Toxicity of Polychlorinated Biphenyl with Special Reference to Porphyrin Metabolism

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Oral administration of a commercial PCB mixture to chickens caused a hepatic-type porphyria characterized by hepatic accumulation and urinary excretion of uroporphyrin. To clarify the mechanism of the porphyrinogenic activity of these PCBs, we studied the structural requirement of synthetic PCB for porphyrinogenic activities by using the cultured chick embryo liver cells and examined the relationship between induction of δ-aminolevulinic acid (ALA) synthetase and inhibition of uroporphyrinogen decarboxylase. We established that the porphyrinogenic effect of PCBs exhibits a sharply defined structure–activity relationship in that only 3,4,3',4'-tetrachlorobiphenyl and 3,4,5,3',4',5'-hexachlorobiphenyl produced a marked accumulation of uroporphyrin. We also demonstrated that in ALA-supplemented cultures, these same compounds lead to accumulation of a large amount of uroporphyrin III, whereas with other PCBs, which were weak inducers of porphyrin synthesis, the accumulated porphyrin was mostly protoporphyrin.

Kinetic studies of the sequential decarboxylation of uroporphyrinogen with purified uroporphyrinogen decarboxylase were performed. The 3,4,3',4'-tetrachlorobiphenyl and 3,4,5,3',4',5'-hexachlorobiphenyl strongly inhibit uroporphyrinogen decarboxylase directly at two steps, i.e., first in the formation of heptacarboxylic porphyrinogen III from heptacarboxylic porphyrinogen III and second in the formation of heptacarboxylic porphyrinogen III from uroporphyrinogen III. The results confirmed that porphyrinogenic PCBs primarily inhibit uroporphyrinogen decarboxylase, leading to a depletion of heme as a result of which synthesis of ALA synthetase increased.

In 1968, Yusho (polychlorinated biphenyl [PCB] poisoning) occurred in the western part of Japan (1), but disturbances of porphyrin metabolism due to PCBs were not investigated at that time. In 1970, Vos and Koeman (2) first reported that oral administration of commercial PCB to chickens caused hepatic-type porphyria, which was characterized by excretion and hepatic accumulation of uroporphyrin (3). We studied the structural requirement of synthetic PCBs for inducing porphyrin in cultured chick embryo liver cells (4) and the inhibitory effect of PCB isomers on uroporphyrinogen decarboxylase, clarifying the mechanism of porphyrinogenic activity of PCB.

Heme Synthesis

A schematic outline of porphyrin and heme biosynthesis is presented in Figure 1 (5). The first step is the condensation of succinyl-CoA and glycine to form δ-aminolevulinic acid (ALA). This reaction is catalyzed by ALA synthetase, which is regulated by feedback repression of heme, i.e., the end product of the biosynthetic pathway. Two molecules of ALA then condense to form porphobilinogen (PBG) and then four molecules of PBG condense to form uroporphyrinogen III. Uroporphyrinogen decarboxylase catalyzes the decarboxylation of uroporphyrinogen into coproporphyrinogen through hepta-, hexa-, and pentacarboxylic porphyrinogen intermediates. Protoporphyrinogen is formed by oxidative decarboxylation of propionate side chains of coproporphyrinogen III to vinyl groups. Protoporphyrin is formed by enzymatic oxidation of protoporphyrinogen. Finally, iron chelation to the protoporphyrin ring gives heme.

Synthesis of PCB Isomers and Their Properties

Commercial PCBs are prepared by chlorination of biphenyl in the presence of iron powder [eq. (1)].

\[
\begin{array}{c}
\text{\text{O}} \quad \text{\text{O}} \\
\text{\text{Fe}} \text{Cl}_2 \\
\text{\text{Cl}_n} \quad n = 1 \text{~to~} 10
\end{array}
\]

Symmetrical PCB isomers are synthesized as shown in eq. (2). Chloroaniline was treated with sodium nitrate to give the corresponding diazonium derivative, which was replaced by iodine, followed by Ullmann condensation (4).

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Biliverdin reductase

NADPH

Biliverdin IXα

NADP

Heme Oxygenase System

Microsome

Outer membrane

Matrix space

Cristae

Mitochondrion

Inner membrane

Cytosol

ALA dehydrase

Formiminoglutamate

Ferrochelatase

ALA synthetase

Heme

Glycine

Succinyl CoA

CO₂

H₂O

NH₃

NADP⁺

NADPH

Eurone oxidase

Coproporphyrinogen IX

Uroporphyrinogen III

Coproporphyrinogen III

Figure 1. Outline of heme biosynthesis.

