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INDUCTION OF PORPHYRIA IN THE RAT BY CHRONIC VERSUS ACUTE EXPOSURE TO 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN

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Abstract—Chronic oral administration of 1 $\mu g \cdot kg^{-1} \cdot week^{-1}$ of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) to female rats for 16 weeks resulted in hepatic porphyria. In contrast, administration of single oral doses as high as 30 μg/kg did not produce porphyria, either acutely or 16 weeks later. Activities of hepatic drug-metabolizing enzymes [aryl hydrocarbon hydroxylase (AHH) and glucuronyl transferase] were increased by chronic oral doses of TCDD as low as 0.01 $\mu g \cdot kg^{-1} \cdot week^{-1}$. When animals were dosed with TCDD chronically and then allowed to recover for 6 months, AHH and glucuronyl transferase activities returned toward normal (98 and 86% recovery). However, animals showed only partial recovery from TCDD-induced porphyria. Hepatic porphyrin levels did decrease during this period, but urinary porphyrins and the rate-limiting enzyme in porphyrin synthesis, δ-aminolevulinic acid synthetase, remained maximally elevated during the 6-month recovery period. It is concluded that single doses of TCDD do not produce porphyria in the rat, but that TCDD is porphyrogenic when given chronically. Moreover, when TCDD administration is stopped, recovery from the porphyrogenic effects of TCDD is very slow and does not correlate with the biological half-life of TCDD in the rat.

The chlorinated dibenzodioxins and dibenzofurans are contaminants of a number of environmental chemicals and are among the most potent hepatotoxic, chloracneogenic, and teratogenic agents known [1]. The most potent of these compounds is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a contaminant of the herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). TCDD has an LD_{50} of 45 μg/kg in the female rat [2]. Administration of TCDD to rats increases the activity of several hepatic enzymes including glucuronyl transferase and enzymes associated with cytochrome P-448, including aryl hydrocarbon hydroxylase (AHH) and biphenyl 2-hydroxylase [3]. Cytochrome P-448 is a form of cytochrome P-450 which is induced by some polycyclic hydrocarbons such as 3-methylcholanthrene, and benzo[*a*]pyrene and by TCDD [4, 5]. TCDD is 30,000 times more potent than 3-methylcholanthrene as an inducer of AHH. Induction has been noted at doses as low as 0.002 μg/kg [6]. Induction of AHH is maximal 3 days after a single dose, but it is still apparent 73 days after dosing [3]. The long-lasting effects of TCDD are probably a consequence of its long half-life (31 days in the rat) [7]. TCDD does not induce mixed-function oxidases which are induced by the phenobarbital class of inducers (aminopyrine *N*-demethylase) [3].

Polychlorinated biphenyls (PCBs), chlorinated benzenes, and 2,4,5-T have been reported to produce hepatic porphyria [8–10]. The chloracne and porphyria reported in workers in a 2,4,5-T factory have been attributed to the presence of TCDD as a contaminant [11]. This possibility was strengthened by the finding that TCDD is a potent inducer of δ-aminolevulinic acid (ALA) synthetase, the rate-limiting step in heme synthesis, in the chick embryo [12]. However, a number of chemicals that are known to be porphyrogenic in the chick embryo are not porphyrogenic in mammals [13]. Woods [14] reported that administration of large doses of TCDD (5, 25 or 100 μg/kg) did not induce ALA synthetase or cause an accumulation of porphyrins in the liver as indicated by fluorescence at periods up to 30 days after oral administration to male rats. In contrast, TCDD was found to be porphyrogenic in male C57BL/6 mice given four weekly doses of 25 μg/kg of TCDD and killed 1 week after the last dose [15]. There was a 200-fold increase in uroporphyrins (7- and 8-carboxyporphyrins) in the livers of these animals. The C57BL/6 mouse is known to be more sensitive to TCDD (induction of hepatic AHH, thymic atrophy, and teratological effects) than certain other strains of mice [5, 15]. TCDD has not yet been shown to be porphyrogenic in other species. The rat is known to respond to a number of porphyrogenic chemicals including hexachlorobenzene (HCB) and PCBs [9, 16]. However, the porphyria induced by these chemicals occurs only after chronic exposure (1–4 months). Although an increase in ALA synthetase is seen in PCB-induced porphyria [16], this increase occurs after the onset of the porphyria and is not generally believed to be the primary defect in this type of porphyria. The porphyria induced by this class of chemicals has also been

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reported to persist after administration of the chemical is discontinued [9].

