Molecular and micellar associations in the pH-dependent stable and metastable dissolution of unconjugated bilirubin by bile salts

J. Donald Ostrow,[1,*] Lillian Celic,[*] and Pasupati Mukerjee[†]

Gastroenterology Section, Department of Medicine,[*] Veterans Administration Lakeside Medical Center and Northwestern University Medical School, Chicago, IL, and School of Pharmacy,[†] University of Wisconsin, Madison WI

Abstract Unconjugated bilirubin (UCB) is almost insoluble in water at neutral pH, but appears in normal human gallbladder bile at concentrations up to 35 μM. We therefore determined whether conjugated bile salts could increase the dissolved concentration ($[B_t]$) of UCB over the pH range 3.0–11.0. Using crystalline UCB, $[B_t]$ was higher with less ordered crystals, with increasing pH and bile salt concentration, and with taurocholate (TC) micelles compared to taurodehydrocholate (TDHC) dimers. Plots of $[B_t]$ versus pH from pH 3.0–9.3 fit the equation, $[B_t] = A(1 + K'_1/[H]^+ + K'_1 \cdot K'_2/[H^+]^2)$, where $A = [B_t]$ at pH < 4.0, and K'_1 and K'_2 are the two apparent ionization constants of UCB. Estimated pK'_1 values in NaCl, TC, and TDHC were 6.8, 6.0, and 5.6, respectively; pK'_2 was ≥ 9.3 in each system. Acidification of disodium bilirubinate to pH < 8.5 produced high, metastable $[B_t]$ in 50 mM TC; this was absent in 0.15 M NaCl, and minor in 50 mM TDHC. In all solutions, maximum $[B_t]$ of 60–65 mM was attained at pH ≥ 10.5. This work helps explain the immense variation among reported $[B_t]$ values, indicates that UCB monoanion predominates at the pH range of bile, and suggests that bile salt monomers, dimers, and micelles enhance the solubility of UCB in bile.—**Ostrow, J. D., L. Celic, and P. Mukerjee.** Molecular and micellar associations in the pH-dependent stable and metastable dissolution of unconjugated bilirubin by bile salts. *J. Lipid Res.* 1988. **29:** 335–348.

Supplementary key words micelles • solubility of bilirubin • taurocholate • pigment gallstone etiology • disodium bilirubinate • metastable supersaturation • pK'a values of bilirubin • taurodehydrocholate

Though unconjugated bilirubin (UCB), crystallized from organic solvents (e.g., chloroform), is virtually insoluble in buffered aqueous solutions (1, 2), it is found in normal human gallbladder bile at concentrations up to 2 mg/dl (35 μM) (3–5). Elevated proportions and concentrations of UCB appear in the bile of many patients (4–8) or mice (9) with pigment gallstones; these stones contain UCB as a major component in the form of calcium bilirubinate salts or a black pigment polymer (10, 11). UCB precipitates from human bile as a Ca^{2+} salt when the conjugates are hydrolyzed by incubation with β-glucuronidase (12). Moreover, UCB precipitates in an undefined physical state in the small bile ducts of jaundiced Gunn rats when the concentration of UCB in bile is increased markedly by phototherapy and the concentration of bile salts is depleted simultaneously by external biliary drainage (13). These observations suggest that, like cholesterol, UCB may be solubilized by the lipid components of bile, especially bile salts (14), and that the concentrations of UCB in relation to bile salts might be a factor in the pathogenesis of pigment gallstones.

To address this issue, a number of investigators (1, 2, 15–20) have studied the solubility of UCB in model aqueous systems, with or without bile salts, akin to the studies performed with cholesterol by Carey and Small (21). However, as summarized in three recent reviews (22–24), there is little quantitative agreement among the various studies as to the absolute solubilities of UCB at various pH values, or the apparent pK'a values for the two carboxyl groups of UCB, though all agree that increasing pH and bile salt concentration enhance the aqueous solubility of UCB. We have therefore studied the solubility of UCB in relation to pH in solutions of various conjugated and unconjugated bile salts, including taurodehydrocholate (TDHC), which forms dimers but not micelles (25). Solubility was approached both from the dissolution of various forms of crystalline UCB diacid, and from the supersaturated conditions obtained by acidification of disodium bilirubinate. The results reveal marked dependency of solubility on the form of UCB

Abbreviations: TLC, thin-layer chromatography; UCB, unconjugated bilirubin; Na_2B, disodium bilirubinate; BH_2, UCB diacid; BH^-, UCB monoanion; $B^=$, UCB dianion; TC, sodium taurocholate; TDHC, sodium taurodehydrocholate; GC, glycocholic; TCDC, taurochenodeoxycholic.

[1]To whom reprint requests should be addressed at: V.A. Lakeside Medical Center (111G), 333 East Huron Street, Chicago, IL 60611.

used, convey understanding regarding the diverse solubilities reported by others, and provide evidence that the pK'a values of UCB are widely separated and much higher than appreciated previously.

MATERIALS AND METHODS

Materials

Crystalline UCB (lots #2391752 and 6544770, produced by British Drug Houses, Poole, Dorset, U.K.), purchased from Gaillard-Schlesinger Corp., New York, NY, was used without further purification. No colored impurities were extracted from their chloroform solutions by 0.1 M Na_2CO_3, and their extinction coefficients in chloroform at 453 nm were 59.3 and 59.9 $\times 10^3$ $M^{-1}cm^{-1}$. By thin-layer chromatography (TLC) (26, 27), the lots contained 99.7 and 99.9% UCB, respectively, and consisted of at least 95% IXα isomer. Lot #6544770 was used for all the detailed studies of the relationships of $[B_t]$ to pH (see Figs. 3, 7, 9, and 11–13), whereas lot #2391752 was used for all the preliminary studies of the relationship of $[B_t]$ to crystal type and amount (see Figs. 1 and 2), and to bile salt concentration (see Figs. 4–6, 8 and 10).

Piperazine-N,N'-bis (2-ethane sulfonic acid) sodium salt (PIPES), and [4-(2-hydroxyethyl)]-1-piperazine ethanesulfonic acid (HEPES), were obtained from Behring-CalBiochem Corp., La Jolla, CA. From the same firm were obtained the grade A sodium salts of taurocholic (TC), glycocholic (GC), taurochenodeoxycholic (TCDC), and taurodehydrocholic (TDHC) acids; all were over 98% pure by TLC (28), and contained no detectable unconjugated bile salts. Anhydrous sodium ethoxide, over 97% pure, was purchased from Aldrich Chemicals, Milwaukee, WI. All other chemicals were reagent grade, obtained from J. T. Baker Co., Phillipsburg, NJ. Precoated 0.25-mm thin-layer plates of silica gel G and H were purchased from Analtech, Inc., Newark, DE. Water, free of ions and organic contaminants, was prepared by passage of distilled water through mixed-bed deionizing and charcoal columns (Continental-Millipore-Waters Co., Broadview, IL); its conductivity was less than 10^{-6} Ω^{-1} cm^{-1}.

Forms of bilirubin crystals

Four forms of solid bilirubin were tested: *a)* DMSO-UCB: UCB, 5 mg/ml in dimethyl sulfoxide (DMSO), was added dropwise with stirring to 30 volumes of water. Addition of several drops of 50% (v/v) acetic acid led to sudden flocculation of a loose precipitate, which was washed thrice with water, and dried in vacuo for 3 days over anhydrous $CaSO_4$, yielding a brown powder; *b)* $CHCl_3$-UCB: UCB crystals were shaken with chloroform, to make a concentrated solution (ca. 20 mg/dl, 0.35 mM). This was sonicated for 10 min at 55 kHz and 125 watts in an ultrasonic cleaner (Model B-220, Branson Ultrasonics Corp., Shelton, CT). After filtration through chloroform-saturated Whatman #1 paper, the $CHCl_3$ was evaporated under a stream of 99.97% pure nitrogen at 50°C, and the crystals were stored for 3 days in vacuo over anhydrous $CaSO_4$. *c)* PHC-UCB: A 0.35 mM solution of UCB in chloroform was added dropwise, with stirring, to 10 vol of petroleum hydrocarbon (bp 30–60°C), leading to precipitation of the UCB. After centrifugation, the residue was washed twice with fresh petroleum hydrocarbon, dried under a stream of nitrogen at 50°C, and finally desiccated in vacuo for 3 days over paraffin shavings; *d)* BDH-UCB: The UCB crystals, which had been kept at least 4 years in the original sealed vial within a vacuum desiccator, were used directly. These crystals were also the source material for all other UCB preparations. Before use, all crystals were finely ground with an agate mortar and pestle.

For X-ray diffraction patterns, UCB crystals were packed into 1.0-mm diameter Lindemann glass capillaries (0.01-mm wall thickness) and mounted into a Debye-Scherrer camera of radius 57.8 mm. Photographic exposures were made over 10 hr, using Ni-filtered Cu-K_α radiation generated at 40 kV and 20 mA with a Rigaku Geiger-flex unit. These X-ray patterns revealed identical diffraction lines in all four preparations (**Fig. 1**), but with increasing sharpness, reflecting an increasing degree of order in the crystal structure, from DMSO-UCB (least ordered) < $CHCl_3$-UCB < PHC-UCB < BDH-UCB (most ordered).

Dissolution of UCB diacid (BH_2) crystals by bile salts

To a measured amount of crystalline UCB in sterile, 10-ml actinic glass flasks was added the desired concentration of bile salt, dissolved in 1.0 ml of sterile buffer–NaCl solution at the desired pH. Final buffer concentrations ranged from 0.02 to 0.2 M, and the total ionic strength ranged from 0.15 to 0.30, though most experiments were done with 0.1 M buffer and ionic strength = 0.20. The flasks were flushed with nitrogen, sealed with a Teflon stopper, and incubated with shaking (120 cycles per min) at 37°C, in the dark, under a nitrogen atmosphere. The mixtures were sonicated at 37°C for 10 min every 24 hr, up to 24 hr before the flasks were opened for analysis. Such treatment only hastened the attainment of constant $[B_t]$, without affecting its value.

