

**Sn-PROTOPORPHYRIN BLOCKS THE INCREASE IN SERUM
BILIRUBIN LEVELS THAT DEVELOPS POSTNATALLY IN
HOMOZYGOUS GUNN RATS**

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It has been demonstrated in previous studies that tin (Sn⁴⁺)-protoporphyrin (SnPP), a potent competitive inhibitor in vitro of the activity of heme oxygenase (1, 2), the rate-limiting enzyme in the degradation of heme to bile pigments (3), blocks the in vivo production of bilirubin and suppresses hyperbilirubinemia in neonatal rats (4-7), mice with severe genetically determined hemolytic anemia (8), newborn primates exhibiting physiologic hyperbilirubinemia (9), normal volunteers (10), patients with elevated levels of plasma bilirubin due to hepatic dysfunction (11), and term infants with direct Coombs-positive ABO incompatibility (12). The mechanism of action of SnPP has been unequivocally shown to involve an inhibition of the in vivo catabolism of heme to bilirubin (4-7).

Jaundice is common in human newborn and a significant number of infants require clinical intervention for this condition because bilirubin is potentially a neurotoxin. At present, effective clinical management of neonatal hyperbilirubinemia is limited to phototherapy and to exchange transfusion (13); both treatment modalities are associated with possible side effects, and in the case of exchange transfusion, mortality. Furthermore, these therapies are designed to remove preformed bilirubin at a time when blood levels of this toxic bile pigment are either already at high levels or increasing rapidly in newborn infants; and the means for applying these treatments are, in any case, often not available to many babies in third-world countries.

An alternate method of treatment, which is designed to diminish the production of bilirubin rather than to dispose of the bile pigment after its formation and tissue distribution, has been proposed using the heme oxygenase inhibitor SnPP (1, 2). SnPP, because of its ability to bind more avidly to the heme-binding site of heme oxygenase than heme itself (1, 2), inhibits heme degradation and the production of bilirubin, and thereby suppresses hyperbilirubinemia, thus offering a new and potentially useful therapy for the clinical management of hyperbilirubinemia in the newborn.

To further examine the actions of SnPP on naturally occurring forms of hyperbilirubinemia, we have examined the effect of the compound in the congenitally jaundiced newborn Gunn rat, an animal model of severe hyperbilirubinemia in which UDP-glucuronyl transferase activity for bilirubin is, for

genetic reasons, absent. This enzyme deficiency mimics the transient deficiency in bilirubin-conjugating ability that characterizes the newborn and the genetically determined deficiency of bilirubin glucuronide conjugation from which patients with the Crigler-Najjar syndrome suffer. The results of this study indicate that SnPP when administered shortly after birth can block the rapid rise in serum bilirubin levels that characterizes the Gunn rat in the postnatal period. These data provide the first demonstration that the severe jaundice that develops in animals completely deficient in UDP-glucuronyl transferase activity for bilirubin can be ameliorated by a synthetic heme analogue acting as a competitive inhibitor of heme oxygenase activity.

Materials and Methods

Rats. 37 newborn homozygous Gunn rats were injected subcutaneously with 50 $\mu\text{mol/kg}$ body weight SnPP at 24–30 h old immediately after blood was drawn for serum bilirubin determinations. Of the 37 newborn rats, 14 were reinjected at 6 d old with an additional 50 $\mu\text{mol/kg}$ body weight SnPP and their serum bilirubin concentrations determined again at 12 d old. 28 untreated newborn homozygous Gunn rats served as controls; in these neonates serum bilirubin concentrations were determined at 24–30 h old, at 6 d, and again ($n = 23$) at 12 d old. All newborn rats were raised with their dams in an environmentally controlled vivarium so that nutrition and nurturing were not interrupted. Serum bilirubin concentrations were determined by an AO Bilirubinometer.

In the bile duct-cannulated animal studies, one control and one SnPP-treated animal were studied at the same time, as previously described (4). SnPP was administered intravenously through a jugular vein cannula at a dose of 10 $\mu\text{mol/kg}$ body weight. Control animals were administered an equivalent volume of saline.

Tissue Preparation. Livers were perfused in situ with ice-cold 0.9% NaCl and homogenized in 3 vol of 0.1 M potassium phosphate buffer, pH 7.4, containing 0.25 M sucrose, and the microsomal fractions were prepared as previously described (1) for the determination of heme oxygenase activity and cytochrome P450 content. Spleen and kidney microsomes were prepared in an identical manner. The cytosolic fraction obtained from the liver of control animals served as a source of biliverdin reductase.

Assays. The activity of heme oxygenase in all tissues was determined as previously described (1). Bilirubin formation was calculated using an absorption coefficient of 40 $\text{mM}^{-1}\text{cm}^{-1}$ between 464 and 530 nm. Cytochrome P450 was measured by the method of Omura and Sato using an absorption coefficient of 91 $\text{mM}^{-1}\text{cm}^{-1}$ between 450 and 490 nm (14). Bile bilirubin content was measured by the fluorometric method of Roth (15) and protein content was determined by the method of Lowry et al. (16) using crystalline BSA as standard. The data were analyzed by Student's *t* test.

