Behavior of Cholesterol and Spin-Labeled Cholestane in Model Bile Systems Studied by Electron Spin Resonance and Synchrotron X-Ray

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ABSTRACT The behavior of mixed bile salt micelles consisting of sodium taurocholate, egg phosphatidylcholine, and cholesterol has been studied by ESR spin labeling and synchrotron x-ray scattering. Consistent with published phase diagrams, pure and mixed bile salt micelles have a limited capacity to incorporate and, hence, solubilize cholesterol. Excess cholesterol crystallizes out, a process that is readily detected both by ESR spin labeling using 3-doxyl-5α-cholestane as a probe for cholesterol and synchrotron x-ray scattering. Both methods yield entirely consistent results. The crystallization of cholesterol from mixed bile salt micelles is indicated by the appearance of a magnetically dilute powder spectrum that is readily detected by visual inspection of the ESR spectra. Both the absence of Heissenberg spin exchange and the observation of a magnetically dilute powder spectrum provide evidence for the spin label co-crystallizing with cholesterol. In mixed bile salt micelles containing egg phosphatidylcholine, the solubility of cholesterol is increased as detected by both methods. With increasing content of phosphatidylcholine and increasing mole ratio cholesterol/phosphatidylcholine, the anisotropy of motion of the spin probe increases. The spin label 3-doxyl-5α-cholestane is a useful substitute for cholesterol provided that it is used in dilute mixtures with excess cholesterol: the cholesterol/spin label mole ratio in these mixtures should be greater than 100. Despite the structural similarity between the two compounds, there are still significant differences in their physico-chemical properties. These differences come to the fore when cholesterol is totally replaced by the spin-label: 3-doxyl-5α-cholestane is significantly less soluble in bile salt and mixed bile salt micelles than cholesterol and, in contrast with cholesterol, it interacts only very weakly, if at all, with phosphatidylcholine. The potential of the ESR method for detecting cholesterol crystal growth in human bile is discussed.

INTRODUCTION

Mixed bile salt micelles as well as phospholipid bilayers as vesicles have been demonstrated to act as cholesterol carriers in human bile (Small, 1967; Carey and Small, 1978; Mazer et al., 1980; Sömjen and Gilat, 1983; Sömjen et al., 1986). The main cholesterol carriers in unsaturated human bile are small, spheroidal or disk-shaped mixed micelles consisting of bile salt, phospholipid, and cholesterol plus simple micelles consisting mainly of bile salt, which may contain cholesterol (Hjelm et al., 1988; Walter et al., 1991). The solubility of cholesterol in bile and the crystallization of excess cholesterol from bile are two important properties intimately related to the pathogenesis of cholesterol gallstones. Here we use as a model system dispersions of mixed bile salts containing cholesterol and egg PC spin-labeled with 3-doxyl-5α-cholestane and test whether the crystallization of cholesterol from these dispersions can be detected by ESR spectroscopy. As a second independent method, x-ray diffraction was applied to detect the crystallization of cholesterol from model bile systems. Synchrotron radiation was used to enhance the sensitivity of the diffraction measurement. Similar results were obtained with the two methods. ESR spin labeling using 3-doxyl-5α-cholestane as a cholesterol analog is a new and convenient method to detect crystal formation of cholesterol in model systems. The method might lend itself to application in human bile.

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Abbreviations used: PC, phosphatidylcholine; EDTA, disodium ethylenediaminetetraacetic acid.

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MATERIALS AND METHODS

Cholesterol (Sigma Chemical Co., St. Louis, MO) and Na^+-taurocholate (Sigma) were twice recrystallized from hot ethanol and ethanol and ether (Pope, 1967), respectively. Egg phosphatidylcholine (PC) (Sigma) and spin-labeled cholestane, 4,4′-dimethylspiro[5α-cholestane-3,2′-oxazolidine]-*N*-oxyl (3-doxyl-5α-cholestane) (Aldrich, Steinheim, Germany) were used without further purification. All lipids used in this study were pure by thin layer chromatography standard. The lipids were dissolved in $CHCl_3/CH_3OH$ (2:1 v/v) in the proportions given in Table 1, dried at room temperature, lyophilized overnight, and kept under argon until used. Unless stated otherwise, lipid dispersions for ESR and x-ray scattering were prepared by suspending the dried lipids in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and incubating the suspension at 55°C for 1 h. Lipid suspensions were kept at room temperature before the measurement, which was carried out within less than 24 h. In the first series of experiments, 3-doxyl-5α-cholestane was admixed to cholesterol so that the final cholesterol/spin label mole ratio was 100; in the second series of experiments, cholesterol was totally replaced by 3-doxyl-5α-cholestane. All lipid dispersions were checked routinely for the presence of cholesterol crystals by polarized light microscopy.

ESR spectra were recorded with a temperature-controlled Varian X-band spectrometer (Model E-104A) operated in the continuous wave mode at 9.2 GHz (3280 G). Spin-labeled lipid dispersions (20–50 μl) were filled into 100-μl glass capillaries (~1 mm internal diameter) with a Hamilton syringe, and the capillaries were flame-sealed and immediately inserted into the ESR cavity. ESR spectra were recorded at room temperature using an incident

microwave power of less than 20 mW, 100 kHz field modulation, 1 G modulation amplitude, 100 or 200 G field sweep, and a sweep rate of 12.5 G/min. The spin label concentration of lipid dispersions containing cholesterol/3-doxyl-5α-cholestane (mole ratio 100:1) varied between 10 and 30 μM, whereas in lipid dispersions devoid of cholesterol the concentration of 3-doxyl-5α-cholestane ranged from 10 to 30 mM. The temperature of the sample was monitored with a thermocouple before and after recording the ESR spectra and was maintained to within ±0.5°.

