EFFECT OF PCB 126 ON LIVER ENZYMES AND THE THYROID AXIS IN THE MALE RAT

by

TARA LYNN ALMEKINDER

(Under the Direction of Jeffrey W. Fisher)

ABSTRACT

PCB 126 is known to interfere with thyroid hormone (TH) homeostasis by inducing microsomal enzymes and perturbing thyroid hormones. The aim of the present study is to evaluate the temporal effects of PCB 126 (3, 3', 4, 4', 5-pentachlorobiphenyl) on the thyroid axis, as mediated by induction of liver cytochrome P450 (total P450 and CYP1A1). A pharmacodynamic model for PCB 126-induced effects on the thyroid axis is under development in our laboratories. Adult male Sprague-Dawley rats were administered a single oral bolus dose of 0, 7.5, 75, or 275 ug PCB 126/kg bw dissolved in corn oil. The rats were sacrificed periodically over 59 days for evaluation. PCB 126 caused a dose-related decrease in body weight gain. Total P450 levels were elevated for 59, 22, and 5 days for the 275, 75, and 7.5 ug/kg dosage groups, respectively. Ethoxyresorufin-O-deethylase (EROD) activity, a marker for CYP1A1, was increased for 1, 5, and 35 days in the 7.5, 75, and 275ug/kg groups, respectively. Serum thyroxine (T4) and triiodothyronine (T3), reverse T3 (rT3), and thyroid stimulating hormone (TSH) were measured by radioimmunoassay. Free T4, the active unbound form, was measured by direct equilibrium dialysis. PCB decreased Total T4 compared to controls at days 1, 5, 9 and 22. Free T4 was decreased throughout the study period. Total T3 and reverse T3

(rT3) showed no discernable trend by dosing. TSH was increased through day 9 and 22 day period compared to control levels. Thyroid glands were analyzed histologically for altered colloid/epithelial ratios on days 20, 35, and 59 at all doses measured. Hepatic Type 1 5'-deiodinase activity was decreased in a dose-related fashion throughout the study. These data demonstrate that PCB 126 interferes with thyroid homeostasis with reduction of free T4 persistent for at least 59 days after dosing.

INDEX WORDS: PCB 126, thyroid hormones, P450, EROD, Rat, and deiodinase activity.

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DEDICATION

I would like to dedicate this thesis to my family and friends for all their support and guidance through the years. Without their constant love and motivation, I would not have been able to accomplish such goals.

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CHAPTER 1

INTRODUCTION

History of PCBs

Polychlorinated biphenyls (PCBs) are toxic, lipophilic, and persistent chemicals in the environment. Production of PCBs originated in 1929 at the Swann Chemical Company until 1935, when purchased by the Monsanto Company (St. Louis, MO) and sold under the trade name Aroclor. Approximately 1.3 billion pounds of PCBs were manufactured before their toxic effects halted production in 1977. PCB mixtures exist in the form of congeners. Named and associated by their percentage of chlorination, PCBs are a family of synthetic chemicals produced by chlorination of the biphenyl molecule, and possess unique chemical properties that offer thermal stability, low flammability, and resistance to acids and bases, and low water solubility making them appealing for industrial and commercial uses (Martin 2002). There are 209 possible PCB congeners marketed by their percentage of chlorination by weight. Potential industrial uses include: fire-resistant dielectric fluid in transformers and capacitors, plasticizers, hydraulic and lubricants, heat-transfer fluid, components of paints, paper coatings, carbonless copy paper, and some packaging materials. Manufactured PCBs were produced in the form of capacitors (50%), transformers (27%), plasticizers (9%), hydraulics and lubricants (6%), and carbonless copy paper (4%), and other uses (4%) (EHSC 8930, UGA).

It has been noted that over 70% of the global production of PCBs is still in the environment today (Hileman 1993), and is of concern for human health because of industrial discharges and emissions (Ahne and Jarre 2002).

Toxic Effects of PCBs

Studies have shown that PCBs elicit toxic effects in both humans and animals. The first study that portrayed the toxic effects of PCBs was published in 1968 when 1800 Japanese people suffered ‘Yusho disease’ after use of PCB-contaminated rice-oil (Rogan et al. 1988). Later, the

chlorinated chemicals were found to cause several toxic effects, such as reproductive (Augustowska et al. 2001), behavioral, and cognitive function abnormalities (Bushnell and Rice 1999), possible carcinogenicity, and disruption of the endocrine system (Li and Hansen 1997). A key determinant of a PCB's toxicity is its percent-chlorine content. Toxic effects have been shown to increase as the percent of chlorination increases, making the highly chlorinated PCBs the most toxic as exhibited to the widely studied 2, 3, 7, 8- tetrachlorodibenzo-p-dioxin (TCDD). Federal Government regulations and guidelines for PCBs have been set by such organizations as the Environmental Protection Agency (EPA), Occupational Safety and Health Administration (OSHA), and the Food and Drug Administration (FDA), the Agency for Toxic Substances and Disease Registry (ATSDR), and National Institute for Occupational Safety and Health (NIOSH). Current EPA regulations set PCB regulating limits in the environment at 0.5 parts per billion (ppb) for drinking water, and 0.17 parts per trillion (ppt) for other water sources such as lakes, ponds and marine dwellings.

The FDA has regulated PCB levels at no more than 0.2 - 3 parts per million (ppm) depending on the food category (i.e. junior foods, milk and eggs). During the era of PCB production, the primary exposure route was dermal exposure to factory workers, but since the termination of PCB production, the main exposure now follows dietary intake. Bioaccumulation in marine biota accounts for potential exposure in humans today (Safe, 1994).

Thyroid Gland as a Target of PCB Toxicity.

The thyroid gland is one of the most important hormonal glands of our body and is the largest endocrine gland in humans. Its secretion of thyroid hormones maintains and influences basal metabolism, heart rate, blood pressure, and body temperature, among other functions in the human body (Capen 2000). The thyroid gland is a butterfly shaped gland that is found at the base

of the neck just below the larynx. The thyroid consists of follicles of varying size that contain colloid produced by the follicular cells. These follicles grow during development and by stimulation of thyroid stimulating hormone (TSH). TH homeostasis is regulated in the body by the hypothalamic-pituitary-thyroid axis in which the thyroid hormones act via a negative feedback loop of the free unbound serum form of the hormones. Alteration of thyroid homeostasis involves potential disruptions in thyroid hormone synthesis, serum binding, transmembrane transport, uptake, metabolism and/or excretion (Capen 1997).

Without iodide there is no thyroid hormone synthesis. The thyroid hormones are iodothyronines, i.e., the result of two coupled iodotyrosines, and are the only iodine-containing hormones in vertebrates (Carrasco 1993). The two main thyroid hormones produced by the thyroid gland are thyroxine (T4) and triiodothyronine (T3) with stimulation from pituitary thyroid stimulating hormone (TSH). The function of the thyroid gland is to take dietary iodine and convert it into thyroid hormones. Transport of iodide into the thyroid follicular cell is made possible by the Na+/I- Symporter (NIS). Trapped iodide is oxidized by TPO (thyroid peroxidase) in the presence of hydrogen peroxide and then incorporated into the tyrosine residues of thyroglobulin (Tg). Tg is a large glycoprotein located within the colloid of the thyroid follicle. Tg is synthesized on the rough endoplasmic reticulum and packaged in the Golgi apparatus of the follicular cells (Capen 2000). Tyrosine residues are enzymatically coupled by TPO within Tg to produce both T3 and T4.

The major thyroid hormone secreted by the thyroid gland is thyroxine and contains four iodine atoms. TSH controls the amount of T4 produced by the thyroid gland. T3 is structurally the same as T4, but with one less iodine molecule. The majority of T3 present on circulation is produced through the conversion of T4 in the extrathyroidal tissues (Zoeller 2003).

Hypothalamic-Pituitary-Thyroid Axis.

