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Mechanistic studies of the inhibition of hepatic uroporphyrinogen decarboxylase in C57BL/10 mice by iron–hexachlorobenzene synergism

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Porphyria was induced in C57BL/10 mice with iron overload by a single oral dose (100 mg/kg) of hexachlorobenzene (HCB). Within 2 weeks hepatic uroporphyrinogen decarboxylase (EC 4.1.1.37) was inhibited, reaching a maximum (> 95%) at 6–8 weeks. There was no recovery by 14 weeks, despite a fall in liver HCB concentrations to only 6% of the day-3 value. The major rise in hepatic porphyrin levels occurred after 4 weeks and secondary inhibition of uroporphyrinogen synthase (EC 4.2.1.75) was inferred from the progressively greater proportion of uroporphyrin I present relative to the III isomer. Plasma alanine aminotransferase (EC 2.6.1.2) activity was also elevated. Although, in further studies, total microsomal cytochrome *P*-450 content and ethoxyphenoxazone de-ethylase activity reached a peak a few days after dosing and had declined significantly at the time of maximum inhibition of the decarboxylase, additional treatment of HCB-dosed mice with a cytochrome P_1-450 inducer, β-naphthoflavone, enhanced the inhibition, whereas piperonyl butoxide, an inhibitor of cytochrome *P*-450, partially protected. Uroporphyrinogen decarboxylase was not radiolabelled *in vivo* by [^{14}C]HCB. There was no major difference in the ability to hydroxylate HCB between hepatic microsomes from induced C57BL/10 mice and those from the insensitive DBA/2 strain. By contrast, lipid peroxidation, in the presence of NADPH, was 8-fold greater in control C57BL/10 microsomes than in DBA/2 microsomes and was stimulated by iron treatment (although not by HCB). The results suggest that the inhibition of hepatic uroporphyrinogen decarboxylase is unlikely to be due to a direct effect of a metabolite of HCB but to another process requiring a specific cytochrome *P*-450 isoenzyme and an unknown iron species.

INTRODUCTION

Porphyria cutanea tarda (PCT) is a human disease characterized by an abnormality of haem biosynthesis in which uroporphyrinogen decarboxylase (EC 4.1.1.37) activity is depressed (Kappas *et al.*, 1983). There are at least two classes of the disease. In the first there is a family history of the condition, familial PCT, and enzyme activity is low in all tissues that have been examined (Kappas *et al.*, 1983). The types of PCT in this class arise by expressions of mutations of the decarboxylase gene (Elder *et al.*, 1985; Sassa *et al.*, 1983; de Verneuil *et al.*, 1984). In the second class, referred to as sporadic or acquired PCT, the depression of decarboxylase activity is restricted to the liver (Elder *et al.*, 1985; Kappas *et al.*, 1983). It is the most common of all forms of porphyria and occurs mainly in patients with high or moderately-high intakes of alcohol and who have no family history of PCT (Kappas *et al.*, 1983). The mechanism of the enzyme defect in sporadic PCT is unknown but remission can be achieved by lowering hepatic iron stores (Kappas *et al.*, 1983; Elder *et al.*, 1985). A similar disease has occurred in people poisoned by hexachlorobenzene (HCB) and related chemicals (Elder, 1978; Smith & De Matteis, 1980; Kappas *et al.*, 1983). Even with sporadic and HCB-induced PCT there may be a genetic predisposition to the disease as alcohol-induced liver damage or exposure to HCB does not necessarily cause the development of porphyria in the majority of individuals (Dogramaci, 1964; Smith & De Matteis, 1980; Kappas *et al.*, 1983).

Rats, like humans, become porphyric by depression of uroporphyrinogen decarboxylase activity when they are fed HCB continuously (Ockner & Schmid, 1961; De Matteis *et al.*, 1961; Elder, 1978) and there is evidence for potentiation by iron (Taljaard *et al.*, 1972; Louw *et al.*, 1977; Smith *et al.*, 1979). This has been studied by many workers as a model for human PCT. Mice exposed to HCB appeared to be too susceptible to neurotoxic effects to be a model for PCT (De Matteis *et al.*, 1961) but they can become highly sensitized to the porphyrogenic actions of HCB after iron overload (Smith & Francis, 1983) perhaps via a free radical-induced or related process (De Matteis & Stonard, 1977; Jones *et al.*, 1981). What is more, this susceptibility is genetically determined and seems to be associated with, but not wholly dependent on, the responsiveness of the mice to the induction of certain microsomal enzymes by some drugs and environmental chemicals (Eisen *et al.*, 1983; Smith & Francis, 1983; Smith *et al.*, 1986*a*). Here we report that porphyria can be induced in susceptible inbred mice preloaded with iron by a single exposure to HCB. This protocol has allowed us to follow the sequence of enzymic and other changes that occur leading to overt porphyria and to examine possible mechanisms of enzyme inhibition.

Abbreviations used: HCB, hexachlorobenzene; PCT, porphyria cutanea tarda; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

A preliminary account of these data was presented at the conference on Porphyrins and Porphyrias, Paris, 1985.

