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FATE OF HEXACHLOROBENZENE IN C57BL/10 MICE WITH IRON OVERLOAD

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Abstract—The distribution of radioactivity in male C57BL/10 mice dosed with [^{14}C]hexachlorobenzene (HCB) was followed over 21 days and found to be high in adipose tissue and adrenals, moderate in thymus whereas liver was relatively poorly labelled. A predose of iron (500 mg/kg), which greatly promotes the porphyrogenic action of HCB in this strain, had only a small effect on the distribution of radioactivity in tissues and excreta. Iron induced excretion of urinary metabolites from HCB by C57BL/10 mice but not by the insensitive DBA/2 strain. However, there was no such difference in faecal metabolites, total metabolism was only slightly increased and there was no correlation between liver porphyrin levels and urinary excretion of metabolites by individual mice. At the end of 4 weeks exposure of iron-treated C57BL/10 mice to HCB urinary metabolites fell while porphyrin excretion continued to rise. Thus the considerable sensitisation of the C57BL/10 strain after iron overload to the induction of porphyria by HCB cannot be ascribed simply to enhancement of total metabolism but must be caused either by the formation of a specific undetected metabolite or induction of some other toxic process.

Hexachlorobenzene (HCB) is one of many polyhalogenated aromatic chemicals that elicit a variety of toxic responses in humans and animals [1]. Some of these chemicals cause a hepatic porphyria due to depression of the activity of uroporphyrinogen decarboxylase, an enzyme in the haem biosynthetic pathway, with massive accumulation of uroporphyrin [2, 3]. Many people were poisoned in Turkey between 1955 and 1961 after accidentally consuming HCB [4, 5], and besides hepatic porphyria other toxic effects occurred such as arthritis, thyroid enlargement and high mortality in the very young [5] with evidence for prolonged effects even after twenty-five years [6].

The rat has been the species most used for experimental studies of the toxicity of HCB [1, 2]. Mice have not been used to the same extent because of their sensitivity to the neurotoxic actions [7], although they could be extremely useful since a great deal is known of the responses and genetics of drug metabolism in this species [8]. However, recently we reported that C57BL/10 mice are sensitized to the porphyrogenic action of HCB after iron overload whereas DBA/2 mice do not respond [9]. Although the influence of iron on HCB-induced porphyria in rats has been described [10–12], results have not always been clear [13], whereas the mouse model is far more dramatic and reproducible. One explanation of the iron effect might be a stimulation of HCB metabolism. We have therefore examined the influence of iron overload on the distribution of HCB in male C57BL/10 mice and on the excretion of some of the primary metabolites [14, 15].

MATERIALS AND METHODS

Chemicals. HCB (Organic Analytical grade) was purchased from B.D.H. Chemicals Co. Ltd. (Poole, U.K.) and [^{14}C]HCB (12 mCi/mmole) from California Bionuclear Corp. (Sun Valley, CA). Sources of other chlorinated compounds were as previously described [16, 17]. Solvents (pesticide grade), Imferon (50 mg of Fe/ml) and its carbohydrate precursor dextran C were obtained from Fisons Co. Ltd. (Loughborough, U.K.).

Mice and treatments. Mice were bred on site or purchased from OLAC (1976) Ltd. (Bicester, U.K.). Iron as Imferon (0.25 ml/25 g, 500 mg/kg) or dextran C (0.2 ml/25 g of a 200 mg/ml solution) were given by s.c. injection 3 days before HCB (liver iron contents rise 20–30-fold, unpublished data). Unlabelled HCB (0.02% of a powdered 41BM diet containing 2% arachis oil) was fed to mice housed in a negative pressure isolator. [^{14}C]HCB was given to C57BL/10 mice p.o. in arachis oil (2 μCi/ml). For excretion studies mice were placed in a plastic metabolic cage on a stainless steel mesh floor directly above a 24 cm Whatman No. 1 filter paper. Urine was absorbed and faeces were left on the surface with no apparent cross contamination since faecal pellets were generally deposited in a separate area to urine without staining the paper. Mice were killed either with diethyl ether or by decapitation. Samples were stored at −40° until analysed.

