AN ABSTRACT OF THE THESIS OF

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Di-ortho polychlorinated biphenyls (PCBs) are prominent environmental contaminants and their biological activity in fish and interactions with other environmental contaminants may be more significant than previously thought. This work investigated di-ortho PCB pretreatment induction of hepatic metabolizing enzymes and the disposition of a subsequent dose of 7,12 [^{3}H]dimethylbenz[a]anthracene (DMBA) or the same PCB.

Hepatic ethoxyresorufin-O-deethylase activity (EROD) activity was approximately one and two orders of magnitude greater than controls in fish receiving a single intraperitoneal (ip) injection of 50 and 250 ug 2,2',4,4',5,5'-hexachlorobiphenyl (2HxCB)/g fish, respectively. Hepatic microsomal CYP1A2 mRNA levels were approximately 3-fold greater in fish treated with 250 ug 2HxCB/g fish than controls while 50 ug 2HxCB/g fish were unchanged. There was no increase of CYP1A1 mRNA in 2HxCB treated fish.

Fish were ip injected with 0, 50 or 250 ug 2HxCB/g and then four weeks later received an ip injection of 10 nmols [^{3}H]DMBA/g. Livers and gallbladders were removed 16 hours later. There was a subtle increase in biliary excretion of [^{3}H]DMBA. After rainbow trout were fed 2HxCB for 4, 8, or 12 weeks fish were ip injected with 10 nmols [^{3}H]DMBA/g. Fish fed 2HxCB had greater biliary concentrations of [^{3}H]DMBA than controls 16 hours after [^{3}H]DMBA injection.

Rainbow trout received an ip injection of 0, 0.5 or 5.0 ug 2,2′,5,5′-tetrachlorobiphenyl (TCB)/g fish 14, 28, and 42 days before ip injection with 10 nmols [^{14}C]TCB/g fish. Average muscle tissue [^{14}C]TCB concentrations increased approximately 3-fold 28 days after pretreatment with 5 ug TCB/g fish while carcass levels decreased 24 hours after injection. Hepatic microsomal EROD activities were unchanged for fish 28 days after treatment with 5 ug TCB/g fish. Total hepatic [^{3}H]DMBA (nmols) decreased 16 hours after [^{3}H]DMBA ip injection in fish pretreated with 5.0 ug TCB/g fish.

2HxCB induced EROD activity and CYP1A protein in rainbow trout and altered disposition of a subsequent dose of [^{3}H]DMBA that was associated with increased CYP1A2 mRNA levels. However, TCB altered tissue distribution of a subsequent dose of [^{14}C]TCB or [^{3}H]DMBA was unrelated to hepatic EROD activity indicating a mechanism of action different from 2HxCB.

Congener-Specific Disposition of Polychlorinated Biphenyls in Rainbow Trout

by

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CONTRIBUTION OF AUTHORS

Dr. Lawrence R. Curtis was involved in the design, analysis, and writing of each manuscript. Dr. Nicholas J. Vrolijk and Dr. Thomas T. Chen were responsible for CYP1A1 and CYP1A2 mRNA analysis.

DEDICATION

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INTRODUCTION

Although the manufacture of polychlorinated biphenyls (PCBs) was discontinued, PCBs remain common environmental contaminants due to widespread use and release to the environment. PCB world production was estimated to be 1.2 million tons with an estimated 372,000 tons already released to the environment and 780,000 tons in use, storage or landfills (Tanabe, 1988). PCBs are hydrophobic, lipophilic compounds that are resistant to biological and chemical degradation. Because of their physical chemical characteristics, PCBs can co-occur in the aquatic environment with other pollutants that have similar characteristics. Polycyclic aromatic hydrocarbons (PAHs) and PCBs were found in sediments from lakes, rivers, and estuaries (Bolton et al., 1985).

PAHs such as 7,12-dimethylbenz[a,h]anthracene (DMBA) (Bieri et al., 1986), benzo[a]pyrene, and phenanthrene were detected in sediments collected from natural waters (Curtis et al., 1993). In addition, PAHs were identified as carcinogenic to wild and laboratory fish. PAHS in sediments were associated with hepatic tumor incidence in fish collected from marine waters (Malins et al.,, 1985a; Malins et al., 1985b; Malins et al., 1987) and rainbow trout hatched from eggs

treated with water-borne DMBA had increased incidence of hepatic tumors 9 months after exposure (Fong et al., 1993).

The classic PAH carcinogenesis model consists of parent PAH metabolized to a reactive intermediate which can bind nucleic acids of DNA to form adducts, mutations, and eventual neoplasia. Reactive metabolites can be formed by cytochrome P450 metabolism. DMBA is a weak carcinogen that can be metabolized by the cytochrome P450 and epoxide hydrolase systems to potent carcinogenic diols such as 7-hydroxymethyl-12-methylbenz[a]anthracene and 3,4-dihydrodiol dimethylbenz[a]anthracene (Yang and Dower, 1975; DiGiovanni, et al., 1983). The latter has been identified in the bile of rainbow trout treated with DMBA (Schnitz et al., 1993).

PCB induction of the cytochrome P450 system may affect PAH metabolism, disposition, and ultimately toxicity. Pre-exposure to commercial PCB mixtures or PAHs alter PAH metabolite formation but effects of individual PCB congeners have not been extensively studied in fish. Increased benzo[a]pyrene metabolism by the hepatic S9 fraction from mullet pretreated with Aroclor 1254 (Tan et al., 1981) and from brown bullhead, black bullhead, and goldfish pretreated with 3-methylcholanthrene was reported (Swain and Melius, 1984).

The toxicity of complex mixtures of compounds that have similar chemical structure and mechanisms of action, such as the Ah-receptor agonists 2,3,7,8 substituted dioxins and

furans, have been studied in fish (Zabel et al., 1995). Interactions of compounds with minimal effect on metabolizing enzyme systems have been less extensively studied but their interactions may be important.

Such is the case with dieldrin, an organochlorine insecticide that does not induce cytochrome P4501A in fish. Fish treated with dieldrin for 10 and 12 weeks were administered a subsequent dose of [^{14}C]dieldrin and had increased levels of hepatic and biliary [^{14}C]dieldrin without induction of CYP1A protein or EROD activity (Gilroy et al., 1993). Induction of cytosolic lipoprotein complexes or ligandin were suggested as possible mechanisms for increased biliary excretion.

2HxCB, a di-ortho PCB commonly detected in environmental samples (McFarland and Clarke, 1989), induced cytochrome P4502B1 (CYP2B1) in rats while fish were refractory to CYP2B1 induction and showed no other biochemical response to 2HxCB exposure (Klienow et al., 1990, James and Little, 1981; van der Weiden et al., 1994). The lack of biochemical response in fish was attributed to 2HxCB's low affinity for the Ah-receptor (reviewed in Safe, 1994). However, recent long term studies feeding 2HxCB to rainbow trout reported increased hepatic EROD and arylhydrocarbon hydroxylase activities (da Costa and Curtis, 1995).

Induction of CYP1A isozymes may occur through Ah receptor dependent and independent pathways and EROD activity may be

associated with more than one CYP1A isozyme. Classic Ah receptor agonists increase CYP1A1 levels but the 4S PAH-binding protein has been identified as another pathway for induction (Sterling et al., 1994). EROD induction in mice exposed to benzo[a]pyrene was associated with increased levels of CYP1A1 and CYP1A2 isozymes (Tsyrlov and Duzchak, 1990) while EROD induction in 2HxCB exposed mice was due to the CYP1A2 and not the CYP1A1 isozymes (De Jongh et al., 1995). CYP1A2 has been reported in mammals and recent evidence indicates two CYP1A genes in rainbow trout, but the existence of CYP1A2 in fish is debated (Berndston and Chen, 1994; Nelson et al., in press; Morrison et al., 1995).

Although highly chlorinated PCBs were considered to be resistant to metabolism with minimal metabolite formation (reviewed in Safe 1989), PCB methylsulfonyl metabolites were identified in tissues from humans, whales, and fish (Haraguchi et al., 1989). Some methylsulfonyl metabolites have biological activity and can bind with cellular macromolecules (Lund et al., 1985; Larsen et al., 1991). The methylsulfonyl metabolites of a tetra- and penta- chlorinated biphenyl increased 7-ethoxycoumarin-O-deethylase and benzo[a]pyrene hydroxylase activities in rat liver tissues (Kato et al., 1995). In addition, PCB hydroxy metabolites bound thyroxine specific binding sites in rat liver nuclear extracts (McKinney et al., 1987) and inhibited the binding of thyroxine with transthyretin (Brouwer et al., 1990). The rate of PCB

metabolite formation was related to degree of chlorination with decreasing metabolism as chlorination increased (Ghiasuddin et al., 1976).

The objectives of this study were to determine if fish exposed to di-ortho PCBs would have increased levels of hepatic cytochrome P450 enzyme and what effect this would have on the disposition of a subsequent dose of a PAH or PCB. Following long term exposure of rainbow trout to di-ortho PCBs hepatic cytochrome P450 levels were measured or the altered tissue disposition of a subsequent dose of a PAH or PCB were measured. The effects of highly chlorinated and lesser chlorinated di-ortho PCBs were compared by using 2HxCB and TCB during the study.

CHAPTER 1

2,2′,4,4′,5,5′-Hexachlorobiphenyl and 3,3′,4,4′,5,5′-Hexachlorobiphenyl Hepatic Induction of Ethoxyresorufin-O-deethylase Activity, CYP1A and Physiological Evidence for Hepatic CYP1A2 in Rainbow Trout (*Oncorhynchus mykiss*)

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ABSTRACT

Di-ortho polychlorinated biphenyls (PCBs) are prominent environmental contaminants and their biological activity in fish may be more significant than previously thought. Four weeks after intraperitoneal (ip) injection with 50 or 250 ug 2,2′,4,4′,5,5′-hexachlorobiphenyl (2HxCB)/g or 5 or 25 ug 3,3′,4,4′,5,5′-hexachlorobiphenyl (3HxCB)/g, rainbow trout livers were removed and frozen at -80°C or microsomes were prepared. Microsomal ethoxyresorufin-O-deethylase activity (EROD) activity was approximately one and two orders of magnitude greater than controls in fish treated with 50 and 250 ug 2HxCB/g fish, respectively. The mean EROD activity was an order of magnitude greater in fish treated with 5 ug 3HxCB/g fish but unchanged in fish treated with 25 ug 3HxCB/g fish. Hepatic microsomal CYP1A2 mRNA levels were approximately 3-fold greater in fish treated with 250 ug 2HxCB/g fish than controls while 50 ug 2HxCB/g fish were unchanged. There was no increase of CYP1A1 mRNA in 2HxCB treated fish. The study showed: 2HxCB induced hepatic EROD activity and CYP1A protein; physiological evidence of a trout hepatic CYP1A2 isozyme; and 3HxCB inhibition of CYP1A activity.

INTRODUCTION

Polychlorinated biphenyls (PCBs) are ubiquitous in the environment due to widespread use and release, lipophilicity, and resistance to environmental degradation. PCB world production has been estimated to be 1.2 million tons with an estimated 372,000 tons already released to the environment and 780,000 tons in use, storage, or landfills (Tanabe, 1988). Approximately half of the 209 PCB congeners were detected in environmental samples with the di-ortho PCB, 2,2′,4,4′,5,5′-hexachlorobiphenyl (2HxCB), the most frequently detected congener (McFarland & Clarke 1989). Although the non-ortho PCB, 3,3′,4,4′,5,5′-hexachlorobiphenyl (3HxCB), was found in commercial mixtures of PCBs (Albro et al., 1979) it was infrequently detected in environmental samples (McFarland and Clarke, 1989).

While 2HxCB induced CYP2B1 in rats, fish were refractory to CYP2B1 induction and showed no other biochemical response to 2HxCB exposure (Klienow et al., 1990; James and Little, 1981; van der Weiden et al., 1994). The lower toxicity and lack of hepatic biochemical response to di-ortho PCBs were attributed to low Ah receptor affinity. Therefore, di-ortho PCBs were considered a low hazard to humans, wildlife, and aquatic life (reviewed in Safe, 1994; Zabel et al., 1995). However, recent studies with mink show that 2HxCB can adversely affect reproduction (Patnode and Curtis, 1994) and long term feeding of 2HxCB to rainbow trout increased hepatic

ethoxyresorufin-O-deethylase (EROD) and arylhydrocarbon hydroxylase activities (da Costa and Curtis, 1995). Non Ah receptor mediated pathways of toxicity and the toxicological significance of 2HxCB in the environment may be underestimated.

The induction of CYP1A isozymes may occur through Ah receptor dependent and independent pathways and EROD may be associated with more than one CYP1A isozyme. Classic Ah receptor agonists increase CYP1A1 levels but the 4S PAH-binding protein has been identified as another pathway for induction (Sterling et al., 1994). EROD induction in mice exposed to benzo(a)pyrene was associated with increased levels of CYP1A1 and CYP1A2 isozymes (Tsyrlov and Duzchak, 1990) while EROD induction in 2HxCB exposed mice was due to the CYP1A2 and not the CYP1A1 isozyme (De Jongh et al., 1995). The human CYP1A2 gene contains a response element which responds to 3-methylcholanthrene but not TCDD (Quattrochi et al., 1994). CYP1A2 has been reported in mammals and recent evidence indicates two CYP1A genes in rainbow trout, but the existence of CYP1A2 in fish is debated (Berndston and Chen, 1994; Nelson et al., in press; Morrisson et al., 1995).

CYP1A protein, CYP1A1 and CYP1A2 mRNA, and EROD levels were measured following long term exposure to a single ip injection of 2HxCB. 3HxCB exposure was used for characteristic Ah receptor agonist effects.

METHODS

Experimental Animals

Sexually immature Shasta strain rainbow trout (20-60 g) were obtained from the Food Toxicology & Nutrition Laboratory, Oregon State University. Two fish receiving the same PCB treatment were kept in aerated glass aquaria (61x32x20 cm; 23 L total volume), separated by a partition, and received a continuous flow of well water (100 ml/min @ 14±2°C). Fish were fed a 3% body weight (dry wt fish/dry wt diet) ration of Oregon Test Diet/day given as three feedings per week. Fish weights were measured weekly and rations were adjusted accordingly. Tank debris was removed 3 times/week. A 12:12 hr light:dark cycle was maintained throughout the study.

Dosage

Fish received a single ip injection of 50 or 250 ug 2HxCB/g or 5 or 25 ug 3HxCB/g in stripped menhaden oil (5 ml/kg) obtained from the Fish Oils Test Material Program. Controls received menhaden oil only.

