On the Acid Dissociation Constants of Bilirubin and Biliverdin

pKₐ VALUES FROM ¹³C NMR SPECTROSCOPY*

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David A. Lightner‡§, Darren L. Holmes§, and Antony F. McDonagh¶

From the §Departments of Chemistry and Biochemistry, University of Nevada, Reno, Nevada 89557-0020 and the ¶Division of Gastroenterology, Box 0538, University of California, San Francisco, California 94143-0538

Biliverdin and bilirubin are naturally-occurring tetrapyrrolic bile pigments containing two propionic acid side chains. These side chains, and their propensity for ionization, are critical in the biological disposition of the pigments. Surprisingly, accurate dissociation constants for the propionic acid groups of biliverdin are unknown, and a wide range of values, extending over some 4 orders of magnitude, has been suggested for the Kₐ values of the propionic acid groups of bilirubin in aqueous solutions. Recently, pKₐ values of 6.7–9.3 have been reported for bilirubin—values much greater than the value of ~ 5 typical of propionic acid groups. These curiously high values, currently being used to explain the biological transport and metabolism of bilirubin and related compounds, have been attributed to intramolecular hydrogen bonding. We have determined the pKₐ values of 99% ¹³C-enriched (¹³C₂O₂H) [83,123-¹³C2]-mesobilirubin-XIIIα, the corresponding biliverdin, and several monopropionic model compounds by ¹³C NMR spectroscopy. This technique allows direct observation and quantitative measurement of the carboxylic acid and carboxylate anion carbon signals. Analysis of the variation of carboxyl ¹³C NMR chemical shift with pH gave rubin pKₐ values of 4.2 and 4.9 and verdin pKₐ values of 3.9 and 5.3 in aqueous buffers containing only a very small quantity (0.086 mol fraction) of dimethyl sulfoxide. When extrapolated to water, the pKₐ values are essentially unchanged. The data provide the first experimentally—determined pKₐ values for a biliverdin. They indicate that intramolecular hydrogen bonding has little effect on the acid dissociation of bilirubin and suggest that the equilibrium acidity of the bilirubin carboxylic acid groups is not abnormally high but similar to the thermodynamic acidity found in other carboxylic acids, as originally suggested by Overbeek et al. (Overbeek, J. T. G., Vink, C. L. J., and Deenstra, H. (1955) Rec. Trav. Chim. Pays-Bas 74, 81–84).

Bilirubin and biliverdin are blue-green and yellow-orange pigments formed from heme during normal metabolism in humans and other animals (1, 2). Like heme, biliverdin and bilirubin have two propionic acid groups and low solubility in water (1–3). The acidity constants of bilirubin have been determined many times during the past 40 years (4–13), but those of heme and biliverdin have not been measured (3). A mean pKₐ of ~ 5.3 has been predicted for porphyrin dicarboxylic acids (14), and pKₐ values of ~ 5.0 and ~ 7.2 have been estimated for biliverdin (3). A wide range of pKₐ values has been reported for bilirubin, with most investigators favoring a pKₐ of 6.2 to 6.5 (15), but recent determinations suggest that the true pKₐ values are even higher (6.8 to ~ 9.3) because of intramolecular hydrogen bonding (11, 12). Since most carboxylic acid pKₐ values are ~ 5 and values ~ 6 are seldom encountered (16–18), these recent determinations imply a remarkable and unprecedented effect of intramolecular hydrogen bonding on proton dissociation. To investigate this effect, we have used ¹³C NMR to study the dissociation of hydrogen-bonded and non-hydrogen-bonded pyrrolic acids and to determine the pKₐ values of mesobilirubin-XIIIα (1) and mesobiliverdin-XIIIα (2), two synthetic surrogates that differ from bilirubin and biliverdin, respectively, only by trivial differences in the nature, i.e. vinyl groups reduced to ethyl, and sequence of alkyl side chains on the lactam rings. Being remote from the propionic acid side chains, the alkyl substituents on the lactam rings are expected...
to have little influence on the dissociation constants of the carboxyl groups.

