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Inhibition of uroporphyrinogen decarboxylase activity

The role of cytochrome *P*-450-mediated uroporphyrinogen oxidation

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It was previously shown that uroporphyrinogen oxidation is catalysed by a form of cytochrome *P*-450 induced by 3-methylcholanthrene [Sinclair, Lambrecht & Sinclair (1987) Biochem. Biophys. Res. Commun. **146**, 1324–1329]. We have now measured uroporphyrinogen oxidation and uroporphyrinogen decarboxylation simultaneously in 10000 *g* supernatants from the livers of methylcholanthrene-treated mice and chick embryos incubated with an NADPH-generating system. We found that uroporphyrinogen oxidation is associated with inhibition of uroporphyrinogen decarboxylase activity. The decreased uroporphyrinogen decarboxylase activity was not due to depletion of substrate, since decarboxylase activity was not increased by a 2.6-fold increase in uroporphyrinogen. Uroporphyrinogen oxidation and the associated inhibition of decarboxylase activity were also observed with liver supernatant from methylcholanthrene-treated chick embryo; both actions required the addition of 3,3′,4,4′-tetrachlorobiphenyl. Uroporphyrinogen oxidation catalysed by microsomes from a methylcholanthrene-treated mouse inhibited the uroporphyrinogen decarboxylase activity in the 100000 *g* supernatant. Ketoconazole, an inhibitor of cytochrome *P*-450, prevented both uroporphyrinogen oxidation and the inhibition of uroporphyrinogen decarboxylation. The addition of ketoconazole to mouse supernatant actively oxidizing uroporphyrinogen inhibited the oxidation and restored decarboxylation. The latter finding suggested that a labile inhibitor was formed during the oxidation. These results suggest uroporphyrinogen oxidation may be important in the mechanism of chemically induced uroporphyria.

INTRODUCTION

Exposure to certain polyhalogenated aromatic hydrocarbons (PHAs) causes uroporphyria in humans and experimental animals (Marks, 1985; Sweeney, 1986). This uroporphyria is characterized by accumulation of uroporphyrin and hepta-carboxyporphyrin in the liver and increased excretion of these compounds in the urine. PHAs that cause this uroporphyria include hexachlorobenzene, planar polychlorinated biphenyls and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Recently, some non-halogenated aromatic hydrocarbons, including 3-methylcholanthrene (MC) and β-naphthoflavone (Francis & Smith, 1987; Urquhart *et al.*, 1988), as well as acetone (Sinclair *et al.*, 1989) have also been shown to cause uroporphyria in animals.

The mechanism by which these compounds cause uroporphyrin accumulation is not known, but it has been suggested that this mechanism involves: (1) decreased activity of uroporphyrinogen decarboxylase, (2) increased oxidation of uroporphyrinogen to uroporphyrin by cytochrome *P*-450 (*P*-450), or (3) a combination of these two processes. Decreased uroporphyrinogen decarboxylase activity has been regarded as the primary cause of chemically induced uroporphyrin accumulation in rodents (Elder, 1978; Sweeney, 1986). Rats made porphyric with hexachlorobenzene have decreased hepatic uroporphyrinogen decarboxylase activity (Elder *et al.*, 1976). Similarly, mice made porphyric with PHAs such as TCDD (Jones & Sweeney, 1980) or non-halogenated compounds such as MC (Francis & Smith, 1987; Urquhart *et al.*, 1988) also have decreased hepatic uroporphyrinogen decarboxylase activity. It has been suggested that this inhibition results from oxidative damage to the thiol groups in the uroporphyrinogen decarboxylase enzyme (Sweeney & Jones, 1981; Ferioli *et al.*, 1984), or from the generation of a specific heat-stable inhibitor (Cantoni *et al.*, 1984; Billi *et al.*, 1986; Smith & Francis, 1987). However, under certain conditions, Japanese-quail and cultured chick-embryo hepatocytes treated with PHAs have been reported to accumulate uroporphyrin without a detectable decrease in uroporphyrinogen decarboxylase activity (Lambrecht *et al.*, 1988). Furthermore, the accumulation of uroporphyrin in cultured chick-embryo hepatocytes is stopped by the addition of piperonyl butoxide or other inhibitors of *P*-450 (Sinclair *et al.*, 1986), strongly suggesting that irreversible inhibition of uroporphyrinogen decarboxylase is not the sole cause of uroporphyrin accumulation in this system.

