

Hepatic Conversion of Bilirubin Monoglucuronide to Diglucuronide in Uridine Diphosphate-Glucuronyl Transferase-Deficient Man and Rat by Bilirubin Glucuronoside Glucuronosyltransferase

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ABSTRACT The microsomal enzyme uridine diphosphate (UDP) glucuronate glucuronyltransferase (E.C. 2.4.1.17) catalyzes formation of bilirubin monoglucuronide from bilirubin and UDPglucuronic acid. Bilirubin glucuronoside glucuronosyltransferase (E.C. 2.4.1.95), an enzyme concentrated in plasma membrane-enriched fractions of rat liver, converts bilirubin monoglucuronide to bilirubin diglucuronide. Bilirubin glucuronoside glucuronosyltransferase activity was studied in homogenates of liver biopsy specimens obtained from patients with the Crigler-Najjar syndrome (Type I) and in subcellular liver fractions of rats homozygous for UDPglucuronate glucuronyltransferase deficiency (Gunn strain). In patients with the Crigler-Najjar syndrome (Type I) and in Gunn rats, hepatic UDPglucuronate glucuronyltransferase activity was not measurable; however, bilirubin glucuronoside glucuronosyltransferase activity was similar to that in normal controls. The subcellular distribution of bilirubin glucuronoside glucuronosyltransferase activity in Gunn rat liver was similar to the distribution observed in normal Wistar rat liver.

When bilirubin monoglucuronide was infused intravenously into Gunn rats, $29 \pm 5\%$ of the conjugated bilirubin excreted in bile was bilirubin diglucuronide. After transplantation of normal Wistar rat kidney, which contained UDPglucuronate glucuronyltransferase activity, in Gunn rats, the serum bilirubin concentration decreased by 80% in 4 days. The major route of bilirubin removal was biliary excretion of conjugated bilirubin, approximately 70% of which was bilirubin

diglucuronide. Although patients with the Crigler-Najjar syndrome (Type I) and Gunn rats lack UDPglucuronate glucuronyltransferase, their livers enzymatically convert bilirubin monoglucuronide to diglucuronide in vitro. Conversion in bilirubin monoglucuronide to diglucuronide was demonstrated in Gunn rats in vivo.

INTRODUCTION

Bilirubin diglucuronide is the major pigment in human and rat bile (1). Uridine diphosphate (UDP)glucuronate glucuronyltransferase (E.C.2.4.1.17)¹ is a microsomal enzyme which catalyzes formation of bilirubin monoglucuronide from unconjugated bilirubin and UDPglucuronic acid. Bilirubin diglucuronide formation requires a second enzyme, bilirubin glucuronoside glucuronosyltransferase (E.C.2.4.1.95), which is concentrated in a plasma membrane-enriched fraction of rat liver, and catalyzes transglucuronidation of 2 mol of bilirubin monoglucuronide to 1 mol of bilirubin diglucuronide and 1 mol of unconjugated bilirubin (2).

Patients with the Crigler-Najjar syndrome (Type I) and homozygous Gunn rats lack hepatic UDPglucuronate glucuronyltransferase activity with bilirubin as a substrate. Their bile lacks bilirubin glucuronides and contains only small amounts of unconjugated bilirubin (3-5). The present study was performed to determine whether patients with the Crigler-Najjar syndrome (Type I) and Gunn rats convert bilirubin monoglucuronide to diglucuronide in vitro and in vivo.

METHODS

Homozygous Gunn rats of both sexes, 250-300 g (Albert Einstein College of Medicine, Bronx, N. Y.) and female

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¹ Abbreviations used in this paper: UDP, uridine diphosphate; α_0 , unconjugated azopigment; δ , conjugated azopigment.

