An electron spin resonance study of cholestane spin label in aqueous mixtures of biliary lipids

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Abstract The effect of cholesterol on the fluidity of the phospholipid matrix in mixed micelles derived from bile salts and lecithin has been determined by the paramagnetic probe technique. It was found that correlation times for the cholestane spin label were discontinuous functions of cholesterol content and that these dicontinuities correlate with the equilibrium solubility limit for cholesterol in this quaternary system. The origin of these discontinuities is attributed to the existence of another aggregate in addition to the disc-shaped mixed micelle in lipid solutions supersaturated with cholesterol.

Supplementary key words biliary mixed micelles · cholesterol supersaturation · egg yolk lecithin · glycocholate · glycochenodeoxycholate

The complex phase behavior of aqueous mixtures of bile salts and lecithin has been characterized by Small, Bourges, and Dervichian (1). The phases produced by this ternary system range from an isotropic mixed micellar solution to a variety of lyotropic paracrystalline phases. On the basis of results from proton magnetic resonance (2), light scattering (3), analytical centrifugation (4), and on empirical grounds (3, 4), the proposed structure of the mixed micelle derived from the ternary system is a disc-shaped bimolecular leaflet of lecithin with bile salts hydrophobically associated with the exposed hydrocarbon chains on the perimeter of the disc. The homogeneity of the mixed micellar phase has been questioned, and recent data (3, 5) suggest that both simple bile salt micelles and mixed micelles constitute this phase. Cholesterol, primarily in association with the phospholipids, can be incorporated in all the phases without major alterations in their structure (6, 7); however, the phase boundaries are substantially modified. The mixed micellar solution derived from the quaternary system has been of considerable interest, since these lipid aggregates are thought to be the predominant mode by which cholesterol is solubilized in bile. The boundary for the mixed micellar phase has been implicated to have pathogenic significance with respect to cholesterol gallstone disease (8). This spontaneous phase separation boundary has been redefined by an equilibrium solubility limit for cholesterol in this aqueous lipid system (9, 10). The structure and properties of the quaternary system in the region between these phase boundaries, a metastable labile zone, are unknown.

The paramagnetic probe technique has been applied extensively to the study of the interactions of phospholipids and cholesterol on a molecular level (11). The majority of work has been devoted to phospholipids in multibilayers (12), aqueous dispersions (13) and membrane fragment systems (14). This study is concerned with the application of the spin label technique to the complex lipid system of bile salts and phospholipids where, in contrast to previous studies, the phospholipids are in a highly dispersed and, presumably, bilayer state.

The primary objective was to measure the influence of cholesterol on the motion of a steroid spin label in this complex aqueous lipid system analogous to bile. Data have been analyzed in terms of a simple correlation time, which reflects the fluidity of the environment of the probe. It has been found that this motion parameter is a discontinuous function of cholesterol concentration for particular bile salt:phospholipid ratios and that these discontinuities correlate with the equilibrium phase boundary. The implications of these results with respect to the structure of the isotropic mixed micellar phase in the metastable labile zone are discussed.

MATERIALS AND METHODS

Sodium glycocholate and glycochenodeoxycholate were purchased from Calbiochem (Grade A) (La

Abbreviations: CSL, cholestane spin label, 3-spiro-[2′-(*N*-oxyl-4′.4′-dimethyloxazolidine)] derivative of 5α-cholestan-3-one; GLC, gas–liquid chromatography; ESR, electron spin resonance; G, Gauss.

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Jolla, CA) and were used without further purification. Egg yolk lecithin was prepared by the method of Singleton (15). The lecithin chromatographed as one spot in thin-layer chromatography using silica gel H plates developed in chloroform–methanol–water 65:25:4, and chloroform–methanol–ammonium hydroxide (11 N) 50:25:4. Cholesterol was obtained from Calbiochem (Reference Standard) and was used without further purification. The cholestane spin label (CSL), the 3-spiro-[2′-(*N*-oxyl-4′,4′-dimethyloxazolidine)] derivative of 5α-cholestan-3-one was synthesized by the method of Keana (16) and was purified by thin-layer chromatography on silica gel H plates developed in ether. The spin label was stored at −20°C and freshly prepared as a chloroform solution before each experiment.