It has recently been shown that the oxidation of uroporphyrinogen to uroporphyrin can be catalysed by *P*-450 (Sinclair *et al.*, 1987; De Matteis *et al.*, 1988). In MC-treated rats, this oxidation is specifically catalysed by P-450IA$_2$ (Jacobs *et al.*, 1989*a*), a form of *P*-450 that is induced by all porphyrinogenic PHAs. These observations suggest that *P*-450-catalysed oxidation of uroporphyrinogen may be important in the mechanism of chemically induced uroporphyria.

The purpose of the present study was to determine whether *P*-450-catalysed uroporphyrinogen oxidation could effectively compete with uroporphyrinogen decarboxylation. We measured the rates of *P*-450-catalysed uroporphyrinogen oxidation and uroporphyrinogen decarboxylation in 10000 *g* supernatants, since these supernatants contain both microsomes and uroporphyrinogen decarboxylase. We report that uroporphyrinogen oxidation decreased decarboxylation by a mechanism that does not involve substrate depletion.

Abbreviations used: DMSO, dimethyl sulphoxide; MC, 3-methylcholanthrene; *P*-450, cytochrome *P*-450; PHAs, polyhalogenated aromatic compounds; TCB, 3,3′,4,4′-tetrachlorobiphenyl; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

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METHODS

Chemicals

MC, NADPH (Type III), sodium isocitrate, isocitrate dehydrogenase, nicotinamide, dithiothreitol and ketoconazole were purchased from Sigma (St. Louis, MO, U.S.A.). Uroporphyrin III was purchased from Porphyrin Products (Logan, UT, U.S.A.). 3,3′,4,4′-Tetrachlorobiphenyl (TCB) was purchased from Ultra-Science (Hope, RI, U.S.A.).

Animals, treatments and preparation of supernatants

Male C57BL/6 mice (20–24 g body wt) were obtained from Charles River Breeding Laboratories (Wilmington, MA, U.S.A.) and were housed in a temperature- and humidity-controlled animal facility with a 12 h light/12 h dark cycle. Mice were provided with Purina RMH Laboratory Chow and tap water *ad lib*. MC was dissolved in warm corn oil (12 mg/ml) and given as a single intraperitoneal injection (130 mg/kg) 48 h before the mice were killed by cervical dislocation. Controls received corn oil only. White-Leghorn-chick embryos, 15–16 days old, were injected with 0.5 mg of MC in DMSO or with DMSO alone (0.2 ml) 48 h before they were killed. Livers were homogenized in 0.25 M-sucrose/0.05 M-Hepes/1 mM-EDTA, pH 7.4 (20%, w/v), and the homogenates were centrifuged at 10000 ***g*** for 10 min. Microsomes and 100000 ***g*** supernatants were prepared from these 10000 ***g*** supernatants as previously described (Sinclair *et al*., 1979).

Assays

Typical incubation conditions for the measurement of uroporphyrinogen oxidation and decarboxylation were as follows: to a 25 ml Ehrlenmeyer flask were added 2.7 ml of 0.25 M-sucrose/0.05 M-Hepes/1 mM-EDTA, pH 6.8 (sucrose/Hepes/EDTA buffer), 0.3 ml of 10000 ***g*** liver supernatant (final protein concn. 3 mg/ml), 0.025 ml of NADPH-generating system [125 mg of sodium isocitrate, 40 mg of nicotinamide, 35 mg of NADPH, 70 mg of $MgCl_2,7H_2O$, 0.2 ml of isocitrate dehydrogenase (20 units) and 0.75 ml of sucrose/Hepes/EDTA buffer] and 0.06 ml of uroporphyrinogen III solution (final concn. 5 μM). Uroporphyrinogen III was freshly prepared from uroporphyrin III (in 5 mM-NaOH and 32 mM-dithiothreitol) using 4% sodium amalgam (Sinclair *et al*., 1987). Chick-embryo liver samples were assayed with a final concentration of 34 μM-TCB added in DMSO or with DMSO added alone. Ketoconazole [dissolved in 50% (v/v) ethanol, and diluted with an equal volume of 50% (v/v) acetone] was added to a final concentration of 50 μM. Reactions were initiated by the addition of uroporphyrinogen, and flasks were incubated in the dark at 37 °C in a shaking water bath. The rate of uroporphyrinogen oxidation was measured by removing 0.1 ml samples, diluting them with 0.9 ml of buffer (sucrose/Hepes/EDTA, pH 7.4), and measuring fluorescence immediately in a Perkin–Elmer 650-10S fluorescence spectrophotometer (excitation wavelength, 397 nm; emission wavelength, 617 nm; excitation slit, 2 nm; emission slit, 15 nm). Decarboxylation was assayed by simultaneously removing a second 0.1 ml sample from the incubation flask, acidifying it with 0.4 ml of 0.1 M-HCl and photo-oxidizing the porphyrinogens to porphyrins. The amount of coproporphyrin present was subsequently measured by using an h.p.l.c. system with a fluorescence detector as previously described (Lambrecht *et al*., 1988). The rate of coproporphyrinogen formation was linear with respect to incubation time and protein concentration under these conditions. Protein was determined by the method of Lowry *et al*. (1951), with BSA as standard.

