Effects of pH on the absorption, emission and light scattering spectroscopy of bilirubin and xanthobilirubic acid in sodium taurocholate solution

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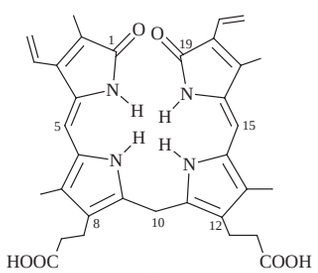
Electronic absorption, differential absorption and emission spectra, as well as light scattering intensities, have been obtained for solutions of the bile pigment bilirubin in the presence of the bile salt sodium taurocholate over the pH range 5.0–8.0. Similar measurements were performed on the dipyrrinone analog compound xanthobilirubic acid. The results are interpreted in terms of hydrophobic solubilization of monomeric bilirubin at alkaline pH, and formation of hydrophobic mixed aggregates of pigment and bile salt at lower pH, with the size of the bilirubin aggregates increasing as the pH decreases. The data are consistent with a model of co-micellization of pigment and bile salt, and also suggest that the chemistry of these complexes is determined mainly by the structural properties of the tetrapyrrole.

Bilirubin IXα (**1**), the end product of heme degradation in



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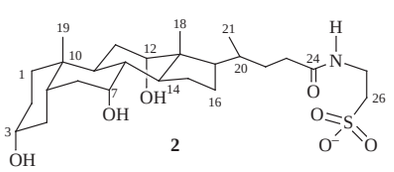
humans, is a cytotoxic pigment which is responsible for jaundice in a variety of clinical disease states.[1] In normal individuals, bilirubin is first converted to more soluble mono- and diester derivatives prior to excretion in the biliary tract. However, the free dicarboxylic acid, commonly referred to as unconjugated bilirubin, is often involved in pathological conditions associated with this substance. Bilirubin is usually found as one of the components of gallstones. Pigment gallstones contain various calcium salts of unconjugated bilirubin and/or bilirubin polymers.[2] Because of the wide occurrence of bilirubin in gallstones, it is generally assumed that factors which affect the physical chemistry of the pigment in bile are important in the pathogenesis of gallstone disease.[3]

In bile, **1** is solubilized by interaction with various amphiphilic lipid species, primarily the bile salts. The latter are present in bile as glycine or taurine esters such as sodium taurocholate (NaTC, **2**). The interaction of **1** with a variety of bile salts has



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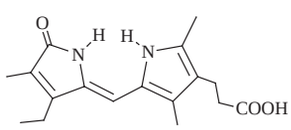
been investigated *in vitro* by several different methods, and the results of these prior investigations have been extensively reviewed.[4] However, a comprehensive model for the solubilization of **1** in aqueous bile salt solutions still does not exist. The influence of pH, temperature and ionic strength on the properties of **1** in such solutions has not been extensively studied. We have previously studied the interaction of **1** and a model compound xanthobilirubic acid (XBR, **3**) with taurocholate at pH



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8.0 using visible absorption and emission spectroscopy.[5] We have now extended these studies to cover a wider pH range, and report here our results and their implications for the solubilization of bilirubin by taurocholate, and the nature of the complexes which are formed.

Experimental

Chemicals and solution preparation

Bilirubin IXα (Sigma) was purified by recrystallization from chloroform–methanol.[6] Xanthobilirubic acid was prepared by hydrolysis of the corresponding methyl ester.[7] The latter was a generous gift from Dr David Lightner of the University of Nevada, Reno, Nevada. Sodium taurocholate was obtained from Calbiochem (Ultrol grade) or Sigma, and was used without purification. All other chemicals were reagent grade (Fisher). Phosphate–borate buffers (0.1 mol dm^{-3}), ranging in pH from 5.0 to 9.0, were used except where noted. Buffers were usually degassed immediately prior to use by flushing with nitrogen. Stock solutions of pigment were prepared by predissolution of a weighed amount in a minimum volume of dimethyl sulfoxide (DMSO, typically 20–50 mm^3) followed by dilution with buffer or taurocholate solution (total volume 10–25 cm^3). Sodium taurocholate was dissolved directly in buffer. Stock solutions and samples prepared as described above were used immediately, and experiments were generally completed within one hour of sample preparation. Experiments were conducted in dim red light, and at room temperature.

