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The Effect of 3,4,3′,4′-Tetrachlorobiphenyl on Plasma Retinol and Hepatic Retinyl Palmitate Hydrolase Activity in Female Sprague–Dawley Rats

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The Effect of 3,4,3′,4′-Tetrachlorobiphenyl on Plasma Retinol and Hepatic Retinyl Palmitate Hydrolase Activity in Female Sprague–Dawley Rats. POWERS, R. H., GILBERT, L. C., AND AUST, S. D. (1987). *Toxicol Appl. Pharmacol.* **89**, 370–377. A single ip dose of 1, 5, or 15 mg/kg 3,4,3′,4′-tetrachlorobiphenyl (TCB) caused a dose-dependent depression of plasma retinol levels 24 hr after treatment of female Sprague–Dawley rats. The loss of plasma retinol appeared to be a function of depressed levels of the retinol–retinol-binding protein (RBP)–transthyretin ternary complex. No free retinol–RBP was observed in plasma from treated animals. Hepatic retinyl palmitate hydrolase (RPH) activity was also depressed and highly and positively correlated to the plasma retinol levels. TCB was determined to be a noncompetitive inhibitor of partially purified RPH with a K_I of 91 μM. Incubation of TCB with liver microsomes and NADPH decreased the inhibition of RPH. Doses of either 2,4,5,2′,4′,5′-hexachlorobiphenyl (HCB) or 3,4,5,3′,4′,5′-HCB equimolar to the 15 mg/kg TCB dose failed to cause a similar depression of plasma retinol in treated female rats. We conclude that, unlike other polychlorinated biphenyl congeners, TCB causes a depression of plasma retinol by inhibition of hepatic RPH. © 1987 Academic Press, Inc.

3,4,3′,4′-Tetrachlorobiphenyl (TCB) is a member of the class of polychlorinated biphenyls (PCB), which have long been recognized as significant environmental contaminants. Many PCB mixtures, as well as certain individual congeners, are capable of causing a toxic response in animals similar to that caused by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; Poland and Knutson, 1982). TCDD-type toxicity typically results in a depletion of hepatic vitamin A reserves and symptoms characteristic of a vitamin A deficiency (Thunberg, 1983a). TCB causes a rapid depletion of hepatic retinoids in mice (Brouwer and van den Berg, 1983) similar to TCDD and the toxic PCB and PBB (polybrominated biphenyl) congeners (Innami *et al.*, 1974; Cecil *et al.*, 1973). In marked contrast to TCDD, however, TCB causes a rapid and pronounced depression of plasma vitamin A (retinol) levels in treated animals (Brouwer and van den Berg, 1984, Brouwer *et al.*, 1985). Also in contrast to TCDD and many of the toxic PCB congeners, TCB is subject to rapid metabolism presumably at least started by the hepatic cytochrome *P*-450 mixed-function oxidase system (Millis *et al.*, 1985).

In a recent paper, Brouwer and van den Berg (1986) proposed a mechanism for the depression of plasma vitamin A (retinol) levels caused by TCB. It was suggested that a metabolite of TCB, which has a high affinity for transthyretin (TTR), binds to and destabilizes the ternary complex consisting of retinol, retinol-binding protein (RBP), and

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transthyretin. This ternary complex is responsible for the transport of retinol in plasma (Goodman, 1984). Presumably, TCB-caused dissociation of the ternary complex allows the retinol–RBP binary complex to be filtered and subsequently degraded by the kidney.

An alternative explanation for the observed depression of plasma retinol levels following a TCB dose would be the inhibition, by either TCB or its metabolite, of the enzyme responsible for the release of retinol from stored retinyl esters within the hepatic parenchymal cells, retinyl palmitate hydrolase (RPH; Goodman and Blaner, 1984). Inhibition of this enzyme could limit the amount of retinol available for complexation with apo-RBP, normally present in excess in the liver (Goodman, 1984). Inhibition of RPH has been shown to correlate with depressed plasma retinol levels in rats treated with nonadecafluorodecanoic acid (NDFDA; Powers and Aust, 1986). NDFDA, while structurally dissimilar from PCB and dioxins, does cause a toxic response in rats similar in many respects to that caused by TCDD (Olson *et al.*, 1982).

