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Efflux of Bilirubin into Plasma following Hepatic Degradation of Exogenous Heme (40805)[1,2]

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The liver is the major site for biosynthesis of heme[4] required for formation of hepatic hemoproteins. Hepatic degradation of heme and hemoproteins, in turn, is responsible for at least 25% of the total daily bilirubin (BR) production (2–4). The BR derived from hepatic heme is excreted ultimately as conjugated BR in bile, where it appears as the first peak of the early labeled bile pigment fraction (5–7). In rats, this peak is observed 60–90 min after injection of radiolabeled heme precursors (7), suggesting that this BR fraction arises from a hepatic heme pool with a rapid turnover rate (8).

From analysis of plasma BR disappearance curves, it has been postulated that hepatic uptake of BR from plasma is the net result of bidirectional flux across the plasma membrane (9–11). Kinetic studies using radiolabeled δ-aminolevulinic acid (ALA) as heme precursor also have suggested that a major portion of the unconjugated BR derived from hepatic heme effluxes into plasma before its conjugation and excretion into bile (4, 12). The conclusions drawn from these studies are based on two reasonable, albeit unproven, assumptions implicit in the experimental design, (i) ALA is utilized exclusively for hepatic heme synthesis, and (ii) radioactivity extracted from plasma by the Weber–Schalm procedure represents only unconjugated BR (13, 14).

When radiolabeled heme is injected in rats, at least 60% of the radioactivity is recovered in bile as BR (15, 16). In the liver, parenterally administered heme represses heme synthesis (17–20), is incorporated into hemoproteins such as tryptophan pyrrolase (21) and cytochrome *P*-450 (22, 23), and enhances heme degradation (24, 25). It appears likely, therefore, that exogenous heme has the same physiological functions and metabolic fate as heme synthesized in the liver. Hence we have used exogenous radiolabeled heme as a probe to examine the metabolic fate of BR formed from hepatic heme. The results of these experiments demonstrate that a substantial fraction of BR formed in the liver effluxes into plasma before its ultimate biliary excretion.

Materials and methods. Heme was obtained as crystalline hemin chloride from Sigma Chemical Company. BR, purchased from Koch–Light, Colnbrook, United Kingdom, was purified by the method of McDonagh and Assisi (26) and contained at least 96% bilirubin IXα. Radioisotopes of ALA (2,3-[^{3}H]ALA, 31 Ci/mmole, and 4-[^{14}C]ALA, 24.6 mCi/mmole) were obtained from Research Products International Corporation and New England Nuclear, respectively. [^{3}H]Heme (17.2 to 65.2 mCi/mmole) was synthesized from 2,3-[^{3}H]ALA incubated with rat reticulocyte lysate (27) and crystallized by the method of Labbe and Nishida (28).

Unless otherwise indicated, male rats weighing 380 ± 30 g were used. Sprague–Dawley rats were obtained from Simonsen Labs, Inc., Gilroy, California, and Gunn rats were bred in the Animal Care Facility of the University of California, San Francisco. Homozygous Gunn rats exhibit com-

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[4] The word heme is used throughout this paper to denote Fe-protoporphyrin IX, irrespective of its redox state.

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plete absence of hepatic BR UDP-glucuronyltransferase activity and consequently have an unconjugated hyperbilirubinemia (29). Heterozygous Gunn rats, on the other hand, have about half of the normal enzyme activity, but a plasma BR concentration which is normal, as determined by conventional methods (30). All animals were allowed food and water *ad libitum*. The left jugular vein and carotid artery were cannulated (PE-10 and PE-50, respectively) under ether anesthesia. The animals were placed in restraining cages, maintained at 37°C, and hydrated by intravenous infusion of 0.87% NaCl (1.2 ml/hr). Two-stage total hepatectomy, leaving the spleen intact, was performed on 560 to 580-g male Sprague–Dawley rats using the procedure of Bollman (31), and the animals were maintained with 10% glucose administered intravenously. Control experiments were performed in weight-matched intact rats subjected to sham laparotomy (see Table, control rats, 4 and 5).

