

Solubility of calcium salts of unconjugated and conjugated natural bile acids

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Abstract The approximate solubility products of the calcium salts of ten unconjugated bile acids and several taurine conjugated bile acids were determined. The formation of micelles, gels, and/or precipitates in relation to Ca^{2+} , Na^+ , and bile salt concentration was summarized by "phase maps." Because the ratio of Ca^{2+} to bile salt in the precipitates was ca. 1:2, and the activity of Ca^{2+} but not that of bile salt (BA^-) could be measured, the ion product of $a_{\text{Ca}^{2+}} [\text{BA}^-]^2$ was calculated. The ion product ($= K_{\text{sp}}$) ranged over nine orders of magnitude and the solubility thus ranged over three orders of magnitude; its value depended on the number and orientation of the hydroxyl groups in the bile acid. Ion products (in units of 10^{-9} mol/l)³ were as follows: cholic ($3\alpha\text{OH}, 7\alpha\text{OH}, 12\alpha\text{OH}$) 640; ursocholic ($3\alpha\text{OH}, 7\beta\text{OH}, 12\alpha\text{OH}$) 2300; hyocholic ($3\alpha\text{OH}, 6\alpha\text{OH}, 7\alpha\text{OH}$) 11; ursodeoxycholic ($3\alpha\text{OH}, 7\beta\text{OH}$) 91; chenodeoxycholic ($3\alpha\text{OH}, 7\alpha\text{OH}$) 10; deoxycholic ($3\alpha\text{OH}, 12\alpha\text{OH}$) 1.5; 12-epideoxycholic (lagodeoxycholic, $3\alpha\text{OH}, 12\beta\text{OH}$) 2.2; hyodeoxycholic ($3\alpha\text{OH}, 6\alpha\text{OH}$) 0.7; and lithocholic ($3\alpha\text{OH}$) 0.00005. The critical micellization temperature of the sodium salt of murideoxycholic acid ($3\alpha\text{OH}, 6\beta\text{OH}$) was $>100^\circ\text{C}$, and its Ca^{2+} salt was likely to be very insoluble. Taurine conjugates were much more soluble than their corresponding unconjugated derivatives: chenodeoxycholytaurine, 384; deoxycholytaurine, 117; and cholytaurine, $>10,000$. Calcium salts of unconjugated bile acids precipitated rapidly in contrast to those of glycine conjugates which were metastable for months. ■ Thus, hepatic conjugation of bile acids with taurine or glycine not only enhances solubility at acidic pH, but also at Ca^{2+} ion concentrations present in bile and intestinal content.—Gu, J. J., A. F. Hofmann, H-T. Ton-Nu, C. D. Schteingart, and K. J. Mysels. Solubility of calcium salts of unconjugated and conjugated natural bile acids. *J. Lipid Res.* 1992. 33: 635-646.

Supplementary key words glycine • taurine • phase equilibria • micelles

Bile acids are amphipathic steroids that are the end products of cholesterol metabolism. After their biosynthesis from cholesterol in the liver, bile acids are conjugated with glycine or taurine, and secreted into bile. After facilitating the transport of cholesterol in bile and the transport of dietary lipids in small intestinal content, bile acids are actively absorbed from the terminal ileum, returned to the liver, and resecreted in bile, this pathway

being termed the enterohepatic circulation (1, 2). The small fraction of bile acids not absorbed from the terminal ileum passes into the large intestine where the molecules undergo bacterial deconjugation and 7-dehydroxylation to form secondary bile acids, as distinguished from primary bile acids that are biosynthesized in the liver from cholesterol. In humans, fecal bile acids are predominantly secondary bile acids and occur almost exclusively in unconjugated form (3).

Conjugation of a bile acid with glycine or taurine decreases its pK_a value (4) and thus greatly increases its aqueous solubility at the pH present in the small intestine (5); [see also the review in the beginning of this issue (6)]. The enhanced ionization at intestinal pH also reduces passive intestinal absorption from the small intestine, thereby promoting a high intraluminal concentration that is essential for efficient solubilization of lipolytic products (5). Dehydroxylation and deconjugation of bile acids, as occurs in the large intestine, has long been considered to promote bile acid precipitation, since unconjugated secondary di- and mono-hydroxy bile acids are poorly soluble at physiological pH (7).

In addition to pH, a second factor that might limit bile acid solubility is calcium ion activity (Ca^{2+}), since the solubility products of the calcium salts of many anionic detergents are quite low. We have previously reported the

Abbreviations: CMC, critical micellization concentration; CMT, critical micellization temperature; HPLC, high performance liquid chromatography; GLC, gas-liquid chromatography; TLC, thin-layer chromatography; UDC, ursodeoxycholic acid; CDC, chenodeoxycholic acid; DC, deoxycholic acid; C, cholic acid; HC, hyocholic acid. See also Table 1.

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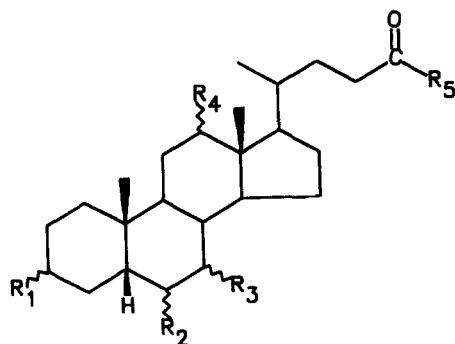
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approximate solubility products of the calcium salts of the common glycine conjugated bile acids and described a general method for characterizing the effect of Ca^{2+} on the behavior of dilute bile salt solutions (8). The findings reported in that paper provided an explanation for the absence of precipitation of calcium salts of glycine-conjugated bile acids in bile and digestive intestinal content despite high Ca^{2+} and high bile salt concentrations in the face of solubility products as low as about 10^{-9} M^3 .

In this paper, we report measurements of the solubility of the calcium salts of ten unconjugated bile acids, as well as of three of the taurine conjugates of these bile acids.

The latter were performed in order to complement our previous studies on glycine-conjugated bile acids (8) with the hope of developing a general understanding of the relationship between bile acid structure and the solubility of calcium bile salts. In addition, such studies were considered of potential biomedical significance, since formation of insoluble calcium bile salts in the large intestine has been proposed as a mechanism that decreases the intrinsic cytotoxicity of the major secondary bile acids (9, 10). As in the previous study, the effect of Ca^{2+} and Na^+ ion concentration on the phases present in dilute bile salt systems was also recorded.