EXPERIMENTAL

Chemicals

HCB (organic analytical grade) and phenobarbital were purchased from BDH. [U-^{14}C]HCB (106 mCi/mmol) was from Amersham International. Other chemicals were purchased from the following suppliers: Imferon (50 mg of Fe/ml) from Fisons; NADPH, sodium dichloroindophenol, thiobarbituric acid and butylated hydroxyanisole from Sigma; β-naphthoflavone from Aldrich; isosafrole (*cis*+*trans*) and piperonyl butoxide from Fluorochem; uroporphyrin I, uroporphyrin III, pentacarboxyporphyrin I and coproporphyrin I from Porphyrin Products. Heptacarboxyporphyrin III was isolated from the livers of rats treated with HCB (Jackson *et al.*, 1976) and found to be > 90% the *d* isomer by n.m.r. (Professor A. H. Jackson, personal communication). Ethoxyphenoxazone was synthesized as described elsewhere (Smith *et al.*, 1986*a*).

Mice and protocols

Male C57BL/10ScSn and DBA/2Nimr mice (7–10 weeks old) were bred in these laboratories and fed Breeder Diet no. 3 expanded (Special Diet Services, Witham, Essex, U.K.). Iron as Imferon was given by subcutaneous injection (10 ml/kg) 3 days before the beginning of an experiment. HCB was dissolved in warm corn oil (10 mg/ml) and given to mice by oral intubation (100 mg/kg; 0.35 mmol/kg). Phenobarbital (60 mg/kg), β-naphthoflavone (100 mg/kg), isosafrole (100 mg/kg), piperonyl butoxide (100 mg/kg) and butylated hydroxyanisole (150 mg/kg) were given by intraperitoneal injection dissolved in physiological saline or corn oil (4 ml/kg) as appropriate and when indicated. Animals that received HCB were kept in a negative pressure isolator. Mice were killed either with CO_2 or by cervical dislocation. In experiments involving the assay of uroporphyrinogen decarboxylase, livers were frozen in liquid N_2 and stored at −70 °C until analysed. Microsomal fractions were prepared from fresh tissue.

Analyses

Plasma alanine aminotransferase (EC 2.6.1.2) was assayed on blood obtained by heart puncture using a kit (No. 57-UV) purchased from Sigma (Greig *et al.*, 1984).

Livers were homogenized in 0.25 M-sucrose (1:4, w/v). Porphyrin concentrations were estimated by fluorescence spectroscopy and are expressed in terms of uroporphyrin (Granick *et al.*, 1975; Smith *et al.*, 1979). Proportions of uroporphyrin and heptacarboxyporphyrin and analysis of uroporphyrin isomers I and III were determined by h.p.l.c. (Smith & Francis, 1983; Francis & Smith, 1985). HCB levels were estimated by g.l.c. (Rizzardini & Smith, 1982; Smith & Francis, 1983). Non-haem iron concentrations in livers were determined by a modified method of Torrance & Bothwell (1968).

To assay uroporphyrinogen decarboxylase activity, homogenate was centrifuged at 40000 *g* for 30 min and the supernatant incubated with pentacarboxyporphyrinogen I (5 μM) in 0.1 mM-EDTA/3 mM-dithiothreitol/50 mM-sodium succinate buffer (pH 5.4) under N_2 in the dark at 37 °C for 10 min (Francis & Smith, 1984). Incubations were then acidified to 0.5 M-HCl, centrifuged at 1000 *g* for 5 min, and the coproporphyrinogen I formed per mg of protein was quantified (as the porphyrin analogue) by reversed-phase h.p.l.c. (Francis & Smith, 1984, 1985). For a given enzyme source and batch of pentacarboxyporphyrin I the reproducibility of the assay showed < 5% variation.

Microsomes were prepared from homogenate in two ways. In the time study of cytochrome *P*-450 induction, microsomal fractions were prepared by single centrifugation at 100000 *g* of the post-mitochondrial fraction (Smith *et al.*, 1986*a*) and used immediately. Cytochrome *P*-450 content was estimated by the method of Omura & Sato (1964) and ethoxyphenoxazone de-ethylase as in Smith *et al.* (1986*a*). Cytosolic NAD(P)H quinone reductase [diaphorase (EC 1.6.99.2)] was assayed with 2,6-dichloroindophenol and NADH as substrates (Smith *et al.*, 1986*a*). For other studies washed microsomes were prepared essentially as described in Stewart & Smith (1986) and stored in a glycerol/0.1 M-Na_2HPO_4/NaH_2PO_4 buffer, pH 7.4 (1:4, v/v) at −70 °C until used.

Proteins in supernatants and microsomal fractions were estimated with bovine serum albumin (Sigma) as a standard essentially by the method of Lowry *et al.* (1951) (Stewart & Smith, 1986).

All results are means ± S.E.M. and significance was assessed by Student's *t*-test.

Microsomal metabolism of HCB and peroxidation studies

Incubations with HCB-induced microsomes were based on those described for rats (Stewart & Smith, 1986) and consisted of 3.4 ml of 0.1 M-Na_2HPO_4/NaH_2PO_4 buffer (pH 7.4), 13 mg of microsomal protein and 100 nmol of HCB added in 30 μl of acetone. Following a 4 min preincubation period at 37 °C, incubations were initiated with an NADPH-regenerating system. After 30 min some reactions were terminated by extraction with ethyl acetate and the metabolism of HCB estimated by the formation of pentachlorophenol (Stewart & Smith, 1986). The limit of detection was 0.05 pmol/min per mg of microsomal protein. Other incubations were placed in ice and malondialdehyde formed by peroxidation of lipid was estimated with thiobarbituric acid (Slater & Sawyer, 1971). Similar incubations at 20 °C were used to measure NADPH oxidation at 340 nm and O_2 consumption in control microsomes.