Small angle x-ray scattering of the model bile systems was performed on the storage ring DORIS using a double-focusing monochromator-mirror camera (Koch and Bordas, 1983) installed at the X33 station of HASYLAB (EMBL Outstation, DESY, Hamburg, Germany). All measurements were made at λ = 0.15 nm. Diffraction patterns from 100-μl samples were recorded at 23 ± 1°C with exposure times of 5 min. Before the evaluation, background scattering caused by the buffer was subtracted from the diffraction pattern. Further details of the data acquisition and evaluation systems can be found elsewhere (Boulin et al., 1986). Data are presented as scattered intensity I vs. s, where $s = 2 \sin \theta/\lambda$.

Auxiliary methods

Polarized light microscopy was used routinely to monitor the crystallization of cholesterol or spin-labeled cholestane from bile salt dispersions. Freeze-fracture electron microscopy was used as a second routine method to show that the particles present in pure and mixed bile salt dispersions are predominantly small micelles.

RESULTS

ESR spin labeling

A representative ESR spectrum of bile salt micelles labeled with a small amount of 3-doxyl-5α-cholestane is shown in Fig. 1 *A*. The spectral shape is characteristic of fast, but anisotropic, motion with a maximum hyperfine splitting of $2A_\perp$ = 38 G. Similar ESR spectra were obtained with cholate, taurocholate, and glycocholate micelles (all as the sodium salt; data not shown) (cf. Stevens, 1977). The average maximum hyperfine splitting measured with 3-doxyl-5α-cholestane in various bile salt micelles was $2A_\perp$ = 37.5 ± 0.7 G. Fig. 1 *B* shows the ESR spectrum of a dispersion of sodium taurocholate containing cholesterol/3-doxyl-5α-cholestane (mole ratio 100:1, cf. sample 1, Table 1[1]). This is a composite ESR spectrum. The lineshape of the main spectral component is very similar to the ESR spectrum shown in Fig. 1 *A* and is characteristic of spin-labeled cholestane present in bile salt micelles. The second minor component is an immobilized ESR spectrum characterized by a maximum hyperfine splitting $2A_{max}$ = 71 G (see *arrows* in Fig. 1 *B*). This value of the maximum hyperfine splitting, which actually exceeds the A_{zz} component of the hyperfine splitting tensor, indicates that we are dealing with a magnetically dilute powder spectrum. ESR spectra were recorded of bile salt micelles to which increasing quantities of cholesterol/3-doxyl-5α-cholestane (mole ratio 100:1) were added. With increasing cholesterol/3-doxyl-5α-cholestane content, the relative intensity of the immobilized component with $2A_{max}$ = 71 G increased proportionally (data not shown). However, no contribution from Heissenberg spin exchange was detected in the ESR spectra of this series of samples,

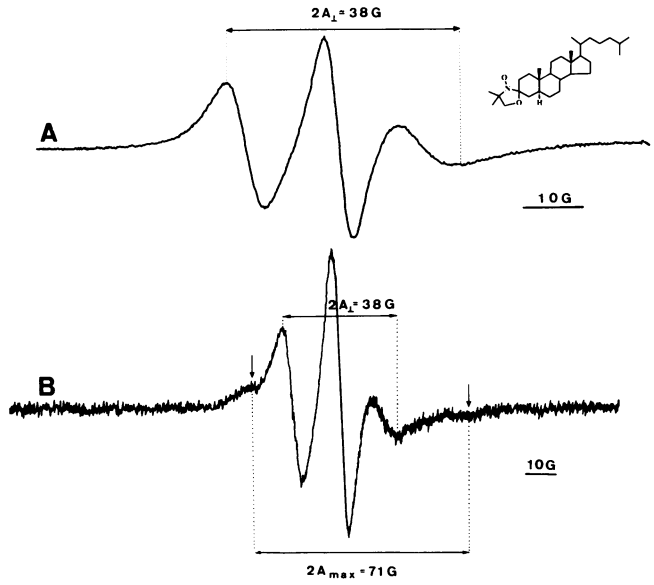

FIGURE 1 (*A*) ESR spectrum of sodium deoxycholate micelles spin-labeled with 3-doxyl-5α-cholestane. The bile salt (50 mM, ~2.1%) containing the spin label at a mole ratio of 250:1 was dispersed in 10 mM sodium phosphate buffer pH 7.3, 140 mM NaCl, 25 mM EDTA. The maximum hyperfine splitting $2A_\perp$ was derived directly from the ESR spectrum as indicated. Sweep width = 100 G. The chemical structure of 3-doxyl-5α-cholestane is shown as an inset. (*B*) ESR spectrum of a lipid dispersion (sample 1 of Table 1) containing 100 mM sodium taurocholate (~5.6%) and 20 mM cholesterol/3-doxyl-5α-cholestane (mole ratio 100) in 50 mM Tris-HCl buffer pH 8.0, 150 mM NaCl. The maximum hyperfine splittings of the two component spectra were derived from this spectrum as indicated. Sweep width = 200 G.

consisting of bile salt micelles and increasing quantities of cholesterol/3-doxyl-5α-cholestane (mole ratio 100:1, cf. Fig. 1 *B*). Computer simulations lend support to this interpretation. The experimental spectrum of Fig. 1 *B* can be simulated by the sum of two component spectra characterized by maximum hyperfine splittings of $2A_\perp$ = 38 G and $2A_{max}$ = 72 G, and the simulations confirm the conclusion that there is no contribution from spin exchange (data not shown). The immobilized spectra are probably indicative of slow motional contributions to the lineshape and the effective hyperfine splittings. However, rigorous simulations of these spectra and a detailed lineshape analysis are not required for the interpretation of these spectra given here.

