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P. J. Quinn [a] & L. J. Lis [b]

[a] Department of Biochemistry, King's College London, Campden Hill, London, W8 7AH, UK

[b] Department of Physics, The Liquid Crystal Institute, Kent State University, Kent, Ohio, 44242, USA

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A Characterization of the Phase Transitions in Saturated Monogalactosyldiacylglycerol-Water Systems Using Real-Time X-Ray Diffraction Methods

P. J. QUINN

Department of Biochemistry, King's College London, Campden Hill, London, W8 7AH, UK

and

L. J. LIS

Department of Physics and The Liquid Crystal Institute, Kent State University Kent, Ohio 44242, USA.

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The phase behavior of fully saturated monogalactosyldiacylglycerol prepared from total polar lipid extracts of spinach leaves and dispersed in excess water has been examined by X-ray diffraction methods Synchrotron radiation was used to determine the real-time structural changes in transitions between the different phases driven by temperature scans of the speed conventionally used in differential scanning calorimetry. The high-temperature α-phase of the lipid recorded at 84°C gave rise to a single, rather broad low-angle Bragg reflection at about 2.8 nm and a broad reflection at 0.48 nm indicating that the hydrocarbon chains were in a disordered configuration. Freeze-fracture electron microscopy of the α-phase was found to be consistent with an amorphous phase formed directly from gel-phase bilayers upon heating above the gel to liquid-crystalline phase transition temperature. The α-phase transforms to the lamellar-β phase on cooling i.e. a liquid-crystalline to gel-phase transition; there was no evidence of an intermediate liquid-crystalline lamellar phase in the lipid. Diffraction maxima which are interpreted to correspond to spacings of the galactose residues are observed in the region of 0.6-0.7 nm. The hydrocarbon chains pack in subcells giving rise to characteristic reflections for each of the lamellar phases in the wide-angle region of the dispersion. Static diffraction patterns of two lamellar crystalline phases, designated L_{C1} and L_{C2} respectively, have been obtained. Transitions between the crystalline phases and the lamellar-gel and α-phase driven by temperature scans of between 1.25° and 10° · min^{-1} have been monitored in real-time and the structural changes correlated with earlier calorimetric studies of this lipid-water system. Thermal transitions are found to correspond to major structural rearrangements in the lipid-water system.

Keywords: monogalactosyldiacylglycerol, galactolipid, x-ray diffraction, lipid phase behavior, phase transitions, synchrotron radiation

INTRODUCTION

Biological membranes consist of an assembly of different lipids and proteins. Model membranes formed by phospholipids and/or glycolipids in aqueous systems have been extensively studied in an attempt to establish the structural and functional

role of individual lipid components. The major lipid constituent of the photosynthetic membrane of higher plant chloroplasts and many photosynthetic algae is monogalactosyldiacylglycerol. It has been characterized as a natural derivative[1] with completely saturated acyl chains[2] and in mixtures involving different galactosyl species[3] or phospholipids mixed with galactolipids.[4] Some information is available on the static structures of monogalactosyldiacylglycerol-water systems as a function of temperature and other environmental factors,[1,5] but there is as yet little known about dynamic processes involved in transitions between phases except for the qualitative determination that such a transition occurs using differential scanning calorimetry. Furthermore, there is no information on the structural configuration of the fully saturated lipid at high temperature although, by analogy with the unsaturated species found in the photosynthetic membrane, it is thought to be hexagonal-II. Real-time studies of the structural changes associated with these transitions are required to understand the role of this or any other lipid in membrane-associated processes.

Monogalactosyldiacylglycerols appear in many respects to behave like the phosphatidylethanolamines in that the naturally-occurring, unsaturated derivatives form lamellar phases at low temperature and undergo transitions to hexagonal-II phases at high temperature.[6,7] In the photosynthetic membrane of the chloroplast, monogalactosyldiacylglycerol is the major polar lipid component and represents more than half the total polar lipid of the membrane. The lipid is believed to fulfill a number of structural roles in the photosynthetic membrane including the packaging of intrinsic membrane proteins into efficient oligomeric complexes[8] and the organization of the photochemical reaction centers with their associated light-harvesting chlorophyll-protein complexes.[9] The characteristic feature of the native lipid is that the fatty acyl chains are polyunsaturated and this has a dominant effect on the polymorphic phase behavior.[10] More saturated species of monogalactosyldiacylglycerols are found in many microorganisms including the thermophilic blue-green alga, *Anacystis nidulans*, and the phase behavior of these lipids[11] as well as hydrogenated monogalactolipids derived from higher plant chloroplasts[12] are markedly altered as a consequence of the change in fatty acyl composition.

