Polychlorinated Biphenyl-Induced Alterations of Thyroid Hormone Homeostasis and Brain Development in the Rat

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Dennis C. Morse

STELLINGEN

1. Zowel neuronale en gliale celtypen zijn betrokken bij de ontwikkelings-neurotoxiciteit van PCBs.

   -dit proefschrift

2. Dalingen in plasma schildklierhormoon niveaus zijn niet betrokken bij PCB-geinduceerde veranderingen in de hersenen tijdens vroege blootstelling.

   -dit proefschrift

3. De accumulatie van gehydroxyleerde PCB metabolieten in het foetale brein is van groter belang dan de hiermee gepaard gaande schildklierhormoon-verlagingen voor de mogelijkheid tot verstoringen van de hersenontwikkeling.

   -dit proefschrift

4. Eenvoudigweg meten van thyroxine en retinol in plasma geeft geen relevante informatie over de gevolgen van PCB blootstelling op weefsel concentraties van de biologisch actieve vormen T3 en retinylzuur.

   -dit proefschrift

5. Vroege blootstelling aan PCBs veroorzaakt veranderingen in hersenontwikkeling, die toenemen met de leeftijd van de dieren. De effecten op het serotonerge systeem verdienen aandacht vanwege de betrokkenheid van dit systeem in stemmings veranderingen.

   -dit proefschrift

6. Het testen van individuele verbindingen op ontwikkelings- of andere vormen van toxiciteit is van beperkt nut voor het voorspellen van effecten van chronische blootstelling aan complexe mengsels. Daarom moet onderzoek geinitïeerd worden waarbij gebruik wordt gemaakt van relevante extracten of synthetische mengsels.

7. De mogelijke rol van xenobiotica in dementie en de ziekte van Parkinson is ten onrechte verwaarloosd bij experimenteel onderzoek.

8. Voor verrassend weinig teratogenen zijn de mechanismen opgehelderd ondanks de enorme hoeveelheid onderzoekstijd en geld die hieraan besteed zijn.

9. Er is geen ruimte in Nederland voor motorcross fanaten.

10. Kinderen van gereformeerde gezinnen hebben een grotere kans om hun creatieve vermogens te ontwikkelen omdat ze niet urenlang voor de TV of achter een spelcomputer zitten.

11. Het feit dat 5 mei nog steeds veel geld oplevert is te danken aan valse sentimenten.

12. Promovendi die tijdens het schrijven van een proefschrift te veel spanning ondervinden, moeten geen ondernemer worden.

Stellingen behorende bij het proefschrift "PCB-induced alterations of thyroid hormone homeostasis and brain development in the rat", Dennis C. Morse, Wageningen, 26 april, 1995.

For my parents,

who gave me the freedom to make mistakes
and then taught me how to learn from them

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CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

Context of the current study

The research presented in this thesis was conducted within the framework of the Dutch Mother's Milk Study. The Dutch Mother's Milk Study is a cooperative effort between clinical and pre-clinical scientists to investigate the effects of placental and lactational exposure to polychlorinated biphenyls (PCBs) and dioxins (PCDDs) on thyroid hormone homeostasis and neurological development of human infants and rats. There is public concern that the current level of human exposure to PCBs and PCDDs may result in developmental alterations, in particular in endocrinological and neurological systems. The work in this thesis pertains to the metabolism and distribution of PCBs in pregnant rats, the effects of maternal PCB exposure on retinoid and brain thyroid hormone homeostasis and neurochemical development in the rat.

Sources of polyhalogenated aromatic compounds

Polychlorinated biphenyls (PCBs), -dibenzo-*para*-dioxins (PCDDs) and -dibenzofurans (PCDFs) and their brominated analogs are halogenated aromatic compounds that are widespread and persistent environmental pollutants. Their chemical structures are shown in Figure 1.1. PCBs consist of a biphenyl ring which may be substituted with chlorine in the *ortho*, *meta* and *para* positions, yielding 209 different congeners.

PCBs were produced commercially in mixtures varying in the degree of chorination, yielding products with a wide range of applications. Their major use was as a dielectric fluid in transformers and capacitors. Total production of PCBs is estimated at 1.5 million metric tons. Through either direct discharge or leakage from discarded electrical equipment an unknown quantity of these compounds has entered the environment (reviewed by De Voogt and Brinkman, 1989).

In 1966 the Swedish chemist Søren Jensen detected PCBs in environmental samples (Jensen, 1966) during routine pesticide analysis and by the early 1970s the global extent of the contamination had become apparent (reviewed by Ballschmitter *et al.* 1989). Evidence that PCBs were toxic to wildlife species such as cormorants (Koeman *et al.* 1973) and mink (Auerlich *et al.* 1971) was also found in the early 1970s.

The chemical and physical characteristics that made PCBs a useful chemical product (e.g. chemical and thermal stability, and lipophilicity) also resulted in their environmental persistence and accumulation in higher trophic levels in the food chain. Although their production has now been phased out in western countries, and environmental levels have decreased, the contamination of the environment with PCBs is still widespread. The recirculation of PCBs already present in the environment by volatilisation followed by atmospheric deposition may prevent further dramatic decreases in environmental PCB levels (Harrad *et al.* 1994). There is evidence that atmospheric

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deposition of PCBs in the Baltic Sea has resulted in steady state levels of these compounds in cod liver from the Baltic area (Falandysz, 1994). In the North Sea PCB levels in cod liver oil also remained constant between 1979 and 1987 (de Boer, 1988).

The relatively high persistence of PCBs in human tissue and the cross-generational transfer of PCBs by breast feeding may slow the decline of PCB levels in humans. Several recent studies indicate that currently the levels of PCBs in human blood and milk are declining very slowly (Hovinga *et al.* 1992, Norén and Lundén, 1991, Duarte-Davidson *et al.* 1994), and may even be increasing in Eastern European countries, (Sikorski *et al.* 1994).

Chlorinated dibenzo-*para*-dioxins and dibenzofurans have never been intentionally produced except in small quantities for analytical and experimental purposes. These compounds are formed during combustion processes and some organochlorine synthesis as an unwanted byproduct (Rappe and Buser, 1989, review). Like PCBs, variations in the degree and site of chlorination result in 75 PCDD congeners and 135 PCDF congeners. Because of their similar chemical properties to PCBs, they have also been found as wide-spread environmental pollutants, and undergo similar long-range atmospheric transport (Eitzer and Hites, 1989).

The main source of human exposure to PCBs, PCDDs and PCDFs is via the diet (Theelen *et al.* 1993). Major dietary sources are meat, dairy products and fish (Theelen *et al.* 1993, Beck *et al.* 1989). For several relatively volatile lower chlorinated PCBs the major source for humans is likely to be via inhalation or via the consumption of leafy
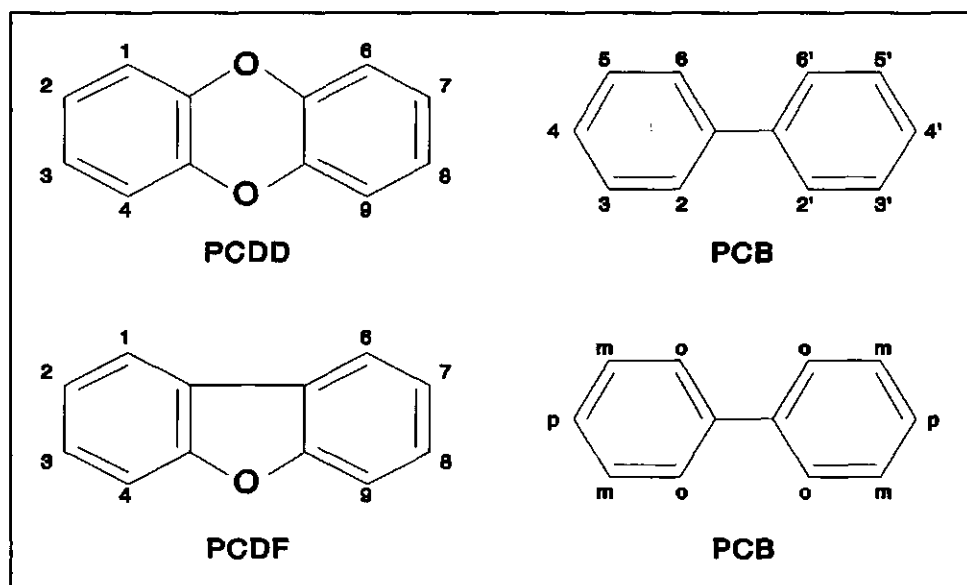
Figure 1.1 Structure and ring positions of PCDD (polychlorodibenzo-*para*-dioxin), PCDF (polychlorodibenzofuran) and PCB (polychlorobiphenyl). o: *ortho*, m: *meta*, p: *para*.

vegetables contaminated by atmospheric deposition (Bush *et al.* 1985, Harrad *et al.* 1994). Human newborns already have a low level of these compounds in their tissues (Masuda *et al.* 1978, Jacobson *et al.* 1984, Schecter *et al.* 1990), and subsequent breast-feeding can significantly increase the levels of PCBs in infants (Yakushiji, 1988).

PCBs, PCDDs and PCDFs are potent developmental toxins in laboratory animals and wildlife species, and following the consumption of rice oil accidentally contamina-ted with PCBs and PCDFs were also found to be developmental toxins in humans, producing delays in physical, neurological and cognitive development. Because of the biological persistence and relatively high lactational transfer of these compounds, there is concern that the exposure to background levels may also result in developmental delays in humans. For risk evaluation of the developmental toxicity of PCBs, PCDDs and PCDFs, an understanding of the metabolism, transplacental and lactational transfer, toxic mechanisms and structure-activity-relationships of these compounds is necessary.

Kinetics and metabolism of PCBs during pregnancy and lactation.

It is important to note that the pattern of individual PCB congeners stored in the adipose tissue of environmentally exposed mammals does not match that of commercial PCB mixtures (Schultz *et al.* 1989, Safe *et al.* 1985, Muir *et al.* 1988). The differing behavior of individual PCB congeners during a variety of physico-chemical processes (partitioning, uptake, photolysis) and during metabolism by microbes and animals alters the composition of PCB mixtures present in the environment.

The kinetics and metabolism of PCBs, PCDDs and PCDFs in adult animals have been reviewed in detail (Matthews and Dedrick, 1984, Safe, 1989, Van den Berg *et al.* 1994). These compounds are readily absorbed from the gastrointestinal tract, and depending on the chlorine substitution pattern may be preferentially retained in the liver and adipose tissue with a long half-life. Lateral substitution of the rings generally increases tissue retention. The presence of two adjacent carbon atoms without a chlorine substitution, preferably in the lateral positions, greatly enhances the hydroxylation of PCBs by mixed-function oxidases and thereby reduces the tissue half-life of the parent compound. The hydroxylated metabolite is not necessarily excreted rapidly, for relative-ly high levels of *para*-hydroxylated PCB metabolites have been found in the plasma of environmentally exposed humans and marine mammals (Bergman *et al.* 1994).

Following hydroxylation, the metabolite can be conjugated with either glutathione or glucuronic acid and excreted via the bile into the intestine. In feces, PCB metabolites are generally found as unconjugated hydroxylated PCBs, which suggests that the glucuronic acid conjugate is deconjugated during intestinal passage. The further metabolism of PCB-glutathione conjugates via the mercapturic acid pathway can result in the formation of methylsulfonyl metabolites which are highly retained in some tissues.

The bioaccumulation of some lower chlorinated PCBs may have been prevented by their metabolism in animals, decreasing their relative contribution to PCB concentrations in tissues. This is illustrated by the observation that mothers exposed occupationally to the commercial PCB mixture Kanechlor 300 and their children have relatively large amounts of tri- and tetrachlorinated PCBs in their blood relative to the

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general population, who are exposed mainly through the diet (Yakushiji, 1988). Also some higher chlorinated PCB congeners lacking *para* and *meta* chlorine substitutions in one or both rings, like 2,2',3,3',6,6'-hexachlorobiphenyl, can be rapidly eliminated as hydroxylated metabolites (Matthews and Tuey, 1980). There also appear to be temporal trends in the congener-specific composition of human milk following restictions on the production and use of PCBs, which show an increase in the relative amounts of more persistent PCB congeners, like 2,2',4,4',5,5'-hexachlorobiphenyl (Norén *et al.* 1990)

The alteration of the PCB congener pattern may have toxicological consequences. A synthetic, reconstituted PCB mixture based on the major PCB contaminants in milk was 7 times more effective in inducing rat hepatic microsomal aryl hydrocarbon hydroxylase than the commercial PCB mixture Kanechlor 500 (Parkinson *et al.* 1980). There is some evidence that commercial PCB mixtures contain PCB congeners that have an antagonistic effect on some biochemical and toxicological endpoints in rodents (Safe *et al.* 1994).

Placental transport

Placental transfer of PCBs has been documented in rodents, monkeys and humans (Curley *et al.* 1973, Takagi *et al.* 1976, Allen and Barsotti, 1976, Akiyama *et al.* 1975, Masuda *et al.* 1978b). Dietary exposure of pregnant mice to Kanechlor 500 resulted in the transfer of 0.1 to 0.2 % of the dose to late gestational fetuses (Masuda *et al.* 1978). The amount of the administered maternal dose of individual PCB congeners transferred to the fetus in mice was less than 0.3% (Masuda *et al.* 1979). However, when examined in terms of the maternal body burden, two lower chlorinated PCBs, 2,4,4'-trichlorobiphenyl and 2,3',4',5-tetrachlorobiphenyl were transferred relatively efficiently to the fetus (4.6 and 13.3%, respectively). Evidence of selective accumulation of PCB congeners in the fetal rat has also been reported following dietary exposure of the dams to Aroclor 1254 (Shain *et al.* 1986). There is insufficient data on the total PCB body bureden of fetal non-human primates or human fetuses to draw conclusions on the degree of transplacental transfer in higher mammals. An estimate by Duarte-Davidson and Jones (1994) suggests that currently in the U.K. less than 1.7% of the body burden of a 20 year old adult will be transferred to the fetus. Some data is available on the levels of individual PCB congeners in fetal cord blood, showing positive correlations between maternal serum PCB levels with those in cord sera or in the placenta (Jacobson *et al.* 1984, Bush *et al.* 1984, Ando *et al.* 1985). There is also evidence that PCDDs and PCDFs cross the placenta and are present in fetal human liver (Schecter *et al.* 1990).

The presence of hydroxylated PCB metabolites in fetal mice and rats following maternal exposure to a limited number of PCB congeners has been reported (Lucier *et al.* 1978, Darnerud *et al.* 1986). The presence of significant amounts of hydroxylated PCBs in adult human plasma indicates that hydroxylated PCB metabolites may also accumulate in the human fetus, but this has not been investigated at present. Methylsulphonyl metabolites of 2,4',5-trichlorobiphenyl have been found to accumulate in the uterine luminal fluid of pregnant mice (Brandt *et al.* 1982), indicating transplacental transport of these apolar metabolites.

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Lactational transfer

The major route of exposure of the developing mammal to PCBs and related compounds is via lactation (Curley *et al.* 1973, Takagi *et al.* 1976, Masuda *et al.* 1978, Van den Berg *et al.* 1987, Nau *et al.* 1986). In humans, the level of PCBs in milk fat exceeds that of other foodstuffs, and is regarded as the major source of PCBs to the nursing infant (Yakashiji, 1988). PCBs, PCDDs and PCDFs have been shown to be readily absorbed from the digestive tract in a nursing infant (McLachlan, 1993). Concentrations of total PCBs or PCB congeners reported in human milk vary, and comparisons are further complicated by differences in analytical methods and the manner of reporting results (on whole milk or lipid basis). PCB concentrations in human milk increase with maternal age (Sikorski *et al.* 1990), and decrease with time spent breast-feeding and the number of children nursed (Rogan *et al.* 1986).

In Europe, similar levels of total PCBs have been found in milk fat. For example, total PCB levels of 0.62, 0.6, 0.9 and 1.2 mg/kg milk fat have been found in Norway, the Netherlands, Germany and Poland, respectively (Skaare and Polder, 1990, Koopman-Esseboom *et al.* 1994, Fürst *et al.* 1994, Sikorski *et al.* 1990). In Canada, Inuit women from the Hudson Bay area had significantly higher PCB levels in their milk fat than caucasian women living in Southern Quebec (3.60 vs 0.77 mg PCB/kg milk fat, Dewailly *et al.* 1989). In the Lake Michgan study, children born to mothers with a PCB concentration greater than 1.25 mg/kg milk fat exhibited decreased performance in the McCarthy memory scales (Jacobson *et al.* 1990).

Polychlorinated aromatic compounds and central nervous system development

Accidental exposure

PCBs, PCDDs and PCDFs are teratogenic, induce late fetal death, cause reproductive disorders in male and female offspring and alter the development of the central nervous system in experimental animals (Peterson *et al.* 1993, review). In humans, one of the best documented effects of exposure to PCBs and related compounds are delays in central nervous system development.

The first evidence that PCBs and related compounds may alter neurological development was found in Japan after the consumption of rice oil in 1968 which had been contam2inated with PCBs and their thermal degradation products, PCDFs and polychlorinated quaterphenyls (PCQTs) (Harada *et al.* 1976, Masuda *et al.* 1985, Kuratsune, 1989). This incident was termed Yusho, which means "oil disease". A similar incident occured in Taiwan in 1979, and was termed Yucheng. Exposure to contaminated rice oil resulted directly in various symptoms including chloracne, pigmentation of the nails and skin thickening, which was attributed to the presence of the highly toxic PCDFs (Hara, 1985). Similar mean blood PCB levels were found in Yusho and Yucheng patients (45 ppb and 39 ppb) as in occupationally exposed workers in Japan (99 ppm in female workers handling KC-300 and KC-500), however, health effects were minor in the workers in comparison to the Yusho and Yucheng patients (Hara, 1985, Kashimoto *et al.* 1985).

Children born to mothers exposed in the Yucheng incident have been carefully followed, revealing a consistent trend of the Yucheng children to score significantly lower on intelligence tests than matched controls (Hsu *et al.* 1989, Chen *et al.* 1992, Chen and Hsu, 1994). Intra-uterine growth was decreased in Yucheng babies (Yen *et al.* 1994), and there is evidence of increased respiratory problems and delayed growth in early childhood (Rogan *et al.* 1987). The majority of the Yucheng children were not breastfed, so the observed effects are most likely to be due to prenatal exposure to PCBs and/or their thermal degradation product, PCDFs (Yu *et al.* 1991). It is not known what the relative contributions of the PCBs and PCDFs were to the observed developmental effects (Yu *et al.* 1990). Occupational exposure to PCBs has been reported to reduce birth weight, an effect partially mediated by reduced length of gestation (Taylor *et al.* 1989).

Background exposure:

In addition to high-level accidental exposure to PCBs and their thermal degradation products, the effects of background levels of PCBs on the neurological and cognitive development have also been investigated. Research on the relationship between the consumption of PCB-contaminated fish from Lake Michigan and neurological development (the "Lake Michigan study") revealed a negative association between greater fish consumption and neuromuscular maturity in infants (Fein *et al.* 1984). In the same study significant lower birth weight, head circumference and gestational age were associated with higher cord serum PCB levels (Fein *et al.* 1984). A follow-up of the Lake Michigan study found that at 7 months the development of visual recognition memory (Fagan test) was impaired in infants that had elevated cord serum PCB levels (Jacobson *et al.* 1984). Postnatal exposure from breastfeeding was unrelated to visual recognition memory performance at 7 months (Jacobson *et al.* 1985).

At 4 years of age prenatal PCB exposure predicted poorer short-term memory performance and decreased body weight in a dose-dependent fashion (Jacobson and Jacobson, 1993). Maternal body burden estimated as mg PCB/kg milk fat, but not cumulative lactational exposure, predicted poorer memory performance, but was a less accurate predictor than cord blood PCB levels (Jacobson *et al.* 1990). The 4-year body burden (based on the children's serum PCB levels) was negatively associated with the composite activity rating (Jacobson *et al.* 1990, Jacobson and Jacobson, 1993).

A second U.S. study based in North Carolina with a different experimental design also reported negative associations between "gestational" PCB exposure and neuromuscular development (Rogan *et al.* 1986, Rogan *et al.* 1988). Actually, the effects of gestational exposure were not explicitly examined in the North Carolina study by measuring cord blood PCB levels, rather PCB concentrations in milk fat were used to estimate gestational exposure (Rogan *et al.* 1986). Postnatal exposure, estimated by PCB levels in milk fat and the duration of breast feeding was not related to neuromuscular development (Rogan *et al.* 1988). Effects on cognitive function were not found, and psychomotor alterations were transient (Gladen and Rogan, 1991).

Recently in the Netherlands, studies have been conducted on the relationship between prenatal and postnatal PCB exposure, postnatal PCDD and PCDF exposure and neurological and cognitive development in human infants. The results of these studies

have not yet been published.

Laboratory studies

The developmental neurotoxicity of PCBs has been most intensively examined in laboratory animals in relation to behavioral parameters. Only limited information is available on the biochemical effects of PCBs in the developing brain and the mechanisms involved are not clear.

The effect of pre- and postnatal PCB exposure on activity levels have been reported in mice, rats and rhesus monkeys (Tilson *et al.* 1979, Lilienthal *et al.* 1990, Erikson *et al.* 1991, Bowman *et al.* 1978, Bowman and Heironimus, 1981). In general, pre- and postnatal PCB exposure results in increased activity in the offspring, although the effect is dependent on the length of the activity test. In monkeys, pre- and postnatal exposure to Aroclor 1248 resulted in hyperactivity in juveniles and hypoactivity in adolescents (Bowman and Heironimus, 1981).

Activity-related behaviors, such as active avoidance learning in mice and rats (Storm *et al.* 1981, Tilson *et al.* 1979, Lilienthal *et al.* 1990) and operant behavior in rats and monkeys (Pantaleoni *et al.* 1988, Lilienthal *et al.* 1990, Mele *et al.* 1986) are also affected by pre- and postnatal PCB exposure. It has been suggested that the effects observed on active avoidance learning and operant behavior may be secondary to PCB-induced changes in activity levels (Lilienthal *et al.* 1990).

Cognitive effects have also been observed in the offspring of PCB-exposed monkeys. Maternal dietary exposure to Aroclor 1248 before gestation and lactation produced deficits in delayed spatial alternation learning in the offspring at 3-4 years of age (Levin *et al.* 1988). Higher PCB body burdens (Aroclor 1248) in infant monkeys had previously been shown to be correlated with increased errors in learning tasks up to 2 years of age (Bowman *et al.* 1978).

Possible mechanisms involved in the developmental neurotoxicity of PCBs

Ah-receptor

The mechanisms involved in the developmental neurotoxicity of PCBs and related compounds are not clear at present. Almost all of the other known toxic effects of PCBs, PCDDs and PCDFs in laboratory animals can be explained by the interaction of these compounds with a cytosolic receptor protein, the aryl hydrocarbon (Ah) receptor (Safe, 1994). These Ah-receptor dependent effects include teratogenicity, hyperkeratinization of epithelia and immunotoxicity. It has been demonstrated that once the ligand has bound the Ah-receptor, the ligand-receptor complex translocates to the cell nucleus and binds to dioxin responsive enhancers in the DNA (Okey *et al.* 1994, Nebert *et al.* 1993). It has been proposed that the binding of the ligand-receptor complex to DRE sequences results in the transcription of genes that mediate the toxicity of these compounds. To date, 26 genes have been identified as having a DRE upstream, although the molecular mechanisms involved in toxicity following their transcription remain elusive (Sutter and Greenlee, 1992).

One fundamental aspect of the Ah-receptor theory is that compounds which

exhibit the highest binding affinity for the Ah-receptor are the most toxic (Safe 1994). A structure-activity relationship has been developed for this interaction. PCBs, PCDDs and PCDFs with 4 lateral chlorine substitutions are the most potent, and increasing chlorine substitution decreases the affinity of the compound for the Ah-receptor and hence its toxic potential. The planarity of the compound is also essential for determining its binding to the Ah-receptor. The dioxins and dibenzofurans are by their chemical nature planar compounds, while PCBs may rotate around the bond connecting the 2 biphenyl rings. Increasing chorine substitution in the *ortho* postitions around the bond connecting the biphenyl rings reduces the planarity of the PCB and hence its affinity for the Ah-receptor.

Although the structure activity relationships for developmental neurotoxicity have not been elucidated, there is evidence that the Ah-receptor may contribute to but is not essential for developmental neurotoxicity. The most toxic polyhalogenated aromatic compound with the highest affinity for the Ah-receptor, 2,3,7,8-tetrachlorodibenzo-*para*-dioxin (TCDD), induces behavioral alterations in monkeys exposed pre- and postnatally (Schantz and Bowman, 1989, Schantz *et al*. 1992). On the other hand, a commercial PCB mixture (Aroclor 1016) which exhibits little Ah-receptor mediated toxicity (Safe, 1994) may induce behavioral alterations in monkeys which have been exposed via gestation and lactation (Levin *et al*. 1988, Schantz *et al*. 1989) and transiently alters striatal dopamine levels in rats following pre- and postnatal exposure (Seegal, 1994).

Inhibition of dopamine synthesis

Seegal and Shain (1992) have proposed that PCBs inhibit dopamine synthesis in the basal ganglia of rodents and non-human primates, and suggested that the decreases in dopamine levels may also play a role in the developmental neurotoxicity of these compounds. In adult rodents and macaques, the dopaminergic system is the most sensitive for sub-chronic exposure to PCBs of the neurotransmitter systems studied to date (Seegal, review), although other neurotransmitter systems (serotonergic, noradrenergic) may be affected (Seegal *et al*. 1986a, 1986b, 1986c).

In PC12 cells, PCB congeners which have little or no affinity for the Ah-receptor are remarkably more effective in reducing cellular dopamine levels than the PCB congener with the highest affinity for the Ah-receptor, 3,3',4,4',5-pentachlorobiphenyl. In adult macaques, the major PCB congeners accumulating in the brain after exposure to Aroclor 1016 (2,4,4', 2,2',4,4', and 2,2',5,5') produce no Ah-receptor mediated toxicity (Safe, 1994), yet long-term decreases in dopamine levels were observed in the basal ganglia. In cell-free homogenates, di-*ortho* PCBs inhibit dopamine synthesis in a concentration-dependent fashion, suggesting that they directly inhibit the activity of tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis. Together, *in vitro* and *in vivo* data suggest that low chlorinated di-*ortho* substituted PCB congeners may be the most neuroactive congeners in adult animals. However, the *in vitro* data should be interpeted with caution, because the presence or absence of a functional Ah-receptor pathway has not been examined in the PC-12 cell cultures used in those studies.

There is some evidence that PCBs alter dopamine function in laboratory animals following pre- and postnatal exposure. Gestational exposure of mice to a relatively high dose of 32 mg 3,3',4,4'-tetrachlorobiphenyl per kg body weight resulted in decreased

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striatal dopamine and dopamine receptor levels in adult offspring (Agrawal *et al.* 1981). Recently, it has been reported that *in utero* and lactational exposure to Aroclor 1016 resulted in transient increases in striatal dopamine levels in rats (Seegal, 1994).

Hormonal alterations

Several other hypotheses have been proposed to explain the developmental neurotoxicity of PCBs. The possible involvement of thyroid hormones or estrogens has been discussed (Rogan *et al.* 1986, Lillienthal and Wieneke, 1991). Both hormones play a role in neurochemical and behavioral development (McEwen, 1992) and PCBs may alter plasma levels of these hormones or influence their action during brain development (Collins and Capen, 1980a, Lundkvist and Kindahl, 1989). However, there has been no direct assessment of the effects of PCB exposure on the levels and metabolism of these hormones in the developing brain.
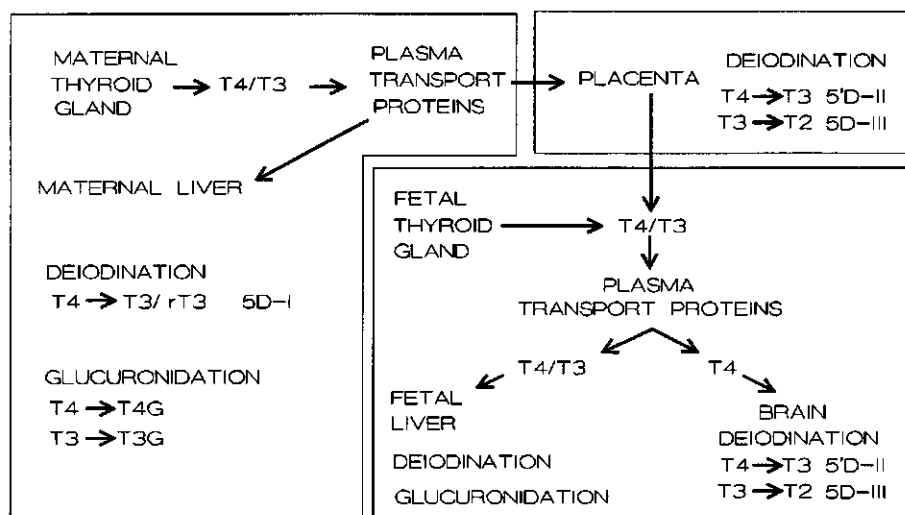
Steroid hormones

The effects of PCBs or PCDDs on estradiol homeostasis has not been intensively studied. There is some evidence that coplanar PCBs and TCDD are anti-estrogenic, while *ortho*-substituted PCBs and some hydroxylated PCBs are estrogenic (Jansen *et al.*1992, Korach *et al.* 1988, Spink *et al.* 1990). Environmental exposure occurs to a mixture of PCDDs, PCDFs, coplanar and *ortho*-substituted PCBs, followed by metabolism of some of these compounds to hydroxylated metabolites which accumulate in the plasma. Due to a lack of information on the relative estrogenic or anti-estrogenic potencies of these compounds *in vivo* and their possible interactive effects, it is currently impossible to predict what the net effect of perinatal exposure to a complex mixture of these compounds will be on estrogenic systems and subsequently on brain development.

Thyroid hormones

There is evidence that reductions in neonatal brain thyroid hormone levels or maternal plasma thyroid hormones before the onset of fetal thyroid hormone secretion result in permanent alterations in behavior and brain maturation (Porterfield and Hendrich, 1993, review). The developmental regulation of thyroid hormone status in the rat has been investigated in detail by the group of Morreale de Escobar and Escobar del Rey (1993, review). The early fetal period is the most sensitive for alterations in thyroid hormone homeostasis. Up to day 18 of gestation the fetus is mainly dependent on the transplacental transport of maternal T_4. The fetal thyroid begins secreting T_4 and T_3 between day 17 and 18 of gestation, and by gestational day 21 is dependent on maternal thyroid hormones only to a limited extent. The major source of the biologically most active hormone, triiodothyronine T_3, in the brain is the local deiodination of T_4 to T_3 by type II 5'-thyroxine deiodinase, an enzyme which catalyses outer ring deiodination of iodothyronines. The induction of 5'D-II activity is a compensatory mechanism to maintain brain T_3 levels when the transport of T_4 into the brain is decreased in fetal, neonatal and adult rats. After fetal thyroid secretion begins and the hypothalamic-pituitary axis and the enzyme 5'D-II are able to respond to decreases in brain T_4 levels,

the fetus becomes less sensitive for decreases in plasma T_4 levels (Ruiz de Oña *et al.* 1988, 1991, Morreale de Escobar *et al.* 1988, 1989, 1990).

The pre- and postnatal administration of compounds (methimazole, propylthioura-cil) which inhibit the synthesis of T_3 and T_4 by the thyroid gland can also severely reduce brain T_3 levels in fetuses and neonates, thereby adversely affect somatic and brain development in the rat (Morreale de Escobar *et al.* 1993, review). Therefore the transplacental and lactational exposure of mammals to PCBs which reduce plasma thyroid hormone levels during development (Collins and Capen, 1980a) may also adversely affect brain thyroid hormone homeostasis and subsequently normal brain development.

Figure 1.2 Schematic representation of thyroid hormone production, transport and metabolism in the late gestational maternal and fetal rat. T_4: thyroxine, 3,3',5,5'-tetraiodothyronine, T_3:, 3,3',5-triiodothyronine, rT_3: reverse T_3, 3,3',5'-triiodothyronine, T2: 3,3'-diiodothyronine, T_4G: T_4-glucuronide, T_3G: T_3 glucuronide, 5D-I: type I 5-deiodinase, 5'D-II: type II 5'-deiodinase, 5D-III: type III 5-deiodinase.

Mechanisms of altered thyroid hormone homeostasis by PCBs

The mechanisms of the decrease of plasma thyroid hormones has been extensive-ly investigated in adult animals. Initial work by Bastomsky (1974, 1977) indicated that the PCB or TCDD-mediated induction of hepatic glucuronidation of thyroxine (T_4) and subsequent elimination of the glucuronide via the bile may play a role in the decrease of plasma T_4 levels. The role of induced T_4 glucuronidation in reducing plasma T_4 levels is also supported a study in which thyroidectomized rats were implanted with an osmotic pump to supply T_4 (Barter and Klaassen, 1992).
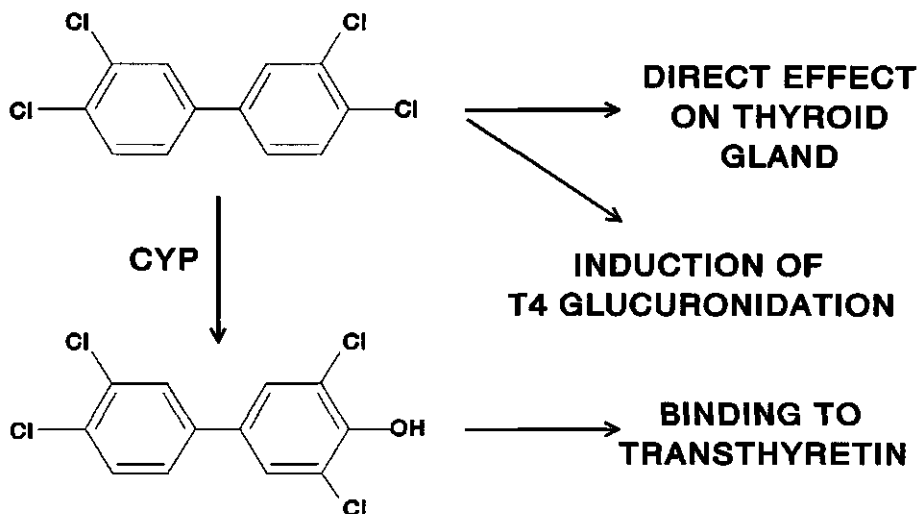
Figure 1.3 Potential mechanisms of PCB-induced reductions in plasma T4 concentrations with 3,3',4,4'-tetrachlorobiphenyl as model compound. CYP: cytochrome P450.

However, research by Collins and Capen (1980b) demonstrated that hepatic glucuronidation of T_4 may be a contributing factor but is not essential for decreases in plasma T_4 levels. Similar decreases in plasma T_4 levels were observed in heterozygous Gunn rats (sufficient in T_4 glucuronidation) and homozygous Gunn rats (deficient in T_4 glucuronidation) following dietary exposure to the commercial PCB mixture Aroclor 1254 (1980b).

Ultrastructural examination of the thyroid following exposure of adult rats to Aroclor 1254 revealed that the observed alterations did not resemble the effects of TSH stimulation, iodine deficiency or thyroidectomy, indicating a direct effect of PCBs on the thyroid gland (Collins and Capen 1980c). The authors suggested that the secretion of thyroid hormones by the thyroid gland is inhibited by PCB exposure (Collins and Capen, 1980c).

The role of *para*-hydroxylated PCB metabolites in the decrease of plasma thyroid hormones was demonstrated *in vivo* and *in vitro* by several research groups (Richenbacher *et al.* 1986, Brouwer and van den Berg, 1986, Brouwer *et al.* 1990). These metabolites bear a strong structural resemblance to T_4 and exhibit competitive binding for transthyretin, the major thyroid hormone transport protein in the plasma of adult rodents (Lans *et al.* 1993). Some hydroxylated PCB metabolites have a stronger affinity for transthyretin than T_4 itself, and therefore displace T_4 from the binding protein, decreasing total plasma T_4 levels (Brouwer *et al.* 1990, Lans *et al.* 1993).

Only limited information is available on the effects of PCBs on thyroid hormone

homeostasis in the developing rat. Gestational and lactational PCB exposure results in decreases in plasma T_4 levels in the newborn and weanling rat (Collins and Capen 1980a, Ness *et al.* 1993). Alterations in the ultrastructure of the fetal rat thyroid have been observed following prenatal PCB exposure, indicating that the fetal rat is also sensitive for PCB-induced alterations in thyroid hormone homeostasis (Collins and Capen, 1980a).

Retinoids

PCB-induced alterations in retinoid homeostasis may also be involved in the developmental neurotoxicity of PCBs. PCBs and related compounds have been shown to alter retinoid homeostasis in laboratory animals and diverse wildlife species (reviewed by Zile, 1992, review). Retinoids induce malformations in the central nervous system when administered during embryogenesis. Functional (behavioral) neuroteratogenicity has been observed following the administration of retinoids during organogenesis (Adams, 1993, review). However, the administration of PCBs to laboratory animals generally results in decreases in retinoid stores in the liver (the major storage organ for retinoids) and some other extrahepatic organs. It has been shown that PCB administration increases the mobilization of hepatic retinoid stores, which can result in an increase of retinoid levels in the kidney. Little is known about the effects of PCBs on retinoid homeostasis in the developing mammal. Neonatal exposure of rats to TCDD results in the decrease of hepatic retinoid stores. (Håkansson *et al.* 1987)

Scope of the present investigation

The aim of the present study was to investigate the relationship between the metabolism and transport of PCBs in pregnant rats, alterations of thyroid hormone status during development and biochemical alterations in brain development. It was hypothesized that the metabolism of PCBs to hydroxylated metabolites would result in an accumulation of these metabolites in fetal and neonatal rat plasma, resulting in reductions in plasma thyroid hormone levels. The reductions in plasma thyroid hormone levels could result in a decrease in brain T_3 levels, thereby inducing permanent alterations in brain development.

The effect of pre- and postnatal exposure to PCBs (3,3',4,4'-tetrachlorobiphenyl and 3,3',4,4',5,5'-hexachlorobiphenyl and the commercial PCB mixture Aroclor 1254) on plasma T_4 and T_3 levels, brain Type II thyroxine 5'-deiodinase activity and hepatic T_4-UDPGT activity was investigated in rats (Chapter 2, 4 and 5). Brain levels of T_4 and T_3 were investigated in the fetuses and weanling rats from dams exposed to Aroclor 1254 (Chapter 5). As the effects on retinoids by PCBs can be related to the effects of PCBs on plasma thyroid hormones, retinoid homeostasis was investigated in the offspring of dams exposed to Aroclor 1254 during gestation (Chapter 6).

PCB metabolism was first investigated in pregnant rats using 3,3',4,4'-tetrachlorobiphenyl as a model compound for a rapidly metabolizable PCB (Chapter 2 and 3), which has been shown to be teratogenic and a developmental neurotoxicant. The accumulation of PCBs and hydroxylated PCB metabolites in the plasma of dams and the plasma and brains of their offspring was investigated with Aroclor 1254 (Chapter 5).

To evaluate the developmental neurotoxicity of Aroclor 1254, the levels of a gliotypic protein (glial fibrillary acidic protein) and a neurotypic protein (synaptophysin) were measured in discrete brain regions following maternal exposure to Aroclor 1254. Glial fibrillary acidic protein and synaptophysin have been previously used to invesitigate the developemntal neurotoxicity of alkyltins (O'Callagan and Miller, 1988, O'Callaghan and Miller, 1989, O'Callaghan and Jensen, 1992). In addition the effect of maternal exposure to Aroclor 1254 on regional brain levels of biogenic amines in the offspring was investigated.

CHAPTER 2

METABOLISM AND BIOCHEMICAL EFFECTS OF 3,3',4,4'-TETRACHLORO-BIPHENYL IN PREGNANT AND FETAL RATS

Abstract

The metabolism and distribution of a single oral dose of 25 µmol [^{14}C]-3,3',4,4'-tetrachlorobiphenyl (TCB) were investigated in pregnant female Wistar rats and their fetuses. TCB was administered on day 13 of gestation and the elimination was followed for 7 days. Non-pregnant rats were treated similarly for comparison. Fecal elimination of [^{14}C]-TCB derived radioactivity was significantly lower in pregnant rats than in non-pregnant rats. The major metabolite found in adult liver and plasma, placental tissue, whole fetuses and fetal plasma was 3,3',4',5-tetrachloro-4-biphenylol (4-OH-tetraCB). Tissue levels (liver, abdominal fat, skin, skeletal muscle, kidney and plasma) of [^{14}C]-TCB-derived radioactivity declined by 65 to 85% over a 7 day period following administration in the adult animals. However, [^{14}C]-TCB-derived radioactivity accumulated more than 100-fold in the fetuses over the same time period, and GC/MS analysis revealed that the fetal accumulation in radioactivity was due primarily to 4-OH-tetraCB, and not the parent compound. On day 20 of gestation, concentrations of 4-OH-tetraCB were 14 times greater in fetal plasma than maternal plasma.

Treatment with [^{14}C]-TCB significantly reduced plasma thyroxine levels by at least 28% up to 7 days after administration in non-pregnant animals and up to 4 days after administration in pregnant rats (31% decrease). By 7 days after administration plasma thyroxine levels had returned to control levels in the TCB-treated pregnant rats. However, fetal plasma thyroxine levels were significantly decreased by 35% in fetuses from [^{14}C]-TCB-treated dams 7 days after TCB administration. Hepatic microsomal ethoxyresorufin-O-deethylase (EROD) activity was significantly induced in TCB-treated dams relative to controls at 4 and 7 days after administration, while no EROD activity was detected in hepatic microsomes from control or TCB treated fetal rats at day 20 of gestation. These data suggest that hydroxylated metabolites of polychlorinated biphenyls may play a role in the development toxicity of these compounds.

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Introduction

Polychlorinated biphenyls are widespread environmental contaminants which are also found in human breast milk and can cross the placenta to a minor extent (McFarland and Clarke, 1989, Bush *et al.* 1984). These compounds have been shown to be developmental toxins in both laboratory animals and man, and the effects include congenital malformations, increases in fetal mortality and neurobehavioral alterations (Peterson *et al.*, 1993, review). 3,3',4,4'-Tetrachlorobiphenyl (TCB) is one of the most toxic PCB congeners (Safe, 1990, review) and has been shown to be a developmental neurotoxin (Tilson *et al.*, 1979, Agrawal *et al.*, 1981, Eriksson, 1988, Eriksson *et al.* 1991).

The excretion and metabolism of TCB has been extensively examined in adult rats (Abdel-Hamid *et al.*, 1981, Yoshimura *et al.*, 1987, Koga *et al.*, 1989) and mice (Klasson Wehler *et al.*, 1989), but there is only one report on the identification of TCB metabolites in pregnant mice and their fetuses, which shows that phenolic and methyl sulfonyl metabolites accumulated in the fetal compartment (Darnerud *et al.*, 1986). TCB is rapidly metabolized in rodents, and it is not clear whether the parent compound or a metabolite is responsible for developmental neurotoxicity. A metabolite of TCB, 3,3',4',5-tetrachloro-4-biphenylol, (4-OH-tetraCB) has a high affinity to transthyretin, the major thyroid hormone binding protein in the rat (Lans *et al.*, 1993, Brouwer *et al.*, 1990). This 4-OH-tetraCB has been found in the plasma of both adult mice and rats treated with TCB (Brouwer *et al.*, 1990, Klasson Wehler, 1989). Because of the structural resemblance of 4-OH-tetraCB to thyroxine (T_4), this metabolite can displace T_4 from TTR, resulting in decreased plasma T_4 concentrations (Brouwer *et al.*, 1986). There is some evidence that prenatal administration of TCB to rats can reduce fetal plasma thyroid hormone levels and alter fetal brain thyroid hormone metabolism (Morse *et al.*, 1993). Since thyroid hormones are essential for normal brain development, decreases in circulating thyroid hormone levels during development can have long-lasting adverse neurological effects (Smith, 1981, Hendrich *et al.*, 1984).

The aim of this study was to examine the metabolism of TCB in pregnant and non-pregnant rats along with effects on plasma thyroid hormone concentrations in the dams and fetuses. Ethoxyresorufin-O-deethylase activity (EROD) activity was determined in fetal and maternal hepatic microsomes as an indication of hepatic metabolic activity for TCB.

Materials and Methods

Chemicals

The [^{14}C]-3,3',4,4'-tetrachlorobiphenyl (TCB, 37.1 μCi/μmol, radiochemical purity > 95%) was purchased from Sigma Chemical Company, St Louis, MO. Unlabelled TCB (> 99% pure, dibenzofuran and dibenzo-p-dioxin free) was obtained from Promochem, Germany. The TCB metabolite standards, 3,3',4,4'-tetrachloro-2-- biphenylol, 3,3',4',5-tetrachloro-4-biphenylol, 3,3',4,4'-tetrachloro-5-biphenylol and 3,3',4,4'-tetrachloro-6-biphenylol were synthezised as described by Klasson Wehler *et al.* (Klasson Wehler *et al.*, 1989, Klasson Wehler *et al.*, 1990).

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Animals

Non-pregnant and timed pregnant Wistar WU rats, 12 weeks old, were obtained from Charles River Wiga GmbH, Sulzfeld, Germany. The animals were maintained at 21°C, 50% humidity with a 12 h light cycle, food (TNO stock diet) and tap water were supplied ad libitum. After an acclimatization period of 1 week the rats were housed in metabolism cages 3 days prior to administration of TCB or the vehicle. On day 13 of gestation 15 pregnant rats were given a single oral dose of 25 µCi [14C]-TCB per kg body wt diluted with unlabelled TCB for a total dose of 25 µmol TCB/kg body weight. The [14C]-TCB was dissolved in corn oil, 25 µmol/2ml. After a similar acclimatization period 12 non-pregnant rats recieved an identical dose of [14C]-TCB in cornoil. Control rats (n=9) for biochemical assays were housed identically and received the vehicle only.

Three rats per group were sacrificed by bleeding via the aorta under ether anesthesia 1, 2, 4 and 7 days after [14C]-TCB administration, (with the exception that 5 pregnant rats treated with [14C]-TCB were sacrificed 7 days after treatment). The following tissues or organs were removed from the adult rats: liver, kidneys, spleen, lungs, heart, thymus, brain, skeletal muscle and a strip of abdominal skin (including subcutaneous fat). In addition, abdominal fat was quantitatively collected by trimming fat from organs from the abdominal cavity and from the abdominal wall. Organs, fetuses and placentas were rinsed with 0.9% NaCl, blotted dry with tissue paper, weighed and stored at -20° C. The carcass of the adult rats was stored at -20° C. Feces and urine were collected daily and cages were rinsed with 200 ml of 0.1% Triton X-100 at the end of the experiment to determine losses of [14C]-TCB derived radioactivity.

In a parallel experiment to determine the effect of TCB on maternal and fetal hepatic microsomal EROD induction, pregnant rats were treated with 25 µmol unlabelled TCB/kg body weight on day 13 of gestation. The rats were sacrificed 4 and 7 days after TCB administration. Livers were rapidly removed, stored at -80°C until microsomes were prepared as previously described and stored at -80 °C until analysis (Morse *et al.*, 1993).

