Molecular Crystals and Liquid Crystals Science and Technology. Section A. Molecular Crystals and Liquid Crystals

Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/gmcl19

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Version of record first published: 04 Oct 2006

To cite this article: P. W. Westerman, R. Jacquet, B. Quinn, P. L. Rinaldi, W. A. Daunch & Y. Sun (1997): Physicochemical Characterization of Liquid Crystalline Phases in Model Bile and Lipid Digestive Mixtures: A ^{2}H Nmr Study, Molecular Crystals and Liquid Crystals Science and Technology. Section A. Molecular Crystals and Liquid Crystals, 299:1, 287-292

To link to this article: http://dx.doi.org/10.1080/10587259708042006

Mol. Cryst. Liq. Cryst., 1997, Vol. 299, pp. 287–292
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PHYSICOCHEMICAL CHARACTERIZATION OF LIQUID CRYSTALLINE PHASES IN MODEL BILE AND LIPID DIGESTIVE MIXTURES: A ^{2}H NMR STUDY

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Abstract Phase properties of aqueous dispersions of model bile mixtures containing cholesterol, lecithin and bile salts and model mixed lipid digestive mixtures containing cholesterol, fatty acid, monoglyceride, lecithin and bile salts have been characterized by ^{2}H NMR. We have chemically incorporated a deuteriomethyl (CD_3) group into one or two lipid components and utilized differences in motionally-averaged quadrupole splittings ($\Delta\nu$) and CD_3 chemical shifts, to determine directly by peak-area integration, for a number of compositions on the phase diagram, the distribution of a ^{2}H-labeled component between micellar, lamellar and solid phases, as well as the chemical compositions of these phases. Our data show that increasing cholesterol stabilizes lamellar phases whereas bile salts disrupt them and favor micelle formation.

INTRODUCTION

To understand the factors which influence the dietary absorption of cholesterol, fatty acids, monoglycerides and fat soluble vitamins, it is necessary to know the physical state in the upper gastrointestinal tract of bile as well as the products formed by the enzymatic breakdown of dietary triglycerides in the presence of detergent bile salts.[2] The traditional approach to physically characterize aqueous dispersions of lipid mixtures has been to determine phase diagrams of model systems and use this information to estimate the distribution of lipid components between micellar, vesicular and solid phases.[3] In much of the work already performed in this area, lipid compositions of the various aggregates have been determined chemically after their separation by techniques which suffer from the disadvantage that artifacts are created by demulsification and the spontaneous transfer of lipids among the several aggregate structures during the separation process.

We describe here ^{2}H NMR techniques that enable both the physical states and chemical compositions of the various phases in model bile and gastrointestinal lipids to be directly determined without separation of the different phases. Our procedure utilizes a non-perturbing deuteriomethyl group which is incorporated synthetically into one of the lipid components of the mixture. ^{2}H NMR of the mixture enables the quantitative determination of the distribution of the ^{2}H-labeled molecule between micellar, liquid

crystalline and solid phases. By systematically examining the same system with the -CD_3 on each of the components of the lipid mixture, it was possible to determine the chemical composition of each aggregate state in the system.

MATERIALS AND METHODS

[26,26,26,27,27,27-$^2H_6$]-Cholesterol (chol-d_6) was obtained commercially (Medical Isotopes, Concord, NH). ω-Deuterated myristic acid was prepared as reported[4]. Phosphatidylcholine, ^2H-labeled in the methyl groups of the choline moiety, was prepared by methylation of phosphatidylethanolamine using C^2H_3I. 1-Myristoylglycerol-ω-d_3 was prepared by the condensation of the acetonide derivative of glycerol with deuterated myristoyl chloride. The protecting group was removed by mild acid treatment.

Mixed bile salt stock (MBS) solutions, patterned after the average composition of human bile, were prepared in methanol. Mixed intestinal lipid (MIL) solutions (myristic acid:DMPC:monomyristin = 5:1:1) were prepared volumetrically from stock solutions of individual lipid components in $CHCl_3$. Systems containing all three components (cholesterol/MIL/MBS) with a single constituent carrying a deuterium label were prepared by coprecipitation of the lipid mixtures from $CHCl_3/CH_3OH$ mixtures. Organic solvents were evaporated under N_2 and then under high vacuum. Aqueous buffer was added and the lipids dispersed by vortex stirring. The aqueous buffer was 33 mM NaH_2PO_4 (pH 6.5) in ^2H-depleted H_2O with sufficient NaCl to give a final $[Na^+]$ = 150 mM. Model bile samples were made by the same procedure with lecithin replacing the MIL component.

