

Research article

Low solubility of unconjugated bilirubin in dimethylsulfoxide – water systems: implications for pK_a determinations

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Abstract

Background: Aqueous pK_a values of unconjugated bilirubin are important determinants of its solubility and transport. Published pK_a data on an analog, mesobilirubin-XIII α , studied by ¹³C-NMR in buffered solutions containing 27 and 64 vol% (C²H₃)₂SO because of low aqueous solubility of mesobilirubin, were extrapolated to obtain pK_a values in water of 4.2 and 4.9. Previous chloroform-water partition data on bilirubin diacid led to higher estimates of its pK_a , 8.12 and 8.44, and its aqueous solubility. A thermodynamic analysis, using this solubility and a published solubility in DMSO, suggested that the systems used to measure ¹³C-NMR shifts were highly supersaturated. This expectation was assessed by measuring the residual concentrations of bilirubin in the supernatants of comparable DMSO-buffer systems, after mild centrifugation to remove microprecipitates.

Results: Extensive sedimentation was observed from numerous systems, many of which appeared optically clear. The very low supernatant concentrations at the lowest pH values (4.1-5.9) were compatible with the above thermodynamic analysis. Extensive sedimentation and low supernatant concentrations occurred also at pH as high as 7.2.

Conclusions: The present study strongly supports the validity of the aqueous solubility of bilirubin diacid derived from partition data, and, therefore, the corresponding high pK_a values. Many of the mesobilirubin systems in the ¹³C-NMR studies were probably supersaturated, contained microsuspensions, and were not true solutions. This, and previously documented errors in pH determinations that caused serious errors in pK_a values of the many soluble reference acids and mesobilirubin, raise doubts regarding the low pK_a estimates for mesobilirubin from the ¹³C-NMR studies.

Background

The saturation status of unconjugated bilirubin (UCB) is relevant to understanding the pathophysiology of jaun-

dice and to interpreting experiments with UCB [1]. UCB, in its diacid form (H₂B), has a low solubility (S₀) of 51

nM in water [2]. The total solubility (S) at any pH is determined by S_o , pK_a values, and pH [2]:

$$S = [H_2B] + [HB^-] + [B^{2-}] = S_o \cdot (1 + K_{a1}/[H^+] + K_{a1} \cdot K_{a2}/[H^+]^2) \quad (Eq. 1)$$

Self-association of UCB dianion, B^{2-} , can also increase S [2].

Beginning in 1995, a series of papers reported the use of ^{13}C -NMR to study the ionization of a close analogue of bilirubin-IX α , mesobilirubin-XIII α (MBR), along with several soluble reference acids, in buffered mixtures of $(C^2H_3)_2SO$ and water [3–7]. Due to the very low aqueous solubility of MBR, data were obtained only at two high concentrations of $(C^2H_3)_2SO$ (64 and 27 vol%). Its pK_a values in "water" (actually in 1 vol% of $(C^2H_3)_2SO$), obtained by an extrapolation procedure based on the behavior of soluble reference acids, were 4.2 and 4.9, far lower than values of 8.12 and 8.44 obtained by solvent partition between chloroform and buffered aqueous solutions [2].

These serious discrepancies led us to reexamine several experimental aspects of the ^{13}C -NMR studies and the implications of their purported low pK_a values for the solubility of UCB. Inaccuracies in the measurements of buffer pH and of the pK_a values of the reference ^{13}C -carboxylic acids, related to failure to correct for the strong effects of DMSO on the pK_a values of weak acids, have been previously noted [8] and acknowledged [9]. In addition, as discussed later, a thermodynamic theory about solubility in mixed solvents, using the S_o values of 51 nM in water [2] and 10 mM in pure DMSO [10], suggested that S_o of UCB would be only about 0.15 μM in 27 vol% DMSO and 2.2 μM in 64 vol% DMSO, which are well below concentrations used in the MBR studies. This implies serious supersaturation effects. In fact, turbidity was reported for some of the systems used in the ^{13}C -NMR papers [4], indicating the formation of coarse suspensions. By definition, as stressed in the Conclusions section, pK_a determinations are valid only for monomeric species and require that solutions are below saturation at all pH values studied. Even reversible aggregation of monomers in undersaturated solutions is known to affect pK_a estimates [2]. If supersaturation leads to the formation of fine sols or coarse suspensions, the data are unacceptable for determination of the pK_a values of monomers.

