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BILIRUBIN DIGLUCURONIDE FORMATION BY RAT LIVER MICROSOMES: DEMONSTRATION BY AFFINITY AND THIN LAYER CHROMATOGRAPHY OF BILE PIGMENT TETRAPYRROLES

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The conjugates formed in vitro by bilirubin UDP-glucuronyl transferase were studied by examining reaction products as intact tetrapyrroles, rather than as dipyrrolic azoderivatives. Bile pigments were extracted from conventional microsomal enzyme reaction mixtures by affinity chromatography over albumin-agarose, eluted with 50% ethanol, and separated by a silica gel thin layer chromatographic system. In the presence of UDPGA, native and activated microsomal preparations all formed both bilirubin mono- and diglucuronides from unconjugated bilirubin, and bilirubin diglucuronide from bilirubin monoglucuronide. No significant non-enzymatic conversion of mono- to diglucuronide occurred without UDPGA, or in the presence of denatured enzyme. Hence, bilirubin diglucuronide is a major product of bilirubin-UDP-glucuronyl transferase.

Bilirubin is excreted in the bile of normal man and rat as a mixture of glucuronide conjugates, of which bilirubin diglucuronide is the predominant compound (1). Conversion of bilirubin to its more polar glucuronide conjugates results from the activity of the microsomal enzyme bilirubin-UDP-glucuronyl transferase (EC 2.4.1.17) (UDPGT), in a reaction requiring UDP-glucuronic acid (UDPGA) as a cofactor (2). Reports that the exclusive products of the activity of this enzyme are the vinyl and isovinyl isomers of bilirubin-IXα monoglucuronide (BMG) (2,3) led to a search for an alternative enzymatic process for formation of bilirubin diglucuronide (BDG). Formation of BDG from BMG by a UDPGA independent plasma membrane transesterase has been reported (4-6), but subsequently disputed (7-9). The original conclusion that UDPGT forms exclusively the two isomeric bilirubin monoglucuronides was based largely on methods involving the coupling of enzymatically formed conjugated bilirubin to diazotized ethylantranilate at pH 2.7, conditions at which unconjugated bilirubin was reportedly unreactive, followed by thin layer chromatographic (TLC) analysis of the

Abbreviations: UDPGA: uridine diphosphate glucuronic acid, UDPGT: bilirubin-UDP-glucuronyl transferase, BMG: bilirubin-IXα-monoglucuronide, BDG: bilirubin-IXα-diglucuronide, TLC: thin layer chromatography, BSA: bovine serum albumin.

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resulting dipyrrolic azopigments (10). Accordingly, we have reexamined this issue by employing TLC methods allowing examination of the reaction products of UDPGT as intact tetrapyrroles.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats, 270-290 g (Charles River Breeding Laboratories, Wilmington, MA), were housed in a temperature controlled room (22°) with alternating 12 hour light/dark cycles for at least 7 days. They were fed Purina Laboratory Chow ad libitum with free access to water, and were sacrificed on the morning of study without a prior fast.

Bile Pigments. Crystalline unconjugated bilirubin (Sigma, St. Louis, MO) with a molar extinction coefficient of 59,000-61,000, and containing > 90% of the IXα isomer (11), was used without further purification. A weighed amount was initially dissolved in a few drops of 0.25 N NaOH, and rapidly transferred to a solution prepared from crystallized and lyophilized bovine serum albumin (BSA). The final solution contained 0.5 mg/ml of bilirubin in 2.5% albumin, and was adjusted to a pH of 7.9 (22°). This solution remained clear, with no change in extinction at 450 nm, total diazotizable bilirubin concentration, or in isomer composition (11) for at least 72 hours when stored in the dark at 4°C. Unconjugated ^{14}C-bilirubin (SA 15.9 mCi/mmole), prepared by biosynthesis from δ-aminolevulinic acid-4-^{14}C (12), was purchased from Amersham (Arlington Heights, IL), and repurified by alumina column chromatography and recrystallization from chloroform with methanol (12). The final product had a molar extinction coefficient of 61,000, and > 96% of the radioactivity migrated with the unconjugated bilirubin band on thin layer chromatograhy (13,14). Pure BDG and ^{14}C-BMG were isolated from rat bile as previously described (14), and stored at -20° in the dark, in vacuo until needed. BDG, previously shown to be stable for up to 3 months (14), was used within this period. ^{14}C-BMG was used within 7 days of either its initial isolation or of its repurification by TLC (14).

