Acid Dissociation Constants of Bilirubin and Related Carboxylic Acid Compounds in Bile Salt Solutions

William E. Kurtin†, Jason Enz†, Celeste Durnmoor†, Neil Evans†, David A. Lightner*

†Department of Chemistry, Trinity University, San Antonio, TX, 78212 and the *Department of Chemistry and Biochemistry, University of Nevada, Reno, NV 89557-0020

Corresponding author:

William E. Kurtin
Department of Chemistry
Trinity University
715 Stadium Drive
San Antonio, TX 78212
Telephone: (210) 999-7382
Email: wkurtin@trinity.edu
Fax: (210) 999-7569

(Running Title: pKₐ of Bilirubin in Bile Salt Solution)
Summary

Bilirubin, the yellow-orange tetapyrrole pigment of jaundice, is essentially insoluble in pure water, but is much more soluble in solutions of bile salts such as sodium taurocholate. The biophysical chemistry of bilirubin in bile salt solutions is affected by changes in the pH of the solution in the range 5-9, suggesting that interactions with bile salt molecules and micelles may alter the acidity of the pigment. We have examined this possibility by determining the apparent pK\textsubscript{a} values for a series of carboxyl $^{13}$C-enriched model compounds, including the bilirubin analog mesobilirubin XIII\textsubscript{α}, in solutions of sodium taurocholate and sodium taurodeoxycholate. Apparent pK\textsubscript{a} values were determined by $^{13}$C NMR titrations in dimethyl sulfoxide-water mixtures. The results show that the acidity of all compounds is decreased, or pK\textsubscript{a} increased, in micellar bile salt solution relative to pure water, and that the effect is greatest for the larger, less water-soluble compounds. We have proposed a model to explain these results, and discussed the implications of these findings for the biophysical chemistry of bilirubin in bile.
Introduction

Bilirubin is the yellow-orange tetrapyrrole pigment that is formed in humans primarily by the oxidative degradation of the heme from senescent red blood cells (1-2). It is best known because of its role in various hepatobiliary diseases, especially kernicterus and gallstone disease. More recently, it has been found to have a beneficial antioxidant function (3).

Like heme, bilirubin has two propionic acid substituents, and the state of ionization of these functional groups can be assumed to play a crucial role in determining the conformation, solubility and transport of the molecule in the various tissues of the biliary tract. The apparent acid dissociation constants of these groups are difficult to obtain because of the very low water solubility of bilirubin. Nevertheless, numerous investigators, using a variety of methods, have reported apparent pKₐ values for the propionic acid groups, and the reported values have ranged between 4.4 and 9.3 (4-9). Recently, a series of mono-, di-, and tetrapyrrole model compounds containing carboxyl substituents with 99% $^{13}$C-enrichment were synthesized, and the apparent pKₐ values determined by $^{13}$C NMR in aqueous solutions containing various amounts of dimethylsulfoxide (10). Furthermore, the validity of this method was proven in a similar study that examined the effect of dimethylsulfoxide on the apparent pKₐs of a series of slightly soluble carboxylic acids (11). The conclusions from these studies are that the acidities of the propionic acid substituents of bilirubin are similar to those of most aliphatic carboxylic acids in aqueous solution, and the estimated pKₐ values in water are ~ 5.

In bile, bilirubin is solubilized mainly by interaction with the steroidal surfactants known as bile salts. Various spectroscopic and chromatographic properties of bilirubin in bile salt solutions have been reported to vary with pH (12-17). Furthermore, the pH dependence of these
observations suggests that the acidity of the propionic acid substituents may be affected as a result of the interaction with bile salts. To investigate this possibility, we have determined the pKₐs of a series of carboxyl $^{13}$C-enriched model compounds in bile salt solution using NMR spectroscopy. Specifically, we have determined the apparent pKₐ values for benzoic acid-carboxy – $^{13}$C (1), [1- $^{13}$C]phenylacetic acid (2), a carboxy-$^{13}$C butanoic acid-substituted monopyrrole (3), the dipyrrinone [8$^{3}$-$^{13}$C]xanthobilirubin acid (4), and the bilirubin analog [8$^{3}$, 12$^{3}$-$^{13}$C$_{2}$]mesobilirubin XIIIα (5) (see Figure 1) in solutions of the bile salts sodium taurocholate (TC)$^{1}$ and sodium taurodeoxycholate (TDC) in aqueous solutions containing 10-20%(v/v) dimethylsulfoxide (DMSO).
Materials and Methods

99% carboxyl $^{13}$C-enriched benzoic acid (1) and phenylacetic acid (2) were purchased from Sigma-Aldrich Chemical Co. and used without further purification.

