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Phase structures and transitions were monitored in real time for a fully hydrated 1 : 1 mixture of saturated and native monogalactosyldiacylglycerol using an x-ray beamline at the Daresbury (U.K.) Synchrotron Lab. A single phase characterized by a single diffraction peak at *ca.* 3.18 nm is observed over all temperatures studied. The initial crystalline acyl chain phase is equivalent to the LC_2 subcell packing observed in fully saturated mixtures. The transition from the LC_2 to the liquid crystalline phase may involve the appearance of intermediate states. Subsequent temperature changes do not produce a change in the liquid crystalline acyl chain phase over a time interval of hours.

INTRODUCTION

Biological membranes consist of a heterogenous mixture of lipids and proteins which in various combinations provide the scope of functions associated with the membrane. In addition, phase transformations and segregation within and between membrane domains are inferred to be an essential part of the mechanisms involved in these functions. Studies of model membranes have centered on either the characterization of individual phases, or the effect of additives on the liquid crystalline model membrane (bilayer) phase. An essential question to be answered is whether the effect of the proposed phase transformations within or between membrane domains on the liquid crystalline (fluid) bilayer phase is necessary for the general function of the biological membrane. Can these localized phase changes either alter the liquid crystalline bilayer phase in the membrane, and/or is the temporal relaxation of the liquid crystalline phase greater or less than the transit times for the localized phase transitions?

Time resolved x-ray diffraction (TRXRD) using x-rays from synchrotron sources has recently been used to examine phase transitions in phospholipid[1-2] and galactolipid systems.[3-5] During the course of our examination[3-5] of the phase transitions in fully saturated monogalactosyldiacylglycerol (MGDG), we also studied a mixture of a fully saturated MGDG and the naturally derived (some unsaturated acyl chains) MGDG. The fully saturated MGDG preparation was previously shown to produce an unusual phase above the liquid crystalline phase transition,[4,5] which produced a single mesophase diffraction peak, and an amorphous freeze fracture electron microscopy texture. The mixture of the fully saturated and naturally derived MGDG's produced the same phase over the entire temperature range studied. In addition, the liquid crystalline acyl chain phase was found to persist over long periods of time after its initial formation and even upon subsequent cooling to 5°C.

EXPERIMENTAL

Total lipid extracts of market spinach leaves were prepared as described previously.[6] The acyl chains in this extract are primarily linolenoyl.[13] Fully saturated monogalactosyldiacylglycerol was prepared by hydrogenating the natural lipid extract 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.[7] Saturation of the fatty acyl residues was verified by gas chromatography of the methyl ester derivatives as described by Restell *et al.*[8] The fatty acyl composition after hydrogenation of the monogalactosyldiacylglyceride was found to be 75% (by mass) stearoyl and 25% palmitoyl residues.

Lipid mixtures were made by combining the individual lipid species in chloroform. The chloroform was then removed under a flowing stream of N_2. X-ray samples were prepared by mixing the lipid with five fold excess by weight of water and allowing them to equilibrate at 20°C for over three 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 using a monochromatic (0.150 nm) focussed x-ray beam at station 7.3 of the Daresbury Synchrotron Laboratory as previously described.[9] A cylindrically bent single crystal of Ge[10] and a long float glass mirror were used for monochromatization and horizontal focussing. A Keele flat plate camera was used with a linear detector constructed at the S.E.R.C. Daresbury Laboratory. The data acquired was stored in a VAX-11/750 computer, and used to correct and analyze data sets. Data processing was performed using an OTOKO program which allowed smoothing of the raw data and subsequent plotting. X-ray scattering recorded in 255 consecutive time frames has been plotted as a function of reciprocal space, $s = 2\sin\theta/\lambda$ using teflon (0.48 nm) as a calibrated standard.[11] The dead-time between data acquisition frames during the time resolved experiments was 50 μs, with the temporal resolution of each frame of 2.0 s. All mesophase and subcell spacings were calculated using Bragg's Law.[12]

Temperature scans were produced by water baths connected internally to the sample mount of the x-ray camera. The rate of change of temperature was 10°C/

min. The temperature of the sample was monitored internally using a thermocouple placed adjacent to the sample in the x-ray sample holder.

