Enzymatic oxidation of unconjugated bilirubin to assess its interactions with taurocholate

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

Department of Surgery,* and Gastroenterology Section,** Department of Medicine, Northwestern University and VA Lakeside Hospital, Chicago, IL 60611

Abstract The rate of peroxidation of unconjugated bilirubin (UCB), catalyzed by horseradish peroxidase (HRP), has been employed by Jacobsen (1969. *FEBS Lett.* **5:** 112–114) to assess the fraction of unbound UCB in the presence of serum albumin. We used this method to examine the interactions of UCB with taurocholate (TC) at pH 8.2, assuming solubilization of UCB by TC is due to pigment binding and/or to partitioning into the micelle, thus rendering UCB unavailable for peroxidation. Inhibition of UCB peroxidation conformed with predictions based on these assumptions and demonstrated significant interaction of UCB with both monomeric and micellar TC. Although significant inhibition of UCB peroxidation was seen with TC monomer, inhibition was even greater with TC micelles. In contrast, pyrogallol, another substrate of HRP, acted very differently in the presence of TC. Inhibition of pyrogallol peroxidation by TC was much less than with UCB and occurred primarily with monomeric TC, with little further inhibition in the micellar range. The results of this study suggest that at taurocholate concentrations above 50 mM, similar to the physiologic bile salt concentrations in human bile, at least 99% of UCB is bound to bile salt, dramatically decreasing the concentration of unbound UCB. Since bile salts also bind Ca^{2+}, they play a dual role in protection against the precipitation of calcium bilirubinate from bile. Therefore, bile salts are a major factor in the prevention of the formation and growth of pigment gallstones.—**Rege, R. V., C. C. Webster, and J. D. Ostrow.** Enzymatic oxidation of unconjugated bilirubin to assess its interactions with taurocholate. *J. Lipid Res.* 1987. **28:** 673–683.

Supplementary key words bile salts • horseradish peroxidase • peroxidation of bilirubin • pigment gallstones

Bile pigments, in the form of calcium bilirubinate or of an insoluble black pigment polymer, are major components of pigment gallstones, which comprise 27–32% of gallstones in Western society (1–5). Thus, the precipitation of unconjugated bilirubin (UCB) from bile is important in the pathogenesis of pigment gallstone disease. The solubility of UCB in water at pH 7.0 is only 1 nM (6), yet normal bile contains approximately 3500 times that amount (7). Studies of the solubilization of UCB by bile salt solutions suggest that bile salts are the most important of factors which increase bilirubin solubility in bile (7).

Assessment of the saturation of bile with calcium bilirubinate requires knowledge of the concentrations of both free, ionized calcium ($[Ca^{2+}]$) and of unbound (free) UCB, including protonated UCB diacid, UCB monoanion, and UCB dianion. Calcium activity is easily measured with a Ca^{2+}-sensitive electrode (8), but a bilirubin electrode is not available and is not likely to be developed. Therefore, the measurement of small concentrations of unbound UCB is technically difficult, and calculation of free UCB is impossible since the binding constants for components of bile with UCB are not known. Furthermore, the proportions of protonated UCB, of UCB monoanion, and of UCB dianion cannot be determined since the pKa' values of UCB in bile have not been measured (7).

A similar problem with the measurement of free UCB in UCB–albumin solutions, where the concentration of free UCB is very low, was solved by Jacobsen (9) who used a kinetic method that assumed that UCB bound to albumin was unavailable for peroxidation by horseradish peroxidase (HRP). The rate of peroxidative degradation of UCB by HRP was used to estimate the concentration of free UCB in the presence and absence of albumin. He was thus able to calculate the dissociation constant (K_d) for the UCB-albumin complex.

The concentration of free UCB is extremely low in bile salt solutions since it cannot be measured by dialysis or ultracentrifugation (1). Thus, it is thought that most UCB is associated with bile salts by binding or by partitioning into the micelle. If the interaction of UCB with bile salts renders it unavailable to react with HRP, free UCB in these solutions could be measured by the kinetic method described by Jacobsen (9). We have adapted the kinetic method of Jacobsen (9) to examine the interactions of UCB with sodium taurocholate (TC).

Abbreviations: UCB, unconjugated bilirubin; HRP, horseradish peroxidase; TC, taurocholate; CMC, critical micellar concentration.

THEORY

UCB peroxidation by HRP is described kinetically by the Michaelis-Menten equation without product inhibition (10). When the initial concentration of UCB ($[UCB]_o$) is well below the Michaelis constant (K_m) of HRP for UCB (800 μM), the Michaelis-Menten equation simplifies to:

$$V = -d[UCB]/dt = k \times [HRP] \times [UCB] \qquad Eq.\ 1)$$

where k is the linear rate constant. In fact, [UCB] was kept well below K_m throughout this study and eq. 1 is therefore applicable to all our results. Since UCB absorbance (A) at 440 nm is defined as:

$$A = E \times [UCB] \qquad Eq.\ 2)$$

where E is the molar extinction coefficient for UCB at 440 nm in a 1-cm light path, and since the colorless peroxidation products do not absorb at this wave length, V can be expressed as a function of A:

$$V = \frac{-dA/dt}{E} = k \times [HRP] \times A/E. \qquad Eq.\ 3)$$

In taurocholate solutions where much of the UCB is associated with bile salt and does not react with [HRP]:

$$V' = -d[UCB]/dt = k' \times [HRP]' \times [UCB_f] \qquad Eq.\ 4)$$

or:

$$V' = k' \times [HRP]' \times [UCB] \times f \qquad Eq.\ 5)$$

where $[UCB_f]$ is the amount of free bilirubin and $f = [UCB_f]/[UCB]$, the fraction of free UCB. In terms of absorbance:

$$V' = (k' \times [HRP]' \times A' \times f)/E' \qquad Eq.\ 6)$$

where E' is the extinction coefficient of UCB in taurocholate solution at 440 nm.

The initial concentrations of UCB that react with HRP in aqueous solution, $[UCB]_o$, and in bile salt solutions $[UCB_f]_o$, can be determined by measuring the initial velocities, V_o and V'_o of the peroxidation of UCB by HRP. Thus:

$$V_o = k \times [HRP] \times A_o/E \qquad Eq.\ 7)$$

and

$$V'_o = (k' \times [HRP] \times A'_o \times f)/E' \qquad Eq.\ 8)$$

where A_o and A'_o are the initial absorbances of bilirubin in each solution at a wavelength of 440 nm.

