Stellingen

behorende bij het proefschrift "Interactions of polyhalogenated aromatic hydrocarbons with thyroid hormone metabolism" van Gerlienke Schuur
te verdedigen op dinsdag 17 november 1998

1. De schildklierhormoonverlaging die gevonden wordt in plasma van proefdieren na blootstelling aan sommige organohalogenen is niet betrokken bij een toename in de omzetting van deze organohalogenen.
(*dit proefschrift*)

2. De expressie en activiteit van cytochroom P4501A worden niet gereguleerd door schildklierhormoon.
(*dit proefschrift*)

3. Ondanks het feit dat blootstelling aan organohalogenen effecten heeft op verschillende routes van schildklierhormoon-metabolisme, wordt de concentratie van aktief schildklierhormoon in de hersenen niet aangetast.
(*dit proefschrift, proefschrift D.C. Morse*)

4. Pentachloorfenol is behalve een goede sulfateringsremmer ook in staat de hoeveelheid sulfotransferase enzym in de lever van ratten te verhogen.
(*dit proefschrift*)

5. In het onderzoek naar pseudo-oestrogenen worden de mogelijke effecten op het metabolisme van oestrogenen onderschat.

6. De omvang van onderzoek naar hormoonverstorende effecten van industriële chemicaliën is in tegenspraak met de veel grotere blootstelling aan natuurlijke hormoonverstorende stoffen via het dieet.

7. De remmende werking van stoffen uit rode wijn op fenolsulfotransferase activiteit kan hoofdpijn veroorzaken.
(*J.T. Littlewood* et al., *Lancet 1 (8585), p. 558, 1988 and A.L. Jones* et al., *Eur. J. Clin. Pharmacol. 49, p. 109, 1995*)

8. Een milde jodide deficiëntie, onder andere veroorzaakt door het eten van te weinig brood, kan mogelijk effecten hebben op de foetale ontwikkeling bij de algemene populatie.
(*J.G. Hollowell & W.H. Hannon, Teratology 55, p. 389, 1997)*

9. TCDD is niet alleen vanuit toxicologische oogpunt een interessante stof maar is daarnaast een handvat voor het onderzoeken van vele werkingsmechanismen.
(*M. Holloway, Scientific American, november, p. 16, 1990*)

10. Een RARE ("retinoic acid response element") is niet zo zeldzaam of vreemd als het klinkt.

11. De bezuiniging, die via de studietijdverkorting is bereikt, resulteert later in een hogere uitgave van de overheid aan ziekteverzuim en werkloosheidsuitkeringen.
(naar aanleiding van een Loesje poster "Twijfelen doe je maar na je studie")

12. Het goed bedoelde advies van de overheid aan vrouwen om kinderen voor je dertigste te krijgen is in conflict met de eerder gebrachte slogan "een slimme meid is op haar toekomst voorbereid".

13. Het toenemend gebruik van Email is de oorzaak van meer spelfouten en een verslechtering van de stijl door de gebruikers.

14. Naamswijzigingen binnen een organisatie zijn vaak een aanwijzing voor bezuinigingen.

15. Nu vaatwassers zuinig zijn met water en energie, is de mogelijkheid van het houden van een goed gesprek tijdens de afwas een laatste argument om ze niet aan te schaffen.
(nav Volkskrant 1 maart 1997)

INTERACTIONS OF POLYHALOGENATED AROMATIC HYDROCARBONS WITH THYROID HORMONE METABOLISM

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INTERACTIONS OF POLYHALOGENATED AROMATIC HYDROCARBONS WITH THYROID HORMONE METABOLISM

Aline Gerda Schuur

Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van de Landbouwuniversiteit Wageningen,
dr. C.M. Karssen,
in het openbaar te verdedigen
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des namiddags te vier uur in de Aula.

The research described in this thesis was carried out at the Department of Toxicology, Agricultural University Wageningen, The Netherlands in cooperation with the Department of Internal Medicine III, Erasmus University Medical School, Rotterdam, The Netherlands.

Title Interactions of polyhalogenated aromatic hydrocarbons with thyroid hormone metabolism

Author A.G. Schuur

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Science should be made as simple as possible
but not simpler.

Albert Einstein

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

The research described in this thesis concerns the possible interactive effects between thyroid hormones and polyhalogenated aromatic hydrocarbons (PHAHs), with emphasis on metabolism. In particular, the possible regulatory role of thyroid hormone on PHAH-induced biotransformation enzymes. Secondly, the possible inhibition of thyroid hormone sulfation by hydroxylated PHAHs was investigated. This research was performed at the department of Toxicology, Agricultural University Wageningen in close collaboration with the department of Internal Medicine III of the Erasmus University Rotterdam.

In this *Chapter*, some information on the toxic effects of PHAHs is given, followed by an introduction into thyroid hormone metabolism. In addition, the effects of PHAHs on thyroid hormone metabolism are discussed. Finally, the outline of the thesis is given.

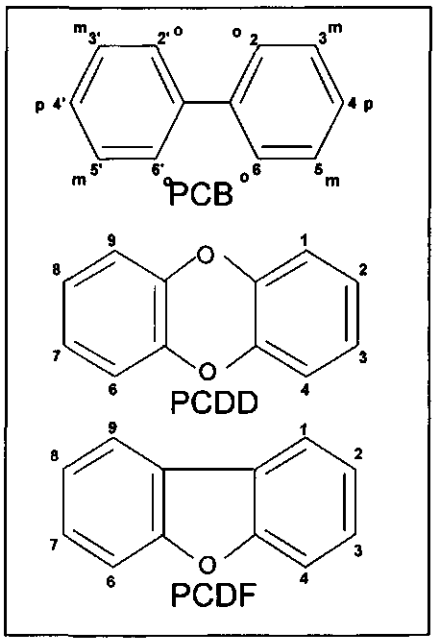

Figure 1.1 The chemical structures of PCBs, PCDDs and PCDFs.

Polyhalogenated aromatic hydrocarbons (PHAHs)

Polyhalogenated aromatic hydrocarbons such as polychlorinated biphenyls (PCBs), dibenzo-p-dioxins (PCDDs) and dibenzofurans (PCDFs) are persistent environmental pollutants (for general structures see Figure 1.1). PCBs were used as plasticizers, flame retardants and as dielectric fluids in transformers and capacitors, but are banned since the early eighties (De Voogt and Brinkman, 1989). PCDDs and PCDFs are formed as unwanted byproducts during organochlorine synthesis or during waste incineration, but recent measures taken have reduced their emissions. All PCB, PCDD and PCDF structures consist of two halogenated phenyl rings, with respectively 209, 75 and 135 possible congeners dependent on the degree and position of chlorine substituents.

PCDDs and PCDFs are rigid planar structures, while the phenyl rings of the PCBs can rotate along the central C-C bond.

PHAHs are highly lipophilic and extremely resistant to breakdown by acids, alkali, heat, and hydrolysis. Therefore, they are very persistent and widely present in the environment, including fish, wildlife and humans. The accumulation of PHAHs depends on their metabolism, which is dependent on the degree of chlorination (Safe, 1989). Hydroxylation by cytochrome P450 enzymes is the main route of metabolism of PHAHs and occurs preferentially at the para position. The presence of two vicinal unsubstituted carbon atoms facilitates oxidative metabolism. Hydroxylated metabolites can be further metabolized to glucuronic acid or sulfate conjugates. The formation of mercapturic acid metabolites of PCBs is thought to start with the reaction of glutathione with an PCB-arene oxide, followed by metabolization to the cysteine-adduct, and thiol-adduct. This thiol-adduct is converted to the methylthio-derivative, followed by oxidation to first a methylsulfinyl- and ultimately to a methylsulfonyl-metabolite. (Sipes and Schnellmann, 1987; Safe, 1989)

PHAHs induce a number of toxic effects, including body weight loss, thymic atrophy, impairment of immune responses (Tryphonas, 1994), hepatoxicity, chloracne and related dermal lesions, tissue-specific hypo- and hyperplastic responses, carcinogenesis, developmental toxicity (Peterson *et al.*, 1993), neurobehavioral responses and neurotoxicity (Seegal, 1996; Schantz, 1996), teratogenicity, and reproductive toxicity (Peterson *et al*, 1993). These toxic effects are congener-, species-, sex- or age-dependent (Safe, 1990; 1994; DeVito and Birnbaum, 1994). In Table 1.1, biochemical effects caused by PHAHs are summarized.

Table 1.1 PHAH induced biochemical effects.

induction of CYP1A1, CYP1A2, and CYP4A1
suppression of CYP2C11
induction of GSTs
induction of UDP-glucuronyl transferases
increased cytochrome P450 levels
induction of epoxide hydrolase
inhibition of uroporphyrinogen decarboxylase activity
induction of ALA synthetase activity
decreased ALA dehydratase
decreased thyroxine levels
decreased hepatic/ plasma vitamin A levels
induction of c-Ha-ras, c-raf, C-yes, c-erbA and c-erbB protooncogen
increased serum lipids and HMG CoA reductase
decreased serum chlolesterol
increased aldehyde dehydrogenase
increased serum SGPT, SGOT

The common mechanism of action for PHAH toxicity is generally accepted to be the Ah receptor pathway (Poland and Knutson, 1982; Okey *et al.*, 1994). PCDDs, PCDFs and all planar PCBs have been shown to be ligands of this cytosolic receptor. After binding of a ligand, the Ah receptor complex loses its heat shock proteins and it is transported together with the Ah receptor nuclear translocator (ARNT) protein into the nucleus where it binds to specific DNA enhancer sequences, the dioxin response elements (DRE). This binding results in the increased expression of certain proteins, including CYP1A1/2, UGT1A6 (Owens, 1977; Münzel *et al.*, 1996) and GST1 (Pimental *et al.*, 1993) (Safe, 1995). Planar structures such as 2,3,7,8-chlorine substitution of PCDDs and PCDFs, non-ortho substituted PCBs as well as mono-ortho PCBs are shown to be Ah-receptor agonists (Safe, 1992).

Non-planar, di- to tetra-ortho PCBs which do not exhibit Ah-receptor agonistic activity, and have been shown to induce CYP2B and the UGT1A2 isozyme (Safe, 1995). These phenobarbital-like PCBs induce toxic effects such as alterations in plasma thyroid hormone (McClain *et al.*, 1989; Barter and Klaassen, 1992a; 1994), neurotoxicity (Seegal *et al.*, 1990; Shain *et al.*, 1991), and tumor promotion (Silberhorn *et al.*, 1990). The mono-ortho substituted PCBs are able to induce both PB-like and Ah-receptor-like biotransformation enzymes.

In addition, effects caused by hydroxylated PHAH metabolites may add to the spectrum of toxic effects. These effects include 1) competitive inhibition of thyroxine (T4) binding to transthyretin (TTR), a thyroid hormone transport protein, as demonstrated *in vitro* (McKinney *et al.*, 1985; Rickenbacher *et al.*, 1986; Lans *et al.*, 1993; 1994) and *in vivo* (Brouwer and van den Berg, 1986; Lans, 1995), 2) reduction of vitamin A and retinol-binding protein (RBP) levels in rat plasma as a result of the binding of PHAH-OHs to TTR, which causes a weakening of the plasma protein TTR-RBP complex carrying both retinol and T4, (Brouwer *et al.*, 1988), 3) uncoupling of mitochrondrial phosphorylation (Lans *et al.*, 1990; Narasimhan *et al.*, 1991), 4) estrogenic or anti-estrogenic activity (Korach *et al.*, 1988; Connor *et al.*, 1997; Fielden *et al.*, 1997), 5) inhibition of gap junctional intercellular communication (GJIC) (De Haan *et al.*, 1994), and 6) inhibition of cytochrome P450 activities. For methylsulfonyl PHAH metabolites also some distinct effects have been shown, such as 1) induction of cytochrome P450 activities (Kato *et al.*, 1995) and 2) the binding to a specific protein in lung (Stripp *et al.*, 1996).

Thyroid hormone metabolism

Thyroid hormone is essential for the normal fetal development of several organs, and for the regulation of growth and basal metabolism throughout life. The active hormone is 3,3',5-triiodothyronine (T3), which acts through binding to a nuclear thyroid hormone receptor, followed by binding to a thyroid hormone responsive element (TRE). Its precursor, thyroxine

(T4) is produced mostly by the thyroid gland and is transported via plasma to the various tissues. Because of the importance of thyroid hormone in numerous processes, the intracellular thyroid hormone levels are tightly regulated in various ways, e.g. plasma transport, cellular metabolism and synthesis and feed-back regulation via the hypothalamus-pituitary-thyroid gland axis. The metabolism of iodothyronines is mediated by several different metabolic enzyme systems: 1) deiodinases, 2) UDP-glucuronyltransferases, and 3) sulfotransferases (Visser, 1990), as is presented in Figure 1.2.

The first metabolic pathway is deiodination, which has been reviewed several times (Köhrle *et al.*, 1987; Visser, 1990; Braverman, 1994; Köhrle, 1996; Larsen, 1997; St. Germain and Galton, 1997). Three types of membrane-bound deiodinases are known. Type I deiodinase (D1) is present especially in liver, but also in kidney and thyroid. D1 is able to deiodinate both the outer and inner ring of iodothyronines, with rT3 as the preferred substrate. It is a selenocysteine-containing enzyme, and can be inhibited by 6-propylthiouracil (PTU). It is most important for the production of plasma T3 levels and for the removal of plasma rT3. In hypothyroidism, D1 activity is decreased, following a reduced T3 concentration in plasma and tissue.

Type II deiodinase (D2) is present in the CNS, pituitary, brown fat, placenta, and in humans in thyroid, skeletal muscle and heart (only proven by mRNA detection). It deiodinates only the outer ring of thyroid hormones, with T4 as the preferred substrate. D2 is also an selenocysteine-containing enzyme, but is insensitive to PTU. Its function is to provide intercellular T3 in brain and pituitary. During hypothyroidism, D2 activity is increased, which can be returned to normal only with T4 infusion (Escobar-Morreale *et al.*, 1997).

Type III deiodinase (D3) is present in CNS, placenta, skin and in fetal tissues. It performs only inner ring deiodination, with T3 and T4 as the preferred substrates. The function of D3 is predominantly the degradation of T3 and T4, thereby regulating local T3 levels.

Iodothyronines are also conjugated by glucuronidation or sulfation. Glucuronic acid delivered by the cofactor uridine diphosphate (UDP)-glucuronic acid (UDPGA) is transferred to the hydroxyl group of iodothyronines. Glucuronidation is catalysed by membrane bound UDP-glucuronyltransferases (UGTs). So far, three different UGT isozymes are known to be involved in glucuronidation of iodothyronines. Phenol UGT preferentially glucuronidates rT3, but is also able to glucuronidate T4. Bilirubin UGT also conjugates T4 and rT3. Finally, androsterone UGT prefers T3 as a substrate. When iodothyronines are glucuronidated, they are excreted into the bile. After hydrolysis in the intestine by β-glucuronidases, at least part of the iodothyronines is reabsorbed. (Visser, 1990; 1994a)

A second conjugation reaction is sulfation. Sulfotransferases represent a family of homologous enzymes which catalyse the sulfation of various compounds with overlapping substrate specificity, using 3'-phosphoadenosine-5'-phosphosulfate (PAPS) as the sulfate donor. Sulfotransferases are soluble isozymes, some of which exist as monomer and others as

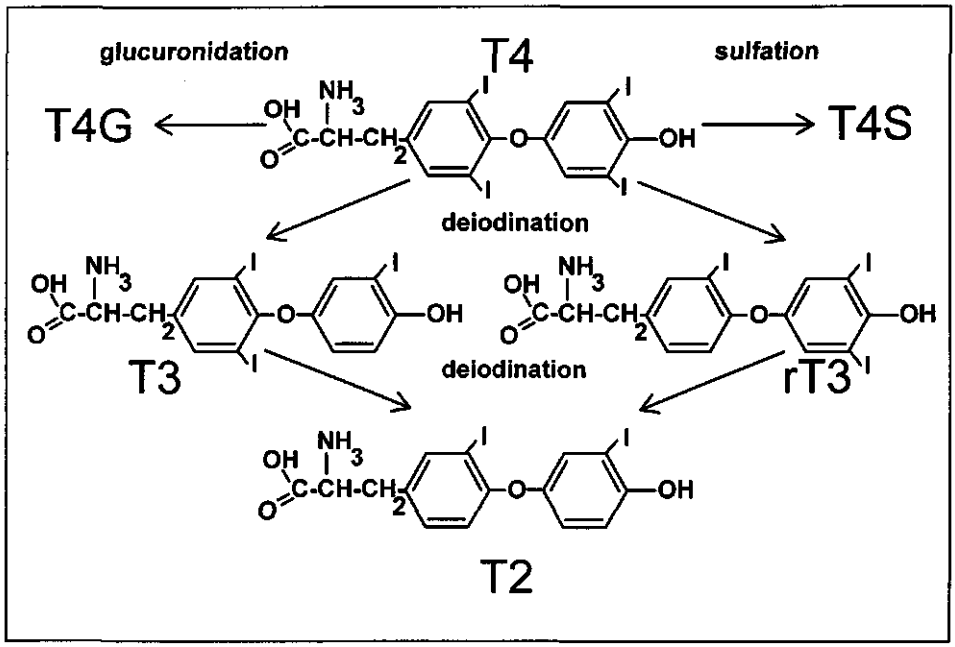

Figure 1.2 Thyroid hormone metabolism pathways. Glucuronidation and sulfation is only presented for T4, but T3, rT3 and T2 are also conjugated.

homodimer or even multimeric complexes, which are localized in the cytoplasm of different tissues (Matsui and Homma, 1994). Iodothyronines are sulfated by phenol sulfotransferases, in the order 3,3-T2 >> T3 > rT3 > T4 by rat liver. *In vitro*, it was demonstrated that the sulfotransferase isozymes rat SULT1C1 and human SULT1A1 and SULT1A3 were able to sulfate iodothyronines (Visser *et al.*, 1996a; 1998a/b).

Small amounts of thyroid hormone sulfates appear normally in bile or serum, because they are rapidly deiodinated in the liver. The inner ring deiodination by D1 is strongly enhanced for iodothyronine sulfates compared to non-sulfated iodothyronines themselves. However, high concentrations of iodothyronines sulfates have been found in plasma, bile and amniotic fluid of fetal sheep and humans (Chopra *et al.*, 1992; Wu *et al.*, 1992a/b; 1993; 1995; Santini *et al.*, 1993; 1994; Polk *et al.*, 1994). When D1 activity is low, e.g. during fetal development, non-thyroidal illness or fasting in rats, iodothyronine sulfate levels are increased. Non-sulfated hormones may then be recovered by sulfatase activity in tissues. This is suggested to play an important role in regulating thyroid hormone levels during fetal development (Santini *et al.*, 1992a). (Visser, 1990; 1994b)

Different enzymes important in carbohydrate metabolism are regulated by thyroid status. Examples of such thyroid hormone-dependent enzymes are malic enzyme activity and α-glycerolphosphate dehydrogenase activity, both have been demonstrated to possess a TRE

in their promoter sequence (Oppenheimer *et al.*, 1977; Pellizas *et al.*, 1996). These activities can be used as a measure for thyroid hormone status in the tissue.

Effects of PHAHs on thyroid hormone metabolism

One aspect of the toxicity of PHAHs is their effect on thyroid hormone levels and metabolism. PHAHs are known to affect the thyroid hormone system on at least three different levels: 1) thyroidal hormone synthesis, 2) plasma transport, and 3) hepatic metabolism (for review, see Brouwer *et al.*, 1998).

Exposure to PHAHs results in a decrease in plasma total and free T4 levels in rats, mice and marmoset monkeys. This has been observed with TCDD (Bastomsky, 1977; Potter *et al.*, 1983, 1986; Pazdernik and Rozman, 1985; Henry and Gasiewicz, 1986, 1987; Jones *et al.*, 1987; Roth *et al.*, 1988; Pohjanvirta *et al.*, 1989; Lans *et al.*, 1990), Aroclor 1254 (Bastomsky, 1974; Byrne *et al.*, 1987; Brouwer, 1989; Beetstra *et al.*, 1991; Gray *et al.*, 1993) and PBBs (Gupta *et al.*, 1983). However, the T3 levels were sometimes reported to be increased (Bastomsky, 1977; Potter *et al.*, 1986) or decreased (Pazdernik and Rozman, 1985), but most reports show that serum T3 is unchanged after PHAH treatment (Henry and Gasiewicz, 1987; Gorski and Rozman, 1987; Jones *et al.*, 1987; Roth *et al.*, 1988; Muzi *et al.*, 1989; Lans, 1995; Morse, 1996).

Firstly, PCBs and PCDDs could act directly on the thyroid gland. Gupta *et al.* (1973) and Rozman *et al.* (1986) described degenerative and necrotic changes in thyroid follicles in rats after TCDD treatment. Aroclor 1254 also showed effects on thyroid function and morphology in Gunn rats, and adult cynomolgus monkeys (Collins *et al.*, 1977, Collins and Capen, 1980a/b/c; Sepkovic and Byrne, 1984; Tryphonas *et al.*, 1984). The effects of PHAHs on thyroid gland function may be direct or secondary to decreases in plasma thyroid hormone levels, resulting in an increased TSH stimulation (suggested by Ness *et al*, 1993). However, the ultrastructural and functional alteration of rat thyroid glands after Aroclor 1254 exposure were dissimilar to thyroid gland alteration found after iodide excess or deficiency, or after a TSH or T4 administration (Collins and Capen, 1980c), suggesting an additional or specific effect of Aroclor 1254 exposure.

Secondly, PHAHs are able to interfere with plasma thyroid hormone transport. McKinney *et al.* (1985) and Rickenbacher *et al.* (1986) described interactions of PHAHs and hydroxylated metabolites with TTR using *in vitro* binding studies and modelling studies. Brouwer and Van den Berg (1986) demonstrated a mechanism based on disturbed plasma transport for the decrease in plasma T4 levels in rats exposed to PCB77, a coplanar PCB congener. Lans *et al.* (1993; 1994) demonstrated that *in vitro* PHAH-OHs are able to inhibit T4 binding to TTR, but not to thyroxine-binding globulin (TBG). Lans (1995) confirmed the

binding of a PCB-OH to TTR, both by X-ray diffraction analysis of a crystal of the TTR-PCB-OH complex and computer-assisted modelling studies.

Finally, PHAHs are known for their modulating effects on thyroid hormone metabolism. Glucuronidation can be induced by PHAHs; planar and mono-ortho PCBs have been shown in rats to induce the phenol type (UGT1A6), while PB and PB-like inducers have been shown to induce the bilirubin type (UGT1A2) (Rozman *et al.*, 1985a/b, 1988; Henry and Gasiewicz, 1987; Beetstra *et al.*, 1991; Saito *et al.*, 1991; De Sandro *et al.*, 1992; Eltom *et al.*, 1992; Barter and Klaassen, 1992a/b; 1994; Visser *et al.*, 1993a; Van Birgelen *et al.*, 1995). In Gunn rats, which are deficient in UGT activity towards bilirubin, phenolic compounds and T4 (Visser *et al.*, 1993a), the T4 UGT activity could be induced by Aroclor 1254 to some extent in the heterozygous rats but not in the homozygous rats. However, the decrease in plasma T4 levels were similar in both strains, suggesting additional effects of the compound (Collins and Capen, 1980a).

D1 activity in liver has been shown to be decreased *in vivo* following treatment of rats with several Ah-receptor agonists, such as 3-methylcholanthrene, 3,3',4,4'-TCB, TCDD and Aroclor 1254 (Adams *et al.*, 1990; Eltom *et al*, 1992; Visser *et al.*, 1993a; Lans, 1995; Raasmaja *et al.*, 1996), but also after PB treatment (De Sandro *et al.*, 1991). Hydroxylated PHAH metabolites also inhibit D1 activity *in vitro* using rat hepatic microsomes (Rickenbacher *et al.*, 1989; Adams *et al.*, 1990; Lans, 1995). In addition, brain D2 activity is increased in fetal and neonatal rats exposed to 3,3',4,4',5,5'-HCB or Aroclor 1254 (Morse *et al.*, 1993; 1996). This increase is probably mediated by the severely reduced plasma and brain T4 levels.

Outline of this thesis

The major aim of this study is focussed on the possible interactions between thyroid hormones and PHAHs, with particular emphasis on metabolism. Two separate research questions were put forward and studied.

Question I. Is it possible that the PHAH-induced decrease in plasma T4 is a mechanism to regulate the induction of biotransformation enzymes that metabolize the PHAHs?

PHAH exposure results in the induction of biotransformation enzymes such as cytochrome P450 (CYP), glutathione S-transferases (GSTs) and UDP-glucuronyltransferases (UGTs). The mechanism for the induction is general accepted to be the Ah-receptor pathway. However, not all toxic effects caused by PHAH can be explained by this mechanism, such as hypovitaminosis A and neurotoxicity.

Exposure to PHAHs also results in lower plasma T4 levels, while many of the signs of TCDD-induced toxicity resemble those observed for thyroid dysfunction, including body

weight loss, dermal toxicity and changes in cholesterol and triglyceride levels. It was demonstrated that thyroidectomy (Rozman *et al.*, 1984; 1985a/b) offers some protection against mortality. Furthermore, it was reported that thyroid hormone is able to regulate cytochrome P450 isozymes (Kato and Takahashi *et al.*, 1968; Rumbaugh *et al.*, 1978; Leakey *et al.*, 1982; Müller *et al.*, 1983a/b; Skett, 1987; Yamazoe *et al.*, 1989; Arlotto and Parkinson, 1989), UGTs (Chowdhury *et al.*, 1983; Moscioni and Gartner, 1983; Pennington *et al.*, 1988; Goudonnet *et al.*, 1990), and GSTs (Williams *et al.*, 1986; Pimental *et al.*, 1993). Therefore, we proposed that the induction of biotransformation enzymes by PHAHs via the Ah-receptor route is modulated by the PHAH-induced decreases in thyroid hormone levels.

Approach: Thyroidectomized (Tx) rats, Tx rats replaced with T3 or T4 and control rats were exposed to TCDD. Thyroid hormone serum levels together with thyroid hormone parameters such as hepatic D1 activity, hepatic malic enzyme activity and brain D2 activity were measured (*Chapter 2*). In this same animal experiment, biotransformation enzyme activities such as CYP1A1 activity, UGT activity, GST activity, and sulfotransferase activities were analysed for their possible regulation by thyroid state (*Chapter 3*).

To obtain more information about thyroid hormone regulation of different types of cytochrome P450, we designed an animal experiment with hypothyroid (Tx), euthyroid (Eu) and hyperthyroid (T3-treated) Sprague-Dawley rats and exposed them to a CYP1A inducer, PCB 126, and a CYP2B inducer, PCB 153. Thyroid hormone serum levels, thyroid hormone status parameters as well as CYP1A and CYP2B activities, protein and mRNA levels were investigated (*Chapter 4*).

Question II. Are hydroxylated PHAHs able to inhibit thyroid hormone sulfation *in vitro* as well as *in vivo*?

Hydroxylated metabolites of PHAHs are known for their competitive inhibition of thyroid hormone binding to proteins such as TTR and D1 (Rickenbacher *et al.*, 1989; Adams *et al.*, 1990; Lans *et al.*, 1993; 1994; Lans, 1995). Due to the structural resemblance of hydroxylated PHAHs with thyroid hormones, it was proposed that phenol sulfotransferase, which is another protein that binds iodothyronines, may be inhibited by PHAH-OHs.

Approach: To start, thyroid hormone sulfation was assayed *in vitro*, using rat liver cytosol as enzyme source and T2 as substrate in the presence of different hydroxylated PHAHs. Some structure-activity relationships of PHAH-OHs for T2 sulfation inhibition were investigated (*Chapter 5*). More information was obtained about the role of different isozymes in inhibition of thyroid hormone sulfation by PHAH-OHs, using different enzyme sources, e.g. cytosolic preparations with specific SULT isozymes. The nature of the T2 sulfotransferase activity inhibition by PHAH-OHs was also investigated (*Chapter 6*). Secondly, a rat and human hepatoma cell line were used to investigate the *in vitro* inhibition of T2 sulfation by PHAH-OHs in cells (*Chapter 7*). Finally, the possible inhibition of thyroid hormone sulfation was

tested *in vivo* in rats, using an experimental setup with prenatal exposure to PCBs or pentachlorophenol, which is a potent sulfotransferase inhibitor (*Chapter 8*).

PART I

THE EFFECTS OF THYROID HORMONE ON THE INDUCTION OF BIOTRANSFORMATION ENZYMES BY POLYHALOGENATED AROMATIC HYDROCARBONS

CHAPTER 2

EXTRATHYROIDAL EFFECTS OF 2,3,7,8-TETRACHLORODIBENZO-*P*-DIOXIN ON THYROID HORMONE TURNOVER IN MALE SPRAGUE-DAWLEY RATS

Abstract

Treatment of rats with different polyhalogenated aromatic hydrocarbons strongly decreases plasma T4, with little or no decrease in plasma T3. The extrathyroidal effects of TCDD on thyroid hormone turnover were studied by ip administration of a single dose of 10 μg TCDD/kg bw or vehicle (corn oil) to euthyroid (Eu) rats, thyroidectomized (Tx) rat, and Tx rats infused with 1 μg T4 (Tx+T4) or 0.4 μg T3 (Tx+T3) per 100 g bw per day by osmotic minipumps. Tx rats showed decreased plasma T4 and T3 and increased plasma TSH levels, decreased hepatic D1 and malic enzyme activities, and increased brain D2 activities. All parameters were largely restored to Eu levels in Tx+T4 rats and, except for plasma T4 and brain D2 activity, in Tx+T3 rats, validating the thyroid hormone-replaced Tx rats as models to study the peripheral effects of TCDD. Three days after TCDD administration, plasma T4 and FT4 levels were significantly reduced in Eu rats and in Tx+T4 rats, and plasma T3 was significantly reduced in Tx+T3, but not in Eu or Tx+T4 rats. Plasma TSH was not affected by TCDD in any group. Hepatic T4 UGT activity was induced approximately 5-fold by TCDD while T3 UGT activity was only increased by about 20% (p=NS) in the different groups. TCDD produced an insignificant decrease in liver D1 activity in Tx rats and an insignificant increase in brain D2 activity in Tx rats and hormone-replaced Tx rats. Hepatic malic enzyme activity was significantly increased by TCDD in all groups, except Tx rats. These results strongly suggest that the thyroid hormone-decreasing effects of TCDD are predominantly extrathyroidal and mediated by the marked induction of hepatic T4 UGT activity.

based on A. Gerlienke Schuur, Franklin M. Boekhorst, Abraham Brouwer, and Theo J. Visser (1997). Extrathyroidal effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on thyroid hormone turnover in male Sprague-Dawley rats. Endocrinology ***128***, *3727-3734.*

Introduction

It is well known that PHAHs, such as polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs), alter thyroid hormone levels and metabolism in rodents. Administration of TCDD to rats or mice results in reduced plasma T4 levels (Potter *et al.*, 1983; 1986; Roth *et al.*, 1988). The mechanisms involved in the thyroid hormone-decreasing effects of PHAHs are still not fully understood. TCDD markedly increased the biliary clearance of T4 (Bastomsky, 1977), which is associated with an increase in hepatic T4 glucuronidation due to the profound induction of UGTs by PHAHs (Beetstra *et al.*, 1991; Saito *et al.*, 1991; De Sandro *et al.*, 1992; Barter and Klaassen, 1992a; Visser *et al.*, 1993a). On the other hand, several investigations have presented histological evidence for a direct damaging effect of PHAHs on the thyroid gland (Gupta *et al.*, 1973; Rozman *et al.*, 1986). Finally, hydroxylated metabolites of PHAHs were found to compete with T4 for binding to plasma transthyretin, resulting in an increased plasma free T4 fraction and, hence, a decreased plasma total T4. The latter effect is, however, highly dependent on the extent of metabolic conversion of PHAHs, and negligible for compounds such as TCDD (Brouwer *et al.*, 1990; Lans *et al.*, 1993). Perhaps, the most remarkable effect of PHAHs is their potent induction of hepatic detoxification enzymes, both phase I enzymes such as CYP isoenzymes, and phase II enzymes such as UGTs and GSTs (Safe, 1990; Sutter and Greenlee, 1992).

Thyroid hormones influence a variety of metabolic processes in most mammalian tissues, and may also have a significant effect on rates of drug metabolism. Studies of Rumbaugh *et al.* (1978) established a dose-dependent stimulating effect of thyroid hormones on hepatic mixed-function oxidases. CYP activities were lower in thyroidectomized (Tx) male and female Sprague-Dawley rats compared with those in euthyroid (Eu) controls. Substitution of Tx rats with T4 restored these activities to Eu levels. Müller *et al.* (1983a/b) showed that T3 treatment increases CYP1A activities in rat liver. This suggests that the effects of PHAHs on biotransformation enzymes may be modulated by the altered thyroid status associated with PHAH treatment.

To further elucidate the peripheral mechanisms mediating the effects of PHAHs on thyroid hormone turnover, we investigated the effects of TCDD in Tx rats substituted with T4 or T3. In addition, this model was used to study the role of changes in thyroid hormone status on the induction of hepatic cytochrome P450 isozymes and other biotransformation enzymes by PHAHs. For this purpose we used Tx male Sprague-Dawley rats infused with substitution doses of T3 (Tx+T3) or T4 (Tx+T4) by osmotic minipumps. Here, we describe the validation of this model as well as the effects of TCDD on plasma and tissue thyroid state-dependent parameters in Eu, Tx, Tx+T3, and Tx+T4 rats. The modulating effects of thyroid hormone on

the induction of hepatic phase I (CYP) and phase II (UGT and GST) detoxification enzymes are presented in *Chapter 3* (Schuur *et al.*, 1998a).

Materials and methods

Chemicals

TCDD (>99% pure) was purchased from Promochem (Wesel, Germany). Iodothyronines, dithiothreitol (DTT), uridinediphosphoglucuronic acid (UDPGA), propyl-2-thiouracil (PTU), and BSA were obtained from Sigma Chemicals Co. (St. Louis, MO). Malic acid was obtained from Janssen Chimica (Tilburg, the Netherlands). BioRad protein reagent was obtained from Bio-Rad Laboratories (BioRad, Richmond, CA). [^{125}I]T4, [^{125}I]T3, and [^{125}I]rT3 were obtained from Amersham (Buckinghamshire, UK); they were purified on Sephadex LH-20 (Pharmacia, Woerden, the Netherlands) before each assay (Rutgers *et al.*, 1989). All other chemicals were of analytical grade.

Animals and treatment

Male Sprague-Dawley rats, surgically thyroidectomized (Tx) or sham-operated by the supplier at 4 weeks of age, were purchased from Harlan/CPB (Zeist, the Netherlands). The complete resection of the thyroid in the Tx rats was confirmed at the end of the experiment by autopsy. The rats were obtained at 6 weeks of age and allowed to acclimatize for 2 weeks before the experiment. They were maintained at 50% humidity and 21 °C on bedding in plastic cages with a 12-h light/12-h dark cycle. Rat chow (Hope Farms, Woerden, the Netherlands) and tap water with 0.5% $CaCl_2$ were supplied *ad libitum*. Model 2002 Alzet minipumps (Charles River Wiga, Sulzfeld, Germany), delivering 0.4 µg T3/100 g bw/day (Tx+T3 rats, n=15) or 1.0 µg T4/100 g bw/day (Tx+T4 rats, n=15) in 0.1 M NaOH in 0.9% NaCl, were implanted ip at day 0 under ether anaesthesia. Five sham operated rats, 15 non-operated Eu rats, and 15 Tx rats received pumps with solvent only. Water and food consumption was recorded daily, and body weight was recorded twice a week. On day 7 after osmotic minipump implantation, 5 rats from each group (Eu, Tx, Tx+T3, and Tx+T4) were given an ip injection of 10 µg TCDD/kg bw in corn oil (5 ml/kg). Of each group, 5 control and 5 pair-fed control rats were given an ip injection with corn oil only. On the day before pump implantation (day -1), day 3, day 7, and day 10, blood (≈1 ml) was collected by orbital puncture in heparinized tubes and stored on ice until separation of plasma. On day 10, all rats were killed under ether anaesthesia. Livers were perfused with saline, dissected, weighed and frozen in 3 portions. Kidneys, thymuses, and brains were removed, weighed and frozen on dry ice. All tissues were stored at -80 °C and plasma at -20 °C until analysis. All procedures were approved by the Animal Welfare Committee of the Agricultural University Wageningen.

Tissue preparation

Whole brains were homogenized in 8 volumes ice-cold 0.1 M Tris-HCl buffer, pH 7.5, containing 1 mM DTT, using a Potter tube, and stored at -80°C until analysis. Livers were homogenized on ice in 3 volumes ice-cold 0.1 M Tris-HCl buffer, pH 7.5, containing 0.25 M sucrose, using a Potter tube, and the homogenate was centrifuged for 30 min at 9,000x*g* and 0-4°C. The resulting supernatant was centrifuged for 90 min at 105,000x*g* and 0-4°C, and the microsomal pellet was resuspended in ice-cold 0.1 M phosphate buffer, pH 7.5. Microsomes and cytosol were stored in aliquots at -80°C until further analysis. Protein levels of tissue fractions were determined using BioRad Protein reagent (Bradford, 1976) and BSA as a standard.

Thyroid hormone analysis

Plasma T4, FT4, and T3 were analyzed in duplicate using the Amerlite chemiluminescence kits (Amersham) according to the protocol of the supplier with the following modifications: the T4 and T3 assay buffer was diluted five times with demineralized water, and the standard curve for T4 ranged from 0 to 120 nmol T4/l. It was ascertained that TCDD does not interfere in the Amerlite assays. Plasma TSH was determined by RIA with the materials and protocols of the NIDDK, NIH, using TSH RP-2 as a standard.

Enzyme assays

Type I iodothyronine deiodinase (D1). Hepatic D1 activity was determined as previously described (Mol and Visser, 1985). In short, microsomes (20 μg protein/ml) were incubated for 30 min at 37°C with 1 μM rT3 and ≈100,000 cpm [^{125}I]rT3 in 0.1 M phosphate buffer, pH 7.2, 2 mM EDTA, and 5 mM DTT. The reaction was stopped on ice by the addition of 750 μl 0.1 M HCl. The tubes were centrifuged, and radioiodide was determined in the supernatant by Sephadex LH-20 chromatography as described previously (Rutgers *et al.*, 1989).

Type II iodothyronine deiodinase (D2). Brain D2 activity was analyzed essentially as described by Visser *et al.* (1982) with slight modifications as described by Morse *et al.* (1993). The final incubation conditions were 0.8 mg brain homogenate protein/ml, 2 nM T4 with ≈50,000 cpm [^{125}I]T4, 1 mM PTU, 0.5 μM T3, 25 mM DTT, and 1 mM EDTA in 200 μl 0.1 M phosphate buffer, pH 7.2. After incubation for 60 min at 37°C, the reaction was stopped on ice by the addition of 100 μl 7% (wt/vol) BSA, followed by 500 μl 10% (wt/vol) trichloroacetic acid. The tubes were centrifuged and the radioiodide released was further isolated from the supernatant by Sephadex LH-20 chromatography as described previously (Mol and Visser, 1985).

UDP-glucuronyltransferases (UGTs). Hepatic T4 and T3 UGT activities were determined essentially according to the method of Beetstra *et al.* (1991). Microsomes (1 mg protein/ml) were incubated for 30 min at 37°C with 1 μM T4 or T3 (plus ≈50,000 cpm ^{125}I-labeled compound), 3.75 mM $MgCl_2$, and 0.125% BSA in the presence or absence (blank) of 5 mM

UDPGA in 200 µl 75 mM Tris-HCl, pH 7.8 (Visser *et al.*, 1993b). Reactions were stopped by the addition of 0.2 ml ice-cold methanol. After centrifugation, 0.2 ml supernatant was mixed with 0.8 ml 0.1 M HCl and analyzed for glucuronide formation on Sephadex LH-20 minicolumns (Beetstra *et al.*, 1991).

Malic enzyme. Hepatic malic enzyme activity was determined according to the method of Hsu and Lardy (1967) by incubating 0.33 mg cytosolic protein/ml for 10 min at 25°C with 2 mM malic acid, 1.13 mM NADP, and 16 mM $MnCl_2$ in 0.13 M triethanolamine buffer, pH 7.4, the formation of NADPH was determined at 340 nm using a 96-well plate spectrophotometer (Molecular Devices Corp., Menlo Park, CA).

Statistics

Treatment-related effects were first evaluated with a one-way analysis of variance followed by a least significant difference test ($p<0.05$) using the statistical software package SPSS/PC+™ (SPSS Inc, Chicago, IL).

Results

No significant differences were found for any of the parameters determined between sham-operated and non-operated Eu rats. Therefore, only data from the latter will be presented. Body weights of the different groups are shown in Table 2.1.

Table 2.1 Body weights and weight gains of the rats on the day before minipump implantation and on day 10 of the experimental period.

	body weight (g)	body weight gain (g)		body weight (g) on day 10	
	on day 0	corn oil	TCDD	corn oil	TCDD
Eu	232.1 ± 4.4	26.8 ± 2.6	26.9 ± 1.5	259.3 ± 5.5	258.1 ± 8.7
Tx	164.9 ± 1.8*	23.2 ± 2.6*	23.6 ± 2.4*	188.5 ± 3.6*	187.8 ± 5.7*
Tx+T3	165.9 ± 2.1*	34.5 ± 3.5*#	37.8 ± 2.6*#	201.5 ± 4.6*	201.7 ± 4.8*
Tx+T4	165.5 ± 3.1*	34.1 ± 4.0*#	35.7 ± 1.5*	201.1 ± 4.9*	198.6 ± 4.1*

Note. Data are presented as means ± SEM. N=15 for Tx and Eu groups, and N=14 for Tx+T3 and Tx+T4 groups. N=5 for all TCDD treated groups. *) Significantly different ($p<0.05$) from Eu group. #) Significantly different ($p<0.05$) fromTx group.

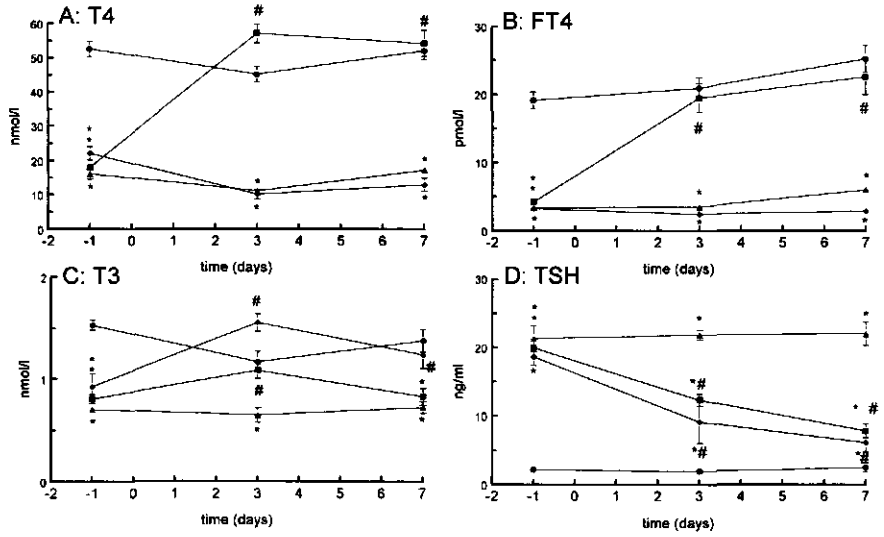

Figure 2.1 Plasma T4 (A), FT4 (B), T3 (C), and TSH (D) levels in Eu (●), Tx (▲), Tx+T3 (◆), and Tx+T4 (■) rats. The levels were measured before and 3 and 7 days after osmotic minipump implantation (day -1). Results represent the means ± SEM of 5 rats per group. *) Significantly different from Eu rats ($p<0.05$). #) Significantly different from Tx rats ($p<0.05$).