RESULTS AND DISCUSSION

The phase relationships in a variety of monogalactosyldiacylglycerols have been wholly or partially characterized. The species isolated from bean and spinach photosynthetic membranes which contain primarily the linolenoyl acyl chain derivatives[13] can exist in an hexagonal (H_{II}) phase. The extent of acyl chain unsaturation has been shown to determine the conditions under which the lipid exists in the H_{II} phase.[14] The completely saturated acyl chain derivatives of MGDG have been shown to primarily form lamellar phases.[5,6,7,15] The distearoyl derivatives of MGDG has been completely characterized using calorimetry, freeze fracture electron microscopy and x-ray diffraction.[6,15] A variety of gel phases and the liquid crystalline phase were characterized and the conditions for inducing specific phase transitions elucidated. In addition, a mixture of saturated acyl chain MGDG species has been shown using calorimetry[7] and time resolved x-ray diffraction[3–5] to mimic the transitions observed for the pure distearoyl derivative.

Figure 1 indicates that the combination of mixtures from the naturally derived MGDG and the fully saturated MGDG at 25°C produces a single x-ray reflection at small angles characteristic of a d-spacing of 3.18 nm. We have previously interpreted this pattern using freeze fracture electron microscopy as being derived from an unusual mesophase packing structure[5] which can be described as being from an amorphous or perhaps liquid like phase.[16] An isotropic, disordered phase consisting of isolated small diffracting units of single (or nearly so) vesicles, bilayers, etc. could cause such a pattern to appear. The wide angle x-ray scattering for the MGDG mixture (saturated and unsaturated chains) consists of a number of reflections (0.79, 0.69, 0.61, 0.56, 0.46, 0.42, and 0.39 nm) which can be correlated to subcell packing of the acyl chains and head groups of the LC_2 phase for the fully saturated MGDG mixture (references 4, 5, and Figure 1). We can thus assign the two main diffraction peaks at 0.69, and 0.56 nm, and the shoulder peaks at 0.79 and 0.61 nm to the head group packing subcell, and the two main diffraction peaks at 0.46 and 0.39 nm and the shoulder peak at 0.42 nm to the acyl chain subcell.

The sample was then driven by a rate of 10°/min to higher temperatures. Figure 2 shows the diffraction patterns that were recorded every 2 sec as the temperature scan of 10°C min^{-1} proceeded. The initial diffraction pattern was identical to the static pattern shown in Figure 1, indicating that the initial structures were the same. As the scan proceeded, the relative intensities of the wide angle x-rays scattering peaks changed in a relatively random fashion. This could be indicative of subtle changes in the molecular orientation within the bilayer which would affect the Fourier series describing the atomic positions within the bilayer and thus influence the intensity distributions.[16] Other possible causes of these intensity fluctuations are the flow of material through the x-ray beam or rotation of the vesicles in the phase within the beam. Spurilous peaks observed in the diffraction patterns at high values of S when the sample temperature is 50 or 60°C are probably due to poor

