# Interactions of unconjugated bilirubin with bile salts

**Robert V. Rege, Cecile C. Webster, and J. Donald Ostrow**

Department of Surgery and Gastrointestinal Section, Department of Medicine, Northwestern University, and Veterans Administration Lakeside Hospital, Chicago, IL 60611

**Abstract** The rate of peroxidation of unbound, unconjugated bilirubin (UCB) was used to assess the interactions of UCB with four taurine-conjugated bile salts at pH 8.2, 37°C, and an ionic strength of 0.15. Each of the four structurally different bile salts markedly decreased the rate of peroxidation of UCB in the presence of horseradish peroxidase (HRP); 30% of UCB was bound even at low, premicellar bile salt concentrations (1 mM). At high bile salt concentrations (75 mM), taurocholate (TC) and tauro-3α,7α-dihydroxy-12-oxo-5β-cholan-24-oate (T12-OXO) exhibited the highest degree of inhibition of UCB peroxidation; only 0.6% and 1.1% of UCB were unbound, respectively. Taurochenodeoxycholate (TCDC) yielded somewhat less inhibition with 2.0% of UCB unbound. Taurodehydrocholate (TDHC), a bile salt that does not form micelles but does form dimers, was comparable to TC and T12-OXO with unbound UCB of 1.0%. With TC and T12-OXO, apparent affinity for UCB was at least four times greater above the published critical micellar concentration (CMC) than in the premicellar range. TCDC was only studied above its CMC value and only one region of UCB binding was noted. Interaction of UCB with TDHC was similar to premicellar interactions with TC and T12-OXO below 25 mM, but increased to values intermediate between monomer and micelle above 40 mM TDHC, compatible with formation of TDHC dimers above 20 mM. These data show that there are differences in the ability of bile salts to bind UCB. Thus, alterations in bile salt profile in bile might lead to higher concentrations of free UCB in bile predisposing to pigment gallstones.—**Rege, R. V., C. C. Webster, and J. D. Ostrow.** Interactions of unconjugated bilirubin with bile salts. *J. Lipid Res.* 1988. **29:** 1289–1296.

**Supplementary key words** taurocholate • taurochenodeoxycholate • pigment gallstones • horseradish peroxidase • peroxidation of bilirubin • tauro-3α,7α-dihydroxy-12-oxo-5β-cholan-24-oate • taurodehydrocholate

Pigment gallstones contain a significant proportion of bile pigment, either as calcium bilirubinate salts or as an insoluble polymeric residue formed from unconjugated bilirubin or related pyrroles (1–6). This suggests that precipitation of bilirubin from bile is important in the formation and growth of these stones. Though conjugated bilirubin accounts for over 98% of the bilirubin in bile (7), its precipitation is unlikely since it is highly water-soluble and does not form insoluble calcium salts (8). In contrast, unconjugated bilirubin (UCB), though it comprises only 1–2% of total bilirubin in bile (7), is thought to be important in pigment gallstone formation since its solubility in simple aqueous solution is only about 1 μM at pH 7.0 (9, 10). Concentrations of UCB reach 10 μM in normal hepatic bile and 35 μM in normal gallbladder bile due to interactions with bile salts which increase the solubility of UCB by several orders of magnitude (10). Nonetheless, bile is thought to be nearly saturated with UCB under normal conditions (10). The association of UCB and bile salts in bile could thus be an important factor in preventing UCB precipitation from bile.

Thermodynamically, precipitation of UCB from bile at any given pH is possible only when the concentration of free UCB in solution increases above the saturation point of UCB. UCB saturation might be exceeded under three circumstances: *1*) an increase in the concentration of total UCB, *2*) a decrease in binding of UCB by biliary components, or *3*) a combination of *1* and *2*. An assessment of the amount of free UCB in bile is thus important in determining the degree of saturation of bile with respect to bilirubin.

We have recently adapted the kinetic method of Jacobsen (11), which uses the selective peroxidation of unbound UCB to colorless products, to determine the unbound fraction of UCB in solutions of taurocholate at pH 8.2 (12). These studies showed that at taurocholate concentrations greater than 50 mM less than 1% of UCB was free (unbound) in solution; the remainder was associated with both taurocholate monomer and micelles. Although these data with taurocholate support the concept that bile salts protect against UCB precipitation by decreasing the concentrations of free UCB in solution, extension of these studies to other bile salts is needed. In the present report, we compare the binding of UCB to four bile salts—taurocholate (TC), tauro-3α,7α-dihydroxy-12-oxo-5β-cholan-24-oate (T12-OXO), taurochenodeoxycholate (TCDC), and taurodehydrocholate (TDHC)—with different structures, and therefore different CMC values and aggregation numbers (13).

