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Uroporphyria produced in mice by 20-methylcholanthrene and 5-aminolaevulinic acid

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Iron-loaded male C57BL/6 mice allowed free access to an aqueous solution of 5-aminolaevulinic acid (ALA) (2 mg/ml) as their only drink, develop severe uroporphyria within 9 days of a single intraperitoneal dose of 20-methylcholanthrene (MC) (125 mg/kg). At 21 days, uroporphyrinogen decarboxylase (EC 4.1.1.37) activities are less than 10% of control activities. The porphyria is not dependent on pretreatment with iron and persists for at least 21 days after withdrawal of ALA. The same intraperitoneal dose of MC does not produce porphyria within 21 days when given without ALA. Continuous administration of ALA markedly accelerates the onset of porphyria in iron-loaded male C57BL/6 mice after a single intraperitoneal dose of hexachlorobenzene (200 mg/kg); mice given phenobarbitone and ALA do not become porphyric. MC with ALA does not produce porphyria in iron-loaded male DBA/2 mice. At least two separate events are needed to produce uroporphyria in mammals: induction of a specific form of cytochrome *P*-450 and stimulation of the formation of intermediates of haem biosynthesis in the liver. These results show that severe, persistent porphyria can be produced in mammals by compounds other than polyhalogenated aromatic hydrocarbons and suggest that a similar mechanism underlies the porphyrogenic action of halogenated and non-halogenated compounds.

INTRODUCTION

Certain polyhalogenated aromatic hydrocarbons, notably 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, hexachlorobenzene and some polyhalogenated biphenyls (Elder, 1978; Sassa *et al.*, 1986), produce chronic uroporphyria in mammals. The porphyria is characterized by a sustained decrease in the activity of uroporphyrinogen decarboxylase (EC 4.1.1.37) in the liver, and by increased excretion and hepatic accumulation of uroporphyrin and other acetic acid-substituted porphyrins derived from the intermediates of the reaction catalysed by this enzyme (Elder, 1978). The mechanism by which these compounds decrease uroporphyrinogen decarboxylase activity is unknown.

The susceptibility of mammals to the porphyrogenic action of polyhalogenated hydrocarbons varies, being directly related both to the level of hepatic iron stores (Taljaard *et al.*, 1972; Smith *et al.*, 1979; Sweeney *et al.*, 1979; Smith & Francis, 1983) and to the inducibility of certain forms of cytochrome *P*-450. All porphyrogenic polyhalogenated aromatic hydrocarbons induce in the liver those forms of cytochrome that are classified (Nebert *et al.*, 1987) in the cytochrome *P*-450 I family (Sinclair *et al.*, 1984; Linko *et al.*, 1986) and whose induction is mediated by the *Ah* receptor (Poland & Knutson, 1982). In inbred strains of mice, susceptibility to uroporphyria partially correlates with expression of the *Ah*-responsive phenotype (Greig *et al.*, 1984).

Immunochemical measurements indicate that the decrease in uroporphyrinogen decarboxylase activity in uroporphyria is the result of a process which inactivates the enzyme without decreasing the concentration of immunoreactive enzyme protein (Elder & Sheppard, 1982). Two different explanations for the relationship between cytochrome *P*-450 induction and the development of uroporphyria have been proposed that are compatible with this type of enzyme defect. First, polyhalogenated hydrocarbons may be converted by a cytochrome *P*-450-dependent reaction to metabolites which bind to the active site of the enzyme (Debets *et al.*, 1981; de Verneuil *et al.*, 1983; Wainstock de Calmanovici *et al.*, 1984). Secondly, polyhalogenated hydrocarbons may interact with cytochrome *P*-450 to stimulate the production of reactive oxygen species which inhibit uroporphyrinogen metabolism by substrate oxidation, inactivation of the enzyme or some other oxidative process that is iron dependent (Ferioli *et al.*, 1984; Elder *et al.*, 1986; Sinclair *et al.*, 1986, 1987; Smith *et al.*, 1986). However, compounds such as 20-methylcholanthrene (MC), which induce the same forms of cytochrome *P*-450 as the porphyrogenic compounds, and similarly stimulate the production of reactive oxygen species (A. Urquhart & G. Elder, unpublished work), are not known to cause uroporphyria; nor has the mechanism whereby oxidative damage leads to specific inactivation of uroporphyrinogen decarboxylase been elucidated.

