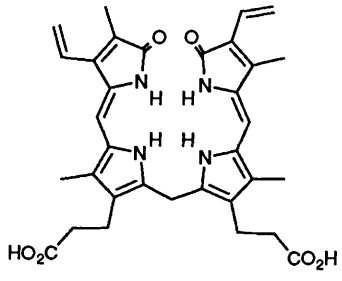
Absorption and Emission Spectral Studies of Bilirubin IXα Complexes with Sodium Taurocholate in Aqueous Buffer, pH 8.0

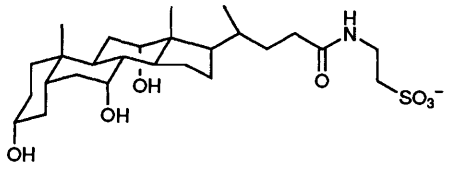
William E. Kurtin,* Denise Reck, Jennifer Lafontaine, Maricel DeLeon and Nhung Tran
Department of Chemistry, Trinity University, San Antonio, Texas 78212, USA

The absorption and fluorescence emission properties of (4Z,15Z)-bilirubin IXα and xanthobilirubic acid in aqueous buffer at pH 8.0 and in the presence of the bile salt sodium taurocholate in the concentration range 1–40 mmol dm^{-3} have been measured. The results are consistent with the formation of a complex in which an intramolecularly hydrogen-bonded conformer of bilirubin is bound in a hydrophobic microenvironment of a taurocholate aggregate, and in which molecular rotations and vibrations of the pigment are relatively unrestrained.

(4Z,15Z)-Bilirubin IXα (**1**), the yellow-orange pigment associated with jaundice, is the catabolic product of heme degradation in liver. It is also known to be an important component of pigment gallstones, in which it occurs as either the calcium salt or as part of an ill-defined polymeric matrix.[1] It is believed that precipitation of bilirubin from bile is an important step in the chemistry leading to gallstone formation in abnormal bile.[2] The aqueous solubility of **1** is practically negligible at pH values near neutrality, due to the propensity of the molecule to form intramolecularly hydrogen-bonded conformers similar to that found in the solid state.[3] In normal bile, the pigment is solubilized to concentrations in the micromolar range by interactions with the bile salts. Bile salts are amphiphilic steroid derivatives, formed by metabolism of cholesterol in the liver, and secreted into bile to function in solubilization and transport of lipids. Several different types of bile salts occur in human bile, but sodium taurocholate (**2**) is the species normally present in the highest amounts. The understanding of the molecular interactions of bilirubin and bile salts in aqueous media is important to the understanding of the mechanisms involved in gallstone formation.

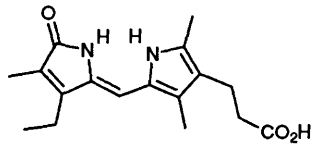


1



2

Prior studies of bilirubin–bile salt interactions have focused on two types of approach. Ostrow's group has used enzymatic and other methods to study the solubility of **1** in various bile salt solutions.[4] They have shown greatly enhanced solubility of the pigment by bile salts, but significant differences among the various types of bile salts in this regard. Several other groups have used circular dichroism to study the possible enantioselective complexation of **1** by bile salts. Perrin and Wilsey,[5] Reisinger and Lightner,[6] and Campanelli *et al.*[7] all reported an induced circular dichroism for the binding of **1** to sodium deoxycholate in aqueous media. More recently, Kano's group also used induced circular dichroism to study binding of **1** to a variety of bile salts in water at pH 10.8.[8] They proposed hydrogen bonding as the main mechanism for stabilization of the complexes. ^{1}H NMR was used in another study of bilirubin solubilization by sodium cholate,[9] and conclusions were similar to those based on circular dichroism studies. Hydrophobic interactions were believed to be important. Finally, Carey and Koretsky reported on studies of the interaction of **1** and taurocholate in carbonate–hydrogen carbonate buffer at pH 10, although this was not the main focus of that study.[10] We have observed an increased fluorescence of **1** in taurocholate solutions, and we have since conducted more detailed investigations of the absorption and emission properties of bilirubin in such media. We present here our results on the optical properties of bilirubin and a model compound, xanthobilirubic acid (**3**), in the presence of pre-micellar and micellar concentrations of sodium taurocholate in phosphate–borate buffer at pH 8.0.



