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The Etiology of Pigment Gallstones

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Pigment gallstones are of two major types, black and earthy brown, each consisting of calcium salts of bilirubin and other anions, along with an unmeasured residue that is largely mucin glycoproteins. Studies in model systems indicate that the small proportion of unconjugated bilirubin in bile is solubilized by bile salts and that the ionized bilirubin is more soluble than the protonated diacid. Solubility is decreased by added lecithin but is unaffected by cholesterol. At the pH of bile, unconjugated bilirubin exists mainly as a monoanion with sufficient solubility in mixed micelles not to precipitate, were it not for the presence of calcium, which forms highly insoluble salts with unconjugated bilirubin anions. Supersaturation of bile with calcium bilirubinates is inhibited by bile salts, which bind calcium, reducing the activity of free calcium ions. When supersaturation occurs, usually due to increased concentrations of bilirubinate anion, nucleation may be initiated by binding of calcium bilirubinate to mucin glycoproteins in bile.

In earthy brown stones, which form mainly in the bile ducts, the pigment is mostly calcium bilirubinate, combined with calcium palmitate. These components form due to hydrolysis, by enzymes in infecting bacteria, of conjugated bilirubin and lecithin, respectively. In black stones, which form mainly in the gallbladder, the pigment is mostly a highly cross-linked network polymer of bilirubin, which is insoluble in all solvents. Concomitant polymerization and oxidation of calcium bilirubinate probably occur in the solid state, after precipitation of the pigment due to hydrolysis of conjugated bilirubin by endogenous β-glucuronidase from the biliary tract and/or liver. This may result from a diet-related decrease in inhibitors of β-glucuronidase in bile. In hemolytic states, increased concentrations of conjugated bilirubin in bile, providing more substrate for hydrolysis, may contribute also to black stone formation. Black stones also contain coprecipitated calcium phosphate and/or carbonate, but the insoluble polymer renders them resistant to physical dissolution therapy.

This communication will summarize current concepts of the pathogenesis of pigment gallstones. Two major types of pigment gallstones have been described (Table 1), which have different characteristics and, likely, different etiologies: black pigment stones (BP) and earthy calcium bilirubinate stones (CB) (1–3).

BP are dark brown to black in color, with an amorphous appearance and powdery consistency, and are formed primarily in the gallbladder (4–6). They are the major stone type found in patients with chronic hemolysis (e.g., due to sickle cell disease, thalassemia, hereditary spherocytosis, cardiac valve prostheses) or cirrhosis, although most patients with BP do not have either condition (2, 7). In both the Orient and Western world, the surrounding bile and stones are usually sterile, except in the presence of associated acute cholecystitis (8).

CB, by contrast, are earthy brown to orange in color, often laminated, have a soft, greasy consistency and are formed primarily in the bile ducts (1, 9–11). They occur most commonly in association with the chronic infectious cholangitis that is almost unique to the Orient (9–11); in the West, CB are uncommon except in patients with primary sclerosing cholangitis or in relation to nonabsorbable suture material retained in the common bile duct after cholecystectomy. The surrounding bile is infected in more than 85% of cases, most often with *Escherichia coli* (1, 8).

In keeping with their differing appearance, site of formation, geographic distribution, and disease associations, BP and CB differ in composition (Table 2). BP contain much insoluble black pigment that is probably a pigment polymer (12–15; Ohkubo, H. et al., *Gastroenterology* October 1984; 87: in press). This is mixed with calcium bilirubinate and calcium carbonate and/or phosphate (4–6, 16–18). By contrast, CB consist mainly of calcium bilirubinate, calcium soaps of fatty acids derived from bile lecithin (19, 20), small and variable amounts of polymer and a significant proportion of cholesterol (1, 9, 19). Both types contain 10 to 60% by weight of an unmeasured residue, believed to consist mainly of a mucin glycoprotein matrix (21–25), and both types contain

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TABLE 1. CHARACTERISTICS OF TWO MAJOR TYPES OF PIGMENT GALLSTONES

Characteristics	Stone type	
	Black pigment	Calcium bilirubinate
Color	Black	Brown to Orange
Consistency	Amorphous, powdery	Soft, laminated
Anatomic location	Gallbladder ± ducts	Bile ducts
Geography	West and Orient	Mostly Orient
Disease association	Hemolysis Cirrhosis	Cholangitis, ova, parasites, sutures
Cultures of bile	Usually sterile	Infected (*E. coli*)
Principal components	Pigment polymer Calcium phosphate and/or Calcium carbonate	Calcium bilirubinate Calcium soaps of fatty acids Cholesterol
Etiology	Increased excretion or hydrolysis of CB	Bacterial hydrolysis of CB[a] to UCB[b]

[a] CB, conjugated bilirubin.
[b] UCB, unconjugated bilirubin.