The compound 3,5-dimethyl-2-methoxycarbonyl-1H-pyrrole-4-[4-$^{13}$C]-butanoic acid (3) was synthesized from 3,5-dimethyl-2-ethoxycarbonyl-1H-pyrrole-4-[4-$^{13}$C]-butyronitrile using a procedure based on that reported previously (18). Thus, 912 mg of the starting nitrile were added to a 100 mL r.b. flask, to which 15 mL of ethyl alcohol, 15 mL of water, and 3.64 g of KOH were added. Then the mixture was refluxed for 40 hr. Excess solvent was removed by rotary evaporation, and the remaining solution cooled in an ice bath, producing a tan solid. The solid was taken up in 12 mL of 50%(w/v) NaNO$_3$. The suspension was cooled in a dry ice - acetone bath. 4.5 mL of cold conc. HNO$_3$ were added dropwise so that the temperature did not exceed -10°C during addition. The resulting solid was filtered, and washed with ice cold water, affording the diacid as a lavender solid, which was dried in vacuo. The diacid was taken up in 10 mL of methanol, cooled in an ice bath, and 40 mL of ethereal diazomethane were added, and stirred for 5 min. The solvent was then removed by rotary evaporation. The residue was taken up in 10 mL of CHCl$_3$, and the dimethyl ester was isolated by radial chromatography, using hexane:CH$_2$Cl$_2$ (1.2:1). Then 405 mg of the diester were placed in a 25 mL r.b. flask, to which 8 mL of methanol and 2 mL of 1M NaOH were added, and the solution was refluxed for 18 hr. The excess methanol was removed by rotary evaporation. The resulting orange-brown solution was diluted with 8 mL water, cooled in an ice bath, and 2 mL of 1.1M HCl were added. The resulting pink precipitate was filtered, washed with ice cold water, and dried in vacuo. The desired product (348 mg) was obtained in an overall yield for the four-step synthesis of approximately 40%. The
compound appeared to be pure by proton NMR, and showed only a single peak in the COOH region in the C-13 NMR.

\[ {\text{[}}{\text{8}}^{3}{\text{,13}}^{13}{\text{C}}^{\text{]}Xanthobilirubin acid (4) was synthesized using a modification of a published procedure (19). Thus, 102 mg (0.32 mmol) of }{\text{[}}{\text{8}}^{3}{\text{,13}}^{13}{\text{C}}^{\text{-xanthobilirubin acid methyl ester were placed in a 25 mL round bottom flask equipped with a stirring bar and reflux condenser. Eight mL of CH}}_{3}{\text{OH and 2 mL of 10% (w/v) NaOH were added to the flask, and the mixture was heated at reflux for 5 hours. The resulting dark orange solution was cooled to room temperature, and the CH}}_{3}{\text{OH removed by rotary evaporation to yield a yellow-orange paste. Ten mL of 10% (w/v) HCl were cooled in an ice bath while flushing with nitrogen gas. The HCl was then added to the rotovapped saponification product in an ice bath, and the suspension was stirred for 20 minutes under nitrogen flushing. The suspension was filtered, and the solid was washed with ice cold water. The solid was dried in vacuo, yielding 77.8 mg (0.26 mmol) of the yellow dipyrrinone (80% yield). The melting point was 270-275°C (dec), and the compound appeared to be pure by proton NMR. The compound yielded only a single line in the COOH region upon C-13 NMR.}\]

\[ {\text{[}}{\text{8}}^{3}{\text{,12}}^{3}{\text{-13}}^{13}{\text{C}_{2}}^{\text{]}Mesobilirubin-XIIIa (5) was synthesized as described previously (18).}\]

Sodium taurocholate and sodium taurodeoxycholate were purchased from Sigma-Aldrich Co., and were used without further purification.

