review

Bile acid solubility and precipitation in vitro and in vivo: the role of conjugation, pH, and Ca^{2+} ions

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Abstract The principles governing the in vitro solubility of the common natural conjugated and unconjugated bile acids and salts in relation to pH, micelle formation, and Ca^{2+} concentration are considered from a theoretical standpoint and then correlated first with experimental observations on model systems and second with the formation of precipitates containing bile acids in health and disease. In vitro, taurine-conjugated bile acids are soluble at strongly acidic pH; glycine-conjugated bile acids are poorly soluble at moderately acidic pH; and many of the common, natural unconjugated bile acids are insoluble at neutral pH. For both glycine-conjugated and unconjugated bile acids, solubility rises exponentially, with increasing pH, until the concentration of the anion reaches the critical micellization concentration (CMC) when micelle formation occurs and solubility becomes practically unlimited. In vivo, in health, conjugated bile acids are present in micellar form in the biliary and intestinal tract. Unconjugated bile acids formed in the large intestine remain at low monomeric concentrations because of the acidic pH of the proximal colon, binding to bacteria, and absorption across the intestinal mucosa. In diseases in which proximal small intestinal content is abnormally acidic, precipitation of glycine-conjugated bile acids (in protonated form) occurs. Increased bacterial formation of unconjugated bile acids occurs with stasis in the biliary tract and small intestine; in the intestine, unconjugated bile acids precipitate in the protonated form. If the precipitates aggregate, an enterolith may be formed. In vitro, the calcium salts of taurine conjugates are highly water soluble, whereas the calcium salts of glycine conjugates and unconjugated bile acids possess limited aqueous solubility that is strongly influenced by bile acid structure. Precipitation occurs extremely slowly from supersaturated solutions of glycine-conjugated bile acids because of metastability, whereas supersaturated solutions of unconjugated bile acids rapidly form precipitates of the calcium salt. In systems containing Ca^{2+} ions and unconjugated bile acids, pH is important, since it is the key determinant of the anion concentration. For bile acids with relatively soluble calcium salts (or with a low CMC), the concentration of the anion will reach the CMC and micelles will form, thus precluding formation of the insoluble calcium salt. For bile acids, with relatively insoluble calcium salts (or with a high CMC), the effect of increasing pH is to cause the anion to reach the solubility product of the calcium salt before reaching the CMC so that precipitation of the calcium salt occurs instead of micelle formation. In vivo, in health, precipitation of the calcium salts of the common, natural conjugated bile acids does not occur. In experimental animals, concretions composed chiefly of insoluble calcium bile salts (unconjugated or conjugated) can be induced to form in the biliary tract by chronic oral administration of uncommon secondary bile acids (or their precursors) whose calcium salts are extremely insoluble (lithocholate, murideoxycholate, allodeoxycholate). It is concluded that the pattern of bile acid hydroxylation and conjugation (with taurine or glycine) of the common natural primary bile acids has resulted in formation of recycling anionic surfactants that are quite resistant to formation of insoluble calcium salts under the usual physiological conditions where they function as effective solubilizers of biliary and digestive lipids. —**Hofmann, A. F., and K. J. Mysels.** Bile acid solubility and precipitation in vitro and in vivo: the role of conjugation, pH, and Ca^{2+} ions. *J. Lipid Res.* 1992. **33:** 617–626.

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Bile acids are not only the water-soluble end products of cholesterol metabolism, but are also amphipathic molecules with multiple physiological functions. Bile acids are secreted into bile in the form of their N-acyl conjugates with glycine or taurine. In bile, they solubilize cholesterol as mixed micelles, enhancing its elimination; in small intestinal content, bile acids solubilize dietary lipids and their digestion products in mixed micelles, enhancing their absorption (1). After promoting the absorption of lipids from the small intestine, bile acids are actively absorbed from the terminal ileum and resecreted into bile; because of their efficient intestinal conservation, a large pool of bile acids accumulates and undergoes a number of enterohepatic cycles daily (2).

Calcium ions are ubiquitous and present at relatively high concentrations (mM) in extracellular fluids. Calcium ions form insoluble salts with many inorganic and organic anions including bile acid anions. Precipitation of insoluble calcium salts of bile acids or other anions in the biliary tract is of medical interest primarily because of the mech-

Abbreviations: C, cholic; LC, lithocholic; CDC, chenodeoxycholic; DC, deoxycholic; CMC, critical micellization concentration; CMpH, critical micellization pH; CMT, critical micellization temperature.