TABLE 2. COMPOSITION OF TWO MAJOR TYPES OF PIGMENT GALLSTONES[a]

Component[a]	Stone type	
	Black pigment (%)	Calcium bilirubinate (%)
Bile pigment, total (major form)	40 (10–90)[b] (insoluble polymer)	50 (28–79) (Ca bilirubinate)
Calcium	15 (3–40)	5 (3–9)
Calcium salts		
Carbonate	13 (0–65)	None
Phosphate	5 (0–32)	<1
Palmitate + stearate	1 (0–3)	23 (11–67)
Cholesterol (unesterified)	3 (1–13)	10 (2–28)
Unmeasured residue	24 (10–73)	12 (0–30)

[a] Values are given as a percentage of dry weight of the stone, based on Trotman (4, 5), Wosiewitz and Schroebler (6), Nakayama (19), Ohkubo (*Gastroenterology* October 1984; 87, in press), Mukaihara and Tanamura (National Institutes of Health Workshop on Pigment Gallstones, Philadelphia, 1981) and their coworkers.
[b] Values are means, with range in parentheses.

an increased proportion of divalent cations (especially Fe^{2+} and Cu^{2+}) in comparison with bile (25–28).

A third type of pigment stone, originating in Japan, has been described in our laboratory (Ohkubo, H. et al., *Gastroenterology* October 1984; 87: in press). These friable concretions are composed of granules of black pigment, either embedded in a crystalline, pale yellow matrix that is high in cholesterol, or dispersed with granules composed principally of calcium carbonate. Thus, they have the appearance of small black and white particles mixed together and have, therefore, been called "salt and pepper" stones. As with the other types of pigment stones, they have an average 40% of unmeasured residue. Their geographic and disease associations have not been defined, and they will not be discussed further here.

NATURE OF THE BLACK PIGMENT

Over 15 years ago, Suzuki (14) showed that the insoluble black material, remaining after exhaustive extraction of other components of BPs, yielded infrared spectra resembling closely that of calcium bilirubinate. Suzuki produced similar black materials by heating bile pigments with strong acid. Based on its insolubility, he and others postulated that the black material is a polymer of bilirubin or of dipyrroles derived therefrom (6, 13–15). The first clear evidence for this proposal was obtained recently in our laboratory, by demonstration that BPs exhibit the property of equilibrium swelling characteristic of cross-linked network polymer structure (12; Ohkubo, H. et al., *Gastroenterology* October 1984; 87: in press). Whereas monomers and linear chain polymers will dissolve in appropriate solvents, because the molecules or chains can separate from each other and disperse in the solvent, cross-linked chains cannot disperse (12). Rather, solvent penetrates the polymer network, causing it to expand. With time, the network will swell to a maximum equilibrium volume, V_{eq}. The ratio of V_{eq} to the initial volume (V_o) of the packed dry polymer is defined as the equilibrium swelling ratio $Q_{eq} = V_{eq}/V_o$. In a pure polymer, V_{eq} is determined by the frequency of, and interval between, cross-links along the chains. In a gallstone, which contains a mixture of network polymers with linear polymer and nonpolymer material, Q_{eq} will depend also on the proportion of network polymer in the stone (12).

