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The liver excretes large amounts of heme into bile when heme oxygenase is inhibited competitively by Sn-protoporphyrin

(neonatal jaundice/metalloporphyrins/enzyme inhibition/biliary heme excretion)

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ABSTRACT **Tin^{IV}-protoporphyrin IX (Sn-protoporphyrin) potently inhibits heme degradation to bilirubin *in vitro* and *in vivo*, and it completely suppresses neonatal hyperbilirubinemia in experimental animals, including primates. It also reduces plasma bilirubin levels in certain naturally occurring or induced forms of jaundice in animals and man. We have examined in this study the fate of that fraction of heme whose degradation to bile pigment is inhibited *in vivo* by administration of this heme oxygenase (EC 1.14.99.3) inhibitor. In bile-duct-cannulated rats, infused exogenous heme is rapidly converted to biliary bilirubin; a small amount of the infused heme is excreted into bile as well. Sn-protoporphyrin, administered with the exogenous heme, markedly increased (3- to 4-fold) the amount of heme excreted into bile and greatly diminished biliary output of bilirubin. The increase in biliary heme output exceeded the decrease in bilirubin excretion elicited by the inhibitor metalloporphyrin. In the same experimental model, Sn-protoporphyrin substantially decreased the conversion of heme, derived from heat-damaged erythrocytes, to biliary bilirubin. This decrease in biliary bilirubin output was accounted for entirely by a prompt and marked increase in biliary excretion of unmetabolized heme. The enhanced biliary excretion of unmetabolized heme following administration of Sn-protoporphyrin is a newly defined and biologically important response associated with use of this synthetic heme analogue. The features of the action of this compound *in vivo*—suppression of formation of the potentially neurotoxic metabolite, bilirubin; enhancement of disposal of the untransformed substrate (heme) of the enzyme that it inhibits; and its own elimination without metabolic alteration—define some of the characteristics of a therapeutically useful chemical.**

The synthetic heme analogue tin^{IV}-protoporphyrin IX (Sn-protoporphyrin) is a potent competitive inhibitor of heme oxygenase [heme, hydrogen-donor:oxygen oxidoreductase (α-methene-oxidizing, hydroxylating); EC 1.14.99.3] (1–5), the rate-limiting enzyme in degradation of heme to bile pigment (6). In addition, this metalloporphyrin can entirely suppress hyperbilirubinemia in neonatal animals (1, 2, 7) and reduce plasma bilirubin levels in a variety of forms of naturally occurring (8) or experimentally induced jaundice in animals and man (9, 10). Tissue distribution studies in animals, done using a sensitive fluorometric method (11) for measurement of Sn-protoporphyrin, indicate that the compound is localized primarily in the liver, spleen, and kidney, where it produces marked inhibition of heme oxygenase activity for prolonged periods (12). It is likely that Sn-protoporphyrin suppresses jaundice primarily by reducing bilirubin production (1, 10).

It seemed important to define the fate of the fraction of heme that remains unmetabolized when heme oxygenase activity is blocked by Sn-protoporphyrin, since this or related compounds potentially might be used to suppress severe hyperbilirubinemia in newborn humans. Also, there may be adverse effects, as yet undefined, of large cellular accumulations of heme, if no alternative means were available for its disposal. Heme has been essentially innocuous when administered in pharmacological doses for therapeutic purposes in patients with neurological exacerbations of the genetic liver disease, acute intermittent porphyria (13, 14). Moreover, in contrast to the recognized neurotoxicity of bilirubin, heme is known to stimulate neurite growth in cultured neuroblastoma cells (15) and, recently, high concentrations of heme were found to protect against the toxic demyelinating action of lead in a cultured peripheral neuronal cell system (16). Nevertheless, it would be useful to have information on the possible cellular accumulation or the metabolic disposition of heme in the whole animal after Sn-protoporphyrin administration. In a previous report (9) from this laboratory, it was noted that amounts of heme sufficient to transiently saturate the heme-dependent enzyme tryptophan pyrrolase (tryptophan 2,3-dioxygenase, EC 1.13.11.11) accumulate in rat liver cells after administration of Sn-protoporphyrin. The transience of the Sn-protoporphyrin effect on tryptophan pyrrolase suggested, however, that the liver does not excessively accumulate heme when heme oxygenase activity is inhibited but rather disposes promptly of the unmetabolized heme in some manner.