3

Experimental

Bilirubin-IXα (Sigma) was purified by recrystallization from chloroform–methanol.[11] Xanthobilirubic acid was prepared by hydrolysis of the corresponding methyl ester.[12] The latter was a generous gift from Dr David Lightner of the University of Nevada, Reno, Nevada. Sodium taurocholate (Ultrol grade) was obtained from Calbiochem, and was used without purification. All other chemicals were reagent grade. Phosphate–borate buffer (0.1 mol dm^{-3}), pH 8.0, was used throughout. Buffer was usually degassed immediately prior to use by bubbling with nitrogen. Stock solutions of pigments were prepared by predissolution of a weighed amount in a minimum volume of dimethyl sulfoxide (typically 20–50 mm^3) followed by dilution with buffer or taurocholate solution. Sodium taurocholate was dissolved directly in buffer. Stock solutions and samples prepared in this fashion were used immediately, and experiments

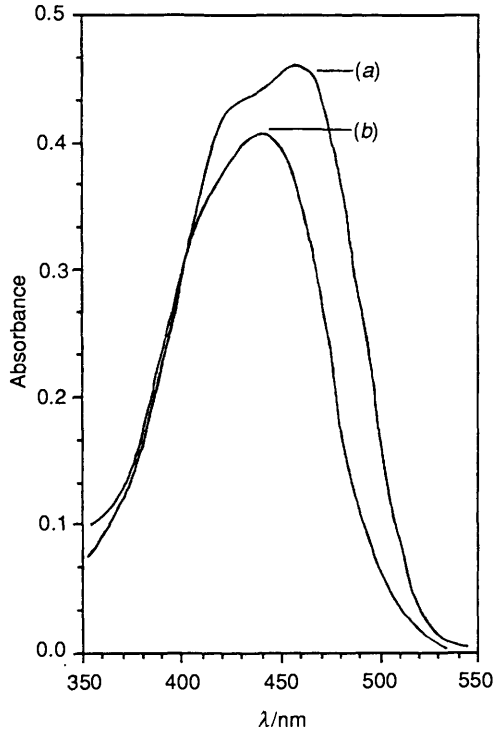


Fig. 1 Long wavelength absorption band of (*a*) 8 μmol dm^{-3} **1**, and (*b*) 8 μmol dm^{-3} **1** with 20 mmol dm^{-3} sodium taurocholate

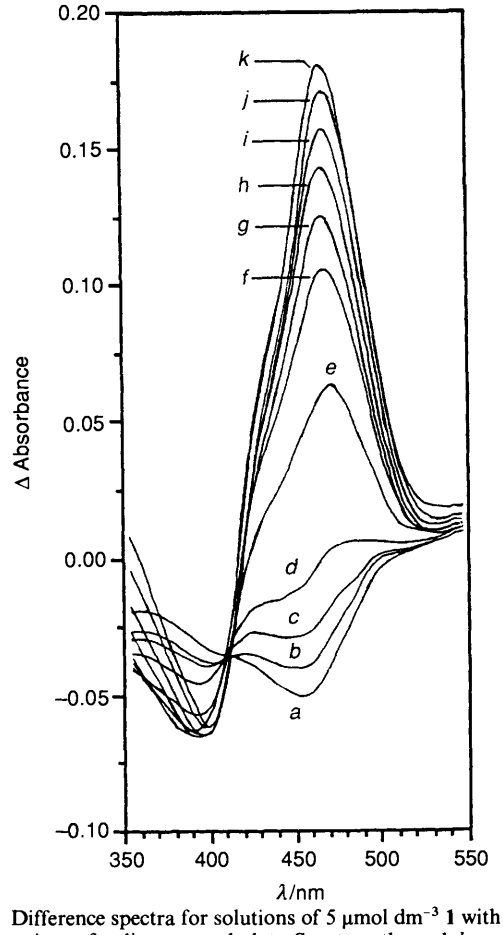


Fig. 2 Difference spectra for solutions of 5 μmol dm^{-3} **1** with varying concentrations of sodium taurocholate. Spectra *a* through *k* correspond to taurocholate concentrations of 1, 2, 3, 5, 10, 15, 20, 25, 30, 35 and 40 mmol dm^{-3}, respectively

were generally completed within 1 h of sample preparation. Experiments were conducted in dim light, and at room temp.