All organic solvents used during this study were of high purity and were redistilled and fractionated before use. Stock solutions of individual lipids, bile salts in methanol, lecithin in chloroform, and cholesterol in ethanol were stored at −20°C. Aqueous solutions of lipid mixtures were prepared by the following procedure. The desired quantities of each lipid solution, including the spin label, were thoroughly mixed and brought to dryness at room temperature using a rotary evaporator. The tubes were placed on a vacuum manifold and evacuated at approximately 10^{-3} mm Hg for 48 hr. The lipids were hydrated using nitrogen-saturated deionized water, which had been prefiltered through a 0.45 μm millipore filter (Millipore Corp., Bedford, MA) and whose pH had been adjusted to 10 with 1N NaOH. The pH of the resulting aqueous lipid mixtures was 7.4 ± 0.2. The solutions were allowed to equilibrate for at least 2 hr under a nitrogen atmosphere before electron spin resonance (ESR) measurements were made.

ESR measurements were made on 50 μl of solution contained within a sealed Pyrex capillary tube using a Varian E9 spectrometer (Palo Alto, CA). Spectra were recorded in the first derivative mode, taking precautions to avoid saturation and overmodulation. The optimal spectrometer settings for minimal distortion and best signal-to-noise ratio were found to be a microwave power of 5 milliwatts, modulation amplitude of 1.25 Gauss (G) and a sweep time of 12.5 G per minute. All spectra were recorded at room temperature. The laboratory temperature was carefully monitored during the experiments and was stable to within ±0.5°C.

The total lipid concentration used for these experiments was ca. 50 mg/ml. The amount of CSL doping was approximately 0.1 mol % of the total lipid in solution. Spectra were recorded for spin label in a series of lipid solutions at fixed bile salt and lecithin concentrations with increasing cholesterol content up to the spontaneous phase separation boundary derived by Small (8). Bile salts were quantitated by a modification of the 3α-steroid dehydrogenase method of Talalay (17), phospholipids by the method of Bartlett (18), and cholesterol by the method of Abell et al. (19) or by GLC (20) with 3% SP-2250 on 100/120 mesh Supelcoport (Supelco Inc., Bellefonte, PA) using a Packard 7400 series gas chromatograph (Downers Grove, IL).

RESULTS AND DISCUSSION

Spin labels have been employed in the elucidation of structural (21) and dynamic (22) properties of micelles. In this study, we are concerned with motional characteristics of a spin label within a mixed micelle and have attempted to minimize other effects that contribute to the observed spectral line shape—e.g., exchange of probe between the micellar and bulk aqueous phase and magnetic dipole–dipole interactions.

Previous studies have indicated that the CSL is a reasonably sensitive probe for the study of oriented phospholipid arrays (12) and will orient in an analogous fashion to cholesterol in bilayer (23) and monolayer systems (24). This spin label has added advantages in the sense that it has a low intrinsic water solubility and has little tendency to migrate within lipid arrays to more fluid regions (25). Preliminary experiments were carried out to ensure that this label would satisfy some of the criteria mentioned above. Variation of the probe concentration to a 20-fold excess of the standard concentration resulted in no significant change in the observed spectrum and, hence, we conclude that, for the experimental conditions, magnetic dipole–dipole interactions are minimal. The bulk viscosity of the aqueous phase was varied by the addition of glycerol to a mixed micellar system. Measurable changes in spectral parameters were noted only above 20% v/v. Therefore, we believe that the ESR spectra reflect primarily the motion of the probe in its environment and contributions from the behavior of the lipid aggregate in the bulk phase are minimal.

A typical spectrum of the CSL in an aqueous dispersion of bile salts and egg yolk lecithin is shown in **Fig. 1**. This spectrum may be compared to two limiting cases: (*a*) free isotropic rotation of the spin labels, label molecules dissolved in organic solvent, and (*b*) fast anisotropic rotation of spin labels (13). In such a comparison, these labels are an intermediate case but lie closer to the situation of isotropic rotation and

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are similar to spectra observed for steroid spin labels in liposomes (26) and single-walled vesicles (27). The observed approximate hyperfine coupling constant, a_N, of ca. 15 G, which is of the order of 1/3 the trace of the hyperfine tensor (14.1 G) for this radical (13, 28) also supports this view. The magnitude of a_N is indicative of the nitroxide moiety being located in a polar environment and suggests that the spin labels are positioned in the micelles in a similar fashion as in liposomes (26) and multibilayer systems (12).

Approximate "correlation times", τ_c, have been calculated according to the equation (29, 30),

$$\tau_c = 6.45 \times 10^{-10}[(h_0/h_{+1})^{\frac{1}{2}} + (h_0/h_{-1})^{\frac{1}{2}} - 2]W_o \quad \text{Eq. 1}$$

where h_{+1}, h_0 and h_{-1} are the amplitudes of the high, center, and low field lines, respectively, and W_0 is the width of the center line at full height in Gauss. The calculated correlation times for the spin label in this study are greater than 10^{-9} sec and are outside the limits where Eq. 1 is strictly valid. Nevertheless, we believe that this expression may be used to provide a reasonable empirical parameter to characterize such ESR spectra and the fluidity of the local environment of the probe.

