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The induction of lamellar stacking by cholesterol in lecithin–bile salt model systems and human bile studied by synchrotron X-radiation

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Small angle X-ray scattering (SAXS) with synchroton radiation was used to investigate interactions among lipid particles in lecithin–bile salt model systems and in native gallbladder biles. In model systems in the absence of cholesterol, isotropic, continuous spectra were found, indicating the absence of periodic structures. In the presence of excess cholesterol, interaction in the form of lamellar stacking was detected by the appearance of discrete diffraction peaks. In the supersaturated cholesterol region of the commonly accepted phase diagram [1], where cholesterol crystals were expected, we found lamellar stacking. The high proportion of cholesterol to bile salts seems to be the common denominator of these models. The lamellar stacking was also found in native unprocessed bile. This effect of cholesterol on lipid structure has not been previously described. Lamellar stacking may contribute to cholesterol solubilization. Its influence on the kinetics of cholesterol crystallization is presently unknown.

Cholesterol; Phospholipid; Bile salt; X-ray scattering; Lamella; Bile

1. INTRODUCTION

Characterization of lipid aggregates in human bile and measurement of their interactions and stability are important for an understanding of the precipitation of cholesterol in bile, which is a crucial step in gallstone formation [1]. According to phase diagrams of quaternary systems of lecithin–bile salt–cholesterol–water, lipid aggregates in the form of mixed micelles, liquid crystals and crystals may coexist [2,3]. In bile, cholesterol was initially thought to be solubilized solely in mixed micelles [1–4]; subsequently unilamellar phospholipid vesicles were identified [5–7]. These vesicles may be the vehicle for cholesterol secretion into bile [8] and are thought to participate in cholesterol crystallization [9]. Recently, phospholipid lamellar structures were described by electron microscopy and SAXS in human gallbladder bile [10]. These particles may be an intermediate between vesicles and micelles. Their role in biliary pathophysiology has not yet been characterized. The influence of cholesterol on the morphology of lipid particles in human bile as well as in model systems containing phospholipids and exceptionally high concentrations of bile salts, is incompletely characterized [3,6,11]. The high intensity of the synchroton generated X-ray beam has enabled us to observe, for the first time, in bile models and in native bile an interaction of bilayers which formed as a result of increasing cholesterol and decreasing bile salt concentrations.

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2. EXPERIMENTAL

2.1. *Model lipid systems*

Cholesterol (Sigma) was twice recrystallized from hot ethanol; bile salts (Sigma) were twice recrystallized from ethanol and ether [12]. Egg phosphatidylcholine (Sigma), cholesterol and a mixture of bile salts were solubilized in a chloroform–methanol solution in the proportions described in Table I, dried at RT and lyophilized overnight. Bile salt mixtures contained Na-cholate, Na-chenodeoxycholate and Na-deoxycholate both as taurine and glycine conjugates in molar proportions of 4:4:2:6:6:3 to mimic a physiological situation [11]. All specimens were solubilized in 150 mM NaCl and 50 mM Tris-HCl pH 8.0, incubated at 55°C for 1 h; then kept at RT and examined within less than 24 h.

Human gallbladder biles were obtained after cholecystectomy and kept for less than 24 h in a thermostable vessel at 37°C until examined. The lipid composition of the biles was determined chemically as previously described [10].

2.2. *Diffraction measurments*

SAXS measurements of the model lipid systems were performed at the outstation of the European Molecular Biology Laboratory on the double focusing monochromator-mirror camera X33 [13] at the Hamburg synchroton radiation facility (HASYLAB) on the storage ring DORIS of the Deutsches Elektronen Synchrotron (DESY). All measurements were made at a wavelength of 0.15 nm. Diffraction patterns from 100 μl samples were recorded at 23 ± 1°C with exposure of 5 min. A background pattern (the buffer) was subtracted. Further details of the data acquisition and evaluation systems can be found elsewhere

[14]. SAXS measurement of the human gallbladder biles was performed on beamline 2.1 at the Synchrotron Radiation Source (SRS), Daresbury, UK as described by Towns-Andrews et al. [15]. The wavelength was 0.154 nm. Diffraction patterns were recorded as described above. For background subtraction, the samples were centrifuged for 40 min at RT at 100 000 × g_{max}. The diffraction pattern of the supernatant solution was subtracted as background for each specimen. The floating lipid layer obtained after this centrifugation [10] from bile 3.2 (Table I) was also examined.

