Hepatic Disposition and Biliary Excretion of Bilirubin and Bilirubin Glucuronides in Intact Rats

Differential Processing of Pigments Derived from Intra- and Extrahepatic Sources

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Abstract

Mechanisms for transport of bilirubin and its conjugates in hepatocytes have not been defined. We investigated the hepatic processing of bilirubin glucuronides and their precursors, and characterized the disposition of bile pigments arising from intra- versus extrahepatic sources. Tracer doses of purified radiolabeled biliverdin, bilirubin, bilirubin monoglucuronide (BMG) or diglucuronide (BDG) were administered intravenously to intact normal or jaundiced homozygous Gunn rats. Rapid sequential analysis of radiolabeled BMG and BDG in bile revealed comparable excretion patterns following biliverdin and bilirubin injection, with BDG as the major pigment. Biliary excretion of radiolabeled conjugates from injected BMG was more rapid, with BMG predominating. Excretion of injected BDG in normal rats and BMG or BDG in Gunn rats was virtually identical to that of unaltered BMG in normal rats. Model independent analysis by deconvolution provided objective comparison of the disposition of radiolabeled pigments from the different sources. These findings indicate that bilirubin glucuronides formed in the liver from endogenous (hepatic) and exogenous (extrahepatic) sources of bilirubin follow a similar excretory pathway. BMG formed endogenously is converted preferentially to BDG, whereas circulating BMG is excreted predominantly unchanged. Exogenous conjugated bilirubins are excreted more rapidly than those generated intrahepatically, by a transcellular pathway that is largely independent of the conjugation system.

Introduction

The metabolism and disposition of heme and its degradation products is a pivotal function of the mammalian liver (1), culminating in biliary excretion of the hydrophilic conjugates of bilirubin-IXα (bilirubin). Under physiologic conditions, 65–75% of daily bilirubin production occurs in the extrahepatic reticuloendothelial system by degradation of the hemoglobin-heme of senescent erythrocytes. Following its release into the circulation, unconjugated bilirubin is bound firmly to albumin from whence it is taken up efficiently by the liver. The remaining 25–35% of bilirubin production results from turnover of hepatic heme and hemoproteins, of which the family of cytochromes P-450 is the most important (1–3). In both situations, the protoporphyrin heme moiety is degraded initially to the green-colored intermediate, biliverdin-IXα (biliverdin) by the microsomal heme oxygenase system (4), and then to bilirubin via biliverdin reductase in the cytosol or at the interface of the endoplasmic reticulum and cytosol (5). Bilirubin conjugation occurs predominantly in the liver, and is catalyzed by uridine diphosphate (UDP)-glucuronyltransferase in the smooth and rough endoplasmic reticulum (6, 7). The end products of this reaction, bilirubin mono- and diglucuronide, are transported to the canalicular pole of the hepatocyte and excreted into bile by mechanisms that are poorly understood (1).

In cholestasis associated with hepatobiliary diseases, a portion of the bilirubin glucuronides destined for excretion in bile reflux back into the circulation. In this situation, unconjugated bilirubin may also accumulate in plasma, presumably from hepatocellular reflux following deconjugation of bilirubin glucuronides formed in the liver (8, 9). The plasma from such patients thus contains a variety of pigment species, namely unconjugated bilirubin, bilirubin diglucuronide, and bilirubin monoglucuronide (with the latter predominating; 10), as well as variable amounts of a conjugated fraction that is bound covalently to albumin (11, 12). With the exception of the covalently bound fraction, correction of the underlying disorder results in rapid clearance of these pigments from the circulation by hepatic uptake and subsequent biliary excretion (13). Thus, the liver simultaneously processes unconjugated and conjugated bilirubins derived from both intrahepatic or "endogenous" and extrahepatic or "exogenous" sources, but it remains to be shown whether these various bile pigment fractions share the same excretory pathways. Indeed, preliminary kinetic evidence suggests that mixing between endogenously and exogenously derived bilirubin (14) and bilirubin monoglucuronide (15) may be incomplete, and that compartmentation of these intermediates may occur within hepatocytes. The present study was undertaken to systematically evaluate the hepatic disposition and biliary excretion of the major mammalian bile pigments arising from intra- and extrahepatic sources.

Methods

Purified and radiolabeled bile pigments. Radiolabeled bilirubin (specific activity 5–30 μCi/μmol for ^{3}H and 5–20 μCi/μmol for [^{14}C]bilirubin) was prepared biosynthetically (16) from the bile of rats injected with δ-amino [3,5-^{3}H]levulinic acid (1.8 Ci/mmol) or δ-amino [5-^{14}C]levulinic acid (49 mCi/mmol; New England Nuclear, Boston, MA). Purified [^{3}H]- or [^{14}C]bilirubin was 96–98% IXα isomer by thin-layer chromatography (TLC; 17). [^{3}H]- or [^{14}C]Biliverdin-IXα of comparable specific activity

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was prepared from crystalline radiolabeled bilirubin (18). TLC analysis (18) demonstrated 97–98% purity of biliverdin with < 1% contamination with bilirubin.

[^{3}H]Bilirubin mono- and diglucuronide were extracted and purified from bile by modification of an existing procedure (6). Bile (5 ml) obtained from rats infused with ^{3}H- or ^{14}C-labeled δ-aminolevulinic acid was extracted with 5 ml of pure ethanol/chloroform (1:1 vol/vol) and 5 ml of 0.1 M hydrochloric acid buffered with glycine to pH 1.8 and saturated with sodium chloride; 50 mg of ascorbic acid was added as an antioxidant. After phase separation by centrifugation, the organic layer was evaporated to ~ 1 ml under a stream of argon. The extract was then plated on a 20 × 20 cm tapered preparative Silica gel plate with a preadsorbent zone (Analtech, Newark, DE) and developed for 1 h in chloroform/methanol/water, 60:30:6 vol/vol. The bilirubin mono- and diglucuronide bands (R_f values 0.5 and 0.2, respectively) were scraped separately into 4 ml acidified methanol (1% glacial acetic acid in methanol), shaken vigorously and centrifuged for 20 s (centrifuge 5414, Eppendorf, Westbury, NY). The supernatants were filtered separately through 5 × 5 mm columns of diatomaceous earth to remove residual silica particles, and the eluant evaporated to dryness under argon. The entire isolation procedure was completed in < 2.5 h and was performed at 4°C in subdued light. A second chromatographic run was necessary for the isolation of pure bilirubin diglucuronide, but this was not essential for the preparation of bilirubin monoglucuronide. The bilirubin diglucuronide and monoglucuronide (a mixture of C8 and C12 isomers) were 98–99% pure as assessed by high-performance liquid chromatography (HPLC; 19), with specific activities of 40 to 55 μCi/μmol. It was shown previously that δ-amino [5-^{14}C]levulinic acid did not comigrate with the bilirubin glucuronides on TLC, and that no bile salts were detectable in these preparations by enzymatic assay (20). The pigments were stored in the dark under argon at −70°C, and in this condition were stable for at least 14 d.

