Chemically-induced formation of an inhibitor of hepatic uroporphyrinogen decarboxylase in inbred mice with iron overload

Andrew G. SMITH and Jean E. FRANCIS

MRC Toxicology Unit, Medical Research Council Laboratories, Woodmansterne Road, Carshalton, Surrey SM5 4EF, U.K.

An inhibitor of hepatic uroporphyrinogen decarboxylase (EC 4.1.1.37) was demonstrated in heat-treated extracts of livers from C57BL/10ScSn mice with iron overload after a single dose (100 mg/kg; 350 μ mol/kg) of hexachlorobenzene (HCB). Inhibition was not due to accumulated uroporphyrin since this could be removed by a SEP-PAK C₁₈ cartridge without affecting inhibitor activity. The presence of the inhibitor could be first demonstrated 2 weeks after mice received HCB and before major elevation of hepatic porphyrin levels. Maximum inhibitory potential was reached at about 8 weeks and was still detected 25 weeks after the chemical, thus paralleling the depression of enzyme activity reported previously [Smith, Francis, Kay, Greig & Stewart (1986) *Biochem. J.* 238, 871–878]. The inhibitor was not detected following treatment of mice with either iron or HCB alone or after the decarboxylase activity was destroyed *in vitro* by the combination of uroporphyrin and light. The formation of the inhibitor by inbred mouse strains nominally *Ah*-responsive (C57BL/6J, C57BL/10ScSn, BALB/c, C3H/HeJ, CBA/J and A/J) and *Ah*-nonresponsive (SWR, AKR, 129, SJL, LP and DBA/2) did not correlate fully with their reported *Ah*-phenotype. There was a correlation amongst the *Ah*-responsive strains only, with hepatic ethoxyphenoxazone de-ethylase activity induced in parallel experiments by treatment with β -naphthoflavone. De-ethylase activity induced by HCB, however, was considerably less than that with β -naphthoflavone, which has not been reported as porphyrogenic. Other polyhalogenated chemicals, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, 2,3,4,2',3',4'-hexachlorobiphenyl and hexabromobenzene, also caused the formation of the inhibitor of uroporphyrinogen decarboxylase.

INTRODUCTION

Some polyhalogenated aromatic chemicals cause a depression of hepatic uroporphyrinogen decarboxylase (EC 4.1.1.37) activity in humans, experimental animals (Elder, 1978; Kappas *et al.*, 1983; Marks, 1985) and chick embryo hepatocytes (de Verneuil *et al.*, 1983; Swain *et al.*, 1983). The resulting porphyria in mammals closely resembles the sporadic form of porphyria cutanea tarda which occasionally develops in patients with moderately high intakes of ethanol or steroids (Kappas *et al.*, 1983). Whether a common mechanism exists to explain all of these deficiencies in decarboxylase activity is not known. A variety of explanations have been proposed; some encompass all experimental and human uroporphyrinurias, whereas others are restricted to the actions of specific chemicals.

The most powerful porphyrogen is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Heat-treated extracts of livers from C57BL/6 mice given TCDD have been reported to inhibit uroporphyrinogen decarboxylase of control liver cytosol (Cantoni *et al.*, 1984) as have extracts from rats fed hexachlorobenzene (HCB) (Billi *et al.*, 1986). HCB will also cause porphyria in C57BL/6 and C57BL/10 mice if they are first given iron (Smith & Francis, 1983; Smith *et al.*, 1986b). We have recently refined this model of porphyria cutanea tarda to produce sustained depression of the decarboxylase after a single dose of HCB (Smith *et al.*, 1986a). By exploiting this protocol we now show that HCB and other polyhalogenated chemicals will also cause the formation of an

inhibitor of uroporphyrinogen decarboxylase in mice and that the response is modulated by genetic factors.

EXPERIMENTAL

Chemicals

HCB (Organic Analytical grade) was purchased from BDH. Hexafluorobenzene, hexabromobenzene, resorufin and β -naphthoflavone were from Aldrich and Firemaster BP-6 was from Greyhound Chromatography. 3,4,3',4'-Tetrachlorobiphenyl was obtained from Ultra Scientific (Hope, RI, U.S.A.) and uroporphyrin III and pentacarboxyporphyrin I from Porphyrin Products (Logan, UT, U.S.A.). 2,3,4,2',3',4'-Hexachlorobiphenyl, 2,4,5,2',4',5'-hexachlorobiphenyl, Aroclor 1254 and TCDD were gifts from Dr. J. B. Greig, Carshalton (Stonard & Greig, 1976). Ethoxyphenoxazone was synthesized as described previously (Smith *et al.*, 1986b).

