Inhibition of calcium phosphate precipitation by bile salts: a test of the Ca2+-buffering hypothesis

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Abstract The ability of bile salts to inhibit the precipitation of either calcium hydroxyapatite or its precursor, amorphous calcium phosphate, by reducing Ca2+ activity or poisoning nascent crystals was determined. When apatite precipitated rapidly (1-4 h), glycocholate and taurine-conjugated bile salts (up to 100 mM) had little effect on apatite formation, but prevented amorphous calcium phosphate precipitation by lowering Ca2+ activity. In contrast, glycodeoxycholate and glycochenodeoxycholate (2-3 mM) inhibited apatite formation for at least 24 h by poisoning embryonic apatite. When apatite precipitated slowly (> 24 h), all the dihydroxy bile salts prevented apatite formation for at least 4 days. At constant initial supersaturation, the phosphate concentration determined the degree of inhibition caused by the six bile salts mixed together in physiologic proportion. At low phosphate concentrations (1.2 mM) total inhibition was achieved by poisoning embryos (~5 mM total bile salt), but with 4.0 mM phosphate only ~60% inhibition was attained (150 mM bile salt) by a combination of poisoning and Ca2+-buffering.

Thus, at low supersaturation all dihydroxy bile salts can prevent apatite formation by reducing free Ca2+ (taurine and glycine conjugates) or poisoning embryos (glycine conjugates). With mixtures of bile salts at higher supersaturation, inhibition of apatite depends on a combination of poisoning and reduction of free Ca2+, mainly caused by glycodeoxycholate and glycochenodeoxycholate. --Crowther, R. S., and M. Okido. Inhibition of calcium phosphate precipitation by bile salts: a test of the Ca2+-buffering hypothesis. J. Lipid Res. 1994. 35: 279-290.

Supplementary key words calcium hydroxyapatite • gallstones • calcification • biomineralization

Calcium salts of bilirubin, carbonate, phosphate, and fatty acids are common constituents of all types of gallstones (1, 2) and understanding the factors that promote or inhibit precipitation of these salts is necessary for the development of strategies to prevent gallstone formation. Precipitation of ionic salts is governed by well-established physical laws, and it therefore appears a trivial undertaking to try to understand the formation of these salts in bile. However, bile is a complex fluid that undergoes both physical and chemical compositional changes, any one of which may influence the precipitation of Ca2+-sensitive anions. Because bile salts are the major biliary solutes, initial attempts to understand the regulation of Ca2+ salt formation in bile have focused on the role of these anions. Seminal work by Williamson and Percy-Robb (3, 4), subsequently confirmed by others (5-13), showed that bile salts bind Ca2+ ions and lower Ca2+ activity. This observation led Moore, Celic, and Ostrow (6) to propose the "Ca2+-buffering" hypothesis: inhibiting Ca2+-sensitive anion precipitation in bile by reducing Ca2+ activity is a major physiological function of bile salts. However, Ca2+-binding per se will not necessarily cause physiologically significant inhibition of Ca2+ salt formation. Any reduction of Ca2+ activity may alter the kinetics of salt formation (i.e., alter the onset of precipitation), but long-term, thermodynamic prevention of precipitation requires that the bile salt concentration and its Ca2+ binding affinity must together be sufficient to make bile unsaturated with respect to the target Ca2+ salt. Unfortunately, although many groups have studied bile salt-Ca2+ binding, relatively few investigators have examined the consequences of this binding for Ca2+ salt precipitation. Thus the Ca2+-buffering hypothesis has not been adequately tested.

Angelico et al. (14) demonstrated in the bile-fistula rat model that the precipitation of mixed Ca2+ salts of mono- and unconjugated bilirubin and palmitate occurred under conditions of bile salt depletion. Precipitation was promoted when Ca2+ was added to bile salt-depleted bile, but not when added to bile salt-rich bile. Conversely, precipitation was inhibited by the addition of micellar concentrations of bile salts, but it was uncertain whether this effect resulted from Ca2+ buffering or from the solubilization of excess unconjugated bilirubin. In vitro

Abbreviations: ACP, amorphous calcium phosphate; CMC, critical micellar concentration; DSNa, degree of saturation with respect to hydroxyapatite; GC, glycocholate; GCDC, glycochenodeoxycholate; GDC, glycodeoxycholate; HAP, calcium hydroxyapatite; Pi, inorganic phosphate; TC, taurocholate; TCDC, taurochenodeoxycholate; TDC, taurodeoxycholate.

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studies of fatty acid precipitation from micellar bile salt solutions showed that calcium palmitate precipitation was promoted when Ca\(^{2+}\) was bound to the bile salt/palmitate mixed micelles (15). Glycine-conjugated bile salts were shown to inhibit calcium carbonate formation in vitro, but it was concluded that an unknown mechanism, unrelated to the reduction of Ca\(^{2+}\) activity, was responsible (16). Thus, of the few studies performed, none have verified the Ca\(^{2+}\)-buffering hypothesis, and some have suggested that other mechanisms may be involved in inhibiting Ca\(^{2+}\)-sensitive anion precipitation.

