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Effects of polychlorinated biphenyl compounds, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, phenobarbital and iron on hepatic uroporphyrinogen decarboxylase

Implications for the pathogenesis of porphyria

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Treatment of cultured chick embryo hepatocytes with phenobarbital, polychlorinated biphenyl compounds and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin resulted in increased δ-aminolaevulinate synthase and decreased uroporphyrinogen decarboxylase activities and porphyrin accumulation; uroporphyrin and heptacarboxyporphyrin predominated. Iron had no effect on these changes. Simultaneous treatment of cultures with dioxin and phenobarbital produced a synergistic response in δ-aminolaevulinate synthase induction, uroporphyrinogen decarboxylase inhibition and porphyrin accumulation. These data suggest that an inhibitor of uroporphyrinogen decarboxylase may be generated in the liver from polychlorinated biphenyl compounds or dioxin by metabolic activation. Additionally these findings bear on the postulated role of these and related chemicals in determining the low levels of uroporphyrinogen decarboxylase activity in porphyria cutanea tarda patients.

PCT in man is characterized by an accumulation of uroporphyrin and heptacarboxyporphyrin in the liver and is associated with decreased activity of uroporphyrinogen decarboxylase (Kushner *et al.*, 1976; de Verneuil *et al.*, 1978; Elder *et al.*, 1978; Felsher *et al.*, 1982). In experimental animals, hexachlorobenzene (De Matteis *et al.*, 1961; Taljaard *et al.*, 1971; Elder *et al.*, 1976; San Martin de Viale *et al.*, 1977), TCDD (Goldstein *et al.*, 1973; Jones & Sweeney, 1977; Smith & Francis, 1980; Smith *et al.*, 1981) and PCB (Vos & Koeman, 1970; Goldstein *et al.*, 1974) have been shown to produce a form of hepatic porphyria associated with an inhibition of hepatic uroporphyrinogen decarboxylase activity. The development of porphyria in animals is usually a slow process requiring several weeks of treatment with these chemicals (Elder, 1978), whereas treatment of chick embryo liver cells in culture with PCB induces a rapid accumulation of uroporphyrin in the cells within 24h (Sinclair & Granick, 1974; Kawanishi *et al.*, 1978). Thus cultured chick embryo cells are a useful model system for examining porphyrinogenic processes induced by these and related chemicals. In the present paper we describe the inhibition of uroporphyrinogen decarboxylase activity in cultured chick embryo hepatocytes treated with phenobarbital, TCDD or PCB. In addition we have studied the effect of iron on PCB- and TCDD-induced porphyria in the cultured liver cells in an attempt to clarify the role of this metal in the porphyrinogenic process.

Abbreviations used: PCB, polychlorinated biphenyl compounds; TCDD, 2,3,7,8-tetrachlorobenzo-*p*-dioxin; PCT, porphyria cutanea tarda; ALA, δ-aminolaevulinate.

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Materials and methods

Chemicals

Aroclor 1254, a mixture of PCB predominantly consisting of four, five and six chlorine atoms per molecule, was a gift from Monsanto Chemical Co. Sodium phenobarbital was purchased from Mallinckrodt. TCDD was a gift from Dr. Alan Poland, McArdle Laboratory for Cancer Research, Madison, WI, U.S.A. β-Naphthoflavone was a gift from Dr. Arleen B. Rifkind, Cornell Medical College, New York, NY, U.S.A. Pentacarboxyporphyrin I methyl ester was purchased from Porphyrin Products, Logan, UT, U.S.A.

Cultures

Cell cultures were prepared from 17-day-old chick

embryos and incubated in a humidified CO_2 incubator at 37°C with CO_2/air (1:19) as described previously (Sassa & Kappas, 1977). The modified F12 medium contained 1 μg of bovine insulin, 50 ng of cortisol and 1 μg of tri-iodothyronine per ml. After 24 h of incubation, the medium was replaced and addition of chemicals was made. Cells grown in a 15 cm (diameter) dish (Falcon no. 3025) were used for the uroporphyrinogen decarboxylase assay and 6 cm dishes (Falcon no. 3060) were used for the ALA synthase assay. Cells were incubated with the chemical for 24 h unless otherwise stated.

