Metastable and equilibrium phase diagrams of unconjugated bilirubin IXα as functions of pH in model bile systems: Implications for pigment gallstone formation

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Berman MD, Carey MC. Metastable and equilibrium phase diagrams of unconjugated bilirubin IXα as functions of pH in model bile systems: Implications for pigment gallstone formation. Am J Physiol Gastrointest Liver Physiol 308: G42–G55, 2015.—Metastable and equilibrium phase diagrams for unconjugated bilirubin IXα (UCB) in bile are yet to be determined for understanding the physical chemistry of pigment gallstone formation. Also, UCB is a molecule of considerable biomedical importance because it is a potent antioxidant and an inhibitor of atherogenesis. We employed principally a titrimetric approach to obtain metastable and equilibrium UCB solubilities in model bile systems composed of taurine-conjugated bile salts, egg yolk lecithin (mixed long-chain phosphatidylethanolamines), and cholesterol as functions of total lipid concentration, biliary pH values, and CaCl2 concentrations. Metastable and equilibrium precipitation pH values were obtained, and average pK_a values of the two carboxyl groups of UCB were calculated. Added lecithin and increased temperature decreased UCB solubility markedly, whereas increases in bile salt concentrations and molar levels of ursodeoxycholic acid were strongly influenced by pH. Reliable literature values for UCB compositions of native gallbladder biles revealed that biles from hemolytic mice and humans with black pigment gallstones are markedly supersaturated with UCB and exhibit more acidic pH values, whereas biles from nonstone control animals and patients with cholesterol gallstone are unsaturated with UCB.

taurine-conjugated bile salts; egg yolk lecithin; cholesterol; metastable and equilibrium solubilities; mean pK_a values; pathophysiological correlations with unconjugated bilirubin; lecithin; native biles

THE SOLUBILITY OF UNCONJUGATED BILIRUBIN IXα (UCB), the deconjugation product of bilirubin glucuronides derived from the catabolism of heme (protoporphyrin IX) especially in bile salt-rich media, is an important biomedical and physical chemical question. Although secreted by the liver principally as glucuronate acid conjugates, UCB is the major component of pigment gallstones, principally as the calcium salt of its mono-anion (HUCB−) (8, 15, 36). Moreover, UCB solubility in the bile salt-rich medium of the distal intestine, especially in bile salt malabsorption syndromes, influences its capacity for passive enterohepatic cycling (4, 53). In turn, this may lead to hyperbilirubinemia, the principal biliary risk factor for black pigment gallstone formation (54). The fully undissociated form of UCB (H2UCB) exhibits only trace aqueous solubility at physiological pH (6, 41) by virtue of the involvement of all its polar functions in six internal hydrogen bonds (2, 3). However, UCB solubility is increased markedly in bile salt monomer and micellar solutions as well as in alkaline media (11, 44, 45, 55). The studies of Ostrow, Mukerjee, and colleagues (23, 32, 33, 37, 40, 44, 45) provide detailed information on the quantitative interactions of UCB with different bile salt species and concentrations, as well as UCB ionization, binding capacities, and influence of pH and pK_a values. However, these studies do not provide a systematic pathophysiological basis or biophysical rationale for constructing equilibrium or metastable phase diagrams (24, 47, 48, 50) to enable key relative compositions of bile to be plotted as functions of total and relative bile salt, phospholipid, and cholesterol and calcium concentrations, as well as variations in bile salt molecular species. Moreover, to gain insight into the molecular mechanisms of bile salt/UCB interactions, such studies would require at a minimum pH-dependent UV-Vis spectra of UCB in bile salt systems, the influences of concentrations of NaCl and the chaotropic agent urea, as well as temperature variations (9, 16, 17, 29, 49).

Micellar or indeed monomeric solubility of a sparingly soluble molecule in aqueous systems can be approached by allowing supersaturated solutions to equilibrate. Alternatively, equilibrium can be approached from the unsaturated state via bulk dissolution of the solute preferably from the amorphous state (29). When performed rigorously, the solubilities by both approaches should agree (29). When the crystal energy of the solute is high, as is the case with UCB (2, 3), the necessity for long equilibration times could pose problems because of the risk of chemical degradation despite prolonged O2 exclusion and pH control and both a heavy metal-free and light-free environment (reviewed in Ref. 41). In the current work, we took advantage of the weakly acidic nature of UCB (6, 11), the strong acidic nature of common taurine-conjugated bile salts (9, 48), and the marked changes in both aqueous and micellar solubilities, as pH is systematically lowered from alkaline to acidic (11). Our approach used HCl titration of Na2UCB to H2UCB in such a bile salt-rich environment (10), where the solubilizing lipid components, principally taurine-conjugated bile salts and, when present, long-chain (egg yolk) lecithin and cholesterol, were nontitratable with the common mineral acid. We took advantage of the fact that this pH range encompassed the full titration range of UCB in the systems, whereas taurine-conjugated bile salts are not titratable within this range (11, 48). Provided experimental times are relatively rapid, a number of important properties for UCB in these systems can be...
estimated, particularly metastable and equilibrium UCB solubilities and precipitation pH values and mean pKₐ values of the two carboxylate groups of UCB (3, 33). This information, in turn, was employed as a framework to determine the UCB (not taking into consideration Ca²⁺) supersaturated nature of acidic gallbladder bile from pigment lithogenic humans and from a hemolytic mouse model, as well as the stable nature of UCB in hepatic bile of the bilirubin UDP-glucuronosyl transferase-deficient Gunn rat (22), a model for the Crigler-Najjar Syndrome (20).

**MATERIALS AND METHODS**

Commercial bilirubin IXα (British Drug Houses) was obtained from Gallard-Schlesinger (Carle Place, NY). Following silica-gel high-performance thin-layer chromatography (TLC) (100-μg applications) in a solvent system of CHCl₃/CH₃COOH (49:1, vol/vol), we found a baseline residue and minor amounts of two common structural isomers of UCB (bilirubin IIIα and bilirubin IIIβ). The material was purified by the NaHCO₃ plus CHCl₃/MeOH (1:1, vol/vol) washing and recrystallization procedure of McDonagh and Assisi (30), producing a 42% yield.

The sodium salts of taurocholic acid (3α, 7α, 12α-trihydroxy-5β-cholanoyltaurine, NaTC), taurodeoxycholic acid (3α, 12α-dihydroxy-5β-cholanolyltaurine, NaTDTC), taurodeoxycholic acid (3α, 7α, 12α-triexo-5β-cholanolyltaurine, NaTDHC), tauroursodeoxycholic acid (3α, 7β, dihydroxy-5β-cholanoyltaurine, NaTUDDC), and glycocholic acid (3α, 7α, 12α-trihydroxy-β-cholangoyleglycine, NaGC) were purchased from Gallard-Schlesinger (Carle Place, NY). Following silica-gel thin-layer chromatography (C₃H₇OH/CH₃CN; 1:1, vol/vol) with UV detection at 210 nm.

After recrystallization from hot EtOH, sterol purity was collected from 1 to 6 h post-ALA infusion. This fraction (1) was used in our study, was wrapped in aluminum foil and dried in an oven at 100°C.

To each of a pair of chronically catheterized homozgyous Gunn rats (Harvard University Institutional Review Board for protection of Laboratory Animals, and the study was approved by the appropriate experimental ethical committee). All bile salts used in the study gave a single spot by TLC (200-μg applications). Grade 1 propranolol (99.998% purity) from Matheson (East Rutherford, NJ) and was 99.998% pure.