Carboxylic acid \( K_a \) values were determined by acid-base titration in DMSO-H\( _2 \)O solutions (10-20% by volume DMSO). Typically, 2-15 mg of the target acid were dissolved in 1-3 mL of DMSO. To this solution was added a volume of H\( _2 \)O or an aqueous solution of bile salt at an appropriate concentration. The pH of the resulting solution was then measured using an Orion Model 810 or 920 pH meter equipped with a glass combination electrode and a
temperature compensation probe. The pH of the solution was then adjusted to 2.5-3 using dilute HCl in DMSO-H2O, and a sample taken for NMR purposes. The sample was then titrated using dilute NaOH in DMSO-H2O, and an aliquot was taken for NMR purposes approximately every 0.5 pH unit increase. Back titrations were performed in some cases to verify the reversibility of the system. The concentrations of HCl and NaOH were such that the total volume of acid and base added during the titration was less than 5% of the starting sample volume.

It was found that 4 was not soluble enough in 10% DMSO to permit a titration without added bile salt. In a solution of 40 mM bile salt, the dipyrrinone was significantly more soluble. But even under these conditions, 4 precipitated in samples with pH<6 after the samples had been standing for 1-2 hr. Therefore, titrations of 4 were performed also at higher concentrations of DMSO in the solvent, and the data extrapolated to 10% DMSO. To obtain an approximate value for the pK\textsubscript{a} with no bile salt present, values were determined at several different bile salt concentrations, and the data extrapolated to zero bile salt.

Compound 5 was found to be only very slightly soluble in 10% DMSO, 40 mM bile salt, and very long acquisition times were necessary to obtain titration data points. For samples of pH<5, overnight scanning (>8000 transients) was required. The compound was much more soluble in 20% DMSO, and the pK\textsubscript{a} determinations were done in this solvent.

NMR measurements of the carboxyl chemical shift were obtained on either a Varian Unity Plus 500-MHz (frequency, 125.706 MHz; spectral width, 28,368.8 Hz; acquisition time, 2.000 s; pulse width, 5.0 \mu s; decouple, $^1$H; line broadening, 1.8 Hz; temperature, 25°C) or a Varian Unity Plus 400 MHz (frequency, 100.580 MHz; spectral width, 25,000.0 Hz; acquisition time, 1.000 s; pulse width, 6.0 \mu s; decouple, $^1$H; line broadening, 1.5 Hz; temperature, 25°C) spectrometer. The decoupler was continuously on for all experiments, and the number of
transients varied depending on the sample concentration. For 1 and 2 the carboxyl C-13 chemical shift was determined relative to a reference of deuterodimethylsulfoxide [(CD<sub>3</sub>)<sub>2</sub>SO], which was used in place of non-deuterated DMSO in sample preparation. For all other compounds, the chemical shift was determined relative to that for deuteracetone [(CD<sub>3</sub>)<sub>2</sub>CO], which was placed in the NMR tube in a sealed capillary insert.

The observed C-13 chemical shift of the acid was plotted versus the pH of the sample, and the data were fit using the equation

\[
y = \frac{a + b\left(\frac{K_a}{[H^+]}\right)}{1 + \left(\frac{K_a}{[H^+]}\right)}
\]

where \(a\) and \(b\) are the carboxyl chemicals shifts of the fully protonated and deprotonated forms of the compound, respectively. The curve fitting was done by non-linear regression using the program Sigmaplot (SPSS, Inc.). For the dicarboxylic acid 5 the equation above was expanded to

\[
y = \frac{a + b\left(\frac{K_{a1}}{[H^+]}\right) + c\left(\frac{K_{a1}K_{a2}}{[H^+]^2}\right)}{1 + \left(\frac{K_{a1}}{[H^+]}\right) + \left(\frac{K_{a1}K_{a2}}{[H^+]^2}\right)}
\]

where \(a\), \(b\), and \(c\) are the chemical shifts of the acid, monoanion, and dianion, respectively. It was not possible experimentally to determine the chemical shift of the monoanion. Therefore, the value for this parameter was estimated using the method reported previously (10).
Results

Benzoic acid (1), phenylacetic acid (2), and the pyrrole-butanoic acid compound (3) were completely soluble in 10% DMSO over the whole pH range used in the titrations. Sample titration curves with no added bile salt are presented in Figure 2. These titration data were fit very well by non-linear regression, with typical values of $r^2$ for the fit exceeding 0.99, and the standard error in the estimated value of the dissociation constant being less than 2%. The mean values of the pK$_a$ determined for 1, 2, and 3 in this manner were 4.34±0.04, 4.42±0.02, and 4.99±0.05, respectively.

