REDUCTION OF THYROXINE LEVELS IN THE CIRCULATION AND IN THE BRAIN OF HEXACHLOROBENZENE-EXPOSED RATS

Daling van thyroxine spiegels in de circulatie en in de hersenen van met hexachloorbenzeen behandelde ratten

PROEFSCHRIFT

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Jeroen Adrianus Gerardus Maria van Raaij

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PROMOTIECOMMISSIE:

Promotor:	*Prof.dr. W.R.F. Notten*
Co-promotor:	*Dr.K.J. Van den Berg*
Overige leden:	*Prof.dr.ir. T.J. Visser*
	Prof.dr. J.F. Koster
	Prof.dr.P.Th. Henderson

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To my parents

CONTENTS

LIST OF ABBREVIATIONS

AHHs	aromatic halogenated hydrocarbons
DIT	diiodotyrosine
EROD	ethoxyresorufin-O-deethylase
HCB	hexachlorobenzene
MC	3-methylcholanthrene
MFO	mixed function oxydase
MIT	monoiodotyrosine
PBBs	polybrominated biphenyls
PCBs	polychlorinated biphenyls
PCDDs	polychlorinated dibenzo-p-dioxins
PCDFs	polychlorinated dibenzo-furans
PCP	pentachlorophenol
PCTP	pentachlorothiophenol
rT3	reverse T3
TCHQ	tetrachlorohydroquinone
T4	3,3',5,5'-tetraiodothyronine; thyroxine
T3	3,3',5-triiodothyronine
TBG	thyroxine-binding globulin
TCB	3,3',4,4'-tetrachlorobiphenyl
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TRH	thyrotropin-releasing hormone
TSH	thyroid stimulating hormone
TTR	transthyretin
UDPGT	uridine-diphosphate-glucuronyltransferase

CHAPTER I

INTRODUCTION

Contents

A: General Introduction

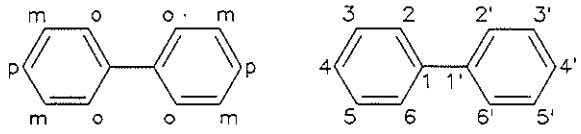
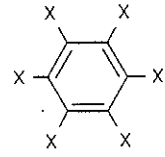
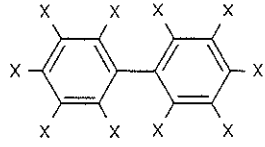
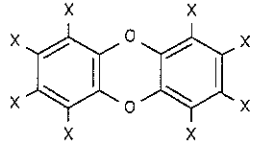
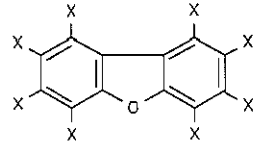
From studies described in literature, it has become clear that many industrial aromatic halogenated hydrocarbons (AHHs) may pose a threat to the health of humans, particularly of those in the occupational environment. These compounds have found a broad environmental distribution and are contaminating food at low levels. The AHHs share the property of a high fat and low water solubility, a high persistancy to biodegradation and a low turnover rate. Hence, they are deposited in the body in adipose (fatty) tissue, and accumulate in the food chain (plants, fish, mammalian species). The group of AHHs (Table 1), consist of the halogenated benzenes, halogenated biphenyls, halogenated dibenzo-p-dioxins and halogenated dibenzofurans (Fig. 1).

Table 1. Toxic accidents with halogenated aromatic hydrocarbons (AHHs)

Chemical class	Toxic compounds	Source	Location	Neuro-toxicity	Thyroid-toxicity	References
Polychlorinated benzenes	Hexachloro-benzene	Contaminated grain	Turkey	+ +	+	Peters et al (1982) Cripps et al (1984)
Polychlorinated biphenyls (PCBs)	PCB mixture	Rice oil	Yusho (Japan)	+	 +	Kuratsune (1972,1976,1980) Mural et al (1987)
			YuCheng (Taiwan)	 +	+	Hsu et al (1984) Rogan et al (1988,1992)
Polybrominated biphenyls (PBBs)	(Firemaster BP-6)	food supplement	Michigan (US)	+	 +	Dunckel (1975) Bahn et al (1986)
Polychlorinated dibenzo-p-dioxins (PCDDs)	TCDD[1)]	2,4,5-trichlorophenol	Weesp (Netherlands) Seveso (Italy) West Virginia (US)	+	 +	Hay (1979) Bertazzi et al (1989)
	TCDD[1)]	Contaminated horse arenas	Missouri (US)	– +	–	Reggiani (1980) Ashe et al (1953)

1) 2,3,7,8-tetrachlorodibenzo-p-dioxin

Fig 1. Chemical structures of AHHs

Chemical	Structure
Backbone(*)	
Chlorinated benzenes[1)]	
Polychlorinated biphenyls[1)]	
Polychlorinated dibenzo-p-dioxins[1)]	
Polychlorinated dibenzofurans[1)]	

(*) The structures indicate the possible positions of the chlorines in terms of ortho (o), meta (m), or para (p) site, and of 1, 2, 3, 4, 5 and 6 positions. 1): X=chlorine.

Toxic episodes with sometimes large-scale exposure of people to AHHs have revealed a number of different signs and symptoms of toxicity, such as dermal lesions (chloracne, e.g hyperkeratosis of the epidermis), hepatomegaly, thymus involution, and alopecia. Neuro- and thyroid-toxicity were found to be associated with the major part of the AHHs (Table 1). All taken together it appears that, despite the differences in chemical backbone of the various AHHs, the signs and symptoms of intoxication with these compounds share a great deal of homology. Apart from the acute toxic effects of these chemicals, there is concern about the long-term effects on human health. In particular, toxicity of AHHs with respect to carcinogenesis, reproduction and development (of the nervous system) are the subject of a number of recently completed or ongoing investigations in the Netherlands and internationally.

In the following sections, information on occurrence, toxicokinetics and toxicodynamics of AHHs is given with special emphasis on polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins (PCDDs) and hexachlorobenzene (HCB). In the outline of this thesis, the aim of the study is presented.

B: Toxicology of aromatic halogenated hydrocarbons with an emphasis on thyroid-and neuro-toxicity: a review

Polychlorinated biphenyls (PCBs) and polychlorinated dibenzo-p-dioxins (PCDDs)

Occurrence

Industrial chemicals which have become widespread contaminants of the environment are PCBs and PCDDs (see reviews of Safe, 1984, George et al, 1988, McFarland et al, 1989, Safe et al, 1990). PCBs were used for over 50 years in numerous industrial processes as additives to open systems, such as paints, plastics, rubber, carbonless paper, printing inks, and insecticides. In addition, PCBs were also extensively applied as insulating materials in electrical capacitors and as coolants in closed systems, such as transformers and condensators (Hutzinger et al, 1974). Theoretically, 209 PCB isomers are possible, and they are classified according to the number and position of chlorine-atoms (Ballschmiter et al, 1980, McFarland et al, 1989).

PCBs have not been produced or used since the end of the seventies,

but the properties that guaranteed their usefulness to industry (i.e., resistance to fire, acids and bases, and oxidation, including suitable viscosity-temperature relationships and low vapour pressure), has resulted in occurrence of considerable amounts in the environment (Tanabe, 1988). Major routes by which PCBs enter the environment are inadequate destruction of PCB containing materials by incineration, leakage from landfills, and leaking cooling water used in transformer manufacturing.

Dioxins (PCDDs) are formed as unwanted by-products during the synthesis of PCBs, chlorophenols (pentachlorophenol, 2,4,5-trichlorophenol), and herbicides. Furthermore, PCDDs are predominantly introduced into the environment as by-products of incinerations, such as fly ash and flue gases (Olie et al, 1977).

Toxicokinetics

PCBs accumulate in skin and muscle, but mainly in adipose tissue, due to their low solubility in water and high accumulation coefficients. PCBs leave the body slowly. Animal species of the aquatic environment (fish, marine mammals, fish eating birds), and land living mammals (also humans) contain large quantities of PCBs, which has often been ascribed to their lipophilicity and stability.

PCBs and other compounds belonging to the AHHs are metabolized by the so- called phase I- and phase II (conjugation) enzymes. The former introduce polar groups into lipophilic substrates by oxidation, reduction or hydrolysis, and the latter couple hydrophylic compounds to the polar group, leading to more water soluble molecules. When highly reactive intermediate (arene) oxides and/or hydroxylated compounds are produced, toxicity may be increased (bioactivation). Xenobiotics with more polar character will be mainly metabolized by phase II enzymes. The phase I enzymes include the hepatic and extrahepatic aryl hydrocarbon-metabolizing mixed function oxidases (MFOs). These are hemoproteins which are localised in membranes of the smooth endoplasmatic reticulum of the cell.

One of the most important enzymes with mono-oxygenase activity, i.e., the capacity to reduce molecular oxygen to water and to insert a second oxygen atom in the substrate, is cytochrome P450. There exist various iso-enzymes, which all possess the capacity to transform various types of substrates (Hahn et al, 1988). Roughly, two classes of MFO inducers, based

on their degree and capacity of induction of different types of cytochrome P450, resembling phenobarbital (PB) or 3-methylcholanthrene (MC) are known.

The oxidative metabolic pathways for PCBs and related compounds are catalyzed by various types of MFOs (see review McFarland et al, 1989). The induced type of MFO and the toxicity of PCBs depends on the structure of the PCB congener. It has been suggested that PCB congeners with strong MC-inducible activity, such as most non-ortho-coplanar PCB congeners with at least two halogen atoms in lateral positions, have the greatest toxic potential (Goldstein, 1979, Poland et al, 1979, Safe, 1984). These congeners mainly induce activities of enzymes, such as arylhydrocarbonhydroxylase (AHH) or ethoxyresorufin-O-deethylase (EROD), both associated with cytochrome P450-1A1 (Sawyer et al, 1982, Kimbrough, 1987). PCB-congeners with the less toxic ortho-chlorine substituents, for instance the non planar di-ortho PCBs, however, do not induce cytochrome P450-1A1 (Safe, 1984, 1987, 1990), but mainly cytochrome P450-2B1/2, like phenobarbital. High levels of induction of cytochrome P450-1A1 and P450-1A2 were observed in rats exposed to dietary mixtures of PCBs (Aroclor 1254), (Dragnev et al, 1994).

Elimination of PCBs is dependent on their degree of lipophilicity and its rate of metabolism. The latter occurs in several organs, but the liver is considered as the major organ of biodegradation of PCBs. From many studies with PCB isomers, it became evident that the degree and position of chlorination on the biphenyl ring, as well as the animal species, determine the extent of metabolism and rate of excretion. Congeners with less chlorination do not tend to accumulate and are readily metabolized and eliminated (Goldstein et al, 1977). For instance, 50 % of 3,3',4,4'-tetrachlorobiphenyl (TCB) is metabolized in rats within three days, while 3,3',4,4',5,5' hexachlorobiphenyl is hardly metabolized at all. In addition, it appeared in dogs that the excretion percentage of 2,2',3,3',6,6'-hexachlorobiphenyl after three days was about 17-fold higher than 2,2'3,3'5,5'-hexachlorobiphenyl (Safe, 1987).

Dioxins (PCDDs) are hardly metabolized in animal species. If dioxins are metabolized, it will mainly occur by hydroxylations at lateral (2,3,7,8) positions (as with PCBs), presumably with arene oxides as intermediates. In addition, the presence of two non-substituted carbon atoms accelerates metabolism. Biotransformation studies with PCDDs have mainly been carried out with

2,3,7,8-tetrachloro-dibenzo-p-dioxin (TCDD). This type of dioxin is one of the most toxic congeners of PCDDs in animals and an extremely strong inducer of MC-like MFOs.

Hexachlorobenzene (HCB)

Occurrence

HCB has been extensively manufactured in the past as a fungicide. However, HCB may still be unintentially produced as a by-product in the manufacturing of many chlorinated hydrocarbons (Menzie, 1986, Tobin, 1986, Jacoff et al, 1986). For instance, HCB may be formed as an impurity during the production of perchloroethylene, trichloroethylene, vinylchloride and pentachloronitro-benzene (Blackwood et al, 1979). In addition, release of HCB may occur during combustion processes such as municipal incinerations (Carpenter et al, 1986). Former applications of HCB were its use as an additive for pyrotechnic compositions for the military, as an intermediate in dye manufacturing and as a wood preservative.

In most Western countries the production and use of HCB as a pure compound is banned (Morris et al, 1986). There is evidence that it has not been commercially available since the late 1970s (Carpenter et al, 1986). However, in the U.S, 4130 tons of HCB are still produced annually as a contaminant in the production of pesticides or chlorinated solvents according to an estimation of the Environmental Protection Agency (EPA).

The physico-chemical properties of HCB include a high lipophilicity (Verschueren, 1983), a high persistency (Beall, 1976, Mansour et al, 1986), and a slight photosensitivity. Evidence for release of HCB from chemical industries into the water and air, and deposition in soil has been established (Schoch et al, 1985, Spigarelli et al, 1986, Slooff et al, 1991).

Toxicokinetics

The major route of human exposure to HCB is contamination via food, since HCB can be taken up by plants and further transported through the food cycle (EPA, 1987). After intestinal uptake, HCB is transported in the bloodstream to adipose tissue (Robinson et al, 1990). Estimates of exposure of humans to HCB from the general environment, using the exposure

commitment method, showed a daily dietary intake of 0.2 μg by ingestion, and an accumulation of 0.047 mg/year in fatty tissue (Bennett, 1981, Cam et al, 1963, Burton et al, 1987). In addition, the body burden of HCB in humans appeared to be 0.7 mg, based on a concentration of 0.04 mg/kg in fatty tissue.

In several laboratory animals exposed to HCB, adipose tissue contained the greatest concentration of HCB, although bone marrow, skin and intestinal tract were also shown to contain HCB (Avrahami, 1975, Mehendale et al, 1975, Iatropoulos et al, 1975, Ingebrigtsen et al, 1983). A number of studies showed evidence for accumulation of HCB in fetal tissue, particularly brain, after placental transfer in several species of laboratory animals (Villeneuve et al, 1974, 1975, Courtney 1979, Courtney et al, 1985a, 1985b, Cripps, 1990, Goldey et al, 1991, 1992. Furthermore, rapid transfer of HCB from maternal adipose tissue via the milk to suckling neonatal rats and mice has been demonstrated (Courtney et al, 1985b, Goldey, 1991). In addition, placentas and cord blood of pregnant Japanese women contained levels of HCB (Ando et al, 1985).

HCB is a MC- and PB-like type inducer of MFOs (Goldstein et al, 1982). The preferential induction of rats exposed to HCB involves MC-inducible forms of cytochrome P450, namely cytochrome P450-1A2 and P450-1A1, with a preference for the former (Linko et al, 1986, Li et al, 1986, Goldstein et al, 1986, Nebert et al, 1989). Rozman et al (1986) obtained evidence for HCB also as a potent inducer of cytochrome P450-2B1 in rats, which is associated with PB-inducible forms of cytochrome P450. Recently, it was found that cytochrome P450-1A1 and P450-1A2 were highly induced in female F344 rats that were exposed to HCB for 30 weeks (Smith et al, 1990).

Several *in vitro* and *in vivo* metabolic studies indicate that the conversion of HCB to pentachlorophenol (PCP) and tetrachlorohydroquinone (TCHQ) is a major metabolic route, and that cytochrome P450-3A1 and/or cytochrome P450-3A2 mediate this metabolic pathway (Van Ommen et al, 1985, 1989). Den Besten et al (1993) found evidence in support of the latter pathway as subchronic co-treatment of rats with triacetyloleandomycin (TAO), a selective inhibitor of the oxidative biotransformation of HCB, did result in diminished levels of PCP and TCHQ in urine. On the other hand, a high degree of PCP urinary excretion correlated well with the degree of HCB-induced porphyria. Earlier studies also showed that the microsomal oxidation of HCB to PCP and TCHQ was related to the porphyrinogenic action of HCB (Debets et al,

1980, Carpenter et al, 1986, Van Ommen et al, 1986, 1989). Koss et al (1986) demonstrated that 80-90 % of daily urinary PCP in man appeared to be formed from HCB. Kinetic studies with [^{14}C]HCB in rats demonstrate that almost half of the amount of HCB was excreted as metabolites over a period of 4 weeks after administration (Koss et al, 1976), and that 20% of the metabolites were identified as PCP, while 3% and 16% were TCHQ and the sulphur-containing metabolite pentachlorothiophenol (PCTP) respectively. PCTP arises from a conjugation reaction of HCB to glutathione (GSH) as a phase II detoxification pathway for HCB, resulting in subsequent formation of the intermediate N-acetyl-S-(pentachlorophenyl)cysteine (PCPNAc), which is hydrolyzed to PCTP (Den Besten, 1992).

There are indications that the elimination of HCB by GSH conjugation is sex-dependent. For instance, D'Amour and Charbonneau (1992) demonstrated that male Sprague-Dawley rats treated with five doses of HCB (500 mg/kg) over 3 days had decreased hepatic GSH levels compared with controls, while in female rats no differences were observed. Furthermore, in male rats dosed with HCB, a higher biliary excretion of PCTP compared with females was observed. However, urinary concentrations of PCTP were higher in female rats after treatment with HCB. In F344/N rats, urinary excretion of PCTP, PCP, and 2,3,5,6-tetrachlorobenzene-1,4-diol was higher in females than in males after 10 weeks of HCB-treatment by intubation (Rizzardini et al, 1982).

Besides PCP, other chlorophenols have been demonstrated as metabolites of HCB. For instance, 2,3,4,6-tetrachlorophenol, 2,3,5,6-tetrachlorophenol and 2,4,5-trichlorophenol were detected in urine of Wistar rats orally treated with HCB for 19 days (Engst et al, 1976). Urine of Sprague Dawley rats that received [^{14}C]HCB in oil orally, contained 0.85% of the original dose, with 2,4,5-trichlorophenol, PCP and several unidentified chlorinated benzenes as metabolites (Mehendale et al, 1975). A daily treatment of Rhesus monkeys with [^{14}C]HCB for 78 weeks resulted in about 50 % urinary excretion of PCP, but pentachlorobenzene (25 %) was also found (Rozman et al, 1977). In feces, 99 % of the label was found as HCB, and 1% as pentachlorobenzene. From most studies which involve elimination of HCB, it has become clear that the route of administration of HCB (i.p, orally, i.v.), the period of treatment and the species caused the enormous variations in its half-life (weeks-years), (see Roper, 1990).

Toxicity of PCBs and PCDDs

PCBs and PCDDs

In contrast to the relative lack of data on toxicity of HCB in humans, the toxic effects of PCBs on different organ systems in animals have been well characterised. The toxicity of PCBs is dependent on the compound, dose, duration of exposure and the species involved (see reviews McConnell 1980, George et al, 1988, McFarland et al, 1989, Safe et al, 1990). Important indications of their systemic and, particularly, skin toxicity (chloracne) in humans became clear from examining people in Japan and Taiwan (respectively Yusho and YuCheng episodes) who were exposed to PCBs via contaminated cooking rice oil (Kuratsune et al, 1972, 1976, Urabe et al, 1977, Hsu et al, 1984, Kunita et al, 1984). Skin lesions also were clearly observed in humans involved in the manufacture of PCBs (Umeda et al, 1978).

Symptoms observed in several species of animals chronically exposed to PCBs include tumours, immunosuppression, hepatomegaly, porphyria and impairment of reproduction (Strik et al, 1980, McConnell, 1980, George et al, 1988, Gray et al, 1993). With respect to skin lesions, it appeared that rodents were rather insensitive, while monkeys were highly sensitive to (mixtures of) PCBs (Brouwer, 1987, Van den Berg et al, 1988a, 1988b, Arnold et al, in press).

It is thought that PCB congeners which induce both the MC- and PB type MFOs are toxic (Yoshimura et al, 1979, McFarland et al, 1989), with the former primarily responsible for the carcinogenic or mutagenic response. These signs of toxicity are related to formation of highly reactive, electrophilic epoxides that arise from oxygen insertion into conformationally hindered sites of planar molecules (Parke, 1985). Co-planar PCBs, with at least two chlorine substituents at the meta positions and at the para site appear to be the most toxic forms. This phenomenon is related to the fact that this conformation facilitates binding to the intracellular cytosolic aryl hydrocarbon (Ah) receptor (Nebert et al, 1979), which can result in a greatly increased MFO activity. A good correlation was also found between immuno- and hepato-toxic potency of coplanar PCBs and induction of cytochrome-P450-1A1 in rodents (Safe et al, 1990).

Most studies involving the toxicity of dioxins have been done with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) as a prototype. TCDD is one of the most

toxic coplanar congeners of the PCDDs, and a very potent inducer of various types of MFOs, particularly cytochrome P4501A1 in mammals (Safe et al, 1990). The symptoms that indicate the toxic action of TCDD closely resemble those induced by PCBs. In humans who were exposed to unknown levels of TCDD after an industrial accident, chronic symptoms such as porphyria, chloracne, and hepatic damage were observed (Ashe et al, 1953). Acute symptoms included respiratory tract irritation, dyspnea and nausea. In exposed animals, reproductive disturbances including fetotoxicity and teratogenicity are the major signs, although hepatic damage, immunosuppression and an increased tumour incidence have also been shown (IARC vol 15, 1977, Poland et al, 1984).

As with PCBs, the toxic properties of PCDDs and structurally related compounds may be associated with an interaction with the Ah receptor (Roberts et al, 1985), the coplanarity of the phenyl rings of TCDD facilitating this binding. An excellent relationship has been observed between binding affinity of TCDD for this receptor (Safe et al, 1984) and its potency as an inducer of mono-oxygenase activities associated with cytochrome P450-1A1 (AHH and EROD).

Neurotoxicity in adults

Neurotoxic symptoms from the episode in Japan (Yusho) included headaches, numbness and weakness in limbs and lowered peripheral nerve-conduction velocities (Kuratsune et al, 1972). Various signs of neurotoxicity similar to those of PCBs appeared several specific types of halogenated aromatics, such as phenoxy-acids, dithiocarbamates, organochlorines and various solvents (Bainova, 1982).

Experimental animal studies also reported neurotoxic effects of PCBs, and it appeared that particularly dopaminergic related systems were highly sensitive to PCBs. For instance, pig-tailed-macaques that were treated daily with the PCB mixture Aroclor-1016 for 20 weeks had decreased levels of neurotransmitters (mainly of dopamine) in various brain regions, (Seegal et al, 1991). In addition, evidence was obtained that the degree of chlorination of the PCBs determined whether dopamine levels altered (Seegal et al, 1988). Studies with cultured rat pheochromocytoma-cells (PC 12), which are capable of dopamine and noradrenaline synthesis, including the release and uptake of dopamine, indicated that PCBs induce decreases in cell dopamine

content (Seegal et al, 1989).

From a variety of *in vivo* studies, including those using cultured cells (see review Seegal et al, 1992) it could be concluded that the ortho-substituted PCB-congeners were mainly responsible for the decrease in dopamine concentrations in the brain. This phenomenon may be due to a direct action on tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis.

Neurotoxic effects of PCDDs in humans are hardly reported in literature. A study of birth defects in TCDD-polluted areas in Seveso indicated a possible association of neural tube defects and TCDD occurence (Bruzzi, 1983). In West Virginia, people became exposed to TCDD after an industrial accident (Ashe et al, 1953) and showed acute symptoms of neurotoxicity, such as headache, dizziness, fatigue, nervousness, decreased libido, and intolerance to cold. Chronic symptoms were mainly polyneuropathies.

A central action of TCDD was proposed from animal studies (rats) since serotonin and histamine levels were increased in brain regions directly involved in regulation of food intake (Rozman et al, 1991, Tuomisto et al, 1991). Recently, Stahl et al (1990) could not find evidence in rats for direct effects of TCDD on areas in the brain which regulate food intake, and suggested that TCDD is more likely to be a peripherally acting anorectic agent.

Neurotoxicity during development

From the episode in Taiwan and Japan, it has become clear that infants who were exposed to PCBs either *in utero* or during breast feeding had a delayed psychomotor activity, decreased visual recognition memory and intelligence scores, and impaired mental functions (Rogan et al, 1988, Tilson et al, 1990, Lin Yu et al, 1991, Rogan et al, 1992, Seegal et al, 1992). Hypotonia and hyporeflexia were also observed in these children. Jacobson et al (1984) reported abnormally weak reflexes, low responsiveness, and uncoordinated movements in children born to mothers who had eaten fish products from Lake Michigan (U.S). Furthermore, these children had impaired visual memory recognition at 7 months of age (Fagan et al, 1986).

In animal behavioral and related biochemical studies evidence was found for neurotoxicity of PCBs, particularly with respect to dopaminergic changes. For instance, exposure of mice to 3,3',4,4'-tetrachlorobiphenyl (TCB) induced in the pups an altered response to dopaminergic agonists and antagonists

(Chou et al, 1979). These pups developed a syndrome of a rapid, uncoordinated circling. Agrawal et al (1981) found in 1 year old offspring of TCB-treated pregnant mice lowered levels of dopamine and dopamine receptors in brain. In 6-week old rat pups born to dams that were exposed to mixtures of PCBs (PCB 169 and 77), increased levels of metabolites of dopamine were found. These effects disappeared after 12 months (Sloot, personal communication). Behavioral studies after amphetamine challenge, however, indicated latent differences in the dopaminergic neuronal system after 12 months (Jaspers, personal communication).

A further remarkable finding is a dramatic reduction in the reproductive capacity in offspring of rats, both males and females, after perinatal exposure (Smits van Prooije et al, 1993). Furthermore, exposure of 10-day old mice to TCB resulted in effects on the development of the cholinergic system (Eriksson, 1988), a system involved in processes such as memory and learning, hearing, vision and aggression (Karczmar, 1975). Studies with monkeys exposed to mixtures of PCBs also indicated that behavioral and/or cognitive deficits occurred in the offspring (Schantz et al, 1989). Recently, it had been demonstrated that prenatal exposure of Wistar WU rats to Aroclor 1254 did result in increased levels of astrocytic proteins in cerebellum of 90-days old male offspring (Morse et al, 1993a).

In another recent study (Morse et al, 1993b), it was proposed that PCB-induced neurotoxic responses during development (fetal and neonatal stage) could be due to reductions of thyroid hormones, since the latter are essential for brain maturation.

Toxicity of HCB

HCB

The first indications of toxicity of HCB in humans became evident from an incident in Turkey in the early fifties (Cam et al, 1963, Courtney, 1979, Peters et al, 1982, Cripps et al, 1984), when HCB-treated grain was consumed during a period of crop failure. The amount of HCB consumed was 0.05-0.2 g per day per person during one month. About 5000 people suffered from a disease resembling porphyria cutana tarda, which is characterised by blistering and fragility of the skin, frequently associated with porphyria. The latter is characterised by accumulation and excretion of

intermediate products of the hepatic heme synthesis, caused by a selective inhibition of the enzyme uroporphyrinogen decarboxylase (Elder et al, 1976, Kerklaan et al, 1977, Strik et al, 1980, Debets et al, 1981). Other symptoms were bone abberations, such as arthritis, osteomyelitis and osteoporosis of the limbs. These symptoms were observed in 76 % of exposed males and in 81 % of exposed children between 4 and 14 years of age. A mortality rate of 95 % was observed in suckling infants. These children were probably pre- and postnatally exposed to extremely high levels of HCB by placental transfer and breast milk. Persistent dermatic, hepatic, arthritic, and porphyritic effects of HCB became clear from symptoms twenty to thirty years after the HCB incident, such as hyperpigmentation, hirsutism, fragile skin, painless arthritis, hepatomegaly, jaundice and elevated uroporphyrin excretion in urine and faeces.

Comparable symptoms were seen in animal studies. For instance, (sub)chronic exposure of rats to HCB induced hepatomegaly often in combination with porphyria (Ockner et al, 1961, Strik et al, 1980, Rozman et al, 1986, Kleinman De Pisarev et al, 1989, 1990, Rizzardini et al, 1990), skin lesions and neurological dysfunctioning (Koss et al, 1978). Furthermore, HCB can be a carcinogenic, mutagenic, immunotoxic, or teratogenic compound depending on the species used (Khera, 1974, Cabral et al, 1977, 1979, 1986, Brusick, 1986, Vos, 1986, Barnett et al, 1987).

Neurotoxicity in adults

HCB may induce neurotoxic symptoms in humans as became apparent from an incident in Turkey, where people became exposed to HCB by food (see previous page). The symptoms included weakness, myotonia, paraesthesia, and sensory shading, and some of these were still observed twenty to thirty years after the initial exposure.

Signs of neurotoxicity observed in laboratory animals (mainly rodents) subchronically exposed to HCB by diet included tremors, alopecia, scabbing, ataxia, and convulsions. Brain lesions (hyperaemia and haemorrhage) have also been seen (Kuiper-Goodman et al, 1977, Cabral et al, 1979, Headly et al, 1981). Female beagle dogs that daily received HCB for 3 weeks showed physiologic alterations in the brain, as appeared from dysrhytmias in the electroencephalogram (Sundlof et al, 1981). In female ferrets, who received HCB in their diet for 47 weeks, signs of abnormal aggressiveness and

hyperexcitability were observed (just prior to death) (Bleavins et al, 1984). In the same study, elevated levels of neurotransmitters, such as serotonin, norepinephrine, and dopamine in various brain regions were observed.

Neurotoxicity during development

There are indications that the developing nervous system may also be a target for HCB. For instance, a recent study indicated that oral exposure of pregnant Sprague Dawley rats to HCB at two weeks before partus, resulted in hyperactive behaviour in pups screened over a period of at least 6 days postnatally (PND 6) (Goldey et al, 1992). The hyperactivity was observed for negative geotaxis (PND 6-10), olfactory discrimination (PND 9-11), and exploratory behaviour (PND 15-20). Furthermore, it was suggested that there was a relationship between the neurotoxic action of HCB and the induced deficits of myelin in the brain. It was also postulated that human fetuses and suckling infants exposed to HCB via placental transfer and breast milk, respectively, are at risk for development of HCB neurotoxicity (Goldey et al, 1992).

Thyroid hormones

Structure, function and action of thyroid hormones.

The thyroid gland secretes two hormones, thyroxine (3,3',5,5'-tetraiodo-L-thyronine; T4) and 3,3',5-triiodo-L-thyronine; T3. T4 is considered as a prohormone, while T3 is the active form in most animal species and in humans (Oppenheimer et al, 1976). The chemical structures of these hormones are presented in figure 2.

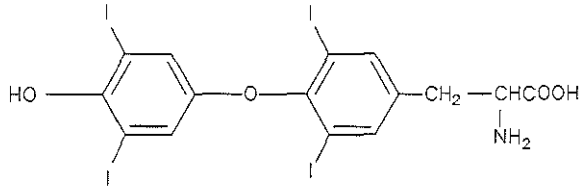
Thyroid hormones are essentially factors for maintenance of homeostasis in warm-blooded mammals by regulating cellular metabolic processes. For instance, these hormones stimulate $Na+/K^+$ ATPase activity which result in heat production (calorigenesis) and increased respiratory oxygen consumption in several organs (Van Hardeveld, 1986, Bjorkman et al, 1990, Kvetney et al, 1990). Other actions include osmoregulation, and stimulation of production and breakdown of proteins, carbohydrates and fatty acids. In general, the stimulating effect of thyroid hormone on breakdown of these compounds is greater than the production. Increased levels of serum lipids

and cholesterol are usually detected in patients with hypothyroidism (Abrams et al, 1981). Symptoms that might be observed in these patients are fatigue, slowness, cold -intolerance, dry skin, heavy weight, hypertensy, bradycardia, and, in severe cases, an alveolar hypoventilation (Laurberg, 1990).

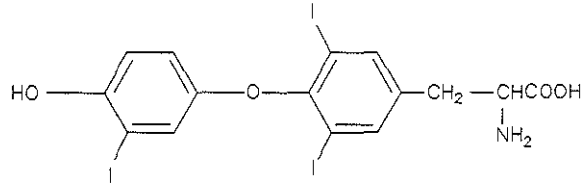
For thyroid hormones to get access to target organs, they have to pass cellular membranes. The latter occurs by sodium and energy dependent

Fig 2. Structure of thyroxine (T4) and 3,3',5-triiodo-L-thyronine (T3)

T4



T3



active transport systems (Docter et al, 1990, Pontecorvi et al, 1989). Effect on cellular differentiation, growth and energy consumption are mainly due to T3 (and much less to T4) via binding to specific, limited capacity, binding sites within the chromatin of the cell nucleus in the target tissues (nuclear receptors), (Eberhardt et al, 1976; Oppenheimer et al, 1983, 1986; Silva et al, 1986; Nikodem et al, 1990). Binding of thyroid hormones to proteins in cell membranes, cytosol and mitochondria have also been described (see review Ichikawa et al, 1991).

Role in cerebral functions

Thyroid hormones are crucial for normal growth, differentiation and development of tissues of higher vertebrates (Dratman, 1978), and are particularly involved in maintenance of a normal development and functioning of the central nervous system during the fetal and neonatal stage of mammalian development (Morreale de Escobar et al, 1983, 1986). Therefore, an adequate and efficient transport of these hormones to the brain is indispensable. An insufficient thyroid activity during the early years of life can result in dwarfism, often combined with mental retardation (cretinism). Learning and memory deficits were observed in young infants with congenital hypothyroidism (Maenpaa, 1972, Macfaul et al, 1978, Sutan-Assin, 1990). Other characteristics of this syndrome are behavioral abnormalities such as lethargy and loss of initiative and energy.

Disturbance of cerebral functions in adult humans with severe hypothyroidism can also develop, such as psychosis, delusions, hearing loss and visual hallucinations, and disorientation (see review of Laurberg, 1990). Relationships of hypothyroidism with depression have also been established (Gold et al, 1981, Tappy et al, 1987).

From micro anatomical studies it appeared that thyroid hormone deficiency may lead to disturbed maturation of several types of organelles (see for review Dussault et al, 1987). For instance, in rats with hypothyroidism, the maturation of synaptosomes and nuclear T3 receptors of the brain were altered (Mussa et al, 1990). During the critical period of rat brain development, the maturation of neuronal synaptic plasma membranes is highly dependent on thyroid hormones. Reductions in neuronal interactions or neurite outgrowth were observed in neonatal rats with inadequate thyroid hormone levels (Eayrs 1964, Legrand, 1977). Rats with hypothyroidism at an age of 12 days, i.e. the period of rapid brain maturation and of maximal uptake of T4 by the brain, developed delayed myelinogenesis (Dalal et al, 1971, Eberhardt et al, 1976). Neurological impairment was also detected in rats when intracellular T3 levels were insufficient during the 'critical period' for brain development, which starts in utero and ends about 10-12 days after birth (Hendrich et al, 1984). Table 2 summarizes specific effects of hypothyroidism on the developing brain of laboratory animals.

Table 2. Biochemical effects of hypothyroidism in the developing brain.

Parameter	Observations[1)]	References
Energy metabolism	Delay in developmental changes in glucose transport rate and ketone body metabolizing enzymes after weaning	Moore et al, 1973 Patel, 1979
Microtubules	Decreased tubulin-tyrosine ligase. Decreased content of tubulin. Defect of assembly.	Gonzalez et al, 1978 Takahashi et al, 1981
Myelin	Decreased content. Delay in development of myelin associated enzymes.	Balazs et al, 1969 Valcana et al, 1975
Cellular types	Delay in maturation of Purkinje cells of cerebellum.	Legrand 1979, 1986
Receptors	Delay of developmental changes in GABA-ergic, muscarinic and ß-adrenergic receptors. Delay in T3 receptors in brain.	Patel et al, 1980 Smith et al, 1980 Mussa et al, 1990

1) See reviews of Nunez (1984) and Dussault et al (1987).