We have therefore hypothesized that the primary mechanism by which TCB rapidly causes a depression in plasma retinol levels in treated rats is the inhibition of hepatic RPH. We have evaluated this hypothesis by determining the effect of a single ip dose of 0, 1, 5, or 15 mg/kg TCB to female Sprague–Dawley rats on plasma and hepatic retinol, renal retinol and retinyl palmitate levels, hepatic RPH activity, and the distribution of retinol among plasma protein fractions. In addition, we have examined the ability of TCB to inhibit partially purified RPH *in vitro* and the effect of microsomal metabolism on the ability of TCB to inhibit RPH. We have also examined the ability of two nonmetabolized PCB congeners, 2,4,5,2′,4′,5′- and 3,4,5,3′,-4′,5′-hexachlorobiphenyl (HCB) to cause depressed plasma retinol levels, when given to rats at a dose equimolar to that of the 15 mg/kg TCB dose.

METHODS

Animal treatment. Female Sprague–Dawley rats, 175–195 g, were procured from Charles River Co. (Portage, MI) and acclimated to standard rodent diet (Rodent-Blox(R); Wayne Feeds, Chicago, IL) and tap water *ad libitum.* Three rats were randomly assigned to each of four treatment groups. All rats were given a single ip dose of 3,4,3′,4′-tetrachlorobiphenyl (Analabs, North Haven, CT) at 0, 1.0, 5.0, or 15.0 mg/kg in corn oil (10 ml/kg). In a separate experiment, rats were similarly treated with 0 or 18.5 mg/kg of 2,4,5,2′,4′,5′- or 3,4,5,3′,4′,5′-hexachlorobiphenyl (Analabs). All rats were fasted overnight and killed 24 hr after dosing by exsanguination via cardiac puncture following CO_2 anesthesia. Blood was drawn into 7-ml EDTA Vacutainer tubes (Becton–Dickinson, Rutherford, NJ) and stored on ice. Plasma was prepared by centrifugation (1500*g*, 10 min at 4°C) and stored at −20°C until analyzed for retinol. Liver was perfused *in situ* with 0.9% NaCl, excised, and stored in the same solution until homogenization. Kidneys were excised, frozen on dry ice, and stored at −20°C prior to homogenization. Livers were weighed and homogenized in 4 ml 0.9% sodium chloride per gram wet weight in a Potter–Elvehjem homogenizer. Kidneys were later thawed, weighed, and homogenized in 9 ml 0.9% NaCl per gram wet weight, as per liver. Homogenates thus prepared were frozen and stored at −20°C prior to analysis for retinol and RPH activity.

Retinoid levels. Serum, hepatic, and renal retinol levels were determined as described previously (Powers and Aust, 1986). Briefly, a 200-μl aliquot of plasma or tissue homogenate was mixed with 2 ml of sodium chloride–saturated water and 2 ml of reagent-grade ethanol. Retinol was extracted into 1.0 ml of HPLC-grade hexane. Phase separation was facilitated by centrifugation (2500 *g*, 2 min). The supernatant was analyzed for retinol by HPLC using a fluorescence detector (Shimadzu RS-530-S, λ_{ex} = 330 nm, λ_{em} = 470 nm) and a 4 × 250-mm column packed with Lichrosorb SI-60 (Alltech, Deerfield, IL). Isocratic elution of retinol was performed using 25% hexane in chloroform (v/v). Retinol was eluted at a retention time of 5.5 min with a detection limit of 500 pg. Retinyl palmitate in 1.0 ml of kidney homogenate samples was determined by the same method, with the exception that the mobile phase used was 8% chloroform in hexane (v/v). Retinol and retinyl palmitate standards (Sigma Chemical Co., St. Louis, MO) were prepared in ethanol and verified by $E^{1\%}_{324\ nm}$ = 1835 and $E^{1\%}_{328\ nm}$ = 975, respectively (Windhols, 1976). HPLC standards of 1.0 and 0.1 ng/μl for both compounds were prepared by dilu-

tion of the stock standard with hexane. All standards were stored at −20°C and remained stable for at least 6 months.

Fractional distribution of retinol in plasma. Plasma proteins were separated on the basis of molecular weight with HPLC using a Spherogel TSK G3000 SW column (Beckman, Berkeley, CA) and a mobile phase of 1.15% potassium chloride, 0.04% sodium azide. Aliquots of plasma (100 μl) from each rat per treatment group were combined and mixed with an equal volume of mobile phase. Aliquots of 20 μl of the combined diluted plasma were injected into the HPLC and column effluent was monitored for absorbance at 280 nm with a Schoeffel SF770 uv/visible detector and for fluorescence (λ_{ex} = 330 nm, λ_{em} = 470 nm) with a Shimadzu RF-530-S detector, connected in series. The peak corresponding to the retinol–RBP–TTR complex was identified on the basis of retinoid fluorescence and the estimated molecular weight. The chromatographic system was standardized by the use of molecular weight marker proteins analyzed on separate chromatographic runs.