SnPP was purchased from Porphyrin Products, Logan, UT. All other chemicals were of the highest grade available from either Sigma Chemical Co., St. Louis, MO, or Fisher Scientific Co., Pittsburgh, PA.

Results

The mean serum bilirubin concentrations of the 28 control newborn rats at 24–30 h old was 6.87 ± 0.5 mg/dl, and at 6 d old the mean value had increased to 8.06 ± 0.6 mg/dl (Fig. 1). The mean serum bilirubin concentration of the 37 pups that were injected with SnPP 24–30 h after birth was 6.56 ± 0.4 mg/dl, a value comparable to the controls; at 6 d old, SnPP-treated pups had a mean serum bilirubin of 6.54 ± 0.4 mg/dl, unchanged from that at birth, whereas the serum bilirubin levels in the control pups, as noted, underwent the normal postnatal increase in bilirubin observed in these animals. The difference in mean values was statistically significant ($p < 0.05$) at 6 d postpartum.

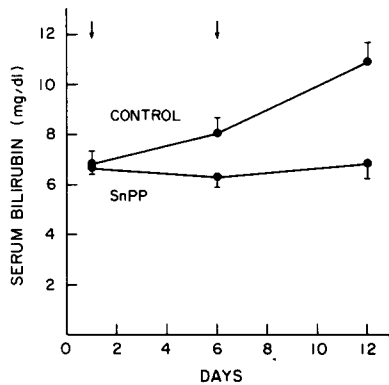


FIGURE 1. Mean serum bilirubin concentrations of newborn Gunn rats injected with SnPP, $n = 14$, 24–30 h after birth and at 6 d old (↓) and with saline (control), $n = 28$. Mean \pm SE.

TABLE I
Effect of SnPP on Bilirubin Production in Bile of Bile Duct-cannulated Gunn Rats

Treatment	Bilirubin		Change
	Before	After	
	$\mu\text{g}/\text{min}$	$\mu\text{g}/\text{min}$	%
Saline	0.87 ± 0.05	0.70 ± 0.02	-19.5
SnPP	1.07 ± 0.05	$0.73 \pm 0.01^*$	-31.8

Mean \pm SE, $n = 7$. Bilirubin measured 6 h before and 15 h after SnPP was administered intravenously at $10 \mu\text{mol}/\text{kg}$ body weight.

* $p < 0.01$ compared to control period.

The serum bilirubin concentrations of the 23 controls were again measured at 12 d old. The serum concentration of the bile pigment, as expected, had continued to rise to a mean value \pm SE of $10.98 \pm 0.6 \text{ mg}/\text{dl}$ (Fig. 1). The serum bilirubin levels of the 14 pups injected with SnPP at 24–30 h old, and reinjected at 6 d old were also determined at 12 d. In contrast to the controls, the mean serum bilirubin level \pm SE of the treated neonates was $6.85 \pm 0.6 \text{ mg}/\text{dl}$ (Fig. 1). Thus, the SnPP-treated animals maintained a constant serum bilirubin concentration after two treatments with SnPP postpartum, whereas the serum bilirubin levels in control animals underwent the rapid, substantial increase characteristic of these UDP-glucuronyl transferase-deficient animals. The values at 12 d were significantly lower for the SnPP-treated pups compared with controls ($p < 0.001$).

The output of bilirubin in bile in seven bile duct-cannulated rats treated with SnPP, $10 \mu\text{mol}/\text{kg}$ body weight, compared with seven similar controls treated with saline, is shown in Table I. This dose of SnPP was the lowest effective dose of the metalloporphyrin that was shown in previous studies to diminish biliary bilirubin output in normal bile duct-cannulated rats (4). SnPP administration resulted in a statistically significant ($p < 0.01$) decrease in the output of bilirubin in the bile of SnPP-treated Gunn rats as compared with the decline in biliary bilirubin output usually observed in the postsurgical period in control animals. The average net decrease in bilirubin output produced by SnPP was 31.8% compared with 19.5% for the untreated animals. The activity of heme oxygenase in liver, kidney, and spleen of SnPP-treated bile duct-cannulated animals was significantly lower than identically treated control animals administered saline

TABLE II
Effect of SnPP on Tissue Heme Oxygenase Activity in the Bile Duct-cannulated Gunn Rat

Tissue	Heme oxygenase (nmol bilirubin/mg per h)		Change %
	Saline	SnPP	
Liver	3.97 ± 0.38	1.08 ± 0.23*	-73
Kidney	1.34 ± 0.31	0.72 ± 0.15	-46
Spleen	10.44 ± 1.26	4.77 ± 0.42‡	-54

Mean ± SE, $n = 7$; SnPP was administered intravenously at 10 $\mu\text{mol/kg}$ body weight.

* $p < 0.001$ when compared with control.

‡ $p < 0.01$ when compared with control.

(Table II). In addition, the levels of hepatic cytochrome P450 remained unchanged in the bile duct-cannulated Gunn rat after SnPP administration (control, 0.59 ± 0.04 nmol/mg; SnPP 0.64 ± 0.03 nmol/mg; mean ± SE, $n = 7$).