MATERIALS AND METHODS

[8,12,13C]Mesobilirubin-XIIIα (1), [8,12,13C]mesobiliverdin-XIIIα (2), [8,13C]xanthobilirubin acid (3, Table 1), 12-[8,13C]despropionic acid-12-ethylmesobilirubin-XIIIα (4), 12-[13C]carbonoxy-3,5-dimethyl-1H-pyrrole-4-propanoic acid (5), and 2-[13C]butylammonium salt to aqueous buffers. The stocksolutions were prepared as described previously (19, 20) or, for compounds 4 and 5, by modification of reported methods (21). Their tetra-n-butylammonium salts were prepared as before (22) from the acid and tetra-n-butylammonium hydroxide. NMR solutions ranging from $8 \times 10^{-4}$ to $2 \times 10^{-2}$ M were prepared in buffered deionized water or water/(CD$_3$)$_2$SO mixtures. The (CD$_3$)$_2$SO (99.9% d$_6$) was obtained from Cambridge Isotope Labs (Andover, MA). NMR samples were prepared in NMR tubes by adding standard aliquots of a stock solution of acid or its tetra-n-butylammonium salt to aqueous buffers. The stock solutions were prepared as $6 - 8 \times 10^{-2}$ M solutions in either H$_2$O or in (CD$_3$)$_2$SO and protected from light and oxygen. Buffers were 0.1 M sodium acetate (for pH 3.2–6.8) and 0.1 M Tris (for pH > 6.8). At pH < 3.2, either 0.1 M acetic acid, 0.1 M acetic acid–HCl, or 0.2 M HCl were used (non-buffered). Phosphate buffers (0.1 M) were used to compare carboxyl δ values derived from 0.1 M Tris buffer. No difference was detected at the same pH. 10–15 sample solutions were prepared at various pH values for use in a complete titration curve. pH was determined using an Orion model 811 pH/mV microprocessor pH meter calibrated with standard-diluted buffers.

Aqueous solutions were prepared by adding 50 μl of a 6–8 × $10^{-3}$ M stock solution of acid or its salt dissolved in deionized H$_2$O to 500 μl of buffer. Aqueous dimethyl sulfoxide solutions were prepared by adding a 50-μl aliquot of a 6–8 × $10^{-3}$ M stock solution of acid or its salt in (CD$_3$)$_2$SO to an aliquot of buffer. Final sample concentrations typically ranged from $8 \times 10^{-4}$ to $2 \times 10^{-2}$ M, with the chemical shift (δ$_{obs}$) for the carboxyl group being independent of concentration in this range. Only a single, well-demarcated line appears in the carboxyl region of the $^{13}$C NMR spectrum: that of the labeled carbon. Only trivial UV-visible spectral changes, 0–2 nm bathochromic shifts, were detected in the pigment solutions with added (CD$_3$)$_2$SO, from 1.8 to 27 volume %, in pH 7.4 Tris buffer, and solutions were usually optically clear. At 64 volume % (CD$_3$)$_2$SO, the mesobilirubin absorption band half-width decreases slightly, causing a 20% increase in molar absorptivity and a 10-nm bathochromic shift of the absorption maximum. Mesobilirubin-XIIIα (1) and mesobiliverdin-XIIIα (2) were less soluble than dipyrrole 3 and monopyrrole 6 at low pH. Their solutions were prepared and analyzed 1 or 2 orders of magnitude more dilute, and the $^{13}$C NMR spectra were run in 10-mm tubes with overnight scanning. Occasionally at low pH, some turbidity developed; however, this did not interfere with obtaining useful δ$_{obs}$ values for the dissolved pigment.

NMR measurements of δ$_{obs}$ for carboxyl groups were obtained with 60° pulse widths and duty cycles approximately 5 times $T_1$ on a Varian Unity Plus 500-MHz spectrometer. Three separate determinations were carried out for each acid; uncertainties in the measured δ$_{obs}$ values were ± 0.05. The NMR instrument settings and parameters specific to the Varian Unity Plus were as follows: frequency, 125.706 MHz; spectral width, 28,368.8 Hz; acquisition time, 2.000 s; relaxation time, 0.000 s; pulse width, 5.0 μs; decouple, H; high power, 40; decoupler continuously on; Waltz 16 modulated software; double precision acquisition; line broadening, 1.8 Hz; temperature, 25°C. The number of acquisitions varied depending on sample concentration. A sealed melting point capillary insert filled with 50 μl of (CD$_3$)$_2$SO was used as the lock and internal reference to standardize all samples to an independent environment reference.