UV-VIS absorption spectra were obtained on a Cary 2315 spectrophotometer. Difference spectra were obtained by using as the reference a solution of the pigment at the same concentration as that of the sample. Repetitive scans showed that such reference solutions were stable during the measurement period. All fluorescence measurements were performed on a Photon Technology LS-100 instrument. Fluorescence spectra were automatically corrected for lamp and detector wavelength response. Solutions of sodium taurocholate, especially at the higher concentrations employed, exhibited significant light scattering, as expected due to micelle formation. In order to account for spectral abnormalities due to scattered light, sample spectra were corrected by subtraction of spectra run on blank samples containing identical concentrations of taurocholate but no pigment. Fluorescence quantum yields were determined by measuring the integrated fluorescence emission of samples against that of an acridine yellow standard in ethanol which was matched in absorbance at the appropriate wavelength.

Results

A comparison of the long wavelength UV-VIS absorption band of **1** in buffer and sodium taurocholate (TC) solution is shown in Fig. 1. In buffer alone, the slightly asymmetric band had a maximum at 442 nm, and a shoulder at about 400 nm. In the presence of 20 mmol dm^{-3} TC, there was an overall red shift of the band, with a maximum at approximately 460 nm. The shorter wavelength shoulder at 420 nm was more pronounced, and the overall intensity of the entire region was somewhat increased. To more fully characterize the changes which occurred in the presence of bile salt, the difference spectra for a series of solutions containing a constant concentration of **1** and varying amounts of TC were recorded. The results are shown in Fig. 2. As the TC concentration was increased in the range 1–40 mmol dm^{-3}, a positive difference band with a maximum at 474 nm appeared at concentrations of 10 mmol dm^{-3} or higher, while a negative difference band in the region 350–400 nm accompanied the changes at longer wavelength. A shoulder at approximately 420 nm was observed in all spectra. At concentrations less than 10 mmol dm^{-3}, a negative difference band was found, but the changes were less pronounced, and no consistent minima or maxima in the negative difference band were observed.

Hypothesizing that the difference band at 474 nm was due to the formation of a unique complex of **1** with taurocholate monomer or a taurocholate aggregate of fixed composition, we analyzed the absorption data using the Benesi–Hildebrand approach.[13] The data were plotted according to eqn. (1), where:

$$\frac{[BR]}{\Delta A} = \frac{1}{K\varepsilon l} \times \frac{1}{[TC]} + \frac{1}{\varepsilon} \quad (1)$$

[BR] = molar concentration of bilirubin; ΔA = height of difference band peak at band maximum; K = formation constant for the complex; l = pathlength of cell; [TC] = molar concentration of sodium taurocholate; and ε = molar absorptivity of the complex at band maximum. The results of the analysis of the full range of data by eqn. (1) are shown in Fig. 3. As expected, a linear result was not found, especially at low concentrations of TC. However, when only the data for TC ≥ 10 mmol dm^{-3} were analysed, the results were highly linear (Fig. 4). Repeated analyses of such plots yielded mean values of 0.060 ± 0.019 dm^3 mol^{-1} and 44 500 ± 5100 dm^3 mol^{-1} for K and ε, respectively.

The fluorescence of **1** in the presence of TC was also measured, and typical emission and excitation spectra are shown in Fig. 5. The emission was characterized by a broad, unstructured band

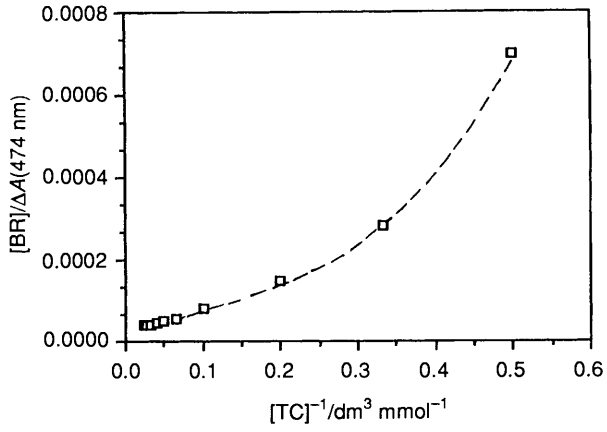


Fig. 3 Plot of the differential absorption data from Fig. 1 according to eqn. (1)