Regulation of thyroid hormone activity

Thyroglobulin synthesis, colloidal uptake of thyroglobulin by the follicular cells of the thyroid gland, and the rate of proteolysis of thyroglobulin for secretion of thyroid hormones, are processes that are under control of thyrotropin (TSH), which is produced by the mammalian adenohypophysis (see review Wilber et al, 1990). If there is a temporary depletion of (free) thyroid hormone supply to tissue, an increase of the secretion of TSH by the

pituitary follows (Emerson et al, 1989). When normal plasma levels of (free) thyroid hormones are reached, the pituitary suppresses TSH production via a coordinated feedback relationship between the pituitary and the thyroid The TSH release is controlled by the thyrotropin-releasing hormone (TRH), a tripeptide produced by the hypothalamus. It is thought that TSH binds receptors on the thyroid membranes, followed by stimulation of adenyl-cyclase and formation of cyclic adenosine monophosphate (cAMP). The latter mediates processes involved in production of thyroid hormones.

Under conditions of hypothyroidism (for instance via iodine deficiency, iodine overdose, treatment with thyreostatic drugs, auto-immune thyroiditis, strumectomy, defects in incorporation of iodine in the thyroid gland, direct damage of the thyroid, pituitary tumours, insensitive thyroid etc), an increased TSH delivery from the pituitary results in an increased vascularisation and hypertrophic enlargement (goitre) of the thyroid gland. Determination of plasma TSH levels is a reliable indirect method to detect disturbances of thyroid homeostasis. A slight increase of plasma TSH level, in combination with normal plasma T3 or T4 levels, without complaints and symptoms is characterised as subclinical hypothyroidism. Clinical hypothyroidism is diagnosed when decreased plasma T4 levels as well as increased TSH levels are detected, in combination with complaints and symptoms.

Transport of thyroid hormones

Nearly all of circulating thyroid hormone is noncovalently bound to transport proteins (Glinoer et al, 1985, De Nayer et al, 1985). The unbound hormone fraction is generally considered as the active hormones at the level of target organs (Oppenheimer, 1968). The primary function of thyroid hormone binding proteins is to act as a buffer system to prevent sudden changes in free thyroid hormone levels.

Three human T4-binding proteins have been described: T4-binding globulin (TBG), transthyretin (TTR) and albumin. Binding characteristics, concentrations and hormone distribution of these proteins, including plasma concentrations in humans are presented in Table 3 and Table 4. The major human thyroid hormone binding protein in plasma is TBG, an acidic glycoprotein which carries about 70 % of all T3 (TT3) and all T4 (TT4) in plasma under physiological conditions (Hollander et al, 1963, Robbins et al,

1978, 1986a, 1986b, Cheung 1985, Murata et al, 1986). The L-alanine side chain conformation, the presence of the phenolic hydroxyl group, and iodines in the inner-and outer ring of thyroid hormones appeared to be essential for optimal binding to TBG (Snyder et al, 1976). TBG is a protein with a molecular weight of 57,000 and an electrophoretic mobility between the alpha 1 and alpha 2-globulins (Oppenheimer, 1968). TBG appears to be composed of a single polypeptide chain with one binding site for thyroid hormones per molecule (Sterling et al, 1971, Cheng, 1977, Gershengorn et al, 1977). For a long time, it was thought that TBG is not present in the circulation of rats. However, Savu et al (1991) found expression and re-expression of the TBG gene during postnatal development and senescence respectively. In this study, TBG was detected in the circulation during postnatal maturation and in old animals. Under hypothyroidic or starvation conditions in young rats, plasma TBG levels increase (Visser, personal communication).

In humans, about 10-15 % of plasma T4 is transported by thyroxine-binding prealbumin (TBPA), also known as transthyretin (TTR). The name TTR was used instead of TBPA to indicate its role in the transport of thyroid hormones in plasma, and to avoid possible confusion with the precursor form of albumin, proalbumin. Not only thyroid hormone, but also retinol-binding protein is transported in plasma by TTR (Kanai et al, 1968).

Human TTR is a stable tetrameric protein (Mw 55 Kd) consisting of four identical subunits (Blake et al, 1974), which are connected by hydrogen bonding and hydrophobic interactions. High-resolution X-ray crystallographic analysis gave insight into the tertiary and quaternary structures of human TTR. With the same technique, analysis of complexes of TTR with thyroid hormones indicated that T4 as well as T3 exclusively interact with two symmetrical sites located deeply within a central channel (Blake et al, 1977, 1978). By allosterical effects, T4 reduces the affinity of the second site for T4 and it is actually suggested that only one T4 molecule binds (Ferguson et al, 1975, De Nayer et al, 1985). Andrea et al (1980) found by using halogen substituents that all four iodine atoms favourably contributed to binding, and suggested a hydrogen-bonding and/or charge transfer interaction between the halogen and TTR. Recently, the interaction of T4 with the T4-binding site of TTR had been described according to a "forward" and a "reverse" binding model (de la Paz et al, 1992). The forward model suggests that interactions are possible between water molecules and the phenolic ring inside the

Table 3. Affinity constants (Ka) and bound percentages for T4 and T3 to human TBG, TTR and albumin.

Plasma-protein	Ka for T4 (M^{-1})	% bound T4	Ka for T3 (M^{-1})	% bound T3
TBG	$1.0\text{-}2.3 \times 10^{10}$	± 65-75	$5.0\text{-}6.0 \times 10^{8}$	80
TTR*	$0.7\text{-}1.0 \times 10^{8}$ (k_1)	± 10-15	$0.9\text{-}1.4 \times 10^{7}$ (k_1)	10
	$7.0\text{-}9.5 \times 10^{5}$ (k_2)		6.0×10^{5} (k_2)	
albu-min#	$5.0\text{-}7.0 \times 10^{5}$ (k_1)	± 10-20	1.0×10^{5} (k_1)	10
	5.0×10^{4} (k_2)		5.0×10^{3} (k_2)	

*: Two values are represented since TTR has two identical binding sites for thyroid hormones.
#: The k_1 values correspond to one binding site for T4 and T3, and the k_2 values correspond to the other (two to six) binding sites. The presented data have been derived from Nilsson et al (1975), Robbins (1976), Robbins et al (1979, 1986a, 1986b) and from Hennemann et al (1990).

protein, under the condition that the phenolic ring is deeply hidden into the TTR-binding site, and the phenyl ring with the amino side chain close to the binding site entrance. The "reverse" model suggests that the hydrogen atoms of the phenolic ring interact with charged residues at the entrance, and that the amino side chain is positioned near the core of the binding site.

The major sites of synthesis of TTR are the liver, the choroid plexus of the brain (Dickson et al, 1985a,b, 1986a,b, Herbert et al, 1986, Kato et al,

1986), the visceral yolk sac (Soprano et al, 1985), and the pancreatic islets (Jacobsson et al, 1979). In rats, the liver is the major organ of TTR degradation, accounting for 36-38% of total body TTR degradation, followed by muscle and skin which account for 12-15% and 1-8% respectively of the total body TTR degradation (Makover et al, 1988).

Table 4. Plasma concentrations of human TBG, TTR and albumin, including of hormone bound complexes

Plasma protein or hormone binding complex	[nmol/l]
TBG	280-510
TTR	2250-4300
albumin	6.4×10^5
T4	110
TBG-T4	82.5
TTR-T4	16.5
Albumin-T4	11
T3	2.3
TBG-T3	1.84
TTR-T3	0.23
Albumin-T3	0.23

The presented data have been derived from Robbins (1976), Robbins et al (1986a, 1986b), and from Hennemann et al (1990).

Besides TBG and TTR, a less specific binding protein that interacts with the thyroid hormones is albumin. It has one strong binding site for T4 and T3 with different K_a values compared with the other (two to six) binding sites

(Table 3). The high concentration of albumin in human plasma results in the binding of about 15 % of circulating T4 (Table 3).

Little information is available about affinities of T4 or T3 for serum binding proteins in laboratory animals. Sutherland et al (1976) found that rat albumin ($K_a = \pm 6.1 \times 10^5\ M^{-1}$) bound T4 significantly more strongly than rabbit albumin ($K_a = \pm 2.3 \times 10^5\ M^{-1}$). In Table 5, the K_a values for the thyroid hormone binding proteins of the rat are presented. cDNA nucleotide sequence analysis demonstrated 83 % similarity between the primary structures of human and rat TTR (Sundelin et al, 1985) and 73 % between human- and rat albumin (Sargent et al, 1981, Dugaiczik et al, 1982). In rats, TTR is the major T4 carrying plasma protein since it binds 70 % of all T4 (Sutherland et al, 1976).

Table 5. Affinity constants (Ka)* for binding of T4 and T3 to rat TBG, TTR and albumin*.

Plasma-protein	Ka for T4 (M^{-1})	Ka for T3 (M^{-1})
TBG	$>1 \times 10^9$	1×10^9
TTR	$0.4\text{-}3.5 \times 10^8$ (k_1)	1×10^6
	9.5×10^5 (k_2)	
albumin	6.1×10^5	0.9×10^5 (#)

*: See Ferguson et al (1975), Sutherland et al (1976) and Savu et al (1989).
(#) = estimated value.

With affinity chromatography on thyroxine-Sepharose, a new thyroxine binding protein was isolated from human blood plasma, namely a 25 Kda

apo-lipoprotein A-1 (apo A-1), a component of high-density lipoproteins. Various forms of apo A-1 had different T4-binding activity (K_a's 4-7.5. 10^7 M^{-1}). The interaction of apo A-1 with T4 involves mechanisms that differ from the complex formation of iodothyronines with albumin, TTR,and TBG (Sviridov et al, 1991).

Metabolism of thyroid hormones by glucuronyltransferases and deiodinases

The metabolism of thyroid hormones occurs through a variety of pathways in several organ systems. In mammals, conjugation of the phenolic hydroxyl group with glucuronic acid or sulphate are important metabolic routes of T4 and T3 (Visser, 1988a, Visser 1990a, Visser et al, 1990b). These processes are dependent on UDP-glucuronyltransferase (UDPGT) generating glucuronide derivates. The thyroid hormone-UDPGTs are homologous enzymes that vary in their substrate specificities and their activity after treatment with microsomal enzyme inducers, such as 3-methylcholanthrene, phenobarbital, PCBs, and dioxins (Visser et al, 1993b, Curran et al, 1991).

Recent studies suggest that T4 is glucuronidated by both clofibrate-inducible bilirubin and MC-inducible phenol UDPGT, and that androsterone-UDPGT is responsible for the glucuronidation of T3 (Visser et al, 1993a, Beetstra et al, 1991). Visser et al (1993b) discovered three different classes of UDPGT-isozymes responsible for glucuronidation of thyroid hormone. Type 1-UDPGT conjugates T4, rT3 (3,3',5'-triiodothyronine, reverse T3) and bilirubin, and is induced *in vivo* by clofibrate and *in vitro* by the detergent Brij 56. Type 2-UDPGT has conjugation activity towards T4, rT3 and phenols, and is induced *in vivo* by MC-type compounds but inhibited *in vitro* by Brij 56. Type 3-UDPGT conjugates androsterone and T3, and is hardly influenced by comparable *in vivo* or *in vitro* treatments.

The metabolic reactions that use UDPGTs with thyroid hormones as a substrate, occur mainly in the liver and kidney and lead to inactivation and facilitation of excretion of the hormones by increasing their water solubility. A part of the produced thyroid hormone glucuronides are hydrolyzed by microbial gut flora, whereafter free hormones become available again via enterohepatic circulation (Rutgers et al, 1989). Besides glucuronidation, sulfation of the phenolic hydroxylgroup is possible. T3-sulfate (T_3S) is rapidly deiodinated, resulting in a biliary excretion of T3 mainly as T3-glucuronide

(T_3G).

Another route of metabolism of thyroid hormones is the conversion of T4 to T3 by the hepatic type 1 deiodinase that accounts for approximately 80 % of T3 production in humans, the remainder being produced by the thyroid gland (Engler et al, 1984, De Nayer et al, 1985, Visser et al, 1988b). In the central nervous system at least 50% of intracellular T3 is derived from local deiodination of T4, while in liver and kidney circulating T3 is the major source of intracellular T3 (Davies et al, 1991). Another product of deiodination of T4 is reverse T3, which is produced by removal of an iodine in the 5'-position. Deiodination of T3 and rT3 forms three possible diiodothyronines (T2); 3,3'-T2, 3',5'-T2 and 3.5-T2 (Engler et al, 1984).

At least three different iodothyronine deiodinases occur in rats : type 1 is capable of deiodination of both the inner and outer ring of T4 and is susceptible to inhibition by 6-propyl-2-thiouracil (PTU), type 2 has only outer ring deiodinase activity and is not sensitive to PTU while type 3 facilitates only inner ring deiodination and is not sensitive to PTU (Visser et al, 1990a,b). Sulfation of T3 seems to be a prerequisite before it is deiodinated (De Herder et al, 1988, Visser et al, 1990a,b).

Metabolism of T4 or T3 by oxidative deamination and decarboxylation of the alanine chain leads to acetic acid analogues (tetraiodothyroacetic acid and triiodothyroacetic acid), but there is no evidence that they are physiological relevant. The metabolic pathway via cleavage of the ether bridge, yielding diiodotyrosine, occurs mainly during infections (Burger et al, 1981). Under normal conditions this route is of minor importance compared with conjugation and deiodination (Engler et al, 1984).

Transport mechanisms of thyroid hormones with brain as target

General aspects

The brain is an important target for thyroid hormones (Shambaugh, 1986), especially during embryonal development (Morreale de Escobar, 1983, 1986). Thyroid hormones enter the brain through the blood brain barrier, which can be divided into a cerebral capillary barrier on the one hand, and a barrier formed by cerebroventricular organs on the other. The latter is formed for a major part by the epithelial cells of the choroid plexus (CP). In mammals, including humans, the CP is able to produce cerebrospinal fluid

(CSF), (Cserr, 1971, Nilsson et al, 1992) and possesses highly specific mechanisms for the transport of essential nutrients required for adequate brain function (Neuwelt, 1989). In humans, the production of CSF occurs for 30 % in the walls of the subarachnoidal space, for 33 % at the ependymal surface of the ventricles, and for 37 % at the CP (Cserr, 1971). In figure 4, the structural and functional relationships of blood supply, CSF and extracellular and intracellular fluid compartments of the brain are presented.

In the CP of many animal species, several highly specific mechanisms for transport (active, carrier-mediated, facilitated transport) of compounds essential for brain function, such as vitamins, nucleosides, purines and amino acids, have been identified (Spector, 1989, Neuwelt, 1989). In addition, the CP also possesses a specific carrier-mediated system for removal of toxic organic acids out of the brain (Rapoport, 1976). A few *in vivo* studies involving mechanisms of transport of thyroid hormones from blood to the brain are known.

With respect to the fast uptake of T3 into the brain by cerebral capillaries(<15 s), using bolus injections into the carotid arteries of rats, a saturable, facilitated (not active) transport mechanism for T3 operating at the capillary blood-brain barrier was found (Pardridge, 1979). It has been suggested that this route of transport is very important, since the surface area of the cerebral capillaries is 5000-fold higher compared with those of the cerebroventricular organs (Crone, 1971).

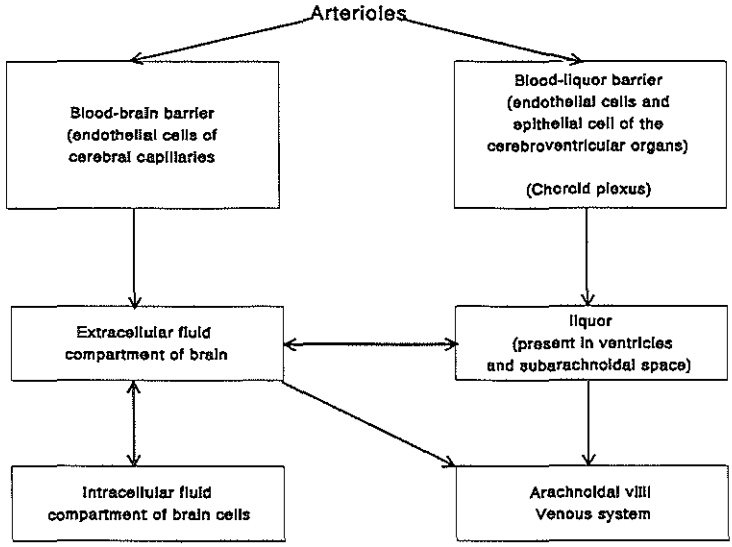
Several studies indicate that T4 and T3 pass also blood-CSF barriers. For instance, indications were found for a saturable carrier-mediated transport using infusions of [^{125}I]T4 in dogs (Hagen et al, 1974), based on an influx from blood into CSF against a concentration gradient. The latter was based on much higher levels of free T4 in CSF compared with plasma. However, Banks et al (1985) demonstrated in mice carrier-mediated systems for the transport of T4 from CSF to blood, and no carrier-mediated system was found for the opposite route. Recently, a saturable uptake of [^{125}I]T3 at the blood - and CSF side of perfused sheep CP was demonstrated (Preston et al 1992).

Role of choroid plexus and TTR in thyroid hormone transport

A special role for TTR as a carrier protein for transport of T4 from blood to the brain has been suggested. Various studies indicated *de novo*

synthesis of TTR in the rat and human brain, independent of hepatic TTR, and this particularly occurs in the CP (Soprano et al, 1985, Kato et al, 1986, Dickson et al, 1985a, b, 1986a). High levels of mRNA coding for TTR or could be identified in the CP of rats (Dickson et al, 1985a, b, 1986a, Stauder et al, 1986) and humans (Dickson et al, 1986b). In addition, CSF collected from the surface of perfused sheep CP was shown to contain highly radioactive TTR after addition of [^{14}C]leucine to the perfusion medium. Furthermore, TTR was released at the apical side (towards the brain, and not at the blood side) of the epithelial cells of the CP. These phenomena indicated a high-capacity uptake of T4 in the CP, and an unidirectional transport of TTR from the CP

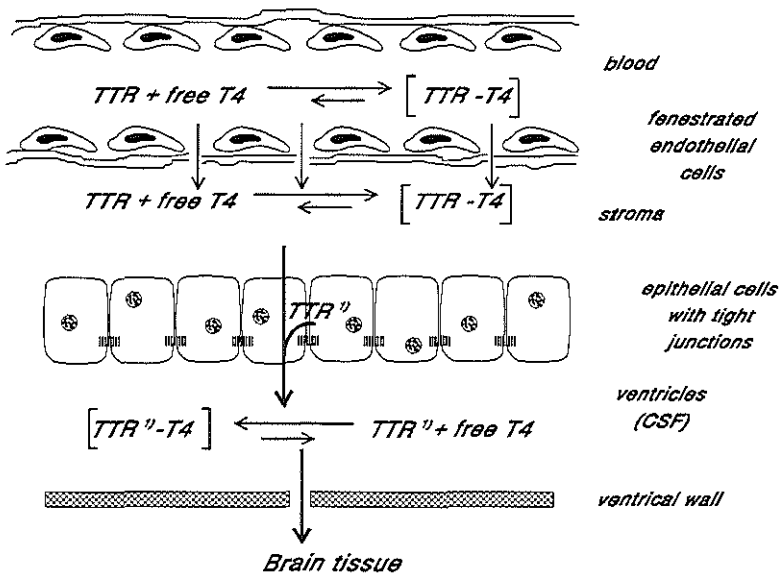
Fig. 4. Relationships between the major compartments of the brain



to the CSF (Schreiber et al, 1990). An earlier study (Dickson et al, 1987) demonstrates that i.v. administered [^{125}I]T4 in rats not only accumulated in the liver, blood, kidney and pituitary, but also in the CP. Furthermore, a delay in uptake of T4 in several brain structures was observed compared with CP.

No accumulation of label was observed in CP or brain after an intravenous injection of [^{125}I]T3. Isolated CP of rabbits also accumulated T4, but not T3, from CSF medium (Spector et al, 1975).

Fig.5. Model for transport of thyroxine (T4) via choroid plexus.



1) TTR synthesized by epithelial cells of choroid plexus

Such observations led to several assumptions involving transport of T4 to the brain (Schreiber et al, 1990). Firstly, the fenestrated capillaries of the CP give access of T4 or T4-TTR complexes to the interstitium of the CP, where available free T4 passes membranes of the epithelial cells of the CP. In the CP, T4 binds to newly synthesized TTR (Fig. 5). The exact place of binding, i.e intracellular, near membranes or extracellular, has still to be identified. The TTR-T4 complex is further transported by the flow of CSF through the ventricles and the sub-arachnoidal space of the brain.

TTR derived from the CP (and probably also TTR in CSF) has a similar primary structure as hepatic TTR (Duan et al, 1989). The amounts of TTR-bound thyroid hormone in CSF are essentially different from plasma. For instance, in euthyroid persons, 12 % of T3 and 44 % of T4 is bound to TTR

in the CSF, while 3% of T3 and 17% of T4 is bound to plasma TTR (Hagen et al, 1973). It is interesting to mention that patients with central hypothyroidism and severe depression had lowered levels of TTR in CSF (Hatterer et al, 1993).

Chanoine et al (1992) found experimental support for TTR in CSF of rats being primarily derived from CP, and not from plasma. In addition, it was shown that transfer of T4 from plasma via CP to CSF was determined by the free T4 levels in plasma and the amount of binding of T4 to TTR derived from the CP.

From a toxicological point of view, a TTR-mediated transport system for T4 in the choroid plexus may be of interest since a number of industrial aromatic compounds have affinity for the T4-binding site of TTR. Therefore, T4 transport to the brain may be disturbed by these compounds. In the thesis, this aspect is elucidated in Chapter VIII and IX.

Toxicity of PCBs and PCDDs for thyroid homeostasis

Human studies

Information about an altered thyroid hormone status in humans induced by PCBs or PCDDs is scarce, but in industrial workers chronically exposed to organochloropesticides, or polybrominated biphenyls (PBBs), evidence for disturbed thyroid physiology was found (Wassermann et al, 1971, Bahn et al, 1986, Marshall et al, 1968). Many subjects showed decreases in plasma total T4 (TT4) and increases in TSH levels. Significant decreases in serum TT4 levels were also observed in transformer maintenance workers exposed to Aroclor 1260 for 4 years (Emmett et al, 1988). However, examination of thyroid function in subjects 16 years after the incident in Yusho revealed significantly raised serum levels of both total T3 (TT3) and TT4 with no differences in TSH and TBG levels in serum (Murai et al, 1987). Ten years after an accident in Seveso (Italy), where a wide area became contaminated with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) by an explosion in a chemical factory, an increased incidence of thyroid cancer has been observed in women who lived in the neighbourhood of the accident (Bertazzi et al 1989).

Animal studies

A direct sign of thyroid toxicity in non-human primates (marmoset monkeys) was the dramatic reduction in serum TT4 levels (99 %) (over the whole exposure period) during repeated dosing of 3,3',4,4'-tetrachlorobiphenyl (TCB) for 18-23 weeks (Van den Berg et al, 1988a,b). Other indicative parameters of thyroid hormone status, such as the free T4 index and serum TT3 levels, were decreased, while TSH and T3 resin uptake were increased. The follicular cells of the thyroid glands of exposed animals appeared highly hyperplastic, and this phenomena was closely correlated with decreased serum TT4 and increased TSH levels. These effects took place at relative low doses and occurred early after exposure (Van den Berg, 1988a,b). Tryphonas et al (1986) demonstrated also histopathological alterations in thyroids of Rhesus monkeys exposed to Aroclor 1254 (a mixture of PCBs) for two years.

Reduced thyroid hormone and/or vitamin A levels in plasma were also observed in rodents exposed to PCBs or PBBs (Sleight et al, 1978, Brouwer et al, 1985, 1986a, 1986b, Brouwer, 1987, 1989). Studies with eight week old male Osborne-Mendel rats exposed to Aroclor 1254 for 12 weeks indicated ultrastructural lesions in thyroid follicular cells associated with lowered serum TT4 levels (Collins et al, 1977, 1980). A post-exposure period of 35 weeks was necessary to get TT4 values back to base levels and follicular cells in a normal condition. Enlarged thyroids induced by PCBs were also observed in fish-eating animals such as salmon and seagulls (Barsano, 1989). Other wildlife species, such as cormorant chicks and harbour seals, that lived in Dutch areas contaminated with PCBs, had decreased plasma thyroid hormone levels (Craane et al, 1990, Brouwer 1991). Pups derived from Sprague-Dawley rats daily exposed to 2,3',4,4'-5-pentachlorobiphenyl or 2,2',4,4',5,5'-hexachlorobiphenyl/kg/day had decreased serum TT4 levels in conjunction with increased follicular cell vacuolization and nuclear vesiculation within thyroids (Ness et al, 1993). Daily exposure of 31-day old Fisher rats to a very low dose of Aroclor 1254 (0.1 mg/kg) for 15 weeks resulted in highly significant decreases of TT4 levels in serum (Gray et al, 1993). In general, determination of TT4 levels in serum may possibly be a suitable and/or sensitive biomarker for (sub)chronic exposure of living organisms to PCBs. Direct evidence for thyroid toxicity was also found in rodents that were exposed to PBBs or pesticides (Gupta et al, 1983, Bainova,

1982). Furthermore, prenatal exposure of pregnant rats to PCBs (3,3',4,4',5,5'-hexachlorobiphenyl) resulted in decreased plasma levels of TT4 and free T4 (FT4) in maternal and neonatal animals (day 21 postpartum), (Morse et al, 1993b).

Indications of disturbed thyroid physiology by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) have been derived from many animal experimental studies. For instance, a single oral dose of TCDD (25 μg/kg) to rats resulted in 50 % reduction of serum TT4 levels, and in elevated serum TSH concentrations, nine days after treatment (Bastomsky, 1977). Male Sprague Dawley rats and male Syrian golden hamsters, which were exposed to several i.p doses of TCDD (0-500 μg/kg), developed changes in serum TT4 levels (Henry et al, 1987). The response of serum TT4 levels appeared species dependent, since in hamsters these levels were increased, and in rats decreased by TCDD. Furthermore, rats appeared more sensitive to TCDD-induced alterations in TT4 levels. Potter et al (1983) demonstrated that one week after administration of TCDD to male Sprague Dawley rats, serum TT4 levels declined to 46 % of controls. In this study, a possible relationship existed between the TCDD-induced hypothermia and lowered serum TT4 levels. These observations could not account for the observed lowered food uptake by TCDD.

Rozman et al (1984) showed that thyroid hormones may mediate TCDD-induced toxicity. This finding was based on the observations that male thyroidectomised Spraque Dawley rats, exposed to TCDD, developed a much slower loss of body weight than nonthyroidectomised-euthyroid rats or thyroidectomised, T4-substituted rats. These results suggest that thyroidectomy may be protective against lethal effects of TCDD.

Disturbances of thyroid homeostasis by PCBs or PCDDs, in particular the reductions of plasma TT4 levels, may have consequences for proper brain function of adults, and for the development of the brain of growing animals.

Toxicity of HCB for thyroid homeostasis

Human studies

Most information about disturbed thyroid physiology in humans has been obtained from an incident in Turkey, where people had eaten food contaminated with HCB (Table 1). In addition to other toxic signs, reduced

plasma TT4 levels were found in conjunction with enlarged thyroids (Peters et al, 1982). The latter occurred in 60 % of the screened females, and in 27 % of the men. In 50 % of the females, very low values of serum TT4 were detected. Recently, Grimalt et al (1994) found possible indications for HCB-induced thyroid neoplasms.

Animal studies

Enlarged thyroids were observed in pigs exposed to HCB for 8 weeks (Den Tonkelaar et al, 1978). In Syrian golden hamsters, that were daily exposed to HCB by diet, thyroid adenomas were observed at the end of life (Cabral et al, 1977, 1986). Increased thyroid weights were observed when Syrian golden hamsters received HCB for 6 weeks in the diet (Smith et al, 1987). Reduced TT4 and TT3 levels in blood were also detected in Sprague-Dawley rats, which received dietary HCB for 55 days (Rozman et al, 1986). Termination of the treatment did not result in a return to normal serum TT4 values. Significant reductions of TT4 and free T4 (FT4) plasma levels were observed in female Wistar rats exposed to HCB (0.03 % in diet) for 5 or 13 weeks (Den Besten et al, 1993). Compounds structurally similar to HCB, such as 1,2-dichlorobenzene, 1,4-dichlorobenzene, 1,2,4-trichlorobenzene, 1,2,4,5-tetrachlorobenzene and pentachlorobenzene also induced a reduction in plasma TT4 levels in male Wistar rats after a single dose (Den Besten et al, 1992). In addition, subchronic oral exposure of rats to several trichlorobenzene isomers (Côté et al, 1988) or tetrachlorobenzene isomers (Chu et al, 1984) led to an altered thyroid morphology.

Mechanisms of toxicity of aromatic halogenated hydrocarbons (AHHs)

Interaction with the Ah receptor

An important non-specific cellular mechanism underlying the capacity of AHHs to induce MFOs by AHHs is the interaction with the Ah (Aryl hydrocarbon) receptor (Brunstrom, 1992). The latter is an intracellular protein that is produced by a structural gene, associated with the Ah locus. After binding of a xenobiotic compound (e.g. a halogenated aromatic carbon) to the Ah receptor, a set of regulatory, structural and possibly temporal genes, involved in controlling many species-dependent and tissue-specific

responses (Poland et al, 1976, Safe, 1985, Safe et al, 1990) may become simultaneously activated. Thereafter, a number of toxic responses may be induced (pleiotropic response), (Safe, 1984). In detail, the Ah-receptor-ligand complex translocates to the nucleus and interacts with the so called dioxin responsive element (DRE), localised in the 5' upstream region of genes, which become activated (Denison et al, 1989, Safe et al, 1990). This is followed by enhanced transcription and translation of mRNAs that code for proteins (mainly MFOs) that are capable of biotransformation of the xenobiotic compound involved (Nebert et al, 1979, Roberts et al, 1985, Safe et al, 1990).

Binding of AHHs with the Ah receptor is highly dependent on their structure. For instance, coplanar dioxins or PCBs show in general a much stronger interaction than non-coplanar structures. In case of binding of coplanar PCBs or TCDD, conversion of the parent compound to less or rather more toxic metabolites occurs primarily by cytochrome P450-1A1.

Interference of AHHs with thyroid and/or vitamin-regulated systems has been suggested to be a mechanism of induction of various toxic responses (Neal et al, 1979, Poland et al, 1982, Rozman et al, 1984, Thunberg et al, 1984). It appeared that most signs of toxicity, mainly observed in exposed rodents and non-human primates, closely resembled clinical symptoms observed under thyroid hormone or vitamin A-deficient conditions. For instance, PCBs and PCDDs are efficient inducers of body weight loss by reduced feed intake (wasting syndrome), heart beat disturbances, skin lesions (this symptom is species dependent), increases of serum total lipid levels, and reductions of vitamin A and thyroid hormone levels in plasma (Innamini et al, 1974, McConnell, 1980, Brouwer et al, 1986a,1986b, Van den Berg et al, 1988a,b, Brouwer, 1991).

Prolonged reductions of thyroid hormone levels may pose a threat to maintenance of important physiological processes in fetal, neonatal, and adult organisms, such as growth and differentiation, organ development (brain), and reproduction (Schwartz, 1983, Legrand 1977, 1986). Furthermore, induced hypothyroidism may contribute to enhancement of the neurotoxic effects of AHHs, since major clinical symptoms in patients include behavioral changes (depression), psychological abnormalities and even seizures (Laurberg, 1990).

Numerous studies indicate increased thyroid hormone metabolism by AHHs via induction of phase II enzymes as an important event for

explanation of decreased thyroid hormone levels in blood. With respect to PCBs and PCDDs, increases in several metabolic parameters, such as hepatic T4-glucuronyltransferase activity, biliary T4 glucuronide production, bile flow, and biliary hormone clearance had been demonstrated in exposed animals (Bastomsky, 1974, 1976a,b, Henry et al, 1987, Beetstra et al, 1991).

Similar effects were also observed in animals dosed with HCB. For instance, female Wistar rats that were treated with high doses of HCB (1000 mg/kg) for 4 weeks (Kleinman de Pisarev et al, 1989) showed a 6-fold increase in the metabolic clearance of T4. In addition, serum thyrotropin (TSH) levels and ^{125}I-thyroidal uptake were also highly increased and indications of interference with deiodinative metabolism of T4 were obtained. Recently, Van Raaij et al (1993) found a relationship between HCB-induced reductions of TT4 levels in serum and increased activity of T4-glucuronyltransferase. In another study, HCB-induced reductions of TT4 levels were not correlated with an impaired thyroid hormone synthesis, indicating an extrathyroidal effect on thyroid homeostasis (Kleinman De Pisarev et al, 1990).

Also mixtures of PCBs enhance T4 metabolism. For instance, reductions of TT4 and FT4 levels in serum were observed in thyroidectomised Sprague Dawley rats that received replacement therapy with T4 and T3 to normal euthyroid levels following a dose of Aroclor 1254 (Barter et al, 1992). These observations indicated enhanced activity of T4 metabolizing enzymes. Bastomsky (1977) obtained evidence for a rapid increase of biliary clearance of plasma [^{125}I]T4 after a single dose of 2,3,7,8-dibenzo-p-dioxin (TCDD) to rats. In contrast, TCDD did not alter biliary excretion of T3. One study of Byrne et al (1987) indicated evidence that exposure of rats to Aroclor 1254 for 5 months induced decreases of TT4 and TT3 levels in serum over the whole exposure period, due to damage of the thyroid itself. An increased hepatic or peripheral catabolism of T4 was not involved since the T4 metabolic clearance rates were not altered by this treatment.

Interaction with thyroid hormone binding proteins

During the past few years, interactions of AHHs with thyroid hormone binding proteins as a possible mechanism by which disturbances of thyroid homeostasis could be induced have become of interest. Special attention has been paid to transthyretin (TTR), a transport protein for thyroid

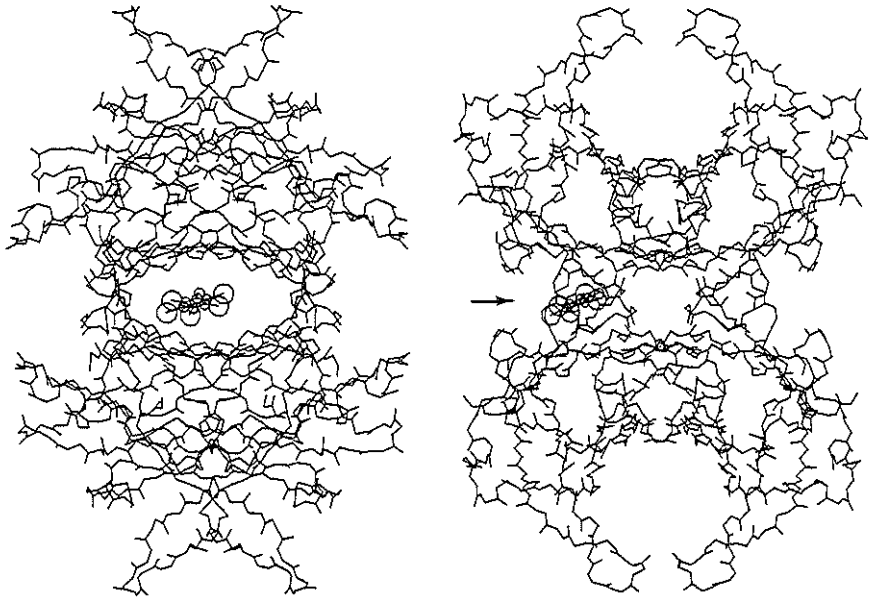
hormones and the retinol-retinol binding (retinol-RBP) complex in mammals, including humans (Brouwer et al, 1986a, 1986b, Brouwer, 1987).