Carboxylic acid pK$_a$ values were determined either graphically or by nonlinear regression analysis. In the first method, titration curves were constructed by plotting δ$_{obs}$ versus pH (19, 23). The pK$_a$ values for monocarboxylic acids were read directly as the pH when δ$_{obs}$ = 0.5 (δ$_{COOH} -$ δ$_{CO_2}$). For a dicarboxylic acid, the pK$_a$ values were interpolated from the titration curves and literature (16) pK$_a$ values of the known dicarboxylic acid adipic acid, as described below. Alternatively, a modified Hill equation (24) was solved by nonlinear regression techniques. Solving the Hill equation for compounds 3 and 6 gave best fits for 0.86
13C NMR titration curves were determined for the following 99% 13CO2H-enriched compounds: mesobilirubin-XIIIα (1) and mesobiliverdin-XIIIα (2); their tetrapyrrole counterparts bearing only one propionic acid (compounds 4 and 5, respectively); and di- and monopyrrole calibration compounds, 3 and 6, which cannot form intramolecular hydrogen bonds of the type seen in bilirubin. The titration curves of all of these compounds showed two plateaux, at low and high pH respectively, separated by ~5 ppm. These plateaux clearly represent the un-ionized and the fully ionized species, respectively, and the chemical shift difference corresponds to the titration shift, \( \Delta = \delta_{\text{CO2H}} - \delta_{\text{COOH}} \), where \( \delta_{\text{CO2H}} \) is the chemical shift of the carboxylate anion and \( \delta_{\text{COOH}} \) is the chemical shift of the carboxylic acid. The titration shifts observed (Table I) are consistent with those reported for a variety of carboxylic acids (20, 21) and were found to be independent of the number of carboxylic acid groups and almost unaffected by added dimethyl sulfoxide, up to about 30 volume %.

Monocarboxylic Acids—All of the monocarboxylic compounds gave typical sigmoidal (\( pK_a \)) versus pH plots with one plateau corresponding to no ionization and the other to complete ionization as exemplified in Fig. 1. The \( pK_a \) values (Table II) were determined by reading the pH at which \( \delta_{\text{obs}} = 0.5 (\delta_{\text{COOH}} + \delta_{\text{CO2H}}) \). Analysis of the data by the Hill equation (24) gave essentially identical results.

As noted previously for compound 6 and other carboxylic acids (19), the presence of (CD3)2SO cosolvent influences the titration curves by displacing them slightly downward with increasing % (CD3)2SO. Yet their shapes and the corresponding \( pK_a \) values vary only modestly over the range of H2O/(CD3)2SO solvent mixtures used. For example, for dipyrrenone acid 3 (Fig. 1), the \( pK_a \) values varied by only 0.13 pH units over the range 20–64 volume % (2.5–31 mol %) (CD3)2SO (Table II). This surprisingly small variation is probably due to the fact that in aqueous solutions containing as much as 64 volume % dimethyl sulfoxide, water is still the principle solvent (and base) on a molar scale. In fact, equimolar solutions are not reached until the volume % (CD3)2SO reaches ~80%. Thus, when the solvent is mainly water, especially when the volume % of (CD3)2SO \( \leq 27 (\approx 91 \text{ mol \% H}_2\text{O}) \), aqueous dimethyl sulfoxide solutions can be used for estimating the \( pK_a \) values of water-insoluble carboxylic acids.

Previously, we noted that \( pK_a \) values determined in aqueous dimethyl sulfoxide solutions could be extrapolated accurately to known \( pK_a \) values in water (19). For compound 6, the small correction to 100% water was based on the excellent linear correlation between the \( pK_a \) values and the logarithm of the volume % (CD3)2SO. Similar empirical correlations have been noted previously (19). The extrapolated \( pK_a \) values obtained (\( pK_a = 4.7 \)) is in perfect agreement with that measured directly in water (\( pK_a = 4.7 \)). For xanthobilirubinic acid (3), the linear correlation (Fig. 1) led to a corrected value of 4.6 for the apparent \( pK_a \) in water. Similarly, from the titration curves of the tetrapyrrole acids 4 and 5 (Fig. 2), which are monopropionic acid analogs of biliverdin and bilirubin, the apparent \( pK_a \) values were found to be 4.5 and 4.8, respectively, in aqueous dimethyl sulfoxide (31 mol %), which extrapolated to 4.3 and 4.5, respectively, for water (Table II).

Dicarboxylic Acids—Exact determinations of the two individual \( pK_a \) values of a dicarboxylic acid are usually difficult (25) unless the titration curve exhibits an inflection lying between the plateaux; i.e., unless \( pK_{a1} = 3 + pK_{a2} \) (for \( K_{a1} \approx 10^3 K_{a2} \)), a condition that applies to few dicarboxylic acids (16). For mesobilirubin-XIIIα (1) and mesobiliverdin-XIIIα (2), inflection points were not detected, as expected. However, limits on the apparent \( pK_a \) values can readily be set from plots of \( \delta_{\text{obs}} \) versus pH. Thus, it is evident from the titration curves of pigments 1 and 2 (Fig. 2), which are similar to those of their monocarboxylic acid analogs, that the \( pK_a \) value of each propionic acid lies between ~3.5 and 5.5. Fig. 2 also indicates that, despite the differing polarity and solubility properties of 1 and 2, their actual \( pK_a \) values are similar to the intrinsic acidity constant of propionic acid, \( pK_a = 4.88 \) (16), as found for analogs 3–6 (Table II).