Spectral measurements

UV–VIS absorption spectra were obtained on a Cary 2315 or a Hitachi U-2000 spectrophotometer. Difference spectra were

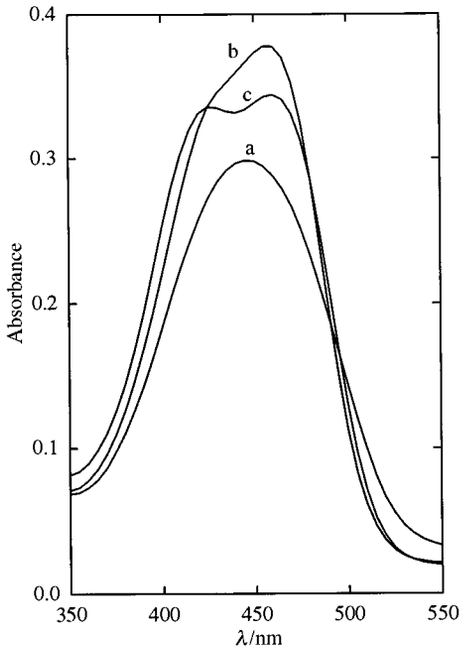


Fig. 1 Visible absorption spectra of bilirubin (**1**) in phosphate–borate-buffered NaTC solution at pH (a) 5.0, (b) 6.5 and (c) 8.0. The concentration of **1** was 6.5 μmol dm^{-3}, and the NaTC concentration was 20 mmol dm^{-3} in each case.

obtained on the same instruments by using as the reference a solution of the pigment at the same concentration as that of the sample, along with a low concentration of NaTC (0.1–1.0 mmol dm^{-3}) which was added in order to stabilize the sample. Repetitive scans showed that such reference solutions were stable during the measurement period. All fluorescence and light scattering measurements were performed on a Photon Technology LS-100 instrument. Fluorescence spectra were automatically corrected for lamp and detector wavelength response. In order to account for light scattering due to aggregation of taurocholate, spectra run on blank samples containing identical concentrations of taurocholate but no pigment were subtracted from sample fluorescence spectra. Light scattering intensity measurements were performed using a similar instrument configuration to that for fluorescence, except that excitation and emission monochromators were both set at 600 nm.

Molecular modeling

Molecular modeling software (Sybyl Version 6.1, Tripos Associates) was used on a Silicon Graphics Personal Iris workstation to determine the most stable interaction energy formed by association of a NaTC monomer and a monomer of **1** or **3**. The Tripos force field was used, and charges were calculated by the Gastgeiger method. Solvent molecules were not included. Coordinates for modeling of the monomer of NaTC were obtained from Campanelli *et al*.,[8] and those for bilirubin from Bonnett *et al.*[9] Coordinates for the dipyrrinone, xanthobilirubic acid, were based on those for the similar unit in bilirubin.

Results

Typical visible absorption spectra of equimolar concentrations of **1** at pH 5, 6.5 and 8 in 20 mmol dm^{-3} NaTC solution are shown in Fig. 1. These spectra were reproducible and stable if the samples were analyzed within 1 h of preparation. At pH 5, however, the shape and intensity of the spectrum showed some variability upon prolonged standing.

The potential effect of ionic strength on the absorption spectra was examined by addition of sodium chloride to equalize the ionic strength for all solutions, and this had no significant effect on the results, up to a total ionic strength of 0.2, and over a bilirubin concentration range of 1–30 μmol dm^{-3}. The nature of the buffering species was varied for each pH region examined, and the absorption spectra were found to be essentially the same at lower pH values (< 7) for all buffer species, but the shape of the curve was affected slightly at higher pH (results not shown).

The difference spectra for **1** in NaTC solution as a function of the concentration of the bile salt are shown at pH 5, 6.5 and 8 in Fig. 2A, 2B and 2C, respectively. At the lower pH values, a positive difference band with a maximum at about 455 nm is observed, but this band is somewhat more asymmetric at pH 6.5 than at pH 5. In addition, the minima of the negative difference bands occur at slightly different wavelengths. An isosbestic point occurs in both cases between the positive and negative bands. In contrast, the positive difference band at pH 8 occurs at 474 nm, and a much smaller negative difference band occurs on the short wavelength side of the positive band. No isosbestic point was observed at pH 8. In separate experiments, the concentration of NaTC was increased to as high as 100 mmol dm^{-3}, resulting in further small increases in difference band intensity at each pH, but no changes in band position or shape.