In this paper we show that MC causes severe, persistent uroporphyria in mice, provided the haem precursor, 5-aminolaevulinic acid (ALA) is given concurrently.

EXPERIMENTAL

Materials

Synthetic pentacarboxylic porphyrin III with the acetic acid substituent at position 5 was obtained from Professor A. H. Jackson, Department of Chemistry,

Abbreviations used: ALA, 5-aminolaevulinic acid; MC, 20-methylcholanthrene.

University College, Cardiff, U.K. Coproporphyrin I (dry standardized vials) was from Sigma. All other porphyrins were from Porphyrin Products, Logan, UT, U.S.A. 7-Ethoxyresorufin (ethoxyphenoxazone) was from Pierce, Rockford, IL, U.S.A. ALA dihydrochloride and MC were from Sigma. Hexachlorobenzene (organic analytical standard) was from BDH Ltd. Imferon (iron dextran injection B.P.) was from Fisons Ltd. Bovine serum albumin (as a standard for protein assay) was obtained from ICN Biomedicals Ltd., High Wycombe, Bucks., U.K.

Animals

Male C57BL/6 and DBA/2 mice (22–28 g body wt.) (Harlan Olac Ltd., Bicester, Oxon, U.K.) were allowed free access to a standard diet and to either tap water or to a solution of ALA in tap water (2 mg/ml) which was freshly prepared every 4 days. Iron was given as Imferon (0.25 ml, containing 12.5 mg of iron) by intraperitoneal injection 3 days before the start of each experiment. MC (125 mg/kg body wt., in corn oil), hexachlorobenzene (200 mg/kg body wt., in corn oil), corn oil (0.5 ml) or sodium phenobarbital (80 mg/kg body wt., in 0.9% (w/v) saline) were all given as a single intraperitoneal injection.

Mice were killed by cervical dislocation. Livers were removed, rinsed in saline, and homogenized in 0.25 M-sucrose containing 20 mM-Tris/HCl buffer (pH 7.4) or 50 mM-Hepes/HCl, 1 mM-EDTA (pH 7.6). Supernatant fractions were prepared from homogenates (20%, w/v) by centrifugation for 5 min at 15000 *g*.

Methods

Uroporphyrinogen decarboxylase was measured in either homogenates or supernatant fractions using pentacarboxylate porphyrinogen as substrate as described by Elder & Wyvill (1982). Ethoxyresorufin deethylase was assayed in 2% (w/v) liver homogenates in the presence of 0.01 mM-dicoumarol (Lubet *et al.*, 1985). Cytochrome *P*-450 concentrations were measured in 1% (w/v) homogenates (Schoene *et al.*, 1972). Experiments with added uroporphyrin showed that, at the maximum concentrations of endogenous uroporphyrin obtained, uroporphyrin accounted for less than 13% of the measured cytochrome *P*-450 concentration.

Total porphyrin in liver homogenates was measured fluorimetrically after extraction into methanol/1 M-$HClO_4$ (1:1, v/v) using coproporphyrin I as standard. Individual porphyrins were measured by h.p.l.c. (Bonkovsky *et al.*, 1986) or by the method of Grandchamp *et al.* (1980). Faecal porphyrins were analysed by t.l.c. (Smith, 1978). Protein was measured using bovine serum albumin as standard (Lowry *et al.*, 1951).

The significance of differences was assessed by the Mann–Whitney test.