The structure of TTR consists of four identical subunits, which form a central channel. Two identical T4-binding sites lined with hydrophobic amino acid side chains form polarizable pockets for halogenated benzene interactions. Van den Berg (1990) showed with respect to the chlorophenols that affinity for the T4-binding site of TTR was predominantly determined by the position and number of chlorine atoms. The most efficient binding occurred when chlorine was present in both ortho positions to the hydroxyl group. Furthermore, according to Scatchard analysis it appeared that the interaction of pentachlorophenol (PCP) with TTR was competitive with T4. It was concluded that the combination of hydroxyl and chlorine groups did result in a higher affinity for the T4-binding site of TTR than each separate group. Within the chlorophenols, PCP had the highest relative affinity for this site. Den Besten et al (1991) confirmed the latter phenomenon, and showed a correlation between relative binding affinities of phenolic metabolites of various chlorinated benzenes for the T4-binding site of TTR and the magnitude of plasma TT4 reduction in male Wistar rats. A clear increase in affinity for TTR was demonstrated from dichlorobenzene, via trichlorobenzene to HCB. No interaction was detectable for monochlorophenol or pentachlorobenzene (PECB). Furthermore, it was suggested that cysteine residues were not involved in binding of T4 to TTR, since tetrachloro-benzoquinone, that possess a strong affinity for protein thiols, had no affinity for T4-binding sites on TTR. Figure 6 shows a PCP molecule fitted into the T4-binding pocket of a TTR tetramer using a molecular modelling program and crystallographic data from Blake and coworkers (1974, 1978).

Also with respect to PCBs, there is evidence that interactions of the hydroxylated metabolites with T4-binding sites of plasma proteins play a role in the mechanism responsible for reductions of plasma TT4 or vitamin A levels. This has become apparent from studies with rats that were exposed to one of the most toxic coplanar PCB congeners, i.e. 3,3',4,4'-tetrachlorobiphenyl (TCB). It could be demonstrated that the 5-monohydroxy-derivative (5-OH-TCB) had a relative high affinity for the T4-binding site of TTR. In addition, serum TT4 levels in marmoset monkeys exposed to TCB, remained lowered as long as 5-OH-TCB was present (Brouwer, 1987). It further became clear that hydrolytic dechlorination at the meta or para position of a phenyl ring facilitated the interaction as was demonstrated with

hydroxylated metabolites of TCB (Brouwer et al, 1990). Rickenbacher et al (1986) demonstrated that the interaction of hydroxylated PCBs with TTR was 3-8 times more effective compared with the interaction of T4 itself, and that the highest binding affinity was observed for the laterally substituted PCBs (3,3',5,5'-), which closely matched the structure of the diiodophenolic ring of T4. Chlorine substitution on nonlateral (2,2',6,6'-) positions resulted in a decrease in binding capacity. A planar structure of PCBs, with a hydroxyl-group and chlorines in lateral positions appeared an ideal condition to interact with the T4-binding site of TTR.

Fig. 6. Interaction of pentachlorophenol (PCP) with the T4-binding site of TTR



Front view Side view

With data from computer molecular modelling studies using X-ray crystallographic measurements, and from experimentally measured binding affinities, it was also demonstrated that hydroxylated derivatives of dioxins and dibenzofurans, or PCB isomers and hydroxy-derivatives of PCBs, fitted

well into the T4-binding-pocket of TTR (McKinney et al, 1985a,b, Rickenbacher et al, 1986). It was generally demonstrated that AHHs containing one phenyl ring, or linear molecules with multiple rings, had a much higher affinity for TTR than normal angular diphenyl-ether bridged thyroid hormone analogues. Comparable phenomena were observed when hepatic nuclear extracts were tested for binding of PCBs (McKinney et al, 1987). From a recent study that used competitive binding assays (Lans et al, 1993), it has become apparent that interaction of hydroxy-PCBs with the T4-binding site of human TTR was most favourable under the following conditions; 1) the hydroxygroup substituted on the *meta/para* positions, and 2) one or more chlorine atoms substituted adjacent to the hydroxy group on either or both aromatic rings. In the same study, hardly any difference was observed in T4-displacement potency between the non-*ortho*-, mono-*ortho*- as well as di-*ortho*-hydroxy-PCBs, and coplanarity was not an essential condition for competitive binding of hydroxy-PCBs to TTR.

Since TTR has a T4-binding site that is complementary to the DNA double helix (Blake et al, 1977), it has been postulated that the thyroid hormone agonistic and/or antagonistic properties of PCDDs, TCDD in particular, were due to interactions with nuclear thyroid hormone receptors in tissue (Blake et al, 1977, McKinney et al, 1985b). This event might be an additional mechanism underlying toxicity besides their high potency to interact with the Ah receptor. A later study however, indicated that TCDD has no intrinsic thyromimetic activity (Roth et al, 1988). Not only hydroxylated PCBs or chlorophenols, but also other types of industrial halogenated aromatics, such as chlorophenoxyacids and dichloro-diphenyl-trichloroethanes (DDTs) appeared to be effective competitors for the T4-binding pocket of TTR (this thesis).

Information about interactions of AHHs with other thyroid hormone-binding proteins, such as albumin or thyroxine-binding globulin (TBG) is scarce. Van den Berg (1990) demonstrated competitive interactions of PCP for the T4-binding site of TBG and albumin, although the affinity was much lower compared with TTR (relative affinities amounted to 0.001, 0.25 and 1.7 respectively). Furthermore, interactions of *o,p'*-DDD with TBG were demonstrated (Marshall et al, 1968). Equilibrium dialysis experiments showed that some benzene derivatives, such as phenols and benzoates were able to displace T4 from the low-affinity T4 site on human serum albumin (HSA), which was dependent on 1) the presence of highly polarizable substituents,

such as iodine atoms, and 2) the presence of anionic groups (Tabachnick et al, 1970). In addition, the interaction of phenols with T4-binding sites on HSA increased with the number of halogen substituents associated with reduction of the pKa value. The herbicide 3,5-diiodo-4-hydroxybenzonitrile (ioxynil) interacts with TTR, but no affinity for TBG or albumin was found (Ogilvie et al, 1988)

The observations described above led to the hypothesis that interaction of hydroxylated AHHs with TTR are for a large part responsible for reductions of T4 or vitamin A levels in plasma (Brouwer et al, 1986a,1986b). Brouwer (1987) suggests that the plasma T4 reductions are the result of binding of the hydroxylated metabolite with the T4-binding site of TTR and a rapid removal of T4 levels from the circulation. The reductions of plasma vitamin A levels may be due to subsequent conformational changes of the TTR bound retinol-RBP complex (Brouwer et al, 1988), destabilization of binding and finally loss of this complex.

Outline and aim of the thesis

From various previous sections (part B), it is clear that exposure of several mammalian species, including humans, to aromatic halogenated hydrocarbons (AHHs) may cause toxic effects. In particular, symptoms of neurotoxicity in exposed juveniles and adults may be induced. In addition, studies report altered thyroid homeostasis induced by AHHs. This results mainly in reductions of thyroxine levels in blood. Since thyroid hormones are required for proper brain function and for development of brain during neonatal and early life, inadequate thyroxine levels may contribute to various neurotoxic responses.

In this thesis, hexachlorobenzene (HCB) was chosen as a model compound for AHHs to give insight in 1) mechanisms underlying altered thyroid hormone levels by HCB in the circulation, 2) the relative contribution of enhanced metabolism and competitive interactions in reduction of thyroxine levels, 3) the consequences of exposure to HCB for thyroxine transport to the brain, 4) the question whether transport of thyroxine and pentachlorophenol (PCP), i.e. the major hydroxylated metabolite of HCB, into cerebrospinal fluid is mediated by a transport mechanism based on transthyretin. These four aspects comprehend the aim of the study.

The major reasons to chose HCB were: HCB has a the most simple

structure in the class of the AHHs and shows comparable physical-chemical properties with PCBs. Furthermore, HCB exhibits a large similarity with other AHHs concerning toxic effects, including neuro-and thyroid toxicity observed in humans and several animal species (see previous sections of part B).

The first experiments (Chapter II) focused on the question whether repeated exposure of rats to HCB lead to reductions of serum thyroid hormone levels, or morphologic changes of thyroid glands. In addition, the activity of hepatic microsomal mixed function oxidases and serum HCB and PCP concentrations were determined, since hydroxylated metabolites of AHHs are possibly causal factors for induced alterations of thyroid hormone levels in blood.

In Chapter III, it is described whether HCB as parent compound, or rather its major metabolites, such as PCP and tetrachlorohydroquinone are able to affect rat serum thyroid hormone levels after a single equimolar i.p dose.

Interactions of PCP or HCB with thyroxine binding sites of serum proteins, and their involvement in altered serum thyroxine levels induced by HCB or PCP are described in Chapter IV. For these experiments, sera or serum extracts were obtained from HCB-or PCP-treated rats to investigate occupation of thyroxine binding sites.

The interaction with serum proteins, in particular transthyretin , is possibly a characteristic of several groups of industrial halogenated compounds, particularly solvents and pesticides. A binding assay was used to estimate the degree of interference of chemical compounds with the thyroxine binding sites of serum proteins (Chapter V).

In Chapter VI, effects of HCB on the activity of hepatic thyroid hormone metabolizing enzymes, such as thyroxine and triiodothyronine UDP-glucuronyltransferases, are described. Furthermore, it was investigated whether biliary excretion of thyroxine and/or bile flow were altered by HCB.

Experiments were performed (Chapter VII) to investigate the significance of competitive interactions of PCP with thyroid hormone binding proteins in HCB-induced alterations of total or free thyroxine levels in serum. Therefore, rats were repeatedly exposed to high doses of HCB followed by determination of PCP and thyroxine levels in serum.

Interactions of AHHs with transthyretin may have consequences for the supply of thyroxine from blood to the brain, since an active role for transthyretin in thyroxine transport from blood to the brain has recently been proposed (Schreiber et al, 1990). When this assumption is valid, it may also

implicate that AHHs with high affinity for the thyroxine binding site of transthyretin enter the brain by a manner comparable with thyroxine. In Chapter VIII, it was investigated in an experimental rat model whether thyroxine transport to cerebrospinal fluid and brain was influenced by HCB or PCP. In addition, this was associated with determination of HCB and PCP in brain and cerebrospinal fluid. Furthermore, entrance of PCP into cerebrospinal fluid was determined in separate experiments.

Experiments described in Chapter IX concerned the role of transthyretin in transport of thyroxine and various AHHs through the blood-cerebrospinal fluid barrier. Therefore, various compounds which possess a high affinity for the thyroxine binding site of transthyretin were given to rats in the presence of [^{125}I]thyroxine, whereafter the uptake of label into cerebrospinal fluid was determined. Finally, in addition to experiments concerning the blood-cerebrospinal fluid barrier, it was investigated whether transthyretin (and also other thyroid hormone binding proteins) played a role in transport of thyroxine through the capillary blood brain barrier during a single pass (Bradbury et al, 1975, Oldendorf et al, 1976).

The conclusions of this thesis and an evaluation of its toxicological significance to man are presented.

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CHAPTER II

EFFECTS OF HEXACHLOROBENZENE ON THYROID HOMEOSTASIS IN RATS

J.A.G.M. van Raaij[1,2], and K.J. van den Berg[2]

1) Institute of Public Health and Social Medicine,
Erasmus University Rotterdam, The Netherlands
2) TNO Medical Biological Laboratory, Rijswijk, The Netherlands

ABSTRACT

In order to characterize the effects of hexachlorobenzene (HCB) on thyroid homeostasis, rats were orally dosed with 3.5 mmol HCB/kg three times a week, for four weeks. With this dosing regimen, few signs of general toxicity, e.g body temperature and body weight were noted. Serum levels of total thyroxine (TT4) were reduced by 46 % after 15 days and remained permanently decreased. Total triiodothyronine (TT3) levels were temporarily decreased in the second and third week of dosing (45% reduction at day 15). The thyroid gland showed signs of increased activity as was evident from increased weight, altered morphology and an increased uptake of $^{131}I^-$. HCB gradually accumulated in serum to 0.049 mmol/l and pentachlorophenol (PCP), the major oxidative metabolite of HCB to 0.011 mmol/l at the end of the dosing period. Induction of hepatic mixed function oxygenases was apparent from a 13-fold increased activity ethoxyresorufin-O-deethylase (EROD) activity.

It is concluded that a repeated dose of HCB to rats results in a hyperactive thyroid, and in decreased thyroid hormone levels. The gradual build up of PCP in serum may play a role in HCB-induced reductions of thyroid hormone levels.

INTRODUCTION

From a vast amount of data in literature, it has become apparent that aromatic halogenated hydrocarbons (AHHs), such as hexachlorobenzene (HCB), polychlorinated biphenyls (PCBs), and polychlorinated-dibenzo-p-dioxins (PCDDs) may pose a threat to the health of humans. Many of these compounds may be formed during incineration processes, or production of

organic solvents and are released into the environment (Olie et al, 1977, Blackwood et al, 1979, Carpenter et al, 1986, Safe et al, 1990).

From a few human studies, and a number of animal studies, the major signs of toxicity of AHHs include dermal lesions, thymic atrophy, and hepatotoxicity (see chapter I). The thyroid may be a target organ for AHHs since this class of chemicals was found to induce disturbances of thyroid homeostasis in laboratory animals and wildlife species. In particular, reductions of plasma thyroxine (T4) levels were demonstrated in rodents or monkeys that were exposed to PCBs and PCDDs (see Chapter I). In contrast to a large amount of data on thyroid effects of PCBs and PCDDs, less information is available on toxic effects of HCB on thyroid homeostasis in mammals, including humans. There is one episode known about humans in Turkey, who became poisoned with HCB via contaminated food, which resulted in reductions of T4 levels in blood and enlarged thyroids (Peters et al, 1982). Some animal studies demonstrated comparable effects with HCB (Rozman et al, 1986, Den Tonkelaar et al, 1978, Smith et al, 1987).

The aim of the present study was to characterize effects on thyroid homeostasis of WAG rats repeatedly dosed with HCB for a period of four weeks. The functional status of the thyroid was assessed on the basis of weight, morphology, and iodine uptake. Furthermore, the activity of hepatic microsomal mixed function oxidases, including serum HCB and PCP levels of the experimental and control animals were determined, since major hydroxylated metabolites of aromatical halogenated hydrocarbons, particularly PCBs, may be causally involved in affecting thyroid hormone levels in blood (Brouwer et al, 1986, Brouwer, 1987).

MATERIALS AND METHODS

Chemicals

HCB was purchased from Aldrich, Brussels, Belgium. $^{131}I^-$ (specific activity 40 mCi/ml respectively) including T4 and T3 RIA kits (Amerlex-M) were acquired from Amersham, U.K. NADPH and (ethoxy)resorufin were obtained from Sigma, St Louis, MO, U.S.A. BCA protein kits were obtained from Pierce, Illinois, U.S.A. Tween-20 was obtained from Bio Rad Laboratories, Richmond, California.

Animals and Treatments

Male WAG/MBL rats (250-300 g) of about 12 weeks of age, were used. The animals, which had free access to water and food, were housed in a room with a 12 h light/dark cycle and an ambient temperature of 24°C. Control animals were dosed by gavage with 2x3 ml vehicle, while experimental animals received 2x3 ml of 3,5 mmol HCB/kg (i.e 1 g/kg), three days per week for 4 weeks. HCB was used as an emulsion of 0.14 mmol/ml in water containing 0.5% Tween-20. Blood was collected by tail bleeding at preselected times. During the period of treatment, the general health of the animals was monitored three times a week by measuring rectal body temperature and body weight. One week after the last dose, animals were killed by an overdose of CO_2, whereafter the thyroid glands, livers and sera were collected for further histological and biochemical studies.

Histopathology

The thyroid glands were weighed, and fixed in 3.5% formalin solution until use for microscopical examination. Histological slides were prepared and stained with hematoxylin-phloxine-saffron (HPS).

Thyroid function

Rats received 50 μCi $^{131}I^-$ in 1 ml of saline i.p (day 46). Approximately 6 h later, the animals were killed by decapitation. Uptake of $^{131}I^-$ in the thyroid glands was determined after gamma counting.

Thyroid hormones, HCB and PCP in sera

Total T4 (TT4) and T3 (TT3) levels were determined in sera of control and HCB-treated rats with a radioimmunoassay. HCB and PCP concentrations in the same sera were analyzed using HPLC as described earlier (van Raaij et al, 1991). The detection limit for PCP in serum was 0.14 μmol/l and for HCB 0.35 μmol/l. The extraction efficiencies for both PCP and HCB were more than 95%.

Microsomal enzymes

The frontal portion of the liver was homogenised in 3 weight volumes of 100 mM KH_2PO4/K_2HPO_4, pH 7,4 (buffer A). Liver microsomes were isolated by ultracentrifugation (9000 x g, for 30 min) of the homogenates. The supernatant was centrifuged once again (100000 x g, for 90 min) whereafter the pellet was resuspended in one volume of buffer A. Aliquots of microsomes were frozen at -70°C until use. The measurement of ethoxyresorufin-deethylase (EROD) activity was used as an indicator of microsomal mixed function oxidative (MFO) activity. EROD was measured according to published procedures using ethoxyresorufine as a specific substrate (Burke et al,1974). Protein concentrations were determined using the BCA method (Smith et al, 1985).

Statistics

Student's t-test was utilised for statistical evaluation of mean values of various parameters between experimental and control animals. Results are presented as means ± SEM.

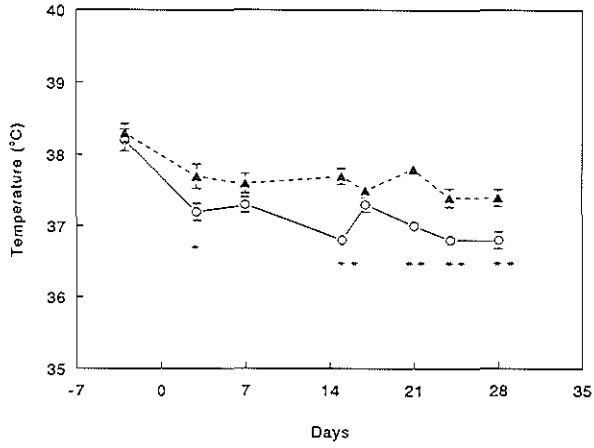
RESULTS AND DISCUSSION

Body temperatures of rats treated with HCB were slightly decreased by about 0.5°C, when compared with control rats during the experimental dosing period, although at termination, the difference was not significant (Fig.1). Body weights in animals receiving HCB were somewhat increased during the first days of dosing and then recovered to follow a normal growth curve (Fig.2). These observations indicate low systemic toxicity of HCB and possibly indicate that effects of HCB on thyroid homeostasis are not a result of altered efficiency of energy utilization.

Serum TT4 levels (Fig. 3) significantly decreased beginning after three days of exposure. A maximum decrease was found after 15 days as compared with control animals (33.9 nmol/l). TT4 levels were declined in animals treated with HCB to 18.2 nmol/l (i.e 46 % decrease). Thereafter, TT4 levels remained at a steady decreased level throughout the rest of the dosing period.

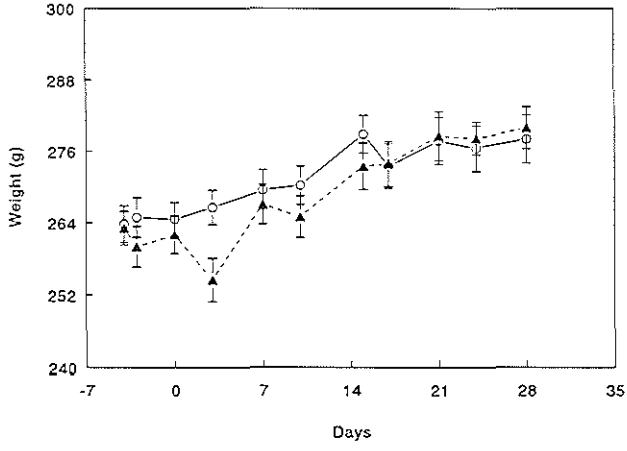
There appeared to be some change in course of control values of serum

Fig.1. Effect of HCB on body- temperature



Rats were repeatedly exposed to HCB for 4 weeks. At preselected times, rectal body temperature of the animals was determined. Significance: *,p< 0.05, **,p< 0.01. HCB: o-o, CON; ▲-▲

Fig.2. Effect of HCB on body- weight

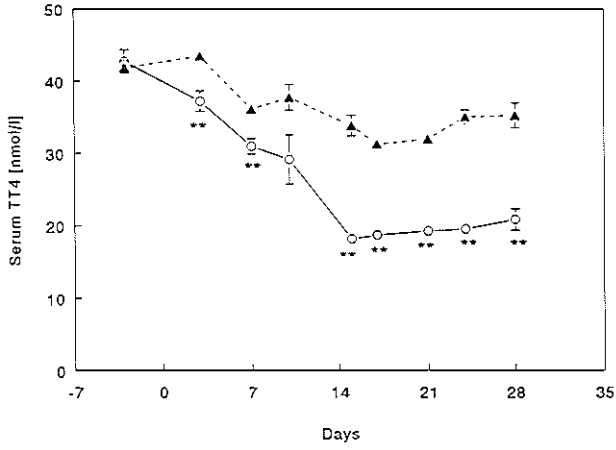


Rats were repeatedly exposed to HCB for 4 weeks. At preselected times, the animals were weighed. Significance: *,p< 0.05. HCB: o-o, CON; ▲-▲

TT4 levels during the whole exposure period with a minimum at about 2

weeks. The time course of TT4 values as a result of exposure to HCB shows a similar effect superimposed on the decrease of TT4 levels. This is maintained by HCB during the whole dosing period. An explanation may be that the combination of preventive measures for HCB handling (e.g. cages with special filter caps, less frequent cleaning of cages, forced oral dosing by stomach tube) induce in the animals in the beginning some stress to which they adapt later on. It is known that thyroid hormones are stress sensitive (De Groot, 1979).

Fig.3. Time course of TT4 levels



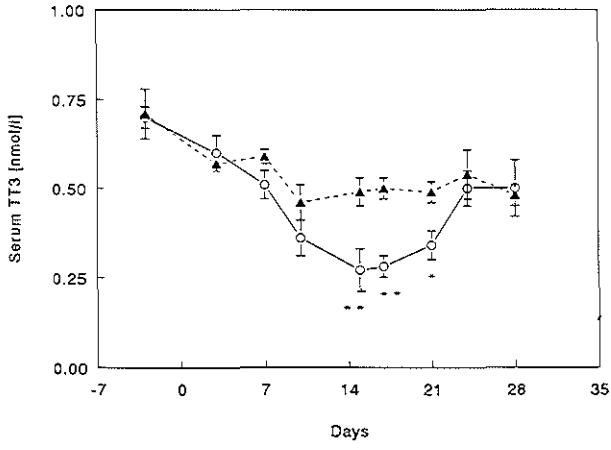
Rats were dosed with HCB for 4 weeks. At the indicated times TT4 levels were determined by radioimmunoassay in the same sera as used for the determination of HCB and PCP levels. Significance: **,p< 0.01. HCB: o-o; CON:▲-▲

TT3 levels in serum were also found to decrease as an effect of HCB-treatment (Fig.4). The decrease (44.9%) appeared to be at a maximum after 15 days. Thereafter, TT3 levels gradually returned to baseline levels. The observations confirm and extend observations of Kleinman de Pisarev (1989, 1990) who found decreased TT4 levels but no significant alterations in TT3 serum levels of female Wistar rats, when measured one, and eight weeks after the start of HCB dosing. The transient decrease in TT3 levels is a novel finding. Den Besten and coworkers (1993) demonstrated significant reductions of TT4, but also of TT3 in serum of female Wistar rats that were

exposed to HCB by diet over a 13 weeks period of exposure. In this study, serum TT3 levels did not return to normal values in rats exposed to HCB, but remained decreased over almost the whole period of exposure.

Differences in effects on serum TT3 levels after HCB treatment may be explained by possible differences in sex, dosing procedure, and animal strains. There are indications for strain specific differences in potential of glucuronidation of T3. For instance, WAG rats, Wistar LA rats and Fisher rats as well show an impaired T3 conjugation with glucuronic acid. This phenomenon may be due to a defect in androsterone-UDP-glucuronyltransferase, e.g an enzyme involved in conjugation of T3 (Beetstra et al, 1991, Visser et al, 1991, Van Raaij et al, 1993). Therefore, the

Fig 4. Time course of TT3 levels



Rats were dosed with HCB for 4 weeks. At the indicated times TT3 levels were determined by radioimmunoassay in the same sera as used for the determination of HCB, PCP and TT4 levels. Significance:*,p<0.05, **, p< 0.01. HCB: o-o; CON:▲-▲

temporary reduction of serum TT3 levels by HCB in WAG rats is probably not based on enhanced hepatic metabolism of T3. Another explanation may be a non permanently altered binding of T3 to serum proteins since PCP is capable of binding to thyroid hormone binding proteins (Van den Berg, 1990, Van den Berg et al, 1991). The return to baseline levels may also be attributed to an increased production of T3 by the thyroid gland as a classic

thyromimetic response (Th. Visser, personal communication), or to an enhanced activity of hepatic deiodinases.

Free T4 (FT4) levels in sera of the experimental animals were decreased by more than 70 % compared with controls (see Chapter VII). As a reaction of the pituitary upon this phenomenon, serum TSH levels were increased (56.6 %, Chapter VII). Thyroid glands of the animals dosed with HCB were significantly enlarged by 27.3 % (Table 1) compared with control animals. Histological examination of slides from thyroids of animals dosed with HCB revealed hypertrophy and hyperplasia (Fig. 5). Uptake of $^{131}I^-$ by the thyroid gland of the HCB-treated animals was greatly increased (5.5-fold). These results suggest that the pituitary-thyroid axis is not impaired by HCB. Although the present findings do not imply an impaired function of the thyroid gland, a direct effect of HCB on the thyroid may not be excluded.

The liver/body weight ratio was 1.7-fold increased by HCB (Table 2). In addition, hepatic EROD activity (associated with cytochrome P450-1A1) was substantially increased (13-fold), (Table 2). These findings indicate stimulation of mixed function oxygenase (MFO) activity as a result of HCB administration. Induction of microsomal cytochrome P450-1A1 (and also of P450-1A2) associated enzymes after HCB exposure in rats had also been demonstrated by other investigators (Mehendale et al, 1975, Linko et al, 1986, Smith et al, 1990). Rozman and coworkers (1986) demonstrated that

Table 1. Effects on thyroid function

Parameter	HCB	N	Control	N
Thyroid gland (mg)	19.1 ± 0.9*	5	15.0 ± 1.2	5
$^{131}I^-$ uptake	1366 ± 213**	5	249 ± 72	4

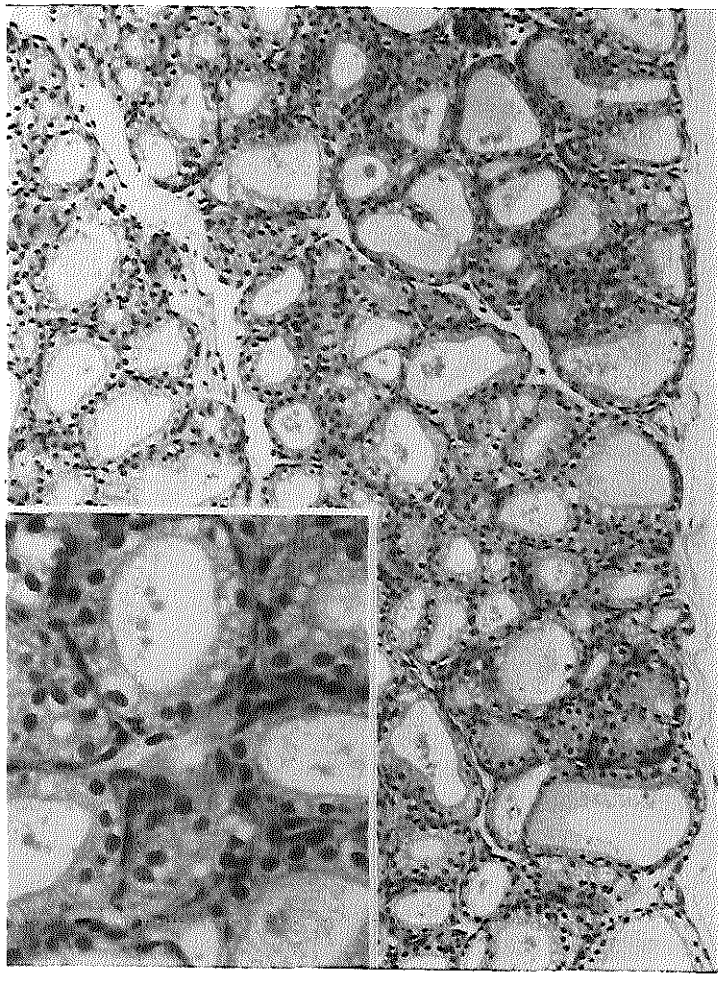
Treatment of animals with HCB is described in the Material and Methods section. Rats received 50 μCi $^{131}I^-$ in 1 ml saline i.p. Uptake of $^{131}I^-$ in the thyroid is expressed as cpm.10^3/mg wet weight. Significance: * $p<0.05$, ** $p<0.001$.

the hepatic EROD activity of rats chronically exposed to HCB was 10-fold increased at the end of a fourteen week period of exposure.

There was a progressive accumulation of HCB in serum up to the fourth

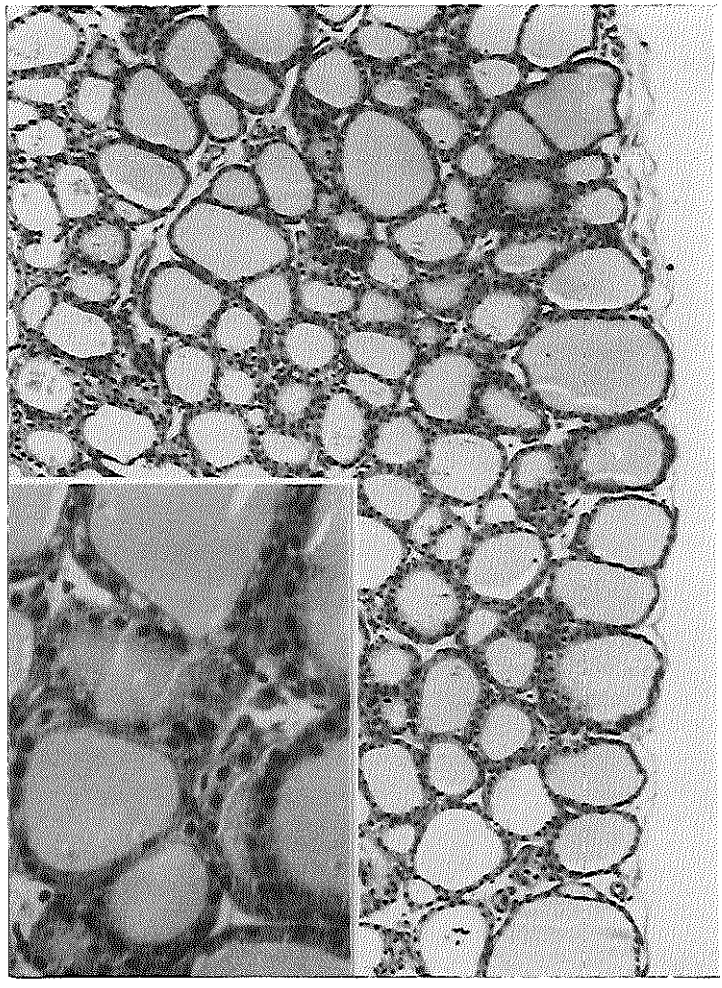
Fig 5. Effect of HCB (A) or vehicle (B) on thyroid morphology

A



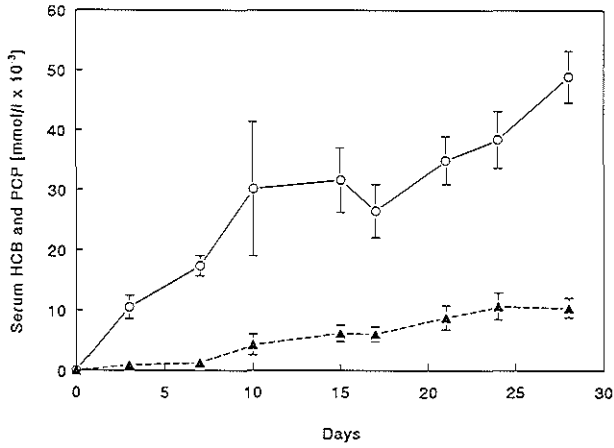
Rats were dosed with HCB for 4 weeks and the thyroid glands were isolated, fixed in formalin solution 3.5 % and stained with hematoxylin-phloxin-saffron (HPS). After treatment (A), follicles are smaller and the lining follicular cells are more cylindrical than in the untreated controls (B). Furthermore, colloid is fine granular and faintly eosinophilic in A compared with the hyaline and more intense eosinophilic staining observed in B. (HPS), X 210; inset, X 500.

B



Rats were dosed with vehicle for 4 weeks and the thyroid glands were isolated, fixed in formalin solution 3.5 % and stained with hematoxylin-phloxin-saffron (HPS). The follicles are relatively large and the lining follicular cells are much less cylindrical than in the experimental animals.

Fig.6. Time course of HCB and PCP levels



Rats were dosed with HCB for 4 weeks. Dosing started at day 0. Blood samples were taken at the indicated times. Concentrations of HCB and PCP in sera were determined with HPLC analysis. HCB: o-o; PCP: ▲-▲

week after repeated administration of HCB (Fig.6). A maximum concentration of 0.049 mmol HCB/l in serum was reached at day 28. After cessation of treatment, the serum levels of HCB declined to 0.039 mmol HCB/l over the two-week post-exposure period.

As a consequence of enhanced MFO activity, there was a gradual build-up in serum of PCP, the major metabolite of HCB (Koss et al, 1976, Van Ommen et al, 1985, 1986). PCP levels in serum increased with dosing of HCB and achieved maximum values of 0.011 mmol/l (day 24) and then remained at a fairly constant level. Control animals had no detectable levels of HCB or PCP.

PCP may possibly be responsible for the decrease of serum TT4 levels after administration of HCB since hydroxylated metabolites of halogenated aromatic compounds, such as PCBs, are able to interfere with thyroid hormone binding proteins (Van den Berg 1990). This aspect is described in following chapters (III-VII) of the thesis.

In conclusion, the results of the present study indicate that repeated dosing of rats to HCB results in 1) time-dependent reduction in both TT4 and

TT3 levels in serum, 2) an activated thyroid gland, 3) enhanced activity of hepatic microsomal enzymes and 4) accumulation of PCP in serum.