After incubation (for 96 hr in detailed experiments), the suspensions were filtered (0.22 μm Millipore filter at 37°C) to remove undissolved bilirubin, and the final pH value of the filtrate was determined immediately, using an expanded scale pH meter (Digital Model 110, Corning Co., Corning, NY). The filtrate was then analyzed for bile salt concentration by the 3-hydroxysteroid dehydrogenase method (29), using enzyme from Worthington Biochemicals Corp., Freehold, NJ, and measuring the formation of NADH fluorimetrically. $[B_t]$ was determined by the Michaelsson diazo method (30) when it exceeded 1.0 μM (azopig-

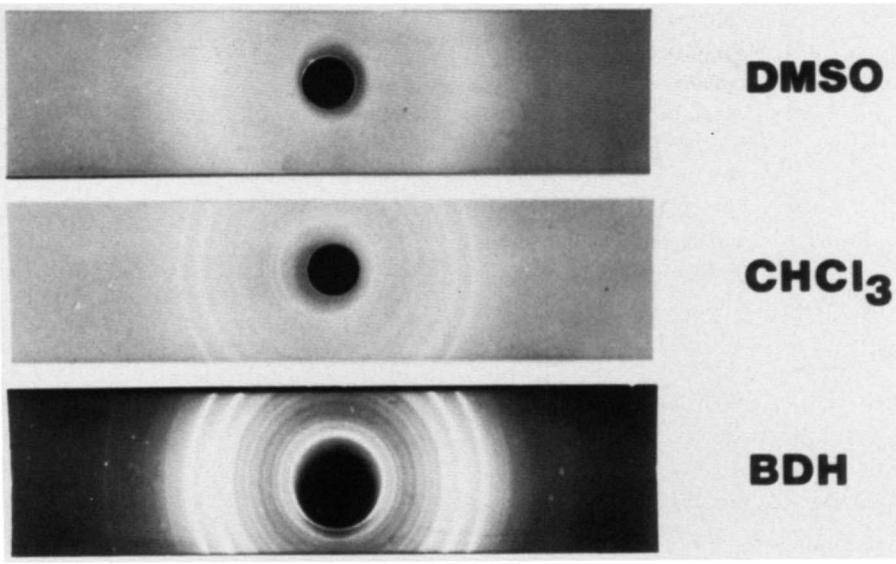

Fig. 1. X-ray diffraction patterns of three different batches of bilirubin crystals, prepared as detailed in text. DMSO, flocculated from dimethylsulfoxide (DMSO) by addition of water; $CHCl_3$, precipitated by drying chloroform solution under nitrogen at 50°C; BDH, original batch of crystals (lot #2391752) from British Drug Houses. The same lines occur with increasing definition from above to below. A fourth type of crystal (PHC), flocculated from chloroform by addition of petroleum hydrocarbons, gave a pattern (not shown) nearly as ordered as BDH.

ment–blank absorbance at 600 nm was > 0.02). $[B_t]$ from 0.1 to 1.0 μM was measured by spectral absorbance of UCB itself at 450 nm, and by a more sensitive diazo procedure. In this, 1.5 ml of filtrate was adjusted to pH 5.0, and the UCB was extracted into chloroform, which was then evaporated at 50°C under nitrogen. The UCB residue was dissolved in 0.2 ml of DMSO and assayed for diazoreactivity by Michaelsson's method, yielding results in agreement with the $A_{450\ nm}$ of UCB. All three methods were standardized with weighed amounts of pure UCB crystals, dissolved in the appropriate initial solutions.

Preparation of disodium bilirubinate

Disodium bilirubinate (Na_2B) was prepared by titration of a saturated solution of UCB in chloroform by dropwise addition of sodium ethoxide, 1.95 mol per mol UCB, dissolved in one-sixth volume of dry methanol. After evaporation of the solvent under vacuum at 50°C, the dry brown powder was washed twice with $CHCl_3$ to remove untitrated UCB, and the $CHCl_3$ was removed under vacuum. The residue was then dissolved in deionized water containing twice the desired concentration of bile salt, and the solution was then brought to the desired pH and ionic strength by rapid addition of an equal volume of buffer. Incubations for 96 hr and analyses were performed as described for the studies with UCB crystals (see above).

Statistical analyses (31)

Best-fit linear and hyperbolic regressions were determined using the PDP-10 computer (Digital Equipment Co.). Using the Cyber computer at the Vogelback Computer Center at Northwestern University with program AR, version 85, for derivative-free nonlinear regression (BMDP Statistical Software Inc., University of California, 1964 Westwood Blvd., Suite 202, Los Angeles, CA 90025), data for log $[B_t]$ versus pH were fitted to the function $[B_t] = A \cdot (1 + K'_1/[H^+] + K'_1 \cdot K'_2/[H^+]^2)$ (1). This equation assumes that the value of A, the BH_2 concentration in solution, is controlled by dissolution of the crystals, which are composed only of BH_2. The other two terms contributing to $[B_t]$ arise from the extremely rapid ionizations of the dissolved BH_2, and from interactions of BH_2, B^-, and $B^=$ with each other and with bile salts, if present. A was taken as the mean ± SD of the 9 to 11 values for $[B_t]$ obtained between pH 3.0–4.0 for that experiment, and had coefficients of variation ≤ 2.5%. Using this A value, the computer then estimated best fit values for pK'_1 and pK'_2, with a coefficient of variation of < 2% in each instance.

RESULTS

Validation of experimental methods

Replicate incubations, sampled at 24- to 72-hr intervals for up to 240 hr, revealed that dissolved bilirubin concentrations were constant from 72 hr on. However, in some instances, $[B_t]$ initially increased to a maximum value at 24 hr that was up to twice the stable $[B_t]$ attained after 72 hr. Analyses in definitive studies were therefore performed after 96 hr of incubation. Repassage of the filtrates through a

fresh Millipore filter revealed no alteration in the concentrations of dissolved bile acids or UCB due to ultrafiltration. TLC of the solutions after incubation revealed no decomposition or isomerization of the bilirubin (26, 27) or bile acids (28), except at pH values above 8.5, and especially above 9.5. In this range, the only visible degradation was conversion of up to 1.6% of the dissolved UCB to mesobiliverdin (presumably by internal transfer of protons from the middle methylene bridge to one of the vinyl sidechains (32)). Incubations for 96 hr of unsaturated solutions of UCB at pH values up to 11.0 revealed loss of no more than 4.8% of the diazoreactivity. At pH $\leq$ 9.5, duplicate incubations yielded $[B_t]$ which agreed within 10%, and pH remained within 0.04 units of its initial value.

Effect of type and amount of bilirubin crystals (Fig. 2)

The solubility of the four different forms of UCB crystals varied inversely with the degree of order seen on X-ray diffraction, with DMSO-UCB most soluble > $CHCl_3$-UCB > PHC-UCB > BDH-UCB. The $CHCl_3$-UCB was used for all detailed studies, since it yielded $[B_t]$ that agreed with those obtained by acidification of Na_2B. Maximum solubility was attained at $\geq$ 3.0 mg UCB/ml solution at pH values below 8.0 (Fig. 2), but as much as 60 mg solid UCB was required at the highest pH values studied. However, even with lesser amounts of solid UCB, much undissolved UCB remained and no further dissolution occurred on prolonging the incubation beyond 96 hr. In each detailed incubation, the quantity of UCB used (3 to 60 mg/ml) was at least 20% more than the amount required to achieve maximum saturation.

Effects of buffer strength and total ionic strength on dissolution of UCB crystals

There were no significant differences in UCB solubility at total ionic strengths from 0.10 to 0.30, with buffer strengths varying from 0.02 to 0.2 M, at any pH value or [TC] studied. However, addition of 0.4–2.0 M NaCl produced moderate increases in UCB solubility that were related linearly to the increases in ionic strength. At pH 7.70, the slope of the linear plot of $[B_t]$ (μM) versus [NaCl] (M) was 55.6 in 30 mM TC and 170 in 130 mM TC ($r > 0.91$, $P < 0.01$ for each [TC]).

Solubility of UCB in buffered sodium chloride solutions (Fig. 3)

In the absence of bile salts, solubility of UCB was similar from dissolution of UCB crystals and after acidification of Na_2B solutions. $[B_t]$ was unmeasurable at pH values below 6.5, 0.25 μM at pH 7.0, 1.6 μM at pH 8.0, and 22 μM at pH 9.0, but rose steeply at pH $\geq$ 9.1 to reach about 10 mM at pH 9.6, and about 40 mM at pH 10.0. At pH $\geq$ 10.5, $[B_t]$ attained constant concentrations averaging 65 mM that were similar to those obtained in 50 mM TC or TDHC (see below).

Effect of taurocholate concentration ([TC]) on dissolution of UCB crystals at various pH values (Fig. 4, Fig. 5, Fig. 6)

At both pH 3.0 and 4.0 (Fig. 4), dissolution of UCB crystals was undetectable at [TC] up to its apparent critical micellar concentration of 6 to 8 mM (33), but increased in linear proportion to [TC] from 9 to 50 mM. At pH 5.0

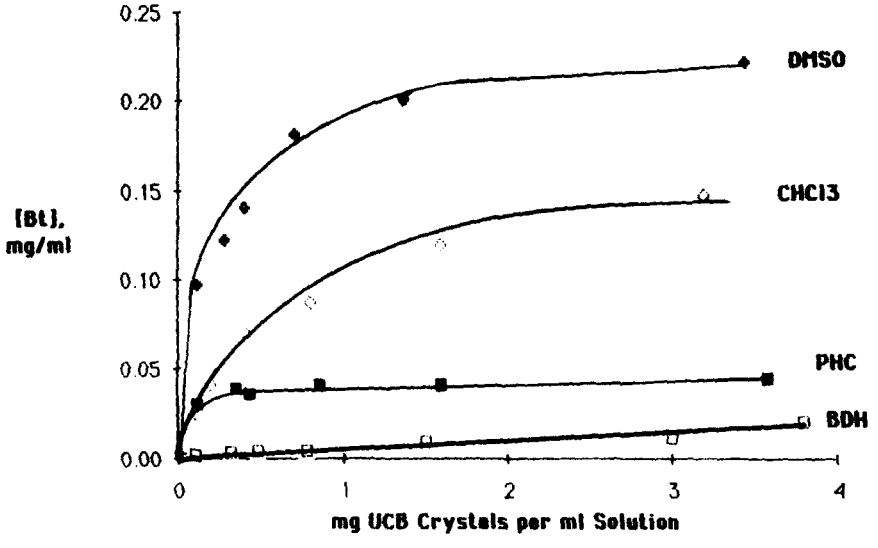

Fig. 2. Effect of increasing amounts of bilirubin crystals on its solubility ($[B_t]$) in 1.0 ml solution of 150 mM taurocholate (TC) in 0.05 M phosphate buffer, pH 7.3. Similar patterns (not shown) were seen with 50 mM TC, and at pH values of 6.2 and 7.8. The maximum $[B_t]$ was inversely correlated with the degree of order of the crystals (abbreviations as in Fig. 1).