RESULTS

Fig. 1 shows that the uroporphyrinogen decarboxylase activity of iron-loaded C57BL/6 mice given MC and ALA fell progressively to 6% of the control activity at 21 days. Activities remained at this level for a further 21 days after stopping ALA administration. Enzyme activity decreased to a similar extent when administration of ALA was delayed for 7 days after injection of MC (Fig. 1). The small decrease in uroporphyrinogen decarboxylase activity ($P < 0.05$, compared with iron-loaded mice) in animals given only MC (Fig. 1) was not accompanied by an increase in hepatic porphyrin concentration (mean 0.26 nmol/g, range 0.23–0.29 nmol/g versus 0.25 nmol/g, 0.20–0.30 nmol/g for iron-loaded mice). Iron-loaded C57BL/6 mice given phenobarbitone and ALA did not become porphyric (Table 1).

The porphyrins that accumulated in the livers of

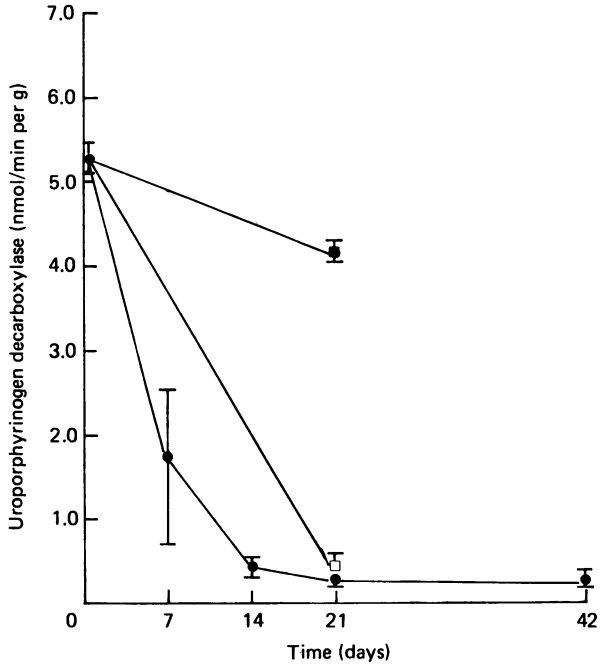

Fig. 1. Effect of MC and ALA on uroporphyrinogen decarboxylase activity

Male C57BL/6 mice, pretreated with iron, received an intraperitoneal injection of MC (125 mg/kg) at the start of the experiment. They were then allowed free access to water for 21 days (■), to ALA solution (2 mg/ml) for 21 days (●) followed by water for 21 days or to water for 7 days followed by ALA for 14 days (□). Results are means and ranges for three or four animals.

Table 1. Effect of phenobarbitone and ALA on hepatic uroporphyrinogen metabolism

Male C57BL/6 mice, pretreated with iron, received intraperitoneal injections of phenobarbitone (80 mg/kg, on days 1, 2, 3, 8, 9 and 10) before being killed 21 days after the first injection. ALA was given for 21 days as described in the Experimental section. Results are means and ranges. Significantly different from iron-treated group: $*P < 0.03$.

Treatment	No. of mice	Uroporphyrinogen decarboxylase (nmol/min per g wet wt.)	Total porphyrin (nmol/g wet wt.)
Iron	3	5.30 (5.10–5.47)	0.25 (0.20–0.30)
Iron/ALA	3	5.04 (3.96–5.62)	2.95 (0.40–6.85)*
Iron/phenobarbitone/ALA	4	4.69 (4.08–5.17)	1.49 (0.25–4.75)*

Table 2. Effect of MC and ALA on hepatic porphyrin concentration

Groups of male C57BL/6 mice received iron, MC and ALA in the combinations indicated. Mice, which had or had not been pretreated with iron, received a single intraperitoneal injection of MC (125 mg/kg) in corn oil at the start of the experiment and were allowed free access to drinking water containing ALA (2 mg/ml) throughout the experiment. Total porphyrin and uroporphyrin concentrations (means and ranges) were measured fluorimetrically as described in the Experimental section. H.p.l.c. analyses are for one or two (mean and range) animals from each group. *Differences between these groups are not statistically significant.