We report here that when heme oxygenase is inhibited by Sn-protoporphyrin administration *in vivo*, the liver responds by rapidly excreting large amounts of heme via the biliary system into the gut. Excretion of large amounts of unmetabolized heme into bile after inhibition of heme oxygenase by Sn-protoporphyrin is a newly defined and biologically important property of this synthetic heme analogue.

MATERIALS AND METHODS

Male Sprague–Dawley rats (Taconic Farms, Germantown, NJ) of 330–360 g were used in these studies. Sn-protoporphyrin IX HCl (Porphyrin Products, Logan, UT) was dissolved in a small amount of 0.2 M NaOH, 3.5 volumes of 0.9% NaCl were added, and the pH was adjusted to 8.0 by dropwise addition of 0.5 M HCl (1). Heme (Sigma) was dissolved in 0.1 ml of 0.05 M NaOH, and 1.9 ml of cold rat serum was added dropwise with stirring (17). Other chemicals utilized were reagent grade and were obtained from Sigma or Fisher.

Bile-duct and jugular-vein cannulations were carried out in animals anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg; Abbott). The animals were kept in restraining cages, and before each experiment, the animals were allowed to recover from surgery for 3–4 hr so that bile flow and bilirubin output could stabilize. The jugular vein

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catheter was infused throughout the experiment with a solution containing equal amounts of 0.45% (wt/vol) NaCl and 5% (wt/vol) glucose at a constant rate of 1.1 ml/hr using an infusion pump (Harvard Apparatus, Millis, MA).

Heat-damaged erythrocytes were prepared from rat blood obtained by translumbar puncture of the inferior vena cava. Washed erythrocytes were resuspended to the original volume in 0.9% NaCl and heated at 49.5°C as described by Harris *et al.* (18) and Jandl *et al.* (19), except that the cells were heated for only 40 min; longer periods of heating were likely to produce various degrees of hemolysis. Hemoglobin concentration in an aliquot of the erythrocyte preparation was measured by the cyanomethemoglobin method (20). All experiments were carried out in subdued light. Bile was collected for 60-min periods in tared plastic tubes, weighed to determine the amount collected, and stored in the dark at 4°C. Bilirubin in bile was determined within 24 hr by the fluorometric method of Roth (21).

The concentration of heme in bile was determined by the pyridine hemochromogen method (22) by using the reduced-minus-oxidized difference in absorption between 557 nm and 540 nm and an absorption coefficient of 20.7 $mM^{-1}cm^{-1}$. The pyridine hemochromogen of pure Sn-protoporphyrin by this method exhibited an absorption peak at 578 nm with troughs at 560 nm and 588 nm. The absorption coefficient of Sn-protoporphyrin between 578 nm and 588 nm was 0.89 $mM^{-1}cm^{-1}$, and between 578 nm and 560 nm it was 0.60 $mM^{-1}cm^{-1}$. Bile samples used for heme determination could be stored at −20°C for up to 3 months without loss of heme. Sn-protoporphyrin added to bile at a concentration of 140 nmol/ml of bile, which is greater than those found in these experiments, did not interfere with the determination of heme in bile by the pyridine hemochromogen method. Spectral studies were performed with an Aminco-Chance DW2A spectrophotometer in the split-beam mode.

For thin layer chromatography (TLC), heme and Sn-protoporphyrin were extracted from bile samples (0.5 ml) with ethyl ether as described by Healey *et al.* (23). After the final extraction step, the ethyl ether was evaporated to dryness under a stream of air at room temperature. The dried residues, which contained heme and Sn-protoporphyrin, were redissolved in 0.1 ml of chloroform/methanol (1:1, vol/vol) and applied to silica gel G plates (Merck). The plates were developed with benzene/methanol/formic acid (85:15:1.3, vol/vol). Sn-protoporphyrin (R_f = 0.31) was readily separated from heme (R_f = 0.24) in this system and identified by its red fluorescence under long-wave length ultraviolet illumination. Heme did not fluoresce but could be identified visually. Studies were conducted in four animals (two treated with heat-damaged erythrocytes alone and two treated with heat-damaged erythrocytes and Sn-protoporphyrin). Bile samples that contained the highest concentration of heme, as determined by the pyridine hemochromogen method, were selected for TLC separation.