The hypothalamic-pituitary-thyroid hormone negative feedback axis regulates thyroid hormone production (Kaptein et al. 1994). Hormones produced by two other organs influence the thyroid gland: the pituitary and the hypothalamus. The pituitary is located at the base of the brain and produces TSH. TSH binds to the thyroid follicular cells and activates adenylate cyclase with accumulation of cyclic adenosine monophosphate (cAMP), thereby increasing the rate of biochemical reactions concerned with the synthesis of TH (Wynford-Thomas et al. 1987). The hypothalamus, a small part of the brain innervating the pituitary, produces thyrotropin-releasing hormone (TRH). The hypothalamus and the pituitary monitor the concentration of the free thyroid hormone (T4, T3) in the circulation. When TRH is released from the hypothalamus, the pituitary is then stimulated to release TSH. In thyroid hormone deficiency, increased levels of TSH then stimulate the thyroid to produce thyroid hormone, and return the levels of thyroid hormone in the blood to a normal state (Zoeller 2003). These three glands and the hormones they produce make up the "Hypothalamic - Pituitary - Thyroid Axis." The state of normal thyroid function is called euthyroidism. In addition to TSH stimulating the thyroid to produce thyroid hormones it also has a second action - it causes growth of thyroid cells. Thyroid follicular cells are cubiodal to columnar, and their hormone is stored on the large protein thyroglobulin in lumen of the follicles with secretion resulting in hormone release from the basolateral surface into the circulation. Polarity of follicular cells is important in iodine uptake as well as required for synthesis of TH (Ericson and Nilsson 1992). In response to long-term stimulation of follicular cells by TSH, which can occur from chronic iodide deficiency as well as low levels of circulating thyroid hormones, both lobes of the thyroid enlarge in order to capture the essential nutrients (i.e.

iodine) to maintain homoeostasis and to synthesize necessary iodothyronine to restore their free circulating concentration to normal (Capen 2000).

The potential disruption of the hypothalamic-pituitary-thyroid axis is important to understand the potential impact of thyrotoxicants on normal development and human health. Thyroid hormone parameters that can be measured in order to identify disruption include plasma total and free T4 (TT4 and FT4, respectively), plasma T3 and rT3, and TSH. But in the laboratory the plasma half-life of T4 is considerably shorter (12-24 hours) than in humans (5-9 days) (Deshler et al. 1979).

Thyroid Hormone Distribution and Transport.

T4 and T3 are released into the blood stream from the thyroid gland. They are transported throughout the body where they control oxidative metabolism. Thyroid hormones are transported through the circulation via serum transport proteins. T4 circulates in the blood in two forms: T4 bound to serum proteins accounts for 99.9% of serum total T4 in the rat. There may also be a facilitating role of these proteins to enhance the rate of cellular uptake. In human, 75% of T4 is bound to thyroid binding globulin (TBG), 15% of T4 is bound to transthyretin (TTR) protein, and the remainder of T4 is bound to albumin (Schussler 2000). This protein bound hormone in serum can be liberated for entry into cells. Free T4, the unbound fraction, enters target tissues to exert its effects and accounts for approximately 0.1% of serum total T4 concentration in the rat. T3 is also protein bound in serum but is 99.7% bound and 0.3% free in total serum concentration. All T4 is produced in the thyroid, while the majority of T3 (approximately 80% in humans, 50% in rats) and reverse T3 (rT3), (its biologically inactive co-product hormone) are produced in the extrathyroidal tissues of the body by the removal of one

iodide atom from T4 (Zoeller 2003). Conversion of T4 to T3 is important because T3 is 3-5 times more potent and may then be considered an activation step (Martin 2002).

Deiodination.

Deiodination of T4 occurs when iodine atoms are removed from either the phenolic (outer) or tyrosyl (inner) ring. In 5'-deiodination, an iodine is removed from the outer ring of T4, yielding T3 (the most active hormone), and in 5-deiodinases, an iodine atom is removed from the inner ring, yielding rT3 (which is inactive).

This bioactivating reaction involves the removal of the 5'- (or 3'-) iodide atom from the phenolic ring of iodothyronines. There are three types of deiodination. There are two "outer-ring" 5'-deiodinases (Type I and Type II) and an "inner-ring" 5'-deiodination (Type III). Type I 5'-deiodination tissues include the thyroid gland, liver, kidney and have a primary role in maintaining circulating levels of T3. Type II 5'-deiodination tissues are represented in the brain, pituitary, brown fat and have a critical role in providing local intracellular conversion of T4 toT3 (Murakami 2001). The rat has been used extensively as a model for the effects of illness on T_4-to-T_3 conversion in humans. Type I and Type II can be distinguished by their sensitivity to 6-*n*-propyl-2-thiouracil (PTU). Type I 5' deiodination is completely susceptible to PTU whereas Type II 5'deioindation being completely insensitive to its presence, but is sensitive to the radiocontrast agent, iopanoic acid.

Conjugative Metabolism of Iodothyronines.

Conjugation is a Phase II drug metabolism step involving largely sulfation and glucuronidation (Clarke and Burchell 1997). In the process of glucuronidation or sulfation, lipophilic compounds are made to be more hydrophilic and are more readily excreted in the urine or bile. Excretion rates are determined by molecular weight. In general, those compounds

having a molecular weight greater then 325, including iodothyronines, are excreted in the bile (Caldwell 1985). Glucuronidation is catalyzed by a group of enzymes known as uridine diphosphate glucuronyltransferases (UDPGTs). These UDPGTs exist primarily in the liver, but also in the skin, kidney, lungs, testis, brain, intestines and other tissues (Borghoff and Birnbaum 1985, Yokota et al. 1999). In mammals, several UDPGT groups have been recognized. UDPGT1 which is responsible for glucuronidation of bilirubin, phenols and T4, and the UDPGT2 group which is responsible for glucuronidation of steroids, bile acids and T3 (Findlay et al. 2000, Viollon-Abadie et al. 2000). Glucuronide conjugates of T3 and T4 are the predominant metabolites of the thyroid hormones excreted into the bile (Visser 1990). Fecal clearance of thyroid hormones have been found to be much less in humans than in rats leading to evidence that humans have a more effective enterohepatic circulating system of thyroid hormones (Rutgers et al. 1989).

Conjugation with sulfate is another pathway for thyroid hormones metabolism (Visser 1994). Sulfation increases water solubility and facilitates excretion into urine and bile (Mulder 1986). There are numerous isoforms in the superfamily of sulfotransferases (Glatt et al. 2001) and thyroid hormones are sulfated by at least four different isoforms in humans and at least two isoforms in rats (Glatt et al. 2001, Schuur et al. 1998). Sulfation of thyroid hormones interacts with their deiodination, such that sulfation facilitates the inactivation of thyroid hormones through Type I 5'-deiodinase and thus offers extra protection against high concentrations of T3. If sulfotransferases are present, sulfation can also be a manner to construct a reserve of sulfated T4 or T3, which can again be converted into active T3. It appears sulfation is the primary route of metabolism for lower iodinated compounds such as T2, and not as important for T4 (Leonard and Koehrle 1996, Visser 1990). To study the complete regulation and function of thyroid

hormones, we cannot limit the parameters measured to only hormone concentrations in plasma, but also need to pay attention to the deiodination and sulfation of thyroid hormones, their transport and the interaction with different receptors.

PCB Congeners.

With 209 possible congener formations of PCB they can include 1 to 10 chlorines. The structural organization determines the toxicity, affinity and binding properties of each congener. In addition to their degree of chlorine content, their toxic potency is related to their planarity, which results in different degrees of cytochrome P-450 enzyme induction (Machala et al. 2003). Congeners are categorized into 3 different groups according to their ortho substitutions, and in relation to their 'dioxin-like' similarities to 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD). The three groups are coplanar, non-coplanar, and mono-ortho chlorine congeners. The coplanar congeners have no chlorines in the ortho positions of the biphenyl rings, have a high affinity to AhR, induce cytochrome P-450 (CYP1A1) and are referred to as TCDD-type congeners. They also induce immunological and reproductive effects as well as shows signs of hepatic toxicity (Haag-Gronlund et al. 1998, Van der Plas et al. 1999). The non-coplanar congener have at least 2 ortho chlorine substitutions, have a lower affinity to the AhR, induce CYP2B, and are called phenobarbital-type congeners. The mono-ortho chlorine congeners are mixed type PCB congeners that induce both enzymes CYP1A and CYP2B. This research is focused on the non-reproductive endocrine effects of the coplanar TCDD-type congener PCB 126 (Van Birgelen 1994).