In the present paper, we assess whether some of the systems used in the ^{13}C -NMR studies were supersaturated with MBR. Our experimental work on sedimentation was done with unconjugated bilirubin (UCB) and we used DMSO instead of its deuterated analogue, $(C^2H_3)_2SO$. It is known that, when alkaline aqueous UCB is acidified to neutral or low pH's, "Usually a colloid suspension of bi-

lirubin is formed and the solution remains clear, as observed by the naked eye, thus inviting an erroneous interpretation" [11]. Such systems, which simulate true solutions, often show sedimentation on centrifugation [12–16]. We here report that sedimentation of UCB from apparently clear solutions can likewise be extensive when UCB in DMSO is diluted with aqueous buffers to final mole fractions (N) of DMSO = 0.025, 0.086 and 0.31, corresponding to 9, 27, and 64 vol% of DMSO. A thermodynamic theory is used to examine the effect of added DMSO on S_o . Our findings, consistent with our partition-derived S_o , S and pK_a values of UCB in aqueous buffers [2], indicate that the recently reported low pK_a values of MBR in comparable $(C^2H_3)_2SO$ -water systems [3–6], were determined above saturation.

Results

Residual UCB in the supernatants after centrifugation

Many systems initially appeared optically clear, but well over 90% of initial UCB sedimented on centrifugation of most samples below pH 7.2. Overall recoveries (UCB in supernatant + UCB in redissolved precipitate) were between 90 and 100%. Only residual [UCB] in supernatants are reported.

Figs. 1A,1B,1C plot the measured residual [UCB] in the supernatant vs. the initial [UCB] at $N_{DMSO} = 0.31$, 0.086 and 0.025. Deviations of their ratio below unity (dotted lines) represent a decrease in [UCB], mainly from precipitation, but possibly also from limited degradation. At $N = 0.31$ (Fig. 1A), [UCB] after sedimentation varied from 2.8 μM at pH 5.86 to 166 μM at pH 8.38. At $N = 0.086$ (Fig. 1B), [UCB] ranged from 0.3 to 2.4 μM at pH 4.50 and from 1.4 to 6.4 μM at pH 7.05 and most of the UCB sedimented. By contrast, at pH 7.56 and 7.70 (phosphate), only minor precipitation was observed. At $N = 0.025$ (Fig. 1C); [UCB] ranged from 0.1 to 2.7 μM at all three pH values (4.15 to 7.18).

Sedimentation of bilirubin-albumin complexes

The supernatant from the original supersaturated UCB-HSA system, when diluted with 1/8th vol. of buffer and centrifuged again, showed more sedimentation and a progressive rise in [UCB] as one moved down the column of fluid. By contrast, after dilution with 1/8th volume of DMSO to decrease UCB saturation, the supernatants produced no further sedimentation and there were no significant differences in [UCB] or protein concentrations along the axis of the fluid column. Thus, the UCB-HSA complex, which constitutes over 99.9% of the UCB in this system [17], did not sediment.