Table 1 presents the results for the CSL in a series of lipid solutions at constant total lipid concentration with various glycocholate:lecithin mol ratios in the absence of cholesterol. These data were derived from at least eight scans per sample, and a calculated correlation time was computed from average values of the appropriate parameters.

Two hours were found to be sufficient for equilibration of the sample, and the only change noted after 48 hr was a diminution of the spectral intensity, presumably due to spin label decomposition. The width of the center line, W_0, which may be taken as an estimate of the velocity of motions of the spin label (31), decreases with decreasing ratios of glycocholate:lecithin. The smaller W_0, the faster the velocity of motions. It has been reported that for the lecithin–bile salt system, the size of the mixed micelles increases with decreasing bile salt:lecithin ratios (3). Our measurements suggest that in the larger micelles the probe moves more rapidly, despite the similarity in correlation times, which implies that the phospholipid arrays in the smaller micelles present a more restricted environment compared to the larger aggregates. The correlation times do not exhibit any consistent variation for these lipid mixtures. This may in part reflect the inadequacies of Eq. 1 to describe the spectra, but it also serves to emphasize that, in these systems, τ_c does not characterize the dimensions of the aggregate.

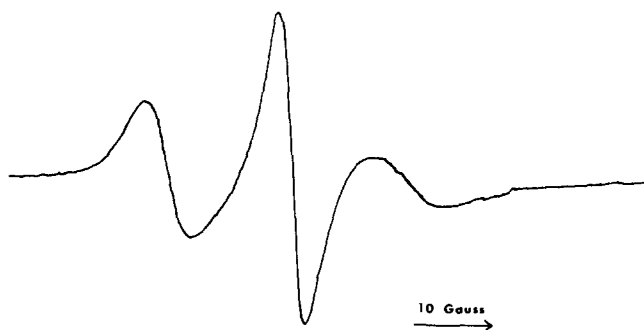

Fig. 1. ESR spectrum of CSL in aqueous glycocholate and lecithin, mol ratio 3.3:1 at 27°C; total lipid concentration 87 mM.

TABLE 1. Correlation times and W_0 values for ternary system with different glycocholate: egg yolk lecithin ratios[a]

Glycocholate: Lecithin	W_0, G	$\tau_c \times 10^9$ sec
0.76	3.20 (0.03)	4.6 (0.03)
1.19	3.30 (0.02)	4.5 (0.04)
1.90	3.40 (0.05)	4.7 (0.03)
3.33	3.63 (0.04)	4.7 (0.03)
7.04	3.75 (0.04)	4.7 (0.04)

[a] At 27 ± 0.5°C. The numbers in parentheses represent the standard error of mean; the maximum uncertainty in W_0 values are ca. 0.05 G.; total lipid concentration 87 ± 0.5 mM.

The quaternary system was studied at three glycocholate:lecithin ratios. Cholesterol could be incorporated in these systems to give optically clear (by polarized light microscopy) solutions up to levels approximating the original isotropic phase limit defined by Small (8) (see **Fig. 2**). Solutions whose compositions were within the metastable labile zone did not exhibit optical or spectral changes after 48 hr. Solutions above the Small phase limit (8) were clearly composed of at least two phases, viz., isotropic solution, lyotropic paracrystalline phases, and crystalline cholesterol. The properties of the CSL in condensed phases derived from this system are currently being pursued. Also, some preliminary results indicate that crystalline cholesterol is not always present in some of these anisotropic dispersions, which is in agreement with an earlier report (32).

The effect of cholesterol on the correlation time of CSL up to the phase boundary is shown in **Fig. 3.** At each glycocholate:lecithin ratio, τ_c was found to be a discontinuous function of cholesterol concentration. The discontinuity is most clearly revealed by the glycocholate:lecithin ratio of 3.3 and is located between 4.0 and 5.5 mol % cholesterol. The discontinuities for the other lipid ratios are less distinct, occurring between 1.5–3 and 7–9 mol % cholesterol for glycocholate:lecithin ratios of 7 and 1.2, respectively. The precision of the measurements does not permit more reliable estimates of their location. The composi-