Chemicals

Fish used for EROD and Western immunoblot analysis were treated with 2HxCB (Accustandard, New Haven CT) that was >99% pure and had <1% mono-ortho PCB detected by gas chromatograph/mass spectrometry (da Costa and Curtis, 1995). No other PCBs, dioxins, or dibenzofurans were detected. 2HxCB

(purity >99%) used for mRNA assays, which did not alter hepatic EROD activity in C57BL/6J mice (Biegel et al., 1989), was a gift from Dr. S. Safe. 3HxCB (Accustandard, New Haven CT) purity was >99%. The primary antibody used in Western immunoblot was a generous gift from Dr. D. Williams. All other chemicals used were of the highest grade available.

Microsomal Preparation and Biochemical Analysis

Microsomes were prepared according to Carpenter et al. (1990) and stored at -80°C until use. Microsomal protein content was measured according to Lowry et al. (1951). EROD and pentoxyresorufin-o-deethylase (PROD) activity were measured as described earlier (Prough et al., 1978; Burke and Mayer 1974; Lubet et al., 1985). Microsomal samples were prepared from pooled livers from two fish of the same treatment.

Immunoquantitation

Microsomal CYP1A protein was measured using Western immunoblot techniques according to Towbin et al., (1979) with modifications. Briefly, SDS-Page electrophoresis was performed using 8% polyacrylamide precast mini-gels (Novex, San Diego CA). Membranes were prepared according to manufacturers recommendations (Amersham International, Little Chalfort England) and proteins were transferred for 60 min at 190 milliamps followed by incubation with trout-anti-rabbit primary antibody. Membranes were rinsed with PBS-tween and

incubated for 60 min with the secondary antibody (anti-rabbit) and enzyme linked chemiluminescence was used for expressing antibodies on photographic film. Quantification was performed with a Hewlett Packard Scan Jet II cx/T. Reference microsomes were prepared from pooled livers of rainbow trout that received an ip injection of beta naphthaflavone and sampled 7 days later.

CYP1A1/1A2 mRNA Analysis

Whole livers were sampled and stored at -80°C until mRNA analysis was performed according to Berndston and Chen (1994) with modifications.

Data Analysis

Difference between treatments were detected using 1-way ANOVA and Least Significant Difference multiple comparison test ($p<0.05$). Data was transformed when data failed test of normal distribution. Log EROD was regressed against CYP1A2 mRNA and ROD and log ROD was regressed against square root (sqrt) CYP1A2 mRNA using simple linear regression.

RESULTS

Hepatic Enzyme Activities

EROD activities were dose dependent for 2HxCB but not 3HxCB treated fish (Table 1.1). 2HxCB 50 ug/g and 250 ug/g treatments increased EROD activities one and two orders of magnitude above controls, respectively. Mean EROD activity

for 3HxCB 50 ug/g treatment were increased above controls while 3HxCB 25 ug/g treated fish remained unchanged. PROD activity was not detected (data not shown).

Table 1.1. Hepatic EROD activity (nmol/min*mg protein) and Western blot relative optical density (ROD) four weeks after ip injection with 2HxCB or 3HxCB.

2HxCB (ug/g):	0	50	250		
3HxCB (ug/g):	0			5	25
EROD:	0.03±0.01	0.34±0.10*	3.44±0.79**	0.45±0.26*	0.17±0.04
CYP1A (ROD):	14.4±5.3	204±52.6*	1205±20.2**		
CYP1A (ROD):	3.40±1.5			348.2±85.4*	645.8±110*
LSI:	0.67±0.07	0.69±0.02	0.79±0.05**	0.79±0.03*	0.90±0.07*

EROD n=3 ±(SE)
LSI: Liver Somatic Index (n=6; 25 3HxCB n=5)
*Significantly higher than controls
**Significantly higher than controls and low dose

Increases in liver/body weight ratio (LSI) were not dose dependent after 2HxCB or 3HxCB treatments (Table 1.1). The mean LSI for 50 ug 2HxCB/g treatment was unchanged from controls while 250 ug 2HxCB/g increased above control and 50 ug 2HxCB/g values. The mean LSI of 0.90 for 25 ug 3HxCB treated fish was greater than controls but was not significantly different from the LSI of fish receiving 5 ug 3HxCB/g.

Hepatic CYP1A Isozyme Levels

Western immunoblot CYP1A ROD increased with dose for 2HxCB but not 3HxCB (Table 1.1, Figure 1.1 and Figure 1.2).

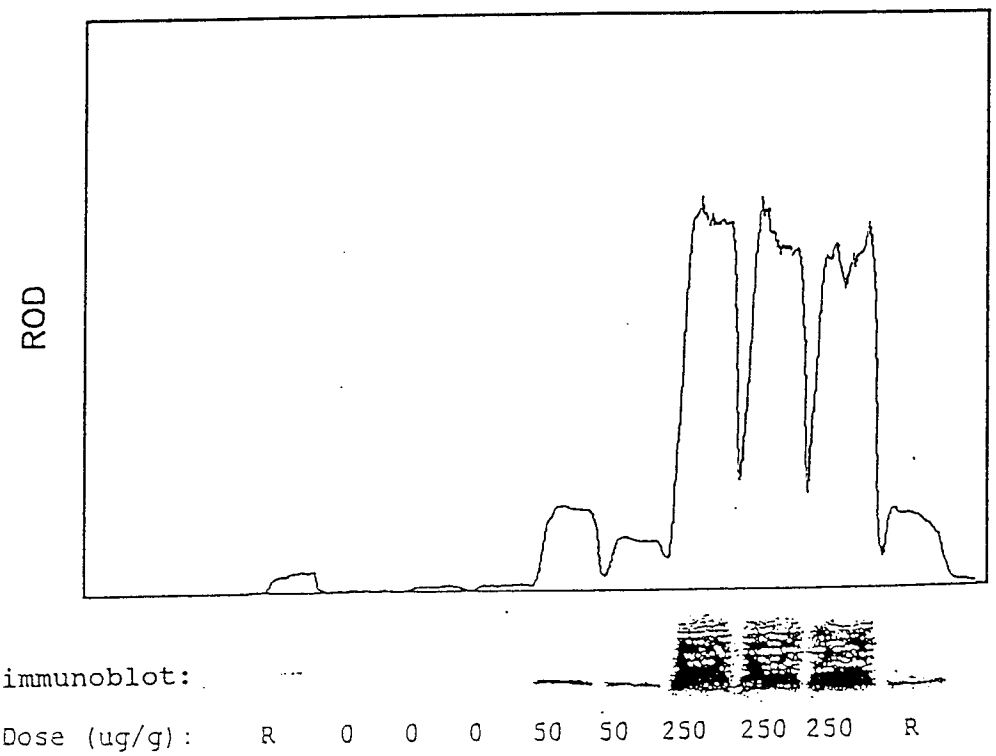

Figure 1.1. Western immunoblot and ROD of hepatic microsomes four weeks after ip injection with 2HxCB (R=beta-naphthaflavone treated fish).

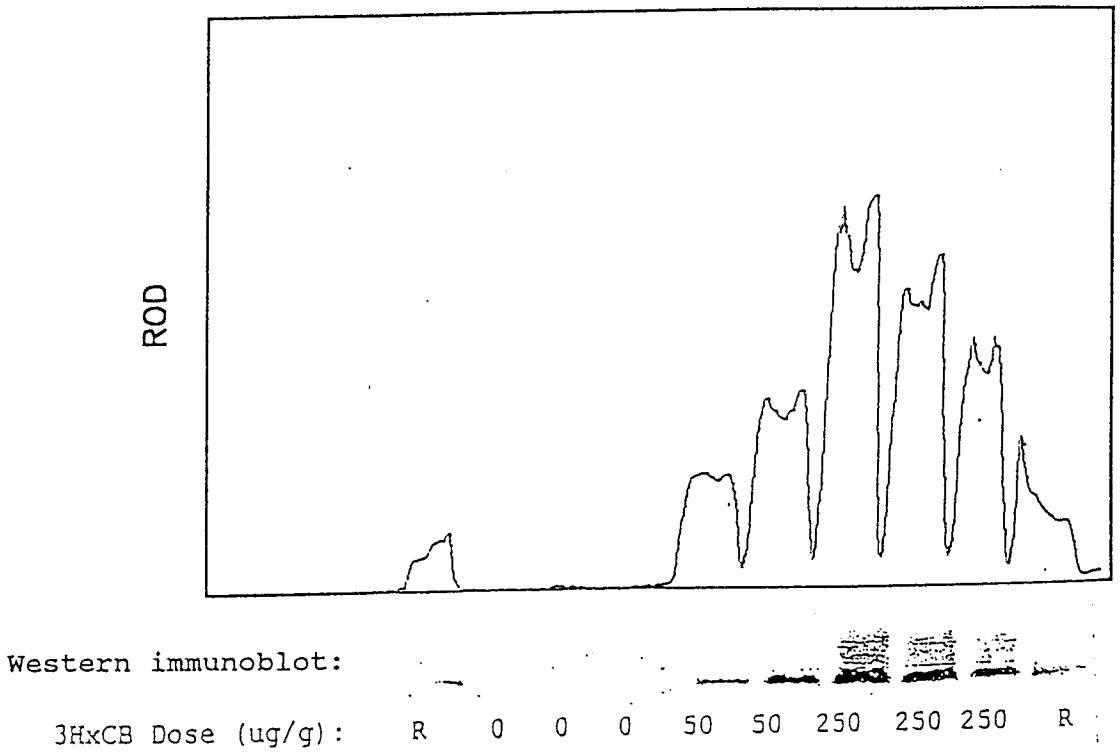

Figure 1.2. Western immunoblot and ROD of hepatic microsomes four weeks after ip injection with 3HxCB (R=beta-naphthaflavone treated fish).

The mean ROD for the 50 ug 2HxCB/g treatment increased approximately 14-fold above controls. The mean ROD for 250 ug 2HxCB/g treatment were approximately 83-fold and 6-fold above controls and 50 ug 2HxCB/g treatment, respectively. Mean RODs were approximately 100 and 190-fold greater than controls for 5 and 25 ug 3HxCB/g treatments but there was no significant difference between 3HxCB treatments.

Hepatic CYP1A2 and CYP1A1 mRNA Levels

CYP1A2 mRNA was detected in all groups tested while CYP1A1 mRNA was detected in one fish (Table 1.2). 250 ug 2HxCB/g treatment increased mean CYP1A2 mRNA concentrations approximately 3-fold while 50 ug 2HxCB/g treated fish were not significantly different from controls. Mean CYP1A1 mRNA concentrations were unchanged with treatment.

Table 1.2. Hepatic CYP1A1 and CYP1A2 mRNA (ng mRNA/ug total RNA) induction after 2HxCB Treatment.

2HxCB (ug/g)(n=3):	0	50	250
CYP1A1:	ND	ND	0.045±0.045
CYP1A2:	0.025±0.005	0.040±0.025	0.086±0.010*

ND below detection limit
*Significantly higher than controls

Correlations between EROD Activity, CYP1A1/1A2, and ROD

Simple linear regression (SLR) was used to express EROD activity as a function of ROD for 2HxCB and 3HxCB treated fish. There was a linear correlation for 2HxCB but not 3HxCB treated fish (Figure 1.3).

EROD activity and ROD for 2HxCB treated fish were expressed individually as a function of CYP1A2 or CYP1A1 mRNA using SLR. There was a significant correlation between EROD activity and CYP1A2 mRNA concentration (Figure 1.4) and between ROD and CYP1A2 mRNA (Figure 1.5) but not with CYP1A1 (results not shown).

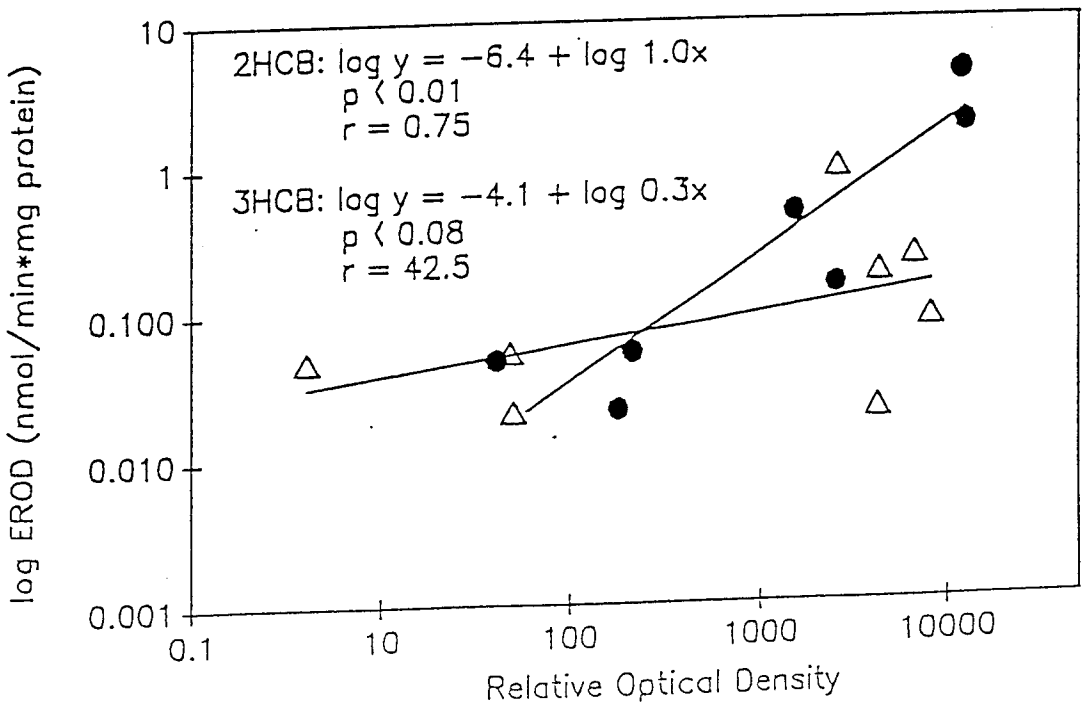

Figure 1.3. Simple linear regression of hepatic microsomal log EROD activity as a function of Western immunoblot ROD for 2HxCB (●) and 3HxCB (△) treated fish.