Treatment	Length of treatment (days)	No. of mice	Total porphyrin (nmol/g)	Uroporphyrin (nmol/g)	Porphyrins (% total) by h.p.l.c.					
					Uro	Hepta	Hexa	Penta	Copro	Proto
Iron	15	4	0.35 (0.31–0.40)	0.12 (0.09–0.17)	41.7	13.9	3.5	3.0	4.2	34.7
Iron/MC	15	4	0.39 (0.32–0.48)	0.13 (0.09–0.18)	56.6	18.9	3.0	3.0	3.0	18.9
Iron/ALA	15	4	0.87 (0.76–1.0)	0.24 (0.11–0.39)	27.5	13.0	1.0	1.0	1.6	56.5
Iron/MC/ALA	9	5	71 (22–179)*	60 (19–143)*	83.7±4.7	15.6±4.2	< 0.2	< 0.2	< 0.2	< 0.2
Iron/MC/ALA	15	7	143 (16–331)*	130.6 (14–280)*	86.3±0.5	12.5±0.8	0.2	0.2	0.2	1.0±0.2
MC/ALA	15	4	72 (6–177)*	67 (5–160)*	90.3±1.3	8.0±0.1	< 0.2	< 0.2	< 0.2	1.5±1.1

animals given MC and ALA were almost entirely uroporphyrin and heptacarboxylic porphyrin (Table 2), as in hexachlorobenzene-induced porphyria in mice (Smith & Francis, 1983). Pretreatment with iron had little effect on the degree of porphyrin accumulation (Table 2). Analysis of faecal porphyrins revealed large amounts of isocoproporphyrin and other acetic acid-substituted porphyrins distributed in a pattern similar to that found in the uroporphyria produced by hexachlorobenzene and other polyhalogenated aromatic compounds (Elder, 1978).

Table 3 shows that the activity of ethoxyresorufin deethylase, which is a measure of induction of the MC-inducible forms of cytochrome *P*-450 (Goldstein *et al.*, 1982), and hepatic cytochrome *P*-450 concentrations were markedly increased by injection of MC and that a small increase in enzyme activity persisted for at least 15 days. At this time, cytochrome *P*-450 concentrations were not increased by continuous administration of ALA. These observations are consistent with previous reports that the peak induction of enzyme activities associated with these forms of cytochrome *P*-450 occurs about 4 days after a large intraperitoneal dose of MC and is followed by a gradual decline to reach control activities by 30 days (Kleeberg *et al.*, 1975), and that ALA does not increase hepatic cytochrome *P*-450 concentrations (Druyan & Kelly, 1972). The relationship between the induction of MC-inducible forms of cytochrome *P*-450 and the development of uroporphyria was investigated by comparing the response of different strains of mice. Table 4 shows that iron-loaded male mice of the *Ah*-non-responsive strain, DBA/2, did not develop porphyria when given MC and ALA according to a protocol that was porphyrogenic for the *Ah*-responsive C57BL/6 strain (Tables 1 and 2; Fig. 1).

To investigate the relationship between the mechanisms by which hexachlorobenzene and MC produce uroporphyria, we examined the effect of ALA on the development of porphyria after a single intraperitoneal injection of hexachlorobenzene. Fig. 2 shows that continuous administration of ALA accelerated the onset of porphyria. After 16 days, uroporphyrinogen decarboxylase activity had fallen to a level that was not reached until 35 days in animals that had not received ALA.

DISCUSSION

Our experiments show that severe, chronic uroporphyria can be produced in mice by compounds other than polyhalogenated aromatic hydrocarbons, the only class of chemicals previously reported to have this effect in mammals. The uroporphyria described here closely resembles that produced by polyhalogenated aromatic hydrocarbons. The gradual onset of the fall in uroporphyrinogen decarboxylase activity, its persistence and the pattern of porphyrin accumulation and excretion are all similar to those observed in polyhalogenated aromatic hydrocarbon porphyria in mice (Goldstein *et al.*, 1973; Smith & Francis, 1983, 1986).

