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-p-dioxin (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 0.001 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 porphyrins 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 δ-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 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, porphyrin, 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. Porphyrin 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 uroporphyrinogen. 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 2,4,5-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 polychlorinated aromatic hydrocarbons such as polychlorinated dibenz-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 δ-aminolevulinic 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 anhydride carbon hydroxylase (Ah) inducibility, indicating that the anhydride carbon (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 Kanecchlor-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 high-level 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 PCDDs (25), mixtures of TCDD and PCBs (26,27), and by in vitro studies with mixtures of PCBs and PCDFs (28). The Ah receptor-mediated 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 synergy in hepatic tumor promotion.

By using interim TEF values (17,35,36), some co-planar and mono-ortho-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-ortho-substituted, 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 regimen 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), and 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 Porphyrim 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: SV 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 13-week 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 Pluss 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 diethyl ether 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 ± SE, n = 8) |
|-----------------------------------------------|
| **Amount in diet** | **Dose** | **PCB 126** | **PCB 156** | **TCDD** | **PCB 126** | **PCB 156** | **Porphyrins** |
| (µg/kg) | (µg/kg) | (µg/kg) | (µg/kg) | (µg/kg) | (µg/kg) | (µg/kg) | (µg/g liver) |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.494 ± 0.059b |
| 0.2 | 0 | 0 | 0 | 0 | 0 | 0 | 0.686 ± 0.066 |
| 0.4 | 0 | 0 | 0 | 0 | 0 | 0 | 0.827 ± 0.073a |
| 20 | 0 | 0 | 0 | 0 | 0 | 0 | 0.954 ± 0.053a |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.889 ± 0.059a |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.764 ± 0.117 |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.853 ± 0.086b |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.694 ± 0.061 |
| 0.4 | 0 | 0 | 0 | 0 | 0 | 0 | 0.841 ± 0.075a |
| 0.4 | 0 | 0 | 0 | 0 | 0 | 0 | 0.105 ± 0.10** |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.01 ± 0.06*** |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.999 ± 0.062 |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.12 ± 0.10 |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.913 ± 0.037 |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.607 ± 0.061 |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.365 ± 0.098b |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.729 ± 0.11* |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.14 ± 0.09 |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.118 ± 0.08 |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.118 ± 0.15 |

*Dose daily is estimated average values based on estimated food consumption, dietary level of compound, and average body weight over the 13-week feeding period.

n = 7.

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

Significant from 0.5 µg TCDD/kg alone (least significant difference test, p < 0.05).

Significant from 1.2 mg PCB 156/kg alone (least significant difference test, p < 0.05).
Table 2. Dose groups, daily doses of PCB 153 and/or TCDD, and hepatic porphyrin levels after 13 weeks on diets containing PCB 153 and/or TCDD (means ± SE, n = 9)

| Amount in diet | Daily dose* | Porphyrins (µg/g liver) |
|----------------|-------------|------------------------|
| TCDD (µg/kg)  | PCB 153 (mg/kg) | TCDD (ng/kg/day) | PCB 153 (mg/kg/day) |
| 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*  |
| 0             | 100         | 0                     | 6.61                | 3.0 ± 0.9   |
| 0.5           | 10          | 33.4                  | 0                   | 1.9 ± 0.5   |
| 0.5           | 30          | 33.9                  | 0.68                | 300 ± 203*  |
| 0.5           | 100         | 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 ± 383* |

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

**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 total whole-liver homogenate, prepared as described earlier (26). Fluorescence was measured in 96-well multiplates at λex 409 nm and λem 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 was analyzed as described earlier (50), after a porphyrin extraction-step of liver homogenates (51) using reverse-phase 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 (MORD), using the same protocol as previously described for ethoxyresorufin-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/µg 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 MORD 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 mg/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. Two-way 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, co-administration 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 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 μg/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 μg 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 μg TCDD/kg co-administered with 100 mg PCB 153/kg had CYP1A2 activities of 516 ± 57 nmol/mg/min (mean ± SE). The non-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 co-exposed 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 right 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 co-treated with TCDD. The reason for the differences between the control groups in