Thyroidectomy performed 4 weeks earlier, *i.e.* at 4 weeks of age, caused an approximately 29% decrease ($p<0.05$) in body weight at the start of the infusion period. The increase in body weight of Tx rats infused with replacement doses of T3 (Tx+T3) or T4 (Tx+T4) during the 7-day period was greater than that of Tx or Eu rats ($p<0.05$). Body weights of Tx+T3 and Tx+T4 rats after 10 days of infusion were in between those of Tx and Eu rats. During the entire period, the daily food intake of Tx rats was about 70% of that of Eu, Tx+T3, and Tx+T4.andNo differences were found in water intake between the different groups.

Plasma concentrations of T4, FT4, T3, and TSH in Eu, Tx, Tx+T3, and Tx+T4 in the 7-day period preceding TCDD treatment are shown in Figure 2.1. Eu rats maintained constant levels of plasma T4 (≈50 nM), T3 (≈1.3 nM), FT4 (≈20 pM) and TSH (2.3 ng/ml) over the 7-day period following osmotic minipump implantation. In the Tx rats, strongly reduced plasma T4 (to ≈30% of that in Eu rats) and FT4 (to ≈20% of that in Eu rats) levels were observed. Plasma T3 levels were less affected in Tx rats and maintained at about 50% of Eu values throughout the 7 day period preceding TCDD treatment. Plasma TSH levels were increased approximately 9-fold in Tx *vs.* Eu rats throughout this period, indicating a strongly decreased

negative feedback on hypophyseal TSH secretion.

Infusion of Tx rats with 1 μg T4/100 g bw/day (Tx+T4 rats) resulted in a restoration of T4 and FT4 levels to almost Eu levels at both 3 and 7 days. There was also a significant increase in plasma T3 levels to almost Eu levels after 3 days of T4 replacement, but a subsequent decrease was observed after 7 days. Infusion of Tx rats with T3 had no effect on plasma T4 and FT4 levels. However, T3 replacement caused a quick restoration of plasma T3 levels to slightly above normal Eu levels on day 3 and to Eu levels on day 7. Plasma TSH levels in Tx rats were progressively decreased to almost Eu levels after 3 and 7 days of substitution therapy with T3 or T4.

No differences were found between pair-fed and *ad libitum* fed, corn oil-treated controls in all groups. Therefore, only data derived from pair-fed controls are presented. Treatment with TCDD had no significant effect on food intake, body weight or body weight gain compared with control rats in any of the groups. Thymus weight was decreased significantly by 6-20% and liver weight was increased significantly by 30-50% after TCDD treatment in the different groups (not shown). Results are presented as enzyme activities per mg protein. The significance of the effects of thyroid state and TCDD treatment were similar if results were expressed per g liver or per whole liver. No significant differences were found for brain and kidney weights between TCDD-treated rats and controls.

The effects of TCDD on plasma thyroid hormone levels are presented in Figure 2.2. In the Eu and the Tx+T4 groups, TCDD significantly reduced plasma T4 levels by 38% and 52%, respectively, compared with their respective control values. No significant effect of TCDD treatment was found on the low plasma T4 levels in the Tx and Tx+T3 groups (Figure 2.2A). TCDD treatment caused a significant 28% decrease and an insignificant 38% decrease in plasma FT4 levels in Eu and Tx+T4 animals, respectively, compared with the corresponding controls (Figure 2.2B), whereas no effect was observed in the Tx and Tx+T3 groups. TCDD exposure resulted in a decrease in plasma T3 concentrations in the Tx, Tx+T3, and Tx+T4 rats, which was significant only for the Tx+T3 group (51% of control Tx+T3 rats; Figure 2.2C). TCDD tended to increase plasma T3 in Eu rats, but this was not significant. No TCDD-related changes were observed in plasma TSH in any of the groups (Figure 2.2D).

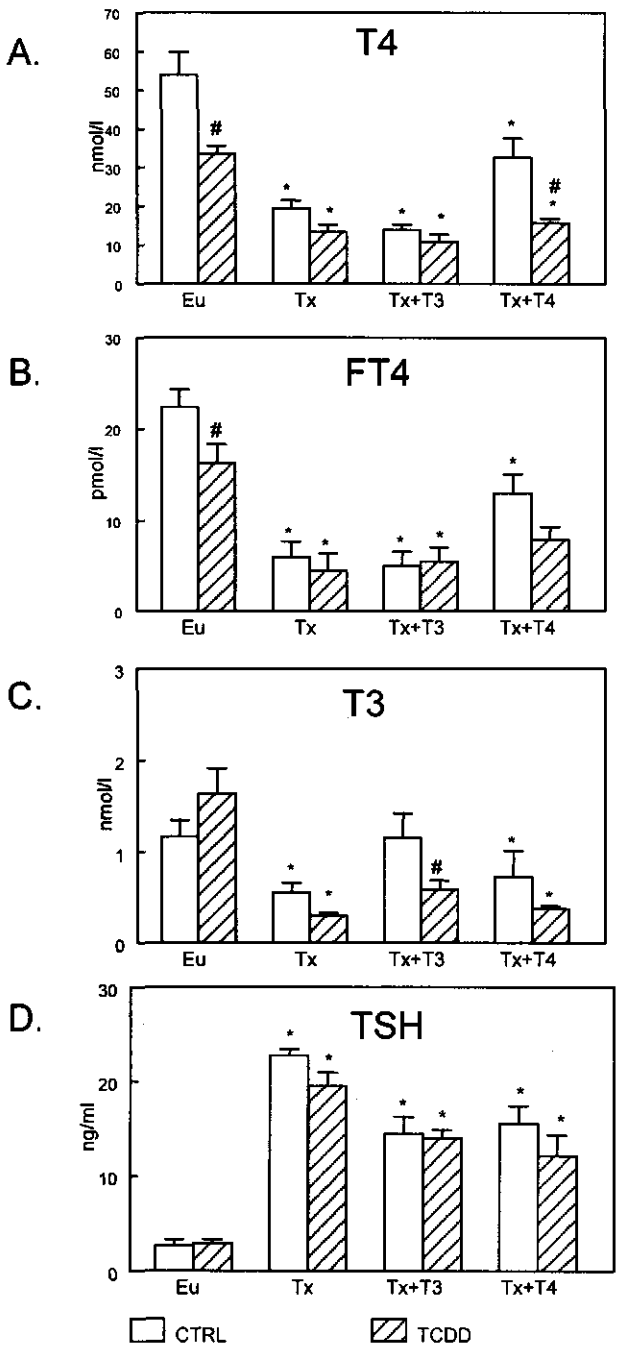

Figure 2.2. Effects of TCDD treatment on plasma T4 (A), FT4 (B), T3 (C), and TSH (D) levels in Eu, Tx, Tx+T3, and Tx+T4 rats. The data shown are the values determined on day 10, 3 days after administration of 10 μg TCDD/kg bw in corn oil, or corn oil alone (CTRL). Results are presented as the means ± SEM of 5 rats per group. *) Significantly different from Eu rats ($p<0.05$). #) Significantly different from CTRL rats ($p<0.05$).

Hepatic D1 activity was insignificantly decreased in control Tx rats compared with that in control Eu rats (Figure 2.3). In control Tx+T3 and Tx+T4 rats, hepatic D1 activity was the same as in Eu rats. TCDD treatment resulted in a further insignificant decrease in D1 activity in Tx rats. No effects of TCDD on D1 activity were observed in Eu, Tx+T3, and Tx+T4 rats.

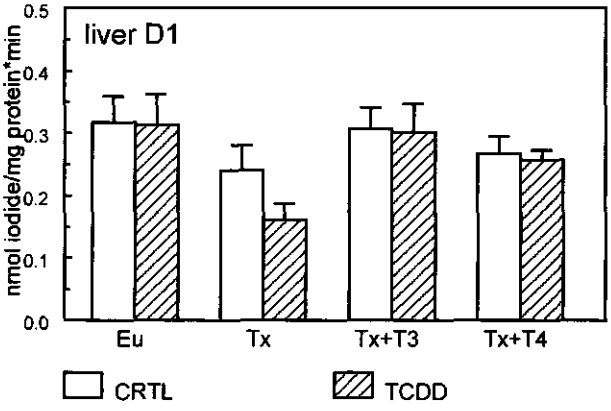

Figure 2.3 Effects of TCDD treatment on hepatic D1 activity in Eu, Tx, Tx+T3, and Tx+T4 rats. Livers were isolated on day 10, 3 days after administration of TCDD in corn oil, or corn oil alone (CTRL). Results are presented as the means ± SEM of 5 rats per group.

Brain D2 activity was increased significantly in control Tx and Tx+T3 rats to about 250% of activity in control Eu rats (Figure 2.4). T4 replacement resulted in a return of D2 activity to control Eu levels. TCDD treatment tended to increase brain D2 activity in the Tx, Tx+T3, and Tx+T4 rats, although the differences with the respective controls were not significant.

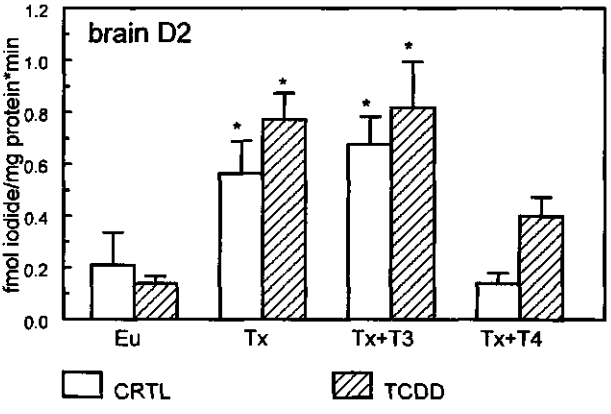

Figure 2.4 Effects of TCDD treatment on brain D2 activity in Eu, Tx, Tx+T3, and Tx+T4 rats. Brains were isolated on day 10, 3 days after administration of TCDD in corn oil, or corn oil alone (CTRL). Results are presented as the means ± SEM of 5 rats per group. *) Significantly different from Eu rats ($p<0.05$).

Hepatic UGT activities were determined using T4 or T3 as substrates (Figure 2.5). With either substrate, UGT activities were similar in control Eu and Tx rats. T4 UGT activity was increased ≈5 fold in all groups treated with TCDD. In contrast, T3 UGT activity was slightly but not significantly increased by TCDD treatment independent of thyroid state.

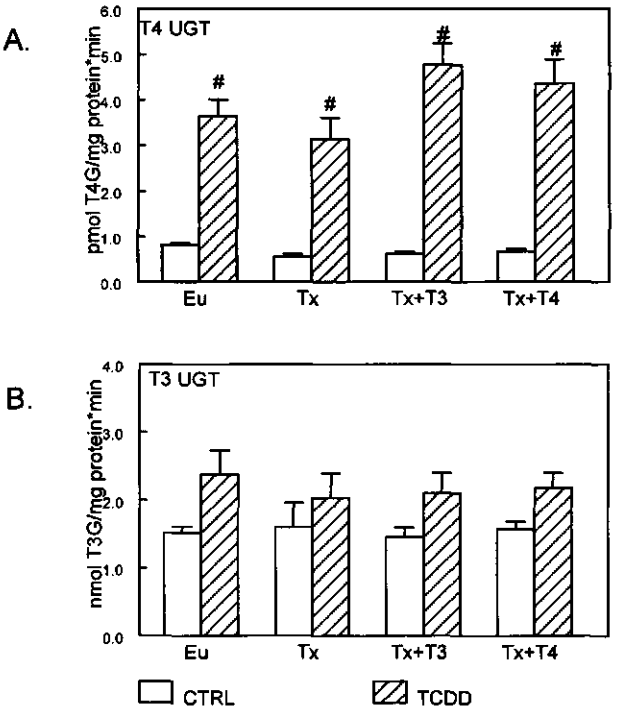

Figure 2.5 Effect of TCDD treatment on hepatic T4 (A) or T3 (B) UGT activities in Eu, Tx, Tx+T3, and Tx+T4 rats. Livers were isolated on day 10, 3 days after administration of TCDD in corn oil, or corn oil alone (CTRL). Results are presented as the means± SEM of 5 rats per group. #) Significantly different from CTRL rats ($p<0.05$).

The activity of the T3-responsive malic enzyme was measured in liver cytosol (Figure 2.6). Malic enzyme activity was decreased by 67% in control Tx rats *versus* control Eu rats. Malic enzyme activity was partially restored in control Tx+T3 and Tx+T4 rats, with values in between those in control Tx and Eu rats. TCDD treatment increased the malic enzyme activity significantly in Eu, Tx+T3, and Tx+T4 rats to 148%, 244%, and 162%, respectively, of the corresponding controls. However, no effect of TCDD treatment on malic enzyme activity was found in the Tx group.

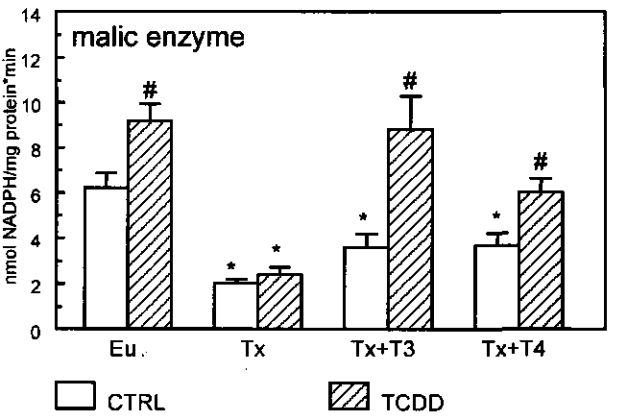

Figure 2.6 Effects of TCDD treatment on hepatic malic enzyme activity in Eu, Tx, Tx+T3, and Tx+T4 rats. Livers were isolated on day 10, 3 days after administration of TCDD in corn oil, or corn oil alone. Results are presented as the means ± SEM of 5 rats per group. *) Significantly different from Eu rats ($p<0.05$). #) Significantly different from CTRL rats ($p<0.05$).

Discussion

This study focussed on the extrathyroidal effects of PHAHs on thyroid hormone turnover using thyroid hormone-substituted Tx rats (Tx+T3, Tx+T4) male Sprague-Dawley rats as a model. In this model, Tx rats were continuously infused with replacement doses of T3 or T4 using osmotic minipumps. Plasma T4, T3, and TSH were restored to levels approximately equal to those observed in Eu rats after 3 and 7 days of hormone replacement, suggesting that a near-euthyroid state was achieved. However, on day 10, *i.e.* 3 days after corn oil administration, plasma T4 and T3 levels showed a significant decrease and plasma TSH a significant increase in the control Tx+T3 and Tx+T4 rats. This may be due to 1) increased body weight gain in the hormone-replaced Tx rats and, thus, decreased T3 and T4 infusion rates per 100 g bw, 2) increased peripheral thyroid hormone metabolism in the hormone-replaced Tx rats (Christenson *et al.*, 1995), and/or 3) the administration of a rather large volume of corn oil, acting as a depot for the T3 and T4 released from the minipumps.

Tissue thyroid state was assessed by measuring hepatic malic enzyme and D1 activities, which are under positive control of thyroid hormone (Oppenheimer *et al*, 1977; Kaplan, 1986), as well as brain D2 activity, which is down-regulated by thyroid hormone, in particular T4 (Leonard *et al.*, 1984). Hepatic malic enzyme activity was very low in Tx rats, indicating functional hypothyroidism. In Tx+T3 and Tx+T4 rats, malic enzyme activity was partially restored back to Eu levels. Smaller, insignificant changes were observed for hepatic D1 activity, suggesting that malic enzyme activity is a more sensitive parameter for hepatic thyroid state. Brain D2 activity was highly elevated in the Tx group, whereas T4, but not T3, replacement resulted in a decrease in D2 activity back to Eu values.

As thyroidal secretion of T4 and T3 in the hormone-replaced rats is negligible, these rats are a suitable model for the study of the extrathyroidal effects of xenobiotics on thyroid hormone status and metabolism. T4 and FT4 levels were reduced to a greater extent by TCDD in Tx+T4 rats than in Eu rats, indicating that the TCDD-induced plasma T4 reduction is mainly due to an extrathyroidal mechanism. This is in agreement with findings reported by Barter and Klaassen (1992a) in Tx+T3/T4 rats after treatment with Aroclor, a PCB mixture. No effect of TCDD was found on plasma T3 levels in Eu rats, which is in agreement with other studies (Potter *et al.*, 1983; Gorski and Rozman, 1987; Henry and Gasiewicz, 1987; Jones *et al.*, 1987; Beetstra *et al.*, 1991), although plasma T3 has also been reported to decrease (Pzadernik and Rozman, 1985; Rozman *et al.*, 1985a) or increase (Potter *et al.*, 1986; Bastomsky, 1977) after TCDD treatment. In contrast to Eu rats, Tx+T3 rats showed a marked decrease in plasma T3 after treatment with TCDD in this study, which may be explained by a TCDD-induced increase in the clearance of plasma T3. The variable effects of TCDD on plasma T3 in Eu rats reported in the different studies may be explained by the varying extents of the inhibition of hepatic D1 activity, the decrease in plasma T4 substrate levels, and the increase in plasma TSH and, hence, the stimulation of thyroidal T3 secretion. No significant effects of TCDD were observed on plasma TSH levels regardless of thyroid state, which has also been shown previously (Henry and Gasiewicz, 1987). It is not known why the TCDD-induced decrease in plasma T4 and FT4 levels does not provoke an increase in TSH secretion. One explanation is a possible damaging effect of TCDD and PCBs on the hypothalamus and/or pituitary (Collins and Capen, 1980a; Gorski *et al.*, 1988; Byrne *et al.*, 1987; Liu *et al.*, 1995). However, chronic administration of TCDD has been shown to increase plasma TSH, which may even be associated with development of thyroid tumors in rats (Sewall *et al.*, 1995).

T4 is glucuronidated in rat liver by at least 2 different UGT isozymes: bilirubin UGT and phenol UGT. Phenol UGT activity is inhibited *in vitro* in the presence of Brij 56, whereas bilirubin UGT activity is stimulated *in vitro* by this detergent (Visser *et al.*, 1993a/b). T4 UGT activity was measured in this study in the absence of Brij 56 and, thus, largely reflect glucuronidation of T4 by phenol UGT. The results showed that TCDD treatment induced an approximately 5-fold increase in T4 UGT activity in Eu, Tx, and Tx+T3 or Tx+T4 rats. These results are in agreement with previous studies, showing that hepatic phenol UGT activity is potently induced by 3-methylcholanthrene-like inducers, such as TCDD and PCBs (Beetstra *et al.*, 1991; De Sandro *et al.*, 1992; Barter and Klaassen, 1992a/b; Visser *et al.*, 1993a), which probably represents an increase in UGT gene expression (Münzel *et al*, 1994). This marked increase in hepatic T4 UGT activity is most likely responsible for the reduction of plasma T4 and FT4 levels, as suggested previously (Beetstra *et al.*, 1991; Barter and Klaassen, 1992a). This is further supported by the significant negative correlation between T4 UGT and plasma T4 levels found in this study (not shown) and in a previous study (Liu *et al.*, 1995) employing different hepatic enzyme inducers. However, TCDD treatment does not decrease residual serum

T4 levels in Tx and Tx+T3 rats. In this respect it should be realized that glucuronidation is not an irreversible pathway of T4 disposal, as T4 glucuronide is hydrolyzed in the intestine and at least part of the liberated T4 is resorbed (enterohepatic cycle) (Visser, 1994a). A possible explanation for the lack of a TCDD-induced decrease in serum T4 in the Tx and Tx+T3 rats is a more efficient recovery of biliary-excreted T4 glucuronide in these animals.

Perhaps the most remarkable result of our study is the decrease in plasma T3 levels after TCDD treatment of Tx+T3 rats. As mentioned above, this may be explained by a TCDD-induced increase in plasma T3 clearance. In contrast to the strong increase in T4 UGT activity, TCDD produced only a small and insignificant increase in hepatic T3 UGT activity. Even if the approximately 30% increase in liver weight is taken into account, TCDD induces only about a 50% increase in hepatic T3 UGT capacity in contrast to a more than 5-fold increase in T4 UGT capacity. The TCDD-induced increase in plasma T3 clearance may, therefore, be due to an increase in alternative pathways of T3 metabolism.

TCDD did not affect hepatic D1 activity in this study, although in Tx rats an insignificant decrease was observed. In other studies TCDD treatment resulted in a significant decrease in liver D1 activity in rats (Visser *et al.*, 1993a; Lans, 1995). Such a decrease in D1 activity has also been found after TCDD treatment of Tx and Tx+T3 rats (Eltom *et al.*, 1992). A possible explanation for this discrepancy is the difference in dose and duration of TCDD exposure between the different studies. If it occurs, the decrease in hepatic D1 activity may be an indirect effect mediated by a TCDD-induced decrease in thyroid hormone bioactivity (Kaplan, 1986). In our study the lowest D1 activity was observed in TCDD-treated Tx rats which also had the lowest serum T3 levels. However, direct inhibition of D1 activity by TCDD-like compounds *in vitro* has also been reported (Rickenbacher *et al.*, 1989).

In this study no statistically significant effects of TCDD were found on brain D2 activity in Eu rats, although in Tx, Tx+T3, and Tx+T4 rats TCDD increased D2 activity to 122-286% of controls. Increased brain D2 activity after treatment of rats with TCDD or PCB mixture has been demonstrated by Lans (1995) and Morse *et al.* (1996). This increase in brain D2 activity suggests a physiological response to decreased plasma T4 levels to maintain constant T3 levels in the brain (Silva and Matthews, 1984). A possible explanation for the relatively small TCDD-induced increases in brain D2 activity in the present study is the relatively small decrease in plasma T4 in comparison with the findings of Lans (1995), who used a higher dose of TCDD and a longer exposure time.

Another important finding of the present study is the increased hepatic malic enzyme activity observed after TCDD treatment in all groups, except Tx rats. Kelling *et al.* (1987) and Roth *et al.* (1988) also reported on an increase in malic enzyme activity after treatment with TCDD, but also only in the presence of thyroid hormone. Thyroid hormone is, therefore, a permissive factor for the induction of malic enzyme by TCDD. Since malic enzyme is also controlled by the peroxisome proliferator-activated receptor (PPAR) (Castelein *et al.*, 1994),

it appears therefore that malic enzyme expression is regulated by multiple nuclear receptors, including the T3 receptor, PPAR, and the Ah receptor. Both the Ah receptor and PPAR mediate the induction of different hepatic CYP isoenzymes by their ligands. The induction of malic enzyme by both receptor-ligand systems may serve the purpose of providing the necessary NADPH required for the induced CYP activities (Kelling *et al.*, 1987).

In conclusion, T4 or T3 was infused by osmotic minipumps into Tx rats restoring plasma T3 and T4 to near-euthyroid levels; this provided a model in which the peripheral effects of TCDD on thyroid hormone turnover can be investigated without confounding effects on thyroid function. TCDD induced greater decreases in plasma T4 levels in Tx+T4 rats than in Eu rats, indicating that these changes are caused by an extrathyroidal mechanism in which increased hepatic T4 glucuronidation by induction of phenol UGT activity plays an important role.

Acknowledgements

We thank M.A.W. Faassen-Peters for her skillful technical assistance with the implantation of the osmotic minipumps and help with the animals. We thank P.J. Tacken for measuring UGT activities, and H. van Toor (Internal Medicine III, Erasmus University Rotterdam) for measuring plasma TSH concentrations.

CHAPTER 3

MODULATING EFFECTS OF THYROID STATE ON THE INDUCTION OF BIOTRANSFORMATION ENZYMES BY 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN

Abstract

In this study we investigated to what extent the induction of detoxification enzymes by TCDD is modulated by concomitant TCDD-induced changes in thyroid state. Euthyroid (Eu) male Sprague-Dawley rats, surgically thyroidectomized (Tx) rats and Tx rats receiving substitution doses of 3,3',5-triiodothyronine (Tx+T3) or thyroxine (Tx+T4) by osmotic minipumps were treated with a single ip injection of 10 μg TCDD/kg body weight or with vehicle (corn oil). Three days after TCDD administration, rats were sacrificed, and blood and livers were collected for analysis. Total hepatic cytochrome P450 content was increased by ≈50% by TCDD in all groups but was not affected by thyroid state. In Eu rats, TCDD increased CYP1A1/1A2 activity 90-fold, CYP1A1 protein content 52-fold, and CYP1A1 mRNA levels ≈5.8-fold. Similar findings were obtained in Tx, Tx+T3 and Tx+T4 rats except that TCDD-induced CYP1A1 activity was significantly decreased in Tx rats. NADPH cytochrome P450 reductase activity was not affected by TCDD but was decreased in Tx rats, which may explain the diminished TCDD-induced CYP1A1 activity in Tx rats. Hepatic *p*-nitrophenol UGT activity was induced ≈4-fold by TCDD in Eu rats. Similar basal and TCDD-induced activities were observed in Tx+T3 and Tx+T4 rats, but TCDD-induced activities were significantly lower in Tx rats. TCDD did not have a significant effect on overall GST activity or hepatic GST 2-2, 3-3 or 4-4 protein levels but produced a marked increase in GST 1-1 protein levels. Thyroid state did not affect basal or TCDD-induced GST activity or subunit pattern. Iodothyronine sulfotransferase activity was not affected by TCDD treatment and was slightly but not significantly lower in Tx rats than in Eu, Tx+T3, and Tx+T4 rats. These results suggest that the changes in thyroid hormone levels associated with TCDD treatment have little modulating effects on the induction of hepatic detoxification enzymes in Sprague-Dawley rats exposed to this compound.

based on A.Gerlienke Schuur, Paul J. Tacken, Theo J. Visser, and Abraham Brouwer (1998). Modulating effects of thyroid state on the induction of biotransformation enzymes by 2,3,7,8-tetrachlorodibenzo-p-dioxin, ETAP **5**, 7-16.

Introduction

TCDD is the most toxic PHAH known and prototypical for the effects induced by this class of compounds. TCDD induces a variety of toxic responses in animals and humans, including chloracne, immunotoxicity, teratogenicity, liver lesions, and carcinogenicity (Poland and Knutson, 1982). A well-known biochemical effect of TCDD and related compounds is the induction of both phase I biotransformation enzymes, especially CYP1A isozymes (Safe, 1986), and phase II conjugation enzymes, such as UGTs (Owens, 1977) and GSTs (Kirsch *et al.*, 1975).

It is also well known that PHAHs may alter thyroid hormone metabolism in rodents (for review, see Brouwer *et al.*, 1998). A single dose of TCDD was found to strongly increase biliary excretion of T4 glucuronide in rats (Bastomsky, 1977). Other reports have also shown marked decreases in plasma T4 and free T4 levels and induction of hepatic T4 UGT activity after TCDD administration to rats (Visser *et al.*, 1993a; Van Birgelen *et al.*, 1995).

Thyroid state may affect a variety of metabolic processes in most mammalian tissues, including the hepatic metabolism of drugs. Rumbaugh *et al.* (1978) reported on a dose-dependent stimulation of hepatic mixed-function oxidases by thyroid hormones in rats. Skett and Weir (1982) demonstrated sex- and substrate-dependent effects of thyroidectomy and thyroid hormone replacement on drug metabolism. More recent studies suggest that thyroid hormone may act as a natural suppressor of the transcription of the CYP2B1, CYP2B2, CYP1A2 and CYP2A1 genes (Yamazoe *et al.*, 1989; Murayama *et al.*, 1991; Arlotto and Parkinson, 1989). In contrast, Eltom *et al.* (1992) found lower CYP1A activities in thyroidectomized (Tx) rats than in Tx rats treated with substitution doses of thyroid hormone. Moreover, thyroidectomy was found to partially protect rats against the lethal effects of 100 μg TCDD/kg body wt per day in a 45-day exposure study (Rozman *et al.*, 1984; 1985a).

We have tested the hypothesis that the decrease in plasma T4 levels by PHAHs represents an adaptation mechanism, modulating the activities of biotransformation enzymes and, perhaps, protecting against the toxic effects induced by these compounds. This was done by studying the effects of TCDD in euthyroid (Eu) rats, Tx rats, Tx rats substituted with T4 (Tx+T4) and Tx rats substituted with T3 (Tx+T3). Thyroid state parameters and thyroid hormone metabolism determined in this experimental model are reported elsewhere (Schuur *et al.*, 1997; *Chapter 2*). Here, we describe the possible effects of thyroid state on basal and TCDD-induced expression of biotransformation enzymes in the liver, *i.e.* CYP1A1/1A2 activity, *p*-nitrophenol (PNP) UGT activities, GST activity, and iodothyronine SULT activity.

Materials and methods

Chemicals.

TCDD, >99% pure, was obtained from Promochem (Wesel, Germany); T4, T3, uridine-diphosphoglucuronic acid (UDPGA), 3'-phosphoadenosine-5'-phosphosulfate (PAPS), Brij 56, PNP, cytochrome c and bovine serum albumin (BSA) from Sigma Chemicals Co. (St. Louis, MO, USA); resorufin (RR) from Janssen Chimica (Tilburg, the Netherlands); BioRad protein reagent from BioRad Laboratories (Richmond, CA, USA); NADPH and glutathione (GSH) from Boehringer Mannheim GmbH (Mannheim, Germany); Trizol and sodium dodecyl sulfate (SDS) from Gibco BRL (Breda, the Netherlands); 3,3'-diiodothyronine (T2) and 3-iodothyronine (T1) from Henning Berlin GmbH (Berlin, Germany); and 1-chloro-2,4-dinitrobenzene (CDNB) from Aldrich Chemie (Bornem, Belgium). [^{125}I]T2 was produced by radioiodination of T1 as described before (Visser *et al.*, 1978). All other chemicals were of analytical grade.

Animals and treatment.

Male Sprague-Dawley rats, surgically thyroidectomized (Tx) or sham-operated by the supplier at 4 weeks of age, were purchased from Harlan/CPB (Zeist, the Netherlands). The rats were obtained at 6 weeks of age and allowed to acclimatize for 2 weeks before the experiment. They were maintained at 50% humidity and 21 °C on bedding in plastic cages with a 12-h light/12-h dark cycle. Rat chow (Hope Farms, Woerden, the Netherlands) and tap water with 0.5% $CaCl_2$ were supplied *ad libitum*. Model 2002 Alzet minipumps (Charles River Wiga, Sulzfeld, Germany), delivering 1 μg T4/100 g body wt per day (Tx+T4 rats, n=15) or 0.4 μg T3/100 g body wt per day (Tx+T3 rats, n=15) in 0.1 M NaOH in 0.9% saline, were implanted ip at day 0 under ether anaesthesia. Five sham-operated rats, 15 non-operated Eu rats, and 15 Tx rats received pumps with solvent only. Water and food consumption were recorded daily, and body weight was recorded twice a week. On day 7, following osmotic minipump implantation, 5 rats of each group (Eu, Tx, Tx+T3 and Tx+T4) were given an ip injection of 10 μg TCDD/ kg body wt in corn oil (5 ml/ kg). Of each group, 5 control and 5 pair-fed control rats were given an ip injection with corn oil only. On the day before pump implantation (day -1), day 3, day 7 and day 10, blood (≈1 ml) was collected by orbital puncture in heparinized tubes and stored on ice until separation of plasma. On day 10 all rats were sacrificed under ether anaesthesia. Livers were perfused with saline, dissected, weighed and frozen in three portions. Tissue was stored at -80 °C until analysis. All procedures were approved by the Animal Welfare Committee of the Agricultural University Wageningen.

Tissue preparation

Livers were homogenized on ice in three volumes ice-cold 0.1 M Tris-HCl buffer, pH 7.5,

containing 0.25 M sucrose, using a Potter tube and the homogenate was centrifuged for 30 min at 9,000x*g* and 0-4 °C. The resulting supernatant was centrifuged for 90 min at 105,000x*g* and 0-4 °C, and the microsomal pellet was resuspended in ice-cold 0.1 M phosphate buffer, pH 7.5. Microsomes and cytosol were stored in aliquots at -80 °C until further analysis. Protein levels of tissue fractions were determined using BioRad Protein reagent (Bradford, 1976) and BSA as a standard. Another part of the liver was used for RNA-isolation using Trizol reagent according to the suppliers protocol.

Enzyme assays

Total cytochrome P450. Cytochrome P450 content in liver microsomes was estimated from the carbon monoxide-reduced difference spectrum in 0.1 M potassium phosphate (pH 7.4) containing 1 mg/ml hepatic microsomal protein (Omura and Sato, 1964).

NADPH cytochrome c reductase. The reduction of cytochrome c was followed spectrophotometrically at 550 nm for 1 min at room temperature. The incubation mixture contained 40 µM cytochrome c, 100 µM NADPH and 12.5 µg/ml microsomes in 0.3 M phosphate buffer (pH 7.7). Results were calculated using an extinction coefficient of 21 $mM^{-1}*cm^{-1}$ (Vermilion and Coon, 1978).

Ethoxyresorufin O-deethylase. EROD activity was measured according to the method of Burke *et al.* (1977) adapted for use with 96 wells plates and a fluorospectrophotometric plate reader (Cytofluor 2350, Millipore, Etten-Leur, the Netherlands). Reaction mixtures contained 0.4 µM 7-ethoxyresorufin (ER), 0.1 mM NADPH, 1 mg/ml BSA and 2.5-100 µg/ml microsomal protein in 200 µl 0.1 M Tris-HCl, pH 7.8. After preincubation for 2 min at 37°C, reactions were started by the addition of NADPH and after 5 min at 37°C they were stopped by adding 50 µl 1 M NaOH. The formation of the product resorufin (RR) was detected fluorimetrically (excitation 530 nm, emission 590 nm) and compared with a calibration curve obtained using 0-150 nM RR in 0.08 M Tris-HCl, 0.8 mg BSA/ml and 0.8 M NaOH. Incubations were carried out in duplicate, and results were corrected for blanks without NADPH.

CYP1A1 protein levels. A cytochrome P450 1A1 ELISA system from Amersham (Amersham, UK) was used to determine CYP1A1 protein levels. The kit was used exactly according to the protocol of the supplier.

CYP1A1 mRNA analysis. Mouse P450 1A1 (ATCC-no. 63006) and human GAPDH (ATCC-no. 57234) cDNA probes were obtained from ATCC (Rockville, MD). Serial dilutions of total RNA (0.176-22.5 µg) were dot-blotted using a mini-blot apparatus (Minifold, Schleicher and Schuell, Den Bosch, the Netherlands) on Hybond-N+ nitrocellulose membrane (Amersham, UK). Hybridization was carried out at 65°C in 0.5 M sodium phosphate (pH 7.2), 7% SDS and 1 mM EDTA using random-primed ^{32}P-labelled probes. Membranes were washed at 65°C successively with 2x SSC/0.1% SDS, 1xSSC/0.1% SDS and 0.3xSSC/0.1% SDS (1x SSC= 150 mM NaCl, 15 mM sodium citrate, pH 7.0). MRNA signals were quantified using a

Phosphor Imager (Molecular Dynamics, Sunnyvale, CA), and CYP1A1 mRNA was standardized relative to GAPDH mRNA.

PNP UDP-glucuronyltransferase. PNP UGT activity was measured by incubation of 1 mM PNP for 15 min at 37°C with 0.25 mg microsomal protein/ml, 5 mM UDPGA in 100 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$ and 0.005% Brij 56 (Bock *et al.*, 1973; Beetstra *et al.*, 1991).

Glutathione S-transferase. GST activity was measured according to Habig *et al.* (1974) adapted for use with 96 wells plates and a spectrophotometric plate reader (Thermo Max Microplate Reader, Molecular Devices Corp, Menlo Park, CA) as described by Van Iersel *et al.* (1996). Reaction mixtures contained 1 mM GSH, 1 mM CDNB and 1 mg cytosolic protein/ml in 250 µl 0.1 M potassium phosphate buffer (pH 6.5) and 2 mM EDTA. After preincubation for 2 min at 25°C, the reaction was started by the addition of CDNB, and conjugate formation was followed for 1 min at 25°C at 340 nm. Separation and quantitation of GST subunits were done as described by Bogaards *et al.* (1989).

Iodothyronine sulfotransferase. SULT activity was determined by incubation of 1 µM T2 and ≈80,000 cpm [^{125}I]T2 for 30 min at 37°C with 50 µM PAPS and 25 µg rat liver cytosol protein/ml in 200 µl 75 mM phosphate (pH 7.2) and 1.5 mM EDTA (Visser *et al.*, 1996a). The reaction was stopped on ice by adding 750 µl 0.1 M HCl, and T2 sulfate formation was determined by Sephadex LH-20 chromatography (Rutgers *et al.*, 1989).

Statistics.

Data are presented as means ± SEM. Treatment-related effects were first evaluated with a one-way analysis of variance followed by a least significant difference test ($p<0.05$) to find significant differences between the treatment groups. The statistical software package SPSS/PC+™ (SPSS Inc, Chicago) was used.

Results

Since the various parameters determined did not differ between the pair-fed and *ad libitum* fed groups, only the data of the TCDD-treated rats and the pair-fed controls given corn oil alone are presented. The effects of thyroidectomy and thyroid hormone replacement on plasma thyroid parameters (T4, T3 and TSH), and tissue thyroid state parameters (hepatic type I deiodinase and malic enzyme activities and brain type II deiodinase activity) are presented elsewhere (Schuur *et al.*, 1997; *Chapter 2*). TCDD had no effect on body weight and food and water intake (Schuur *et al.*, 1997; *Chapter 2*).

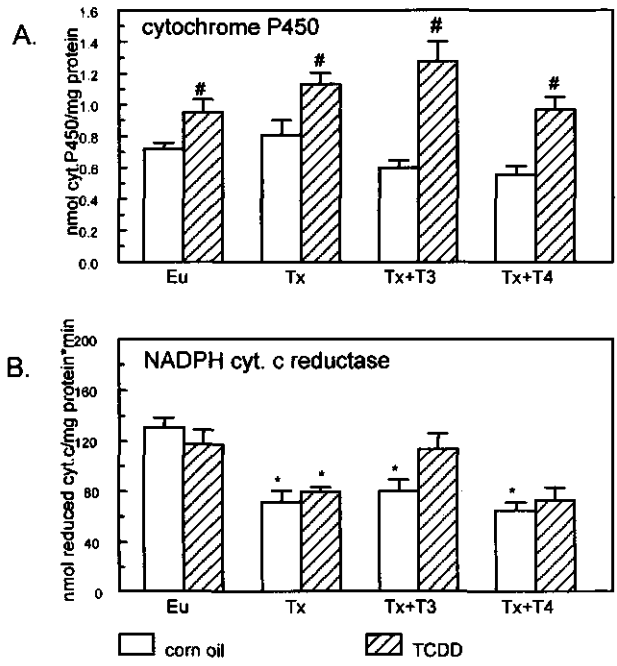

Figure 3.1 Effects of TCDD treatment on hepatic cytochrome P450 content (A) and NADPH cytochrome c reductase activity (B) in Eu, Tx, Tx+T3, and Tx+T4 rats. Results are presented as means ± SEM of 5 rats. *) Significantly different ($p<0.05$) from Eu rats. #) Significantly different ($p<0.05$) from corn oil-treated rats.

Cytochrome P450

The effects of TCDD and thyroid state on total hepatic CYP450 content are shown in Figure 3.1A. TCDD exposure significantly increased the amount of CYP450 by 30-110% in the different groups. No significant effects of thyroidectomy and thyroid hormone replacement were observed on basal as well as TCDD-induced CYP450 levels.

The NADPH cytochrome P450 reductase activity (Figure 3.1B) was measured using cytochrome c as an electron acceptor. Thyroidectomy significantly decreased the cytochrome c reductase activity by almost 50% but replacement with T3 or T4 did not restore this activity back to Eu control levels. TCDD exposure did not affect cytochrome c reductase activity in the different groups, although an insignificant increase was observed in Tx+T3 rats.

Cytochrome P4501A1

Basal CYP1A1/1A2 activity, measured in the EROD assay, was not significantly different between Eu, Tx, Tx+T3 and Tx+T4 rats (Figure 3.2A). TCDD induced EROD activity 65- to 160-fold in the different groups. TCDD-induced EROD activity was similar in Eu, Tx+T3 and Tx+T4 rats but significantly lower in Tx rats.

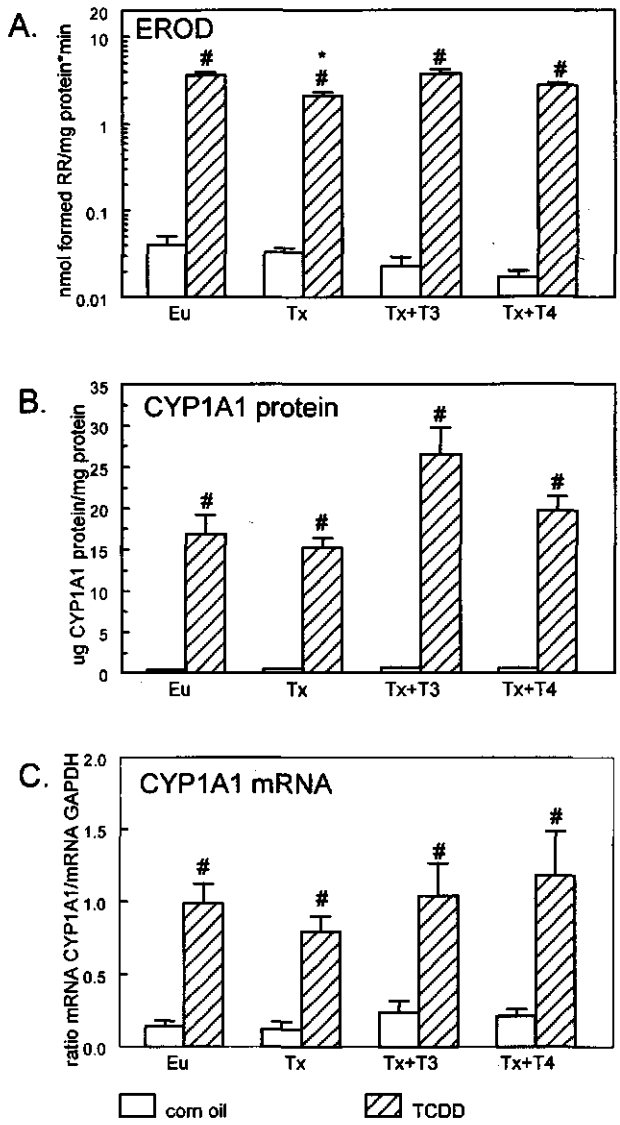

Figure 3.2 Effects of TCDD treatment on hepatic EROD activity (A), CYP1A1 protein (B) and CYP1A1 mRNA (C) levels in Eu, Tx, Tx+T3 or Tx+T4 rats. Results are presented as means ± SEM of 5 rats. *) Significantly different ($p<0.05$) from Eu rats. #) Significantly different ($p<0.05$) from corn oil-treated rats.

CYP1A1 protein levels were measured by a specific ELISA (Figure 3.2B). No significant effects of thyroidectomy or thyroid hormone replacement were found on basal levels. TCDD caused a 31- to 52-fold induction of CYP1A1 protein levels in the different groups. TCDD-induced CYP1A1 protein levels were not significantly affected by thyroid state.

CYP1A1 mRNA levels were also measured in this study (Figure 3.2C). TCDD treatment resulted in a 4.4- to 7.3-fold increase in CYP1A1 mRNA levels in the different groups. Neither thyroidectomy nor thyroid hormone replacement significantly affected basal or TCDD-induced CYP1A1 mRNA levels.

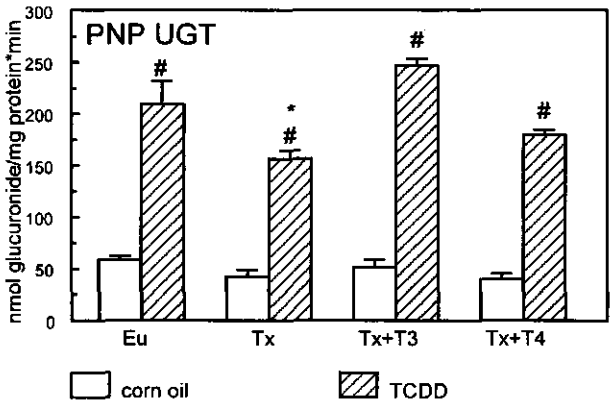

Figure 3.3 Effects of TCDD treatment on hepatic PNP UGT activity in Eu, Tx, Tx+T3 or Tx+T4 rats. Results are presented as means ± SEM of 5 rats. *) Significantly different ($p<0.05$) from Eu rats. #) Significantly different ($p<0.05$) from corn oil-treated rats.