The data do not permit an accurate determination of \( pK_{a1} \) and \( pK_{a2} \) for 1 and 2. However, the values can be estimated.
from the titration curve of a calibration standard, adipic acid. From the known acidity constants, $pK_a^1 = 4.44$ and $pK_a^2 = 5.44$ (16), and the measured $\delta_{obs}$ versus pH titration curve, we found that $pK_a$ for adipic acid corresponds to the value of $\delta_{obs}$ when $\delta_{obs} = \delta_{(COOH)2}^1 + 0.354\Delta$, and $pK_a$ corresponds to the value of $\delta_{obs}$ when $\delta_{obs} = \delta_{(COOH)2}^1 + 0.785\Delta$, where $\Delta = \delta_{(COOH)} - \delta_{(COOH)2}$, viz., the difference between the chemical shift of the diacid and the chemical shift of the diacid (see Table I). Use of these correction factors gave $pK_a^1 = 4.2$ and $pK_a^2 = 4.9$ for rubin (1), and $pK_a^1 = 3.9$ and $pK_a^2 = 5.3$ for verdin (2) in 100% water (Table II). More accurate estimates of the $pK_a$ values would require knowledge of the exact carboxyl chemical shifts of the individual verdin and rubin monoanions, which would be difficult to measure (25).

**DISCUSSION**

Biliverdin and bilirubin are water-insoluble pigments that occur widely in nature (1). Although similar in constitution, they have markedly different physicochemical properties largely because of their different three-dimensional structures and modes of hydrogen bonding. Biliverdins tend to assume helical, "lock-washer," conformations in solution that are stabilized by intramolecular hydrogen bonding between NH groups and the unprotonated nitrogen atom (26, 27); whereas, bilirubin adopts enantiomeric conformations that are shaped like ridge tiles and are stabilized by intramolecular hydrogen bonds between the pyrrole/lactam functions and the propionic carboxyl (or carboxylate) groups (26, 28) (Fig. 3). In solution, enantiomeric conformers of bilirubin interconvert via nonplanar intermediates in which the hydrogen bonding network is never completely broken (28, 29) (not via flat, non-hydrogen-bonded conformers as proposed recently by Ostrow et al. (30)), and their chirality depends on their backbone shape (not on the unsymmetrical methyl-vinyl substitution pattern as erroneously stated by the same authors (30, 48)). The ridge tile conformation is the only one that has been observed in crystals of bilirubin and its carboxylate salts (31–33), and spectroscopic, particularly NMR, studies supported by energy calculations strongly indicate that similar conformers prevail in solution, even in the dipolar protophilic solvent dimethyl sulfoxide (22, 28, 34, 35). The preferred conformation of bilirubin in protein-free aqueous solutions is not known, but calculations and the absorption spectra of freshly-made dilute solutions indicate that it is similar to that in dimethyl sulfoxide (29, 36).

**TABLE II**

| Carboxylic Acid | $pK_a$ in 0-31 Mole % (CD$_3$)$_2$SO |
|-----------------|-----------------------------------|
|                 | 0  | 2.5 | 8.6 | 31  |
| CH$_3$O$_2$C     | 6: | 4.69 (4.68) | 4.78 (4.78) | 4.79 (4.82) | 4.81 (4.84) |
|                 | 3: | 4.57$^b$ | 4.70 | 4.77 | 4.83 |
| CH$_3$O$_2$C     | 4: | 4.5$^b$ | 4.6$^b$ | 4.7$^b$ | 4.8 |
|                 | 5: | 4.3$^b$ | 4.4$^b$ | 4.5$^b$ | 4.5 |
| CH$_3$O$_2$C     | 1: | 4.2$^b$$^c$ | 4.2$^b$$^c$ | 4.9$^b$$^c$ | 4.9 | 5.0 |
|                 | 2: | 3.9$^b$$^c$ | 3.9$^b$$^c$ | 4.0 | 4.1 |

$^a$ Determined from pH-dependent $^{13}$C NMR data using the modified Hill equation and nonlinear regression analysis. Values in parentheses come from reading pH from the curves at $\delta_{obs} = 0.5 (\delta_{(COOH)} + \delta_{CO2})$. Measured values are the average of three independent determinations, ± 0.05 pK units; extrapolated $pK_a$ values are ± 0.1.

$^b$ Insoluble; extrapolated value using the slope in Fig. 2.