Table 2. Induction of hepatic microsomal enzymes

Parameter	HCB	N	Control	N
liver/body weight (x 10^{-2})	5.9 ± 0.1**	5	10.0 ± 0.4	5
EROD (nmol/min/mg)	257.3 ± 15.1**	5	20.1 ± 2.5	4

Dosing of animals, isolation of livers and preparation of hepatic microsomes was carried out as described in the Material and Methods section. Significance: ** $p<0.001$

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CHAPTER III

EFFECTS OF HEXACHLOROBENZENE AND ITS METABOLITES PENTACHLOROPHENOL AND TETRACHLOROHYDROQUINONE ON SERUM THYROID HORMONE LEVELS IN RATS

J.A.G.M van Raaij[1,2], K.J. van den Berg[1], R. Engel[1], P.C. Bragt[1] and W.R.F. Notten[1,2]

1) TNO Medical Biological Laboratory, Rijswijk, The Netherlands
2) Erasmus University Rotterdam, The Netherlands

(Keywords:Thyroxine, triiodothyronine, hexachlorobenzene, pentachlorophenol, tetrachloro-p-hydroquinone, serum levels)

ABSTRACT

Effects of administration of equimolar doses of hexachlorobenzene (HCB) and its metabolites pentachlorophenol (PCP) and tetrachlorohydroquinone (TCHQ) serum thyroxine (TT4) and triiodothyronine (TT3) levels in rats were studied. Furthermore it was investigated whether the observed effects were related to the serum levels of HCB or PCP. Rats received either corn oil (controls) or HCB, PCP or TCHQ in a single equimolar intraperitoneal dose of 0.056 mmol/kg. Results indicated that HCB did not alter serum TT4 and TT3 levels for a period up to 96 h after dosing. In contrast, PCP and TCHQ were both capable of reducing serum TT4 levels with a maximum effect between 6 and 24 h after exposure. TCHQ was more effective in repressing TT3 than TT4 blood levels. Dose-response experiments were carried out in order to obtain insight in the sensitivity of the observed effects. Rats received different doses of PCP or TCHQ intraperitoneally. The reductions of TT4 levels by PCP were inversely related to serum PCP levels in exposed animals, based on the toxicokinetics and dose-response profiles. Furthermore, PCP serum levels after HCB administration appeared too low to cause an effect. The results of this study indicate that not HCB itself, but rather its metabolites PCP and TCHQ may be involved in reduced serum thyroid hormone levels after HCB administration.

INTRODUCTION

Hexachlorobenzene (HCB) has been used in industrial applications mainly as an antimicrobial agent (fungicide) for wood preservation [1]. Symptoms of HCB-induced toxicity includes disturbances of thyroid hormone homeostasis. In one human poisoning episode in Turkey, for example, persons poisoned via consumed grain contaminated with HCB, exhibited a number of toxic signs including decreased thyroxine levels and an enlarged thyroid [2]. Similar phenomena have also been observed in rodents following HCB exposure [3,4]. Recently reported effects of HCB in rats deal with an increase of hepatic thyroxine metabolism and thyroxine dehalogenation accompanied with decreased thyroxine levels in blood and an elevated excretion of porphyrinogen in urine [5].

Other chlorinated aromatic hydrocarbons have also been found to interfere with thyroid homeostasis: Exposure of primates and rodents to polychlorinated (PCB) or to polybrominated biphenyls (PBBs) resulted in decreased plasma thyroxine levels and altered thyroid morphology [6-10]. Organochlorine insecticides were found to decrease protein bound iodine levels in workers [11]. Dioxin (2,3,7,8-tetrachlorodibenzo-p-dioxin) appeared to reduce serum T4 levels and was capable of inducing thyroid tumours in rodents [12,13].

Evidence is accumulating that metabolites of halogenated aromatics such as PCBs may play a role in disturbed thyroid hormone plasma levels [14,15]. Whether metabolites also are involved in HCB-induced decrease of thyroid hormone levels is essentially unknown. The in vitro and in vivo metabolism of HCB and PCP is well established [16-20]: In the liver, HCB is first metabolized to the main metabolite pentachlorophenol (PCP) and in a second step partly to tetrachloro-p-hydroquinone (TCHQ) which is further converted to trichlorohydroquinone. Experiments of Koss et al. with labelled HCB revealed that in rats almost half of the amount of HCB is eliminated as metabolites [19]. Part of the metabolites appears in the urine in the form of PCP-glucuronide or tetra/trichloro-p-hydroquinone glucuronide [16,17,21]. Recently, Van den Berg demonstrated interference of PCP with thyroid hormone carriers in vitro [22].

In this paper we investigated the influence of HCB and its metabolites PCP and TCHQ on serum thyroid hormone levels in rats after single i.p dosing. Additionally, HCB or PCP serum concentrations were measured and

correlated with serum concentrations of thyroid hormones.

MATERIALS AND METHODS

Materials

HCB was obtained from Serva, Heidelberg, Germany, PCP from Aldrich Chemical Company, Brussels, Belgium and TCHQ from Sigma, St Louis, MO U.S.A. These chemicals were dissolved in pure corn oil. T4 and T3 RIA kits, (Amerlex-M), were acquired from Amersham, England.

Methods

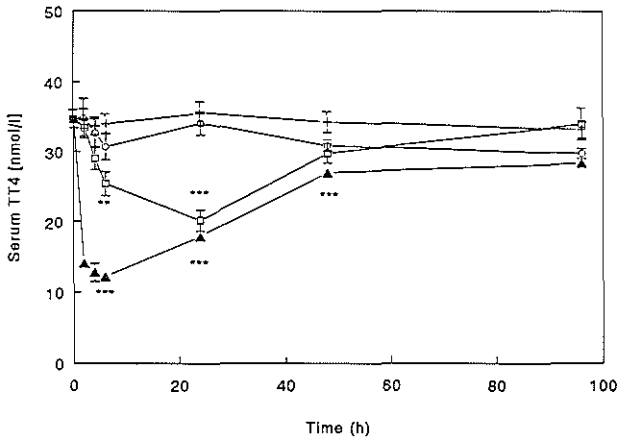
Twelve-week old male WAG/MBL rats, weighing 250-300 gram, were used. The animals were housed in a 12 h lighted room maintained at a temperature of 24°C and a humidity of 50-70 %. Water and food were available ad libitum. In the toxico-kinetic experiments, the animals were injected intraperitoneally with 1 ml of a single equimolar dose of 0.056 mmol/kg HCB, PCP or TCHQ (i.e. 16, 15, or 14 mg/kg respectively). Control rats received a single 1 ml injection of corn oil. Blood samples were taken under ether anaesthesia from the tail just before exposure (t=0) and at 2,4,6,24,48 and 96 h after dosing. Each chemical was administered to a group of 4-5 animals.

In the dose response experiments, PCP and TCHQ were tested using 6 and 4 different doses respectively. The highest dose of PCP administered was 0.105 mmol/kg (28 mg/kg) and that of TCHQ, 0.052 mmol/kg (13 mg/kg) both in a volume of 2.5 ml. Higher doses of TCHQ were not possible because of the appearance of toxic signs, such as hypothermia and diarrhoea. Each dose was tested in groups consisting of 4 animals. Control rats (300 g) received 2.5 ml corn oil. In these dose-response-experiments blood was collected 6 h after exposure, when effects appeared to be at a maximum.

Total T4 (TT4) and total T3 (TT3) levels in sera were analyzed by radioimmunoassay according to the manufacturer's instruction. Pilot studies indicated that neither HCB, PCP, or TCHQ disturbed the RIA assay methods, nor was there an effect of corn oil on TT4 or TT3 levels compared to saline injected rats.

Total HCB and PCP levels in rat sera (i.e. free + protein-bound fractions) were determined by HPLC analysis: a 100 μl sample of 1 mol/l hydrochloric acid was added to 100 μl of serum. The sample was further diluted to 1 ml with water and subsequently extracted with 4 ml of n-hexane. An aliquot of the n-hexane phase (3.5 ml) was evaporated under a gentle nitrogen flow at room temperature, in the presence of propanediol, to prevent evaporation of HCB and PCP. The residue was taken up in methanol and a 20 μl sample was injected into the HPLC system. Chromatography was carried out with a Waters RCM 8x10 cartridge holder containing a 8NVC18-4μ cartridge. Elution was performed with methanol, containing 10 ml of water, 4 ml of acetic acid and 2 g of sodium perchlorate per litre at a flow rate of 1.5 ml/min.

Fig.1. Time course of TT4 levels



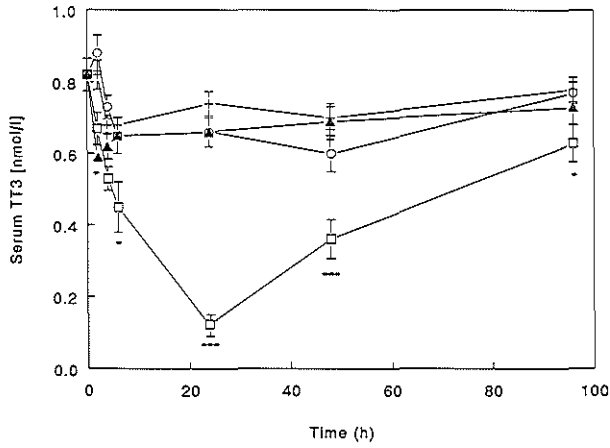
Rats were injected i.p with 1 ml of corn oil, HCB (hexachlorobenzene), PCP (pentachlorophenol) or TCHQ (tetrachlorohydroquinone) in doses of 0.056 mmol/kg. Blood samples were taken at the indicated times after exposure.
Statistical significance:*, $p<0.05$, **, $p<0.005$, ***, $p<0.001$. CON +-+; HCB o-o; PCP ▲-▲; TCHQ;□-□

Detection was carried out by means of two detectors in series. The use of two detectors, consisting of a coulometric detector (ESA model 5100 A; set to + 0.63 V) for the detection of PCP and a UV detector (Perkin-Elmer model LC-95, set to 254 nm) for the detection of HCB allowed for the

determination of HCB and PCP in one run. Extraction efficiencies for HCB and PCP were 100%. No interfering peaks were detected when serum samples from animals not treated with HCB or PCP were analyzed. Detection limits were 0.15 μmol/l and 0.35 μmol/l for PCP and HCB respectively. TCHQ could not be detected with the procedures described above. On addition of TCHQ to control serum, TCHQ levels were undetectable, which made further extraction and recovery impossible.

Statistical analysis of effects was performed using Student's t tests. The variation in the concentrations of thyroid hormones, HCB or PCP is represented by the standard error of the mean.

Fig.2. Time course of TT3 levels.



The same chemicals as described in the legends of Fig. 1 were i.p injected in rats and serum TT3 levels were determined.

RESULTS

The time course of changes in serum TT4 after exposure to an equimolar dose of HCB, PCP or TCHQ is presented in Fig. 1. After HCB administration no significant changes in serum TT4 levels were detected over a 96 h period. In contrast, PCP caused a marked decrease in TT4 levels compared with the control values. This effect was strongest between 6 and 24 h after exposure. The average serum TT4 level after PCP injection dropped from 34.3 ± 1.8 to

12.2 ± 0.8 nmol/l (i.e. 64.4 % decrease) at the time of peak effect. Thereafter, serum TT4 levels slowly returned to control levels. TCHQ also caused significant reductions of serum TT4 levels after 6 and 24 h, although the peak magnitude of the effect was less compared with PCP (i.e. 25.9% decrease). After 48 h, serum TT4 levels following TCHQ were back to normal values.

Serum TT3 levels, as shown in Fig. 2, did not change after HCB administration. However, PCP treatment resulted in a slight but significant decrease of 28% after 2 h. TCHQ caused greater reductions in TT3 levels between 6 and 24 h than did PCP. After 24 h, TT3 concentrations appeared to be at a minimum (i.e. 85.4 % decrease). Thereafter TT3 levels slowly increased, but still were significantly lower than control values up to 96 h.

To obtain more insight into the sensitivity of TT4 and TT3 responses to these chemicals, dose-response experiments were done. Because effects appeared at their maximum at 6 h after exposure, this time point was selected. The effects of different doses of PCP and TCHQ on TT4 and TT3 levels are presented in Table 1. A significant decrease in serum TT4 level was demonstrated at 0.007 mmol PCP/kg (1.75 mg/kg). Effects on TT4 levels after administration of TCHQ were less as compared to PCP, with a dose of 0.034 mmol TCHQ/kg (8.4 mg/kg) being the lowest effective dose. In addition, the no-effect level was 5-10 times higher for TCHQ than PCP (i.e. 0.026 mmol TCHQ/kg vs 0.002 mmol PCP/kg).

With respect to TT3 levels, the chosen range of PCP dosing had no obvious effects except at doses higher than 0.052 mmol/kg. TCHQ appeared to have an effect on TT3 (and TT4 levels) if a minimum dose of 0.034 mmol/kg (8.4 mg/kg) was used.

The time course of the HCB levels and the levels of its metabolite PCP after exposure of 0.056 mmol HCB/kg is represented in Fig. 3. Up to 4 h after HCB dosing, HCB levels were hardly detectable. From 6 h on, however, HCB was detectable (0.63 μmol/l), reaching to a steady-state level of about 1.58 μmol/l HCB at 24 h. The PCP levels after HCB administration reached a final mean level of 0.20 μmol/l after 96 h.

Figure 4 represents the PCP concentrations in serum after a single dose of 0.056 mmol PCP/kg. Maximum PCP concentrations (varying from 0.19 to 0.16 mmol/l) were found at 2, 4 and 6 h after administration. At 24, 48 and 96 h after exposure the level decreased to less than 0.0002 mmol/l.

Figure 5 shows the relationship between serum PCP levels and the dose of PCP administered.

Table 1. Effects of the dose of PCP and TCHQ on serum TT4 and TT3 levels.

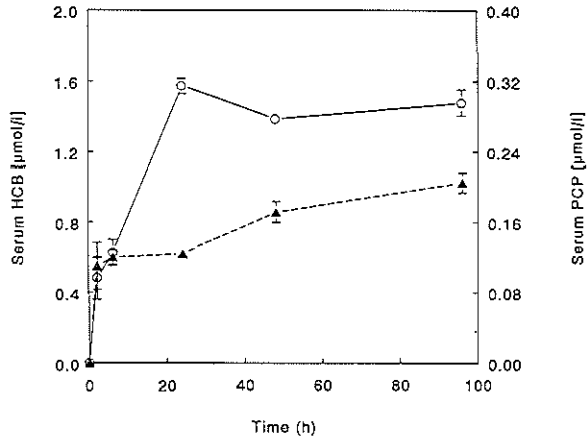
Dose of chemical (mmol/kg)	TT4 (nmol/l)	TT3 (nmol/l)
PCP		
0	31.2 ± 0.92	0.68 ± 0.04
0.002	32.0 ± 0.48	0.53 ± 0.01
0.007	20.0 ± 1.60***	0.61 ± 0.02
0.013	17.3 ± 0.70***	0.69 ± 0.02
0.026	17.1 ± 1.55***	0.69 ± 0.10
0.052	13.6 ± 0.46***	0.62 ± 0.02
0.105	9.4 ± 0.70***	ND
TCHQ		
0	31.2 ± 0.92	0.68 ± 0.04
0.026	32.0 ± 2.70	0.67 ± 0.04
0.034	26.1 ± 1.40*	0.55 ± 0.02
0.042	21.4 ± 1.10***	0.34 ± 0.04***
0.052	26.2 ± 1.75*	0.41 ± 0.02**

Serum TT4 and TT3 levels of rats i.p. injected with 2.5 ml of PCP or TCHQ in 6 or 4 different doses, respectively. Blood samples were taken at 6 h after exposure. Control animals received corn oil only. Data are expressed as mean ± S.E.M. ND= not done. Statistical significance is indicated as in Fig.1.

DISCUSSION

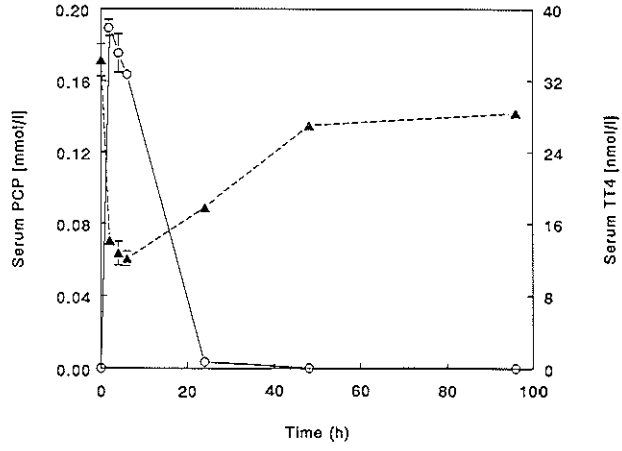
The results presented in this study show that HCB administered intraperitoneally (i.p) is rather inefficient in decreasing thyroid hormone levels in rats as compared with the i.p administration of its metabolites PCP and TCHQ after a single equimolar dose of 0.056 mmol/kg. Whereas PCP caused an almost 65% reduction of TT4 levels between 6 and 24 h, and TCHQ a reduction of TT3 levels of about 85% after 24 h, a similar dose of HCB had neither effect on TT4 or TT3 levels. Other HCB studies, where HCB was daily administered to rats in much higher concentrations (60x) compared to the

Fig. 3. Serum HCB and PCP levels after a single dose of 0.056 mmol HCB/kg (16 mg/kg), determined with HPLC analysis.



The PCP values after 2, 4 and 6 h. are values "less than" (<), which is also the case at HCB values after 2 and 4 h. HCB o-o PCP ▲-▲

Fig. 4. Time course levels of serum PCP levels.



Concentrations were analyzed by HPLC as reported in Materials & Methods. The mean PCP concentrations are represented by a black line. Mean serum TT4 levels from Fig. 1 are superimposed (dotted line).

present study, showed a decrease in TT4 levels in blood [5]. Studies with rats which were fed with a HCB enriched diet for 55 days, exhibited significant decreases in thyroxine and triiodothyronine levels [4].

The difference in toxicokinetics of the compounds is probably a determining factor in affecting thyroid hormone levels. After i.p injection of HCB, dissolved in corn oil, it will be released more slowly into the circulation than PCP, because of the higher oil-water partition coefficient of HCB. Furthermore, HCB is easily trapped by fatty tissue and the liver where it accumulates. It is known that adipose tissue HCB levels rise rapidly after exposure, being 200-500 fold higher than serum levels [23]. Measurements of serum HCB levels indicated comparatively low levels (Fig. 3). PCP dosing, however, resulted in substantial concentrations in the circulation as demonstrated in this study (Fig. 4). The toxicokinetic profile of PCP was essentially similar as reported by Braun [21] who determined the elimination rate of a single 10 mg/kg dose of radiolabelled PCP (half-life value being 20 h) to rats and found also plasma peak of PCP at 4 to 6 h after exposure. During this time the availability of PCP and protein binding appeared at a maximum. Plasma protein binding and retention by the liver slowed the removal of PCP from the body by urinary excretion. Ninety percent of the administered PCP was eliminated within 3 days while the remaining part was eliminated more slowly [21].

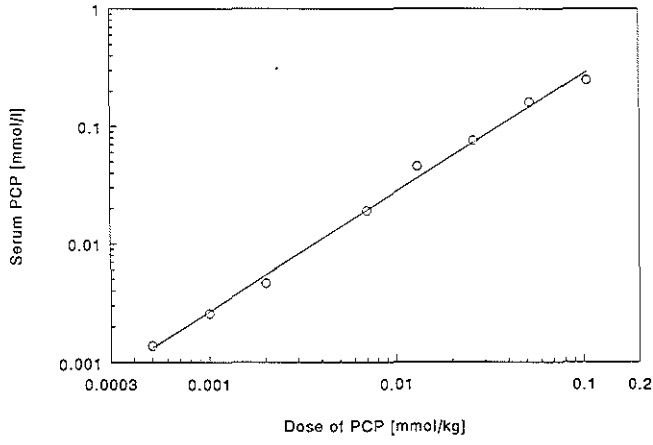
TCHQ is one of the metabolites of PCP, and was found decrease TT3 serum levels to a much higher degree than TT4 levels. This was an unexpected finding since most halogenated aromatics studied thus far with respect to thyroid hormone levels, e.g. PCBs, PBBs, phenoxy-acids, TCDD, HCB, have their main effect on TT4 levels, usually the TT3 levels being less affected [4,8,10,12,24,25]. Attempts to relate serum TCHQ levels to decrease of serum TT4 and TT4 levels were unsuccessful. HPLC methods for TCHQ analysis failed, possibly because of covalent binding to serum proteins [20,26]; alternatively, TCHQ and TCHQ glucuronide may be rapidly excreted via the urine and therefore do not persist in the circulation [21].

One interpretation of the present findings is that actually PCP, the major metabolite of HCB, is the cause of the decreased serum TT4 levels in chronically HCB-fed or poisoned individuals. There is an inverse correlation between serum concentrations of PCP, and decrease of serum TT4 levels as is obvious when Table 1 and Fig. 5 are compared. Upon inspection of the time course of TT4 level and the toxicokinetics of PCP (Fig. 4) these two

curves are near mirror images; serum PCP levels peak when serum TT4 levels are at a minimum. After a single dose of HCB, circulatory PCP reached rather low levels (Fig. 3) (less than 0.00014 mmol/l), while a serum level of about 0.0188 mmol/l was at least required for a decreased serum TT4 6 h after dosing (Table 1). Multiple dosing and chronic feeding however may lead to a considerable liver storage of HCB. The induction of hepatic P-450 microsomal enzymes by HCB [1,27] may result in PCP levels high enough to affect thyroid hormone homeostasis.

The decreased thyroid hormone levels by PCP and TCHQ may be caused by a disturbed synthesis, a diminished release of thyroid hormones by the thyroid, and an accelerated uptake by other organs. In addition,

Fig. 5. Dependency of serum PCP levels on administered dose.



Doses of 0.0005, 0.001, 0.002, 0.007, 0.013, 0.026, 0.052 and 0.105 mmol PCP/kg were injected i.p. Sera were taken after 6 h. and analyzed by HPLC.

several specific mechanisms have been advanced in connection with halogenated aromatics such as: (1) accelerated metabolism of the thyroid hormones via a rapid conjugation followed by excretion into the bile [7,12,28,29]; (2) accelerated deiodination of thyroxine [5]; and (3) interference with thyroid hormone binding proteins. With respect to the last possibility, recent studies indicate that a number of xenobiotics possess the property to bind to thyroid hormone transport proteins leading to decreased

thyroid hormone levels [15,22,30]. Whether such a phenomenon exists in HCB or PCP treated animals is under investigation.

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CHAPTER IV

HEXACHLOROBENZENE AND ITS METABOLITES PENTACHLOROPHENOL AND TETRACHLOROHYDROQUINONE: INTERACTION WITH THYROXINE BINDING SITES OF RAT THYROID HORMONE CARRIERS EX VIVO AND IN VITRO

J.A.G.M van Raaij[1,2], K.J. van den Berg[1], and W.R.F. Notten[1,2]

1) TNO Medical Biological Laboratory, Rijswijk, The Netherlands
2) Erasmus University Rotterdam, The Netherlands

(Keywords: Hexachlorobenzene, pentachlorophenol, tetrachlorohydroquinone, carrier, thyroxine, binding site, occupation)

ABSTRACT

Previous results have indicated that hexachlorobenzene (HCB)-induced hypothyroidism may be caused by its main metabolite pentachlorophenol (PCP), and by tetrachlorohydroquinone (TCHQ), rather than by the parent compound. In the present experiments it was investigated whether hormone displacement from serum carriers could be a factor in the development of this hypothyroidism.

In an *in vitro* competition assay PCP was an effective competitor for the thyroxine (T4)-binding sites of serum carriers, whereas HCB was ineffective. *Ex vivo* experimental results demonstrated occupation of T4-binding sites in sera from PCP-exposed animals but not in sera from HCB- or TCHQ-treated animals. Competing ability for T4-binding sites was still present in sera of PCP-exposed animals but was absent in HCB or TCHQ-exposed animals. The results suggest that thyroid hormone displacement by the major metabolite PCP may play a role in HCB-induced hypothyroidism.

INTRODUCTION

It is well known that exposure to a number of halogenated aromatic compounds such as polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs), and hexachlorobenzene (HCB) may induce alterations of the thyroid function in several species. Hyperplasia and hypertrophy, as well

as decreases in thyroxine levels in the circulation, have been reported [1-8]. Evidence is accumulating that interaction of hydroxylated metabolites of these compounds with thyroid hormone transport carriers may play an important role in the reduction of thyroxine levels. It was found *in vivo* that the hydroxylated metabolite of 3,4,3',4'-tetrachlorobiphenyl specifically binds to transthyretin (TTR), a transport carrier of thyroid hormones and vitamin A [9]. Furthermore, *in vitro,* various hydroxylated PCBs have been shown to compete with the thyroxine (T4)-binding site of TTR [10,11]. Hydroxylated PCBs with a linear, and symmetrical molecular shape (i.e. a rigid planar structure), and a lateral halogenation of the molecule appear to have a favourable configuration for high-affinity interaction with the T4-binding site of TTR [10].

Several derivatives of tetrachloro-dibenzo-p-dioxins, tetrachloro-dibenzo-furans also strongly interact in a competitive manner with the T4-binding site [10,12,13]. In addition, a number of other halogenated aromatic industrial chemicals, i.e. chlorophenols, chlorinated phenoxy acids, brominated and iodinated benzonitrils, have been found to compete with the T4-binding site of TTR and were able to reduce serum T4 levels *in vivo* [8,14,15]. Recently, it was demonstrated that pentachlorophenol (PCP), the major metabolite of HCB, as well as tetrachlorohydroquinone (TCHQ), the minor metabolite, were much more efficient in the reduction of serum T4 levels than the parent compound, HCB [8], an effect that may be similar to that of 3,4,3',4'-tetrachlorobiphenyl and its metabolites [11].

In the present study experiments were carried out to determine the extent of thyroid hormone displacement from its carriers by HCB or its metabolites. Firstly, the interaction of HCB, PCP and TCHQ with the T4-binding sites of serum carriers was studied *in vitro.* Secondly, the occupation of carrier-sites and T4-displacement ability in serum extracts from animals dosed with HCB, PCP or TCHQ was investigated in *ex vivo* experiments.

MATERIALS AND METHODS

Materials

HCB was obtained from Serva (Heidelberg, F.R.G.), PCP from Aldrich Chemical Company (Brussels, Belgium), TCHQ and L-thyroxine from Sigma (St Louis, MO, U.S.A.) and L-[3',5'-^{125}I]thyroxine (spec act 1500 μCi/μg, 55

MBq/μg) from Amersham (Buckinghamshire, U.K.).

Methods

To examine the ability of HCB, PCP and TCHQ to displace T4 from the T4-binding sites of thyroid hormone serum carriers, sera of WAG/-MBL rats (250-300 g) were taken for *in vitro* competitive binding studies, as described earlier [8]. Different concentrations of HCB, PCP or TCHQ (range 1 nM - 0.1 mM), all dissolved in ethanol 100%, were incubated with ^{125}I-labelled thyroxine, in phosphate buffered saline (PBS:0.01 M phosphate, pH 7.3, 0.154 M NaCl), and diluted serum (1/50 in PBS) for 50 minutes at room temperature to obtain maximum T4-binding. As a reference, different concentrations of unlabelled thyroxine were incubated instead of the chemicals. After incubation, bound T4 was determined with gamma counting after separation the free T4 from bound T4, using G-25 Sephadex column chromatography [16]. In every competition assay an excess of T4 (i.e 10 μM) or ethanol 100% was added instead of the chemicals to determine the non-specific bound T4, or the maximum T4-binding to serum respectively. Non-specific binding was always lower than 6%.

To study the competitive effects *ex vivo*, groups of 3-4 rats were intraperitoneally injected with 1 ml of an equimolar dose (0.052 mmol/kg) of HCB (16mg/kg), PCP (15 mg/kg) or TCHQ (14 mg/kg), dissolved in corn oil. Control rats received 1 ml corn oil. Several times after injection blood samples were taken by tail bleeding where after the sera were stored at -20°C.

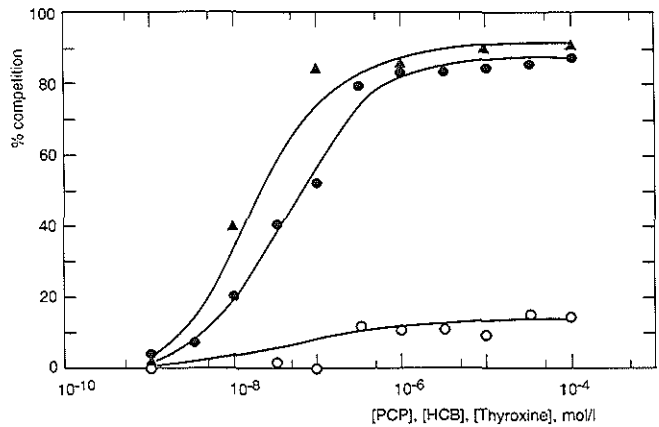
Occupation of binding sites was determined in separate serum samples, diluted 1/30 in PBS. To obtain evidence that compounds from sera of animals treated with aromatic compounds were capable of competing with T4 for binding sites of carrier proteins, an organic acid extraction was performed from pooled sera from animals treated with corn oil, HCB or PCP (0.052 mmol/kg) as described by Van Raaij et al [8]. The extraction efficiency was 100 % for HCB and PCP. Concentration of the serum HCB extracts was done using propanediol/methanol 2:8 as described previously (extraction procedure A) [8]. To correct for a competitive effect of propanediol, the latter was also added to serum of corn oil-treated rats, whereafter the extraction procedure was performed (extraction procedure B). The specific competition caused by HCB was determined by subtraction of the competition

determined by extraction procedure B from that determined by extraction procedure A. Different dilutions of the extracted material were tested in the standard competition assay. To compare competing effects in propanediol containing extracts of sera of corn oil-treated animals with those in extracts of corn oil-treated-animals, the sera of corn oil-treated animals were extracted without propanediol.

RESULTS AND DISCUSSION

Results from the *in vitro* studies indicated that HCB was hardly able to compete with thyroxine for binding sites of serum carriers, i.e. less than 15% competition at 10^{-5} M (Fig. 1). PCP however, had a high potency to displace

Fig.1. Competition of HCB, PCP, and T4 with T4-binding sites of serum carriers.



Different concentrations of HCB (o-o), PCP (●-●) or T4 (▲-▲), as indicated on the x-axis, were incubated with [^{125}I]T4 and diluted rat serum under standard conditions. Competition was determined as described in Materials and Methods.

thyroxine from carriers (IC_{50} about 10^{-8} M). T4 competed more efficiently (IC_{50} about 10^{-9} M) than PCP. Competition experiments with TCHQ gave evidence of interference as described previously [15], but the results which were obtained did not allow the construction of a proper displacement curve.

While at concentrations of TCHQ from 10^{-2} to $3.3.10^{-4}$ M there was a dose dependent inhibition of T4-binding, at concentrations above 10^{-4} M more radioactivity (up to 50 % more) was eluted in the void volume than in the control incubation without anything added.

Due to the reactivity of hydroquinones, these compounds may have reacted with components of the assay system, TTR, T4 or the column material. The strong competitive interaction of PCP with the pool of serum thyroxine carriers confirms previous work that showed strong separate interactions with 3 major human carriers, namely TTR, thyroxine binding globulin and albumin [17]. In this study little interaction of HCB with the pool of T4 carriers, in rats predominantly albumin and TTR [18], could be detected, but some affinity of HCB for the T4 site of human TTR has been reported by us in previous studies [15].

In the *ex vivo* experiments, where animals were treated with a single dose of HCB, little evidence of reduced thyroid hormone levels (Table 1) could be detected. However, an equimolar dose of PCP caused a drastic fall in total thyroxine (TT4) levels (Table 1). Previous studies indicated that an equimolar dose of TCHQ did not have an early effect on TT4 levels, but at later time

Table 1. TT4 levels and relative availability of T4-binding sites in sera from rats treated with corn oil, HCB, PCP or TCHQ.

Treatment	N	[TT4] nmol/l	% Binding
Corn oil	5	32.0 ± 3.9	100.0
HCB	4	29.9 ± 3.2	112.3 ± 6.4
PCP	4	13.4 ± 1.2***	18.2 ± 2.4 ***
TCHQ	4	27.0 ± 3.6	115.7 ± 9.3

Rats were injected i.p with 2.5 ml of corn oil (control animals), HCB, PCP or TCHQ (0.052 mmol/kg). Blood samples were taken by tail bleeding at 6 h after exposure. Occupation of T4 sites in serum carriers was determined as described in Materials and Methods. Results are expressed as mean ± standard-deviation. Statistical analysis of effects was done with Student's t-tests. Significance:*, $p<0.05$, **, $p<0.005$, ***, $p<0.001$

points especially TT3 but also TT4 levels were lowered [8]. In the *ex vivo* experiments presented in Table 1, the occupancy of T4 carrier sites in sera of these treatment groups was examined. Sera were taken at the time of the lowest observed levels of thyroid hormone. The results showed that occupation of T4 carrier sites, as determined by the binding of radiolabelled T4 to sera of HCB- or TCHQ- treated animals, was similar to that of control sera (Table 1). In contrast, in sera of animals treated with an equimolar dose of PCP, significantly less radiolabelled thyroxine was able to bind to serum carriers (18.2 % of control values, $p<0.001$). These results suggest that in animals treated with PCP, circulating T4 sites on serum carriers may be available only in very limited numbers while at the same time serum TT4 levels are strongly decreased. An alternative explanation is a drastic reduction in the number of carrier protein molecules. Data from experiments with 3,4,3',4'-tetrachlorobiphenyl, which induces a similar hypothyroidism and interaction with T4 carriers, however, did not show alterations in serum TTR levels [19].

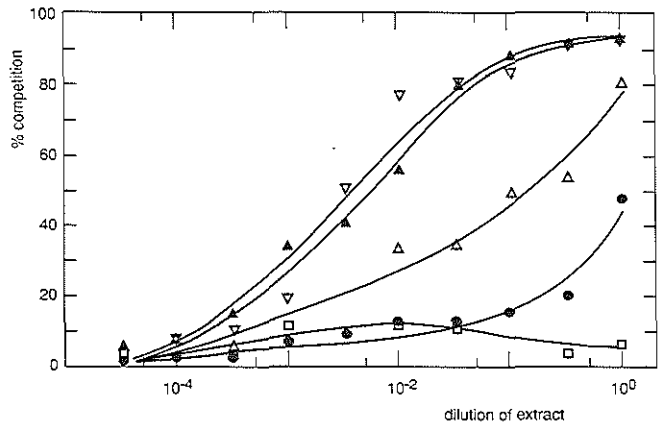
In a second series of *ex vivo* experiments it was studied whether sera of animals exposed to HCB, PCP or TCHQ contained compounds that were able to compete for T4 carrier sites. Sera were extracted according to procedures that allowed maximal recovery of HCB and PCP as described previously [8]. Current procedures, however, have failed to recover TCHQ from sera of treated animals as well as from spiked control serum, possibly due to covalent protein binding of TCHQ [20].

Extracts were tested in the competition assay with the T4 site of serum carriers. The results presented in Figure 2 show that extracts from sera of HCB-treated animals had about the same low competing activity as extracts from control sera. No competitive ability of the serum extracts at 2, 6, or 24 h after HCB administration, as compared with the control sera, was found. Extracts of sera early (2 and 6 h) after administration of PCP were highly efficient competitors while extracts obtained after 24 h were less competitive. These results indicate the presence of components able to occupy the T4-binding sites of serum carriers in animals dosed with PCP and which are present only in very low amounts or are absent in animals given an equimolar dose of HCB. Previously, HPLC analysis of sera from the HCB-groups revealed low levels of HCB and PCP (0.63 μmol/l and <0.14 μmol/l, respectively) while relatively high levels of PCP (190 μmol/l) were present in animals treated with an equimolar dose of PCP. The 24 h serum levels of

PCP were also found to be much lower than the 2 and 6 h levels [8]. These results are indicative of a close relationship between competing ability *in vivo* and serum levels of PCP in animals treated with a single equimolar dose of PCP or HCB.

In conclusion, the present results demonstrate that: (1) PCP is a much more potent competitor for the T4-binding sites of serum carriers than HCB; (2) T4-binding sites of thyroid hormone carriers are occupied in sera of PCP-dosed but not in HCB- or TCHQ-dosed animals; (3) serum levels of PCP in PCP- or HCB-dosed animals are correlated with competing ability of extracts

Fig.2. Competing ability of extracts from serum of rats treated with HCB, PCP or corn oil.