Specific activities of the purified radiolabeled pigments were obtained assuming $E_{377} = 49.7 \times 10^3$ liter · mol^{-1} · cm^{-1} for biliverdin in methanol (18); $E_{453} = 62 \times 10^3$ liter · mol^{-1} · cm^{-1} for bilirubin in chloroform (21) and $E_{453} = 60 \times 10^3$ liter · mol^{-1} · cm^{-1} for bilirubin mono- and diglucuronide in methanol (8). All preparative and analytical procedures were carried out in subdued light to minimize photodegradation of pigments.

Animal preparation. Male Sprague-Dawley rats (Charles River Breeding Laboratories, North Wilmington, MA) weighing 304±15 g (mean±SD) and heterozygous and homozygous Gunn rats (282±27 g) were maintained on Purina chow ad lib. Under light ether anesthesia the left jugular vein was cannulated with 40 cm of PE-10 polyethylene tubing (Clay Adams Div., Becton, Dickinson & Co., Parsippany, NJ), and the bile duct with a 19-cm length of tubing. Surgery was commenced between 7 and 8 a.m., and total anesthesia time was ~ 30 min. The rats were placed in wire restraining cages with access to rat chow and were hydrated by infusion of 0.15 M saline at 1.5 ml/h for the duration of the experiment. Animals were warmed continuously by infrared lamps. The rats were allowed a 45- to 75-min recovery period following surgery, during which time constant bile flow was achieved with rates of 1.3±0.3 ml/h. Bile flow remained stable throughout the subsequent 120-min experimental period. The duration of the recovery period was selected to optimize the opposing effects of recovery from ether anesthesia versus sustained biliary drainage on both bilirubin glucuronidation (22, 23) and bile salt depletion (24). No difference was observed in the patterns of bilirubin conjugates excreted in animals allowed recovery periods of 45 min or 75 min. In separate experiments, results, obtained for rats either anesthetized with pentobarbital (45 mg/kg i.p.) or allowed a period of 20 h to recover from ether anesthesia prior to the administration of radiolabeled pigments (25), were virtually identical to those reported in this study.

Administration of radiolabeled pigments. Aliquots of biliverdin or bilirubin were solubilized in 30 μl 0.05 M sodium hydroxide and immediately brought up in normal rat serum; bilirubin mono- or diglucuronide were reconstituted directly in rat serum. Mixing of pigments for dual-label experiments was performed just before injection. Radiolabeled pigments were injected as a bolus over 20 s into normal rats, either individually using ^{3}H-labeled pigments; [^{3}H]biliverdin (n = 5), [^{3}H]bilirubin (n = 4), [^{3}H]bilirubin monoglucuronide (n = 4), [^{3}H]bilirubin diglucuronide (n = 1), or in combination with a second radiolabeled pigment; [^{3}H]biliverdin/[^{14}C]bilirubin (n = 4), [^{14}C]bilirubin/[^{3}H]bilirubin monoglucuronide (n = 3), [^{14}C]bilirubin/[^{3}H]bilirubin diglucuronide (n = 3). For paired heterozygous and homozygous Gunn rats, [^{14}C]bilirubin monoglucuronide/[^{3}H]bilirubin diglucuronide (n = 6) was injected. Purity of the radiolabeled pigments was verified (see above) immediately after injection into rats from the material remaining in the syringe. There was no significant degradation of any of the radiolabeled pigments. [^{3}H]Biliverdin was used in normal rats to rapidly generate radiolabeled unconjugated bilirubin within hepatocytes. Previous studies (26) have demonstrated that plasma clearance of biliverdin in hepatectomized rats occurs at one-eighth the rate of plasma elimination in intact animals. In addition, using hepatocytes and nonparenchymal cells (27) isolated from rat liver, we have demonstrated that the nonparenchymal cell uptake of radiolabeled biliverdin constitutes < 7% of the rapid hepatic uptake of biliverdin (unpublished observations). Radiolabeled biliverdin thus provided a convenient probe for rapidly generating radiolabeled unconjugated bilirubin within hepatocytes.

Tracer doses (8–12 μg) of radiolabeled pigments were employed in order to closely approximate the physiologic state. Individual radiolabeled pigments were excreted identically whether injected alone or in combination with an equal amount of a second radiolabeled pigment (i.e., dual-label experiments), reflecting a lack of competition or apparent perturbation of hepatocyte transport processes at these concentrations. Moreover, in separate experiments (data not shown) paired normal rats (n = 4) were injected with either 10 or 30 μg each of [^{3}H]biliverdin/[^{14}C]bilirubin; biliary excretion of radiolabeled bilirubin mono- and diglucuronide (expressed as percent administered dose per minute) was indistinguishable for these two groups of rats, thus demonstrating the ability of the animals to handle three times the standard dose of injected pigment (60 μg total) without impairment or modification of the pattern of biliary excretion. Finally, there was no difference in the results obtained from injected [^{3}H]- or [^{14}C]bilirubin. In subsequent data analysis, therefore, no distinction was made between the excretion curves from experimental subgroups injected with single or dual-label pigments.