$^c$ Extrapolated values calibrated to adipic acid; pigments not soluble over the entire pH range in H$_2$O.
Bilirubin and Biliverdin pKₐ

The acid-base properties of biliverdin and bilirubin are undoubtedly important determinants of their transport, metabolism, and distribution within organisms, and the pKₐ values of the carboxyl groups of bilirubin are thought to be a key factor in its hepatic transport and neurotoxicity and in the formation of pigment gallstones (30, 37, 38). In view of the extensive literature on bile pigments and related compounds, it is surprising that there are no reliable measurements of the pKₐ values of biliverdin and that the pKₐ values of the carboxyl groups of bilirubin are controversial. There are several probable reasons for this dearth of definitive data. First, until recently there has been no persuasive reason to suppose that the carboxyl pKₐ values are anomalous or substantially different from the expected values of ~4.5–5.5. Second, commercial preparations of bilirubin and biliverdin are generally impure, making them unsuitable for accurate pKₐ measurements, and difficult to purify (1). Third, bilirubin is prone to photosomerization, even in dim light (39), and unstable in alkaline aqueous solutions in the dark, undergoing rapid radical reactions (1) that have often been neglected in pKₐ studies. Fourth, ionization of the carboxyl groups has little effect on the chromophores of biliverdin and bilirubin, making spectrophotometric methods of pKₐ determination insensitive and unreliable. Last, but probably the main reason, is the low solubility of the pigments in water (1, 3), particularly at pH < 7, which can cause precipitation and phase separation during pKₐ determinations.

There appear to be only two reports in the literature concerning the pKₐ values of biliverdin. Carey and Spivak (3) estimated pKₐ₂ to be −5.0 and 7.2, respectively, but precipitation of pigment at −pH 6.6 during acidimetric titration banjaxed accurate measurements (3). Gray et al. (5) were similarly unsuccessful. Many more measurements of the pKₐ values of bilirubin have been published (Table III). Several groups have used potentiometric or spectrophotometric backtitrations in water, methanol/water, or water/detergent (5, 7, 9, 11). However, precipitation of pigment, causing breaks or inflections in the titration curves, is a major problem with these methods, and spectrophotometric titrations are further complicated by autooxidation and aggregation of bilirubin and by the requirement for accurate extinction coefficients for the un-ionized and ionized forms of the pigment in water, which are not available. Using the spectrophotometric method, Gray et al. (5) were unable to obtain accurate pKₐ values for bilirubin, but Moroi et al. (11) found values of 6.1–6.5 for pKₐ₁ and 7.3–7.6 for pKₐ₂, ascribing these remarkably high values, without explanation, to intramolecular hydrogen bonding (11). In contrast, using similar methods, Kolosov and Shapovalenko (9) estimated pKₐ₁ and pKₐ₂ for bilirubin to be 4.5 and 5.9, respectively.

Because of the problems in working with aqueous solutions, Lee et al. (8) used dimethylformamide, in which bilirubin is soluble, as cosolvent for spectrophotometric titrations (8). They estimated pKₐ₁ and pKₐ₂ values for bilirubin in water to be 4.3 and 5.3, respectively. Hansen et al. (10) followed the ionization of bilirubin in dimethyl sulfoxide by natural abundance ¹³C NMR and estimated the mean pKₐ of the two carboxyl groups, by extrapolation, to be 4.4 in water (10). These values are close to those expected for aliphatic carboxyl groups (16). More recently, Harman et al. (40) attempted to measure pKₐ values for bilirubin by micellar electrokinetic capillary chromatography but obtained inconclusive results.

Mori et al. (11) and Ostrow et al. (12) used solubility methods to determine the pKₐ values for bilirubin (11, 12). In general, the solubility method is less accurate than potentiometric, spectrophotometric, or conductimetric methods and is particularly prone to error if the acid contains impurities, especially impurities that are more soluble than the acid itself. Moroi et al. (11) used analytically pure bilirubin and estimated solubilities spectrophotometrically, whereas Ostrow et al. (12) used poorly characterized bilirubin preparations derived from unpurified commercial material and estimated bilirubin solubilities spectrophotometrically and by the nonspecific and rather insensitive diazo reaction. Both groups were unable to accurately measure the intrinsic solubility of un-ionized bilirubin and had to use estimates in calculating pKₐ values. Moroi et al. (11)
estimated $pK_{a_1}$ to be 6.0–6.5 and $pK_{a_2}$ to be 7.3–7.7, whereas Ostrow et al. (12) found $pK_{a_1}$ to be 5.6–6.8 and $pK_{a_2}$ to be >9.2. The latter incredibly high and widely separated $pK_a$ values were attributed to retarded proton dissociation caused by intramolecular hydrogen bonding. Recognizing the methodological deficiencies (13, 30, 41) of their earlier determinations, Ostrow and co-workers (13) subsequently remeasured dissociation constants for bilirubin using a complicated solvent partitioning technique involving extraction of chloroform solutions with aqueous buffer, back-extraction of aqueous extracts with chloroform, evaporation of the extracts, and diazo assay of the residues in dimethyl sulfoxide containing sodium taurocholate and sodium EDTA (13). They concluded that the two $pK_a$ values of bilirubin are not widely separated, as they had reported previously, but are similar, with values of $pK_{a_1} = 8.12$ and $pK_{a_2} = 8.44$. Derivation of these values was based on questionable assumptions regarding the solubilities, aggregation, and phase-transfer properties of bilirubin species. Again intramolecular hydrogen bonding was invoked as the cause of such remarkably high values.