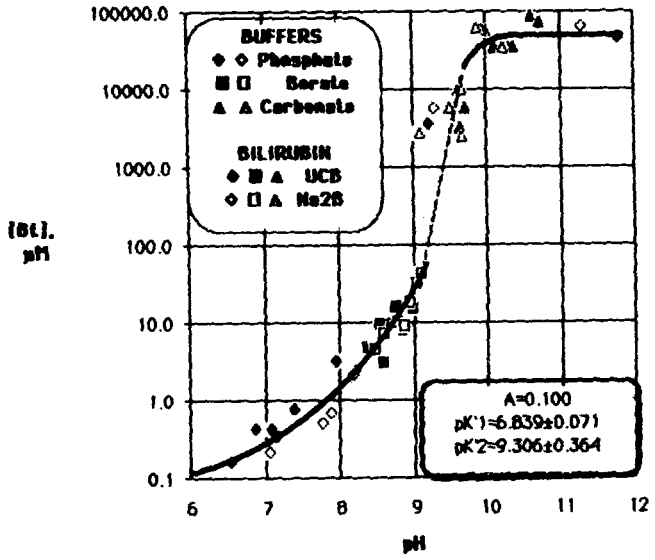

Fig. 3. Effect of pH on solubility of UCB in buffered saline, comparing $CHCl_3$-UCB crystals (filled symbols) and acidified Na_2B (open symbols), each prepared from the same lot of UCB (# 6544770). Results were similar from both forms of UCB. Buffers were 0.02 M and total ionic strength 0.15. At pH < 6.4, $[B_t]$ was below the measurable limit of 0.2 mM. The computer-derived parameters (mean ± SD) and theoretical curve from pH 6.5 to 9.1 were derived with A set at 0.10, which gave the best fit. When A was set at 0.20, pK'_1 = 7.253 ± 0.085 and pK'_2 = 8.982 ± 0.257. Computer analyses that included the values above pH 9.1 did not converge for the pK'a values, indicating that the data with carbonate belonged to a different parametric group.

and 5.5 (Fig. 4), dissolution was undetectable up to [TC] of 3 mM and 2 mM, respectively, with a curvilinear increase in $[B_t]$ at higher [TC]. At pH 6.4 and 7.0 (Figs. 5 and 6), $[B_t]$ was < 0.2 and 0.6 μM in the absence of bile salt, increased hyperbolically from 0 to 8 mM TC, and then increased linearly as [TC] increased from 10 to 250 mM. At pH 7.8 (Fig. 6), $[B_t]$ increased hyperbolically throughout, from a concentration of 1.7 μM in the absence of bile salt to 543 μM at [TC] = 280 mM. At this and all higher pH values (not shown), the plots of $[B_t]$ versus [TC] fit a curvilinear function of the type given in the legend to Fig. 6.

Effect of pH on dissolution of UCB crystals at [TC] = 50 mM

Fig. 7 shows $[B_t]$ in 50 mM TC in relation to pH. From pH 3.0 to 3.8, $[B_t]$ was essentially constant at an average value of 1.9 μM. From pH 4.0 to 6.8, log $[B_t]$ increased gradually with pH. From pH 6.8 to 8.5, log $[B_t]$ increased pseudolinearly with pH, with a slope of 0.98. Solubilities in Tris buffer were significantly ($P < 0.05$) less than those in PIPES or borate buffer at corresponding pH values, but the mean slope was close to 1.0 in each buffer in this pH range. From pH 8.5 to 9.8, the slope increased toward a value of 2.0. Above pH 9.8, $[B_t]$ increased little with increasing pH, and above pH 10.0, became essentially constant at a mean value of about 60 mM, similar to the $[B_t]$ seen without bile salts in this pH range (Fig. 3).

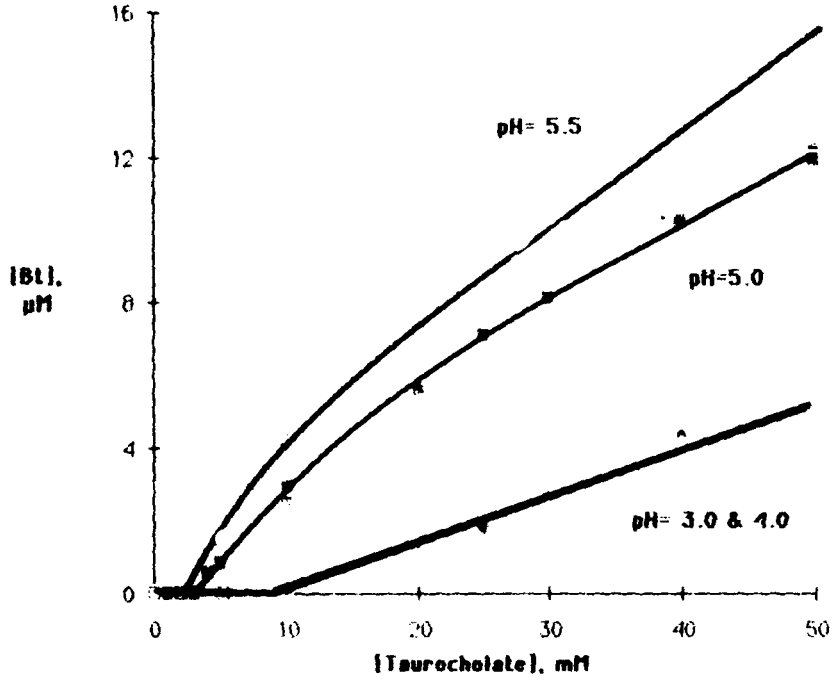

Fig. 4. Effect of increasing taurocholate concentration on dissolution of UCB crystals (lot # 2391752) at low pH values. No dissolution was detected below [TC] = 9 mM at pH 3.0 or 4.0, below [TC] = 3 mM at pH 5.0, and below [TC] = 2.0 mM at pH 5.5.

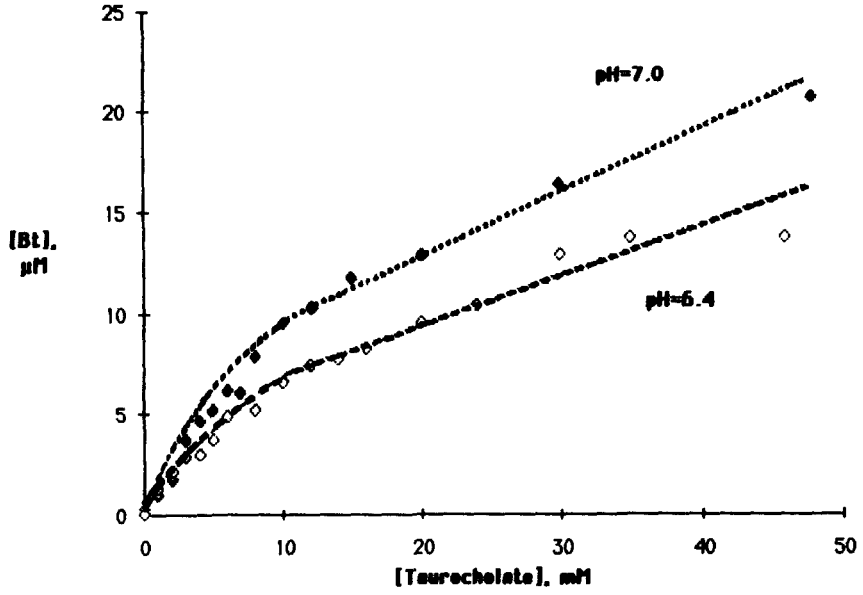

Fig. 5. Effect of increasing taurocholate concentration on dissolution of UCB crystals (lot #2391752) at pH 6.4 and 7.0, in 0.05 M phosphate buffer and total ionic strength 0.30. In the absence of bile salt, $[B_t]$ was < 0.2 and 0.61 μM, respectively. Hyperbolic increases in $[B_t]$ were seen as [TC] increased from 0 to 8 mM, and then linear increases from 9 to 250 mM TC at each pH value.

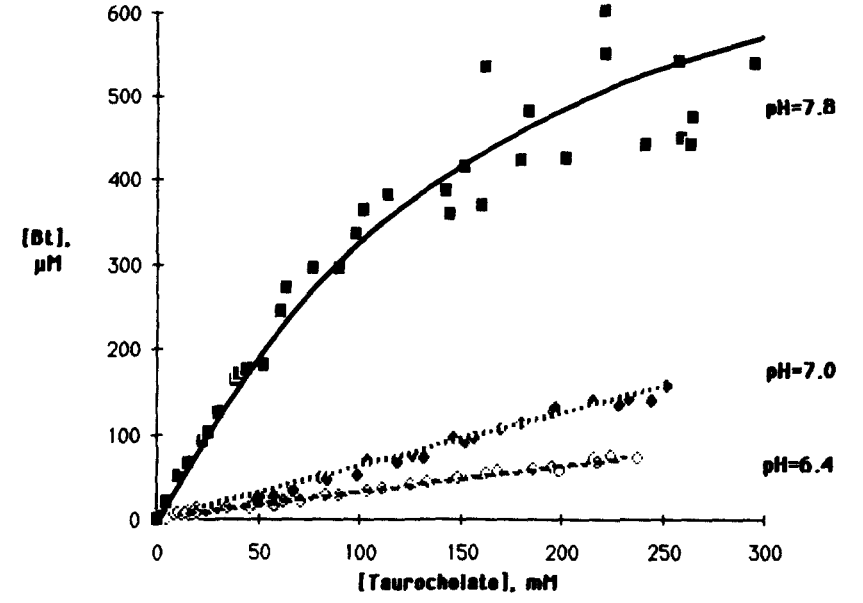

Fig. 6. Effect of increasing taurocholate concentration on dissolution of UCB crystals (lot #2391752) in 0.05 M phosphate buffer at pH 6.4, 7.0, and 7.8, at total ionic strength 0.30. Solubility at pH 7.8 in the absence of bile salt was 1.7 μM, with a hyperbolic increase in $[B_t]$ as [TC] increased from 0.25 to 280 mM {$[B_t]$ (μM) = [TC] (mM)/(0.1971 + 0.001136 · [TC]), $P < 0.005$}. The linear portions of the graphs at pH 7.0 and 6.4 ([TC] = 9–250 mM) were described by the regressions: at pH 7.0, $[B_t]$ (μM) = 0.6139 · [TC] (mM) −2.44, $r = 0.99$, $P < 0.005$; at pH 6.4, $[B_t]$ (μM) = 0.3061 · [TC] (mM) + 2.17, $r = 0.99$, $P < 0.005$. See Fig. 5 for enlargement of initial portions of graphs at pH 6.4 and 7.0.