Bile collection. Bile was collected continuously into tared tubes in the dark on ice. Bile flow was determined gravimetrically and total radioactivity measured in 5- to 40-μl aliquots from each bile sample before further analysis. The calculated dead volume of the biliary cannula was 12 μl, and since this introduced a lag time of only 35–40 s at the observed rates of bile flow, no adjustment was made for this time in the computation of data.

Analytical procedures. Alkaline methanolysis of radiolabeled pigments in bile and TLC of the methyl derivatives (28) was performed on each bile sample after the addition of 12 μl bilinoid-enriched bile as carrier (obtained from rats infused with 20 mM bilirubin i.v. in 3.2% bovine serum albumin). Radioactivity in the separated bands was determined by solubilizing samples in 0.1 ml isopropyl alcohol and 0.5 ml Soluene-350 (Packard Instrument Co., Downers Grove, IL), bleaching with 0.1 ml 30% hydrogen peroxide for 2 h under incandescent light, adding 5 ml of Dimiscint scintillation cocktail (Packard Instrument Co.) and then counting in a liquid scintillation spectrometer (Beckman LS7500, Beckman Instruments, Fullerton, CA). All samples were counted for 20,000 counts or 5 min to insure an error in net count rate of < 2%.

Statistical analysis. Plots of radiolabeled bilirubin monoglucuronide and diglucuronide output (percent administered dose/minute) vs. time were constructed for individual rats within each administered substrate group, and the respective populations of output curves examined for homogeneity. Tests for normality revealed a normal distribution of counts at each time point, permitting their representation as mean±SD vs. time. The resulting curves (Figs. 1 *A–D* and 5) all had a similar pattern, a steep rise followed by a gradual fall to zero, but they differed with respect to maximum value (excretory rate max), time to achieve maximum output (t_{max}) and total recovery (cumulative output). These parameters, calculated for each rat in all substrate groups, were found to have normal distributions, permitting use of parametric comparison methods. The param-

Table I. Biliary Excretion of Radiolabeled Bilirubin Monoglucuronide and Diglucuronide in Normal and Homozygous Gunn Rats

Administered radiolabeled pigment	Radiolabeled bilirubin conjugate in bile	Strain of rat	n	t_{max} (min)	Excretory rate max (% administered dose/min)	Cumulative output (% administered dose)
[^{3}H]BV	BMG	Normal	9	8.8±1.5	2.0±0.3	31.7±3.5
[^{3}H]- or [^{14}C]BR	BMG	Normal	14	7.6±1.1	2.3±0.4	32.7±4.4
[^{3}H]BMG	BMG	Normal	7	5.5±1.3*	4.8±1.1*	45.1±4.9*
[^{14}C]BMG	BMG	Gunn	3	4.8±0.6	8.8±0.6	86.9±6.4
[^{3}H]BV	BDG	Normal	9	9.9±1.6	3.1±0.5	54.2±5.7
[^{3}H]- or [^{14}C]BR	BDG	Normal	14	9.4±1.3	3.2±0.7	50.8±6.9
[^{3}H]BMG	BDG	Normal	7	7.9±1.1‡	2.8±0.5	36.0±7.6*
[^{3}H]BDG	BDG	Normal	4	4.8±1.0§	7.9±1.2§	80.2±7.6§
[^{3}H]BDG	BDG	Gunn	3	3.8±0.6	10.7±1.0	90.3±0.6

The numerical parameters for the experiments presented in Figs. 1, 2, 5, and 6 are summarized. BV denotes biliverdin; BR, bilirubin; BMG, bilirubin monoglucuronide; BDG, bilirubin diglucuronide; n, number of animals in each experimental group; t_{max}, time elapsed in minutes between injection of radiolabeled pigment and maximal rate of excretion of radiolabeled bilirubin conjugates in bile; excretory rate max, maximum rate of radiolabeled bilirubin conjugate excretion in bile, expressed as percentage of the administered dose of radiolabel per min; cumulative output, cumulative recovery of radiolabeled bilirubin conjugates over the 120 min experimental period, expressed as percentage of the administered dose of radiolabel. All values are expressed as mean±SD. * $P < 0.01$ when compared with administered radiolabeled biliverdin or bilirubin. ‡ $P < 0.05$ when compared with administered radiolabeled biliverdin or bilirubin. § $P < 0.01$ when compared with administered radiolabeled biliverdin, bilirubin, or bilirubin monoglucuronide.

eters obtained from each substrate group were compared using one-way analysis of variance (ANOVA)[1] followed by Newman-Keuls multiple range tests for pairwise comparisons (29). The parameters from each substrate group and the results of comparison testing are shown in Table I.

Deconvolution analysis of curves. Further characterization of the observed differences in the hepatic processing of injected pigments was obtained by employing deconvolution analysis of biliary excretion curves of radiolabeled bilirubin mono- and diglucuronides. The advantage of this model independent technique is that no inherent assumptions are necessary regarding metabolic pathways or functional compartmentation. Deconvolution analysis examines the entire relationship between two curves through the derivation of an intermediate "weighting function," and thus supplements comparisons of isolated parameters of those curves. Specifically, two time-dependent functions are designated "input" and "output," or I(t) and O(t), respectively (30). A weighting function [G(t)] can be derived which, when convolved on I(t) using the LaPlace transform technique, yields O(t). This computation is best performed using numerical methods of approximation (e.g., least squares), which can be readily programmed for computers and are particularly stable to data noise in the input and output functions (31–33). Methods for performing these computations are well described (34).

In the present study, the biliary excretion curve for radiolabeled bilirubin monoglucuronide was considered the input function, I(t), and that for radiolabeled bilirubin diglucuronide the output function, O(t), for normal rats injected with radiolabeled biliverdin, bilirubin, or bilirubin monoglucuronide. Preliminary calculations demonstrated that the weighting functions had high initial values and approached zero asymptotically. Thus, for subsequent calculations the weighting functions, G(t), were constrained to monotonic nonnegative values. A weighting function was computed for each rat using a least-squares method of approximation (34, 35). All substrate groups of derived weighting functions had a normal distribution without outliers. Descriptive statistics were computed for each group and these data are presented graphically in Fig. 4. The three curves were compared using both blocked ANOVA for those rats that received dual-injection of radiolabeled pigments, and rank sum testing for the entire population.

