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Non-enzymic hydrolysis of bilirubin mono- and diglucuronide to unconjugated bilirubin in model and native bile systems

Potential role in the formation of gallstones

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Pigment gallstones contain considerable amounts of unconjugated bilirubin (UCB) in the form of calcium bilirubinate and/or bilirubin polymers. Since more than 98% of bile pigments are excreted as conjugates of bilirubin, the source of this UCB needs to be identified. By using a rapid h.p.l.c. method, we compared the non-enzymic hydrolysis of bilirubin monoglucuronide (BMG) and bilirubin diglucuronide (BDG) to UCB in model bile and in native guinea-pig bile. Model biles containing 50 μM solutions of pure BMG and BDG were individually incubated in 25 mM-sodium taurocholate (NaTC) and 0.4 M-imidazole/5 mM-ascorbate buffer (TC-BUF) at 37 °C. Over an 8 h period, BMG hydrolysis produced 4–6 times more UCB than BDG hydrolysis. At pH 7.4, 25% of the BMG was converted into UCB, whereas only 4.5% of BDG was converted into UCB. Hydrolysis rates for both BMG and BDG followed the pH order 7.8 > 7.6 $\simeq$ 7.4 > 7.1. Incubation with Ca^{2+} (6.2 mM) at pH 7.4 in TC-BUF resulted in precipitated bile pigment which, at 100 × magnification, appeared similar to precipitates seen in the bile of patients with pigment gallstones. At pH 7.4, lecithin (crude phosphatidylcholine) (4.2 mM) was a potent inhibitor of hydrolysis of BMG and BDG. The addition of a concentration of cholesterol equimolar with that of lecithin eliminated this inhibitory effect. Guinea-pig gallbladder bile incubated with glucaro-1,4-lactone (an inhibitor of β-glucuronidase) underwent hydrolysis similar to the model bile systems. The non-enzymic hydrolysis of bile pigments, especially BMG, may be an important mechanism of bile-pigment precipitation and, ultimately, of gallstone formation.

INTRODUCTION

Bilirubin IXα (UCB) is the major degradation product of haem catabolism in most mammals. Since UCB is non-polar and water-insoluble at physiological pH (Brodersen, 1979), before its excretion in hepatic bile, UCB is rendered polar by enzymic conjugation of one or both of its propionic acid groups with a polar sugar, the most common conjugating sugar in man being glucuronic acid (Fevery *et al.*, 1977). The action of hepatic glucuronyltransferase, the enzyme responsible for conjugation, results in the formation of BDG, BMG and a variety of other di- and mono-conjugates of bilirubin (Spivak & Carey, 1985). These conjugates of bilirubin make up more than 98% of hepatic bile pigments, with less than 2% being UCB (Fevery *et al.*, 1983; Boonyapisit *et al.*, 1976; Spivak & Carey, 1985; Spivak & Yuey, 1986).

Once excreted, bile pigments play an important role in the formation of gallstones. Biliary sludge, a precursor to gallstones, appears to be primarily made of precipitated calcium bilirubinate and small amounts of conjugated bilirubin (Allen *et al.*, 1981). Although conjugated bilirubin is water-soluble, UCB, calcium bilirubinate and bilirubin polymers are relatively insoluble and therefore precipitate from bile. Cholesterol gallstones almost always have a central bile pigment–protein nidus around which cholesterol appears to coalesce and solidify (Lamont *et al.*, 1984). Pigment gallstones contain very little cholesterol, but are made up of calcium bilirubinate, UCB, bilirubin polymers, proteins and inorganic and organic calcium salts.

The source of UCB for the development of pigment stones is problematic, since less than 2% of bile pigment is normally excreted in the unconjugated form. In the non-cholestatic human, hepatic canalicular bile pigment must be soluble (otherwise obstruction to hepatic bile flow would occur and cholestasis would ensue), but gallbladder bile somehow becomes saturated with insoluble bile pigment. Pigment gallstone formation in the setting of bacterial infection of the biliary tree is undoubtedly related to bacterial β-glucuronidase, which hydrolyses conjugated bilirubin to UCB with subsequent bile-pigment precipitation. Such bacterial infection of the biliary tree is associated with both intrahepatic and ductular gallbladder pigment stones (Nagase *et al.*, 1978). However, most biles are not infected and therefore this mechanism cannot generally explain UCB production in bile (Goodhart *et al.*, 1978).

Animal models of gallstone formation, such as the prairie dog, deer mouse, guinea pig and hamster, have bile containing either BMG as the exclusive, or the predominant, bile pigment (Spivak & Carey, 1985). Although man has BDG as the predominant bile

Abbreviations used: BMG, bilirubin monoglucuronide; BDG, bilirubin diglucuronide; UCB, unconjugated bilirubin (bilirubin IXα); NaTC, sodium taurocholate; TC-BUF, 25 mM-sodium taurocholate/0.4 M-imidazole/5 mM-ascorbate buffer at the designated pH.

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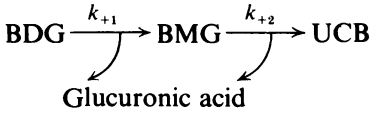
pigment, with BMG making up most of the remaining conjugated pigment, there is evidence that disorders associated with increased BMG production may be associated with gallstones (Allen *et al.*, 1981; Masuda & Nakayama, 1979; Soloway *et al.*, 1977).

We have previously shown that BMG undergoes non-enzymic hydrolysis and that this hydrolysis can lead to bile-pigment precipitation (Spivak *et al.*, 1986). In the present paper we analyse the rates of hydrolysis of BMG and BDG to UCB at low bile-pigment concentration (50 μM) in model biles containing 25 mM-NaTC in an attempt to isolate the kinetics of hydrolysis from the possible interference that precipitation of bile pigment may have on the hydrolysis curves. We also demonstrate that non-enzymic hydrolysis occurs in guinea-pig gallbladder bile and that such hydrolysis can lead to bile-pigment precipitation.