Incubations of cytosol with control or HCB-induced C57BL/10 microsomes (5 days) were conducted as above for 20 min except that 1.5 ml of the 0.1 M-Na_2HPO_4/NaH_2PO_4 buffer was substituted with 1.5 ml of control 100000 *g* cytosol. Decarboxylase activity in a portion of the mixtures was then estimated as above.

Partial purification of uroporphyrinogen decarboxylase after [^{14}C]HCB

Liver from C57BL/10 mice with iron overload that had received [^{14}C]HCB (see the Results section) was homogenized in 0.25 M-sucrose (1:4, w/v), gently mixed with cellulose phosphate P11 ion exchanger (Francis & Smith, 1984) (1:10, w/v) and centrifuged at 40000 *g* for 30 min at 4 °C. The supernatant was applied to a 20 cm × 3 cm DEAE-Sephacel column (Pharmacia) and eluted with 2–3 column vol. each of 10, 50 and

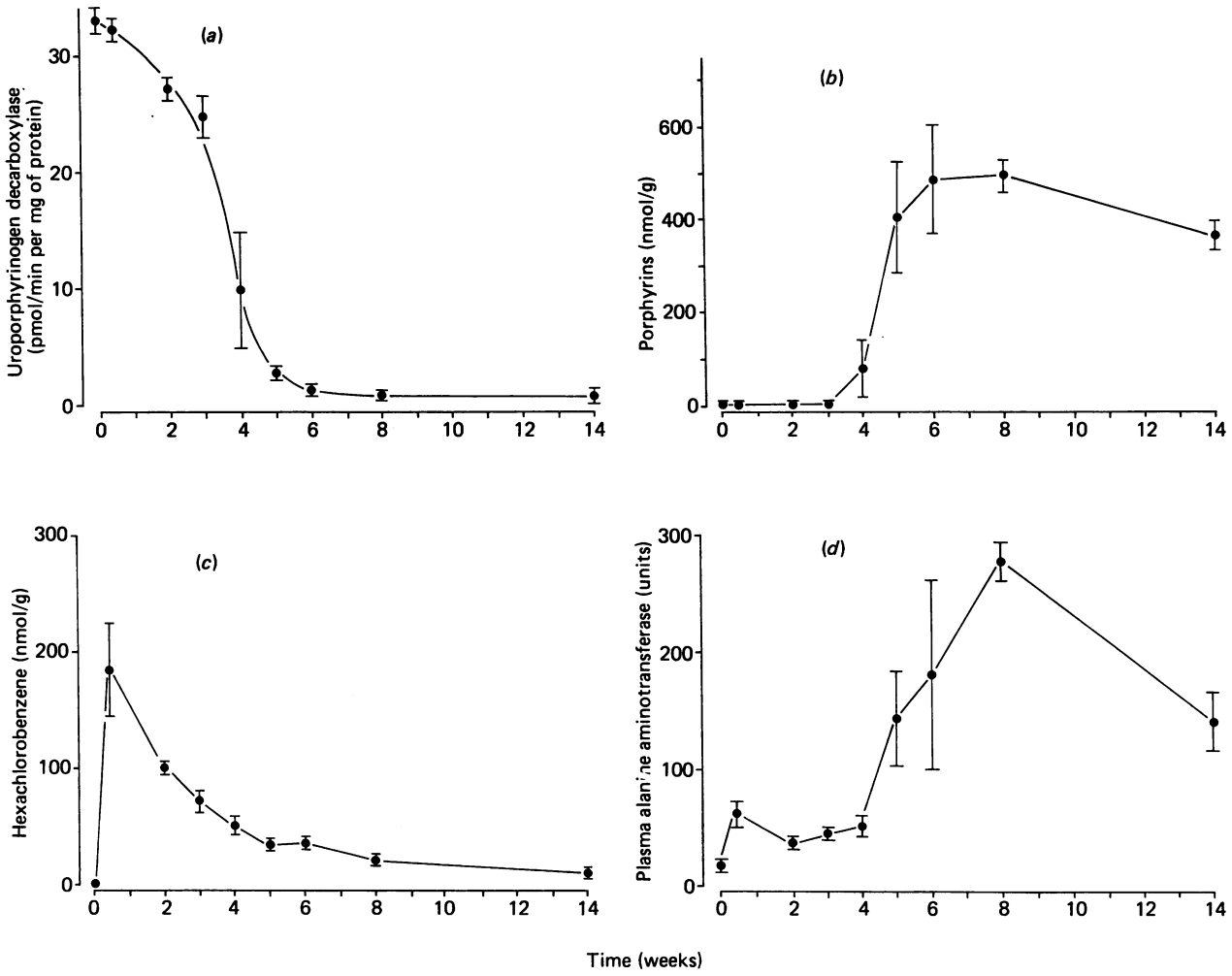

Fig. 1. Influence of a single oral dose of HCB to C57BL/10 mice on (*a*) hepatic uroporphyrinogen decarboxylase activity, (*b*) hepatic porphyrin levels, (*c*) hepatic HCB concentrations, and (*d*) plasma alanine aminotransferase activity

Mice predosed with Imferon (10 ml/kg 3 days previously) received HCB (100 mg/kg; 0.35 mmol/kg) and were killed at intervals. Hepatic and plasma parameters were determined as described in the Experimental section. Results are means ± S.E.M. for four mice per group. Decarboxylase activities were measured in four groups consisting of one liver from each time point. At 2 weeks enzyme activities were significantly different from zero time values ($P < 0.01$), as were every subsequent time. Porphyrin levels were not significantly different from zero time until 5 weeks ($P < 0.01$).