Sutor and Percival (17) showed that small volumes of bile inhibited calcium phosphate formation in vitro, and this effect was reproduced by low concentrations of mixed taurocholate/phosphatidylcholine micelles. We recently showed that glycochenodeoxycholate (GCDC) inhibited calcium phosphate precipitation by "poisoning" calcium hydroxyapatite (HAP) embryos, which prevented the transformation of amorphous calcium phosphate (ACP) into HAP (18). Moore et al. (19) calculated that bile was normally undersaturated with respect to CaHPO\(_4\), and they suggested that this mineral phase could precipitate in bile only when excess inorganic phosphate was produced by phospholipid hydrolysis. CaHPO\(_4\) is one of the possible forms of ACP. Although ACP was once believed to be an obligate precursor of HAP, this is now known to be false (20). Because HAP is much more insoluble than CaHPO\(_4\) (21), it may precipitate from solutions that are undersaturated with respect to CaHPO\(_4\) and therefore phospholipid hydrolysis may not be necessary for HAP to precipitate in bile. The major forms of calcium phosphate in gallstones are HAP and whitlockite (1), and the object of the current work was to study the effects of the major human bile salts on the formation of HAP and its sometime precursor ACP, and to deduce the mechanism of inhibition in each case.

**MATERIALS AND METHODS**

CaCl\(_2\), Na\(_2\)HPO\(_4\), and NaCl were obtained from J. T. Baker Chemical Co. (Phillipsburg, NJ). Sodium salts of GCDC, glycocholic (GC), taurocholic (TC), glycodeoxycholic (GDC), taurodeoxycholic (TDC), and taurochenodeoxycholic (TCDC) acids were obtained from Sigma Chemical Co. (St. Louis, MO), Steraloids Inc. (Wilton, NH), and Calbiochem (La Jolla, CA). Except where noted, experiments were performed with bile salts obtained from Sigma. Tetrahydrofuran and 1,6-diphenyl-1,3,5-hexatriene were obtained from Aldrich Chemical Co. (Milwaukee, WI). HAP (type VI) and all other reagents were from Sigma Chemical Co. HAP was analyzed for calcium and phosphate content and the Ca/P ratio was 1.69 ± 0.01, which is consistent with HAP, Ca\(_5\)(PO\(_4\))\(_3\)OH, (1.67).

**Calcium phosphate precipitation**

Stock solutions of NaCl, Na\(_2\)HPO\(_4\), and bile salts, all dissolved either in 50 mM Tris or 10 mM HEPES buffer, pH 7.5, were mixed together in polystyrene microcentrifuge tubes. The NaCl concentration was adjusted in inverse proportion to the bile salt concentration to keep the ionic strength approximately constant. Solutions were not purged of CO\(_2\) because previous experience had shown that precipitation of CaCO\(_3\) did not occur under these experimental conditions (18). To initiate the reaction, CaCl\(_2\) in buffer was added to give a final volume of 1.0 ml and the tubes were vortexed. In different series of experiments the initial CaCl\(_2\) and Na\(_2\)HPO\(_4\) concentrations were adjusted so that HAP would precipitate either directly or through its ACP precursor. For each experiment the initial concentrations of reactants after mixing are given in the figure legends. After mixing, 200 µl of solution was removed and the absorbance at 405 nm was measured (Titertek Multiskan Plus plate reader, Flow Labs, McLean, VA). The samples were returned to their respective tubes, which were capped and incubated at 37°C. During temperature equilibration the pH of the solutions fell to 7.3 when Tris buffer was used and to 7.4 when HEPES buffer was used. These pH values were used when initial saturations of solution with respect to HAP were calculated (see Calculation of solution saturation). At intervals the tubes were vortexed and the absorbance of a 200-µl aliquot of solution was measured as before. After 24 h incubation the solutions were centrifuged at 11,000 g for 5 min and the supernatants were assayed for inorganic phosphate (Pi) and, in some cases, for total calcium and bile salt (see Analytical methods). In some experiments the pellets were washed by resuspending them twice in 1 ml of deionized water, and the washed pellets were placed on glass slides and dried at 100°C. A sample of the dried pellet was transferred to a BaF\(_2\) window, and the infrared spectrum of the precipitate was obtained at a resolution of 2 cm\(^{-1}\) using a UMA300A infrared microscope attached to an FTIR spectrometer (Bio-Rad, Digilab Division, Cambridge, MA).

**Analytical methods**

HAP precipitation was quantitated from the amount of Pi, remaining in solution after centrifugation. Phosphate was measured by the malachite green/phosphomolybdate method (22), which was specifically developed to measure Pi in bile. Preliminary experiments demonstrated that bile salts did not interfere with the assay. Total calcium in supernatants was measured by atomic absorbance spectrophotometry (model 5100, Perkin-Elmer Corp., Norwalk, CT) after dilution of samples with 5% trichloroacetic acid/1% lanthanum chloride. Supernatant bile salt was measured by reverse-phase HPLC on a C\(_18\) column (Nova-Pak Radial-Pak cartridge, Waters, Milford, MA) by the method of Rossi, Converse, and Hofmann (23).
We previously showed that the amounts of ACP formed in these experiments were too small to measure by chemical means (18). Accordingly, the absorbance at 405 nm was used as an indicator of ACP precipitation. To validate this method, we mixed together equal volumes of Na₂HPO₄ and CaCl₂ (each 20 mM in 10 mM HEPES buffer, pH 7.5), producing an instantaneous precipitate of ACP. After 5 min, we serially diluted the precipitate with buffer and measured the absorbance of 200-μl aliquots at 405 nm. The optical density was proportional to the ACP concentration in the range 0-0.15 absorbance units (r² = 0.996), and all the experimental values fell within this range. However, because the absorbance is a function of particle size as well as the total amount of precipitate, this measurement provides only a relative estimate of the amount of ACP precipitated.