The 28 BP stones examined by us have shown maximum Q_{eq} values at pH 10.5 ranging from 1.3 to 3.2, even though the mean pigment content is only 30% by weight. This indicated significant network polymer content in the pigment. Swelling occurred in aqueous buffers but not organic solvents, indicating that the interstices of the network were lined by polar groups. Moreover, the degree of swelling increased with increasing pH values from 5.5 to 10.5, suggesting that the carboxyl side chains of bilirubin were intact but involved in secondary (noncovalent) cross-links within the network. Increased swelling when ethylenediamine tetraacetic acid (EDTA) was added suggested that Ca^{2+} and other divalent cations were the likely bridges between the carboxyl groups. A close correlation between the degree of swelling (Q_{eq}) and the loss of the vinyl groups of the pigment (determined by infrared spectroscopy) suggested strongly that the network was formed primarily by covalent polymerization of the vinyl groups. This relationship has been observed also in CB and "salt and pepper" stones, although they had more intact vinyl groups and less swelling than BP stones, in keeping with their lesser content of black pigment.

Taken together, these data suggest that the black pigment in BP is a highly cross-linked polyvinyl network polymer of bilirubinate. However, solid state nuclear magnetic resonance spectroscopy suggests that the vinyl side chains may not be involved (29, 30). Moreover, the putative mucoprotein matrix in the "unmeasured residue" of these stones presumably could contribute also to the swelling. Thus, although the presence of polymer has been established, the role of the vinyl groups is disputed.

CHARACTERISTICS OF BILE IN PATIENTS WITH PIGMENT GALLSTONES

Analyses of bile from gallbladders that contain pigment gallstones, compared with those that contain cholesterol gallstones or no stones, reveal that only unconjugated bilirubin (UCB) concentrations and β-glucuronidase activity are abnormally elevated. Thus, UCB concentrations rarely exceed 2 mg per dl (35 μ*M*) or 2% of total bilirubin in normal bile, but are higher than this in most biles with BP and more strikingly increased in bile containing CB stones (31–39). Except in patients with BP related to chronic hemolysis, total bilirubin concentrations are usually normal or low, although the proportion of monoconjugates to diconjugates of bilirubin may be increased also (32, 34, 39, 40). Total calcium concentrations are generally normal (37, 41), and the limited data on unbound, ionized calcium suggests that this is normal as well (41, 42). In bile surrounding stones containing calcium carbonate or bilirubinate, ultrafilterable calcium may be increased, but this fraction includes soluble complexes of calcium with small anions and may actually decrease the ionized free calcium fraction (41). pH values are generally in the physiological range of 6.2 to 7.8 (37), and there are no consistent changes in the composition or concentrations of bile acids, phospholipids or cholesterol (30–32, 34, 35). Increased concentrations of free fatty acids, resembling in composition those of the bile lecithin, are found in many patients with CB but not BP stones (19, 20, 43). There are no good data on carbonate or phosphate concentrations.

These data suggest that the major factor in the pathogenesis of pigment gallstones, as proposed originally by Maki (1) and his coworkers at Tohoku University in Sendai, is the appearance of an excess concentration of UCB in bile, generated by hydrolysis of unconjugated bilirubin by β-glucuronidase. Therefore, we will next examine the determinants of the solubility of UCB in bile.

PHYSICAL CHEMISTRY OF SOLUBILIZATION OF UCB *IN VITRO* (SEE ALSO OSTROW AND CELIC, THIS ISSUE)

In bilirubin, the two protonated carboxyethyl side chains form trios of internal hydrogen bonds (44) that suppress the ionization of the carboxyl groups and monopolize all of the polar groups in the bilirubin molecule. As a consequence, the crystalline UCB dissolves poorly in buffered aqueous solutions at physiologic pH values and has an *apparent* pKa value in the weakly alkaline range (45, 46). In such aqueous solutions, there is a linear relationship between the log of UCB solubility and pH values, with solubility of about 0.1 μ*M* at pH 8.0 (46).

By contrast, in the dianion salt of UCB present at more alkaline pH values and in bilirubin conjugates, these internal hydrogen bonds are not formed, and the pigment is in a flexible, open configuration with a tendency to stack as dimers or large aggregates that shield the nonpolar regions of the molecules from the aqueous solvent (47, 48). As a consequence, the dianion and conjugates exhibit much greater aqueous solubility, and the pKa values are near 4.4, as expected of carboxyl groups (49).