Bilirubin Conjugation. Details of the methods for preparation of rat liver microsomes and of the UDPGT incubation system have been described elsewhere (15). Briefly, unconjugated bilirubin was glucuronidated in vitro by UDPGT in the form of untreated or UDP-N-acetyl glucosamine (UDPNAG)- or digitonin-activated rat liver microsomes, employing a standard incubation mixture (15). The pH of the reaction mixture was buffered to 7.7, and all incubations were for 30 minutes at 37°. For studies employing UDPNAG, the allosteric activator was added to the incubation mixture at a concentration of 3.07 mM. For studies employing digitonin at its optimal concentration of 0.65% (15) as the enzyme activator, thawed microsomes were resuspended in sucrose/EDTA containing the appropriate concentration of digitonin, and maintained in an ice bath for 30 minutes before addition to the reaction mixture.

All incubations were carried out in the presence of subdued light. Multiple replicates of each reaction mixture were always prepared. The amount of conjugated bilirubin formed was assayed in one tube of each set by diazotization with ethylanthranilate at pH 2.7 (2), and in a second tube by extraction and diazotization with sulfanilic acid (16). These two assays have been shown to provide equivalent results (15). Calculation of the quantities of bilirubin conjugated (nmoles conjugated/10 min/mg protein) was carried out using a value of 37.8 for $E^{1\,cm}_{570nM}$ of the conjugated sulfanilic acid azopigment, and of 38.5 for $E^{1\,cm}_{530nM}$ of the conjugated ethylanthranilate azodipyrrole (15).

Studies of the Bilirubin Conjugates Formed. In order to determine which conjugates of bilirubin were formed by 'native', UDPNAG- and digitonin-activated enzyme, the remaining replicates of various reaction mixtures - after 30 minutes incubation - were placed on small (4 ml) columns containing albumin-coupled agarose gel (17). The columns were washed with 0.02 M phosphate buffered saline, pH 7.0, until the washes were colorless. Bile pigments bound to the gel were then eluted in 50% ethanol, and subjected to TLC on silica gel plates as previously described (13,14). For studies employing UDPNAG- or digitonin-activated enzyme, the pigment bands were identified by spraying the plates with diazotized sulfanilic acid, and the relative quantities of each pigment determined with a densitometric TLC scanner (Helena Laboratories,

Beaumont, TX). For studies employing untreated microsomes, which formed smaller quantities of conjugated products, product identification was facilitated by adding 100,000 dpm of unconjugated ^{14}C-bilirubin to the reaction mixture at the start of incubation. Following TLC of the reaction products as described above, locations of the pigment bands were determined by fluorography, employing pre-flashed X-ray film (18). Relative quantities of radiolabeled pigments were then determined by densitometric scanning of the fluorograms.

Incubations with ^{14}C-Bilirubin-Monoglucuronide. Incubations were also conducted employing 100,000 dpm ^{14}C-BMG as a substrate (SA 6.0 mCi/mmole). In these studies, bilirubin was omitted, but the incubation mixture was otherwise identical to that described above. In some studies employing ^{14}C-BMG, the enzyme was activated with 1% deoxycholate, as previously reported (19). To analyze the reaction products in these studies, bile pigments were extracted with albumin-agarose and subject to TLC and fluorography, as described above.

Control Incubations with Inactivated Enzyme. In control studies, UDPGA was omitted and/or the microsomal preparations were inactivated by boiling prior to incubation. Some control studies were done with ^{14}C-BMG, but no unconjugated bilirubin, in order to determine whether any ^{14}C-BDG was formed by a non-enzymatic disproportionation reaction (20) under the conditions of the experimental incubations. In other control incubations which contained unconjugated ^{14}C-bilirubin, diazotization with ethylanthranilate was carried out after adjustment of the pH to 2.7 with glycine/HCl buffer; unreacted bilirubin was extracted with chloroform; and any azopigments formed were then extracted from the aqueous reaction mixture into 2 pentanone:n-butylacetate (2,10). The latter extract was subjected to TLC to identify any observed azodipyrroles (2,10), and the presence of azopigment bands, which were not always identifiable by inspection or densitometry, was sought by fluorography as described above.