Analysis of total [14C] radioactivity

The concentration of [14C] radioactivity in fecal samples and individual organs was determined using a Packard 307 sample oxidizer and liquid scintillation counting (LSC) with a LKB Wallac 1410 scintillation counter. Samples were weighed and then dried overnight in a stove at 35° C prior to sample oxidation. Feces was homogenized with 4 parts water (w/w) prior to sample drying. Radioactivity in cage washes, urine and plasma samples was determined directly with LSC by mixing 500 µl cage wash or urine and 100 µl plasma with 4.5 ml scintillation fluid (Safe-Fluor, Packard). Particulate matter in the urine was allowed to settle before taking a sample for LSC. The carcass was dissolved overnight in 1.5 M NaOH containing 45% ethanol (v/v). After homogenization a 500 µl aliquot was added to 10 ml scintillation fluid compatible with high ionic solutions (Hionic-Fluor, Packard) and counted with LSC. Three fetuses and placentas were pooled for each pregnant rat for determination of total [14C] radioactivity. Total [14C] radioactivity per organ was analysed separately for each non-pregnant and pregnant rat. In order to estimate total plasma, skin and skeletal muscle radioactivity the following assumptions were made for both the pregnant and non-pregnant rats: skeletal muscle is 40%, skin is 10% and plasma is 4% of the total body weight.

Analysis of TCB and metabolites

The amount of TCB and its major metabolites were determined in the plasma and liver from the pregnant and non-pregnant rats, as well as in the fetuses and placentas. One volume of methanol was added to plasma which was then extracted three times with two volumes of diisopropylether. The ether was evaporated under nitrogen and the residue was methylated with diazomethane. The plasma extract was then resuspended in hexane and partitioned with concentrated sulfuric acid. The hexane phase was washed with water, passed over sodium sulfate and evaporated to dryness with nitrogen.

Fetal or maternal tissues from 3 animals were pooled for each sacrifice point and freeze-dried. TCB and its metabolites were extracted from the adult and fetal rat tissues as described by Klasson Wehler *et al.* (Klasson Wehler *et al.*, 1990). Briefly, freeze dried tissues were ground with a mortar and pestle and then extracted by Sohxlet extraction for 5 h with chloroform-ethanol (1:1). The extraction efficiency was determined from the [^{14}C] contents of the extracts and the dried tissue residue. The compounds of interest were separated from lipids by gel permeation chromatography (GPC) with Bio-Beads SX-3 (BioRad) using cyclohexane-dichloromethane (1:1, v/v) as a mobile phase. The level of [^{14}C]-TCB derived radioactivity was monitored in the GPC fractions with LSC and the lipid-free fractions containing [^{14}C] were pooled and methylated with diazomethane and then analyzed with GC/ECD and GC/MS.

Gas chromatography/mass spectrometry (GC/MS) was performed on an ITS40 instrument (Finnigan). The GC (Varian 3400) was equiped with a fused silica capillary column (DB5, 30 m x 0.25 mm i.d., J&W, Scientific Inc., CA, USA). The temperature program was 80°C for 2 min, then 10°C/min to 240°C and maintained at 240°C for 20 min. The injector temperature was 260°C and the injections were made in the splitless mode, using an autosampler (CTC A200S, Finnigan MAT). The mass spectrometer was operated in the electron impact mode and the manifold temperature was 220°C.

Gas chromatography/electron capture detection (GC/ECD) analyses were performed with a Varian 3770 gas chromatograph equipped with an on column injector (Okla and Wesén, 1984), a DB5$^+$ fused silica capillary column (30 m x 0.32 mm i.d., J&W Scientific Inc.) and a ^{63}Ni electron capture detector opertated at 300°C. The column temperature was programmed as follows: initial temperature 60°C, then 20°C/min to 160°C, then 160°C for 5 min, followed by 5°C/min to 280°C and maintained at 280°C for 10 min. The chromatograms were recorded and stored by a Shimadzu R-C3A integrator.

Thyroid Hormone Analysis

Plasma total T_4 (TT_4), total T_3 (TT_3) and free T_4 (FT_4) were determined with the Amerlite chemiluminesence assay (Amersham, UK) according to the protocol of the supplier with the following modification: the TT_4 assay buffer was diluted five times with demineralized water; the standard curve for TT_4 ranged from 0 to 120 nmol TT_4/liter.

Ethoxyresorufin-O-deethylase (EROD) activity

EROD activity was measured in maternal and fetal hepatic microsomes according to the method of Burke *et al.* (1977), with slight modifications. The reaction was carried out at 37° C with a final concentration of ethoxyresorufin and NADPH of 2 and 0.1

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μM, respectively. The final microsomal protein concentration was 100 μg/ml for pregnant and non-pregnant rats, and 1 mg/ml for fetal rats. To detect the deethylation product, resorufin (RR), the excitation wavelength of the fluorimeter was set at 530 nm, while the emission wavelength was 580 nm. Protein concentrations were determined using the Bio-Rad coomassie blue assay (Bio-Rad, Richmond, CA).

Results

Fecal and urinary [14C]-TCB excretion

The average total recovery of radioactivity per rat (feces, urine, cage washings, organs and carcass) was 90.3 ± 5.5 %. Cumulative fecal excretion of [14C]-TCB derived radioactivity over 1 week, shown in Figure 2.1A, was significantly lower in pregnant rats than in non-pregnant animals. The difference in cumulative fecal excretion was due primarily to the excretion during the first 24 h after TCB administration: non-pregnant rats excreted 43 ± 3% of the dose, while pregnant rats excreted 30 ± 2% of the dose. Seven days after [14C]-TCB administration, the fecal elimination of [14C]-TCB in non-pregnant and pregnant rats was 85 ± 1.3% and 78 ± 1.5% of the total dose, respectively.

Cumulative urinary excretion of radioactivity was also lower in pregnant rats than in non-pregnant rats, however the differences were not significant for all time points (Figure 2.1B). The elimination of [14C]-TCB derived radioactivity in the urine was only a small fraction (<5%) of the total radioactivity eliminated from the rats.
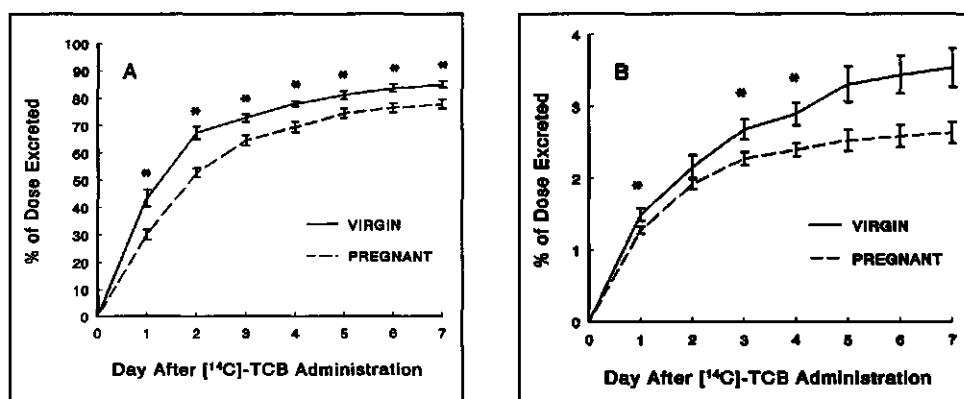
Figure 2.1 A and B.
Cumulative fecal (A) and urinary (B) excretion of [14C]-TCB derived radioactivity from pregnant and non-pregnant rats after an oral dose of 25 μmol [14C]-TCB/kg, * indicates a significant difference between values from pregnant and non-pregnant rats, $P \leq 0.05$, student's t-test, see Materials and Methods for number of animals per time point.

[^{14}C]-TCB tissue levels:

The significantly higher retention of radioactivity in the residual carcass of pregnant rats than non-pregnant rats 1 and 2 days after [^{14}C]-TCB administration (Table 2.1) is in accordance with the slower fecal excretion of radioactivity by pregnant rats compared to with non-pregnant rats. Levels of radioactivity (dpm/g) from tissues with the highest concentration of radioactivity (abdominal fat and skin, including subcutaneous fat) were not significantly different between pregnant and non-pregnant rats at any time point (Table 2.2). However, when corrected for total tissue wt the amount of [^{14}C]-TCB derived radioactivity (% dose per tissue or organ) was significantly higher in the abdominal fat of the pregnant rats than non-pregnant rats either 1 and 2 days after [^{14}C]-TCB-administration (Table 2.1).

Table 2.1
Tissue distribution of [^{14}C]-TCB derived radioactivity in pregnant and non-pregnant rats (expressed as percentage of dose)

	Day after [^{14}C]-TCB administration			
	1	2	4	7
Plasma				
Non-pregnant	1.06±0.03	0.63±0.07	0.43±0.09	0.37±0.12
Pregnant	0.87±0.23	0.50±0.03	0.34±0.03	0.15±0.03
Liver				
Non-pregnant	1.47±0.02	0.69±0.23	0.48±0.02	0.31±0.03
Pregnant	1.33±0.05	0.74±0.09	0.43±0.03	0.22±0.04*
Kidney				
Non-pregnant	0.10±0.03	0.06±0.02	0.04±0.00	0.04±0.01
Pregnant	0.10±0.01	0.06±0.01	0.03±0.00	0.02±0.00*
Abdominal Fat				
Non-pregnant	2.47±0.56	1.47±0.21	2.00±0.18	0.91±0.08
Pregnant	3.51±0.27*	3.15±0.39*	1.93±0.44	1.34±0.51
Skin				
Non-pregnant	5.55±2.22	2.74±1.64	1.35±0.20	0.85±0.15
Pregnant	7.64±3.42	4.68±2.24	1.49±0.13	0.74±0.34
Skeletal Muscle				
Non-pregnant	1.88±0.60	0.83±0.15	0.70±0.21	0.65±0.55
Pregnant	1.15±0.73	0.55±0.20	0.30±0.15*	0.17±0.08*
Carcass				
Non-pregnant	40.9±5.0	13.3±2.8	8.4±0.8	4.1±0.7
Pregnant	50.1±2.0*	27.7±2.6*	11.4±1.0	5.1±0.5

Pregnant and non-pregnant rats received an oral administration of 25 µmol [^{14}C]-TCB/kg. Data are the mean±S.E.M., * indicates a significant difference between values from pregnant and non-pregnant rats, n=3, P≤0.05, 2 sided student's t-test.

Table 2.2
Tissue distribution of [^{14}C]-TCB derived radioactivity in pregnant and non-pregnant rats (expressed as dpm x 10^3 per g tissue)

|	Day after [^{14}C]-TCB administration			
Tissue/Organ	1	2	4	7
Plasma				
Non-pregnant	18.3±0.2	11.0±0.9	6.2±1.6	6.5±1.3
Pregnant	11.8±1.9*	7.1±0.2*	4.7±0.2	2.1±0.2*
Liver				
Non-pregnant	21.0±0.9	10.2±2.1	6.8±0.5	4.7±0.2
Pregnant	15.5±0.4*	9.5±0.5	4.8±0.2*	2.6±0.2*
Kidney				
Non-pregnant	9.5±1.8	5.5±1.0	3.9±0.1	2.9±0.4
Pregnant	8.0±0.3	5.1±0.4	2.6±0.1*	1.5±0.1*
Spleen				
Non-pregnant	4.0±1.5	2.2±0.6	1.2±0.1	1.1±0.0
Pregnant	1.8±0.1	1.2±0.1	0.7±0.0*	0.5±0.0*
Lung				
Non-pregnant	6.9±1.1	3.1±0.6	2.1±0.2	1.8±0.3
Pregnant	3.9±0.1	2.7±0.0	1.6±0.1	0.8±0.1*
Heart				
Non-pregnant	3.5±0.3	2.4±0.4	1.2±0.0	1.2±0.1
Pregnant	4.0±0.4	1.8±0.1	0.9±0.0*	0.5±0.0*
Brain				
Non-pregnant	1.1±0.1	0.4±0.1	0.4±0.1	0.3±0.0
Pregnant	0.6±0.0*	0.4±0.0	0.2±0.0	0.1±0.0*
Abdominal Fat				
Non-pregnant	109±13.9	73.1±11.5	68.2±3.4	2.7±3.5
Pregnant	105±5.3	82.4±13.0	58.3±1.8*	35.4±2.3
Skin				
Non-pregnant	38.6±9.2	25.3±5.4	9.1±.7	6.0±0.6
Pregnant	41.5±10.1	26.6±7.1	8.3±0.5	4.1±0.8
Skeletal Muscle				
Non-pregnant	3.3±0.7	1.5±0.2	1.2±0.2	0.6±0.0
Pregnant	1.9±0.7	0.8±0.2*	0.4±0.1*	0.2±0.0*

Pregnant and non-pregnant rats received an oral administration of 25 µmol [^{14}C]-TCB/kg with a specific activity of 1 µCi/µmol TCB. The data are the mean ± SEM, * indicates a significantly lower concentration of radioactivity in tissue from pregnant compared with non-pregnant rats, n=3, P≤0.05, student's t-test.

Despite an initial lower fecal elimination of radioactivity, some tissue levels of radioactivity were generally lower in the pregnant than non- pregnant animals. In pregnant rats significantly lower concentrations of [^{14}C]-TCB derived radioactivity (dpm per g tissue) were found in the plasma, skeletal muscle, liver and kidneys than in non-pregnant rats 7 days after [^{14}C]-TCB administration (Table 2.2). Tissue concentrations of radioactivity were also significantly lower in the spleen, brain, heart and thymus of pregnant rats than in non-pregnant rats 7 days after [^{14}C]-TCB administration (Table 2.2).

The only compartments showing accumulation of radioactivity from 1 to 7 days after TCB administration, as expressed as % of dose were the placenta and fetus (Figure 2.2A). The percentage of the dose in the fetus increased more than 100 fold and in the placenta 5 fold by the end of the experiment. Also the concentration of [^{14}C]-TCB derived radioactivity in the fetus increased from 880 $\pm$ 290 to 2390 $\pm$ 540 dpm/g fresh wt between 1 and 7 days after [^{14}C]-TCB administration (Figure 2.2b). No increase was observed in the concentration of placental [^{14}C]-TCB derived radioactivity during the experiment (Figure 2.2b). Seven days after [^{14}C]-TCB administration, the percent of the [^{14}C] dose present in the pooled fetuses (0.410% $\pm$ 0.153) had increased to above the amount retained in the maternal liver (0.218% $\pm$ 0.036), even though the liver is the maternal organ (aside from adipose tissue) exhibiting the highest concentration (dpm/g) and total amount (% of dose) of radioactivity.

Identification of [^{14}C]-TCB metabolites:

The relative amount of chloroform/ethanol unextractable compounds in livers increased with time after [^{14}C]-TCB administration (Table 2.3). In pregnant rats unextractable [^{14}C]-TCB derived radioactivity in the liver increased from 13 % of the
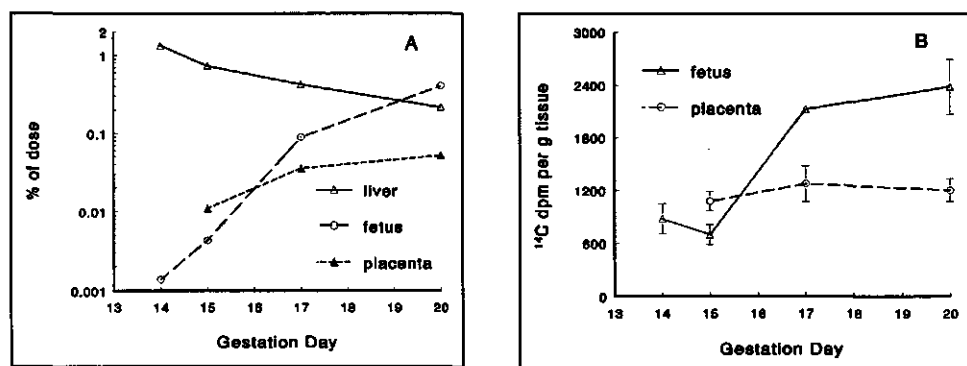
Figure 2.2 A and B.
A: Percentage of dose of [^{14}C]-TCB derived radioactivity in pooled fetuses, placentas and maternal liver from pregnant rats administered an oral dose of 25 μmol [^{14}C]-TCB on day 13 of gestation, B: Concentration of [^{14}C]-TCB derived radioactivity (dpm/g) fresh wt in fetuses and placentas from pregnant rats, n=3.

Figure 2.3. Levels of 4-OH-[^{14}C]-TCB *in the plasma of non-pregnant* and pregnant rats as well as fetuses (day 20 of gestation only) after oral administration of 25 μmol [^{14}C]-TCB. Pregnant rats were dosed on day 13 of gestation, * indicates a significant difference between values from pregnant and non-pregnant rats, P≤0.05, student's t-test.
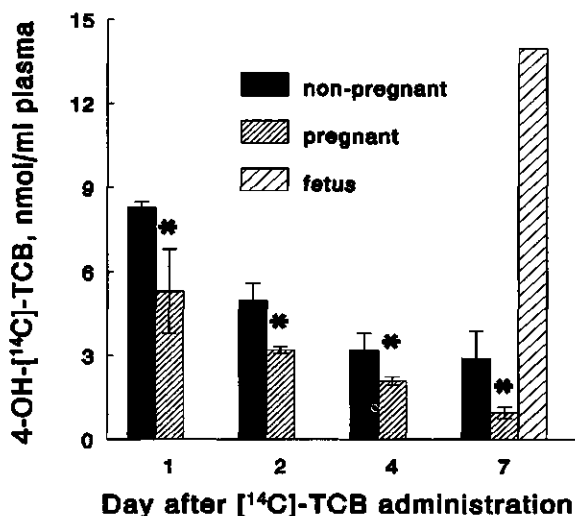
Table 2.3
Percentage of [^{14}C]-TCB derived radioactivity extracted from tissue

Day after treatment	Fetus	Placenta	Liver Pregnant	Liver Non-pregnant
1			87	76
2	60	51	68	65
4	87	55	72	55
7	70	54	53	59

The data are the results of chloroform/ethanol extractable [^{14}C]-TCB derived radioactivity in pooled tissue samples.

total radioactivity in the sample 24 h after exposure to 47 % at 7 days after exposure. In non-pregnant rats the increase in unextractable compounds in the liver was from 24% to 41% of total radioactivity between day 1 and 7 after exposure, respectively.

GC-MS analysis of the extracts of pooled fetal samples revealed that the major compound (> 95%) accumulating in the fetuses and placenta was 3,3',4',5-tetrachloro-4-biphenylol (4-OH-tetraCB) at 4 and 7 days after [^{14}C]-TCB administration (Table 2.4). Traces of the parent compound and 3,3',4,4'-tetrachloro-5-biphenylol (5-OH-TCB) were present in the placenta according to GC/ECD analysis although the presence of 5-OH-

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Table 2.4
Levels of [^{14}C]-TCB and metabolites (nmol/g lipid) in livers from non-pregnant and pregnant rats and their fetuses and placentas.

		Day after TCB administration			
		1	2	4	7
Compound	Tissue	Concentration (nmol/g lipid)			
TCB	liver, NP	43	32	9.9	7.5
	liver, P	89	31	12	6.7
	placenta			ND	1.5
	fetus			ND	2.5
4-OH-tetraCB	liver, NP	17	10	3.8	7.5
	liver, P	18	9.1	6.5	4.7
	placenta			23	16
	fetus			58	65
5-OH-TCB	liver, NP	3.4	1.1	0.2	0.4
	liver, P	3.4	1.2	0.2	ND
	placenta			ND	ND
	fetus			ND	ND
Ratio 4-OH-tetraCB/TCB	liver, NP	0.32	0.31	0.38	1.0
	liver, P	0.20	0.29	0.54	0.7
	placenta			3.1	
	fetus				26.0

The data are from analysis of pooled samples (n=3) for each time point. The calculations are based on the relative amount of the compounds determined by GC/ECD analysis in the extractable radioactivity from the tissue sample. NP:non-pregnant rats, P:pregnant rats, ND: not detected, 4-OH-tetraCB:3,3',4',5-tetrachloro-4-biphenylol, 5-OH-TCB: 3,3',4,4'-tetrachloro-5-biphenylol.

TCB could not be confirmed by GC/MS analysis due to a low concentration. In the plasma of non-pregnant and pregnant rats only 4-OH-tetraCB was detected by GC/ECD at all time points. GC/ECD analysis of fetal plasma from day 20 of gestation revealed that only 4-OH-tetraCB was present, and that the amount of 4-OH-tetraCB in fetal plasma was more than 14 times greater than that in maternal plasma (14 nmol/ml versus 0.95 nmol/ml, Figure 2.3). The level of 4-OH-tetraCB was significantly lower in the plasma of pregnant rats compared to non-pregnant rats at all time points (Figure 2.3).

Livers from both non-pregnant and pregnant rats contained an increasing amount of 4-OH-tetraCB relative to TCB after treatment when results are expressed as nmol 4-

OH-tetraCB/g extracted lipid (Table 2.4). Seven days after [^{14}C]-TCB administration a large fraction of the extractable compounds present in the liver were in the form of 4-OH-tetraCB in both pregnant and non-pregnant rats (50 and 41%, respectively). Only minor amounts of 5-OH-TCB and 3,3',4,4'-tetrachloro-2-biphenylol (2-OH-TCB) were detected in liver samples (data not shown). The amount of 4-OH-tetraCB nmol/g lipid is greater in the placenta (3 fold) and fetus (13 fold) than in the maternal liver on day 20 of gestation. Also the amount of 4-OH-tetraCB relative to TCB on day 20 of gestation increases greatly from the materal liver (0.7) through the placenta (3.1) to the fetus (26.0).

Hepatic ethoxyresorufin-O-deethylase (EROD) activity:
   Maternal hepatic EROD activity was significantly elevated relative to cornoil-treated controls on day 17 and 20 of gestation following TCB exposure (12 fold and 10 fold, respectively, Table 2.5). The level of EROD activity was significantly lower (48%) in TCB-treated pregnant rats on day 20 of gestation than on day 17 of gestation. EROD activity was undetectable in microsomes from control and TCB-treated fetuses on both day 17 and day 20 of gestation.

Table 2.5
Hepatic EROD activity in pregnant rats

Gestation Day	Treatment	EROD (nmol RR/min*mg protein)	
		Maternal	Fetal
17	Control	0.027±0.005a	ND
	TCB	0.330±0.042b	ND
20	Control	0.017±0.004a	ND
	TCB	0.170±0.043c	ND

RR: resorufin, values with different superscripts are significantly different from each other, Tukey's honest difference test, P≤ 0.05. Three to 5 animals per group recieved 25 μmol 3,3',4,4'-tetrachlorobiphenyl per kg or cornoil alone on day 13 of gestation. ND: not detected.

Plasma thyroid hormone levels:
   Plasma TT_4 levels were significantly depressed 4 days after TCB administration in pregnant rats and 7 days after TCB administration in non-pregnant rats (Table 2.6). Control plasma TT_4 levels decreased during gestation from 44.2 ± 6.9 nmol/liter to 22.1 ± 2.6 nmol/liter, while levels of TT_4 in non-pregnant rat plasma remained constant after

vehicle administration. Plasma TT_3 concentrations were significantly decreased in non-pregnant rats relative to controls 1 and 2 days after TCB administration and were significantly increased in pregnant rats at the same time points. Plasma FT_4 levels were significantly decreased relative to controls in pregnant rats treated with TCB from 1 to 4 days after TCB exposure. Fetal FT_4 and TT_4 levels were significantly reduced relative to controls on day 20 of gestation by maternal TCB administration. Fetal plasma TT_3 was undetectable in the assay as performed.

Table 2.6
Effect of [^{14}C]-TCB administration on thyroid hormone levels in non-pregnant, pregnant rats and fetuses

Animal	Treatment	Day after [^{14}C]-TCB administration			
		1	2	4	7
		TT_4, nM			
NP	Control	49.8±6.5	50.3±6.5	51.6±3.6	42.5±5.0
NP	TCB	14.5±1.6*	18.7±3.9*	24.5±4.7*	30.8±2.8*
P	Control	44.2±6.9	31.9±5.0	28.4±1.1	25.0±2.4
P	TCB	18.7±2.7*	15.2±2.5*	19.5±2.8*	25.4±7.8
F	Control	NA	NA	NA	5.9±0.6
F	TCB	NA	NA	NA	3.8±0.9*
		TT_3, nM			
NP	Control	1.40±0.07	1.34±0.28	1.08±0.14	1.36±0.02
NP	TCB	0.67±0.12*	0.66±0.11	1.29±0.08	1.39±0.20
P	Control	0.75±0.08	0.54±0.02	0.83±0.01	0.79±0.16
P	TCB	1.13±0.27*	0.98±0.14*	0.63±0.10	0.91±0.24
		FT_4, pM			
P	Control	25.2±1.7	16.4±7.3	16.5±1.6	14.3±0.6
P	TCB	15.3±2.0*	9.8±1.0*	9.2±0.8*	14.9±4.8
F	Control	NA	NA	NA	4.5±0.1
F	TCB	NA	NA	NA	2.0±0.5*

Pregnant and non-pregnant rats received an oral administration of 25 μmol [^{14}C]-TCB/kg. Pregnant rats were dosed on day 13 of gestation. Data are the means±S.E.M., NP: non-pregnant, P: pregnant, F: fetus, TT_4: total thyroxine, TT_3, total triiodothyronine, FT_4: free thyroxine, * significantly different from controls, n=3, P≤0.05, 2 sided student's t-test, NA: data not available.

Discussion

[¹⁴C]-TCB was rapidly eliminated from both non-pregnant and pregnant rats mainly via the feces, although fecal and urinary excretion was lower in pregnant rats than in non-pregnant rats. Previous studies have also shown that TCB is rapidly metabolized and excreted via the feces in mice and rats (Yoshimura *et al.*, 1987, Klasson Wehler *et al.*, 1990). The significantly lower fecal and urinary elimination of [¹⁴C]-TCB-derived radioactivity in pregnant rats compared to non-pregnant rats may be due to the increase in body weight and adipose tissue and hence distribution volume during pregnancy. Another explanation is that hepatic metabolism of TCB during pregnancy may be decreased, although we did not address this issue in our experiments. Sinjari *et al.* reported that basal EROD activity is significantly lower in control pregnant C57BL mice on day 17 of gestation compared to non-pregnant mice, however the induction of EROD activity by TCB was not affected by gestation.

The results demonstrate that there is extensive metabolite formation in both pregnant and non-pregnant rats following TCB exposure. A large portion of the [¹⁴C] radioactivity in the livers was not extractable, and 7 days after administration of [¹⁴C]-TCB, 50% of the extractable radioactivity was in the form of metabolites. The percentage of non-extractable [¹⁴C] radioactivity in the livers of pregnant rats increased from 13% to 47% of the total radioactivity from 1 to 7 days after [¹⁴C]-TCB administration, which can be consistent with the observed covalent binding of TCB-metabolites to hepatic proteins (Shimada and Sawabe, 1984).

The major metabolite found in the plasma and livers of non-pregnant and pregnant rats, placentas and fetuses is 3,3',4',5-tetrachloro-4-biphenylol (4-OH-tetraCB). Low amounts of 5-OH-TCB were found in the livers of non-pregnant and pregnant rats, and the ratio of 4-OH-tetraCB/5-OH-TCB increased with time, indicating that 4-OH-tetraCB is either produced in a greater amount or is selectively retained. There is some evidence that supports both of these possibilities. The amount of 4-OH-tetraCB produced in incubations of TCB with rat hepatic microsomes is more than two times greater than that of 5-OH-TCB (Ishida *et al.*, 1991), while similar amounts of both metabolites are excreted in rat feces after TCB administration (Yoshimura *et al.*, 1987). Furthermore, when either metabolite is administered to rats, 5-OH-TCB is excreted much more rapidly than 4-OH-tetraCB (Yoshimura *et al.*, 1987). It should be noted that the livers analysed in the current experiments were not perfused, so that part of the 4-OH-tetraCB present in the liver extracts is due to the presence of blood in the livers prior to homogenization.

Although 4-OH-tetraCB is found to bind to transthyretin, the plasma thyroid hormone transport protein, this cannot in itself explain its increased retention relative to 5-OH-TCB, because both compounds exhibit similar binding affinities for transthyretin (Lans *et al.*, 1993). A possible explanation for the difference in excretion is that 5-OH-TCB is readily conjugated with glucuronic acid in the liver, while the chlorines flanking the hydroxy group in 4-OH-tetraCB form a steric hinderence for conjugation.

Surprisingly high levels of [¹⁴C] radioactivity accumulated in the fetus with time, and the extractable radioactivity was present mainly as 4-OH-tetraCB, with negligible levels of TCB itself. This is in contrast to a previous study, in which was reported that the major phenolic metabolite accumulating in mouse fetuses was 3,3',4,4'-tetrachloro-2-

biphenylol (Darnerud *et al.*, 1986). This metabolite was not detected in any of the tissues examined in our study. However, a re-evaluation of the mouse study shows results more similar to our study, in that 4-OH-tetraCB is the major fetal metabolite and the *ortho*-hydroxylated metabolite is present in only small amounts (Dr. P.O. Darnerud, personal communication).

It is unlikely that the hydroxylated metabolites are formed in the fetal compartment, because EROD activity was undetected in fetal microsomes after maternal TCB exposure. *In vitro* metabolism studies with TCB have shown that EROD activity is closely related to TCB metabolism (Mills *et al.*, 1985), and that the enzyme CYP1A, which catalyzes the deethylation of ethoxyresorufin, is able to hydroxylate TCB (Ishida *et al.*, 1991).

Since hepatic EROD activity was induced more than 10-fold in TCB-treated pregnant rats and undetectable in fetuses, it is most likely that maternal production of TCB metabolites is the main source of hydroxylated metabolites found in the fetus. The 13 fold greater concentration of 4-OH-tetraCB in the whole fetus as compared to the maternal liver on a lipid weight basis and the 14 fold greater concentration in the fetal plasma on day 20 of gestation suggests a specific mechanism of accumulation.

In a previous study, it was suggested that hydroxylation of PCBs in the maternal compartment and glucuronidation in the fetal liver was necessary for the accumulation of PCB metabolites in the fetal rat intestine (Lucier *et al.*, 1978). Various PCBs hydroxylated in either the *para* or *meta* position exhibit a high affinity for TTR (Lans *et al.*, 1993). 4-OH-tetraCB has a higher affinity for human TTR *in vitro* than T_4 itself, and was found to be bound to TTR in the plasma of rats treated with TCB (Brouwer *et al.*, 1990, Brouwer *et al.*, 1986). It is therefore plausible that the accumulation of hydroxylated PCBs in the rat fetus is dependent in part on TTR for transplacental transport. The study from Lucier *et al.* (1978) also revealed a high ratio of fetal to maternal blood radioactivity after prenatal TCB administration.

Plasma TT_4 levels were decreased in TCB treated non-pregnant and pregnant rats, as well as their fetuses, when compared with cornoil treated controls. The decreases in maternal plasma T_4 concentrations may be due to the binding of 4-OH-tetraCB to TTR, thereby displacing T_4. The selective retention of 4-OH-tetraCB in maternal plasma further supports the role of this metabolite in the reduction of maternal plasma T_4 levels.

Also other mechanisms are probably involved, for it has been shown that the coplanar PCBs 3,3'4,4',5,5'-hexachlorobiphenyl and 3,3',4,4'-TCB can induce hepatic T_4-glucuronyltransferase activity in pregnant rats (Morse *et al.*, 1993), which may also contribute to decreases in plasma T_4 levels by increased excretion of T_4 (Bastomsky, 1977, Barter and Klaassen, 1991). Furthermore, there is ultrastructural and biochemical evidence that PCBs inhibit the release of thyroid hormones from the thyroid gland itself in adult rats (Collins and Capen, 1980b, Collins and Capen, 1980c).

The response of plasma TT_3 levels to TCB treatment was markedly different in pregnant and non-pregnant rats. While plasma TT_3 concentrations increased in pregnant rats following TCB exposure, they decreased in non-pregnant rats relative to controls. The mechanism for these differences is unclear.

TCB-induced reductions were observed in fetal plasma TT_4 and FT_4 concentrations when the plasma TT_4 and FT_4 concentrations in the TCB-treated dams had returned to control levels, which suggests that reduced transplacental transport of

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maternal T_4 was not involved in the reduction of fetal plasma thyroid hormones. Furthermore, the thyroid gland in the fetal rat begins to secrete thyroid hormones between day 17 and 18 of gestation, and at 21 days of gestation only 18% of the extrathyroidal T_4 pool of the fetuses is of maternal origin (Morreale de Escobar *et al.*, 1990). Previous results provide evidence that in the rat the increased hepatic glucuronidation of T_4 is related to decreases in maternal or neonatal circulating T_4 concentrations, but not to fetal T_4 concentrations following maternal PCB exposure because the levels of fetal hepatic microsomal T_4 glucuronidation are 5-10 fold lower than maternal or neonatal levels (Morse *et al.*, 1993). Therefore the decreases in fetal plasma TT_4 and FT_4 concentrations following maternal TCB exposure are probably a consequence of the accumulation of 4-OH-tetraCB in the fetal compartment, in particular the fetal plasma.

While the hydroxylated metabolites of TCB, 4-OH-tetraCB and 5-OH-TCB, have been shown to be relatively non-toxic compared to the parent compound in adult rats (no alteration of organ weights) following a bolus injection (Yoshimura *et al.*, 1987), and in chicken embryos (no increased mortality) (Klasson Wehler *et al.*, 1990), they are not biologically inactive compounds. The capacity of 4-OH-tetraCB to reduce plasma thyroid hormone levels by binding specifically to TTR and the high accumulation in the fetal compartment, especially the blood, poses a threat to the maintainance of sufficient brain triiodothyronine concentrations derived from T_4. As thyroid hormones are essential for normal brain development, the accumulation of 4-OH-tetraCB in fetal plasma could adversely affect neurological parameters.

CHAPTER 3

ß-NAPHTHOFLAVONE- AND SELF-INDUCED METABOLISM OF 3,3',4,4'-TETRACHLOROBIPHENYL IN HEPATIC MICROSOMES OF THE MALE, PREGNANT FEMALE AND FETAL RAT

Abstract

The *in vitro* metabolism of 3,3',4,4'-tetrachloro-[^{14}C]-biphenyl ([^{14}C]-TCB) by hepatic microsomes from Wistar rats was investigated with liver microsomes from the male, pregnant female and fetal Wistar rat. Three hydroxylated metabolites (4-OH-3,3',4,5'-tetrachlorobiphenyl, 5-OH-3,3',4,4'-tetrachlorobiphenyl and 6-OH-3,3',4,4'-tetrachlorobiphenyl) were identified by high performance liquid chromatography and gas chromatography-mass spectrometry after incubations of liver microsomes from ß-naphthoflavone pretreated male rats and TCB-treated pregnant rats. No metabolites of [^{14}C]-TCB were found after incubation with fetal liver microsomes from dams pretreated with TCB. The results indicate that the *in vivo* accumulation of 4-OH-tetraCB in the fetal compartment is probably due to transplacental transport rather than the formation of this metabolite in the fetus.

Pretreatment of male rats with ß-naphthoflavone substantially induced the formation of hydroxylated metabolites, but pretreatment with phenobarbital and dexamethasone was without effect. Based on *in vitro* incubations of liver microsomes from ß-naphthoflavone pretreated male rats, an apparent Km and Vmax of 4.5 μM and 240 pmol/mg protein/min), respectively, was determined for the metabolism of [^{14}C]-TCB. The formation of phenolic metabolites of [^{14}C]-TCB was most likely dependent on cytochrome P4501A induction.

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Introduction

The wide range of biological activity exhibited by polychlorinated biphenyls may be caused by a variety of mechanisms involving the interaction of the parent compound or a metabolite thereof with a specific receptor protein (Safe, 1994). The most toxic PCB congeners, 3,3',4,4'-tetrachlorobiphenyl (TCB), 3,3',4,4',5-pentachlorobiphenyl and 3,3',4,4',5,5'-hexachlorobiphenyl have a coplanar structure and have a high affinity for the Ah (arylhydrocarbon) receptor (Bandiera *et al.* 1982), which mediates most of the toxic effects of these coplanar PCBs (Safe, 1994). The binding of coplanar PCBs to the Ah receptor can result in the induction of cytochrome P4501A enzymes and the associated ethoxyresorufin-O-deethylase (EROD) activity in microsomes of animal tissues (Safe, 1994).

Several reports indicate that the cytochrome P4501A1 isoenzyme may catalyze the hydroxylation of PCBs, including TCB (Mills *et al.* 1985, Ishida *et al.* 1991). TCB is readily metabolized to phenolic metabolites *in vivo* as well as *in vitro* (Ishida *et al.* 1991, Yoshimura *et al.* 1987, Klasson Wehler *et al.* 1989). In contrast, 3,3',4,4',5-pentachlorobiphenyl and 3,3',4,4',5,5'-hexachlorobiphenyl are hardly metabolized in animals (Koga et al 1990, Mills *et al.* 1985). Other non-planar, *ortho*-substituted PCBs may also be metabolized, resulting in the formation of hydroxylated (Bergman *et al.* 1994) or methylsulfonated compounds (Mitzutani *et al.* 1978, Bergman *et al.* 1992). These metabolites have a wide range of biological activity, including interactions of hydroxylated PCBs with thyroid hormone binding proteins (Lans *et al.* 1993) and the eostrogenic activity of hydroxylated PCBs (Jansen *et al.* 1993, Korach *et al.* 1988) or the binding of methylsulfonyl PCBs to $\alpha_{2\mu}$-globulin (Larsen *et al.* 1990). Moreover, the induction of several cytochrome P450 isoenzymes by methylsulphone derivatives of PCBs has been demonstrated (Kato *et al.* 1993).

Both mice and rats rapidly metabolize TCB, resulting in the formation of three major metabolites, namely the 1,2-shift metabolite, 4-OH-3,3',4',5-tetrachlorobiphenyl (4-OH-tetraCB), 5-OH-3,3',4,4'-tetrachlorobiphenyl (5-OH-TCB) and 6-OH-3,3',4,4'-tetrachlorobiphenyl (6-OH-TCB) (Yoshimura *et al.* 1987, Klasson Wehler *et al.* 1989, Morse *et al.* 1995). Interestingly only 4-OH-tetraCB accumulates in both mouse and rat plasma (Morse *et al.* 1995a, Klasson Wehler, 1989, Brouwer *et al.* 1990), whereas 5-OH-TCB is excreted in greater amounts than 4-OH-tetraCB in the feces (Yoshimura *et al.* 1987). A re-evaluation of a previously published study which demonstrated the accumulation of phenolic metabolites of TCB in fetal mice (Darnerud *et al.* 1986) revealed that the major hydroxylated metabolite of TCB in the fetus was 4-OH-tetraCB (Eva Klasson Wehler, personal communication). Recent research from our laboratory has confirmed that 4-OH-tetraCB is the major compound accumulating in late gestational rat fetuses following maternal TCB exposure (Morse *et al.* 1995a). The selective retention of 4-OH-tetraCB in rat plasma is explained by competitive binding to transthyretin (TTR), the major plasma thyroid hormone binding protein in the rat and mouse (Brouwer and Van den Berg, 1986, Savu *et al.* 1989, Vrancks *et al.* 1990), as a result of the structural resemblance of 4-OH-tetraCB to thyroxine (T_4) (Lans *et al.* 1993) . The accumulation of 4-OH-tetraCB in the fetal plasma results in a decrease of the binding capacity of TTR for T_4 (unpublished observations, P.O. Darnerud and D.C. Morse) and consequently in decreases in plasma T_4 levels (Morse *et al.* 1995a).

However, it was not clear from the *in vivo* studies above if 4-OH-tetraCB in the fetus is maternally derived or formed in the fetus itself.

The aim of the present study was to enable the use of [^{14}C]-TCB as a model compound to investigate the metabolic capacity of adult, fetal and maternal tissues. Moreover, it was considered to be of interest to determine which pretreatment with inducers of hepatic mono-oxygenases resulted in the greatest induction of [^{14}C]-TCB metabolism. ß-Naphthoflavone and phenobarbital (inducers of PCB metabolism, Mills et al. 1985) and dexamethasone (inducer of chlorinated benzene metabolism, (den Besten *et al.* 1991) as well as TCB itself were used as pretreatments *in vivo*. The reaction conditions for the incubation of [^{14}C]-TCB with hepatic microsomes from male rats were validated using high performance liquid chromatography (HPLC). Gas chromatography-mass spectrometry was used to confirm the identity of the extractable radioactive products. The HPLC technique was then used to determine the source of phenolic metabolites found in the fetus *in vivo* by examining [^{14}C]-TCB metabolism with hepatic microsomes obtained from TCB-exposed pregnant rats and their fetuses .

Material and Methods

Materials

3,3',4,4'-tetrachloro-[^{14}C]-biphenyl (37.1 µCi/µmol, radiochemical purity 95%) and bovine serum albumin (96-99% pure) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Unlabelled 3,3',4,4'-tetrachlorobiphenyl (>99% pure) was obtained from Promochem, Wesel, Germany. 2-OH-3,3',4,4'-tetrachlorobiphenyl, 4-OH-3,3',4',5-tetrachlorobiphenyl, 5-OH-3,3',4,4'-tetrachlorobiphenyl and 6-OH-3,3',4,4'-tetrachloro-biphenyl (chemical purity >99%) were synthesised according to Klasson Wehler *et al.* (1990). The following chemicals were purchased from Merck, Darmstadt, Germany: Tris, NaOH, Folin-Ciocalteu phenol reagent, $MgCl_2$, acetone, diisopropyl ether, methanol, ethanol, ethyl acetate, and diethyl ether (all solvents were analytical grade). Methanol (HPLC quality) was purchased from Janssen Chimica, Tilburg, The Nether-lands. Glucose-6-phosphate, glucose-6-phosphate-dehydrogenase, NADP$^+$ and NADPH were obtained from Boehringer Mannheim, Almere, The Netherlands.

Animals

Hepatic microsomes were prepared from Wistar rats from two different experi-mental situations.

Experiment 1. Male Wistar WU rats (300 g) were treated with dexamethasone (DEX, 4 consecutive daily oral administrations of 300 mg/kg body weight dissolved in 2% Tween 80), ß-naphthoflavone (ß-NF, 3 daily ip injections of 30 mg/kg dissolved in cornoil) or phenobarbital (PB, 0.1% w/v, in the drinking water for 7 days). Control rats were of a similar weight but were untreated. One day after the last treatment, the rats were sacrificed under ether anesthesia and the livers removed. The livers of three rats per treatment group were pooled. Microsomes were prepared as previously described and stored at -80°C (Morse *et* al. 1993). Two different batches of ß-NF microsomes were used for the experiments, and are designated as ß-NF(1) and ß-NF(2). The specific enzyme activity (ethoxyresorufin-O-deethylase) of both batches are presented in the

results.

Experiment 2. Pregnant female Wistar WU rats (16 weeks old) were treated with 25 μmol 3,3',4,4'-tetrachlorobiphenyl per kg body wt dissolved in corn oil (2 ml/kg body wt) on day 13 of gestation. On day 20 of gestation the pregnant rats were sacrificed under ether anesthesia and both the fetal and maternal livers removed. Fetal livers were pooled from each dam. Microsomes were prepared and stored as above.

Purification of [^{14}C]-3,3',4,4'-tetrachlorobiphenyl (TCB)

[^{14}C]-TCB was purified using semi-preparative high performance liquid chromatography. Fifty μCi of [^{14}C]-TCB was dissolved in 400 μl methanol and injected in 200 μl aliquots on a Zorbax ODS reverse phase column (Dupont, 4.6 mm i.d. x 25 cm) and eluted with a mixture of 85% methanol (v/v) and 15% water at a flow rate of 2 ml/min. Fractions were collected every minute for 30 minutes and uv absorbance monitored at 262 nm. A 100 μl aliquot of each fraction was counted by liquid scintillation counting (LSC) using Ultima Gold (Packard) as the scintillation fluid and the main fractions containing [^{14}C]-TCB radioactivity were pooled. The recovery of total radioactivity from the column was greater than 99%. The column had previously been calibrated using unlabelled TCB as a standard.

The water content of the pooled HPLC fractions was adjusted to 50% and a final volume of 90 ml. The methanol:water (1:1) phase was then extracted 4 times with 10 ml hexane by thorough mixing in a volumetric flask and removing the hexane after the phases had separated. Extraction efficiency for [^{14}C]-TCB in this latter case was greater than 99%. The hexane extract was evaporated under nitrogen, redissolved in 2 ml toluene and stored at 4°C. In order to check the purification, 1 μCi of the purified extract was redissolved in 100 μl methanol and 50 μl was injected on the same column using the same mobile phase conditions as described above. Fractions were collected every 30 seconds and when counted by LSC the radiochemical purity was greater than 99.8%. No contaminating peaks were found by monitoring the absorbance at 262 nm.

Incubation of [^{14}C]-TCB with hepatic microsomes

Initial experiments were carried out with ß-NF(1) microsomes because previously published research indicated that inducers of cytochrome P4501A activity resulted in an induction of TCB metabolism in rat hepatic microsomes (Ishida *et al.* 1991). Incubations were carried out in glass tubes at 37 °C in a shaking water bath with 0.5 μCi [^{14}C]-TCB per tube in a total volume of 2 ml with a 0.1 M (final concentration) Tris-HCl buffer, pH 7.5. Microsomal protein concentrations were varied between 0.1 and 2.0 mg protein/ml. The final concentration of TCB used varied between 1 and 50 μM. Unlabelled TCB was added to [^{14}C]-TCB to obtain the desired stock solution, which was dissolved in acetone and added to the pre-incubation mixture containing the microsomes and buffer (25 μl acetone/ml of incubation mixture) and vortexed for 1 min. NADPH was used as a cofactor in concentrations of 1 and 2 mM. An NADPH regenerating system was also used which consisted of 0.3 mM NADP$^+$, 10 mM glucose-6-phosphate, 0.25 units of glucose-6-phosphate dehydrogenase and 7.5 mM MgCl$_2$ (final concentrations). The incubation mixtures were prepared in tubes placed in an ice water bath. Incubations were pre-incubated for 2 minutes at 37°C and the reaction initiated by addition of NADPH, or in the case of the NADPH regenerating system with glucose-6-

phosphate. Reaction time was varied between 0 and 30 min. In order to investigate the effect of glucuronidation on the appearance of phenolic metabolites in the incubation mixture, ß-NF(2) microsomes (1 mg/ml) were incubated for 15 minutes with 10 µM [^{14}C]-TCB, 1 mM NADPH, with and without 1 mM uridine-diphosphoglucuronic acid.

Reactions were stopped by the addition of 2 ml of ice-cold methanol to the incubation mixture and vortexed for 30 sec. After the addition of methanol, the incubation mixtures were extracted 3 times with 4 ml of diisopropyl ether by vortexing for 30 s, centrifuged at 1000g for 5 minutes and then removal of the diisopropyl ether. Blanks were carried out by performing identical incubations with heat-inactivated microsomes (boiling water bath for 10 min). The ether extracts were pooled and dried under nitrogen and stored at 4°C until analysis. The lower phase containing the microsomal pellet and the water/methanol mixture was also stored at 4°C until further analysis. For HPLC analysis of the water/methanol phase it was first centrifuged at 1000g for 5 min, the supernatant removed, evaporated under nitrogen and resuspended in 100 µl water:methanol (1:1).

