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Chemical Pharmacology, Vol. 26, pp. 2043–2047. Pergamon Press, 1977. Printed in Great Britain

IN VITRO INTERACTIONS OF DI-, TETRA- AND HEXA-CHLOROBIPHENYL WITH RABBIT LIVER MONOOXYGENASE

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(*Received 22 December 1976; accepted 31 March 1977*)

Abstract 2,4,6,2',4',6'-Hexa-, 2,5,2',5'-tetra- and 2,2'-dichloro-biphenyl (HCB, TCB and DCB) caused a type-I-binding spectrum with liver microsomes of phenobarbital treated rabbits. They had a high affinity to cytochrome P-450, which increased with decreasing chlorine content of the compounds. In parallel, the *O*-demethylation of *p*-nitroanisole was most effectively inhibited by DCB, to a smaller extent by TCB and HCB. Inhibition was competitive. While DCB was rapidly metabolized, metabolism of TCB was slow. No metabolites have been detected of HCB. Although HCB was not metabolized it stimulated the microsomal NADPH- and oxygen-consumption and also the reduction of cytochrome P-450. The stoichiometry of the NADPH- and O_2-consumption of 2:1 in the presence of HCB is compatible with the notion that NADPH is oxidized to H_2O, i.e. HCB may act as uncoupler of electron transport from monooxygenation.

Polychlorinated biphenyls (PCBs) are potent inducers of the hepatic monooxygenase(s) in mammals and birds and their stimulatory effect on the metabolism of numerous drugs has been documented [1–3]. Some observations indicate that PCBs may inhibit drug metabolism as well [4–6]. Fuhremann and Lichtenstein [4] observed that the technical PCB-mixture Aroclor 1248 decreased the toxicity of parathion and increased the toxicity of paraoxon in house flies. The toxic effects of parathion on insects are primarily due to the formation of paraoxon in the insect body [7]. Therefore the Aroclor effects have been interpreted as an inhibition of the *in vivo* conversion of parathion to paraoxon and the detoxification of paraoxon itself. Reinmoldt *et al.* [5] found the *p*-nitroanisole-*O*-demethylase to be competitively inhibited by Aroclor 1248 in rat liver microsomes. They also observed that after administration to rats PCBs can reach levels in the liver which are inhibitory in microsomes *in vitro*. Aroclors of differing degrees of chlorination caused a biphasic effect on pentobarbital sleeping time in Japanese quail [6]. During the initial phase they exerted a prolongation of the pentobarbital anaesthesia which the authors attributed to a depressed drug metabolizing enzyme activity.

The investigations on the inhibitory effects of PCBs have been performed with technical mixtures of low and high chlorinated compounds and it has not been determined which component of the mixtures is the inhibitory agent. Since the lower chlorinated compounds are substrates of the monooxygenase [8–10] it is likely that they will compete with endogenous and exogenous substrates of this enzyme system. The inhibitory activity of the higher chlorinated biphenyls, which are poorly or not at all metabolized by the monooxygenase [11], is more difficult to assess. They might be inert as inhibitors due to their virtual lack of metabolism. However it appears possible that they bind to the microsomal oxidase without being catalytically converted and function as inhibitors of the classical competitive type.

Binding of substrates to the terminal oxidase, cytochrome P-450, may involve an increased rate of reduction of the cytochrome-substrate complex [12] and is followed by the consumption of NADPH and O_2 [13]. This may also occur with compounds that bind to the enzyme but are not subjected to catalytic conversion as shown by Ullrich and Diehl for perfluorohexane [14]. The highly chlorinated biphenyls may belong to this type of compounds.

In the present investigations 2,2'-di-, 2,5,2',5'-tetra- and 2,4,6,2',4',6'-hexa-chlorobiphenyl served as model compounds to examine the relationships between chlorine content of the molecule, binding to cytochrome P-450, metabolism and inhibitory properties. Furthermore we studied the effects of the biphenyls on the reduction of cytochrome P-450 and on NADPH- and O_2-consumption in liver microsomes of phenobarbital pretreated rabbits.