1. *Abbreviations used in this paper:* ANOVA, analysis of variance.

Results

After injection of radiolabeled biliverdin, bilirubin, or bilirubin mono- or diglucuronide, radioactivity appeared in bile within 60 s. Biliary excretion of radioactivity in either bilirubin mono- or diglucuronide achieved maximum values 4 to 10 min after the injected dose and, in all rats, radioactivity was essentially zero by the end of the 120 min experimental period. Total recovery in bile of the injected ^{3}H- or ^{14}C-radioactivity as bilirubin mono- and diglucuronide was consistently 80–90%. Radioactivity recovered in unconjugated bilirubin was < 2% of the injected dose, and hence is not reported.

For normal rats, the biliary excretion curves of radiolabeled bilirubin monoglucuronide and diglucuronide obtained following injection of radiolabeled biliverdin, bilirubin, bilirubin monoglucuronide or diglucuronide are shown in Fig. 1. The cumulative output of radiolabeled bilirubin mono- and diglucuronide for these experiments is presented in Fig. 2. The computed parameters describing these curves and statistical comparisons of the data are shown in Table I. In rats injected with radiolabeled biliverdin, bilirubin, or bilirubin monoglucuronide (Fig. 1 *A–C*), peak bilirubin monoglucuronide excretion occurred approximately 2 min before that of diglucuronide excretion, consistent with a brief delay imposed by hepatocyte conjugation of bilirubin mono- to diglucuronide. Maximal excretion of radiolabeled bilirubin monoglucuronide occurred at approximately the same time following administration of either radiolabeled biliverdin or bilirubin, but was significantly earlier for injected bilirubin monoglucuronide (see t_{max}, Table I). The maximum output (excretory rate max) of radiolabeled bilirubin monoglucuronide also was significantly greater after injection of bilirubin monoglucuronide.

Biliary excretion of radiolabeled bilirubin diglucuronide exhibited a pattern comparable to that of monoglucuronide; the t_{max} observed following administration of biliverdin or bilirubin was similar, but was significantly earlier following injection of

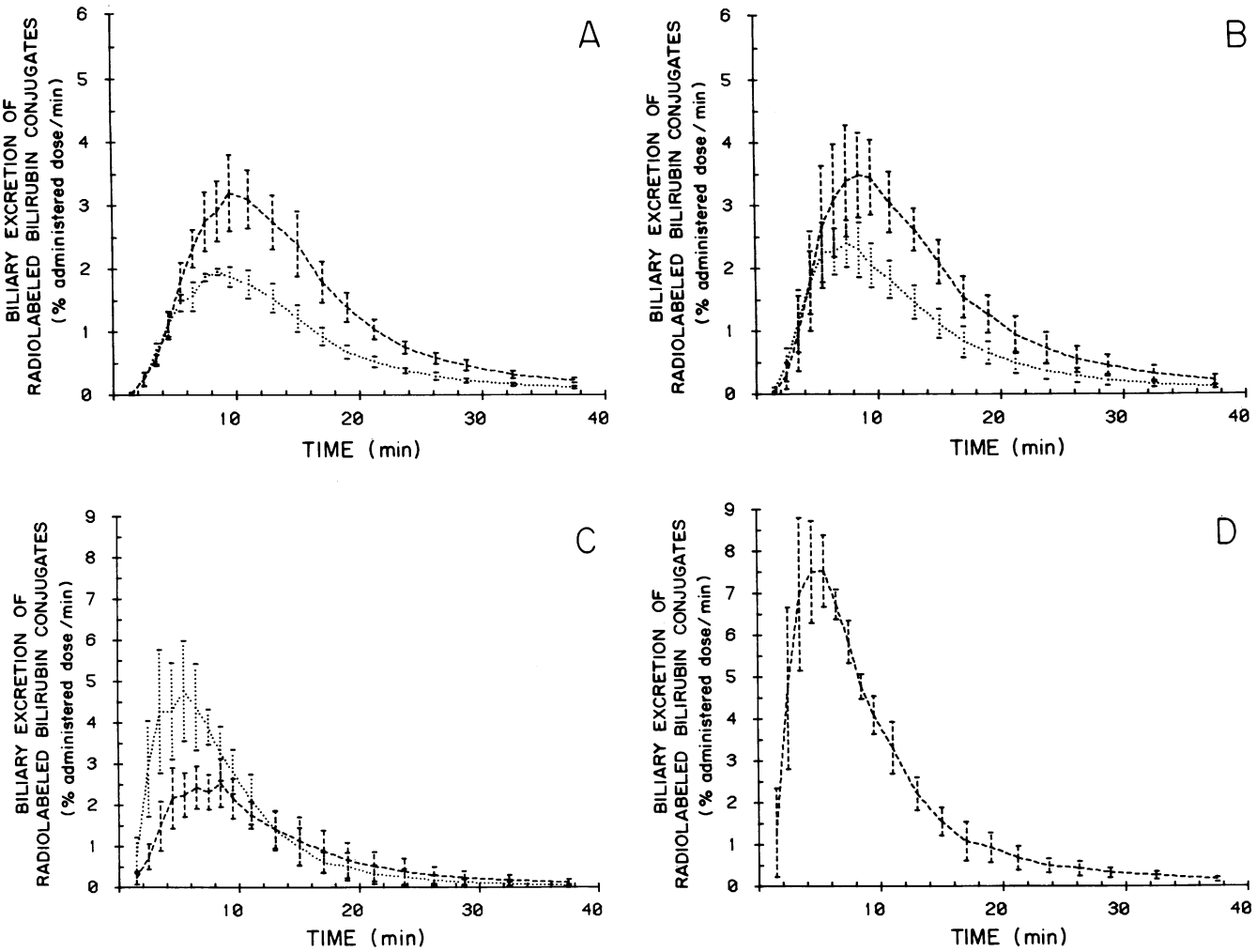

Figure 1. Rate of biliary excretion of radiolabeled bilirubin monoglucuronide (*dotted line*) and bilirubin diglucuronide (*dashed line*) in normal rats after intravenous administration of radiolabeled biliverdin (*A*), bilirubin (*B*), bilirubin monoglucuronide (*C*), or bilirubin diglucuronide (*D*). Tracer doses (8–12 μg) of [^{3}H]- or [^{14}C]biliverdin (n = 9), bilirubin (n = 14), bilirubin monoglucuronide (n = 7) or bilirubin diglucuronide (n = 4) of high specific activity and purity were injected intravenously as a bolus. The rates of biliary excretion of radiolabeled bilirubin monoglucuronide and bilirubin diglucuronide were measured for 120 min. Biliary excretion of radiolabel, expressed as percentage of the injected dose excreted per minute, was negligible beyond 40 min and hence is not shown. Data are plotted as the mean±SD for the mid-point of each measured time interval. Note: In (*D*), excretion of radiolabeled bilirubin monoglucuronide was undetectable after injection of radiolabeled bilirubin diglucuronide.

