Aqueous dissociation constants of bile pigments and sparingly soluble carboxylic acids by ^{13}C NMR in aqueous dimethyl sulfoxide: effects of hydrogen bonding

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Abstract pK_as for the acid dissociation of the carboxyl groups of bilirubin in water have been reported recently to be 8.1–8.4, or higher. These high values were attributed to intramolecular hydrogen bonding. They have led to suggestions that monoanions of bilirubin predominate at physiologic pH and are the species transported most readily into hepatocytes by carriers. Such high aqueous pK_as are inconsistent with recent ^{13}C nuclear magnetic resonance (NMR) measurements on mesobilirubin XIIIα, done on aqueous solutions containing dimethyl sulfoxide. To investigate whether the presence of dimethyl sulfoxide leads to unreliable values when using ^{13}C NMR spectroscopy to determine pK_as of carboxylic acids that can undergo intramolecular hydrogen bonding, we measured the pK_as of ^{13}C-labeled fumaric, maleic, and phthalic acids in solutions containing up to 27 vol % dimethyl sulfoxide. In addition, we used ^{13}C NMR to estimate the pK_as of 2,2′-methylenebis[5-carbomethoxy-4-methylpyrrole-3-[1-^{13}C]propanoic acid], a model for the two central rings of bilirubin. Our results show that ^{13}C NMR of aqueous dimethyl sulfoxide solutions can be used with confidence to measure pK_as of intramolecularly hydrogen-bonded carboxylic acids. They support our previous estimates for the pK_as of bilirubin and confirm that intramolecular hydrogen bonding has little effect on the acidity of bilirubins in water. Together with previous studies and chemical arguments they strongly suggest that reported aqueous pK_as of >8, or even >6, for the carboxyl groups of bilirubin are incorrect and that arguments used to rationalize them are questionable.—**Trull, F. R., S. Boiadjiev, D. A. Lightner, and A. F. McDonagh.** Aqueous dissociation constants of bile pigments and sparingly soluble carboxylic acids by ^{13}C NMR in aqueous dimethyl sulfoxide: effects of hydrogen bonding. *J. Lipid Res.* 1997. **38:** 1178–1188.

Supplementary key words bilirubin • biliverdin • fumaric acid • gallstones • jaundice • maleic acid • organic anions • phthalic acid • pK_a

Best-known as a colorful herald of hepatobiliary disease, bilirubin (BR) (**1, Fig. 1**) is the toxic agent in kernicterus, the major component of pigment gallstones, and an endogenous inhibitor of free-radical injury (1–3). Like its blue-green precursor biliverdin (**2**), it is a dicarboxylic acid. Accurate values for the acid dissociation constants (K_as) of biliverdin and BR have been difficult to ascertain because both pigments are only very sparingly soluble in water at physiologic pH and below (4–7). Until about 1980 most estimates and determinations of the K_as of BR indicated them to be about 5.0×10^{-5} M (pK_a 4.3)–1.3×10^{-6} M (pK_a 5.9) (7–13), which is within the range expected for aliphatic carboxylic acids (14). Recent studies (15–17), however, have concluded that they are about 10^{-8} M or less because of intramolecular hydrogen bonding (H-bonding). These extraordinarily low values are gradually becoming accepted in the literature (18–22).

Recently, we used ^{13}C nuclear magnetic resonance (NMR) spectroscopy to measure the K_as of several synthetic compounds related to BR (23–25). These included mesobilirubin (MBR) XIIIα (**3**), mesobiliverdin XIIIα (**4**), the two mono-pronionic bile pigments (**5**) and (**6**), and the di and monopyrrolic acids (**7**) and (**8**). For each compound we found K_as in the range expected for aliphatic carboxylic acids and no evidence for a large effect of intramolecular H-bonding. Since ^{13}C-NMR spectroscopy is a sensitive and accurate method for determining dissociation constants (26–29), our results led us to question the high pK_a values

Abbreviations: BR, bilirubin IXα; H-bond (ed, ing), hydrogen bond (ed, ing); HPLC, high pressure liquid chromatography; MBR, mesobilirubin; NMR, nuclear magnetic resonance.

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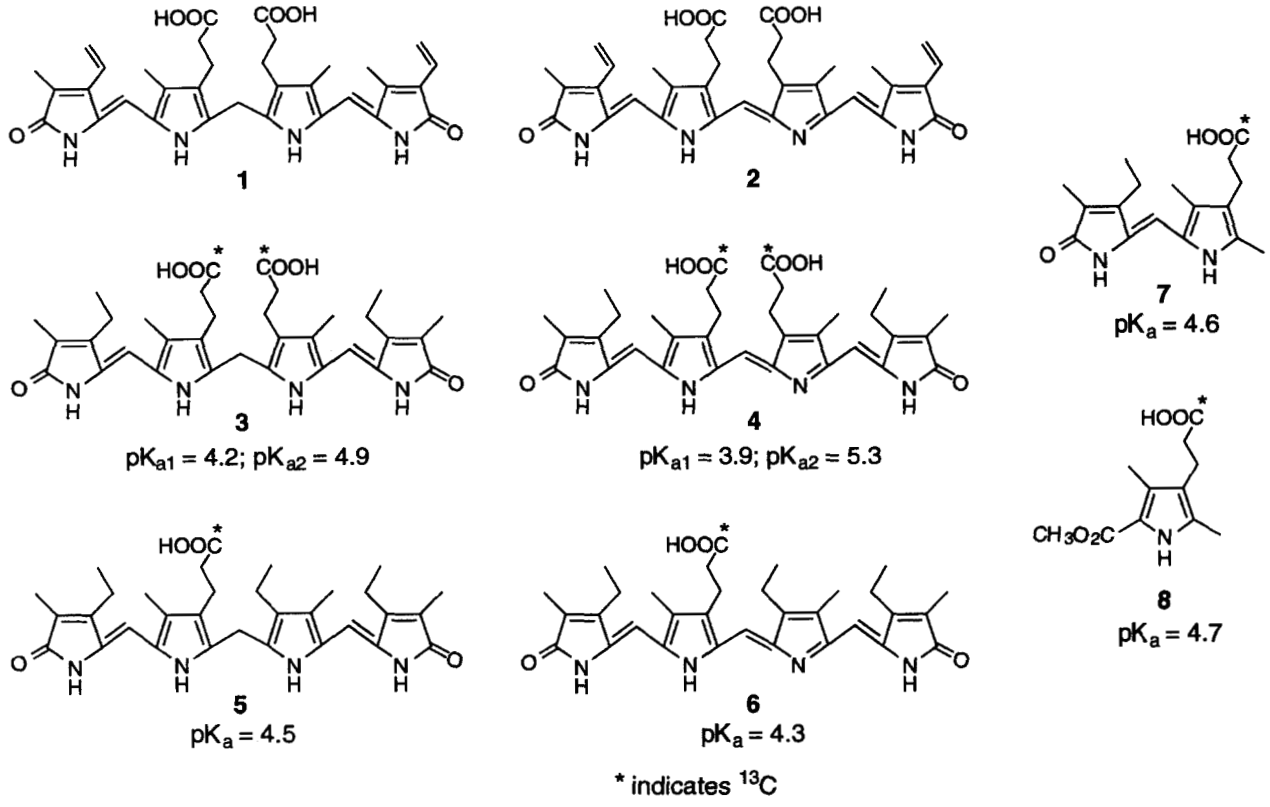