100 mM-Na_2HPO_4/NaH_2PO_4 buffers (pH 6.8). The fractions eluted by the last buffer that contained decarboxylase activity were precipitated with $(NH_4)_2SO_4$ (75% satd.) and then applied in 100 mM-phosphate buffer to a 15 cm × 2 cm phenyl-Sepharose (Pharmacia) column equilibrated with 0.8 M-$(NH_4)_2SO_4$. After elution with this $(NH_4)_2SO_4$ solution followed by 2 mM-Na_2PO_4/NaH_2PO_4 buffer (pH 6.8) enzyme activity was eluted with 50% ethylene glycol. Combined appropriate fractions were applied to a second DEAE-Sephacel column (15 cm × 1 cm) and then eluted as above. Radioactivity was monitored throughout this procedure (Khanna & Smith, 1985). The high levels of ferritin and other iron-containing proteins present in the experimental livers caused some interference with the purification process. One unit of enzyme activity was defined as 1 nmol of pentacarboxyporphyrinogen I converted to coproporphyrinogen I per min under the conditions of assay described under 'Analyses'.

RESULTS

Inhibition of uroporphyrinogen decarboxylase after a single ingestion of HCB

Previously it has been demonstrated that C57BL/10 mice are dramatically sensitized to the porphyrinogenic actions of HCB in the diet by pretreatment by iron (Smith & Francis, 1983). Further studies showed that hepatic porphyria in C57BL/10 mice with iron overload could be induced by a single oral dose of HCB (100 mg/kg; 0.35 mmol/kg) (Fig. 1). Smaller doses were less effective and experiments with larger quantities of HCB were restricted by the solubility of the chemical in oil (A. G. Smith, unpublished work). Within 2 weeks following the single ingestion of HCB, hepatic uroporphyrinogen decarboxylase activity began to decline, reaching a maximum 97% inhibition by about 6 weeks (Fig. 1*a*). Remarkably, after 14 weeks no recovery of enzyme activity was detected. In contrast, hepatic

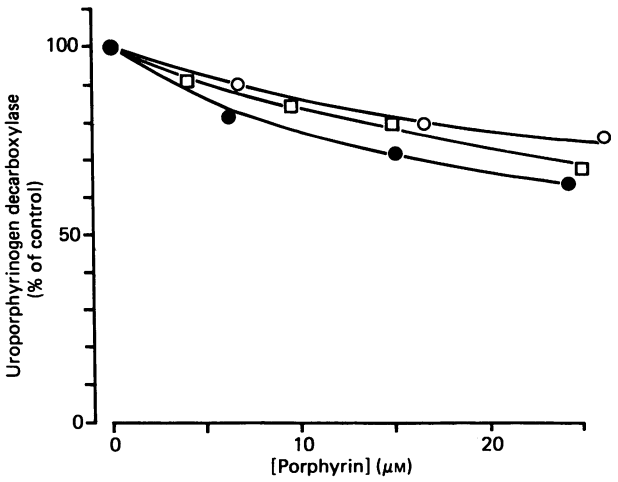

Fig. 2. Inhibition of the formation of coproporphyrinogen I from pentacarboxyporphyrinogen I by supernatant from the liver of a control mouse in the presence of uroporphyrin I (●), uroporphyrin III (○) and heptacarboxyporphyrin III (□)

Incubations were performed as described in the Experimental section immediately following addition of porphyrin.

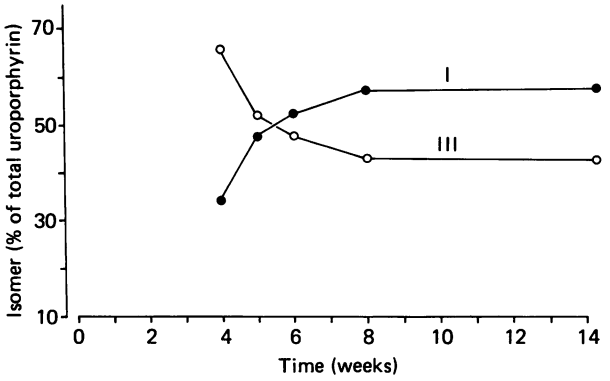

Fig. 3. Isomeric composition of the uroporphyrins accumulated in the liver during the course of the porphyria shown in Fig. 1(*b*)

Uroporphyrin I (●) and uroporphyrin III (○) were determined by h.p.l.c. of 40000 *g* supernatant as described previously (Smith & Francis, 1983; Francis & Smith, 1985).

porphyrin levels did not increase markedly until 4 weeks by which time decarboxylase activity was inhibited by 70% (Fig. 1*b*). Maximum porphyrin levels were observed 8 weeks after the dose of HCB and decreased slightly after 14 weeks. The porphyrin that accumulated at 8 weeks consisted of uroporphyrin (80–90%) and heptacarboxyporphyrin III. Observations of differences in fluorescence between livers at the time of killing and again when analysed suggested that during the earlier stages of porphyria much of the porphyrin was present as porphyrinogen. In another experiment the catalytic activity of the decarboxylase after 18 weeks showed a small recovery but was still only 6% of the initial value and porphyrin levels continued to be elevated (513 ± 36 nmol/g).