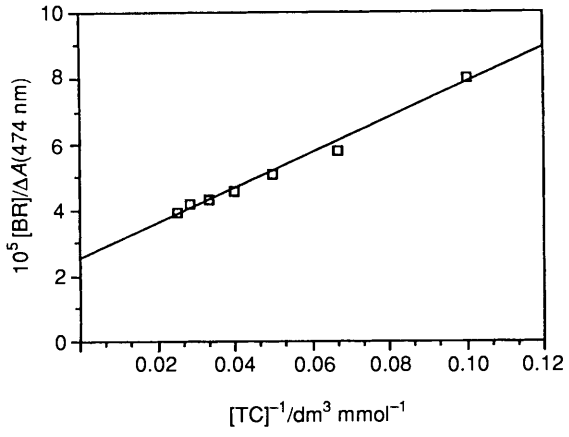


Fig. 4 Plot of the differential absorption data from Fig. 1 according to eqn. (1), but only for those points where [TC] $\geq$ 10 mmol dm^{-3}

with a maximum centred at 540 nm. The excitation spectrum was a more asymmetric band with a maximum at *ca.* 470 nm, and a shoulder at about 420 nm. The emission spectrum was obtained with varying excitation wavelengths in the range 420–480 nm, and while small variations in the maximum were found because of the inherent noise in the signal, the broad emission maximum was always centred at 540 nm. The excitation maximum similarly varied little with emission wavelength, but the excitation spectra were more difficult to measure due to the amount of light scattering in the concentrated bile salt solutions. The fluorescence quantum yield of **1** in 20 mmol dm^{-3} TC solution was measured by comparison with acridine yellow standard, and was found to be 0.0017. The lifetime of the fluorescence was < 0.5 ns, the lower lifetime resolution limit of the available instrument. The signal intensity remained unchanged for a period of at least 30 min, under conditions of repeated excitation.

The integrated fluorescence intensity of **1** as a function of TC concentration is shown in Fig. 6. At concentrations of the bile salt above 5 mmol dm^{-3}, the area of the emission spectrum increases above background levels to a value that is approximately a four-fold increase at 40 mmol dm^{-3} TC. The increase is not linear with the increase in TC concentration, but has a slight curvature in this regard. The increase does appear to be related to the increase in absorbance at 474 nm, as shown by the representative data in Fig. 7. The fluorescence area data were also treated using the same approach as for the absorbance difference data [eqn. (1)], yielding essentially the same results (results not shown).

The long wavelength absorption spectrum of xanthobilirubic acid (**3**) in taurocholate solution is shown in Fig. 8. At low bile

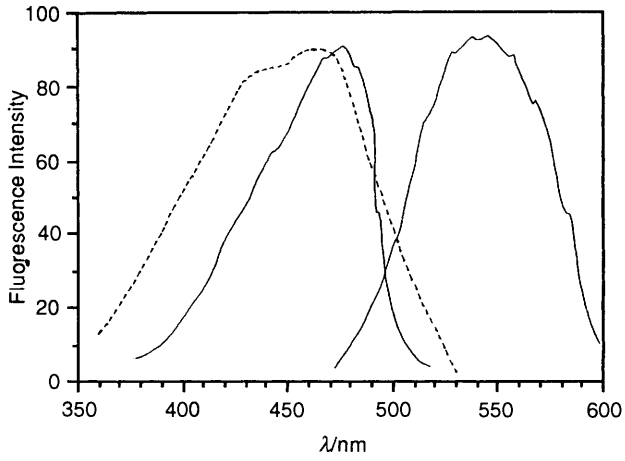


Fig. 5 Solid curves: corrected fluorescence emission (right, λ_{exc} = 470 nm) and fluorescence excitation (left, λ_{em} = 540 nm) spectra for 5 μmol dm^{-3} **1** with 20 mmol dm^{-3} sodium taurocholate. Dashed curve: the corresponding absorption spectrum is overlaid for reference. The vertical axis is given in arbitrary units.

salt concentrations (< 8 mmol dm^{-3}) the spectrum was almost symmetric, with a maximum at 412 nm. At higher bile salt concentrations, there was a progressive red shift of the maximum, with little change in the shape of the band. At the highest concentration of bile salt used, the maximum was 423 nm. There was also an increase in intensity of the band with increase in concentration of TC up to 40 mmol dm^{-3}. Difference spectra showed the emergence of a positive difference band at 436 nm, and a negative difference band with a minimum at 390 nm (Fig. 9). For solutions with [TC] $\geq$ 10 mmol dm^{-3}, an isosbestic point was located at approximately 410 nm. Treatment of the absorbance difference data as before [eqn. (1)] yielded values of 0.018 ± 0.005 dm^3 mmol^{-1} for the formation constant K and 39 800 ± 3300 dm^3 mol^{-1} cm^{-1} for ε.