If the linear rate constants in eqs. 7 and 8 are equal (k = k'), i.e., the unbound UCB is oxidized at the same rate whether or not bile salts are present:

$$\frac{(V_o \times E)}{([HRP] \times A_o)} = \frac{(V'_o \times E')}{([HRP]' \times A'_o \times f)} \qquad Eq.\ 9)$$

and:

$$f = \frac{V'_o \times [HRP] \times A_o \times E'}{V_o \times [HRP]' \times A'_o \times E} \qquad Eq.\ 10)$$

Thus, the fraction of free UCB in bile salt solutions can be measured by comparing peroxidation reaction rates in the presence and absence of bile salts.

Below the critical micellar concentration (CMC) of sodium taurocholate (TC), monomers and possibly dimers are present, while above the CMC the bile salt molecules aggregate to form micelles. Although the type of interaction between UCB and TC has not been characterized, the hydrophobic behavior of UCB in aqueous solution would predict a different interaction between UCB and bile salt micelles as compared to bile salt monomers. Since the total concentration of UCB in bile and in aqueous solutions is never more than micromolar while that of the bile salt is millimolar, the stoichiometry of UCB and TC interactions is always much less than 1:1. Therefore, UCB–bile salt interactions do not follow classic theory of binding or partitioning which would predict that there is at least one binding site per bile salt binding unit or that each micelle will accommodate at least one molecule of UCB in its center.

METHODS

Horseradish peroxidase (HRP, crude, essentially salt-free, RZ approx. = 0.3, Sigma Chemical Co., St. Louis, MO) was dissolved in deionized, charcoal-treated water to a final concentration of approximately 0.44 mg of crude enzyme per ml. Enzyme activity was determined by assay with the substrate pyrogallol (10). The enzyme solutions were diluted to concentrations that maintained a linear decrease in UCB concentration as UCB was peroxidized by HRP. The concentrations of enzyme used varied considerably depending upon the concentration of bile salt present.

UCB (Gallard-Schlesinger, Carle Place, NY) was dissolved in $CHCl_3$ to a final concentration of approximately 4 mg/ml and 7.0 ml of this solution was mixed with 1.0 ml of 50 mM Na_4 EDTA (Eastman Kodak Co., Rochester, NY) in methanol. One- to 2-ml aliquots were dried under N_2 at 40°C and were stored under vacuum until use. The

UCB remained stable under these conditions for periods of 48 hr and was easily dissolved in the buffer solutions used to prepare the experimental solutions described below.

Standard solutions used to calculate V_o in the absence of bile salts were prepared with 0.15 M Tris buffer (Fisher Scientific Co., Fair Lawn, NJ) and NaCl to a final pH of 8.2 and an ionic strength of 0.15. Test solutions containing sodium taurocholate were prepared in Tris buffer at pH 8.2 and were adjusted to ionic strength 0.15 with NaCl. The sodium salt of taurocholate was purchased from Calbiochem, La Jolla, CA and was greater than 98% pure, and contained no phospholipids, cholesterol, or unconjugated bile salts. Both standard and test solutions were prepared by dissolving the previously dried UCB-EDTA mixture in 0.867 ml of 50 mM Na_4EDTA and diluting to 10 ml final volume with the appropriate buffer or buffer-taurocholate solution. Thus, the final solutions had a pH of 8.2, ionic strength of 0.15, [EDTA] = 5.0 mM, and taurocholate concentrations ranging from 0 to 75 mM.

The concentration of UCB which varied in each experiment was measured spectrophotometrically at 440 mM. At low [UCB], absorbance was linearly related to [UCB] (extinction coefficient = 0.973), but became nonlinear as [UCB] increased. Therefore, a standard curve was constructed from actual measurements of [UCB] by modified Michaelsson reaction (11) compared with absorbance on a Lambda I UV/VIS (Perkin-Elmer, Norwalk, CT) spectrophotometer **(Fig. 1).** This curve was used to estimate [UCB] in the reaction mixtures, and to convert the change in absorbance with time (dA/dt), as UCB was peroxidized, to a change in [UCB] with time (d[UCB]/dt). UCB concentrations, and thus rates of reaction, were corrected for E'/E when bile salt was present (eq. 3 and 4).

Before assay, each solution was filtered through a 0.22-μm Millipore filter (Millipore Corp., Bedford,MA) to remove undissolved UCB aggregates. To avoid subsequent precipitation of the metastable, standard UCB solutions (lacking bile salts), they were assayed immediately after filtration and equilibration to temperature (25°C or 37°C). Test solutions containing taurocholate were more stable and were allowed to equilibrate for at least 30 min at the same temperature before assays were done to insure complete binding of UCB to the bile salt.

Immediately before assay, 0.5 ml of 0.3% H_2O_2 was added to 10 ml of filtered standard or test solution, and the reaction was initiated by adding 1 ml of this solution to a cuvette containing 10 to 100 μl of HRP solution. After rapid mixing by inversion of the cuvette, the reaction was monitored continuously in a Lambda I UV/VIS spectrophotometer (Perkin-Elmer, Norwalk, CT) at a wavelength of 440 nm and the absorbance was recorded against time on a chart recorder. The rate of reaction (dA/dt) and the initial absorbance (A_o) were calculated by linear regression of data points obtained automatically by the spectrophotometer at 5-sec intervals throughout the assay. Assay time was 1 to 2 min. All plots were checked for linearity, visually and by the correlation coefficient of the regression analysis.

The inhibition by TC of the activity of HRP against

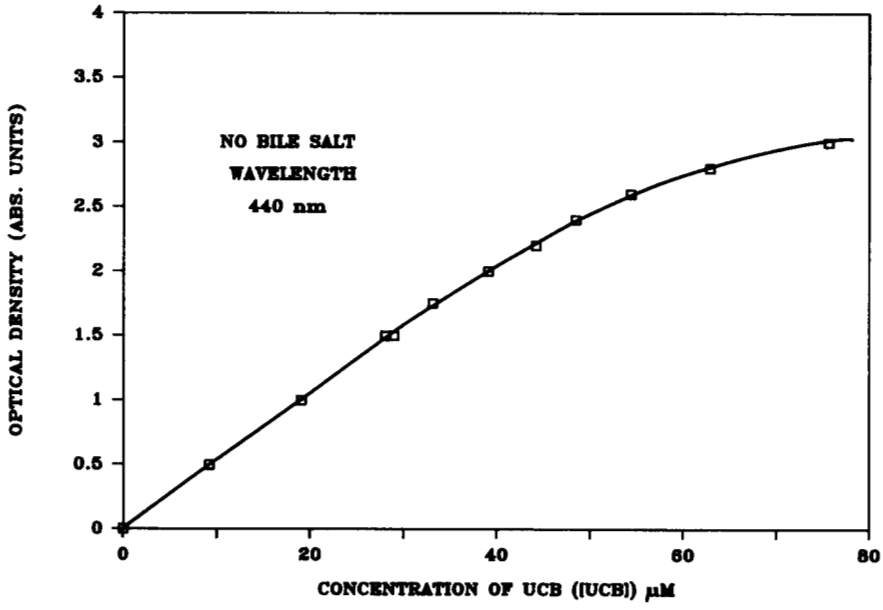

Fig. 1. Absorbance of UCB in aqueous solution. The absorbance of UCB at 440 nm (absorbance units) is plotted against the concentration (μM) of UCB in solution. Note that the relationship is linear to [UCB] = 28 μM (absorbance = 1.5), but deviates from linearity above this point. Both [UCB] and the velocity of the reaction (d[UCB]/dt) were corrected for this deviation above 28 μM.