RESULTS

Fig. 1 shows the results of a typical experiment in which 10000 ***g*** supernatants from the livers of control or MC-treated mice were incubated with uroporphyrinogen III (5 μM) and an NADPH-generating system. As shown previously (Jacobs *et al*., 1989*a*), the supernatant from the MC-treated mouse was much more active than the control in oxidizing uroporphyrinogen to uroporphyrin (Fig. 1*a*). In contrast, the supernatant from the MC-treated mouse showed almost no uroporphyrinogen decarboxylase activity, whereas the control sample actively converted uroporphyrinogen into coproporphyrinogen (Fig. 1*b*). In the present paper, uroporphyrinogen decarboxylase activity is reported as the formation of coproporphyrinogen, the final decarboxylation product of this enzyme. H.p.l.c. analysis also measured the formation of hepta-, hexa- and penta-carboxyporphyrinogens. The increase in the sum of all the decarboxylation products was also linear with time, and heptacarboxyporphyrinogen was the only other major product apart from coproporphyrinogen. Total recovery of porphyrin after oxidation at the end of the reaction was typically greater than 90% of the porphyrinogen added.

In a subsequent experiment, uroporphyrinogen oxidation and decarboxylation were measured using uroporphyrinogen concentrations of 5 or 13 μM. With either of these substrate concentrations, 10000 ***g*** supernatants from the livers of MC-treated mice had very low decarboxylation rates (less than 0.25 pmol of coproporphyrinogen/min per mg of protein), indicating that this decreased rate of decarboxylation by supernatants from MC-treated mice was not due to lack of substrate. These results suggest that oxidation of uroporphyrinogen inhibited the decarboxylation of this substrate.

To determine whether microsomal *P*-450 is required to cause a decrease in the rate of coproporphyrinogen formation, 10000 ***g*** supernatants from MC-treated mice were prepared and further separated into a 100000 ***g*** supernatant (containing the uroporphyrinogen decarboxylase) and a microsomal preparation (containing the *P*-450). In Fig. 2, uroporphyrinogen oxidation and decarboxylation were measured in these fractions, and also

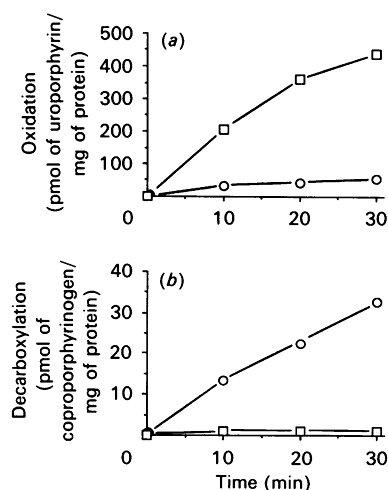

Fig. 1. Effect of MC pretreatment on uroporphyrinogen oxidation and decarboxylation in 10000 *g* supernatants from mouse liver homogenates

Male C57BL/6 mice were treated with corn oil (○) or MC (□), and uroporphyrinogen oxidation (*a*) and decarboxylation (*b*) were measured as described in the Methods section.

