Proc. Indian Acad. Sci. (Chem. Sci.), Vol. 98, Nos 5 & 6, June 1987, pp. 453–468.

Solubilization of bilirubin by cholate micelles. Spectroscopic and gel permeation studies

KASTURI LAHIRI PURANAM and P BALARAM
Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560 012, India

Abstract. The interactions of bilirubin with bile salts have been studied using fluorescence, circular dichroism and ^{1}H NMR methods. Enhancement of bilirubin fluorescence and induction of optical activity in bilirubin in the presence of cholate has been observed. Fluorescence enhancement is pronounced above the critical micelle concentration, while induced CD bands are detectable even in the premicellar region. Dehydrocholate and deoxycholate did not cause a fluorescence increase, but induced CD bands were observed for bilirubin in these cases. Gel permeation chromatography on Sephadex G-50 yielded a single bilirubin-cholate species at alkaline pH, while two species were obtained at neutral pH. ^{1}H NMR and CD spectral characterizations of these complexes are reported. A 4:1 cholate-bilirubin mixture has been analysed by difference (nuclear Overhauser effect) NOE spectroscopy. Observation of strong, negative NOE, both intermolecular and intramolecular leads to the conclusion that specific methyl groups on bilirubin and cholate are proximal in the mixed micelle.

Keywords. Bilirubin; bile salts; micelles; induced circular dichroism; fluorescence; nuclear Overhauser effects.

1. Introduction

An important aspect of biliary physical chemistry is the physical state of organic anions in bile. Bilirubin is one of the most thoroughly studied anions because of its biological and clinical importance. Other anions are drugs and dyes like indocyanine green, bromsulphphthalein, rose bengal etc. Many of these are incorporated into mixed micelles of lipids and bile acids (Bickel and Minder 1970; Scharschmidt and Schmid 1978; Reuben *et al* 1982). A physical association with lipid micelles helps in the effective excretion of the xenobiotics in bile (Reuben 1984).

Bilirubin IX α is the end product of heme degradation in mammals (Brown and Troxler 1982) which has necessarily to be excreted as it serves no useful purpose and is neurotoxic. Bilirubin is practically insoluble under physiological conditions and is transported in blood as the serum albumin complex (Meuwissen and Heirwegh 1982) to the liver, where it undergoes conjugation with glucuronic acid to form the more soluble diglucuronides. In the case of bilirubin, the rate of biliary secretion has been shown to rise with an increase in biliary output of bile salts (Goresky *et al* 1974). The solubility of bilirubin has been found to be enhanced in the presence of bile salts (Carey and Koretsky 1979; Ostrow and Celic 1984). Bilirubin is therefore thought to be associated with mixed micelles of lecithin and bile salts. However, structural details of such an interaction between bilirubin and

bile salts are lacking and that has prompted us to undertake a spectroscopic investigation of this system. This report summarizes fluorescence, circular dichroism (CD), gel permeation and ^{1}H NMR studies of bilirubin-bile salt complexes.

2. Materials and methods

Bilirubin obtained from Sigma Chemical Co. was purified according to the procedure of McDonagh and Assisi (1972). Cholic acid, dehydrocholic acid and sodium deoxycholate were also from Sigma. Both cholic acid and dehydrocholic acid were converted into their sodium salts by adding a molar equivalent of sodium hydroxide and subsequent lyophilization.

Aqueous stock solutions of bilirubin were prepared using sodium hydroxide for complete dissolution. The concentration of such stock solutions was determined using $\varepsilon_{438} = 47{,}000\ M^{-1}cm^{-1}$ (Carey and Koretsky 1979). The solutions were used within two to three hours of preparation. Samples containing bilirubin and cholate for ^{1}H NMR studies were made by addition of solid bilirubin to the cholate solution in D_2O and then increasing the pD of the solution to $\backsim$ 10·5 using NaOD, when all the bilirubin dissolves. Such samples could be stored in the dark, at 4°C, for at least two days without the occurrence of bilirubin photodegradation.