bilirubin monoglucuronide or diglucuronide (more so for the latter). No difference was noted in the maximum output of radiolabeled bilirubin diglucuronide following administration of biliverdin, bilirubin, or bilirubin monoglucuronide, however, maximum output was considerably higher following injection of radiolabeled bilirubin diglucuronide.

There were notable differences in the cumulative excretion of radiolabeled bilirubin mono- and diglucuronide after the injection of radiolabeled biliverdin, bilirubin and bilirubin monoglucuronide (Fig. 2, Table I). Monoglucuronide excretion was less than that of diglucuronide after injection of biliverdin or bilirubin, but the reverse relationship was observed following the administration of bilirubin monoglucuronide. The cumulative excretion of bilirubin diglucuronide following injection of this pigment was substantially greater than that for the other three substrates, since it was the only significant radiolabeled species excreted in bile (Fig. 1 *D*).

The differential excretion of bilirubin mono- versus diglucuronide after injection of radiolabeled precursors is illustrated in Fig. 3, which shows the ratio of bilirubin diglucuronide to bilirubin monoglucuronide output in bile. For administered bilirubin monoglucuronide this ratio was significantly lower over the initial 20-min period (during which the majority of radiolabel was excreted into bile) than that observed for injected bilirubin or biliverdin. Interestingly, for all three injected pigments the initial excretion of radiolabeled bilirubin monoglucuronide exceeded that of diglucuronide; this was evident for the first 5–6 min for injected biliverdin and bilirubin, and over the first 14 min for bilirubin monoglucuronide.

These findings demonstrate distinct differences in the hepatic processing of trace amounts of radiolabeled biliverdin and bilirubin versus bilirubin monoglucuronide and bilirubin diglucuronide, based simply on the biliary excretion curves arising from the different injected radiolabeled substrates. Further objective comparison was gained by analyzing the relationship between the bilirubin mono- and diglucuronide excretion curves

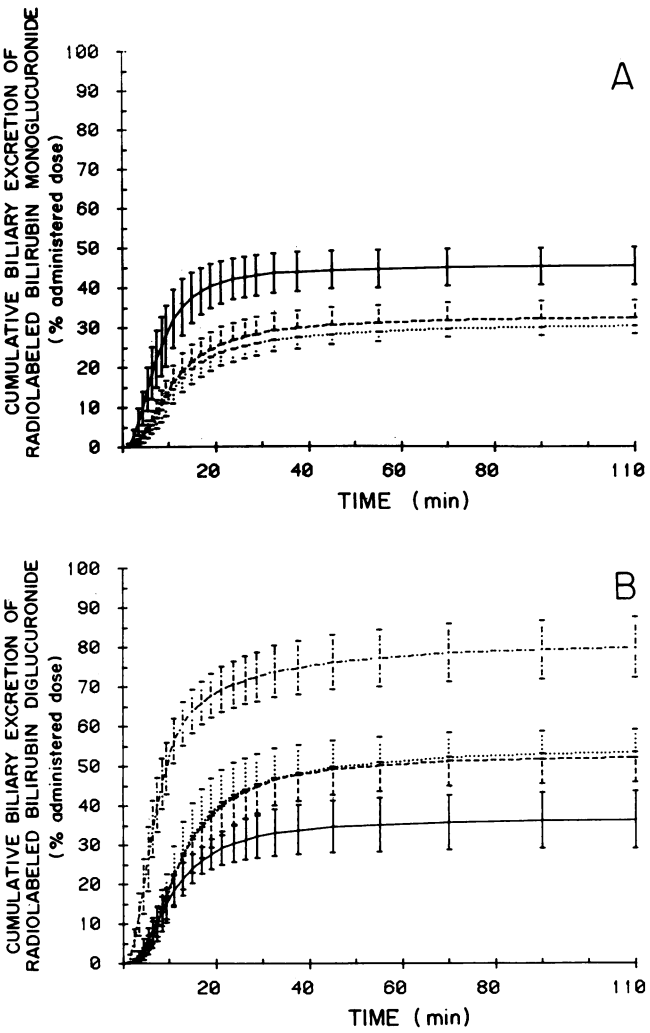

Figure 2. Cumulative biliary excretion of radiolabeled bilirubin monoglucuronide (A) and bilirubin diglucuronide (B) in normal rats following intravenous administration of radiolabeled biliverdin (*dotted line*), bilirubin (*dashed line*), bilirubin monoglucuronide (*solid line*) or bilirubin diglucuronide (*dashed-dot line*). Cumulative excretion of radiolabeled bilirubin glucuronides is shown for the experiments depicted in Fig. 1. Excretion of radiolabel is expressed as percentage of the administered dose (mean±SD).