Mice and protocols

Male mice (6–10 weeks old) were obtained from the following sources. C57BL/6J, C57BL/10ScSn, DBA/2, BALB/c and C3H/HeJ were bred in these laboratories. CBA/J, A/J, SWR, LP and AKR (for induction studies) were obtained from the National Institute of Medical Research, U.K. SJL, 129 and AKR (for inhibitor studies) were purchased from OLAC (1976) Ltd. The histories of these strains are available on request. Mice were fed Breeder Diet No. 3 expanded (Special Diet Services, Witham, Essex, U.K.). Iron, as Imferon (Fisons

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

Pharmaceuticals) containing 50 mg of Fe/ml, was given by subcutaneous injection (12 ml/kg; 600 mg/kg) 3 days before the beginning of an experiment. HCB and other polyhalogenated chemicals, dissolved in arachis oil, were given by oral intubation (usually in 10 ml of oil/kg body wt.). Animals were killed by cervical dislocation and livers for inhibitor experiments were frozen in liquid N₂ and stored at -40 °C until analysed.

Analyses

For the standard assay of inhibitor activity livers were homogenized in water (1:4, w/v) and a portion was centrifuged at 40000 *g* for 30 min. This *g* value was obtained at the maximum speed of an MSE High-Spin 21 centrifuge and gave a nearly clear supernatant below a surface lipid layer without recourse to a higher speed instrument and longer centrifugation times. Approx. 4 ml of supernatant was heated at 90 °C for 5 min, cooled and centrifuged at 1000 *g* for 5 min. An aliquot (0.5 ml) of the resulting clear supernatant was applied to a SEP-PAK C₁₈ cartridge (Waters Associates, Milford, MA, U.S.A.) which had been prepared by passage of methanol (5 ml) and water (5 ml). The sample was eluted with 1.5 ml of water and the eluate was freeze-dried overnight. The material was then reconstituted with 0.5 ml of 50 mM-sodium succinate buffer, pH 5.4, giving a clear, pale straw-coloured solution containing < 3% of the protein present in the supernatant. A portion (0.2 ml) was added to a standard decarboxylase assay incubation mixture, but without substrate, containing 0.2 ml of control liver 40000 *g* supernatant (prepared in 0.25 M-sucrose) from untreated C57BL/10ScSn mice (Francis & Smith, 1984; Smith *et al.*, 1986a). After an aerobic preincubation at 37 °C for 10 min in the dark, pentacarboxyporphyrinogen I (5 nmol) was added and the conversion to coproporphyrinogen I determined under anaerobic conditions as described previously (Smith *et al.*, 1986a). H.p.l.c. of porphyrins was conducted using LDC/Milton Roy ConstaMetric 3000 pumps and a Spherisorb 5 µm ODS-1 column (25 cm × 4.6 mm internal diam.). A gradient elution system was constructed from mixtures of 0.05 M-lithium citrate (pH 3.0) and methanol controlled by an Apple IIe computer (Francis & Smith, 1984, 1985). Detection was by absorbance at 400 nm with a LDC/Milton Roy SpectroMonitor 3000.

Porphyrin contents of liver homogenates were assayed by fluorescence spectroscopy and are expressed in terms of uroporphyrin (Granick *et al.*, 1975). The induction of ethoxyphenoxazone *O*-de-ethylase activity was determined using hepatic 9000 *g* supernatants in the presence of 20 µM-dicumarol [to inhibit NAD(P)H-diaphorase activity] essentially as reported by Lubet *et al.* (1985). Protein was assayed with bovine serum albumin as a standard (Lowry *et al.*, 1951). Results are means ± S.E.M. and significance was usually assessed by Student's *t*-test.

Inactivation of uroporphyrinogen decarboxylase by light/uroporphyrin

Liver 40000 *g* supernatant (0.3 ml) from control C57BL/10ScSn mice was diluted to 1.5 ml with 0.1 mM-EDTA / 3 mM-dithiothreitol / 50 mM-sodium succinate buffer (pH 5.4) in the presence or absence of 25 µM-uroporphyrin (a mixture of the I and III isomers isolated from rats treated with HCB; Smith & Francis, 1981). Samples were either exposed to laboratory fluorescent

Fig. 1. H.p.l.c. analyses of the porphyrins in 40000 *g* liver supernatants from C57BL/10ScSn mice given (a) oil or (b) HCB and the effects of heat-treated SEP-PAK C₁₈-purified extracts of (c) oil or (d) HCB supernatants on the conversion of pentacarboxyporphyrinogen I to coproporphyrinogen I by control hepatic uroporphyrinogen decarboxylase