To examine the effect of the DMSO co-solvent on the dissociation constant, and to get an estimate of the pK$_a$ values in 100% water, additional titrations were performed with varying amounts of DMSO up to 20%(v/v). The results for 1, 2, and 3 are shown in Figure 3. The general effect of the increasing concentration of DMSO was to move the chemical shift of both the acid and conjugate base forms of these compounds upfield, with the difference in chemical shift in 10% and 20% DMSO being 0.2-0.3 ppm. Thus, there was an overall shift of the titration curves downward in the vertical direction, and to the right in the horizontal direction, resulting in an increase in the apparent pK$_a$. It was found that the pK$_a$ was linearly proportional to the vol% DMSO over this small range of percentages (Fig. 3). The extrapolated values of the pK$_a$s in water (0% DMSO) were 4.14 (1), 4.27 (2), and 4.67 (3). The literature values of the pK$_a$s for 1 and 2 in water are 4.19, and 4.28, respectively. The pK$_a$ for 3 has not been determined previously, but the value for butanoic acid (4.83) represents a reasonable approximation. These literature values in water are in good agreement with the extrapolated values from this work. The results obtained with these compounds are quite similar to those obtained previously concerning
the effects of added DMSO (20). The one difference is that the pKₐ was found to be linearly correlated with the vol% DMSO in this work, instead of the log vol% as was found in the earlier studies. The reason for this difference has been explained recently and is associated with the difference in methodology (21).

The effect of DMSO co-solvent on the pKₐ for 1 is in very good agreement with the work of Rubino and Berryhill (22). Those authors attributed the effects of the co-solvent on the pKₐ to both electrostatic and non-electrostatic medium effects. Theory suggests that the pKₐ should be inversely proportional to the dielectric constant of the medium, and the increase in apparent pKₐ that is observed in DMSO is consistent with that idea (23). However, the pKₐ of 2 was also determined in 10%(v/v) N-methylformamide (dielectric constant 170), and was found to be 4.39, compared to 4.49 in 10% DMSO. Although the change in pKₐ is in the right direction, it might be expected that the value would be less than that for pure water, which was not the case. Thus, additional factors beyond a simple medium effect appear to be involved.

The effects of bile salts on the apparent acidities of 1, 2, and 3 were determined next, and the results for 3 in the absence and presence of 40mM taurodeoxycholate (TDC) are shown in Fig. 4. The effect of the bile salt on chemical shift was somewhat different from that of DMSO. The chemical shift of the anion was not greatly affected, while that of the acid was shifted upfield by approximately 0.6 ppm. The calculated pKₐ was higher than the value without bile salt, by about 0.30 pH units, and this difference was statistically significant. The results suggest significant interaction of fully protonated 3 with TDC, but little interaction of the anionic conjugate base with the bile salt. The interaction with the anion is presumably weaker because of charge repulsion between it and the negatively charged bile salt monomers and micelles. The reason for the upfield shift of the carboxyl resonance in bile salt solution is not clear, but might
be due to disruption of hydrogen-bonded dimers of the carboxylic acid by interaction with the bile salt. This explanation was proposed for the solvent effect on the carboxyl chemical shift of acetic acid (24). Results with 1 and 2 were very similar to those for 3, but the magnitudes of the shifts were smaller than those observed for 3. Results using the bile salt taurocholate (TC) were very similar to those using TDC.

To examine whether the observed effects were due to interactions with bile salt monomers and/or micelles, the carboxyl chemical shifts of 1, 2, and 3 were determined in a series of solutions of varying bile salt concentration in 10% DMSO/90% 0.1M acetic acid (pH=3.0), and 10% DMSO/90% phosphate (pH=8). In acid solution, the chemical shift decreased slowly up to approximately 5 mM TDC or 15 mM TC, and then decreased more rapidly as bile salt concentration was increased further, up to 40 mM. Representative results are shown in Figure 5 for 3. The results for 1 and 2 were very similar, and the data for all three compounds are consistent with micellar solubilization in these solutions. The cmc's for TC and TDC in aqueous solutions are known to be 8-10 mM and 2-4 mM, respectively (25). The effect of the DMSO co-solvent on the cmc of these bile salts has not been investigated. However, it might be expected that the cmc would be increased slightly due to the decreased polarity of the medium. As would be expected from the results shown in Figure 4, the effect of bile salt concentration on chemical shift at pH 8 was much smaller in all cases.