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anical effects caused by the presence of fine precipitates (sludge) or stones (gallstones). In addition, formation of precipitates of calcium bile salts could remove bile acids from solution, thus lowering their concentration and decreasing their solubilizing activity. Lastly, precipitation of bile acids in the large intestine has been thought to decrease the intrinsic cytotoxicity of bile acids and thus influence the turnover of colonic epithelial cells (3). This could occur because of formation of the insoluble calcium salt or by a less direct mechanism proposed by van der Meer et al. (4), in which bile acid anions adsorb to calcium phosphate particles present in colonic content.

Bile acids, as many weak organic acids whose anions are amphipathic, can exist in several states in biological systems: an insoluble, protonated acid; a simply dissolved protonated acid and/or its anion; a simple micelle; a constituent of mixed micelles or vesicles; or an insoluble calcium salt. A paper published elsewhere in this issue (5) and a previous publication (6) from this laboratory reported the solubility of the calcium salts of a number of unconjugated bile acids and their corresponding N-acyl glycine and taurine conjugates. Those studies also described, by means of phase maps, the behavior of dilute aqueous solutions when bile acid concentration, sodium concentration, and calcium activity were varied simultaneously. Those studies showed that the solubilities of the calcium salts of bile acids varied widely and were influenced by the number, position, and orientation of hydroxylic substituents on the steroid moiety as well as by the side chain structure (unconjugated, glycine-conjugated, or taurine-conjugated).

This paper is a review of the general problem of bile acid solubility and precipitation in vitro in model systems and in vivo in health and disease. The emphasis is mainly on bile acid concentration and type, but also considers the role of pH, micelle formation, and Ca^{2+} concentration, all of which are potentially key determinants of bile acid solubility. The review is based on our own work as well as the work from other laboratories on the thermodynamic and kinetic aspects of the solubility of bile acids and their calcium salts. As will be shown, precipitation of bile acids in the form of the insoluble, protonated acid is mainly controlled by pH, whereas precipitation of the insoluble calcium salt is controlled by the activity of Ca^{2+} ions and by the concentration and structure of the monomeric bile acid anion (BA^-). In turn, the activity of Ca^{2+} ions in the biliary and gastrointestinal tract is controlled by many factors including sodium ion concentration, Donnan equilibrium effects (because of the presence of micelles, vesicles, and proteins), as well as the presence of anions such as phosphate, carbonate, and fatty acid anions that may complex with bile acids in solution or form insoluble calcium salts. Accordingly, Ca^{2+} activity shall be considered an independent variable, and the behavior of various bile acids in its presence will be discussed. The emphasis is largely on events in the biliary and intestinal tract in mammals, with particular emphasis on humans. The chemical structures of the bile acids (and salts) under consideration are shown in Fig. 1 of the accompanying paper (5).

Overview of the problem

In general terms, the solubility in water of the common natural C_{24} unconjugated bile acids and of their corresponding glycine conjugates, that is, of their protonated form, is small (7, 8). For the four major bile acids present in humans, the solubility of the unconjugated bile acids ranges from 0.05 μM (lithocholic, LC) to 28 μM (chenodeoxycholic, CDC and deoxycholic, DC) to 273 μM (cholic, C) (7). Whatever is dissolved is in equilibrium with bile salt anions as well as with hydrogen ions. Hence, as pH increases, the concentrations of these anions increases exponentially if the supply of the bile acid is sufficient. This pattern may be broken by the formation of micelles, which practically removes the limit on solubility, or by precipitation as an insoluble salt such as a calcium salt, which stops the increase. All of the above equilibria are reached rapidly (within a few hours or much less) except for the precipitation in vitro of the calcium salts of the common natural glycine-conjugated bile acids for which equilibrium may not occur for months (6).

In the presence of Ca^{2+} ions with an activity equal to that present in plasma, bile, or small intestinal content (1–3 mM), the equilibrium concentrations of the bile acid anions in a saturated solution vary over a broad range depending on their structure, specifically on the number, position, and orientation of hydroxy or other substituents, as well as the mode of conjugation (with glycine, with taurine, or unconjugated).

Effect of pH on aqueous solubility

In vitro. Taurine-conjugated bile acids are sulfonic acids and therefore extremely strong acids. The common taurine-conjugated bile acids are likely to be freely soluble in water when added in the form of the protonated acid, in contrast to the protonated form of bile acids conjugated with glycine or to the unconjugated bile acids. The aqueous solubility of a number of unconjugated bile acids has been shown by Roda and Fini (7) to range from the submicromolar to the millimolar; solubility increases as the number of hydroxyl groups increases from one to three. (These values are given in **Table 1**.) According to these workers, for a given bile acid, the solubility of the protonated form of the glycine-conjugated bile acids is still lower by a factor of three than that of its corresponding unconjugated derivative (8).