Fig. 1. Constitutional structures of bilirubin (**1**), biliverdin (**2**), mesobilirubin XIIIα (**3**), mesobiliverdin XIIIα (**4**) mono-carboxylic analog of mesobilirubin XIIIα (**5**), mono-carboxylic analog of mesobiliverdin XIIIα (**6**), xanthobilirubinic acid (**7**), and 2,4-dimethyl-5-carbethoxy-pyrrole-3-propionic acid (**8**).

reported recently (15, 16, 30, 31) for BR and to suspect that the transport and distribution models (17) based on them might be incorrect.

The side-chain carboxyl (COOH) groups of BR can form intramolecular hydrogen bonds (H-bonds) with neighboring dipyrrinone NH and C=O groups (**Fig. 2**) (32, 33). These weak bonds have been invoked to explain the anomalously low dissociation constants (high pK$_a$s) found by some investigators in water (15–17, 30, 34–36). In our ^{13}C-NMR measurements we used $(CD_3)_2SO$ (deuterated dimethyl sulfoxide, d_6-DMSO) as a co-solvent to facilitate the preparation of solutions and increase solute solubility over a wide pH range. DMSO allegedly interferes with H-bonding in BR, thereby increasing the acidity of the carboxyl groups (15–17, 34, 35). On this basis it could be argued that the DMSO used in our NMR measurements might have interfered with H-bonding in MBR XIIIα and model compound **5** and markedly enhanced the COOH dissociation. Such an effect might explain the very large difference between the NMR-derived K$_a$s for bilirubins (13, 24) and those reported recently for BR (15, 16, 30, 31). More generally, it might invalidate the use of DMSO as a co-solvent for measuring K$_a$s of hydrogen-bonded (H-bonded) carboxylic acids. To investigate this possibility, we have studied the effect of DMSO on the carboxyl ^{13}C-NMR spectra of maleic (**9, Fig. 3**), phthalic (**10**) and fumaric (**11**) acids in water. The pK$_a$s of maleic and phthalic acids are markedly influenced

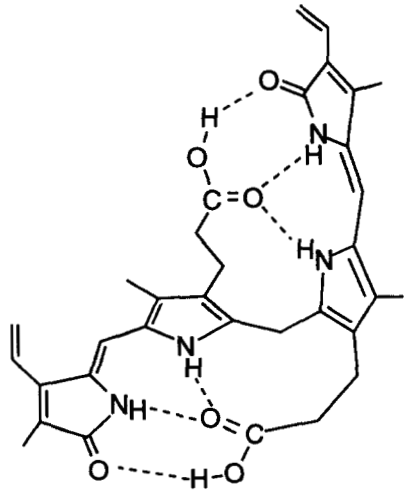

Fig. 2. Intramolecular hydrogen bonding in bilirubin.

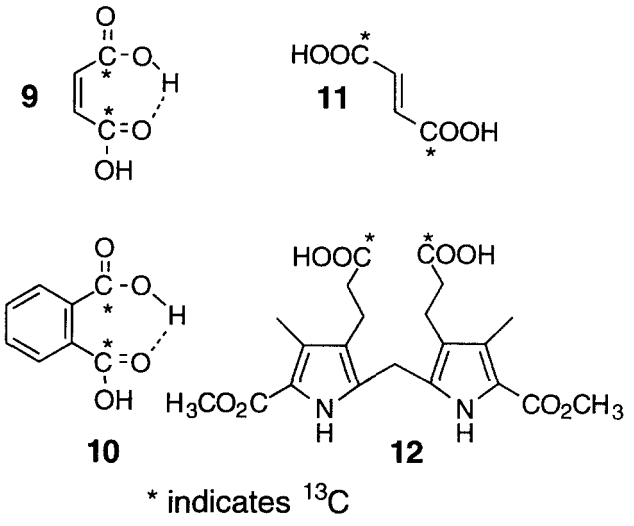

Fig. 3. Constitutional structures of maleic acid (**9**), phthalic acid (**10**), fumaric acid (**11**), and 2,2′-methylenebis[5-carbomethoxy-4-methylpyrrole-3-[1-^{13}C]-propanoic acid] (**12**), a model for the central two rings of bilirubin.

by intramolecular H-bonding (14, 37, 38), in contrast to those of fumaric acid. In addition we have used ^{13}C-NMR to measure the K_as of the ^{13}C-labeled dipyrrolic diacid **12** (Fig. 3). Diacid **12** is a structural model for the central rings of BR, but lacks the lactam NH and C═O groups that are essential for complete intramolecular H-bonding of the carboxyl groups.