**Solutions.** Aqueous solutions of bile salts, lecithin, and cholesterol of intended molar proportions were prepared by coprecipitation from a mutual organic solvent (14). Briefly, following dry weight analyses, lecithin with or without cholesterol (including 14C-cholesterol) in CHCl₃/MeOH (2:1 by vol) was added to the desired concentration of bile salts in MeOH. After being mixed, the solvent was evaporated under a stream of dry N₂ at 40°C, producing a viscous paste. The tubes were then plugged with gauze and dried under reduced pressure in a Freezemobile 12 (Virtis, Gardiner, NY). The dried bile salt, bile salt-lecithin, or bile salt-lecithin-cholesterol mixtures were stored for short periods of time under Ar at 21–22°C. Aqueous lipid solutions were then composed on a rigorous wt/vol basis by adding distilled and deionized water containing specific concentrations of pure NaCl and CaCl₂. Recrystallized bilirubin IXα (30) was dissolved in each mixed lipid solution with addition of a few μl of 2 M NaOH to achieve a final UCB concentration of ~600 μM.

**Potentiometric titrations.** The pH of all solutions was raised to approximately pH 11 by adding a few μl of 2 M NaOH, thereby solubilizing all purified UCB. Titrations were carried out slowly with additions of μl 0.5 M HCl via a microsyringe to achieve final equilibrium conditions (1, 48). The pH was measured with a Radiometer Radiometer, Copenhagen, Denmark). Exactly 5 ml of a fully dissolved UCB-biliary lipid solution was placed in the glass cupt of a manual titration assembly (TTTI, Radiometer, Copenhagen, Denmark), through which water saturated with Ar was circulated. Constant solution temperature was maintained by circulating water from a Haake Model FE water bath (Haake, Saddlebrook, NJ) through a calibrated thermostatic water jacket enclosing the titration assembly. Continuous stirring was maintained with a Teflon-coated magnetic stirrer. The solution pH was monitored following each HCl addition until a constant reading was obtained. The time required for complete titration of UCB to pH 3.5 ranged from 4 to 10 h. The precipitation pH (pHₚₑₜ) was recorded visually with the aid of a laser beam that illuminated the sample intermittently. UCB solubilities and mean pKₐ values were calculated according to the modified method of Back and Steenbergen (1), as detailed elsewhere (48). Fully titrated solutions were then centrifuged at 30,000 revolution/min for 30 min in a Beckman Model L5–65 ultracentrifuge (Beckman Coulter, Palo Alto, CA). The supernatant was assayed for UCB by the method of Michaelsson (31). In experiments where UCB was titrated in NaTC plus-lecithin mixtures with 14C-cholesterol, micellar cholesterol solubility was determined by counting tritium in an aliquot of the supernatant (Beckman LS-230 Liquid Scintillation Spectrometer, Beckman Coulter). All radioactive samples were bleached overnight, and a channels-ratio method was employed to correct for quenching.

**Dissolution experiments.** Determinations of the pH dependence of UCB solubilities in micellar NaTC were carried out by dissolution to equilibrium, using minor modifications of an earlier method employed for crystalline cholesterol (25). To obtain rigorous pH control, 14C-
UCB as well as recrystallized cold UCB were adjusted to integer pH values between 2 and 12 in H2O and freeze dried. We composed NaTDC as a 2.5 g/dl solution in 0.2 M ionic strengths, with or without 10 mM CaCl2, employing the following buffers: glycine-HCl for pH 2 and 3, acetic acid/acacetate for pH 4, citrate/NaH2PO4 for pH 5, 6, and 7 in the absence of calcium, imidazole for the same pH range with CaCl2, and Tris-HCl for pH 8 and 9. Solutions with pH values of 10, 11, and 12 were left unbuffered and equilibrated to pH 9.8. During 96 h of dissolution, pH values of all systems did not differ from the original. All dissolution studies employed an excess of amorphous or microcrystalline 14C-UCB or cold UCB (total concentration: 0.01 to 0.05 to 15.0 mM, depending on pH) and were shaken vigorously in the dark at 37°C under Ar using O2-free H2O. Each dissolution system was sampled daily for 96 h, and all were found to have achieved an equilibrated state by 48 h. Between pH 2 and 7, UCB solubilities were typically low, e.g., 0.08 μM in 2.5 g/dl NaTC solution (5, 6); therefore, for all samples in this pH range, we employed 14C-UCB, with scintillation counting of daily aliquots after threefold microfiltration through 0.22-μm filters. Between pH 7 and 9.8, the Michaelsson assay method (31) was employed after a similar microfiltration procedure. At the end of these experiments, UCB was shown by TLC to exhibit no appreciable degradation.

Absorption spectra. A Cary 118C recording UV-Visible Spectrophotometer (Varian Associates, Palo Alto, CA) was employed. As determined from solubilized UCB concentrations, we used matched glass cuvettes to keep UCB absorbances between 0.05 and 2 units. Spectral bandwidths of the light beam were 10–13 nm per mm of slit width, with the latter approximating 0.05 nm. For both experimental and blank cuvettes, the spectrophotometer was fitted with thermostatically controlled jackets, through which water at 37°C was recirculated using a Lauda K-2/RD water bath (Brinkmann Instruments, Westbury, NY). Scans of absorption spectra from 350–550 nm were obtained in ~25 s to avoid possible light-induced molecular decomposition or configurational isomerization in UCB solutions.

**RESULTS**

Titration curves. Representative equilibrium titration curves for the taurine-conjugated bile salt sodium NaTDC, alone and with UCB, are shown in Fig. 1A.

Only an initial inflection point is observed in the TDC curve (Fig. 1A), indicating that the sulfonate (SO3−) group of taurine is not titratable to pH 2 with HCl (9, 48). Nonetheless, HCl titrates the two propionic carboxylate groups of UCB (solid curve) in the presence of the taurine-conjugated bile salt. There is evidence of two inflection points encompassing the titration of the fully ionized groups (2, 3) of UCB ([UCB]TOT). After the initial inflection point (first vertical line) at approximately pH 8.2–8.3, the titration curve becomes concave upward. Between the second vertical line and the point labeled pHppt-met (metastable precipitation pH) where the system suddenly becomes two phases, the solution is supersaturated with UCB that is presumably bound principally to bile salt monomers and micelles. Without further addition of HCl, a pHppt-eql (equilibrium precipitation pH) point is reached. Thereafter, all further additions of HCl lead to a plateau in the titration curve because the added protons are removed from solution by continuous phase separation of the insoluble diacid H2UCB, which acts as a physical chemical buffer (48). Titration of all UCB is complete by the second inflection point (indicated by the third vertical line). Extrapolation of the plateau portion of the curve (light dashed line) intersects the titration curve at the second...
vertical line. The equivalents of UCB titrated between this point and the first inflection point represent the equilibrium solubility ([UCB]eq) of the mono- and diacid forms of UCB in the NaTDC micellar solution at equilibrium, i.e., in the theoretical absence of supersaturation. The metastable UCB solubility ([UCB]met) in the text) is the equivalent amount of HCl consumed between pHppt-met and the first inflection point of the titration curve. [UCB]tot represents the total equivalents of UCB titrated by HCl between the inflection points and corresponds to the gravimetric weight used in the experiment (±2%). A similarly shaped curve is obtained with NaTDC, NaTDHC, NaTC, or NaTUDC and NaGC, each plus lecithin and/or cholesterol because the PO4 or OH of the latter lipids are also not titratable with HCl (examples not shown in Fig. 1A). Because the ratio of the total moles of HCl required to titrate the moles of UCB present in solution at the start of the experiment approximates 200% (range observed was 184–212%), it is apparent that UCB is protonated according to the following equation: Na3UCB + 2 HCl → NaHUCB + NaCl + HCl → H2UCB + 2 NaCl (Eq. 1).

In contrast to dianionic UCB, which is moderately H2O soluble (≈8 mM), undissociated UCB is extremely insoluble in pure aqueous media (5, 11, 23) with an estimated value of ≈66 mM (23). In 2.5 g/dl bile salt micellar systems, this value increases to 0.08–0.10 μM, still a very low range for the right side of Eq. 1, i.e., H2UCB. We obtained several important electrochemical and solubility values from the titration curves. These include the solubility of UCB in the bile salt system under both metastable and equilibrium conditions as well as metastable and equilibrium pHppt values (1, 9, 48). The average pK⁺ values of the two propionyl carboxylate groups of UCB can then be calculated using a modification (9, 48) of the Back and Steenberg formula (1): pK⁺ = pHppt + log([Soluble H₂UCB]/[Total UCB − Soluble H₂UCB]) + [(0.5√μ)/(1 + √μ)] (Eq. 2), in which the quotient on the far right is a correction for ionic strength.

