# Synergistic Effect of 2,2',4,4',5,5'-Hexachlorobiphenyl and 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin on Hepatic Porphyrin Levels in the Rat

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We studied the effect of polychlorinated biphenyls (PCBs) on hepatic porphyrin accumulation in female Sprague-Dawley rats by feeding them diets containing 2,3,7,8-tetrachlorodibenzo-pdioxin (TCDD), 2,2',4,4',5,5'-hexachlorobiphenyl (PCB 153), 2,3,3',4,4',5-hexachlorobiphenyl (PCB 156), 3,3',4,4',5-pentachlorobiphenyl (PCB 126), or combinations of the single PCB congeners with TCDD for 13 weeks. A dose-dependent increase in hepatic porphyrin accumulation occurred after TCDD, PCB 126, or PCB 156 administration, reaching maximal levels of about twice control values. The lowest dose levels for which a significant increase in hepatic porphyrin accumulation was found were 0.7 µg TCDD/kg diet, 50 µg PCB 126/kg diet, or 6 mg PCB 156/kg diet. These doses are equivalent to 47 ng TCDD/kg/day, 3.2 µg PCB 126/kg/day, and 365 µg PCB 156/kg/day. Relative potencies for hepatic porphyrin accumulation, using TCDD as a reference, ranged from 0.015 to 0.06 for PCB 126 and from 0.0001 to 0.0003 for PCB 156. CYP1A2 activities significantly correlated with hepatic porphyrin levels, with coefficients of 0.629, 0.483, or 0.808 for TCDD, PCB 126, or PCB 156, respectively. Administration of PCB 153 alone did not result in hepatic porphyrin accumulation. Co-administration of PCB 153 and TCDD revealed a strong synergistic effect on porphyrin accumulation (about 800 times control levels). This synergistic effect was significant in rats fed diets containing any combination of PCB 153 with TCDD. Uroporphyrin III and heptacarboxylic porphyrin were accumulated in porphyrinogenic livers. These results suggest that TCDD induction of CYP1A2 may be involved, leading to oxidation of uroporphyrinogen III to uroporphyrin III, in combination with an increase in  $\delta$ -aminolevulinic acid synthetase induced by PCB 153. Under porphyrinogenic conditions, an inhibitor of CYP1A2 activity may also be formed. The interactive effects on porphyrin accumulation after co-administration of dioxinlike and non-dioxinlike compounds may have significant implications for the risk assessment of these chemicals. Key words: Ah-receptor, chemical synergism, polychlorinated biphenyl, porphyria, porphyrins, 2,3,7,8-tetrachlorodibenzo-p-dioxin. Environ Health Perspect 104:550-557 (1996)

The porphyrias are a group of clinical symptoms associated with inherited or induced disturbances in heme biosynthesis. Porphyria cutanea tarda (PCT), the most common hepatic porphyria, occurs in humans with an inherited deficiency of hepatic uroporphyrinogen decarboxylase (UROD), an enzyme involved in the decarboxylation of uroporphyrinogen to coproporphyrinogen. The excess of hepatic uroporphyrinogen resulting from this deficiency is eventually excreted in the urine as uroporphyrin. However, alcohol ingestion is a common precipitating cause (1-3).

Following a disastrous hexachlorobenzene (HCB) poisoning in southeastern Turkey in the 1960s, victims displayed signs of disturbed heme synthesis, resulting in massive urinary excretion of porphyrins and hepatic accumulation of porphyrins, consistent with PCT (4,5). PCT was also reported in industrial workers producing the herbicides 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid (6,7). 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) was found to be a contaminant in these herbicides. TCDD is a potent porphyrinogenic agent in rats and mice (8-10). The Seveso accident in Italy involving human exposure to TCDD revealed an increase in urinary coproporphyrin (11). Human exposure to a complex mixture of polychlorinated dibenzofurans (PCDFs) and biphenyls (PCBs) in the Taiwanese Yu-Cheng poisoning resulted in elevated urinary excretion of uroporphyrin and aminolevulinic acid (ALA), both precursors of heme synthesis (12).

Porphyrin accumulation has also been observed under field conditions and in experimental animals. Herring gulls (*Larus argentatus*) from colonies throughout the Great Lakes showed elevated hepatic porphyrin concentrations in comparison to colonies from coastal areas (13). The Great Lakes have been associated with a high contamination of polyhalogenated aromatic hydrocarbons such as polychlorinated dibenzo-p-dioxins (PCDDs), PCDFs, and PCBs.