Other similarities suggest that the mechanism responsible for the decrease in hepatic uroporphyrinogen decarboxylase activity may also the same. Neither *Ah*-responsive mice given phenobarbitone with ALA (Table 1), nor *Ah*-non-responsive mice given MC and ALA (Table 4) became porphyric, suggesting that induction of

Table 3. Effect of MC and ALA on hepatic cytochrome *P*-450 concentrations and ethoxyresorufin de-ethylase activities

Experimental conditions were as described in the legend to Table 2. Values are means and ranges for three to seven animals. Significance of differences from iron-treated group: $*P < 0.01$.

Treatment	No. of mice	Length of treatment (days)	Liver weight (g)	Cytochrome *P*-450 (pmol/mg of protein)	Ethoxyresorufin de-ethylase (pmol/min per mg of protein)
Iron	4	15	1.58 (1.18–1.96)	197 (163–241)	14 (14–17)
Iron/ALA	4	15	1.82 (1.62–2.09)	170 (163–180)	10 (9–12)
Iron/MC	4	15	1.78 (1.69–1.91)	181 (141–213)	33 (27–47)*
Iron/MC/ALA	3	2	1.67 (1.53–1.83)	485 (454–530)*	276 (249–293)*
Iron/MC/ALA	5	9	–	380 (286–449)*	222 (80–326)*
Iron/MC/ALA	7	15	1.74 (1.30–2.04)	179 (135–232)	32 (23–45)*
MC/ALA	4	15	1.43 (1.31–1.51)	230 (202–272)	36 (24–44)*

cytochromes of the *P*-450 I family is an essential requirement for the development of this type of porphyria, as it is for polyhalogenated aromatic hydrocarbon uroporphyria in mammals (Poland & Knutson, 1982; Linko *et al.*, 1986; Smith *et al.*, 1986) and in chick embryo hepatocyte cultures (Sinclair *et al.*, 1984). Furthermore, the onset of hexachlorobenzene-induced porphyria was accelerated by ALA (Fig. 2), which suggests that both MC and hexachlorobenzene share an action that, when supplemented by ALA, leads to inactivation of uroporphyrinogen decarboxylase in the liver.

In mice, MC induces two forms of cytochrome *P*-450, P_1-450 and P_3-450 (Negishi & Nebert, 1982), which respectively correspond to *P*-450c and *P*-450d of rat liver (Kimura *et al.*, 1984). Whether one or both of these is involved in the development of porphyria is not known; our experimental model, by allowing the porphyrogenic action of a wide range of cytochrome *P*-450 inducers to be compared, may help to solve this problem.

What is the role of MC-inducible forms of cytochrome *P*-450 in the production of porphyria? Our findings add to existing evidence (Sinclair *et al.*, 1986; Smith *et al.*, 1986; Stewart & Smith, 1987) against the hypothesis that polyhalogenated compounds are converted by cytochrome *P*-450-dependent reactions to metabolites that directly inhibit uroporphyrinogen decarboxylase (Debets *et al.*, 1981; de Verneuil *et al.*, 1983; Wainstok de Calmanovici *et al.*, 1984). The structure and metabolism of MC and polyhalogenated porphyrogens are different and MC did not cause porphyria in the absence of ALA (Fig. 1; Table 1), even though the extent of induction of cytochrome *P*-450-dependent enzymes was unaffected by the haem precursor (Table 3). Another explanation for the role of cytochrome *P*-450, that has recently been favoured because it may also explain the

Table 4. Effect of MC and ALA on hepatic uroporphyrinogen decarboxylase activities and porphyrin concentrations of DBA/2 mice

Male DBA/2 mice, pretreated with iron, were given an intraperitoneal injection of MC (125 mg/kg) and received a solution of ALA (2 mg/ml) as their only drink until they were killed 21 days after the injection. Mice that received neither compound were killed at the same time. Results are means and ranges for four animals. Differences between the groups were not statistically significant.