MATERIALS AND METHODS

Chemicals. *p*-Nitroanisole was purchased from Fluka, Buchs, Switzerland and recrystallized before use. Enzymes and coenzymes were obtained from Boehringer, Mannheim, Germany. We gratefully acknowledge the synthesis of the three pure chlorinated biphenyls, DCB, TCB, HCB, by the Institut für Ökologische Chemie, Gesellschaft für Strahlen- und Umweltforschung, München, Germany. Benzphetamine was a gift of The Upjohn Comp., Kalamazoo, MI, USA. Metyrapone and SKF 525-A (2-diethylaminoethyl 2,2'-diphenylvalerate) were gifts of Dr. Hildebrandt, Freie Universität Berlin, resp. Dr. Kampffmeyer, Universität München, Germany. All

Abbreviations. PCB polychlorinated biphenyl; DCB dichloro-biphenyl; TCB 2,5,2',5'-tetrachloro-biphenyl; 2,4,6,2',4',6'-hexachloro-biphenyl; ICDH isocitrate dehydrogenase (EC 1.1.1.42).

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other chemicals were of analytical grade and were products of E. Merck, Darmstadt, Germany.

Animals. Experiments were conducted with male rabbits of the "White Russian" strain (Fa. Gassner, Sulzfeld, Germany, or our own animal breeding station). All animals received water and a normal laboratory diet (Altromin®, Lage, Lippe, Germany) *ad lib*. The animals were pretreated with phenobarbital (50 mg/kg per day i.p.) for 10 days.

Microsomes. Rabbits were killed by a blow on the neck and exsanguination. After perfusion with ice-cold saline the livers were minced with scissors, further disrupted by a tissue press [15] and homogenized in a Potter Elvehjem homogenizer in a 4-fold volume of 0.25 M sucrose. Centrifugation was performed as described [16]. The pellets of the last centrifugation were resuspended and recentrifuged in 0.15 M KCl. The final pellets were suspended in sufficient volumes of 0.05 M Tris·HCl buffer, pH 7.5, containing 0.25 M sucrose to obtain a protein concentration of 10–20 mg/ml. The suspensions were frozen in liquid nitrogen and stored at −22° until use. Protein concentration was determined by the modified biuret-method described by Szarkowska and Klingenberg [17], with dried bovine serum albumin (Behring Werke, Marburg, Lahn) as standard. The turbidity arising from insoluble material in this method is eliminated by subtracting the absorption after decolorisation with KCN from the initial reading at 546 nm.

Solubilisation of the polychlorinated biphenyls. Ultrasonic suspensions of DCB, TCB and HCB were prepared, since organic solvents or detergents like Tween 80, which are frequently used for solubilisation may inhibit microsomal hydroxylase activity [18].

Approximately 20 mg of PCBs were dissolved in 50 µl of chloroform in a test tube and 5 ml water added dropwise. The chloroform solution at the bottom was sonified for 10–20 sec by the microtip of a Branson sonifier at 50 W output. After evaporating the chloroform and filtering, a suspension being stable for at least 15 min was obtained. The concentration was determined by the u.v. absorption in methanol.

Microsomal assays. If not stated otherwise microsomal incubations were carried out with 1 mg microsomal protein per ml of 0.1 M potassium phosphate buffer, pH 7.5, containing 5 mM $MgCl_2$, 10 mM isocitrate and 0.4 units of ICDH.

Assay of p-nitroanisole-O-demethylation. *p*-Nitroanisole-O-demethylation was determined by the method of Netter and Seidel [19], with the exception, that *p*-nitroanisole was omitted from the reference cuvette, and the reaction was started by addition of 0.13 µmoles of NADP in 0.01 ml H_2O to both cuvettes. When inhibitors were used, these were added to the microsomes before incubation. The absorption increase at 420 nm due to the formation of *p*-nitrophenol (E_{420nm} = 12.7 $mM^{-1} \cdot cm^{-1}$) was followed with a Hitachi Model 556 spectro-photometer. I_{50} values were determined at a *p*-nitroanisole concentration of 0.2 mM. I_{50} values were obtained by plotting the percentage of inhibition versus the logarithm of the inhibitor concentrations. Five to ten concentrations of each inhibitor were used.