Discussion

The results of the experiments described above provide confirmation of our previous studies showing that SnPP inhibits the *in vivo* catabolism of heme and suppresses hyperbilirubinemia in a wide variety of experimentally induced and naturally occurring forms of jaundice in animals and man, including newborn infants (12). In this study, however, the inhibition was demonstrated in the Gunn rat, an animal completely deficient in the enzyme activity, UDP-glucuronyl transferase for bilirubin, and thus in the ability to conjugate and excrete this bile pigment in normal fashion. The resulting severe hyperbilirubinemia is a pathologic condition, permanent rather than transient, but which mimics in certain respects the jaundice that develops in human neonates, who at birth are characterized by a cholestatic state, have a transient deficiency in UDP-glucuronyl transferase activity, and thus display an impaired ability to conjugate and excrete bilirubin at normal rates. This developmentally dependent deficiency in bilirubin-conjugating ability coupled with the excessive rate at which heme is catabolized postnatally to bile pigments accounts in large part for the severe hyperbilirubinemia that develops in many newborn infants.

As these studies show, in the naturally occurring, genetically determined form of severe neonatal jaundice in the Gunn rat, SnPP acts effectively to prevent the marked increase in serum bilirubin concentration that occurs in the immediate postnatal period, in the same manner as it does in normal neonatal animals and in adult animals treated with exogenous heme (4). Moreover, a repeat injection of SnPP at 6 d old maintained the inhibition of bilirubin production and prevented the rapid increase in blood levels of this bile pigment that would otherwise be anticipated during the initial 12 d of the postnatal period in the Gunn neonates. On the basis of earlier work in mice, severely jaundiced as a result of genetically determined hemolytic anemias, the SnPP effect would be expected to be sustained as long as the inhibitor was being administered (8). It is possible that SnPP would not control serum bilirubin levels in mature Gunn rats as effectively as it does in neonates, since early administration of the

compound in the newborn would be expected to diminish the accumulation of bilirubin in tissue sites, which characterizes the adult Gunn rat.

The administration of the metalloporphyrin resulted in a significant decrease in bilirubin output in bile (Table I) in the adult homozygous Gunn rat, an action similar to that of the compound in normal experimental animals (4, 7) and in normal human volunteers (10). This decrease in biliary bilirubin output occurred without an increase in plasma bilirubin levels, clearly indicating that SnPP behaves in the Gunn rat in a manner similar to that observed in animals with normal bilirubin-conjugating ability (4, 7). Since essentially all bilirubin formed from heme catabolism undergoes biliary excretion, based on these experiments and the extensive data presented in other reports (4, 7), the decreased output of biliary bilirubin is most reasonably explained by an SnPP inhibition of production of the bile pigment. The substantial diminution in the activities of hepatic, splenic, and renal heme oxygenase in the SnPP-treated Gunn rats support this view (Table II).

It is interesting to note that SnPP administration had no effect on the levels of cytochrome P450 in the bile duct-cannulated Gunn rat. The dose of SnPP administered (10 $\mu\text{mol/kg}$ body weight) was substantially (15–20-fold) greater than that used to significantly diminish the increases in plasma bilirubin that occur in the immediate postnatal period in term infants with direct Coombs positive ABO incompatibility (12). Thus, within the range of the small doses of SnPP that might conceivably be used clinically, based on the studies described above, no significant influence of the compound on cytochrome P450 content or function would be expected to occur.

The demonstration that even in the Gunn rat—an animal species characterized by a genetically determined absence of UDP-glucuronyl transferase activity for bilirubin—SnPP administration can substantially block the marked increases in plasma bilirubin levels that occur in the immediate postnatal period affirms the potency of this enzyme inhibitor to block bilirubin production *in vivo*. These findings also raise the possibility that SnPP, or a related heme oxygenase inhibitor (17), might prove useful in maintaining low plasma levels of bilirubin in patients with the Crigler-Najjar syndrome, a genetic disorder in which there is a partial deficiency of UDP-glucuronyl transferase, and in which patients are at substantial risk of neurotoxicity due to the sustained severe hyperbilirubinemia associated with this syndrome.

Summary

Administration of Sn-protoporphyrin to Gunn rats that are characterized by a genetically determined absence of UDP-glucuronyl transferase activity for bilirubin, 24–30 h after birth, prevented the marked increase in serum bilirubin concentration that occurs in these animals in the postnatal period. A second administration of Sn-protoporphyrin at day 6 maintained serum bilirubin levels in the neonates at the initial level for an additional 6 d. In contrast, in untreated Gunn neonates, serum bilirubin levels increased substantially as expected during the immediate 2-wk period after birth. Studies in adult Gunn rats demonstrated that Sn-protoporphyrin administration diminished biliary bilirubin output, decreased tissue heme oxygenase activity, and did not alter hepatic cytochrome P450 levels. These findings raise the possibility that Sn-protoporphyrin may

prove clinically useful in maintaining low levels of serum bilirubin in congenitally jaundiced individuals, such as patients with the Crigler-Najjar syndrome.

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