**Influence of bile salts on the solution and electrochemical properties of bilirubin IXα.** When bilirubin was titrated with HCl in the absence of bile salts, its pHppt-eql was 8.3–8.9, with a saturation solubility in H2O of 7 nM at pH 7.4 (5). We find here (Table 1) that, when UCB is titrated with HCl in conjugated bile salt micelles (2.5 g/dl total lipids, 0.15 M NaCl, 37°C), the pHppt-eql of UCB is reduced to 7.6 with NaTC or NaTDC and to 7.5 with NaGC. Because NaGC exhibits a pK⁺ of ≈3.8–4.1 and a pHppt-eql of 4.3 (48), UCB is also the only titratable component of the NaGC-UCB system (viz. Fig. 1A). As anticipated, micellar H2UCB solubilities are increased by orders of magnitude compared with those in H2O, reaching 57–63 μM. In contrast, equivalent concentrations of NaTDHC, a bile salt that only dimerizes in solution, and NaTUDC, the most hydrophilic of the common bile salts (9), decrease the pHppt-eql of UCB from that in H2O (11) to 8.1 and 7.9, respectively. Nonetheless, solubilities of H2UCB are 12 μM in NaTDHC and 10 μM in NaTUDC, which are considerably higher than in H2O (5) but lower than in micelles of the common bile salts, NaTC, or NaTDC (Table 1). The mean pK⁺ values of the two propionyl groups of UCB range from 5.9 to 6.4 in the conjugated bile salt systems (Table 1). This contrasts with the appreciably higher pK⁺ values (8.1 and 8.4) reported by Hahm et al. (23) for aqueous UCB at 21–25°C and physiological ionic strength. However, much lower pK⁺ values of 4.2 and 4.9 were suggested for UCB based on 13C nuclear magnetic resonance (NMR) of mesobilibrubin XIIIα in

| Table 1. Titrimetric properties of unconjugated bilirubin in bile salt and bile salt-lecithin systems |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Mol % Lecithin | pK⁺ | pHppt-eql | pHppt-met | Solubility, eql. μM | Solubility, met. μM | Saturation Ratios |
| NaTC | 0.00 | 6.33 ± 0.01 | 7.55 ± 0.03 | 6.98 ± 0.02 | 56.7 ± 3.3 | 190 ± 5 | 825 ± 52 |
| | 5.00 | 6.44 ± 0.01 | 7.84 ± 0.03 | 7.31 ± 0.02 | 39.3 ± 1.2 | 115 ± 5 | 1228 ± 20 |
| | 10.00 | 6.47 ± 0.02 | 7.88 ± 0.04 | 7.46 ± 0.01 | 36.7 ± 1.7 | 113 ± 6 | 1200 ± 50 |
| | 20.00 | 6.53 ± 0.03 | 8.04 ± 0.03 | 7.58 ± 0.01 | 30.3 ± 0.3 | 110 ± 5 | 1253 |
| | 30.00 | 6.69 ± 0.02 | 8.18 ± 0.02 | 7.76 ± 0.03 | 32.3 ± 1.5 | 111 ± 5 | 1058 ± 103 |
| | 40.00 | 6.84 ± 0.01 | 8.31 ± 0.02 | 7.89 ± 0.02 | 30 | 90 | 869 |
| | 50.00 | 6.81 ± 0.04 | 8.35 ± 0.03 | 8.01 ± 0.01 | 30 | 97 ± 12 | 698 |
| | 60.00 | 6.83 ± 0.03 | 8.41 ± 0.05 | 8.11 ± 0.03 | 25 ± 5 | 87 ± 7 | 665 ± 78 |
| NaTDC | 0.00 | 6.41 ± 0.07 | 7.58 ± 0.04 | 7.18 ± 0.05 | 63.3 ± 6.7 | 217 ± 18 | 778 ± 91 |
| | 5.00 | 6.45 ± 0.01 | 7.80 ± 0.02 | 7.44 ± 0.02 | 41.3 ± 1.3 | 133 ± 9 | 1185 ± 37 |
| | 10.00 | 6.53 ± 0.02 | 7.90 ± 0.02 | 7.47 ± 0.03 | 38.3 ± 1.7 | 135 ± 3 | 1184 ± 54 |
| | 20.00 | 6.61 ± 0.00 | 8.17 ± 0.04 | 7.76 ± 0.05 | 28.3 ± 1.7 | 101 ± 2 | 1407 ± 74 |
| | 30.00 | 6.73 ± 0.05 | 8.22 ± 0.05 | 7.82 ± 0.04 | 28.3 ± 1.7 | 90 ± 5 | 1146 ± 73 |
| | 40.00 | 6.84 ± 0.07 | 8.35 ± 0.03 | 8.03 ± 0.04 | 30 | 78 ± 2 | 881 ± 1 |
| | 50.00 | 6.82 ± 0.08 | 8.41 ± 0.05 | 8.06 ± 0.06 | 26.7 ± 3.3 | 97 ± 7 | 826 ± 12 |
| NaTDHC | 0.00 | 6.16 | 8.05 | 7.51 | 12 | 20 | 3916 |
| | 10.00 | 6.26 | 8.24 | 7.80 | 10 | 60 | 4380 |
| | 20.00 | 6.26 | 8.29 | 7.83 | 10 | 50 | 3820 |
| NaTUDC | 0.00 | 5.93 | 7.91 | - | 10 | 40 | 4792 |
| NaGC | 0.00 | 6.20 | 7.49 | 7.15 | 60 | 100 | 855 |

Applicable values are means ± SE. Conditions are 2.5 g/dl, 0.15 M NaCl, 37°C. Mean pK⁺ of the propionic carboxylate groups of unconjugated bilirubin (UCB) in the bile salt (and lecithin) micellar systems. Saturation ratios are of bile salt to UCB molecules at equilibrium (eq) solubility in each system. pHppt-met= metastable precipitation pH; NaTC, sodium taurocholate; NaTDC, sodium taurodeoxycholate; NaTDHC, sodium taurodehydrocholate; NaTUDC, sodium tauroursodeoxycholate; NaGC, sodium glycocholate.
dimethylsulfoxide (27), but these values are not considered valid for a highly polar solvent, such as H$_2$O (33).

**Influence of bile salt-plus-lecithin mixed micelles on solution properties of bilirubin IXa.** Table 1 and Fig. 1B show that, at constant total lipid concentration (2.5 g/dl total lipids in 0.15 M NaCl at 37°C), added lecithin decreases by nearly 50% the capacity of NaTC and NaTDC bile salt micelles to solubilize UCB. Purified UCB was titrated (see MATERIALS AND METHODS and Fig. Legend 1) to equilibrium in Fig. 1A, Na(TC, sodium taurocholate) and Fig. 1B, B (NaTDC) as functions of increasing mole percentage of lecithin, all at a total lipid concentration of 2.5 g/dl in 0.15 M NaCl at 37°C, means ± SE (μM). Both metastable (met) and equilibrium (eq) solubilities of UCB are appreciably higher in pure NaTDC (Fig. 1B, B) compared with NaTC (Fig. 1B, A) and are decreased markedly with additions of lecithin. This is the case even when as little as 5 mol % lecithin is employed; above 20 mol % lecithin, relatively constant UCB solubility values are obtained. In all experiments, metastable solubilities are appreciably lower than equilibrium UCB solubilities (Table 1).

Figure 1C plots metastable and equilibrium pH$_{\text{ppt}}$ values of UCB, which increase curvilinearly with added lecithin and are accompanied by monotonic increases in mean pK’$_{\text{a}}$ values (6.3 to 6.8) for the two carboxyl groups of UCB in NaTDC (Fig. 1C, a) and NaTDC (Fig. 1C, b) as functions of increasing mole percentage of lecithin. Both pH$_{\text{ppt(eq)}}$ at pH$_{\text{ppt(met)}}$ increase markedly in each system and are accompanied by curvilinear increases in mean pK’$_{\text{a}}$ values for the carboxyl groups of UCB.