another substrate (12), pyrogallol, was examined at various taurocholate concentrations (0–75 nM). Because pyrogallol undergoes spontaneous oxidation at pH 8.2, the net rate of oxidation at this pH was estimated by difference spectroscopy (Model 533 UV/VIS double beam scanning spectrophotometer, Perkin-Elmer, Norwalk, CT) at 420 nm with enzyme-free pyrogallol in the blank cuvette. Unlike UCB peroxidation experiments, the assays with pyrogallol were done at only one concentration of substrate. Five percent (400 mM) pyrogallol was mixed with the other reagents diluting the pyrogallol to a final concentration of 120 mM in the reaction vessel.

The data reported in all figures are the means of at least three assays. Usually, five assays were performed in each series, but assays that were not linear over the assay period were excluded. Occasionally the assays were inconsistent due to air bubbles in the reaction cuvette, most commonly at higher bile salt concentrations; these were also excluded. Thus handled, the results were highly reproducible from day to day, and the standard error of the mean was consistently below 10%.

RESULTS

Initial velocity of UCB peroxidation at 25 and 37°C

The initial velocity (V_o) of UCB peroxidation was linearly related to low initial concentrations of UCB in standard solutions (no bile salt) at both 25 and 37°C **(Fig. 2).** The regressions in the linear range: $V_o = 62.05 \times [UCB] + 0.97$, $r = 0.98$ at 37°C, and $V_o = 48.16 \times [UCB] - 0.372$, $r = 0.99$ at 25°C were used to calculate standard V_o at the total [UCB] measured in the test (taurocholate) solutions. The slope of the regression lines, 62.05 and 48.12 (μmol of HRP × min)$^{-1}$, are the linear rate constants, k, in eq. 1 at each temperature.

Data were obtained at 37°C to validate our spectrophotometric method of determining free UCB since published kinetic data are available for UCB at this temperature. Our value of 62.05 (μmol of HRP × min)$^{-1}$ is essentially the same as the value of 62.5 (μmol of HRP × min)$^{-1}$ calculated from the kinetic data of Brodersen and Bartels (10). Moreover, the slower rate at 25°C is appropriate for the 12°C decrease in reaction temperature. Above

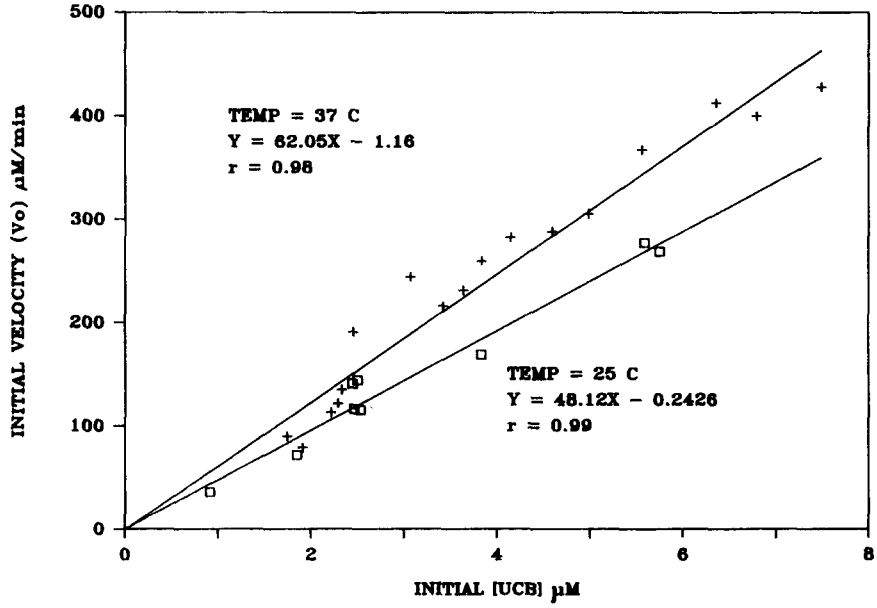

Fig. 2. The initial velocity of UCB peroxidation in the absence of taurocholate. The initial velocity of the peroxidation of UCB is plotted against the initial concentration of substrate UCB at both 37°C and 25°C. The slope of the linear regressions: $V_O = 62.05 \times [UCB] - 1.16$, $r = 0.98$ and $V_O = 48.12 - 0.2426$, $r = 0.99$ give the rate constant k of the reaction at each temperature, respectively. The value of 62.05 at 37°C is essentially the same as the value calculated from the kinetic data of Brodersen and Bartels (10) for HRP and UCB. The lower value at 25°C is consistent with the 12°C drop in reaction temperature.

[UCB] = 10 μM at 25°C and 5 μM at 37°C, a value still well below the K_m of HRP for UCB (800 μM), V_o of the reaction deviated slightly from linearity, consistent with the dimer and multimer formation known to occur at [UCB] in this range in aqueous solution (13). Thus, our data obtained spectrophotometrically are consistent with previously reported data on UCB peroxidation by HRP (9, 10), but the spectrophotometric method may actually be more sensitive to UCB aggregation (self-binding).

Initial velocity of UCB peroxidation in the presence of taurocholate monomer (below the CMC)

The inhibition of UCB peroxidation by TC monomer is illustrated in **Fig. 3.** Similar to standard solutions (line labeled no bile salt), solutions containing 2 and 5 mM TC (below the CMC) demonstrated a linear relationship between V_o and [UCB]. However, the linear regressions: $V_o = 23.68 \times [UCB] - 3.72$, $r = 0.99$ and $V_o = 12.61 \times [UCB] + 1.53$, $r = 0.99$ indicate that the linear rate constants of 23.68 and 12.61 (μmol of HRP × min)$^{-1}$, respectively, progressively decrease from the standard value as [TC] increases in the monomeric range.