The fluorescence emission spectrum of **3** in taurocholate solution is presented in Fig. 10. The approximately symmetrical band had a maximum at 484 nm. The excitation spectrum (not shown) was similar in shape to that shown for **1** (Fig. 5), but with a maximum at 432 nm, and a shoulder at approximately 410 nm. The area of the fluorescence emission spectrum increased with TC concentration above 5 mmol dm^{-3}, and did not reach a maximum up to 40 mmol dm^{-3} bile salt. The fluorescence quantum yield of **3** in 20 mmol dm^{-3} taurocholate was 0.0065.

Discussion

The absorption and differential absorption spectra of **1** in aqueous sodium taurocholate at pH 8.0 suggest very weak and relatively non-specific interactions of pigment and bile salt at concentrations of the steroid below 8–10 mmol dm^{-3}, but formation of a 1 : 1 complex of **1** and a taurocholate micellar aggregate at higher bile salt concentrations. The observation of a stable difference band at 474 nm when the taurocholate concentration exceeds 8–10 mmol dm^{-3} is evidence for the formation of a complex of fixed stoichiometry at these higher concentrations. Although taurocholic acid does not seem to have a well-defined critical micelle concentration (cmc) in water, this value is generally assumed to be in the range 3–6 mmol dm^{-3}.[14] As judged by the magnitude of the calculated formation constant, the complex between **1** and TC micelles is a relatively weak one. The mathematical analysis assumes equilibrium binding between **1** and a single taurocholate micellar species. However, since the actual stoichiometries are not known, the constant K cannot be equated with an actual binding constant, and the value of K is useful only for relative comparisons. The

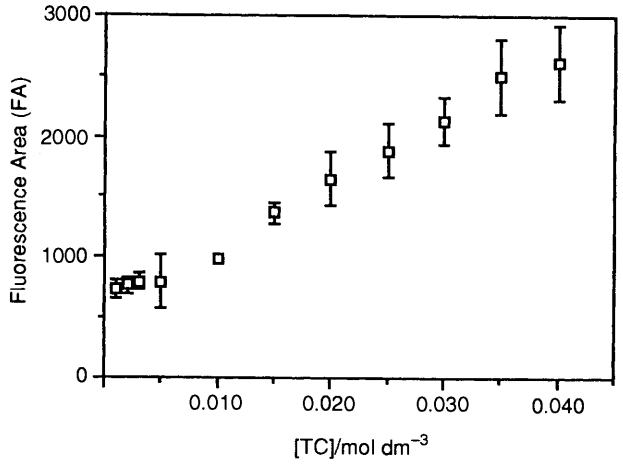


Fig. 6 Integrated fluorescence emission intensity curves for solutions of 5 μmol dm^{-3} **1** with varying concentrations of sodium taurocholate between 1–40 mmol dm^{-3} (λ_{exc} = 470 nm). The data points are the averages for six separate experiments. The fluorescence area is given in arbitrary units.

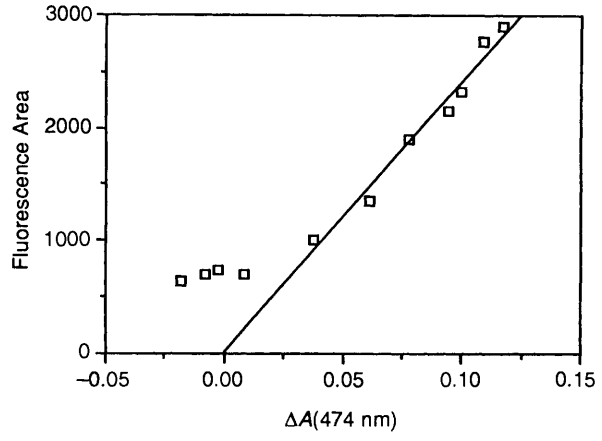


Fig. 7 Representative data showing the relationship between the integrated fluorescence emission (λ_{exc} = 470 nm) and the differential absorption at 474 nm for a series of solutions containing 5 μmol dm^{-3} **1** and 1–40 mmol dm^{-3} taurocholate