The kinetics of the crystallization of cholesterol from mixed bile salt micelles can be derived from the time course of the appearance of the magnetically dilute powder spectrum with $2A_{max}$ = 72 G. The increase in signal intensity of this spectrum with time parallels the crystal growth as monitored by polarized light microscopy (data not shown). For instance, sample 1 (Table 1) containing 3-doxyl-5α-cholestane (cholesterol/spin label mole ratio = 100) at time 0 gave rise to an ESR spectrum identical to that of Fig. 1 *A*. Between 1 and 2 h after dispersion of this sample, the second spectral component characterized by $2A_{max}$ = 72 G was first detected and, eventually, the composite ESR spectrum shown in Fig. 1 *B* was obtained. Between 24 and 48 h, the signal intensity of the second spectral component reached its

TABLE 1 Composition and maximum hyperfine splittings of mixed bile salt micelles

Sample No.	Cholesterol (mM)	Egg PC (mM)	Sodium taurocholate (mM)	Total lipid conc. (g/dl)	Cholesterol (mol %)	Cholesterol/ egg PC (mole ratio)	CSI*	Maximum hyperfine splittings (2 A_{max}‡) Mobile component (G)	Maximum hyperfine splittings (2 A_{max}‡) Immobile component (G)
1	20		100	5.8	16.7	∞	n.d.	37.8	71.0
2	10		100	5.4	9.1	∞	n.d.	37.5	72.5
3	10	15	100	6.5	8.0	0.67	180	43.1 ± 0.8	n.d.
4	10	20	100	6.8	7.7	0.50	146	43.5 ± 1.0	n.d.
5	10	30	100	7.6	7.1	0.33	106	42.9 ± 0.3	
6	10	40	100	8.4	6.7	0.25	87	42.1 ± 0.3	
7	10	50	100	9.2	6.3	0.20	77	41.8 ± 0.6	
8	20	50	100	9.6	11.8	0.40	143	43.5 ± 0.4	
9	30	50	100	9.9	16.7	0.60	201	43.5 ± 1.0	
10	10	50	150	11.6	4.8	0.20	63	41.0 ± 0.3	

*CSI, cholesterol saturation index derived according to Carey (1978).
‡For ESR measurements, all samples contained cholesterol/3-doxyl-5α-cholestane (mole ratio 100:1). For composite ESR spectra consisting of two component spectra, two maximum hyperfine splittings are given. The mobile component refers to the ESR spectrum of 3-doxyl-5α-cholestane present in bile salt micelles, and the immobilized component is due to the spin label present in cholesterol crystals.
n.d., not determined.

maximum (data not shown). The determination of the crystal growth by this ESR method is subject to a large error due to growing crystals sedimenting in the ESR tube. The kinetics of the crystal growth of cholesterol from different bile salt dispersions and the methodology involved in this measurement will be the subject of a separate publication.

In the second series of ESR experiments, mixed bile salt micelles of the same lipid composition as in Table 1 were used except that cholesterol was replaced by spin-labeled cholestane. A representative ESR spectrum of this series is shown in Fig. 2 (corresponding to sample 1, Table 1). This ESR spectrum is also composed of two spectral components; one spectrum is very similar to that shown in Fig. 1 *A* and is characteristic of the spin label present in bile salt micelles. As seen in Fig. 2, this spectrum is apparently superimposed on a spin exchange spectrum. Consistent with x-ray diffraction experiments discussed below (cf. Figs. 5, 6 *C*, and 7 *C*), the ESR spectra of all samples of this series showed a significant contribution from spin exchange, indicating that a

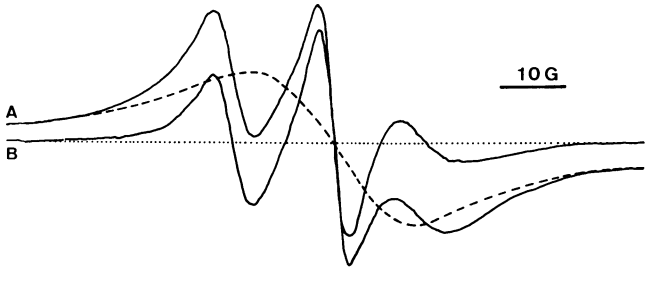

FIGURE 2 ESR spectrum of sodium taurocholate micelles (100 mM, ~5.6%) containing 20 mM 3-doxyl-5α-cholestane dispersed in 50 mM Tris-HCl buffer, pH 8.0, 150 mM NaCl. The experimental spectrum (*A*, ——) can be computer-fitted by the sum of a spin exchange spectrum (- - - -) and an anisotropic spectrum (*B*) characteristic of the spin label in bile salt mixed micelles with a maximum hyperfine splitting of $2A_{\perp}$ = 38 G. Spectral baseline (· · · ·).

sizable proportion of the spin label crystallized out under these conditions. This was true for bile salt micelles both in the absence and presence of PC (cf. Fig. 4) and is taken as evidence that the solubility of 3-doxyl-5α-cholestane in pure and mixed bile salt micelles is rather limited.

ESR spectra of sodium taurocholate micelles containing a fixed quantity of cholesterol/3-doxyl-5α-cholestane (mole ratio 100:1) and increasing amounts of PC are depicted in Fig. 3 (samples 2–5, Table 1). Inspection of the ESR spectra of Fig. 3 reveals two effects of increasing the PC content in mixed bile salt micelles: (1) the intensity of the immobilized spectrum with $2A_{max}$ = 72 G (see *arrows*) decreased with increasing PC content; at PC concentrations >15 mM, this spectral component is barely detectable; (2) the incorporation of PC into bile salt micelles caused an increase in the maximum hyperfine splitting $2A_{\perp}$ from about 38 G, characteristic of 3-doxyl-5α-cholestane present in pure bile salt micelles to 40–40.5 G, characteristic of the spin label present in PC bilayers. As shown in Table 1, the value of $2A_{\perp}$ increased approximately linearly with cholesterol content, leveling off at $2A_{\perp}$ = 43.5 G.