Eq. 10 can be used to calculate the fraction of UCB that was free in each solution tested at [TC] = 2 and 5 mM. The initial [UCB_f] was then obtained since [UCB] is known in each case and was plotted against the ratio of the concentration of "bound" UCB to taurocholate [UCB_b]/[TC] in **Fig. 4.** The plots for [TC] = 2 mM (□) and 5 mM (+) both fall on essentially the same regression line: [UCB_f] = 1913 × [UCB_b]/[TC], $r = 0.99$. This relationship can be rearranged to the form:

$$K_{mon} = 522 \text{ l/M} = \frac{[UCB_b]}{[UCB_f] \times [TC]} \qquad \textit{Eq. 11)}$$

where the molar concentrations are used to obtain K_{mon}. Thus, the association of UCB with taurocholate in the monomeric range can be described empirically by the constant K_{mon}.

Inhibition of UCB peroxidation in the presence of taurocholate micelles

Above the CMC of TC, the proportion of micelles increases progressively as [TC] is increased, since the concentration of monomer remains essentially constant while that of micelles increases. The effect of TC micelles on UCB peroxidation is illustrated in **Fig. 5.** Again, V_o is linearly related to [UCB] above the CMC, and rate constants determined from the slopes of linear regressions decrease from 4.57 to 0.24 (μmol of HRP × min)$^{-1}$ as the concentration of TC is increased from 10 to 75 mM.

Again [UCB_f] was plotted against [UCB_b]/[TC] for the micellar solutions **(Fig. 6).** The relationships were again linear, but there was a progressive decrease in the slope of the regressions as [TC] increased from 10 to 20 mM. There was then little change between regressions at 20

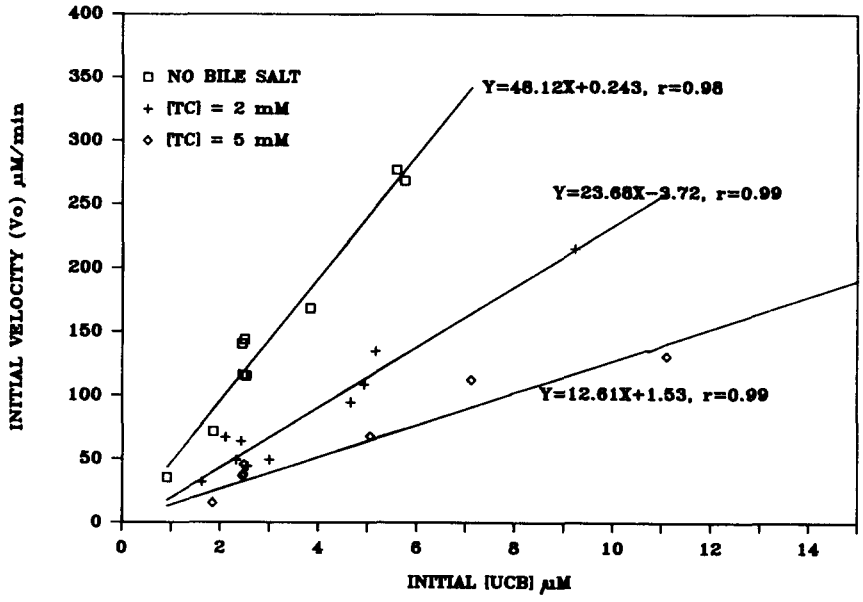

Fig. 3. Initial velocity of UCB peroxidation in the presence of taurocholate monomer. The relationship between initial velocity of UCB peroxidation and [UCB] at 25°C is shown for two concentrations of taurocholate (2 and 5 mM) in the monomeric range and is compared to the values in the absence of taurocholate (□). The linear regressions in the presence of taurocholate were: 2 mM TC, $V_O = 23.68 \times [UCB] - 3.72$, $r = 0.99$ and 5 mM TC, $V_O = 12.61 \times [UCB] + 1.53$, $r = 0.99$. The apparent rate constant in the presence of taurocholate is less than in standard solutions and decreases from 23.68 to 12.61 (μM HRP × min)$^{-1}$ as [TC] increases from 2 to 5 mM.

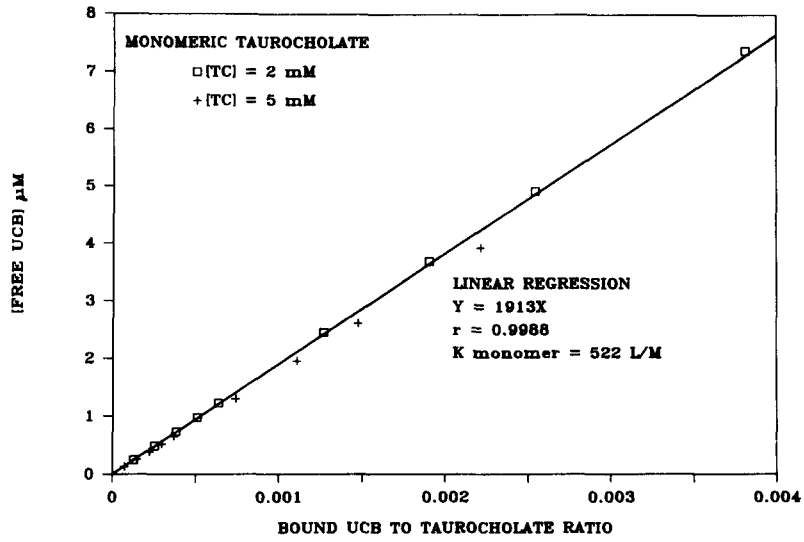

Fig. 4. Relationship of free UCB to the ratio of bound UCB and taurocholate in the monomeric range. Plots of calculated [UCB_f] against the ratio of [UCB_b]/[TC] are not significantly different at [TC] = 2 (□) and 5 mM (+). This indicates that the interaction of UCB with TC is governed by a single constant, K_{mon} = [UCB_b]/[UCB_f] × [TC]), and that there is no significant reaction of bound UCB, and no inhibition of the enzyme HRP over the monomeric range of [TC]. The line in the graph is the average plot of [UCB_f] and [UCB_b]/[TC], with K_{mon} equal to 522 l/M(1/slope of this line).