The continuing disagreement over the $pK_a$ values of bilirubin (41, 42), the increasing use of high values in the biomedical

| $pK_a$ | Solvent | Year | Method | Reference |
|--------|---------|------|--------|-----------|
| (4.4 and 5.0)$^a$ | H$_2$O | 1955 | emf,$^b$ solubility | Overbeek et al. (4) |
| 7.1 | H$_2$O/CH$_3$OH | 1961 | Spectrophotometry$^c$ | Gray et al. (5) |
| < 7 | H$_2$O/(CH$_3$)$_2$CO | 1961 | emf$^b$ | Lucassen (6) |
| 7.55 | H$_2$O-Triton X-100 | 1973 | emf$^b$ | Krasner and Yaffe (7) |
| 4.3 and 5.4 | (CH$_3$)$_2$NCHO | 1974 | $^{13}$C NMR,$^d$ emf,$^b$ | Lee et al. (8) |
| 4.50 and 5.90 | H$_2$O | 1977 | Spectrophotometry$^f$ | Kolosov and Shapolovenko (9) |
| 5.1 | (CD$_3$)$_2$SO$^d$ | 1979 | Spectrophotometry$^f$ | Hansen et al. (10) |
| 6.7 and 7.5 | H$_2$O | 1985 | Solubility | Moroi et al. (11) |
| 6.8 and 9.3 | H$_2$O | 1988 | Solubility | Ostrow et al. (12) |
| 8.1 and 8.4 | H$_2$O/CHCl$_3$ | 1992 | Solvent partition$^f$ | Hahm et al. (13) |

$^a$ Suggested values, not measured experimentally.
$^b$ Potentiometric titration using pH meter.
$^c$ UV-visible spectrophotometric titration.
$^d$ Co-titration with hydroxybenzoic acid standards.
$^e$ Extrapolated to $pK_{a_1} = 4.4$ in water from dimethylsulfoxide using the Born equation.
$^f$ Solvent partition between CHCl$_3$ phase and aqueous buffer phase.
literature (30), and the unprecedented effects being ascribed to intramolecular hydrogen bonding led us to reinvestigate the problem using $^{13}$C NMR. This method is rapid and sensitive, allows direct observation of the carboxyl groups undergoing deprotonation (43), and, unlike partitioning and solubility methods, is insensitive to traces of polar impurities in the acid and does not require extensive manipulation or extraction of solutions. Although the technique has not been used extensively, it is particularly useful for bile pigments, because it focuses specifically on the carboxyl group. Ionization of pyrrole or lactam groups, which may occur at extreme pH values and can complicate spectrophotometric titrations, is not detected (21). Natural abundance $^{13}$C NMR (10) has already been used to estimate pK$_a$ values for bilirubin, but the results have been dismissed because of the use of dimethyl sulfoxide as solvent (12, 13, 30). In our studies, we used highly labeled compounds and high field NMR to increase sensitivity and allow accurate measurements on dilute solutions. Since $^{13}$COOH-labeled bilirubin and biliverdin are not easy to synthesize, we used the corresponding available meso-XIIIa analogs as surrogates. The symmetrical side chain substitution pattern of these compounds simplifies the NMR spectra and amplifies the $^{13}$C signals but is expected to have negligible effect on the intramolecular hydrogen bonding or pK$_a$ values compared with the natural analogs. We also measured the pK$_a$ values of a dipyrrolic pigment that cannot undergo intramolecular hydrogen bonding, of tetrpyrrole analogs with only one propionic acid side chain, and of the dicarboxylic acid standard adipic acid. Use of this interrelated set of compounds allowed us to examine specifically the effects of intramolecular hydrogen bonding and electrostatic interactions on pK$_a$ values. We used perdeuterated (CD$_3$)$_2$SO as a cosolvent to facilitate the rapid preparation of solutions and to ensure that solutions remained homogeneous over a wide pH range, and we kept the concentration of cosolvent in the final solutions to $\leq$ 31 mol%. Solutions were optically clear and devoid of colloidal or particulate material, except at low pH, where there was sometimes some turbidity. However, any insoluble pigment present is not detected by the NMR under the conditions used, nor does it seem to interfere with the determination of the carboxyl chemical shift of the dissolved pigment. Although pK$_a$ values determined by $^{13}$C NMR chemical shifts in (CD$_3$)$_2$SO may differ markedly from those determined in water, pK$_a$ values of aryl and pyrrolic carboxylic acids derived by NMR in aqueous solutions containing up to 31 mol% (CD$_3$)$_2$SO differ little from values determined in the absence of the organic cosolvent (28). Thus, the caveat that pK$_a$ values measured in aqueous organic solvents cannot be extrapolated to give reliable pK$_a$ values for water (46) does not apply to the present $^{13}$C NMR method when aqueous dimethyl sulfoxide solutions containing excess water are used.