Treatment	Uroporphyrinogen decarboxylase (nmol/min per g)	Total porphyrin (nmol/g)
Iron	6.42 (6.09–6.88)	0.27 (0.16–0.35)
Iron/MC/ALA	5.72 (4.63–6.25)	0.96 (0.29–2.78)

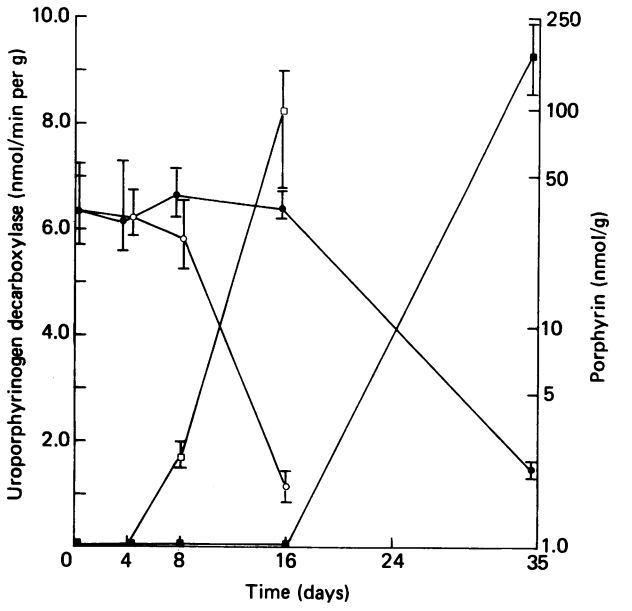

Fig. 2. Effect of hexachlorobenzene and ALA on uroporphyrinogen decarboxylase activity and porphyrin concentration

Male C57BL/6 mice, pretreated with iron, were given a single intraperitoneal injection of hexachlorobenzene (200 mg/kg) at the start of the experiment and then allowed free access to either water (●,■) or ALA solution (2 mg/ml) (○,□) as their only drink. Uroporphyrinogen decarboxylase activity (●,○); porphyrin concentration (■,□). Measurements are means and ranges for three to five animals. Hepatic porphyrin concentrations in the mice that did not receive ALA were less than 1.0 nmol/g until after 16 days.

involvement of iron in this type of porphyria (Ferioli *et al.*, 1984), invokes the generation of reactive oxygen species by cytochrome *P*-450s that become partially uncoupled by bound porphyrogens as the event that initiates the development of uroporphyria (Sinclair *et al.*, 1986; Smith *et al.*, 1986; Sinclair *et al.*, 1987). Both hexachlorobenzene and MC at the doses used in our experiments stimulate the production of reactive oxygen species by microsomes to a similar extent (A. Urquhart & G. Elder, unpublished work).

However, our experiments show that induction of the appropriate form of cytochrome *P*-450 in the presence of adequate iron stores is not sufficient by itself to cause porphyria, even when the animals are pretreated with iron. For porphyria to develop, a second action is required that, in our experiments, was provided by continuous administration of the haem precursor, ALA. This compound enters the liver where it is metabolized to haem and haem precursors, but does not increase the concentration of cytochrome *P*-450 and other haemoproteins (Druyan & Kelly, 1972). Enhanced production of haem precursors and haem in the liver, therefore, seems to be a second essential requirement for the development of porphyria. For porphyrogenic polyhalogenated aromatic compounds, this action may be provided through their ability to induce hepatic ALA synthase (EC 2.3.1.37) activity. Thus, for the most potent porphyrogen, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, there is a direct relationship between the dose required to produce porphyria and to induce ALA synthase in mice (Goldstein *et al.*, 1973). With less potent compounds, such as hexachlorobenzene, there is some evidence that the rate of hepatic haem synthesis is increased to a small extent (Rajamanickam *et al.*, 1972) before increased ALA-synthase activity becomes readily detectable at the time that porphyria develops (Stonard, 1974; Wainstok de Calmanovici *et al.*, 1984). Combined treatment of rats with phenobarbitone and hexachlorobenzene both increases ALA-synthase activity and accelerates the onset of porphyria (Wainstok de Calmanovici *et al.*, 1984). It thus seems likely that the main factor determining the porphyrogenicity of a compound that induces cytochromes of the *P*-450 I family is its effectiveness as a long-term stimulator of ALA formation in the liver.