C57BL/10ScSn mice predosed with Imferon (12 ml/kg 3 days previously) received orally either oil (10 ml/kg) or HCB (100 mg/kg; 350 µmol/kg). After 5 weeks 40000 *g* liver supernatants were prepared and analysed for porphyrins by h.p.l.c. (a,b). Aliquots were then heat-treated, purified from uroporphyrin via SEP-PAK C₁₈ cartridges and tested for their potential to inhibit uroporphyrinogen decarboxylase in liver supernatants from untreated C57BL/10ScSn mice using 5 µM-pentacarboxyporphyrinogen I as substrate (see the Experimental section) (c,d). The coproporphyrinogen I product was determined (as the porphyrin) by h.p.l.c. using a 5 µm ODS-1 column and elution with a methanol/50 mM-lithium citrate (pH 3) gradient. The four chromatograms represent initial equivalent amounts of test liver supernatants, however, (c) and (d) are at twice the sensitivity used for (a) and (b). Peak 8, uroporphyrin isomers; peak 7, heptacarboxyporphyrin III; peak 5, pentacarboxyporphyrin I; peak 4, coproporphyrin I.

lights at 0 °C for 2 h or kept in the dark. Some samples were heat-treated, purified via SEP-PAK C₁₈ cartridges and freeze-dried as described in the previous section. The effects of uroporphyrin and exposure to light on uroporphyrinogen decarboxylase activity and inhibitory potential were then determined.

Fig. 2. Inhibition of control hepatic uroporphyrinogen decarboxylase activity by extracts of liver from C57BL/10ScSn mice up to 25 weeks after receiving HCB

Mice predosed with Imferon received HCB (100 mg/kg; 350 μ mol/kg) and were killed at intervals. Heat-treated extracts of livers were separated from uroporphyrin as described in the Experimental section and were tested for inhibitory potential against uroporphyrinogen decarboxylase activity of 40000 g liver supernatant from untreated C57BL/10ScSn mice ($\circ$). Porphyrin contents in the livers were also determined ($\bullet$). Results are means $\pm$ S.E.M. of four or eight (2 weeks) mice per time point. Both residual decarboxylase activity and porphyrin levels at 2 weeks were significantly different from those in zero time animals ($P < 0.001$ and $P < 0.05$ respectively) as were all subsequent groups.

RESULTS

Release of an inhibitor of uroporphyrinogen decarboxylase from HCB livers

Hepatic uroporphyrinogen decarboxylase activity was inhibited by 67% compared with controls in C57BL/10ScSn mice with iron overload 4 weeks after a single dose of HCB (cf. Smith *et al.*, 1986a). Equal mixtures of 40000 g supernatants from the HCB-treated and control mice gave activities expected from the mean of control and inhibited decarboxylases, i.e. control 42.7 ± 0.4 , HCB 14.3 ± 2.0 , control+HCB 26.0 ± 3.1 , expected control+HCB 28.6 ± 1.1 pmol/min per mg of protein ($n = 4$), illustrating that there was no free additional inhibitory activity. When heat-treated extracts of the HCB liver supernatants were prepared an inhibitor was present which after preincubation with control supernatant and subsequent assay of the decarboxylase caused a 75% decrease in enzyme activity. Extracts of livers from mice given iron alone had no effect. Inhibitory power was unaffected by extraction of heat-treated supernatants with ethyl acetate or diethyl ether. Uroporphyrins and heptacarboxyporphyrin III are inhibitors of rat and mouse hepatic uroporphyrinogen decarboxylase (Smith & Francis, 1981; Smith *et al.*, 1986a) but levels of these porphyrins from porphyric livers in incubations were too low to account for the degree of inhibition observed. Furthermore, a 'clean up' procedure was developed using SEP-PAK C_{18} cartridges which eliminated most of the uroporphyrin and heptacarboxyporphyrin III, but not the inhibitor, present in extracts of 40000 g HCB supernatants (see the Experimental section). For instance, the homogenate and

supernatant from a porphyric liver contained 89.6 and 62.7 nmol of porphyrin/ml respectively, whereas the reconstituted SEP-PAK eluate contained < 0.5 nmol/ml. Fig. 1(b) illustrates by h.p.l.c. the presence of uroporphyrins in liver supernatants from a HCB liver. After heat treatment and elution through a SEP-PAK C_{18} cartridge the uroporphyrins had been removed by $> 98\%$ and the reconstituted eluate was a potent inhibitor of control liver uroporphyrinogen decarboxylase activity as followed by the conversion of pentacarboxyporphyrinogen I to coproporphyrinogen I (Fig. 1d).