TABLE 1. Chemical structure of bile acids used to measure solubility of their calcium salts



Trivial Name	Abbrev.	R ₁ (C ₃)	R ₂ (C ₆)	R ₃ (C ₇)	R ₄ (C ₁₂)	R ₅ (C ₂₄)	CMC of Na ⁺ Salt ^a		Source
							Water	Na ⁺ 0.15 M	
Unconjugated bile acids									
Chenodeoxycholic	CDC	αOH		αOH		O^-	9	4	<i>b</i>
Ursodeoxycholic	UDC	αOH		βOH		O^-	19	7	<i>b</i>
Deoxycholic	DC	αOH			αOH	O^-	10	3	<i>c</i>
Lagodeoxycholic ^c	LDC	αOH			βOH	O^-	18	10	<i>b</i>
Hyodeoxycholic	HDC	αOH	αOH			O^-	14	6	<i>d</i>
Murideoxycholic ^c	MDC	αOH	βOH			O^-	<i>i</i>	<i>i</i>	<i>b,f</i>
Cholic	C	αOH		αOH	αOH	O^-	13	11	<i>g</i>
Hyocholeic	HC	αOH	αOH	αOH		O^-	17	8	<i>d</i>
Ursocholeic ^c	UC	αOH		βOH	αOH	O^-	60	39	<i>h</i>
Lithocholic	LC	αOH				O^-	<i>i</i>	<i>i</i>	<i>d</i>
Conjugated bile acids									
Chenodeoxycholyltaurine	CDC-T	αOH		αOH		$\text{NH}(\text{CH}_2)_2\text{SO}_3^-$	7	3	<i>j</i>
Deoxycholyltaurine	DC-T	αOH			αOH	$\text{NH}(\text{CH}_2)_2\text{SO}_3^-$	6	2.4	<i>j</i>
Cholyltaurine	C-T	αOH		αOH	αOH	$\text{NH}(\text{CH}_2)_2\text{SO}_3^-$	10	6	<i>j</i>
Chenodeoxycholylglycine	CDC-G	αOH		αOH		$\text{NHCH}_2\text{CO}_2^-$	6	1.8	<i>j</i>
Ursodeoxycholylglycine	UDC-G	αOH		βOH		$\text{NHCH}_2\text{CO}_2^-$	12	4	<i>j</i>
Deoxycholylglycine	DC-G	αOH			αOH	$\text{NHCH}_2\text{CO}_2^-$	6	2.0	<i>j</i>
Cholylglycine	C-G	αOH		αOH	αOH	$\text{NHCH}_2\text{CO}_2^-$	12	10	<i>j</i>

^aCMC values are taken from Roda, Hofmann, and Mysels (21).

^bDiamalt AG, Raubling, Germany.

^cSigma Chemical Co., St. Louis, MO.

^dCanada Packers, Limited, Toronto, Canada.

^eThe trivial names, ursocholeic and murideoxycholic, have not been approved by IUPAC, but are in common usage by workers in the field. The trivial name lagodeoxycholic acid has recently been proposed for $3\alpha,12\beta$ -dihydroxy- 5β -cholan-24-oic acid because (as its glycine conjugate) it is present in rabbit bile (34). Another name for this bile acid is 12-epideoxycholic acid.

^fGift from E. H. Mosbach, Beth Israel Medical Center, New York, NY.

^gAldrich Chemical Co., Milwaukee, WI.

^hGipharmex S.p.A., Milan, Italy.

ⁱThese bile salts do not have a CMC that is easily measured because their Kraft point (critical micellization temperature) is $>60^\circ\text{C}$. Other CMC values were taken from Roda et al. (21).

^jSynthesized in this laboratory by the method of Tserng et al. (11).

MATERIALS AND METHODS

Unconjugated bile acids were purchased or received as gifts from colleagues. The chemical structure, trivial name, abbreviation, critical micellization concentration (CMC), and source are summarized in **Table 1**. Bile acids were purified by chromatography and crystallization until purity by HPLC, TLC, and GLC was estimated to be >98%. Glycine and taurine conjugates were prepared using a minor modification of the EEDQ-catalyzed synthesis described by Tserng, Hachey, and Klein (11), starting with a pure unconjugated bile acid; the final product was isolated by freeze drying or crystallization. Samples were chromatographed when necessary using silicic acid columns and stepwise elution with increasing concentrations of methanol in chloroform. All samples of conjugated bile acids were at least 98% pure by TLC (12) and HPLC (13).

NaCl, CaCl₂, and arginine were reagent grade chemicals. Orange OT (1-O-tolylazo-2-naphthol) was synthesized in this laboratory as described by Abu-Hamdiyyah and Mysels (14). The material was purified by column chromatography.

Preparation of samples

The procedures used were similar to those described previously (8). Individual mixtures were prepared in 20-ml borosilicate glass vials (sold for liquid scintillation counting) that had plastic screw-top closures lined with aluminum foil. To prepare samples, appropriate aliquots of concentrated solutions of calcium chloride, sodium bile salt, sodium chloride, and arginine were added, followed by the appropriate amount of deionized water. The resulting overall sample concentration is referred to as "initial concentration." Vials were capped, shaken, and kept at room temperature.

The systems were designed to form a matrix (as shown, for example, in Fig. 1) with initial bile salt concentration being the x axis, the overall sodium ion concentration (sodium ion comes from both the sodium bile salt and the sodium chloride) being the y axis, and the initial calcium ion concentration being the z axis.

Bile salt concentrations were 0, 1, 2, 3, 5, 10, and 20 mM, since these concentrations were judged likely to include those present in the intestinal lumen. Initial calcium concentrations were usually 2.5, 5.0, or 10.0 mM. These concentrations were selected so that precipitation was absent or minimal at the lowest concentration and considerable at the highest concentration. With bile salts whose solubility product was very high, much higher Ca²⁺ concentrations were used. Arginine was selected as a zwitterionic buffer with a pK_a of 9.0; the pH of the solutions was adjusted with NaOH to 8.5, sufficiently alkaline that the unconjugated bile acids would be fully ionized, but not so alkaline that CaCO₃ precipitation occurred. [The

unconjugated bile acids have a pK_a in monomeric solution of about 5 (4, 15); the effective pK_a increases about 0.6–1.0 pK_a unit when the concentration is sufficiently great that self-association to form micelles occurs (16, 17).]

Analyses of supernatants

For dihydroxy and trihydroxy bile salts, total bile salt concentration was determined by an endpoint enzymatic method using 3-hydroxysteroid dehydrogenase (18). The enzyme reacted equally with all of the bile salts used. For lithocholic acid, a monohydroxy bile acid, the aqueous concentration was too low to measure by this method; accordingly, an ultrasensitive enzymatic procedure based on bioluminescence was used (19).

Calcium activity measurements were made using a micro calcium ion-selective electrode (PVC membrane containing a neutral ionophore) and a Ag/AgCl/KCl reference electrode; both electrodes were purchased from Microelectrodes, Inc. (Nashua, NH). Standard solutions were prepared with Ca²⁺ concentration ranging from 0.1 to 100 mM; the activity coefficient *f* was calculated for each of these solutions using the equation:

$$-\ln f = \frac{0.512 \times \text{Ca}^{2+} \times \sqrt{I}}{1 + 0.328 \times 6 \times \sqrt{I}}$$

where $I = 1/2 \sum m_i z_i^2$, m_i is the molar concentration; z_i is the charge of the *i* ion species. Then f_+ was calculated using the equation $f = f_+ \times f_-^2$ with the assumption that $f_+ = f_-^4$ (20). Using these values for f_+ and the millivolt reading for each standard, a fresh standard curve of $a_{\text{Ca}^{2+}} = [\text{Ca}^{2+}] \cdot f_+$ versus millivolts was made for each series of measurements. Standard curves were prepared for each set of sodium ion concentrations.

Total calcium concentrations were analyzed using a fluorescent chelating dye titration procedure (Calcette ultra-micro calcium analyzer, Precision Systems, Inc., Natick, MA).

