Thyroid hormone binding proteins as novel targets for hydroxylated polyhalogenated aromatic hydrocarbons (PHAHs): possible implications for toxicity

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Front cover: Transthyretin with hydroxylated PCB metabolites in binding site, general view

Back cover: Transthyretin with hyroxylated PCB metabolites, view towards binding channel

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Stellingen

1 De structurele overeenkomst van hydroxy-metabolieten van PCBs, PCDDs en PCDFs met het schildklierhormoon verklaart de selectieve retentie in het plasma via binding aan TTR (Dit proefschrift).

2 De aanwezigheid van een *meta*- of *para*-hydroxy-groep met aangrenzende chlooratomen vormt een voorwaarde voor binding van hydroxy-metabolieten van PCBs, PCDDs en PCDFs aan TTR (Dit proefschrift).

3 De waterstofbrug-vorming tussen de hydroxygroep van een PCB metaboliet en het aminozuur-residu Serine 117 in de bindingsplaats van het TTR, verklaart de sterkere binding van deze hydroxy-PCB metaboliet dan thyroxine aan TTR (Dit proefschrift).

4 Tot dusver zijn de *in vivo* effecten van hydroxy-PCB metabolieten, waarvan is aangetoond dat zij selectief in het bloed van verschillende diersoorten en de mens aanwezig blijven, onderbelicht.

5 Voor de gangbare hypothese dat de toxische werking van dioxines en verwante stoffen in hoofdzaak tot stand komt via Ah receptor-gemedieerde processen, is de mechanistische onderbouwing mager.

6 Het toeschrijven van de afname van de gemiddelde spermaproductie van de man aan de blootstelling aan oestrogene stoffen in het milieu is voorbarig.
(Skakkebaek en Sharpe (1993), Lancet 341, 1392-1395).

7 Bij het leggen van claims voor de gezondheidsbeschermende werking van individuele voedingscomponenten wordt te vaak voorbijgegaan aan de mitigerende invloed van de voedselmatrix.

8 Het gebruik van niet gevalideerde modellen in toxicologisch onderzoek draagt bij aan onjuiste beeldvorming omtrent de gevaren van chemische stoffen in de samenleving.

9 Het ongegrond in stand houden van een "milieuprobleem" teneinde onderzoeksgelden te vergaren, mag als onethisch worden beschouwd.

10 De betekenis van het woord mens als "redelijk aards wezen" (Prisma Woordenboek Nederlands) gaat in deze samenleving niet op.

11 Zingeving is vaak uit de lucht gegrepen.

12 Ook aan het gedrag van mannen tijdens wetenschappelijke congressen waaraan ook vrouwen deelnemen (waaronder het jaarlijkse Dioxine congres), is niet te merken dat de milieuverontreiniging met oestrogene stoffen van invloed is op de mens.

13 There are two kinds of silence. One in which no word is spoken. The other where perhaps a torrent of words is employed.
(H. Pinter)

Stellingen behorende bij het proefschrift "Thyroid hormone binding proteins as novel targets for hydroxylated polyhalogenated aromatic hydrocarbons (PHAHs): possible implications for toxicity", door Martine C. Lans, te verdedigen op dinsdag 17 Oktober, 1995 te Wageningen.

Creatie

Uit 'n overrompelende slaap ontwaakt
in de holen van de nacht.
De muren suizen. Spiegel die blaakt
van een geheimzinnig vuren op haar ziel.
Uit welk heelal? En met welk licht?
Wat wordt hier al uren verricht?
Er viel
iets uit de hoeken naar omlaag.
De kamer snikt, omdat er traag
en genadeloos iets uit haar glijdt,
dat zich genadeloos bevrijdt,
waarna de lege morgen daagt.

Gerrit Achterberg (1905-1986)

Contents

CHAPTER 1

General introduction

General introduction

Over the past decades extensive research was performed to elucidate the toxic mechanism of polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs) and biphenyls (PCBs). These compounds belong to the class of polyhalogenated aromatic hydrocarbons (PHAHs) and cause a broad range of toxic effects in different species, eg. dermal, immuno- and hepato-toxicity, carcinogenic, teratogenic, neurobehavioural and endocrine effects as well as diverse biochemical responses, like the induction of several drug metabolizing enzymes (for reviews see: Ahlborg *et al.*, 1992, Safe, 1994, 1992, 1990, Van den Berg *et al.*, 1994, Peterson *et al.*, 1993, McConnell, 1980, Brouwer *et al.*, 1995).

A common mechanism of action, the Ah-receptor pathway, has already been described in detail (Poland and Knutson, 1982). The interaction of PHAHs with this Ah-receptor can result in induced expression of numerous Ah-receptor responsive genes, which may result in the observed toxic effects. In addition, effects of PHAHs have recently been described that may not be directly mediated through the Ah-receptor pathway (Ahlborg *et al.*, 1992; Brouwer, 1991). Among the observed effects of PHAHs were changes in endocrine systems, essential for many physiological functions. In this thesis particular emphasis will be put on the interference of PHAHs and especially their hydroxylated metabolites with the thyroid hormone endocrine system.

Polyhalogenated aromatic hydrocarbons: PCDDs, PCDFs and PCBs

Sources and structures

As early as in the 1960s PCBs were discovered in environmental samples (Jensen *et al.*, 1966) and diverse wildlife species like cormorants (Koeman *et al.*, 1969). Since then numerous studies reported the occurrence of PCDDs, PCDFs and PCBs in the global environment including air, water, sediments, fish, wildlife and human tissues (McFarland and Clarke, 1989, Rappe and Buser, 1989, Duinker *et al.*, 1989, Kannan *et al.*, 1989). Long range atmospheric and oceanic transport are important pathways of global (re)distribution of PHAHs (Tanabe and Tatsukawa, 1986), leading to the ubiquitous presence throughout the world, including Arctic and Antarctic ecosystems (Norstrom *et al.*, 1990).

PCDDs and PCDFs are entering the environment mainly as unwanted persistent byproducts in industrial chemicals like chlorophenols, chlorophenoxy-herbicides and PCBs that are still in use (Rappe *et al.*, 1991) and by combustion processes of chlorinated organic- or inorganic compounds in municipal or hospital waste incinerators and car engines (Hutzinger and Fiedler, 1989). The wide spread presence of PCDDs and PCDFs in environment and foodstuffs for human consumption, like cow's milk, led to

strict regulations on the emission of PCDDs and PCDFs from municipal and chemical waste incinerators in the Netherlands. Unlike PCDDs and PCDFs, PCBs mixtures have been commercially produced and used for industrial purposes from the early 1930s. The intrinsic properties that made PCBs suitable for the use in transformers and capacitors, eg. inflammability, chemical stability and dielectric properties, simultaneously led to the bioaccumulation through the foodchain of these persistent lipophilic compounds to high and sustained levels in top-predators like humans, sea-mammals, fish eating birds (McFarland and Clarke, 1989). The bioaccumulation and long half life of certain PCBs and simultaneously growing knowledge on their potential toxicity, resulted in the ban of production of PCBs in the early eighties.

However, it is estimated that two-thirds of PCBs produced worldwide are still in use or entering the environment through leakage from closed systems and disposal of PCB-contaminated materials so their environmental levels are unlikely to decline fast in the near future (Loganathan and Kannan, 1994). In addition to the industrialized countries in Europe, North America and Japan, PCBs are also detected in food for human consumption in developing countries. Nevertheless, declines in PCB levels were documented in environmental compartments, like some foods for human consumption, human adipose tissue, shellfish and various fish species especially between the late 1970s and the mid-1980s. These declines are expected to continue but at much slower rates (Fensterheim, 1993). Still, the main source of human exposure to PHAHs is via the diet.

All PCDD, PCDF and PCB structures consist of two halogenated phenylrings, with resp. 75, 135 and 209 possible congeners dependent on the degree and place of chlorine substituents (Fig. 1). PCDDs and PCDFs are rigid planar structures, while the two phenyl rings of the PCBs can rotate along the central C-C bond.

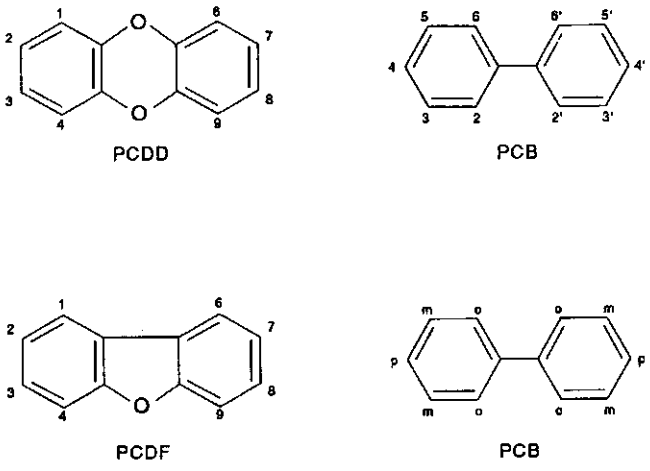

Figure 1 Structure and ring positions of PCDD (polychlorodibenzo-*para*-dioxin), PCDF (polychlorodibenzofuran) and PCB (polychlorobiphenyl). o = *ortho*, m = *meta*, p = *para*.

Non-*ortho*-substituted PCBs can adapt a planar conformation and have a 3-methylcholantrene (3-MC) type of induction of biotransformation enzymes, like cytochrome P450 1A1 and 1A2 isoenzymes. *Ortho*-substituted PCB congeners with chlorine substituents on two or more *ortho*-positions may preferably adapt a non-planar conformation and consequently are phenobarbital (PB) type of inducers of biotransformation enzymes, like cytochrome P450 2B isoenzymes. Mono-*ortho* substituted PCBs can adapt both co-planar and non-planar conformations (mixed-type inducer). The non-planarity of PCBs is dependent on the degree of chlorine substituents on the *ortho*-positions of the phenylrings; the more *ortho*-chlorine substituents the lower the possibility to adopt a planar conformation.

Metabolism of PCBs, PCDFs and PCDDs takes place mainly by cytochrome P450 mediated oxidation, preferentially on the lateral positions. This oxidation is mediated through an arene-oxide intermediate which can spontaneously rearrange to a phenolic metabolite or readily react with glutathione to form a sulfur-containing metabolite. In addition a concommitant shift (NIH-shift) is possible of chlorine substituents from the site of hydroxylation to the next neighbour carbon atom in the aromatic ring. The presence of 2 vicinal unsubstituted carbon atoms greatly facilitates metabolic hydroxylation (Safe, 1989). The hydroxylated metabolites can further be conjugated to glucuronic acid or sulfate, facilitating excretion in urine, bile and faeces. Subsequently the sulfur-containing metabolites can be further metabolised to methylsulphone-metabolites via the mercapturic acid pathway (Safe, 1989, Bakke *et al.*, 1982).

In the environment, PCDDs and PCDFs that are at least 2,3,7,8-substituted are the most persistent and slowest metabolisable PCDD/F congeners due to the lateral chlorine substitutions and consequently are selectively retained and bioaccumulated in the foodchain (Van den Berg *et al.*, 1994, Tulp and Hutzinger, 1978). The relative concentrations of the 2,3,7,8-substituted PCDD or PCDF congeners in biota increase with increasing chlorination.

Metabolism of PCB congeners is slow when they have no adjacent unsubstituted carbon atoms on at least one of the rings, chloro-substituents on the lateral positions and increasing number of chloro-substituents (Safe, 1989). This results in the presence of non-planar, di-*ortho* substituted 2,2',4,4',5,5'-hexaCB, 2,2',3,4,4',5'-hexaCB and 2,2',3,4,4',5,5'-heptaCB in most species in the ecosystem. The bioaccumulation through the foodchain of individual PCB isomers may depend on the species, their exposure situation or dietary intake and the ability of PCB metabolism (Sipes and Schnellman, 1987, Murk *et al*, 1994). This results in specific PHAH congener patterns in the species of various trophic levels that are different from PCB congener patterns present in commercial PCB mixtures. The ease of metabolism of PCBs seems to decrease in the order terrestrial mammals > marine mammals > birds > fish (Safe, 1989, Tanabe *et al*;., 1987, 1988). Coplanar non- and mono-*ortho* PCB congeners were detected in relatively high levels in shell-fish, fish and marine mammals (McFarland and Clarke, 1989; Kannan *et al.*, 1989). In terrestrial mammals, including humans, di-*ortho* PCB congeners concentrations were relatively high compared to non-and mono-*ortho* PCB congeners. In both marine and terrestrial mammals, nearly equal PCDD and PCDF concentrations were detected (Kannan *et al.*, 1989).

Toxicity and mechanisms of action.

In animal species PHAHs can cause thymic atrophy, immunotoxicity , hepatotoxicity and porphyria, reproductive and developmental toxicity, endocrine responses, tissue specific hypo- and hyper-plastic responses and carcinogenesis. In humans and primates PHAH exposure can also cause chloracne and related dermal lesions (McConnell *et al.*, 1989). Risk assessment of PHAH exposure for humans has been focused on the potential carcinogenity of these compounds, since carcinogenic effects of TCDD were observed in experimental studies (Kociba *et al.*, 1978). In addition PHAHs were suggested to act as human carcinogens after occupational exposure or in the Yu-cheng incident in Taiwan, the Yusho incident in Japan or the Seveso incident in Italy although no conclusive evidence has been obtained (Safe, 1994).

However, recently observed effects of low levels of PHAHs on immune system function (Neubert *et al.*, 1992) and behavioural and reproductive development of perinatally exposed animals and human infants (Brouwer *et al.*, 1995, Peterson, 1993) has led to the re-evaluation of the risk assesment of TCDD and related compounds in the US (EPA report) and the Netherlands. Two Dutch human studies revealed decreased birth weights, changes in the thyroid hormone endocrine system and effects on neurological development in perinatally exposed children from mothers with background body burdens of PCDDs, PCDFs and PCBs (Koopman-Esseboom *et al.*, 1994, Pluim *et al.*, 1993). In addition, in children from mothers exposed to relatively high dietary levels of PCBs, PCDDs or PCDFs, defects in cognitive and neuromotor functions were found (Jacobson *et al.*, 1985, 1990, Rogan *et al.*, 1988).

Developmental toxicity and changes in neurochemistry and endocrine systems, like thyroid hormones, have also been found in experimental animals (Peterson, 1993, Tilson *et al.*, 1990, Morse, 1995). Recently disturbances in thyroid hormones after exposure to PCBs, led to increased sperm production and testes size in rats (Stone, 1995). In wildlife species exposed to PHAHs, like seals and cormorants, effects on plasma thyroid hormone levels were found (Brouwer, 1991), while seals also showed impaired reproduction and immune system functions possibly related to PHAH exposure (Reynders, 1986, De Swart, 1995).

Ah-receptor pathway and Ah-receptor mediated toxicity

A majority of the toxic and biochemical effects of PHAHs are explained by a single mechanism of action, the arylhydrocarbon (Ah) receptor pathway (Poland and Knutson, 1982). The compounds acting through this Ah-receptor mediated pathway are the planar laterally substituted 2,3,7,8-PCDDs and PCDFs, with 2,3,7,8-TCDD as the most potent congener, and related coplanar and mono-*ortho* substituted PCB isomers. The Ah-receptor (AhR) is present in cytosol as a complex with the chaperone protein hsp90, which dissociates when a ligand binds to the Ah-receptor. Subsequently the ligand-Ah-receptor complex is translocated to the nucleus by the Ah-receptor nuclear translocator (Arnt) protein. In the nucleus the ligand-AhR-Arnt complex binds selectively to dioxin-responsive elements (DRE) on the DNA. This interaction induces expression of DRE-regulated genes, like the marker gene CYP1A1, which causes an increased expression of cytochrome P450 1A1 mRNA and protein, a phase 1 biotransformation enzyme. Likewise expression of other genes under control of the Ah-receptor pathway can be induced after activation by TCDD, such as CYP1A2 and phase 2 biotransformation enzymes like UDP-glucuronyltransferase I and glutathion-S-transferase π (Sutter and

Greenlee, 1992). Recently, mice lacking the Ah-receptor by knocking out the gene encoding the receptor protein were found to suffer severe liver damage and decreased immune function, suggesting that the Ah-receptor may play a physiological role in the development of the liver and immune system (Fernandez *et al.*, 1995).

The induction of the cytochrome P450 1A1 isoenzyme, which can be measured by deethylation of the model substrate ethoxyresorufin (EROD-activity), and the Ah-receptor binding affinity show a good correlation with certain toxic effects, like thymic atrophy, hepatotoxicity and body weight reduction, for a broad range of PCDD, PCDF and PCB congeners. Therefore a toxic equivalency factor (TEF) concept for Ah-receptor-mediated effects based on additivity could be developed, which allows the expression of the toxic potency of a complex mixture into one toxic equivalency (TEQ) value, relative to the potency of TCDD, the most toxic congener (Safe, 1990). TEF values of individual toxic PCDD, PCDF and PCB congeners were set internationally (Ahlborg *et al.*, 1994, Nato/CCMS, 1988).

However, antagonistic or synergistic effects of combined exposures of specific PCB congeners and TCDD indicated that the additivity of the TEF concept may under- or overestimate toxic effects of complex PHAH mixtures (Van Birgelen *et al.* 1994a,b, Bannister *et al.*, 1987, Leece *et al.*, 1987). Neurobehavioural, neurochemical and carcinogenic effects and endocrinological changes were found in experimental animals after exposure to planar PHAHs, suggesting these may be Ah-receptor mediated effects. Yet exposure to non-planar mono-*ortho* and di-*ortho* PCB congeners, PCB metabolites and PCB mixtures can also result in comparable classes of effects (for review see: Ahlborg *et al.*, 1994, Brouwer *et al.*, 1995). These findings make the use of the TEF concept for risk assesment of PHAHs for specific endpoints like reproductive and developmental toxicity and endocrine disturbances difficult.

Effects of PHAHs that may not be Ah-receptor mediated

Recently more attention is paid to effects of PHAHs that may not directly be mediated by the Ah-receptor pathway. Non-planar PCBs can cause various toxic effects. For instance, neurological disturbances are found in non-human primates after exposure to Aroclor 1016, a PCB mixture that contains low-chlorinated di-*ortho* substituted PCB isomers (Schantz and Bowman, 1989) while mainly changes in dopamine concentrations were found in rats and primates exposed to Aroclor 1016 (Seegal, 1992). In experimental animals both coplanar and nonplanar PCB isomers can be tumour promotors (Silberhorn *et al.*, 1990, Sargent *et al.*, 1992). Especially 2,2',4,4',5,5'-hexaCB, a PB type PCB, has been described as a potent tumour promoting agent by Buchmann *et al.* (1986, 1991). So carcinogenic effects of PCBs cannot be solely explained by Ah-receptor mechanisms of action. Changes in vitamin K dependent blood coagulation are described in rats exposed to either 2,2',4,4',5,5-hexaCB or TCDD, again suggesting a not strictly Ah-receptor mediated pathway (Bouwman *et al.*,1990).

In general biotransformation of xenobiotics lead to detoxification and enhanced excretion, PHAH metabolites, however, are described to own specific biological activities. Although the Ah-receptor mediated induction of CYP1a1/2 can be necessary for the formation of hydroxylated and methylsulphone metabolites, these metabolites may cause non-Ah-receptor mediated responses. Methylsulphone PCB metabolites can accumulate in adrenals and lungs and binds to specific endogenous proteins, eg. renal and urinary alpha2u-globulin and intestinal fatty acid binding protein (Larsen *et al.*,

1992, 1994). Furthermore these metabolites can also alter AHH activity *in vitro* and *in vivo* and strongly induce PB-type biotransformation enzymes in rats (Kato *et al.*, 1995).

A hydroxylated 3,3',4,4'-tetraCB metabolite, 4-OH-3,3',4',5-tetraCB, was described to interact with transthyretin (TTR), the major plasma thyroid hormone transport protein, in rats. This led to decreased plasma thyroid hormone, retinol and retinol-binding protein (RBP) levels (Brouwer and van den Berg, 1986) through displacement of T_4 from TTR and disruption of the TTR-RBP complex by the binding of 4-OH-3,3',4',5-tetraCB metabolite to TTR (Brouwer, 1987).

Thyroid hormone endocrine system and effects of PHAHs.

Disruption of the thyroid endocrine system after exposure to PHAHs has been found in several species, including experimental animals, wildlife species, like seal (Brouwer et al.,1989, Brouwer 1991), and man (Koopman-Esseboom *et al.*, 1994, Pluim *et al.*, 1993). Changes in thyroid hormone homeostasis can also be caused by Ah-receptor mediated changes in thyroid hormone excretion after exposure to PCBs, PCDFs and PCDDs. However, hydroxylated PCB metabolites may also have an effect on thyroid hormone transport, thereby decreasing plasma thyroid hormone levels.In the following paragraphs thyroid hormone synthesis and function, metabolism and plasma transport and the effects of PHAHs on the thyroid hormone system are described in more detail.

Synthesis and function of thyroid hormones

Thyroid hormones, also known as iodothyronines, play an important role in cellular metabolic processes and growth, differentiation and development processes of vertebrates like the development of the brain and central nervous system (for review see: Legrand, 1986). Thyroid hormones are synthesized in the follicular cells of the thyroid gland. In mammals the thyroid mainly secretes 3,3',5,5'-tetraiodo-L-thyronine (thyroxine, T_4, fig. 2), which is regarded as a prohormone since it has relatively low bioactivity (Chopra, 1991). Less than 20 % of the circulating 3,3',5-triiodo-L-thyronine (T_3, fig. 2), e.g. the bioactive hormone, is produced by the thyroid. The majority of T_3 present in the blood is mainly generated by mono-deiodination of T_4 in liver and kidney. Local conversion of T_4 to T_3 is also found in other extrathyroidal tissues like brain, pituitary and brown adipose tissue.

Genomic mechanisms of thyroid hormone action resemble steroid hormone action. The free T_3 hormone is the physiologically active thyroid hormone and is transported to the nucleus where it acts at the transcriptional level through binding to nuclear T_3 receptor (TR) (Oppenheimer and Schwartz, 1986, Oppenheimer, 1991). The binding affinity of T_4 for TR is 10 times lower than the binding affity of T_3 for TR. There are several different TRs which are products of C-erbA proto oncogenes (Weinberger *et al.*, 1986, Sap *et al.*, 1986). The T_3-TR complex binds to specific genetic sequences on the DNA, the thyroid hormone responsive elements (TRE), resulting in expression of thyroid hormone responsive genes.

Non-genomic mechanisms of action of thyroid hormones, like the interaction with plasma membranes, mitochondrial membranes and cytoplasmatic proteins are described by Davis (1991) and Segal and Ingbar (1986).

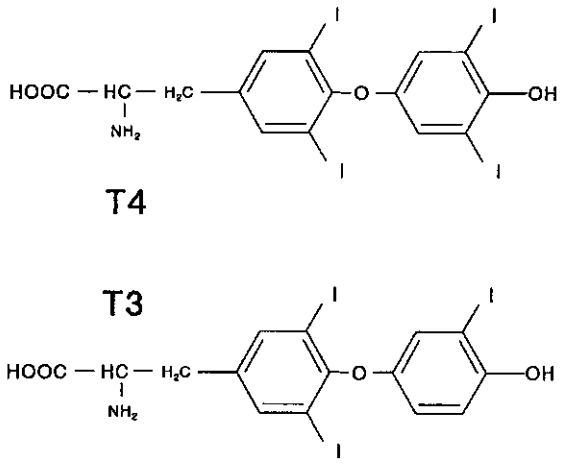

Figure 2 Structures of 3,3',5,5'-tetraiodo-L-thyronine (thyroxine, T_4) and 3,3',5-triiodo-L-thyronine (T_3)

The thyroid hormone levels in blood are not only dependent on the rate of synthesis but also on the rate of excretion from the circulation. Reduced levels of circulating thyroid hormones or the release of thyrotropin releasing hormone (TRH) by the hypothalamus can induce the release of thyroid stimulating hormone (TSH) into the blood by the pituitary gland, successively leading to increased thyroid hormone synthesis and secretion in blood by the thyroid gland (Taurog, 1991).

Metabolism of thyroid hormones

Thyroid hormones can be metabolized through various pathways in several organs and tissues (for review see: Köhrle *et al.*, 1991). These metabolic processes have a role in control of hormonal activity by regulating the amount and form of thyroid hormone present in the cell. The mechanisms of deiodination and conjugation of thyroid hormones wil be described in more detail.

Thyroid hormone deiodination

As stated before, mono-deiodination of the phenolic ring (outer ring deiodination (ORD)) of T_4 is necessary for production of the majority of bioactive T_3 present in the body (Leonard and Visser, 1986). However, deiodination of the tyrosyl ring of T_4 (inner ring deiodination (IRD)) results in the inactive reverse T_3 (3,3',5'-triiodothyronine, rT_3). All other forms of thyroid hormone metabolism, like further deiodination of T_3 to T2, lead to inactivation of the thyroid hormones.

At least three iodothyronine deiodinases are present in mammals, mainly as membrane associated proteins, located in the tissue micosomal fraction. They require sulfhydryl cofactors, like dithiotreitol for their catalytic activity *in vitro*. However, these enzymes show clear differences in iodothyronine substrate specificity, tissue distribution and amount of cofactor required. Furthermore they are dissimilar in susceptibility to inhibitors and regulation by thyroid hormone status. Type 1 iodothyronine deiodinase (ID-I) is abundantly present in liver, kidney and thyroid, and capable of both ORD and

IRD. This enzyme is important for both production of plasma T_3 and clearance of plasma rT_3. Type 2 deiodinase (ID-II), mainly present in pituitary, brown adipose tissue and the central nervous system (neurons of cerebral cortex and cerebellum), only deiodinates the outer ring (ORD) of T_4, and therefore plays a role in the local production of T_3. Type 3 deiodinase (ID-III) can only deiodinate the inner ring, and thus inactivates T_4 by rT_3 production in the central nervous system (glial cells of cerebral cortex, placenta, skin, fetal rat intestine and chick embryo liver). Rat and human hepatic ID-I deiodination and rat and human placental ID-II and ID-III deiodination seem to be strikingly similar. During a hypothyroid status, ID-I activity is decreased while ID-II activity is increased, suggesting a regulation to maintain constant T_3 levels in tissues (Kaplan, 1986).

Thyroid hormone conjugation

Another important pathway in the metabolism of thyroid hormones is conjugation of the phenolic hydroxyl group with glucuronic acid (UDPGA) or sulfate, both so-called phase-II biotransformation reactions (Burger, 1986, Köhrle *et al.*, 1991). Glucuronidation of thyroid hormones by UDP-glucuronyltransferases (UGTs) is mainly found in the microsomal fractions of liver, but also of kidney, intestines and other tissues.

UGTs can be induced by a broad range of xenobiotics or endogenous compounds, in order to increase elimination of these compounds in bile, faeces and urine. T_4 and rT_3 appear to be glucuronidated by the 3-methylcholantrene inducible p-nitrophenol-UGT and the clofibrate inducible bilirubin-UGT isoenzymes, while T_3 is glucuronidated by the androsterone-UGT isoenzyme in rat liver (Beetstra *et al.*, 1991, Visser *et al.*, 1993). The produced inactive glucuronides (T_4G and T_3G) are better water soluble and subsequently excreted in bile.

Plasma thyroid hormone transport

The majority of thyroid hormones present in blood of mammals are non-covalently bound to specific transport proteins (for reviews see Robbins, 1991, Robbins and Bartalena, 1986). These proteins function as a circulating reservoir of thyroid hormones to buffer sudden changes in thyroid hormone levels and to avoid high levels of free thyroid hormones in circulation, which are considered to be directly available to tissues and organs. The main thyroid hormone carrier proteins in man are thyroxine-binding globulin (TBG), transthyretin (TTR) and albumin, while in rat mainly TTR and albumin are present.

TTR was formerly known as prealbumin due to its ability to migrate faster than serum albumin on gel-electrophoresis of whole plasma. TTR has a molecular weight of 55000 D, and is composed of four identical polypeptide chains, each of 127 amino acid residues. Not only the complete amino acid sequence of TTR is known (Kanda *et al.*, 1974), but also its full three-dimensional structure has been resolved by X-ray crystallography (Blake *et al.*, 1978). Only one molecule of T_4 binds to TTR with high affinity (10^7-10^8 M^{-1}), while the second binding site has a ten to hundred fold lower affinity for T_4 (10^6 M^{-1}) due to negative cooperativity (De la Paz *et al.*, 1992). TTR is important not only for the TRANSport of THYroxine, but also of RETINol through the co-transportation of retinol binding protein (RBP). The interaction of TTR with T_4 appears not to be affected by binding of the retinol-RBP complex (Robbins *et al.*, 1978, Raz and Goodman, 1969). The precise binding site of RBP on TTR is not known yet, although

recent studies suggest the interference of the Ile 84 amino acid residue located on the outside of the TTR molecule in RBP binding (Berni *et al*, 1994).

TTR is a highly conservative plasma protein and present in plasma of most mammalian species and birds, and in reptiles at very low concentrations but is absent in bloodplasma of fish and amphibians (Larsson *et al.*, 1985, Schreiber *et al.*, 1993). TTR is synthesized mainly in liver (Dickson *et al.*, 1985). Secretion of TTR from liver to blood is not dependent on thyroid hormone binding (Robbins and Bartalena, 1986). Degradation of TTR takes place in the liver, muscle and skin (Makover *et al.*, 1988). TTR has a higher production and disappearance rate that TBG, resulting in a half-live in plasma of 1-2 days.

In addition TTR is synthesized in the visceral yolk sac (Soprano *et al.*, 1985) and choroid plexus of the brain (Kato *et al*, 1986, Harms *et al.*, 1991), even at a very early stage in development of rats (Thomas *et al.*, 1988). High levels of mRNA coding for TTR were found in the choroid plexus of rats and humans (Schreiber *et al.*, 1990). TTR gene expression was alo detected in choroid plexus of reptiles, but not in liver, indicating that the expression of TTR in choroid plexus appeared earlier in evolution than TTR expression in liver (Richardson *et al.*, 1994, Harms *et al.*, 1991).

Most T_4 in CSF is bound to this locally synthesized TTR which does not appear to reach circulation (Schreiber, 1987). It was suggested that TTR played a role in the transport of T_4 from blood to the brain through the blood-CSF barrier. However, recent studies suggest that T_4 is transported to the brain primarily through the blood brain barrier and not via the choroid plexus (Blay *et al.*, 1993). Moreover, the T_4 transport to CSF takes place not through TTR binding, but depends on free T_4 levels (Van Raay, 1994; Chanoine *et al.*, 1992).

TBG is a high affinity, low-capacity T_4 transport protein, synthesized in the liver of some higher mammals, inculding man. It has a molecular weight of 54000 D (Robbins and Bartalena, 1986) and is an acidic glycoprotein consisting of a single polypeptide chain with one thyroid hormone binding site per molecule (Korcek and Tabachnik 1976). TTR and TBG have different T_4 binding properties; in human plasma TBG transports a higher percentage of T_4 (50-80 %) than TTR (10-40 %) (Petterson, 1989, Larsson *et al.*, 1985). Despite the low serum concentration TBG (15 mg/l) carries the bulk of the thyroid hormones in humans because of its high affinity for these compounds. TTR, with a 20 fold higher concentration than TBG in human plasma (300 mg/l), has a 100 fold lower thyroid hormone binding affinity and acts as a secondary carrier protein in man, but as the primary carrier protein in rodents. Not only humans but also rats and mice possess TBG, which is very similar to their human counterpart. The rodent TBG was recognized only recently because it is a dramatically development-regulated protein, highly expressed during post-natal growth but virtually undetectable in adults except in hypothyroidal state (Rouaze-Romet *et al.*, 1992, Savu *et al.*, 1989, Vranckx *et al.*, 1990). The total absence of TBG in some individuals due to genetic polymorphism, constitutes evidence that TBG does not play an essential role in thyroid hormone action. No genetic polymorphism resulting in the absence of TTR is described in mammalian species, suggesting that TTR may be important during development (Robbins and Bartalena, 1986). However, recently produced transgenic mice lacking the TTR gene, showed normal development and reproduction, although the levels of thyroid hormones, retinol and RBP were low (Episkopou *et al.*, 1993).

Albumin carries a relatively small portion of thyroid hormones (10-20 %) due to

the low binding affinity, despite high serum concentrations. Other T_4 binding plasma proteins, apo-lipoprotein apoA-1, apoB and apoE, were recently described by Benvenga *et al.* (1989, 1993).

Effects of PHAHs on thyroid hormone homeostasis in man and experimental animals

Changes in plasma thyroid hormone levels

The thyroid hormone endocrine system can be disrupted by exposure to PHAHs in diverse species, including experimental animals and man. In pregnant women exposed to background PHAH levels a negative correlation between the levels of PCDD/F and PCB congeners in human milk and plasma thyroid hormone levels was found. In the newborn infants higher plasma TSH levels were detected when they were exposed to relatively high PCDD/F and PCB concentrations through maternal body burden (Sauer *et al.*, 1994, Koopman-Esseboom *et al.*, 1994). In addition, increases in plasma TSH and T_4 levels in newborn babies exposed to increasing levels of PCDD/Fs were reported by Pluim *et al.* (1992). Changes in human serum T_4 levels following occupational or accidental exposure to high levels of PCBs or polybrominated biphenyls (PBBs) were found by Emmett *et al.* (1988), Murai *et al.* (1987), Bahn *et al.* (1980) and Kreiss *et al.* (1982).

In experimental animals, like rats or mice, disruption of the thyroid hormone endocrine system was found after exposure to different PHAHs. Exposure to TCDD resulted in reduced plasma T_4 levels while plasma T_3 levels were reported to be increased, unchanged or decreased after TCDD exposure (Bastomsky, 1977, Potter *et al.*, 1983, Lans *et al.*, 1990, Potter *et al.*, 1986, Roth *et al.*, 1988, Jones *et al.*, 1987, Henry and Gasiewicz, 1986, 1987, Pohjanvirta *et al.*, 1989, Pazdernik and Rozman, 1985). These contradicting effects on plasma thyroid hormones after TCDD exposure, did not give clear evidence on the thyroid status of the exposed animals. In turn thyroid hormone status may modulate TCDD toxicity in rodents as was described by Rozman *et al.* (1984, 1985, 1987), Pazdernik and Rozman (1985) and Lamb IV *et al.* (1986). Thyroidectomized rats exposed to TCDD seemed to be partly protected for loss of body weight when compared to TCDD exposed non-thyroidectomized rats.

Analogous to TCDD, several individual PCB congeners can decrease plasma T_4 levels in rodents, like 3,3',4,4'-tetraCB (TCB) (Brouwer, 1989; Murk *et al.*, 1991), 3,3',4,4',5,5'-hexaCB (Morse *et al.*, 1993a), 2,3,3',4,4',5-hexaCB (Van Birgelen *et al.*, 1994a) 3,3',4,4',5-pentaCB (Van Birgelen *et al.*, 1994b) and 3,3',4,4',5,5'-hexabromobiphenyl (Spear *et al.*, 1994). In primates, eg. marmoset monkeys, exposure to TCB caused hypothyroidism (Van den Berg *et al.*, 1988). Commercial PCB mixtures like Aroclor 1254 also strongly decreased plasma T_4 levels in rodents (Gray *et al.*, 1993; Byrne *et al.*, 1987, Murk *et al.*, 1991) while plasma T_3 levels were not decreased (Brouwer, 1989; Bastomsky, 1974; Beetstra *et al.*, 1991). Commercial PBB mixtures decreased serum T_3 and T_4 levels and the rate of T_4 production in rats leading to hypothyroidism (Byrne *et al.*, 1987) that was also observed in mice after exposure to a PBB mixture (Gupta *et al.*, 1983).

The observed changes in plasma thyroid hormone levels after exposure to PHAHs have been explained by different mechanisms, eg. impaired thyroid gland function, changes in thyroid hormone metabolism and possible disturbed plasma transport of thyroid hormones.

Changes in thyroid gland function and morphology

Direct effects of PHAHs on thyroid gland function may result in disturbed plasma thyroid hormone levels. On the contrary, disturbed thyroid gland structure and function by PHAH exposure may be caused by changes in plasma thyroid hormone levels, leading to a feedback stimulation of the thyroid gland. A possible increase in incidence of thyroid cancer in women exposed to high levels of TCDD during the Seveso accident (Bertazzi *et al.*, 1989, Pesatori *et al.*, 1993) and histological changes in the thyroid of TCDD treated rats could be interpreted as sustained thyroid activation, probably due to lowered plasma T_4 levels (Gupta *et al.* 1973; Rozman *et al.*, 1986). Pups from pregnant rats exposed to the PCB congener 2,3',4,4',5-pentaCB showed histological changes in the thyroid suggestive of sustained TSH elevation (Ness *et al.*, 1993) while another individual PCB congener, 3,3',4,4',5-pentaCB, caused mild thyroid alterations in rats (Chu *et al.*, 1994). Likewise, effects on thyroid function and morphology in adult, perinatal and Gunn rats and adult cynomolgus monkeys by Aroclor 1254 exposure are described by Collins and Capen (1980a,b,c), Collins *et al.* (1977), Sepkovic and Byrne (1984), Capen and Martin (1989) and Tryphonas *et al.* (1984). Aroclor 1254 and a PBB mixture caused ultrastructural lesions in thyroid follicular cells which appeared to interfere with synthesis and secretion of T_4 (Kasza *et al.*, 1978). The ultrastructural and functional alterations of rat thyroid glands after Aroclor 1254 exposure were dissimilar to thyroid gland alterations found after iodide excess and deficiency and thyrotropin or thyroxine administration (Collins and Capen, 1980c), suggesting an additional or specific effect of Aroclor 1254 exposure.

Changes in thyroid hormone metabolism

Changes in thyroid hormone metabolism are also suggested to lead to the observed decreased plasma T_4 levels after PHAH exposure. Increase in T_4 excretion by TCDD was caused by the induction of T_4-UGT activity, an Ah-receptor mediated pathway (Beetstra *et al.*, 1991; Lucier *et al.*, 1975, Sutter and Greenlee, 1992, Van den heuvel and Lucier, 1994). TCDD can induce phenol-UGT activities in the rat towards *p*-nitrophenol (Rozman *et al.*, 1985b, 1987), 1-naphtol (Eltom *et al.*, 1992) and T_4 (Henry and Gasiewicz, 1987) and highly induce phenol-UGT mRNA levels in rat liver (Münzel *et al.*, 1994). However, TCDD did not or weakly induce the UGT activity towards bilirubin, testosterone or estrone as substrates (Visser *et al.*, 1993; Umbreit *et al.*, 1989, Lucier *et al.*, 1975), resulting in increased T_4 glucuronidation, but no change in T_3-glucuronidation. These findings agree with the early observations of Bastomsky (1977), who found an increase in biliary excretion of T_4 (mostly T_4-glucuronide) but not T_3.

T_4 glucuronidation is also induced after exposure to other individual halogenated biphenyl congeners, like hexachlorobenzene (HCB) (Van Raay *et al.*, 1993), TCB (Visser *et al.*, 1993), 3,3',4,4',5,5'-hexabromobiphenyl (Spear *et al.*, 1990) and 3,3',4,4',5,5'-hexaCB (Morse *et al.*, 1993), compounds which simultaneously decrease plasma T_4 levels. In addition exposure to commercial PCB mixtures like Aroclor 1254 and Kanechlor 400 increased T_4 glucuronidation and bile flow in rats (Bastomsky, 1974; Beetstra *et al.*, 1991; Saito *et al.*, 1991; Bastomsky and Murthy, 1976; Barter and Klaassen, 1992, 1994).

Another enzyme involved in thyroid hormone metabolism, hepatic ID-1, was inhibited in rats *in vivo* following treatment of rats with several Ah-receptor agonists, such as 3-methylcholantrene, TCB and TCDD (Visser *et al.*, 1993; Eltom *et al.*, 1992;

Rickenbacher *et al.*, 1986, Adams *et al.*, 1990). Hydroxylated TCB metabolites could also inhibit ID-1 activity *in vitro* using rat hepatic microsomes (Adams *et al.*, 1990, Rickenbacher *et al.*, 1989). Furthermore brain ID-II activity was increased in fetal and neonatal rats exposed to 3,3',4,4',5,5'-hexaCB, which had severely reduced plasma T_4 levels, probably to compensate and maintain T_3 levels in brain (Morse *et al.*, 1993a).

Changes in thyroid hormone transport

Effects of PHAH exposure on plasma transport of thyroid hormones leading to altered plasma thyroid hormone levels, were already described by Bastomsky (1974). He proposed a possible reduction of thyroid hormone binding to plasma proteins in Aroclor 1254 exposed rats. Kanechlor 400, another commercial PCB mixture, increased T_4 glucuronidation in rats but still the authors suggested an additional role of disturbed plasma T_4 transport in the plasma T_4 decreases (Saito *et al.*, 1991). Moreover in Gunn rats, deficient in UGT activity towards bilirubin, phenolic compounds and T_4 (Visser *et al.*, 1993), the T_4-UGT activity could be induced by Aroclor 1254 to some extent in the heterozygous rats but not in the homozygous rats. However, the decreases in plasma T_4 levels were similar in both strains, suggesting an additional interference with plasma transport proteins (Collins and Capen, 1980a). Rickenbacher *et al.* (1986) and McKinney *et al.* (1985) described interactions of PHAHs, like TCDD and 3,3',4,4',5,5'-hexaCB, and some hydroxylated metabolites, with thyroxine binding prealbumin (TBPA, synonymus to TTR) using in vitro binding studies and graphics modelling studies.

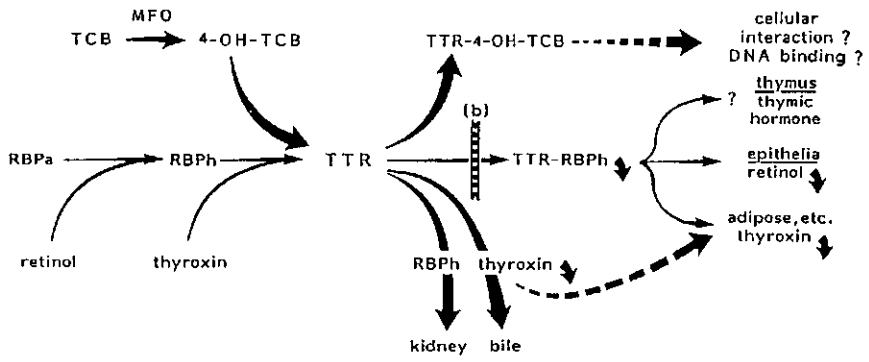

Figure 3 Model for interference of thyroxine (T_4) and retinol transport in serum after binding of a PCB metabolite to transthyretin (TTR), and its effect on T_4, retinol and retinol binding protein (RBP) levels (Brouwer, 1987). The thin lines represent the normal situation with TTR transporting T_4 and retinol (bound to RBP), the solid and interrupted bold lines represent the interaction of the 4-OH-3,3',4',5-tetraCB (4-OH-TCB) metabolite with TTR after exposure of rats to 3,3',4,4'-tetraCB (TCB) and subsequent predicted effects. MFO: mixed function oxidases, b: blockage.

Brouwer and Van den Berg (1986), suggested a mechanism based on disturbed plasma transport for the decrease in plasma T_4 levels in rats exposed to TCB, a coplanar PCB-congener. A hydroxylated TCB metabolite (4-OH-3,3',4',5-tetraCB) present in plasma interacted with TTR *in vivo*, resulting in the competitive displacement of T_4 from TTR. In addition the interaction of 4-OH-3,3',4',5-tetraCB with TTR, diminished RBP-TTR binding (Brouwer, 1987) which successively led to decreases in plasma RBP and retinol (Brouwer and Van den Berg, 1986, Brouwer *et al.*, 1988). This proposed mechanism (Fig. 3) was the basis for the work on the interactions of hydroxylated PHAH metabolites with thyroid hormone binding proteins described in this thesis.

Objectives and approaches

Objectives

Based on the interaction of the 4-OH-3,3',4',5-tetraCB metabolite with TTR in serum of rats exposed to 3,3',4,4'-tetraCB (Brouwer and van den Berg, 1986, Fig. 3) and the structural resemblance of this metabolite with thyroxine (T_4), we hypothesized that hydroxylated metabolites of related PHAHs could also interact with TTR. Moreover, hydroxylated PHAH metabolites may interact with thyroid hormone binding proteins other than TTR. Consequently hydroxylated PHAH metabolites may possibly attribute to the observed plasma T_4 decreases and changes in thyroid hormone metabolism observed in experimental animals after PHAH exposure in vivo.

Approaches

Structural requirements for binding to TTR by hydroxylated metabolites of PCBs, PCDDs and PCDFs and parent compounds were determined using in vitro T_4-TTR binding competition studies (Chapter 2). Analogous to TTR, binding of hydroxylated metabolites of PCBs, PCDDs and PCDFs and parent compounds to other thyroxine binding proteins eg. thyroxine-binding globulin (Chapter 3) and type-1-deiodinase (Chapter 4) were investigated in vitro and structural requirements for interactions were determined. In addition, *in vivo* animal experiments were carried out to differentiate the effects on thyroid hormone metabolism and plasma transport by possible hydroxylated metabolites of a complex PCB mixture (Aroclor 1254) (Chapter 5) and the persistent 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Chapter 6). Finally, the exact structural requirements of hydroxylated PCB metabolites for binding to TTR were determined using computer assisted graphics modelling and structure analysis of a protein-complex of TTR with a hydroxylated PCB metabolite, using X-ray crystallography (Chapter 7).

CHAPTER 2

Structure dependent, competitive interaction of hydroxy-polychlorobiphenyls, -dibenzo-*p*-dioxins and dibenzofurans with human transthyretin

Abstract

Previous results from our laboratory indicated specific and competitive interactions of hydroxylated metabolites of 3,3',4,4'-tetrachlorobiphenyl with the plasma thyroid hormone transport protein, transthyretin (TTR), in rats *in vivo* and with human TTR *in vitro*. In the present study the structural requirements for competition with thyroxine (T_4) for TTR-binding were investigated in more detail. Several hydroxylated polychlorinated biphenyls (PCBs), dibenzo-p-dioxins (PCDDs) and dibenzofurans (PCDFs) were tested in an *in vitro* competitive binding assay, using purified human TTR and ^{125}I-T_4 as a displaceable radioligand. All hydroxylated PCBs, but not the single PCB tested, competitively displaced ^{125}I-T_4 from TTR with differential potency. The highest competitive binding potency was observed for hydroxylated PCB congeners with the hydroxygroup substituted on *meta* or *para* positions and one or more chlorine atoms substituted adjacent to the hydroxy-group on either or both aromatic rings (IC_{50} range 6.5 - 25 nM; K_a range: 0.78 - 3.95 * 10^8 M^{-1}). The relative potency of all *meta* or *para* hydroxylated PCBs was higher than that of the physiological ligand, T_4 (relative potency range: 3.5 - 13.6 compared to T_4). There were no marked distinctions in TTR-T_4 competitive binding potencies between the *ortho*- and non-*ortho*-chlorine substituted hydroxy-PCB congeners tested. Marked differences in TTR-T_4 binding competition potency were observed between the limited number of hydroxylated PCDDs and PCDFs tested. The hydroxy-PCDD/Fs, with chlorine substitution adjacent to the hydroxy-group i.e., 7-OH-2,3,8-trichlorodibenzo-*p*-dioxin, 2-OH-1,3,7,8-tetrachlorodibenzo-*p*-dioxin and 3-OH-2,6,7,8-tetrachlorodibenzofuran, all showed a similar or higher relative binding potency, i.e. 1, 4.4 and 4.5 times higher respectively, than T_4. No detectable ^{125}I-T_4 displacement was observed with 2-OH-7,8-dichlorodibenzofuran, 8-OH-2,3,4-trichlorodibenzofuran and 8-OH-2,3-dichlorodibenzo-*p*-dioxin, which did not contain chlorine substitution adjacent to the OH-group. These results indicate a profound similarity in structural requirements for TTR binding between hydroxy-PCB, -PCDD and -PCDF metabolites and the physiological ligand, T_4, e.g., halogen substitution adjacent to the *para* or *meta* hydroxy-group, while planarity does not seem to influence the ligand binding potency.