Fluorimetry of porphyrins

Porphyrins were extracted into a mixture of 1 M-$HClO_4$/methanol (1:1, v/v) and their concentrations were determined spectrofluorimetrically in a Hitachi–Perkin–Elmer MPF-4 fluorescence spectrophotometer (Sassa & Kappas, 1977).

Protein content of the cells was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Enzyme assays

ALA synthase activity was assayed by the modified method of Sinclair & Granick (1977) as described previously (Sassa & Kappas, 1977). Uroporphyrinogen decarboxylase assay (de Verneuil *et al.*, 1978) was modified for a smaller scale. Specifically, the reaction mixture contained, in a final volume of 200 μl, 100 mM-potassium phosphate buffer (pH 6.0), 5 mM-dithiothreitol, 10–15 μM-pentacarboxyporphyrinogen I and the enzyme preparation (0.5–1.5 mg of protein from a 12000 *g* supernatant of homogenized cells). The porphyrinogen substrate was prepared from its corresponding porphyrin methyl ester by hydrolysis in 6 M-HCl followed by reduction with sodium amalgam (de Verneuil *et al.*, 1978). Incubation was carried out in the dark for 1 h at 37°C. The reaction was stopped by adding 40 μl of 3 M-HCl. After mixing, the tubes were left under u.v. light for 20 min to allow oxidation of porphyrinogens to porphyrins. Porphyrins were converted into their methyl esters by incubation overnight with 3 ml of methanol/H_2SO_4 (9:1, v/v). Porphyrin methyl esters were extracted into chloroform and then dried by an air stream. The dried porphyrin methyl esters were redissolved in chloroform and separated by high-performance liquid chromatography (on a 250 mm × 2.6 mm column packed with 10 μm silica) using, as a detector, a Hitachi MPF-4 fluorescence spectrophotometer equipped with an R-928 photomultiplier (Perkin–Elmer, Norwalk, CT, U.S.A.) (excitation wavelength, 400 nm; emission wavelength, 625 nm). Porphyrins were eluted by a linear gradient in 6 min from 10% to 22% of chloroform/methanol/aq. 2 M-NH_3 (850:150:1, by vol.) in hexane at a flow rate of 2 ml/min.

Results

Time course of the porphyrinogenic response to PCB

Fig. 1 shows the levels of porphyrin, and activities of uroporphyrinogen decarboxylase and ALA synthase after treatment of liver cell cultures with PCB (30 μM). Uroporphyrinogen decarboxylase activity was decreased after 4 h and remained at a low level for at least 24 h (Fig. 1). ALA synthase activity increased within 4 h after the chemical treatment and

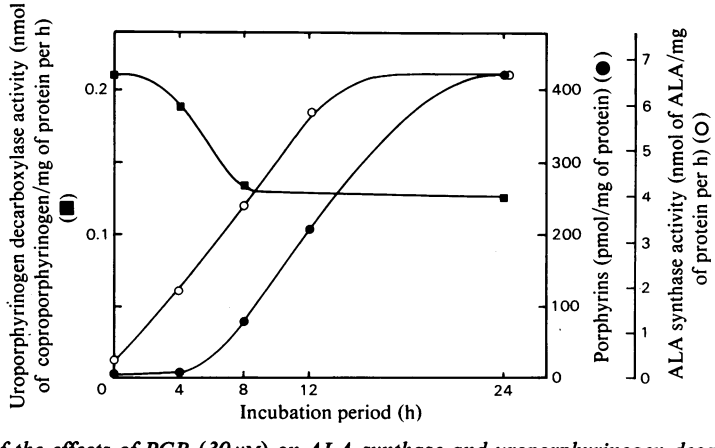

Fig. 1. *Time course of the effects of PCB (30 μM) on ALA synthase and uroporphyrinogen decarboxylase activities and porphyrin formation in chick embryo liver cell cultures*

After 24 h of incubation at 37°C in CO_2/air (1:19), the medium was removed and fresh medium was added with the chemicals. Incubations were terminated at various times, from 0 to 24 h. Assays were done in duplicate.

became maximal after 24h (Fig. 1). Porphyrin concentration increased significantly at 8h and continued to rise over 24h (Fig. 2). Among the total porphyrins accumulated, uroporphyrin and heptacarboxyporphyrin accounted for approx. 90% (Fig. 2).