HPLC analysis of extractable radioactivity
Metabolite analysis was conducted using a Gilson 300 series HPLC system fitted with a Perkin-Elmer 3 µm C-18 column (4.6 mm i.d. x 83 mm). The uv absorbance was monitored at 262 nm, which yielded the best average response of the standards used according to a wavelenth scan (200-400 nm) in 78% methanol/22% water with a Beckman DU-64 spectrophotometer. The diisopropyl ether extracts were redissolved in 100 µl methanol containing 2.5 µg of the following standards: 2-OH-3,4,3',4'-tetrachlorobiphenyl, 4-OH-3,3',4',5-tetrachlorobiphenyl, 5-OH-3,4,3',4'-tetrachlorbiphenyl and 6-OH-3,4,3',4'-tetrachlorobiphenyl. Twenty µl of the ether extract or the concentrated water/methanol phase was injected on the column. The mobile phase was 78% (v/v) methanol, 22% water (containing 0.01% w/v Na-azide) for 20 minutes, followed by a gradient to 100% methanol in 5 minutes, and 100% methanol for another 10 minutes. The flow rate was 1 ml/min. The column was allowed to equilibrate at 78% v/v methanol, 22% water (0.01% w/v Na-azide) for at least 10 minutes between injections. Fractions were collected every 0.4 minutes for 35 minutes with a Redifrac fraction collector (Pharmacia). Five ml of scintillation fluid (Ultima Gold, Packard) was added to each fraction and was then counted in a liquid scintillation counter (Tri-Carb 1600, Packard). Identification of the metabolites was based on the coelution of radioactivity with authentic standards.

Gas-chromatographic-mass spectrometric analysis of extractable metabolites
Once the pattern of the metabolites formed had been firmly established on the basis of coelution with authentic standards using HPLC analysis, further confirmation of the identity of the metabolites formed in the *in vitro* incubations of hepatic microsomes with [^{14}C]-TCB was provided by gas chromatography with electron-capture detection (GC-ECD) and gas chromatography-mass spectrometry (GC-MS). The ether extracts from 3 incubations were pooled. The incubation conditions were: 2 ml final volume with 10 µM [^{14}C]-TCB, 1 mg microsomal protein (ß-NF(2)) per ml, 1 mM NADPH in 1 mM Tris-HCl buffer, pH 7.5 and a 10 minute incubation time. The pooled extracts were resuspended in 200 µl methanol and 20 µl was injected on the C-18 column that was

routinely used for analysis of the extracts. One ml fractions were collected in glass tubes and 50 µl of each fraction was counted using LSC as above. The fractions containing the radioactivity were pooled separately for each peak and the solvent was evaporated under nitrogen. The dried residue was redissolved in 1 ml n-hexane and methylated with diazomethane. Unreacted diazomethane and the hexane were then evaporated under nitrogen.

GC-MS was performed on an ITS40 instrument (Finnigan). The GC (Varian 3400) was equipped with a fused silica capillary column (DB5, 30 m x 0.25 mm i.d., J&W Scientific Inc. CA, USA) and a split-splitless injector. The temperature program was 80°C for 2 min, then 10°C/min to 240°C and maintained at 240°C for 20 min. The injector temperature was 260°C and the injections were made in the splitless mode, using an autosampler (CTC A200S, Finnigan). The mass spectrometer was operated in the electron impact mode and the manifold temperature was 220°C. GC-ECD analyses were performed with a Varian 3400 gas chromatograph equipped with a DB5$^+$ fused silica column (30 m x 0.25 mm i.d., J&W Scientific Inc. CA, USA), a split-splitless injector operated at 250°C, and a ^{63}Ni electron capture detector (ECD) operated at 300°C. The samples were injected in the splitless mode and the column temperature was programmed as follows: initial temperature 80°C for 2 min, followed by 10°C/min up to 300°C.

Covalent binding to microsomal protein

Covalent binding of metabolites to microsomal protein was determined as previously described (den Besten *et al.* 1991) after diisopropyl ether extraction of the incubation mixture. The water/methanol phase containing the microsomal proteins was centrifuged at 1000g for 5 min and the supernatant removed. The pellet was resuspended and washed succesively with 2 ml of solvent of declining polarity (water, methanol, ethanol and ethyl acetate). The microsomal pellet was recovered by centrifugation at 1000g for 5 min and removal of the solvent. After the final wash with ethyl acetate, the pellet was resuspended in 1 ml 1 N NaOH. Radioactivity was determined in the wash solvents and 500 µl of the resuspended pellet by LSC. The remaining 500 µl of the resuspended pellet was used for a protein determination with bovine serum albumin as a standard (Lowry *et al.* 1951).

The radioactivity in the water/methanol phase was analysed for the presence of glucuronic acid conjugates and phenolic metabolites by incubation of 50 µl aliquots of the water:methanol phase (after concentration to 200 µl) with and without 150 µl of a ß-glucuronidase solution (1000 units/ml 0.1 potassium phosphate buffer, pH 6.8) for 30 minutes at 37°C (Shen *et al.* 1991) or 4 N HCl at 100°C for 1 h (6), followed by diisopropyl ether extraction and HPLC analysis as described above.

Ethoxyresorufin-O-deethylase (EROD) activity

EROD activity was determined using a final concentration of 1 µM ethoxyresorufin, 0.1 mM NADPH and 100 µg microsomal protein per ml at 37°C (Burke *et al.* 1977). To detect the deethylation product resorufin, the excitation wavelength of the fluorimeter was 530 nm, and the emission wavelength was 580 nm. Initial reaction velocities were used to calculate EROD activity.

Results

Optimization of incubation conditions

Incubations were first optimized for cofactor requirements, length of incubation, microsomal protein concentration and TCB concentration by examining the total amount of extractable metabolites formed with HPLC. Calculations of total extractable metabolites formed were based on the percentage of the total radioactivity in the HPLC radiochromatogram eluting in the metabolite fractions, adjusted for the total amount of TCB (unlabelled and labelled) present in the incubation. Recoveries in the diisopropyl ether extracts were always greater than 95% for 5 minute incubations.

In order to determine whether the addition of NADPH or an NADPH regenerating system yielded the highest activity, ß-NF(1) microsomes (1 mg/ml) were incubated for 15 and 30 minutes with 1 or 2 mM NADPH and an NADPH regenerating system with 1.7 μM [^{14}C]-TCB. Since the amount of extractable metabolites formed at both time points was highest with 1 mM NADPH (data not shown), this concentration was therefore used for further experiments. Because the reaction was non-linear over 30 minutes, a second experiment was conducted with 1 mM NADPH, 1 mg microsomal protein/ml and 1.5 μM [^{14}C]-TCB and the amount of extractable metabolites formed was determined at 1, 5, 10, 15 and 30 minutes, and revealed that the reaction was linear for the first 5 minutes at this TCB concentration (data not shown).
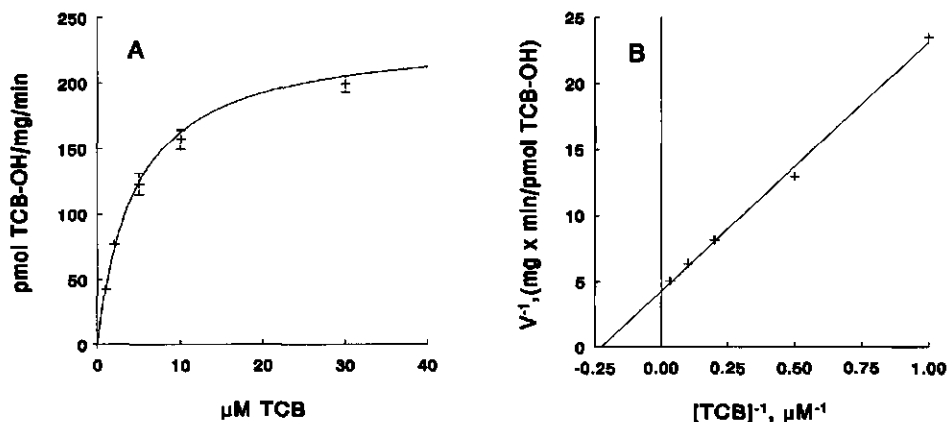
Figure 3.1 A and B.
A) The influence of substrate concentration on the formation of diisopropyl ether-extractable metabolites (TCB-OH) of [^{14}C]-TCB with microsomes from ß-naphthoflavone-treated rats (1 mg protein/ml) with 1 mM NADPH and an incubation time of 5 min. The results (mean ± range) are the average of duplicate incubations. If no error bar is visible it is smaller than the marker. The curve is fitted by computer for first order kinetics with an apparent K_m and V_{max} of 4.5 μM and 240 pmol/mg protein/min), respectively. B) Lineweaver-Burke plot of the mean data from Figure 3.1A.

In order to determine the effect of substrate concentration on metabolite production, incubations were performed with 1 to 50 μM [^{14}C]-TCB, 1 mg microsomal protein/ml and 1 mM NADPH for 5 minutes. At 50 μM [^{14}C]-TCB the solubility of the substrate was poor, yielding large variations in the amount of extractable metabolites formed. The poor solubilty of 50 μM [^{14}C]-TCB was confirmed by the finding that there were large variations in the amount of radioactivity recovered when five 100 μl aliquots of the incubation mixture were quantified with liquid scintillation counting (LSC) prior to extraction, and therefore the data from the incubations with 50 μM [^{14}C]-TCB were not used for the Lineweaver-Burke plot. Saturating concentrations of [^{14}C]-TCB were not completely achieved by 32 μM (Figure 3.1A). From the Lineweaver-Burke plot (Figure 3.1B) the apparent K_m and V_{max} values were determined to be 4.5 μM and 240 pmol/mg protein/ min), respectively, as calculated from the amount of diisopropyl ether-extractable metabolites formed.
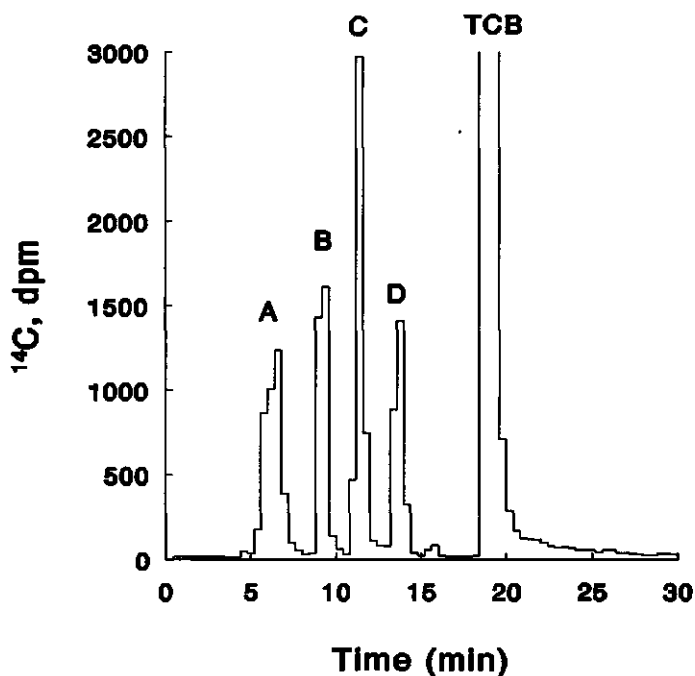
Figure 3.2.
A typical histogram of reversed-phase HPLC analysis of diisopropyl ether-extractable metabolites of 10 μM [^{14}C]-TCB with microsomes from ß-naphthoflavone-treated rats (1 mg protein/ml) with 1 mM NADPH and an incubation time of 5 minutes. A: 4-OH-3,3',4',5-tetrachlorobiphenyl, B: 5-OH-3,3',4,4'-tetrachlorobiphenyl, C: 6-OH-3,3',4,4'-tetrachlorobiphenyl, D: unidentified peak in the HPLC radiochromatogram.

The effect of microsomal protein concentration on the formation of extractable metabolites was examined at 2.5 and 10 μM [^{14}C]-TCB, 1 mM NADPH and an incubation time of 5 minutes (data not shown). At 10 μM [^{14}C]-TCB, the formation of extractable metabolites (pmol/min) increased linearly with the protein concentration up to 1 mg microsomal protein/ml. At the lower substrate concentration used (2.5 μM), the formation of extractable metabolites was not a linear function of the protein concentration.

Identification of metabolites

HPLC fractionation followed by LSC of the ether-extractable radioactivity revealed four radioactive metabolite peaks. A typical histogram of a reverse-phase HPLC fractionation of the ether extractable radioactivity following the incubation of [^{14}C]-TCB with ß-NF-microsomes is shown in Figure 3.2. The first three radioactive peaks coeluted with the metabolite standards 4-OH-tetraCB, 5-OH-TCB and 6-OH-TCB, respectively. The fourth radioactive peak did not coelute with any of the authentic metabolite standards and no radioactive peak coeluted with 2-OH-TCB.

GC-ECD and GC-MS analysis of the methylated fractionated peaks after HPLC separation of pooled incubation extracts confirmed the identity of the radioactive peaks that coeluted with the authetic standards for 4-OH-tetraCB, 5-OH-TCB and 6-OH-TCB, both the retention time and mass spectra of the isolated metabolites after methylation corresponded to the methylated authentic standards (data not shown). No 2-OH-TCB was detected by GC-ECD as its methylated derivative in the HPLC fraction collected where the authentic standard would elute or in an unfractionated diisopropyl ether extract of incubation with ß-NF microsomes (data not shown). The radioactive peak that did not coelute with any of the authentic standards (Peak D, Figure 3.2) yeiled two compounds after methylation when analysed with GC-MS with molecular ions of 320 and 306, both with an isotopic cluster corresponding to 4 chlorine ions (data not shown).

Extracting the incubations 3 times with diisopropyl ether effectively removed all phenolic metabolites and TCB from the aqueous lower phase. When the lower phase was evaporated and resuspended in methanol, followed by reversed phase HPLC analysis, only one radioactive peak was observed that eluted close to the front of the chromatogram and comprised approximately 10% of the total metabolites formed. Treatment of the lower phase with ß-glucuronidase or 4 N HCl did not change the position of this peak in the radiochromatogram (data not shown).

The formation of ether-extractable, polar and covalently bound metabolites was followed over a period of 15 minutes in an incubation of ß-NF(2) microsomes (1 mg/ml) with 10 μM [^{14}C]-TCB and 1 mM NADPH. Over 15 minutes the rate of formation of 4-OH-tetraCB (17.1 $\pm$ 0.5 pmol/mg protein/min) was more than 50% greater than that of the other two identified phenolic metabolites, 5-OH-TCB and 6-OH-TCB (11.4 $\pm$ 0.6 and 12.5 $\pm$ 0.5 pmol/mg protein/min), (Figure 3.3A). The radioactive peak containing unidentified extractable metabolites was formed at a rate similar to that of 5-OH-TCB and 6-OH-TCB (13.3 pmol/mg protein/min).

Of the metabolites that were non-extractable in ether, the largest amount formed was in the form of one or more water soluble compounds (23.4 $\pm$ 0.1 pmol/mg protein/min) during the first 5 min (Figure 3.3B). The formation rate of water soluble compounds was even higher during the second five minutes of incubation (36.4 $\pm$ 5.3

pmol/mg protein/min). However, between 10 and 15 minutes the concentration of water soluble compounds present in the incubation mixture decreased from 150 ± 2.6 pmol/ml to 106 ± 22.3 pmol/ml. Although we were unable to demonstrate the presence of phenolic metabolites in the water/methanol phase by acid or enzymatic hydrolysis, the addition of 1 mM UDGPA to the incubation mixture significantly reduced the amount of 5-OH-TCB in the incubation mixture after 15 minutes from 86 ± 4.4 to 59 ± 7.3 pmol/ml ($P<0.05$, student's t-test, n=3). The levels of the other phenolic metabolites in the incubation mixture were unaffected by the addition of 1 mM UDPGA (data not shown).

Covalently bound metabolites were formed over the first 5 minutes of the incubation at a low rate (4.3 ± 0.9 pmol/mg protein/min) which increased to 9.3 ± 3.9 pmol/mg protein/min) in the second 5 minutes of the incubation and remained at the same rate (9.3 ± 0.4 pmol/mg protein/min) during the third 5 minutes.
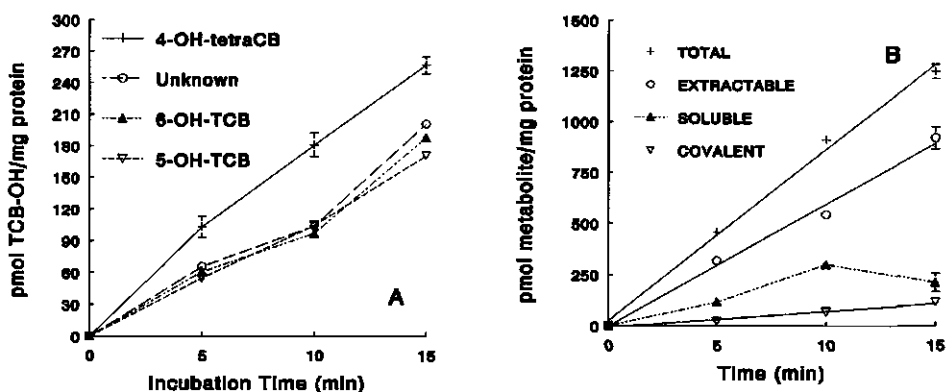
Figure 3.3 A and B.
A: The formation of individual diisopropyl ether-extractable metabolites of 10 μM [^{14}C]-TCB with microsomes from ß-naphthoflavone-treated rats (1 mg protein/ml) with 1 mM NADPH and an incubation time of 5, 10 and 15 min. The results (mean ± SD) are the average of three incubations. For the clarity of the figure, error bars are presented only for 4-OH-tetraCB. 4-OH-tetraCB: 4-OH-3,3',4',5-tetrachlorobiphenyl, 5-OH-TCB: 5-OH-3,3',4,4'-tetrachlorobiphenyl, 6-OH-TCB: 6-OH-3,3',4,4'-tetrachlorobiphenyl, unknown: unidentified peak in the HPLC radiochromatogram.
B: The formation of diisopropyl ether-extractable, water soluble and covalently bound and total metabolites of 10 μM [^{14}C]-TCB with microsomes from ß-naphthoflavone-treated rats (1 mg protein/ml) with 1 mM NADPH and an incubation time of 5, 10 and 15 min. The results (mean ± SD) are the average of three incubations. If no error bar is visible, it is smaller than the marker.

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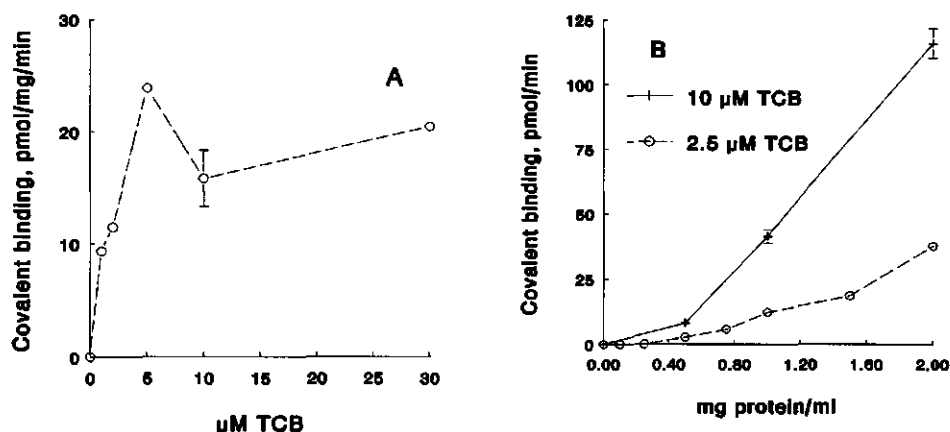
Figure 3.4A and B.
A: Influence of substrate concentration on the formation of covalently bound metabolites of [14C]-TCB with microsomes from ß-NF-treated rats (1 mg protein/ml) with 1 mM NADPH and an incubation time of 5 min. The results are the mean ± range of duplicate incubations.
B: The influence of microsomal protein concentration on the formation of covalently bound metabolites of 2.5 and 10 μM [14C]-TCB with microsomes from ß-naphthoflavone-treated rats with 1 mM NADPH and an incubation time of 5 min. The results (mean ± range) are the average of duplicate incubations. If no error bar is visible, it is smaller than the marker.

The production of metabolites covalently bound to microsomal proteins was investigated in relation to substrate concentration and protein concentration. After 5 minute incubations of 1-32 μM [14C]-TCB with 1 mg microsomal protein (ß-NF1)/ml and 1 mM NADPH, the amount of covalently bound metabolites formed increased in a non-linear fashion with the [14C]-TCB concentration (Figure 3.4A). When the microsomal protein concentration was varied in 5 minute incubations with 10 μM [14C]-TCB and 1 mM NADPH, the amount of covalently bound metabolites in the microsomal pellet increased linearly bewteen 0.5 and 2.0 mg protein/ml (Figure 3.4B). However, at a lower substrate concentration (2.5 μM [14C]-TCB), the amount of covalently bound metabolites did not increase in a linear fashion (Figure 3.4B).

Influence of pretreatment and development
The induction of TCB metabolism by various inducers of hepatic mono-oxygenases was investigated in male Wistar rats. The incubation conditions were 1 mg microsomal protein/ml, 10 μM [14C]-TCB, 1 mM NADPH and an incubation time of 5 min. The only pretreatment that resulted in the induction of TCB metabolism (formation of extractable metabolites) was ß-naphthoflavone (Table 3.1). ß-NF treatment also resulted in the greatest induction of EROD activity relative to untreated rats. Although pretreatment with phenobarbital induced EROD levels 4-fold relative to controls, neither treatment with phenobarbital nor dexamethasone had any effect on hepatic microsomal TCB metabolism.

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Table 3.1
Microsomal metabolite production from [^{14}C]-TCB after different pretreatments of male Wistar rats.

Treatment	TCB Metabolism pmol OH-TCB/mg protein/min	EROD activity nmol RR/mg protein/min
Control	3.9±0.4	0.05±0.01
Phenobarbital	3.6±0.7	0.20±0.05
Dexamethasone	2.1±0.4	0.06±0.01
ß-Naphthoflavone (1)	157±7.2	6.13±0.60
ß-Naphthoflavone (2)	62.9±3.8	2.29±0.24

The incubation conditions were: 10 μM [^{14}C]-TCB, 1 mg microsomal protein/ml, 1 mM NADPH, 37°C for 5 minutes. The rate of metabolism is based on the amount of ether-extractable metabolites formed in the incubations. The results are the mean±SD of triplicate incubations. EROD: ethoxyresorufin-O-deethylase, RR: resorufin

Table 3.2
Microsomal metabolite production from [^{14}C]-TCB in control and TCB-treated pregnant Wistar rats and fetuses.

Treatment	TCB Metabolism pmol OH-TCB/mg protein/min	EROD activity nmol RR/mg protein/min
Control dams	ND	0.02±0.00
Control fetuses	ND	ND
TCB-dams	43.9±8.5	1.70±0.43
TCB-fetuses	ND	ND

The incubation conditions were: 10 μM [^{14}C]-TCB, 1 mg microsomal protein/ml, 1 mM NADPH, 37°C for 5 minutes. The rate of metabolism is based on the amount of ether-extractable metabolites formed in the incubations. The results are the mean±SD of duplicate incubations of microsomes from 4 individual dams or their fetuses. ND: not detected. EROD: ethoxyresorufin-O-deethylase, RR: resorufin

Table 3.3

Comparison of the production of phenolic metabolites of [^{14}C]-TCB by microsomes from ß-NF-treated male and TCB-treated pregnant female Wistar rats.

Metabolite	ß-NF(2)-treated males	TCB-treated dams
	pmol OH-TCB/mg protein/min	
4-OH-tetraCB	13.5±2.3	20.7±2.0
5-OH-TCB	7.1±1.1	10.9±0.7
6-OH-TCB	8.7±1.3	12.1±0.6
Unknown	6.1±1.1	13.1±1.4

The incubation conditions were: 10 µM [^{14}C]-TCB, 1 mg microsomal protein/ml, 1 mM NADPH, 37°C for 5 minutes. The rate of metabolism is based on the amount of ether-extractable metabolites formed in the incubations. The results are the mean±SD of duplicate incubations of microsomes from 4 individual dams or triplicate incubations of ß-NF(2) microsomes. ß-NF: ß-naphthoflavone.

The capacity of fetal and maternal rat microsomes from day 20 of gestation to metabolize [^{14}C]-TCB or ethoxyresorufin after pretreatment on day 13 of gestation with either unlabelled TCB (25 µmol/kg) or the vehicle alone (corn oil, 2 ml/kg) was also investigated. The incubation conditions were 1 mg microsomal protein/ml, 10 µM [^{14}C]-TCB, 1 mM NADPH and an incubation time of 5 min. There was no detectable formation of extractable metabolites in incubations with microsomes from corn oil-treated dams and fetuses or with microsomes from TCB-exposed fetuses. Only incubations of [^{14}C]-TCB with microsomes from TCB-treated dams resulted in the production of ether-extractable metabolites (Table 3.2). The level of EROD activity with microsomes from TCB-treated dams was 100-fold higher than that of corn oil-treated dams, and fetal EROD activity was undetectable irrespective of the pretreatment. EROD activity was also 3-fold lower with microsomes from corn oil-treated dams (17 pmol/mg protein/min) than untreated male rats (53 pmol/mg protein/min). The relative amounts of phenolic PCB metabolites and the extractable unknown compound(s) formed was nearly identical in incubations of [^{14}C]-TCB with microsomes from ß-NF-treated male Wistar rats and TCB-treated pregnant female Wistar rats (Table 3.3).

Discussion

The major phenolic metabolites of TCB formed in the incubation mixture (4-OH-3,3',4',5-tetraCB, 5-OH-TCB and 6-OH-TCB) were resolved by HPLC and their identity confirmed by coelution with authentic standards on a reverse-phase HPLC column. Definite confirmation of the identity of the phenolic compounds in the HPLC fractions was given by GC-ECD and GC-MS analysis after methylation of the fractions. The reaction conditions were partially optimized for cofactor requirements, substrate and

microsomal protein concentration. Ideally a substrate concentration should be chosen which will result in V_{max} conditions, however, at 30 µM [^{14}C]-TCB this V_{max} conditions was not attained and at 50 µM [^{14}C]-TCB the solubility of the substrate in the incubation mixture was poor at a microsomal protein concentration of 1 mg/ml. The use of 10 µM [^{14}C]-TCB forms a reasonable compromise, for the production of metabolites was a linear function of time (up to 15 minutes) at 10 µM [^{14}C]-TCB using highly induced microsomes (1 mg protein/ml).

In male rats, only treatment with the cytochrome P4501A inducer, ß-NF, resulted in a significant induction of [^{14}C]-TCB metabolism relative to control microsomes. This is in agreement with previous studies (Mills *et al.* 1985, Ishida *et al.* 1991), which demonstrated the cytochrome P4501A1 dependence of TCB metabolism. Phenobarbital treatment has been shown to induce the metabolism of 2,2',5,5'-tetrachlorobiphenyl, but not 3,3',4,4'-tetrachlorobiphenyl (Ishida *et al.* 1991). In rats dexamethasone is an extensive inducer of cytochrome P4503A1 (Wrighton *et al.* 1985) and the hydroxylation of various chlorinated benzenes such as hexachlorobenzene (Van Ommen, 1987), pentachlorobenzene and 1,2,4-trichlorobenzene (den Besten *et al.* 1991), 1,2-dichlorobenzene and 1,4-dichlorobenzene (den Besten *et al.* 1992). However, in the current study the pretreatment of rats with dexamethasone did not result in the increased hepatic microsomal metabolism of [^{14}C]-TCB, indicating that the presence of a phenyl ring in place of a chlorine (as in 1,2,4-trichlorobenzene) effectively inhibits the hydroxylation of [^{14}C]-TCB by cytochrome P4503A1.

In the current study, 3 phenolic metabolites, 4-OH-3,5,3',4'-tetraCB, 5-OH-TCB and 6-OH-TCB, were positively identified in the ether extracts. A fourth radioactive peak in the in the HPLC fractions of the ether-extracts appeared to contain two compounds upon GC-MS analysis, although we were unable to identify the structure of these latter compounds.

Interestingly, two *in vivo* studies (Yoshimura *et al.* 1987, Koga *et al.* 1989), and one *in vitro* study (Ishida *et al.* 1991), all using male Wistar rats or microsomes, did not find any fecal excretion or *in vitro* production of 6-OH-TCB, despite the use of an authentic standard. 6-OH-TCB is one of the most lipophillic of the phenolic metabolites, as judged by its relatively long retention time on a C-18 HPLC column relative to the other phenolic metabolites formed, and may thus be poorly excreted into the feces following its formation. This hypothesis is supported by the report that when C57BL mice were treated with [^{14}C]-TCB, only trace amounts of 6-OH-TCB were detected in the feces, but 6-OH-TCB comprised 16% of total radioactivity in the adipose tissue (Klasson Wehler, 1989). It is unclear why the production of 6-OH-TCB was not detected in the study using microsomes from 3-methylcholanthrene-treated Wistar rats (Ishida *et al.* 1991). One significant difference in the analytical methods is that in this study high resolution capillary GC-ECD and GC-MS was used and in the previous study (Ishida *et al.* 1991) packed column GC was used, which could result in a difference in the resolution of the metabolites on the column.

One minor metabolite previously identified *in vivo* in chicken embryos and mouse urine, 2-OH-TCB (Klasson Wehler, 1989, Klasson Wehler *et al.* 1990), was not found in the ether extracts of incubations with highly induced microsomes, either by HPLC or GC-MS analysis. Similarly 2-OH-TCB was also not found in the feces of Wistar rats treated with TCB (Yoshimura *et al.* 1987), which may indicate species

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specific metabolism of TCB.

In the current study the nature and relative amounts of the ether-extractable metabolites formed in incubations with hepatic microsomes from TCB-treated dams were identical to those formed in incubations with microsomes from male rats pretreated with ß-NF. 4-OH-tetraCB and 5-OH-TCB have been also been found in the livers of pregnant rats treated with TCB (Morse *et al.* 1995).

When we examined the metabolism of [^{14}C]-TCB with microsomes from TCB-treated pregnant rats and their fetuses, the formation of phenolic metabolites was observed only in incubations of maternal microsomes. Even when the incubation time was extended to 30 minutes, no phenolic metabolite production was observed in incubations with fetal microsomes. In addition, no induction of fetal hepatic microsomal EROD activity was observed following maternal TCB treatment. This data supports the hypothesis that 4-OH-tetraCB and other phenolic metabolites are produced in the maternal liver, only 4-OH-tetraCB is selectively retained in the maternal plasma bound to TTR, then crosses the placenta and accumulates in the fetal compartment. A recent study has shown that the accumulation of 4-OH-tetraCB in fetal rats is associated with decreases in fetal plasma TT_4 levels in the absence of effects on maternal TT_4 levels at the same time of gestation (Morse *et al.* 1995). This finding suggests that maternally produced metabolites may affect fetal thyroid hormone status, although there are no obvious effects on maternal thyroid status.

Although TCB is only a minor component of commercial PCB mixtures and environmental extracts, other PCBs may be metabolized to hydroxylated metabolites, of which a limited number, mainly with an 1,2-shift hydroxy-substitution in the *para*-position, are selectively retained in the blood of rats and environmentally exposed animals (Bergman *et al.* 1994). Recent research has shown that, analogous to the fetal accumulation of 4-OH-tetraCB in the rat, the major metabolite (4-OH-2,3,5,3',4'-pentachlorobiphenyl) found in adult rat, mouse and mink plasma following Aroclor 1254 exposure (Bergman *et al.* 1994, Klasson Wehler *et al.* 1993) also accumulates in fetal rat plasma following Aroclor 1254 exposure to pregnant rats (Dr. Eva Klasson Wehler, personal communication).

We were unable to identify the nature of the water soluble metabolites formed in incubations of [^{14}C]-TCB with ß-NF microsomes. Although no compounds coeluting with the metabolite standards were produced by acid hydrolysis or ß-glucuronidase treatment of the lower phase, the addition of UDPGA to the incubation mixture selectively reduced the amount of 5-OH-TCB present in the ether-extracts, which may indicate the formation of glucuronic acid conjugates. The presence of conjugated metabolites of TCB has been indicated in the urine of mice after an oral dose of TCB (Klasson Wehler *et al.* 1989).

The covalent binding of [^{14}C]-TCB metabolites to microsomal or erythrocyte membrane proteins has been previously demonstrated in *in vitro* studies (Shimada and Sawabe, 1983, Shimada *et al.* 1985). Metabolites of [^{14}C]-TCB also bind to cellular macromolecules in several organs of the rat *in vivo* (Shimada and Sawabe, 1984), although the significance of this covalent binding for toxicity is not clear. It has been suggested that the high retention of [^{14}C]-TCB derived radioactivity in the blood (Shimada *et al.* 1985) is due to covalent binding to blood components and that the retention in the blood is related to the high toxicity of TCB. However, in a recent study,

it has been demonstrated that there is a selective accumulation of one metabolite, 4-OH-3,5,3',4'-tetrachlorobiphenyl in the plasma of rats following [^{14}C]-TCB exposure (Morse *et al.*, 1995a), which is due to competitive binding of this metabolite to TTR, the major thyroid hormone transport protein in rat plasma (Brouwer *et al.* 1990, Brouwer *et al.* 1986).

Although the metabolism of TCB appears to be largely similar between rats and mice and is related to the induction of P4501A enzymes, this may not be the case for some other species. A qualitative interspecies variation in the production of phenolic metabolites of TCB by hepatic microsomes from mammals and birds has recently been observed (Murk *et al.* 1994). The most notable result was that environmentally exposed marine mammals (seal and porpoise) showed a very similar metabolite pattern to ß-NF-treated rats, whereas eider duck microsomes (exposed to either Clophen A50 or TCB) produced almost exclusively 5-OH-TCB.

In conclusion, we have developed a technique for the HPLC analysis of ether-extracts of hepatic microsomes with [^{14}C]-TCB which can provide qualitative and quantative information on the formation of hydroxylated metabolites of TCB. The reaction kinetics of [^{14}C]-TCB with hepatic microsomes have been partially characterised to allow for optimal incubation conditions. The technique is useful for the investigation of the metabolic capacity in different developmental stages, following various pretreatments (this study), or in different animal species (Murk *et al.* 1994).

CHAPTER 4

INTERFERENCE OF POLYCHLORINATED BIPHENYLS IN HEPATIC AND BRAIN THYROID HORMONE METABOLISM IN FETAL AND NEONATAL RATS

Abstract

The effects of prenatal oral administration of 0.2, 0.6 and 1.8 mg/kg body weight of 3,3',4,4',5,5'-hexachlorobiphenyl (HCB) on day 1 of gestation and a combination of 1 mg/kg 3,3',4,4'-tetrachlorobiphenyl (TCB) from day 2 to day 18 with 0.6 mg HCB/kg bw on day 1 of gestation on thyroid hormone status and peripheral thyroid metabolism were studied in pregnant Wistar rats, their fetuses and offspring. Plasma total thyroxine (TT_4) and free thyroxine (FT_4) levels were reduced by the highest HCB dose in pregnant rats (day 12 and 20 of gestation) and neonates (day 21 post partum), while only a combined dose of HCB and TCB were effective in decreasing fetal thyroid hormone levels by 65 % on day 20 of gestation. The activity of type II thyroxine 5'-deiodinase (5'D-II), the enzyme responsible for the deiodination of thyroxine (T_4) to biologically active triiodothyronine (T_3) in the brain, was examined in whole brain homogenates in fetuses and neonates. Decreases in plasma thyroid hormones were accompanied by significant increases, up to 100%, in 5'D-II activity in brain homogenates from fetuses (day 20 of gestation, combined dose only) and neonates (day 7 and 21 post partum). Increases in 5'D-II activity in female neonates were observed in the high HCB and combined dose groups, while the increases in male neonatal 5'D-II were restricted to the high HCB group. The glucuronidation of $^{125}I\text{-}T_4$ by hepatic microsomes was increased by at least 100% relative to control levels by all treatments in fetuses (day 20 of gestation) and increased at least 40% in neonates (day 7 and 21 post partum) by a dose of 0.6 and 1.8 mg HCB/kg and the combined dose. These data indicate that prenatal HCB and/or TCB administration result in increased peripheral T_4 metabolism. The increase in 5'D-II activity suggests that local hypothyroidism occurs in the brains of fetal and neonatal rats exposed to HCB and/or TCB. Since these effects occur during a period in which thyroid hormones play an important role in brain maturation, they may help explain the mechanism of developmental neurotoxicity induced by polychorinated biphenyls.

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Introduction

Polychlorinated biphenyls (PCBs) are persistent environmental contaminants found in human adipose tissue and mother's milk, which can also cross the placenta to a minor extent (Jensen and Sundström 1974, Mes *et al.* 1984, Bush *et al.* 1984, Bush *et al.* 1985). The pre- or postnatal exposure of mice, rats, monkeys and humans to PCBs results in neurodevelopmental changes in the offspring (reviewed by Tilson *et al.* 1990). Prenatal high-level exposure of humans to a mixture of PCBs and their thermal degradation products in Japan and Taiwan resulted in a variety of physical problems and developmental delay in motor and mental function (Rogan *et al.* 1988). Two U.S. studies on the effects of perinatal exposure to background levels of PCBs revealed an association between elevated cord serum PCB levels and smaller birth size in one study, while in both studies there was an effect on neurodevelopmental parameters (Fein *et al.* 1984, Jacobson *et al.* 1985, Gladen *et al.* 1988, Rogan *et al.* 1986).

However, the mechanism by which PCBs interfere with neurological de-velopment remains unknown. Lower chlorinated di-ortho PCB congeners, such as 2,4,4'-trichlorobiphenyl, lead to reductions in dopamine levels in pheochromocytoma cells (Shain *et al.*, 1991) or in specific brain regions in macaque monkeys (Seegal *et al.*, 1990). These di-ortho PCBs do not have the structural characteristics of the most potent coplanar PCB congeners with a dioxin-like toxicity (e.g. cytochrome P4501A1 induction, thymus atrophy, bodyweight loss). Nonetheless, coplanar compounds are not devoid of developmental neurotoxicity, for perinatal exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin leads to neurotoxicity in monkeys (Schantz *et al.* 1989) and prenatal (Tilson *et al.* 1979) and postnatal (Eriksson *et al.* 1991) administration of 3,3',4,4'-tetrachlorobiphenyl results in neurotoxicity in mice.

In view of the significant role thyroid hormones play in brain maturation in man and other species (Smith 1981), a possible explanation is that alterations in thyroid hormone transport and metabolism caused by PCBs may lead to abnormal brain development. The dietary administration of Aroclor 1254 to pregnant rats has been shown to reduce plasma thyroxine (T_4) levels and induce thyroid hyperplasia in newborn and neonatal rats (Collins and Davis 1980). In adult rats, plasma T_4 levels were de creased not only by PCBs, but also by polybromobiphenyl (PBB, Byrne *et al.* 1987) mixtures as well as by individual congeners, such as 3,3',4,4'-tetrachlorobiphenyl (TCB, Brouwer and van den Berg 1986), 3,3'4,4',5,5'-hexabromobiphenyl (HBB, Spear *et al.* 1990) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, Bastomsky 1977), compounds which also induce hepatic glucuronidation of T_4. In addition to the rat, reduced plasma T_4 levels caused by exposure to TCB has been documented in marmoset monkeys and by PBBs in the mouse (Van den Berg *et al.* 1988, Gupta *et al.* 1983) In man, the effects of occupational exposure to PBBs or prenatal exposure to thermally degraded PCBs suggest interference in thyroid function (Bahn *et al.* 1980, Murai *et al.* 1987).

Fetal and neonatal rats respond to hypothyroidism with an increase in brain Type II thyroxine 5' deiodinase (5'D-II), the enzyme responsible for the local conversion of thyroxine to triiodothyronine (T_3), the biologically most active hormone (Ruiz de Oña *et al.* 1988, Silva and Larsen 1982, Silva and Matthews 1984). It is therefore of interest to determine if PCB-induced decreases in plasma T_4 levels in fetal and neonatal rats result in increased 5'D-II activity in the brains of fetal and neonatal rats, as plasma T_3 can not

protect the fetal or neonatal rat brain from hypothyroidism (Calvo *et al.* 1990, Silva and Matthews 1984).

In this investigation we have examined the effects of the prenatal administration of 3,3',4,4',5,5'-hexachlorobiphenyl (HCB) alone and in combination with 3,3',4,4'-tetrachlorobiphenyl (TCB) to pregnant rats on maternal, fetal and neonatal plasma total and free thyroxine hormones and hepatic microsomal T_4 glucuronidation. We also studied the effects of HCB and TCB administration on brain 5'D-II activity, a specific response of the developing rat brain to hypothyroidism. The combined dose of HCB and TCB was used to determine if the additional exposure to a rapidly metabolizable PCB congener like TCB would elicit effects on thyroid homeostasis in the fetus. Due to the short biological half life of TCB (Yoshimura *et al.* 1987), we used multiple low doses during gestation. HCB is a congener that is poorly metabolized, thus a single dose at the beginning of pregnancy should be sufficient to induce effects during pregnancy and lactation.

Material and Methods

Chemicals.
The 3,3',4,4',5,5'-hexachlorobiphenyl and 3,3',4,4'-tetrachlorobiphenyl (99% pure, dibenzo-p-dioxin and -furan free) were obtained from Promochem, Wesel, Germany.

Animals
One hundred and twenty female and 60 male Wistar W.U. rats (Charles River Wiga GmbH, Sulzfeld, Germany) were allowed at least a 5 day acclimatization period. When the rats were about 14 weeks old, they were paired by placing one male with 2 females per disposable plastic cage. The animals were kept on a 12 hr light cycle, the temperature was 22 ± 3 °C and relative humidity was 30-70%. A powdered stock diet (TNO Toxicology and Nutrition Institute, Zeist, The Netherlands) and tap water were offered ad libitum. When sperm was found in a vaginal smear the female was housed individually and this was termed as day 0 of gestation. Animals were distributed into 5 dose groups on a rotating basis. Pregnant rats were dosed orally by gavage with either 0.2, 0.6 or 1.8 mg HCB per kg body weight or the vehicle (corn oil, 2 ml/kg body weight) on day 1 of the gestation. A fifth group of pregnant rats received a single dose of 0.6 mg HCB/kg body weight on day 1 and then 1.0 mg/kg of TCB daily from day 2 to day 18 of the gestation.

Autopsy and sample preparation
Three to 5 pregnant rats per dose group were sacrificed on day 12 and 20 of gestation, and 4 to 8 nests were sacrificed per group 7 and 21 days after birth under ether anesthesia. Fetal (day 20 of gestation) and neonatal trunk blood was collected by decapitation in heparinized tubes and maternal blood was drawn from the vena cava. Plasma was prepared by centrifugation at 1000 g and stored at -20 °C.

Fetal brains, livers and plasma were pooled for each pregnant animal, while tissues from male and female neonates of each nest as well as maternal samples were collected separately.

Fetal and neonatal whole brains were homogenized on ice in a Potter tube with 0.1 M Tris-HCl buffer, pH 7.5 containing 1 mM dithiothreitol (DTT, 8 ml buffer per g tissue) and stored at -80 °C until analysis. Livers were immediately frozen in 0.1 M Tris-HCl buffer, pH 7.5 containing 0.25 M sucrose (3 ml/g liver) at -80 °C until microsomes were prepared within 1 week. Microsomes were prepared by centrifuging liver homogenates at 9,000 g, for 30 min, the resulting supernatant was centrifuged at 105,000 g for 90 min, and the pellet was resuspended in 0.1 M phosphate buffer, pH 7.5. Microsomes were stored at -80 °C until further analysis.

Hormone analysis

Plasma total T_4 (TT_4) and free T_4 (FT_4) were determined with the Amerlite system (Amersham, UK) according to the protocol of the supplier with slight modifications. The TT_4 assay buffer was diluted 5 times with demineralized water, the standard curve for TT_4 ranged from 0 to 120 nmol TT_4/l.

Brain Type II thyroxine 5'-deiodinase

Brain 5'D-II activity was measured essentially as described by Visser *et al.* (1982). The final reaction conditions were 100 mM phosphate buffer pH 7.2, 25 mM DTT, 1 mM propyl-2-thiouracil, 1 mM EDTA, 2 nM T_4 with 10^5 cpm ^{125}I-T_4 (Amersham, U.K.), 100 nM triiodothyronine and brain homogenate (0.8 mg protein) in a total volume of 200 μl. The addition of 100 nM triiodothyronine (T_3) was necessary to inhibit the inner-ring deiodination of T_4 by type III deiodinase, which is present in significant amounts in fetal and neonatal rat brains (Kaplan and Yaskoski 1981, 1982). T_3 is the preferred substrate for type III deiodinase (Kaplan *et al.*, 1983). Incubations were carried out at 37 °C for 60 min. The reaction was stopped on ice by the addition of 100 μl BSA (70 mg/ml) in order to bind the remaining substrate and T_3, followed by 500 μl of 10% TCA w/v to precipitate the protein. The tubes were then centrifuged at 4000 rpm in an eppendorf centrifuge for 5 min, then 500 μl supernatant containing mainly $^{125}I^-$ liberated from the ^{125}I-T_4 was removed. The amount of $^{125}I^-$ present in the supernatant was determined using Sephadex LH-20 chromatography (Otten *et al.* 1984). Blanks were carried out using brain homogenates inactivated by boiling for 10 min. Results are expressed as fmol T_4 deiodinated per hour per mg protein.

Hepatic microsomal T_4 glucuronyltransferase (T_4G)

Hepatic T_4G activity was determined according to Beetstra *et al.* (1991). The final reaction conditions were 0.1 M Tris-HCl buffer, pH 7.8, 5 mM uridine-5'-diphosphoglucuronic acid, 3.75 mM $MgCl_2$, 0.25% (w/v) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate (CHAPS), 0.125% (w/v) BSA, 1 μM T_4 with 50,000 cpm ^{125}I-T_4 and 0.2 mg microsomal protein in a total volume of 200 μl. The reaction was carried out at 37 °C for 30 minutes because preliminary experiments showed that the reaction was linear for up to 30 minutes for microsomes exhibiting the greatest induction in T_4G activity. The reaction was stopped with 200 μl ice-cold methanol and after centrifugation 200 μl supernatant was combined with 750 μl 0.1 N HCl. The amount of ^{125}I-T_4 glucuronide in the supernatant was analyzed with Sephadex LH-20 chromatography (Rutgers *et al.* 1989). Protein levels were determined using the

Bio-Rad assay (Richmond, California).

Ethoxyresorufin-O-deethylase activity

Ethoxyresorufin-O-deethylase (EROD) activity was measured in fetal hepatic microsomes according to the method of Burke *et al.* (1977), with slight modifications. The reaction was carried out at 37° C with a final concentration of ethoxyresorufin and NADPH of 2 μM and 0.1 mM, respectively. The final microsomal protein concentration was 100 μg/ml for all dose groups. To detect the deethylation product, resorufin (RR), the excitation wavelength of the fluorimeter was set at 530 nm while the emission wavelength was 580 nm.

Statistics

The statistical evaluation of plasma thyroxine levels and thyroid hormone metabolism was made with a one sided Student's t-test. The t-values were adjusted for non-homogeneity of the variances.

Results

Effects on plasma T_4 levels.

Maternal plasma TT_4 and FT_4 levels were significantly reduced at a dose of 0.6 and 1.8 mg HCB/kg bw on day 12 of gestation and by the combined dose of 0.6 mg HCB/kg bw with 1 mg/kg bw TCB daily (Figure 4.1 and 4.2). The combined dose of HCB and TCB resulted in a significantly larger decrease in plasma TT_4 levels (25% of controls) than the same dose of HCB alone (75% of controls,) or the highest HCB dose group (65% of controls, Figure 4.1). On day 20 of gestation only the highest HCB dose and the combined dose of HCB and TCB significantly decreased maternal plasma TT_4 and FT_4 levels (Figure 4.1 and 4.2) , while 7 days after giving birth, plasma TT_4 levels had recovered in all groups except for the high HCB dose group (Figure 4.1).
Fetal plasma TT_4 and FT_4 levels on day 20 of gestation (Figure 4.3 and 4.4) exhibited a similar response to the treatments as the maternal levels, although only the combined dose of HCB and TCB resulted in a significant reduction of plasma TT_4 levels (Figure 4.3 and 4). Control levels of both TT_4 and FT_4 were 6 times lower in the fetus than in the maternal rat.