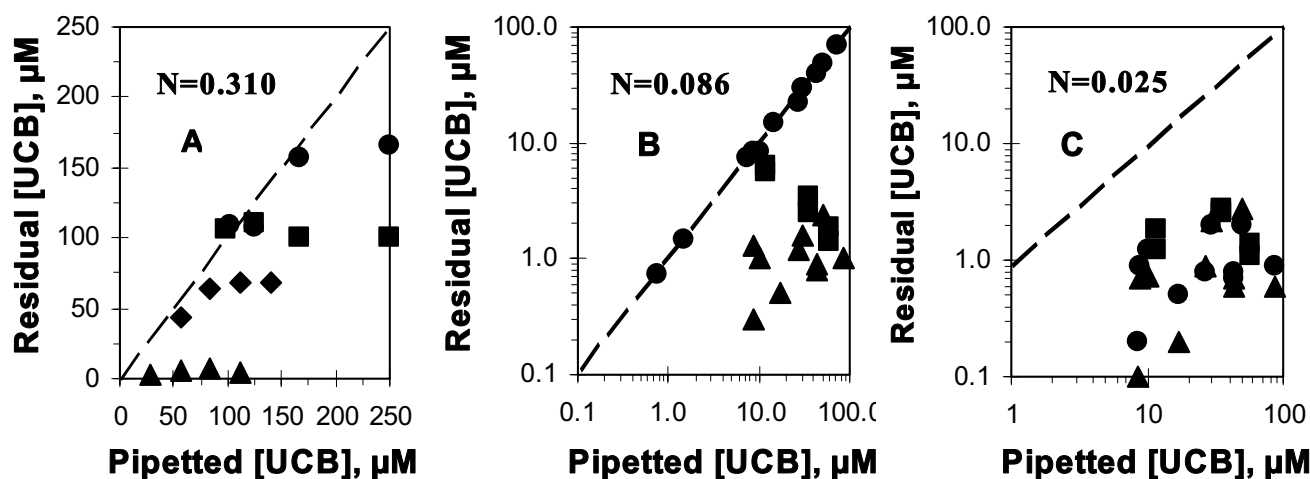


Figure 1

A-C. Residual vs. initial UCB concentrations in buffered DMSO/water systems. Measured residual [UCB] in supernatants of buffered DMSO/water systems, after centrifugation for 5 min at 14,000 g, are plotted against the pipetted, initial UCB concentrations before centrifugation. Mole fractions (N) of DMSO were 0.310 (A), 0.086 (B) and 0.025 (C). Panels B and C are log-log plots. The dashed unity line indicates complete retention of UCB in supernatant. The buffers used and the corrected pH values of the DMSO-buffer systems were: Panel A – ● Phosphate pH 8.4, ■ Tris pH 7.1, ♦ Acetate pH 6.9, ▲ Acetate pH 5.9; Panel B – ● Phosphate pH 7.6–7.7, ■ Tris pH 7.1, ▲ Acetate pH 4.5; Panel C – ● Phosphate pH 7.2, ■ Tris pH 7.0, ▲ Acetate pH 4.15. [UCB] in each supernatant was determined from A at 458 nm, using the extinction coefficient of $0.0634 \mu\text{M}^{-1}\text{cm}^{-1}$.

Discussion

Findings and their relation to thermodynamic theory

Supersaturated aqueous systems of UCB, that are optically clear before centrifugation, may exhibit considerable variation in the extent of sedimentation [12–14,18]. Although sedimentation is often extensive, it is generally incomplete and may not be observed at all. Our data in DMSO-water show the same features, which are expected from the complex kinetics of nucleation and growth of insoluble aggregates of UCB diacid (H_2B), leading to the formation of a new solid phase [19,20]. Our centrifugation, 5 min at 14,000 g, was quite mild, and the short 20-minute period between preparation of UCB-DMSO-water systems and centrifugation severely limited the time-dependent growth to large aggregates. Lack of sedimentation of the UCB-HSA complex (mol. wt. 68,000) indicates that fine colloids composed of 100 UCB molecules would be too small to sediment. Thus, supersaturated systems lacking coarse, insoluble aggregates may not show sedimentation, but any sedimentation observed indicates their presence.

To evaluate the important effect of pH on sedimentation efficiency, we calculated S in water using chloroform-water partition data on UCB and the best measure of S_0 in chloroform, 0.88 mM [2]. S at any pH, e.g. 62 nM at pH

7.4 and 0.32 μM at pH 8.5, can be calculated from the fitted partition data, or, equivalently, from Eq. 1, using the partition-derived S_0 in water of 51 nM and pK_a values of 8.12 and 8.44 [2]. In aqueous systems [12], the lowest [UCB] in water, below which no sedimentation was observed at $100,000 \times g$ for a few hours, was 100 nM at pH 7.4, modestly higher than our partition-derived S of 62 nM [2]. Even under such vigorous centrifugation, the lowest [UCB] increased rapidly with increasing pH, to 17 μM (150 times S) at pH 8.05 and 34 μM (230 times S) at pH 8.2 [12]. This indicates increasing charge-stabilization of fine, non-sedimenting colloids of H_2B by adsorbed UCB anions [12,19,20]. In contrast, below pH 6.7, sedimentation of 10 μM UCB was nearly complete [13]. This is compatible with a dearth of stabilizing UCB anions at this pH, as expected from the high pK_a values of 8.12 and 8.44 [2].