Analysis of RPH activity. RPH activity in hepatic homogenates was determined by a modification of the method described by Prystowsky *et al.* (1981). A 200-μl aliquot of the hepatic homogenate described above was added to 1.78 ml of Mops–cholate buffer (50 mM 3-(*N*-morpholino)-propane sulfonic acid, pH 7.2, 2.0% sodium cholate, 0.02% sodium azide) and 20 μl of 10 nmol/μl retinyl palmitate (in ethanol) and incubated for 20 min at 37°C in a shaking water bath. The reaction was terminated by the addition of 1 ml of ethanol and 1 ml of sodium chloride–saturated water. Retinol in the incubation mixture was extracted and quantitiated by HPLC as described above for plasma retinol. Samples were corrected for the amount of retinol present in unincubated samples. Protein concentration of the homogenate was determined by a bicinchoninic acid microassay (Redinbaugh and Turley, 1985).

Partial purification of RPH. Retinyl palmitate hydrolase was partially purified from rat livers as previously described (Powers and Aust, 1986). Briefly, an acetone-dried powder was prepared by homogenization of liver in acetone (0.02 g/ml) and removal of the acetone by evaporation. The acetone-dried powder was solubilized in Mops–cholate buffer at 2 ml/g original liver weight. Material precipitating between 33 and 70% ammonium sulfate saturation of this mixture was collected by centrifugation and resolubilized in and dialyzed against Mops–cholate and used in the experiments described below.

In vitro inhibition of RPH by TCB. The ability of TCB to inhibit RPH *in vitro* was evaluated by examining the effect of TCB on the kinetics of the hydrolysis of retinyl palmitate by the enzyme preparation described above. Control incubations were performed in triplicate with 200 μl of partially purified RPH (2.2 mg/ml) and substrate (retinyl palmitate) supplied at 0, 2.5, 10, 20, and 40 μM. The K_m and V_{max} were calculated from a Lineweaver–Burk plot of the data. The effect of TCB on RPH activity was determined by repeating the above assays in the presence of 100 μM TCB (added to the incubation buffer in 5 μl of ethanol). K_m and V_{max} for the inhibited RPH activity were calculated from a Lineweaver–Burk plot, as above.

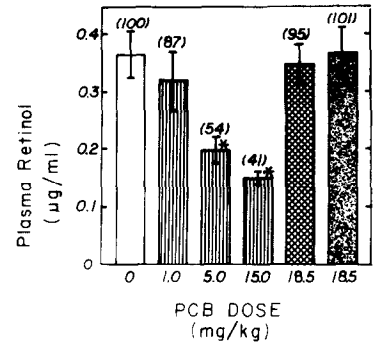

FIG. 1. Effect of a single ip dose of corn oil (□), or corn oil containing 3,4,3′,4′-tetrachlorobiphenyl (▥), 2,4,5,2′,4′,5′-hexachlorobiphenyl (▩), or 3,4,5,3′,4′,5′-hexachlorobiphenyl (⊡) on plasma retinol concentrations in female Sprague–Dawley rats 24 hr post-treatment. Each bar represents the mean ± SD of three rats and asterisks denote significant difference from controls ($p < 0.05$). Numbers in parentheses at the top of each bar represent the percentage of control value.

Effect of microsomal incubation on the ability of TCB to inhibit RPH in vitro. Microsomes were prepared from the livers of rats treated for 3 days at 40 mg/kg/day, po, with the 3-methylchloanthrene (MC)-type inducer β-naphthoflavone (BNF) as previously described (Millis *et al.*, 1985). Reaction mixtures consisted of 40 μl of resuspended microsomes (25 mg of protein/ml), 1 ml of PGE buffer (10 mM phosphate, pH 7.4, 20% glycerol, and 0.1 mM EDTA), TCB (100 μM), and/or 100 μl of an NADPH-generating system and were incubated for 1 hr at 37°C in a shaking water bath, under conditions previously shown to produce metabolic degradation of TCB (Millis *et al.*, 1985). Following this incubation, the mixture was evaluated for endogenous RPH activity by the addition of 100 μl of 20% sodium cholate and 20 μl of 10 nmol/μl retinyl palmitate and incubation in the dark at 37°C for 20 min in a shaking water bath. The reaction was terminated, retinol was extracted, and the amount of retinyl palmitate hydrolysis was determined as described above for RPH activity analyses.