Phase behavior determination

Samples were observed at 1 day and at 30 days; there was little change in behavior during this time, indicating that metastability, which had been a striking feature of glycine-conjugated bile salts (7), did not occur with unconjugated bile acids. Phases present were assessed using a magnifying glass and polarizing filters. For separation of the aqueous phase, samples were filtered through a 220-nm pore size fluorocarbon polymer membrane filter (Millex, G. V. Filter Units, Millipore Corp., Bedford, MA). All filtrates that were analyzed were clear.

Precipitates were separated by centrifugation and examined by light microscopy using a cell culture microscope and photographed at 400 × magnification.

The presence (or absence) of micelles was determined qualitatively by adding Orange OT crystals and noting

visually whether the supernatant was orange in color. Orange OT is an uncharged aromatic dye that is virtually insoluble in water, but dissolves readily in bile salt micelles. It has been used extensively to measure the CMC of detergents and has been fully validated as an ideal micellar solubilize (14, 21–23).

Composition of precipitates

For direct measurement, precipitates of the calcium salts of deoxycholic and lagodeoxycholic (12-epideoxycholic) acid were isolated by centrifugation, washed with distilled water, and dried. They were then dissolved in ethanol-water 1:1 (v/v). One aliquot was used for Ca^{2+} determination by a fluorescent chelating dye procedure. A second aliquot was used for bile acid determination by the end point enzymatic procedure.

Initially it was assumed that the precipitates were $\text{Ca}(\text{BA})_2$, i.e., that the BA/Ca ratio, $n = 2$. Attempts to check this assumption by direct analysis yielded values varying between 2.3 and 2.8 for deoxycholic (DC) and lagodeoxycholic (LDC), suggesting that the composition may have been altered during the separation from the mother liquor.

An attempt was then made to obtain this information from the analysis of the equilibrated solutions. Two approaches were used. Both assumed that other compositions, and therefore other BA/Ca ratios, were possible and evaluated how well these could account for the experimental data. As wide variations in Na^+ and Cl^- concentrations had little effect, the incorporation of these ions in the precipitates was considered unlikely and only acid and basic salts were taken into account. The possible compositions and the corresponding ratios (n) are as follows: CaOHBA , 1; $\text{Ca}_2\text{OH}(\text{BA})_3$, 1.5; $\text{Ca}_3\text{OH}(\text{BA})_5$, 1.66; $\text{Ca}(\text{BA})_2$, 2; $\text{Ca}_3\text{H}(\text{BA})_7$, 2.33; $\text{Ca}_2\text{H}(\text{BA})_5$, 2.5; and $\text{CaH}(\text{BA})_3$, 3. Thus n was varied from 1 to 3. In both approaches, only clear nonmicellar and gel-free supernatants of systems equilibrated for 30 days were used for analysis.

In the first approach, the composition of the precipitate was calculated from the difference between the initial composition of the solutions and that of the equilibrium supernatant. From the experimental Ca value, that of BA corresponding to a given n was calculated and compared with the experimental one. Then the F value for that n was computed (24). The F value is the ratio of variances of two distributions, that is, $F = [(x_1^2)\nu_2/(x_2^2)\nu_1]$. Whether two distributions differ significantly at a given confidence level is given by tables that take into account their degrees of freedom (25). The best-fitting n was thus obtained by inspection and those that differed significantly from it at the 99% confidence level were deduced from the F values.

In the second approach, the analysis of the supernatant was used directly to calculate the best-fitting ion product. Its F value was calculated for each n ; and the fits were compared, again using the 99% confidence level.

Graphics

Phase maps were drawn using an isometric representation of the cubic matrix with the two horizontal coordinates being the initial bile salt and sodium concentrations; as only two or three calcium concentrations were used, these were represented as horizontal planes parallel to the base of the cubic space (8). As in the previous study (8), when two adjoining samples had two different phases, it was assumed that the phase boundary was halfway between the two samples. Since the upper bile acid concentrations were widely separated (i.e., 5, 10, or 20 mM), the concentration limit of micellar phase, e.g., 7.5 or 15 mM, may differ considerably from the CMC values obtained by standard methods that pinpoint the CMC by extrapolation or titration.

Ionic product and approximate solubility product

Ionic products were calculated as described (7).

$$a_{\text{Ca}^{2+}}[\text{BA}^-]^2 = a_{\text{Ca}^{2+}} \cdot (a_{\text{BA}^-})^2 / f_{\text{BA}^-^2}$$

where f denotes the activity coefficient of the bile acid anion (BA^-). As noted there, the activity coefficient of the bile anion is not far from unity, especially in those samples with low bile acid concentration and low Na^+ concentration; the activity of bile acid anions can be reduced only slightly by monomeric binding of calcium (25), and the activity coefficient should always be less than unity in these dilute systems. The "ionic product" is thus an approximate measure of the solubility of the insoluble calcium salt and is slightly larger than the true value of the solubility product.

RESULTS

Common phase behavior of the bile salt systems

In the absence of added Ca^{2+} ion, all systems were clear isotropic solutions, either monomeric or micellar. Increasing salt concentration favored micellization by common ion effect. In the presence of Ca^{2+} ions, some solutions remained clear, showing that the solubility limit of the calcium salt was not exceeded either because of its solubility in water or its solubilization by micelles. Other solutions gelled or contained a precipitate, probably the $\text{Ca}(\text{BA})_2$ salt as discussed below. The gelled solutions all contained precipitate after 30 days, showing that the latter is more stable. On the other hand, analysis of the liquid separated from crystals and from the gel showed little difference, indicating that they may be closely related. In view of the fibrous nature of many of the precipitates, it is likely that the gels were formed by elongated microcrystals similar to those of the precipitate yet slightly less stable because of their higher specific surface area.

The presence of NaCl affected the precipitation of cal-

cium salts mainly by favoring micellization and thus the solubilization of the precipitates, as can be seen in the phase maps.

Specific behavior of the bile salt systems

Specific unconjugated bile salts. The behavior of individual systems is discussed in detail only when it deviated significantly from the common phase behaviors of all systems, as described above. Concentrations at which phase changes occurred and the kinds of aggregates present are shown in the individual phase maps. Data are given in Tables 1 and 3.

Chenodeoxycholic acid (CDC) system. Precipitation occurred at low bile salt concentrations (2–5 mM) at both 2.5 and 5.0 mM initial Ca^{2+} concentrations (Fig. 1). At 10 mM bile salt concentration a micellar phase coexisted with a precipitate which, by light microscopy, consisted of spheroids (Fig. 2, A).

At the highest bile salt concentration (20 mM), gelation occurred; the gel, which solubilized Orange OT, coexisted with a precipitate. The effect of addition of Na^+ depended on both bile acid and Ca^{2+} concentration. At 2.5 mM Ca^{2+} concentration and at low bile acid concentrations (2–5 mM), the addition of Na^+ led to solution of the precipitate. At higher bile salt concentrations, the addition of Na^+ had no effect until the highest Na^+ concentration of 250 mM, which caused disappearance of both gel and precipitate and formation of a micellar solution. This was accompanied by a fall in Ca^{2+} activity and a rise in total bile salt concentration.

The ionic product was calculated to be $10.1 \times 10^{-9} \text{ M}^3$.