Abbreviations: UCB, unconjugated bilirubin; TC, taurocholate; T12-OXO, tauro-3α,7α-dihydroxy-12-oxo-5β-cholan-24-oate; TCDC, taurochenodeoxycholate; TDHC, taurodehydrocholate; CMC, critical micellar concentration; HRP, horseradish peroxidase.

## METHODS

TC, TCDC, and TDHC were purchased from Calbiochem, La Jolla, CA, and were each more than 98% pure, containing no phospholipids, cholesterol, or unconjugated bile salts. T12-OXO, donated by Dr. Alan Hofmann's laboratory at the University of California, San Diego, CA, was prepared by methods described elsewhere (13) and was more than 99% pure. UCB (99% pure, Gaillard-Schlesinger, Carle Place, NY) was converted to the disodium salt in the presence of $Na_4EDTA$ (12), and dissolved in buffer with or without bile salt shortly before use. Horseradish peroxidase (HRP, crude, essentially salt-free) was purchased from Sigma Chemical Co., St. Louis, MO. Tris, NaCl, and $Na_4EDTA$ were reagent grade (Fisher Chemical Co., Fairlawn, NJ). Deionized, charcoal-treated water was used throughout.

The interaction of UCB with the four bile salts was determined, as described previously (12), by comparison of the peroxidation of UCB in the presence or absence of bile salt. The fraction of UCB bound to bile salt is not oxidized (12). Peroxidation of UCB to colorless products was estimated spectrophotometrically from the linear decrease in UCB absorbance at 440 nm (extinction coefficient 0.973) during the 2 min after addition of HRP at pH 8.2 and 37°C. As in our previous study (12), an assay pH of 8.2 was chosen because HRP activity is optimal and this is the lowest pH at which one can prepare metastable solutions of UCB without bile salts. These UCB solutions are used to measure the rate of reaction of UCB with HRP in the absence of bile salts. Concentrations in the assay systems were: bile salt, 0–75 mM; UCB, 5 ± 0.5 μM; $H_2O_2$, 0.015%; HRP, 4.4–44 μg; EDTA, 5.0 mM; and Tris–NaCl buffer to a final ionic strength of 0.015.

The data reported are the mean of at least three assays at each concentration of bile salt, normalized to [HRP] = 1.0 μM by correction for the enzyme activity measured with pyrogallol as substrate (14), and for the enzyme concentration used. Usually, five assays were performed, but nonlinear assays, resulting from the presence of air bubbles in the cuvette after mixing, were excluded (12). The results were highly reproducible from day to day, with the standard error consistently less than 10% of the mean. Duplicate samples on the same day agreed within 2%.

Since the exact mechanisms and stoichiometry of the interactions of UCB with TC have not been determined, it was not possible to calculate true thermodynamic affinity or partition constants. However, to access the relative interactions of UCB with TC monomers and micelles (12), an empirical constant, *K*, that resembles a binding constant, was defined. This constant *K* was defined by the following equation:

$$K = \frac{[UCB_b]}{[UCB_f] \times [TC]} \qquad Eq.\ 1)$$

where $[UCB_b]$ is the amount of UCB associated with bile salt and is calculated as total UCB minus free UCB ($[UCB] - [UCB_f]$). The constant K as defined will have the dimensions of liters/mole (L/M). Rearrangement of equation 1 to:

$$[UCB_b]/[UCB_f] = K \times [TC] \qquad Eq.\ 2)$$

demonstrates that a plot of $[UCB_b]/[UCB_f]$ versus [TC] should be linear and that the slope of the plot will be equal to the constant, *K*. In this study, similar constants are calculated for each bile salt studied. For any given bile salt, an increase in the slope of the plot (and therefore of *K*) as bile salt concentration increases will indicate an increase in the interaction between UCB and that bile salt.