Saturation ratios (Table 1), the number of conjugated bile salt molecules required to solubilize one molecule of H$_2$UCB (calculated from the titration curves), approximate 800:1 for the two common bile salts NaTC and NaTDC. When lecithin is added, the saturation ratios of both systems show marked increases in the number of bile salt molecules (1,200–1,400) required to solubilize one molecule of H$_2$UCB. The equilibrium and metastable pH$_{\text{ppt}}$ values of the NaTDHC system (Table 1) are also increased with the addition of lecithin, indicative of some bile salt dimeric solubility of H$_2$UCB. In the cases of NaTDC and NaTUDC, the saturation ratios of bile salt to H$_2$UCB molecules reach ~4,000:1. As anticipated for the two-phase NaTDHC system consisting of bile salt dimers and phase-separated liquid crystals of lecithin, the saturation ratio of bile salt to solubilized H$_2$UCB molecules was only marginally altered by the addition of lecithin (Table 1).

When total lipid concentrations are held constant, the shapes of the NaTC and NaTDC curves are quite similar (Fig. 1B and C). The most notable difference occurs in the abrupt drop in UCB solubility in the NaTC compared with NaTDC system when lecithin is added (Fig. 1B), possibly reflecting the smaller size of NaTC compared with NaTDC micelles (49) under those conditions. With respect to pH$_{\text{ppt}}$ values, H$_2$UCB solubilities, and pK’$_{\text{a}}$ values, there are no significant differences between the two systems (Fig. 1C).

Figure 1D illustrates that the diminution in micellar H$_2$UCB solubility in NaTDC micelles with added lecithin is not attributable to decreases in the proportions of bile salt molecules in the systems because, in this experiment, the [bile salt] is held constant, whereas the total lipid concentration is increased by adding lecithin from 2.5 to 6.2 g/dl to reach its maximum micellar solubility in the NaTDC-lecithin system (14). Especially at the higher molar percentage of lecithin, the pH$_{\text{ppt}}$ values are reduced slightly, but UCB solubilities and mean pK’$_{\text{a}}$ values are not altered appreciably (see also Table 1). As Fig. 1D, b shows, H$_2$UCB solubilities decline to an equilibrium value of 30 μM and a metastable value of 110 μM. The solubilization ratio of bile salt to H$_2$UCB more than doubles from 800 to 1,700:1 when the bile salt concentration is held constant (Table 1). This suggests that H$_2$UCB molecules are more likely associated with the hydrophobic surface of bile salt molecules, and H$_2$UCB becomes displaced as more and more lecithin molecules interact with presumably the same binding sites.

**No influence of cholesterol on solubility or electrochemical properties of bilirubin IXa in mixed bile salt-lecithin micellar solutions.** Bilirubin IXa was titrated in mixed micelles of NaTDC and lecithin (2.5 g/dl total lipids, 20 mol % lecithin, 0.15 M NaCl, 37°C) with cholesterol added to attain both maximum equilibrium solubility as well as metastable supersaturation (10 mol/100 mol of total lipid) (14). Figure 2A displays that, despite progressive increases in micellar cholesterol concentrations, neither the equilibrium, metastable pH$_{\text{ppt}}$ values of UCB, mean pK’$_{\text{a}}$ values, nor solubilities of UCB are altered from the values without cholesterol. Figure 2B demonstrates that this is also the case for metastably supersaturated concentrations of micellar cholesterol. Specific activity plotted against mole percentage of cholesterol (up to 10 mol %) follows the line of identity. This indicates that, when diadic UCB precipitates from an artificial bile system and the system has no other additives, it does not coprecipitate with cholesterol molecules, even when the micellar system is supersaturated with cholesterol.

**Influence of increasing total bile salt and total biliary lipid concentrations on the solubility and electrochemical properties of bilirubin IXa.** Summarized in Table 2 are the solubilities and pH$_{\text{ppt}}$ values of UCB, showing that, as NaTC concentration is increased at constant temperature (37°C) and added neutral electrolyte concentration (0.15 M NaCl), UCB becomes increasingly soluble, and both pH$_{\text{ppt}}$ values decline. With a total lipid concentration of 10 g/dl typical of gallbladder bile (14), 674 μM H$_2$UCB is soluble in the system of NaTC micelles at equilibrium, and the solubilization ratio of bile salt to H$_2$UCB falls correspondingly. As NaTC concentration is increased, however, the mean pK’$_{\text{a}}$ values do not vary appreciably.

Figure 3A illustrates that, at a constant NaTC-lecithin molar ratio of 8:2, increases in total lipid concentration from 1.25 to 10 g/dl produce marked decreases in pH$_{\text{ppt}}$ coupled with increases in mean pK’$_{\text{a}}$ values of UCB (Fig. 3A, a). For 1.25, 2.50, 5.00, and 10.00 g/dl total lipid concentrations, the UCB-containing bile salt micellar systems were composed at constant NaTC-lecithin molar ratio (8:2) in 0.15 M NaCl at 37°C. Marked decreases in pH$_{\text{ppt}}$ and increases in pK’$_{\text{a}}$ occur (Fig. 3A). These are accompanied by an eightfold increase in UCB solubility over the displayed range of total lipid concentrations (Fig. 3B). Figure 3C illustrates that, although increases in lecithin content decrease UCB solubilities, marked increases in total lipid concentration reverse this trend and are accompanied by increased UCB solubilities.

**Influence of added univalent electrolyte concentration on solution and electrochemical properties of bilirubin IXa.** In Fig. 4, UCB was titrated in 2.5 g/dl NaTC solution at 37°C as a function of increasing NaCl concentration from 0–0.9 M. With all additions of NaCl, pH$_{\text{ppt}}$ and pK’$_{\text{a}}$ values decline.
progressively (Fig. 4A), but UCB solubilities and the delta values between [UCB]_{met} and [UCB]_{eql} do not change (Fig. 4B). Over the NaCl concentration range studied, the sizes of NaTC micelles vary from aggregation numbers, \( n \) (i.e., number of monomers per micelle) from about \( \approx 4 \) to 9 and NaTDC from 6 to \( \approx 47 \) (13, 48, 49). Table 3 summarizes the numerical values showing that the saturation ratios of bile salts in UCB in the NaTDC systems display modest fluctuations as functions of increasing [NaCl] from 778:1 to 1,198:1.

**pH dependencies of micellar bilirubin IXα solubilities in pure NaTC systems by dissolution to equilibrium.** Amorphous samples of H\(_2\)UCB, NaHUCB, and Na\(_2\)UCB, prepared at the required pH in different buffers by titration and freeze drying, greatly facilitated equilibration times by dissolution. In all cases, equilibration was reached by 48 h because no further changes in the quantity of UCB dissolved in any of the bile salt systems were found at 72 or 96 h (data not displayed). Figure 5 illustrates that, in these systems, there were only small changes in UCB solubility from pH 2.0 to \( \approx 7.0 \), with or without 10 mM CaCl\(_2\). Without added calcium, UCB solubilities climb markedly at higher pH values, whereas solubilities in the presence of calcium are only augmented slightly with increasing pH. In systems differing only in the ionic strength of solutions and solubility of UCB in NaTC systems. Figure 7A illustrates that, in these systems, there were only small changes in UCB solubility from pH 2.0 to \( \approx 7.0 \), with or without 10 mM CaCl\(_2\). Without added calcium, UCB solubilities climb markedly at higher pH values, whereas solubilities in the presence of calcium are only augmented slightly with increasing pH. In systems differing only in the ionic strength of NaCl, the solubility of UCB by titration (shown by an “X”) at a pH value corresponding to the pH_{ppt-eql} shows good concordance (\( \approx 60 \mu M \) at pH 7.6) with the dissolution data. Above pH 8.0–8.5, there is a marked divergence in values, with the curve for no calcium reaching UCB solubilities of \( \approx 10 \mu M \) at pH 9.8. In contrast, over the same pH range with added 10 mM CaCl\(_2\), only a slight augmentation in the solubility of UCB occurs, with [CaUCB] being 70 \mu M at pH 12.0. At the lower pH values, the solubility of UCB in the absence of calcium increases from 4 \mu M at pH 2.0 to \( \sim 7 \mu M \) at pH 8.0.