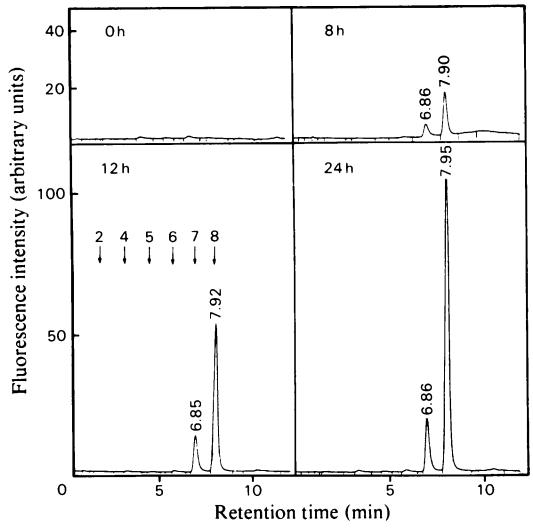

Fig. 2. *High-performance-liquid-chromatography tracing of porphyrins (as methyl ester derivatives) accumulated in the cells at 0, 8, 12 and 24h after treatment with PCB*

Porphyrins were esterified by adding 3ml of methanol/H_2SO_4 (9:1, v/v) to 0.2ml of the 12000*g* supernatant used for enzyme assay. Arrows indicate the retention time of the markers (methyl esters): 2, protoporphyrin; 4, coproporphyrin; 5, pentacarboxyporphyrin; 6, hexacarboxyporphyrin; 7, heptacarboxyporphyrin; 8, uroporphyrin.

Effect of iron on the porphyrinogenic response to PCB

To investigate the role of iron in the porphyrinogenic action of polychlorinated hydrocarbons, we compared porphyrin accumulation, ALA synthase induction and uroporphyrinogen decarboxylase inhibition by PCB in the absence and in the presence of added iron (Table 1). No significant potentiation of the PCB effect on porphyrin formation, ALA synthase induction and uroporphyrinogen decarboxylase inhibition was found after treatment of cultures with the metal. Similar results were obtained when TCDD was used at concentrations of 0.1–1 μM in the presence or in the absence of iron (Fig. 3).

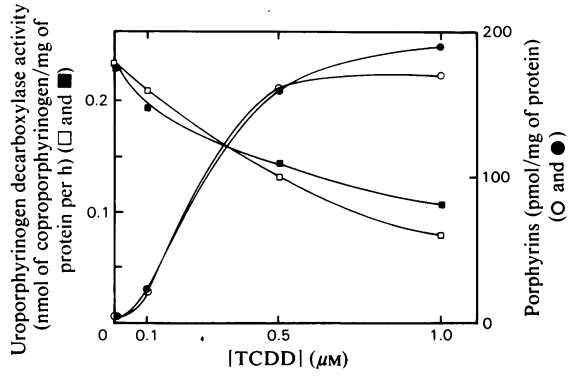

Fig. 3. *Effects of TCDD with or without iron on porphyrin formation and uroporphyrinogen decarboxylase activity in the cultured liver cells*

Porphyrin accumulation and uroporphyrinogen decarboxylase activity were measured after 24h of incubation in the presence of different doses of TCDD. Data are means of three determinations.

Table 1. *Effects of PCB and iron on porphyrin formation, uroporphyrinogen decarboxylase and ALA synthase activities in cultured liver cells*

Porphyrin accumulation, ALA synthase activity and uroporphyrinogen decarboxylase activity were measured after 24h as described in the Materials and methods section. The control value for uroporphyrinogen decarboxylase was 0.19nmol of coproporphyrinogen/mg of protein per h. Abbreviation used: n.d., not determined.

	Porphyrins (pmol/mg of protein per 24h)	ALA synthase (nmol of ALA/mg of protein per h)	Uroporphyrinogen decarboxylase (% of control)
Control	5	0.40	100
$(NH_4)_2Fe(SO_4)_2$ (400μM)	12	0.40	105
PCB (15μM)	455	2.0	76
PCB (15μM) + $(NH_4)_2Fe(SO_4)_2$ (400μM)	640	2.05	83
PCB (30μM)	871	3.40	62
PCB (30μM) + $(NH_4)_2Fe(SO_4)_2$ (400μM)	746	3.38	72
PCB (60μM)	924	4.70	50
PCB (60μM) + $(NH_4)_2Fe(SO_4)_2$ (400μM)	1060	n.d.	55

Table 2. *Effects of iron-chelating agents (desferrioxamine and CaMgEDTA) on porphyrin formation induced by PCB in cultured liver cells*

A portion (0.5 ml) of 1:150 cell suspension in modified F12/insulin/cortisol/tri-iodothyronine was placed in wells of 24-well cluster dishes. After 24 h of incubation, the medium was replaced and addition of chemicals was made. Porphyrins were measured 24 h later as described in the Materials and methods section. Results for total porphyrins are means ± S.D. (four determinations).