In laboratory animals, a clear induction of PCT-like signs, i.e., accumulation of hepatic uroporphyrinogen and heptacarboxylic porphyrin, induction of  $\delta$ -aminole-

vulinic acid synthetase (ALAS) activity, and inhibition of UROD activity, have been observed after exposure to TCDD and related compounds. Jones and Sweeney (14) studied the porphyrinogenic action of TCDD in genetically responsive C57BL/6 and nonresponsive DBA mice. Susceptibility to porphyria correlated with aryl hydrocarbon hydroxylase (AHH) inducibility, indicating that the aryl hydrocarbon (Ah) receptor may be involved in this response. In addition, chronic exposure of rats to TCDD caused hepatic porphyria, which could not be established after acute exposure to TCDD (9). Accumulation of hepatic porphyrins in mice during a 10-week feeding study was also found after exposure to 3,3',4,4',5,5'hexachlorobiphenyl (PCB 169) or Kanechlor-500 administration (15). After 3 weeks of dietary exposure, porphyrins were manifest, ALAS activity was increased, and UROD activity was decreased.

In spite of a broad range of adverse human health effects observed after highlevel exposure (16, 17), the major public concern involves the protection of the general population against these compounds due to background exposure. The worldwide occurrence of PCDDs, PCDFs, and PCBs has led to concern and a need for risk assessment with regard to the general population. This need is strengthened by the adverse effects on various (neuro)developmental parameters at low-level, background exposure (18-21).

The mechanism of action of TCDD and related compounds involves an initial

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binding to the Ah receptor (22,23). A clear correlation between the Ah receptor-mediated biochemical and toxic effects was observed in a number of laboratory studies (16,17). As a result, quantitative structure-activity relationships were obtained, subsequently leading to the development of the toxic equivalency concept for this group of compounds. In this concept, each compound is assigned a toxic equivalency factor (TEF) that reflects its relative toxic and biochemical potency to TCDD, the most potent compound, assigned a TEF value of 1. A prerequisite for the TEF concept is additive toxicity, which is supported by in vivo studies with mixtures of PCDDs and PCDFs (24), mixtures of PCDFs (25), mixtures of TCDD and PCBs (26,27), and by in vitro studies with mixtures of PCBs and PCDFs (28). The Ah receptormediated toxic potency of mixtures of PCDDs, PCDFs, and PCBs is therefore calculated by the summation of the toxic equivalents (TEQs), i.e., the product of the concentration and the TEF value of each individual congener.

However, antagonistic as well as synergistic effects have been found after co-administration of binary mixtures in single-dose, short-term experiments in rodents using hepatic cytochrome P450 isozymes, immunotoxicity, and teratogenicity as endpoints (29-33). After chronic exposure to 2,2',4,4',5,5'- and 3,3',4, 4',5,5'-hexabromobiphenyl in rats, Jensen and Sleight (34) found synergism in hepatic tumor promotion.

By using interim TEF values (17,35,36), some co-planar and monoortho-substituted PCBs account for a significant contribution to the total TEQ in biotic samples (37-39). On this basis, and as a consequence of the nonadditive effects reported, toxicity studies involving mixture exposure using PCBs are important for risk assessment. For the PCB congeners, relevant PCBs were chosen from each PCB group (di-ortho-substituted, mono-orthosubstituted, and co-planar PCBs). This choice was based on high relative toxicity and concentration in human milk and fat tissue (40-42).

Additionally, a subchronic dosing regime was chosen in view of extrapolation for risk assessment of these compounds. Since risk assessment based on intake dose does not involve kinetic aspects of the compound to be evaluated, extrapolation based on intake dose will fail when large kinetic differences exist between species (43,44).

In the present study, subchronic effects on hepatic porphyrin accumulation were studied in rats fed diets containing 2,2',4, 4',5,5'-hexachlorobiphenyl (PCB 153), 2,3,3',4,4',5-hexachlorobiphenyl (PCB 156), 3,3',4,4',5-pentachlorobiphenyl (PCB 126), or TCDD. At the same time, the single PCB congeners were co-administered with TCDD to study possible interactive effects. The concentration ratios used were comparable with those found in human milk and fat samples.