Rats were given i.p a single equimolar dose of 0.052 mmol/kg of HCB, or PCP, or corn oil only (controls). Blood samples were taken after 2,6, and 24 h. Sera from animals were pooled and extracted as described in Methods and Materials. Competition was determined in different dilutions of extracts, which were incubated in the presence of [^{125}I]T4 and diluted rat serum under standard conditions. At different times after exposure, blood was taken from animals, where after the sera were pooled, and extracted. +-+ = control extract, 6 h after exposure. □-□ = HCB extract 24 h after exposure. ▽-▽, ▲-▲ and △-△, PCP extracts 2,6 and 24 h after exposure.

from those sera; (4) at present, the effect of the minor HCB-metabolite TCHQ on serum TT4 and TT3 levels cannot be explained on the basis of competition with thyroid-hormone-binding-sites. Taken together, these results suggest the displacement of thyroid hormone carriers by the major

metabolite PCP as a possible factor in hypothyroidism due to chronic HCB administration. Experiments are in progress specifically aimed at testing this hypothesis.

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CHAPTER V

INTERACTIONS OF HALOGENATED INDUSTRIAL CHEMICALS WITH TRANSTHYRETIN AND EFFECTS ON THYROID HORMONE LEVELS IN VIVO

K.J. van den Berg[1], J.A.G.M. van Raaij[1,2], P.C. Bragt[1] and W.R.F. Notten[1,2]

1) TNO Medical Biological Laboratory, Rijswijk, The Netherlands
2) Erasmus University Rotterdam, The Netherlands

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ABSTRACT

Previous results in experimental systems have suggested that hydroxylated PCBs may decrease thyroid hormone levels through associative interaction with transthyretin. In the present paper it was investigated whether this property was also shared by various industrial chemicals, mainly pesticides. In total, 65 compounds from 12 chemical groups were analyzed for direct interference with the T4-binding site of transthyretin using a competitive binding assay. Sixty per cent of the compounds were competitive at a concentration level of 100 μM. Relatively strong interactions were observed by several chlorophenols, chlorophenoxy acids and nitrophenols, as well as by individual compounds such as hexachlorobenzene, dicofol, bromoxynil and tetrachlorohydroquinone. Examples from these chemical groups, e.g. pentachlorophenol, 2,4-dichlorophenoxybutyric acid, dinoseb and bromoxynil, also reduced plasma TT4 levels in rats. In addition, bromoxynil decreased plasma TT3 levels.

The results suggest the existence of a number of halogenated industrial chemicals with a potential for lowering plasma thyroid hormone levels through interference with hormone transport carriers.

INTRODUCTION

Exposure of man and experiment animals to a number of xenobiotics,

including halogenated organic chemicals, may lead to changes in thyroid hormone homeostasis. This is generally characterised by reduced plasma hormone levels and increased levels of TSH through feedback regulating mechanisms of the pituitary-thyroid axis. The normal morphology of the thyroid is altered towards hyperplasia and hypertrophy. The toxicological consequences of an induced long term stimulatory pressure on the thyroid and the pituitary have been reviewed recently (Zbinden 1987; Hill et al. 1989).

Only a few cases of occupational exposure to halogenated chemicals concerning effects on the thyroid are known. Workers handling organochlorine insecticides had lower protein-bound iodine levels than non-exposed workers (Wassermann et al. 1971). Among workers in a plant manufacturing polybrominated biphenyls (PBBs), a high prevalence of primary hypothyroidism was encountered (Bahn et al. 1980). Although non-occupational treatment of patients with the organochlorine pesticide *o,p'*-DDD decreased serum protein-bound iodine (Danowski et al. 1964). Long term toxic effects of consumed grain treated with hexachlorobenzene included reduced plasma thyroid hormone levels accompanied by thyromegaly (Peters et al. 1982).

In experimental animal systems, rodents as well as primates, interference in thyroid homeostasis by polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs), 2,3,7,8-tetrachloro-*p*-dioxin (TCDD), HCB and DDT is well documented (Wassermann et al. 1973; Hurst et al. 1974; Bastomsky 1977; Gupta et al. 1983; Rozman et al. 1986; Van den Berg et al. 1988).

The mechanism of decreased plasma thyroid hormone levels by these chemicals is not clear. Enhanced hepatic thyroxine metabolism may in part be responsible for reductions of T4 levels by TCDD (Bastomsky 1977; Henry and Gasiewicz 1987). With respect to PCBs, we have recently obtained evidence that in vivo a metabolite was associated specifically with transthyretin (TTR), a carrier of both vitamin A and thyroid hormones (Brouwer and Van den Berg 1986). This has led to the suggestion that PCBs may cause a decrease in plasma vitamin A and thyroid hormone levels through displacement of the physiological compounds from their binding sites by hydroxylated metabolites (Brouwer and Van den Berg 1986; Brouwer 1989). Direct evidence for competition of hydroxylated PCBs for the T4-binding site of TTR in vitro has recently been provided (Rickenbacher et al. 1986).

In an attempt to determine whether this was a unique property of PCB metabolites or a more general phenomenon among industrial chemicals, a number of compounds from various chemical origins were investigated for the ability to occupy the T4-binding site of TTR. In addition, the effect of a few of these industrial chemicals on plasma thyroid hormone levels in vivo was studied.

MATERIALS AND METHODS

Materials

Chemicals were of the highest purity commercially available and were obtained from Aldrich, Brussels, Belgium; from Sigma, St. Louis, Mi USA and from Serva, Heidelberg, Germany. Stock solutions (0.023 M) of the compounds were made in ethanol, except for 3,4,3',4'-tetrachlorobiphenyl (ethanol/DMSO,1:1). Human transthyretin was obtained from Sigma, St Louis, Mi USA. L-[3',5'-^{125}I]thyroxine (specific activity about 1500 μCi/μg, 55 Mbq/μg) was purchased from Amersham, England. All other chemicals used were of analytical grade.

Methods

Competition assay. The capacity of various compounds to compete with T4-binding sites was studied essentially as described by Somack et al. (1982) with modifications as described in detail elsewhere (Van den Berg, 1990). Briefly, an aliquot of the compound was added to a reaction mixture consisting of phosphate buffered saline (PBS, 0.01 M phosphate pH = 7.3 + 0.154 M NaCl), radiolabelled thyroxine (about 0.2 μCi/7,4 kBq) and transthyretin (TTR 1.1 μg/ml, final concn) in total volume of 460 μl. The reaction mixture was allowed to reach equilibrium for 30 min at room temperature. A 200 μl aliquot of the reaction mixture was placed on a 1 ml Sephadex G-25 column kept at 0°C. TTR-bound radioactivity was eluted with 300 μl ice-cold PBS using slight overpressure. The column was subsequently eluted with 1.0 ml PCS to assess the amount of free iodide (about 10%). Replicate 200 μl samples were processed on separate columns. Radioactivity in eluate fractions was determined in a gamma counter. For determination of maximum binding ethanol was added instead of the test compound.

Protein-bound radioactivity amounted to 15% of the total amount of label in the reaction mixture. Non-specific binding was also determined in each series of experiments, by addition of cold T4 to 10 μM final concentration and was less than 10%.

Animal studies. Chemicals were dissolved in corn oil and administered i.p. to WAG/MBL rats (male, 12 weeks old, 250-300 g, 4-6 animals/dose group) in a single dose. Control animals received 1 ml corn oil i.p. After 6 h blood was taken from the tail vein under ether anaesthesia. Plasma samples were prepared and stored at -20°C for analysis of thyroid hormones. Determination of plasma TT4 and TT3 levels was done using commercial RIA kits (Amerlex-M, England). It was ascertained that the chemicals did not interfere in the thyroid hormone assays.

Statistics. Statistical difference between the means of the groups dosed with chemicals and the control group was determined using Student's *t*-test.

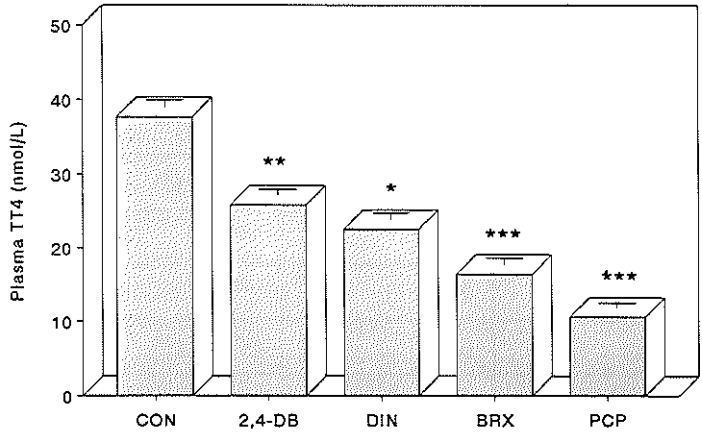
RESULTS AND DISCUSSION

A total of 65 industrial compounds from 12 different chemical groups were analyzed with respect to a possible interference with the thyroxine binding site of TTR. The results, presented in Table 1, show that 39 compounds (60%) were competitors for T4 binding at a concentration level of 100 μM. A global differentiation in potency of the compounds was made over three ranges of competition. It reveals that 21 compounds (32%) are moderate to potent competitors, 10 (15%) weak and 8 (12%) very weak competitors.

The competition assay used is very sensitive owing to the high specific radioactivity of T4, since a 50% competition level (IC_{50}) could be attained by 4×10^{-8} M cold T4 under standard assay conditions. Additional studies with chlorophenols (Van den Berg 1990) indicate that relative affinities [defined as $IC_{50}(T4)/IC_{50}(Chemical)$] of 0.001-1 are in the highest (+++) group, while the middle (++) group harbours compounds with a relative affinity of about 0.0001. In the scale of competition used in the table very weak interactions (<0.0001 relative affinity) are apparent in the + group. The classification of competition levels in the table may therefore be somewhat over-representing low affinity interactions.

The compounds with the highest level of competition were found among the chlorophenols. This group of chemicals has recently been studied in more detail (Van den Berg 1990). The results indicate that i) interaction with the T4 site is dependent on the degree of chlorination, ii) the combination of hydroxyl and chlorine groups is more competitive than either group separately, iii) displacement of T4 from the binding site is by a competitive type of interaction. In order to investigate in vivo effects with respect to thyroid hormone levels, rats were dosed with pentachlorophenol (PCP, 0.06 mmol/kg i.p.). The results (Fig. 1) show a reduction of plasma TT4 level by PCP of 72% as compared with the control values. Plasma TT3 concentrations were not significantly changed by PCP (Fig. 2).

Fig.1. Effect of industrial chemicals on plasma TT4 levels.



The chemicals were administered to rats and plasma samples were obtained for determination of TT4 levels as described in the Materials and Methods section. *CON* (control), 2,4-*DB* (2,4-dichlorophenoxybutyric acid, 0.06 mmol/kg), *DIN* (dinoseb, 0.03 mmol/kg), *BRX* (bromoxynil, 0.06 mmol/kg). *PCP* (pentachlorophenol, 0.06 mmol/kg). Values are expressed as means ± SEM. Statistical significance: $^{*}P < 0.01$, $^{**}P < 0.005$, $^{***}P < 0.0001$

Additional industrial chemicals such as chlorophenoxy acids (Table 1, group 2) and nitrophenols (Table 1, group 3) were also effective competitors for the T4-binding site. Theoretical modelling studies by Cody (1985) have

Table 1. Competition of industrial chemicals for the T4-binding site of transthyretin (TTR)

Chemical group	Number of Compounds tested	-[a]	+[a]	++[a]	+++[a]
1. Chlorophenols	10	1[1]		2[2]	7[3]
2. Phenoxy acids	8			1[4]	7[5]
3. Nitrophenols	3				3[6]
4. Chlorobenzenes	3	1[7]		1[8]	1[9]
5. DDTs	4	1[10]	2[11]		1[12]
6. Organophosphates	5	2[13]	1[14]	2[15]	
7. Biphenyls	3		2[16]	1[17]	
8. Chloroalkanes	9	7[18]	1[19]	1[20]	
9. Phenyldimethylurea	5	3[21]	1[22]	1[23]	
10. Phenols	8	7[24]		1[25]	
11. Organotin	2	2[26]			
12. Others	5	2[27]	1[28]		2[29]
Totals	65	26	8	10	21

a: Competition; - = <10 %, + = 11-40 %, ++ = 41-70 %, +++ = 71-100 %.

All compounds were tested at 100 μM in the standard assay as described in Materials and Methods. Compounds are specified below by superscripts 1-29.

1 4-chlorophenol
2 2-chlorophenol, 3-chlorophenol
3 Pentachlorophenol, hexachlorophene, 2,4,6-trichlorophenol, 2,4,5-trichlorophenol, 2,3 dichlorophenol, 2,4-dichlorophenol, 2,6-dichlorophenol
4 2,4,5-trichlorophenoxyacetic acid methyl ester
5 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4-

dichlorophenoxybutyric acid (2,4-DB), 2-(2,4-dichlorophenoxy)propionic acid (Dichlorprop), 4-(chloro-*o*-tolyoxy) acetic acid (MCPA), 4-(4-chloro-2-methylphenoxy) butyric acid (MCPB), 2-(2,4,5-trichlorophenoxy) propionic acid (Fenoprop)

6 2,4-dinitrophenol, 2,4-dinitro-6-methylphenol (DNOC), 2,4-dinitro-6-*sec*-butylphenol (Dinoseb)

7 dichlorobenzene

8 trichlorobenzene

9 hexachlorobenzene

10 methoxychlor

11 p,p'-DDD, *o,p'*-DDD

12 1,1,1-trichloro-2,2-bis(chlorophenyl)ethanol (Dicofol)

13 paraxon, ethyl-azinphos

14 malathion

15 ethyl-parathion, ethyl-bromophos

16 3,4,3',4'-tetrachlorobiphenyl, 4-hydroxybiphenyl

17 2-hydroxybiphenyl

18 trichloroethylene, perchloroethylene, tetrachloroethane, chloroform, chloral hydrate, hexachloroethane, tetrachloromethane

19 trichloroacetic acid

20 hexachlorocyclohexane (-lindane)

21 monuron, diuron, neburon

22 linuron

23 chloroxuron

24 phenol, catechol, resorcinol, *o*-cresol, 3-aminophenol, 4-*tert*-butylphenol, 2,6-di-*tert*-butyl-4-methylphenol (BHT)

25 pyrogallol

26 trimethyltin, triethyltin

27 benzene, endosulfan

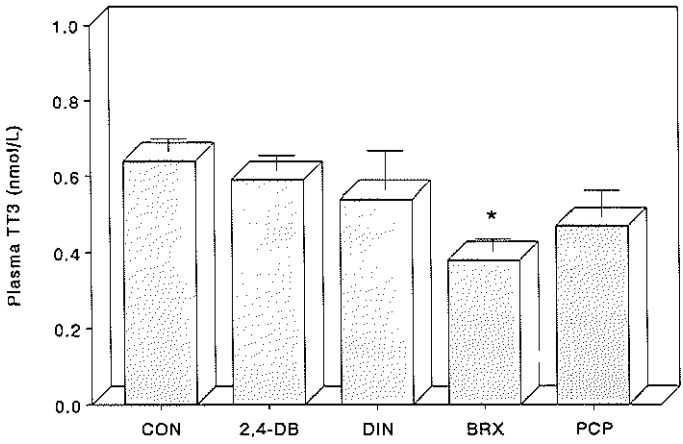
28 dioctylphtalate

29 3,5-bibromo-4-hydroxybenzonitril (Bromoxynil), tetrachlorohydroquinone

suggested potential fitting of chlorophenoxy acids in the binding pocket of transthyretin. Little is known about in vivo effects of chlorophenoxy acids and nitrophenols with respect to thyroid hormone levels in man or experimental animals. Therefore, rats were dosed with 2,4-dichlorophenoxybutyric acid (2,4-DB, 0.06 mmol/kg) or 2,4-dinitro-6-*sec*-butylphenol (dinoseb, 0.03 mmol/kg). The results show that plasma TT4 levels were significantly decreased by 2,4-DB (by 32%) or dinoseb (by 40 %, Fig. 1). No significant changes of plasma TT3 levels were observed by 2,4-DB or dinoseb (Fig. 2).

With respect to the group of chlorobenzenes (Table 1, group 4) a clear increase in binding ability was observed from dichlorobenzene (compound 7), via trichlorobenzene (compound 8) to hexachlorobenzene (compound 9).

Fig.2. Effect of industrial chemicals on plasma TT3 levels.



Plasma TT3 levels were determined as described in Materials and Methods. Other details are the same as in the legends of Fig.1

Hexachlorobenzene (HCB) poisoning in man has led to a number of toxic symptoms including thyromegaly along with lowered thyroid hormone levels, especially in women (Peters et al. 1982). Furthermore, chronic HCB treatment of experimental animals has been shown to result in significant reductions in plasma thyroid hormone levels (Rozman et al. 1986). Experiments are currently in progress to determine the respective contributions of HCB and its metabolites pentachlorophenol and tetrachlorohydroquinone (Ahlborg et al. 1978; Van Ommen et al. 1986) in lowering thyroid hormone levels in vivo.

A few DDT-like chemicals such as *p,p'*-DDD, *o,p'*-DDD and dicofol, in particular, were found to interact with thyroid hormone carrier (group 5, compounds 11/12). In man, *o,p'*-DDD has been found to lower serum protein bound iodine by intervening with thyroid hormone carrier (Marshall and Tompkins 1968). Modelling studies also indicate a fit of DDT in the T4-binding pocket of transthyretin (Cody 1985). The thyroid hormone-lowering effect of DDT, the parent compound, in a few experimental animals systems is well known (Bastomsky 1974; Hurst et al. 1974). To date, very limited data is available on effects of dicofol with respect to plasma thyroid hormone levels. Bromoxynil, a brominated benzonitril herbicide, was found to be an

efficient competitor for the T4-binding site of TTR (Table 1, group 12, compound 29). In rats dosed with bromoxynil (0.06 mmol/kg) a significant 56% reduction of plasma TT4 levels was found (Fig. 1). In addition, plasma TT3 levels (Fig. 2) were significantly decreased by bromoxynil (by 41 %). Recently, a related compound, ioxynil (an iodinated benzonitril), showed binding specifically to the T4 site of transthyretin and may affect the thyroid of experimental animals (Ogilvie and Ramsden 1988).

A number of compounds among organophosphates, (halogenated) biphenyls, chloroalkanes, and phenyldimethylurea showed definite ability for specific competition with thyroid hormone carrier albeit with low affinity (Table 1, groups 6-9). There are indications that malathion may decrease serum protein-bound iodine in experimental animals (Balasubramanian et al. 1986). The effect of halogenated biphenyls on the thyroid is supported by a vast amount of data mainly in experimental animal systems both in rodents and in primates (Wassermann et al. 1973; Hurst et al. 1974; Collins et al. 1977; Sleight et al. 1978; Gupta et al. 1983; Byrne et al. 1987; Van den Berg et al. 1988). In man, occupational exposure to polybrominated biphenyls (PBBs) may result in reduced plasma thyroid hormone and increased thyrotropin levels (Bahn et al. 1980). Metabolites of PCBs have been found as a complex with transthyretin and are thought to be involved in reduced serum thyroid hormone and vitamin A levels in experimental animals (Brouwer and Van den Berg 1986; Brouwer 1989). Using similar in vitro binding assays as in the current work, Rickenbacher et al. (1986) demonstrated binding of hydroxylated PCBs to the thyroxine site of transthyretin as well as to the nuclear receptor. Besides the interaction of PCBs with thyroid hormone carriers, these compounds may induce hepatic thyroxine metabolism, e.g. glucuronidation. This may also contribute to decreased plasma hormone levels (Bastomsky 1977; Henry and Gasiewicz 1987).

Most compounds tested in the group of chloroalkanes were not competitors at 100 μM, except for trichloroacetic acid and Υ-hexachlorocyclohexane (Υ-lindane) (Table 1, group 8, compounds 19/20) that exhibited a low to moderate level of interaction. In vivo data concerning thyroid effects of these compounds is not at hand. Of the phenyldimethylurea pesticides investigated, linuron and chloroxuron were weak to moderate competitors (Table 1, group 9, compounds 22/23). Effects of urea pesticides on the thyroid in experimental studies have been reported [cited in Bainova (1982)].

The present results show that examples from a variety of halogenated pesticides with different chemical backbones are able to interact with the T4-binding site of transthyretin, one of the carriers of thyroid hormones in the circulation. It should be noted, however, that in man TBG is the major carrier of thyroid hormones but recent studies have also provided evidence for interaction of chlorophenols with the T4-binding site of TBG and albumin in addition to transthyretin (Van den Berg 1990).

The interaction of number of industrial chemicals with circulatory carriers may have several consequences. Firstly, total plasma thyroid hormone levels may be altered. Although it is too early to make final conclusions, there appears to be an indication of a relationship between interference of chemicals at the carrier level and a concomitant reduction of plasma thyroxine levels in vivo. More combined in vitro/ in vivo studies are required to establish a firm relationship that may lead to a relatively simple in vitro tool to predict thyroid effects of industrial/occupational chemicals. In occupational settings with possible exposure to halogenated organic chemicals of various sorts it may be valuable to determine thyroid parameters.

Secondly, interaction of chemicals with transthyretin may also have implications for neurobiological/toxicological processes in exposed individuals. Transthyretin is one of the few proteins identified in the cerebrospinal fluid (CSF), that is synthesized in the choroid plexus (Kato et al. 1986) and may have a function in the transport of thyroxine through the blood-CSF barrier (Dickson et al. 1987). It is possible that chemicals interacting with transthyretin may affect the transport function of the choroid plexus with possible consequences for brain functions. A large proportion of the chemicals with affinity for thyroid hormone carrier (Table 1) appear to have neurotoxic properties (Bainova 1982). In fact, recent evidence indicates that neurotoxic chemicals such as 2,4,5-T and 2,4-D (Table 1, group 8, compound 5) affect choroid plexus transport (Pritchard 1980; Kim et al. 1983, 1987).

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CHAPTER VI

INCREASED GLUCURONIDATION OF THYROID HORMONE IN HEXACHLOROBENZENE-TREATED RATS

J.A.G.M van Raaij[1,2], E.Kaptein[3], T.J. Visser[3] and K.J van den Berg[2]

1) Institute of Public Health and Social Medicine,
Erasmus University Medical School, Rotterdam, The Netherlands
2) TNO, Medical Biological Laboratory, Rijswijk, The Netherlands
3) Department of Internal Medicine III,
Erasmus University Medical School, Rotterdam, The Netherlands

ABSTRACT

Metabolism of thyroid hormones was investigated in WAG/MBL rats that had been exposed to hexachlorobenzene (HCB). Serum thyroxine (T4) levels were lowered by 35.5 %, whereas triiodothyronine (T3) levels were not changed. Bile flow, as well as T4 excretion in bile were increased by HCB-treatment. Analysis of bile by HPLC revealed a more than 3-fold increase of T4-glucuronide (T4G) and a concomitant reduction of non-conjugated T4. T4-UDP-glucuronyltransferase activity (T4 UDPGT) activity in hepatic microsomes was increased more than 4.5-fold in animals exposed to HCB. *p*-nitrophenol (PNP) UDPGT showed a comparable increase by HCB. Both T3 and androsterone UDPGT activities were low in WAG/MBL rats compared with normal Wistar rats. T3 UDPGT activity was increased 2.5-fold by HCB, but androsterone UDPGT activity was unchanged. These results suggest that T4 is a substrate for HCB-inducible PNP UDPGT and T3 for androsterone UDPGT. In the absence of the latter, T3 is also glucuronidated to some extent by PNP UDPGT. Type 1 iodothyronine deiodinase activity was decreased by HCB treatment.

It is concluded that decreased T4 levels in serum of animals after exposure to HCB may be due to a combined effect of displacement of T4 from carriers, an increased glucuronidation of T4 and enhanced bile flow.

INTRODUCTION

It is well known that the fungicide hexachlorobenzene (HCB) affects the

thyroid system. Chronic exposure of different species of animals but also of humans leads to a decrease of thyroid hormone levels in the circulation (1-7). Several chlorinated aromatic compounds, such as polychlorinated biphenyls (PCBs) and dioxins have also been found to cause hypothyroidism (8-11). The mechanisms responsible for reduced thyroid hormone levels after chronic exposure of rats to HCB are not completely understood. There are indications that the major metabolite of HCB, i.e. pentachlorophenol (PCP), may play a role because PCP is more potent in decreasing serum thyroid hormone levels in rats than an equimolar dose of HCB (3). In addition, *in vitro* and *ex vivo* observations suggest that PCP interacts strongly with serum thyroxine (T4) carrier proteins as compared to HCB (4,12,13). PCP was found to interact competitively with the T4-binding site of transthyretin, while the affinity of PCP is about 2-fold higher than that of T4 (12). This competition with thyroid hormone carriers may contribute to the lowered blood T4 levels (4,12,13).

Enhanced hepatic metabolism of T4 in HCB-treated rats might be an additional mechanism for reducing serum T4 levels (5,6,14,15). The most prominent metabolic routes for T4 are the deiodination pathways and hepatic conjugation of the phenolic hydroxyl group with glucuronic acid or sulfate. Recent studies reported that multiple UDP-glucuronyltransferase (UDPGT) isozymes are involved in the glucuronidation of thyroid hormones, and suggested that T4 is glucuronidated by *p*-nitrophenol (PNP) and bilirubin UDPGTs, and triiodothyronine (T3) specifically by androsterone UDPGT (16,17).

In the present study, the role and identity of enzymes involved in the metabolic clearance of thyroid hormones in HCB- or vehicle exposed rats were investigated by determining (1) bile flow and biliary excretion of thyroid hormone glucuronides (2) T4 and T3 UDPGT and type 1 deiodinase activities in liver microsomes in parallel with PNP and androsterone UDPGT activities.

MATERIALS AND METHODS

Chemicals

HCB was obtained from Aldrich (Brussels, Belgium); [^{125}I]T4 (SA >1200 μCi/μg), [^{125}I]T3 (SA 2800 μCi/μg), [^{125}I]rT3 (SA> 1200 μCi/μg), T4 and T3 radioimmunoassay (RIA) kits (Amerlex-M) from Amersham (Amersham, U.K);

androsterone from Steraloids (Wilton, NH, U.S.A); [^{3}H] androsterone (SA 116 μCi/μg) from New England Nuclear (Boston, MA, U.S.A); UDP glucuronic acid (UDPGA) from Boehringer (Mannheim, F.R.G.); bovine serum albumin (BSA), 3,3-cholamidopropyl-dimethylammonio-1-propanesulfonate (CHAPS), di-thiothreitol (DTT), PNP, T4, and T3 from Sigma Chemical Co. (St Louis, MO, U.S.A); 3,3',5'-triiodothyronine (rT3) from Henning (Berlin, F.R.G.); Sephadex LH-20 from Pharmacia (Uppsala, Sweden). Radiolabelled T4 and T3 were purified on Sephadex LH-20 before incubation.

Animals and Treatments

Male WAG/MBL (WAG) rats, weighing 200-300 g were maintained on regular diet and tap water *ad libitum*. They were housed in a constant environment with a 12 h light : 12 h dark cycle, a temperature of 24°C and a humidity of 50-70 %. Animals were treated orally three times a week for 4 weeks with 3 ml of either 1 g HCB/kg (40 mg/ml, 0.5 % Tween-20 in water) or water plus 0.5% Tween-20 only. After the last dose, liver and sera were collected from animals after killing by decapitation. Livers were frozen at -70°C until preparation of microsomes. Sera were also stored at -70°C until analysis of T4 and T3 by commercial radioimmunoassay (RIA) kits.

Bile flow and biliary clearance

Animals were injected i.p with 25 μCi [^{125}I]T4 in 1 ml saline. After about 5 h, bile ducts were cannulated under halothane anaesthesia. Bile was collected in fractions of 10 min for a 1 h period. After termination of the experiment, serum samples were collected. Radioactivity in serum and bile samples was determined in a gamma counter.

HPLC analysis of bile samples

Representative chromatograms of T4 glucuronide (T4G) excretion were obtained by injecting 20 μl of pooled bile from HCB- or vehicle-treated rats into a reverse phase HPLC C18 system, eluting with linear gradients of acetonitrile in ammonium acetate (pH 4), as described earlier (18). This procedure results in the separation of iodide, sulfated, glucuronidated and non-conjugated iodothyronines (18). Fractions were collected and counted

for radioactivity. Recovery of applied radioactivity amounted to 80 and 100 %.

Microsomal preparations

Livers from WAG rats were homogenised in 5 volumes of 0.25 M sucrose, 10 mM Hepes, 1 mM DTT (pH 7.0) at 4°C. Microsomes were obtained by centrifugation for 10 min at 25000 x g and subsequent centrifugation of supernatants for 60 min at 100000 x g. The microsomal pellets were suspended at a protein concentration of 10-20 mg/ml in 0.1 M phosphate (pH 7.2), 2 mM EDTA and 1 mM DTT, and frozen in 0.5 ml aliquots at -80°C. Protein contents in microsomes were measured with the bicinchoninic acid procedure (18), or with the Bio-Rad assay (Richmond, CA, U.S.A), using BSA as the standard.

Assays

T4 and T3 UDPGT activities were assayed essentially as previously described (16) in incubations of 1 μM labelled T4 or T3 with 1 mg/ml protein and 5 mM UDPGA in 75 mM Tris (pH 7.8), 3.75 mM $MgCl_2$, containing 0.125 % BSA and 0.025 % CHAPS. PNP UDPGT (20), androsterone UDPGT (21) and type I deiodinase activity (22) were also measured according to published methods.

Statistics

Bile flow and biliary T4 clearance in control and HCB-treated rats were statistically evaluated by analysis of variance and covariance with repeated measures. Student's t-tests were used for comparing other effects in control and HCB-treated animals.

RESULTS AND DISCUSSION

When animals had been exposed to HCB for 4 weeks, serum T4 levels were decreased by 35 % compared with control animals (Table 1). Serum levels of T3 were not different between both groups (Table 1). In earlier studies (5), a transient decrease in serum levels of T3 during the second and

third week of dosing was observed.

To investigate whether lowered T4 levels after dosing with HCB were the result of an increased metabolism, biliary T4 clearance was analysed. It appeared that bile flow was significantly increased ($p<0.05$) by more than 100 % after HCB treatment compared with control values (Fig. 1). This may

Table 1. Serum T4 and T3 levels of rats treated with HCB

Hormone	HCB	N	Control	N
T4 (nmol/L)	12.9 ± 0.58*	5	20.0 ± 2.00	5
T3 (nmol/L)	0.63 ± 0.16	5	0.65 ± 0.15	5

Rats were exposed to HCB for 4 weeks. After the last dose, serum levels of T4 and T3 were determined by radioimmunoassay. Results are given as mean ± SEM. *, $p<0.001$.

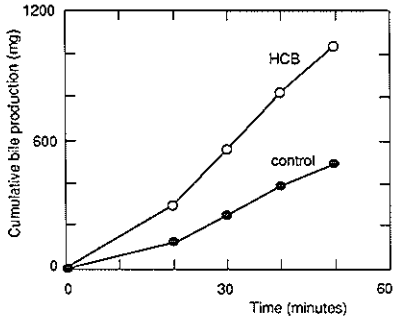
be explained by enlargement of the liver, which was significantly increased in weight by HCB: livers of rats exposed to HCB or vehicle weighed 16.7 ± 0.5 g and 10.0 ± 0.4 g respectively ($p< 0.001$). Recently, Cuomo et al.(23) reported an increased bile acid independent flow (BAIF) by HCB.

In order to examine excretion of T4 in bile, animals received [^{125}I]T4. Radioactivity excreted into bile fluid was also significantly increased (more than 100%, $p<0.01$) by HCB (Fig. 2, Table 2). Therefore, it may be concluded that HCB increases the elimination of T4 by an increased bile flow.

The composition of biliary radioactivity was analysed by HPLC. A chromatogram of bile from control animals showed the label predominantly in association with T4G and to a minor extent with non-conjugated T4 (Fig.3A). Some iodide was present in the void volume fractions. A similar chromatogram of bile from animals exposed to HCB revealed a greater extent of T4 glucuronidation (Fig.3B). Excretion of T4G was increased 3.7-fold by HCB exposure, whereas non-conjugated T4 was decreased 2.6 fold (Table 2).

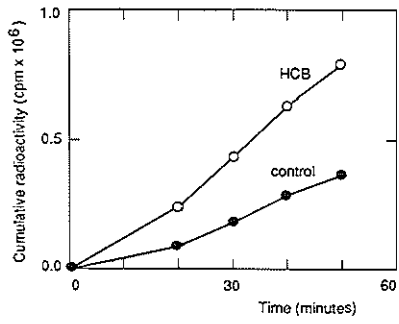
HCB stimulation of biliary T4G excretion may be due to an enhanced T4 UDPGT activity. Indeed, a 4.7-fold increase of T4 UDPGT activity was observed (Table 3), which appeared somewhat more substantial than the

Fig 1. Bile flow in control and HCB-treated rats.



Bile ducts were cannulated from rats exposed to HCB (N=4) or to vehicle (N=3). Bile was collected in 10 min fractions and weighed. Results are expressed as the cumulative weight of bile collected.

Fig 2. Cumulative biliary T4 clearance in control and HCB-treated rats.



Rats exposed to HCB (N=4) or to vehicle (N=3) received [^{125}I]T4 i.p. About 5 h later bile collection was started. Bile samples were weighed and counted.

Table 2. Biliary T4 clearance in control and HCB- treated rats

Parameter	HCB (% of dose)	N	Control (% of dose)	N
Bile radioactivity	2.9 ± 0.12*	4	1.3 ± 0.22	3
T4G	1.5 ± 0.06**	4	0.4 ± 0.08	3
T4	0.07 ± 0.01	4	0.18 ± 0.05	3

Rats were exposed for 4 weeks to HCB or vehicle. At the end of the experiment, the animals were injected i.p with 25 μCi [^{125}I]T4. About 5 h later, bile ducts were cannulated for collection of bile. Total bile was analyzed by HPLC as described in Materials and Methods. Results are given as means ± SEM. *, $p < 0.01$. **, $p < 0.001$

excretion of T4G into bile (Table 2). Increased levels of T4G in bile were also demonstrated after exposure of rats to PCBs, associated with a strong induction of T4 UDPGT (16). Thus, HCB induces processes involved in the metabolism of T4.

There are indications that in rats T4 and PNP are substrates for a common UDPGT isozyme and that xenobiotics, such as PCBs, methylcholantrene, dioxines, and other various hepatic microsomal enzyme inducers, are potent inducers of T4 and PNP glucuronidation (14-16, 24-26). PNP UDPGT activity in the WAG rats used in the present study was increased 4.5-fold by HCB (Table 3). In view of the broad substrate specificity, this enzyme could also be involved in glucuronidation of PCP, the major metabolite of HCB. Our findings suggest that also in the WAG rat the enzyme responsible for enhanced T4 glucuronidation by HCB may be identical to the HCB-induced PNP UDPGT.