Aryl Hydrocarbon Receptor.

The aryl hydrocarbon receptor (AhR) is a ligand-dependent transcription factor regulating gene expression. AhR mediates toxicity of several environmental contaminants. It has been demonstrated that CYP1A1 activity and expression are inducible through activation of AhR (Whitlock 1999). AhR is a member of the basic helix-loop-helix family of nuclear transcription factors, and this receptor was initially identified by its high affinity binding to the environmental toxicant, 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD). Once the dioxin binds to the receptor it leads to the induction of microsomal aryl hydrocarbon hydroxylase (AHH) activity. Hydroxylase activity is the first step in the metabolisms of fat-soluble polycyclic hydrocarbons to water-soluble products. When a dioxin binds to an Ah receptor in the cytoplasm of the cell, the complex moves into the nucleus, where it associates with a third protein and activates gene transcription.

PCBs have been known to initiate their effects of toxicity via the activation of the AhR. The main mechanism of action is the binding ability of the toxic chemical to the AhR. When bound to the ligand, AhR associates with the AhR nuclear translocator (ARNT) in the cell nucleus. This complex binds to the xenobiotic-responsive element (XRE), a specific gene regulatory sequence. PCBs are then able to modulate hormone synthesis and metabolism at receptor levels.

Studies on the effect of PCB 126 to P-450 activity, specifically the inductions of CYP1A1 (as measured by EROD), have shown inductions in response to doses starting as low as 0.4 ug/kg in female rats (Craft, 2002). In the same study, UDPGT, the enzyme that catalyses

glucuronidation of thyroid hormones for biliary elimination, were induced following PCB 126 exposure at levels starting at 12ug/kg doses (Craft, 2002).

Metabolite (M-1).

PCBs are metabolized in the liver by microsomal P-450 enzymes to less lipophilic metabolites that can undergo conjugation with glutathione or glucuronic acid (Martin 2002). The rate of metabolism of PCBs depends on 1) degree of ring chlorination and 2) chlorine ring substitution pattern (ATSDR Tox Profile PCBs 2000). Those PCB congeners with low chlorine content form hydroxylated derivatives and are eliminated in the urine. PCB congeners that are high in chlorine content are retained in the body and stored in fat and peripheral tissues or excreted in the feces either as a metabolite of unchanged (Koga et al. 1990). One study investigating the metabolism of PCB 126 has identified a metabolite called 4'-hydroxy-3,4,5,3',4'- Pen CB (also known as M-1) as analyzed by GC-MS (Koga et al. 1990). The structure of this metabolite requires chlorine shift at the 4' position to the 5' position, indicating that a 4', 5'-epoxide is formed. PCB 126 is accumulated in the fat and liver. The metabolite is only detectable in small amounts in the liver with an apparent low binding affinity for the AhR receptor as measured by P-450 induction. PCB 126 has been shown to bind with high affinity to the AhR (Bandiera et al. 1982). M-1 is considered to be a detoxification product and is quickly eliminated from the liver of the PCB treated animals (Koga et al., 1990).

Influence of PCBs interaction on the Hypothalamic-Pituitary-Thyroid Axis.

Many studies evaluating PCB congeners and their effects on thyroid hormone homeostasis have been performed. The most readily quantifiable measures of hormone homeostasis are concentrations of circulating thyroid hormones in serum. Reduced plasma T4 and T3 levels have been documented extensively among PCB studies involving human and

animal studies. Occupational exposure studies of thirty-eight transformer repairmen exposed to PCBs for an average of four years at levels ranging between 0.00001mg/m^3 and 0.012mg/m^3 revealed that serum TT4 and FT4 concentrations fell by 10% compared to control workers (Emmett et al. 1988). Numerous animal studies have evaluated the effect of PCB exposure in rats, or thyroid hormone concentrations. Reduced serum FT4 and TT4 concentrations in rats from PCB exposures have been observed (Barter and Klaassen 1994, Hood et al. 1999, Price et al. 1988, Collins et al. 1977), while the more active hormone T3 is generally not reduced (Barter and Klaassen 1992; Bastomsky et al. 1976; Price et al. 1988) or reduction is minimal (Collins et al. 1997; Hood et al. 1999; Liu et al, 1995). One study carried out a 35 week control diet in male rats following the 4 week PCB diet and found recovery serum TT4 concentrations, to suggest that the effect of decreased TT4 levels can be irreversible once the PCB diet returned to control diet (Collins et al. 1977) at the previous doses raging from 0.44-44mg/kg Aroclor 1254.

Induced T4UGT activity in liver is also associated with PCB exposure in female rats (Desaulniers, et al, 1997). Previous studies showed a correlation between PCB exposure and reduction in plasma total thyroxine (TT4) concentrations and induction of the microsomal phase II enzyme UDP-glucuronyltransferase, performed using T4 as a substrate (Van Birgelen, 1995).

PCB 126 Effects on the Hypothalamic-Pituitary-Thyroid Axis.

Several studies have evaluated the effects of PCB 126 on the thyroid hormones and shown comparable results (Craft et al., 2002; Desaulniers et al., 1999; Van Birgelen et al., 1994; Martin 2002). Parameters commonly evaluated in these studies include terminal body weight, hepatic microsomal activity, thyroid hormone serum concentrations, and metabolic pathways such as glucuronidation and deiodination.

Previous studies have significant effects of PCB 126 on terminal body weights. Decreases in body weight gain have been shown for cumulative doses of PCB 126 ranging from 10-100ug/kg in female rats (Craft et al., 2002), but starting at 400ug/kg in male rats (Desaulniers et al., 1999). A study monitoring food consumption and this effect found a decrease in body weight gain in male rats during a 13-week subchronic feeding study correlated with a 22% reduction in food consumption. (Van Birgelen et al., 1994).

Hepatic microsomal enzyme induction is also a valuable parameter for assessing the effects of the binding affinity to the AhR of the "dioxin-like" PCBs. PCB 126 has shown a remarkable induction capacity for total P450 and more specifically CYP1A1 (as measured by EROD). CYP1A1 is considered a key indication of "dioxin-like" potential. EROD activity has been induced for doses ranging from 0.1-100 ug/kg in female rats and 0.3 – 100 ug/kg in male rats (Craft et al., 2002; Desaulniers et al., 1999; Van Birgelen et al., 1994; Martin, 2002).

Serum Total T4 concentration are significantly reduced in response to PCB 126 starting as low as 4 ug/kg for female rats and 12 ug/kg for male rats (Craft et al., 2002; Desaulniers et al., 1999; Van Birgelen et al., 1994; Martin, 2002). Free T4 has also shown to be decreased in serum concentration levels during a 13-week subchronic feeding study at both a 50 and 180 ug/kg PCB 126 diet (Van Birgelen et al., 1994). Also a Ph.D. dissertation from Rutgers showed a decrease in thyroxine levels starting at 35ug/kg dose of PCB 126 (Martin 2002).

Total T3 and Free T3 have also been studied but not as extensively, but results are more variable and because of peripheral deiodination effects are less indicative of direct effects on the Hypothalamic-Pituitary-Thyroid Axis. No effect from a 13-week feeding study at doses ranging from 7-180ug/kg was seen in rat subjects (Van Birgelen et al, 1994). One unpublished dissertation by Martin with a cumulative dose of 70ug/kg showed a decrease in Free T3 levels,

but there was no associated dose-related pattern (Martin 2002), and the procedure used was not the "gold standard" equilibrium dialysis method.

TSH is a key parameter of pituitary's interpretation of adequacy of circulating thyroid hormones. Studies that deal with the effect of PCB 126 exposure that have analyzed TSH at doses ranging from 13-800 ug/kg have shown no effect (Desaulniers et al., 1999; Martin, 2002).