UDP-glucuronyltransferase activities

Hepatic UGT activities were measured using PNP as a substrate (Figure 3.3). PNP is a model substrate for one of the two UGT isoenzymes which also catalyze glucuronidation of T4 (Schuur *et al.*, 1997; *Chapter 2*). PNP UGT activity was induced 3.7- to 4.6-fold by TCDD treatment in the different groups. TCDD-induced, but not basal, PNP UGT activity was significantly lower in Tx rats than Eu rats. There was no significant difference in basal or TCDD-induced PNP UGT activities between Eu, Tx+T3 and Tx+T4 rats.

Glutathione S-transferase

GST activity was measured using CDNB as a substrate (Figure 3.4A). Thyroidectomy, thyroid hormone replacement, and TCDD treatment were all without significant effects on GST activity, although there was a slight increase in GST activity after TCDD treatment in Tx, Tx+T3 and Tx+T4 rats. TCDD induced a marked increase in GST protein subunit 1-1 levels in all groups, although the difference was not significant in Tx+T4 rats (Figure 3.4B-E). TCDD did not produce significant changes in the amount of GST protein subunits 2-2, 3-3 or 4-4 in the different groups. Thyroidectomy and thyroid hormone replacement were not associated with significant differences in basal or TCDD-induced GST protein subunit levels.

Iodothyronine sulfotransferase

Thyroid state had no significant effect on iodothyronine ST activity, although a small, insignificant decrease was observed in Tx rats (Figure 3.5). TCDD treatment did not affect iodothyronine ST activity in any group.

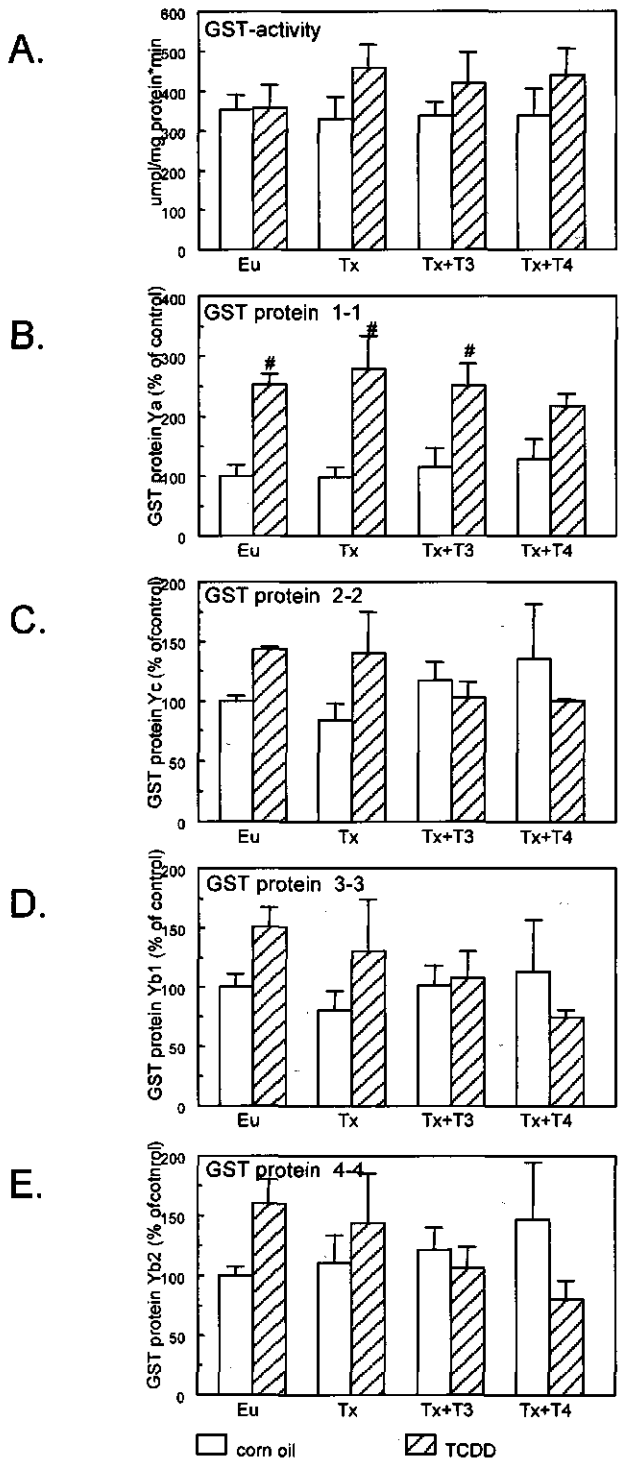

Figure 3.4 Effects of TCDD treatment on hepatic GST activity (A) and GST protein subunit levels (B-E) in Eu, Tx, Tx+T3 or Tx+T4 rats. GST subunit levels are expressed as percentage of that the corn oil-treated Eu rats. Results are presented as means ± SEM of 5 rats. #) Significantly different ($p<0.05$) from corn oil-treated rats.

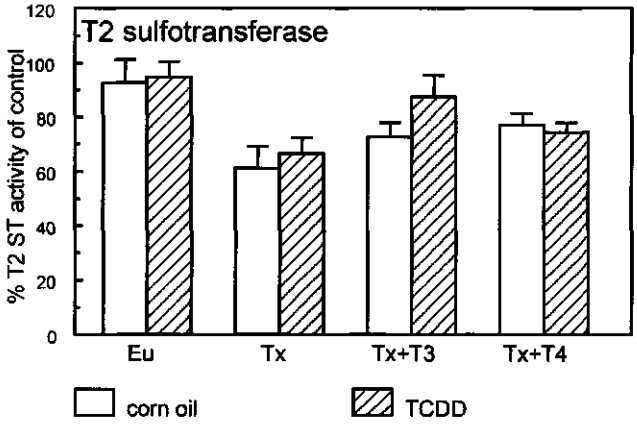

Figure 3.5 Effects of TCDD treatment on hepatic T2 ST activity in Eu, Tx, Tx+T3 or Tx+T4 rats. T2 ST activity is expressed as percentage of that in the corn oil-treated Eu rats. Results are presented as means ± SEM of 5 rats.

Discussion

In this study, we tested the hypothesis that the induction of biotransformation enzymes by PHAHs is modulated by concomitant changes in plasma thyroid hormone levels. This hypothesis is based on the observations that many PHAHs interfere with the metabolism of thyroid hormone, usually resulting in strongly reduced plasma T4 levels, and that thyroid hormone affects many metabolic pathways, including the activities of biotransformation enzymes. The hypothesis was tested by comparing the effects of TCDD on different hepatic phase I and phase II biotransformation enzymes in Eu rats, in Tx rats, and in Tx rats infused with replacement doses of T4 or T3. The plasma thyroid hormone levels and tissue iodothyronine deiodinase activities in these animals are described elsewhere (Schuur *et al.*, 1997; *Chapter 2*), showing that the hypothyroid state of the Tx rats is normalized by substitution with T4 or T3.

The major conclusion of the present study is that there is little influence of thyroid state on induction of biotransformation enzymes by TCDD in male Sprague-Dawley rats. This applies to total cytochrome P450 levels, CYP1A1 protein levels and expression, GST activity and protein levels as well as iodothyronine ST activity. The only indications for a significant interaction between thyroid state and TCDD treatment were the decreased TCDD-induced PNP UGT and CYP1A1 activities in Tx rats as compared to Eu control rats. In addition, basal NADPH cytochrome c reductase activity and, although not significantly, basal iodothyronine ST activity were somewhat reduced in Tx rats.

The finding that basal and TCDD-induced total hepatic cytochrome P450 content were not affected in Tx rats is in agreement with other studies (Rozman *et al.*, 1985b; Henry and Gasiewicz, 1986; Eltom *et al.*, 1992). However, other reports have shown an increase in basal

P450 levels after thyroidectomy and a decrease after treatment with T3 or T4 (Rumbaugh *et al.*, 1978; Leakey *et al.*, 1982; Müller *et al.*, 1983a; Goudonnet *et al.*, 1990). The discrepancies between these findings may be related to possible differences in severity and duration of the hypo- and hyperthyroid states of the animals investigated.

NADPH cytochrome c reductase activity is a measure for the NADPH cytochrome P450 reductase activity (Ram and Waxman, 1992). The lower reductase activities we found in Tx versus Eu rats are in agreement with earlier reports that hypophysectomy and methimazole-induced hypothyroidism result in decreased NADPH cytochrome P450 reductase activities and protein levels in rats (Kato and Takahashi, 1968; Waxman *et al.*, 1989; Ram and Waxman, 1992). Surprisingly, the decreased reductase activity in Tx rats was not restored by T3 or T4 substitution, perhaps because this requires longer periods of replacement.

TCDD was found to induce CYP1A1 activity in all rats. However, in Tx rats the degree of induction was significantly (≈30%) lower than in Eu rats, which was reversed by substitution of Tx rats with either T3 or T4. This suggests a permissive effect of thyroid hormone on TCDD-induced CYP1A1 activity, in agreement with earlier reports (Eltom *et al.*, 1992; Henry and Gasiewicz, 1986). However, although hyperthyroidism may lead to increased basal CYP1A1 activities (Müller *et al.*, 1983a), it has also been reported to impair TCDD induction of this isoenzyme (Eltom *et al.*, 1992). In contrast, Rozman *et al.* (1985b) found no effect of thyroidectomy on induction of EROD by a lethal TCDD dose (100 μg/kg). Roth *et al.* (1988) showed that the effects of thyroidectomy on the induction of EROD activity indeed depends on the TCDD dose; low doses of TCDD produced smaller effects in Tx than in normal rats, and the opposite was found at higher TCDD doses. However, at the TCDD dose used in our experiment (10 μg/kg) they found a higher EROD induction in Tx than in control rats. This discrepancy may be explained by differences in duration of TCDD exposure.

The decreased TCDD-induced CYP1A1/1A2 activity we found in Tx versus Eu rats was not associated with decreased CYP1A1 protein or mRNA levels, suggesting that this effect was not exerted at the level of CYP1A1 gene expression. A possible explanation for the diminished TCDD-induced CYP1A1/1A2 activity in Tx rats is the decreased NADPH cytochrome P450 reductase activity that was observed in these animals. This is supported by the study of Waxman *et al.* (1989) who reported that cytochrome P450-catalyzed hydroxylations are stimulated by supplementation of microsomal incubations with NADPH cytochrome P450 reductase, suggesting that the latter is rate-limiting.

The possible modulation by thyroid state of the induction of UGT isoenzymes by TCDD was studied using PNP as a substrate for one of the UGTs involved in the glucuronidation of T4 (Visser *et al.*, 1993a; 1996b). PNP UGT activity was strongly induced by TCDD treatment in agreement with previous studies using TCDD and related compounds (Beetstra *et al.*, 1991; Saito *et al.*, 1991; Visser *et al.*, 1993a; Van Birgelen *et al.*, 1995). We found that thyroidectomy suppressed TCDD-induced PNP UGT activity, which was restored by substitution of Tx rats

with T3 or T4. These findings are in agreement with previous studies showing that basal and TCDD-induced phenol UGT activities are decreased in Tx rats and increased in T3-treated rats (Graham and Skett, 1987; Pennington *et al.*, 1988; Moscioni and Gartner, 1983; Chowdhury *et al.*, 1983; Goudonnet *et al.*, 1990; Masmoudi *et al.*, 1996). Therefore, PNP UGT activity appears to be positively regulated by thyroid hormone.

TCDD treatment induced a small increase in GST activity and a marked increase in GST subunit 1-1 levels in agreement with findings reported by others (Pimental *et al.*, 1993). Neither basal nor TCDD-induced GST activity and subunit levels were affected by thyroid hormone state in our study. Williams *et al.* (1986) reported on an increase in GST activity with CDNB or DCNB as substrate and a decrease with 1,2-epoxy-3-(*p*-nitrophenoxy)propane as substrate in mice made hypothyroid by treatment with propylthiouracil. These effects were reversed by treatment of the hypothyroid mice with T3. Beckett *et al.* (1988) found a decrease in GST activity with CDNB as substrate after treatment of rats with T3 or T4 for 4 weeks, which was associated with decreases in GST subunit 1-1, 2-2 and 3-3 levels.

In the present study, hepatic iodothyronine ST activity was assayed utilizing T2 as the preferred substrate (Visser *et al.*, 1996a). We observed a decrease in iodothyronine ST activity in Tx versus Eu rats, which was restored by infusion of replacement doses of T4 or T3. These findings are in agreement with the decrease in hepatic T3 ST activity in Tx rats and its reversal by T3 substitution reported before by Gong *et al.* (1992). TCDD treatment had no effect on T2 ST activity. It has previously been reported that treatment of rats with 3-methylcholanthrene results in a decreased hepatic expression of hydroxysteroid ST (Runge-Morris and Wilusz, 1994), but it is unlikely that this isoenzyme is involved in the sulfation of iodothyronines.

In conclusion, we found little support for the hypothesis that the induction of biotransformation enzymes by TCDD is modulated by the marked changes in thyroid hormone levels that are associated with TCDD treatment. The variable responses of the basal and TCDD-induced activities of these enzymes to changes in thyroid state observed in our study and those published by others may be due to differences in strain and sex of the animals, the severity and duration of the hypo- and hyperthyroid states induced as well as the duration and dose of TCDD treatment.

The most obvious indications for an interaction between thyroid state and TCDD treatment were the decreased TCDD-induced CYP1A1/1A2 and PNP UGT activities in Tx rats. It remains unknown, however, if the marked decrease in plasma T4 level associated with TCDD treatment is a limiting factor in the induction of these biotransformation enzymes. In this respect it should be realized that the plasma level of the bioactive hormone T3 shows a much smaller decrease in response to TCDD treatment than the plasma level of the prohormone T4.

Acknowledgements

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CHAPTER 4

EFFECT OF THYROID STATE ON POLYCHLOROBIPHENYL-INDUCED CYTOCHROME P450 1A AND 2B ISOZYME EXPRESSION

Abstract

Earlier reports have suggested a potential modulating effect of thyroid state on cytochrome P450 expression. PCBs induce a decrease in plasma T4 and are potent inducers of CYP1A and 2B isozymes. In this study we investigated the potential interaction between thyroid hormone and PCBs in the regulation of CYP1A1 and CYP2B expression. Hypothyroid (surgically thyroidectomized, Tx) male Sprague-Dawley rats, euthyroid (Eu) rats, and rats made hyperthyroid by infusing 10 μg T3/100 g BW/day (Eu+T3) were treated with a single ip injection of 75 mg 2,2',4,4',5,5'-hexachlorobiphenyl (PCB 153)/kg BW, 100 μg 3,3',4,4',5-pentachlorobiphenyl (PCB 126)/kg BW or with vehicle (corn oil). Four days after PCB administration, rats were sacrificed, and blood and livers were collected for analysis.

The thyroid state of the rats was confirmed by measurement of plasma T4, T3 and TSH and of functional parameters such as hepatic type I deiodinase activity, malic enzyme activity and α-glycerolphosphate dehydrogenase activity. Total hepatic cytochrome P450 content was increased by about 50% by PCB treatment in all groups, but was not affected by thyroid state. NADPH cytochrome P450 reductase activity was decreased in Tx rats and increased in Eu+T3 rats, while PCB treatment had no effect. PCB 126 specifically induced T4 UGT activity, measured in the absence of detergent, and CYP1A activity, protein and mRNA levels, whereas PCB 153 induced T4 UGT activity, measured in the presence of the detergent Brij 56, and CYP2B activity, protein and mRNA levels. Thyroid state did not significantly affect T4 UGT activity or CYP1A and CYP2B activities, protein or mRNA levels.

In conclusion, hepatic NADPH cytochrome P450 reductase activity is dependent on thyroid state, whereas both total cytochrome P450 as well as CYP1A1 and CYP2B are not or only marginally affected. The results from this study are different from earlier reports suggesting a modulating effect of thyroid state on CYP1A and CYP2B. In our hands, the PCB-induced decrease in T4 was not enough to create an effect on the activity of the biotransformation enzymes tested.

based on A.Gerlienke Schuur, Marylse C. Vis, Mira A.M. Wenker, Abraham Brouwer, and Theo J. Visser (1998). Effects of thyroid state on polychlorobiphenyl-induced induction of cytochrome P450 1A and 2B isozyme expression, submitted.

Introduction

PCBs and PCDDs are potent inducers of biotransformation enzymes in the liver. PCB congeners can be divided into three distinct groups, based on their induction of specific isozymes of the cytochrome P450 system. The first group consists of the non-ortho coplanar PCBs which induce CYP1A1/2, i.e. a 3MC- or dioxin-type of induction. Secondly, the di-ortho substituted PCBs which, like PB, induce CYP2B1/2. The final group consists of the mono-ortho coplanar PCBs which induce both CYP1A and CYP2B isozymes (Safe, 1994).

PCBs and PCDDs are also potent thyroid state-modulating environmental toxicants (for review see Brouwer *et al.*, 1998). PCDDs and PCBs cause a decrease in plasma total and free T4 levels in rats, mice and marmoset monkeys (for TCDD:Bastomsky, 1977; Potter *et al.*, 1983; 1986; Pazdernik and Rozman, 1985; Henry and Gasiewicz, 1986; 1987; Jones *et al.*, 1987; Roth *et al.*, 1988; Pohjanvirta *et al.*, 1989; Lans, 1995; for Aroclor: Bastomsky, 1974; Byrne *et al.*, 1987; Gray *et al.*, 1993; Brouwer, 1989; Beetstra *et al.*, 1991). This decrease in plasma T4 levels is accomplished by at least three different mechanisms. Firstly, PCBs and PCDDs induce hepatic UGT towards T4 which results in an increased excretion of T4 glucuronide (Bastomsky, 1974; Beetstra, 1991). Secondly, PCBs and PCDDs directly inhibit thyroid hormone secretion (Gupta *et al.*, 1973; Rozman *et al.*, 1986; Collins and Capen, 1980b; Sepkovic and Byrne, 1984). Thirdly, T4 binding to plasma transthyretin (TTR) is competitively inhibited by hydroxylated metabolites of different PCDDs and PCBs (Brouwer and van den Berg, 1986; Lans *et al.*, 1993; 1994).

However, it is still unclear whether this decrease in plasma thyroid hormone levels after treatment with PCBs and PCDDs should be considered as a toxicological effect, or an adaptive response of the animal to a toxic stressor. It has been reported that thyroidectomy has a partially protective effect on TCDD-induced toxicity (Pazdernik and Rozman, 1985; Rozman *et al.*, 1984). Alteration of thyroid hormone levels may also have a modulating effect on the induction of biotransformation enzymes. Eltom *et al.* (1992) found a suppressive effect of Tx on TCDD-induced CYP1A1 activity, measured as EROD activity, in rat liver. However, Rozman *et al.* (1985b) found no effect of Tx on EROD activity after TCDD treatment. We have previously also investigated the hypothesis that thyroid hormones modulate the induction of biotransformation enzymes (Schuur *et al.*, 1997; 1998a; *Chapter 2 and 3*). We found that induction of EROD activity by TCDD was slightly decreased in Tx vs normal rats. Yet, no effect of Tx was found on CYP1A1 protein or mRNA levels.

This lack of a clear effect of thyroid hormones on CYP1A1 activity differs from other reports on the endocrine regulation of different cytochrome P450 isozymes. Arlotto and Parkinson (1989) found a suppressive effect of thyroid hormones on hepatic CYP2A1 mRNA and protein levels. Murayama *et al.* (1991) also found lower levels of hepatic CYP2B1/2 mRNA and protein in hyperthyroid rats. Ram and Waxman (1991) established that thyroid

hormones can differentially regulate the expression of three male-specific, growth hormone-regulated hepatic P450s. CYP3A2 was shown to be under negative control of T4 and CYP2C11 under positive control, while CYP2A2 was not appreciably affected by thyroid state.

Here we report on further studies of the interaction between thyroid hormone and PCBs in the regulation of CYP1A1 and CYP2B expression. 2,2',4,4',5,5'-Hexachlorobiphenyl (PCB 153) was chosen as a CYP2B inducer and 3,3',4,4',5-pentachlorobiphenyl (PCB 126) as a CYP1A1 inducer. The experiment involved both Tx rats and rats made hyperthyroid by T3 infusion using osmotic minipumps. The thyroid state of the rats was confirmed by measurement of plasma T4, T3, and TSH levels and of functional parameters such as type I deiodinase (D1) activity, α-glycerolphosphate dehydrogenase (α-GPD) activity and malic enzyme activity in liver.

Materials and methods

Chemicals.

PCB 153 and PCB 126 were obtained from Schmidt BV (Amsterdam, The Netherlands); T4, T3, rT3, DTT, UDPGA, Brij 56, PTU, 2-iodophenyl-3-nitrophenyl-5-phenyltetrazolium chloride (INT) and BSA from Sigma Chemicals Co. (St. Louis, MO, USA); malic acid and resorufin (RR) from Janssen Chimica (Tilburg, The Netherlands); NADPH from Boehringer Mannheim GmbH (Mannheim, Germany); Trizol and sodium dodecyl sulfate (SDS) from Gibco BRL (Breda, The Netherlands) and BioRad protein reagent from Bio-Rad Laboratories (Richmond, CA, USA). [^{125}I]T4, [^{125}I]T3 and [^{125}I]rT3 were obtained from Amersham (Buckinghamshire, UK); they were purified on Sephadex LH-20 (Pharmacia, Woerden, The Netherlands) before each assay. All other chemicals were of analytical grade.

Animals and treatment.

Male Sprague-Dawley rats, surgically thyroidectomized or sham-operated by the supplier at 4 weeks of age, were purchased from Iffa Credo (L'Arbresle Cedex, France). The rats were obtained at 6 weeks of age; they were allowed to acclimatize for 2 weeks and were maintained at 50% humidity and 21°C on bedding in plastic cages with a 12 h light-12 h dark cycle. Rat chow (Hope Farms, Woerden, The Netherlands) and tap water with 0.5% $CaCl_2$ were supplied *ad libitum*. Model 2002 Alzet minipumps (Charles River Wiga, Sulzfeld, Germany) were implanted intraperitoneally (ip) in sham-operated, euthyroid (Eu) rats at 8 weeks of age (day 0) under ether anaesthesia, delivering 10 μg T3/100 g BW per day in 0.1 N NaOH in 0.9% NaCl (Eu+T3 group; n=15). All 15 Tx and 15 other Eu rats received pumps with solvent only. Body weight was recorded twice a week. On day 6 following osmotic minipump implantation, 5 rats of each group (Tx, Eu and Eu+T3) were given an ip injection of 100 μg PCB 126 or 75 mg PCB

153 in corn oil/kg BW or with corn oil alone (controls; 5 ml/kg BW).

On the day before the PCB injection (day 5) and on day 10, blood (≈1 ml) was collected by orbital puncture in heparinized tubes and stored on ice until plasma was prepared by centrifugation. All rats were sacrificed under ether anaesthesia on day 10. Livers were perfused with saline, isolated, weighed and frozen in 3 portions on dry ice. Kidneys, thymus and brains were isolated, weighed and frozen on dry ice. All tissues were stored at -80°C and plasma at -20°C until analysis. All procedures were approved by the Animal Welfare Committee of the Agricultural University Wageningen.

Tissue preparation.

Livers and brains were homogenized and centrifuged as described before (Schuur *et al.*, 1997; 1998a; *Chapter 2 and 3*). Mitochondria, cytosol and microsomes were prepared by differential centrifugation and stored at -80°C. Protein levels of tissue fractions were determined using the Bio-Rad protein reagent (Bradford, 1976) and BSA as a standard. Another part of the liver was used for RNA isolation using Trizol reagent according to the suppliers protocol.

Thyroid hormone analysis.

Plasma TT4, FT4 and TT3 were analysed in duplicate using the Amerlite chemiluminescence kits (Amersham, Little Chalfont, UK) according to the protocol of the supplier with the following modifications: the TT4 and TT3 assay buffers were diluted five times with demineralized water; the standard curve for TT4 ranged from 0 to 120 nmol/l. TSH was determined by RIA with the materials and protocols of the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), using TSH RP-2 as a standard.

Enzyme assays.

Type I iodothyronine deiodinase (D1). Hepatic D1 activity was determined as previously described (Mol and Visser, 1985). Shortly, microsomes (20 µg protein/ml) were incubated for 30 min at 37°C with 1 µM rT3 and ≈100,000 cpm [^{125}I]rT3 in 0.1 M phosphate buffer, pH 7.2, 2 mM EDTA and 5 mM DTT. The reaction was stopped on ice by the addition of 750 µl 0.1 M HCl. The reaction mixtures were centrifuged and radioiodide was analyzed in the supernatant by Sephadex LH-20 chromatography as described before (Rutgers *et al.*, 1989).

UDP-glucuronyltransferase (UGTs). Hepatic T4 UGT activity was determined essentially according to Beetstra *et al.* (1991). Microsomes (1 mg/ml) were incubated for 30 min at 37°C with 1 µM T4 (50,000 cpm [^{125}I]T4), 3.75 mM $MgCl_2$, 0.1 mM PTU in the presence or absence (blank) of 5 mM UDPGA in 200 µl 75 mM Tris-HCl, pH 7.8 (Rutgers *et al.*, 1989). Reactions were stopped by the addition of 0.2 ml ice-cold methanol. After centrifugation, 0.2 ml supernatant was mixed with 0.75 ml 0.1 M HCl and analyzed for glucuronide formation by chromatography on Sephadex LH-20 minicolumns (Beetstra *et al.*, 1991). T4 UGT was

determined in the absence and in the presence of the detergent 0.05% Brij 56 (Visser *et al.*, 1993b).

Malic enzyme. Malic enzyme activity in liver cytosol was determined according to Hsu and Lardy (1967) by following the formation of NADPH spectrophotometrically for 10 min. at 340 nm using a 96-wells plate spectrophotometer (Thermo Max Microplate Reader, Molecular Devices, Menlo Park, CA, USA). Incubation mixtures contained 16 mM $MnCl_2$, 1.13 mM NADP, 0.1 mM malic acid, 0.33 mg cytosolic protein/ml in 0.13 M triethanolamine buffer (pH 7.4).

α-Glycerolphosphate dehydrogenase. αGPD activity in liver mitochondria was measured according to Garrib and McMurray (1984) with a continuous spectrophotometrical method monitoring the reduction of INT. The incubation mixture contained 0.25 mM INT, 0.8 mM potassium cyanide, 30 µl of a saturated menadione solution in 0.2% BSA, 24 mM L,D-glycerol phosphate and 0.2-1 mg protein/ml of mitochondrial pellet in a total volume of 250 µl in 0.25 M potassium phosphate buffer (pH 7.5). The absorption of INT at 490 nm was measured every 10 sec for 10 min. An extinction coefficient of 11.5 $nM^{-1}*cm^{-1}$ was used.

Total cytochrome P450. Cytochrome P450 content in liver microsomes was estimated from the CO-reduced difference spectrum in 0.1 M potassium phosphate (pH 7.4) containing 1 mg microsomal protein/ml (Omura and Sato, 1964).

NADPH cytochrome c reductase. The reduction of cytochrome c was followed spectrophotometrically at 550 nm for 1 min at room temperature. The incubation mixture contained 40 µM cytochrome c, 100 µM NADPH and 12.5 µg microsomal protein/ml in 0.3 M phosphate buffer (pH 7.7). Results were calculated using an extinction coefficient of 21 $mM^{-1}*cm^{-1}$ (Vermilion and Coon, 1978).

Ethoxyresorufin and pentoxyresorufin O-deethylase. EROD was measured according to the method of Burke *et al.* (1977) adapted for use with 96 wells plates and a fluorospectrophotometric plate reader (Cytofluor 2350, Millipore, Etten-Leur, The Netherlands) (Schuur *et al.*, 1998a; *Chapter 3*). PROD was measured following the same procedure as for EROD with a final concentration of 2 µM pentoxyresorufin (PR) and 25-100 µg microsomal protein/ml.

CYP1A1 and CYP2B protein levels. Cytochrome P4501A1 and 2B1/2 ELISA systems from Amersham were used to determine CYP1A1 or 2B1/2 protein levels according to the protocol of the supplier.

CYP1A1 or CYP2B mRNA analysis. Mouse CYP1A1 (ATCC-no. 63006) and human GAPDH (ATCC-no. 57234) cDNA probes were obtained from ATCC (Rockville,MD, USA). Rat CYP2B cDNA probe was a generous gift from Dr. M. Adesnik (Kumar *et al.*, 1983). Serial dilutions of total RNA (0.176-22.5 µg) were dot-blotted using a mini-blot apparatus (Minifold, Schleicher and Schuell, Den Bosch, The Netherlands) on Hybond-N+ nitrocellulose membrane (Amersham). Hybridization was carried out overnight at 65 °C in 0.5 M sodium phosphate (pH

7.2), 7% SDS and 1 mM EDTA using random-primed ^{32}P-labelled probes. Membranes were washed for 30 min at 65°C with successively 2xSSC/0.1% SDS, 1xSSC/0.1% SDS and 0.3xSSC/0.1% SDS (1xSSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0). Messenger RNA signals were quantified using a Phosphor Imager (Molecular Dynamics, Sunnyvale, CA), and CYP1A1 or CYP2B mRNA was standardized relative to GAPDH mRNA.

Statistics.

The data were first tested on normality and homogeneity of variances with respectively the Chi-square test and the Bartlett test. The data were log-transformed to obtain normality and homogeneity. The log-transformed data were evaluated with a 2-way ANOVA with the thyroid state (Tx, Eu and Eu+T3) and the PCB treatment (vehicle and 75 mg PCB 153/kg BW or vehicle and 100 µg PCB 126/kg BW) as the two factors. Treatment-related effects were further determined using ANOVA with a least significant difference test ($p<0.05$) to find significant differences between the treatment groups. $P<0.05$ was considered significant. All analyses were performed with the statistical software package SAS. Data are presented as the mean ± SEM.

Results

Body weights of Tx rats were 74% of Eu controls prior to osmotic minipump implantation (day 0) and 72% of both Eu and Eu+T3 rats at the end of the experiment (day 10). Liver and thymus weights were not significantly different between the various treatment groups on day 10 (data not shown).

Plasma thyroid hormone levels

Plasma concentrations of TT4, FT4, TT3 and TSH in corn oil-treated control rats on day 5 (one day prior to PCB treatment) and day 10 (end of the experiment) after osmotic minipump implantation are presented in Table 4.1. Eu rats had higher levels of TT4 on day 10 than on day 5, but TSH, TT3 and FT4 levels remained fairly constant over the 6 day period in Eu rats.

Tx rats showed a significant (62%) decrease in plasma TT3 levels as compared to Eu rats on day 10 but not on day 5. TT4 and FT4 plasma levels in Tx rats were significantly decreased to ≈15% of Eu rats on day 5 and 10. Plasma TSH concentrations were increased ≈10 fold in Tx rats compared with Eu rats, also on both days.

Treatment of Eu rats with T3 caused ≈4 fold increase in plasma TT3 concentrations on day 5, compared with Eu rats. Plasma TT3 levels in Eu+T3 rats were lower on day 10 than on day 5, and approached the levels in Eu rats. TT4 and FT4 plasma levels were strongly decreased in Eu+T3 rats to ≈10% of Eu levels on day 5, and to ≈35% of Eu levels on day 10. TSH levels in Eu+T3 rats were significantly lower, ≈6-fold on day 5 and ≈2-fold on day 10 than in Eu rats.

Table 4.1 Plasma thyroid hormone levels in Tx, Eu, and Eu+T3 rats.

	Tx		Eu		Eu+T3	
	day 5	day 10	day 5	day 10	day 5	day 10
TT3 (nmol/l)	1.13 ± 0.08	0.44 [ac] ± 0.02	1.14 ± 0.08	0.86 ± 0.04	4.10 [ab] ± 0.70	0.93 ± 0.02
TT4 (nmol/l)	6.72 [a] ± 1.00	8.76 [a] ± 1.47	35.20 ± 3.28	48.83 ± 1.75	2.64 [a] ± 0.24	13.24 [a] ± 4.67
FT4 (pmol/l)	4.96 [ac] ± 0.52	3.19 [a] ± 0.53	21.65 ± 1.96	20.15 ± 1.34	2.32 [a] ± 0.38	5.44 [a] ± 1.78
TSH (ng/l)	25.41 [a] ± 4.80	24.68 [a] ± 2.65	3.07 ± 0.67	2.60 ± 0.65	0.48 [a] ± 0.14	1.24 [a] ± 0.45

Note. Euthyroid male Sprague-Dawley rats were infused with T3 (10 μg/100g BW/day) via an osmotic minipump from day 0 on (Eu +T3). Thyroidectomized (Tx) and euthyroid (Eu) male Sprague-Dawley rats received a minipump with solvent only. On day 6 these rats were given an injection with corn oil (as a control). Blood was taken on day 5 and on day 10, the final day of the experiment. Data are presented as mean ± SEM (n=5 for all groups, except Eu n=4). a) denotes a significant difference ($p<0.05$) with the Eu group, b) a significant difference ($p<0.05$) with the Tx group, and c) a significant difference ($p<0.05$) with the Eu+T3 group.

The effects of PCB 153 or PCB 126 on plasma thyroid hormone levels are presented in Table 4.2. No effect of either the di-ortho PCB 153 or the coplanar PCB 126 treatment was found on plasma TT3 concentrations on day 10 in any of the groups.

In Tx rats, plasma TT4 levels were not significantly lower after PCB 153 treatment to 53% of corn oil-treated Tx rats. In PCB 126 treated Tx and Eu rats, plasma TT4 concentrations were also lower, to 64% and 75% of corn oil treated rats, respectively. Little or no change in plasma TT4 was observed in the PCB 153 or PCB 126 treated other groups.

Plasma FT4 levels were changed in a similar manner as TT4 levels after PCB treatment. PCB 153 caused a not-significant decrease in plasma FT4 levels in Tx rats (to 51% of corn oil Tx rats), no obvious effect in Eu rats and not-significant higher levels in Eu+T3 rats (to 166% of corn oil treated Eu+T3 rats). FT4 concentrations were somewhat lower in all groups after PCB 126 treatment compared with corn oil-treated rats, but this was only significant in Tx rats (to 66% of corn oil-treated Tx rats).

Plasma TSH concentrations were not significantly changed by PCB treatment in any of the groups.

Table 4.2 Plasma thyroid hormone levels in Tx, Eu, and Eu+T3 rats after treatment with PCB 153 and PCB 126.

		TT3 (nmol/l)	TT4 (nmol/l)	FT4 (pmol/l)	TSH (ng/l)
Tx	Corn oil	0.44 ± 0.02 [ac]	8.76 ± 1.47 [a]	3.19 ± 0.53 [a]	24.68± 2.65 [a]
	PCB 153	0.34 ± 0.06 [ac]	4.64 ± 0.34 [a]	1.63 ± 0.15 [a]	21.60± 2.44 [a]
	PCB 126	0.54 ± 0.09 [ac]	5.64 ± 0.58 [a]	2.10 ± 0.19 [a]	19.38± 2.98 [a]
Eu	Corn oil	0.86 ± 0.04	48.83 ± 1.75	20.15 ± 1.34	2.60± 0.66
	PCB 153	0.91 ± 0.06	43.50 ± 1.34	18.28 ± 1.03	3.30± 0.53
	PCB 126	0.90 ± 0.03	36.46 ± 1.93 [d]	17.52 ± 1.35	4.15± 0.36
Eu+T3	Corn oil	0.93 ± 0.02	13.24 ± 4.67 [a]	5.44 ± 1.78 [a]	1.24± 0.45 [a]
	PCB 153	0.90 ± 0.06	19.94 ± 4.64 [ab]	9.02 ± 2.25 [ab]	1.38± 0.34 [a]
	PCB 126	1.00 ± 0.09	11.32 ± 5.63 [a]	5.33 ± 2.37 [a]	1.17± 0.45 [a]

Note. Thyroidectomized (Tx), euthyroid (Eu), and Eu rats infused with T3 (10 μg/ 100 g BW/day; Eu +T3) from day 0 were treated with 75 mg PCB 153/kg BW, 100 μg PCB 126/kg BW, or with the vehicle corn oil on day 6. The experiment was terminated on day 10. Data are presented as mean ± SEM (n=5 for all groups, except Eu n=4). a) denotes a significant difference ($p<0.05$) with the Eu group, b) a significant difference ($p<0.05$) with the Tx group, c) a significant difference ($p<0.05$) with the Eu+T3 group, d) denotes a significant difference ($p<0.05$) with the corn oil treated rats in the same group.

Thyroid hormone metabolism

Figure 4.1 shows thyroid state-dependent enzyme activities in livers isolated at day 10 after the implantation of the osmotic minipumps, i.e. 4 days after PCB treatment. Hepatic D1 activity was lower in Tx rats compared with Eu rats, in particular following PCB treatment. In Eu+T3 rats, a 2-fold elevation of D1 activity was observed compared with Eu rats, although the difference was smaller after PCB 153 treatment and not significant after PCB 126 treatment. PCB treatment had no significant effect on D1 activity.

Hepatic mitochondrial αGPD activity was decreased in all Tx rats to ≈30% of that in Eu rats. In Eu+T3 rats, αGPD activity was ≈2.6-fold higher than in Eu rats. No effect was observed on αGPD activity by PCB 126 and PCB 153 in all groups.

Thyroidectomy caused a decrease in hepatic malic enzyme activity to ≈30% of that in Eu rats. Malic enzyme activity was increased ≈2.4-fold in Eu+T3 rats compared with Eu rats. No significant effects were found on malic enzyme activity by PCBs in any group.

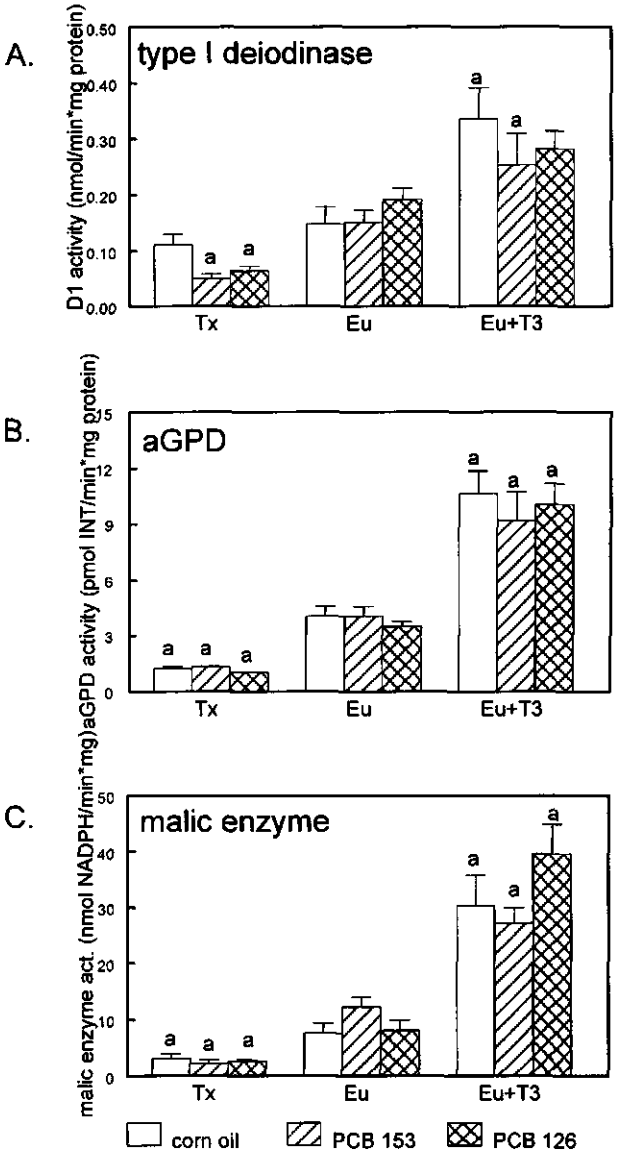

Figure 4.1 Functional thyroid hormone status parameters in Tx, Eu and Eu+T3 rats Hepatic type I deiodinase activity (A), α-glycerol phosphate dehydrogenase (α-GPD), (B) and malic enzyme (C) were measured on day 10 after minipump implantation, i.e. at 4 days after treatment with corn oil, PCB 153 or PCB 126. Results are presented as mean ± SEM (for all groups n=5, only Eu corn oil group n=4). a) denotes a significant difference ($p<0.05$) with the corresponding Eu group.

T4 UGT activities were measured in the absence or presence of Brij 56 (Figure 4.2). No significant effects of thyroid state on T4 UGT activities were found, although a downward trend was observed from Tx to Eu+T3 rats. PCB 126 treatment, but not PCB 153 treatment, caused a significant increase in T4 UGT activities measured in the absence of Brij 56 in all groups. In contrast, T4 UGT activities measured in the presence of Brij 56 were increased significantly by PCB 153 treatment, but not by PCB 126 treatment, in all groups.

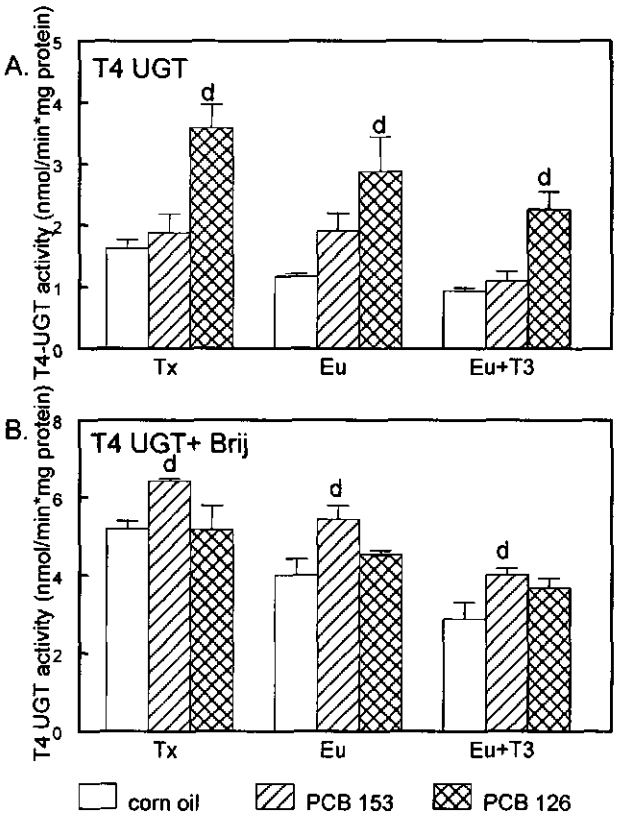

Figure 4.2 Hepatic T4 UGT activities in Tx, Eu and Eu+T3 rats,measured in the absence (A) or presence of Brij 56 (B), on day 10 after minipump implantation, ie at 4 days after treatment with corn oil, PCB 153 or PCB 126. Results are presented as mean ± SEM (for all groups n=5, only Eu corn oil group n=4). d) denotes a significant difference ($p<0.05$) with the corn oil treated rats in the same group.

Effects on cytochrome P450 parameters

Effects of thyroid state on basal and PCB-induced cytochrome P450 parameters are presented in Table 4.3 and Figures 4.3, 4.4, and 4.5.

Total amounts of cytochrome P450 were not significantly different between Tx, Eu and Eu+T3 rats (Figure 4.3A). Total cytochrome P450 levels were increased significantly after treatment with PCB 153 and PCB 126 irrespective of thyroid state. The increase was over 2-fold with PCB 153 and about 1.5-fold with PCB 126. NADPH cytochrome P450 reductase was measured by following the reduction of cytochrome c (Figure 4.3B). NADPH cytochrome c reductase was significantly decreased in Tx rats and significantly increased in Eu+T3 rats compared to Eu rats. NADPH cytochrome c reductase activity was not significantly affected by PCB treatment.