RESULTS

Bilirubin UDPGT Activity. Under the conditions employed, UDPGT activity in six studies averaged 3.7±0.4 (SD), 6.1±0.8 and 14.8±1.6 nmoles/10 min/mg protein in untreated, UDPNAG- and digitonin-activated microsomes, respectively, when assayed by the ethylanthranilate diazotization procedure. Corresponding values by the sulfanilic acid diazotization procedure were 3.9±0.5, 6.5±0.7 and 13.7±2.2 nmoles/10 min/mg protein, respectively.

Product Formation. The bile pigments extracted by affinity chromatography from the reaction mixtures incorporating 'native', UDPNAG- or digitonin-activated enzyme and unconjugated bilirubin-IXα as substrate always included both BMG and BDG as determined by their migration on a well characterized TLC system (13,14) (Figure 1). Of the conventional 1-O-acyl glucuronide conjugates detected, the proportion of BDG ranged from 38-60%, the remainder being BMG. Occasionally, bands presumed to represent 2,3 or 4-O-acyl conjugates (20) were observed. There was no consistent difference in the relative proportions of the two principal glucuronide conjugates as a function of whether 'native', UDPNAG- or digitonin-activated enzyme was employed.

Incubations containing non-denatured enzyme, UDPGA and ^{14}C-BMG invariably led to the formation of ^{14}C-BDG. No quantitatively appreciable non-enzymatic conversion of

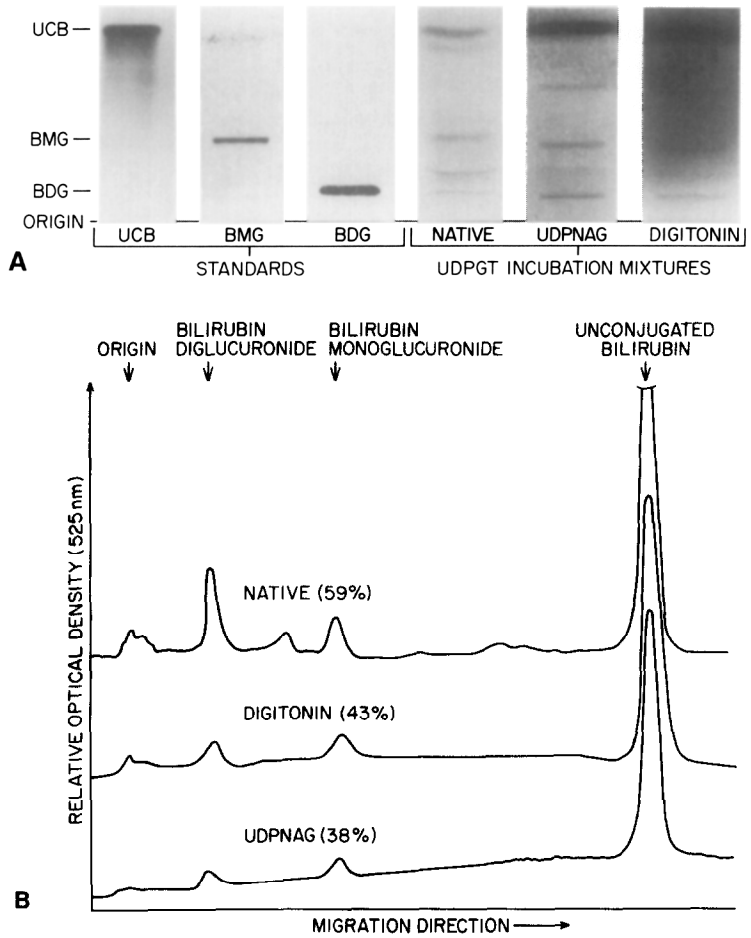

Figure 1. A. Thin layer chromatograms of the bile pigments extracted from various UDPGT incubation mixtures. TLC strips for each of the standards and for the UDPNAG- and digitonin-treated incubation mixtures represent photographs of TLC plates after spraying with diazotized sulfanilic acid. Strip designated 'native' is a fluorogram illustrating the radioactive bile pigments produced by untreated microsomes after incubation with unconjugated ^{14}C-bilirubin. UCB = unconjugated bilirubin; BMG = bilirubin monoglucuronide; BDG = bilirubin diglucuronide.