Microsomal metabolism of DCB, TCB and HCB. Ultrasonic suspensions of the PCBs were incubated with liver microsomes and the NADPH-regenerating system in a shaking water bath at 33° for 10 min. In a total volume of 80 ml the substrate concentrations were 0.1 mM DCB, 0.03 mM TCB and 0.05 mM HCB. Extraction with ethyl acetate terminated the reaction. The solvent was evaporated and after thin layer chromatography on Kieselgel G (Merck, Darmstadt) with chloroform as solvent, the phenolic metabolites were stained with $FeCl_3$-K_3-$Fe(CN)_6$ reagent. After elution, u.v.-spectra in methanol were recorded and the bathochromic shift of the maximal wavelength was observed after addition of NaOH.

NADPH-oxidation. NADPH-oxidation was measured determining spectrophotometrically the absorbance decrease at 340 nm with a Zeiss PMQ II spectrophotometer. The reaction mixture contained 0.2 or 0.4 mg protein/ml and 0.25 mM NADPH instead of the NADPH-regenerating system. After recording the NADPH-oxidation due to endogenous substrate for 3–5 min, 0.05 to 0.10 ml of an aqueous suspension of DCB, TCB or HCB were added to a final concentration of 0.1 mM.

Oxygen consumption. The oxygen consumption was measured with an oxygen electrode (YSI model 53 Biological Oxygen Monitor, Yellow Springs Instrument Co.). To 3.0 ml of the microsomal incubation 0.05 to 0.1 ml of an aqueous suspension of DCB, TCB or HCB were added to a final concentration of 0.1 mM or an adequate volume of aqua dest. After temperature equilibration to 33° the reaction was started by 0.39 µmoles NADP in 0.03 ml H_2O.

Lipid peroxidation. Microsomes were incubated in the presence or absence of NADP (0.13 mM) for various times up to 20 min. Lipid peroxidation was estimated by measuring the formation of malondialdehyde with the thiobarbituric acid reagent as described by Placer *et al.* [20].

Cytochrome P-450. Cytochrome P-450 content was calculated from the carbon monoxide difference spectrum of reduced microsomes ($E_{450} - E_{490nm}$) using an extinction coefficient of 91 $mM^{-1} \cdot cm^{-1}$ [21]. The rate of cytochrome P-450 reduction was determined using a Gilford 240-spectrophotometer according to Gigon *et al.* [12] as modified by Diehl *et al.* [22].

Substrate-induced difference spectra. Difference spectra were recorded using a Hitachi Model 556 spectrophotometer. The microsomal suspension contained 1.5 mg protein/ml. After a baseline had been recorded an aqueous suspension of the PCB compound (1–100 µM final concentrations) was added to the sample cell. Corresponding volumes of water were added to the reference cell. The difference spectra were recorded between 500 and 370 nm.

RESULTS

Interaction of polychlorinated biphenyls with the type I binding site of the microsomal cytochrome P-450. Addition of DCB, TCB and HCB to microsomes caused a characteristic type-I-spectrum with a maximum at 385 nm and a minimum at 423 nm. With equimolar concentrations of HCB and DCB the difference spectra were similar (Fig. 1). Figure 2 represents the Lineweaver–Burk plot of DCB-, TCB- and HCB-binding to microsomes. The maximal spectral shift caused by the three PCBs was independent of

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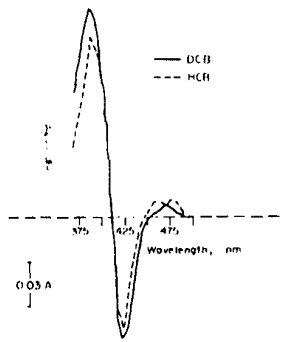

Fig. 1. Substrate binding spectra of rabbit liver microsomes with 0.1 mM DCB and HCB. For assay conditions see Methods.