Figs. 3 and 4 show the effect of 3 mol dm^{-3} urea on the difference spectra of **1** in 20 mmol dm^{-3} NaTC solution at pH 6.5 and 8, respectively. In both cases there is a decrease in the intensity of the positive and negative difference bands at longer wavelength, in favor of an increased contribution from the shorter wavelength positive difference band (approx. 420 nm). At pH 6.5, this latter band becomes stronger than the longer wavelength band. At pH 5, the changes produced by added urea were not reproducible, but generally resulted in a decrease in intensity of the main difference band.

The effect of pH on the corrected fluorescence emission band of **1** in 20 mmol dm^{-3} NaTC is shown in Fig. 5. The emission maximum shifts from approximately 540 nm at pH 8 to about 530 nm at pH 5. The intensity of the band increases also, with the integrated intensity at pH 5 being approximately four times that at pH 8. The maxima of the emission bands at pH < 7 were found to vary with excitation wavelength, and the excitation spectra did not closely resemble the absorption spectra.

The relative light scattering intensity of solutions of **1** and NaTC is shown as a function of pH in Fig. 6. The scattering intensity remains low and relatively constant between pH 9 and 7, and then increases gradually by a factor of almost 10 between pH 7 and 5. This change in light scattering intensity was not observed in solutions of NaTC with no bilirubin present. Based on the hypothesis that the observed changes in light scattering are directly connected to changes in the state of ionization of **1**, theoretical titration curves were calculated and compared with the observed light scattering–pH profile. The dashed line in Fig. 6 represents the relative concentration of the fully protonated form of a diprotic acid with $pK_{a1} = 5.4$ and $pK_{a2} = 6.0$.

At a given pH, the light scattering intensity depended on the bilirubin and bile salt concentrations, and typical results are shown in Figs. 7 and 8. Below a 'critical' bilirubin concentration, the intensity stayed relatively constant, and then increased sharply at higher concentrations. When the NaTC concentration was varied at a constant concentration of **1**, the scattering was low and relatively constant above 20 mmol dm^{-3} bile salt, and then increased gradually by a factor of more than 10 at 1 mmol dm^{-3}. A similar dependence on pigment and bile salt concentrations was observed at all pH values, but the 'critical' concentration of the pigment varied with the bile salt concentration, as expected. Changes in light scattering intensity of the magnitude shown in Fig. 8 were not observed in the absence of bilirubin.

The effects of pH on the differential absorption spectra of XBR (**3**) in NaTC solution were more subtle, and the spectra at pH 4, 5 and 6.5, and at 20 mmol dm^{-3} are shown in Fig. 9.