Ursodeoxycholic acid (UDC) system. The ursodeoxycholic acid system was similar to the chenodeoxycholic acid system, except that the domain of the isotropic, monomeric phase was considerably larger (Fig. 3), indicating a much higher solubility of the calcium salt. Addition of Na^+ ion resulted in the system changing from a nonmicellar solution coexisting with a precipitate to a clear micellar solution at 2.5 mM Ca^{2+} , and to a coexistent micellar phase and precipitate at 5.0 mM Ca^{2+} concentration. At higher bile salt and Na^+ concentrations, a viscous gel, which solubilized orange OT, was the sole phase present.

The calculated ionic product was $91 \times 10^{-9} \text{ M}^3$ for crystals having the composition of $\text{Ca}(\text{UDC})_2$. However, the ratio of Ca^{2+} to bile acid in crystals was 1.5 instead of the expected ratio of 2. Thus, these crystals are likely to have a composition different in structure than those formed by other bile acids (see below).

Deoxycholic acid (DC) system. The deoxycholic acid system formed a precipitate at low bile salt concentration (between 1 and 2 mM for a Ca^{2+} concentration of 2.5 mM; <1 mM for Ca^{2+} of 5.0 mM). At initial sodium concentrations >10 and <200 mM a micellar phase co-existed (Fig. 4). No gelation occurred. The effect of increasing

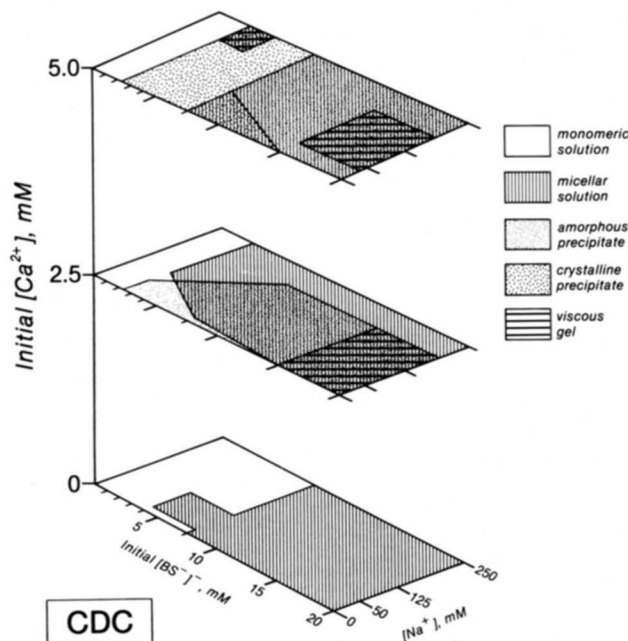


Fig. 1. Phase map for the Na^+ - Ca^{2+} -CDC system 30 days after preparing the samples. The key indicates the type of phases that were formed.

sodium ion concentration was to extend the region of the micellar phase (coexisting with the precipitate) to intermediate bile acid concentrations. By light microscopy, the precipitate appeared as fibers (Fig. 2, B).

The ionic product was calculated to be $1.5 \times 10^{-9} \text{ M}^3$. **12-Epideoxycholic (lagodeoxycholic, LDC) acid system.** At a Ca^{2+} concentration of 5 mM, precipitation was so complete that neither a micellar phase nor gel phase was present (Fig. 5). Na^+ concentration influenced the form of insoluble material: by light microscopy, the precipitate was in the form of batonnets up to 150 mM Na^+ ions; at a Na^+ concentration of 250 mM, the precipitate was in the form of rectangular crystals (Fig. 2, C).

The ionic product was calculated to be $2.2 \times 10^{-9} \text{ M}^3$.

Hyodeoxycholic acid (HOC) system. The hyodeoxycholic acid system formed a precipitate at the lowest bile salt concentration (1 mM) (Fig. 6), but was generally similar to the deoxycholic acid system. In contrast to the deoxycholic acid system, no gelation occurred, and there was no effect of Na^+ concentration. By light microscopy, the precipitate was in the form of well-defined crystals (Fig. 2, D). An approximate ionic product of $0.7 \times 10^{-9} \text{ M}^3$ was obtained.

Murideoxycholic acid system. This dihydroxy bile salt had a Krafft point (CMT) above 100°C , and a stable, isotropic solution of its sodium salt could not be prepared. Thus, an ionic product of the calcium salt was not obtained. Based on the behavior of the lithocholic acid system, it is likely to be several orders of magnitude less than that of the other dihydroxy bile acids.