Neonatal plasma TT_4 and FT_4 levels increased to adult levels by day 21, and there was essentially no difference between male and female values within a given group (Figure 4.3 and 4.4). Relative decreases in plasma TT_4 7 days after birth were greatest (66% of controls) in the group recieving the combined dose of HCB and TCB, while 21 days after birth the largest decreases in TT_4 levels (68% of controls) were observed in the highest HCB dose group (Figure 4.3). Decreases in plasma FT_4 levels were similar on day 7 and day 21 postpartum for both the 1.8 mg HCB/kg bw and combined HCB and TCB dose groups (66-78% of controls, Figure 4.3). The prenatal administration of 0.2 and 0.6 mg HCB/kg bw had no effect on neonatal plasma TT_4 and FT_4 levels at the time of observation (Figure 4.3 and 4.4).
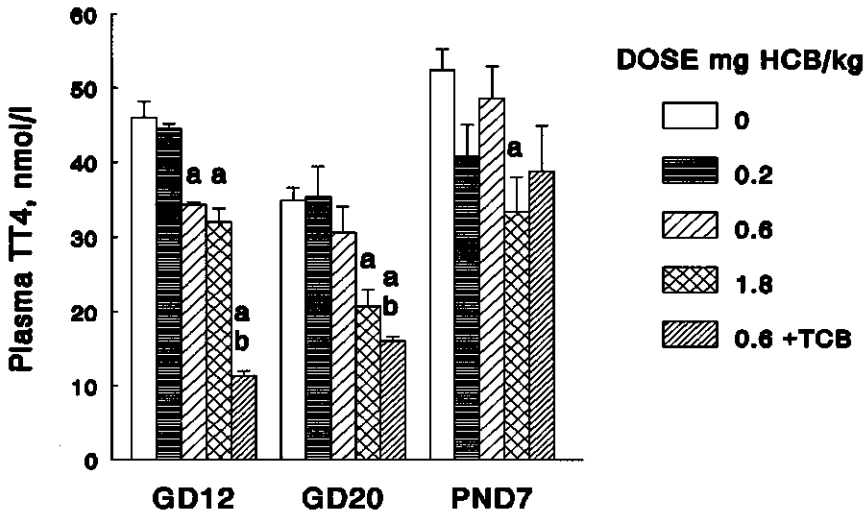
Figure 4.1
Plasma levels of maternal total thyroxine (TT_4) in pregnant and lactating rats following prenatal HCB/TCB exposure. (a) indicates values significantly different from control values (P<0.05), (b) indicates a significant difference between the combined dose group of 0.6 mg HCB/kg and TCB and the group that recieved 0.6 mg HCB alone (P<0.05), GD: gestation day, PND: postnatal day.

Figure 4.2
Plasma levels of maternal free thyroxine in pregnant and lactating rats following prenatal HCB/TCB exposure. (a) indicates values significantly different from control values (P<0.05), (b) indicates a significant difference between the combined dose group of 0.6 mg HCB/kg and TCB and the group that recieved 0.6 mg HCB alone (P<0.05).

Figure 4.3
Plasma levels of total thyroxine (TT_4) in fetal and neonatal rats following prenatal HCB/TCB exposure. (a) indicates values significantly different from control values (P<0.05), (b) indicates a significant difference between the combined dose group of 0.6 mg HCB/kg and TCB and the group that recieved 0.6 mg HCB alone (P<0.05), GD: gestation day, PND: postnatal day.
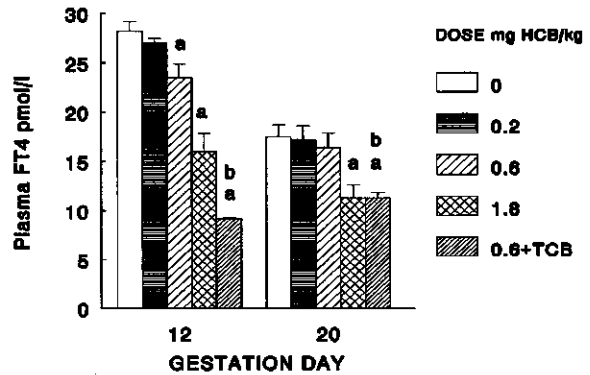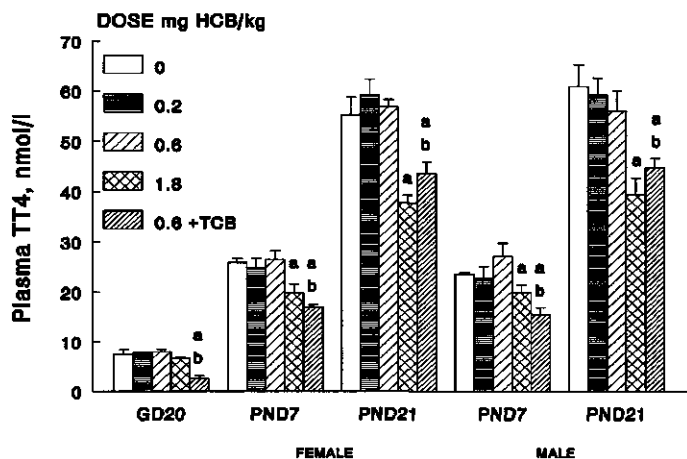

Figure 4.4
Plasma levels of free thyroxine (FT_4) in fetal and neonatal rats following prenatal HCB/TCB exposure. (a) indicates values significantly different from control values (P<0.05), (b) indicates a significant difference between the combined dose group of 0.6 mg HCB/kg and TCB and the group that recieved 0.6 mg HCB alone (P<0.05), GD: gestation day, PND: postnatal day.

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Hepatic T_4 Glucuronidation.

Specific maternal hepatic microsomal T_4 glucuronyl transferase (T_4G) activity (pmol T_4 glucuronide formed per min per mg protein) was measured only on day 12 of gestation. The induction of T_4G increased from 150% in the 0.2 mg HCB/kg bw group to over 400% in the 1.8 mg HCB/kg bw and the combined HCB and TCB dose group (Figure 4.5). The daily administration of 1 mg TCB/kg bw resulted in a significant additional induction of T_4G than 0.6 mg HCB/kg bw alone (Figure 4.5).

Fetal T_4 glucuronyl transferase activity on day 20 of gestation was significantly increased relative to controls in all dose groups (Figure 4.6). Levels of fetal T_4G were 15-20% of maternal T_4G for each dose group. There was a dose dependent increase in neonatal T_4G activity by prenatal HCB administration 7 and 21 days after birth (Figure 4.6) similar to that observed in microsomes from maternal livers from day 12 of gestation. The co-administration of TCB and HCB resulted in additional induction of T_4G relative to the administration of HCB alone on day 7 postpartum in both male and female neonates, but not on day 21 postpartum for female neonates. Control levels of T_4G activity were similar for male and female neonates on day 7 to day 21 postpartum, and were 5 times higher than fetal control levels (Figure 4.6).

Brain Type II thyroxine 5'-deiodinase

Maternal type II thyroxine 5'-deiodinase (5'D-II) activity was significantly increased relative to controls in whole brain homogenates from pregnant rats (day 20 of gestation) which had recieved 1.8 mg HCB/kg or the combined dose of HCB and TCB (Figure 4.7). On day 7 postpartum levels of maternal 5'D-II were generally lower than day 20 of gestation. Maternal 5'D-II levels on day 7 postpartum remained increased relative to controls in the highest HCB dose group and the combined dose group.

Figure 4.5
Specific T_4 glucuronidation activity in hepatic microsomes from pregnant rats on day 12 of gestation following HCB/TCB administration. (a) indicates values significantly different from control values (P<0.05), (b) indicates a significant difference between the combined dose group of 0.6 mg HCB/kg and TCB and the group that recieved 0.6 mg HCB alone (P<0.05).

Figure 4.6
Specific T_4 glucuronidation activity in hepatic microsomes from fetal and neonatal rats following maternal HCB/TCB administration. (a) indicates values significantly different from control values (P<0.05), (b) indicates a significant difference between the combined dose group of 0.6 mg HCB/kg and TCB and the group that recieved 0.6 mg HCB alone (P<0.05), GD: gestation day, PND: postnatal day).
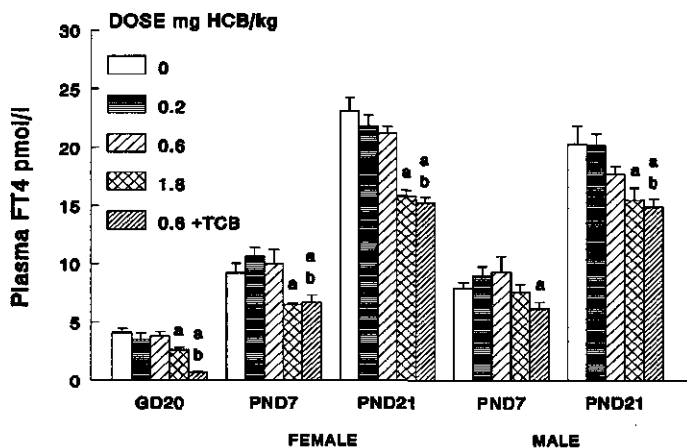
Figure 4.7
Specific type II thyroxine 5'-deiodinase activity in whole brain homogenates from pregnant and lactating rats following prenatal administration of HCB/TCB. (a) indicates values significantly different from control values (P<0.05), (b) indicates a significant difference between the combined dose group of 0.6 mg HCB/kg and TCB and the group that recieved 0.6 mg HCB alone (P<0.05).

An effect on 5'D-II activity in whole brain homogenates of fetuses from gestational day 20 was observed only in the combined HCB/TCB dose group (Figure 4.8). The increase (110%) was significant compared to controls as well as the group that received 0.6 mg HCB/kg alone. Neonatal brain 5'D-II was increased in whole brain homogenates from male neonates from the highest HCB group, while female neonatal 5'D-II was increased in both the highest HCB dose group as well as in the combined
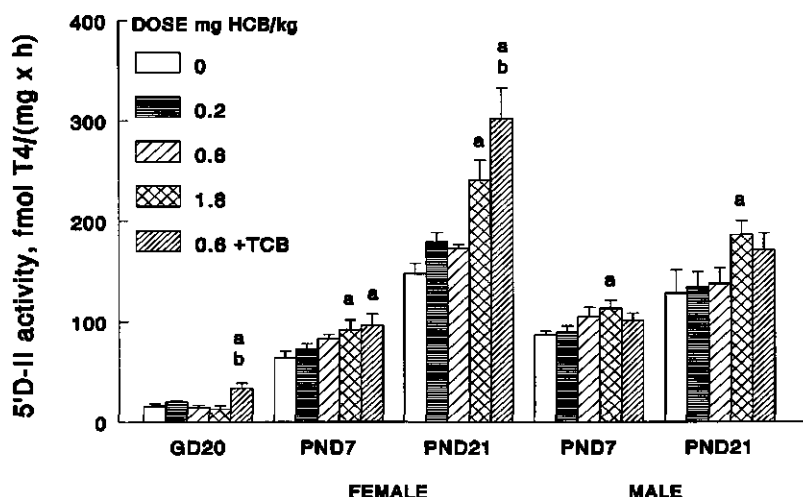
Figure 4.8
Specific type II thyroxine 5'-deiodinase activity in whole brain homogentes from fetal and neonatal rats following prenatal administration of HCB/TCB.(a) indicates values significantly different from control values (P<0.05), (b) indicates a significant difference between the combined dose group of 0.6 mg HCB/kg and TCB and the group that recieved 0.6 mg HCB alone (P<0.05), GD: gestation day, PND: postnatal day.

dose group on postnatal day 7 and 21 (Figure 4.8). Seven days after birth, female neonatal 5'D-II activity was significantly higher than controls) in the highest HCB and combined dose group, while for males the only relative increase (130% of controls) was in the 1.8 mg HCB/kg dose group. On postnatal day 21, the greatest increase in brain 5'D-II was observed in female neonates in the combined dose group (100%) while in male neonates only the 1.8 mg HCB group exhibited a significant increase in 5'D-II.

Ethoxyresorufin-O-deethylase activity.
        EROD activity was not detected with fetal microsomes from the control and the lowest dose level. Low levels of EROD activity (0.002 ± 0.001 nmol RR per mg per min) were detected in fetal microsomes from the group that recieved 0.6 mg HCB/kg, while relatively high similar EROD activities were detected in fetal microsomes from the highest HCB dose (0.069 ± 0.015 nmol RR per mg per min) and the combined HCB and TCB dose group (0.081 ± 0.021 nmol RR per mg per min).

Discussion

        Exposure of pregnant rats to either a single dose of HCB alone or to a combination of HCB and daily doses of TCB caused significant, but differential

reductions in fetal and neonatal plasma thyroid hormones, and increases in hepatic T_4 glucuronidation as well as brain 5'D-II activity.

One reason for the apparent differential effects between the single and combined exposure may be that highly chlorinated, poorly metabolizable PCBs, like 3,3',4,4',5,5'-HCB and 2,2',4,4',5,5'-hexachlorobiphenyl are efficiently transferred to the neonate by lactation, while placental transfer is limited (Vodicnik and Lech 1980, 1982). This suggests that HCB exerts its major effects after lactational transfer to the neonate. However, the combined total of the TCB dose, 17 mg, is higher than the HCB dose, and the TCB was administered for the greater part of the gestation, which may in part explain the effect of the combined dose on the rat fetus. Nonetheless, the observation that EROD levels were comparable in the high HCB dose group and the combined HCB and TCB dose group suggests that the toxic potency of parent compound reaching the fetus was similar.

On the other hand, TCB is rapidly metabolized in Wistar rats (Yoshimura *et al.* 1987), and quickly eliminated from neonatal mice (Lucier *et al.* 1978a and Eriksson 1988). Phenolic metabolites accumulate in late gestational fetal C57BL mice (Darnerud *et al.* 1986) and TCB-derived radioactivity accumulates in CD-1 fetal rat plasma (Lucier *et al.* 1978b). Unpublished results from our laboratory indicate that prenatal administration of TCB leads to a substantial accumulation of 3,3',4',5-tetrachloro-4-biphenylol in the fetal Wistar rat. This metabolite, which competes with T_4 for transthyretin binding sites, has been indicated as the causitive agent of the severe plasma thyroid hormone reductions observed in TCB-exposed adult rats (Brouwer and Van den Berg 1986, Brouwer *et al.* 1990).

In fetuses, only the combined dose of HCB and TCB caused a severe reduction of plasma TT_4 levels, while induction of hepatic microsomal T_4G activity reached a plateau by the lowest dose group, 0.2 mg HCB/kg bw. The levels of T_4G in the fetus are 5 times lower than in neonates and 10 times lower than in pregnant rats from day 12 of gestation. These results suggest that T_4 glucuronidation plays a minor role in the reduction of plasma T_4 levels in the fetus. The severe reductions in maternal TT_4 levels induced by the combined dose of TCB and HCB may have contributed to low fetal TT_4 levels due to a reduction of transplacental T_4 delivery, while the transport of phenolic metabolites of TCB to the fetus will aggrevate this effect.

However, in the maternal and neonatal situation, HCB concentrations should be much higher than in the fetus, which in combination with higher basal levels of T_4G leads to much higher T_4G activities in HCB-treated animals. Therefore it is anticipated that the observed decline in neonatal and maternal plasma TT_4 levels by HCB are caused by increased T_4 glucuronidation.

The observed induction of 5'D-II activity in fetal and neonatal rats following prenatal HCB and TCB administration suggests a compensatory mechanism to maintain brain T_3 levels while plasma and tissue T_4 levels are depressed (Ruiz de Oña *et al.* 1988, Silva and Matthews 1984). Fetal rat brain T_3 levels are entirely dependent on local deiodination of T_4 (Ruiz de Oña *et al.* 1988, Calvo *et al.* 1990). The treatment of maternal rats with methimazole (MMI), a compound which inhibits thyroid hormone synthesis and can cross the placenta, results in decreased fetal brain T_4 and T_3 levels accompanied by increased 5'D-II activities (Ruiz de Oña *et al* 1988). Only when dams were infused

with T_4 did the fetal brain T_3 levels from MMI treated dams reach control levels. It is therefore possible that situations may occur where an increase in 5'D-II activity following maternal HCB and/or TCB administration is insufficient to maintain normal T_3 levels in the developing rat brain unless extra-physiological T_4 is supplied. In this experiment, since we did not measure levels of brain thyroid hormones, we can only suggest that the observed increase in 5'D-II activity indicates a local hypothyroidism in the brain.

Since these effects are observed during a period when thyroid hormones play an essential role in brain development, it is proposed that alterations in fetal and neonatal thyroid hormone levels may be involved in some of the developmental neurotoxicity of PCBs and related chemicals. Further studies are in progress to relate low brain thyroid hormone levels and selected neurochemical parameters indicative of structural and biochemical aspects of brain development.

CHAPTER 5

ALTERATIONS IN RAT BRAIN THYROID HORMONE STATUS FOLLOWING PRE- AND POSTNATAL EXPOSURE TO POLYCHLORINATED BIPHENYLS

Abstract

The effects of oral maternal exposure to 0, 5 or 25 mg/kg body weight of a polychlorinated biphenyl (PCB) mixture (Aroclor 1254) on day 10 to 16 of gestation on plasma and brain thyroid hormone concentrations and peripheral thyroid hormone metabolism were examined in fetal and weanling rats. Plasma thyroid hormone levels and hepatic microsomal thyroid hormone glucuronidation were also examined in pregnant rats and the adult offspring. Plasma and brain levels of PCBs and hydroxylated PCB metabolites were analysed in fetal, weanling and adult offspring.

Maternal exposure to Aroclor 1254 significantly decreased fetal and neonatal plasma total thyroxine (T_4) and free thyroxine levels in a dose dependent manner. Effects of maternal Aroclor 1254 exposure on plasma total and free T_4 concentrations were less pronounced in offspring at 21 days of age and absent 90 days after birth. Plasma concentrations of thyroid stimulating hormone were unaltered in the offspring following maternal treatment with Aroclor 1254. The concentration of T_4 was severely depressed in the forebrain and cerebellum of fetal rats on day 20 of gestation following maternal Aroclor 1254 exposure. Brain triiodothyronine (T_3) concentrations in the Aroclor exposed fetuses were significantly decreased relative to control values only in the low dose group. On day 21 postpartum T_4 concentrations were significantly decreased in the forebrains of female weanling rats from the 25 mg Aroclor 1254/kg dose group, and no reductions were observed in forebrain T_3 concentrations in male or female neonates.

The deiodination of T_4 to T_3 was significantly increased by both PCB treatments in fetal forebrain homogenates. In female weanling brain homogenates the deiodination of T_4 to T_3 was significantly decreased in the low dose group, and unaltered in the high dose group. No alterations in brain thyroid hormone metabolism were observed in forebrain homogenates from adult offspring exposed pre- and postnatally to Aroclor 1254. Hepatic microsomal T_4 glucuronidation was significantly decreased in fetal microsomes following perinatal PCB exposure, and significantly increased in weanling hepatic microsomes in a dose dependent manner.

A significant accumulation of one PCB metabolite, 2,3,3',4',5-pentachloro-4-biphenylol was observed in fetal plasma and forebrain on gestation day 20, and in neonatal and weanling rat plasma on postnatal day 4 and 21. Although PCB levels were relatively high in the weanling rat forebrain, no hydroxylated PCB metabolites were detected. On day 90 postpartum, plasma levels of PCBs and 2,3,3',4',5-pentachloro-4-biphenylol were still elevated in the offspring of PCB-treated dams relative to controls.

These results indicate that the accumulation of hydroxylated PCB metabolites in fetal plasma can reduce fetal plasma T_4 levels and accordingly fetal brain T_4 levels. However, in late gestational fetuses, the induction of brain 5'D-II activity compensates for decreases in brain T_4 levels, so that reductions in brain T_3 levels are minimal.

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Introduction

It has been hypothesized that the ability of polychlorinated biphenyls to induce hypothyroxinemia in mammals may be related to some aspects of the developmental neurotoxicity of these compounds (Rogan *et al.* 1986, Morse *et al.* 1993a). PCB exposure has been associated with alterations in thyroid function in transformer repair workers (Emmett *et al.* 1988) and in human infants (Pluim *et al.* 1992, and Koopman-Esseboom *et al.* in press). Both PCB mixtures, such as Aroclor 1254 (Collins and Capen, 1980a) and individual PCB congeners of varying structure, such as 3,3',4,4'-tetrachlorobiphenyl, 2,3',4,4',5-pentachlorobiphenyl, 2,2',4,4',5,5'-hexachlorobiphenyl and 3,3',4,4',5,5'-hexachlorobiphenyl (Ness *et al.* 1993, Morse *et al*, 1993) are capable of reducing plasma thyroxine in neonatal and weanling rats.

Recent research from this laboratory (Morse *et al.* 1993a) has shown that perinatal exposure to coplanar PCBs (3,3',4,4',5,5'-hexachlorobiphenyl and 3,3',4,4'-tetrachlorobiphenyl) results in decreased fetal, neonatal and weanling plasma total thyroxine (T_4) and free T_4 concentrations accompanied by an increase in the activity of type II thyroxine 5'-deiodinase (5'D-II) in brain homogenates, the enzyme responsible for the conversion of T_4 to triiodothyronine (T_3, Visser *et al.* 1982, Kaplan *et al.* 1983). The observed increase in activity of 5'D-II is a compensatory response of the brain to maintain brain T_3 levels when T_4 concentrations are decreased, for the brain is dependent on the local deiodination of T_4 for the production of T_3 (Calvo *et al.* 1990). We therefore suggested that following pre- or postnatal PCB exposure a situation can occur in which the increase in 5'D-II can no longer compensate for decreased T_4 levels in the brain, resulting in decreases in T_3 concentrations. T_3 is currently regarded as the biologically most active hormone (Davis, 1991).

In order to test our hypothesis, we administered the technical PCB mixture Aroclor 1254 to pregnant rats on day 10 to 16 of gestation and examined the effects on brain T_4 and T_3 levels in fetuses and weanlings, as well as plasma thyroid hormone levels and brain thyroid hormone metabolism in fetuses, weanlings and adult offspring. We chose to use a technical PCB mixture instead of individual PCB congeners, because a wide range of PCB congeners can reduce plasma T_4 concentrations. To gain insight in the mechanisms involved in the decrease of plasma thyroid hormone levels we measured hepatic microsomal T_4 glucuronidation and the plasma and brain levels of PCBs and

hydroxylated PCB metabolites.

Material and Methods:

Animals

Wistar WU rats, 100 females and 50 males, (14 weeks old) were purchased from Charles River Sutzfeld, Germany. The rats were allowed to acclimatize for 2 weeks and were maintained at 50% humidity and 21°C on bedding in plastic cages with a 12 hour light cycle. Rat chow (Hope Farms, Woerden, the Netherlands) and tap water were supplied *ad libitum*. After the acclimatization period two females were placed in a cage with one male overnight from 17:00 to 9:00 the next day. The females were examined each morning by vaginal smear. When spermatozoa were found the animal was housed separately and this was termed day 0 of gestation. Animals were pre-assigned to a particular dose group on a rotating basis. Maternal body weight gain was monitored throughout gestation and lactation. On day 10 of gestation the pregnant female rats were transferred to a macrolon cage with a grated steel support to facilitate the collection of PCB-contaminated feces and to prevent contamination of the animal facilities. Pregnant females received a daily oral dose of 0, 5 or 25 mg Aroclor 1254 per kg body weight dissolved in cornoil (2 ml/kg body weight) from day 10 of gestation to day 16 of gestation. Rats were weighed each day before administration of Aroclor 1254 or corn oil alone.

On day 20 of gestation 6 rats were sacrificed per treatment group under ether anesthesia, maternal blood was collected from the vena cava. Fetuses were removed, washed in 0.9% NaCl, blotted dry and weighed. Fetal blood was collected by decapitation and the fetal livers were removed, blotted dry, weighed and frozen on dry ice. Fetal brains were removed, the forebrain was separated from the cerebellum. Both brain regions were weighed and then frozen on dry ice. Fetal cerebella were pooled for thyroid hormone analysis. One fetal forebrain was saved separately for thyroid hormone analysis, the remaining forebrains were pooled for analysis of thyroid hormone metabolism. Fetal blood was pooled for thyroid hormone analysis. Blood was collected in heparinized tubes and stored on ice until plasma was prepared by centrifugation. Livers and brain tissue were stored at -80 °C and plasma at -20 °C until analysis.

The remaining rats were transferred to bedding material on day 20 of gestation and were given paper tissues to make a litter. The rats were inspected each morning at 8:00 and afternoon at 18:00 for litters. Pups found during the morning inspection were termed 1 day old, pups born between the morning and afternoon inspection were termed 0 day old. Pups were examined for sex, weighed on day 0 postpartum when possible, and always on day 1, 4, 7 postpartum and thereafter on a weekly basis. On day 4 postpartum litters were reduced to 4 males and 4 females. When this was not possible, litters were made up of a total of 8 males and females from the same dam, and if necessary, an extra pup from another litter from the same treatment group and same age was used to achieve a total of 8 pups per litter. Pups were individually marked by incision in the ear, and pups transferred from one litter to another were never used for any analysis. Plasma was collected from pups sacrificed on day 4 postpartum for analysis of thyroid hormones.

On day 21 postpartum, 8-10 litters were sacrificed per treatment group. Dams

and weanlings were sacrificed by decapitation, trunk blood was collected in heparinized tubes, and livers were removed and stored as described above. Weanling livers were pooled per sex for analysis. Trunkblood was collected separately for each weanling. Weanling brains were rapidly removed, and the forebrains were separated from the cerebellum and stored separately at -80°C.

The offspring from the remaining litters were weaned on postpartum day 21 and male and female rats were housed separately in groups of 2-4 rats per cage. The remaining animals (10 litters per treatment group) were sacrificed 90 days after birth by decapitation. Forebrains, cerebella and trunk blood were collected separately for each rat, and 1 liver per sex was analysed per litter.

Thyroid hormone analysis

Plasma total T_4 (TT_4), free T_4 (FT_4) and total T_3 (TT_3) were analysed in duplicate using a chemiluminesence kit and plasma thyroid stimulating hormone (TSH) concentrations were analysed in duplicate with a specific rat TSH radioimmunoassay kit, both kits were purchased from Amersham, Amersham, U.K.

Brain concentrations of T_4 and T_3 were determined by extraction and purification of the hormones, followed by a radioimmunoassay according to Morreale de Escobar *et al*. (1985). Briefly, tissues were homogenized in methanol containing approximately 2000 cpm of $[^{131}I]T_4$ and $[^{125}I]T_3$ as internal standards. Tissue lipids were removed by extraction of the homogenate with chloroform, followed by a back-extraction of thyroid hormones with an aqueous phase. The aqueous phase was further purified on Bio-Rad AG 1-X2 resin, 200-400 mesh (Bio-Rad, Richmond, CA), and the iodothyronines were eluted with 70% acetic acid, which was then evaporated to dryness. Each sample was then analysed in duplicate at two dilutions in a highly specific radioimmunoassay for T_4 or T_3. The limit of sensitivity was 2.5 pg T_4 and 1.5 pg T_3 per tube.

In this experiment, fetal brains from 6 dams per treatment group were analysed for T_3 and T_4. One fetal forebrain from two different dams from the same treatment group were pooled prior to extraction, while all fetal cerebella were pooled from each dam and each pool was extracted separately. One male and one female weanling forebrain (postnatal day 21) per litter (8-10 litters per group) were extracted separately.

Thyroid hormone metabolism

Brain type II thyroxine 5'-deiodinase activity was analysed essentially as described by Visser *et al*. (1982) with slight modifications (Morse *et al*. 1993a). The final incubation conditions were 100 mM phosphate buffer, pH 7.2, 25 mM dithiotreitol, 1 mM propyl-2-thiouracil, 1 mM EDTA, 2 nM T_4 with 10^5 cpm $[^{125}I]$-T_4, 500 nM T_3 and brain homogenate (0.8 mg protein/ml) in a total volume of 200 μl. Preliminary experiments using reversed phase high preformance liquid chromatography and Sephadex LH-20 analysis of the reaction products revealed that the addition of 500 nM T_3 to the incubation mixture inhibited the inner-ring deiodination of T_4 to reverse triiodothyronine (rT_3) without decreasing the outer-ring deiodination of T_4 to T_3.

Inhibition experiments were carried out by adding the rT_3, or the PCBs (Aroclor 1254, 3,4,3',4'-tetrachlorobiphenyl (PCB 77), or hydroxylated PCB metabolites, 3,3',4',5-tetrachloro-4-biphenylol (metabolite of PCB 77, Yoshimura *et al*. 1987) and 2,3,3',4,5'-pentachloro-4'-biphenylol (metabolite of PCB 105, Klasson Wehler *et al*.

1993) (0.1 and 1 µM, final concentration) in 10 µl methanol to 400 µl incubations with homogenates of forebrains pooled from control female neonates (day 21 postpartum) and the activity was compared with that of incubations with methanol only. The addition of 2.5% methanol (v/v) had no effect on the deiodinase activity compared to control incubations.

Hepatic microsomal T_4 uridine diphosphoglucuronyl transferase (T_4-UDPGT) activity was determined in microsomal incubations with [^{125}I]-T_4 as described by Beetstra *et al.* (1991) with slight modifications (Morse *et al.* 1993a).

Analysis of PCBs and hydroxy-PCB metabolites in plasma and brain

Trunk blood was collected from the dams and offspring and plasma was prepared as described above and pooled per treatment group for the dams and fetuses. Plasma from neonatal, weanling and adult offspring were pooled per sex and treatment group. Forebrain homogenates prepared for deiodinase activity measurements was pooled per treatment group for fetuses and per sex and treatment group for weanling and adult offspring. Plasma and forebrain homogenates were freeze-dried prior to extraction.

Freeze-dried plasma (ca. 1 g dry weight, corresponding to ca. 20 ml) was dissolved in 5 ml water, then 5 ml methanol was added and the samples were extracted and analysed according to a previously described method (Bergman *et al.* 1994). The freeze-dried brains (ca. 7 g dry weight) were soaked in water (5 ml) and extracted and analysed by the same procedure as the plasma samples. Total PCB levels were calculated on the basis of the following peaks: CB-99, CB-118/149, CB-146, CB-153, CB-105, CB-138, CB-128, CB-180, CB-170 and when the concentration was sufficient CB-183 and CB-187 were included. Since all chromatograms were nearly identical with respect to the congener pattern, the PCB data are presented as the level of CB-153 in the plasma or brain. CB-153 constituted 20.3 $\pm$ 3.7% (mean $\pm$ SD) of total PCBs (mass basis) in plasma samples and 20.6 $\pm$ 3.9% (mean $\pm$ SD) in brain samples.

Statistics

Dose dependent effects were first examined with a one-way analysis of variance followed by a modified least significant difference test, and correlations between hepatic glucuronidation and plasma thyroid hormone levels were evaluated using Pearson correlation coefficients. The statistical software package SPSS was used for the analysis.

Results

Plasma and brain PCB and hydroxy-PCB levels

The major hydroxylated PCB metabolite, determined as its methylated derivative, found in all plasma samples and the fetal brain samples, was 4-MeO-2,3,5,3',4'-pentachlorobiphenyl (4-MeO-pentaCB). 4-MeO-pentaCB constituted more than 73% of all phenolic metabolites (mass basis) present in the samples until day 90 postpartum. Plasma levels of 4-MeO-pentaCB and CB-153 are presented in Table 5.1 and brain levels of 4-MeO-pentaCB and CB-153 are presented in Table 5.2.

There was a substantial accumulation of 4-MeO-pentaCB in the fetal compartment, with a fetal/maternal plasma ratio of 3.5 in the 5 mg/kg dose group and of 2.1 in

Table 5.1
Pooled plasma levels (ppb fresh weight) of 4-MeO-2,3,3'4',5-pentachlorobiphenyl (4-MeO-pentaCB) and 2,2',4,4',5,5'-hexachlorobiphenyl (CB-153) in dams, fetuses, neonates, weanling rats and young adult offspring.

	Dose (mg Aroclor 1254/kg per day)		
	0	5	25
Fetus (GD20)			
4-MeO-pentaCB	2	600	1600
CB-153	3	40	110
Dam (GD20)			
4-MeO-pentaCB	<1	170	750
CB-153	1	45	170
Male (PND4)			
4-MeO-pentaCB	8	510	1600
CB-153	5	120	590
Female (PND4)			
4-MeO-pentaCB	<2	410	1200
CB-153	10	140	670
Male (PND21)			
4-MeO-pentaCB	150	260	120
CB-153	26	26	52
Female (PND21)			
4-MeO-pentaCB	94	300	150
CB-153	31	31	56
Dams (PND21)			
4-MeO-pentaCB	15	45	100
CB-153	8	4	13
Male (PND90)			
4-MeO-pentaCB	<0.1	5	4
CB-153	0	3	4
Female (PND90)			
4-MeO-pentaCB	<0.2	4	3
CB-153	0	1	5

Pregnant rats were treated with Aroclor 1254 on day 10-16 of gestation. GD20: gestation day 20, PND4: postnatal day 4, PND21: postnatal day 21, PND90: postnatal day 90.

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the 25 mg/kg dose group. In the fetal plasma the concentration of 4-MeO-pentaCB (0.6 and 1.6 ppm, fresh wt. basis in 5 and 25 mg/kg dose groups, respectively) exceeded the concentration of the persistent PCB congener 2,4,5,2',4',5'-hexachlorobiphenyl (CB 153) by a factor of 15. Although the concentration of 4-MeO-pentaCB was approximately 10-fold lower in the fetal brains than plasma (fresh wt. basis), it was still 4.2 and 2.4 fold greater than the concentration of CB-153 in the brain in the 5 and 25 mg/kg dose groups, resepectively. On day 20 of gestation maternal plasma also exhibited a high ratio of 4-MeO-pentaCB to CB 153 (3.8 and 4.4 in the low and high exposure groups, respectively).

The highest plasma concentrations of CB 153 were observed in neonatal plasma from day 4 postpartum (approximately 0.13 and 0.63 ppm, fresh weight basis, 5 and 25 mg/kg dose group, respectively). Plasma concentrations of 4-MeO-pentaCB were similar in 4 day old pups and fetuses from day 20 of gestation.

Table 5.2
Brain levels of 4-MeO-pentaCB and CB-153 in pooled samples from fetuses, weanling rats and young adult offspring.

	Dose Aroclor (mg/(kg x day)	CB-153 ppm lipid	CB-153 ppb fw	4-MeO-pentaCB ppm lipid
			GD20	
Fetus	0	0.04	0.8	nd
	5	1.0	24	4.2
	25	3.9	66	9.3
			PND21	
Male	0	0.08	3.2	nd
	5	1.2	36	nd
	25	3.9	156	nd
Female	0	0.04	1.9	nd
	5	1.1	55	nd
	25	4.0	200	nd
			PND90	
Male	0	0.003	0.1	nd
	5	0.15	13	nd
	25	0.15	13	nd
Female	0	0.006	0.4	nd
	5	0.12	7.3	nd
	25	0.45	35	nd

4-MeO-pentaCB: 4-MeO-2,3,3'4',5-pentachlorobiphenyl, CB 153: 2,2',4,4',5,5'-hexachlorobiphenyl, nd: not detected, less than 2 ppb lipid weight, GD20: gestation day 20, PND21: postnatal day 21, PND90: postnatal day 90.

Both plasma CB 153 and 4-MeO-pentaCB concentrations decreased 10-fold between day 4 and day 21 postpartum in neonates from the high exposure group and decreased 5-fold in neonates from the low exposure group (plasma 4-MeO-pentaCB concentrations: 0.28 and 0.14 ppm fresh wt. in the 5 and 25 mg/kg dose groups, respectively). However, control plasma CB 153 and 4-MeO-pentaCB levels increased over the same period, and were similar to concentrations observed in PCB-exposed animals from the 5 mg/kg exposure group. Furthermore, 4-MeO-pentaCB plasma levels were a factor 2 higher in the low dose group than in the high exposure group. Weanling levels of CB 153 (1.1 and 4.0 ppm lipid wt basis, 5 mg/kg and 25 mg/kg dose group, respectively) on day 21 postpartum were similar to fetal brain levels. 4-MeO-pentaCB was below the limit of detection (< 2 ppb) in weanling brains 21 days after birth from all treatment groups.

A 10-fold decrease in plasma CB 153 and 4-MeO-pentaCB concentrations in both Aroclor exposed groups was observed between postnatal day 21 and 90, resulting in concentrations in the low ppb range (2 and 4 ppb, fresh wt. in the 5 and 25 mg/kg dose groups, respectively). Brain levels of CB 153 remained elevated in the Aroclor 1254 exposed groups, averaging 140 ppb and 300 ppb on a lipid wt. basis in the 5 mg/kg and 25 mg/kg dose groups, respectively.

Plasma thyroid hormones

The results of plasma thyroid hormone analysis are presented in Table 5.3. Maternal plasma total thyroxine (TT_4) and free thyroxine (FT_4) levels were unaffected by the low PCB dose and significantly reduced relative to controls (TT_4: 50%, FT_4: 58%) only by the highest PCB dose on day 20 of gestation. Maternal plasma total triiodothyronine (TT_3) levels were reduced by 27% relative to controls in the highest PCB exposure group. Fetal plasma thyroid hormone levels were more sensitive to maternal PCB exposure than maternal thyroid hormone levels. Significant reductions of 50% and 74% were observed in fetal TT_4 levels and fetal plasma FT_4 was reduced by 70% and 80% relative to controls in the low and high PCB dose group, respectively.

Four days after birth, male and female neonatal TT_4 levels were significantly reduced relative to controls by both PCB treatments. FT_4 levels were not affected by PCB treatment in male neonates on day 4 postpartum, while female neonatal FT_4 levels were significantly decreased by 50% relative to controls by the high PCB dose. Plasma TT_3 levels were decreased in both male and female neonates from the highest PCB treatment group on day 4 postpartum, but this reduction was significant only in the males.

On day 21 postpartum, maternal TT_4 and FT_4 levels were unaffected by the administration of PCB during gestation, and a slight but significant decrease in plasma TT_3 concentrations was observed only in dams from the low PCB exposure group. Plasma TT_4 were significantly reduced by 36% and 32% respectively in male and female weanlings from the highest PCB dose group 21 days postpartum. Plasma FT_4 levels were significantly reduced (25%) only in female weanlings from the highest dose group on day 21 postpartum. Male and female weanling TT_3 concentrations were significantly reduced by approximately 16% in the high PCB dose group, and female weanling TT_3 was also significantly decreased (10%) in the low PCB dose group.

Plasma thyroid hormone levels were generally unaltered relative to controls in

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Table 5.3
Effect of maternal Aroclor 1254 exposure on thyroid hormone levels in the dams and offspring.

| | Age | Dose Aroclor 1254, mg/kg body weight per day | | |
		0	5	25
		TT_4 nM		
maternal	GD20	25.3±1.24	23.5±2.0	12.7±1.2*
fetal	GD20	5.4±0.1	2.6±0.4*	1.4±0.3*
male	PND4	21.9±1.9	17.4±0.8*	11.7±0.8*
female	PND4	22.9±2.1	15.7±1.9*	8.2±1.2*
maternal	PND21	34.5±2.5	33.7±1.6	31.4±2.3
male	PND21	42.3±4.2	38.4±2.1	26.9±3.3*
female	PND21	41.9±3.6	39.9±1.2	28.5±3.6*
male	PND90	63.4±2.2	71.0±2.3*	67.6±2.0
female	PND90	52.3±3.3	50.8±3.5	48.3±3.7
		FT_4 pM		
maternal	GD20	13.4±1.3	10.6±1.4	5.6±1.3*
fetal	GD20	3.7±0.1	1.0±0.1*	0.7±0.1*
male	PND4	4.4±0.3	4.1±0.4	3.9±0.5
female	PND4	6.7±1.2	3.8±0.6*	3.3±0.4*
maternal	PND21	13.9±1.2	13.1±0.6	12.7±1.1
male	PND21	14.2±1.5	14.5±0.5	11.5±1.1
female	PND21	16.5±0.6	16.8±0.4	12.3±1.5*
male	PND90	21.2±1.0	21.1±0.7	21.2±0.8
female	PND90	20.4±1.5	19.1±1.1	18.4±1.5
		TT_3 nM		
maternal	GD20	0.95±0.03	0.86±0.06	0.70±0.06*
male	PND4	0.32±0.02	0.27±0.03	0.15±0.02*
female	PND4	0.28±0.03	0.19±0.05	0.18±0.03
maternal	PND21	0.83±0.03	0.71±0.02*	0.79±0.04
male	PND21	1.28±0.07	1.18±0.05	1.05±0.04*
female	PND21	1.29±0.05	1.15±0.02*	1.09±0.05*
male	PND90	1.03±0.06	1.10±0.07	1.03±0.05
female	PND90	1.05±0.04	0.95±0.05	1.05±0.04

The data are presented as the mean±SEM, an asterix (*) indicates a statistically significant difference with controls (P<0.05). N=6 on gestation day 20 (GD20), N=6-10 on postnatal day 4 (PND4), N=8-10 on postnatal day 21 (PND21), N=10 on postnatal day 90 (PND90).

Table 5.4
Plasma concentration of thyroid stimulating hormone following perinatal exposure of rats to Aroclor 1254.

	Dose Aroclor 1254, mg/kg body weight per day		
	0	5	25
	TSH (ng/ml plasma)		
Gestation day 20			
fetal	6.13 ± 0.05	6.09 ± 0.56	5.96 ± 0.12
Postnatal day 4			
male	5.74 ± 0.11	4.45 ± 0.35	5.43 ± 0.23
female	6.69 ± 0.19	7.10 ± 0.56	6.67 ± 0.25
Postnatal day 21			
male	5.38 ± 0.37	5.52 ± 0.18	5.12 ± 0.28
female	5.51 ± 0.25	5.46 ± 0.27	5.64 ± 0.34
Postnatal day 90			
male	9.10 ± 0.50	9.50 ± 0.67	9.40 ± 0.56
female	7.35 ± 0.61	6.71 ± 0.43	6.07 ± 0.54

The data are presented as the mean$\pm$SEM. N=6 on gestation day 20, N=6-10 on postnatal day 4, N=8-10 on postnatal day 21, N=10 on postnatal day 90.

adult offspring (postnatal day 90) exposed pre- and postnatally to PCB, with the exception of a slight, but statistically significant elevation of plasma TT_4 concentrations (12%) in male offspring from the lowest PCB treatment group.

Plasma levels of thyroid stimulating hormone (TSH) in fetal, neonatal and adult offspring were unaltered by maternal exposure to Aroclor 1254, despite severe reductions in fetal TT_4 and FT_4 levels (Table 5.4). Male levels of plasma TSH were significantly higher than female levels on day 90 postpartum.

Brain thyroid hormone concentrations
T_4 concentrations were reduced by 50% in the fetal cerebella exposed to the low PCB dose, and were below the limit of detection in the cerebella of fetuses exposed to the high PCB dose (Table 5.5). T_3 concentrations in cerebella of control fetuses were 5 fold lower than T_4 concentrations in the fetal cerebella. No significant effect was observed of PCB exposure on T_3 concentrations in the fetal cerebella, although T_3 levels were lower with an increasing dose of Aroclor 1254. In the forebrain T_4 concentrations were also decreased by 60% and 85%, although these decreases were not analysed statistically due to the small group size. One sample in the control group was

Table 5.5

Fetal and neonatal brain levels of thyroid hormones

	Dose Aroclor 1254, mg/kg body weight per day		
	0	5	25

	ng T_4 or T_3/g tissue		
Gestation day 20			
Fetal Forebrain T_4	1.83±0.70(2)	0.67±0.34(3)	0.24±0.01(3)
Fetal Forebrain T_3	0.53±0.02(3)	0.43±0.01(3)*	0.49±0.02(3)
Fetal Cerebella T_4	0.36±0.05(6)	0.17±0.02(6)*	ND(6)
Fetal Cerebella T_3	0.07±0.02(6)	0.06±0.01(6)	0.05±0.01(6)
Postnatal day 21			
Female Forebrain T_4	2.03±0.17(8)	1.64±0.14(7)	1.13±0.15(8)*
Female Forebrain T_3	3.34±0.14(8)	3.22±0.16(7)	3.58±0.25(8)
Male Forebrain T_4	2.68±0.12(8)	2.42±0.15(7)	2.38±0.15(8)
Male Forebrain T_3	3.45±0.19(8)	3.15±0.29(7)	3.11±0.19(8)

The data are presnted as mean±SEM, followed by the number of samples in parentheses, ND: not detected, an asterix (*) indicates a statistically significant difference with controls (P<0.05).

contaminated with T_4 during processing and excluded from the data analysis, resulting in N=2. The concentration of T_3 was significantly decreased (20%) in the forebrain of fetuses in the low exposure group, and unaltered in the fetal forebrains from the high exposure group.

Concentrations of T_4 were reduced by 44% in the forebrains of female weanlings from the high PCB exposure group. The concentration of T_4 was unaffected by PCB treatment in the forebrains of male weanlings, and no reductions in T_3 concentrations were observed in the forebrains of male and female weanlings on day 21 postpartum.

Brain Type II 5'-thyroxine deiodinase activity (5'D-II)

The activity of 5'D-II in fetal forebrain homogenates was significantly increased relative to controls by 35% and 100% in the low and high PCB dose group, respectively (Figure 5.1). In forebrain homogenates from female neonates from the low PCB treatment group on day 21 of gestation 5'D-II activity was significantly decreased by 48% relative to controls and the high dose group. A similar pattern was seen in 5'D-II activity in the forebrains of male neonates, however the activity in the low dose group was significantly lower only from the high dose group. The level of 5'D-II activity was unaffected by perinatal PCB exposure in the forebrains of adult offspring.

Inhibition studies were conducted to investigate the effect of several model compounds on 5'D-II activity, because the pattern of 5'D-II activity in female weanling forebrain homogenates following perinatal PCB exposure was unexpected (decreased
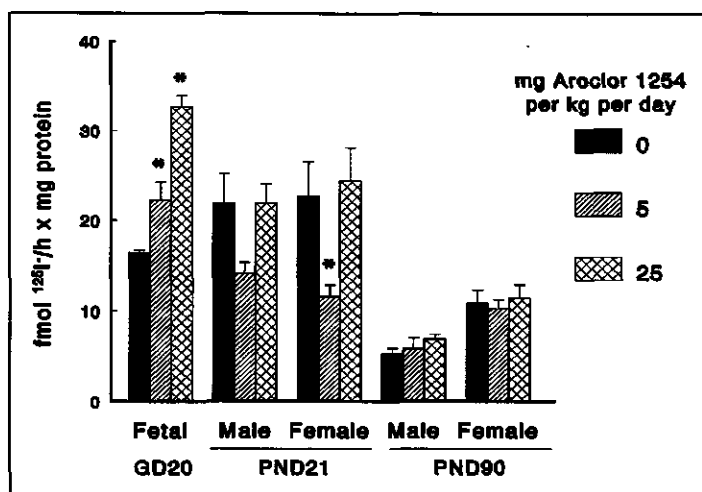
Figure 5.1
Type II thyroxine 5' deiodinase (5'D-II) activity in forebrain homogenates from fetuses, neonates and adult offspring following maternal exposure to Aroclor 1254 on day 10 to 16 of gestation. An asterix (*) indicates a statistically significant difference with controls (P<0.05). GD20: gestation day 20, PND21 postnatal day 21, PND90, postnatal day 90.