In Fig. 4, ESR spectra are presented of mixed bile salt micelles of composition analogous to samples 7–9 of Table 1, except that in these dispersions cholesterol was totally replaced by spin-labeled cholestane. As discussed for Fig. 2, composite ESR spectra are obtained under these conditions consisting of a spin exchange spectrum and a three-line spectrum characteristic of spin-labeled cholestane present in bile salt micelles. The intensity of the spin exchange spectrum obtained by spectral decomposition as described for Fig. 2 increased linearly with the spin label content (data not shown).

In the series of micellar dispersions corresponding to samples 3–7 of Table 1 in which cholesterol was totally replaced by spin-labeled cholestane, we found that the spin exchange contribution in the ESR spectra only

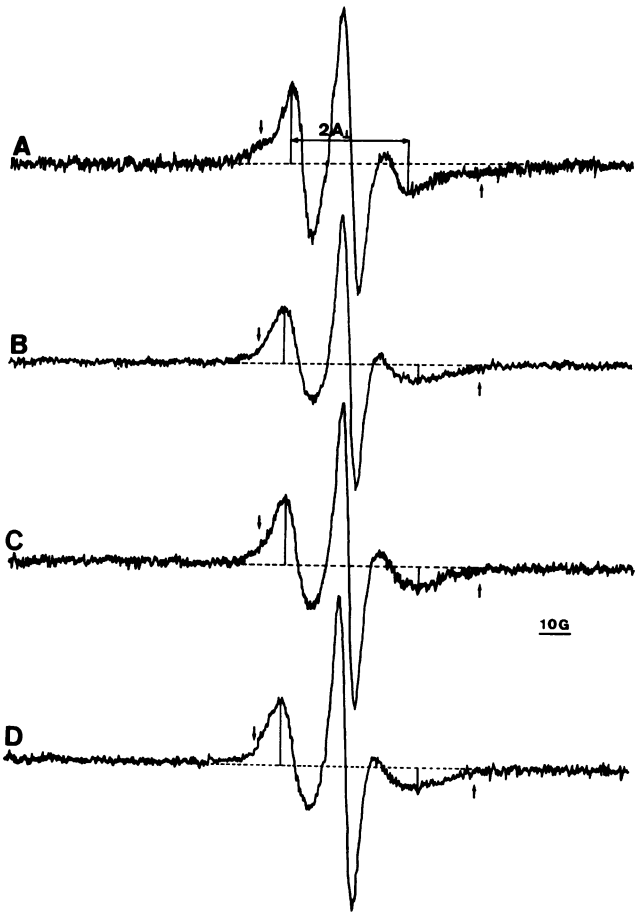

FIGURE 3 ESR spectra of mixed bile salt micelles. All samples contained 100 mM (~5.6%) sodium taurocholate, 10 mM cholesterol/3-doxyl-5α-cholestane (cholesterol/spin label mole ratio 100), and increasing quantities of egg PC except for *A*, which contained no egg PC (sample 2 of Table 1). The mixed bile salt dispersions contained 15 mM egg PC (*B*) (sample 3 of Table 1), 20 mM egg PC (*C*) (sample 4 of Table 1), 30 mM egg PC (*D*) (sample 5 of Table 1). Sweep width = 200 G. Arrows indicate the maximum hyperfine splitting $2A_{max}$ of the immobilized spectrum. (- - - -) Spectral baseline. Vertical solid lines indicate the maximum hyperfine splitting $2A_{\perp}$ characteristic of the spin label present in bile salt micelles.

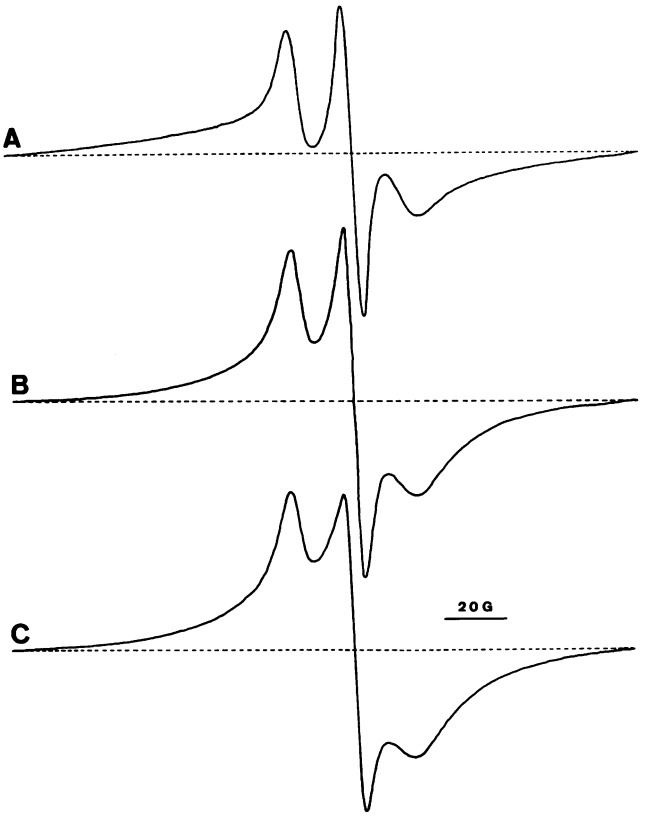

FIGURE 4 ESR spectra of mixed bile salt micelles containing 100 mM (~5.6%) sodium taurocholate, 50 mM egg PC, and increasing quantities of 3-doxyl-5α-cholestane; the concentration of spin label was 10 mM (*A*), 20 mM (*B*), and 30 mM (*C*). Bile salt dispersions were made in 50 mM Tris-HCl buffer, pH 8.0, 150 mM NaCl. Sweep width = 200 G. The spectral gain used was 1.6×10^3, 1.25×10^3, and 1.0×10^3 for spectra *A–C*, respectively.

slightly decreased going from sample 3 to 7 (data not shown). This result indicated that increasing contents of PC in mixed bile salt micelles have only a marginal effect on the solubility of the cholestane spin label in these micelles. As far as the interaction with PC is concerned, spin-labeled cholestane therefore behaved significantly differently from cholesterol.