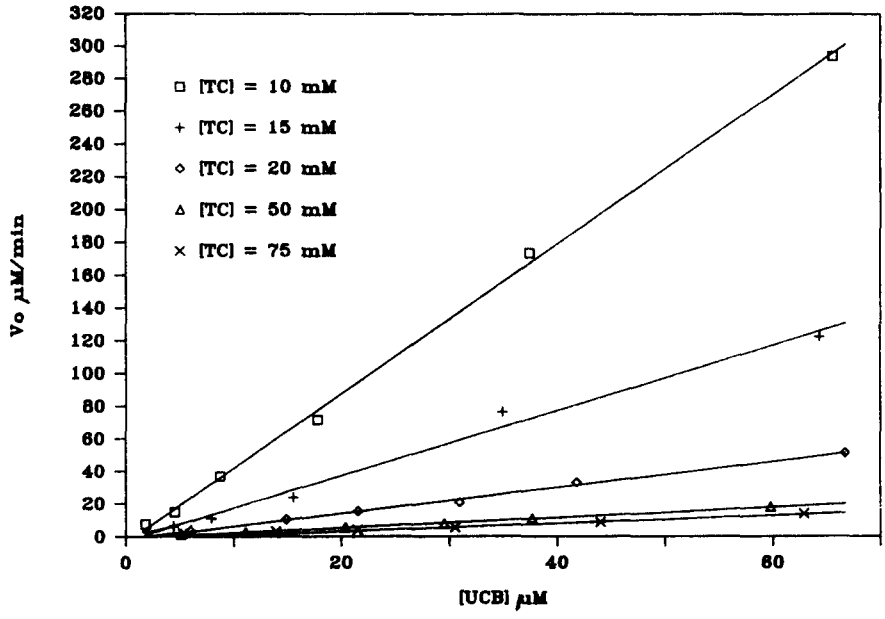

Fig. 5. Inhibition of UCB peroxidation in the presence of taurocholate micelles. The relationships between initial velocity of UCB peroxidation and [UCB] is shown at concentration of TC in the micellar range from [TC] of 10 to 75 mM. The linear regression lines for the peroxidation in the presence of micellar TC were: 10 mM TC, $V_O = 4.57 \times [UCB] - 3.54$, $r = 0.99$; 15 mM TC, $V_O = 2.00 \times [UCB] - 2.24$, $r = 0.99$; 20 mM TC, $V_O = 0.79 \times [UCB] - 1.50$, $r = 0.99$; 50 mM TC, $0.31 \times [UCB] - 0.68$, $r = 0.99$; and 75 mM TC, $V_O = 0.24 \times [UCB] - 1.43$, $r = 0.99$. In micellar solutions there were further decreases in the slope of the linear regressions from 4.57 to 0.24 indicating further inhibition of the peroxidation of UCB as the concentration of TC micelles increases. At [TC] = 50 mM, the reaction rate is inhibited by over 99%.

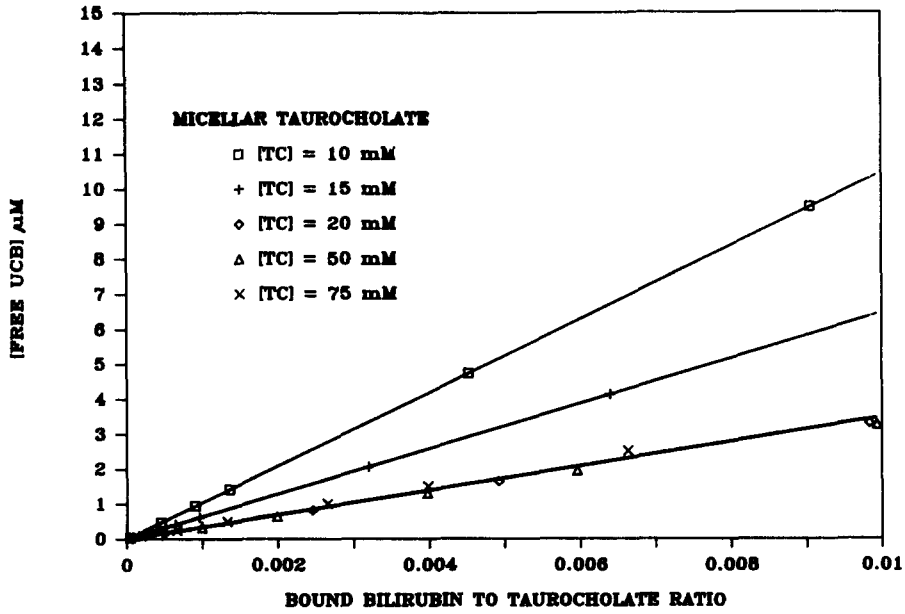

Fig. 6. Relationship of free UCB to the ratio of bound UCB to taurocholate in the micellar range. The calculated values of $[UCB_f]$ are plotted against $[UCB_b]/[TC]$, but the slope of the plots decreased progressively from [TC] = 10 to 20 mM. There was little change in slope for [TC] = 20 to 75 mM. Thus, at each [TC] the interaction of UCB and TC could be described by a constant, $K = [UCB_b]/([UCB_f] \times [TC])$.

and 75 mM. At each [TC] in the micellar range, the regressions can be rearranged in the form:

$$K = \frac{[UCB_b]}{[UCB_f] \times [TC]} \qquad Eq.\ 12)$$

which is analogous to eq. 11. It would at first appear that the association of UCB with TC is a constantly changing function in the micellar range.

However, one should realize that the constant K in eq. 12 depends on the amount of UCB that is associated with micelles and with monomer since both are present in micellar bile salt solutions. Since the concentration of TC monomer remains approximately the same above the CMC, the proportion of TC micelles to monomer constantly rises. The effect of the changing concentrations of TC monomer and micelles can be described mathematically as:

$$K = X \times K_{mon} + Y \times K_{mic} \qquad Eq.\ 13)$$

where X is the fraction of monomer ($[TC]_{mon}/[TC]$) and Y is the fraction of micelles ($([TC]_{mon})/[TC]$) and:

$$X + Y = 1. \qquad Eq.\ 14)$$

This equation predicts that K should approach K_{mic} from K_{mon} as [TC] increases above the CMC. Since the slopes of the regressions of $[UCB_f]$ versus $[UCB_b]/[TC]$ at 50 and 75 nM are essentially the same, K_{mic} can be estimated from the average values observed at these concentrations to be 2875 l/M.

The changes in K with [TC] are illustrated in **Fig. 7** by plotting both the expected (eq. 13) and observed (eq. 12) overall K values at each [TC] studied. If K_{mon} is 522 l/M, and K_{mic} is 2875 l/M, the predicted values of K (□) closely approximate the observed values when a value of 7.5 mM is chosen for the CMC of TC. This value of CMC is in the range of reported values of CMC in the literature (6 to 9 mM (14)) which have been measured by other methods.