Since some chemical porphyrias appear to develop only after relatively long-term exposure, we examined the effects of chronic (4 months) versus acute exposure to TCDD on ALA synthetase, hepatic mixed-function oxidases, and hepatic porphyrin accumulation and excretion in rats to determine whether TCDD would produce porphyria in this species. We also examined the extent to which these variables returned to normal after a 6-month recovery period. The recovery period was selected on the basis of the half-life of TCDD (31 days) [7], to allow tissue levels to decrease by more than 90%. We were particularly interested in whether rats would develop porphyria after chronic exposure to TCDD, and whether the disease would regress after the termination of exposure.

MATERIALS AND METHODS

Animals. Fifty-six 4-week-old female Sprague–Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) were distributed randomly into seven groups (eight rats per group). Five groups of rats were dosed orally once a week for 16 weeks with 0, 0.01, 0.1, 1 or 10 μg/kg TCDD in acetone–corn oil (1:7). All animals dosed chronically with 10 $\mu g \cdot kg^{-1} \cdot week^{-1}$ of TCDD died or were killed moriband after eight to twelve doses. The remaining groups were killed 1 week after the last dose for the chronic study (dosing schedule A in Fig. 1). Two groups of rats (recovery experiment) were given 0 or 1 $\mu g \cdot kg^{-1} \cdot week^{-1}$ for 16 weeks and were allowed to recover for 6 months before being killed (dosing schedule B, Fig. 1). Urine was collected in metabolic cages for 24 hr, at 2, 12 and 24 weeks after the last dose. Five additional groups of rats (four per group) were given a single oral dose of 0, 1, 3, 10 or 30 μg/kg and killed 3 days (dosing schedule C) or 16 weeks (dosing schedule D) after dosing to test whether single oral doses would be as effective in producing porphyria as chronic doses.

Dosing solution. TCDD was dissolved in acetone and corn oil added to give a final concentration of 6 μg/ml in acetone–corn oil (1:7). This solution was diluted appropriately immediately before dosing.

Enzyme assays. ALA synthetase was assayed in whole liver homogenates [17], aminopyrine *N*-demethylase in 9000 *g* supernatant fractions [18] with the substitution of HEPES* for Tris buffer, and AHH in 9000 *g* supernatant fractions [19]. Microsomes were prepared by $CaCl_2$ precipitation [20]. *p*-Nitrophenol glucuronyl transferase was assayed in microsomes equivalent to 10 mg wet weight of liver for 5 min in the presence of 0.3% digitonin [21]. Microsomal cytochrome P-450 [22] and ethyl isocyanide (ETNC) difference spectra [23] were determined using an Aminco DW-2 spectrophotometer. Urinary ALA and porphobilinogen (PBG) were assayed immediately after collection [24]. Urine was

* HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

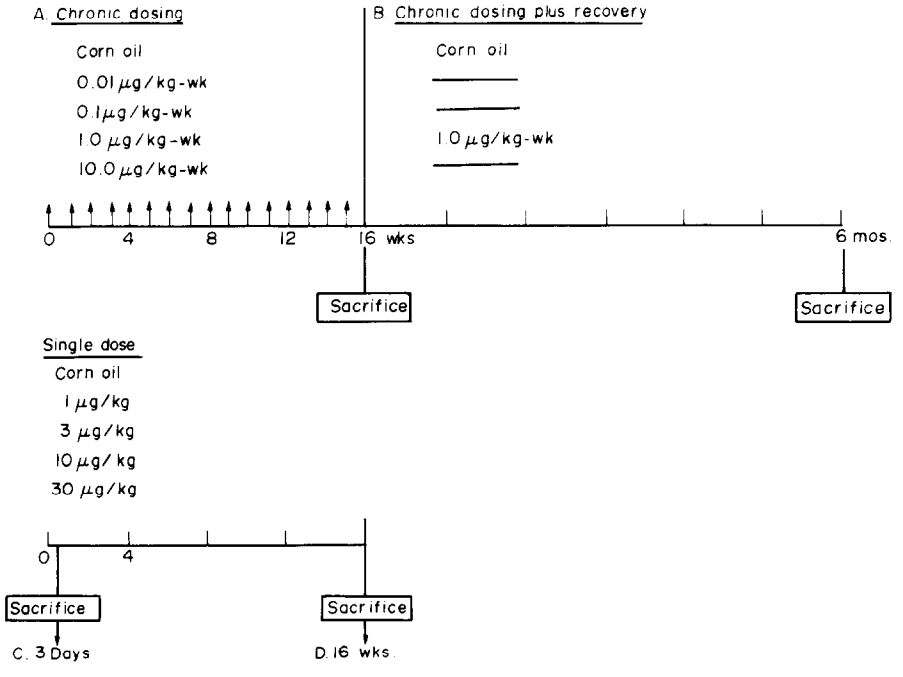

Fig. 1. Dosing schedule for TCDD treatment. Four-week-old female Sprague–Dawley rats were dosed p.o. once weekly for 16 weeks with the indicated doses of TCDD and killed 1 week after the last dose (A: chronic dosing) or allowed to recover for 6 months before sacrifice (B: chronic dosing plus recovery). A second group of rats was given a single dose of TCDD and killed 3 days (dosing schedule C) or 16 weeks (dosing schedule D) later.

frozen for 24 hr before determination of urinary porphyrins [16]. Recoveries of coproporphyrin from urine averaged 85–100%, and uroporphyrin 50–55%. Tissue porphyrins were determined using uroporphyrin as a standard [25].

RESULTS

Body weight was decreased significantly by 10 $\mu g \cdot kg^{-1} \cdot week^{-1}$ of TCDD (1 week) and 1 $\mu g \cdot kg^{-1} \cdot week^{-1}$ (13 weeks) (Fig. 2); those receiving 10 $\mu g \cdot kg^{-1} \cdot week^{-1}$ died or were moribund within 8–12 weeks. TCDD produced hepatic porphyria when administered weekly at doses of 1 μg/kg (Fig. 3). Hepatic porphyrin levels were elevated 1000-fold. Not every animal was porphyric; the incidence was 7/8 animals. At 0.1 $\mu g \cdot kg^{-1} \cdot week^{-1}$, one of eight rats had a discrete fluorescent area in the liver which contained increased amounts of porphyrins. None of the animals given 0.01 $\mu g \cdot kg^{-1} \cdot week^{-1}$ of TCDD were porphyric. Moreover, none of the animals given lethal doses of 10 $\mu g \cdot kg^{-1} \cdot week^{-1}$ were porphyric at death. In contrast to chronic exposure, single oral doses of 1–30 μg/kg of TCDD did not increase hepatic porphyrin levels 3 days or 16 weeks after dosing. This is in contrast to the fact that a chronic dose of 1 $\mu g \cdot kg^{-1} \cdot week^{-1}$ (16 μg total) was porphyrogenic. After 6 months recovery from chronic exposure to 1 $\mu g \cdot kg^{-1} \cdot week^{-1}$ of TCDD, hepatic porphyrin concentrations were still elevated 100-fold. However, these porphyrin levels were only 10% of the value observed immediately after chronic exposure.

ALA synthetase was increased 4-fold in porphyric rats given weekly doses of 1 $\mu g \cdot kg^{-1} \cdot week^{-1}$ of TCDD (Fig. 4), and 40% by 0.1 $\mu g \cdot kg^{-1} \cdot week^{-1}$. ALA synthetase was still maximally elevated 6 months after the last dose of TCDD (1 $\mu g \cdot kg^{-1} \cdot week^{-1}$). There was no significant difference between pre- and post-recovery values, even though hepatic porphyrin levels decreased 90% during the recovery period. In contrast to the effects of chronic doses, no alterations in ALA synthetase were seen 3 days after single doses. Slight changes were seen 16 weeks after a single dose, but they were not of the magnitude usually associated with porphyria.