Bile acids, which have some capacity to interfere with hydrogen bond formation, increase by four orders of magnitude (to about 1 m*M* at pH 8.0) the dissolution of UCB in aqueous solutions (48, 50; Berman, M. D. et al., *Gastroenterology* 1980; 78:1141; Ostrow, J. D. et al., *Gastroenterology* 1980; 78:1315, Abstracts). As in simple aqueous solutions, the logarithm of the solubility of UCB increases linearly with pH value, and the ionized form is more soluble than the protonated UCB (Ostrow, J. D., and Celic, L. *Gastroenterology* 1984; 86:1334, Abstract). However, the linear plot of log solubility vs. pH is biphasic, exhibiting a 5-fold greater slope at pH values above, as compared to below, 6.8. This suggests that different moieties are being titrated, most likely the protonated and monoanion forms below pH 6.8 and the monoanion and dianion at pH values above 6.8 (Ostrow, J. D., and Celic, L. *Gastroenterology* 1984; 86:1334, Abstract). The solubilization increases with increasing bile acid concentration, but all of the common micelle-forming bile acids are about equally effective in enhancing UCB solubilization. By contrast, solubilization of UCB is much less enhanced by nonmicellar bile acids, such as taurodehydrocholate (50; Ostrow, J. D., and Celic, L. *Gastroenterology* 1984; 86:1334, Abstract). Added lecithin inhibits, but added cholesterol does not affect, the solubilization of UCB by bile acids (50; Berman, M. D. et al., *Gastroenterology* 1980; 78:1141, Abstract). These relationships suggest that the enhanced solubilization of UCB may be related to interactions of the pigment with bile acid monomers and the surface of micelles, a view supported by studies with quasielastic light scattering at pH 10.0 (48).

INTERACTIONS WITH CALCIUM (SEE ALSO MOORE, THIS ISSUE)

Bilirubin glucuronides are not precipitated by calcium ions (49). However, since the bilirubin in pigment gallstones is present almost entirely as the calcium salts of UCB (5, 16–18), the interactions of calcium and UCB in bile acid solutions are of importance. Recent data from our laboratory (Ostrow, J. D., and Celic L., *Gastroenterology* 1984; 86:1334, Abstract) indicate that added calcium, 4 to 12 m*M*, decreases the equilibrium solubilization of bilirubin crystals in 50 m*M* taurocholate at all pH values above 4.0. The inhibition of solubilization increases with increasing pH above 4.0 and is more than 90% above pH 6.8, and 98% above pH 9.0. This is in keeping with the suggestion that UCB in taurocholate solution is all in the mono- and dianion form above pH 6.8, but that an increasing proportion of protonated diacid bilirubin, that cannot form calcium salts, is present as the pH is decreased below 6.8. Below pH 4.0, all bilirubin is in the diacid form and, thus, not precipitated by calcium. Over the physiological range of bile pH values from 6.0 to 8.0 (37), the solubilization of bilirubin in 50 m*M* taurocholate in the presence of 4 to 12 m*M* calcium is 10 to 15 μ*M* (Ostrow, J. D., and Celic, L. *Gastroenterology* 1984; 86:1334, Abstract), which is in the range of

maximum concentrations of UCB present in normal human gallbladder bile (31–39). Berman et al. (*Gastroenterology* 1980; 78:1141, Abstract) have obtained similar values from curves derived by titration with HCl of disodium bilirubinate dissolved in bile salt solutions containing calcium.

Nonetheless, the precipitation of bilirubinate from bile acid solutions, on addition of calcium, is much less than expected (Ostrow, J. D., and Celic, L. *Hepatology* 1982; 2:165, Abstract). This is likely due to interactions of Ca^{2+} ions with bile acids (51–54), the cation showing greater avidity for bile acid monomers than for micelles (51–54). Thus, bile acids may serve as an important "buffer" that reduces the proportion of unbound calcium cations available to form insoluble salts with bilirubinate, carbonate, phosphate and fatty acids in bile (51, 55). Calcium also is likely to form complexes with mucoproteins, proteins, citrate and oxalate in bile (53, 55), further reducing the available activity of Ca^{2+}. However, the much higher concentrations of bile acids, as compared to these other moieties, suggests that bile acids are quantitatively the most important in this role as a calcium "buffer" (51).