MATERIALS AND METHODS

General procedures

Nuclear magnetic resonance (NMR) spectra were determined on a 500 MHz Unity Plus spectrometer (Varian Associates, Palo Alto, CA) as previously described (24) and are reported in δ (ppm) downfield from $(CH_3)_4Si$. A sealed capillary insert filled with 50 μl of d_6-DMSO was used as the lock and external reference to standardize all samples to an independent of environment reference. Measurements of pH were determined at 22°C on a microprocessor pH/millivolt meter (Model 811, Orion Research, Inc., Boston, MA). Melting points were determined on a Thomas-Hoover Uni-Melt capillary apparatus and are uncorrected. Analytical TLC was carried out on 125-μm thickness silica gel IB-F plates (J. T. Baker Co., Phillipsburg, NJ), using $CHCl_3$–MeOH 10:1 (v/v) as mobile phase. Solvents were HPLC grade. Maleic anhydride-1,4-$^{13}C_2$ (99 atom % ^{13}C) and 99.9% deuterated d_6-DMSO were from Cambridge Isotope Laboratories Andover, MA). Phthalic acid-α,α′-$^{13}C_2$ (99.4% atom % ^{13}C) was from Isotec Inc., Miamisburg, OH. Maleic anhydride (mp 60°C), maleic acid (mp 140–142°C), fumaric acid (mp 299–300°C; sealed tube) and phthalic acid (mp 210–211°C), all of 99% purity, were from Aldrich Chemical Co. (Milwaukee, WI). Maleic acid-1,4-$^{13}C_2$ was obtained in quantitative yield from malic anhydride-1,4-$^{13}C_2$ as previously reported for the unlabeled material (39). Its thin-layer chromatography, melting point and infrared characteristics were identical to those of unlabeled material. Fumaric acid-1,4-$^{13}C_2$ was obtained by heating maleic acid-1,4-$^{13}C_2$ in a sealed tube in a glycerine bath to 140°C for 1 h, as previously reported for the unlabeled compound (39). This treatment gave a mixture (by thin-layer chromatography) of ^{13}C-labeled maleic anhydride, maleic acid, and fumaric acid. The most polar fumaric acid was isolated by washing the mixture repeatedly with $CHCl_3$, to remove the anhydride, followed by $CHCl_3$ containing 1% (v/v) added MeOH, to remove maleic acid. The thin-layer chromatography characteristics, melting point, and infrared spectrum of the product were identical to those of authentic unlabeled material.

Synthesis of 2,2′-methylenebis[5-carbomethoxy-4-methylpyrrole-3-[1-^{13}C]propanoic acid] (12)

The title compound was made by a modification of a published synthesis of the unlabeled compound (40). To a solution of 0.6 g (2.5 mmol) of 2,4-dimethyl-5-methoxycarbonyl-1*H*-pyrrole-3-propanoic acid methyl ester, 99% ^{13}C-enriched at the propanoic acid carboxyl carbon (23), in 10 ml of dry ethyl acetate was added a solution of 0.13 ml (2.5 mmol) of bromine in 2.5 ml of ethyl acetate over 5 min. The mixture was warmed and heated at reflux for 1.5 h. The solvent was removed under vacuum to dryness. The residue was then dissolved in 10 ml of methanol; 3 drops of conc. HCl were added, and the mixture was heated at reflux for 4.5 h. The mixture was cooled for 2 h at −10°C and precipitated solid was collected by filtration and recrystallized from methanol to afford 406 mg (70%) of colorless crystals of the tetramethyl ester, with mp 146–148°C [lit. (41) mp 145–146°C]. It had ^{1}H-NMR ($CDCl_3$) δ: 2.28 (6H, s), 2.53/2.56 (4H, dt, J = 7.0, 6.8 Hz), 2.77/2.78 (4H, dt, J = 6.9, 5.8 Hz), 3.68 (6H, d, J = 3.8 Hz), 3.76 (3H, OCH_3, $^3J_{CH}$ = 3.8 Hz), 3.76 (6H, s, OCH_3), 3.97 (2H, s), 9.16 (2H, brs, NH) ppm and ^{13}C-NMR ($CDCl_3$) δ: 10.63 (q), 19.19 (t), 22.32 (q), 33.93/34.69 (d, $^1J_{CC}$ = 57.5 Hz), 50.87 (q), 51.75/51.78 (d, $^2J_{CC}$ = 2.2 Hz), 118.14 (s), 120.00/120.01 (d, $^3J_{CC}$ = 1.4 Hz), 126.7 (s), 130.6 (s), 161.9 (s), 174.0 (s, ^{13}COOH) ppm.

The tetra-ester above was selectively hydrolyzed at its two propanoic ester groups. To a solution of 232 mg (0.5 mmol) of tetramethyl ester in 5 ml of methanol was added 1 M NaOH (1.1 ml, 1.1 mmol), and the mixture was stirred for 14 h. The methanol was removed under vacuum; then 1 ml of H_2O was added, and the mixture was acidified at 0°C with 10% HCl. The re-

sulting solid product was removed by filtration, washed (3 × 3 ml) with water and recrystallized at 0°C from methanol–H_2O to afford 209 mg (96%) of the ^{13}C-labeled diacid with mp 204–206°C (decomp.) [lit. (42) mp 201–202°C for the diethyl ester]. It had ^{1}H-NMR ($(CD_3)_2SO$) δ: 2.01/2.04 (4H, dt, J = 7.0, 7.8 Hz), 2.12 (6H, s), 2.49 (under solv.), 3.72 (6H, s), 3.83 (2H, s), 11.17 (2H, s, HH), 11.98 (2H, s, COOH) ppm and ^{13}C-NMR ($(CD_3)_2SO$) δ: 10.24 (q), 19.00 (br t), 22.01 (t), 34.24/34.96 (d, $^1J_{CH}$ = 56.1 Hz), 50.62 (q, OCH_3), 116.3 (s), 119.6/119.7 (d, J = 4.1 Hz), 125.5 (s), 131.4 (s), 161.2 (s, *C*OOMe), 173.9 (s, ^{13}COOH) ppm.