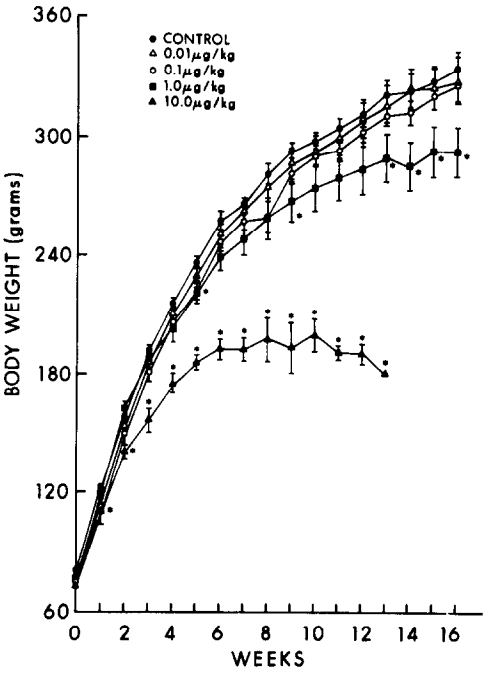

Fig. 2. Effect of chronic treatment with TCDD on body weight. Animals were dosed orally with 0, 0.01, 0.1, 1, or 10 $\mu g \cdot kg^{-1} \cdot week^{-1}$ of TCDD for 16 weeks. Values are means ± S.E. (N = 8). Key: (*) significantly less than controls ($P < 0.05$).

Figure 5 shows the urinary excretion of porphyrins during a 6-month recovery period after chronic exposure to TCDD. Chronic exposure to 1 $\mu g \cdot kg^{-1} \cdot week^{-1}$ of TCDD for 16 weeks produced a massive increase (100-fold) in the excretion of urinary uroporphyrins and a small increase in urinary coproporphyrin (6-fold). Chronic exposure to TCDD also produced a large increase in the excretion of porphobolinogen (PBG) in the urine and a lesser increase in ALA. The excretion of uropor-

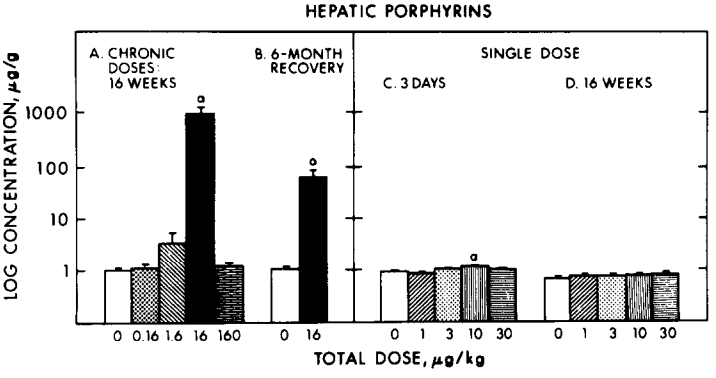

Fig. 3. Effects of acute versus chronic administration of TCDD on liver porphyrin content. Rats were dosed weekly for 16 weeks with TCDD and killed 1 week after the last dose (A) or allowed to recover for 6 months before sacrifice (B), or given single oral doses and killed 3 days (C) or 16 weeks later (D), as described in the legend of Fig. 1. The dose shown is the total cumulative dose. Values are means ± S.E. (N = 8). Key: (a) significantly greater than controls, $P < 0.05$.

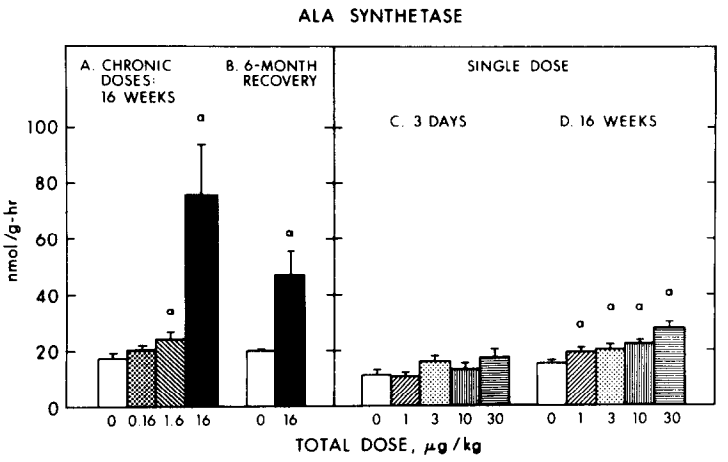

Fig. 4. ALA synthetase activity in rats treated acutely or chronically with TCDD. Animal treatment is described in the legend of Fig. 1. The dose shown is the total cumulative dose. Values are means ± S.E. (N = 8). Key: (a) significantly greater than controls, $P < 0.05$.