Table 5.6
Effect of rT_3, PCBs or PCB metabolites on Type II thyroxine 5' deiodinase activity in forebrain homogenates

Compound	Concentration Added (μM)	Activity (% of control)
Aroclor 1254	0.1	110
	1.0	93
3,3',4,4'-tetrachlorobiphenyl	0.1	98
	1.0	105
3,3',4',5-tetrachloro-4-biphenylol	0.1	101
	1.0	111
2,3,3',4,5'-pentachloro-4'-biphenylol	0.1	103
	1.0	97
3,3',5'-triiodothyronine (rT_3)	0.1	44
	1.0	22

The results are the average of duplicate incubations.

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activity when brain T_4 levels were similar to controls and no increase in activity when brain T_4 levels were depressed).

The activity of 5'D-II in incubations of control female weanling brain homogenates was unaffected by the addition of 0.1 or 1.0 μM Aroclor 1254, 3,3',4,4'-tetrachlorobiphenyl, 3,3',4',5-tetrachloro-4-biphenylol and 2,3,3',4,5'-pentachloro-4'-biphenylol to the incubation mixture (Table 5.6). The positive control, rT_3, added to the incubations at a concentration of 0.1 and 1.0 μM reduced 5'D-II activity by 56% and 77%, respectively.

Hepatic microsomal T_4 glucuronidation

Maternal (day 20 of gestation) hepatic microsomal T_4 uridine diphosphoglucuronyl transfrase (T_4-UDPGT) activity was significantly induced (520% of controls) only by the highest maternal PCB dose (Figure 5.2). A significant negative correlation was observed between maternal T_4-UDPGT activity and plasma TT_4 levels (R: -0.83, P<0.001) and plasma FT_4 levels (R: -0.71, P<0.001). Fetal (day 20 of gestation) hepatic microsomal T_4-UDPGT activity was significantly reduced by 56%
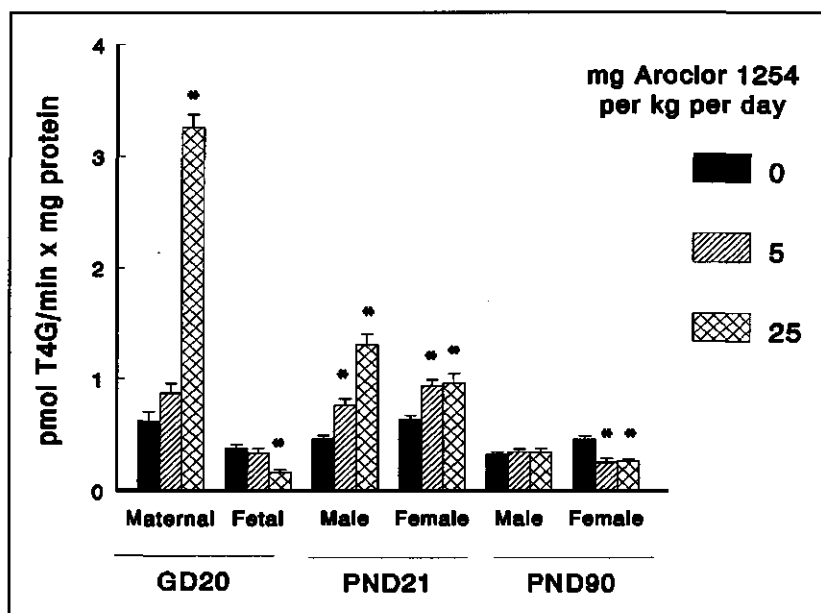
Figure 5.2
Hepatic microsomal T_4 uridine diphosphoglucuronyl transferase (T_4-UDPGT) activity from dams, fetuses, neonates and adult offspring following maternal exposure to Aroclor 1254 on day 10 to 16 of gestation. The activity is expressed as pmol T_4-glucuronide (T_4G) formed per minute per mg microsomal protein (mean + SEM). An asterix (*) indicates a statistically significant difference with controls (P<0.05). GD20: gestation day 20 (N=6), PND21: postnatal day 21 (N=8-10), PND90: postnatal day 90 (N=10).

relative to controls by the highest maternal PCB treatment. At 21 days postpartum male weanling hepatic microsomal T_4-UDPGT activity was significantly induced relative to controls by both the low and high PCB exposure (30% and 120% increase, respectively). Female weanling was significantly increased relative to controls by 30% in both PCB-treatment groups. On day 90 postpartum, no treatment related effects were observed in T_4-UDPGT activity from male offspring, while T_4-UDPGT activity was significantly lower than controls by more than 40% in females from both PCB-exposed groups. No significant correlations were observed between fetal, weanling and young adult T_4-UDPGT activity and plasma thyroid hormone levels.

Discussion

In this study we examined the hypothesis that PCB induced hypothyroxinemia during the perinatal period may result in decreases in brain T_3 levels in the rat. The mechanisms involved in the reduction of plasma and brain thyroid hormone levels were also examined by measuring brain and plasma PCB and hydroxy-PCB levels as well as hepatic microsomal T_4 glucuronidation.

Brain and plasma PCB and hydroxy-PCB levels

The results demonstrate that the exposure of pregnant rats to the technical PCB mixture Aroclor 1254, results in the highly selective accumulation of one hydroxylated PCB metabolite, 4-OH-2,3,5,3',4'-pentachlorobiphenyl (4-OH-pentaCB), in maternal, fetal and neonatal plasma. The placenta does not form a barrier for this compound, for the concentrations were higher in fetal plasma than in maternal plasma on day 20 of gestation. Compared to the levels of 2,2',4,4',5,5'-hexachlorobiphenyl (CB 153), 4-OH-pentaCB readily enters the brain of fetal rats, however, this metabolite could not be detected in the brains of Aroclor 1254-exposed neonatal or adult offspring. This observation indicates that after birth the blood-brain barrier effectively excludes the accumulation of 4-OH-pentaCB in the brain and that 4-OH-pentaCB that had entered the fetal brain is highly diluted in neonates due to rapid postnatal brain growth and/or is mobilized from brain tissue.

The high levels of 4-OH-pentaCB in the plasma relative to the total PCB concentration suggest that 4-OH-pentaCB also has a high affinity binding site in the plasma. We hypothesize that 4-OH-pentaCB is transported over the placenta to the fetus, and that the fetus is then not able to eliminate the compound from its circulation, resulting in the accumulation in the fetal plasma. The accumulation of hydroxylated PCB metabolites in the fetus has been previously demonstrated after maternal exposure to 4-chlorobiphenyl (Lucier *et al.* 1978) and 3,3',4,4'-tetrachlorobiphenyl (Darnerud *et al.* 1986, Morse *et al.* 1995).

In a study by Shain *et al.* (1986) total brain PCB levels (based on a congener specific analysis of 67 different PCB congeners) in newborns was reported as 788 ppb and in day 21 weanlings as 3000 ppb on a fresh wt basis following continous dietary exposure to 30 ppm Aroclor 1254 from day 0 of gestation. The dosing regimen in this study resulted total PCB levels in the fetal brain that are comparable to the newborn rat data from Shain *et al* (1986): 275 ppb and 1235 ppb on a fresh wt basis in the fetal brain following 5 and 25 mg/kg on day 10 to 16 of gestation. However, in the current

study levels of total PCBs in the brain of weanlings are considerably lower than those previously reported (Shain *et al.* 1986), in which continous dietary exposure to 30 ppm resulted in 3000 ppb on a fresh wt basis. This difference is due to the fact that in the current study dams were exposed only during gestation, while in the former study exposure occured during gestation and lactation.

Effect of PCB exposure on plasma thyroid hormone levels

Maternal PCB exposure produced a transient decrease in plasma total thyroxine (TT_4), free thyroxine (FT_4) and triiodothyronine (T_3) in the dams and their offspring. Despite that lactational transfer of PCBs is much greater than placental transfer (Shain *et al.* 1986), the reductions of plasma TT_4 and FT_4 were most severe in the fetus following maternal PCB exposure. Plasma levels of CB-153 in this study also indicate that although substantial levels of PCBs were present in fetal blood, neonatal exposure via lactation was considerably higher.

The reductions of fetal plasma TT_4 and FT_4 were not secondary to reductions in maternal plasma TT_4 and FT_4 levels, for the maternal transfer of T_4 accounts for only 17% of the fetal T_4 pool in the late gestational Wistar rat fetus (Calvo *et al.* 1990, Morreale de Escobar *et al.*, 1989). Furthermore, major reductions in fetal plasma TT_4 and FT_4 levels were observed after maternal exposure to the low dose of Aroclor 1254, a dose which resulted in only minor reductions in maternal plasma thyroid hormone levels.

The selective accumulation in the fetal plasma of a hydroxylated PCB metabolite, 2,3,3',4',5-pentachloro-4-biphenylol (4-OH-pentaCB) is probably responsible for the decreases observed in fetal plasma TT_4 and FT_4 levels. *In vitro*, meta and para hydroxylated PCB metabolites bearing a structural resemblance to thyroxine can displace thyroxine from transthyretin (TTR), the major plasma thyroxine binding protein in the rat (Lans *et al.* 1993, Rickenbacher *et al.* 1986). Unpublished observations from our laboratory indicate that 4-OH-pentaCB binds to TTR with a 10-fold higher affinity than T_4. Decreased binding of T_4 to TTR due to the presence of 3,3',4',5-tetrachloro-4-biphenylol in plasma following exposure to 3,3',4,4'-tetrachlorobiphenyl has been demonstrated in the rat (Brouwer and van den Berg, 1986, Brouwer *et al.* 1990). Recent research from our laboratory shows that gestational exposure of rats to 3,3',4,4'-tetrachlorbiphenyl leads to a significant accumulation of 3,3'4',5-tetrachloro-4-biphenylol in fetal plasma, resulting in decreased fetal plasma TT_4 and FT_4 levels in the absence of decreases in maternal plasma TT_4 and FT_4 levels. (Morse *et al*, 1995). High levels of 4-OH-pentaCB were also found in neonatal plasma on postnatal day 4, and may also have contributed to the decreases observed in neonatal plasma TT_4 and FT_4 levels.

Although not addressed in the current study, the secretion of T_4 by the fetal thyroid may also have been depressed following maternal PCB exposure. In a previous study, the dietary exposure of pregnant and lactating rats to Aroclor 1254 resulted in ultrastructural alterations in the fetal thyroid gland indicating interference in the colloid droplet-lysozyme interaction, which would impair thyroid hormone secretion (Collins and Capen, 1980a). Further support for the decreased secretion of T_4 and T_3 by the thyroid following PCB exposure has been given in studies which examined the kinetics of $[^{125}I]$-T_4 and $[^{125}I]$-T_3 in rats (Byrne *et al.* 1987, Sepkovic and Byrne, 1984).

Hepatic microsomal T_4 glucuronidation

In the current study, the reductions in maternal and neonatal plasma TT_4 and FT_4 levels may in part be due to the observed induction of hepatic microsomal T_4 glucuronidation in maternal and neonatal rats. The administration of Aroclor 1254 to thyroidectomized rats implanted with osmotic pumps to deliver T_4 results in decreased plasma thyroxine levels (Barter and Klasson, 1992) presumably by increased bilary excretion of T_4 glucuronides (Bastomsky, 1977). However, the relationship between hepatic microsomal T_4 glucuronidation and plasma TT_4 levels was weak in weanling rats, indicating that the contribution of hepatic glucuronidation was relatively minor.

In contrast to a previous study in which T_4-UDPGT activity was induced in fetal rat hepatic microsomes following maternal exposure to coplanar compounds, (Morse *et al.* 1993a) in this study fetal T_4-UDPGT activity was significantly reduced by maternal exposure to Aroclor 1254. Therefore, hepatic glucuronidation of T_4 in the fetus did not play a role in decreasing fetal plasma TT_4 levels in the current study.

It has been previously shown that PCB exposure results in a decrease of plasma T_4 levels in the absence of increased T_4 glucuronidation in non-thyroidectomized rats. The work by Collins and Capen (1980b) demonstrated that reductions in plasma T_4 levels in homozygous Gunn rats were identical to the reductions found in heterozygous Gunn rats following Aroclor 1254 exposure. Since the homozygous Gunn rats are severely deficient in T_4 glucuronidation and heterozygous Gunn rats exhibited a 5 fold increase in bilary T_4 glucuronides after PCB exposure, the authors suggested that also the secretion of T_4 by the thyroid is reduced by PCBs.

Effect of PCB exposure on the regulation of brain thyroid hormones

The results show that while reductions in fetal or weanling rat plasma TT_4 or FT_4 levels result in decreased brain T_4 levels following maternal PCB exposure, brain T_3 levels are affected only to a minor extent (Figure 5.3). The reductions in brain T_4 levels were most severe in the fetus, and the induction of 5'D-II activity in the fetal forebrain can account for the maintenance of forebrain T_3 levels in the fetus. The induction of forebrain 5'D-II activity is a well charactereized response of the rat brain to decreased T_4 levels (Silva and Matthews, 1984, Ruiz de Oña et al. 1988, Obregón et al. 1991). It has been proposed that type III thyronine 5-deiodinase (5-D-III) regulates cerebellar T_3 levels by regulating the deiodination of T_3 to T2 (Silva and Matthews, 1984).

In female weanling rats normal forebrain T_3 levels were maintained, despite a significant reduction in forebrain T_4 levels and the absence of induced 5'D-II activity in forebrain homogenates following in utero and lactational exposure to the high dose of Aroclor 1254. In addition, female weanling forebrain T_3 levels were also maintained despite slight decreases in forebrain T_4 levels and a decrease in 5'D-II activity following in utero and lactational exposure to the low dose of Aroclor 1254. The maintenance of forebrain T_3 levels in female weanlings is therefore probably not regulated by 5'D-II activity following maternal PCB exposure. As in the cerebellum, a decrease in 5-D-III activity may maintain T_3 levels in the forebrain when 5'D-II activity is improperly regulated.

The decrease in 5'D-II activity in brain homogenates from weanling rats from the low PCB dose group is difficult to explain. We eliminated competitive inhibition by PCBs or metabolites by examining 5'D-II activity with brain homogenates from control

weanling rats with concentrations of PCBs or metabolites that were more than 1000-fold higher than the concentrations found in brain tissue in this study. It is also possible that PCBs or their metabolites may act on the inactivation of 5'D-II by stimulating the binding of the enzyme to F-actin filaments, and thus increasing the internalization of the enzyme, which is a well-characterized response of the enzyme to T_4 (Farwell and Leonard, 1992, Farwell *et al.* 1993, Safran *et al.* 1993). However, preliminary results using the primary astrocyte system described by Farwell and Leonard (1992) for detecting 5'D-II inactivation or inhibition revealed no effects of Aroclor 1254, individual PCB congeners or PCB metabolites on 5'D-II activity at concentrations of 1 mM in the culture medium.

Plasma TSH levels

No increase of plasma TSH levels was found as a response to decreased plasma thyroid hormones in fetal or neonatal rats. Elevated plasma TSH levels have been found in adult male hooded rats exposed to Aroclor 1254 and 2,3,7,8-TCDD, in Rhesus monkeys exposed to 2,3,4,7,8- pentachlorodibenzofuran, and marmoset monkeys exposed to 3,3',4,4'-tetrachlorobiphenyl (Bastomsky, 1976, Bastomsky, 1977, Van den Berg *et al.* 1988, Brewster *et al.* 1988). It has been recently demonstrated that the TSH elevation observed after dietary exposure of adult male Sprague-Dawley rats is lower than expected on the basis of plasma TT_4 and FT_4 reductions (Barter and Klaasen, 1994). We know of no previous reports in the litertature of the effects of pre- and postnatal exposure to a polychlorinated biphenyl mixture on TSH concentrations in rats. Fetal Wistar rats have been shown to respond to chemically induced (methimazole) hypothyroidism with 5-fold increases in plasma TSH on day 21 of gestation (Morreale de Escobar *et al.* 1993). There is no apparent reason to explain the lack of increased TSH secretions in the current experiment on the basis of the plasma thyroid hormone levels, because plasma T_4 and not T_3 levels are thought to be primarily responsible for regulating fetal plasma TSH levels (Morreale de Escobar *et al.* 1993), and the levels of fetal plasma TT_4 and FT_4 were severely reduced in the high dose group. Furthermore, in male neonates (day 4 postpartum) 50% reductions in both plasma TT_4 and TT_3 levels did not result in elevated TSH concentrations. The abnormal regulation of of TSH secretion in fetal and neonatal rats, and of 5'D-II activity in the brains of neonatal rats cannot be explained by our current knowledge of processes involved in the interactions between PCBs and thyroid hormones. It is conceivable that either PCB congeners in the Aroclor mixture or their metabolites interact with the nuclear T_3 receptor in the pituitary and modify the response of TSH release. Interactions of polyhalogenated cyclic compounds with T_4 binding have been demonstrated in crude nuclear extracts (McKinney *et al.* 1987) and both Aroclor 1254 and the PCB congener 3,3',4,4',5-pentachlorobiphenyl reduce the level of c-erbA mRNA which encodes for the thyroid hormone nuclear receptor *in vitro* (Hornhardt *et al.* 1994).

Significance of the findings

If one assumes that T_3 is the biologically active thyroid hormone during brain development, one can question if the slight but significant decreases in fetal brain T_3
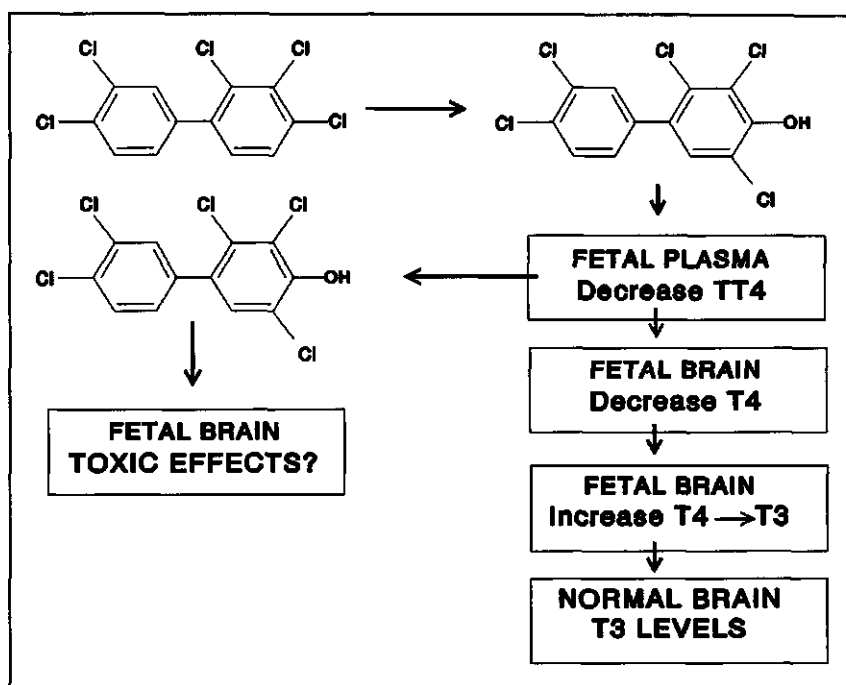
Figure 5.3
Schematic representation of the effects on thyroid hormone homeostasis following the accumulation of the PCB metabolite 2,3,4',5',5-pentachloro-4-biphenylol in fetal rat plasma. The metabolite binds to fetal transthyretin, displacing T_4 and thereby decreasing plasma TT_4 and FT_4 concentrations. Subsequently, the transfer of T_4 to the fetal brain is limited, decreasing brain T_4 concentrations, with an increase in Type II thyroxine 5'-deiodinase activity, so that brain T_3 concentrations are maintained. Due to the lack of a functional blood-brain barrier, 2,3,4',5',5-pentachloro-4-biphenylol also accumulates in the fetal brain with unknown toxicological consequences.

levels on day 20 of gestation observed in this study only in the low dose group following maternal PCB exposure are sufficient to result in alterations in brain development. However, several considerations should be made before concluding that PCB-induced hypothyroxinemia is not involved in PCB-induced developmental neurotoxicity.

The thyroidectomy of dams results in the thyroid hormone deficiency of embryos prior to the onset of fetal thyroid hormone production (Morreale de Escobar *et al.* 1985, Porterfield and Hendrich, 1992). Once fetal thyroid hormone secretion begins after day 17 of gestation only marginal effects are observed on the concentration of T_4 and T_3 in the fetal brain despite maternal thyroidectomy (Ruiz de Oña *et al.* 1991) because the fetus is then only partially dependent on maternal thyroid hormones (Morreale de

Escobar *et al.*, 1988, 1989, 1990). However, the progeny of thyroidectomized dams exhibit permanent behavioral defects (Hendrich *et al.* 1984, Attree *et al.* 1992), and alterations in brain biochemistry (Pickard *et al.* 1993, Ruiz de Elvira *et al.* 1989, Hadjzadeh *et al.* 1990), which indicate a requirement for thyroid hormones before the onset of fetal thyroid secretion. Considering the higher sensitivity of the fetus than the dam for the induction of hypothyroxinemia on day 20 of gestation, we can not rule out that brain levels of T_3 were significantly decreased at previous time points. This issue should be addressed by examining the effects of prenatal PCB exposure on the levels of thyroid hormones in the embryonic and fetal tissues up to day 18 of gestation. In addition, levels of brain thyroid hormones should also be examined between 10 to 15 days after birth, a period in which hypothyroidism can result in decreased myelinisation (Rodriguez-Peña *et al.* 1993).

Regardless of whether or not functional decreases in brain thyroid hormone concentrations occur, the transport to and accumulation of hydroxylated PCB metabolites in the fetal brain may also pose a threat to brain development. Para-hydroxylated PCB metabolites bind 10-fold stronger to T_4 receptors in nuclear extracts than T_4 itself (McKinney *et al.* 1987) en bezitten estrogene eigenschappen (Jansen *et al.* 1993, Korach *et al.* 1988). Therefore these metabolites may directly alter brain cell differentiation and/or proliferation.

In conclusion, maternal exposure to the PCB mixture Aroclor 1254 results in the substantial accumulation of mainly one hydroxylated metabolite (2,3,3',4',5-pentachloro-4-biphenylol) in the fetal plasma, and thereby probably contributes to the decreases observed in fetal plasma thyroxine levels. Decreases in plasma T_4 levels resulted in marked decreases in fetal brain T_4 levels, which are compensated in part by increased 5'D-II activity, so that decreases in brain T_3 levels are less dramatic. Based on the current knowledge of the developmental regulation of thyroid hormone function, the developing central nervous system may be at risk for PCB induced alterations in brain thyroid hormone concentrations. Also the selective accumulation of 4-OH-pentaCB in the fetal brain warrants toxicological investigation.

CHAPTER 6

FETAL, NEONATAL AND LONGTERM ALTERATIONS IN HEPATIC RETINOID LEVELS FOLLOWING MATERNAL POLYCHLORINATED BIPHENYL EXPOSURE IN RATS

Abstract

This study was undertaken to investigate the effects of perinatal polychlorinated biphenyl (Aroclor 1254) exposure on hepatic and plasma retinoid levels in fetal rats, their dams and neonatal and adult offspring. Pregnant Wistar rats were treated with 0, 5, or 25 mg Aroclor 1254/kg body weight from day 10 to 16 of gestation. Hepatic retinoids (retinol, retinyl palmitate and retinyl stearate) levels were determined in fetuses and dams from day 20 of gestation, in male and female neonates 21 days postpartum and in young adult offspring 90 days after birth. Retinol levels were determined in fetal and maternal plasma (gestation day 20) and plasma from the offspring 21 and 90 days after birth.

Maternal and fetal plasma retinol levels were decreased by 35% and 38% on day 20 of gestation following exposure to the highest dose of Aroclor 1254. Male, but not female neonatal plasma retinol levels were significantly decreased (23%) in the high dose group. No effects of PCB treatment were seen on plasma retinol levels in the offspring 90 days after birth.

Only slight reductions in fetal and maternal hepatic retinol and retinyl palmitate concentrations were observed after prenatal PCB exposure. Male neonatal hepatic retinyl palmitate levels were reduced by 25% and 50% in the 5 mg and 25 mg Aroclor 1254/kg dose group, respectively, while female neonatal hepatic retinyl palmitate levels were significantly reduced only in the high dose group. Ninety days after birth, male hepatic retinyl palmitate levels were only slightly reduced in the highest dose group, however, hepatic retinol concentrations were significantly reduced by 50% in both PCB treatment groups. Female adult offspring exhibited significant reductions in hepatic retinyl palmitate levels (25%) in both PCB treatment groups, while hepatic retinol levels exhibited an unusual increase of more than 100% of controls in the low dose group, while levels in the high dose group were similar to controls. This study demonstrates that even a relatively low maternal dose of Aroclor 1254 results in long-term alterations in retinoid status of the offspring in the rat.

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Introduction

Polychlorinated biphenyls, -dibenzo-*p*-dioxins and -dibenzofurans have been demonstrated to alter retinoid status in various experimental animals and wildlife species (reviewed by Zile, 1992). In general, exposure to polyhalogenated aromatic hydrocarbons (PHAHs) results in decreased hepatic stores of retinol and retinyl esters while extrahepatic stores (with the exception of the kidney) of retinoids appear to be affected to a lesser extent (Håkansson *et al.* 1991, Brouwer *et al.* 1988a). The relative effects of PHAH exposure on tissue retinoid levels are dependent on retinoid status (Håkansson *et al.* 1991a), the species or strain of experimental animal used (Puhvel *et al.* 1991, Håkansson *et al.*, 1991b), and the time after exposure (Azais *et al.*, 1987). The structure-activity relationship for the effects of PHAHs on tissue retinoid levels is not yet clear (Chen *et al.*, 1992), and is likely to involve multiple mechanisms, such as the modulation of hepatic retinol ester hydrolase activity (Mercier *et al.* 1990), acylCoA:retinol acyl transferase (Jensen *et al.* 1987) and interactions of hydroxylated PCB metabolites with the plasma retinol-binding protein-transthyretin complex (Brouwer and Van den Berg, 1986, Brouwer *et al.* 1988b).

Some of the effects of exposure to PHAH resemble those of retinoid deficiency, for example negative reproductive outcome, (Peterson *et al.* 1993, Takahashi *et al.* 1975), keratinisation of epithelia (Chopra, 1983) and impaired immune function (Safe, 1994, Mark *et al.* 1983). Specific targets in the developing organism, such as the craniofacial area and the central nervous system, are sensitive to retinoid and PHAH induced teratogenicity (Abbott and Birnbaum, 1989, Tilson et al, 1990, Peterson *et al.* 1993, Durston *et al.*, 1989). In addition, PHAHs are well known tumor promotors (Silberhorn, 1990), while retinoid status can modulate PHAH induced carcinogenesis (Flodstrom et al, 1991). Both polyhalogenated aromatic hydrocarbons and retinoic acid effect cellular responses through the regulation of gene expression mediated by their respective and distinct nuclear receptors (Petkovitch, 1992, Poland and Knutson, 1982). In addition, the coadministration of retinoic acid to pregnant mice increases the incidence of cleft palate by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (Abbott and Birnbaum, 1990). Moreover, retinoic acid and retinol are potent inhibitors of dioxin-induced differentiation of human keratinocytes in vitro (Berkers *et al.* in press).

Pre- and postnatal exposure to PHAHs forms a potential risk for alterations in retinoid homeostasis during a period of tissue differentiation. However, very little information is available on the effect of PHAH exposure on plasma retinol and hepatic retinoid stores during development in mammals. The exposure of lactating rats to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), results in the reduction of hepatic retinoid stores in neonates, an effect resembling that of the exposure of adult rats to TCDD (Håkansson *et al.* 1987, Lans *et al.* 1990). To our knowledge, no information is available on the effects of maternal PHAH exposure on the plasma and hepatic retinoid levels in the fetal or adult offspring.

In this study we examined the effects of perinatal exposure to the polychlorinated biphenyl mixture Aroclor 1254 on circulating and hepatic retinoid levels in pregnant rats and their fetuses, as well as neonatal and adult offspring. Retinol was analysed in the plasma, and retinol, retinyl palmitate and stearate were analysed in the livers. Body weight gain, relative organ weights and reproductive parameters were also monitored.

Material and Methods

Chemicals

Aroclor 1254 was kindly donated by Dr. Martin van den Berg, Research Institute of Toxicology, University of Utrecht, The Netherlands. The following chemicals were purchased from Merck, Darmstadt, Germany: Tris, diisopropylether, HCl, NaCl and 2,6-di-*tert*-butyl-4-methylphenol (BHT, chemicals were analytical grade). Methanol (HPLC quality) was purchased from Janssen Chimica, Tilburg, the Netherlands. Retinol, retinyl acetate and retinyl palmitate were purchased from Fluka Chemie, Bornem, Belgium.

Animals

Wistar WU rats, 100 females and 50 males, (14 weeks old) were purchased from Charles River Sutzfeld, Germany. The rats were allowed to acclimatize for 2 weeks and were maintained at 50 % humidity and 21°C on bedding in macrolon cages with a 12 hour light cycle. Rat chow (Hope Farms, Woerden, the Netherlands) and tap water were supplied *ad libitum*. After the acclimatization period two females were placed in a cage with one male overnight from 17:00 to 9:00 the next day. The females were examined each morning by vaginal smear. When spermatazoa were found the animal was housed separately and this was termed day 0 of gestation. Animals were pre-assigned to a particular dose group on a rotating basis. Maternal body weight gain was monitored throughout gestation and lactation. On day 10 of gestation the pregnant female rats were transferred to a macrolon cage with a grated steel support to facilitate the collection of PCB-contaminated feces and to prevent contamination of the animal facilities.

Pregnant females received a daily oral dose of 0, 5 or 25 mg Aroclor 1254 per kg body weight dissolved in cornoil (2 ml/kg body weight) from day 10 of gestation to day 16 of gestation. Rats were weighed each day before administration of Aroclor 1254 or corn oil alone. On day 20 of gestation (GD20) 6 pregnant rats were sacrificed per treatment group under ether anesthesia. Maternal blood was collected from the vena cava, and maternal livers were removed, washed in 0.9% NaCl, blotted dry, weighed and frozen in liquid nitrogen. Fetuses were removed, sex was determined, the placental cord was cut and clamped, and the fetuses were washed in 0.9% NaCl, blotted dry and weighed. Fetal blood was collected by decapitation and the fetal livers were removed and frozen in liquid nitrogen. Fetal livers and blood were pooled per dam for analysis. Blood was collected in heparinized tubes and stored on ice until plasma was prepared by centrifugation. Livers were stored at -80 °C and plasma at -20 °C until analysis.

The remaining rats were transferred to bedding material on GD20 and were given paper tissues to make a nest. The rats were inspected each morning at 8:00 h and afternoon at 18:00 h for nests. Pups found during the morning inspection were termed 1 day old, pups born between the morning and afternoon inspection were termed 0 day old. Pups were examined for sex, weighed on day 0 postpartum when possible, and always on day 1, 4 and 7 postpartum and thereafter on a weekly basis. On postnatal day 4 (PND4) nests were reduced to 4 males and 4 females. When this was not possible, nests were made up of a total of 8 males and females from the same dam, and if necessary, an extra pup from another nest from the same treatment group and same age was used to achieve a total of 8 pups per nest. Pups were individually marked by incision in the ear, and pups transferred from one nest to another were never used for

any analysis.

On postnatal day 21 (PND21), 8-10 nests were sacrificed per treatment group. Dams and neonates were sacrificed by decapitation, trunk blood was collected in heparinized tubes, and livers were removed and stored as described above. Neonatal livers were pooled per sex for analysis. Trunkblood was collected separately for each neonate. The thymus was also removed from each rat and weighed.

The neonates from the remaining nests were weaned on PND21 and male and female rats were housed separately in groups of 2-4 rats per cage. The remaining animals (10 nests per treatment group) were sacrificed 90 days (PND90) after birth. Trunk blood was collected separately for each rat, and 1 liver per sex was analysed per nest. Pooled fetal plasma and livers were analysed for retinoids. For day 21 postpartum one of the plasma and pooled liver samples per sex were used for retinoid analysis. For day 90 postpartum one plasma and one liver sample were used for retinoid analysis. The animal used for retinoid analysis had been assigned on day 4 postpartum to eliminate bias.

Extraction and analysis of retinoids

Plasma and hepatic retinoids were analysed according to Brouwer *et al.* (1989) with some modifications. For the extraction of hepatic retinoids, livers were homogenized in 50 mM Tris-HCl buffer, pH 7.5, 3 ml buffer/g liver using an ultra-Turrax followed by homogenization in a Potter tube with a teflon pestle. Plasma samples or liver homogenates (50 µl) were vortexed with 50 µl methanol containing the internal standard (500 ng retinyl acetate/ml for plasma, 1 µg/ml for livers) and 0.1 % BHT (w/v) as an anti-oxidant. Plasma and liver retinoids were then extracted overnight at -20 °C with 100 µl diisopropylether. The ether phase was removed and filtered over a 0.45 µm filter (Millipore, Etten Leur, the Netherlands), then evaporated under nitrogen and resuspended in 80 µl methanol (0.1% BHT). Extractions were carried out in duplicate and some series of measurements were repeated to verify the accuracy of the data. Extraction efficiencies were routinely above 80%.

Twenty µl aliquots of resuspended extracts were analysed with HPLC using a C18 analytical column (Pecosphere, 3 µm particle size, 3.3 cm length and 4.6 mm internal diameter, Perkin Elmer). A Merck-Hitachi HPLC system was used consisting of a L6200 pump, L4200 uv-vis detector, AS-2000A autosampler and a D2500 integrator. A wavelength of 326 nm was used for the detection of retinoids. Plasma extracts were analyzed isocratically with 85% methanol and 15% water with a flow rate of 1 ml/min and data collection for 10 minutes. Hepatic retinoids were analysed by 85% methanol, 15% water for 1.5 minutes, followed by a gradient to 100% methanol for 2.5 minutes, and subsequent elution of the retinyl esters at 100% methanol for 12 minutes. The column was then re-equilibrated at 85% methanol, 15% water for 6 minutes. Retinol was quantified in the plasma extracts, while retinol, retinyl palmitate and retinyl stearate were quantified in liver extracts based on calibration curves using retinol or retinyl palmitate as standards.

Statistical analysis

Treatment effects were analysed by one-way analysis of variance follwed by Duncan's multiple range test ($p < 0.05$) and differences between control values of male

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and female animals were analysed with the student's t-test using the software package SPSS/PC+™ (SPSS Inc., Chicago, IL). The data are presented as the means ± SEM.

Results

Body weights, organ weights and reproduction

No effect of Aroclor 1254 treatment was observed on the number of implantations, resorptions, late fetal death, mean fetal body weight, nest size, sex ratio or postnatal survival (data not shown). No gastrointestinal bleeding was observed in the control or Aroclor 1254 exposed fetuses. The relative liver weight in fetuses was not affected by maternal Aroclor 1254 exposure. There were no treatment related effects on maternal body weight during gestation or after birth. Maternal relative liver weights on GD20 were increased by 7% and 14% by the 5 mg/kg and 25 mg/kg dose group, respectively, however the differences were not statistically significant (data not shown).

Male and female body weight gain of the offspring were slightly, but significantly, reduced by maternal exposure to 25 mg Aroclor 1254/kg body weight from PND4 to PND90 (Figure 6.1, only male data is shown). Body weight gain in the male and female offspring from the 5 mg/kg dose group was identical to controls at all time points and the data is not shown. Body weight reductions relative to controls in male offspring
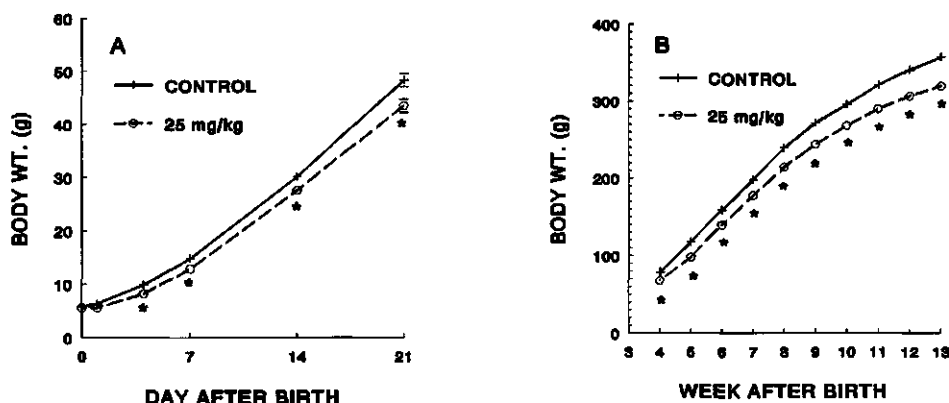
Figure 6.1
Body weights of male offspring from control and Aroclor 1254-exposed dams, mean ± SEM, following maternal exposure to 0 or 25 mg Aroclor 1254/kg on day 10-16 of gestation. A) neonatal body weight (N=18-20) up to PND21, B) body weight (N=10) from week 4 (PND28) to week 13 (PND90). An asterisk (*) indicates a significant difference from controls, $P<0.05$.

Figure 6.2 Cumulative food consumption of male and female offspring (from PND28 to PND90), N=10, mean ± SEM, following maternal exposure to 0, 5 or 25 mg Aroclor 1254/kg on day 10-16 of gestation. An asterisk (*) indicates a significant difference from controls, P<0.05.
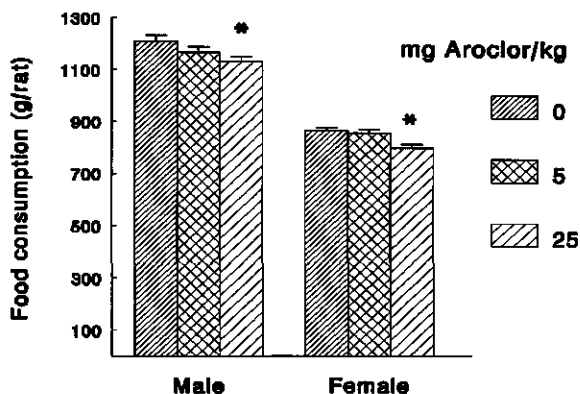
from the 25 mg Aroclor 1254/kg dose group were 17% on PND4 and 11% on PND90 (Figure 6.1A and B). Total food consumption from 28 to 90 days of age was significantly lower in both male (6.2%) and female (8.3%) offspring from the high dose group than controls (Figure 6.2). Effects on female body weight gain (data not shown) in the 25 mg Aroclor 1254 dose group were similar to those in male offspring.

Relative liver weights were significantly increased by the high dose of Aroclor 1254 on PND21 in both male and female offspring (29% and 30% respectively), but not 8on PND90 (Table 6.1). Relative thymus weight was slightly reduced in male and female neonates on PND21 in the high dose group (14% and 9.8%, respectively) but no effect of maternal treatment with Aroclor 1254 was observed on relative thymus weight 90 days after birth in the offspring (Table 6.1). Relative liver weights were unaffected on PND90 after maternal Aroclor 1254 exposure, however, absolute liver weights were significantly lower than controls in male and female adult offspring from the high dose group (data not shown) as a result of significantly lower body weight gain.

Plasma and hepatic retinoids

Control values of hepatic retinoids are given in Table 6.2. Hepatic retinol concentrations increase from 2.09 µg/g liver in the fetus to 4.85 µg/g (females) and 5.83 µg/g (males) on day 21 after birth, with an additional 4 fold increase by day 90 after birth. The most dramatic increase of retinoids during development is seen in the hepatic retinyl esters. Concentrations of retinyl palmitate increase more than 10 fold from fetal values to 21 day old neonates, and then again more than 10 fold from day 21 to day 90 after birth. The hepatic concentration of retinyl stearate shows a similar pattern of development as retinyl palmitate. While concentrations of retinyl palmitate and retinyl stearate are nearly identical for both sexes on day 21 postpartum, female control offspring exhibit significantly higher concentrations than their male littermates 90 days after birth. Retinyl palmitate concentrations were 66% higher and retinyl stearate concentrations were 54% higher in female relative to male adult offspring.

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Table 6.1
Relative organ weights of male and female offspring following maternal exposure to Aroclor 1254 on day 10 to 16 of gestation.

Dose mg Aroclor 1254/kg	PND21		PND90	
		Liver (%body weight)		
	Male	Female	Male	Female
0	4.11±0.05	4.14±0.04	3.94±0.10	3.36±0.06
5	4.40±0.06	4.34±0.05	3.90±0.06	3.28±0.06
25	5.30±0.10*	5.39±0.11*	4.00±0.08	3.37±0.05
		Thymus (%body weight)		
	Male	Female	Male	Female
0	0.50±0.02	0.51±0.02	0.16±0.01	0.20±0.01
5	0.49±0.01	0.53±0.01	0.18±0.01	0.20±0.01
25	0.43±0.01*	0.46±0.02*	0.17±0.01	0.18±0.01

Pregnant rats were treated with Aroclor 1254 on day 10-16 of gestation, An asterisk (*) indicates a significant difference from controls, $P<0.05$, N=8-10. PND21: postnatal day 21, PND90: postnatal day 90.

Maternal hepatic retinol and retinyl stearate concentrations were unaffected by Aroclor 1254 exposure (Figure 6.3 and 6.4). Maternal hepatic retinyl palmitate concentrations were significantly decreased by 20% on day 20 of gestation by exposure to 25 mg/kg Aroclor 1254 (Figure 6.5). When the results were adjusted for liver weight, no significant effects were observed in the maternal livers. Fetal hepatic retinol concentrations were decreased 24% and 15% relative to controls by maternal treatment with 5 and 25 mg Aroclor 1254 per kg, respectively, but this decrease was significant only in the low dose group (Figure 6.3). Fetal retinyl palmitate and stearate concentrations were significantly decreased in the 25 mg Aroclor 1254/kg (Figure 6.4 and 5). Also, total fetal hepatic stores of retinol, retinyl palmitate and retinyl stearate were all significantly decreased relative to controls by about 30% in fetal livers from the 25 mg/kg treatment group (Table 6.3). Fetal and maternal plasma retinol concentrations were significantly reduced relative to controls (38% and 35%, respectively) following exposure to 25 mg Aroclor 1254/kg (Table 6.4).

Table 6.2
Control levels of hepatic retinoids from maternal rats and their fetal, neonatal and adult offspring.

	retinol	retinyl palmitate	retinyl stearate	N
		µg/g liver		
GD20				
Maternal	12.6±1.1	817±20	89.7±2.9	6
Fetal	2.1±0.2	7.36±0.8	1.1±0.1	6
PND21				
Male	5.8±0.5	80.7±2.5	8.8±0.3	8
Female	4.9±0.6	75.7±5.0	8.2±0.7	10
PND90				
Male	21.5±1.6	733±30	105±5	10
Female	18.5±1.4	1215±65*	162±9*	10

The values presented are the means ± SEM. An asterisk (*) indicates a significant difference between male and female offspring, $P < 0.05$. GD20: gestation day 20, PND21: postnatal day 21, PND90: postnatal day 90.

Significant reductions of 35% in male and female neonatal (PND21) hepatic retinol concentrations (Figure 6.3) were observed in the high Aroclor dose group. However, although there is a trend for a decrease on PND 21, no significant reductions in total retinol stores per liver were observed (Table 6.3), probably as a consequence of increased liver size. Significant decreases in retinyl palmitate (Figure 6.5) and retinyl stearate (Figure 6.4) concentrations (50% and 46%, respectively) as well as total stores (42% and 38%, respectively, Table 6.3) were observed in male neonates from the highest dose group. In female neonates only the total store of hepatic retinyl palmitate was significantly reduced by 38% relative to controls in the highest dose group (Table 6.3), while both retinyl palmitate and retinyl stearate concentrations were significantly reduced in the highest dose group (49% and 36%, respectively, Figure 6.4 and 6.5). A slight but significant reduction (23%) of plasma retinol relative to controls was observed in male neonatal offspring from the high exposure group, however the decrease in female neonates was not significant (Table 6.4).

Table 6.3

Hepatic retinoid content in maternal rats and their fetal, neonatal and adult offspring following maternal Aroclor 1254 exposure.

Dose Aroclor 1254 mg/kg	retinol	retinyl palmitate	retinyl stearate	
Maternal (GD20)	µg/liver	mg/liver	mg/liver	N
0	135±12	8.75±0.27	0.96±0.05	5
5	116±12	7.99±0.37	0.91±0.06	6
25	136± 6	7.92±0.87	1.00±0.07	6
Fetal (GD20)	µg/liver	µg/liver	µg/liver	
0	0.33±0.04	2.28±0.32	0.33±0.04	6
5	0.28±0.02	1.83±0.07	0.28±0.02	6
25	0.24±0.01*	1.62±0.08*	0.24±0.01*	6
Male (PND21)	µg/liver	µg/liver	µg/liver	
0	11.6±1.5	161±13	17.4±1.4	8
5	11.0±2.4	146±13	16.5±1.2	8
25	8.6±2.3	93± 9*	10.8±1.2*	8
Female (PND21)	µg/liver	µg/liver	µg/liver	
0	9.0±1.1	141±14	15.3±1.6	10
5	8.7±0.6	142±10	18.4±1.4	8
25	6.9±0.3	88± 9*	12.0±1.3	8
Male (PND90)	µg/liver	mg/liver	mg/liver	
0	298±27	10.2±0.6	1.45±0.08	10
5	176±17*	9.7±0.5	1.31±0.06	10
25	167±20*	8.2±0.4*	1.14±0.07*	10
Female (PND90)	µg/liver	mg/liver	mg/liver	
0	131± 7.5	8.69±0.30	1.16±1.6	10
5	297±34.1*	6.82±0.39*	1.26±1.4	10
25	129±18.0	6.62±0.40*	1.18±1.3	10

Pregnant rats were treated with Aroclor 1254 on day 10-16 of gestation. The data presented are the means ± SEM, an asterisk (*) indicates a significant difference from controls, $P < 0.05$. GD20: gestation day 20, PND21: postnatal day 21, PND90: postnatal day 90.

Figure 6.3 Hepatic retinol concentrations, expressed as percentage of control values, mean ± SEM, from dams, their fetuses, male and female offspring (N=6-10), following maternal exposure to Aroclor 1254 on day 10-16 of gestation. An asterisk (*) indicates a significant difference from controls, P<0.05. GD20: gestation day 20, PND21: postnatal day 21, PND90: postnatal day 90.
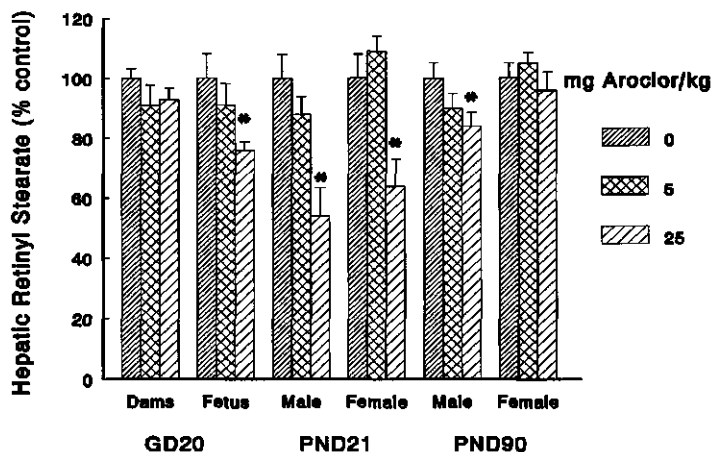
Figure 6.4 Hepatic retinyl stearate concentrations, expressed as percentage of control values, mean ± SEM, from dams, their fetuses, male and female offspring (N=6-10), following maternal exposure to Aroclor 1254 on day 10-16 of gestation. An asterisk (*) indicates a significant difference from controls, P<0.05. GD20: gestation day 20, PND21: postnatal day 21, PND90: postnatal day 90.