The pK_a of all the common natural C_{24} unconjugated bile acids is close to 5.1, based on measurements reported by Roda and Fini (7). The glycine

TABLE 1. Effect of monomer solubility, Ca^{2+} activity, pH, CMC, and micelle formation on aqueous solubility of common unconjugated natural bile acids

						Activity of Ca^{2+}			
						1.0 mM		0.1 mM	
	Solubility[b] (BAH)	CMC[c]	CMpH[d]	K_{sp} of $Ca(BA^-)_2$ L/M^3 ($\times 10^{-9}$)[e]	Maximum Permissible $a_{Ca^{2+}}$ at CMC[f]	$[BA^-]$ in Saturated Solution[g]	pH_{ppt} for $Ca(BA^-)_2$[h]	$[BA^-]$ in Saturated Solution[g]	pH_{ppt} for $Ca(BA^-)_2$[h]
	μM	mM			mM	mM		mM	
Dihydroxy bile acids									
CDC	27	4	7.22	10	0.625	3.2	7.12	CMC	M
CDC[i]		(2)	6.97	(10)	(2.50)	(CMC)	(M)	(CMC)	M
DC	28	3	7.08	1.4	0.155	1.2	6.68	CMC	M
UDC	9	7	7.94	91	1.86	CMC	M	CMC	M
HDC	15	6	7.65	0.55	0.015	0.74	6.75	2.3	7.25
Trihydroxy bile acids									
C	273	11	6.65	630	5.2	CMC	M	CMC	M
HC	45	8	7.30	12	0.19	3.5	6.94	CMC	M

Abbreviations: CMC, critical micellar concentration; CMpH, critical micellization pH; BAH, protonated bile acid; M, micelles.

[a]For chemical structure, see Table 1 in paper by Gu et al. (5).

[b]Solubility values published by Roda and Fini (7).

[c]CMC values published by Roda et al. (32) (for Na^+ = 0.15 M).

[d]pH value at which concentration of monomer $[BA^-]$ reaches the CMC. Additional $[BA^-]$ will form micelles and the concentration of $[BA^-]$ will remain constant at the CMC.

[e]K_{sp} values from ref. 2. For this table, the K_{sp} has been equated with the ion product.

[f]$[BA^-]$ at which solution is saturated with calcium salt of that bile acid for the indicated Ca^{2+} ionized concentration (activity).

[g]$[BA^-]$ in saturated solution for $a_{Ca^{2+}}$ of 1.0 mM or 0.1 mM. If this concentration is above the CMC, micelles form and the monomeric concentration of BA is the CMC.

[h]pH value at which $[BA^-]$ reaches saturation for the solubility of its calcium salt (for the Ca^{2+} indicated). If $pH_{ppt} > CMpH$, micelles will form, indicated by M. $[Ca^{2+}]$ will decrease because of binding of Ca^{2+} ions to micelles. If $pH_{ppt} < CMpH$, the calcium bile salt will form and micelles will not form, as the pH is increased.

[i]Values in parentheses show the effect of lowering the CMC of CDC to 2 mM, a situation that might occur in the presence of other amphiphilic molecules that formed mixed micelles with the CDC anions. The effect is to lower the CMpH, to increase the maximum permissible $a_{Ca^{2+}}$ at the CMC, and to abolish formation of the calcium bile salt when $a_{Ca^{2+}}$ is 1.0 mM.

conjugates are moderately stronger acids with a pK_a of 3.7 (9). These pK_a values indicate, of course, the pH value at which the concentration in solution of the protonated bile acid molecules is equal to that of the bile acid anion. The anion concentration (and total solubility) rises exponentially with increasing pH.

More quantitatively, the dissociation constant of an unconjugated bile acid is:

$$^{BA}K'_D = ([H^+][BA^-])/[BAH] \qquad Eq.\ 1)$$

where K'_D is the apparent dissociation constant of the carboxylic acid group, $[BA^-]$ is the concentration of the bile acid anion, and [BAH] is the concentration of the protonated acid. As noted, the value of $^{BA}K'_D$ is 9×10^{-6} (M/l).

In the presence of an excess of solid, the protonated acid dissolves until the aqueous concentration of the acid is equal to its aqueous solubility:

$$^{BA}K_{sol}^* = [BAH] \qquad Eq.\ 2)$$

The asterisk indicates the presence of the solid phase. Combining these two equations gives:

$$[BA^-] = (^{BA}K_D\ ^{BA}K_{sol})/H^+ \qquad Eq.\ 3)$$

This can be rearranged to:

$$\log [BA^-] = pH - pK_a - {}^{BA}pS \qquad Eq.\ 4)$$

where ^{BA}pS is the negative logarithm of the aqueous solubility of the protonated acid. Equation 4 shows that as long as excess solid is present, the concentration of bile acid anions of any given unconjugated or glycine conjugated bile acid depends on three factors. The first is pH. The second is the pK_a of the bile acid (3.7 for glycine-conjugated bile acids; 5.1 for unconjugated bile acids). The third is the water solubility of the protonated form.