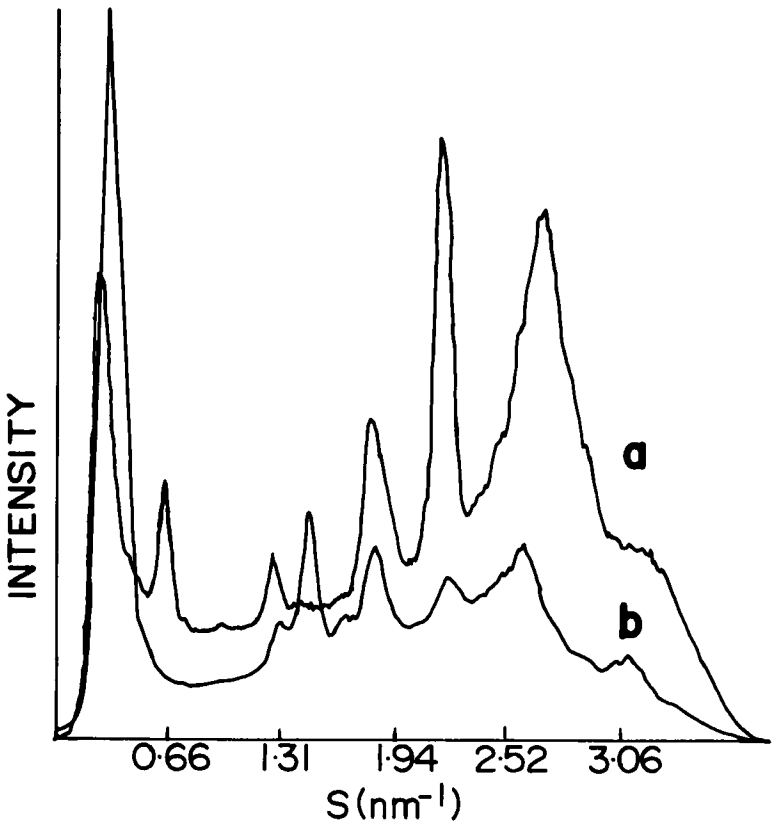

FIGURE 1 X-ray diffraction patterns for a) a fully saturated mixture of monogalactosyldiacylglycerol and b) a fully hydrated 1:1 mixture of saturated and native monogalactosyldiacylglycerol at 25°C. Diffraction intensity is plotted as a function of reciprocal spacing $s = 2\sin\theta/\lambda$.

detector response at the higher diffraction angles. These peaks *do not* fluctuate in intensity nor do they appear in consecutive patterns. The breadth of the transition as determined from Figure 2 is consistent with a previously reported thermogram.[7] The final state of our mixture is described by the x-ray diffraction pattern taken at 68°C after the phase transition to the liquid crystalline acyl chain subcell occurs. The single diffraction peak at a spacing of 3.2 nm is observed and again characterized as due to a mesophase packing from an amorphous or liquid like structure.[16] The acyl chains pack in a disordered subcell characteristic of the liquid crystalline or fluid phase with a spacing of 0.48 nm. Subsequent cooling and heating cycles over the span of hours did not result in a change in the acyl chain packing from the liquid crystalline phase. It may not be surprising that a mixture of lipids with high and low transition temperatures may prefer to remain in this disordered chain packing when cooled to below the transition temperature of the mixtures. A temporal hysteresis has been observed for phospholipid mixtures[17] upon cooling below the acyl chain transition temperature. However, the transition of these bilayers from the liquid crystalline to a gel state occurs within seconds of reaching the

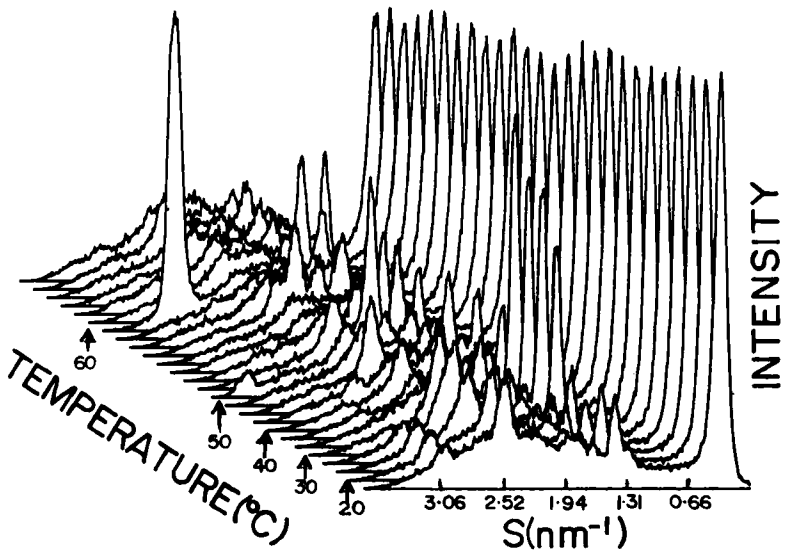

FIGURE 2 Three dimensional representation of diffraction patterns collected over 510 sec for a fully hydrated 1:1 mixture of saturated and native monogalactosyldiacylglycerol undergoing a temperature scan of 10°C/min. Every tenth frame of the total ensemble of 255 frames is indicated in this figure.