^2H NMR spectra were recorded at 37°C, using either a "home-built" spectrometer operating at 30.87 MHz or a Varian Unityplus 600 MHz spectrometer operating at 92 MHz. A modified quadrupole echo sequence was used for data acquisition. Spectra were obtained "on-resonance." With a 25 mg lipid sample, specifically labeled on a single component in a single position, adequate signal to noise was obtained from 5,000–40,000 transients. Each sample was allowed to equilibrate for 4 days before recording its spectrum.

RESULTS AND DISCUSSION

Model Bile Mixtures

The equilibrium phase diagram for the ternary lipid system lecithin-cholesterol-MBS is shown in Fig. 1. NMR samples examined contain either 1,2-dimyristoyl phosphatidylcholine (DMPC) or 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) as the lecithin and either 4 or 10 mol % cholesterol. The total sample compositions examined by ^2H NMR are indicated by region A and points B-G on the phase diagram. The 30.87 MHz ^2H NMR spectra of 10 wt % DMPC-d_9/MBS/4 mol % cholesterol mixtures in ^2H-depleted H_2O were recorded (37°C) for varying DMPC/MBS ratios (Region A). A single motionally averaged quadrupole splitting ($\Delta\nu$) was observed and its magnitude is plotted versus the mole fraction of MBS (Fig. 2(a)). Representative spectra are shown.

Addition of the detergent bile salts to DMPC bilayers produces an almost linear reduction in the observed $\Delta\nu$. When 40 mol % MBS has been added, $\Delta\nu = 0$. Closer examination of this sample (Point B) using both chol-d_6 and DMPC-d_9 gave the 92 MHz

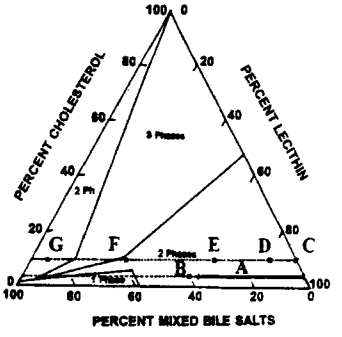

FIGURE 1 Phase Diagram for Lecithin-Cholesterol-MBS-H_2O (90 wt %) Mixtures.

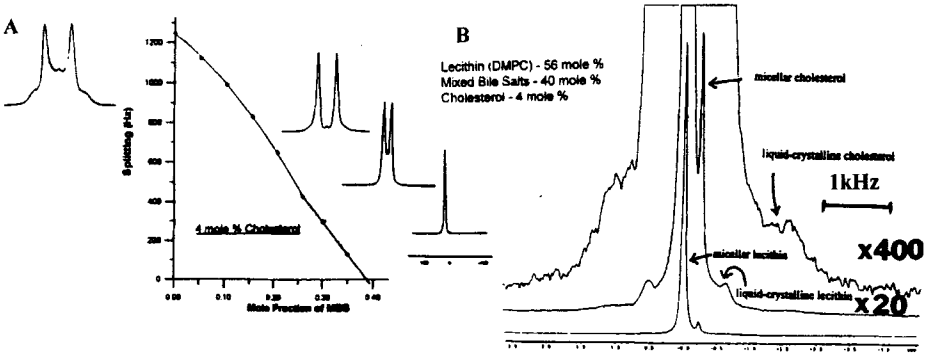

FIGURE 2 a) Quadrupole splittings vs Mole fraction of MBS in DMPC-d_9/ cholesterol/MBS/H_2O Mixtures; b) ^2H NMR spectrum of DMPC-d_9 (56 mol %)/ cholesterol-d_6 (4 mol %)/MBS (40 mol %)/H_2O Mixture.

NMR spectrum shown in Fig. 2b. Two sharp peaks in the center with a separation of 2.35 ppm were assigned to micellar DMPC-d_9 and cholesterol-d_6. Integration of these peaks allows the relative amounts of cholesterol and DMPC in the micelles to be estimated. The bile salt composition could not be determined. ^2H NMR experiments with ^2H-labeled bile salt (sodium glycodeoxycholate-d_2) showed a single sharp peak, indicating fast exchange between simple MBS micelles and mixed DMPC/cholesterol/MBS micelles. Also evident in the above spectrum (Fig. 2b) are powder patterns assigned to DMPC-d_9 ($\Delta\nu = 0.9$ kHz) and chol-d_6 ($\Delta\nu \approx 2$ kHz), arising from multilamellar vesicles (MLVs), composed of approximately equimolar amounts of DMPC and cholesterol.