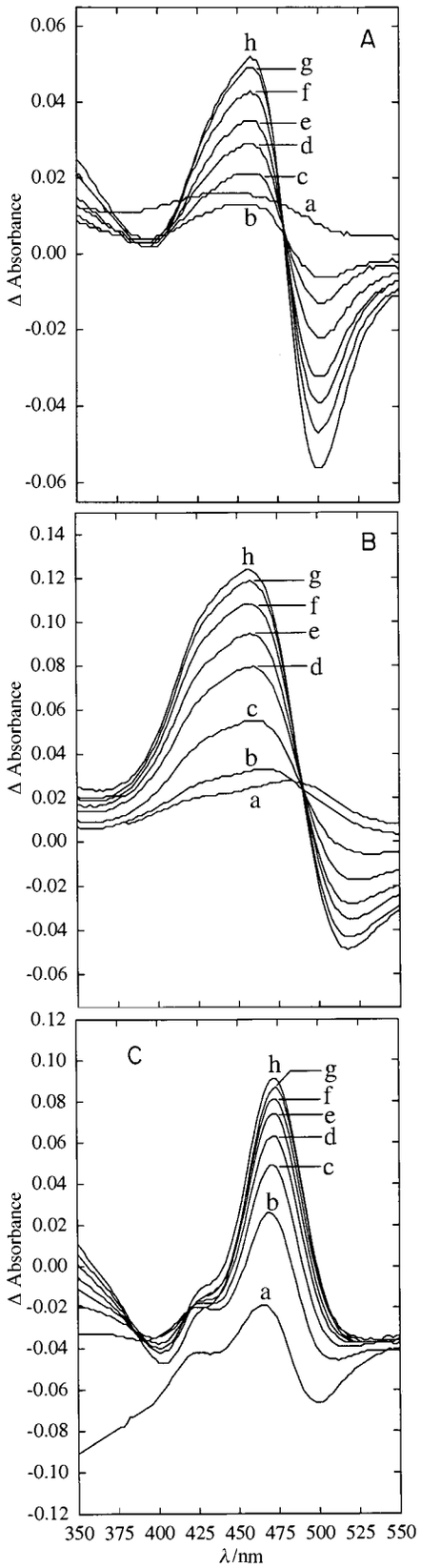


Fig. 2 Differential absorption spectra of **1** as a function of NaTC concentration at (A) pH 5.0, (B) pH 6.5 and (C) pH 8.0. The concentration of **1** was constant at approximately 8 μmol dm^{-3} for each experiment. The concentration of NaTC was 0.5 mmol dm^{-3} in the reference solution, and spectra (a) through (h) correspond to 5, 10, 15, 20, 25, 30, 35 and 40 mmol dm^{-3} NaTC, respectively, in the sample solution.

A positive difference band with a maximum near 430 nm is observed. The spectra at all pH values are similar, and resemble the shape of the difference spectrum of **1** at pH 8. In all cases,

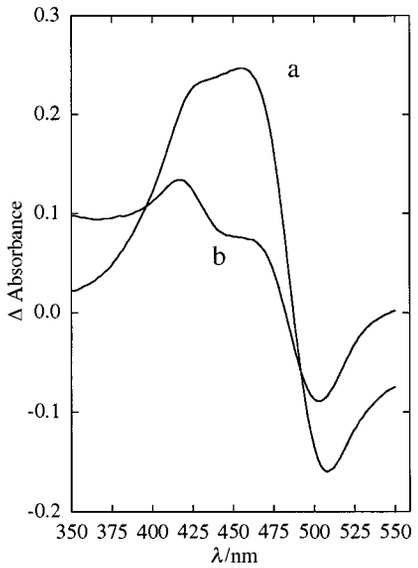


Fig. 3 Differential absorption spectra of **1** in NaTC solution at pH 6.5, (a) without and (b) with added urea to a concentration of 3.0 mol dm^{-3}. The sample conditions were approximately the same as shown in Fig. 2B, with the NaTC concentration in the sample at 20 mmol dm^{-3}.

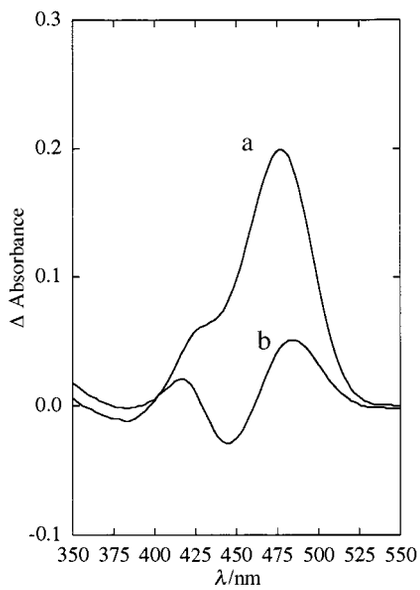


Fig. 4 Differential absorption spectra of **1** in NaTC solution at pH 8.0, (a) without and (b) with added urea to a concentration of 3.0 mol dm^{-3}. The sample conditions were approximately the same as shown in Fig. 2C, with the NaTC concentration in the sample at 20 mmol dm^{-3}.

there was very little effect observed below a bile salt concentration of 10 mmol dm^{-3}. As the NaTC concentration increased above this value, there was a gradual increase in the intensity of the band, but no shift of the maximum.

Solutions of **3** exhibited no dependence of light scattering intensity on pH in the range 4–8, and only small increases with NaTC concentration above 10 mmol dm^{-3} (results not shown).