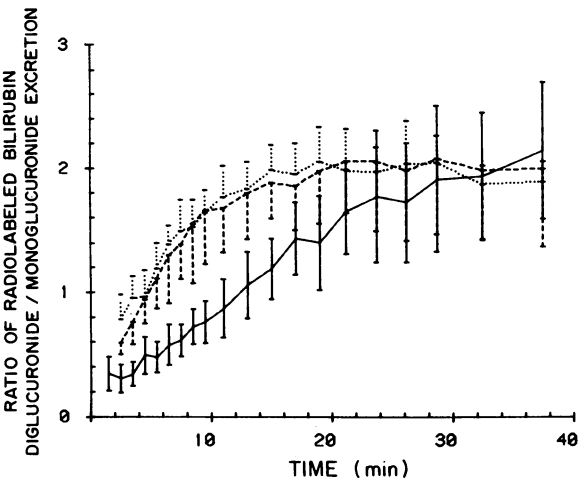

Figure 3. Relative rate of biliary excretion of radiolabeled bilirubin diglucuronide versus bilirubin monoglucuronide in normal rats following intravenous administration of precursor radiolabeled pigments. Excretion is shown as the ratio (mean±SD) of radiolabeled bilirubin diglucuronide to bilirubin monoglucuronide (BDG/BMG) present in bile (i.e., a ratio of 1.0 represents equal output of radiolabeled bilirubin mono- and diglucuronide) following intravenous administration of [^{3}H]biliverdin (*dotted line*), [^{3}H]- or [^{14}C]bilirubin (*dashed line*) or [^{3}H]bilirubin monoglucuronide (*solid line*) for the experiments outlined in Fig. 1 *A–C*. Beyond 40 min, the BDG/BMG excretion ratio remained constant at ~2.0±0.5 for all injected pigments and hence the data are not shown.

by deconvolution analysis (see Methods). As shown in Fig. 4, the weighting curves for injected biliverdin and bilirubin appeared comparable, although rank-sum testing indicated a significant difference between the two composite sets of curves ($P < 0.01$), with the biliverdin-derived functions being slightly higher than those for bilirubin. However, the curves obtained for bilirubin monoglucuronide injected animals were substantially lower than those for either biliverdin or bilirubin ($P < 0.005$). Rigorous analysis of the form of the weighting curves after normalization to either area under curve or initial value revealed no significant differences between the three substrate groups ($P > 0.05$, data not shown). Thus, these curves differed in magnitude rather than functional form.

To further define the hepatocellular transport and excretion of bilirubin conjugates independent of the conjugating system, radiolabeled bilirubin mono- and diglucuronide were injected into homozygous Gunn rats, which exhibit no measurable hepatic bilirubin UDP-glucuronyltransferase activity (36). The biliary excretion curves for unmodified pigments are presented in Fig. 5, and the cumulative outputs for these experiments are shown in Fig. 6. The computed parameters describing these

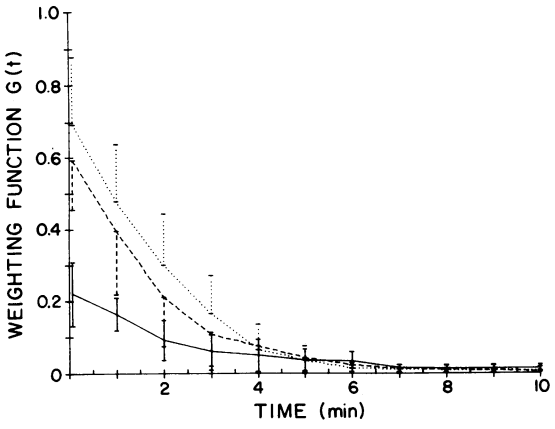

Figure 4. Weighting functions [G(t)] derived by deconvolution analysis of biliary excretion curves for radiolabeled bilirubin monoglucuronide and diglucuronide in normal rats. A weighting function was derived for individual rats by deconvolution analysis (see Methods), relating the rate of biliary output of radiolabeled bilirubin monoglucuronide and bilirubin diglucuronide after bolus administration of intravenous tracer doses of [^{3}H]biliverdin (*dotted line*), [^{3}H]- or [^{14}C]bilirubin (*dashed line*) or [^{3}H]bilirubin monoglucuronide (*solid line*) for the experiments shown in Fig. 1 *A–C*. Data are presented as the computed values (mean±SD) for G(t) at each time point for rats in each substrate group. Because the weighting functions were essentially zero after the initial 10 min, data are not shown beyond that time.

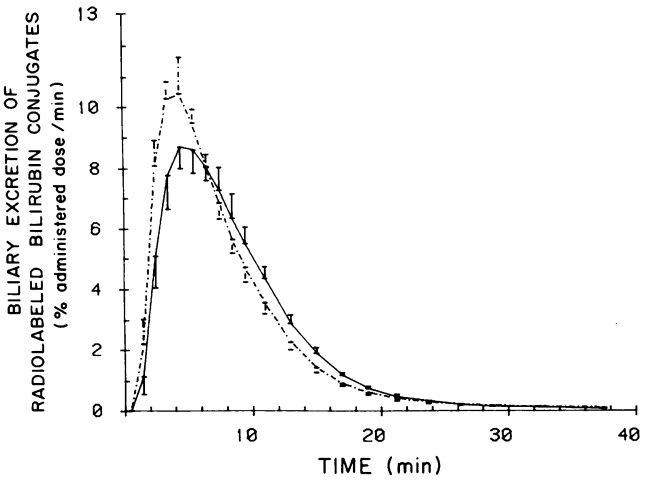

Figure 5. Rate of biliary excretion of intravenously administered radiolabeled bilirubin monoglucuronide (*solid line*) and diglucuronide (*dashed-dot line*) in homozygous Gunn rats. Tracer doses (8–10 μg) of [^{14}C]bilirubin monoglucuronide and [^{3}H]bilirubin diglucuronide were injected simultaneously into homozygous Gunn rats (n = 3). The bilirubin glucuronides were excreted in bile without apparent metabolism or modification. Radiolabeled bilirubin glucuronide excretion was measured for 90 min, and the results expressed as percent of injected dose excreted per min (mean±SD). Biliary excretion of radiolabel was negligible beyond 40 min and hence is not shown.

curves are presented in Table I. The pattern of biliary excretion of glucuronides was virtually identical to that for unmetabolized bilirubin mono- and diglucuronides in normal rats, with no delay apparent in the time elapsed to maximum excretion (see t_{max}, Table I). Since the injected pigments were excreted without alteration, the only differences related to magnitude (i.e., excretory rate max and cumulative output). A similar series of experiments were performed using heterozygous Gunn rats, which exhibit only a partial reduction in their bilirubin conjugating capacity (37), to ensure that inherent differences between the parent Wistar strain and the normal Sprague-Dawley rats used in this study did not influence the handling of bile pigments. No differences were observed in the processing of injected bilirubin mono- or diglucuronide between the two strains (data not shown).