# **Materials and Methods**

Chemicals. TCDD (purity 99%) was synthesized by Dow Chemical (Midland, Michigan). PCB 126 (purity 99%) as used in the subchronic study was obtained from Schmidt B.V. (Amsterdam). PCB 153 (purity >99.9%) as used in the subchronic study was synthesized according to Hutzinger and Safe (45) as described earlier (46). PCB 126 and PCB 153 (purities >98%) as used in the in vitro inhibition experiment were from Ultra Scientific. PCB 156 was synthesized according to Mullin and co-workers (47) as previously described (26). Uroporphyrinogen I, coproporphyrin III, (copro III), and a marker kit containing 2-, 4-, 5-, 6-, 7-, and 8-carboxyl porphyrin isomers I were obtained from Porphyrin Products Inc. (Logan, Utah). Acetanilide, 4-hydroxyacetanilide, 3-hydroxyacetanilide, and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, Missouri). Methoxyresorufin was purchased from Molecular Probes Inc. (Eugene, Oregon). The other chemicals used were of analytical grade and were obtained from Merck AG (Darmstadt, Germany), BDH Chemicals Ltd. (Poole, Dorset, UK), or Sigma.

Animals and treatment. Female Sprague-Dawley rats [Iva: S/V 50 (SD)], Ivanovas (Kissley, Germany), 7 weeks old, weighing about 150 g, were kept on a standard laboratory diet (Nafag 890, Gossau, Switzerland) for 1 week before the experiment. One day before the start of the 13week feeding experiments, the rats were randomly divided into groups of eight (first experiment) or nine (second experiment) animals with a similar mean and standard deviation in body weights. The diets, in pulverized form, were prepared according to Pluess and co-workers (24) and contained PCB 153, PCB 156, PCB 126, TCDD, or combinations of the PCBs with TCDD (Tables 1 and 2). Water and food were given ad libitum. The rats were housed three or four per cage and held under controlled conditions of temperature (20°C) and lighting (12-hr light/dark cycle).

After termination of the experiments, the animals were killed under diethylether anesthesia by taking blood of the inferior vena cava. The liver was removed, rinsed in physiological saline solution, and weighed. Parts of the liver were frozen in liquid nitrogen and stored at -70°C until cytochrome

**Table 1.** Dose groups, daily doses of PCB 156, PCB 126, and/or TCDD, and hepatic porphyrin levels after 13 weeks on diets containing PCB 126, PCB 156, and/or TCDD (means  $\pm$  SE, n = 8)

	Amount in diet	t		Daily dose <sup>a</sup>		
TCDD (µg/kg)	PCB 126 (μg/kg)	PCB 156 (mg/kg)	TCDD (ng/kg/day)	PCB 126 (µg/kg/day)	PCB 156 (µg/kg/day)	Porphyrins (µg/g liver)
0	0	0	0	0	0	$0.494 \pm 0.059^{b}$
0.2	0	0	14	0	0	0.494 ± 0.039
0.4	0	0	26	0	0	0.686 ± 0.066
0.7	0	0	47	0	0	0.827 ± 0.073*
5	0	0	320	0	0	0.954 ± 0.053*
20	0	0	1024	0	0	0.889 ± 0.059*
0	7	0	0	0.47	0	0.764 ± 0.117
0	50	0	0	3.18	0	0.853 ± 0.086*
0	180	0	0	10.1	0	0.841 ± 0.075*
0.4	<u>`</u> 7	0	27	0.48	0	0.694 ± 0.061
0.4	50	0	26	3.25	0	1.05 ± 0.10**
0.4	180	0	23	10.4	0	1.01 ± 0.06 <sup>c</sup> **
5	7	0	315	0.44	0	0.899 ± 0.062
5	50	0	306	3.06	0	1.12 ± 0.10
5	180	0	269	9.68	0	0.913 ± 0.037
0	0	1.2	0	0	81	0.607 ± 0.061
0	0	6	0	0	365	0.956 ± 0.098 <sup>b*</sup>
0	0	12	0	0	729	1.17 ± 0.11*
5	0	1.2	317	0	76	1.14 ± 0.09 <sup>†</sup>
5	0	6	305	0	366	1.19 ± 0.08
5	0	12	290	0	696	1.18 ± 0.15 <sup>b</sup>

<sup>a</sup>Daily doses are estimated average values based on estimated food consumption, dietary level of compound, and average body weight over the 13-week feeding period.

<sup>b</sup>n = 7.

<sup>c</sup>n = 6.