PROPOSED PATHOGENESIS OF PRECIPITATION OF BILIRUBIN FROM BILE

The above observations suggest that the internally bonded form of UCB is poorly soluble in bile at physiological pH values, whereas the pigment is highly soluble in the open configuration, which can be stabilized by bile acids. Therefore, it is relevant that UCB is likely to occur in bile mainly in the open configuration. The overwhelming majority of UCB in bile arises from hydrolysis of excreted bilirubin conjugates (31). The other source of UCB in bile is from excretion of Z-E photoisomers of bilirubin into bile, with subsequent spontaneous reversion of these unstable geometric isomers back to the natural Z,Z configuration (56). This process occurs even under ordinary indoor lighting conditions (57). It is noteworthy that, in each instance, the UCB precursor (bilirubin monoglucuronide or the IXα-Z,E photoisomer of UCB) is half in the open and half in the internally bonded configuration. It is suggested that, due to the stabilizing influence of bile acids which are present in high concentrations, this configuration is maintained during hydrolysis or isomeric reversion of the respective precursor of UCB, just as occurs during acid titration of the bilirubinate anion in bile acid solutions. If this is so, then UCB in bile at physiological pH values would always be in the very soluble monoanion or dianion form and would never precipitate, were it not for the presence of Ca^{2+} ions to form the insoluble calcium bilirubinate salts. Thus, the precipitation of bilirubin from bile can be considered primarily in terms of supersaturation of bile with the calcium salts of UCB. The apparent solubility product of the monoanion salt (Ca^{++} activity $\times$ [total bilirubin]^2) has been estimated to be ca. 5×10^{-13} *M* (Ostrow, J. D., and Celic, L. *Hepatology* 1982; 2:165, Abstract).

FACTORS LEADING TO SUPERSATURATION OF BILE WITH CALCIUM BILIRUBINATE

Bile could become supersaturated with calcium bilirubinate by one of three mechanisms: an increase in the concentration of bilirubinate anions, an increase in the activity of unbound Ca^{2+} cations or a decrease in factors which solubilize calcium bilirubinate.

Increased bilirubinate anion concentrations may occur in bile, without any increase in the concentration of UCB, as a result of an increase in the pH of bile; however, no clinical or experimental examples of this mechanism have been described. However, at a given pH value, increased bilirubinate anion concentrations can result from an increased total concentration of UCB. This can occur, without an accelerated fractional rate of hydrolysis of conjugated bilirubin, secondary to an increased excretion of total (mainly conjugated) bilirubin in bile. Such is the likely mechanism of the formation of the black pigment gallstones characteristic of conditions associated with chronic hemolysis, including hepatic cirrhosis. Trotman's elegant studies, of an inbred strain of mice with congenital hemolytic anemia, have documented clearly that the increased concentrations of UCB precede, and are prerequisite to, stone formation (58).

Another mechanism for increased UCB in bile, without increased hydrolysis of conjugates, is augmented photoisomerization of UCB. This could be due either to increased concentrations of UCB in the cutaneous sites accessible to light or to increased surface, intensity or duration of light exposure. An example of the latter is the precipitation of UCB sludge in Gunn rat bile during phototherapy (59). Theoretically, one might expect a higher frequency of pigment gallstones in sunny and warm climates, where more skin surface is exposed to more radiant energy, than in cool, cloudy environments; relevant epidemiologic correlations have not been reported.

The best documented, and possibly most common, cause of increased UCB in bile is augmented hydrolysis of conjugates due to increased activity of β-glucuronidase in bile, as proposed originally by Maki's group in Sendai, Japan (1). His laboratory demonstrated that the formation of earthy, usually intraductal, CB stones was related to increased activity of β-glucuronidase, originating from *E. coli* or other bacteria (60, 61), cultured from the bile in more than 90% of such cases (1, 8). This is accompanied by increased hydrolysis of lecithins by bacterial phospholipases, accounting for the high content of fatty acid-calcium soaps in CB stones (43). However, gallbladder biles from Western subjects with BP stones exhibit increased enzymatic hydrolysis of conjugated bilirubin (31), presumably due to β-glucuronidase (62), in the absence of bacterial infection. This enzymatic hydrolysis was most active at pH 4.5 and was presumably traceable to β-glucuronidase derived endogenously from the hepatocytes or biliary epithelium (62). There is no evident enzyme activity in bile from normal gallbladders or in most biles from patients with cholesterol gallstones (31, 62). However, Japanese workers have suggested that normal bile does contain β-glucuronidase, whose activity is nullified by inhibitors, such as bile acids (63) and glucaric acid (64, 65). Changes in levels of glucaric acid are related to diet (64) in both experimental animals and in man, suggesting a mechanism whereby dietary manipulations can engender pigment stone formation. The activity of β-glucuronidase could also be enhanced by acidification of the bile (e.g., in infected bile), shifting the ambient pH values closer to the optimum for the