One of the major tools for investigating the structure of lipid-water phases is X-ray diffraction.[13] Recently high intensity X-rays from a synchrotron source have been successfully used to determine the structures involved in lipid phase transitions in real time.[14–18] The three dimensional packing of the lipid mesophase can be determined from the small angle X-ray scattering, and the two dimensional packing of the lipid hydrocarbon chains can be obtained concurrently from the wide angle X-ray scattering.[13] Transition kinetics in the range of about 100 ms can be measured and used to probe the mechanisms involved in these processes as well as to characterize the rate limiting step(s). In addition, transient intermediates or phases can be identified by this technique.

In this study, real-time X-ray methods have been used to describe the phase transitions previously reported for fully saturated mono-galactosyldiacylglycerol dispersed in excess water. The structural polymorphism of this lipid as a function of temperature is confirmed. Furthermore, structural changes can be detected that

are not associated with significant changes in enthalpy and hence a complete description of the phase changes in these lipid-water systems appears to be more complex than hitherto indicated.

MATERIALS AND METHODS

Total lipid extracts of market spinach leaves were prepared by the method described previously.[5] Purified monogalactosyl-diglyceride was hydrogenated in the presence of Adam's catalyst (Johnson-Matthey Chemicals, U.K.) in benzene. The hydrogenated lipids were separated from the catalyst and possible degradation products by preparative thin layer chromatography.[19] Saturation of the fatty acyl residues was verified by gas chromatography of the methyl ester derivatives as described by Restall *et al.*[20] Pentadecanoic acid was added to lipid samples as an internal standard before methylation for quantitative estimations of galactolipid concentration. The fatty acyl composition after hydrogenation of the monogalactosyldiglyceride was 85% (by mass) stearoyl and 15% palmitoyl residues.

Samples were prepared by mixing the liquid with a five-fold excess by weight of water and allowing them to equilibrate for over 3 days. The samples were then mounted between mica sheets 1 mm apart in an X-ray sample holder.

The X-ray experiments were carried out by using a monochromatic (0.15 nm) focussed X-ray beam at station 7.3 of the Daresbury Synchrotron Radiation Laboratory as previously described.[21] A cylindrically bent single crystal of Ge[22] and a long float glass mirror were used for monochromatization and horizontal focussing, providing 2×10^9 photons $\cdot s^{-1}$ down a 0.2 mm colimator at 2.0 GeV and 100 to 200 mA of electron beam current. The sample holder was mounted in a Keele flat-plate camera. Scattered X-rays were recorded with a linear detector constructed at the Daresbury laboratory. The dead-time between successive data aquisition frames was 50 μs, with the temporal resolution of each frame varying from 100 ms to 6 sec. for temperature scans. X-ray scattering has been plotted as a function of reciprocal spacing, $s = 2 \sin \theta/\lambda$ using teflon (0.48 nm) as a calibration standard.[23] No corrections were applied to path distances from the sample to the linear detector consequently wide-angle spacings will be slightly longer than measured directly by the detector. Spacings for the mesophases and subcells were determined by Bragg's Law.[24] The acyl chain subcell packing was classified as previously described.[25,26]

The temperature scans were produced by water baths connected internally to the sample mount of the X-ray camera. The rate of temperature change in the scans was adjusted by regulating the flow of water through the sample holder and was recorded concomitantly with the diffraction patterns. The temperature of the sample was monitored internally using a thermocouple placed adjacent to the sample region of the X-ray sample holder. We expect that the thermal diffusion through our samples was approximately the same as that observed in experiments reported by[16] since our sample thickness was also 1 mm.

RESULTS AND DISCUSSION

The structure of low-temperature lamellar phases formed by saturated monogalactosyldiacylglycerol in aqueous systems has been investigated by X-ray diffraction and freeze-fracture electron microscopy.[1,2,9] Surface-area isotherms of the lipid have also been reported using monomolecular film techniques.[27-29] Transitions between the different lamellar phases and between lamellar phases and a high-temperature, so called α-phase, have been characterized by differential scanning calorimetry[5] and fluorescence probe methods.[1,19] At least four different phases have been identified on the basis of these studies and the relationship between the phases formed by saturated monogalactosyldiacylglycerol from spinach is illustrated in Figure 1. Three phases are in a lamellar configuration with sharp reflections in the wide angle region of the X-ray diffraction pattern indicating that in all cases the hydrocarbon chains are packed into relatively ordered lattices. There is no structural information about the long-range order in the high-temperature phase which has been designated the α-phase because the hydrocarbon chains are in a disordered liquid-crystalline conformation. The pathways of interconversion between the various phases have been deduced from calorimetric studies. These have shown that the transitions L_{C2} to L_{C1} and L_β to L_{C1} are both exothermic whereas L_β to α and L_{C1} to α are both endothermic transitions.