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**Table 3. Rat hepatic CYP1A2 activity, measured as methoxyresorufin-O-demethylase, and protein levels after 13 weeks on diets containing TCDD and PCB 153.**

| Amount in diet | CYP1A2 activity (pmol/mg/min) | CYP1A2 protein (optical density/μg protein) |
|----------------|-----------------------------|------------------------------------------|
| TCDD (μg/kg)  | PCB 153 (mg/kg)             |                                          |
| 0              | 0                           | 185 ± 5                                   |
| 0              | 10                          | 163 ± 16                                 |
| 0              | 30                          | 156 ± 23                                 |
| 0              | 100                         | 123 ± 19                                 |
| 0.5            | 0                           | 1195 ± 45*                               |
| 0.5            | 10                          | 1258 ± 59                                |
| 0.5            | 30                          | 628 ± 142**                              |
| 0.5            | 100                         | 441 ± 100**                              |
| 5              | 0                           | 1864 ± 50*                               |
| 5              | 10                          | 2021 ± 125                               |
| 5              | 30                          | 706 ± 392**                              |
| 5              | 100                         | 888 ± 221**                              |

\[ \text{ANOVA : } F(4, 20) = 12.74, p < 0.001 \]

\[ \text{Mean difference : } t(10) = 4.32, p < 0.001 \]

\[ \text{Significant from control (least significant difference test, } p < 0.05) \]

\[ \text{Significant from corresponding TCDD dose alone (least significant difference test, } p < 0.05) \]
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

asay since all porphyrin samples were ana-
alyzed (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 poly-
halogenated aromatic hydrocarbons is well
known (8-10,14,58). Jones and Sweeney
(14) postulated the involvement of an Ah
receptor-mediated mechanism in the accu-
mulation of hepatic porphyrins by TCDD.

Using TCDD as a reference compound and
hepatic porphyrin accumulation as a mark-
er, the relative potencies derived from no-
observed-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-medi-
ated effects using the same experimental
design (26,27,59). In mice, relative potent-
cies 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 hep-
atic 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 receptor-
mediated mechanism in hepatic porphyrin
accumulation involves the induction of
CYP1A2. Strong evidence for a CYP1A2-
related mechanism in the oxidation of uro-
porphyrinogen III to uroporphyrin III has
been reported by Lambrecht and co-work-
ers using hepatic rat microsomes and puri-
ified mouse CYP1A2 (62,63). In our study,
CYP1A2 activities were correlated with
hepatic porphyrin levels after administra-
tion of TCDD, PCB 126, or PCB 156
(Fig. 2). At the highest dose levels of these
compounds, CYP1A2 activities were max-
imally 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 com-
petitive high-affinity binding inhibitor
(27,44,64,65). In addition, hepatic por-
phyrin levels were slightly but not signifi-
cantly decreased in rats treated with 20 µg
TCDD/kg diet compared to 5 µg

TCDD/kg diet. TCDD (5 µg/kg diet) co-
administered 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 accumula-
tion might be based on an Ah receptor
CYP1A2-mediated oxidation of uroporphyr-
ogenogen III to uroporphyrin III.

A strong synergistic porphyrin accumu-
lation 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 experi-
ment). The accumulated hepatic por-
phyrins were uroporphyrin III and hep-
tacarboxylic porphyrin, which indicate
PCT-like effects (11,66). In contrast, co-
administration 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 accumu-
lation in our study has been reported previ-
ously also after co-administration of pheno-
obarbital-type and 3-methylcholanthrene-type
inducing compounds. Co-administration of
2,2',4,4',5,5'- and 3,3',4,4',5,5'-hexa-
bromobiphenyls or phenobarbital and
TCDD in cultured chick embryo hepatoc-
cytes resulted in synergistic porphyrin
accumulation (67,68). Additionally, induc-
tion of ALAS activity and inhibition of
UROD activity was synergistically affected
after co-administration of TCDD and pheno-
obarbital in these chick hepatocytes (68).
In female Sprague-Dawley rats, a synergis-
tic response on hepatic porphyrin accumu-
lation 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-admin-
istration 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

| Compound | NOAEL (µg/kg diet) | LOAEL (µg/kg diet) | NOAEL (µg/kg diet) | LOAEL (µg/kg diet) |
|----------|--------------------|--------------------|--------------------|--------------------|
| PCB 126  | 0.026              | 0.047              | 1                  | 1                  |
| PCB 156  | 0.47               | 3.18               | 0.06               | 0.14               |
| Estimated relative potency                      |                     |                    |                    |                    |
| PCB 126  | 81                 | 365                | 0.0003             | 0.0001             |

enzyme activity in rat hepatocytes (70-73).
As PCB 153 and phenobarbital are well-
known CYP2B-inducing compounds, a
similar ALAS induction may have occurred
in our study. Both mechanisms, i.e., induc-
tion of ALAS by PCB 153 and enhanced
oxidation of uroporphyrinogen III to uro-
porphyrin 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-associa-
ted activity of benz(a)pyrene hydroxyla-
tion (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 activi-
ties, 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
cotreatment with PCB 153. All this infor-
mation suggests that under porphyrino-
genic 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, sug-
gest 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 micromolar-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 con-
ditions, 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 dose-dependent 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 co-treatment 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 dioxin-like induced CYP1A2 mechanism is involved, leading to oxidation of uroporphyrin 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 porphyrogenic 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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