Typical titration data for 4 in 40mM TDC and TC solutions (20%DMSO) are presented in Figure 6. For 4, the mean pK\textsubscript{a} values were 5.87 and 5.51 in TDC and TC solutions, respectively. From the dependence of the pK\textsubscript{a} on the bile salt concentration (results not shown), an extrapolated pK\textsubscript{a} value with no bile salt was determined to be 4.93. Thus the shift in apparent pK\textsubscript{a} in the presence of TDC is almost 1 pH unit. From the dependence of the pK\textsubscript{a} of 4 on the
Vol% DMSO in the solvent (results not shown), extrapolated values in 10%(v/v) DMSO were obtained, and were found to be 5.74 and 5.27 for 40mM TDC and 40mM TC, respectively. Thus the estimated ΔpK values in 10% DMSO are significantly higher than those for 1, 2, and 3 under the same conditions. Table 1 shows the effects of bile salt on the carboxyl chemical shift of 4 at low and high pH. First, the changes in chemical shift are significantly larger than those observed for 3, indicating greater interaction of both the anionic and neutral forms of 4 with bile salt than was the case with the monopyrrole. Secondly, TDC has a greater effect than TC, but the effects are much more pronounced at low pH than at high pH.

Titration data for 5 in 40mM TDC, 20% DMSO are shown in Figure 7. Since it was not possible to determine the chemical shift of the monoanion from the titration data, this data was analyzed three different ways. Using the curve-fitting procedure, and allowing the monoanion chemical shift to take on a best-fit value, pK_{a1} and pK_{a2} values of 6.2 and 6.5 were obtained. However, the dependence on the monoanion chemical shift was very high, and errors in the estimation of pK_{a1} and pK_{a2} were between 10 and 20%. Prior work on 5 and other dicarboxylic acid compounds showed that the titration data could be fit reasonably well by using correction factors obtained from the titration of adipic acid. (10, 11) Applying that approximation to our data for 5, values of pK_{a1} and pK_{a2} were estimated to be 6.4 and 7.0. Lastly, an assumption was made that the chemical shift of the monoanion is approximately the average of those for the acid and dianion. Using this approximation, estimated pK_{a1} and pK_{a2} values were 6.3 and 6.9. The errors in all these estimated values are at least 10%. However, it is clear from the titration curve that both pK_{a} values are in the range 6.0 - 7.3, and we estimate pK_{a1} to be 6.2-6.4 and pK_{a2} to be 6.4-7.0. We did not determine the pK_{a} values for this compound with no bile salt present, because of solubility problems. However, these values in comparable solvents were reported
previously, and, for example, were approximately 5.2 and 5.9 in 27 vol% DMSO (10). By varying the vol% DMSO in the solvent, we were able to estimate values for pK\textsubscript{a1} and pK\textsubscript{a2} of 5 in 10% DMSO, 40 mM TDC of 5.9 and 6.5. Thus, in 10% DMSO, the bile salt at 40mM concentration causes a shift in apparent pK\textsubscript{a} of about 1 pH unit for the tetrapyrrrole. In pure water we would expect the pK\textsubscript{a1} and pK\textsubscript{a2} values with 40mM TDC to be similarly shifted, with values between 5.6-6.6.

We were unable to obtain a complete titration curve for 5 in 20% DMSO in the presence of 40mM TC. Below pH 6.5, the carboxyl\textsuperscript{13}C peak was not observed, even after overnight scanning. However, the data obtained at pH>6.5 are plotted in Fig. 7. From these data it appears that the pK\textsubscript{a} shift for 5 in TC solution would be less than that in TDC solution, as was found for compounds 1 through 4.