Initial experiments were undertaken to characterize the α-phase. An X-ray diffraction profile of the lipid dispersed in excess water recorded at 84°C is shown in Figure 2. Only one diffraction order in the low-angle region is observed which has a spacing of about 2.8 nm. The inset containing a 10-fold expanded scattering intensity axis confirms the absence of higher order Bragg diffractions. The absence of higher order Bragg reflections was confirmed using a conventional rotating anode X-ray generator and the spacing corresponding to the low-angle reflection was found to be dependent on temperature above the phase transition temperature. Furthermore, diffraction patterns recorded over periods of up to 24 hours indicated that the high temperature phase is relatively stable. Two broad bands centered at about 0.67 and 0.59 nm are attributed to the sugar residues of the polar head group of the lipid. While assignment of these diffraction bands to sugar spacings is not

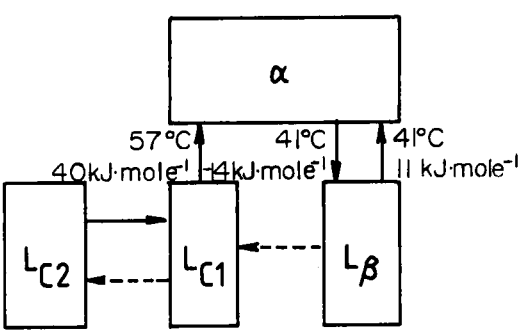

FIGURE 1 Schematic of the structures and phase transformations in fully hydrated saturated monogalactosyldiacylglycerol in water.

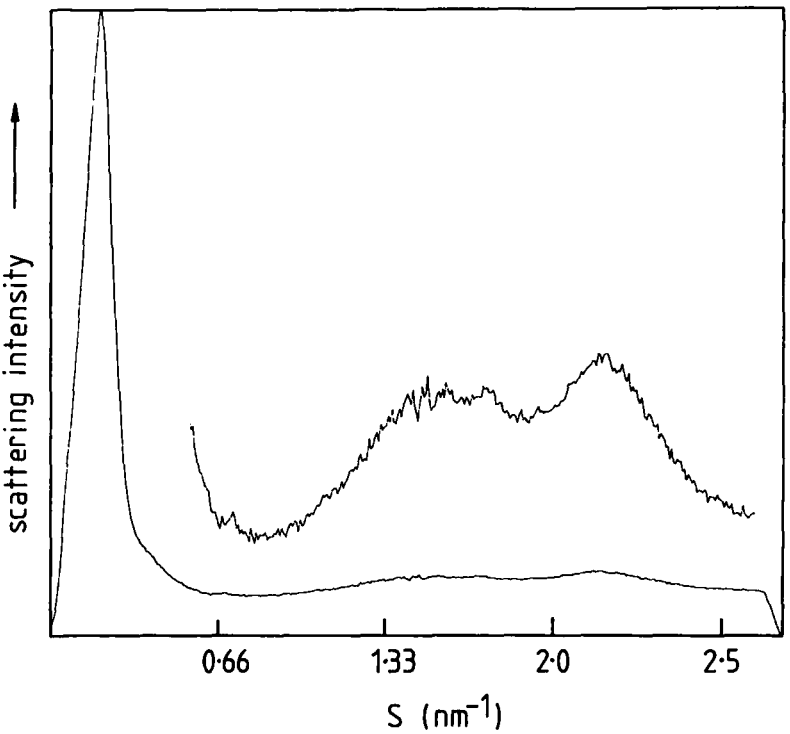

FIGURE 2 Scattered X-ray intensity as a function of scattering angle for saturated monogalactosyl-diacylglycerol in water at 84°C. The data were collected at constant temperature for 100 seconds. The pattern is characteristic of the liquid-crystalline phase in this system. A ten-fold expanded intensity scale in the wide-angle region of the X-ray dispersion is also shown. The inset gives the relationship between diffraction maxima of the low-angle band and temperature.

unequivocal, single crystal studies of simple aklyl-D-glucopyranosides[30,31] and cerebroside[32] suggest that this assignment is not unreasonable. A broad diffuse diffraction band at 0.48 nm is characteristic of disordered hydrocarbon chains associated with an α-phase.

