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HEPATIC TOXICITY AND UROPORPHYRINOGEN DECARBOXYLASE ACTIVITY FOLLOWING A SINGLE DOSE OF 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN TO MICE

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Abstract—A single, low-lethality, oral dose of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (75 μg/kg) induces an hepatic porphyria in C57BL/10 mice of either sex. The hepatic porphyrin levels are maximal 4–6 weeks after dosing and are still elevated 12 weeks after the dose. The activity of hepatic uroporphyrinogen decarboxylase is depressed within one week of the dose and this appears to precede the onset of porphyria. Mice of the DBA/2 strain show no changes of the same magnitude at doses of 1200 μg/kg, for porphyrin levels, or 75 μg/kg, for decarboxylase activity. In both strains there is an increase in hepatic iron content 3 weeks after the 75 μg/kg dose. Female C57BL/10 mice are more resistant than males to the lethal effects of TCDD.

INTRODUCTION

The human disease *porphyria cutanea tarda* (PCT)*, in which there is an accumulation of uroporphyrins, is caused by a decreased activity of one of the enzymes of haem biosynthesis, uroporphyrinogen decarboxylase, and has at least two aetiologic factors. One of these is genetic and in certain families this disease is hereditary [1]. Also, the same condition may be seen following exposure to toxicants and an episode of poisoning with HCB in Turkey was responsible for a persistent outbreak of PCT in 1955 [2]. Subsequently it was shown that feeding of HCB could cause a similar porphyria in rats [3] with an accompanying inhibition of the hepatic decarboxylase [4, 5].

Many other chlorinated organic compounds will also produce this type of porphyria in experimental animals [6, 7]. In the rat certain hexachlorobiphenyl isomers can produce the lesion following prolonged dietary administration of the compounds [8]; and multiple doses of TCDD are particularly effective in inducing the porphyric condition [9, 10]. Some strains of mice are more sensitive than others to the development of this hepatic porphyria following TCDD treatment. By giving C57BL/6 mice, a sensitive strain, a single dose of 150 μg/kg of TCDD, which corresponded to an LD_{93} dose, Goldstein and her co-workers were able to produce massively elevated hepatic porphyrin levels 3–4 weeks after treatment [11]. Also, by giving smaller doses of TCDD weekly, she was able to obtain a similar effect. Sweeney has used this latter schedule in mice to show the strain dependence of the development of porphyria and its association with suppression of uroporphyrinogen decarboxylase activity [12, 13].

In order to study both the early changes which precede the overt porphyria, and also the recovery from this condition, a model is needed in which a single dose of an agent results in a high incidence of hepatic porphyria without any undue mortality. This work has been carried out to develop and use such a model.

METHODS

Precautions. TCDD is highly toxic to many animal species [14] and is a potent chloracnegen in man. It is chemically and biologically very stable. Safety procedures appropriate to radioactive materials should be used when handling it. All carcasses, animal litter, tissues, gloves, bench covering etc. were bagged for high temperature incineration. Glassware, syringes, needles etc. were thoroughly washed with acetone. Acetone washings and surplus dosing solutions were allowed to concentrate by evaporation in a fume cupboard where they were held for subsequent destruction.

TCDD. The preparation of the TCDD used in these experiments has been described elsewhere [15]. A stock solution (100 μg/ml in arachis oil) was prepared and was diluted with oil gravimetrically to the concentrations required for dosing.

Animals. Mice of the C57BL/10 ScSn/Lac and DBA/2J/Lac strains were bred in these laboratories. One group of DBA/2 mice was obtained from Bantin & Kingman, Aldbrough, U.K. Animals had access to pellet diet MRC 41B and water at all times. TCDD was given as a single dose of the oil solution (10 ml/kg body wt) by oesophageal intubation, when the mice were 42–121 days old. Controls received oil alone. Thereafter the animals were housed in filter boxes to prevent the spread of contaminated litter.

Analysis of liver constituents. Mice were killed by

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

Table 1. 45-Day mortality of C57BL/10 mice dosed with TCDD

Dose of TCDD (μg/kg body weight)	No. of deaths/Animals treated	
	Males	Females
85	1/5	0/5
107	0/5	0/5
135	2/5	0/5
170	4/5	0/5
213	4/5	1/5
269*	-	0/4
338*	-	1/4
426*	-	1/4
536*	-	2/4

* These dosages were administered in a separate experiment.

cervical dislocation and then were decapitated and allowed to bleed out. The liver was removed, washed in ice-cold deionised water, blotted dry and weighed. Two samples of the left lateral lobe were weighed and then were digested in conc. nitric/72% perchloric acids (4:1, v/v). The residue was dissolved in 0.7% aq HCl (w/v) and the solution was analysed for metal content on a Perkin–Elmer 460 atomic absorption spectrophotometer.