CYP1A1 activity was determined as EROD activity (Figure 4.4A). Basal and PCB-induced CYP1A1 activity was not influenced by the thyroid state. There was also no effect of thyroid state on CYP1A1 protein (Figure 4.4B) and mRNA (Figure 4.4C) levels. PCB 126

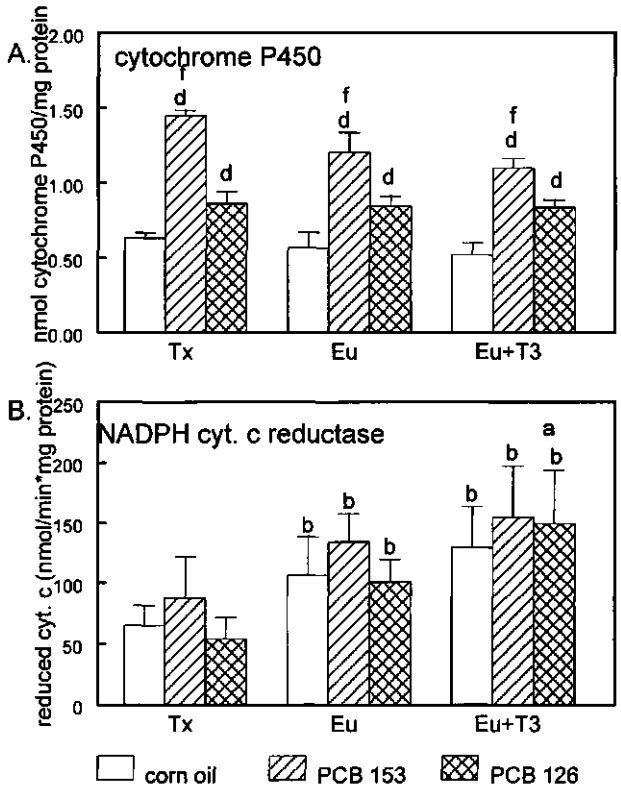

Figure 4.3 Total hepatic cytochrome P450 (A) and NADPH cytochrome c reductase activity (B) in Tx, Eu and Eu+T3 rats, measured on day 10, 4 days after exposure to corn oil, PCB 153 or PCB 126. Results are presented as mean ± SEM (for all groups n=5, only Eu corn oil group n=4). a) denotes a significant difference ($p < 0.05$) with corresponding Eu group, b) a significant difference ($p < 0.05$) with corresponding Tx group, d) a significant difference ($p<0.05$) with corn oil treated rats of the same group and f) a significant difference ($p<0.05$) with PCB 126 treatment group.

induced the EROD activity 200-fold in all groups compared with corresponding corn oil-treated controls.

CYP1A1 protein amounts were also highly induced by PCB126 in all thyroid state groups. The fold induction of CYP1A1 protein levels by PCB 126 was about 22 times in Tx, 37 times in Eu and 20 times in Eu+T3 rats. PCB 126 treatment induced CYP1A1 mRNA levels about 6-fold in all groups. PCB 153 did not affect CYP1A1 activity, protein or mRNA levels (Figure 4.4).

CYP2B activity was determined as PROD activity. Basal as well as PCB-induced PROD activity, CYP2B protein and mRNA levels were not significantly affected by thyroid state (Figure 4.5A-C and Table 4.3). PCB 153 treatment caused an induction of PROD activity in all groups, although the fold induction depended on the thyroid state. In Tx rats PROD was induced 9 fold, in Eu rats 13 fold, and in Eu+T3 rats 26 fold. CYP2B1/2 protein levels were also increased by PCB 153 in all groups. The fold induction was 10 in Tx rats, 13 in Eu rats and 9 in Eu+T3 rats. CYP2B1/2 mRNA levels were also significantly induced by PCB 153 in

all groups, i.e. 31-fold in Tx, 19-fold in Eu, and 12-fold in Eu+T3 rats. PCB 126 also caused an increase in PROD activity. The induction varied between 7 and 14 fold compared with the corn oil-treated controls. CYP2B1/2 protein and mRNA levels were not changed by PCB 126 treatment in any of the groups (Figure 4.4C).

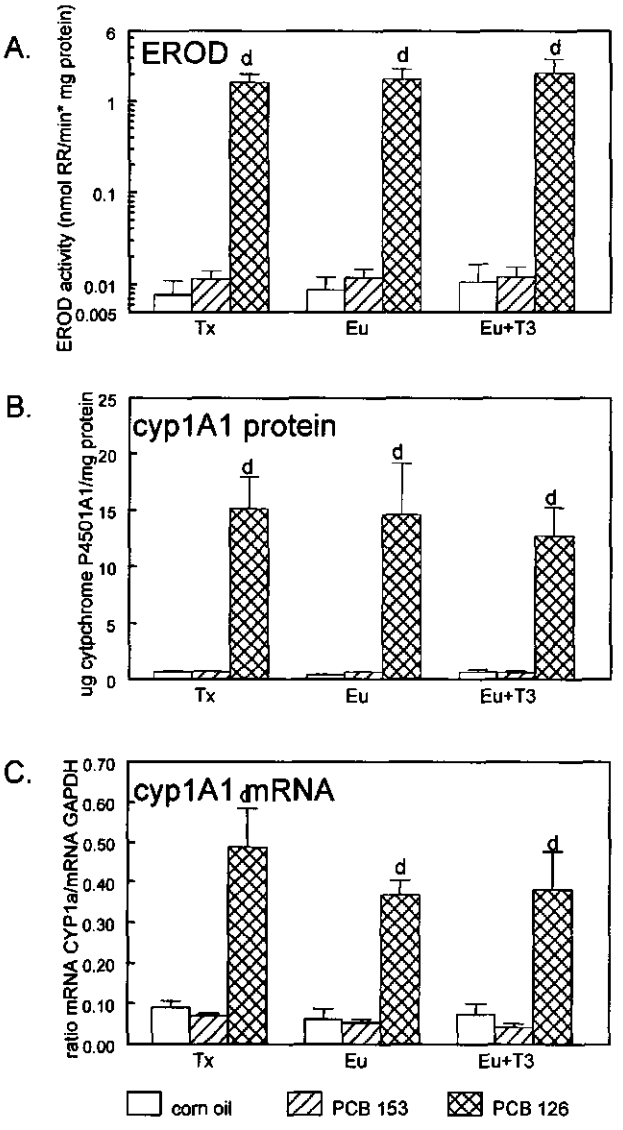

Figure 4.4 CYP1A1 parameters in Tx, Eu and Eu+T3 rats. Liver microsomal EROD activity (A), CYP1A1 protein levels (B) and CYP1A1 mRNA levels (C) were measured on day 10, 4 days after treatment with corn oil, PCB 153 or PCB 126. Results are presented as mean ± SEM (for all groups n=5, only Eu corn oil group n=4). d) denotes a significant difference ($p < 0.05$) with corresponding corn oil treated group.

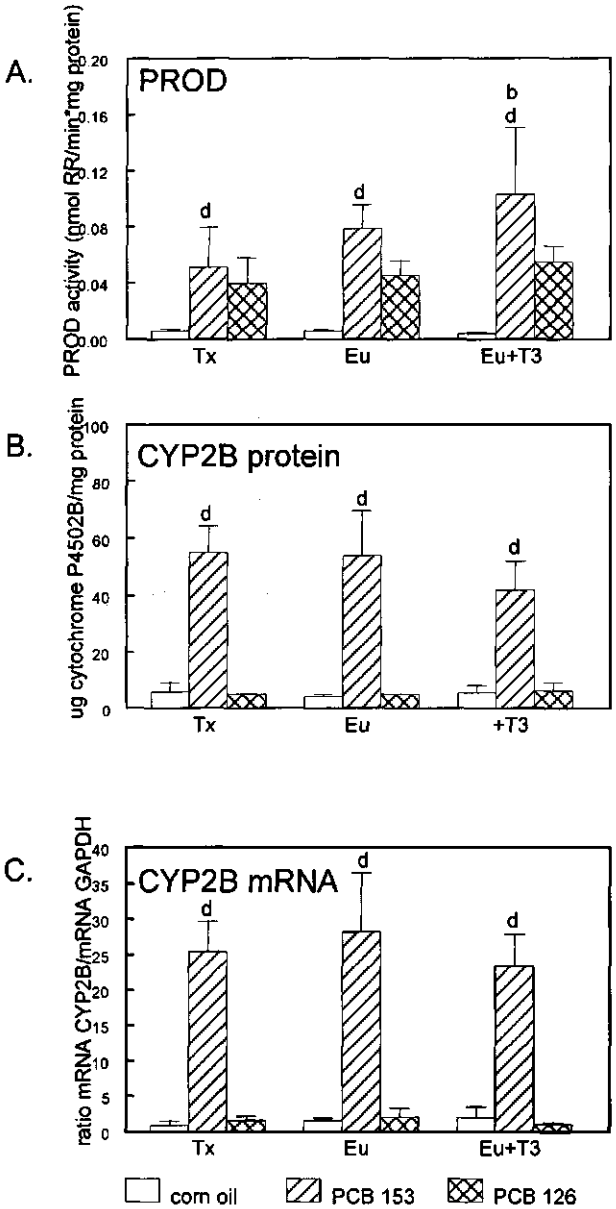

Figure 4.5 CYP2B1/2 parameters in Tx, Eu and Eu+T3 rats Liver microsomal PROD activity (A), CYP2B1/2 protein levels (B) and CYP2B1/2 mRNA levels (C) were measured on day 10, 4 days after treatment with corn oil, PCB 153 or PCB 126. Results are presented as mean ± SEM (for all groups n=5, only Eu corn oil group n=4). b) denotes a significant difference ($p<0.05$) with Tx group, d) a significant difference ($p<005$) with corresponding corn oil treated group.

The effects of thyroid state and PCB treatment on all cytochrome P450 parameters are summarized in Table 4.3, computed by a two-way ANOVA, with PCB treatment and thyroid state as factors.

It is shown that PCB 126 treatment caused a significant increase in total cytochrome

Table 4.3 PCB 126/153 and thyroid state effects on cytochrome P450 parameters (analysed by 2-way ANOVA).

		PCB effect			Thyroid effect	hormone	state
		corn oil (n=14)	PCB 153 (n=15)	PCB 126 (n=15)	Tx (n=15)	Eu (n=14)	Eu+T3 (n=15)
t otal cytochrome P450	(nmol/mg protein)	0.571	1.251 [df]	0.847 [d]	0.981	0.893	0.817
NADPH cyt. P450 reductase	(nmol/min*mg protein)	99.91	125.48 [d]	101.30	68.75 [a]	114.38 [b]	144.52[ab]
EROD activity	(nmol/min*mg protein)	0.009	0.012	1.804 [d]	0.544	0.633	0.689
CYP1A1 protein	(µg/mg protein)	0.586	0.652	14.12 [d]	0.550	0.554	0.464
CYP1A1 mRNA	(ratio to GAPDH mRNA)	0.082	0.077	0.412 [de]	0.216	0.181	0.166
PROD activity	(nmol/min*mg protein)	0.005	0.077 [df]	0.046 [d]	0.032	0.046	0.054
CYP2B1/2 protein	(µg/mg protein)	5.126	50.24 [d]	5.270	21.89	20.95	17.79
CYP2B1/2 mRNA	(ratio to GAPDH mRNA)	1.479	25.65 [df]	1.564	9.854	11.98	9.333

Note. Thyroidectomized (Tx) rats, normal euthyroid (Eu) rats, and normal rats treated with T3 (10 µg/100 g BW/day; Eu+T3) were treated with corn oil, PCB 153 (75 mg/kg BW) or PCB 126 (100 µg/kg BW). The PCB effect was computed over all different thyroid state groups together. The thyroid state effect, however, was computed over corn oil and both PCB treatments. a) denotes a significant difference ($p<0.05$) with the Eu group, b) a significant difference ($p<0.05$) with the Tx group, d) a significant difference ($p<0.05$) with the corn oil treated group, e) a significant difference ($p<0.05$) with the PCB 153 treated group, and f) a significant difference ($p<0.05$) with the PCB 126 treated group.

It is shown that PCB 126 treatment caused a significant increase in total cytochrome P450 content, EROD activity, CYP1A1 protein and mRNA levels. PCB 153 significantly increased total cytochrome P450 content, PROD activity, CYP2B protein and mRNA levels and NADPH cytochrome c reductase activity. Thyroid state only caused a significant change in NADPH cytochrome c reductase activity.

Discussion

The main objective of the present study was to investigate the possible role of thyroid state on PCB-induced cytochrome P450 1A1 and 2B isozymes expression in liver. The study was carried out in rats made hypothyroid by thyroidectomy, in euthyroid rats, and in rats made hyperthyroid by minipump infusion of excess T3. Tx rats had decreased plasma TT3, TT4 and FT4 concentrations, increased TSH levels, as well as lowered hepatic thyroid state sensitive parameters (eg D1, malic enzyme and αGPD activities), indicating that they were functionally hypothyroid. Treatment of Eu rats with T3 showed increased plasma TT3 and decreased TSH levels, although this was less clear on day 10 than on day 5. However, increased hepatic D1, malic enzyme and αGPD activities were clear signs of a functional hyperthyroidism. This was accompanied by strongly reduced TT4 and FT4 levels, as a consequence of suppressed TSH secretion.

T4 is glucuronidated in rat liver by at least two different UGT isozymes: bilirubin UGT and phenol UGT. Phenol UGT activity is inhibited *in vitro* in the presence of Brij 56, whereas bilirubin UGT activity is stimulated *in vitro* by this detergent (Visser *et al.*, 1993a/b). T4 UGT activity in the absence of detergent (the phenol type) was induced after treatment with PCB 126, while PCB 153 treatment caused an induction of T4 UGT activity in the presence of Brij (the bilirubin type). This is in agreement with other reports showing an induction of phenol UGT activity by 3MC inducers such as PCBs and TCDD and an induction of bilirubin UGT activity by PB inducers such as di-ortho PCBs (Moscioni and Gartner, 1983; Beetstra *et al.*, 1991; Saito *et al.*, 1991; Barter and Klaassen, 1992a; De Sandro *et al.*, 1992). In this study, thyroid state had no effect on basal and PCB-induced UGT activities. In earlier reports, thyroidectomy led to a slight increase in bilirubin UGT activity in rats and treatment with T3 resulted in a decrease in this activity (Chowdhury *et al.*, 1983; Moscioni and Gartner, 1983; Pennington *et al.*, 1988; Visser *et al.*, 1996b). In contrast, thyroidectomy had a suppressive effect on phenol UGT activity, while treatment with T3 or T4 resulted in a restoration of UGT activity up to Eu or even higher levels (Chowdhury *et al.*, 1983; Moscioni and Gartner, 1983; Graham and Skett, 1987; Pennington *et al.*, 1988; Goudonnet *et al.*, 1990; Visser *et al.*, 1996b; Masmoudi *et al.*, 1996).

No significant effect of thyroid state was observed on basal or PCB-induced total cytochrome P450 levels. Other investigations have reported conflicting effects of thyroid state on cytochrome P450 levels in rat liver. Thyroidectomy was found to increase (Rumbaugh *et al.*, 1978; Goudonnet *et al.*, 1990) or to have no effect (Kato and Takahashi, 1968; Rozman *et al.*, 1985b; Henry and Gasiewicz, 1987; Eltom *et al.*, 1992; Schuur *et al.*, 1998a), whereas T3 treatment was reported to cause an increase (Müller *et al.*, 1983a) or a decrease in total cytochrome P450 levels (Rumbaugh *et al.*, 1978; Leakey *et al.*, 1982; Müller *et al.*, 1983b; Goudonnet *et al.*, 1990; Rosenberg *et al.*, 1995). The opposite effects of T3 on basal cytochrome P450 levels in the two studies by Müller *et al.* (1983a/b) were suggested to be due to different doses and administration routes or different ages of the animals.

In this study, NADPH cytochrome P450 reductase activities were diminished in Tx rats and elevated in Eu+T3 rats compared with Eu rats. This is in agreement with previous reports (Kato and Takahashi, 1968; Rumbaugh *et al.*, 1978; Leakey *et al.*, 1982; Rozman *et al.*, 1985b; Waxman *et al.*, 1989; Ram and Waxman, 1992; Schuur *et al.*, 1998a). PCB 153 slightly increased NADPH cytochrome P450 reductase activities in all thyroid state groups in our experiment. PCB 153 is regarded as a PB-type microsomal enzyme inducer, and PCB treatment has also been shown to stimulate NADPH cytochrome P450 reductase activity (Shephard *et al.*, 1982). The dioxin-like PCB congener, PCB 126, had no effect on NADPH cytochrome P450 reductase activities. This is in accordance with Rozman *et al.* (1985b) who found no effect of TCDD.

In this study, no effect of thyroid state was observed on basal and PCB-induced EROD activity, CYP1A1 protein levels nor on CYP1A1 mRNA levels. This is in contrast with our hypothesis and with other reports. In our previous study a decrease in EROD activity was found in Tx rats (Schuur *et al.*, 1998a). Eltom *et al.* (1992) found a decrease in basal and TCDD-induced EROD activity after thyroidectomy, which was reversed by treatment with T3. Rumbaugh *et al.* (1978) also found a decrease in benzo(a)pyrene hydroxylase activity, which is also catalyzed by CYP1A1, in Tx rats which was reversed by treatment with T4. Roth *et al.* (1988) showed that the modulation of TCDD-induced EROD activity by thyroidectomy depended on the TCDD dose. Rozman *et al.* (1985b) found no effects of thyroidectomy and T3 or T4 treatment on basal and TCDD-induced EROD activity. However, benzo(a)pyrene hydroxylase activity was significantly decreased in control but not in TCDD-induced Tx rats, while this effect was reversed by T3 treatment but not by T4 treatment.

The differences between the results obtained in the various studies could probably be explained by differences in the dose and duration of TCDD treatment and the thyroid state.

CYP2B activity, CYP2B protein and mRNA levels were induced after treatment with PCB 153 in all rats. PCB 126 also induced PROD activity, but not CYP2B protein or mRNA levels. This PCB 126-induced PROD activity reflects induction of CYP1A as pentoxyresorufin is also moderately metabolized by CYP1A (Burke *et al.*, 1994).

In our study, PROD activity showed an upward tendency from the hypothyroid to the hyperthyroid state, although this was only significant for the Eu+T3 vs Tx group after PCB 153 treatment. Thyroid state had no significant effect on CYP2B1/2 protein or mRNA levels. These small effects of thyroid state on CYP2B in this study are in conflict with results published earlier. Rosenberg *et al.* (1995) found a decrease in CYP2B1 (to 20%) and CYP2B2 (to 10%) protein levels after T3 treatment of Tx male Sprague-Dawley rats. Murayama *et al.* (1991) found 14 times higher basal CYP2B1 plus CYP2B2 levels after thyroidectomy or 6-propyl-2-thiouracil (PTU) treatment of male Sprague-Dawley rats, which was reversed by T3 treatment. After treatment with PB, hypothyroid rats showed only a 10% increase in CYP2B levels. Also in hypophysectomized male rats, administration of T3 or growth hormone (GH) reversed a 10-50 fold increase in CYP2B1 and CYP2B2 levels (Yamazoe *et al.*, 1989). Yamazoe and his group suggest that both CYP2Bs are regulated by GH and thyroid hormone, with CYP2B2 being more responsive for T3 and CYP2B1 more responsive to GH (Murayama *et al.*, 1991).

There is not a simple explanation for the discrepancy in effect of thyroid hormones on constitutive as well as induced CYP2B levels between our study and earlier reports. The fact that we used PCB 153 instead of PB is an unlikely cause of this difference. Two reports have suggested a common mechanism for the induction of CYP2B by di-ortho substituted PCBs and PB (Connor *et al.*, 1995; Ikegwuonu *et al.*, 1996). Another possible explanation is the difference in rat strains used. Larsen *et al.* (1994) reported on differences in basal and PB-induced CYP2B protein levels between 3 strains; Wistar Furth inbred rats, Wistar Kyoto rats and F344 rats. Those differences were greater in female rats than in male rats. These variations between the strains largely disappeared after hypophysectomy, whereas they were enhanced by feeding intact rats a carbohydrate-rich diet. Larsen and Jefcoate (1995) also reported that methimazole (MMI)-induced hypothyroidism was as effective as hypophysectomy in elevating PB-induced CYP2B1 and CYP2B2 levels, whereas basal CYP2B2 levels were not elevated by MMI treatment. However, rats of the same strain and sex (male Sprague-Dawley rats) were used by Yamazoe's group (Yamazoe *et al.*, 1987; Yamazoe *et al.*, 1989; Murayama *et al.*, 1991), Rosenberg *et al.* (1995) and in our study. Thus, the differences in modulation of CYP2B by thyroid hormone are not likely explained by strain differences, although the Sprague-Dawley strain is an outbred strain. Finally, it is not excluded that the discrepancy between the present study and other reports is due to differences in the degree of hypo- and hyperthyroid states of the experimental animals.

In conclusion, hepatic NADPH cytochrome P450 reductase activity is dependent on thyroid state. However, in our hands, basal and PCB-induced total cytochrome P450 as well as CYP1A1 and CYP2B levels are not or only marginally affected by thyroid state. Therefore, the PCB-induced decrease in plasma T4 is of no importance in regulating the expression of biotransformation enzymes induced by toxic PCBs.

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PART II

INHIBITION OF THYROID HORMONE SULFATION BY HYDROXYLATED METABOLITES OF POLYHALOGENATED AROMATIC HYDROCARBONS

CHAPTER 5

IN VITRO INHIBITION OF THYROID HORMONE SULFATION BY HYDROXYLATED METABOLITES OF POLYHALOGENATED AROMATIC HYDROCARBONS

Abstract

Earlier studies in our laboratory showed that hydroxylated metabolites of PCBs, PCDDs and PCDFs competitively inhibit T4 binding toTTR and D1 activity. In this study we investigated the possible inhibitory effects of hydroxylated metabolites of PHAHs on iodothyronine sulfotransferase activity. Rat liver cytosol was used as a source of sulfotransferase enzyme in an *in vitro* assay with ^{125}I-labelled T2 as a model substrate. Increasing amounts of hydroxylated PCBs, PCDDs or PCDFs, or extracts from incubation mixtures of PHAHs with induced liver microsomes were added as potential inhibitors of T2 sulfotransferase activity. Hydroxylated metabolites of PCBs, PCDDs and PCDFs were found to be potent inhibitors of T2 sulfotransferase activity *in vitro* with IC50 values in the low micromolar range (0.2-3.8 μM). The most potent inhibitor of T2 sulfotransferase activity within our experiments was the PCB metabolite 3-hydroxy-2,3',4,4',5-pentachlorobiphenyl with an IC50 value of 0.2 μM. A hydroxyl group in the para or meta position appeared to be an important structural requirement for T2 sulfotransferase inhibition by PCB metabolites. Ortho hydroxy PCBs were much less potent and none of the parent PHAHs was capable of inhibiting T2 sulfotransferase activity. In addition, the formation of T2 sulfotransferase-inhibiting metabolites of individual brominated diphenyl ethers and nitrofen as well as from some commercial PHAH mixtures (e.g. Bromkal, Clophen A50 and Aroclor 1254) was also demonstrated.

These results indicate that hydroxylated PHAHs are potent inhibitors of thyroid hormone sulfation. Since thyroid hormone sulfation may play an important role in regulating free hormone levels in the fetus, and PCB metabolites are known to accumulate in fetal tissues after maternal exposure to PCBs, these observations may have implications for fetal thyroid hormone homeostasis and development.

based on A. Gerlienke Schuur, Fransje F. Legger, Marieke E. van Meeteren, Mariëlle J.H. Moonen, Ingeborg van Leeuwen-Bol, Åke Bergman, Theo J. Visser, and Abraham Brouwer (1998). In vitro *inhibition of thyroid hormone sulfation by hydroxylated metabolites of halogenated aromatic hydrocarbons.* Chemical Research in Toxicology, ***11****, 1075-1081.*

Introduction

PCBs, PCDDs and PCDFs are persistent environmental pollutants that induce a broad spectrum of toxic effects in mammals (Safe, 1990). One aspect of the toxicity of these compounds is their effect on thyroid hormone levels and metabolism. TCDD and PCBs are known to affect the thyroid hormone system on at least at three different levels: 1) thyroid gland, 2) plasma transport, and 3) hepatic metabolism (for review; Brouwer *et al.*, 1998). Exposure to TCDD or PCBs resulted in increased glucuronidation and biliary clearance of thyroxine (T4) and, thus decreased serum T4 levels in laboratory mammals (Potter *et al.*, 1986; Roth *et al.*, 1988; Beetstra *et al.*, 1991). In addition, it has been shown that hydroxylated metabolites of PCBs, PCDDs and PCDFs competitively inhibit T4 binding to TTR, but not to TBG, both plasma thyroid hormone transport proteins (Lans *et al.*, 1993; 1994). The same hydroxylated compounds were also demonstrated to inhibit D1 involved in the conversion of T4 to the active hormone T3 or to the inactive metabolite rT3 (Adams *et al.*, 1990; Lans, 1995).

Sulfotransferase isoenzymes catalyzing the sulfation of thyroid hormone are also possible targets for hydroxylated polyhalogenated aromatic hydrocarbons (PHAH-OHs), based on the earlier observation that PCP is a potent inhibitor of phenol sulfotransferase activity (Meerman *et al.*, 1983). Sulfation is an important inactivation pathway for thyroid hormones. Iodothyronine sulfates have been demonstrated to be better substrates for hepatic D1 than the non-sulfated iodothyronines (Visser, 1994a/b). Thyroid hormones have been shown to be sulfated by several rat and human phenol sulfotransferases (Young *et al.*, 1988; Gong *et al.*, 1992). Phenol sulfotransferases constitute one of two known sulfotransferase families, the other being the hydroxysteroid sulfotransferase family. Highest phenol sulfotransferase activity is found in the liver (Matsui and Homma, 1994).

The major objective of this study was to investigate the possible inhibition of iodothyronine sulfotransferase activity by hydroxylated metabolites of PCBs, PCDDs and PCDFs. In addition, the iodothyronine sulfotransferase inhibitory potential of some individual PHAHs and mixtures of PHAHs was tested after metabolic conversion. For this purpose microsomes obtained from rats treated with phenobarbital (cytochrome P4502B) or ß-naphthoflavone (a cytochrome P4501A inducer) were used. A second aim of this study was to obtain more information about structural requirements of hydroxylated PCBs for inhibition of thyroid hormone sulfation. In our *in vitro* sulfation assay, we used 3,3'-T2 as a model substrate for T3, the active form of thyroid hormone. T2 has been shown to be the best substrate of all iodothyronines tested (Visser *et al.*, 1996a). The PHAH-OHs tested in this study have been identified as major metabolites in blood samples from environmentally or experimentally exposed mammals.

Materials and methods

Materials

The structures of the PCB-, PCDD- and PCDF-metabolites tested are shown in Figure 5.1. They include: A) 2-OH-3,3',4,4'-tetrachlorobiphenyl (TCB), B) 4-OH-3,3',4',5-TCB, C) 5-OH-3,3',4,4'-TCB, D) 6-OH-3,3',4,4'-TCB, E) 4,4'-$(OH)_2$-3,3',5,5'-TCB, F) 4-OH-2',3,3',4',5-pentachlorobiphenl (PeCB), G) 4-OH-3,3',4',5,5'-PeCB, H) 4-OH-2',3,4',5,5'-PeCB, I) 4-OH-2,3,3',4',5-PeCB, J) 4-OH-3,3',4'-trichlorobiphenyl (TriCB), K) 4-OH-2',3,3',4',5,5'-hexachlorobiphenyl (HCB), L) 2-OH-2',3,3',4,4'-PeCB, M) 3-OH-2',3',4,4',5-PeCB and N) 3-OH-2,3',4,4',5-PeCB. They were synthesized according to the method described elsewhere (Klasson-Wehler *et al.*, 1990; Bergman *et al.*, 1995) and were at least 98% pure. 4-OH-3,3',5,5'-TCB (O) and 5-OH-3,3',4,4',5'-PeCB (P) were a generous gift from Dr. H. Kuroki, Daiichi College of Pharmaceutical Sciences (Fukuoka 815, Japan). 3,3'-$(OH)_2$-4,4'-dichlorobiphenyl (DCB) (Q) was a generous gift from Dr. S.H. Safe, Texas A&M University (Texas, USA).

The hydroxylated polychlorinated dibenzofurans (PCDF-OHs) used in the present study were: a) 8-OH-2,3,4-trichlorodibenzofuran (TriCDF), b) 2-OH-7,8-dichlorodibenzofuran (DCDF), c) 3-OH-2,6,7,8-tetrachlorodibenzofuran (TCDF), d) 3-OH-2,4,7,8-TCDF, e) 2-OH-1,3,7,8-TCDF, f) 1-OH-2,4,7,8-TCDF, g) 3-OH-2,4,7,8,9-pentachlorodibenzofuran (PCDF), h) 4-OH-1,3,6,7-TCDF (see Figure 5.1 for corresponding structures). PCDF-OHs a to c were a generous gift from Dr. S.H. Safe, and PCDF-OHs d to h were a generous gift from Dr. H. Kuroki.

The hydroxylated polychlorinated dibenzo-*p*-dioxins (PCDD-OHs) used in the present study were: i) 8-OH-2,3-dichlorodibenzo-*p*-dioxin (DCDD), j) 7-OH-2,3,8-trichlorodibenzo-*p*-dioxin (TriCDD) and k) 2-OH-1,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (see Figure 5.1for structures). All PCDD-OHs were obtained from Dr. S.H. Safe.

Other hydroxylated compounds (for structures see Figure 5.2) tested in the present study were 2-OH-2',4,4'-trichlorodiphenylether (2-OH-TriCDE), obtained from Ultra Science (N. Kingstown, RI), and pentachlorophenol (PCP), bisphenol A (4,4'-isopropylidenediphenol), 3,3',5,5'-tetrachlorobisphenol A and 3,3',5,5'-tetrabromobisphenol A, which were all obtained from Aldrich Chemical Co. (Bornem, Belgium). In addition, the parent PCB congeners 3,3',4,4'-TeCB (PCB 77) and 3,3',4,4',5-PeCB (PCB 126), which were obtained from Promochem (Wesel, Germany) were also tested.

The following compounds (for structures, see Figure 5.2) were tested for T2 sulfotransferase inhibition after metabolic activation by incubation with liver microsomes and NADPH: Nitrofen (2,4-dichloro-4'-nitrodiphenylether, a generous gift from Prof. D. Tibboel, Sophia Children's Hospital Rotterdam, the Netherlands), 3,3',4,4'-tetrachlorodiphenyl ether (TCDE), 2,2',4,4'-brominated diphenyl ether (PBDE) mixture, Bromkal 70-5-DE (obtained from Chemische Fabrik Kalk, Germany) and three pure PBDE congener's 2,2',4,4'-

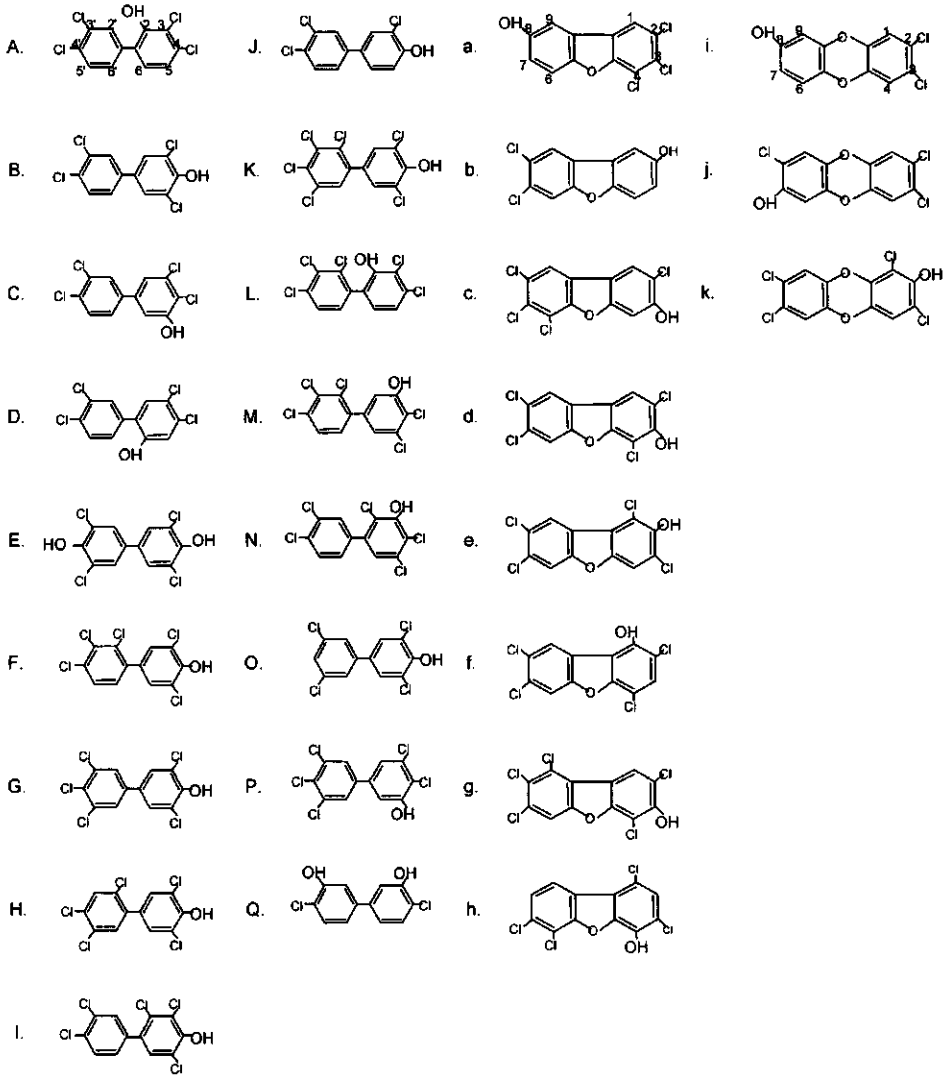

Figure 5.1 Structures of the hydroxylated PCBs, PCDFs and PCDDs used as possible inhibitors in the T2 sulfotransferase activity assay. Names corresponding to the alphabetic indicators are given in the Materials and methods section.

tetrabromodiphenyl ether (TBDE), 2,2',4,4',5-pentabromodiphenyl ether (PeBDE), and 2,2',4,4',5,5'-hexabromodiphenyl ether (HBDE), which were synthesized according to the method described by Örn *et al.* (1996). TCDE, Aroclor 1254 (obtained from Ultra Science (N. Kingstown, RI)), a technical mixture of PCBs. Phenobarbital (PB) was obtained from Fluka Chemie (Buchs, Switzerland). β-naphthoflavone (NF) was obtained from Janssen Chimica

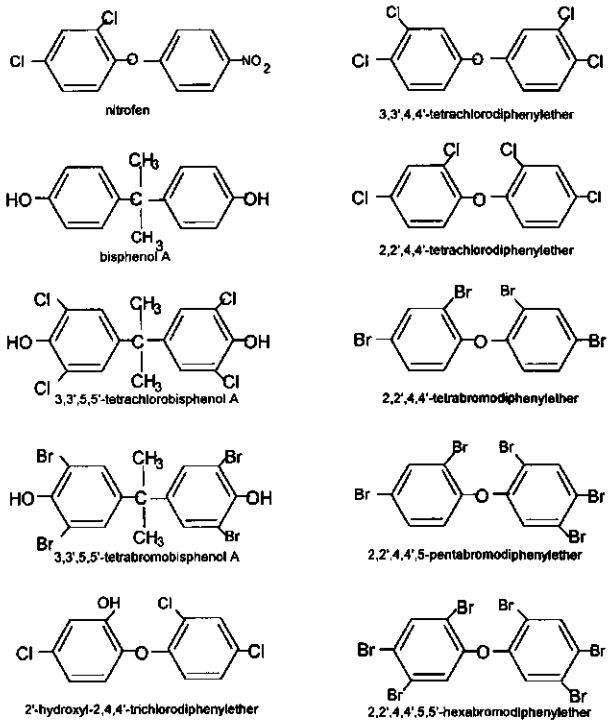

Figure 5.2 Structures of other halogenated aromatic compounds used as possible inhibitors in the T2 sulfotransferase activity assay.

(Tilburg, The Netherlands). 3,3'-Diiodothyronine (T2) and 3-iodothyronine (T1) were obtained from Henning (Berlin, Germany). 3,[3'-^{125}I]T2 was produced by radioiodination of T1 as described before (Visser *et al.*, 1978). NADPH was obtained from Boehringer (Mannheim, Germany). Sephadex LH20 was purchased from Pharmacia (Woerden, The Netherlands). 3'-phosphoadenosine-5'-phosphosulfate (PAPS) was purchased from Sigma Chemicals Co. (St. Louis, MO, USA). All other chemicals were of analytical grade.

Cytosolic and microsomal preparations.

Male Wistar rats (about 250 g of body weight) were treated with NF (3 daily ip injections of 30 mg/kg body weight dissolved in corn oil) or PB (0.1% w/v in the drinking water for 7 days). Rats were sacrificed under ether anesthesia, one day after the last NF treatment, and the livers were removed. The livers of three rats per treatment group were pooled. Livers were homogenized on ice in 3 volumes of ice-cold 0.1 M Tris-HCl buffer, pH 7.5 containing 0.25 M sucrose, using a Potter-Elvehjem tube. The homogenate was centrifuged for 30 min at 9,000xg and 0-4°C. The resulting supernatant was collected and centrifuged for 90 min at 105,000xg and 0-4°C. The microsomal pellet was suspended in 0.1 M potassium phosphate

buffer, pH 7.5, and stored at -80°C until use.

A cytosolic preparation from the liver of a non-treated 8 week old male Wistar rat was used as a source of sulfotransferase activity. Protein levels were determined with the Bio-Rad assay, using BSA as a standard (Bradford, 1976).

Metabolism of PHAHs by induced hepatic microsomes.

Incubations were carried out in glass tubes at 37°C in a shaking water bath in a final volume of 1 ml 0.1 M Tris-HCl buffer, pH 7.5, as described before (Morse *et al.*, 1995) with modifications. A mixture containing 1 mg/ml microsomal proteins and 10 μM PHAH was vortexed for 1 minute and pre-incubated for 2 minutes at 37°C. The reaction was initiated by adding NADPH in a final concentration of 1 mM. The incubation was continued for 15 min at 37°C. Reactions were stopped by the addition of 2 ml of ice-cold methanol. After centrifugation (1200xg, 5 min), the supernatants were extracted 2 times with 2 ml of diisopropyl ether by vortexing for 30 s and the diisopropyl ether fractions were collected following centrifugation at 1000xg for 3 min. Control incubations were done without NADPH or without PHAH. The pooled diisopropyl ether extracts were dried under nitrogen, dissolved in 30 μl DMSO and stored until analysis.

Liver cytosolic T2 sulfotransferase activity assay.

T2 sulfotransferase activity was determined using radiolabelled T2 as substrate. In short, rat liver cytosol (25 μg/ml final protein concentration) was incubated with 1 μM T2 and 80,000 cpm [^{125}I]T2, 50 μM PAPS in 75 mM Tris-HCl, 1.5 mM EDTA (pH 7.2) in a total volume of 200 μl. Incubations were carried out for 30 min at 37°C. The reaction was stopped on ice by adding 750 μl of 0.1 M HCl. The T2 sulfate formed was analyzed by Sephadex LH-20 chromatography as described by Rutgers *et al.* (Rutgers *et al.*, 1989).

Stock solutions of the various hydroxylated PCBs, PCDDs and PCDFs as well as the extracts of the *in vitro* metabolism experiments were made up in DMSO. PHAH-OHs were diluted in 75 mM Tris-HCl, 1.5 mM EDTA (pH 7.2) and added in a concentration series from 0.01 to 10 μM. Extracts were diluted in buffer and added to the T2 sulfotransferase assay mixture, such that the concentration of formed metabolites could be maximally 1.7 μM. The maximum percentage of DMSO in the assay mixture did not exceed 0.5 %. Inhibition curves are presented as T2 sulfotransferase activity (as percentage of uninhibited control; means ± SD of duplicates) versus the log concentration of inhibitor. The concentrations causing 50% inhibition of control activity (IC50) were obtained from the inhibition curves in at least two experiments.

Results

Hydroxylated PCBs, PCDDs and PCDFs

The inhibition of T2 sulfation by four different hydroxy metabolites of the parent compound 3,3',4,4'-TCB is presented as an example in Figure 5.3. A clear dose-dependent almost complete inhibition of T2 sulfotransferase activity was observed by 4-OH-3,3',4',5-TCB (B), 5-OH-3,3',4,4'-TCB (C) and 6-OH-3,3',4,4'-TCB (D) with IC50 values of 1.4, 3.8 and 6.9 μM respectively. No inhibition of T2 sulfotransferase activity was found for the parent compound 3,3',4,4-TCB as well as its ortho hydroxy metabolite 2-OH-3,3',4,4'-TCB (A).

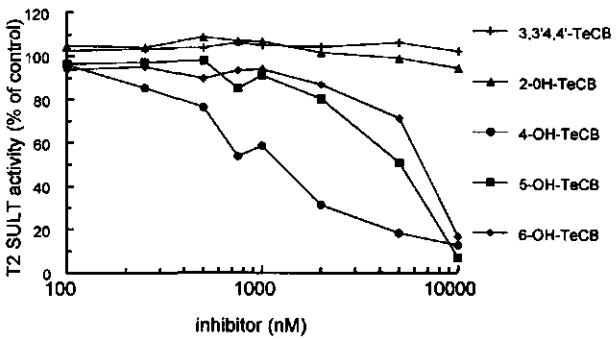

Figure 5.3 Inhibition of T2 sulfotransferase activity by 3,3',4,4'-TeCB and it metabolites using rat liver cytosol. T2 sulfotransferase activity is presented as percentage of control. Data are given as the mean.

In addition to the metabolites of 3,3',4,4'-TCB, a series of other hydroxylated organohalogens was tested for their inhibition of T2 sulfotransferase activity (Table 5.1). All of the hydroxylated PCBs, except for the ortho hydroxy substituted 2-OH-3,3',4,4'-TCB (A) and 2-OH-2',3,3',4,4'-PeCB (L), inhibited T2 sulfotransferase activity with IC50 values in the low micromolar range. The most potent inhibitors of T2 sulfotransferase activity with IC50 values between 0.2 and 1.0 μM were the meta and para hydroxy-substituted 3-OH-2,3',4,4',5-PeCB (N), 4-OH-2,3,3',4',5-PeCB (I), 4-OH-2',3,3',4',5-PeCB (F), and the di-hydroxy-metabolites 3,3'-$(OH)_2$-4,4'-DiCB (Q) and 4,4'-$(OH)_2$-3,3',5,5'-TeCB (E). The other PCB-OHs had IC50 values ranging from 1.3-6.9 μM. The tested hydroxylated PCDDs and PCDFs were also capable of inhibiting T2 sulfotransferase activity except for 1-OH-2,4,7,8-TeCDF (f). They had IC50 values in the same concentration range as the PCB-OHs, i.e. from 0.2 to 3.0 μM.

From these data, we can obtain some information about the structural requirements of PHAH-OHs for inhibition of T2 sulfotransferase. Firstly, hydroxylation of the PHAHs is an essential requirement since both parent PCBs 3,3',4,4'-TCB (Figure 5.3) and 3,3',4,4',5-PeCB (data not shown) were incapable of inhibiting T2 sulfotransferase activity. Substitution of the OH group in the meta and para positions appeared to be necessary, while ortho-hydroxy

Table 5.1 *In vitro* inhibition of T2 sulfotransferase activity by the tested hydroxylated PCBs, PCDFs and PCDDs using rat liver cytosol.