For the fluorescence and CD experiments the final concentration of bilirubin used was always less than 1×10^{-5} M. 1 cm pathlength cuvettes were used for the fluorescence experiments, while for the CD experiments a 5 cm pathlength, cylindrical cell was employed. Fluorescence studies were carried out on Perkin Elmer MPF 44A and Hitachi 650–60 fluorimeters and CD studies on a JASCO J20 spectropolarimeter.

Gel permeation studies were done on a Sephadex G-50 column of dimensions 1·1 cm × 70 cm. Blue dextran (Sigma) was used to check the column packing as well as to determine the void volume. The column was calibrated using proteins like melittin (monomeric), cytochrome c from horse heart and ovalbumin. The sample volume loaded varied between 150 and 300μl. In the case where ^{1}H NMR spectra of column fractions are reported, the appropriate fractions were pooled, lyophilized and redissolved in D_2O.

^{1}H NMR spectra were recorded on a Bruker WH-270 FT NMR spectrometer at the Sophisticated Instruments Facility, Indian Institute of Science, Bangalore. No external standard was added to the sample, the 4·76 ppm resonance of H_2O (in D_2O) was taken as an internal reference. All experiments were done at 293 K. Difference nuclear Overhauser effect (NOE) spectra were obtained by sequential recording of perturbed and normal spectra (8 K memory each) using low power on-resonance shifting of the irradiation frequency, respectively. A delay time of 3·0 sec was used between transients. The difference free induction decay was multiplied by a decaying exponential, prior to Fourier transformation.

3. Results

The linear structure of bilirubin IX α, the physiologically important bilirubin isomer, is shown in figure 1. Also shown in figure 1 is the structure of cholate, the

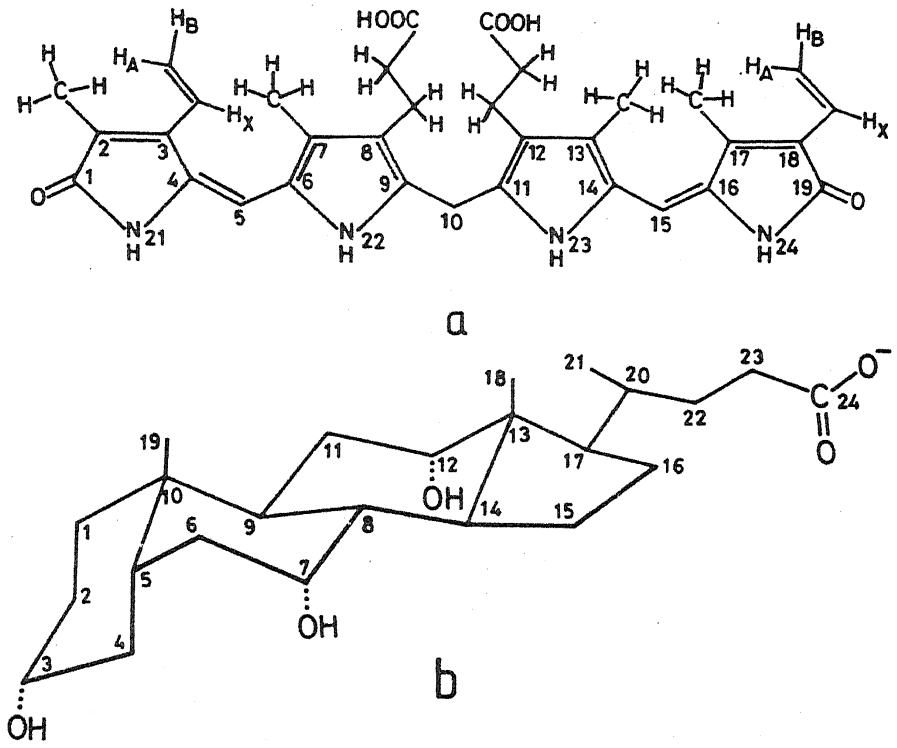

Figure 1. (a) Structure of bilirubin IX α (4Z, 15Z configuration) with numbering scheme. (b) Structure of cholate with numbering scheme.

bile salt which is the subject of most of the studies described in this report. The other bile salts studied are deoxycholate which lacks a hydroxyl group at position 7 and dehydrocholate, where all the three hydroxyl groups are replaced by keto groups.

