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CARBON MONOXIDE GENERATION FROM TIN- AND ZINC-PROTOPORPHYRIN BY TISSUE HOMOGENATES

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SUMMARY. Both heme and tin-protoporphyrin (TP), but not zinc-protoporphyrin (ZP), supported significant NADPH-stimulated, concentration-dependent CO production in all tissues. These rates, for 400 μM substrate, ranged: for heme 0.52 (intestine) to 4.18 (spleen); for TP 0.08 (kidney) to 0.71 (liver); and for ZP 0.01 (liver) to 0.25 (kidney) nmoles CO/hr/mg protein. All three metalloporphyrins (400 μM) supported concentration-dependent CO production in the absence of NADPH. The rates ranged: for heme 0.31 (kidney) to 0.80 (spleen); for TP 0.41 (kidney) to 1.04 (intestine); and for ZP 0.12 (kidney) to 0.51 (spleen) nmoles/hr/mg protein. We conclude that both TP and ZP are subject to in vitro degradation by 13,000 x g supernatants of adult rat organs via CO-producing reactions.

INTRODUCTION. Heme oxygenase (EC 1.14.99.3) has been reported to be the rate-limiting step in the heme degradative pathway leading to bilirubin formation (1). Tin-protoporphyrin (TP) and zinc-protoporphyrin (ZP) have been shown to be competitive inhibitors of heme oxygenase (HO) and both substances also lower plasma bilirubin concentrations (2). TP (3,4) and ZP (5) also decrease bilirubin production as measured by the pulmonary CO excretion rate (VeCO).

Anderson et al., studying the distribution time course of TP in the rat were able to account for only ~50% of the administered dose (6). The results could support the hypothesis that TP was metabolized, thus providing an unmeasured sink. We recently reported on a gas chromatographic assay for the determination of heme oxygenase activity (7,8). This method is also suitable for the determination if TP and ZP can serve as substrates for heme oxygenase or are otherwise metabolized by tissue homogenates to CO.

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MATERIALS AND METHODS. Tissue preparation. The liver, spleen, kidneys and intestine were removed from decapitated, fed, adult female Wistar rats (250 to 300 g). The content of the intestine was removed by flushing the lumen with KPO_4 buffer. All tissues were homogenized with a Biohomogenizer (Biospec Products, Inc., Bartlesville, OK 74005) in 4 volumes of 0.1 M KPO_4, pH 7.4. The homogenate was centrifuged for 15 minutes at 13,000 x g and the supernatant was utilized for the reactions.
NADPH, 4.5 mM. Four mg of Na_3NADPH, 3.5 H_2O, (Sigma Chemical Co., St. Louis, MO) was dissolved in 1 ml 0.1 M KPO_4, pH 7.4
Protoporphyrin solutions, 1 mM. Iron- (6.5 mg), tin- (7.5 mg) and zinc-protoporphyrin (6.3 mg) (Porphyrin Products, Logan, UT 84321) were dissolved in 500 µl 1 N NaOH under mixing for 5 min. Two and one half milliliter 0.4 M Na_3PO_4, was added and the mixing was continued for 5 min. After addition of 2.5 ml distilled water, the pH was gradually adjusted to 7.4 with 20-µl aliquots 1 N HCl. The final volume was adjusted to 10.0 ml with distilled water. Dilutions were made with 0.1 M KPO_4 buffer. All procedures were carried out in subdued light. Each solution was used within 4 hrs of preparation.
CO determination. The heme oxygenase activity assay described in a previous report (8) was used to determine rates of CO production. Supernatants of tissue homogenates (10 µl) were incubated with 20 µl of the substrate (0-400 µM, final concentration) in the presence (total) and absence (blank) of NADPH in septum-sealed vials at 37° C for 15 min. The reactions were terminated by transfer of the vials (2 ml) to -78° C (acetone/dry ice). The generated CO was determined by analyzing the entire headspace volume by gas chromatography, using a Reduction Gas Analyzer (Trace Analytical, Inc., Menlo Park, CA 94305). The limit of detection was 1 pmol CO/vial. The analyzer was calibrated with a mixture of CO in nitrogen (Airco Rare and Specialty Gases, Santa Clara, CA 95051). CO production is expressed as nmoles/hr/mg protein. The CO production rates in the presence of heme, TP and ZP have been corrected for the background rate (no substrate) to reflect CO production rates due to the added substrate. Protein concentrations were determined by the method of Lowry, et al. (9) with bovine serum albumin as standard. NADPH-supported CO production is defined as the difference between total and blank CO production.

RESULTS AND DISCUSSION. The rates of CO production supported by the metalloporphyrins added to the reaction mixtures with tissue supernatants are listed in Table 1. The values for total (reaction with NADPH), blank (no NADPH), are listed as well as the difference between these two reactions (NADPH-supported, heme oxygenase-like CO production).