\*Significant from control (least significant difference test, p <0.05).

\*\*Significant from 0.4  $\mu$ g TCDD/kg alone (least significant difference test, p <0.05).

<sup>†</sup>Significant from 1.2 mg PCB 156/kg alone (least significant difference test, *p* < 0.05).

Table 2	2. Dose groups,	daily doses	of PCB	153 and/or	TCDD,	and hepatic	porphyrin	levels after	· 13 weeks
on diet	s containing PC	B 153 and/or	TCDD (I	means ± SE	E, <i>n</i> = 9)				

Amount in diet		Daily		
TCDD (µg/kg)	PCB 153 (mg/kg)	TCDD (ng/kg/day)	PCB 153 (mg/kg/day)	Porphyrins (µg/g liver)
0	0	0	0	1.9 ± 0.5
0	10	0	0.72	2.8 ± 1.6
0	30	0	2.07	1.4 ± 0.3 <sup>b</sup>
0	100	0	6.61	3.0 ± 0.9
0.5	0	33.4	0	1.9 ± 0.5
0.5	10	33.9	0.68	300 ± 203*
0.5	30	32.6	1.95	969 ± 270*
0.5	100	32.0	6.40	1223 ± 326*
5	0	320	0	2.5 ± 0.3
5	10	318	0.64	22.0 ± 6.4*
5	30	301	1.81	1527 ± 480*
5	100	293	5.85	1094 ± 393*

<sup>e</sup>Estimated average value based on estimated food consumption, dietary level of compound, and average body weight over the 13-week feeding period.

<sup>b</sup>n = 8.

\*Significant from PCB 153 or TCDD at corresponding dose alone (least significant difference test, p <0.05).

P450 activity measurements, or at -20°C until porphyrin analyses. The same part of the liver was used for the same type of analysis for all animals.

Determination of porphyrins. Total hepatic porphyrin content was determined according to the method of Schwartz et al. (48), as modified by Debets et al. (49), using an acid-ethanol solution of chloranil (2,3,5,6-tetrachloro-1,4-benzoquinone) and 100-µl samples of whole total-liver homogenate, prepared as described earlier (26). Fluorescence was measured in 96well multiplates at  $\lambda_{ex}$  409 nm and  $\lambda_{em}$ 645 nm using a cytofluor multiwell plate reader (Millipore B.V., Etten-Leur, the Netherlands) and coproporphyrin III as a standard.

The pattern of porphyrins accumulated in the livers were analyzed as described earlier (50), after a porphyrin extraction-step of liver homogenates (51) using reversephase HPLC (LKB-Produkter, Bromma, Sweden).

Cytochrome P450 measurements. Liver microsomes were prepared according to the method of Burke and Mayer (52). Microsomal CYP1A2 activities in the first experiment (PCB 126/PCB 156/TCDD) were measured by the 4-hydroxylation of acetanilide (4-OH-AA) according to Liu et al. (53) as described earlier (26). Microsomal CYP1A2 activities in the second experiment (PCB 153/TCDD) were measured by the demethylation of methoxyresorufin (MROD), using the same protocol as previously described for ethoxyrosorufin-O-deethylase (54). The substrate concentration used was 1.5 nM methoxyresorufin. Western blot analysis of CYP1A2 protein was determined in selected samples of the second experiment as described previously (55), with the exception that data are expressed as optical densities/ $\mu$ g protein. Protein levels were spectrophotometrically measured according to Bradford (56) by using a Bio-Rad Model 3550 microplate reader and BSA as a standard.

In vitro inhibition of CYP1A2 activity. Liver microsomes of rats treated with 100 mg PCB 153/kg diet in co-administration with 5 µg TCDD/kg diet were used for the in vitro inhibition of CYP1A2 measurement using MROD as a marker. To test the effect of substrate on possible competition with an unknown binding inhibitor in vitro, the concentration of methoxyresorufin ranged from 1.5 nM to 30 nM using microsomes of a responder and a nonresponder. To test the effect of PCB 126 and PCB 153 on in vitro inhibition of CYP1A2 activity, the methoxyresorufin concentration used was 1.5 nM. PCB 153 or PCB 126 was dissolved in dimethyl sulfoxide (DMSO) to a final concentration ranging from 1.3 ng/ml to 13 µg/ml. Samples were preincubated with 6 µl DMSO or 6 µl of the tested PCB in DMSO for 4 min. Reaction was started with NADPH and followed for 2-3 min. All measurements were performed in duplicate. Data are expressed as a percentage of CYP1A2 activity using 6 µl of DMSO.