[^{3}H]Heme, dissolved in a small volume of 0.1 *M* NaOH, neutralized with 0.1 *M* HCl, and buffered to a pH 7.4 with 0.1 *M* potassium phosphate, was mixed with 1.0 ml of rat serum to yield a final heme concentration of 100 μg/ml. In this preparation, it was assumed that over 90% of the heme was bound to albumin and the remainder to hemopexin (32). Plasma disappearance of heme was examined following injection of [^{3}H]heme as an intravenous bolus (240–360 nmole/kg). Twelve plasma samples of 100 μl each were obtained at intervals during the subsequent 5 hr. To compare hepatic BR production from exogenous and endogenous heme, 7.8–107 nmole [^{3}H]heme and 42–120 nmole [^{14}C]ALA were mixed in buffered rat serum and injected simultaneously.

Heme and BR were extracted from plasma into a mixture of chloroform and methanol (1/2.1, v/v) containing hexadecyltrimethylammonium bromide (cetrimide, 5 mg/ml) (Dr. Colin Berry, personal communication). Unlabeled carrier heme and bilirubin IXα were dissolved in 0.1 *M* NaOH and added to plasma samples before extraction. Heme and unconjugated BR in the organic phase were separated by tlc (silica gel 60, 5763, 0.25 mm, Merck, Darmstadt, Germany) using a chloroform/methanol/water (10/5/1, v/v/v) solvent system (33). The R_f values for heme and BR in this system are 0.36 and 0.89, respectively. The BR radioactivity also migrated as a single band in chloroform/acetic acid (99/1, v/v) (26). In both solvent systems, the R_f value for radioactive BR extracted from plasma corresponded to those of a bilirubin IXα standard (kindly supplied by Dr. Antony McDonagh). Heme and BR were identified on tlc plates by their characteristic brown or yellow colors and were scraped and eluted into methanol or chloroform, respectively. Eluted BR was measured by absorbance at 454 nm in chloroform (using $\epsilon = 62\ mM^{-1}cm^{-1}$) and heme by the pyridine hemochromogen method (34). Eluates were evaporated under N_2 to dryness, digested, and decolorized with 1.0 ml Soluene 350 (Packard Instrument Co.) containing 0.2 ml 30% hydrogen peroxide and 0.2 ml isopropyl alcohol. After addition of 10 ml Dimilume (Packard Instrument Co.), radioactivity was determined in a liquid scintillation spectrometer (Beckman LS-250). The plasma concentrations of [^{3}H]heme and [^{3}H]BR were expressed as nanomoles per milliliter, assuming that the specific activity of [^{3}H]BR corresponded to that of the injected parent heme. In dual-label experiments, the amounts of [^{14}C]BR and [^{3}H]BR in plasma were determined separately using a standard quench correction technique (4). The plasma fractional disappearance rate (k) of injected heme was calculated by the method of least squares and the half-life ($t_{\frac{1}{2}}$) was derived from the ratio $0.693/k$. The absolute plasma disappearance rate (V) of heme was calculated from the formula $V = kD$, where D is the dose of heme injected (nmole/kg body wt).

Results and discussion. The plasma fractional disappearance of [^{3}H]heme was monoexponential and similar in both strains of rat (Table I; $k = 0.014 \pm 0.003$ min^{-1}, $t_{\frac{1}{2}} = 50 \pm 12$ min). In two hepatectomized rats, V was greatly reduced (Table I) indicating that at least 93% of administered heme was taken up by the liver. This is in agreement with previous observations that the liver is the primary site of heme removal from the plasma (15, 35).

In normal rats, unconjugated [^{3}H]BR ap-

TABLE I. PLASMA DISAPPEARANCE OF [^{3}H]HEME IN RATS[a]

	Sprague–Dawley rats				Gunn rats			
	Control		Hepatectomy		Heterozygous		Homozygous	
Rat No.	k	V	k	V	k	V	k	V
1	0.012	3.4	0.001	0.30	0.018	5.0	0.014	4.9
2	0.017	5.3	0.001	0.22	0.018	4.9	0.015	4.4
3	0.011	3.0			0.016	5.8	0.011	3.2
4[b]	0.008	1.9						
5[b]	0.012	3.4						

[a] Plasma fractional disappearance rate (k, min^{-1}) and disappearance rate (V, μmole/kg/min) following intravenous administration of [^{3}H]heme (240–360 nmole/kg) in individual rats.