Inhibition of pyrogallol activity by taurocholate

The activity of HRP for pyrogallol is significantly decreased by TC monomer, but little additional inhibition occurs as the concentration of TC micelles is increased **(Fig. 8).** This could be due to interactions of pyrogallol with TC or to direct inhibition of HRP by the bile salt. The exact mechanism cannot be determined from this study. However, so that we might compare inhibition by TC of UCB and pyrogallol peroxidation, we assumed pyrogallol-TC interactions and calculated constants in the monomeric and micellar regions from eqs. 11, 12, and 13 as was done for UCB **(Fig. 9).** Clearly, interactions between TC, pyrogallol, and HRP were highest in the monomeric range, although tenfold lower than that seen for UCB, and TC micelles did little to further inhibit the reaction. Thus pyrogallol and UCB act very differently with TC and/or HRP. Since the physical-chemical proper-

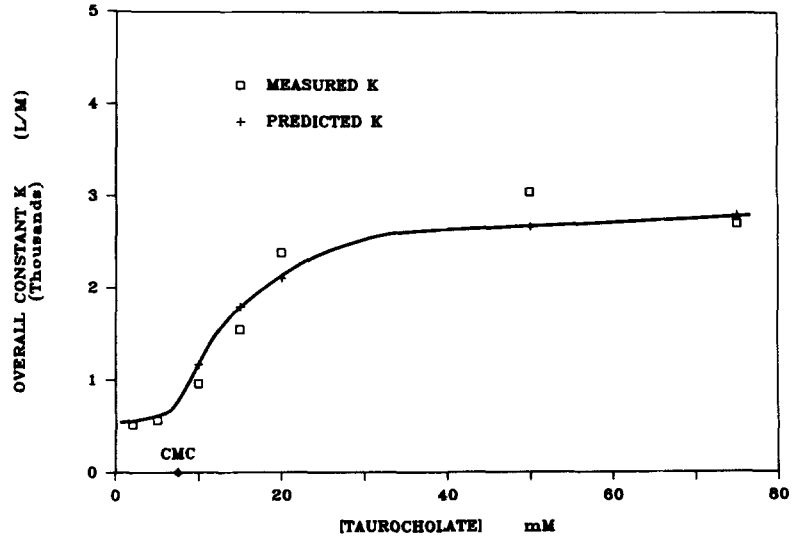

Fig. 7. Calculation of K_{mic}. The value of K in the micellar range depends on the value of K_{mon}, K_{mic}, and the proportion of bile salt monomers and micelles. The values of K were calculated from the slopes of the plots in Fig. 6 and are plotted against the concentration of TC (□). K_{mic} was approximated from the average of K at [TC] = 50 and 75 mM to be 2875 l/M, since K will approach K_{mic} as [TC] increases. The observed values of K are compared to predicted values (+) calculated from the equation: $K_{overall} = X \times K_{mon} + Y \times K_{mic}$ using $K_{mon} = 522$ l/M, $K_{mic} = 2875$ l/M, and CMC for TC = 7.5 mM. The intermediate values of K match nicely, demonstrating the expected change in K as the proportion of TC micelles increases.

ties of UCB predict that it should be more closely associated with the micelle, these data are consistent with: *1*) inhibition of the peroxidation of UCB due to UCB-TC interactions; *2*) inhibition of pyrogallol oxidation due to another mechanism; and *3*) very little, if any, direct inhibition of the enzyme HRP by the bile salt.

DISCUSSION

Although UCB comprises only 1–3% of the total bilirubin in bile, bile is thought to be close to saturation with UCB (1, 7). Small changes in the concentration of UCB or in the ability of bile to bind UCB could, therefore,

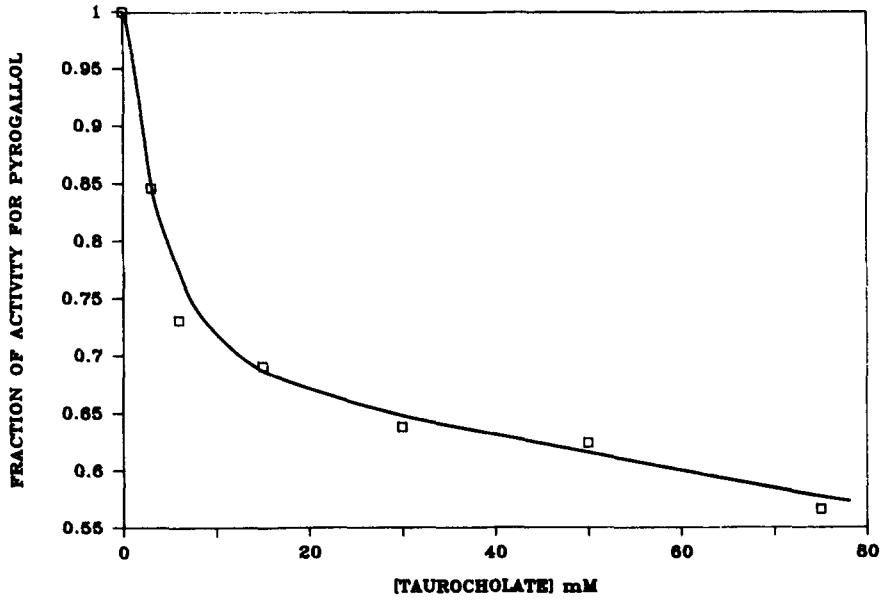

Fig. 8. Inhibition of the peroxidation of pyrogallol by horseradish peroxidase. The fraction of activity of HRP for the substrate pyrogallol (1,2,3-trihydroxybenzene) is plotted against the concentration of taurocholate. Note that the activity drops in the monomeric range of concentrations, but not to the degree seen with UCB. There is little further decrease in activity as [TC] increases in the micellar range.

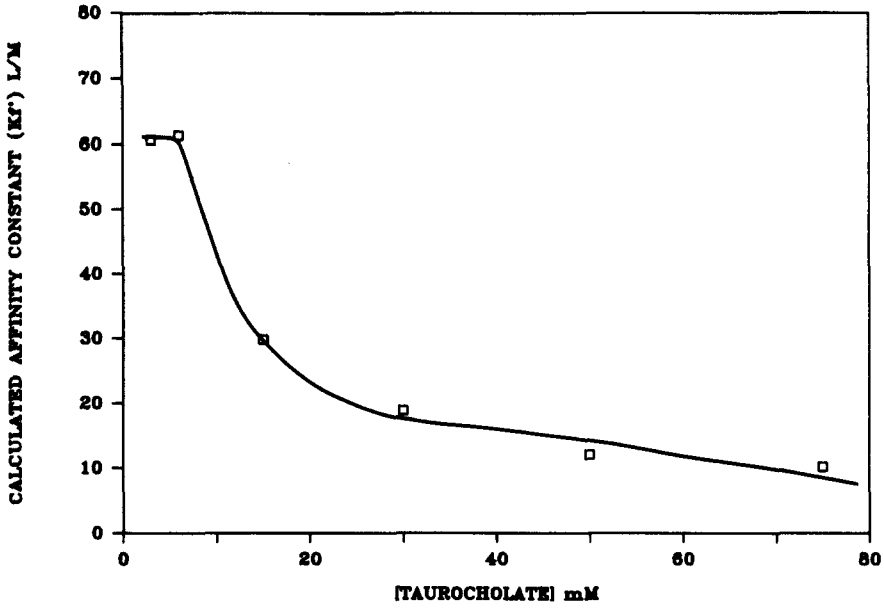

Fig. 9. Calculation of pyrogallol data assuming pyrogallol-TC interactions. The data obtained for the inhibition of HRP activity for pyrogallol was compared to that for UCB by assuming that it interacts with TC. Note that this gives a pattern very different from UCB. K_{mon} for pyrogallol is lower than K_{mon} for UCB and higher than K_{mic} for pyrogallol, demonstrating that the interactions of pyrogallol and/or HRP with TC monomer are less than with UCB and that, unlike UCB, there is essentially no interaction with micelles.