**Calculation of solution saturation**

The ionic strength, activities of the relevant ions (taking into account ion-pair formation), and the degree of saturation of solutions with respect to HAP (DSHAP) were calculated by an iterative computer program, I on-Product (24), that uses the equation of Varughese and Moreno (25) to calculate DSHAP:

$$DSHAP = \left( \frac{(Ca^{2+})^6 (PO_4^{2-})^2 (OH^-)}{K_{sp \ HAP}} \right)^{1/9}$$

where $K_{sp}$ is the solubility product and the round brackets denote activities. When DSHAP = 1, the solution is saturated; if DSHAP is greater or less than 1, the solution is supersaturated or undersaturated, respectively.

**Effect of bile salts on free Ca²⁺ concentration**

Free Ca²⁺ concentration was measured using the Ca²⁺-sensitive dye murexide (11, 15, 26). Solutions contained 4 mM CaCl₂, 0-100 mM NaCl, 0-100 mM bile salt and were buffered to pH 7.5 with 50 mM Tris. The NaCl concentration was adjusted in inverse proportion to the bile salt concentration to keep the ionic strength approximately constant. The standards consisted of 0-4 mM CaCl₂ in 100 mM NaCl/50 mM Tris, pH 7.5. For each solution a specific blank was prepared that had the same composition, but was deprived of Ca²⁺. To 1.0 ml of each sample or blank solution, 20 μl of murexide was added to give a final concentration of 50 μM. Solutions were then incubated at 37°C for 30 min. The absorbance of each solution at 470 and 540 nm was measured against the appropriate blank in quartz cuvettes maintained at 37°C (Ultrospec K spectrophotometer, LKB Biochrom, Cambridge, UK). Free Ca²⁺ concentration was proportional to log (absorbance₄₇₀ - absorbance₅₄₀). Apparent formation constants ($K_f$) were calculated for the Ca²⁺-bile salt complexes. Each Ca²⁺ was assumed to be bound by two bile salt molecules and $K_f$ was calculated according to the formula given by Moore et al. (6).

**Bile salt binding to HAP**

A series of solutions containing 50 mM Tris, pH 7.5, 0-30 mM bile salt, and sufficient NaCl to make I = 0.15 were mixed with 20 mg/ml of HAP and incubated at room temperature for 2 h. The suspensions were then centrifuged at 11,000 g for 4 min; the supernatants were assayed for bile salt by the 3α-hydroxysteroid dehydrogenase method (27); and the amount of bound bile salt was calculated by difference. Initial experiments with GCDC demonstrated that 20 min incubation was sufficient to approximate equilibrium, because bile salt binding changed only ~6% between 20 min and 10 days of incubation.

**Determination of bile salt critical micellar concentration**

The apparent critical micellar concentration (CMC) of each bile salt was measured fluorimetrically using the apolar probe diphenylhexatriene (28). A 1-μl aliquot of 10 mM diphenylhexatriene in tetrahydrofuran was added to different concentrations of bile salts dissolved in 2.0 ml of Tris/saline buffer, pH 7.5, I = 0.15. The tubes were incubated in the dark at room temperature for 30 min and the resulting fluorescence was measured at an excitation wavelength of 358 nm and an emission wavelength of 430 nm (LS-5B Luminescence Spectrometer, Perkin-Elmer Corp.). For GCDC, the CMC was also determined after incubation for 30 min at 37°C and the fluorescence was measured in cuvettes also maintained at 37°C.

**RESULTS**

**Inhibition of calcium phosphate precipitation**

When the initial CaCl₂ and Na₂HPO₄ concentrations were both 4.0 mM, the precipitation of calcium phosphate occurred in two stages (Fig. 1A). The initial stage corresponded to ACP precipitation and the second, to HAP formation. We have previously confirmed the identity of these mineral phases by infrared spectroscopy (18). The time at which HAP formation was initiated is referred to as the induction time (18, 29), which for the example shown occurred at 65 min. This sequential precipitation is governed by the Ostwald-Lussac Law of Stages: in a system capable of precipitating multiple phases, the least stable (most soluble) phase will precipitate first (30). At the induction time the solution is no longer supersaturated with respect to ACP, and HAP precipitation commences. The formation of the first few HAP nuclei sequesters free ions and causes the solution to become undersaturated with respect to ACP. In response,
Fig. 1. Mineral phase formation monitored by optical density changes. (A) HAP formed through ACP precursor: CaCl₂ and Na₂HPO₄ were mixed together, giving 4.0 mM concentrations of each, and were incubated at 37°C, pH 7.3, ionic strength = 0.171, DSₖ = 33.52. At intervals the tubes were vortexed, 200 μl was removed, and the optical density was measured. (B) HAP formed directly: initial CaCl₂ and Na₂HPO₄ concentrations after mixing were 8.0 and 1.2 mM, respectively; T = 37°C, pH 7.3, ionic strength = 0.176, DSₖ = 33.03. Points are the mean ± SD of three independent measurements.