enzyme; it is unclear if this is an important factor in natural pigment stone disease in man.

Increased unbound calcium ion (Ca^{2+}) in bile could explain the presence in pigment gallstones of multiple calcium salts, including those of bilirubinate, carbonate, phosphate and fatty acids (5, 16, 17, 19, 25, 41). Such an increase in the activity of calcium ions in bile could come from relative decreases in the concentrations of bile salts and other agents that complex Ca^{2+} or from an increase in total calcium.

In guinea pigs, unconjugated bile acids more readily diffuse from the gallbladder than conjugated bile salts; thus, a selective decrease in bile salt concentrations could occur as a result of bacterial deconjugation of bile salts in infected bile (66). Bacteria might also metabolize other putative calcium buffers, such as oxalate or citrate. Selective permeability changes in the gallbladder, favoring absorption of bile salts relative to calcium, might occur also. There is evidence, in both guinea pigs and man, that nonfunctioning gallbladders may exhibit increased absorption of bile acids, to which they are normally relatively impermeable. However, Sutor and Wilkie (41) reported that the concentration of total calcium in bile is decreased also in nonfunctioning gallbladders in man and that the proportion of Ca^{2+} to total calcium is constant at 8 to 15% in both gallbladder and hepatic bile of patients with gallstones (42); this may be due to the fact that the biliary secretion of calcium is coupled to secretion of bile salts (Cummings, S., and Hofmann, A. F. *Gastroenterology* 1983; 84:1369, Abstract). As noted earlier, the presence of increased ultrafilterable calcium in the biles surrounding stones composed of a large proportion of calcium bilirubinate or phosphate might reflect an increase in soluble calcium complexes with small anions, rather than an increase in unbound Ca^{2+}. Thus, there is no current evidence for increases in the Ca^{2+} activity of pathologic gallbladder bile, although more extensive studies with a calcium electrode are clearly needed to evaluate critically this possibly important pathogenetic mechanism.

A role for solubilizers of calcium bilirubinate is suggested by the findings that, in the presence of calcium, solubility of bilirubinate is increased by bile acids and decreased by added lecithin (50; Berman, M. D. et al., *Gastroenterology* 1980; 78:1141, Abstract). These effects could be due simply to the complexing of calcium and/or bilirubin *per se* by bile salts and/or interference with this complexing by lecithin, altering the ion products of calcium cations with bilirubinate anions. On the other hand, there might be also some degree of physical solubilization of calcium-bilirubinate salts by mixed micelles in bile, influencing the limited aqueous solubility of these salts. Thus, a decrease in bile salts or increase in lecithin concentrations in bile might contribute to precipitation of calcium bilirubinate by several mechanisms, yet to be scrutinized experimentally.

MECHANISMS FOR PRECIPITATION OF CALCIUM BILIRUBINATE

Having considered how bile might become supersaturated with calcium bilirubinate, we must finally discuss the factors that lead to its precipitation and, in the case of BP stones, to its polymerization. These aspects have been little studied for pigment stones, so analogies with cholesterol gallstone formation must be sought.

For cholesterol gallstone formation, it is known that supersaturated bile is a prerequisite, but not solely sufficient, condition for the precipitation of cholesterol from bile. Rather, some nidus might be present on which the nucleation of cholesterol can occur (67, 68). In a model of diet-induced cholesterol gallstone formation in the prairie dog, the nucleation was apparently promoted by secretion of mucosubstances by the gallbladder and prevented by blockade of the mucous secretion with an inhibitor of prostaglandin synthesis (69). Recent evidence indicates that there are inhibitors of nucleation in human bile from both normal subjects and those with supersaturated bile but no gallstones (Gollish, S. H., and Strasberg, S. M. *Hepatology* 1981; 1:512; Howell, J. H. et al. *Hepatology* 1982; 2:728, Abstracts).

