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THE ROLE OF BILIARY CALCIUM IN GALLSTONE PATHOGENESIS

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1. ABSTRACT

Calcium is an element that is extremely important in the pathogenesis of gallstones. It is present in bile in large quantities since components of bile bind the calcium avidly. Precipitated calcium salts with the calcium sensitive ions, bilirubinate, carbonate and phosphate are major components of pigment gallstones and are present in high concentrations in the centers and rims of cholesterol gallstones. An understanding of the solubility of calcium in bile is thus essential if one is to understand the process that leads to gallstone formation. In addition, calcium also has a very important role in the function of the gallbladder epithelial cell. This manuscript discusses what is known about biliary calcium, the factors that regulate total and free calcium concentrations in bile, the solubility of calcium in both normal and lithogenic bile, and the role calcium plays in epithelial cell function during gallstone formation.

2. INTRODUCTION

Calcium is a critical element for survival and function of living organisms. On cellular and subcellular levels, it regulates processes such as absorption and secretion, endo- and exocytosis, and integrity of the cell cytoskeleton. It is particularly important for normal contractility in muscle cells and generation of action potentials in nerve cells. In addition, calcium salts are the major components of exoskeletons (shells) in invertebrates and skeletons (bone) in vertebrates. It is not surprising then that calcium homeostasis is one of the most tightly controlled processes in higher organisms. On the other hand, the concentrations of calcium in body fluids required to maintain these functions carry a clear disadvantage. Body fluids are normally supersaturated with calcium salts, such as calcium carbonate and phosphate, and the potential for pathological precipitation of calcium salts is therefore always present. Although protective mechanisms evolved to ameliorate this tendency, pathologic calcium precipitation manifests itself with aging and in several disease states by abnormal tissue calcification, calcified vascular plaques, and formation of calcium-containing calculi in excretory systems. This discussion will focus on the roles played by calcium in formation and growth of biliary calculi, a problem that affects approximately 10% of individuals in the United States.

In humans, gallstones are classified by visual inspection, chemical analysis, and IR spectroscopy (1-7) as cholesterol (near pure or mixed) or pigment (black or "brown" calcium bilirubinate) gallstones. As indicated by name, cholesterol stones are composed largely of cholesterol crystals (50-99%), but also contain other

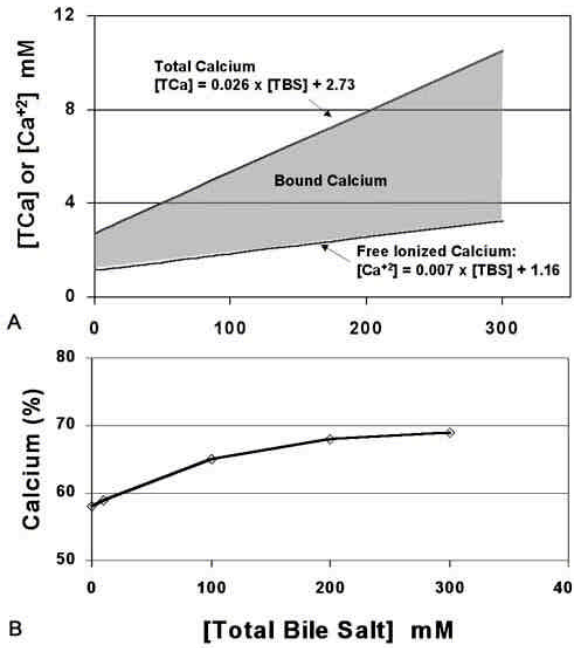

Figure 1. A: Total (TCa) and free ionized (Ca^{+2}) calcium concentrations in canine bile are plotted against total bile salt concentration ([TBS]). Note that both [TCa] and [Ca^{+2}] were correlated with total bile salt concentration with the linear relationships: [TCa] = 0.026 x [TBS] + 2.73, r^2 = 0.96 and [Ca^{+2}] = 0.007 x [TBS] + 1.16, r^2 = 0.91. Note that the intercepts of both relationships are approximately the values in canine plasma and that the shaded area between the lines represents bound calcium. B: The percentage of calcium that is bound in canine bile is plotted against total bile salt concentration. As bile salt concentration increases in common bile duct bile or in the gallbladder as bile is concentrated, the percent of bound calcium increases from about 58% to about 69%. This demonstrated the strong affinity of bile salts for calcium.

substances forming a central calcium nidus, pigmented regions, and/or calcified shells with composition resembling pigment gallstones (1;8;9). Pigment gallstones are composed of calcium carbonate, phosphate, bilirubinate, and free fatty acids imbedded in an insoluble residue of mucus and a polymer of bilirubin or a bilirubin-like substance (1-5, 8-11). They may also contain up to 10% cholesterol. The composition of pigment gallstones and the distribution of calcium in cholesterol stones led to the hypothesis that calcium precipitation is critical in formation and growth of all types of gallstones (8). For example, the central nidus of calcium in cholesterol gallstones suggests that calcium salts act as nucleating agents for precipitation of cholesterol from cholesterol vesicles in supersaturated bile.

