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Inhibitory Effect of Bile Acids on the Activity of Human β-Glucuronidase at Its Optimal pH (41170)

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Abstract. The effect of bile acids on β-glucuronidase activity was studied with centrifuged and dialyzed urines as the source of enzyme and phenolphthalein glucuronide as the substrate. The enzyme activity was measured at 56° in acetate buffer pH 5.2. All unconjugated bile acids tested showed no effect on the enzyme activity, primarily due to their low solubility in water. The glycine and taurine conjugates of cholic acid also showed no significant influence on the enzyme. Conjugated deoxycholic and chenodeoxycholic acids, on the other hand, were profoundly inhibitory to the enzyme within the physiological range of concentration in bile. Kinetic studies revealed that taurine conjugates of these two bile acids were pure competitive inhibitors of the enzyme for the substrate; whereas, their glycine conjugates exhibited a mixed competitive and noncompetitive inhibition. Since both urine and bile enzymes have an identical narrow pH activity curve, we conclude that pH and bile acids are two major determinants of β-glucuronidase activity in bile and their alteration may predispose to bilirubin pigment gallstone formation.

β-Glucuronidase (EC 3.2.1.31), a lysosomal acid hydrolase, is believed to play an important role in the formation of bilirubin pigment gallstones for the enzyme deconjugates bilirubin diglucuronide to form water-insoluble bilirubin (1–3). The source of β-glucuronidase in the bile is thought to be primarily, if not entirely, from the intestinal bacteria which invade the biliary tract (1, 2). We have, however, recently demonstrated that the bile from both animals (4) and human (5) contains a large quantity of β-glucuronidase of endogenous origin. The major difficulty encountered in the determination of the β-glucuronidase activity in the bile is due to the presence of abundant bilirubin diglucuronide which, as one of the natural substrates for the enzyme, exerts a competitive inhibition to the enzyme for the substrate such as phenolphthalein glucuronide used in the assay, resulting in a falsely low value of enzyme activity (4, 5).

Recently, we have also found that the pH stability and pH activity curves of biliary β-glucuronidase and a Michaelis constant of 1.46 mmol/liter of phenolphthalein glucuronide are identical to that of the urinary enzyme in man (6). It is, therefore, reasonable to assume that both are the same enzyme. This enzyme has a pH optimum between 5.0 and 5.2 and a single dissociation constant at pH 5.6 in its active site. The enzyme is generally quite inactive in the hepatic duct bile which has a mean pH of 7.7, ranging from as high as 8.6 to as low as 6.9. In the gallbladder, part of the bicarbonate is absorbed; as a result of this and also because of the effect of Donnan membrane equilibrium (7), bladder bile is nearly neutral or even becomes acidic. The gallbladder bile of fasting dogs can be as acid as pH 5.18 to 6.00 (8). The latter is approaching the pH optimum of β-glucuronidase.

Other than conjugated bilirubin and pH, bile acids, one of the major constituents of the bile, may influence β-glucuronidase activity. Our preliminary study in rats, indeed, shows that the biliary β-glucuronidase activity is affected by various bile acids in different ways (4). It is the purpose of the present study to investigate the effect of individual bile acids on the activity of β-glucuronidase and to elucidate the mechanisms of such effect in man. In order to avoid the contamination of conjugated bilirubin and bile acids, human urine, instead of bile, is used as the source of the enzyme. The reaction was carried out at its pH optimum of 5.2.