5'-Deiodinase activity following PCB 126 has not, to our knowledge, been previously analyzed. However, studies involving PCB exposure (Aroclor 1254) have shown a decrease in Type I 5' deiodinase activity up to 72% in rats dosed with 200ppm (Hood and Klaassen, 2000). The overall hypothesis to be addressed in this thesis is that PCB 126 has distinct effects on thyroid hormone homoeostasis and consistent with the "dioxin-like" potential that has previously been reported. This study will also evaluate the temporal responses of PCB 126 on serum T4, T3, rT3, TSH and hepatic Type I 5'-deiodinase in male rats over the period of the study after single oral bolus exposure.

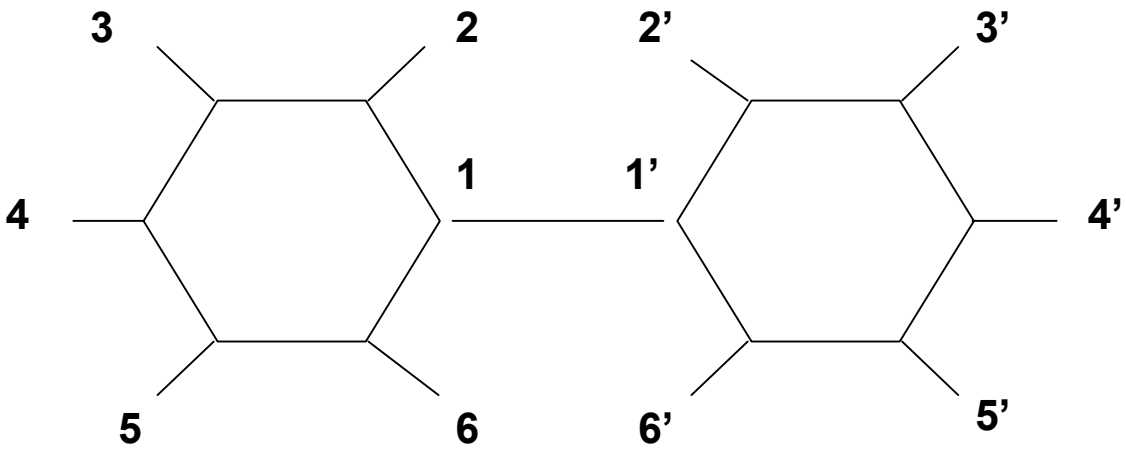
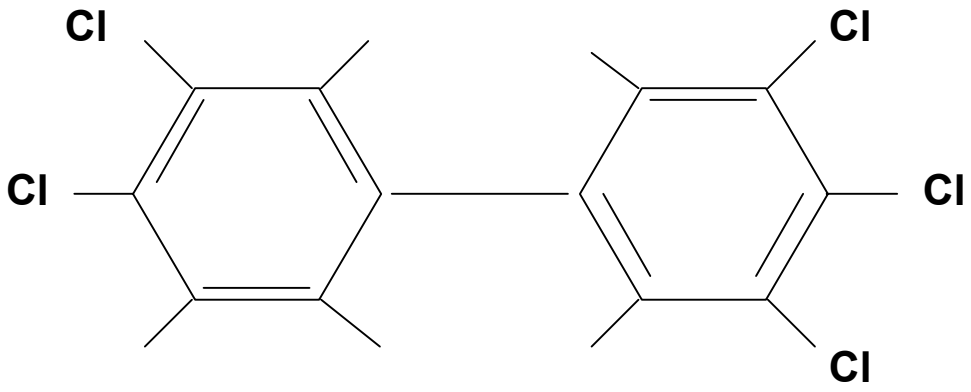


Figure 1.0 The General PCB Congener Structure

Figure 1.1 Chemical Structure of 3, 3', 4, 4', 5-pentachlorobiphenyl (PCB 126)



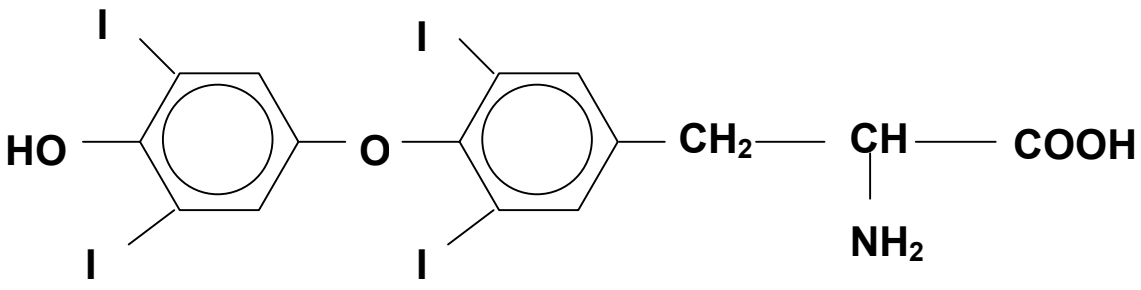


Figure 1.2 THYROXINE (T_4)

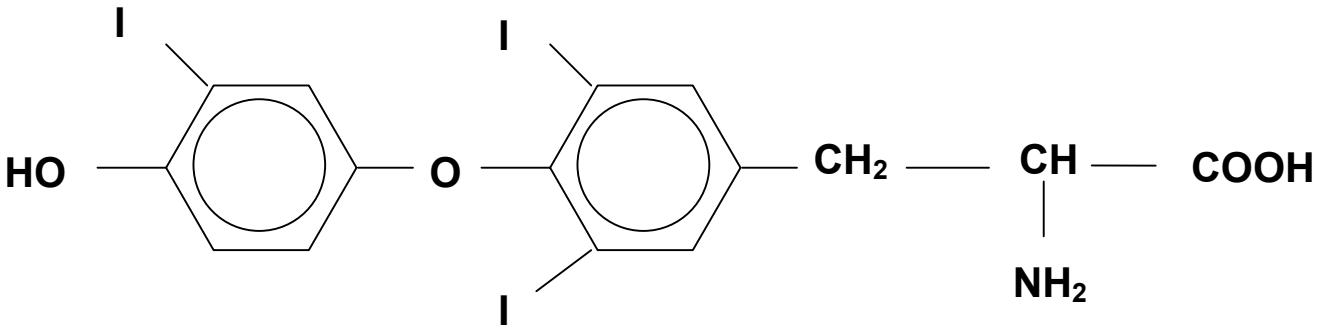


Figure 1.3 TRIIDOTHYRONINE (T_3)

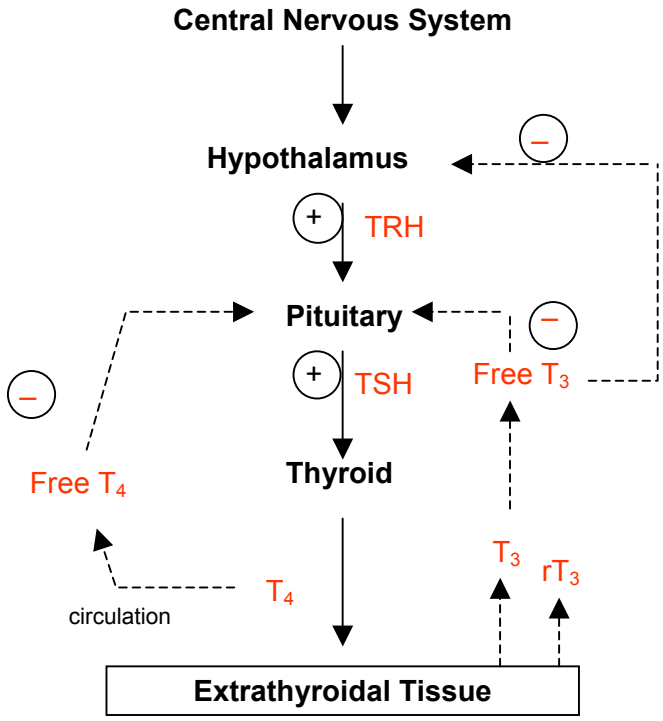


Figure 1.4 Normal Hypothalamic-Pituitary-Thyroid Axis

Neurons whose cell bodies reside in the hypothalamic paraventricular nucleus (PVN) synthesize the tripeptide Thyrotropin-Releasing Hormone (TRH). TRH is delivered by the pituitary-portal vasculature to the anterior pituitary gland to stimulate the synthesis and release of Thyroid Stimulating Hormone (TSH). Pituitary TSH binds to the receptors on the surface of the thyroid follicle cells stimulating adenylate cyclase. The effect of increased cAMP is to increase the uptake of iodide on to thyroid cells, iodination of tyrosyl residues on Tg by TPO, synthesis and oxidation of thyroglobulin (Tg), Tg uptake from the thyroid colloid, and hydrolysis and secretion of the iodothyronines T4 and T3.