Wistar rats, 300–350 g (Marland Farm, Peekskill, N. Y.) were used. Uridine 5'-monophosphoric acid and D-glucose-6-phosphate were obtained from Sigma Chemical Co., St. Louis, Mo. Glucaro-1, 4-lactone, and *p*-nitrophenyl thymidine 5'-phosphate were from Calbiochem, San Diego, Calif. Ethyl anthranilate was obtained from Eastman Kodak Co., Rochester, N. Y. Lauryl sodium sulfate was from Schwarz/Mann Div., Orangeburg, N. Y., digitonin from J. T. Baker Chemical Co., Phillipsburg, N. J., and Triton X-100 from New England Nuclear, Boston, Mass. For thin layer chromatography, glass plates precoated with silicagel 60 (0.25 mm, 20×20 cm) were used (Merck & Co., Inc., Rahway, N. J.).

Liver biopsy specimens. Control liver specimens were obtained by aspiration biopsy from four patients during elective surgery for cholelithiasis. The tissues were obtained and studied with written permission of the patients. Results of conventional liver function tests and histology of liver sections were normal. Two unrelated 1.5- and 19-yr-old patients with life-long nonhemolytic unconjugated hyperbilirubinemia were studied. Each had serum bilirubin concentrations persistently >18 mg/100 ml since birth. Over 98% of serum bilirubin gave an indirect van den Bergh reaction. Detailed chemical, morphologic, and hematologic studies revealed no evidence of hemolysis or liver injury. Aspiration liver biopsies were obtained with parental consent.

A portion of each liver biopsy was sent for histopathological examination. The rest was immediately weighed and homogenized in ice-cold Tris HCl, 0.005 M, pH 7.8 at 37°C with sucrose, 0.25 M, to give a homogenate containing 25 mg tissue per ml. Conversion of bilirubin monoglucuronide to diglucuronide (2) and UDPglucuronate glucuronyltransferase activity were then determined (6).

Preparation of subcellular fractions. Male homozygous Gunn rats and normal Wistar rats were fasted for 18 h and given water ad libitum before decapitation. The livers were perfused, homogenized and fractionated according to Touster et al. (7). Nuclear (N), mitochondrial and lysosomal (M + L), microsomal, and cytosol fractions were prepared (7). The nuclear and microsomal fractions were subfractionated on discontinuous sucrose gradients (7). The fraction at the 7.5–37.2% interface (N_2), the 37.2% sucrose fraction, including the 37.2–47% interface (N_3), and the 47% sucrose fraction including the pellet (N_4) were collected. From the microsomal fraction, P_2 fraction was collected from the 7.5–34% interface, P_3 was the part of the 34% sucrose fraction above the thick band at the 34–49% interface, and P_4 contained this band and the 49% sucrose fraction.

Conversion of bilirubin monoglucuronide to diglucuronide (bilirubin glucuronoside glucuronosyltransferase) and activities of marker enzymes were measured in fractions stored at 4°C for not longer than 3 days. This treatment had no effect on enzyme activity when studied in liver fractions from Wistar rats.

Enzyme assays. For assay of bilirubin glucuronoside glucuronosyltransferase activity, bilirubin monoglucuronide was prepared biosynthetically (2). Liver homogenate or subfractions containing 50 μ l enzyme, 1 μ mol glucaro-1,4 lactone, and 20 μ mol sodium phosphate in a final volume of 0.2 ml, at pH 6.4 were incubated at 25°C for 60 min. Glucaro-1,4 lactone was included to inhibit β -glucuronidase present in homogenates or cell fractions. The reaction was started by adding bilirubin monoglucuronide, 28–34 μ mol in 50 μ l Tris HCl buffer 0.1 M, pH 7.8. Blanks contained enzyme preparations inactivated by placing in boiling water for 10 min (2). After incubation for 3 min at 37°C, the tubes were transferred to ice and 2 ml ethyl anthranilate diazo reagent was added (8). After 30 min at 25°C, the reaction was terminated by