Experimentally, with a number of common unconjugated bile acids, Roda and Fini (4) observed that

$$^{BA}K^*_{sol} = 9 \text{ to } 45 \times 10^{-6}\ M \qquad Eq.\ 5)$$

substituting in equation 3, one obtains

$$[BA^-] = [9 \times 10^{-6}]\,[(9 \text{ to } 45) \times 10^{-6}\ M] = [(8 \text{ to } 40) \times 10^{-11}]/H^+ \quad \textit{Eq. 6)}$$

The effect of N-acylation with glycine on the anion concentration of a given bile acid is small—about 20%—as a result of the opposing effects of their being stronger acids yet possessing a lower aqueous solubility.

Effect of pH on aqueous solubility

In vivo in health. In order to solubilize biliary and dietary lipids, bile salts must be sufficiently soluble to be present in micellar concentration. The pH of hepatic and gallbladder bile in humans ranges from 6.5 to 8 (10). The pH of small intestinal content distal to the duodenal bulb averages 6 and increases to about pH 8 in the distal ileum (11, 12). The glycine and taurine conjugates of the predominant bile acids present in humans (C, CDC, DC) are fully soluble at these pH values. Accordingly, precipitation of bile salts has been found not to occur to any appreciable extent during digestion in the small intestine in humans, and bile acid anions are present at several times their critical micellization concentration (CMC) (13).

Unconjugated bile acids are not present to any appreciable extent in gallbladder bile (14) or in proximal small intestinal content (15, 16).

In vivo in disease. In certain diseases, gastric acid secretion is too great to be neutralized by the secretion of bicarbonate in pancreatic, biliary, and intestinal secretions. When this occurs, the proximal small intestinal pH falls below pH 6. [This may occur because of excessive gastric acid secretion (17) (caused by the presence of neoplasms that secrete gastrin, the hormone that induces gastric acid secretion) or deficient pancreatic bicarbonate secretion (caused by intrinsic pancreatic disease) (18, 19).] In these pathological conditions, glycine-conjugated bile acids precipitate from solution in the form of the protonated acid (17–19); the result is that bile acids are often present at submicellar concentrations, precluding micellar solubilization of dietary lipids.

Some unconjugated bile acids are formed by bacterial deconjugation in the distal small intestine (2, 15, 16), presumably because of reflux of bacteria from the large intestine across the ileo-cecal valve. The unconjugated bile acids thus formed are also absorbed from the small intestine and are reconjugated in the liver (2). In the large intestine, bile acids undergo deconjugation and 7-dehydroxylation (20). The pH in the cecum (pH 5 to 6) is considerably more acidic than that of the distal ileum (11, 12), at least in part because of organic acid production by the anaerobic bacterial flora (21). Based on measurements in cecal aspirates (22) or in the supernatant of centrifuged feces (22–24), the concentration of bile acid in aqueous solution is low. Presumably, this is not only because of the acidic pH, but also because of binding to bacteria, as well as absorption across the colonic mucosa.

The proportion of bile acids present in unconjugated form in the small intestine may increase greatly in disease conditions in which there is impaired intestinal propulsion. In such conditions, bacteria proliferate in the stagnant segment causing extensive deconjugation of bile acids (25), and sizeable intestinal concretions termed enteroliths may then form (26, 27). These are composed of the protonated acids that are insoluble at the prevailing pH.

In patients with bile acid malabsorption, the concentration of bile acids in the colonic lumen has been shown to increase markedly and to play a role in the secretory diarrhea present in such patients. The aqueous concentration is pH-dependent (22), indicating that the aqueous concentrations of unconjugated bile acids are determined by the presence of precipitated protonated bile acids.

Effect of micellization and pH on aqueous solubility

If the concentration of bile acid anions is large enough, the molecules self-associate to form micelles containing from perhaps 4 to 40 molecules (28, 29). Micelle formation can be regarded as a phase separation, but this approximation is less valid for bile salts than ordinary ionic surfactants because bile salts self-associate more gradually (30). The solubility of this phase, in terms of its anions, is actually a point within a fairly narrow concentration range; that point is defined as CMC (31). Below the CMC, added bile salt molecules dissolve in the form of monomers; above the CMC, added bile salt molecules form micelles leaving the monomeric concentration essentially constant. The value of the CMC depends on the structure of the bile acid anion with the number of hydroxyl groups being the primary factor, although their position and orientation are also important (32). Values for the common, natural bile acids at a Na^+ concentration of 0.15 M are given in Table 1. The CMC is also decreased by the presence of other lipophilic molecules (cf. 33), and is influenced by the concentration and type of cations (34). The micelle has a surface of multiple negative charges and can also bind polyvalent cations; the micelle can act as an anion exchanger (35).