Thyroid hormones are carried in the blood by serum proteins, including transthyretin (TTR) and albumin. Thyroid hormones exhibit a negative feedback effect on the release of pituitary TSH and on the activity of the hypothalamic TRH neurons. T4 and T3 are actively

transported into target tissues. T4 can be converted to T3 by the action of outer-ring Deiodination (ORD) or 5'-deiodinase. Thyroid hormones are cleared form the blood in the liver following glucuronidation by UDPGT. These modified thyroid hormones are then eliminated through the bile. T4 and/or T3 are actively concentrated in target cells about 10-fold over that of the circulation, although this is tissue dependent (Zoeller 2003).

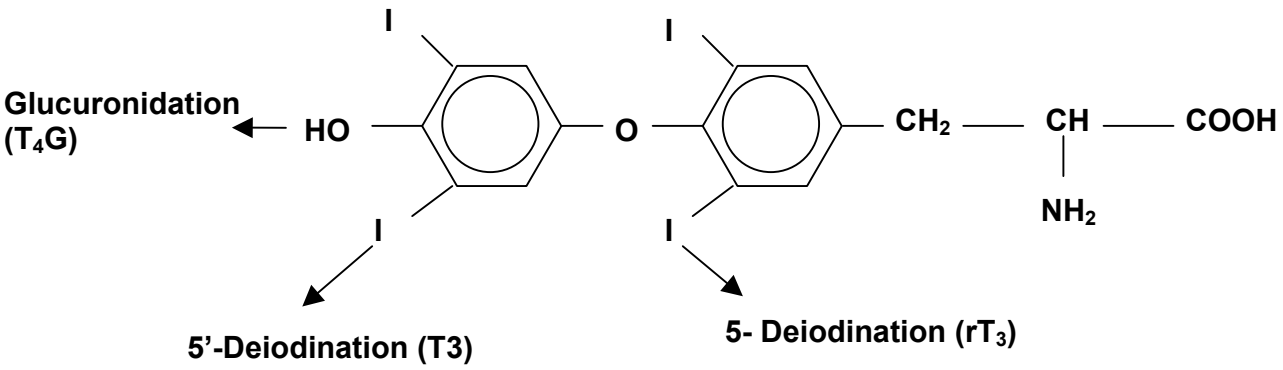


Figure 1.5 THYOXINE METABOLISM

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CHAPTER 2

EFFECTS OF PCB 126 ON LIVER ENZYMES AND THE THYROID AXIS[1,2]

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INTRODUCTION

Polychlorinated biphenyls (PCBs) are a family of 209 synthetic congeners produced by chlorination of a biphenyl molecule. They were produced form the years 1929-1977 for their unique chemical properties such as thermal stability, resistance to acids and bases, and low water solubility. Production was halted due to increasing awareness of health effects associated with PCB exposure. PCBs are very lipophilic and persistent in the environment. PCBs congeners or mixtures (Aroclors) have been associated with various toxic responses in humans and laboratory animals including hepatotoxicity, gastrointestinal and respiratory disturbances, neurotoxicity, immunotoxicity, reproductive and developmental effects, and endocrine disruption including thyrotoxicity (Li and Hanson 1997).

Congeners are categorized into 3 groups according to their ortho substitutions: coplanar, non-coplanar, and mono-ortho chlorine congeners. The coplanar congeners have no chlorines in the ortho positions of the biphenyl rings. Non-ortho coplanar PCB congeners have a high affinity for the aryl hydrocarbon receptor (AhR) protein located in the cytoplasm of the cell , which ultimately leads to induction of several enzymes in the liver including cytochrome P4501A (CYP1A) and uridine diphosphate glucuronyl transferases (UDPGTs) (Bager et al. 1995 and Schurr 1997). PCBs that bind to the AhR are referred to as "TCDD-like" congeners and represent the most toxic congeners (Desaulniers et al., 1999). 3,3',4,4',5-Pentachlorobiphenyl (PCB 126), is a non-ortho coplanar congener and is considered the toxicologically most significant PCB congener present in the environment because it accounts for approximately 40-70% of total toxic potency of PCBs that have "dioxin-like" activity (Hong et al. 1992).
The measurement of circulating hormone concentrations is an important diagnostic tool in evaluating endocrine function and effects of microsomal enzyme inducers.

In laboratory animals, cumulative PCB 126 doses ranging from as low as 10ug/kg have resulted in decreased body weight gain and food consumption (Van Birgelen et al. 1994; Desaulniers 1999) as well as induction of P-450 activity and ethylresorufin-o-deethylase (EROD) activity (Van Birgelen 1994, Desaulniers 1999, Craft 2002, Martin 2002). Also, as with other PCBs (Barter and Klaassen 1994, Schurr 1997), a cumulative PCB 126 dose of 280 ug/kg (40 ug/kg x 7 days) caused about a 6 fold increase in T4 glucuronidation (Martin 2002) and is thought to be the primary mechanism by which PCB 126 disturbs the thyroid axis. Cumulative PCB 126 doses ranging from 35 to 800 ug/kg cause a marked decline in plasma concentrations of free thyroxine (FT4) and total thyroxine (TT4) in rats (Van Birgelen et al. 1994; Martin 2002; Craft et al., 2002; Desaulniers et al., 1999). PCB 126 does not readily affect serum T3 and rT3 concentrations. Serum T3 and rT3 levels are reported to decline modestly or not significantly (Martin 2002; Sridhar 2004; Barter and Klaassen 1994). Thyroid stimulating hormone (TSH), the pituitary hormone which increases in response to low concentrations of circulating free thyroid hormones, has also been shown to increase in the presence of PCB 126 exposure (Barter and Klaassen 1994).

Another mechanism for PCBs to alter thyroid function is altering non-deiodinative metabolism. Since the majority of T3 found in serum is derived from 5' (outer-ring) deiodination (ORD) of T4, increased deiodination may play a role in maintaining serum T3 concentration in rats treated with microsomal enzyme inducers (Hood and Klaassen 2000). However, 5'- deiodinase activity following administration of 50 ppm of Aroclor (a commercial PCB mixture) was less than controls.

In the present study, we hypothesized that PCB 126, doses as low as 7.5ug/kg and an estimated half-life in liver of 30 days (Yoshimura et al. 1985), would result in disruption of

thyroid hormone homeostasis by increased elimination of TH from circulation. The endocrine-disrupting effects of PCB 126 were tested in adult male rats, by measuring the concentrations levels of T4, Free T4, T3, rT3 and TSH. Histopathological analysis including morpho-metric analysis of colloid and epithelial cell volume was performed to identify histological changes potentially induced by TSH. Hepatic Type I 5-deiodinase activity was also measured.
The temporal pattern and dose-response characteristics of PCB 126 induced perturbations in the thyroid axis in the male rat were characterized as part of a larger research program to develop biologically based model of toxicant effects on the thyroid axis.

MATERIALS AND METHODS

Chemicals and Reagents. PCB 126 was obtained from Accustandard Corporation (New Haven, CT) at purity greater than 99%. Stock solutions were prepared by dissolving PCB 126 in n-hexane, mixing the n-hexane with the corn oil (Mazola™, Best Foods, Englewood Cliffs, NJ), and then removing the n-hexane by evaporation (adapted from DeVito *et al*., 1993). Concentrations of PCB 126 in stock solutions were checked by GC/FED. All solutions were prepared by dilution of equal amounts of hexane or hexane with corn oil. Four dosing solutions were prepared (0.0, 7.5, 75, 275 ug/kg) with final concentrations of 0.0, 0.002, 0.020, and 0.0599 mg/ml, respectively.