adding 1 ml 20% ascorbic acid; azopigments were extracted in 0.5 ml methylpropyl ketone/butyl acetate (17/3, vol/vol), and 0.2-ml aliquots were applied to precoated silica gel plates. The plates were developed in chloroform/methanol/water (65/25/3, vol/vol/vol). Two spots appear which contain unconjugated (α_0) or conjugated (δ) azopigments. The spots were scraped off, and extracted in 1 ml methanol. Azopigments were quantitated spectrophotometrically at 530 nm (maximal absorbance). Unconjugated azopigment (α_0) is derived from bilirubin monoglucuronide only, whereas glucuronidated azopigments are derived from the conjugated half of bilirubin monoglucuronide and from bilirubin diglucuronide. The conversion rate of bilirubin monoglucuronide to bilirubin diglucuronide was calculated from $(\delta - \alpha_0)$ reaction minus $(\delta - \alpha_0)$ blank, utilizing E_{530} for bilirubin, 44.4×10^3 M per cm (8). When tested on rat liver, the assay is linear for the duration of incubation and in the range of amount of tissue homogenate or subcellular fractions used.

Bilirubin monoglucuronide formation (UDPglucuronate glucuronyltransferase activity) was determined as previously described (9). Glucose-6-phosphate activity was determined according to Swanson (10). 5'-nucleotidase activity with adenosine monophosphoric acid as substrate, and phosphodiesterase-I activity with *p*-nitrophenyl 5'-thymidilate as substrate were assayed as described by Touster et al. (7). Protein was determined by the method of Lowry et al. (11) with bovine serum albumin as standard. Serum total and conjugated bilirubin were determined by the method of Malloy and Evelyn (12), and serum creatinine was determined as described by Owen et al. (13).

Studies in vivo. Under light ether anesthesia, five male Gunn rats were provided with polyethylene canulae (Intramedic PE 10) in their jugular veins and bile ducts. During a 1-h control period, the rats received 5% dextrose 0.33% sodium chloride intravenously at 0.3 ml/min with a pump. Only rats which produced a minimum of 0.2 ml bile per 10-min period were used. Bilirubin monoglucuronide (346–498 nmol in Tris HCl buffer 0.1 M, pH 8), 4.8 ml, was infused for 16 min. Bile was collected for 3 20-min intervals during the control period, and for 12 20-min periods after infusion. Total conjugated bilirubin was determined and diazo pigments were analyzed by thin layer chromatography for quantitation of bilirubin monoglucuronide and diglucuronide (14).

Two female Gunn rats underwent bilateral nephrectomy under light ether anesthesia. Normal female Wistar rats served as kidney donors. The donor kidney was removed immediately before transplantation and cooled with ice-cold 0.9% NaCl during the procedure. With vascular occlusion clamps, the renal vein and artery of the donor organ were anastomosed with the aorta and vena cava, respectively, of the recipient animal. The ureters were connected with silastic tubing. Operated rats were kept in metabolic cages in a dark room, and were given rat chow and water ad libitum. Urine was collected in 12-h specimens for 4 days. Serum concentrations of bilirubin, conjugated bilirubin, and creatinine were determined daily starting 2 days before surgery. 96 h after surgery, the urinary bladder was catheterized through the urethra with a silastic catheter and the bile duct was cannulated with a polyethylene (PE 10) tubing. Bile and urine were collected for 3 h; the animal was decapitated, and the transplanted kidney was removed. The renal cortex was removed with a razor blade, homogenized in 3 ml/g tissue of Tris HCl buffer, pH 8, 0.005 M with sucrose 0.25 M, and UDPglucuronyl transferase activity was determined (8). Bilirubin monoglucuronide and diglucuronide concentrations in bile and urine were determined as described (2, 14).

RESULTS

Studies in vitro

The distributions of bilirubin glucuronoside glucuronosyltransferase, UDPglucuronate glucuronosyltransferase, and other marker enzymes were measured in subcellular fractions of Gunn rat liver. The distributions were compared to those in normal Wistar rat liver fractions as previously described (2).