## RESULTS

### Inhibition of the peroxidation of unconjugated bilirubin by bile salts

Each of the four bile salts, though differing in steroid ring substituents, markedly decreased the rate of peroxidation of UCB by HRP. This is illustrated in **Fig. 1** where the fraction f of UCB which is unbound (free), f = $UCB_f$/total UCB, is plotted against total bile salt concentration. Note that even at the lowest concentrations of each bile salt (1 mM), f was no greater than 0.70. In each case, f declined greatly at first as bile salt concentration increased, and then reached a plateau at higher bile salt concentrations.

The micelle-forming bile salts TC and T12-OXO exhibited the highest degree of inhibition of UCB peroxidation; only 0.6% and 1.1% of UCB were free at bile salt concentrations of 75 mM, respectively. TCDC, which forms larger micelles and has a lower CMC value (13), yielded somewhat less inhibition of UCB peroxidation with 2.0% unbound UCB at [TCDC] = 75 mM. This fraction of unbound UCB was actually greater than the 1.0% observed at 75 mM TDHC, a bile salt which does not form micelles.

### Inhibition of UCB peroxidation by TC and T12-OXO

Plots of $[UCB_b]/[UCB_f]$ versus [total bile salt], for TC (**Fig. 2**) and T12-OXO (**Fig. 3**), were linear, with transitions from lower to higher slope at bile salt concentrations near their respective CMC values (13). The slopes corresponded to the constant *K* (Eq. 2). Thus, with each bile salt, *K* was lower for monomers (below CMC) than for micelles.

For TC concentrations between [TC] 1 and 8 mM (Fig. 2), $[UCB_b]/[UCB_f] = 0.55 \times [TC] - 0.30$, $r = 0.972$. This was different from the values for [TC] at or above 10 mM: $[UCB_b]/[UCB_f] = 2.30 \times [TC] - 19.82$, $r = 0.985$. The *K* constants of 550 L/M for the premicellar and 2300 L/M for the micellar regions corresponded well with the values (522 and 2875 L/M) reported by us previously for TC; the

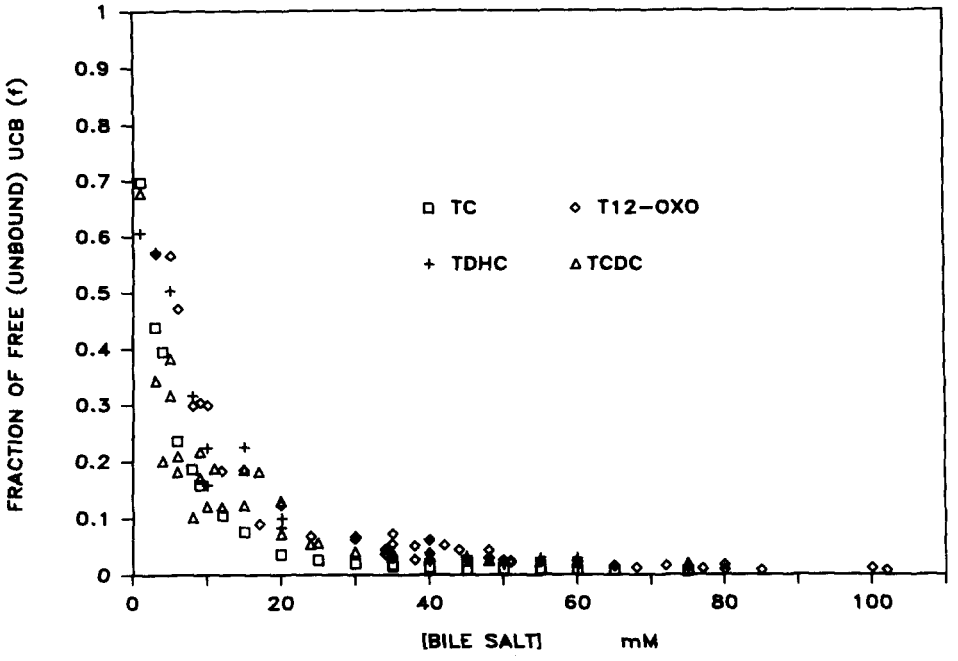


**Fig. 1.** The fraction of unconjugated bilirubin (UCB) free in bile salt solutions of varying concentration. The fraction of free UCB present in solutions of four bile salts that differ in structure and in physical properties is shown at bile salt concentrations from 1 to 75 mM. All measurements were made at pH 8.2, 37°C, and an ionic strength of 0.15. At low bile salt concentrations (1 mM), f was never greater than 0.7 indicating that, regardless of structure, each bile salt efficiently decreased the amount of free UCB in solution. At 75 mM, 0.6% and 1.1% of UCB were free in the presence of the micellar bile salts TC and T12-OXO, respectively, while only 2% of UCB was free in the presence of TCDC, a bile salt that forms larger micelles. It is interesting that TDHC, which does not form micelles, was as effective at binding UCB (1.0% free UCB) as TC and T12-OXO.