The structure of the α-phase was further investigated by freeze-fracture electron microscopy. An electron micrograph of the sample used for X-ray study which had been thermally quenched from 70°C is shown in Figure 3. Several replicas were prepared and all consisted of an amorphous structure. The electron micrograph shown in Figure 3 depicts one of the very isolated regions where an apparently amorphous structure transforms directly into bilayer sheets typical of the lamellar phases.[1] The lamellar phase could not be detected in X-ray diffraction patterns consistent with the relatively rare occurrence observed in the replicas. The location of these bilayer regions deep within the lipid phase suggested they may have formed as a result of slow thermal quenching since the thermal conductivity within the phase is relatively low. The rate of thermal quenching was determined directly by insertion of a thermocouple into the specimen and was found to be greater than 2000 K · s^{-1} over the temperature range 0° to −100°C.

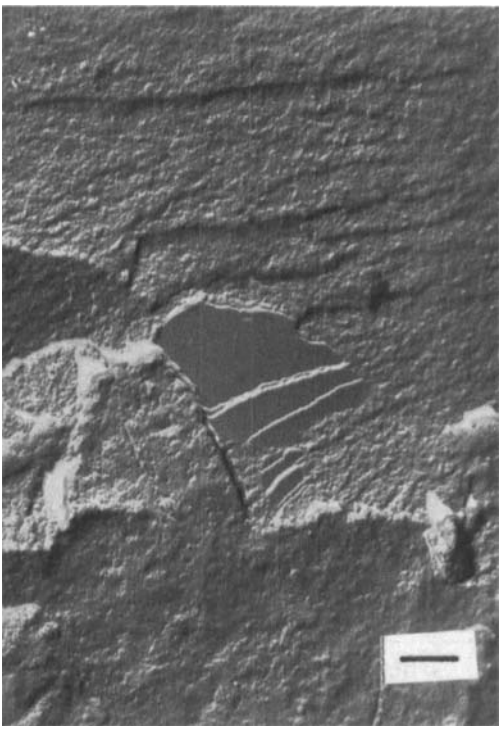

FIGURE 3 An electron micrograph of a freeze-fracture replica prepared from the α-phase of the saturated monogalactosyldiacylglycerol-water system. The sample was thermally quenched from 70°C. Bar represents 10 μm.

The transition between the α-phase and the L_β phase was examined in a lipid dispersion cooled at $10° \cdot min^{-1}$ from 60°C. Differential scanning calorimetry of the mixed acyl-chain lipid showed the transition to be exothermic with an onset temperature of about 41°C and a midpoint at about 35°C.[19] The corresponding changes in the X-ray diffraction profile over the temperature of the transition are presented in Figure 4. Changes in the lipid structure as reflected by changes in the low-angle scattering, are seen to take place while the sample is cooled from 60°C. This is evidenced by a reduction in intensity of the single low-angle diffraction band and a progressive shift in the diffraction maxima to longer spacings which continues until commencement of the transition at about 40°C. The α to L_β phase transition thus appears to proceed by the progressive replacement of the amorphous α-phase by the gel-phase bilayer structure with no evidence for the existence of any ordered intermediate phase. At the rate of temperature change employed in the experiment the overall transit time is about 75 s. Calorimetric scans of this lipid at $5° \cdot min^{-1}$ produced similar transition enthalpys as those obtained at $10° \cdot min^{-1}$[19] suggesting that the sample used in the X-ray experiment shown in Figure 4 was close to thermal equilibrium. This is also consistent with the static X-ray diffraction measurements shown in the inset to Figure 2. The formation of a lamellar structure in the L_β