Materials and Methods. *Determination*

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of β-glucuronidase activity. Fresh, early morning urines collected between 7 AM and 9 AM from a healthy adult male were the source of β-glucuronidase used in all experiments. The urine sample was centrifuged at 1000*g* for 10 min and the supernatant was dialyzed against cold tap water at a flow rate of 25 to 50 ml for 2 hr for removal of dialyzable inhibitor, namely D-glucaro-1,4-lactone (6). Phenolphthalein mono-β-glucuronic acid (Sigma Chemical Co., St. Louis, Mo.) was chosen as the substrate. The β-glucuronidase activity in the dialyzed urine was determined by incubation of a mixture of 0.3 ml of 0.2 *M* acetate buffer, pH 5.2; 0.03 *M*, 0.2 ml of substrate, pH 5.2; and 0.5 ml of urine in a 56° water bath for 1 hr under constant shaking. The reaction was stopped by adding 5 ml of 0.1 *M* 2-amino-2-methyl-1-propanol buffer, pH 11. The intensity of the resulting red color of phenolphthalein in alkali at the end of incubation was measured in a Bausch and Lomb Spectronic 20 spectrophotometer at a wavelength of 55 nm versus water as reference. The activity was expressed as micromoles of phenolphthalein liberated per minute per milliliter of urine.

Effect of bile acids on β-glucuronidase activity. Three major bile acids, namely cholic, deoxycholic, and chenodeoxycholic acids, and their taurine and glycine conjugates in crystalline form of 98 to 99% purity were purchased from Sigma Chemical Company. Since the unconjugated bile acids have a very limited solubility in water, their saturated solutions were prepared with 0.2 *M* acetate buffer, pH 5.2, at room temperature. The bile acid concentrations in such saturated solutions were determined enzymatically using 3α-hydroxysteroid dehydrogenase (9) (Worthington Biochemical Corp., Freehold, N.J.), and were found to be 0.60 μmol/ml for cholic acid (CA), 0.56 μmol/ml for deoxycholic acid (DCA), and negligibly minute for chenodeoxycholic acid (CDCA). Four twofold sequential dilutions with the same acetate buffer were made from the saturated solutions of both CA and DCA. The acetate buffer containing bile acid was used as the buffer for determination of β-glucuronidase activity as described.

We have found in our previous studies (10, 11) that the total bile acid concentration ranges from 3 to 20 μmol/ml in human hepatic bile and 194 ± 65 μmol/ml (mean ±SD) in the gallbladder bile. Since the ratio of CA:CDCA:DCA is roughly 2:2:1 in man (12), we prepared a stock solution of 133.3 μmol/ml with the acetate buffer for each individual conjugated bile acid. This was equivalent to a final concentration of 40 μmol/ml in the reaction medium. Six twofold serial dilutions of stock solution were made with acetate buffer. These solutions were used as the buffer in the determination of β-glucuronidase activity.

Enzyme kinetic studies. Those bile acids which exhibited either a stimulatory or an inhibitory effect on β-glucuronidases were then subjected to enzyme kinetic study as below. The β-glucuronidase activity in a dialyzed urine was determined with six different concentrations of substrate including the stock solution of 0.03 *M* and its five twofold sequential dilutions in the absence or presence of various amounts of bile acids. The maximal velocity or V_{max} and the Michaelis constant or K_m of the enzyme at each concentration of bile acid were then determined from the Lineweaver–Burk reciprocal plot of the enzyme velocity against substrate concentration (13).

Results. *Effect of unconjugated bile acids.* The β-glucuronidase activity was totally unaltered by the presence of unconjugated CA, DCA, and CDCA. This apparently due to the low solubility of these unconjugated bile acids, particularly CDCA which is practically insoluble in water.

Effect of conjugated bile acids. The β-glucuronidase activity was not affected by glycocholic acid (GCA) even at a concentration as high as 40 μmol/ml (Fig. 1). The activity was slightly lowered to a mean of 94% in the presence of taurocholic acid (TCA). However, the degree of suppression varied greatly and did not correlate with the concentration of this bile acid. Therefore, the effect of TCA on β-glucuronidase was considered as minimal.