Statistical analysis. Statistical analysis was performed using Student's *t* test with the Bonferroni correction for multiple comparisons (Godfrey, 1985). All references to statistical significance were at the $p < 0.05$ level.

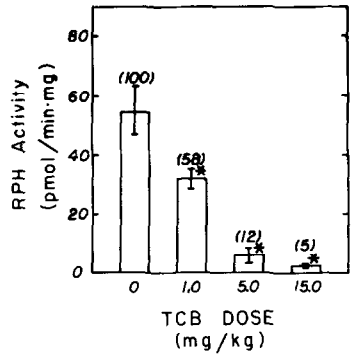

FIG. 2. Effect of a single ip dose of 3,4,3',4'-tetrachlorobiphenyl on the hepatic RPH activity of female Sprague–Dawley rats 24 hr post-treatment. Each bar represents the mean ± SD of three rats and asterisks denote significant difference from control animals ($p < 0.05$). Numbers indicated at the top of each bar represent percentage of control value.

RESULTS

Plasma, Hepatic, and Renal Retinoid Levels

A single ip dose of TCB caused, at 24 hr a depression in plasma retinol levels that was dose-dependent and significantly different from control rat levels at the two highest dose groups, as shown in Fig. 1. Neither the 2,4,5,2',4',5'- nor 3,4,5,3',4',5'-HCB congeners caused a similar depression of plasma retinol levels. No significant differences were noted in hepatic, renal, retinol, or renal retinyl palmitate levels in any of the groups treated with 3,4,3',4'-tetrachlorobiphenyl when compared to control group values (data not shown).

Hepatic RPH Activity

The TCB dose caused a significant and dose-dependent depression of hepatic RPH activity of all treated groups when compared to control group animals, as shown in Fig. 2. Further, the hepatic RPH activity was positively correlated ($R = 0.90$) with plasma retinol as shown in Fig. 3. This relationship could be considered as a linear (plasma retinol = $0.164 + 3.69 \times 10^{-3}$ (RPH activity); SD of fit = 3.56×10^{-2}) or a second-degree polynomial function (plasma retinol = $0.133 + 8.81 \times 10^{-3}$ (RPH activity) + 9.08×10^{-5} (RPH activity); SD of fit = 7.47×10^{-3}).

In Vitro Inhibition of RPH by TCB

TCB significantly inhibited the activity of retinyl palmitate hydrolase when included in an RPH assay incubation at 100 μM, as shown in Fig. 4. The K_m for retinol (8.6 μM), determined by analysis of a double-reciprocal plot of the substrate–velocity data, appeared to be unaffected by TCB. However, the apparent V_{max} (17 pmol/min–mg) was significantly lower than that for control incubations (41 pmol/min–mg), indicating that the inhibition was noncompetitive, with a K_I of 91 μM.

Fractional Distribution of Retinol Among Plasma Proteins

The analysis of combined plasma samples from the different treatment groups by HPLC yielded a fluorescent peak corresponding to

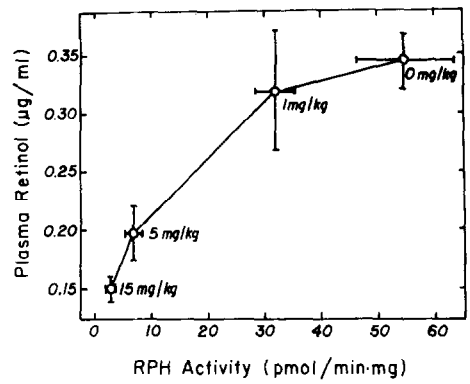

FIG. 3. Relation of plasma retinol levels and hepatic RPH activity in female Sprague–Dawley rats 24 hr following a single ip dose of 3,4,3',4'-tetrachlorobiphenyl. Each point represents the mean ± SD of three rats for each parameter.