None of the Gunn rats had detectable glucuronosyltransferase activity in any liver fraction (Table I). About 80% of glucose-6-phosphatase activity was recovered in the microsomal fraction; after subfractionation the highest specific activity for this enzyme occurred in fraction P₄ (Table I).

The distribution of bilirubin glucuronoside glucuronosyltransferase was similar to the distribution of 5'nucleotidase and phosphodiesterase I activities. These enzymes have a bimodal distribution with highest recovery in the nuclear and microsomal fractions (Table I). The highest specific activities for conversion of bilirubin monoglucuronide to diglucuronide, 5'nucleotidase, and phosphodiesterase I occurred in fractions N₂ and P₂ (Table I).

Liver from normal human controls and patients with the Crigler-Najjar syndrome. Livers from the two patients with the Crigler-Najjar syndrome (Type I) had no detectable glucuronosyltransferase activity. Bilirubin glucuronoside glucuronosyltransferase activities in control livers and in livers from patients with the

TABLE I
Specific Enzyme Activities and Enzyme and Protein Recoveries in Subcellular Fractions of Gunn Rat Livers

	UDPglucuronosyl- transferase	Glucose-6- phosphatase	5'nucleotidase	Phospho- diesterase I	Bilirubin- glucuronoside glucuronosyl- transferase	Protein recovery %
Homogenate	0	0.41±0.2	2.0±0.4	0.49±0.05	1.3±0.35	100
Nuclear	0	0.11±0.01 (2.2±0.6)	1.8±0.22 (33.3±3.0)	0.85±0.23 (29.0±3.0)	2.0±0.1 (34.5±3.5)	15.9±4.8
M + L	0	0.9±0.35 (10.1±1.0)	2.8±0.2 (3.5±1.2)	1.8±0.06 (4.0±0.09)	0.09±0.01 (3.2±1.5)	30.02±5.1
Microsomal	0	0.9±0.35 (79.2±6.5)	2.8±0.2 (58.1±3.1)	1.8±0.06 (61.5±9.3)	1.3±0.08 (47.8±8.4)	21.9±9.0
Supernate	0	0.02±0.02 (1.0±0.06)	0.2±0.02 (2.0±1.1)	0.03±0.02 (1.1±0.42)	0.11±0.03 (0.8±0.3)	28.1±3.8
N ₂	0	0.01±0.005 (0.15±0.05)	70.2±11.0 (4.61±0.08)	6.3±0.55 (4.1±0.78)	22.6±4.0 (4.21±1.0)	0.24±0.08
N ₃	0	0.33±0.03 (1.1±0.4)	9.2±0.9 (22.95±2.5)	1.0±0.38 (16.12±2.0)	19.1±7.0 (14.15±4.1)	7.12±0.6
N ₄	0	0.02±0.01 (1.0±0.45)	4.28±1.1 (6.9±1.02)	0.3±0.05 (6.3±1.2)	1.5±0 (6.10±1.8)	7.0±0.6
P ₂	0	0.58±0.12 (0.80±0.21)	30.2±3.1 (20.08±2.1)	12.1±3.1 (24.6±3.5)	17.2±5.0 (12.22±3.1)	1.25±0.2
P ₃	0	2.1±0.12 (11.2±1.2)	8.2±1.5 (11.10±1.5)	7.0±0.6 (20.08±3.5)	5.1±1.0 (14.11±4.9)	5.8±1.2
P ₄	0	3.8±0.9 (67.6±5.2)	2.4±0.4 (20.1±2.0)	1.2±0.4 (17.12±3.3)	1.28±0 (10.1±4.8)	14.8±1.3

Subcellular fractions were prepared from Gunn rat liver homogenates. Enzyme activities and protein were determined as described in Methods. The data are means±SE of six experiments. Enzyme activities are expressed per milligram protein in the following manner: UDPglucuronosyltransferase, nmol/20 min; bilirubin glucuronoside glucuronosyltransferase, nmol/min; glucose-6-phosphatase, μmol/20 min; phosphodiesterase I, μmol/15 min; and 5'nucleotidase, μmol/20 min. Recovery of enzyme activity or protein is expressed as percent of total homogenate enzyme activity or protein, and appears in parentheses.