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Introduction

Polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) are ubiquitous environmental pollutants that induce a broad spectrum of toxic signs and lesions in animals and man (McConnell, 1980). One aspect of the toxicity of these chemicals is their profound effect on thyroid hormone metabolism, which may play a role in the onset of certain toxic endpoints, such as the wasting syndrome, neurotoxicity and carcinogenicity.

There are several reports on the interaction of halogenated biphenyls with thyroid hormone metabolism. Administration of, for instance, 3,3',4,4'-tetrachlorobiphenyl (TCB) (Brouwer and Van den Berg, 1986) and 3,3',4,4',5,5'-hexabromobiphenyl (Spear *et al.*, 1990) to rats caused changes in thyroid hormone metabolism and decreased plasma thyroxine (T_4) levels. In addition, exposure to TCB is known to induce T_4, but not triiodothyronine (T_3) glucuronidation in rats (Beetstra *et al.*, 1991). Exposure of marmoset monkeys to TCB caused hypothyroidism in these animals (Van den Berg *et al.*, 1988). Rats treated with Aroclor 1254, a commercial PCB mixture, exhibited decreased T_4 but no changes in T_3 levels in serum and increased biliary T_4 excretion was observed (Bastomsky, 1974). Saito *et al.* (1991) found that Kanechlor 400 also increased T_4 glucuronidation in rats. Chronic exposure of rats to commercial PCB and polybrominated biphenyls (PBB) mixtures led to decreases in serum T_3 and T_4 levels and a decrease in the rate of T_4 production which led to hypothyroidism (Byrne *et al.*, 1987). Commercial PBB mixtures have also been reported to cause hypothyroidism in the mouse (Gupta *et al.*, 1983). Moreover, changes in human serum T4 levels following exposure to PCBs or PBBs are described in several studies (Emmett *et al.*, 1988; Murai *et al.*, 1987; Bahn *et al.*, 1980, Kreiss *et al.*, 1982).

Other related compounds, such as PCDDs and PCDFs are known to affect thyroid hormone metabolism in a similar way. For example, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) markedly increased the biliary excretion of T_4 (Bastomsky, 1977). The increase in T_4 excretion was most likely due to induction of T_4 glucuronyltransferase activity by TCDD (Beetsta *et al.*, 1991, Lucier *et al.*, 1975). Administration of TCDD to rats resulted in reduced serum T_4 levels but unchanged serum T_3 levels (Potter *et al.*, 1983). The induction of T_4 glucuronidation by certain PCBs, PCDDs and PCDFs may explain, in part, the hypothyroidism induced by these chemicals.

However, Brouwer and Van den Berg (1986) have reported another possible pathway for the interaction of PCBs and related compounds with thyroid hormone metabolism. Hydroxylated metabolites of TCB have a marked structural resemblance to T_4, the natural ligand for transthyretin (TTR), the major thyroid hormone transport protein in rat plasma. Due to this resemblance T_4 could be specifically displaced from TTR by the hydroxylated metabolites, causing a decrease in plasma T_4 levels. In the blood of rats administered TCB the TCB metabolite 4-OH-3,3',4',5-tetrachlorobiphenyl (4-OH-TCB) was identified (Brouwer, 1989). The metabolite exhibited strong binding affinity with TTR, thus decreasing in plasma T_4 levels.

Furthermore Rickenbacher *et al.* (1986) found that both PCBs and hydroxylated derivatives of PCBs competitively interacted with TTR, using computer modelling and *in vitro* binding studies. However, in other *in vitro* TTR-T_4 competition studies it was shown that only hydroxylated metabolites of TCB, but not the parent compound were able to compete with T_4 for binding to TTR (Brouwer *et al.*, 1990). Similar results were

obtained for halogenated benzenes and their phenolic metabolites (Van den Berg, 1990, Den Besten *et al.*, 1991) where it was shown that the presence of a hydroxy-group on the parent compound was essential for TTR binding. In contrast, McKinney *et al.* (1985) showed that an adipamide-derivative of TCDD competed with T_4 for binding to TTR using computer modelling.

The major objective of the present study was to further investigate the structural requirements of hydroxylated PCB, PCDD and PCDF metabolites and a parent compound for interaction with TTR. Various synthetic hydroxylated PCB, PCDD and PCDF congeners were used for *in vitro* competitive binding studies, using purified human TTR. Similar biochemical properties of TTR were found in human, rat and other species (Larsson *et al.*, 1985; Herbert *et al.*, 1986). In another study rat TTR was compared with human TTR and was found to be highly conserved at the binding sites for T4 (Dickson *et al.*, 1985). Therefore and for practical reasons we used human TTR as a model protein for rats.

Two different classes of compounds were chosen for this study (Fig. 1), namely (i) PCB and PCDD metabolites which have been identified from *in vivo* studies (Klasson-Wehler *et al.*, 1990; Koga *et al.*, 1990, Mason and Safe, 1986) (compounds B, C, D, E, I, J, K, P, Q) and (ii) congeners which vary both in their chlorine and hydroxyl group substitution patterns (compounds F, G, H, L, M, N, O). The first group of compounds (i) contains 2,3,3',4,4'-pentachlorobiphenyl (PeCB 105, A in Fig. 1) and its hydroxylated metabolites (B, C and D). This PeCB 105 is a major mono-ortho PCB with a relatively high toxicological impact, in addition a hydroxylated metabolite (C) appeared to have a selective retention in rat-, polar bear- and seal-blood and is also found in human serum samples (Klasson-Wehler *et al.*, 1992). The binding affinities of the latter group of compounds (ii) can more accurately define the structural basis for the interaction of hydroxylated PCBs, PCDDs and PCDFs with TTR.

Materials and methods

Materials

Radiolabeled L-3',5'-[^{125}I] thyroxine (46 μCi/μg thyroxine) was purchased from Amersham, Buckinghamshire, England and contained 5-10% free iodide, determined by Sephadex LH-20 (Pharmacia LKB, Woerden, the Netherlands) gel filtration. TTR (human prealbumin, 98% pure) and 3,3',5,5'-L-thyroxine (T_4) were obtained from Sigma Chemical Company (St. Louis, MO, USA).

The tested hydroxylated chlorodibenzo-*p*-dioxins and chlorodibenzofurans (2-OH-7,8-diCDF (L), 8-OH-2,3,4-triCDF (M), 3-OH-2,6,7,8-tetraCDF (N), 8-OH-2,3-diCDD (O), 7-OH-2,3,8-triCDD (P) and 2-OH-1,3,7,8-tetraCDD (Q)), shown in Figure 1, were synthesized as previously described (Mason and Safe, 1986; Denomme *et al.*, 1985; Denomme *et al.*, 1986). 2,3,3',4,4'-Pentachlorobiphenyl (CB 105) (A, Fig. 1) was purchased from Promochem GmbH, Wesel, Germany. The hydroxylated PCBs (Fig. 1) used in the present study: 2-OH-2',3,3',4,4'-pentaCB (B), 4-OH-2',3,3',4',5-pentaCB (C), 5-OH-2',3,3',4,4'-pentaCB* (D), 4-OH-3,3',4'-triCB (E), 4-OH-2,3,3',4'-tetraCB (F), 4-OH-2,2',3,3',4'-pentaCB (G), 4,4'-$(OH)_2$-2,3,3',5,5'-pentaCB (H), 4,4'-$(OH)_2$-3,3',5,5'-tetraCB (I), 4-OH-3,3',4',5,5'-pentaCB (J) and 5-OH-3,3',4,4'-tetraCB* (K) were obtained by demethylation of the corresponding methoxy-PCBs (Klasson-Wehler *et*

al., 1990). The methoxy-PCBs were prepared and structures verified as described elsewhere (Bergman *et al.*, 1995; Safe and Safe, 1984).
Methanol (HPLC grade) was obtained from Merck Chemical Company (Darmstadt, Germany). Biogel P-6DG desalting gel was obtained from Bio-Rad Laboratories (Richmond, CA, USA).

* The numbering of the hydroxy-substituents on the biphenyl does not follow IUPAC rules but has been chosen in order to facilitate comparison of the structures.

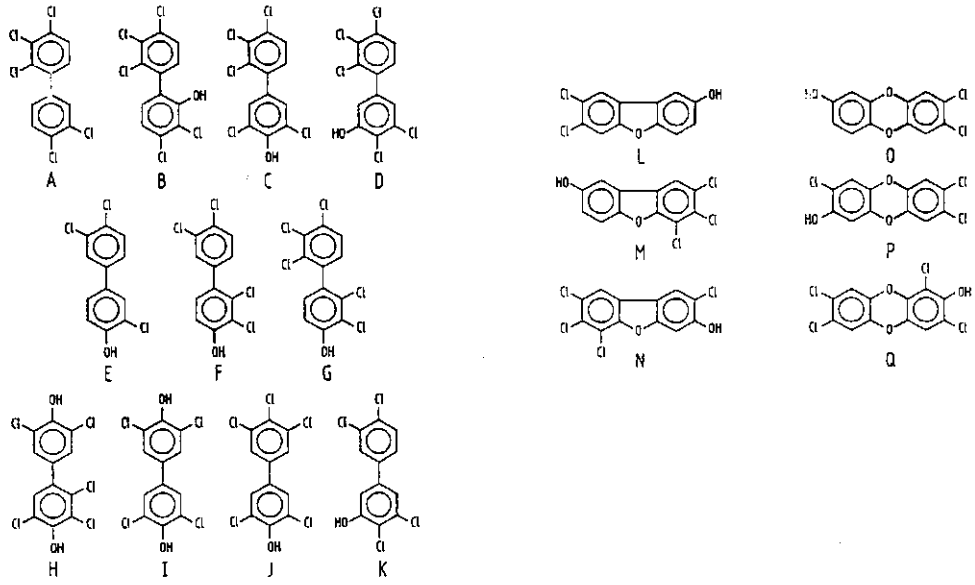


Figure 1 Structures of hydroxylated PCBs, PCDDs and PCDFs tested in the TTR-T_4 competition binding assay and synthesized as described in Materials and Methods. CB 105: 2,3,3',4,4'-pentaCB (A). Hydroxylated PCBs: 2-OH-2',3,3',4,4'-pentaCB (B), 4-OH-2',3,3',4',5-pentaCB (C), 5-OH-2',3,3',4,4'-pentaCB (D), 4-OH-3,3',4'-triCB (E), 4-OH-2,3,3',4'-tetraCB (F), 4-OH-2,2',3,3',4'-pentaCB (G), 4,4'-$(OH)_2$-2,3,3',5,5'-pentaCB (H), 4,4'-$(OH)_2$-3,3',5,5'-tetraCB (I), 4-OH-3,3',4',5,5'-pentaCB (J) and 5-OH-3,3',4,4'-tetraCB (K). Hydroxylated PCDDs and PCDFs: 2-OH-7,8-diCDF (L), 8-OH-2,3,4-triCDF (M), 3-OH-2,6,7,8-tetraCDF (N), 8-OH-2,3-diCDD (O), 7-OH-2,3,8-triCDD (P) and 2-OH-1,3,7,8-tetraCDD (Q).

In vitro T_4 competition-binding assays on TTR

The gel-filtration procedure as decribed by Somack *et al.* (1982) with minor modifications was used for the T_4 competition-binding assay. The assay, using human TTR, was performed as follows: TTR (30 nM, dissolved in 0.1 M Tris-HCl, 0.1 mM NaCl, 1 mM EDTA buffer, pH 8) was incubated with a mixture of ^{125}I-T_4 and unlabeled T_4 (70.000 cpm, 55 nM) in Tris-HCl buffer, and competitors (T_4, parent or hydroxylated PCBs, PCDDs or PCDFs) were added in increasing concentrations (dissolved in methanol, volume added was 5 µl). Control incubations were made by adding 5 µl methanol instead of competitor. Total ^{125}I-radioactivity added to each of the incubation mixtures (200 µl total volume) was checked by gamma-counting (Multi Prias, Packard Instrument co., USA). The incubations were allowed to reach binding equilibrium overnight at 4 °C. Protein-bound ^{125}I-T_4 and free ^{125}I-T_4 were separated on 1 ml Biogel P-6DG columns (prepared in a 1 ml disposable syringe, equilibrated in Tris-HCl buffer) that were equilibrated with 300 µl 10 % (W/V) saccharose-Tris-HCl buffer) and centrifuged for 1 min at 1000 rpm (100 g) in a precooled centrifuge (Difuge, Hereaus). The columns were spin-forced eluted with an additional 200 µl Tris-HCl buffer. The first 2 eluate fractions, containing the protein bound ^{125}I-T_4 fraction, were combined; radioactivity was counted and compared to the control incubations. Free T_4 was bound to the Biogel matrix and therefore was not present as a contaminant in the fractions eluted. Competition binding curves were made by plotting relative ^{125}I-T_4-protein binding (% of control) against added log competitor concentration. Competition binding assays with unlabeled T4 were used as reference assays to make comparisons between different experiments.

Analysis of binding data

Binding characteristics are given as relative potency: the ratio of inhibitor concentration at 50 % inhibition (IC_{50}) of OH compounds vs the IC_{50} of unlabeled T_4 [IC_{50}(competitor)/IC_{50}(T_4)]. Affinity constant analysis was performed with the Ligand-program (obtained from Dr. K.J. van den Berg, MBL-TNO, Rijswijk, the Netherlands) (Munson and Rodbard, 1980).

Statistics

Data are given as means with standard deviations (S.D.). Significant differences were determined by statistical analysis using the Student's t-Test (with a probability value $p < 0.05$).

Results

CB 105 and its hydroxy-metabolites

The results in Figure 2 summarize the competitive binding of different hydroxylated metabolites of CB 105 (B,C,D) and the parent compound CB 105 (A) using ^{125}I-T_4 as the displaceable radioligand. The parent compound did not show any interaction with TTR, whereas the hydroxylated metabolites all competitively displaced ^{125}I-T_4 from TTR. The 2-OH-2',3,3',4,4'-pentaCB metabolite (B) competed with ^{125}I-T_4 for TTR binding, but with lower potency than unlabeled T_4 (IC_{50} 950 nM and 88.3 nM, respectively).

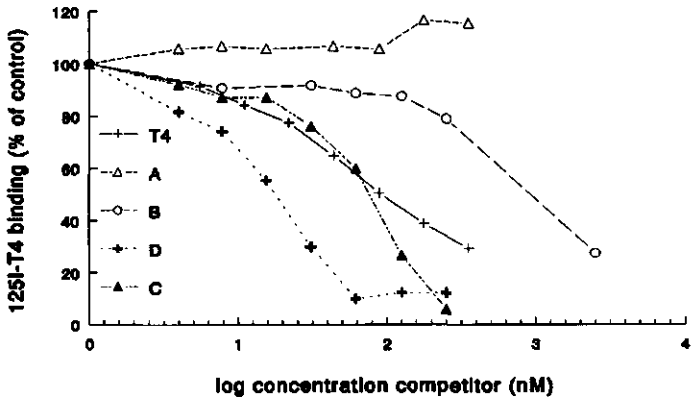


Figure 2 Competitive binding of CB 105 (A) and its hydroxy metabolites (B,C and D), as measured by the TTR-T_4 competition-binding assay (Materials and Methods). Datapoints are mean values of duplicate incubations. Relative ^{125}I-T_4-TTR binding (% of control value) is plotted against the log concentration (nM) of competitor or T_4 added.

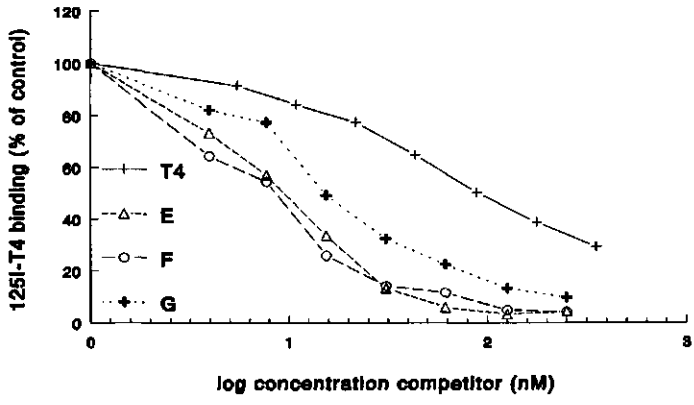


Figure 3 Competitive binding of non-*ortho* (E), mono-*ortho* (F) and di-*ortho* (G) chlorine substituted *para*-hydroxylated PCBs, as measured by the TTR-T_4 competition-binding assay (Materials and Methods). Datapoints are mean values of duplicate incubations. Relative ^{125}I-T_4-TTR binding (% of control value) is plotted against log concentration (nM) of competitor or T_4 added.

Table 1 Competitive ^{125}I-T_4-TTR binding inhibition concentrations (IC_{50}), relative potencies and TTR binding affinity constants (Ka) of parent and hydroxylated polychlorobiphenyls (PCBs).

No	Structure	IC_{50} (nM)	Rel. Pot.	Ka (M^{-1}) (means ± s.d.)
	Thyroxine	88.3	1	2.05 ± 0.57 * 10^7
A	2,3,3',4,4'-pentaCB	>1000	<< 1	N.D.
B	2-OH-2',3,3',4,4'-pentaCB	950	0.09	8.85 ± 3.98 * 10^{4*}
C	4-OH-2',3,3',4',5-pentaCB	15.0	5.9	3.41 ± 0.72 * 10^{8*}
D	5-OH-2',3,3',4,4'-pentaCB	19	4.6	7.78 ± 2.02 * 10^{7*}
E	4-OH-3,3',4'-triCB	10.5	8.4	8.28 ± 2.15 * 10^{8*}
F	4-OH-2,3,3',4'-tetraCB	8.8	10.2	5.11 ± 1.38 * 10^{8*}
G	4-OH-2,2'3,3',4'-pentaCB	17.5	5.0	6.22 ± 1.18 * 10^{7*}
H	4,4'-$(OH)_2$-2,3,3',5,5'-pentaCB	6.5	13.6	3.95 ± 2.17 * 10^{8*}
I	4,4'-$(OH)_2$-3,3',5,5'-tetraCB	16.5	5.4	4.31 ± 1.60 * 10^{8*}
J	4-OH-3,3',4',5,5'-pentaCB	10.3	8.5	4.65 ± 1.86 * 10^{8*}
K	5-OH-3,3',4,4'-tetraCB	25	3.5	6.80 ± 2.0 * 10^{7*}

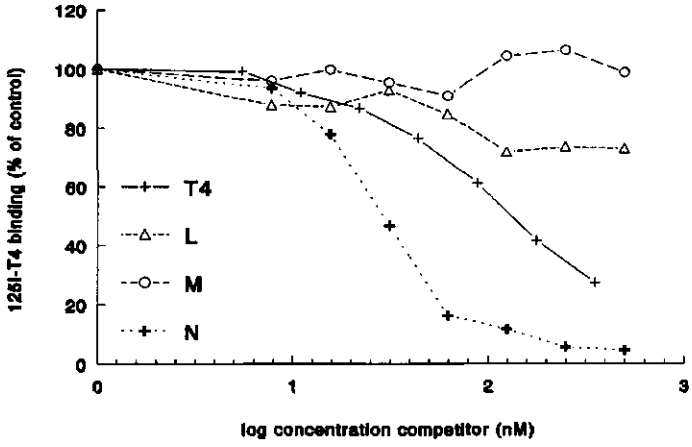
Note: Compounds are numbered as in Figure 1. Relative potency is given as the ratio of $IC_{50}(T_4)/IC_{50}$(competitor). Binding affinity constants (Ka) are determined by non-linear curve fitting of T_4 displacement curves. Results shown are means ± S.D. of triplicate measurements. Values that differ significantly (Student's t-Test) from the natural ligand, T_4 are indicated: $^*p < 0.05$

In contrast the 4-OH-2',3,3',4',5-pentaCB (C) and 5-OH-2,3,3',4,4'-pentaCB (D) were more potent than unlabeled T_4 in this assay system (IC_{50} 15.0 and 19 nM, respectively, Table 1). The binding affinity constants (Ka's) deduced from the displacement curves by non-linear curve fitting showed that *para-* (C) and *meta-* (D) hydroxylated metabolites of CB 105 had higher binding affinities to TTR than the *ortho*-hydroxy-metabolite (B) (Table 1).The non-*ortho*-chlorine substituted 4-OH-3,3',4',5,5'-pentaCB (J), a metabolite of 3,3',4,4',5,-pentaCB (CB 126) was also a potent inhibitor of TTR-T_4 binding (IC_{50}: 10.3 nM, Ka: 4.65 * 10^8 M^{-1})

Hydroxylated PCBs with different degree of coplanarity

The ease with which the biphenylrings can adopt a co-planar configuration is dependent on the degree of *ortho*-chlorine substitution of PCBs. Therefore the influence of the degree of *ortho*-chlorine substitutions of *meta-* or *para*-hydroxylated PCBs on the TTR-

A



B

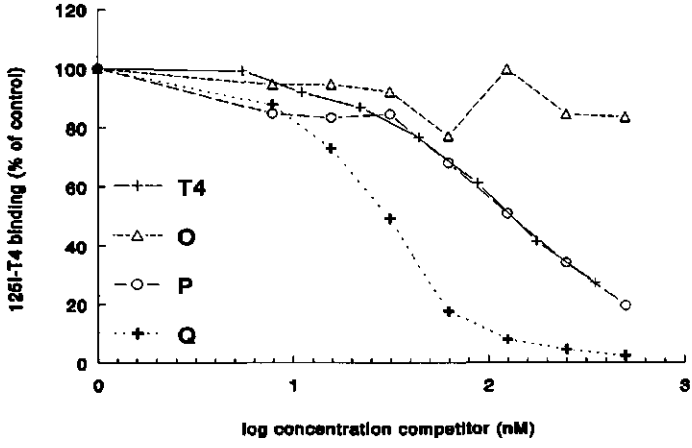


Figure 4 A) Competitive binding of hydroxylated PCDFs (L,M,N) and B) hydroxylated PCDDs (O,P,Q) measured by the TTR-T_4 competition-binding assay (Materials and Methods). Datapoints are mean values of duplicate incubations. Relative ^{125}I-T_4-TTR binding (% of control value) is plotted against the log concentration (nM) of competitor or T_4 added.

T_4 displacement potency of the compounds was investigated. The TTR-T_4 displacement potency and TTR affinity constants of all non- (E), mono- (C,D,F,H), and di-*ortho* (G) chlorine substituted hydroxy-PCBs were within the same order of magnitude i.e., IC_{50} and Ka values ranging from 6.5 - 17.5 nM and 6.2 * 10^7 - 8.3 *10^8 M^{-1} (Table 1). The data indicate that these groups of hydroxy-PCBs were more potent than the natural ligand T_4 (Fig. 3). The binding affinities of non- and mono-*ortho* chlorine substituted PCB-metabolites 4-OH-3,3',4'-triCB (E) and 4-OH-2,3,3',4'-tetraCB (F) were similar (IC_{50} resp. 8.8 and 10.5 nM), while the di-*ortho* chlorine substituted compound 4-OH-2,2',3,3',4'-pentaCB (G) exhibited lower potency.

The binding characteristics of 5 other hydroxylated PCB congeners with a different degree in *ortho*-chlorine substitution are summarized in Table 1. Both the two dihydroxylated PCBs, the 4,4'-$(OH)_2$-3,3',5,5'-tetraCB (H) and the 4,4'-$(OH)_2$- 2,3,3',5,-5'-pentaCB (I) had a high affinity for TTR (IC_{50} 16.5 and 6.5 nM) and again the degree of *ortho*-chlorine substitution did not seem to have much influence on binding to TTR. Hydroxylation on both *para* positions (H,I) did not result in significantly stronger TTR binding. The binding affinities and Ka values for the 2 *meta* hydroxy-PCBs, 5-OH-3,3'4,4'-tetraCB (K) and 5-OH-2',3,3',4,4'-pentaCB (D) (Table 1) were also similar (6.8 * 10^7 and 7.78 * 10^7 M^{-1}, respectively).

Table 2 Competitive ^{125}I-T_4-TTR binding inhibition concentrations (IC_{50}), relative potencies and TTR binding affinity constants (Ka) of hydroxylated polychlorodibenzo-*p*-dioxins (PCDDs) and polychlorodibenzofurans (PCDFs).

No	Structure	IC_{50} (nM)	Rel. Pot.	Ka (M^{-1}) (mean ± s.d.)
	Thyroxine	138	1	2.27 ± 0.13 * 10^7
L	2-OH-7,8-diCDF	>1000	<<1	N.D.
M	8-OH-2,3,4-triCDF	>1000	<<1	N.D.
N	3-OH-2,6,7,8-tetraCDF	30.2	4.5	5.35 ± 1.44 * 10^{8*}
O	8-OH-2,3-diCDD	>1000	<<1	N.D.
P	7-OH-2,3,8-triCDD	136	1.0	4.33 ± 1.17 * 10^7
Q	2-OH-1,3,7,8-tetraCDD	31.6	4.37	6.33 ± 4.93 * 10^{8*}

Note: Compounds are numbered as in Figure 1. Relative potency is given as the ratio of $IC_{50}(T_4)/IC_{50}$(competitor). Binding affinity constants (Ka) are determined by non-linear curve fitting of T_4 displacement curves. Results shown are means ± S.D. of triplicate measurements. Values that differ significantly (Student's t-Test) from the natural ligand, T_4 are indicated: $^*p < 0.05$

Hydroxylated PCDDs and PCDFs

In addition to hydroxylated PCB compounds, a number of hydroxylated PCDDs and PCDFs were also tested in the TTR competitive binding assay. Large differences were observed in T_4-TTR binding inhibition potencies and affinity constants between the various hydroxylated PCDDs and PCDFs (Fig. 4a,b; Table 2). 2-OH-1,3,7,8-tetraCDD (Q) and 3-OH-2,6,7,8-tetraCDF (N) exhibited the highest TTR-T_4 binding inhibition potential with IC_{50} values of 31.6 and 30.2 nM, respectively. These 2 compounds were both substituted with one or two chlorine atoms adjacent to a OH-group. A significant difference in potency was noted between 7-OH-2,3,8-triCDD (P) and 3-OH-2,6,7,8-tetraCDF (N), the latter more highly chlorinated compound was approximately 4 times more potent than the former (Table 2).

The hydroxylated PCDFs, 2-OH-7,8-diCDF (L) and 8-OH-2,3,4-triCDF (M) did not possess chlorine atoms substituted adjacent to the OH-group and did not interact with TTR at the concentrations tested in this study (Fig 4a). The TTR binding affinities of the hydroxylated chloro-dibenzo-*p*-dioxins 7-OH-2,3,8-triCDD (P) and 2-OH-1,3,7,8-tetraCDD (Q) were equal or higher than that observed for T_4 whereas 8-OH-2,3-diCDD (O), lacking adjacent chlorine and hydroxy groups was inactive in this binding assay. In addition the parent compound 2,3,7,8-tetraCDD (TCDD) was unable to compete in the TTR-T_4 binding assay. No inhibition of T4 binding to TTR was found, even at high concentrations (595 nM TCDD in incubation, data not shown). The potencies of the hydroxylated PCDDs and PCDFs (N,P,Q) in the TTR-T_4 binding assay were similar to those observed for the hydroxylated PCBs (Table 1 and Table 2).

Discussion

The main objective of this study was to gain further insight in the structural requirements of hydroxy metabolites of PCBs and related compounds as competitors for T_4 binding to TTR.

The *in vitro* TTR-T_4 competitive binding data obtained in this study indicate an essential requirement of a hydroxy-group on the PCB molecule for displacement of ^{125}I-T_4 from the TTR protein. The hydroxy-PCBs with the hydroxy-group substituted on either *meta*, or *para* positions (compounds C - K) showed a much higher competitive binding potency (35 - 136 fold) than the corresponding *ortho* hydroxy substituted compound, 2-OH-2',3,3',4,4'-pentaCB (B). In addition, there is an essential requirement of at least one, but preferably more chlorine substitutions adjacent to the *meta*, or *para* hydroxy-group on the PCB molecule. However, coplanarity of the hydroxy-PCB molecule does not appear to be a requirement for competitive binding to TTR, since there is little or no difference in ^{125}I-T_4 displacement potency between the non-, mono- and di-*ortho* hydroxy-PCB congeners tested (E, F, G). This result is not surprising in view of the non-planar structure of the natural ligand (T_4) for TTR.

Some hydroxylated derivatives of PCDD and PCDF also showed a potent competitive ^{125}I-T_4 displacement capacity on TTR. Similar to the hydroxy-PCBs; chlorine substitution adjacent to the hydroxy-group on the PCDD and PCDF molecule (compounds N,P,Q) appeared to be an essential requirement for competitive binding to TTR. Again, the most toxic PCDD isomer, TCDD, did not show any competition for T4 due to the absence of a hydroxy-group. The relative binding potencies of the hydroxy-

PCDD/F compounds N,P,and Q were in the same range as the *meta/para*-hydroxylated PCBs (compounds C - K) and one to four fold higher than the TTR binding potency of the natural ligand, T_4. Apparently, the rigid, planar structure of the hydroxylated PCDDs and PCDFs (N,P,Q) did not negatively influence the TTR-T_4 competitive binding capacity, compared to the more flexible structures of the *meta/para*-hydroxylated PCBs.

The structure-activity data on competitive binding to TTR of hydroxy-PCBs, PCDDs and PCDFs obtained in this study are in good agreement with previous studies on hydroxylated metabolites of 3,3',4,4'-tetrachlorobiphenyl (CB 77) (Brouwer and Van den Berg, 1986,; Brouwer *et al.*, 1990), of several other PCB congeners (Rickenbacher *et al.*, 1986; Brouwer *et al.*, 1990) and of chlorinated benzenes and their phenolic metabolites (Van den Berg, 1990; Den Besten *et al.*, 1991). Favourable structural features for ^{125}I-T_4 displacement on TTR of CB 77 metabolites involved *meta/para* hydroxylation with one or more chlorine substitutions adjacent to the hydroxy-group. Similarly, competitive binding studies with chlorinated benzenes and -phenols indicated an essential requirement for adjacent chlorine and hydroxy substitution (Van den Berg , 1990). Rickenbacher *et al.* (1986) also observed that substitution of PCBs with a hydroxy-group, in particularly on the *meta*, or *para* positions with adjacent chlorine substitution greatly facilitated competitive binding to TTR.

However, there are several differences between the results of our studies (Brouwer and Van den Berg, 1986, Brouwer *et al.*, 1990) and the reports of Rickenbacher *et al.* (1986) and McKinney and coworkers (1985). For example, they reported that laterally substituted PCBs showed the highest affinity for TTR and further *ortho*-chlorine substitution lowered the binding affinity of these compounds to a great extent. This was not observed in this study, where non-*ortho*, mono-*ortho* and di-*ortho* chlorine substituted hydroxy-PCBs showed similar competitive binding to TTR. Furthermore, using computer modelling the potentially high competitive binding of TCDD and its adipamide-derivative to TTR was also reported (McKinney *et al.*, 1985), but we were unable to find any competitive binding potency of TCDD in our *in vitro* TTR-T_4 competitive binding assay. In fact, no ^{125}I-T_4 displacement on TTR has been observed using either parent PCB congeners (CB 77, CB 105), a commercial PCB mixture Aroclor 1254, TCDD or several different chlorobenzenes. This indicates an essential requirement of a, preferably lateral substituted hydroxy-group for competitive binding. In contrast, Rickenbacher *et al.* (1986) reported high competitive binding of the parent PCBs, 3,3',5,5'-tetraCB and 3,3',4,4',5,5'-hexaCB to thyroxine binding prealbumin (TBPA, synonymus to TTR), which suggests that there may be no strict requirement for a laterally substituted hydroxy-group. The reason for this discrepancy is unknown, and will be further investigated in our laboratory.

The structural requirements of hydroxylated PCBs, PCDDs and PCDFs for competitive binding to TTR observed in this study resemble those for T_4 binding to TTR (Blake and Oatley, 1977). Halogen-substitution on the phenolic ring adjacent to the *para* hydroxy-group enhance the binding of T_4 to TTR (Andrea *et al.*, 1980), similar to the effects of chlorine substitution adjacent to the *meta/para* hydroxy-group observed for the hydroxylated PCBs, PCDDs and PCDFs investigated in this study. Also the diphenyl ether structure of T_4 is not necessary for strong TTR binding (Pages *et al.*, 1973), which suggests that there is flexibility in the T_4-binding pocket on the TTR molecule thus facilitating the binding of compounds with markedly different three-dimensional

conformation.

T_4 can bind to TTR in probably 2 different modes; the "forward" and "reverse" mode (De la Paz *et al.*, 1992). In the "forward" mode the phenolic ring is buried deep in the TTR binding site while the phenyl-ring with amino-side chain is close to the binding site entrance. In this way the phenolic ring can form hydrogen-bonds with watermolecules inside the protein. In the "reverse" binding mode the amino-side chain is closest to the centre of the binding site, while the phenolic ring hydrogen-binds with the charged residues at the entrance. Hydrogen-bonds seem to be important for T4 binding. This may explain the hydroxy-group requirement for TTR binding of PCBs and related compounds, as shown in this study. The actual entrance and docking of hydroxy-PCBs in the T_4-binding pocket of TTR will be further investigated.

The close structural resemblance between T_4 and the hydroxy-PCBs, PCDDs and PCDFs, and the high *in vitro* competitive ^{125}I-T_4 displacement potency of all *meta/para* hydroxylated PCBs and the hydroxy-PCDD/F compounds (N,P,Q), suggests that hydroxy-metabolites of these compounds may also play a role in the *in vivo* observed marked alterations in thyroid hormone metabolism, following exposure of rodents and other species to these compounds. In fact, high affinity, competitive binding of 4-OH-3,3',4',5-tetraCB to TTR, accompanied by marked plasma total T_4 reductions was observed in rats, mice (Brouwer and Van den Berg, 1986, Brouwer, 1989) and marmoset monkeys (Beetstra *et al.*, 1991, Brouwer, 1987), following exposure to CB-77.

For other PCB congeners and commercial PCB mixtures, such as Aroclor 1254, it is still unknown whether hydroxy-metabolites also occupy T_4-binding sites on plasma TTR to such an extent that they may play a role in plasma thyroid hormone reductions observed, following exposure to these chemicals. However, several of the tested hydroxy PCBs and related compounds have been identified *in vivo*, such as the hydroxy metabolites of CB 105 (compounds B,C and D) which have been identified in mice following exposure to this compound (Klasson-Wehler *et al.*, 1990). Recent results on exposure of rats to Aroclor 1254, indicate the presence of considerable amounts of several hydroxy-PCB metabolites in the blood, including the 4-OH-2',3,3',4',5-pentaCB (Klasson-Wehler *et al.*, 1992). The latter hydroxylated PCB compound was tested in our study and found to possess a potent competitive binding affinity to TTR (IC_{50}: 15.0 nM). Furthermore, 4-OH-3,3',4',5,5'-pentaCB (J), a metabolite of 3,3',4,4',5-pentachlorobiphenyl (CB 126), which is shown to have a high competitive TTR-binding affinity, has been detected after *in vivo* exposure of rats to CB 126 (Koga *et al.*, 1990). The 2 major hydroxy-metabolites of 2,3,7,8-tetraCDD (compounds P and Q) identified in mammalian species (Mason and Safe, 1986; Ramsey *et al.*, 1982) were found to be potent TTR-T_4 binding competitors.

Due to the important function of TTR in rodent T_4 plasma transport, T_4 decreases found in rodents after PCB exposure *in vivo* can be caused by TTR blockage by hydroxylated metabolites. Still, one cannot exclude the role of induced T_4 glucuronidation by PCBs in plasma T_4 decrease. The extent of involvement of hydroxy-metabolites of PCBs and related compounds in the observed reduction in plasma T_4 levels will be investigated further in several laboratory and wildlife species, including man. Other studies in our laboratory, showed marked decreases in plasma T_4 levels, and alterations in thyroid hormone metabolism in TCB exposed pregnant rats, their fetuses and their offspring (Morse *et al.*, 1993a). The possible neurotoxic consequences of PCB exposure in fetal and neonatal rats may be caused by interference of PCBs in thyroid hormone

metabolism. Although TTR is not the major human T_4 transport protein (10-40 % T_4 binding in plasma) when compared to thyroxin binding globulin (TBG, 50-80 % T_4 binding in plasma) (Larsson *et al.*, 1985), TTR is essential for T_4 transport over the blood-brain barrier in mammals (Herbert *et al.*, 1986). The role of T_4-TTR displacement in T_4 levels in man may be marginal, but the suggested role of TTR in the brain may selectively put the brain at risk for toxic consequences.

In conclusion, highly potent, competitive interactions of hydroxylated PCBs, PCDDs and PCDFs with the thyroid hormone transport protein TTR, were found *in vitro* using human TTR. The structural requirements for this competitive binding resembled those observed for the natural TTR ligand, T_4. The possible role of hydroxylated metabolites in the toxicity of PCBs, PCDDS and PCDFs is unknown. However based on the data presented herein it is possible that hydroxylated PCBs, PCDDs, PCDFs and related compounds may contribute to some of the toxic responses of these chemicals, especially those that may relate to reduced thyroid hormone levels, such as wasting syndrome, developmental neurotoxicity and carcinogenicity.

Acknowledgements

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CHAPTER 3

Different competition of thyroxine binding to transthyretin and thyroxine binding globulin by hydroxy-polychloro-biphenyls, -dibenzo-*p*-dioxins and -dibenzofurans

Abstract

In a previous study several hydroxylated polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) competitively displaced ^{125}I-thyroxine (T_4) from transthyretin with different potencies. Transthyretin is the major T_4 transport protein in plasma of rodents. In man, however, thyroxine-binding globulin transports most T_4 in blood. In this study, hydroxylated PCBs, PCDDs and PCDFs were tested in an in vitro competitive binding assay, using purified human thyroxine-binding globulin and ^{125}I-T_4 as the displaceable radioligand. None of the tested hydroxylated PCBs, PCDDs and PCDFs inhibited ^{125}I-T_4 binding to thyroxine-binding globulin. In addition, some T_4 derived compounds; e.g. tyrosine, mono-iodotyrosine, di-iodotyrosine and tri-iodophenol were tested on both transthyretin and thyroxine-binding globulin to investigate possible differences in structural characteristics determining T_4 binding to thyroxine-binding globulin and transthyretin. The T_4 derived compounds also did not inhibit ^{125}I-T_4 binding to thyroxine-binding globulin as tested in the in vitro assay. However, tri-iodophenol and to a lesser extent di-iodotyrosine inhibited ^{125}I-T_4-transthyretin binding. These results indicate a marked difference in T_4 binding to thyroxine-binding globulin or transthyretin. The hydroxylated PCBs, PCDDs and PCDFs can inhibit T_4 binding to transthyretin, but not to thyroxine-binding globulin, and thus may cause different effects in rodents and man.

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Introduction

Polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) are widespread, persistent environmental contaminants (Ballschmitter et al., 1989; Rappe and Buser, 1989). These compounds are present in higher levels in top predators like fish-eating birds, marine mammals and man due to the biomagnification through the foodchain. Background levels found in human breastmilk range from 0.3 to 2.8 ppm total PCBs in milk fat whereas in human blood (lipid) levels of 650-7700 ppt PCBs, 150-1000 ppt PCDDs and 25-190 ppt PCDFs are found (Ahlborg *et al.*, 1992).

PCBs, PCDFs and PCDDs cause a range of common toxic effects in laboratory animals such as hepatotoxicity, thymic atrophy (McConnell, 1980), chloracne and related dermal lesions, immunotoxicity, reproductive and developmental toxicity and different endocrine responses including disturbances in thyroid hormone homeostasis (Ahlborg *et al.*, 1992, McConnell, 1989). Exposure to mixtures of PCBs (Bastomsky, 1974, Saito *et al.*, 1991) and polybrominated biphenyls (PBBs) (Byrne *et al.*, 1987, Gupta *et al.*, 1983, Spear *et al*, 1990) changed thyroid hormone metabolism in rats and mice and especially induced thyroxine (T_4) glucuronidation. Certain PCBs and PCDDs like 3,3',4,4'-tetrachlorobiphenyl (TCB) (Brouwer and Van den Berg, 1986, Brouwer, 1989, Beetstra *et al.*, 1991) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Bastomsky, 1977, Lucier *et al.*, 1975, Potter *et al.*, 1983, Lans *et al.*, 1990) reduced plasma total (TT_4) and free T_4 levels and simultaneously induced T_4-glucuronidation and inhibited thyroxine-5'-deiodinase type 1 activity in rats (Adams *et al.*, 1990). Exposure of marmoset monkeys to TCB caused hypothyroidism in these animals (Van den Berg *et al.*, 1988). In man, several studies indicate changes in human serum T_4 levels after exposure to PCBs or PBBs (Emmett *et al.*, 1988, Murai *et al.*, 1987, Bahn *et al.*, 1980, Kreiss *et al.*, 1982).

The decreases in plasma T_4 levels after exposure of rats and mice to PCBs, PCDDs and PCDFs are caused in part by increased hepatic T_4 glucuronidation. In addition, a disturbance of plasma T_4 transport may be involved in the reduction of plasma T_4 concentrations by PCBs. Exposure of rats (Brouwer, 1989) and mice (Brouwer and Van den Berg, 1986) to TCB *in vivo* severely reduced plasma T_4 levels. Hydroxylated metabolites of TCB specifically interacted with the T_4 binding site of transthyretin, the major plasma thyroid hormone transport protein in rodents, and inhibited T_4-transthyretin binding causing T_4 levels to drop (Brouwer, 1989). Different hydroxylated PCBs, PCDDs, PCDFs (Lans *et al.*, 1993, Brouwer *et al.*, 1990) and related chlorophenols (Van Den Berg, 1990, Den Besten *et al.*, 1991) specifically inhibited T_4-transthyretin binding *in vitro*.

Transthyretin is a highly conserved plasma T_4 transport protein present in most mammalian species (Larsson *et al.*, 1985). Another important plasma T_4 binding protein, thyroxine binding globulin, is present in higher mammals, including man. Thyroxine-binding globulin, however, is not present in rats and mice, except during the first 3 weeks after birth or in hypothyroidal state (Savu *et al.*, 1989).

Thyroxine-binding globulin is a high affinity, low capacity T_4 transport protein and levels in human blood circulation (15 mg/l) are lower than transthyretin levels (300 mg/l, De la Paz *et al.*, 1992). Transthyretin and thyroxine-binding globulin have different T_4 binding properties; in human plasma thyroxine-binding globulin transports a higher percentage of T_4 (50-80 %) than transthyretin (10-40 %) (Larsson *et al.*, 1985). The T_4 binding affinity for thyroxine-binding globulin is higher than for transthyretin

(Ka's: 10^{10} M^{-1} and 10^{7}-10^{8} M^{-1} respectively). In addition thyroxine-binding globulin has only one T_4 binding site while transthyretin has two T_4 binding sites with different binding affinities.

Different structural characteristics of the T_4 molecule seem to be important for binding to thyroxine-binding globulin as compared to transthyretin. The phenolic hydroxyl group and iodine substituents on the phenolic ring of T_4 are clearly important for binding to transthyretin (Andrea *et al.*, 1980). The necessary structural characteristics of T_4 for binding to thyroxine-binding globulin are the iodine-substituents on the inner amino-phenyl ring and the outer phenolic ring, the L-configuration of the alanyl side chain and the presence of a 4'-hydroxyl-group (Snyder *et al.*, 1976, Terry and Blake, 1992).

In vitro competitive interactions of hydroxylated PCBs, PCDDs and PCDFs with purified human transthyretin have already been observed in an earlier study (Lans *et al.*, 1993). However, thyroxine-binding globulin appears to be quantitatively more important than transthyretin for T_4 transport in man. It was considered important to investigate the potential competitive binding affinity of the hydroxylated metabolites of these compounds to thyroxine-binding globulin to be able to predict possible reductions in plasma T_4 levels in man, following exposure to PCBs, PCDDs and PCDFs.

Several hydroxylated PCB, PCDD and PCDF metabolites and parent compounds (Fig. 1,3 and 4) were tested in this study for T_4 displacement potential on thyroxine-binding globulin *in vitro* using competitive binding assays. Additionally, T_4-derived compounds were tested *in vitro* to investigate possible differences in structural characteristics determining T_4 binding to thyroxine-binding globulin and transthyretin. Tyrosine (Tyr), mono-iodotyrosine (MIT) and di-iodotyrosine (DIT) are structurally related to the amino-phenylring of T_4, with a phenolic hydroxyl-group present. 2,4,6-Triiodophenol (TIP) is structurally related to the iodophenolic ring of T_4.

Materials and methods

Materials

Radiolabeled L-[3',5'-^{125}I] thyroxine (spec. act. 46 μCi/μg) was purchased from Amersham, Buckinghamshire, England and contained 5-10% free iodide, as determined with Sephadex LH-20 (Pharmacia LKB, Woerden, the Netherlands) gel filtration. Transthyretin (human prealbumin, 98% pure), thyroxine-binding globulin (human, 100% pure) and 3,3',5,5'-L-thyroxine (T_4) were obtained from Sigma Chemical Company (St. Louis, MO, USA). Methanol (Lichrosolv) was obtained from Merck Chemical Company (Darmstadt, Germany). Biogel P-6DG desalting gel was obtained from Bio-Rad Laboratories (Richmond, CA, USA).

Tyrosine (Tyr), mono-iodotyrosine (MIT) and di-iodotyrosine (DIT) were kindly provided by Dr. T.J. Visser, Erasmus University Rotterdam, the Netherlands. 2,4,6-triiodophenol (TIP) was purchased from Aldrich Chemical Co. Ltd., Brussels, Belgium.

2,3,3',4,4'-Pentachlorobiphenyl (CB 105) (A, Fig. 3) and 3,3',4,4'-tetrachlorobiphenyl (TCB or CB 77) (a, Fig. 1) were purchased from Promochem GmbH, Wesel, Germany. The hydroxylated metabolites of TCB (fig.1) eg. 2-OH-3,3',4,4'-tetraCB (b), 4-OH-3,3',4',5-tetraCB (c), 5-OH-3,3',4,4'-tetraCB (d) and 6-OH-3,3',4,4'-tetraCB (e) were synthesized as previously described (Lans *et al.*, 1993, Klasson-Wehler *et al.*, 1990). The other hydroxylated PCBs (fig. 3) used in the present study: 2-OH-2',3,3',4,-

4'-pentaCB (B), 4-OH-2',3,3',4',5-pentaCB (C), 5-OH-2',3,3',4,4'-pentaCB (D), 4-OH-3,3',4'-triCB (E), 4-OH-2,3,3',4'-tetraCB (F), 4-OH-2,2',3,3',4'-pentaCB (G), 4,4'-$(OH)_2$-2,3,3',5,5'-pentaCB (H), 4,4'-$(OH)_2$-3,3',5,5'-tetraCB (I), 4-OH-3,3',4',5,5'-pentaCB (J) and 5-OH-3,3',4,4'-tetraCB (K)* were obtained as a gift from Dr. E. Klasson-Wehler, Environmental Chemistry, Wallenberg Laboratory, Stockholm University, S-10691 Stockholm, Sweden. *The numbering of the hydroxy-substituents on the biphenyl does not follow IUPAC rules but has been chosen in order to facilitate comparison of the structures.

The tested hydroxylated polychloro-dibenzo-*p*-dioxins and polychloro-dibenzofurans (2-OH-7,8-diCDF (L), 8-OH-2,3,4-triCDF (M), 3-OH-2,6,7,8-tetraCDF (N), 8-OH-2,3-diCDD (O), 7-OH-2,3,8-triCDD (P) and 2-OH-1,3,7,8-tetraCDD (Q)), shown in figure 3, were synthesized as previously described (Denomme *et al.*, 1985, 1986, Mason and Safe, 1986) and kindly donated by prof. dr. S. Safe, Department of Veterinary Physiology and Pharmacology, A & M University, College Station, Texas, U.S.A.