The median lobe of the liver was homogenised in 0.1 M Na_2HPO_4–NaH_2PO_4 buffer (pH 6.8) containing 0.1 mM EDTA (1:9 w/v). Portions of the homogenate were then analysed for porphyrins and uroporphyrinogen decarboxylase. Porphyrins were estimated (as uroporphyrin) by fluorescence spectroscopy with a Perkin–Elmer MPF-3 instrument [16]. Uroporphyrinogen decarboxylase was assayed using uroporphyrinogen substrate (8 μM) generated from the uroporphyrin (mainly isomer III) isolated from the livers of rats made porphyric with hexachlorobenzene [17]. Incubations were performed for 1 hr under N_2 and the activity is expressed as nmoles of coproporphyrinogen formed per hr/g wet tissue.

Statistics. Probit analysis [18] was performed using a computer programme adapted from that of Davies [19]. Values are expressed as means ±1 S.E.M. or as ranges, as appropriate. The statistical significance of differences between groups was assessed either by Student's *t*-test or, when the data was not normally distributed, by Wilcoxon's two-sample rank test.

RESULTS

Sex differences in the toxicity of TCDD

Single oral doses of TCDD ranging from 85 to 213 μg/kg body weight were administered to groups

Table 2. Dose response for the porphyrogenic action of TCDD in male and female mice of the C57BL/10 and DBA/2 strains

Strain/dose of TCDD (μg/kg body weight)	N	Hepatic porphyrins (nmoles/g liver) Males	Females
C57BL/10			
0	11	0.29 (0.12–0.72)	0.21 (0.10–0.45)
5	5	0.29 (0.15–0.40)	0.30 (0.24–0.41)
15	5	0.34 (0.23–0.39)	0.38 (0.20–0.84)
50	5	17 (0.25–31)* †	1.8 (0.29–5.1) †
75	7	80 (15 – 141) †	65 (3.1 –197) †
DBA/2			
0	12	0.29 (0.24–0.35)	0.25 (0.16–0.31)
75	7	0.36 (0.20–0.46)	0.44 (0.34–0.54) †‡
150	5	0.44 (0.42–0.46) †‡	0.41 (0.27–0.46) †
300	5	0.51 (0.46–0.63) †	0.43 (0.38–0.49) †
600	6	3.0 (0.39–15) †	0.39 (0.31–0.50) †
600 §	3	0.53 (0.48–0.53) †	0.39 (0.35–0.45) †
1200	4	0.81 (0.52–1.5) †	0.34 (0.29–0.41) †

Values are the means and ranges, significance of differences from control values was assessed by Wilcoxon's two-sample rank test.

* $P < 0.05$.

† $P < 0.01$.

‡ One animal less in this group.

§ These groups were measured 5 weeks after dosing, all others were measured 3 weeks after dosing.

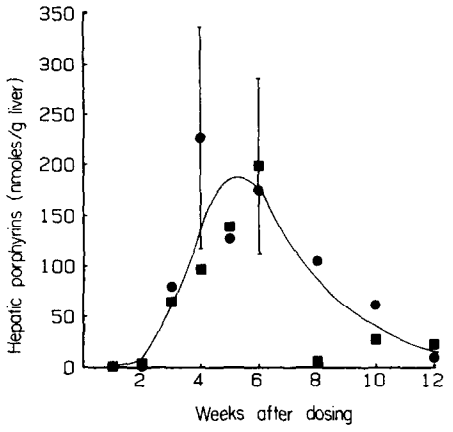

Fig. 1. Time course of the hepatic porphyria in male (■) and female (●) C57BL/10 mice following a single oral dose of TCDD (75 μg/kg). Points represent the means of groups of 3–7 animals killed after the appropriate interval. Because of the high variability of the results, illustrated by the bar representing ±1 standard error of the highest group mean in each sex, one curve has been approximated to the points.