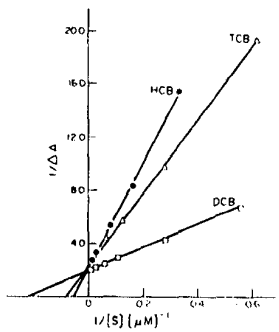

Fig. 2. Determination of the spectral dissociation constants (K_s) of DCB, TCB and HCB by Lineweaver Burk plots.

the chlorine content as is indicated by the common intersection of the straight lines with the ordinate. For all three compounds a low spectral dissociation constant (K_s) was determined indicating a high affinity to cytochrome P-450. The K_s values increased with increasing number of chlorine atoms. DCB had the lowest K_s value of about 5 μM. The K_s value of TCB (13 μM) was more than 2-fold higher while that of HCB (18 μM) was 3–4 times that of DCB.

Inhibition kinetics of the p-nitroanisole-O-demethylation. The *O*-demethylation of *p*-nitroanisole was chosen to investigate PCB mediated inhibition of the metabolism of a type-I-substrate, because the initial velocities of the formation of *p*-nitrophenol can be determined [19]. With liver microsomes of phenobarbital treated rabbits inhibition of *p*-nitroanisole-*O*-demethylase activity by TCB is competitive as shown by Lineweaver Burk and Dixon plots (Fig. 3). The low K_i of 7 μM for TCB compared to the K_M of 40 μM for *p*-nitroanisole-*O*-demethylation indicates that TCB is an effective inhibitor. When DCB was used, Lineweaver-Burk plots also showed competitive inhibition, when initial velocities were determined during the first 2–4 min.

The effect of DCB on the *p*-nitroanisole-*O*-demethylation was stronger than that of TCB as shown by the I_{50} values in Table 1. Twelve μM of DCB were sufficient to cause a 50 per cent inhibition, whereas 28 μM of TCB and 140 μM of HCB were necessary to cause the same effect. Thus inhibition was stronger with the lower chlorinated compounds DCB and TCB than with HCB. The inhibition of *p*-nitroanisole-*O*-demethylation was compared to the effect of two known inhibitors of monooxygenases, metyrapone and SKF 525-A. The I_{50} value of metyrapone (3 μM) was lower than those of all three PCBs whereas SKF 525-A with an I_{50} value of 100 μM was in the range of HCB.

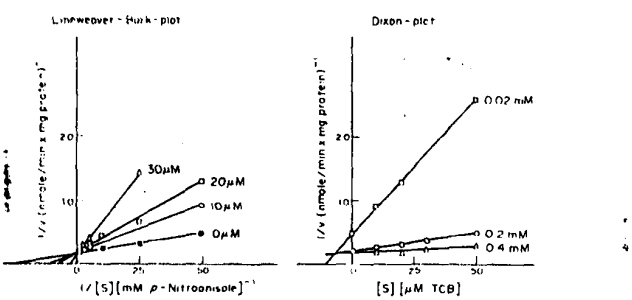

Fig. 3. Inhibition of *p*-nitroanisole-*O*-demethylation by TCB in liver microsomes. Incubation mixtures contained 1 mg protein in a final volume of 1 ml 0.1 M potassium phosphate buffer, pH 7.85.

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Table 1. *In vitro* inhibition of *p*-nitroanisole-*O*-demethylase in rabbit liver microsomes

Inhibitor	I_{50} (μM)*	*n*
DCB	12† ± 6	(4)
TCB	28 ± 6	(5)
HCB	140 ± 17	(3)
SKF 525 A	100 ±	(1)
Metyrapone	3 ± 2	(3)

* mean ± S.D.

† *p*-nitroanisole concentration = 0.2 mM.