In vitro T_4 competition-binding assays for TBG and TTR

The gel-filtration procedure as decribed by Somack *et al.* (1982) with minor modifications was used for the T_4 competition-binding assay. The assay, using human transthyretin or thyroxine-binding globulin, was performed as described by Lans *et al.*, 1993. Transthyretin or thyroxine-binding globulin (30 nM, dissolved in 0.1 M Tris-HCl, 0.1 mM NaCl, 1 mM EDTA buffer, pH 8) was incubated with a mixture of ^{125}I-T_4 and unlabeled T_4 (70.000 cpm, 55 nM) in Tris-HCl buffer, and competitors (T_4, monoiodotyrosine, di-iodotyrosine, tyrosine, tri-iodophenol and parent or hydroxylated PCBs, PCDDs or PCDFs) in increasing concentrations or methanol (control) were added. Total ^{125}I-radioactivity added to each of the incubation mixtures (200 µl total volume) was checked by gamma-counting (Cobra Auto-gamma, Packard Instrument co., USA). The incubations were allowed to reach binding equilibrium overnight at 4 °C. Protein-bound ^{125}I-T_4 and free ^{125}I-T_4 were separated on 1 ml Biogel P-6DG columns and were spin-forced eluted with Tris-HCl buffer. The first 2 eluate fractions, containing the protein bound ^{125}I-T_4 fraction, were combined; radioactivity was counted and compared to the control incubations. Non-protein bound T_4 was retained on the Biogel matrix. Competition binding curves were made by plotting relative ^{125}I-T_4-protein binding (% of control) against added log competitor concentration.

Analysis of binding data

Binding characteristics are given for transthyretin as relative potencies: the ratio of inhibitor concentration at 50 % inhibition (IC_{50}) of unlabeled T_4 vs the IC_{50} of hydroxylated metabolites of PCBs, PCDDs and PCDFs.

Statistics

Data are given as means with standard deviations (S.D.). Significant differences were determined by statistical analysis using the Student's t-test (with a probability value $p < 0.05$).

Results

Effects of different PCB metabolites on T_4 binding to TTR and TBG

The competitive binding potency of unlabeled T_4 expressed as IC_{50} was 52.2 - 85 nM for thyroxine-binding globulin (data not shown) and 88.3 - 138 nM for transthyretin (Table 1 and 2) in the *in vitro* T_4 binding competition assays, in accordance with the higher T_4 binding affinity of thyroxine-binding globulin. TCB and its OH metabolites (Fig. 1) did not compete with T_4 for binding to thyroxine-binding globulin (Fig. 2a) in the *in vitro* binding studies; only very high concentrations of 6-OH-3,3',4,4'-tetraCB (e) and 5-OH-3,3',4,4'-tetraCB (d) inhibited T_4 binding to thyroxine-binding globulin (IC_{50}: 1380 and 8000 nM respectively). On the contrary, all OH-metabolites of TCB inhibited T_4-transthyretin binding, but with different inhibition potencies of : 5-OH-3,3',4,4'-tetraCB = 4-OH-3,3',4',5-tetraCB > T_4 > 2-OH-3,3',4,4'-tetraCB > 6-OH-3,3',4,4'-tetraCB > TCB (with relative potencies : 2.7, 2.6, 1, 0.5, 0.05 and <<1, respectively) (Fig. 2b). TCB apparently did not inhibit T_4-transthyretin binding.

Several other hydroxylated PCB metabolites (Fig. 3) tested on thyroxine-binding globulin did not inhibit T_4 binding to thyroxine-binding globulin (>> 90% binding at 250 nM competitor added) (Table 1). All hydroxylated metabolites of 2,3,3',4,4'-PeCB (Fig. 3, B,C,D) showed T_4-transthyretin binding competition in contrast to the parent compound 2,3,3',4,4'-PeCB (Table 1). The 2-OH-2',3,3',4,4'-pentaCB metabolite (B) had a higher IC_{50} than T_4 (950 and 88.3 nM respectively), while the other metabolites 4-OH-2,3,3',4',5-pentaCB (C) and 5-OH-2,3,3',4,4'-PeCB (D) had IC_{50}'s lower than T_4 (15 and 19 nM).

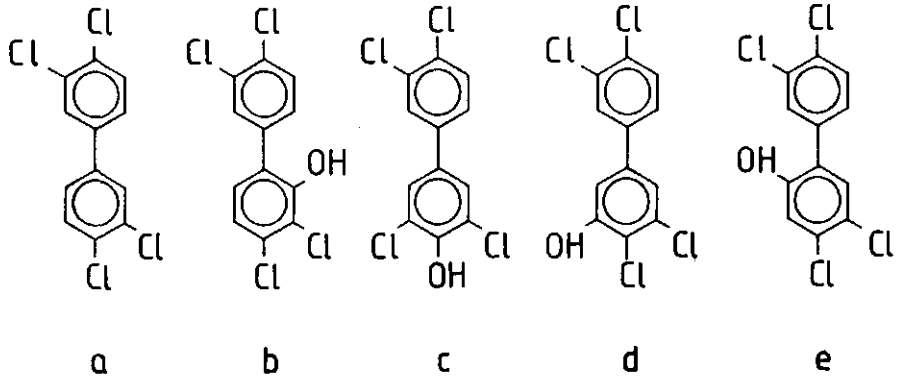
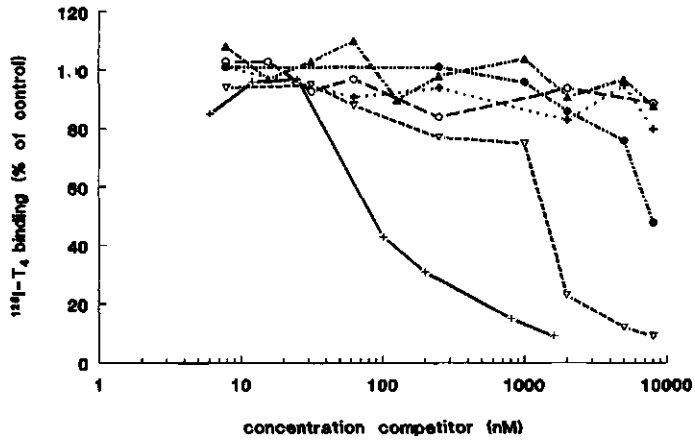


Figure 1 3,3',4,4'-Tetrachlorobiphenyl (TCB, a) and its hydroxymetabolites, 2-OH-3,3',4,4'-tetraCB (b), 4-OH-3,3',4',5-tetraCB (c), 5-OH-3,3',4,4'-tetraCB (d) and 6-OH-3,3',4,4'-tetraCB (e) as tested in the T_4-transthyretin or T_4-thyroxine-binding globulin competition binding assays and synthesized as described in Materials and Methods.

A



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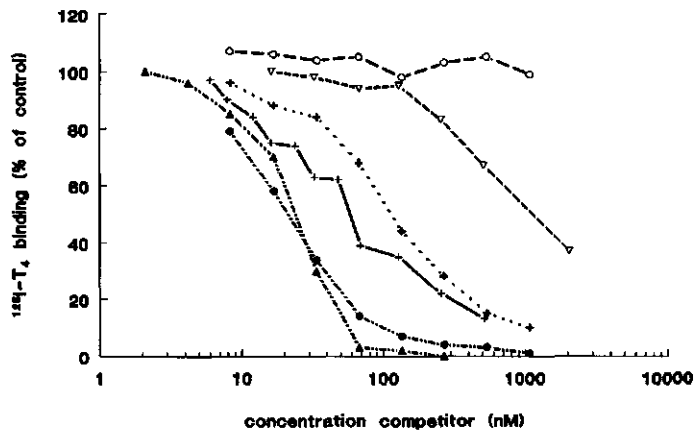
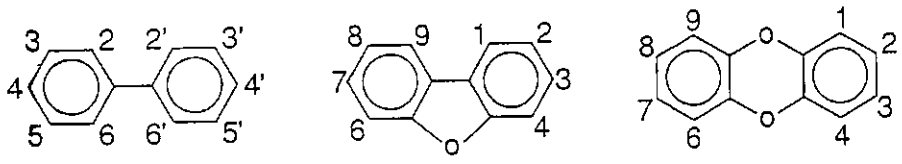


Figure 2 A) Competitive binding to thyroxine-binding globulin or B) competitive binding to transthyretin of T_4(+), TCB (fig.1:a(○)) and its hydroxy metabolites (fig.1: b(+),c(▲),d(●) and e(▽)), as measured by the *in vitro* competition-binding assay (Materials and Methods). Datapoints are mean values of duplicate incubations. Relative ^{125}I-T_4-thyroxine-binding globulin or ^{125}I-T_4-transthyretin binding (% of control value) is plotted against the log concentration (nM) of competitor or T_4 added.



I II III

R	2	3	4	5	6	2'	3'	4'	5'	6'
A	Cl	Cl	Cl				Cl	Cl		
B	OH	Cl	Cl			Cl	Cl	Cl		
C		Cl	OH	Cl		Cl	Cl	Cl		
D		Cl	Cl	OH		Cl	Cl	Cl		
E		Cl	OH				Cl	Cl		
F	Cl	Cl	OH				Cl	Cl		
G	Cl	Cl	OH			Cl	Cl	Cl		
H	Cl	Cl	OH	Cl			Cl	OH	Cl	
I		Cl	OH	Cl			Cl	OH	Cl	
J		Cl	OH	Cl			Cl	Cl	Cl	
K		Cl	Cl	OH			Cl	Cl		

R	1	2	3	4	6	7	8	9
L		OH				Cl	Cl	
M		Cl	Cl	Cl			OH	
N		Cl	OH		Cl	Cl	Cl	

R	1	2	3	4	6	7	8	9
O		Cl	Cl				OH	
P		Cl	Cl			OH	Cl	
Q	Cl	OH	Cl			Cl	Cl	

Figure 3 Structures of hydroxylated PCBs, PCDDs and PCDFs tested in the T_4-transthyretin or T_4-thyroxine-binding globulin competition binding assay and synthesized as described in Materials and Methods.
I: (hydroxylated) polychlorobiphenyls with substituents R (OH or Cl).
2,3,3',4,4'-pentaCB (CB 105) (A), 2-OH-2',3,3',4,4'-pentaCB (B), 4-OH-2',3,3',4',5-pentaCB (C), 5-OH-2',3,3',4,4'-pentaCB * (D), 4-OH-3,3',4'-triCB (E), 4-OH-2,3,3',4'-tetraCB (F), 4-OH-2,2',3,3',4'-pentaCB (G), 4,4'-$(OH)_2$-2,3,3',5,5'-pentaCB (H), 4,4'-$(OH)_2$-3,3',5,5'-tetraCB (I), 4-OH-3,3',4',5,5'-pentaCB (J) and 5-OH-3,3',4,4'-tetraCB (K).
II: hydroxylated PCDFs with substituents R (OH or Cl).
2-OH-7,8-diCDF (L), 8-OH-2,3,4-triCDF (M), 3-OH-2,6,7,8-tetraCDF (N).
III: hydroxylated PCDDs with substituents R (OH or Cl).
8-OH-2,3-diCDD (O), 7-OH-2,3,8-triCDD (P) and 2-OH-1,3,7,8-tetraCDD (Q).

Table 1 T_4-transthyretin binding inhibition potencies (IC_{50}), relative potencies of transthyretin-binding (Lans *et al.*, 1993) and ^{125}I-T_4 binding to thyroxine-binding globulin (% bound of control at 250 nM competitor added) of parent and hydroxylated polychlorobiphenyls (PCBs). Compounds are numbered as in fig. 2. Relative potency is given as the ratio of $IC_{50}(T_4)/IC_{50}$(compound). Results shown are means ± S.D. of triplicate measurements. Values that differ significantly (Student's t-test) from the natural ligand thyroxine (T_4), are indicated: $^a p < 0.05$.

No	Structure	TTR IC_{50} (nM)	TTR relative potency	TBG % ^{125}I-T_4 bound
	Thyroxine	88.3	1	17.0 ± 2.0
A	2,3,3',4,4'-pentaCB	>1000	<<1	96.0 ± 1.3[a]
B	2-OH-2',3,3',4,4'-pentaCB	950	0.09	94.2 ± 2.5[a]
C	4-OH-2',3,3',4',5-pentaCB	15.0	5.9	99.2 ± 2.0[a]
D	5-OH-2',3,3',4,4'-pentaCB	19	4.6	95.2 ± 5.6[a]
E	4-OH-3,3',4'-triCB	10.5	8.4	93.9 ± 8.1[a]
F	4-OH-2,3,3',4'-tetraCB	8.8	10.2	95.6 ± 1.7[a]
G	4-OH-2,2'3,3',4'-pentaCB	17.5	5.0	99.7 ± 2.6[a]
H	4,4'-$(OH)_2$-2,3,3',5,5'-pentaCB	6.5	13.6	97.7 ± 4.3[a]
I	4,4'-$(OH)_2$-3,3',5,5'-tetraCB	16.5	5.4	97.9 ± 3.7[a]
J	4-OH-3,3',4',5,5'-pentaCB	10.3	8.5	96.1 ± 3.6[a]
K	5-OH-3,3',4,4'-tetraCB	25	3.5	94.1 ± 6.8[a]

Effects of different hydroxylated PCDFs and PCDDs on T_4 binding to TTR and TBG

Consistent with the hydroxylated PCB metabolites, the hydroxylated PCDFs and PCDDs tested on thyroxine-binding globulin (Table 2) did not inhibit T_4 binding to thyroxine-binding globulin. Only 3-OH-2,6,7,8-tetraCDF, 7-OH-2,3,8-triCDD and 2-OH-1,3,7,8-tetraCDD (Fig. 3, N, P and Q) (Table 2) inhibited T_4-transthyretin binding.

Effects of T_4-derived structures on T_4 binding to TTR and TBG

The T_4-derived structures tyrosine (Tyr), mono-iodotyrosine (MIT) and di-iodotyrosine (DIT) (Fig. 4) have been tested on competition of T_4 binding to transthyretin and thyroxine-binding globulin to further investigate the difference in binding characteristics of T_4. Tyrosine and mono-iodotyrosine did not inhibit binding of T_4 to transthyretin (Fig. 5b) nor thyroxine-binding globulin (Fig. 5a). However, tri-iodophenol and to a lesser extent di-iodotyrosine, inhibited T_4 binding to transthyretin (Fig. 5b) but not to thyroxine-binding globulin (Fig. 5a).

Discussion

The tested hydroxylated PCBs, PCDDs and PCDFs that inhibited T_4-transthyretin binding, did not inhibit T_4 binding to thyroxine-binding globulin. Moreover, the parent compounds 3,3',4,4'-tetraCB (TCB) and 2,3,3',4,4'-pentaCB (CB 105) did not show T_4 binding competition with transthyretin nor thyroxine-binding globulin. The T_4-derived structures, tyrosine, mono-iodotyrosine, di-iodotyrosine and tri-iodophenol did not interact with thyroxine-binding globulin, while tri-iodophenol and to some extent di-iodotyrosine competed with T_4 for transthyretin.

These data indicate that thyroxine-binding globulin, unlike transthyretin, is not a target protein for hydroxylated metabolites of PCBs, PCDDs and PCDFs. Furthermore, there are remarkable differences in ligand-protein interactions which may reflect different structural organisation of the T_4 binding pockets of transthyretin and thyroxine-binding globulin. The following features of thyroid hormones are important for optimal binding to thyroxine-binding globulin: (a) the L-alanine side chain conformation, (b) the presence of a 4'-hydroxyl group, (c) the presence of two substituents on the phenolic outer ring and the amino-phenyl inner ring (positions 3, 5, 3' and 5') and (d) the presence of either bromine or iodines on the inner amino-phenyl ring and iodines on the outer phenolic ring (Snyder *et al.*, 1976). Especially the inner ring iodines seem to be important for T_4 binding to thyroxine-binding globulin. This suggests the structural need for a second halogenated phenyl-ring of compounds inhibiting T_4 binding to thyroxine-binding globulin. Binding to thyroxine-binding globulin also requires the L-configurated ammonium-ion and the negative charged carboxylate ion of the amino-sidechain of T_4 (Snyder *et al.*, 1976). Of lesser importance is the presence of an oxygen atom in the ether position of T_4.

Table 2 T_4-transthyretin binding inhibition potencies (IC_{50}), relative potencies of transthyretin binding (Lans *et al.*, 1993) and ^{125}I-T_4 binding to thyroxine-binding globulin (% bound of control at 250 nM competitor added) of hydroxylated polychlorodibenzo-p-dioxins (PCDDs) and polychlorodibenzofurans (PCDFs). Compounds are numbered as in fig. 2. Relative potency is given as the ratio of $IC_{50}(T_4)/IC_{50}$(compound). Results shown are means ± S.D. of triplicate measurements. Values that differ significantly (Student's t-test) from the natural ligand thyroxine (T_4), are indicated: $^a p < 0.05$

No	Structure	TTR IC_{50} (nM)	TTR relative potency	TBG % ^{125}I-T_4 bound
	Thyroxine	138	1	17.0 ± 2.0
L	2-OH-7,8-diCDF	>1000	<<1	113.4 ± 4.8[a]
M	8-OH-2,3,4-triCDF	>1000	<<1	105.3 ± 0.5[a]
N	3-OH-2,6,7,8-tetraCDF	30.2	4.5	98.1 ± 3.0[a]
O	8-OH-2,3-diCDD	>1000	<<1	102.1 ± 1.1[a]
P	7-OH-2,3,8-triCDD	136	1.0	105.9 ± 8.3[a]
Q	2-OH-1,3,7,8-tetraCDD	31.6	4.37	95.3 ± 10.3[a]

The halogen substitutions on the outer phenolic ring appear to have two roles in binding to thyroxine-binding globulin: one is to increase the ionization of the 4'-hydroxyl group and the other is to contribute directly to hydrophobic bonding. According to Snyder *et al.* (1976) substitution of a smaller halogen (like bromine) for iodine on the inner amino-phenyl ring (positions 3 and 5) allows a closer approximation of this ligand to its binding site on thyroxine-binding globulin while bromines on the outer phenolic ring (positions 3' and 5') results in a marked decrease in thyroxine-binding globulin binding potency. Chlorine or bromine instead of iodine substitutions on ligands give weaker interactions with thyroxine-binding globulin due to the aromatic character of the binding site (Terry and Blake, 1992). So the tested (hydroxylated) PCBs, PCDDs and PCDFs may be unable to compete for T_4 binding to thyroxine-binding globulin as is shown in this study due to the chlorine substituents and the absence of a charged amino-sidechain and a tyrosine ring.

Additional experiments on thyroxine-binding globulin and transthyretin with thyroid hormone derived structures showed that tri-iodophenol and to a lesser extent di-iodotyrosine competively inhibited T_4-transthyretin binding, but not the binding of T_4 to thyroxine-binding globulin. These binding studies using mono-aromatic ring derivatives of T_4 indicate the importance of the phenolic ring with iodine atoms adjacent to the OH-group for binding to transthyretin but not to thyroxine-binding globulin. This explains why transthyretin is a target protein for the OH-metabolites of PCBs, PCDDs and PCDFs, while thyroxine-binding globulin is not; e.g. these compounds do contain a phe-

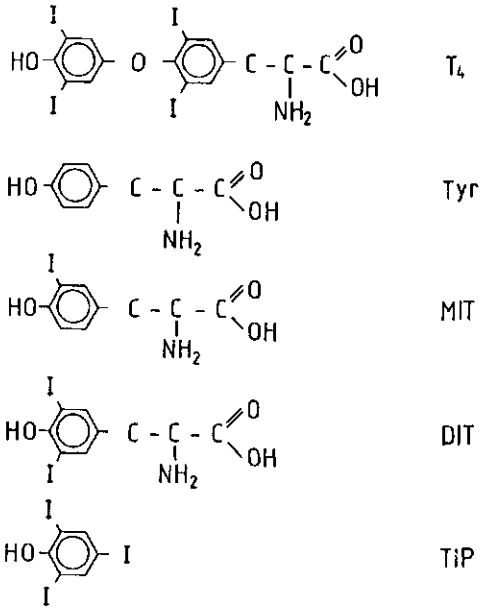
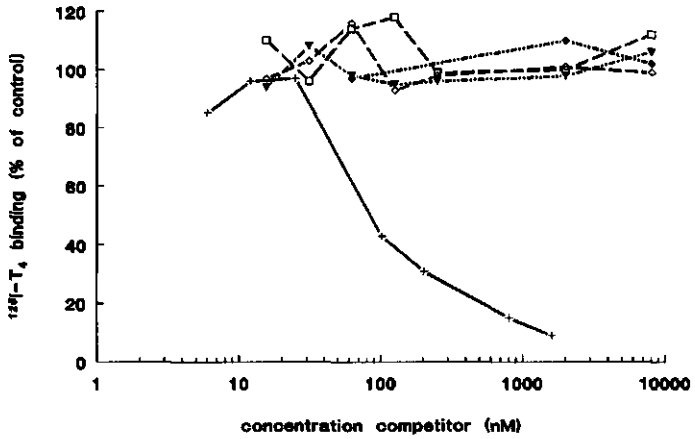


Figure 4 Thyroxine (T_4) and derived structures: tyrosine (Tyr), mono-iodotyrosine (MIT), di-iodotyrosine (DIT) and 2,4,6-triiodophenol (TIP), as tested in the *in vitro* competition binding assays, described in Materials and Methods.

A



B

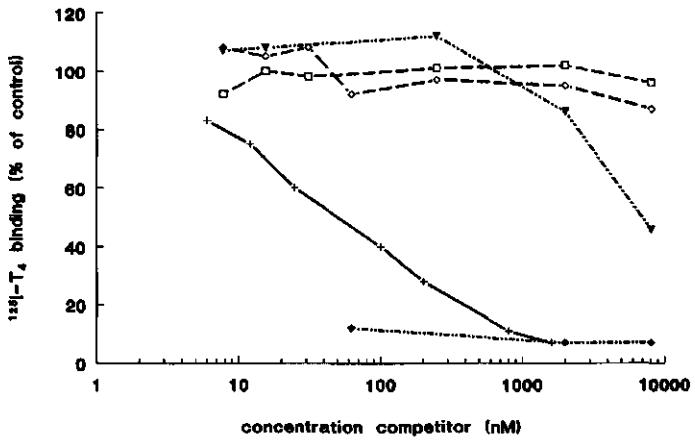


Figure 5 A) Competitive binding to thyroxine-binding globulin and B) competitive binding to transthyretin of T_4(+), tyrosine(◇), mono-iodotyrosine(□), di-iodotyrosine(▼) and tri-iodophenol(◆) (fig. 4) as measured by the *in vitro* competition binding assay (Materials and Methods). Datapoints are mean values of duplicate incubations. Relative ^{125}I-T_4-thyroxine-binding globulin or ^{125}I-T_4-transthyretin binding (% of control value) is plotted against the log concentration (nM) of competitor or T_4 added.

nolic ring with halogen atoms adjacent to the OH-group. T_4-transthyretin binding competition is also found with other phenolic compounds like chlorophenols (Vanden-Berg, 1990, Den Besten *et al.*, 1991).

The increase in T_4-transthyretin binding competition, as found with increasing numbers of iodines adjacent to the hydroxyl group (di-iodotyrosine and tri-iodophenol) confirm the importance of the outer phenolic ring iodines of T_4 for transthyretin binding as found by Andrea *et al.* (1980). The inability of tyrosine and mono-iodotyrosine to bind to transthyretin may be explained by the lack or inadequate number of iodine substitutions.

The presence of the amino acid side chain in addition to the phenolic ring does not suffice for binding of the mono-cyclic aromatic analogues of T_4 to thyroxine-binding globulin, indicating the need for other structural characteristics of compounds for binding to thyroxine-binding globulin. Major differences in binding to transthyretin or thyroxine-binding globulin are also found for various acidic drugs which had structural similarity to T_4 (Munro *et al.*, 1989), the drugs being more potent inhibitors of T_4-transthyretin than T_4-thyroxine-binding globulin binding.

The binding of T_4 to thyroxine-binding globulin or transthyretin is accomplished through diverse mechanisms with different structural elements of the natural ligand T_4. In addition there seems to be a major difference in the role of transthyretin and thyroxine-binding globulin in rodents and man with regard to T_4 transport in blood. Interaction of hydroxylated PCBs, PCDDs and PCDFs with thyroid hormone transport proteins will be limited to T_4-transthyretin binding inhibition according to the results from this study. Some, especially lower chlorinated PCB, PCDD or PCDF congeners can be metabolized to hydroxylated compounds in rats and mice (Klasson-Wehler *et al.*, 1989, Klasson-Wehler *et al.*, 1993, Koga *et al.*, 1990, Mason and Safe, 1986, Ramsey *et al.*, 1982). Metabolites of 2,3,3',4,4'-pentachlorobiphenyl have been found in the blood of different environmentally exposed species like seal, polar bear and man (Klasson-Wehler *et al.*, 1992). These hydroxylated metabolites of PCBs, PCDDs and PCDFs may be partially involved in the observed plasma reductions in thyroid hormone levels in several laboratory species exposed to these compounds.

The differences in T_4 plasma transport in rodents and man may cause less extensive disturbances in T_4 homeostasis after exposure to PCBs, PCDDs or PCDFs in man compared to rat. However, since T_4 transport to the brain is thought to be mediated specifically by transthyretin (Herbert *et al.*, 1986, Dickson *et al.*, 1985), the T_4-transthyretin binding inhibition or the specific retention of hydroxylated PCB, PCDD and PCDF metabolites due to transthyretin binding may be of relevance to the neurotoxic effects of PCBs and related compounds in rats (Morse *et al.*, 1993b) and man.

Acknowledgements

We kindly thank prof. dr. S. Safe from the Department of Veterinary Physiology and Pharmacology, A & M University, College Station Texas for providing us the hydroxylated PCDDs and PCDFs and dr. E. Klasson-Wehler from the Department of Environmental Chemistry, Wallenberg Laboratory, Stockholm University, S-10691 Stockholm, Sweden for providing us the hydroxylated PCBs.

CHAPTER 4

In vitro inhibition of thyroxine type 1 deiodinase by hydroxylated polychloro-biphenyls, -dibenzo-*p*-dioxins and -dibenzofurans

Abstract

In earlier studies hydroxylated metabolites of polychloro-biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) were found to competitively inhibit thyroxine (T_4) binding to transthyretin (TTR) but not to thyroxine-binding globulin (TBG), both plasma thyroid hormone transport proteins. In this study we investigated the possible inhibition of thyroxine type-1-deiodinase (ID-1), another T_4 binding protein involved in the enzymic conversion of T_4 to T_3 and/or reverse T_3 (rT_3), by several hydroxylated PCBs, PCDDs and PCDFs. Rat hepatic microsomes were used in an *in vitro* ID-1 activity assay, with labeled rT_3 as the substrate and increasing amounts of hydroxylated PCBs, PCDDs and PCDFs were added as inhibitors. The formation of free labeled iodide by the conversion of rT_3 to T_2 in hepatic microsomal incubations was used as a measure for outer ring ID-1 activity. Only the dihydroxylated metabolites (4,4'-$(OH)_2$-3,3',5,5'-tetraCB, 4,4'-$(OH)_2$-2,3,3',5,5'-pentaCB and 4,4'-$(OH)_2$-3,3'-dibromobiphenyl strongly inhibited ID-1 activity. The inhibition constants (K_i) of these compounds ($3.7*10^{-8}$ - $2.7*10^{-7}$ M) were in the same order of magnitude as the K_m value for the preferred natural substrate rT_3 ($6.4*10^{-8}$ M). Monohydroxylated metabolites of all classes of compounds inhibited ID-1 activity only at concentrations 10 to 100 times higher. The inhibition of ID-1 activity by these hydroxylated PCB metabolites was of competitive nature as was shown for 5-OH-3,3',4,4'-tetraCB and 4,4'-$(OH)_2$-3,3',5,5'-tetraCB. The data presented in this study indicate that the structural requirements for ID-1 inhibition by metabolites of PCBs, PCDDs and PCDFs are preferentially dihydroxylation and halogen substitution adjacent to (at least) one hydroxy-group. The observed inhibition of ID-1 activity by hydroxylated PCBs, PCDDs and PCDFs with this *in vitro* system suggests that hydroxylated metabolites may play a role in the decrease in hepatic ID-1 activity found in rats after exposure to PCBs and related compounds.

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Introduction

Polychloro-biphenyls (PCBs), -dibenzofurans (PCDFs) and -dibenzo-*p*-dioxins (PCDDs) are contaminants ubiquitously present in the environment. Due to their lipophilic character these compounds bio-accumulate through the foodchain, resulting in relatively high levels in top-predators like fish-eating birds, marine mammals and man. Toxic effects observed in animals and man after exposure to these compounds include developmental and reproductive toxicity, dermal lesions, hepatotoxicity, carcinogenesis, endocrine effects and induction of various drug-metabolizing enzymes (McConnell, 1980; Safe, 1992).

Disturbances in thyroid hormone levels and metabolism are among the endocrine effects observed in animals following exposure to PCBs and related compounds. Exposure of rats to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Bastomsky, 1977; Lucier *et al.*, 1975; Potter *et al.*, 1983; Lans *et al.*, 1990), 3,3',4,4'-tetrachlorobiphenyl (TCB) (Beetstra *et al.*, 1991; Brouwer and Van den Berg, 1986), 3,3',4,4',5,5'-hexachlorobiphenyl (Morse *et al.*, 1993a), Aroclor 1254 (Bastomsky, 1974), Kanechlor 400 (Saito *et al.*, 1991) and several polybrominated biphenyls (Byrne *et al.*, 1987; Gupta *et al.*, 1983; Spear *et al.*, 1990) resulted in increased glucuronidation and biliary clearance of thyroxine (T_4) and decreased plasma T_4 levels. TCDD and TCB are also reported to decrease thyroxine ID-1 activity in rat liver *in vivo* (Adams *et al.*, 1990, Eltom *et al.*, 1992).

In addition to the effects of these compounds on thyroid hormone metabolism, a hydroxylated TCB-metabolite (4-OH-3,3',4',5-TCB) was found to interact with transthyretin (TTR), a thyroid hormone binding transport protein in the blood, after exposure of rats to TCB (Brouwer and Van den Berg, 1986; Brouwer, 1989). *In vitro* studies showed that several different hydroxylated PCB, PCDF and PCDD metabolites but not the parent compounds could displace T_4, the natural ligand, from TTR (Brouwer *et al.*, 1990; Lans *et al.*, 1993). This was explained by the striking structural resemblance of these hydroxylated metabolites with T_4. However, the same hydroxylated metabolites did not interact with thyroxine-binding globulin (TBG), the major thyroid hormone plasma transport protein in man (Lans *et al.*, 1994).

Another T_4-binding protein, type-1-deiodinase (ID-1), present in rat liver was inhibited *in vitro* by hydroxy-metabolites of TCB, but not by TCB itself (Adams *et al.*, 1990). The potency of the different TCB-metabolites was considerably lower for inhibition of the ID-1 activity as compared to the inhibition of T_4 binding to TTR. Köhrle *et al.* (1986) and Auf'mkolk *et al.* (1986) however showed strong correlations between the structural requirements for inhibition of T_4 binding to TTR and inhibition of rat liver ID-1 activity by thyronine analogues without iodines on the phenolic ring and aurone derivatives.

ID-1 is one of three known thyroid hormone deiodinases, present in animals and man, with different substrate specificities and organ distribution. ID-1 is mainly present in liver, kidney and brain, prefers reverse T_3 (3,3',5'-triiodothyronine, rT_3) as a substrate over T_4 and requires reduced dithiols as cofactor for deiodination *in vitro*. ID-1 can deiodinate the inner and outer ring of T_4, resulting in the biologically active T_3 or the inactive rT_3, respectively (Leonard, 1990; Visser, 1991). Due to these important characteristics of ID-1 activity, possible inhibition by hydroxylated PCB, PCDD and PCDF metabolites could play a role in the disturbances in thyroid hormone levels and metabo-

lism found in animals exposed to PCBs and related compounds.

When comparing the various thyroid hormone binding proteins, e.g. TTR, TBG and ID-I, inhibitors of ligand binding or enzyme activity apparantly seem to require different structural characteristics. Due to the structural resemblance of hydroxylated PCB, PCDD and PCDF metabolites with T_4, we continued to focus our attention on T_4 binding proteins as possible target proteins for these compounds that may mediate toxic responses. The reported effects of TCB and TCDD on the ID-1 activity in rat liver *in vivo*, and the *in vitro* effects of hydroxylated TCB-metabolites, prompted us to investigate the inhibitory potency of different hydroxylated PCB, PCDD and PCDF metabolites on ID-1 activity in an *in vitro* system.

Materials and methods

Materials

The hydroxylated PCBs and PBB (Fig. 1) used in the present study: 2-OH-2',3,3',4,4'-pentaCB (A), 4-OH-2',3,3',4',5-pentaCB (B), 5-OH-2',3,3',4,4'-pentaCB* (C), 2-OH-3,3',4,4'-tetraCB (D), 4-OH-3,3',4',5-tetraCB (E), 5-OH-3,3',4,4'-tetraCB* (F), 4-OH-3,3',4'-triCB (G), 4-OH-2,3,3',4'-tetraCB (H), 4-OH-2,2',3,3',4'-pentaCB (I), 4,4'-$(OH)_2$-3,3',5,5'-tetraCB (J), 4,4'-$(OH)_2$-2,3,3',5,5'-pentaCB (K) and 4,4'-$(OH)_2$-3,3'-diBB (L) were obtained by demethylation of the corresponding methoxy-PCBs or PBB (Klasson-Wehler *et al.*, 1990). The methoxy-PCBs were prepared and structures verified as described elsewhere (Lans *et al.*, 1993). *The numbering of the hydroxy-substituents on the biphenyl does not follow IUPAC rules but has been chosen in order to facilitate comparison of the structures.)

The tested hydroxylated chlorodibenzo-*p*-dioxins and chlorodibenzofurans 2-OH-1,3,7,8-tetraCDD (N), 7-OH-2,3,8-triCDD (O), 8-OH-2,3-diCDD (P), 3-OH-2,6,7,8-tetraCDF (Q), 8-OH-2,3,4-triCDF (R) and 2-OH-7,8-diCDF (S), shown in Figure 1, were synthesized as previously described (Mason and Safe, 1986; Denomme *et al.*, 1985; Denomme *et al.*, 1986) and kindly provided by prof. S. Safe of the Department of Veterinary Physiology and Pharmacology, Texas A&M University, College Station, Texas, U.S.A.. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) (M) was commercially obtained from Schmidt B.V., Amsterdam, The Netherlands.

Methanol (HPLC grade) was obtained from Merck Chemical Company (Darmstadt, Germany). Reverse T_3 was obtained from Henning (Berlin, Germany); Sephadex LH-20 was purchased from Pharmacia (Woerden, The Netherlands). 3,3',5'-^{125}I-triiodothyronine ([^{125}I]rT_3) was purchased from Amersham, Aylesbury, Buckinghamshire, United Kingdom, (specific activity 1200 μCi/μg rT_3).

Microsomal preparations

Microsomal preparations were made according to Visser *et al.* (1979) from livers of 2 female Wistar rats (16 weeks old). Livers were perfused with 0.9 % (w/v) NaCl, homogenized in 10 mM Tris, pH 7 with 0.25 M sucrose and 1 mM DTT and centrifuged at 9000*g* for 30 minutes. The supernatant was collected and centrifuged at 105.000*g* for 90 minutes. The resulting pellet was resuspended in 10 mM Tris, pH 7.4, 3 mM EDTA, 1 mM DTT and microsomes were stored at -80 °C until further use.

Hepatic microsomal type 1 rT_3 deiodinase assay

Hepatic type 1 deiodinase (ID-1) activity was determined in triplicate in microsomal preparations by the formation of $^{125}I^-$ from [^{125}I]rT_3 according to Mol *et al.* (1985). 50 µl of 100 µg microsomal protein/ml in 200 mM phosphate-buffer pH 7.4, containing 4 mM EDTA and 2 mM DTT was incubated for 30 minutes at 37 °C with 50 µl 0.2 µM [^{125}I]rT_3, 50 µl incubation buffer and 50 µl of the test compounds (0-40 µM) stock solutions dissolved in methanol, or methanol as control to the reaction mixtures (methanol concentration in incubation mixture was 1%). The reaction was started by adding the microsomal protein and stopped by addition of 750 µl 0.1 M HCl. The $^{125}I^-$ produced was separated from the reaction mixture by Sephadex LH-20 chromatography according to Otten *et al.* (1984) and counted on a Cobra Auto Gamma counter (Canberra-Packard). Microsomes boiled for 10 minutes were used as blanks. The percentage of deiodination was determined by dividing the number of counts from the $^{125}I^-$ fraction by the total number of counts in the incubation. Controls were used to determine 100 % ID-1 activity. Inhibition curves indicate the % of ID-I activity inhibition compared to controls against the log concentration of competitor added (means ± standard deviations of triplicates).

To determine competitive or non-competitive inhibition by 4,4'-$(OH)_2$-3,3',5,5'-tetraCB and 5-OH-3,3',4,4'-tetraCB increasing amounts of rT_3 (0 - 3.2 µM in the incubation) were used in the *in vitro* ID-1 activity assay, with constant amounts of 4,4'-$(OH)_2$-3,3',5,5'-tetraCB and 5-OH-3,3',4,4'-tetraCB or no inhibitor and 25 µg/ml microsomal protein in the final incubation. In order to determine a good inhibitor concentration, the inhibition of ID-1 activity by 5-OH-3,3',4,4'-tetraCB and 4,4'-$(OH)_2$-3,3',5,5'-tetraCB metabolite was again tested using higher concentrations (0-20 µM) than in the inhibition studies described earlier. A constant inhibitor concentration of 2 µM was chosen for the incubations with increasing amounts of substrate (rT_3). ID-1 activity was calculated in nmol I^-/min/mg protein. Lineweaver Burk plots were plotted with $1/[rT_3]$ in μM^{-1} on the X-axis and 1/V ($nmol^{-1}$*min*mg) on the Y-axis. In the plots of the incubations without inhibitor the Y-axis intercept represents $1/V_{max}$, while the X-axis represents $-1/K_m$. In the plots of the incubations with an inhibitor the Y-axis intercept also represents $1/V_{max}$, while the X-as intercept represents $-1/K_m(K_i/(K_i+[I]))$. So V_{max}, K_m and K_i can be calculated from these Lineweaver Burk plots.

Analysis of inhibition potency

Curve-fitting of the inhibition curves using non-linear regression, using Graphpad Inplot, was performed and IC_{50} and IC_{80} values (molar concentrations of competitor which inhibit ID-1 activity to 80% and 50% of control) were calculated using the fitted curve. K_i values were calculated using the rT_3 concentration of 50 nM (L) and the K_m value for rT_3 of 64 nM (K) (33) with the formula: $K_i = EC_{50}/(1+L/K)$

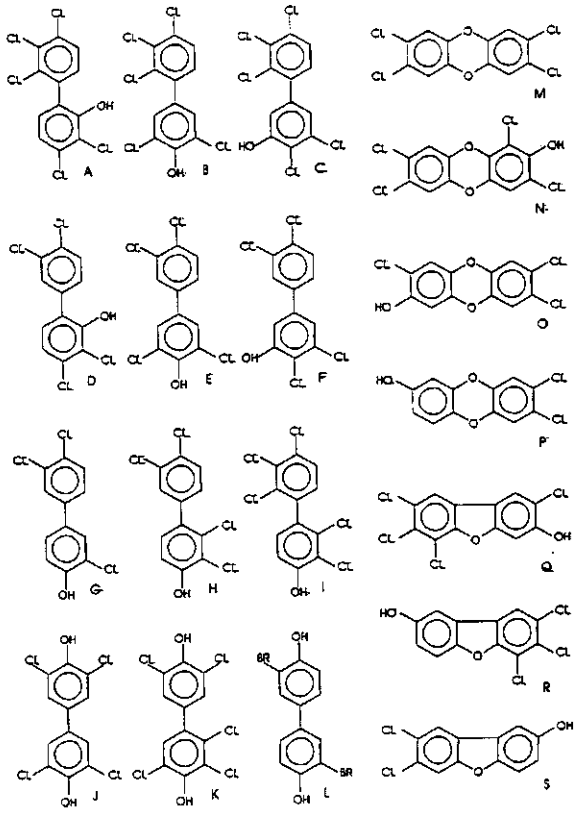


Figure 1 Structures of hydroxylated PCBs, PCDDs and PCDFs tested in the ID-1 activity assay and synthesized as described in Materials and Methods; 2-OH-2',3,3',4,4'-pentaCB (A), 4-OH-2',3,3',4',5-pentaCB (B), 5-OH-2',3,3',4,4'-pentaCB (C), 2-OH-3,3',4,4'-tetraCB (D), 4-OH-3,3',4',5-tetraCB (E), 5-OH-3,3',4,4'-tetraCB* (F), 4-OH-3,3',4'-triCB (G), 4-OH-2,3,3',4'-tetraCB (H), 4-OH-2,2',3,3',4'-pentaCB (I), 4,4'-$(OH)_2$-3,3',5,5'-tetraCB (J), 4,4'-$(OH)_2$-2,3,3',5,5'-pentaCB (K), 4,4'-$(OH)_2$-3,3'-diBB (L), TCDD (M), 2-OH-1,3,7,8-tetraCDD (N), 7-OH-2,3,8-triCDD (O), 8-OH-2,3-diCDD (P), 3-OH-2,6,7,8-tetraCDF (Q), 8-OH-2,3,4-triCDF (R) and 2-OH-7,8-diCDF (S). *The numbering of the hydroxy-substituents on the biphenyl does not follow IUPAC rules but has been chosen in order to facilitate comparison of the structures.)

Results

The inhibition of ID-1 activity by the tested hydroxylated PCBs, PCDDs and PCDFs (Fig. 1) is summarized in Table 1. For most compounds an IC_{80} value was calculated, IC_{50} values were only available for the most potent inhibitors (compounds F, J, K, L). Compounds B, C, I, M, P, R and S did not inhibit ID-1 activity to 80% of control at the highest concentration added ($1*10^{-5}$ M), therefore no IC_{50} or IC_{80} could be calculated.

The dihydroxylated PCB compounds (J, K, L) strongly inhibited ID-1 activity (Table 1). 4,4'-$(OH)_2$-2,3,3',5,5'-PentaCB (K) had the strongest inhibitory potency (IC_{50}: $6.44*10^{-8}$ M), followed by 4,4'-$(OH)_2$-3,3',5,5'-tetraCB (J, IC_{50}: $1.92*10^{-7}$ M) and 4,4'-

$(OH)_2$-3,3'-diBB (L, IC_{50}: $5.95*10^{-7}$ M). The inhibition curves are shown in Figure 2. In Table 2 the calculated inhibition constants (K_i) of the dihydroxylated PCB compounds are listed. All K_i's found are between $3.7*10^{-8}$ M and $2.7*10^{-7}$ M, which is in the range of the K_m for rT_3 ($6.4*10^{-8}$ M) (Visser *et al.*, 1984).

Table 1 Inhibition of ID-1 activity by tested hydroxylated PCBs, PCDDs and PCDFs.

Compound	Code	IC_{80} (M)	IC_{50} (M)
2-OH-2',3,3',4,4'-pentaCB	A	$5.16\ 10^{-6}$	-
4-OH-2',3,3',4',5-pentaCB	B	-	-
5-OH-2',3,3',4,4'-pentaCB	C	-	-
2-OH-3,3',4,4'-tetraCB	D	$1.25\ 10^{-6}$	-
4-OH-3,3',4',5-tetraCB	E	$3.12\ 10^{-7}$	-
5-OH-3,3',4,4'-tetraCB	F	$8.25\ 10^{-7}$	$3.63\ 10^{-6}$
4-OH-3,3',4'-triCB	G	$1.17\ 10^{-5}$	-
4-OH-2,3,3',4'-tetraCB	H	$1.10\ 10^{-5}$	-
4-OH-2,2',3,3',4'-pentaCB	I	-	-
4,4'-$(OH)_2$-3,3',5,5'-tetraCB	J	$3.57\ 10^{-8}$	$1.92\ 10^{-7}$
4,4'-$(OH)_2$-2,3,3',5,5'-pentaCB	K	$1.47\ 10^{-8}$	$6.44\ 10^{-8}$
4,4'-$(OH)_2$-3,3'-diBB	L	$1.31\ 10^{-7}$	$5.95\ 10^{-7}$
2,3,7,8-tetraCDD	M	-	-
2-OH-1,3,7,8-tetraCDD	N	$1.29\ 10^{-6}$	-
7-OH-2,3,8-triCDD	O	$4.11\ 10^{-6}$	-
8-OH-2,3-diCDD	P	-	-
3-OH-2,6,7,8-tetraCDF	Q	$2.42\ 10^{-6}$	-
8-OH-2,3,4-triCDF	R	-	-
2-OH-7,8-diCDF	S	-	-

Note: IC_{80} and IC_{50} values (molar concentrations of inhibitor resulting in inhibition of ID-1 activity to 80% and 50% of control (no inhibitor)) are calculated by non-linear regression curve fitting of inhibition curves (not shown). The compounds are coded as in Figure 1. (- = no calculation of IC_{80} or IC_{50} possible due to little or no inhibition of ID-1 activity).

Of the mono-hydroxylated 2',3,3',4,4'-pentaCB metabolites, only 2-OH-2',3,3',4,4'-pentaCB (A) inhibited ID-1 activity to 80% of control at a high concentration ($5.16*10^{-6}$ M). The 4-OH-2',3,3',4',5-pentaCB (B) and 5-OH-2',3,3',4,4'-tetraCB (C) showed no inhibitory potency. The hydroxylated 3,3',4,4'-tetraCB metabolites all inhibited ID-1 activity with different potencies (Fig. 3); 5-OH-3,3',4,4'-tetraCB (F) was the strongest inhibitor (IC_{50}: $3.63*10^{-6}$ M) followed by 4-OH-3,3',4',5-tetraCB (E) and 2-OH-3,3',4,4'-tetraCB (D) with IC_{80}s of $3.12*10^{-7}$ and $1.25*10^{-6}$ M, respectively (Table 1). The non- and mono-*ortho*-chlorine substituted 4-OH-PCB metabolites 4-OH-3,3',4'-triCB (G) and 4-OH-2,3,3',4'-tetraCB (H) weakly inhibited ID-1 activity (IC_{80}: $1.17*10^{-5}$ and $1.10*10^{-5}$ M) while the di-*ortho*-chlorine 4-OH-pentaCB, 4-OH-2,2',3,3',4'-pentaCB (I), did not inhibit ID-1 activity (Table 1).

Of the tested hydroxylated PCDDs only the two hydroxylated metabolites of TCDD, 2-OH-1,3,7,8-tetraCDD (N) and 7-OH-2,3,8-triCDD (O), weakly inhibited ID-1 activity with IC_{80}s of $1.29*10^{-6}$ and $4.11*10^{-6}$ M (Table 1). The parent compound TCDD (M) and 8-OH-2,3-diCDD (P) showed no inhibitory action on type 1-D activity. 3-OH-2,6,7,8-tetraCDF (Q) had an IC_{80} of $2.42*10^{-6}$ M, whereas the other hydroxylated PCDFs, 8-OH-2,3,4-triCDF (R) and 2-OH-7,8-diCDF (S) did not inhibit ID-1 activity (Table 1).

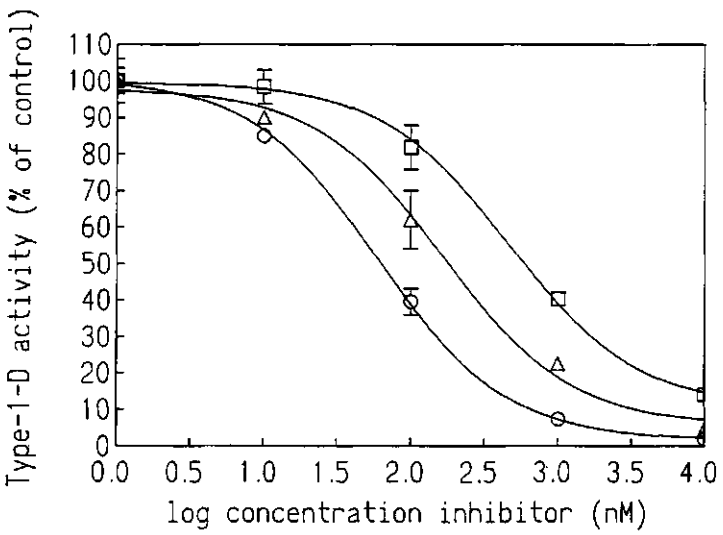


Figure 2 Inhibition of ID-1 activity by dihydroxylated compounds 4,4'-$(OH)_2$-3,3',5,5'-pentaCB (J, △), 4,4'-$(OH)_2$-2,3,3',5,5'-pentaCB (K, ○), 4,4'-$(OH)_2$-3,3'-diBB (L, □). Datapoints are mean values of triplicates with standard deviation. Control ID-1 activity was 60.15 ± 0.44 pmol I/min/mg. Percentage of ID-1 inhibition compared to control is plotted against log concentration of inhibitor (nM).