NMR sample preparation

NMR samples were prepared in 5-mm NMR tubes by adding aliquots of stock solutions of carboxylic acid to aqueous buffers. Concentrations of d_6-DMSO are given as % by volume. Note that 27–29% d_6-DMSO contains >90 mole % water. Stock solutions of acids were prepared immediately before use as 0.007–0.009 M solutions in either water or d_6-DMSO. Buffered solutions were based on 0.2 M KCl + 0.2 M HCl (for pH ~0.8 to 2.1), 0.1 M acetic acid (for pH ~3.2; non-buffered), 0.1 M acetic acid + 0.1 M sodium acetate (for pH ~4 to 6.3), 0.1 M phosphate (for pH ~6.4 to 6.9), and 0.1 M Tris base (for pH above ~6.9). δ values measured in 0.1 M acetate buffer were identical to those measured in 0.1 M phosphate buffer of the same pH (6.3). In general, aqueous solutions were prepared by adding 50 µl of a 0.007–0.009 M stock solution of diacid in deionized water to 50 µl of buffer and aqueous d_6-DMSO solutions were prepared by adding an aliquot of a similar stock solution of diacid in d_6-DMSO to an aliquot of buffer followed by further addition of d_6-DMSO as required. The solution of $^{13}C_2$-dipyrrole (**12**) containing 2.7% d_6-DMSO was prepared by dissolving 3.7 mg in 300 µl of d_6-DMSO, diluting the solution to 1 ml with water and warming to 70°C to completely dissolve the diacid; aliquots (50 µl) of this solution were then added to NMR tubes containing 500 µl of buffer. pH values were measured in NMR tubes with a microelectrode. Curves for the variation of carboxyl ^{13}C chemical shift with pH were generated using the software program Kaleidograph (Version 3.0, Synergy Software, Reading, PA) using a smoothing function to fit the data points, and were not specifically fit to an idealized Henderson-Hasselbach curve.

RESULTS

Figure 4 shows the variation of chemical shift with pH for the sharp single carboxyl carbon peak in the ^{13}C-NMR spectra of dilute solutions (~10^{-3} M) of [1,4-$^{13}C_2$]fumaric acid (**11**, Fig. 3) in water and aqueous solutions containing 9% and 29% d_6-DMSO. This peak reflects the time-averaged signal from the $^{13}CO_2H$ and $^{13}CO_2^-$ carbon atoms. The curves have the typical and expected sigmoidal shapes in which the lower plateau corresponds to 100% unionized acid and the upper plateau to 100% dianion. Titration shifts were 6.0, 5.9, and 5.6 ppm in water, 9% and 29% d_6-DMSO/H_2O, respectively. The known pK_as for fumaric acid are 3.02 and 4.38 (14). As expected for a dicarboxylic acid whose pK_as differ by less than 3 units, there are no inflections in the curves. The presence of d_6-DMSO had little effect on the general shapes of the curves, but resulted in a downward displacement, with respect to the curve for water without d_6-DMSO, and no displacement parallel to the abscissa. It is obvious from the curves that both pK_{a1} and pK_{a2}, where K_{a1} and K_{a2} are the first and second carboxyl dissociation constants, must fall within the range of 2.6–5.5 for all three solutions. As ^{13}C chemical shift data for the fumarate monoanion are not available, exact pK_as for the first and second ionizations cannot be calculated from the curves without extensive curve-fitting procedures. However, if the chemical shift of the monoanion is the average of those of the diacid and dianion, as reported (43) for succinic acid ($HO_2CH_2CH_2CO_2H$), then pK_{a1} is equal to the pH at which the observed chemical shift (δ_{obs}) equals $[\delta_{CO_2H} + 0.25(\delta_{CO_2^-} - \delta_{CO_2H})]$ and pK_{a2} is equal to the pH at which $\delta_{obs} = [\delta_{CO_2H} + 0.75(\delta_{CO_2^-} - \delta_{CO_2H})]$. Using this approximation, we derived values of 3.2 and 4.4 for the two pK_as of fumaric acid in water and 3.3 and 4.4 for the corresponding values in 29% d_6-DMSO/H_2O solution. These are close to the accepted literature measurements of 3.02 and 4.38. Curves similar to those shown in Fig. 4 were obtained using natural-abundance ^{13}C NMR of unlabeled fumaric acid at higher concentra-

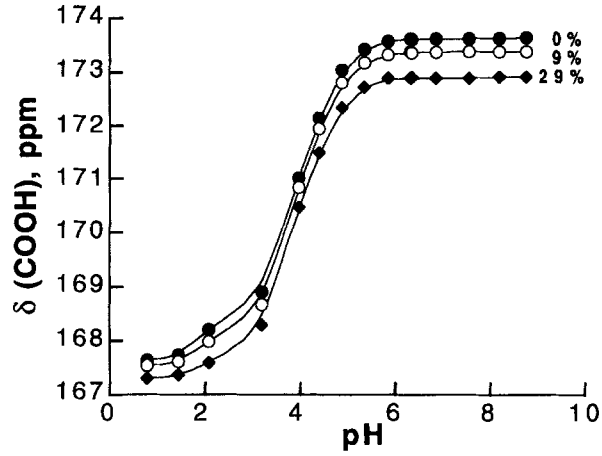

Fig. 4. ^{13}C-NMR titration curves for fumaric acid-1,4-$^{13}C_2$(6–8 × 10^{-4} M) in aqueous buffers containing 0%, 9%, and 29% d_6-DMSO.

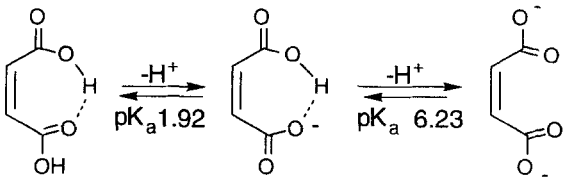

Fig. 5. Intramolecular hydrogen bonding in maleic acid and its mono- and dianions.

tions (~0.1 M) and using only dilute NaOH and HCl solutions to adjust the pH, rather than buffer.

The carboxylic acid groups in fumaric acid (**11**), being *trans*, are sterically unable to H-bond with each other. In contrast, those in its *cis* stereoisomer maleic acid (**9**, Fig. 3) are favorably disposed for intramolecular H-bonding interactions and provide suitable models for testing the effects of DMSO on the dissociation constants of H-bonded carboxyl groups (14, 38, 44, 45). It is known that intramolecular H-bonding of the carboxyl groups in the acid form of maleic acid does not retard dissociation of the first proton (14, 37, 38), as has been suggested for BR (15–17, 30, 34–36). Rather, stabilization of the corresponding monoanion by H-bonded sharing of the remaining proton between the two carboxyl groups (**Fig. 5**) results in increased acidity compared to fumaric acid and an unusually small pK_{a1} of 1.92 (14). However, the pK_a for dissociation of the remaining proton, 6.23, is greater than pK_{a2} for fumaric acid because of electrostatic and/or H-bonding effects (14, 37, 38). **Figure 6** shows δ versus pH curves for [1,4-$^{13}C_2$]maleic acid in water and aqueous d_6-DMSO solutions. Unlike the plain fumaric acid curves, these curves show two inflection points. The clear parallelism between the curve for water and those for aqueous d_6-DMSO solutions demonstrates that the presence of the organic co-solvent has no significant influence on intramolecular H-bonding. Titration shifts (dianion–diacid) were 5.7, 5.6, and 5.5 ppm for water, 9% d_6-DMSO/H_2O, and 28% d_6-DMSO/H_2O, respectively. These values are close to the 5.5 ppm reported by Perrin and Thoburn (43) for maleic acid in water. The apparent pK_{a1} and pK_{a2} values determined from the curves in Fig. 6 were 1.8 and 6.0, 1.9 and 6.2, and 1.9 and 6.4 for water, 9% d_6-DMSO/H_2O and 28% d_6-DMSO/H_2O, respectively. The NMR values for maleic acid in water are in good agreement with the literature values of 1.92 and 6.23, and the values determined in the presence of up to 28% d_6-DMSO are close to these.