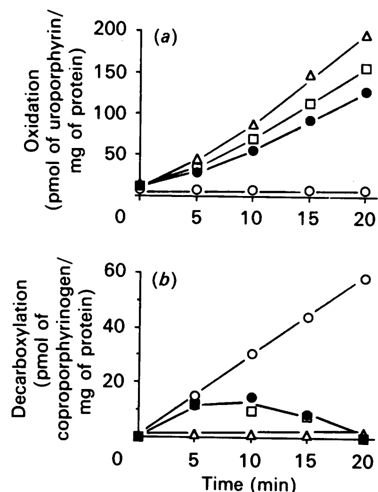

Fig. 2. Effect of fractionation of mouse liver homogenates on uroporphyrinogen oxidation and decarboxylation

A liver homogenate from MC-treated mice was fractionated into a 10000 *g* supernatant (□), a 100000 *g* supernatant (○) and a microsomal pellet (△). Uroporphyrinogen oxidation and decarboxylation were measured in these three fractions, along with the combination of 100000 *g* supernatants and microsomes (●). The amount of protein used in these assays was chosen to approximate the relative proportions of soluble and microsomal proteins present in the original 10000 *g* supernatant.

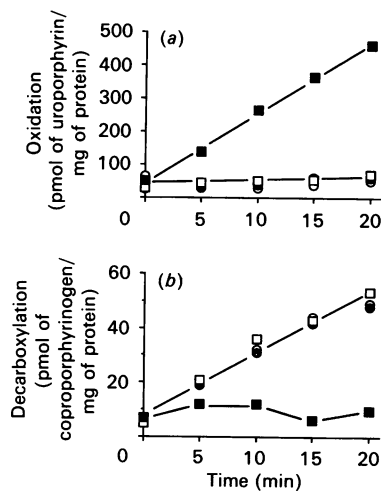

Fig. 3. Effect of MC pretreatment on uroporphyrinogen oxidation and decarboxylation in 10000 g supernatants from chick-embryo livers

Chick embryos were treated with corn oil (○, ●) or MC (□, ■), and uroporphyrinogen oxidation and decarboxylation were measured in the presence (filled symbols) or absence (open symbols) of 34 μM-TCB.

in a combination of 100000 *g* supernatant and microsomes. Oxidation rates were highest in fractions which contained *P*-450 (10000 *g* supernatant, microsomes and the combination of 100000 *g* supernatant plus microscomes) (Fig. 2*a*). In contrast, coproporphyrinogen formation was highest with the 100000 *g* supernatant alone, and was much lower in fractions which had high oxidation activity (Fig. 2*b*). Some coproporphyrinogen was formed in the first 5 min with the 10000 *g* supernatant and with the combination of microsomes plus 100000 *g* supernatant. This

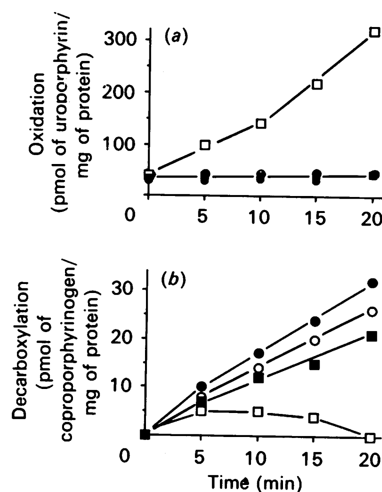

Fig. 4. Effect of ketoconazole on uroporphyrinogen oxidation and decarboxylation

Mice were treated with corn oil (○, ●) or MC (□, ■), and uroporphyrinogen oxidation and decarboxylation were measured in the presence (filled symbols) or absence (open symbols) of 50 μM-ketoconazole.

may indicate that some time is required before decarboxylation becomes inhibited.

We have previously demonstrated that liver preparations from MC-treated chick embryos oxidize uroporphyrinogen only in the presence of TCB (Sinclair *et al.*, 1987). We investigated whether the inhibition of uroporphyrinogen decarboxylase is also dependent on addition of TCB. In the experiment shown in Fig. 3, 10000 *g* supernatants from control and MC-treated chick embryos were incubated in the presence or absence of TCB. The sample with the greatest rate of uroporphyrinogen oxidation (supernatant from MC-treated chick embryo plus TCB) had the lowest rate of coproporphyrinogen formation. In the absence of TCB, supernatants from MC-treated chick embryos had the same rates of oxidation and decarboxylation as supernatants from untreated chick embryo. Thus incubation of 10000 *g* supernatant from MC-treated chick embryos with uroporphyrinogen and NADPH in the absence of oxidation did not lead to inhibition of decarboxylation.