In vitro metabolism of DCB, TCB and HCB. Incubation of DCB with microsomes yielded two metabolites which could be distinguished by thin layer chromatography and exhibited R_f values of 0.5 and 0.25 with chloroform as developing agent. The bathochrome shift of the maximal u.v.-absorption after addition of NaOH and the molecular peak of 238 in the mass spectra established their identities as monohydroxy-derivatives of DCB. Data of others indicate [23] that the rate of DCB hydroxylation is about 2/3 of that of biphenyl, which under conditions comparable to ours is metabolized at a rate of 3 nmoles·min^{-1}·mg protein [24].

Using TCB as substrate again two phenolic metabolites with R_f 0.6 and 0.25 were detected. Two monohydroxy products have also been isolated from rabbits fed TCB [9]. On the basis of the maximal u.v.-absorption of the DCB- and TCB-metabolites the hydroxylation rate of TCB appeared to be 10 per cent of that of DCB.

No phenolic metabolites were detected with HCB as substrate. This agrees with the very slow metabolism *in vivo* of hexachlorobiphenyls [11, 25].

NADPH- and oxygen-consumption in the presence of PCBs. The endogenous rate of NADPH consumption was 8.7 nmoles·min^{-1}·mg protein. It was increased by 260 per cent in the presence of DCB and 160 per cent by TCB and by HCB (Fig. 4).

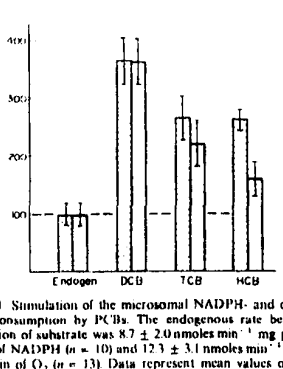

Fig. 4. Stimulation of the microsomal NADPH- and oxygen-consumption by PCBs. The endogenous rate before addition of substrate was 8.7 ± 2.0 nmoles·min^{-1}·mg protein of NADPH (n = 10) and 12.3 ± 3.1 nmoles·min^{-1}·mg protein of O_2 (n = 13). Data represent mean values of at least 6 determinations ± standard deviations. Hatched bars = NADPH, dotted bars = O_2.

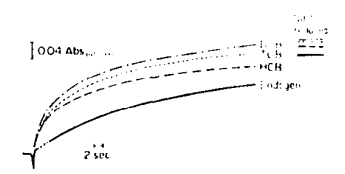

Fig. 5. Effects of DCB, TCB and HCB on the NADPH-cytochrome P-450 reductase. The reaction mixture contained per ml: 1.2 mg protein, 0.9 mM glucose, 60 U catalase in 0.05 mM Tris HCl buffer. In a special anaerobic mixing cuvette (Aminco, Silverspring, MD, USA) the solution was gassed for 5 min with carbon monoxide. 100 μg of glucose oxidase and an aqueous suspension of the PCB compound were added equivalent to a final PCB-concentration of 0.1 mM. The cell was gassed again with carbon monoxide. After temperature equilibration to 30° the reaction was started with 0.1 μmoles of NADPH. The rate of cytochrome P-450 reduction was recorded by the absorbance increase at 450 nm with a Gilford 240 spectrophotometer.

The rate of oxygen consumption (endogenous rate 12.3 nmoles·min^{-1}·mg protein) was increased in the presence of DCB by 270 per cent, of TCB by 120 per cent and of HCB by 60 per cent above the endogenous rate. Thus, the cofactor consumption due to HCB was 7 nmoles·min^{-1}·mg protein of O_2 and 14 nmoles·min^{-1}·mg protein of NADPH, resulting in a NADPH/O_2 ratio of 2:1. This additional NADPH- and O_2-consumption was not a consequence of a stimulated lipid peroxidation. In agreement with other reports [26] lipid peroxidation was nearly absent in rabbit liver microsomes and was not stimulated by PCBs (data not shown).

The reduction kinetics of cytochrome P-450 show that the first rapid phase is increased by all three PCBs (Fig. 5). This increase, which is dependent on the chlorine content, indicates, that in the presence of HCB as well as of the other PCBs, the electron flow to the cytochrome is enhanced. This corresponds to the higher NADPH- and oxygen utilization.