3. RESULTS

X-Ray diffraction patterns of all dispersions composed of lecithin and bile salts without cholesterol (Table I, models 1.1–1.4), revealed only isotropic and continuous spectra without discrete peaks. Fig. 1a shows a typical diffraction pattern for these models, of a dispersion containing twice as much lecithin as bile salts (model 1.1). There is a clear intensity minimum at 12.9 nm and a secondary maximum at 4.4 nm. The presence of cholesterol above saturation (Table I, models 1.5–1.7) caused a shift in the position of the minimum and maximum to 7.2 and 4.0 nm respectively (Fig. 1b). In model systems containing 12 and 17 mol% cholesterol (Table I, models 1.11 and 1.12, Fig. 1c and 1d) distinct sharp peaks were seen at a distance of 8.1 and 8.0 nm respectively. Weak second order reflections are found at distances of 3.8 and 3.9 nm. This indicates the presence of some relatively long range periodicity consistent with lamellar stacking. An additional reflection was found at 3.4 nm in the dispersion which was richest in cholesterol (model 1.12), probably indicating the presence of crystalline cholesterol domains [16].

Model bile systems with lipid concentrations equal to those found in native gallbladder biles (Table I, group II) demonstrated, in six of the seven cases, peaks with repeat distances ranging from 8.0 to 8.9 nm. The only model bile not showing particle interactions had a much higher bile salt to cholesterol ratio (11.7) and a lower cholesterol concentration (6 mol%).

The above data indicate that lamellar stacking was found only in model systems containing ≥ 10 mol% cholesterol and having a bile salt to cholesterol molar ratio of <10. All these models were above saturation (11); but supersaturation was not a sufficient condition for lamellar stacking. The water content of all model systems was ≥ 88 WT%.

Six native gallbladder biles were studied; two of these biles were highly concentrated (Table I, Group III). One

Table I

Lipid composition and SAXS analyses of specimens studied

	Ch	PL	BS	Lipids (g/dl)	CSI	d spacings	
	(mM)					(nm)	
Group I							
1.1	0	50	25	5.1			
1.2	0	50	50	6.3			
1.3	0	50	100	8.8			
1.4	0	50	150	11.3			
1.5	10	15	100	6.5	180		
1.6	10	20	100	6.8	146		
1.7	10	30	100	7.6	106		
1.8	10	40	100	8.4	87		
1.9	10	50	100	9.2	77		
1.11	20	50	100	9.6	143	8.1	3.8
1.12	30	50	100	9.9	201	8.0	3.9
1.13	10	50	150	11.6	63		
Group II							
2.1	20.8	56.9	97.0	10.0	143	8.0	
2.2	13.3	34.6	64.9	6.4	153	8.5	
2.3	11.4	28.7	75.6	6.4	137	8.2	
2.4	13.2	43.7	154.0	11.5	89		
2.5	28.1	54.8	61.4	8.3	252	8.9	6.8
2.6	10.0	30.7	60.0	5.7	132	8.7	
2.7	13.2	37.1	76.0	7.1	134	8.4	
Group III							
3.1	17.2	81.3	264.5	20.0	59		
3.2	39.7	110.7	298.9	24.8	99	6.3	

Ch, cholesterol; PL, phospholipids; BS, bile salts; CSI, cholesterol saturation index [23]. Groups I are model systems prepared with bile salt mixtures as described in section 2. Groups II are model systems prepared with Na-taurocholate. Group III are gallbladder biles.

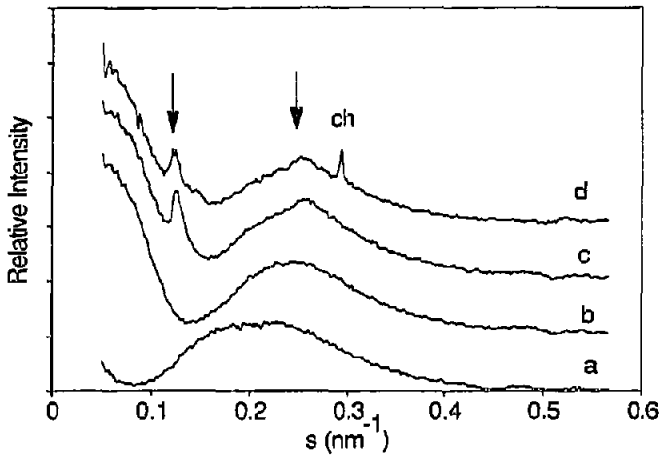

Fig. 1. The X-ray scattering profiles of model systems: (a) model 1.1; (b) model 1.9; (c) model 1.11; (d) model 1.12. All X-ray diffraction patterns represent the relative intensities and are plotted versus the parameter $s = 2 \sin \theta/\lambda = 1/d$ with θ, scattering angle, λ, wavelength, d, spacing ratios of the reflections. The peaks indicating lamellar stacking are marked by arrows. The crystalline cholesterol peak is marked with ch.

had a low cholesterol concentration and no stacked lamellae were seen (Table I, bile 3.1, Fig. 2a). In the second bile the cholesterol concentration was higher (9 mol%) and stacked lamellae were seen (Table I, bile 3.2, Fig. 2b). The interlamellar distance was 6.3 nm and the second reflection was seen at 3.3 nm. A floating lipid layer obtained from this bile following centrifugation showed a much more pronounced peak of stacked lamellae, with a similar interlamellar distance of 6.3 nm, a second order reflection at 3.2 nm and a reflection at 3.4 nm again probably corresponding to crystalline cholesterol (Fig. 2c). The other four biles in this group had unusually low lipid concentrations (<3 g/dl) and no lamellar stacking was observed.