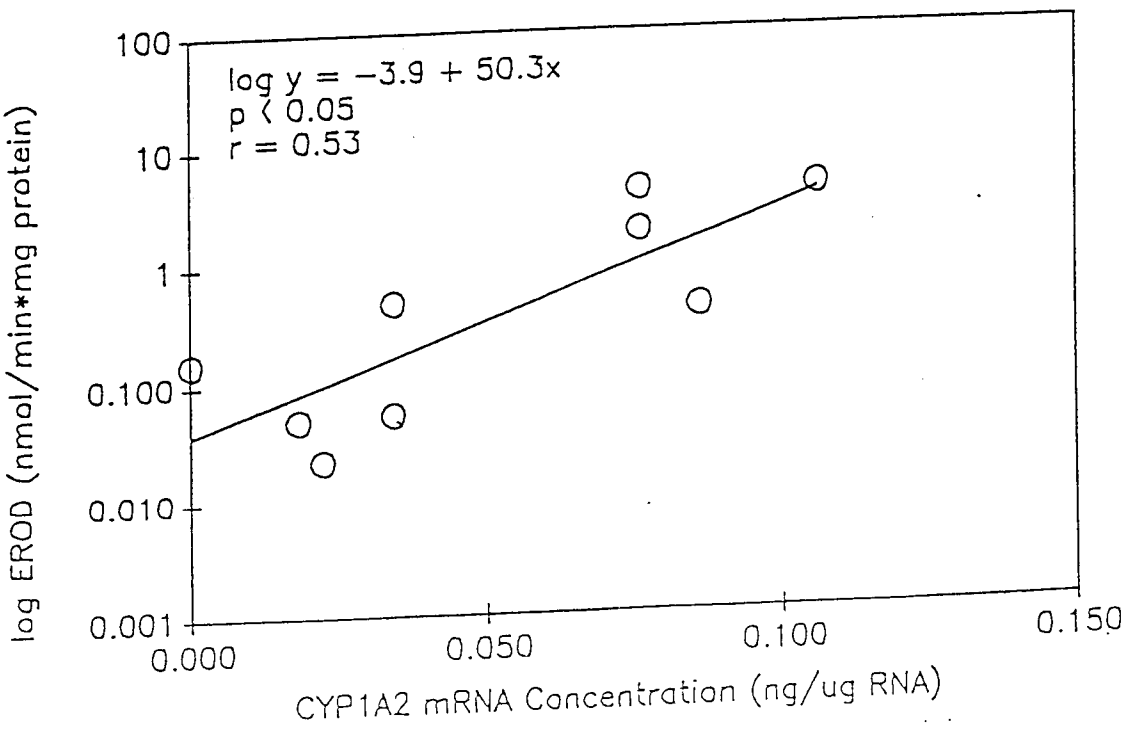

Figure 1.4. Simple linear regression of hepatic log EROD activity as a function of CYP1A2 mRNA concentration for 2HxCB treated fish.

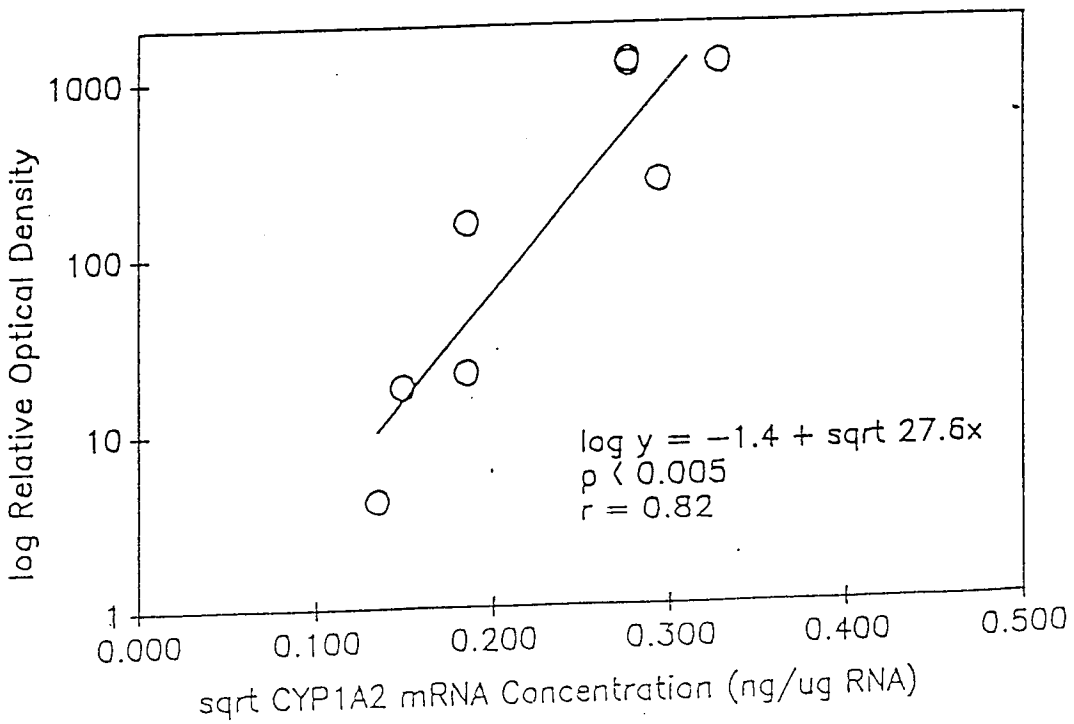

Figure 1.5. Simple linear regression of hepatic microsomal Western immunoblot log ROD as a function of square root (sqrt) CYP1A2 mRNA for 2HxCB treated fish.

DISCUSSION

The results of this study demonstrated that hepatic CYP1A protein and EROD were induced in rainbow trout treated with 2HxCB or 3HxCB.

Surprisingly, 2HxCB treated fish had approximately 10-times greater EROD activity and double the hepatic microsomal Western immunoblot ROD than the 3HxCB treated fish. The EROD activity for 3HxCB 25 mg/kg treated fish was unchanged and CYP1A ROD was elevated indicating inhibition of CYP1A mediated metabolism. 3HxCB inhibited the EROD activity, while CYP1A mRNA was increased and CYP2K1 mRNA was unchanged, for rainbow trout receiving an ip injection of 3HxCB and sampled 12 weeks later (Donohoe et al., 1995). Other Ah receptor agonists such as 3,3′4,4′-tetrachlorobiphenyl and beta naphthaflavone have been reported to inhibit EROD activity in fish (Gooch et al., 1989; Haasch et al., 1993).

CYP1A1 and CYP1A2 mRNA analysis was performed on hepatic tissues of fish treated with purified 2HxCB to verify the EROD and CYP1A protein data. The expression of CYP1A2 mRNA increased in trout treated with the purified 2HxCB confirming the EROD and CYP1A protein results. CYP1A1 and CYP1A2 were identified in mammalian species (Jaiswal et al., 1985; Ikeya et al., 1989; Gonzalez et al., 1985; Strom et al., 1992). CYP1A1 was considered the ancestral CYP1A gene and since mammalian and fish divergence was thought to have occurred

prior to the development of CYP1A2, the working hypothesis was that fish would have one CYP1A gene (Nebert and Gonzalez, 1987; Jaiswal et al., 1985). Recently, Berndston and Chen 1994, described the nucleotide sequence for two CYP1A genes in rainbow trout which they identified as CYP1A1 and CYP1A2. There has been disagreement over the nomenclature (Nelson et al., in press) and whether the CYP1A2 gene identified was the same as in mammalian species or a cytochrome P450 unique to rainbow trout (Morrisson et al., 1995).

EROD activity and CYP1A protein ROD were positively correlated with CYP1A2 mRNA levels in 2HxCB treated trout. The 2HxCB induction of CYP1A2 mRNA and EROD in rainbow trout were consistent with CYP1A2 isozyme and EROD induction in mice exposed to 2HxCB (De Jongh et al., 1995). EROD activity has been associated with the CYP1A2 isozyme in mice treated with 2HxCB (De Jongh et al., 1995) and benzo(a)pyrene (Tsyrlov and Duzchak 1990). The primary antibody used for Western immunoblot in this study probably cross reacted with CYP1A1 and CYP1A2 isozymes. The lack of CYP1A1 mRNA in trout hepatic microsomes indicated that the ROD increase was due to the CYP1A2 isozyme.

The low toxicity of 2HxCB has been attributed to low Ah receptor affinity due to a noncoplanar conformation of the PCB congener (reviewed in: Safe, 1984; Safe 1990). However, CYP1A1 and CYP1A2 isozymes may be regulated by Ah receptor dependent (reviewed in Safe, 1994; Quattorchi et al., 1994) or

independent pathways (Sterling et al., 1994; Cook and Hodgson, 1986; Quattrochi et al., 1994). The model Ah receptor agonist 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induced CYP1A1 and CYP1A2 mRNA in rainbow trout (Curtis et al., 1996). Absence of CYP1A1 mRNA in 2HxCB treated fish indicates a non Ah receptor pathway for the 2HxCB induction of CYP1A2 or the induction of CYP1A1 was less sensitive than CYP1A2. The fact that the trout CYP1A2 gene was constitutively expressed, as was the human CYP1A2 gene (Quattrochi et al., 1994), suggested some regulatory similarity.

2HxCB induction of CYP1A activity in early studies (Forlin and Lidman, 1979) was attributed to dioxin contamination of the 2HxCB (James and Little, 1981). Subsequent studies with short term exposures of trout and carp to 2HxCB failed to establish induction of EROD and CYP1A (Klienow et al., 1990, van der Weiden et al., 1994). However, pregnant mink exposed to 2HxCB had increased embryo resorption and surviving embryos had decreased weight and size (Patnode and Curtis 1994). In addition, long term feeding of 2HxCB to rainbow trout increased EROD and AHH activity which was positively correlated with 2HxCB liver concentration (da Costa and Curtis, 1995) indicating that 2HxCB absorption and target organ dose were contributing to activity variability.

The major findings of this study were: 2HxCB ip injection induced hepatic CYP1A; physiological evidence of a trout hepatic CYP1A2 isozyme; and 3HxCB induction of hepatic CYP1A

and inhibition of catalytic activity. Long term fish studies may identify interactions between 2HxCB and traditional Ah receptor agonists.

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CHAPTER 2

2,2′,4,4′,5,5′- and 3,3′,4,4′,5,5′-Hexachlorobiphenyl Pretreatment Alters the Biliary and Hepatic Disposition of a Challenge Dose of 7,12-[^{3}H]Dimethylbenz[a]anthracene in Rainbow Trout (*Oncorhyncus mykiss*)

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ABSTRACT

Di-ortho PCBs and polycyclic aromatic hydrocarbons (PAHs) are prominent environmental contaminants and their interactions may be more significant than previously thought. Four weeks after ip injection with 50 or 250 ug 2,2′,4,4′,5,5′-hexachlorobiphenyl (2HxCB)/g or 5 or 25 ug 3,3′,4,4′,5,5′-hexachlorobiphenyl (3HxCB)/g, rainbow trout were ip injected with 10 nmols [^{3}H]7, 12-dimethylbenz[a]-anthracene (DMBA)/g. Livers and gallbladders were removed 16 hours later. There was a subtle increase in biliary excretion of [^{3}H]DMBA for 2HxCB and 3HxCB treated fish. In a second experiment, rainbow trout were fed 60 or 220 ng 2HxCB/g fish/day or 1.9 or 7.8 ng 3HxCB/g fish/day for 4, 8, or 12 weeks. After these treatments fish were ip injected with 10 nmols [^{3}H]DMBA/g. Sixteen hours later liver, bile, mesenteric fat, stomach, muscle, kidney, and plasma were sampled. Fish fed 220 ng 2HxCB/g fish/d for four weeks and 60 or 220 ng 2HxCB/g fish/d for eight weeks had greater biliary concentrations of [^{3}H]DMBA than controls. Biliary [^{3}H]DMBA increased for fish fed 7.8 ng 3HxCB/g fish/d for four weeks. Cytochrome P450 1A induction may partially explain di-ortho PCB altered [^{3}H]DMBA biliary excretion. (Supported by USPHS grant ES05543).

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) are two classes of environmental contaminants which have been found in sediments from lakes, rivers and estuaries (Bolton, et al., 1985). PAHs and PCBs co-occur in the aquatic environment because of similar physical and chemical characteristics such as hydrophobicity, lipophilicity, and resistance to biological and chemical degradation, which affect their fate and transport. Carcinogenic PAHs such as 7,12-dimethylbenz[a,h]-anthracene (DMBA) (Bieri et al., 1986), benzo[a]pyrene, and phenanthrene were detected in sediments collected from natural waters (Curtis et al., 1993). Commercial PCBs contained complex mixtures of di-ortho and non-ortho congeners (Albro et al., 1981) with the di-ortho non coplanar, 2,2′,4,4′,5,5′-hexachlorobiphenyl (2HxCB) detected most frequently and the non-ortho coplanar 3,3′,4,4′,5,5′-hexachlorobiphenyl (3HxCB) rarely detected in environmental samples (McFarland and Clark 1989). Exposure of PAHs and subsequent toxic metabolite formation represent a threat to the health of fish species.

PAHs have been identified as carcinogenic to wild and laboratory fish. PAHs in sediments were associated with hepatic tumor incidence in fish collected from marine waters (Malins et al., 1985a; Malins et al., 1985b; Malins et al., 1987) and rainbow trout hatched from eggs treated with water-borne DMBA had increased incidence of hepatic tumors 9 months

after exposure (Fong et al., 1993). The classic PAH carcinogenesis model consists of parent PAH metabolized to a reactive intermediate which can bind with nucleic acids of DNA to form adducts, mutations, and eventual neoplasia. Reactive metabolites can be formed by cytochrome P450 metabolism. DMBA is a weak carcinogen that can be metabolized by the cytochrome P450 and epoxide hydrolase systems to potent carcinogenic diols such as 7-hydroxymethyl-12-methylbenz[a]anthracene and 3,4-dihydrodiol dimethylbenz[a]anthracene (Yang and Dower, 1975; DiGiovanni, et al., 1983). The latter has been identified in the bile of rainbow trout treated with DMBA (Schnitz et al., 1993).

2HxCB exhibited low biological activity and toxicity which was attributed to low Ah-receptor affinity due to a noncoplanar conformation (reviewed in: Safe 1990; Safe 1994). Low Ah-receptor affinity was supported by absence of EROD induction in fish exposed for short durations to 2HxCB (van der Weiden et al., 1994; Kleinow et al., 1990). However, long term exposures of fish to 2HxCB increased EROD activity which was correlated with increased expression of cytochrome P4501A2 mRNA (CYP1A2) (Foster et al., 1996). CYP1A2 induction may be regulated through Ah- or non Ah-receptor mechanisms (Quattrochi et al., 1994; Sterling et al., 1994; Cook and Hodgson, 1986). PCB induction of the cytochrome P450 system, reflected by increased EROD activity, may increase PAH metabolism affecting disposition and ultimately toxicity.

Pre-exposure to commercial PCB mixtures or PAHs alter PAH metabolite formation, but effects of individual PCB congeners have not been extensively investigated in fish. Benzo(a)pyrene metabolism by the hepatic S9 fraction from mullet pretreated with Aroclor 1254 (Tan et al., 1981) and from brown bullhead, black bullhead, and goldfish pre-treated with 3-methylcholanthrene increased (Swain and Melius, 1984). In addition, pretreatment of rainbow trout with non EROD inducing organochlorine altered the disposition of a challenge dose. Pre-exposure of fish to dieldrin altered the hepatic and biliary concentrations of a [^{14}C]dieldrin challenge dose without an increase in hepatic EROD activity (Shubat and Curtis, 1986; Gilroy et al., 1993).