3.1 *Fluorescence*

Figure 2 shows the enhancement of bilirubin fluorescence in the presence of sodium cholate. Bilirubin is practically non-fluorescent in solution at room temperature, the quantum yield being less than 10^{-4} (Matheson *et al* 1975). Binding to serum albumin results in fluorescence enhancement. This increase has been attributed to translational and vibrational immobilization of bilirubin in its bound form (Beaven *et al* 1973). Bilirubin has also been shown to exhibit enhanced fluorescence when it binds to gramicidin S (Marr-Leisy *et al* 1985) and symmetrical alkyl diamines (Lahiri and Balaram 1987). Increase of bilirubin fluorescence in the presence of other macromolecules can thus be considered as indicating interaction leading to binding. It is interesting to note in figure 3 that the enhancement of bilirubin fluorescence in the presence of varying concentrations of cholate exhibits a sharp discontinuity around 13 mM, followed by a further rise. The critical micelle concentration (CMC) of cholate is known to be between 11 and 13 mM (Roda *et al* 1983), depending on the presence of salts like sodium chloride. It thus appears that bilirubin binds preferentially to micelles of cholate. There is no observed increase in the fluorescence of bilirubin in the presence of the other bile salts, deoxycholate and dehydrocholate. While the CMC of deoxycholate is $\backsim$ 10 mM, that of dehydrocholate is $>$ 250 mM (Roda *et al* 1983).

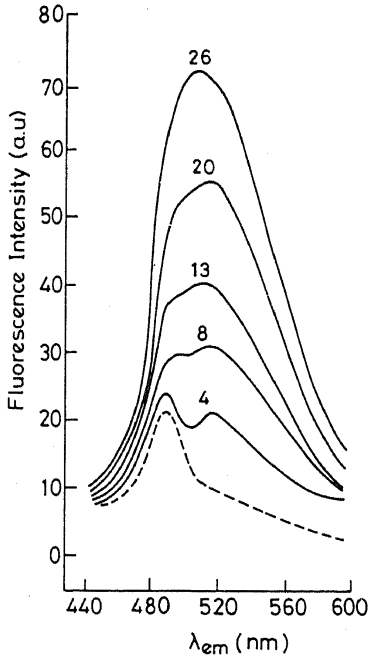

Figure 2. Enhancement of bilirubin ($4{\cdot}8 \times 10^{-6}$ M) fluorescence (arbitrary units) in the presence of cholate in H_2O at pH 10·9, 150 mM NaCl. Number over each trace indicates the cholate concentration in mM λ_{ex} = 420 nm.

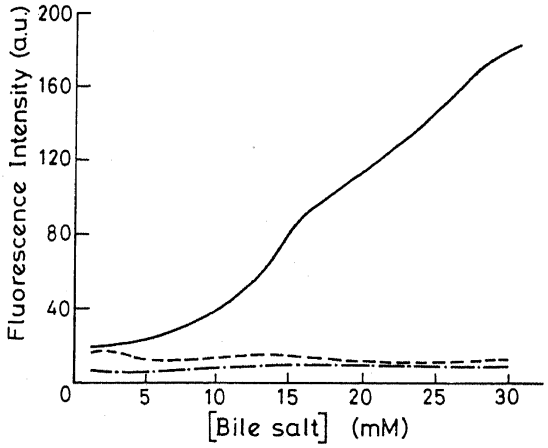

Figure 3. Dependence of bilirubin fluorescence on the concentration of bile salts in H_2O, pH 10·8, 150 mM NaCl. λ_{ex} = 420 nm, λ_{em} = 520 nm. (—— $4{\cdot}6 \times 10^{-6}$ M bilirubin in cholate; — — — $7{\cdot}3 \times 10^{-6}$ M bilirubin in deoxycholate; –·– $7{\cdot}3 \times 10^{-6}$ M bilirubin in dehydrocholate.)