transition between 8 and 10 mM TC is in close agreement with the published CMC values of TC (13) at this ionic strength.

For [T12-OXO] below 60 mM (Fig. 3), $[UCB_b]/[UCB_f] = 0.87 \times [T12\text{-}OXO] - 5.68$, $r = 0.963$, whereas for [T12-OXO] of 60 to 80 mM, $[UCB_b]/[UCB_f] = 3.00 \times [T12\text{-}OXO] - 136.74$, $r = 0.894$. Thus, the $K$ constants of 870 for premicellar and 3000 L/M for micellar T12-OXO were different and the change in slope occurred between 45 and 60 mM T12-OXO, close to the published CMC values for this bile salt (13).

### Inhibition of UCB peroxidation by TCDC and TDHC

TCDC which has a CMC of about 1 to 3 mM (13) was only studied in the micellar range. A single linear relationship was obtained: $[UCB_b]/[UCB_f] = 0.74 \times [TCDC] - 1.51$, $r = 0.979$, (**Fig. 4**). The observed value of K for TCDC micelles was therefore 740 L/M.

TDHC (**Fig. 5**), which does not form micelles but forms dimers at concentrations above 25 mM (15), also inhibited UCB peroxidation. The interactions of UCB with TDHC could be described by a single linear regression: $[UCB_b]/[UCB_f] = 1.12 \times [TDHC] - 8.41$, $r = 0.957$, with a value of $K$ of 1120 L/M, or by a bilinear plot, with a transition at 20 to 40 mM. In the latter analysis, the slope was lower in the monomeric range below 25 mM with little scatter of the data points. In the clearly dimeric range above 40 mM, the slope was greater, but there was marked scatter. The respective linear regressions in these regions were: $[UCB_b]/[UCB_f] = 0.889 \times [TDHC] - 4.26$, $r = 0.89$ ([TDHC] between 0 and 20 mM) and $[UCB_b]/[UCB_f] = 1.63 \times [TDHC] - 37.47$, $r = 0.850$ ([TDHC] between 40 and 75 mM). These plots gave separate $K$ values of 798 and 1630 L/M, respectively, similar to the other bile salts studied. The transition point occurred between [TDHC] of 20 and 40 mM, the range where a bile salt electrode (15) has shown dimerization to occur.

## DISCUSSION

UCB may precipitate from aqueous solution in its fully protonated form, $H_2B^0$, or as calcium salts of either UCB monoanion, $HB^-$, and dianion, $B^{2-}$. Precipitation of $H_2B^0$ becomes possible when the total concentration of UCB exceeds the solubility of UCB in water. The resultant precipitate would then consist of crystals of bilirubin diacid. However, bilirubin in pigment stones is associated with calcium, suggesting that precipitation of calcium salts of bilirubin

