

SUPPRESSION OF HYPERBILIRUBINEMIA IN THE RAT  
NEONATE BY CHROMIUM-PROTOPORPHYRIN

Interactions of Metalloporphyrins with  
Microsomal Heme Oxygenase of Human Spleen\*

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We have in recent studies (1-3) described the ability of tin-protoporphyrin to act as a potent competitive inhibitor of heme oxygenase, the rate limiting enzyme of heme degradation, both in vitro and in vivo. This synthetic metalloporphyrin, administered to newborn rats, can block the major increases in heme oxygenase activity that occur in various tissues after birth and can completely prevent the development of the hyperbilirubinemia that normally occurs postnatally.

We report here that another synthetic metalloporphyrin, chromium (Cr) -protoporphyrin, also markedly inhibits the oxidative degradation of heme to bile pigment and can, when administered in single doses to neonatal animals immediately after birth, prevent the hyperbilirubinemia that characterizes the early postnatal period. The competitive interaction of Cr-protoporphyrin, as well as other synthetic metalloporphyrins, with the human splenic heme oxygenase system is also described. The results of these studies indicate that synthetic metalloporphyrins have the potential for inhibiting the rate of heme degradation to bile pigment in human as well as animal tissues.

**Materials and Methods**

*Animals.* Male (140-160 g) and 15-d pregnant female Sprague Dawley rats purchased from Taconic Farms, Germantown, NY and Holtzman, Madison, WI, respectively, were used. Pregnancy was synchronized in the latter group of animals so that large numbers of newborn could be studied within the same postnatal time period; the study involved a total of 700 neonates divided approximately equally among the control and experimental groups described below.

*Chemicals.* The synthetic metalloporphyrins used in this study were purchased from Porphyrin Products, Logan, UT. All other chemicals were of the highest grade obtainable from either Sigma Chemical Co., St Louis, MO, or Fisher Scientific Co., Pittsburgh, PA.

*Animal Treatment and Preparation of Microsomes.* Male rats were injected at subcutaneous sites in the nuchal region with various doses of Cr-protoporphyrin up to and including 50  $\mu\text{mol/kg}$  body weight (bw). To prepare Cr-protoporphyrin in solution, the metalloporphyrin was taken up in a small volume of 0.2 M NaOH (0.2 ml/1.0 ml of final volume of the metalloporphyrin solution) adjusted to pH 7.4 with 1 M HCl, and made up to final volume with 0.9% NaCl. Control animals were injected with an equivalent volume of saline. The animals were allowed free access to water but were starved for 16 h before killing. Microsomes were prepared as previously described (4).

At birth neonates received a single subcutaneous injection of Cr-protoporphyrin (10  $\mu\text{mol/}$

\* Supported by a grant from the Dyson Foundation, New York; grant I-804 from the March of Dimes Birth Defects Foundation; and grant ES-01055 from the U. S. Public Health Service.

kg bw) prepared as described above, but in a final volume of 0.1 ml. Control neonates received an equivalent volume of saline at birth. The neonates remained with their mothers throughout the experiment; groups of neonates (6–30 animals per group) were killed at the times indicated in the figures; assays were performed for serum bilirubin and heme oxygenase as described below.

*Preparation of Microsomes from Human Spleen.* Samples of human spleen, obtained during the course of operative procedures requiring spleen removal, were provided to us through the Tissue Procurement Service at the Memorial Sloan-Kettering Cancer Center, New York. The tissue was washed thoroughly with 0.9% saline to remove excess blood and then minced, homogenized in 3 vol of 0.1 M potassium phosphate buffer pH 7.4, containing 0.25 M sucrose, and the microsomal fraction was prepared as described previously (4).

*Enzyme Assays.* Heme oxygenase was assayed as described (1), and bilirubin formation was calculated on the basis of an extinction coefficient of  $40 \text{ mM}^{-1} \text{ cm}^{-1}$  and the difference in absorbance between 464 nm and 530 nm. Heme oxygenase activity was expressed as nanomoles bilirubin formed per milligrams microsomal protein per h. All assays were performed in duplicate on an Aminco Chance DW2A spectrophotometer in the split-beam mode.

Total bilirubin was estimated fluorometrically by the method of Roth (5); the variation in replicate serum samples was <5%. Cr-protoporphyrin did not interfere with bilirubin determination in this assay. Protein concentration was determined using crystalline serum bovine albumin as standard by the method of Lowry et al. (6). The data were analyzed by the standard *t* test and the value  $P < 0.05$  was regarded as significant.