**Influence of pH on the UV-visible absorption spectra of bilirubin IXα in NaTC solutions.** To obtain an estimate of the microlocation of UCB in/on bile salt micelles, spectral wavelengths at absorption maxima (\( \lambda_{\text{max}} \)) were measured as a function of pH and are listed in Table 4 for 6.8–85 \mu M UCB concentrations in 2.5 g/dl NaTC (0.15 M NaCl, 37°C) with decrements in pH from 10 to 4. Selected absorption spectra for four UCB concentrations are shown in Fig. 6 with the integer pH values (4 to 10) labeled on the spectra.

Original data are shown in overlapping format with the response factor being varied to match the full spectra between 550 and 350 nm to the chart paper. At pH values greater than 8, there are two essentially symmetrical peaks in the spectra, one centered at 456–459 nm and the other at 422–428 nm. As pH is decreased, the shorter wavelength peak disappears entirely at pH 7, and the longer wavelength peak shifts to 443–458 nm, a value that is consistent with the diminished micellar solubility of these H\(_2\)UCB concentrations in NaTC micelles, especially as the pH falls into the strongly acidic range (see Fig. 5).

**Influence of temperature variation on pH_{ppt} and pK'_a values and solubility of UCB in NaTC systems.** Figure 7A summarizes the pH_{ppt} values of UCB in 2.5 g/dl NaTC systems (0.15 M

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**Table 2. Solution properties of unconjugated bilirubin in increasing concentrations of NaTC**

<table>
<thead>
<tr>
<th>[NaTC], g/dl</th>
<th>pK'_{a}</th>
<th>pH_{ppt-met}</th>
<th>pH_{ppt-eql}</th>
<th>Solubility, eql, ( \mu M )</th>
<th>Solubility, met, ( \mu M )</th>
<th>Saturation Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.25</td>
<td>6.21</td>
<td>7.91</td>
<td>7.33</td>
<td>18</td>
<td>90</td>
<td>1320</td>
</tr>
<tr>
<td>2.5</td>
<td>6.33</td>
<td>7.55</td>
<td>6.99</td>
<td>57</td>
<td>190</td>
<td>825</td>
</tr>
<tr>
<td>5</td>
<td>6.32</td>
<td>7.05</td>
<td>6.70</td>
<td>160</td>
<td>240</td>
<td>590</td>
</tr>
<tr>
<td>10</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Conditions were 0.15 M NaCl, 37°C. Shown are the mean pK’_a of the two carboxylic acid groups of UCB. Saturation ratio is the molar ratio of bile salt to UCB molecules at micellar saturation.
NaCl) as temperature is increased from 5 to 52°C. Both pH\textsubscript{ppt} values display marked elevations with increasing temperature. However, only modest changes occur in the pK\textsubscript{a} values and then only at the highest temperature. Figure 7B illustrates that both UCB solubilities (met and eql) decrease markedly over the same temperature range. At 52°C, solubility of UCB\textsubscript{eql} is lower by a factor of four compared with values at 5°C. These data suggest that hydrophilic interactions, possibly via hydrogen bonds, are predominant in micellar NaTC-UCB systems in the pH range of 6.6 to 7.7 (see Fig. 7A). Typical error bars ± SE are shown. Because of the hydrogen bond rupturing effects of increases in temperature, these data are consistent with both strong hydrophobic and hydrophilic interactions between H\textsubscript{2}UCB and NaTC in the pH 6.6–7.7 range, further suggesting that UCB is ∼50% ionized in this system (see Table 1).

Influence of urea on electrochemical properties of bilirubin IX\alpha in NaTC solutions. With a NaTC concentration of 2.5 g/dl and physiological conditions (0.15 M NaCl, 37°C) and the addition of up to 8 M concentration of the chaotropic agent, urea produces little effect on the pH\textsubscript{ppt-eql} of UCB, whereas the pH\textsubscript{ppt-met} becomes monotonically elevated from 7.0 to 7.3 (data not displayed). Urea did not affect the mean pK\textsubscript{a} of UCB appreciably until its concentration reached 8 M (data not displayed). Despite these relatively small effects of urea on electrochemical properties, the influence of the chaotropic agent on increasing the solubility of UCB was striking (Fig. 8). Bile salt micelles are disaggregated essentially completely in 8 M urea (49); therefore, the most likely explanation for these data is that the internally hydrogen-bonded network of UCB is broken (11) with the most likely exception that the π-orbital

**Fig. 3.** A: influence of total lipid concentration (1.25–10.00 g/dl) covering the physiological range on pH\textsubscript{ppt} and mean pK\textsubscript{a} (a) and solubilities (met and eql) of UCB (b) in NaTC and in NaTC-lecithin mixed micellar solutions. B: influence of increasing mole percentage of lecithin and total lipid concentration (inscribed on curves) on the pH\textsubscript{ppt} (eql and met) of UCB in micellar NaTC-lecithin systems. C: influence of mole percentage of lecithin and total lipid concentrations (1.25–10.00 g/dl) on the solubility of UCB in NaTC-lecithin systems (0.15 M NaCl, 37°C).

**Fig. 4.** A: influence of increasing NaCl concentration (0–0.9 M) on pH\textsubscript{ppt} and mean pK\textsubscript{a} values of UCB in 2.5 g/dl NaTC solutions at 37°C. With additions of NaCl, a near linear decline occurred in pH\textsubscript{ppt} and mean pK\textsubscript{a} values (a), but solubilities and the differences between [UCB]\textsubscript{met} and [UCB]\textsubscript{eql} do not change (b). B: influence of increasing NaCl concentrations (0–0.5 M) on pH\textsubscript{ppt} and mean pK\textsubscript{a} values of UCB in 2.5 g/dl NaTDC solutions at 37°C. With all additions of NaCl, the pH\textsubscript{ppt} and mean pK\textsubscript{a} values decline (a). Apart from 0.5 M NaCl when salting out of NaTDC occurs (b), UCB solubility values (met and eql) increase and remain relatively constant.
hydrogen-bonded interactions between bile salt monomers and UCB remain intact in a hydrophobic environment wherein they are the strongest bonds (46).

Pathophysiological correlations of \([UCB]\)-pH \(_{ppt}\)-\% lecithin phase diagram with UCB levels in Gunn rat bile. Because UCB is present in small amounts in homozygous Gunn rat bile (21), we measured the pH, total diazo-positive pigments, and percentage of lecithin content of native hepatic bile from these animals that are totally deficient in hepatic glucuronosyl transferase activity for bilirubin IX \(_\alpha\) (20). We then plotted the values on the UCB solubility-pH \(_{ppt}\)-\% lecithin truncated binary phase diagrams at 37°C and 0.15 M NaCl, ignoring calcium content because the latter, in typical biliary concentrations, does not influence the solubility of UCB, at least up to pH 7.0 to 7.5 (Fig. 5). Of note (Fig. 9A) is that the native pH values of all Gunn rat biles were above the pH \(_{ppt}\)-met values for UCB observed in this work; furthermore, the pH values of most biles were at, or above, the pH \(_{ppt}\)-eq\(_l\) determined for UCB (Fig. 9A). The exception was a bile with a [UCB] of 172 μM, but, because its pH value was 8.2, the solution was thermodynamically stable and biliary calcium levels had no effect on the solubility of UCB. UV-visible absorption spectra from four of these biles (data not displayed) revealed \(\lambda_{\text{max}}\) for bilirubin of 411 nm (the minor one being considerably lower than shown for the pure systems in Table 4) and 453 nm (major), consistent with both hydrophobic and hydrophilic interactions in the model UCB- NaTC spectra (Fig. 6, Table 4) for the same pH and UCB concentrations. These results provide strong positive pathophysiological correlations because the carboxyl groups of bilirubin are always free (i.e., unconjugated) in Gunn rat bile (21). Moreover, because many of the diazo-positive pigments are further oxidized by additional hydroxyl groups (21), the true solubility of all diazo-positive materials is likely to be slightly higher than the monocarboxylic acid form of UCB (HUCB\(^-\)), which remains the predominant molecular species of bile pigment in Gunn rat bile (21).