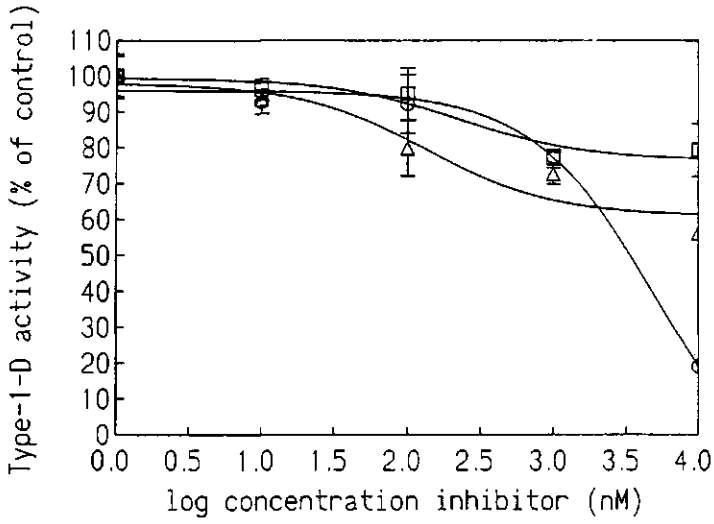


Figure 3 Inhibition of ID-1 activity by hydroxylated metabolites of 3,3',4,4'-TCB; 2-OH-3,3',4,4'-tetraCB (D, □), 4-OH-3,3',4',5-tetraCB (E, △), 5-OH-3,3',4,4'-tetraCB (F, ○). Datapoints are mean values of triplicates with standard deviation. Control ID-1 activity was 60.15 ± 0.44 pmol I/min/mg. Percentage of ID-1 inhibition compared to control is plotted against log concentration of competitor (nM).

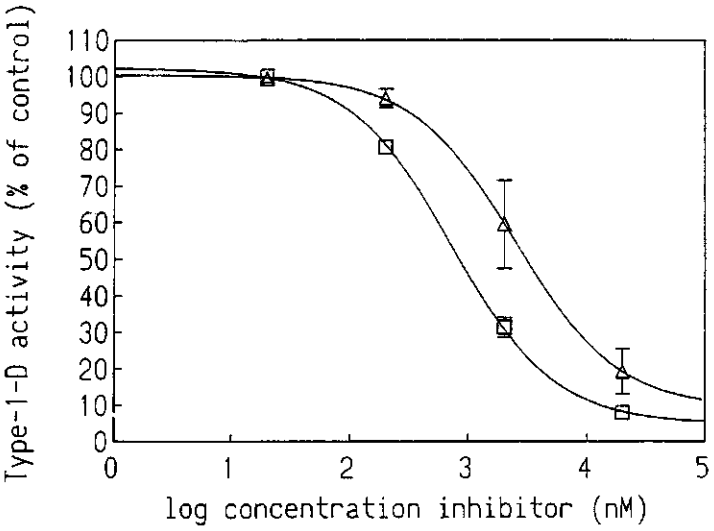


Figure 4 Inhibition of ID-1 acitivity by the hydroxylated PCB metabolites of 5-OH-3,3',4,4'-tetraCB (F, △) and 4,4'-$(OH)_2$-3,3',5,5'-tetraCB (J, □). Datapoints are mean values of triplicates with standard deviation. Control ID-1 activity was 60.45 pmol I/min/mg. Percentage of ID-1 inhibition compared to control is plotted against log concentration of competitor (nM).

To determine whether the inhibition of ID-1 activity by the hydroxylated PCBs and related compounds was of competitive or non-competitive nature, additional experiments were carried out in incubations using a constant amount of the inhibitors 4,4'-$(OH)_2$-3,3',5,5'-tetraCB or 5-OH-3,3',4,4'-tetraCB and increasing amounts of rT_3 as the substrate. In order to determine a good inhibitor concentration, the inhibition of ID-1 activity by 5-OH-3,3',4,4'-tetraCB and 4,4'-$(OH)_2$-3,3',5,5'-tetraCB metabolite was again tested in an *in vitro* ID-1 activity assay, using higher concentrations (0-20 μM) than in the inhibition studies described earlier. The inhibition curve is shown in figure 4. Using curve-fitting by non-linear regression the K_i's and IC_{50} values were calculated for 5-OH-3,3',4,4'-tetraCB (K_i: 1.39 10^{-6} M^{-1}; IC_{50}: 3.05 10^{-6} M) and 4,4'-$(OH)_2$-3,3',5,5'-tetraCB (K_i: 0.41 10^{-6} M^{-1}; IC_{50}: 0.84 10^{-6} M), which were in the same range as the K_i's and IC_{50} listed in Table 1 and 2 for these two compounds. In figure 5A the double reciprocal Lineweaver Burk plots determined by linear regression are shown of the ID-1 activity assay incubations using constant amounts of inhibitors. V_{max}'s, determined from the y-axis intercept, are almost identical for the incubations with 5-OH-3,3',4,4'-tetraCB, 4,4'-$(OH)_2$-3,3',5,5'-tetraCB and without inhibitor (respectively 0.59, 0.69 and 0.80 nmol/min/mg), indicating a competitive inhibition of ID-1 activity. The K_i's calculated using the X-intercepts of the Lineweaver-Burk plots were 1.80 and 0.57 * 10^{-6} M^{-1} for 5-OH-3,3',4,4'-tetraCB and 4,4'-$(OH)_2$-3,3',5,5'-tetraCB, respectively. When linear regression was performed on the Lineweaver-Burk plots using the average V_{max} (1/Y-axis intercept: 0.71 * 10^{-6} M^{-1}), assuming competitive inhibition, the K_i's found were 1.31 and 0.51 * 10^{-6} M^{-1}, for 5-OH-3,3',4,4'-tetraCB and 4,4'-$(OH)_2$-3,3',5,5'-tetraCB, respectively (Fig. 5B).

Discussion

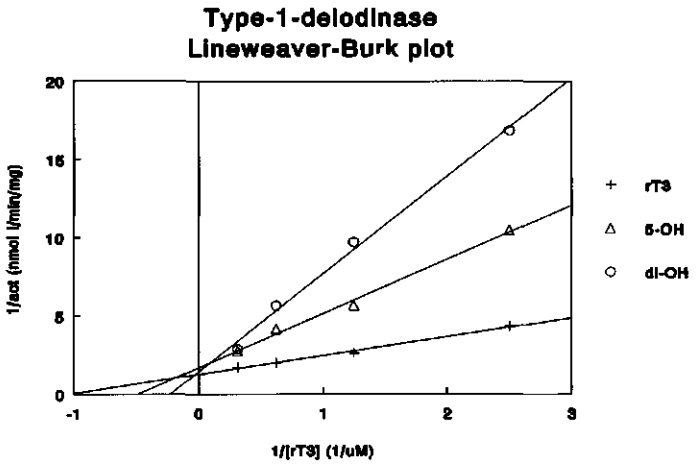
Of all hydroxylated PCBs, PCDDs and PCDFs tested only 4 were strong inhibitors of ID-1 activity, namely 4,4'-$(OH)_2$-3,3',5,5'-tetraCB, 4,4'-$(OH)_2$-2,3,3',5,5'-pentaCB, 4,4'-$(OH)_2$-3,3'-diBB and 5-OH-3,3',4,4'-tetraCB. All other compounds were not able to inhibit ID-1 activity by 50% at concentrations up to 10 μM. TCDD was not able to inhibit ID-1 activity and its major metabolite *in vivo*, 2-OH-1,3,7,8-tetraCDD (Mason

Table 2 Inhibition constants (K_i) of tested dihydroxylated PCBs (Fig. 1: J,K,L).

Compound	K_i (M)
4,4'-$(OH)_2$-2,3,3',5,5'-pentaCB (K)	3.70 10^{-8}
4,4'-$(OH)_2$-3,3',5,5'-tetraCB (J)	1.13 10^{-7}
4,4'-$(OH)_2$-3,3'-diBB (L)	2.69 10^{-7}

Note: K_i's are calculated by non-linear regression curve fitting of the inhibition curves shown in Figure 2, using the formula $K_i = EC_{50}/(1 + L/K)$, with L = 50 nM (rT_3 concentration) and K = 64 nM (K_m of rT_3).

A



B

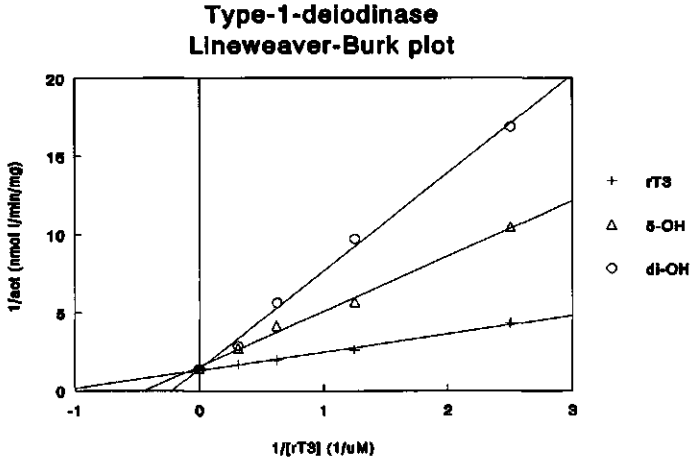


Figure 5 Double reciprocal Lineweaver-Burk plots of inhibition of ID-1 activity using increasing concentrations of substrate rT3 (0-3.2 μM) in the presence of 2 μM 5-OH-3,3',4,4'-tetraCB (F, Δ), 2 μM 4,4'-$(OH)_2$-3,3',5,5'-tetraCB (J, ○) or without inhibitor (+). 1/ID-1 activity (min/mg/nmol I) is plotted against 1/[rT3] substrate concentration (1/μM) without average Vmax (A) and with average Vmax (B). Y-axis intercept represents 1/Vmax, while X-axis intercept represents -1/Km. Datapoints are means of triplicates.

and Safe, 1986; Ramsey *et al.*, 1982), only weakly inhibited ID-1 activity at concentrations above 0.1 μM. Lineweaver-Burk plots showed a competitive inhibition of ID-1 activity by 5-OH-3,3',4,4'-tetraCB and 4,4'-$(OH)_2$-3,3',5,5'-tetraCB.

The dihydroxylated 4,4'-$(OH)_2$-2,3,3',5,5'-pentaCB was the strongest inhibitor of ID-1 activity, followed by 4,4'-$(OH)_2$-3,3',5,5'-tetraCB and the 4,4'-$(OH)_2$-diBB. The bromine substituted dihydroxy-congener was as potent as the chlorine substituted dihydroxylated PCBs for ID-1 inhibitors, indicating no major influence of chlorine or bromine substitutions on inhibitory potency. The K_i's calculated for the dihydroxylated compounds have values ranging between the K_m for rT_3 (64 nM), the preferred natural substrate for ID-1, and the K_m for T_4 (1.9-2.3 μM), another natural substrate (Visser *et al.*, 1984; Visser, 1990). The presence of two hydroxyl group substitutions appears to be important for the strong inhibitory action, because related mono-hydroxymetabolites (4-OH-3,3',4',5-TCB and 4-OH-2,3,3',4',5-PeCB) are only weak inhibitors of ID-1 activity. *Ortho*-chlorine substitution does not influence inhibitory action for dihydroxylated metabolites; the $(OH)_2$-pentaCB is the most potent inhibitor. However, for the monohydroxylated PCB metabolites, *ortho*-chlorination seemed to decrease inhibitory potency of these metabolites.

In an earlier *in vitro* study by our laboratory on the effects of TCB and its hydroxylated metabolites on ID-1 activity (Adams *et al.*, 1990), the same structure activitity relations as in this study were found for the 2-OH-3,3',4,4'-, 4-OH-3,3',4',5- and 5-OH-3,3',4,4'-tetraCB metabolites. In both studies 5-OH-3,3',4,4'-tetraCB was found to be a stronger inhibitor than the 4-OH-3,3',4',5- and 2-OH-3,3',4,4'-tetraCB metabolites. The 6-OH-3,3',4,4'-tetraCB, not tested in this study, also inhibited ID-1 activity, so *ortho*-hydroxylated compounds could also act as inhibitors as was found for the 2'-OH-2,3,3',4,4'-pentaCB metabolite in this study. Adams *et al.* (1990) found no inhibition of ID-1 activity by TCB, while in this study no inhibition by TCDD was found. This confirms the requirement of hydroxylation of the inhibitor, with chlorine(s) substituted adjacent to the hydroxyl group. The inhibition of ID-1 by the tested monohydroxylated compounds is of competitive nature as was found in this and other studies (Adams *et al.*, 1990; Rickenbacher *et al.*, 1989), with an inhibition constant in the micromolar range for 5-OH-3,3',4,4'-tetraCB which equals the K_m for T_4 (1.9-2.3 μM), but is higher than the K_m of rT_3 (64 nM) (Visser *et al.*, 1984, Visser, 1990), both physiological substrates for ID-1.

The differences in inhibition potencies of the hydroxylated PCDDs and TCDD suggest that at least one hydroxyl group is necessary for (weak) inhibitory action on ID-1 activity. TCDD did not inhibit ID-1 activity, but the hydroxylated tetraCDD and triCDD, metabolites of TCDD *in vivo*, did. The hydroxylated PCDDs and PCDFs that had no chlorines next to the hydroxyl group, had no inhibitory potency indicating that halogen-substitution adjacent to the hydroxyl group may increase inhibition of ID-1 activity.

Several structural requirements for the competitive inhibitory action on ID-1 activity by these halogenated aromatic compounds are suggested in this study. At least one hydroxyl group substitution is essential, while adjacent chlorine substitution seems to increase inhibitory potency on ID-1 activity by these compounds. Dihydroxylated poly-halogenated biphenyls with hydroxyl groups on both *para*-positions were very potent inhibitors, suggesting that the polarity or high degree of hydroxylation of these compounds may attribute to the inhibitory action.

Other halogenated dioxin and biphenyl derivatives were tested on inhibition of ID-1 activity *in vitro* by Rickenbacker *et al.* (1989), showing substrate competitive inhibition. 4,4'-$(OH)_2$-3,3',5,5'-TetraCB was also tested in their study and was the strongest competitive inhibitor with an IC_{50} of 0.07 μM, comparable to the IC_{50} of the same compound found in this study (0.19 μM). Other hydroxylated PCB compounds tested by Rickenbacher *et al.* were 4,4'-$(OH)_2$-2,3,5,6-tetraCB, 4-OH-3,4',5-triCB, 4'-OH-2,4,6-triCB. Like the structural requirements found for ID-1 inhibitors in this study, Rickenbacher *et al.* (1989) found that the hydroxylated PCBs inhibited ID-1 with different potencies, suggesting an important role for dihydroxylation and chlorine substitution next to the hydroxyl group for good inhibitory action. *Ortho* chlorine substitutions on one phenyl ring resulted in low inhibitory potency for the monohydroxylated 4'-OH-2,4,6-triCB, but did not decrease inhibitory action of the dihydroxylated 4,4'-$(OH)_2$-2,3,5,6-tetraCB. Thus, like in this study, *ortho*-chlorination only seemed to influence the inhibition potency of mono-hydroxylated PCB metabolites. The dibenzo-*p*-dioxin-adipamide and 3,7,8-trichlorodibenzo-*p*-dioxin-adipamide derivates tested by Rickenbacher *et al.* (1989) also showed that chlorine substitutions and a polarizable group on these TCDD related compounds were necessary for inhibition of ID-1 activity, like was found for TCDD and its hydroxylated metabolites in this study.

The ligand binding site of ID-1 is described by Köhrle *et al.* (1986), who determined the ligand binding characteristics of ID-1 by dose response inhibition of T_4 deiodination using iodothyronine analogues. The optimal structural requirements for a ID-1 substrate or inhibitor were a negatively charged side chain, a 4'-substituent with a phenolic pKa or hydrogen bonding capacity, at least one halogen on the tyrosyl ring and at least one polarizable group on the phenolic ring. These structural characteristics resemble the requirements of the presence of strong polarizable and at least one hydroxy group with adjacent halogenation on the hydroxylated PCBs and related compounds for ID-1 inhibition potency. However, it was not strictly necessary for the hydroxylated PCBs to have a hydroxyl group on the 4 (*para*)-position on the phenolic ring. *Ortho* (2')-iodine substituents of iodothyronine analogues were found to increase ID-1 inhibition, while *ortho* chlorination of monohydroxylated PCBs was found to decrease inhibitory potency in this study. Köhrle *et al.* (1986) found inhibition of ID-1 by thyronine analogues without iodines on the phenolic ring. This would mean that no halogen substituents are necessary for ID-1 activity inhibition.

In another study by Auf'mkolk *et al.* (1986) the interaction of aurone derivatives with the ID-1 binding site were evaluated by inhibition studies. Two mechanisms of ID-1 activity inhibition were suggested, a cofactor competitive mechanism where hydroxylated, non-halogenated aurones can bind to to the oxidized form of the deiodinase and prevent enzyme regeneration by the reduced dithiol cofactors. Another mechanism is the inhibition of ID-1 activity by iodinated aurones that compete with the substrate binding site of the native reduced enzyme. Iodination of ID-1 inhibitors seem to enhance their potency as substrate competitive inhibitors. The hydroxylated PCB, PCDD and PCDF metabolites tested in this study seem to inhibit ID-1 activity by a substrate competitive mechanism as was shown in the Lineweaver Burk plots, confirming the enhancing effect of the presence of halogen groups next to the hydroxyl group on ID-1 inhibitory action of these compounds.

Other substrate or cofactor specific inhibitors of ID-1 activity are iodinated phenols and aniline derivatives like the X-ray contrast reagent iopanoic acid and ipodate,

(halogenated) dyes like bromophenol blue, erythrosine and rose-bengal, thiourea derivatives and catecholamine (ant)agonists, usually all aromatic substances with halogen substituents adjacent to hydroxyl or amino-groups (Leonard and Visser, 1986). In addition to these known inhibitors of ID-1 activity, this study shows that OH-metabolites of PCBs, PCDDs or PCDFs are also capable of inhibiting ID-1 in a substrate competitive manner.

The compounds tested on ID-1 inhibitory action in this study have also been tested on inhibition of T_4 binding to transthyretin (TTR) and thyroxine binding globulin (TBG) in *in vitro* binding competition studies (Lans *et al.*, 1993, 1994). The major differences found were the almost essential requirement of dihydroxylation for ID-1 inhibitory action, while monohydroxylated PCBs could be potent inhibitors of T_4-TTR binding. The influence of *ortho*-chlorine substitution of monohydroxylated PCBs, which seemed to decrease ID-1 inhibition, did not affect T_4-TTR binding inhibition potency of the compounds. Köhrle *et al.* (1986) found that ID-1 showed no stereospecificity when D-T_4 and L-T_4 are used as substrates, whereas for the TTR binding site stereoselectivity has been found. Also the binding affinities of the naturals ligand are different for type 1-D (rT_3 >> T_4) and TTR (T_4 >> rT_3). Moreover, it is difficult to compare inhibition of enzymic activity with ligand binding, as two modes of enzyme activity inhibition are suggested. Thus one should be careful using the TTR binding site as a model for the ID-1 active site in order to design or model other deiodinase inhibitors (Köhrle *et al.*, 1986, Auf'mkolk *et al.*, 1986). It is clear however, that the binding site of ID-1 is more related to the TTR binding site than the TBG binding site, because hydroxylated PCBs, PCDDs and PCDFs showed no inhibition at all of T_4-TBG binding in *in vitro* studies (Lans *et al.*, 1994).

ID-1 activity is important for the metabolism of rT_3 and the activation or inactivation of T_4 to resp. T_3 or rT_3 in the liver. The choice of rT_3 as a substrate for ID-1 activity in these inhibition studies with hydroxylated PCBs, PCDDs and PCDFs directs the enzyme to only perform outer ring deiodination (ORD) of the substrate. Exposure to PCBs and related compounds may result in metabolite formation in the liver *in vivo* (Darnerud *et al.*, 1986; Klasson-Wehler *et al.*, 1993, Abdel-Hamid *et al.*, 1981; Koga *et al.*, 1990). These metabolites could then inhibit ID-1 ORD activity, so rT_3 may not be metabolized to T_2 which can lead to increasing amounts of rT_3. Because rT_3 is the most potent ID-1 substrate, T_4 may not be deiodinated to T_3 because of the lowered ID-1 activity and the increasing amount of rT_3 present in the liver. In parallel these data can indicate that the conversion of T_4 to T_3, which also involves ID-1 ORD, may directly be reduced resulting in a decrease of T_3 formation. Both pathways could lead to decreased amounts of bioactive T_3 in the liver, affecting metabolism and T_3 regulated processes. On the other hand, conversion of rT_3 to T_2 may not be inhibited due to the high affinity of rT_3 for ID-1. This could imply that the physiological function of ID-1 in liver to eliminate extrahepatically formed rT_3 from circulation is not influenced by these metabolites.

Exposure of rats to TCDD and TCB leads to inhibition of ID-1 activity in the liver (Adams *et al.*, 1990; Eltom *et al.*, 1992). This could be due to the metabolites formed from these compounds in the liver, directly inhibiting ID-1 activity. In addition the exposure to PCBs and related compounds can decrease thyroid hormone levels in blood, which may also explain the observed reduction in ID-1 activity due to the modulating physiological effects of thyroid hormone levels in blood on hepatic ID-1

activity (Kaplan, 1986). Further experiments are necessary to distinguish between those causes that may lead to the decreased ID-1 activity in liver after exposure of rats to PCBs and related compounds.

Acknowledgements

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CHAPTER 5

In vivo alterations in thyroxine metabolism and plasma transport by Aroclor 1254 in rats

Abstract

Previous studies in our laboratory indicated that hydroxylated polychlorinated biphenyls (PCBs), -dibenzo-*p*-dioxins (PCDDs) and -dibenzofurans (PCDFs) could specifically inhibit thyroxine (T_4) binding to transthyretin (TTR), a plasma thyroxine transport protein, and decrease type 1 thyroxine deiodinase activity in rat liver microsomes *in vitro*. In the present study the *in vivo* effects of exposure to Aroclor 1254 on plasma transport and hepatic metabolism of T_4 was investigated in adult Wistar rats. Rats were exposed to a single i.p. dose of 50, or 500 mg Aroclor 1254/kg b.w. and killed and dissected after 3 or 8 days, while part of the pretreated rats were i.p. injected with ^{125}I-T_4 one day before section on day 3 or 8, to determine thyroid hormone binding capacities of plasma proteins by polyacrylamide gelelectrophoresis (PAGE). Plasma total and free T_4 levels were decreased in the high dosed, but not in the low dosed group, while total T_3 levels were not changed by either treatment. Hepatic EROD activity was increased in the low and high dosed group, but cytochrome P450 levels and relative liver weight were only increased in the high dose group. No clear changes were found in hepatic type 1 T_4 deiodinase (ID-I) activity. T_4 glucuronidation was increased in the high dosed groups at day 3 and 8 after Aroclor 1254 administration. Ratios of ^{125}I-T_4 binding to TTR vs. albumin were significantly decreased in the high dosed group at day 8. In this treatment group a hydroxylated PCB-metabolite, 4-OH-2,3,3',4',5-pentaCB, present in high concentrations in plasma, could interact with TTR in the blood and competitively inhibit T_4 binding to TTR. In conclusion, both reduction of T_4 binding to the plasma transport protein TTR and increased hepatic T_4 glucuronidation appeared to play a role in the decrease in plasma T_4 levels by Aroclor 1254. In addition, selective plasma accumulation was shown of a specific hydroxylated PCB metabolite, e.g. 4-OH-2,3,3',4',5-pentaCB in rats exposed to the commercial PCB mixture Aroclor 1254.

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Introduction

Polychlorinated biphenyls (PCBs) are a group of widespread and persistent environmental contaminants (McConnell, 1980; McFarland and Clarke, 1989; Safe, 1990, 1994). Due to their chemical stability and widespread use they have bioaccumulated in the foodchain during the last decades. The commercial PCB mixtures, which have been banned from industrial use since the early 1980s, contain PCB-congeners with a different degree of chlorination. The various congeners present in these mixtures can cause a broad range of adverse effects, such as alterations of epithelial differentiation, embryonal development, energy metabolism, reproduction and endocrine function (Ahlborg *et al.*, 1992; Safe, 1994). These effects are highly dependent on age, sex, species and strain of animal used and the composition of the PCB-mixture, or the individual PCB-congener.

Effects of PCBs on thyroid hormone metabolism are extensively described. Exposure to Aroclor 1254, a commercial PCB mixture containing 54 % chlorine (by weight), decreased plasma thyroxine (T_4) levels (Gray *et al.*, 1993; Byrne *et al.*, 1987; Brouwer, 1989) and increased T_4 glucuronidation and bile flow in rats while plasma 3,3,5-triiodothyronine (T_3) levels were not decreased (Bastomsky, 1974; Beetstra *et al.*, 1991). In addition, effects of Aroclor 1254 on thyroid function and morphology are described by Collins and Capen (1980a,b,c). Saito *et al.* (1991) found that Kanechlor 400, another commercial PCB mixture, also increased T_4 glucuronidation in rats. Chronic exposure of rats to commercial PCB and polybrominated biphenyls (PBB) mixtures caused decreases in serum T_3 and T_4 levels and a decrease in the rate of T_4 production which led to hypothyroidism (Byrne *et al.*, 1987). Commercial PBB mixtures have also been reported to cause hypothyroidism in the mouse (Gupta *et al.*, 1983). Moreover, changes in human serum T_4 levels following exposure to PCBs or PBBs are described in several studies (Emmett *et al.*, 1988, Murai *et al.*, 1987, Bahn *et al.*, 1980, Kreiss *et al.*, 1982).

Decreased plasma T_4 levels in rodents after PCB exposure are commonly explained as the result of increased excretion and metabolism (increased glucuronidation and bile flow) or decreased synthesis (thyroid damage) of thyroid hormones. Brouwer and Van den Berg (1986), suggested a third mechanism for the decrease in plasma T_4 levels in rats exposed to 3,3',4,4'-tetrachlorobiphenyl (TCB), a coplanar PCB-congener. A hydroxylated TCB metabolite (4-OH-3,3',4',5-TCB) interacted with transthyretin (TTR), the major thyroid hormone plasma transport protein in rodents, *in vivo* and *in vitro* (Brouwer, 1989). Other hydroxylated PCB metabolites also inhibited T_4 binding to TTR *in vitro* (Lans *et al.*, 1993). Due to the structural resemblance of these metabolites with T_4, they could competitively inhibit T_4 binding to TTR. In addition, type 1 iodothyronine deiodinase (ID-I), which converts T_4 to active T_3 or inactive reverse T_3 in liver, could be inhibited by hydroxylated PCB metabolites *in vitro* using rat hepatic microsomes (Adams *et al.*, 1990, Rickenbacher *et al.*, 1989, Lans *et al.*, 1994). The parent PCBs themselves did not inhibit T_4-TTR binding or ID-I activity in these *in vitro* studies.

In the environment most species, including man, are not exposed to single PCB congeners but to complex PCB-mixtures (Safe, 1994). In this study the contribution of decreased plasma transport and increased metabolism of thyroid hormones to alterations in plasma thyroid hormone levels was investigated by *in vivo* exposure of rats to a commercial PCB mixture, Aroclor 1254, which resembles the real life exposure situation.

Materials and methods

Materials

Aroclor 1254 was kindly donated by Dr. M. van den Berg of the Research Institute for Toxicology, University of Utrecht, The Netherlands. 4-OH-2,3,3',4',5-pentachlorobiphenyl was synthesized by Dr. E. Klasson-Wehler of the Wallenberg Laboratory, Stockholm University, Sweden, according to the methods described in Lans *et al.* (1993). ^{125}I-T_4 (1500 μCi/μg T_4), ^{125}I-rT_3 (1200 μCi/μg rT_3) and TT_4, FT_4 and TT_3 Amerlite kits were obtained from Amersham (Amersham, Aylesbury, Buckinghamshire, United Kingdom). T_4, T_3, 6-propyl-2-thiouracil (PTU), uridine-5'-diphosphoglucuronic acid (UDPGA), (3[{3-cholamidopropyl} dimethylammonio]-1-propane sulphonate) (CHAPS) and dithiotreitol (DTT) were purchased from Sigma (St. Louis, MO, USA); rT_3 was obtained from Henning (Berlin, Germany); Sephadex LH-20 was purchased from Pharmacia (Woerden, The Netherlands). Biorad protein reagent was obtained from Biorad Laboratories (Bio-rad, Richmond, CA, USA). ^{125}I-T_4 and ^{125}I-rT_3 were purified on Sephadex LH-20 immediately before each assay according to Beetstra *et al.* (1991). Pure human TTR and BSA were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Experimental design

42 Female wistar rats (16 weeks old) were dosed once i.p. with 0, 50 and 500 mg Aroclor 1254/ kg b.w. in corn oil (14 rats per dose, 5 ml/kg b.w.). After 2 and 7 days 3 rats of each dose-group were i.p. injected with 25 μCi ^{125}I-T_4 (21 pmol T_4). Blood samples were collected by tail bleeding at 3, 6 and 24 hours after ^{125}I-T_4-injection. Plasma was prepared from blood by centrifugation at 1000*g* and stored at -20 °C. At 24 hours after ^{125}I-T_4 injection the rats exposed to Aroclor 1254 (for 3 and 8 days in total) were killed and dissected and blood, plasma, livers and brain were collected and stored at -80 °C. At day 3 and 8, four other, non ^{125}I-T_4 treated rats per dose-group were killed and plasma, liver and brains were collected and stored at -80° C. Of all non ^{125}I-T_4 treated rats per dose-group blood samples were collected on day 1 and 6 of Aroclor 1254 exposure.

Sample preparations of non ^{125}I-T_4 treated rats

Livers were divided in two for preparation of microsomes in two different buffers e.g. for EROD-activity and T_4-glucuronidation and for hepatic type 1 deiodinase (ID-I) activity. Microsomes for EROD/T_4 glucuronidation were prepared by homogenizing liver on ice in a Potter-tube in 0.1 M Tris-HCl buffer, pH 7.5 containing 0.25 M sucrose (2 ml/g liver), followed by centrifugation at 9000*g* for 30 min. The resulting supernatant was centrifuged at 105,000*g* for 90 min. and the pellet was resuspended in 0.1 M phosphate buffer, pH 7.5. Microsomes for ID-I activity were prepared by homogenizing liver in 10 mM Tris-HCl, pH 7.4, containing 0.25 M sucrose and 1 mM DTT. After differential centrifugation at 9000 and 105,000*g* the microsomal pellet was resuspended in 10 mM Tris-HCl, pH 7.4, containing 1 mM DTT and 3 mM EDTA. All hepatic microsomes were stored at -80 °C until further use. Protein concentrations were determined using the Bio-rad coomassie blue assay.

Cytochrome P-450 levels

Hepatic cytochrome P450 levels were measured using the method of Omura and Sato (1964) with modifications according to Rutten *et al.* (1987). Briefly hepatic microsomes

(used for EROD and T_4 glucuronidation) were diluted in 980 µl of 0.1 M phoshate-buffer, pH 7.4 to a final concentration of 1 mg/ml. Carbon-monoxide gas was run through the incubation. An absorption spectrum from 400 - 500 nm was collected before and after addition of 20 µl saturated sodium-dithionite solution in phosphate buffer to the incubation. The difference spectrum showed a peak at 450 nm from which cytochrome P450 content could be calculated in nmol/mg microsomal protein.

Ethoxyresorufin-O-deethylase activity

Ethoxyresorufin-O-deethylase (EROD) activity was determined in hepatic microsomes using a modified method of Burke *et al.* (1977). In short the reaction mixture, containing final concentrations of 2 µM ethoxyresorufin and 100 µg/ml microsomal protein in 0.1 M Tris-HCl buffer, pH 7.8, was pre-incubated for 2 minutes at 37 °C. The reaction was started by adding 0.1 mM NADPH to the incubation mixture. To detect the deethylation product, resorufin (RR), the excitation and the emission wavelength of the fluorimeter were 530 and 586 nm, respectively. The results are expressed as nmol RR formed per minute per milligram of microsomal protein.

Hepatic T_4-UDP-glucuronyltransferase activity (T_4-UGT)

Hepatic microsomal T_4-UGT activity was determined in duplicate according to Beetstra *et al.* (1991) by the formation of ^{125}I-T_4-glucuronide from ^{125}I-T_4. The final concentrations in the reaction mixture were 0.1 M Tris-HCl buffer, pH 7.8, 5 mM UDPGA, 3.75 mM $MgCl_2$, 0.25 % (w/v) CHAPS , 0.125% (w/v) BSA, 1 µM T_4 with 50,000 cpm ^{125}I-T_4, and 0.2 mg micosomal protein in a total volume of 200 µl. The reaction mixture was incubated at 37 °C for 30 min. The reaction was stopped with 200 µl ice-cold methanol and after centrifugation 200 µl of supernatant was added to 750 µl 0.1 M HCl. The amount of ^{125}I-T_4 glucuronide (T_4G) in the supernatant was isolated using Sephadex LH-20 chromatography and quantified using a Cobra AutoGamma counting system (Canberra-Packard, USA) (Rutgers *et al.*, 1989). Boiled microsomes were used as blanks. The results are expressed as nmol T_4G formation per minute per mg of microsomal protein.

Hepatic type 1 rT_3 deiodinase (ID-I) activity

Hepatic ID-I activity was determined in duplicate in microsomal preparations by the formation of $^{125}I^-$ from ^{125}I-rT_3 according to Mol and Visser (1985). A sample of 100 µl of 50 µg microsomal protein/ml in 200 mM phosphate-buffer pH 7.4, containing 4 mM EDTA and 2 mM DTT was incubated for 30 minutes at 37 °C with 100 µl of 0.2 µM rT_3 and 100,000 cpm ^{125}I-rT_3 in 4 mM NaOH, which did not affect the pH of the reaction mixture. The reaction was started by adding the microsomal protein. The reaction was stopped by addition of 750 µl 0.1 M HCl. The $^{125}I^-$ produced was separated from the reaction mixture by Sephadex LH-20 chromatography according to Otten *et al.* (1984) and counted on a Cobra Auto Gamma counting system (Canberra-Packard, USA). Boiled microsomes were used as blanks. The percentage of deiodination was determined by dividing the amount of counts from the $^{125}I^-$ fraction by the total amount of counts in the incubation.

Thyroid hormone analysis

Plasma total T_4 (TT_4), total T_3 (TT_3) and free T_4 (FT_4) were measured with the Amerlite chemiluminescence system (Amersham, U.K) using the standard protocol from the supplier, with minor modifications. For TT_4 measurements the standard curve ranged from 0 to 120

nM TT_4 and the assay buffer was diluted five times with demineralized water.

In vivo thyroxine binding inhibition study (T_4 binding capacity of plasma proteins)

At several time points after ^{125}I-T_4 injection, blood was collected from the rats by tail-bleeding. Aliquots of 25 µl serum were mixed 1:1 with electrode buffer 5 mM Tris, 38 mM glycine buffer, pH 8.3 containing 10 % glycerol. These samples were subjected to native polyacrylamide gelelectrophoresis (PAGE) using a 10% separating gel and run during 5 hours at a constant current of 50 mA at 4 °C according to the method of Brouwer and Van den Berg (1986). The acrylamide gel was cut into 1 mm pieces, which were counted for ^{125}I-radioactivity in a Cobra Auto Gamma counter (Canberra-Packard). PAGE gel-profiles were made by plotting the ^{125}I-radioactivity against the migration distance on the gel. ^{125}I-Radioactivity (cpm) bound to the different serum proteins on the gels was used to calculate ^{125}I-T_4 binding ratios between transthyretin (TTR) and albumin. Pure human TTR and BSA and control rat serum were used to identify the plasma proteins on PAGE by comparing migration distances.

TTR levels

Radioimmunochemical analyses of TTR were performed at the laboratory of Dr. W.S. Blaner, Institute of Human Nutrition (Columbia University, New York, USA) according to the method of Muto *et al.* (1972) and Brouwer (1987), using immunosorbent purified, mono-specific rabbit anti-rat TTR (Navah *et al.*, 1977). The antibody was radiolabeled with ^{125}I via the Chloramine-T method. Samples of serum (25 times diluted in 50 mM Tris buffer pH 8.6, with 1% BSA (w/v)) were incubated with the specific TTR antibody . After precipitation with polyethylene-glycol buffer, the tubes were centrifuged for 30 minutes and the amount of TTR was determined by counting ^{125}I-radioactivity. The limit of detection was 20 ng/ml.

Analysis of hydroxylated Aroclor 1254 metabolites in plasma

PCBs and hydroxylated PCB metabolites (OH-PCBs) were analysed according to the method of Bergman *et al.* (1994). Plasma samples were diluted with one volume of methanol and one volume of water. After acidification, PCBs and OH-PCBs were extracted with hexane:methyl *tert*-butyl ether (MTBE). The extract was partitioned with 1 M KOH (in 50 % ethanol). The alkaline phase was acidified and the OH-PCBs were re-extracted in hexane:MTBE. The OH-PCBs were methylated with diazomethane and co-extracted lipids removed by treatment with concentrated sulphuric acid (%). The PCB fraction was similarly treated with concentrated sulphuric acid, while the hexane phase contained PCBs.
The samples were analyzed by GC, a Varian 3400 equipped with an electron capture detector (ECD) and a fused silica capillary column DB5+, 30 m by 0.25 mm i.d. with a film thickness of 25 µM (J&W Scientific Inc.), with a temperature program of 80 °C(2 min.)-10 °C/min-300 °C(10 min.), injector temperature was 250 °C and detector temperature was 360 °C. The individual congeners were identified by comparison of relative retention time to the internal standards (2,3,3',4,4',5,5'-heptaCB (PCB 189) and 4-OH-2,3,3',4',5,5',6-heptaCB, respectively) with authentic standards. The quantifications were done individually using the internal standard and comparing with a standard also containing the internal standard. The OH-PCB was quantified only for the 4-OH-2,3,3',4',5-pentaCB. A comparison was made of amount of the 4-OH-pentaCB metabolite with the amount of

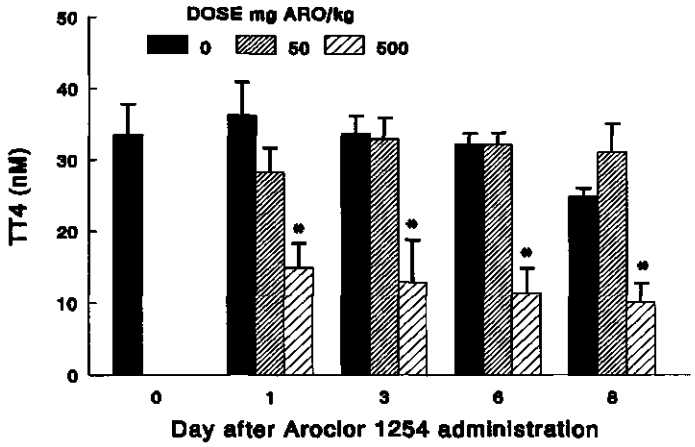

Figure 1 Plasma levels of total thyroxine in control and Aroclor 1254 exposed rats (50 and 500 mg/kg b.w.) on day 0 before i.p. injection with corn-oil (control) or Aroclor 1254, and on exposure day 1, 3, 6 and 8. Results are means ± SEM. *Indicates values significantly different from controls ($p < 0.05$).

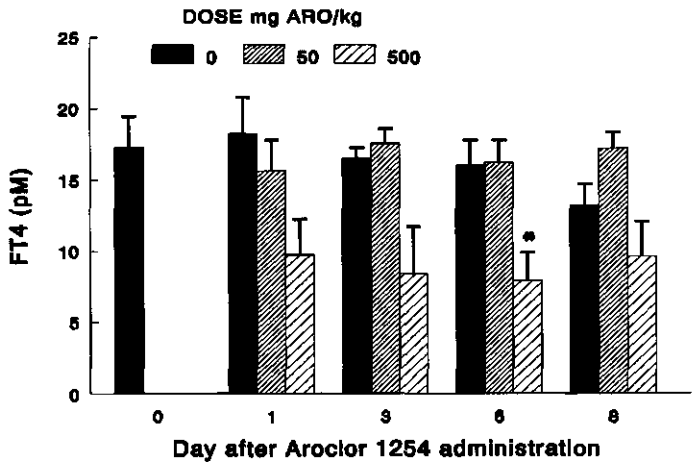

Figure 2 Plasma levels of free thyroxine in control and Aroclor 1254 exposed rats (50 and 500 mg/kg b.w.) on day 0 before i.p. injection with corn-oil (control) or Aroclor 1254, and on exposure day 1, 3, 6 and 8. Results are means ± SEM. *Indicates values significantly different from controls ($p < 0.05$).

2,2',4,4',5,5'-hexachlorobiphenyl (PCB 153) present in the plasma and a ratio was calculated.

In vitro T_4 competition-binding assays on TTR

The gel-filtration procedure as decribed by Somack *et al.* (1982) with minor modifications as described in Lans *et al.* (1993) was used for the *in vitro* T_4 competition-binding assay. The assay, using human TTR, was performed as follows: TTR (30 nM, dissolved in 0.1 M Tris-HCl, 0.1 mM NaCl, 1 mM EDTA buffer, pH 8) was incubated with a mixture of ^{125}I-T_4 and unlabeled T_4 (70.000 cpm, 55 nM) in Tris-HCl buffer, and competitors (T_4 or hydroxylated 4-OH-2,3,3',4',5-pentaCB) were added in increasing concentrations (dissolved in methanol, volume added was 5 µL). Control incubations were made by adding 5 µl methanol instead of competitor. Total ^{125}I-radioactivity added to each of the incubation mixtures (200 µl total volume) was checked by gamma-counting. The incubations were allowed to reach binding equilibrium overnight at 4 °C. Protein-bound ^{125}I-T_4 and free ^{125}I-T_4 were separated on 1 ml Biogel P-6DG columns (prepared in a 1 ml disposable syringe, equilibrated in Tris-HCl buffer) that were equilibrated with 300 µl 10 % (W/V) saccharose-Tris-HCl buffer) and centrifuged for 1 min at 1000 rpm (100 g) in a precooled centrifuge (Difuge, Hereaus). The columns were spin-forced eluted with an additional 200 µl Tris-HCl buffer. The first 2 eluate fractions, containing the protein bound ^{125}I-T_4 fraction, were combined; radioactivity was counted and compared to the control incubations. Free T_4 was bound to the Biogel matrix and therefore was not present as a contaminant in the fractions eluted. Competition binding curves were made by plotting relative ^{125}I-T_4-protein binding (% of control) against added inhibitor concentration. Competition binding assays with unlabeled T_4 were used as reference assays to make comparisons between different experiments.

Statistics

Data were analysed, after testing for homogeneity and normality, with one way analysis of variance (ANOVA) and the least significant difference test (LSD; $p < 0.05$ or 0.005) for differences to controls. A significant difference of $p < 0.05$ or $p < 0.005$ is indicated in the figures and tables, showing means and standard errors of means (SEM) or standard deviations (S.D.). All data analysed are results of duplicate, or triplicate measurements.

Results

Effects on plasma thyroid hormone levels

Total thyroxine (TT_4) levels were significantly decreased to 41, 38, 35 and 41 % of controls, in the high dose Aroclor 1254 group (500 mg/kg b.w.) on respectively day 1, 3, 6, and 8 after exposure (Fig. 1). The low total T_4 level (14.9 ± 6.9 nM) found at day 1 after exposure in the high dose Aroclor 1254 group did not further decrease but leveled off at day 3, 6 and 8. No decrease of plasma total T_4 was found in the low dose Aroclor 1254 group (50 mg/kg b.w.). Exposure to 500 mg Aroclor 1254/kg b.w. also resulted in reductions in plasma free T_4 concentrations to 9.7 nM (53 % of control), 8.4 nM (51 % of control), 7.9 nM (49 % of control) and 9.6 nM (73 % of control) at day 1, 3, 6 and 8 after exposure (Fig. 2), while no reduction of free T_4 was found after exposure to 50 mg Aroclor 1254/kg.

Plasma T_3 levels (not shown) were not changed significantly in the low and high dose group at either timepoint of analysis.

Effects on plasma protein binding of T_4

Polyacrylamide gel-electrophoresis (PAGE) was used to determine the amount of ^{125}I-T_4 derived radioactivity bound to plasma proteins. Examples of PAGE profiles of ^{125}I-T_4 binding proteins in plasma from the control, low and high dose groups are shown in Figures 3a-c. Two peaks of ^{125}I-T_4 derived radioactivity could be identified on the gels that co-migrated with the reference samples of purified TTR and albumin, respectively.

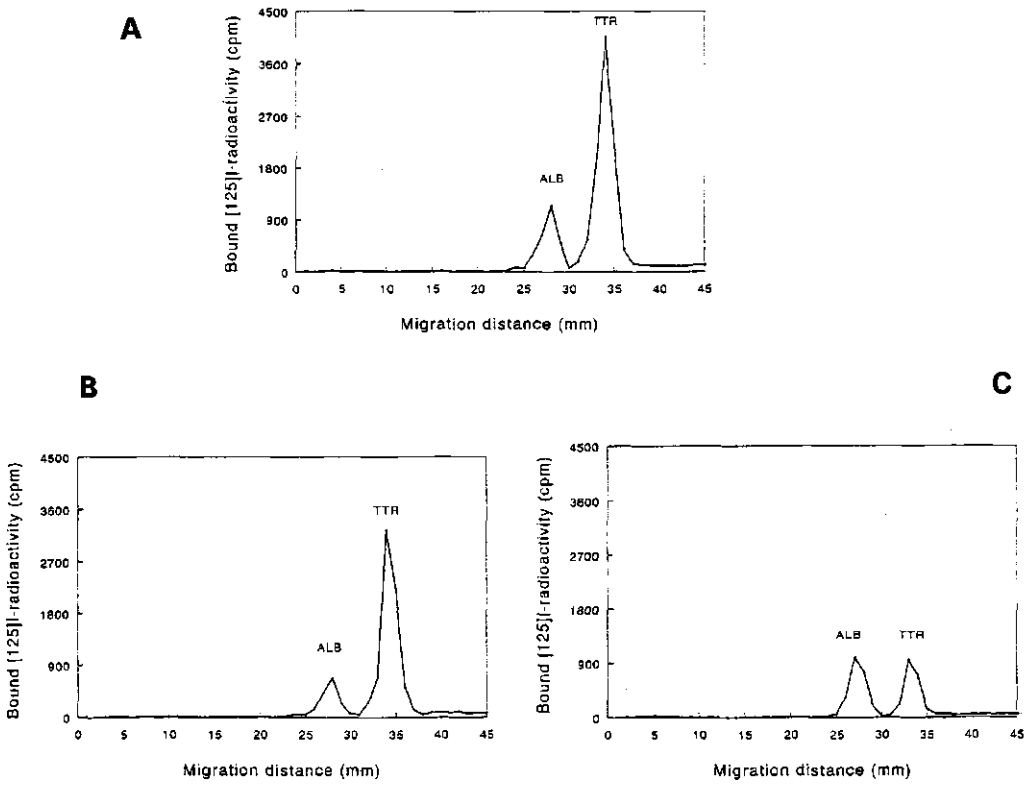

Figure 3 Examples of PAGE profiles of ^{125}I-T_4 derived radioactivity bound to plasma proteins as described in Materials and Methods. Transthyretin (TTR) and albumin (ALB) were identified by comparison of migration distances, using pure human TTR and BSA and rat serum proteins. PAGE profile of serum of control, corn-oil treated rats (A), 50 mg Aroclor 1254/kg b.w. (B) and 500 mg Aroclor 1254/ kg b.w. (C) treated rats, on exposure day 7, 6 hours after i.p. injection with ^{125}I-T_4 (25 μCi).