Maximum levels of HCB were observed at 3 days and thence decreased (Fig. 1*c*). A logarithmic plot of the data illustrated that the decline consisted of two phases with half-lives of about 3 and 6 weeks. At 14 weeks HCB levels were only 6% of the values at 3 days and thus did not correlate with decarboxylase inhibition.

Plasma levels of alanine aminotransferase were also determined as an estimate of liver damage during the course of the experiment (Fig. 1*d*). The main rise in aminotransferase activity approximately corresponded with the accumulation of porphyrins and not with the depression of uroporphyrinogen decarboxylase activity.

Although hepatic non-haem iron levels fell from 48.9 ± 0.8 μmol/g of liver at the time of HCB ingestion to 34.2 ± 1.2 μmol/g after 14 weeks, mice were still in an overt iron overload condition at the end of the experiment (control values 0.5–0.8 μmol/g). Non-haem iron levels and the severity of porphyria were not potentiated by predosing with larger quantities of iron, although smaller doses were less effective.

To determine how much of the observed inhibition of uroporphyrinogen decarboxylase was due to endogenous porphyrins present in supernatant fractions, control enzyme was assayed in the presence of added porphyrins. Uroporphyrins I and III and heptacarboxyporphyrin III inhibited the decarboxylation of pentacarboxyporphyrinogen I to coproporphyrinogen I; however, this only accounted for 3% of the inhibition of the enzyme estimated in supernatants from 4-week mice and less than 15% in those at 5 weeks (Fig. 2). Thus neither the loss of decarboxylase activity which was observed up to 5 weeks after HCB nor the continued inhibition at 14 weeks could be accounted for solely by endogenous uroporphyrins interfering in the assay procedure.

Isomeric composition of uroporphyrin

In the experiment shown in Fig. 1(*b*) the ratio of uroporphyrin I to III was determined from the time of the first accumulation of porphyrins at 4 weeks to 14 weeks. Between 4 and 8 weeks the proportions of these isomers were reversed so that, as the porphyria developed, uroporphyrin I became the major isomer present (Fig. 3).

Induction of ethoxyphenoxazone de-ethylase

The induction of porphyria by polyhalogenated aromatic chemicals has been associated, at least partly, with the induction of the cytochrome *P*-450 isoenzymes induced by polycyclic aromatic hydrocarbons such as 3-methylcholanthrene and β-naphthoflavone (Jones & Sweeney, 1980; Sinclair *et al.*, 1984; Smith *et al.*, 1986*a*). A good enzymic marker for this induction is microsomal ethoxyphenoxazone de-ethylase (Burke & Mayer, 1983).

Induced levels of cytochrome *P*-450 and ethoxyphenoxazone de-ethylase activity by HCB were found to be greatest at 5 days (Figs. 4*a* and 4*b*) at approximately the same time as the level of HCB (Fig. 1*c*). However, even after 8 weeks both cytochrome *P*-450 and de-ethylase were still significantly elevated over pre-dose values. In contrast, the induction of cytosolic diaphorase activity, which is partly associated with cytochrome *P*-450 induction of this type (Kumaki *et al.*, 1977), returned to control values by 5 weeks (Fig. 4*c*).

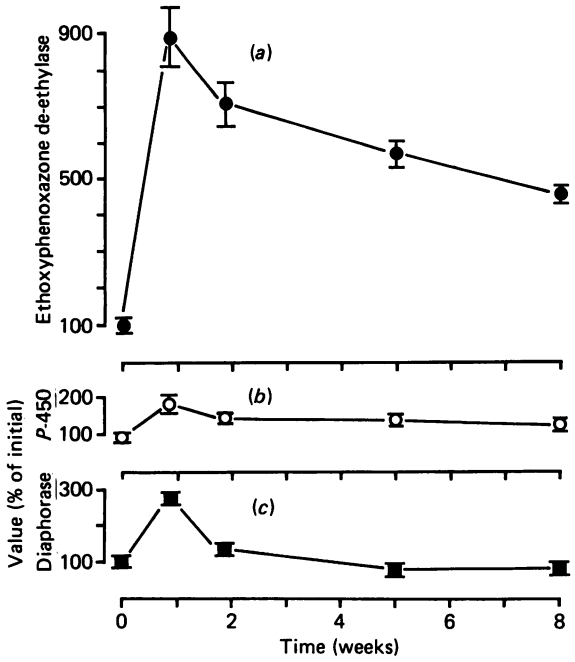

Fig. 4. Effect of a single oral dose of HCB (100 mg/kg) on (*a*) microsomal ethoxyphenoxazone de-ethylase activity, (*b*) cytochrome *P*-450, and (*c*) cytosolic NAD(P)H diaphorase activity in the livers of C57BL/10 mice

Enzymes were assayed as described in the Experimental section. Results are means ± S.E.M. for four mice per group.