The results show that when no substrate was added to the reaction mixtures, all tissues produce rates of CO (background) which are lower than those when any of the tested substrates were present. This is true for the reactions in the absence of added NADPH (blank) as well as for the

TABLE I: CO production by rat tissue supernatants without the addition of substrate (background) and CO production due to the presence of 400 μM heme, tin-protoporphyrin (TP), or zinc-protoporphyrin (ZP). The reactions were performed with ("Total") and without ("Blank") added NADPH (1.8 mM). The NADPH-supported CO production rate was obtained by subtraction of the Blank rate from the Total rate. The rates listed for heme, TP, and ZP have been corrected for the background rate (No substrate). CO production rates are in **bold** with standard errors (*italic*).

SUBSTRATE	CO PRODUCTION (nmoles/hr/mg protein)											
	LIVER (n = 6)			SPLEEN (n = 6)			KIDNEY (n = 3)			INTESTINE (n = 3)		
	Total	Blank	NADPH	Total	Blank	NADPH	Total	Blank	NADPH	Total	Blank	NADPH
NONE	**0.25** *0.04*	**0.20** *0.03*	**0.05** *0.02*	**0.63** *0.07*	**0.78** *0.30*	**-0.15** *0.27*	**0.58** *0.36*	**0.49** *0.19*	**0.09** *0.23*	**0.50** *0.17*	**0.57** *0.32*	**-0.07** *0.16*
HEME	**2.44** *0.31*	**0.42** *0.08*	**2.01** *0.25*	**4.98** *1.28*	**0.80** *0.40*	**4.18** *1.12*	**1.02** *0.58*	**0.31** *0.26*	**0.72** *0.37*	**1.03** *0.66*	**0.51** *0.34*	**0.52** *0.20*
TP	**1.23** *0.42*	**0.52** *0.25*	**0.71** *0.28*	**1.62** *0.42*	**1.02** *0.37*	**0.59** *0.52*	**0.49** *0.52*	**0.41** *0.43*	**0.08** *0.26*	**1.50** *0.34*	**1.04** *0.56*	**0.45** *0.23*
ZP	**0.11** *0.09*	**0.10** *0.06*	**0.01** *0.04*	**0.54** *0.31*	**0.51** *0.36*	**0.04** *0.41*	**0.13** *0.43*	**-0.12** *0.21*	**0.25** *0.31*	**0.32** *0.29*	**0.30** *0.50*	**0.02** *0.22*

total- and NADPH-supported reactions. However, in contrast to reactions with added substrate, the presence of NADPH did not significantly increase the NADPH-supported rates.

As expected, heme proved to be the most suitable substrate for CO production by all tissues in the presence of NADPH. Spleen tissue had the greatest HO activity and the other tissues had lower, but similar activities as has been shown before with this assay as well as the spectrophotometric method (8,9). The present absolute HO activity values are expected to be somewhat lower than those reported previously (8) on account of the less than optimum heme concentration used. Heme also increased the blank rates as has been shown previously (8).

When TP was incubated, all four tissue preparations generated CO both in the absence and presence of NADPH. This indicates that this protoporphyrin can serve as substrate for heme oxygenase, as well as for other unidentified CO-producing reactions. This is an unexpected finding. Anderson, et al., investigated the in vitro metabolism of TP, by incubating the compound for 6 hr at 37° C with several rat liver fractions (6). No disappearance of TP from the reaction mixtures could be detected. However, it has been shown that the rate of bilirubin formation, in vitro, by spleen HO decreases to zero within one hour (11). Thus, much longer incubation times may not necessarily produce a sufficient decrease in TP to be detected.

ZP also proved to support CO production by all four tissue preparations. In contrast to the results with TP, the CO was produced

primarily via non-NADPH-dependent reactions. Except for the kidney, no tissue produced significant amounts of CO in presence of NADPH. Furthermore, the total rates of CO production were at least 3 times lower than for TP (spleen). More significantly, the rate for liver was about 9% of the rate determined for TP.
When lower concentrations of TP and ZP (100, 200, 300 μM, final concentrations) were incubated, the blank- and NADPH-supported rates increased linearly with the concentration of each compound and all tissues (not shown).

The source of CO in the background reactions (no substrate) is not known, but it is possibly due to the presence of endogenous substrate and NADPH. The nature of the blank rate (no NADPH) has not been established. The presence of NADPH in tissue preparations has been suggested (12), and lipid peroxidation may be another source of CO (13). Other products of the TP and ZP reactions have not yet been identified. Preliminary results with extraction and HPLC analysis of reaction mixtures do not suggest that bilirubin is a product of either the blank or the NADPH-supported reaction.

In conclusion, rat liver, spleen, kidney and intestine preparations all produce CO from the HO substrate, heme, and its inhibitors, TP and ZP. This CO is produced by all substrates via NADPH-independent reactions. In addition, heme and TP yield CO via a NADPH-dependent reaction. Both types of reactions are substrate concentration-dependent in the range tested (0-400 μM). The metabolic rates are also tissue-dependent. Spleen has the greatest activity for all three compounds.

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