3.2 *Circular dichroism*

CD is a useful technique to monitor bilirubin binding to other molecules since the observation of induced optical activity in the otherwise optically inactive bilirubin is a positive indication of the occurrence of such interactions (Blauer 1983). Such induced optical activity is observed when bilirubin binds to proteins like serum albumins (Brodersen 1982), ligandin (Bhargava *et al* 1980), aminoazodye-binding protein A (Tipping *et al* 1976) and chymotrypsin and lysozyme (Blauer 1986). Bilirubin also exhibits induced CD when bound to chiral amines and basic

polypeptides like poly-L-lysine, melittin and gramicidin S (Marr-Leisy *et al* 1985). Induced optical activity has been observed for bilirubin in the presence of sodium deoxycholate (Perrin and Wilsey 1971). Figure 4 shows the induced CD in $7{\cdot}6\times10^{-6}$ M bilirubin in the presence of 2×10^{-2} M cholate, deoxycholate and dehydrocholate at pH 10·5. The characteristic bisignate CD pattern is observed with long wavelength positive and short wavelength negative bands, reminiscent of the bilirubin-human serum albumin 1:1 spectrum at ~pH 7 (Blauer and Harmatz 1972). In the case of bile salts, the long wavelength bands lie between 450 and 470 nm and the short wavelength band between 375 and 400 nm. The CD parameters for bilirubin in the presence of various bile salts are summarized in table 1. The parameters for bilirubin-human serum albumin are also given for comparison. Figure 5 shows the induced CD spectra for bilirubin in presence of increasing concentrations of cholate. The cholate concentrations studied range from 1 to 20 mM. Figure 6 shows a plot of the CD band intensities at 400 and 455 nm as a function of cholate concentration. The results suggest that induced optical activity is detectable even at premicellar cholate concentrations.

3.3 *Gel permeation chromatography*

To characterize the size of the complex formed by bilirubin and cholate, gel permeation chromatography was performed on a Sephadex G-50 column. A 4:1 cholate-bilirubin mixture in water at pH 10·5 was loaded on the column, the eluant

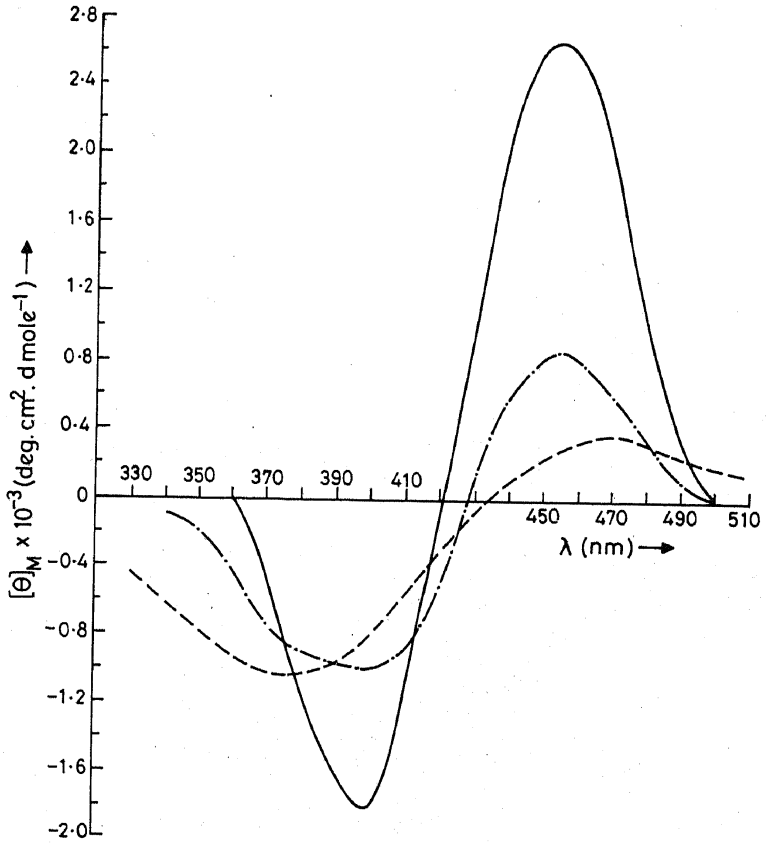

Figure 4. Induced CD spectra of bilirubin ($7{\cdot}6\times10^{-6}$ M) in the presence of 20 mM bile salts in H_2O, pH ~ 10·5, 150 mM NaCl. Cholate (—·—), deoxycholate (— — —), dehydrocholate (——).