TABLE II
UDPglucuronyltransferase and Bilirubin Glucuronoside Glucuronosyltransferase Activities in Human Liver Homogenates

	UDPglucuronyltransferase		Bilirubin glucuronoside glucuronosyltransferase	
	Per g tissue	Per mg protein	Per g tissue	Per mg protein
	nmol/20 min		nmol/min	
Control (mean±SE of four specimens)	652.5±25.8	3.32±0.10	290±25	1.48±0.33
Crigler-Najjar syndrome, type I (two cases)	0, 0	0, 0	301, 298	1.50, 1.51

Results are expressed as mean±SE or as individual values.

Crigler-Najjar syndrome (Type I) were not significantly different (Table II).

Studies in vivo

Bilirubin monoglucuronide infusion. After intravenous infusion of 475±25 nmol bilirubin monoglucuronide, which contained only 0–2 nmol bilirubin diglucuronide, 316±20 nmol conjugated bilirubin was recovered in bile in 4 h. Thin layer chromatography of the azo pigments from control Gunn rat bile revealed no glucuronide band (δ), a faint α₀, and an unidentified band immediately behind the α₀ band. For calculation of bilirubin diglucuronide and monoglucuronide (Table III) in Gunn rat bile following infusion of bilirubin monoglucuronide, it was assumed that the contribution to the α₀ band by unconjugated bilirubin in bile remained constant. Of conjugated bilirubin excreted in bile, 29±5% (SE) was bilirubin diglucuronide (Table III).

Renal transplantation. Serum total bilirubin and creatinine concentrations before and after renal transplantation in Gunn rats are shown in Fig. 1. Serum creatinine concentration remained within normal limits and serum bilirubin concentrations decreased by 80%. Because of lability of conjugated bilirubin, urine collected in metabolic cages could not be analyzed quantitatively for its pigments; however, analysis

obtained on termination of the experiment revealed that urinary excretion of bilirubin was much less than was biliary excretion of bilirubin (Table IV). UDPglucuronyltransferase activity in cortical homogenates from transplanted kidneys was 310 and 375 nmol/20 min per g kidney tissue (wet weight). Renal cortical homogenates from six normal Wistar rats had glucuronyltransferase activity of 210±25 nmol/20 min per g wet weight. No glucuronyltransferase activity was detected in homogenates of renal cortex from four control Gunn rats. Bilirubin glucuronoside glucuronosyltransferase activity in renal cortical homogenates in six normal and four Gunn rats was 202±28 nmol/g per min and 216±32 nmol/g per min, respectively.

DISCUSSION

Conversion of bilirubin to a more polar conjugate is essential for its biliary excretion (15). Recent studies (2) of the bilirubin conjugating system in rat and human liver reveal that two distinct enzymes are required for formation of bilirubin diglucuronide. UDPglucuronate glucuronyltransferase the microsomal enzyme required for conversion of bilirubin to bilirubin monoglucuronide, is present in the renal cortex (16), small intestinal mucosa (17), and liver. Bilirubin monoglucuronide transglucuronidation activity is present in rat liver and renal cortex (18), but not in erythrocyte

TABLE III
Biliary Excretion of Conjugated Bilirubin after Infusion of Bilirubin Monoglucuronide in Five Gunn Rats

	Bilirubin monoglucuronide	Bilirubin diglucuronide	Total conjugated bilirubin
	nmol		
Control bile (1 h)	0	0	1.8±0.4
Injected	475±25	0–2	475±27
Recovered in bile in 4 h	222±18	94±12	316±20

Results are expressed as mean±SE.