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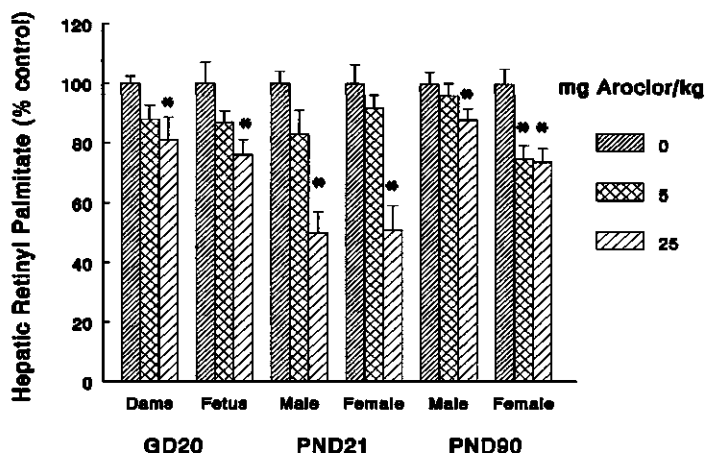
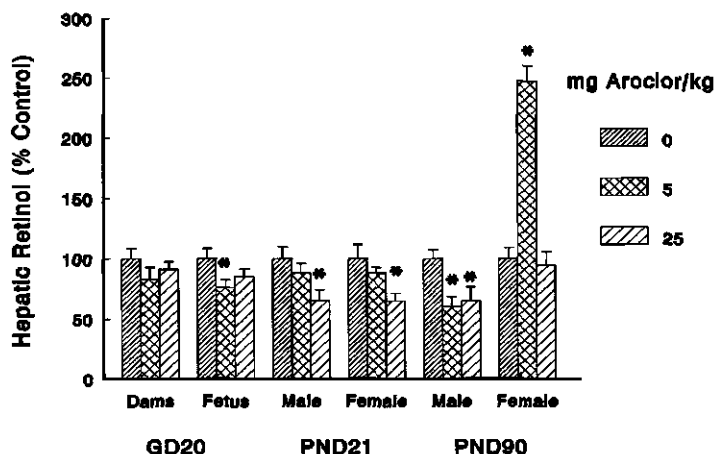**Figure 6.5**. Hepatic retinyl palmitate concentrations, expressed as percentage of control values, mean ± SEM, from dams, their fetuses (N=6), male and female neonates (N=8-10), and adult offspring, (N=10), following maternal exposure to 0, 5 or 25 mg Aroclor 1254/kg on day 10-16 of gstation. An asterisk (*) indicates a significant difference from controls, P<0.05. GD20: gestation day 20, PND21: postnatal day 21, PND90: postnatal day 90.

Effects from perinatal exposure to Aroclor 1254 on retinoid homeostasis in male and female offspring persisted into adulthood. In male offspring from the high dose group, the hepatic retinyl palmitate and retinyl stearate concentrations were significantly lower than controls by 16% (Figure 6.4 and 6.5). Total male hepatic stores of retinyl palmitate and stearate were significantly decraesed by 20% and 21%, respectively (Table 6.3). The concentration of male hepatic retinol was significantly decreased (40% and 35%, Figure 6.3) and the total amount of hepatic retinol was decreased (41% and 44%) relative to controls by maternal treatment with 5 and 25 mg Aroclor 1254/kg, respectively (Table 6.3). In adult females the concentration of retinyl palmitate, but not retinyl stearate, were significantly decreased by 25 and 26% relative to controls in the low and high exposure group (Figure 6.4 and 6.5). Decreases in total hepatic stores of retinyl palmitate in adult female offspring were 22% and 24% in the 5 mg and 25 mg Aroclor 1254/kg treatment groups, respectively (Table 6.3). Suprisingly, hepatic retinol concentrations and total retinol were increased by 230% in the lowest exposure group relative to controls or the high exposure group (Figure 6.3). When new aliquots of adult female liver homogenates were extracted and analyzed by HPLC to confirm the data, nearly identical results were obtained (data not shown).

Table 6.4
Plasma retinol concentrations in maternal rats and their fetal, neonatal and adult offspring following maternal Aroclor 1254 exposure.

|	Dose (mg Aroclor 1254/kg)		
	0	5	25
		ng retinol/ml plasma	
GD20			
Maternal	233±19	263±18	151±25*
Fetal	383±29	332±34	237±10*
PND21			
Male	193± 6	184± 9	148±10*
Female	279±21	289±13	236±16
PND90			
Male	297±26	301±22	322±11
Female	369±67	339±43	247±15

Pregnant rats were treated with Aroclor 1254 on day 10-16 of gestation. The data presented are the means ± SEM, an asterisk (*) indicates a significant difference from controls, $P<0.05$. GD20: gestation day 20, N=6, PND21: postnatal day 21, N=8-10, PND90: postnatal day 90, N=10.

Discussion

This study demonstrates that pre- and postnatal exposure of rats to a mixture of polychlorinated biphenyls produces prenatal, postnatal and long-term alterations in hepatic retinoid status. Plasma retinol concentrations were decreased by maternal Aroclor 1254 exposure in fetal and neonatal offspring, but not in adult offspring. Alterations in hepatic retinoid levels were observed in the fetus, but the most prominent alterations were observed postnatally. The observed decreases in hepatic retinoid stores are not simply a result of decreased food intake, for decreases in hepatic retinoids were also observed in offspring from the 5 mg Aroclor 1254/kg dose group in which food intake and body weight gain were the same as in control animals. The low dose used (5 mg Aroclor/kg) exerted no overt toxicity in the offspring, for the relative thymus and liver weights were unaffected in this treatment group. However, similar doses of Aroclor 1254 (5 repeated doses of 8 mg/kg) administered postnatally have a negative effect on reproductive parameters in male (Sager *et al*. 1987) and female rats (Sager and Girard, 1994). Lower doses of Aroclor 1254 administered to pregnant monkeys (0.1 and 0.4 mg/(kg x day)) are fetotoxic (Truelove *et al*. 1982).

By examination of the individual retinoids in the livers of male or female rats, sex-related differences in control levels and the response to perinatal PCB exposure could be observed. For example, female hepatic retinol concentrations were increased to 240% of controls, while male hepatic retinol concentrations decreased significantly relative to controls in the adult offspring exposed perinatally to 5 mg Aroclor 1254/kg. Sex related differences in plasma retinol concentrations have been reported in control rats, however, liver retinoid concentrations (measured as total retinol) did not differ between sexes (Pasatiempo *et al.* 1989).

Despite the direct exposure of the dams to repeated doses of Aroclor 1254 during gestation, no effect was observed in total hepatic retinol or retinyl esters in the dams 4 days after the last administration. The decreases in maternal hepatic retinyl palmitate concentrations were offset by increases in maternal liver weight. Decrease in maternal plasma retinol levels may also be responsible for the decreases observed in fetal plasma retinol concentrations by reduced transplacental transport of retinol. The reductions in fetal plasma retinol levels were accompanied by slight reductions in the hepatic storage of retinol, retinyl palmitate amd retinyl stearate in the fetuses. Retinoid homeostasis is highly regulated in pregnant mice and rats to control the passage of retinol to the offspring (Moore, 1971), which may explain the difference in response to PCB exposure between the neonates in this study and the pregnant and fetal rats. The increased sensitivity of neonatal rats relative to the fetuses for decreases in hepatic retinoid stores following maternal Aroclor 1254 exposure could also be due to the limited placental transfer of PCBs in comparison to lactational exposure (Shain *et al.* 1985).

In this experiment we observed a selective accumulation of hydroxylated PCB metabolites in maternal and fetal plasma (unpublished observations) which may explain the decreases in maternal and fetal plasma retinol concentrations. Decreases in plasma retinol concentrations have been shown to occur as a result of the binding of an hydroxylated metabolite of 3,3',4,4'-tetrachlorobiphenyl to transthyretin, the major thyroid hormone transport protein in the plasma of rats (Brouwer and Van den Berg, 1986, Brouwer *et al.* 1988b). TTR normally forms a complex with retinol binding protein, however, the binding of 4-OH-3,3',4',5-tetrachlorobiphneyl to TTR disturbs the TTR-RBP binding, leading to decreased plasma levels of thyroid hormones, RBP and retinol (Brouwer *et al.* 1988b).

Following maternal rat TCDD exposure significant decreases in maternal and neonatal hepatic retinoid concentrations have also been observed (Lans *et al.* 1990, Håkansson *et al.* 1987), however, in the one report in which it was studied, decreases in hepatic retinoid concentrations were not accompanied by decreases in total hepatic retinoid content until after weaning (Håkansson *et al.*, 1987). The study was hampered by low group size which made finding a statistically significant effect difficult, and therefore it cannot be excluded that pre- and postnatal exposure to TCDD will also result in early decreases in retinoid homeostasis.

We know of no study in which the residual effects of pre- and postnatal PHAH exposure on retinoid status in adult offspring have been examined. A previous study in which Sprague-Dawley rats were contiuously exposed to diet containing 100 ppm of a polybromiated biphenyl mixture (Firemaster BP-6) from day 8 of gestation up to 16 weeks after birth revealed significant decreases in total retinoids from 4 to 14 weeks after birth (McCormack *et al.* 1982). The concentration of individual retinoids was not

investigated. Since in that experiment dietary PBB exposure was continued after weaning resulting in significant liver enlargement up to 16 weeks postpartum, the decreases in total hepatic retinoid concentrations may actually reflect a dilution of the retinoids present and not altered storage of retinol and a reduction of the total amount of retinoids present.

The mechanisms resulting in decreased hepatic retinoids following the activation of the Ah receptor by the binding of PCBs or TCDD have not been clearly elucidated, although enzymes involved in the storage and mobilization of retinol from the liver have been implicated (for a review see Zile, 1992). However, reductions in plasma retinol appear not to be related to Ah-receptor responsiveness, for TCB induces reductions in plasma retinol in both C57BL and DBA2 mice, and the DBA2 strain is the most sensitive (Brouwer and Van den Berg, 1984, Brouwer *et al.* 1985). Furthermore, when administered in equimolar doses, several PCBs which do not bind to the Ah-receptor, like 2,2',5,5'-tetrachlorobiphenyl, 3,3',5,5'-tetrachlorobiphenyl, 2,2',3,3',5,5'-hexachlorobiphenyl reduce plasma retinol concentrations (Chen *et al.* 1992). TCDD administration generally results in the increase of plasma retinol in experimental animals (Zile, 1992).

The analysis of storage and transport forms of vitamin A (retinyl esters and retinol) in the liver and plasma does not address the question whether gene transcription mediated by the retinoic acid receptor is actually affected by exposure to polyhalogenated aromatic compounds. In the current study there would appear to be sufficient hepatic reserves of retinoids to maintain normal tissue concentrations of retinoic acid, one of the biologically active forms of the vitamin. However, extrahepatic tissue concentrations of retinol can also be altered by PCB or TCDD administration (Brouwer *et al.* 1988a, 1989), and the in vitro glucuronidation of retinoic acid has also been shown to be induced following TCDD exposure in rats (Bank *et al.* 1989). It is possible that the most sensitive period for mammals for toxic effects related to altered vitamin A homeostasis is during development. Vitamin A (measured as total retinoids) accumulates in three phases during fetal development in the rat, the highest concentration per conceptus is reached between days 7-9 of gestation, while only late in gestation vitamin A is stored in the liver (Takahashi *et al.* 1977). An analysis of biologically active forms of specific embryonal/fetal tissue retinoids (all-trans-retinoic acid, oxo-retinoic acid metabolites) throughout gestation following PCB or PCDD exposure could reveal alterations in retinoid homeostasis with direct relevance for developmental toxicity.

CHAPTER 7

LONG-TERM ALTERATIONS OF NEUROCHEMICAL MARKERS IN THE OFFSPRING OF RATS EXPOSED TO POLYCHLORINATED BIPHENYLS

Abstract

Pregnant Wistar WU rats were exposed to 0, 5 and 25 mg of the technical polychlorinated biphenyl (PCB) mixture Aroclor 1254 per kg body weight on day 10 to 16 of gestation. Pregnant rats were sacrificed on gestation day 20 to observe effects on fetal body and brain weights. Male and female offspring were sacrificed on postnatal day 21 and 90 (PND21 and PND90) and examined for treatment related effects on neurochemical parameters. The concentrations of the neuronal and glial cell markers, synaptophysin and glial fibrillary acidic protein (GFAP), were measured in diverse brain regions from the offspring using immunochemical techniques. The level of calcineurin activity was measured in cerebellar homogenates. In addition, ethoxyresorufin-O-deethylase activity was determined in hepatic microsomes as a measure of a well-characterized response to PCB-exposure.

The major alterations of GFAP levels following maternal PCB treatment were significant increases in the lateral olfactory tract (LOT) and the cerebellum (CB) and significant decreases in the brainstem (BS) of the offspring on PND21 and 90. Synaptophysin levels were significantly decreased relative to controls in the LOT, prefrontal cortex (PFC) and striatum (ST) of the offspring on PND90. In the BS, synaptophysin levels were significantly decreased relative to controls in male and female weanlings on PND21 and males on PND90, however significant increases were observed in the BS of females on PND90. No effect of maternal PCB treatment was observed on levels of GFAP and synaptophysin in the dorsal hippocampus on PND21 and 90. Calcineurin activity was decreased in the female CB on PND21, but a significant increase in activity was observed in the female CB on PND90. No effect of maternal PCB treatment was observed on the cerebellar calcineurin activity in male offspring on PND21 and 90. EROD activity was highly induced in maternal microsomes from both PCB treatment groups, but only slightly induced in fetal hepatic microsomes. On PND21 weanling hepatic microsomal EROD activity was highly induced following gestational and lactational PCB exposure, however on PND90 EROD activity was unaffected by maternal PCB treatment in male offspring and significantly decreased in female offspring.

The results of the present study indicate that gestational and lactational exposure to the technical PCB mixture results in long term alterations in a neuronal and glial cell markers in specific brain regions of rats. These marker proteins may be useful for determining the strucure-activity relationships in PCB-induced developmental neurotoxicity.

Dennis C. Morse, Annemieke Plug, Wendelien Wesseling, Kor J. van den Berg, Abraham Brouwer.

Introduction

The pre- and postnatal exposure of laboratory animals to polychlorinated biphenyls (PCBs) results in developmental behavioral neurotoxicity, and is one of the most sensitive functional responses to PCB (Tilson *et al.* 1990, review). In Taiwan, human exposure to rice oil containing PCBs and polychlorinated dibenzofurans (PCDFs), with subsequent transplacental exposure of the offspring, resulted in neurodevelopmental effects that have persisted up to 12 years after birth (Chen and Hsu, 1994). Studies on the effects of background exposure to PCBs in the United States (Rogan *et al.* 1986, Gladen *et al.* 1988, Jacobson *et al.* 1985, Jacobson and Jacobson, 1993) have demonstrated associations with the level of exposure and delays in neurological and cognitive development in human infants.

The mechanisms and structure-activity relationships involved in the developmental neurotoxicity of PCBs and PCDFs are unknown, but may be distinct from the Aryl hydrocarbon (Ah) receptor mediated toxic effects in adult animals. The Ah-receptor mediates almost all of the toxic effects of the coplanar PCBs, PCDDs and PCDFs (Poland and Knutson, 1982), but there is evidence that the Ah receptor may not be involved in some aspects of neurotoxicity in adult animals (Seegal *et al.* 1992).

Several hypotheses have been forwarded to explain the developmental neurotoxicity of these compounds, for example that pre- and postnatal alterations in brain thyroid function may affect neuromuscular development (Rogan *et al.*, 1986, Morse *et al.* 1993) and the interaction of di-ortho substituted PCBs with the enzyme tyrosine hydroxylase, resulting in decreases in dopamine levels *in vitro* and *in vivo* in adult animals. (Seegal *et al.* 1992).

In order to test these hypotheses it is helpful to identify specific markers for different brain cell populations which are sensitive to gestational and lactational PCB exposure. Recent advances in the use of specific marker proteins in neurotoxicology (O'Callaghan and Jensen, 1992, Goldey *et al.* 1994, Gramsbergen and van den Berg, in press) and developmental neurobiology (Rami and Rabié, 1990, Leclerc *et al.* 1989), can be applied to investigation of cellular and biochemical responses in developmental neurotoxicology. The regional brain levels of glial fibrillary acidic protein (GFAP) and synaptophysin can be used to follow the normal ontogeny of astrocytes and synaptogenesis as well as the influence of hormonal imbalances like hypothyroidism or a developmental neurotoxin (triethyltin) on brain development (Rami and Rabié, 1990, O'Callaghan and Miller, 1988).

To gain insight in the cellular and biochemical processes of PCB-induced developmental neurotoxicity we investigated the effect of transplacental and lactational exposure of the rat to the technical PCB mixture Aroclor 1254 on the development of GFAP and synaptophysin levels in homogenates of discrete brain regions. A marker for the effects of prenatal hypothyroidism in the cerebellum, calmodulin-dependent phosphatase activity (Ruiz de Elvira *et al.*, 1989) was also studied. A well characterized response of laboratory animals to PCB exposure, the induction of hepatic microsomal ethoxyresorufin-O-deethylase activity (Safe, 1994) was measured for comparison with the neurochemical effects.

Material and Methods

Materials

Aroclor 1254 was kindly donated by Dr. Martin van den Berg, RITOX, Utrecht, the Netherlands. Bovine serum albumin (Fraction V) was purchased from Sigma Chemical Co., St. Louis, U.S.A. The following chemicals were purchased from Merck, Darmstadt, Germany: isopropanol, diethylether, sodium acetate, sodium bicarbonate, sodium carbonate, sodium chloride, sodium hydroxide, Tris, Tween 20 and glycine.
Bovine glial fibrillary acidic protein (GFAP, 981150), monoclonal anti-GFAP (814369), NADPH and p-nitrophenylphosphate were purchased from Boehringer, Mannheim, Germany. Alkaline phosphate conjugate (315-055-003) was purhased from Jackson Immunoresearch. Polyclonal anti-GFAP (Z334), monoclonal mouse anti-synaptophysin (M776), rabbit anti-mouse (Z259) was purchased from Dako, Denmark. [^{125}I]-protein A and Hybond nitro-cellulose were purchased from Amersham, Amersham U.K.

The BCA protein assay reagent was obtained from Pierce. The Bio-Rad protein assay reagent, SDS and 2-mercaptoethanol were purchased from Bio-Rad Laboratories, Munchen, Germany. Calmodulin from bovine brain, methylumbilleferyl phosphate and methylumbilleferone were purchased from Fluka Chemie, Buchs, Switzerland.

Animals

Wistar WU rats, 100 females and 50 males, (14 weeks old) were purchased from Charles River Sutzfeld, Germany. The rats were allowed to acclimatize for 2 weeks and were maintained at 50 % humidity and 21°C on bedding in macrolon cages with a 12 hour light cycle. Rat chow (Hope Farms, Woerden, the Netherlands) and tap water were supplied *ad libitum*. After the acclimatization period two females were placed in a cage with one male overnight from 17:00 to 9:00 the next day. The females were examined each morning by vaginal smear. When spermatazoa were found the animal was housed separately and this was termed day 0 of gestation. Animals were pre-assigned to a particular dose group on a rotating basis. Maternal body weight gain was monitored throughout gestation and lactation. On day 10 of gestation the pregnant female rats were transferred to a macrolon cage with a grated steel support to facilitate the collection of PCB-contaminated feces and to prevent contamination of the animal facilities.

Pregnant females received a daily dose by gavage of 0, 5 or 25 mg Aroclor 1254 per kg body weight dissolved in cornoil (2 ml/kg body weight) from day 10 of gestation to day 16 of gestation. Rats were weighed each day before administration of Aroclor 1254 or corn oil alone. Pregnant rats were transferred to bedding material on gestation day 20 (GD20) and were given paper tissues to make a nest. The rats were inspected each morning at 8:00 h and afternoon at 18:00 h for litters. Pups found during the morning inspection were termed 1 day old, pups born between the morning and afternoon inspection were termed 0 day old. Pups were examined for sex, weighed on day 0 postpartum when possible, and always on day 1, 4 and 7 postpartum and thereafter on a weekly basis. On postnatal day 4 (PND4) litters were reduced to 4 males and 4 females. When this was not possible, litters were made up of a total of 8 males and females from the same dam, and if necessary, an extra pup from another litter from the same treatment group and same age was used to acheive a total of 8 pups per litter. Pups were individually marked by incision in the ear, and pups transferred from one litter to another

were never used for any analysis.

On GD20, 6 pregnant females per treatment group were sacrificed by exsanguination under ether anethesia. The fetuses were removed, blotted dry and weighed. The fetal brains were removed, separated into the forebrain and cerebellum and weighed. Fetal livers and maternal livers were removed and frozen on a block of dry ice, then stored at -80°C until microsomes were prepared. On postnatal day (PND21), 8-10 litters were sacrificed per treatment group. Dams and weanlings were sacrificed by decapitation, and livers were removed, weighed, snap-frozen in liquid nitrogen and stored at -80 °C. Weanling livers were pooled per sex for analysis. Brains were rapidly removed and dissected on an ice-cold plate in lateral olfactory tract, prefrontal cortex, striatum, hypothalamus, dorsal hippocampus, cerebellum and brainstem. The brain regions were immediately frozen on a block of dry-ice, weighed and stored at -80°C. Weanlings from the remaining litters were weaned on PND21 and male and female rats were housed separately in groups of 2-4 rats per cage. The remaining animals (10 litters per treatment group) were sacrificed 90 days (PND90) after birth and livers and brain regions were removed, weighed and stored as described above.

Ethoxyresorufin-O-deethylase (EROD) activity

Hepatic microsomes were prepared as previously described (Morse *et al.* 1993). EROD activity was measured with hepatic microsomes essentially according to Burke *et al.* (1977). adapted for use as with 96 well plates and a fluorospectrophotometric plate reader (CytoFluor 2350, Millipore). The reaction conditions (in a total volume of 200 μl) were 0.1 Tris-HCl buffer, pH 7.8, 1 mg BSA/ml, 0.1 mM NADPH, 0.4 μM ethoxyresorufin (ER), microsomal protein concentrations were varied between 100 μg/ml for controls and 1 μg/ml for weanlings from the 25 mg Aroclor 1254/kg treatment group. The reaction mixtures were preincubated at 37°C for 2 minutes, the reaction was started by the addition of ER, and incubations were stopped after 5 minutes at 37°C by the addition of 50 μl 1 N NaOH. The formation of the product resorufin was detected fluorimetrically and compared to a calibration curve (0-150 nM resorufin) made up in in the Tris-HCl buffer with 0.8 mg BSA/ml and 0.8 N NaOH. Incubations were carried out in duplicate and corrected for a blank without NADPH. Hepatic microsomal protein concentrations were determined according to Bradford (1976).

Calmodulin-dependent phosphatase activity

Calmodulin-dependent phosphatase activity was determined fluorimetrically in cerebellar supernatants using methylumbilleferone phosphate as a substrate according to Ruiz de Elvira *et al.* (1988). Cerebella were homogenized in a Potter tube with 10 volumes (v/w) ice-cold Tris-HCl buffer, pH 7.0, 0.2 mg BSA/ml, 0.01% (v/v) mercaptoethanol and 2 mM EDTA. The homogenate was centrifuged at 1500g and 4°C for 30 min. The supernatant was removed and stored at -80°C until analysis. Final incubation conditions (in 400 μl) were 10 μg supernatant protein/ml, 12.5 mM Tris-HCl buffer, pH 7.0 and 15 μM methylumbelliferyl phosphate as substrate for basal phosphatase activity, while 60 nM calmodulin (previously saturated with Ca^{2+}) was added to detect the sum of calmodulin-dependent and basal phosphatase activity. The reaction mixtures were preincubated at 24°C for 2 minutes, the reaction was started by the addition of the substrate, and after 5 min at 24°C, the reaction was terminated by the

addition of 2 ml ice-cold 0.2 M NaOH-glycine buffer, pH 10.4. The production of the dephosphorylated product, methylumbelliferone, was detected fluorimetrically with an excitation wavelength of 361 nm and an emmision wavelength of 444 nm and compared to a calibration curve produced by incubating 0-1 μM methylumbelliferone with 10 mg BSA/ml and 12.5 mM Tris-HCl buffer, pH 7.0 as above and terminating the incubation after 5 minutes in the same manner. The difference between the total activity with calmodulin and the basal phosphatase activity represents the calmodulin-dependent phosphatase activity. Incubations were carried out in duplicate. Protein concentrations were determined according to Bradford (1976).

Glial fibrillary acidic protein (GFAP)

GFAP concentrations were determined in several brain regions (lateral olfactory tract: LOT, prefrontal cortex: PFC, striatum: ST, hypothalamus: HT, dorsal hippocampus: HC, cerebellum: CB, brainstem: BS) in duplicate using a sandwich ELISA method adapted from O'Callaghan (1991) and described in detail by Van den Berg and Gramsbergen (1993). Tissue samples were homogenized in 10 volumes (w/v) of a hot (90°C) 1% (w/v) SDS solution with a sonicator and stored at -20°C until analysis. The samples were diluted before analysis with phosphate buffered saline containing 0.02% v/v Tween-20. The dilutions used for the brain regions were: cerebellum, 793 fold; striatum, 143 fold; brainstem, 533 fold; hypothalamus, 143 fold for PND21 and 861 fold for PND90; lateral olfactory tract, 506 fold; prefrontal cortex, 231 fold. The calibration curve ranged from 4 to 250 ng GFAP/ml. Protein concentrations were determined with the bicinchoninic acid method from Pierce.

Synaptophysin

Synaptophysin levels in brain regions (LOT, PFC, ST, HT, CB, BS) were determined in triplicate using a modification of the dot-blot method described by Brock and O'Callagan 1987). All steps were carried out at room temperature unless otherwise stated. The same tissue homogenates described above (except the dorsal hippocampus) were diluted to a protein concentration of 50 μg/ml in PBS and 20 μl of the diluted homogenate was absorbed to Hybond nitrocellulose in a Dot Blot apparatus (Biorad, Richmond CA). Samples from all treatment groups from one sex, one brain region and one timepoint were incubated per filter. The proteins were fixed to the filter by incubation with a 2.5% glutaraldehyde (w/v) solution in 0.2 M Na-acetate solution for 15 minutes. The filter was washed 5 times with demineralized water and then once with PBS (5 min). The remaining sites on the filter were blocked by an incubation of 1 h (37°C) with PBS containing 2.5% w/v BSA. The filter was then incubated with monoclonal mouse anti-synaptophysin, (diluted 1:200 with PBS) with 0.1% (v/v) Tween-20 and 1% (w/v) BSA. The filter was then washed 3 times with PBS (5 min). Subsequently, the filter was incubated with the second antibody, rabbit-anti-mouse, (diluted 1:100 with PBS) with 0.1% (v/v) Tween-20 and 1% (w/v) BSA for 30 min at 37°C. The filter was then washed 3 times with PBS (5 min). The incubation with ^{125}I-protein A (8 μCi/8 ml PBS with 0.1% v/v Tween-20) was carried out at room temperature for an hour. The filter was washed 3 times 5 minutes and 2 times 15 minutes with PBS, then dried at 40°C. The radioactivity on the filter was quantified directly with a Matrix 96 counter (Packard) and corrected for background counts. The fixation of the proteins, subsequent washing and blokking of the

filter was carried out in a plastic box with 50 ml of the respective solutions. The incubations with antibodies and subsequent wash steps were carried out in a slowly rotating 500 ml plastic bottle with an 8 ml volume of the respective solutions. As purified synaptophysin was unavailable for a standard, the results obtained are expressed as percentage of control values based on the cpm ^{125}I-protein A/μg protein.

Statistical analysis

Treatment-related effects were first evaluated with a one-way analysis of variance followed by a least significant difference test to find significant differences between the treatment groups. Data are presented as the mean±SEM.

Results

Reproduction, body weight, relative and absolute brain weight

Reproductive parameters, including maternal body weight gain before and after birth, fetal body weight, number of live fetuses, late gestational death, number of resorptions, number of live pups born, male to female ratio and postnatal death were not affected by maternal PCB exposure (data not shown).

Male and female fetal body weights, absolute and relative liver, forebrain and cerebellum weights were unaltered by in utero exposure to Aroclor 1254 on gestation day 20 (GD20, data not shown). Maternal exposure to the lower dose (5 mg/kg) of Aroclor 1254 on day 10 to 16 of gestation did not result in significant effects on body weights, absolute or relative forebrain and cerebellum weights of male and female offspring 21 and 90 days after birth (Table 7.1). On PND21 slight but significant reductions in male weanling forebrain and cerebellum weights (4.6 and 5.8% respectively) were observed in offspring from dams exposed to 25 mg Aroclor 1254/kg bw. Relative forebrain and cerebellum weights were unaffected by maternal PCB exposure on PND21 in both male and female offspring.

On PND90 male and female body weights were significantly reduced by 7.1% and 8.7% respectively (P<0.01) following maternal exposure to 25 mg Aroclor 1254/kg bw. Absolute forebrain weight was significantly reduced in male offspring from the high dose group (6.4%, P<0.0001), reductions in forebrain weights from female offspring from the high dose group were lower (2.5%) and did not attain statistical signifigance. Relative forebrain weight were significantly increased (8.6%, P<0.05) in females from the high dose group on PND90. Both absolute and relative cerebellum weight was unaffected by maternal PCB exposure on PND90 in both male and female offspring.

EROD activity

The effect of maternal PCB exposure on EROD activity of hepatic microsomes from maternal rats and their fetal, weanling and adult offspring is presented in Table 7.2. Maternal hepatic microsomal EROD activity was induced 20 fold by the lowest PCB dose and more than 200 fold by the highest PCB dose relative to controls on GD20. Fetal (GD20) EROD activity was barely detectable in microsomes from the control and low dose group (0.2 pmol/(mg protein x min)), but was induced 5 fold in the

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Table 7.1
Body weight, absolute and relative brain weight in the offspring of PCB exposed dams

	Dose mg Aroclor 1254/kg body weight		
	0	5	25
	PND21		
male offspring			
body wt (g)	47.8±1.56	48.5±1.5	45.2±1.3
forebrain wt (g)	0.952±0.010	0.948±0.014	0.908±0.001**
cerebellum wt (g)	0.172±0.002	0.173±0.003	0.162±0.004*
rel. FB wt (%)	1.997±0.076	1.981±0.060	2.053±0.066
rel. CB wt(%)	0.364±0.001	0.359±0.001	0.368±0.001
female offspring			
body wt (g)	46.27±1.753	47.13±1.233	43.46±1.286
forebrain wt (g)	0.915±0.014	0.910±0.009	0.884±0.010
cerebellum wt (g)	0.167±0.003	0.163±0.003	0.152±0.003
rel. FB wt (%)	2.033±0.096	1.911±0.070	2.042±0.065
rel. CB wt (%)	0.367±0.002	0.350±0.001	0.368±0.006
	PND90		
male offspring			
body wt (g)	352.4±5.0	353.6±9.3	327.5±3.8**
forebrain wt (g)	1.323±0.011	1.304±0.013	1.238±0.013****
cerebellum wt (g)	0.273±0.004	0.264±0.006	0.260±0.007
rel. FB wt (g)	0.382±0.005	0.373±0.013	0.395±0.006
rel. CB wt (g)	0.077±0.001	0.075±0.002	0.080±0.002
female offspring			
body wt (g)	214.9±3.4	213.3±6.0	196.2±3.5**
forebrain wt (g)	1.217±0.012	1.201±0.025	1.186±0.016
cerebellum wt (g)	0.248±0.005	0.247±0.006	0.238±0.005
rel. FB wt (%)	0.568±0.009	0.581±0.012	0.617±0.015*
rel. CB wt (%)	0.116±0.003	0.118±0.004	0.118±0.003

FB: forebrain, CB: cerebellum, * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$, N=8-10 on postnatal day 21 (PND21) and N=10 on postnatal day 90 (PND90). Pregnant rats were treated with 0, 5 or 25 mg Aroclor 1254/kg body weight on day 10 to 16 of gestation.

Table 7.2
EROD activity in hepatic microsomes from dams, fetuses, weanling and adult offspring following exposure of pregnant rats to PCBs.

	Dose mg Aroclor 1254/kg body weight		
	0	5	25
	GD20		
fetal[a]	0.20±0.04	0.17±0.03	1.03±0.19***
maternal[b]	0.051±0.004	1.04±0.19	11.1±0.8****
	PND21		
male[b]	0.068±0.011	3.33±0.18**	11.60±0.18****
female[b]	0.063±0.011	3.41±0.33****	9.30±0.34****
	PND90		
male[b]	0.019±0.002	0.019±0.001	0.023±0.001
female[b]	0.047±0.004	0.021±0.003****	0.036±0.003*

a) pmol RR/(min x mg protein), b) nmol RR/(min x mg protein), * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$, N=6 on gestation day 20 (GD20), N=8-10 on postnatal day 21 (PND21) and N=10 on postnatal day 90 (PND90). Pregnant rats were treated with 0, 5 or 25 mg Aroclor 1254/kg body weight on day 10 to 16 of gestation.

high dose group (1.0 ±0.2 pmol resorufin/mg x min). The induction of EROD activity in male and female weanling (PND21) hepatic microsomes was nearly identical, an induction of 50 fold and 150 fold was observed in the low and high dose groups, respectively. Maternal PCB exposure had no effect on hepatic EROD activity in adult male offspring (PND90), although in adult female offspring hepatic EROD activity was decreased by 55% ($P<0.0001$) and 23% ($P<0.05$) by the low and high PCB dose, respectively.

Calmodulin-dependent phosphatse activity
        Effects on the level of calmodulin-dependent phosphatase activity were observed only in the cerebella of the female offspring (Figure 7.1). On PND21 a significant decrease in cerebellar calmodulin-dependent phosphatase activity was observed of 38%

Figure 7.1 Calmodulin-dependent phosphatase activity (nmol methylumbelliferone (MU) formed/(min x mg protein) in cerebellar supernatants from weanling and adult offspring from pregnant rats treated with 0, 5 or 25 mg Aroclor 1254/kg body weight on day 10 to 16 of gestation. ** P<0.01, *** P<0.001, N=8-10 on postnatal day 21 and N=10 on postnatal day 90.
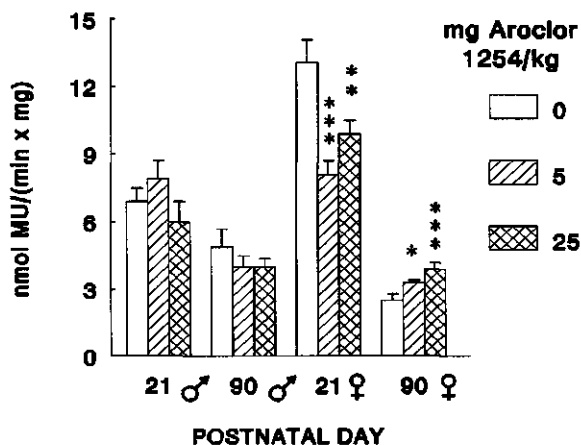
(P<0.001) and 24% (P<0.01) in female weanlings from the low and high dose group, while in adult female offspring (PND90) calmodulin-dependent phosphatase activity increased 31% (P<0.05) and 54% (P<0.001) in the low and high dose group.

Glial Fibrillary Acidic Protein (GFAP)

The effects of maternal PCB exposure on the levels of GFAP (µg GFAP/mg total protein in the SDS brain homogenates) in brain regions from weanling and young adult offspring are presented in Table 7.3. For illustrative purposes, the major effects of maternal PCB exposure on regional GFAP levels (lateral olfactory tract, cerebellum and brainstem) of weanling and adult offspring are presented in Figure 7.2a-c as percentage of control values. No treatment-related effects were observed in GFAP levels in the hypothalamus and dorsal hippocampus.

Lateral olfactory tract (LOT)

On PND21 the most consistent effects on GFAP levels were observed in the LOT, for maternal PCB exposure resulted in significant increases in GFAP concentrations of 22% (P<0.01) and 42% (P<0.001) in the LOT from female weanlings from the low and high PCB dose group, respectively. In male weanlings a significant increase (35%, P<0.05) in GFAP concentrations in the LOT were observed in the low PCB group, while a slight and statistically non-significant increase (18%) was observed in GFAP levels in the LOT from male weanlings from the high dose group. On PND90 increases in GFAP levels relative to controls in the LOT from male offspring were highly significant in both the low (34%, P<0.001) and high (32%, P<0.001) PCB treatment groups. In female offspring on PND90 GFAP levels in the LOT were increased significantly increased relative to controls (63%, P<0.001) only in the high PCB dose group.

Cerebellum (CB)

On PND21 a significant increase in GFAP concentrations (35% relative to controls, P<0.001) in the CB were observed only in female weanlings from the high dose group. However, on PND90 levels of GFAP were significantly elevated relative to controls in female offspring from both the low (29%, P<0.0001) and high exposure group (35%, P<0.0001) and in young adult males from the high dose group (33%, P<0.001).

Brainstem (BS)

In contrast to the LOT and CB, decreases were observed in GFAP concentrations in the BS of offspring from PCB-exposed dams. The only significant decrease observed on PND21 in GFAP concentrations in the BS was in females from the low dose group (23% relative to controls, P<0.05). On PND90 significant decreases in BS GFAP concentrations were observed in male and female offspring from the low dose group (42% and 31%, respectively) and from the high dose group (39% and 29%, respectively). While BS GFAP levels increased 56% and 22% in male and female control offspring between PND21 and 90, no increases in BS GFAP concentrations were observed in the PCB-exposed offspring over the same period (Table 7.3).

Prefrontal cortex (PFC) and striatum (ST)

Less consistent alterations in GFAP levels were observed in the PFC and ST in the offspring following maternal PCB exposure. GFAP concentrations were elevated in the PFC in male and female weanlings, but these increases were not statistically significant. On PND90 GFAP levels were significantly increased in the PFC of male weanlings from the low dose group (31%, P<0.01) and of female weanlings from the high dose group (46%, P<0.05).

Figure 7.2a. Relative levels of glial fibrillary acidic protein (GFAP, % of controls) in the lateral olfactory tract of weanling and adult offspring from pregnant rats treated with 0, 5 or 25 mg Aroclor 1254/kg body weight on day 10 to 16 of gestation. * P<0.05, ** P<0.01, *** P<0.001, N=8-10 on postnatal day 21 and N=10 on postnatal day 90.
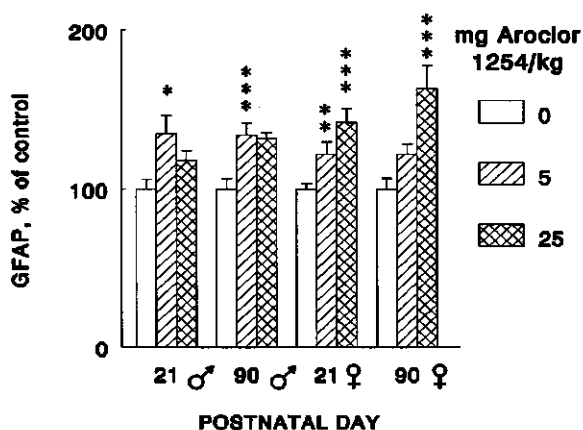
Significant increases in ST GFAP levels were observed in male, but not female weanlings from both the low and high PCB treatment groups (50%, P<0.05 and 74%, P<0.01, respectively). On PND90 reductions in ST GFAP levels were observed in both male and female offspring from the high dose group (19% and 28%, P<0.05, respectively).

Figure 7.2b Relative levels of glial fibrillary acidic protein (GFAP, % of controls) the cerebellum of weanling and adult offspring from pregnant rats treated with 0, 5 or 25 mg Aroclor 1254/kg body weight on day 10 to 16 of gestation. *** P<0.001, **** P<0.0001, N=8-10 on postnatal day 21 and N=10 on postnatal day 90.
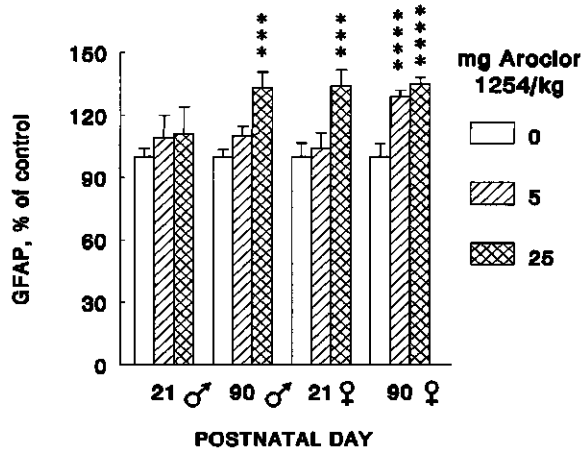
Figure 7.2c Relative levels of glial fibrillary acidic protein (GFAP, % of controls) in the brainstem of weanling and adult offspring from pregnant rats treated with 0, 5 or 25 mg Aroclor 1254/kg body weight on day 10 to 16 of gestation. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001, N=8-10 on postnatal day 21 and N=10 on postnatal day 90.

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Table 7.3
Regional brain GFAP levels (µg/mg protein) in weanling and adult offspring following exposure of pregnant rats to PCBs.

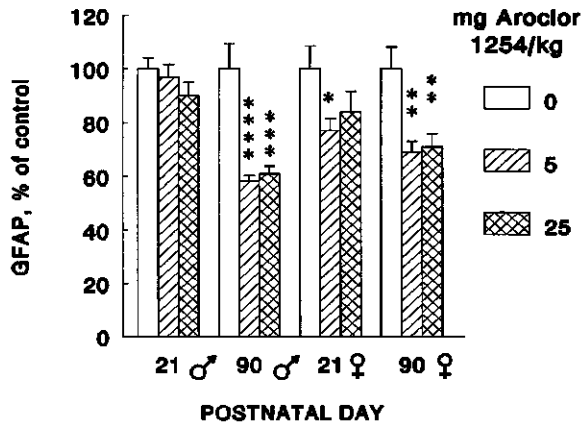|	Dose mg Aroclor 1254/kg body weight		
	0	5	25
PND21, Males			
LOT	1.31±0.08	1.77±0.15*	1.55±0.08
PFC	0.97±0.06	1.19±0.08	1.15±0.11
STR	0.34±0.03	0.51±0.07*	0.59±0.09**
HC	1.83±0.23	1.69±0.29	1.72±0.31
HTH	0.74±0.05	0.77±0.03	0.71±0.04
CB	1.00±0.04	1.09±0.06	1.11±0.13
BS	2.01±0.08	1.95±0.09	1.80±0.10
PND90, Males			
LOT	2.06±0.13	2.77±0.15***	2.71±0.07***
PFC	1.41±0.09	1.85±0.10**	1.65±0.06
STR	0.69±0.03	0.65±0.04	0.56±0.03*
HC	1.59±0.18	1.54±0.13	1.60±0.16
HTH	3.30±0.37	4.15±0.29	3.71±0.42
CB	3.24±0.11	3.58±0.16	4.31±0.25***
BS	3.14±0.29	1.83±0.07****	1.93±0.09***
PND21, Females			
LOT	1.16±0.04	1.41±0.09**	1.65±0.10***
PFC	0.76±0.07	0.77±0.08	0.94±0.07
STR	0.52±0.04	0.56±0.07	0.50±0.06
HC	1.28±0.08	1.39±0.11	1.39±0.13
HTH	0.59±0.06	0.64±0.05	0.75±0.05
CB	0.76±0.05	0.79±0.07	1.02±0.06***
BS	2.23±0.19	1.71±0.10*	1.87±0.17
PND90, Females			
LOT	1.44±0.10	1.76±0.09	2.34±0.21***
PFC	0.82±0.06	1.09±0.11	1.20±0.15*
STR	0.76±0.07	0.71±0.04	0.55±0.04*
HC	1.18±0.06	1.24±0.17	1.47±0.18
HTH	3.29±0.45	3.21±0.46	3.14±0.52
CB	4.78±0.30	6.18±0.15****	6.45±0.16****
BS	2.72±0.22	1.89±0.11**	1.93±0.13**

LOT: lateral olfactory tract, PFC: prefrontal cortex, ST: striatum, HTH, hypothalamus, HC: dorsal hippocampus, CB: cerebellum, BS: brainstem, * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001, N=8-10 on postnatal day 21 and N=10 on postnatal day 90. Pregnant rats were treated with 0, 5 or 25 mg Aroclor 1254/kg body weight on day 10 to 16 of gestation.

Synaptophysin.

Relative concentrations of synaptophysin in the LOT, PFC and BS of offspring on PND21 and 90 after maternal PCB exposure are shown in Figure 7.3a-c, while data from the female ST and hypothalamus (HTH) are shown in Table 7.4.

Lateral olfactory tract

In the LOT synaptophysin concentrations were decreased by 26% (P<0.001) and 18% (0.001) in male and female weanlings (PND21) from the high PCB dose group (Figure 7.3a). ON PND90 reductions were still observed in synaptophysin concentrations in the LOT from female offspring from the high dose group (27% relative to controls, P<0.001), but a slight, but significant increase was observed in LOT synaptophysin concentrations (15%, P<0.05) in male offspring.

Prefrontal Cortex

On PND90 synaptophysin levels in the PFC (Figure 7.3b) were significantly decreased by 17% (P<0.0001) and 31% (P<0.0001) in male offspring from the low and high PCB dose group respectively, while in female offspring from PND90 significant decreases in PFC synaptophysin concentrations were observed only in the high PCB dose group (14%, P<0.001). No effects of maternal PCB exposure were observed on synaptophysin concentrations in the PFC on PND21 in either male or female weanlings.

Brainstem

In the BS synaptophysin concentrations (Figure 7.3c) were significantly decreased on PND21 in male weanlings from the high dose group (38%, P<0.0001) and in female weanlings from both the low (29%, P<0.01) and high dose groups (18%, P<0.01).

Figure 7.3a Relative levels of synaptophysin (% of controls) in the lateral olfactory tract of weanling and adult offspring from pregnant rats treated with 0, 5 or 25 mg Aroclor 1254/kg body weight on day 10 to 16 of gestation. * P<0.05, ** P<0.01, *** P<0.001, N=8-10 on postnatal day 21 and N=10 on postnatal day 90.

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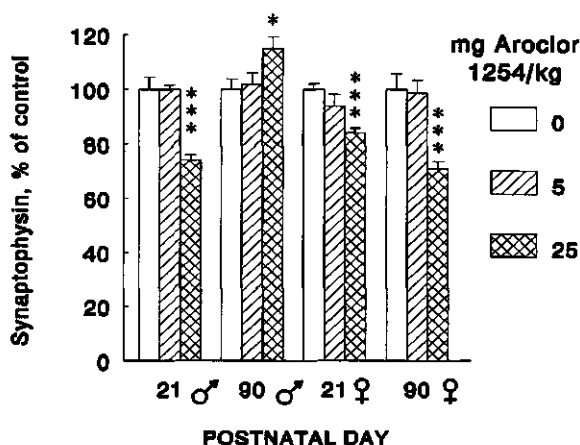
On PND90 synaptophysin levels were lower relative to controls in the BS of male weanlings from both the low and high PCB dose groups (20%, P<0.05 and 31%, P<0.01, respectively). In contrast to the male offspring, on PND90 synaptophysin levels in the BS of female offspring were significantly increased by 47% (P<0.05) and 109% (P<0.0001) relative to controls in the low and high PCB dose group, respectively.