	Compound	IC50 (μM)
A.	2-OH-3,3',4,4'-tetrachlorobiphenyl	>10.0
B.	4-OH-3,3',4',5-tetrachlorobiphenyl	1.4 ±1.0
C.	5-OH-3,3',4,4'-tetrachlorobiphenyl	3.8 ±1.6
D.	6-OH-3,3',4,4'-tetrachlorobiphenyl	6.9 ±0.8
E.	4,4'-$(OH)_2$-3,3',5,5'-tetrachlorobiphenyl	0.8 ±0.23
F.	4-OH-2',3,3',4',5-pentachlorobiphenyl	0.6 ±0.03
G.	4-OH-3,3',4',5,5'-pentachlorobiphenyl	3.0 ±1.0
H.	4-OH-2',3,4',5,5'-pentachlorobiphenyl	1.3 ±0.25
I.	4-OH-2,3,3',4',5-pentachlorobiphenyl	0.6 ±0.08
J.	4-OH-3,3',4'-trichlorobiphenyl	2.8 ±0.25
K.	4-OH-2',3,3',4',5,5'-hexachlorobiphenyl	2.1 ±0.40
L.	2-OH-2',3,3',4,4'-pentachlorobiphenyl	>5
M.	3-OH-2',3',4,4',5-pentachlorobiphenyl	3.3 ±0.75
N.	3-OH-2,3',4,4',5-pentachlorobiphenyl	0.2 ±0.02
O.	4-OH-3,3',5,5'-tetrachlorobiphenyl	2.8 ±1.30
P.	5-OH-3,3',4,4',5'-pentachlorobiphenyl	2.4 ±0.05
Q.	3,3'-$(OH)_2$-4,4'-dichlorobiphenyl	0.5 ±0.02
a.	8-OH-2,3,4-trichlorodibenzofuran	2.1
b.	2-OH-7,8-dichlorodibenzofuran	1.3
c.	3-OH-2,6,7,8-tetrachlorodibenzofuran	1.8±0.05
d.	3-OH-2,4,7,8-tetrachlorodibenzofuran	1.9 ±0.00
e.	2-OH-1,3,7,8-tetrachlorodibenzofuran	0.9 ±0.06
f.	1-OH-2,4,7,8-tetrachlorodibenzofuran	>10
g.	3-OH-2,4,7,8,9-pentachlorodibenzofuran	2.2±0.35
h.	4-OH-1,3,6,7-tetrachlorodibenzofuran	0.2 ±0.07
i.	8-OH-2,3-dichlorodibenzo-*p*-dioxin	3.0 ±0.03
j.	7-OH-2,3,8-trichlorodibenzo-*p*-dioxin	1.4 ±0.57
k.	2-OH-1,3,7,8-tetrachlorodibenzo-*p*-dioxin	1.2 ±0.08

Note. Structural formulas of congeners as represented in Figure 5.1. IC50 values are the micromolar concentrations of inhibitor resulting in inhibition of T2 sulfotransferase activity to 50% of control (no inhibitor).

substituted PCBs did not inhibit T2 sulfotransferase activity. The number of halogen atoms on the phenolic ring did not seem to be a determining factor for T2 sulfotransferase inhibition potency e.g., 3,3'-$(OH)_2$-4,4'-DiCB (Q) with one chlorine atom on each phenolic ring was as potent an inhibitor as 3-OH-2,3',4,4',5-PeCB (N) with 3 chlorine atoms on the phenolic ring. The planarity of the molecule does not seem to play a major role either, since the rigid planar structures of the PCDD-OHs and PCDF-OHs had a similar T2 sulfotransferase inhibitory potency as the hydroxylated PCBs, where there is freedom of axial rotation of the aromatic rings along the central C-C axis. Chlorine substitution on the non-hydroxylated phenyl ring slightly affected the inhibition potency, as is apparent when comparing compounds with the same chlorine substitution pattern on the phenolic-ring but with a different chlorine substitution pattern on the other ring, namely: 4-OH-3,3',4',5-TCB (A), 4-OH-2',3,3',4',5-PeCB (F), 4-OH-3,3',4',5,5'-PeCB (G), 4-OH-2',3,4',5,5'-PeCB (H), 4-OH-2',3,3',4',5,5'-HeCB (K), and 4-OH-3,3',5,5'-TCB (O).

Related hydroxylated halogenated compounds

Some other hydroxylated halogenated compounds, related to the group of hydroxylated PCBs, PCDDs and PCDFs, were also tested for their inhibition of T2 sulfation (Table 5.2). The well-known phenol sulfotransferase inhibitor PCP was by far the most potent inhibitor of T2 sulfotransferase activity with an IC50 value of 0.005 μM. The plasticizer bisphenol A did not inhibit T2 sulfation. However, the halogenated bisphenol A compounds were potent inhibitors of T2 sulfotransferase activity with IC50 values of 1.5 μM for 3,3',5,5'-tetrachlorobisphenol and 4.3 μM for 3,3',5,5'-tetrabromobisphenol. The only commercially available hydroxylated metabolite of a halogenated diphenyl ether (2-OH-2',4,4'-TriCDE) was tested and had an IC50 value of 3.1 μM.

Table 5.2 *In vitro* inhibition of T2 sulfotransferase activity by different hydroxylated compounds using rat liver cytosol.

Compound	IC50 (μM)
bisphenol A	>10.0
3,3',5,5'-tetrachlorobisphenol	1.5 ±0.5
3,3',5,5'-tetrabromobisphenol	4.3 ±1.1
2'-OH-2,4,4'-trichlorodiphenylether	3.1 ±0.7
pentachlorophenol	0.005 ±0.0003

Note. Structures of these compounds are shown in Figure 5.2. IC50 values are the micromolar concentrations of inhibitor resulting in inhibition of T2 sulfotransferase activity to 50% of control (no inhibitor).

T2 sulfotransferase inhibition following metabolism of PHAHs

Some individual PHAH congeners as well as PHAH mixtures were tested after incubation with cytochrome P450s for their possible inhibition of T2 sulfotransferase activity (Table 5.3). Control incubations without NADPH or without PHAH did not produce T2 sulfotransferase inhibiting compounds. Incubation of NF-microsomes (CYP1A-enriched) with 2,2',4,4',5-PeBDE or 2,2',4,4'-TeCDE in the presence of NADPH resulted in the formation of T2 sulfotransferase-inhibiting metabolites. Incubation of these compounds with PB-microsomes (mostly CYP2B-enriched) also resulted in T2 sulfotransferase inhibiting metabolites. CYP2B but not CYP1A-enriched microsomes catalyzed the formation of T2 sulfotransferase-inhibiting metabolites from the parent compounds nitrofen, 2,2',4,4'-TeBDE and 3,3',4,4'-TeCDE in the presence of NADPH. Incubation of the PCB mixtures Aroclor 1254 and Clophen A50 but not the PBDE mixture Bromkal with either PB- or NF microsomes also resulted in production of metabolites that inhibited T2 sulfotransferase activity.

Table 5.3 *In vitro* inhibition of T2 sulfotransferase activity, using rat liver cytosol, of various PHAH compounds after incubation with liver microsomes enriched with CYP1A or CYP2B and NADPH.

Compound	with CYP1A	with CYP2B
nitrofen	-	++
Aroclor 1254	+	+
Clophen A50	+	++
bromkal	-	-
2,2',4,4'-tetrabromodiphenylether	-	+
2,2',4,4',5-pentabromodiphenylether	+	+
2,2',4,4',5,5'-hexabromodiphenylether	-	-
2,2',4,4'-tetrachlorodiphenylether	++	++
3,3',4,4'-tetrachlorodiphenylether	-	+

Note. Structures of these compounds are shown in Figure 5.2. Results are presented as qualitative data: -: no inhibition of T2-SULT activity; +: inhibition between 20 -60%; ++: more than 60% inhibition in comparison with control incubations (without NADPH or without compound).

Discussion

The results from this study clearly indicate that the sulfotransferase isozyme(s) catalyzing thyroid hormone sulfation is (are) also a target protein for inhibition of binding by hydroxylated PHAHs. PCB-OHs, PCDD-OHs, PCDF-OHs as well as other hydroxylated halogenated compounds were shown to be potent inhibitors of *in vitro* T2 sulfation using rat liver cytosol as enzyme source. In addition, a number of individual PHAH congeners as well as mixtures of PHAHs could be converted *in vitro* into T2 sulfation-inhibiting substances by incubation with both CYP1A and CYP2B enriched microsomes and NADPH.

To compare the inhibiting potency of the various chemicals, IC50 values were calculated. The most potent PCB-OH with respect to inhibition of T2 sulfation was the metabolite 3-OH-2,3',4,4',5-PeCB (N) with an IC50 value of 0.2 μM. The IC50 values of all other PHAH-OHs inhibiting T2 sulfotransferase activity were in the low micromolar range, within a relatively small range of 0.2 and 6.9 μM. The most potent chemical tested in this study was PCP (with an IC50 value of 0.005 μM), which is a well-known phenol sulfotransferase inhibitor, *in vitro* (Duffel and Jakoby, 1981) as well as *in vivo* (Meerman *et al.*, 1983).

With the results obtained in this study, it is possible to make some statements about structural requirements for the PHAH-OHs that inhibit T2 sulfation *in vitro*. The first and very important structural requirement for the inhibition of T2 sulfation is the presence of a hydroxyl group. As shown in Figure 5.3, the parent compound 3,3',4,4'-TeCB itself did not cause any inhibition of T2 sulfotransferase activity, while all OH-metabolites of 3,3',4,4'-TeCB were inhibitors. Similar conclusions were reported by Eaton *et al.* (1996) for inhibition by flavonoids of phenol sulfotransferase activity, measured with *p*-nitrophenol, minoxidil or acetaminophen as a substrate. The 7-hydroxyl substituent seemed to be the most important structural feature of flavonoids for inhibition of phenol sulfotransferase activity. Other natural phenolic compounds and food additives were also found to be potent inhibitors of phenol sulfotransferase activity (Gibb *et al.*, 1987; Bamforth *et al.*, 1993).

A second structural aspect which appeared to have a major impact on the T2 sulfotransferase inhibition potency of the tested PHAH-OHs, is the position of the OH-group in PHAH-OHs. This study indicates that for PCB-OHs a hydroxyl group in the para or meta position is necessary; ortho hydroxylated PCBs caused no T2 sulfation inhibition in the tested concentration range. However, the only commercially available, ortho hydroxylated chlorinated diphenyl ether (2-OH-2',4,4'-TriCDE) inhibited T2 sulfotransferase activity with an IC50 value of 3.1 μM. The affinity of this compound for the sulfotransferase active site may be explained by its diphenyl ether structure. For PCDF-OHs, it is possible to conclude that an OH-group in position 1 is not favorable for T2 sulfation inhibition. Secondly, an OH-group in position 4 resulted in the lowest IC50 value found, 0.2 μM.

Another structural property that has an influence on the T2 sulfotransferase inhibition is the halogen substitution pattern. This applies especially to the bisphenol A derivatives, where it was shown that they were inhibitors of T2 sulfation only when substituted with bromines or chlorines. However, for PCDD-OHs a chlorine atom adjacent to the OH groups is not required for T2 sulfation inhibition, as is shown by the potent inhibitor 8-OH-2,3-DiCDD (i). It was also demonstrated that PCB-OHs with a similar chlorine substitution pattern on the hydroxylated phenyl ring but with a different chlorine substitution pattern on the other phenyl ring had slightly different IC50 values.

As a last structural property, it can be stated that the planarity of the molecule does not seem to play a major role since rigid planar structures of the PCDD-OHs and PCDF-OHs have similar T2 sulfotransferase inhibitory potencies as the hydroxylated PCBs.

Some other chemicals were also tested for their possible inhibition on T2 sulfotransferase activity after incubation with NADPH and microsomes enriched with CYP1A or CYP2B. Mammals are not exposed to individual congeners but to mixtures of PHAHs, which can possibly be hydroxylated by hepatic cytochrome P450 isozymes. It was demonstrated that the PCB mixtures Aroclor 1254 and Clophen A50 caused T2 sulfotransferase activity inhibition after incubation with NADPH and liver microsomes enriched with CYP1A or CYP2B after *in vivo* NF or PB treatment, respectively. Obtaining quantitative information about the potency of these compounds is not possible, but our results strongly suggest that hydroxylated metabolites of other PHAHs such as nitrofen and brominated diphenyl ethers are able to inhibit T2 sulfotransferase activity *in vitro*.

The inhibition potency of T2 sulfotransferase activity by PHAH-OHs may be compared with their potency to interfere with other thyroid hormone-binding proteins such as TTR and D1. In general, inhibition of T2 sulfotransferase activity by PHAH-OH is less potent than inhibition of T4 binding to TTR, which is characterized by IC50 values in the low nanomolar range (Lans *et al.*, 1993; 1994). Secondly, the range of IC50 values for T4-TTR competition is much broader for the various PCB metabolites, i.e. 6-1000 nM. It is remarkable how small the range of IC50 values is for T2 sulfotransferase inhibition. Yet, the structural requirements for both inhibitions are very similar, both prefer an OH-group on the para or meta position.

Similar IC50 values are observed for the inhibition of D1 activity and T2 sulfotransferase activity by PCB-OHs. IC50 values for D1 inhibition by the various PCB-OHs were also in the micromolar range (Adams *et al.*, 1990; Lans, 1995). A remarkable difference, however, is that dihydroxylated PCB-OHs are more potent inhibitors of D1 activity than of T2 sulfotransferase activity.

The results on T2 sulfotransferase inhibition by hydroxylated PHAHs as reported here, were all obtained by *in vitro* methods, using hepatic cytosol as source of sulfotransferase(s) and liver microsomes as source of CYP1A or CYP2B. It is still a question whether the sulfotransferase inhibitory effects of PHAH-OHs would also occur *in vivo*. For that to happen, sufficient amounts of PHAH-OHs should be able to reach the intracellularly located sulfotransferase proteins. Relatively high levels of para-hydroxylated PCB metabolites from penta-, hexa- and hepta-chlorinated biphenyls have been found in the plasma of rats exposed to Aroclor 1254 and in environmentally exposed humans and marine mammals (Bergman *et al.*, 1994). Other metabolites (2-OH-2',3,3',4,4'-PeCB (L) and 4-OH-2',3,3',4',5-PeCB (F)) were identified in mice following exposure to 2,3,3',4,4'-PeCB (PCB 105; Klasson-Wehler *et al.*, 1990). Exposure of rats to Aroclor 1254 resulted in the presence of considerable amounts of several hydroxy PCB metabolites in blood, including the 4-OH-2',3,3',4',5-PeCB (F, Klasson-Wehler *et al.*, 1992). Furthermore, 4-OH-3,3',4',5,5'-PeCB (G) has been detected after *in vivo* exposure to 3,3',4,4',5-PeCB (PCB 126; Koga *et al.*, 1990). There was also a significant accumulation of 4-OH-2,3,3',4',5-PeCB (I) in the plasma of late gestational fetuses from pregnant rats exposed to 25 mg/kg Aroclor 1254 for 7 days (concentrations up to 4.6 μM). In addition, relatively large amounts of this metabolite were found in the fetal, but not weanling rat brain (Morse *et al.*, 1996). Two of the major hydroxy metabolites of 2,3,7,8-TCDD (compounds j and k) were identified in mammalian species (Mason and Safe, 1986; Ramsey *et al.*, 1982). Summarizing the reports above, PCB and PCDD metabolites are found in blood of animals treated with the parent compound but also in free living mammals. Concentrations of some of the mentioned metabolites may be up in micromolar range, especially in fetal plasma and tissues. These metabolites were shown in the present study to be potent T2 sulfotransferase inhibitors with IC50 values also in the micromolar range.

In this study, only male rat liver cytosol was used in the *in vitro* T2 sulfotransferase assay. It is known that iodothyronine sulfotransferase activity is higher in male than in female rat liver cytosol (Kaptein *et al.*, 1997). Iodothyronines are sulfated in rats by at least two isozymes, *i.e.* rat SULT1B1 and rat SULT1C1 (Sakakibara *et al.*, 1995; Fujita *et al.*, 1997; Visser *et al.*, 1998a). Whereas hepatic expression of SULT1B1 is independent of sex, SULT1C1 is only expressed in male but not in female rat liver (Nagata *et al.*, 1993; Araki *et al.*, 1997). Therefore, the sulfation of T2 analyzed in the present study represents the activity of both SULT1B1 and SULT1C1. However, similar results were obtained in experiments utilizing female rat liver cytosol, where T2 sulfation is only catalyzed by SULT1B1 (Schuur *et al.*1998b; *Chapter 6*).

In the healthy adult, sulfation results in the irreversible inactivation of thyroid hormone, since sulfation strongly facilitates the inner ring deiodination of T4 and T3 by D1 (Visser, 1994b). T3S is barely detectable in serum of healthy human adults (<0.1 nM) but is much higher in fetuses of different stages (Chopra *et al.*, 1992; Santini *et al.*, 1993). It has been

suggested that sulfation of iodothyronines is an important pathway when D1 activity is low, such as in non-thyroidal illness and during fetal development (Santini *et al.*, 1992a). This is supported by Huang *et al.* (1996), who reported on similar time courses in serum T3 levels and hepatic T3 sulfatase activity in the fetal rat, suggesting that sulfation-desulfation of T3 plays a role in the regulation of thyroid hormone metabolism in developing mammals. Based on these findings, the hypothesis is postulated that prenatal exposure of mammals to PHAHs, resulting in the accumulation of hydroxylated PHAHs in the fetal compartment, causes an inhibition of thyroid hormone sulfation with potential thyroid hormone-disrupting consequences.

Acknowledgements

We like to thank Peter H. Cenijn and Willeke M.C. Jong for performing part of the sulfotransferase activity assays.

CHAPTER 6

IN VITRO INHIBITION OF THYROID HORMONE SULFATION BY POLYCHLOROBIPHENYLOLS: ISOZYME SPECIFICITY AND INHIBITION KINETICS

Abstract

It was recently demonstrated by our laboratory that hydroxylated metabolites of polychlorinated biphenyls (PCB-OHs) are inhibitors of thyroid hormone sulfation. In this study, a more detailed investigation on sulfotransferase isozyme specificity and the kinetics of inhibition was performed. Thyroid hormone sulfation was determined using 3,3'-T2 as a substrate, and various sources of sulfotransferase enzyme were used, e.g. female and male rat liver cytosol, male brain cytosol and cytosolic preparations of V79 cells transfected with rat SULT1C1, human SULT1A1 and human SULT1A3.

The inhibition pattern and IC50 values were very similar for male and female rat liver and rSULT1C1 and hSULT1A1. PCB-OHs were not able to inhibit the T2 sulfotranferase activity using hSULT1A3. Metabolite 3-hydroxy-2,3',4,4',5-pentachlorobiphenyl did not inhibit T2 sulfotransferase activity in male brain cytosol, while it was a very potent inhibitor in male and female rat liver cytosol. IC50 values for the tested PCB-OHs were not different with either T2 or T3 as substrate, supporting the hypothesis that T2 is the preferred iodothyronine substrate for the sulfotransferases catalyzing the sulfation of the active hormone T3. The Lineweaver-Burk plot obtained with rat liver cytosol and T2 suggested that the nature of the T2 sulfation inhibition by 4-hydroxy-2',3,3',4',5-pentachlorobiphenyl is competitive. Finally, it was demonstrated that tested hydroxylated polychlorinated dibenzo-*p*-dioxins and biphenyls were, albeit poorly, sulfated by sulfotransferases as measured by the production of ^{35}S-labeled metabolites.

based on A. Gerlienke Schuur, Ingeborg van Leeuwen-Bol, Willeke M.C. Jong, Åke Bergman, Michael WH. Coughtrie, Abraham Brouwer, and Theo J. Visser (1998). In vitro *inhibition of thyroid hormone sulfation by polychlorobiphenylols: isozyme specificity and inhibition kinetics.* Toxicological Sciences, *in press*.

Introduction

Sulfation is one of the major conjugation reactions for drugs and environmental chemicals as well as for endogenous compounds. Sulfate conjugation is catalyzed by multiple forms of sulfotransferases present in cytosol, which transfer a sulfuryl group from 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to hydroxyl and amino groups of numerous substrates (Mulder and Jakoby, 1990). Recently, accumulating evidence indicates that sulfotransferases exist as a multigene family, each possessing distinct but closely related catalytic properties. Sulfotransferases may be classified into phenol (Family 1) and hydroxysteroid (Family 2) sulfotransferase families based on amino acid sequence identity and catalytic function (reviewed in Falany, 1991; Matsui and Homma, 1994; Falany, 1997; Weinshilboum *et al.*, 1997).

Thyroid hormones belong to the endogenous substrates of the phenol sulfotransferases as firstly characterized by Sekura *et al.* (1981). Thyroid hormones are sulfated by at least four different human isozymes (Young *et al.*, 1988; Visser, 1994b; Visser *et al.*, 1998a; Fujita *et al.*, 1997); two forms of a phenol-preferring sulfotransferase (P-PST; SULT1A1 and SULT1A2), a monoamine-preferring phenol sulfotransferase (M-PST; SULT1A3) and SULT 1B1. In rat, iodothyronine sulfation was only demonstrated for SULT1B1 (Sakakibara *et al.*, 1995) and SULT1C1 but not for SULT1A1 (Visser *et al.*, 1996a; 1998b). Iodothyronine sulfation rates in rat liver cytosol were in the order 3,3'-T2 >> T3 ≈ rT3 > T4 (Visser *et al.*, 1996a; 1998b). Inhibition profiles of well-known sulfotransferase inhibitors (Rein *et al.*, 1982) may distinguish between the different isozymes. It was shown that T3 sulfation in rat liver cytosol was inhibited by low concentrations of 2,6-dichloro-4-nitrophenol (DCNP; IC50= 5.5 μM) and PCP (IC50= 0.065 μM) (Gong *et al.*, 1992), suggesting that T3 sulfation was catalyzed by a P-PST like-isozyme. We have shown previously (Schuur *et al.*, 1998b; *Chapter 5*) that hydroxylated metabolites of polychlorinated biphenyls (PCB-OHs), polychlorinated dibenzo-p-dioxins (PCDD-OHs) and polychlorinated dibenzofurans (PCDF-OHs) are also able to inhibit thyroid hormone sulfation *in vitro*, using T2 as a substrate.

In this study, the inhibition kinetics and the isozyme specificity of iodothyronine sulfotransferase inhibition by PCB-OHs were investigated in more detail. Firstly, different sulfotransferase enzyme sources were used: male and female rat liver cytosol, male rat brain cytosol and cytosol from V79 cells transfected with rat SULT1C1 (Nagata *et al.*, 1993; Glatt *et al.*, 1996), with a variant of human SULT1A1 (Jones *et al.*, 1995) or with human SULT1A3 (Jones *et al.*, 1995). Secondly, T2 and T3 were compared as substrates in the sulfotransferase assay, to validate the used of T2 as a model substrate for the active hormone T3. Thirdly, T2 sulfotransferase inhibition kinetics of 4-hydroxy-2,3,3',4',5-pentachlorobiphenyl were investigated using rat liver cytosol. Fourthly, we investigated if PCB-OHs or PCDD-OHs were sulfated themselves by rat liver cytosol.

Materials and methods

Materials

The following hydroxylated polychlorinated biphenyls (PCB-OHs) were tested (see Figure 6.1 for structures): A) 2-hydroxy-2',3,3',4,4'-pentachlorobiphenyl (2-OH-2',3,3',4,4'-PeCB), B) 4-OH-2',3,4',5,5'-PeCB, C) 4-OH-2,3,3',4',5-PeCB and D) 3-OH-2,3',4,4',5-PeCB. They were obtained by demethylation of the corresponding methoxy-PCBs synthesized according to the method described by Klasson-Wehler *et al.* (1990). They were at least 98% pure and their structural identification was performed using mass spectrometry as reported by Bergman *et al.* (1995). The following PCDD-OHs were used: a) 8-OH-2,3-dichlorodibenzo-*p*-dioxin (8-OH-2,3-DCDD), b) 7-OH-2,3,8-trichlorodibenzo-*p*-dioxin (7-OH-2,3,8-TriCDD) and c) 2-OH-1,3,7,8-tetrachlorodibenzo-*p*-dioxin (2-OH-1,3,7,8-TCDD) (structures not shown). They were a generous gift of Dr. S.H. Safe, Texas A&M University (Texas, USA). The PCDD-OHs were synthesized according to Mason and Safe (1986) and their structural identity was checked as reported by Safe and Safe (1984).

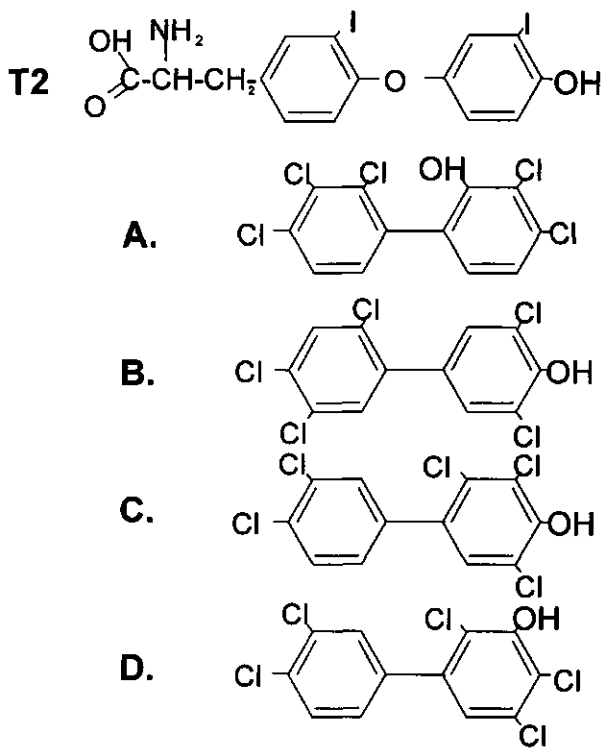

Figure 6.1 Structures of the hydroxylated PCBs used as inhibitors in the T2 sulfotransferase activity assays. For structural formulas see Materials and methods.

Pentachlorophenol (PCP) and *p*-hydroxybiphenyl were obtained from Aldrich (Bornem, Belgium). Bovine serum albumin (BSA), 3'-phosphoadenosine-5'-phosphosulfate (PAPS) and 3,3',5-triiodothyronine (T3) were purchased from Sigma (Zwijndrecht, The Netherlands). 3,3'-diiodothyronine (T2) and 3-iodothyronine (T1) were obtained from Henning (Berlin, Germany). ^{125}I-labeled T2 was produced by radioiodination of T1 as described before (Visser *et al.*, 1978). ^{125}I-labeled T3 was obtained from Orange Medical (Tilburg, The Netherlands). [^{35}S]PAPS was obtained from Du Pont-NEN (Den Bosch, The Netherlands). Sephadex LH-20 was purchased from Pharmacia (Woerden, The Netherlands). Dimethylsulfoxide (DMSO) was obtained from Acros Chimica (Den Bosch, The Netherlands).

Cytosolic preparations

Cytosol was prepared from the liver and brain of an 8 weeks old untreated male and from the liver of a 32 weeks old female Wistar rat. Tissue was homogenized on ice in 3 volumes ice-cold 0.1 M Tris-HCl buffer, pH 7.5, containing 0.25 M sucrose, using a Potter tube. The homogenate was centrifuged for 30 min at 9,000xg and 4°C. The resulting supernatant was centrifuged for 90 min at 105,000xg and 4°C. The high-speed supernatant was stored at-80°C until analysis.

Cytosol was also prepared from cultured V79 cells stably expressing specific sulfotransferase isozymes, e.g. rat SULT1C1 (Nagata *et al.*, 1993; Glatt *et al.*, 1996), a variant of human SULT1A1 (Jones *et al.*, 1995), and human SULT1A3 (Jones *et al.*, 1995). These cells were cultured in DMEM (Gibco Life Technologies, Breda, The Netherlands) supplemented with 10% FCS (Sigma) in a 37°C, 5% CO_2 humidified air environment. Confluent cell layers were washed in PBS, scraped from the bottom in 0.1 M Tris-HCl, 2 mM EDTA buffer (pH 7.2), homogenized and centrifuged for 90 min at 105,000xg and 4°C. The resulting supernatant (cytosol) was stored at -80°C until analysis.

Protein levels were determined with the Bio-Rad assay (Bio-Rad Laboratories, Munich, Germany), using BSA as a standard (Bradford, 1976).

Iodothyronine sulfotransferase assay

Iodothyronine sulfotransferase activity was determined as described previously (Visser *et al.*, 1996a; Schuur *et al.*, 1997; *Chapter 2*) using radiolabelled [^{125} I]T2 or [^{125}I]T3 as substrate. In short, cytosol was incubated with 1 μM T2 or T3 and 80,000 cpm [^{125}I]T2 or [^{125}I]T3, 50 μM PAPS in 75 mM Tris-HCl and 1.5 mM EDTA (pH 7.2) in a total volume of 200 μl. The protein concentrations at which the different enzyme preparations were tested varied between 25 μg/ml (female and male liver and male brain cytosol) and 50-200 μg/ml for the V79 cytosolic preparations. Incubations were carried out for 30 min at 37°C. The reaction was stopped on ice by adding 750 μl 0.1 M HCl. The T2 (or T3) sulfate formed was analyzed by Sephadex LH-20 chromatography as described by Rutgers *et al.* (1989).

Stock solutions of the hydroxylated PCBs were prepared in DMSO, further diluted

in buffer and added in the concentration range of 0.01 to 10 μM to the T2 sulfotransferase incubation mixture, such that the DMSO concentration did not exceed 0.5% (v/v). Inhibition curves are presented as T2 sulfotransferase activity (% of control) versus the log concentration of inhibitor (nM). The concentrations of inhibitor causing 50% inhibition (IC50) were estimated from the inhibition curves obtained in at least two experiments.

The kinetics of inhibition of T2 sulfotransferase by PCB-OHs were investigated by incubation of male rat liver cytosol (25 μg protein/ml) with 0.1-10 μM radiolabelled T2 in the absence or presence of 0.5, 1 and 2 μM 4-OH-2,3,3',4',5-PeCB. The results were analysed by Lineweaver-Burk analysis.

Sulfation of PCB-OHs and PCDD-OHs

A concentration series of 0.05-10 μM of PCB-OHs or PCDD-OHs were incubated with male rat liver cytosolic (25 μg protein/ml) and 0.1 μM [^{35}S]-PAPS in a total volume of 150 μl 10 mM potassium phosphate buffer (pH 7.4). A modified assay (Foldes and Meek, 1973; Pacifici *et al.*, 1991) was used and optimized using *p*-hydroxybiphenyl as substrate. Other substrates tested were: the PCB-metabolites A, B, C and D (Figure 6.1) as well as the PCDD metabolites 8-OH-2,3-DiCDD, 7-OH-2,3,8-TriCDD and 2-OH-1,3,7,8-TeCDD and PCP. The reaction mixture was incubated for 30 min at 37°C. [^{35}S]PAPS and protein were precipitated with 200 μl each of 0.1 M barium acetate, 0.1 M barium hydroxide and 0.1 M zinc sulfate. After vortexing and centrifugation, 500 μl of the supernatant together with 4 ml scintillation liquid (Ultima Gold, Packard, Groningen, The Netherlands) was counted in a liquid scintillation counter (Tri-Carb 1600, Packard). The amount of sulfated product was calculated after correction for the radioactivity recovered from blank reaction mixtures containing the solvent DMSO but no substrate.

Results

Source and isozyme specificity

Hydroxylated PCBs were previously reported to markedly reduce T2 sulfotransferase activity in male rat liver cytosol (Schuur *et al.*, 1998b; *Chapter 5*). Inhibition of iodothyronine sulfotransferase activity by PCB-OHs was extended to other sources of enzyme, including female rat liver, male rat brain and V79 cells transfected with human SULT1A1, human SULT1A3 and rat SULT1C1.

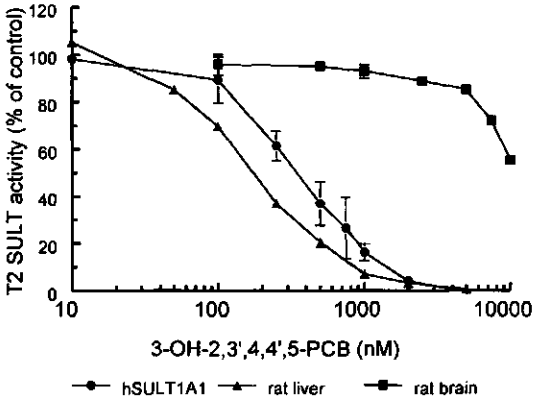

Figure 6.2 Dose-dependent inhibition of T2 sulfotransferase activity (% of control) by 3-OH-2,3',4,4',5-PeCB (D) using different enzyme sources. The enzyme sources used were male rat liver cytosol, male rat brain cytosol and cytosol obtained from V79 cells containing human SULT1A1. The control T2 sulfotransferase activity was 324.2, 44.9 and 80.3 pmol T2 sulfate formed/mg protein*min respectively. Data are presented as mean % of control ± SD, obtained from one experiment and performed in duplicate.

IC50 values of PCB-OHs for T2 sulfotransferase activity are summarized in Table 6.1. Even at 10 μM, the ortho hydroxylated metabolite 2-OH-2',3,3',4,4'-PeCB (A) was unable to inhibit T2 sulfation by any of the enzymes tested . The meta and para hydroxylated metabolites (B, C and D) inhibited all sulfotransferase activities except that of human SULT1A3. IC50 values for metabolites B, C and D were slightly higher with female rat liver cytosol than with male rat liver cytosol, although the order of potency remained the same. The meta hydroxylated 3-OH-2,3',4,4',5-PeCB (D) did not inhibit T2 sulfotransferase activity in male brain cytosol, even though it was the most potent inhibitor using male or female rat liver cytosol (also demonstrated in Figure 6.2). Both para hydroxylated compounds B and C had similar IC50 values in male brain and liver cytosol (Table 6.1).

The recombinant sulfotransferases were also used to obtain more information about isozyme specificity of sulfotransferase inhibition by PCB-OHs. Rat SULT1C1 isozyme (Table 6.1 and Figure 6.2) showed almost the same pattern for T2 sulfotransferase inhibition by PCB-OHs as rat liver cytosol. Human SULT1A3, on the other hand, was not inhibited at all by any of the PCB metabolites. Human SULT1A1 behaves very similar to rat SULT1C1 which suggests a close resemblance between their substrate specificities.

Table 6.1 *In vitro* inhibition of sulfotransferase activity by PCB-OHs using different enzyme sources and T2 or T3 as substrates.

substrate cytosol source	T3 ♂ liver	T2 ♂ liver	T2 ♀ liver	T2 ♂ brain	T2 hSULT1A1	T2 hSULT1A3	T2 rSULT1C1
A.	>5.0	>5.0	>10.0	>10.0	>5.0	>5.0	>5.0
B.	3.6	1.3	2.6	3.4	1.9	>5.0	1.2
C.	N.A.	0.6	2.2	1.1	1.0	>5.0	0.7
D.	0.3	0.2	0.6	>10.0	0.8	>5.0	0.2

Note. The data are presented as IC50 values in μM, obtained from 2-4 different experiments. The single isozyme preparations were obtained from V79 cells transfected with these specific isozymes. The standard deviations of the presented IC50 values never exceeded 25%.
A. 2-OH-2',3,3',4,4'-pentachlorobiphenyl,
B. 4-OH-2',3,4',5,5'-pentachlorobiphenyl,
C. 4-OH-2,3,3',4',5-pentachlorobiphenyl
D. 3-OH-2,3',4,4',5-pentachlorobiphenyl,
N.A. not analysed

Substrate dependence of sulfotransferase inhibition by PCB-OHs

In the above experiments, T2 was tested as the preferred substrate for iodothyronine sulfotransferases but T3 is the physiologically relevant, active thyroid hormone. Therefore, we have tested the validity of T2 sulfotransferase data for T3 sulfation. As shown in Table 6.1 and Figure 6.3 (example), the IC50 values for T3 sulfotransferase inhibition by PCB-OHs were in the same range as the IC50 values using T2 as a substrate. Both order and potency of sulfotransferase inhibition by PCB-OHs were very similar using either T3 or T2 as a substrate, supporting the idea that T2 is a good model substrate for T3 in sulfotransferase inhibition studies.

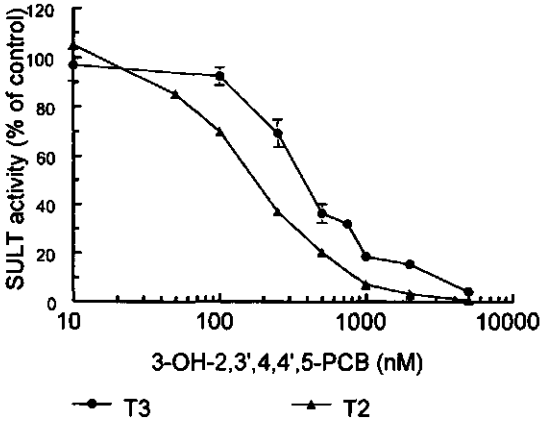

Figure 6.3 Comparison of a dose-response curve of sulfotransferase inhibition by 3-OH-2,3',4,4',5-PeCB (D) using T2 or T3 as substrates and male rat liver cytosol. Data are presented as mean % of control ± SD, obtained from one experiment and performed in duplicate.

T2 sulfotransferase inhibition kinetics by PCB-OHs

The mode of inhibition of T2 sulfotransferase activity was investigated using male rat liver cytosol, a range of [^{125}I]T2/T2 substrate concentrations and increasing 4-OH-2,3,3',4',5-PeCB concentrations. As shown in Figure 6.4A, the substrate concentration-dependent increase in sulfotransferase activity in the absence of inhibitor followed Michaelis-Menten kinetics, with a maximum sulfation rate being reached at 5 μM of T2. The sulfotransferase activities were reduced at all T2 concentrations by 4-OH-2,3,3',4',5-PeCB in a dose-dependent manner. Double-reciprocal conversion of the data from Figure 6.4A into Lineweaver-Burk plots is shown in Figure 6.4B. The apparent Km and Vmax values are 1.3 μM and 0.6 nmol/mg*min. The slope and x-axis intercept of the linear Lineweaver-Burk plots were much more affected in the presence of inhibitor than the y-axis intercept, suggesting that the mode of inhibition was largely competitive. The calculated apparent Ki for 4-OH-2,3,3',4',5-PeCB is 0.09 μM.

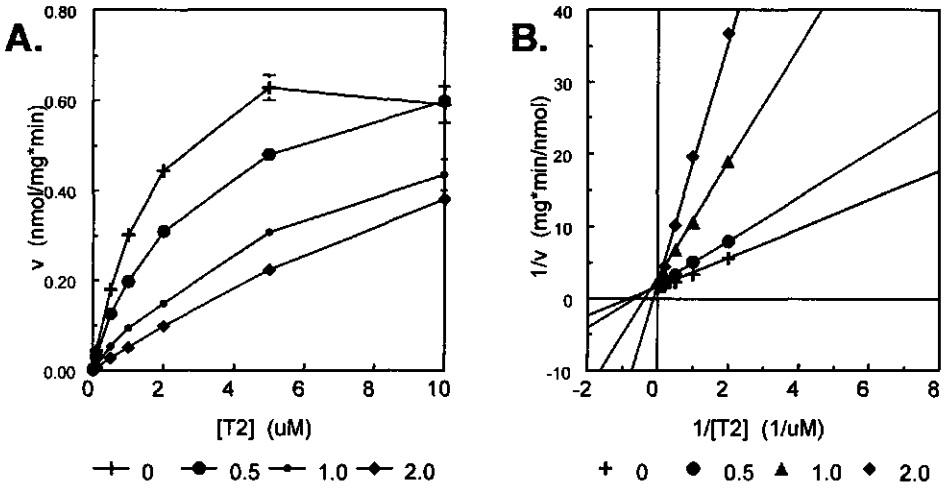

Figure 6.4 Kinetics of sulfotransferase inhibition by 4-OH-2,3,3',4',5-PeCB metabolite (C). Rat liver cytosol was used as enzyme source and T2 as substrate.
A. T2 sulfotransferase activity versus T2 concentration.
B. Lineweaver-Burk plot.

Are hydroxylated PCB inhbitors also potential substrates for sulfotransferases?

The PCB-OH inhibitors used in this study and some PCDD-OHs were also tested as possible substrates for sulfotransferases In Table 6.2, the amount of sulfated products formed in 20 minutes, is presented. These data indicate that the tested PCB metabolites were poorly sulfated, the best one being metabolite D. The tested PCDD-OHs as well as PCP were somewhat better substrates, although the activities were still very low.

Table 6.2 Sulfation of PCB-OHs and PCDD-OHs measured with [^{35}S]-PAPS using rat liver cytosol.

PHAH metabolite	pmol sulfated product/ mg protein/20 min
2-OH-2',3,3',4,4'-PeCB (A)	17 ± 11
4-OH-2',3,4',5,5'-PeCB (B)	55 ± 9
4-OH-2,3,3',4',5-PeCB (C)	44 ± 26
3-OH-2,3',4,4',5-PeCB (D)	109 ± 9
8-OH-2,3-DCDD	235 ± 54
7-OH-2,3,8-TriCDD	390 ± 32
2-OH-1,3,7,8-TCDD	127 ± 13
PCP	127 ± 27

Note. Data are obtained from 2-3 different experiments, using 0.1 μM PAPS and 25 μg cytosolic protein/ml. The maximum enzyme rate is given.

Discussion

It was shown previously that T2 sulfation in rat liver cytosol is inhibited by hydroxylated PCBs (Schuur *et al.*, 1998b; *Chapter 5*). In the present study, the kinetics, isozyme and substrate specificity of iodothyronine sulfotransferase activity inhibition by PCB-OHs were investigated in more detail.

Phenol sulfotransferase isozyme specificity

To facilitate comparison of the different enzyme sources for sulfotransferase, IC50 values were calculated for the four tested PCB-OHs with different sulfotransferase enzyme sources. IC50 values of the PCB-OHs for the recombinant sulfotransferase isozymes human SULT1A1 and rat SULT1C1 were quite similar and followed the same order as with male rat liver cytosol. However, T2 sulfation by SULT1A3 was not inhibited at all by the tested PCB-OHs.

Other known sulfation inhibitors such as DCNP and PCP were also found to inhibit human P-PST (SULT1A1) but not M-PST (SULT1A3) (Rein *et al.*, 1982; Campbell *et al.*, 1987). In this study, we tested the variant of SULT1A1 cloned by Jones *et al.* (1995), which is 99% identical with the SULT1A1 cloned by Falany's group (Wilborn *et al.*, 1993; Ganguly *et al.*, 1995). However, the apparent Km value of the SULT1A1 variant (Jones *et al.*, 1995)

for T2 was about 10-fold higher compared with the variant cloned by Ganguly *et al.* (1995) (Visser *et al.*, 1998a). Further studies are required to investigated the role of other human isozymes such as SULT1A2, the recently cloned SULT1B1 (Fujita *et al.*, 1997; Wang *et al.*, 1998) and SULT1C1 (Her *et al.*, 1997).

For rat, it was demonstrated *in vitro* that thyroid hormones are sulfated by rat SULT1C1 and SULT1B1 (Sakakibara *et al.*, 1995), but not by rat SULT1A1 (Visser *et al.*, 1996a; 1998b). The rat isozyme SULT1C1 may be responsible for the inhibition of T2 sulfotransferase activity by PCB-OHs in male rat liver cytosol. However, SULT1C1 is a male-dominant sulfotransferase and is expressed at very low levels in female liver (Nagata *et al.*, 1993; Liu and Klaassen, 1996), and in this study female rat liver cytosol showed similar IC50 values for PCB-OHs as male rat liver cytosol, rat SULT1C1 and human SULT1A1. SULT1B1 is equally expressed in female and male rat liver (Yamazoe *et al.*, 1994; Sakakibara *et al.*, 1995), and is probably responsible for T2 sulfation in female liver cytosol. SULT1B1 could, thus, be the main target for inhibition by PCB-OHs in female liver. Our results, therefore, suggest similar substrate specificities for rat SULT1B1 and SULT1C1.

T2 sulfotransferase activity in male rat brain cytosol was not inhibited at all by the meta-hydroxylated metabolite D, even though it was the most potent inhibitor using rat liver cytosol. A possible cause of this difference could be a variation in isozyme pattern between rat liver and brain. Dunn and Klaassen (1996) demonstrated SULT1C1 expression only in rat liver, kidney and spleen, but not in rat brain. Apparently, the iodothyronine sulfotransferase(s) in rat brain is not identical with rat SULT1B1 or SULT1C1. However, it is not excluded that the lack of inhibition of T2 sulfation in rat brain by compound D is due to sequestration of the inhibitor by non-specific binding to other proteins in brain cytosol.