Table 2 lists the experimentally determined pK\textsubscript{a} values for all five compounds in solutions without bile salt present compared to those in samples containing either TC or TDC (40 mM). The magnitude of the increase in pK\textsubscript{a} in the presence of bile salt is smallest for the simple aromatic acids 1 and 2, and increases as the size of the parent acid increases, and the corresponding water solubility decreases. The increase in pK\textsubscript{a} is always greatest for TDC solutions compared to TC solutions, and this difference in the effect of the two bile salts on pK\textsubscript{a} increases as the size and nonpolar character of the compounds increases.
Discussion

A model consistent with the observed effect of bile salts on pK\textsubscript{a} may be proposed as follows:

\begin{align*}
\text{BRH}_2 & \rightleftharpoons K_{a1} H^+ + \text{BRH}^- \rightleftharpoons K_{a2} H^+ + \text{BR}^{2-} \\
\text{M:BRH}_2 & \rightleftharpoons K_{a1}' H^+ + \text{M:BRH}^- \rightleftharpoons K_{a2}' H^+ + \text{M:BR}^{2-}
\end{align*}

where \text{BRH}_2, \text{BRH}^-, and \text{BR}^{2-} represent bilirubin diacid, monoanion, and dianion, respectively, and \text{M} represents a bile salt micelle. The three different \( K_m \) symbols are the micellar association constants for the three different states of ionization of the pigment. For this model, it may be shown that

\[
K_{a1}' = K_{a1} \frac{K_{m2}}{K_{m1}} \quad K_{a2}' = K_{a2} \frac{K_{m3}}{K_{m2}}
\]

If \( K_{m1} > K_{m2} > K_{m3} \), then \( K_{a1}' < K_{a1} \) and \( K_{a2}' < K_{a2} \)

and \( pK_{a1}' > pK_{a1} \) and \( pK_{a2}' > pK_{a2} \)

The model is presented for bilirubin diacid, but a similar model may be applied to any monocarboxylic or dicarboxylic acid. In this model, the amphipathic carboxylic acid partitions between the aqueous and micellar phases. The interaction occurs between the acid and a bile salt
micelle. This interaction could also be with bile salt monomer, but the results indicate that monomer interaction is probably insignificant, and this is supported by our prior spectroscopic investigations (17). Complexation in the micellar phase is assumed to occur by hydrophobic interactions, analogous to the model proposed by McGown for bile salt solubilization of polycyclic aromatic hydrocarbons (26), and also suggested by our prior studies of bilirubin and xanthobilirubic acid with bile salts (17, 27). It is assumed that all the equilibria depicted are established rapidly, and that an observed carboxyl chemical shift is the time average of the chemical shifts of all acid and conjugate base species present in the sample. Since the acid is in a less polar environment in the micellar phase, the observed chemical shift of the carboxyl carbon should decrease, i.e., the resonance should be shifted upfield, analogous to the reported solvent effects on C-13 chemical shifts of carbonyl groups (28). This “solvent effect” might also occur by interaction of the acid with bile salt monomers. However, as shown in Figure 5, the changes in chemical shift did not become significant until the concentrations of the bile salts were increased to levels that exceeded the known cmc values for these surfactants.

Since the acid partitions between the aqueous and micellar phases, the relative acidity will be decreased, i.e., the apparent pK_a will increase, because of the decrease in activity of the acid in the bulk solvent. As the water solubility of the series of acids decreases, the relative pK_a values should increase because of greater partitioning of the more nonpolar compounds into the micellar phase. The presence of bile salt monomers in the bulk solvent is expected to decrease the dielectric constant of the medium. This also would produce an increase in apparent pK_a due to the medium effect. However, this effect is assumed to be small because of the relatively low concentration of the bile salts in these experiments.
It is possible that both the acid and conjugate base forms of these compounds partition into the micellar phase, although the complexation of the anions would be less likely due to electrostatic repulsion. The NMR data clearly indicate that there is little, if any, interaction of the anions of 1, 2, and 3 with bile salt aggregates, but that there is interaction of the anions of 4 and 5 with the micelle phase. In the case of 4 and 5, the magnitudes of the chemical shift changes indicate weaker interaction of the conjugate bases compared to the parent acids, as expected. In any case, the magnitude of the shift in pKₐ should increase with the relative propensity of the compound to be sequestered in the micellar phase. This should be the least for 1 and greatest for 5, and is consistent with the experimental results.