The following discussion examines our current understanding of factors regulating total and free calcium concentrations in bile, and summarizes our understanding of calcium solubility in both normal and lithogenic bile. The nucleating properties of calcium for other substances such as cholesterol and the role played by calcium in gallbladder epithelial cellular function will also be discussed.

3. TOTAL AND FREE IONIZED CALCIUM CONCENTRATIONS IN BILE

The total concentration of calcium in bile is much higher than the plasma concentration due to calcium binding by other components of bile, especially bile salts. The binding-affinity of bile salts for calcium ions is particularly strong and accounts for the majority of bound calcium in bile. Thus, total calcium in bile may be separated into two fractions, bound and free calcium. Although the latter species is often termed "free ionized calcium", it should be understood that all calcium in bile is in the ionized state. Free ionized calcium therefore is synonymous with unbound calcium. Free ionized calcium is difficult to measure in bile, but can be quantitated using ultrafiltration of bile and calcium-sensitive electrodes protected from the toxic effects of bile salts (8;9;12-15).

Moore, Celic, and Ostrow (16) studied Ca^{+2}-binding to six major bile salts in human bile using a calcium-sensitive electrode covered by a protective membrane to eliminate bile salt interference. All six bile salts demonstrated high-affinity premicellar binding and relatively low-affinity micellar binding. Both premicellar and micellar binding were strongly dependent on the number and position of the cholanic ring hydroxy groups of the bile salts – premicellar binding to dihydroxy bile salts was 6-8 times that of trihydroxy (cholate) conjugates, while micellar binding was 3-6 times greater. Binding of calcium by both naturally occurring and synthetic (exotic) bile salts involved the 7- and 12-OH cholanic ring group and terminal carboxyl or sulfonic side chain groups.

The effects of bile salts on calcium concentrations *in vivo* were studied by examining canine bile collected over a wide range of bile flow and after variable degrees of concentration in the gallbladder (17). Bile in this species contains only taurine conjugates of three major bile salts with calcium binding capacity –cholate, deoxycholate and chenodeoxycholate. As illustrated in (Figure 1), total ([TCa]) and free ionized ([Ca^{+2}]) calcium concentrations in bile were always higher than plasma values reflecting calcium binding in bile. When common bile duct and gallbladder bile calcium concentrations were plotted against total bile salt concentrations ([TBS]), linear relationships: [TCa] = 2.73 + 0.26 [TBS], r = 0.96 (upper line, left panel) and [Ca^{+2}] = 1.16 + 0.007 [TBS], r = 0.91 (lower line, left panel), were obtained. The shaded area between the lines in the graph on the left then represents concentrations of bound calcium at each bile salt concentration. The intercepts of both relationships at [TBS] = 0 are approximate canine plasma calcium concentrations indicating that, in the absence of bile salts, bile calcium concentrations would be approximately the same as those in canine plasma. Bile salts bind approximately 58% of calcium in bile at lower bile salt concentrations (Figure 1, right panel) and percent binding increases to as high as 69% at full concentration of bile in the canine gallbladder ([TBS] between 280 and 300

mM). The increase in bound calcium as bile salt concentration rises demonstrates the binding affinity of the bile salts for calcium and their ability to buffer the concentration of free ionized calcium, as bile is concentrated in the gallbladder. However, free ionized calcium concentrations still rise with increasing bile salt concentration demonstrating dual effects of bile salts with respect to calcium. While buffering of calcium by bile salts tend to lower free ionized calcium concentration at any given total calcium concentration, their binding affinity brings more calcium into bile than would be there if they were not present.

The situation is similar in normal human bile although [TCa], [Ca^{+2}], and [TBS] are somewhat lower than in canine bile (18), reflecting a different mix of bile salts in humans. When bile samples from 62 patients (40 with cholesterol gallstones, 12 with pigment gallstones, and 10 with normal gallbladders) undergoing operation were examined, both [TCa] and [Ca^{+2}] were linearly related to [TBS] with the relationships: [TCa] = 2.58 + 0.07 x [TBS], r = 0.70 and [Ca^{+2}] = 1.15 + 0.0034 x [TBS], r = 0.89. Note again that the intercepts are approximately the same as in human plasma measured using the same technique, but that slopes differ from dog reflecting a different bile salt composition in the two species. When groups of patients were compared, no differences in calcium concentrations were found between control patients and patients with either type of gallstone. However, it should be understood that operation for symptomatic gallstones occurs years after gallstone formation. Calcium elevations might be transient and these data could not exclude calcium abnormalities during active formation of gallstones.

In one sense, binding of calcium by bile salts can be viewed as a protective mechanism; bile salts act as calcium buffers lowering free ionized calcium concentrations. On the other hand, once precipitation begins, bile salt-calcium complexes in bile act as a calcium "sink", fueling the process by releasing calcium as free calcium is consumed by precipitation.