Table 1 presents the melting points, gas chromatographic retention times and K band of the symmetrical PCB isomers (4). The relative retention times were correlated with the lipophilicity of the synthetic compounds. The 3,4,3',4'-teta- and 3,4,5,3',4',5'-hexachlorobiphenyl isomers showed the highest retention times among the tetrachloro- and hexachlorobiphenyls investigated, respectively.

Spectroscopic observations on K bands and ε values were in good agreement with those reported by MacNeil et al. (6). In a chlorobiphenyl isomer with two or more chlorine atoms at the ortho and iortho' positions, considerable steric hindrance was expected. The shift of the K band toward shorter wavelengths was observed.
Porphyrin-Inducing Activity of Synthetic and Commercial Polychlorinated Biphenyls

Chick embryo liver cell cultures were prepared by using a slightly modified procedure of Granick (4,7). Table 2 presents the porphyrin-inducing activities of synthetic and commercial polychlorinated biphenyls. It was found that 3,4,3',4'-tetrachlorobiphenyl and 3,4,5,3',4',5'-hexachlorobiphenyl isomers were the most active inducers. 3,5,3',5'-Tetrachlorobiphenyl was a moderate inducer. The 4,4'-dichloro, 2,3,2',3'-, 2,4,2',4'-, and 2,6,2',6'-tetrachloro- and 2,3,4,2',3',4'- and 2,4,6,2',4',6'-hexachlorobiphenyl isomers were weak inducers; the 2,5,2',5'-tetrachloro- and decachlorobiphenyl isomers were inactive. Kanechlor-400 was found to be the strongest inducer among the commercial polychlorinated biphenyl samples tested. On the basis of these data, we established that requirements for porphyrin-inducing activity of PCB are a structure of the type I.

![Diagram](image)

First, a coplanar structure of the chlorobiphenyl is essential for porphyrogenic activity, as shown in the most potent inducers such as 3,4,3',4'-tetrachloro- and

| Compound added       | Porphyrin, pmole/mg protein |
|----------------------|----------------------------|
| None                 | 13 ± 1.5                   |
| Biphenyl             | 15 ± 2.4                   |
| 4,4'-Dichlorobiphenyl| 56 ± 20                    |
| 2,3,2',3'-Tetrachlorobiphenyl | 80 ± 20  |
| 2,4,2',4'-Tetrachlorobiphenyl | 81 ± 29  |
| 2,5,2',5'-Tetrachlorobiphenyl | 15 ± 3.0  |
| 2,6,2',6'-Tetrachlorobiphenyl | 62 ± 16  |
| 3,4,3',4'-Tetrachlorobiphenyl | 3400 ± 480 |
| 3,5,3',5'-Tetrachlorobiphenyl | 420 ± 170  |
| 2,3,4,2',3',4'-Hexachlorobiphenyl | 200 ± 65  |
| 2,4,6,2',4',6'-Hexachlorobiphenyl | 140 ± 35  |
| 3,4,5,3',4',5'-Hexachlorobiphenyl | 2600 ± 410 |
| Decachlorobiphenyl   | 48 ± 24                    |
| Kanechlor-200        | 280 ± 75                   |
| Kanechlor-300        | 550 ± 50                   |
| Kanechlor-400        | 1600 ± 190                 |
| Kanechlor-500        | 250 ± 60                   |
| Kanechlor-600        | 50 ± 8.0                   |
| DDC                  | 120 ± 33                   |

*Liver cells were prepared and incubated as described in the literature (4,7). After the medium was changed, a series of chlorobiphenyl isomers dissolved in 25 μL dimethyl sulfoxide were added. The concentrations of chemicals were in all cases 2 μg/mL. Porphyrin and protein were measured after 24-hr incubation. Values presented are the means ± SE for five experiments.