Our present data on residual [UCB] in DMSO-water systems likewise show decreased sedimentation with increasing pH (Fig. 1A,1B,1C). At each N_{DMSO} , the lowest [UCB] were at the lowest pH values: 0.1 μM ($N = 0.025$, pH 4.15); 0.3 μM ($N = 0.086$, pH 4.5); and 2.8 μM ($N = 0.31$, pH 5.9). As in water, these are likely to be closest to the S_0 values at each N. Indeed, they are only moderately higher than the corresponding S_0 values of 0.07 μM , 0.15 μM and 2.2 μM , respectively, calculated from Equation 2 us-

ing S_o values of 51 nM in water [2] and 10 mM in DMSO [10].

$$\log S_{o,mixed} = \log S_{o,water} + (\log S_{o,DMSO} - \log S_{o,water}) \times N \quad (Eq. 2)$$

Equation 2 is a thermodynamic relationship based on assumptions of complete ideality of mixing [21]. In general, a roughly linear variation of $\log S_o$ with N at low N is expected. For example, data from 1-naphthoic acid in DMSO-water [22] show that $\log S_o$ is a linear function of N up to $N = 0.35$. Such a relationship leads to a relatively small effect of low N values on S_o . Thus, according to Equation 2, S_o increases by a factor of only 1.4 at $N = 0.025$ and 2.9 at $N = 0.086$, but by a relatively larger factor of 44 at $N = 0.31$. This would markedly reduce the supersaturation factor ($[UCB]/S_o$), which is a measure of the tendency of UCB to come out of solution at $N = 0.31$. This explains in part the relatively high $[UCB]$ at high pH at $N = 0.31$ (Fig. 1A).

The pH effects on $[UCB]$ at each N_{DMSO} are of interest also. The lowest $[UCB]$ at each pH registered relatively small increases with significant increases in pH: for example from 0.1 μM (pH 4.14) to 0.2 μM (pH 7.0) at $N = 0.025$; from 0.3 μM (pH 4.5) to 1.4 μM (pH 7.1) at $N = 0.086$; and from 111 μM (pH 7.1) to 166 μM (pH 8.4) at $N = 0.31$. These increases are probably caused mainly by increasing charge-stabilization of colloidal aggregates, as in aqueous media [12,19,20]. If, instead, the relatively small increases are ascribed entirely to increases in true solubility (S) at the high pH (Eq. 1), the required pK_a values are about 7 at $N = 0.025$ and 0.086, and 8.5 at $N = 0.31$. The true pK_a s of UCB in DMSO-water are thus probably significantly higher.

We note that some variability in sedimentation results from our short-term experiments, most evident at the low residual $[UCB]$ in Figs. 1B and 1C, in part magnified by the log-log scale used. Some variability is expected, however, because of the complexity of the kinetic processes of nucleation, growth and flocculation that precede sedimentation. In Fig. 1A, the difference between acetate and Tris buffers is quite small (note the linear scale), compatible with the 58% higher $[H^+]$ in the acetate buffer. In Fig. 1B, the markedly lower sedimentation from phosphate buffers at pH 7.6–7.7, as compared to Tris buffer at pH 7.1, can be ascribed mainly to the much higher pH values and ionic strength of the phosphate systems. Another significant factor may be the difference in charge between the buffer salts; phosphate is anionic whereas Tris is cationic and zwitterionic. The cationic species of Tris can, in principle, reduce the negative charges on the surface of the colloidal H_2B sufficiently to facilitate the formation of coarser particles and, thus, increase sedimentation.