Fig. 2. Inhibition of ACP formation. The effect of GC and taurine-conjugated bile salts on ACP precipitation, under the conditions described for Fig. 1A, was estimated from the maximum absorbance of the ACP phase. Points are the mean ± SD of three independent measurements.

Fig. 3. Effect of bile salts on the onset of indirect HAP formation. HAP was formed via ACP under the conditions described in Fig. 1A in the presence of increasing bile salt concentrations and the induction time was determined from plots of absorbance at 405 nm versus time. Points are the mean ± SD of three independent measurements.

sufficient ACP dissolves to restore saturation and the released ions are incorporated into the precipitating HAP. This process continues until all the ACP has been dissolved and reprecipitated as the less soluble HAP. We showed by infrared spectroscopy that the transition to HAP was completed within 2-4 h (18) and therefore measurement of Pₐ precipitated at 24 h indicates the amount of HAP formed.

In Fig. 2 the maximum absorbance at 405 nm of the ACP phase (i.e., the absorbance at the induction time) is plotted as a function of bile salt concentration for GC, TC, TDC, and TCDC. With the exception of TC, these bile salts were equally effective inhibitors of ACP formation. Low concentrations of TC increased the maximum absorbance of the ACP phase, but this was probably caused by effects on ACP aggregation (31) rather than by a true increase in the amount precipitated. With these bile salts the maximum absorbance of the ACP phase always occurred within 40 to 150 min, but in the presence of GDC or GCDC (2-3 mM) the absorbance of the ACP phase rose slowly for at least 24 h, at which time the absorbance was ~ 0.08 (not shown). Fig. 3 and Fig. 4 show the effects of all bile salts on the onset of HAP precipitation and on the amount of HAP precipitated at 24 h, respectively. At concentrations < 1.5 mM, GDC and GCDC paradoxically accelerated HAP precipitation, but 2-3 mM of either bile salt prevented HAP formation for at least 24 h (Fig. 3). In contrast, much higher concentrations of taurine-conjugated bile salts or GC had only
versus respective control) (Fig. 4). Very slight effects that ranged from no inhibition with 100 mM GDC or GCDC, for which analysis was not possible because of insufficient precipitate (not shown). The infrared spectra of each experiment showed the presence of calcium phosphate (32), except in the presence of 2-3 mM GDC or GCDC (P < 0.001), but the infrared spectra of each bile salt approximately doubled the induction time (P < 0.01 for each bile salt versus respective control). For each experiment the infrared spectrum of the mineral phase at 24 h was always consistent with poorly crystalline HAP (32), except in the presence of 2-3 mM GDC or GCDC, for which analysis was not possible because of insufficient precipitate (not shown). The infrared spectra did not contain peaks at ~1400 cm⁻¹, which demonstrated that no CaCO₃ had precipitated (33). Henceforward for brevity, and based on the apparent selectivity of inhibition, GDC and GCDC will be referred to as HAP inhibitors and GC, TC, TDC, and TCDC will be referred to as ACP inhibitors.

In human gallbladder bile, the calcium concentration is normally greater than the inorganic phosphate concentration (19, 34-39), and a calcium concentration of 8 mM CaCl₂ and 1.2 mM Na₂HPO₄. Under these conditions, the ACP phase was no longer observed (Fig. 1B), but the divergent behavior of the two groups of bile salts concerning HAP formation was unchanged (Table 1). The ACP inhibitors had little effect on the onset of HAP formation or, with the exception of GC, on the amount of HAP precipitated at 24 h. GDC or GCDC (2 mM) prolonged the induction time > 24 h and effectively prevented precipitation. Some of these experiments were repeated using bile salts from alternative sources, and the same results were obtained. The only exception was for TDC obtained from Steraloids, which gave significantly more inhibition of HAP formation than did the same material from Sigma (Table 1).

In the above experiments HAP was formed much faster than may be the case in vivo. Accordingly, we investigated the ability of bile salts to inhibit precipitation from solutions that required > 24 h for HAP to form (Fig. 5). Under these conditions, GDC or GCDC (3 mM) and TDC or TCDC (100 mM) prevented precipitation for at least 4 days. TC or GC (100 mM) did not prevent precipitation, but did reduce by ~40% the amount of HAP formed at equilibrium.