	Total porphyrins (pmol/mg of protein per 24 h)	Uroporphyrin (%)	Coproporphyrin (%)	Protoporphyrin (%)
Control	4.5 ± 0.3	9	21	70
PCB (30 μM)	465 ± 36	85	8	7
Desferrioxamine (1.5 mM)	74 ± 21	5	3	92
CaMgEDTA (5 mM)	43 ± 5	5	2	93
PCB (30 μM) + desferrioxamine (1.5 mM)	380 ± 38	40	2	58
PCB (30 μM) + CaMgEDTA (5 mM)	323 ± 75	39	2	59

The effects of iron-chelating agents on the hydrocarbon-induced porphyrinogenesis were also examined. As shown in Table 2, incubation of the cells with desferrioxamine or CaMgEDTA caused the accumulation (16- and 9-fold greater than the control value respectively) predominantly of protoporphyrin (approx. 90%). Treatment with PCB led to a marked increase of porphyrin (100 times the control value, 85% of which consisted of uroporphyrin). Incubation of the cells with PCB and CaMgEDTA or desferrioxamine produced similar results, but the accumulated porphyrins represented a mixture of uroporphyrin (40%) and protoporphyrin (58%). These results are consistent with those reported by Sinclair & Granick (1974) utilizing a comparable liver cell culture system.

Inhibition of uroporphyrinogen decarboxylase activity by phenobarbital

Addition of phenobarbital to chick embryo liver cell cultures led to a significant accumulation (40-fold) of porphyrins (Fig. 4; Table 3). The porphyrin pattern was identical with that obtained with PCB (Fig. 5) or TCDD alone (results not shown), i.e. uroporphyrin and heptacarboxyporphyrin are the major porphyrins accumulated. Consistent with these findings, uroporphyrinogen decarboxylase activity was decreased by phenobarbital treatment in a dose-related manner (Fig. 4); the highest dose of phenobarbital used (1 mM) led to a 20% inhibition of the enzyme.

Effect of TCDD on phenobarbital and β-naphthoflavone-pretreated liver cells

Table 3 shows the effects of TCDD addition on porphyrin accumulation and ALA synthase and uroporphyrinogen decarboxylase activities in cultures pretreated with phenobarbital or β-naphthoflavone. The effects of phenobarbital and TCDD on porphyrin accumulation, ALA synthase induction and uroporphyrinogen decarboxylase inhibition were synergistic (Table 3). In contrast there was no potentiation of the porphyrinogenic action of TCDD by pretreatment of the cells with β-naphthoflavone (Table 3).

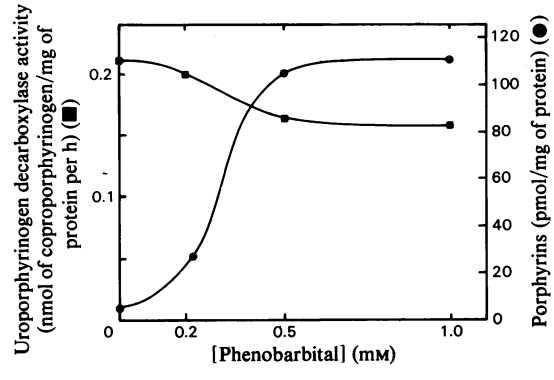

Fig. 4. *Porphyrin formation and uroporphyrinogen decarboxylase activity in chick embryo liver cells exposed to different doses of phenobarbital*

Experimental conditions were as described in the legend to Fig. 3. The porphyrin pattern is shown in Fig. 2.