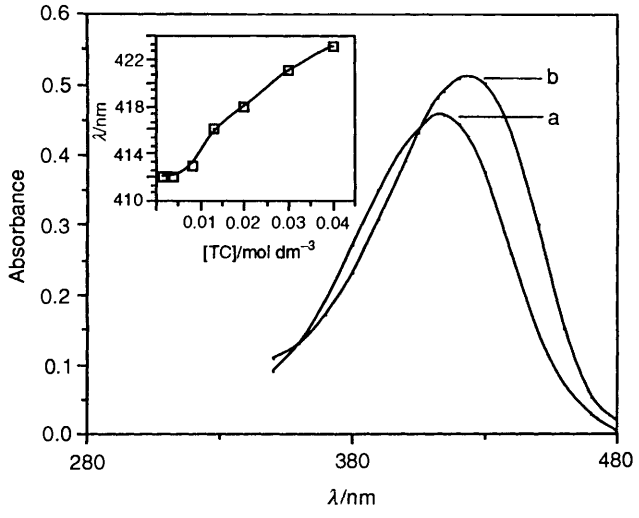


Fig. 8 Long wavelength absorption band of (*a*) 6 μmol dm^{-3} **3**, and (*b*) 6 μmol dm^{-3} **3** with 20 mmol dm^{-3} sodium taurocholate. Inset: dependence of the long wavelength absorption maximum of solutions of 6 μmol dm^{-3} **3** and 1–40 mmol dm^{-3} taurocholate on the bile salt concentration.

fact that the height of the difference band continued to increase, even at the highest concentrations of bile salt employed, suggests that at least one other species is always present in these

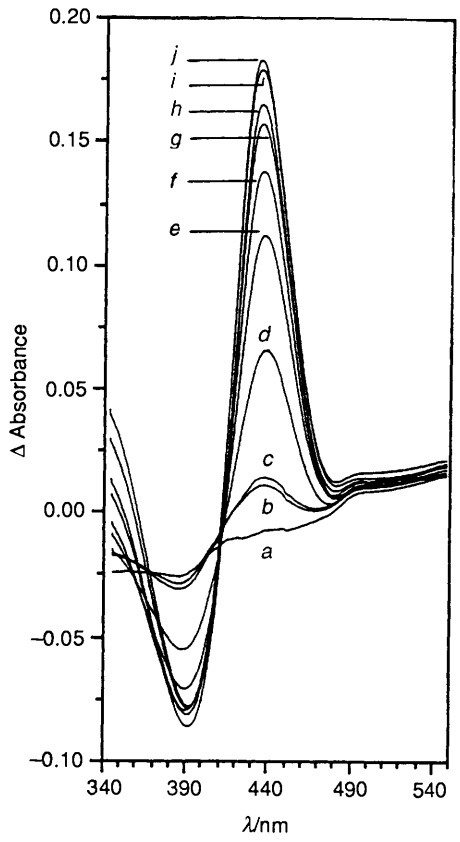


Fig. 9 Difference spectra for solutions of 6 μmol dm^{-3} **3** with varying concentrations of sodium taurocholate. Spectra *a* through *j* correspond to taurocholate concentrations of 1, 3, 5, 10, 15, 20, 25, 30, 35, and 40 mmol dm^{-3} respectively.

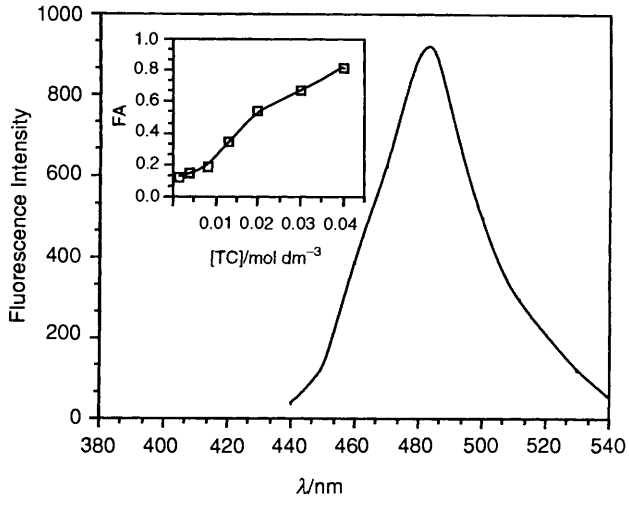


Fig. 10 Corrected fluorescence emission spectrum of 6 μmol dm^{-3} **3** with 20 mmol dm^{-3} sodium taurocholate (λ_{exc} = 430 nm). Vertical axis is given in arbitrary units. Inset: dependence of the integrated fluorescence emission intensity on the concentration of sodium taurocholate in the range 1–40 mmol dm^{-3}.