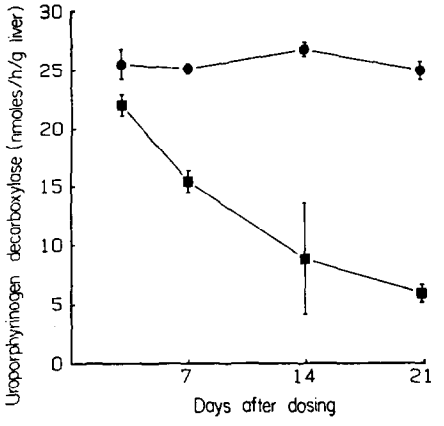

Fig. 3. Time course of the decrease in activity of hepatic uroporphyrinogen decarboxylase in male C57BL/10 mice following a single oral dose of TCDD (75 μg/kg). Values are the means ±1 S.E.M. of groups of 4 control (●) or treated (■) animals. Only the 3 day treated value was not significantly different by Student's *t*-test from that of its control group. The mean and ranges of hepatic porphyrin levels in the treated groups after 3, 7, 14 or 21 days were 0.32 (0.23–0.37), 0.41 (0.29–0.55), 28 (0.29–79), and 142 (7.5–382) nmoles/g respectively. The overall mean control level was 0.21 (0.14–0.29) nmoles/g.

of male and female C57BL/10 mice. The animals were then observed daily for 45 days. From the number of deaths occurring at each dose level probit analysis was used to estimate an LD_{50} of 146 μg/kg, with 95% confidence limits of 111–211 μg/kg, for the males. Only one female, in the highest dosage group, died. Despite the addition of higher dose groups, see Table 1, it was not possible to calculate an LD_{50} for the females. However it is clear that the LD_{50} of TCDD in female C57BL/10 mice is significantly different from that in males and is probably greater than 450 μg/kg.

Most deaths occurred between 22 and 26 days after dosing, 11/16 for both sexes combined. The remaining deaths were scattered in the period up to 38 days. In all animals found dead post-mortem revealed an enlarged liver with a roughened surface. On exposure to long wave u.v. light red fluorescence was commonly seen in the liver, gall bladder, bone and, less frequently, in the urine. Subcutaneous oedema was often observed and, in the animals dying later, was of greater extent and was associated with the finding of peritoneal and pleural exudates. Intestinal haemorrhage was a common observation.

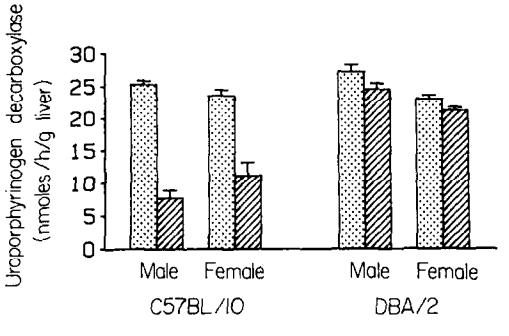

Fig. 2. Strain difference between C57BL/10 and DBA/2 mice of both sexes in the activity of hepatic uroporphyrinogen decarboxylase measured 3 weeks after a single oral dose of oil (stippled cells) or TCDD (75 μg/kg, hatched cells). Values are means +1 S.E.M. of groups of 4 animals. Only the treated C57BL/10 groups were significantly different from their controls by Student's *t*-test.

Dose response of different mice strains to TCDD

Groups of C57BL/10 and DBA/2 mice, both males and females, were given doses of TCDD in the range of 0–75 μg/kg body wt for the C57BL/10 mice, or 0–1200 μg/kg for the DBA/2 mice. After 3 weeks the animals were killed and the livers were analysed for porphyrins. The levels are shown in Table 2 and illustrate four main points. Firstly, the development of porphyria in the animals is erratic, with a large range of values in some groups, despite their coming from inbred strains. Secondly, there appears to be no sex difference in the porphyrinogenic response of the animals to TCDD. Thirdly, there is a clear difference in the response of the two strains to the induction of porphyria by a single dose of TCDD. The DBA/2 strain is at least 20 time less sensitive than the C57BL/10 strain. Finally, the lack of response of the DBA/2 strain does not appear to be due to a delay in the onset of the porphyria, as after dosing with 600 μg/kg porphyrin levels were still normal 5 weeks later.