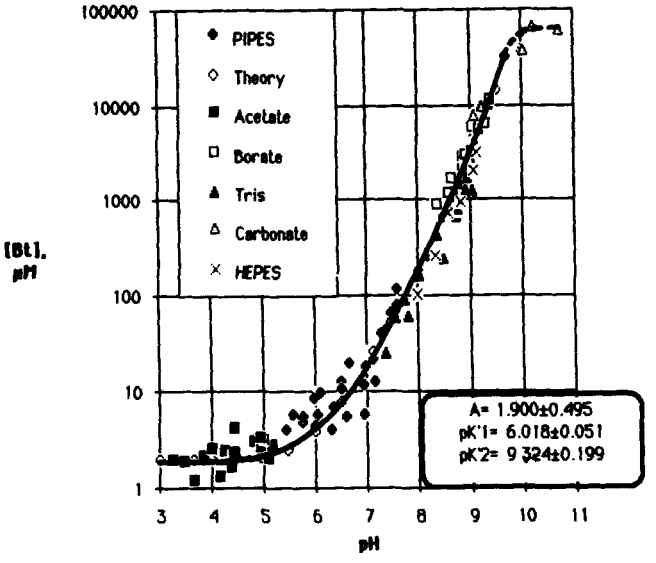

Fig. 7. Effect of pH on dissolution of UCB crystals (lot #6544770) in 50 mM taurocholate solutions with 0.1 M buffer and total ionic strength 0.20. The semilogarithmic plot is approximately linear from pH 6.8 to 8.5, with a slope of 0.978 (r = 0.94 and $P < 0.01$). The box in the lower right lists the computer-derived parameters of the plotted curve (means ± SD), using values obtained in all the buffers except carbonate. Values, obtained with data from Tris buffers excluded were pK'_1 = 5.975 ± 0.051 and pK'_2 = 9.332 ± 0.253.

A comparable study, using UCB lot #2391752 in 50 mM TC, yielded $[B_t]$ values that were higher by almost a factor of three at each pH value (curve not shown). This analysis indicated that the A value (BH_2 concentration) arising from the BH_2 crystals in this lot was three times that of lot #6544770, but that the computed values for pK'_1 and pK'_2 were almost the same in both lots.

Dissolution of UCB crystals by taurodehydrocholate (TDHC)

Fig. 8 shows that increasing concentrations of TDHC at pH 6.4, 7.0, and 7.8 increased the solubilization of UCB in a manner similar to TC (Fig. 6), but the solubilities were much less in TDHC. The changes in $[B_t]$ with increasing pH in 50 mM TDHC (**Fig. 9**) likewise exhibited the pattern seen with 50 mM TC (Fig. 7), but the solubilization of UCB was much lower throughout, especially at pH 3.0–3.8, where $[B_t]$ was only 0.45 mM in TDHC, or 24% of the values in TC. However, above pH 10.2 (not shown), $[B_t]$ in 50 mM TDHC was constant at about 60 mM, similar to the $[B_t]$ seen in TC or NaCl solutions.

Dissolution of UCB crystals by other bile salts

Fig. 10 shows that the solubilization of UCB by glycocholate (GC) and taurochenodeoxycholate (TCDC) was almost the same as by TC at two pH values that span the physiological pH range of bile. At pH 7.7, solubilization of UCB by the unconjugated bile salt, chenodeoxycholate (not shown) was as effective as its taurine conjugate (TCDC). The unconjugated bile salt was not studied at lower pH values because of limited solubility at acidic pH values (34).

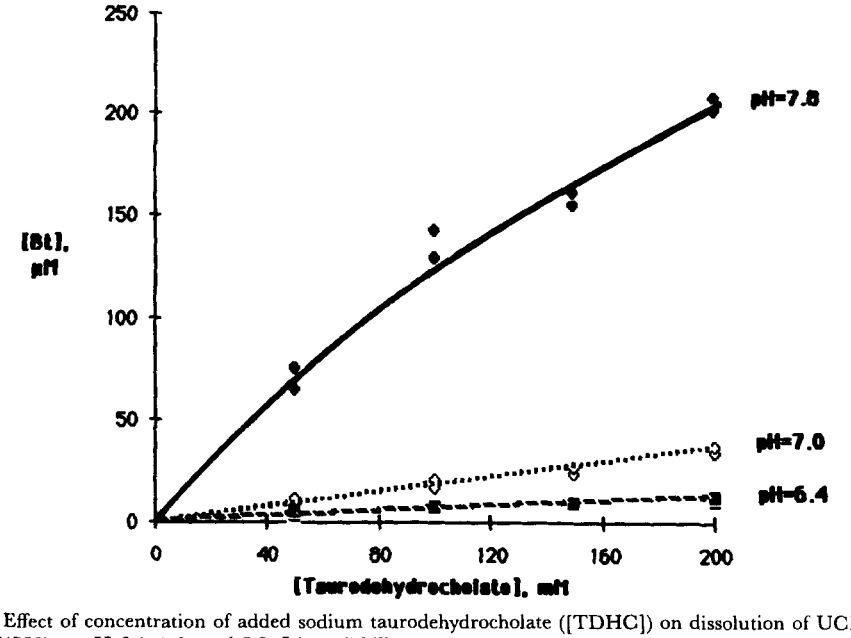

Fig. 8. Effect of concentration of added sodium taurodehydrocholate ([TDHC]) on dissolution of UCB crystals (lot #2391752) at pH 6.4, 7.0, and 7.8. The solubility curves are similar in shape, but much lower in $[B_t]$, compared to those obtained with taurocholate at the same pH values (Fig. 6). Studies done with phosphate buffers of ionic strength 0.05 and total ionic strength = 0.30. Regressions were: at pH 7.8, $[B_t]$ (μM) = [TC] (mM)/(0.596 + 0.002033 · [TC]), $P < 0.01$; at pH 7.0, $[B_t]$ (μM) = 0.1796 · [TC] (mM) + 0.137, r = 0.98, $P < 0.005$; at pH 6.4, $[B_t]$ (μM) = 0.0513 · [TC] (mM) + 0.063, r = 0.99, $P < 0.005$.

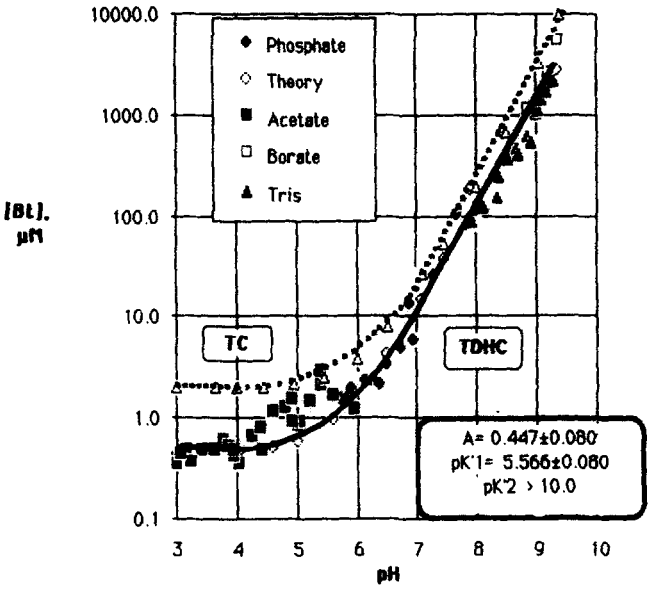

Fig. 9. Effect of pH on dissolution of UCB crystals (lot #6544770) in 50 mM taurodehydrocholate (TDHC) with 0.1 M buffers and total ionic strength 0.20 (solid line). The curve resembled that obtained for TC (dashed line) with the same lot of UCB crystals (see Fig. 7), but the $[B_t]$ with TDHC were much lower throughout. For TDHC, the semilogarithmic plot of $[B_t]$ versus pH was essentially linear from pH 6.8 to 9.0, with a slope of 1.01 ($r = 0.96$, $P < 0.01$). Theoretical curve and pK'a values (mean ± SD) shown for TDHC include data for Tris. Values with Tris data excluded were $pK'_1 = 5.463 \pm 0.053$ and $pK'_2 = 9.811 \pm 0.643$.

Solubility after acidification of Na_2B in TC and TDHC solutions

When disodium bilirubinate was dissolved initially in unbuffered TC solutions, and the pH then reduced from 11.0 by rapid addition of buffer (**Fig. 11**), the resultant $[B_t]$ was only slightly higher than the solubility of UCB crystals at final pH values above 9.0. As the final pH declined from 8.0 to 6.0, $[B_t]$ actually *increased* from about 3 mM to 15 mM, and was always much greater than $[B_t]$ from crystals. From pH 6.0 to 4.0, $[B_t]$ was level at 10–15 mM, or about 10^4 times the maximal solubility of UCB crystals in this pH range. Below pH 4.0, $[B_t]$ again declined, but still remained almost three orders of magnitude higher than dissolution of UCB crystals. These high solubilities were not decreased by addition of crystalline UCB to the incubation mixtures.