Discussion

The results of this study suggest that bilirubin and NaTC form molecular complexes over the pH range 5–8, but that the conformation and/or state of aggregation of the solubilized pigment changes significantly with pH. The complexation appears to involve hydrophobic interactions with bile salt molecules to form mixed aggregates, depending on pH, bile salt concentration and pigment concentration. The observed increases in light scattering of solutions of **1** are interpreted to be due

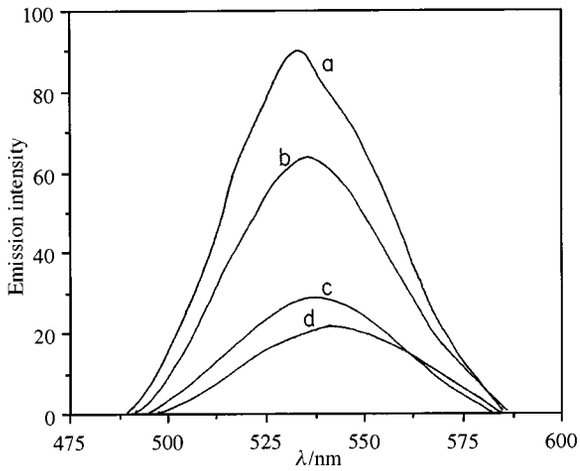


Fig. 5 Fluorescence emission spectra of **1** in 20 mmol dm^{-3} NaTC at (a) pH 5.0, (b) pH 6.0, (c) pH 7.0 and (d) pH 8.0. The concentration of **1** was 5 μmol dm^{-3}. The excitation wavelength was 440 nm.

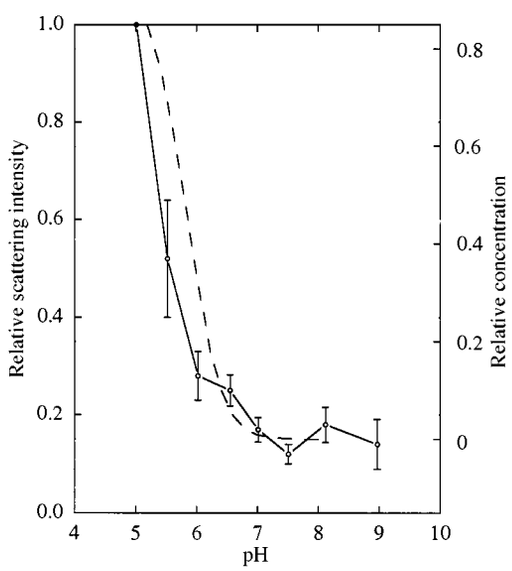


Fig. 6 (——) Light scattering intensity at 600 nm for solutions of **1** (10 μmol dm^{-3}) in NaTC (20 mmol dm^{-3}) solution as a function of pH. The light scattering intensities have been normalized to the value obtained at pH 5. (– – –) Calculated relative concentration of the fully protonated form of a diprotic acid with $pK_{a1} = 5.4$ and $pK_{a2} = 6.0$.

to aggregation of the pigment, since these effects were not observed if **1** was not present, and since others have shown that bile salt aggregation is not affected by pH in this range.[10] The pH dependence of the light scattering suggests that multimers of increasing size are formed as the pH decreases below 7, which is most likely associated with charge neutralization of the propionic acid groups. At pH 5 these aggregates apparently become large enough to make effective solubilization by NaTC more difficult, and the solution is metastable. The results support the idea that the solubilization mechanism does not involve entrapment of bilirubin molecules in pre-formed bile salt micelles, but rather hydrophobic stabilization of a polydisperse array of bile salt and pigment molecules in a type of co-micellization, an idea which has been advanced by others.[4]

Our results at pH 8 are similar to those reported by other groups at higher pH.[11,12] Only weak interactions are observed at NaTC concentrations < 10 mmol dm^{-3}. Above this concentration, the appearance of a positive difference band, the intensity of which increases uniformly as the bile salt concentration increases, indicates the formation of a complex between a unique conformation of the dianion of **1** and taurocholate molecules. First, our data suggest that, at alkaline pH, the pigment

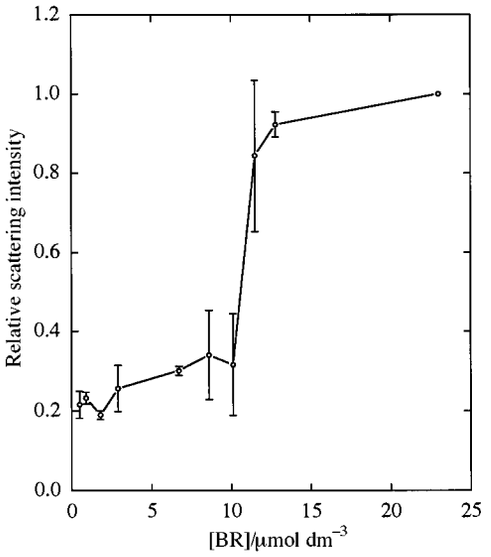


Fig. 7 Light scattering intensity at 600 nm for solutions of **1** (BR) in NaTC (20 mmol dm^{-3}) solution at pH 7. The light scattering intensities have been normalized to the value obtained at the highest concentration of **1**.