Time course of the porphyric response of C57BL/10 mice to a single dose of TCDD

Groups of 3–7 male or female C57BL/10 mice were administered a dose of TCDD (75 μg/kg body wt), and were killed after intervals of 1–12 weeks. The levels of hepatic porphyrins were measured and are shown in Fig. 1. Despite the wide scatter of the results caused by interanimal variation it is obvious that the porphyrinogenic effect of TCDD is maximal

Table 3. Liver weights and content of Fe, Cu and Zn in C57BL/10 and DBA/2 mice following TCDD treatment

Strain & sex	Treatment* (N)	Days after dosing	Liver weight† (g/100g BW)	Metal content (μg/g liver) Fe	Cu	Zn
C57BL/10 ♂	Oil (4)	3	5.72 ± 0.26	57 ± 4	6.3 ± 0.4	28 ± 1
	TCDD (4)		5.82 ± 0.39	65 ± 7	6.6 ± 0.3	26 ± 0.6
C57BL/10 ♂	Oil (4)	7	4.88 ± 0.10	62 ± 4	6.4 ± 0.1	28 ± 0.6
	TCDD (4)		6.60 ± 0.32 §	69 ± 5	6.2 ± 0.4	24 ± 0.9 §
C57BL/10 ♂	Oil (4)	14	5.27 ± 0.17	54 ± 2	6.1 ± 0.2	26 ± 0.3
	TCDD (4)		6.27 ± 0.06 §	87 ± 7 §	5.0 ± 0.1 §	21 ± 0.5 §
C57BL/10 ♂	Oil (4)	21	5.17 ± 0.24	74 ± 14	6.4 ± 0.2	27 ± 0.4
	TCDD (4)		6.31 ± 0.08 §	103 ± 12	4.7 ± 0.1 §	22 ± 0.5 §
DBA/2 ♂	Oil (8)	21	5.22 ± 0.08	109 ± 6	5.0 ± 0.1	29 ± 0.3
	TCDD (7)		5.76 ± 0.08 §	142 ± 6 §	4.6 ± 0.1 §	31 ± 0.9
DBA/2 ♀	Oil (6)	21	5.29 ± 0.13	191 ± 5	4.1 ± 0.1	33 ± 0.8
	TCDD (5)		5.85 ± 0.28	225 ± 11 ‡	4.2 ± 0.1	30 ± 0.7

* TCDD was administered p.o. at 75 μg/kg body weight in arachis oil (10 ml/kg body wt). Oil was given at the same dosage to control mice.

† All values represent means ±S.E.M. Metal analyses were performed on duplicate samples. Significance of differences from control values was assessed by Student's *t*-test.

‡ $P < 0.05$.

§ $P < 0.01$.

in both sexes at some time between 4 and 6 weeks after dosing. In addition the porphyrin levels are still significantly elevated after 12 weeks. There is no apparent difference in the response of the sexes.

Of the 22 mice of each sex which were given this dose and were kept for more than 3 weeks only one male and one female died before the day on which they were scheduled to be killed.

Influence of TCDD on hepatic uroporphyrinogen decarboxylase in C57BL/10 and DBA/2 mice

The activity of hepatic uroporphyrinogen decarboxylase was measured in groups of male or female C57BL/10 or DBA/2 mice 3 weeks after a dose either of TCDD (75 μg/kg) or of oil. The lack of any significant effect on the DBA/2 mice of either sex and the depression of enzyme activity in C57BL/10 mice of both sexes is shown in Fig. 2. To demonstrate that this depression of the uroporphyrinogen decarboxylase was not due to inhibition by elevated levels of endogenous porphyrins [20] samples of homogenates were gently swirled with the powdered resin Zerolit FF (ip). This procedure removed more than 96% of the porphyrins but did not affect the decarboxylase activity, which remained depressed in the homogenates of porphyric livers.

The time course of the decrease in activity of the decarboxylase was investigated in groups of male C57BL/10 mice given either TCDD (75 μg/kg) or arachis oil. Animals were killed after 3, 7, 14 or 21 days and the livers analysed for the levels of uroporphyrinogen decarboxylase and porphyrins. The steady decrease with time in levels of the decarboxylase in the treated animals is shown in Fig. 3; the concomitant increases in hepatic porphyrin levels are listed in the figure legend.