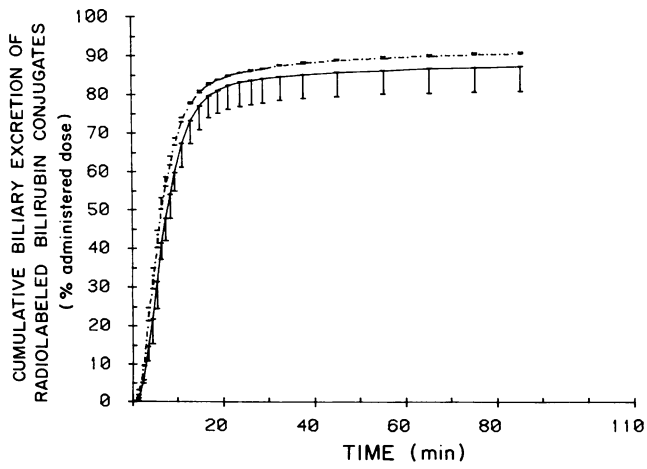

Figure 6. Cumulative biliary excretion of radiolabeled bilirubin monoglucuronide (*solid line*) and diglucuronide (*dot-dashed lines*) in homozygous Gunn rats. Cumulative biliary excretion of radiolabeled bilirubin glucuronides is plotted for the experiments shown in Fig. 5. Excretion of radiolabel is expressed as percentage of the injected dose (mean±SD).

Discussion

The metabolism and disposition of bilirubin have been the subject of intense scrutiny over the past two decades. The demonstration of hepatic conversion of bilirubin to bilirubin glucuronides (38, 39) was followed by elegant studies of the plasma elimination and hepatic uptake of bilirubin precursors (2, 40, 41), bilirubin (2, 8, 40–42) and bilirubin conjugates (13, 43). Kinetic analysis and mathematical modeling techniques have provided quantitation and valuable insights into the functional compartmentation of bile pigment physiology in both humans and intact animals (44, 45). However, definition of the intracellular processes involved in hepatic transport and elimination of bilirubin and its conjugates remain elusive (1).

In this study, we have systematically examined the hepatic transport and biliary excretion of all of the major mammalian bile pigments. A high degree of precision was achieved by the use of essentially pure (~ 99%) radiolabeled pigments of high specific activity, prepared by modification of existing techniques (6, 17, 18, 21). Application of recent advances in the analysis of purified pigments (19, 20) and of native bilirubin mono- and diglucuronides in bile (28), combined with the administration of dual-labeled pigments, yielded highly reproducible data amenable to rigorous mathematical analysis. The validity of our tracer conditions was verified experimentally, both by comparison of data from single- and dual-label experiments, and by demonstrating that a threefold increase in the injected dose of pigments did not alter their pattern or rate of biliary excretion. Deconvolution analysis, which has been used specifically in modeling studies of bilirubin metabolism (2, 40) and to characterize insulin (46) and lipoprotein (47) transport, was employed in this study in a model-independent fashion, so as to avoid the assumptions implicit in constructing compartmental models (48, 49). By closely approximating physiologic conditions in our experimental design, we believe that the findings provide new insight into the hepatocellular disposition of bile pigments. Specifically, substantial differences have been documented in the intracellular processing of conjugated bilirubins derived from intrahepatic versus extrahepatic sources.

Heme is the primary endogenous source of bilirubin in mammals, but biliverdin is an essential intermediate and the immediate precursor of unconjugated bilirubin (1, 5). On the basis of preliminary experiments with isolated parenchymal cells (see Methods), exogenous biliverdin was used as an efficient source of endogenously generated bilirubin within hepatocytes. As shown in Figs. 1 and 2 and Table I, there was no significant difference in the biliary excretion of radiolabel derived from biliverdin and bilirubin. The primary excretory product was bilirubin diglucuronide. When radiolabeled bilirubin monoglucuronide was injected, an entirely different pattern of bilirubin conjugate excretion resulted. In normal rats, only a minor portion of exogenous (or circulating) bilirubin monoglucuronide underwent further hepatic conjugation to bilirubin diglucuronide, whereas the majority was excreted unchanged. Moreover, injected radiolabeled monoglucuronide was transported through the liver and excreted into bile more rapidly than radiolabel

from either biliverdin or bilirubin. This was evident both for radiolabel appearing in bile as unaltered bilirubin monoglucuronide, as well as for that excreted as bilirubin diglucuronide. However, the slight delay (< 2 min) in excretion of radiolabel as bilirubin diglucuronide relative to monoglucuronide, and the similar excretory rate max exhibited for bilirubin diglucuronide derived from all three administered radiolabeled precursors, suggest that a final common pathway is operative for the intracellular transport (i.e., endoplasmic reticulum to canalicular membrane) and biliary excretion of all endogenously generated bilirubin diglucuronide.

Deconvolution analysis confirmed the observation that hepatic conversion of exogenously administered or circulating bilirubin monoglucuronide to diglucuronide is relatively inefficient. The computed weighting functions shown in Fig. 4 represent the time-dependent conversion of bilirubin monoglucuronide to diglucuronide within the hepatocyte. The low magnitude of the weighting function for exogenously administered bilirubin monoglucuronide, compared with that for endogenously derived monoglucuronide (from biliverdin or bilirubin), clearly documents the less efficient conversion of exogenously administered or plasma-derived bilirubin monoglucuronide to diglucuronide. Given the similar forms of the weighting functions however, it is unlikely that the differences in hepatic handling of endogenously and exogenously generated bilirubin monoglucuronide reflect different mechanisms of enzymatic conversion. Rather, the lower magnitude of the weighting function is more likely due to decreased access of plasma-derived bilirubin monoglucuronide to the endoplasmic reticulum. This concept is supported by preliminary in vitro studies with rat liver microsomes, which demonstrate preferential conversion to bilirubin diglucuronide of monoglucuronide formed in situ from bilirubin compared to bilirubin monoglucuronide added exogenously (50). The decreased access of exogenous or plasma bilirubin monoglucuronide to the sites of glucuronidation in hepatocytes may reflect both efficient mechanisms for hepatocellular transport and biliary excretion of circulating bilirubin monoglucuronide, and more favorable partitioning of unconjugated bilirubin into the endoplasmic reticulum and hence to the active site of the conjugating enzyme.