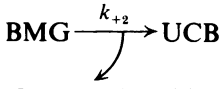
THEORY AND EXPERIMENTAL DESIGN

We theorize that non-enzymic hydrolysis of BMG to UCB may be a more rapid process than that of BDG to UCB, since the former requires the hydrolytic cleavage of only one ester bond whereas the latter requires the cleavage of two ester bonds. Since UCB is not very soluble in bile, especially in the presence of calcium (Berman *et al.*, 1980; Ostrow & Celic, 1984), non-enzymic hydrolysis of BMG may lead to bile-pigment precipitation in the gallbladder between periods of gallbladder emptying, whereas the same concentrations of BDG would form less UCB and therefore be less likely to precipitate or form less of a precipitate.

The hydrolysis of BDG to UCB can be viewed as a two-step process:



The hydrolysis of BMG alone would then be:



Accordingly, the amount of UCB produced during BMG hydrolysis, when plotted against time, would give a typical first-order curve and such a curve would be exponential in shape, whereas UCB production from BDG plotted against time would give a curve sigmoidal in shape and typical of *consecutive* first-order reactions (Moore, 1983). Alternatively, if $k_{+1} \gg k_{+2}$, then the BDG curve might not appear sigmoidal but exponential, and appear similar to the BMG curve, since production of BMG would be extremely rapid.

Although true equilibrium for hydrolysis takes longer, we have chosen an 8 h period for analysis of hydrolysis curves; 8 h represents a typical average inter-meal period of time between major gallbladder emptyings. Analysis of 8 h periods also minimizes the possible effect of slower side reactions, such as oxidation or 2-*O*-, 3-*O*- or 4-*O*-acyl migration, on hydrolysis rates. In spite of this it appears that side reactions do affect hydrolysis rates (see the Results and Discussion sections).

METHODS

Chemicals

All chemicals and buffers used for h.p.l.c. analysis were of the same grade and obtained from the same distributors as previously described (Spivak & Carey, 1985; Spivak & Yuey, 1986). Cholesterol (> 99% pure) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and was recrystallized thrice from hot 95% (v/v) ethanol and stored under vacuum before use. Egg lecithin (phosphatidylcholine; > 97% pure) was obtained from Calbiochem, La Jolla, CA, U.S.A. Imidazole, glucaro-1,4-lactone, ascorbic acid and $CaCl_2$ (dried under vacuum at 70 °C before use) were from Sigma and of the highest quality obtainable.

H.p.l.c. apparatus and elution

As previously described (Spivak & Yuey, 1986), all h.p.l.c. apparatus was obtained from Perkin–Elmer (Elmwood Park, NJ, U.S.A.), except as noted.

Bile-pigment preparation and analysis

Preparative separation of BMG and BDG was performed by the method of Spivak & Carey (1985). Individual eluates containing BMG and BDG were isolated by preparative h.p.l.c. using a 5 μ Altex Ultrasphere ODS column as previously described (Spivak & Carey, 1985). The eluates were evaporated to 0.3 ml by using a Speedvac centrifuge evaporator (Savant Instruments, Hicksville, NY, U.S.A.), and then placed on separate Sep-Pak C_{18} cartridges (Waters Associates, Milford, MA, U.S.A.). The Sep-Pak cartridge with adherent bile pigments was washed with 30 ml of distilled water to remove the bulk of formate ion. The bile pigments were then eluted from the Sep-Pak Cartidge with 3–5 ml of 1% (v/v) acetic acid in methanol. This methanol solution was then evaporated to dryness and the pigments stored at −10 °C for up to 2 weeks.

Bile-pigment analysis was performed by using the h.p.l.c. method of Spivak & Yuey (1986). However, in order to increase the sensitivity of UCB detection to 0.25 μM, the injector volume was increased from 10 μl to 100 μl in the present study. As a result, some loss of resolution of BMG isomers (C-8 and C-12 and 2-*O*-, 3-*O*- and 4-*O*-acyl) was noted. Except for this change, h.p.l.c. elution was performed as previously described (Spivak & Yuey, 1986). H.p.l.c. solvent A was methanolic 0.04 M-sodium acetate, and solvent B was 1% ammonium acetate buffer, pH 4.5. H.p.l.c. elution involved a linear gradient of 65% A/35% B to 95% A in 7 min, 95% A to 75% A in 5 min and then continuing 75% A for an additional 3 min. BDG, BMG and UCB were quantified by using a UCB standard curve (Spivak & Yuey, 1986).

Hydrolysis experiments

***(a)* Preparation of model bile systems.** All aqueous solutions were bubbled with argon before use. For solutions containing bile salt without cholesterol or lecithin, TC-BUF was adjusted at room temperature (24 °C) to 0.25 pH unit above the desired pH at 37 °C (to compensate for the pH decrease at 37 °C). For solutions containing bile salt and lecithin with or without cholesterol, 0.2689 g of NaTC was dissolved in ~2 ml of methanol and then added to 635 μl of lecithin (1 g/10 ml) in chloroform/methanol (3:2, v/v). In experiments with cholesterol, 0.0322 g of cholesterol in ~0.5–1 ml of

chloroform was then added to this solution and the solution was then dried under a steady stream of argon at 45 °C. Then 10 ml of water containing 0.0176 g of ascorbic acid to prevent oxidation of lipids and molecular scrambling of bilirubin to XIIIα and IIIα isomers (Jansen, 1973) was added, followed by 10 ml of 0.8 M-imidazole buffer. These solutions, containing the desired concentration of bile salt, lecithin and cholesterol, were then added to, and mixed with, the solid pure BMG or BDG. The final NaTC concentrations was always 25 mM and the final imidazole buffer concentration was always 0.4 M. When present, the final lecithin and cholesterol concentrations were both 4.2 mM. Before actual incubation at 37 °C was performed, the lipids in solution were allowed to equilibrate for 30 min at 4 °C. Since preliminary experiments demonstrated that the addition of 5 mM-glucaro-1,4-lactone did not change hydrolysis rates, it was not subsequently added to the model bile systems.