The important species of calcium for assessment of calcium salt saturation in bile is [Ca^{+2}], not total calcium (see below – Supersaturation of Bile with Calcium), and [Ca^{+2}] in bile is normally higher than plasma values. The question thus arose: Is biliary calcium elevated during the formation and growth of gallstones? To answer this question, investigators (19-23) examined animal models of gallstone formation and humans actively forming gallstones (18;24-26).

We (27) examined gallbladder bile obtained by needle puncture of the gallbladder at laparotomy from dogs fasted 12 hours before and 6 weeks after a lithogenic high carbohydrate, low methionine diet. No dogs had stones at initial laparotomy, but all developed pigment gallstones by 6 weeks after lithogenic diet. Total bile salt concentrations in gallbladder bile were similar before and after diet, 257 ± 7 versus 241 ± 9 mmol/L, demonstrating similar degrees of bile concentration by the gallbladder during the fasting period. Therefore, differences in calcium concentrations were not due to differences in bile salt concentration or to different degrees of concentration of bile by the gallbladder. Statistically significant increases were observed in [Ca^{+2}] (3.71 ± 0.17 versus 3.02 ± 0.07 mmol/L) and [TCa] (13.16 ± 0.57 versus 10.16 ± 0.19 mmol/L) in dogs with pigment gallstones compared to their pre-diet samples. These data suggest that elevated biliary calcium plays a role in the development of pigment gallstones in the canine model of pigment gallstones. Trotman (20) measured ionized calcium concentrations in common duct bile from nb/nb hemolytic mice that form pigment gallstones. Ionized calcium concentrations were significantly elevated compared to control mice. When bone marrow was transplanted into histocompatible W/Wv mice, ionized calcium in common duct bile remained significantly elevated, although somewhat lower than in nb/nb mice. These data demonstrate that ionized calcium plays a role in pigment gallstones associated with hemolytic anemia in mice and that concentrations of ionized calcium are in part controlled by genetic factors. Increased total and free ionized calcium have also been observed in prairie dogs forming cholesterol and pigment gallstones (21;22). In all of these models, increases in calcium concentration appear to be transient occurring early during gallstone formation and growth. Once stones form, calcium levels return to normal, consistent with findings in most humans at a time when they require operation.

Obese patients undergoing gastric bypass operations for weight loss present a unique opportunity to study humans as they form gallstones. Thirty-eight percent of these patients develop gallstones and 12% biliary sludge within months of gastric bypass surgery for obesity. As many as a third of the patients with gallstones or sludge require cholecystectomy for symptoms within 6 months of operation during the period of rapid weight loss. Gastric bypass patients at the Medical College of Virginia underwent of bile aspiration from the gallbladder at their initial gastric operation and at the time of cholecystectomy if they developed symptomatic gallstones. Biliary calcium was examined in 68 obese and 27 non-obese patients. Considerable overlap was noted between obese and non-obese patients and between patients with and without gallstones. When all patients were considered, free ionized and bound calcium increased linearly with increasing bile salt concentrations. No differences in the slopes of these relationships were noted in obese patients or in patients with gallstones, although gallstone patients exhibited a greater concentration of free ionized calcium at all concentrations of bile salt. (28) [Ca^{+2}] increased before, and [Ca^{+2}] and mucin concentrations significantly after, formation of stones and sludge (25;26). These studies suggest that increased [Ca^{+2}] is present in humans during, but not late, after gallstone formation and may be related to or associated with increased concentrations of mucin in bile.

4. CALCIUM ENTRY INTO BILE

The mechanism of entry of calcium into bile has important implications for the therapeutic modulation of

calcium in bile. If calcium is actively secreted into bile, inhibition of the process might be used to reduce biliary calcium concentrations. On the other hand, passive entry indicates that calcium will always be present in bile in high concentrations and, if possible, must be reduced by chelation within the biliary lumen. In 1983, Cummings and Hofmann (29;30) studied the mode of entry of calcium into canine bile over a wide range of bile flow induced by bile salt infusion. Calcium output was tightly linked to bile acid output and, similar to bile flow, could be separated into bile salt-dependent and bile salt-independent fractions. The results were thought to be consistent with passive entry of calcium into bile, but they could not exclude active transport into bile.

Subsequent studies in dogs (31) compared entry of calcium over a wide range of bile flow to entry of potassium, a small ion known to be passively distributed across bile duct epithelium (32). Free ionized calcium concentration increased in a curvilinear fashion as both bile flow and bile acid output increased. The concentration of free ionized calcium rose with increasing bile salt concentration and bile flow below a bile salt concentration of about 40 mM. It then became relatively constant (~ 1.5 mM) at higher bile salt concentrations and rates of bile flow suggesting that entry of calcium into the biliary system is flow limited. Calcium and potassium outputs were tightly linked to bile flow and to each other. A plot of potassium output versus bile flow produced a linear relationship: potassium output = -0.002 + 0.0056 x bile flow, r = 0.99. This relationship demonstrates tight linkage of potassium entry with convective water movement into the duct and an intercept of approximately 0, findings expected for a passively distributed ion. Likewise, a plot of calcium output versus bile flow yielded a linear relationship: calcium output = -0.0018 + 0.0032 x bile flow, r = 0.99. Again, calcium entry was tightly linked to convective water movement and the intercept was near zero, suggesting passive entry. Plotting calcium output against potassium output further emphasized the similarities between calcium and potassium. A very close linear relationship was observed: calcium output = 0.000 + 0.566 x potassium output, r = 0.98. The tight correlation and the intercept of 0 support passive entry of both ions. Note that the slope of 0.566 is higher than the plasma ratio of calcium to potassium of 1.22/4.5 = 0.27 reflecting substantial binding of biliary calcium by bile salts.