Statistics. Data were analyzed for differences from controls with one-way analysis of variance and the least significant difference test (p < 0.05 for groups exposed to one compound). Groups treated with mixtures of two compounds were compared to the corresponding compounds alone. Twoway analysis of variance was used to determine possible interactive effects (p < 0.05). Correlations between CYP1A2 activities and hepatic porphyrin levels were examined by using Student's *t*-test (p < 0.05).

## Results

Total hepatic porphyrin levels are summarized in Table 1 for groups fed diets containing PCB 126 or PCB 156, with or without co-administration of TCDD (first experiment). Table 2 shows the hepatic porphyrin levels in rats fed on diets containing PCB 153 and/or TCDD (second experiment).

TCDD showed a dose-dependent increase in hepatic porphyrin levels after 13 weeks of dietary exposure in the first experiment. This increase was also found for PCB 126 or PCB 156, reaching maximum levels about twice the control values (Table 1). However, it should be noted that the control values of the first experiment were lower than the control values of the second experiment. The lowest dietary dose levels for which a significant increase in hepatic porphyrin accumulation was found were 0.7 µg TCDD/kg, 50 µg PCB 126/kg, and 6 mg PCB 156/kg. In addition, co-administration of 50 or 180 µg PCB 126/kg and 0.4 µg TCDD/kg resulted in a significant increase in porphyrin accumulation as compared to TCDD alone. Similarly, coadministration of 1.2 mg PCB 156/kg and 5 µg TCDD/kg in the diet resulted in an additional accumulation (1.9-fold) compared to 1.2 mg PCB 156 alone.

In the second experiment, both PCB 153 and TCDD, when administered alone, did not alter hepatic porphyrin levels compared to controls up to levels of 100 mg PCB 153/kg or 5 µg TCDD/kg, respectively. Co-administration of any dose of PCB 153 and TCDD resulted in a strong accumulation of hepatic porphyrins, which was statistically different from control groups and from the corresponding PCB or TCDD group alone. The highest hepatic porphyrin accumulation was 1500 µg/g liver (i.e., about 800 times control levels). At the highest dose groups (i.e., 100 mg PCB 153/kg in co-administration with 5 µg TCDD/kg), four rats showed hardly any porphyrin accumulation (84 ± 2 µg/g liver), whereas in six rats a porphyrin level was found which was nearly 1200 times above control levels, i.e., 2215 ± 640 µg/g liver. This extreme individual variation was only observed in the experimental group fed on diets containing 100 mg PCB 153/kg co-administered with 5 µg TCDD/kg.

A qualitative HPLC analysis revealed uroporphyrin III and heptacarboxylic porphyrin as the major accumulated porphyrins in porphyrinogenic livers (data not shown).

Dose-dependent induction of 4-hydroxylation of acetanilide (4-OH-AA) by TCDD, PCB 126, and PCB 156 has been reported before (26,27,57). Figure 1 shows significant correlations between hepatic CYP1A2 activities, measured as 4-OH-AA, and hepatic porphyrin levels after TCDD (Fig. 1A), PCB 126 (Fig. 1B), or PCB 156 (Fig. 1C) administration. The correlation coefficients were 0.629 (p < 0.001), 0.483 (p < 0.01), and 0.808 (p < 0.001) for TCDD, PCB 126, and PCB 156, respectively.

CYP1A2 activity, measured as MROD, was increased 10-fold after TCDD (5  $\mu$ g/kg diet) exposure in experiment 2 (Table 3). PCB 153 (100 mg/kg diet) gave no increase in MROD activity compared to controls. Co-administration of 0.5 or 5  $\mu$ g TCDD/kg with 30 or 100 mg PCB 153/kg resulted in a decrease in MROD activity compared to the corresponding TCDD dose alone (Table 3). The porphyrinogenic rats exposed to 5  $\mu$ g TCDD/kg co-administered with 100 mg PCB 153/kg had CYP1A2 activities of 516  $\pm$ 57 nmol/mg/min (mean  $\pm$  SE). The non-



**Figure 1.** Correlations between hepatic microsomal CYP1A2 activities, measured as 4-hydroxylation of acetanilide and hepatic porphyrins after 13 weeks on diets containing (A) TCDD, (B) PCB 126, or (C) PCB 156. Correlation coefficients were 0.629 (p < 0.001), 0.483 (p < 0.01), and 0.808 (p < 0.001) for TCDD, PCB 126, and PCB 156, respectively.

responding rats in this combined treatment group had CYP1A2 activities of 1410 ±171 nmol/mg/min.