Uroporphyrinogen oxidation was previously shown to be catalysed by *P*-450 and to be prevented by inhibitors of *P*-450, such as piperonyl butoxide (Sinclair *et al.*, 1987) or ketoconazole (Jacobs *et al.*, 1989*b*). As shown in Fig. 4, in 10000 *g* supernatants from MC-treated mice ketoconazole inhibited uroporphyrinogen oxidation when added immediately before uroporphyrinogen. In the presence of ketoconazole the decarboxylation proceeded at rates similar to that in supernatants from untreated mice. Ketoconazole had no effect on uroporphyrinogen oxidation or decarboxylation in supernatants from control mice.

In the experiment illustrated in Fig. 5 we investigated whether ketoconazole would restore decarboxylase activity if added after oxidation had proceeded for 15 min. Addition of ketoconazole completely stopped the oxidation of uroporphyrinogen and restored decarboxylation. This result was confirmed in several experiments, although there was some delay before coproporphyrinogen was detectable, and the length of this delay varied from experiment to experiment. These results show that the inhibition of uroporphyrinogen decarboxylation was reversible and confirm that the inhibition of decarboxylation was not due to lack of substrate, since after ketoconazole was added the decarboxylation resumed.

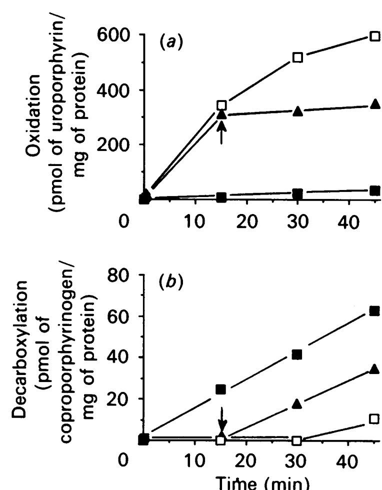

Fig. 5. Effect of addition of ketoconazole before or after uroporphyrinogen on uroporphyrinogen oxidation and decarboxylation

Mice were treated with MC, and uroporphyrinogen oxidation and decarboxylation were measured in the absence of ketoconazole (□), with ketoconazole added to the incubation flask immediately before the uroporphyrinogen (■), and with ketoconazole added 15 min after the uroporphyrinogen (▲), as indicated by the arrows.

DISCUSSION

The major finding of the present study is that *P*-450-mediated uroporphyrinogen oxidation inhibits uroporphyrinogen decarboxylation *in vitro*. This conclusion is based on several observations: (*a*) when 10000 ***g*** supernatants from MC-treated mice catalyse uroporphyrinogen oxidation, uroporphyrinogen decarboxylation was minimal (Figs. 1, 2, 4 and 5); (*b*) microsomal *P*-450 was required for decreased decarboxylation (Fig. 2), and inhibition of *P*-450-catalysed activity prevented the decrease (Figs. 4 and 5); (*c*) in 10000 ***g*** supernatants from chick embryos treated with MC, oxidation of uroporphyrinogen and inhibition of decarboxylase activity were both dependent on the addition of TCB (Fig. 3). The finding that *P*-450-mediated uroporphyrinogen oxidation inhibits uroporphyrinogen decarboxylation was observed in at least 15 separate experiments. Each of the experiments illustrated in the Figures was conducted at least twice, and the pattern of uroporphyrinogen oxidation and inhibition of uroporphyrinogen decarboxylation was consistent between experiments.

The most obvious explanation for the effect of uroporphyrinogen oxidation on decarboxylase activity was that the oxidation reaction limited the amount of substrate available. However, increasing the concentration of uroporphyrinogen from 5 μM to 13 μM did not increase decarboxylase activity in 10000 ***g*** supernatants from MC-treated mice. Both of these concentrations are well above the K_m values of uroporphyrinogen decarboxylase reported for uroporphyrinogen III, which range from 0.5 to 1.8 μM in rat liver (Smith & Francis, 1979; Mukerji & Pimstone, 1986). The total amount of added substrate converted into uroporphyrin or into decarboxylation products was typically less than 15%, implying that most of the added uroporphyrinogen was still present. Furthermore, when ketoconazole was added to 10000 ***g*** supernatant from MC-treated mice that was actively catalysing uroporphyrinogen oxidation, decarboxylase activity resumed (Fig. 5), indicating that sufficient uroporphyrinogen was still present.