solutions. Enzymatic studies of bilirubin binding to taurocholate at pH 8.2 showed that a significant amount of unbound pigment existed at concentrations of TC like those used in our experiments.[4b] Our results are similar to those reported by Carey and Koretzky, who carried out their measurements in carbonate–hydrogen carbonate buffer at pH 10.[10]

The spectral changes at low bile salt concentrations are almost negligible, and there is no clear pattern to the observed changes. Kano *et al.* recently reported studies of the interaction of **1** with a variety of bile salts in water at pH 10.8 and in

methanol.[8] Based on induced circular dichroism of the pigment in bile salt solutions at concentrations of 1–10 mmol dm^{-3}, they concluded that **1** enantiomerically complexes with the steroids in water by hydrogen bonding between the carboxylate ions of **1** and hydroxy groups of the bilt salts. Their results are not directly comparable with ours since they used unconjugated bile salts and unbuffered aqueous solutions for their studies. However, the magnitude of the reported induced CDs suggest that relatively weak and non-specific complexes were formed.

The fluorescence results support the conclusion that a unique, but weak complex of **1** and taurocholate exists at micellar concentrations of the bile salt. The fluorescence increases uniformly above the cmc region, and the excitation maximum is close to the difference absorption maximum. However, the fluorescence quantum yield of **1** in these solutions increases only slightly compared to that in organic solutions.[15] The most prominent pathway for deexcitation of bilirubin in a variety of media has been shown to be $Z \rightarrow E$ photoisomerization in the exocyclic double bonds.[16] It has been well established that this process involves a very rapid twisting of the excited molecules about these double bonds, resulting in a decrease in the separation of the ground and excited state energy surfaces, and low fluorescence yields. In the binding of **1** to taurocholate micelles, the pigment is apparently not held 'tightly' in the micelle environment, or in such a way that excited state twisting is significantly retarded. This suggests that weak interactions are involved. The fluorescence results are similar to those reported for solution of various polycyclic aromatic hydrocarbon probes in aqueous sodium taurocholate.[17]

It seems likely that, in its interaction with taurocholate micelles, the pigment prefers to exist in an intramolecular lipophilic hydrogen-bonded conformation like the so-called ridge tile structure which has been shown to exist in a variety of solvents.[18] One piece of evidence for this is the shift to longer wavelength upon formation of the complex. This is more characteristic of the absorption of the pigment in non-polar solvents. In addition, it has been shown that **1** is enantiomerically complexed in this folded conformation in micellar solutions of sodium deoxycholate.[5–7] However, the shape of the absorption spectrum of the complex also suggests that the conformation of the pigment may be somewhat distorted from the ridge tile structure. The shape of the long wavelength band of **1** when bound to various macromolecules has been interpreted in terms of a molecular exciton model, and varies with the dihedral angle between the two planar dipyrromethenone halves of the molecule.[19] Since the spectrum of the complex of **1** and taurocholate exhibits a maximum at about 460 and a shoulder at about 420 nm, it is possible that the binding site in the micelle causes a distortion of the bound pigment that results in exciton splitting to produce a shoulder in the absorption spectrum. At pH 8.0, we assume that **1** exists as the dianion, based on our own evidence and that of others.[20] Although the bilirubin dianion has been shown to prefer the ridge tile structure in many solvents,[21] the negative charge of the pigment may inhibit interaction with bile salt micelles due to charge repulsion, and the latter may also result in alterations of the pigment conformation. Preliminary experiments at lower pH values indicate that, upon protonation of the pigment, the absorption spectrum shifts further to the red, and fluorescence intensity is further increased.[22] In addition, electrophoresis studies indicate a higher affinity of **1** for taurocholate micelles at lower pH.[20b] These results are all consistent with our interpretations. Further experiments to probe the nature of the binding environment in this complex are in progress.

Our results on the spectral properties of xanthobilirubic acid (**1**) in taurocholate solution support the interpretations on the interactions of **1** with TC. Little interaction occurs with the bile salt below the cmc region. At micellar concentrations, a complex with a red-shifted absorption maximum is formed, but the interaction is also relatively 'loose', as the fluorescence is increased only to a small extent. No shoulder appears in the absorption spectrum at shorter wavelengths, most likely because there is only one chromophore in this case. It is interesting to note that the fluorescence quantum yield of **3** is higher than that of **1** in the complex. It has been noted that, in general, mechanisms which decrease hydrogen bonding in bilirubin lead to increased fluorescence.[16b] This is consistent with the suggestion that binding of **1** to taurocholate leads to a distortion from the perfect ridge tile hydrogen-bonded conformer.