Pathophysiological correlations of \([UCB]\)-pH \(_{ppt}\)-\% lecithin phase diagram with gallbladder bile composition of wild-type and hemolytic nb/nb mice with or without black pigment gallstones. Gallbladder bile compositions (mean values) were derived from the published work of Trotman and colleagues on hemolytic deer mice (52) and are plotted on the same truncated binary phase diagram at 37°C (Fig. 9B). The biles of wild-type mice (controls) display very low concentrations of UCB well below equilibrium solubility limits. Moreover, the mean pH is below the pH \(_{ppt}\)-eq\(_l\). Therefore, all control biles are thermodynamically stable in terms of UCB content. In pigment gallstone biles, the biliary pH values are lower than in WT controls and plot within the metastable pH region for their specific lecithin concentrations. Additionally, the UCB concentrations of pigment gallstones are the strongest bonds (46).

Table 3. Influence of added NaCl concentration on the electrochemical and solubility properties of UCB in NaTDC

<table>
<thead>
<tr>
<th>[NaCl], M</th>
<th>pK(_{a})</th>
<th>pH (_{ppt})-eq(_l)</th>
<th>pH (_{ppt})-met</th>
<th>eq(_l) μM</th>
<th>met, μM</th>
<th>Saturation Ratio</th>
</tr>
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<tbody>
<tr>
<td>0.01</td>
<td>6.43</td>
<td>7.85</td>
<td>7.47</td>
<td>40</td>
<td>180</td>
<td>1198</td>
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<tr>
<td>0.05</td>
<td>6.58</td>
<td>7.79</td>
<td>7.37</td>
<td>60</td>
<td>190</td>
<td>799</td>
</tr>
<tr>
<td>0.1</td>
<td>6.47</td>
<td>7.74</td>
<td>7.37</td>
<td>55</td>
<td>185</td>
<td>871</td>
</tr>
<tr>
<td>0.15</td>
<td>6.41 ± 0.07</td>
<td>7.57 ± 0.04</td>
<td>7.18 ± 0.05</td>
<td>63.3 ± 6.7</td>
<td>216.6 ± 17.6</td>
<td>778 ± 91</td>
</tr>
<tr>
<td>0.2</td>
<td>6.47</td>
<td>7.65</td>
<td>7.30</td>
<td>60</td>
<td>185</td>
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<td>7.11</td>
<td>60</td>
<td>200</td>
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<tr>
<td>0.5</td>
<td>6.13</td>
<td>7.47</td>
<td>7.09</td>
<td>40</td>
<td>160</td>
<td>1198</td>
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</tbody>
</table>

Applicable values are means ± SE. Conditions were 2.5 g/dl, 37°C. Shown are the mean pK\(_{a}\) of the two carboxylate groups of UCB. Saturation ratio is the number of bile salt molecules required to solubilize one H\(_2\)UCB molecule at micellar saturation.

Fig. 5. pH dependence of micellar UCB solubilities assessed by dissolution of amorphous UCB in 2.5 g/dl NaTTC (0.15 M NaCl) at 37°C with and without addition of 10 mM CaCl\(_2\). The data point denoted by X is UCB\(_{eq\(_l\)}\) solubility at the indicated pH by titration. True divergence of the data with and without Ca\(^{2+}\) is only observed at pH 8.0 and higher.

Table 4. Absorption maxima of bilirubin in NaTC solution

<table>
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<tr>
<th>pH</th>
<th>(\lambda_{\text{Maxima}})</th>
<th>6.8</th>
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</table>

Conditions were 2.5 g/dl bile salt concentration, 0.15 M NaCl, 37°C.
stone biles are 10-fold larger than in control biles. Biles of hemolytic nb/nb mice without pigment stones contain even higher UCB concentrations than those with pigment stones, indicating that nucleation and phase separation had not taken place. The UCB concentrations in control mice biles are low plotting well below those of black pigment stone and nb/nb biles without stones, and these biles are clearly stable because they plot well below the metastable or equilibrium UCB solubility limits. The UCB concentrations are as much as 10-fold larger in pigment stone, i.e., highly supersaturated, than in control mouse biles.

Pathophysiological correlation of [UCB]-pH ppt-% lecithin phase diagram with gallbladder bile composition of human black pigment stone and cholesterol stone biles. These data (Fig. 9C) are taken from the work of Tritapepe and colleagues (51) with pH values of the same biles communicated by the authors. Here, the UCB concentrations are plotted for an estimated 20 mol % lecithin at 37°C and 0.15 M NaCl, typical of human gallbladder bile, and cholesterol content was ignored because the sterol does not influence the micellar solubility of UCB (see Fig. 2A). As shown in Fig. 9C, all biles are acidic, with the mean pH values of control (i.e., cholesterol gallstone) biles plotting below both pH_ppt boundaries, and hence would be supersaturated save for the fact that the UCB concentration is less than the equilibrium solubility limit of UCB; these biles are therefore thermodynamically stable. Black pigment stone biles also display pH values below both pH_ppt boundaries, but their UCB concentrations plot above the metastable solubility limits for UCB in our model systems. UCB concentrations of the black pigment stone biles are as much as 8- to 10-fold larger than in disease control (i.e., cholesterol gallstone) biles, rendering them highly supersaturated and thermodynamically unstable with UCB. Phase diagrams and data plots from human biles in relation to cholesterol gallstones (14) and upper small intestinal content postprandially have been developed by our group in the past (24, 50), but in neither case was pH considered an important variable to be taken into account within the (patho)physiological pH range. It is important to emphasize that the phase boundaries in Fig. 9 are drawn for 2.5% NaTC in 0.15 M NaCl at 37°C, corresponding exactly to the relevant data in Fig. 1 for these conditions. Employing the data plotted in Fig. 3, an interested investigator can construct similar phase diagrams for other physiological conditions in hepatic or gallbladder bile with typical total lipid concentration ranges of 1.25 to 10.00 g/dl (37°C, 0.15 M NaCl).

DISCUSSION

The pH-solubility relationships of UCB were explored systematically in model bile systems, and information to construct metastable and equilibrium phase diagrams was collected. We found that solubility of the diacid species was very poor with only minor differences induced by the common bile salt species, but bile salt-lecithin ratios were of enormous importance because lecithin even in small doses diminished the poor solubility of UCB markedly. Total lipid concentration was clearly important, but cholesterol content had no effect on solubility and electrochemical properties of UCB. The model bile systems studied are relevant to the solubility of UCB in bile and other bile salt-rich media, such as proximal and distal large intestines in bile salt malabsorption syndromes (53). One of the more curious findings in this study is that Ca^{2+} did not form insoluble salts with UCB until pH exceeded 7.5 (Fig. 5). Because hepatic bile is mildly alkaline but gallbladder bile is invariably acidic, we were able to ignore the influence of Ca^{2+} especially when we plotted the bile UCB compositions and pH of gallbladder bile. Furthermore, the physical chemistry of the solubilization process itself was investigated at the molecular level. We inferred from these data that monomeric UCB can bind to bile salt molecules by either hydrophobic or hydrophilic interactions depending on ambient pH and that the resulting systems can be uniquely metastable. Furthermore, we found that this binding could either be on the α-surface, i.e., hydrophobic backbone, or OH-studded β-surface of the bile salt monomer in solution or in micelles. Finally, pathophysiological correlations were performed utilizing the [UCB], pH,
and mole percentage of lecithin phase diagrams at 37°C and 0.15 M NaCl and ignoring Ca²⁺ for model bile systems and relating them to the UCB concentrations, lecithin content, and pH values from bile of homozygous Gunn rats (22), which are totally deficient in hepatic glucuronosyl transferase for UCB (20), with the use of high-quality published literature values. We also established pathophysiological correlations between UCB solubilities in model bile systems with those in gallbladder biles from healthy control mice and from hemolytic nb/nb mice with or without black pigment gallstones (52). Lastly, we correlated the solubilities of UCB in model biles with human gallbladder biles from both pigment and cholesterol gallstone subjects (51). We employed phase boundaries for 2.5 g/dl NaTC in Fig. 1, but clearly the data plotted in Fig. 3 can be used to construct phase diagrams for lower and higher total lipid concentrations.