Reduction of free Ca²⁺ versus poisoning of HAP embryos

In the following experiments the amount of Ca²⁺ bound by bile salts in solutions of the same composition as in the

![Fig. 4. Inhibition of indirect HAP formation. HAP was formed indirectly under the conditions described in Fig. 1A in the presence of increasing bile salt concentrations. After 24 h, HAP was pellet by centrifugation at 11,000 g for 5 min and the remaining supernatant P, was measured. Points are the mean ± SD of three independent experiments. In some instances the error bars were smaller than the symbol size.](image)

**TABLE 1.** Effect of bile salts on direct HAP formation

<table>
<thead>
<tr>
<th>Bile Salt</th>
<th>Onset*</th>
<th>HAP pptd. at 24 h</th>
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<tbody>
<tr>
<td>None</td>
<td>215 ± 1</td>
<td>1.20 ± 0</td>
</tr>
<tr>
<td>GC</td>
<td>2 mM</td>
<td>185 ± 0</td>
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<tr>
<td></td>
<td>100 mM</td>
<td>196 ± 27</td>
</tr>
<tr>
<td></td>
<td>100 mM'</td>
<td>300 ± 0</td>
</tr>
<tr>
<td>TC</td>
<td>2 mM</td>
<td>185 ± 0</td>
</tr>
<tr>
<td></td>
<td>100 mM</td>
<td>188 ± 21</td>
</tr>
<tr>
<td></td>
<td>100 mM'</td>
<td>300 ± 50</td>
</tr>
<tr>
<td>TDC</td>
<td>2 mM</td>
<td>185 ± 0</td>
</tr>
<tr>
<td></td>
<td>100 mM</td>
<td>272 ± 85</td>
</tr>
<tr>
<td></td>
<td>100 mM'</td>
<td>245 ± 0</td>
</tr>
<tr>
<td>GDC</td>
<td>2 mM</td>
<td>&gt; 24 h</td>
</tr>
<tr>
<td></td>
<td>3 mM</td>
<td>&gt; 24 h</td>
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<tr>
<td></td>
<td>3 mM'</td>
<td>&gt; 24 h</td>
</tr>
<tr>
<td>GCDC</td>
<td>2 mM</td>
<td>&gt; 24 h</td>
</tr>
<tr>
<td></td>
<td>3 mM</td>
<td>&gt; 24 h</td>
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<tr>
<td></td>
<td>3 mM'</td>
<td>&gt; 24 h</td>
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</tbody>
</table>

*Initial CaCl₂ and Na₂HPO₄ concentrations were 8.0 and 1.2 mM, respectively; T = 37°C, pH = 7.3, f = 0.176, DS₅₀AP = 33.03.

*Absorbance was measured at 60-min intervals, therefore induction times less than 60 min apart are considered to be identical.

*Significantly different from control (P < 0.0001, t-test).
precipitation experiments, but omitting the phosphate, was measured and correlated with their inhibition of ACP or HAP formation.

The ability of the ACP inhibitors to diminish ACP precipitation was proportional to their reduction of free Ca\(^{2+}\) \((r^2 = 0.850, P < 0.001)\), and no ACP formed when free Ca\(^{2+}\) was < 3.5 mM (Fig. 6). GDC (3 mM) and GCDC (2 mM) had very little effect on the free Ca\(^{2+}\) concentration when the total calcium was 4.0 mM (3.88 ± 0.02 and 3.92 ± 0.02 mM, respectively) or on the maximum absorbance of the ACP phase (within 20% of the absorbance without bile salt), and yet they greatly slowed the rate of ACP formation.

To determine whether bile salts inhibited HAP precipitation by reducing the free Ca\(^{2+}\) concentration, we first established the intrinsic effect of free Ca\(^{2+}\) concentration on HAP formation. A fixed concentration of phosphate (4.0 mM) was mixed with 2.0–4.0 mM CaCl\(_2\), with no bile salt present, and the amount of HAP precipitated at 24 h is given by the line in Fig. 7. Ca\(^{2+}\)-phosphate ion pair formation was ignored and the initial free Ca\(^{2+}\) concentration of each solution was assumed to equal the total calcium concentration. Neglecting ion pair effects overestimated the free Ca\(^{2+}\) concentration by ~10%. Ion pair formation was neglected to allow comparison with the following experiment in which Ca\(^{2+}\) binding to bile salts was measured in the absence of phosphate. Bile salts in the concentrations detailed in the legend to Fig. 7 were mixed with 4.0 mM CaCl\(_2\) and the free Ca\(^{2+}\) concentration was measured with murexide. The resulting free Ca\(^{2+}\) concentrations were then plotted against the amount of HAP precipitated at equilibrium in the presence of the same bile salt concentrations (from Fig. 4). The results for the ACP inhibi-
tors (100 mM) all fell above the line representing the intrinsic effect of free Ca\(^{2+}\) concentration on HAP precipitation, which demonstrated that these bile salts had a smaller effect on the amount of HAP precipitated than would be predicted from their reduction of free Ca\(^{2+}\). However, GC and TCDC were more effective than were TC and TDC, which suggested that a fraction of the Ca\(^{2+}\) bound to GC and TCDC was held with sufficient affinity for it to be unavailable to HAP, but that all the TC- or TDC-bound Ca\(^{2+}\) was available. In contrast, GDC and GCDC (2-3 mM) had almost no effect on free Ca\(^{2+}\) and yet they prevented HAP formation. Clearly, under these conditions, GDC and GCDC inhibited HAP formation by a mechanism that did not involve reducing the free Ca\(^{2+}\) concentration. For GC, TC, TDC, and TCDC, the mean micellar \(K_f\) values (liters/mol) were: 9.4 ± 1.7, 3.06 ± 0.4, 12.2 ± 1.0, and 11.6 ± 0.2, respectively. Ca\(^{2+}\) binding to GCDC and GDC was measured at a single concentration (2 and 3 mM, respectively) and the \(K_f\) values were 22.2 and 22.4. These results are consistent with those obtained by Moore and Sanyal (13) using a Ca\(^{2+}\)-sensitive electrode.