(*b*) Preparation of guinea-pig gallbladder bile. Guinea-pig gallbladder bile was obtained by the method of Spivak & Carey (1985). After the bile was layered with argon, solid glucaro-1,4-lactone and solid ascorbic acid were dissolved in the bile using gentle agitation to give a final concentration of 10 mM-ascorbic acid and 5 mM-glucaro-1,4-lactone. The bile was frozen (−20 °C) overnight and then thawed at room temperature the next morning and immediately used for the hydrolysis experiment.

(*c*) Model bile and gallbladder bile incubation. A 1.4 ml portion of freshly prepared model or guinea-pig bile was placed in a sealed 1.5 ml translucent Eppendorf test tube prelayered with argon. This test tube was utilized for all h.p.l.c. injections. A 300 μl sample of the same bile was placed in a sealed 1 mm-path-length optical quartz cuvette prelayered with argon. The cuvette was utilized for observation of a precipitate. At zero time 100 μl of bile was withdrawn from the Eppendorf tube for h.p.l.c. determination of bile-pigment concentration. The Eppendorf test tube was then placed in a dark, temperature-controlled, circulating water bath that maintained temperature at 37 °C and provided continuous gentle agitation. Samples (100 μl) were withdrawn from the Eppendorf test tube and immediately injected on the h.p.l.c. at $t \simeq 0$, 30, 60, 90, 120, 240, 360 and 480 min. At zero time the cuvette was placed in a thermostatically controlled (37°) cuvette holder. Every 30 min the cuvette was manually agitated for a few seconds and observed under a 5 ×-power magnifying glass for the presence of a precipitate or turbidity (this correlated well with precipitates seen at 100 × magnification). Paired experiments demonstrated that the concentrations of individual bile pigments were equal in the Eppendorf tube and the cuvette during hydrolysis; therefore, if the transparent cuvette demonstrated a precipitate, the translucent Eppendorf tube was centrifuged at 4000 rev./min for 3 min and the 100 μl of supernatant was removed for each subsequent h.p.l.c. injection.

RESULTS

A typical h.p.l.c. analysis of hydrolysis of 50 μM-BDG and -BMG in model biles at pH 7.4 containing 25 mM-NaTC with 5 mM-ascorbic acid is shown in Figs. 1(*a*) and 1(*b*) respectively. Note the steady decrease in BDG in Fig. 1(*a*) and BMG in Fig. 1(*b*) with the concomitant rise in UCB. The peaks near BMG represent 2-*O*-, 3-*O*- and 4-*O*-acyl derivatives of BMG that form during alkaline hydrolysis (Blanckaert *et al.*, 1978; Compernolle *et al.*, 1978). These peaks are not well resolved, owing to the large (100 μl) injection volume. Because of the 0.4 M-concentration of the buffer, the pH of the solution did not change significantly (< 0.04 pH unit) during the hydrolysis. Fig. 1(*c*) demonstrates non-enzymic hydrolysis of BMG occurring in native guinea-pig bile containing 11.6 μM-BMG in 10 mM-ascorbic acid and 5 mM-glucaro-1,4-lactone (an inhibitor of β-glucuronidase). Note how similar this hydrolysis is to that of pure BMG. In none of these hydrolysis reactions was a precipitate formed. However, when solid BMG was dissolved in the same guinea-pig bile before hydrolysis to give a final concentration of BMG of 93 μM, bile pigment precipitated after 4 h of hydrolysis. (The precipitate appeared similar to the precipitate shown below in Fig. 6*b*.) In Fig. 2 the percentage conversion of BMG into UCB is plotted against time. Note that, in model biles, BMG hydrolysis is six times faster than BDG hydrolysis and that UCB production from BMG increases exponentially, as one would expect from the first-order reaction:

$$\text{BMG} \rightarrow \text{UCB} + \text{glucuronic acid}$$

On the other hand, BDG hydrolysis shows a sigmoidal curve typical of consecutive first-order reactions, implying:

$$\text{BDG} \rightarrow \text{BMG} \rightarrow \text{UCB} + \text{glucuronic acid}$$

Guinea-pig bile-pigment hydrolysis (same sample as in Fig. 1*c*) gives an exponential curve similar to model-bile BMG, although at a slower rate. Figs. 3(*a*) and 3(*b*) demonstrate the effect of pH on hydrolysis of BMG and BDG respectively. The rate of hydrolysis follows the pH order $7.8 > 7.6 \simeq 7.4 > 7.1$. BMG forms 4–6 times more UCB at any given pH than BDG. The sigmoidal nature of the BDG curve (except at pH 7.1, where the sigmoidal shape of the curve is not apparent because of the low quantity of UCB formed) and the exponential nature of the BMG curve appears to be pH-independent. Fig. 4(*a*) demonstrates the effect of biliary lipids on BMG and BDG hydrolysis at pH 7.4 in 25 mM-NaTC/5 mM-ascorbic acid. The concentration of UCB formed from BMG in solutions containing 25 mM-NaTC plus 4.2 mM-lecithin is $\sim 40\%$ less at 240 min and $\sim 25\%$ less at 480 min than that in solutions containing bile salt alone. Lecithin has a similar effect on BDG hydrolysis (Fig. 4*b*). The addition of 4.2 mM-cholesterol to give a 6:1:1 bile salt/lecithin/cholesterol molar ratio negates the inhibitory effect of lecithin on BMG and BDG non-enzymic hydrolysis, and appears to enhance slightly the hydrolysis during the first 6 h (Figs. 4*a* and 4*b*). Although the addition of lecithin and cholesterol affects the rate of hydrolysis, the curves still remain sigmoidal and exponential for BDG and BMG. Bile-pigment precipitates were not present at the end of 8 h, nor were they present at the end of 24 h in any of the previous experiments, except when additional BMG was added to guinea-pig bile.