Omission of the preincubation step lessened the observed inhibition but there was no change if it was conducted under anaerobic conditions. Preincubation in the light gave slightly less effect than in the dark. Inhibition was approximately proportional to the volume of reconstituted eluate from SEP-PAK cartridges in the range 20–250 μ l.

A time study of the presence of this inhibitor in liver extracts showed significant levels 2 weeks after HCB, when porphyrin concentrations had only risen by 1.4-fold (Fig. 2). Maximum inhibitory activity was observed after 8 weeks at the peak of porphyrin accumulation. Remarkably, extracts from livers 25 weeks after HCB were just as potent towards the decarboxylase although liver porphyrin levels had declined. In a separate study uroporphyrinogen decarboxylase activity and inhibitory activity were determined at an early time-point after HCB (18 days). Both were significantly different from controls ($P < 0.001$) and equivalent to 16.7% and 14.7% inhibition respectively, i.e. decarboxylase control 37.5 ± 0.2 , HCB 31.3 ± 0.2 , inhibitor assay 31.1 ± 0.4 , HCB 26.6 ± 0.2 pmol/min per mg of protein ($n = 4$).

Light-induced inactivation

Uroporphyrinogen decarboxylase is inactivated by uroporphyrin in the presence of light (Elder *et al.*, 1986). In agreement with these findings, only 8% of decarboxylase activity remained when C57BL/10ScSn supernatant was left in buffer containing 25 μ M-uroporphyrin under lights for 2 h at 0 $^{\circ}$ C. Light alone had little effect (94%) and uroporphyrin in the dark inhibited activity to the extent previously observed (60% of controls; Smith *et al.*, 1986a). Inactivation of DBA/2 mouse hepatic decarboxylase occurred equally as well as that of C57BL/10ScSn liver. Despite these losses in enzyme activity induced by the combination of uroporphyrin and light, evidence could not be obtained for the formation of an inhibitor in this system. In addition there was no marked inhibitory activity when livers from iron-loaded C57BL/10 mice were homogenized in the presence of 125 μ M-uroporphyrin (equivalent to 500 nmol/g of tissue) and processed by heat treatment, centrifugation and SEP-PAK C_{18} cartridges, suggesting that the inhibitor was unlikely to be an artefact of processing porphyric tissue.

Genetic variation

Twelve inbred strains of mice were compared for the formation of the inhibitor and porphyria 5 weeks after a single dose of HCB (Table 1). Strains have been classified as *Ah*-responsive (C57BL/6J, C57BL/10ScSn, BALB/c, C3H/HeJ, CBA/J and A/J) and *Ah*-nonresponsive (SWR, AKR, 129, SJL, LP and DBA/2) based on the ease of inducibility by polycyclic aromatic

Table 1. Assay of liver extracts from inbred mice given HCB for the inhibitor of uroporphyrinogen decarboxylase

Male mice received Imferon (0.3 ml/25 g mouse) and 3 days later HCB (100 mg/kg; 350 μ mol/kg) in oil (10 mg/ml). After 5 weeks extracts of livers were prepared as described in the Experimental section and preincubated with untreated C57BL/10ScSn 40000 g liver supernatant for 10 min at 37 °C. Pentacarboxyporphyrinogen I (final concentration 5 μ M) was then added and the conversion to coproporphyrinogen was determined after a further 10 min incubation period (residual decarboxylase activity). Mouse strains are classified as *Ah*-responsive or nonresponsive as reviewed by Nebert *et al.* (1982). Inhibitory activity is expressed as the percentage decrease in residual decarboxylase activity compared with incubations with extracts from livers of C57BL/10ScSn which received only Imferon and oil (control). Results are means $\pm$ S.E.M. of four or eight ($\dagger$) mice per group. Significantly different from control: * P < 0.001, ** P < 0.005, *** P < 0.05.

Strain	Residual decarboxylase activity (pmol/min per mg of protein)	Inhibitory activity (% inhibition compared with control)	Liver porphyrins (nmol/g of tissue)
<i>Ah</i> -responsive			
C57BL/6J	3.5 $\pm$ 1.1*	88.9	411 $\pm$ 130***
C57BL/10ScSn	7.1 $\pm$ 1.2*	77.6	691 $\pm$ 77*
BALB/c $\dagger$	28.2 $\pm$ 0.4*	10.8	2.36 $\pm$ 0.33**
C3H/HeJ	29.8 $\pm$ 0.8	5.7	1.06 $\pm$ 0.07
CBA/J	30.1 $\pm$ 0.7	4.7	0.56 $\pm$ 0.11**
A/J	31.0 $\pm$ 0.5	1.9	0.78 $\pm$ 0.02**
<i>Ah</i> -nonresponsive			
SWR	23.7 $\pm$ 4.8	25.0	39.8 $\pm$ 19.8
AKR	24.3 $\pm$ 0.5*	23.1	4.82 $\pm$ 3.48
129	27.8 $\pm$ 1.2***	12.0	1.24 $\pm$ 0.08
SJL	30.1 $\pm$ 0.6	4.7	0.93 $\pm$ 0.10
LP	31.3 $\pm$ 0.6	0.9	0.87 $\pm$ 0.12
DBA/2	32.1 $\pm$ 0.4	0	0.62 $\pm$ 0.07**
Control C57BL/10ScSn	31.6 $\pm$ 0.5	0	1.06 $\pm$ 0.07