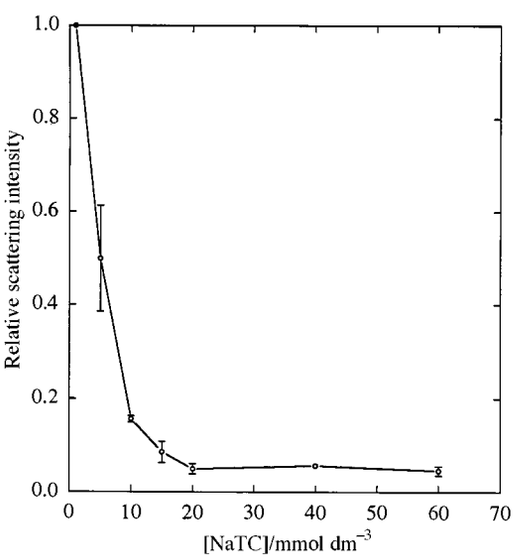
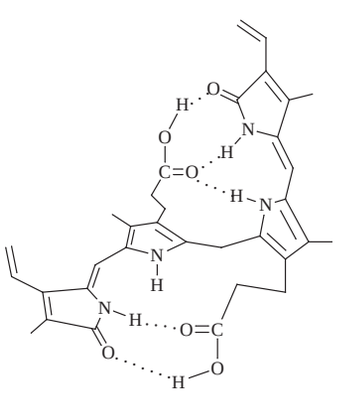


Fig. 8 Light scattering intensity at 600 nm for solutions of **1** (10 μmol dm^{-3}) in NaTC solution at pH 7. The light scattering intensities have been normalized to the value obtained at [NaTC] = 1 mmol dm^{-3}.



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exists in a conformation similar to that of the well-documented ridge-tile conformer (**4**). The long wavelength band in bilirubin has been shown to arise from exciton splitting involving the two dipyrrinone chromophores in the two halves of the tetrapyrrole.[13,14] The dianion of **1** has been shown to prefer a ridge-

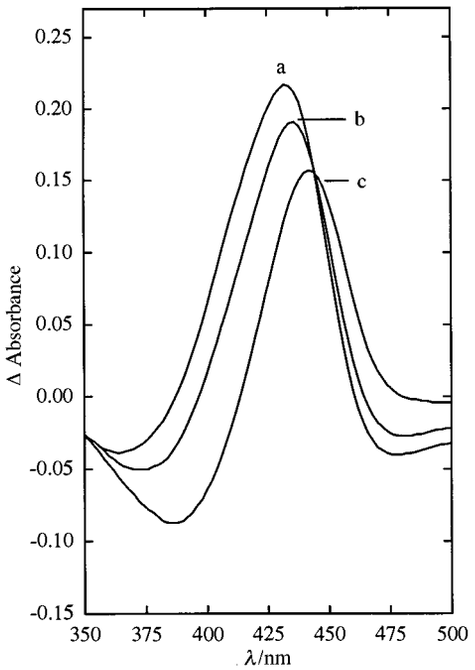
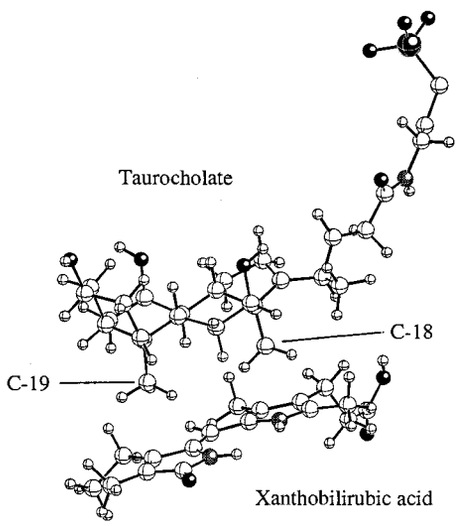