Gleeson, Murphy and Dowling (33) studied the effects of serum calcium on biliary calcium levels in patients with t-tubes. A 10% calcium gluconate infusion was used to vary serum calcium concentrations. Serum total calcium increased from 2.08 to 3.18 mM and free ionized calcium from 1.13 to 1.68 mM. Biliary total and free ionized calcium increased proportionately with total calcium rising from 1.90 to 2.8 mM and from 0.7 to 1.19 mM. The findings were consistent with passive entry of calcium and partial restriction of calcium entry at the bile flow rates studied. The authors concluded that serum calcium levels were one determinant of biliary calcium concentrations.

These data indicate that entry of the majority of calcium into bile is a passive event. If significant active transport was present, intercepts would not have been 0 and plots of calcium and potassium output would not have been so similar. Tracking of plasma calcium concentrations by biliary calcium also suggests passive entry. The implication of these studies is that calcium will always be present in bile since it enters with convective water movement. It is unlikely that there is any important active cellular secretory mechanism to inhibit that is sufficient to decrease the amount of calcium entering bile. Control of the saturation of calcium salts in bile must therefore be directed toward chelation of calcium in the lumen or reduction in the concentration of calcium sensitive anions in bile. The success of chelation then depends on whether or not calcium enters bile freely. The studies outlined seem to indicate that the movement of calcium across biliary epithelium is somewhat restricted.

Movement of calcium ions across gallbladder epithelium was studied in normal, isolated, cystic duct-ligated guinea pig gallbladder by inducing convective water movement across the membrane with intraluminal solutions of varying osmolality (34). Calcium movement (ΔQ^{Ca}) was directly and linearly related to water flow, presumably across paracellular channels with the relationship: $\Delta Q^{Ca} = 0.602 \times \Delta V - 1.27$, r = 0.71. The slope of the relationship of 0.602 ($\Delta Q^{Ca}/\Delta V$) represents the concentration of calcium translocating the membrane and is only half of plasma or luminal concentration. The mean sieving concentration (1-r) calculated from this slope is approximately 0.5 indicating that the epithelium is only moderately permeable to calcium ions. These results confirm that calcium ions move across the membrane in response with convective water movement, and likely with diffusion gradients, but that movement is somewhat restricted.

5. CALCIUM ABSORPTION FROM THE GALLBLADDER

The fate of calcium in the canine gallbladder was studied and compared to absorption of potassium, an ion passively distributed across gallbladder epithelium (35). Gallbladder bile from 14 dogs obtained at laparotomy after a 24-hour fast and 12 samples of common duct bile collected from 3 dogs during infusion of sodium taurocholate were examined. Mass balance equations, based on the assumptions that bile salts were not absorbed from the gallbladder and no emptying of gallbladder contents during the fasting period, utilized gallbladder volume and gallbladder and common duct concentrations of bile salts, calcium, (35) and potassium to calculate the volume of common duct bile delivered to the gallbladder. The amounts of an electrolyte presented to and remaining in the gallbladder were calculated as the product of its concentration and the respective volume and absorption of the electrolyte as the difference between the amount of electrolyte presented to and remaining in the gallbladder. If bile salts were absorbed or the gallbladder emptied part of its contents, this methodology underestimates calcium absorption. Therefore, the results of this study represent