To obtain an estimate of the turnover of uroporphyrinogen decarboxylase in the liver the effect of an inhibitor of protein synthesis on this enzyme was determined. The administration of cycloheximide (40 mg/kg, 20 mg/ml in saline, i.p.) had no effect on the hepatic levels of the decarboxylase in the male C57BL/10 mouse within 10 hr. The activity of the decarboxylase was 30.5 ± 1.0 nmoles/hr/g liver (N = 4) 10 hr after cycloheximide, and was not significantly different from the values of control groups, 32.2 ± 0.4 at this time or 31.4 ± 0.6 nmoles/hr/g liver at time zero. Also, the differences were not significant when the results were expressed per mg protein.

Influence of TCDD on the hepatic levels of Fe, Cu *and* Zn

Samples of the liver from animals which had been killed for estimation of hepatic porphyrins and uroporphyrinogen decarboxylase (see above) were analysed for their content of iron, copper and zinc. Additional liver samples from male and female DBA/2 mice, which had received either oil or TCDD in oil (75 μg/kg body wt), were also analysed. The results are shown in Table 3. For the C57BL/10 mice the increase in liver weight as a response to TCDD was followed by an increase in the iron concentration in the liver; levels of copper and zinc, expressed per gram of liver, were decreased. Similar changes, without the reduced zinc levels, were found in male DBA/2 mice; in female DBA/2 mice only a slight increase in the total hepatic iron content was seen.

DISCUSSION

In seeking to develop a system in which the onset of porphyria follows a single, low-lethality dose of TCDD it was natural to use strains of mice which were either responsive or non-responsive to the induction of aromatic hydrocarbon hydroxylase by TCDD and polycyclic aromatic hydrocarbons [21, 22, 23]. It has been shown [24] that the dose response of other signs of toxicity of TCDD in mice segregates with the gene or genes controlling responsiveness to

AHH induction. The C57BL/6 and DBA/2 strains, responsive and non-responsive respectively, have been the subjects of studies on the induction of porphyria using multiple doses of TCDD; the toxicology of TCDD in the former strain has been described [25–27].

Since C57BL/6 mice were not readily available, animals of the C57BL/10 subline were used instead. These lines have been derived from a common stock and both carry the Ah^b allele, giving a responsive phenotype to the induction of AHH*. They have only been found to differ at one locus, *Lv*, which controls the amount of 5-aminolaevulinate dehydratase present in liver and other tissues [29]. In the C57BL/10 line the level of this enzyme is intermediate between that of the C57BL/6 (low) and DBA/2 (high) strains. This enzyme is not rate limiting for porphyrin biosynthesis and it was felt that its level would not influence the results.

Confirmation of the similarity of the effects of TCDD in the two sublines was obtained whilst measuring the oral, single-dose LD_{50} of TCDD in male C57BL/10 mice. The value of 146 μg/kg body wt with 95% confidence limits of 111–211 μg/kg is not significantly different from values which have been determined in male C57BL/6 mice, i.e. 114 μg/kg [25] and 126 μg/kg with 95% confidence limits of 86–183 μg/kg [26]. In addition the time to death and post-mortem findings are similar in both sublines.

It was not possible to measure an LD_{50} in female C57BL/10 mice. The pattern of deaths in the different dosage groups was more variable, an effect in mice which has been described previously [30], the dose response curve was apparently less steep and these combined to suggest that the LD_{50} is much greater in females of this strain. The observation of a sex difference in the toxicity of TCDD has earlier been made in rats [31]; however, there the male was the more resistant sex. Other results in the rat study indicated that detoxification of TCDD might occur through hepatic metabolism by the mixed-function oxidases. Whereas in the rat the male has the higher basal and inducible levels of these oxidases, in the mouse the situation is reversed [32], therefore increased metabolism of TCDD might explain its lower toxicity to the female C57BL/10 mouse.

When the response of the two strains of mice to different doses of TCDD was measured in terms of the hepatic porphyrin content 3 weeks after administration of the dose the DBA/2 mice were clearly less susceptible than the C57BL/10 mice (see Table 2). There was no indication of any sex difference in either strain. These observations are in accord with those made in multiple dosing experiments [13]. A dose of 75 μg/kg to the C57BL/10 males corresponds to an approximate LD_5, it would be less than this to the females or the DBA/2 mice. Accordingly this dose was used for additional studies on the induction of porphyria.

By giving a dose of TCDD at this level it has been possible to avoid untoward lethality and to study the time course of hepatic porphyria (Fig. 1). The elevation of porphyrin levels persisted up to 12 weeks after a single dose, a period comparable to that in which elevation of hepatic enzymes such as AHH and reduced-NAD(P): menadione oxidoreductase has been reported [33]. By measuring the hepatic porphyrins it is possible to detect the porphyria at an earlier stage than is possible if urinary porphyrins are assayed [13].