transition temperature. Even the transition from the liquid crystalline state to a more ordered acyl chain packing in mixtures of saturated monogalactosyldiacylglycerol only[18] requires minutes to occur once the sample temperature has been cooled below T_m. The long time necessary for the relaxation from the liquid crystalline chain packing for the mixture of saturated and unsaturated monogalactosyldiacylglycerols is thus unusual.

There was no evidence in any of our data sets that the LC_2 to liquid crystalline phase transition occurred via a gradual change in the subcell packing arrays as observed for the saturated chain MGDG mixture undergoing a 10°C/min scan.[5] However, subtle changes in the peak intensities may be indicative of the formation of intermediate states as observed for a saturated chain MGDG mixture undergoing a fast temperature jump.[4] We can thus infer that this phase transition proceeds via a non-two state or second order mechanism.

CONCLUSIONS

Our results indicate that for a multicomponent system consisting of mixtures of naturally derived (*i.e.*, unsaturated acyl chains), and fully saturated monogalactosyldiacylglycerol in water, the lipids pack in an unusual mesophase array at all temperatures. In addition, the liquid crystalline acyl chain packing subcell is found to have a temporal relaxation time of hours. These results suggest that membrane processes involving phase transitions and segregations do not affect the overall acyl chain order in a membrane because the transit time for the transition from the

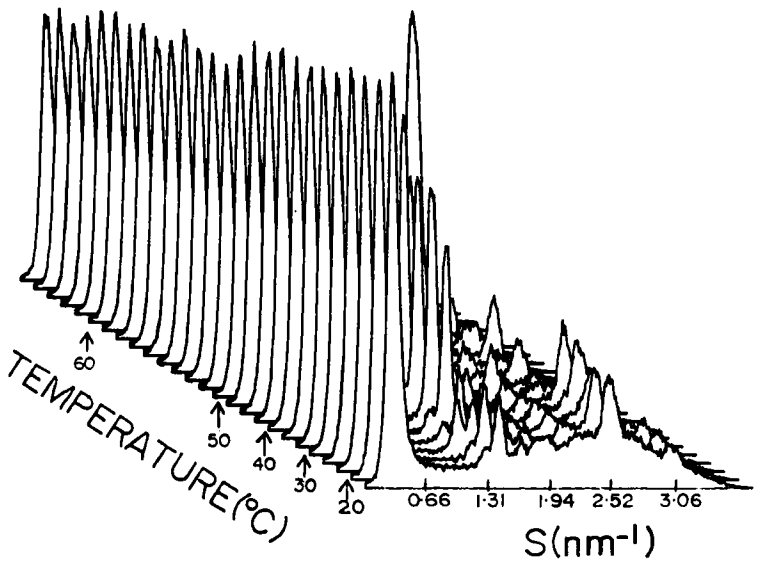

FIGURE 3 The same data as shown in Figure 2 but viewed from a different perspective to illustrate changes in the low angle diffraction region of the pattern.

liquid crystalline chain packing in a lipid mixture containing glycolipids with unsaturated chains is orders of magnitude slower than, for example, transitions involving a change from the liquid crystalline to gel bilayer state in saturated chain MGDG[5] or from hexagonal to lamellar mesophases in phospholipids.[2] The composition of components in a lipid mixture are shown to influence the phase structures, the transition mechanisms and the kinetics of the relaxation processes leading to a phase transition. We cannot at this time hypothesize why MGDG with unsaturated acyl chains has a greater effect on the relaxation from the alpha phase than the other lipids studied so far.

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