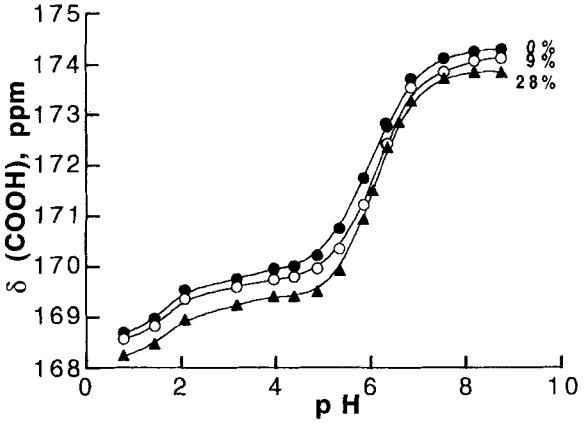

Fig. 6. ^{13}C-NMR titration curves for maleic acid-1,4-$^{13}C_2$ (1.1×10^{-3} M) in aqueous buffers containing 0%, 9%, and 27% d_6-DMSO.

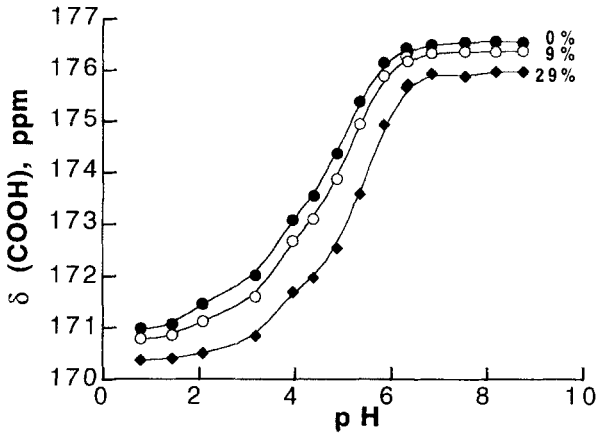

Fig. 7. ^{13}C-NMR titration curves for phthalic acid-α,α′-$^{13}C_2$ ($6–9 \times 10^{-4}$ M) in aqueous buffers containing 0%, 9%, and 29% d_6-DMSO.

Phthalic acid (**10,** Fig. 3), is another example of a dicarboxylic acid in which the carboxyl groups are favorably juxtaposed for intramolecular H-bonding of carboxylate protons (38). This causes a lowering of the first ionization constant relative to, say, benzoic or terephthalic (*p*-phthalic) acids, in which there is no intramolecular H-bonding. However, the difference between pK_{a2} and pK_{a1} (ΔpK) for phthalic acid is much smaller than for maleic acid (2.46 versus 4.31, respectively) (14). **Figure 7** show δ versus pH curves for [α,α′-$^{13}C_2$]phthalic acid in aqueous solutions with and without d_6-DMSO co-solvent. Again, the curves are parallel. Consistent with the Δp*K* of <3, clear inflections corresponding to successive ionizations of the carboxyl groups are not clearly present, though there is a hint of an inflection in each curve. The curves show that both pK_{a1} and pK_{a2} have values between ~2.5 and 6.2. The ^{13}C chemical shift for the phthalate monoanion has been reported to be the average of the chemical shifts of the unionized and dianionic forms (43). Therefore, to estimate the two individual ionization constants, we used the same approximations that we used for fumaric acid. This gave apparent pK_{a1} and pK_{a2} values of 3.4 and 5.3, 3.6 and 5.4, and 4.1 and 5.8 for water, 9% d_6-

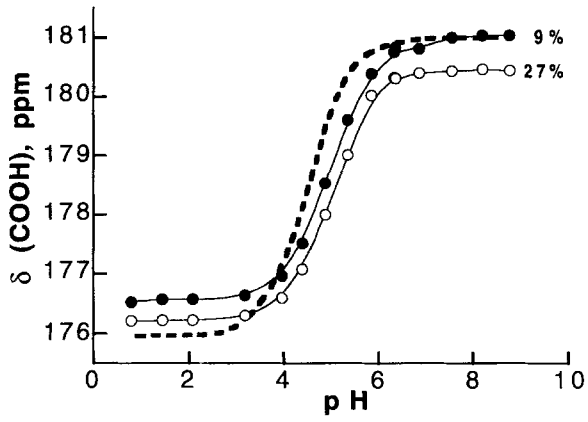

Fig. 8. ^{13}C-NMR titration curves for 2,2′-methylenebis[5-carbomethoxy-4-methylpyrrole-3-[1-^{13}C]-propanoic acid] (**12**) (7–8 × 10^{-4} M) in aqueous buffers containing 9% and 27% d_6-DMSO. The dotted line is the titration curve for ^{13}C-labeled mesobilirubin XIIIα (**3**) in buffer containing 27% d_6-DMSO taken from reference 24.

DMSO/H_2O, and 29% d_6-DMSO/H_2O, respectively. The literature values for water are 2.95 and 5.41 (14).