[b] Weight-matched, sham-operated control rats for hepatectomized animals.

peared in the plasma within 10 min of [^{3}H]heme administration (Fig. 1), and its concentration rose progressively, reaching a maximum after 60–90 min; thereafter, the plasma concentration decreased slowly over the next 3 hr (Fig. 1). The essential role of the liver in the conversion of injected [^{3}H]heme to [^{3}H]BR was demonstrated in the two rats which had undergone total hepatectomy. Under these circumstances, not only was [^{3}H]heme plasma disappearance rate negligible (see above), but the amount of BR which appeared in the plasma was markedly reduced (Fig. 2). Homozygous Gunn rats were used as controls for the hepatectomy experiments because they resemble hepatectomized rats in having a substantially reduced capacity for plasma BR clearance (2, 9), but like normal rats retain the ability to convert heme to bilirubin. The finding that plasma [^{3}H]BR appearance was almost abolished by hepatectomy (Fig. 2) indicates that extrahepatic tissues are responsible for only a small proportion of the BR formed from exogenous heme. Hence, most of the BR appearing in plasma after [^{3}H]heme administration to intact rats has been formed in the liver.

It is not possible to quantitate from the present data the magnitude of the BR fraction derived from injected heme that effluxed into plasma. Certain additional measurements necessary for this computation, such as a simultaneous plasma bilirubin disappearance curve, were not obtained. It was apparent, however, that BR efflux into plasma was appreciable, as [^{3}H]BR was readily detected in plasma when only trace amounts of [^{3}H]heme had been injected. Based on a compartmental model derived from human studies, Jones

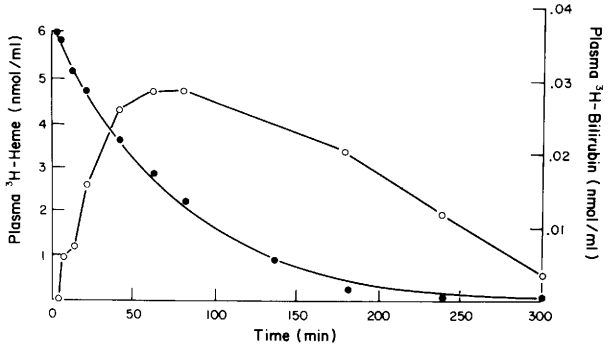

FIG. 1. Plasma heme (●) disappearance and plasma [^{3}H]bilirubin (○) appearance in a normal rat following intravenous administration of 120 nmole [^{3}H]heme. This study is representative of six individual experiments (see Table I, control Sprague–Dawley rats 1–3 and heterozygous Gunn rats 1–3). Note the different scale for BR and heme.

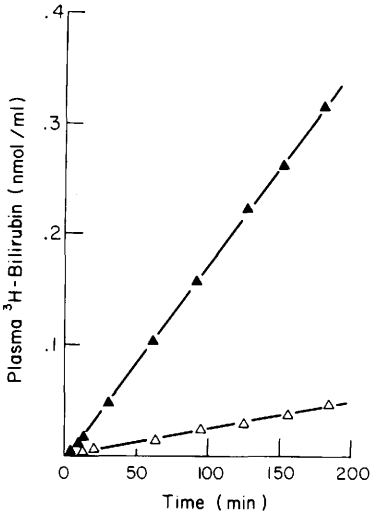

FIG. 2. Plasma appearance of [^{3}H]bilirubin following injection of 108 nmole [^{3}H]heme to an intact 375-g homozygous Gunn rat (▲), and 166 nmole [^{3}H]heme to a hepatectomized 580-g Sprague-Dawley rat (△).

et al. have estimated that about 70% of the BR derived from hepatic heme passes to plasma (12). This is consistent also with our recent observation that a major portion of the BR formed in the liver from trace amounts of administered biliverdin (an intermediate in heme degradation) effluxes into plasma before its biliary excretion (36).

In order to conclude that passage of hepatic BR into plasma is a physiological occurrence, it was necessary to demonstrate that injected exogenous heme and hepatic heme synthesized in the liver are catabolized through the same metabolic pathways. Hence, trace amounts of [^{14}C]ALA, to label endogenous hepatic heme, and [^{3}H]heme were injected simultaneously. In three Sprague-Dawley rats, serial determination of plasma concentrations of [^{14}C]- and [^{3}H]BR over the subsequent 5 hr revealed a qualitatively similar temporal profile for the appearance of radiolabeled BR derived from the two precursors (Fig. 3A). Moreover, in two homozygous Gunn rats the cumulative plasma levels of each BR radioisotope were proportional (Fig. 3B). These findings suggest that endogenous hepatic heme and exogenous heme do share the same routes of hepatic degradation to BR.