Table 3. Effects of HCB on UDPGT and type I deiodinase activities

Enzyme activity	HCB	N	Control	N
T4 UDPGT (pmol/min/mg)	1.26 ± 0.07**	5	0.27 ± 0.02	5
PNP UDPGT (nmol/min/mg)	278 ± 8.94**	5	62 ± 2.68	5
T3 UDPGT (pmol/min/mg)	0.88 ± 0.04**	5	0.35 ± 0.01	5
Androsterone UDPGT (nmol/min/mg)	0.52 ± 0.01*	5	0.47 ± 0.02	5
Deiodinase (pmol/min/mg)	442 ± 90*	5	641 ± 131	5

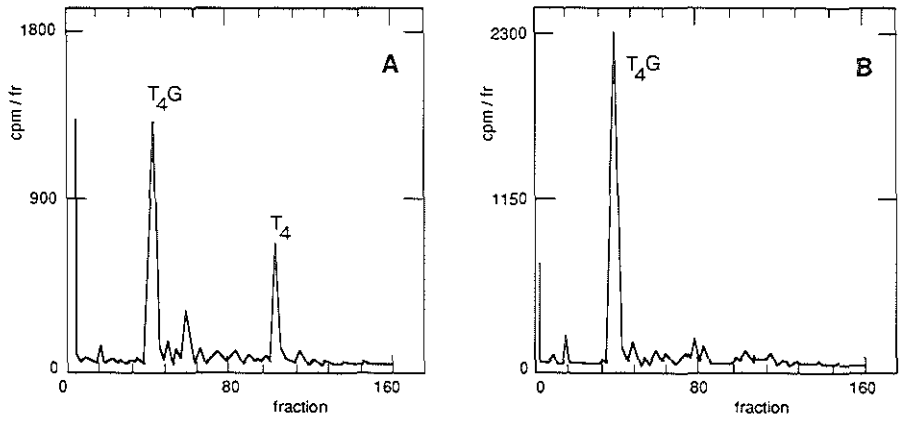
Dosing of animals, isolation of livers, preparation of hepatic microsomes and enzyme assays were carried out as described in Materials and Methods. *, $p < 0.05$. **, $p < 0.001$

Androsterone UDPGT activity in untreated WAG rats was ≈10% of that measured in livers of normal Wistar (HA) rats and similar to the androsterone

UDPGT activity in Wistar LA and Fisher rats, which have a genetic defect in the gene coding for this isoenzyme (16,17). Like Wistar LA and Fisher rats, T3 UDPGT activity in WAG rats is only about one-third of that in Wistar HA rats, suggesting that T3 is a substrate for androsterone UDPGT.While HCB has little influence on the (low) androsterone UDPGT activity in WAG rats, it increases liver microsomal T3 glucuronidation 2.5-fold (Table 3). This suggests that in the absence of androsterone UDPGT, T3 is glucuronidated to some extent by the HCB-inducible PNP UDPGT.

In conclusion, decreased serum T4 levels in animals exposed to HCB may be explained by a combination of factors: displacement of T4 from serum proteins and induction of T4 UDPGT activity. The resultant increase in thyroid activity apparently does not fully compensate for the increased T4 clearance.

Fig 3. HPLC analysis of bile from control (A) and HCB-treated (B) rats.



Bile samples were analyzed by HPLC as described in the Methods section. Results are expressed as radioactivity per fraction. Note differences in scale of the vertical axis.

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CHAPTER VII

HEXACHLOROBENZENE-INDUCED HYPOTHYROIDISM: INVOLVEMENT OF DIFFERENT MECHANISMS BY PARENT COMPOUND AND METABOLITE

J.A.G.M. van Raaij[1,2], C.M.G. Frijters[2], and K.J. van den Berg[2]

1) Institute of Public Health and Social Medicine,
Erasmus University Rotterdam, The Netherlands
2) TNO Medical Biological Laboratory, Rijswijk, The Netherlands

ABSTRACT

Rats received repeated oral treatment with different doses of hexachlorobenzene (HCB) (0 - 3.5 mmol/kg) for 2 or 4 weeks. Measurements of thyroid hormone status after 2 weeks showed a dose-dependent decrease of total thyroxine (TT4) levels, decreased free thyroxine (FT4) levels and little change of total triiodothyronine (T3) levels. The effects on thyroid hormone status were more pronounced after 4 weeks and also included increased thyroid stimulating hormone (TSH) levels. These conditions suggest that HCB had induced hypothyroidism in these animals. Indications for occupation of thyroid hormone binding proteins were found in serum of exposed animals. The major metabolite pentachlorophenol (PCP) also caused, by competitive interactions with thyroid hormone binding proteins in serum, a rapid and dose-dependent decrease of TT4 and FT4 levels, but not of TT3 levels in serum. The decrease of serum TT4 levels by repeated dosing with 3.5 mmol HCB/kg for 4 weeks could be attributed to competitive interactions of PCP with hormone serum binding protein and to increased metabolism induced by HCB to an equal degree. At lower dose levels or with shorter dosing periods, increased metabolism of T4 is the main cause of decreased TT4 serum levels.

This is the first indication that a similar effect is caused at the same time by parent compound and metabolite through different and independent mechanisms.

INTRODUCTION

Hexachlorobenzene (HCB), a polyhalogenated aromatic compound, has

been used in industry mainly as a fungicide and occurs as a by-product of industrial aromatic chlorination processes (1). Accidental poisoning of humans to HCB by consumption of HCB-treated grain has been reported and toxic effects observed included enlargement of the thyroid gland and decreased blood thyroxine (T4) levels (2).

In experimental animal systems a number of biological and biochemical effects of HCB on thyroid homeostasis have been described. Chronic feeding of laboratory animals with HCB was found to induce thyroid adenomas (3), thyromegaly, and hypothyroidism (4-6). Similar effects concerning the thyroid have been observed in rodents and primates chronically exposed to other halogenated aromatics such as polychlorinated biphenyls (PCBs) or polybrominated biphenyls (PBBs), (7-10).

The mechanism(s) involved in HCB-induced hypothyroidism is not clear. Microsomal enzymes are strongly induced by HCB (4,6,11) and this could lead to an increased T4 catabolism. Recently, it was shown that HCB strongly enhanced glucuronidation of T4 through induction of several UDP-glucuronyltransferases (UDPGTs) (12), resulting in an augmented disappearance of T4 from the body (12). In animals exposed to PCBs and dioxins, such a mechanism has also been advanced (13-16).

An additional factor that might contribute to depressed thyroid hormone levels in HCB-induced thyroid changes is pentachlorophenol (PCP), the major oxidative metabolite of HCB (17-19). PCP has been found more effective in reducing thyroid hormone levels than HCB itself in acute *in vivo* experiments (20). Furthermore, *in vitro* studies have demonstrated competitive interactions of PCP and T4 for binding sites on T4-binding serum proteins with a greater specificity for binding to transthyretin (TTR) than either thyroid binding globulin (TBG) or albumin (21,22). Comparable results have been found in animals exposed to chlorinated benzenes or 3,4,3',4'-tetrachlorobiphenyl (TCB), suggesting that not only the hydroxylated form of chlorinated aromatic compounds might be responsible for lowered T4 levels in serum through competitive interactions with T4-binding serum proteins (23-26).

Other studies showed that hydroxylated PCBs, but also the hydrophylic derivates of dioxins and dibenzofurans are capable to interact with the T4-binding sites of TTR and nuclear thyroid hormone receptors (27-30). In addition, it was demonstrated by molecular modelling that DDT (dichlorodiphenyltrichloroethane), an insecticide that also lowers thyroid

hormone levels, fitted into the T4-binding sites of TTR (31).

The aim of the present study was to determine the relative contributions of 1) increased microsomal catabolism of T4 by HCB and 2) interference of PCP at the thyroid hormone binding protein level in hypothyroidism induced by HCB. For this purpose, animals were repeatedly dosed with HCB to induce hypothyroidism as determined from measurements of thyroid hormone status. In addition, serum concentrations of HCB and PCP in these animals were analyzed. In separate experiments the capacity of PCP to decrease thyroid hormone levels by competitive interactions was determined. The combined results were used to estimate the relative role of both types of mechanisms in hypothyroidism induced by HCB.

METHODS

Chemicals

HCB and PCP were purchased from Aldrich (Brussels, Belgium). Commercial TT4 (Amerlex-M), TT3 (Amerlex-M) and TSH RIA kits (Amerlex-M, code RPA 554) were purchased from Amersham (Amersham, U.K). The FT4 and FT3 assays (Amerlex-MAB) were obtained from Kodak Clinical Diagnostics Ltd, (Cardiff, U.K). Tween-20 was obtained from Bio Rad Laboratories, Richmond, California.

Animals and Treatments

Male rats of the inbred Wistar strain (WAG-RIJ), weighing 200-300 g and approximately 12 weeks of age, were used. They were housed in animal quarters with a 12-h light/dark cycle and an ambient-temperature of 24°C and a humidity of 50-70%. Water and food were freely accessible. Animals were treated with oral doses of either vehicle (control animals) or different doses of HCB (3.5, 2.6, 1.7 and 0.9 mmol/kg, 5 animals per dose group), 3 days per week over a period of 2 weeks and with a dose of 3.5 mmol/kg for 4 weeks. These experiments (2 or 4 weeks dosing) were conducted at a different time point. HCB was administered as an emulsion of 0.14 mmol/ml in water containing 0.5% Tween-20. Approximately 2 days after cessation of dosing, blood was collected from the tail vessel, and the prepared serum stored at -20 °C for further analysis. During the period of HCB dosing, the

general health of the animals was monitored three times a week by measuring rectal body temperature and body weight.

In experiments with PCP, the compound was dissolved in corn oil and administered i.p on an acute basis at doses between 0 and 100 μmol/kg. Each dose of PCP was tested in groups consisting of four animals. Serum was collected 6 h after exposure, when effects on thyroxine levels are at a maximum (20).

Biochemical analysis

TT4, TT3, free T4 (FT4), free T3 (FT3) and TSH levels were determined in sera of control and experimental rats using appropriate RIAs (Amersham). HCB and PCP concentrations in sera were analyzed using HPLC as described earlier (20). The detection limit for PCP in serum was 0.075 μmol/l and for HCB 0.351 μmol/l. The extraction efficiencies for both PCP and HCB were more than 95%.

Competitive interactions with T4-binding proteins

Occupation of thyroid hormone binding sites by HCB and/or PCP was studied by a competitive binding assay as described previously (21,32). Sera were obtained from control and experimental animals (day 39) and diluted with phosphate-buffered saline (PBS) (1:30, v/v) before competitive binding of radiolabelled T4 was determined using mini-Sephadex G-25 columns. Radioactivity of eluate fractions was determined in a gamma counter.

Statistics

Student's t-test was used for statistical evaluation of mean values of various parameters between experimental and control animals. Results are presented as means ± SD.

RESULTS

Effects of sub-chronic dosing with HCB on general health parameters

Rectal body temperature of rats treated with repeated doses of HCB (3.5

mmol/kg) for a period of 4 weeks was not altered on the last day of the dosing period (38.0°C ± 0.41 vs 37.7°C ± 0.49 in control- and experimental animals respectively). Body weights of the experimental and control animals were not different (285 g ± 11.7 vs 285 g ± 14.7 in control- and experimental animals, respectively), and also followed a normal growth curve (results not shown).

Effect of HCB on thyroid hormone status

Treatment of rats for 2 weeks with 3.5 or 2.6 mmol/kg HCB produced a significant reduction ($p<0.05$) of serum TT4 levels (36.9 % and 24.8 % respectively, Table 1). Levels of FT4 in serum were also significantly reduced ($p<0.05$) at a dose of 2.6 mmol/kg HCB (19.3 % decrease, Table 1). After 4

Table 1. Effect of HCB on thyroid hormone status

HCB (mmol/kg)	N	Expt (wks)	TT4[1)]	FT4[2)]	TT3[1)]	TSH[3)]
0.0	4-5	2	21.4 ± 2.1	14.7 ± 2.2	0.44 ± 0.06	3.5 ± 0.5
0.9	3-5	2	23.7 ± 2.6	17.2 ± 2.2	0.44 ± 0.08	4.9 ± 1.1
1.7	3-5	2	19.2 ± 2.7	13.3 ± 1.9	0.42 ± 0.08	5.1 ± 1.6
2.6	5	2	16.1 ± 3.7*	11.8 ± 0.7*	0.33 ± 0.13	4.6 ± 1.6
3.5	4-5	2	13.5 ± 1.2*	12.1 ± 1.2	0.38 ± 0.07	4.8 ± 1.8
0.0	3-5	4	35.3 ± 3.8	9.2 ± 1.3	0.48 ± 0.06	7.6 ± 0.4
3.5	4-5	4	20.9 ± 3.4*	2.7 ± 0.6*	0.50 ± 0.17	11.6 ± 2.9*

Groups of rats (N=3-5) were orally dosed three times a week with different doses of HCB for a period of 2 or 4 weeks. Within 24 h after the last dose, blood was collected from the tail, and thyroid hormone parameters were determined. #: The doses of HCB expressed in mg/kg are 0, 250, 500, 750 and 1000, respectively. Statistical significance: * $p<0.05$. 1), 2) and 3): concentrations in nmol/L, pmol/L and ng/mL respectively.

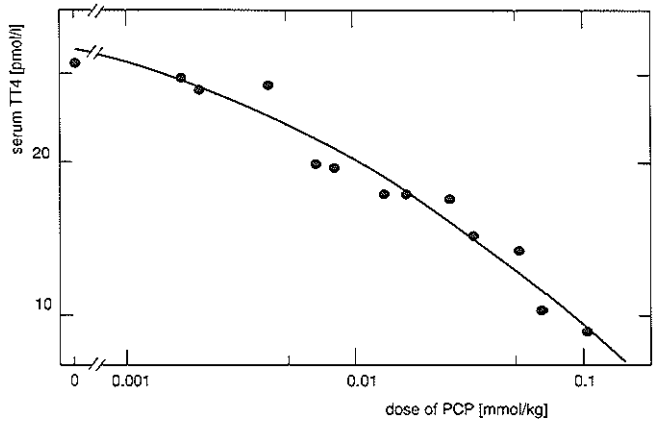
weeks of dosing, TT4 and FT4 levels in animals exposed to HCB were even more depressed (41 % for TT4, and 70.7 % for FT4). In contrast, no significant decrease of TT3 levels in serum were found (Table 1) after a 2 or

4 week dosing period with a dose of up to 3.5 mmol HCB/kg. A trend of increased TSH levels in serum by HCB was noted after 2 weeks. TSH levels were significantly increased by 56.6 % after a dosing period of 4 weeks with 3.5 mmol HCB/kg. An increasing effect of at least 31 % was found comparing control values of TSH (Table 1).

Effects of administered PCP on serum (free) thyroxine levels

In separate dosing experiments investigating the competitive interactions of PCP with thyroid hormone serum binding proteins, results indicated that PCP caused a rapid and substantial reduction in TT4 concentration in serum that was highly dose dependent (Fig.1). When rats were exposed to the

Fig.1. Effect of the dose of PCP on serum TT4 levels.



Groups of rats (N=4) received a single i.p injection of different doses of PCP (0-0.105 mmol/kg in corn oil). Control rats received corn oil only. Blood samples were taken at 6 h after exposure. TT4 levels in serum were determined with a RIA. The results represent combined data from two independent animal experiments.

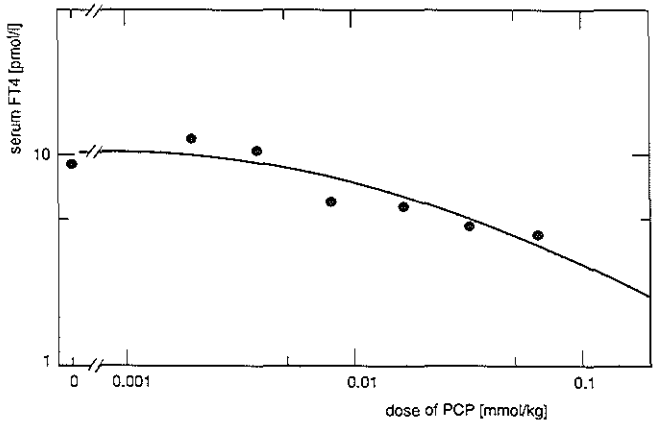
highest dose of PCP (0.10 mmol/kg), TT4 concentrations in serum were reduced by more than 65 % (Fig.1). FT4 levels in serum were also strongly reduced (by 60 %) after dosing with PCP compared with control values (Fig.2). PCP had neither clear effects on serum levels of (F) T3 nor on TSH

levels (data not shown).

HCB and PCP levels in serum during HCB treatment

Accumulation of HCB and its major metabolite PCP in serum after repeated exposure of animals to different doses of HCB was determined by HPLC methods. A clear dose-response relationship was found between the serum concentrations of HCB or PCP reached and the administered dose of HCB after 2 weeks (Table 2). At a given dose of HCB (3.5 mmol/kg) doubling of the exposure period from 2 to 4 weeks generally resulted in higher serum concentrations of both HCB and PCP, as also appeared from a separate time-course study (results not shown). Control animals had no detectable levels of HCB or PCP at any time point.

Fig. 2. Effect of the dose of PCP on serum FT4 levels.



Single i.p injections of different doses of PCP were given to groups of rats (N=4). Blood was collected 6 h after administration of PCP. FT4 levels in serum were determined with a RIA.

Occupancy of serum binding proteins.

Sera of animals exposed to 3.5 mmol HCB/kg for 4 weeks did bind significantly less T4 (20 % less) than sera of control animals, indicating partial occupancy of binding sites as a consequence of dosing with HCB (Table 3).

Table 2. HCB and PCP concentrations in serum of animals exposed to HCB

Dose of HCB (mmol/kg)	Dosing period (weeks)	HCB (μmol/L)	PCP (μmol/L)
0.0	2	DL	DL
0.9	2	9.0 ± 0.9	1.1 ± 0.51
1.7	2	14.5 ± 3.3	1.7 ± 0.58
2.6	2	23.6 ± 4.0	2.7 ± 0.45
3.5	2	23.2 ± 2.3	2.6 ± 0.52
3.5	4	49.0 ± 8.7	10.4 ± 3.25

Groups of rats (N=4-5) were orally dosed three times a week with HCB for a period of 2 or 4 weeks. Within 24 h after the last dose, blood was collected by tail bleeding. HCB and PCP concentrations in serum were analyzed by HPLC. DL, Detection limit (see Materials and Methods).

Table 3. Occupancy of binding sites after HCB-dosing

Incubation conditions	N	cpm	% inhibition
HCB sera	5	37463 ± 1321*	20
Control sera	5	46662 ± 866	0
Control sera + T4 (100 μM)	2	3083 ± 194	93

Sera were obtained from animals dosed with 3.5 mmol HCB/kg for 4 weeks or without HCB. Binding of a standard amount of [^{125}I]T4 to serum binding proteins was determined as described in the Materials and Methods. Significance: * $p<0.05$

PCP levels in serum after PCP treatment

The relationship between the dose of PCP administered and the concentration of PCP in serum reached, as determined by HPLC analysis,

was found to be linear (Fig.3) up to an administered dose of 100 μmol/kg.

DISCUSSION

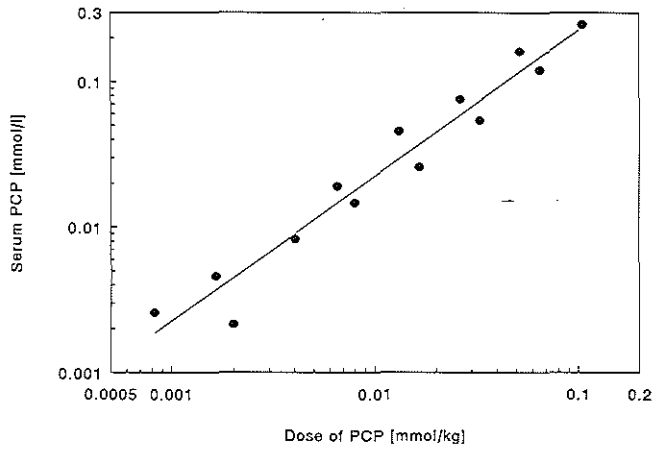
The results of the present experiments demonstrate that both HCB and its major metabolite PCP are able to produce a decrease in serum thyroid hormone levels. HCB produced significant reductions of TT4 in a dose-dependent manner (2 or 4 weeks exposure), while levels of TT3 were not found to be substantially altered. However, in short term experiments (2 weeks) there appeared to be no large dose-dependent effect on FT4, and hence on TSH levels (because the TSH respons is to FT4 rather than to TT4). During longer exposure to HCB (4 weeks), a greater reduction of TT4 levels was observed, including decreased FT4 and hence increased TSH levels. An explanation may be that during the shorter period (2 weeks) the buffering capacity of serum binding proteins on T4 is still large enough to compensate the FT4 pool. With prolonged exposure this capacity may become exhausted through competitive effects of PCP, the FT4 levels can no longer be maintained and therefore TSH levels go up. TT3 levels have hardly been observed to be altered with PCBs, dioxins, chlorinated benzenes etc. These observations confirm and extend results of other investigators (4-6) who also found decreased TT4 levels but no significant alterations in TT3 serum levels by HCB. Recently however, evidence had been obtained for HCB-induced significant reductions of plasma TT3 levels (26). The hypothalamus-pituitary axis is probably not impaired as indicated by an increased TSH response, while preliminary results have shown evidence for an increased activity of the thyroid gland as appeared histologically by hyperplasia in combination with increased iodine uptake in the thyroid. The combination of decreased TT4 and FT4 levels in sera and an increased TSH response after subchronic treatment with HCB suggest the condition of hypothyroidism.

Repeated dosing with HCB leads to induction of microsomal enzymes, e.g. P450 (11), ethoxyresorufin-deethylase (11) and UDPGT levels (12). Enhanced activity of several UDPGTs have recently been shown to give rise to increased levels of T4-glucuronides and to an increased bile flow (12), the combined effect could lead to a rapid removal of T4 from the circulation (12).

A further consequence of hepatic microsomal enzymatic activity is a build-up in serum of PCP, the major metabolite of HCB (17-19). PCP has

been causally implicated in HCB-induced reductions of thyroid hormone levels because of its greater effectiveness in reducing TT4 serum levels (20). The present experiments confirm the rapid decrease of TT4 serum levels in animals exposed to PCP. In addition, FT4 levels in serum were also equally affected. No significant alterations of TSH levels were observed, but the time period of 6 h may not be long enough for proper operation of the hypothalamus-pituitary-thyroid feed-back mechanism.

Fig. 3. Relationship between serum PCP levels and administered dose.



Different doses of PCP were injected i.p. and blood was collected 6 h later. PCP concentration in serum was analyzed by HPLC. These results represent combined data from two independent animal experiments.

In the present study, evidence for significant competitive interactions (19.7 % competition in the standard gel filtration assay) was observed in sera of animals dosed with HCB. In contrast, no alterations in serum binding of T4 in sera of animals exposed to HCB were reported using an electrophoretic technique (6). Possible differences in sensitivity of the techniques being applied may account for the different findings. In earlier studies it was found that there was very little binding of HCB to T4-binding sites of serum proteins. PCP however, is a very avid binder of these sites. Among the serum binding proteins PCP has relatively the highest binding to TTR, even higher than T4 itself, followed by albumin and lowest binding to TBG. By Scatchard

analysis it is apparent that PCP alters the affinity of T4-binding and not the number of binding sites on serum proteins (21,22,25,32).

One of the unresolved issues concerns the question to what extent the possible mechanisms, (i.e competitive interactions and increased metabolism), are involved in the decrease of thyroid hormone by HCB and related polyhalogenated aromatics. The present experiments allow an estimation of the respective contribution of either of these mechanisms. It was possible to estimate the contribution of PCP to the decrease of TT4 levels through competitive interactions, because, relationships between administered doses of PCP versus serum concentrations of PCP on the one hand (Fig.3) and versus serum TT4 levels on the other were established (Fig.1). After dosing for 4 weeks with 3.5 mmol HCB/kg, PCP levels in serum reached 0.01 mmol/l (Table 2). This serum level of PCP would correspond with an administered dose of 0.0044 mmol/kg PCP (by extrapolation in Fig. 3) that would be able to induce a decrease of 19.5 % in serum TT4 levels (and a marginal decrease of FT4 levels) through competitive interactions (by extrapolation in Fig. 1). Since the present data showed a maximum reduction of TT4 levels of 41 % with a sub-chronic dosing of 3.5 mmol HCB/kg (Table 1) it can be calculated that PCP may decrease TT4 levels through competition at the hormone binding protein level by about 48 % (i.e. 19.5/41 x 100 %). The remaining 52 % decrease of TT4 levels is attributed to other causes such as increased hepatic metabolism. At the earlier time of 2 weeks, serum levels of PCP were not high enough to cause a decrease of TT4 levels by competitive interactions with thyroid hormone binding proteins in serum and the effect on TT4 levels may be caused solely by the metabolic pathway.

The present findings suggest that the decrease of T4 levels by repeated dosing of HCB may initially be caused by induction of metabolic pathways for T4 only. With prolonged dosing, HCB accumulates, for instance, in the liver. As hepatic microsomal metabolism is also increased, PCP levels in the circulation can reach levels that are high enough to compete with thyroid hormone for binding sites of thyroxine binding proteins such as TTR and albumin (21,22,25,26). In this phase both competitive interactions and metabolism work in conjunction to lower T4 serum levels. As far as we know, this may be the first example of a common effect (decrease of serum TT4 levels) caused by parent compound and metabolite at the same time through different and independent mechanisms.

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CHAPTER VIII

REDUCTION OF THYROXINE UPTAKE INTO CEREBROSPINAL FLUID AND RAT BRAIN BY HEXACHLOROBENZENE AND PENTACHLOROPHENOL

J.A.G.M. van Raaij[1,2], C.M.G. Frijters[2], L. Wong yen Kong[2], K.J van den Berg[2], and W.R.F. Notten[1,2]

1) Institute of Public Health and Social Medicine,
Erasmus University Rotterdam, The Netherlands
2) TNO Medical Biological Laboratory, Rijswijk, The Netherlands

Keywords:Hexachlorobenzene (HCB), Pentachlorophenol (PCP), Cerebrospinal fluid (CSF), Transthyretin (TTR), Thyroxine (T4).

ABSTRACT

In the present study the effects of hexachlorobenzene (HCB) and the metabolite pentachlorophenol (PCP) were investigated with respect to uptake of thyroxine (T4) into cerebrospinal fluid (CSF), and brain structures of rats. [^{125}I]T4 was taken up into CSF of control rats by a relatively slow process, reaching a steady state after about 3 h. Both repeated dosing of HCB and single doses of PCP caused decreased uptake of [^{125}I]T4 into CSF, total brain tissue as well as specific brain structures, such as occipital cortex, thalamus, and hippocampus. Although HCB-treatment caused a build-up of HCB and PCP levels in serum, in brain only HCB was present in significant amounts (16 % of the serum level). In CSF, both HCB and PCP concentrations were both below detection levels. Separate experiments with PCP showed, however, a dose- and time dependent uptake of PCP into CSF.

The present results indicate that PCP and the parent compound HCB are able to affect brain supply of T4. This may have consequences for an adequate development of the brain or proper brain function in adults. The exact mechanisms of interference of PCP and/or HCB in brain uptake of T4 remain to be established.

INTRODUCTION

From several studies it has become clear that hexachlorobenzene (HCB), which is produced as a by-product in industrial processes and formerly used as a fungicide, may cause disturbances of thyroid homeostasis of several species including humans (1-6). Other chlorinated aromatics such as polychlorinated biphenyls (PCBs), polybrominated biphenyls and dioxins induce similar effects (7-12). In fact, reductions of serum thyroid hormone levels are among the most sensitive indicators of exposure to these compounds in experimental studies (6,12,13).

A major mechanism responsible for decreases of thyroxine (T4) in the circulation after exposure to HCB or PCBs appears to be increased metabolism of T4 through an increased biliary clearance (4, 14-19) and induction of several types of T4 UDP-glucuronyltransferases (4,20-22). Interference of pentachlorophenol (PCP), the major oxidative metabolite of HCB (23-26) with T4-binding to serum proteins has also been found to play a role (27-29). *In vitro* studies demonstrated that this interaction was of a competitive nature, and that the highest affinity was observed with transthyretin (TTR) (28,30), a major transport protein for T4 in rats. Additional evidence for a competitive mechanism was also obtained from *in vivo* and *ex vivo* studies (3,5,29). Recent evidence suggests that after repeated exposure of rats to HCB both types of mechanisms, i.e. enhanced metabolism by HCB and competitive interactions by the metabolite PCP, may operate independently and to an equal degree in reducing serum T4 levels (5).

As a consequence of decreased peripheral thyroid hormone levels, target organs and tissues may be insufficiently supplied with thyroid hormone. Of special interest is the brain because it has been suggested that TTR might play a role for the transport of T4 through the blood-cerebrospinal fluid (CSF) barrier (31-33). The choroid plexus forms part of the blood-CSF barrier and a considerable amount of data indicate that TTR is newly synthesized by the epithelial cells of rat and human CP independently from hepatic production of TTR (34-41). In addition, evidence for a saturable transport system for T3 in the blood-CSF barrier (42) has been described. HCB and/or PCP could alter brain supply of thyroid hormones through various routes, e.g. via decreased blood levels or via interference with transport systems. Alternatively, transport into the CSF and the brain of xenobiotic compounds that have a high affinity for TTR, e.g. PCP, might be

facilitated.

Thus far, little information is available concerning effects of these and other compounds with respect to the brain supply of thyroid hormones or their specific uptake into the brain. This could be relevant for the interpretation of neurotoxicity and developmental toxicity associated with halogenated aromatic compounds (43-46).

The purpose of the present study was to investigate in an experimental rat model whether exposure to HCB or PCP would influence T4 uptake into CSF and brain structures. This was correlated with measurements of HCB and PCP in brain and CSF. Finally, entry of PCP into CSF was determined separately.

MATERIALS AND METHODS

Chemicals

[^{125}I]T4 (SA 1500 μCi/μg), was obtained from Amersham (England), HCB and PCP from Aldrich Company (Brussels, Belgium).

Animals, housing and treatment

In all experiments, male WAG-RIJ rats of about 14-15 weeks old, weighing 200-250 g were used. They were housed in groups of 3-4 on a 12 h light-dark cycle with free access to food and water. For experiments with HCB, groups of rats (N=3-5) were repeatedly treated (three times a week) with an emulsion of HCB (40 mg/ml, 0.5 % Tween-20 in water), or vehicle (control animals) by gavage for two (exp 1) or four weeks (exp 2), using different doses (0-3.5 mmol HCB/kg). Following the last dose (day 14 and day 46 of exp 1 and exp 2 respectively), brain uptake of T4 was determined. In experiments with PCP, different doses (0-0.10 mmol/kg), as a solution in corn oil (1 ml) were administered i.p to groups of rats (N=3-4). Control animals received corn oil only.

Uptake of T4 into CSF

[^{125}I]T4 from a stock solution in 70 % ethanol and 30 % water was gently concentrated by a nitrogen flow to remove ethanol. The remainder was taken

into PBS (8 mM $Na_2HPO_4.2H_2O$, 1.5 mM KH_2PO_4, 140 mM NaCl, 2.5 mM KCl). In order to characterize the uptake of T4 in the CSF, rats were brought under ether anaesthesia, and the jugular vein was prepared for injection of 5-10 μCi [^{125}I]T4 (200 -300 μl). The open wound was closed with special woundclips. For collection of CSF, puncture of the cisterna magna was done at several times (0.5-24 h) after injection. Only CSF samples not contaminated with blood were further analyzed. In all experiments, radioactivity in CSF was determined by gamma counting.

Uptake of T4 in brain

Uptake of T4 in brain structures was determined by i.p. injection of animals with 25 μCi [^{125}I]T4 in 1 ml of saline. Approximately 1 h (exp 1) or 6 h (exp 2) later, the animals were killed by decapitation for isolation of brains. Brains were dissected (or not) in several brain structures, e.g occipital cortex (OC), hippocampus (HP) and thalamus (TH). Structures were weighed and radioactivity was determined in a gamma counter.

Tissue levels of HCB and PCP

HCB and PCP concentrations in CSF and sera from exposed animals were analyzed by HPLC as described earlier (3). For determination of brain levels of HCB and PCP, brains were isolated and homogenised in 4 volumes of cold saline. HPLC analysis was carried out as described before (3) except that HCB was extracted with ethylacetate (5 ml), and PCP with n-hexane (4 ml) under acidic conditions. The detection limit for analysis of PCP in sera or CSF was 0.14 μmol/l and for HCB 0.35 μmol/l, with recoveries of 100 %. The detection limit for analysis of PCP in brain homogenates was 0.05 nmol/g wet weight tissue, and for HCB 0.16 nmol/g, with recoveries of 97 % and 92 % respectively.

Statistical evaluation

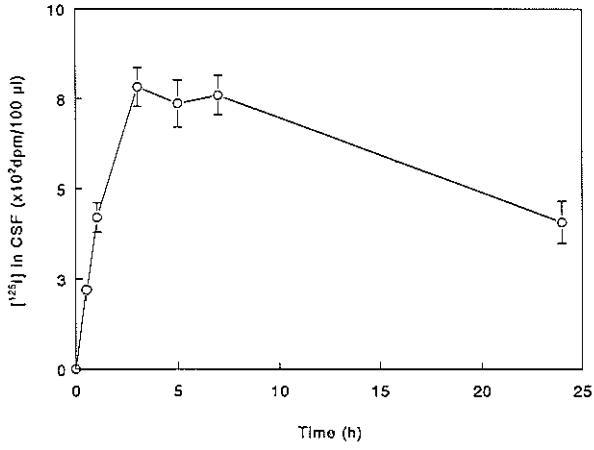
In order to determine differences between control and experimental animals, statistical evaluation was done with Student's t-test.

RESULTS

Uptake of radiolabelled T4 into CSF was first examined in non-treated control animals. The kinetics revealed a gradual increase of radioactivity into CSF during the first hours after injection (Fig.1). The highest levels in CSF were found between 3 and 7 h and thereafter CSF radioactivity slowly declined.

When [^{125}I]T4 was administered to rats after repeated treatment with two different doses of HCB for two weeks, significantly decreased amounts

Fig. 1. Uptake of T4 into CSF.



[^{125}I]T4 was administered i.v. to rats (N=3-4). At several times after injection, CSF was sampled and radioactivity in a volume of 100 μl was determined. Results are expressed as mean ± SEM.

of label in CSF were observed (30 % and 37 % decrease respectively, $p<0.05$) compared to control values but no clear dose-response relationship was found (Table 1).

Treatment of rats with a single injection of PCP, did result in a dose-dependent decrease of uptake of [^{125}I]T4 into CSF (34-75 % decrease, Table 2).

The effect of HCB-treatment on the uptake of [^{125}I]T4 in whole brain tissue and selected brain structures is shown in Table 3. In whole brain tissue, significant reductions (by ± 46-55 %, $p<0.05$) in the content of label

Table 1. Effect on uptake of T4 into CSF in rats dosed with HCB

Dose (mmol/kg)[1)]	CSF (dpm/100 μl)	Percent Change
0	826.9 ± 98.0	
2.6	524.2 ± 74.2*	-37
3.5	582.5 ± 124.2*	-30

Groups of rats (N=3-5) were repeatedly dosed with HCB or vehicle as indicated for a period of 2 weeks. Following the last dose (day 14), [^{125}I]T4 was injected i.v., CSF was collected after 1 h and animals were exsanguinated. Radiolabel in CSF was determined by gamma counting. Results are expressed as mean values ± SD. Statistical significance: * $p<0.05$.
1) Dose in mg/kg: 750 and 1000 respectively.