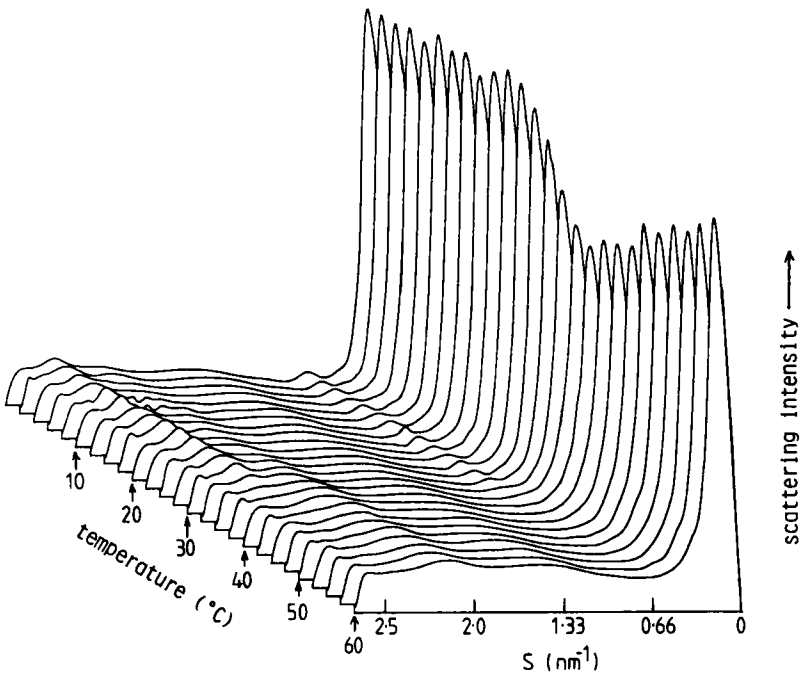

FIGURE 4 Three-dimensional plot of scattered X-ray intensity vs reciprocal spacing as a function of temperature for saturated monogalactosyldiacylglycerol dispersed in excess water. Temperature was scanned at 10°C per minute between 60° and 10°C. A total of 255 frames of 1.5 second duration each were recorded. Every tenth frame of the complete data set is presented in this figure. The patterns are characteristic of the transition from the liquid-crystal phase to gel phase bilayer (L_β) structures in this system.

phase can be seen with the growth of the fourth order Bragg reflection and the increase in diffraction intensity of the first order peak. The onset of these changes in low-angle reflections is first detected at 35°C coinciding with the midpoint of the exothermic transition observed by calorimetry. Corresponding changes in the wide-angle region of the diffraction pattern show a replacement of the broad band centered at 0.48 nm, commencing at about the same temperature as the changes signalling the formation of the lamellar phase, by a single sharp band at a spacing of 0.41 nm. There are no obvious changes in the broad bands corresponding to spacings of between 0.6-0.7 nm to indicate any change in spacing of the sugar residues, however, careful examination of individual frames of the data set through the transition indicate a progressive increase of the two spacings by about 0.02 nm takes place over the temperature range 25° to 35°C.

A static diffraction pattern obtained from the sample immediately on cooling the dispersion to 4°C from the α-phase is shown in Figure 5. The characteristic feature of the low-angle region is a single intense scattering band centered at 6.25 nm and a fourth order Bragg reflection indexing the lamellar repeat spacing. The inset showing an expanded scattering intensity scale in the wide-angle region confirms that the relative spacings for diffraction maxima from the sugar residues (0.69

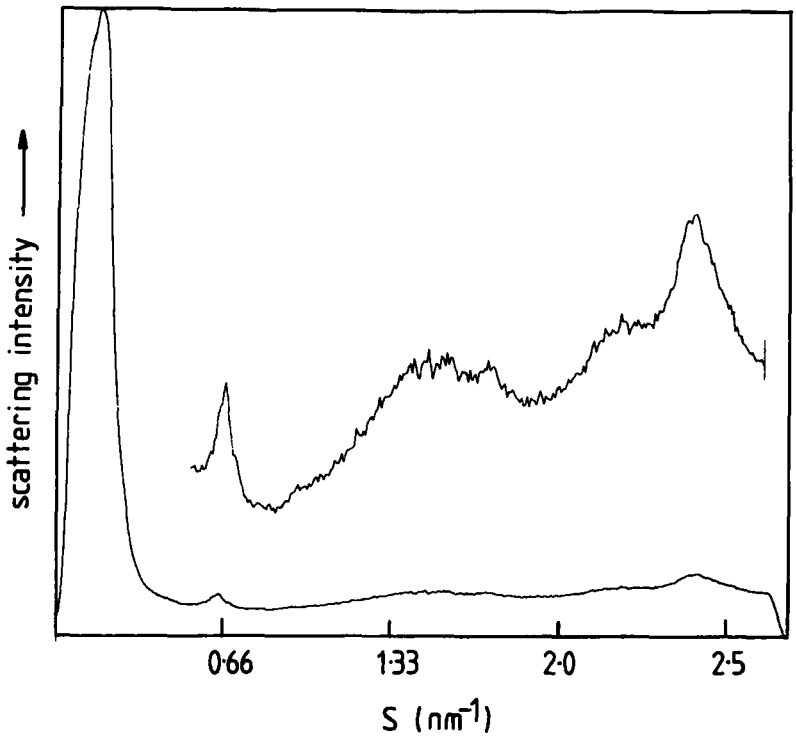

FIGURE 5 Scattered X-ray intensity as a function of reciprocal spacing for saturated monogalactosyldiacylglycerol in excess water at 4°C. The data were collected at constant temperature for 100 seconds. The pattern is characteristic of the gel bilayer phase (L_β) in this system. Inset shows ten-fold expanded intensity scale in the wide-angle region of the X-ray dispersion.