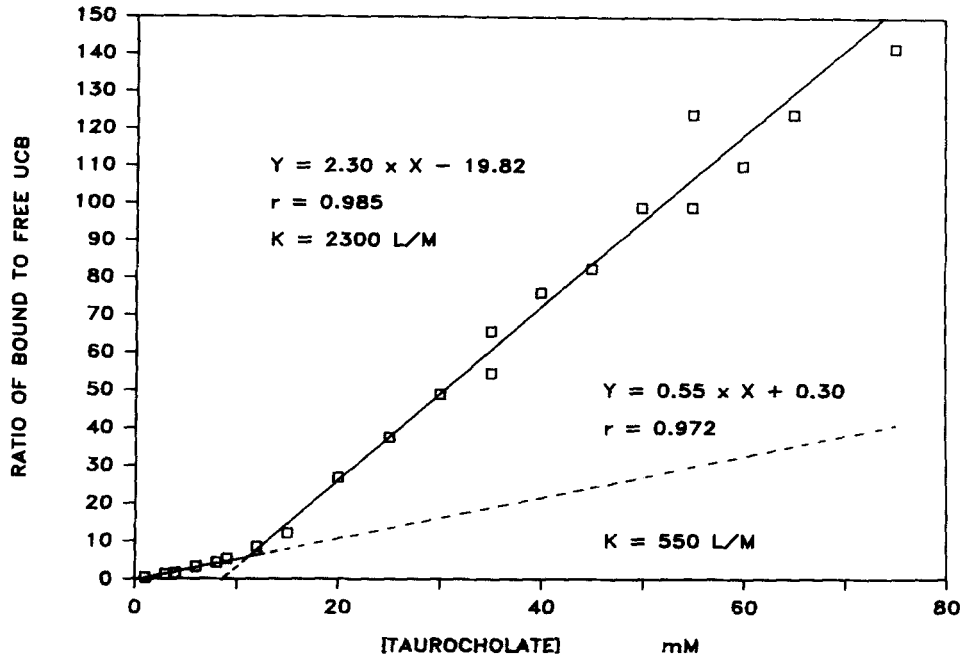


**Fig. 2.** The ratio of bound UCB to free UCB ($UCB_b/UCB_f$) as a function of taurocholate (TC) concentration. $UCB_b/UCB_f$ and TC correlated linearly in both the premicellar and micellar ranges with the respective regressions: $UCB_b/UCB_f = 0.55 \times [TC] - 0.30$, $r = 0.972$, and $UCB_b/UCB_f = 2.30 \times [TC] - 19.82$, $r = 0.985$. Thus the K constants calculated from the slope of these plots (see Methods) were 550 and 2300 L/M, respectively. Note that the transition point is between 8 and 10 mM in agreement with the published CMC of TC.

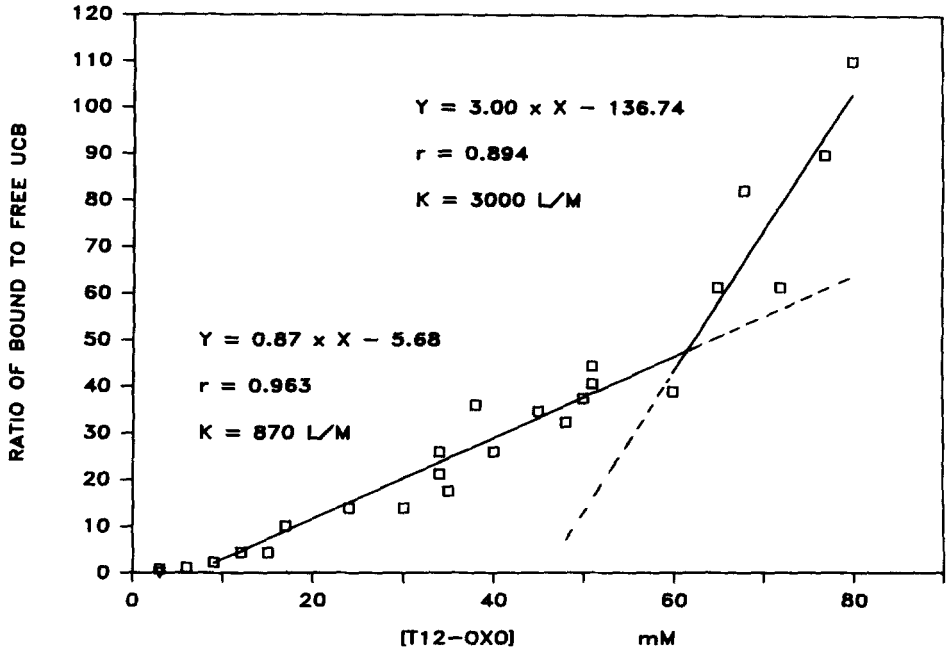


**Fig. 3.** The ratio of bound UCB to free UCB ($UCB_b/UCB_f$) as a function of tauro-3$\alpha$,7$\alpha$-dihydroxy-12-oxo-5$\beta$-cholan-24-oate (T12-OXO) concentration. Separate linear regressions were found at premicellar and micellar concentrations of T12-OXO: $UCB_b/UCB_f = 0.87 \times [T12\text{-}OXO] - 5.68$, $r = 0.963$ and $UCB_b/UCB_f = 3.00 \times [T12\text{-}OXO] - 136.74$, $r = 0.894$. $K$ would therefore be 870 and 3000 L/M in the premicellar and micellar regions, respectively. The transition between regressions between 45 and 60 mM is close to the published CMC values for T12-OXO.