Relationship of Structure to Accumulation of PCB isomers in the Liver

Is porphyrin-inducing activity due to the direct action of added chemicals or to some active metabolite formed by the cells? To answer this question, we first studied what kind of PCB isomers would accumulate in mouse liver after feeding a diet containing PCB isomers. Table 3 shows the relationship of structure of PCB isomers to accumulation in liver. On the basis of these data, we classified PCB isomers into three types based

| Compound administered          | Number of deaths | Concentration in liver, μg/g |
|--------------------------------|------------------|----------------------------|
| 2,3,2',3'-Tetrachlorobiphenyl | 0                | < 0.5                      |
| 2,4,2',4'-Tetrachlorobiphenyl | 0                | 2.6 ± 1.6                 |
| 2,5,2',5'-Tetrachlorobiphenyl | 0                | < 0.5                      |
| 2,6,2',6'-Tetrachlorobiphenyl | 0                | < 0.5                      |
| 3,4,3',4'-Tetrachlorobiphenyl | 0                | < 0.5                      |
| 3,5,3',5'-Tetrachlorobiphenyl | 0                | 2500 ± 1200               |
| 2,3,4,2',3',4'-Hexachlorobiphenyl | 0        | 12 ± 4                    |
| 2,4,6,2',4',6'-Hexachlorobiphenyl | 4        | 1800 ± 200               |
| 3,4,5,3',4',5'-Hexachlorobiphenyl | 4        | 650 ± 130               |
| Kanechlor-400                | 1                | 86 ± 95                   |
| Kanechlor-600                | 2                | 480 ± 95                  |

*The mice were fed a diet containing 300 ppm of each of chlorobiphenyl isomers and commercial polychlorinated biphenyls. After 14 weeks the residue levels in liver were determined by gas-liquid chromatography. Values presented are the means ± SE for four mice.
on PCB metabolism as shown in Figure 2. The 3,4,3',4'-, 2,3,2',3', 2,5,2',5', and 2,6, 2',6'-tetrachlorobiphenyls do not accumulate appreciably in mice liver. These four compounds have unsubstituted pairs of carbon atoms in the molecule, and hence they are likely to be metabolized to the hydroxylated derivatives. Steric hindrance cannot be expected in the molecule (see Table 1). Similar results were reported by Gage and Holm (10); the rate of loss of polychlorinated biphenyl isomers from mouse fat was influenced by unsubstituted pairs of carbon atoms in the molecule. On the other hand, 3,5,3',5'-tetra-, and 2,4,6,2',4',6'- and 3,4,5,3',4',5'-hexachlorobiphenyl isomers markedly accumulated in mouse liver. These compounds contain only isolated unsubstituted carbon atoms, and this situation may account for the difficulty of metabolic conversion to hydroxylated derivatives. The 2,4,2',4'-tetrachloro- and 2,3,4,2',3',4'-hexachlorobiphenyl isomers possess unsubstituted pairs of carbon atoms; however, they are not so readily metabolized, since they have chlorine atoms at the ortho-ortho' positions, resulting in the formation of sterically hindered structures. In fact, 3,4,5,3',4',5'-hexachlorobiphenyl was one of the most active agents, although it was less metabolized. Therefore, it seems likely that porphyrin induction in the chick embryo liver system is due to the orginal chemicals and not to their metabolites.

### Relationship between Induction of ALA Synthetase and Inhibition of Uroporphyrinogen Decarboxylase by PCB

The next question arises: why is porphyrin synthesis induced by PCB? In order to clarify the mechanism of the porphyrinogenic activity of these PCBs, we used cultured chick embryo liver cells supplemented with ALA and examined the relationship between induction of ALA synthetase and inhibition of uroporphyrinogen decarboxylase (11).

When cultured chick embryo liver cells were supplemented with ALA, a large amount of protoporphyrin accumulated, primarily in the medium (Table 4). A small amount of uroporphyrin, usually <10% of total porphyrins, and some coproporphyrin were also formed. Addition of a strong inducer such as 3,4,3',4'-tetrachlorobiphenyl or 3,4,5,3',4',5'-hexachlorobiphenyl resulted in the accumulation of a large amount of uroporphyrin, while protoporphyrin accumulation was greatly reduced. Moreover, much of the uroporphyrin remained intracellular (Table 4). With a moderate inducer such as 3,5,3',5'-tetrachlorobiphenyl, the increase in uroporphyrin and the decrease in protopor-