## Results

*The Dose-dependent Effect of Cr-Protoporphyrin on Heme Oxygenase Activity in Liver.* Cr-protoporphyrin administered in doses ranging from 0.5 to 50  $\mu\text{mol/kg}$  bw was examined in adult male rats for its effects in vivo on heme oxygenase, the rate limiting enzyme (7, 8) of heme degradation (Table I). Hepatic heme oxygenase activity, measured 16 h after Cr-protoporphyrin administration, was lowered in a dose-dependent manner. Even at the lowest dose of the metalloporphyrin studied (0.5  $\mu\text{mol/kg}$  bw), heme oxygenase activity was diminished by >50%. Splenic and renal heme oxygenase activities were also lowered in a dose-dependent manner; however, at the lowest dose of Cr-protoporphyrin examined (0.5  $\mu\text{mol/kg}$  bw), no significant decrease in enzyme activity was detected in either organ (results not shown). This effect of Cr-protoporphyrin on enzyme activity was long lasting. A single administration of the metalloporphyrin at a dose of 1  $\mu\text{mol/kg}$  bw resulted in a rapid (within 5 h) lowering of hepatic heme oxygenase activity to a level, by 8 h, <50% of the initial control level ( $1.16 \pm 0.13$  and  $2.56 \pm 0.22$ , respectively) where it remained for the succeeding 14 d.

*Competitive Inhibition of Heme Oxygenase Activity In Vitro by Cr-Protoporphyrin.* The apparent  $K_m$  for heme in the rat spleen microsomal heme oxygenase system was determined to be 7.68  $\mu\text{M}$ , which is within the range reported previously (9, 10). The addition of Cr-protoporphyrin (0.025  $\mu\text{M}$ ) to splenic heme oxygenase resulted in a marked competitive inhibition of enzyme activity, with a greater than threefold increase (7.68–25.64  $\mu\text{M}$  [Fig. 1]) in the apparent  $K_m$  for heme. The  $K_i$  (inhibition constant) for Cr-protoporphyrin was determined to be 0.011  $\mu\text{M}$ . This value is identical to the  $K_i$  value reported previously for tin (Sn-) protoporphyrin (1).

On the basis of these findings (Table I, Fig. 1) Cr-protoporphyrin was administered to neonatal rats at birth to determine if this metalloporphyrin was capable of blocking the development of hyperbilirubinemia in the postnatal period. The dose of Cr-protoporphyrin chosen (10  $\mu\text{mol/kg}$  bw), to be administered in a single dose at birth to neonatal rats, was sufficient to block splenic as well as hepatic and renal heme oxygenase activity in adult animals.

TABLE I  
Effect of Cr-Protoporphyrin on Heme Oxygenase Activity in Rat Liver

| Dose                    | Heme oxygenase                |
|-------------------------|-------------------------------|
| $\mu\text{mol/kg b.w.}$ | $\text{nmol bilirubin/mg ph}$ |
| Control                 | $2.27 \pm 0.18$               |
| 0.5                     | $1.06^* \pm 0.06$             |
| 1                       | $0.89^* \pm 0.26$             |
| 5                       | $0.51^* \pm 0.11$             |
| 50                      | $0.29^* \pm 0.04$             |

Adult male rats (three to six animals) were administered Cr-protoporphyrin subcutaneously at the doses indicated. Animals were starved for 16 h before killing. Microsomal fractions were prepared and heme oxygenase assayed as described in Materials and Methods.

\*  $P < 0.05$  compared with control.

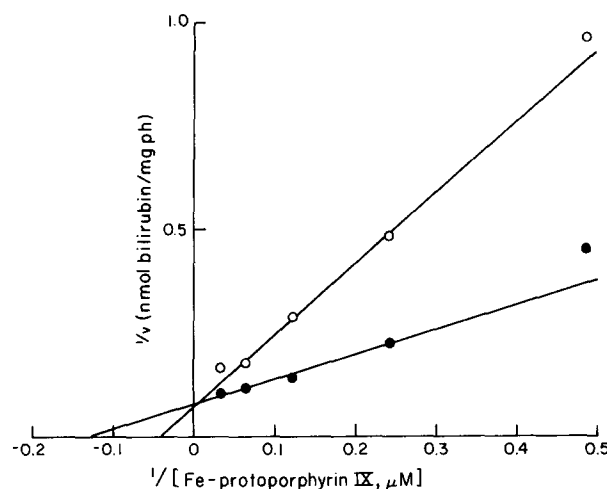


FIG. 1. Double reciprocal plot of substrate concentration vs. velocity of rat spleen microsomal heme oxygenase (●) and in the presence of Cr-protoporphyrin ( $0.025 \mu\text{M}$ ) (○).