Table 1. CD spectral parameters of bilirubin in presence of bile salts.

Bile salt (serum albumin)[a]	Solvent	Bile salt (HSA) concentration (M)	Bilirubin concentration (M)	λ_{nm} ($[\theta]_M$)[b]
Cholate	H_2O, pH 10·6	2×10^{-2}	$7{\cdot}63\times10^{-6}$	$400(-1{\cdot}0\times10^3)$; $455(0{\cdot}86\times10^3)$
Deoxycholate	H_2O, pH 10·5	2×10^{-2}	$7{\cdot}68\times10^{-6}$	$375(-1{\cdot}04\times10^3)$; $472(0{\cdot}38\times10^3)$
Dehydrocholate	H_2O, pH 10·5	2×10^{-2}	$7{\cdot}68\times10^{-6}$	$398(-1{\cdot}82\times10^3)$; $453(2{\cdot}66\times10^3)$
HSA	H_2O, pH 8·7	$3{\cdot}46\times10^{-5}$	$3{\cdot}46\times10^{-5}$	$397(-1{\cdot}45\times10^5)$; $448(1{\cdot}97\times10^5)$

[a] Data taken from Marr-Leisy *et al* 1985; [b] $[\theta]_M$ values as deg. cm^2. d mole^{-1}.

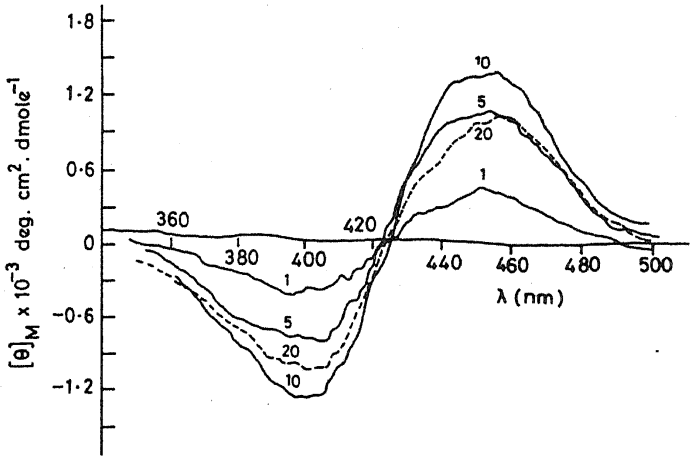

Figure 5. Induced CD spectrum of $6{\cdot}9\times10^{-6}$ M bilirubin with increasing cholate concentrations in H_2O at pH 9·2 150 mM NaCl. Number over each trace is the concentration of cholate in mM.

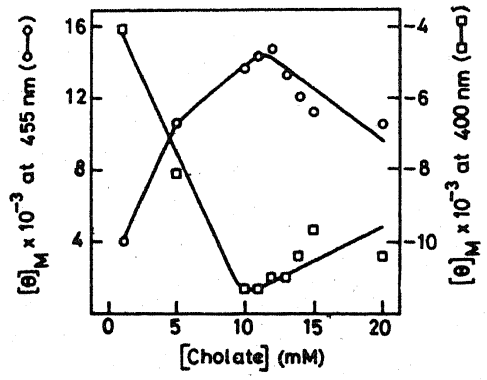

Figure 6. Change in molar ellipticity ($[\theta]_M$) of the bilirubin CD bands at 400 and 455 nm as a function of cholate concentration. ($[\theta]_M$ values as deg. cm^2. dmole^{-1}).