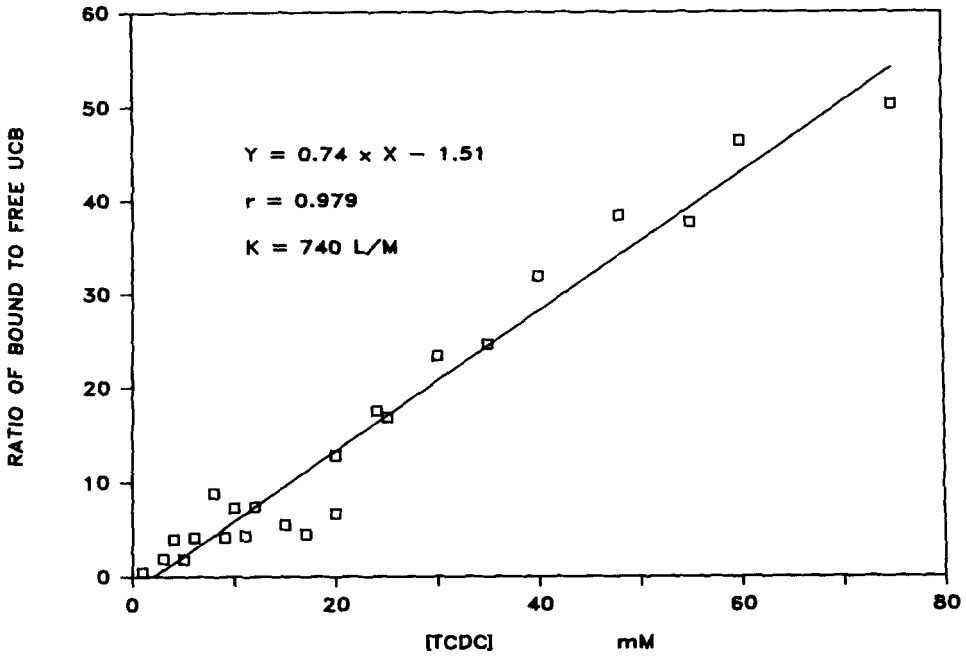


**Fig. 4.** The ratio of bound UCB to free UCB ($UCB_b/UCB_f$) as a function of taurochenodeoxycholate (TCDC) concentration. The micellar bile salt, TCDC, was only studied above its CMC, and $UCB_b/UCB_f$ was linearly related to [TCDC] at all concentrations studied: $UCB_b/UCB_f = 0.74 \times [TCDC] - 1.51$, $r = 0.979$. $K$ was thus 740 L/M.

from bile is important in the formation of pigment gallstones, and that UCB per se may not be the species that precipitates. The present study reinforces our prior work (10, 12) since it confirms that whatever species precipitates to form gallstones, failure to precipitate under normal conditions is due largely to the interaction of UCB with bile salts.

In agreement with data from our laboratory on the solubilization of UCB by bile salts (10), the present studies show interaction of UCB with bile salt monomers, dimers, and micelles. A large portion of UCB was associated with bile salt monomer. Thus, even at low concentrations of bile salts (1 mM), only 70% of UCB was free in solution, regardless of bile salt structures that differed in ring substituents. As bile salt concentration was increased, higher affinity binding to micelles decreased free UCB in solution to extremely low concentrations. At concentrations of bile salts exceeding 50 mM, simulating the concentrations found in hepatic bile (16), our data indicate that less than 3% of UCB in solution is free.

This study suggests two regions of UCB-bile salt interaction; lower affinity interactions with bile salt monomers and higher affinity association with bile salt micelles. The samples were prepared in this study by addition of buffered bile salt solutions to UCB that had been converted to its disodium salt in the presence of $Na_4EDTA$. They are thus akin to the metastable solutions in our previous study (10) which examined the association of UCB with bile salts upon acidification of disodium bilirubinate dissolved in 50 mM TC or TDHC. It was shown in that study that micelles were much more effective than bile salt dimers or monomers in stabilizing such metastable microsuspensions; the results of the present study are in agreement.