*Effects of Cr-Protoporphyrin on Heme Oxygenase Activity and Serum Bilirubin Levels in the Neonate.* The effect of a single dose ( $10 \mu\text{mol/kg}$ ) of Cr-protoporphyrin administered at birth on hepatic and spleen heme oxygenase activities is shown in Fig. 2. This enzyme activity rose rapidly in liver after birth, as expected, reaching a level fourfold above normal adult levels at 5 d before commencing to decline to adult levels. In neonates receiving Cr-protoporphyrin, this liver enzyme activity declined promptly, where it remained at levels well below normal adult levels throughout the 14-d period of study. This dose of Cr-protoporphyrin also immediately and profoundly lowered ( $\sim 80\%$ ) renal heme oxygenase activity, to a point where it remained throughout the entire period of study (results not shown). Splenic heme oxygenase activity in untreated neonates followed the normal course of postnatal development, being low at birth and increasing rapidly in the postnatal period to reach normal adult levels by day 14 (Fig. 2). Administration of Cr-protoporphyrin ( $10 \mu\text{mol/kg bw}$ ) prevented this increase; indeed the splenic heme oxygenase activity declined markedly until day 5, when it began to increase towards the levels characteristic of the normal adult animal.

The total bilirubin concentration in the serum of untreated neonates increased after birth, as expected, to a maximum at day 2, before commencing to decline towards the range typical of the normal adult animal (Fig. 3). Administration of Cr-protoporphyrin ( $10 \mu\text{mol/kg bw}$ ) at birth prevented the increase in serum bilirubin at day 2; thereafter serum bilirubin levels remained lower than those in the control group for

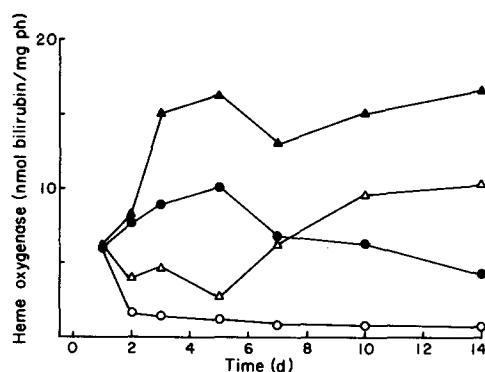


FIG. 2. The activity of heme oxygenase in liver and spleen of the neonate after a single injection of Cr-protoporphyrin ( $10 \mu\text{mol/kg b.w.}$ ) immediately after birth. Hepatic heme oxygenase in control (●), and Cr-protoporphyrin (○)-treated neonates and splenic heme oxygenase in control (●), and Cr-protoporphyrin (○)-treated neonates was assayed in duplicate as described in Materials and Methods. Each point represents the average of three litters (10–12 animals/litter). The values for hepatic heme oxygenase at day 5 were  $10.13 \pm 0.82$  and  $1.22 \pm 0.25$  nmol bilirubin formed/mg microsomal protein per h in control and Cr-protoporphyrin-treated neonates, respectively.

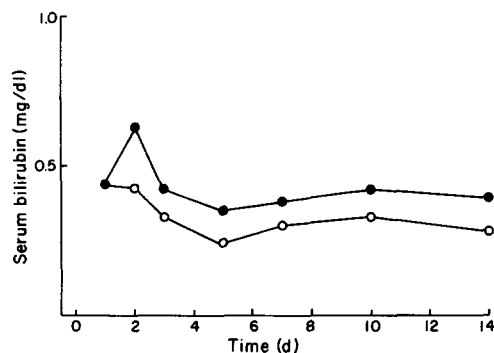


FIG. 3. The levels of serum bilirubin in control (●) and Cr-protoporphyrin-treated (○) neonates. Each time point represents the average of pooled serum samples. The mean values for serum bilirubin from day 2 through day 14 were significantly lower for Cr-protoporphyrin-treated neonates than for the respective controls ( $P < 0.05$  at each time point).

the initial 14 d after birth. The same dose of Sn-protoporphyrin was shown earlier (3) to prevent the prompt postnatal rise of serum bilirubin levels in the newborn rat. At the dose studied ( $10 \mu\text{mol/kg bw}$ ), Cr-protoporphyrin produced no detrimental effects in the newborn animals.