Whether these concepts apply to pigment stone formation and the precipitation of calcium salts of bilirubinate, carbonate, phosphate and free fatty acids has not been studied. It may be relevant that Maki and Suzuki (70) were able to coagulate fine suspensions of calcium carbonate or calcium bilirubinate into large concretions *in vitro* by addition of polyanion polymers. These polyanions, such as alginate from seaweed, have properties that resemble the mucoproteins that likely form the matrix of pigment and cholesterol gallstones and, therefore, are presumed to be important in the nucleation and accretion of these stones. Smith and LaMont (71) have demonstrated recently that bilirubin binds to the mucin glycoproteins of gallbladder bile, a process that may involve calcium also. Clinically, calcium bilirubinate stones, found in the common duct after cholecystectomy, even without infected bile, usually have retained nonabsorbable suture material at their center. Likewise, it is not uncommon for CB stones from the Orient to have parasites or their ova as a nidus. The inflammatory debris in infected gallbladders may serve as a nucleus for CB stones, and precipitates of calcium phosphate and/or carbonate may function similarly in black pigment stones, but this remains to be documented directly.

MECHANISMS OF POLYMER FORMATION

Neither the exact structure nor the mechanism of formation of the pigment polymer in BP stones are known. Also undetermined is whether polymerization of pigment occurs in solution in bile, leading to precipitation of polymer, or whether calcium bilirubinate precipitates as a monomer and then polymerizes in the solid state. We have observed an increase in Q_{eq} and a decrease in IVG values during prolonged storage of BP stone powders at room temperature in the dark *in vacuo*. The presence of calcium bilirubinate sludge in human gallbladders that contain BP stones (31) also suggests that polymerization may follow precipitation of calcium bilirubinate. Also in favor of this concept is our observation that CB stones contain a variable proportion of polymer (Ohkubo, H., et al. *Gastroenterology* October 1984; 87: in press). The initiators that trigger the polymerization are likewise unknown. Superoxides and peroxides, which are formed ubiquitously and require special mechanisms to

protect against their destructive effects *in vivo*, are logical candidates (72). Such a mechanism could account also for the fact that the polymer is brown or black, since peroxidation of bilirubin (e.g., in alkaline solutions) leads to formation of multicolored derivatives resulting from oxidation of the methene bridges (73). Mixtures of multiple colored pigments are brown or black (74).

If it may be assumed that solid state polymerization is a slow process, then the rate of stone growth, influencing the interval to development of symptoms, could affect the polymer content of pigment stones removed at surgery. A reasonable hypothesis is that all pigment stones are initiated by nucleation of monomeric calcium bilirubinate on a mucoprotein matrix (71) and that polymerization occurs subsequently in the solid state. The slower the subsequent accretion, the more time is available for polymer formation and the blacker the stones. In infected bile, very high concentrations of bacterial β-glucuronidase would lead to rapid formation of UCB and precipitation of calcium bilirubinate, with rapid growth of stones to symptomatic size. With little time for polymer formation, such stones are of the CB type. Lower activities of β-glucuronidase would engender slower precipitation and accretion of calcium bilirubinate, providing time for polymer formation and the development of BP stones. Experimental evaluation of this hypothesis is needed.

THERAPEUTIC DISSOLUTION OF PIGMENT GALLSTONES

Since the black pigment polymer is virtually insoluble in all known solvents, except when degraded by strong alkali at elevated temperatures (21), there seems little hope of achieving physical dissolution of this component of BP stones *in vivo*. This is consonant with the known resistance of BP stones to dissolution during ductal perfusion with bile acids, monooctanoin, EDTA or combinations thereof (75). The only potential approach may be the injection into the biliary tree of microorganisms or enzymes, tailored to digest preferentially the polyvinyl chains or the methylene bridges linking the dipyrrolic halves of the pigment repeat units in the polymer. Experimentally, disruption of the methylene bridges by diazotization does alter the degree of cross-linking of BP stones, as revealed by a decrease in the Q_{eq} (Ohkubo, H., et al. *Gastroenterology* 1983; 84:1388, Abstract).