Other brain regions

Effects on striatal syanaptophysin levels were observed in female offspring from the high PCB dose group, with reductions of 24% (P<0.05) in the high dose group on PND21 and reductions of 23% (P<0.05) and 26% (P<0.01) on PND90 in the low and high PCB dose group, respectively. Also in the hypothalamus reductions in synaptophysin concentrations were observed in the female offspring, with a 27% (P<0.01) and 35% (P<0.001) decrease relative to controls on PND21 in the low and high PCB dose group, respectively, and a 29% (P<0.05) decrease in the low PCB dose group on PND90. Relative levels of synaptophysin in the male ST and HTH were unaffected by maternal PCB exposure on PND21 and 90 (data not shown). Cerebellar concentrations of synaptophysin were unaltered following maternal PCB exposure in both male and female offspring on PND21 and 90 (data not shown).

Figure 7.3b Relative levels of synaptophysin (% of controls) in the prefrontal cortex of weanling and adult offspring from pregnant rats treated with 0, 5 or 25 mg Aroclor 1254/kg body weight on day 10 to 16 of gestation. * P<0.05, **** P<0.0001, N=8-10 on postnatal day 21 and N=10 on postnatal day 90.
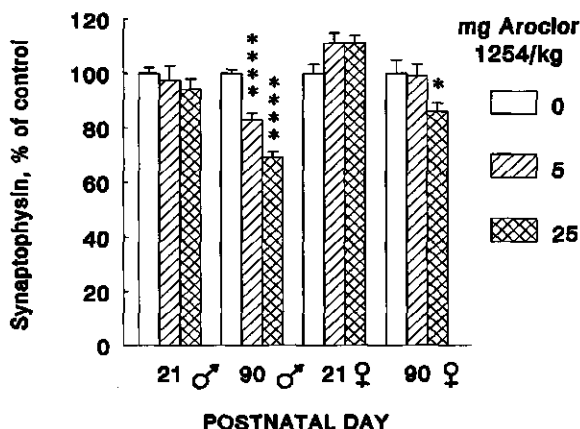
Figure 7.3c Relative levels of synaptophysin (% of controls) in brainstem of weanling and adult offspring from pregnant rats treated with 0, 5 or 25 mg Aroclor 1254/kg body weight on day 10 to 16 of gestation. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001, N=8-10 on postnatal day 21 and N=10 on postnatal day 90.
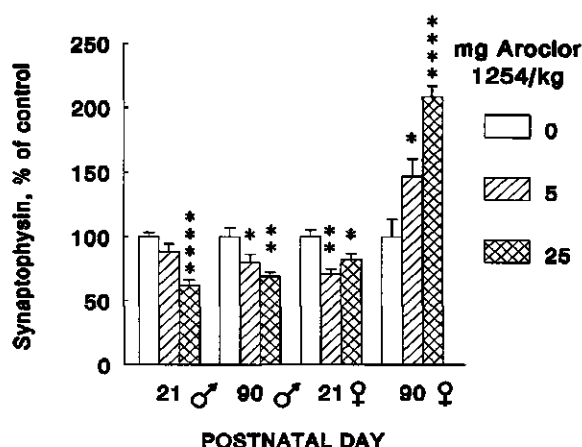
Table 7.4
Regional brain synaptophysin levels (% of control) in weanling and adult female offspring, following exposure of pregnant rats to PCBs.

	Dose mg Aroclor 1254/kg body weight		
	0	5	25
		PND21	
Striatum	100+9	102+8	76+5*
Hypothalamus	100+5	73+3	65+8.2***
		PND90	
Striatum	100+6	77+7*	74+5**
Hypothalamus	100+7	71+8*	83+19

* P<0.05, ** P<0.01, *** P<0.001, N=8-10 on postnatal day 21 (PND21) and N=10 on postnatal day 90 (PND90). Pregnant rats were treated with 0, 5 or 25 mg Aroclor 1254/kg body weight on day 10 to 16 of gestation.

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Discussion

The current study was undertaken to evaluate the sensitivity of several neurochemical markers for the developmental neurotoxicity of the polychlorinated biphenyl mixture (Aroclor 1254) in male and female offspring of the rat. Long term effects (up to PND90) of maternal PCB exposure were observed in the regional brain levels of glial fibrillary acidic protein, synaptophysin concentrations and calmodulin-dependent phosphatase activity. These effects were observed at a PCB dose that had no effect on the reproductive parameters examined (5 mg Aroclor 1254/kg bw) and at a PCB dose which had a slight effect on body weight gain, food consumption and forebrain weight in the offspring (25 mg Aroclor 1254/kg bw). However, it is unlikely that the biochemical effects observed at the higher maternal PCB dose are due to undernutrition, for undernutrition affects the growth rate of all processes in the brain to the same extent (Peeling and Smart, 1994, review). A sensitive and well characterized response of experimental animals to PCB exposure, the induction of hepatic microsomal ethoxyresorufin-O-deethylase (EROD) activity (Safe, 1994), occurred in fetal, maternal and weanling rats, but this effect was absent in young adult rats, indicating that residual PCB levels were no longer causing direct effects in the liver. However, residual effects of PCBs in the brain cannot be ruled out.

In this study the differential effects on synaptophysin and GFAP levels in the diverse brain regions indicates that several mechanisms are involved in the alteration of brain development, following maternal PCB exposure. The alterations in regional brain GFAP and synaptophysin levels can be evaluated in terms of 1) reactive gliosis following direct damage of the neurons or glia, or 2) as a hormone-dependent alteration in the regulation of brain development. Correspondingly, it has been previously proposed that the developmental neurotoxicity of PCBs may be due to direct effects of PCBs on neuronal cells (Seegal *et al.* 1992) or indirectly due to PCB-induced endocrine imbalances (Rogan *et al.* 1986).

Reactive gliosis can occur as a response to either mechanically induced trauma (Eng, 1988) or chemically induced neuronal loss (O'Callaghan and Jensen, 1992) resulting in increases in GFAP levels and decreases in synaptophysin levels. Increases in GFAP concentrations accompanied by decreases in neurotypic proteins following neurotoxicant treatment have been associated with neuronal damage in the developing (O'Callaghan and Miller, 1988, 1989) and adult central nervous system (Brock and O'Callaghan, 1987).

The effects in GFAP and synaptophysin levels that best resemble the result of neuronal damage or death are found primarily in the lateral olfactory tract and prefrontal cortex and to a lesser extent in the cerebellum. The lack of effect of transplacental and lactational PCB exposure on synaptophysin levels in the presence of significant increases in GFAP levels in certain brain regions may indicate that neuronal damage was limited to a small subpopulation of neurons, or to neurons containing low levels of synaptophysin.

The mechanisms involved in the neuronal damage and/or delayed synaptogenesis and increased GFAP levels in the LOT and PFC following maternal PCB exposure are unclear. The effects observed in the current study in the lateral olfactory tract and the

prefrontal cortex, which are indicative of neuronal damage or death and subsequent astrogliosis, could be due to a developmental delay of neurogenesis and astrogliogenesis in the brainstem. The brainstem is one of the first brain regions to exhibit a neuronal growth spurt (Rodier, 1980, 1988) and contains raphe nucleus cell bodies which project to the frontal cortex (Kosofsky and Molliver, 1987). A delay of the innervation of cortical neurons could result in their premature death, manifesting itself as decreased concentrations of synaptophysin and increased concentrations of GFAP. This hypothesis is in part supported by the observation that serotonin metabolism is primarily affected in the LOT and PFC (unpublished data) in this study. The serotonergic system is one of the main systems projecting from the brainstem to the frontal cortex and lateral olfactory tract (Molliver, 1987). In the current study the relatively low levels of GFAP on PND90 and of synaptophysin on PND21 in the brainstem may be indicative of delayed development following maternal PCB exposure.

The alterations in GFAP and synaptophysin levels in the LOT and PFC observed in this study may be a result of direct effects of PCBs in these brain regions. *in vitro* studies have demonstrated direct effects of PCBs on dopamine metabolism in the neuroendocrine cell line PC12 (Seegal *et al.* 1992), which may correspond to decreases in regional brain dopamine levels *in vivo* in adult rats and macaques (Seegal *et al.*, 1991, Seegal *et al.*, 1994). The mechanistic significance of this finding for the developing nervous system has not yet been determined, and no significant decreases of regional brain dopamine levels were observed in this study (unpublished observations). Disruption of calcium homestasis in rat cerebellar granular cells (Kodavanti *et al.* 1993) and adenosine triphosphatse activity in mitochondrial and synaptosomal fractions (Maier *et al.* 1994) has been demonstrated *in vitro* with relatively high PCB concentrations (in the µM range), while no *in vivo* counterpart of these effects by PCBs has yet been demonstrated. Although PCB congeners induced biochemical effects in PC12 and cerebellar granular cells, no cytotoxicity was observed with cell culture media concentrations up to 200 µM (Kodavanti *et al.* 1993, Seegal *et al.* 1991), which exceeds the highest brain PCB concentration in this study by a factor of 60,000 (Eva Klasson Wehler, personal communication). It is therefore unlikely that brain PCB residues resulted directly in neuronal degeneration, although alterations in neuronal migration and differentiation caused directly by PCBs cannot be ruled out.

It has been proposed that PCB-induced alterations in pre- and postnatal thyroid hormone levels and metabolism may be involved in PCB induced developmental neurotoxicity (Rogan *et al.*, 1986, Morse *et al.* 1993). Maternal PCB exposure has been associated with decreased plasma thyroid hormones, (Collins and Capen, 1980, Morse *et al.* 1993, Ness *et al.*, 1993) and altered brain thyroid homone metabolism (Morse *et al.* 1993) in fetal and weanling rats. Decreases in fetal and weanling brain thyroid hormone levels have also been observed in this study (Morse *et al.* manuscript submitted for publication). In humans, negative correlations have been observed between PCB, PCDD and PCDF concentrations in milk fat and maternal plasma thyroid hormone levels during gestation and lactation, and higher PCB, PCDD and PCDF levels were associated with decreases in neonatal plasma thyroid hormone levels (Koopman-Essenboom *et al.* 1994).

Hormonal modifications of regional brain GFAP levels have been observed in both developing and adult rodents. Decreases in GFAP immunoreactivity in the hippocampus and cerrebellum, (Faivre-Sarrailh *et al.* 1991), and the parietal cortex

(Granholm *et al*. 1985) and the delayed synaptogenesis in the dentate gyrus as evaluated by synaptophysin immunoreactivity have been associated with delayed brain maturation following neonatal hypothyroidism.

Additional evidence for endocrine-related effects of PCBs on brain development can be derived from effects observed in the hypothalamus in the current study. We found no evidence for reactive gliosis in male or female offspring from PND21 or 90, while synaptophysin levels were significantly reduced in the hypothalamus of female offspring on PND21 and 90. This effect may reflect the influence of some PCB congeners with estrogenic properties (Korach *et al*. 1988, Jansen *et al*. 1993) on neuronal plasticity in the hypothalamus (McEwen, 1992). Furthermore, hippocampal GFAP surface density is lower in adult female rats than in males, but this difference can be abolished by androgenization of female weanlings (Garcia-Segura *et al*., 1988), thereby establishing a role of gonadal hormones in GFAP levels. Estradiol also modulates GFAP immunoreactivity in the arcurate nucleus in adult female rats (Garcia-Segura, 1994).

Although it has been demonstrated that maternal PCB exposure can result in decreased circulating thyroid hormones in the fetal, neonatal and weanling rats (Morse *et al*. 1993) and that postnatal hypothyroidism can result in alterations in synaptogenesis and the distribution of GFAP immunoreactivity in the rat brain, the comparison of the effects reported in this study with previous reports on postnatal hypothyroidism is fraught with difficulties. The most significant problem is the existence of significant inconsitencies in the results of studies on the effect of postnatal hypothyroidism on the development of GFAP immunoreactivity in the rat brain (Kálmán *et al*. 1991). For example, while little effect was observed on GFAP immunoreactivity in serial sections from rats from an unspecified strain thyroidectomized on postnatal day 3 to 5, (Kálmán *et al*. 1991), propylthiouracil treatment of Wistar rat dams from GD18 to PND35 resulted in significant decreases in GFAP concentrations in the cerebellum of the offspring on PND35 (Faivre-Sarrailh, 1991). It is therefore possible that the results on GFAP levels after hypothyroidism are affected by the rat strain or the protocol for the induction of hypothyroidism, and an intrinsic neurotoxicity of propylthiouracil cannot be excluded.

Partial support for the role of thyroid hormones in PCB-induced developmental neurotoxicity is that the activity of calmodulin-dependent phosphatase was significantly increased in the cerebellum of female, but not male offspring on PND21 and 90. This may be an indication of developmental alteration in the female cerebellum, for prenatal hypothyroidism results in increased calmodulin-dependent phosphatase activity in young adult rats (Ruiz de Elvira *et al*., 1989).

In conclusion, this study demonstrates that gestational and lactational PCB exposure results in long-term alterations in glial and neuronal cell marker proteins in rats. The mechanisms involved in the alterations of these proteins are unclear, but may involve endocriene mechanisms. In addition, GFAP and synaptophysin may be useful markers for the investigation of the structure-activity relationships of the developmental toxicity of PCBs both *in vivo* and *in vitro*.

CHAPTER 8

LONG-TERM ALTERATIONS IN REGIONAL BRAIN SEROTONIN METABOLISM FOLLOWING MATERNAL POLYCHLORINATED BIPHENYL EXPOSURE IN THE RAT

Abstract

Pregnant Wistar WU rats were administered PCBs (0, 5 or 25 mg Aroclor 1254 per kg body weight) by gavage on day 10 to 16 of gestation. Levels of biogenic amines were measured in the lateral olfactory tract, prefrontal cortex, striatum, hippocampus and hypothalamus in male and female offspring 21 and 90 days after birth. 5-hydroxyindole acetic acid (5-HIAA) concentrations and the ratio of 5-HIAA/5-hydroxytryptamine (5-HT, serotonin) were significantly increased in the lateral olfactory tract, prefrontal cortex and hippocampus on postnatal day 90 in male and female offspring following maternal PCB treatment. No effects were observed on regional brain levels of dopamine, 3,4-dihydroxyphenylacetic acid, norepinephrine and homovanillic acid. The results indicate that pre- and postnatal exposure to Aroclor 1254 results in regionally specific long-term alterations in the serotonergic system.

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Introduction

Transplacental and/or lactational exposure to background levels of polychlorinated biphenyls has been associated with cognitive and neurological alterations in human infants (Jacobson *et al.*, 1990, Rogan *et al.*, 1986). The pre- and postnatal exposure of laboratory animals to either individual PCB congeners or various commercial mixtures has confirmed the developmental neurotoxicity of PCBs (for a review see Tilson *et al.* 1990). However, the majority of the studies on the developmental neurotoxicity of PCBs has focussed on behavioral effects, so there is little information available on the neurochemical alterations underlying the behavioral changes. Neonatal exposure to a single oral dose of 41 mg 3,3',4,4'-tetrachlorobiphenyl per kg body weight has been reported to decrease neonatal and increase adult muscarinic receptor levels in the hippocampus of the mouse, which was accompanied by alterations in open field activity (Eriksson, 1988, Eriksson *et al.* 1991). The repeated oral exposure of CD-1 mice to 32 mg 3,3',4,4'-tetrachlorobiphenyl per kg body weight on day 10 to 16 of gestation resulted in decreases in striatal dopamine levels and receptor binding sites in the adult offspring (Agrawal *et al.* 1981). However, 3,3',4,4'-tetrachlorobiphenyl is toxic to the conceptus at a maternal dose of 4 mg/kg and reduces maternal body weight gain at 16 mg/kg in CD-1 mice (Marks *et al.* 1989), which suggests that the dose used by Agrawal *et al.* (1981) was too high to specifically investigate developmental neurotoxicity.

Subchronic exposure to commercial PCB mixtures with either a low or high degree of chlorination specifically affects the dopaminergic neurons in the basal ganglia of adult rats and primates (Seegal *et al.* 1991, Seegal *et al.* 1994), resulting in decreases in dopamine levels. Acute exposure to highly chlorinated PCB mixtures has a more diffuse effect, altering dopaminergic, serotonergic and adrenenergic neurotransmitter systems (Seegal *et al.*, 1985, 1986a, 1986b). It has been reported that in utero and gestational exposure to Aroclor 1016, a commercial PCB mixture with a low degree of chlorine substitution, resulted in transient increases in striatal dopamine levels (Seegal, 1994). However, in the majority of reports on the behavioral developmental toxicity of PCBs in experimental animals commercial PCB mixtures with a high degree of chlorination were used. We therefore investigated the effect of maternal exposure to the commercial PCB mixture Aroclor 1254 on regional brain biogenic amine levels in weanling and young adult rats.

Methods

Animals

Wistar WU rats, 100 females and 50 males, (14 weeks old) were purchased from Charles River Sutzfeld, Germany. The rats were allowed to acclimatize for 2 weeks and were maintained at 50 % humidity and 21°C on bedding in macrolon cages with a 12 hour light cycle (lights on from 08.00 to 20.00). Rat chow (Hope Farms, Woerden, the Netherlands) and tap water were supplied *ad libitum*. After the acclimatization period two females were placed in a cage with one male overnight. When spermatozoa were found the females were housed separately and this was termed day 0 of gestation.

Animals were pre-assigned to a particular dose group on a rotating basis. Maternal body weight gain was monitored throughout gestation and lactation.

Pregnant females received a daily oral dose by gavage of 0, 5 or 25 mg Aroclor 1254 per kg body weight dissolved in corn oil (2 ml/kg body weight) from day 10 of gestation to day 16 of gestation. Rats were weighed each day before administration of Aroclor 1254 or corn oil alone. The rats were inspected each morning at 8:00 h and each afternoon at 18:00 h for litters. Pups found during the morning inspection were defined as 1 day old, pups born between the morning and afternoon inspection were termed 0 day old. Pups were examined for sex, weighed on day 0 postpartum when possible, and always on day 1, 4 and 7 postpartum and thereafter on a weekly basis. On postnatal day 4 (PND4) litters were reduced to 4 males and 4 females.

On postnatal day 21 (PND21), 8-10 litters were sacrificed per treatment group and on PND 90, 10 litters were sacrificed per treatment group. Dams and offspring were sacrificed by decapitation. Brains were rapidly removed and dissected by hand on an ice-cold plate into the following regions: lateral olfactory tract, prefrontal cortex, striatum, hypothalamus and dorsal hippocampus. The brain regions were immediately frozen on a block of dry-ice, weighed and stored at -80°C. Neonates from the remaining litters were weaned on PND21 and male and female rats were housed separately in groups of 2-4 rats per cage. The remaining animals were sacrificed 90 days (PND90) after birth and brain regions were removed, weighed and stored as described above.

Measurement of biogenic amines

Brain regions (lateral olfactory tract, prefrontal cortex, striatum, hypothalamus and dorsal hippocampus) were dissected, frozen, weighed and then stored at -80°C as described above until processing. The brain regions were thawed by the addition of 10 volumes of ice cold 0.2 N perchloric acid containing 100 mg/l of ethylene glycol-bis-(B-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), then homogenized with an ultrasonic tissue disrupter (Vibra cell™, Sonics&Materials Inc. Danbury, CT, USA) for 30 seconds.

The regional brain levels of the neurotransmitters dopamine (DA), 5-hydroxytryptamine (5-HT), norepinephrine (NE) and their respective metabolites dihydroxyphenylacetic acid, 5-hydroxyindoleacetic acid (5-HIAA) and homovanillic acid (HVA) were determined by HPLC separation and electrochemical analysis as previously described (Seegal *et al.* 1986c).

Statistical analysis

Data on regional brain biogenic amine levels were obtained from one male and one female offspring from 8-10 litters per treatment group and sacrifice time. Treatment-related effects on male and female offspring were analysed separately. and were first evaluated with a one-way analysis of variance followed by a least significant difference test to find significant differences between the treatment groups. Data are presented as the mean+SEM.

Results

Reproduction and body weight gain

Maternal exposure to either 5 or 25 mg Aroclor 1254/kg had no effect on maternal body weight gain during gestation or lactation, the number of pups born or neonatal deaths. The low dose, 5 mg Aroclor 1254/kg, had no effect on body weight gain of the offspring, while 25 mg Aroclor 1254/kg resulted in slightly, but significantly lower body weights (approximately 8%) in both male and female offspring over the entire postnatal period (see Chapter 6).

Regional brain biogenic amine concentrations

Only data on the indolamines (5-HT and 5-HIAA) are presented, because there were no consistent significant effects found on the concentrations of dopamine, dihydroxyphenylacetic acid, norepinephrine and homovanillic acid or on DOPAC/DA ratios in the brain regions examined from offspring following maternal PCB exposure.

Lateral olfactory tract (LOT)

The effects of maternal exposure to Aroclor 1254 on indoleamine concentrations in the LOT are presented in Table 1. On PND 21 5-HIAA concentrations in the LOT of male and female neonates from the high PCB dose group were increased by 29% ($P<0.05$) and 18% ($P<0.05$), respectively. In the female neonates the increases in LOT 5-HIAA levels were accompanied by a significant increase (16%, $P<0.05$) in the 5-HIAA/5-HT ratio. On PND 90 the 5-HIAA levels in the LOT were increased by 76% ($P<0.0001$) and 86% ($P<0.001$) respectively in male and female offspring from the high dose group, while in female offspring a 55% increase ($P<0.01$) in 5-HIAA levels was found in the low PCB dose group. 5-HIAA/5-HT ratios were also significantly increased by 61% ($P<0.005$) and 68% ($P<0.001$) in the LOT from male offspring from the low and high PCB dose group, respectively, and in female offspring by 47% ($P<0.05$) and 77% ($P<0.005$) in the low and high PCB dose group, respectively. 5-HT levels in the LOT were generally unaffected by maternal PCB exposure, with the exception of an isolated decrease (35%, $P<0.05$) in male offspring from the low PCB dose group on PND 90.

Prefrontal Cortex (PFC)

Indoleamine concentrations in the PFC, following maternal exposure to Aroclor 1254 are presented in Table 2. On PND 90 significant increases in 5-HIAA concentrations were observed in male offspring (67%, $P<0.0001$ and 125%, $P<0.0001$) and in female offspring (57%, $P<0.01$ and 79%, $P<0.001$) in the low and high PCB treatment groups, respectively. 5-HIAA/5-HT ratios were also elevated in the PFC on PND 90 in male offspring (47%, $P<0.005$ and 44%, $P<0.005$, low and high PCB dose group, respectively) and in female offspring (51%, $P<0.005$) from the high PCB dose group. In addition, 5-HT levels were also increased in the PFC from male offspring from the high dose group (58%, $P<0.0001$) and female offspring from the low PCB dose group (30%, $P<0.05$) on PND 90. No effects were observed on indoleamine concentrations in the PFC of the male and female neonates on PND 21 (data not shown).

Table 1.

Lateral olfactory tract indolamine levels (ng/ mg tissue, fresh wt.) in offspring following maternal exposure to Aroclor 1254

	Dose, mg Aroclor 1254/kg		
	0	5	25
PND 21			
Male			
5-HIAA	0.024±0.002	0.023±0.001	0.031±0.003*
5-HT	0.031±0.002	0.031±0.003	0.031±0.002
5-HIAA/5-HT(%)	79.63±6.32	78.31±19.30	89.06±8.41
Female			
5-HIAA	0.028±0.001	0.027±0.001	0.033±0.002*
5-HT	0.023±0.002	0.024±0.001	0.020±0.002
5-HIAA/5-HT(%)	131.0±9.62	114.8±6.81	152.4±11.8*
PND 90			
Male			
5-HIAA	0.025±0.009	0.025±0.07	0.044±0.01*****
5-HT	0.054±0.004	0.035±0.003*	0.059±0.007
5-HIAA/5-HT(%)	42.69±3.54	68.59±5.02***	71.18±6.59****
Female			
5-HIAA	0.022±0.002	0.034±0.002**	0.041±0.004****
5-HT	0.064±0.007	0.067±0.003	0.068±0.006
5-HIAA/5-HT(%)	36.81±4.56	54.11±3.31*	65.00±7.46***

5-HIAA: 5-hydroxyindole acetic acid, 5-HT: 5-hydroxytryptamine, * $P<0.05$, ** $P<0.01$, *** $P<0.005$, **** $P<0.001$, ***** $P<0.0001$, N=8-10 on postnatal day 21 (PND21) and N=10 on postnatal day 90 (PND90).

Striatum and dorsal hippocampus

The only significant effects observed in the striatum on indolamine concentrations observed were an increase in 5-HIAA levels (57%, $P<0.001$) in male offspring from the high PCB dose group on PND 90. The concentration of 5-HIAA increased in the striatum of female offspring exposed maternally to PCBs by 13% and 38% in the low and high PCB dose group respectively, but these increases were not statistically significant.

Table 2.
Prefrontal cortex indolamine levels (ng/ mg tissue, fresh wt.) in offspring following maternal exposure to Aroclor 1254

	Dose, mg Aroclor 1254/kg		
	0	5	25
		PND90	
Male			
5-HIAA	0.012+0.001	0.020+0.001*****	0.027+0.001*****
5-HT	0.019+0.001	0.022+0.001	0.030+0.001*****
5-HIAA/5-HT(%)	63.46+6.07	93.06+7.79***	91.28+4.58***
Female			
5-HIAA	0.014+0.001	0.022+0.003**	0.025+0.002****
5-HT	0.020+0.001	0.026+0.008*	0.024+0.005
5-HIAA/5-HT(%)	69.53+5.29	88.07+10.5	105.0+8.80***

5-HIAA: 5-hydroxyindole acetic acid, 5-HT: 5-hydroxytrytamine, * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001, ***** P<0.0001, N=10 on postnatal day 90 (PND90).

Table 3.
Striatal and hippocampal HIAA levels (ng/ mg tissue, fresh wt.) in offspring following maternal exposure to Aroclor 1254

	Dose, mg Aroclor 1254/kg		
	0	5	25
		PND90, Striatum	
Male	0.028+0.002	0.032+0.006	0.044+0.003****
Female	0.032+0.002	0.036+0.004	0.044+0.005
		PND90, Hippocampus	
Male	0.018+0.003	0.020+0.002	0.026+0.002*
Female	0.019+0.001	0.035+0.004**	0.030+0.002****

5-HIAA: 5-hydroxyindole acetic acid, * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001, N=10 on postnatal day 90 (PND90).

A 44% increase in hippocampal 5-HIAA levels was observed in male offspring from the high PCB dose group, while a 84% (P<0.05) and a 58% (P<0.001) increase was observed in female offspring from the low and high PCB dose groups, respectively. No significant alterations of indolamine concentrations or 5-HIAA/5-HT ratios were observed in the hypothalamus on PND 21 and 90.

Discussion

This study demonstrates that gestational and lactational exposure to the commercial PCB mixture Aroclor 1254 at very low doses results in alterations in 5-hydroxytryptamine (5-HT, serotonin) metabolism in several macrodissected brain regions in the offspring of the rat. The effects are characterized by increased concentrations of hydroxyindoleacetic acid (5-HIAA), the principle metabolite of 5-HT, and increased 5-HIAA/5-HT ratios, indicative of an increase in 5-HT turnover rather than a decrease in 5-HT synthesis (Seegal *et al.* 1986b). However, it is difficult to interpet our results solely in terms of increased neuronal activity, because the observed effects result from a combination of synthesis, release, re-uptake and metabolism of 5-HT. The increases in 5-HIAA levels and 5-HIAA/5-HT ratios were seen mainly on PND 90 in the lateral olfactory tract (LOT) and prefrontal cortex (PFC), while in the striatum and hippocampus only increases in 5-HIAA levels were observed. On PND 21 slight increases in 5-HIAA levels were seen in the LOT in male and female offspring from the 25 mg/kg dose group. The levels of other biogenic amines, norepinephrine, 3,4-dihydroxyphenylacetic acid, dopamine and homovanillic acid were unaffected by maternal PCB exposure, suggesting a selective effect on the ontogeny of the serotonergic system.

The LOT and frontal cortex are highly innervated by serotonergic projections from both the dorsal and median raphe nuclei in the brainstem and are among the first neuronal systems to project to the cortex during brain development in the rat (Kosofsky and Molliver, 1987). The striatum is innervated primarily by the dorsal raphe nucleus, while the hippocampus and hypothalamus are innervated by both the dorsal and median raphe nucleus (Kosofsky and Molliver, 1987). Since 5-HIAA levels were affected to the greatest extent in the LOT and PFC by maternal PCB exposure, it may be the timing of serotonergic innervation or axonal length that determines the sensitivity of a given brain region for the developmental neurotoxicity of Aroclor 1254. 5-HT neurons projecting from the dorsal raphe appear to play an important role in control of the emotional state and may be involved in perceptual integration (Molliver, 1987). Possible consequences of impairment of the serotonergic system include alterations of mood-related behaviors, such as appetite, sleep and attention (Willner, 1985).

It seems highly unlikely that one specific mechanism is responsible for the complete spectrum of developmental neurotoxicity observed following maternal exposure to a mixture of PCBs, because PCBs and their metabolites produce a broad

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spectrum of structure-dependent alterations in endocrine systems (Safe, 1994) which may influence brain development (McEwen, 1992) and hence alter the developmental expression of important neurotransmitters.

One endocrine system that may be involved in PCB induced developmental neurotoxicity is the thyroid system. It has been suggested that PCB-induced decreases in fetal and neonatal brain thyroid hormone levels may result in altered brain development (Morse *et al.* 1993). Several studies have demonstrated that maternal PCB exposure results in decreased circulating thyroid hormone levels in either fetal or neonatal rats (Collins and Capen, 1980, Ness *et al.*, 1993, Morse *et al.* 1993a, Morse *et al.* 1995a) and the importance of thyroid hormones for brain development is widely recognized (Porterfield and Hendrich, 1993). Significant decreases in fetal brain thyroxine levels were observed in this study (Chapter 5).

Although we did not directly assess the relationship between PCB exposure, thyroid hormones and regional levels of 5-HT and 5-HIAA in this study, the effects observed on the levels of indoleamines in this study partly resemble the effects of pre- and postnatal hypothyroidism in the rat. Neonatal hypothyroidism resulted in significant increases in 5-HT and 5-HIAA levels in many discrete brain nuclei in the forebrain, midbrain and hindbrain (Savard *et al.* 1984). In larger brain regions decreases of 5-HT accompanied by an increase in 5-HIAA levels were observed in the cerebellum, midbrain and striatal regions in contrast to an accumulation of 5-HT and 5-HIAA in the pons-medulla (Rastogi and Singhal, 1978).

In addition, the estrogenic system may also be involved in PCB-induced developmental alterations in brain development. The coplanar PCB, 3,3',4,4'-tetrachloro-biphenyl and 2,3,7,8-tetrachlorodibenzo-para-dioxin possess anti-estrogenic properties (Jansen *et al.* 1993, Spink *et al.* 1990), while some hydroxylated metabolites and diortho-PCBs are weakly estrogenic (Jansen *et al.* 1993, Korach *et al.* 1988). Estrogen receptors have been found in the dorsal raphe nucleus during fetal brain development (Stumpf *et al.* 1983), and it has been reported that neonatal treatment with sex steroids cause different alterations in 5-HT levels in the male and female rat brain (Giulian *et al.* 1973). However, it is currently unclear if the effects observed on regional brain indoleamine concentrations in the current study are due to estrogenic or anti-estrogenic effects of PCBs and their metabolites. Firstly, the alterations in regional brain indoleamine concentrations were quantitatively and qualitatively similar in both male and female offspring. Secondly, Aroclor 1254 contains coplanar PCBs with anti-estrogenic properties, di-ortho PCBs that are potentially estrogenic, and many congeners are metabolized in rodents to hydroxylated compounds that may be estrogenic (Bergman *et al.* 1994), making a prediction of the overall effect of a PCB mixture on the estrogenic system extremely difficult.

Although acute PCB administration can increase serotonin turnover, it is plausible that the alterations in serotonin metabolism observed on PND21 and 90 are not due to direct effects of residual PCB levels for two reasons. The direct effects of PCBs *in vitro* or in adult animals following chronic and subchronic exposure are primarily

associated with alterations in dopamine metabolism (Seegal, 1992). The striatum is one of the most sensitive regions for alterations in dopamine metabolism following PCB exposure. In this study there was no evidence for alterations in dopamine metabolism in the striatum of PCB exposed offspring, either directly after weaning or in young adults. Secondly, brain PCB concentrations are significantly higher in weanling rats than in adult offspring following pre- and postnatal exposure (Shain *et al.* 1986) but the alterations in serotonin metabolism in this study are observed predominantly in young adult rats rather than weanling rats. Furthermore, acute administration of a mixture of Aroclor 1254 and 1260 to the rat resulted in transient increases in serotonin metabolism in the prefrontal cortex, hippocampus, medial-basal hippocampus and brainstem, but not in the lateral olfactory tract (Seegal *et al.* 1986b), the latter brain region being the most sensitive for persistent increases in serotonin metabolism in this study. In combination, the results from this study demonstrate that *in utero* and lactational exposure to low doses of Aroclor 1254 result in alterations in the normal developmental expression of the serotonergic system.

The lack of effect on the dopaminergic and noradrenergic amines observed in the current study could be explained in several ways. Firstly, effects may be only observed in discrete brain nuclei and not in macrodissected brain regions. Secondly, pre- and postnatal exposure to Aroclor 1254 affects the developing brain by a different mechanism than in the adult brain. Lastly, the administered dose was too low or Aroclor 1254 does not contain (sufficient) amounts of PCB congeners that alter the concentration of dopaminergic and noradrenergic amines. For example, 3,3',4,4',5,5'-hexachlorobiphenyl, a poorly metabolized coplanar PCB, increases striatal dopamine levels in the offspring of pregnant rats treated with 1.8 mg/kg on day 1 of gestation (Dr. Beverly Kulig, personal communication). This PCB congener is present in very low levels in Aroclor 1254 (less than 1 ppm, Kannan *et al.* 1987). Nonetheless, the negative data from the current study do not exclude effects on the dopaminergic and noradrenergic systems, for receptor concentrations and activity could be altered in the absence of effects on the neurotransmitter concentration.

In conclusion, pre- and postnatal exposure to the commercial PCB mixture Aroclor 1254 results in alterations in 5-HT metabolism but not in dopamine metabolism, which indicates a significant difference between the mechanisms of Aroclor 1254-induced developmental neurotoxicity and neurotoxicity induced in the adult animal.

CHAPTER 9

GENERAL DISCUSSION

Introduction

The work described in this thesis was undertaken to gain insight in the processes involved in the developmental neurotoxicity of polychlorinated biphenyls. It has been previously hypothesized that the alteration of thyroid hormone status by PCBs may be in part responsible for the developmental neurotoxicity of these compounds in humans (Rogan *et al.* 1986). This is a logical hypothesis, given the well-described effects of PCBs on plasma thyroid hormone levels in adult animals, and the indisputable importance of thyroid hormones in brain development.

Therefore the first goal was to determine the nature and mechanism of PCB-induced decreases in circulating and brain thyroid hormone levels in fetal and neonatal rats (Chapter 2,3,4 and 5). We examined the effects of maternal PCB administration on the metabolism of thyroid hormone in the brain and liver of fetal, neonatal and adult offspring in relation to the level of thyroid hormone in the plasma and brain. Vitamin A status, which may be linked to thyroid hormone status following PCB exposure, was also examined in one reproduction study (Chapter 6).

Since the kinetics and metabolism of PCBs may play a pivotal role in the alteration of thyroid hormones, a radiolabelled and easily metabolized PCB congener was used in *in vivo* (Chapter 2) and *in vitro* experiments (Chapter 3) to examine the kinetics and metabolism of a model compound in pregnant and fetal rats. The relevance of this model compound for complex PCB mixtures was ascertained following the administration of a commercial PCB mixture to pregnant rats (Chapter 5).

Lastly, neurochemical analysis were conducted on the brains of the offspring following maternal PCB exposure to determine which brain regions, cell types and neurotransmitter systems are affected during brain development (Chapter 7 and 8).

Biotransformation of PCBs to thyroid hormone antagonists

The metabolism and distribution of $[^{14}C]$-3,3',4,4'-tetrachlorobiphenyl ($[^{14}C]$-TCB) was examined in pregnant rats and their fetuses (Chapter 2 and 3, Morse *et al.*, 1995). The major metabolite found in adult liver and plasma, placental tissue, whole fetuses and fetal blood was 3,3',4',5-tetrachloro-4-biphenylol (4-OH-tetraCB). While maternal tissue levels of $[^{14}C]$-TCB derived radioactivity significantly decreased by 65-85% over a 7 day period, radioactivity in the fetus accumulated more than 100-fold over the same period. The fetal accumulation of radioactivity was due primarily to 4-OH-tetraCB, and on day 20 of gestation, fetal plasma levels of 4-OH-tetraCB were 14 times higher than maternal plasma levels (14 µM vs 1 µM).

In order to determine the source of 4-OH-tetraCB in the fetus, *in vitro* studies were carried out by incubating $[^{14}C]$-TCB with maternal and fetal rat microsomes and analysing the reaction products with high pressure liquid chromatography and gas

chromatographic/mass spectrometric analysis. First, incubation conditions were optimized using male rat microsomes. Under optimal incubation conditions, hepatic microsomes from pregnant rats pretreated with TCB produced 4-OH-tetraCB as the major metabolite, while no metabolites were detected in incubations with microsomes from fetuses from pregnant rats pretreated with TCB. The results indicate that 4-OH-tetraCB found in the fetal compartment is due to transplacental transport from maternally formed 4-OH-tetraCB. This is in agreement with the observation that the biotransformation of TCB is dependent on CYP1A1 induction, and no CYP1A1 activity was observed in fetal microsomes after maternal treatment with TCB.

In late gestation, the high levels of 4-OH-tetraCB found in the fetal plasma were asssociated with decreases in fetal plasma thyroid hormone levels in the absence of significant decreases in maternal plasma thyroid hormones. 4-OH-tetraCB has a high affinity for transthyretin (the major plasma thyroid hormone transport protein in the rat) and competitively displaces thyroxine from this protein (Brouwer *et al.* 1990, Lans *et al.* 1993). It was therefore concluded that the accumulation of 4-OH-tetraCB in the fetus is due to the high affinity of this metabolite for transthyretin, and results in significant decreases in fetal plasma thyroxine levels.

Since the model compound 3,3',4,4'-TCB is present in only very low levels in the environment, it was of interest if the exposure of pregnant rats to a commercial PCB mixture (Aroclor 1254) would also result in the accumulation of phenolic metabolites in the fetal plasma (Chapter 5). Relatively high levels of hydroxylated PCB metabolites from penta, hexa and hepta-chlorinated biphenyls have been found in the plasma of rats exposed to Aroclor 1254 and in environmentally exposed humans (Bergman *et al.* 1994). A significant accumulation of 4-OH-2,3,3',4',5-pentachlorobiphenyl (4-OH-pentaCB) was found in the plasma of late gestational fetuses from pregnant rats exposed to Aroclor 1254 (up to 4.6 µM). This PCB metabolite has a 10-fold higher binding affinity for TTR than thyroxine, thereby confirming the relevance of work with the model compound, 3,3',4,4'-tetrachlorobiphenyl. In addition, relatively large amounts of 4-OH-pentaCB were found in the fetal (0.46 µM), but not weanling rat brain, indicating that in the absence of a functional blood-brain barrier hydroxylated PCB metabolites may enter the brain. The toxicological significance of this finding deserves investigation.

Effects of PCB exposure on thyroid homone levels and metabolism

T_4-Uridine-diphospho-glucuronyl transferase

Decreases in plasma thyroid hormone levels in adult rodents may also be caused by the induction of the hepatic glucuronidation of thyroxine (Bastomsky, 1974, Barter and Klaasen 1992). The effect of a single maternal dose of 3,3',4,4',5,5'-hexachlorobiphenyl (HCB) on day 1 of gestation and in combination with repeated maternal doses of TCB (day 2 to 18 of gestation) on maternal, fetal and neonatal hepatic microsomal and brain thyroxine metabolism is described in Chapter 4. The results indicated that although maternal administration of coplanar PCBs may result in the induction of fetal hepatic microsomal T_4 glucuronidation, this induction did not cause the reductions in fetal plasma T_4 levels. Only the combined dose of HCB with TCB resulted in significant decreases in fetal plasma T_4 levels. This indicates that decreased

placental transport of maternally-derived T_4 and the blockage of fetal thyroid hormone transport by 4-OH-tetraCB resulted in the decrease of fetal T_4 levels. In neonates and dams, however, the induction of T_4 glucuronidation by lactational exposure to coplanar PCBs may contribute to the observed decreases in plasma thyroxine levels.

Maternal exposure to the commercial PCB mixture Aroclor 1254 also induced T_4-UDPGT activity in hepatic microsomes from pregnant and weanling rats, but not in the fetus (Chapter 5). Since only the induction of maternal hepatic microsomal T_4-UDPGT correlated with reductions in plasma thyroid hormones, it was concluded that the induction of T_4-UDPGT activity played only a minor role in the reductions of plasma thyroid hormones in fetal and weanling rats. Large reductions in plasma thyroid hormones have also been observed following dietary Aroclor 1254 exposure in homozygous Gunn rats, which are deficient in T_4-UDPGT activity (Collins and Capen, 1980a). The only long-term effect on thyroid hormone metabolism observed following maternal PCB exposure was a significant decrease in female hepatic microsomal T_4 glucuronidation in young adult offspring.

Type II thyroxine 5'-deiodinase

As most of the biologically active hormone triiodothyronine (T_3) is derived from T_4 by deiodination in the brain by Type II thyroxine 5'-deiodinase (5'D-II, Silva and Larsen, 1982, Kaplan *et al.* 1983), it was of interest to examine the effects of PCB-induced reductions in plasma T_4 levels on 5'D-II activity. Decreases in brain T_4 levels result in a slower turnover of the enzyme, yielding a higher activity per unit protein in brain homogenates (Leonard *et al.* 1984). This regulatory mechanism is important in maintaining brain T_3 levels. In Chapter 4, the significant decreases in fetal, neonatal and weanling rat plasma T_4 levels following coplanar PCB exposure were accompanied by significant increases in 5'D-II activity in brain homogenates. It was concluded that the increases in 5'D-II activity were in compensation for low T_4 levels in the developing rat brain, which could be detrimental for normal brain development if insufficient T_3 was formed from T_4.

Following maternal exposure to Aroclor 1254, reductions in fetal plasma T_4 were also accompanied by increases in brain 5'D-II activity. However, in contrast to the effects observed with coplanar PCBs in weanling rats, 5'D-II activity was decreased in weanling rats with normal plasma and brain T_4 levels, and equal to control values when plasma and brain T_4 levels were decreased. This can not be explained by the current knowledge of 5'D-II regulation.

Plasma and brain thyroid hormone levels

In the current study, the effect of maternal PCB exposure on plasma thyroid hormone levels was transient, with only mild effects observed in weanling rats. Despite the significant lactational transfer of PCBs to the neonate, the effects on neonatal thyroid hormone homeostasis are less severe in neonates as in the fetus. Several mechanisms appear to be involved that may explain the difference in responses between fetuses and weanling rats: the induction of maternal hepatic T_4 glucuronidation late in gestation, the accumulation of hydroxylated PCB metabolites in the fetus, and reduced placental transfer of T_4. Also the dilution of the tissue PCB levels during postnatal growth and the fecal and urinary excretion of PCBs may reduce the severity of plasma T_4 reductions in

weanling rats following gestational PCB exposure. For example, the continuous postnatal dietary exposure of maternal rats to Aroclor 1254 results in low plasma T_4 levels throughout the weaning period (Collins and Capen, 1980b).

Despite severe decreases in fetal plasma and brain T_4 levels following maternal PCB exposure, only marginal decreases were observed in fetal brain T_3 levels. This indicates that the late gestational rat fetus can maintain brain T_3 levels by an increase in 5'D-II activity, and is at little risk for PCB-induced hypothyroidism, at least in the brain.

The observation that plasma TSH levels did not increase following PCB-induced decreases in plasma T_4 levels in the fetus and plasma T_3 and T_4 levels in the neonate suggests that the developing brain may have been euthyroid. However, the decreases in plasma T_4 levels themselves could be expected to result in an increase in TSH levels. Similar decreases in plasma T_4 levels in late gestational fetal Wistar rats following maternal treatment with methimazole have been shown to result in an 600% increase in plasma TSH levels (Morreale de Escobar *et al.* 1993), and it is likely that fetal TSH levels are modulated predominately by plasma T_4 rather than T_3. In adult rats, significant increases in plasma TSH levels have been observed following dietary exposure to Aroclor 1254 that resulted in that decreases in plasma T_4, but not T_3 at the same time point (Barter and Klaassen, 1994). A weak effect of PCBs on TSH secretion has been observed following a relatively high dietary exposure to Aroclor 1254, after which the rise in plasma TSH was suprisingly low in comparison to the rise in plasma TSH following dietary exposure to polychlorinated naphthalenes, which induced similar decreases in plasma T_4 levels as PCBs (Barter and Klaassen, 1994).

In conclusion, maternal PCB exposure during gestation results in a large decrease of fetal brain T_4 levels, but only marginal decreases in T_3 levels in the late gestational rat fetus. It is possible that earlier in gestation, before 5'D-II activity can compensate for decreases in brain T_4 levels, significant reductions in brain T_3 levels are induced by maternal PCB treatment.

Retinoids

Analogous to thyroid hormones, retinoids play a crucial role in brain development, although their most important effects are during early and mid-gestation (Adams, 1993). To evaluate retinoid status, plasma and hepatic retinol and retinylesters were determined following maternal Aroclor 1254 exposure. The reductions in plasma retinol levels may be caused by the accumulation of 4-OH-pentaCB in the plasma, analogous to the disruption of the binding of retinol binding protein to transthyretin by 4-OH-tetraCB following exposure to 3,3',4,4'-tetrachlorobiphenyl. Although the effects of maternal PCB exposure on retinoid homeostasis in the fetus, neonate and young adult offspring appear to be minor, the regulation of retinoid homeostasis exhibited long-term alterations in the PCB exposed group.

Alterations in neurochemistry

In Chapter 8 and 9 the effects of maternal PCB (Aroclor 1254) exposure were examined on the ontogeny of biogenic amines, a glial cell marker (glial fibrillary acidic protein, GFAP) and a neuronal cell marker (synaptophysin) in diverse brain regions.

Biogenic amines

Of the biogenic amines examined, only the levels of 5-hydroxytryptamine (5-HT, serotonin) and its metabolite, 5-hydroxy-indoleacetic acid (5-HIAA) were altered by pre- and postnatal PCB exposure. It is notable that in adult animals the dopaminergic system is the most sensitive for exposure to commercial PCB mixtures, while we found no effects on the levels of dopamine or its major metabolite in the brains of PCB-exposed offspring (Seegal *et al.* 1985, 1986a, 1986b, 1991). Pre- and postnatal exposure to the lightly chlorinated PCB mixture Aroclor 1016 resulted in transient increases in striatal dopamine levels (Seegal, 1994). Therefore the effects of PCB exposure on regional brain monoamine metabolism during development do not resemble the effects in adult animals.

In general, the effects can be characterized by an increase in 5-HIAA concentrations and the 5-HIAA/5-HT ratio in the lateral olfactory tract and prefrontal cortex, and an increase in 5-HIAA levels in the striatum and hippocampus on postnatal day 90. Since the effects on the serotonergic system are almost absent on day 21 postpartum when exposure to PCBs via lactation ceased, there appears to be a delayed effect on the ontogeny of serotonin metabolism.