Discussion

The present study demonstrates that treatment of avian embryo liver cells maintained in a chemically defined medium with PCB, TCDD or phenobarbital results in the inhibition of uroporphyrinogen decarboxylase activity and the accumulation of uroporphyrin and heptacarboxyporphyrin in the cells. ALA synthase activity was, additionally, increased

Table 3. *Effects of TCDD on chick embryo liver cells pretreated with phenobarbital and β-naphthoflavone*
Cultured cells were treated with phenobarbital or β-naphthoflavone at zero time. After 24h of incubation, the medium was changed and then TCDD was added. The different parameters were measured 24h later. The control value for uroporphyrinogen decarboxylase activity was 0.21 nmol of coproporphyrinogen/mg of protein per h. Abbreviation used: n.d., not determined. Data are means of two, three and four determinations for uroporphyrinogen decarboxylase, ALA synthase and porphyrins respectively. Variations of individual values were within 10% of the mean value.

	Porphyrins		ALA synthase	Uroporphyrinogen
	(pmol/mg of protein per 24h)	Increase caused by TCDD (pmol/mg of per 24h)	(nmol of ALA/ mg of protein per h)	decarboxylase (% of control)
Control	5	—	0.67	100
TCDD (0.1 μM)	20	—	1.10	82
TCDD (1 μM)	165	—	1.56	47
Phenobarbital (1 mM)	216	—	16.3	84
Phenobarbital (1 mM) + TCDD (0.1 μM)	354	138	21.6	42
Phenobarbital (1 mM)) + TCDD (1 μM)	541	325	22.5	32
β-Naphthoflavone (18 μM)	38	—	1.29	104
β-Naphthoflavone (18 μM) + TCDD (0.1 μM)	48	10	2.19	69
β-Naphthoflavone (18 μM) + TCDD (1 μM)	224	186	2.50	n.d.

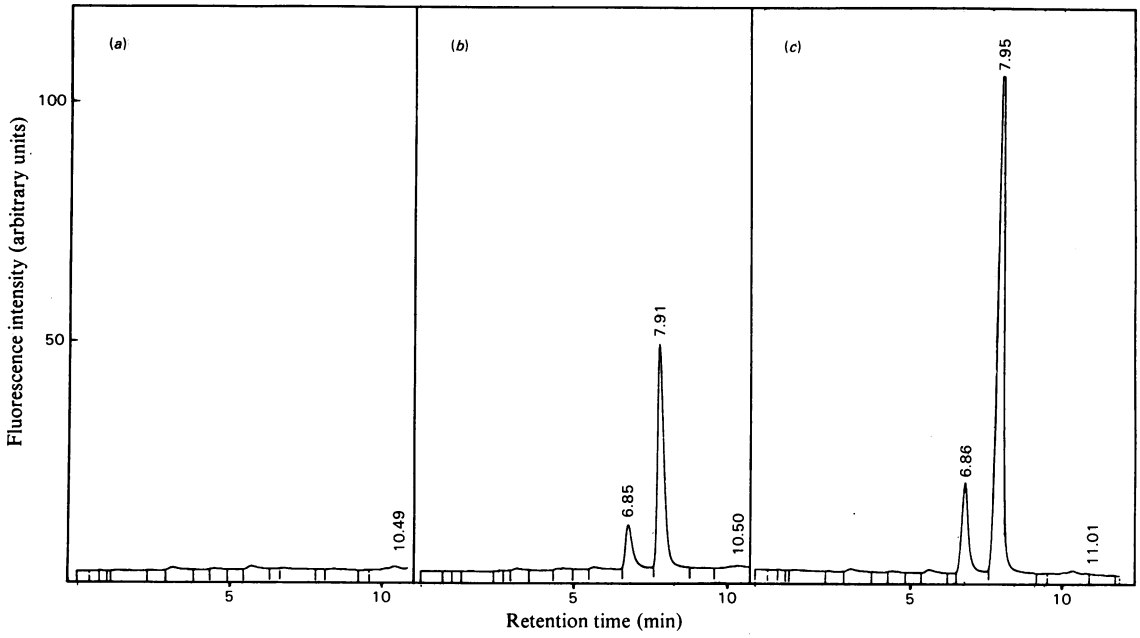

Fig. 5. *High-performance-liquid-chromatographic analysis of porphyrins accumulated in cultured liver cells after treatment with phenobarbital or PCB*
Chick-embryo liver cells were incubated with the compound for 24h. Porphyrin analyses were carried out as described in Fig. 2. (*a*), Control; (*b*), phenobarbital (1 mM); (*c*), PCB (30 μM-Aroclor).

in a dose-dependent manner after the chemical treatments. Inhibition of uroporphyrinogen decarboxylase and the increase of ALA synthase occurred within 4h of treatment, whereas significant porphyrin accumulation was observed only 8h after treatment.