By contrast, experiments with acidification of Na_2B in 50 mM TDHC solution yielded solubility curves (**Fig. 12**) that resembled those obtained with dissolution of UCB crystals by TC and TDHC (Figs. 7 and 9). Nonetheless, $[B_t]$ from acidification of Na_2B in TDHC averaged six times greater than dissolution of UCB crystals by TDHC, although the derived pK'_1 and pK'_2 values were similar.

Mathematical modelling of the solubility curves

Except for the data obtained from acidification of Na_2B in 50 mM TC (Fig. 11), all the curves of UCB solubility versus pH yielded a reasonable fit to the equation $[B_t] = A \cdot (1 + K'_1/[H^+] + K'_1 \cdot K'_2/[H^+]^2)$, where A = the constant $[B_t]$ at pH below 4.0, and K'_1 and K'_2 are the apparent ionization constants of the first and second carboxyl groups of UCB. At pH 4.5–6.3, experimental values for $[B_t]$ tended to be slightly higher than calculated $[B_t]$. However, computer-derived pK'a values changed less than 0.1 unit when data in this region were excluded, and the fit was extremely close above and below this pH range. Computer-derived best fits for pK'_1 ranged from 6.0 to 6.2 in TC and from 5.4 to 5.6 in TDHC; best fits for pK'_2 were 9.3–9.5 in TC and 9.8 or higher in TDHC (Figs. 7, 9, 12). In each case, the higher values were obtained when Tris-buffered solutions were included in the data. In the absence of bile salts, the derived pK'_1 was 6.8 to 7.2 and pK'_2 was 9.3 to 9.0, for A values from 0.1 to 0.2 μM. Since, in buffered saline solutions, measured $[B_t]$ at pH < 6.5 was too low to be reliable, A values had to be estimated by extrapolation to pH ≥ 4.0, rather than measured.

Using the computed pK'_a values from Fig. 7, we calculated the proportions of BH_2, BH^-, and $B^=$ in 50 mM solutions (**Fig. 13**). The diacid (BH_2) constituted virtually all the dissolved UCB below pH 5.9, but was ≤ 1% of total UCB above pH 8.0. At pH 6.2, the solution contained equal amounts of BH_2 and the monoanion, BH^-. The monoanion constituted over 90% of the UCB from pH 6.95 to 8.4. The dianion ($B^=$) constituted less than 1% of total UCB below pH 8.05, but became the dominant anion above pH 9.2. Thus, if the solubilization of UCB crystals is an appropriate model for bile, BH^- accounted for over 95% of the *ionized* UCB at physiological pH values.

DISCUSSION

Though there have been many prior studies of the aqueous solubility in UCB, with and without bile salts (1, 2, 15–20), this is the first to comprehensively consider: the form and quantity of UCB crystals; the effects of pH, ionic strength, the type and concentration of bile salts, and the presence or absence of micelles; and to compare solubilities obtained by dissolution of UCB crystals versus acidification of dissolved disodium bilirubinate (Na_2B). We also provided evidence that $[B_t]$ attained a relatively constant value many days beyond the 96-hr period at which analyses were performed, and that the Millipore filtration did not selectively remove individual dissolved components. Though we excluded light and oxygen, up to 4.8% of the dissolved UCB was degraded to diazo-negative products above pH 8.5, and especially above pH 9.5. However, it is considered unlikely that interactions of this small proportion of degradation products with the bile salts (23), or the bilirubin, greatly altered the solubility of UCB.

The major new findings and concepts from our work in-

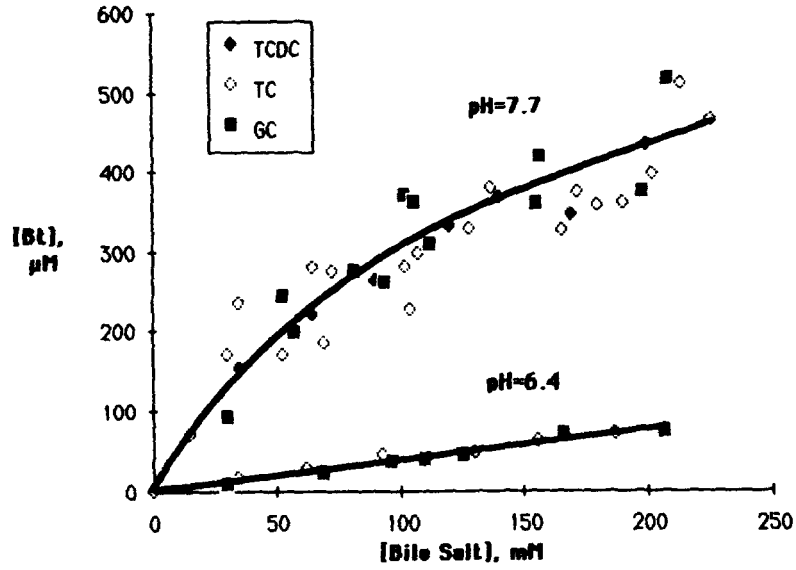

Fig. 10. Effect of varying concentrations of sodium taurocholate (TC), sodium glycocholate (GC), and sodium taurochenodeoxycholate (TCDC) on dissolution of UCB crystals (lot #2391752) by bile salts in 0.05 M phosphate buffers, pH 6.4 and 7.7, at total ionic strength = 0.30. There are no significant differences among the three conjugated bile salts. Regressions at pH 7.7 are: $[B_t]$ (μM) = [TC] (mM)/(2.222 + 0.0325 · [TC]), $P < 0.01$; $[B_t]$ (μM) = [GC] (mM)/(3.356 + 0.0191 · [GC]), $P < 0.01$; $[B_t]$ (μM) = [TCDC] (mM)/(2.996 + 0.0273 · [TCDC]), $P < 0.01$. Regressions at pH 6.4 are: $[B_t]$ (μM) = 0.0209 · [TC] (mM) + 0.085, $r = 0.99$, $P < 0.005$. $[B_t]$ (μM) = 0.0225 · [GC] (mM) − 0.047, $r = 0.99$, $P < 0.005$.

cluded: the dramatic differences in solubility among different forms of UCB crystals, and of crystals compared with acidified Na_2B; the possibly important role of particle size effects; and the wide separation of the two apparent pK'a values of UCB in the presence or absence of bile salts. We also corroborated the findings, obtained by other methods (15, 35, 36), that $[B_t]$ increases with increasing pH values, and with increasing bile salt concentrations at all pH values up to about 10, indicating that salts of BH^- and $B^=$ were soluble, and that bile salts interacted with all three species of UCB.

Apparent solubilities of UCB in the absence of bile salts

Over the physiological pH range of bile (6.0–8.0) (37), our solubilities from dissolution of UCB crystals (Fig. 3) were one or more orders of magnitude higher than the values reported by Brodersen (2), and by Moroi, Matuura, and Hisadome (15). On the other hand, our solubilities were much lower at pH 8, and higher at pH 6, than those calculated by Overbeek, Vink, and Deenstra (1) from potentiometric titration data. However, those investigators completed their experiments in only a few hours; we have documented the need for more prolonged experiments. The concordance, within experimental error, between the $[B_t]$ values we obtained from undersaturated solutions (UCB crystals), and supersaturated solutions (acidification of Na_2B) (Fig. 3), meets an essential criterion of the attainment of equilibrium.

Apparent solubilities of UCB in the presence of bile salts

Our data indicate that establishment of solubility equilibrium is a more complex problem in the presence of bile salts. First, at all pH values and bile salt concentrations, large excesses of UCB crystals were required to obtain reproducible values of $[B_t]$, especially at low pH

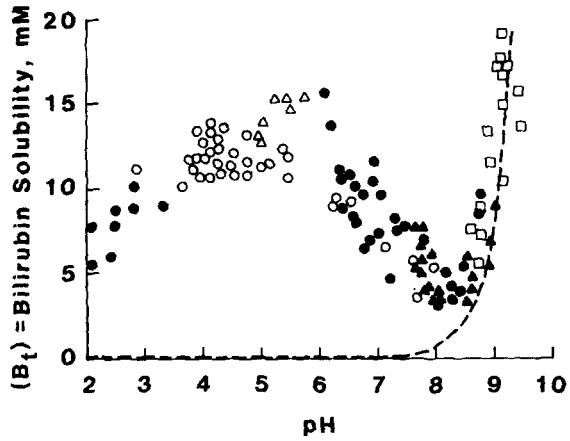

Fig. 11. Effect of pH on solubility of UCB obtained by acidification of solutions of disodium bilirubinate (Na_2B) in 50 mM taurocholate (TC) in 0.1 M buffers and total ionic strength 0.20. Note the linear scale on the ordinate. The dashed line is the curve from Fig. 7, showing dissolution by taurocholate of UCB crystals of the same lot (#6544770) used to prepare the Na_2B. Buffers were: (●) phosphate, (○) acetate, (△) citrate, (▲) Tris, and (□) borate. Though not shown, the $[B_t]$ above pH 9.3 were identical to those obtained from UCB crystals in 50 mM TC (Fig. 7).