Earlier work has demonstrated that there is a decreased activity of uroporphyrinogen decarboxylase associated with the induction of hepatic porphyria by TCDD [12]. This study has shown that the strain difference which was observed in the effect on this enzyme following multiple dosing [34] can also be seen when responsive and non-responsive strains receive a single dose of TCDD. Removal of excess porphyrins from liver homogenates of the mice fails to restore the activity of the decarboxylase, a similar observation has been made in hexachlorobenzene-poisoned rats [35]. This coupled with the rapid reduction in the enzyme activity which follows a single dose of TCDD and precedes major increase in porphyrin levels (Fig. 3) suggests that the lowered decarboxylase activity is responsible for the porphyria and not consequent on it. It is relevant that when the decarboxylase levels were measured in mice 8 or 12 weeks after TCDD dosage (results not presented here) those animals which had the highest hepatic porphyrin content all still had enzyme levels less than half of control values.

At present the immediate cause of the reduction of uroporphyrinogen decarboxylase activity in the C57BL/10 mice is not known. Mechanisms involving the formation of an inhibitor or altered metabolism of the enzyme are all feasible. It has been suggested [36] that prolonged saturation of high-affinity cytosolic receptor with TCDD is responsible for the various signs of toxicity. The strain difference seen with DBA/2 mice is then due to a lowered affinity of the receptor protein in this strain [23, 37]. Is there then sufficient TCDD in the livers of C57BL/10 mice 12 weeks after dosing to explain the continuing lowered activity of the enzyme?

Hepatic uptake of TCDD by C57BL/6 and DBA/2 mice has been measured over a 2 week period [38] and, following a single i.p.injection, is approximately 20% of the administered dose in both strains 14 days after dosing. An earlier study [39] recorded a hepatic content of 10% of the dose 20 days after administration to Swiss–Webster mice. It is therefore conceivable that, in the C57BL/10 strain at 12 weeks, there still exists a high enough concentration of TCDD to cause the decreased decarboxylase activity.

An alternative explanation for the prolonged action of TCDD is that the half-life of the decarboxylase enzyme in the liver is very long and, once synthesis of the enzyme has been blocked for a period, resynthesis of the enzyme and recovery of normal activity proceed only slowly. The experiment with cycloheximide indicates that the half-life of the

* The C57BL/6 strain is the prototype of the responsive phenotype, the C57BL/10 line has been shown to be responsive in the dimethylbenzanthracene skin ulceration test (Dr. J. Hilkens, personal communication to J.B.G., and *Mouse News Letter* **54**, 19 (1976) [28]). This test is believed to correlate with methods of assignment of the *Ah* phenotype [21].

enzyme is considerably greater than 1 day and does not exclude this hypothesis.

Changes of hepatic iron content have been reported as being associated with TCDD-induced porphyria [11]. Analysis of the levels of iron, copper and zinc in the livers of treated and control animals revealed an increase in total iron, not only in male C57BL/10 mice, but also in both male and female DBA/2 animals. This may not be related to the disturbances of haem metabolism but could merely be a reflection of an increase in intestinal absorption of iron which has been reported in rats and mice*. Additional data, from an experiment not reported here, showed that the basal levels of total iron in female C57BL/10 mice were higher than those of the males of this strain. It appears that there is an inverse correlation between the toxicity of TCDD and the hepatic iron content of these mice. This is at variance with studies that show a protective effect of diets low in iron on TCDD-mediated hepatotoxicity in mice and requires further investigation. The hepatic levels of copper and zinc are reduced following TCDD treatment; however, because of the increased liver weights, it is possible that this is accounted for by hepatocellular enlargement.

The observation that porphyria can be induced in C57BL/10 mice at sub-lethal doses of TCDD and also that identical doses in the resistant DBA/2 strain cause increases in hepatic iron levels suggests that these changes are not directly related to the mechanisms by which lethality is caused in mice.

In conclusion, we have shown that a long-lasting hepatic porphyria in mice, which resembles the human condition PCT, can be produced by the administration of a single, minimally-lethal dose of TCDD.

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* The data from mice is recorded in Ref. [40]. The mice used were 8-week-old males of the random-bred CF/1 strain (Dr. J. Manis, personal communication to J.B.G.).