High performance liquid chromatography (HPLC) analysis of bilirubin conjugates and heme in bile was also carried out. An HPLC series 3 apparatus (Perkin–Elmer) and a μBondapak C_{18} column (3.9 × 300 mm, Waters Associates) with a guard column (C_{18} reversed-phase, 3 × 30 mm), equipped with a Rheodyne 7105 septumless injector, were used. Bilirubin glucuronides were extracted from bile samples (500 μl), which had been previously treated with ammonium sulfate (400 mg) and L-ascorbic acid (20 mg), with isopropanol/methanol/Me_2SO (6:3:1, vol/vol) (24). The extracted bilirubin glucuronides were injected into the HPLC apparatus, eluted with a methanol gradient (40–90% by volume) in 0.1 M sodium acetate, pH 4.0/5 mM heptanesulfonic acid (25) at a flow rate of 1 ml/min, and detected with an LC75 spectrophotometer (Perkin–Elmer) set at 436 nm. HPLC analysis of heme in bile was conducted by using the same apparatus and columns but with a methanol gradient (40–90% by volume) in 10 mM potassium phosphate, pH 6.0/5 mM tetrabutylammonium hydroxide at a flow rate of 0.8 ml/min and detection at a wavelength of 410 nm. Under these conditions, heme was completely separated from Sn-protoporphyrin and the bilirubin derivatives; Sn-protoporphyrin and the bilirubin conjugates were not clearly separated. Studies were conducted with two pairs of animals (two animals treated with heme alone and two treated with Sn-protoporphyrin plus heme).

RESULTS

Effect of Sn-Protoporphyrin on Excretion of Heme and Bilirubin in Bile After Infusion of Heat-Damaged Erythrocytes in Bile-Duct-Cannulated Rats. Heat-damaged erythrocytes injected into animals have been reported to be taken up primarily in the spleen and other reticuloendothelial cell-containing tissues; heme is then oxidized to bile pigment by heme oxygenase in these cells (6, 26, 27). The objective of the experiments described below, therefore, was to determine the extent to which Sn-protoporphyrin affected the metabolism of heme in reticuloendothelial cells and the biliary excretion of heme after administration of heat-damaged erythrocytes. The output of bilirubin and heme in bile was followed for a control period of 5 hr in 16 bile-duct-cannulated rats. Subsequently, 8 of the animals (defined as controls) received an intravenous bolus of 8.9 × 10^9 heat-damaged erythrocytes/kg of body mass, equivalent to 8.5 μmol of heme/kg, and the other 8 received, in addition, Sn-protoporphyrin (10 μmol/kg) injected intravenously at the same time.

For 7 of the 8 control animals receiving heat-damaged erythrocytes alone, no increase in heme output in bile was detected in the 12–14 hr following the erythrocyte infusion; for 1 control animal (Fig. 1*B*) an increase in heme output was detected but it was negligible. The results of three experiments that typify the results for all 8 Sn-protoporphyrin-treated animals are shown in Fig. 1 *A–C*. In the 8 animals receiving both heat-damaged erythrocytes and Sn-protoporphyrin, there was, in each instance, a prompt increase in biliary heme excretion (Fig. 1 *A–C*) as measured by the pyridine hemochromogen method; this was confirmed by TLC analysis at times throughout the study period shown (data not shown). As expected, a marked and rapid increase in biliary bilirubin output developed in each animal (Fig. 2 *A–C*) infused with heat-damaged erythrocytes. This increase was greatly diminished in all but one Sn-protoporphyrin-treated animal (Fig. 2*C*).

In these experiments with heat-damaged erythrocytes, the combined amounts of heme plus bilirubin in bile in the two groups of animals (control and Sn-protoporphyrin treated) were similar, averaging about 50% of the dose of heme administered (as hemoglobin in the heat-damaged erythrocytes). However, in the animals treated with the erythrocytes alone, almost all of the ≈50% of the heme metabolized was recovered as bilirubin; in the Sn-protoporphyrin-treated group, on the other hand, the decline in biliary bilirubin output was accounted for almost entirely by an increase in heme excretion in bile. TLC analysis of biliary heme in these animals confirmed the pyridine hemochromogen findings in the same bile samples.