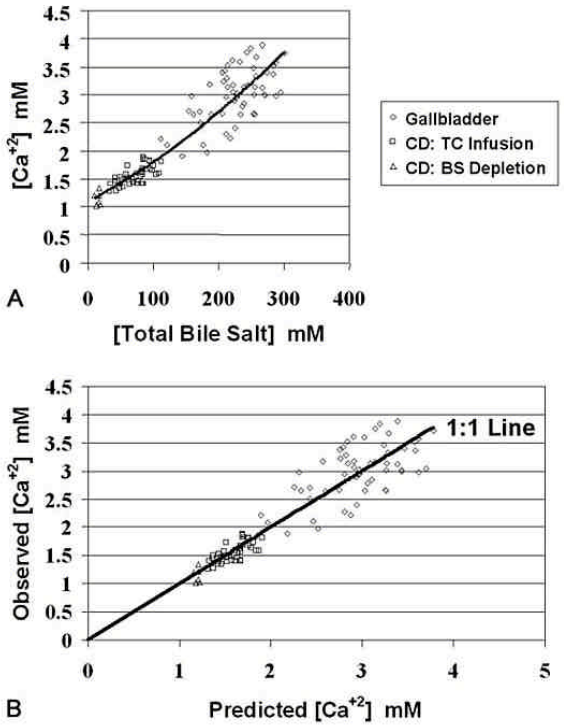

Figure 2. A: Observed concentrations of free ionized calcium ($[Ca^{+2}]$) obtained from canine gallbladder and common bile duct bile are plotted against total bile salt concentration. The line represents predicted values of calcium calculated using the Gibbs-Donnan effects of bile salts. Note that the observed values straddle the line indicated that Ca^{+2} ions tend toward Gibbs-Donnan equilibrium values. B: Observed $[Ca^{+2}]$ is plotted against predicted $[Ca^{+2}]$, calculated using the Gibbs-Donnan effects of bile salts. The values nicely straddle the 1:1 line representing perfect correlation, again emphasizing that Ca^{+2} ions are subject to Gibbs-Donnan forces in bile.

the minimum values of absorption of ions from the canine gallbladder.

The canine gallbladder reduced the volume of bile presented to it by at least 5.3-fold. Bile pH decreased from a mean of 7.7 ± 0.04 in the common duct to 6.09 ± 0.10 in the gallbladder, essentially eliminating biliary bicarbonate during concentration and acidification. Biliary potassium, total calcium, and free ionized calcium concentrations increased from 4.7 ± 0.3, 3.8 ± 0.2, and 1.5 ± 0.1 mM respectively in the common duct to 10.8 ± 0.2, 9.8 ± 0.4, and 2.8 ± 0.1 mM in the gallbladder. Despite the increase in concentrations, net absorption of potassium (57%) and calcium (51%) was observed. Absorption of at least 50% of calcium by the canine gallbladder is important to gallstone pathogenesis. Without absorption, calcium concentration would be at least twice as high as the observed value at full concentration in the gallbladder. Therefore, without absorption the ion product and degree of saturation of each calcium salt in bile would be increased at least two-fold.

6. DISTRIBUTION OF CALCIUM IONS ACROSS BILIARY EPITHELIUM

Data demonstrating passive entry of calcium into bile and net calcium absorption of calcium in the face of rising concentrations of bound and free calcium seems to be contradictory. The answer to this paradox resides in the linear relationship of free ionized calcium to total bile salt concentration and the physiochemical properties of bile salts. The gallbladder is essentially impermeable to bile salt anions and they accumulate in the gallbladder lumen as the gallbladder concentrates bile. If a membrane separating two solutions is impermeable to electronically charged molecules, but freely permeable to water and small ions, electrochemical and osmotic forces are generated which result in an unequal distribution of ions across the membrane. This situation is referred to a Gibbs-Donnan equilibrium. The biliary tract is thus a classic Gibbs-Donnan system with bile containing high concentrations of impermeant bile salt molecules being separated from plasma by the semipermeable gallbladder epithelium.

By Gibbs-Donnan theory, the distribution of ions g across the membrane at equilibrium is defined as:

$$g = \left(\frac{[Na^+]_b}{[Na^+]_a}\right)^{1/1} = \left(\frac{[K^+]_b}{[K^+]_a}\right)^{1/1} = \left(\frac{[Cl^-]_a}{[Cl^-]_b}\right)^{1/1} = \left(\frac{[Ca^{+2}]_b}{[Ca^{+2}]_a}\right)^{1/2}$$

where α and β are the respective sides of the membrane. My collaborator, the late Edward Moore, measured Gibbs-Donnan effects generated by bile salts using equilibrium dialysis (unpublished) and found the following relationship conforming to Gibbs-Donnan theory:

Predicted g = 1 + (0.0027 x [Total Bile Salt]) eq. 2

where g is the Gibbs-Donnan ratio defined above. Using this equation and actual data from canine bile, we were able to plot predicted and observed concentrations of calcium in common duct and gallbladder bile.

The concept was tested first with potassium, a small cation that is known to be passively distributed across biliary epithelium. Observed concentrations of potassium were plotted against predicted values calculated using equations 1 and 2. Predicted and observed values nicely straddled the 1:1 line as expected if it was subjected to the Gibbs-Donnan effects of bile salts. Although is understood that bile is never in true equilibrium, it appears that potassium values approach the values predicted by Gibbs-Donnan theory. Like calcium, net absorption of potassium occurs during concentration of bile in the gallbladder lumen and its concentration rises.

Predicted calcium concentrations were calculated in a similar manner and were plotted in two ways (Figure 2). Observed values are plotted against total bile salt concentration (Figure 2, left panel). Note that observed values nicely straddle the line, which represents the expected curvilinear relationship between free ionized calcium and total bile salt concentrations calculated using equations 1 and 2. A plot of observed versus predicted free ionized calcium concentrations (Figure 2, right panel)

demonstrates the excellent fit of data around the expected 1:1 line. The excellent correlation between observed and predicted free ionized calcium concentrations in canine bile (Figure 2) indicate that calcium, likely potassium, is passively distributed throughout the biliary tract and subject to Gibbs-Donnan effects of bile salts.