cause bile to become supersaturated with either calcium bilirubinate or with UCB itself, and precipitation may then occur. Increased [UCB] has been demonstrated in the bile of patients with black pigment gallstones related to hemolytic anemia (1) and in *nb/nb* mice with congenital hemolytic anemia, an animal model of pigment gallstone disease (15). On the other hand, a decreased ability to solubilize UCB is suggested by data in Japanese patients with pigment gallstone disease who have both a decrease in total bile salt concentrations and an altered bile salt profile in their gallbladder bile (16). The extent of in vitro solubilization of UCB by bile salts indicates that the latter are probably the major factor in bile responsible for solubilization of UCB (17).

Studies of the solubilization of UCB have shown an increase in UCB solubility of about four orders of magnitude when bile salts are present (17). The solubility of UCB increases with increasing a bile salt concentration, but bile salt micelles are much more efficient than monomers. It is felt that bile salts increase the solubility of UCB by binding to it or by partitioning the hydrophobic UCB molecule into the center of the bile salt micelle (17). However, like cholesterol, UCB interactions with bile salts differ from other substances that do bind to bile salts or partition into bile salt micelles in that much less than one molecule of UCB interacts with each bile salt molecule or micelle. The actual nature of UCB-bile salt interactions has not been determined. It is, therefore, not possible to describe these interactions mathematically with classic binding or partitioning theory.

This study employed a modification of a method used previously to determine the dissociation constant (K_d) of the UCB-albumin complex (9) by measuring the initial velocity of the peroxidative degradation of UCB to colorless products. Since the initial velocity depends on the amount of UCB free to react, the measurement of free UCB in taurocholate solutions by this method depends on several assumptions: *a*) the rate of dissociation of UCB from taurocholate is more rapid than the rate of UCB peroxidation and is not the rate limiting step; *b*) free UCB, but not bound UCB, reacts with HRP; and *c*) taurocholate does not directly inhibit the enzyme HRP. The validity of these assumptions is addressed as follows.

The rate of peroxidation of UCB was controlled by decreasing the concentration of HRP until a plot of absorbance versus time was linear and V_o was linearly dependent on both the initial concentrations of UCB and HRP. Since free UCB concentration is very low, a rate of peroxidation that is faster than the rate of dissociation of UCB from taurocholate would result in a nonlinear plot of absorbance versus time. Thus, the first assumption is justified by the conditions imposed upon the study.

The linearity of the plot of the concentration of free UCB against fixed ratios of "bound" UCB to taurocholate (Figs. 4 and 6) validates the other two assumptions. If the UCB associated with TC monomer and micelles reacted with HRP, the calculated concentration of free UCB would not be the same at fixed ratios of $[UBC_b]/[TC]$ over a range of [TC]. Although the $[UCB_b/[TC]$ ratio is the same, $[UCB_b]$ would be much greater at higher as

compared to lower TC concentrations. The contribution of the UCB_b to the initial velocity would consequently be greater at high [TC] and [UCB_f] versus [UCB_b/[TC] would have a higher than predicted slope at high [TC].

Likewise, direct inhibition of the enzyme HRP by the bile salt taurocholate would decrease the linear rate constant k to k' (eq. 1) and the value of [UCB_f] would be much lower than predicted for [UCB_f] at higher bile salt concentrations. The plots of [UCB_f] versus [UCB_b]/[TC] would consequently have lower slopes than predicted as taurocholate concentration increased.

In actuality, the slopes of the plot of [UCB_f] versus [UCB_b]/[TC] were identical for 2 and 5 mM taurocholate indicating a single constant, K_{mon}, of 522 l/M (eq. 11) in the monomeric range and excluding reaction of bound UCB or direct enzyme inhibition. In the micellar range, slopes of [UCB_f] versus [UCB_b]/[TC] decreased, and thus the constant, K, increased progressively with a rise in [TC] from 10 to 50 mM. The slopes and constant were then essentially the same at 50 and 75 mM indicating that K was approaching K_{mic} with a value of about 2875 l/M. A plot of overall K versus [TC] (Fig. 7) again demonstrated the compliance of our micellar data with the behavior theorized for solutions containing both bile salt monomers and micelles (eq. 13).

In contrast, examination of the inhibition by TC of the activity of HRP for another substrate, pyrogallol (1,2,3-trihydroxybenzene), was very different. The structure of pyrogallol suggests that, compared to UCB, it would interact very differently, if at all, with TC. In fact, inhibition of pyrogallol activity was much less than with UCB and occurred only in the monomeric range with very little contribution by micelles. This contrasted with the high monomer and, even higher, micellar inhibition of UCB peroxidation by TC.

The results of this study are as predicted by equations derived assuming that the inhibition of the peroxidation of UCB in the presence of TC is due to TC-UCB association and not due to enzyme inhibition, suggesting that we are indeed estimating free UCB by this kinetic method. This is further supported by the very different behavior of UCB as compared to pyrogallol. Some contribution of direct enzymatic inhibition cannot, however, be excluded, especially in the monomeric range.

If pyrogallol does not bind to TC, a reasonable scenario considering its structure, and if the inhibition of the oxidation of pyrogallol in the presence of taurocholate is due entirely to direct inhibition of the enzyme by the bile salt, correction of our UCB data for this direct enzymatic inhibition gives qualitatively the same results. Quantitatively, the constants, K_{mon} and K_{mic}, would decrease to 390 and 1765 l/M. The amount of free UCB at 50 mM would, thus, be about 1% of the total as opposed to about 0.6% as reported in this study.