Effect of Sn-Protoporphyrin on the Excretion of Heme and Bilirubin in Bile After the Infusion of Heme in Bile-Duct-Cannulated Rats. Heme administered to animals is taken up mostly by hepatic parenchymal cells, where it is rapidly metabolized to bile pigment (28–30). In the present experiments, the effect of Sn-protoporphyrin on the metabolism of exogenous heme to bilirubin as well as on the excretion of heme in bile was studied. Bilirubin and heme excretion in bile were monitored for a control period of 3 hr in 10 bile

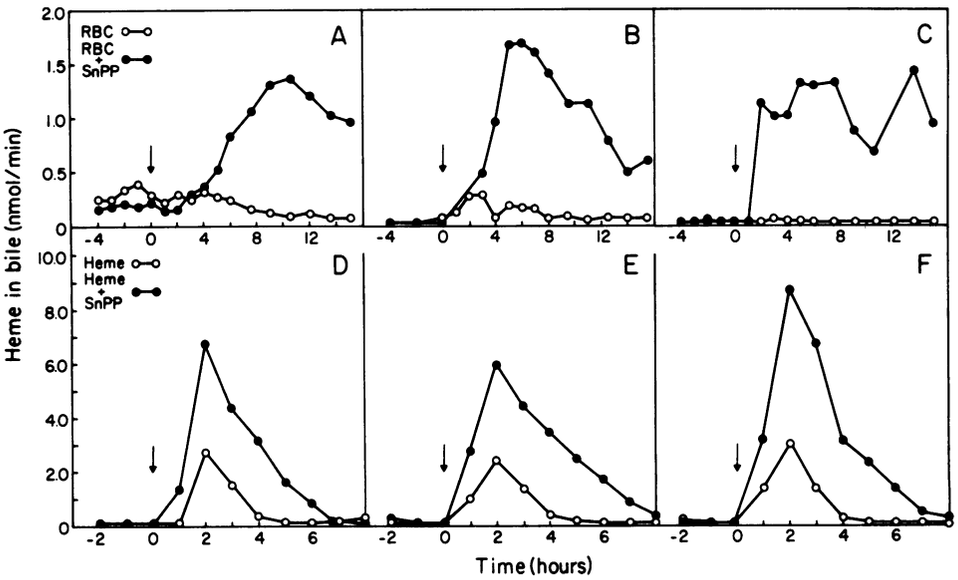

FIG. 1. Effect of Sn-protoporphyrin (SnPP) (10 μmol/kg of body mass, i.v.) on heme excretion in bile of bile-duct-cannulated rats. Bile was collected during 1-hr intervals from bile-duct-cannulated male rats before and after treatment (arrows) with heat-damaged erythrocytes (RBC), with or without Sn-protoporphyrin (*A–C*) (8 pairs of animals), or with heme, with or without Sn-protoporphyrin (*D–F*) (5 pairs of animals). Each pair of control (open circles) and Sn-protoporphyrin-treated animals (closed circles) was studied on the same day and the data shown are from three representative experiments in each group.

duct-cannulated rats. Then, 5 of the animals were given an intravenous bolus of heme (6.1 μmol/kg) and the other 5 received the same dose of heme plus Sn-protoporphyrin (10 μmol/kg), also injected intravenously, at the same time. The results of three studies, which typify those in all studies carried out, are depicted graphically in Fig. 1 *D–F*. In all animals, little or no heme could be detected in bile collected before heme infusion. Injection of heme alone resulted in the appearance of some heme in bile in all animals studied (Fig. 1 *D–F*).