The data in Fig. 2a are interpreted in terms of the following model. In the absence of bile salts the lecithin exists as MLVs containing 4 mol % cholesterol. Bile salt addition leads to its binding on the bilayer surface in the DMPC headgroup region, resulting in increased bilayer curvature. Probably large linear defects or tears first develop in the bilayers. With increasing concentration of bile salts the sample becomes increasingly transparent and viscous. When the mole fraction of MBS is 0.20 we postulate the existence of long cylindrical "worm-like" micelles of variable length, with the

surface of the cylinders coated with the hydrophilic faces of the bile salts and the polar headgroups of DMPC molecules, and the interiors of the cylinders consisting of DMPC acyl chains. We were unable to obtain x-ray scattering data to indicate a hexagonal packing of these cylindrical micelles. Further addition of bile salt results in increased breakage of the long micelles into small micelles which tumble rapidly on the NMR time scale. The formation of these smaller particles would account for the observed decrease in sample viscosity between MBS mole fractions of 0.25 and 0.40.

The 92 MHz ^{2}H NMR spectra of 10 wt % POPC-d_9/MBS/10 mol % chol-d_6 mixtures in ^{2}H-depleted water were recorded (37°C) for varying POPC/MBS ratios (Points C–G, Fig. 1). Representative spectra are shown in Fig. 3. In the absence of bile salts only a single Pake doublet with $\Delta\nu$ = 1.21 kHz for POPC is observed, indicative of a lamellar (L_α) phase. An additional out-of-phase powder pattern from chol-d_6 ($\Delta\nu \approx$ 2kHz) is evident upon the addition of 10 mol % MBS (Point D). Addition of more MBS gives a sample (30 mol % MBS, 60 mol % POPC-d_9) whose spectrum (Point E) is the superposition of two sharp lines separated by 2.35 ppm. These peaks were assigned to POPC-d_9 and chol-d_6 in micellar particles, whose POPC to cholesterol molar ratio is 22:1. Two out-of-phase powder patterns with $\Delta\nu$ = 1.03kHz and ≈ 2kHz were also observed and assigned to lamellar POPC and cholesterol. Although it was not possible to determine the bilayer composition by spectral integration, this quantity was calculated from the total sample and micellar compositions and the relative intensity of the powder pattern from POPC-d_9 (55 mol % POPC; 45 mol % cholesterol). It is apparent from this spectrum that POPC bilayers containing 10 mol % cholesterol respond differently to the

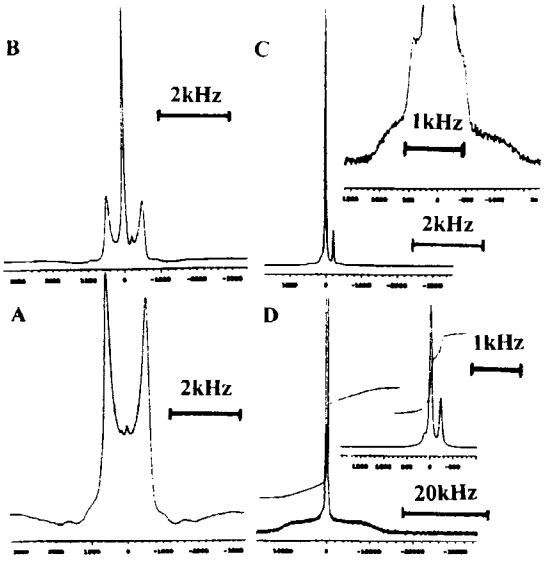

FIGURE 3 92 MHz ^{2}H NMR spectra of 10 wt % POPC-d_9/MBS/10 mol % cholesterol-d_6 dispersions in H_2O. A) Point D, 80 mol % POPC:10 mol % MBS; B) Point E, 60 mol % POPC:30 mol % MBS; C) Point F, 30 mol % POPC:60 mol % MBS; D) Point G, 5 mol % POPC:85 mol % MBS. Note different scale in D. Points D–G refer to Fig. 1.

increasing amounts of MBS than do those containing 4 mol % cholesterol. Rather than single Pake doublet with a decreasing splitting observed in the spectrum of the latter we find the superposition of a sharp line on a Pake doublet for the samples containing 10 mol % cholesterol. The addition of cholesterol to the bilayer slows down the rate of exchange of POPC-d_9 between lamellar and cylindrical micellar environments; that is, cholesterol stabilizes the POPC lipid bilayer, and counteracts the disruptive effects of detergent bile salts.

Further addition of MBS gives samples whose spectra show the presence of both micellar and lamellar aggregates. The molar ratio, POPC/cholesterol in the micellar particles decreases from 7:1 to 5:2 between total sample compositions of 40 mol % POPC; 50 mol % MBS and 5 mol % POPC:85 mol % MBS. In the last sample, there is insufficient MBS and POPC in the sample to solubilize all the chol-d_6 which precipitates out as polycrystalline material. This is the origin of the broad spectral component of width ~28 kHz observed in Fig. 3, point G.