In control rats the amount of ^{125}I-T_4 derived radioactivity bound to TTR was approximately 2.5 fold higher on day 2/3 (data not shown) and 5.5 fold higher on day 7/8 than the ^{125}I-T_4 derived radioactivity associated with albumin. On day 2 and 3 after Aroclor 1254 exposure the ratio of ^{125}I-radioactivity bound to TTR over albumin, was not significantly different between the control and Aroclor 1254 groups (Fig. 4). On day 7 and 8 after Aroclor 1254 administration the high dose Aroclor 1254 group had significantly (3.5-4.1 fold) lower TTR/albumin ratios of bound ^{125}I-T_4 radioactivity than controls, while the level of radioactivity associated to albumin remained essentially the same (Fig. 4). The low dose Aroclor 1254 group showed no significant differences in TTR/albumin ratios on day 7/8. The control TTR/albumin ratios of bound ^{125}I-T_4 radioactivity were higher in the group injected with ^{125}I-T_4 on day 2/3 when compared with the group treated on day 7/8.

Plasma TTR levels

Analysis of plasma TTR concentrations using specific anti-TTR antibodies indicated that the TTR levels were decreased significantly on day 3 in the low dose Aroclor 1254 group to 48% (22.9 mg TTR/dl plasma; 4.2 μM) of control and in the high dose group to 63 % (29.9 mg TTR/dl; 5.4 μM) of control (47.5 mg TTR/dl; 8.6 μM) (Fig. 5). On day 8 of Aroclor 1254 exposure, however, TTR levels were not different from controls (38.0 mg TTR/dl; 6.9 μM) in high (35.9 mg TTR/dl; 6.5 μM) and low dosed Aroclor 1254 groups (38.9 mg TTR/dl; 7.1 μM), but were increased with 20 % and 70 %, respectively, compared to day 3 of exposure. Levels of plasma TTR were slightly lowered to 80 % at day 8 compared to day 3 in corn-oil treated controls.

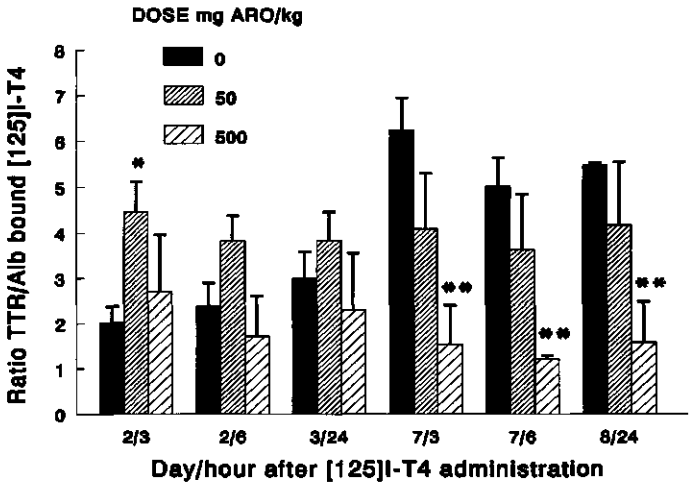

Figure 4 Ratio of ^{125}I-T_4 radioactivity bound to TTR over albumin, calculated as described in Materials and Methods, in rat serum of control and Aroclor 1254 exposed rats (50 and 500 mg/kg b.w.) on different exposure days 2 and 7, at 3, 6 and 24 hours after ^{125}I-T_4 injection (day after Aroclor 1254 exposure/hour after ^{125}I-T_4 injection). Results shown are means ± SEM of triplicate measurements. *Indicates values significantly different from controls ($p < 0.05$). **Indicates values significantly different from controls ($p < 0.005$)

Presence of hydroxylated PCB-metabolites in plasma

In the Aroclor 1254 treated rats, GC analysis of plasma extracts indicated the presence of relatively large amounts of hydroxylated PCB-metabolites at exposure day 3 and 8. GC-MS analysis using synthetically prepared OH-PCB-metabolite reference compounds revealed the presence of mainly one major metabolite e.g. 4-OH-2,3,3',4',5-pentachlorobiphenyl (4-OH-pentaCB) metabolite (data not shown), which can be formed by para-hydroxylation of 2,3,3',4,4'-pentaCB (PCB 105) or 2,3,3',4',5-pentaCB (PCB 118), both present in Aroclor 1254. Quantification of the amount of 4-OH-pentaCB is represented in Figure 6. High levels of 4-OH-pentaCB up to respectively 758 ± 110 and 1020 ± 17 ppb (fresh weight), corresponding to 2.22 ± 0.32 and 2.98 ± 0.05 μM, were observed in the plasma of 500 mg Aroclor 1254/kg dosed rats at day 3 and 8 after Aroclor 1254 administration. About 6-fold lower (on day 3: 133 ± 102 ppb, 0.39 ± 0.30 μM) and 17-fold lower concentrations (on day 8: 62 ± 66 ppb, 0.18 ± 0.18 μM) of 4-OH-pentaCB were observed in the 50 mg Aroclor 1254/kg dosed rats. The molar ratios of TTR levels over the amount of 4-OH-pentaCB metabolites were 10.8 on day 3 and 40 on day 8 for the low dosed group, while for the high dosed group ratios were 2.4 on day 3 and 2.2 on day 8.

In addition the amount of PCB 153 (2,2',4,4',5,5'-hexachlorobiphenyl) was quantified and ratios were calculated of levels of metabolite vs. PCB 153 in plasma, which ranged from 7.5 to 12 for the low dosed group and 3.1 to 12.7 for the high dosed group, indicating that relative large amounts of metabolite compared to the persistent PCB 153 congener were present in the blood of both low and high Aroclor 1254 exposed rats. PCB 153 is almost always present in ca. 20 % (15-25%) of the total PCBs in biological samples.

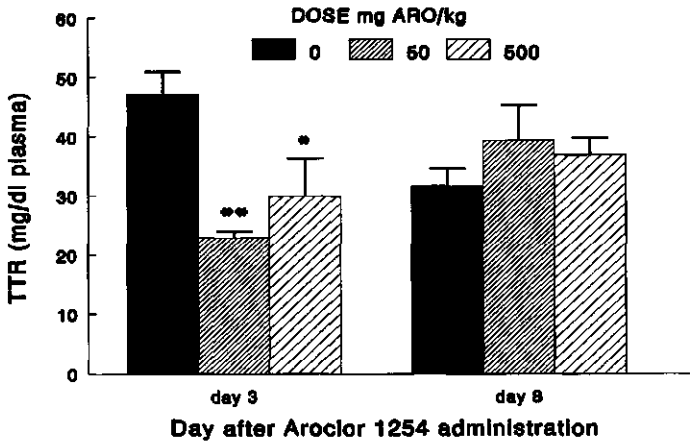

Figure 5 Plasma TTR levels of control rats and Aroclor 1254 exposed rats (50 and 500 mg/kg b.w.) on exposure day 3 and 8. Results are means ± SEM of duplicate measurements. *Indicates values significantly different from controls ($p < 0.05$). **Indicates values significantly different from controls ($p < 0.005$).

In vitro competition of T_4 binding to TTR by the hydroxylated PCB metabolite
The 4-OH-2,3,3',4',5-pentaCB metabolite as was present in the plasma of the Aroclor 1254 exposed rats, was synthesized and used in an *in vitro* T_4-TTR binding competition study. The competition curves of the 4-OH-pentaCB metabolite and T_4 are shown in Figure 7. The IC_{50}'s (the concentration inhibiting 50 % of T_4-TTR binding) of 4-OH-PentaCB and T_4 (resp. 15.5 nM and 101.6 nM) show that 4-OH-pentaCB inhibits T_4-TTR binding 6.5 times more potent than T_4, the natural ligand, itself.

Effects on hepatic thyroid hormone metabolism
Hepatic T_4-glucuronidation was significantly increased to 2.9 and 6.1 times control values in the high dose Aroclor 1254 group at day 3 and 8 after Aroclor 1254 administration, respectively (Table 1). A slight, but not significant increase in hepatic T_4-UGT activity was observed on day 3 (1.4 times control value) in the low dose Aroclor 1254 group. Hepatic type 1 reverse T_3 deiodinase (ID-I) activity was not significantly different between controls and the low and high dosed Aroclor 1254 groups at either day 3 or day 8 after exposure (Table 1), except for the low dose group at day 3.

Hepatic cytochrome P450 levels and EROD activity
Cytochrome P450 levels were increased in the high dose group at day 3 and 8 after Aroclor 1254 administration (1.4 and 1.5 times control values) but not in the low dose group (Table 2). Hepatic microsomal EROD-activity, however, was markedly increased at day 3 (not significantly) and 8 after exposure in both 50 mg Aroclor 1254/kg (13.1 and 6.3

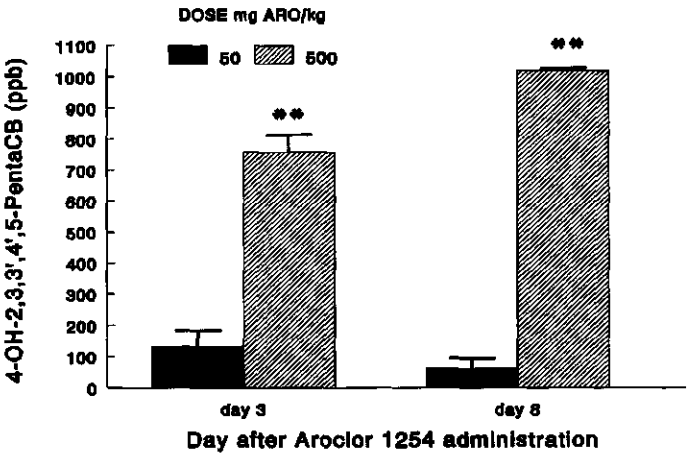

Figure 6 Plasma concentrations of 4-OH-2,3,3',4',5-pentachlorobiphenyl (ppb of fresh plasma weight) in 50 and 500 mg Aroclor 1254/kg b.w. exposed rats, on exposure day 3 and 8. Results are means ± SEM of duplicate measurements. **Indicates values significantly different from 50 mg Aroclor 1254/kg dose group ($p < 0.005$).

Table 1 Hepatic T_4 glucuronidation (T_4-UGT) activity and hepatic type 1 deiodinase (ID-I) activity in control, 50 and 500 mg Aroclor 1254/kg b.w. exposed rats (n=4) on day 3 and 8 after administration.

Enzyme activity	Day	Control (corn oil)	Aroclor 1254 (50 mg/kg)	Aroclor 1254 (500 mg/kg)
T_4-UGT activity	3	0.82 ± 0.09	1.11 ± 0.27	2.36 ± 0.47[a,b]
(pmol T_4/min/mg)	8	0.78 ± 0.20	0.85 ± 0.23	4.79 ± 1.31[a,b]
ID-I activity	3	60 ± 5.5	85 ± 6.0[a]	60 ± 5.5
(pmol rT_3/min/mg)	8	72 ± 4.0	78 ± 5.0	60 ± 7.5

Note: Results are means ± SEM of duplicate measurements. [a]Indicates values significantly different from controls ($p < 0.05$). [b]Indicates values significantly different from 50 mg Aroclor 1254/kg dose group ($p < 0.05$).

Table 2 Cytochrome P450 levels and EROD activity in hepatic microsomes of control and Aroclor 1254 exposed rats (50 and 500 mg Aroclor 1254/kg b.w., n=4) on day 3 and 8 after exposure.

	Day	Control (corn oil)	Aroclor 1254 (50 mg/kg)	Aroclor 1254 (500 mg/kg)
Cytochrome P450	3	1.77 ± 0.33	1.84 ± 0.47	2.43 ± 0.14
(nmol/mg)	8	1.65 ± 0.30	1.18 ± 0.13	2.43 ± 0.31[a]
EROD activity	3	0.036 ± 0.001	0.469 ± 0.231	2.336 ± 0.513[a,b]
(nmol RR/min/mg)	8	0.035 ± 0.001	0.221 ± 0.074	2.241 ± 0.328
Rel. liver weight	3	3.13 ± 0.14	3.25 ± 0.15	3.72 ± 0.14[a,b]
(g/100 g b.w.)	8	3.13 ± 0.06	3.19 ± 0.06	3.70 ± 0.21[a,b]

Note: Relative liver weights are given as ratio of liver weight per 100 g b.w.. Results are means ± SEM of duplicate measurements. [a]Indicates values significantly different from controls ($p < 0.05$). [b]Indicates values significantly different from 50 mg Aroclor 1254/kg dose group ($p < 0.05$).

times control) and 500 mg Aroclor 1254/kg group (65.2 and 63.8 times control) (Table 2). The increased EROD activity in the high dose Aroclor 1254 group was also significantly different from the low dose Aroclor 1254 group at day 3 and 8 after exposure (resp. 5 and 10 fold increased compared to low dose group).

No changes in body weights were observed in the low and high exposed Aroclor 1254 group compared to controls. Relative liver weights were only significantly increased in the high dose group (1.2 times) at day 3 and 8 compared to controls and low dose group (Table 2).

Discussion

Exposure of Wistar rats to Aroclor 1254 (50 and 500 mg Aroclor 1254/kg b.w.) resulted in decreased plasma total and free thyroxine (T_4) levels. Both reduced binding of T_4 to TTR in plasma and increased hepatic glucuronidation of T_4 were observed in this study, suggesting the participation of both mechanisms of action in the reduction of circulating T_4 levels. In addition, selective accumulation of the 4-OH-2,3,3',4',5-pentaCB metabolite was found in this study, i.e. a PCB-metabolite that has the structural requirements to bind to TTR *in vitro* and strongly displace T_4 from TTR as found in this study and by Lans *et al.* (1993). The observed diminished T_4 binding capacity to TTR (as demonstrated on PAGE by the low ratio of T_4 binding to TTR over albumin) may indicate that the decrease in plasma T_4 found at day 8 in highly exposed rats in this study, may partially be caused by inhibition of T_4 binding to TTR by the 4-OH-pentaCB metabolite present in plasma.

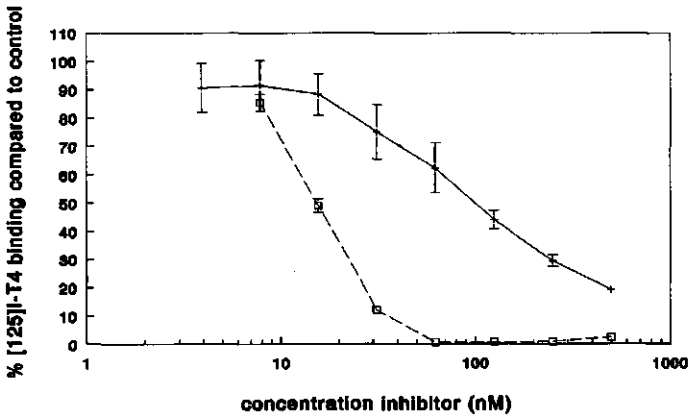

Figure 7 *In vitro* competition of T_4 binding to TTR by 4-OH-2,3,3',4',5-pentaCB (□) and T_4 (+). Competition curves show the percentage (means ± S.D.) of *radiolabeled* T_4 bound to TTR compared to control (no inhibitor) at different inhibitor concentrations (nM).

Like the decreased T_4 binding to TTR after Aroclor 1254 exposure found in this study, Brouwer and Van den Berg (1986) found a comparable selective decrease of radiolabeled T_4 bound to TTR in plasma after exposure of rats to 3,3',4,4'-tetrachlorobiphenyl (TCB). In both studies the amount of labeled T_4 bound to albumin remained unaltered and TTR levels were not decreased. Exposure of rats to radiolabeled TCB resulted in TCB-derived radioactivity, later identified as the 4-OH-3,3',4',5-tetraCB metabolite, which was selectively bound to TTR in the plasma and displaced T_4 from TTR (Brouwer, 1989). Consistent findings of high blood concentrations of TCB derived radioactivity, mainly in the form of a TCB-metabolite, were described after exposure to radioactive TCB for mice by Lucier *et al.* (1978b) and for rats and monkeys by Abdel Hamid *et al.* (1981). Also in pregnant rats exposed to TCB, a selective accumulation of the 4-OH-3,3',4',5-tetraCB metabolite was found in the fetus and fetal plasma (Morse *et al.*, 1995b).

In this study the 4-OH-2,3,3',4',5-pentaCB metabolite was identified as the major PCB-metabolite in the plasma of Aroclor 1254 exposed rats. This metabolite can theoretically be formed by para-hydroxylation of 2,3,3',4,4'-pentaCB (PCB 105), or 2,3,3',4',5-pentaCB (PCB 118). Both CB 105 and 118 are present in Aroclor 1254 mixtures in relatively high amounts (resp. 4 and 6 % by weight) (Schulz *et al.*, 1989). As shown by the T_4-TTR inhibition study, the 4-OH-pentaCB was a potent inhibitor structurally resembling T_4. As found for other hydroxylated PCB metabolites (Lans *et al.*, 1993, 1994) the potency of inhibiting T_4-TTR binding *in vitro* was even stronger than T_4, the natural ligand. The difference in 4-OH-pentaCB levels on day 3 and 8 in the high dosed Aroclor 1254 group may be enough for reaching a threshold level for displacement of T_4 from TTR. In combination with the high T_4 glucuronidation on day 8 this may explain the decrease in the ratio of T_4 bound to TTR/albumin at day 8 and the lack of effect on the ratio on day 3 in the high dosed group.

Hydroxylated Aroclor 1254 metabolites were also found in adult rats *in vivo* in another study (Bergman *et al.*, 1994) where rats were dosed with Aroclor 1254 at lower levels (25 mg/kg for 3 subsequent days) and plasma and tissues like lung, kidney, liver and adipose tissue were analysed by GC-MS. In plasma 12 hydroxylated PCB metabolites were found, the major peak was identified as the 4-OH-2,3,3',4',5-pentaCB metabolite. Hydroxylated PCB metabolites were found in rat lung and liver, whereas no hydroxylated PCB metabolites were detected in adipose tissue. Selective retention of several hydroxylated PCB metabolites in plasma of environmentally exposed species, like man and seal, was described by Bergman *et al.* (1994), showing the presence of the 4-OH-2,3,3',4',5-pentaCB metabolite and a hydroxylated hexaCB in seal blood and the 4-OH-2,3,3',4',5-pentaCB and 4-OH-2',3,3',4',5,5'-hexaCB (a PCB 156 metabolite) in human serum. So the 4-OH-pentaCB found in rat plasma after Aroclor 1254 exposure in this study, can also be present in plasma after background exposure to PCBs present in environment.

TTR plasma levels measured in this study were decreased in the low and high dosed Aroclor 1254 group on day 3. However, a direct effect of the PCB congeners present in the Aroclor mixture on TTR synthesis is not likely because no clear dose-dependent response was found. Moreover, TTR synthesis in the liver is not changed by differences in thyroid hormone status (Blay *et al.*, 1994). On day 2/3 after Aroclor 1254 exposure, the low dosed group showed the lowest TTR levels, while on day 3 the TTR/albumin ratio of bound ^{125}I-T_4 was elevated in the low dose group compared to control. Opposite effects are found in the control group of day 3 which shows high TTR levels, but a low ratio of TTR/albumin

bound ^{125}I-T_4. No clear mechanism can be found explaining these contradicting results. On the contrary, high TTR levels would suggest a concommitant high ratio of TTR/albumin bound ^{125}I-T_4. Plasma TTR levels at day 8 were equal in all exposure groups, while the strongest reduction in the ratio of TTR/albumin bound ^{125}I-T_4 was observed at this time point in the high dose group. This clearly suggests that Aroclor 1254 exposure interfered with T_4 binding to TTR, most likely by competitive displacement of T_4 by the 4-OH-pentaCB metabolite present in the blood.

The other mechanism studied in this paper that is possibly involved in plasma T_4 reduction is hepatic T_4 metabolism (glucuronidation and deiodination). T_4 glucuronidation was significantly increased in the high dose group on day 3 and 8 after Aroclor 1254 administration, suggesting a concomittantly increased biliary T_4 excretion. In the low dosed Aroclor 1254 group no changes in plasma in T_4 levels were observed while T_4-UGT activity was not increased either. Changes in total T_4 as found on day 1 and 3 in the high dosed Aroclor 1254 group, are probably caused by increased T_4 glucuronidation, because no effects on plasma T_4 transport was found in the high dose group at day 3. Several other studies on Aroclor 1254 exposure in rats have shown increased T_4 glucuronidation and excretion (Bastomsky, 1974; Barter and Klaassen, 1992). Kanechlor 400, another commercial PCB mixture increased T_4 glucuronidation to the same extent as other inducers like 3-methylcholanthrene and beta-naphtoflavone, but TT_4 levels in plasma were decreased more (Saito *et al.*, 1991). The authors suggest that decreased T_4 binding to plasma proteins could be causing this difference. T_4 glucuronidation is also increased after exposure to other halogenated aromatic compounds, like hexachlorobenzene (HCB), TCB, TCDD (Visser *et al.*, 1993), 2,3,3',4,4',5-hexachlorobiphenyl (PCB 156) (Van Birgelen *et al.*, 1994a), polybromobiphenyl (PBB, Byrne *et al.*, 1987), 3,3',4,4',5-hexabromobiphenyl (Spear *et al.*, 1990) and 3,3',4,4',5,5'-hexachlorobiphenyl (Morse *et al.*, 1993a), compounds which simultaneously decrease plasma T_4 levels.

Hepatic type 1 reverse T_3 deiodinase (ID-I) activity was not affected in this study. Inhibition of ID-I activity by hydroxylated PCB and TCB metabolites has been found in *in vitro* studies (Adams *et al.*, 1990; Rickenbacher *et al.*, 1989; Lans *et al.*, 1994). Hydroxylated PCB metabolites apparently do have the capacity to inhibit ID-I activity. However, in this study even the high dose Aroclor 1254 group, with relatively high levels of hydroxylated PCB metabolites in plasma, did not show inhibition of ID-I *in vivo*. On the other hand, *in vivo* inhibition of ID-I activity (to 50%) has been observed following treatment of rats with several Ah-receptor agonists, such as 3-methylcholantrene, TCB and TCDD (Visser *et al.*, 1993). Likewise, Adams *et al.* (1990) found a decrease in hepatic ID-I activity in rats after exposure to 50 mg TCB/kg *in vivo*. This decrease in ID-I activity could be caused by direct inhibiting effects of the parent compound PCBs and TCDD, or as a secondary effect of hypothyroidism caused by these compounds (Kaplan, 1986). Hence inhibition of ID-I activity by both hydroxylated PCB metabolites and parent compounds may occur. The lack of effect by Aroclor 1254 suggests that there may be antagonists present in the PCB mixture that obscure effects of parent compounds or hydroxylated metabolites on ID-I activity, or that the exposed rats have no functional hypothyroid status.

In conclusion, in this study a hydroxylated PCB metabolite selectively accumulated in blood of rats exposed to Aroclor 1254 and inhibited T_4 binding to TTR. In addition T_4 glucuronidation was strongly increased. Consequently decreases in plasma thyroid hormone levels after Aroclor 1254 exposure are most likely caused by a combined effect of different mechanisms e.g. disturbances in thyroid hormone metabolism and selective inhibition of

thyroid hormone plasma transport in rodents. We cannot exclude that a direct effect on the thyroid gland by Aroclor 1254 has also occurred, similar to results discussed by Collins and Capen (1980a,b,c) that may additionally contribute to the reductions in circulating thyroid hormone levels.

The presence and selective retention of a specific major hydroxy-PCB metabolite, 4-OH-2,3,3',4',5-pentaCB, in plasma given the exposure of rats to a complex PCB mixture, deserves further attention in terms of occurence of hydroxylated PCB metabolites in human and wildlife and its potential toxic effects, especially on brain or neurologic development and reproduction through thyroid hormone disturbances (Morse *et al.*, 1993b).

Acknowledgements

We thank dr. W.S. Blaner, Institute of Human Nutrition (Columbia University, New York, USA) for his advice and assistence on the TTR quantification assays.

CHAPTER 6

In vivo alterations in thyroxine metabolism and plasma transport by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in rats

Abstract

Polyhalogenated aromatic hydrocarbons such as polychlorobiphenyls (PCBs) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) are potent environmental goitrogens, that cause marked reductions in plasma thyroid hormone levels. In earlier studies at our laboratory hydroxylated polychloro-dibenzo-*p*-dioxin (PCDD), polychloro-dibenzofuran (PCDF), and PCB-metabolites inhibited thyroxine (T_4) binding to transthyretin (TTR), the major thyroid hormone plasma transport protein in rodents, *in vitro*. *In vivo*, hydroxylated PCB metabolites were found to inhibit T_4 binding to TTR after exposure of rats to 3,3',4,4'-tetrachlorobiphenyl or Aroclor 1254, thereby decreasing plasma T_4 levels. *In vivo* exposure of rats and mice to TCDD has been described to cause major changes in plasma T_4 levels. In this study the contribution of distinct modes of interaction of TCDD with thyroid hormone metabolism and plasma transport, i.e. changes in hepatic glucuronidation and deiodination and inhibition of T_4-TTR binding, to the observed plasma T_4 reductions were investigated. Female Wistar rats were dosed with a single i.p. injection of TCDD (25 μg/kg) or cornoil (vehicle control). Four days after TCDD exposure part of the control and TCDD-exposed rats were injected with ^{125}I-T_4 (25 μCi). Blood samples were taken after 3, 6, and 24 hours after ^{125}I-T_4 injection. All rats were killed at t=24 h after ^{125}I-T_4 injection (day 5 after TCDD exposure) and liver and brains were collected.

Plasma total T_4 levels were significantly decreased with 56 % and 46 % in TCDD-treated rats at resp. 4 (non ^{125}I-T_4 injected rats) and 5 days after exposure, plasma free T_4 levels were non-significantly decreased at 4 and 5 days after exposure. No alterations were observed in plasma total T_3 levels. Polyacrylamide gelelectrophoresis (PAGE) revealed two peaks of ^{125}I-T_4-derived radioactivity in plasma which were identified as TTR and albumin. The ratio of ^{125}I-T_4 binding to TTR over albumin was calculated for PAGE plasma profiles of control and TCDD exposed rats as an indication of T_4-TTR binding capacity. This ratio was the same in control and TCDD treated rats 3 hours after ^{125}I-T_4 injection, but was decreased at 6 hours and significantly at 24 hours after ^{125}I-T_4 injection. However, no hydroxylated TCDD-metabolites, which could inhibit T_4 binding to TTR *in vitro*, were detected in plasma, while plasma TTR levels were not

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changed either after TCDD exposure. The parent compound TCDD was present in plasma, but is not likely to inhibit T_4 binding to TTR. Hepatic cytochrome P450 levels and cytochrome P4501A1 (EROD) activity were significantly induced resp. 1.8 and 212 fold in TCDD exposed rats. In addition, TCDD exposure resulted in an increased hepatic T_4 glucuronidation (3.9-fold) while hepatic type 1 deiodinase activity was decreased to 82% of control values. Brain type 2 deiodinase activity was increased 2-fold following TCDD exposure indicating a hypothyroid status of the rats. Based on these results one can conclude that reduced T_4-TTR binding by hydroxylated TCDD-metabolites does not play a role in the decreases of plasma T_4 levels, while enhanced hepatic elimination of thyroid hormone may be the major cause of the plasma thyroid hormone reductions found in rats after TCDD exposure.

Introduction

Polychlorinated dibenzo-*p*-dioxins (PCDDs) and related compounds, like polychlorinated dibenzofurans (PCDFs) and biphenyls (PCBs), are ubiquitous environmental contaminants. In contrast with the commercially produced PCBs, PCDDs and PCDFs are unwanted byproducts of waste-combustion and the production of diverse chlorinated aromatics, like PCBs and hexachlorobenzene in the past, and chlorinated phenoxybenzenes and phenols (Safe, 1990; Van den Heuvel and Lucier, 1993; Van den Berg *et al.*, 1994).

The 2,3,7,8-substituted congeners of PCDDs and PCDFs are selectively bioaccumulated in the foodchain, and are especially abundant in higher trophic levels like fish eating birds, marine mammals and man (Ahlborg *et al.*, 1992). PCDDs and PCDFs cause a wide range of toxic responses including teratogenic, reproductive, immunotoxic, hepatotoxic and neuro-endocrine effects and are known carcinogens in experimental animals (Safe, 1990). These toxic responses are congener-, dose-, species- and tissue specific (Peterson *et al.*, 1993). The lateral substituted congeners (2,3,7,8-substituted) PCDDs and PCDFs are causing most if not all their effects via a specific cytosolic receptor, the Ah-receptor. The most toxic congener is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD).

Effects of TCDD on plasma thyroid hormone levels and metabolism in liver have been described by several authors. TCDD markedly increased the biliary excretion of thyroxine (T_4) (Bastomsky, 1977). The increase in T_4 excretion was most likely due to induction of T_4 glucuronyltransferase (T_4-UGT) activity by TCDD (Beetstra *et al.*, 1991; Lucier *et al.*, 1975). Administration of TCDD to rats or mice resulted in reduced plasma T_4 levels (Potter *et al.*, 1983; Lans *et al.*, 1990; Potter *et al.*, 1986; Roth *et al.*, 1988). Plasma 3,3',5-triiiodothyronine (T_3) levels, however, increased (Bastomsky, 1977; Potter *et al.*, 1986), did not change (Potter *et al.*, 1983; Henry and Gasiewicz, 1987; Lans *et al.*, 1990; Muzi *et al.*, 1989; Gorski and Rozman, 1987; Jones *et al.*, 1987) or decreased (Pazdernik and Rozman, 1985) after exposure of rodents to TCDD.

Induction of T_4 glucuronidation by certain PCDDs, PCDFs and PCBs may partly explain the decrease in plasma T_4 levels. A second mechanism that may be involved in this decrease is the inhibition of T_4 transport in plasma after exposure to these compounds. In *in vitro* studies in our laboratory, hydroxylated PCDD, PCDF and PCB metabolites could inhibit T_4 binding to transthyretin (TTR), the plasma T_4 transport

protein in rodents (Lans *et al.*, 1993). This was confirmed by *in vivo* studies where plasma thyroid hormone transport via TTR was inhibited by hydroxylated PCB-metabolites of 3,3',4,4'-tetrachlorobiphenyl (TCB or CB77) and Aroclor 1254 in rats and mice (Brouwer, 1989; Lans *et al.*,1994). However, hydroxylated PCDD, PCDF and PCB metabolites did not inhibit *in vitro* T_4 binding to thyroxine-binding globulin (TBG), the major plasma thyroid hormone transport protein in man (Lans *et al.*, 1994). Another possible pathway of interference in thyroid hormone levels is through modulation of hepatic type 1 deiodinase (ID-I) activity, another thyroxine binding protein which converts T_4 to T_3 or 3,3',5'-triiodothyronine (rT_3) in the liver. Conversion of T_4 by ID-I was competitively inhibited *in vitro* by hydroxylated PCDD, PCDF and PCB metabolites (Lans *et al.*, 1994; Rickenbacher *et al.*, 1989). Moreover, TCDD exposure decreased ID-I activity in rats *in vivo* (Eltom *et al.*, 1992; Rickenbacher *et al.*, 1986).

All of these interactions of TCDD with thyroid hormone metabolism have been studied and reported separately. The present study was carried out to investigate the contribution of the various interaction pathways to the reduction of plasma T_4 by TCDD in the same experiment. Corn oil or TCDD-pretreated rats were injected with ^{125}I-T_4 to determine the T_4 binding capacity of the various plasma proteins, like TTR and albumin. TCDD and hydroxylated TCDD metabolites were analysed in plasma by GC/MS analysis. In addition, parameters of hepatic T_4 metabolism, eg. T_4-UGT and ID-I activity were measured. Moreover, brain type 2 deiodinase (ID-II) activity was measured as an indication of functional hypothyroidism.

Materials and methods

Materials

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (>99 % pure) was obtained from Promochem, Wesel, Germany. The hydroxylated TCDD metabolites, 2-OH-3,7,8-TriCDD and 2-OH-1,3,7,8-TCDD were kindly donated by Dr. S.H. Safe of the Department of Veterinary Physiology and Pharmacology, Texas A&M University, College Station, Texas, USA. ^{125}I-T_4 (1500 μCi/μg T_4), ^{125}I-rT_3 (1200 μCi/μg rT_3) and TT_4, FT_4 and TT_3 Amerlite kits were obtained from Amersham (Amersham, Aylesbury, Buckinghamshire, United Kingdom). T_4, T_3, 6-propyl-2-thiouracil (PTU), uridine-5'-diphosphoglucuronic acid (UDPGA), (3[{3-cholamidopropyl} dimethylammonio]-1-propane sulphonate) (CHAPS) and dithiotreitol (DTT) were purchased from Sigma (St. Louis, MO, USA); rT_3 was obtained from Henning (Berlin, Germany); Sephadex LH-20 was purchased from Pharmacia (Woerden, The Netherlands). Biorad protein reagent was obtained from Biorad Laboratories (Bio-rad, Richmond, CA, USA). Pure human TTR and BSA were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Experimental design

Female wistar rats (16 weeks old) were dosed with a single i.p. injection of 0 or 25 μg TCDD/kg b.w. in corn oil (8 rats per dose, 5 ml/kg b.w.). After 4 days four rats of each dose-group were injected i.p. with 25 μCi ^{125}I-T_4 (21 pmol). Blood samples were collected by tail bleeding at 3, 6 and 24 hours after ^{125}I-T_4 injection. Plasma was prepared from blood by centrifugation at 1000*g* and stored at -20 °C. At 24 h after ^{125}I-T_4 injection (day 5 after TCDD exposure) the rats were killed and dissected and blood

plasma, livers and brain were collected and stored at -80 °C. At day 5 after TCDD exposure four other, non ^{125}I-T_4 treated rats per dose-group were killed and plasma, liver and brains were collected and stored at -80 °C. Of all non ^{125}I-T_4 treated rats per dose-group blood samples were collected by tail-bleeding on day 1, 4 and 5 after TCDD exposure.

Sample preparations of non ^{125}I-T_4 treated rats

Livers were divided in two pieces for preparation of microsomes in two different buffers e.g. for EROD-activity and T_4-glucuronidation and for hepatic type 1 deiodinase (ID-I) activity. Microsomes for EROD/T_4 glucuronidation were prepared by homogenizing liver in a Potter-tube on ice with 2 ml/g liver of 0.1 M Tris-HCl buffer, pH 7.5 containing 0.25 M sucrose, followed by centrifugation at 9000*g* for 30 min. The resulting supernatant was centrifuged at 105,000*g* for 90 min. and the pellet was resuspended in 0.1 M phosphate buffer, pH 7.5. Microsomes for ID-I activity were prepared by homogenizing liver in 10 mM Tris-HCl, pH 7.4, containing 0.25 M sucrose and 1 mM DTT. After differential centrifugation at 9000 and 105,000*g* the microsomal pellet was resuspended in 10 mM Tris-HCl, pH 7.4, containing 1 mM DTT and 3 mM EDTA. All hepatic microsomes were stored at -80 °C until further use. For brain type 2 iodothyronine deiodinase (ID-II) measurements, brains were homogenized on ice in a Potter-tube with 8 ml/gram of 100 mM phosphate-buffer, pH 7.4, containing 4 mM EDTA and 25 mM DTT and stored at -80 °C until further use. Protein concentrations were determined using the Bio-rad coomassie blue assay.

Cytochrome P-450 levels

Hepatic cytochrome P450 levels were measured using the method of Omura and Sato (1964) with modifications according to Rutten *et al.* (1987). Briefly hepatic microsomes (used for EROD and T_4 glucuronidation) were diluted in 980 µl of 0.1 M phoshate-buffer, pH 7.4 to a final concentration of 1 mg/ml. Carbon-monoxide gas was run through the incubation. An absorption spectrum from 400 - 500 nm was collected before and after addition of 20 µl saturated sodium-dithionite solution in phosphate buffer to the incubation. The difference spectrum showed a peak at 450 nm from which cytochrome P450 content could be calculated in nmol/mg microsomal protein.

Ethoxyresorufin-O-deethylase activity

Ethoxyresorufin-O-deethylase (EROD) activity was determined in hepatic microsomes using a modified method of Burke *et al.* (1977). In short the reaction mixture, containing final concentrations of 2 µM ethoxyresorufin and 100 µg/ml microsomal protein in 0.1 M Tis-HCl buffer, pH 7.8, was pre-incubated for 2 minutes at 37 °C. The reaction was started by adding 0.1 mM NADPH to the incubation mixture. To detect the deethylation product, resorufin (RR), the excitation and the emission wavelength of the fluorimeter were 530 and 586 nm, respectively. The results are expressed as nmol RR formation per minute per milligram of microsomal protein.

Hepatic T_4-UDP-glucuronyltransferase activity (T_4-UGT)

^{125}I-T_4 was purified on Sephadex LH-20 immediately before each assay according to Beetstra *et al.* (1991). Hepatic microsomal T_4-UGT activity was determined in duplicate according to Beetstra *et al.* (1991) by the formation of ^{125}I-T_4-glucuronide from ^{125}I-T_4.

The final concentrations in the reaction mixture were 0.1 M Tris-HCl buffer, pH 7.8, 5 mM UDPGA, 3.75 mM $MgCl_2$, 0.25 % (w/v) CHAPS , 0.125% (w/v) BSA, 1 µM T_4 with 50,000 cpm ^{125}I-T_4, and 0.2 mg micosomal protein in a total volume of 200 µl. The reaction mixture was incubated at 37 °C for 30 min. The reaction was stopped with 200 µl ice-cold methanol and after centrifugation 200 µl of supernatant was added to 750 µl 0.1 M HCl. The amount of ^{125}I-T_4 glucuronide (T_4G) in the supernatant was isolated using Sephadex LH-20 chromatography and quantified using a Cobra AutoGamma counting system (Canberra-Packard, USA) (Rutgers *et al.*, 1989). Boiled microsomes were used as blanks. The results are expressed as nmol T_4G formation per minute per mg of microsomal protein.

Hepatic type 1 rT_3 deiodinase (ID-I) activity

^{125}I-rT_3 was purified on Sephadex LH-20 immediately before each assay according to Beetstra *et al.* (1991). Hepatic ID-I activity was determined in duplicate in microsomal preparations by the formation of $^{125}I^-$ from ^{125}I-rT_3 according to Mol and Visser (1985). A sample of 100 µl of 50 µg microsomal protein/ml in 200 mM phosphate-buffer pH 7.4, containing 4 mM EDTA and 2 mM DTT was incubated for 30 minutes at 37 °C with 100 µl of 0.2 µM rT_3 and 100,000 cpm ^{125}I-rT_3 in 4 mM NaOH, which did not affect the pH of the reaction mixture. The reaction was started by adding the microsomal protein. The reaction was stopped by addition of 750 µl 0.1 M HCl. The $^{125}I^-$ produced was separated from the reaction mixture by Sephadex LH-20 chromatography according to Otten *et al.* (1984) and counted on a Cobra Auto Gamma counting system (Canberra-Packard, USA). Boiled microsomes were used as blanks. The percentage of deiodination was determined by dividing the amount of counts from the $^{125}I^-$ fraction by the total amount of counts in the incubation.

Brain type 2 thyroxine deiodinase activity (ID-II)

^{125}I-T_4 was purified on Sephadex LH-20 immediately before each assay according to Beetstra *et al.* (1991). Brain ID-II activity was determined in duplicate as described by Visser *et al.* (1982) by the formation of $^{125}I^-$ from ^{125}I-T_4. Briefly the final concentrations in the reaction mixture were 100 mM phosphate buffer, pH 7.2, 25 mM dithiotreitol (DTT), 1 mM 6-propyl-2-thiouracil (PTU), 1 mM EDTA, 2 nM T_4 with 100,000 cpm 125I-T_4, 500 nM T_3 and 0.4 mg protein (brain homogenate) in a final volume of 200 µl. The addition of T_3 was necessary to inhibit inner-ring deiodination of T_4 by brain type 3 deiodinase (ID-III) (Kaplan *et al.*, 1983). Reaction mixtures were incubated at 37 °C for one hour. The reaction was stopped on ice by the addition of 100 µl 70 mg/ml BSA in order to bind the remaining substrate and T_3, followed by adding 500 µl of 10 % TCA (w/v) to precipitate the protein. The tubes were then centrifuged at 4000 rpm for 5 min., and 500 µl supernatant was removed, containing the free $^{125}I^-$ liberated from the ^{125}I-T_4. The amount of $^{125}I^-$ present in the supernatant was determined using Sephadex LH-20 chromatography (Otten *et al.* 1984). Boiled brain homogenates were used as blanks. Results are expressed as fmoles T_4 deiodinated per hour per mg of protein.

Thyroid hormone analysis

Plasma total T_4 (TT_4), total T_3 (TT_3) and free T_4 (FT_4) were measured with the Amerlite chemiluminescence system (Amersham, U.K) using the standard protocol from the supplier, with minor modifications. For TT_4 measurements the standard curve ranged from 0 to 120 nM TT_4 and the assay buffer was diluted five times with demineralized water.

In vivo T_4 binding capacity of plasma proteins

At several time points after ^{125}I-T_4 injection, blood was collected from the rats by tail-bleeding. Aliquots of 25 µl serum were mixed 1:1 with electrode buffer 5 mM Tris, 38 mM glycine buffer, pH 8.3 containing 10 % glycerol. These samples were subjected to native polyacrylamide gelelectrophoresis (PAGE) using a 10% separating gel and chromatographed during 5 hours at a constant current of 50 mA at 4 °C according to the method of Brouwer and Van den Berg (1986). The acrylamide gel was cut into 1 mm pieces, which were counted for ^{125}I-T_4-radioactivity in a Cobra Auto Gamma counter (Canberra-Packard). PAGE gel-profiles were made by plotting the ^{125}I-T_4-radioactivity against the migration distance (relative to the free T_4 peak used as front) on the gel. ^{125}I-T_4-Radioactivity (cpm) bound to the different serum proteins on the gels was used to calculate ^{125}I-T_4 binding ratios between transthyretin (TTR) and albumin. Pure human TTR and BSA and control rat serum were used to identify the plasma proteins on PAGE by comparing migration distances.

TTR levels

Radioimmunochemical analyses of TTR were performed at the Institute of Human Nutrition (Columbia University, New York, USA) according to the method of Muto *et al.* (1972) and Brouwer (1987) using immunosorbent purified, mono-specific rabbit anti-rat TTR (Navah *et al.*, 1977). The antibody was radiolabeled with ^{125}I via the Chloramine-T method. Samples of serum (25 times diluted in 50 mM Tris buffer pH 8.6, with 1% BSA (w/v)) were incubated with the specific TTR antibody . After precipitation with polyethylene-glycol buffer, the tubes were centrifuged for 30 minutes and the amount of TTR was determined by counting ^{125}I-radioactivity. The limit of detection was 20 ng/ml.

GC/MS analysis of hydroxylated TCDD metabolites

Clean-up and GC/MS procedures of rat plasma were performed according to the methods used by Dr. M. Van den Berg (Research Institute of Toxicology, University of Utrecht, The Netherlands). Serum was mixed with H_20 and methanol (2:1:1), acidified to pH 3 with H_2SO_4 and ^{13}C-labeled 2,3,7,8-TCDD (Cambridge Isotope Laboratories, Woburn, MA, U.S.A.) was added as an internal standard. This mixture was extracted three times with hexane:tert-butyl ether (1:1) by mixing and centrifugation. The organic phase was collected, concentrated and dissolved in aceton. The putative TCDD-metabolites were methylated by adding K_2CO_3 and CH_3I, and refluxing during 15 hours. K_2CO_3 was filtered and the remaining aceton was evaporated, hexane was added next and concentrated. The extract was run through an hexane pre-eluted aluminium-oxide column, eluted with dichloromethane CH_2Cl_2, and concentrated by evaporation. The standard hydroxylated metabolites, 2-OH-3,7,8-TriCDD and 2-OH-1,3,7,8-TCDD were methylated (to Cl_3-DD-OCH_3 and Cl_4-DD-OCH_3, respectively) using the described method.

GC-MS procedure

A J&W deltaB5 60m*0.22mm column was used, on a QMD 1000 high resolution gas chromatography/low resolution mass spectrometer, operating in electron impact mode. The temperature program used was T_1=120 °C, 1 min. to T_2=250 °C; rate 30 °C/min. First a full scan of 2,3,7,8-TCDD, Cl_3-DD-OCH_3 and Cl_4-DD-OCH_3 was made to obtain retention times and full mass-spectra. Next a multiple ion run (MI) was carried out scanning: 2,3,7,8-[^{13}C]/[^{12}C]-TCDD on M^+ and M^+ +2; Cl_3-DD-OCH_3 and Cl_4-DD-OCH_3 on M^+, M^+ +2, M^+ -15 and (M^+ +2)-15. Cl_3-DD-OCH_3 mass were 315.9; 317.9; 302.9; 300.9, and Cl_4-DD-OCH_3 mass were 351.9; 349.9; 336.9; 334.9.

Statistics

Data were analysed, after tesing for homogeneity and normality, with one way analysis of variance (ANOVA) and the least significant difference test (LSD; p <0.05 or 0.005) for differences to controls. A significant difference of $p < 0.05$ or $p < 0.005$ is indicated in the figures and tables, showing means and standard errors of means (SEM). All data analysed are results of duplicate or triplicate measurements.

Results

Effects on plasma thyroid hormone levels

Total plasma T_4 levels were significantly reduced in TCDD treated rats at day 4 and 5 after exposure to respectively 43.8 % and 53.6 % of their controls at the respective days (Fig. 1a). TT_4 levels at day 1 of exposure showed a tendency of reduction, but this was not significant at this time point. Plasma FT_4 levels showed a similar decrease at day 4 and 5 to 55.2 % and 63.3 % of controls, respectively (Fig. 1b), but this did not reach the level of significance. No changes were observed in plasma TT_3 levels following exposure of rats to 25 µg/kg TCDD at either time point of sampling (Fig. 1c).

Effects on plasma protein binding of T_4

^{125}I-T_4 binding proteins in plasma of control and TCDD pretreated rats were separated by gel electrophoresis (Figs. 2a-f). Three peaks of radioactivity were identified on PAGE, the first two representing albumin and transthyretin (TTR), the last one represented free T_4. The identification was based on co-migration with purified albumin and TTR. The amount of T_4 radioactivity bound to TTR or albumin was determined by gamma-counting and, subsequently the TTR/albumin ratio of ^{125}I-T_4 binding was determined. At 3 hrs after the ^{125}I-T_4 injection the TTR/albumin ^{125}I-T_4 binding ratio was equal in TCDD exposed and control rats (Table 1). This ratio decreased with time at 6 hrs (79 % of control), and was significantly lower that controls on 24 hrs (54 % of control) after ^{125}I-T_4 injection in the TCDD exposed rats (Table 1). In order to exclude that the decreased TTR/albumin ^{125}I-T_4 binding ratio was caused by reduced TTR levels following TCDD treatment, TTR was quantified by a specific radioimmuno-assay for rat TTR. TTR levels were not significantly decreased compared to controls after 4 days of TCDD exposure (resp. 69.65 ± 6.38 and 55.94 ± 7.00 mg TTR/dl plasma).