As noted earlier, the $K$ constants are only arbitrarily defined values that cannot be translated to true association constants ($K$'f) unless the stoichiometry of the UCB-bile salt interactions is known. It is of interest that the aggregation numbers of TCDC and TC are approximately 20 and 5–6, respectively (13). Thus, there are more than three times as many bile salt molecules in TCDC micelles as compared with TC micelles, and our $K$ value for TC micelles is a little more than three times that for TCDC micelles. This suggests that the true micellar affinity constants (corrected for the aggregation number of each bile salt) of these two bile salts are similar, in agreement with the similar solubilization of UCB crystals by these two bile salts (10).

We found relatively small differences in the binding of UCB among the bile salts we studied, suggesting that changes in the composition of the bile salts might have little effect on UCB solubility. However, since [UCB] in normal bile appears to be close to saturation (10), even small increases in free [UCB] could result in supersaturation of bile with UCB or calcium bilirubinate. For example, the proportion of free UCB in solutions of T12-OXO or TDHC was almost twice (1.1 and 1.0%), and TCDC three times (1.8%), that in TC solutions (0.6%). These increases in free [UCB] would translate directly into corresponding increases in each species of UCB in bile; $H_2B$, $HB^-$, and $B^{2-}$.

Since interactions with bile salts alone appear to account

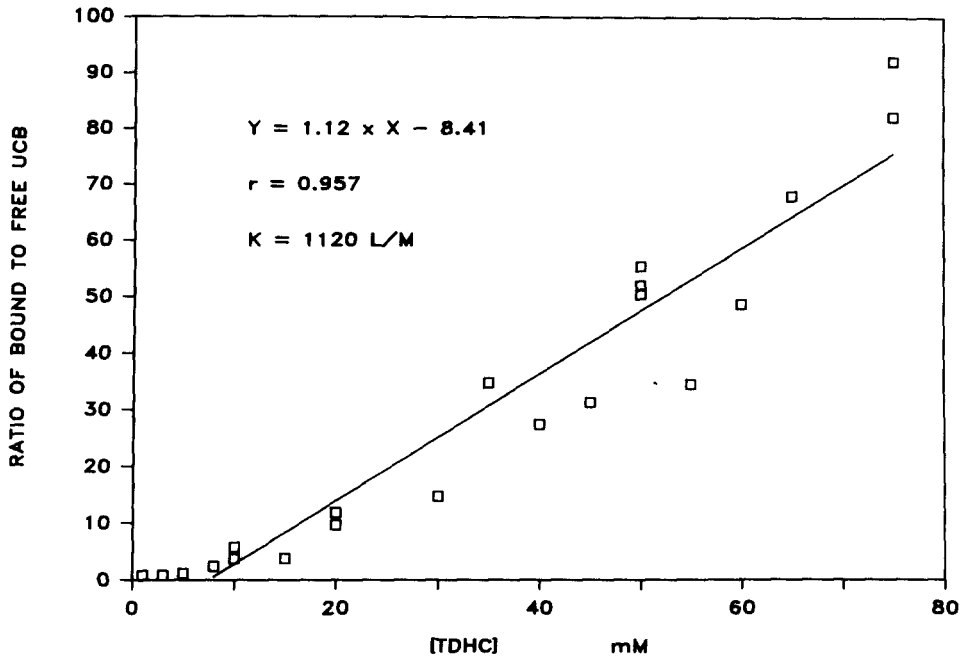


**Fig. 5.** The ratio of bound UCB to free UCB ($UCB_b/UCB_f$) as a function of taurodehydrocholate (TDHC) concentration. TDHC, which does not form micelles, could be described by one linear correlation: $UCB_b/UCB_f$ = 1.12 × [TDHC] − 8.41, $r$ = 0.957 and one constant $K$ = 1120 L/M. However, there appeared to be a transition point between 20 and 40 mM, the range of TDHC concentration where dimerization has been noted with a bile salt electrode. Thus, TDHC interactions with UCB may also be described by bilinear plots: $UCB_b/UCB_f$ = 0.89 × [TDHC] − 4.26, $r$ = 0.889 below 20 mM and $UCB_b/UCB_f$ = 1.63 × [TDHC] − 37.47, $r$ = 0.850 above 40 mM. This would correspond to monomeric and dimeric constants of 890 and 1630 L/M, respectively.