Fig. 9 Differential absorption spectra of **3** in 20 mmol dm^{-3} NaTC solution at (a) pH 4.0, (b) pH 5.0 and (c) pH 6.5. The concentration of **3** was constant at 7 μmol dm^{-3}. The concentration of NaTC was 0.5 mmol dm^{-3} in the reference solution.

tile type conformation in solution.[15] The resemblance of the spectra for **1** in alkaline NaTC solutions to those for **1** in serum albumin solutions, and to that reported for **1** in sodium deoxycholate solution at alkaline pH, where the ridge-tile conformer is preferred, suggests that the conformation of the pigment is similar in these cases.[16,17] Thus, we ascribe the long wavelength difference band at pH 8 to a taurocholate-solubilized dianion of **1** in a ridge-tile conformation. Furthermore, we suggest that **1** exists as a monomer in this complex, in agreement with the interpretation of Carey and Koretsky.[11] Bilirubin dianion is known to form dimers in aqueous solution, at concentrations above 10 μmol dm^{-3}.[18] Carey and Koretsky observed a hypsochromic shift of the long wavelength maximum of **1** at concentrations > 10 μmol dm^{-3} at pH 10.0, and suggested this was due to aggregation of the pigment.[11] Thus, in solutions of NaTC, the shorter wavelength feature in the absorption and differential absorption spectra at pH 8 may indicate the presence of dimers or higher order multimers. This feature decreases in intensity as bile salt concentration increases. At higher concentrations of **1**, this feature becomes more prominent (results not shown). We suggest that, as the bile salt concentration increases, the hydrophilic–hydrophobic balance of the solution shifts so as to favor bilirubin–bile salt association over dimerization or mutimerization of the tetrapyrrole. However, since no isosbestic point is observed in the difference spectra at pH 8, the solution may comprise bilirubin monomers and multimers in several different microenvironments. At higher bile salt concentrations, the solubilized monomer form of the pigment appears to be the predominant species in solution. We also conclude that the complex between **1** and taurocholate is stabilized by hydrophobic interactions, as suggested by others.[11] Evidence to support this idea is found in the effect of the chaotropic agent, urea, on the difference spectra. A high concentration of urea caused a decrease in the band associated with the solubilized monomer, and a concomitant increase in the shorter wavelength band, which is probably associated with dimers/multimers. Kano *et al.*, in a study of bile acid-induced circular dichroism of **1** in protic solvents, concluded that the enantioselection was due to hydrogen-bonded interaction of the carboxy groups of **1** with hydroxy groups on the polar face of the steroid molecules.[19] However, we found that the difference spectra for solutions of **1** and **3** in NaTC at high pH were very similar. From this, we conclude that the modes of interaction for these two pigments with the bile salt are similar. Assuming that **1** retains the folded ridge-tile conformation, it is difficult to envision a model in which hydrogen bonding plays a primary role, and yields similar results for the dipyrrole and tetrapyrrole.

A hydrophobic interaction between **1** and taurocholate would probably involve association of the non-polar face of the steroid with the dipyrrinone units of the tetrapyrrole. Molecular modeling of this interaction with xanthobilirubic acid yielded the structure **5**. The bile salt molecule is oriented so



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that its steroid skeleton is roughly parallel to the plane of the dipyrrinone unit, and the C-19 methyl group of the steroid is nestled near the center of the lactam ring of the dipyrrinone, while the C-18 methyl is over the pyrrole ring. Complete solubilization of this molecule by taurocholate would probably involve association of a second molecule of the bile salt on the other face of the pigment in a similar manner to form a sandwich-like complex. Modeling of this type of interaction with **1** in a ridge-tile conformation produced a very similar result for the orientation of the bile salt with respect to the outer faces of the dipyrrinone units. This model is consistent with the NMR results of Puranam *et al.*, who observed NOE enhancements indicative of interactions between peripheral olefinic protons on bilirubin and the C-18 and C-19 methyl groups on cholic acid, the parent steroid of taurocholic acid, in alkaline aqueous solution.[20]

It is now generally accepted that taurocholate forms aggregates of several different sizes at concentrations above 8–10 mmol dm^{-3},[21] and McGown has proposed a model that invokes stepwise aggregation of this bile salt in hydrophobic solubilization of planar polycyclic aromatic compounds.[22] Our results for **3** are explained very well by this model, with the pigment sandwiched between two taurocholate molecules. Extrapolation of this model to solubilization of a bilirubin ridge-tile conformer is more complicated, and more information on the size of the mixed aggregates is necessary.