Modification *in vivo* of the inhibition of uroporphyrinogen decarboxylase

Multiple treatments of mice with inducers of cytochrome *P*-450 and other chemicals were investigated to determine their influence on the inhibition of uroporphyrinogen decarboxylase caused by HCB (Table 1). β-Naphthoflavone significantly enhanced the inhibition of the enzyme and the accumulation of porphyrins. By itself β-naphthoflavone had no detectable effect on decarboxylase activity, although a slight rise in porphyrins of the uroporphyrin type was observed by fluorescence spectroscopy (Granick *et al.*, 1975). The influences of phenobarbital and isosafrole (results not shown) were less certain. Piperonyl butoxide, an inhibitor of cytochrome *P*-450-mediated enzyme activities, partially protected against decarboxylase inhibition whereas an antioxidant, butylated hydroxyanisole, was ineffective.

Experiment *in vivo* with [^{14}C]HCB

To determine whether inhibition of the decarboxylase was caused by binding of a carbon-containing metabolite of HCB, eight C57BL/10 mice preloaded with iron received 100 mg/kg of the chemical. After 3 weeks each mouse was given 1 μCi of [^{14}C]HCB and left for 5 days. A combined homogenate of the livers was mixed with an equal volume of control homogenate, centrifuged at 40000 *g*, precipitated with $(NH_4)_2SO_4$ and chromatographed on DEAE-Sephacel and phenyl-Sepharose until the uroporphyrinogen decarboxylase had been purified 120-fold (see the Experimental section). Discrete peaks of radioactivity were observed during chromatography but the specific radioactivity of the decarboxylase fraction fell from 4073 d.p.m./unit of enzyme activity in the mixed homogenate to < 0.2 d.p.m./unit at the final step. This final value represented a level of measured radioactivity (< 1 d.p.m.) which was not significantly different from background.

Incubations of HCB-induced microsomes with control cytosol

Microsomes from control and HCB-induced C57BL/10 mice with iron overload were incubated with control 100000 *g* cytosol and HCB (30 μM) for 20 min. Pentachlorophenol did not accumulate with control microsomes as opposed to 27 ± 5 pmol/mg of microsomal protein ($n = 4$) with HCB-induced microsomes. Although subsequent assay of uroporphyrinogen decarboxylase suggested that there was a slight loss of enzyme activity in the HCB-induced group (control 38.4 ± 1.1, HCB 36.0 ± 0.5 pmol/min per mg of protein), this was not statistically significant.

Table 1. Modification of the inhibition of uroporphyrinogen decarboxylase induced by iron–HCB following repeated treatments with various chemicals

Male C57BL/10 mice received Imferon (0.25 ml/25 g mouse) and then 3 days later oil (controls) or HCB (100 mg/kg) in oil (10 mg/ml). During the next 4 weeks groups of mice received ten doses (i.e. every 2 or 3 days) of various modifiers as described in the Experimental section; phenobarbital (60 mg/kg), β-naphthoflavone (100 mg/kg), piperonyl butoxide (100 mg/kg), butylated hydroxyanisole (150 mg/kg). Uroporphyrinogen decarboxylase activity and porphyrin levels were estimated as described in the Experimental section. Results are means ± S.E.M. for four mice except where indicated: *three mice per group, **seven mice per group, ***nine mice per group. Significantly different from control: †$P < 0.001$, ††$P < 0.01$. Significantly different from HCB group: ‡$P < 0.001$, ‡‡ < P 0.005, ‡‡‡ < P 0.01, ‡‡‡‡ < P 0.05.

Treatment	Uroporphyrinogen decarboxylase (pmol/min per mg of protein)		Porphyrins (nmol/g)	
	Control	HCB	Control	HCB
None	40.0 ± 0.2	14.1 ± 1.6***†	0.78 ± 0.08	73.6 ± 23.1***††
Phenobarbital	39.9 ± 0.7	8.6 ± 2.4‡‡‡‡	0.52 ± 0.03	88.2 ± 41.0
β-Naphthoflavone	39.4 ± 1.1	4.6 ± 1.4‡‡	1.65 ± 0.26‡‡‡	829 ± 167‡‡
Piperonyl butoxide	38.6 ± 1.0	23.2 ± 2.7**‡‡‡	0.45 ± 0.01	23.5 ± 10.4**
Butylated hydroxyanisole	41.0 ± 0.2*	8.1 ± 3.2**	0.83 ± 0.03*	381 ± 100**

Table 2. Comparison between lipid peroxidation and metabolism of HCB in hepatic microsomes of C57BL/10 and DBA/2 mice