Conventional methods of examining the type of inhibition of enzymatic reactions, such as Lineweaver-Burk plots, are not possible with TC and UCB. Each type of inverse plot requires given substrate concentrations near the K_m value of the enzyme for that substrate, in the case of HRP and UCB, 800 μM. Although UCB concentrations in this range can be obtained in metastable solutions and the K_m and V_{max} of HRP have been measured in the absence of inhibitor (10), even higher concentrations of UCB are required to accurately measure the apparent K_m and V_{max} of the reaction when it is significantly inhibited by TC. These concentrations could not be reached, even when the method of Jacobsen (9) was used instead of the spectrophotometric method. We could only study UCB concentrations that were at maximum 1/10 K_m by the spectrophotometric method. At concentrations this low, Lineweaver-Burk plots give intercepts on both the x- and y-intercepts that are too close to the origin to be accurate. Thus, the physical-chemical properties of UCB preclude full determination of mechanisms of inhibition of peroxidation of UCB by HRP.

The empirical constants derived from plots of [UCB_f] versus [UCB_b]/[TC] have been presented in a form that resembles equilibrium binding constants (eqs. 11 and 12), but it must be emphasized that they are not binding constants. Calculation of the true formation constants, K_f, for the interaction of UCB with bile salt monomer or micelle requires knowledge of the stoichiometry of UCB-bile salt binding. Morever, interactions in the micellar range of bile salt might not involve binding alone, but rather involve partitioning of UCB into the center of the micelle. Clearly, eqs. 11 and 13 are useful for describing our data and for comparing UCB-TC interactions in the monomeric and micellar ranges of bile salt concentration, but they in no way imply any information concerning the stoichiometry or affinity of interactions which is occurring between these two substances.

The actual affinity constants that describe the association of UCB and taurocholate await accurate descriptions of the complex solution of UCB and taurocholate. This must include full knowledge of the type of interaction and stoichiometry of the association of UCB with bile salts. Moreover, we would expect that each UCB species, protonated UCB, UCB monoanion, and UCB dianion, would interact differently with bile salts and would have different affinity constants, greatly complicating the analysis of UCB-TC interactions. This last assumption was not tested in the present work since the pKa' values of UCB are not known, but examination of the interactions between bile salts and UCB at different pH values should now be done to begin to examine this assumption.

In summary, the inhibition by taurocholate of UCB peroxidation catalyzed by the enzyme HRP appears to be secondary to interactions between UCB and TC; direct inhibition of the enzyme is less likely. Moreover, the peroxidation of UCB associated with TC appears insig-

nificant in comparison to the reaction of free UCB. Therefore, this kinetic method is applicable in examining the interactions of UCB with taurocholate, and possibly with other bile salts, in aqueous solution.

UCB appears to interact with both TC monomer and micelle, but interaction is most striking in the micellar range. At the physiologic pH value of 8.2 and at [TC] > 50 mM, the amount of free UCB estimated by this method is less than 1%. Thus taurocholate and, probably, bile salts in general interact with significant amounts of UCB, explaining the high concentration of UCB found in bile, despite its minimal solubility in aqueous solution. By maintaining [UCB_f] well below total [UCB], TC buffers the concentration of free UCB available to react with other substances in bile. Since bile salts are known to also bind calcium, and thus to reduce the concentration of free Ca^{2+} in bile (18), they would play a dual role in limiting the values of [UCB] and [Ca^{2+}] in bile and in preventing the precipitation as calcium bilirubinate. They would therefore play a critical role in preventing the initiation and growth of pigment gallstones.

The authors appreciate the technical assistance of Kelli Ann Mosher who assisted in the performance of these studies. This work was supported by Grant 1-R01-AM 32130, NIDDK, National Institutes of Health, and the Otho S. A. Spraque Fund at Northwestern University.

Manuscript received 29 July 1985, in revised form 23 June 1986, and in re-revised form 1 December 1986.

REFERENCES

1. Soloway, R., B. W. Trotman, and J. D. Ostrow. 1977. Pigment gallstones. *Gastroenterology.* **72:** 167–182.
2. Ostrow, J. D. 1984. The etiology of pigment gallstones. *Hepatology.* **4:** 215s–222s.
3. Wosiewitz, U., and S. Schroebler. 1978. On the chemistry of 'black' pigment stones from the gallbladder. *Clin. Chim. Acta.* **89:** 142.
4. Wosiewitz, U., and S. Schroebler. 1978. 'Polymer pigments' in human gallstones. *Naturwissenschaften.* **65:** 162–163.
5. Ohkubo, H., S. H. Carr, J. D. Ostrow, and R. V. Rege. 1982. Polymer networks in pigment gallstones assessed by equilibrium swelling and infrared spectroscopy. *Gastroenterology.* **87:** 805–814.
6. Brodersen, R. 1979. Bilirubin: solubility and interaction with albumin and phospholipid. *J. Biol. Chem.* **254:** 2364–2369.
7. Ostrow, J. D., and L. Celic. 1984. Bilirubin chemistry, ionization, and solubilization by bile salts. *Hepatology.* **4:** 38s–45s.
8. 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. *Hepatology.* **4:** 228s–243s.
9. Jacobsen, J. 1969. Binding of bilirubin to human serum albumin—determination of the dissociation constants. *FEBS Lett.* **5:** 112–114.
10. Brodersen, R. and P. Bartels. 1969. Enzymatic oxidation of bilirubin. *Eur. J. Biochem.* **10:** 468–473.
11. Michaelsson, M. 1961. Bilirubin determination in serum and urine. *Scand. J. Clin. Lab. Invest. Suppl.* **56:** 40–50.
12. Theorell, H. 1951. The iron-containing enzymes. B. Catalases and peroxidases "Hydroperoxidases". *In* The Enzymes. Vol. 2, Part 1. J. B. Summer and K. Myrbach, editors. Academic Press Inc., New York. Chap. 56, 397–427.
13. Carey, M. C., and A. P. Koretsky. 1979. Self-association of unconjugated bilirubin-IX in aqueous solutions at pH 10.0 and physical-chemical interactions with bile salt monomers and micelles. *Biochem. J.* **179:** 675–689.
14. 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.
15. Trotman, B. W., S. E. Bernstein, W. F. Balistreri, G. D. Wert, and R. A. Martin. 1981. Hemolysis-induced gallstones in mice: increased unconjugated bilirubin in hepatic bile predisposes to gallstone formation. *Gastroenterology.* **81:** 232–236.
16. Higasi, Y., M. Nagase, H. Tanimura, R. Shioda, M. Setoyama, N. Kobayashi, S. Mokaihara, T. Kamata, K. Maruyama, H. Kato, and K. Mori. 1980. Epidemiology and etiology of gallstones. *Arch. Jpn. Chir.* **49:** 555–571.
17. Carey, M. C. 1984. Physical-chemical methods for determining bilirubin solubilities in simulated bile systems. *Hepatology.* **4:** 223s–227s.
18. 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:** 1079–89.