7. SUPERSATURATION OF BILE WITH CALCIUM

A prerequisite for precipitation of any substance from solution is supersaturation of the solution with the substance. The saturation of salts in aqueous solution is well understood. Below the saturation point, precipitation cannot occur; above this point, the solution is supersaturated and precipitation becomes thermodynamically possible. The point of saturation may be defined by a solubility product, Ksp', where:

$$Ksp' = [cation^{+x}]^x * [anion^{-y}]^y$$

Where $[cation^{+x}]$ and $[anion^{-y}]$ are the concentrations of the cation and anion forming the salt with the valences x and y, respectively (8;9).

These general principles apply to the saturation of bile with calcium. Saturation of bile with at least one calcium salt is a prerequisite for calcium precipitation; that is, the ion product $[Ca^{+2}]^2$ x $[anion^{-x}]^x$ in solution must exceed Ksp' for that salt. In bile, carbonate, phosphate, bilirubinate, and free fatty acids, the substances commonly found in gallstones, form insoluble calcium salts (low Ksp') and are the anions of interest to investigators examining gallstone pathogenesis. Note that the ion product of a calcium salt may be increased due to an increase in calcium concentration, an increase in the concentration of the calcium sensitive anion, or increases in both (8;9). Therefore, saturation of bile with calcium might be due to problems with calcium, calcium-sensitive anions, or both.

From data on calcium concentrations in canine and human bile (see above), increasing $[Ca^{+2}]$ as bile is concentrated in the gallbladder would result in a greater degree of calcium saturation (higher ion product for each salt). For example, normal concentration of bile by the canine gallbladder results in a two to three-fold increase in $[Ca^{+2}]$ (Figure 1), and thus the ion product of each calcium salt. During pigment gallstone formation in dogs and humans, $[Ca^{+2}]$ is further increased above normal exacerbating this tendency. However, saturation of a calcium salt requires knowledge about the concentration of the anion in question. No conclusions about saturation can be made unless data for both the cation (calcium) and anion are known.

To explore this further, ion products in canine bile (17)were calculated for calcium carbonate from common bile duct and gallbladder bile. Despite increasing $[Ca^{+2}]$, the ion product fell precipitously from an high of approximately 6.5×10^{-7} to 2.0×10^{-10}, values well below the Ksp' of 3.76×10^{-8} for this salt, calculated using similar methodology. The lesson learned from these studies is that although calcium concentrations rise in bile during concentration, the concentrations of calcium-sensitive anions in bile decrease under normal conditions as they are absorbed or in the case of carbonate, as the critical species ($CO_3^{=}$), decreases due to bile acidification.

Studies in humans and experimental animals models of pigment gallstones support the concept that supersaturation of bile with calcium bilirubinate is important for pigment gallstone formation and that increased concentrations of unconjugated bilirubin (UCB) are important in the pathogenesis of these stones. (1;19;36-42). Increased concentrations of UCB is a consistent finding in humans, dogs, prairie dogs and mice with pigment gallstones, and must play a key role in each circumstance. In humans, increased concentrations of unconjugated bilirubin is due to hydrolysis of conjugated bilirubin (38) or to hemolytic anemia, while nb/nb mice (20;36;37) have congenital hemolytic anemia, which leads to increased hepatic secretion of UCB. In dogs, the cause of increased biliary [UCB] is not known. Likewise, biliary [UCB] increases in children (43) and pigs receiving parenteral nutrition, in a pigment gallstone model in prairie dogs (44), and with Crohn's disease (45). UCB secretion also increases in cirrhosis and with hemolytic anemia. The incidence of pigment gallstones is high in each case (1). Although these studies emphasize the importance of biliary anions in supersaturation of bile with calcium salts, the concentration of calcium is also elevated in many of these circumstances compared to normal patients or control animals. Therefore, it is likely that supersaturation of bile for calcium salts is due to both increases in biliary calcium and calcium-sensitive anion concentrations.

These principles were illustrated in a study examining the degree of calcium bilirubinate saturation in dogs with pigment gallstones. Comparison of 15 normal dogs with 15 dogs with pigment gallstones induced by 6 weeks of methionine-deficient diet revealed that there was minimal alteration in the gallbladder's ability to acidify or concentrate bile during stone formation. Both bile $[Ca^{+2}]$ and unconjugated bilirubin concentrations increased significantly. It is not possible to measure the concentration of unbound bilirubinate in bile accurately to calculate the Ksp' for calcium bilirubinate. Therefore, the total unconjugated bilirubinate were compared to equilibrium values in model bile salt solutions constructed with biliary lipid concentration similar to the canine samples. In normal bile, unconjugated bilirubin concentrations were equal to or lower than those measured in model bile solutions with comparable $[Ca^{+2}]$. On the other hand, unconjugated bilirubin concentrations exceeded those found at equilibrium in model bile solutions indicating that bile from dogs actively forming stones was supersaturated with calcium bilirubinate. Supersaturation was due to a combined increase in calcium and unconjugated bilirubin concentrations.