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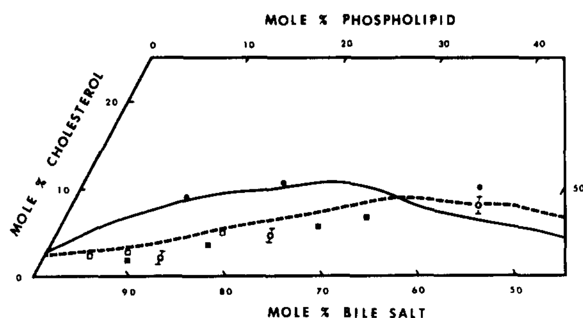

Fig. 2. A portion of the total triangular coordinate system (7): the solid and broken lines represent the two solubility limits as defined by Admirand and Small (8) and Holzbach (10), respectively; □ and ■, the equilibrium solubility of cholesterol in glycocholate:lecithin mixtures reported by Dam (9) and Saunders and Wells (33), respectively; ●, the spontaneous phase separation for glycocholate:lecithin mixtures in the presence of CSL; ○, the composition at the observed discontinuity in correlation time for CSL as a function of cholesterol (the bars indicate maximum uncertainty in location).

tions of the lipid solutions at these points are shown in Fig. 2. The boundary defined by these compositions is lower but correlates well with the equilibrium solubility for cholesterol in similar systems using a mixture of conjugated bile salts (9, 10). Other data for glycocholate systems (33, 34) would suggest that glycocholate is less efficient than mixtures of bile salts in terms of equilibrium solubility levels of cholesterol. These data were derived for different total lipid concentrations and by varying methodologies such that absolute comparisons are unwise.

The results for CSL in the quaternary system using glycochenodeoxycholate as the bile salt are shown in **Fig. 4**, along with those for the glycocholate system at a similar bile salt: lecithin ratio. The results are qualitatively similar to those for the glycocholate system. The correlation time is a discontinuous function of cholesterol. The dependence of τ_c on cholesterol content is not as marked as in the case of glycocholate and the discontinuity is shifted to higher cholesterol concentrations. If this discontinuity is indicative of equilibrium solubility, then these data suggest that the equilibrium solubility of cholesterol in a glycochenodeoxycholate system is higher. Although there is no directly comparable data in the literature, studies have demonstrated that chenodeoxycholate is a more effective component than cholate in solubilizing cholesterol (9, 34). Full interpretation of the present observations requires more detailed knowledge of the equilibrium solubility of cholesterol in this lipid system, the size of the mixed micelles, and the distribution of glycochenodeoxycholate between mixed and simple micelles.

The influence of cholesterol on lecithin in oriented multibilayer systems and aqueous dispersions has been

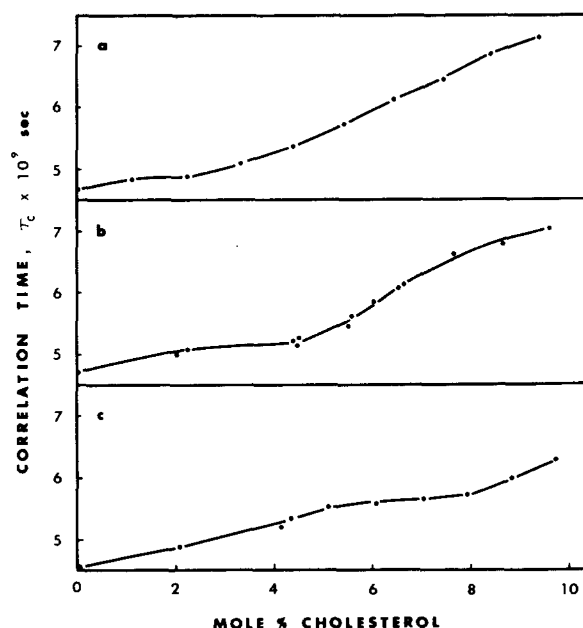

Fig. 3. Variation of τ_c for CSL as a function of cholesterol content for different glycocholate:lecithin ratios at 27 ± 0.5°C.; concentration of glycocholate and lecithin were (a) 76 mM and 10.8 mM, (b) 66.5 mM and 20 mM, (c) 47.5 mM and 40 mM, respectively.

studied using probes that exhibit rapid anisotropic motion and permit the evaluation of an order parameter (35). The CSL in oriented multibilayer systems produces angularly dependent spectra, allowing estimates of both fluidity and order to be made. Both parameters are shown to increase monotonically as a function of cholesterol content, rising without discontinuities to a maximum at 33 mol % cholesterol (36). A similar study (37) also reports similar findings for this system to 70 mol % cholesterol which is in excess of the stable equimolar ratio. Similar studies of fatty acid spin labels, where the nitroxide moiety is attached to