phyrins in the urine did not decrease appreciably over a 6-month recovery period. In fact, one rat which was not porphyric immediately after dosing became porphyric during the recovery period. On the other hand, urinary excretion of ALA and PBG decreased during the recovery period (arrows, Fig. 5).

Chronic exposure to weekly doses of 0.01, 0.1, and 1 $\mu g \cdot kg^{-1} \cdot week^{-1}$ of TCDD (total doses of 0.16, 1.6, and 16 μg/kg) produced a significant elevation in AHH (3-, 16- and 48-fold) and glucuronyl transferase activities (Fig. 6). Six months after the last dose (1 $\mu g \cdot kg^{-1} \cdot week^{-1} \times 16$), glucuronyl transferase and AHH activities were still elevated 2-fold, but recovery was 86 and 98%.

Relatively high doses of TCDD have been reported to decrease activity of aminopyrine *N*-demethylase, which is a cytochrome P-450 associated enzyme, while lower doses have no effect [3]. In the present study, chronic exposure to 0.01 to

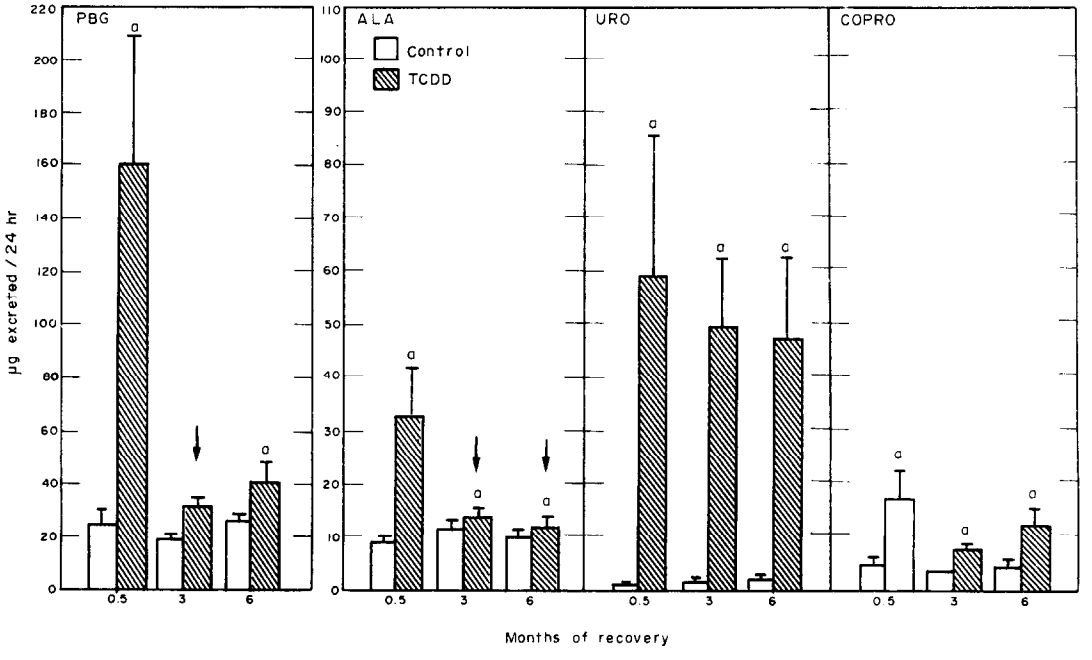

Fig. 5. Urinary excretion or porphyrins and porphyrin precursors in female rats treated chronically with TCDD (1 $\mu g \cdot kg^{-1} \cdot week^{-1} \times 16$ weeks) immediately after dosing and during a 6-month recovery period. Animal treatment (dosing schedule B) is described in the legend of Fig. 1. Values are means ± S.E. (N = 8). Arrows indicate a significant decrease compared to treated animals at 0.5 months. Key: (a) significantly greater than controls, $P < 0.05$.