The taurine and glycine conjugates of both DCA and CDCA, on the contrary, showed profound inhibitory effect on the β-glucuronidase. The inhibition was ob-

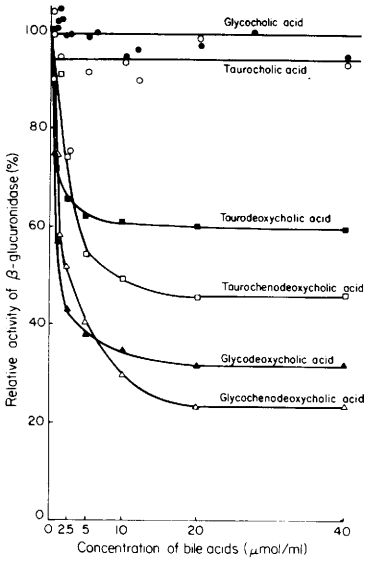

FIG. 1. Effect of glycine and taurine conjugates of cholic, deoxycholic, and chenodeoxycholic acids on β-glucuronidase activity determined at 56° in acetate buffer, pH 5.2, containing 0.006 mmol/ml of the substrate, phenolphthalein glucuronide.

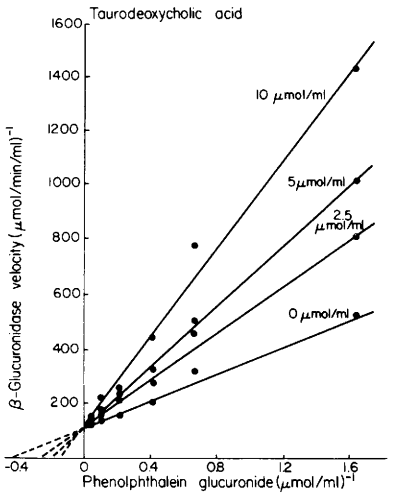

FIG. 2. Lineweaver–Burk reciprocal plot of the velocity of β-glucuronidase against substrate concentration in the presence of 0, 2.5, 5, and 10 μmol/ml of taurodeoxycholic acid. Note that the y intercepts (or $1/V_{max}$) of the four plots are identical; whereas, the x intercepts (or $-1/K_m'$) vary with the concentration of TDCA.

served at a concentration of these bile acids as low as 1.25 μmol/ml, and reached maximum at a concentration 10 to 20 μmol/ml (Fig. 1). In general, the glycine conjugates exerted a greater inhibition than the taurine conjugates, also the conjugated CDCA showed a greater inhibition than the conjugated DCA.

Kinetics of inhibition. The Lineweaver–Burk plots for taurodeoxycholic acid (TDCA) and taurochenodeoxycholic acid (TCDCA) are shown in Figs. 2 and 3. Both plots revealed that all $1/V_{max}$ values derived from the enzyme velocity at three different concentrations of either TDCA or TCDCA were identical to each other and to that of the control in the absence of bile acids; whereas, the apparent Michaelis constant or K_m' increased proportionally with the increase of bile acid concentration. Thus, both TDCA and TCDCA are classified as competitive inhibitors of the substrate for the enzyme.

The relationship between the apparent Michaelis constant (K_m', μmol/ml) and the concentration of either TDCA or TCDCA (i, μmol/ml) is expressed in the following equation: $K_m' = K_m(i/K_i + 1)$ or $K_m' = K_m + (K_m/K_i)i$, where K_m and K_i are the Michaelis constant for phenolphthalein glucuronide and the dissociation constant of the enzyme–bile acid complexes, respectively. By plotting K_m' (y) against i (x), the following equations were obtained:

$$y = 2.465 + 0.474x \quad \text{for TDCA, and}$$
$$y = 2.270 + 1.269x \quad \text{for TCDCA.}$$

Since K_m and K_i are reciprocal of the affinity of β-glucuronidase for substrate and

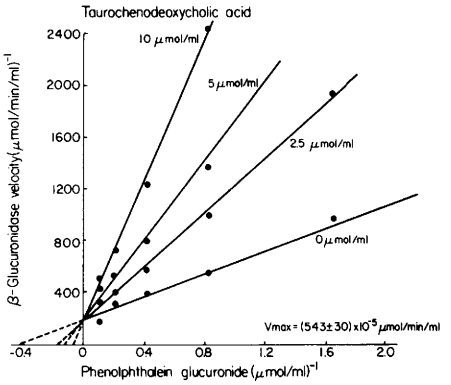

FIG. 3. Lineweaver–Burk plot in the presence of various amounts of taurochenodeoxycholic acid. Note the similar pattern as shown in Fig. 2.