As found previously (21) (Table II), values for the pK$_a$ of the monopyrrolic model compound 6 in aqueous (CD$_3$)$_2$SO solutions were within about 0.1 pK$_a$ unit of the value measured in water, and extrapolation from the aqueous (CD$_3$)$_2$SO data gave a value for water that was identical within the experimental error to the measured value of 4.7. Similarly, the water-insoluble dipyrromethane xanthobilirubin acid 3 showed typical sigmoid pH versus chemical shift curves in aqueous (CD$_3$)$_2$SO solutions (Fig. 1). Apparent pK$_a$ values, determined from the midpoints of these curves varied by only 0.13 pK$_a$ units over the range 2.5–31 mol% (CD$_3$)$_2$SO and were similar to the corresponding values for monopyrrolic acid 6. In addition, the slopes of the pK$_a$ versus log volume % (CD$_3$)$_2$SO lines were similar for compounds 3 and 6, allowing an extrapolated value of 4.6 to be determined for the apparent pK$_a$ of xanthobilirubinic acid in water.

The biliverdin analog 4 with one propionic acid showed a typical sigmoid titration curve in 31 mol% (CD$_3$)$_2$SO (Fig. 2), qualitatively similar to those obtained with the mono and dipyrrolic acids 3 and 6. This curve undoubtedly corresponds to ionization of the lone carboxyl group. The apparent pK$_a$ for 4 in 31 mol% (CD$_3$)$_2$SO was 4.5—close to, but slightly lower than the corresponding values for 3 and 6. Assuming that the slope of the pK$_a$ versus log volume % (CD$_3$)$_2$SO plot for 4 would be similar to that for xanthobilirubinic acid, we estimated the pK$_a$ for 4 in water to be $\sim$4.3. Thus, the measured and extrapolated pK$_a$ values for the three monocarboxylic model compounds, 3, 4, and 6, for which intramolecular hydrogen bonding of the type seen in bilirubin is impossible, were similar and within the range expected for propionic acid groups. These results indicate that the presence of mono-, di-, or tetrapyrrole components has no unusual effect on propionic side chain acidity, and they lend further support to the reliability and utility of $^{13}$C NMR for determining the pK$_a$ values of bile pigments.