The spectral results for **1** in NaTC solution below pH 7 indicate that the form of the pigment solubilized is quite different from that at alkaline pH. Prior work in other laboratories has shown that dimerization of bilirubin occurs in aqueous media without bile salts, and leads to a decrease in the intensity of the main visible absorption band, and introduction of a shoulder at longer wavelengths (500–520 nm).[23] Our difference spectral data at pH < 7, showing loss of absorbance in this region, can be interpreted as the result of solubilization of dimers and possibly higher order aggregates of the pigment by interaction with

the bile salt. The effect of added urea again suggests that the interaction is hydrophobic in nature, in agreement with Carey and Spivak.[24] The isosbestic points in the difference spectra suggest that a single form of solubilized pigment is being produced, which could be the dimer. Protonation of the propionic acid groups in **1** should enhance the possibility of aggregation of the pigment. We suggest that in solutions of **1** and taurocholate below pH 7, the pigment exists as polydisperse aggregates, with preferential solubilization of one or a limited set of these aggregates (dimers) due to preferred packing arrangements with bile salt molecules. The most likely arrangement of bilirubin molecules in such aggregates would be similar to that found in the solid state.[9] Puranam and Balaram, using gel permeation chromatography, reported formation of a higher molecular weight mixed aggregate of **1** and cholic acid at pH 6.6 compared to pH 10.8.[25]

The fluorescence results support the conclusions from the absorbance and light scattering results. It has been shown by others that the most efficient process in the excited state photophysics of **1** in a variety of media is $Z \rightarrow E$ photoisomerization in the exocyclic double bonds, resulting in a low fluorescence yield.[26,27] Binding of **1** to taurocholate does not result in a large increase in fluorescence at any pH, and thus the complexes do not greatly affect the deexcitation pathways described above. However, it appears that, at lower pH, **1** is complexed in a way that restricts its molecular motions to a greater extent than at alkaline pH. This would be consistent with the idea of stacked multimers of **1** at lower pH *versus* solubilized monomers at alkaline pH.

The observed changes in spectral properties of **1** with pH appear to be associated with changes in the state of ionization of the propionic acid substituents. In the ridge-tile conformation, the two carboxy groups are separated by a distance of approximately 8 Å, and the molecule can be expected to exhibit the acid–base behavior of a simple dicarboxylic acid of appropriate dimensions. Lightner *et al*. have shown that the pK_a values for **1** are 4.2 and 4.9 in aqueous solution containing a small amount of DMSO, showing that the pigment behaves as a typical dicarboxylic acid in that solvent.[28] Others have shown that the apparent pK_a values of protonic acids are increased upon binding to neutral or anionic micelles.[29] Our previous results using capillary electrophoresis showed that the mobility of **1** in NaTC solution began to decrease dramatically between pH 7.0–7.5.[30] In addition, D'Alagni *et al*. reported the effects of pH (in the range 7–9) on the circular dichroic (CD) spectra of **1** in sodium taurodeoxycholate solution,[31] and they observed a large increase in CD intensity below pH 8. All these results suggest that **1** behaves as a somewhat weaker acid in bile salt solution. The pH dependence of the light scattering studies is consistent with the ionization of a dicarboxylic acid with pK_a's of approximately 5–6. This estimate is very similar to the results from capillary electrophoresis experiments, which predicted values of 6–6.4.

The implications of these results are important for the understanding of the chemistry of bilirubin in bile. The pH of bile varies from 6.5–7.8. Our results imply that bilirubin will exist mainly in its anionic forms in bile fluid, but that acidification of the bile to a pH less than 7 would increase the concentration of protonated forms with more limited solubility. Taurocholate appears to be able to solubilize some multimeric forms of bilirubin. However, overall, the effects of pH on the biophysical chemistry of bilirubin in taurocholate solution appear to be similar to the effects seen in aqueous solution of the tetrapyrrole without bile salt, and are determined mainly by the preferred conformation of the tetrapyrrole in solution.

Acknowledgements

This work was supported by grants from the National Science Foundation (CHE-9300130, CHE-9531357) and from the Camille and Henry Dreyfus Foundation.

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Paper 7/08487K
Received 24*th November* 1997
Accepted 20*th April* 1998