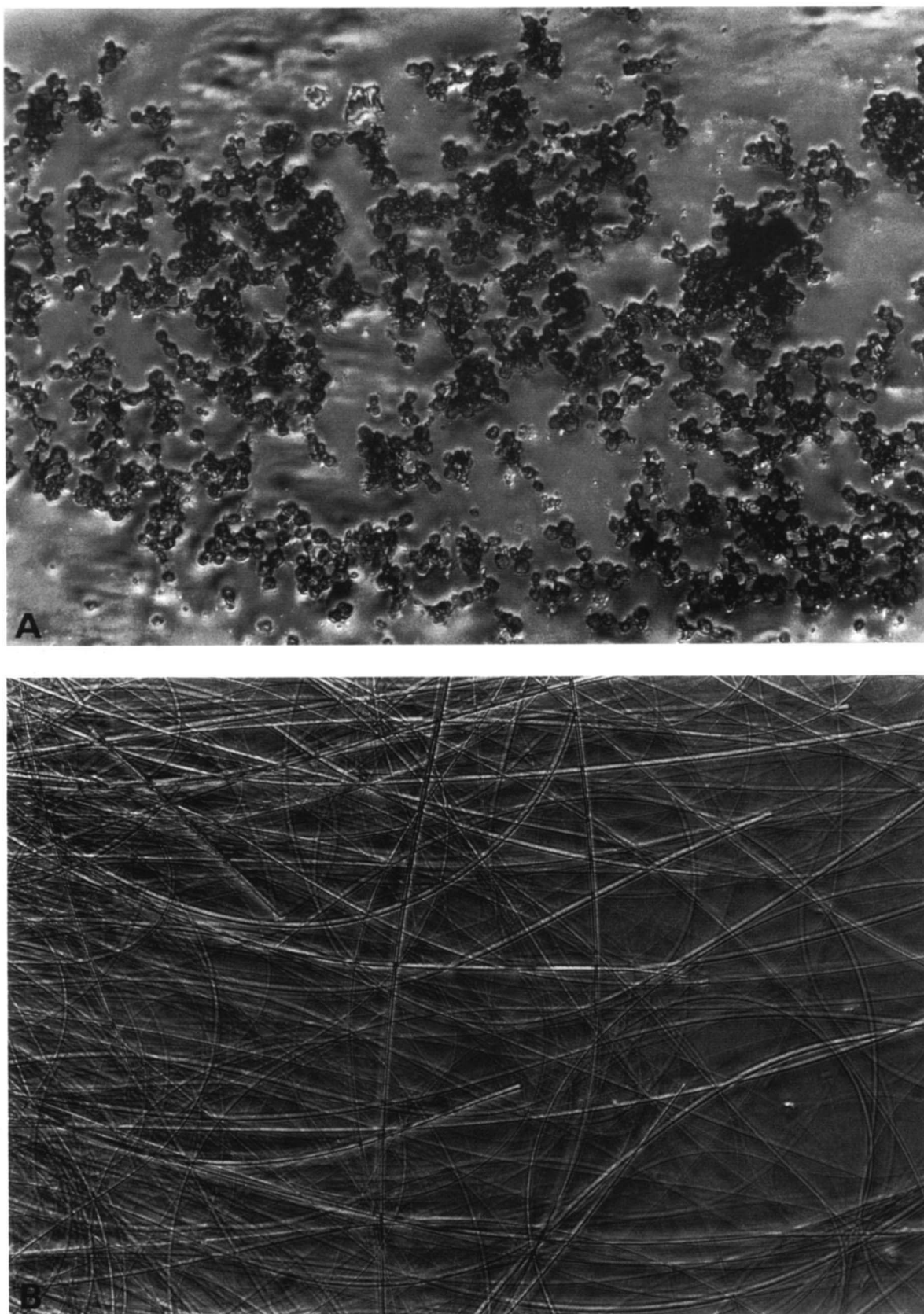
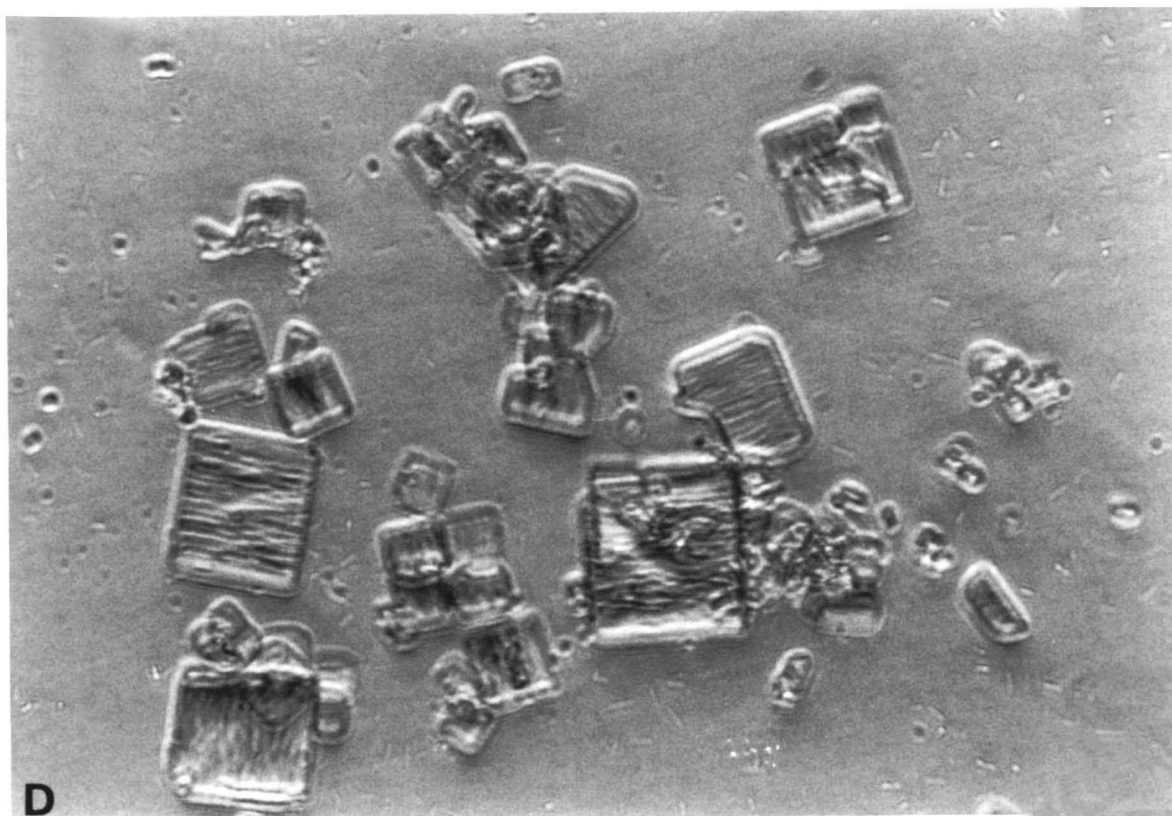
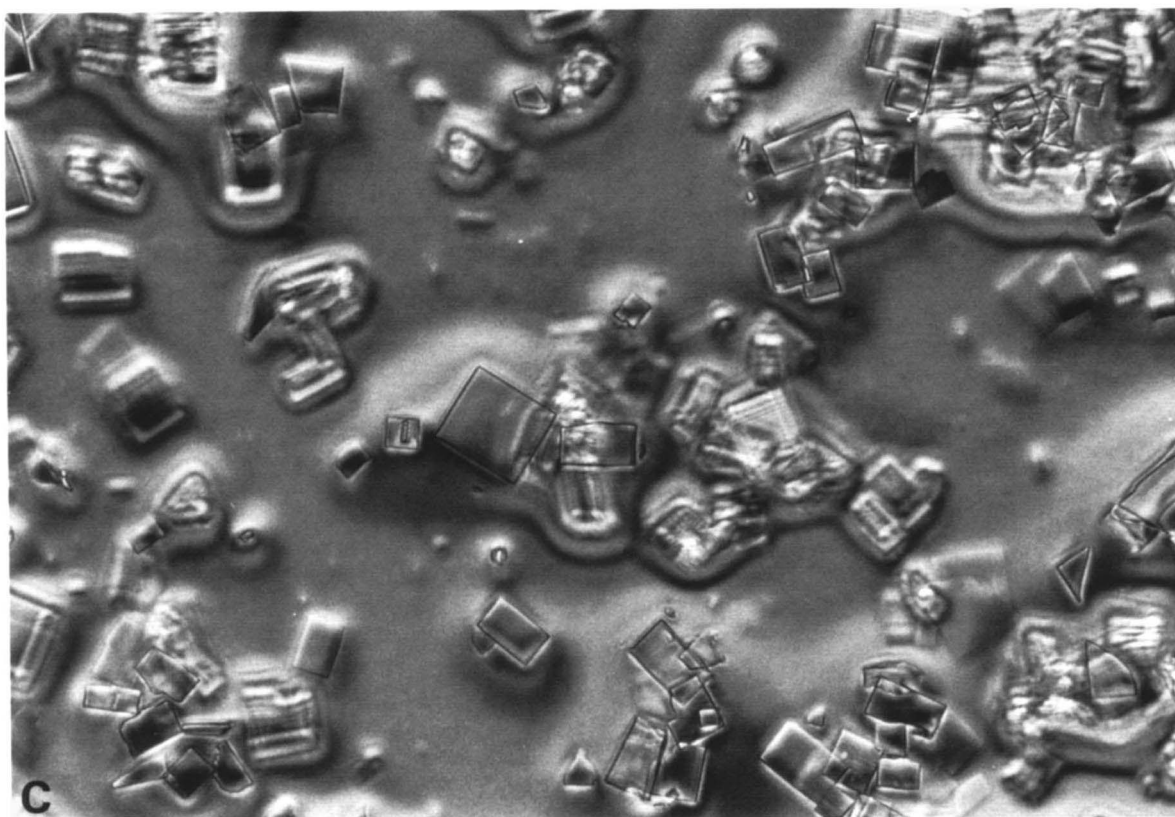


Fig. 2. Light microscopic appearance of the precipitates formed by calcium salts of dihydroxy bile acids. A, calcium chenodeoxycholate [$\text{Ca}(\text{CDC})_2$]; B, calcium deoxycholate [$\text{Ca}(\text{DC})_2$]; C, calcium 12-epideoxycholate (lagodeoxycholate) [$\text{Ca}(\text{LDC})_2$]; D, calcium hyodeoxycholate [$\text{Ca}(\text{HDC})_2$].