### Table 4. Effect of PCBs on porphyrin formation from exogenous ALA in cultured chick embryo liver cells.

| PCB added                    | Porphyrins, pmole/mg protein | Prevalent type of porphyrin |
|------------------------------|------------------------------|-----------------------------|
| None                         | Cells | Medium |
| 49 ± 4                       | 240 ± 13  | Proto          |
| 2,3,2',3'-Tetrachlorobiphenyl | 54 ± 4  | 230 ± 14   | Proto          |
| 2,4,2',4'-Tetrachlorobiphenyl | 53 ± 9  | 230 ± 10   | Proto          |
| 2,5,2',5'-Tetrachlorobiphenyl | 54 ± 6  | 230 ± 6    | Proto          |
| 2,6,2',6'-Tetrachlorobiphenyl | 51 ± 4  | 240 ± 4    | Proto          |
| 3,4,3',4'-Tetrachlorobiphenyl | 210 ± 9 | 89 ± 6     | Uro + Proto   |
| 2,3,4,2',3',4'-Hexachlorobiphenyl | 130 ± 10 | 150 ± 16  | Uro + Proto   |
| 2,4,6,2',4',6'-Hexahlorobiphenyl | 65 ± 9  | 240 ± 17   | Proto          |
| 3,4,5,3',4',5'-Hexachlorobiphenyl | 58 ± 4  | 250 ± 9    | Proto          |
| 190 ± 9                      | 91 ± 4    | Uro          |

*Chick embryo liver cells were cultured in 2 mL Falcon dishes in Eagle's minimum essential medium containing 10% fetal calf serum for 20 hr. After the original medium was replaced, various PCBs in 4 μL DMSO (final concentration 2 μg/mL) were added and the mixture was incubated for 1 hr. 0.2 mole ALA was then added and the incubation continued for another 3 hr. Total porphyrin content of cells and medium was quantified and the major porphyrins were characterized by HPLC. Porphyrin content of cells and medium was expressed/mg cell protein. Values represent the means ± SD of 3–6 individual experiments.
Porphyrin were more moderate. Addition of a weak inducer such as 2,3,2',3'-, 2,4,2',4'-, 2,5,2',5'- or 2,6,2',6'-tetrachlorobiphenyl and 2,3,4,2',3',4'- or 2,4,6,2',4',6'-hexachlorobiphenyl had little or no effect on porphyrin accumulation after 3 hr incubation (Table 4), but extension of the incubation time led to accumulation of uroporphyrin to some extent over the control in the cells.

The time course of porphyrin accumulation with 3,4,3',4'-tetrachlorobiphenyl is shown in Figure 3. Whereas ALA supplementation alone (Figure 3A) resulted mainly in protoporphyrin accumulation, addition of 3,4,3',4'-tetrachlorobiphenyl (Figure 3B) reversed the pattern, in that mostly uroporphyrin was formed at the expense of protoporphyrin. The uroporphyrin formed was almost entirely the type III isomer. These results suggested that the same PCBs which most actively induce porphyrin synthesis also inhibit uroporphyrinogen decarboxylase.

Inhibitory Effect of PCB Isomers on Purified Uroporphyrinogen Decarboxylase

In order to confirm the inhibition of uroporphyrinogen decarboxylase by PCB, we purified the enzyme from chicken erythrocytes (12). Purification was accomplished by chromatography on DEAE-cellulose, ammonium sulfate fractionation, chromatography on Sephadex G-100, and chromofocusing. The most purified preparation was homogeneous on polyacrylamide gel electrophoresis and had a specific activity of 1420 units/mg of protein, the highest value so far reported.

The molecular weight, as determined by Sephadex G-150 gel chromatography, was 79,000. The subunit molecular weight, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, was 39,700, suggesting that uroporphyrinogen decarboxylase is dimeric. The purified enzyme had an isoelectric point of 6.2 and a pH optimum of 6.8. Sulfhydryl reagents inhibited the enzyme activity, but neither metal ions nor cofactor requirements could be demonstrated.