B. Densitometric scans of typical diazo-treated plates (digitonin, UDPNAG) or fluorograms (native). Figures in parenthesis indicate the proportion of bilirubin glucuronides represented by bilirubin diglucuronide in the scans illustrated.

^{14}C-BMG to BDG was observed in the control incubation mixtures containing denatured enzyme (Figure 2) or lacking UDPGA.

Diazotization of Unconjugated Bilirubin by Ethylanthranilate at pH 2.7. In the control incubation mixtures containing both unlabeled (0.214 mM) and ^{14}C-labeled unconjugated bilirubin, addition of diazotized ethylanthranilate after adjustment of the pH to 2.7 invariably led to the formation of small quantities of azodipyrrole. These were

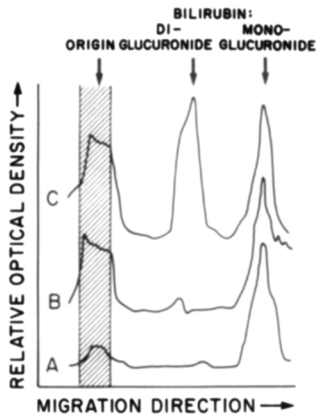

Figure 2. Densitometric scans of fluorograms of reaction products employing deoxycholate-activated UDPGT and ^{14}C-bilirubin monoglucuronide as substrate. A) ^{14}C-bilirubin monoglucuronide standard; B) heat inactivated enzyme; C) viable enzyme.

identified fluorographically as the α_o (unconjugated) azopigment by their migration with an R_f of 0.76 on a well standardized TLC system (2,10). No other azodipyrrole bands were observed. The quantity of azopigments formed under these circumstances was too small to be consistently detected by visible inspection or densitometric scanning of the TLC plates, or by spectrophotometry of the 2-pentanone:n-butylacetate extract at 530 nM.

DISCUSSION

The results described above indicate, not surprisingly, that the reported failure of unconjugated bilirubin to undergo diazotization with ethylanthranilate at pH 2.7 is not absolute. Under conditions which obtain in the conventional assay system for UDPGT (2), some α_o azopigments are likely to be derived from the excess of unconjugated bilirubin present. This would result in an overestimate of the relative amount of monoconjugate formed, and a corresponding underestimate of the amount of diconjugate produced (2,10).

Our studies also demonstrate the formation of both BMG and BDG from unconjugated bilirubin by the reaction mixture, whether 'native', UDPNAG- or digitonin-activated enzyme was employed. Enzymatic conversion of BMG to BDG was also demonstrated. Since the affinity chromatography procedure used to demonstrate these findings may preferentially extract one bilirubin conjugate more efficiently than the other, the quantitative aspects of these observations should not be overemphasized. However, whereas

others have concluded that BMG is the sole product of microsomal UDPGT, based principally on chromatographic analysis of product azoderivatives (2,3), our study, which separated the intact tetrapyrrolic conjugates by TLC; that of Gordon and Goresky (21), which utilized HPLC for the same purpose; and the report of Blanckaert, Gollan and Schmid (7), which employed TLC of the tetrapyrrole derivates formed by alkaline methanolysis, provide incontrovertible evidence for the formation of at least some bilirubin diglucuronide by microsomal enzyme preparations. In the reports of Blanckaert et al (7) and Gordon and Goresky (21), maximal relative bilirubin diglucuronide formation was observed at low bilirubin concentrations (< 35 μM), and predominantly bilirubin monoglucuronide was formed at substrate concentrations in excess of 100 μM. In contrast, we observed appreciable BDG formation at a substrate bilirubin concentration of 214 μM. While the explanation for this difference is unclear, neither of these other studies incorporated albumin in their incubation mixtures to improve solubilization of the bilirubin. Our inclusion of albumin is likely to have significantly reduced the effective or 'available' substrate concentration.

Although the qualitative demonstration of the formation of BDG by rat liver microsome preparations removes the need to postulate the existence of a second bilirubin conjugating enzyme, these results are not incompatible with the existence of a separate plasma membrane enzyme capable of converting BMG to BDG by a transesterification reaction (4-6). The relative significance in vivo of these two potential mechanisms for BDG formation is controversial.

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