In conclusion, we suggest that human SULT1A1 and rat SULT1C1 are involved in the inhibition of T2 sulfation by PCB-OHs, as well as a sulfotransferase isozyme responsible for T2 sulfation in female liver cytosol, probably rat SULT1B1. However, it is necessary to obtain more information about the various isozymes involved in iodothyronine sulfation in humans as well as in rats. Only then is it possible to investigate more specifically which of these isozymes are sensitive to inhibition by PCB-OHs.

Substrate specificity

In this study, T2 was used as a model substrate in the *in vitro* inhibition studies. *In vivo* however, T3 is the most important and active form of thyroid hormone. The IC50 values as well as the ranking order of sulfotransferase activity inhibition by the tested PCB-OHs were almost the same for the substrates T2 and T3. Overall, we conclude that T2 sulfation is a suitable model substrate to assess the potency of T3 sulfation inhibitors.

Type of inhibition

In this study, kinetic analysis suggested a competitive mode of inhibition of T2 sulfation by PCB-OHs. On the basis of the close structural resemblance between PCB-OHs and iodothyronines, it is not unexpected to observe competitive inhibition. Other thyroid hormone-binding proteins such as type I deiodinase and transthyretin are also competitively inhibited by hydroxylated PCBs (Adams *et al.*, 1990; Rickenbacher *et al.*, 1989; Lans *et al.*, 1994; Lans, 1995).

An explanation for the inconclusive results of the kinetic analysis may be the involvement of multiple sulfotransferases in the sulfation of T2 in cytosol which may be inhibited differently. To get more decisive results, single purified isozymes should be tested for the inhibition kinetics of T2 sulfation by PCB-OHs. Another explanation is that the inhibitor PCB-OH is a substrate for sulfotransferase itself, and may, therefore, be sulfated itself, thereby lowering its concentration.

Different types of inhibition of phenol sulfotransferase activity have been reported for different inhibitors in rat and human tissues. For instance, vanillin, a substrate for phenol sulfotransferase, showed a partial non-competitive or mixed-type inhibition in rat liver cytosol and phenol, *p*-nitrophenol or dopamine as substrates (Cruickshank and Sansom, 1993). In human liver cytosol, the flavonoid quercetin was also found to be a non-competitive inhibitor of *p*-nitrophenol, acetaminophen and minoxidil sulfation (Walle *et al.*, 1995; Eaton *et al.*, 1996). Another well-known inhibitor, DCNP, demonstrated in rat liver cytosol competitive inhibition vs substrates (Seah and Wong, 1994). However, in human brain cytosol (Whittemore *et al.*, 1986) and human liver cytosol or partially purified P-PST (Walle *et al.*, 1995), DNCP was demonstrated to be a non-competitive inhibitor of phenol sulfation. Furthermore, the inhibitor PCP was found to be a competitive inhibitor vs the substrate (Duffel and Jakoby, 1981). Inhibitors are mostly alternative substrates, as we also demonstrated for PCB-OHs and PCDD-OHs (this study; Schuur *et al.*, 1998b; *Chapter 5*). It should also be mentioned that phenol sulfotransferases are subject to substrate inhibition, which results in complex inhibition mechanisms (Binder and Duffel, 1988).

Relevance

The potential for inhibition of thyroid hormone sulfation by PCB-OHs *in vivo* is difficult to ascertain. DCNP has been shown to inhibit iodothryonine sulfation *in vivo* (De Herder *et al.*, 1988). PCBs are known neurotoxicants exerting effects on fetal development (Brouwer *et al.*, 1995). It was demonstrated before that PCB-OHs accumulate up to low micromolar concentrations in fetal tissues after exposure of pregnant rats to Aroclor 1254, a PCB mixture (Morse *et al.*, 1996).

Sulfation is an important step in the irreversible inactivation of thyroid hormone, since iodothyronine sulfates are rapidly degraded by the type I iodothyronine deiodinase (D1). It has

been suggested that sulfation of iodothyronines is an important reversible pathway of thyroid hormone inactivation when D1 is low, such as in non-thyroidal illness and during fetal development (Visser *et al.*, 1994b). Santini *et al.* (1992a) postulated that T3 sulfation has an important function during fetal development since it allows recovery of active T3 from T3 sulfate by action of sulfatases in tissues at the time when hormone action is required. Taken together, it is important to investigate the possible *in vivo* inhibition of the thyroid hormone sulfation by PCB metabolites during fetal development.

Acknowledgements

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CHAPTER 7

EFFECTS OF PENTACHLOROPHENOL AND HYDROXYLATED POLYCHLORINATED BIPHENYLS ON THYROID HORMONE CONJUGATION IN A RAT AND HUMAN HEPATOMA CELL LINE

Abstract

It was previously demonstrated in our laboratory that hydroxylated metabolites of polychlorinated biphenyls (PCB-OHs) inhibit the sulfation of iodothyronines in rat liver cytosol. In this study, the inhibition of 3,3'-diiodothyronine (T2) sulfation by pentachlorophenol (PCP) and PCB-OHs was investigated in hepatoma cell lines in relation to the cellular uptake of these compounds, providing a more appropriate model of the *in vivo* situation. The human HepG2 hepatoma cell line was shown to conjugate T2 almost exclusively by sulfation, glucuronidation being negligible. The rat FaO hepatoma cell line on the other hand produced 37% T2 sulfate and 63% T2 glucuronide. It was demonstrated that PCP inhibited T2 sulfation in both cell lines, although it was 10^3 times less potent in cells than in rat liver cytosol. Remarkably, 10 μM PCP inhibited the sulfation and glucuronidation of T2 by FaO cells to the same extent. Micromolar concentrations of 4-hydroxy-3,3',4',5-tetrachlorobiphenyl or 4-hydroxy-2',3,3',4',5-pentachlorobiphenyl hardly affected T2 conjugation in FaO cells, but both PCB-OHs reduced T2 sulfate formation in HepG2 cells. Inhibition of T2 sulfation was stronger using medium without FCS than medium with 5% FCS. This was due to a lower uptake of inhibitor by the cells in the presence of serum, as demonstrated using radiolabeled PCP. In conclusion, this study confirms the inhibition of T2 sulfation by PCP and PCB-OHs previously observed in rat liver cytosol in a rat and a human hepatoma cell line. Thus, it seems reasonable to assume that iodothyronine sulfation is also inhibited by PCB metabolites and PCP *in vivo*.

based on A. Gerlienke Schuur, Åke Bergman, Abraham Brouwer, and Theo J. Visser (1998). Effects of pentachlorophenol and hydroxylated polychlorinated biphenyls on thyroid hormone conjugation in a rat and a human hepatoma cell line. submitted.

Introduction

The main metabolic pathways for thyroid hormone are deiodination, glucuronidation and sulfation (Visser, 1990; 1994b). Sulfation has been viewed as an essential step in the metabolism of iodothyronines, increasing their water solubility and thus stimulating their excretion in bile and urine. Furthermore, 3,3',5-triiodothyronine (T3) sulfate does not bind to the T3 receptor and is thus unable to mimic thyroid hormone activity (Spaulding *et al.*, 1992). Moreover, iodothyronine sulfates are rapidly degraded by inner ring deiodination by the type I deiodinase (D1) (Otten *et al.*, 1983; Visser *et al.*, 1984; Rutgers *et al.*, 1989). It has been suggested that thyroid hormone sulfation is an important reversible inactivation step when D1 is low such as occurs in the fetal situation (Santini *et al.*, 1992a/b). In the fetus, active T3 may be recovered from T3S through the action of sulfatases in tissues where hormone action is required.

Environmental pollutants such as polychlorinated biphenyls (PCBs) are known to interfere with thyroid hormone metabolism through multiple mechanisms (for review: see Brouwer *et al.*, 1998). One way of interference is through competive inhibition of thyroid hormone binding to its specific binding proteins, such as transthyretin and D1, by hydroxylated metabolites of polychlorinated biphenyls (PCB-OHs) (Adams *et al*, 1989; Lans *et al.*, 1993, 1994). Recently, it was demonstrated that sulfotransferases are also targets for hydroxylated PCBs and related compounds (Schuur *et al.*, 1998b; *Chapter 5*). Hydroxylated PCBs were found to inhibit iodothyronine sulfation in rat liver cytosol. This inhibition was mainly competitive in nature and sulfotransferase isozyme-specific, e.g. rat SULT1C1 and human SULT1A1, but not human SULT1A3, were inhibited by PCB-OHs (Schuur *et al.*, 1998c; *Chapter 6*).

In the present investigation, the inhibition of thyroid hormone sulfation by pentachlorophenol (PCP) and hydroxylated PCB metabolites was investigated using rat and human hepatoma cell lines. Such a study using intact cells is thought to be more representative of the *in vivo* situation. PCP and the model hydroxylated PCBs, 4-hydroxy-2',3,3',4',5-pentachlorobiphenyl (PeCB-OH) and 4-hydroxy-3,3',4',5-tetrachlorobiphenyl (TeCB-OH), were used to examine their potential impact on iodothyronine sulfation in the rat FaO hepatoma cell line and the human HepG2 hepatoma cell line, using 3,3'-diiodothyronine (T2) as a model substrate. [^{14}C]PCP was used to estimate the cellular uptake of this phenol.

Materials and methods

Materials

4-Hydroxy-2',3,3',4',5-pentachlorobiphenyl (PeCB-OH) and 4-hydroxy-3,3',4',5-tetrachlorobiphenyl (TeCB-OH) were synthesized according to the method described elsewhere (Klasson-Wehler *et al.*, 1990; Bergman *et al.*, 1995). Pentachlorophenol (PCP) was obtained from Aldrich (Bornem, Belgium). [^{14}C]PCP, 6-propyl-2-thiouracil (PTU) and 3'-phosphoadenosine-5'-phosphosulfate (PAPS) were obtained from Sigma (St. Louis, MO). 3,3'-diiodothyronine (T2) and 3-iodothyronine (T1) were obtained from Henning (Berlin, Germany). [^{125}I]T2 was produced by radioiodination of T1 as described before (Visser *et al.*, 1978). Sephadex LH-20 was purchased from Pharmacia (Woerden, The Netherlands). DMSO was obtained from Acros Chimica (Den Bosch, The Netherlands). Phosphate-buffered saline (PBS) was obtained from Oxoid Ltd (Basingstoke, UK). Cell culture media were obtained from Gibco Life Technologies (Breda, The Netherlands). Fetal calf serum (FCS) was obtained from Sigma. Cell culture flasks and 6-well plates were obtained from Costar Europe Ltd (Badhoevedorp, The Netherlands).

Cell culture

HepG2 cells were obtained from ATCC (no HB 8065, Rockville, MD) and FaO cells from ECACC (no 89042701, Salisbury, UK). Both cell types were cultured in DMEM/F12 supplemented with 10% FCS in a 37°C, 5% CO_2 humidified air environment. For the experiments, confluent cell layers were obtained in 6-well plates, washed with PBS and incubated with 2 ml of medium which consisted of DMEM/F12 without phenol red, containing 100 μM PTU, 1 μM T2 with ≈100,000 cpm [^{125}I]T2. PTU is an inhibitor of D1 (Visser, 1990) and was added to inhibit the possible breakdown of the T2 sulfate formed. This medium was used without or with 5% FCS. The PCB-OHs and PCP were dissolved in DMSO and diluted in the medium with a maximal final DMSO concentration of 1%. The cells were incubated for 4 h in an atmosphere of 37°C and 5% CO_2. To determine the amount of PCP present in the cells following exposure, [^{14}C]PCP was added to the wells. In individual experiments each condition was tested in duplicate, and untreated controls (with maximally 1% DMSO) and blanks (medium without cells) were included. Each experiment was performed 2-5 times.

T2 sulfotransferase activity

Cells were washed with PBS, and scraped in 0.1 M Tris-HCl, 2 mM EDTA (pH 7.2). After centrifugation for 75 min at 105,000xg and 0-4°C, protein levels were determined with the Bio-Rad assay (Bio-Rad Laboratories, Munich, Germany), using BSA as a standard (Bradford, 1976). T2 sulfotransferase activity was measured as described by Kaptein *et al.* (1997). In short, cell cytosol (500 μg/ml final protein concentration) was incubated with 1 μM T2 and 80,000

cpm [^{125}I]T2, 50 μM PAPS in 75 mM Tris-HCl, 1.5 mM EDTA (pH 7.2) in a total volume of 200 μl. Incubations were carried out for 30 min at 37°C. The reaction was stopped on ice by adding 750 μl of 0.1 M HCl. The T2 sulfate formed was analyzed by Sephadex LH-20 chromatography as described by Rutgers *et al.* (1989).

T2 conjugate analysis

After 4 h of exposure, 0.5 ml culture medium was taken and 0.5 ml 1 M HCl was added to the sample. The remainder of the medium was removed and cells were extracted in 0.5 ml 0.1 M NaOH and 0.5 ml 1 M HCl was added. The acidified medium and cell samples were counted for ^{125}I radioactivity using a Cobra AutoGamma counting system (Canberra-Packard, Groningen, The Netherlands). Conjugated [^{125}I]T2 was isolated by Sephadex LH-20 chromatography as described by Rutgers *et al.* (1989) and counted for radioactivity.

In a separate experiment, the nature of the conjugated T2, i.e. the ratio between T2 glucuronide (T2G) and T2 sulfate (T2S), was determined. For this purpose, medium and cell samples were analyzed by Sephadex LH-20 chromatography without and after treatment for 1 h at 80°C with 1 M HCl. T2S is hydrolyzed during this treatment, while T2G is stable (Mol and Visser, 1985).

Presence of PCP in the cells

After 4 h of exposure to [^{14}C]PCP, 1 ml of medium was harvested and 4 ml of scintillation liquid (Ultima Gold, Packard) was added. The cells were extracted in 1 ml 0.1 M NaOH and 4 ml of scintillation liquid was added. All samples were counted using a liquid scintillation counter (Tri-Carb 1600, Packard).

Results

Initially, we tested the sulfotransferase activity in the cytosol of the hepatoma cell lines using 1 μM [^{125}I]T2 as substrate and 50 μM 3'-phosphoadenosine-5'-phosphosulfate as cofactor. In the rat FaO hepatoma cell line this amounted to 286 pmol T2S formed/mg protein per 30 min and in the human HepG2 hepatoma cell line 702 pmol/mg protein per 30 min.

Thereafter, we tested the ability of intact hepatoma cell lines to conjugate T2 to T2S and T2G. Figure 7.1 shows the total amounts (medium plus cells) of T2 conjugates as well as of T2S and T2G produced by FaO and HepG2 cells. In FaO cells (Figure 7.1A), T2 conjugates consisted for 63% of T2G and 37% of T2S. Total T2 conjugation was reduced by ≈40% by addition of 10 μM PCP and by only ≈10% in the presence of 10 μM TeCB-OH. Similar effects of PCP and TeCB-OHwere found on T2S and T2G formation. In HepG2 cells (Figure 7.1B), total T2 conjugation was almost completely accounted for by sulfation. T2S formation was

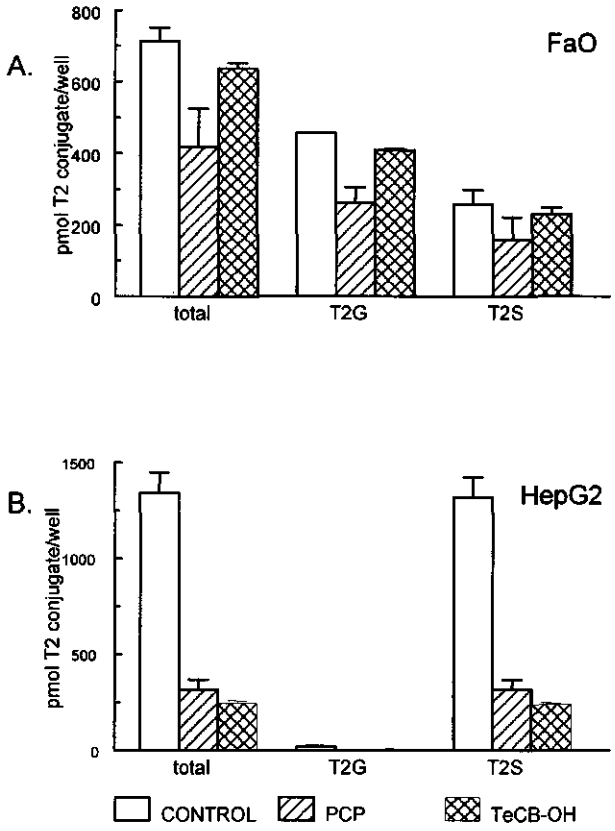

Figure 7.1 Effect of pentachlorophenol (PCP) and 4-OH-3,3',4',5 tetrachlorobiphenyl (TeCB-OH) on T2 conjugation in intact hepatoma cells. Total T2 conjugation as well as T2 glucuronides and T2 sulfates were measured in medium and cells of the rat hepatoma cell line FaO (A) and the human hepatoma cell line HepG2 (B) after in 4 h of exposure to 10 μM PCP or TeCB-OH in medium without 5% FCS. Results presented are from one experiment performed in duplicate.

inhibited by ≈75% by 10 μM PCP and by ≈80% by 10 μM TeCB-OH. In addition, the amount of non- and conjugated T2 present in cells was not changed in the presence of PCP or PCB-OHs, as is shown in Table 7.1.

Dose-dependence of T2 sulfation inhibition by PCP or PeCB-OH

Inhibition studies were performed in intact cells coincubated with [^{125}I]T2 and PCP or PeCB-OH. The control value of total T2 conjugate formed in 4 h by FaO cells was about 0.5 nmol per well in medium with 5% FCS and 0.9 nmol per well in medium without FCS for most of the incubations. In untreated control HepG2 cells, the total amount of T2 conjugate formed in 4 h was about 0.9 nmol per well in medium with 5% FCS and about 1.2 nmol per well in medium without FCS. Irrespective of the presence of serum, most T2 conjugate was found in the medium. For this reason, further data represent T2 conjugates determined in the medium.

Table 7.1 T2 conjugation in the rat hepatoma cell line FaO or the human hepatoma cell line HepG2. The amount of T2S and T2G formation in medium and cells is given, in the absence or presence of 10 μM PCP or 4-OH-2',3,3',4',5-pentachlorobiphenyl (PeCB-OH).

FaO	-FCS	+FCS	-FCS	+FCS	**HepG2**	-FCS	+FCS	-FCS	+FCS
T2 pmol/well	medium	medium	cells	cells	T2 pmol/well	medium	medium	cells	cells
DMSO	1118	1417	79	85	DMSO	439	737	104	113
PCP	1368	1439	121	55	PCP	1172	941	433	219
PeCB-OH	1199	1443	68	56	PeCB-OH	1154	915	518	88
T2S pmol/well					T2S pmol/well				
DMSO	259	184	5	5	DMSO	1114	955	211	126
PCP	162	181	1	0	PCP	290	652	31	105
PeCB-OH	227	201	9	0	PeCB-OH	219	876	29	67
T2G pmol/well					T2G pmol/well				
DMSO	436	267	37	15	DMSO	27	40	10	3
PCP	251	244	26	13	PCP	12	38	4	2
PeCB-OH	407	263	18	14	PeCB-OH	16	25	4	2

Figure 7.2 shows the results of one example experiment in which the effects of increasing concentrations of PCP and PeCB-OH on the conjugation of T2 in FaO cells are demonstrated. PCP decreased T2 conjugate formation in FaO cells incubated in medium without FCS by up to 80% (Figure 7.2A). Repeated experiments with FaO cells showed a high degree of variation, with IC50 values ranging between 0.5 and 8 μM. However, when medium with 5% FCS was used, formation of T2 conjugates in FaO cells was not reduced by up to 10 μM PCP. PeCB-OH caused a slight (20%) reduction of T2 conjugation by FaO cells in the absence of FCS but had no effect in the presence of 5% FCS (Figure 7.2B).

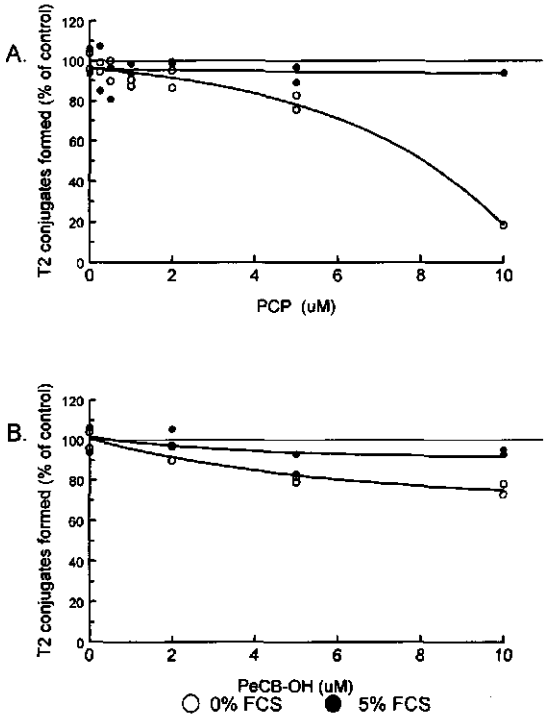

Figure 7.2 Dose-dependency of T2 conjugate reduction by pentachlorophenol (PCP; A) and 4-OH-2',3,3',4',5-pentachlorobiphenyl (PeCB-OH; B) exposure for 4 h in rat hepatoma FaO cells, in medium with or without 5% FCS. The percentage of formed T2 conjugate in medium is expressed as percentage of DMSO control. Results presented are from one experiment performed in duplicate.

Figure 7.3 shows the results of one separate experiment in which the inhibitory effect of PCP and PeCB-OH on T2S formation by HepG2 cells are presented. PCP was found to clearly reduce the formation of T2S in HepG2 cells in a dose-dependent manner in the absence but not in the presence of 5% FCS (Figure 7.3A). IC50 values for the reduction of T2S formation by PCP varied between 0.8 and 3.9 μM in medium without FCS. PeCB-OH decreased T2S formation by HepG2 cells to the same extent as PCP (Figure 7.3B), with an IC50 value of 3.6

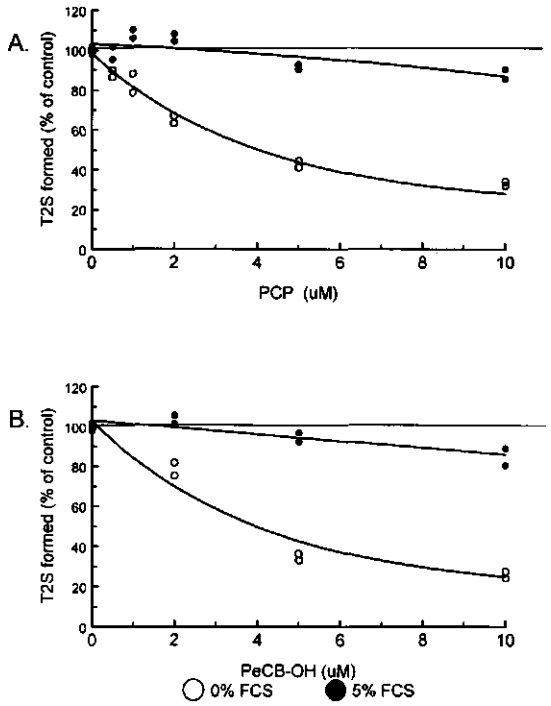

Figure 7.3 Dose dependency of T2 conjugate reduction by pentachlorophenol (PCP; A) and 4-OH-2',3,3',4',5-pentachlorobiphenyl (PeCB-OH; B) exposure for 4 h in human hepatoma HepG2 cells, in medium with or without 5% FCS. The percentage of formed T2 conjugate in medium is expressed as percentage of DMSO control.

μM in the absence of FCS. However, in the presence of 5% FCS, no effect on T2S formation by PeCB-OH was observed up to 10 μM.

[^{14}C]PCP cellular uptake

The difference in reduction of T2 conjugation in cells exposed to inhibitors in medium without and with 5% FCS could be caused by a difference in the amount of the inhibitor taken up by the cells. To investigate this, [^{14}C]PCP was used to determine the amount of inhibitor present in the cells after 4 h of exposure. In HepG2 cells, 8 and 22% of labelled PCP was present, in medium with and without FCS respectively. The percentages of PCP present in FaO cells accounted for 2.5 and 8% in medium with and without FCS respectively. Figure 7.4 shows the amount of PCP present in FaO or HepG2 cells after 4 h of exposure versus the added concentration of PCP in the medium. It was found that in the presence of 5% FCS the amount of PCP taken up in the cell was up to 70% lower than that in the absence of 5% FCS. Furthermore, the amount of PCP taken up in HepG2 cells was about 3 times higher compared to the uptake in FaO cells.

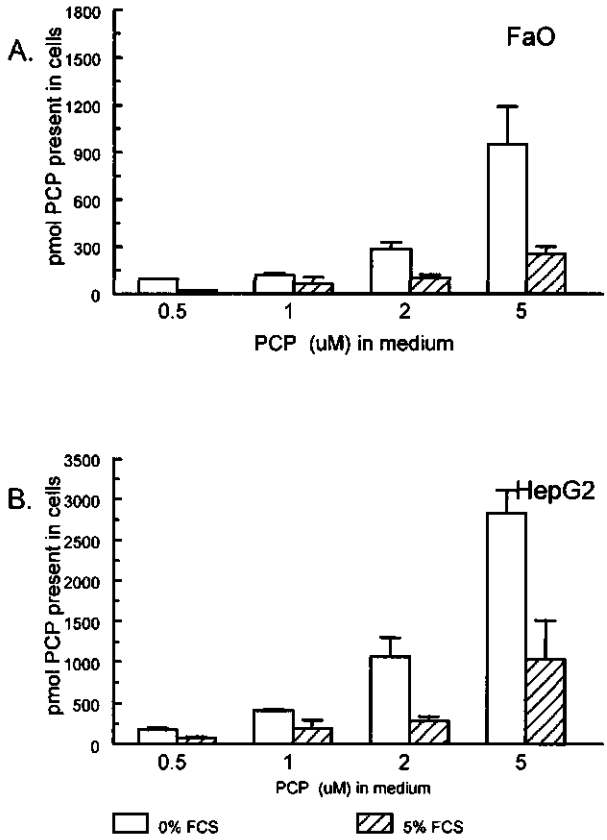

Figure 7.4 The uptake of PCP in the rat hepatoma cell line FaO (A) or the human hepatoma cell line HepG2 (B) in medium with or without 5% FCS. The amount of PCP (measured with ^{14}C-PCP) present in the cells is plotted against the PCP concentration added in the medium. Results presented are from one experiment performed in duplicate.

Discussion

Recently, it was shown that phenolic organohalogens, such as PCP and hydroxylated PCBs, were potent inhibitors of the iodothyronine sulfotransferase activity in rat liver cytosol (Schuur *et al.*, 1998a; *Chapter 5*). The aim of the present study was to investigate the sulfotransferase inhibitory effect of PCP and PCB-OH in intact cells using the rat FaO hepatoma cell line and the human HepG2 hepatoma cell line. Although hepatoma cells are different from normal hepatocytes, studies using these cells were thought to be more representative of the *in vivo* situation than experiments using subcellular fractions.

The T2 conjugates produced by FaO cells consisted for one-third of T2S and for two-third of T2G. This is in agreement with another report demonstrating low or even undetectable 2-naphthol sulfation but significant 1-naphthol glucuronidation in FaO cells (Utesch *et al.*, 1992). Addition of PCP or TeCB-OH to the culture medium of FaO cells produced a parallel

decrease in the production of T2S and T2G.

Little or no formation of T2G was found in HepG2 cells, and T2 sulfation was ≈2-fold higher than in FaO cells. Other reports do not give consistent results on sulfation in HepG2 cells. Dawson *et al.* (1985) found very little ethoxycoumarin sulfation. Van Stralen *et al.* (1993) reported that T3 is poorly sulfated in HepG2 cells, while De Jong *et al.* (1995) demonstrated that both T2 and Triac were sulfated in HepG2 cells, with only a small percentage of T2 being glucuronidated. Due to the sulfotransferase isozymes present, HepG2 cells may represent a good model for sulfate conjugation in normal human liver cells (Shwed *et al.*, 1992). We found that PCP and PeCB-OH are equipotent inhibitors of T2 sulfation in HepG2 cells.

We have shown that PCP has a similar potency in inhibiting T2 sulfation in HepG2 and FaO cells, with IC50 values of ≈4 μM. Whereas PeCB-OH is equally potent in reducing T2S formation in HepG2 cells, PeCB-OH is a much weaker inhibitor in FaO cells. The IC50 values for PCP in both FaO and HepG2 cells are much greater than the IC50 values of 5 nM and 2 nM for PCP inhibition of T2 sulfation in rat and human liver cytosol, respectively (Schuur *et al.*, 1998b; *Chapter 5*; Visser *et al.*, 1998a). The IC50 value for PeCB-OH in HepG2 cells is much closer to its IC50 value of ≈1 μM for inhibition of T2 sulfation by rat liver cytosol and human SULT1A1 (Schuur *et al.*, 1998c; *Chapter 6*). Other groups also reported on the difference between *in vitro* assays using cytosolic preparations or cells. For example, the flavonoid quercetin and the well-known phenol sulfotransferase inhibitor DCNP were found to be much less potent inhibitors of PNP sulfation in HepG2 cells (with 10% FCS) than with cell-free phenol sulfotransferase solutions (Eaton *et al.*, 1996). The difference may be caused by binding of the inhibitor to serum proteins, relative membrane impermeability and the conversion to inactive metabolites in cells. In addition, the composition of sulfotransferase isoenzymes may be different between tumor cells and normal tissue.

We demonstrated that in medium with 5% FCS, IC50 values were higher for both inhibitors and both cell lines than in medium without FCS. This is probably explained by the lower availability of the inhibitor in the presence of serum due to protein (albumin) binding. It was indeed proven using radiolabelled PCP, that the amount of inhibitor present in the cell was higher after incubation without FCS than in the presence of 5% FCS. The amount of PCP taken up by HepG2 cells was higher than that taken up by FaO cells. This is in agreement with the more strongly reduced formation of T2S in HepG2 cells than in FaO cells.

The potential for inhibition of thyroid hormone sulfation by PCB-OHs *in vivo* is difficult to estimate. Similar to PCP and DNCP (Mulder and Scholtens, 1977; Koster *et al.*, 1982; Meerman *et al.*, 1983), PCB-OHs may also be active inhibitors of thyroid hormone sulfation *in vivo*. The effectiveness *in vivo* will depend on the bioavailability of the compounds. Quercetin has been shown to have no effect on the sulfation of harmol in the perfused rat liver (Shali *et al.*, 1991), although quercetin was a potent inhibitor in human liver cytosol and in HepG2 cells (Walle *et al.*, 1995; Eaton *et al.*, 1996). Uptake and tissue distribution of PCB-OHs

may be similar to other phenolic halogenated compounds such as PCP and DNCP, due to similar physico-chemical properties. PCB-OHs were demonstrated before in human plasma, and in plasma and tissues of PCB-exposed animals (Bergman *et al.*, 1994), and at even higher levels in rat fetuses of PCB-exposed mothers (Morse *et al.*, 1996).

The parallel reduction of T2 sulfation and glucuronidation in FaO cells by PCP is surprising, since sulfotransferases are generally regarded as high-affinity enzymes and UDP-glucuronyltransferases as low-affinity enzymes. These findings suggest that a common step preceding sulfation and glucuronidation of T2 in FaO cells is inhibited, such as transport of T2 across the cell membrane. However, in the presence of PCP accumulation of non-conjugated T2 was observed in both FaO cells and HepG2 cells (not shown), suggesting direct inhibition of the conjugating enzymes and not inhibition of cellular uptake of T2.

Further studies in our laboratory are aimed at determining *in vivo* effects of PCB-OH exposure on fetal thyroid hormone metabolism including sulfation and the potential toxicological impact for the developing animal.

Acknowledgements

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CHAPTER 8

EFFECT OF PRENATAL EXPOSURE TO PENTACHLOROPHENOL OR AROCLOR 1254 ON MATERNAL AND FETAL THYROID HORMONE METABOLISM, ESPECIALLY SULFATION, IN WISTAR RATS

Abstract

Polyhalogenated aromatic hydrocarbons (PHAHs) are known to interfere with several aspects of thyroid hormone metabolism. Recently, the inhibition of thyroid hormone sulfation by hydroxylated PHAH metabolites was demonstrated *in vitro*. The present study was undertaken to investigate whether PCB metabolites are also able to inhibit thyroid hormone sulfation *in vivo*. Pregnant Wistar rats were exposed to Aroclor 1254, to the well-known phenol sulfotransferase (SULT) inhibitor pentachlorophenol (PCP), or to corn oil (vehicle) from day 10 of gestation (GD10) until GD18. Dams and fetuses were sacrificed on GD20, and maternal and fetal blood, liver and brain were collected. Thyroid hormone and thyroxine sulfate (T4S) levels were measured in serum; T4 UDP-glucuronyltransferase (UGT), type I deiodinase (D1) and 3,3'-iodothyronine (3,3'-T2) SULT activities were analysed in liver; and type II deiodinase (D2) and 3,3'-T2 SULT activities were analysed in brain.

Aroclor 1254 exposure resulted in decreased total T4 (TT4) and free T4 (FT4) levels in dams as well as fetuses. Treatment with PCP resulted in a decrease in serum TT4 but an increase in serum FT4 in dams and fetuses. The FT4/TT4 ratio was increased after both treatments. Fetal T4S levels were very low in contrast to previously reported high levels in fetal serum ofhuman and sheep. Treatment with PCP or Aroclor 1254 did not significantly change the T4S levels in both dams and fetuses. In maternal and fetal brain, 3,3'-T2 SULT activity was not affected after treatment with Aroclor 1254 or PCP. Maternal and fetal liver 3,3'-T2 SULT activity was not affected as well by Aroclor 1254. However, PCP caused an increase in 3,3'-T2 SULT activity in maternal liver and a decrease in fetal liver. Further data showed an induction of hepatic T4 UGT activity in dams after treatment with Aroclor 1254, but not with PCP. D1 activity was low in fetal versus maternal liver, and was decreased in both dams and fetuses after treatment with Aroclor 1254 and PCP. Brain D2 activity was increased in both dams and fetuses after exposure to Aroclor 1254.

In conclusion, perinatal exposure to PCP resulted in placental transfer of PCP to fetal rat tissues. Thyroid hormone levels and metabolism were affected after PCP exposure. The effect of PCP and Aroclor 1254 on thyroid hormone sulfation was not clear, and will be dependent on the *in situ* balance between the concentrations of substrate thyroid hormone, the enzyme sulfotransferase and the competitive inhibitor PCP/PCB-OH. Administration of PCP or Aroclor 1254, overall did not result in a decrease in fetal serum T4S levels, perhaps because degradation of T4S by hepatic D1 is simultaneously decreased. Moreover, in fetal rats, sulfation seems to play a less important role in the metabolism of thyroid hormone than in fetal humans or sheep, considering the low T4S levels in fetal serum.

based on A. Gerlienke Schuur, Peter H. Cenijn, Maria A.W. Faassen-Peters, Dennis C. Morse, Hans van Toor, Åke Bergman, Eva Klasson-Wehler, Peter J. van Bladeren, Theo J. Visser, and Abraham Brouwer (1998). Effect of prenatal exposure to pentachlorophenol or Aroclor 1254 on maternal and fetal thyroid hormone metabolism, especially sulfation, in Wistar rat. submitted.

Introduction

PCBs and related compounds are known to interfere with thyroid hormone metabolism (Brouwer *et al.*, 1998). Exposure of laboratory animals to PCBs results in increased hepatic UGT activity and, consequently, decreased serum TT4 levels (e.g. Beetstra *et al.*, 1991). Hydroxylated metabolites of PCBs and related compounds compete with thyroid hormones for binding to different thyroid hormone-binding proteins such as TTR (Lans *et al.*, 1993; 1994), D1 (Adams *et al.*, 1990; Lans, 1995) and phenol sulfotransferases. Inhibition of thyroid hormone sulfation *in vitro* was studied using 3,3'-T2 as a substrate and rat liver cytosol as enzyme source (Schuur *et al.*, 1998b; *Chapter 5*). More recently, inhibition of 3,3'-T2 sulfation by 4-hydroxy-2',3,3',4',5-pentachlorobiphenyl (4-OH-PeCB) as well as by PCP was also demonstrated in a rat and a human hepatoma cell line (Schuur *et al.*, 1998d; *Chapter 7*).

In adult rats, thyroid hormone sulfation is considered an irreversible inactivation pathway. Not only does sulfation impair the binding of the active hormone T3 to the T3 receptor, it also accelerates the inner ring deiodination of T3 and its precursor T4 by hepatic D1 to inactive 3,3'-T2 and rT3, respectively. It has been suggested that sulfation is a reversible inactivation pathway in the fetal situation, when D1 activity is low (Santini *et al.*, 1992a; Visser *et al.*, 1994b). In this respect, T3 sulfate (T3S) is regarded as a reservoir of inactive hormone from which active T3 may be recovered by action of sulfatases in tissues where hormone action is required.

It has been demonstrated before that prenatal exposure to Aroclor 1254, a mixture of

PCBs, results in the accumulation of PCB metabolites in fetal tissues (Morse *et al.*, 1996). The aim of this study is to investigate if thyroid hormone sulfation in the fetus is inhibited by these PCB metabolites. This hypothesis was tested by exposing pregnant Wistar rats to Aroclor 1254 from day 10 to day 18 of gestation. The well-known *in vitro* and *in vivo* phenol SULT inhibitor PCP (Duffel and Jakoby, 1981; Meerman *et al.*, 1983) was used in the same study as a positive control, although it has never been tested as SULT inhibitor in the fetal situation. Serum T4S and T3S levels were determined as well as iodothyronine SULT activities in fetal and maternal liver using 3,3'-T2 as the preferred substrate (Visser *et al.*, 1998a). The possible effect of the inhibition of iodothyronine SULT activity on thyroid hormone levels was studied by measurement of fetal serum T4, T3 and rT3 concentrations. In addition, hepatic D1 and T4 UGT activities, and brain D2 activities were determined in maternal and fetal tissues. Moreover, the amounts of PCP and 4-OH-PeCB were analysed in pooled maternal and fetal blood and tissues.

Materials and methods

Chemicals

PCP (99% pure) was obtained from Riedel-de Haën AG (Seelze, Germany), and Aroclor 1254 was kindly donated by Dr. M. Van den Berg (Ritox, University of Utrecht, Utrecht, The Netherlands). T4, T3, rT3, DTT, UDPGA, PTU, PAPS and bovine serum albumin (BSA) were obtained from Sigma Chemicals Co. (St. Louis, MO, USA); resorufin from Janssen Chimica (Tilburg, The Netherlands); NADPH from Boehringer Mannheim GmbH (Mannheim, Germany) and Bio-Rad protein reagent from Bio-Rad Laboratories (Richmond, Ca, USA). [^{125}I]T4, [^{125}I]T3 and [^{125}I]rT3 were obtained from Amersham (Buckinghamshire, UK); they were purified on Sephadex LH-20 (Pharmacia, Woerden, The Netherlands) before each assay. 3,3'-T2 and 3-iodothyronine (3-T1) were obtained from Henning (Berlin, Germany). ^{125}I-labelled 3,3'-T2 was produced by radioiodination of 3-T1 as described before (Visser *et al.*, 1978). All other chemicals were of analytical grade.

Animals and treatment

Wistar WU rats (14 weeks old) were purchased from Charles River (Sulzfeld, Germany). The rats were allowed to acclimatize for 2 weeks and were maintained at 50% humidity and 21°C on bedding in plastic cages with a 12-light/12-h dark cycle. Rat chow (Hope Farms, Woerden, The Netherlands) and tap water were supplied *ad libitum*. After the acclimatization period breeding was started by placing two females in a cage with one male overnight. The females were examined each morning by vaginal smear. When spermatozoa were found, the animal was housed separately, and this was termed day 0 of gestation (GD0). Animals were assigned

at random to the different treatment groups. Maternal body weight was monitored daily throughout gestation. On GD10 the pregnant rats were transferred to a macrolon cage with a grated steel support to facilitate the collection of PCB and PCP-contaminated feces. Pregnant females were untreated or received a daily oral dose of corn oil (vehicle), 25 mg Aroclor 1254 or 30 mg PCP per kg body weight in corn oil (2 ml/kg body weight) from GD10 until GD18.

On GD20, rats were sacrificed under ether anesthesia, and maternal blood was collected from the vena cava. Maternal brains and livers as well as fetuses were isolated and weighed. Fetal blood was collected by decapitation, and the fetal livers and brains were isolated, weighed and frozen on dry ice. Fetal blood was pooled per mother and serum was prepared for thyroid hormone analysis. Pooled fetal liver and brain tissues as well as maternal liver and brain tissues were stored at -80°C. Serum was stored at -20°C until analysis. All procedures were approved by the Animal Welfare Committee of the Agricultural University Wageningen.

Tissue preparation

Livers and brains were homogenized, and cytosol and microsomes were prepared as described before (Schuur *et al.*, 1997; *Chapter 2*). Protein levels of tissue fractions were determined using the Bio-Rad protein reagent (Bradford, 1976) and BSA as a standard.

Thyroid hormone analysis

Serum TT4, TT3 and FT4 were analysed using the Amerlite chemiluminescence kits (Amerlite, Amersham, Buckinghamshire, UK) according to the protocol of the supplier with the following modifications: the TT4 and TT3 assay buffer were diluted five times with demineralized water; the standard curve for TT4 ranged from 0 to 120 nmol TT4/l. Serum rT3 was measured by specific RIA as described before (Eelkman Rooda *et al.*, 1989). Plasma T4S and T3S were determined by specific RIAs using antisera kindly donated by Dr. Sing-Yung Wu (Veterans Affairs Medical Center, Long Beach, CA, USA) essentially as described by Wu *et al.* (1995).

Analysis of PCP and 4-OH-PeCB in serum, liver and brain

Serum from a PCP-treated dam and serum from an Aroclor 1254-treated dam and its fetuses were used for the analysis. In addition, 3 pooled samples of brain and liver tissues from all PCP treated dams and their fetuses were used for the analysis. The method used will be described in detail elsewhere (Hovander *et al.*, 1998). In brief, serum samples (±5 g) were denaturated by HCl and 2-propanol after which the compounds were extracted with hexane:methyl-tert-butyl ether. The extract was separated into phenol and neutral compounds, by partitioning between KOH (0.5 M in 50% ethanol) and hexane. The phenolic compounds were derivatized with diazaomethane prior to a lipid removal step. The extract was dissolved in hexane and lipids were removed by partitioning with sulphuric acid. Analysis was performed by GC (ECD) (Bergman *et al.*, 1994).

Enzyme assays

Ethoxyresorufin O-deethylase (EROD) activity. EROD activity was measured according to the method of Burke *et al.* (1977) adapted for use with 96-well plates and a fluoro-spectrophotometric plate reader (Cytofluor 2350, Millipore) (Schuur *et al.*, 1998a; *Chapter 3*).

T4 UGT activity. Hepatic T4 UGT activity was determined essentially according to Beetstra *et al.* (1991). Microsomes (1 mg/ml) were incubated for 30 min at 37°C with 1 µM (50,000 cpm) [^{125}I]T4 in the presence or absence (blank) of 5 mM UDPGA in 200 µl 75 mM Tris-HCl (pH 7.8), containing 3.75 mM $MgCl_2$ and 0.1 mM PTU. Reactions were stopped by the addition of 0.2 ml ice-cold methanol. After centrifugation, 0.2 ml supernatant was mixed with 0.75 ml 0.1 M HCl and analyzed for glucuronide formation on Sephadex LH-20 minicolumns (Beetstra *et al.*, 1991).

D1 activity. Hepatic D1 activity was determined as previously described (Mol and Visser, 1985). Shortly, microsomes (20 µg protein/ml) were incubated for 30 min at 37°C with 1 µM (100,000 cpm) [^{125}I]rT3 in 0.1 M phosphate buffer (pH 7.2), containing 2 mM EDTA and 5 mM DTT. The reaction was stopped on ice by the addition of 750 µl 0.1 M HCl. The tubes were centrifuged and radioiodide was determined in the supernatant by Sephadex LH-20 chromatography as described before (Rutgers *et al.*, 1989).