When bile salt binding to mature HAP crystals was measured, in each case the fraction bound initially increased with bile salt concentration but then declined as saturation was approached (binding curve for GDC shown in Fig. 8, inset). The maximum value of bound/free was taken as a measure of the HAP-binding affinity of each bile salt and was plotted against the cognate equilibrium bile salt concentration (Fig. 8). GDC and GCDC had 6- to 36-fold greater affinity for HAP than had the bile salts of the ACP-inhibiting group. Thus, the ability of GDC and GCDC to inhibit HAP formation correlated with their HAP-binding affinity, and not with their reduction of free Ca\(^{2+}\). This result agrees with previous work that suggested inhibition of HAP formation by GCDC depended on the poisoning of HAP embryos (18).

The apparent CMCs of the bile salts were measured with an apolar fluorescent probe. The CMCs of GDC and GCDC (2.2 and 2.4 mM, respectively) were greater than the equilibrium bile salt concentrations at which maximum binding affinity was observed (1.2 mM for both). Conversely, the remaining bile salts tended to reach their maximum binding affinity at free bile salt concentrations slightly above their CMC: the CMCs of GC, TC, TDC, and TCDC were determined to be ~10, ~9, 1.8, and 2.0 mM, respectively, and their maximum binding affinities were observed at 14, 13.3, 2.1, and 2.9 mM, respectively. These CMC values agree with our previous determinations (40) and are within the ranges measured with other techniques (41, 42).

**Effect of DSHAP on inhibition by GCDC**

Because the total calcium concentration in human gall-bladder bile varies over a wide range (34–36, 39), but the phosphate concentration varies much less (19, 37, 38), we examined the effect of calcium concentration on the ability of GDC to inhibit HAP formation. When the initial phosphate concentration was fixed at 2.0 mM and the initial calcium was varied from 4 to 15 mM, inhibition of HAP precipitation as a function of GCDC concentration reached a plateau that depended on the initial calcium concentration (Fig. 9A). When HAP was precipitated, the equilibrium GCDC concentration was lower than the initial concentration because of bile salt binding to HAP (Fig. 9B). Binding affinity tended to increase with increasing free Ca\(^{2+}\) concentration, and the approximate upper and lower limits of binding are indicated by the least-squares fitted lines drawn through the points representing initial Ca\(^{2+}\) concentrations of 15 and 6 mM. The maximum level of inhibition for every initial Ca\(^{2+}\) concentration was always attained in equilibrium with GCDC concentrations that were below the CMC (Fig. 9A), which suggested that premicellar aggregates were responsible for the inhibition. The equilibrium DSHAP values for the points on the inhibition plateaus were calculated and plotted against the initial calcium concentration (Fig. 10). Regardless of the initial DSHAP (circles), all the solutions reached the same equilibrium DSHAP (squares; 32.6 ± 1.19) in the presence of sufficient GCDC. The only exception to this result was the lowest initial calcium concentration, for which the initial and maximum equilibrium DSHAP values were the same because...
precipitation was totally prevented. Thus, inhibition of HAP precipitation by GCDC premicellar aggregates was effective only in solutions with $DS_{HAP}$ less than ~33.

Effect of mixed bile salts on HAP precipitation

The six major human bile salts were mixed together in typical physiologic proportions and tested for their ability to inhibit calcium phosphate precipitation. When the initial $CaCl_2$ and $Na_2HPO_4$ concentrations were both 4.0 mM, a complex pattern of inhibition was observed (Fig. 11). At 5 mM total bile salt the amount of HAP precipitated decreased by ~20%, but no further inhibition was observed until the total bile salt concentration exceeded 50 mM, at which point a steady decline in HAP precipitation began that reached ~60% inhibition with 150 mM total bile salt. The maximum absorbance at 405 nm of the ACP phase increased slightly at bile salt concentrations up to 10 mM, probably because of altered aggregation, but then declined to approximately zero at 50 mM total bile salt (Fig. 11). However, when the initial $CaCl_2$ and $Na_2HPO_4$ concentrations were 8.0 mM and 1.2 mM, respectively, the mixed bile salts rapidly caused complete inhibition of phosphate precipitation, even though the initial $DS_{HAP}$ was very similar to the previous experiment (Table 2).

DISCUSSION

For the past decade the Ca$^{2+}$-buffering hypothesis has been an important concept in the investigation of gall-
The inhibition of ACP precipitation by GC and the taurine conjugates was directly proportional to their concentration.

**TABLE 2. Effect of mixed bile salts on HAP precipitation**

<table>
<thead>
<tr>
<th>Total Bile Salt</th>
<th>HAP precipitated at 24 h</th>
<th>μmol P&lt;sub&gt;i&lt;/sub&gt;</th>
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<tr>
<td>mM</td>
<td></td>
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<tr>
<td>0</td>
<td></td>
<td>1.20 ± 0.00</td>
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<td>5</td>
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<tr>
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<td>150</td>
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*Initial CaCl<sub>2</sub> and Na<sub>2</sub>PO<sub>4</sub> concentrations were 8.0 and 1.2 mM, respectively; T = 37°C; pH = 7.4, I = 0.176, DS<sub>HAP</sub> = 36.95.