The effect of the addition of Ca^{2+} (6.3 mM), with 25 mM-NaTC at pH 7.4 on BMG and BDG hydrolysis is demonstrated in Fig. 5. The hydrolysis curves during the first 6 h are very similar, but at 8 h, when a

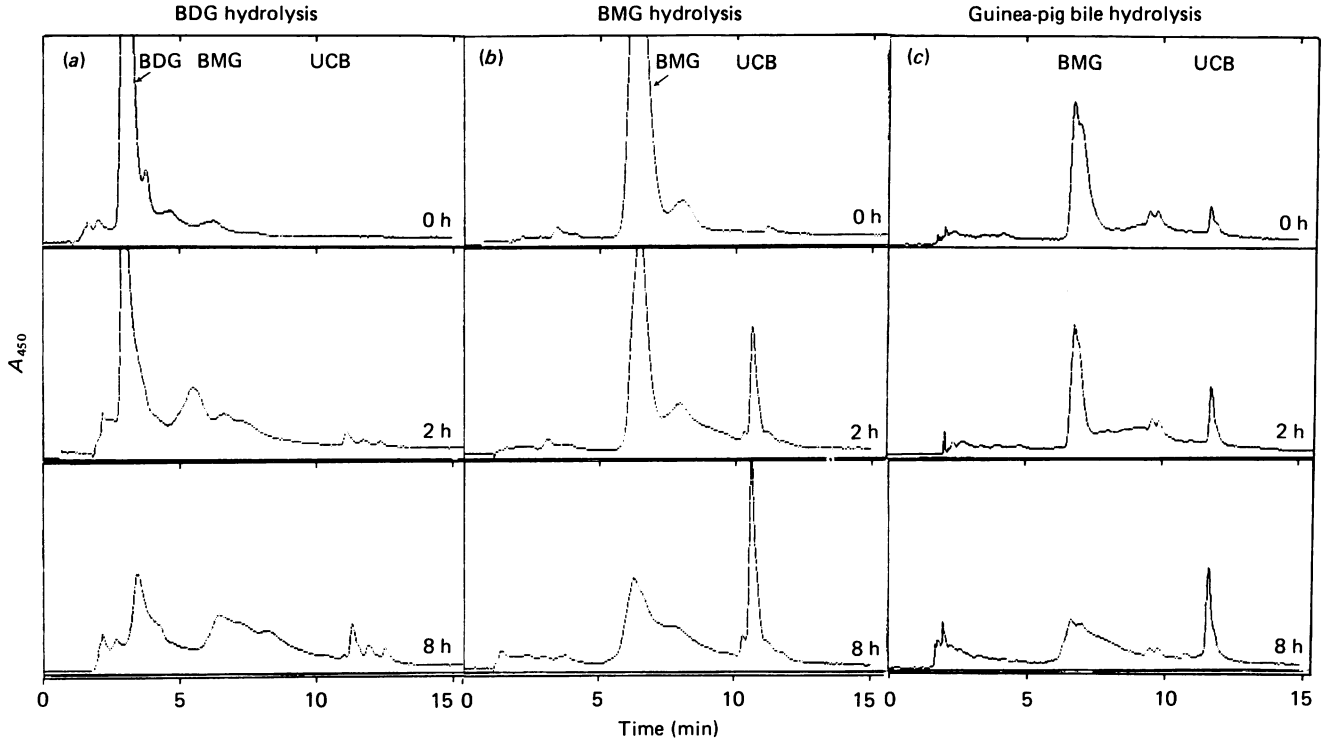


Fig. 1. H.p.l.c. elution profiles from non-enzymic bile-pigment hydrolysis

(*a*) Model bile containing 50 μM-BDG; (*b*) model bile containing 50 μM-BMG; (*c*) guinea-pig gallbladder bile containing 11.2 μM-BMG. In addition to pure bile pigment, model biles contained TC-BUF at pH 7.4. The guinea-pig bile was also at pH 7.4. All biles were incubated at 37 °C with gentle agitation. Note that BDG in model bile hydrolyses to BMG and UCB; however, UCB production from BDG is several times slower than from BMG.

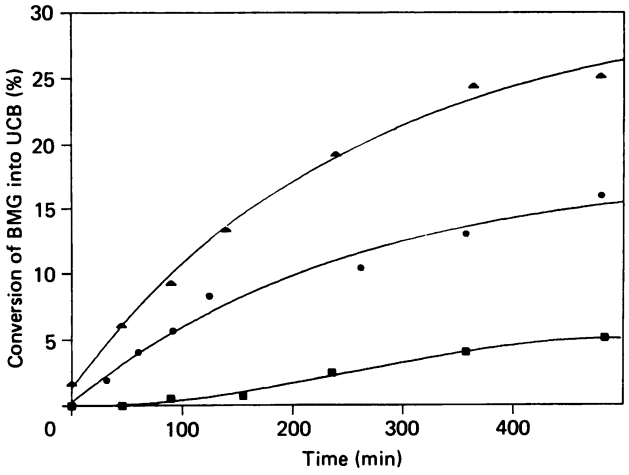


Fig. 2. Comparative hydrolysis of bile pigments, plotted by using computer-generated least-squares regression curves