Animals. Male Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA) weighing 151-175 grams. Animals were housed two per "shoe-box" style arrangement and were allowed to acclimate for 5 days before use. Animals had unrestricted access to PMI #5001 rodent chow (PMI feeds, St. Louis, MO) and water. Animals were kept in

a humidity/climate-controlled facility with a 12-hour light/dark cycle. Rats were weighed upon dosing and then weekly until sacrifice. Rats were euthanized by CO_2 asphyxiation.

Dosing Protocol. Rats were given a single oral bolus of PCB 126 in corn oil (7.5,75,or 275ug/kg) (n=8) or corn oil control (n=8). The dosing volume was adjusted to give 1.0ml/kg body weight. After dosing, tissues and blood were collected for PCB 126 analysis and thyroid axis perturbations at 8hr, 1d, 5d, 9d, 22d, 35d (275 ug/kg group only), and 59d (275 ug/kg group only) between 9:30- 12:30 AM. Additional rats were killed at other time points (n=4) for analysis of PCB 126. The pharmacokinetic data for PCB 126 in blood, perirenal fat and liver are reported elsewhere.

Tissue Collection and Preparation. Blood was collected from the inferior vena cava using an 18½-gauge needle (Becton Dickinson) and 10cc syringe (Becton Dickinson). Blood was placed into serum separator tubes, allowed to clot and centrifuged for 15min at room temperature. Serum aliquots were stored at -80ºC until analysis of TSH and thyroid hormones. Livers were excised and portions of the liver divided for analysis of P450 enzymes, P450 CYP1A1, and Type I 5'-deiodinase activity.

Liver microsomes were prepared by taking five grams of liver sample from each animal, homogenizing in 0.02 M Tris-HCl containing 0.15 M KCl (pH 7.4) and centrifuging at 10,000g for 30 min at 4ºC. The pellet was then washed, resuspended in 0.25 M sucrose containing 10mM EDTA and 1.15% KCl (pH 7.4) and stored at -80°C. Liver homogenates for Type I 5'-deiodinase activity were prepared by homogenizing 5 grams of liver in 5mls of 100mmol/L potassium phosphate, pH 7.0, containing 1mmol/L Ethylenediamine tetraacetate (EDTA), and 1mmol/L dithiothreitol (DTT) and stored at 4ºC. Pituitary tissues were pooled 4 per tube and homogenized in 5x the pituitary weight volume of 100mmol/L potassium

phosphate, pH 7.0, containing 1mmol/L EDTA, and 20mmol/L DTT. Protein concentrations for homogenates were done using the Bradford Method (Bradford, 1976). Thyroids were excised, weighed and placed in 10% formalin for histopathology to determine follicle/ colloid epithelium volume ratios.

Enzyme Analysis. The standard methods of Lowry *et al*. (1951) and Omura and Sato (1964) were used to quantify hepatic microsomal protein and total P450 levels, respectively. Ethoxyresorufin-O-deethylation (EROD) was measured as indicator of activity of CYP1A1. The fluorimetric technique of Burke and Mayer (1974), as modified by Lubet *et al*. (1985), was used to quantify EROD activity.

Hormone Analysis. Serum free thyroxine (FT4) analysis was carried out at first thaw of the serum aliquots. FT4 serum concentrations were measured by direct dialysis using a radioisotopic Nichols Kit cat. # 44-2210. (Nichols Diagnostic, Inc.) Serum TT4, T3 concentrations were quantified by radioimmunoassay (Chopra 1980, El Tom et al. 1992). Rat Serum rT3 was measured by radioimmunoassay as described by Ferguson and Peterson (1992). Labeled hormones T3 ([^{125}I] triiodothyronine, high specific activity)(IM321-20uCi) and T4 (1-[3',5'-^{125}I] thyroxine, high specific activity)(IM141-50uCi) were obtained from Amersham Biosciences. Serum TSH was measured by radioimmunoassay kit ICN Diagnostics #07C-90102 (Orangeburg, NY).

Type 1 5'-Deiodinase activity was measured by placing 10ug of liver homogenate into 20ul of buffer and samples put in 12x75mm glass tubes in triplicate in a 37°C water bath. The reaction was started with the addition of 100ul of reaction buffer containing (100mmol/L

potassium phosphate, pH 7.0, containing 1mmol/L Ethylenediamine tetraacetate (EDTA), 2mmol/L dithiothreitol(DTT), 3ul/ml ^{125}I -rT3, and 5.0ug/ml rT3 cold) buffers for 2 minutes and 12 minutes each for a total of 12 tubes per sample. The reaction was carried out with and without PTU. The reaction was terminated with the addition of 500ul of fetal bovine serum (FBS) and immediately incubated on ice for 30 minutes. After incubation, 500ul of 10% TCA was added to each tube, vortexed and centrifuged at 3,000 rpm for 15minutes at 4°C. 500ul was taken from supernatant and placed in a fresh tube. Pellet and supernatant were counted for radioactivity with a gamma counter.

Thyroid Colloid/Epithelium Volume Ratios. Quantitative thyroid analysis was performed by computerized digital volumetry. Both thyroid glands from each rat were fixed in neutral buffered 10% formalin solution, embedded in paraffin, sectioned sagittally at 3μm, and stained with hematoxylin and eosin. The volume fractions of follicular epithelial cells and of colloid were measured by a steriometric technique using light microscopy and an eyepiece reticle (Russ, 1986). Volume fractions were expressed as percent of tissue volume occupied by follicular epithelial cells and percent of tissue volume occupied by colloid.

Statistical Analysis. Treatment-related effects were first evaluated with a two-way ANOVA followed by a least significant difference test ($P < 0.5$), unless otherwise noted, using the statistical software package SASv8.2 (SAS Institute, Cary, NC). Multiple regression was also used to test for significant relationships with dose and day.

RESULTS

Control Rats

Liver: Mean total P450 control amounts varied from about 0.3 to1.1 nmole/mg protein over the 59 days of the study (Fig. 2) with Days 35 and 59 showing the greatest variation. The mean EROD control activity (Fig. 3) over the 59-days ranged from 13.6 to19.4 nmoles /min/mg protein. Mean Type I 5'- deiodinase control activities were similar on Days 35 and 59 (Table 1), but the Day 9 activity was nearly two fold greater. The reason for this is unknown.

Thyroid Function: Mean free T4 (Fig. 4) serum concentrations showed little variation over 59 days with values ranging from 2.5-3.0 ng/dl, while mean total T4 serum concentrations varied more with values that ranged from 4.8 to 7.6 ug/dl (Fig. 5). Mean total T3 and rT3 control concentrations (Fig. 7 and 8), ranged from 63.5 to 88.2 ng/dl and 2.8 to 7.0 ng/dl, respectively, over 59 days. TSH serum concentrations ranged from 4.5 to 8.3 ng/ml over 59 days of study (Fig. 9).

PCB 126 Treated Rats

PCB 126 doses of 7.5, 75 or 275 ug/kg, did not significantly alter body weight gain, although the mean body weight gain for the 275 ug/kg dose was lower on Day 35 compared to controls (Fig. 1). Liver P450 and EROD activities were increased in all dose groups (Figs. 2 and 3). Over 22 days of treatment the 75 ug/kg dose group P450 activity was elevated, with peak induction on Day 5 post dosing. The 7.5 ug/kg dose group peak P450 activity was also on Day 5, but P450 activity returned to near control values by Day 9 post dosing. EROD activity peaked on Day 1 and 5 post dosing for the 7.5 and 75 ug/kg dose groups, respectively, and then declined throughout the remainder for of 22 day study period. Over a 59 day study period the P450 and EROD activities remained elevated in the 275 ug/kg dose group with peak mean enzyme

activities on Day 22 for P450 and Day 5 for EROD. On Day 9 there as a noticeable decline in liver enzyme activities for this group.