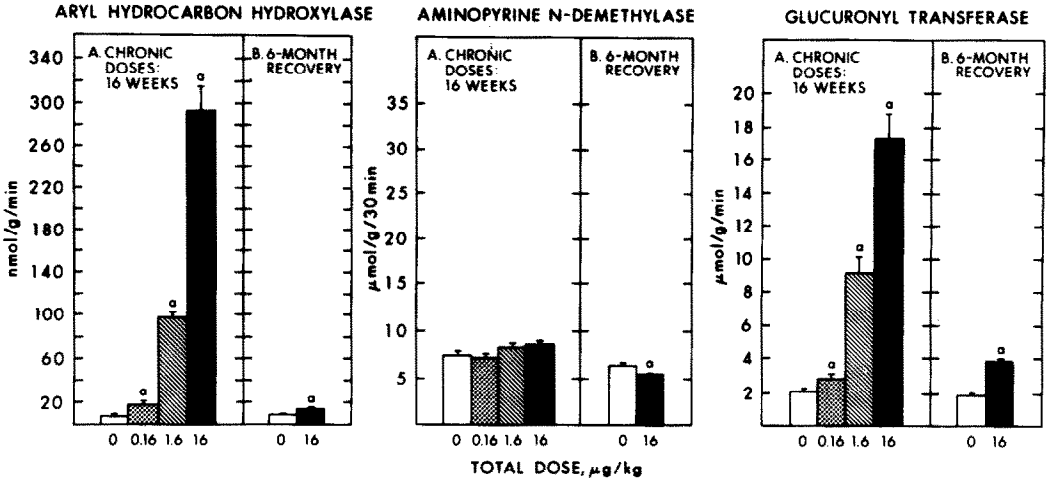

Fig. 6. Hepatic AHH aminopyrine *N*-demethylase and glucuronyl transferase activities in female rats exposed chronically (16 weeks) to TCDD before and after a 6-month recovery period. Animal treatment is described in the legend of Fig. 1 (dosing schedules A and B). Each value is the mean ± S.E. (N = 8). Key: (a) significantly greater than controls, $P < 0.05$.

$1\ \mu g \cdot kg^{-1} \cdot week^{-1}$ of TCDD did not affect this enzyme. A slight decrease was seen after 6 months recovery (Fig. 6).

Cytochrome P-450 was elevated 40% by weekly doses of 0.1 μg/kg TCDD, and 100% by $1\ \mu g \cdot kg^{-1} \cdot week^{-1}$ TCDD (Fig. 7). The peak of the CO-difference spectrum was shifted to 448 nm, indicating a preferential increase in cytochrome P-448 relative to other subspecies of cytochrome P-450. A significant decrease in cytochrome P-450 occurred after 6 months recovery from chronic dosing at the high dose. When ethyl isocyanide was used as the ligand for the reduced microsomes, the ratio of the 455/430 peaks was increased by weekly doses of 0.1 and 1 μg/kg (Fig. 7). This increase was 90% less after 6 months recovery, but was still significantly higher than controls.

DISCUSSION

The present study shows that TCDD is porphyrogenic to the rat when administered chronically. Exposure to $1\ \mu g \cdot kg^{-1} \cdot week^{-1}$ of TCDD for 16 weeks produced hepatic porphyria in seven of eight rats, and $0.1\ \mu g \cdot kg^{-1} \cdot week^{-1}$ produced porphyria in one of eight rats. These rats showed an accumulation of porphyrins in the liver and a dramatic increase in the excretion of urinary uroporphyrins. Surprisingly, chronic administration of a lethal dose of TCDD did not produce porphyria. In contrast to chronic exposure, equivalent single doses of TCDD (10 and 30 μg/kg) did not produce porphyria. This difference explains the earlier negative report by Woods [14] who studied the effects of single doses of TCDD in rats. The porphyria produced by TCDD resembles