GFAP and Synaptophysin levels

The most consistent effects of maternal PCB exposure on GFAP levels were observed in the lateral olfactory tract and the brainstem. Increases in GFAP concentrations were observed in both male and female offspring 21 and 90 days after birth. Increases were also observed in cerebellar GFAP levels on 21 and 90 days postpartum. In the brainstem of male and female offspring maternal PCB exposure prevented the increase in GFAP concentrations that was observed in control offspring, indicating a delay in the ontogeny of brainstem GFAP expression.

Synaptophysin levels in the brain of the offspring were affected in a more complex manner than GFAP following maternal PCB exposure. Following maternal PCB exposure, the most sensitive brain regions from both sexes for decreases in synaptophysin concentrations on postnatal day 21 were the lateral olfactory tract and the brainstem. However, in young adult animals brainstem synaptophysin levels were significantly decreased in males and significantly increased in females. Synaptophysin levels were also significantly decreased in the striatum and hypothalamus of female, but not male offspring following maternal PCB exposure.

The mechanisms involved in the alterations of GFAP and synaptophysin levels in the brains of the PCB-exposed offspring are not yet been elucidated. Increases in GFAP levels accompanied by decreases in synaptophysin levels in the lateral olfactory tract and prefrontal cortex are characteristic of reactive gliosis following neuronal loss (O'Callaghan and Miller, 1989). The decreases in GFAP and synaptophysin levels in the brainstem in weanling rats may be indicative of a developmental delay in brainstem maturation. The raphe nuclei in the brainstem contain serotonergic neurons which project to the lateral olfactory tract and the prefrontal cortex (Kosofsky and Molliver, 1987). It is therfore conceivable that the alterations in serotonergic metabolism as well as GFAP and synaptophysin levels in the lateral olfactory tract and prefrontal cortex result from a developmental delay in the serotonergic innervation of these brain regions.

Relevance of the conducted research for human development and future toxicological research

Thyroid hormones

In a recently published study of 105 mother-infant pairs, elevated maternal body burdens of polychlorinated dibenzo-p-dioxins, dibenzofurans and biphenyls (levels in milk fat) were shown to be associated with alterations of human thyroid hormone status (Koopman-Esseboom *et al.* 1994). The effects are characterized as negative correlation of PCDD, PCDF and PCB congeners with maternal plasma TT_3 before delivery and maternal plasma TT_4 and TT_3 after delivery, and a positive correlation with plasma TSH levels in the infants in the second week and third month after birth. In infants with a higher exposure, plasma TT_4 levels were significantly lower (10%) and TSH levels were significantly higher (37%). Maternal body burden of three PCB congeners (CB 118, CB 138 and CB 153) was positively correlated with umbilical plasma TSH levels. In a similar study with 38 mother-infant pairs an increase in infant plasma TSH levels and plasma TT_4 levels was observed in infants with a higher exposure to the total toxic equivalents of PCDDs and PCDFs (Pluim *et al.*, 1992).

The relative effects of PCBs, PCDFs and PCBs on plasma thyroid hormone levels observed by Koopman-Esseboom *et al.* (1994) in mother-infant pairs are generally the same as observed in adult mice, rats and monkeys at much higher doses. In contrast to the decreases observed in late gestational fetal rat plasma TT_4 and FT_4 following maternal exposure to Aroclor 1254, no effect was observed of maternal body burden on umbilical cord TT_4 and FT_4 levels.

It is unlikely that the alterations in thyroid hormone homeostasis associated with maternal PCB and PCDD levels observed in human newborns and infants will result in developmental alterations of the nervous system. Compensatory mechanisms, such as the induction of Type II 5'-thyroxine deiodinase in the brain, should offset decreases in plasma and brain T_4 levels. In the late gestational rat, near normal brain T_3 levels were maintained despite severe decreases in plasma and brain T_4 levels following maternal PCB exposure. However, the potential exists for significant reductions in fetal thyroid hormone levels earlier in gestation before compensatory mechanisms have fully developed or in specific brain regions not examined in this study.

There are several important aspects in which the thyroid hormone transport differs between humans and rats with possible consequences for the effects of PCBs. The main thyroid hormone transport protein in humans is TBG, while in rats TTR is the major protein (Robbins, 1991). Although under certain circumstances TBG may be present in rats, it has a low affinity for T_4 (Rouaze-Romet *et al.* 1992). Hydroxylated PCB metabolites bind only very weakly to TBG, so it is possible that the impact of hydroxylated PCB metabolites on plasma T_4 levels in humans may be minor. However, the fetal mouse has both TTR and TBG as transport proteins, and mouse TBG has similar binding properties to human TBG (Vrancks *et al.* 1990), so the mouse may be a better model than the rat for studying the effects of PCBs on thyroid hormone transport. Recent research has shown that following maternal TCB exposure 4-OH-tetraCB accumulates in the fetal mouse, binds to TTR, resulting in the decrease of fetal plasma T_4 levels (unpublished results, D.C. Morse and P.O. Darnerud). Therefore, it is possible that transplacental transport of hydroxylated PCBs in humans results in the decrease

plasma and brain thyroid hormone levels before the rise of fetal hypothalamic-pituitary function in mid-gestation.

While it is generally accepted that thyroid hormone deficiency in neonates and in late gestation has a negative effect on brain development in the rat as well as humans, the effects of thyroid hormone deficiency earlier in gestation are not clearly understood (Morreale de Escobar *et al.* 1993, review, Porterfield and Hendrich, 1993, review). Thyroid hormone and their receptors have been found in human fetuses by 10 weeks of gestation (Fisher, 1985), although the functional significance of these observations is currently unclear. The finding of Pharoah *et al.* (1972) that neurological damage of endemic cretinism could be prevented if iodized oil was given to the mother before the second trimester of pregnancy supports a role of thyroid hormones in brain development in this period.

Therefore several questions remain to be answered: does maternal PCB exposure result in significant decreases in brain thyroid hormones in early and mid-gestation in rodents and humans, and whether such decreases are relevant for brain development.

Effects on neurochemical development

Gestational and lactational exposure to a commercial PCB mixture, Aroclor 1254 resulted in long-term effects on the neurochemical development of the progeny of rats (Chapter 7 and 8). The study does not support the hypothesis that PCB-induced pre- or postnatal hypothyroidism was the cause of the neurochemical alterations (Chapter 5). The study does give an indication which neurotransmitter systems, which cell types and which brain areas may be affected by in utero and lactational exposure to a higher chlorinated PCB mixture, providing a solid base for further research.

One of the questions which has interested researchers in PCB-induced toxicity for nearly 20 years is which PCB congeners are responsible for the toxicity of these compounds. This question has only been adequately answered for the immunotoxicity and some developmental endpoints (teratogenesis and fetotoxicity) in which the interaction of the PCB congeners with the Ah-receptor plays an important role.

To date, reports have been published on the behavioral neurotoxicity of only two individual PCB congeners, 3,3',4,4'-tetrachlorobiphenyl and 3,3',4,4',5-pentachlorobiphenyl, both of which are coplanar PCBs with a high affinity for the Ah-receptor. 3,3',4,4'-tetrachlorbiphenyl was shown to be a developmental neurotoxin in mice following high dose gestational exposure, reducing striatal dopamine and dopamine receptor levels in mice, delaying advoidance behavior and inducing neuropathological alterations in the cranial roots (Tilson *et al.* 1979, Chou *et al.* 1979, Agrawal *et al.* 1981). Postnatal exposure of mice to 3,3',4,4'-tetrachlorobiphenyl has also been shown to affect hippocampal muscarinic receptor levels and alter spontaneous activity (Eriksson, 1988, Eriksson *et al.* 1991). Due to the rapid metabolism of 3,3',4,4'-tetrachlorobiphenyl and accumulation of hydroxylated metabolites in the fetus no conclusions can be drawn as to the role of the parent compound or its metabolites in the developmental neurotoxicity of this PCB congener (Morse *et al.* 1995).

On the other hand, exposure of pregnant rats to poorly metabolizable 3,3',4,4',5-pentachlorobiphenyl delayed the onset of spontaneous activity and neuromuscular maturation in the offspring, which was related to delay in body weight gain. However, the development of reflexes and visual discrimination was not affected by maternal

exposure to 3,3',4,4',5-pentachlorobiphenyl (Bernhoft *et al.* 1994). Taken together, these data may indicate that highly toxic coplanar PCBs may not be direct developmental neurotoxins in rodents. If this is the case, it is questionable if the use of individual PCB congeners in studies on the effects on neurochemical and behavioral development will resolve novel structure-activity relationships within the same time-frame as the structure-activity relationships for immunotoxicity or CYP1A1 induction in adult animals. First, the reproductive studies involved are much more lengthy and costly than acute studies with adult animals. Secondly, there is no general agreement on experimental protocols (timing and length of PCB administration, neurochemical and behavioral endpoints) between researchers working in this field, frustrating the comparison of data. Thirdly, it is very likely that complex interactions of Ah-receptor binding, PCB metabolism, fetal accumuation of metabolites and hormonal alterations affect brain development *in vivo*, so that the structure-activity relationships for individual congeners will not predict the effects of complex mixtures.

Therefore it may be more useful to characterize the effects of environmentally relevant mixtures in terms of dose-response studies, neurochemical and behavioral endpoints and species sensitivity. Environmentally relevant mixtures can be obtained by extracting contaminated foodstuffs or constructing mixtures using synthetic standards. Although mixtures will vary somewhat in their composition, the results of such studies may be more relevant for regulatory purposes than data based on studies with individual congeners. Cell culture techniques using glial cell or dissociated neural cell cultures may be useful in investigating structure-activity relationships of the direct effects of individual PCB congeners on brain development. Parameters that have been first demonstrated to be affected *in vivo* should be analysed *in vitro*.

Main conclusions:

1) PCB congeners (3,3',4,4'-tetrachlorobiphenyl, 2,3,3',4,4'-pentachlorobiphenyl and 2,3',4',4,5-pentachlorobiphenyl) can be metabolized to hydroxylated metabolites which accumulate in the fetal plasma and brain and cause severe reductions in late gestational fetal plasma and brain thyroxine levels in rats.

2) The reductions in brain T_4 levels in late gestational fetal rats are effectively compensated by increases in Type II thyroxine 5'-deiodinase activity, so that only marginal decreases in brain T_3 levels are observed following maternal exposure to a commercial PCB mixture. This is an indication that PCB-induced decreases in plasma T_4 levels are not responsible for alterations in the development of the central nervous system.

3) Maternal exposure to the commercial PCB mixture (Aroclor 1254) specifically alters the development of serotonin metabolism in the brain of the offspring in rats. Since the dopamine metabolism exhibits a greater sensitivity and persistency for the administration of Aroclor 1254 in adult rodents and macaques than serotonin metabolism, the mechanism of PCB-induced developmental neurotoxicity is distinct from the mechanism of alterations in biogenic amine metabolism in adult animals.

4) The development of both neuronal and glial cells is affected in the brains of offspring from pregnant rats treated with Aroclor 1254. The alteration in astrocyte development in the brainstem of PCB-exposed offspring is not a response to neuronal death, for levels of glial fibrillary acidic protein (GFAP) are decreased, while increased neuronal death is generally accompanied by increases in GFAP expression. It is therefore likely that PCBs affect brain development by altering cell differentiation and proliferation.

5) Since the brainstem is one of the first structures to develop in the brain, the observed alterations in brainstem development following pre- and postnatal PCB exposure probably have a negative effect on the subsequent development of other brain structures.

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SAMENVATTING

Inleiding

Het onderzoek dat in dit proefschrift beschreven wordt, is uitgevoerd om inzicht te verkrijgen in de processen die een rol spelen bij de neurotoxiciteit van PCB's tijdens de ontwikkeling. Er werd al eerder verondersteld dat de veranderingen in schildklierhormoon status na blootstelling aan PCB's voor een deel verantwoordelijk zijn voor de ontwikkelings-neurotoxiciteit van deze stoffen in mensen (Rogan *et al.*, 1986). Dit is een logische hypothese, gezien de goed omschreven effecten van PCB's op plasma schildklierhormoon-niveaus in volwassen dieren, en het onomstreden belang van schildklierhormonen op de hersenontwikkeling.

Het eerste doel van dit onderzoek was om de aard en het mechanisme van PCB-geïnduceerde verlagingen van schildklierhormoon-spiegels in plasma en hersenen van foetale en neonatale ratten te bepalen (Hoofdstuk 2,3,4 en 5). De effecten van maternale PCB blootstelling op het schildklierhormoon-metabolisme in de hersenen en lever van foetale, neonatale en volwassen nakomelingen werden onderzocht in samenhang met de schildklierhormoon-niveaus in plasma en hersenen. De vitamine A status, die gekoppeld kan zijn aan de schildklierhormoonstatus, werd eveneens onderzocht in een reproductiestudie (Hoofdstuk 6).

Omdat de kinetiek en het metabolisme van PCB's een belangrijke rol kunnen spelen bij de effecten op schildklierhormoon-niveaus, werd een radioactief gelabelde en snel metaboliseerbare PCB congeneer als modelstof gebruikt in *in vivo* (Hoofdstuk 2) en *in vitro* experimenten (Hoofdstuk 3). In deze experimenten werd de kinetiek en het metabolisme van de modelstof onderzocht in zwangere en foetale ratten. De toepasbaarheid van deze stof als model voor complexe PCB-mengsels werd onderzocht in een experiment waarin zwangere ratten blootgesteld werden aan een commercieel PCB mengsel (Hoofdstuk 5).

Tot slot werden neurochemische analyses uitgevoerd in de hersenen van nakomelingen na maternale PCB blootstelling om vast te stellen welke hersenregio's, celtypen en neurotransmittersystemen beïnvloed werden tijdens de hersenontwikkeling (Hoofdstuk 7 en 8).

Biotransformatie van PCB's tot schildklierhormoon antagonisten

Het metabolisme en de distributie van $[^{14}C]$-3,3',4,4'-tetrachlorobiphenyl ($[^{14}C]$-TCB) werden onderzocht in zwangere ratten en hun foetussen (Hoofdstuk 2 en 3, Morse *et al.*, 1995). De "belangrijkste" metaboliet die gevonden werd in maternale lever en plasma, placentaal weefsel, foetussen en foetaal bloed was 3,3',4',5-tetrachloro-4-biphenylol (4-OH-tetraCB). Ondanks een afname van 65-85% van radioactiviteit in de maternale weefsels binnen 7 dagen, werd in dezelfde periode een 100-voudige toename in radioactiviteit waargenomen in de foetus. De foetale accumulatie van radioactiviteit was grotendeels toe te schrijven aan 4-OH-tetraCB. Op dag 20 van de dracht waren foetale plasma concentraties van 4-OH-tetraCB 14 maal hoger dan maternale plasma

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concentraties (14 μM versus 1 μM).

In vitro studies werden uitgevoerd door [^{14}C]-TCB te incuberen met maternale en foetale lever-microsomen waarna de reactieproducten geanalyseerd werden met hoge druk vloeistof chromatografie en gas chromatografie/mass spectrometrie om de herkomst van 4-OH-tetraCB in de foetus te bepalen. De in vitro incubatie condities werden hiervoor geoptimaliseerd met microsomen van mannelijke ratten. Met deze optimale in vitro incubatie omstandigheden, produceerden lever-microsomen van drachtige ratten voorbehandeld met TCB, het 4-OH-tetraCB als de voornaamste metaboliet van TCB. Lever-microsomen van foeten, die maternaal blootgesteld waren aan TCB, waren niet in staat om TCB om te zetten tot fenolische metabolieten. Deze resultaten bevestigen de hypothese dat het 4-OH-tetraCB dat gevonden werd in de foetus afkomstig is van het moederdier. Dit is in overeenstemming met de observatie dat de biotransformatie van TCB afhankelijk is van cytochroom P4501A1 (CYP1A1) inductie, en geen CYP1A1 activiteit werd gevonden in foetale microsomen na behandeling van zwangere ratten met TCB.

Tegen het eind van het dracht waren de hoge concentraties 4-OH-tetraCB in de foetale plasma geassocieerd met verlagingen in de foetale plasma schildklierhormoon-niveaus terwijl de maternale plasma schildklierhormoon-niveaus niet verlaagd waren. 4-OH-tetraCB heeft een hoge affiniteit voor transthyretine (TTR, het belangrijkste transporteiwit voor schildklierhormoon in het plasma van de rat) en verdringt het schildklierhormoon (thyroxine, T$_4$) van dit eiwit (Brouwer et al. 1990, Lans et al. 1993). Er werd geconcludeerd dat de accumulatie van 4-OH-tetraCB in de foetus veroorzaakt wordt door de hoge affiniteit van deze metaboliet voor het TTR, waardoor verlagingen in foetale schildklierhormoon niveaus optreedt.

Omdat de modelstof 3,3',4,4'-tetrachlorobifenyl (TCB) in zeer kleine hoeveelheden in het milieu aanwezig is, was het van belang om te bepalen of de blootstelling van drachtige ratten aan het commerciële PCB mengsel (Aroclor 1254) eveneens resulteerde in de accumulatie van fenolische metabolieten in het foetale plasma. Relatief hoge concentraties van gehydroxyleerde PCB-metabolieten die afkomstig waren van penta-, hexa- en hepta-gechloreerde biphenylen, zijn gevonden in plasma van volwassen ratten die blootgesteld waren aan Aroclor 1254 en in plasma van mensen met achtergrondsblootstelling aan PCB's (Bergman et al. 1994). Een significante accumulatie van 4-OH-2,3,3',4',5-pentachlorobifenyl (4-OH-pentaCB) werd gevonden in het plasma (tot 4.6 μM) van foeten van drachtige ratten blootgesteld aan Aroclor 1254 (Hoofdstuk 5). Deze PCB metaboliet heeft een 10 maal hogere bindingsaffiniteit voor TTR dan schildklierhormoon, vergelijkbaar met de 4-OH-TCB metaboliet, waarmee tevens de relevantie van de kinetiek van de modelstof TCB in zwangere ratten wordt bevestigd (Hoofdstuk 2). Verder werden relatief grote hoeveelheden 4-OH-pentaCB in foetale hersenen (0.46 μM) gevonden, maar niet in de hersenen van pas gespeende ratten. Dit suggereert dat in de afwezigheid van een functionele bloed-hersenbarriere gehydroxyleerde PCB metabolieten de hersenen kunnen binnendringen. Het toxicologisch belang van deze bevinding verdient verder onderzoek.

Invloed van PCB's op schildklierhormoon niveaus en metabolisme

T$_4$-uridine-difosfo-glucuronyl transferase

Verlagingen in plasma schildklierhormoon-niveaus kunnen veroorzaakt worden

door de inductie van glucuronidatie van thyroxine (T_4) in de lever (Bastomsky, 1974, Barter and Klaassen, 1992, 1994). Het effect op het maternale, foetale en neonatale schildklierhormoon-metabolisme in de hersenen en lever na een éénmalige maternale dosering met 3,3',4,4',5,5'-hexachlorobifenyl (HCB) op dag 1 van de dracht en in combinatie met herhaalde doseringen van TCB (dag 2 tot en met 18 van de dracht) is beschreven in Hoofdstuk 4. De resultaten duiden erop dat maternale blootstelling aan een coplanaire PCB de foetale T_4-UDPGT activiteit induceert, hoewel deze inductie niet verantwoordelijk is voor de verlagingen in foetale plasma T_4 niveaus. Alleen de gecombineerde dosis van HCB en TCB resulteerde in significante verlagingen in T_4 niveaus in foetaal plasma. Dit suggereert dat het verlaagde transplacentale transport van maternaal T_4 in combinatie met de blokkade van foetaal plasma T_4 transport door 4-OH-tetraCB resulteert in de verlaging van foetale plasma T_4 niveaus. In tegenstelling tot de foetus, werden verlagingen van maternale en neonatale plasma T_4 concentraties door HCB waarschijnlijk wel veroorzaakt door de inductie van T_4-UDPGT in de lever.

Maternale blootstelling aan het commerciële PCB mengsel Aroclor 1254 resulteerde ook in een inductie van T_4-UDPGT in maternale en neonatale ratten, maar niet in de foetus (Hoofdstuk 5). Omdat de inductie van T_4-UDPGT activiteit alleen in zwangere ratten correleerde met verlaagde plasma T_4 waarden, werd er geconcludeerd dat de inductie van T_4-UDPGT activiteit een ondergeschikte rol speelt in de verlaging van plasma T_4 concentraties in foetale, neonatale en gespeende ratten. Sterke verlagingen van plasma T_4 concentraties zijn ook gevonden als een gevolg van Aroclor 1254 blootstelling in homozygote Gunn ratten, een rattestam die deficiënt is in T_4-UDPGT activiteit (Collins and Capen, 1980). Het enige lange-termijn effect op T_4-UDPGT activiteit in de PCB-blootgestelde nakomelingen was een significante verlaging in microsomale T_4-UDPGT activiteit in levers van net volwassen vrouwtjes.

Type II thyroxine 5'-dejodase activiteit

De belangrijkste bron van het biologisch actieve hormoon trijodothyronine (T_3) in de hersenen is de dejodering van T_4 door het type II thyroxine 5'-dejodase enzym (5'D-II, Silva and Larsen, 1982, Kaplan *et al.* 1983). Verlagingen in T_4 niveaus in de hersenen leiden tot een lagere omzetting van dit enzym, zodat een hogere specifieke activiteit wordt bereikt (Leonard *et al.* 1984). Deze regulatie is belangrijk voor het handhaven van T_3 niveaus in de hersenen. Het was daarom interessant om te bepalen wat het effect was van PCB-geïnduceerde verlagingen in plasma T_4 niveaus op 5'D-II activiteit in de hersenen. Zoals beschreven in Hoofdstuk 4 leidden verlagingen in plasma T_4 niveaus door coplanaire PCB's in foetale, neonatale en pas gespeende ratten tot een significante verhoging van 5'D-II activiteit in hersenhomogenaten. Er werd geconcludeerd dat de verhogingen in 5'D-II activiteit als compensatie dienden voor de verlaagde T_4 niveaus in de hersenen. Indien de verhoging in 5'D-II activiteit in hersenen onvoldoende is om normale T_3 concentraties te handhaven, kan dit nadelig zijn voor de hersenontwikkeling.

Ook na maternale blootstelling aan Aroclor 1254, gingen verlagingen van foetale plasma T_4 spiegels gepaard met verhogingen in 5'D-II activiteit in de hersenen van foetale ratten. In tegenstelling tot het effect van coplanaire PCBs, leidde maternale Aroclor 1254 blootstelling echter tot verlagingen in 5'D-II activiteit in de hersenen van pas gespeende ratten met normale plasma T_4 niveaus (lage dosis), terwijl de 5'D-II

activiteit gelijk bleef aan controlewaarden bij verlaagde plasma T_4 niveaus (hoge dosis). Deze tegenstrijdige effecten in pas gespeende ratten kunnen niet verklaard worden met de huidige kennis van de regulatie van 5'D-II activiteit.

Schildklierhormoon niveaus in de hersenen en plasma.

In de huidige studie waren de effecten van maternale Aroclor 1254 blootstelling op plasma schildklierhormoon niveaus niet langdurig, met slechts kleine verlagingen in pas gespeende ratten, terwijl geen effect meer werd gevonden in de volwassen nakomelingen. Ondanks een substantiële overdracht van PCB's tijdens de lactatie, waren de effecten op schildklierhormoon-niveaus in pas gespeende ratten veel minder dramatisch dan in de foetus. Verschillende mechanismen kunnen dit verschil in respons tussen foetale en pas-gespeende ratten verklaren: de inductie van maternale lever T_4-UDPGT activiteit, verlaagd placentaal transport van schildklierhormoon, en de accumulatie van gehydroxyleerde PCB metabolieten in de foetus. Ook het uitverdunnen van PCB-niveaus in weefsels tijdens postnatale groei en het uitscheiden van PCB's en metabolieten kunnen de verlagingen van plasma T_4 spiegels doen verminderen. Continue pre- en postnatale maternale blootstelling aan Aroclor 1254 via het dieet resulteerde bijvoorbeeld in constante, lage plasma T_4 gehaltes tijdens de eerste drie weken na geboorte.

Ondanks sterke verlagingen van T_4 niveaus in foetale plasma en hersenen na maternale PCB blootstelling, werden geen verlagingen van T_3 niveaus gevonden in foetale hersenen. Hieruit blijkt dat de foetus laat in de dracht de T_3 niveaus in de hersenen kan handhaven door een verhoging van de 5'D-II activiteit, waardoor weinig risico voor PCB-geïnduceerde hypothyroidie in de hersenen bestaat.

De waarneming dat plasma TSH (schildklierstimulerend hormoon) spiegels niet verhoogd waren na PCB-geïnduceerde verlagingen in plasma T_4 niveaus in de foetus en neonaat suggereert dat het ontwikkelende brein euthyroid was. Vergelijkbare dalingen in plasma T_4 niveaus in foeten van Wistar ratten behandeld met methimazole resulteren wel in 6-voudige verhogingen van plasma TSH spiegels (Morreale de Escobar, 1993), zodat het waarschijnlijk is dat foetale plasma TSH spiegels worden gemoduleerd door T_4, maar niet door T_3 niveaus in plasma. In volwassen ratten werden eveneens verhogingen van plasma TSH niveaus en verlagingen in plasma T_4, maar niet in T_3 niveaus waargenomen na Aroclor 1254 blootstelling (Barter and Klaassen, 1994). Eveneens waren deze verhogingen in plasma TSH spiegels relatief gering na PCB blootstelling, in vergelijking met de sterke verhogingen in plasma TSH spiegels na polychloornaftaleen blootstelling, terwijl de dalingen in plasma T_4 niveaus gelijk waren voor beide behandelingen. Het is dus niet ondenkbaar dat PCB's een directe invloed kunnen hebben op de TSH afgifte.

Er kan geconcludeerd worden dat maternale PCB blootstelling een sterke verlaging van T_4 niveaus in foetale hersenen veroorzaken, terwijl slechts marginale verlagingen in T_3 niveaus worden gevonden in foetussen laat in de dracht. Het is mogelijk dat maternale PCB blootstelling vroeger in de dracht, voordat 5'D-II activiteit T_4 verlagingen in hersenen kan compenseren, significante verlagingen in T_3 niveaus in hersenen zou kunnen induceren.

Invloed van maternale PCB-blootstelling op vitamine A status

Analoog aan schildklierhormonen, spelen retinoiden een belangrijke rol in hersen-ontwikkeling. Hun belangrijkste invloed is vroeg of midden in de dracht waargenomen (Adams, 1993). Om het effect van vroege PCB belasting op de retinoid-homeostase te onderzoeken, werden retinol in plasma en retinol en retinylesters in de lever bepaald na maternale Aroclor 1254 blootstelling. De waargenomen verlagingen in plasma retinol kunnen het resultaat zijn van de accumulatie van 4-OH-pentaCB in het plasma dat door binding aan het TTR, de binding van retinol-binding (RBP) eiwit aan TTR verstoort.
Dit is vergelijkbaar met de eerder beschreven verstoring van TTR-RBP binding door de 4-OH-tetraCB metaboliet van 3,3',4,4'-tetrachloorbifenyl (Brouwer *et al.* 1986) Ondanks het feit dat de effecten van maternale PCB blootstelling op retinoid-homeostase in foeten, neonaten en volwassen nakomeling klein waren, was de verstoring langdurend. Omdat de retinoid-reserves niet uitgeput waren door maternale PCB-blootstelling, kan er waarschijnlijk wel genoeg retinylzuur (de meest actieve retinoid-vorm) gevormd worden voor celregulatie.

Veranderingen in neurochemie

In Hoofdstuk 8 en 9 werden de effecten van maternale PCB blootstelling op de ontogenie van enkele neurotransmitters, gliale en neuronale cel-eiwitten in diverse hersenregio's onderzocht.

Neurotransmitters

Maternale blootstelling aan Aroclor 1254 resulteerde in langdurige veranderingen in de gehaltes van 5-hydroxytryptamine (5-HT, serotonine) en de bijbehorend metaboliet, 5-hydroxy-indoolazijnzuur (5-HIAA) in specifieke hersenregio's van de nakomelingen. Gehaltes aan dopamine en noradrenaline waren niet veranderd na maternale blootstelling aan Aroclor 1254. Het is opmerkelijk dat het dopaminerge systeem het meest gevoelig blijkt te zijn voor blootstellingen aan PCB-mengsels (inclusief Aroclor 1254) in volwassen dieren, wat gekenmerkt wordt door een verlaging van dopamine gehaltes in de basale ganglia (Seegal *et al.*, 1985, 1986a,1986b, 1991). Pre- en postnatale blootstelling aan het laag gechloreerde PCB-mengsel Aroclor 1016 resulteerde in een tijdelijke stijging van dopamine gehaltes in de striatum van de nakomelingen (Seegal, 1994). Hieruit kan geconcludeerd worden dat tijdens ontwikkeling het effect van PCBs op de gehaltes van biogene amines in bepaalde hersenregio's verschilt van het effect op volwassenen, en afhankelijk is van de chloreringsgraad van het PCB-mengsel.

In het algemeen worden de effecten van vroege Aroclor 1254 blootstelling gekenmerkt door een toename van 5-HIAA concentraties en de 5-HIAA/5-HT ratio in de laterale olfactory tractus (LOT) en de prefrontale cortex, en een toename in 5-HIAA concentraties in het striatum en de hippocampus op dag 90 postnataal. Omdat deze effecten bijna afwezig zijn op dag 21 postnataal, is er waarschijnlijk een vertraagd effect op de ontwikkeling van het metabolisme van serotonine.

Concentraties van glial fibrillary acidic protein (GFAP) en synaptophysine

Het meest consistente effect van maternale PCB blootstelling op GFAP concentraties werd waargenomen in de laterale olfactory tractus (LOT) en de hersenstam. GFAP concentraties waren verhoogd in de LOT van zowel PCB-blootgestelde mannelijke en vrouwelijke nakomelingen 21 en 90 dagen na geboorte. Verhogingen in GFAP concentraties traden eveneens op in het cerebellum van PCB-blootgestelde dieren op 21 en 90 dagen na geboorte. In tegenstelling tot de LOT en het cerebellum, remde PCB-blootstelling de toename van GFAP concentraties in de hersenstam van de nakomelingen. Dit effect op de hersenstam is een indicatie dat er een vertraging in de ontwikkeling van astrocyten in de hersenstam optreedt.

Synaptophysine concentraties in de hersenen van nakomelingen werden door maternale PCB blootstelling op een complexere manier beïnvloed dan GFAP concentraties. De meest gevoelige hersenregio's voor verlagingen in synaptophysine concentraties na maternale PCB-blootstelling op dag 21 na de geboorte, waren de LOT en de hersenstam. In volwassen nakomelingen leidde pre- en postnatale PCB blootstelling tot een verlaging van synaptophysine gehaltes in de hersenstam van mannelijke nakomelingen, maar tot een verhoging in vrouwelijke ratten. Synaptophysine concentraties waren na maternale PCB blootstelling ook verlaagd in het striatum en de hypothalamus van vrouwelijke, maar niet van mannelijke nakomelingen.

De mechanismen die betrokken zijn bij de veranderingen in GFAP en synaptophysine expressie in de hersenen van de PCB-blootgestelde nakomelingen zijn nog niet opgehelderd. De waargenomen verhogingen van GFAP en verlagingen van synaptophysine concentraties in de LOT en prefrontale cortex zijn karakteristiek voor gliosis (hypertrofie van astrocyten) als reactie op neuronale sterfte (O'Callaghan and Miller, 1989). De verlagingen van zowel GFAP en synaptophysine concentraties in de hersenstam kunnen een indicatie zijn voor een vertraagde ontwikkeling van de hersenstam. De raphe nuclei in de hersenstam bevatten serotonerge neuronen met uitlopers naar de LOT en de prefrontale cortex (Kosofsky and Molliver, 1987). Het is niet ondenkbaar dat de veranderingen in serotonine-metabolisme en GFAP en synaptophysine concentraties in de LOT en prefrontale cortex een gevolg zijn van een vertraagde serotonerge innervatie van deze hersenregio's.

Relevantie van het onderzoek voor humane ontwikkeling en toekomstig toxicologisch onderzoek.

Schildklierhormoon

In een recent gepubliceerd onderzoek van 105 moeder-kind paren werd een verband gevonden tussen verhoogde maternale belasting aan polychloor-dibenzo-*p*-dioxines (PCDD's), dibenzofuranen (PCDF's) en bifenylen (PCBs) en veranderingen in schildklierhormoonstatus (Koopman-Esseboom *et al.*, 1994). De veranderingen worden gekenmerkt door een negatieve correlatie van verschillende PCDD, PCDF en PCB congeneren met maternale plasma T_3 niveaus voor geboorte en maternale T_3 en T_4 niveaus na geboorte, en een positieve correlatie met plasma TSH van de kinderen twee weken en drie maanden na geboorte. In zuigelingen met een verhoogde blootstelling waren plasma TT_4 (total T_4) niveaus significant verlaagd (10%) en plasma TSH spiegels

significant verhoogd (37%). Maternale belasting met drie PCB congeneren (PCB 118, 138 en 153) correleerde positief met TSH concentraties in de navelstreng. In een vergelijkbare studie met 38 moeder-kind paren werden verhogingen van plasma TSH en TT_4 concentraties waargenomen in kinderen met een hogere blootstelling aan totale toxische equivalenten van PCDD's en PCDF's (Pluim *et al.*, 1992).

Indien er sprake is van een oorzakelijk verband, lijken de effecten van PCDD's, PCDF's en PCB's op plasma schildklierhormoon niveaus bij mensen, zoals bescreven door Koopman-Esseboom *et al.* (1994), in het algemeen op de effecten waargenomen in volwassen muizen, ratten en apen bij hogere doseringen. In tegenstelling tot de verlagingen in TT_4 en FT_4 (free T_4) in plasma van rattefoetussen laat in de dracht na maternale PCB blootstelling aan Aroclor 1254 (dit proefschrift), werd geen effect waargenomen van maternale PCB belasting op TT_4 en FT_4 concentraties in de menselijke navelstreng (Koopman-Esseboom *et al.*, 1994).

Het is onwaarschijnlijk in de mens dat de veranderingen in schildklierhormoon-homeostase die samenhangen met maternale PCB en PCDD belasting zullen resulteren in ontwikkelingsstoornissen van het centrale zenuwstelsel. Compensatiemechanismen, waaronder de inductie van 5'D-II activiteit in de hersenen, kunnen de T_3 concentraties waarschijnlijk op peil houden. In de foetale rattehersenen waren T_3 spiegels niet beïnvloed na PCB blootstelling, ondanks sterke verlagingen van T_4 concentraties in plasma en hersenen. Het is echter nog steeds mogelijk dat verlagingen in T_3 concentraties in foetale hersenen kunnen optreden voordat de compensatie mechanismen volledig zijn ontwikkeld. Eveneens is niet volledig uit te sluiten dat zeer lokale T_3 verlagingen kunnen optreden door onvoldoende compensatiemechanismen.

Transport van schildklierhormonen in de rat en de mens verschillen van elkaar in sommige aspecten die consequenties kunnen hebben voor de mogelijke effecten van PCBs. Het belangrijkste schildklierhormoon-transporteiwit in de mens is thyroxine binding globulin (TBG), terwijl in ratten TTR het belangrijkste transporteiwit is (Robbins, 1991). Hoewel TBG onder bepaalde omstandigheden aanwezig is in ratteplasma, heeft dit eiwit een lage affiniteit voor T_4 (Rouaze-Romet *et al.* 1992). Gehydroxyleerde PCB metabolieten binden zeer zwak aan humaan TBG (Lans *et al.*, 1994), dus is het waarschijnlijk dat de effecten van gehydroxyleerde PCB metabolieten op de humane plasma T_4 niveaus in volwassenen zeer gering zullen zijn. De foetale muis heeft zowel TTR en TBG als transporteiwitten, waarbij muize-TBG vergelijkbare T_4 bindende eigenschappen heeft als humaan TBG (Vrancks *et al.* 1990). Hierdoor is de muis een geschikt modeldier voor deze effecten van PCB's op de mens. Recent onderzoek heeft echter aangetoond dat na toediening van TCB aan zwangere muizen het 4-OH-tetraCB metaboliet accumuleert in het plasma van de foetus. Hier bindt het aan TTR, wat vervolgens resulteert in verlagingen van foetale plasma T_4 concentraties (Darnerud *et al.*, submitted). Het is daarom mogelijk dat het transplacentale transport van gehydroxyleerde metabolieten die aanwezig zijn in het humane plasma resulteert in de accumulatie van deze metabolieten in de foetus. Een accumulatie van metabolieten in de foetus kan leiden tot verlagingen in schildklierhormoonniveaus in plasma en hersenen voordat in het tweede trimester de foetale hypothalamus-hypofyse functies toenemen.

Terwijl algemeen wordt aangenomen dat een gebrek aan schildklierhormonen laat in de zwangerschap of in neonaten een negatieve invloed kan hebben op hersenontwikkeling, zijn de effecten van schildklierhormoon-deficiëntie eerder in de

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zwangerschap niet uitgebreid onderzocht (Morreale de Escobar *et al.* 1993, review, Porterfield and Hendrich, 1993, review). In ratten resulteert thyroidectomie van de moederdieren in veranderingen in het gedrag en de neurochemie van de nakomelingen, wat suggereert dat schildklierhormoon nodig is voor de hersenontwikkeling voordat foetale schildklierhormoon secretie begint (Hendrich *et al.* 1984, Pickard *et al.* 1993). Schildklierhormonen en schildklierhormoon-receptoren zijn na week 10 van de zwangerschap aanwezig in de hersenen van humane foeten, maar het functionele belang hiervan is niet bewezen. De bevinding van Pharoah *et al.* (1972) dat de neurologische schade door endemisch cretinisme (tengevolge van een jodium tekort) voorkomen kon worden indien de moeders gejodeerde olie toegediend kregen voor het tweede trimester, ondersteunt de rol van het schildklierhormoon bij de hersenontwikkeling tijdens deze periode van de zwangerschap.

Meerdere vragen moeten beantwoord worden voordat de rol van een veranderde schildklierhormoon-homeostase in PCB-geïnduceerde neurotoxiciteit tijdens de ontwikkeling duidelijk wordt. Eerst zal vastgesteld moeten worden of maternale PCB blootstelling leidt tot verlagingen in de concentraties van T_3 in de hersenen vroeger in de zwangerschap, en vervolgens of deze verlagingen van betekenis zijn voor de hersenontwikkeling. Ongeacht of PCB blootstelling functionele veranderingen in schildklierhormoon-gehaltes tot gevolg heeft, leidt het transport van gehydroxyleerde PCB's naar de foetus en de accumulatie daarvan in het plasma en de hersenen tot een andere vraag: wat is de biologische activiteit van deze metabolieten in het hersenweefsel? Gehydroxyleerde PCB's binden 10 maal sterker aan kernextracten dan T_4 (McKinney *et al.*, 1987) en bezitten estrogene eigenschappen (Jansen *et al.* 1993, Korach *et al.* 1988).

Invloed op neurochemische ontwikkeling

Pre- en postnatale blootstelling van ratten aan een commercieel PCB mengsel, Aroclor 1254, resulteert in lange-termijn effecten op de nakomelingen. Deze studie geeft informatie over welke neurotransmittersystemen, celtypen en hersenregio's beïnvloed worden na blootstelling aan een hoog gechloreerd PCB mengsel. Dit kan van belang zijn voor verdere studies naar structuur-activiteits relaties en het bepalen van een NOAEL (no observed adverse effect level).

Een van de vragen die onderzoekers al meer dan 20 jaar lang bezig houdt, is welke PCB congeneren verantwoordelijk zijn voor de toxiciteit van deze verbindingen. Deze vraag is voldoende beantwoord voor immunotoxiciteit en sommige ontwikkelingseffecten (teratogeniteit en foetale sterfte) waarbij de interactie van PCB congeneren met de Ah-receptor een belangrijke rol speelt.

Tot op heden zijn er publicaties verschenen over de functionele neurotoxiciteit van slechts twee individuele PCB congeneren, namelijk 3,3',4,4'-tetrachlorobifenyl (TCB) en 3,3',4,4',5-pentachlorobifenyl, beiden coplanaire PCB congeneren met een hoge affiniteit voor de Ah-receptor. TCB bleek neurotoxisch te zijn in muizen na een hoge maternale blootstelling, en veroorzaakte verlagingen in dopamine en dopamine receptor concentraties in het striatum, neuropathologische veranderingen in de "cranial roots" en een vertraging van "avoidance" gedrag (Tilson *et al.* 1979, Chou *et al.* 1979, Agrawal *et al.* 1981). Postnatale blootstelling van muizen aan TCB beïnvloedde de muscarinerge receptor concentraties in de hippocampus en de spontane activiteit

(Eriksson, 1988, Eriksson *et al.* 1991). Door het snelle metabolisme van TCB en de accumulatie van gehydroxyleerde metabolieten in de foetus kunnen geen conclusies worden getrokken over de rol van de uitgangsstof of zijn metaboliet in de ontwikkelings-neurotoxiciteit van deze PCB congeneer.

Blootstelling van zwangere ratten aan de slecht metaboliseerbare 3,3',4,4',5-pentachloorbifenyl vertraagde de ontwikkeling van spontane activiteit en de neuromusculaire ontwikkeling in de nakomelingen. Deze effecten waren gerelateerd aan een groeivermindering. De ontwikkeling van reflexen en visuele discriminatie werd echter niet beïnvloed door maternale blootstelling aan 3,3',4,4',5-pentachloorbifenyl (Bernhoft *et al.* 1994). Tesamen suggereren deze data dat de toxische coplanaire PCB congeneren niet direct ontwikkelings-neurotoxisch zijn in knaagdieren. Hierdoor is het onwaarschijnlijk dat het gebruik van individuele PCB congeneren voor het bestuderen van de effecten op neurochemische parameters en gedrags-ontwikkeling nieuwe structuur-activiteits relaties zal voortbrengen op dezelfde termijn als de structuur-activiteits relaties voor imuunotoxiciteit of CYP1A1 inductie in volwassen dieren. Ten eerste zijn reproductie-studies veel langduriger en duurder dan subacute studies met volwassen dieren. Ten tweede is er geen algemene overeenstemming voor experimentele protocollen (tijstip en duur van PCB blootstelling, neurochemische en gedrags eindpunten) tussen onderzoekers in dit veld, wat het vergelijken van data bemoeilijkt. Ten derde is het zeer waarschijnlijk dat complexe interacties van Ah-receptor binding, PCB metabolisme en hormonale veranderingen de hersenontwikkeling beïnvloeden, zodat de structuur-activiteits relaties voor individuele congeneren geen voorspelling zullen geven over het effect van complexe mengsels.

Het verdient daarom de voorkeur om milieu-relevante mengsels te gebruiken voor dosis-respons studies, en om neurochemische- en gedrags-parameters en species-gevoeligheid te bepalen. Milieu-relevante mengsels kunnen verkregen worden door het extraheren van PCB-gecontamineerde voedingscomponenten (b.v. visolie) of door het samenstellen van synthetische mengsels. Ondanks variaties in de samenstelling, kunnen de resultaten van studies met zulke mengsels relevanter zijn voor beleidsgerichte doeleinden dan resultaten van studies met individuele congeneren. Dit vraagt echter om een effect-gerichte normstelling en risicoschatting in plaats van risicoschatting op basis van individuele componenten. Celkweek-technieken die gebruik maken van gliale, neuronale of gemengde neurale cellen kunnen nuttig zijn voor het onderzoek naar structuur-activiteits relaties van de individuele PCB congeneren of directe effecten van PCB congeneren op de neurochemische ontwikkeling. Na het vaststellen van effecten van mengsels *in vivo* kan dan gebruik gemaakt worden van verwante effect-parameters *in vitro*.

CONCLUSIES

1) Diverse PCB congeneren (3,3',4,4'-tetrachloorbifenyl, 2,3,3',4,4'-pentachloorbifenyl en 2,3',4,4',5-pentachloorbifenyl) kunnen gemetaboliseerd worden tot gehydroxyleerde metabolieten welke accumuleren in plasma en hersenen van de foetus. Vervolgens kunnen zij dramatische verlagingen in thyroxine concentraties in foetaal plasma en hersenen laat in de dracht veroorzaken.

2) De verlagingen in thyroxine niveaus in de hersenen van de foetus worden effectief gecompenseerd door verhogingen in Type II thyroxine 5'-dejodase activiteit. Als gevolg hiervan zijn alleen marginale verlagingen in trijodothyronine concentraties in de foetale hersenen waargenomen na maternale blootstelling aan het commerciële PCB-mengsel Aroclor 1254. Dit is een indicatie dat PCB-geïnduceerde verlagingen in plasma T_4 niveaus niet verantwoordelijk zijn voor storingen in de ontwikkeling van het centrale zenuwstelsel.

3) Maternale blootstelling aan Aroclor 1254 verandert specifiek de ontwikkeling van het serotonine-metabolisme in de hersenen van de nakomelingen. De blootstelling van volwassen ratten en makaken aan Aroclor 1254 veroorzaakt echter veranderingen in het dopamine-metabolisme die gevoeliger en langduriger zijn dan veranderingen in het serotonine-metabolisme. Het mechanisme van PCB-geïnduceerde neurotoxiciteit tijdens de ontwikkeling is derhalve verschillend van het mechanisme van veranderingen in biogene amine-metabolisme in volwassen dieren.

4) De ontwikkeling van zowel neuronale als gliale cellen wordt beïnvloed in de hersenen van nakomelingen van zwangere ratten die behandeld zijn met Aroclor 1254. De veranderingen in astrocyt-ontwikkeling in de hersenstam door PCB-blootstelling zijn niet het gevolg van neuronale sterfte, omdat de concentraties van glial fibrillary acidic protein (GFAP) verlaagd zijn, terwijl over het algemeen neuronale sterfte samengaat met verhogingen in GFAP. Daarom is het waarschijnlijk dat PCB's de hersenontwikkeling beïnvloeden via veranderingen in cel-differentiatie en proliferatie en niet door het direct induceren van neuronale sterfte.

5) Aangezien de hersenstam een van de vroegst ontwikkelende hersenstructuren is, kunnen de veranderingen op de hersenstam zoals waargenomen na pre- en postnatale PCB-blootstelling, een negatieve invloed hebben op de verdere ontwikkeling van andere hersenstructuren.

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Curriculum vitae

Dennis Carlyle Morse was born in Gloucester, Massachusetts on August 22, 1962. Between 1978 and 1980 he was enrolled in Phillips Exeter Academy. He finished the academic year of 1980 at Rockport High School and recieved his high school diploma from Gloucester High School. He attended Salem State College for 1 year in 1981, following the B.Sc. program. He then attended Hampshire College between 1982 and 1984, specializing in the biochemistry of nitrogen-fixing bacteria under the supervision of Dr. Larry Winship and Prof. Lynn Miller. In 1984 he conducted additional research for 6 months at the Department of Microbiology at the Agricultural University in Wageningen for a B.A. thesis under the supervision of Dr. Anton Akkermans. After transferring to the Agricultural University in 1985, he followed the M.Sc. program voor environmental hygiene and graduated in 1989 with a specialization in Toxicology under the supervision of Dr. Bram Brouwer and Prof J.H. Koeman. Between December 1989 and November 1993 he worked as a Ph.D. student at the Department of Toxicology in Wageningen on a collaborative study on the effects of PCBs and dioxins on the newborn financed by the Dutch Toxicology Research Promotion Program and the Dutch Health Research Stimulation Program, with the supervision of Dr. Bram Brouwer. In December 1993 he was employed by the Research School for Environmental Chemistry and Toxicology to continue research on the developmental neurotoxicity of PCBs and dioxins at the Department of Toxicology in Wageningen. The work described in this thesis is based on research carried out between 1989 and 1994.