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Dissociation constants of water-insoluble carboxylic acids by ^{13}C-NMR. pK_as of mesobiliverdin-XIIIα and mesobilirubin-XIIIα

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Abstract. High-field ^{13}C-NMR of ^{13}C-enriched compounds in dilute aqueous d_6-Me_2SO solutions provides a simple, accurate method for measuring pK_as of sparingly soluble carboxylic acids. Using this method, we found the pK_as of mesobilirubin-XIIIα to be 4.2 and 4.9, much lower values than reported recently for bilirubin, and of mesobiliverdin-XIIIα to be 3.9 and 5.3.
Key words. Bilirubin; biliverdin; dimethylsulfoxide; dicarboxylic acids.

Biliverdin (**1**) and bilirubin (**2**) (fig. 1) are naturally-occurring tetrapyrrolic pigments containing two propanoic acid side-chains. These side-chains and their propensity for ionisation are critical in the biological disposition of biliverdin and bilirubin[1,2]. Surprisingly, accurate dissociation constants for the propanoic acid groups of **1** are not available and a wide range of values, extending over some four orders of magnitude, has been suggested for the pK_as of the propanoic acid groups of **2** in aqueous solutions[1–7]. Recently, pK_as of 6.7–9.3 have been reported for bilirubin[6,7] – values much greater than the value of ~5 typical of propanoic acid groups[8]. These curiously high values, thought to be caused by intramolecular hydrogen-bonding[6,7], are currently being used as a basis for theories of the biological transport and metabolism of **2** and related compounds[9].

Both **1** and **2** have very low solubility in water at neutral or acidic pH[1,10,11]. This has hampered determination of their dissociation constants by electrochemical methods. ^{13}C-NMR spectroscopy offers an alternative approach since the chemical shift of the carboxyl carbon in aqueous environments is highly sensitive to dissociation of the carboxyl hydrogen[12]. In water and in aqueous Me_2SO, COO^- chemical shifts (δ_{COOH^-}) are typically ~5 ppm more deshielded than those of ^{13}COOH (δ_{COOH})[12–14]. This 'titration shift' allows direct monitoring of acid dissociation equilibria and determination of pK_a. However, when samples contain only natural abundance (~1.1%) ^{13}C, the method is insensitive and requires high sample concentrations. These drawbacks can be overcome by using ^{13}C-enriched samples and high field (500 MHz) instruments. Thus, Holmes and Lightner[14] showed recently that the dissociation constants of several carboxylic acids, including water-insoluble mono- and bilirubin, can be readily determined by ^{13}C-NMR measurements on dilute solutions (10^{-5}–10^{-6} mol dm^{-3}) of derivatives highly enriched with ^{13}C in their COOH groups[14]. Additionally, using ^{13}C-labeled phenyl-

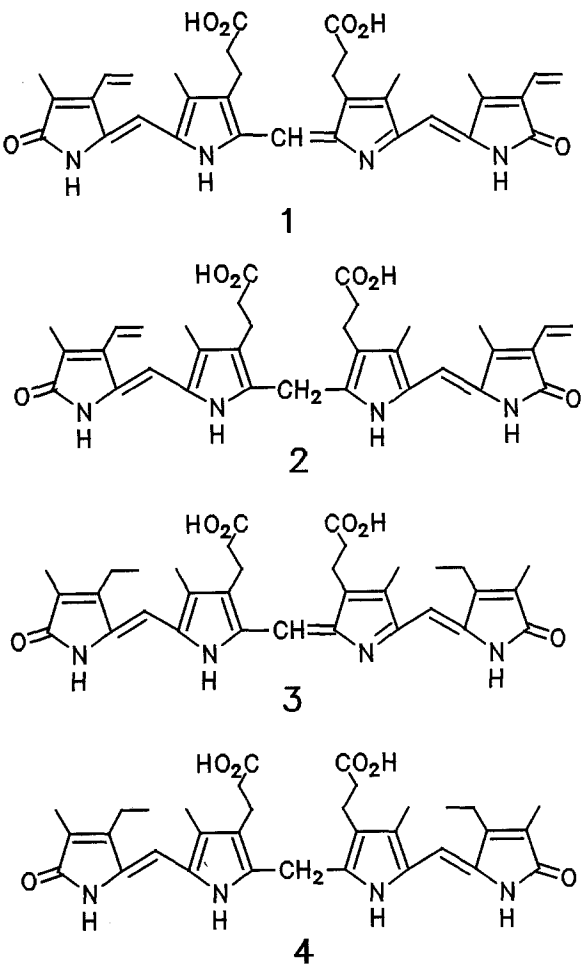

Figure 1. Linear representations of **1** (biliverdin), **2** (bilirubin), **3** (mesobiliverdin-XIIIα) and **4** (mesobilirubin-XIIIα).