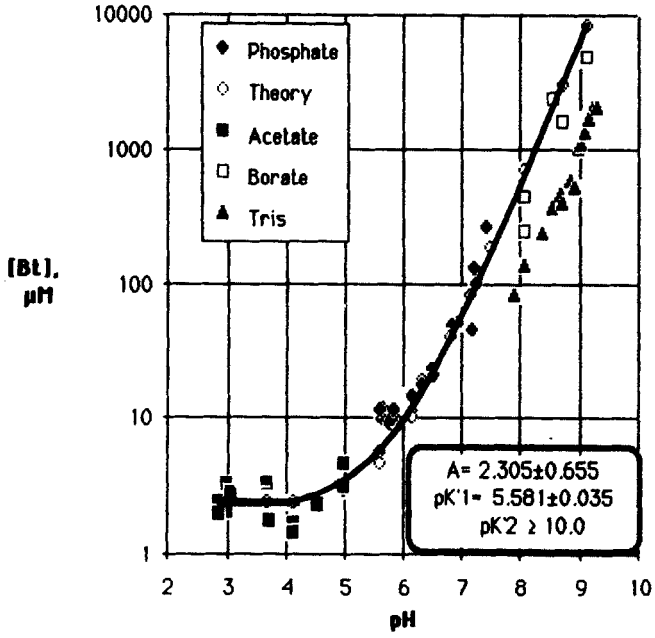

Fig. 12. Effect of pH on solubility of UCB obtained after acidification of solutions of Na_2B in 50 mM taurodehydrocholate (TDHC) in 0.1 M buffers and total ionic strength 0.20. Ordinate scale is logarithmic. Compare with curves for solubility from acidified disodium bilirubinate (Fig. 11) and UCB crystals (Fig. 7) in taurocholate (TC), and of UCB crystals in TDHC (Fig. 9), all derived from the same lot of UCB (#6544770). The computer-derived theoretical curve, and its parameters (mean ± SD) exclude data for Tris. With Tris included, no convergence was obtained for either pK'_1 or pK'_2.

values. Second, (Figs. 1 and 2), greater stable solubility values were reached with crystals demonstrated by X-ray diffraction to be less ordered, although the identity of X-ray diffraction lines (Fig. 1) indicated that no more than one crystalline form was present. Third, in some incubations, $[B_t]$ was much higher at 24 hr than at 48 and 72 hr. Finally, in 50 mM TC solutions below pH 8.5, we found much higher apparent solubilities from acidified Na_2B, than from dissolution of UCB crystals at the same final pH values (Fig. 11). Similar solubilities from acidification of Na_2B were reported by Nakama and his coworkers (17, 18) and by Wosiewitz and Schroebler (19).

Possible factors in these phenomena include: the effects of bile salts on crystal dissolution rates; the effects of different amounts, and of heterogeneous forms (38–40) and sizes (41) of crystals; formation of solvates; and effects of bile salts in prolonging metastability. Mysels and McBain, with six different preparations of aluminum dilaurate crystals in water (38), and Brodersen (2), with UCB itself in methylisobutyl ketone, reported higher apparent solubilities with increasing quantities of crystals. Brodersen's work (2) indicates that, for UCB, these phenomena reflect intrinsic properties of the crystals, and are unrelated to the presence of bile salts or micelles, or to the formation of hydrates.

Inhomogeneities may arise from different forms and/or different sizes of crystals. The early, temporary overshoot in $[B_t]$, mentioned above, strongly suggests that, during incubation, finer or less ordered (and thus more soluble) crystals gradually became coarser or more ordered, thus resulting in less-soluble microsuspensions, as observed by Rosoff et al. (41). Reorganization with time has been detected by serial X-ray diffraction during maturation of dry UCB crystals over a 5-week period (42).

The prolonged metastability in 50 mM TC (Fig. 11) is likely to have arisen from two factors: the small fraction of dissolved bilirubin that is in the form of unbound BH_2, which is the only species involved in crystal growth and dissolution, and the presence of a surface-active species. Supporting this argument is the fact that TDHC, which is less surface-active than TC, and is also less efficient in solubilizing UCB crystals, was vastly less effective in stabilizing the metastable microsuspension of UCB (Fig. 12 vs. Fig. 11). Thus, interactions of UCB species with bile salts, especially in micelles, apparently were instrumental in the formation of these metastable UCB solutions. The rapidity of acidification of the Na_2B also was a factor, since, in bile salt solutions, our solubilities of UCB crystals agreed closely with solubilities obtained by *gradual* precipitation of UCB from alkaline solutions of Na_2B upon *very slow* titration with HCl (Berman, Koretsky, and Carey (20)).

Separation of the two pK'a values of UCB

The analysis of our data on $[B_t]$ versus pH indicated that the classical model for dissolution of crystals of UCB (1, 2, 24) could be applied to our systems at pH values between 3.0 and 9.2. In the absence of bile salts, this model assumes that the dissolved BH_2 species, that is in equili-

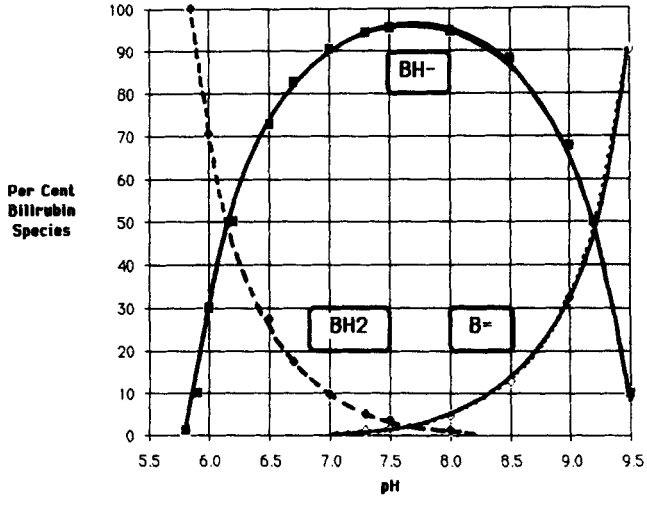

Fig. 13. Proportions of UCB diacid (BH_2), monoanion (BH^-), and dianion ($B^=$) in 50 mM taurocholate solutions from pH 5.8 to 9.5, calculated from the theoretical curve and the parameters in Fig. 7. BH^- is the dominant anion throughout the physiological pH range of bile (6.0 to 8.0).

brium with the crystals of the diacid, ionizes in solution to an extent determined by the pH and the intrinsic pKa values. If bile salts are present, the UCB species then associate with the monomers and aggregates (e.g., dimers and micelles) of bile salts. The mathematical expression of this model, $[B_t] = A \cdot (1 + K'_1/[H^+] + K'_1 \cdot K'_2/[H^+]^2)$ does not assume equilibration among multiple solid forms of UCB crystals, and does not have separate terms to describe either the aggregation of the dissolved BH_2, BH^-, and $B^=$ with each other or their interactions with bile salts. These effects are included in the A, K'_1 and K'_2 parameters. For example, the constant A denotes the total solubility of all dissolved BH_2 species, whereas the term $K'_1/[H^+]$ includes all species containing BH^-, arising from the dissolved BH_2. The relative constancy of BH_2 activity, over the entire range of pH values, accounts also for the attainment of stable, reproducible values of $[B_t]$ for each batch of crystals, even when impurities or crystal effects render that activity anomalously high.

As calculated from our data using this model, the apparent pK'_1 of UCB ranged from 5.6 to 6.8, and the apparent pK'_2 exceeded 9.2. In the absence of bile salts, the discordant pK'a values derived from this model by Brodersen (2, 24), and by Moroi et al. (15), likely reflected the brevity of their incubations (see above). However (Fig. 3), our calculated pK'_1 value was dependent on the considerable experimental uncertainty of the estimated A constant, and the accuracy of our calculated pK'_2 was affected by the scatter of the data at pH 9.1–9.8 in the carbonate buffers. Moreover, in this pH range, the presence of a slope much steeper than 2.0 is strongly indicative of increasing self-association of $B^=$ (35) as its concentration increases. Neglect of this self-association in our mathematical model results in an underestimation of the value of pK'_2. Above pH 10, despite some degradation of UCB, the level values of $[B_t]$ suggest that UCB solubilities here are controlled by the solubility of $Na^+_2B^=$, which comprises almost all the dissolved UCB in this pH range.

In the presence of bile salts (Figs. 7 and 9), the terms $K'_1/[H^+]$ and $K'_1 \cdot K'_2/[H^+]^2$ together clearly increase with pH from nearly zero at pH $\leq$ 4.0 to about 10 μM at pH 7, about 10^3 μM at pH 9, and over 10^4 μM above pH 10. Since all equilibria involving ionization and other interactions among the dissolved species are expected to be extremely facile (43), the problems of attainment of thermodynamic equilibria in relation to the crystals are confined to the determination of the constant A, the activity of dissolved BH_2. Apparently, undetected impurities or different particle sizes, in different batches of crystals, affect only this parameter. These arguments help explain why two different lots of UCB crystals in 50 mM TC yielded $[B_t]$ values that differed by a factor of 3 over the whole pH range, the result of a change in the value of A in solution, with little alteration in the apparent pK'_1 and pK'_2 values. Our values for pK'_2 are probably overestimated due to the reduction in the solubilizing power of TC and TDHC at pH values above 9.2 (see below), but underestimated due to the aforementioned strong self-association of $B^=$, which also occurs in the presence of bile salts (35). The values for pK'_1 are more reliable, because the self-association of BH^- (pH $\leq$ 8.5) is much less avid (44), and because $[B_t]$ is low, giving relatively "ideal" conditions.

These unexpectedly high and widely separated pK'a values for UCB, which would render BH^- the dominant UCB anion in bile (Fig. 13), applied even in the absence of bile salts (Fig. 3), and are therefore intrinsic properties of the UCB diacid, likely related to the internal hydrogen bonding of the -COOH groups in UCB (42, 45) that retards the dissociation of the bonded protons. In corroboration, pK'a values below 5.0, expected of carboxylic acids, are obtained when UCB is titrated in solvents (e.g., DMSO or dimethylformamide) which interfere with formation of hydrogen bonds (22, 46). The apparent pK'_1 of UCB, that we obtained in the presence of both bile salt micelles (Fig. 7) and dimers (Fig. 9), is lower than our estimated pK'_1 in the absence of bile salts (Fig. 3). This is opposite to reports that micellar binding increases the pK'a of organic monoanions, such as fatty acids (47, 48) or indicator dyes (49). Theory predicts such an increase in pK'a, compared with the bulk phase, due to the lower effective dielectric constant at the surface of the micelle (43). This in turn results from dielectric saturation, the high concentration of counterions, and the proximity of the hydrocarbon core of the micelle to the micellar surface "layers" (43). The contrary behavior of UCB may derive from a major conformational change as the pigment binds to micelles, akin to that which occurs when UCB binds to albumin (24).

Interactions between bilirubin species and bile salts at various pH values

The solubility functions of log $[B_t]$ versus pH (Figs. 7 and 9) reflect the molecular interactions among the three species of bilirubin (BH_2, BH^-, and $B^=$) and the bile salt monomers, dimers, and micelles. At pH < 4.0, $[B_t]$ was minimal until [TC] exceeded its critical micellar concentration of 6–8 mM (33) (Fig. 4), and was about fourfold greater in 50 mM TC (Fig. 7) than in 50 mM TDHC (Fig. 9). These findings suggested that dissolution of the crystals of BH_2 diacid was promoted by micelles and/or dimers of bile salts, and that micellar dissolution was more effective.