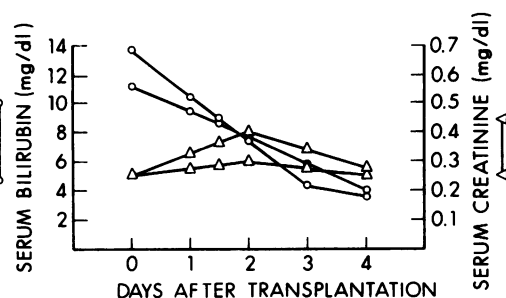


FIGURE 1 Serum bilirubin (○ — ○) and creatinine (▲ — ▲) concentrations of the two Gunn rats before and after transplantation with normal Wistar rat kidneys.

TABLE IV
Biliary and Urinary Excretion of Conjugated Bilirubin by Two Gunn Rats 4 Days after Transplantation of a Normal Wistar Rat Kidney

	Bilirubin monoglucuronide	Bilirubin diglucuronide	Total conjugated bilirubin
	nmol/h		
Excretion in bile	137, 177	357, 429	502, 598
Excretion in urine*	4.8, 7.2	31.6, 44.4	38, 50

* Collected via catheter for 3 h before termination of experiment.

membranes or homogenates of heart or striated muscle. After subcellular fractionation of Wistar rat liver by the method of Touster et al. (7), the highest specific activity for bilirubin monoglucuronide transglucuronidation occurred in fraction N₂. By electronmicroscopy, this fraction consisted of sheets of plasma membrane and was enriched in the activities of several plasma membrane marker enzymes, including 5' nucleotidase and phosphodiesterase I (2).

The distribution of bilirubin glucuronoside glucuronosyltransferase activity in subcellular fractions of Gunn rat liver is similar to its distribution in normal Wistar rat liver fractions (2). A subfraction of Wistar rat liver plasma membrane which was prepared by zonal centrifugation and is highly enriched in bile canaliculi, had a specific enzyme activity three times greater than was present in other plasma membrane fractions.²

After intravenous infusion of bilirubin monoglucuronide, conversion to bilirubin diglucuronide occurred in Gunn rats in vivo. Bilirubin can be excreted in bile as a mono- or diglucuronide conjugate. When an equimolar mixture of [³H]bilirubin monoglucuronide and [³H]bilirubin diglucuronide was injected intravenously in Sprague-Dawley rats, 20% of labeled conjugated bilirubin was excreted in the first 10 min, primarily as bilirubin diglucuronide. Subsequently, the two glucuronide conjugates were excreted in the same proportion as when injected. 90% of labeled conjugated bilirubin was excreted in bile in 60 min. Approximately 30% of injected bilirubin monoglucuronide was recovered in bile as bilirubin diglucuronide.³ This observation suggests that bilirubin diglucuronide may be cleared more readily by the liver than is bilirubin monoglucuronide.

The present study confirms the presence of glucuronyltransferase activity in normal rat kidney (19). Glucuronyltransferase activity in the cortex of the trans-

planted kidney was about 60% higher than that in the renal cortex of normal Wistar rats. Similar findings were observed by Foliot et al. (19). The mechanism for this effect is unknown. After renal transplantation from a normal rat into a Gunn rat, conjugated bilirubin was excreted predominantly in bile and, to a lesser extent, in urine. The transplanted normal rat kidney served as an internal source of glucuronyltransferase. The conjugate excreted in bile was predominantly bilirubin diglucuronide. Since the kidney from normal and Gunn rats has bilirubin glucuronoside glucuronosyltransferase activity, it is not possible to determine from these experiments whether the diglucuronide excreted in bile is produced in the transplanted kidney or in the liver of the Gunn rat. Correction of the metabolic defect in Gunn rats by transplantation of normal rat kidney suggests that renal transplantation may benefit patients with the Crigler-Najjar syndrome (Type I) who usually die of kernicterus in infancy or early childhood (5). Further studies are required to characterize UDPglucuronyltransferase in human kidney before this can be attempted.

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