The apparently paradoxical acceleration of HAP formation with GDC or GCDC concentrations < 1.5 mM also results from their ability to bind to HAP. Binding to HAP occurs through interaction with the surface Ca<sup>2+</sup> ions (40), and the bile salt aggregates are presumed to possess groups (-OH and/or -COO-) that are correctly arranged to mirror the spacing of Ca<sup>2+</sup> ions on the HAP surface. If the bile salt aggregates bind free Ca<sup>2+</sup> ions instead of Ca<sup>2+</sup> ions at the HAP surface then these ions will be held at an appropriate spacing for inclusion into a HAP crystal, and this will favor HAP nucleation. With slightly higher bile salt concentrations HAP nucleation will still be promoted, but sufficient bile salt will be in solution to bind to the remaining surface of the resulting embryo, so that the embryo is poisoned.

When HAP was formed indirectly (DS<sub>HAP</sub> = 33.5), the ACP inhibitors displayed varied effects on the onset of HAP formation. TC slightly accelerated HAP formation; TDC consistently retarded HAP formation; and GC or TCDC initially accelerated, but at higher concentrations retarded, HAP precipitation. We propose that the spacing of the bile salt-bound Ca<sup>2+</sup> ions governs the outcome of the binding. For TC the spacing is always favorable for promotion of HAP nucleation. For GC and TCDC the spacing is initially favorable, but as the bile salt concentration increases the bile salt micelles may rearrange so that the spacing of the Ca<sup>2+</sup> ions no longer matches the...
spacing in a HAP crystal. For TDC the spacing is always unfavorable. The lowered Ca²⁺ activity resulting from unfavorable Ca²⁺ binding reduces the probability of sufficient Ca²⁺ and phosphate ions converging to form a HAP embryo, producing prolonged induction times. But when an embryo eventually forms through random thermodynamic fluctuations, it begins to compete with the bile salt for free Ca²⁺. It seems that most of the Ca²⁺ bound by TC and TDC was held with insufficient affinity to deny Ca²⁺ to the nascent embryo and growth occurred almost unchecked. GC and TCDC slightly decreased the amount of HAP precipitated, which suggested that a fraction of the Ca²⁺ bound by these bile salts was unavailable to HAP. However, when less saturated solutions were studied (DSHAP = 25.8), TDC and TCDC bound sufficient Ca²⁺ to prevent HAP formation for at least 4 days, while GC and TC reduced the amount of HAP formed by ~40%. Therefore, under conditions that are likely to occur in vivo, Ca²⁺ buffering can be an effective mechanism of inhibiting HAP formation.

Understanding the structural requirements for high affinity bile salt-apatite binding is hindered by our ignorance of the aggregation number of the bile salt species that is bound (dimer, trimer, etc.) and by the lack of any generally accepted model for the structure of bile salt aggregates. Addadi and colleagues (45-47) have shown that the acidic proteins that control biomineralization of invertebrate exoskeleton or rat dentin appear to recognize a common structural motif that is present in a variety of crystals, such as calcite, calcium phosphate esters, and calcium dicarboxylates. This motif consists of rows of calcium ions and phosphates (or carbonates or carboxylates) arranged in a plane defined by two free oxygens emerging perpendicular to the crystal face. These oxygen atoms are assumed to be arranged to optimally cooperate with the acidic proteins in forming the coordination polyhedron of Ca²⁺. It may be that such a motif is also present in HAP perpendicular to the crystal face. These oxygen atoms are than do their glycine-conjugated counterparts.

Calcium dicarboxylates. This motif consists of rows of calcium ions and phosphates (or carbonates or carboxylates) arranged in a plane defined by two free oxygens emerging perpendicular to the crystal face. These oxygen atoms are assumed to be arranged to optimally cooperate with the acidic proteins in forming the coordination polyhedron of Ca²⁺. It may be that such a motif is also present in HAP perpendicular to the crystal face. These oxygen atoms are than do their glycine-conjugated counterparts. Previous work showed that as well as having a higher affinity for HAP, GDC and GCDC were also bound to a greater extent than were the other bile salts (at least 2-fold more bile salt bound at saturation), which suggested that more binding sites were available to the glycine-conjugated dihydroxy bile salts (40). The excess binding sites were considered to comprise “steps” or discontinuities on the crystal surface, which are the sites of crystal growth (51), and the exclusion of GC and the taurine conjugates from these binding sites would explain their apparent inability to poison HAP embryos. This exclusion may have its root in the fact that these bile salts tended to display their maximum binding affinity at concentrations above their CMC, while GDC and GCDC were bound with greatest affinity as premicellar aggregates. This result suggests that GDC and GCDC have a higher affinity for HAP than they do for self-association, while the opposite tends to be true for the other bile salts. Although the taurine conjugates and GC are not necessarily binding mainly as micelles, the greater average size of the bound aggregates of these bile salts, compared with those of GDC and GCDC, may preclude their binding to the crucial step binding sites on steric grounds.