Distribution of [^{14}C]*HCB.* Male C57BL/10 mice which received [^{14}C]HCB (20 μCi/kg) were killed after 1, 3, 7, 14 or 21 days. Whole or portions of tissue were weighed and solubilized in 1 ml of Beckman tissue solubilizer-450 (Beckman Ltd., High Wycombe, U.K.). Extracts were decolourized with 0.25 ml of H_2O_2 solution and mixed with 10 ml of

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Ready Solv MP liquid scintillation cocktail containing 1% acetic acid. Carcasses of 21 day mice were digested with 1 M NaOH for 3 days, neutralized with HCl and the radioactivity contents determined by liquid scintillation counting with a Searle Analytic Inc. Mark III system. For autoradiography mice were given [^{14}C]HCB (125 μCi/kg) in 10 ml arachis oil/kg and after 26 hr they were killed with CO_2 and submerged in hexane at −70°. Whole body sagittal sections 20 μ thick were cut in a Brights wholebody cryostat and exposed to Agfa Osray M film for 5 weeks.

Cumulative excretion of radioactivity from [^{14}C]-*HCB.* Mice were given [^{14}C]HCB (20 μCi/kg) and urine and faeces collected as described above every 3 or 4 days. Areas of filter papers containing urine were cut into small pieces and mixed with 50 ml of 0.5 M NaOH. Faeces were treated similarly and filtered samples of both extracts assayed. Combined recoveries of radioactivity from organs, carcass and excreta were 91–93%.

Metabolism of [^{14}C]*HCB.* Urine and faeces were collected from C57BL/10 mice for 3 days after [^{14}C]HCB (20 μCi/kg). HCB, pentachlorophenol, tetrachloro-1,4-benzenediol, pentachlorobenzenethiol and tetrachloro-1,4-benzenedithiol (0.1 mg of each) were added as carriers to 2 ml of extracts of urine or faeces prepared as above. These were hydrolysed, extracted, methylated [16] and subjected to chromatography on aluminium-backed silica gel HF_{254} t.l.c. sheets with chloroform/hexane (5:95 v/v). Metabolites were viewed under u.v. light, cut out, mixed with scintillation fluid and radioactivities determined.

Metabolism of unlabelled HCB. C57BL/10 and DBA/2 mice were fed HCB in the diet. Extracts of faeces and urine (2 ml) were hydrolysed, extracted into toluene, methylated and analysed by electron capture g.l.c. [16, 17]. Porphyrins were assayed in terms of uroporphyrin by spectrofluorimetry [18].

RESULTS

Tissue distribution, excretion and metabolism of [^{14}C]*HCB*

After [^{14}C]HCB (20 μCi/kg) the distribution of radioactivity in tissues was followed over 21 days (Table 1). Levels were highest in most tissues after 3 days but thereon steadily declined although the thymus retained label longer than other organs. Adipose tissue and adrenals contained the highest concentrations of radioactivity whereas the liver was relatively poorly labelled. Radioactivity contents of tissues from dextran-treated animals (data not shown) were approximately 5–20% lower than in those given iron. The percentage of the administered dose remaining in mice after 21 days was about 40% but only 0.5–0.8% was in the liver the remainder being mostly in skin (3–4%) or remaining carcass (33–35%). Autoradiography confirmed these results and in addition showed that the nasal region was highly labelled.

The excretion of ^{14}C in urine and faeces was also followed over 21 days (Fig. 1). In the first 3 days approximately 25% of the administered [^{14}C]HCB was excreted in the faeces, some of this excretion probably representing unabsorbed compound and was consistently 4% greater in dextran-treated mice. In contrast, urinary excretion of radioactivity was greater in those animals given iron. Half-lives for the elimination of [^{14}C]HCB from the whole body after the initial rapid loss were estimated as 29 and 32 days for dextran- and iron-treated mice respectively.

Table 1. Distribution of radioactivity in some of the tissues of male C57BL/10 mice with iron overload following a single oral administration of [^{14}C]hexachlorobenzene*