The effect of Sn-protoporphyrin given concurrently with the heme infusion was to elicit in all animals a prompt and marked increase in biliary heme excretion, which lasted for ≈6 hr, as shown for the three typical experiments depicted in Fig. 1 *D–F*. A significant increase in bilirubin output also occurred (Fig. 2 *D–F*) in all animals treated with heme alone. The Sn-protoporphyrin treatment completely or almost completely eliminated the increase in biliary bilirubin resulting from the infusion of heme in all animals studied (Fig. 2 *D–F*). The total amount of heme excreted in the bile of the control (i.e., injected with heme alone) animals averaged 14 ± 3% (SD) of the dose injected, and an average of 18 ± 6% (SD) was excreted as bilirubin. This degree of conversion of exogenous heme to biliary bilirubin is within the range described by others (30). Therefore, the total amount of exogenous heme recovered in the bile of control animals as both heme and bilirubin averaged 32 ± 7% (SD) of the dose administered. The amounts of heme excreted in bile in the animals

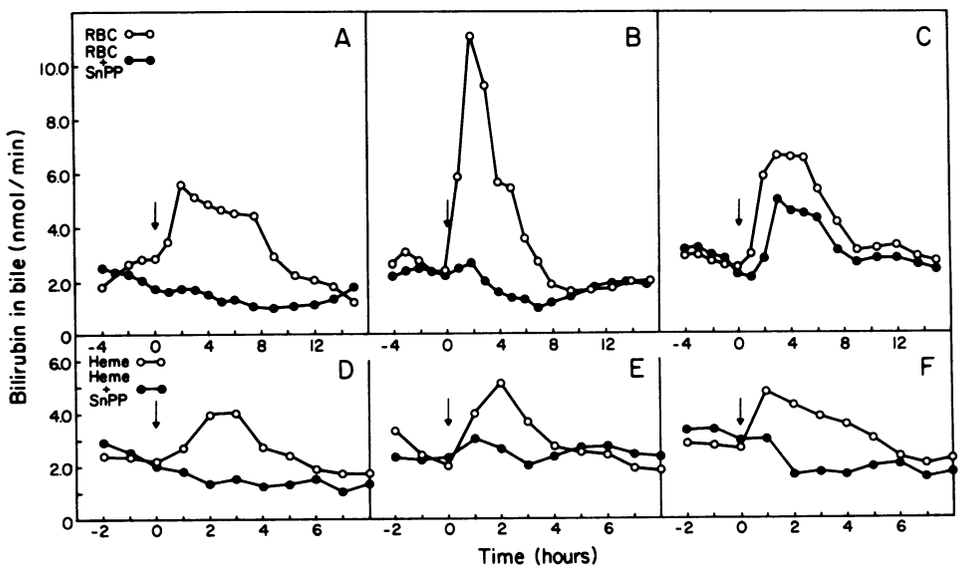

FIG. 2. Effect of Sn-protoporphyrin (SnPP) (10 μmol/kg of body mass, i.v.) on bilirubin excretion in bile of bile-duct-cannulated rats. Bile was collected during 1-hr intervals from bile-duct-cannulated male rats before and after treatment (arrows) with heat-damaged erythrocytes (RBC), with or without Sn-protoporphyrin (*A–C*) (8 pairs of animals), or with heme, with or without Sn-protoporphyrin (*D–F*) (5 pairs of animals). Open circles represent control animals and closed circles represent Sn-protoporphyrin-treated animals in three representative experiments in each group.

treated with Sn-protoporphyrin and heme were 3- to 4-fold greater than the amounts of heme in bile in the control animals treated with heme alone (means of 51% and 14%, respectively, of the dose of heme injected). In all cases, the increased output of heme in bile elicited by Sn-protoporphyrin exceeded the decrease in biliary bilirubin content produced by the synthetic metalloporphyrin.

HPLC analysis of bile for heme and of bilirubin conjugates was carried out for 2 rats treated with heme alone (controls) and 2 rats treated with Sn-protoporphyrin and heme. Bile obtained during pretreatment periods and during several intervals (0–1, 1–2, and 3–4 hr) after treatment was analyzed. In control animals (i.e., heme treatment alone), a small but distinct increase in biliary heme was detected within 1 hr; the mono- and diglucuronide derivatives of bilirubin, which were well resolved, increased markedly after heme administration. Concurrent administration of Sn-protoporphyrin was accompanied by a prompt and marked increase in the output of biliary heme and greatly diminished the heights of both peaks of the bile pigments (Fig. 3).