bile acid, respectively, the affinity of the substrate for the enzyme is thus about twice (K_i/K_m = 2.11) as high as that of TDCA but is only about 80% that of TCDCA (K_i/K_m = 0.79). The stronger inhibitory effect of TCDCA than TDCA is also reflected in Fig. 1.

Instead of pure competitive inhibition, the mechanism of enzyme inhibition by the glycine conjugates of both DCA and CDCA is somewhat more complicated. In both cases, not only the K_m' but also the V_{max} increased with the increase in the concentration of these bile acids (Figs. 4 and 5). In other words, it was a case of mixed competitive and noncompetitive inhibition.

Discussion. Our previous study has shown that the biliary β-glucuronidase in rats has a strong affinity for bilirubin diglucuronide (4). Such affinity is 163 times higher than that for phenolphthalein glucuronide. Although the concentration of β-glucuronidase in the bile is quite high and its affinity for conjugated bilirubin very strong, the prevalence of bilirubin pigment gallstones remains low in the Western world. Therefore, under normal physiological condition, this enzyme must be quite inactive in bile.

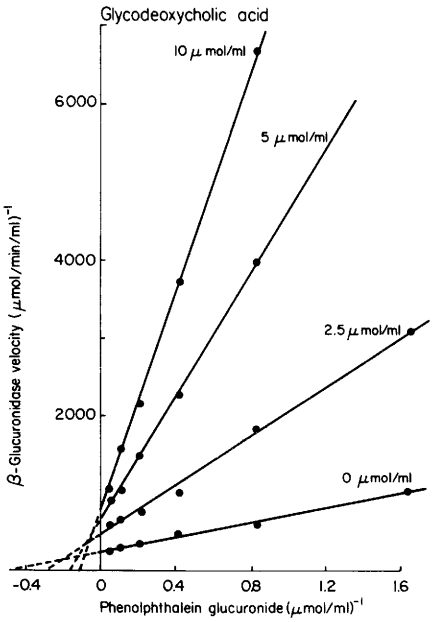

FIG. 4. Lineweaver–Burk plot in the presence of various amounts of glycodeoxycholic acid. Note that both y intercepts (or $1/V_{max}$) and x intercepts (or $-1/K_m'$) vary with the concentration of GDCA.

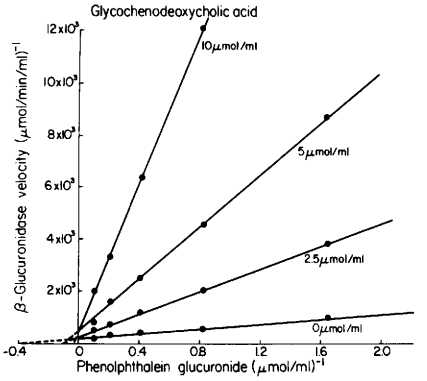

FIG. 5. Lineweaver–Burk plot in the presence of various amounts of glycochenodeoxycholic acid. Note that both y and x intercepts vary with the concentration of GCDCA.

The enzyme has a pH optimum between 5.0 and 5.2 and is only active within a narrow range of pH (4, 6). The high pH of the bile is one of the major factors suppressing the activity of β-glucuronidase.