This work was initiated to study the interaction of persistent environmental contaminants. The study compared the effects of 2HxCB and 3HxCB on [^{3}H]DMBA disposition in rainbow trout.

METHODS

Experimental Animals

Immature Shasta strain rainbow trout (15-60 g) were obtained from the Food Toxicology & Nutrition Laboratory, Oregon State University. Two fish receiving the same pretreatment dose of PCB were kept in aerated glass aquaria (61x32x20 cm; 23 L total volume), separated by a partition, and received a continuous flow of well water (100 ml/min @

14±2°C). Fish administered the PCB in food were fed a 5% body weight (dry wt fish/dry wt diet) ration of Oregon Test Diet (OTD)/day given as three feedings per week. Fish ip injected with PCBs were fed a 3% body weight (dry wt fish/dry wt diet) ration of OTD/day given as three feedings per week. Fish weights were measured every four weeks for fish fed PCBs and weekly for ip injected fish with rations adjusted accordingly. Tank debris was removed 3 times/week. A 12:12 hr light:dark cycle was maintained throughout the study.

Chemicals

Fish were treated with 2HxCB (Accustandard, New Haven CT) that was >99% pure and had <1% mono-ortho PCB detected by gas chromatograph/mass spectrometry (da Costa and Curtis, 1995). No other PCBs, dioxins, or dibenzofurans were detected. 3HxCB (Accustandard, New Haven CT) purity was >99%. [^{3}H]DMBA (Amersham Corp., Arlington Heights IL) (specific activity 63 Ci/mMol) and DMBA (Aldrich Chemical Co., Milwaukie WI) cleanup were performed according to DePierre et al., 1975.

[^{3}H]DMBA Time Course

Fish were ip injected with 10 nmols [^{3}H]DMBA, and 4, 8, 16, 18, and 32 hrs later bile, liver, mesenteric fat, kidney, plasma, and stomach were sampled. Whole tissue or subsamples of 4:1 water:tissue homogenates were solubilized in Soluene 350 (Packard, Downers Grove IL) and [^{3}H]DMBA was measured using a Packard 1600CA TriCarb liquid scintillation analyzer

and Ultima Gold scintillation cocktail (Packard, Downers Grove IL).

[^{3}H]DMBA Disposition After PCB Pretreatment

Fish were given a single ip injection of 50 or 250 ug 2HxCB/g or 5 or 25 ug 3HxCB/g in stripped menhaden oil (5 ml/kg) and sampled four weeks later. Controls received menhaden oil only. In another experiment, fish were fed a diet of 0, 60 or 220 ng 2HxCB/g-fish/day or 1.9 or 7.8 ng 3HxCB/g-fish/day administered three times per week for 4, 8, and 12 weeks. Following PCB treatment fish were ip injected with 10 nmols [^{3}H]DMBA/g fish and sampled 16 hrs later. Bile, liver, mesenteric fat, stomach, skeletal muscle, kidney, and plasma were sampled from fish fed PCBs while bile and liver were sampled from PCB ip injected fish. Tissues were prepared and analyzed for [^{3}H]DMBA as described above.

Data analysis

Difference between treatments were detected using 1-way ANOVA and Least Significant Difference multiple comparison test (one-tailed $p<0.05$) unless otherwise specified.

RESULTS

[^{3}H]DMBA Deposition in Bile and Liver

[^{3}H]DMBA in bile and liver increased with time and contained less than 3.0% and 0.7% of the [^{3}H]DMBA administered dose, respectively (Table 2.1) SLR was used to express

[^{3}H]DMBA as a function of time. There was a significant positive correlation of [^{3}H]DMBA with time for biliary and liver nmols, nmols/g tissue, and percent dose (Table 2.1). Kidney, mesenteric fat, plasma, and stomach [^{3}H]DMBA were not correlated with time (data not shown).

PCB Altered [^{3}H]DMBA Deposition in Bile and Liver

Fish ip injected with 50 ug 2HxCB/g or 25 ug 3HxCB/g had a subtle decrease in biliary concentration of [^{3}H]DMBA (Table 2.2). The mean biliary concentration of [^{3}H]DMBA (% dose/g) for fish fed 220 ng 2HxCB/g fish/d for four weeks was approximately 3-times greater, while fish fed 60 and 220 ng 2HxCB/g fish/d was approximately 1.5-times greater than controls, respectively (Table 2.3). Mean hepatic [^{3}H]DMBA (% dose/g) approximately doubled in fish fed 220 ng 2HxCB/g fish/d for four weeks (Table 2.3). Fish fed 7.8 ng 3HxCB/g fish/d for four weeks had increased biliary [^{3}H]DMBA (% dose/g) with no change in hepatic tissues (Table 2.4). The LSI for 3HxCB fed fish increased in fish treated at eight and twelve weeks for fish fed 7.8 and 1.9 ng 3HxCB/g fish/d, respectively while there was no change in LSI for 2HxCB fed

Table 2.1. Mean biliary and liver [^{3}H]DMBA equivalents 4 to 32 hours after ip injection with 10 nmols [^{3}H]DMBA/g fish.

Time (hours)	4	8	16	18	32	a	b	p	r^2
nmols in bile:	0.44 (0.14)	0.80 (0.24)	2.49 (0.59)	2.44 (0.90)	4.14 (0.88)	-5.33	3.95	<0.000	79.7
nmols/g bile:	13.72 (5.18)	25.88 (3.50)	56.75 (17.03)	61.21 (8.02)	123.87 (14.51)	-0.05	0.13	<0.000	57.9
% dose in bile:	0.28 (0.09)	0.45 (0.09)	1.61 (0.32)	1.19 (0.28)	2.17 (0.24)	0.05	0.07	<0.000	69.0
nmols in liver:	0.59 (0.17)	0.51 (0.09)	0.61 (0.05)	0.93 (0.93)	1.17 (0.22)	2.78	0.11	<0.002	42.4
nmols/g liver:	3.85 (0.91)	3.40 (0.22)	3.91 (0.21)	4.95 (0.26)	6.75 (1.19)	0.40	0.02	<0.005	39.2
% dose in liver:	0.35 (0.08)	0.30 (0.02)	0.41 (0.06)	0.51 (0.02)	0.62 (0.07)	0.26	0.01	<0.001	51.6

Results are means of % dose (SE) for n=4. a = intercept, b = slope, and p = two-tailed p-value are results of simple linear regression.

Table 2.2. Mean biliary and hepatic tissue [^{3}H]DMBA equivalents 16 hours after ip injection with 10 nmols [^{3}H]DMBA/g fish that were ip injected with 50 or 250 ug 2HxCB/g fish or 5 or 25 ug 3HxCB/g fish four weeks earlier.

2HxCB ug/g:	0	50	250		
3HxCB ug/g:	0			5	25
nmols in bile:	6.52 (0.9)	6.44 (1.2)	8.68 (1.0)	8.32 (0.9)	6.79 (1.9)
nmols/g bile:	135.6 (32.1)	72.8[1] (15.5)	95.2 (15.0)	100.6 (20.1)	56.7[2] (12.4)
nmols in liver:	2.34 (0.3)	1.86 (0.3)	2.24 (0.3)	2.16 (0.3)	1.77 (0.5)
nmols/g liver:	7.19 (0.9)	5.41 (0.7)	5.53 (1.0)	5.31 (1.0)	8.65 (4.6)
LSI:	0.67 (0.02)	0.69 (0.02)	0.79* (0.05)	0.80* (0.03)	0.90* (0.07)

*Different from control at a one-tailed p-value<0.05.
[1]Different from control at a one-tailed p-value<0.09.
[2]Different from control at a one-tailed p-value<0.06.
Results are mean [^{3}H]DMBA equivalents (SE) for n=6 except for 25 ug 3HxCB/g n=5 for liver and n=4 for bile.

Table 2.3. Mean biliary and hepatic tissue [^{3}H]DMBA equivalents 16 hours after ip injection with 10 nmols [^{3}H]DMBA/g fish that were fed 60 or 220 ng 2HxCB/g fish/d for four, eight, or twelve weeks.

Week:		4		8		12	
2HxCB ng/g/d:	0	60	220	60	220	60	220
% dose in bile:	2.93 (0.38)	2.37 (0.50)	3.88 (2.11)	3.98 (0.38)	2.23 (0.64)	2.29 (0.60)	3.09 (0.21)
% dose/g bile:	52.03 (7.28)	63.14 (13.8)	150.95* (98.9)	90.58* (9.94)	79.84* (9.24)	37.09 (10.7)	55.88 (14.2)
% dose in liver:	0.73 (0.04)	0.74 (0.16)	1.02 (0.13)	0.73 (0.09)	0.65 (0.06)	0.56 (0.09)	0.61 (0.06)
% dose/g liver:	3.82 (0.56)	6.05 (1.57)	8.15* (3.19)	4.28 (0.55)	4.52 (0.48)	2.74 (0.58)	2.39 (0.20)

*Significantly different from controls which are a grand mean of week 8 and week 12 controls as week 4 controls were not available. Results are mean % dose (SE) for: controls n=8; week 4 220 ng/g fish/d n=2; and, all others n=4.

Table 2.4. Mean biliary and hepatic tissue [^{3}H]DMBA equivalents 16 hours after ip injection with 10 nmols [^{3}H]DMBA/g fish that were fed 1.9 or 7.8 ng 3HxCB/g fish/d for four, eight, or twelve weeks.

Week:	4			8			12		
3HxCB ng/g/d:	0	60	220	0	60	220	0	60	220
% dose in bile:	2.10 (0.25)	2.62 (0.42)	2.16 (0.48)	1.38 (0.62)	2.22 (0.22)	2.65 (0.27)	1.51 (0.19)	2.05 (0.26)	2.00 (0.18)
% dose/g bile:	29.50 (4.94)	42.11 (6.17)	55.04* (7.67)	35.17 (25.2)	28.52 (3.85)	29.42 (6.76)	17.50 (4.03)	14.95 (1.84)	16.49 (1.47)
% dose in liver:	0.68 (0.10)	0.81 (0.11)	0.54 (0.16)	0.73 (0.22)	0.71 (0.16)	0.82 (0.10)	0.62 (0.19)	0.61 (0.05)	0.35 (0.05)
% dose/g liver:	3.32 (0.56)	3.40 (0.69)	2.58 (0.96)	3.45 (1.28)	2.94 (0.83)	2.81 (0.44)	1.87 (0.65)	1.83 (0.15)	0.93 (0.14)

*Significantly different from controls Results are mean % dose (SE) n=4 except for biliary controls for week 8 n=3.

fish (data not shown). There were no changes in kidney, mesenteric fat, plasma, stomach, or muscle [^{3}H]DMBA concentrations with PCB treatment (data not shown).

DISCUSSION

Previous work in this laboratory demonstrated decreased hepatic and increased biliary accumulation of [^{14}C]dieldrin following four weeks of dieldrin pretreatment of rainbow trout (Shubat and Curtis, 1986). Subsequent work described increased hepatic and biliary [^{14}C]dieldrin levels following 10 and 12 weeks of dieldrin pretreatment of rainbow trout without induction of hepatic CYP1A protein or EROD activity (Gilroy et al., 1993). Examination of other non Ah-receptor environmental xenobiotics and their effects on xenobiotic tissue disposition with prior treatment was warranted.

This study investigated the effect of 2HxCB or 3HxCB treatment on [^{3}H]DMBA biliary and liver disposition. The linear correlation of tissue [^{3}H]DMBA concentration with time following a single ip injection of [^{3}H]DMBA was used to select a time point for the PCB/[^{3}H]DMBA interaction experiments. The study demonstrated altered biliary and liver [^{3}H]DMBA disposition with 2HxCB treatment and increased hepatic [^{3}H]DMBA with 3HxCB treatment. Altered biliary and hepatic [^{3}H]DMBA disposition may be partially explained by increased levels of hepatic CYP1A2 protein.

Liver and bile results from the ip injection [^{3}H]DMBA time course experiments were comparable to the results reported for other exposure methods, PAHs, and environmental samples. The percent [^{3}H]DMBA administered dose, recovered from bile (<2%) or liver (<1%), 16 after ip injection were consistent with the results reported for fish orally exposed to DMBA (O'Connor, et al., 1988; Schnitz, et al., 1987). DMBA appears to be more slowly excreted than benzo(a)pyrene in rainbow trout. The mean [^{3}H]DMBA biliary concentration 32 hours after ip injection was 123.87 nmols/g, while bile from fish ip injected with 10 nmols [^{3}H]benzo[a]pyrene/g fish and then sampled 24 hours later had approximately 700 nmols [^{3}H]benzo[a]pyrene/g, respectively (Curtis et al., 1990). In addition, [^{3}H]DMBA liver concentrations in this study were approximately an order of magnitude higher than those found in brown trout (*Salmo trutta*) and white sucker (*Catastomus commersoni*) and approximately two orders of magnitude lower than phenanthrene muscle tissue levels for lamprey (collected from the Hershey River (McElroy et al., 1989)

Biliary [^{3}H]DMBA increased for fish fed 2HxCB or 3HxCB while liver [^{3}H]DMBA was increased in 2HxCB fed trout. Increased biliary deposition could be due to increased metabolism resulting in a change in the biliary metabolite profile. Aroclor 1254 treatment of mullet increased the production of 9, 10-dihydro-9,10-dihydroxybenzo[a]pyrene, 4,5-dihydro-4,5,-dihydroxybenzo[a]pyrene, 7,8-dihyrdo-7,8-

dihydroxybenzo[a]pyrene, and unidentified quinones in the hepatic S9 fraction incubated with benzo[a]pyrene (Tan et al., 1981). Biliary 3,4-dihydrodiol dimethylbenz[a]anthracene was identified from multiple doses and 2-hydroxy-DMBA and 3-hydroxy-DMBA were identified from a single dose of DMBA to rainbow trout (Schnitz et al., 1993).

The cytochrome P450 system is responsible for the primary oxidative metabolism of PAHs (Buhler and Williams, 1989), and DMBA metabolism would be expected to increase with induction of the system if metabolism was rate limiting. 2HxCB, a di-ortho non-co-planar PCB, did not increase EROD activity in fish exposed for a short duration (van der Weiden et al., 1994; Kleinow et al., 1990), and increased cytochrome P450 levels (Forlin and Lidman, 1979) were attributed to dioxin contamination of the 2HxCB (James and Little, 1981). Long term exposure of trout to 2HxCB increased hepatic CYP1A and CYP1A2 mRNA, CYP1A protein, EROD and AHH activities (Foster et al., 1996; da Costa and Curtis, 1995). 3HxCB increased hepatic CYP1A and CYP1A1 mRNA protein and EROD activity in trout (Donohoe et al., 1995).