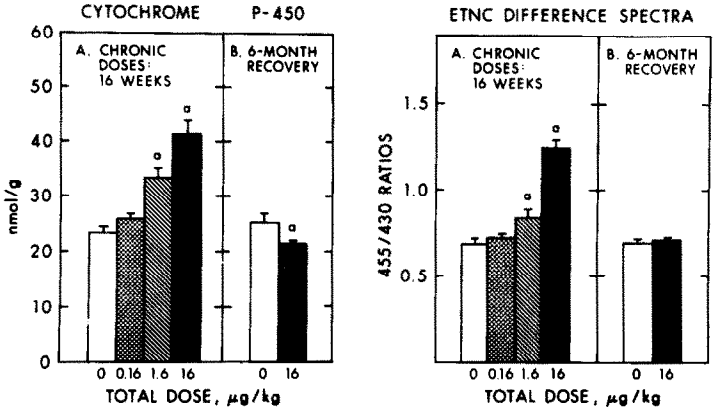

Fig. 7. Hepatic cytochrome P-450 and ETNC difference spectra of microsomes from female rats exposed chronically (16 weeks) to TCDD before and after a 6-month recovery period. Animal treatment (dosing schedules A and B) is described in the legend of Fig. 1. Values are means ± S.E. Key: (a) significantly different from controls, $P < 0.05$.

that produced by HCB or PCBs. As seen with TCDD, the porphyrins excreted in the urine of HCB or PCB-treated animals were primarily uroporphyrins [16, 20]. PCBs and HCB also produce porphyria in rats only after 2–6 months of continuous administration [6, 16]. Acute exposure to high levels of PCBs (1 week) did not produce porphyria, even though the amount of PCBs in the livers of these animals was equal to that found in porphyric animals exposed to a lower dose of PCBs for 6 months [26].

In the present study, the rate-limiting enzyme in heme synthesis (ALA synthetase) was not increased acutely by TCDD. Moreover, ALA synthetase was elevated after 16 weeks of chronic exposure only in those animals which were porphyric. We have noted that an increase in ALA synthetase activity occurs only after the onset of porphyria induced by PCBs [16]. We therefore suggested that the increase in ALA synthetase, while perhaps important in the development of the disease state, is not the primary defect. A decrease in uroporphyrinogen decarboxylase activity has been postulated to be the primary defect in porphyria induced by halogenated aromatics [27]. However, the decrease in the decarboxylase also occurs only after a lag period of several weeks administration of HCB [28].

Our data suggest that porphyria is not the result of a direct effect of TCDD on enzymes in the biosynthetic pathway for heme (via inhibition, activation or repression). Otherwise, a single high dose of this chemical should be effective. It is not clear why chronic exposure is necessary. Presumably, a high concentration of the chemical must be maintained in the liver for an extended period of time. Unfortunately, the concentrations of TCDD in the liver were not determined in the present study. However, some predictions may be made from pharmacokinetic studies [7] which have estimated the rate-constant for elimination (K_e) of TCDD in the Sprague–Dawley rat. This rate constant was similar for single and multiple daily doses. Moreover, the K_e for liver was identical to that for the whole body. Utilizing the values calculated by Rose *et al.* [7] for K_e (0.023 ± 0.06/day) and F (the fraction of the dose absorbed) (0.86), we calculate that the amount of TCDD remaining in the body of rats given a single dose of 30 μg/kg of TCDD (correcting for changes in body weight) should decrease from 26 μg/kg at day 1 to approximately 4.7 μg/kg (week 4), 1.7 μg/kg (week 8), 0.8 μg/kg (week 12) and 0.4 μg/kg (week 16). In contrast, the average weekly concentration of TCDD in the body of rats given weekly doses of 1 μg/kg is estimated to be 1.9 μg/kg (week 4), 3.1 μg/kg (week 8), 3.9 μg/kg (week 12), and 4.5 μg/kg (week 16). Therefore, the concentration of TCDD in the livers of rats given a single dose of 30 μg/kg would be predicted to fall below that in the livers of rats dosed chronically with 1 $\mu g \cdot kg^{-1} \cdot week^{-1}$ after week 6. It is not clear why the liver concentration of TCDD must remain high for several weeks in order to produce porphyria. However, studies by Sweeney *et al.* [29], which implicate iron in the pathogenesis of TCDD-induced porphyria, suggest that the alterations in enzymes in the heme biosynthetic pathway which result in porphyria may be secondary to other cumulative changes in the liver such as a progressive increase in lipid peroxides.