Table 2. Effect on uptake of T4 into CSF in rats dosed with PCP

Dose (mmol/kg)[1)]	CSF (dpm/100 μl)	Percent Change
0	670.8 ± 105.5	
0.026	441.7 ± 29.1*	-34
0.052	266.2 ± 52.6*	-60
0.105	165.2 ± 18.1*	-75

Groups of rats (N=3-4) received a single injection of different doses of PCP i.p. After a period of 4 h following dosing, [^{125}I]T4 was injected i.v., CSF was collected after another 2 h and animals were exsanguinated. Radiolabel in CSF was determined by gamma counting. Results are expressed as mean values ± SD. Statistical significance: * $p<0.05$.
1) Dose in mg/kg: 7, 14 and 28 respectively.

were observed. In addition, strongly reduced levels of label (by ± 42-56 %, $p<0.05$) were found in each of the specific structures examined, e.g., occipital cortex, hippocampus and thalamus (Table 3). No specific differences in the magnitude of the reductions were found between the various brain structures on the one hand, and between brain structures and total brain tissue on the other.

The effect of treatment with HCB on the accumulation of HCB and PCP in rat brain, CSF and in serum is shown in Table 4. After a period of 4 weeks, a substantial concentration of HCB could be measured in brain tissue. The concentration of PCP in brain, however, was below the detection limit. In CSF, neither HCB nor PCP were found in concentrations above the detection limit. In serum there was a time-dependent accumulation of HCB as well as of PCP. PCP levels in serum were on the average 11 % of the serum HCB

Table 3. Effect on uptake of T4 in total brain and brain structures in rats dosed with HCB

Dose	Total Brain	Brain structures[1]		
		OC	HP	TH
(mmol/kg)[2]	dpm/mg	dpm/mg	dpm/mg	dpm/mg
0.0	9.90 ± 0.08	50.0 ± 6.3	57.7 ± 19.7	67.7 ± 20.4
2.6	5.35 ± 1.28*	ND	ND	ND
3.5	4.46 ± 1.87*	22.2 ± 5.5*	30.6 ± 6.9*	39.2 ± 17.3*

Groups of rats (N=3-5) were repeatedly treated with different doses of HCB for a period of 2 weeks (for total brain) or 4 weeks (brain structures). Following the last dose, [^{125}I]T4 was administered, and brains were isolated and dissected, or not (see methods for details). Differences in radiolabel levels in total brain and brain structures of control animals are due to different amounts of total label injected within two separate experiments. Results are expressed as mean values ± SD. Statistical significance: * $p<0.05$.

1) OC, occipital cortex, HP, hippocampus, and TH, thalamus.

2) Dose in mg/kg: 750 and 1000 respectively. ND, not determined.

concentrations after a 2 week exposure period and increased to 22 % if the exposure period was prolonged to 4 weeks. The HCB concentration in brain tissue (Table 4) was about 16 % of the HCB levels in serum after exposure to 3.5 mmol HCB/kg for a period of 4 weeks.

Next it was investigated whether the blood-CSF barrier would pose a strict limitation for entry of PCP into CSF. Different doses of PCP up to 64 μmol/kg (17 mg/kg = 1/3 LD_{50}) were given as a single dose to rats and PCP levels were determined in CSF after 6 h by HPLC. The results, shown in Fig.2, demonstrate that uptake of PCP into CSF was linearly related to the administered dose.

The time course of PCP uptake into CSF (Fig.3), following a single dose of 64 μmol/kg, indicates a gradual increase to a steady state level that was achieved after about 6 h. Thereafter, PCP levels in CSF declined and, after 24 h, were essentially below the detection limit.

Table 4. HCB and PCP concentrations in brain, CSF and serum of rats exposed to HCB

Dose[a]	CSF		Brain		Serum	
Mmol HCB/kg	HCB[b]	PCP[b]	HCB[c]	PCP[c]	HCB[b]	PCP[b]
1.7	< 0.2	<0.1	ND	ND	15.5 ± 2.8	1.8 ± 0.57
2.6	< 0.1	<0.1	ND	ND	23.6 ± 4.0	2.7 ± 0.45
3.5	< 0.1	<0.1	ND	ND	23.2 ± 2.3	2.6 ± 0.52
3.5*	ND	ND	6.0 ± 1.1	<0.05	38.5 ± 7.2	8.5 ± 3.57

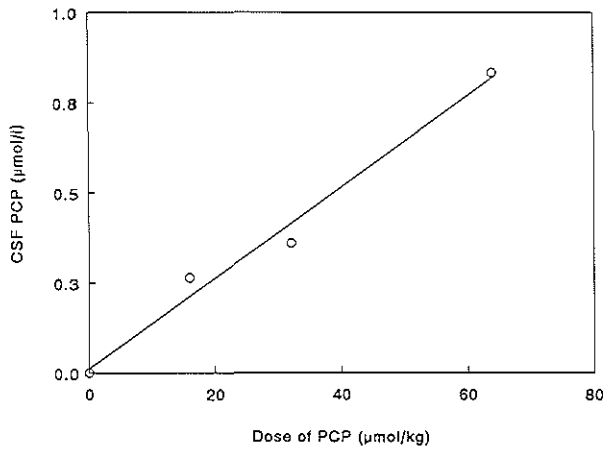
Groups of rats (N=3-5) were repeatedly treated with different doses of HCB for a period of 2 or 4 (*) weeks. HCB and PCP concentrations in CSF, brain and serum were determined by HPLC analysis as described in Materials and Methods. Levels in brain are expressed on the basis of wet weight. Results represent mean values ± SD. ND, Not determined.
a) Dose in mg/kg: 500, 750 and 1000 respectively. b) concentration in μmol/l. c) concentration in nmol/g.

DISCUSSION

A vast amount of data is available now to indicate reductions of thyroid hormone levels, and more specifically T4, in experimental studies following exposure to halogenated aromatic compounds such as HCB (2,5,18,27). One of the consequences of decreased plasma levels of T4 may be that in certain target tissues levels of the biological active T3 are not adequately met. This may in particular hold for the brain as a target organ since more than 90 % of T3 content of the brain is derived from intracerebral conversion of T4 to T3 by deiodinating enzymes, rather than from plasma T3 (47,48). In view of this, levels of T3 in the brain are dependent on the supply of T4 from the circulation as well as upon activity of type II 5'-deiodinase.

The results from the present study indicate that exposure of rats to a polyhalogenated aromatic compound such as HCB has implications for a proper supply of T4 to target tissues such as the brain. A repeated exposure

Fig. 2. Dose-dependent uptake of PCP into CSF

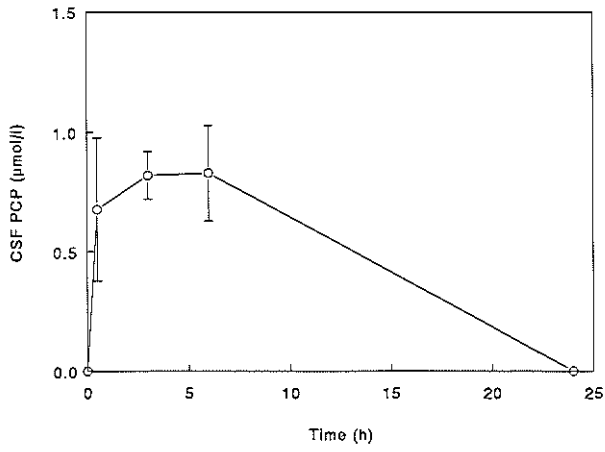


Different doses of PCP were administered i.v. to rats (N=3-4). After 6 h, CSF was collected and PCP levels were determined by HPLC. Results are expressed as the mean values.

regimen of rats to HCB did result in a strongly impaired uptake of peripherally administered T4 into CSF (by 37 %). In addition, uptake of T4

was decreased by HCB in total brain tissue as well as in a few selected brain regions (by 42-56 %). Previously it has been shown that PCP, the major metabolite of HCB, was more effective in altering plasma levels of T4 than the parent compound (3). The present results indicate that the same principle may apply for brain uptake of T4 since single doses of PCP did cause a large reduction of T4 uptake into CSF (up to 75 %).

Fig. 3. Time-dependent uptake of PCP into CSF



Rats (N=3-4) received a single dose of 0.064 mmol (17 mg) PCP/kg. At several times after administration. CSF was collected and PCP concentration was analyzed by HPLC. At 24 h, PCP concentration in CSF was < 0.2 µmol/l. Results are expressed as mean ± SEM.

The mechanism(s) by which HCB and PCP inhibit brain uptake of T4 is not clear at present. With respect to T4 uptake into CSF it has been suggested that a separate pool of TTR, synthesized in the choroid plexus, is involved (33). Since PCP is known to interact with the T4-binding site of TTR, it could compete with T4 for uptake into CSF. Furthermore, the present results indicate that PCP may enter CSF (Fig.2 and 3), possibly facilitated by the same mechanism. By occupying TTR binding sites for T4 in CSF, PCP could further impair intracerebral distribution of T4. Using different compounds with binding ability to TTR, for example a synthetic flavenoid (EMD 21388), arguments for such a mechanism have been obtained (49).

Further studies with PCP are in progress to adress this aspect more specifically. It cannot be excluded at this stage, however, that impaired cerebral uptake of T4 by HCB and PCP reflects to some extent the decreased plasma T4 levels.

A reduced availabity of thyroid hormones in the brain may have consequences for the normal functioning of biochemical and physiological processes in the brain of adult and particularly juvenile animals. For example, insufficient levels of thyroid hormones during pre- and post natal development of the brain can result in decreased protein synthesis, decreased myelogenesis, disturbed maturation of synaptic plasma membranes, and disturbed ramification of axons in the brain (50, 51). Furthermore, dendritogenesis and regeneration of sympathetic neurons may be affected. Both processes depend on nerve growth factor (NGF), and it is known that thyroid hormones are regulating factors of NGF biosynthesis (51). If early postnatal replacement therapy with thyroid hormones is not applied in congenital hypothyroidism, all these phenomena may lead to impairment of intellectual performance.

It has become clear that transfer of maternal T4 via the placenta protects the fetus from deficiency of T4, and possibly deficiency of T3. Whether during pregnancy the intracellular availability of thyroid hormone of the fetal brain may be impaired by compounds such as HCB or PCP, depends possibly on the period of maternal exposure. For instance, it has been suggested that inadequate levels of maternal T4 might result in damage to the developing fetal brain early in gestation (52). The maternal thyroid function and TSH response as a reaction of exposure to HCB *in utero* will for a large part determine fetal thyroid hormone status in blood and brain as well. In the fetal brain, the adaptive response in deiodinating capacity for local production of T3 from T4 may be a factor that determines whether a deficiency of T3 in the developing brain will develop. For the fetus, deiodination in the brain is a very active and important process during a period of hypothyroidism (47), since maternal transfer of T3 to the fetus does not protect the fetus from T3 deficiency (52). Recent studies with perinatal exposure of rats to PCBs have indicated decreased fetal and neonatal plasma levels of T4 in association with increased type II 5'-deiodinase activity in brain tissue (53).

In adults, disturbed brain function as a result of severe hypothyroidism becomes apparent from behavioral symptoms such as depression, paranoia and sleepiness. Studies in adult rats with hypothyroidism have demonstrated

morphological changes in the thyroid hormone sensitive pyramidal cells of the hippocampus (54), a neural area associated with cognitive processes. In view of the above mentioned general symptoms during hypothyroidism, it is possible that a reduced T4 supply to the adult brain might enhance the neurotoxicity of HCB or PCP.

In conclusion, the reduction of serum T4 levels by repeated administration of HCB or a single dose of the metabolite PCP appeared to directly affect the brain supply of T4. This may have consequences for proper brain development in exposed juveniles and neurological functions in exposed adults. Additional studies are required to more precisely determine the mechanism of T4 and PCP transport into CSF, including a possible role of TTR, and interference of PCP (HCB) in this process.

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CHAPTER IX

THYROXINE TRANSPORT INTO THE BRAIN: ROLE OF TRANSTHYRETIN (TTR)

J.A.G.M. van Raaij[1,2], C.M.G Frijters[2], L. Wong Yen Kong[2], K.J. van den Berg[2] and W.R.F. Notten[1,2]

1) Institute of Public Health and Social Medicine,
Erasmus University Rotterdam, The Netherlands
2) TNO Medical Biological Laboratory, Rijswijk, The Netherlands

ABSTRACT

The role of TTR in thyroxine (T4) transport into cerebrospinal fluid (CSF) and brain structures was studied using *in vivo* experiments. Rats received [^{125}I]T4 i.v in the presence of compounds with affinity for the T4-binding site of transthyretin (TTR), and uptake of [^{125}I]T4 in CSF was determined. No significant decrease in uptake was observed in the presence of either pentachlorophenol, 2,4,5-trichlorophenol, 2,4-dichlorophenol, hexachlorophene or cold T4. In contrast, significant increases were observed with 2,4,5-trichlorophenol (33 %), 2,4-dichlorophenol (36 %) or bromoxynil (27 %). Inhibitors of transport systems for vitamins and organic acids in the choroid plexus (CP) such as ascorbate, salicylate or probenecid did not impair T4 uptake into CSF. These results are interpreted in a diffusion model rather than in a transport model on the basis of TTR.

T4 transport through the capillary blood brain barrier (BBB) was also investigated. Brain uptake indices (BUIs) did not alter after carotid injections of [^{125}I]T4 in the presence of an excess of cold T4 or pentachlorophenol. Addition of T4-binding serum proteins to the injection solution did lead to a decreased BUI of T4. These observations indicate the absence of high affinity T4 transport systems in the BBB. T4 may enter the brain as a free molecule by a nonsaturable process.

A model is proposed to interpret transfer from T4 from serum to CSF over the blood-CSF barrier on the basis of free T4 gradients. T4 uptake from serum through the BBB may be based on the same principle.

INTRODUCTION

It is well established that the brain requires an adequate supply with thyroid hormone for proper cerebral development and functioning. Thyroid hormones from the circulation have to pass various brain barriers, e.g. capillary blood-brain barrier (BBB) and/or blood-cerebrospinal fluid (CSF) barrier in order to be taken up into the brain compartment.

The mechanisms involved in the transport of thyroid hormones across the different barriers are not completely understood. Although transport of triiodothyronine (T3) through the BBB appears to be carrier-mediated (1), the situation with respect to thyroxine (T4) is less clear. Recently it has been suggested that T4 transport through the blood-cerebrospinal fluid (CSF) barrier may involve transthyretin (TTR), a major serum thyroid hormone binding protein in mammals. This was based on the observation that high levels of i.v administered [^{125}I]T4 preferentially accumulated in isolated choroid plexus (CP), i.e a major CSF producing organ (2-5). Molecular biological studies have provided additional evidence that in the CP of rats, but also of humans, high levels of mRNA coding for TTR are produced indicating active synthesis of TTR (6-10). In CSF, a high proportion of T4 appears to be bound to TTR (11,12).

A more active role of TTR in transport of T4 from blood to the brain has been proposed in a model by Schreiber et al (3). In this model TTR, having a high affinity for T4 and being synthesized in the CP epithelial cells and excreted in the CSF, transports T4 from the bloodstream to the brain. Pardridge has advanced the hypothesis that plasma protein-binding may facilitate transport of thyroid hormones into tissues via microcirculatory barriers (1,13,14).

Few experimental in vivo data are available to substantiate a TTR-based model for transport of T4 into brain. Recently it was shown that T4 covalently bound to TTR hardly entered CSF when administered i.v.(15). The results of this study further indicated the existence of separate and independent pools for hepatic- and CP-derived TTR. Furthermore, it was suggested that binding to TTR in the CP might be more important for transport of T4 to the brain than binding to circulatory TTR. Circumstantial evidence using compounds that bind to the T4-binding site of TTR suggests also some role of TTR in brain transport of T4. For instance, a synthetic flavonoid (EMD 21388) was found to decrease TTR binding of T4 in CP and CSF as well as uptake of T4

in CSF and brain structures (15).

In vivo treatment of rats with pentachlorophenol (PCP) has recently been observed to result in reduced uptake of T4 from blood into the CSF as well as in brain structures (16). In addition, PCP was found to be taken up into CSF with similar kinetics as T4 (16). PCP has been shown to interact competitively with the T4-binding site of TTR at a higher affinity than T4 itself (17).

In the present communication, an attempt was made to clarify the participation of TTR in transport of T4 and xenobiotics such as PCP through the blood-CSF barrier. For this purpose, experiments were done to investigate whether various compounds that are known to interact with the T4-binding site of TTR competed directly with uptake of T4 into CSF. In addition to the blood-CSF barrier, attention was focussed on the BBB in order to establish an eventual role of TTR.

MATERIALS AND METHODS

Chemicals

L-[^{125}I]T4 (specific activity 1500 μCi/μg), [^{3}H]-H_2O (specific activity 5 mCi/ml) and [^{3}H]inulin (specific activity 442 μg/mg) were obtained from Amersham, England. Human serum albumin (HSA), thyroxine binding-globulin (TBG), human transthyretin (TTR), L-thyroxine and 2,4,5-trichlorophenol were obtained from Sigma Chemical Co (St Louis, MO, U.S.A). Salicylic acid, pentachlorophenol, bromoxynil and 2,4-dichlorophenol were obtained from Aldrich (Brussels, Belgium). Probenecid and hexachlorophene were purchased from Serva (Heidelberg, Germany). Ascorbinic acid was obtained from Merck (Darmstadt, Germany).

Animals and housing

Male WAG/RIJ rats, weighing 200-300 g were used. The rats were housed in groups of 3-4 on a 12 h light-dark cycle with free access to food and water.

Brain uptake of T4

For determination of T4 uptake in CSF, rats were brought under ether anaesthesia and the jugularic vein was exposed for injection. [^{125}I]T4 was taken into PBS (8 mM $Na_2HPO_4.2H_2O$, 1.5 mM KH_2PO_4, 140 mM NaCl, 2.5 mM KCl) to 25-50 μCi/ml. The animals were injected in the jugularic vein with 200 μl of [^{125}I]T4 in the presence of competing compounds (0-3000 μM in PBS). Control rats received [^{125}I]T4 in PBS only. After injection, the open wound was closed with woundclips. CSF samples were taken by cisterna magna punction. Only CSF samples not contaminated with blood were further analyzed. Radioactivity in CSF was determined by a gamma counter and was expressed as dpm/100 μl CSF.

The Oldendorf procedure (18-20) was used to investigate transport of T4 through the BBB. Briefly, rats were brought under anaesthesia with 0.1 ml hypnorm (i.e. 0.2 mg fentanyl/ml) s.c and 0.2 ml hypnorm i.m. The carotid artery of animals was exposed for rapid injection of a 200 μl sample of mixture A. Mixture A was Krebs-Ringer buffer (127 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 2 mM $MgSO_4$, 1.25 mM NaH_2PO_4, 10 mM glucose, 26 mM $NaHCO_3$, pH 7.4), containing 2.5 -5.10^6 dpm T4/ml (1-2 μCi) and [^{125}I]T4/ml and 25.10^6 dpm ^3H_2O/ml (10 μCi). The experimental animals were injected with 200 μl mixture A containing different concentrations of T4- binding proteins, cold T4 or PCP. After a 15 sec period, to allow unidirectional flow into the brain, the animals were decapitated. Brains were dissected into striatum, occipital cortex, and thalamus. Dissection was done at the side ipsilateral to the injected bolus. The structures were weighed, dissolved in tissue solubilizer (Soluene, Packard) whereafter radioactivity was determined by double-isotope liquid-scintillation counting with quench correction. The brain uptake index (BUI) was calculated from the expression:

$$BUI = \frac{\text{dpm/mg T4/3H in tissue} \cdot 100}{\text{total dpm T4/3H}}$$

In order to estimate the contribution of arterial contamination in brain tissue, [^{3}H]inulin was used because this protein is not supposed to cross the blood brain barrier (21). About 5.10^6 dpm of the isotope was injected. After decapitation, the brain was dissected and blood was collected. Radioactivity in tissues was determined as described above. The contribution of tracer in

serum in the dissected brain structures was found to be < 5 %.

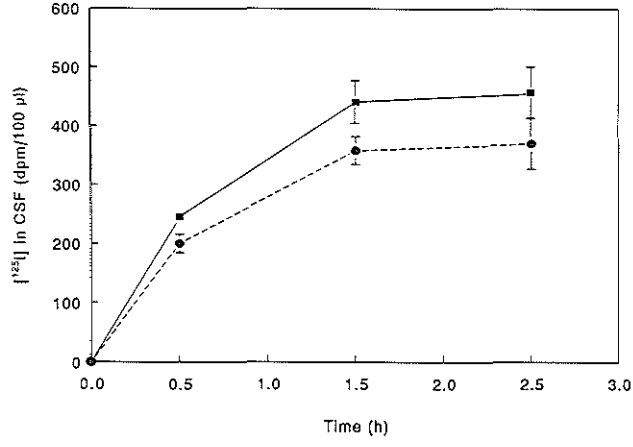
Statistical evaluation

In all experiments, statistical evaluation was done with Student's t test, and results expressed as mean ± sd, except in figure 1, where mean ± sem (standard error of the mean) is presented.

RESULTS

The time course of T4 levels in CSF of control animals is presented in Fig.1. [^{125}I]T4 uptake into CSF increased for a period of 1.5 h and then remained at a steady state for at least 1 h. The effect of excess PCP (3000 μM) on the kinetics of [^{125}I]T4 in CSF is also given in Fig.1. It appeared that the uptake of [^{125}I]T4 into CSF was not inhibited in animals dosed with PCP, compared with controls.

Fig.1. Time course of [^{125}I]T4 levels in CSF of rats in the presence of pentachlorophenol.



Uptake of T4 into CSF without of with 3000 μM PCP was determined as reported in Materials and Methods. Results are expressed as mean ± sem. ●-● = Controls; ■-■ = PCP.

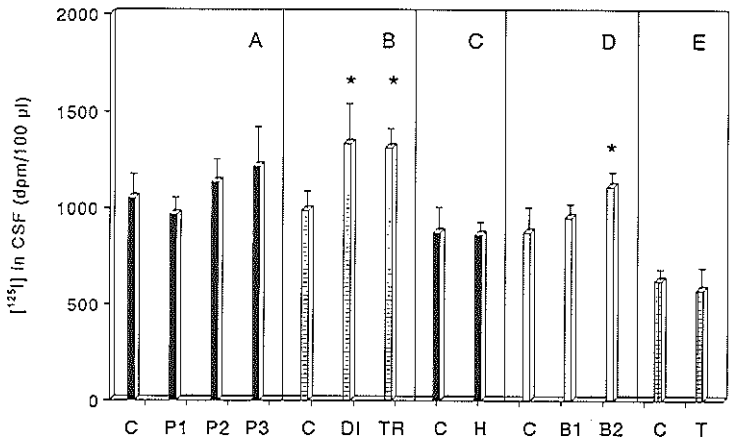
Other compounds, previously shown to interact with the T4-binding site of TTR, such as 2,4-dichlorophenol or 2,4,5-trichlorophenol (Fig.2B), hexachlorophene (2,2'-methylenebis-(3,4,6-trichlorophenol) (Fig.2C), and

bromoxynil (3,5-dibromo-4-hydroxybenzonitril) (Fig.2D) were not able to compete effectively with T4 for entry into CSF. In contrast, T4 uptake into CSF was found to be slightly increased in the presence of 2,4-dichlorophenol or 2,4,5-trichlorophenol (Fig.2B), and bromoxynil (Fig.2D). Furthermore, the uptake of [^{125}I]T4 into CSF was not inhibited in the presence of cold T4 (Fig.2E).

A summary of the competing ability of these chemicals with T4 for binding to TTR, the relative binding affinities of these chemicals for the T4-binding site of TTR and their effect on T4 uptake into CSF is given in Table 1. The results show essentially an absence of a relationship between the ability to occupy binding sites of TTR and effect on T4 uptake into CSF.

It is well established that the blood-CSF barrier also contains transport systems in the choroid plexus for a number of vitamins and organic acids, for example ascorbate and salicylate. Other active transport systems in the choroid plexus deal with efflux of specific compounds, e.g. neurotransmitter metabolites (22-26). It was investigated whether transport of T4 into or out

Fig.2. Effect of TTR-binding compounds on uptake of T4 into CSF



Rats were i.v. injected with [^{125}I]T4 in the presence of different concentrations of pentachlorophenol **(A)**, (P1, P2 and P3: 333, 1000 and 3000 μM resp.), 2,4-Dichlorophenol (DI: 600 μM) and 2,4,5-Trichlorophenol **(B)**, (TR: 600 μM), Hexachlorophene **(C)**, (H: 30 μM), Bromoxynil **(D)**, (B1 and B2: 300 and 3000 μM resp.), and T4 **(E)**, (T: 500 μM). CSF was sampled after 1 h (T4), 2 h (2,4-Dichlorophenol and 2,4,5-Trichlorophenol) or 3 h (pentachlorophenol, hexachlorophene and bromoxynil). C= control values. Statistical significance: * $p<0.05$.

of the CSF could be linked to any of the systems involved in the transport of vitamins, organic acids or efflux. Using representative compounds for each of these systems, e.g. salicylate (Fig. 3A), probenecid (Fig. 3B) and ascorbate (Fig. 3C), neither one of these compounds was found to significantly affect levels of radiolabelled T4 in CSF.

The effect of various serum binding proteins on the transport of T4 through the BBB was determined using the single pass procedure (18-20). Addition of TTR to the incubation medium did not result in enhanced brain uptake of T4 (Table 2). Rather, a tendency towards decreased BUI of T4 was observed. With the highest amount of TTR (30 μg) a significant decrease in BUI (by 32 %, $p<0.05$) was found in striatum. In the presence of thyroxine binding globulin (TBG) a significant decrease in BUI of T4 was observed in striatum, occipital cortex and thalamus (by $\pm$ 34 - 41 %, $p< 0.05$). With albumin added to the incubation medium the effect on BUI of T4 was less pronounced with a tendency to decreased BUI at the highest concentration (statistically not significant).

Finally, it was investigated whether BUI of T4 could be altered by an

Table 1. Compounds binding to TTR and their effect on T4 uptake into CSF

Compound	Competing effect[a] in vitro (%)	Relative affinity[b]	[^{125}I]T4 Uptake into CSF
Pentachlorophenol	96	1.7	Not inhibited[1]
2,4,5-trichlorophenol	94	0.15	Increased[2]
2,4-dichlorophenol	94	0.003	Increased[2]
Hexachlorophene	93	0.7	Not inhibited[3]
Bromoxynil	87	ND	Increased[1]
T4	98	1	Not inhibited[4]

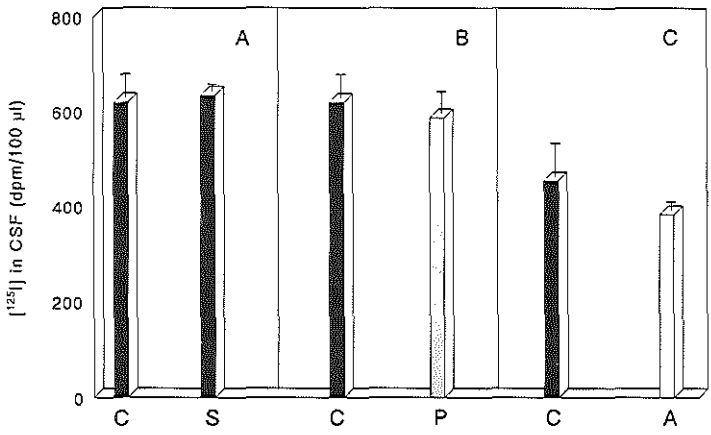
[a] Competition of compounds with T4 for binding to TTR *in vitro* was determined as described previously (17).
[b] Data on relative affinity of compounds for the T4-binding site of TTR were taken from Van den Berg et al (1991), (27). ND, Not determined. [1] at 3000 μM; [2] at 600 μM; [3] at 30 μM; [4] at 500 μM.

excess of unlabelled T4. The results, shown in Table 3, indicate that the BUI of [^{125}I]T4 was not found to be significantly decreased by an excess of unlabelled T4 or an excess of PCP. Rather a trend towards an upward BUI was observed, being significant for T4 with respect to uptake in the thalamus ($p< 0.05$).

DISCUSSION

In vitro studies of transport of T4 through the blood-CSF barrier have suggested that T4 may be directly transported via TTR-mediated systems localised in the CP (2,3). Several observations from the present study bring into question the *in vivo* relevance of TTR as a possible membrane transport carrier molecule for the CSF-uptake of T4. T4 uptake into CSF was found to be unaffected in the presence of an excess of several compounds such as T4 itself, PCP, 2,4,5-trichlorophenol, 2,4-dichlorophenol, hexachlorophene and bromoxynil (Fig. 2) that were known to interact specifically with the T4-binding site of TTR (Table 1). PCP has previously been shown to have an even higher affinity for that site than T4 (17).

Fig.3. Effect of salicylate, probenecid and ascorbate on uptake of T4 into CSF



Rats were i.v. injected with [^{125}I]T4 in the presence of salicylate **(A)**, (S: 500 μM), probenecid **(B)**, (P: 500 μM), or ascorbate **(C)**, (A: 500 μM). One hour later, CSF was sampled. Radioactivity was determined. C= control values.

If TTR was a rate limiting factor of T4 uptake into CSF, inhibition of T4 uptake would have been proportional to the interacting capacities of these compounds. Furthermore, in the rat model T4 uptake into CSF over the blood-CSF barrier was a fairly slow process that required several hours to reach a steady state (Fig.1). The final ratio of accumulation of T4 in CSF versus blood has previously been found to be much smaller than unity (16). Also these data are not in favor of an active transport of T4 through the

Table 2. Effect of T4-binding proteins on brain uptake of T4

Binding protein (μg)	BUI (ST)	BUI (OC)	BUI (TH)
TTR			
0	33.6 ± 5.7	38.9 ± 11.5	39.5 ±13.9
7.5	30.7 ± 2.7	28.3 ± 3.0	39.4 ± 4.5
15	29.2 ± 2.6	ND	25.0 ± 3.3
30	22.8 ± 4.4*	22.2 ± 0.9	21.0 ± 1.7
TBG			
0	13.6 ± 0.9	12.8 ± 1.6	11.2 ± 0.7
6.6	8.0 ± 2.6*	8.2 ± 0.9*	7.4 ± 2.7*
Albumin			
0	40.1 ± 15.4	35.3 ± 9.9	27.5 ± 7.1
15	29.2 ± 5.0	35.1 ± 6.3	27.7 ± 6.2
45	33.4 ± 8.5	30.4 ± 10.8	25.9 ± 1.4

[^{125}I]T4 in the presence of different amounts of T4-binding serum proteins was injected into the carotid artery of rats. The Brain Uptake Index (BUI) was determined in different brain structures (ST, striatum; OC, occipital cortex; TH, thalamus) as described in the Methods section. ND, not determined. Significance: *, $p<0.05$.

the blood-CSF barrier, where a ratio higher than unity would have been expected. Using a somewhat different approach, Chanoine et al (15) recently observed that T4 covalently bound to TTR was hardly taken up into CSF and brain as compared to unbound T4. This strongly indicates that at least circulatory TTR is not a facilitating factor in blood-CSF transport of T4.

Table 3. Effect of competing T4 and PCP on brain uptake of radiolabelled T4.

Competing compound (μM)	BUI (ST)	BUI (OC)	BUI (TH)
T4			
0	25.3 ± 3.7	31.0 ± 5.5	23.9 ± 1.5
0.8	28.7 ± 7.7	32.8 ± 7.9	26.6 ± 5.3
4	27.1 ± 5.8	26.9 ± 1.7	25.8 ± 1.0
20	29.3 ± 3.2	32.4 ± 2.2	32.3 ± 2.4*
PCP			
0	25.2 ± 7.3	24.9 ± 2.4	24.4 ± 5.0
1877	28.7 ± 12.6	26.2 ± 6.2	33.2 ± 13.5

[^{125}I]T4 in the presence of different concentrations of unlabelled T4 or PCP was injected into the carotid artery of rats. The Brain Uptake Index (BUI) was determined in different brain structures (ST, striatum; OC, occipital cortex; TH, thalamus) as described in the Methods section. Significance: *, $p<0.05$.

In the CP, several systems are involved in the transport of nutrients, e.g. amino acids, vitamins, nucleosides etc. as well as a specific system for removal of toxic organic acids, such as biogenic amines and their acidic metabolites (22-26). Specific compounds acting on these systems, for example ascorbate, salicylate and probenecid, were not very effective in lowering T4 uptake into CSF (Fig. 3), suggesting that neither of these transport systems may be involved in T4 transport into or out of the CSF. A rather consistent finding was a tendency for somewhat *increased* T4 levels

in the presence of high concentrations of PCP, 2,4-dichlorophenol, 2,4,5-trichlorophenol and bromoxynil. It cannot be excluded that these compounds may inhibit efflux of T4 from the brain, a process in which TTR may or may not participate. This efflux system is supposedly different from the organic acid type of efflux system that is inhibited by probenecid (an alternative explanation based on FT4 gradients is given below). Taken together, the present results with respect to the *in vivo* mechanism of T4 uptake from blood into CSF tend to exclude several possibilities for carrier-mediated transport systems and leaves the option of uptake by diffusion.

The role of transport systems for T4, and the involvement of T4-binding proteins, TTR in particular, was also studied with respect to the BBB using a single pass unidirectional flow (18-20) technique. The results indicate that neither TTR nor any other T4-binding proteins, such as TBG or albumin, did facilitate T4 uptake through the BBB into various brain structures, e.g. striatum, thalamus and occipital cortex. In contrast, with TTR as well as TBG the BUI of T4 into brain structures was decreased as compared to uptake of T4 alone (Table 2). The suggestion from these results is that serum binding proteins rather act merely as circulatory buffers of T4 than that they are involved in the passage of T4 over the BBB into various brain structures. A further suggestion from these findings is also that only free T4 molecules pass are able to pass the cerebral capillaries of the brain. The inability of an excess T4 to decrease effectively the BUI of radiolabelled T4 tend to eliminate possibilities for the existence of carrier-mediated transport systems for T4 in the cerebral capillaries that form the blood-brain barrier. This leaves the alternative of uptake by diffusion.

On the bases of the foregoing results, a revision of the Schreiber model is proposed regarding a TTR-based transport system for T4 into CSF. T4 bound to TTR or other serum binding proteins is delivered as a complex or as free T4 (FT4) through the fenestrated endothelial cells into the stroma of the CP. In the stroma there exists a normal equilibrium between the TTR-T4 and FT4. Transport of FT4 takes place across the basolateral and apical membranes of the epithelial cells that constitute the CP to reach the CSF. The mechanism of transport may involve a process of diffusion on the basis of lipid partioning. During passage of the epithelial cells, T4 is not able to interact with TTR that is newly synthesized, for as yet unknown reasons that may have to deal with intracellular sequestration and/or processing of TTR. Only in the CSF complexes are allowed to be formed of FT4 and newly synthesized TTR. In this model there is no active role of plasma- or CP-derived TTR involving transport of T4 into CSF. The driving force could be the rate of CSF production and the continuous CSF flow that should create

a concentration gradient of FT4 between the blood and CSF compartment.

The differential effects observed with respect to T4 uptake into CSF after pretreatment of rats with PCP (16) compared with simultaneous addition of PCP (this paper) may be explained with this new model. Pretreatment of animals with PCP was previously found to result in strongly decreased blood levels of TT4 and FT4 (28) as well as decreased uptake of T4 into CSF (16). The kinetics of this process have revealed a maximum decrease after about 6 h (29). The decreased FT4 levels, of course, lower the inward gradient, e.g. direction CSF, and hence the rate of diffusion. If PCP is added simultaneously or is present for only a limited amount of time, the FT4 concentration gradient is insufficiently lowered to decrease T4 flow into CSF. Rather the opposite effect may occur because the strong competitive effect of PCP with the T4-binding site of TTR in particular may lead to temporarily increased serum FT4 levels, an increased FT4 gradient and enhanced inflow of T4 into CSF. The apparant increase in T4 CSF uptake by 2,4,5-trichlorophenol, 2,4-dichlorophenol and bromoxynil may also be explained on this basis. Using the T4 flavonoid analog (EMD 21388), with affinity for the T4-binding site of TTR, it could be shown that T4 transfer from serum to CSF was dependent on the serum FT4 level (15).