DISCUSSION

We have shown previously that Sn-protoporphyrin administration reduced the production of bilirubin from endogenous as well as exogenous heme *in vivo*, as manifested by a decreased output of bilirubin in bile of bile-duct-cannulated rats (10, 31). The eventual fate of unmetabolized heme when heme oxygenase, the rate-limiting enzyme in the catabolism of heme to bilirubin, is inhibited by Sn-protoporphyrin has been clarified in the present study by the discovery of marked increases in the output of heme in bile after administration of the synthetic metalloporphyrin to animals treated with exogenous heme. Trace amounts of heme in bile of bile-duct-cannulated rats during control periods (Fig. 1) were detected in some animals in this study. This was not a consistent finding and it was not possible to quantitate accurately this excretion of endogenous heme. However, after administration of exogenous heme, there was a readily detectable increase in biliary excretion of heme in all animals studied; this increase was minimal or did not occur at all in the animals treated with heat-damaged erythrocytes alone. After Sn-protoporphyrin administration, heme excretion in bile increased markedly in both groups of animals as compared with control periods. The effect of Sn-protoporphyrin on heme excretion in bile was more prolonged in animals treated with heat-damaged erythrocytes than in animals treated with heme (Fig. 1 *A–C*), but the total amount of heme excreted in bile was greater in the animals treated with heme and the inhibitor metalloporphyrin (Fig. 1 *D–F*).

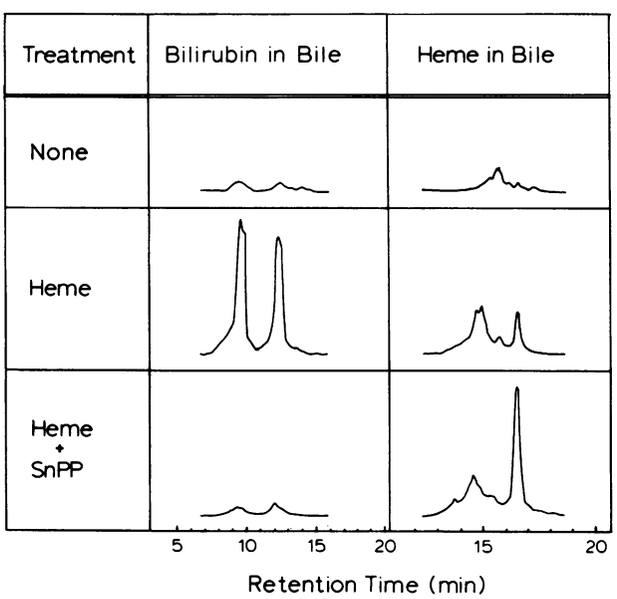

FIG. 3. Effect of heme alone and Sn-protoporphyrin (SnPP) plus heme on the excretion of bilirubin conjugates and heme in bile-duct-cannulated rats. One pair of animals each was used for analyses of biliary bilirubin and heme by HPLC. Data shown are representative HPLC profiles for bile specimens collected between 1 hr and 2 hr after injection of compounds. Conditions for HPLC analyses are described in *Materials and Methods*. Peaks with retention times of 8.7 min and 11.1 min in the bilirubin profiles correspond to bilirubin mono- and diglucuronide, respectively, and peaks with retention times of 14–15 min and 16.8 min in the heme profiles correspond to bilirubin glucuronides and heme, respectively. Sn-protoporphyrin appears at a retention time of 15.3 min under the conditions of the assay but did not significantly contribute to the analysis of heme.

The increase in the output of heme in bile during inhibition of heme oxygenase in rats treated with heat-damaged erythrocytes accounted in large part for the decrease in bilirubin output observed in these animals. This finding indicates that heme released by the breakdown of heat-damaged erythrocytes does not necessarily accumulate in the reticuloendothelial cells responsible for capture and lysis of these red cells and that a substantial portion of such unmetabolized heme can be transported to hepatocytes and be excreted in the bile if heme oxygenase is inhibited. In bile-duct-cannulated animals treated with heme and Sn-protoporphyrin, the total amount of heme excreted in bile exceeded the amount of heme which the inhibitory metalloporphyrin had prevented from being transformed into bilirubin. This fact indicates that in the presence of the enzyme inhibitor, not only is a substantial fraction of exogenous heme that would ordinarily be degraded to bilirubin rapidly excreted in bile but that an additional fraction of heme—of exogenous or endogenous origin, or both—is also disposed of in this manner. Thus, it is apparent that a substantial fraction of heme that remains unmetabolized when heme oxygenase is inhibited is excreted via the biliary system into the intestinal tract, where its degradation products can be disposed of in the feces. These findings establish that Sn-protoporphyrin, though it potently inhibits the degradation of heme to bile pigment, does not cause a substantial or sustained tissue accumulation of undegraded heme, since unmetabolized heme can be promptly excreted into bile. Therefore, concern about potentially deleterious, though as yet undefined, biological effects of large cellular accumulations of heme after Sn-protoporphyrin administration seems unwarranted.