Table 2. Induction of ethoxyphenoxazone de-ethylase by HCB and β -naphthoflavone in the livers of inbred mice

Male mice were given a single oral dose of oil (10 ml/kg), HCB (100 mg/kg; 350 μ mol/kg) or β -naphthoflavone (100 mg/kg). Oil and β -naphthoflavone animals were killed after 2 days and those given HCB after 5 days. Ethoxyphenoxazone de-ethylase activity was estimated on hepatic 9000 g supernatants in the presence of 20 μ M-dicumarol (Lubet *et al.*, 1985) as described in the Experimental section. Results are means $\pm$ S.E.M. from four animals per group. Significantly different from controls: * P < 0.001, ** P < 0.01, *** P < 0.05.

Strain	Ethoxyphenoxazone de-ethylase (pmol/min per mg)			Induction(-fold) over controls	
	Control	HCB	β -Naphthoflavone	HCB	β -Naphthoflavone
C57BL/6J	15 $\pm$ 1	69 $\pm$ 9***	1933 $\pm$ 377**	4.6	129
C57BL/10ScSn	16 $\pm$ 1	73 $\pm$ 3*	1623 $\pm$ 50*	4.6	101
BALB/c	18 $\pm$ 1	61 $\pm$ 2*	541 $\pm$ 103**	3.4	30
C3H/HeJ	24 $\pm$ 3	69 $\pm$ 8**	229 $\pm$ 18*	2.9	9.5
CBA/J	35 $\pm$ 1	91 $\pm$ 13**	135 $\pm$ 5*	2.6	3.9
A/J	20 $\pm$ 1	52 $\pm$ 8**	131 $\pm$ 23**	2.6	6.6
SWR	31 $\pm$ 5	77 $\pm$ 15***	86 $\pm$ 10**	2.5	2.8
AKR	23 $\pm$ 2	58 $\pm$ 3*	45 $\pm$ 6***	2.5	2.0
129	28 $\pm$ 5	57 $\pm$ 5**	45 $\pm$ 7	2.0	1.6
SJL	15 $\pm$ 1	23 $\pm$ 5	47 $\pm$ 5*	1.5	3.1
LP	26 $\pm$ 4	57 $\pm$ 8**	69 $\pm$ 7**	1.4	2.7
DBA/2	20 $\pm$ 3	29 $\pm$ 3	32 $\pm$ 6	1.5	1.6

hydrocarbons of microsomal aryl hydrocarbon hydroxylase mediated by cytochrome P_1 -450, as reviewed by Nebert *et al.* (1982). The *Ah*-responsive strains C57BL/6J and C57BL/10ScSn became highly porphyrin and yielded potent inhibitory extracts. BALB/c mice on the other hand were considerably less sensitive and the remaining nominally *Ah*-responsive strains gave very little inhibitor or associated increases in liver porphyrin concentrations. No inhibitor or porphyria were detected

with the prototype *Ah*-nonresponsive strain, DBA/2, but some other *Ah*-nonresponsive mice (SWR, AKR and 129) gave signs of inhibitor formation and porphyrin accumulation greater than the *Ah*-responsive strains CBA/J and A/J.

The classification of mouse-strains as *Ah*-responsive or *Ah*-nonresponsive based on the inducibility of benzo-pyrene hydroxylase can be complicated by differences in absolute basal and induced levels of enzyme activity

Table 3. Comparison between the ability of halogenated chemicals to induce the formation of an inhibitor of uroporphyrinogen decarboxylase in the livers of C57BL/10ScSn mice

C57BL/10ScSn mice received Imferon (0.3 ml/25 g mouse) and then 3 days later halogenated chemical dissolved in oil (10 ml/kg of body weight) was given as a single oral dose. (An exception was hexabromobenzene which because of its low solubility was given in four portions over 4 days in a total volume of oil of 40 ml/kg.) Mice were left for 5 weeks and then extracts of the livers prepared as in the Experimental section. The potential of these extracts to inhibit hepatic uroporphyrinogen decarboxylase from untreated C57BL/10ScSn mice was determined as described in Table 1. Results are means $\pm$ S.E.M. from four mice per group. Significantly different from controls (oil alone): * $P < 0.001$, ** $P < 0.005$.