Small angle x-ray scattering

In Fig. 5, x-ray diffraction patterns of sodium taurocholate dispersions containing cholesterol/3-doxyl-5α-cholestane (mole ratio 100:1, pattern A) and pure spin-labeled cholestane (pattern B) are compared. The 34.1-Å diffraction peak can be attributed to cholesterol crystals (Craven, 1976). In contrast, in bile salt dispersions containing spin-labeled cholestane without cholesterol, the measured diffraction peak was at 19.5 Å. The presence of PC in the taurocholate dispersions renders the cholesterol soluble in mixed bile salt dispersions, as seen in Fig. 6, *A* and *B*. In these bile salt dispersions, the cholesterol and taurocholate concentrations remained constant, whereas egg PC was varied from 15 to

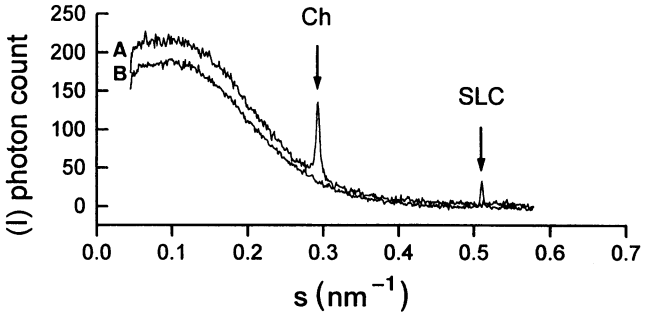

FIGURE 5 X-ray scattering profiles are presented after background subtraction and normalization to a constant beam intensity. Two model bile salt systems are shown. (*A*) 150 mM (~8.3%) sodium taurocholate, 10 mM cholesterol/3-doxyl-5α-cholestane (mole ratio 100:1). (*B*) The same bile salt dispersion as above except that cholesterol was replaced by spin-labeled cholestane. Both dispersions were made in 50 mM Tris-HCl buffer, pH 8.0, 150 mM NaCl as described in Materials and Methods. The first-order diffraction peaks due to cholesterol crystals and crystals of 3-doxyl-5α-cholestane are marked with Ch and SLC, respectively.

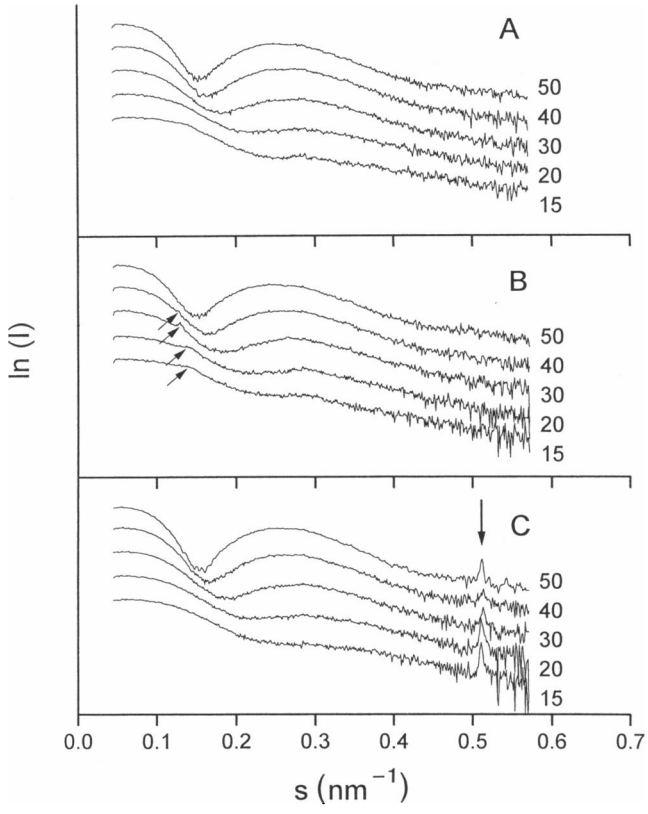

FIGURE 6 X-ray scattering profiles of mixed bile salt dispersions in 50 mM Tris-HCl buffer, pH 8.0, 150 mM NaCl. (*A*) Samples containing 10 mM cholesterol, 100 mM (~5.6%) sodium taurocholate, and 15–50 mM PC (samples 3–7 of Table 1, *bottom to top*); (*B*) Samples 3–7 of Table 1 except that each sample contained 1 mol% 3-doxyl-5α-cholestane (*bottom to top*). (*C*) Samples 3–7 of Table 1 except that cholesterol was replaced by 3-doxyl-5α-cholestane. The lamellar diffraction peaks are marked by arrows in *B*. The diffraction peaks due to crystalline 3-doxyl-5α-cholestane in *C* are marked by an arrow. The numbers on the right of the scattering profiles refer to the egg PC concentration (in mM).

50 mM (samples 3–7 of Table 1). As seen in Fig. 6, *A* and *B*, no cholesterol crystals could be detected. Lamellar stacking is indicated in x-ray scattering by the appearance of relatively sharp diffraction peaks at submultiples of about 80 Å (Sömjen et al., 1991). No lamellar stacking was detected in mixed bile salt dispersions containing pure cholesterol (Fig. 6 *A*). In samples of the same composition but containing 1 mol% 3-doxyl-5α-cholestane, lamellar stacking was detectable (see *arrows* in Fig. 6 *B*). For mixed bile salt micelles containing 15, 20, 30, and 40 mM egg PC, the shoulders or peaks marked by arrows correspond to 71.7, 73.0, 76.3, and 78.1 Å, respectively. A shift of the maxima and minima of the scattering profiles was observed. When the dispersions were prepared with spin-labeled cholestane instead of cholesterol, sharp diffraction peaks at 19.5 Å were observed (see *arrow* in Fig. 6 *C*). This is evidence for the crystallization of the 3-doxyl-5α-cholestane molecules. However, no lamellar stacking was observed in these dispersions. The minima and maxima of the scattering profiles were shifted from their original positions of the cholesterol-containing samples, indicating a change in the micellar size.