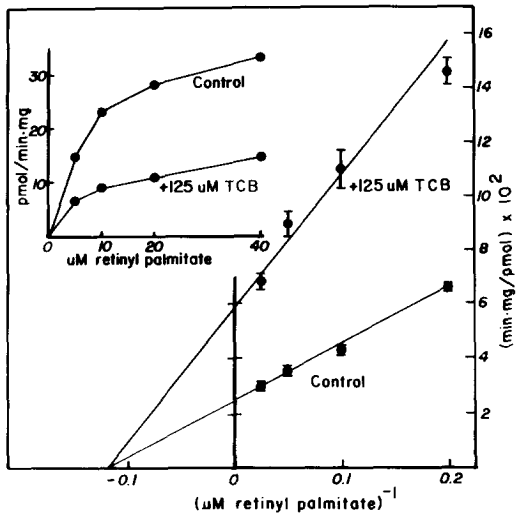

FIG. 4. Inhibition of RPH activity by 3,4,3',4'-tetrachlorobiphenyl. Values are means ± SD of 1/RPH activity (min·mg/pmol) versus 1/retinyl palmintate concentration (1/μM retinyl palmitate]) for three replicate RPH assays, as described under Methods. Insert values are the corresponding means ± SD of RPH activity (pmol/min·mg) versus retinyl palmitate concentration (μM retinyl palmitate) for three replicate assays.

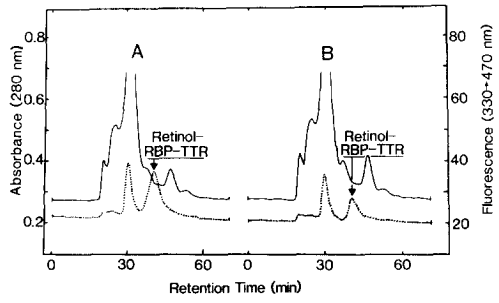

FIG. 5. HPLC analysis of combined plasma samples from rats with 0 (A) or 15 mg/kg (B) 3,4,3',4'-tetrachlorobiphenyl. Samples were 10-μl aliquots of combined plasma diluted 1:1 with mobile phase, as described under Methods. Column effluent was monitored for absorbance at 280 nm (—) and fluorescence at 330 nm excitation, 470 nm emission (· · ·).

the ternary complex of retinol–RBP–TTR. It appeared that the difference in retinol levels between the control and treated groups could be accounted for entirely by changes in the retinol–RBP–TTR peak (Fig. 5.). No peaks corresponding to free retinol–RBP were observed.

Effect of Incubation of TCB with Microsomes on the Inhibition of RPH in Vitro

Inhibition of endogenous microsomal RPH activity by TCB was significantly reduced by an initial incubation of the microsome–TCB mixture with an NADPH-generating system, as shown in Table 1.

DISCUSSION

Vitamin A uptake, storage, and mobilization is a highly regulated process, such that plasma retinol in normally nourished and healthy animals is maintained within well-defined limits (Underwood, 1984). Even in the case of a developing vitamin A deficiency, serum retinol levels tend to be maintained until hepatic retinyl ester stores are nearly exhausted (Underwood *et al.*, 1979). Therefore,

TABLE 1

THE EFFECT OF INCUBATION WITH MICROSOMES AND NADPH ON THE ABILITY OF 3,4,3',4'-TETRACHLOROBIPHENYL TO SUBSEQUENTLY INHIBIT ENDOGENOUS RETINYL PALMITATE HYDROLASE[a]

Incubation conditions	RPH activity: *pmol* min·mg ($\bar{x}$ ± SD)	RPH activity: % Control activity
+NADPH	37.1 ± 0.8	100
+TCB	8.15 ± 0.72[b]	22.0
+NADPH, TCB	32.8 ± 2.0	88.4

[a] Microsomes were incubated for 60 min at 37°C with an NADPH-generating system, TCB (100 μM), or both. RPH activity was measured in a subsequent assay following the addition of 100 μl sodium cholate (20%), as described under Methods.

[b] Significantly different ($p < 0.05$) from either the +NADPH or +NADPH, TCB samples.

the rapid and pronounced drop in plasma retinol levels in both rats and mice given a dose of TCB (Brouwer *et al.*, 1985, Brouwer and van den Berg, 1986) suggests that this PCB congener may provide an interesting means of investigating aspects of vitamin A mobilization, transport, and delivery.

Vitamin A is mobilized from the liver as retinol, bound to RBP, following the RPH-catalyzed hydrolysis of retinyl esters. The control mechanisms for this process have not yet been clearly elucidated (Goodman and Blaner, 1984). Prystowsky *et al.* (1981) found that hepatic RPH activity of control animals varied widely and did not correlate with serum retinol values, suggesting that RPH activity may not be a limiting factor in the control of serum retinol levels. However, Napoli and Beck (1984) demonstrated that RPH was subject to noncompetitive inhibition by vitamin E and phylloquinone. This suggested that inhibition of the enzyme could result in the depression of serum retinol levels by limiting the availability of retinol for complexation with apo-RBP.