Tissue†	DAY 1	DAY 3	DAY 7	DAY 14	DAY 21
Adipose‡	396.8 ± 11.2	489.5 ± 23.4	237.7 ± 16.2	197.5 ± 21.8	129.1 ± 5.5
Adrenal	324.3 ± 11.0	338.4 ± 22.4	171.6 ± 40.7	137.8 ± 32.2	73.2 ± 17.9
Muscle§	77.6 ± 13.3	32.0 ± 10.0	11.5 ± 1.7	8.6 ± 1.8	6.2 ± 1.7
Thymus	58.3 ± 2.2	72.8 ± 6.2	40.5 ± 3.7	37.0 ± 5.9	33.3 ± 5.4
Skin	35.4 ± 4.8	45.8 ± 2.4	36.9 ± 0.6	33.8 ± 5.6	13.2 ± 1.4
Kidney	22.6 ± 2.8	32.1 ± 4.2	15.9 ± 4.6	11.0 ± 0.3	9.5 ± 0.8
Blood‖	20.1 ± 0.7	23.6 ± 2.2	22.4 ± 1.0	10.5 ± 1.0	7.2 ± 0.3
Lung	17.0 ± 0.8	24.9 ± 1.4	17.5 ± 0.5	9.4 ± 0.2	6.7 ± 0.2
Heart	14.7 ± 1.2	12.7 ± 0.3	7.0 ± 0.5	5.7 ± 0.5	4.5 ± 0.1
Stomach¶	14.4 ± 2.7	15.5 ± 0.5	7.2 ± 0.2	5.7 ± 0.5	3.8 ± 0.1
Liver	12.6 ± 0.7	15.1 ± 1.4	9.6 ± 3.1	7.1 ± 0.5	5.0 ± 0.1
Spleen	12.0 ± 0.8	14.8 ± 0.2	7.8 ± 1.8	6.0 ± 0.6	4.9 ± 0.2
Brain**	11.7 ± 1.4	13.9 ± 1.1	7.9 ± 2.4	5.3 ± 0.4	4.2 ± 0.1
Testis	10.8 ± 1.2	12.5 ± 2.5	6.4 ± 0.1	5.1 ± 0.3	4.1 ± 0.3

* Mice received [^{14}C]HCB (20 μCi/kg) 3 days after s.c. injections of iron (500 mg/kg in dextran). Results are expressed as d.p.m. × 10^{-3} per g of tissue and are means ± S.E. from 3 mice per group.

† Tissues were removed and analysed as described in Materials and Methods.

‡ Epididymal fat pad.

§ Removed from upper hind limb.

‖ In studies with Balb/c mice most of the radioactivity in blood was associated with erythrocytes (approximately 90%) as has been reported for other species [19].

¶ Analyses of various parts of the alimentary canal gave similar results to those for stomach.

** Longitudinal half of brain. No difference was observed between cerebellum and cerebrum.

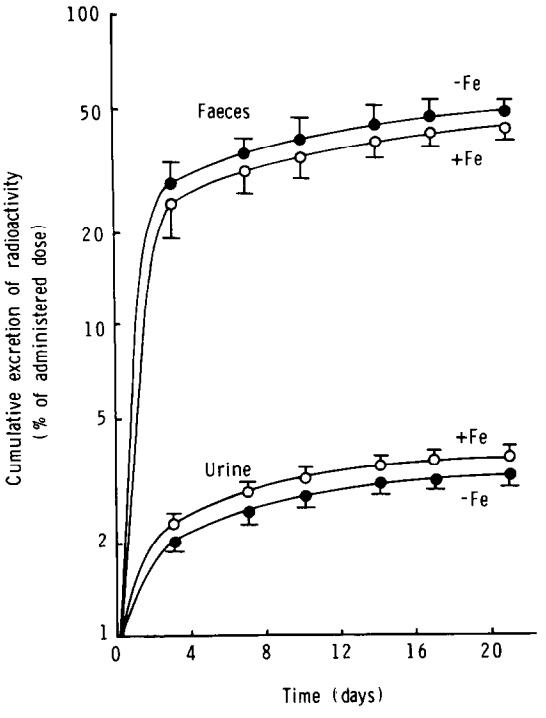

Fig. 1. Cumulative excretion of ^{14}C in urine and faeces by C57BL/10 mice. Animals were given an oral dose of [^{14}C]-HCB (20 μCi/kg) 3 days after pretreatment with iron (○) or dextran (●). Results are means ± S.E. (N = 3).

When [^{14}C]HCB was given to C57BL/10 mice (20 μCi/kg) and then the excreta collected for 3 days radioactivity associated with HCB in the urine was greater in dextran-treated mice than those which received iron (results not shown). In contrast, labelling of urinary pentachlorophenol, pentachlorobenzenethiol, tetrachlorobenzene-1,4-diol and tetrachlorobenzene-1,4-dithiol was increased after pretreatment with iron (combined excretions: iron 0.31 ± 0.06; dextran 0.19 ± 0.02% of dose administered in these metabolites ± S.E.M., N = 4) but not in the faeces (iron 0.32 ± 0.06; dextran 0.35 ± 0.05%). Similar results were obtained when mice were fed HCB for 1 week (0.02% of diet) before receiving [^{14}C]HCB (urine: iron 0.37 ± 0.03; dextran 0.17 ± 0.02; faeces: iron, 0.60 ± 0.1; dextran, 0.52 ± 0.03% of dose ± S.E.M., N = 4).