The capacity of the liver to excrete excess heme in bile when heme oxygenase activity is inhibited defines a new and important relationship between heme oxygenase, competitive inhibitors of the enzyme, and hepatic excretory function. It was shown previously that coproporphyrin isomers (32, 33), protoporphyrin (34, 35), and metalloporphyrins, including heme (36, 37) and Sn-protoporphyrin (12), could be identified in bile (or feces in the case of Sn-protoporphyrin) when individually administered to animals with biliary fistulas or to humans. It had not been demonstrated previously, however, that inhibitors of heme oxygenase could rapidly and markedly increase the excretion of unmetabolized heme into bile. The present study indicates that this biliary excretion represents the major route for heme disposal when heme binding at the catalytic site of heme oxygenase, and perhaps at other cellular sites, is inhibited by Sn-protoporphyrin. Furthermore, the results suggest that the biliary excretion of heme merits further study under a variety of conditions in which there is increased delivery of heme to the liver or in which the levels or activity of heme oxygenase or other heme-binding proteins are altered by chemical exposures, developmental factors, or selected metabolic or pathologic circumstances. Examples of such circumstances include the

great variety of genetic disorders characterized by acute or chronic intravascular hemolysis, environmental exposures in which erythrocyte lysis occurs, and certain of the human hereditary porphyrias that are treated by the intravenous infusion of heme.

The present studies show that when heme oxygenase activity is inhibited by Sn-protoporphyrin, those amounts of exogenous heme that exceed the residual capacity of the heme degradation system to form bile pigments can be excreted into the bile. We have not examined whether the same phenomenon occurs with normally produced amounts of endogenous heme. However, we have shown that Sn-protoporphyrin inhibits the output of biliary bilirubin derived from the catabolism of endogenous heme in normal bile-duct-cannulated rats (10, 31), and Millevile *et al.* (38) have shown that this metalloporphyrin significantly reduces endogenous carbon monoxide production in mice. Moreover, Blumenthal *et al.* (39) earlier demonstrated that the bile of several mammalian species, including human fetuses, contained a brown pigment that was characterized spectroscopically and chromatographically as identical to heme. It is probable, therefore, that the liver of newborn mammals, including humans, is also able to excrete into bile that fraction of endogenous heme that is not degraded to bile pigment when heme oxygenase activity is inhibited by Sn-protoporphyrin. The possibility of minimizing the deposition of excess heme-derived iron in the liver and perhaps in other tissues by enhancing the biliary excretion of heme through appropriate administration of an inhibitor of heme oxygenase is also raised by our present findings.

From a clinical point of view, the response of the liver excretory mechanism to Sn-protoporphyrin administration described in this study supports the rationale of attempting to treat neonatal hyperbilirubinemia by inhibiting heme oxygenase activity rather than by attempting to dispose of bilirubin after the bile pigment has been formed. This is evident since the existence of a biliary excretion mechanism for the substrate heme (9) as well as for the toxic metabolite bilirubin (10) clearly provides a ready means for disposal of unmetabolized heme. Therefore, Sn-protoporphyrin, as a potent inhibitor of heme oxygenase, has the valuable pharmacological property of not only suppressing bilirubin production but also enhancing the biliary excretion of untransformed heme. In addition, the synthetic metalloporphyrin itself undergoes significant biliary disposal in the unmetabolized form (12). These features of the biological action of Sn-protoporphyrin define some of the characteristics of a chemical that could prove to be a prototype of a group of related compounds with potential as therapeutic agents in humans.

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