▲, BMG model bile hydrolysis; ■, BDG model bile hydrolysis; ●, guinea-pig gallbladder-bile hydrolysis. Data is plotted from experiments described in Fig. 1. Note that the ordinate is percentage conversion of BMG into UCB. Both BMG and BDG hydrolyses follow an exponential curve typical of first-order (or pseudo-first-order) reactions (BMG→UCB). The BDG hydrolysis curve is sigmoidal, typical of consecutive first-order reactions (BDG→BMG→UCB). These curves and all subsequent curves were generated by computerized exponential least-squares regression analysis for BMG and third-order polynomial analysis for BDG. BDG data would not fit an exponential curve.

precipitate is noted, the amount of UCB in solution falls off considerably, owing to precipitation of calcium bilirubinate from solution. The fact that precipitation begins to occur from the BDG solution at 2.7 μM implies that the solubility of calcium bilirubinate is extremely limited, although a high degree of supersaturation obviously occurs from the BMG solution. Before precipitation, Ca^{2+} appears to have little effect on the hydrolysis. Fig. 6 demonstrates the similarity between (*a*) native bile pigment precipitates in the gallbladder bile of a patient with pigment gallstones and (*b*) bile-pigment precipitates in the model bile containing BMG plus Ca^{2+}.

DISCUSSION

The hydrolysis of bile pigments can occur by both enzymic and non-enzymic mechanisms. Both bacterial and human β-glucuronidase may play a role in the enzymic hydrolysis; however, since the pH optimum for human β-glucuronidase is 5.0–5.2 (Ho & Ho, 1981), the human enzyme is unlikely to play a major role in bile-pigment hydrolysis, whereas the pH optimum for bacterial β-glucuronidase is closer to that of physiological bile (pH 7.2–8.0). Thus, in infected bile, bacterial enzymic hydrolysis leads to the release of insoluble UCB (Maki, 1981), which may complex with Ca^{2+} to form insoluble calcium bilirubinate (Allen *et al.*, 1981) or may undergo polymerization to a black pigment polymer, both of which are highly insoluble (Black *et al.*, 1982). Alternately, since UCB itself is insoluble at physiological pH, it may precipitate from solution by itself or be

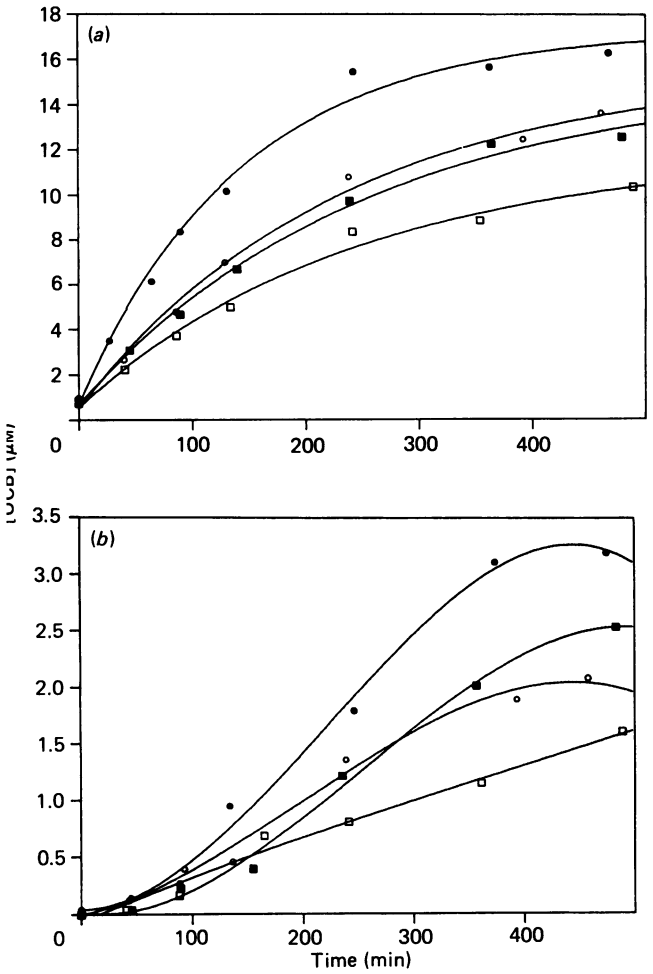


Fig. 3. pH effect on non-enzymic (*a*) BMG and (*b*) BDG hydrolysis

□, pH 7.1; ■, pH 7.4; ○, pH 7.6; ●, pH 7.8. BMG or BDG (50 μM) was incubated in TC-BUF. Samples (100 μl) were removed at the indicated time points and concentration determined by h.p.l.c.