Increased biliary excretion of [^{3}H]DMBA with 2HxCB treatment may be related to hepatic CYP1A2 induction. A second hepatic CYP1A isozyme identified as CYP1A2 was reported recently for trout (Berndston and Chen, 1994). Increased EROD and acetanilide 4-hydroxylation (ACOH) activities were positively correlated with expression of hepatic CYP1A2

protein in rats exposed to 2HxCB while EROD but not ACOH was correlated with CYP1A1 (De Jongh, et al, 1995).

Simple linear regression was used to express biliary and liver [^{3}H]DMBA, for 2HxCB and 3HxCB ip injected fish as a function of hepatic CYP1A protein, CYP1A2 mRNA, and EROD activity (Foster et al., 1996). There was a significant but low correlation between biliary [^{3}H]DMBA (nmols) and CYP1A2 mRNA for 2HxCB ip injected fish ($p<0.01$; $r^2=38\%$). In addition, [^{3}H]DMBA in bile and liver was poorly correlated with hepatic CYP1A protein levels and enzymatic activity for 3HxCB ip injected fish. Significant correlations were expected as 3HxCB increased hepatic CYP1A protein and EROD activity in trout, although high doses of 3HxCB inhibited EROD activity (Foster and Curtis, 1996; Donohoe et al., 1995). The lack of correlation may be due to other rate limiting processes.

Other physiological processes such as biliary excretion and not hepatic enzymatic activity were suggested as rate limiting for pentachlorophenol, hexachlorobenzene, and mirex in rainbow trout (Niimi and Palazzo, 1985) and for benzo(a)pyrene in spiny lobster (Little et al., 1985). Induction of cytosolic lipoprotein complexes or ligandin were suggested as possible mechanisms for the increased biliary excretion of a challenge dose of dieldrin for fish pretreated with dieldrin (Gilroy et al., 1993).

In summary, there was a low potency for 2HxCB or 3HxCB to alter the biliary and liver disposition of a challenge dose of [^{3}H]DMBA. The [^{3}H]DMBA altered disposition may only be partially explained by increased hepatic CYP1A protein levels and other factors may be important. Methylsulfone metabolites of tetra-, penta-, and hexachlorobiphenyls increased cytochrome P450 levels and the activities of 7-ethoxycoumarin and benzo[a]pyrene hydroxylase in rats (Kato et al., 1995). PCB metabolism was affected by the degree of chlorination with metabolite formation increasing with decreasing chlorination (Ghiasuddin et al., 1976). Therefore, PCBs with fewer chlorines than 2HxCB, such as a di-ortho tetrachlorobiphenyl, may have a greater effect on DMBA metabolism.

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CHAPTER 3

2,5,2′,5′-Tetrachlorobiphenyl Pretreatment Altered the Tissue Distribution of a Subsequent Dose of 2,5,2′,5′-[^{14}C]Tetrachlorobiphenyl or 7, 12-[^{3}H]Dimethylbenz[a]anthracene in Rainbow Trout (*Oncorhyncus mykiss*)

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ABSTRACT

2,5,2′,5′-tetrachlorobiphenyl (TCB), a di-ortho polychlorinated biphenyl (PCB), frequently co-occured with polycyclic aromatic hydrocarbons (PAHs) in contaminated aquatic environments. Rainbow trout received an intraperitoneal (ip) injection of 0, 0.5 or 5.0 ug TCB/g fish 14, 28, and 42 days before ip injection with 10 nmol [^{14}C]TCB/g fish. After 24 hours liver, bile, fat, muscle, kidney, plasma, pharyngeal tissue, and carcass were radioassayed. Average muscle and pharyngeal tissue tissue [^{14}C]TCB concentrations (% retained dose/g tissue) increased approximately 3-fold 28 days after pretreatment with 5 ug TCB/g fish while carcass levels decreased. Hepatic microsomal ethoxyresorufin-O-deethylase (EROD) activities were unchanged for fish 28 days after treatment with 5 ug TCB/g fish and there was no evidence of saturation in biliary, hepatic, or fat tissues with increasing dose of [^{14}C]TCB. Total hepatic [^{3}H]7, 12-dimethylbenz[a]-anthracene (DMBA) (nmols) decreased 16 hrs after ip injection with 10 nmols [^{3}H]DMBA/g for fish pretreated with 5.0 ug TCB/g fish. A time course experiment where fish received a single ip injection of [^{14}C]TCB and were sampled at day 1, 3, 7, 14, and 28 thereafter with subsequent solvent fractionation of muscle tissue showed increasing [^{14}C]TCB in the DMSO solvent fraction through day 28. Altered tissue distribution was unrelated to hepatic EROD activity.

INTRODUCTION

The exposure of aquatic organisms to complex mixtures of organic contaminants commonly occurs in polluted environments as evidenced by the co-occurrence of PCBs and PAHs in sediments (Bolton et al., 1985) and chlorinated pesticides, PCBs, dioxins, and furans in fish tissues (Curtis et al., 1993). Bioaccumulation of these compounds in fish represent a potential threat to natural populations and to humans and other animals consuming contaminated fish. The interaction of complex mixtures of arylhydrocarbon (Ah) receptor agonists, such as 2,3,7,8 chlorine substituted dioxins and furans and non-ortho and mono-ortho PCBs, have been studied in fish and mammalian species (Zabel et al., 1995; reviewed in Safe 1994). Di-ortho PCBs, which occur with greater frequency and in higher concentrations in environmental samples have been less extensively studied. However, lake trout (*Salvelinus namaycush*) and chinook salmon (*Oncorhyncus tshawytscha*) sac fry mortality increased when exposed to a water-borne di-ortho PCB, 2,4,5,2′,4′,5′-hexachlorobiphenyl (2HxCB), at a water concentration of 5 ug/l (Broyles and Noveck, 1979). In addition, long term exposure of rainbow trout (*Oncorhyncus mykiss*) to 2HxCB increased hepatic arylhydrocarbon hydroxylase and EROD activities and cytochrome P450 1A2 mRNA levels (da Costa and Curtis, 1995; Foster et al., 1996) while pretreatment of rainbow trout with 2HxCB altered the hepatic

disposition of a subsequent dose of [^{3}H]DMBA (Foster and Curtis, 1996).

Although highly chlorinated PCBs were considered to be resistant to metabolism with minimal metabolite formation (reviewed in Safe 1989), PCB methylsulfonyl metabolites were identified in tissues from humans, other mammals, and fish (Bergman et al., 1994; Haraguchi et al., 1989). Some methylsulfonyl metabolites have biological activity and can bind with cellular macromolecules (Lund et al., 1985; Larsen et al., 1991). The methylsulfonyl metabolites of 2,3′,4′,5-tetrachlorobiphenyl and 2,2′,3′,4′,5- and 2,2′,4,′,5,5′-pentachlorobiphenyl increased 7-ethoxycoumarin O-deethylase and benzo[a]pyrene hydroxylase activities in rat liver (Kato et al., 1995). PCB metabolite formation was related to degree of chlorination with decreasing metabolism as chlorination increased (Ghiasuddin et al., 1976). Therefore, TCB may have higher potency than 2HxCB on physiological processes and tissue distribution of xenobiotics.

This work was initiated to study the interaction of persistent environmental contaminants. The effect of 2,2′,5,5′-tetrachlorobiphenyl (TCB) pretreatment on the disposition of a subsequent dose of [^{14}C]TCB or [^{3}H]7, 12-dimethylbenz[a]anthracene (DMBA) was measured in rainbow trout.

METHODS

Experimental Animals

Immature Shasta strain rainbow trout (15-60 g) were obtained from the Food Toxicology & Nutrition Laboratory, Oregon State University. Two fish receiving the same PCB pretreatment were kept in aerated glass aquaria (61x32x20 cm; 23 L total volume), separated by a partition, and received a continuous flow of well water (100 ml/min @ 14±2°C). Fish were fed a 2% body weight (dry wt fish/dry wt diet) ration or Oregon Test Diet/day administered in 3 feedings per week. Fish weights were measured weekly with rations adjusted accordingly. Tank debris was removed 3 times/week. A 12:12 light:dark cycle was maintained throughout the study. Fish treated with radiolabel were placed in static aerated glass aquaria with a charcoal filter. Fish were fasted 48 hrs prior to treatment. Fish for the [^{14}C]TCB time course experiment were fed ab libitum on day 4.

Chemicals

Fish were treated with TCB (Accustandard, New Haven CT) that was >98% pure. No PCBs, dioxins, or dibenzofurans were detected by gas chromatography/mass spectrometry. [^{14}C]TCB was obtained from Sigma Chemical Co., St. Louis, MO (12.2 mCi/mMol). [^{3}H]DMBA (Amersham Corp., Arlington Heights IL) (specific activity 63 Ci/mMol), and DMBA (Aldrich Chemical

Co., Milwaukie WI) with cleanup performed according to DePierre et al. (1975).

[^{14}C]TCB Time Course

Fish were fasted for seven days and then received a single ip injection of 10 nmol [^{14}C]TCB/g fish and liver, bile, mesenteric fat from around the intestine and stomach, plasma, skeletal muscle, and pharyngeal tissues containing thyroid cells were sampled on day 1, 3, 7, 14, and 28. Whole tissues or subsamples of 4:1 water:tissue homogenates (with the exception of muscle tissue which was solvent fractionated) were solubilized in Soluene 350 (Packard, Downers Grove IL) and [^{14}C]TCB was measured using a Packard 1600A Tricarb liquid scintillation analyzer and Ultima Gold scintillation cocktail (Packard, Downers Grove IL).

[^{14}C]TCB Solvent Fractionation

Muscle tissue from the [^{14}C]TCB time course experiment were solvent fractionated according to Bergman et al., 1992 with some modification. Briefly, muscle tissue was homogenized with 80:20 hexane:acetone with a polytron. Homogenate was vortexed, centrifuged, and hexane phase decanted.

Fractionation for PCB-OH: The hexane phase was extracted with an aqueous solution of 0.5M KOH and Ethanol:water (95:5). The aqueous phase was decanted and the procedure was repeated two more times. The aqueous phase was washed with hexane

which was combined with the extracted hexane phase. The aqueous phase was acidified with HCl (6M), extracted 5 times with MTBE:hexane (1:9), solvent evaporated and lipid weight determined. Lipid was resuspended in acetone and a subsample was counted by liquid scintillation as described above.

Fractionation for Methylsulfonyl PCB: The hexane phase was washed with HCl (1M and EtOH (95%) and extracted 3-times with anhydrous DMSO. The DMSO phase was washed with hexane 3-times with the hexane returned to the extracted hexane phase. The DMSO phase was diluted with water and then extracted 3-times with MTBE:hexane (1:9). Solvents were evaporated, and lipid weight was determined. Lipid was resuspended in acetone and a subsample was counted by liquid scintillation as described above.

Fractionation for PCB: The hexane phase was evaporated and lipid weight determined. Lipid was resuspended in acetone and a subsample counted by liquid scintillation as described above.

PCB Pretreatment

Fish received a single ip injection of 0, 0.5, or 5.0 ug TCB/g fish in stripped menhaden oil (5 ml/kg) obtained from the Fish Oils Test Materials Program. Controls received menhaden oil only.

[14C]TCB Altered Distribution

14, 28, and 42 days after ip injection with TCB fish were

ip injected with 10 nmols [^{14}C]TCB/g fish 24 hrs later, bile, liver, mesenteric fat, kidney, skeletal muscle, stomach, pharyngeal tissue, plasma, and carcass were sampled. Tissues were prepared as described for the [^{14}C]TCB time course experiment. Fish were fasted for 24 hrs prior to ip injection with [^{14}C]TCB.

[^{3}H]DMBA Altered Distribution

7, 14, or 29 days after ip injection with 0, 0.5, or 5.0 ug TCB/g, fish were ip injected with 10 nmols [^{3}H]DMBA/g fish. 16 hrs later, bile, liver, and mesenteric fat were sampled. Tissue were treated as described for the [^{14}C]TCB time course. Fish were fasted for 24 hrs prior to ip injection with [^{14}C]TCB.

[^{14}C]TCB Saturation

3 days after fish were ip injection with 0, 2.5, 5, 10, 20, and 40 nmols [^{14}C]TCB/g fish, bile, liver, and mesenteric fat were sampled with tissues treated as described for the [^{14}C]TCB time course. Fish were fasted 48 hrs prior to PCB treatment and were not fed through the experiment.

Microsomal preparation and biochemical analysis

7 and 14 days after ip injected with 0, 0.5, or 5.0 ug TCB/g fish microsomes were prepared according to Carpenter et al. 1990 and stored at -80°C until use. Microsomal protein content was measured according to Lowry et al. 1951. EROD

activity was measured as described earlier (Prough et al., 1978; Burke and Mayer 1974). Microsomes were prepared from pooled livers from two fish of the same treatment.

[^{14}C]TCB microsomal incubation

Microsomal incubations were performed in duplicate as described by Williams et al., 1989 with modifications. Briefly, microsomes (0.5 mg protein) were incubated with 0.5M [^{14}C]TCB, 1 unit glucose-6-phosphate dehydrogenase, 0.1M KPO4, 5mM MgCl2, and 5mM glucose-6-phosphate to a final volume of 1 ml and incubated at 25°C for 3 minutes at which time 1mM NADPH was added. The mixture was incubated at 25°C for 90 minutes with shaking (100 cycles/min). The reaction was terminated by adding MeOH and rapid cooling. The mixture was centrifuged and the MeOH phase collected for HPLC fractionation and liquid scintillation counting. MeOH, MeOH:acetone, MeOH:acetonitrile, acetone, acetone:acetonitrile, and acetonitrile solvent gradients were used with a SpectraPhysics SP8800 ternary HPLC with Phenomenex Bondex 10 C18 column (300 X 3.9 mm).

Data analysis

Difference between treatments for the same time point and between controls for different time points were detected using 1-way ANOVA and Least Significant Difference multiple comparison test (one-tailed $p<0.05$) unless otherwise specified. The Dixon outlier test (two-tailed p-value<0.01)

was used and resulted in the removal of data for a single fish in the control day 14 TCB pretreated fish that received a subsequent dose of [^{14}C]TCB. Simple linear regression (SLR) was used to express[^{14}C]TCB as a function of time.