In both bile and alimentary tract, the glucuronosyl ester linkages of bilirubin conjugates undergo hydrolysis by endogenous biliary, intestinal mucosal, and bacterial β-glucuronidases (8, 36, 53). Any cause of increased bilirubin loads delivered to the liver (i.e., hemolysis, ineffective erythropoiesis, or induced enterohepatic cycling) (4, 53) can produce hyperbilirubinemia (8, 54). This provides an increased substrate load in bile for endogenous β-glucuronidase hydrolysis and results in increased production of UCB. UCB is a calcium-sensitive anion that, as we have shown here this, depends critically on pH (41), as visualized in Fig. 5 of this work, because only the monoanionic (HUCB⁻) and the dianionic (UCB²⁻) species interact with Ca²⁺. When its formation product at the appropriate pH [Ca(HUCB)₂ at pH values more typical of hepatic than gallbladder bile] exceeds the solubility product in bile, then thermodynamically HUCB⁻, as the acid Ca salt, is capable of nucleating and phase separating from solution. However, the fact that UCB can be measured in solution in most biles, especially in the gallbladder, attests to the fact that Ca²⁺ cannot form a salt and remove UCB from an acidic solution. Hence, the amount of UCB that can be held in solution in bile, as well as in distal small and large intestines, as functions of physical-chemical conditions of pathophysiological relevance, is clearly paramount to health. We have argued elsewhere (53) that excess bile salts within the colon from bile salt malabsorption syndromes can promote passive enterohepatic cycling of UCB. This recently hypothesized cause of hyperbilirubinemia typifies ileal Crohn’s disease and ileectomy but also many other common conditions (53). We have made initial inroads on this problem here by utilizing pure

Fig. 7. A: influence of increases in temperature (5–52°C) on pH and mean pKₐ of UCB in 2.5 g/dl NaTC systems (0.15 M NaCl, 37°C). B: UCB solubilities (met and eq) that show a steep decline with increasing temperature in the same NaTC systems.

Fig. 8. Influence of added urea to a maximum of 8 M on solubilities (met and eq) of UCB in 2.5 g/dl NaTC solutions (0.15 M NaCl, 37°C). Urea, a chaotropic agent, displays a marked increase in [UCB] solubilized by disrupting hydrophobic and hydrophilic interactions.
bile salt model systems as functions of pathophysiological variables found in the distal ileum and proximal colon and also in artificial bile systems with added mixed long-chain lecithins from egg yolk as well as cholesterol. The present study has addressed the relationships involved by defining relevant phase diagrams (under both metastable and equilibrium conditions) required to plot native biliary pigment compositions and pH to determine the phase relations of UCB in bile and in the proximal large intestine. Our study has also initiated fundamental approaches to understanding the complex molecular nature of the micellar solubilization of UCB by bile salts (12, 16–18, 41), which is presently unresolved (16–18), because the physical-chemical state in solution is inferred from crystal structures only, and, in contrast to our work, only pH has been varied systematically (18).

This "ridge-tile" conformation of bilirubin IXα provides the lowest free energy state in the diacidic crystal (2, 3), as well as for the mono- and dianion in alkaline solution (41, 43). The secondary structure of the molecule forces bilirubin IXα to become essentially nonpolar, i.e., an insoluble lipopigment, in aqueous solution at pH values below 8.3–8.9 (11, 41). This phenomenon underscores the pH dependency of the solubility of UCB because the protonation of each carboxylic acid group and internal hydrogen bonding eliminates all possibility of solvation of the polar functions by water molecules. A moderately soluble amphiphile at very alkaline pH (50 mM in H2O) transitions to a binary system of highly insoluble states at pH < 8.3 (11).

Because of the conformational changes involved and their related effects on aqueous solubility, precise measurements of the two pK′α of UCB have proven difficult to measure, with reported values ranging from the alkaline (23) to the acidic (27). In the current work, we used the method of Back and Steenberg (1) to provide derived estimates of the mean pK′α and solubilities of undissociated UCB molecules from potentiometric titrations in taurine-conjugated bile salt solutions, whose - and -faces mimic both a hydrophilic and a hydrophobic environment (9, 48, 49). Using this approach, we established that the mean pK′α values of UCB in pure taurine-conjugated and one glycine-conjugated bile salt system (Table 1) are likely to range from 5.9 to 6.4, increasing to 6.8 in the presence of maximum amounts of solubilized lecithin (Table 1, Fig. 3). UCB is solubilized by a very strong sulfonic acid-like taurine-conjugated bile salt when it should become an even weaker acid. For example, Cabral and colleagues (7) demonstrated by 13C-NMR that, at saturation, cholic acid solubilized in NaTC micelles (1:9 mol/mol) increases its pK′α from 4.6 (a value obtained in H2O below the critical micellar concentration of cholate) to 5.3. Kurtin and colleagues (26) also showed that UCB becomes a weaker acid in bile salt solutions. Both studies are in agreement with this work. Nonetheless, it is now fairly certain that the derived pK′α of UCB by Lightner and colleagues (27) may be realistic for a hydrophobic environment but not for physiologically relevant highly polar aqueous systems, where the pK′α values of 8.1 and 8.4 proposed by Hahm et al. (23) for UCB are more likely correct (32). Unfortunately, the limitations of the Back and Steenberg formula in the present work do not allow us to derive or estimate the pK′α values for individual carboxylic groups of UCB.

Equilibrium solubility of UCB molecules in bile salt micelles was rapid because we approached the solubilization and...
pH_{ppt} of UCB from the supersaturated state, a procedure that fosters an accelerated approach to equilibration (29). We also showed that, by carrying out a UCB dissolution study in bile salt solutions (Fig. 5), the solubility of UCB was similar, within one order of magnitude, to that obtained by the supersaturation approach. The methodology used can be applied only when the ionized groups of the solubilizing micellar amphiphile, such as taurine-conjugated bile salts, remain untitratable with HCl (48) within the pH range in which UCB is titrated to completion (Eq. 1). Not only was this the case in the present work (Fig. 1A), but also this approach allowed both pH_{ppt-equ} and pH_{ppt-met} values as well as UCB solubilities (eql and met) to be obtained (Fig. 1A). Both sets of values are essential for defining the extraordinary metastable behavior and the phase equilibria of UCB systems in model bile. We found (as have others, see summary in Ref. 41) that the solubility of UCB as the diacid is increased by orders of magnitude in pure bile salt solutions over that in aqueous systems. Moreover, our data for equilibrium UCB solubility (57–63 μM) in pure NaTC, NaGC, and NaTDC as 2.5 g/dl solutions (Table 1) at physiological pH with extremely high saturation ratios correlate satisfactorily with other high-quality studies (41), in which ultrapure materials were employed.