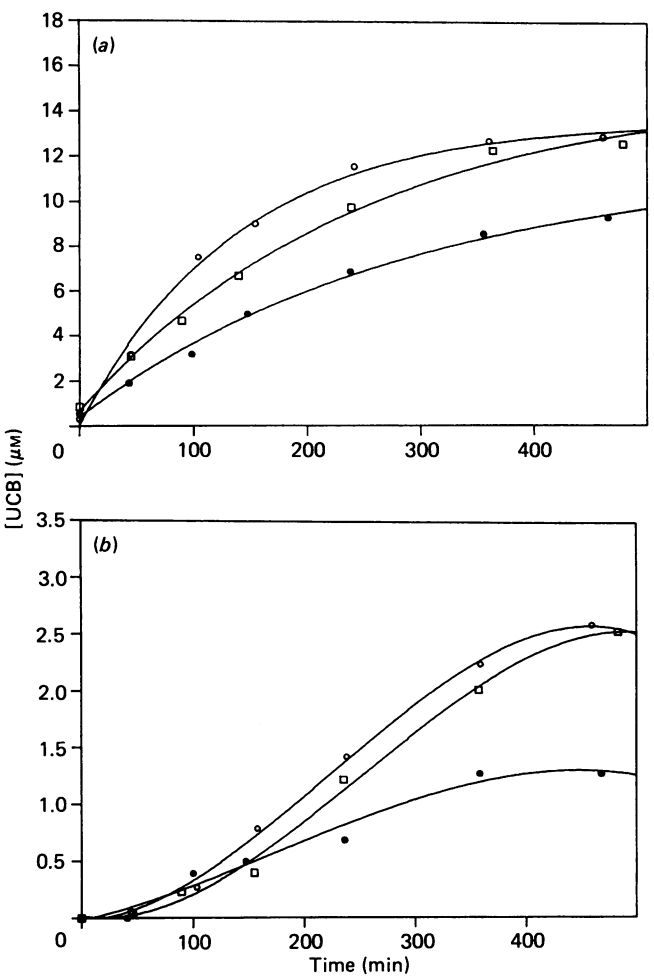


Fig. 4. Cholesterol and lecithin effect on model (*a*) BMG and (*b*) BDG hydrolysis

BMG or BDG (50 μM) was incubated at 37 °C in TC-BUF, pH 7.4. UCB concentrations were determined from 100 μl h.p.l.c. injections. □, No cholesterol or lecithin in TC-BUF; ●, 4.2 mM-lecithin in TC-BUF; ○, 4.2 mM-cholesterol +4.2 mM-lecithin in TC-BUF. Note the inhibitory effect of lecithin but not lecithin+cholesterol on model-bile-pigment hydrolysis. See the text for details of the preparation of mixed micellar solutions.

complexed with bile protein. This initial precipitation forms a biliary sludge which may later form into a pigment stone. If the bile is supersaturated with cholesterol, precipitated bile pigment may form the nidus around which cholesterol forms a cholesterol gallstone (Lamont *et al.*, 1984).

We demonstrate here that non-enzymic hydrolysis occurs readily in model bile systems in the physiological pH range. Glucaro-1,4-lactone, an inhibitor of β-glucuronidase, had no effect on this hydrolysis, so that even if some bacterial contamination of the specimen did occur during sampling, the presence of bacterial β-glucuronidase could not explain this hydrolysis. In addition, we have performed paired experiments using completely aseptic techniques and the same methodology as described here, and have found no difference in the rate of hydrolysis (W. Spivak & W. Yuey, unpublished work). Hence we are convinced that this reaction is non-enzymic.

We purposely used concentrations of bile pigment that are considerably less than those found in most animal models of gallstone formation (except the guinea pig) and in human bile. We avoided higher concentrations of bile pigment because we have found that concentrations of BMG as low as 200 μM would lead to precipitation of bile pigment (Spivak & Yuey, 1985). With a two-phase system (bile pigment in solution and in the solid phase), true hydrolysis curves were not obtainable, since precipitation of pigment lowered the concentration of UCB in solution independent of hydrolysis.

Since production of UCB from BDG depends first on the hydrolysis of BDG to BMG, and since the hydrolysis of BDG to BMG is *not* a rapid process (Fig. 1), BDG hydrolysis to UCB is considerably slower than BMG hydrolysis to UCB. Hence, whereas ~ 25% of BMG is converted into UCB in 8 h at pH 7.4, only ~ 4.5% of BDG is converted into UCB during this same period.

The non-enzymic hydrolysis of bile pigments has been reported by McDonagh *et al.* (1984) and by Spivak & Yuey (1985). This hydrolysis occurs at the weak ester linkage between the 1-*O*-hydroxy group of the conjuga-

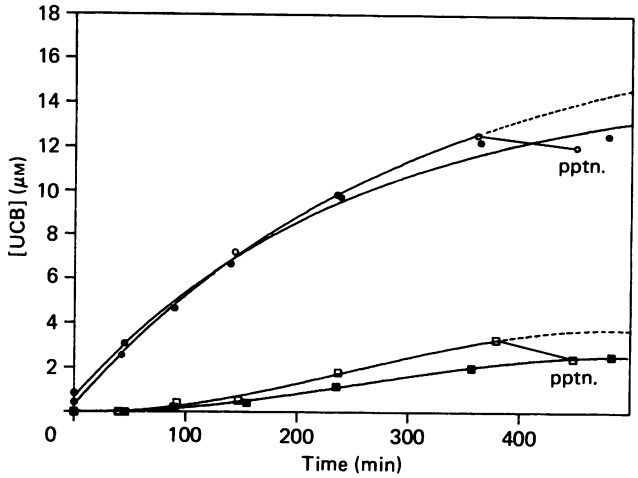


Fig. 5. Effect of Ca^{2+} on BMG and BDG hydrolysis: precipitation of calcium bilirubinate

Hydrolysis of 50 μM-BMG (●) and 50 μM-BDG (■) in TC-BUF, pH 7.4, and hydrolysis of 50 μM-BMG (○) and 50 μM-BDG (□) in TC-BUF containing 6.3 mM-$CaCl_2$. Continuous lines represent the true hydrolysis curves; broken lines represent projected continuation of curves if precipitation ('pptn.') had not occurred. Note that before visible precipitation occurs at $t = 480$ min, the respective BMG and BDG hydrolysis curves with and without Ca^{2+} are similar.