and 0.61 nm) remain unchanged from their position in the α-phase but the dimensions of the subcell is slightly larger. This may indicate that the arrangement of the polar head groups of the lipid is similar in the α and L_β phases and it is the hydrocarbon chains that are transformed from a disordered configuration to pack into an ordered lattice of the gel-phase bilayer. The dominant spacing of the chains in the L_β phase is centered at 0.41 nm indicating an hexagonal subcell packing arrangement of the chains typical of the L_β phase observed in saturated phospholipids and glycolipids.

The calorimetric studies of Sen *et al.*[5] have shown that the exothermic transition from α to L_β is unaffected by the rate of cooling from the α-phase but that the transition from L_β to α depnds markedly on the temperature and length of storage of the lipid before reheating through the transition. The extent of hydration of the sample and the rate of heating were also found to be factors which affect the transition from L_β to α-phase. These studies were all performed using the distearoyl derivative of monogalactosyldiacylglycerol and contributed significantly to the scheme illustrated in Figure 1. One feature of these studies was that in slow heating scans of the fully hydrated lipid in the L_β phase there was evidence for a conversion from L_β to another phase that was inferred to be a lamellar crystalline phase, designated

L_{C1} (a description of this phase is given below). A slow temperature scan (1.25° · min^{-1}) of the mixed acyl chain lipid was performed to see if a conversion from L_β to L_{C1} could be induced in the transition which terminates ultimately in the formation of the α-phase. There was a progressive decrease in intensity of the lamellar repeat diffraction commencing at about 25°C indicating expansion of the lattice which continued with heating up to 41°C when a sharp transition in both the wide and low-angle diffraction peaks was observed. This decrease in diffraction intensity of the first order lamellar repeat spacing is seen also in a broadening of the fourth order lamellar repeat and suggests increasing stacking disorder of the lipid bilayers as the temperature is increased. The most conspicuous changes are observed in the wide-angle region of the diffraction pattern. Increasing temperature between 30° and 40° causes a gradual shift and broadening of the chain diffraction peak at 0.41 nm to a position approximately centered at 0.46 nm. At 41°C this relatively sharp diffraction peak is transformed into the broad diffraction band centered at 0.48 nm characteristic of the disorded chains of the α-phase. Completion of the phase transition at about 42°C is associated with a broadening of this band and loss of higher order Bragg spacings in appearance of the α-phase. It would appear, therefore, at least in the mixed acyl chain lipid that the transition from $L_\beta \rightarrow \alpha$ proceeds in a manner consistent with a reversal of the $\alpha \rightarrow L_\beta$ transition with no evidence of a transition pathway $L_\beta \rightarrow L_{C1} \rightarrow \alpha$.

The L_{C1} phase can, however, be formed by thermal equilibration of the L_β phase at temperature less than about 30°C. Differential scanning calorimetric studies have shown that the rate of formation of the L_{C1} phase depends markedly on the equilibration temperature as well as the extent of hydration of the sample; the rate is faster with partially hydrated specimens because a loss of water from the phase appears to be associated with the transition.[5] Figure 6a shows a diffraction profile of the L_{C1} phase. An examination of the low-angle region of the diffraction pattern on an expanded intensity scale indicates the presence of two superimposed lamellar repeats which index to spacings of 7.41 nm and 6.29 nm. This suggests that the phase may not be homogeneous and there may be some residual L_β structure identified by the 6.29 lamellar repeat and the sharp acyl chain subcell band at 0.40 nm. According to this interpretation, the pure L_{C1} phase would consist of a lamellar repeat of 7.41 nm and a single sharp diffraction at 0.60 nm. We interpret the origin of this diffraction peak as arising from the packing of the galactose residues in an hexagonal subcell.[33] The hydrocarbon chains appear to be arranged in a distorted hexagonal (i.e. orthorhombic) subcell with spacings of 0.40 and 0.38 nm respectively. The transition between L_β and L_{C1} under isothermal conditions has been considered in detail elsewhere.[33,34] The transition involves a rearrangement of the galactose residues of the polar head group from orthorhombic to an hexagonal subcell with a concomitant transformation in the acyl chains from an hexagonal arrangement to a packing arrangement based on an orthorhombic subcell. This requires an apparent rotation of the whole molecule about an axis perpendicular to the bilayer plane and with the rearrangement of the hydrocarbon chains providing the driving force for the transition.