Chemical	Dose (μ mol/kg)	Residual decarboxylase activity (pmol/min per mg of protein)	Liver porphyrins (nmol/g of tissue)
Control	—	31.2 $\pm$ 0.5	0.86 $\pm$ 0.05
Hexafluorobenzene	1000	31.2 $\pm$ 0.5	1.33 $\pm$ 0.12
Hexachlorobenzene	350	2.3 $\pm$ 0.4*	539 $\pm$ 8*
Hexabromobenzene	350	9.7 $\pm$ 1.7*	602 $\pm$ 68*
3,4,3',4'-Tetrachlorobiphenyl	350	26.5 $\pm$ 1.9	3.15 $\pm$ 2.10
2,3,4,2',3',4'-Hexachlorobiphenyl	350	2.0 $\pm$ 0.3*	156 $\pm$ 68
2,4,5,2',4',5'-Hexachlorobiphenyl	350	24.5 $\pm$ 0.3*	1.38 $\pm$ 0.32
Aroclor 1254†	100	6.3 $\pm$ 1.3*	704 $\pm$ 29*
Firemaster BP-6†	100	3.0 $\pm$ 0.3*	226 $\pm$ 25*
TCDD	0.25	4.2 $\pm$ 0.7*	2982 $\pm$ 482**
HCB/dextran‡	350	32.8 $\pm$ 0.6	1.25 $\pm$ 0.06**

† Doses calculated on assumption that the main constituents were the hexahalogenated congeners.

‡ HCB/dextran group did not receive Imferon.

(Thomas *et al.*, 1972; Thomas & Hutton, 1973; Robinson *et al.*, 1974; Nebert *et al.*, 1982). Liver microsomal ethoxyphenoxazone de-ethylase (ethoxyresorufin de-ethylase) is another cytochrome P_1 -450-mediated hydroxylase activity which is highly induced by 3-methylcholanthrene and β -naphthoflavone in C57BL/10 mice (Burke & Mayer, 1983; Smith *et al.*, 1986b), but not in the DBA/2 strain (Lum *et al.*, 1986) and correlates with aryl hydrocarbon hydroxylase activity in Heterogeneous Stock mice (Lang *et al.*, 1981). The induction of hepatic ethoxyphenoxazone de-ethylase in the 12 mouse strains after a single oral dose of β -naphthoflavone was determined and compared with the induction observed with an equivalent dose of HCB (Table 2). All strains classified from the literature as *Ah*-responsive showed higher absolute levels and greater fold induction of ethoxyphenoxazone de-ethylase by β -naphthoflavone than did the *Ah*-nonresponsive strains. However, there was a 14-fold difference in induced activity between C57BL/6J mice and A/J mice. The propensity of *Ah*-responsive strains to inhibitor formation and to the accumulation of porphyrins associated with the induction of ethoxyphenoxazone de-ethylase by β -naphthoflavone (significant correlation coefficient $r = 0.992$, $P < 0.01$) but with *Ah*-nonresponsive strains there was no apparent correlation. At the time of assay, induction by HCB was far lower than with β -naphthoflavone and apparently showed no correlation with inhibitor formation although it was significantly greater with C57BL/6J and C57BL/10ScSn mice than with the resistant DBA/2 strain, as observed previously (Smith *et al.*, 1986b).

Comparison of polyhalogenated chemicals

A range of polyhalogenated aromatic chemicals was compared for their potential to cause the formation of the uroporphyrinogen decarboxylase inhibitor 5 weeks after a single dose (except for hexabromobenzene which

was given in four successive doses) (Table 3). TCDD was > 1400 times more active than HCB and > 400 times more than the commercial polychlorinated and polybrominated biphenyl mixtures (Aroclor 1254 and Firemaster BP-6 respectively). 2,3,4,2',3',4'-Hexachlorobiphenyl was a much more potent agent than the 2,4,5,2',4',5'-isomer (cf. Stonard & Greig, 1976) but surprisingly 3,4,3',4'-tetrachlorobiphenyl was relatively ineffective (probably due to rapid metabolism). No inhibitor was detected after HCB if mice had not received a pre-dose of iron.