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propanoic and phenylacetic acids as standards, they found that pK_a determinations made on dilute aqueous solutions containing up to 31 mole% d_6-Me_2SO differed little from those made in the absence of d_6-Me_2SO and that measurements made in dilute d_6-Me_2SO water mixtures could be extrapolated reliably to give values for water. The ability to use Me_2SO as cosolvent provides a useful method for overcoming the solubility problems that have hitherto hindered pK_a determinations of water-insoluble dicarboxylic acids such as biliverdin and bilirubin[1,2]. We have now used ^{13}C-NMR to measure the acid dissociation constants of 99% enriched ^{13}COOH mesobiliverdin-XIIIα **3** and mesobilirubin-XIIIα **4** (fig. 1). These compounds are structurally similar to the natural analogs **1** and **2** and would be expected to have similar pK_as.

Figure 2 shows the variation in ^{13}C-carboxyl chemical shift (δ_{obs}) with pH for solutions of **3** containing 8.6 mole% (27 vol%) d_6-Me_2SO. Similar curves, shifted slightly downwards, were observed for solutions containing higher concentrations of d_6-Me_2SO (up to 31 mole%). **3** exhibited titration shift values ($\Delta = \delta_{dianion} - \delta_{diacid}$) typical of carboxylic acids: 5.1, 5.1 and 4.8 in water, 2.5 mole% d_6-Me_2SO and 8.6 mole% d_6-Me_2SO respectively. Therefore, the curve of figure 1 reflects a transition from $-(COOH)_2$ to $-(COO^-)_2$. The curve shows no indication of an inflection, as expected[15] for a dicarboxylic acid with individual pK_as (pK_{a1} and pK_{a2}) that differ by less than ~2–3, and it clearly defines the lower and upper limits of pK_{a1} and pK_{a2} as 3 and 6, respectively.

Approximate values for pK_{a1} and pK_{a2} can be derived from the δ_{obs}/pH curve by assuming that δ for the monoanion lies halfway between δ_{diacid} and $\delta_{dianion}$. With this assumption, pK_{a1} is equal to the pH at which $\delta_{obs} = \delta_{diacid} + 0.25\Delta$; and pK_{a2} is equal to the pH at which $\delta_{obs} = \delta_{diacid} + 0.75\Delta$, where Δ is the titration shift. This simple approximation gave pK_{a1} and pK_{a2} values of 3.58 and 5.16, respectively, for **3** in 8.6 mole% d_6-Me_2SO/water, which extrapolated to 3.76 and 5.23, respectively, for water. For a better approximation we measured δ_{obs} vs. pH for ^{13}C-adipic acid, a six-carbon aliphatic dicar-