*Competitive Inhibition of Heme Oxidation in Human Spleen Microsomes by Synthetic Metalloporphyrins.* The apparent  $K_m$  for heme in the human spleen microsomal heme oxygenase system was determined to be  $19.04 \pm 0.08 \mu\text{M}$ , which is within the range reported previously for this tissue (11). Using both Dixon and Lineweaver-Burk plots, the  $K_i$  for each of five synthetic metalloporphyrins in this system was determined; the values for Cr- and Sn-protoporphyrins indicate that these compounds were significantly more potent as competitive inhibitors of heme oxidation in human spleen than manganese, cobalt, or zinc protoporphyrins (Table II).

### Discussion

This study demonstrates that Cr-protoporphyrin, when administered to rats in single, small doses immediately after birth, can markedly inhibit the major increases

TABLE II  
*K<sub>i</sub> of a Series of Metalloporphyrins for Human Spleen Microsomal Heme Oxygenase*

| Metalloporphyrin  | <i>K<sub>i</sub></i><br>μM |
|-------------------|----------------------------|
| Sn-protoporphyrin | 0.018 ± 0.001              |
| Cr-protoporphyrin | 0.033 ± 0.009              |
| Mn-protoporphyrin | 0.530 ± 0.140              |
| Co-protoporphyrin | 0.670 ± 0.190              |
| Zn-protoporphyrin | 2.523 ± 0.720              |

*K<sub>i</sub>* were determined using double reciprocal plots of substrate concentration vs. velocity of heme oxygenase in the presence of the appropriate metalloporphyrin and by Dixon plots. No significant difference was found between *K<sub>i</sub>* determined by the two methods.

that develop in the heme oxygenase activities of various tissues and can also prevent the hyperbilirubinemia that normally occurs postnatally. Cr-protoporphyrin is a powerful competitive inhibitor of heme oxygenase activity in vitro (Fig. 3, Table II). The *K<sub>i</sub>* (0.011 μM) of Cr-protoporphyrin for rat spleen microsomal heme oxygenase is identical to that previously reported for Sn-protoporphyrin (1). The rapidity with which heme oxygenase activity is lowered in vivo after Cr-protoporphyrin administration is undoubtedly attributable to the marked ability of this metalloporphyrin to competitively inhibit heme degradation; this effect cannot be due to inhibition of enzyme synthesis as the half-life of heme oxygenase has been previously reported to be ~16 h (12, 13).

Cr-protoporphyrin inhibition of heme oxygenase in the rat spleen microsomal system is competitive in nature (Fig. 1), with an affinity of the metalloporphyrin for the enzyme identical to that previously determined for Sn-protoporphyrin. Both compounds also competitively inhibit the rate of heme oxidation in the human spleen microsomal heme oxygenase system to a comparable degree (Table II). The marked affinities of these metalloporphyrins for human spleen heme oxygenase, as reflected in the *K<sub>i</sub>* values (Table II), greatly exceeded those of several other metalloporphyrins examined and was consistent with their ability to not only inhibit heme oxygenase activities in various animal tissues in vivo and in vitro, but also to concurrently suppress the hyperbilirubinemia which normally occurs in the immediate postnatal period (1, 2). As we noted earlier (1), the ability of a synthetic metalloporphyrin to inhibit heme oxygenase (1-3, 10, 14, 15) does not of itself guarantee that the compound in question will have the requisite biological characteristics in vivo to suppress hyperbilirubinemia. Whole animal studies are essential to establish this point because of the physiological and pharmacokinetic variables involved in such a response. The ineffectiveness of Mn- or Zn-protoporphyrin in preventing neonatal hyperbilirubinemia (1, 3) and the dual properties (16) of Co-protoporphyrin of inhibiting heme oxygenase in vitro while potently inducing the enzyme in vivo affirm this view.

The possibility of suppressing neonatal jaundice by appropriately regulating the rate of heme oxidation in vivo was proposed in a previous study from this laboratory (17). Our recent studies with Sn-protoporphyrin (1, 2) have shown that this compound can effectively exert such a regulatory action in the rat neonate. Cr-protoporphyrin, as the data in the present report indicate, exerts a similar regulatory action.