Our hypothesis, that the mechanism by which TCB causes lowered plasma retinol levels is the inhibition of hepatic RPH, is supported by several observations. The strong, positive correlation of plasma retinol levels in both control and TCB-treated animals with hepatic RPH activity suggests that the enzyme activity and the depressed plasma levels are linked in a cause–effect relationship. Also, the dose-dependent depression of hepatic RPH activity observed in the treated animals supports the idea that TCB is directly responsible for that depression, as does the kinetic data showing that TCB causes noncompetitive inhibition of the enzyme *in vitro*. Further, incubation of TCB with microsomes, under conditions consistent with the metabolic degradation of TCB (Millis *et al.*, 1984), was shown to ameliorate the subsequent inhibition of RPH. These data suggest that it is the parent compound, rather than a metabolite, that is primarily responsible for the inhibition of RPH. This last point may partially explain the recovery of plasma retinol toward normal levels observed in rats given a single dose of TCB.

Another explanation for the decrease in plasma retinol has been recently published by Brouwer and Van den Berg (1986). They have suggested an interference, by either TCB or one of its metabolites, in the binding of holo-RBP to transthyretin. Such a destabilization of the ternary complex would presumably result in the removal of free plasma RBP–retinol by the kidney and its degradation or storage. A logical consequence of this theory would be the accumulation of retinoids in the kidney, since this organ effectively scavenges any free retinol–RBP complex, and is thought to catabolize RBP (Goodman, 1984). Curiously, both in vitamin A deficiency (Moore and Sharman, 1950) and TCDD toxicity (Thunberg, 1983b), there is a marked tendency of the kidney to store retinoids in the form of retinyl esters. We did not observe any increase in the concentration of retinoids in the kidneys of TCB-treated rats (data not shown), but the degradation of these species could be so rapid or the time course of this experiment too short to allow our observation of such an elevation.

An additional consequence of the above hypothesis would be the increase in the plasma concentration of free retinol–RBP, as a result of ternary complex dissociation. However, upon HPLC analysis of plasma from control and (15 mg/kg) TCB-treated rats, we did not observe any protein peak that would correspond to retinol–RBP. However, it should be noted that renal clearance of the RBP–retinol could be rapid enough to preclude observation of the free retinol–RBP.

TCB is one of the toxic PCB congeners, and like TCDD, has been shown to cause the depletion of hepatic retinyl esters and symptoms of a vitamin A deficiency in treated animals. The depressed plasma retinol levels caused by the inhibition of RPH may then hasten the onset or exacerbate the magnitude

of the vitamin A deficiency apparently caused by TCB and similarly acting compounds.

It is clear, however, that the depressed plasma retinol levels caused by TCB should not be thought of as being characteristic of TCDD-type toxicity for several reasons. The plasma retinol level depression following a dose of TCB is observed both in strains of mice that are considered sensitive (C57/BL6) and insensitive (DBA/2) to TCDD-type toxicity, while the hepatic retinoid depletion occurs only in the "sensitive" strains (Brouwer and van den Berg, 1984, 1985). Also, TCDD itself does not cause this phenomenon; instead it causes a temporary rise in plasma retinol levels (Thunberg *et al.*, 1979). Conversely, while several compounds which do not cause symptoms of dioxin-type toxicity (e.g., retinoic acid (Keilson *et al.*, 1979), endosulfan (Sriram and Misra, 1983), and cadmium (Sugawara and Sugawara, 1978) depress plasma retinol levels, NDFDA does cause a depression of plasma retinol levels and also causes some acute toxic effects similar to those caused by TCDD-type toxicity (Powers and Aust, 1986).

In summary, we have attempted to elucidate the mechanism for one of the toxic effects of the PCB congener 3,4,3′,4′-tetrachlorobiphenyl. Our findings suggest that TCB is an effective inhibitor of RPH activity both *in vitro* and *in vivo,* in addition to causing a depression of plasma retinol levels. This also points out a significant difference between the toxic response of rats to TCB and other PCB congeners. Additionally, the data demonstrate the consequences of the inhibition of hepatic RPH on the levels of the retinol–RBP–TTR ternary complex in plasma.

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