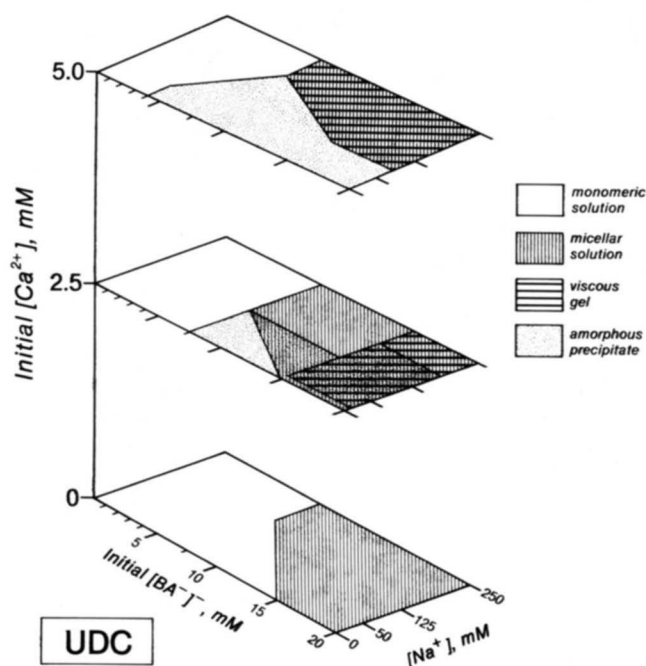


Fig. 3. Phase map for the Na⁺-Ca²⁺-UDC system at 30 days after preparing the samples.

Cholic acid (C) system. Calcium cholate was far more soluble than any of the dihydroxy bile salts. No precipitation occurred at 2.5 or 5.0 initial Ca²⁺ concentration. At 10 mM Ca²⁺, a gel formed with 20 mM bile salt and the three lowest Na⁺ concentrations (20, 45, and 70 mM total Na⁺). With a Ca²⁺ concentration of 20 mM, a gel appeared at 10 mM initial bile salt concentration. An isotropic phase was separated from the gel by ultracentrifugation and used for determination of the ion product.

Because of uncertainty about the validity of this measurement, additional samples were prepared with Ca²⁺ concentrations of 100 mM and 200 mM. With these extremely high Ca²⁺ concentrations a precipitate formed which, by light microscopy, was fiber-like (not shown) and resembled the precipitate formed in the deoxycholic acid systems, which are shown in Fig. 2. At higher bile salt concentrations, the samples gelled.

An isotropic phase was separated by filtration and used for determination of the ion product. The ionic product values obtained from this phase agreed with those separated from the gel at a Ca²⁺ concentration of 10 mM. Both gave a value of approximately $630 \times 10^{-9} \text{ M}^3$.

Ursocholic acid system. The ursocholic acid system was the least prone to precipitate of all the unconjugated bile acids studied. A precipitate could only be obtained by preparing samples with 500 mM Ca²⁺. These formed a precipitate with 30 mM ursocholate.

A very approximate ionic product of ca. $2300 \times 10^{-9} \text{ M}^3$ was obtained.

Hyocholic acid (HC) system. The hyocholic acid system was similar to the hydoxycholic acid system (Fig. 7). It gave precipitates at low bile acid concentrations (2–3 mM) which by light microscopy were spheroids, similar in appearance to those formed by chenodeoxycholic acid and shown in Fig. 2.

An ionic product of $11 \times 10^{-9} \text{ M}^3$ was obtained.

Lithocholic acid system. The calcium salt of lithocholic acid was extremely insoluble, as was anticipated since the Krafft point (critical micellization temperature) of its sodium salt is known to be in the range of 60–80°C (5, 24). The aqueous phase was obtained by centrifugation and its bile salt concentration was determined by an enzymatic bioluminescence procedure (19); it ranged from 3 to 5 μM.

The ionic product was calculated to be $4.8 \times 10^{-14} \text{ M}^3$ ($0.00005 \times 10^{-9} \text{ M}^3$).

Conjugated bile salts. Chenodeoxycholytaurine. No precipitation occurred until Ca²⁺ was added to a concentration of 400 mM. At this concentration, crystals appeared. The approximate ionic product was $350 \times 10^{-9} \text{ M}^3$.

Deoxycholytaurine. No precipitation occurred until Ca²⁺ ion was added to a concentration of 400 mM when crystals appeared. The approximate ionic product was $120 \times 10^{-9} \text{ M}^3$.

Cholytaurine. A solution containing 0.66 M Ca²⁺ and 0.66 M cholytaurine remained isotropic for 48 h. When additional solid CaCl₂ was added to raise the approximate aqueous concentration of Ca²⁺ to 1.3 M, a white precipitate appeared. The ionic product of calcium cholytaurine

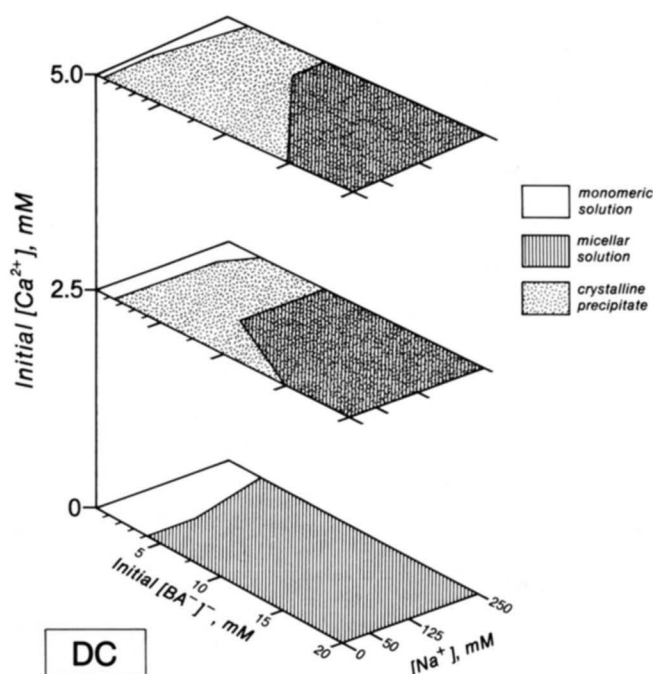


Fig. 4. Phase map for the Na⁺-Ca²⁺-DC system at 30 days after preparing the samples.