If the protonated bile acid is present in solid excess, the concentration of bile acid anions can be increased by increasing the pH. When the concentration of bile acid anions is raised to the CMC, micelle formation occurs and the solubility of the bile salt increases very rapidly with further increase in pH; nonetheless, the anion concentration remains practically constant at the CMC. Thus, there is a critical micellization pH or CMpH (1).

The CMpH can be calculated using equation 4 by replacing the bile acid concentration, ($[BA^-]$), by the CMC,

replacing the pH by the CMpH, and rearranging terms to give:

$$CMpH = \log [CMC] + pK_a + {}^{BA}pS \qquad Eq.\ 7)$$

This equation indicates that the CMpH is directly correlated with the CMC, but inversely correlated with the aqueous solubility of the protonated bile acid. It is of interest to compare the two common, natural isomeric 3,7-dihydroxy bile acids, ursodeoxycholic acid and chenodeoxycholic acid. Ursodeoxycholic acid has a higher CMC (about 7 mM) than chenodeoxycholic acid (about 4 mM). The aqueous solubility of ursodeoxycholic acid (9 μM) is one-third that of chenodeoxycholic acid (27 μM). These factors act additively to make the CMpH of ursodeoxycholic acid (CMpH = 7.94) considerably higher than that of chenodeoxycholic acid (CMpH = 7.22). Calculated CMpH values for the common, natural bile acids are given in Table 1.

A similar phenomenon occurs as the temperature is raised in the presence of excess solid bile salt. Again, the concentration of the anion increases with increasing temperature until it reaches the CMC; the narrow temperature range over which the solution changes from a suspension of the bile salt to a micellar solution may be called the critical micellization temperature (CMT) (1). Historically the term "Krafft point" has been used because of the description of the phenomenon with sodium salts of long chain fatty acids that was made nearly a century ago by Krafft and Wiglow (36).

In vivo, bile acids are present in bile and small intestinal content in the form of mixed micelles and vesicles. In bile, the mixed micelles and vesicles contain phosphatidylcholine as the other major amphipathic species (37); and in small intestinal content, the mixed micelle contains fatty acids (partly ionized) and monoglycerides as the other major amphipathic species (38–40). Because of the cooperativity of these lipids in micelle formation, the concentration of bile acid anions at which mixed micelle formation occurs is considerably below that of the CMC observed when bile salts are the sole amphipathic solute (28, 32, 33). It is common to refer to the monomeric concentration of bile acids in equilibrium with these mixed micelles and vesicles as the intermicellar concentration (41, 42). In vitro in systems containing bile acids and phosphatidylcholine, simple micelles containing mostly bile acid anions may also co-exist with mixed micelles (43, 44) and vesicles. Presumably monomers, simple micelles, mixed micelles, and vesicles coexist in bile.

The mixed micelles present in bile and small intestinal content can solubilize other lipids, either in the interior of the micelle or on its surface. In bile, the solubilized lipid of greatest importance is cholesterol, since defective solubilization of biliary cholesterol may result in cholesterol gallstone formation (45). In small intestinal content, the solubilized lipids of greatest nutritional importance are fat-soluble vitamins; in the absence of micelles, fat-soluble vitamin deficiency may occur.

Addition of Ca^{2+} ions to the system

Overview. As a general rule, the calcium salt of a given anion is less soluble than its sodium salt. Experimentally, this is true of all bile salts for which measurements have been made. This finding permits us to generalize conclusions for those calcium salts whose solubility has not been measured. The low solubility of calcium bile salts raises the possibility that they might form precipitates in vivo.

In vitro. Conjugated bile acids. Experimentally, as reported elsewhere in this issue (5), the calcium salts of the common taurine-conjugated bile acids were found to be very soluble. That paper and a preceding paper from this laboratory (6) contain extensive data on the behavior of dilute aqueous systems of conjugated and unconjugated bile salts as sodium and calcium ion concentrations were varied simultaneously. Most of the results can be accounted for by the precipitation of calcium salts with the structure of $Ca^{2+}(BA^-)_2$ although for some bile acids the formation of acid or basic salts cannot be excluded. The estimated "ion products" (that is, $a_{Ca^{2+}} \times [BA^-]^2$ where $a_{Ca^{2+}}$ denotes calcium activity) are shown in Table 1.

In the studies with conjugated bile acids, the most remarkable finding was that of aqueous dispersions of the common glycine-conjugated bile acids that were supersaturated with respect to the solubility of the calcium salt; an extremely long period ranging from weeks to months was required to reach equilibrium (6). In contrast, when solutions of unconjugated bile acids were supersaturated in Ca^{2+}, insoluble calcium salts formed rapidly (5).