Since the present results indicate that also uptake of T4 through the BBB takes place by a diffusion process, the FT4 concentration gradient from blood to brain over the BBB may be a determining factor for T4 uptake into brain structures. The decrease in both serum TT4 and FT4 levels and T4 uptake in brain structures as previously observed in animals that had been repeatedly dosed with hexachlorobenzene (16) may be interpreted according to this concept.

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EVALUATION

During the investigations presented in this thesis, evidence for two mechanisms for hexachlorobenzene (HCB)-induced reduction of thyroxine levels in the circulation of rats was obtained. 1: Enhanced thyroxine-metabolism caused by HCB (*Chapter VI*). 2: Competitive interaction of pentachlorophenol (PCP), a major metabolite of HCB, with the thyroxine-binding sites of serum proteins (*Chapter IV and V*). It was estimated that in rats exposed to HCB, nearly half of the reduction of serum total thyroxine was caused by interaction of PCP with the thyroxine-binding sites of serum proteins (*Chapter VII*). The other half of the reduction is probably caused by an enhanced metabolism of thyroxine. Effects of HCB on the synthesis of thyroxine in the thyroid gland may not be exluded.

Exposure of rats to HCB or PCP did also result in reductions of thyroxine levels in the cerebrospinal fluid (*Chapter VIII*). This phenomenon could probably not be attributed to specific interference of PCP with transthyretin in the cerebroventricular organs for the following reason: when radiolabelled thyroxine was simultaneously administered with PCP, no decrease of thyroxine uptake into the cerebrospinal fluid was found (*Chapter IX*). Comparable results were found (no blockade of thyroxine uptake) with other aromatic halogenated hydroxylated compounds (AHHs) having a high affinity for the thyroxine-binding site of transthyretin. Furthermore, transport of radiolabelled thyroxine through the capillary blood brain barrier was not inhibited by excess PCP or thyroxine (*Chapter IX*). These findings could not be explained with existing models involving thyroxine transport from blood to the brain (*Chapter I*).

A new model for thyroxine transport is proposed in this thesis (Fig.1), which explains the results on thyroxine transport in the rat brain obtained during the investigations underlying this thesis. This model is based on that of Schreiber et al (1990), who postulated that transthyretin derived from the choroid plexus was responsible for thyroxine transport from the blood to the rat brain. This hypothesis was based on a high-capacity uptake of radiolabelled thyroxine in the choroid plexus, a major part of the blood-cerebrospinal fluid barrier (see review *Chapter I*), after systemic administration. It is possible that this observation is caused by binding of the label to transthyretin at the apical side (cerebrospinal fluid side) of the

choroid plexus, since the epithelial cells possess the capacity to reabsorb apically proteinaceous material from cerebrospinal fluid (Olsson et al, 1973, Smith et al, 1964). Furthermore, in a recent study, it was suggested that T4 reaches the brain primarily through the blood-brain barrier and not via the choroid plexus (Blay et al, 1993). This statement was based on a decreased- and an increased uptake of [^{125}]T4 in the choroid plexus and cerebral cortex, respectively, after treatment of rats by cycloheximide and an i.v. injection of [^{125}]T4.

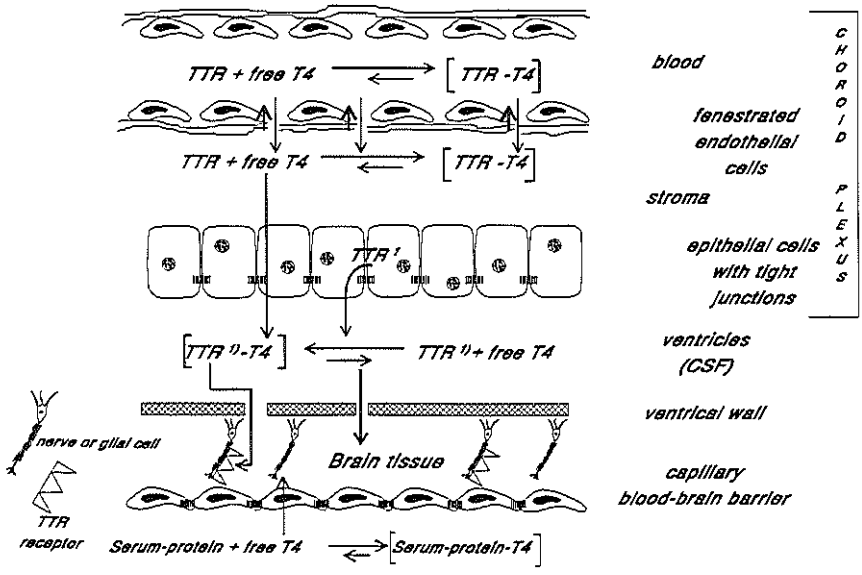
In contrast with the hypothesis of Schreiber (1990), the postulated model (Fig.1) implies no role of transthyretin in the epithelial cells of the choroid plexus. In this model, transthyretin, transthyretin-thyroxine complexes and free thyroxine pass the fenestrated capillaries of the choroid plexus (passive transport) as a first step. The complexes, and also transthyretin are able to return to the blood by the same route. In the stroma of the choroid plexus, there exists an equilibrium between free thyroxine and the transthyretin-thyroxine complex (comparable with the equilibrium in blood). Free thyroxine crosses the basolateral and apical membranes of the epithelial layer respectively by lipid partitioning, whereafter the cerebrospinal fluid is reached. In this compartment, free thyroxine binds to transthyretin which has been produced by the choroid plexus. Thyroxine-transthyretin complexes may interact with transthyretin receptors of the neuronal and glial membranes in brain tissue.

When serum thyroxine-protein complexes make a contact with the blood side of endothelial membranes of the capillary blood brain barrier, thyroxine-binding proteins will deliver free thyroxine into the extracellular space of the brain (Fig.1). Thereafter, free thyroxine migrates slowly from the extracellular space of the brain, where free thyroxine reaches the membranes and cytoplasm of the brain cells (neurons or glial cells). Otherwise, free thyroxine migrates further into the cerebrospinal fluid (where free thyroxine is bound to transthyretin) after free passage of the ventricle wall.

In the postulated model, the possible function of transthyretin produced by the choroid plexus is 1) a carrier for thyroxine in the cerebrospinal fluid for an efficient delivery of thyroxine into the extracellular and/or intracellular compartment of the brain and 2) to allow transfer of thyroxine into the brain cells using transthyretin receptors on membranes. In any case, there is still no evidence that transthyretin is able to interact with free thyroxine in the intracellular compartment of the epithelial cell. This may imply an intracellular

processing of transthyretin in such a manner that thyroxine molecules are not able to reach binding sites of intracellular transthyretin. The main rate-limiting step responsible for the cerebrospinal fluid-thyroxine levels may probably be the rate of cerebrospinal fluid secretion by the epithelial choroid plexus-cells, and not the level of epithelial transthyretin.

Fig. 1. Hypothesis for transport of thyroxine from the blood to the brain



1) transthyretin produced by the choroid plexus

Possible interactions of AHHs with transthyretin in the cerebrospinal fluid or with transthyretin receptors on membranes may explain neurotoxic properties of AHHs by disturbances of mammalian intracerebral thyroxine concentrations. When a possible neurotoxic compound enters the cerebrospinal fluid after crossing the epithelial cells of the choroid plexus as free molecules, it may compete with free thyroxine for the thyroxine-binding site of transthyretin in the cerebrospinal fluid. Such a compound may also interact with transthyretin receptors on neuronal- and glial transthyretin receptors. As a consequence, this may result in a disturbance of the

intracellular availability of thyroxine in the neuronal and glial cells. Effects of AHHs related to interactions with transthyretin in the brain should be of more significance in fetal or neonatal species since AHHs may enter the brain more easily because the blood-brain barrier is not always fully developed during the pre-and postnatal period (Johanson et al, 1976, Johanson, 1980, 1989). An altered thyroid hormone status in the mammalian brain induced by AHHs during critical periods of brain growth and development may act negatively because these processes are essentially dependent on thyroid hormones (see review of *Chapter I*). Experimental studies involving interactions of AHHs with intracerebral transthyretin of juvenile animals could be helpful to understand mechanisms of induced developmental neurotoxicity by local hypothyroidism.

Possible implications for humans exposed to aromatic halogenated hydrocarbons

The percentage of thyroxine bound to human thyroxine-binding globulin in blood is 6-fold, and the affinity of thyroxine for this protein is 100-fold higher compared with transthyretin (*Chapter I*). Therefore, in humans exposed to AHHs, thyroxine-binding globulin in the circulation will prevent fluctuations of free thyroxine resulting from displacement of T4 from transthyretin. Decreased serum thyroxine levels based on interactions of AHHs with the thyroxine-binding site of transthyretin may play a significant role at the moment that thyroxine-binding sites of all serum thyroxine-binding globulin molecules are occupied. However, transthyretin transports only 10-15 % of circulating thyroxine which implies that the highest possible decrease of free T4 due to interactions of AHHs with the thyroxine-binding site of transthyretin and removal from the circulation, will be 10-15 %.

In view of the relevance for risk assessment in occupational toxicology, research is necessary to examine industrial compounds with a high affinity for thyroxine-binding globulin. Examples of relationships between interactions of industrial compounds with thyroxine-binding globulin and reduced thyroxine levels in serum are scarce. However, some pharmacologically important drugs, such as salicylate, or the anticonvulsant diphenylhydantoin and oleate, which possess a high affinity for the thyroid hormone-binding site of human thyroxine-binding globulin (Oppenheimer et al, 1962, Larsen, 1972, Savu et al, 1989), are able to reduce plasma thyroid hormone levels

(Molholm Hansen et al, 1974, De Sandro et al, 1991, Davies et al, 1991).

Interactions of AHHs with transthyretin in the brain may have implications for thyroid hormone homeostasis in the brain of humans exposed to AHHs, because 44 % of thyroxine is bound to transthyretin in cerebrospinal fluid (Hagen et al, 1973). Herbert and coworkers (1986) report even a percentage of ± 80 % bound T4 to human transthyretin in this fluid. If AHHs, such as PCP, penetrate the blood brain-and/or blood cerebrospinal fluid barriers and reach transthyretin in the cerebrospinal fluid, interaction with the thyroxine-binding site is possible. Hence, it may be expected that thyroxine levels in the cerebrospinal fluid and brain will change, i.e. free and bound levels will probably increase and decrease, respectively. Whether these interactions, or those with cellular membrane transthyretin receptors will occur, depend probably on 1) the penetrating capacity of the AHHs through the membranes (Neuwelt, 1989) of the capillary blood brain barrier and of the endothelial and epithelial membranes of the choroid plexus, 2) the rate of migration of AHHs from the extracellular space of the brain to the cerebrospinal fluid, and 3) the concentrations of the AHHs reached in the cerebrospinal fluid.

AHHs as parent compounds will probably pass membranes of the blood-brain barrier more easily than the associated hydroxy-compounds, because the latter possess in general a less lipophylic character (compare HCB versus PCP), (*Chapter VIII*). The presence of cytochrome P450-mediated activity in the human brain (Vijayalakshmi et al, 1990), may possibly result in conversion of the parent compounds (Ravindranath et al, 1989) into hydroxylated compounds (e.g. the biotransformation of HCB into PCP).

Whether these compounds interact with transthyretin (receptors) in the brain and hence disturb thyroxine homeostasis in the brain may be an interesting topic for future investigations.

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SUMMARY

During various industrial processes (incinerations, production of pesticides), aromatic halogenated hydrocarbons (AHHs) such as hexachlorobenzene (HCB), polychlorinated biphenyls (PCBs), and polychlorinated dibenzo-p-dioxins (PCDDs) are still released into the environment. These highly persistent compounds may act negatively on the health of humans via occupational- or environmental exposure. The major general toxic effects of PCBs and related AHHs in humans are: headache, loss of weight, loss of appetite, abdominal pain and nausea. More specific effects include dermal lesions (chloracne), disturbed hepatic function (enlargement, hepatocellular necrosis), and reduction of sensory and motor nerve condition velocity (peripheral).

Toxicological studies in various animal species report "wasting syndrome" (loss of fat), thymic atrophy, impaired reproduction, disturbed embryonal development and dysfunctioning of the central nervous system. Other toxic effects of AHHs include disturbances on vitamin-and hormone levels.

During the last ten years, much attention has been given to effects of PCBs and dioxins on thyroid hormone status (*Chapter I*). A number of animal studies demonstrated that these compounds were able to induce reductions of thyroid hormone levels in blood. Decreased plasma thyroxine (T4) levels were detected in humans, who had consumed food contaminated with HCB. Prolonged inadequate thyroid hormone levels (for example in hypothyroidism) in organs, such as the brain, may lead to disturbances of biochemical processes in organs, such as the brain (*Chapter I*). In particular, brain growth and development during the fetal and neonatal stage is strongly dependent on the availability of thyroid hormone.

In this thesis, mechanisms are identified which may clarify the phenomenon of AHHs-induced reductions of T4 levels in blood and brain of rats. In addition, it is described whether AHHs are able to influence supply of T4 to the brain, and whether interactions of these compounds with T4 transport systems localised in the blood-cerebrospinal barrier or capillary blood-brain barrier are involved. HCB was chosen as a model compound because of its relatively simple chemical structure and the physico-chemical characteristics shared with PCBs; Also the thyroid and neurotoxicological properties of HCB are analogous with those of PCBs; Furthermore,

pentachlorophenol (PCP), an important metabolite of HCB, possibly interferes with T4-binding serum proteins.

The first experiments were designed to characterize effects of HCB on the thyroid gland and thyroid hormone levels (*Chapter II*). Repeated administration of HCB to rats for a period of 4 weeks resulted in reductions of total T4 (TT4) levels in the serum. The total T3 (TT3) levels in serum were only decreased in the second and third week of dosing. The thyroid gland of rats exposed to HCB had an increased iodine uptake (± 5.5-fold) and was clearly hyperplastic compared with controls. These phenomena indicated that the hypothalamus-pituitary-thyroid axis was probably not impaired by HCB. During prolonged exposure, a gradual build up of PCP levels in serum was demonstrated. Cytochrome P450-1A1 appeared to be induced by HCB, since microsomal hepatic ethoxyresorufin-O-deethylase activity was increased in exposed animals (13-fold) compared with controls. Taken together, it was concluded that exposure of rats to HCB resulted in a hyperactive thyroid gland and reductions of thyroid hormone levels.

Experiments were performed to investigate whether the reduced serum thyroid hormone levels by HCB could be attributed to HCB itself, or to its major metabolite PCP (*Chapter III*). A single dose of HCB had no significant effects on TT4 or TT3 within a period of 24 h post exposure. In contrast, an equimolar dose of PCP resulted in a decrease of ± 60 % of TT4 levels in serum. Furthermore, TT3 levels in serum were slightly decreased by PCP. A clear inverse relationship was found between the kinetics of serum PCP and TT4 levels. The results suggested that PCP might be causally involved in decreases of TT4 levels induced by HCB.

In *Chapter IV*, results are described which show to what extent hormone displacement from serum proteins is involved in the PCP-induced reductions of serum TT4 levels. Occupancy of T4-binding sites of serum proteins occurred in sera from rats exposed to PCP, but was hardly detectable in sera from rats exposed to HCB. Competing ability towards T4 was found in serum extracts from PCP-, but hardly from HCB-exposed rats. It was also found that PCP had a higher potency to interact with T4-binding sites of serum proteins than HCB. These results implied that PCP, as metabolite of HCB, might be a major causal factor in HCB-induced reductions of serum TT4 levels, possibly through interaction with T4-binding serum proteins.

Not only PCP and other chlorophenols, but also other groups of industrial compounds, such as nitrophenols, phenoxy acids, chlorobenzenes and

DDTs competed with T4 for binding to transthyretin (TTR) (*Chapter V*). Some of these compounds were also able to cause reductions of TT4 levels in blood. It could be postulated that the ability of a variety of industrial compounds to interact with the T4-binding site of TTR might be related to their potential to reduce TT4 levels in blood.

Exposure of rats to HCB can result in increase of biotransformation processes by induction of cytochrome P450 (phase I reaction). Induction of phase II (conjugation) enzymes by HCB may also occur. Therefore, reduced serum TT4 levels caused by HCB might also be attributed to an enhanced metabolism of T4. It was found that the activity of the T4-UDP-glucuronyltransferase (T4-UDPGT) activity in hepatic microsomal fractions of rats repeatedly exposed to HCB was increased by a factor 5 compared with controls (*Chapter VI*). Furthermore, an almost 3-fold higher production of T4-glucuronides in bile was caused by HCB. A decrease of the TT4 levels in serum by increased activity of the hepatic type 1 deiodinase was excluded since HCB inhibited this enzyme. The results indicated that exposure of rats to HCB leaded to enhanced metabolism of T4 (by increased glucuronidation), which might contribute to decreased serum TT4 levels.

Separate experiments (with HCB or PCP) were designed to estimate the contribution of competitive interaction of PCP with serum T4-binding proteins in decreased TT4 levels caused by HCB (*Chapter VII*). It appeared that a linear relationship existed between the administered dose of PCP and serum levels of PCP. It was also found that the amount of PCP-induced reductions of serum TT4 levels was dependent on the administered dose of PCP. Using these data, it was concluded that after repeated exposure of rats to HCB (with known HCB and PCP levels in serum), 48 % of the total reduction of TT4 in serum was related to interactions of PCP with T4-binding serum proteins. The other part of the reduction might be associated with an increased metabolism of T4 by HCB, although other unknown mechanisms could be involved (for instance a direct effect of HCB on the synthesis of T4 in the thyroid gland).

In *Chapter VIII*, the consequences of exposure of rats to HCB or PCP with respect to the T4 uptake into cerebrospinal fluid (CSF) and brain (structures) are described. It was found that repeated oral exposure of rats to HCB caused a lowered uptake of administered labelled T4 into CSF and various brain structures, compared with controls. A single i.p. dose of PCP resulted in a dose-dependent decrease of T4 uptake into CSF. Since HCB and/or

PCP were able to decrease T4 uptake into CSF, these compounds may act negatively on T4-dependent processes in the mammalian brain.

Experiments were set up to investigate whether a diminished T4 uptake into CSF caused by PCP, might be attributed to specific interference of PCP with T4 transport systems localised in the blood-CSF or capillary blood-brain barriers (*Chapter IX*). Attention is paid to the question whether the interaction of PCP with plasma and/or brain TTR might be responsible for the reduced T4 uptake into the CSF. In addition, it was investigated whether T4 transport to the brain along the cerebroventricular organs (such as choroid plexus) involved transport systems for organic acids or vitamins. It appeared that simultaneous systemic administration of labelled and excess cold T4, PCP and other TTR-binding compounds, did not impair T4 uptake into CSF (*first part of Chapter IX*). Similarly, T4 uptake into CSF could not be inhibited by inhibitors of organic acid transport (probenecid, salicylate) or vitamin transport (ascorbate). The results implied that transport of T4 into CSF was not based on a TTR-mediated system, nor on several other specific transport systems of the choroid plexus. Also, these results may be interpreted as transfer of T4 through blood-CSF barriers as a free molecule, with a diffusion gradient as a driving force.

Finally, experiments were done in order to study whether transport of T4 through the capillary blood brain barrier is based on high affinity systems (*second part of Chapter IX*). Using carotid injections of labelled T4 with an excess of cold T4 or PCP, no significant alterations of "brain uptake indices" (BUIs) were found. Addition of T4-binding proteins (TTR, albumin, or TBG) to the injection mixture resulted in decreased BUIs of T4 in various brain structures. These observations suggested that again only free T4 fractions were available for rapid transport through the capillary blood brain barrier, based on diffusion processes.

Whether HCB-or PCP (or other AHHs)-induced reductions of TT4 levels act negatively on brain functions of adult mammals (also humans) could be an issue for further research. In any case, thyroid hormones are indispensable for a normal growth and development of the brain during the fetal and neonatal stage of humans and animals. Therefore, in view of possible effects of AHHs on thyroid hormone concentrations (maternal, fetal, neonatal, see *Chapter I*), investigations of the consequences of pre-and post natal exposure of AHHs to brain functions of young human infants seems even more appropriate.

SAMENVATTING

Bij veel industriële processen (verbrandingen, pesticiden productie) kunnen gehalogeneerde aromatische verbindingen (AHHs), zoals hexachloorbenzeen (HCB), polychloorbiphenylen (PCBs) en dioxinen (PCDDs) vrij komen in de omgeving. Deze verbindingen bezitten de eigenschap om sterk te persisteren in lichaamsweefsel, en kunnen de gezondheid van mensen die beroepsmatig of via het milieu worden blootgesteld, negatief beinvloeden. De belangrijkste algemene toxische effecten van PCBs en gerelateerde gehalogeneerde aromatische hydrocarbonzuren zijn bij de mens: hoofdpijn, gewichtsverlies, eetlustverlies, buikpijn en misselijkheid. De meer specifieke effecten zijn: huidlaesies (chloracne), leverfunctiestoornissen (vergroting, hepatocellulaire necrose), en afname in sensorische en motorische zenuwgeleidingssnelheid (perifeer).

Toxicologische studies bij verschillende diersoorten rapporteren "wasting syndroom" (verlies van lichaamsvet), thymusatrofie, verslechterde reproductiecapaciteit, gestoorde embryonale ontwikkeling en afwijkend functioneren van het centrale zenuwstelsel. Andere toxische effecten van AHHs zijn verstoringen van vitamine- en hormoon spiegels.

Gedurende de laatste tien jaren is veel aandacht geschonken aan effecten van PCBs en dioxinen op de schildklierhormoon status (*Hoofdstuk I*). Een aantal dierstudies hebben laten zien dat deze verbindingen in staat zijn om dalingen van schildklierhormoon spiegels in bloed te induceren. Bij mensen, die voortdurend waren blootgesteld aan HCB (via gecontamineerd voedsel) zijn dalingen van plasma thyroxine (T4) spiegels beschreven. Een chronisch tekort aan schildklierhormoon (bijvoorbeeld bij hypothyroïdie) kan een aanleiding zijn tot verstoringen van biochemische processen in organen, zoals bijvoorbeeld de hersenen (*Hoofdstuk I*). In het bijzonder zijn groei en ontwikkeling van het brein tijdens de foetale en neonatale periode sterk afhankelijk van de beschikbaarheid van schildklierhormoon.

In dit proefschrift worden enkele mechanismen opgehelderd, die mogelijk ten grondslag liggen aan door AHHs geinduceerde dalingen van T4 spiegels in het bloed en in de hersenen van de rat. Ook wordt beschreven in hoeverre AHHs in staat zijn de T4 toevoer naar de hersenen te beïnvloeden, en in hoeverre interacties van deze verbindingen met transportsystemen voor T4 in de bloed-hersenvocht of bloed-hersen barrières hierbij betrokken zijn. HCB

is als modelstof gekozen om de volgende redenen: HCB heeft binnen de AHHs een relatief eenvoudige chemische structuur; de fysisch-chemische eigenschappen van HCB zijn vergelijkbaar met die van de PCBs; HCB beschikt over analoge eigenschappen wat betreft de schildklier-en neurotoxiciteit van PCBs; Pentachloorfenol (PCP), een belangrijke metaboliet van HCB interfereert mogelijk met T4-bindende serum eiwitten.

In *hoofdstuk II* staan resultaten beschreven die de effecten van HCB op de schildklier en op schildklierhormoonspiegels karakteriseren. Na herhaalde blootstelling van ratten aan HCB gedurende een periode van 4 weken bleek dat in het serum de totale T4 (TT4) spiegels gedaald waren. De totale T3 (TT3) spiegels in het serum waren eveneens gedaald, maar vanaf ± 2 weken na de eerste blootstelling keerden de spiegels weer geleidelijk naar het "normale" niveau. De schildklier van de met HCB behandelde dieren was duidelijk hyperplastisch, en had een verhoogde iodium opname (± 5.5-voudig) ten opzichte van de controle dieren. Deze bevindingen betekenen dat de hypothalamus-hypofyse-schildklier as waarschijnlijk normaal functioneert. Naarmate de blootstelling langer duurde, kon een geleidelijke opbouw van PCP in het serum aangetoond worden. Cytochroom P450-1A1 bleek geïnduceerd te kunnen worden door HCB, zoals bleek uit een 13-voudige toename van de microsomale ethoxy-resorufine-O-deethylase (EROD) activiteit in de lever ten opzichte van de controles. De conclusie kon getrokken worden dat blootstelling van de rat aan HCB leidt tot een hyperactiviteit van de schildklier en tot dalingen van schildklier-hormoonspiegels.

Experimenten werden uitgevoerd om te onderzoeken of de door HCB geïnduceerde dalingen van de TT4 en TT3 spiegels veroorzaakt worden door HCB zelf, of juist door belangrijke metabolieten van HCB, zoals PCP (*hoofdstuk III*). Het bleek dat HCB niet in staat was significante veranderingen in TT4 en TT3 spiegels te veroorzaken binnen een periode van 24 uur na blootstelling. Echter, een equimolaire dosis van PCP leidde al binnen 2 uur na blootsteling tot een sterke daling van serum TT4 spiegels (± 60 %). Verder veroorzaakte PCP een lichte daling van TT3 spiegels. Ook bleek dat het verloop van de serum PCP concentraties duidelijk omgekeerd correleerde met het verloop van de serum TT4 concentraties. Er is dus een mogelijkheid dat PCP causaal betrokken is bij de door HCB geinduceerde dalingen van TT4 concentraties in de circulatie.

In *hoofdstuk IV* staan resultaten beschreven die laten zien in hoeverre

hormoonverdringing van serum eiwitten toe te schrijven is aan door PCP-geinduceerde dalingen van TT4 spiegels in serum. Bezetting van T4-bindingsplaatsen in sera kon aangetoond worden in ratten die blootgesteld waren aan PCP-, maar in veel mindere mate in sera die afkomstig waren van ratten die aan HCB blootgesteld waren. Ook bleken serum-extracten van de met PCP gedoseerde dieren een sterke competerende activiteit (vergeleken met T4) te hebben. In serum-extracten afkomstig van dieren die aan HCB blootgesteld waren was nauwelijks competerende activiteit aanwezig. Verder kon aangetoond worden dat PCP een veel sterker vermogen heeft om T4-bindingsplaatsen te bezetten dan HCB. De resultaten suggereren dat PCP, als metaboliet van HCB, een belangrijke oorzaak kan zijn van de gedaalde TT4 spiegels die door HCB geinduceerd kunnen worden, mogelijk door interacties met T4-bindende serum eiwitten.

Niet alleen PCP en anderen chloorfenolen, maar ook andere groepen van industriële stoffen, zoals bijvoorbeeld nitrofenolen, fenoxyzuren, chloorbenzenen en DDTs blijken te competeren met T4 voor de T4-bindingsplaats van transthyretine (TTR), zoals blijkt uit resultaten die beschreven zijn in *hoofdstuk V*. Sommige van deze verbindingen blijken ook in staat te zijn om dalingen van TT4 spiegels te veroorzaken. De resultaten impliceren dat er een verband bestaat tussen het vermogen van sommige van deze verbindingen te binden aan TTR en hun vermogen om TT4 spiegels te verlagen.

Blootstelling van ratten aan HCB kan resulteren in toename van biotransformatie-processen door inductie van cytochroom P450 (fase I reactie). In het lichaam kan HCB mogelijk ook fase II (conjugatie) enzymen induceren, die mogelijk een rol spelen bij de door HCB-veroorzaakte dalingen van TT4 spiegels. Wanneer namelijk ratten herhaaldelijk blootgesteld werden aan HCB, werd in de hepatische microsomale fracties een 5-voudige verhoging van de T4-UDP-glucuronyltransferase (T4-UDPGT) activiteit ten opzichte van controles gevonden (*Hoofdstuk VI*). Verder was in de blootgestelde dieren de T4-glucuronide uitscheiding in de gal bijna 3 maal verhoogd. Afbraak van T4 gebeurt ook via activatie van type 1 deiodinase in de lever, maar blootstelling aan HCB veroorzaakte een remming van dit enzym. De mogelijke conclusie is dus dat blootstelling van ratten aan HCB leidt tot een verhoogd metabolisme van T4 (door toenmame van de glucuronidering), hetgeen kan bijdragen aan dalingen van de TT4 spiegels.

Experimenten werden uitgevoerd (met HCB en PCP afzonderlijk) om in een situatie van gedaalde TT4 spiegels door inwerking van HCB een schatting te kunnen doen van het relatieve aandeel van interacties van de metaboliet PCP met T4-bindende serum eiwitten en het metabolisme van T4 (*hoofdstuk VII*). Het bleek dat er een lineair verband bestond tussen de toegediende dosis van PCP en de ontstane spiegels van PCP in het serum. Verder kon aangetoond worden dat de hoeveelheid dalingen van de TT4 spiegels afhangt van de toegediende dosis van PCP. Met behulp van de gegevens kon berekend worden dat na herhaalde blootstelling van ratten aan HCB (met bekende serum concentraties HCB en PCP), 48 % van de totale T4 dalingen in het serum te relateren is aan interacties van PCP met T4-bindende serum eiwitten. De rest van de totale T4 daling kan mogelijk gerelateerd worden aan een verhoogd metabolisme van T4 geinduceerd door HCB, hoewel andere op dit moment onbekende mechanismen ook nog van belang kunnen zijn (bijvoorbeeld een mogelijk direct effect van HCB op de synthese van T4 in de schildklier).

In *hoofdstuk VIII* worden de consequenties beschreven voor de normale opname van T4 in cerebrospinaal vocht (CSF) en hersen(strukturen) na blootstelling van ratten aan HCB of PCP. Wanneer HCB herhaaldelijk oraal gegeven werd, bleek dat de opname van toegediend gelabeld T4 in het CSF en hersenstructuren duidelijk verminderd te zijn ten opzichte van de controle dieren. Ook een eenmalige dosering van PCP resulteerde in het CSF in een soortgelijk effect dat dosis- afhankelijk was. Omdat zowel HCB als PCP de opname van T4 in de CSF doen dalen, kunnen deze stoffen dus mogelijk T4-afhankelijke processen in zoogdierhersenen nadelig beïnvloeden.

Of de door PCP veroorzaakte gedaalde T4 opname in het CSF of hersenen gerelateerd kan worden aan specifieke interactie met T4 transportsystemen in de bloed-CSF of capillaire bloed-hersen barrière wordt beschreven in *hoofdstuk IX*. Hierin wordt onder andere aandacht besteed aan de vraag of interactie van PCP met plasma-en/of hersen-TTR verantwoordelijk zou kunnen zijn voor de gereduceerde T4 opname in het CSF. Verder werd onderzocht of het transport van T4 via cerebroventriculaire organen (zoals choroid plexus) naar de hersenen, verloopt via het transport voor organische zuren of vitaminen. Het bleek dat indien overmaat ongelabeld (koud) T4, PCP, of andere TTR-bindende stoffen, simultaan met gelabeld T4 systemisch werd toegediend, de opname van T4 in het CSF niet geremd was (*eerste deel van hoofdstuk IX*). Hetzelfde fenomeen werd

gevonden met probenecid, salicylzuur of ascorbinezuur. De resultaten impliceren dat het transport van T4 naar het CSF niet gebaseerd is op een door TTR-gemedieerd systeem, of op andere specifieke transportsystemen in de choroid plexus. De resultaten kunnen geïnterpreteerd worden door aan te nemen dat T4 getransporteerd word over bloed-CSF barrières als een vrij molecuul, met een diffusiegradient als de drijvende kracht.

Aanvullende experimenten werden uitgevoerd om vast te stellen of het T4 transport via de capillaire bloed hersen barrière naar de hersenen te verzadigen is (*laatste deel hoofdstuk IX*). Gebruik makende van carotis injecties van gelabeld T4 in aanwezigheid van koud T4 of PCP, kon gesteld worden dat het T4 transport over deze barrière onverzadigbaar was, omdat de "brain uptake indices" (BUIs) niet veranderden. Ook werd gevonden dat indien gelabeld T4 toegediend werd in een gebonden vorm (aan albumine, TTR of TBG), de BUIs van T4 in diverse hersengebieden afnamen. Dit betekent mogelijk wederom dat alleen de vrije T4 fractie via diffusie beschikbaar is voor transport over de cerebrale capillairen.

Of de door HCB-of PCP (of andere AHHs) geïnduceerde dalingen van serum thyroxine spiegels negatief inwerken op hersenfuncties van volwassen zoogdieren (ook de mens) kan een relevante vraag zijn voor toekomstig onderzoek. Schildklierhormonen zijn in ieder geval onmisbaar voor een normale groei en ontwikkeling van de hersenen gedurende het foetale en neonatale stadium van mens en dier. Daarom is het gezien de mogelijke effecten van AHHs op schildklierhormoonconcentraties (maternaal, foetaal, neonataal, zie *Hoofdstuk I*) wenselijk om onderzoek te doen naar de consequenties van pre-en post natale blootstelling van AHHs voor de hersenfuncties van zeer jonge kinderen.

CURRICULUM VITAE

Jeroen van Raaij is geboren te Bergen op Zoom op 20-07-1962. Na het behalen van het diploma Atheneum ß aan het Mollerlyceum te Bergen op Zoom, begon hij met de studie Biologie aan de Rijksuniversiteit van Utrecht. Als specialisatie werd de richting Medische Biologie (B5*) gekozen. Het doctoraalexamen werd behaald in juni 1988 met als hoofdvakken Immunologie (Prof.dr.R.E. Ballieux) en Endocrinologie (Prof.dr.J.L. van den Brande/dr. S. van Buul), en als bijvak Moleculaire biologie (Prof.dr.A.D.M.E. Osterhaus/dr P. de Vries).

In aansluiting op de studie deed hij electromyografisch onderzoek bij ponies[1)] aan de afdeling Functionele morfologie van de faculteit der diergeneeskunde (Drs M. Jansen/Prof.dr.W. Hartman). Vanaf oktober 1989 begon hij met het in dit proefschrift beschreven onderzoek (o.l.v. dr.K.J. van den Berg, Medisch Biologisch Laboratorium te Rijswijk). Tijdens dit onderzoek volgde hij tevens de postdoctorale opleiding Toxicologie (SMBWO). Verder is hij co-auteur van 2 recente publicaties[2,3)] die in samenwerking met de afdeling Interne Geneeskunde III (Prof.dr.Ir. T.J. Visser) van de Erasmus Universiteit van Rotterdam werden bewerkt.

1) Quantitative analysis of computer-averaged electromyographic profiles of intrinsic limb muscles in ponies at the walk.
M.O. Jansen, J.A.G.M. van Raaij, A.J. van den Bogert, H.C. Schamhardt, W. Hartman, *American Journal of Veterinary Research. 53:2343-2349, 1992.*

2) Multiple UDP-glucuronyltransferases for the glucuronidation of thyroid hormone with preference for 3,3',5'-triiodothyronine (reverse T3).
T.J. Visser, E. Kaptein, J.A.G.M. van Raaij, C. Tjong Tjin Joe, T. Ebner and B. Burchell. *FEBS Letters 315:65-68, 1993.*

3) Glucuronidation of thyroid hormone in rat liver: Effects of *in vivo* treatment with microsomal enzyme inducers and *in vitro* assay conditions.
T.J. Visser, E. Kaptein, H. van Toor, J.A.G.M. van Raaij, K.J. van den Berg, C.T.T. Joe, J.G.M. van Engelen and A. Brouwer. *Endocrinology 133:2177-2186, 1993.*

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