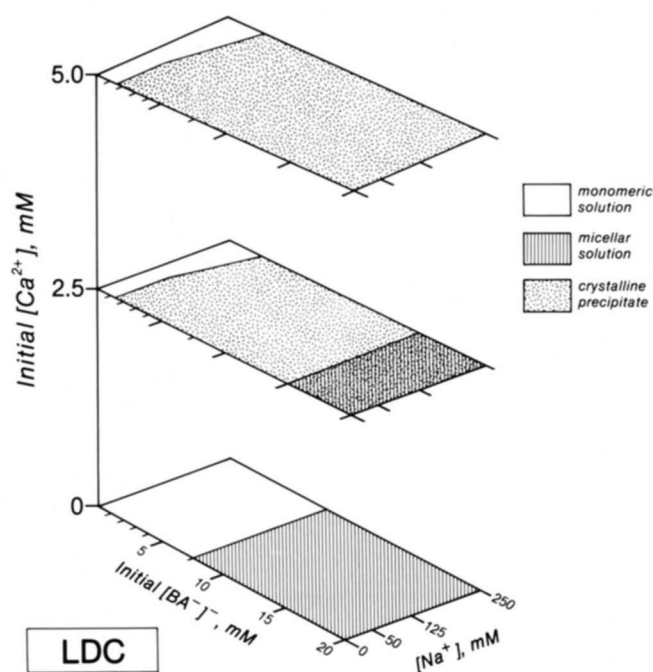


Fig. 5. Phase map for the Na^+ - Ca^{2+} -LDC system at 30 days.

was at least $10,000 \times 10^{-9}$ (M^3). The calcium salt of cholytaurine is thus freely water-soluble.

Thus, under physiological conditions of ionized Ca^{2+} concentration (1–2 mM), formation of insoluble Ca^{2+} salts by any of these taurine conjugated bile acids will not occur (see Discussion).

Cholyglycine was not restudied because of its extraordinary metastability, supersaturated solutions remaining without precipitate for more than a year. Reconsideration of the data reported in the previous paper led to an estimated ionic product of about $400\text{--}600 \times 10^{-9}$.

Composition of precipitates

The calcium salts of deoxycholic and 12-epideoxycholic (lagodeoxycholic) acid were isolated and analyzed as described above. By analysis, the bile acid to calcium ratio for both these compounds ranged from 2.3 to 2.8.

The two approaches used to obtain the ratios for these and the remaining dihydroxy bile acids are described in detail in Methods.

The two methods did not agree well except in one case, that of UDC where $n = 1.5$ gave the best fit by both methods. (This corresponds to a crystal structure of $\text{Ca}_2\text{OH}(\text{BA})_3$ or $\text{Ca}_2\text{Cl}(\text{BA})_3$.) At the other extreme was HDC for which all values of n were rejected by one or the other method. However, in none of the six systems analyzed by both approaches was the normal salt, $n = 2$, rejected by both methods, and for CDC, UDC, DC, and HC it was not rejected by either. For CDC, DC, C, and

HC, $n = 2$ gave the best fit by one of the approaches. In no case was the most probable composition outside of the range from $n = 1.5$ to $n = 2.5$.

In all of the above the tacit assumption was made that the system was at equilibrium, and that the precipitate had a definite composition and was not a mixture of two or more compounds. This seemed to hold for UDC but not necessarily for the other systems.

Thus, our attempts to determine the composition of the precipitate were not successful with discrepancies often much larger than simple analytical error. Nevertheless, the results suggest that the simple di-salt $\text{Ca}(\text{BA})_2$ was the main precipitate for most bile acids, and this was assumed to be the case. This is likely to be close to the real situation and has little effect on the conclusions because of the limited range of conditions over which the results are to be applied. The uncertainty in the ion product values, as shown by the coefficient of variation values (Table 2), indicates the problems involved for the individual bile acids.

DISCUSSION

Influence of pattern of hydroxylic substituents on ionic products

Results, as summarized in Table 2, indicate that, for unconjugated bile acids, the number, location, and configuration of hydroxyl groups are major determinants of the solubility of the calcium salt. The solubility products

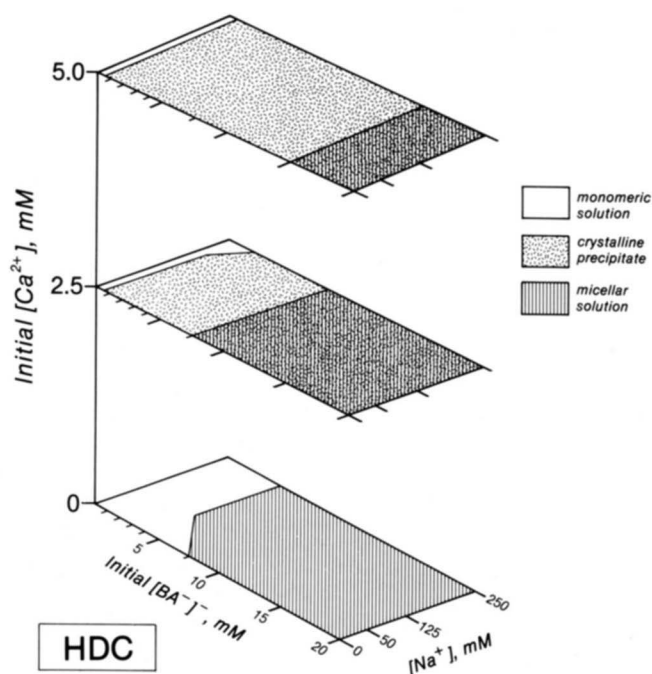


Fig. 6. Phase map for the Na^+ - Ca^{2+} -HDC system at 30 days.

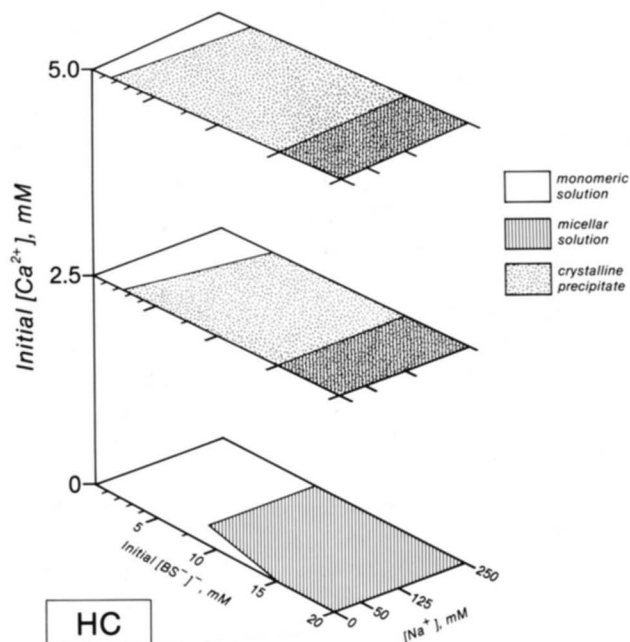


Fig. 7. Phase map for the Na^+ - Ca^{2+} -HC system at 30 days.

of the calcium salt of ten common bile acids ranged over nine orders of magnitude; and the solubilities of the calcium salts thus ranged over three orders of magnitude. The ionic product of the calcium salt of lithocholic acid, the single monohydroxy bile acid studied, was least, being five orders of magnitude lower than that of the dihydroxy bile acids. The sodium salt of lithocholic acid is known to have an extremely low solubility at room temperature, since the critical micellization temperature of this bile acid is $>60^\circ\text{C}$ (26). With the exception of ursodeoxycholic acid, calcium salts of dihydroxy bile acids had lower ionic products than those of trihydroxy bile acids.