8. KINETIC FACTORS IN GALLSTONE PATHOGENESIS

Although precipitation of a substance becomes possible once supersaturation occurs, other factors

determine if and when precipitation occurs. Supersaturated solutions may remain in metastable state for long periods of time. This principle has been well documented with cholesterol gallstones. Many individuals develop bile supersaturated with cholesterol, but most never develop cholesterol gallstones. Therefore, an understanding of the kinetic factors that determine when, if, and how quickly calcium salt precipitation occurs is important in the understanding of gallstone pathogenesis (46-50). Wang (51) mapped crystallization pathways for cholesterol in model solutions and studied the effects of added calcium. Calcium increased the number of solid crystals that formed, but did not influence the appearance of crystals, the crystallization pathway, or the micellar solubility for cholesterol. Teramen (52) examined the effects of biliary concanavalin-A bound glycoproteins and calcium ions on cholesterol crystal growth. Concanavalin-A bound glycoproteins accelerated nucleation time and growth rate and shifted a considerable amount of cholesterol from micelles to vesicles. Calcium only affected nucleation time and enhanced the effects of concanavalin-A bound glycoproteins.

The precipitation of calcium salts to form exo- and endoskeletons is a carefully controlled process, which requires surrounding fluids to be supersaturated with the respective calcium salt. Organisms have developed sophisticated methods of controlling this biomineralization process, including nucleating and anti-nucleating proteins. Thus, it is not surprising that canine bile (50;53) contains potent factor(s), which inhibit calcium carbonate precipitation. Addition of large amounts of calcium to bile increasing saturation indices in bile 4 to 12-fold did not result in calcium carbonate precipitation. In contrast, addition of calcium to NaCl-$NaHCO_3$ solutions constructed with saturation indices comparable to the bile samples resulted in precipitation once a two to three-fold increase in saturation index was reached (35). The addition of only 0.2 ml of canine bile abolishes precipitation of calcium carbonate from supersaturated saline solutions, whereas taurocholate and albumin have little effect. The effect is not due to a decrease in calcium concentration or the degree of calcium carbonate saturation, and remains even when the bile is diluted 10-fold (50). Some of the effect may be due to bile salts that are known to inhibit calcium hydroxyapatite precipitation from solution (54), but inhibition at high dilution suggests that the factor is truly an anti-nucleating agent, probably a protein that is normally secreted into bile. It has also been shown that other normal components of bile, albumin, bilirubin, sodium taurocholate, and phospholipid (55) and polyamines (56) inhibit calcium carbonate precipitation to some extent.

Several investigators (57-67) have isolated calcium-binding proteins from stones and bile and examined their role as nucleating and antinucleating agents. Afdhal *et al.* (61) demonstrated that calcium-binding protein also binds to mucin. They also showed inhibition of calcium phosphate precipitation by mucin and a decrease in the mass of precipitation by calcium binding protein plus mucin. They concluded that calcium-binding protein and mucin participated in a biomineralization process important for gallstone formation and growth. Van den Berg and his co-workers (63) examined the complex relationships between calcium-binding protein and mucin. Calcium phosphate precipitates, and to a lesser extent soluble calcium, stimulated cholesterol crystallization from model bile solutions. Addition of human mucin increased the mass of precipitated cholesterol crystals. Human albumin plus calcium-binding protein together, but not alone, decreased the stimulatory effect of calcium, but not if mucin was also added. It appears that calcium salt precipitates and human mucin are strong nucleating agents for cholesterol precipitation while calcium-binding proteins have markedly less effects. Although the role of calcium in cholesterol precipitation is quite complex and dependent on other factors in bile, studies to date indicate that calcium plays an important role in this process.

It is expected that, as has been suggested for cholesterol, precipitation of calcium from bile depends not only on the degree of saturation, but also on a balance of calcium nucleating and anti-nucleating factors which control whether precipitation actually occurs once the thermodynamic prerequisite of supersaturation is present. These observations may also explain why dogs do not spontaneously develop gallstones, despite marked calcium carbonate supersaturation of bile.