Excretion of metabolites by C57BL/10 and DBA/2 mice

Excreta from C57BL/10 and DBA/2 mice fed HCB for 5 weeks were hydrolysed and analysed for pentachlorophenol, tetrachloro-1,4-benzenediol, and the compounds derived from the mono- and dimercapturates, pentachlorobenzenethiol and tetrachloro-1,4-benzenedithiol [15, 17]. HCB was converted to these products to the same degree by C57BL/10 and the insensitive DBA/2 mice when predosed with dextran (Table 2). After iron overload the urinary levels of pentachlorobenzenethiol and tetrachlorobenzenedithiol were increased 2.5- and 3.4-fold respectively with C57BL/10 mice but this effect was not observed with the DBA/2 strain. However, combined urinary and faecal excretions of the metabolites varied little between strains or treatments. No new products were detected after iron overload, with or without prior hydrolysis, but hepatic porphyrin levels were highly elevated with iron pretreatment of C57BL/10 mice (2.89 ± 0.13 compared with 125 ± 28 nmoles/g) whereas this did not occur with DBA/2 mice (1.22 ± 0.13 and 0.94 ± 0.02 nmoles/g).

To see whether there was any correlation between liver porphyrin levels and metabolites excreted a group of 14 C57BL/10 mice predosed with iron were fed HCB for 4 weeks. Porphyrin levels in the livers ranged from 11 to 261 nmoles/g and pentachlorophenol, pentachlorobenzenethiol and tetrachloro-1,4-benzenedithiol excretion in the urine ranged from 71 to 495, 87 to 311 and 71 to 311 nmoles/24 hr per kg respectively. No correlations between hepatic

Table 2. Excretion of metabolites in urine and faeces after the dietary administration of hexachlorobenzene to male C57BL/10 and DBA/2 mice†

Strain	Iron treatment	Pentachlorophenol (nmoles/24 hr/kg)	Pentachlorobenzenethiol (nmoles/24 hr/kg)	Tetrachloro-benzenedithiol (nmoles/24 hr/kg)	Total (nmoles/24 hr/kg)
Urine					
C57BL/10	−	303 ± 28	63 ± 6	70 ± 24	436 ± 45
	+	459 ± 113	155 ± 21**	241 ± 28*	856 ± 160***
DBA/2	−	402 ± 50	53 ± 8	92 ± 10	546 ± 62
	+	307 ± 24	51 ± 6	76 ± 18	434 ± 35
Faeces					
C57BL/10	−	111 ± 6	200 ± 21	520 ± 67	833 ± 85
	+	94 ± 27	173 ± 31	331 ± 61	600 ± 109
DBA/2	−	212 ± 27	213 ± 27	491 ± 44	915 ± 85
	+	183 ± 15	229 ± 23	481 ± 31	892 ± 59

† Mice were fed HCB as 0.02% of the diet for 5 weeks. Excreta were collected and analysed after hydrolysis as described in Materials and Methods. Results are means ± S.E.M. (N = 4). The amounts of tetrachloro-1,4-benzenediol detected were small and have not been included.

Significantly different from dextran group *$P < 0.005$; **$P < 0.01$; ***$P < 0.05$. The sums of metabolites from urine and faeces were C57BL/10 dextran 1267 ± 103, iron 1456 ± 266; DBA/2 dextran 1461 ± 141, iron 1325 ± 71 nmoles/24 hr/kg with no significant differences between strains or treatments. Liver HCB contents were 586 ± 23, 934 ± 38, 810 ± 67, 571 ± 59 nmoles/g respectively.