In conclusion, absorption, differential absorption, and fluorescence spectral studies of bilirubin IXα dianion in sodium taurocholate solution indicate that the pigment forms a weak complex in which the tetrapyrrole is probably bound to a taurocholate micellar aggregate as an intramolecularly hydrogen-bonded conformer, and in which the complex is most likely stabilized by hydrophobic interactions.

Acknowledgements

We are indebted to the National Science Foundation (Grants No. CHE-8900395, CHE-9200033 and CHE-9119484) for support of this work.

References

1 J. D. Ostrow, *Hepatology*, 1984, **4**, 215s.
2 R. S. Crowther and R. D. Soloway, *Seminars in Liver Disease*, 1990, **10**, 171.
3 R. Brodersen, in *Bilirubin*, ed. K. Heirwegh and S. Brown, CRC Press, Boca Raton, FL, 1982, vol. I, p. 75.
4 (*a*) J. D. Ostrow, L. Celic and P. Mukerjee, *J. Lipid Res.*, 1988, **29**, 335; (*b*) R. V. Rege, C. C. Webster and J. D. Ostrow. *J. Lipid Res.*, 1988, **29**, 1289.
5 J. H. Perrin and M. Wilsey, *J. Chem. Soc., Chem. Commun.*, 1971, 769.
6 M. Reisinger and D. A. Lightner, *J. Inclusion Phenomena*, 1985, **3**, 479.
7 A. R. Campanelli, S. Candeloro De Sanctis, E. Chiessi, M. P. Alagni and E. Giglio, *J. Phys. Chem.*, 1989, **93**, 1536.
8 K. Kano, N. Tsujino and M. Kim, *J. Chem. Soc., Perkin Trans 2*, 1992, 1747.
9 K. L. Puranam, S. Raghothama and P. Balaram, *Biochim. Biophys. Acta*, 1987, **922**, 67.
10 M. C. Carey and A. P. Koretsky, *Biochem. J.*, 1979, **179**, 675.
11 A. F. McDonagh, in *The Porphyrins*, ed. D. Dolphin, Academic Press, New York, 1979, vol. VI, p. 452.
12 D. A. Lightner, J.-S. Ma, T. C. Adams, R. W. Franklin and G. L. Landen, *J. Heterocycl. Chem.*, 1983, **21**, 139.
13 H. A. Benesi and J. Hildebrand, *J. Am. Chem. Soc.*, 1949, **71**, 2703.
14 M. C. Carey and D. M. Small, *Arch. Intern. Med.*, 1972, **130**, 506.
15 A. A. Lamola, J. Eisinger, W. L. Blumberg, S. C. Patel and J. Flores, *Anal. Biochem.*, 1979, **100**, 25.
16 (*a*) A. A. Lamola, in *Optical Properties and Structure of Tetrapyrroles*, de Gruyter, Berlin, 1985, p. 311; (*b*) H. Falk, *The Chemistry of Linear Oligopyrroles and Bile Pigments*, Springer Verlag, Wien, New York, 1989, p. 454, and references therein.
17 S. M. Meyerhoffer and L. B. McGown, *Langmuir*, 1990, **6**, 187.
18 G. Puzicha, Y.M. Pu and D. A. Lightner, *J. Am. Chem. Soc.*, 1991, **113**, 3583.
19 D. A. Lightner, J. K. Gawronski and W. M. D. Wijekoon, *J. Am. Chem. Soc.*, 1987, **109**, 6354.
20 (*a*) J.-S. Hahm, J. D. Ostrow, P. Mukerjee and L. Celic, *J. Lipid Res.*, 1992, **33**, 1123; (*b*) A. D. Harman, R. G. Kibbey, M. A. Sablik, Y. Fintschenko, W. E. Kurtin and M. M. Bushey, *J. Chromatogr.*, 1993, in the press.
21 Y.-M. Pu and D. A. Lightner, *Tetrahedron*, 1991, **47**, 6163.
22 W. E. Kurtin and R. Heo, unpublished results.

Paper 3/03119E
Received 2nd *June* 1993
Accepted 12th *August* 1993