Implications for pK_a values of mesobilirubin-XIII α (MBR)

In the recent ^{13}C -NMR studies of the ionization of the ^{13}C -COOH groups of MBR [3–6], it was assumed that the relevant physical properties of UCB and MBR, and of $(CH_3)_2SO$ (DMSO) and $(C^2H_3)_2SO$, are similar. Actually, as expected from the replacement of two vinyl groups in UCB with two ethyl groups in MBR, MBR is slightly more soluble in organic solvents [23] and has a higher R_f on silica gel t.l.c. [24]; MBR is thus more hydrophobic and should be less soluble in water than is UCB. Our low $[UCB]$ in DMSO-water systems at comparable N , therefore, indicate that many of the $(C^2H_3)_2SO$ /buffer systems used in the ^{13}C -NMR studies [3–6] were likely supersaturated with MBR. In those studies, the MBR concentrations used were stated to be 1 to 100 μM at $N = 0.086$ [3], compared to our lowest $[UCB]$ of 0.3 μM at pH 4.5 and 1.4 μM at pH 7.05. At this N , 9 of 11 MBR data points were obtained at pH below 7.05 and 5 below pH 4.5 [4], so that even 1 μM MBR was likely to be supersaturated. At $N = 0.31$, our lowest $[UCB]$, 2.8 μM at pH 5.9, was close to the lower limit of the 2 to 800 μM range of $[MBR]$ used [4,5]. Thus, many data points, obtained at pH values down to 2 [4,5], were probably from supersaturated systems, despite being optically clear. As noted here and elsewhere [12–16,18], optical clarity gives no assurance of the absence of supersaturation.

Actually, turbidity was reported in some of the ^{13}C -NMR samples [4], indicating that coarse, insoluble aggregates of MBR were present. The claim that such turbidity did not affect ^{13}C -NMR measurements [3,5,6] contrasts with evidence that even small multimers can change NMR chemical shifts [25,26]. It should be noted also that, at high concentrations of B^- , extensive, reversible self-association of B^- can lead to apparently stable supersaturation with no separation of an insoluble phase [2]. For example, at pH 8.5 and a UCB concentration of 20 μM (63 times S), the weight-average aggregation number of UCB has been found to be 7.17 [18], corresponding to a molecular weight of 4,195. The aggregation number remained fairly high, 4.2, in 60% (w/v) ethanol [18]. The successful application of equilibrium ultracentrifugation for that study [18] suggests a complete absence of even small colloidal species of UCB. Self-association of MBR dianions in $(C^2H_3)_2SO$ -water mixtures cannot be ruled out on *a priori* grounds. It has been shown that neglect of self-association of B^- leads to an artefactually low estimate of pK_a values for UCB [2].

In addition to the problems of insolubility, supersaturation and self-aggregation of the MBR systems in $(C^2H_3)_2SO$ -water [3–6], we had shown previously that inaccuracies in the pH measurements affected both the magnitude of ΔpK_a (the change in pK_a on adding $(C^2H_3)_2SO$ to water), as well as the degree of the variation of ΔpK_a

with N [8]. This is important for extrapolating pK_a values in $(C^2H_3)_2SO$ -water to pure water ($N = 0$). Indeed, re-measurement of one soluble acid raised its pK_a by as much as 3 units at $N = 0.31$ [9]. Thus, the inaccuracies in pH measurement produced serious errors in reported pK_a values of more than fifteen soluble acids used as models for MBR, as well as for MBR itself [3–7].

Many methods, using appropriate pH measurements, have been applied in the past to determine thermodynamic pK_a values of soluble acids in non-aqueous or partially aqueous media, including DMSO-water systems [27,28]. Many other relevant references were given in our prior paper [8]. In that paper, our pK_a measurements on acetic acid in DMSO-water systems were based on the potentiometric method, using properly calibrated glass electrodes, which determine the activity of H^+ , and on estimates of the activity coefficients of the acetate ion. This method, which is well established for aqueous solutions, yielded results in good agreement with data from the literature that was based on a very different method, measurements of electrical conductivity [28]. In the ^{13}C -NMR papers, therefore, it was not justified, to assume that pH values do not change on adding DMSO [3–7], or to use uncalibrated pH measurements for determination of the pK_a values of soluble acids [9].