### Summary

The synthetic metalloporphyrin, Cr-protoporphyrin, is a potent competitive inhib-

itor of heme oxygenase activity in rat spleen, liver, and kidney. When administered to neonatal animals in a single dose immediately after birth, Cr-protoporphyrin suppresses postnatal hyperbilirubinemia and produces a marked and sustained lowering of heme oxidation activity in liver, spleen, and kidney. The metalloporphyrin also potently inhibited the rate of heme degradation to bile pigment in human spleen.

The authors wish to thank Ms. Susan Spinner and Ms. Beth Sheard for their technical assistance and Mrs. Heidemarie Robinson for the preparation of this manuscript.

*Received for publication 15 September 1982.*

### References

1. Drummond, G. S., and A. Kappas. 1981. Prevention of neonatal hyperbilirubinemia by tin protoporphyrin IX, a potent competitive inhibitor of heme oxidation. *Proc. Natl. Acad. Sci. U. S. A.* **78**:6466.
2. Kappas, A., and G. S. Drummond. 1982. The regulation of heme oxidation by synthetic metalloporphyrins: experimental and clinical implications. In *Microsomes, Drug Oxidations and Drug Toxicity*. R. Sato, and R. Kato, editors. Japan Scientific Societies Press, Tokyo, Wiley-Interscience, New York. 629-636.
3. Drummond, G. S., and A. Kappas. 1982. Chemoprevention of neonatal jaundice: potency of tin protoporphyrin in an animal model. *Science (Wash. D. C.)*. **217**:1250.
4. Drummond, G. S., and A. Kappas. 1979. Manganese and zinc blockade of enzyme induction: studies with microsomal heme oxygenase. *Proc. Natl. Acad. Sci. U. S. A.* **76**:5331.
5. Roth, M. 1967. Dosage fluorimetric de la bilirubine. *Clin. Chim. Acta.* **17**:487.
6. Lowry, O., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265.
7. Tenhunen, R., H. S. Marver, and R. Schmid. 1968. The enzymatic conversion of heme to bilirubin by microsomal heme oxygenase. *Proc. Natl. Acad. Sci. U. S. A.* **61**:748.
8. Tenhunen, R., H. S. Marver, and R. Schmid. 1969. Microsomal heme oxygenase—characterization of the enzyme. *J. Biol. Chem.* **244**:6388.
9. Tenhunen, R. 1976. The enzymatic conversion of heme to bilirubin *in vivo*. *Ann. Clin. Res.* **8**(Suppl.)17:2.
10. Maines, M. D., and A. Kappas. 1977. Enzymatic oxidation of cobalt protoporphyrin. IX. Observations on the mechanism of heme oxygenase action. *Biochemistry.* **16**:419.
11. Schacter, B. A., B. Yoda, and L. G. Israels. 1976. Human spleen oxygenase in normal, hemolytic and other pathological states. *Ann. Clin. Res.* **8**(Suppl.)17:28.
12. Drummond, G. S., and A. Kappas. 1980. Metal ion interactions in the control of haem oxygenase induction in liver and kidney. *Biochem. J.* **192**:637.
13. Sardana, M. K., S. Sassa, and A. Kappas. 1982. Metal ion-mediated regulation of heme oxygenase induction in cultured avian liver cells. *J. Biol. Chem.* **257**:4806.
14. Yoshinaga, T., S. Sassa, and A. Kappas. 1982. Purification and properties of bovine spleen heme oxygenase: amino acid composition and sites of action of inhibitors of heme oxidation. *J. Biol. Chem.* **257**:7778.
15. Maines, M. D. 1981. Zinc-protoporphyrin is a selective inhibitor of heme oxygenase activity in the neonatal rat. *Biochim. Biophys. Acta* **673**:339.
16. Drummond, G. S., and A. Kappas. 1982. The dual ability of Co-heme (cobalt protoporphyrin IX) to alter heme oxygenase activity; potent induction *in vivo* and profound competitive inhibition *in vitro*. In *Microsomes, Drug Oxidations, and Drug Toxicity*, R. Sato and R. Kato, editors. Japan Scientific Societies Press, Tokyo, Wiley/Interscience, New York. 423.
17. Maines, M. D., and A. Kappas. 1975. Study of the developmental pattern of heme catabolism in liver and the effects of cobalt on cytochrome P-450 and the rate of heme oxidation during the neonatal period. *J. Exp. Med.* **141**:1400.