DISCUSSION

The three chlorinated biphenyls investigated inhibited the *p*-nitroanisole-*O*-demethylation of rabbit liver microsomes. This inhibition appeared to be inversely correlated with the chlorine content of the molecules. Concentrations of 12 μM DCB and 28 μM TCB inhibited the *p*-nitroanisole-*O*-demethylation by 50 per cent, while 140 μM of HCB were required for the same effect (Table 1). Comparison with the commonly used inhibitors metyrapone and SKF 525-A shows a similar inhibitory potency of the PCBs.

The inhibition was competitive (Fig. 3), suggesting that the PCBs combine reversibly at the active site of the enzyme. However, only DCB was rapidly metabolized and rather small amounts of phenolic products were detected of TCB, in agreement with results of others [9]. No phenolic metabolites were found with HCB.

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The binding spectra (Figs. 1 and 2) indicate that the three PCBs form an enzyme-substrate complex [27]. At saturating concentrations the magnitude of the binding spectra with DCB, TCB and HCB were quantitatively and qualitatively similar. The affinity constants however were different depending on the chlorine content. The highly chlorinated compounds had a lower affinity as indicated by higher K_S values and this was paralleled by lower inhibitory effects. The data suggest that the capability of the PCBs to inhibit metabolism of type-I-substrates is due to their high affinity to cytochrome P-450.

Although *in vitro* the inhibitory potency decreases with increasing degree of chlorination, *in vivo* the interference with monooxygenase activity by higher chlorinated homologues might be of greater significance. As the highly chlorinated compounds are not or only very slowly metabolized by the oxidase [11, 25] their inhibitory action may be quite persistent.

Addition of DCB and TCB as well as of HCB caused an increase in oxygen- and NADPH-consumption, although HCB was not metabolized by the oxidase (Fig. 4). We do not know the fate of the oxygen and reducing equivalents in the presence of HCB in microsomes. The increased O_2- and NADPH-consumption seems not to be due to an enhanced ratio of lipid peroxidation (Hesse, unpublished results). It is possible that HCB caused an uncoupling of the microsomal electron flow from the oxygenation of the substrate, which has been postulated for perfluorohexane by Ullrich and Diehl [14]. Substraction of the endogenous rate of O_2- and NADPH-consumption from those in the presence of HCB yields a consumption of 14 nmoles·mg protein^{-1}·min of NADPH and 7 nmoles·mg protein^{-1}·min of O_2, i.e. a ratio of 2:1. This ratio is compatible with the formation of H_2O as suggested to occur during the uncoupling reaction caused by other compounds [14]. However since the degree of involvement of cytochrome P-450 in the endogenous consumption of O_2 and NADPH is not known, this quantification has to be viewed with reservation. The interpretation of the data of TCB and DCB is further complicated by the observation that the ratio of NADPH:O_2 decreases below 1.0 with increasing degree of metabolism of the chlorinated biphenyls. The reason for this phenomenon is not clear. In view of the relatively low metabolism of TCB and DCB a partial uncoupling should lead to a ratio higher than one. It is possible that secondary oxidation of the PCB metabolites consumes additional O_2 thus lowering the NADPH:O_2 ratio.

The involvement of cytochrome P-450 in the "uncoupled" consumption of NADPH (and O_2) is suggested by the observation that the rate of cytochrome P-450 reduction is enhanced in the presence of the non-metabolized HCB (Fig. 5).

It remains to be established whether the drain of reducing equivalents in the presence of highly chlorinated PCBs observed *in vitro* plays a significant role *in vivo* and contributes to the toxicity of these compounds.

Acknowledgements—The authors wish to thank Mrs. M. Merzger, Mrs. H. Wanders and Mr. J. Lichtmannegger for their skilful technical assistance and are indebted to Dr. H. Greim and Dr. F. Wiebel for helpful criticism and advice. Mr. Staudacher is thanked for registration of mass spectra.

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