It seems highly likely, therefore, that BR efflux from liver to plasma is indeed a physiological consequence of hepatic heme degradation. An alternative explanation is that the BR formed from injected heme transiently overwhelmed the BR conjugating capacity of the liver and hence effluxed into plasma. This was unlikely as the amount of administered heme metabolized by the liver during the course of the experiment was considerably less than that normally degraded by the rat liver. To examine this possibility further, [^{3}H]heme, in amounts comparable to those used in normal rats, was injected into heterozygous Gunn rats which have a reduced activity of BR UDPglucuronyltransferase. The time

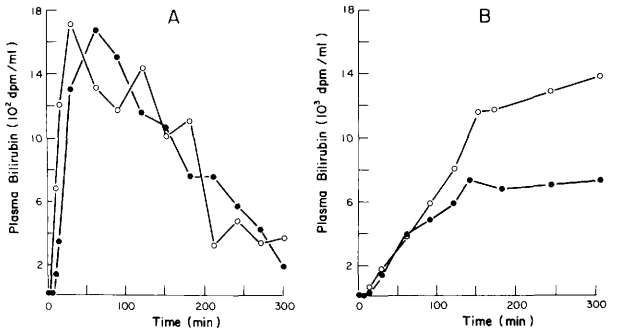

FIG. 3. Plasma concentration of [^{3}H]bilirubin (○) and [^{14}C]bilirubin (●) after simultaneous intravenous injection of [^{3}H]heme and [^{14}C]ALA at time 0. (A) Sprague–Dawley rat given 140 nmole [^{14}C]ALA (24.6 mCi/mmole) and 7.8 nmole [^{3}H]heme (65.2 mCi/mmole). (B) Homozygous Gunn rat given 42 nmole [^{14}C]ALA (24.6 mCi/mmole) and 107 nmole [^{3}H]heme (17.2 mCi/mmole). Note that, compared with panel (A), the scale for plasma bilirubin concentration is a different order of magnitude.

course and magnitude of plasma [^{3}H]BR in heterozygous Gunn rats were similar to those in normal rats (data not shown). This finding indicates that plasma efflux of BR formed in the liver is a phenomenon independent of the hepatic BR conjugating capacity.

Several possible explanations exist for this indirect route of BR excretion. Hepatic BR may be returned to plasma as a simple consequence of the normal bidirectional flux of the pigment across the liver plasma membrane (9–11). A more intriguing possibility, which has been postulated previously on the basis of kinetic studies (4), is that these findings may be accounted for by hepatic compartmentation of BR metabolism. Thus, nascent BR arising from hepatic heme may enter a different pool(s) from that which enters the liver directly from plasma. Such compartmentation could exist within the same hepatocyte or between different populations of hepatocytes within the hepatic lobule (37).

Regardless of the mechanism by which hepatic BR enters the plasma, the present findings have implications for the pathophysiology of unconjugated hyperbilirubinemia. It is now readily apparent that plasma BR levels are influenced not only by the overall rate of BR production and the efficiency of hepatic BR uptake, transport, and secretion into bile, but also by the proportion of the hepatic BR pool which effluxes to plasma. This may be particularly relevant in conditions, such as intravascular hemolysis, where the hepatic heme load is increased.

Summary. In rats, unconjugated [^{3}H]BR appeared in plasma within 10 min and reached a maximum concentration 60–90 min after intravenous administration of trace amounts of [^{3}H]heme. Total hepatectomy virtually abolished plasma heme disappearance and markedly reduced the early plasma appearance of [^{3}H]BR, thereby confirming the central role of the liver in plasma heme disposal and the hepatic origin of BR derived from it. In heterozygous Gunn rats, which have reduced BR UDPglucuronyltransferase activity, results were similar to those in normal rats. This indicates that the efflux of BR from liver to plasma did not result from BR production exceeding the hepatic BR conjugating capacity. After simultaneous injection of trace amounts of [^{14}C]ALA (to label endogenously synthesized hepatic heme) and [^{3}H]heme, both [^{14}C]- and [^{3}H]BR appeared in plasma with strikingly similar temporal profiles. The results of these studies show directly that a considerable proportion of BR derived from hepatic heme passes to the plasma before its ultimate biliary excretion. Moreover, the kinetic profile reflecting hepatic BR production from administered heme conforms closely to the established pattern for that of early-labeled BR. These findings offer further experimental support for the concept that injected heme is incorporated into a labile unassigned heme pool in the liver.

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