Inhibition of hepatic uroporphyrinogen decarboxylase by polychlorinated hydrocarbons has been reported in rats *in vivo* (De Matteis *et al.*, 1961; Taljaard *et al.*, 1971; Elder *et al.*, 1976; San Martin de Viale *et al.*, 1977) and mice (Jones & Sweeney, 1977; Smith *et al.*, 1981). Use of a tissue culture

system of chick embryo liver cells maintained in a chemically defined medium allowed us to demonstrate the inhibitory effects of these environmental chemicals, and also of the drug phenobarbital, on uroporphyrinogen decarboxylase activity in the isolated liver cells. This inhibition remained at a constant level during the 24h incubation with the chemicals, whereas ALA synthase activity continued to rise (Fig. 1). These findings support the hypothesis (Elder, 1978) that the development of porphyria after PCB or TCDD exposure is primarily due to inhibition of uroporphyrinogen decarboxylase activity.

An unexpected finding of the present study was that phenobarbital could also significantly inhibit uroporphyrinogen decarboxylase activity and increase the accumulation of uroporphyrin and heptacarboxyporphyrin in liver cells. The porphyrinogenic action of phenobarbital has previously been attributed to a presumed effect on the induction of apo-(cytochrome *P*-450), which would in turn result in a depletion of the 'regulatory' haem pool leading to a derepression of ALA synthase (De Matteis, 1980). Our findings suggest the additional possibility that phenobarbital may induce ALA synthase by acting at the transcriptional level to increase the synthesis of mRNA for ALA synthase (for review, see Kappas *et al.*, 1982) or by inhibiting the activity of uroporphyrinogen decarboxylase, which could of itself lead to a diminution of the postulated regulatory haem pool for ALA synthase (Sassa & Kappas, 1981; Kappas *et al.*, 1982).

Clinical and experimental evidence suggests that iron plays an important role in the pathogenesis of PCT. For example, iron depletion by phlebotomy often leads to clinical and biochemical remission of the disease, and iron overload accelerates the onset of the related experimental disorder hexachlorobenzene porphyria in rats (Taljaard *et al.*, 1971). Iron has been reported to have variable effects on uroporphyrinogen decarboxylase activity in rat liver (Kushner *et al.*, 1975; Blekkenhorst *et al.*, 1979; Woods *et al.*, 1981). In our recent studies (de Verneuil *et al.*, 1983) we have shown that this metal has no direct inhibitory effect when added to the purified enzyme. In the present study, porphyrinogenesis after treatment of the cultured liver cells with PCB or TCDD was not affected by iron. Nevertheless, the addition of a chelator of the metal counteracted the PCB-induced accumulation of uroporphyrin (Table 2). This raises the possibility that exogenously and endogenously derived iron have differential roles in regulating uroporphyrinogen decarboxylase activity and the latter may be important in the development of the porphyria induced by these chemicals.

The mechanism(s) whereby polychlorinated hydrocarbons inhibit the activity of uroporphyrinogen decarboxylase in liver cells is not known. In the chick embryo liver cultures, it has been demonstrated that phenobarbital and β-naphthoflavone induce distinctive types of cytochrome *P*-450 (Sinclair *et al.*, 1979). Our results show that TCDD-mediated inhibition of uroporphyrinogen decarboxylase is exaggerated in phenobarbital-pretreated liver cells, but not in β-naphthoflavone-pretreated cells, suggesting that a specific phenobarbital-inducible cytochrome *P*-450 may be involved in the activation of TCDD. Kawanishi *et al.* (1981) have reported inhibitory effects of PCB on partially purified uroporphyrinogen decarboxylase. However, we were not able to demonstrate any inhibitory effect of PCB added either to homogenously purified uroporphyrinogen decarboxylase from human erythrocytes (de Verneuil *et al.*, 1983) or to homogenates from chick embryo liver cell cultures. TCDD added directly to homogenates from cultured chick embryo liver cells also did not inhibit the enzyme activity. On the basis of these findings the possibility is raised that the substances inhibitory to the enzyme may be generated in the liver from parent hydrocarbons such as PCB or TCDD, and that such metabolites themselves inhibit uroporphyrinogen decarboxylase activity.

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