Prolonged (2–3 days) storage of the L_{C1} phase of the distearolymonogalactosyldiacylglycerol at temperatures less than about 30°C caused a transition to a low-

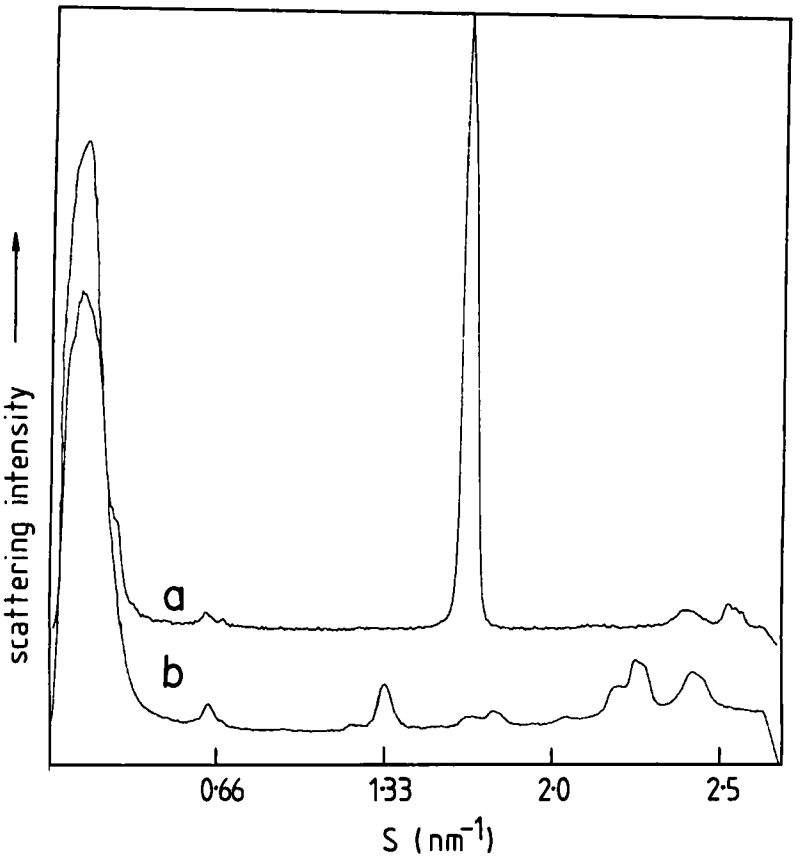

FIGURE 6 Scattered X-ray intensity as a function of reciprocal spacing for saturated monogalactosyldiacylglycerol in excess water (a) recorded at 20°C 10 min after cooling the dispersion from the α-phase. The data were recorded during 6 sec. exposure to the X-ray beam. The pattern is characteristic of the metastable crystalline bilayer (L_{C1}) phase in the system. (b) recorded at 14°C after equilibration at 20°C for 3 days before the data were collected during 100 sec. exposure to the X-ray beam. The pattern is characteristic of the low temperature crystalline bilayer (L_{C2}) phase of this sample.

temperature modified phase designated L_{C2}.[5] Figure 6b shows a diffraction pattern recorded after 3 days storage of the sample from which the X-ray scattering profile shown in Figure 6a was recorded. As with the L_{C1} phase the equilibrium state of the L_{C2} phase may be a mixture of two or even more different phases. Storage of up to 14 days, however, did not significantly alter the diffraction pattern. The residual sugar peak at 0.60, for example, may be due to the existence of some residual L_{C1} phase in the dispersion. Otherwise the profile looks quite distinct with a lamellar phase repeat of about 6.00 nm extending out to four Bragg diffraction orders. We would expect that if the sample was in an intermediate stage of a phase transformation between structures that the structures themselves would vary as a function of time. Since this is not the case, we can conclude that the phase is in equilibrium under the conditions employed in the study.

The peaks at spacings corresponding to 0.83, 0.75, 0.60 and 0.56 nm respectively can be attributed to the packing of the sugar residues. The hydrocarbon chains

appear to be arranged on a subcell of spacings corresponding to 0.43 and 0.40 nm; the origin of the weaker diffraction spacing at 0.45 nm is presumably also derived from the acyl chain packing (i.e. in a monoclinic subcell). The distinctive feature of both crystalline phases of the lipid is that the hydrocarbon chains tend to occupy a distorted hexagonal (i.e. orthorhombic and monoclinic) subcell compared with the hexagonal subcell of the lamellar gel phase.