Our sedimentation data and their interpretation indicate that significant additional uncertainties, not important for the soluble acids investigated, exist for the reported pK_a values of the relatively insoluble MBR in $(CD_3)_2SO$ -water (4.2 and 4.9 at $N = 0.086$ and 4.3 and 5.0 at $N = 0.31$), as well as their extrapolation to obtain pK_a values of 4.2 and 4.9 in water [9]. Indeed, if these low aqueous pK_a values, along with the experimental S values at pH 8.5 of $0.32 \mu M$ [2], or $0.6 \mu M$ [17], are applied to Eq. 1, the calculated extremely low S_o values of UCB diacid of 4 or $8 \times 10^{-15} M$ are seven orders of magnitude lower than the experimental S_o , $5.1 \times 10^{-8} M$ [2]. Applying the S_o of 4 or $8 \times 10^{-15} M$ to Eq. 2, moreover, would indicate massive supersaturation (up to 8 to 10 orders of magnitude) of MBR at the concentrations (1 – $800 \mu M$) used in the ^{13}C -NMR studies [3–6].

Conclusions

The present sedimentation data for UCB in DMSO-water demonstrate that the true solubilities of UCB, even at fairly high pH values, are low at DMSO mole fractions up to 0.31 . The results and related considerations are compatible with similar results in purely aqueous solutions [12–14], and support both the estimated solubility (S_o) of $5.1 \times 10^{-8} M$ for uncharged UCB (H_2B) in water, and the corresponding high aqueous pK_a values of 8.12 and 8.44 , derived from our partition studies [2]. These were performed in undersaturated systems and took into account the self-

association of $B^=$. Our experimental data indicate problems of insolubility, supersaturation and self-aggregation of UCB in DMSO-water mixtures with compositions similar to the MBR systems in $(C^2H_3)_2SO$ -water [3–6]. In $(C^2H_3)_2SO$ -water, DMSO-water [27] or any other medium [8], properly determined pK_a values for the dissociation equilibria of a diacid H_2A ($H_2A \leftrightarrow HA^- + H^+$ and $HA^- \leftrightarrow A^= + H^+$) must pertain to monomeric H_2A , HA^- and $A^=$, the solute species involved in the stated equilibria, and require unambiguous determination of $[H^+]$ or pH. Unless pK_a values are determined for monomeric systems, relative concentrations of H_2A , HA^- and $A^=$ cannot be determined from the pK_a values and the pH. The ^{13}C -NMR data, suggesting low pK_a values for MBR in $(C^2H_3)_2SO$ -water and water [3–6,9], did not meet these essential requirements of proper pH measurements [8] nor provide assurance that the MBR in every system was below saturation and not self-associated [2]. The issues raised are not trivial, since the pK_a and S_o values of UCB are clinically relevant to the effects of pH on the precipitation of calcium bilirubinates in pigment gallstones and the neurotoxicity caused by UCB diacid in severely jaundiced neonates [29].

Materials and Methods

Materials

UCB (Calbiochem) was purified by alkaline extraction of a chloroform solution, recrystallized twice from chloroform-methanol [30], dried under Argon, stored *in vacuo* in the dark and used within 6 weeks. DMSO was spectroscopic grade, 99.8% pure (UVASol, Merck). Human serum albumin (HSA, lot 903635) was from Calbiochem-Boehringer. All other chemicals were reagent grade (Merck). Water used was deionized and distilled. All flasks and tubes were Kimax glass, washed with $0.1 N$ HCl and rinsed 4X with water and then dried before use. Stock buffers, $1.0 M$, were: Tris-HCl, pH 7.01; Na-phosphate, pH 6.85, or 6.99; and Na-acetate, pH 4.01. Stock UCB in DMSO (4 to $6 mM$) and stock HSA, $613 \mu M$ in $0.1 M$ Tris-HCl buffer, pH 7.01, were prepared freshly for each experiment.