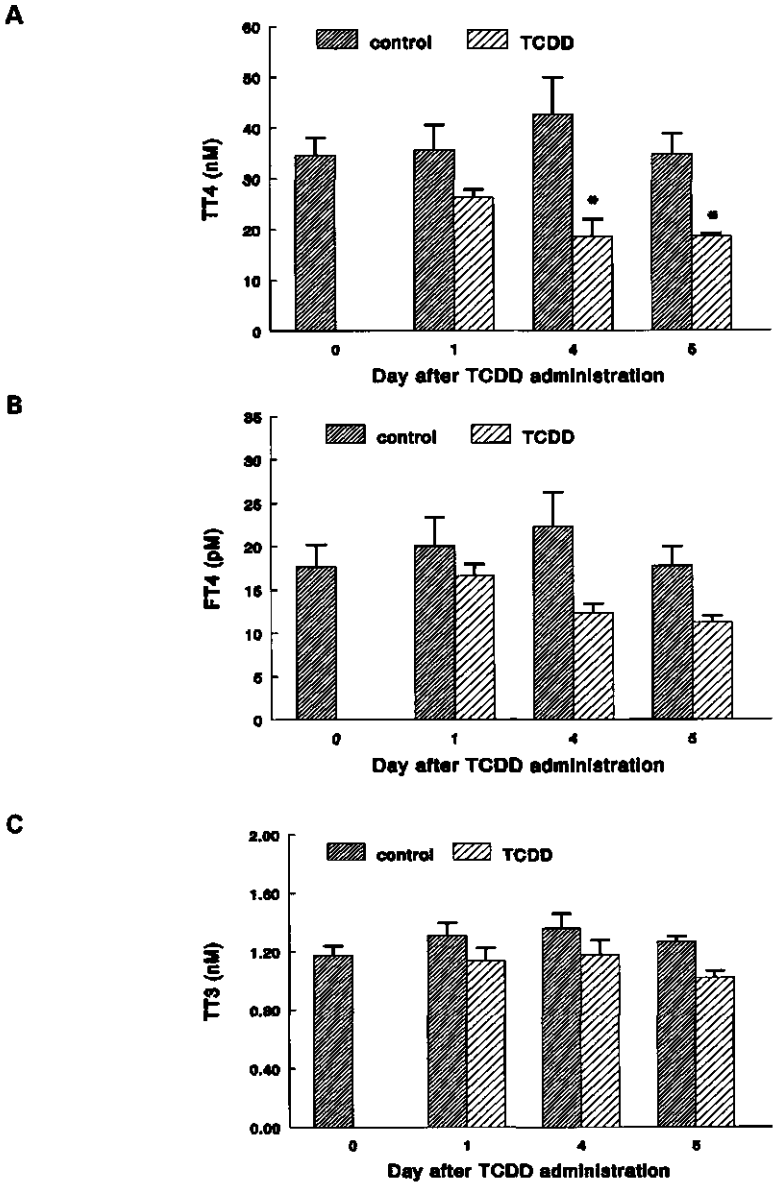

Figure 1 Total plasma thyroid hormone levels of control and TCDD treated rats. Blood samples were collected on 1 day before dosing (day 0), and 1, 4 and 5 days after dosing rats with 25 μg TCDD/kg b.w. or corn oil (n=4). A) total T_4 (nmol/l); B) free T_4 (pmol/l); C) total T_3 (nmol/l). Results are means ± SEM of duplicate measurements. *Indicates values significantly different from controls ($p<0.05$).

Presence of hydroxylated TCDD metabolites in plasma

The decrease in the ratio of TTR/albumin ^{125}I-T_4 ratio may be explained by the presence of compounds in TCDD treated rats, e.g. hydroxy metabolites of TCDD, that can inhibit T_4 binding to TTR *in vitro* (Lans *et al.*, 1993). GC-MS analysis of plasma aliquots of TCDD treated rats revealed the presence of TCDD but no hydroxylated TCDD metabolites in the plasma (data not shown). The MI-scan revealed only TCDD in plasma in a concentration of 0.843 ng/g plasma (ppb). The detection limit for the internal standard 2,3,7,8-[^{13}C]-TCDD was 75 pg, corresponding to 40 pg/g plasma (ppt). If the electron impact response of the Cl_4 and Cl_3 TCDD-metabolites (2-OH-3,7,8-TriCDD and 2-OH-1,3,7,8-TCDD) are assumed to be equal to 2,3,7,8-[^{13}C]-TCDD, the absolute detection limit was 50-100 pg, corresponding to 25-50 pg/g plasma (ppt). Thus the detection limit was low enough to be able to detect levels of TCDD-metabolites if present, representing 5 % of 2,3,7,8-[^{12}C]-TCDD present in plasma.

Hepatic cytochrome P450 levels and EROD activity

Total cytochrome P450 levels were significantly increased in livers of TCDD treated rats to 1.8 times control levels on a mg protein basis and to 2.5 times control values on a total liver content basis (Table 2). Cytochrome P450 1A1 isoenzyme (EROD) activity was concurrently highly induced in TCDD treated rats compared to controls, both on basis of activity/mg protein (212 times control), per nmol total cytochrome P450 (182 times control) and per total liver (497 times control) (Table 2). Liver weight was significantly increased with 28 % in TCDD rats compared to controls (9.71 ± 0.99 vs. 7.59 ± 0.41 g, respectively; $p<0.01$) while body weights were not significantly changed after TCDD exposure: 205.1 ± 11.5 g in controls vs. 198.2 ± 14.2 g in the TCDD treated group.

Table 1 Relative binding of ^{125}I-T_4 to TTR and albumin in plasma of control and TCDD treated rats.

	Ratio of ^{125}I-T_4-TTR / ^{125}I-T_4-albumin	
Time after ^{125}I-T_4 injection (hr)	Control (corn-oil)	TCDD (25 μg/kg)
3	4.2 ± 0.8	4.4 ± 1.0
6	3.9 ± 0.6	3.1 ± 0.3
24	4.6 ± 0.7	2.5 ± 0.4*

Note: for each time point of blood sampling after ^{125}I-T_4 injection, t= 3, 6 and 24 hours, gel profiles were made from plasma of control (n=4) or TCDD-treated rats (n=4). Ratios were calculated as described in Materials and Methods, using ^{125}I-T_4 radioactivity measured on gel profiles as shown in figure 1. Results are means ± SEM. *Indicates values significantly different from control ($p < 0.05$)

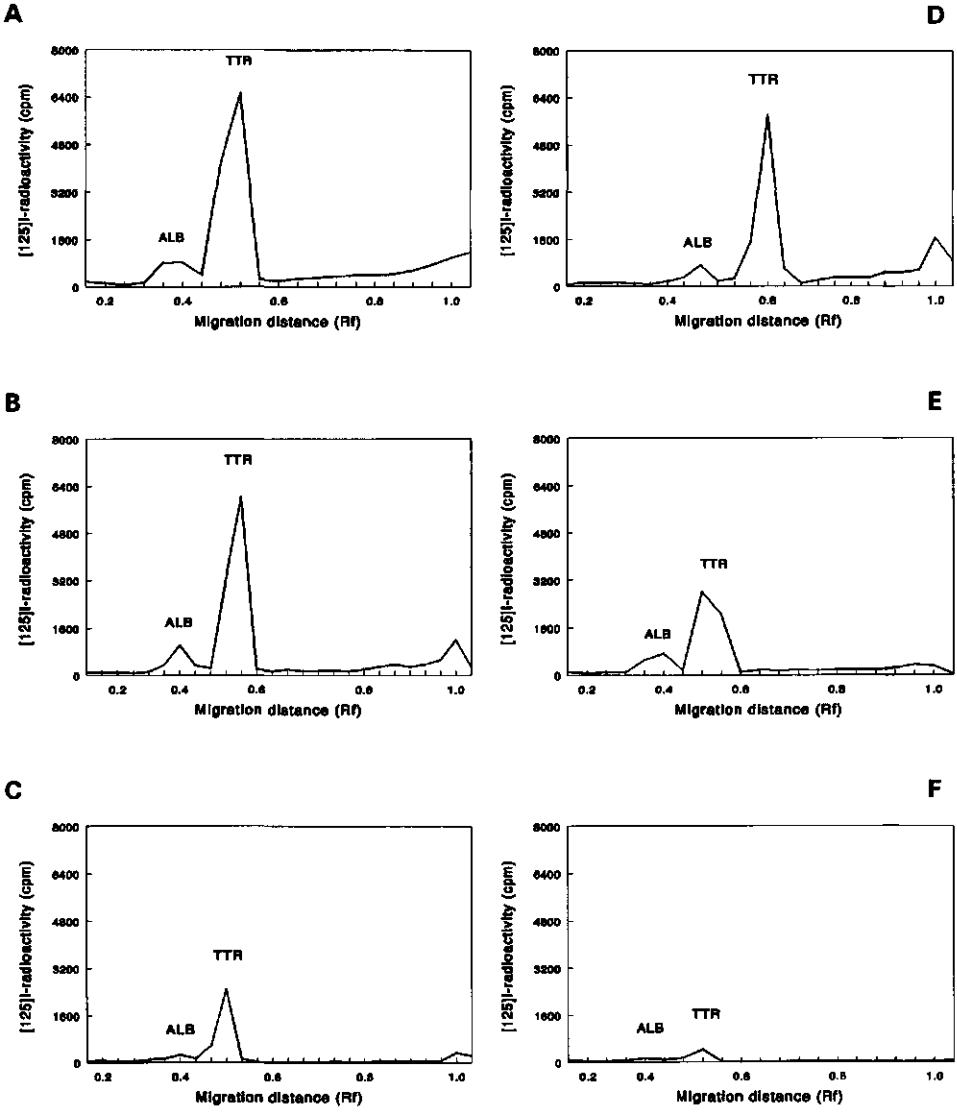

Figure 2 PAGE profiles of ^{125}I-T_4 derived radioactivity bound to plasma proteins as described in Materials and Methods. Transthyretin (TTR) and albumin (Alb) were identified by comparison of migration distances using pure human TTR and BSA and rat serum proteins. Rf values were calculated using the free ^{125}I-T_4 peak as front, Rf is the ratio of migration distance of ^{125}I-T_4 peak vs. front. PAGE profiles are shown of plasma of control, corn-oil treated rats (A: 3hrs, B:6 hrs, C:24 hrs after ^{125}I-T_4 injection) and TCDD-treated (25 μg/kg b.w.) rats (D:3 hrs, E:6 hrs, F:24 hrs after ^{125}I-T_4 injection).

Effects on thyroid hormone metabolism

Hepatic T_4-glucuronidation (T_4-UGT) was significantly increased 3.9 fold control levels (Table 3) on the basis of specific activity. On the contrary hepatic type 1 deiodinase (ID-I) activity was significantly reduced to 82% of control activities (per mg protein) (Table 3). Brain type 2 deiodinase (ID-II) activity was significantly increased to about 2-fold control levels indicating a status of functional hypothyroidism in TCDD treated rats (Table 3).

Table 2 Hepatic cytochrome P450 levels and EROD-activity of control (corn-oil) and TCDD-treated rats (25 μg TCDD/kg).

Cytochrome P450 parameters	Control (n = 4)	TCDD (n = 4)
Cytochrome P450 level (nmol/mg protein)	0.552 ± 0.031	0.991 ± 0.097**
Cytochrome P450 level (nmol/total liver)	64 ± 3	161 ± 35*
EROD activity (nmol RR/min/mg protein)	0.016 ± 0.003	3.390 ± 0.731**
EROD activity (nmol RR/min/nmol P450)	0.022 ± 0.004	4.000 ± 0.864**
EROD activity (nmol RR/min/total liver)	1.31 ± 0.15	651 ± 264*

Note: results are means ± SEM of duplicate measurements. *Indicates values significantly different from control ($p < 0.05$), **Indicates values significantly different from control ($p < 0.005$).

Table 3 Thyroid hormone metabolism enzyme activities of control (corn-oil) and TCDD-treated rats (25 μg TCDD/kg).

Thyroid hormone metabolism parameters	Control (n = 4)	TCDD (n = 4)
T_4-UGT-activity (pmol T_4/min/mg)	1.732 ± 0.125	6.782 ± 1.172*
ID-I-activity (nmol rT_3/min/mg)	97 ± 5	80 ± 6*
ID-II-activity (fmol T_4/hour/mg)	28.90 ± 9.14	57.57 ± 14.79*

Note: hepatic microsomal T_4-UGT activity (pmol/min/mg); hepatic microsomal type-1-deiodinase (ID-I) activity (nmol/min/mg) and brain type-2-deiodinase (ID-II) activity (fmol/hour/mg protein from whole homogenate) are shown. Results are means ± SEM of duplicate measurements. *Indicates values significantly different from control ($p < 0.05$).

Discussion

Exposure of rats to TCCD resulted in distinct decreases in total and free plasma T_4 levels, while T_3 levels were not changed. These plasma thyroid hormone reductions were accompanied by an increased hepatic T_4-glucuronidation which may result in enhanced excretion and a decreased hepatic type 1 (ID-I) deiodination, presumably lowering the conversion of T_4 to active T_3 or inactive rT_3. In addition the TTR/albumin ratio of ^{125}I-T_4 binding in plasma of TCDD exposed rats showed a decrease, which is suggestive for an interference of hydroxylated metabolites of TCDD on T_4 transport by plasma TTR. However, GC/MS analysis revealed that no hydroxylated TCDD-metabolites were present in plasma, despite the highly induced cytochrome P450IAI activities in livers of TCDD exposed rats. TTR plasma levels were not changed by TCDD exposure either, excluding the possibility that plasma T_4 levels were decreased due to disruption of plasma thyroid hormone transport by TCDD exposure.

The decrease in plasma thyroid hormone levels as found in this study has been described in several other studies in which rodents were exposed to TCDD. Administration of TCDD to rats or mice resulted in reduced plasma T_4 levels (Bastomsky, 1977, Potter *et al.*, 1983, Lans *et al.*, 1990, Potter *et al.*, 1986, Roth *et al.*, 1988, Jones *et al.*, 1987, Henry and Gasiewicz, 1986, 1987, Pohjanvirta *et al.*, 1989). Gorski and Rozman (1987) found decreases in total and free T_4 and rT_3 while plasma T_3 and TSH levels did not change in Sprague-Dawley rats exposed to different doses of TCDD, including the dose used in this study (25 μg/kg). Likewise, Henry and Gasiewicz (1987) found a decrease in serum T_4 but not tri-iodothyronine (T_3) in rats. Plasma T_3 levels were reported to be increased (Bastomsky, 1977, Potter *et al.*, 1986), unchanged (Potter *et al.*, 1983, Henry and Gasiewicz, 1987, Lans *et al.*, 1990, Muzi *et al.*, 1989, Gorski and Rozman, 1987, Jones *et al.*, 1987, Roth *et al.*, 1988) or decreased (Pazdernik and Rozman, 1985) after TCDD exposure. These conflicting results on plasma T_3 levels may be attributed to differences in TCCD dose, exposure time and species differences.

The decreased TTR/albumin ratio of ^{125}I-T_4 binding suggests an interference in plasma T_4 transport by TCDD exposure. However we could not find any evidence for the presence of hydroxy metabolites of TCDD in plasma of rats in this study. Therefore the mechanism of interference in plasma T_4 transport is not caused by hydroxy-TCDD metabolites. TCDD, however, was detected in the plasma of exposed rats. Although McKinney and coworkers (1985) suggested that TCDD could be associated to TTR on the basis of structural resemblance to T_4, TCDD did not inhibit T_4 binding to TTR in *in vitro* binding studies (Lans *et al.*, 1993). Hence TCDD is not expected to interfere with plasma T_4 transport in this study. Another possibility would be that TCDD exposure may have enhanced TTR-binding metabolite formation from endo- or exogenous compounds (PCBs, pesticides) already accumulated in the body via diet. The conditions for accelerated metabolite formation are present, since cytochrome P4501A1 activity (EROD), an isozyme known to be involved in hydroxy-metabolite formation of several polyhalogenated arylhydrocarbons, was markedly increased in the TCDD treated rats. This does not, however, explain the lack of difference in the TTR/albumin ^{125}I-T_4 binding ratio at 3 hrs. after ^{125}I-T_4 injection in control and TCDD treated rats.

Since plasma TTR levels were not decreased, the reduced TTR/albumin ^{125}I-T_4 binding ratio seems to indicate some other mechanism of interference with T_4 binding to TTR. No difference was found between control and TCDD rats in the TTR/albumin ^{125}I-

T_4 binding ratio at 3 hrs. after ^{125}I-T_4 injection, while the ratio decreased at 6 hours and significantly at 24 hours after ^{125}I-T_4 injection, suggesting an increased clearance of ^{125}I-T_4 from blood. One possibility would be that the increased hepatic T_4 metabolism and subsequent excretion may have an influence on the bound/free equilibrium of T_4 to its plasma binding proteins. Such a mechanism would have to account for a more rapid dissociation of T_4 from TTR than from albumin, which is not likely to happen, because TTR has an 10-fold higher binding affinity for T_4 than albumin. Since the ^{125}I-T_4 radioactivity in plasma 24 hours after ^{125}I-T_4 injection is much lower than after 3 hrs., and just above detection level, the observed decrease may also be the result of inaccuracy.

Despite the highly induced EROD activity, no metabolites of TCDD could be detected in the plasma. Several reports however, demonstrated that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin is metabolized in mammals to yield a number of hydroxylated products which include, 2-OH-3,7,8-TriCDD, 2-OH-1,3,7,8-TCDD, 1-OH-2,3,7,8-TCDD, other hydroxylated chlorinated dibenzo-p-dioxins and diphenylethers and 4,5-dichlorocatechol (Van den Berg *et al*, 1994, Neal *et al.*, 1984, Poiger and Buser, 1984, Sawahata *et al.*, 1982, Weber *et al.*, 1982, Mason and Safe, 1986). Most of these hydroxylated TCDD metabolites, however, are excreted in bile and faeces as glucuronide conjugates (Ramsey *et al.*, 1982, Weber *et al.*, 1982). Increased UGT activities were also observed in this study, which could have resulted in effective glucuronidation of TCDD-metabolites explaining the absence of these metabolites in plasma in this study.

Hepatic metabolism of T_4 was clearly affected in rats by TCDD treatment in this study. The glucuronidation pathway of T_4 was significantly induced after TCDD exposure. T_4 is glucuronidated by different UDP-glucuronyl-transferase (UGT) isoenzymes, like the bilirubin- and phenol-UGT isoenzymes, while T_3 is glucuronidated by the androsterone-UGT isoenzyme (Visser *et al.*, 1993). It has been reported that TCDD induced UGT activities in the rat 4-7 fold toward *p*-nitrophenol (Rozman *et al.*, 1985b,1987), 1-naphtol (Eltom *et al.*, 1992) and T_4 (Henry and Gasiewicz, 1987) but not or weakly toward testosterone or estrone as substrates (Umbreit *et al.*, 1989, Lucier *et al.*, 1975). In another study exposure of rats to TCDD selectively induced the phenol-UGT activities, but did not change the bilirubin- and androsterone-UGT activity, resulting in increased T_4 glucuronidation, but no change in T_3-glucuronidation (Visser *et al.*, 1993). These findings are similar to the earlier observations of Bastomsky (1977), using the same dose as in this study, who found an increase in biliary excretion of T_4 (mostly T_4-glucuronide) but not T_3, indicating an induction of T_4 but not T_3 glucuronidation. This increase in T_4 glucuronidation could explain the decrease of T_4 plasma levels after TCDD exposure of rats. The in tandem induction of phenol-UGT and cytochrome P450 1A1 activities suggests that both isozymes are regulated via the Ah-receptor (Sutter and Greenlee, 1992).

A second enzyme involved in hepatic T_4 metabolism, type 1 deiodinase (ID-I), was weakly but significantly inhibited by TCDD in this study. Using reverse T_3 as a model substrate for ID-I, only the outer ring deiodination (which converts T_4 into T_3) by ID-I was selectively measured (Köhrle *et al.*, 1991). Inhibition of outer ring deiodination may presumably lower the amount of active T_3 in liver *in vivo* and if severe enough may also result in reduced plasma T_3 levels. This may partly explain why in some studies plasma T_3 is lowered and in others not. Inhibition of ID-I activity by several Ah-receptor agonists, including TCDD, has been reported before (Visser *et al.*, 1993, Eltom *et al.*,

1992, Rickenbacher and McKinney, 1986). Neonatal rats, exposed to TCDD via lactation, showed a significant and sharp decrease in ID-I activity (Adams *et al.*, 1990). In addition exposure of adult rats to 3,3',4,4'-tetrachlorobiphenyl (TCB) resulted in inhibition of ID-I activity (Adams *et al.*, 1990) while it was also observed that hydroxylated TCB metabolites inhibited ID-I activity, *in vitro*. Therefore it was suggested that hydroxylated metabolites of related compounds could also play a role in the *in vivo* inhibition of ID-I activity. Other *in vitro* studies showed only moderate inhibition of ID-I activity by hydroxylated TCDD metabolites (Rickenbacher *et al.*, 1989, Lans *et al.*, 1994). One cannot exclude the possibility that in addition to TCDD also the hydroxylated TCDD metabolites, which may have been formed in the liver, could be partly responsible for the decrease in hepatic ID-I activity *in vivo*.

Decreased hepatic ID-I activity can also be a physiological response to hypothyroidism or low T_4 plasma levels in rats (Kaplan, 1986). Likewise brain type-2-deiodinase (ID-II) activity was increased in this study, suggesting a physiological response to decreased plasma T_4 levels to keep T_3 levels in brain constant (Köhrle *et al.*, 1991). Increased brain ID-II has also been reported in fetal and neonatal rats exposed to 3,3',4,4',5,5'-HCB, that showed severely reduced plasma T_4 levels (Morse *et al.*, 1993a). In this study TCDD exposure of rats caused a decrease in hepatic ID-I activity, an increase in brain ID-II activity and concommitant decreases in plasma TT_4 and FT_4 concentrations, which may indicate that in the TCDD treated rats compensatory mechanisms keep T_3 concentrations in tissue sufficiently high in order to maintain a functional euthyroid status in the rats.

No conclusive evidence on the thyroid status of TCDD-treated rats can be found in the conflicting results reported in *in vivo* studies so far. Effects that indicate a hypothyroid status after TCDD treatment in rats are decreases in serum T_4 levels, free T_4 index, feed intake, growth and increased serum cholesterol concentrations (Bastomsky, 1977, Potter *et al.*, 1983; Potter *et al.*, 1986, Rozman *et al.*, 1985a,b; Moore *et al.*, 1985; Christian *et al.*, 1986; Seefeld *et al.*, 1984; Albro *et al.*, 1978). Hypothyroidism in rats after exposure to TCDD was also suggested by elevated TSH plasma levels and goiters (Bastomsky, 1977). Furthermore Gupta *et al.* (1973) and Rozman *et al.* (1986) found histological changes in the thyroid which may be interpreted as thyroid activation in TCDD treated rats, probably due to lowered plasma T_4 levels. On the other hand effects suggesting an euthyroid state in TCDD-exposed rats are described also by Potter *et al.* (1983, 1986) and Pohjanvirta *et al.* (1989), while Kelling *et al.* (1987) and Roth *et al.* (1988) found contradicting effects on thyroid hormone regulated enzyme activities in rat liver after TCDD exposure.

In conclusion the decreases in plasma T_4 concentrations as found in rats after TCDD exposure in this study, are mainly caused by increased thyroid hormone metabolism. No evidence is found for reduction in binding of T_4 to TTR in plasma by hydroxylated TCDD metabolites or TCDD itself. The changes in plasma thyroid hormone concentrations, with concurrent changes in hepatic and brain deiodination, suggest that compensatory mechanisms in the thyroid hormone regulatory system probably maintain an euthyroid status in the liver and brain tissues of rats exposed to TCDD. The effects of TCDD on the thyroid hormone regulatory system may also be of importance for man, since effects on plasma thyroid hormone concentrations were also found in pregnant woman and newborn infants exposed to background concentrations of PCDDs, PCDFs and PCBs (Koopman-Esseboom et al., 1994).

CHAPTER 7

Structural basis for the binding of hydroxylated polychlorobiphenyl (PCB) metabolites to human transthyretin

Abstract

This is the first detailed description of the binding of a hydroxylated polychlorinated biphenyl (OH-PCB) metabolite to transthyretin (TTR), both by X-ray analysis of a crystal of the OH-PCB-TTR complex and computer assisted graphics modelling studies. The crystal structure of the complex of 4,4'-$(OH)_2$-3,3',5,5'-tetrachlorobiphenyl (diOH-TCB) with human TTR, a plasma thyroid hormone transport protein, was determined and refined for data to 2.7 Å resolution. *In vitro* the diOH-TCB molecule is a potent inhibitor of thyroxine (T_4) binding to TTR (Lans *et al.*, 1993) as well as an inhibitor of type-1 thyroxine deiodinase activity (Lans *et al.*, 1995a). The crystal of the TTR-diOH-TCB complex was isomorphous to native TTR crystals. Interpretation of the difference Fourier electron density maps revealed the mode of binding of diOH-TCB in the T_4 binding site of the TTR tetramer. The molar ratio of diOH-TCB to TTR was between 1:1 and 2:1 as based on the electron densities present in the two independent T_4 binding sites of the TTR tetramer. The diOH-TCB molecule bound deep in the binding channel near the Serine 117 residue. This allowed the 4-OH group of the diOH-TCB molecule to form a hydrogen bond with the oxygen present on the sidechain of the Ser 117 residue, as was clearly shown by the electron dense bridge between the 4-OH group of diOH-TCB and the two paired Ser 117 residues in the channel centre. The diOH-TCB molecule binds in a non-planar conformation, as was expected on the basis of the minimum energy rotation angle of the molecule (McKinney and Singh, 1988). Comparison with the earlier described interaction of T_4 with TTR, showed that the diOH-TCB molecule was bound deeper into the binding channel than T_4. The mode of diOH-TCB binding observed in this cocrystal TTR complex confirms the results from computer assisted graphics modelling studies on the interactions of hydroxylated PCB metabolites with TTR also described in this study.

In these studies it was also shown that hydroxyl groups on the *meta*-positions of PCB metabolites have the potential to form hydrogenbonds with the Ser 117 residues, explaining the similar binding TTR affinities of *para*- or *meta*-hydroxylated PCB metabolites.

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In addition, the computer modelling studies predicted no structural changes of the TTR molecule after binding of either hydroxy-PCB metabolites or pentachlorophenol (PCP). This did not explain the ability of hydroxylated PCB metabolites but not PCP to disrupt the TTR-retinol binding protein complex as found *in vivo* and *in vitro*.

Introduction

Exposure to the environmental pollutants polychlorinated biphenyls (PCBs), can lead to changes in plasma transport and hepatic metabolism of thyroid hormones in experimental animals. One mechanism to explain the PCB-induced decrease in plasma thyroxine (T_4) levels is by competition of hydroxylated PCB metabolites for T_4 binding to transthyretin (TTR), the major plasma thyroid hormone transport protein in rodents (Brouwer *et al.*, 1989, Lans *et al.*, 1995b). The close structural resemblance of these hydroxylated PCB metabolites with T_4 has been shown to result in competition for the T_4 binding site on TTR in *in vitro* binding studies (Rickenbacher et al., 1986, McKinney et al., 1985, Lans *et al.*, 1993, 1994). In these studies structural requirements of hydroxylated PCB metabolites for T_4-TTR binding competition were determined. *Para-* or *meta*-hydroxylation with at least one adjacent chlorine substitution was necessary for strong inhibition of T_4-TTR binding. *Ortho*-hydroxylated PCB metabolites were poor inhibitors of T_4-TTR binding while di-*ortho*-chlorination only weakly decreased the inhibitory potency of *para*-hydroxylated PCB metabolites, probably due to the non-planar conformation. The tested parent PCB compounds did not inhibit T_4-TTR binding *in vitro* (Lans *et al.*, 1993, Lans *et al.*, 1994). *In vivo* exposure of rats and mice to TCB or Aroclor 1254, showed a selective retention of *para*-hydroxy-substituted PCB metabolites in the blood, bound to TTR (Brouwer 1989, Bergman *et al.*, 1994, Morse *et al.*, 1995b). In addition, selective retention of *para-* or *meta*-hydroxylated PCB metabolites were detected in human and seal blood, exposed to environmental levels of PCBs (Bergman *et al.*, 1994).

TTR not only transports thyroid hormones in plasma of rats and mice, but also forms a complex with retinol binding protein (RBP), which transports retinol, eg. vitamin A (Raz and Goodman, 1969). Binding of the hydroxylated 4-OH-3,3',4',5-tetraCB metabolite to TTR in plasma of rats exposed to 3,3',4,4'-tetraCB, diminished RBP-TTR binding which in turn caused decreases in plasma RBP and retinol (Brouwer and Van den Berg, 1986, Brouwer *et al.*, 1988). The interference of this hydroxylated PCB metabolite with TTR-RBP binding may be explained by conformational changes in the TTR molecule upon binding of the PCB metabolite. However, exposure of rats to a related halogenated phenolic compound, pentachlorophenol (PCP), resulted in decreased plasma T_4 levels due to competitive T_4 displacement from TTR, but not in plasma retinol levels (Den Besten *et al.*, 1991). These data suggest a dissimilar effect of PCP and hydroxylated PCBs on T_4-TTR and the TTR-RBP interaction.

In order to better understand the molecular basis of the structural requirements for hydroxylated PCB metabolites to inhibit T_4-TTR binding and also to elucidate possible differences between PCP and hydroxylated PCB metabolites with respect to TTR-RBP dissociation, computer assisted molecular graphics modelling studies were performed and described in this paper. The 4,4'-$(OH)_2$-3,3',5,5'-tetrachlorobiphenyl (di-OH-TCB) molecule is one of the most potent inhibitors of T_4-TTR binding, eg. 5.4 fold more potent than the natural ligand T_4 (Lans *et al.*, 1993). Thus this di-OH-TCB was suitable as an illustrative

model compound for TTR binding of hydroxylated PCB metabolites and related compounds. The three-dimensional structures of diOH-TCB and TTR, which were based on X-ray crystallographic structure analysis (Blake *et al.*, 1978, McKinney and Singh, 1988), were used for modelling their interaction. In addition several other hydroxylated PCB metabolites and parent compounds were modelled and compared with PCP in order to explain the consequences of ligand binding for TTR-RBP interaction and for conformational changes of the TTR molecule.

To obtain more accurate data on the interaction of TTR with hydroxylated PCB metabolites and compare these with the data collected from graphics modelling in this study, cocrystallisation experiments were performed using human plasma TTR with different hydroxylated PCB metabolites including the diOH-TCB. The three dimensional structure of a diOH-TCB-TTR complex has been determined in this study by X-ray diffraction and is refined for data to a 2.7 Å resolution. Comparisons are made between the crystal structure data and the graphics modelling data on the interaction of TTR with the diOH-TCB molecule.

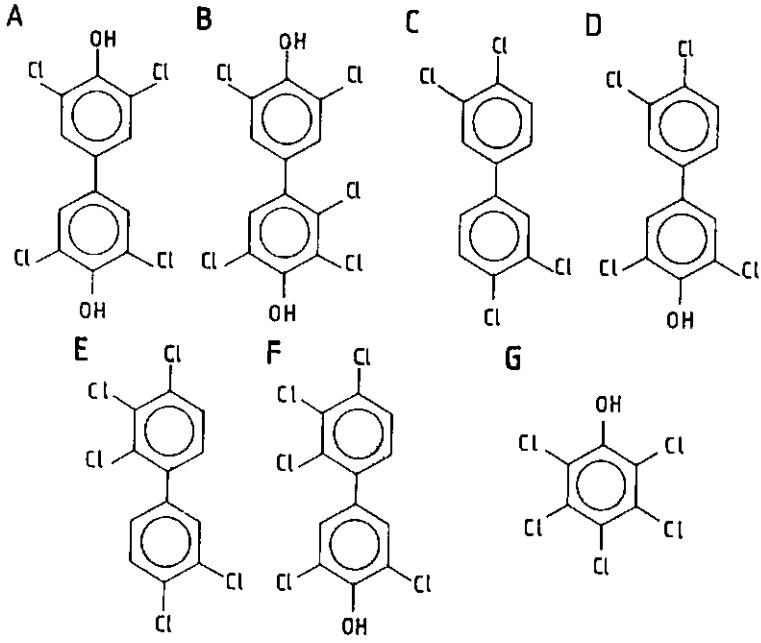


Figure 1 Structures of compounds used in computer assisted graphics modelling studies. Atomic coordinates as available for A:4,4'-$(OH)_2$-3,3',5,5'-tetrachlorobiphenyl (4,4'-diOH-TCB) were used as a basis for the structure of the other hydroxylated PCB structures, B:4,4'-$(OH)_2$-2,3,3',5,5'-pentachlorobiphenyl (4,4'-diOH-PeCB), C:3,3',4,4'-TCB (TCB), D:4-OH-3,3',4',5-tetrachlorobiphenyl (4-OH-TCB), E:2,3,3',4,4'-PeCB (PeCB), F:4'-OH-2,3,3',4,5'-pentachlorobiphenyl (4'-OH-PeCB), and G:pentachlorophenol (PCP).

Materials and methods

Modelling

Modelling compounds

Atomic coordinates as available for 4,4'-$(OH)_2$-3,3',5,5'-tetrachlorobiphenyl (diOH-TCB) (McKinney and Singh, 1988) were used in the graphics modelling study. From this structure other hydroxylated PCB structures, like 4-OH-3,3',4',5-tetrachlorobiphenyl (4-OH-TCB), 4'-OH-2,3,3',4,5'-pentachlorobiphenyl (4'-OH-PeCB), 4,4'-$(OH)_2$-2,3,3',5,5'-pentachlorobiphenyl (4,4'-diOH-PeCB), the parent compounds 3,3',4,4'-tetrachlorobiphenyl (TCB) and 2,3,3',4,4'-pentachlorobiphenyl (PeCB) and pentachlorophenol (PCP) were derived and also used in the graphics modelling studies (Fig. 1). To facilitate comparisons with the OH-PCB metabolites, the OH-group of PCP was defined to be on a 4-position, similar to the *para*-hydroxyl groups on the OH-PCBs.

Graphics modelling system

Graphics modelling was carried out on an Evans and Sutherland PS390 Picture System using the FRODO program suite. Coordinates of the native transthyretin (Blake *et al.*, 1978), obtained from Brookhaven Protein Data Bank were used, while the coordinates of 4,4'-$(OH)_2$-3,3',5,5'-tetrachlorobiphenyl (diOH-TCB) were based on previous crystallographic measurements as described in McKinney and Singh (1988). Atomic distances and contacts of the chlorine and hydroxyl substituents of the modelled compounds with important amino acid residue pairs within the TTR binding site were measured using the FRODO program.

Modelling assumptions

The diOH-TCB molecule has a size of 6.57 by 4.79 by 19.46 Å (x,y,z-axis) with a phenyl ring-ring bond of 1.5 Å. The compound crystallizes in a coplanar state due to intermolecular stacking interactions in the crystal. However, on basis of molecular mechanics calculations the phenyl-rings have an angle of 36° when no intermolecular interactions are present, as was described in McKinney and Singh (1988). This agrees with the minimum energy angles of 35-45° between the phenyl-rings of non-*ortho* substituted biphenyls as found by *ab initio* calculations and gas phase determinations (McKinney *et al.*, 1983, Pedersen *et al.*, 1986). The graphics model of the diOH-TCB-TTR interaction served as a basis for models made of TTR complexes with the other hydroxylated PCB structures (4-OH-TCB, 4'-OH-PeCB, 4,4'-diOH-PeCB, the parent compounds TCB and PeCB and pentachlorophenol (PCP).

Before modelling of diOH-TCB and related test compounds into the binding site of TTR, several assumptions were made. The interaction of the test compounds with TTR was modelled so that their symmetry matched that of the TTR binding site. The two fold axis along the length of a compound were coincident with that of the binding site, while the inner phenyl ring near the channel centre was more or less coplanar with the phenolic ring of T_4. When only one hydroxyl group was present on the compound, it was modelled in a "forward" mode of binding, with the hydroxyl group near the channel centre.

Positions of hydroxyl groups within a distance of ca. 3 Å with hydrogen bonding groups of the amino-acid residues present in the TTR binding channel, could presumably be involved in hydrogen bond formation. Van der Waals interactions could occur between atoms of the modelled compounds and the amino acid residues present in the binding channel, which are 3-4 Å apart.

Cocrystallization experiments

Compounds

The compounds 4-OH-2',3,3',4',5-tetrachlorobiphenyl, 4,4'-$(OH)_2$-2,3,3',5,5'-pentachlorobiphenyl and 4,4'-$(OH)_2$-3,3',5,5'-tetrachlorobiphenyl were synthesized according to the methods described by Bergman *et al.* (1994) and used in the cocrystallisation incubations. Human serum transthyretin was purified according to the method described by Saraiva *et al.* (1983).

Cocrystallisation

Human TTR (20 nM) was incubated with the hydroxylated PCB compounds (200 nM, molar ratio OH-PCB:TTR = 10:1) in 0.165 M sodium-citrate buffer pH 7.0, in 0.25 % (w/v) 1,2,3 heptanetriol (Sigma) for 2 days at 4 °C. Using these incubations of TTR and hydroxylated PCB metabolites, cocrystals were grown using the hanging drop vapor diffusion method, with saturated ammonium sulfate solutions (in 0.165 M sodium-citrate buffer) ranging from 43 to 53 % and pHs ranging from 4.5 to 5.5. A crystal was grown in conditions of 45 % ammonium sulfate solution, pH 4.5.

Data collection

X-ray intensities were collected using an Enraf-Nonius FAST area detector system and graphite-monochromated Cu-kα radiation (λ 1.5418 Å), with a focal spot size of 0.3 x 0.3 mm, from a Nonius FR-571 rotating anode generator operating at 45 kV and 99 mA. A total of 1303 frames 0.15° wide in ϕ were measured with an exposure time of 120 seconds per frame, at two orthogonal crystal orientations from the same crystal. These frames were then processed with the software package MADNES to produce a set of integrated intensities which were scaled and merged together using the CCP4 (Collaborative Computational Project Number 4 (1994), Acta Cryst. D50, 760-763). The crystal diffracted to a maximum resolution of 2.7 Å. In the resolution $27.4 \leq d \leq 3.25$ Å a total of 12539 measurements of 3950 independent reflections were obtained, with a merging R-factor (I) = 0.155 and an average redundancy of 3.2, corresponding to 97.6 % of the theoretically possible within this resolution limit. The total number of observed independent reflections was 4761 corresponding to 70 % of the theoretical value for 2.7 Å resolution.

Crystallographic refinement

The crystals are isomorphous with those of native TTR (Blake *et al.*, 1978) and coordinates of the native protein, obtained from Brookhaven Protein Data Bank, were used to calculate the phases. The initial model, including only residues 10-123 for each monomer, was refined using XPLOR (Brunger, A., 1988, J. Mol Biol. 203, 803-816). First the two monomers were refined as rigid groups and then functional refinement using conjugate gradient minimization was used. Difference Fourier maps were calculated and analysed using FRODO. The major peaks of density were observed in the protein channel along the crystallographic twofold axis. Coordinates obtained from the X-ray diffraction studies on diOH-TCB (McKinney and Singh, 1988) were used to model the PCB molecule in the electron density using FRODO. Conventional positional refinement with two diOH-TCB molecules introduced in the protein channel, resulted in R=23.8 %. Refinement of B-factors for each residue and improvement of the model, using difference Fourier maps and the model-building program FRODO, followed by positional refinement, resulted in R=20%.

Using the program FRODO interatomic distances were calculated between amino

acid residues present in the TTR binding site and the diOH-TCB molecule, in order to compare the actual binding of the diOH-TCB molecule in the T_4 binding site of TTR with the graphics modelling studies of this interaction.

Results

Characteristics of T_4 binding to the TTR binding site

Structural data for TTR, as determined by Blake *et al.* (1978), showed that the TTR tetramer contains a channel that is divided in two symmetry related halves (between monomers A anc C or B and D), each forming a T_4 binding site. Three distinct regions, important for ligand interactions, are formed by the side chains of the symmetry related pairs of amino-acid residues within the binding site (Fig. 2). A hydrophilic region in the centre of the binding channel is formed by the hydroxyl groups of the Ser 112, Ser 115, ser 117 and Thr 119 pairs of residues. The paired residues Leu 17, Thr 106, Ala 108, Leu 110 and Val 121 form a hydrophobic region in the middle of the binding site. Finally, a charged region near the channel entrance was formed by the paired side chains of Lys 15 and Glu 54.

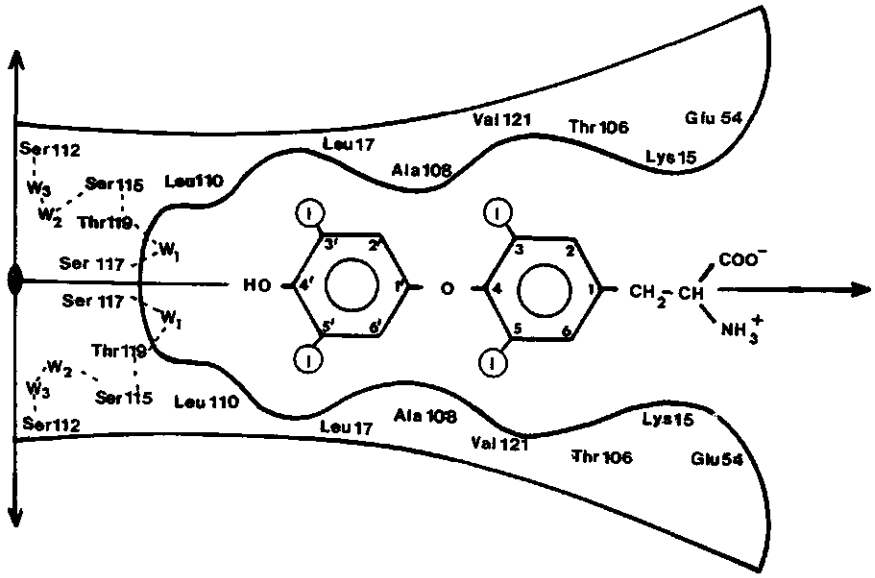


Figure 2 A schematic drawing of the relative positions of the protein side chains and the bound hormone T_4 in the T_4 binding site of TTR. (From De la Paz et al., 1992).

The interaction of the natural ligand T_4 with TTR is described in detail by De la Paz *et al.* (1992). T_4 binds to TTR on a 1:1 molar ratio under physiological conditions. X-ray studies showed that T_4 bound into the TTR molecule along the long axis of the binding channel, with the phenolic hydroxyl group close to the channel centre. The binding site matched the hydrophylic-hydrophobic-ionic character of the T_4 molecule in this way (Blake *et al.*, 1978). In each T_4 binding site six pockets, formed by the grooves between neighbouring beta-strands, are capable of binding an iodine T_4 substituent (Fig. 3); eg. an outer pair of pockets (P1 and P1') and two inner sets the proximal P2 and P2' and the distal P3 and P3'. The 3,5 iodines of the tyrosine ring of T_4 bind to the hydrophobic outer pockets (P1 and P1') while the 3',5' iodines of the phenolic ring bind to the non-symmetry related P2 and P3',respectively (Blaney *et al*, 1982). The phenolic hydroxyl group of T_4 may interact with the Ser 117 and Thr 119 residues either directly or most probably via a water molecule present in the channel centre (De la Paz *et al.*, 1992). At the channel entrance the amino acid side chain of T_4 can interact with the charged Glu 54 and Lys 15 residues, with the carboxylate group playing a more important role than the ammonium group on the T_4 molecule in this interaction (Andrea *et al.*, 1980). In addition to this conventional "forward" mode of binding, also a "reverse" mode of T_4 binding to TTR is described, with the phenolic hydroxyl group of T_4 located near the channel entrance and the amino acid side chain of T_4 located in the channel centre (De la Paz *et al.*, 1992).

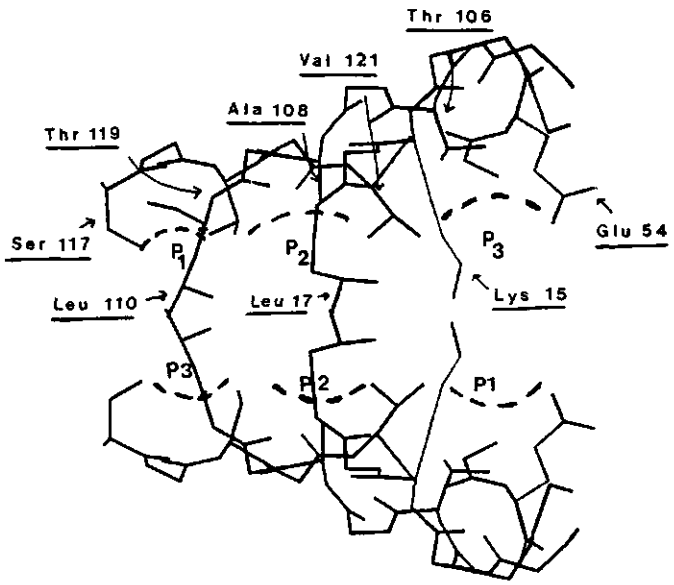


Figure 3 Diagram of the T_4 hormone binding site in TTR showing the side chains which project into the hormone channel. Entry into the channel is on the right. Broken lines represent the three symmetry related pairs of iodine binding pockets; P1, P2 and P3. (From De la Paz et al., 1992)

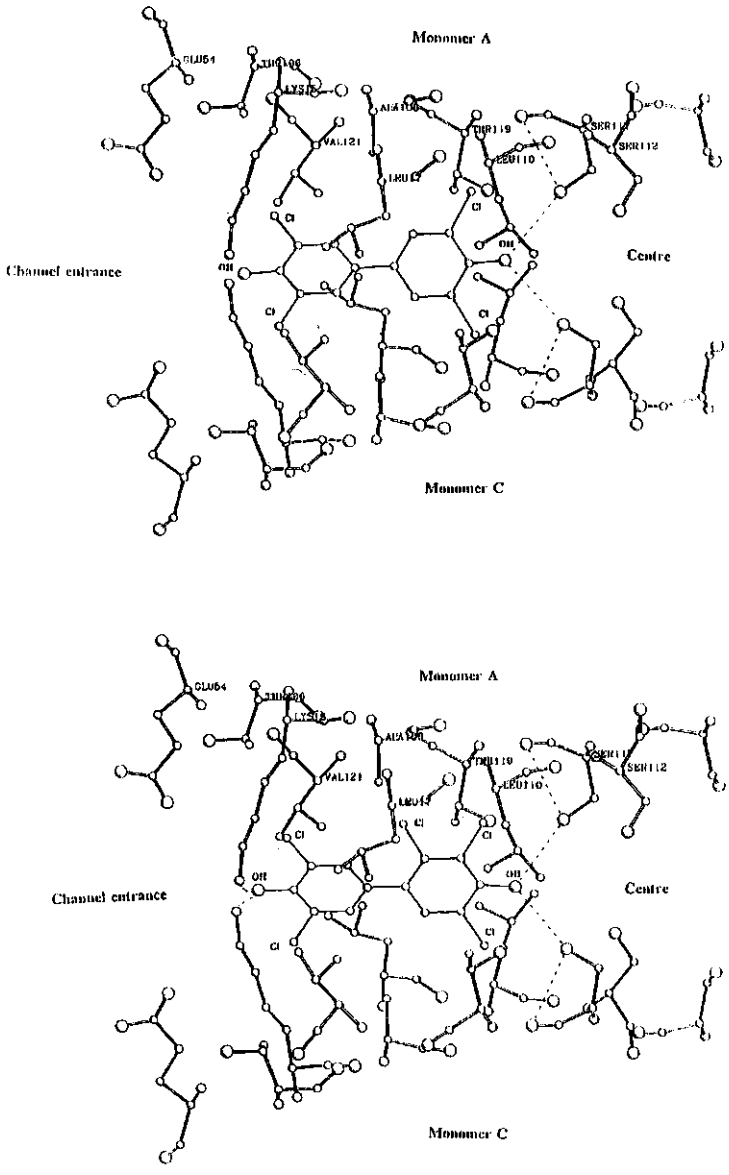
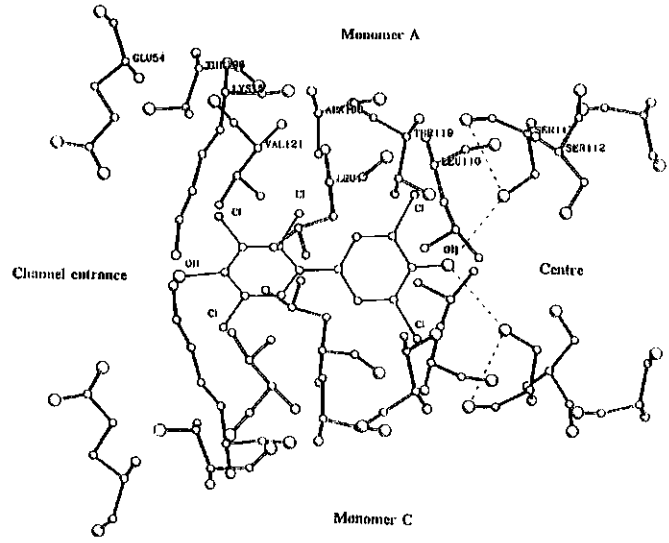
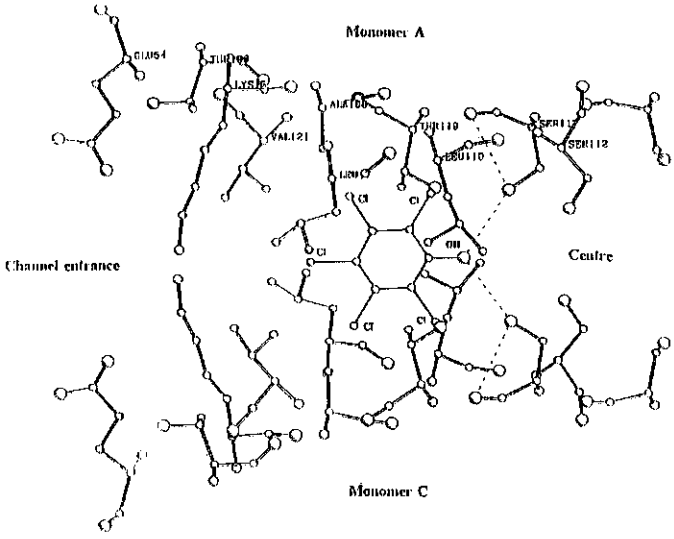


Figure 4 Graphics modelling of the binding of hydroxylated PCB metabolites and PCP in the T_4 binding site of TTR formed by monomers A and C. Hydrogen bond formation is represented by the dotted line. Only side chains of the amino acid residues present in the binding site are shown. No individual hydrogen atoms are drawn. Schematic representations are shown of binding in TTR binding site of: A) 4,4'-$(OH)_2$-3,3',5,5'-tetrachlorobiphenyl (4,4'-diOH-TCB); B) 4,4'-$(OH)_2$-2,3,3',5,5'-pentachlorobiphenyl in forward binding mode (4,4'-diOH-PeCB); C) 4,4'-$(OH)_2$-2,3,3',5,5'-pentachlorobiphenyl in reverse binding mode (4,4'-diOH-PeCB); D) pentachlorophenol (PCP).