D2 activity. Brain D2 activity was measured essentially as described by Visser *et al.* (1982) with slight modifications as described in Morse *et al.* (1993). The final incubation conditions were 100 mM phosphate buffer (pH 7.2), 25 mM DTT, 1 mM PTU, 1 mM EDTA, 2 nM (50,000 cpm) [^{125}I]T4, 0.5 µM T3 and brain homogenate (0.8 mg protein/ml) in a total volume of 200 µl. Incubations were carried out at 37°C for 60 min. The reaction was stopped on ice by the addition of 100 µl BSA (70 mg/ml), followed by 500 µl trichloroacetic acid (10% w/v). The tubes were then centrifuged and the radioiodide released was further isolated from the supernatant by LH-20 chromatography.

SULT activity. Iodothyronine SULT activity was determined using 3,[3'-^{125}I]T2 as described by Kaptein *et al.* (1997). In short, cytosol was incubated for 30 min at 37 °C with 1 µM (80,000 cpm) 3,[3'-^{125}I]T2 and 50 µM PAPS in 200 µl 75 mM Tris-HCl (pH 7.2) and 1.5 mM EDTA. The reaction was stopped on ice by addition of 750 µl 0.1 M HCl. The labelled 3,3'-T2S generated was isolated on Sephadex LH-20 minicolumns (Kaptein *et al.*, 1997) and counted for radioactivity. The cytosolic protein concentrations were 12.5, 25 and 50 µg/ml for maternal liver, 100 µg/ml for fetal liver, 50 µg/ml for maternal brain, and 100 µg/ml for fetal brain.

Statistics

Treatment-related effects were evaluated by one-way analysis of variance followed by a least significant difference test using the SPSS statistical software package. Data are presented as the means ± SEM.

Results

Very similar data were obtained in untreated and corn oil (vehicle)-injected controls. In the following section, findings in animals exposed to Aroclor 1254 or PCP are compared with those obtained in corn oil-treated animals.

Body and organ weights

All body and organ weights of dams on GD20 are presented in Table 8.1. Maternal body weights were decreased significantly by about 10% after treatment with PCP, but no change was observed with Aroclor 1254. Treatment of dams with Aroclor 1254 resulted in significantly higher absolute and relative liver weights. PCP treatment caused only an increase in relative liver weights in dams. Absolute, but not relative, brain weights were decreased in PCP-treated dams. Placenta weight was decreased by both Aroclor 1254 and PCP.

Treatment-related effects were also found on body and organ weights of fetuses (Table 8.2). Body weights of fetuses were significantly decreased by 22% after maternal exposure to PCP, but not after Aroclor 1254 treatment. PCP treatment also caused a significant decrease in absolute brain weights and absolute liver weights, a significant increase in relative brain weights, but had no statistically significant effect on relative liver weights. Exposure of dams to Aroclor 1254 on the other hand resulted in an increase in relative liver weights in the fetuses.

Effects of treatment of dams from GD10 until GD18 with Aroclor 1254 or PCP on the progeny are shown in Table 8.3. The number of implantations and resorptions were not significantly affected by either Aroclor 1254 or PCP treatment. One of nine PCP-treated dams was found to have total (12) resorptions. No visible malformations were detected in the fetuses at autopsy.

Serum and tissue PCP, PCB and 4-OH-PeCB levels

In order to have an estimation of the concentration of compound transferred from the dam to the fetus, GC analyses were performed on single serum and pooled tissue samples. For the Aroclor 1254-treated rats, data were already obtained in an earlier study performed under almost the same conditions. Therefore, only serum samples were analysed to confirm the previous findings (Morse *et al.*, 1996). As in the previous study, 4-OH-2,3,5,3',4'-pentachlorobipheynyl (4-OH-PeCB) was the dominating metabolite and the levels were 1155 and 800 ng/g fresh weight in fetal and maternal serum, respectively. The concentration of 4-OH-PeCB was higher in fetal serum compared with maternal serum, which confirms the earlier finding of high placental transfer of these phenolic metabolites of PCBs (Morse *et al.*, 1996).

In PCP-treated dams, high concentrations of PCP were found in serum (7100 ng/g fresh weight), liver (221 ng/g lipid) and brain (11 ng/g lipid). The liver PCP concentration in fetuses (210 ng/g lipid) was almost equal to that in the treated dam, indicating considerable

transplacental transfer. The PCP concentrations in fetal brain even exceeded the maternal brain levels by almost 4-fold (40 ng/g lipid).

Serum thyroid hormones

Maternal serum levels. Serum thyroid hormone and T4S concentrations are presented in Figures 8.1-8.3. Maternal TT4 serum levels were significantly decreased after treatment with Aroclor 1254 and PCP by 73% and 30%, respectively (Figure 8.1A). Serum FT4 levels in dams were also significantly decreased by 29% after exposure to Aroclor 1254 (Figure 8.1B). PCP exposure, however, caused a significant increase in FT4 levels by 30%. Maternal TT3 levels (Figure 8.2A) were not changed by either Aroclor 1254 or PCP. Serum levels of rT3 in dams were significantly decreased by 52% after exposure to Aroclor 1254 (Figure 8.2B). Treatment with PCP, however, resulted in a large increase in rT3 serum levels to 326% of controls. Serum levels of T4S were very low in control dams, and even somewhat lower, although not significantly, in Aroclor 1254-treated animals (Figure 8.3). In PCP-treated dams the T4S levels were slightly but not significantly higher than in control dams, whereas the T4S/FT4 ratio was not changed.

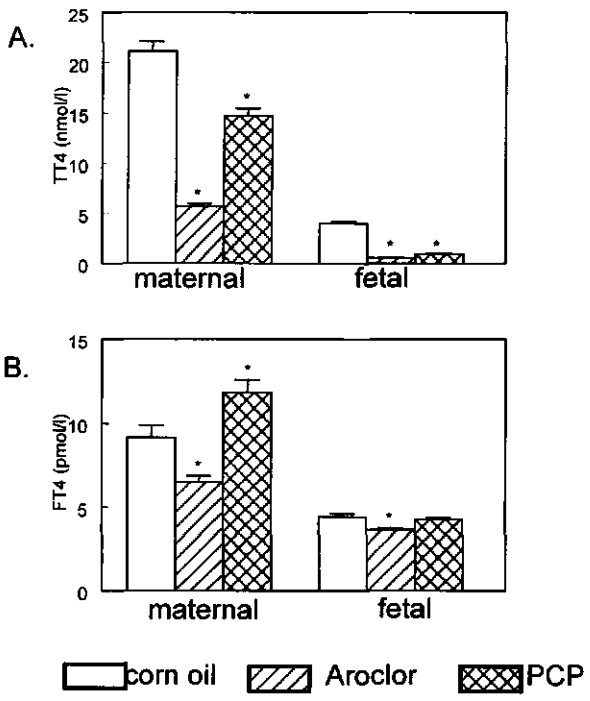

Figure 8.1 Serum total T4 (A) and free T4 (B) levels in dams and fetuses following prenatal exposure to Aroclor 1254 or PCP from GD10-18. Results are presented as mean ± SEM. *) denotes a significant difference ($p<0.05$) with the corn oil (vehicle control) group.

Table 8.1 Body weights and organ weights of dams after prenatal exposure to Aroclor 1254 or PCP from day 10 to 18 of gestation.

	BW (kg)	liver weight (g)	brain weight (g)	relative liver weight (% of BW)	relative brain weight (% of BW)	placenta (g)
Untreated control (N=7)	316.3 ± 8.8	10.45 ± 0.47	1.74 ± 0.01	3.30 ± 0.10	0.55 ± 0.01	0.54 ± 0.03
Corn oil (N=9)	324.1 ± 8.8	10.49 ± 0.49	1.75 ± 0.01	3.22 ± 0.09	0.54 ± 0.02	0.50 ± 0.02
Aroclor (N=9)	313.1 ± 5.7	12.18 ± 0.29 #*	1.68 ± 0.04	3.89 ± 0.08 #*	0.54 ± 0.01	0.44 ± 0.03 *
PCP (N=9)	293.3 ± 4.7 #*	11.32 ± 0.24	1.66 ± 0.03 #	3.86 ± 0.09 #*	0.57 ± 0.02	0.37 ± 0.01 #*

Note. Results are presented as mean ± SEM. *) denotes a significant difference ($p<0.05$) with the untreated control group, #) denotes a significant difference ($p<0.05$) with the corn oil group.

Table 8.2 Mean body weights and organ weights of fetuses after prenatal exposure to Aroclor 1254 or PCP from day 10 to 18 of gestation.

	mean BW (g)	liver weight (g)	brain weight (g)	relative liver weight (% of BW)	relative brain weight (% of BW)
Untreated control (N=7)	3.4 ± 0.2	0.24 ± 0.01	0.15 ± 0.03	7.1 ± 0.3	4.6 ± 0.2
Corn oil (N=9)	3.3 ± 0.1	0.24 ± 0.01	0.15 ± 0.03	7.3 ± 0.2	4.7 ± 0.1
Aroclor (N=9)	3.3 ± 0.1	0.26 ± 0.01	0.15 ± 0.03	8.1 ± 0.2*#	4.7 ± 0.1
PCP (N=8/9)	2.6 ± 0.1 #*	0.20 ± 0.01 *#	0.13 ± 0.02 #*	7.5 ± 0.2	5.1 ± 0.1 #*

Note. Results are presented as mean ± SEM. *) denotes a significant difference ($p<0.05$) with the untreated control group, #) denotes a significant difference ($p<0.05$) with the corn oil group.

Table 8.3 Effects on reproductive outcome after prenatal exposure to Aroclor 1254 or PCP from day 10 to 18 of gestation.

	nest size	alive fetuses (no. per nest)	dead fetuses (no. per nest)
Untreated control (N=7)	10.9 ± 1.1	9.9 ± 1.6	1.0 ± 1.0
Corn oil (N=9)	12.7 ± 0.4 *	12.4 ± 0.4	0.2 ± 0.2
Aroclor (N=9)	12.0 ± 0.4	11.6 ± 0.5	0.4 ± 0.2
PCP (N=8)	12.1 ± 0.5	10.9 ± 1.1	2.4 ± 1.6

Note. Results are presented as mean ± SEM. *) denotes a significant difference ($p<0.05$) from the untreated control group.

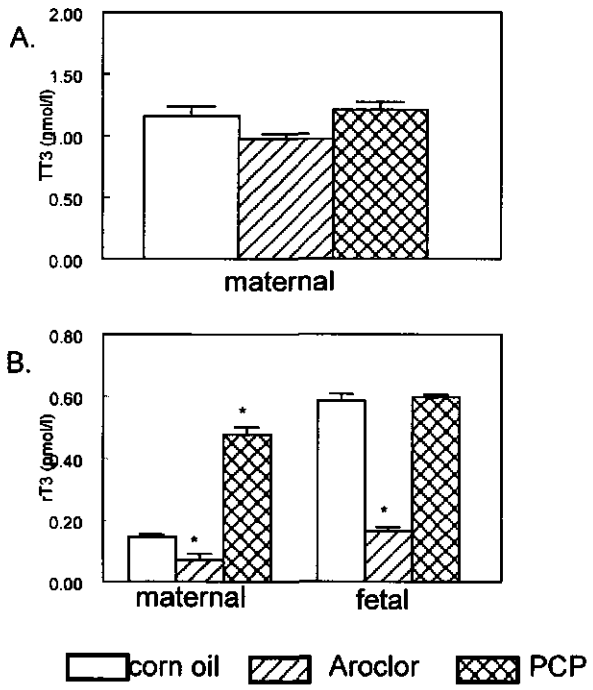

Figure 8.2 Serum total T3 (A) and rT3 (B) levels in dams and fetuses following prenatal exposure to Aroclor 1254 or PCP from GD10 to 18. Results are presented as mean ± SEM. *) denotes a significant difference ($p<0.05$) with the corn oil (vehicle control) group.

Fetal serum levels. Fetal serum TT4 levels were dramatically decreased by treatment with Aroclor 1254 and PCP to 15% and 22% of control, respectively (Figure 8.1A). Fetal FT4 serum levels were decreased by 17% after exposure to Aroclor 1254, whereas PCP treatment had no effect (Figure 8.1B). T3 levels in fetal serum were undetectable (<0.15 nmol/l). Serum rT3 levels were reduced to 28% of control after exposure to Aroclor 1254, whereas PCP treatment had no effect (Figure 8.2B). Serum T4S levels were very low in control fetuses and unaffected by treatment with Aroclor 1254 or PCP (Figure 8.3). T3S levels were found to be even lower than T4S levels in pooled serum samples from untreated fetuses (not shown).

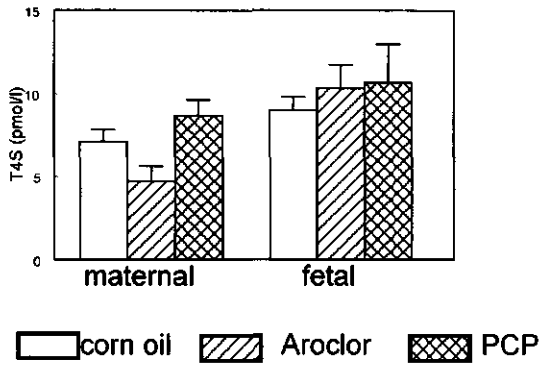

Figure 8.3 Serum T4S levels in dams and fetuses following prenatal exposure to Aroclor 1254 or PCP from GD10 to 18. Results are presented as mean ± SEM. *) denotes a significant difference ($p<0.05$) with the corn oil (vehicle control) group.

Enzyme activities

Hepatic EROD and T4 UGT activities. Maternal exposure to Aroclor 1254 resulted in an 200-fold induction of EROD activity in maternal liver and a 5-fold induction in fetal liver (Figure 8.4A). PCP treatment did not affect maternal or fetal EROD activity.

T4 UGT activity in maternal liver was induced about 4 times by Aroclor 1254 but T4 UGT activity in fetal liver was not affected (Figure 8.4B). PCP treatment did not affect the T4 UGT activity in maternal or fetal liver.

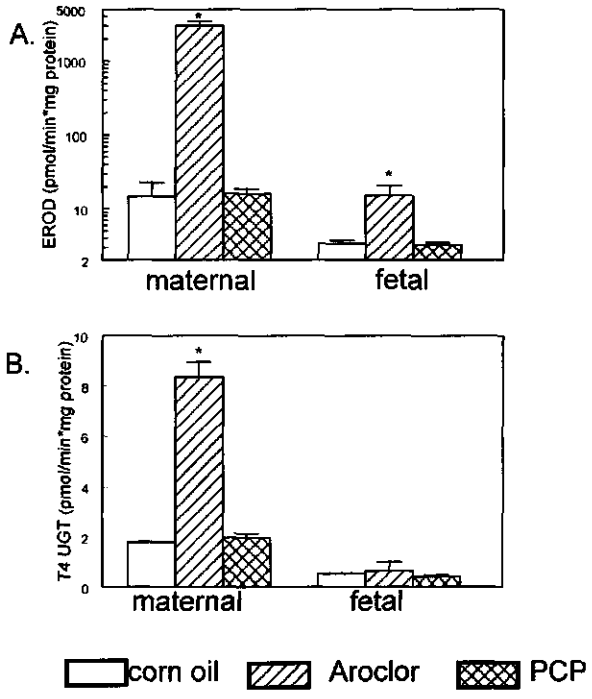

Figure 8.4 Hepatic EROD (A) and T4 UGT (B) activities in dams and fetuses following prenatal exposure to Aroclor 1254 or PCP from GD10 to 18. Data are presented as mean ± SEM. *) denotes a significant difference ($p<0.05$) with the corn oil (vehicle control) group.

Deiodinase activities. Maternal hepatic D1 activity was significantly decreased by 56% after Aroclor 1254 treatment (Figure 8.5A). Exposure to PCP caused a slight but significant decrease in D1 activity in maternal liver. D1 activity was also decreased in fetal liver after maternal exposure to Aroclor 1254 or PCP.

Maternal brain D2 activity was slightly (but not significant) increased in Aroclor 1254-exposed rats (Figure 8.5B). PCP treatment, however, resulted in a significant 58% decrease in maternal D2 activity. In fetal brain, Aroclor 1254 treatment caused a significant 62% increase, whereas PCP exposure resulted in a slight and non-significant decrease in D2 activity.

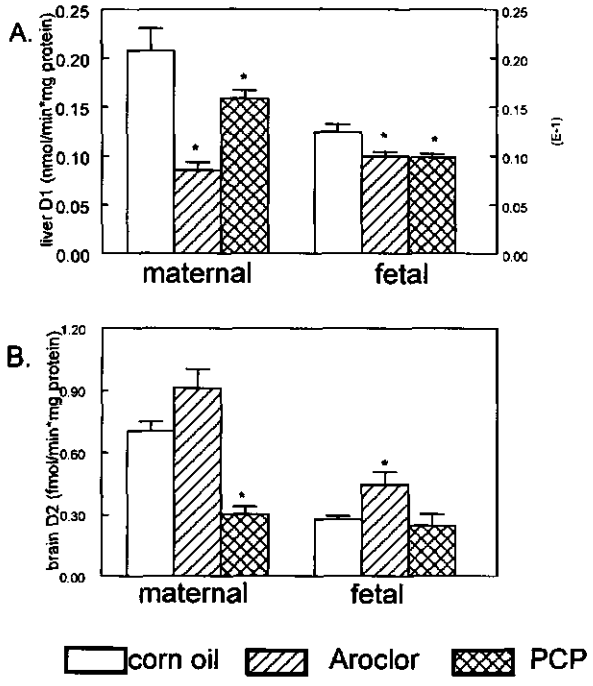

Figure 8.5 Hepatic D1 activity (A) and brain D2 activity (B) in dams and fetuses following prenatal exposure to Aroclor 1254 or PCP from GD10 to 18. Results are presented as mean ± SEM. *) denotes a significant difference ($p<0.05$) with the corn oil group.

SULT activities. Figure 8.6 shows the 3,3'-T2 SULT activities in fetal and maternal liver. In maternal liver, treatment with the SULT inhibitor PCP caused a significant increase in 3,3'-T2 SULT activity, whereas Aroclor 1254 had no effect. In fetal liver, PCP treatment resulted in lower 3,3'-T2 SULT activities, whereas Aroclor 1254 treatment showed no effect. In maternal brain, 3,3'-T2 SULT activity was higher after PCP treatment and unchanged after Aroclor 1254 treatment. SULT activities in fetal brain were not affected by Aroclor 1254 or PCP exposure.

Figure 8.7A shows the 3,3'-T2 SULT activity in pooled (equally from all samples) fetal liver cytosol (final protein concentration 100 μg/ml) as a function of the substrate concentration (0.1-10 μM). Exposure to PCP or Aroclor 1254 resulted in lower SULT activities at most 3,3'-T2 concentrations used. Figure 8.7B shows the 3,3'-T2 SULT activities in maternal liver as a function of the cytosolic protein concentration (12.5-50 μg/ml). In control liver cytosol, the 3,3'-T2 sulfation rate decreases on dilution of the protein concentration. However, in liver from PCP-exposed dams, 3,3'-T2 sulfation did not decrease with the cytosol concentration, suggesting that the effect of the competitive inhibitor PCP is diluted out, and PCP treatment may have resulted in an increased enzyme concentration.

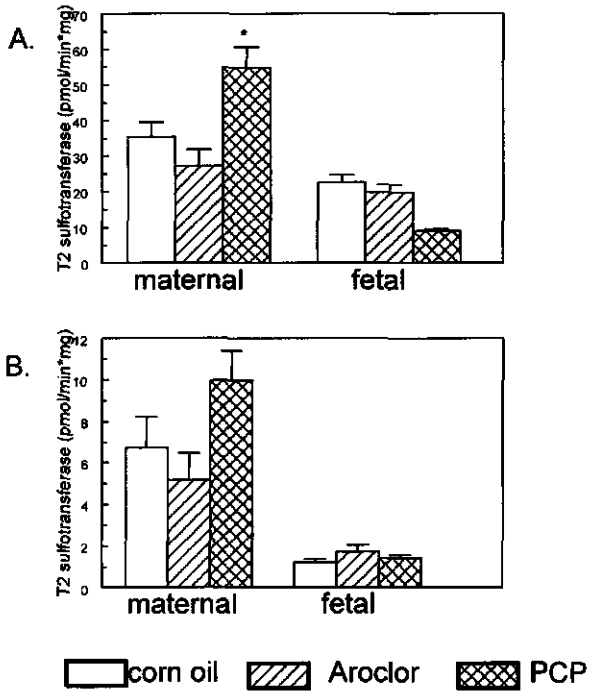

Figure 8.6 Hepatic (A) and brain (B) 3,3'-T2 SULT activity in dams and fetuses following prenatal exposure to Aroclor 1254 or PCP from GD10 to 18. The cytosolic protein concentrations were 25 µg/ml for maternal liver, 100 µg/ml for fetal liver, maternal brain, and fetal brain. Results are presented as mean ± SEM. *) denotes a significant difference ($p<0.05$) with the corn oil group.

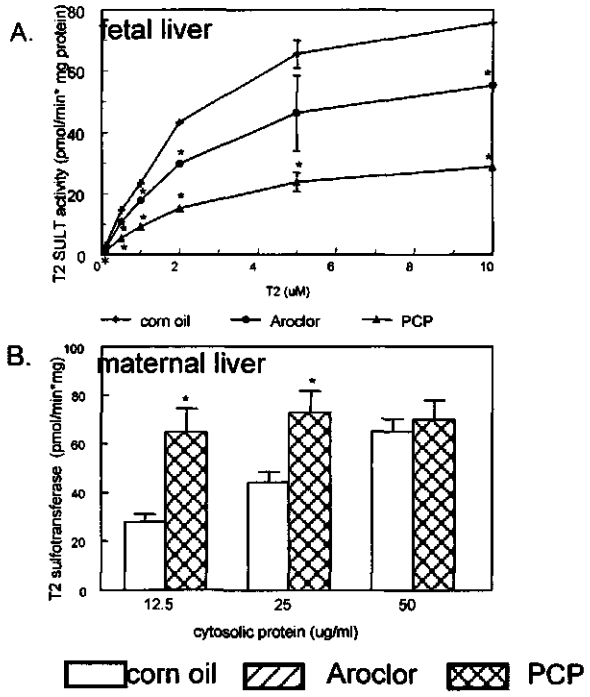

Figure 8.7 Hepatic 3,3'-T2 SULT activity in fetuses (A) and dams (B) following prenatal exposure to PCP or Aroclor 1254 from GD10 to 18. Activities were measured in duplicate using pooled fetal liver samples (100 µg protein/ml) and a concentration series of the 3,3'-T2 substrate (A). Maternal liver cytosol samples (N=8-9) were used in different cytosolic protein concentrations and 1 µM 3,3'-T2. Results are presented as means ± SEM. *) denotes a significant difference ($p<0.05$) with the corn oil group.

Discussion

Sulfation is an important metabolic pathway for the elimination of thyroid hormone in the adult rat. However in fetal rats, sulfation is suggested to play an important role in the regulation of T4 and T3 levels, which may be recovered by desulfation of T4S and T3S by sulfatases (Santini *et al.*, 1992a; Spaulding *et al.*, 1992). Recently, we have demonstrated that hydroxylated metabolites of PCBs as well as PCP are potent inhibitors of 3,3'-T2 sulfation *in vitro* (Schuur *et al.*, 1998b; *Chapter 5*). Moreover, perinatal exposure to PCBs results in neurodevelopmental changes in the offspring (reviewed by Tilson *et al.*, 1990; Brouwer *et al.*, 1995; Jacobson and Jacobson, 1997). Taken together, the possible inhibition of thyroid hormone sulfation may be a probable mechanism for PCBs to disrupt thyroid hormone.

Thus, the major aim of this study was to investigate the potential impact of prenatal exposure to Aroclor 1254 or PCP on the thyroid hormone metabolism of both dams and fetuses, with special emphasis on the sulfation of thyroid hormone. This discussion is divided into 3 parts, starting with some general aspects of the animal experiment, followed by a summary of effects of PCBs on thyroid hormone metabolism and levels. Finally, the effect of the well-know phenol sulfation inhibitor PCP on *in vivo* thyroid hormone sulfation is discussed, and compared to the effect of Aroclor treatment. Finally, the discussion is concluded with some remarks on the importance of these effects.

Overall, no gross toxic effects were observed neither in dams nor in fetuses following Aroclor 1254 exposure. PCP treatment produced some mild toxic effects, such as a slightly reduced maternal body weight and brain weight, and a non-statistically significant increase in fetal death. Induction of liver weight was observed in dams both after Aroclor 1254 and PCP treatment. EROD activity, a well-known marker of Ah receptor-mediated responses (Safe, 1994) was highly induced in maternal and fetal liver, whereas no effects were observed with PCP. The induction of EROD activity in fetal liver after maternal exposure to PCBs has been reported before (Morse *et al.*, 1996).

Our findings indicate a high placental transfer of both PCP and 4-OH-PeCB. The PCP concentration in fetal liver was almost equal to that in maternal liver. More striking is the 4-fold higher concentration of PCP in fetal brain compared with maternal brain. 4-OH-PeCB has previously been identified as one of the major metabolites retained in blood after exposure to Aroclor 1254 and was also found to accumulate in fetal brain tissue (Morse *et al.*, 1996). The higher 4-OH-PeCB concentration in fetal than in maternal serum is also in keeping with the latter study. Other reports have also demonstrated that hydroxylated PCB metabolites accumulate in the fetal rat after maternal exposure to 4-chlorobiphenyl (Lucier *et al.*, 1978) or 3,3',4,4'-tetrachlorobiphenyl (Darnerud *et al*, 1996; Morse *et al.*, 1995).

Thyroid hormone metabolism and levels are affected by treatment with PCBs in different ways as shown in Figure 8.8. The reduction of maternal serum TT4 and FT4 levels by Aroclor 1254 treatment are in agreement with previous studies (Bastomsky, 1974; Gray *et al.*, 1993; Byrne *et al.*, 1987; Brouwer, 1989; Beetstra *et al.*, 1991). These changes are most likely caused by an increased hepatic T4 glucuronidation as well as competitive inhibition of T4 binding to TTR by hydroxylated PCB metabolites (Rickenbacher *et al.*, 1986; Lans *et al.*, 1993; 1994: Lans, 1995). The reduction of fetal serum TT4 by Aroclor 1254 treatment is also in line with earlier observations by Morse *et al.* (1996). High serum levels of the hydroxylated PCB metabolite 4-OH-PeCB were detected in this and the previous study (Morse *et al.*, 1996). Decreased maternal supply of T4, as well as competitive inhibition of T4 binding to fetal TTR by 4-OH-PeCB, may contribute to the low fetal serum T4 levels. Fetal hepatic T4 glucuronidation was not induced in Aroclor 1254-exposed animals.

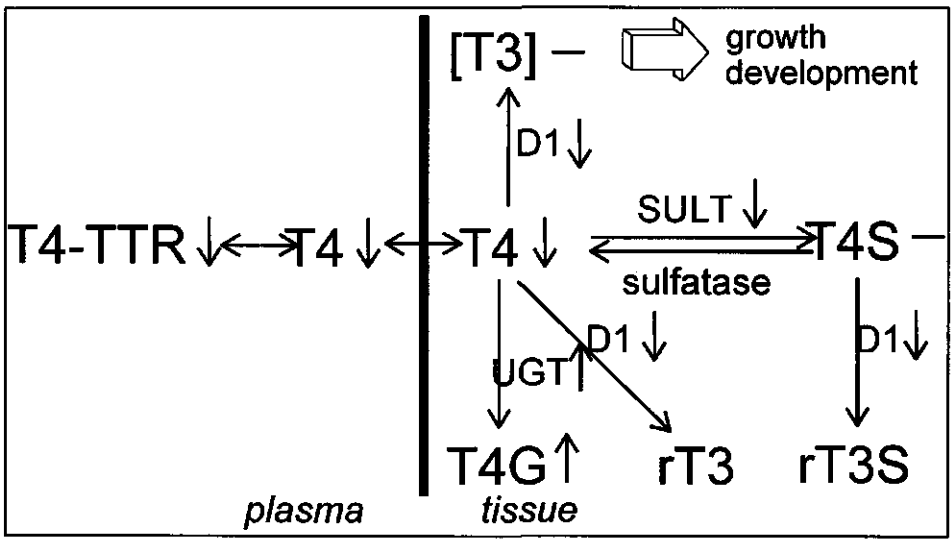

Figure 8.8 Overview of the interaction sites of PHAHs and their metabolites with the thyroid hormone metabolism. The vertical arrows indicate the effects of PHAHs on the serum levels or hepatic enzyme activities.

PCP treatment also decreased T4 levels in damsand fetuses, probably due to inhibition of T4 binding to TTR (Van den Berg *et al.*, 1991; Den Besten *et al.*, 1991). This is supported by the even higher FT4 levels in maternal serum after exposure to PCP. T4 glucuronidation was not changed by PCP in both dams and fetuses and, therefore, does not play a role in decreasing serum T4 levels. PCP is probably transported by TTR to the fetal compartment, where it also

serum T4 levels. PCP is probably transported by TTR to the fetal compartment, where it also causes a decrease in TT4 without changing the serum FT4. Jekat *et al.* (1994) reported on decreased serum T4 and T3, accompanied by decreased FT4 and FT3 levels. TSH was unexpectedly decreased, suggesting an interference of PCP at pituitary or hypothalamic levels, which was supported by reduced intrathyroidal hormone stores.

The decreases in hepatic D1 activity in both dams and fetuses by Aroclor 1254 or PCP treatment may be due to direct inhibition of D1 by hydroxylated PCB metabolites (Adams *et al.*, 1990; Rickenbacher *et al.*, 1989; Lans, 1995) and PCP. Hepatic D1 activity has also been shown to be reduced after treatment with 3,3',4,4'-tetrachlorobiphenyl (Adams *et al.*, 1990; Visser *et al.*, 1993a) or with the Ah-receptor agonists 3-methylcholanthrene and TCDD (Visser *et al.*, 1993; Raasmaja *et al.*, 1996). However, other studies involving treatment with Aroclor 1254 (Lans, 1995) or TCDD (Schuur *et al.*, 1997; *Chapter 2*) have shown no effect on hepatic D1 activity in adult rats. Treatment of adult male rats with hexachlorobenzene, resulting in high PCP levels, also decreases hepatic D1 activity (Van Raaij *et al.*, 1993). In addition to the inhibition of D1 activity by the administered PHAHs or their metabolites, D1 expression may also be decreased by the hypothyroid state possibly induced by these chemicals (Visser *et al.*, 1993a).

Reduction of D1 activity usually results in increased serum rT3 levels (e.g. Eelkman Rooda *et al.*, 1989). Indeed, PCP treatment resulted in highly increased maternal serum rT3 levels, which may be explained by the decrease in hepatic D1 activity in combination with the increased FT4 (precursor) concentration. However, Aroclor 1254 treatment resulted in decreased rT3 concentrations in both fetal and maternal serum despite the decrease in hepatic D1 activity, which may in part be due to the decrease in FT4 levels.

Brain D2 activity is under negative control of serum T4 which is thought to be an adaptative response to maintain T3 levels in brain constant when thyroid function changes (Silva and Matthews, 1984; Ruiz de Ona *et al.*, 1988; Obregon *et al.*, 1991). D2 activity in maternal and fetal brain was significantly increased after treatment with Aroclor 1254, which is explained by the decreased serum FT4 levels. Such increased brain D2 activities were reported before in adult rats after treatment with TCDD (Lans, 1995) and in fetal and neonatal rats after treatment with 3,3',4,4',5,5'-hexachlorobiphenyl (Morse *et al.*, 1993) and Aroclor 1254 (Morse *et al.*, 1996). In fact, despite the dramatic reduction in brain T4 levels, the T3 levels in fetal brain remained unaffected, supporting an increased conversion of T4 into T3 by D2 (Morse *et al.*, 1996). PCP treatment significantly reduced D2 activity in maternal brain in agreement with the increase in serum FT4 concentration.

The well-known sulfation inhibitor PCP was included in this animal experiment as a positive control. Remarkably, PCP treatment resulted in significantly higher 3,3'-T2 SULT activities in maternal liver at low but not at high cytosol concentrations. The results suggest that PCP

not only competitively inhibits 3,3'-T2 SULT activity but also increases the hepatic expression of 3,3'-T2 SULT(s), probably indirectly. Using almost similar concentrations of PCP compared with our study, Meerman *et al.* (1983) were able to lower the amount of harmol sulfates formed, using *ex vivo* techniques. Singer *et al.* (1984) reported on increased hepatic sulfotransferase activity after treatment with PCP. Extrapolating our results obtained with 1 μM 3,3'-T2 and different cytosolic protein concentrations to the *in situ* conditions with undiluted cytosol, these opposing effects may result in a net reduction in iodothyronine sulfation in the liver of PCP-treated dams *in vivo*. In the fetal liver, PCP treatment caused a clear inhibition of the 3,3'-T2 SULT activity, demonstrated best in the pooled samples using a range of substrate concentrations. The different effects of PCP treatment on 3,3'-T2 SULT activity in maternal and fetal liver is in agreement with the higher cytosolic protein concentration (and thus higher PCP concentration) required to assay fetal liver SULT activity. This is in agreement with the concentration of PCP detected in fetal and maternal liver, which was about the same per ng lipid,with lower lipid levels in fetal liver. In fetal brain, no effect of PCP on 3,3'-T2 SULT activity was shown under the *in vitro* assay conditions used.

Aroclor 1254 treatment did not affect 3,3'-T2 SULT activity in maternal liver and brain. In the fetal liver, Aroclor 1254 treatment caused a clear inhibition of the 3,3'-T2 SULT activity, demonstrated best in the pooled samples using a range of substrate concentrations. In fetal brain, no effect of Aroclor 1254 on 3,3'-T2 SULT activity was shown under the *in vitro* assay conditions used.

Inhibition of fetal hepatic iodothyronine SULT activity by maternal exposure to Aroclor 1254 or PCP did not result in a decrease in fetal serum T4S levels, even though serum FT4 was also decreased in Aroclor 1254-treated animals. A possible explanation for this is that the clearance of T4S is simultaneously decreased by inhibition of fetal hepatic D1 activity. Although the regulation of thyroid hormone bioactivity is directly affected by the reversible sulfation/desulfation of T3, we did not attempt to analyze the effects of SULT inhibitors on fetal serum T3S, since we found that T3S levels in untreated fetal rats are even lower than T4S levels.

These low iodothyronine sulfate levels are in contrast to the high levels found in fetal human and sheep serum (Wu *et al.*, 1992a; 1992b; 1993a; 1993b; Santini *et al.*, 1993). They cannot be explained by low hepatic iodothyronine SULT activity or high hepatic D1 activity, which catalyzes the breakdown of T4S and T3S, in the 20-day old rat fetus. In our study and that of Hurd *et al.* (1993), it was shown that fetal hepatic 3,3'-T2 or T3 SULT activities are about half the activities in maternal liver on GD20. Since SULT1C1 is only expressed in adult male liver (Dunn and Klaassen, 1996; Liu and Klaassen, 1996), the isoenzyme responsible for iodothyronine sulfation in fetal liver may be SULT1B1, but this remains to be demonstrated. Iodothyronine SULT activity in fetal brain is 6-10 times lower than in maternal brain (Hurd

et al., 1993; this study). Fetal hepatic D1 activity has been reported to be very low during fetal life, starting to increase only at GD20 (Harris *et al.*, 1978; Ruiz de Ona *et al.*, 1991). We indeed found that D1 activity on GD20 was still 20 times lower in fetal than in maternal liver.

Sulfation of iodothyronines in adult male rat liver is catalyzed by both SULT1B1 and SULT1C1 isoenzymes (Yamazoe *et al.*, 1994; Sakakibara *et al.*, 1995; Visser *et al.*, 1998a). SULT1B1 is equally expressed in male and female liver (Yamazoe *et al.*, 1994; Sakakibara *et al.*, 1995), whereas SULT1C1 is not expressed in female liver (Nagata *et al.*, 1993; Dunn and Klaassen, 1996; Liu and Klaassen, 1996). Therefore, SULT1B1 is the only isoenzyme known to be involved in the sulfation of iodothyronines in female liver. Neither SULT1B1 nor SULT1C1 is expressed in rat brain (Sakakibara *et al.*, 1995; Dunn and Klaassen, 1996). Therefore, the isoenzyme(s) responsible for thyroid hormone sulfation in rat brain is (are) unknown. We previously showed similar patterns for the inhibition of 3,3'-T2 sulfation by four hydroxylated PCB metabolites in male rat liver cytosol, female rat liver cytosol and recombinant rat SULT1C1 (Schuur *et al.*, 1998c; *Chapter 6*). However, it was demonstrated that 3-hydroxy-2,3',4,4',5-pentachlorobiphenyl was much less potent in inhibiting 3,3'-T2 SULT activity in rat brain than in male or female rat liver cytosol or SULT1C1. These results are explained by the above findings that the SULT isoenzyme(s) involved in the sulfation of thyroid hormone in brain differ(s) from those (SULT1B1 and SULT1C1) catalyzing the sulfation of iodothyronines in rat liver.

In conclusion, perinatal exposure to PCP resulted in placental transfer of PCP to fetal rat tissues. Thyroid hormone levels and metabolism were affected after PCP exposure, including decreased FT4 and TT4 levels in dams and fetuses, decreased fetal and maternal D1 activity, and decreased maternal D2 activity. The effect of PCP and Aroclor 1254 on thyroid hormone sulfation is not unequivocal, and may be dependent on the *in situ* balance between the concentrations of substrate thyroid hormone, the enzyme sulfotransferase and the competitive inhibitor PCP/PCB-OH. The prenatal administration of PCP or Aroclor 1254 in this study did not result in a decrease in fetal serum T4S levels, perhaps because degradation of T4S by hepatic D1 is simultaneously decreased. Moreover, in fetal rats, sulfation seems to play a less important role in the metabolism of thyroid hormone than in fetal humans or sheep, considering the low T4S levels in fetal serum.

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CHAPTER 9

SUMMARY AND CONCLUDING REMARKS

This thesis deals with the possible interactions of polyhalogenated aromatic hydrocarbons and/or their metabolites with thyroid hormone metabolism. This chapter summarizes firstly the effects of thyroid hormone on the induction of biotransformation enzymes by PHAHs. Secondly, the results on the inhibition of thyroid hormone sulfation by hydroxylated metabolites of PHAH are summarized. Some conclusions and remarks on the overall implications of the results are given at the end of this chapter.

The effects of thyroid hormone on the induction of biotransformation enzymes by polyhalogenated aromatic hydrocarbons

The first part of this thesis focussed on the question whether or not the PHAH-induced decrease of plasma T4 is an adaptive endocrine response of the animal to cope with the onset of toxic effects by PHAHs. For this purpose, the possible regulatory effect of thyroid hormones on biotransformation enzymes was investigated, using rats differing in thyroid state which were exposed to TCDD or PCBs as model inducers of biotransformation enzymes.

In *Chapter 2*, the thyroid state of euthyroid (Eu), thyroidectomized (Tx) and Tx rats in which T3 or T4 levels are restored using osmotic minipumps were compared. The decreased circulatory levels of plasma T4 and T3, the increased pituitary feedback response (plasma TSH levels), as well as changed functional responses (decreased hepatic D1 and malic enzyme activities, and increased brain D2 activities) in Tx rats were largely restored to Eu levels in Tx+T4 rats and, except for plasma TT4 and brain D2 activity, in Tx+T3 rats. These results indicated that the thyroid hormone-replaced Tx rats were valid models to study peripheral effects of TCDD. Three days after exposure to 10 μg TCDD/kg body weight, plasma TT4 and FT4 levels were significantly reduced in Eu rats and in Tx+T4 rats, and plasma T3 was significantly reduced in Tx+T3 but not in Eu or Tx+T4 rats. Hepatic T4 UGT activity was induced by TCDD while T3 UGT activity was only slightly increased in the different exposed groups. These results strongly suggest that the thyroid hormone-decreasing effects of TCDD are predominantly extrathyroidal and mediated by the marked induction of hepatic T4 UGT activity.

The effects of thyroid state modulation on the induction of detoxification enzymes by TCDD in experimental animals are described in *Chapter 3*. In all rats, TCDD largely induced CYP1A1/1A2 activity (EROD), CYP1A1 protein content, and CYP1A1 mRNA levels.

TCDD exposure also resulted in higher total hepatic cytochrome P450 content, hepatic p-nitrophenol UGT activity, and GST 1-1 protein levels, but had no effect on hepatic NADPH cytochrome P450 reductase activity, overall GST activity and GST 2-2, 3-3, and 4-4 protein levels and iodothyronine sulfotransferase activity. Thyroid state did not affect the total cytochrome P450, and GST activity and protein levels, but slightly decreased CYP1A1/2 activity, NADPH cytochrome P450 reductase activity, PNP UGT activity and iodothyronine sulfotransferase activity were demonstrated in Tx rats, as compared to Eu rats.

In the second animal experiment, the interaction between thyroid state and PCBs in the regulation of CYP1A1 and CYP2B expression is described (*Chapter 4*). Male Tx Sprague-Dawley rats, Eu rats, and rats made hyperthyroid by infusing T3 were treated with a single ip dose of the CYP2B inducer PCB 153 and the CYP1A inducer PCB 126. The thyroid states of the rats were confirmed by measurement of plasma T4, T3 and TSH and of functional parameters such as hepatic D1 activity, malic enzyme activity and α-glycerolphosphate dehydrogenase activity. Total hepatic cytochrome P450 content was increased by PCB treatment in all groups, but was not affected by thyroid state. NADPH cytochrome P450 reductase activity was decreased in Tx rats and increased in hyperthyroid rats, while PCB treatment had no effect. PCB 126 specifically induced T4 UGT activity, measured in the absence of detergent, and CYP1A activity, protein and mRNA levels, whereas PCB 153 induced T4 UGT activity, measured in the presence of the detergent Brij 56, and CYP2B activity, protein and mRNA levels. Thyroid state, neither hypo nor hyper, significantly affected T4 UGT activity or CYP1A and CYP2B activities, protein or mRNA levels.

The almost complete lack of response of basal and PCB- or TCDD-induced activities of biotransformation enzymes to changes in thyroid state observed in our studies is in contrast to effects published by others (Kato and Takahashi *et al.*, 1968; Rumbaugh *et al.*, 1978; Leakey *et al.*, 1982; Müller *et al.*, 1983a/b; Skett, 1987; Yamazoe *et al.*, 1989; Arlotto and Parkinson, 1989; Murayama et al., 1991; Chowdhury *et al.*, 1983; Moscioni and Gartner, 1983; Pennington *et al.*, 1988; Goudonnet *et al.*, 1990; Williams *et al.*, 1986; Pimental *et al.*, 1993). This may be due to differences in strain and sex of the animals, the severity and duration of the hypo- and hyperthyroid states induced as well as the duration and dose of TCDD/PCB treatment. Overall, it can be concluded that hepatic NADPH cytochrome P450 reductase activity is dependent on thyroid state, whereas total cytochrome P450 as well as CYP1A1 and CYP2B together with UGT, GST and sulfotransferase activities show little or no thyroid hormone dependence. These slight effects are unlikely to represent an endocrine adaptation to a chemical stressor (TCDD). Therefore, the PHAH-induced decreased T4 levels , as well as other aspects of PHAH-induced alterations in thyroid hormone metabolism, are most likely a direct reflection of the developing toxicological response of the animals toward PHAH exposure.

Inhibition of thyroid hormone sulfation by hydroxylated metabolites of polyhalogenated aromatic hydrocarbons.

The second part of this thesis focussed on the question whether or not hydroxylated metabolites of PHAHs (PHAH-OHs) are able to inhibit thyroid hormone sulfation *in vitro* as well as *in vivo*.