The inhibitory effects of synthetic PCBs on the purified enzyme are summarized in Table 5. All the PCBs tested inhibited uroporphyrinogen decarboxylase. The strongest porphyrin inducers such as 3,4,3',4'-tetrachlorobiphenyl and 3,4,5,3',4',5'-hexachlorobiphenyl strongly inhibited the enzyme (80 and 94%, respectively). The moderate inducer, 3,5,3',5'-tetrachlorobiphenyl, moderately inhibited the enzyme (77%). Addition of weak inducers such as 2,3,2',3'-, 2,4,2',4'-, 2,5,2',5'-, and 2,6,2',6'-tetrachlorobiphenyl and 2,3,4,2',3',4'- and 2,4,6,2',4',6'-hexachlorobiphenyl, however, resulted in a partial inhibition of the enzyme (34–69%). These results indicate that there is a good relationship between the inhibition of uroporphyrinogen decarboxylase in cultured liver cells (Table 4) and inhibition of purified uroporphyrinogen decarboxylase. However, the inhibition of the enzyme by weak inducers seems to be slightly stronger than that observed in cultured liver cells. This may be due to low permeability of the weak porphyrinogenic PCB isomers, which have low lipophilicity, through the membrane of liver cells. Their low lipophilicity was inferred from the observation that they showed lower retention times on gas-liquid chromatography than the strong inducers (Table 1).
In order to clarify which steps of the decarboxylation from uroporphyrinogen to coproporphyrinogen are inhibited by PCB isomers, we studied the kinetics of the sequential decarboxylation using purified enzyme. We developed a simple and sensitive enzyme assay based on the separation and quantification of free porphyrins with 8- to 4-carboxylic acids using HPLC equipped with a spectrofluorometric detector.

The typical inhibitory effect of PCB on the enzyme was studied with the use of 3,4,5,3',4',5'-hexachlorobiphenyl at two different concentrations. As shown in Figure 4, in the control experiment (A), uroporphyrinogen III was almost quantitatively converted into coproporphyrinogen III. With a low concentration of PCB (5.5 μM), a large amount of heptacarboxylic porphyrinogen III accumulated and a small amount of coproporphyrinogen III was formed (B), whereas the addition of a higher concentration of PCB (8.3 μM) resulted in the complete inhibition of the decarboxyla-

tion (C). These results show that 3,4,3',4'-tetrachlorobiphenyl and 3,4,5,3',4',5'-hexachlorobiphenyl which specifically induce ALA synthetase also strongly inhibit uroporphyrinogen decarboxylase directly at two steps, i.e., first in the formation of hexacarboxylic porphyrinogen III from heptacarboxylic porphyrinogen III and second in the formation of heptacarboxylic coproporphyrinogen from uroporphyrinogen III.

**Acquired Porphyria in C57BL/6 Mice Due to PCB Isomer Intoxication**

Finally we report the results of our recent genetic studies on mice. In order to investigate the influence of genetic factors on porphyria induction by PCB, we used C57BL/6 mice. Livers of these mice treated with 3,4,5,3',4',5'-hexachlorobiphenyl were larger than those of ddY mice. Under ultraviolet light, we observed a pronounced red fluorescence in livers of C57BL/6 mice treated with the PCB isomer. Table 6 summarizes the porphyrin content in the livers of mice treated with 3,4,5,3',4',5'-hexachlorobiphenyl. There is a clear difference in the reponse of the two strains to porphyrin induction by PCB. A large amount of uroporphyrin fraction accumulated in the livers of C57BL/6 mice treated with PCB but not in the livers of ddY mice.

It is well known that C57BL/6 mice carry the $A_{h}{b}$ allele, giving a responsive phenotype to the induction of aromatic hydrocarbon hydroxylase (13). This suggests

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**Table 6. Porphyrin content in the liver of mice treated with PCB.***

| Strain       | Treatment                        | Uro μg/g liver | Copro μg/g liver | Proto μg/g liver |
|--------------|----------------------------------|----------------|------------------|-----------------|
| ddY          | Control                          | 0.12 ± 0.06    | 0.06 ± 0.02      | 0.17 ± 0.07     |
| C57BL/6      | 3,4,5,3',4',5'-Hexachlorobiphenyl| 0.26 ± 0.05    | 0.06 ± 0.02      | 0.24 ± 0.13     |

*Male mice (7 weeks old) were treated with a diet containing 300 ppm 3,4,5,3',4',5'-hexachlorobiphenyl. After 3 weeks the animals were killed and the porphyrins in liver were determined.

**Mean ± SD of four mice.
that induction of apocytochrome P-450 may take part in inducing porphyrin synthesis.

**Mechanism of Porphyrinogenic Activity of PCB**

On the basis of these results, we propose a possible mechanism of porphyrin accumulation caused by PCB as shown in Figure 5. Porphyrinogenic PCB's primarily inhibited uroporphyrinogen decarboxylase, leading to a depletion of heme. In addition, induction of apocytochrome P-450 by PCB may contribute to a decrease of heme. As a result, synthesis of ALA synthetase increased leading to an accumulation of uroporphyrin in liver.

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