Consistent with the observation that GCDC binds to HAP as premicellar aggregates, the inhibition of HAP formation by GCDC reached a maximum in equilibrium with submicellar concentrations of bile salt. The failure of micellar concentrations of GCDC to produce greater inhibition was unexpected. When premicellar aggregates became bound to embryos it would be anticipated that the micelles would dissociate, thus replenishing the reservoir of premicellar aggregates and enhancing the degree of inhibition. Failure of this enhancement to occur suggests that micellar dissociation and diffusion of the resulting premicellar aggregates to a new embryo is outstripped by the rate of embryo formation and growth to a critical nucleus. The maximum degree of inhibition that could be produced by GCDC was inversely proportional to the initial DSHAP. Equilibrium DSHAP values were calculated for each solution displaying maximal inhibition, and each solution had the same equilibrium DSHAP (~33), regardless of the initial DSHAP. This result showed that GCDC can only prevent HAP formation by poisoning embryos in solutions that have DSHAP < 33. If the initial DSHAP exceeds 33, sufficient HAP must precipitate for DSHAP to fall below this threshold value before inhibition can commence.

However, when bile salts were mixed together in typical physiologic proportions, we observed that total inhibition was possible in solutions with DSHAP > 33. This result could be due to synergy between the bile salts or, more likely, to the lower phosphate concentration used in this experiment. We previously showed that phosphate ions...
inhibit, and Ca\textsuperscript{2+} ions promote, GCDC binding to HAP (40); therefore, the phosphate ion concentration might more powerfully affect the ability of GCDC to inhibit than does the Ca\textsuperscript{2+} concentration. Although increases in the concentration of either ion will increase $D_{\text{HAP}}$, for phosphate the effect of this increase will be exacerbated by the decreased HAP-binding ability of GCDC. In support of this interpretation, when the phosphate concentration was increased and Ca\textsuperscript{2+} was decreased, keeping $D_{\text{HAP}}$ approximately constant, the ability of the mixed bile salts to inhibit was much less. In this case, the inhibition of phosphate precipitation occurred in three phases: 1) with 5 mM total bile salt there was a small decline in HAP precipitation, presumably caused by poisoning HAP embryos; 2) between 10 and 50 mM total bile salt, the abolition of ACP precipitation occurred, without a further decrease in the amount of HAP formed; and 3) above 50 mM bile salt, HAP precipitation was inhibited further, and we attribute this to the reduction of free Ca\textsuperscript{2+} activity by binding mainly to GDC and GCDC.

In summary, inhibition of calcium phosphate precipitation by bile salts is a complex event that occurs by either the reduction of Ca\textsuperscript{2+} activity or the poisoning of HAP embryos (i.e., binding to the embryo in competition with phosphate). The ability to poison HAP embryos was restricted to GDC and GCDC, and only low concentrations (2–3 mM) of bile salt were necessary. The taurine-conjugated bile salts and GC could also significantly inhibit HAP formation, but higher concentrations (~100 mM) were required and they were effective only in solutions from which HAP precipitated slowly ($D_{\text{HAP}} = 25.8$). This inhibition was presumably due to the reduction of free Ca\textsuperscript{2+} concentration, which was the mechanism by which these bile salts also prevented ACP formation. When HAP was formed via ACP and inhibition was caused by the six bile salts mixed together, the inhibition resulted from a combination of poisoning embryos and reducing the free Ca\textsuperscript{2+} concentration, and most of the activity was attributed to GDC and GCDC. The preeminence of GDC and GCDC for the inhibition of Ca\textsuperscript{2+}-sensitive anion precipitation has previously been postulated by Moore and Sanyal (52). The relative importance of each mechanism of inhibition appears to depend on the solution saturation, and particularly on the phosphate concentration. This dependency requires further study. The role of other biliary lipids should also be investigated because, although we could show no effect with TC alone, Sutor and Percival (17) inhibited HAP formation with mixed TC/phosphatidylcholine micelles. As well as lipids, biliary proteins are likely to play an important role in regulating the precipitation of calcium salts, and an acidic peptide that powerfully inhibits calcium carbonate precipitation in vitro has recently been isolated from human cholesterol (53) and black pigment (54) gallstones. The inhibitory lipids and proteins may interact with each other to modify their effects, and full understanding of the regulation of calcium salt precipitation in bile will require knowledge of the behavior of all the potential promoters and inhibitors of precipitation.

We wish to thank Dr. Roger D. Soloway for many helpful discussions. This work was supported by grant AM-16549 from the National Institutes of Health and grants from the Sealy and Smith and M. D. Anderson Foundations. Dr. R. Peter Shellis, The Dental School, Bristol, UK, kindly supplied the ionproduct computer program.

 Manuscript received 22 April 1993 and in revised form 23 August 1993.

REFERENCES

7. Moore, E. W. 1984. The role of calcium in the pathogenesis of gallstones: Ca\textsuperscript{2+} electrode studies of model bile salt solutions and other biologic systems. With an hypothesis on structural requirements for Ca\textsuperscript{2+} binding to proteins and bile acids. Hepatology. 4: 228S–243S.