Mice were pretreated with iron (500 mg/kg 3 days before any other treatment), HCB (100 mg/kg 5 days before preparation of microsomes) or β-naphthoflavone (100 mg/kg 2 days before preparation of microsomes) as described in the Experimental section. Cytochrome *P*-450 levels were determined at the time of microsomal preparation. Incubations were based on those described previously (Stewart & Smith, 1986) and consisted of 3.4 ml of 0.1 M-Na_2HPO_4/NaH_2PO_4 buffer (pH 7.4), approx. 13 mg of microsomal protein and an NADPH regenerating system. HCB (100 nmol) was added in acetone (30 μl). After 30 min at 37 °C lipid peroxidation as malondialdehyde formation and HCB metabolism as pentachlorophenol accumulation were estimated in different incubations but the same microsomal fraction. n.d., not detected at 0.05 pmol/min per mg of microsomal protein. Zero time malondialdehyde values were subtracted from peroxidation results. C57BL/10 and DBA/2 microsomes incubated in the absence of HCB gave the following malondialdehyde values: control, C57BL/10 114±5, DBA/2 8±3†; iron, C57BL/10 211±11, DBA/2 21±4† pmol/min per mg of protein. Results are means±S.E.M. for four or five mice per group. Significantly different from controls: *$P < 0.001$, **$P < 0.005$, ***$P < 0.05$; significantly different from C57BL/10 group: †$P < 0.001$.

Strain	Pretreatment	Cytochrome *P*-450 (nmol/mg of protein)	Peroxidation (pmol/min per mg of protein)	HCB metabolism (pmol/min per mg of protein)
C57BL/10	Control	0.98±0.01	104±8	n.d.
	Iron	0.75±0.03	228±8*	n.d.
	HCB	1.14±0.12	75±3***	1.03±0.18
	HCB+iron	1.09±0.08	191±26	0.88±0.14
	β-Naphthoflavone	1.33±0.03	84±3	n.d.
DBA/2	Control	0.89±0.05	13±2†	n.d.
	Iron	0.87±0.02	27±2*†	n.d.
	HCB	0.97±0.02	9±1***†	0.37±0.06
	HCB+iron	0.81±0.08	36±6†	0.66±0.14
	β-Naphthoflavone	0.90±0.03	5±1**	n.d.

Comparison between HCB metabolism and lipid peroxidation in C57BL/10 and DBA/2 microsomes

The metabolism of HCB to pentachlorophenol was not detected in control or iron overload microsomes from either C57BL/10 or DBA/2 mice. Pretreatment with HCB induced pentachlorophenol formation in both strains, but this was not significantly stimulated by the combination of iron and HCB to any greater extent than by HCB alone. There was only a small difference between the strains (Table 2). In contrast, the amount of malondialdehyde formed was 8-fold more by microsomes from control C57BL/10 mice than by those from DBA/2 animals. Iron treatment yielded greater amounts of malondialdehyde with both strains but the strain difference was still maintained (Table 2). HCB, given either as a pretreatment to mice or added to incubations, had no, or even an inhibitory, effect on malondialdehyde production in this system. β-Naphthoflavone did not affect the metabolism of HCB to pentachlorophenol or stimulate the production of malondialdehyde. In additional experiments, control C57BL/10 microsomes oxidized NADPH and consumed O_2 at much faster rates than did control DBA/2 microsomes. [NADPH oxidation: C57BL/10 8.21±0.80, DBA/2 2.98±0.26 nmol/min per mg of protein (significantly different, $P < 0.005$). O_2 consumption: C57BL/10 45.3±1.0, DBA/2 9.4±1.0 nmol/min per mg of protein ($P < 0.001$).]

DISCUSSION

Sequence of the induction of porphyria

Previously it had been assumed that the induction of hepatic porphyria in rodents by HCB required continual exposure for a prolonged period (Elder, 1978). In contrast, the findings here show that a single ingestion of HCB will cause a progressive inhibition of uroporphyrinogen decarboxylase in siderotic C57BL/10 mice probably beginning within a few days and lasting many weeks. Although a time lag of some weeks before the enzyme in rodents is inhibited by HCB has been reported (Elder, 1978; Smith *et al.*, 1979, 1986*a*) these results and other published work (Smith *et al.*, 1981; Smith & Francis, 1983; Wainstok de Calmanovici *et al.*, 1984) demonstrate that the lag phase can be much shorter. Prolonged inhibition of the enzyme does not appear to be directly correlated with levels of HCB in the liver or with inductions of total cytochrome P-450 and microsomal ethoxyphenoxazone de-ethylase activity. If these parameters are involved in the inhibition they must still be sufficient after 14 weeks to maintain the depression of uroporphyrinogen decarboxylase activity (Koss *et al.*, 1983; Khanna & Smith, 1985; Smith *et al.*, 1985). The accumulation of liver porphyrins is probably potentiated by induction of aminolaevulinate synthase (EC 2.3.1.37) (Wainstok de Calmanovici *et al.*, 1984) partly explaining the disparities between decarboxylase inhibition and porphyrin levels that were sometimes observed. This underlines the importance of measuring decarboxylase activity in studies of this kind.

A specific type of liver damage is seen in sporadic PCT patients (Kappas *et al.*, 1983). The elevation of plasma alanine aminotransferase activity at approximately the same time as hepatic porphyrin levels suggests that in the mice the latter may be one cause of liver damage. Whether similar effects occur in human PCT is unknown.

