2 phase led to a mixture of $L\beta$ and Lc phases. A comparison between semi-synthetic 1,2-distearoylgalactosylglycerol and synthetic 1,2-distearoylglucosylglycerol shows similarities in the phase sequence, although the temperatures of the lamellar to non-lamellar transformation are correspondingly higher for the galactolipid [22, 23]. However, the temperatures of the transition from lamellar to non-lamellar phase for 1,2-distearoyl MGDG are considerably lower than those of the phosphatidylethanolamine with corresponding acyl chain substituents [24, 25]. These similarities in the structural and thermal properties of the non-lamellar forming lipids can be related to their putative functional roles in biomembranes. Non-lamellar forming lipids are believed, for example, to package intrinsic proteins into the lipid bilayer matrix and to seal the protein-lipid interface to the leakage of solutes. There is also a suggestion that in the photosynthetic membrane, the MGDG is required to promote the association between the light harvesting chlorophyll-protein complexes with their respective photochemical reaction centre complexes.

The research was supported by a grant from the FEBS and AFRC Plant Molecular Biology programme. We were grateful for the assistance of W. Bras at the Daresbury Laboratory.

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