Intravenous administration of radiolabeled bilirubin diglucuronide resulted in its rapid excretion into bile without detectable modification, as shown in Figs. 1 *D* and 2, and excretion occurred earlier than bilirubin diglucuronide synthesized from other sources (Table I). Moreover, the excretion curves of unmetabolized bilirubin monoglucuronide (Fig. 1 *C*) and bilirubin diglucuronide (Fig. 1 *D*) were similar, differing only in magnitude, due to conversion of a fraction of the injected monoglucuronide to diglucuronide. It thus appears likely that bilirubin mono- and diglucuronide circulating in plasma are transported through hepatocytes and excreted by comparable (or identical) mechanisms, with only a minor portion of exogenous bilirubin monoglucuronide gaining access to the endogenous pathway and being converted to bilirubin diglucuronide.

This concept of exogenous bilirubin glucuronides having a distinct transcellular pathway for delivery to the canaliculus is supported by the experiments in homozygous Gunn rats; injected bilirubin mono- and diglucuronide were excreted directly into bile with the same time course as that for unmetabolized bilirubin glucuronides in normal rats (Fig. 5 and Table I). Because bilirubin UDP-glucuronyltransferase activity is absent in these animals (36), excretion of plasma bilirubin glucuronides must reflect intracellular transport mechanisms that are independent of the conjugating system. Intuitively, it seems likely that exogenous or plasma bilirubin mono- and diglucuronide share identical intracellular transport mechanisms. However, in both normal and Gunn rats there was a slight but consistent lag in the trailing portion of the monoglucuronide excretion curves relative to diglucuronide excretion (see Fig. 5, from 8 to 20 min after injection). Although speculative, this may be due to the fact that bilirubin monoglucuronide is more hydrophobic than diglucuronide (1), and hence more liable to partition into lipid-based microenvironments within the hepatocyte en route to biliary excretion.

The hepatocellular metabolism and transport of all bile pigments studied was extremely rapid. The calculated dead volumes of the bile cannula (12 μl) and of the rat intrahepatic biliary tree (~10–12 μl; 51) accounted for an estimated 60–80-s delay in the appearance of radiolabel in bile. Yet, radiolabeled pigments were measurable in bile within 1 min of intravenous injection of bilirubin conjugates (Fig. 5), with peak excretion within 5 min (Table I). Rapid hepatic transit of bile acids from the sinusoidal compartment to bile has also recently been documented (52). The specific mechanisms responsible for such rapid directed hepatocyte transport of organic anions from plasma to bile have not been defined, but are generally presumed to involve transfer via cytosolic binding proteins (53). It is unlikely that vesicular fluid phase transport is involved in the transcellular transport and excretion of bilirubin glucuronides, since fluid phase markers appear in bile after a lag period of approximately 12 min (54). Vesicular transport may, however, play a role in the transfer of

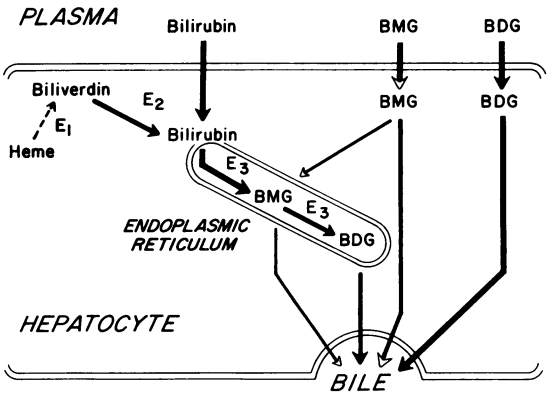

Figure 7. Hepatocellular transport and excretory pathways for bile pigments are represented schematically, illustrating differences in the processing of endogenously and exogenously generated bilirubin and bilirubin conjugates. Pigments present in plasma may include bilirubin, and bilirubin mono- and diglucuronide (BMG and BDG, respectively). Intrahepatic biliverdin is derived from hepatocellular sources of heme via the heme oxygenase system (E_1), and is converted rapidly to bilirubin by biliverdin reductase (E_2). Intracellular bilirubin is converted to BMG or BDG by bilirubin UDP-glucuronyl-transferase (E_3) in the endoplasmic reticulum. Both BMG and BDG generated by this pathway are excreted efficiently into bile. BMG taken up from plasma is excreted predominantly unchanged, with a minor fraction entering the endoplasmic reticulum for glucuronidation to BDG. Plasma-derived BDG is excreted directly into bile without modification. The relative weight of the arrow lines indicates the relative flux through each pathway. The dashed arrow between heme and biliverdin indicates that this pathway was not examined in this study. The open arrowheads designate BMG transport pathways.

endogenously derived bilirubin glucuronides to the canalicular membrane (25).

In conclusion, these findings demonstrate distinct differences in the manner in which the liver handles bilirubin glucuronides arising from different sources. The complex interrelationships between the intracellular pathways of endogenously and exogenously derived bile pigments are illustrated schematically in Fig. 7. It should be noted that our data do not permit assignment of these putative pathways to specific intracellular components. Bilirubin glucuronides formed in the liver from endogenous sources of bilirubin (i.e., hepatic heme) and from bilirubin generated extrahepatically follow a similar excretory pathway, with preferential excretion of bilirubin diglucuronide. Circulating bilirubin glucuronides are excreted more rapidly than those generated in the liver. Moreover, exogenous bilirubin monoglucuronide appears to have impaired access to the conjugating enzyme in the endoplasmic reticulum, and is excreted in bile predominantly unchanged. Thus, in contrast to bilirubin glucuronides synthesized in the liver, conjugated bilirubins taken up directly from the circulation traverse the hepatocyte by a transcellular pathway that is largely independent of the conjugating system.

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