Preparation of UCB-DMSO-buffer systems

Test systems ($4.0 mL$) of UCB were prepared in duplicate: to $0.4 mL$ of the stock buffer were added successively the appropriate volumes of water, DMSO and, finally, up to $150 \mu L$ of stock UCB/DMSO solution. To minimize UCB oxidation, all tubes and solutions were deoxygenated with Argon and kept in the dark [31]. To determine the [UCB] in the UCB/DMSO stock, $6.0 \mu L$, was added to $3.0 mL$ of the HSA stock. The absorbance, A , at $460 nm$ was read against a blank containing $2.0 mL$ of HSA stock plus $4.0 \mu L$ DMSO, and the [UCB] calculated using the extinction coefficient, ϵ , $47,000 M^{-1} \cdot cm^{-1}$ [18]. The pH measurement of each final DMSO/aqueous buffer system included an

electrode calibration using the strong acid, HClO_4 [8]. The 0.1 M phosphate buffer in $N = 0.31$ DMSO was centrifuged because of partial insolubility [8] and only the supernatant was used.

Centrifugation and analysis of residual UCB in supernatants

Fifteen min after mixing, samples were assessed visually for turbidity or precipitation. After Vortex-mixing, duplicate 1.8 mL aliquots were transferred to polypropylene tubes and centrifuged for 5 min at 25°C and 14,000 g (Mikroliter centrifuge, Hettich, Tuttlingen, Germany). The supernatants were assayed spectrophotometrically within 20 min. The precipitates were washed once with 1.8 mL of water, again centrifuged, the water aspirated, and the packed precipitate dissolved in DMSO for spectrophotometry. Absorbance (A) was measured in triplicate at 458 nm on each sample, diluted, when necessary, with DMSO or DMSO/buffer mixture to A of 0.2 to 0.8; a comparable medium without UCB was used as a blank. In all systems, including the redissolved UCB sediments, we applied the extinction coefficient of $0.0634 \mu\text{M}^{-1}\text{cm}^{-1}$ at the peak wavelength of 458 nm for UCB in pure DMSO [18]. Preliminary calibration studies of the effects of DMSO concentration and buffer composition on A had confirmed this value for pure UCB in pure DMSO and in DMSO/buffer systems containing 64 vol% DMSO. In the systems containing 27 and 9 vol% DMSO, the spectrum developed a plateau between 458 and 450 nm, but A at 458 nm remained within $\pm 10\%$ of the value expected from applying the extinction coefficient of $0.0634 \mu\text{M}^{-1}\text{cm}^{-1}$ to the measured quantity of UCB dissolved in each system. The variability is in part due to degradation, discussed above, and in part due to the low absorbances at [UCB] below the saturation limit in some buffer/DMSO systems. For these reasons, no corrections were made for these minor differences in A .

Sedimentation of bilirubin-albumin complexes

To determine if UCB bound to HSA would sediment, we prepared a system containing HSA, 300 μM , and UCB 170 μM , in 0.1 M Tris-HCl buffer, pH 7.01. Microcentrifugation for 5 min yielded a small amount of precipitated UCB. Three aliquots of the clear supernatant were diluted with 1/8th volume of DMSO and a fourth aliquot diluted with 1/8th volume of buffer. The diluted samples (in duplicate) were then microcentrifuged for another 10 min and 25 μL samples taken from the top, middle and bottom of the fluid column in each tube, using a Hamilton syringe. Protein concentrations were determined with the Bio-Rad bicinchonic acid method, which is unaffected by bilirubin. After dilution with 1.8 mL DMSO, triplicate A readings were taken at 458 nm.

Abbreviations

UCB, unconjugated bilirubin; H_2B , UCB diacid; $\text{B}^=$, UCB dianion; DMSO, dimethylsulfoxide; MBR, mesobilirubin XIII α ; N = mole fraction of DMSO in DMSO-aqueous buffer systems; NMR, nuclear magnetic resonance; S , solubility of UCB or MBR at a given pH; S_0 , solubility of UCB diacid.

Authors' note

An abstract of this work has been published (Gastroenterology 2000; 118:A1477)

Authors' contributions

All three authors collaborated in the conception, design and writing of this study. The work was performed by JDO while he was a visiting professor at the Academic Medical Center in Amsterdam, the Netherlands. All authors read and approved the final manuscript.

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