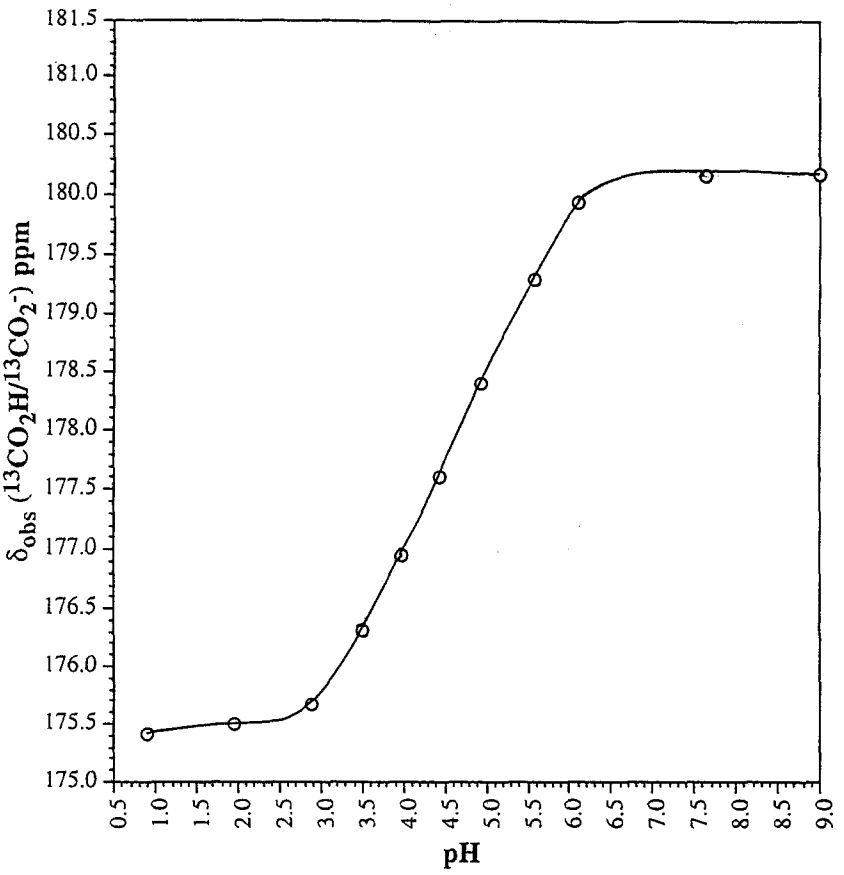

Figure 2. ^{13}C-NMR titration curve showing the pH-dependent behaviour of the carboxyl carbon (δ_{obs}) for 99% $^{13}CO_2H$ enriched **3** in aqueous buffer containing 8.6 mole% d_6-Me_2SO. Solutions (8×10^{-5}–2×10^{-6} mol dm^{-3}) of **3** and **4** (ref. 29) and their tetra-*n*-butylammonium salts[30] in aqueous buffers containing Me_2SO (99.9% d_6) were prepared as described previously[14] and used for 500 MHz proton-decoupled ^{13}C-NMR measurements at 25 °C. Below pH 3.2, solutions were unbuffered and 0.1 mol dm^{-3} MeCOOH or MeCOOH/HCl or 0.2 mol dm^{-3} HCl were used to adjust pH. Above pH 3.2, solutions were buffered with 0.1 mol dm^{-3} sodium acetate (pH ~ 3.2–6.8) or 0.1 mol dm^{-3} Tris (pH > ~6.8). Complete δ_{obs}/pH curves, each based on 10–15 sample solutions, were measured for solutions containing 8.6 and 31 mole% d_6-Me_2SO. A sealed capillary insert containing d_6-Me_2SO was used to standardize chemical shifts and a glass electrode calibrated with standardized buffers was used to determine pH.

boxylic acid with well-established pK_as (4.44 and 5.44)[8], and found that pK_{a1} was equal to the pH at which $\delta_{obs} = \delta_{diacid} + 0.354\Delta$; and that pK_{a2} was equal to the pH at which $\delta_{obs} = \delta_{diacid} + 0.785\Delta$. Using these calibrated correction factors we obtained pK_{a1} and pK_{a2} values of 3.95 and 5.33, respectively, for 3 in 8.6 mole% d_6-Me_2SO/water and 3.92 and 5.28, respectively, for **3** in water. These values are as expected for a dicarboxylic acid containing independent, non-interacting, spacially-separated aliphatic carboxyl groups[8,15]. They are considerably lower than estimates ($pK_{a1} \sim 5.0$, $pK_{a2} \sim 7.2$) based on acidimetric titration curves in water, during which **1** precipitated[2].

In an entirely analogous way, pK_a values for **4** were found to lie between 3.5 and 5.5 and estimated to be 4.2 and 4.9 in water. These values are similar to those (4.4 and 5.0) originally suggested[10] for **2** in 1955 on the basis of neglected, and often misinterpreted[1,16], titrimetric solubility studies, and they are close to later estimates [4.3 and 5.4 (ref. 16), 4.5 and 5.9 (ref. 3) average 4.4 (ref. 4)] obtained by several different methods, including natural abundance ^{13}C-NMR (ref. 4). They differ substantially from recent pK_a estimates for bilirubin derived from more error-prone solubility and partitioning experiments[6,7] and calculated on the basis of a mathematical model limited by arguable assumptions, particularly regarding the solubilities, aggregation and phase-transfer properties of bilirubin species.