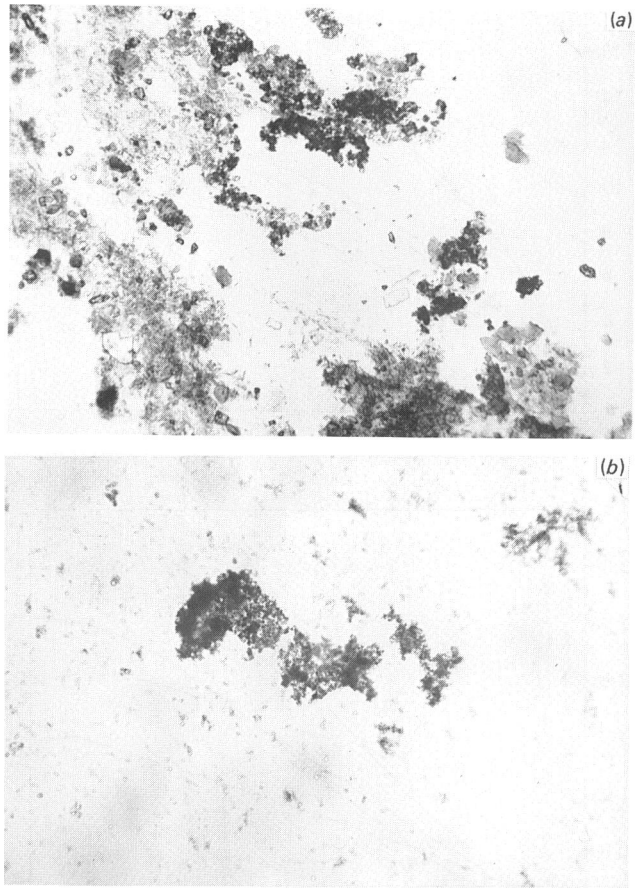


Fig. 6. Comparison of human and model bile pigment precipitates

(*a*) Pigment precipitates seen in gallbladder bile of patient with pigment gallstones. (*b*) Calcium bilirubinate precipitate after 24 h of hydrolysis in model bile containing TC-BUF + 6.3 mM-$CaCl_2$ + 50 μM-BMG at zero time (magnification × 54).

ting sugar and the carboxylic acid group(s) of bilirubin. Presumably nucleophilic attack of OH^- ion at the carbonyl group of the alkali-labile glycosidic ester linkage leads to hydrolysis (pathway A, Scheme 1); similar nucleophilic attack appears to be the mechanism for the formation of methanolic and amide derivatives of bilirubin conjugates in basic ammonia solutions (Jansen & Billing, 1971). Alternately, nucleophilic attack in basic solution may lead to the formation of an ortho acid cyclic intermediate (Compernolle *et al.*, 1978; McDonagh *et al.*, 1984) that then forms the 2-*O*-acyl glycosidic derivatives of UCB (pathway B, Scheme 1).

Although hydrolysis of BMG follows first-order kinetics, the reaction is not truly first-order, since it is pH-dependent. This is further evidence that OH^- ion is important in the hydrolysis reaction. We have elected to monitor UCB appearance in plotting the reaction against time because of the difficulty in plotting BMG disappearance (BMG forms 2-*O*-, 3-*O*- and 4-*O*-acyl isomers that result in broad BMG peaks with time, making it difficult to integrate these peaks). Because the hydrolysis reaction really proceeds as:

$$\text{BMG-1-}O\text{-acyl} + OH^- \xrightarrow{k_{+2}} \text{UCB} + \text{GlcA}$$

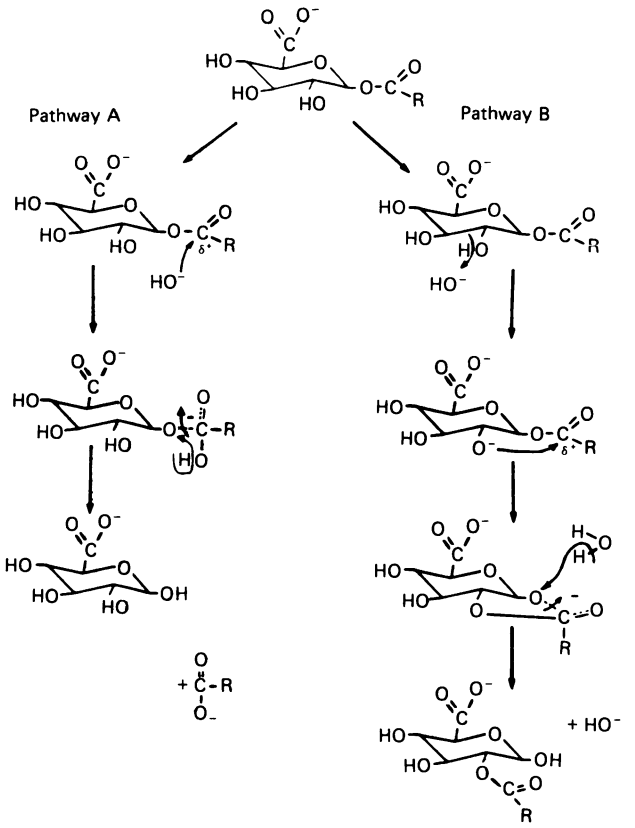
$$\xrightarrow{k_{+3}} \text{BMG-2-}O\text{-acyl}$$

$$\xrightarrow{k_{+4}} \text{BMG-3-}O\text{-acyl}$$

$$\xrightarrow{k_{+5}} \text{BMG-4-}O\text{-acyl}$$

(where GlcA is glucuronic acid) with $k_{+2} > k_{+3} + k_4 + k_{+5}$ the reaction appears to be first-order. The OH^- concentration affects not only the hydrolysis, but also the rate of BMG-acyl-derivative formation. These acyl derivatives are less susceptible to enzymic (Blanckaert *et al.*, 1978) and non-enyzmic hydrolysis (W. Spivak & W. Yuey, unpublished work). Thus the fact that hydrolysis is approximately the same at pH 7.4 as it is at pH 7.6 is probably due to the fact that although 1-*O*-acyl glycosidic hydrolysis is more rapid as the pH rises, so is acyl-derivative formation (Blanckaert *et al.*, 1978). Since the acyl derivatives are less susceptible to hydrolysis, the overall rate of UCB formation decreases.