When animals were dosed chronically with TCDD and allowed to recover for 6 months, the amount of uroporphyrins excreted in the urine remained maximally elevated throughout the 6-month recovery period. Hepatic porphyrin levels decreased appreciably during this period (90%), but they were still elevated 100-fold over control levels. Conceivably, the animals might have recovered fully if the recovery period had been extended longer. However, ALA synthetase remained maximally elevated during the 6-month recovery period. For this reason, it is uncertain whether complete recovery would take place. In contrast to ALA synthetase, activities of mixed-function oxidases returned toward normal during the 6-month recovery period (90–100% recovery). One would predict from the half-life of TCDD [7] that the amount of TCDD in the liver should decrease by approximately 98% during a 6-month recovery period. The recovery of the mixed-function oxidases was consistent with this prediction. The fact that ALA synthetase remained elevated while other enzymes recovered suggests that the elevation did not depend on the amount of TCDD in the liver. Perhaps the alteration in amounts of intermediates in the heme biosynthetic pathway resulted in the sustained induction of ALA synthetase.

Subsequent to a preliminary report of the present study [30], Cantoni *et al.* [31] dosed female rats identically with 0.01, 0.1 and 1 $\mu g \cdot kg^{-1} \cdot week^{-1}$ of TCDD for 10 months and also found uroporphyrinuria at a dose of 1 $\mu g \cdot kg^{-1} \cdot week^{-1}$. In their study, urinary porphyrins were determined at several time points. Small changes in coproporphyrin excretion were seen at 3 months at all doses. However, dramatic increases in urinary porphyrins indicative of porphyria were not seen until 6 months and then only at the high dose. At this time, the porphyrins found in the urine were predominantly uroporphyrins. Kociba *et al.* [32] did not find a similar increase in porphyrins in the urine of rats dosed with 1.0 $\mu g \cdot kg^{-1} \cdot day^{-1}$ of TCDD 5 days/week for 13 weeks. The time period may have been too short. Surprisingly, they also did not find porphyria in rats fed a dose of 0.1 $\mu g \cdot kg^{-1} \cdot day^{-1}$ of TCDD for 4, 12, or 23 months [33]. The reason for the negative findings in their study is now clear. However, urines were analyzed for porphyrins in another laboratory. In our study, urine samples were analyzed almost immediately, and recoveries of uroporphyrin and coproporphyrin from urine were determined. We found that uroporphyrin recoveries were quite variable depending on the techniques utilized (unpublished data).

Kociba *et al.* [32] reported morphological changes in the liver and thymus of animals given 0.1 and 1.0 μg/kg TCDD/day, 5 days/week for 13 weeks. In the present study, the increases in AHH, glucuronyl transferase, and cytochrome P-448 in livers of animals treated with total chronic doses as low as 0.16 μg/kg TCDD over 16 weeks were compatible with increases reported after single doses of 0.2 μg/kg [6] and indicate that the effects were cumulative within this time period as would be expected from the 31-day half-life of this compound [7]. The enzy-

matic changes produced by TCDD appeared to be the most sensitive indicator of exposure.

Porphyria curtanea tarda (PCT) has been found in factory workers exposed to 2,4,5-T contaminated by TCDD [10, 11] and in people in Turkey who accidentally ingested hexachlorobenzene [9]. This type of porphyria is characterized by massive excretion of uroporphyrins in the urine. A relatively large number of people were recently exposed to TCDD as the result of an accident in a chemical plant near Seveso, Italy. Minor alterations in the pattern of porphyrins in urine samples of some of these people have been reported [34]; apparently large increases in porphyrin excretion such as those seen after the accidental ingestion of hexachlorobenzene [9] have not yet been reported. However, this study indicates that this type of chemically induced porphyria occurs only after chronic exposure, at least in some species. A requirement for chronic exposure could explain the relatively large incidence of porphyria in factory workers exposed chronically to 2,4,5-T [10, 11] and the absence of this disease after the relatively brief exposure to TCDD at Seveso [34].

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