RESULTS

[^{14}C]TCB Tissue Distribution and Solvent Fractionation

[^{14}C]TCB concentration in liver, plasma, and muscle increased linearly with time while biliary and fat concentration plateaued after day 3 and for pharyngeal tissue after day 14 (Table 3.1). SLR was used to express [^{14}C]TCB as function of time. There was a significant positive correlation of nmols [^{14}C]TCB/g tissue with time for liver and plasma and for nmols [^{14}C]TCB/g lipid for muscle (Table 3.1). The largest percentage of the administered dose was found in muscle (4.6%) on day 28 and fat (1.2%) on day 3 while liver, bile, and pharyngeal tissue contained less than 1% at all time points (Figure 3.1).

The hexane fraction contained higher [^{14}C]TCB concentrations than the DMSO fraction for all time points except day 7. The [^{14}C]TCB concentration (nmols/g lipid) in both the hexane and DMSO fractions increased with time (Table 3.2). [^{14}C]TCB was not detected in the acidic fraction with the exception of two samples which had trace levels of [^{14}C]TCB (data not shown).

Table 3.1. Mean [^{14}C]TCB equivalents (nmols and nmols/g) in selected tissues 1 to 28 days following ip injection with 10 nmols [^{14}C]TCB/g fish.

Time	bile		liver		muscle		pharyngeal		plasma		fat	
(day)	nmols	nmols/g	nmols	nmols/g	nmols	nmols/g*	nmols	nmols/g	nmols	nmols/g	nmols	nmols/g
1	0.47 (0.05)	16.78 (3.03)	0.27 (0.01)	1.85 (0.10)	3.03 (0.41)	17.42 (4.03)	0.26 (0.03)	1.38 (0.11)	0.06 (0.01)	0.45 (0.09)	1.43 (0.31)	103.8 (34.9)
3	0.86 (0.26)	22.42 (2.18)	0.32 (0.01)	2.22 (0.11)	4.49 (0.32)	29.33 (8.67)	0.50 (0.08)	2.63 (0.39)	0.03 (0.01)	0.24 (0.03)	2.36 (0.33)	154.3 (27.1)
7	0.33 (0.18)	10.17 (4.52)	0.34 (0.09)	1.96 (0.49)	5.36 (1.52)	15.76 (5.39)	0.63 (0.16)	2.98 (0.66)	0.05 (0.01)	0.45 (0.14)	1.99 (0.89)	153.8 (58.3)
14	0.85 (0.10)	20.29 (3.71)	0.59 (0.09)	4.18 (0.57)	8.78 (1.32)	30.44 (7.41)	0.81 (0.12)	4.61 (0.64)	0.11 (0.03)	0.65 (0.10)	2.17 (0.86)	177.4 (52.3)
28	0.75 (0.13)	24.70 (2.80)	0.86 (0.11)	7.34 (0.48)	9.25 (1.92)	72.31 (35.33)	0.46 (0.12)	3.45 (0.92)	0.14 (0.02)	1.18 (0.30)	0.88 (0.44)	129.3 (48.8)
a	0.39	11.09	0.17	0.90		8.88		1.60		0.20		98.1
b	0.02	0.53	0.03	0.23		2.11		0.10		0.03		2.45
p	<0.06	<0.02	<0.00	<0.00		<0.00		<0.00		<0.00		<0.23
R^2	15.9	23.9	74.3	87.2		35.0		32.0		58.8		6.6

*nmols per gram of lipid. (SE) n=4 except for bile day 28 n=3. Average [^{14}C]TCB at day 0 <0.01.
a = intercept, b = slope, p = p-value, R^2 = adjusted r^2 for linear regression model y = a + bx.

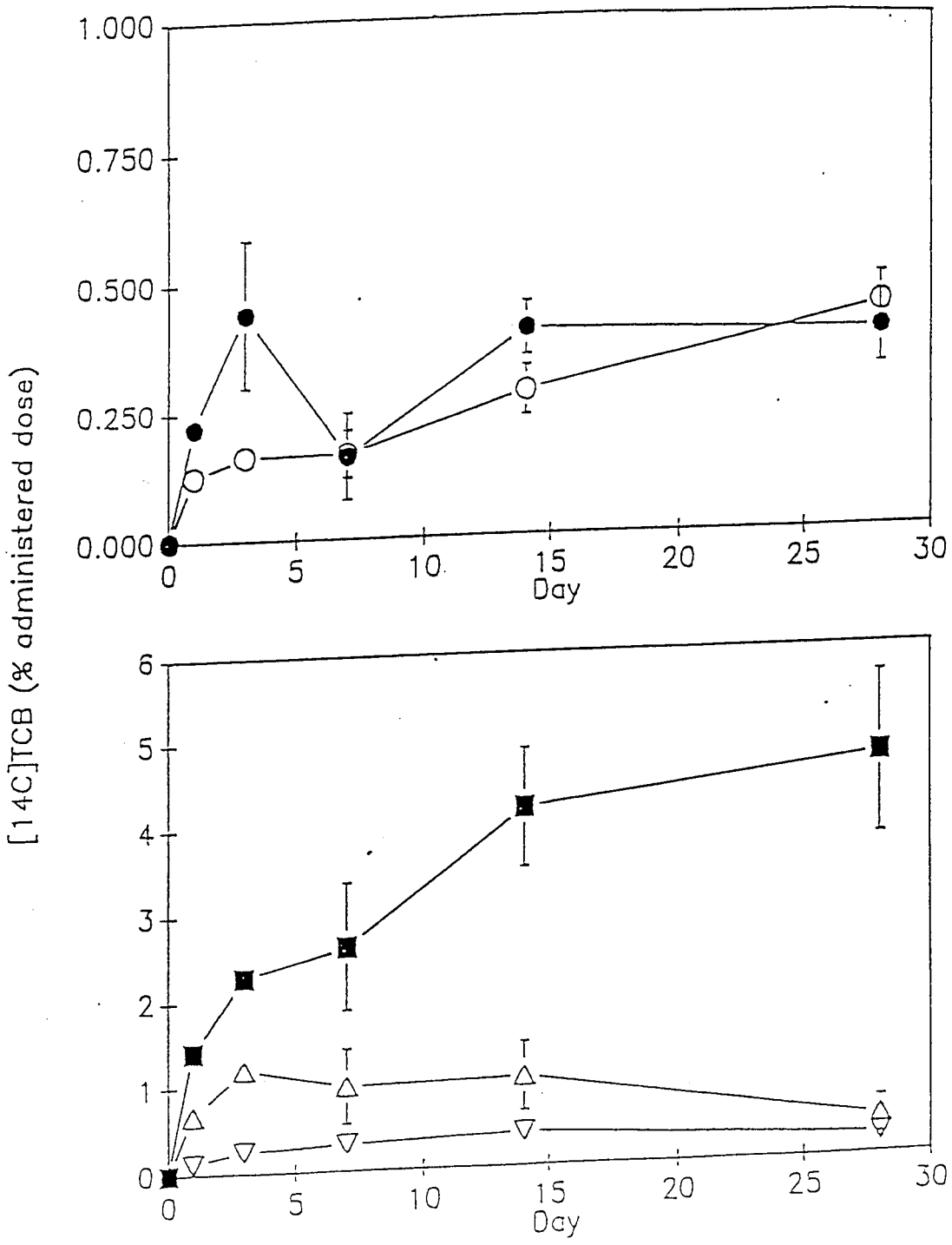

Figure 3.1 The [^{14}C]TCB % administered dose in liver (○), bile (●), fat (△), muscle (■), and pharyngeal (▽) tissues 1 to 28 days after ip injection with 10 nmols [^{14}C]TCB/g fish.

Table 3.2. Mean muscle [^{14}C]TCB equivalents (nmols/g lipid) in the hexane fraction and the DMSO fraction 0 to 28 days after ip injection with 10 nmol [^{14}C]TCB/g fish.

Day	Hexane	DMSO
0	0	0
1	28.8 (8.9)	7.5 (2.6)
3	50.9 (20.9)	8.0 (0.9)
7	17.4 (6.2)	81.8 (40.1)
14	36.6 (8.8)	19.4 (8.8)
28	119.3 (69.5)	55.2 (17.4)

(SE) n = 4

Altered [^{14}C]TCB Tissue Distribution with TCB Pretreatment

Pretreatment of rainbow trout with TCB altered the tissue distribution of a subsequent dose of [^{14}C]TCB. The average concentration of [^{14}C]TCB (% retained dose/g tissue) in muscle and pharyngeal tissue increased approximately 3-fold 28 days after pretreatment with 5 ug TCB/g fish (Figures 3.2 and 3.3). Total [^{14}C]TCB in the carcass (% retained dose) decreased 28 days after pretreatment with 5 ug TCB/g fish (Figure 3.4). Also at day 28, there was a suggestion of increased hepatic

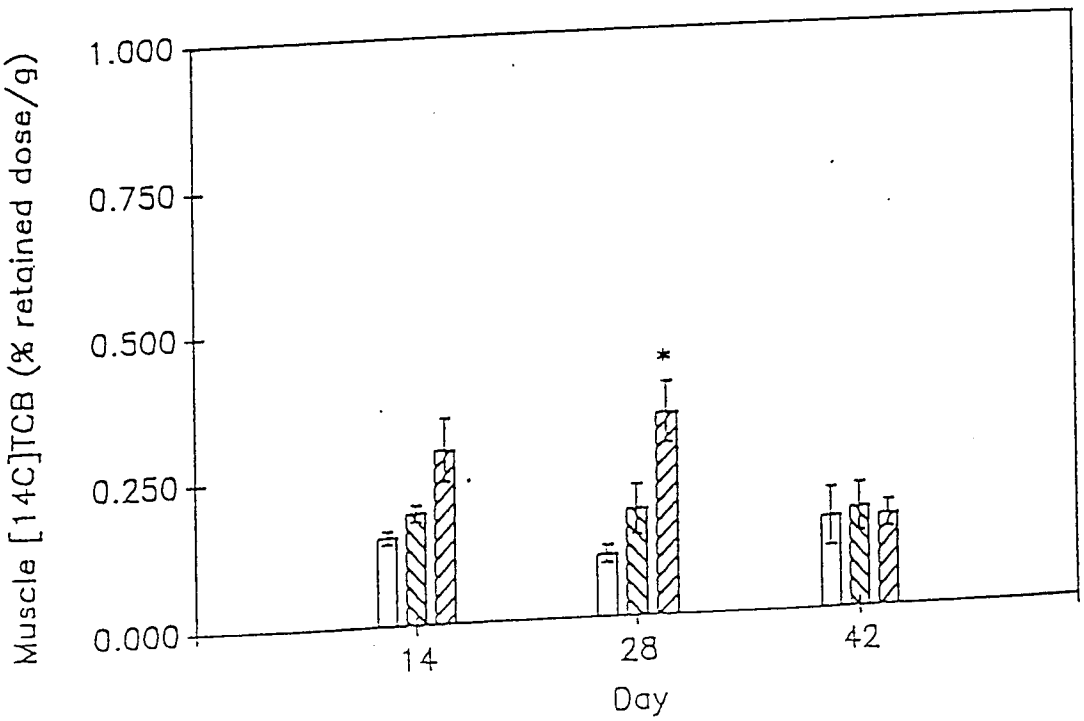

Figure 3.2. Mean muscle [^{14}C]TCB (% retained dose/g) in fish 24 hours after ip injection with 10 nmols [^{14}C]TCB/g 14, 28, and 42 days after treatment with 0, 0.5, and 5 ug TCB/g. *Significantly different from control, $p<0.05$. No difference between control groups.

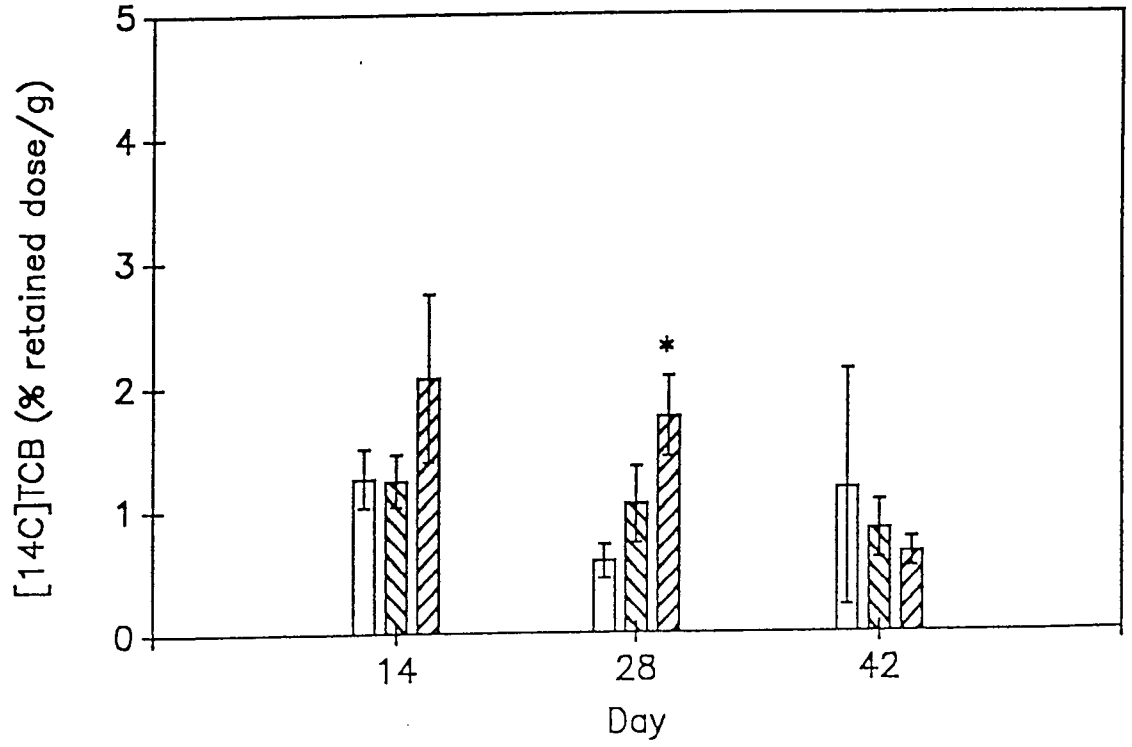

Figure 3.3. Mean pharyngeal tissue [^{14}C]TCB (% retained dose/g) in fish 24 hours after ip injection with 10 nmols [^{14}C]TCB/g 14, 28, and 42 days after treatment with 0, 0.5, and 5 ug TCB/g. *Significantly different from control, $p<0.05$. No difference between control groups.