To explain the dual need for an increased supply of ALA and for induction of a specific form of cytochrome *P*-450 in the liver, we propose the following mechanism for this type of porphyria. Activation of oxygen by the induced cytochrome *P*-450 system produces a reactive species that reacts with a haem precursor, or possibly haem itself, to produce an active-site-directed irreversible inhibitor of uroporphyrinogen decarboxylase. Continuous administration of ALA and potent inducers of ALA synthase each substantially increase the concentration of the haem precursor from which the inhibitor is derived and, therefore, produce porphyria more rapidly than compounds that enhance ALA formation to only a small extent.

The specificity of the inhibitor for uroporphyrinogen decarboxylase can be explained by postulating that it is derived from a substrate for this enzyme and, thus, like other active-site-directed inhibitors, is a substrate analogue. Heat-stable, irreversible inhibitors of uroporphyrinogen decarboxylase have been partially purified from the livers of animals made porphyric with hexachlorobenzene (Rios de Molina *et al.*, 1980) or tetrachlorodibenzodioxin (Cantoni *et al.*, 1984), but their structures have not been determined. The inhibitor may be uroporphyrin which is known to inhibit uroporphyrinogen decarboxylase (Smith & Francis, 1979) and may be produced by a cytochrome *P*-450-dependent oxidation *in vivo* (Ferioli *et al.*, 1984; Sinclair *et al.*, 1986). Cytochrome *P*-450 from chick embryo hepatocytes treated with MC catalyses an NADPH-dependent oxidation of uroporphyrinogen, provided a porphyrogenic tetrachlorobiphenyl isomer is present (Sinclair *et al.*, 1987). The same reaction is catalysed by hepatic microsomes from MC-treated rats and mice, but does not require the presence of a halogenated biphenyl (P. Sinclair, J. Jacobs, R. Lambrecht & J. Sinclair, unpublished work). However, uroporphyrin is not a sufficiently effective inhibitor to explain the low enzyme activities found in uroporphyria in rodents, nor does removal of porphyrin re-activate uroporphyrinogen decarboxylase (Rios de Molina *et al.*, 1980) or decrease the inhibition produced *in vitro* by inhibitory fractions isolated from porphyric liver (Cantoni *et al.*, 1984). It therefore seems more likely that the inibitor is not uroporphyrin or a related porphyrin, but an unidentified compound that is also produced by oxidation of a porphyrinogen substrate *in vivo*.

The mechanism proposed above suggests that there are two main sites at which iron may act. First, iron overload may increase the activity of hepatic ALA synthase directly (Bonkovsky *et al.*, 1980; Cantoni *et al.*, 1986) or by potentiating its induction by porphyrogenic chemicals (Stein *et al.*, 1970; Wainstok de Calmanovici *et al.*, 1986). Secondly, iron may be required for formation of the inhibitor either as a catalyst of the oxidative reaction that produces it or as a component of its structure. The active site of uroporphyrinogen decarboxylase is sensitive to oxidative inactivation and a planar iron-containing porphyrin analogue bound to the active site might act as a catalyst for the local production of damaging hydroxyl radicals (Elder *et al.*, 1986). The experimental model described here should allow the contribution of each of these sites to be assessed, since, if iron acts exclusively at the level of ALA synthase, the porphyria produced by ALA and MC should be uninfluenced by iron status.

Finally, in the related human uroporphyria, porphyria cutanea tarda, overt porphyria is also associated with inactivation of the active site of uroporphyrinogen decarboxylase by a process that is prevented by depleting hepatic iron stores (Elder *et al.*, 1985). Our results suggest that this process may be initiated by a combination of acquired factors that separately stimulate ALA formation and induce the appropriate form of cytochrome *P*-450 in the liver or otherwise promote the generation of reactive oxygen species.

Note added in proof (3 May 1988)

Francis & Smith (1987) reported that chronic exposure of iron-loaded mice to 20-methylcholanthrene or other cytochrome P_1-450 inducers causes inhibition of hepatic uroporphyrinogen decarboxylase.

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