Mean serum total and free T4 values were depressed in a dose dependent manner (Figs., 4 and 5) over 22 Days in the 75 ug/kg dose group and 59 Days in the 275 ug/kg dose group, although for the total T4 serum concentrations on Days 35 and 59 were not statistically different from control values for the 275 ug/kg dose group. A decline in serum total T4 concentration occurred by 24 hours in the 275 ug/kg dose group and continued through Day 9 and then the serum levels increased throughout the remainder of the 59 day study period approaching control serum concentrations. Recovery of serum total T4 concentrations was also evident in the 75 ug/kg dose group by Day 22. In the 7.5 ug/kg dose group mean total and free T4 serum levels were not significantly different from controls over the 22 Day study period. Mean serum TSH concentrations were increased by PCB 126 administration with statistically significant changes occurring on Days 9 and 22 in the 75 and 275 ug/kg dose groups (Fig. 6).

Serum Total T3 levels was not altered appreciably by PCB 126 administration (Fig. 7). Although on Day 22 the 75 and 275 ug/kg dose group serum total T3 levels were significantly less than controls, but not in a dose-related manner. Mean rT3 serum concentrations were somewhat variable throughout the study and found to be significantly different from control values on Day 5, but not in a dose related manner.

PCB 126 caused a decrease in mean liver type 1 5′ deiodinase activities compared to corresponding controls (Table 1) with statistically significant reductions only on Day 59 in the 275 ug/kg dose group. Table 2 provides the thyroid follicle: colloid volume ratios for rats on Days 22 dosed with 7.5, 75 and 275 ug/kg PCB 126 and on Days 35 and 59 dosed with 275 ug/kg PCB 126. Statistically significant reductions in the ratios were found for all dose groups.

DISCUSSION

The effects of exposure to PCB 126 were assessed by measuring the dose response of liver drug metabolizing enzymes and serum hormone levels in adult male rats. These results demonstrate that circulating FreeT4, T4 and TSH levels are the key endocrine indicators of PCB 126 exposure. PCB-126 treatment decreased T4 (Fig. 2.4, Fig.2.5, Fig 2.6b) and increased TSH (Fig. 2.9). These findings are somewhat inconsistent with previous studies (Table 3) which have shown the same dose related decreases in serum T4 concentrations levels, yet have not found the negative feedback effect of increased TSH levels (Desaulniers et al., 1999; Martin 2002). Previous studies have showed that serum levels of T4 decline at cumulative doses of PCB 126 at 35, 40, 50ug/kg (Desaulniers et al., 1999; Craft et al., 2002; Martin, 2002), while TSH levels did not significantly increase (Desaulniers et al., 1999; Martin, 2002). Studying the time course of PCB 126 induced perturbations of the thyroid axis allowed for examining both the onset and recovery of the thyroid axis. Examples of this are given in Fig 2.5 and Fig 2.6b with serum T4. These results are explained by the hypothalamus and the pituitary's detection of low free TH concentrations in the serum, resulting in a negative feedback leading to the production of TSH to stimulate TH production by the thyroid. The response of serum T3 and rT3 serum concentrations (Fig 2.7, Fig 2.8) were minimal but may be complicated by the 5' and 5- peripheral tissue deiodinase enzyme activities. A study by Desaulniers et al. (1999) has shown significant reductions in TT3 levels when a cumulative dose of 200ug/kg PCB 126 was achieved.

Inductions of hepatic microsomal enzymes, specifically EROD (a marker for CYP1A1) and uridine diphosphate glucuronyl transferases (UDPGT) have been found to be important mechanistic indicators related to decreased serum T4 levels (Craft et al., 2002; Martin, 2002). The effect of PCB 126 on P450 enzymes, specifically CYP1A1, was clearly induced starting by

day 1 in all dose groups and remained induced to day 59 for the 275ug/kg dose (Fig 2.2, Fig 2.3). The induction of CYP1A1 through day 59 of the study suggests that PCB 126 is still persistent in the body and further analyses of thyroid hormones at all doses beyond 59 days would be useful. Induction of the CYP1A1 activity was expected and consistent with other findings following PCB 126 administration (Martin, 2002; Craft et al., 2002; Desaulniers et al., 1999). The observed decreases in serum concentrations of FT4 suggest that PCB 126 alters thyroid function by increased phase II conjugation of T4 as previously reported by McCleary (2001). Previous studies have shown significant increases in T4-UDPGT activity have been associated with increased biliary excretion of radiolabeled T4 (Martin 2002; Craft et al., 2002). UDPGT is the primary enzyme responsible for increasing the water solubility of lipophilic steroid and thyroid hormones. When biliary T4 clearance is increased in the rat hypothyroidism may result. This study indirectly supports this mechanism of action (Fig 2.6a). Analysis of T4-UDPGT in liver of animals treated with PCB 126 is required. The persistently low FT4 concentrations (Fig 2.4) with no increase in T3 or rT3 concentrations are highly suggestive that non-deiodinative thyroxine metabolism is enhanced.

In the present study a histopathology analyses of the thyroid gland tissue revealed a typical pattern decreases of follicular colloid/ follicular epithelium ratio for TSH stimulated thyroid gland. Desaulniers et al. (1999) analyzed the histology of the thyroid glands of PCB 126 treated animals and revealed that the follicular colloid is less dense than in control animals which is consistent with depletion by TSH stimulation. Important changes in thyroid histology are usually associated with significant changes in TSH levels. Perhaps the most sensitive endocrine indicator of PCB exposure is free serum thyroxine concentrations (Desaulniers et al., 1999). The present study is a therefore consistent with the findings the decrease in FT4 concentrations

on days 5 and 22 at the 75 and 275ug/kg doses which may be caused by increased biliary clearance and is corroborated by the observed reduction in thyroid colloid follicle ratios.

Since the majority of T3 found in serum is derived from outer-ring deiodination (ORD) of T4, increased deiodination is a potential physiological mechanism that may play a role in maintaining serum T3 concentration in rats treated with microsomal enzyme inducers (Hood and Klaassen 2000). It has been shown that TSH stimulated type-I ORD activity in the thyroid (Wu et al., 1985). Authors of a previous study of the effects of Aroclor 1254 suggested that to maintain levels of T3 following exposure to microsomal enzyme inducers, Type I 5'-deiodinase activity would be induced (Hood and Klaassen 2000). However, both the previous and present studies showed that Type I 5'- deiodinase activity in the liver was reduced in a dose-related manner (Hood and Klaassen 2000) following PCB exposure (Table 2). ORD activities in the liver are regulated by thyroid hormones, and therefore the reduced ORD activities are a physiological response to the hypothyroid state (Hood and Klaassen 2000) or, as we have shown for TCDD, an indirect effect of the chemical.

Because T3 serum concentrations did not significantly change with dosage, we speculate that adaptive responses occurred to maintain T3 concentrations such as a decrease in 5' Type I deiodinase activity in liver due to hypothyroidism or increased production of T3 by the thyroid (Schurr et al., 1997). Type II 5' deiodinase activity has been previously measured in rat tissue (Hood and Klaassen 2000), and showed that when TH concentrations are low, brain Type II 5'deioination activity increases.

To our knowledge this was one of the first studies to apply the technique of FT4 measurements by equilibrium dialysis to studies of PCB effects on thyroid hormone metabolism. Many studies of PCBs and the thyroid axis in non-primate species show declines in serum FT4

levels using non-dialysis methodology. This probably results in an underestimation of the FT4 levels in serum. Non-dialysis procedures are generally not accurate in non-primate species because of the lower amount of binding affinity of TBG in these species. Also, PCBs have been shown to be inhibitors of transthyretin, which may contribute to the under reporting of FT4 levels.

The present study is the first to evaluate single oral bolus doses of PCB 126 to evaluate the onset and temporal TH concentrations. Although exposure to one specific congener of PCB is not likely, understanding the effects and potential thyroid implications of the most toxic PCB congener may help in assessing the upper bounds of toxicity.

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FIGURE LEGEND

Figure 2.1. Effects of vehicle only, 7.5, 75, or 275ug/kg bw single oral bolus dose at day 0 on PCB 126 dose-related weight gain. Data are expressed in grams. Results represent the mean ± standard error of 8 rats.

Control groups are represented by the solid black column, 7.5ug/kg/bw dose is represented by the gray/white striped column, 75ug/kg/bw dose is represented by the checkered column, and the 275ug/kg/bw dose is represented by the solid gray column.