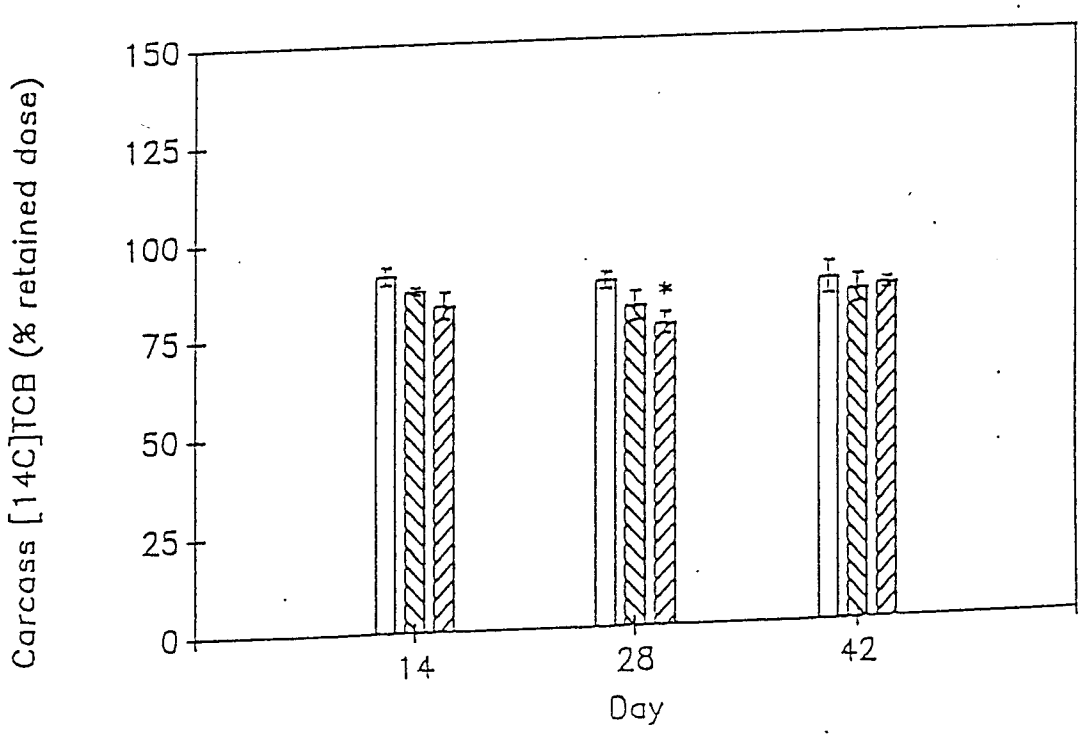

Figure 3.4. Mean carcass [^{14}C]TCB (% retained dose/g) in fish 24 hours after ip injection with 10 nmols [^{14}C]TCB/g 14, 28, and 42 days after treatment with 0, 0.5, and 5 ug TCB/g. *Significantly different from control, $p<0.05$. No difference between control groups.

[^{14}C]TCB (% retained dose in liver) in 5 ug TCB/g treated fish (data not shown; 1-way ANOVA at a one-tailed p-value<0.08 with a 90% confidence interval). There were no differences between treatments at other time points or tissues, with the exception of decreased [^{14}C]TCB concentration in fat (% retained dose/g tissue) 14 days after pretreatment with 0.5 and 5 ug TCB/g fish (data not shown).

[^{14}C]TCB Tissue Concentrations Increased with [^{14}C]TCB Dose

There was a linear increase in [^{14}C]TCB concentration in bile and liver with dose. SLR was used to express [^{14}C]TCB as a function of dose. There was a significant positive correlation for bile, liver, and mesenteric fat (Table 3.3). [^{14}C]TCB concentrations increased through all time points with no evidence of saturation for the tissues sampled.

TCB Altered [^{3}H]DMBA Tissue Distribution

Fish 29 days after treatment with 5 ug TCB/g fish had decreased total nmols of [^{3}H]DMBA in the liver but there was no change with TCB pretreatment for [^{3}H]DMBA concentration (nmols/g) in bile, liver, mesenteric fat or lsi (Figure 3.5).

TCB Effect on EROD Activity and Microsomal Metabolism of [^{14}C]TCB

There was no change in EROD activity with treatment and all sample activities were below detection (data not shown). HPLC retention times for [^{14}C]TCB incubated with microsomes

from beta naphtha-flavone treated rainbow trout were unchanged from controls (data not shown).

Table 3.3. Average [^{14}C]TCB concentrations (nmols/g) in rainbow trout bile, liver, or fat 3 days after receiving a single ip injection of 0, 2.5, 5, 10, 20, or 40 nmols [^{14}C]TCB/g fish.

Dose	Bile	Liver	Fat
0	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
2.5	5.0 (1.7)	1.0 (0.4)	16.2 (3.5)
5	11.5 (1.4)	1.2 (0.3)	47.1 (9.6)
10	16.6 (5.7)	2.0 (0.5)	56.5 (12.3)
20	49.7 (15.9)	8.1 (3.2)	148.2 (35.5)
40	76.6 (16.5)	8.7 (0.4)	444.6 (135.8)
intercept	0.02	0.20	29.7
slope	0.13	0.06	1.57
p-value	0.00	0.00	0.00
Adjusted R^2	88.4	82.4	75.2

(SE) n=3. Coeffients for linear regression model
square root [nmols/g] = intercept + slope * square root [dose]

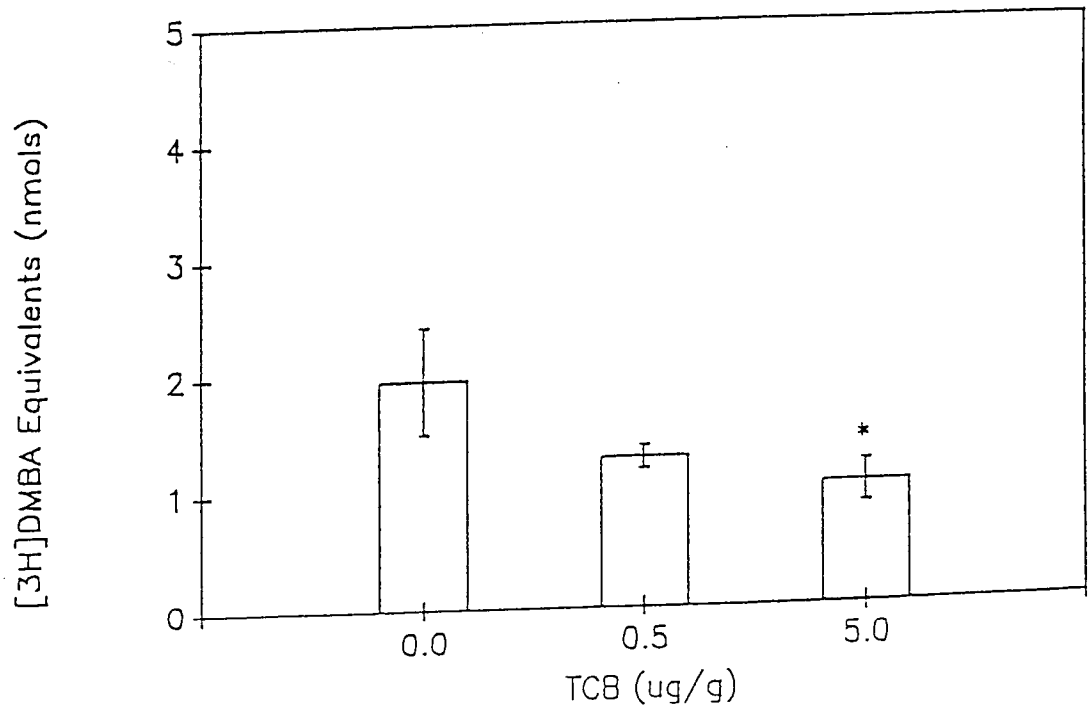

Figure 3.5. Mean liver [^{3}H]DMBA equivalents (nmols) 16 hours after ip injection with 10 nmols [^{3}H]DMBA/g fish 29 days after ip injection with 0, 0.5, or 5 ug TCB/g fish. *Significantly different from control.

DISCUSSION

Long term exposure of rainbow trout to non Ah-receptor agonists have altered the tissue distribution of a subsequent dose of the same compound or a PAH. Treatment with dieldrin for 10 and 12 weeks increased hepatic and biliary concentrations of a subsequent dose of [^{14}C]dieldrin (Gilroy et al., 1993) while hepatic [^{3}H]DMBA increased four weeks after pretreatment with 2HxCB (Foster and Curtis 1996). The altered tissue distribution from treatment with 2HxCB could not be fully explained by increased hepatic CYP1A protein levels and PCB methylsulfonyl metabolites were suggested as a possible factor influencing tissue distribution. The rate of PCB metabolite formation can be affected by the degree of chlorination with lesser chlorinated metabolized faster than more highly chlorinated PCBs (Ghiasuddin et al., 1976).

This study demonstrated a linear correlation of tissue [^{14}C]TCB concentration with time following a single ip injection of [^{14}C]TCB and the effect of TCB treatment on tissue distribution of a subsequent dose of [^{14}C]TCB or [^{3}H]DMBA. Hepatic EROD activity and the formation of PCB metabolites were investigated as potential mechanisms of altered tissue disposition. The [^{14}C]TCB time course experiment was performed to establish a time for investigating the TCB interaction with [^{14}C]TCB or [^{3}H]DMBA.

[^{14}C]TCB concentrations increased linearly through day 3 for all tissues (Table 3.1). An apparent steady state was reached for fat and bile after day 3. This was comparable to the skin, visceral, and carcass of rainbow trout and yellow perch exposed orally to [^{14}C]TCB (Guiney and Peterson 1980). However, rainbow trout exposed orally to [^{14}C]TCB had a greater percentage of the administered dose in muscle tissue, approximately 15% (Guiney and Peterson, 1980) compared to the 3% to 5% found in this study. The low recoveries of [^{14}C]TCB from tissues during the [^{14}C]TCB time course experiment, which has also been reported for TCDD administered by ip injection (Curtis et al., 1996), may be due to branchial or renal excretion prior to passage through the liver. There was a decrease in [^{14}C]TCB tissue concentrations at day 7 which may be due to feeding fish at day 4 of the experiment and ingested food competing with [^{14}C]TCB for plasma protein transport.

The study demonstrated that chronic exposure of rainbow trout to a low dose of TCB altered the tissue distribution of a subsequent dose of [^{14}C]TCB or [^{3}H]DMBA. TCB induced a redistribution of a challenge dose of [^{14}C]TCB from the carcass to muscle and pharyngeal tissue but not to mesenteric fat. These results were similar to pretreatment of rainbow trout with dieldrin and the redistribution of a subsequent dose of [^{14}C]dieldrin from the carcass, but differed in that the dose was then redistributed to the liver, bile, and mesenteric fat (Gilroy et al., 1993). The difference in redistribution

pattern may be due to [^{14}C]TCB having a high affinity for rainbow trout muscle tissue (Guiney and Peterson, 1980). Redistribution of [^{14}C]TCB with TCB pretreatment was not correlated with increased hepatic EROD activity.

However, constitutive hepatic enzymatic levels produced conjugated TCB metabolites in rainbow trout (Melancon and Lech 1976) and methylsulfonyl PCBs were detected in wild fish (Haraguchi et al., 1989). PCB methylsulfonyl metabolites can be formed by metabolism of glutathione conjugates through the mercapturic acid pathway (Bakke et al., 1982). Both UDP-glucuronosyltransferase and glutathione S-transferase activity are inducible (Anderson et al., 1985) and may have contributed to the metabolism and redistribution of [^{14}C]TCB. Increased [^{14}C]TCB in the DMSO solvent fraction through day 28 of the time course experiment supports this notion (Table 3.2). The DMSO fraction would contain the methylsulfonyl metabolites (Bergman et al., 1992). The increase of [^{14}C]TCB in the DMSO fraction on day 7 may also be explained by the fish being fed on day 4 as metabolizing enzymes can be affected by nutritional status.

HPLC profiles of [^{14}C]TCB were similar after [^{14}C]TCB was incubated with microsomes from control or beta-naphthaflavone treated fish. There were no hydroxylated metabolites of TCB detected in the excreta of brook trout (*Salvelinus fontinalis*) treated with TCB (Hutzinger et al., 1972). However, goldfish (*Carassius auratus*) liver homogenates incubated in a NADPH

generating system with 2,3,2′-[^{14}C]trichlorobiphenyl produced very polar and somewhat polar metabolites while incubations with rainbow trout (*Oncorhyncus mykiss*) and bullhead (*Ictalurus sp.*) liver homogenates produced negligible amounts of metabolites (Hinz and Matsumura, 1977). This would indicate that metabolizing enzymes requiring additional components not included in the incubation mixture, such as glutathione, may be important in rainbow trout metabolism of TCB. The effects of a cytosolic protein, such as glutathione S-transferase, on [^{14}C]TCB metabolism and redistribution remains unknown.

Methylsulfonyl and hydroxy PCB metabolites have biological activity. PCB methylsulfonyl metabolites increased rat hepatic cytochrome P450 2B1 and 2B2 content (Kato et al., 1995), bound with high affinity to a cytosolic binding protein in mouse lung clara cells (Lund et al., 1985), and also to fatty acid binding protein in rat intestinal mucosa (Larsen et al., 1991). PCB hydroxy metabolites bind to thyroxine specific binding sites in rat liver nuclear extracts (McKinney et al., 1987) and inhibited the binding of thyroxine with transthyretin (Brouwer et al., 1990). The effect of PCB metabolites on PCB tissue distribution remains to be determined.

Decreased hepatic [^{3}H]DMBA due to TCB pretreatment was contrary to earlier work performed that pretreated rainbow trout with 2HxCB and caused increased hepatic [^{3}H]DMBA after

a subsequent dose of [^{3}H]DMBA (Foster and Curtis 1996). Altered hepatic [^{3}H]DMBA due to 2HxCB pretreatment was weakly correlated with increased hepatic cytochrome P450 1A2 mRNA levels (Foster et al., 1996) while in TCB pretreated fish there was no evidence of cytochrome P450 induction, indicating different mechanisms of action.

In summary, we have shown that pretreatment with TCB, a di-ortho PCB, altered the disposition of a subsequent dose of [^{14}C]TCB or [^{3}H]DMBA. The redistribution of [^{14}C]TCB was unrelated to hepatic EROD activities but may be influenced by other hepatic metabolizing enzymes, such as glutathione S-transferase, or changes in physiological processes due to the effects of TCB metabolites.

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SUMMARY

Di-ortho PCBs are prominent environmental contaminants in aquatic systems and their effects on fish are more significant than previously thought. The di-ortho PCB, 2HxCB, was one of the most frequently detected PCB congeners in environmental samples (McFarland and Clarke, 1989). The low toxicity of 2HxCB, and di-ortho PCBs in general, was attributed to low Ah receptor affinity due to a noncoplanar conformation of the PCB congener (reviewed in Safe, 1984; Safe, 1990). 2HxCB induction of cytochrome P450 in early studies (Forlin and Lidman, 1979) was attributed to dioxin contamination of the 2HxCB (James and Little, 1981). Subsequent studies with short term exposures of trout and carp to 2HxCB failed to establish induction of EROD activity and CYP1A (Klienow et al., 1990; van der Weiden et al., 1994). However, long term feeding of 2HxCB to rainbow trout increased EROD and AHH activities which were positively correlated with 2HxCB liver concentration (da Costa and Curtis, 1995).