Although the polymer cannot be dissolved, the other components of pigment stones may be, as demonstrated by Leuschner et al. (75). By means of ductal perfusion with several multicomponent solutions, they were able to dissolve more than 70% of mixed and CB stones. The active components in the perfusates were: disodium EDTA to chelate calcium and convert the precipitated calcium salts to soluble sodium salts; bile acids and glyceryl monooctanoate to dissolve the lipid-soluble cholesterol, fatty acids and UCB, and trypsin or papain to digest the mucoprotein matrix of the stones. The solutions were buffered to alkaline pH with carnosine, to maximize the chelating effect of the EDTA and the activity of the proteolytic enzymes.

Future approaches to medical treatment of pigment gallstones may stress prevention in subjects at high risk. Dietary adjustments to increase inhibitors of β-glucuronidase in bile, development of cholephilic chelating agents that would be secreted into and decrease Ca^{2+} activity in bile, and inhibitors of mucoprotein secretion in the gallbladder, are among approaches that might be explored.

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DISCUSSION

Nancollas: Actually, if you do not use a buffer and do a pH and calcium titration simultaneously, you can get all of this information without measuring bilirubin. If you know your pKa values and do the speciation properly . . .

Ostrow: But we do not know our pKa values. That is our problem.

Nancollas: The pKa for?

Ostrow: Bilirubin. We do not really know that. That is one of the real touchy issues that we are still working on.

Nancollas: Yes, you must have that first.

Ostrow: That is why we have not used that approach.

Hofmann: You know the pKa. You just do not know relation to physical state, right?

Ostrow: Let's say we know the ideal abstract pKa of bilirubin. But when there is internal hydrogen bonding, when bilirubin is intercalated in the micelles and so forth, the apparent pKa is quite different.

Nancollas: Yes. If one works below the critical micellar concentration, it is a little more accessible.

Ostrow: I would think so.

Hofmann: Do you think the techniques of Dr. Lindblom, where he looks at the order effects in the phospholipids, could tell you, in the mixed system, whether the bilirubin is going into the mixed micelles or the simple bile acid multimers?

Ostrow: Yes. I think they could.

Hofmann: Dr. Lindblom has a mixture of bile acids and lecithin, and he puts it in bilirubin. The lecithin decreases the solubility of the bilirubin. One way to interpret that is that the bilirubin is dissolving in the simple multimers and not in the mixed phospholipid bile acid micelles. Can you use your order parameter to get any information on whether the bilirubin is going in next to the phospholipid or is present only in the phospholipid-poor aggregates?

Lindblom: I think so.

Hofmann: It seems like it would be nice to do that.

Ostrow: I think somebody has to really study these systems with the techniques that have been applied for cholesterol. I do not have the expertise or the equipment to do this. I am hoping to stimulate a superb basic scientist to do this sort of thing because these issues are important. These gallstones are the major types of gallstones seen everywhere but in the Western world.

Small: I was not quite sure that I followed the lecithin story. When you started at a given pH with the bilirubin in undissolved form, then lecithin actually decreased the solubility at a fixed pH?

Ostrow: Right. This was true irrespective of pH within the physiological range and irrespective of the bile acid concentration.

Small: If you started at a high pH and lowered it to pH 7.9, then adding lecithin . . .

Ostrow: Lecithin was all in there before we lowered the pH. I want to emphasize that. Whenever we do the experiments where we start with sodium bilirubinate and then bring the pH down, we have all of the components in solution before we bring the pH down. The exception to that is calcium, which we add at the end.

Small: That actually increased the solubility.

Ostrow: That increased the solubility in one circumstance, and it decreased it in another, depending on the bile acid:phospholipid ratio.

Small: Do you know that the phospholipid is not degrading at that high pH?

Ostrow: Degrading in the sense of?

Small: Forming lysolecithin and fatty acid.

Ostrow: Yes. It is not at high pH for more than a few minutes. The highest pH I used long term was 7.9. I have chromatographed the phospholipids at the end of the incubation, and I found no degradation. I have done that with all of the components. The only thing that degrades in these systems is the bilirubin, due to internal transfer of protons forming mesobiliverdin.