Above pH 4.0, the slope of the plot of log $[B_t]$ versus pH gradually increased. From pH 6.8 to 8.5, the slope was approximately constant at about 1.0, reflecting titration primarily of the first -COOH group of bilirubin (2, 22, 24). At pH above 6.2, where BH^- predominates, $[B_t]$ was also increased by TC at premicellar concentrations (Figs. 5 and 6) and by monomers of TDHC (Fig. 8). Thus, unlike BH_2, BH^- also interacts with bile salt monomers. At pH 8.2, where over 90% of the dissolved UCB is BH^- (Fig. 13),

the aqueous solubility of UCB crystals was increased approximately 100-fold by addition of 50 mM TC (Figs. 7 vs. 3), consistent with the findings of Rege, Webster, and Ostrow (36) who have shown, by an independent method, that UCB is over 99% bound to bile salts.

At pH 8.5–9.8, the slope increased toward a value of 2.0, compatible with the progressive ionization of the second -COOH group of UCB. However, above pH 10, the $[B_t]$ values leveled off in both 50 mM TC and 50 mM TDHC (Figs. 7 and 9) to about the same value as was observed with bile salts. The minimal effect of the addition of 50 mM TC in this pH range (Fig. 7) may be explained as follows. At pH values from 3.0 up to about 8.5, where $[B_t]$ was less than 2% of the concentration of the bile salt, the association of BH_2, BH^-, and $B^=$ with bile salts occurred at almost constant activities of BH_2, Na^+, and of the interacting bile salt monomers and aggregates. Above pH 8.5, however, aggregation of the bile salt species with UCB anions presumably decreased the activity of the bile salts, and thus reduced their ability to solubilize UCB, especially at pH $\geq$ 10.0, where $[B_t]$ actually exceeded the concentration of the bile salt. The small residual solubilizing influence of bile salts presumably counterbalanced the decrease in $[B_t]$ expected from the one-third higher Na^+ concentration used in the bile salt experiments. Near-exhaustion of the availability of TC at pH > 9, decreasing its thermodynamic activity and surface activity, would increase the proportion of unbound BH_2, promoting crystallization; this may explain the absence of metastability of UCB at the higher pH values (Fig. 11).

Molecular models of UCB monoanions (BH^-) (23) reveal a folded, biplanar configuration, with an ionized sidechain protruding from the concave surface. Although the convex surface of the biplane is hydrophobic, the polar -CONH and >NH groups, arranged along the perimeter of the dipyrromethenone halves of the UCB molecule, may restrict intercalation of BH^- and $B^=$ into any existing micelle; when these polar groups are fully masked by internal bonding, as in BH_2, insertion into micelles may be feasible. However, similar slopes were obtained with UCB in TC and TDHC, which does not form micelles, and the curves for $[B_t]$ versus [bile salt] (Figs. 5, 8, and 10) did not show a critical micellization effect at pH 6.2–7.8, at which BH^- predominated. Therefore, we propose that BH^- interacts hydrophobically with one or two TC or TDHC monomers to produce mixed aggregates that may differ in structure from existing aggregates produced by self-association of TC or TDHC. Dissolution of the semi-rigid BH^- by complete bile salt micelles may also involve specific interaction with an optimum number of rigid bile salt anions (50).

Implications for UCB solubility in bile

Our results indicate that the true aqueous solubilites of bilirubin are difficult to determine from systems involving crystals, and in large part explain the great variation in reported solubilities of UCB, especially between studies starting with BH_2 crystals versus acidified Na_2B. Our aqueous UCB solubilities of 0.21 μM at pH 6.5, and about 1 μM at pH 8.0, are probably maximum estimates, and are far below the maximum UCB concentration of about 35 μM found in normal human gallbladder bile (4, 5) and of 10 μM in hepatic bile (3). Our studies indicate that bile salts can account for the enhanced solubility of UCB in bile as compared to water–NaCl systems, and that changes in the relative composition of bile salts in the bile would little affect the solubility of UCB at any given total bile salt concentration (Fig. 10).

Fig. 6 indicates that UCB may have similar solubilities in gallbladder and hepatic bile since, during modification of bile in the gallbladder, the decrease in pH is compensated by an increase in bile salt concentration. The lowest solubilities obtained from dissolution of UCB crystals in 50 mM TC at pH = 6.5 to 7.0 (Fig. 7) straddle the maximum concentrations of UCB (10 μM) found in normal human hepatic bile (3) at similar bile salt concentrations and pH values. Since the addition of lecithin decreases UCB solubility in bile salt solutions (16–20, 51), hepatic bile samples with pH $\leq$ 7.0 may be supersaturated with UCB, whereas alkaline samples would not be saturated. However, most UCB in bile is derived from hydrolysis of bilirubin glucuronides (52), which, like Na_2B are in the open configuration without hydrogen bonds (23), and are highly water-soluble. Biliary micelles mighty well maintain this finely dispersed state of the pigment when UCB is generated by hydrolysis, producing a metastable, supersaturated state akin to that seen with acidification of Na_2B (Fig. 11).

This latter scenario is more compatible with the fact that UCB per se does not precipitate from bile, but is found in pigment gallstones exclusively as calcium salts of bilirubin monoanion, acid soap, and dianion (10, 22, 53, 54). Therefore, the presence of calcium ions and the very low apparent aqueous solubility of calcium bilirubinate, even in the presence of bile salts and lecithin (51), are important factors in the formation of pigment gallstones. As discussed by Moore (55), the precipitation of calcium bilirubinate from bile is buffered by bile salts, which interact with, and thus diminish the free (unbound) fraction of, both Ca^{2+} ions (56–59) and bilirubinate anions (this paper, 15–20, 22, 23, 35, 36). The acidification of gallbladder bile (60) would also reduce the concentrations of the "calcium sensitive" anions (55), BH^- and $B^=$.

In summary, we have demonstrated high and widely separated apparent pK'a values for UCB in aqueous solutions with or without bile salts; we have defined problems caused by the great dependence of dissolution on the type of UCB crystal used, and have documented the formation of extreme metastable systems when Na_2B is acidified in the presence of bile salt micelles. We have thus clarified the wide variations among published values for the solubility

of UCB, and presented the best available estimates of these solubilities over a wide pH range. Elucidation of the nature of the interactions between UCB and bile salts will require the use of systems without a crystalline phase.

We appreciate the help of John Quinn, Dept. of Chemistry, University of Pennsylvania, and Karol J. Mysels, Dept. of Chemistry, University of California, San Diego, in the interpretation of the effects of crystal structure on solubility. The stimulating input of our co-investigators, Edward W. Moore, Medical College of Virginia, and Alan F. Hofmann, University of California, San Diego, was invaluable. The computerized analysis of the $[B_t]$ versus pH data was performed by Dr. Daniel Garside, Senior Programmer, Dept. of Community Medicine, Northwestern University Medical School. Deborah DeBoer and Prof. Stephen H. Carr, Dept. of Materials Sciences, Northwestern University, kindly performed the X-ray diffraction spectra of the various forms of bilirubin crystals. We are indebted also to Thomas Devers, Denise Gallo, and Heidemarie Cheney, who participated in the preliminary studies performed at the V.A. Hospital and University of Pennsylvania in Philadelphia. This work was supported by an NIH grant (1-R01-AM-32130), by a Veterans Administration Merit Review Research Award, and by the Otho S. A. Sprague Foundation at Northwestern University.

Manuscript received 10 October 1986 and in revised form 10 April 1987.