Monomer A
GLU54
VAL121
THR119
SER112
Cl
OH
Channel entrance
Centre
Monomer C

C


Monomer A
GLU54
VAL121
THR119
SER112
Cl
OH
Channel entrance
Centre
Monomer C

D

Table 1 Interatomic distances (Å) of 4,4′-$(OH)_2$-3,3′,5,5′-tetrachlorobiphenyl with amino acid residues of monomers A and C of the TTR tetramer forming a T_4 binding site, as collected by graphics modelling (Fig. 4a).

Substituent position		3 Cl	4 OH	5 Cl	3′ Cl	4′ OH	5′ Cl
		Interatomic distance (Å)					
Residue	Monomer						
O-Ser 117	A	3.41	3.28				
	C		3.27	3.41			
O-Thr 119	A	3.40	4.55				
	C		4.54	3.38			
C-Leu 110	A	5.21	3.84				
	C		3.86	5.28			
N-Lys 15	A				4.36	2.20	
	C					2.40	4.66
N-Glu 54	A					5.36	
	C					5.23	

Table 2 Interatomic distances (Å) of 4,4′-$(OH)_2$-2,3,3′,5,5′-pentachlorobiphenyl (forward mode of binding) with amino acid residues of monomers A and C of the TTR tetramer forming a T_4 binding site, as collected by graphics modelling (Fig. 4b).

Substituent position		2 Cl	3 Cl	4 OH	5 Cl	3′ Cl	4′ OH	5′ Cl
		Interatomic distances (Å)						
Residue	Monomer							
O-Ser 117	A		3.35	3.15				
	C			3.13	3.35			
O-Thr 119	A		5.06	4.60				
	C			4.55	5.07			
C-Leu 110	A		3.82	3.78				
	C			3.82	3.69			
N-Lys 15	A						2.37	
	C						2.35	
C-Ala 108	A	3.12						

Table 3 Interatomic distances (Å) of 4,4'-$(OH)_2$-2,3,3',5,5'-pentachlorobiphenyl (backward mode of binding) with amino acid residues of monomers A and C of the TTR tetramer forming a T_4 binding site, as collected by graphics modelling (Fig. 4c).

Substituent position		3' Cl	4' OH	5' Cl	2 Cl	3 Cl	4 OH	5 Cl
		Interatomic distances (Å)						
Residue	Monomer							
O-Ser 117	A	3.41	3.28					
	C		3.27	3.41				
O-Thr 119	A	3.40	4.55					
	C		4.54	3.38				
C-Leu 110	A	5.21	3.84					
	C		3.86	5.28				
N-Lys 15	A						2.17	
	C						2.31	
C-Ala 108	A				2.30	4.40		
	C							4.28

Table 4 Interatomic distances (Å) of pentachlorophenol (PCP) with amino acid residues of monomers A and C of the TTR tetramer forming a T_4 binding site, as collected by graphics modelling (Fig. 4d). Substituents positions are labeled comparable to OH-PCBs (Table 1,2,3), with OH-group on 4-position.

Substituent position		1 Cl	2 Cl	3 Cl	4 OH	5 Cl	6 Cl
		Interatomic distances (Å)					
Residue	Monomer						
O-Ser 117	A			3.07	3.04		
	C				3.01	2.99	
O-Thr 119	A			4.73	4.59		
	C				4.64	4.84	
C-Leu 110	A			3.91	3.79		
	C				3.72	3.79	
C-Ala 108	A		2.93				
	C						3.14
C-Leu 17	A	3.78					
	C	4.28					

Graphics Modelling of OH-PCBs and PCP binding to TTR

The interatomic contacts of chlorine and hydroxyl substituents of hydroxylated PCBs and PCP with amino acid residues in the TTR binding site as found by graphics modelling, are listed in Tables 1-4. The interaction of the symmetric 4,4'-$(OH)_2$-3,3',5,5'-tetrachlorobiphenyl (diOH-TCB) molecule with TTR was used as the reference model, with the 4-OH group of the diOH-TCB molecule located in the channel centre (Fig. 4A, Table 1). The 4,4'-$(OH)_2$-2',3,3',5,5'-pentachlorobiphenyl (4,4'-diOH-PeCB) was modelled in both possible modes of binding, with the least chlorinated phenyl-ring and 4-OH group in the channel centre (forward, Fig. 4B, Table 2) or towards the channel entrance (reverse, Fig. 4C, Table 3). The monohydroxylated PCB metabolites and PCP (Fig. 4D, Table 4) were modelled in the binding site in a forward binding mode, again meaning the OH group was directed towards the channel centre. Atomic distances or contacts of chlorine and hydroxyl substituents of the other modelled compounds were similar to the analogous substituents on the diOH-TCB and diOH-PeCB and not separately stated (data not shown).

The modelling of the compounds along the binding channel axis and with the inner phenyl ring coplanar to the inner ring of T_4, allows the diOH-TCB to bind deep in the binding pocket of TTR (Fig. 2). This position in the channel centre of the 4-OH group present on the di-OH-TCB molecule makes it possible to interact and presumably form a hydrogen bond with the O-group of the paired Ser 117 residues (3.27 - 3.28 Å, Table 1). No interaction is expected of the 4-OH group with the hydrophobic Leu 110 residues regardless the close distance (3.84-3.86 Å, Table 1).

The 4'-OH substituent of di-OH-TCB shows very close contacts with the N atom of the Lys 15 residue (2.20 - 2.40 Å), which may result in hydrogen bond formation (Table 1; Fig. 4A). The flexible side chain of Lys 15 may assume a different conformation upon ligand binding, similar to the conformational change when T_4 is bound in the binding site (De la Paz *et al.*, 1992). The close contact of 4'-OH group of the OH-PCB compounds can only lead to polar interactions between this OH group and the N-group of the Lys 15 residue if the OH group of diOH-TCB molecule is ionized. No close contact was found between the 4'-OH group with the charged Glu 54 residues near the channel entrance (5.23 Å, Table 1).

The chlorines substituted on the *meta* (3 and 5) positions of the diOH-TCB molecule are within close distance with the oxygen atoms present on Ser 117 (3.41 Å) and Thr 119 residues (3.38-3.40 Å) which could result in electrostatic or charge transfer interactions (Table 1). The 3 and 5 chlorines fitted well into the T_4 iodine binding pockets, also called the P3 and P3' pockets, which are surrounded by residues Ala 108, Ala 109, Leu 110, Ser 117, Thr 118 and Thr 119 (Fig. 4A; Table 1). The chlorine substituents present on the 3' and 5' position of the diOH-TCB molecule are not within close distance with the Lys 15 residue to allow possible interactions (4.36 and 4.66 A, Table 1).

The chlorines substituted on position 3' and 5' positions of the diOH-TCB and 4,4'-diOH-PeCB compounds (located near the channel entrance) may fill the P1 and P1' T_4 iodine binding pockets of the binding site formed by residues Lys 15, Leu 17, Thr 106, Ala 108 and Val 121, or will fit into the P2 and P2' T_4 binding pockets (Fig. 2).

The ring-ring bond region of the diOH-TCB is located in the hydrophobic region of the TTR binding site formed by the paired residues Leu 17, Thr 106, Ala 108, Leu 110 and Val 121 (Fig. 2), however no limiting contacts are made with the residues in this area.

Like diOH-TCB, the 4-OH group of the 4,4'-$(OH)_2$-2,3,3',5,5'-PeCB molecule can interact with the paired Ser 117 residues (3.13-3.15 Å) present in the channel centre when

bound in forward mode (Fig. 4B, Table 2). Likewise the chlorines on positions 3 and 5 can bind into the P3 and P3' T_4 binding pockets while the 3' and 5' chlorines may fit in the P2 and P2', or P1 and P1' T_4 binding pockets. The 4'-OH-group present near the channel centre when diOH-PeCB is bound to TTR in forward mode may also interact with the Lys 15 residue (2.35-2.37 Å) analogous to the 4'-OH group of diOH-TCB (Fig. 4A). In reverse mode of binding similar interactions of the 4-OH and the 4'-OH group can be made as resp. the 4'-OH and 4-OH group in forward binding mode (Table 3, Fig. 4C). The *ortho*-chlorine substituent (2-position) of diOH-PeCB bound forwardly shows close contact (3.12 Å) with the Ala 108 residue, which is also present in the P2 and P2' binding pockets (Table 2, Fig. 4B). When, in reverse mode of binding, the *ortho*-chlorine substitution is present on the outer phenyl ring (2'-position), a very close contact (2.3 Å) is made with the Ala 108 residue, which may result in a repelling force due to steric hindrance and therefore possible a lower binding to TTR (Table 3, Fig. 4C).

Pentachlorophenol also shows the ability for hydrogen bond formation of the hydroxyl group with the O atoms of both Ser 117 residues present in the binding channel (3.01 - 3.04 A, Table 4, Fig. 4D). Pentachlorophenol does not have close contacts with Lys 15 or Glu 54 residues near the binding channel entrance, because the axial-length of PCP is too short to be positioned close enough to the N-lys 15, or N-Glu 54, when an interaction of the 4-OH group with the Ser 117 residue is assumed. In addition the Cl substituents of PCP would prevent a polar interaction with Lys 15 or Glu 54, even when it would meet the minimal distances required. Chlorines substituted on the 1, 2 and 6 position of PCP are in

Figure 5 Fourier electron density maps (blue) of the two T_4 binding sites of TTR as calculated from X-ray analysis for the diOH-TCB-TTR cocrystal to a resolution of 2.7 Å, showing the presence of 2 diOH-TCB molecules. The centre of the binding channel is located in the middle of the picture.

close contact with Ala 108 (2.93-3.14 Å) and Leu 17 (3.78-4.28 Å, Table 4), but still seemed to fit easily into the T_4 binding site. They likely occupy the P3 and P3' and part of the P2 and P2' T_4 binding pockets, which are surrounded by the Lys 15, Leu17, Ala 108, Ala 109 and Leu 110 residues, and the hydrophobic region of the binding site formed by residues Leu 17, Thr 106, Ala 108, Leu 110 and Val 121 (Fig. 3; Table 2).

When parent PCB compounds were modelled into the TTR binding channel (data not shown), it was evident that the lack of hydroxyl groups excluded the two interactions important for binding to TTR; eg. the possible hydrogen bond formation of the 4-OH group with Ser 117 in the channel centre and possible charged interactions of 4'-OH with the Lys 15 residue near the channel entrance. Although the atomic distance of chlorines on the *para* (4) position of PCB molecules to the O atom of Ser 117 will be equal since the chlorine and hydroxyl group are almost similar in size, hydrogen bond formation is not expected due to the differences in chemical properties of the OH and Cl groups. Likewise a close contact but no polar interaction will be formed with the Lys 15 or Glu 54 residues when a chlorine substituent is present on the 4' position of PCB molecules located near the channel centre.

The graphics modelling of the hydroxylated PCB metabolites in the T_4 binding site of TTR, as performed in this study does not clearly reveal the actual conformation of the OH-PCBs assumed upon binding to TTR, because modelling is partly based on assumptions made beforehand. Especially for the rotational angle of the phenyl-rings, which was assumed to be 36°, it cannot be excluded that the compounds may adapt a coplanar state or different rotational angle upon binding to TTR. In addition the possible hydrogen-bond interaction between the 4-OH group and Ser 117, is a consequence of modelling the OH-PCB metabolites deep inside the binding channel. For precise information on the conformation and location of the OH-PCB metabolites in the TTR binding channel after binding, X-ray structure analysis of cocrystallisation experiments was performed.

X-ray crystallographic structure analysis

The co-crystallisation experiments resulted in a crystal of the TTR-diOH-TCB complex usable for X-ray diffraction analysis. The structure of the diOH-TCB-TTR complex is isomorphous to the native TTR structure (Blake *et al.* 1978). The crystals are orthorombic, with space groups $P2_12_12$ with A=42.73 Å, B=85.66 Å and C=65.08 Å. In this study no significant changes in overall TTR and binding site structure were observed upon diOH-TCB binding. However, the resolution of the complex is not good enough to precisely locate all the atoms of the protein side chains, so residues 1-9 and 124-127 were not included, analogous to studies on native TTR (Blake *et al.*, 1978), which include only residues 10-123. A general view of the TTR molecule is shown on the front cover of this thesis, with the two hydroxylated PCB molecules (white) present in the T_4 binding sites and with the symmetric alpha-helices (red), beta-sheets (green) and connecting peptide chains (yellow) that form the tertiary structure of the TTR molecule. In figure 5 the location of the calculated and refined electron density maps to a resolution of 2.7 Å on the TTR molecule are shown in blue. The electron density corresponding to the diOH-TCB was clear so the exact location of the molecule in the T_4 binding site of TTR (formed by monomers A and C or B and D) could be determined. Electron density maps showed the presence of a hydroxylated PCB molecule in each T_4 binding site of TTR, although in the diffraction data the densities were much better for one of the diOH-TCB molecules present in the T_4 binding sites. This indicates that diOH-TCB is bound to TTR in a molar ratio between 1.5:1 and 2:1 (diOH-TCB:TTR).

Table 5 Interatomic distances ($\leq$ 4 Å) of 4,4'-$(OH)_2$-3,3',5,5'-tetrachloro-biphenyl with amino acid residues of resp. A and C monomers of the TTR tetramer forming a T_4 binding site, as collected using FRODO from X-ray crystallographic structure analysis. (--- = hydrogen bond formation)

Monomer A/residue	atom	(Å)	Monomer C/residue	atom	(Å)
Lys 15 -CD	C4B	3.9513	Lys 15-CD	O4B	3.8285
Lys 15 -CD	O4B	3.4472	Lys 15-CE	O4B	3.629
Lys 15 -CE	C4B	3.6410	Leu 17-CD1	C1B	3.9320
Lys 15 -CE	C5B	3.9152	Leu 17-CD1	C2B	3.3707
Lys 15 -CE	O4B	3.2417	Leu 17-CD1	C3B	3.9216
Lys 15 -CE	L5B	3.8046	Ala 108-CB	C2A	3.5262
Leu 17 -CD1	C6B	3.7495	Ala 108-CB	C5B	3.9660
Ala 108-CB	C6A	3.8237	Ala 108-CB	C6B	3.4099
Ala 108-CB	C2B	3.5678	Ala 108-C	Cl3	3.6571
Ala 108-CB	Cl3B	3.9522	Ala 108-O	Cl3A	3.1297
Ala 109-CA	Cl5A	3.9854	Ala 109-N	Cl3A	3.9864
Ala 109-C	Cl5A	3.8343	Ala 109-CA	Cl3A	3.8742
Leu 110-N	Cl5A	3.6431	Ala 109-C	Cl3A	3.9727
Leu 110-CB	Cl5A	3.7363	Leu 110-N	Cl3A	3.6732
Leu 110-CD2	O4A	3.8161	Ser 117-CB	O4A	3.7225
Ser 117-OG	C4A	3.8207	Ser 117-OG	O4A-----	2.7815
Ser 117-OG	C5A	3.9446	Ser 117-C	Cl3A	3.2700
Ser 117-OG	Cl5A	2.8846	Ser 117-O	Cl3A	2.9324
Ser 117-OG	O4A-----	2.8683	Thr 118-N	Cl3A	3.3829
Ser 117-C	Cl5A	3.5666	Thr 118-CA	Cl3A	3.2137
Ser 117-O	Cl5A	3.0907	Thr 118-C	Cl3A	3.5246
			Thr 119-N	Cl3A	3.7994
			Thr 119-OG1	C3A	3.9583
			Thr 119-OG1	Cl3A	3.6128

A detailed representation in figure 6 clearly shows the hydroxylated diOH-TCB molecule, located deep in the T_4 binding site of TTR formed by monomers A and C. The 4-OH-group of the diOH-TCB undoubtedly forms a hydrogen bond with the oxygen present on the Ser 117 residues in the centre of the binding channel, as confirmed by the presence of electron density between the 4-OH-group and the Ser 117 side chains (Fig. 6). The O atoms from the paired Ser 117 residues make close contacts with the O atom of the 4-OH group and the 3 and 5 Cl atoms on the diOH-TCB molecule (Table 5). The electron density map covers the diOH-TCB molecule in a way that verifies the coordinates and size of the diOH-TCB molecule as described by McKinney and Singh (1988). A view into the T_4 binding channel towards the centre of TTR, with the non-planar diOH-TCB molecules (white) located alongside each other (back cover of thesis) also reveals a non-planar conformation of the hydroxylated PCB, although no *ortho* chlorine-substitutions are present on the diOH-TCB molecule. The two phenyl-rings of the diOH-TCB are lined up and shown in the centre with a rotational angle of ~45°. So upon binding to TTR, the diOH-TCB molecule adopts a non-planar conformation as was predicted by molecular mechanics calculations (McKinney and Singh, 1988).

In figure 7 a schematic representation of the diOH-TCB molecule in the T_4 binding site of TTR shows the most important amino acid residues for ligand binding. The interatomic coordinates as calculated from this co-crystal complex structure as stated in Table 5, show general agreements with the interatomic coordinates collected from the

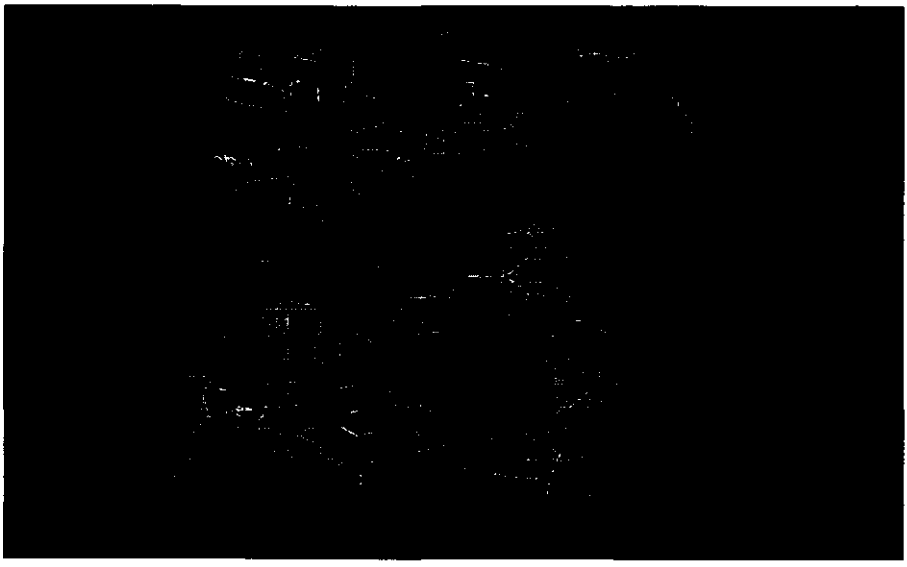


Figure 6 Fourier electron density map (blue) of a bound diOH-TCB molecule present in the T_4 binding site of TTR, formed by monomers A and C. Hydrogen bonds are present between the 4-OH-group of diOH-TCB (red) and the O-atom of the paired Ser 117 residues (red) in the form of electron dense material (blue).

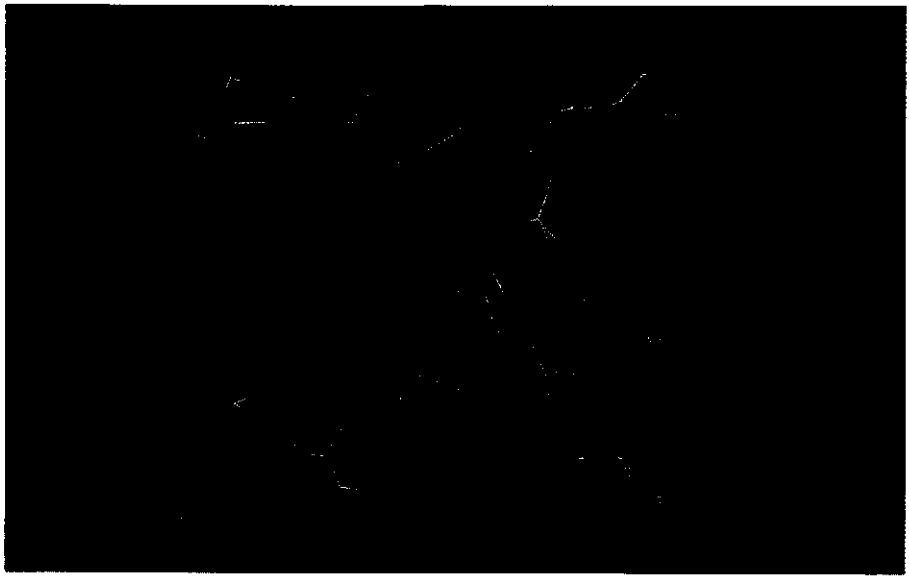


Figure 7 Schematic representation of the location of the diOH-TCB molecule, as based on X-ray diffraction analysis of a cocrystal, within the binding site of TTR formed by monomers A and C, here only showing the peptide backbone and amino acid side chains of the paired amino acid residues Lys 15, Leu 17, Leu 110 and Ser 117.

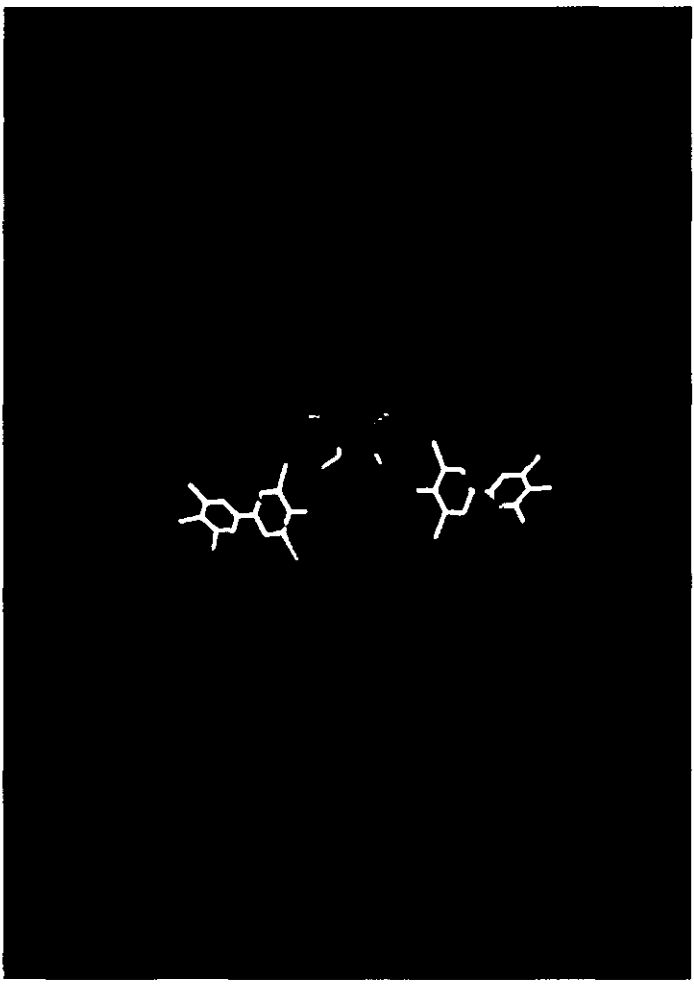


Figure 8 Detailed view into the T_4 binding channel centre of TTR with both two diOH-TCB molecules (white) and the Serine 117 residues of the upper TTR monomers (light blue), and with the symmetric alpha-helices (red), beta-sheets (green) and connecting peptide chains (yellow) that form the tertiary structure of the TTR molecule.

graphics modelling study with the diOH-TCB molecule (Table 1, Fig. 4A). Due to the 2.7 Å resolution small differences in interatomic distances collected from the co-crystallographic experiments and data collected from the graphics modelling studies can only be compared with caution. The most notable differences are found in the distances between the 4'-OH group of diOH-TCB and the N-Lys 15 residue, which is 2.17-2.40 Å in the graphics modelling study and ~ 3 Å in the crystal complex. In figure 8 a detailed view is given into the centre of the binding channel.

Discussion

Computer assisted graphics modelling and co-crystallisation experiments were used to better understand the molecular characteristics of the binding of hydroxylated PCB metabolites to the T_4 binding pocket of TTR. Results from the modelling studies indicate that the tested hydroxylated PCB metabolites and the related compound pentachlorophenol fitted in the TTR binding pocket accurately. These fits were based on the modelled interaction of 4,4'-$(OH)_2$-3,3',5,5'-TCB (diOH-TCB) with TTR, using the coordinates of both ligand and protein derived from X-ray crystallographic analysis (McKinney and Singh, 1988, Blake *et al.*, 1978). In addition X-ray crystallography of a cocrystal revealed the structure of a TTR-diOH-TCB complex, confirming the interactions between hydroxyl- and chlorine-groups present on the diOH-TCB and amino acid residues present in the binding channel as found in the graphics modelling studies.

The tested hydroxylated PCB metabolites which are smaller and more planar than the natural ligand T_4, were able to bind deeper into the binding channel compared to the natural ligand T_4 due to the smaller halogen substituents. This resulted in direct hydrogen bond formation of the *para*-hydroxyl (4-OH) group of the diOH-TCB with the O-atom of the Ser 117 residues, present in the channel centre. When T_4 is bound to TTR, the phenolic hydroxyl group of T_4 presumably only interacts indirectly with the Ser 117 residue through a water molecule (De la Paz *et al.*, 1992).

Co-crystallisations of milrinone (Wojtczak *et al.*, 1993), 3',5'-dibromo-2',4,4',6-tetrahydroxybromoaurone (Ciszak *et al.*, 1992) and 3,3'-diiodo-L-thyronine (Wojtczak *et al.*, 1992) with TTR, indicated that these ligands were also able to bind deeper in the TTR binding site than the natural ligand T_4, which allowed hydrogen bond formation of the 4-OH groups of these compounds with the Ser 117 residue, similar to our modelling studies and crystallographic data.

In earlier studies the structural requirements of hydroxylated PCB metabolites for binding to TTR were investigated using *in vitro* T_4-TTR binding competition assays (Lans *et al.*,1994, 1993, Brouwer *et al.*, 1990). *Para*- or *meta*-hydroxylation and at least one adjacent chlorine substitution on the PCB metabolites were necessary for strong inhibition of T_4-TTR binding. These requirements are consistent with the important role of the hydroxyl and chlorine substitutions on hydroxylated PCB metabolites for close interaction with the TTR binding channel as observed in this study using computer assisted graphics modelling and X-ray crystallographic structure analysis. *Para*-hydroxylation of the phenyl-rings resulted in possible interactions of the hydroxyl group with resp. Ser 117 near the channel centre, or Lys 15 near the channel entrance. *Meta*-hydroxylation of the phenyl-ring located near the channel centre could also lead to hydrogen-bond interactions with Ser 117 because short distances were found in this study between *meta*-substitutions on the phenyl-ring and the Ser 117 residue. These findings are in line with the strong T_4-TTR displacement potency of *meta*-hydroxylated PCB metabolites.

Meta-chlorine substitutions, adjacent to the hydroxyl group on the phenyl-ring located in the channel centre fitted well in the P3 and P3' T_4 iodine binding pockets of the binding channel. *Ortho*-chlorine substitutions on the hydroxylated PCB metabolites tested in the graphics modelling studies did not clearly affect the binding in the T_4 binding channel, although di-*ortho*-chlorine substitution weakly lowered the TTR binding affinity of hydroxylated PCB metabolites (Lans *et al.*, 1993). This could, however, be the result of steric hindrance of the *ortho*-chlorine substitutions on both phenyl-rings with the Ala 108

residue in the channel, as was found by graphics modelling of the diOH-PeCB molecule.

Since the ether bridge of T_4 is not required for binding to TTR (Rickenbacher *et al.*, 1986), the linear polychlorinated biphenyl structures tested in this study could easily bind to TTR, with the phenyl-ring bond fitting into the hydrophobic region of the binding site. However, the rigid 3,3',5,5'-tetrachloro-diphenoquinone structure showed weaker binding (Ka's) than di-OH-TCB (Pedersen *et al.*, 1986), which may indicate that the C-C bond which allows rotation of the phenyl-rings, may be important for the high binding affinities of the hydroxylated PCBs for TTR.

The inability of parent PCB compounds to bind to and displace T_4 from TTR as found by Brouwer *et al.* (1990) and Lans *et al.* (1993, 1994) was confirmed by the graphics modelling fits because non-hydroxylated, parent compounds like TCB and PeCB lack the possibilities for hydrogen bond interactions with the Ser 117 residues present in the binding channel centre. This could result in lower binding affinity of parent PCB compounds compared to the *para*-hydroxylated PCB metabolites. The increase in binding affinities of non-, mono-, and di-*para*-hydroxylated PCB molecules for TTR with increasing numbers of hydroxyl-substituents as found *in vitro* (Lans *et al.*, 1993), may be caused by the additional possibility of interactions of hydroxyl groups with N-Lys 15 next to interactions with Ser 117 that may strengthen binding to TTR.

Pentachlorophenol (PCP) was also found to inhibit T_4-TTR binding *in vitro* (Van den Berg, 1990; Den Besten *et al.*, 1991), which was confirmed in this study where a close fit of the PCP molecule within the TTR binding site was observed, with formation of a hydrogen-bond between the hydroxyl group of PCP with the Serine 117 residues and the chlorines substituted adjacent to the hydroxyl group of PCP fitting in the P3 and P3' and part of the P2 and P2' iodine binding pockets of the T_4 binding site of TTR.

Other graphics modelling studies of the related compounds 3,3',4,4',5,5'-hexaCB and 2,2',4,4',6,6'-hexaCB by Rickenbacher and coworkers (1986), showed a good fit of these molecules in the TTR binding channel. However, by superimposing the inner phenyl ring of these compounds on the place of the inner-phenyl ring of T_4 in the binding channel, these compounds were fitted more towards the channel entrance. The authors concluded that the 3,3',4,4',5,5'-hexaCB was able to bind to TTR while 2,2',4,4',6,6'-hexa-CB showed no good binding due to steric hindrance in the TTR binding pocket (Rickenbacher *et al.*, 1986). However, in the same study it was suggested that *para*-hydroxylation with adjacent *meta*-chlorination of the biphenyls led to the strongest binding to TTR, on basis of *in vitro* T_4-TTR binding assays with related compounds. In another study 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and 2,3,7,8-tetrachloro-dibenzofuran (TCDF) were modelled into the binding site of TTR (McKinney *et al.*, 1985). Only the adipamide derivatives of TCDD and TCDF were tested in *in vitro* binding assays, showing possible binding to TTR. In a earlier study from our laboratory TCDD was found not to inhibit T_4-TTR binding, suggesting no binding to TTR as was found for the other non-hydroxylated PCBs (Lans *et al.*, 1993). Hydrogen-bond interactions may therefore be important requirements for strong binding of PCBs and related compounds to TTR, as was found in this study.

The selective binding of hydroxylated PCB metabolites to TTR has also been observed *in vivo*. Exposure of rats and mice to TCB (Brouwer and Van den Berg, 1986, Brouwer, 1989) resulted in the selective retention of the 4-OH-3,3',4',5-TCB metabolite, bound to TTR in plasma and was indicated to be at least partly responsible for the decrease in plasma T_4 levels by inhibition of T_4-TTR binding. This metabolite was also found to be

selectively retained in rat fetuses after TCB exposure of pregnant rats (Morse *et al.*, 1995b). Another PCB congener 2,3,3',4,4'-PeCB caused a selective retention of a hydroxylated metabolite in plasma of rats exposed to this compound (Klasson Wehler *et al.*, 1993). In plasma of rats exposed to Aroclor 1254, a commercial PCB mixture, the *para*-hydroxylated, *meta*-chlorine substituted PCB metabolite 4-OH-2,3,3',4',5-PentaCB was mainly found (Bergman *et al.*, 1994, Lans *et al.*, 1994), while this metabolite also accumulated in fetuses after Aroclor 1254 exposure of pregnant rats (Morse *et al.*, 1995d). In addition environmental exposure to PCBs caused the selective retention of mainly 4-OH-2,3,3',4',5-pentaCB in seal plasma and both 4-OH-2,3,3',4',5-pentaCB and 4-OH-2,2',3,4',5,5',6-heptaCB in human plasma (Bergman *et al.*, 1994). It was evident that *para*- or *meta*-hydroxylation and adjacent chlorine substitution was necessary for binding to TTR in plasma. No selective retention of the parent compounds by TTR was found in plasma of exposed animals. Thus, all structural requirements for binding to TTR as found in *in vitro* studies (Lans *et al.*, 1993, 1994) and by computer assisted graphics modelling and X-ray crystallography in this study are confirmed by *in vivo* findings, following both experimental and environmental PCB exposures.

The selective retention of di- or tri-*ortho* chlorinated, non-planar hydroxylated PCB metabolites after exposure to commercial PCB mixtures, or environmental exposure (Bergman *et al.*, 1994) suggests that a non-planar conformation does not interfere with binding to TTR. Since di-*ortho* highly chlorinated PCBs are present in substantial levels in the environment (for review see McFarland and Clarke, 1989, Safe, 1994) marine mammals (Kannan et al., 1989) and human milk (Safe *et al.*, 1985), and the metabolism of these compounds is slow (Safe, 1989), this may explain the presence of metabolites of di- or tri-*ortho* chlorine substituted PCBs in plasma of environmentally exposed organisms.

In addition to T_4-binding and transport, TTR also functions in RBP-retinol transport. It was observed by Brouwer *et al.* (1988) that the affinity of TTR for RBP complex formation was reduced by the interaction of the 4-OH-3,3',4',5-TCB metabolite. This suggested that binding of a hydroxylated PCB metabolite did not only disturb T_4-TTR binding but also RBP-TTR binding, probably as a consequence of conformational changes of the TTR protein. Competition of 4-OH-TCB for the RBP binding site on TTR was not likely because the metabolite competed with T_4 for the thyroid hormone binding site on TTR (Brouwer *et al.*, 1990, Lans *et al.*, 1994). The interaction of hydroxylated PCB metabolites with TTR as found in this study does not support the hypothesis of a conformational change causing the decrease in TTR-RBP binding, because binding of a hydroxylated PCB molecule does not seem interfere with the native TTR conformation. Only the Lys 15 residues present near the channel entance may assume a different conformation. This change is analogous to the conformational change of the Lys 15 residues upon T_4 binding, which does not interfere with RBP binding, so no interference of TTR-RBP binding is expected by this mechanism. Recently, the participation of the Ile 84 residues, on the outside of the TTR tetramer, with the TTR-RBP interaction is described (Berni et al., 1994). Interaction of hydroxylated PCB metabolites with TTR are, however, not expected to interfere with this region on the TTR molecule as based on the interaction with the T_4 binding site of TTR described in this study.

In vivo studies with PCP-exposed rats showed a decrease in plasma total T_4 levels that could also be caused by inhibition of T_4-TTR binding as found *in vitro* (Van den Berg, 1990). However, no decrease in plasma retinol was found, suggesting no interference with

RBP-TTR binding (Den Besten *et al.*, 1991). As for the hydroxylated PCB metabolites tested in this graphics modelling, no indication was found for a conformational change in the TTR molecule after binding of PCP, which seemed to fit well in the binding channel of TTR.

The mechanism of the different effects of hydroxylated PCB metabolites and PCP on RBP-TTR interaction is not yet elucidated. Detailed structural information on the PCP-TTR interaction, as could be derived from X-ray crystallograpic studies on the PCP-TTR complex, could help explain the dissimilar effects on RBP-TTR interaction of the related PCP and hydroxylated PCB metabolites.

In addition to disturbances of T_4-TTR binding, several hydroxylated PCB metabolites could also competitively inhibit type-1-deiodinase activity *in vitro* (Lans *et al.*, 1995a), while no disturbances were found *in vitro* of T_4 binding to thyroxin-binding globulin, the major human thyroid hormone transport protein in plasma (Lans *et al.*, 1993), indicating differences in the structural requirements of hydroxylated PCB metabolites for binding to these thyroxine binding proteins.

In conclusion, exact and detailed information derived from X-ray analysis of cocrystallized complexes of TTR and diOH-TCB and the computer assisted graphics modelling of complexes of hydroxylated PCBs with TTR confirmed structural requirements of these compounds for TTR binding as found *in vitro*, and gave insight in the mechanism of selective retention of *para*- or *meta*-hydroxylated PCB metabolites as found *in vivo* in plasma of mammals after experimental and environmental PCB exposure. These techniques can therefore be used for predictions of possible interferences of compounds, structurally related to T_4, with TTR. In ongoing studies, binding energies of the hydroxylated PCB metabolites modelled in the TTR binding site in this study will be calculated using energy minimisation calculations in order to compare binding energies derived from graphics modelled interactions with binding affinities determined *in vitro* (Lans *et al.*, 1993). Detailed information on subtle changes due to diOH-TCB binding in the binding channel of TTR and differences in the binding of T_4 and diOH-TCB to TTR will be obtained by superimposing the X-ray crystallographic structures.

CHAPTER 8

Summary and concluding remarks

Summary

Some toxic effects caused by polyhalogenated aromatic hydrocarbons (PHAHs) develop through alterations in the reproductive and thyroid hormone regulatory systems, thereby affecting (brain) development, reproduction and behaviour of several species (Stone, 1995, Birnbaum, 1994, for review: Brouwer *et al.*, 1995, Peterson *et al.*, 1993). In this thesis we have focused on the effects of different classes of PHAHs, eg. polychlorinated biphenyls (PCBs), dibenzofurans (PCDFs) and dibenzo-*p*-dioxins (PCDDs) and their hydroxylated metabolites on thyroid hormone homeostasis. These changes seem to be partly caused by Ah-receptor mediated changes in thyroid hormone glucuronidation, and effects on the thyroid gland affecting hormone production and secretion. However, hydroxylated metabolites of PCBs, PCDFs and PCDDs may have an additional effect on thyroid hormone transport. Previous studies (Brouwer, 1987) have shown that exposure to 3,3',4,4'-tetrachlorobiphenyl (TCB) can disturb the plasma transport of thyroxine (T_4) and retinol in rats through specific competition of a hydroxylated metabolite, 4-OH-3,3',4',5-tetraCB, with T_4 for the thyroid hormone binding site of transthyretin (TTR), the major thyroid hormone transport protein in rodents. This observation raised the question if structurally related hydroxylated PHAH-metabolites could interact with TTR in the same way, as well as with other thyroxine binding proteins, like thyroxine binding globulin (TBG) and type-1-deiodinase (ID-1), subsequently disturbing thyroid hormone transport and metabolism. Special attention was also paid on the structure-activity relationships of hydroxylated PHAH metabolites for binding to TTR by using *in vitro* and *in vivo* studies and X-ray crystallographic structure analysis.

In vitro studies on interactions of hydroxylated PHAH metabolites with thyroxine binding proteins

In *in vitro* studies the interactions of several hydroxylated PHAH metabolites with 3 different T_4 binding proteins, eg. TTR, thyroxine binding globulin (TBG) and type-1-deiodinase (ID-1), were investigated (Chapter 2, 3 and 4). The inhibition of T_4 binding to TTR by hydroxylated PHAH metabolites was studied using *in vitro* T_4-TTR bindingstudies. These studies revealed the structural requirements for competition of T_4 binding to TTR by hydroxylated PHAH metabolites: *para-* or *meta*-hydroxylation on one or both phenylrings, with one or more adjacent chlorine substitutions (Chapter 2). PHAH metabolites with these structural characteristics showed a remarkable resemblance to T_4, the natural ligand for TTR, consequently displacing T_4 from the T_4 binding site of TTR. Both non-planar, *ortho*-chlorinated hydroxylated PCB metabolites and rigid, planar hydroxylated PCDF or PCDD metabolites could inhibit T_4-TTR binding. However, the *ortho*-hydroxylated PHAH metabolites and parent PHAH compounds, like TCDD, 2,3,3',4,4'-pentaCB and 3,3',4,4'-tetraCB could not inhibit T_4-TTR binding *in vitro*.

In subsequent *in vitro* studies, a wide range of hydroxylated PHAH metabolites

did not inhibit T_4 binding to TBG, the major plasma thyroid hormone transport protein in man (Chapter 3). This indicates that ligand interactions with TTR or TBG are clearly different. Additional studies with iodothyronine derivatives, showed that tri-iodophenol and to a lesser extent di-iodotyrosine could inhibit T_4-TTR binding but not T_4-TBG binding *in vitro*. Finally, the enzymatic activity of hepatic ID-1, which plays a role in the (in)activation of thyroid hormones, could be competitively inhibited mainly by di-*para*-hydroxylated, *meta*-halogenated PHAH metabolites while mono-hydroxylated PHAH metabolites were 10 to 100 times less potent (Chapter 4). The differences between the structural requirements of hydroxylated PHAH metabolites for interactions with TTR, TBG and ID-1, are in line with previous studies in which related hydroxylated PHAH compounds or iodothyronine derivatives were used. In conclusion, specific hydroxylated PHAH metabolites can disturb T_4-TTR interactions, or inhibit ID-1 activity *in vitro*, indicating that hydroxylated PHAH metabolites may play an additional role in the observed disturbances in thyroid hormone transport and metabolism after PHAH exposure *in vivo*.

In vivo studies on effects of Aroclor 1254 and TCDD on thyroid hormone transport and metabolism

Two *in vivo* experiments were carried out to study role that both disturbances in plasma T_4 transport and hepatic T_4 metabolism caused by hydroxylated PHAH metabolites play in the observed decreases in plasma T_4 levels. Rats were exposed to Aroclor 1254, a commercial mixture containing persistent and metabolisable PCB congeners (Chapter 5) or the persistent 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Chapter 6).

In adult Wistar rats, that were exposed to a high dose of Aroclor 1254, plasma T_4 levels were decreased on day 3 and day 8 (Chapter 5). In addition, high levels of a single hydroxylated PCB metabolite, eg. 4-OH-2,3,3',4',5-pentaCB, were detected in plasma of the rats on day 8, while lower concentrations of this metabolite were present in the blood on day 3. However, the T_4 binding capacity in plasma was decreased only in the high dosed group on day 8 but not on day 3, indicating a threshold level for the hydroxylated PCB metabolite to disturb T_4-TTR binding. On both day 3 and 8, hepatic cytochrome P450 1A1 levels and activity were induced, which is essential for the formation of hydroxylated metabolites. Hepatic T_4 glucuronidation was induced simultaneously. The decreased plasma T_4 levels found in all exposure groups could therefore be attributed to both disturbed plasma T_4 transport and/or induced T_4 glucuronidation. No significant changes in plasma T_3 levels were found following Aroclor 1254 treatment. In addition, hepatic ID-1 activity was not decreased, suggesting that the *in vitro* inhibition by hydroxylated PCB metabolites does not occur *in vivo*. However in an earlier study Adams *et al.* (1990), *in vivo* exposure to the easily metabolisable 3,3',4,4'-tetraCB or persistent TCDD could inhibit ID-1 activity. The levels of PCB metabolites with the required structure for inhibition of hepatic ID-1 activity were possibly too low (Chapter 4) liver after Aroclor 1254 exposure *in vivo*.

Another remarkable finding was the selective retention of a single specific hydroxylated PCB metabolite, 4-OH-2,3,3',4',5-pentaCB, in plasma of rats exposed to a complex mixture of PCB congeners (Fig. 1). This was the result of the presence of PCB-congeners that strongly induce cytochrome P4501A1 activity and PCB-congeners that could easily form hydroxylated PCB metabolites in the Aroclor 1254 mixture, and the strict selectivity of the TTR present in plasma retaining only hydroxylated PCB

metabolites that meet the structural requirements as described in Chapter 2. Surprisingly the 4-OH-2,3,3',4',5-pentaCB metabolite which was formed and selectively retained in plasma, has a hydroxy group on the highest chlorinated ring, in contrast to the expected formation of mainly metabolites with a *para*- or *meta*-hydroxy group on the least chlorinated ring. However, the inhibition potencies of T_4-TTR binding for the 4-OH-2,3,3',4',5-pentaCB metabolite (Chapter 5) and the structurally related 4'-OH-2,3,3',4,5'-pentaCB metabolite (Chapter 2) were almost similar. There is no clear indication yet on the mechanism of selective retention of the 4-OH-2,3,3',4',5-pentaCB in rat plasma, although pharmacokinetics and tissue levels of the presumed parent compounds 2,3,3',4,4'-pentaCB (CB 105) or 2,3',4,4',5-pentaCB (CB 118) may play a role.

No detectable levels of hydroxylated metabolites were found by GC-MS analysis of plasma extracts of rats at both day 3 and 8 following exposure to the persistent TCDD, although cytochrome P4501A1 levels and activity were markedly induced (Chapter 6). In addition no unequivocal decrease in T_4-TTR binding in plasma of TCDD-exposed rats was observed. However, hepatic thyroid hormone metabolism was clearly altered: T_4 glucuronidation and brain type-2-deiodinase (ID-2) activity were increased and hepatic ID-1 activity was decreased, which may explain the observed plasma T_4 reductions in the TCDD exposed rats. These changes in thyroid hormone homeostasis suggest a hypothyroxinemic state of the TCDD-exposed rats, although no decreased plasma T_3 levels were found. The decrease in ID-1 activity after TCDD exposure was not likely to be caused by hydroxylated metabolites, as was described in Chapter 4, but may be caused by direct effects of TCDD on ID-1 activity or by the assumed hypothyroid state of the TCDD exposed rats.

While Aroclor 1254 exposure disturbed T_4 plasma transport and increase T_4 glucuronidation (Chapter 5), TCDD exposure only enhanced the hepatic elimination of T_4 (Chapter 6), leading to decreased plasma T_4 levels. Although the *in vivo* studies described in this thesis indicate two different mechanisms for decreases in plasma T_4 levels after PHAH exposure, we can not exclude a third possible mechanism since several studies described changes on thyroid gland histology and thyroid hormone secretion after exposure to Aroclor 1254, TCDD and related compounds.