Model Lipid Digestive Mixtures[5]

The equilibrium phase diagram for aqueous model lipid mixtures resembling those found in the upper small intestine during lipid digestion and absorption in adult human beings is similar to that shown for model bile mixtures (Fig. 1) except that the lecithin component is replaced by a 5:1:1 molar mixture of fatty acid-monoglyceride-lecithin.

The 30.87 MHz ^{2}H NMR spectra of a 10 wt % mixture of 4 mol % cholesterol 76 mol % mixed intestinal lipids (myristic acid-monomyristin-DMPC = 5:1:1) and 20 mol % MBS, in ^{2}H-depleted water (pH 6.5; [Na^+]=150 mM) are shown in Fig. 4. Each of the four lipid components was ^{2}H-labeled on its terminal methyl group, in turn.

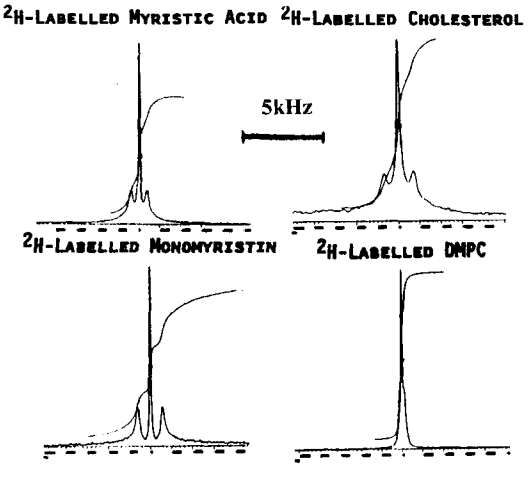

FIGURE 4 ^{2}H NMR spectra of 4 mol % cholesterol/76 mol % MIL/20 mol % MBS.

From the data in Fig. 4, the chemical composition of the micellar and lamellar phases was estimated. Similar calculations were performed in the same manner for three other points (0, 40 and 60 mol % MBS: 96, 56 and 36 mol % MIL:4 mol % cholesterol)

on the phase diagram. The results are shown in Fig. 5a. The dependency of $\Delta\nu$ for each lipid component in the lamellar phase, as a function of the MBS mole fraction is shown in Fig. 5b.

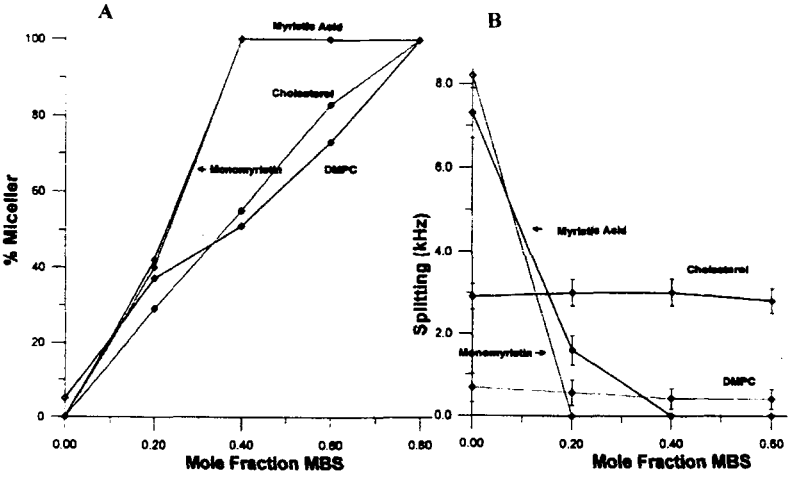

FIGURE 5 a) % of ^{2}H-labeled lipid component present as micelles in MIL/MBS/cholesterol (4 mol %)/H_2O dispersions at several mole fractions of MBS; b) Dependence on MBS mole fraction of $\Delta\nu$ in the ^{2}H NMR spectra of MIL/MBS/cholesterol/H_2O in which a single lipid is ^{2}H-labeled.

The above figures demonstrate the preference of myristic acid and monomyristin for micellar particles and the preference of cholesterol and DMPC for the L_α phase. The composition of the MIL phase changes as a function of MBS content, indicating that it should not be regarded, as we have done, as a single component in the phase diagram.

The above results show that ^{2}H NMR is a powerful technique for the physicochemical characterization of aqueous lipid dispersions resembling bile and mixed intestinal lipids. Chemical compositions of the micellar particles and lamellar phase can be determined and the orientational ordering of each component in the lamellar phase measured.

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