Chapter 5 presents the investigations concerning the possible inhibitory effects of PHAH-OHs on iodothyronine sulfotransferase (SULT) activity. Rat liver cytosol was used as a source of sulfotransferase in an *in vitro* assay with ^{125}I-labelled T2 as a model substrate. Hydroxylated metabolites of PCBs, PCDDs and PCDFs were found to be potent inhibitors of T2 SULT activity *in vitro* with IC50 values in the low micromolar range (0.2-3.8 μM). The most potent inhibitor of T2 SULT activity within our studies was the PCB metabolite 3-hydroxy-2,3',4,4',5-pentachlorobiphenyl with an IC50 value of 0.2 μM. A hydroxyl group in the para or meta position appeared to be an important structural requirement for T2 SULT inhibition by PCB metabolites. Ortho hydroxy PCBs were much less potent and none of the parent PHAHs were capable of inhibiting T2 SULT activity. In addition, the formation of T2 SULT-inhibiting metabolites from individual brominated diphenyl ethers and nitrofen as well as from some commercial PHAH mixtures (e.g. Bromkal, Clophen A50 and Aroclor 1254) by CYP450 catalyzed hydroxylation was also demonstrated.

Consequently, the inhibition of thyroid hormone sulfation by PHAH-OHs was studied in more detail, investigating isozyme specificity and inhibition kinetics (*Chapter 6*). The difference in inhibition pattern demonstrated for SULT activity present in rat liver and brain cytosol, is probably caused by a difference in isozyme pattern. It was shown that PCB-OHs inhibited T2 sulfation by interacting with the rat isozyme SULT1C1 and an additional isozyme responsible for T2 sulfation in female liver cytosol, probably rat SULT1B1, but not SULT1A1. On the other hand, human phenol SULT1A1 was inhibited by PCB-OHs, but not the human isozyme SULT1A3. In conclusion, we suggested that at least human SULT1A1, and rat SULT1C1 and perhaps rat SUL1B1 are involved in the inhibition of T2 sulfation by PCB-OHs. However, more information is needed about the various isozymes involved in iodothyronine sulfation in humans as well as in rats, before definite conclusions can be drawn.

Furthermore, it is shown that T2 is a good model substrate for the active hormone T3 when investigating the inhibition of thyroid hormone sulfation by hydroxylated metabolites of PHAHs. The inhibition kinetics strongly suggested that the nature of the T2 sulfation inhibition by PCB-OHs is competitive. To obtain more decisive information, tests with purified isozymes should be performed. It was also demonstrated that PCDD-OHs and PCB-OHs themselves are substrates -albeit poor- for SULT enzymes, which further supports the competitive inhibition of thyroid hormone sulfation by PHAH-OHs.

To bridge the gap between *in vitro* experiments using cytosol and the *in vivo* situation, we investigated the inhibition of thyroid hormone sulfation in hepatoma cell lines (*Chapter*

7). Two PCB-OHs, 4-hydroxy-2',3,3',4',5-pentachlorobiphenyl and 4-hydroxy-3,3',4',5-tetrachlorobiphenyl, together with the known sulfation inhibitor pentachlorophenol (PCP) were tested in the rat hepatoma cell line FaO and the human hepatoma cell line HepG2. PCP inhibited T2 sulfation *in vitro* in FaO and HepG2 cells, although it was 1000 times less potent in whole cells than in rat liver cytosol. Micromolar concentrations of the two tested PCB-OHs hardly affected T2 conjugation in FaO cells, but reduced T2 sulfate formation in HepG2 cells. Inhibition of T2 sulfation was more pronounced using medium without FCS than in medium with 5% FCS, due to a lower uptake of inhibitor by the cells in the presence of serum, as demonstrated using radiolabeled PCP.

These *in vitro* results indicate that hydroxylated PHAHs are potent inhibitors of thyroid hormone sulfation. Since thyroid hormone sulfation may play an important role in regulating "free" hormone levels in the fetus, and hydroxylated PCB metabolites are known to accumulate in fetal tissues after maternal exposure to PCBs, these observations *in vitro* might have implications for fetal thyroid hormone homeostasis and development.

The *in vivo* experiment in which was tested if PHAH-OHs are able to inhibit T2 sulfation, was described in *Chapter 8*. Pregnant rats were exposed to 25 mg Aroclor 1254/kg body weight or to the well-known phenol sulfation inhibitor PCP (25 mg/kg body weight) from day 10 till day 18 of gestation. Fetuses and dams were sacrificed on gestation day 20 (GD20). PCP and PCB metabolite levels in fetal serum and tissues were high. Aroclor 1254, but not PCP exposure resulted in an induction of hepatic EROD and T4 UGT activity in dams.

PHAHs are known for their disrupting effects on thyroid hormone metabolism, as shown in Figure 9.1. In this animal experiment, Aroclor 1254 exposure caused an increase in T4 UGT activity, resulting in decreased TT4 levels. Treatment with PCP also resulted in decreased serum TT4 levels, but increased FT4 levels, in dams and fetuses. The ratio FT4/TT4 was increased indicating a reduced plasma TTR binding capacity in fetuses and dams following both treatments. D1 activity in liver decreased in dams and fetuses after treatment with Aroclor 1254 and PCP. This decrease is probably caused indirectly by the lowered T4 levels. D2 activity in brain decreased by exposure to PCP in dams but no effect was found in fetuses, and increased by exposure to Aroclor 1254 in fetuses, with no effect in dams. The increasing D2 activity is a response of the brain to low T4 levels, to maintain the T3 homeostasis.

The positive control PCP was shown to increase the T2 SULT activity measured in maternal liver and brain cytosol. Studies using varying T2 concentrations and different protein concentrations suggested competitive inhibition of PCP carried over in the *in vitro* assay as well as true induction of T2 SULT activity. This effect of PCP on thyroid hormone sulfation

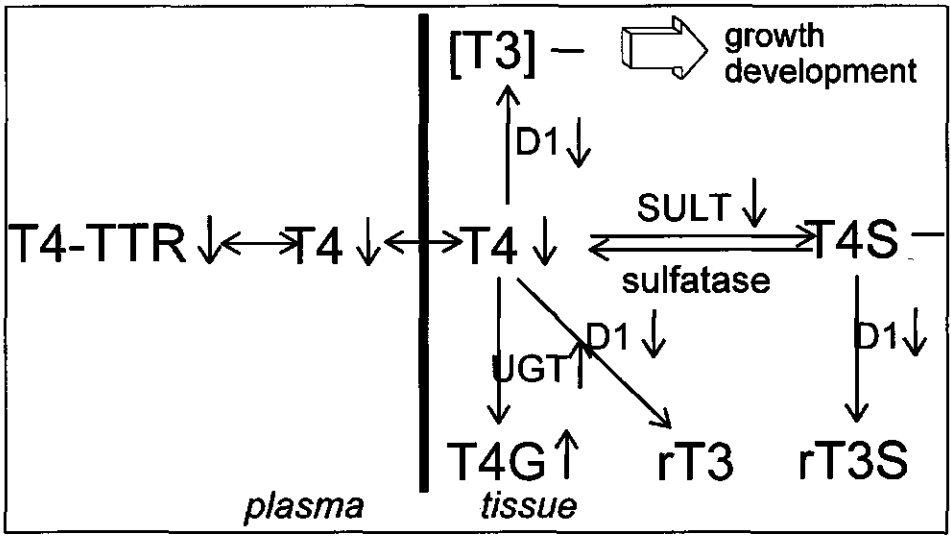

Figure 9.1 Overview of the interaction sites of PHAHs and their metabolites with the thyroid hormone metabolism. The vertical arrows indicate the effects of PHAHs on the serum levels or hepatic enzyme activities.

in vivo apparently did not result in lower levels of the product T4S, since fetal and maternal serum levels of T4S were not changed after treatment with PCP. This negative answer may be explained by an increased availability of substrate (FT4; maternal) together with a reduced D1 activity by PCP treatment, resulting in a reduced enzymatic breakdown of T4S.

Exposure to Aroclor 1254, which resulted in the formation of hydroxylated metabolites, did not significantly change the T2 SULT activity in maternal or fetal brain or liver cytosol, nor the serum levels of T4S.

Remarkably, the T3S and T4S levels were very low in fetal rat serum in this study, especially when compared with the reported high iodothyronine sulfate levels in fetal human and sheep serum. This can not be explained by low SULT activity levels or high D1 activity levels in rat fetuses on day 20.

Overall implications of the observed PHAH effects on thyroid hormone metabolism

PHAHs induce a wide spectrum of toxic effects in rats. Some effects have been suggested to be linked to a hypothyroid situation, such as the "wasting syndrome", decreased feed intake, and increased cholesterol concentrations. Indeed, reduced serum T4 concentrations have been

and increased cholesterol concentrations. Indeed, reduced serum T4 concentrations have been observed following exposure to PHAHs (Bastomsky *et al.*, 1977; Gorski and Rozman, 1987; Hermansky *et al.*, 1988; Brouwer, 1989; Beetstra *et al.*, 1991), and it is tempting to speculate about a relationship between the hypothyroxinemia and the observed toxic responses. However, induction of a hypothyroid situation or a hypothyroxinemia by PHAHs could also be regarded as an adaptive endocrine response to diminish the PHAH-induced toxicity. One argument in support of this interpretation is the observed protective effect of thyroidectomy on TCDD-induced lethality and immune toxicity (Rozman *et al.*, 1985).

In this study, it is proposed that the T4 decrease could well have a regulatory role in the induction of hepatic biotransformation enzymes, as was reported before (see *Chapter 1*). The present investigations suggest that the lowering effects of PHAHs on T4 levels are only a toxic effect of PHAHs and not an adaptive response to regulate the induction of biotransformation enzymes. The differences with other reports on modulating effects of thyroid hormone state on biotransformation enzymes may be explained by differences in the time and dose of inducers as well as by a difference in hypo- or hyperthyroid state. Nevertheless, the T4 decreases in the hypothyroid animals in our study are similar to the PHAH-induced T4 decreases. Therefore, the model was good enough to investigate our hypothesis.

The second part of this thesis demonstrated that the sulfotransferase enzyme is another thyroid hormone-binding protein, besides D1 and TTR, which can be competitively inhibited by hydroxylated metabolites of PHAHs. In a relatively narrow range of low micromolar concentrations, PHAH-OHs were able to competitively inhibit T2 SULT acttivity *in vitro*, in a SULT isozyme and tissue specific manner.

Studies using a perinatal exposure setup were performed to test inhibition of T2 sulfation *in vivo*. It was demonstrated that the well-known sulfation inhibitor PCP was able to indeed competively inhibit T2 SULT activity, but also was able to upregulate the sulfotransferase protein amounts. Aroclor 1254 exposure resulted in a slight inhibition of T2 SULT activity, probably caused by hydroxylated metabolites formed. This inhibition, together with lower substrate (FT4) levels found after Aroclor treatment did not result in decreased serum T4S levels, which is probably caused by a concomitantly decreased inactivation route, i.e. a decreased D1 activity, together with a higher availibility of substrate (FT4) after PCP exposure.

Remarkably, the serum T4S levels in fetal rat are low compared to the levels in sheep and human fetal serum samples (Wu *et al.*, 1992a/b; 1993a/b; Santini *et al.*, 1993). This could not be explained by already higher D1 activities or a relatively low sulfation activity in the control fetus around GD20. For this reason, we concluded that the fetal rat probably is not a very good model for humans in terms of investigating the impact of toxic compounds on fetal thyroid hormone sulfation. However, it should be mentioned that, although PHAHs and their metabolites interfere at many sites with thyroid hormone transport and metabolism, the fetus

apparently is able to cope with those changes and can keep its homeostasis in T3.

Another interesting point deduced from this study, is that PCP, which could be a model for PCB-OHs, itself showed effects on thyroid hormone levels and metabolism, indicating the importance of phenolic organohalogens compounds for disrupting effects on the thyroid hormone system. This also indicates that the disrupting effects of PCBs on the thyroid hormone system are for a large part caused by the hydroxylated metabolites formed. The own toxicity of PCB-OHs and related phenolic organohalogens inducing a separate set of effects together with the recently observed high fetal accumulation of hydroxy-PHAHs, give reason to further investigate the potential toxicity of these compounds on thyroid hormone metabolism and transport (see also Figure 9.1). It is worth mentioning that besides the "old" organohalogen pollutants that have been phased out since the 1980's, there is a wide range of new products on the market, such as brominated diphenylethers (PBDEs), chlorinated benzenes, bisphenol A and so on. PBDEs, which are nowadays used as flame retardants, have been demonstrated at increasing levels in our environment (De Boer *et al.*, 1989; Sellstrom *et al.*, 1996), and are probably able to cause similar effects as PHAHs. Serum T4 decreases have already been reported in rats after exposure to PBDEs (Darnerud *et al.*, 1996) or PCDEs (Rosiak *et al.*, 1997). Also, hydroxylated metabolites of PBDEs have been found to competitively inhibit the T4 binding to TTR *in vitro* (Meerts *et al.*, 1998).

The human diet contains a diverse spectrum of naturally occuring and xeno-compounds that affect thyroid hormone metabolism. These include the organohalogens and related contaminants, and in addition, a large number of food components. Flavones and flavonoids have been reported to interfere with thyroid hormone binding proteins such as D1 (Auf'mkolk *et al.*, 1986; Cody *et al.*, 1989) and TTR (Lueprasitsakul *et al.*, 1990; Köhrle *et al.*, 1986). Flavonoids such as quercetin were similarly found to be able to inhibit phenol sulfotransferase activity *in vitro* (Walle *et al.*, 1995; Eaton *et al.*, 1996), and also other food additives were potent inhibitors of phenol sulfation (Bamforth *et al.*, 1993). The potential adverse human health impact of these compounds depends on a number of factors, including dietary intake, metabolism and pharmacokinetics, compound potency, serum concentrations, relative binding to serum proteins, and interactions or cross-talk with other endocrine pathways. In a risk evaluation, it should be taken into account that humans are exposed to a mixture of compounds with effects on thyroid hormone metabolism. If the mechanism of interference is similar for all these classes of compounds, the effects might very well be additive, or interactive. Additionally, the very persistent PHAHs are probably of more importance from a risk assessment point of view than the natural food components having a higher degradation rate.

The effects of PHAHs on the thyroid hormone system in this study have been obtained in rats, are the results relevant for the human situation. Occupational or accidental exposure to high levels of PCBs or PBBs results in changes in serum T4 levels as was found by Bahn *et al.* (1980), Kreiss *et al.* (1982), Murai *et al.* (1987), and Emmet *et al.* (1988). Moreover, in

pregnant women exposed to background levels of PHAHs mainly through diet, a significant negative correlation was observed between human milk levels of PHAHs and plasma T4 and T3 levels (Koopman-Esseboom *et al.*, 1994). In addition, increases in plasma TSH and both increases and decreases in plasma T4 levels were found in newborn babies following exposure to increasing PHAH levels through in utero and lactational transfer (Pluim *et al.*, 1993; Koopman-Esseboom *et al.*, 1994). Besides, prenatal exposure to PCBs is related to disorders in neurological development of children, found in some in epidemiologic studies (Rogan *et al.*, 1986; Jacobson *et al.*, 1990). It still is however not clear if these effects of PHAHs on thyroid hormone levels and metabolism may have possible effects on (brain) development.

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SAMENVATTING EN SLOTOPMERKINGEN

Dit proefschrift handelt over de interactie van polygehalogeneerde aromatische koolwaterstoffen (PHAKs) met het schildklierhormoon metabolisme. Dit laatste hoofdstuk begint met een samenvatting van de effecten van schildklierhormoon op de door PHAKs geïnduceerde biotransformatie-enzymen. Vervolgens worden de resultaten van de remming van schildklierhormoonsulfatering door gehydroxyleerde metabolieten van PHAKs besproken. Tot besluit worden enkele opmerkingen gemaakt met betrekking tot de implicaties van in deze studie gevonden resultaten.

Effecten van schildklierhormoon op de inductie van biotransformatie-enzymen door polygehalogeneerde aromatische koolwaterstoffen

Het eerste deel van dit proefschrift is gericht op de vraag of de door PHAKs geïnduceerde afname van plasma T4 een hormonale aanpassing van het lichaam is, zodat het dier beter kan omgaan met de toxische effecten veroorzaakt door PHAKs. Om dit uit te zoeken werd de mogelijk regulerende rol van schildklierhormonen op biotransformatie-enzymen bestudeerd, waarbij gebruik werd gemaakt van ratten met een verschillende schildklierhormoonstatus. Hiervoor werden ratten behandeld met TCDD of PCBs als modelinductoren van biotransformatie-enzymen.

Hoofdstuk 2 beschrijft de verschillen in de schildklierhormoonstatus van euthyroide (Eu) ratten, gethyroidectomeerde (Tx) ratten en Tx ratten behandeld met T3 of T4. De verlaagde plasmaniveaus van T4 en T3, de verhoogde hypofyse respons (hogere plasma TSH concentraties) samen met de veranderde functionele respons (verlaagde D1 en malic enzym activiteit in de lever, verhoogde D2 activiteit in de hersenen) in Tx ratten werden in Tx+T4 ratten hersteld tot Eu niveaus. Dit werd tevens in Tx+T3 ratten gevonden met uitzondering van de plasma T4 en D2 activiteit. Uit deze resultaten blijkt dat de met schildklierhormoon behandelde Tx ratten een goed model zijn om perifere effecten van TCDD te bestuderen. Drie dagen na blootstelling aan 10 µg TCDD/kg lichaamsgewicht waren plasma TT4 en FT4 concentraties significant verlaagd in Eu en Tx+T4 ratten. Plasma T3 concentraties waren significant verlaagd in Tx+T3 ratten maar niet in Eu of Tx+T4 ratten. T4 UGT activiteit in de lever werd geïnduceerd door TCDD behandeling, terwijl T3 UGT activiteit alleen licht verhoogd was in de verschillende groepen. Deze resultaten suggereren dat de schildklierhormoonverlaging veroorzaakt door TCDD voornamelijk buiten de schildklier plaats vindt en gemedieerd wordt door de sterke verhoging van T4 UGT activiteit in de lever.

De effecten van modulering van de schildklierhormoonstatus op de inductie van detoxificerende enzymen door TCDD in laboratoriumdieren is beschreven in *Hoofdstuk 3*. In alle ratten induceerde TCDD een sterke verhoging van CYP1A1/1A2 activiteit (EROD), CYP1A1 eiwithoeveelheid en van CYP1A1 mRNA-niveaus. TCDD-blootstelling resulteerde ook in verhoogde totaal cytochroom P450 hoeveelheden, p-nitrofenol UGT activiteit, en GST 1-1 eiwitconcentraties in de lever. Daarentegen werd geen effect van TCDD gevonden op NADPH cytochroom P450 reductase activiteit, totale GST activiteit en GST 2-2, 3-3 en 4-4 eiwitconcentraties en iodothyronine sulfotransferase activiteit in de lever. Schildklierhormoonstatus had geen effect op de totaal cytochroom P450 hoeveelheid, GST activiteit en GST eiwitconcentraties. In Tx ratten werd echter een kleine verlaging geconstateerd in CYP1A1/2 activiteit, NADPH cytochroom P450 reductase activiteit, PNP UGT activiteit en iodothyronine sulfotransferase activiteit in Tx ratten vergeleken met Eu ratten.

In het tweede dierexperiment is de interactie onderzocht tussen de schildklierhormoonstatus en PCBs op de regulatie van CYP1A1- en CYP2B-expressie (*Hoofdstuk 4*). Mannelijke Sprague-Dawley Tx ratten, Eu ratten en hyperthyroide ratten (door behandeling met T3) werden blootgesteld aan een eenmalige ip dosis van de CYP2B inductor PCB 153 en de CYP1A inductor PCB 126. De schildklierhormoonstatus werd gecontroleerd door plasma T4, T3 en TSH concentraties te bepalend en door functionele parameters te meten, zoals D1, malic enzym en α-glycerolfosfaatdehydrogenase activiteiten in de lever. De hoeveelheid totaal cytochroom P450 in de lever was verhoogd in alle groepen na PCB blootstelling, maar werd niet beïnvloed door schildklierhormoonstatus. De NADPH cytochroom P450 reductase activiteit was verlaagd in Tx ratten en verhoogd in hyperthyroide ratten, terwijl de blootstelling aan PCB hierop geen effect had. PCB 126 induceerde specifiek T4 UGT activiteit gemeten in de afwezigheid van detergens, en verhoogde de mRNA-niveuas, eiwitconcentraties en de activititeit van CYP1A. PCB 153 induceerde juist T4 UGT activiteit ge meten in de aanwezigheid van het detergens Brij 56, en verhoogde de mRNA-niveuas, eiwitconcentraties en de activititeit van CYP2B. Schildklierhormoonstatus, zowel een hypo- als een hyperthyroide status, had geen effect op beide T4 UGT activiteiten of op de mRNA-niveaus, eiwitconcentraties of activiteiten van CYP1A en CYP2B.

Het bijna volledig afwezig zijn van een respons van basale of door PCB- of TCDD-geïnduceerde effecten op biotransformatie-enzymen door veranderingen in schildklierhormoonstatus in onze studies is niet in overeenstemming met eerder gevonden effecten (Kato and Takahashi, 1968; Rumbaugh *et al.*, 1978; Leakey *et al.*, 1982; Müller *et al.*, 1983a/b; Skett, 1987; Yamazoe *et al.*, 1989; Arlotto and Parkinson, 1989; Murayama *et al.*, 1991; Chowdhury *et al.*, 1983; Moscioni and Gartner, 1983; Pennington *et al.*, 1988; Goudonnet *et al.*, 1990; Williams *et al.*, 1986; Pimental *et al.*, 1993). Deze verschillen zouden veroorzaakt kunnen worden door verschillen in stam en geslacht van de gebruikte dieren, de

mate en tijdsduur van de hypo- of hyperthyroide status als wel van de mate en tijdsduur van de TCDD/PCB dosering. Concluderend, NADPH cytochroom P450 reductase activiteit in de lever is afhankelijk van de schildklierhormoonstatus, terwijl er geen of bijna geen veranderingen werde waargenomen in de hoeveelheid totaal cytochroom P450 evenals in CYP1A1 en CYP2B, UGT, GST en sulfotransferase activiteiten bij een verschil in schildklierhormoonstatus. Deze kleine verschillen zijn zeer waarschijnlijk geen endocriene aanpassing aan een chemische stressor (TCDD). De door PHAKs geïnduceerde T4 verlaging evenals andere aspecten van schildklierhormoonmetabolisme zijn daarom zeer waarschijnlijk een directe aanwijzing van een zich ontwikkelende toxicologische respons van deze dieren op de PHAK blootstelling.

De remming van schildklierhormoonsulfatering door gehydroxyleerde metabolieten van polygehalogeneerde aromatische koolwaterstoffen

Het tweede deel van dit proefschrift handelt over de vraag of gehydroxyleerde metabolieten van PHAKs in staat zijn om schildklierhormoonsulfatering te remmen, *in vitro* en *in vivo*.

In *Hoofdstuk 5* wordt het onderzoek beschreven naar het mogelijk remmende effect van gehydroxyleerde metabolieten van PHAHs op iodothyronine sulfotransferase activiteit. Rattenlevercytosol werd gebruikt als bron voor sulfotransferase in een *in vitro* methode met ^{125}I-gelabeld T2 als een modelsubstraat. Aangetoond werd dat gehydroxyleerde metabolieten van PCBs, PCDDs en PCDFs sterke remmers zijn van T2 sulfotransferase activiteit *in vitro* met IC50 waarden in het laag micromolaire gebied (0.2-3.8 µM). De PCB-metaboliet 3-hydroxy-2,3',4,4',5-pentachlorobiphenyl was de sterkste remmer van T2 sulfotransferase activiteit in onze studie met een IC50 waarde van 0.2 µM. Een belangrijke structurele voorwaarde voor remming van T2 sulfotransferase activiteit door PCB-metabolieten blijkt een hydroxyl groep op de para of meta positie te zijn. Ortho gehydroxyleerde PCBs zijn veel minder potent, en geen van de geteste PCBs was zelf in staat om T2 sulfotransferase activiteit te remmen. Tevens werd aangetoond dat omzetting van individuele gebromeerde diphenylethers, nitrofen, en commerciële PHAK mengsels (zoals Bromkal, Clophen A50 en Aroclor 1254) met behulp van cytochroom P450 resulteerde in de vorming van gehydroxyleerde metabolieten die in staat waren om T2 sulfotransferase activiteit te remmen.

In *Hoofdstuk 6* is de remming van schildklierhormoon sulfatering door PHAK-OHs in meer detail bestudeerd met behulp van isoenzymen en remmingskinetiek. Het verschil in remmingspatronen voor sulfotransferase activiteit gevonden tussen rattenlever- en rattenhersencytosol wordt waarschijnlijk veroorzaakt door een verschil in isoenzym patroon. T2 sulfatering werd geremd door PCB-OHs via een interactie met SULT1C1 en een ander isoenzym wat verantwoordelijk is voor T2 sulfatering in vrouwelijk levercytosol, waarschijnlijk

rat SULT1B1, maar niet SULT1A1. Daarentegen geldt voor humaan fenol sulfotransferase, dat SULT1A1 geremd werd door PCB-OHs, en niet het isoenzym SULT1A3. Tenslotte wijzen de resultaten erop dat tenminste het humane SULT1A1, en het rat SULT1C1 en misschien ook rat SULT1B1 betrokken zijn bij de remming van T2 sulfatering door PCB-OHs. Het is echter noodzakelijk om meer informatie te verkrijgen over de verschillende isoenzymen die betrokken zijn bij de iodothyronine sulfatering in mensen en ratten.

Tevens is aangetoond dat T2 een goed model substraat is voor het actieve hormoon T3 bij de bestudering van de remming van schildklierhormoonsulfatering door gehydroxyleerde metabolieten van PHAKs. De kinetiek van de remming van T2 sulfatering door PCB-OHs duidt op een competitieve remming. Definitief bewijs wacht op experimenten met gezuiverde isoenzymen. Tenslotte werd aangetoond dat PCDD-OHs en PCB-OHs zelf ook enigzins als substraat kunen dienen voor sulfotransferase enzymen, wat een tweede aanwijzing is voor een competitieve remming.

Om de stap van *in vitro* experimenten met gebruik van cytosol naar de *in vivo* situatie te verkleinen, is de remming van schildklierhormoonsulfatering onderzocht in hepatoma cellijnen (*Hoofdstuk 7*). Twee PCB metabolieten, 4-hydroxy-2',3,3',4',5-pentachlorobiphenyl en 4-hydroxy-3,3',4',5-tetrachlorobiphenyl alsook de bekende sulfateringsremmer pentachlorophenol (PCP) zijn getest in de rattenhepatoma cellijn FaO en de humane hepatoma cellijn HepG2. Aangetoond is dat PCP de T2 sulfatering *in vitro* remt in FaO en HepG2 cellen, hoewel PCP 1000 keer minder aktief was in cellen in vergelijking met rattenlevercytosol. Micromolaire concentraties van 2 geteste PCB-OHs beïnvloedden de T2 conjugatie nauwelijks in FaO cellen, maar reduceerden wel de sulfaatvorming in HepG2 cellen. De remming van T2 sulfatering was sterker in medium zonder serumtoevoeging in vergelijking met medium met 5% serum. Dit laatste wordt veroorzaakt door een lagere opname van de remmer door de cellen in aanwezigheid van serum zoals aangetoond werd met behulp van radioactief gelabeld PCP.

Deze *in vitro* resultaten tonen aan dat gehydroxyleerde PHAKs sterke remmers zijn van de schildklierhormoonsulfatering. Schildklierhormoonsulfatering speelt waarschijnlijk een belangrijke rol in de regulatie van "vrije" hormoonconcentraties in de foetus. Daarnaast is bekend dat gehydroxyleerde PCB metabolieten accumuleren in foetale weefsels na blootstelling van de moeder aan PCBs. De remming van T2 sulfatering zou dus eventueel een effect kunnen hebben op de foetale schildklierhormoonhomeostase en ontwikkeling.

Het *in vivo* experiment waarin de mogelijke remming van T2 sulfatering door PHAK metabolieten werd getest, is beschreven in *Hoofdstuk 8*. Drachtige ratten werden blootgesteld aand Aroclor 1254 of aan de bekende fenolsulfotransferase remmer PCP op dag 10 tot en met dag 18 van de dracht. Op dag 20 van de dracht werd sectie uitgevoerd op foetussen en moederdieren. De concentraties van PCP en PCB metaboliet waren hoog in foetaal serum en weefsels. Aroclor 1254 blootstelling resulteerde in verhoogde EROD en T4 UGT activiteit in de lever van moederdieren, terwijl PCP geen effect had op deze parameters.

PHAKs staan bekend om hun verstorend effect op schildklierhormoonmetabolisme, zoals aangegeven in Figuur 9.1 *(Chapter 9)*. In dit dierexperiment veroorzaakte Aroclor 1254 blootstelling een verhoogde T4 UGT activiteit in de lever, wat resulteerde in verlaagde TT4 niveaus. Blootstelling aan PCP leidde ook tot verlaagde serum TT4 concentraties, maar tot verhoogde FT4 concentraties in zowel foetussen als moederdieren. De ratio FT4/TT4 was verhoogd, wat duidt op een verlaagde bindingscapaciteit voor T4 aan TTR in foetussen en moederdieren als gevolg van de beide behandelingen. D1 activiteit in de lever was verlaagd in moederdieren en foetussen na behandeling met Aroclor 1254 en PCP. Deze verlaging wordt waarschijnlijk indirect veroorzaakt door de verlaagde T4 concentraties. D2 activiteit in de hersenen was verlaagd door blootstelling aan PCP in moederdieren, terwijl er geen effect was in de foetussen. Blootstellling aan Aroclor 1254 verhoogde de D2 activiteit in foetale hersenen, en gaf geen effect te zien bij de moederdieren. De verhoogde D2 activiteit is een respons van de hersenen op lage T4 concentraties, nodig om de T3 homeostase te bewaren.

In tegenstelling met de verwachting liet de positieve controle PCP juist een verhoging van de T2 SULT activiteit in lever- en hersencytosol van de moeder zien. Experimenten waarbij variërende T2 concentraties en verschillende eiwitconcentraties werden gebruikt, suggereren dat PCP in staat is sulfotransferase activiteit competitief te remmen en tevens de hoeveelheid sulfotransferase-eiwit te verhogen. Dit effect van PCP op schildklierhormoonsulfatering leidt blijkbaar niet tot lagere concentraties van het schildklierhormoonsulfaat, aangezien de serum T4S concentraties in moederdieren en foetussen niet verlaagd zijn na behandeling met PCP. Dit negatieve effect zou het gevolg kunnen zijn van een verhoogde beschikbaarheid van het substraat (FT4; in de moederdieren) samen met een verlaagde D1 activiteit door PCP, wat resulteert in een verlaagde enzymatische afbraak van T4S.

Blootstelling aan Aroclor 1254, waarvan gehydroxyleerde metabolieten gevormd worden, veroorzaakte geen significante verschillen in de T2 sulfotransferase activiteit in lever- of hersencytosol van moeders of foeten, noch in T4 serum concentraties.

De T3S en T4S concentraties waren opmerkelijk laag in foetaal rat serum in deze studie, in vergelijking met de hoge waarden van iodothyronine sulfaten in foetaal humaan en schapenserum. Dit kan niet verklaard worden door een lage sulfotranferase activiteit of reeds hoge D1 activiteiten in rattenfoeten op dag 20.

Implicaties van de geobserveerde PHAK-effecten op het schildklierhormoonmetabolisme

PHAKs induceren een breed spectrum aan toxische effecten in ratten. Van sommige effecten, zoals het "wasting syndrome", afname van voedelinname en verhoogde serum cholesterol

concentraties, is gesuggereerd dat ze in verband staan met een hypothyroide situatie. Inderdaad worden er verlaagde serum T4 concentraties gevonden na blootstelling aan PHAKs (Bastomsky *et al.*, 1977; Gorski and Rozman, 1987; Hermansky *et al.*, 1988; Brouwer, 1989; Beetstra *et al.*, 1991). Daardoor is het verleidelijk om te speculeren over een relatie tussen de hypothyroxinemie en de geobserveerde toxische responsen. De inductie van een hypothyroide situatie of een hypothyroxinemie door PHAKs kan echter ook beschouwd worden als een hormonale aanpassing om de PHAK-geïnduceerde toxiciteit op te kunnen vangen. Een argument voor deze interpretatie, is de observatie van een beschermend effect van een thyroidectomie op door TCDD-geïnduceerde lethaliteit en immuuntoxiciteit (Rozman *et al.*, 1985).

In dit onderzoek gaan we uit van de hypothese dat de T4 verlaging een regulerende rol zou kunnen hebben in de inductie van biotransformatie-enzymen in de lever, zoals reeds eerder is gerapporteerd (zie *Hoofdstuk 1*). De resultaten van het huidige onderzoek suggereren dat de verlaging van T4 concentraties door PHAKs alleen maar een toxisch effect is van de PHAKs en geen adaptieve respons om de inductie van biotransformatie-enzymen te reguleren. Het verschil met andere onderzoeken over modulerende effecten van schildklierhormoonstatus op biotransformatie-enzymen kan eventueel verklaard worden door verschillen in tijd en dosis van de inductoren als mede door een verschil in de hypo- of hyperthyroide status. De T4 verlagingen in de hypothyroide dieren in onze studie waren echter vergelijkbaar aan door PHAKs geïnduceerde T4 verlagingen. Daarom was het model goed genoeg om onze hypothese te onderzoeken.

Het tweede deel van dit proefschrift laat zien dat, naast het type I dejodase en transthyretine, het sulfotransferase enzym ook competitief geremd kan worden door gehydroxyleerde metabolieten van PHAKs. In een vrij smal concentratiegebied (μM) zijn PHAK-OHs in staat om T2 sulfotransferase activiteit *in vitro* competitief te remmen op een sulfotransferase isoenzym- en weefselspecifieke manier.

Ratten perinataal blootgesteld aan PCBs werden gebruikt om T2 sulfateringsremming te testen *in vivo*. Er werd aangetoond dat de bekende sulfateringsremmer PCP in staat was de T2 sulfotransferase activiteit te verhogen, waarschijnlijk als een indirect gevolg van competieve remming resulterend in verlaagde niveaus van gesulfateerd product. Door gebruik te maken van verschillende cytosol eiwitconcentraties en een concentratiereeks van het substraat T2 in de *in vitro* assay kon geconcludeerd worden dat PCP de T2 sulfotransferase activiteit competitief remde, hoewel niet zo sterk als verwacht. Aroclor 1254 blootstelling resulteerde in een kleine verlaging in T2 sulfotransferase activiteit, hetgeen waarschijnlijk veroorzaakt wordt door remming door gevormde gehydroxyleerde PCB metabolieten. Deze remming samen met de gevonden verlaagde substraat (FT4) concentratie gevonden na blootstelling aan Aroclor 1254, resulteerde niet in verlaagde T4S serum niveaus. Dit zou verklaard zou kunnen worden

door een tegelijkertijd verminderde afbraakroute, namelijk de verlaagde D1 activiteit, als ook door een verhoogde substraatbeschikbaarheid (FT4) na blootstelling aan PCP.

De serum T4S niveaus in foetale ratten zijn opmerkelijk laag in vergelijking met foetale concentraties in humaan en schapenserum (Wu *et al.*, 1992a/b; 1993a/b; Santini *et al.*, 1993). Dit kan niet verklaard worden uit een al verhoogde D1 activiteit of een relatief lage sulfateringsactiviteit in de foetus rond dag 20 van de dracht. Vanwege die lage concentraties zou de rat wellicht niet zo'n goed model kunnen zijn voor het bestuderen van de effecten van toxische stoffen op foetaal schildklierhormoonsulfatering bij de mens. Er moet wel opgemerkt worden dat ondanks het feit dat PHAKs en hun metabolieten op verschillende wijze kunne interfereren met het schildklierhormoontransport en -metabolisme, de foetus blijkbaar in staat is om te gaan met deze veranderingen en de homeostase kan bewaren.

Een ander interessant punt uit deze studie is dat PCP, wat als model voor een PCB-OH zou kunnen dienen, ook effecten liet zien op schildklierhormoonniveaus en -metabolisme. Dit duidt erop dat de effecten van PCBs op schildklierhormoonniveaus en -metabolisme niet alleen door PCBs zelf maar ook door hun gevormde gehydroxyleerde metabolieten veroorzaakt kunnen worden. De door PCB-OHs en andere fenolische organohalogenen veroorzaakte effecten geeft aanleiding tot meer onderzoek naar de potentiële toxiciteit van deze stoffen op schildklierhormoontransport en -metabolisme. Daarnaast moet ook opgemerkt worden dat naast de "oude" organohalogeenvervuilers die niet meer gebruikt mogen worden sinds de tachtiger jaren, er een breed spectrum aan nieuwe producten op de markt is gekomen zoals gebromeerde diphenylethers (PBDEs), gechloreerde benzenen, bisphenol A enzovoort. Tegenwoordig worden PBDEs veel gebruikt als vlamvertragers, en zijn aangetoond in toenemende hoeveelheden in ons milieu (De Boer, 1989; Sellstrom *et al.*, 1996). Waarschijnlijk zijn ze in staat om soortgelijke effecten als PHAKs te veroorzaken. Serum T4 verlagingen zijn al gerapporteerd in ratten na blootstelling aan PBDEs (Darnerud *et al.*, 1996) of PCDEs (Rosiak *et al.*, 1997). Eveneens werd gevonden dat gehydroxyleerde metabolieten van PBDEs in staat zijn de binding van T4 aan TTR *in vitro* te verhinderen (Meerts *et al.*, 1998).

Tevens is bekend dat een groot aantal voedselcomponenten in staat zijn het schildklierhormoontransport en -metabolisme te verstoren. Over flavonen en flavonoiden is gerapporteerd dat ze interfereren met schildklierhormoon-bindende eiwitten zoals D1 (Auf'mkolk *et al.*, 1986; Cody *et al.*, 1989) en TTR (Lueprasitsakul *et al.*, 1990; Köhrle *et al.*, 1986b). Flavonoiden zoals quercetine zijn in staat tot het remmen van phenol sulfotransferase activiteit *in vitro* (Walle *et al.*, 1995; Eaton *et al.*, 1996), evenals andere voedseladditieven (Bamforth *et al.*, 1993). Het humane dieet bevat een breed spectrum aan van nature voorkomende en non-nutrionele bestanddelen die een effect kunnen hebben op schildklierhormoonmetabolisme. Het mogelijke negatieve effect op de gezondheid van de mens hangt af van een aantal factoren, zoals de voedselinname, metabolisme en pharmacokinetiek, de potentie van de stof, de serumconcentraties, de relatieve binding aan serumeiwitten en van

interacties met andere endocriene routes. In een risico-evaluatie zal meegenomen moeten worden dat mensen blootgesteld worden aan een mengsel van stoffen met effecten of schildklierhormoonmetabolisme. Indien het mechanisme gelijk is voor deze stofgroepen, zouden de effecten additief kunnen zijn. Daarnaast zijn de zeer persistenst PHAKs waarschijnlijk belangrijker voor een risico-evaluatie dan de natuurlijk voorkomende voedselcomponenten, vanwege hun hogere afbreekbaarheidsgraad.

De effecten van PHAKs op het schildklierhormoonsysteem in deze studie zijn allen geobserveerd ratten, maar wat is nu eigenlijk de relevantie voor de mens? Beroepsmatige of accidentele blootstelling aan hoge concentraties van PCBs of PBBS resulteerde in veranderingen in serum T4 concentraties, zoals gevonden door Bahn *et al.* (1980), Kreiss *et al.* (1982), Murai *et al.* (1987) en Emmet *et al.* (1988). Daarnaast werd in zwangere vrouwen blootgesteld aan achtergrondniveaus van PHAKs door voeding, een significant negatieve correlatie gevonden tussen PHAK-concentraties in moedermelk en plasma T3 en T4 niveaus (Koopman-Esseboom *et al.*, 1994). Eveneens werden toenames in plasma TSH en zowel toenames als afname in plasma T4 levels gevonden in pasgeboren babies na blootstelling aan toenemende PHAK-concentraties in de baarmoeder en via borstvoeding (Pluim *et al.*, 1993; Koopman-Esseboom *et al.*, 1994). Daarnaast zijn er neurologische verstoringen gerelateerd aan perinatale blootstelling aan PCBs gevonden in kinderen in epidemiologische studies (Rogan *et al.*, 1986; Jacobson *et al.*, 1990). Het is echter nog steeds niet duidelijk of deze effecten van PHAKs op schildklierhormoonconcentraties en -metabolisme mogelijke effecten kunnen hebben op (hersen)ontwikkeling.

ABBREVIATIONS

3MC	3-methylcholanthrene
Ah-receptor	Arylhydrocarbon receptor
bw	body weight
CNS	central nervous system
CYP	cytochrome P450
D1	type I deiodinase
D2	type II deiodinase
D3	type III deiodinase
EROD	ethoxyresorufin O-deethylase
Eu	euthyroid
FCS	fetal calf serum
FT4	free T4
GD	gestation day
GST	glutathione S-transferase
IC50	inhibition concentration at 50%
ip	intraperitoneaal
PAPS	phospho-adenosine phosphosulphate
PB	phenobarbital
PCB	polychlorinated biphenyl
PCP	pentachlorophenol
PCB 126	3,3',4,4',5-pentachlorobiphenyl
PCB 153	2,2',4,4',5,5'-hexachlorobiphenyl
PCDD	polychlorinated dibenzo-*p*-dioxin
PCDF	polychlorinated dibenzofuran
PHAH	polyhalogenated aromatic hydrocarbon
PHAH-OH	hydroxylated polyhalogenated aromatic hydrocarbon
PNP	p-nitrophenol
rT3	reverse T3; 3,3',5'-triiodothyronine
SULT	sulfotransferase
T2	3,3'-diiodothyronine
T3	3,3',5-triiodothyronine
T4	thyroxine; 3,3',5,5'-tetraiodothyronine
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TSH	thyroid stimulating hormone
TTR	transthyretin
Tx	thyroidectomized
UDP	uridinediphospho
UGT	UDP-glucuronyltransferase

CURRICULUM VITAE

Gerlienke Schuur werd geboren op 10 juni 1968 te Eibergen. In juni 1986 behaalde zij het VWO-diploma aan het Ichthus College in Enschede en in september van dat jaar begon ze met de studie Moleculaire Wetenschappen aan de Landbouwuniversiteit te Wageningen (LUW).Tijdens de doctoraalfase werd door haar onderzoek uitgevoerd op de vakgroep Biochemie (LUW) onder begeleiding van W.A. Balvers en dr. I.M.C.M. Rietjens naar het effect van fosfolipiden op het cytochroom P450 systeem. Daarna werd op de afdeling Biologische Toxicologie van TNO Voeding te Zeist *in vitro* de accumulatie van cadmiumchloride en metallothioneine gebonden cadmium in darm cellijnen bestudeerd onder begeleiding van dr. J.P. Groten. Op de vakgroep Experimentele Diermorfologie en Celbiologie werd geprobeerd MHC-genen van de atlantische zalm te kloneren onder begeleiding van dr. R.J.M. Stet. Tenslotte werd een stageperiode doorgebracht bij het Department of Microbiology, University of Mississippi Medical Center in de Verenigde Staten waar de invloed van 3-methylcholanthrene op meervallymfocyten werd onderzocht onder begeleiding van dr. N.W. Miller en dr. W.A. Cuchens. De studie Moleculaire Wetenschappen werd in september 1992 afgerond.

Half oktober 1992 trad ze in dienst van NWO als onderzoeker in opleiding bij de vakgroep Toxicologie (LUW). Het in dit proefschrift beschreven onderzoek werd uitgevoerd onder begeleiding van dr. A. Brouwer (Toxicologie) en prof. dr. ir. T.J. Visser, vakgroep Interne Geneeskunde III, Erasmus Universiteit Rotterdam. Naast het verrichten van promotie-onderzoek, heeft zij in deze periode enige modules van de postdoctorale opleiding Toxicologie gevolgd. Op het 18th Symposium on Chlorinated Dioxins and Related Compounds in Stockholm (1998) heeft zij de Student Award gekregen voor haar presentatie getiteld: Effect of Aroclor 1254 on thyroid hormone sulfation in fetal and maternal rat liver.

Sinds half oktober 1997 is zij werkzaam als toegevoegd onderzoeker bij de Leerstoelgroep Toxicologie (LUW) op het EU-project "Retinoid (vitamin A) interactions with organohalogen food residues: mechanisms, biomarkers and implications for developmental toxicity".

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