4. DISCUSSION

Multilamellar structures are characteristically formed in concentrated pure phospholipid dispersions in aqueous media [17]. Bile salts solubilize large sheet like structures into discrete bilayered particles [18] and, in excess, induce the formation of mixed phospholipid–bile salt micelles [19]. The influence of the addition of cholesterol to phospholipid–bile salt mixtures has been partially characterized; for mixtures with physiologic proportions of constituents similar to those examined here no structural effects were found [3,20,22]. In the present work we have demonstrated the occurrence of lamellar stacking in the presence of excess cholesterol (>10 mol%) and when the bile salt to cholesterol molar ratio was <10. Both these constraints were previously not recognized as being structural determinants in model bile. In the models supersaturated with cholesterol, investigated in this study, no cholesterol crystals were found except in the most supersaturated solution (model 1.12). Nevertheless, according to the phase diagrams published [2,3,11], cholesterol crystals might have been expected. This absence of crystals may be due to the effect of stacked lamellae on cholesterol solubility. However, there were also differences in equilibration times. In addition, Cabral and Small [1] have commented that if the bile salt to lecithin proportion is <7:3 in solutions supersaturated with cholesterol, a liquid crystalline phase (stacked bilayers?) will be formed and only at higher degrees of supersaturation will cholesterol crystals precipitate.

Certain differences were noted in this study between the stacked lamellar structures in model systems and in human gallbladder bile. Lamellar stacking was found in native bile at lesser cholesterol concentration (9 mol%; CSI = 99). In addition the interlamellar distance was 6.3 nm in the one bile measured and 8.0 nm or more in the model systems. Many components present in bile and not included in the model systems such as proteins, mucins, Ca, as well as different phospholipids could have caused these differences. Osmotic activity of mucins in bile might contribute to the different hydrations observed. Since all these systems have a fairly high water content, no cubic phases would be expected; hexa-

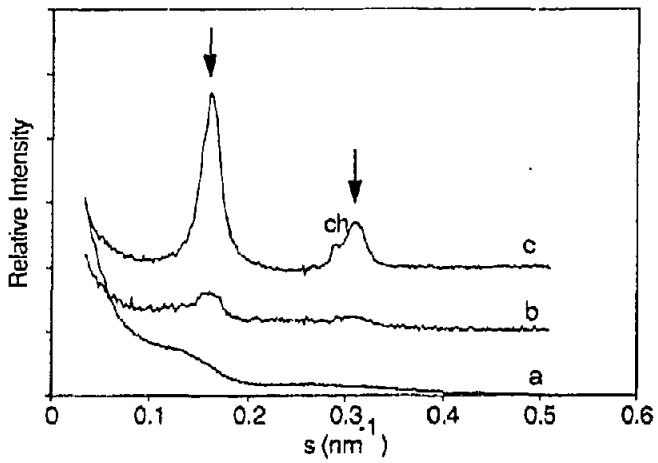

Fig. 2. The X-ray scattering profiles of native gallbladder biles: (a) bile 3.1; (b) bile 3.2; (c) floating lipid layer of bile 3.2.

gonal symmetry as suggested by other investigators [20] was not found, as shown by ratio of the spacings of the reflections (approx. 2:1) measured both in the native bile and in the model systems (Table I).

With the present technique, stacked lamellae were found in six of the seven model systems containing lipid concentrations similar to gallbladder biles in which stacked lamellae were previously demonstrated in floating lipid layers of these biles [10]. This good correlation between the data from these models systems and the extracts from native biles is remarkable. Structures investigated by polarized light microscopy and designated as liquid crystals were previously seen by some investigators [21,22] in supersaturated human biles and in model systems. With the present experimental configuration, vesicles could not be detected; therefore it is not known whether the stacked lamellae are independent structures or are in vesicular form. Lamellar stacking, induced by excess cholesterol, as demonstrated by the present study, has to the best of our knowledge, not been previously described. Stacked lamellae may represent an intermediate structure contributing to cholesterol solubilization. Their effect on the kinetics of cholesterol crystallization in supersaturated biles is being studied.

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