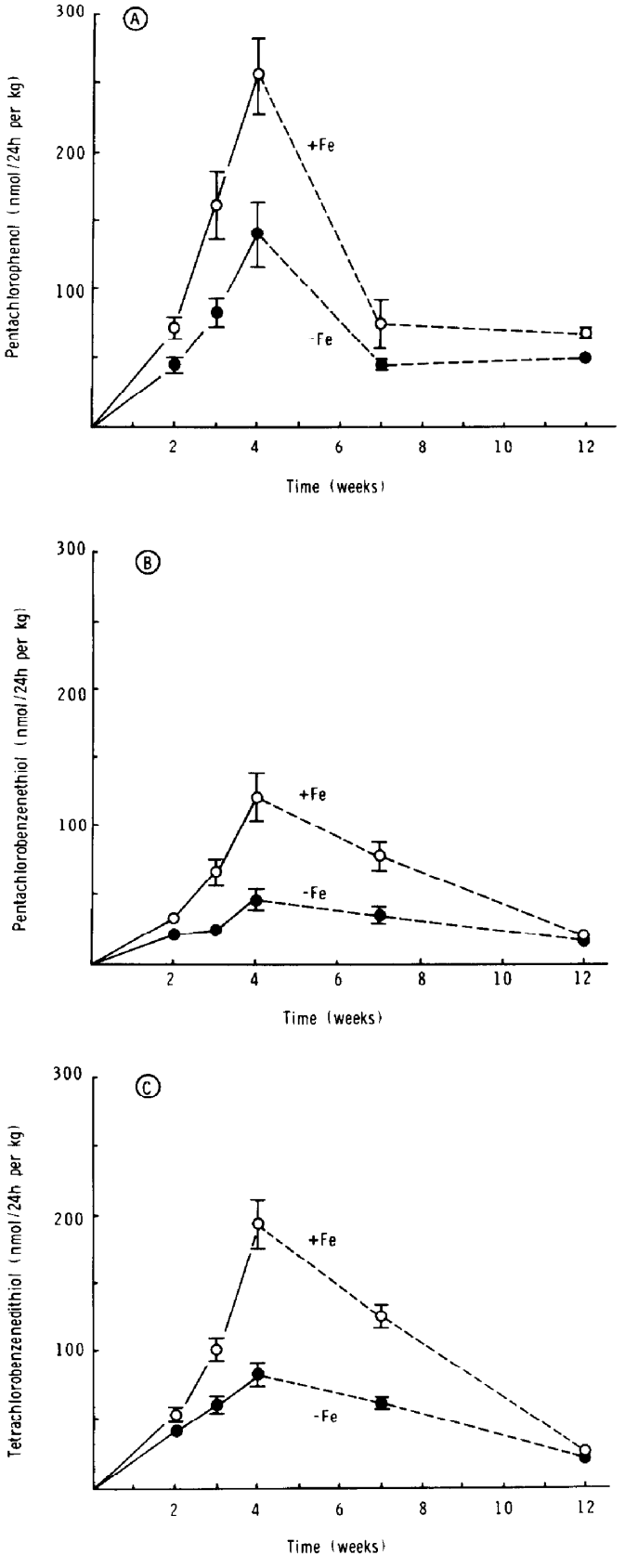

Fig. 2. (*continued*).

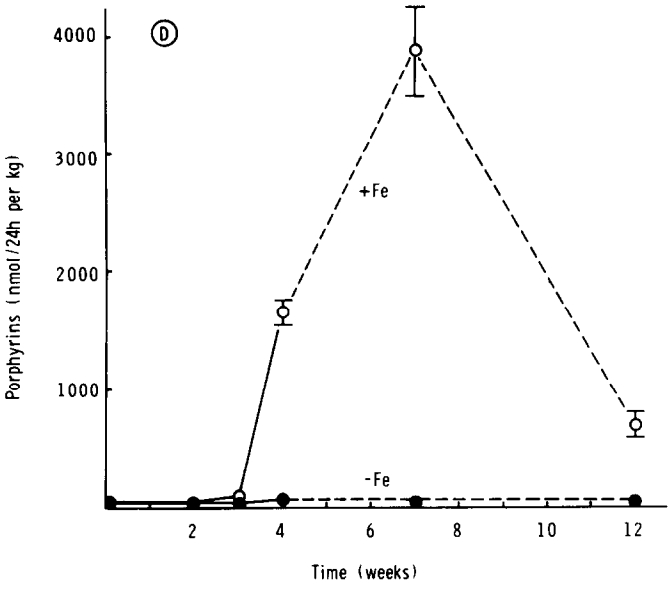

Fig. 2. Analysis of urine from iron-treated (○) and dextran-treated (●) C57BL/10 mice following a diet containing HCB (0.02%) for 4 weeks (——) and a further 8 weeks on control diet (----): (A) pentachlorophenol; (B) pentachlorobenzenthiol and (C) tetrachloro-1,4-benzenedithiol (formed by hydrolyses of the respective mercapturates); (D) porphyrins. Results are means ± S.E. (N = 4). Errors not shown were less than point size.

porphyrin concentrations and excretions of metabolites were found by linear regression analysis.