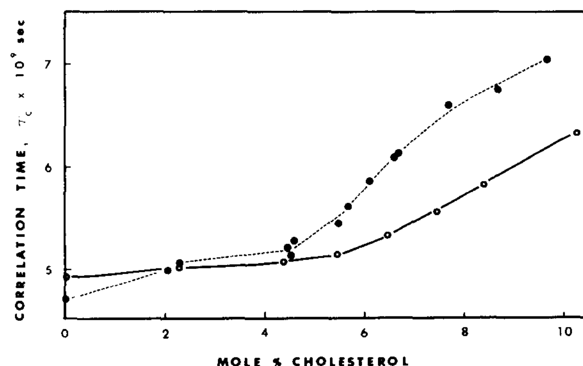

Fig. 4. Variation of τ_c for CSL as a function of cholesterol content at 27 ± 0.5°C; the solid line represents the glycochenodeoxycholate:lecithin system with lipid concentrations of 65.8 mM and 20 mM, respectively; the broken line represents the glycocholate:lecithin system with lipid concentrations of 66.5 mM and 20 mM, respectively.

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the hydrocarbon chain, in aqueous lecithin dispersions exhibit a smooth variation of an order parameter from 0 to 60 mol % cholesterol (38). Although the present data are analyzed in terms of fluidity of the phospholipid matrix, it does not preclude that, at higher cholesterol levels, the motion of the probe is becoming more anisotropic, reflecting an increase in order of the matrix.

In the absence of cholesterol, two possible environments for the probe exist, i.e., simple bile salt micelles and mixed micelles. The observed resonance will report on the average environment of the probe. Introduction of cholesterol produces a decrement in fluidity of the environment for all lipid ratios as expected. The abrupt change in fluidity at the equilibrium mixed micellar phase boundary suggests that the average environment is significantly modified. This may be explained by postulating that the quaternary system retains its integrity with respect to populations of simple and mixed micelles and that supersaturation is manifested by the lipid matrices containing cholesterol in excess of the available thermodynamically stable sites producing a more restricted environment for the probe. The onset of supersaturation would be expected to occur before the stable limit of a lecithin:cholesterol mol ratio of 1:1 is attained, since some of the phospholipid molecules interact with the bile salts (2), which is in harmony with the present results.

Alternatively, supersaturation may be accommodated by the production of a different aggregate in addition to simple and mixed micelles, in which the CSL has markedly different motional characteristics such that the observed spectra reflect a composite of these environments. The absence of discontinuities in ESR parameters for the CSL in multilayers and dispersions of phospholipids and cholesterol would favor such an interpretation.[1] It is of interest to note that many of the time- and temperature-dependent properties ascribed to metastable solutions derived from coprecipitated lipids, e.g., the appearance of liquid crystalline bodies and crystalline cholesterol, are similar to aqueous dispersions of cholesterol and phospholipids alone (39, 40). Thus, these observations may reflect the properties of such an aggregate and not those of a monodispersed system of supersaturated mixed micelles per se.

In summary, these studies show that the equilibrium solubility limit of cholesterol in this quaternary system may be demonstrated prior to attaining formal thermodynamic equilibration. These findings suggest that excess cholesterol in metastable solutions prepared by the coprecipitation method is accommodated by the formation of another aggregate in addition to the disc-shaped mixed micelle. The relevance of these findings to the structure of cholesterol supersaturated bile in humans is unknown but, clearly, the spin label probe technique may provide useful information in the elucidation of effects of other biliary constituents, e.g., conjugated bilirubin, on this complex quaternary lipid system.

The author wishes to express his sincere gratitude to Professor Peter Smith, Department of Chemistry, Duke University, for making available the ESR facility and to Dr. Malcolm Tyor and colleagues of the Division of Gastroenterology, Duke University Medical Center, for their helpful suggestions and encouragement during the course of this work.

Manuscript received 29 September 1976 and accepted 23 January 1977.

[1] We have attempted to resolve this question by gel filtration. Preliminary results for the glycocholate:lecithin system on Sepharose 4B columns have given data compatible with this hypothesis. Aggregates from lipid mixtures whose compositions are below the equilibrium solubility limit are eluted by glycocholate, above the critical micelle concentration, as one species. Under similar conditions, lipid mixtures from the metastable labile zone chromatograph with cholesterol in association with two species. One possesses a similar lipid stoichiometry to the equilibrium system; the other is larger and is composed predominantly of phospholipid and cholesterol but is still associated with bile salt.

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