The pathway of transition from L_{C2} phase to α was investigated by following changes in X-ray diffraction patterns during a temperature scan from 20°C at a heating rate of $10° \cdot min^{-1}$. Thermal studies of L_{C2} phase of the distearoyl derivative of the lipid show a broad exotherm commencing at about 57°C followed by the main endothermic transition at about 82°C.[5] Similar studies of the mixed acyl chain derivative show a much less pronounced exotherm at about 35°C and the major endothermic peak at 57°C.[19] The molar enthalpy values obtained for the main lamellar-α-phase transition of the distearoly derivative ($67 kJ \cdot mole^{-1}$) is somewhat greater than the mixed chain derivative ($40 kJ \cdot mole^{-1}$) and presumably reflects the more homogeneous packing of the chains in the pure molecular species. The pathway of the transition from L_{C2} to is believed to proceed via an intermediate phase of L_{C1} on the basis of the calorimetric studies and subsequently confirmed by time-resolved X-ray diffraction studies employing a temperature jump to drive the transition. Figure 7 shows the corresponding real-time X-ray diffraction profiles obtained during a slow thermal heating at $10° \cdot min^{-1}$.

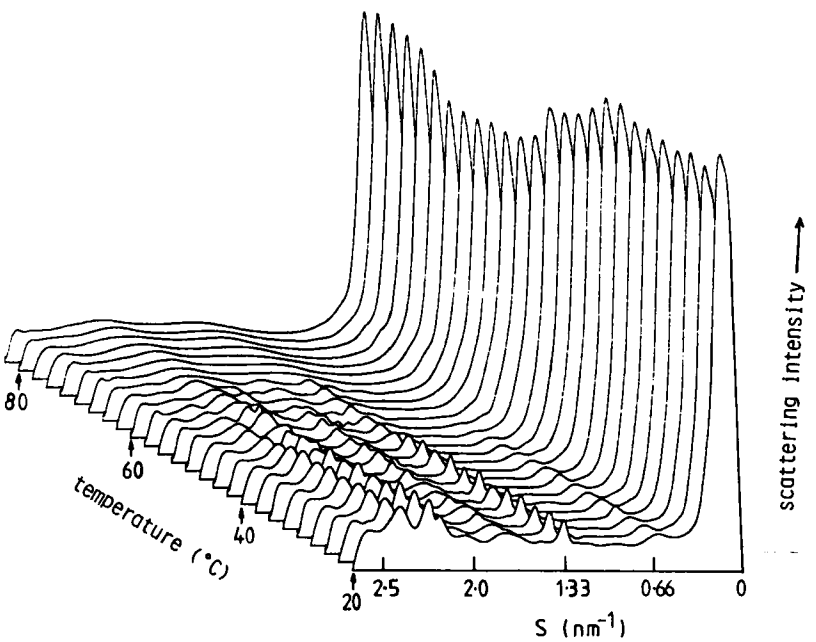

FIGURE 7 Three-dimensional plot of scattered X-ray intensity vs reciprocal spacing as a function of temperature for saturated monogalactosyldiacylglycerol. The temperature of the dispersion was increased from 20° to 80°C at a scan rate of $10° \cdot min^{-1}$. The sample was equilibrated for 3 days at 20°C prior to examination. A total of 255 frames were recorded each of 1.5 sec duration. Every tenth frame of the data set is presented in the figure. The patterns are characteristic of the transition between the crystalline bilayer (L_{C2}) phase and the liquid-crystalline phase of this system.

The slow heating mechanism, however, proceeds solely by a broadening of the sugar and acyl chain diffraction peaks suggesting a continuous disordering of the respective subcells. The loosening of the unit cell dimensions at slow scan rates and the existence of defined intermediate states during fast temperature jumps are both characteristic of phase transitions that are not first order. It is noteworthy that, in this system, the transition mechanism depends on heating rate but the order of the transition remains apparently the same.

The real-time X-ray diffraction study of the monogalactolipid-water system shows that the structural changes involved in transitions between phases is relatively complex. Furthermore, what are generally considered to be homogeneous phases on the basis of thermal studies are shown here to be complex multistructural entities which may not form single structure phases even under precisely defined conditions. It is also obvious from this study that the order of the phase transition can be determined using this technique and that the mechanism of a transition may be rate dependent. Specifically, we have determined that the L_{C1} phase can be observed as an intermediate in the L_{C2} transition only during fast temperature jumps. Slower scan rates allow for fluctuations between states to be averaged into distortions of the unit cell.

Acknowledgments

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