REFERENCES

1. Overbeek, J. T. G., C. L. J. Vink, and H. Deenstra. 1955. The solubility of bilirubin. *Rec. Trav. Chim. Pays-Bas.* **74:** 81–84.
2. Brodersen, R. 1979. Bilirubin solubility and interaction with albumin and phospholipid. *J. Biol. Chem.* **254:** 2364–2369.
3. Fevery, J., N. Blanckaert, P. LeRoy, R. Michiels, and K. P. M. Heirwegh. 1983. Analysis of bilirubins in biological fluids by extraction and thin-layer chromatography of the intact tetrapyrroles: application to bile of patients with Gilbert's syndrome, hemolysis or cholelithiasis. *Hepatology.* **3:** 177–183.
4. Boonyapisit, S. T., B. W. Trotman, and J. D. Ostrow. 1978. Unconjugated bilirubin, and the hydrolysis of conjugated bilirubin, in gallbladder bile of patients with cholelithiasis. *Gastroenterology.* **74:** 70–74.
5. Masuda, H., and F. Nakayama. 1979. Composition of bile pigment in gallstones and bile, and their etiological significance. *J. Lab. Clin. Med.* **93:** 353–360.
6. Inoue, T. 1966. Studies on bilirubin in biliary tract disease. *Fukuoka Acta Med.* **36:** 417–443.
7. Izumi, K. 1965. Studies on the chemical composition of gallbladder bile and gallstone, especially on the difference in the process of gallstone formation between cholesterol stone and bile pigment stone. *Fukuoka Acta Med.* **56:** 488–523.
8. Nakayama, F., and W. Van der Linden. 1971. Bile composition, Sweden vs. Japan: its possible significance in the difference in gallstone incidence. *Am. J. Surg.* **122:** 8–12.
9. Trotman, B. W., S. E. Bernstein, W. F. Balistreri, G. D. Wirt, and R. A. Martin. 1981. Hemolysis-induced gallstones in mice: increased unconjugated bilirubin in hepatic bile predisposes to gallstone formation. *Gastroenterology.* **81:** 232–236.
10. Sutor, D. J., and L. I. Wilkie. 1977. Calcium in bile and calcium salts in gallstones. *Clin. Chim. Acta.* **79:** 119–127.
11. Ohkubo, H., J. D. Ostrow, S. H. Carr, and R. V. Rege. 1984. Polymer networks in pigment and cholesterol gallstones assessed by equilibrium swelling an infrared spectroscopy. *Gastroenterology.* **87:** 805–814.
12. Maki, T. 1966. Pathogenesis of calcium bilirubinate gallstones: role of *E. coli,* β-glucuronidase, and coagulation by inorganic ions, polyelectrolytes, and agitation. *Ann. Surg.* **165:** 90–100.
13. Ostrow, J. D., C. S. Berry, and J. E. Zarembo. 1974. Studies on the mechanism of phototherapy in the congenitally jaundiced rat. *In* Phototherapy of the Newborn; an Overview. G. B. Odell, R. Schaffer, and A. P. Simoupoulis, editors. Natl. Acad. of Sciences, Washington, DC. 74–92.
14. Mazer, N. A., and M. C. Carey. 1983. Quasi-elastic light-scattering studies of aqueous biliary lipid systems. Cholesterol solubilization and precipitation in model bile solutions. *Biochemistry.* **22:** 426–442.
15. Moroi, Y., R. Matuura, and T. Hisadome. 1985. Bilirubin in aqueous solution. Absorption spectrum, aqueous solubility, and dissociation constants. *Bull. Chem. Soc. Japan.* **58:** 1426–1431.
16. Ostrow, J. D., T. J. Devers, and D. Gallo. 1977. Determinants of the solubility of unconjugated bilirubin in bile: relationship to pigment gallstones. *In* Chemistry and Physiology of Bile Pigments. P. D. Berk and N. I. Berlin, editors. National Institutes of Health, DHEW, Fogarty International Conference Proceedings, Bethesda, MD. 404–409.
17. Nakama, T. 1976. Significance of biliary cholesterol and bilirubin in gallstone formation. *Fukuoka Acta Med.* **67:** 413–441.
18. Nakama, T., T. Furusawa, H. Itoh, and T. Hisadome. 1979. Correlation of cholesterol and bilirubin solubilization in bile salt solution. *Gastroenterology. Jpn.* **6:** 565–572.
19. Wosiewitz, U., and S. Schroebler. 1979. Solubilization of unconjugated bilirubin by bile salts. *Experientia.* **35:** 717–718.
20. Berman, M. D., A. P. Koretsky, and M. C. Carey. 1980. Influence of pH on the solubility of unconjugated bilirubin (UCB) in artificial bile solutions. *Gastroenterology.* **78:** 1141 (abstract).
21. Carey, M. C., and D. M. Small. 1978. The physical chemistry of cholesterol solubility in bile. *J. Clin. Invest.* **61:** 998–1026.
22. Ostrow, J. D., and L. Celic. 1984. Bilirubin chemistry, ionization and solubilization by bile salts. *Hepatology.* **4:** 38S–45S.
23. Carey, M. C., and W. Spivak. 1986. Physical chemistry of bile pigments and porphyrins with particular reference to bile. *In* Bile Pigments and Jaundice; Molecular, Metabolic and Medical Aspects. J. D. Ostrow, editor. Marcel Dekker, New York. Chapter 4, 81–132.
24. Brodersen, R. 1986. Aqueous solubility, albumin binding, and tissue distribution of bilirubin. *In* Bile Pigments and Jaundice; Molecular, Metabolic and Medical Aspects. J. D. Ostrow, editor. Marcel Dekker, New York. 157–181.
25. Moore, E. W., Jr., and J. W. Ross, Jr. 1985. The surfactant electrode; a new advance for physiologic and physicochemical studies of bile salt metabolism and structure–activity relationships. I. Sodium taurodehydrocholate. *Gastroenterology.* **88:** 1680 (abstract).
26. Berry, C. S., J. D. Ostrow, and J. E. Zarembo. 1972. Evidence for conversion of bilirubin to dihydroxyl derivatives in the Gunn rat. *Biochem. Biophys. Res. Commun.* **49:** 1366–1375.
27. McDonagh, A. F., and F. Assisi. 1972. The ready isomerization of bilirubin IXα in aqueous solution. *Biochem. J.* **129:** 797–800.
28. Bruusgaard, A. 1970. Quantitative determination of the major 3-hydroxy bile acids in biological material after thin-

layer chromatographic separation. *Clin. Chim. Acta.* **28:** 495–504.
29. Iwata, T., and K. Yamasaki. 1964. Enzymatic determination and thin-layer chromatography of bile acids in blood. *J. Biochem. (Tōkyo)* **56:** 425–431.
30. Michaelsson, M. 1961. Bilirubin determination in serum and urine. Studies on diazo methods and a new copper–azopigment method. *Scand. J. Clin. Lab. Invest.* **13 (Suppl. 56):** 1–80.
31. Snedecor, G. W., and W. G. Cochran. 1967. Statistical Methods. 6th edition. Iowa State University Press, Ames, IA. 59–60, 94–96, 114–118.
32. Gray, C. H., A. Kulczycka, and D. C. Nicholson. 1961. The chemistry of the bile pigments. Part IV. Spectrophotometric titration of the bile pigments. *J. Chem. Soc. (London)* **II:** 2276–2285.
33. Roda, A., A. F. Hofmann, and K. J. Mysels. 1983. The influence of bile salt structure on self-association in aqueous solutions. *J. Biol. Chem.* **258:** 6362–6370.
34. Carey, M. C. 1984. Bile acids and bile salts: ionization and solubility properties. *Hepatology.* **4:** 66S–71S.
35. Carey, M. C., and A. P. Koretsky. 1979. Self-association of unconjugated bilirubin IXα in aqueous solution at pH 10.0, and physical–chemical interactions with bile salt monomers and micelles. *Biochem. J.* **179:** 675–689.
36. Rege, R. V., C. C. Webster, and J. D. Ostrow. 1987. Enzymatic oxidation of unconjugated bilirubin to assess its interactions with taurocholate. *J. Lipid Res.* **28:** 673–683.
37. Tera, H. 1960. Stratification of human gallbladder bile in vivo. *Acta Chir. Scand.* **256: (Suppl.)** 1–85.
38. Mysels, K. J., and J. W. McBain. 1948. Variability and inhomogeneity of albumin dilaurate. *J. Phys. Colloid Chem.* **52:** 1471–1481.
39. Nancollas, G. H. 1984. Crystallization in bile. *Hepatology.* **4:** 169S–172S.
40. Lucassen, J. 1966. Hydrolysis and precipitates in carboxylate soap solutions. *J. Phys. Chem.* **70:** 1824–1830.
41. Rosoff, M., J. H. Schulman, H. Erbring, and W. Winkler. 1967. Surface activity of metastable colloidal solutions. Part I. Experimental results with solutions of aescin. *Kolloid Z. Z. Polym.* **216–217:** 347–355.
42. Bonnett, R., J. E. Davies, and M. B. Hursthouse. 1976. Structure of bilirubin. *Nature (London)* **262:** 326–328.
43. Mukerjee, P., J. R. Cardinal, and N. R. Desai. 1977. The nature of the local microenvironments in aqueous micellar systems. *In* Micellization, Solubilization, and Microemulsions. Vol. 1. K. L. Mittal, editor, Plenum Press, New York. 241–261.
44. Brodersen, R. 1966. Dimerisation of bilirubin anion in aqueous solution. *Acta Chem. Scand.* **20:** 2895–2896.
45. Falk, H. 1986. Molecular structure of bile pigments. *In* Bile Pigments and Jaundice; Molecular, Metabolic and Medical Aspects. J. D. Ostrow, editor. Marcel Dekker, New York. 7–29.
46. Hansen, P. E., H. Thiessen, and R. Brodersen. 1979. Bilirubin acidity; titrimetric and ^{13}C-NMR studies. *Acta Chem. Scand.* **B33:** 281–293.
47. Shankland, W. 1970. The ionic behavior of fatty acids solubilized by bile salts. *J. Colloid Interface Sci.* **34:** 9–25.
48. Hofmann, A. F. 1968. Molecular association in fat digestion. Interaction in bulk of monoolein, oleic acid, and sodium oleate with dilute, micellar bile salt solutions. *In* Molecular Association in Biological and Related Systems. American Chemical Society, Washington, DC. Adv. Chem. Series. **84:** 53–66.
49. Mukerjee, P., and K. Banerjee. 1964. A study of the surface pH of micelles using solubilized indicator dyes. *J. Phys. Chem.* **68:** 3567–3574.
50. Mukerjee, P., Y. Moroi, M. Murata, and A. Y. S. Yang. 1984. Bile salts as atypical surfactants and solubilizers. *Hepatology.* **4:** 61S–65S.
51. Fu, X-P., X-S. Zhou, W-H. Zhang, S-Q. Deng, and X-M. Shao. 1985. "Bilirubin–calcium compound" precipitation and the effect of bile salts on it; the pathogenesis of pigment gallstone. *Chinese Med. J.* **98:** 728–738.
52. Ostrow, J. D. 1984. The etiology of pigment gallstones. *Hepatology.* **4:** 215S–222S.
53. Bogren, H., and K. Larsson. 1963. On the pigment in biliary calculi. *Scand. J. Clin. Lab. Invest.* **15:** 569–572.
54. Sutor, D. J., and L. I. Wilkie. 1977. The crystalline salts of calcium bilirubinate in gallstones. *Clin. Sci. Mol. Med.* **53:** 101–103.
55. Moore, E. W. 1984. The role of calcium in the pathogenesis of gallstones: Ca^{2+} electrode studies of model bile salt solutions and other biologic systems with an hypothesis on structural requirements for Ca^{2+} binding to proteins and bile acids. *Hepatology.* **4:** 228S–243S.
56. Moore, E. W., L. Celic, and J. D. Ostrow. 1982. Interactions between ionized calcium and sodium taurocholate. Bile salts are important buffers for prevention of calcium-containing gallstones. *Gastroenterology.* **83:** 1079–1089.
57. Williamson, B. W. A., and I. W. Percy-Robb. 1979. Interaction of calcium ions with glycocholate micelles in aqueous solution. *Biochem. J.* **181:** 61–66.
58. Rajagopalan, N., and S. Lindenbaum. 1982. The binding of Ca^{2+} to taurine- and glycine-conjugated bile salt micelles. *Biochim. Biophys. Acta.* **711:** 66–74.
59. Rege, R. V., E. W. Moore, and D. L. Nahrwold. 1985. Pathogenesis of calcium-containing gallstones: relationships of total calcium and free ionized Ca^{2+} in canine gallbladder and duct bile. *Surg. Forum.* **36:** 132–134.
60. Rege, R. V., and E. W. Moore. 1987. Evidence for H^+ secretion by the in vivo canine gallbladder. *Gastroenterology.* **92:** 281–289.