Recovery of C57BL/10 mice

In another experiment C57BL/10 mice were fed HCB for 4 weeks and the urine monitored for pentachlorophenol, pentachlorobenzenethiol and tetrachlorobenzenedithiol (Fig. 2A–C). At 4 weeks, excretion of these metabolites by iron overload animals was approximately twice that of mice which received only dextran. The mice were then fed control diet for a further 8 weeks during which time the excretion of the metabolites steadily declined. The levels of porphyrins in the urine of dextran-treated mice did not change but those which received iron excreted more than thirty times more porphyrin, the peak time being not at 4 weeks on termination of HCB administration, but surprisingly at 7 weeks when levels of metabolites had significantly declined (Fig. 2D). Even at 12 weeks (8 weeks after HCB) the urinary excretion of porphyrins in the iron group was still elevated as were levels in the liver (dextran 0.76 ± 0.02; iron 363 ± 33 nmoles/g tissue) although HCB concentrations had fallen considerably (34 ± 2 and 41 ± 8 nmoles/g respectively) and were much lower than those after 5 weeks (Table 2).

DISCUSSION

Iron overload greatly sensitises C57BL/10 mice to the hepatotoxicity of HCB as judged by an inhibition of uroporphyrinogen decarboxylase [9]. The mechanism is unknown. One aspect of the present study was designed to illustrate the tissue distribution of [^{14}C]HCB in mice, a species that has not been investigated in as much detail as rats [20–22], swine [23], quail [24], monkeys [22, 25] and dogs [26] and to determine any major changes associated with iron overload. Although radioactivity contents of tissues from dextran-treated mice were lower than those which received iron the difference does not appear to be sufficient for this to be the mechanism of iron action. At the dose used the liver was not one of the most highly labelled tissues although owing to its size it did contain a significant proportion of retained label. Interestingly, aside from adipose tissue, the adrenals represented the greatest concentration of [^{14}C]HCB relative to the other organs examined. Similar observations have been made for other species and related compounds [20, 23, 25–29] and further studies of the influence of polychlorinated aromatic chemicals on adrenal function may well prove rewarding [30, 31]. The relatively high continued labelling of the thymus (well known for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [32, 33]) may be responsible for some of the immunotoxic actions of HCB [34].

Both types of experiments which investigated the excretion of metabolites, using either [^{14}C]HCB or analysing excreta after feeding HCB, showed that in C57BL/10 mice iron can stimulate the urinary excretion of metabolites and particularly the thiols arising from hydrolysis of glutathione-derived conjugates. However, increases were not observed for faeces suggesting that at least during chronic HCB treatment iron may only be influencing the route of excretion rather than total metabolism. DBA/2 mice, which do not respond to the synergism of iron and HCB [9], showed no such changes in urinary excretion but overall metabolism was not significantly different from the C57BL/10 strain. If the effect of iron on porphyria in C57BL/10 mice is due

to increased metabolism the events induced must thus be associated with a quantitatively small route leading to intermediates or products that have not yet been detected. HCB not only induces its own metabolism but also other microsomal and cytosolic processes (F. P. Stewart and A. G. Smith, unpublished data) thus the onset of porphyria may not be directly associated with metabolism of HCB but rather to the induction of other systems in which iron can play a role. These might involve a free radical mechanism or the formation of toxic ferrous iron [35] which is known *in vitro* to inhibit mouse liver uroporphyrinogen decarboxylase [9].

Whatever the role of iron the elevated excretion of porphyrins and their continual presence in the liver 8 weeks after termination of HCB demonstrates that the symptoms of toxicity are long-lasting and do not correlate with excretion of metabolites and hepatic levels of the chemical. Similar conclusions have been made from studies with rats [36] and fit in with our previous observations that the depression of uroporphyrinogen decarboxylase in mouse liver after exposure to HCB continues even after regeneration following partial hepatectomy [37].

In conclusion, iron which greatly sensitises C57BL/10 mice to the induction of porphyria by HCB, does not greatly alter its disposition or total metabolism. Although there is evidence for increased excretion of metabolites in the urine the relationship between metabolism and the accumulation of liver porphyrins would seem to be complex and may not exist at all.

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