Dipyrrole **12** (Fig. 3) contains two propionic acid side-chains that are in similar chemical environments to the corresponding acid side-chains of MBR XIIIα (**3**) and BR (**1**) (Fig. 1). However, because **12** lacks the lactam rings that are present in both MBR XIIIα and BR, its propionic acid groups cannot form the same network of intramolecular H-bonds that can occur in BR free acid or its anions. Consequently, **12** is a useful model for a partially H-bonded or non-H-bonded BR. **Figure 8** shows ^{13}C-NMR titration curves for **12** in 9% and 27% d_6-DMSO/H_2O. (The curve for **12** in 2.7% d_6-DMSO/H_2O was almost identical to that for 9% d_6-DMSO/H_2O and is not shown in the figure for clarity.) Also included in Fig. 8, for comparison, is the curve for MBR XIIIα in 27% d_6-DMSO/H_2O published previously (24, 25). The curves for **12** and for MBR XIIIα are similar, which indicates that any potential for intramolecular H-bonding in MBR XIIIα has little effect on its titration curve and, thus, on its carboxyl dissociation constants. The curves for **12** show that both of its pK_as lie between 3.8 and 6.1, as expected for a simple propionic acid, with individual values of 4.2 and 5.4 for pK_{a1} and pK_{a2}, respectively, in water. During measurements of the titration curve for **12** in 2.7% d_6-DMSO/H_2O some precipitation and turbidity developed at low pH values, reducing the concentration of **12** in solution. Despite this, a smooth titration curve was obtained. Thus, precipitation of acids during the NMR measurements does not cause significant interference, provided that sufficient material remains in solution for accurate ^{13}C chemical shift measurements.

DISCUSSION

Determining the aqueous K_as of acids such as protoporphyrin, heme, biliverdin, and BR that are barely soluble in water requires sensitive analytical techniques, and aggregation or precipitation (particularly below pH 7) may cause interference. The K_as for protoporphyrin and biliverdin have not apparently been measured, and pK_a for heme has been reported only recently (46). In contrast, there is an extensive confusing literature on the K_as of BR.

The K_as of BR were first discussed by Overbeek, Vink, and Deenstra (7), who assumed that the two COOH groups did not interact and that the K_as are twice and one-half of those expected for pyrrole propionic acid (i.e., pK_{a1} = 4.4; pK_{a2} = 5.0). Since, then several groups have used electrometric and acidimetric titrations to determine the pK_as experimentally, concluding that *1*) the individual pK_as for BR in water are much less than 7 (8,13); *2*) the sum of pK_{a1} and pK_{a2} is 7.55 (9); and *3*) pK_{a1} and pK_{a2} are 4.3 and 5.4 (10). However, the methods used may be unreliable because of aggregation and precipitation near pH 8.3 (6).

Spectrophotometric methods also have been used. These, too, are subject to interference by precipitation and are complicated by dimerization of pigment and by the fact that the individual spectra of dimerized species and of BR monoanions are not known (4, 5, 47, 48). In addition, the method is intrinsically insensitive because ionization of the COOH groups does not result in strong spectra changes. Using spectrophotometry, Gray, Kulczycka, and Nicholson (49) were unable to determine accurate pK_a values, but concluded that (pK_{a1} + pK_{a2}) is ~7.1; Kolosov and Shapovalenko (12) concluded that pK_{a1} and pK_{a2} are 4.50 and 5.90, respectively, and Lee, Daly, and Cowger (10), using non-aqueous solutions, obtained similar values (4.3 and 5.4). Kolosov and Shapovalenko (11) also found with a solubility method that (pK_{a1} + pK_{a2}) is 9.5.

Because of problems with traditional methods, Hansen, Thiessen, and Brodersen (13) used natural abundance ^{13}C NMR. Internal standards were used and aqueous pK_as were calculated from pK_as measured in d_6-DMSO. They concluded that the pK_as for both COOH groups of BR in water are similar and close to 4.4.

Thus, all determinations and estimates published before 1985 are consistent with pK_as within the normal range for aliphatic carboxylic acids and no greater than 6 for the individual COOH groups. Subsequently, increasingly higher, and chemically unusual, values, ranging from ~6 to >9.2, have been reported (15, 16, 30, 31).

^{13}C NMR is one of the most direct spectroscopic methods for determining pK_as (26–29). It relies on the

large difference in ^{13}C chemical shift (δ) between the ionized and unionized forms of the COOH group (50) which is measurable with high accuracy and reproducibility. Unfortunately, the natural abundance of ^{13}C is only 1.1% and ^{13}C NMR is generally too insensitive to measure aqueous K_as of acids with low water solubility. In preliminary studies we found that pK_as of pyrrolic carboxylic acids related to BR can be measured by using ~100% ^{13}C labeling of COOH groups and limited amounts of d_6-DMSO as co-solvent (24, 25). We used the mono- and di-carboxylic acids **5** and **3** (Fig. 1) as models for BR, and to investigate the possible effects of H-bonding we compared the ^{13}C-NMR titration curves for **5** and **3,** in which H-bonding of the BR type is possible, with those for **4, 6, 7,** and **8,** in which it is not. To validate the method we measured pK_as of simple mono- and di-carboxylic acids with well-established pK_as (23, 25). We noted the following results. *1)* pK_as measured on solutions containing <31 mole % d_6-DMSO were within ~0.2 pK_a units of those measured in its absence and could be linearly extrapolated to give accurate values for water. The feeble effect of d_6-DMSO is consistent with observations by others (51–53). *2)* Intramolecular H-bonding of the BR type has little effect on pK_as. For example, mesobiliverdin XIIIα (**4,** Fig. 1) and MBR XIIIα (**3**) have similar pK_as (3.9 and 5.3 for **4** versus 4.2 and 4.9 for **3**) (24). That conclusion is supported by the close similarity of the of pK_as of dipyrrolic acid **12** (Fig. 3) and MBR XIIIα (**3,** Fig. 1) (Fig. 8). The similarity of the curves for **12** and **13** and for the monopropionic bilirubin **5** (Fig. 1) (24) also indicates that the COOH groups in MBR XIIIα behave more or less independently and that ionization of one COOH does not substantially influence subsequent ionization of the other.