In vitro. Unconjugated bile acids. For a given unconjugated bile acid, in the presence of Ca^{2+} ions, its solubility increases exponentially with pH. If the anion concentration reaches the CMC before the system becomes saturated with respect to the calcium salt, micelles will form. The micelles bind Ca^{2+} ions and formation of the calcium salt does not occur. At higher pH values, more micelles are formed, so that formation of the insoluble calcium salt does not occur. This situation is shown graphically for the system cholic acid–sodium cholate–Ca^{2+} (ions) in **Fig. 1.**

In contrast, if the anion reaches the concentration at which the solution becomes saturated with respect to the solubility of its calcium salt and this concentration is below the CMC, the calcium salt will form. At higher pH values, the solubility does not increase because added bile acid molecules form additional calcium salt (provided there is an excess of calcium ions). This situation is shown graphically for the system deoxycholic acid–sodium deoxycholate–Ca^{2+} (ions) in **Fig. 2.**

The addition of Na^+ ions can have a striking effect in such systems mainly because the CMC is decreased by the addition of cations, whereas the solubility of the calcium salt is not changed. If the added Na^+ ions lower the

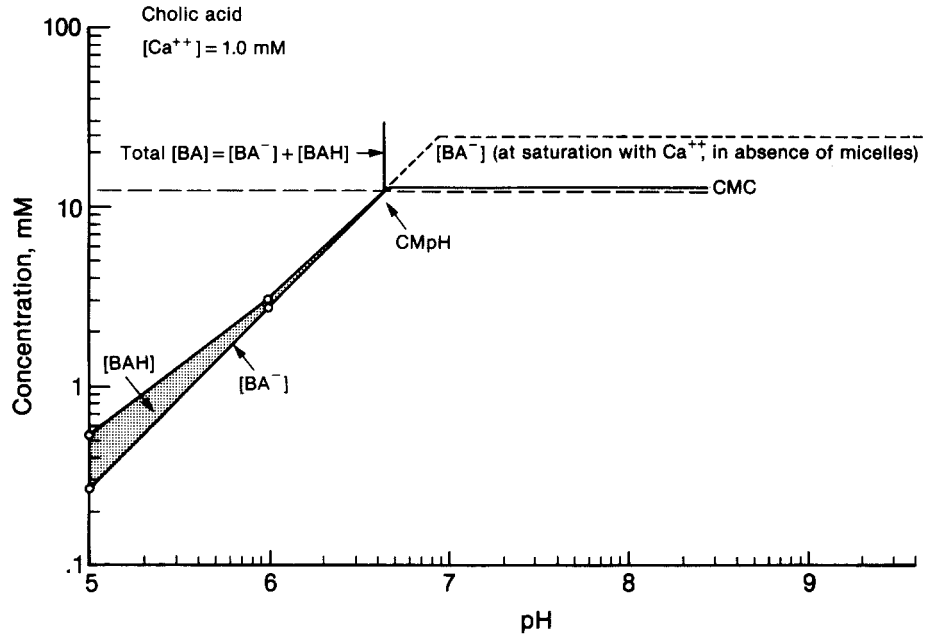

Fig. 1. Solubility of cholic acid (including cholate ions) in relation to pH in a system containing calcium ions at 1.0 mM in concentration (and 0.15 M in Na^+ ions). Both axes are logarithmic. The solubility of the protonated form is indicated by the stippled zone; at higher pH values, its contribution to the total cholate solubility becomes progressively smaller. In contrast, the concentration of cholate anions increases exponentially with pH until it reaches the CMC of the system. The pH at which this occurs is defined as the critical micellization pH. At higher pH values, all added cholate will be present as micelles. The upper horizontal line indicates the cholate concentration at which saturation with its calcium salt occurs. Since this concentration is above the CMC and the CMC defines the approximate monomeric concentration of bile acid anions, calcium salts never form.