Changes in the proportions of uroporphyrin isomers during the development of porphyria is again of considerable interest with respect to human PCT in which uroporphyrin I predominates over the III isomer

(Dowdle *et al.*, 1970). One explanation might be that uroporphyrinogen III synthase (EC 4.2.1.75) is inhibited either by iron (Kushner *et al.*, 1972) or by the accumulating uroporphyrinogens and uroporphyrins (Levin, 1971). The unstable hydroxymethylbilane substrate might then spontaneously cyclize to uroporphyrinogen I rather than be transformed to uroporphyrinogen III. Alternatively, there may be an increased tendency in HCB-treated tissue for hydroxymethylbilane to be cyclized to uroporphyrinogen I and porphyrinogens oxidized to porphyrins. These possibilities should also be considered when equating uroporphyrin accumulation with uroporphyrinogen decarboxylase inhibition in experimental systems.

Mechanism of the inhibition of uroporphyrinogen decarboxylase

There is considerable evidence to suggest that two of the major factors associated with the inhibition of uroporphyrinogen decarboxylase by HCB and other polyhalogenated chemicals are: (*a*) the induction of cytochrome P_1-450 controlled by the *Ah* locus in mice (Jones & Sweeney, 1980; Smith *et al.*, 1986*a*) or related isoenzymes in other experimental models of PCT (Carpenter *et al.*, 1984; Sinclair *et al.*, 1984); (*b*) the presence of some unknown non-haem iron species (Taljaard *et al.*, 1972; Louw *et al.*, 1977; Smith *et al.*, 1979; Jones *et al.*, 1981; Smith & Francis, 1983; Ferioli *et al.*, 1984; Greig *et al.*, 1984). Whether mice become porphyric would seem to depend partly on a balance between liver iron content and the potency of a particular chemical to induce *Ah*-type microsomal mono-oxygenase activities in both *Ah*-responsive and *Ah*-nonresponsive strains (Smith & Francis, 1983; Greig *et al.*, 1984; Smith *et al.*, 1986*a*). In agreement with this, the nonchlorinated *Ah*-type inducers 3-methylcholanthrene and β-naphthoflavone will potentiate the effects of HCB and related chemicals much more than phenobarbital (Table 1; Carpenter *et al.*, 1984; Sinclair *et al.*, 1984) and partial protection is afforded by an inhibitor of cytochrome *P*-450, piperonyl butoxide (Table 1; Sinclair *et al.*, 1984). Induction of a cytochrome P_1-450 isoenzyme alone may not be sufficient for a porphyrogenic response since we were unable to detect any loss of decarboxylase activity after treatment with β-naphthoflavone, although, interestingly, some abnormalities in porphyrin accumulation were observed.

How do these factors cause an inhibition of hepatic uroporphyrinogen decarboxylase? HCB may be metabolized by cytochrome P_1-450 in a process, stimulated by iron, to a product which inhibits or covalently binds with the enzyme. However, incubation of microsomes from the susceptible C57BL/10 strain and the non-susceptible DBA/2 strain (Smith & Francis, 1983) with HCB has not shown convincing evidence for either differential metabolism or stimulation by iron overload (Table 1). Similar results have been obtained *in vivo* (Khanna & Smith, 1985). In addition, no radiolabelling of uroporphyrinogen decarboxylase was detected in experiments with [^{14}C]HCB. These findings agree with studies on rats in which there was no correlation between metabolism of HCB and the marked sex difference in susceptibility to porphyria (Rizzardini & Smith, 1982; Stewart & Smith, 1986).

A free radical-mediated mechanism for the porphyrogenicity of TCDD was proposed by Jones *et al.* (1981) because of the protection afforded by iron depletion and by dietary butylated hydroxyanisole (Sweeney, 1982). We have been unable to find any protective effect of this antioxidant (similar results have been reported for rats; Debets *et al.*, 1981), but the great enhancement by iron overload of both HCB and TCDD porphyrogenicity in mice (Smith & Francis, 1983; Greig *et al.*, 1984) would be consistent with a related mechanism perhaps involving interaction between microsomal electron transport and ferritin (De Matteis & Stonard, 1977; Rowley & Sweeney, 1984). Siderotic mice fed HCB produce more lipid peroxidation products than do controls (Smith *et al.*, 1986*b*) but the finding of a marked difference between hepatic microsomes from C57BL/10 and DBA/2 mice in the production of malondialdehyde, the oxidation of NADPH and O_2 consumption, may be of greater relevance despite the lack of induction by HCB. Cheeseman *et al.* (1985) have shown a similar difference between control microsomes from C57BL/6 and DBA/2 mice with the ADP/Fe^{2+} system, but not with other methods of stimulating microsomal lipid peroxidation. This difference between the mouse strains remains unexplained. A free radical process with production of an active oxygen species might, in the presence of porphyrin, lead to inactivation of uroporphyrinogen decarboxylase at the active site perhaps involving a thiol group (Elder *et al.*, 1985). Immunoreactive decarboxylase is still present in HCB- and TCDD-induced porphyria (Elder & Sheppard, 1982). Alternatively, a high-affinity inhibitor (Cantoni *et al.*, 1984) may be produced which is not a metabolite of HCB.

The relevance of the mechanism of the induction of porphyria in experimental animals by polyhalogenated aromatic chemicals to our understanding of the pathogenesis of human sporadic PCT is still not unequivocably established. However, a mechanism which does not involve inhibition of the enzyme by a metabolite of the chemical would be attractive in linking these two manifestations of depressed hepatic uroporphyrinogen decarboxylase activity.

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