For a given number of hydroxyl groups, the configuration of the hydroxy group strongly influenced the ionic product. The change from a 7α hydroxy group (as in chenodeoxycholic acid) to a 7β hydroxy group (as in ursodeoxycholic acid) was associated with a 9-fold increase in the ionic product of the calcium salt. Similarly, the change from a 7α hydroxy group (as in cholic acid) to a 7β hydroxy group (as in ursocholic acid) was associated with a 30-fold increase in the ionic product of the calcium salt. In contrast, the changing of a 12α hydroxy group (as in deoxycholic acid) to a 12β hydroxy group (as in 12-epideoxycholic acid) was associated with little increase in the ionic product of the calcium salt. The calcium salt of hyodeoxycholic acid, with hydroxy groups at 3 and 6, had an ionic product 180 times lower than that of ursodeoxycholic acid with hydroxy groups at 3 and 7. A remarkable finding was the extremely high Krafft (CMT) point of sodium murideoxycholate ($>100^\circ\text{C}$). By analogy with the

sodium and calcium salts of lithocholic acid, the ionic product of its calcium salt should be extremely low.

The hydration energy of the hydroxyl groups must be a significant contribution to the solid-solution equilibrium and accounts for the general trend of increasing solubility as one goes from zero to one to two and to three hydroxyls in the bile acid molecule. The variations on this theme produced by the different orientations and positions of the hydroxyls are presumably due to differences in crystal structure and energy.

Influence of bile acid structure on phase behavior

Direct and indirect estimates of the composition of the precipitates indicated that the ratio of bile salts to Ca^{2+} had the expected stoichiometric ratio of about two for all bile acids except ursodeoxycholic acid for which the ratio was less than two. For ursodeoxycholic acid, chloride may have been incorporated into the crystal lattice as has been reported for cholate (27). While this work was in progress, Lichtenberg et al. (28) reported that when calcium deoxycholate was precipitated from micellar solutions, the ratio of bile salts to Ca^{2+} was 3 to 1. However, crystal structure was not analyzed. At any rate, the phase maps define the bile acid concentration at which precipitation occurs when Ca^{2+} and Na^+ are varied. Clearly, further study of the nature of the solid phase formed in these systems is warranted.

Only the 3,7 and 3,7,12 substituted bile acids formed gels when their solutions were supersaturated with Ca^{2+} . The glycine conjugates of these bile acids also form gels from solutions supersaturated with Ca^{2+} , which have extraordinary metastability lasting longer than 2 years (8). This finding suggests that calcium interacts with the 7 position to form metastable intermolecular bridges, possibly as extended helices of an arrangement differing from

TABLE 2. Ion products (approximate solubility products) for calcium bile salts of unconjugated bile acids^a

	IP (mean) ² ($\text{M}^3 \times 10^{-9}$)	SD	CV, %	MR
Dihydroxy				
CDC	10.1	1.5	15	2
UDC	91	30	25	2
DC	1.5	0.6	37	2
LDC	2.2	1.0	45	2
HDC	0.7	0.2	27	2
Trihydroxy				
C	635	32	5	2
HC	11.2	1.3	12	2
UC	2300	^b	^b	2
Monohydroxy				
LC	4.8×10^{-5}	0.76×10^{-5}	16	2

^aFor structures of bile acids, see Table 1. Abbreviations: IP, ion product; SD, standard deviation; CV, coefficient of variation [$(\text{mean}/\text{SD}) \times 100$]; MR, molar ratio of Ca^{2+} to BA^- in precipitate.

^bIon product was too high to determine confidence intervals.

TABLE 3. Effect of conjugation with glycine or taurine on ionic products of Ca^{2+} salt ($\times 10^{-9}$)

	Unconjugated	Glycine-Conjugated	Taurine-Conjugated
CDC	10	2 ^a	354
DC	1.5	10 ^a	117
C	630	ca. 600 ^a	> 10,000 ^b

^aSupersaturated solutions are metastable, not forming insoluble calcium salts for months to years.

^bThis is an estimate based on the failure to obtain a precipitate of 2 M cholytaurine by an equal volume of 2 M Ca^{2+} Cl_2 .

that present in the crystalline state (cf. 29, 30); such interaction could well retard crystal formation or growth. The molecular arrangement of crystals of the sodium salt of deoxycholytaurine (31), chenodeoxycholic acid ($3\alpha\text{OH}$, $7\alpha\text{OH}$) (32), and ursodeoxycholic acid ($3\alpha\text{OH}$, $7\beta\text{OH}$) (33) (the latter two in the form of the protonated acid) has been shown to be helical.

The present study indicates that the calcium salts of $3\alpha,6\alpha$ (hyodeoxycholic acid), $3\alpha,6\beta$ (murideoxycholic acid), and $3\alpha,6\alpha,7\alpha$ (hyocholic acid) have far lower solubility products than those of $3\alpha,7\alpha$ (chenodeoxycholic acid), $3\alpha,7\beta$ (ursodeoxycholic acid), $3\alpha,7\alpha,12\alpha$ (cholic acid), or $3\alpha,7\beta,12\alpha$ (ursocholic acid); from the standpoint of increasing the solubility products of Ca^{2+} bile salts, 7α , 7β , or 12α hydroxylation is superior to 6α , which in turn is far superior to 6β hydroxylation.

Influence of conjugation on ionic products

In Table 3, the ionic products of the calcium salts of three glycine-conjugated bile acids that were previously measured are listed, as well as estimated values for cholyglycine and cholytaurine. Conjugation with glycine did not have a consistent or large effect on the ionic product of a bile acid; in contrast, conjugation with taurine was associated with a 30- to 50-fold increase in the ionic product.

Despite the lack of consistent effect of conjugation with glycine on the ionic product as noted previously (8), conjugation with glycine greatly increased the time required in vitro for precipitation of a Ca^{2+} salt for the common bile acids. Thus, for Ca^{2+} of 2.5 mM, chenodeoxycholyglycine did not precipitate until after 100 days, whereas the calcium salt of unconjugated chenodeoxycholic acid precipitated within 1 day; for ursodeoxycholyglycine, there was no precipitate of the calcium salt until after 70 days, whereas the calcium salt of ursodeoxycholate precipitated within 1 day. Similarly, deoxycholyglycine was not precipitated by 2.5 mM Ca^{2+} , whereas the calcium salt of deoxycholic acid showed extensive precipitation of its calcium salt within 1 day.

The increase in the ionic product associated with conjugation with taurine as compared to glycine could be the

result of either replacement of the carboxylic acid group by a sulfonic acid group or the presence of an additional methylene group. Experiments using bile acids conjugated with aminomethanesulfonate or beta alanine should answer this question.

We conclude that hepatic conjugation of bile acids with taurine or glycine not only enhances solubility at acidic pH, but also enhances solubility at the concentrations of Ca^{2+} ions present in bile and small intestinal content. For both of these effects, taurine-conjugated bile acids are superior to glycine-conjugated bile acids. In a brief review published in this issue (6), we have discussed the influence of pH as well as calcium ion activity on unconjugated and conjugated bile acid solubilities using the data developed here and in previous studies. We have also considered the biological and pathological factors bearing on the precipitation of bile acids from solution either as the protonated acid or as the insoluble calcium salt in the biliary or intestinal tract. ■

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