A comparison was made of mixed bile salt dispersions containing constant concentrations of egg PC (50 mM) and sodium taurocholate (100 mM) and increasing concentrations of cholesterol in the absence and presence of spin-labeled cholestane. First-order diffraction peaks were observed at 74.7 and 77.1 Å (Fig. 7 *A*) and at 79.7 and 77.1 Å (Fig. 7 *B*), indicative of lamellar stacking. In this figure, a shoulder centered at a larger value of *s* than that of the main diffraction peak can be seen (cf. *dashed arrow*). In the dispersions containing 1 mol% 3-doxyl-5α-cholestane, this shoulder is already observed at a lower cholesterol content (20 mM) (cf. *dashed arrow*, Fig. 7 *B*).

The dispersions composed of pure cholestane spin label showed strong diffraction peaks at 19.5 Å due to the crystallization of spin-labeled cholestane (see *arrow* in Fig. 7 *C*). The intensities of these peaks appear to be proportional to the amount of 3-doxyl-5α-cholestane in the different dispersions.

Auxiliary measurements

The following measurements were carried out routinely for the purpose of aiding in the interpretation of the ESR and

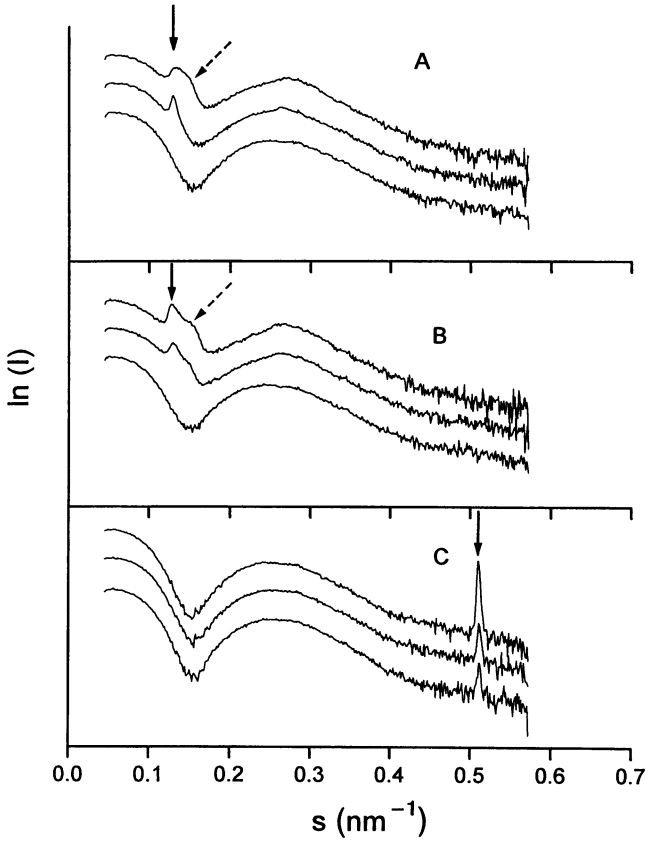

FIGURE 7 X-ray scattering profiles of mixed bile salt dispersions in 50 mM Tris-HCl buffer, pH 8.0, 150 mM NaCl. (*A*) Samples 7–9 of Table 1 (*bottom to top*). (*B*) Samples 7–9 of Table 1 except that each sample contained 3-doxyl-5α-cholestane at a cholesterol/3-doxyl-5α-cholestane mole ratio of 100 (*bottom to top*). (*C*) Samples 7–9 of Table 1 except that all cholesterol was replaced by 3-doxyl-5α-cholestane. The lamellar diffraction peaks are indicated by arrows; shoulders of these diffraction peaks are marked by dashed arrows (*A* and *B*). The arrow in *C* marks the diffraction peaks due to the crystallization of 3-doxyl-5α-cholestane.

x-ray scattering experiments. The crystallization of cholesterol from saturated pure and mixed bile salt micelles was monitored routinely by polarized light microscopy. The crystallization of cholesterol or spin-labeled cholestane from bile salt micelles detected by either ESR spin labeling or x-ray diffraction was confirmed by this routine method.

DISCUSSION

Here we used the spin-labeled cholestane 3-doxyl-5α-cholestane (see *inset* of Fig. 1 *A*) as a probe of cholesterol behavior in bile salt micelles. The same spin label was used by Stevens (1977) to probe glycocholate-mixed micelles containing egg PC and/or cholesterol. This author obtained ESR spectra very similar to that shown in Fig. 1 *A*. However, he treated the spectra as pseudo-isotropic and interpreted them in terms of rotational correlation times.