9. CALCIUM AND GALLBLADDER EPITHELIAL CELL FUNCTION

Giurgiu and co-workers studied formation of cholesterol gallstones in prairie dogs and found increases in biliary lipids and increased concentrations of free ionized calcium associated with altered ion transport. Gallstone formation was divided into three phases – pre-crystal formation, presence of crystals, and presence of gallstones (68). Free ionized calcium concentrations were significantly elevated in the pre-crystal phase and progressively increased as crystals and gallstone appeared. Sodium absorption increased during the pre-crystal phase, normalized as the serosal-to-mucosal fluxes of sodium and chloride increased with appearance of crystals, and then decreased when gallstones formed. The authors speculated that the alteration in gallbladder cell transport might be related to elevated calcium and bile salt concentration in gallbladder bile. Subsequent studies (69;70) demonstrated that biliary calmodulin concentrations were altered during cholesterol gallstone formation in the prairie dog and that gallstone formation diminishes calmodulin-mediated inhibition of gallbladder epithelial cell sodium chloride transport. Lithogenic (1.2% cholesterol) diet increased calmodulin, phospholipid, and cholesterol concentrations in hepatic and gallbladder bile. Gallbladder tissue levels of calmodulin were not elevated indicating that the increased biliary levels of calmodulin originated in the liver and seemed to be related to increased concentrations of the bile salt taurochenodeoxycholate and phospholipid hypersecretion (70). Because free ionized calcium is elevated prior to cholesterol gallstone formation in this species and since Ca^{+2}-calmodulin tonically inhibits gallbladder absorption under normal circumstances, ion transport by prairie dog gallbladders from control animals

and animals feed a lithogenic, 1.2% cholesterol diet were studied *in vitro* in Ussing chambers. Electrophysiological parameters and ion flux were examined in the presence and absence of the calmodulin antagonist trifluoperazine or the calcium ionophore A23187. Trifluoperazine decreased short circuit current, transepithelial potential difference, and tissue conductance, but had less effect on animals fed the high cholesterol diet. These effects were not reversed by A23187. These results are consistent with partial release of basal inhibition of gallbladder ion transport during cholesterol gallstone formation in the prairie dog.

The role of intra- and extracellular calcium in the conversion of gallbladder absorption to secretion during cholesterol gallstone formation in the prairie dog was studied using the calcium ionophore A23187 (71). Sodium, chloride, and water fluxes were measured in the Ussing chamber with varying concentrations of calcium (5-10 mM) in the mucosal bathing solution and in the presence and absence of A23187. The ionophore increased short circuit current, transepithelial potential difference and tissue resistance. These changes were associated with decreases in mucosal-to-serosal flux of chloride and stimulation of serosal-to-mucosal flux of sodium. The net result was net chloride secretion, decreased sodium absorption and conversion of water absorption to water secretion. The effects of A23187 were blunted by low bathing solution calcium concentrations. These studies indicate that intracellular calcium regulates ion transport by the gallbladder epithelial cell and that high intracellular calcium concentrations convert normal absorption to secretion.

The formation of gallstones associated with octreotide is also associated with increased concentrations of biliary calcium. The effects of octreotide on ion transport were therefore studied in prairie dog gallbladder (72). Gallbladders were mounted in Ussing chambers and ion transport was studied in the presence and absence of 50 nmol octreotide. During basal conditions, the gallbladder absorbed sodium and calcium. Serosal octreotide increased net sodium absorption by stimulating mucosal-to-serosal flux of sodium and decreased tissue conductance and short circuit current significantly. In addition, basal calcium absorption was converted to net calcium secretion by stimulating serosal-to-mucosal calcium flux. Calcium secretion stimulated by octreotide may account for the elevated concentrations of gallbladder bile calcium noted with this agent.

10. SUMMARY

Calcium is an important component of bile and of gallstones and studies outlined above indicate that it plays several important roles in the formation and growth of all types of gallstones. An understanding of the factors that control entry of calcium into bile, biliary concentrations of calcium in the bile ducts and gallbladder, absorption of calcium from the gallbladder, and calcium solubility in bile are important for an understanding of precipitation of calcium salts from bile. In addition, the role of calcium in normal and pathological gallbladder epithelial function adds yet another dimension to our understanding of the pathological events leading to gallstone formation in the gallbladder. In addition, failure of gallstone dissolution is often attributed to calcium shells surrounding the stones, and an understanding of calcium solubility is key for the successful non-surgical treatment and prevention of gallstones of all types.

11. PERSPECTIVE

As outlined here, calcium is a critical element in gallstone pathogenesis. The concentration of calcium in bile increases significantly during early gallstone formation contributing to increased saturation of each calcium salt in bile. The causes of these increases have not been determined but may be due to increased secretion of calcium into bile, decreased absorption from the gallbladder lumen through a thickened mucin layer overlying the gallbladder epithelium, or due to impaired gallbladder epithelial cell function, or simply due to increased Gibbs-Donnan forces of biliary anions such as bile salts or mucin which may be abnormal during gallstone formation. However, studies on calcium salt saturation indicate that supersaturation of these salts is even more dependent on the concentration of calcium-sensitive anions in bile, including bilirubinate, carbonate and phosphate. Thus, it is not known if eliminating the observed increases in biliary calcium concentration will prevent gallstone formation. Moreover, studies to date have shown that calcium will always be present in bile in large quantities since it enters freely into the biliary tract and is controlled by passive forces. No calcium excretion process can be inhibited to decrease biliary calcium. Thus, control of biliary calcium will require chelation of calcium in the lumen and may not be easily controlled. To date, this has not been possible, although attempts have been made to synthesize novel bile salts with high calcium binding ability. However, an improved understanding of the factors that control biliary calcium and its role in gallstone formation have greatly improved our understanding of gallstone pathogenesis.

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