The proposed model, while consistent with the results of these studies, is oversimplified, and does not take into account other factors such as electrostatic effects at the micelle-water interface. Fernandez and Fromherz studied the pK shifts of certain pH indicators in micellar solution, and found, for example, a substantial increase in pKₐ for incorporation of hydroxycoumarin in SDS micelles (29). They attributed the pKₐ shift to the reduced polarity at the micelle surface, and also to the effect of the electrical potential at the surface of the charged micelles. The pKₐ shifts that we have observed in bile salt solutions appear to be similar in character to those reported for SDS. However, knowledge of the equilibria involved is incomplete, and the factors contributing to the pKₐ shifts cannot be accurately defined from our results. The effect of bile salt on the pKₐ of these compounds is similar to that recently reported for bile salt solubilization of retinoic acid and derivatives (30). Our results are also similar to those reported earlier for the ionization behavior of cholic acid in micelles and membranes (31), and for the acidity of fatty acids when bound to proteins and membranes (32). The micellar
complexation model that we have used is similar to those proposed for complexation of aromatic carboxylic acids by cyclodextrins (33, 34).

Taurocholate (TC) is a trihydroxy-substituted bile salt, while taurodeoxycholate (TDC) is dihydroxy-substituted. TC is thus somewhat more polar than TDC, as reflected in the higher cmc value for this species. Assuming that micellar solubilization of the compounds in this study occurs by hydrophobic interaction with the non-polar faces of the bile salts, the microenvironments provided by TC and TDC would not be expected to differ greatly. It is known, however, that the size and shape of the micelles formed by these two species are different (35, 36). Based on the relative polarities of the two bile salts, it can be predicted that the observed pKₐ values should be higher in TDC solution than in TC solution. The results are consistent with this prediction. Whether the difference in these values is due to differences in the structure of the micellar complexes or to a more general type of medium effect cannot be determined from these results.

The results of this study help to explain earlier results on the effects of pH on various properties of bilirubin in aqueous solutions of bile salts. Specifically, the light scattering intensity of such solutions, as well as the capacity factor of bilirubin in capillary electrophoresis (CE) experiments, have been shown to have a marked pH dependency. Some of the relevant light scattering data are presented in Figure 8. Plotted along with this data is the relative concentration of the fully protonated form of bilirubin, calculated using the pKₐ₁ and pKₐ₂ values determined in this study. The data strongly suggest that the observed variations in these properties with pH are coincident with the formation of the fully protonated acid. The increase in light scattering intensity would be due to formation of colloidal aggregates of the pigment, a process that would be more favorable for the neutral species. The increase in CE capacity factor might also involve
aggregation, but might also be due to the reduced mobility of the pigment in the electric field upon charge neutralization.

The results also have implications concerning the biophysical chemistry of bile fluid. The pH of hepatic bile varies between 5.7-8.6, with a typical value of about 7.6, while the pH of concentrated gallbladder bile has been reported to be 6.1-8.6, with a typical value of about 6.8 (37). There has been disagreement about the state of ionization of bilirubin in these fluids, due to the lack of widely accepted values for the ionization constants of the pigment in relevant media. Assuming that bilirubin behaves like mesobilirubin XIIIα (5) in bile salt solution, the molecule in bile will be a weaker acid than in pure water. Between pH 6 and 7 monoanionic forms would predominate, but significant amounts of the fully protonated species might also be present. The distribution of the various acid and base forms of the pigment would depend on the respective micellar partition coefficients of these species. In any case, the existence of significant amounts of the protonated forms in gallbladder bile would enhance the likelihood of aggregation and precipitation of the pigment. Bile fluid is a much more complex medium than a simple bile salt solution, but it is expected that similar effects on the acidity of bilirubin will be found when it is solubilized in solutions containing mixed micelles of bile salts and phospholipids.
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References


Footnotes

1The abbreviations used are: TC, sodium taurocholate; TDC, sodium taurodeoxycholate; DMSO, dimethyl sulfoxide.
Figure Legends

Fig. 1 Molecular structures of $^{13}$C-enriched compounds and bile salts used in this study. (1) benzoic acid; (2) phenylacetic acid; (3) 3,5-dimethyl-2-methoxycarbonyl-1H-pyrrole-4-butanoic acid; (4) xanthobilirubin acid; (5) mesobilirubin XIIIα.

Fig. 2 $^{13}$C NMR titration curves for carboxy $^{13}$C-enriched compounds in 10% DMSO, 90% H$_2$O (v/v). Benzoic acid (1, □); carboxy $^{13}$C-enriched phenylacetic acid (2, △); carboxy $^{13}$C-enriched pyrrole butanoic acid (3, ○).