For the interpretation of the ESR spectra, we assume that Brownian tumbling of the whole micelle does not contribute significantly to the averaging of the hyperfine splitting tensor. Hence, the observed ESR lineshape reflects primarily the molecular motion of the spin label. This assumption is reasonable considering the average dimensions of the mixed bile salt micelles. The composite ESR spectrum shown in Fig. 1 *B* arises from the limited solubility of cholesterol and spin-labeled cholestane in pure bile salt micelles (Small et al., 1966a, b). The observation of an immobilized spectral component with an average value for the maximum hyperfine splitting of $2A_{max} = 72 \pm 1$ G is interpreted to indicate that excess cholesterol crystallizes out giving rise to the immobilized spectrum (see *arrows* in Fig. 1 *B*). This immobilized spectrum is a magnetically dilute powder spectrum, indicating that the cholestane spin-label co-crystallizes with cholesterol. Phase separation or demixing of cholesterol and the cholestane spin label in the crystal would give rise to a spin exchange spectrum. Because there was no detectable contribution from spin exchange to the ESR spectrum shown in Fig. 1 *B*, phase separation or demixing can be ruled out. If all cholesterol is replaced by the cholestane spin label, a Heissenberg spin exchange spectrum is observed instead of the magnetically dilute powder spectrum (cf. Figs. 1 *B* and 2). The observation of spin exchange indicates that 3-doxyl-5α-cholestane, like cholesterol, has a limited solubility in bile salt micelles and excess spin label crystallizes out. In summarizing the ESR evidence, we conclude that the crystallization of cholesterol from mixed bile salt micelles is indicated by the appearance of a magnetically dilute, rigid limit powder spectrum; in contrast, the crystallization of 3-doxyl-5α-cholestane from mixed bile salt micelles, in which all cholesterol is replaced by the spin label, is indicated by spin exchange.

We have observed reproducibly that in magnetically dilute, rigid limit powder spectra of 3-doxyl-5α-cholestane the value of the maximum hyperfine splitting $A_{max} = 35$–36 G exceeds significantly the A_{zz} component of the hyperfine splitting tensor. The value published for A_{zz} in the early literature is $A_{zz} = 32$ G (Smith, 1971; Gaffney, 1976; Hsia et al., 1970; Smith and Butler, 1976). The A_{max} value we observe is more in line with the A_{zz} value of 34.5 G used by Freed and his collaborators in the simulation of ESR spectra of 3-doxyl-5α-cholestane present in oriented multilayers of 1,2-dipalmitoyl-*sn*-phosphatidylcholine (Kar et al., 1985). The high A_{max} value cannot be due to an error in the instrumental field scan, which was calibrated with Fremy's salt: measured and nominal values of the hyperfine splitting agreed within 2%. The high A_{max} values are very likely due to hydrogen bonding of the nitroxide group to water. Hydrogen bonding or, more generally, a more polar environment of this group was shown to increase the unpaired spin density on the N-atom of the nitroxide group, leading to an increase in the hyperfine splitting (Griffith et al., 1974; Johnson, 1981; Marsh, 1981; Kar et al., 1985).

Our interpretation of the ESR results is supported by evidence from x-ray diffraction (Fig. 5). Dispersions of 150 mM bile salt micelles containing 10 mM spin-labeled cholestane or 10 mM cholesterol/3-doxyl-5α-cholestane (mole ratio, 100:1) yielded sharp diffraction peaks superimposed on the x-ray scattering profile arising from bile salt micelles. The diffraction peak corresponding to 34.1 Å observed with bile salt dispersions containing cholesterol is consistent with the single-crystal structure of cholesterol monohydrate (Craven, 1976). The cholesterol monohydrate crystallizes in the form of bilayers with a repeat distance $d_{(001)} = 33.9$ Å. For the dispersion, in which cholesterol was totally replaced by the spin label, a sharp diffraction peak corresponding to 19.5 Å was observed (Fig. 5 *B*). The single-crystal structure of the cholestane spin label is not available. The repeat distance of 19.5 Å is significantly smaller than that of the cholesterol monohydrate crystal. The reason for this discrepancy is unknown. One possible explanation is that in the crystal structure of 3-doxyl-5α-cholestane the molecules pack in an interdigitated monolayer. In such a structure, neighboring molecules are packed head-to-tail with the steroid nuclei forming the hydrophobic interior and polar nitroxide groups forming the surface layers on either side of the hydrophobic region.

Although exact values of the solubilities of cholesterol and 3-doxyl-5α-cholestane in sodium taurocholate micelles were not determined here, an estimate of the upper limit of these values can be derived. Both ESR spin labeling and X-ray diffraction show that less than ~6 mol% cholesterol is soluble in taurocholate micelles. This value is even smaller for 3-doxyl-5α-cholestane. From the spin exchange contribution to the ESR spectra shown in Fig. 4, a value of about 5 mM (=3.2 mol%) is derived for the solubility of 3-doxyl-5α-cholestane in taurocholate-PC mixed micelles. At spin label concentrations in excess of 5 mM (=3.2 mol%), spin exchange is observed with mixed taurocholate micelles of composition shown in Fig. 4. At a spin label concentration of 6.3 mol% (cf. ESR spectrum A of Fig. 4) the spin exchange is clearly detectable. The limited solubility of cholesterol in bile salt micelles is in agreement with the ternary phase diagram of bile salt-cholesterol-water as well as the quaternary phase diagram of bile salt-PC-cholesterol-water, according

to which only limited quantities of cholesterol are accommodated in pure and mixed bile salt micelles (Small et al., 1966a, b; Bourges et al., 1967; Carey and Small, 1978). Stevens (1977) observed discontinuities in the plot of correlation times versus cholesterol content of mixed glycocholate micelles. These discontinuities apparently coincide with the phase boundary or solubility limit of cholesterol in the quaternary phase diagram bile salt-PC-cholesterol-water (Bourges et al., 1967) and also with the appearance of magnetically dilute, rigid limit powder spectra in mixed taurocholate micelles of comparable compositions as described here.