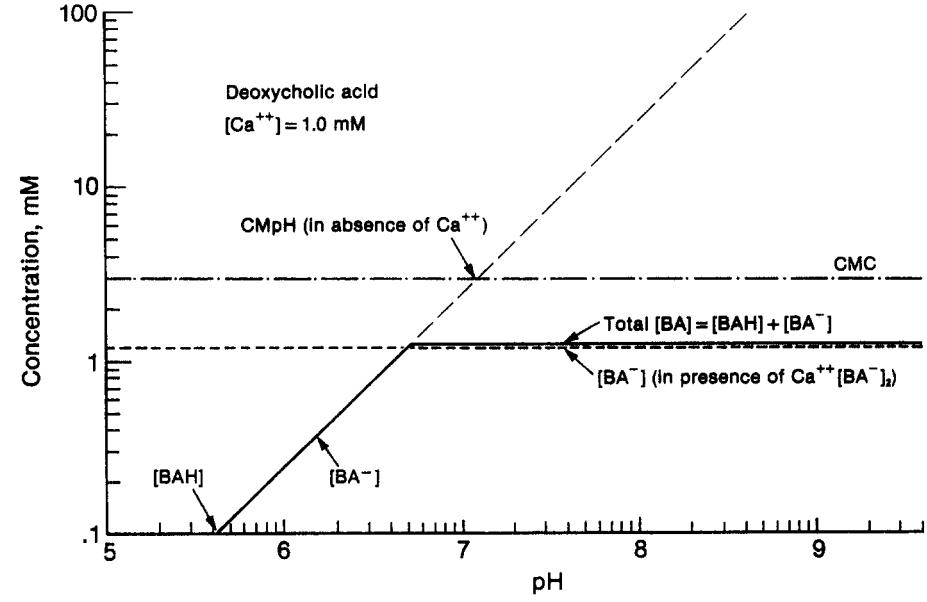

Fig. 2. Solubility of deoxycholic acid (including deoxycholate anions) in relation to pH in a system containing calcium ions at 1.0 mM in concentration (and 0.15 M in sodium ions). Both axes are logarithmic. The solubility of the protonated form is too small to be shown accurately. The concentration of the anion increases exponentially with pH, until it reaches the maximum permitted concentration for a saturated solution of its calcium salt, as defined by its solubility product (lower dashed line). (The amount of protonated species present at this pH is shown in exaggerated form.) Micelles cannot form because the monomeric concentration of anions has never reached the CMC; the pH at which the insoluble calcium salt forms is below the critical micellization pH, which is indicated by the intersection of the extrapolated solubility and the line corresponding to the CMC. The addition of sodium ions will not only lower the CMC but also decrease Ca^{2+} activity. If the monomeric concentration of anions reaches the CMC before saturation with the calcium salt occurs, no precipitation will occur; and the solubility curve will resemble that of cholic acid shown in Fig. 1.

CMC to a concentration below that at which the system is saturated with respect to the calcium salt, micelles will form instead of precipitation of the insoluble calcium salt.

In vivo. In health. calcium salts of the natural common di- and tri-hydroxy conjugated bile acids do not occur. In the large intestine, secondary bile acids in unconjugated form predominate (20). However, as noted above, the aqueous concentration of the anion remains too low for calcium salts to form.

In vivo. In disease. In disease conditions in which there is stasis and bacterial proliferation in the biliary tract or small intestine, unconjugated bile acids are formed rapidly (25). Nonetheless, their proportion has never been reported to exceed that of conjugated bile acids, possibly because they are rapidly absorbed as they are formed. In primary bile duct stones formed because of biliary stasis, unconjugated bile salts constitute $<5\%$ of the duct stone constituents (46). In the small intestine, as noted above, in conditions of stasis, enteroliths may form (26, 27). These are composed of the insoluble protonated acid and not the calcium salt.

Precipitates containing calcium salts of conjugated bile acids can be induced consistently in animal models of gallstone formation. These precipitates involve calcium salts of uncommon bile acids in either conjugated or unconjugated form that are much less soluble than the corresponding salts of the natural di- and tri-hydroxy common bile acids. Since glycine-conjugated bile acids are involved, the supersaturated solutions occurring in vivo cannot have the same kind of remarkable metastability displayed in vitro by slightly supersaturated solutions of the glycine conjugates of the common natural bile acids. Precipitates occur in a matter of weeks in the gallbladders of rabbits and prairie dogs, and in the common bile duct of rats, a species lacking a gallbladder.

Three examples are known and may be briefly summarized.

Cholestanol is the saturated derivative of cholesterol in which the A and B rings are present in the *trans* form (the 5 hydrogen atom is in the α-configuration). When this sterol is administered in the diet to rabbits, it is absorbed and converted in the liver to allocholic acid (the 5α-, A/B *trans* stereoisomer of cholic acid) which in turn is conjugated with glycine. During enterohepatic cycling, the allocholylglycine undergoes bacterial deconjugation and 7-dehydroxylation to form allodeoxycholic acid. Allodeoxycholic acid is absorbed, conjugated with glycine during hepatic transport, and secreted into bile where the calcium salt of allodeoxycholylglycine precipitates. The solubility product of this bile salt has never been determined because of its scarcity. It is likely to be considerably less than that of the calcium salt of deoxycholylglycine (5β, A/B *cis*) that occurs naturally in the rabbit. In addition, solutions that are supersaturated with respect to the calcium salt of allodeoxycholylglycine are not metastable and form an insoluble calcium salt rapidly (47).