This work demonstrated that both 2HxCB and TCB altered tissue distribution of a subsequent dose of a PAH, and TCB altered the tissue distribution of a subsequent dose of [^{14}C]TCB. There are several mechanisms that could affect disposition of a compound some of which are altered biliary excretion and changes in metabolizing enzymes. The data

indicate that 2HxCB and TCB altered disposition were by different mechanisms.

The cytochrome P450 system is responsible for the primary oxidative metabolism of PAHs (Buhler and Williams, 1989) and DMBA metabolism was expected to increase with induction of CYP1A if metabolism was rate limiting. Biliary excretion of [^{3}H]DMBA increased in fish fed 2HxCB for 4 and 8 weeks. In addition, fish ip injected with 2HxCB had increased EROD activity and CYP1A2 mRNA but no change from controls for CYP1A1 mRNA. CYP1A1 and CYP1A2 isozymes may be regulated by Ah receptor dependent (reviewed in Safe, 1993; Quattorchi et al., 1994) or independent pathways (Sterling et al., 1994; Cook and Hodgson, 1986; Quattrochi et al., 1994). EROD induction in mice exposed to 2HxCB was due to the CYP1A2 and not the CYP1A1 isozyme (De Jongh et al., 1995). The model Ah receptor agonist, TCDD, increased CYP1A1 and CYP1A2 mRNA in rainbow trout (Curtis et al., 1996). Therefore, absence of CYP1A1 mRNA in 2HxCB treated fish indicates either a non Ah receptor pathway for the induction of CYP1A2 or the induction of CYP1A1 was less sensitive than CYP1A2. The similarity between mouse and rainbow trout patterns of hepatic metabolizing enzyme induction due to 2HxCB exposure is physiological evidence for the existence of a CYP1A2 isozyme in rainbow trout.

TCB pattern of altered [^{3}H]DMBA disposition was different from 2HxCB treated fish. TCB treated fish had decreased

levels of [^{3}H]DMBA in the liver and biliary [^{3}H]DMBA did not change with treatment while 2HxCB treated fish had no change in liver and increased biliary levels of [^{3}H]DMBA. In addition, EROD activities for TCB treated fish were unchanged from controls. PCB metabolite formation was related to degree of chlorination with decreasing metabolism as chlorination increased (Ghiasuddin et al., 1976). Therefore, TCB would be expected to produce more metabolites than 2HxCB. Some methylsulfonyl metabolites of PCBs increased hepatic enzymatic activities in rats (Kato et al., 1995), bound with high affinity to a cytosolic binding protein in mouse lung clara cells (Lund et al., 1985), and also to fatty acid binding protein in rat intestinal mucosa (Larsen et al., 1991). [^{14}C]TCB levels in the DMSO fraction, which would contain the methylsulfonyl metabolites (Bergman et al., 1992), increased through day 28 and may influence tissue disposition of a subsequent dose of [^{14}C]TCB or [^{3}H]DMBA following pretreatment with TCB.

TCB altered disposition of a subsequent dose of [^{14}C]TCB was similar to the altered disposition of [^{14}C]dieldrin following dieldrin pretreatment (Gilroy et al., 1993). EROD, UDP-glucuronosyl-transferase, and glutathione S-transferase activities were unchanged in the dieldrin treated fish and changes in lipoprotein complexes were suggested as a possible mechanism for altered disposition of a subsequent dose of [^{14}C]dieldrin (Gilroy et al., 1993). EROD activity was

unchanged in TCB treated fish while UDP-glucuronosyl-transferase and glutathione S-transferase activities were unmeasured and can not be ruled out as possible mechanisms of altered [^{14}C]TCB disposition with TCB pretreatment at this time.

In summary, the pattern of 2HxCB induction of EROD activity and CYP1A2 mRNA and the lack of CYP1A1 mRNA expression were consistent with the existence of a CYP1A2 isozyme in rainbow trout. 2HxCB altered the disposition of a subsequent dose of [^{3}H]DMBA which can be partially explained by increased CYP1A2 mRNA levels. TCB altered the disposition of a subsequent dose of [^{14}C]TCB or [^{3}H]DMBA which appears to be mechanistically different from the 2HxCB altered disposition of [^{3}H]DMBA. Future work should directly evaluate the effects of PCB metabolites on altering the disposition of a subsequent dose of a PAH or PCB.

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APPENDIX

APPENDIX

2,2′,4,4′,5,5′- and 3,3′,4,4′,5,5′- Hexachlorobiphenyl Altered Plasma Thyroxine Levels in Rainbow Trout (*Oncorhyncus mykiss*)

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ABSTRACT

Plasma was collected from rainbow trout four week after ip injection with either 50 or 250 ug 2,2′,4,4′,5,5′-hexachlorobiphenyl (2HxCB)/g or 5 or 25 ug 3,3′,4,4′,5,5′-hexachlorobiphenyl (3HxCB)/g. Fish that received 50 ug 2HxCB/g had plasma thyroxine level approximately double that of controls while there was no change in all other treatments. In a second experiment, plasma was collected from rainbow trout 12 weeks after ip injection with either 25 or 100 ug 3HxCB/g. Thyroxine levels were approximately double in 25 ug 3HxCB/g with no change in 100 ug 3HxCB treated fish. Long term exposure of rainbow trout to 2HxCB and 3HxCB altered the plasma thyroxine levels.

INTRODUCTION

Polychlorinated biphenyls (PCBs) were common environmental contaminants with di-ortho PCBs detected more frequently than the more toxic non-ortho PCBs (McFarland and Clarke, 1989). Di-ortho PCBs, such as 2,2′4,4′5,5′-hexachlorobiphenyl (2HxCB), were considered to have low toxicological significance (reviewed in Safe 1994) but recent work exposing rainbow trout to 2HxCB reported increased EROD activity and altered disposition of a subsequent dose of a polycyclic aromatic hydrocarbon (Foster et al., 1996; Foster and Curtis, 1996). In addition, 2HxCB affected plasma thyroid hormones in rats (Soyano et al., 1993).

This study measured plasma thyroid hormones in rainbow trout following treatment with 2HxCB or 3,3′,4,4′,5,5′-hexachlorobiphenyl (3HxCB).

METHODS

Experimental Animals

Sexually immature Shasta strain rainbow trout (20-60 g) were obtained from the Food Toxicology & Nutrition Laboratory, Oregon State University. Two fish of the same PCB treatment were kept in aerated glass aquaria (61x32x20 cm; 23 L total volume), separated by a partition, and received a continuous flow of well water (100 ml/min @ 14$\pm$2°C). Fish were fed a 3% body weight (dry wt fish/dry wt diet) ration of Oregon Test

Diet/day given as three feedings per week. Fish weights were measured weekly and rations were adjusted accordingly. Tank debris was removed 3 times/week. A 12:12 hr light:dark cycle was maintained throughout the study.

Dosage

Fish received a single ip injection of 50 or 250 ug 2HxCB/g or 5 or 25 ug 3HxCB/g in stripped menhaden oil (5 ml/kg) obtained from the Fish Oils Test Materials Program and then sampled 4 weeks later. Controls received menhaden oil only. In a second experiment fish received an ip injection of 25 or 100 ug 3HxCB/g and then sampled 12 weeks later.

Thyroxine analysis

Thyroxine analysis was performed on the plasma samples according to Sullivan et al., 1989.

RESULTS

Thyroxine levels increased in the plasma of fish treated with 50 but not 250 ug 2HxCB/g while thyroxine levels remained unchanged in fish four weeks after treatment with 3HxCB (Figure 4.1). Plasma thyroxine increased in fish twelve weeks after treatment with 25 ug 3HxCB/g but not 100 ug 3HxCB/g (Figure 4.2).

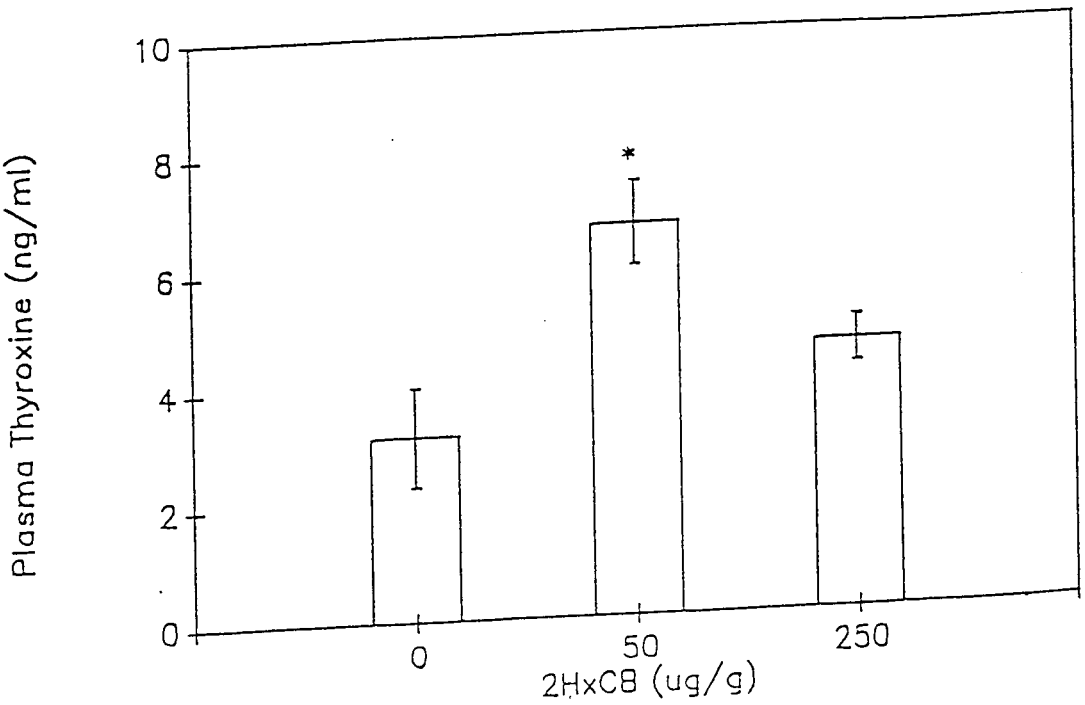

Figure 4.1. Rainbow trout plasma thyroxine levels four weeks after ip injection with 2HxCB. *Significantly different from controls, $p<0.05$.

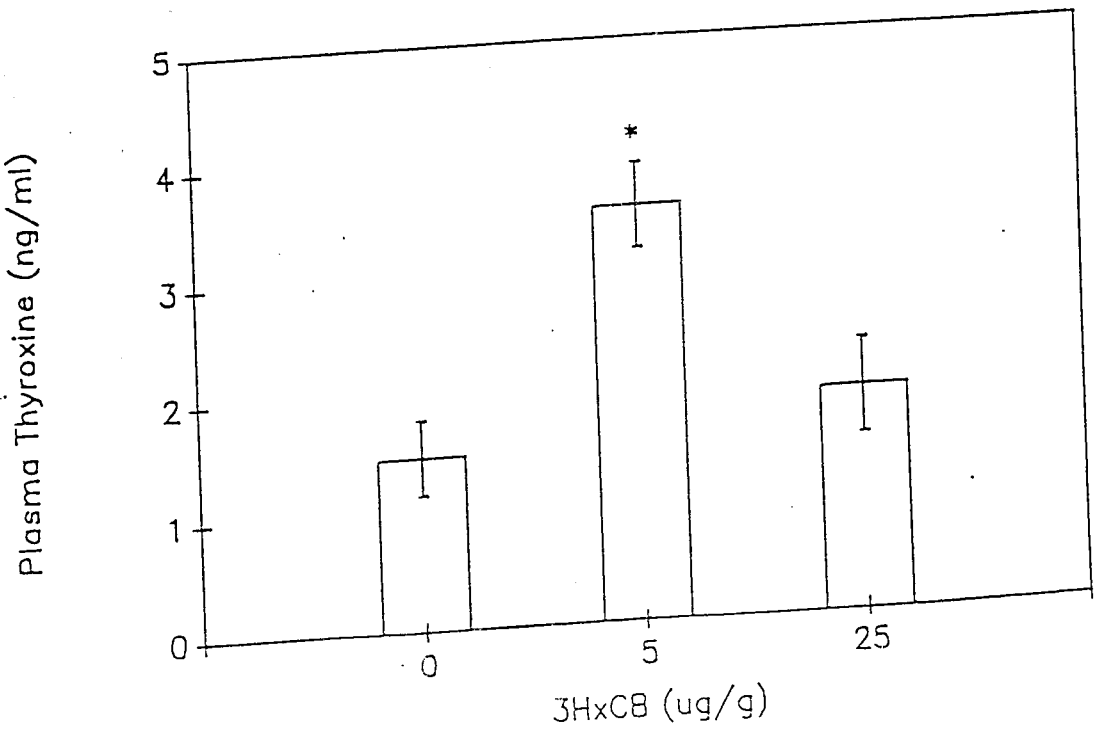

Figure 4.2. Rainbow trout plasma thyroxine levels twelve weeks after ip injection with 3HxCB. *Significantly different from controls, $p<0.05$.

DISCUSSION

This study demonstrated that 2HxCB and 3HxCB can alter plasma thyroxine levels in rainbow trout.

Thyroid hormones are important for proper neurological development in the early life stages of animals and can be important in reproduction. Medaka plasma 3,3'5-triiodothyronine and 17B-estradiol followed similar diurnal patterns and ovarian follicles exhibited enhanced gonadotropin induced 17B-estradiol production with exposure to 3,3'5-triiodothyronine (Soyano et al., 1993).

In addition, 2HxCB exposure altered plasma thyroid hormones in rats. Rats born from females that received 16 or 64 ug 2HxCB/g during pregnancy had decreased plasma thyroxine levels (Ness et al., 1993). The mechanism of 2HxCB action on plasma thyroxine was unknown, but was thought to be related to characteristic 2HxCB induction of cytochrome 2B1. Recent studies with mice showed increased cytochrome 1A2 with 2HxCB treatment (De Jongh et al., 1995). In addition, long term exposures of rainbow trout to 2HxCB resulted in increased EROD activity, CYP1A protein and CYP1A2 mRNA (Foster et al., 1996). As fish are refractory to cytochrome P450 2B1 induction, the effect of cytochrome P450 1A2 induction on plasma thyroxine levels may be important.

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