Fig. 3 together with Figs. 5 to 7 provide evidence that the presence of PC increases the capacity of bile salt micelles to incorporate cholesterol (Wilson and Rudel, 1994). No immobilized component due to the crystallization of cholesterol was detectable in the ESR spectra of bile salt micelles containing more than 15 mM (12 mol%) egg PC keeping the concentrations of bile salt (100 mM) and cholesterol/3-doxyl-5α-cholestane (10 mM) constant (cf. Fig. 3). Consistent with this finding, the x-ray diffraction patterns of these samples are devoid of sharp diffraction peaks characteristic of crystalline cholesterol (cf. Figs. 6, *A* and *B* and 7, *A* and *B*). In contrast, in bile salt dispersions in which cholesterol was totally replaced by the cholestane spin label, sharp diffraction peaks characteristic of crystalline 3-doxyl-5α-cholestane are observed (cf. Figs. 6 *C* and 7 *C*). These results clearly show that the presence of egg PC in mixed bile salt micelles increases the solubility of cholesterol but not of spin-labeled cholestane in these micelles. This finding is consistent with results obtained by Stevens (1977).

The presence of PC in mixed bile salt micelles increases the maximum hyperfine splitting and, hence, the anisotropy of motion (Table 1). There is an initial linear relationship between the maximum hyperfine splitting $2A_\perp$ and the cholesterol/PC mole ratio (cf. data in Table 1). This is interpreted to be a manifestation of the interaction between cholesterol and PC molecules. Cholesterol-PC interactions have been studied extensively and are well documented in the literature (see, e.g., Finean, 1990). In contrast, in mixed bile salt micelles, in which cholesterol was totally replaced by spin-labeled cholestane, an analogous interaction between spin-labeled cholestane and PC was not detected. Upon adding increasing quantities of PC to mixed bile salt micelles (samples 3–7 of Table 1, except that cholesterol was replaced by spin-labeled cholestane), no significant reduction in the intensity of the spin exchange spectrum was observed. At the highest PC concentration (samples 6–10 of Table 1), the $2A_\perp$ values were 40–41 G, characteristic of the spin label present in PC bilayers; all other mixed bile salt dispersions (samples 1–5 of Table 1 with cholesterol being replaced by spin-labeled cholestane) gave ESR spectra with an average $2A_\perp$ value of 38 $\pm$ 0.8 G, characteristic of the spin label present in bile salt micelles. These two lines of evidence that (1) increasing quantities of PC in mixed bile salt micelles containing a fixed amount of 3-doxyl-5α-cholestane have little if any effect on the intensity of the spin exchange spectrum, and (2) the presence of PC has no or little effect on the maximum hyperfine splitting of the mobile spectral component demonstrate that 3-doxyl-5α-cholestane behaves quite differently from cholesterol regarding its interaction with PC. There is no or only a very weak interaction with PC. One explanation for this is that 3-doxyl-5α-cholestane lacks an H-bond donor function and, unlike cholesterol, this molecule cannot form a hydrogen bond to the carbonyl group of fatty acyl chains of PC or membrane phospholipids.

Both ESR and x-ray diffraction data indicate consistently that the physico-chemical properties of spin-labeled cholestane differ from those of cholesterol. Both methods clearly show that spin-labeled cholestane is less soluble than cholesterol in mixed bile salt micelles containing PC. Further, both methods provide evidence that there is only a very weak, if any, interaction between spin-labeled cholestane and PC. Differences in the surface properties of cholesterol and 3-doxyl-5α-cholestane have been reported by Cadenhead et al. (1972, 1975). Despite these obvious differences in the physico-chemical properties of cholesterol and 3-doxyl-5α-cholestane, the data presented still demonstrate the utility of the spin label when it is used in dilute mixtures with cholesterol at cholesterol/spin label mole ratios >100. Under these conditions, the label appears to probe the behavior of cholesterol satisfactorily; moreover it co-crystallizes with cholesterol.

In dispersions of mixed micelles with a relatively high cholesterol content (>7 mol%, Table 1), sharp lamellar diffraction peaks are observed superimposed on the x-ray scattering pattern arising from bile salt micelles (Figs. 6 *B* and 7, *A* and *B*). The sharp diffraction lines are indicative of a periodic structure that was identified previously using small angle x-ray diffraction (Sömjen et al., 1991). Lamellar diffraction peaks were observed by x-ray diffraction with mixed bile salt dispersions 3–6, 8, and 9 of Table I that contained between 6.7 and 16.7 mol% cholesterol. This result suggests that the occurrence of lamellar stacking requires a threshold concentration of cholesterol in the mixed bile salt micelle. The principle governing the stacking was discussed previously (Sömjen et al., 1991).

In conclusion, we find that the two independent methods, ESR spin labeling and synchrotron x-ray scattering, used here to characterize the behavior of mixed bile salt/cholesterol/PC micelles give consistent results. 3-doxyl-5α-cholestane is a reasonably good substitute for cholesterol provided that it is used together with excess cholesterol at cholesterol/3-doxyl-5α-cholestane mole ratios >100. Although there are clear differences in the physico-chemical properties of cholesterol and 3-doxyl-5α-cholestane, the spin label was found to co-crystallize with cholesterol. The data presented here clearly show that the crystallization of cholesterol from bile salt micelles is readily detected by ESR using 3-doxyl-5α-cholestane. When the spin label is used in magnetically dilute dispersions, e.g., as cholesterol/3-doxyl-5α-cholestane mixtures (mole ratio, 100:1) crystallization of cholesterol manifests itself in the appearance of a magnetically dilute powder pattern. If cholesterol is totally replaced

by spin-labeled cholestane, the crystallization of the spin label is indicated by the appearance of a spin exchange spectrum. ESR spin labeling and x-ray diffraction show that bile salt micelles can accommodate about 5 mol% cholesterol and 3 mol% 3-doxyl-5α-cholestane. Excess cholesterol and 3-doxyl-5α-cholestane crystallize out. ESR spin labeling as a relatively inexpensive as well as sensitive method may lend itself as a routine method for detecting the crystal growth of cholesterol in human bile. Native bile would have to be equilibrated with sufficient spin-labeled cholestane so that the crystallization of cholesterol can be detected. Work is currently in progress in our laboratory (H. Hauser) to test the potential of the ESR method in this respect.

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