The second and third examples involve two 5β bile acids, lithocholic acid and murideoxycholic acid, that are usually present in bile in only trace proportions, but whose proportion in biliary bile acids is increased by addition of these bile acids to the diet. In these experimental models of cholelithiasis, lithocholic acid, a 3α-hydroxy bile acid, and murideoxycholic acid, a 3α,6β-dihydroxy bile acid, form insoluble precipitates rich in their calcium salts.

These two bile acids are the only known natural bile acids whose sodium salts have a CMT well above body temperature. The CMT of sodium lithocholate is $>60°C$, and its calcium salt has a solubility product $<10^{-12}$ M^3 (5). Murideoxycholic acid, its 6β-hydroxylation product, has a sodium salt with a CMT $>100°C$ (5), and thus its calcium salt is likely to be extremely insoluble. Even its taurine conjugate has an extremely low aqueous solubility (48).

In the second example, administration of lithocholic acid to the taurine-deficient rat causes enrichment of the circulating bile acids in lithocholic acid and murideoxycholic acid. With time, gallstones form composed of calcium and sodium salts of mixtures of the two bile acids; the bile acids are present in unconjugated form (49) and under some circumstances also as glycine conjugates in addition (50).

In the third example, administration of murideoxycholic acid to the prairie dog leads to enrichment in biliary bile acids of its taurine conjugate. In time, this conjugated bile acid precipitates as mixtures of its calcium and sodium salts, which appear grossly as gallstones (48).

Conclusions

It is axiomatic that precipitation of a calcium salt will not occur if its solubility product (in terms of activities) is not reached. However, the corollary, that precipitation will occur if the solubility product is exceeded, appears to be untrue for glycine conjugates of the common, natural bile acids, at least in vitro, because of the metastability of their supersaturated solutions. This class of conjugates is the major class of bile acid conjugates present in human bile.

At alkaline pH, the concentration of monomeric bile acid anions is not normally limited by the solubility of the acid but is determined by micelle formation. The reported CMC values such as those listed in Table 1 were obtained at a Na^+ concentration of 0.15 M, but Na^+ concentrations up to twice this value occur commonly in gallbladder bile (10), lowering the CMC still more. From these CMC values and the ion products measured under

similar conditions (5), one can calculate directly the maximum activity of calcium ions at the ion product for a saturated solution, that is, the activity of calcium ions that can exist without danger of precipitation. These values are included in Table 1 for Ca^{2+} activities of 1.0 mM and 0.1 mM. Since the activity coefficient of Ca^{2+} at physiological sodium ion concentrations is about 0.3 (6), the total concentration of calcium ions at saturation will be at least three times higher.

Furthermore, in vivo, as noted above, the presence of other amphiphilic molecules lowers the CMC of the bile acid mixture present in the human biliary and intestinal tract. For bile, the monomer concentration is 1–3 mM because of the influence of phosphatidylcholine on micelle formation (28, 43); for small intestinal content, the monomer concentration is about 3 mM, in the presence of mono-oleyl glycerol, which is probably representative of the mixture of lipid digestion products (33). Experimentally, the presence of phosphatidylcholine decreases the formation of an insoluble calcium salt by the glycine conjugate of CDC (6). Thus, the bile acid monomer concentration is likely to be one-third to one-half of the CMC (in the presence of an amphiphilic additive); and since it is the square of the bile acid concentration that is involved in the solubility product of the calcium salt, the maximum permitted calcium activities given in Table 1 must be a considerable underestimate; the total concentration of calcium at saturation might well be one order of magnitude higher. We have shown in Table 1 the effect of lowering the CMC of CDC by one-half on the maximum permitted Ca^{2+} ion activity. The effect is to increase the CMpH, to increase the maximum permissible $a_{Ca^{2+}}$ at the CMC, and to abolish formation of the calcium bile salt when the activity of Ca^{2+} is 1.0 mM.

When a soluble calcium salt such as calcium acetate is administered orally, the predominant insoluble salt that forms in the intestinal lumen is calcium phosphate (51). The formation of such an insoluble calcium phosphate salt is likely to act as a buffer for the activity of intraluminal calcium. Only a small increase in fecal bile acid output occurs in response to large oral calcium loads (4, 52), and it seems reasonable to propose that this increase in bile acid output is explained by adsorption of bile acids to insoluble calcium phosphate, as has been shown to occur in vitro (53).

Thus, although the calcium salts of unconjugated and glycine-conjugated bile acids are quite insoluble, they do not precipitate under healthy conditions because of the low monomer concentration of bile anions, as well as the low activity of calcium ions. In addition, kinetic factors are sometimes likely to prevent any precipitation. Under pathological conditions, a lowering of pH in the small intestinal lumen or an increased concentration of uncommon bile acids in the biliary tract may lead to precipitates composed of the acid in protonated form or as its calcium salt; such precipitation may contain unconjugated or conjugated bile acids or both.

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