Our earlier findings were inconsistent with the hypothesis (15–17, 30, 34–36) that intramolecular H-bonding has a marked effect on the K_as of BR and with pK_as of 6–9 (15, 16, 30, 31). However, there remained the possibility that the co-solvent we used interfered with H-bonding and led to spurious results. Therefore we investigated the effect of d_6-DMSO on the ^{13}C-NMR titration curves of ^{13}C-labeled maleic (**9,** Fig. 3), fumaric (**11**), and phthalic acids (**10**). In maleic acid intramolecular H-bonding decreases the first pK_a and increases the second with respect to fumaric acid, for which the intramolecular H-bonding is sterically impossible. Were d_6-DMSO to rupture H-bonding, as often suggested for BR (15–17, 34, 35), its presence would be expected to increase the pK_{a1} for maleic acid and to lower pK_{a2}, making the titration curve appear like that for fumaric acid. We observed no such effect. pK_as for maleic acid in water (1.8 and 6.0) and 28% d_6-DMSO/H_2O (1.9 and 6.4) were similar and close to the literature values of 1.92 and 6.23 for water. Titration curves for the two solvents were parallel, with no relative displacement along the pH axis (Fig. 6). Similarly, added d_6-DMSO had little effect on the shapes of the titration curves for phthalic acid (Fig. 7). Thus, at the concentrations used in our studies, d_6-DMSO has no significant pK_a-lowering effect. This observation validates our earlier pK_a determinations and the use of d_6-DMSO as co-solvent, even for measurements on carboxylic acids, such as BR and MBR XIIIα, that have the potential for intramolecular H-bonding. Claims that DMSO ruptures all intramolecular H-bonding in BR or that ionization loosens it are not supported by recent NMR data and are misleading. NMR measurements of BR in 100% DMSO indicate that it has a conformation similar to that in chloroform on the solid state, with the COOH groups juxtaposed close to, and possibly H-bonded to, pyrrole and lactam hydrogens (33). To what degree DMSO participates in intramolecular H-bonding of BR or disrupts it is unknown (33, 54, 55) and any such effects are unlikely to be relevant to our studies because we used a large molar excess of water and because DMSO is comparable to water as H-bonding acceptor (56).

In addition to our experimental evidence, there is compelling literature evidence that intramolecular H-bonding is unlikely to suppress ionization of the carboxyl groups in BR or lead to extremely high pK_as. Maleic acid and monoethyl ester (**13, Fig. 9**), in which the carboxyl is intramolecularly H-bonded to the ester carboxyl oxygen, has a pK_a of 2.96 (57), which is slightly lower than that of fumaric acid monoethyl ester (**13,** pK_a 3.23) (57) and similar to that of fumaric acid (**11,** pK_a 3.02) in which the carboxyl groups are not intramolecularly H-bonded (14). Similarly, salicylic acid methyl ether (**15,** pK_a 4.09) and methyl phthalate (**16,** pK_a 3.25) (57) have lower pK_as than benzoic acid (**17,** pK_a 4.20), in which there is no intramolecular H-bonding (14). In salicylic acid itself (**18,** pK_a 2.98) and 2,6-dihydroxybenzoic acid (**19,** pK_a 2.30) intramolecular H-bonding of the carboxyl groups lowers the pK_as substantially with respect to benzoic acid (pK_a 4.20) or 3,4-dihydroxybenzoic acid (**20,** pK_a 4.47) because of stabilization of the carboxylate ion by H-bonding to phenolic groups (14, 37). Thus, the argument (15–17) that intramolecular H-bonding in BR increases its pK_as by several orders of magnitude is specious. In fact, H-bonding might be expected to diminish pK_{a1} because the ionization of either COOH group leads to a carboxylate anion that is stabilized by H-bonding to pyrrole NHs, as in partial structure **22** (**Fig. 10**).

Although the persistently proposed rationale for peculiarly high pK_as (13–16, 30) is clearly insubstantial, that is not to say that H-bonding of COOH protons cannot suppress dissociation and increase pK_as. The phenomenon is well known, and is reflected, for example,

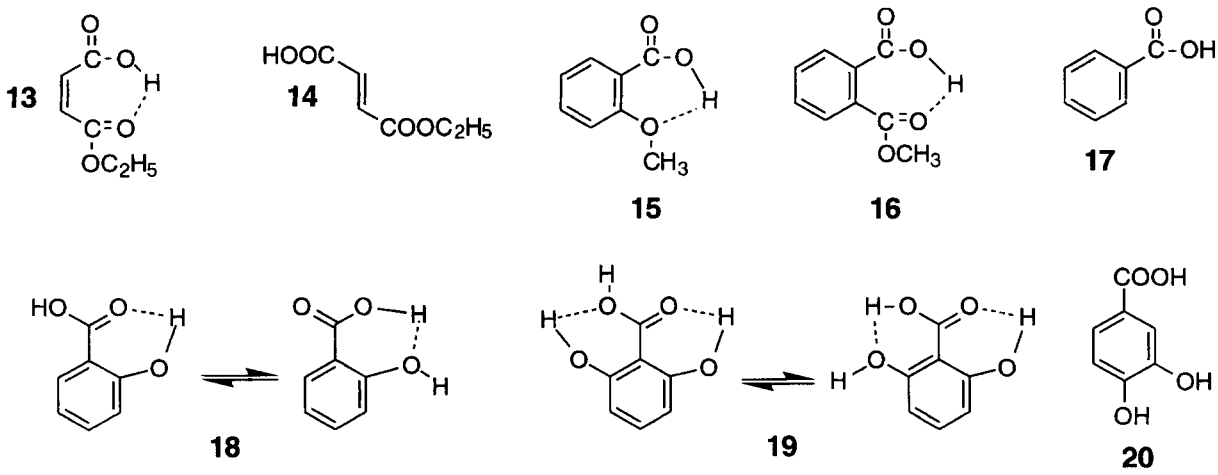

Fig. 9. Chemical structures of ethyl maleate (**13**), ethyl fumarate (**14**), salicyclic acid methyl ether (**15**), methyl phthalate (**16**), benzoic acid (**17**), salicyclic acid (**18**) 2,6-dihydroxy-benzoic acid (**19**) and 3,-4-dihydroxy-benzoic acid (**20**).

in pK_{a2} for maleic acid (6.23), which is considerably lower than that for fumaric acid (4.38). But instances where the pK_a increase is as large as proposed for BR (16) invariably involve carboxyl protons that are H-bonded to negatively charged atoms, such as a carboxylate oxygen, and other structural features that make them inappropriate models for BR (14, 37, 38, 58, 59). In the rather rare examples where intramolecular H-bonding of COOH groups in neutral molecules does increase pK_a the increase is usually less than one pK_a unit (60–64). A striking example of the weak effect of H-bonding on pK_{a1} of a diacid and a large effect on pK_{a2} is diacid **23** (**Fig. 11**), for which pK_{a1} is 4.8 and pK_{a2} is 11.1 in aqueous ethanol (65). In general, however, the effects of intramolecular H-bonding on the ionization of carboxylic acids are weak (66).