The marked depressant effect of lecithin on the solubility of UCB in bile salt solutions (Table 1, Fig. 1, B–D) even at very low phospholipid concentrations has been noted earlier (34, 38). This was verified by our experiment (Fig. 1D), in which the bile salt concentration was kept constant and progressive amounts of lecithin were added, thereby increasing the total lipid concentration by a factor of 2.5. In all experiments with added lecithin, equilibrium pH_{ppt} values moved upward and asymptotically approached the pH_{ppt} of UCB in pure aqueous solution (11). The mean pK_{a} values also displayed a corresponding increase with added lecithin, which is further evidence that UCB is solubilized in an enhanced ionic environment when lecithin is added, as has also been documented for bile acids solubilized in long-chain lecithin vesicles (34). In bile salt-plus-lecithin mixed micelles, molecular dynamics simulations (28) and isothermal monolayer experiments employing the surface balance (19) reveal that the acyl chains of lecithin interact strongly with and are condensed by the β-hydrophobic surfaces of bile salt monomers. This suggests that added lecithin may bind to the same bile salt-binding site that a proportion of H_{2}UCB molecules are bound to when solubilized by bile salt micelles. Hence, not only do saturation ratios increase with added lecithin, but also bile salt structures that have no distinct polarity (TDHC) or poorly defined polarity (TUDC) between α- and β-faces of the steroid nucleus (47–49) display very high saturation ratios with solubilized H_{2}UCB molecules, reaching values of 4–5,000:1 (Table 1). This concept is also supported by the lack of any specific effects of cholesterol (Fig. 2A) because the sterol is buried deep within the micellar cores of the quasicentrosymmetric mixed bile salt-lecithin-cholesterol micelles and not at the surface where the planar bile salt molecules are arrayed (28). When the monovalent counterion NaCl was added, NaTC micellar size doubled and NaTDC micellar size increased by a factor of eight (49), yet UCB solubility did not change in either of these systems with additions of NaCl (Fig. 4, A and B). Therefore, the solubilization of UCB molecules in bile salt micelles has no dependence on micellar size but rather on the absolute number of bile salt molecules present in the total system. This is further supported by the linear dependency of UCB solubilities on total lipid concentration (Fig. 3A) and provides strong evidence for unique H_{2}UCB-bile salt hybrid interactions, presumably mostly hydrophobic, in both the aqueous monomeric state and in micelles.

The UV-visible absorption spectra (Fig. 6) provide insights into the pH-dependent effects of bile salt micelles on the molecular mechanism of UCB solubilization. As noted before by us (12), two absorption maxima are observed for UCB at pH 8 or greater and can be attributed to hydrophilic (blue) as well as hydrophobic (red) interactions with bile salt micelles. The former is presumed to be via π-orbital-OH bonds because these are extremely strong in a hydrophobic environment (46) and, as shown in Fig. 8, were demonstrated to be resistant to breakage with 8 M urea. We found earlier (12) that the shorter wavelength peak at pH 10 in micellar NaTC disappears with increasing temperature, suggesting hydrogen bond breakage, borne out in the current work as temperature was increased from 5 to 52°C (Fig. 7).

Below pH 8, only the “red-shifted” λ_{max} of 443–458 nm is present (Table 4). UCB displays an absorption maximum of ~450 nm in organic solvents (11), whereas, in aqueous solution at pH ≥ 8, its UV-visible absorption also displays a peak at a shorter wavelength. These findings further support the notion that, in alkaline media, UCB^{2−} molecules are located in a hydrophilic bile salt environment as well as at a hydrophobic site in/on bile salt micelles. With lower pH values, the protonated H_{2}UCB is solubilized essentially only by a hydrophobic environment of bile salt micelles.

The concentration-dependent effects of urea on the electrochemical properties and solubility of UCB in NaTC micellar systems are of considerable interest with respect to the mode of micellar solubilization. Our findings displaying dramatic increases in UCB solubility may, in fact, be related not only to the ability of urea to break the intermolecular hydrogen bonds as noted above, but also to its destroying hydrophobic interactions within micelles because NaTC is effectively disaggregated in 8 M urea (49). As shown by our results (Fig. 9), UCB is rendered much more soluble in the aqueous bile salt medium (presumed to be monomeric), most likely by both increased ionization and by binding to bile salt monomers, probably via π-orbital-OH interactions that are totally resistant to the disruptive effects of the high molar concentrations of urea and are strongest in hydrophobic environments (46).

We assumed in this work that, for most “first-order” metastable and equilibrium phase studies, we could use truncated diagrams without incorporating a Ca^{2+} effect because its influence should be minuscule on the micellar solubility of UCB at neutral or acidic pH values. Moreover, our data by titration (Fig. 7) show clearly that, above pH 7.6, nearly all the UCB will precipitate with Ca^{2+} in proportion to increases in UCB concentration, lecithin, and Ca^{2+} content. Therefore, the ionic product of calcium salt formation with UCB is likely to be initiated in alkaline hepatic rather than in acidic gallbladder bile. If this is the case, it would suggest considerable metastability of Ca(HUCB)_{2} salts in dilute native bile salt-lecithin-cholesterol systems.

It is evident from the pathophysiological correlations in this investigation (Fig. 9) that our results have allowed us to construct phase diagrams (Fig. 9, A–C) relating pH_{ppt} and UCB...
solubility to total lipid concentration and percentage of lecithin in bile, which are the crucial factors that govern the solubility of UCB in bile below pH 7.6–8.0. For UCB to precipitate spontaneously or heterogeneously from bile, the pH must be below the metastable pH of precipitation. Our data on Gunn rat bile (Fig. 9A) suggest that the small amount of UCB in these native systems may be in metastable supersaturated solution. The phase equilibrium plot of the data on hemolytic nb/nb mice (52) suggests that mice with pigment gallstones exhibit lower gallbladder bile pH values than those without stones (Fig. 9B). Despite this pH difference, the UCB present was in a supersaturated metastable state in both groups and actually present in higher concentrations in hemolytic nb/nb mice without stones than those with stones (Fig. 9B). We speculate that this occurred because of lack of presumably heterogeneous nucleation with Ca$^{2+}$ salt formation and phase separation that would have deposited UCB molecules onto the growing stones. Similarly, investigations in humans with pigment gallstones (51) revealed that gallbladder bile pH was reduced relative to biles of patients with cholesterol stones (Fig. 9C). Additionally, UCB levels were considerably higher in pigment gallstone patients with cholesterol stones (Fig. 9C). Taking the results of all these studies together, we can hypothesize that, for pigment gallstone formation to occur, a necessary precondition for nucleation and precipitation is for UCB to be held in a metastable supersaturated solution and that the bile salt-lecithin ratio, pH, and Ca$^{2+}$ content are all pivotal factors influencing its stability. This work also raises the possibility that pigment gallstone formation may be initiated in alkaline hepatic bile rather than in gallbladder bile, where the final nucleation of the calcium salt bilirubinate would occur in a slightly acidic medium.

Furthermore, despite the fact that Ca(HUCB)$_2$ is often present at the core of cholesterol gallstones, there was no influence of micellar solutions of cholesterol on the solubility of UCB in model bile solutions (Fig. 2A). Moreover, no precipitation effect of UCB on supersaturated systems of cholesterol was noted (Fig. 2B), and hence neither HUCB$^-$ nor H$_2$UCB would play an initiating physical-chemical role in cholesterol gallstone formation; nonetheless, it might be highly likely that other lithogenic factors, such as mucin gel, for example, could play a role in the initiation of the nucleation process, and indeed our model system should lay down a framework to test these other potential lithogenic factors.

The present work should also provide a basic framework for further studies on the interactions of the biliary molecules that must include UCB as well as its conjugates, especially insoluble salt formation between Ca$^{2+}$ and ionized UCB in both model and native bile systems. In this manner, our results can serve as a framework for the broader pathophysiological phenomenon of human pigment gallstone disease.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**

Author contributions: M.D.B. and M.C.C. conception and design of research; M.D.B. and M.C.C. performed experiments; M.D.B. and M.C.C. analyzed data; M.D.B. and M.C.C. interpreted results of experiments; M.D.B. and M.C.C. prepared figures; M.D.B. and M.C.C. drafted manuscript; M.D.B. and M.C.C. edited and revised manuscript; M.D.B. and M.C.C. approved final version of manuscript.

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