Other than pH, we demonstrate in the present study that certain bile acids in their physiological concentrations also exert profound inhibitory effect on the activity of β-glucuronidase. Among various species of bile acids, the unconjugated CA, DCA, and CDCA show no effect on the enzyme, primarily due to their poor solubility in water. The glycine and taurine conjugates of CA also show no significant influence on the enzyme (Fig. 1). Conjugated DCA and CDCA, on the other hand, are inhibitory to the enzyme (Fig. 1). The inhibitory effect of the conjugated DC and CDC seems to be directly related to their "monomer" concentration. Their critical micellar concentrations (CMC) under our experimental condition are of the order of several micromoles per milliliter (14, 15). The increase in the inhibitors activity is linearly proportional to their concentration only below such CMC, becomes retarded when approaching CMC, and reaches a maximum when above CMC (Fig. 1).

Conjugated CDCA has, in general, stronger inhibitory effect than the conjugated DCA. Apparently the mechanism of inhibition is also influenced by the type of

conjugation. The taurine conjugates of both bile acids are pure competitive inhibitors of the enzyme for the substrate; whereas, their glycine conjugates exhibit a mixed competitive and noncompetitive type of inhibition. In other words, the taurine conjugates prevent the binding of substrate to the enzyme while the glycine conjugates not only compete substrates for binding to the enzyme but also bind to a site on the enzyme, not identical to the substrate-binding site. The resulting enzyme–inhibitor–substrate complex cannot be converted into product. The glycine conjugates, therefore, show a more profound inhibitory effect on the enzyme than the taurine conjugates.

The molecular mechanism of such inhibition is not clear. Although many competitive inhibitors have a structural resemblance to the substrate and bind to the same site on the enzyme, this is not a necessary criterion for competitive inhibition. It is also possible that the bile acids bind at a different site on the enzyme and inhibit the binding of the substrate to the active site through an allosteric mechanism.

Generally speaking, the hepatic duct bile has a higher pH but a lower bile acid content; whereas, the reverse is true for the gallbladder bile due to reabsorption of bicarbonate and concentration of bile in gallbladder. Therefore, the high pH of the hepatic duct bile and the high concentration of conjugated bile acids in the gallbladder bile play the major role in inhibition of biliary β-glucuronidase.

Contrary to the Western world, bilirubin pigment gallstones are still quite prevalent in the Orients (16). Biliary tract infection is thought to be the major cause of stone formation (1–3). Several species of aerobic and anaerobic enterobacteria are capable of splitting the bile acid conjugates (17). The bacterial enzymes are usually active at the pH of native bile because they have a higher pH optimum than human enzyme (1, 18). However, in a study on a group of 100 patients with pigment gallstones, we could isolate bacteria from only 10% of these cases and failed to detect β-glucuronidase activity from the isolated bacteria (unpublished data). Furthermore, in many instances, the muddy pigment stones continued to develop even after the removal of the original stones, performance of sphincteroplasty, insertion of common bile duct T-tube, and adequate antibiotic therapy. In such sterile hepatic duct bile, the β-glucuronidase remains active (5). This enzyme is presumably of endogenous origin and is activated by both lowering of pH of the infected bile and decrease in the concentration of conjugated bile acids through the action of bacterial hydrolytic enzymes (18). The unconjugated bile acids precipitate together with unconjugated bilirubin in the process of stone formation. Indeed, we have found in the chemical analysis of pigment gallstones from 26 patients, that the bile acids constitute 15 to 20% of the dry weight of the stones (20).

Other than biliary tract infection, there are dietary, metabolic, and therapeutic factors involved in the regulation of the pH and the bile acid constituents of the bile. The pH of the hepatic duct bile is generally alkaline, whereas the pH of gallbladder bile is alkaline when obtained shortly after a meal but declines with fasting which can be as low as 5.18–6.00 as demonstrated in dogs (8). The ratio of the primary to the secondary bile acids is mainly determined by the enzymatic action of the intestinal bacteria flora, while the ratio between their taurine and glycine conjugates is influenced by diet, vitamins, and hormones (21). Our recent studies on the effect of pH (4, 6) and the present study on the effect of bile acids on β-glucuronidase provide a guideline for the further investigation of various factors and conditions affecting the activity of endogenous biliary β-glucuronidase and their relation to bilirubin pigment stone formation.

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