The mechanism by which uroporphyrinogen oxidation inhibits uroporphyrinogen decarboxylation is not known. *P*-450 plays an important role in this process, as indicated by the requirement for MC treatment of the animals to induce the appropriate *P*-450, presumably P-450IA2 (Jacobs *et al*., 1989*a*), and the ability of ketoconazole, an inhibitor of *P*-450, to stop this process. We suggest that a uroporphyrinogen oxidation product produced *in vitro* is an inhibitor of the decarboxylase and that this inhibitor is labile, since inhibition of the oxidation by ketoconazole restored the decarboxylation. This product is not uroporphyrin itself, since the addition of ketoconazole does not change the uroporphyrin concentration and yet the uroporphyrinogen decarboxylase activity is restored (Fig. 5). We suggest that this inhibitory intermediate may be derived from unstable partial oxidation intermediates (di- or tetra-hydroporphyrins) described by Mauzerall (1962). In some of our experiments, there is some coproporphyrinogen formation at early time points, indicating that it takes some time to form this putative inhibitor. Francis & Smith (1988) presented evidence for an inhibitor of decarboxylation produced *in vitro*. These workers used an enzymic system (hypoxanthine/xanthine oxidase/Fe-EDTA) to oxidize uroporphyrinogen, presumably by forming hydroxyl radicals, and generated a decarboxylase inhibitor. We do not know the relationship between our results and those for the inhibition of the decarboxylase *in vitro* by oxygen radicals produced in iron-catalysed reactions. Elsewhere we showed that the microsomal reaction in the absence of added iron does not produce reactive oxygen species that oxidize uroporphyrinogen (Jacobs *et al*., 1989*b*).

The results presented here may explain some of the conflicting reports from different laboratories concerning whether treatment of cultured chick-embryo hepatocytes with PHAs results in inhibition of uroporphyrinogen decarboxylase (Lambrecht *et al*., 1988). The results presented here suggest that, in the intact hepatocyte, there is inhibition of uroporphyrinogen decarboxylase caused by *P*-450-mediated uroporphyrinogen oxidation. This inhibition would be readily reversed by cell lysis, as previously suggested (Sinclair *et al*., 1983). Thus, if 10000 ***g*** supernatants are used to measure uroporphyrinogen decarboxylase activity, an apparent decrease in this activity may be observed owing to *P*-450-mediated uroporphyrinogen oxidation in the presence of residual NADPH and TCB. This inhibition would be enhanced by using concentrated supernatants, with a corresponding greater concentration of NADPH and TCB. Inhibition of uroporphyrinogen decarboxylase activity would not occur with control supernatants, which do not have the appropriate form of *P*-450 to support uroporphyrinogen oxidation. This problem of residual uroporphyrinogen oxidation can be avoided by using 100000 ***g*** supernatants or by adding ketoconazole to the reaction mixture.

Our results *in vitro* may have implications for the mechanism of chemically induced uroporphyria *in vivo*. Accordingly, the primary role of the porphyrinogenic agent would be to induce P-450IA$_2$ (Jacobs *et al*., 1989*a*). The induced *P*-450 would catalyse the oxidation of uroporphyrinogen and cause inhibition of uroporphyrinogen decarboxylase activity, analogously to the inhibition seen in Figs. 1–5. This proposed mechanism would be consistent with the suggestion by several authors (Urquhart *et al*., 1988; De Matteis *et al*., 1988; Smith & Francis, 1987) that the inhibition of uroporphyrinogen decarboxylase may be caused by a compound derived from uroporphyrinogen. This oxidation of uroporphyrinogen could also lead to the formation of the heat-stable inhibitor of uroporphyrinogen decarboxylase previously reported by Cantoni *et al*. (1984), Billi *et al*. (1986) and Smith & Francis (1979).

In summary, under conditions obtained *in vitro*, *P*-450-mediated oxidation of uroporphyrinogen can decrease uroporphyrinogen decarboxylase activity.

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