Why recent studies yielded such high unlikely values for the pK_as of BR is puzzling; methodological and technical problems are probably to blame. Using spectrophotometry, Moroi, Matuura, and Hisadome (30) obtained values of 6.1–6.5 and 7.3–7.6 for pK_{a1} and pK_{a2} respectively, whereas Russell et al. (31) obtained ~6 and 8.3. We have already noted some of the potential problems with this method. In addition, both groups assumed that the spectral changes observed were caused only by ionizations of the COOH groups, which is at odds with Brodersen's observation (4) that the absorption spectra of dilute (0.3 μM) solutions of BR at pH 7.0, 8.0, and 9.0 are identical. A priori, wide separations of pK_{a1} and pK_{a2} would not be expected for a molecule containing two spatially separated, non-interacting COOH groups.

Even higher estimates have been obtained by solubility and partitioning procedures (15, 16, 30). Using solubility measurements Kolosov and Shapovalenko (11) found (pK_{a1} + pK_{a2}) to be 9.5 but Moroi et al. (30) obtained 6.01 and 7.60 for the two pK_as of BR while Ostrow, Celic and Mukerjee (15) reported 5.6–6.8 for pK_{a1} and a phenomenal >9.2 for pK_{a2}. Confusingly, the same group (16) have also reported pK_as of 8.12 and 8.44 based on partition studies. Those studies, though superficially rigorous, are technically flawed. They involved considerable sample manipulation; corrections had to be applied for suboptimal recoveries; BR concentrations were measured only by the nonspecific insensitive diazo assay (67, 68); diazo measurements were made at only one wavelength, without adequate controls; no evidence was presented, other than diazo reactivity, that the molecular species extracted into aqueous phases

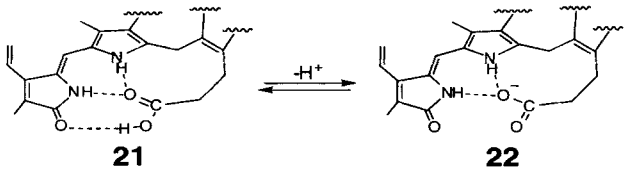

Fig. 10. Partial structures of bilirubin (**21**) and one of its monoanions (**22**) showing possible intramolecular hydrogen bonding.

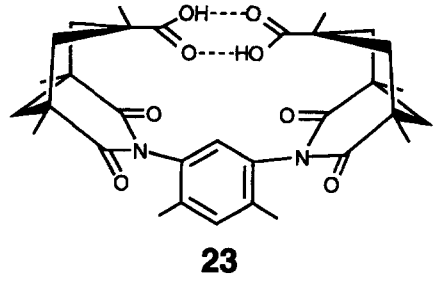

Fig. 11. Forced intramolecular hydrogen bonding in Rebek's diacid **23.**

was, in fact 4Z,15Z-bilirubin IXα, as assumed; comparative control studies with biliverdin were not done; and chloroform, which has been shown to be unsuitable for such studies (69) was used as the organic phase. Earlier partitioning studies, not cited in (16), done with more suitable water-immiscible organic solvents gave data consistent with pK_as of ~4.3 and 5.3 (70), close to our measured values for MBR XIIIα (24). In principle, the solubility and partitioning methods are reliable. However, they require accurate measurement of extremely low concentrations of BR in water at acid pHs, which is difficult, and are prone to interference from traces of water-soluble diazo-reactive bilirubinoid impurities. The solubility method also requires knowledge of the intrinsic water solubility of unionized BR, which has proved difficult to measure accurately and is controversial (15, 16, 30). In contrast, the ^{13}C-NMR technique does not require measurement of concentrations, is insensitive to trace amounts of diamagnetic impurities, and unlike the partitioning procedure used recently (16) requires no evaporation or extensive manipulation and sampling of solutions.

CONCLUSIONS

In this and previous papers (23–25) we have shown systematically: *i*) that ^{13}C NMR in aqueous d_6-DMSO solutions is a reliable method for determining aqueous pK_as of mono- and dicarboxylic acids, even those that are intramolecularly H-bonded; *ii*) that mono-, di-, and tetrapyrroles containing propionic acid side-chains have pK_as similar to those of simple aliphatic acids; *iii*) that acids that can undergo the same type of intramolecular H-bonding as BR have pK_as similar to those that cannot; and *iv)* that the pK_a of BR analog **5,** with only one propionic acid, is 4.3. With that work as foundation we measured the pK_as of the COOH groups of MBR XIIIα and found that both are <6 with pK_{a1} ~4.2 and pK_{a2} ~4.9 (24). As the constitutional and three-dimensional structures of MBR XIIIα and BR are similar, we conclude that the two pK_as of BR also are likely to be in the range ~4.2–4.9. That conclusion is broadly consistent with widely overlooked solvent partitioning studies (69, 70) and all observations on the pK_as of BR made before 1985. Our studies reveal no peculiar effect of DMSO on H-bonding in BR and raise doubts about the accuracy of recent determinations by solubility (15, 26), spectrophotometric (26, 27), and solvent partitioning methods (16) and the significance of biological models based on them (17). Our data indicate that the predominant species present in simple aqueous solutions of BR at physiologic pH is the dianion, as previously pointed out by Brodersen (4, 5, 48) among others. This does not exclude the possibility that BR monoanions play a special role in phase transfer of BR in vivo, as proposed by Wennberg (71). The pK_as of BR will, of course, be dependent on the medium and values for water may differ from those for BR in other environments, such as bile or lipids. Studies on fatty acids and bile acids (27, 28) suggest that the pK of BR in a membrane might be some three units higher than in water. Our studies indicate that the aqueous ionization of BR is not abnormal or unusual or much influenced by intramolecular H-bonding, contrary to earlier unsubstantiated assumptions (15–17). What is odd about BR compared to other dicarboxylic acids such as biliverdin and protoporphyrin is its extraordinary lipophilicity (72) which seems to have a dominant effect on its transport and metabolism (32, 72). But for that, BR probably would not cross the placenta in fetal life (73) or require glucuronidation for hepatic excretion postnatally. Focusing only on ionization, neglecting three-dimensional structure and accepting improbably high aqueous pK_a values is liable to produce a jaundiced perspective of BR transport and metabolism.

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