Figure 2 shows the relationship between hepatic porphyrin accumulation and CYP1A2 activities, using MROD as a marker, in TCDD, PCB 153, and coexposed rats. CYP1A2 activities and hepatic porphyrin levels in the co-exposed animals were significantly correlated (p < 0.05; r = -0.792).

A tight band of CYP1A2 protein was observed in all samples. However, in porphyrinogenic rats, a smear of protein staining throughout the gel was present in addition to the tight CYP1A2 band. Measurements of CYP1A2 protein levels in selected samples in this experiment gave the same optical density for TCDD-treated rats as for rats co-exposed with PCB 153 (Table 3). No difference in CYP1A2 protein levels was observed between the porphyrinogenic rats (responders) and the nonporphyrinogenic rats (nonresponders) after co-treatment with the highest dose levels of TCDD and PCB 153.

Using MROD as a marker and microsomes of a nonresponding and responding (porphyrinogenic) rat in the highest dose group in experiment 2 (TCDD/PCB 153), increasing substrate concentrations of methoxyresorufin up to 30 nM did not restore MROD activities in vitro. In the in vitro inhibition experiment with PCB 153 and PCB 126 using microsomes of a nonresponding and responding (porphyrinogenic) rat, both PCB 153 and PCB 126 were able to inhibit MROD activity. PCB 153 inhibited MROD activity by 50% in both samples at a final concentration of 13 µg/ml. At a final concentration of 13 ng/ml, reductions of 50% and 25% were observed in the

responding and nonresponding rat. A final concentration of 1.3 ng PCB 126/ml resulted in 25% and 15% reduction in MROD activity in the responding and nonresponding rat, respectively.

## Discussion

The work described in this study clearly shows that porphyrins accumulate in the liver after subchronic treatment with TCDD, PCB 126, or PCB 156 in rats. However, co-treatment of TCDD with PCB 153 resulted in enhanced hepatic porphyrin levels, which was not observed in the PCB 126/PCB 156 groups when cotreated with TCDD. The reason for the differences between the control groups in



Figure 2. Correlation between microsomal CYP1A2 activities, measured as methoxyresorufin-Odemethylase, and hepatic porphyrin accumulation after 13 weeks on diets containing TCDD and PCB 153.

Table 3. Rat hepatic CYP1A2 activity, measured as methoxyresorufin-O-demethylase, and protein levels after 13 weeks on diets containing PCB 153 and/or TCDD (means ± SE)

Amount in diet		CVP1A2 activity	CVP1A2 protein	
TCDD (µg/kg)	PCB 153 (mg/kg)	(pmol/mg/min) <sup>a</sup>	(optical density/µg protein)	
0	0	185 ± 5	······································	
0	10	183 ± 18		
0	30	156 ± 23		
0	100	123 ± 19	$0.013 \pm 0.004^{b}$	
0.5	0	1195 ± 45*		
0.5	10	1258 ± 59		
0.5	30	628 ± 142**		
0.5	100	441 ± 100**		
5	0	1864 ± 50*	1.27 ± 0.16 <sup>c</sup>	
5	10	2021 ± 135		
5	30	706 ± 392**	1.20 ± 0.29 <sup>c</sup>	
5	100	888 ± 221**	1.32 ± 0.26 (responders) <sup>c</sup> 1.43 ± 0.10 (nonresponders) <sup>b</sup>	

<sup>a</sup>n = 4 or 5.

<sup>b</sup>n = 2.

<sup>c</sup>n = 3.

\*Significant from control (least significant difference test,  $\rho$  <0.05); \*\*significant from corresponding TCDD dose alone (least significant difference test,  $\rho$  <0.05).

the two experiments, or in general between the two experiments (TCDD effect/lack of effect) is not known. This difference was not due to variability in the porphyrin assay since all porphyrin samples were analyzed (both experiment 1 and 2) at the same time using the same reagent solutions, standards, and (positive) control samples. In addition, the animals in the 5 µg TCDD/kg groups ate the same amount of food in both experiments, so the doses were comparable (Tables 1 and 2).