Structural basis for interactions of hydroxy-PCB metabolites with TTR

The selective retention of a specific hydroxylated PCB metabolite *in vivo* (Chapter 5), inspired us to look into the interactions of hydroxylated PCB metabolites with TTR in more detail, and to try to find a structural basis for the structural requirements for TTR binding as described in Chapter 2. X-ray crystallographic analysis of a complex of TTR with a hydroxylated PCB metabolite, eg. 4,4'-$(OH)_2$-3,3',5,5'-tetraCB, refined to a 2.7 Å resolution, revealed a hydrogen bond formation between a *para*-hydroxy group of the metabolite with the paired Serine 117 amino acid residues, present in the centre of the TTR binding channel (Chapter 7). The location of this hydroxylated PCB metabolite, deep in the binding channel, and the hydrogen bond formation could explain the stronger TTR binding affinity of this metabolite than the natural ligand T_4. The chlorine atoms present on the *meta*-positions of the 4,4'-$(OH)_2$-3,3',5,5'-tetraCB metabolite, fitted easily in the T_4-iodine binding pockets present in the TTR binding channel. Additional computer-assisted graphics modelling studies on the interactions of several hydroxylated PCB metabolites with TTR, showed that hydroxy groups present on *meta*-positions could also form hydrogen bonds with the paired Serine 117 residues in the centre of the

binding channel (Chapter 7). Furthermore, the modelling studies showed no significant differences between the interactions of hydroxylated PCB metabolites and the structurally related pentachlorophenol (PCP) with TTR, although *in vivo* studies by others indicated the disruption of the complex of retinol binding protein (RBP) and TTR after binding of a hydroxylated PCB metabolite but not by PCP in rodents. In conclusion, the detailed structural studies described in Chapter 7 confirmed the necessity of *para-* or *meta*-hydroxylation and adjacent chlorine substituents as structural elements of hydroxylated metabolites of PCBs and related compounds for interactions with TTR (Chapter 2), leading to selective retention of a specific hydroxylated PCB metabolite in plasma of rats exposed to a complex PCB mixture *in vivo* (Chapter 5).

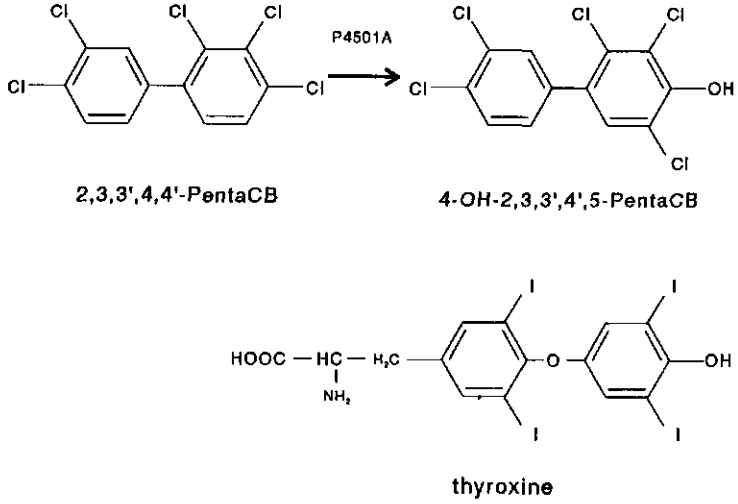


Figure 1 Formation of the 4-OH-2,3,3',4',5-pentaCB metabolite from one possible parent compound, 2,3,3',4,4'-pentaCB, by cytochrome P4501A (P4501A), and its structural resemblance to thyroxine.

Concluding remarks

The outcome of the present study clearly reveals the structural requirements that are essential for interactions of PHAH metabolites and other related chemicals for interactions with TTR. Hydroxylated PHAH metabolites can structurally resemble the thyroid hormone T_4. Overall the structural requirements for TTR interaction were hydroxy-substitution on the *para-* or *meta* positions of one or both of the phenyl rings, with adjacent chlorine substitutions, herewith confirming some of the suggestions for TTR interactions of related hydroxylated PHAH metabolites by Rickenbacher *et al.* (1986). Especially the observation by X-ray crystallographic structure analysis that a hydrogen bond could be formed in the TTR binding channel upon binding of a hydroxylated PCB metabolite, provides strong evidence that *para-* or *meta*-hydroxylation of the PHAH compound forms an essential prerequisite for binding to the T_4 binding site of TTR. No interactions with TTR were found for the tested parent PHAH compounds, contradictory to earlier suggestions of McKinney *et al.* (1985) which were based mainly on computer modelling and few *in vitro* binding studies (Rickenbacher *et al*, 1986). It should be noted that graphics modelling may show that parent PHAH compounds may fit the TTR binding site, but gives little information on binding affinity to TTR. In an affirmative *in vitro* T_4-TTR binding assay, several parent PCB congeners (3,3',4,4'-tetraCB, 3,3',4,-4',5-pentaCB, 3,3',4,4',5,5'-hexaCB, 2,3,3',4,4'-pentaCB, 2,2',5,5'-tetraCB), TCDD and Aroclor 1254, a commercial PCB mixture, were tested at high concentrations and exhibited no inhibition of T_4-TTR binding (unpublished data).

PHAH metabolites that are predicted to have high binding affinities for TTR are indeed detected in plasma of rodents experimentally exposed to PCBs or PCDFs (Morse *et al.*, 1995b, Koga *et al.*, 1990, Kuroki *et al.*, 1993). For instance, exposure to a complex mixture of PCBs, Aroclor 1254, led to the selective retention in blood of a single PCB metabolite, 4-OH-2,3,3',4',5-pentaCB, which met the structural requirements for TTR binding (Bergman *et al.*, 1994).

On the basis of the proposed structural requirements we can give an explanation for the interactions of related compounds with TTR as found by others, for instance pentachlorophenol (Van den Berg, 1990, Van Raay *et al.*, 1994, Den Besten *et al.*, 1991), natural compounds like flavones and halogenated aurones (Cody, 1989; Ciszak *et al.*, 1992) and certain drugs like milrinone (Wojtczak *et al.*, 1993). Moreover, these structural insights make it possible to predict whether other related classes of environmental contaminants can interact with TTR. In addition, due to combined exposure in the environment, one may expect additivity of binding to TTR of hydroxylated PHAH metabolites with other structurally related environmental or natural compounds.

An important question is whether these experimental data can be extrapolated to other species. Two factors are essential for the TTR mediated selective retention of hydroxylated PHAH metabolites namely the presence of TTR in blood and the formation of relevant PHAH metabolites. It is most likely that hydroxylated metabolites can be formed in many species other than rodent. Recently hydroxylated PCB metabolites were identified in human and seal plasma, which were environmentally exposed to background PCB levels (Bergman *et al.*, 1994). The major metabolites were again the 4-OH-2,3,3',4',5-pentaCB metabolite and to a lesser extent 4-OH-2',3,3',4',5-pentaCB in seal and human plasma and 4-OH-2,2',3,4',5,5',6-heptaCB in human plasma. Thus the hydroxylated PCB metabolites detected *in vivo* completely matched the structural

requirements for TTR binding. The PHAH metabolite patterns in plasma of both experimentally and environmentally exposed animals and humans are species specific and depend not only on the structural requirements for binding to TTR, but also on the exposure situation and the capacity of biotransformation of PHAHs of the species.

The species-specific metabolism of PHAHs decreases in the order: terrestrial mammals > aquatic mammals > birds > fish (Safe, 1989). Several mammalian and avian species, like rats, seals, porpoises and eiderducks could form hydroxylated metabolites in *in vitro* microsomal incubations with the model substrate 3,3',4,4'-tetraCB (TCB). However fish, like trout and flounder, could not metabolise TCB, although cytochrome P4501A-like activity, responsible for biotransformation of planar PHAHs, can be induced (Murk *et al.*, 1994, Morse *et al.*, 1995c, Ishida *et al.*, 1991).

The selective retention of specific hydroxylated PHAH metabolites in plasma through binding to TTR is expected in species that both can metabolise PHAHs and posses TTR as a plasma thyroid hormone binding protein. TTR is a evolutionary conservative protein present in plasma of not only rodents but also other placental mammals, birds and to a lesser extent in reptiles. No TTR was detected in the lower species like fish and amphibians. In higher mammals like man, however, not only TTR but also thyroxine binding globulin (TBG), is present in blood as a primary thyroid hormone transport protein. In conclusion, hydroxylated PHAH metabolites can be formed and selectively retained by TTR in blood of a wide variety of species.

The toxicological consequences of the TTR mediated selective retention of hydroxylated PHAH metabolites in plasma are not yet fully understood. The TTR protein plays a primary role in the transport of thyroid hormones in the blood of many species. Although TTR binds less T_4 than TBG in human serum, TTR may be responsible for much of the immediate delivery of T_4 and T_3 to cells due to the lower binding T_4 affinity. Furthermore TTR is important for the transport of retinol in blood by forming a complex with retinol-binding protein (Robbins, 1991).

Disturbances in thyroid hormone plasma levels are found in several species experimentally or environmentally exposed to PHAHs, like rodents, seal (Brouwer *et al.*, 1989) and man (Koopman Esseboom *et al.*, 1994), species in which hydroxylated PCB metabolites were also present in plasma. Disruption of thyroid hormone homeostasis after exposure to PHAHs can however be caused by at least two mechanisms, eg. the disturbed plasma T_4 transport through competitive binding of hydroxylated PHAH metabolites to TTR, but also the Ah-receptor mediated induction of T_4 glucuronidation by parent compounds.

The possible disruption of the TTR-RBP complex upon binding of a hydroxylated PHAH metabolite can also markedly decrease plasma retinol levels in rodents (Brouwer, 1987). It was suggested that seals exposed to PHAHs in the environment, have an impaired function of the immune system (de Swart, 1995), possibly resulting from disturbed retinoid levels (Brouwer, 1991, Brouwer *et al.* 1989). Hydroxylated PHAH metabolites may attribute to these effects on thyroid hormone and retinoid homeostasis through interactions with TTR in plasma. It is not known whether similar effects occur in man.

TTR is the major thyroid hormone binding protein in cerebro-spinal fluid (CSF), suggesting a role in distribution of thyroid hormones in the central nervous system. This TTR is produced in the choroid plexus and is present in high concentrations in CSF of rats and humans, even at a very early stage in development. Moreover, in all species

where TTR is present in blood, TTR has also been detected in brain. Because TTR is an important carrier for T_4 to target tissues, for instance brain, one may expect that it may also act as a facilitated transport system for hydroxylated PHAH metabolites. This is in accordance with observations of a strong accumulation of hydroxylated PCB metabolites of maternal origin in the plasma and brain of late gestational fetuses from pregnant rats or mice exposed to PCBs (Morse *et al.*, 1995b,d, Darnerud *et al.*, 1995). In rat fetuses perinatally exposed to Aroclor 1254 the selective accumulation of the 4-OH-2,3,3',4',5-pentaCB metabolite in maternal plasma and fetal plasma and brain led to decreases in brain T_4 levels, while brain T_3 levels were only lightly changed. In addition plasma and hepatic retinoid concentrations were decreased in fetal and neonatal offspring (Morse *et al.*, 1995a). The hydroxylated metabolites accumulated to high levels in fetal rat brain and may themselves attribute to observed neurochemical changes (Morse, 1995).

Hydroxylated PHAH metabolites have been shown to possess biological activity *in vitro* (Brouwer, 1994). Hydroxylated PCB metabolites can interfere with mitochondrial structure and function *in vivo* and *in vitro* (Lans *et al.*, 1990, Narashimhan *et al.*, 1991). Moreover, they can bind to the Ah-receptor and weakly induce EROD activity. In addition, an *in vitro* marker of tumor promoting potential, the gap-junctional intercellular communication, could be weakly inhibited. Hydroxylated PCB metabolites can also exert (anti)-estrogenic activities *in vivo* (Bergeron *et al.*, 1994) and *in vitro* (Kramer *et al.*, 1994). No clear structure activity relationships for (anti)-estrogenicity could be found for the tested hydroxylated PCB metabolites. However, the hydroxylated PCB metabolites selectively retained in fetal plasma and brain (Morse *et al.*, 1995d) do have a weak (anti)-estrogenic activity. The intrinsic capacity to disrupt endocrine systems, eg. thyroid and estrogen status, and the relatively large accumulating levels of hydroxylated PCB metabolites in late gestational rat fetuses, suggests there is a potential risk for adverse developmental effects by these hydroxylated PHAHs. This possible hydroxy PCB-mediated route of developmental toxicity should be investigated in a sound *in vivo* experimental setup.

Subtle changes in plasma thyroid hormone levels and parameters for neurological development were described in children exposed to background levels of PHAHs in utero and through lactation (Koopman-Esseboom *et al.*, 1994, Sauer *et al.*, 1994, Pluim *et al.*, 1993). Hydroxylated PHAH metabolites did not interact with TBG, the major T_4 binding protein in human plasma (Lans *et al.*, 1994). However, the hydroxylated PCB metabolites which are recently detected in human plasma (Bergman *et al.*, 1994) are mainly bound to TTR, as was found after selective purification of TTR from human plasma (unpublished results). Therefore TTR-mediated accumulation of hydroxylated PCB metabolites or related compounds in fetal plasma and brain and subsequent decreases in T_4 levels, as found in late gestational rat fetuses, may be of concern for fetal growth and (brain) development in a wide variety of species, including man.

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Samenvatting en slotopmerkingen

Samenvatting

Polygehalogeneerde aromatische koolwaterstoffen (PHAK's) kunnen verstoringen in de reproductie- en schildklierhormoon-systemen veroorzaken waardoor uiteindelijk de (hersen)ontwikkeling, reproductie en het gedrag van diverse diersoorten negatief worden beinvloed kan worden. In dit proefschrift is voornamelijk aandacht besteed aan de effecten van verschillende klassen van PHAK's, nl. de polychloor-bifenylen (PCBs), polychloor-dibenzofuranen (PCDFs), polychloor-dibenzo-*p*-dioxinen (PCDDs) en hun gehydroxyleerde metabolieten op het schildklierhormoon-regulerend systeem. De veranderingen van schildklierhormoon-concentraties in het bloed na blootstelling aan deze stoffen lijken gedeeltelijk te worden veroorzaakt door een toename van de glucuronidering van het schildklierhormoon, een Ah-receptor-gemedieerd proces. Bovendien kan door aantasting van de schildklier door PHAK's de hormoon-productie en secretie beinvloed worden. Gehydroxyleerde metabolieten van PCBs, PCDFs en PCDDs zouden daarnaast het schildklierhormoon-transport in het bloed mogelijk kunnen verstoren. In eerder onderzoek uitgevoerd door Brouwer (1987) was reeds aangetoond dat blootstelling aan 3,3',4,4'-tetrachloor-bifenyl (TCB) het plasma transport van thyroxine (T_4) en retinol in ratten verstoort. Dit werd veroorzaakt door de specifieke competitie van een gehydroxyleerde PCB metaboliet, nl. 4-OH-3,3',4',5-tetraCB met het T_4 voor de schildklierhormoon bindingsplaats op het transthyretine (TTR), het belangrijkste transporteiwit voor schildklierhormonen in knaagdieren. Deze waarneming leidde tot de vraag of structureel verwante gehydroxyleerde PHAK metabolieten een vergelijkbare interactie zouden kunnen hebben met TTR, als ook met andere schildklierhormoon bindende eiwitten, zoals thyroxine bindend globuline (TBG) en type-1-deiodinase (ID-1). Hierdoor zou het schildklierhormoontransport en metabolisme na blootstelling aan PHAK's *in vivo*, mede door gehydroxyleerde PHAK metabolieten verstoord kunnen worden. Speciale aandacht is besteed aan de structuur-activiteitsrelaties van gehydroxyleerde PHAK metabolieten voor interacties met TTR door middel van *in vitro* en *in vivo* studies en Röntgen-diffractie eiwitkristallografie.

In vitro experimenten: Interacties van gehydroxyleerde PHAK metabolieten met T_4-bindende eiwitten

In verscheidene *in vitro* experimenten werden de interacties van gehydroxyleerde PHAK metabolieten met 3 verschillende T_4 bindende eiwitten: TTR, thyroxine bindend globuline (TBG) en type-1-dejodase (ID-1), onderzocht (Hoofdstuk 2, 3 en 4). De remming van de binding van T_4 aan TTR door gehydroxyleerde PHAK metabolieten werd onderzocht in *in vitro* T_4-TTR bindingsstudies. Hieruit bleek dat de structurele voorwaarden die nodig waren voor de binding van gehydroxyleerde PHAK metabolieten aan TTR, bestonden uit: de *para-* of *meta*-hydroxylatie van een of beide fenylringen, met aan een of weerszijden chlooratomen (Hoofdstuk 2). De PHAK metabolieten met

deze structurele kenmerken vertonen een opmerkelijke overeenkomst met T_4, het natuurlijke ligand voor TTR, met als gevolg dat deze stoffen het T_4 competitief van de T_4-bindingsplaats op het TTR verdringen. Zowel niet-planaire gehydroxyleerde PCB metabolieten met *ortho*-chloorsubstituties, als planaire, rigide gehydroxyleerde PCDF of PCDD metabolieten kunnen de binding van T_4 aan TTR remmen. De *ortho*-gehydroxyleerde PHAK metabolieten en de PHAK uitgangsstoffen zoals TCDD, 2,3,3',4,4'-pentaCB en 3,3',4,4'-tetraCB remmen de T_4-TTR binding echter niet *in vitro*.

Vervolgens bleek uit *in vitro* bindingsstudies met TBG, het belangrijkste schildklierhormoon-transporteiwit bij de mens, dat de geteste gehydroxyleerde PHAK metabolieten de T_4-TBG binding niet remden (Hoofdstuk 3) Dit wijst erop dat interacties van liganden met TTR en TBG duidelijk verschillend zijn. Aanvullende *in vitro* experimenten met iodothyronine derivaten, toonden aan dat triiodofenol en in mindere mate di-iodotyrosine de T_4-TTR binding maar niet de T_4-TBG binding konden remmen.

Tenslotte kon de enzymatische activiteit van het ID-1 enzym in de lever, dat een rol speelt bij de (in)activatie van schildklierhormonen, competitief geremd worden door di-*para*-gehydroxyleerde, *meta*-gehalogeneerde PHAK metabolieten, terwijl mono-gehydroxyleerde PHAK metabolieten 10 tot 100 keer minder sterk remden (Hoofdstuk 4). De verschillen tussen de structurele voorwaarden van gehydroxyleerde PHAH metabolieten die nodig zijn voor interacties met TTR, TBG en ID-1, zijn in overeenstemming met eerdere studies waarin verwante gehydroxyleerde PHAK metabolieten of iodothyronine derivaten zijn gebruikt. Concluderend: specifieke gehydroxyleerde PHAK metabolieten kunnen *in vitro* de T_4-TTR binding verstoren en ID-1 activiteit remmen, waardoor gehydroxyleerde PHAK metabolieten een additionele rol zouden kunnen spelen in de waargenomen veranderingen in de schildklierhormoonconcentraties in het bloed na blootstelling aan PHAK's *in vivo*.

In vivo experimenten: Effecten van Aroclor 1254 en TCDD op schildklierhormoon-transport en -metabolisme

Er zijn twee *in vivo* experimenten uitgevoerd om de rol te onderzoeken die de verstoringen in het plasma T_4 transport en het T_4 metabolisme in de lever, veroorzaakt door gehydroxyleerde PHAK metabolieten, spelen in de waargenomen verlagingen in plasma T_4 gehaltes na blootstelling aan PHAK's. Ratten werden blootgesteld aan Aroclor 1254, een commercieel PCB mengsel dat zowel persistente als metaboliseerbare PCB congeneren bevat (Hoofdstuk 5), of aan het persistente 2,3,7,8-tetrachloordibenzo-*p*-dioxin (TCDD) (Hoofdstuk 6).

In volwassen Wistar ratten, blootgesteld aan een hoge dosis Aroclor 1254, werden verlaagde plasma T_4 gehaltes gevonden op dag 3 en dag 8 na de blootstelling (Hoofdstuk 5). Tegelijkertijd werden op dag 8 hoge gehaltes van een specifieke gehydroxyleerde PCB metaboliet, 4-OH-2,3,3',4',5-pentaCB, aangetoond in het plasma van de ratten terwijl op dag 3 lagere hoeveelheden van deze metaboliet aangetroffen werden. De T_4-bindingscapaciteit in het plasma van de hoog gedoseerde groep ratten was echter alleen op dag 8 en niet op dag 3 verlaagd. Dit geeft aan dat er vermoedelijk een drempelniveau bestaat voor het verstoren van de T_4-TTR binding door gehydroxyleerde PCB metabolieten. Zowel op dag 3 als dag 8 waren de cytochroom P450 gehaltes en activiteiten in de lever geinduceerd, wat van belang is voor de vorming van de gehydroxyleerde metabolieten. De T_4 glucuronidering in de lever was echter

tegelijkertijd geinduceerd. De verlaagde T_4 concentraties in het plasma die in alle blootgestelde groepen werd waargenomen, kunnen daarom toegeschreven worden aan zowel verstoord plasma T_4 transport door gehydroxyleerde PCB metabolieten en/of aan verhoogde T_4 glucuronidering in de lever. Er werden geen significante verschillen gevonden in plasma T_3 gehaltes na blootstelling aan Aroclor 1254. De ID-1 activiteit in de lever was eveneens niet geremd, wat suggereert dat de *in vitro* remming van ID-1 activiteit door gehydroxyleerde PCB metabolieten niet *in vivo* optreedt. In een eerder experiment (Adams *et al.*, 1990) werd de ID-1 activiteit in de lever echter wel *in vivo* geremd door zowel het gemakkelijke metaboliseerbare 3,3',4,4'-tetraCB als het persistente TCDD. In de lever van de ratten die blootgesteld waren aan Aroclor 1254 *in vivo* (Hoofdstuk 4) zouden geen of te lage hoeveelheden PCB metabolieten met de vereiste structuur-voorwaarden voor ID-1 remming (Hoofdstuk 4), aanwezig kunnen zijn geweest.

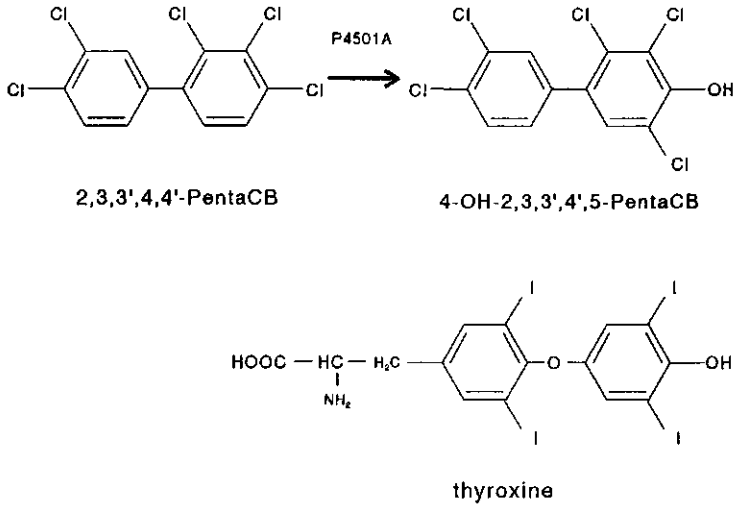
Een opmerkelijke bevinding was dat in ratten die blootgesteld zijn aan een complex mengsel van PCB congeneren, slechts een enkele specifieke gehydroxyleerde PCB metaboliet, nl. 4-OH-2,3,3',4',5-pentaCB, in hoge concentratie in het plasma aanwezig was (Fig. 1). Dit was het gevolg van de gecombineerde aanwezigheid in het Aroclor 1254 mengsel van PCB congeneren die de cytochroom P450 activiteit sterk induceren en PCB congeneren die makkelijk metaboliseerbaar zijn. Daarnaast blijven door de selectiviteit van het TTR eiwit alleen gehydroxyleerde metabolieten aanwezig in het plasma die voldoen aan de structurele vereisten voor TTR binding zoals beschreven in Hoofdstuk 2. Verrassend was dat de 4-OH-2,3,3',4',5-pentaCB metaboliet die werd gevormd en selectief in het plasma aanwezig bleef, een hydroxygroep bezat op de hoogst gechloreerde ring, terwijl verwacht wordt dat voornamelijk metabolieten met een *para*- of *meta*-groep op de minst gechloreerde ring zouden worden gevormd. De binding van T_4 aan TTR werd door de 4-OH-2,3,3',4',5-pentaCB metaboliet (Hoofdstuk 5) en de structureel verwante 4'-OH-2,3,3',4,5'-pentaCB metaboliet (Hoofdstuk 2) met vrijwel gelijke potentie geremd. Er zijn vooralsnog geen duidelijke verklaringen voor de selectieve retentie van de 4-OH-2,3,3',4',5-pentaCB metaboliet in het plasma van de rat. Mogelijk spelen de farmacokinetiek en de weefselgehaltes van de vermoedelijke uitgangsstoffen 2,3,3',4,4'-pentaCB (CB 105) of 2,3',4,4',5-pentaCB (CB 118) hierin een rol.

In ratten, blootgesteld aan TCDD, werden met GC-MS analyse geen detecteerbare hoeveelheden gehydroxyleerde TCDD metabolieten aangetoond in plasma extracten op dag 5 na blootstelling, hoewel de cytochroom P450 gehaltes en activiteit sterk geinduceerd waren (Hoofdstuk 6). Bovendien werd geen eenduidige afname gevonden van de T_4-TTR binding in het plasma van TCDD blootgestelde ratten. Het schildklierhormoon metabolisme was echter wel duidelijk veranderd: zowel de T_4 glucuronidering in de lever als de type-2-deiodinase (ID-2) activiteit in de hersenen waren toegenomen, terwijl de ID-1 activiteit in de lever was afgenomen. Deze veranderingen zouden de verlaagde plasma T_4 gehaltes in de blootgestelde ratten kunnen verklaren. De veranderingen in het schildklierhormoon-metabolisme zouden eveneens kunnen wijzen op een hypothyroide status van de ratten hoewel geen verlagingen in de plasma T_3 concentraties zijn aangetoond. De afname in ID-1 activiteit na TCDD blootstelling werd mogelijk niet veroorzaakt door gehydroxyleerde TCDD metabolieten maar zou veroorzaakt kunnen worden door directe effecten van TCDD op de ID-1 activiteit of door de mogelijke hypothyroide status van de blootgestelde ratten.

Terwijl de verlaagde plasma T_4 waarden na blootstelling aan Aroclor 1254 werden veroorzaakt door zowel het verstoorde plasma T_4 transport en de verhoogde T_4 glucuronidering (Hoofdstuk 5), werd na blootstelling aan TCDD alleen verhoogde T_4 glucuronidering als oorzaak voor plasma T_4 verlagingen vastgesteld. De *in vivo* experimenten zoals beschreven in dit proefschrift, geven aan dat er 2 verschillende mechanismen kunnen zijn voor verlagingen in plasma T_4 concentraties na blootstelling aan PHAK's. Een derde mechanisme kan echter niet uitgesloten worden omdat in verscheidene studies veranderingen in de structuur van de schildklier en secretie van schildklierhormonen bescheven worden na blootstelling van ratten aan Aroclor 1254, TCDD en verwante stoffen.

Structurele onderbouwing voor de interacties van gehydroxyleerde PCB metabolieten met TTR

De selectieve retentie van een specifieke gehydroxyleerde PCB metaboliet in het plasma van ratten *in vivo* (Hoofdstuk 5), leidde ertoe de interacties van gehydroxyleerde PCB metabolieten met TTR in meer detail te onderzoeken en hiermee de structurele kenmerken die nodig zijn voor binding aan TTR, zoals die beschreven zijn in Hoofdstuk 2, te onderbouwen. De drie-dimensionale structuur van een eiwitcomplex van TTR met een gehydroxyleerde PCB metaboliet, 4,4'-$(OH)_2$-3,3',5,5'-tetraCB, werd met behulp van Röntgen-diffractie kristallografie opgehelderd tot een 2.7 Ångstrom resolutie. Hierin werd aangetoond dat een waterstofbrug gevormd kon worden tussen de *para*-hydroxy groep van de metaboliet met de gepaarde Serine 117 aminozuur residuen, die aanwezig zijn in het centrum van het bindingskanaal van het TTR eiwit (Hoofdstuk 7). De locatie van deze PCB metaboliet, diep in de T_4 bindingsplaats van TTR, en de waterstofbrug-vorming verklaarden de sterkere affiniteit voor TTR van deze metaboliet vergeleken met het natuurlijke ligand T_4. De chloor-atomen die aanwezig zijn op de *meta*-posities van de 4,4'-$(OH)_2$-3,3',5,5'-tetraCB metaboliet, pasten gemakkelijk in de bindingsplaatsen voor de jood-atomen van T_4 die aanwezig zijn in het bindingskanaal van TTR. Aanvullende computer-ondersteunde grafische modellering van de interacties van verschillende PCB metabolieten met TTR, toonden aan dat hydroxy-groepen die aanwezig zijn op *meta*-posities eveneens waterstof-bruggen konden vormen met de Serine 117 aminozuur residuen (Hoofdstuk 7). Bovendien toonden deze model-studies aan dat er geen significante verschillen waren in de binding van gehydroxyleerde PCB metabolieten en het structureel verwante pentachloorfenol (PCP) met TTR. Eerdere *in vivo* experimenten met ratten wezen echter op een verstoring van het complex van TTR met retinol bindend eiwit (RBP) na binding van een gehydroxyleerde PCB metaboliet, maar geen verstoring van dit complex door PCP. Concluderend: de gedetailleerde structuuropheldering van het eiwitcomplex van TTR met de PCB metaboliet zoals beschreven in Hoofdstuk 7, onderbouwt de structurele voorwaarden voor PCB metabolieten om aan TTR te binden (Hoofdstuk 2) welke leiden tot de selectieve retentie van een specifieke PCB metaboliet in het plasma van ratten die blootgesteld zijn aan een complex PCB mengsel (Hoofdstuk 5).



Figuur 1 Vorming van de 4-OH-2,3,3',4',5-pentaCB metaboliet van een van zijn uitgangsstoffen, 2,3,3',4,4'-pentaCB, door cytochroom P4501A (P4501A), en zijn structurele overeenkomst met thyroxine

Concluderende slotopmerkingen

In dit proefschrift zijn de structurele kenmerken die nodig zijn voor de interacties van PHAK metabolieten en andere verwante stoffen met TTR duidelijk opgehelderd. Globaal zijn de structurele voorwaarden die nodig zijn voor binding aan TTR: hydroxylering op de *meta*- of *para*-positie van een of beide fenylringen, met aan een of weerszijden chlooratomen. Gehydroxyleerde PHAK metabolieten met deze structurele kenmerken lijken qua structuur sterk op het schildklierhormoon T_4. De hier beschreven structurele voorwaarden bevestigen de resultaten van een onderzoek naar interacties van verwante gehydroxyleerde PCB metabolieten met TTR zoals beschreven door Rickenbacher *et al.*, 1986. Vooral het aantonen van de waterstofbrugvorming na binding van een gehydroxyleerde PCB metaboliet in het TTR bindingskanaal met behulp van Röntgendiffractie eiwitkristallografie, versterkt de bevinding dat *para*- of *meta*-hydroxylatie van de PHAK een essentiele structurele voorwaarde vormt voor de binding van deze stoffen op de T_4 bindingsplaats van TTR.

PHAK metabolieten waarvan voorspeld kan worden dat ze een hoge bindingsaffiniteit voor TTR hebben, zijn inderdaad aangetoond in plasma van ratten die blootgesteld waren aan PCBs of PCDFs (Morse *et al.*, 1995b,d, Koga *et al.*, 1990, Kuroki *et al.*, 1993). Na blootstelling van ratten aan het complexe PCB mengsel Aroclor 1254, werd in het bloeplasma de PCB metaboliet 4-OH-2,3,3',4',5-pentaCB aangetoond, die voldoet aan de structurele kenmerken die nodig zijn voor binidng aan TTR (Bergman *et al.*, 1994).

In *in vitro* studies werden geen interacties van de geteste PHAK uitgangsstoffen met TTR gevonden, wat in tegenspraak is met eerdere bevindingen van McKinney *et al.*, 1985, die voornamelijk gebaseerd waren op computermodelling studies en slechts enkele

in vitro bindingsstudies (Rickenbacher *et al.*, 1986). Nadrukkelijk moet worden gesteld dat computer ondersteunde grafische modellering kan aantonen dat uitgangsstoffen in de bindingsplaats van TTR passen, maar dat deze methode weinig informatie geeft over de bindingsaffiniteit van deze stoffen voor TTR. Daarom zijn in een aanvullende *in vitro* T_4-TTR bindingsstudie verschillende uitgangsstoffen zoals 3,3',4,4'-tetraCB, 3,3',4,4',5-pentaCB, 3,3',4,4',5,5'-hexaCB, 2,3,3',4,4'-pentaCB, 2,2',5,5'-tetraCB, TCDD en Aroclor 1254 tot in hoge concentraties getest, waarbij wederom geen remming van T_4 binding aan TTR gevonden werd (niet gepubliceerde data).

Met de voorgestelde structurele voorwaarden voor gehydroxyleerde PHAK metabolieten voor interacties met TTR kunnen we eveneens de interacties met TTR verklaren van verwante verbindingen zoals pentachloorfenol (PCP) (Van den Berg, 1990, Van Raay, 1994, Den Besten *et al.*, 1991), natuurlijke stoffen zoals flavonen en gehalogeneerde auronen (Cody, 1989; Ciszak *et al.*, 1992) en bepaalde geneesmiddelen zoals milrinone (Wojtczak *et al.*, 1993). Bovendien maken deze structurele inzichten het mogelijk om te voorspellen of andere klassen van milieuvervuilende stoffen eveneens aan TTR zouden kunnen binden. Daarnaast kan men additiviteit voor binding aan TTR verwachten van gehydroxyleerde PHAK metabolieten met andere structureel verwante milieucontaminanten of natuurlijke stoffen.

Een ander belangrijk aspect is of de experimentele gegevens, zoals beschreven in dit proefschrift, geextrapoleerd kunnen worden naar andere diersoorten. Voor de selectieve retentie van gehydroxyleerde PHAK metabolieten door binding aan TTR in bloed zijn twee factoren van belang, namelijk de aanwezigheid van TTR in het bloed en de mogelijke vorming van gehydroxyleerde metabolieten. Waarschijnlijk kunnen andere diersoorten dan knaagdieren eveneens gehydroxyleerde PHAK metabolieten vormen na blootstelling aan PHAK's. Recentelijk zijn gehydroxyleerde PCB metabolieten aangetoond in het bloed van mensen en zeehonden, die blootgesteld waren aan achtergrondniveau's van PCBs (Bergman *et al.*, 1994). De belangrijkste metabolieten zijn wederom de 4-OH-2,3,3',4',5-pentaCB metaboliet en in mindere mate de 4-OH-2',3,3',4',5-pentaCB metaboliet in het plasma van zeehonden en de mens, en de 4-OH-2,2',3,4',5,5',6-heptaCB metaboliet in menselijk plasma. Dus de gehydroxyleerde PCB metabolieten die *in vivo* aangetoond zijn, zowel na experimentele als achtergrond- of milieu-blootstelling, voldoen volledig aan de structurele voorwaarden voor binding aan TTR. De karakteristieke patronen van PHAK metabolieten in het plasma van mens en dier na experimentele of achtergrond-blootstellling, zijn soort-specifiek en hangen ni*et al*leen af van de structurele vereisten voor binding aan TTR maar eveneens van de blootstellingssituatie en de mogelijkheid tot biotransformatie van PHAK's van de diersoort.

Het soort-specifieke metabolisme van PHAK's neemt af in de volgorde: landzoogdieren > zeezoogdieren > vogels > vissen (Safe, 1989). Verscheidene zoogdier- en vogel-soorten, zoals ratten, zeehonden, dolfijnen en eidereenden kunnen gehydroxyleerde metabolieten vormen van het modelsubstraat 3,3',4,4'-tetraCB (TCB) in *in vitro* micocomale incubaties. Vissen, zoals de forel en de bot, kunnen TCB echter niet metaboliseren, hoewel het cytochroom P4501A isoenzym dat verantwoordelijk is voor de biotransformatie van planaire PHAK's, wel geinduceerd kan zijn (Murk *et al.*, 1994, Morse *et al.*, 1995a, Ishida *et al.*, 1991).

De selectieve retentie van specifieke gehydroxyleerde PHAK metabolieten in plasma door de binding aan TTR wordt voornamelijk verwacht in soorten die zowel

PHAK's kunnen omzetten en in het bezit zijn van TTR als transport-eiwit voor schildklierhormonen in plasma. TTR is een, door de evolutie heen, geconserveerd eiwit dat aanwezig in het plasma van knaagdieren, maar ook van andere zoogdieren, vogels en in mindere mate van reptielen. In de lagere diersoorten zoals vissen en amfibiëen werd echter geen TTR in plasma aangetroffen. In de hogere diersoorten zoals de mens, is naast TTR nog een ander, belangrijker transport eiwit in plasma aanwezig, het thyroxine bindend globuline (TBG). Concluderend kunnen de gehydroxyleerde PHAK metabolieten in een groot aantal diersoorten gevormd worden en selectief in plasma aanwezig blijven door binding aan TTR.

De toxicologische gevolgen van de selectieve retentie van gehydroxyleerde PHAK metabolieten in plasma door binding aan TTR, zijn nog niet volledig bekend. Het TTR eiwit speelt een primaire rol bij het transport van schildklierhormonen in het bloed van vele diersoorten. Hoewel TTR minder T_4 bindt dan TBG in humaan serum, is TTR, door de lagere bindingsaffiniteit, waarschijnlijk verantwoordelijk voor het merendeel van de directe levering van T_4 en T_3 aan cellen. Verder is TTR belangrijk voor het transport van retinol in het bloed door het vorming van een complex met het retinol bindend eiwit (RBP) (Robbins, 1991).

In verschillende diersoorten die experimenteel of in het milieu blootgesteld zijn aan PHAK's, zoals knaagdieren, zeehonden en de mens, zijn veranderingen in schildklierhormoon concentraties in plasma aangetoond, terwijl in deze soorten eveneens gehydroxyleerde PHAK metabolieten in het plasma aangetoond zijn. Verstoringen in de schildklierhormoon-homeostase door blootstelling aan PHAK's kunnen echter niet alleen veroorzaakt worden door het verstoorde plasma T_4 transport door competitieve binding van gehydroxyleerde PHAK metabolieten aan TTR , maar eveneens door de Ah-receptor gemedieerde inductie van T_4 glucuronidering door de PHAK uitgangsstoffen.

De mogelijke verstoring van het TTR-RBP complex na binding van een gehydroxyleerde PHAK metaboliet zou eveneens een sterke daling van de retinolgehaltes in het plasma van knaagdieren teweeg kunnen brengen (Brouwer, 1987). Er werd gesuggereerd dat zeehonden die blootgesteld zijn aan PHAK's in het milieu, een verzwakt immuunsysteem bezitten (De Swart, 1995), wat een mogelijk gevolg zou kunnen zijn van verstoorde retinoiden gehaltes (Brouwer, 1991, Brouwer *et al.*, 1989). Gehydroxyleerde PHAK metabolieten kunnen aan deze effecten van PHAK's op de schildklierhormoon- en retinoiden-homeostase bijdragen door interacties aan te gaan met TTR in het plasma. Het is onduidelijk of deze effecten voorkomen in de mens.

TTR is het belangrijkste schildklierhormoon bindende eiwit in de cerebro-spinale vloeistof (CSF) zodat gesuggereerd wordt dat TTR een rol speelt in de distributie van schildklierhormonen in het centraal zenuwstelsel. Dit TTR wordt in de choroid plexus geproduceerd en is, zelfs zeer vroeg in de ontwikkeling, in hoge concentraties aanwezig in het CSF van ratten en mensen. Bovendien is in alle diersoorten waar TTR aanwezig is in plasma, ook TTR in de hersenen aangetoond. Omdat TTR een belangrijke leverencier is van T_4 naar de doelweefsels, zoals de hersenen, kan men verwachten dat TTR eveneens als een gefaciliteerd transport systeem voor gehydroxyleerde PHAK metabolieten werkt. Dit is in overeenstemming met de aangetoonde sterke accumulatie van gehydroxyleerde PCB metabolieten, afkomstig van de moeder, in het plasma en de hersenen van foetussen laat in de gestatie, in zwangere ratten en muizen die blootgesteld zijn aan PCBs (Morse *et al.*, 1995b,d, Darnerud *et al.*, 1995). In rattefoetussen, perinataal blootgesteld aan Aroclor 1254, leidde de ophoping van de 4-OH-2,3,3',4',5-

pentaCB metaboliet in maternaal plasma en foetaal plasma en hersenen, tot dalingen in T_4 concentraties in de hersenen, terwijl de T_3 concentraties in de hersenen slechts weinig veranderde. Bovendien werden verlagingen in de plasma en lever retinolconcentraties waargenomen in de foetale en neonatale nakomelingen (Morse *et al.*, 1995a). De gehydroxyleerde metabolieten die tot hoge concentraties in foetale rattehersenen accumuleerden, zouden kunnen bijdragen aan de neurochemische veranderingen in de foetale hersenen die waargenomen werden na blootstelling van zwangere ratten aan Aroclor 1254 (Morse, 1995).

Gehydroxyleerde PHAK metabolieten bezitten *in vitro* bepaalde biologische activiteiten (Brouwer, 1994). Gehydroxyleerde PCB metabolieten kunnen bijvoorbeeld de functie van mitochondria verstoren (Lans *et al.*, 1990, Narashimhan *et al.*, 1991). Bovendien kunnen ze binden aan de Ah-receptor en in lichte mate de cytochrome P450 1A1 activiteit induceren. Daarnaast kan een *in vitro* marker voor tumor promotie werking, de gap-junction intercellulaire communicatie, zwak geremd worden door gehydroxyleerde PCB metabolieten. Gehydroxyleerde PCB metaboieten vertonen eveneens een (anti)-estrogene werking, zowel *in vivo* (Bergeron *et al.*, 1994) als *in vitro* (Kramer *et al.*, 1994). Er is geen duidelijk structuur activiteitsrelatie gevonden voor de (anti-)estrogeniteit van de geteste gehydroxyleerde PCB metabolieten. De gehydroxyleerde PCB metaboliet die selectief in het plasma en de hersenen van rattefoetussen ophoopt, na blootstelling van zwangere ratten aan Aroclor 1254 (Morse *et al.*, 1995d), heeft echter een zwak (anti-)estrogene activiteit. De intrinsieke eigenschap om endocriene systemen, zoals schildklierhormonen en estrogenen, te verstoren en de relatief grote hoeveelheden gehydroxyleerde PCB metabolieten die accumuleren in de foetussen laat in de gestatie, zouden kunnen betekenen dat er een potentieel risico bestaat voor verstoringen van de ontwikkeling door gehydroxyleerde PHAK's. Deze mogelijke route van ontwikkelingstoxiciteit door gehydroxyleerde PHAK metabolieten zou in een degelijk *in vivo* experiment onderzocht moeten worden.

Subtiele veranderingen in plasma schildklierhormoon concentraties en parameters voor neurologische ontwikkeling werden eveneens in kinderen gevonden die in de baarmoeder en via lactatie blootgesteld waren aan achtergrondniveau's van PHAK's (Koopman-Esseboom *et al.*, 1994, Sauer *et al.*, 1994, Pluim *et al.*, 1993). Hoewel gehydroxyleerde PHAK metabolieten niet binden aan TBG, het belangrijkste T_4 bindende eiwit in humaan plasma (Lans *et al.*, 1994), zijn de gehydroxyleerde PCB metabolieten die recentelijk in humaan plasma aangetoond zijn (Bergman *et al.*, 1994) voornamelijk gebonden aan het TTR, zoals werd gevonden na de opzuivering van TTR uit humaan serum (ongepubliceerde resutaten). De ophoping van gehydroxyleerde PCB metabolieten of verwante stoffen in foetaal plasma en hersenen door binding aan TTR en de daaropvolgende verlagingen in plasma T_4 concentraties, zoals gevonden is in rattefoetussen laat in de gestatie, kunnen daarom zorgwekkend zijn voor de foetale groei en (hersen)ontwikkeling van een groot aantal diersoorten, waaronder de mens.

Appendix

List of abbreviations

3MC	3-methylcholantrene
Ah-receptor	Aryl hydrocarbon receptor
AHH	aryl hydrocarbon hydroxylase
CSF	cerebrospinal fluid
diOH-TCB	4,4'-$(OH)_2$-3,3',5,5'-tetrachlorobiphenyl
DIT	diiodo-tyrosine
DRE	dioxin-responsive element/enhancer
DTT	dithiotreitol
EROD	ethoxyresorufin-O-deethylase
ID-I	type-1 iodothyronine deiodinase
ID-II	type-2 iodothyronine deiodinase
ID-III	type-3 iodothyronine deiodinase
IRD	inner ring deiodination
MFO	mixed function oxidases
MIT	monoiodo-tyrosine
OH-PCB	hydroxy-PCB metabolite
ORD	outer ring deiodination
PAGE	poly-acrylamide gel electrophoresis
PB	phenobarbital
PBB	polybromo-biphenyl
PCB	polychloro-biphenyl
PCDD	polychloro-dibenzo-*p*-dioxin
PCDF	polychloro-dibenzofuran
PCP	pentachlorophenol
PHAH	polyhalogenated aromatic hydrocarbon
RBP	retinol-binding protein
rT_3	3,3',5'-triiodo-L-thyronine, reverse T_3
T_3	3,3',5-triiodo-L-thyronine, triiodothyronine
T_4	3,3',5,5'-tetraiodo-L-thyronine, thyroxine
TBG	thyroxine binding globulin
TBPA	thyroxine binding prealbumin (=TTR)
TCB	3,3',4,4'-tetrachlorobiphenyl
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TEF	toxic equivalency factor
TEQ	toxic equivalency
TIP	2,4,6-triiodophenol
TR	thyroid hormone receptor
TRE	thyroid hormone response element
TRH	thyrotropin releasing hormone
TSH	thyroid stimulating hormone
TTR	transthyretin
Tyr	tyrosine
UDPGA	uridinyl-5'-diphosphoglucuronic acid
UGT	UDP-glucuronyl-transferase

Curriculum Vitae

Martine Lans was born in Amersfoort, the Netherlands, on October 4, 1964 where she lived until 1976, and where she returned in 1978 after a 2 years stay in Jakarta, Indonesia. In May 1983 she graduated from the Eemland-College in Amersfoort and in September she started to study Biology at the Agricultural University Wageningen. In March 1989 she graduated for her M.Sc. in Biology with specialisations in Biochemistry and Toxicology. In April of the same year she started working as a Ph.D. student on a research project investigating the possible role of interactions of polyhalogenated aromatic hydrocarbons and their hydroxylated metabolites with thyroid hormone binding proteins at the Department of Toxicology, Agricultural University Wageningen, and in collaboration with the RITOX (Research Institute of Toxicology, University of Utrecht) under supervision of dr. A. Brouwer and dr. M. van den Berg. Between 1989 and 1993, she also attended the Postgraduate training in Toxicology. In August 1993, she continued doing research as a research scientist at the Department of Toxicology, on an Biomed project financed by the European Community, that investigated the role of mutations on the conformation of the transthyretin protein in hereditary diseases called familiar amyloidogenic polyneuropathies. This multidisciplinary project was under coordination of dr. M.J.M. Saraiva, Centro de Paramiloidose in Porto, Portugal and in collaboration with dr. C.C.F. Blake, Laboratory of Molecular Biophysics, Oxford, Great-Britain, Dr. A. Damas, University of Porto, Portugal and dr. E. Lundgren, University of Umea, Sweden. Subsequently she was employed in February 1995 by the Gezondheidsraad as a scientific advisor to the secretary of the Advisatory committee on the risk assessment of polychlorinated dibenzo-p-dioxins. This thesis is based on research that was conducted between 1989 and 1994.

List of publications

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