DISCUSSION

The previous observations that an inhibitor of uroporphyrinogen decarboxylase is produced in livers of mice after doses of TCDD (Cantoni *et al.*, 1984) have been substantiated and extended to HCB, hexabromobenzene and polyhalogenated biphenyls. We are unable to state categorically that the formation of this inhibitor is the primary event *in vivo* for initiating uroporphyrin, although significant inhibitory activity was found within 2 weeks after the administration of HCB to C57BL/10ScSn mice and prior to the major rise in porphyrin concentrations (Fig. 2). Also the levels of this inhibitor closely paralleled the depression of decarboxylase activity reported previously (Smith *et al.*, 1986a). Just as decarboxylase activity remained depressed for many weeks, so were high levels of the inhibitor detected 6 months after HCB. Although increased liver porphyrin concentrations did not always correlate exactly with estimated inhibitor levels, this might be explained by variances in activities of other enzymes of the haem biosynthesis pathway.

Previous studies on the metabolism of HCB in rats and mice, and attempts to detect radiolabelled enzyme after administration of [14 C]HCB to C57BL/10ScSn mice, have not proved that the decarboxylase is inhibited by a

metabolite of porphyrogenic chemicals (Khanna & Smith, 1985; Stewart & Smith, 1986; Smith *et al.*, 1986a). The insolubility of the inhibitor in ethyl acetate and diethyl ether, together with its lack of retention by SEP-PAK C₁₈ cartridges, suggest that it is a relatively hydrophilic molecule, in contrast with that reported for rats (Billi *et al.*, 1986). Further studies of the nature of this inhibitor produced in mice and other methods of its formation and extraction are necessary.

In crosses between C57BL/6 and DBA/2 mice, the susceptibility to porphyria was inherited with the inducibility of hepatic aryl hydrocarbon hydroxylase activity, i.e. the *Ah*-phenotype (Jones & Sweeney, 1980). The present and previous studies on porphyria caused by TCDD and HCB demonstrate that such a classification cannot be relied on absolutely to predict the susceptibility of other strains (Smith & Francis, 1983; Greig *et al.*, 1984; Table 1). In fact, the wide range of activities of ethoxyphenoxazone de-ethylase induced by β -naphthoflavone amongst mouse strains currently considered *Ah*-responsive (Table 2) shows that the *Ah* terminology must be used cautiously. If cytochrome *P*₁-450 is involved in the porphyrogenic process, probably what may be important is not the degree of induction of aryl hydrocarbon hydroxylase (on which basis *Ah*-responsiveness is defined) but absolute levels of the cytochrome. Even so, induction of cytochrome *P*₁-450 cannot be the only criterion for initiating a porphyrogenic response, since β -naphthoflavone and other polycyclic aromatic hydrocarbons (powerful inducers of cytochrome *P*₁-450) have not so far been reported to cause porphyria in rodents (Sweeney *et al.*, 1984) whereas HCB, a much less potent inducer (Smith *et al.*, 1986b; Linko *et al.*, 1986), is a well-established porphyrogenic agent. One possibility is that polyhalogenated chemicals cause an uncoupling of the cytochrome *P*-450 reductase–cytochrome *P*₁-450 system, releasing activated oxygen to cause porphyria by an unknown mechanism (De Matteis & Stonard, 1977; Ferioli *et al.*, 1984; Sinclair *et al.*, 1984, 1986; Elder *et al.*, 1986), the effects of which are potentiated by the presence of high endogenous iron (Smith & Francis, 1983; Greig *et al.*, 1984). Polyhalogenated aromatic chemicals, rather than polycyclic aromatic hydrocarbons, may initiate porphyria because they cause long-term induction of the system but at the same time do not provide a rapid turnover of substrate. This would explain the potency of TCDD. An alternative mechanism is the interaction between the microsomal enzymes, especially cytochrome *P*₁-450, and ferritin (De Matteis & Stonard, 1977; Rowley & Sweeney, 1984; Sweeney *et al.*, 1984) to release Fe²⁺ catalysing an oxidative or other toxic sequence.

We would like to thank Dr. J. B. Greig and Dr. F. De Matteis for discussions.