The porphyrinogenic activity of polyhalogenated aromatic hydrocarbons is well known (8-10,14,58). Jones and Sweeney (14) postulated the involvement of an Ah receptor-mediated mechanism in the accumulation of hepatic porphyrins by TCDD. Using TCDD as a reference compound and hepatic porphyrin accumulation as a marker, the relative potencies derived from noobserved-adverse-effect levels (NOAELs) and lowest-observed-adverse-effect levels (LOAELs) in the first experiment (Table 4) were in the same range as those reported using other well-known Ah receptor-mediated effects using the same experimental design (26,27,59). In mice, relative potencies of PCDDs, PCDFs, and PCBs derived from hepatic porphyrin accumulation were in the same range as those based on hepatic CYP1A1 and CYP1A2 induction (60). All these results suggest the involvement of an Ah receptor-mediated mechanism in hepatic porphyrin accumulation. Moreover, this is strengthened by the absence of hepatic porphyrin accumulation after subchronic dosing with di-ortho-substituted PCBs, such as PCB 153 (Table 1) (58) or 2,2',3',4,4', 5,5'-heptachlorobiphenyl (PCB 180) (61). However, Stonard and Greig (58) also found hepatic porphyrin accumulation by subchronic administration of 2,2',3, 3',4,4'-hexachlorobiphenyl (PCB 128) and 2,2',3,4,4',5'-hexachlorobiphenyl (PCB 138) in rats.

It can be suggested that an Ah receptormediated mechanism in hepatic porphyrin accumulation involves the induction of CYP1A2. Strong evidence for a CYP1A2related mechanism in the oxidation of uroporphyrinogen III to uroporphyrin III has been reported by Lambrecht and co-workers using hepatic rat microsomes and purified mouse CYP1A2 (62,63). In our study, CYP1A2 activities were correlated with hepatic porphyrin levels after administration of TCDD, PCB 126, or PCB 156 (Fig. 2). At the highest dose levels of these compounds, CYP1A2 activities were maximally induced as reported earlier (26,27,57). The lower correlation for PCB 126 might be a consequence of inhibition of the catalytic activity of CYP1A2 by PCB

126, which has been suggested to be a competitive high-affinity binding inhibitor (27,44,64,65). In addition, hepatic porphyrin levels were slightly but not significantly decreased in rats treated with 20 µg TCDD/kg diet compared to 5 µg TCDD/kg diet. TCDD (5 µg/kg diet) coadministered with 180 µg PCB 126/kg diet resulted in slightly lower hepatic porphyrin levels compared to co-administration with 50 µg PCB 126/kg. The same trend was observed in these specific groups for CYP1A2 activities using 4-OH-AA as a marker (26,27). Additionally, MROD activity was inhibited by PCB 126 and PCB 153 in vitro in the presented study. However, PCB 126 was about 1000 times more potent than PCB 153 for this effect in vitro. The results of this study, combined with the results from the literature, suggest that the relative potencies of the single PCB congeners for hepatic porphyrin accumulation might be based on an Ah receptor CYP1A2-mediated oxidation of uroporphyrinogen III to uroporphyrin III.

A strong synergistic porphyrin accumulation occurred after co-administration of PCB 153 and TCDD, leading to hepatic porphyrin levels as high as 800-fold the level of control animals (second experiment). The accumulated hepatic porphyrins were uroporphyrin III and heptacarboxylic porphyrin, which indicate PCT-like effects (11,66). In contrast, coadministration of PCB 126 or PCB 156 with TCDD yielded no further hepatic porphyrin accumulation compared to the highest single dose of PCB or TCDD congeners (first experiment).

The synergism on porphyrin accumulation in our study has been reported previously after co-administration of phenobarbital-type and 3-methylcholanthrene-type inducing compounds. Co-administration of 2,2',4,4',5,5'- and 3,3',4,4',5,5'-hexabromobiphenyls or phenobarbital and TCDD in cultured chick embryo hepatocytes resulted in synergistic porphyrin accumulation (67,68). Additionally, induction of ALAS activity and inhibition of UROD activity was synergistically affected after co-administration of TCDD and phenobarbital in these chick hepatocytes (68). In female Sprague-Dawley rats, a synergistic response on hepatic porphyrin accumulation was reported after 20-week co-exposure of the 2,2',4,4',5,5'- and 3,3',4, 4',5,5'-hexabromobiphenyls (69).