Fig. 3 Dependence of the experimentally determined pK$_a$ values on the vol% DMSO in the solvent. Benzoic acid (1, □); carboxy $^{13}$C-enriched phenylacetic acid (2, △); carboxy $^{13}$C-enriched pyrrole butanoic acid (3, ○).

Fig. 4 Effect of bile salt on the $^{13}$C NMR titration curve for 3 in 10% DMSO, 90% H$_2$O (v/v). Curve without bile salt (○); curve with 40mM taurodeoxycholate (●).

Fig. 5 Effect of bile salt concentration on the carboxyl-$^{13}$C chemical shift of 3 in 10% DMSO, 90% H$_2$O (v/v). Taurocholate (○); taurodeoxycholate (●).

Fig. 6 Effect of different bile salts on the $^{13}$C NMR titration curves for carboxy $^{13}$C-enriched xanthobilirubin acid (4) in 20% DMSO, 80% H$_2$O (v/v). 40mM taurocholate (○); 40mM taurodeoxycholate (●).
Fig. 7 Effect of different bile salts on the $^{13}$C NMR titration curves for carboxy $^{13}$C-enriched mesobilirubin XIII$\alpha$ (5) in 20% DMSO, 80% H$_2$O (v/v). 40mM taurocholate (○); 40mM taurodeoxycholate (●).

Fig. 8 Comparison of the pH dependence of the normalized light scattering intensity and relative concentration of diacid species for bilirubin in solution with the bile salt taurocholate. Light scattering data from Ref. 16, for bilirubin in 20 mM sodium taurocholate (-○-); calculated relative concentration of bilirubin diacid species, based on estimated pK$_a$ values for bilirubin of 5.8 and 6.4 (-●-). All data are normalized to the value at pH 5.
Table 1. Effect of Bile Salt on Carboxyl Chemical Shift of XBR(4) at Low and High pH

<table>
<thead>
<tr>
<th>Bile Salt</th>
<th>pH</th>
<th>$\Delta \delta^a$ (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC, 40mM</td>
<td>2.5</td>
<td>1.19</td>
</tr>
<tr>
<td>TDC, 40mM</td>
<td>2.5</td>
<td>1.51</td>
</tr>
<tr>
<td>TC, 40 mM</td>
<td>8.5</td>
<td>0.19</td>
</tr>
<tr>
<td>TDC, 40mM</td>
<td>8.5</td>
<td>0.21</td>
</tr>
</tbody>
</table>

$a - \Delta \delta = [\delta \text{(no bile salt)} - \delta \text{(40mM bile salt)}]$
Table 2. Apparent pKₐ Values in DMSO-H₂O

<table>
<thead>
<tr>
<th>Compound</th>
<th>No Bile Salt</th>
<th>TC, 40 mM</th>
<th>ΔpKₑ</th>
<th>TDC, 40 mM</th>
<th>ΔpKₑ</th>
</tr>
</thead>
<tbody>
<tr>
<td>1ᵃ</td>
<td>4.36±0.03</td>
<td>4.44±0.03</td>
<td>0.08</td>
<td>4.50±0.03</td>
<td>0.14</td>
</tr>
<tr>
<td>2ᵃ</td>
<td>4.46±0.03</td>
<td>4.54±0.02</td>
<td>0.08</td>
<td>4.56±0.01</td>
<td>0.10</td>
</tr>
<tr>
<td>3ᵃ</td>
<td>4.99±0.05</td>
<td>5.27±0.02</td>
<td>0.28</td>
<td>5.35±0.05</td>
<td>0.36</td>
</tr>
<tr>
<td>4ᵇ</td>
<td>4.93ᶜ</td>
<td>5.51±0.01</td>
<td>0.58</td>
<td>5.87±0.02</td>
<td>0.94</td>
</tr>
<tr>
<td>5ᵇ</td>
<td>5.2, 5.9ᵈ</td>
<td>nd</td>
<td></td>
<td>6.3, 6.7</td>
<td>~1</td>
</tr>
</tbody>
</table>

a - 10% DMSO, 90% H₂O (v/v)
b - 20% DMSO, 80% H₂O (v/v)
c - Extrapolated value from dependence of pKₐ on [TDC]
d - Values taken from Ref. 10, at 27 vol% DMSO
e - Difference between pKₐ with and without bile salt
nd - not determined