Though similar in constitution, **1** and **2** (and model compounds **3** and **4**) have markedly different three-dimensional structures. **1** and **3** exist in solution predominantly as helical conformers[17,18], whereas **2** and **4** preferentially adopt a folded 'ridge-tile' conformation extensively stabilized by six intramolecular hydrogen bonds between the nitrogenous edge of each pyrromethenone moiety and the carboxyl group of a propanoic side-chain (fig. 3)[19–22]. Crystallographic[23] and spectroscopic[21–24] evidence indicates that a similar folded hydrogen-bonded conformation (with only four, probably stronger, hydrogen bonds) persists in the dianion. Comparison of pK_{a1} for **3** and **4** indicates that intramolecular hydrogen-bonding has only a small effect on the dissociation constant of **4**. It could be argued that Me_2SO, a hydrogen-bond acceptor, disrupts hydrogen-bonding in **4**, lowering the pK_as, relative to water. However, recent NMR NOE studies[22] indicate that, despite possible intercalation of Me_2SO solvent molecules into the structure, a partially intramolecularly hydrogen-bonded ridge-tile conformation is maintained even in Me_2SO.[25] Therefore, effects of Me_2SO in dilute solutions containing a large molar excess of water are likely to be small. Obversely, there appears to be no precedent for intramolecular hydrogen-bonding of unsubstituted aliphatic COOH groups engendering pK_as as high as 9.3 (ref. 6) or even 7.6 (ref. 5) except for examples, such as the maleate monoanion[26], that are not structurally relevant to dissociation of **2** or **4**.

Extending earlier work on monocarboxylic acids[27,28], our studies indicate that high-field ^{13}C-NMR of ^{13}C-enriched compounds in dilute aqueous d_6-Me_2SO solutions affords a simple and accurate method for measuring pK_as of mono- and dicarboxylic acids that have low solubility in water. They suggest that intramolecular hydrogen-bonding has only a small effect on acidity in bilirubins. Mesobilirubin-XIIIα (**4**) and bilirubin (**2**) have similar physico-chemical properties and are metabolized at similar rates in rats. Therefore, despite the small side-chain differences, the pK_as for natural biliverdin and bilirubin are likely to be close to 3.9 and 5.3, and 4.2 and 4.9, respectively, as measured here for the meso analogs **3** and **4** and close to those expected for remote pairs of propanoic acid groups within the same molecule. It seems likely that biliverdin and bilirubin are fully ionized in vivo in the aqueous phase of blood, bile or

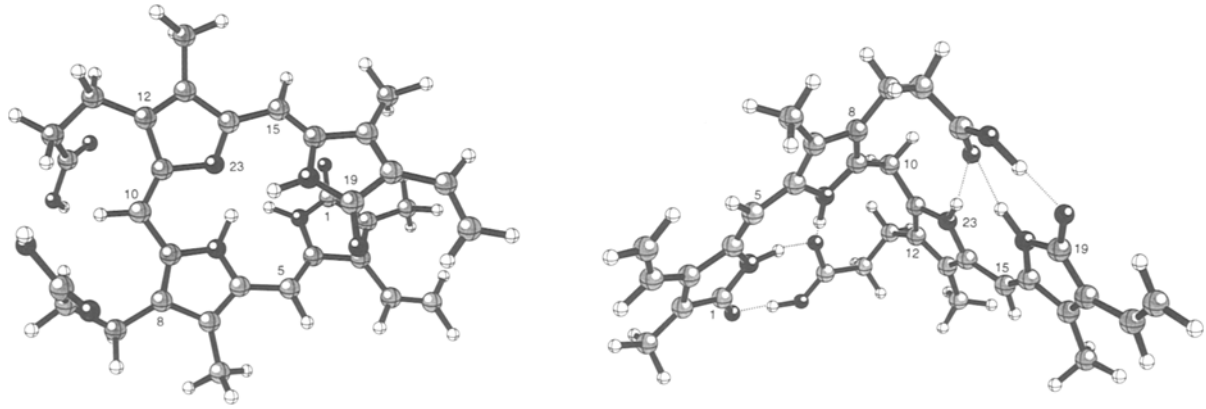

Figure 3. Conformational drawings of **1** (left) and **2** (right) from Müller and Falk's Ball and Stick program (Cherwell Scientific, Oxford, U.K.) for the Macintosh.

cytoplasm and that recent estimates of the pK_as for bilirubin[5–7] are far too high and possibly irrelevant.

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