It can be speculated that the cause for the synergistic response after co-administration with PCB 153 may be found in an effect on the rate-limiting enzyme in heme synthesis, ALAS. Phenobarbital has been reported to induce both ALAS mRNA and

Table 4. No-observed-adverse-effect levels(NOAELs) and lowest-observed-adverse-effectlevels (LOAELs) for porphyrin accumulation after13-week exposure to TCDD, PCB 126, or PCB 156and estimated relative potencies

	(µg/kg	/day)	Estimated relative potency	
Compound	NOAEL	LOAEL	NOAEL	LOAEL
TCDD	0.026	0.047	1	1
PCB 126	0.47	3.18	0.06	0.014
PCB 156	81	365	0.0003	0.0001

enzyme activity in rat hepatocytes (70-73). As PCB 153 and phenobarbital are wellknown CYP2B-inducing compounds, a similar ALAS induction may have occurred in our study. Both mechanisms, i.e., induction of ALAS by PCB 153 and enhanced oxidation of uroporphyrinogen III to uroporphyrin III by CYP1A2, might have led to the strong synergistic effect observed after co-administration of the compounds. This dual mechanism might explain the high porphyrinogenic action of 2,2',3, 3',4,4'-hexachlorobiphenyl (PCB 128) and 2,2',3,4,4',5'-hexachlorobiphenyl (PCB 138), both di-ortho-substituted PCBs, which also induced the Ah receptor-associated activity of benzo[a]pyrene hydroxylation (58). However, it should be noted that the mixed-type inducer PCB 156 revealed hardly any porphyrin accumulation in the liver after 13 weeks of exposure.

Co-administration of PCB 153 and TCDD resulted in lower CYP1A2 activities, using MROD as a marker, compared to TCDD alone (Table 3). In addition, an inverse relationship was found between hepatic porphyrin accumulation and CYP1A2 activities in co-treated animals (Fig. 2). However, TCDD-induced CYP1A2 protein levels were unaffected by co-treatment with PCB 153. All this information suggests that under porphyrinogenic conditions, CYP1A2 activity is decreased while CYP1A2 protein levels remain intact. This decreased activity could not be restored by adding more substrate (methoxyresorufin) during the assay, suggesting that a tight-binding inhibitor has been formed in vivo. Because free PCB 153 decreased CYP1A2 activities in vitro only at the high concentration of 13 µg/ml, it is unlikely that microsomal-bound PCB 153 from co-treated rats was responsible for the inhibition of CYP1A2 (Table 2).

Whether PCB 153 was left over in these microsomes at all, since PCB 153 has more affinity for fat tissue than for liver, is an unanswered question. It has been reported that under porphyrinogenic conditions, a tight-binding inhibitor of UROD is formed (74,75). Based on our results, it can be speculated that under the same conditions, a tight-binding inhibitor of CYP1A2 activity is also formed. This suggestion is strengthened by the dosedependent inhibition in chicken hepatocyte CYP1A activity, using EROD as a marker, after treatment with PCDDs and PCBs (76-78). This dose-dependent inhibition in CYP1A activity occurred at dose levels that caused an increase in porphyrin levels (76,78). Nevertheless, it remains unclear why a large degree of variability was observed in the PCB 153/TCDD cotreatment group at the high dose levels of both compounds. However, it can be excluded that this was due to variability in PCB 153 or TCDD concentrations in hepatic tissue (46).

In summary, an Ah receptor-mediated mechanism most likely plays a role in porphyrin accumulation after single-congener exposure, as relative potencies of PCB 126 and PCB 156 are in the same range as other well-known Ah receptor-mediated effects. However, this porphyrin accumulation is enhanced by the combined effect of PCB 153 and TCDD, leading to an exceptionally high hepatic porphyrin accumulation of rats subchronically exposed to combinations of these compounds. We postulate that in this synergistic process a dioxinlike induced CYP1A2 mechanism is involved, leading to oxidation of uroporphyrinogen III to uroporphyrin III, together with induction of ALAS by PCB 153. In addition, it can be speculated that high levels of TCDD of PCB 126 decrease hepatic porphyrin accumulation by tightly binding to CYP1A2. We suggest that under porphyrinogenic conditions, a binding inhibitor of CYP1A2 is formed. The interactive effect on porphyrin accumulation after co-administration of dioxinlike and non-dioxinlike compounds may have significant implications for the risk assessment of these chemicals.

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