REFERENCES

- Billi, S. C., Wainstok de Calmanovici, R. & San Martin de Viale, S. C. (1986) in *Hexachlorobenzene: Proceedings of an International Symposium* (Morris, C. R. & Cabral, J. R. P., eds.), IARC Scientific Publications no. 77, pp. 487–491, IARC, Lyon
- Burke, M. D. & Mayer, R. T. (1983) *Chem.-Biol. Interact.* **45**, 243–258
- Cantoni, L., Dal Fiume, D., Rizzardini, M. & Ruggieri, R. (1984) *Toxicol. Lett.* **20**, 211–217
- De Matteis, F. & Stonard, M. (1977) *Semin. Hematol.* **14**, 187–192
- de Verneuil, H., Sassa, S. & Kappas, A. (1983) *Biochem. J.* **214**, 145–151
- Elder, G. H. (1978) in *Heme and Hemoproteins* (De Matteis, F. & Aldridge, W. N., eds.), pp. 157–200, Springer-Verlag, Berlin
- Elder, G. H., Roberts, A. G. & Urquhart, A. J. (1986) *Colloq. INSERM* **134**, 147–152
- Ferioli, A., Harvey, C. & De Matteis, F. (1984) *Biochem. J.* **224**, 769–777
- Francis, J. E. & Smith, A. G. (1984) *Anal. Biochem.* **138**, 404–410
- Francis, J. E. & Smith, A. G. (1985) *Trends Anal. Chem.* **4**, 80–86
- Granick, S., Sinclair, P., Sassa, S. & Grienering, G. (1975) *J. Biol. Chem.* **250**, 9215–9225
- Greig, J. B., Francis, J. E., Kay, S. J. E., Lovell, D. P. & Smith, A. G. (1984) *Toxicol. Appl. Pharmacol.* **74**, 17–25
- Jones, K. G. & Sweeney, G. D. (1980) *Toxicol. Appl. Pharmacol.* **53**, 42–49
- Kappas, A., Sassa, S. & Anderson, K. E. (1983) in *The Metabolic Basis of Inherited Disease* (Stanbury, J. B., Wyngaarden, J. G., Frederickson, D. S., Goldstein, J. L. & Brown, M. S., eds.), 5th edn., pp. 1301–1384, McGraw-Hill, New York
- Khanna, R. N. & Smith, A. G. (1985) *Biochem. Pharmacol.* **34**, 4157–4162
- Lang, M. A., Gielen, J. E. & Nebert, D. W. (1981) *J. Biol. Chem.* **256**, 12068–12075
- Linko, P., Yeowell, H. N., Gasiewicz, T. A. & Goldstein, J. A. (1986) *J. Biochem. Toxicol.* **1**, 95–107
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Lubet, R. A., Nims, R. W., Mayer, R. T., Cameron, J. W. & Schechtman, L. M. (1985) *Mut. Res.* **142**, 127–131
- Lum, P. Y., Burke, M. D., Mayer, R. T. & Ioannides, C. (1986) *Cancer Lett.* **32**, 255–262
- Marks, G. S. (1985) *CRC Crit. Rev. Toxicol.* **15**, 151–179
- Nebert, D. W., Jensen, N. M., Shinozuka, H., Kunz, H. W. & Gill, T. J. (1982) *Genetics* **100**, 79–87
- Robinson, J. R., Considine, N. & Nebert, D. W. (1974) *J. Biol. Chem.* **249**, 5851–5859
- Rowley, B. & Sweeney, G. D. (1984) *Can. J. Biochem. Cell Biol.* **62**, 1293–1300
- Sinclair, P. R., Bement, W. J., Bonkovsky, H. L. & Sinclair, J. F. (1984) *Biochem. J.* **222**, 737–748
- Sinclair, P. R., Bement, W. J., Bonkovsky, H. L., Lambrecht, R. W., Frezza, J. E., Sinclair, J. F., Urquhart, A. J. & Elder, G. H. (1986) *Biochem. J.* **237**, 63–71
- Smith, A. G. & Francis, J. E. (1981) *Biochem. J.* **195**, 241–250
- Smith, A. G. & Francis, J. E. (1983) *Biochem. J.* **214**, 909–913
- Smith, A. G., Francis, J. E., Kay, S. J. E., Greig, J. B. & Stewart, F. P. (1986a) *Biochem. J.* **238**, 871–878
- Smith, A. G., Francis, J. E. & Bird, I. (1986b) *J. Biochem. Toxicol.* **1**, 105–117
- Stewart, F. P. & Smith, A. G. (1986) *Biochem. Pharmacol.* **35**, 2163–2170
- Stonard, M. D. & Greig, J. B. (1976) *Chem.-Biol. Interact.* **15**, 365–379
- Swain, M. G., Follows, S. B. & Marks, G. S. (1983) *Can. J. Physiol. Pharmacol.* **61**, 105–108
- Sweeney, G., Basford, D., Rowley, B. & Goddard, G. (1984) *Banbury Rep.* **18**, 225–237
- Thomas, P. E. & Hutton, J. J. (1973) *Biochem. Genet.* **8**, 249–257
- Thomas, P. E., Kouri, R. E. & Hutton, J. J. (1972) *Biochem. Genet.* **6**, 157–168