In contrast to the propionic acid group in verdin 4, the single propionic acid group in rubin 5 can participate in intramolecular hydrogen bonding of the type seen in the ridge tile conformation of bilirubin. Nevertheless, in 31 mol% (CD$_3$)$_2$SO, 5 displayed a sigmoid titration curve (Fig. 2) that resembled the titration curve for the corresponding verdin 4 and yielded an apparent pK$_a$ of 4.8. Extrapolation to zero mol% (CD$_3$)$_2$SO, as for the verdin 4, gave an estimated pK$_a$ of 4.5 for 5 in water. Comparison of the pK$_a$ values of 4 and 5 indicates that intramolecular hydrogen bonding within a ridge-ridge conformation has little effect, if any, on the pK$_a$. Hydrogen bonding may retard the dissociation of the COOH proton slightly, but it does not reduce the dissociation constant by orders of magnitude, as suggested previously (11–13, 30, 41).
that the $pK_a$ values for the two carboxyl groups of monomeric bilirubin in water are close to 4.2 and 4.9, respectively. These values are consistent with the observed solubility properties of bilirubin (4). Our results are in good agreement with previous measurements by Lucassen (6), Hansen et al. (10), and by Lee et al. (8), and with the values originally assumed by Overbeek et al. (4) some 40 years ago. The much higher values that have been reported recently have been rationalized by invoking intramolecular hydrogen bonding (11–13, 30, 41). However, these rationalizations appear to be based on misconceptions concerning the effects of ionization and solvent on the structure of bilirubin in solution and erroneous ideas concerning the effects of hydrogen bonding on acidity. Some misconceptions are that the hydrogen-bonded structure of bilirubin in solution is rigid (30, 47), that intramolecular hydrogen bonding is not maintained in dimethyl sulfoxide (12, 13, 30, 48), that flat (non-hydrogen-bonded) conformations of bilirubin occur in solution (12, 30), that intramolecular hydrogen bonding in the free acid suppresses ionization of the carboxyl groups (11–13, 30, 41, 49, 50), and that the carboxyl groups no longer undergo intramolecular hydrogen bonding after they are ionized (12, 49). Such misconceptions have fueled the view that the acid dissociation constants of bilirubin are abnormally low because of hydrogen bonding. In addition, the focus on deprotonation or dissociation has neglected the potential stabilization of the carboxylate anion by intramolecular hydrogen bonding. In general, the effect of intramolecular hydrogen bonding on the $pK_a$ values of dicarboxylic acids is small except for instances in which intramolecular hydrogen bonding between carboxyl and carboxylate groups occurs in the monoanion (17, 51, 52), which is sterically unlikely for bilirubin, though possible for biliverdin. In such acids, for example maleic or phthalic acid, hydrogen bonding invariability lowers $pK_a$, rather than increases it, as claimed for bilirubin (30). In bilirubin, proton dissociation might be facilitated somewhat by stabilization of the resulting carboxylate anion by intramolecular hydrogen bonding to a dipyrrinone, but this stabilization would be offset by electrostatic repulsion between carboxylate and lactam oxygens. When large effects of intramolecular hydrogen bonding occur in dicarboxylic acids, they are manifested by abnormally large values of $K_{a2}/K_{a1}$ (17, 51, 52), which is clearly not the case for bilirubin.

The biochemical relevance of the earlier measurements by Hansen et al. (10) and by Lee et al. (8) has been cursorily disregarded on the unfounded basis that intramolecular hydrogen bonding within the ridge-tile structure of bilirubin is not maintained in dimethylformamide and dimethyl sulfoxide (12, 13, 30), the solvents in which the measurements were made. For dimethylformamide, there is little evidence to show whether this is true or not, but for dimethyl sulfoxide there is extensive NMR and spectroscopic evidence that intramolecular hydrogen bonding is maintained, albeit slightly altered (34, 35, 53). This evidence indicates that the ridge tile structure prevails, although somewhat flattened, and that, at high sulfoxide concentrations in organic solvents or in pure anhydrous dimethyl sulfoxide, a sulfoxide group intercalates between each propanic carboxyl and its opposite dipyrrinone NH groups (35, 54, 55). Although such solution might influence proton dissociation, it is not likely to increase it by several orders of magnitude. In the present studies, where all measurements were done in solutions containing excess water, solvation or putative hydrogen bond breaking by dimethyl sulfoxide is evidently irrelevant (56), as shown by the weak effects of dimethyl sulfoxide concentration on the carboxyl chemical shifts and the measured $pK_a$ values. This conclusion is supported by measurements of the second ionization constants for adipic, malonic, and maleic acids by the same technique, which gave values identical to those measured in water. Thus, there is no plausible reason to expect that intramolecular hydrogen bonding would decrease the acidity of bilirubin, and arguments invoking hydrogen bonding to explain impossibly large measured $pK_a$ values are specious.

We conclude that the acid dissociation constants of natural bilirubin and biliverdin are likely to be almost the same and within the normal range for aliphatic propionic acids. Consequently, when unbound bilirubin and biliverdin occur in the aqueous phase of living tissues at physiologic pH, the predominant species present will be the di-anions, not the monoanions or un-ionized acids, and current theories of bilirubin distribution, toxicity, and gallstone formation that assume otherwise are implausible and need re-evaluating. We detected no major effects of intramolecular hydrogen bonding on acid dissociation of the bilirubin analog mesobilirubin-XIII$_a$ and only a weak effect on the second ionization of the biliverdin analog mesobiliverdin-XIII$_a$, confounding the prevalent notion that such bonding inhibits proton dissociation from bilirubin. Intramolecular hydrogen bonding is undoubtedly important in the biological chemistry of bilirubin—not because it retards carboxyl dissociation, but because it engenders lipophilicity (57).

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