The addition of lecithin to either the BMG or the BDG system significantly slowed down hydrolysis. We suspect that lecithin incorporation into the NaTC micelle (Carey & Koretsky, 1979) forces conjugated BMG or BDG further into the hydrophobic core of the micelle, thus allowing the electrophilic carbon atom of the carbonyl ester group less exposure to the aqueous alkaline environment. Alternately, the negatively charged phosphodiester region of the polar head groups of lecithin may be closely associated with the electrophilic carbon atom of the carbonyl group of the glycosidic bond of BMG or BDG. The local negative charge surrounding the glycosidic carbonyl atom would be less favourable to the OH^- ion nucleophilic attack from the bulk aqueous environment.



Scheme 1. Proposed pathways for non-enzymic hydrolysis of BMG to UCB (pathway A) and 1-*O*-acyl-BMG conversion into 2-*O*-acyl-BMG (pathway B)

R = unconjugated bilirubin IXα.

Cholesterol, which is closely associated with lecithin in the micelle (Mazer & Carey, 1983), may compete with this bile-pigment–lecithin interaction; therefore, in the presence of cholesterol and lecithin, bile-pigment hydrolysis returns to previous levels or may even be slightly accelerated, as demonstrated in Figs. 4(*a*) and 4(*b*).

In the presence of Ca^{2+}, precipitation of bile pigment occurs, even at the low concentration of UCB formed from BDG. Precipitation was detected by the presence of a very fine solid orange dispersion visible both under 5 × magnification and, with careful inspection, to the naked eye. However, Ca^{2+} does not appear to significantly affect initial hydrolysis rates before precipitation (Fig. 5). This implies that calcium bilirubinate formation is an extramicellar event, for if it were an intramicellar occurrence, one would expect that hydrolysis would be affected by the local removal of product from the hydrolytic environment. Although Ca^{2+} appears to bind to bile salts, perhaps between the SO_3^{2-} of taurine and the OH^- substituent of the cholanic acid ring (Moore, 1984), such binding does not affect the hydrolysis, and it would therefore appear that the ester linkage of bilirubin conjugates is not adjacent to the Ca^{2+} binding area.

The relatively low concentration of soluble UCB found in solution in the presence of Ca^{2+} is consistent with physiological concentrations of UCB found in gallbladder and duodenal bile (Spivak & Carey, 1985). Further studies to analyse the importance of non-enzymic hydrolysis in the formation of bile-pigment precipitates are necessary to determine its physiological role in the pathogenesis of gallstone formation.

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REFERENCES

Allen, B., Berhoft, R., Blanckaert, N., Svanvik, J., Filly, R., Gooding, G. & Way, L. (1981) Am. J. Surg. **141**, 51–56
Berman, M. D., Koretsky, A. P. & Carey, M. C. (1980) Gastroenterology **78**, 1141 (abstr.)
Black, B. E., Carr, S. H., Okhubo, H. & Ostrow, D. (1982) Biopolymers **21**, 601–610
Blanckaert, N., Compernolle, F., Leroy, P., Van Houtte, R., Fevery, J. & Heirwegh, K. P. M. (1978) Biochem. J. **171**, 203–214
Boonyapisit, S., Trotman, W., Ostrow, J. D., Olivieri, P. J. & Gallo, D. J. (1976) J. Lab. Clin. Med. **85**, 857–866
Brodersen, R. (1979) J. Biol. Chem. **254**, 2364–2369
Carey, M. C. & Koretsky, A. P. (1979) Biochem. J. **179**, 675–689
Compernolle, F., Van Hees, G. P., Blanckaert, N. & Heirwegh, K. P. M. (1978) Biochem. J. **171**, 185–201
Fevery, J., Van de Vijver, M., Michiels, R. & Heirwegh, K. P. M. (1977) Biochem. J. **164**, 737–746
Fevery, J., Blanckaert, N., Leroy, P., Michiels, R. & Heirwegh, K. P. M. (1983) Hepatology **3**, 177–183
Goodhart, G. L., Levison, M. G., Trotman, B. W. & Soloway, R. D. (1978) Am. J. Dig. Dis. **23**, 877–882
Ho, K. J. & Ho, L. H. (1981) Proc. Soc. Exp. Biol. Med. **167**, 304–309
Jansen, P. L. M. (1973) Clin. Chem. Acta **49**, 233–240
Jansen, F. H. & Billing, B. H. (1971) Biochem. J. **125**, 917–919
Lamont, J. T., Smith, B. F. & Moore, J. R. L. (1984) Hepatology **4**, 51S–56S
Maki, T. (1981) Ann. Surg. **164**, 90–100
Masuda, H. & Nakayama, F. (1979) J. Lab. Clin. Med. **93**, 353–360
Mazer, N. A. & Carey, M. C. (1983) Biochemistry **22**, 426–442
McDonagh, A. F., Palma, L. A., Louff, J. J. & Wu, T. W. (1984) J. Clin. Invest. **74**, 763–770
Moore, W. J (1983) Basic Physical Chemistry, chapter 13, pp. 277–291, Prentice-Hall, Englewood Cliffs, NJ
Moore, E. W. (1984) Hepatology **4**, 228S–243S
Nagase, M., Tanimura, H., Setoyama, M. & Hikasa, Y. (1978) Am. J. Surg. **135**, 788–790
Ostrow, J. D. & Celic, L. (1984) Gastroenterology **86**, 1334 (abstr.)
Soloway, R. D., Trotman, B. W. & Ostrow, J. D. (1977) Gastroenterology **72**, 167–182
Spivak, W. & Carey, M. C. C. (1985) Biochem. J. **225**, 787–805
Spivak, W. & Yuey, W. (1985) Hepatology **5**, 1019 (abstr.)
Spivak, W. & Yuey, W. (1986) Biochem. J. **234**, 101–109
Spivak, W., DiVenuto, D. & Yuey, W. (1986) Gastroenterology **90**, 1771 (abstr.)

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