for the amounts of UCB commonly observed in bile (12) and since there is some reason to believe that calcium bilirubinate may be the species that precipitates to form gallstones, it is relevant to inquire whether bile salts also affect the precipitation of calcium bilirubinate salts. Precipitation of a calcium salt becomes thermodynamically possible only when the product of calcium and anion concentrations (activities) exceeds the solubility product, K'sp, of that salt (17). The ion product of the monoanionic salt of bilirubin is defined as: $[Ca^{2+}] \times [HB^-]^2$, while that of the dianionic salt is: $[Ca^{2+}] \times [B^{2-}]$. Thus, it can be seen that K'sp for either salt could be exceeded by an increase in $[Ca^{2+}]$, an increase in [anion], or an increase in both species. Since only the unbound (free) concentration of $Ca^{2+}$ and UCB are relevant the calculation of calcium bilirubinate saturation (17, 18), the association of UCB, as well as calcium, with bile salt monomers and micelles would be expected to greatly reduce the ion products and degree of bile saturation with calcium bilirubinate. The ion products of the monoanionic ($[Ca^{2+}] \times [HB^-]^2$) and dianionic ($[Ca^{2+}] \times [B^{2-}]$) calcium salts of UCB would be increased 4 to 9- and 2 to 3-fold, respectively, increasing the likelihood of precipitation of either salt (17).

The interaction between UCB and bile salts is not the sole determinant of unbound UCB. The concentration of unbound UCB would also depend on total [UCB] in solution, the pH of the solution, and the pK'a values for the ionization of UCB (10, 17) and the affinity of other components in bile to associate with UCB. Moreover, the affinity of bile salts for each ionic species of UCB, protonated UCB, monoanion, and dianion, may differ so that UCB-bile salt interactions may vary with pH. Thus, these studies performed at pH 8.2, slightly above pH values observed in hepatic bile, give only relative information about UCB-bile salt interactions in bile. These studies must be extended to pH values in the range observed in hepatic and gallbladder bile (pH 7.8 to 6.0) before these data can be quantitatively applied to the saturation of UCB in actual bile samples. However, the high affinity of bile salts for UCB observed in this study suggests that UCB is highly bound to bile salts at all of the pH values observed in hepatic and gallbladder bile. This concept is supported by our studies of the solubilization of UCB by bile salts (10).

In bile, assessment of UCB solubility is more complex because UCB associates with other components of bile. This association, mainly with bile salts, increases the solubility of UCB in bile to about 3500 times that in aqueous solution. Since bile salts alone would account for the majority of UCB observed in hepatic and gallbladder bile (10), we studied the interactions of UCB and bile salts in solutions of four bile salts that varied widely in physiochemical properties. Clearly, before we can apply the results

of the present study to bile, further studies are needed in more complex solutions that include phospholipids, cholesterol, and calcium (and possibly protein) that more closely approximate the composition of hepatic and gallbladder bile.

In the presence of calcium, assessment of bile salt-UCB interactions becomes quite complex. Formation of soluble complexes of calcium with UCB would be expected and would decrease the concentration of free (unbound) UCB in solution. To accurately analyze data in solutions containing calcium would obviously require physicochemical data regarding calcium-UCB interactions. Moreover, calcium might also alter the affinity of bile salts for UCB by affecting the CMC of bile salts (19), changing the ratios of premicellar and micellar bile salts in the system. Since such data are not available at this time, we are limited to studies such as those presented which measured the interactions of UCB and bile salts in the absence of calcium.

In summary, supersaturation of hepatic and gallbladder bile with UCB might occur for several reasons: *a*) an increase in total [UCB] in bile, as has been observed during hemolysis (20–22), phototherapy (23), or during hydrolysis of bilirubin conjugates (24–26); *b*) an increase in biliary lecithin concentration, which decreases the solubility of UCB in bile salt solutions (16); *c*) a decrease in the concentration of bile salts, which bind both UCB and calcium; or *d*) alterations in the bile salt profile, with a relative decrease in the proportions of bile salts that are most effective in binding UCB and/or calcium. In any case, the marked association of UCB with bile salts, demonstrated in the present report, is probably an important factor in preventing precipitation of UCB or its calcium salts from bile.

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