Figure 2.2. Effects of PCB 126 on rat liver microsome P450 activities. Data are expressed in nmol/mg protein. Rats received either vehicle only, 7.5, 75, or 275ug/kg bw single oral bolus dose at day 0. Results represent the mean ± standard error of 8 rats. Asterisks denote significant differences ($p \leq 0.05$) from control values.

Control groups are represented by the solid black column, 7.5ug/kg/bw dose is represented by the gray/white striped column, 75ug/kg/bw dose is represented by the checkered column, and the 275ug/kg/bw dose is represented by the solid gray column.

Figure 2.3. Effects of PCB 126 on rat liver microsome EROD activities. Data are expressed in nmol/min/mg protein. Rats received either vehicle only, 7.5, 75, or 275ug/kg bw single oral bolus dose at day 0. Results represent the mean ± standard error of 8 rats. An asterix denotes significant differences ($p \leq 0.05$) from control values.

Control groups are represented by the solid black column, 7.5ug/kg/bw dose is represented by the gray/white striped column, 75ug/kg/bw dose is represented by the checkered column, and the 275ug/kg/bw dose is represented by the solid gray column.

Figure 2.4. Effects of PCB 126 on serum free T4 levels. Data are expressed in ng/dl. Rats received either vehicle only, 7.5, 75, or 275ug/kg bw single oral bolus dose at day 0. Results represent the mean ± standard error of 8 rats. An asterix denotes a significant difference ($p \leq 0.05$) from control values.

Control groups are represented by the solid black column, 7.5ug/kg/bw dose is represented by the gray/white striped column, 75ug/kg/bw dose is represented by the checkered column, and the 275ug/kg/bw dose is represented by the solid gray column.

Figure 2.5. Effects of PCB 126 on serum total T4 concentrations were significantly decreased following Data are expressed in ug/dl. Rats received either vehicle only, 7.5, 75, or 275ug/kg bw

single oral bolus dose at day 0. Results represent the mean ± standard error of 8 rats. An asterix denotes a significant difference ($p \leq 0.05$) from control values.
Control groups are represented by the solid black column, 7.5ug/kg/bw dose is represented by the gray/white striped column, 75ug/kg/bw dose is represented by the checkered column, and the 275ug/kg/bw dose is represented by the solid gray column.

Figure 2.6a. Correlation between hepatic EROD activity expressed in nmol/min/mg protein and serum total T4 concentrations expressed in ug/dl in PCB 126 treated rats.
The diamond bullet line is representative of day 5, and the triangle bullet line represent day 22.

Figure 2.6b. Correlation between total T4 levels exporessed in ug/dl and the 275ug/kg dose on all days. The square bullet line represents temporal changes in total T4.

Figure 2.7. Effects of PCB 126 on serum total T3 concentrations. Data are expressed in ng/dl. Rats received either vehicle only, 7.5, 75, or 275ug/kg bw single oral bolus dose at day 0. Results represent the mean ± standard error of 8 rats. An asterix denotes a significant difference ($p \leq 0.05$) from control values.
Control groups are represented by the solid black column, 7.5ug/kg/bw dose is represented by the gray/white striped column, 75ug/kg/bw dose is represented by the checkered column, and the 275ug/kg/bw dose is represented by the solid gray column.

Figure 2.8. Effects of PCB 126 on serum rT3 concentrations Data are expressed in ng/dl. Rats received either vehicle only, 7.5, 75, or 275ug/kg bw single oral bolus dose at day 0. Results

represent the mean ± standard error of 8 rats. An asterix denotes a significant difference ($p \leq 0.05$) from control values.

Control groups are represented by the solid black column, 7.5ug/kg/bw dose is represented by the gray/white striped column, 75ug/kg/bw dose is represented by the checkered column, and the 275ug/kg/bw dose is represented by the solid gray column.

Figure 2.9. Effects of PCB 126 on serum TSH Data are expressed in ng/ml. Rats received either vehicle only, 7.5, 75, or 275ug/kg bw single oral bolus dose at day 0. Results represent the mean ± standard error of 8 rats. An asterix denotes a significant difference ($p \leq 0.05$) from control values.

Control groups are represented by the solid black column, 7.5ug/kg/bw dose is represented by the gray/white striped column, 75ug/kg/bw dose is represented by the checkered column, and the 275ug/kg/bw dose is represented by the solid gray column.

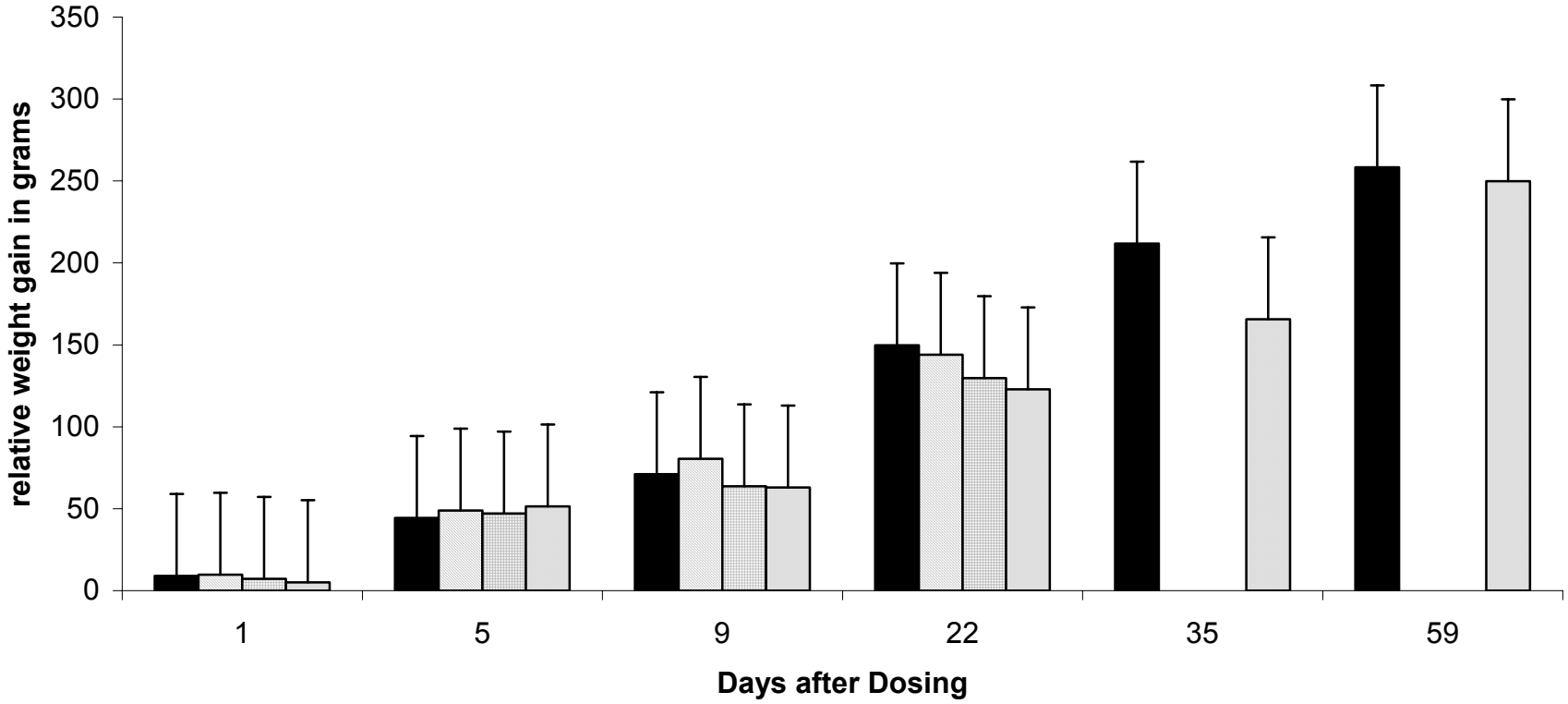
350
300
250
200
150
100
50
0
relative weight gain in grams
1
5
9
22
35
59
Days after Dosing

Figure 2.1

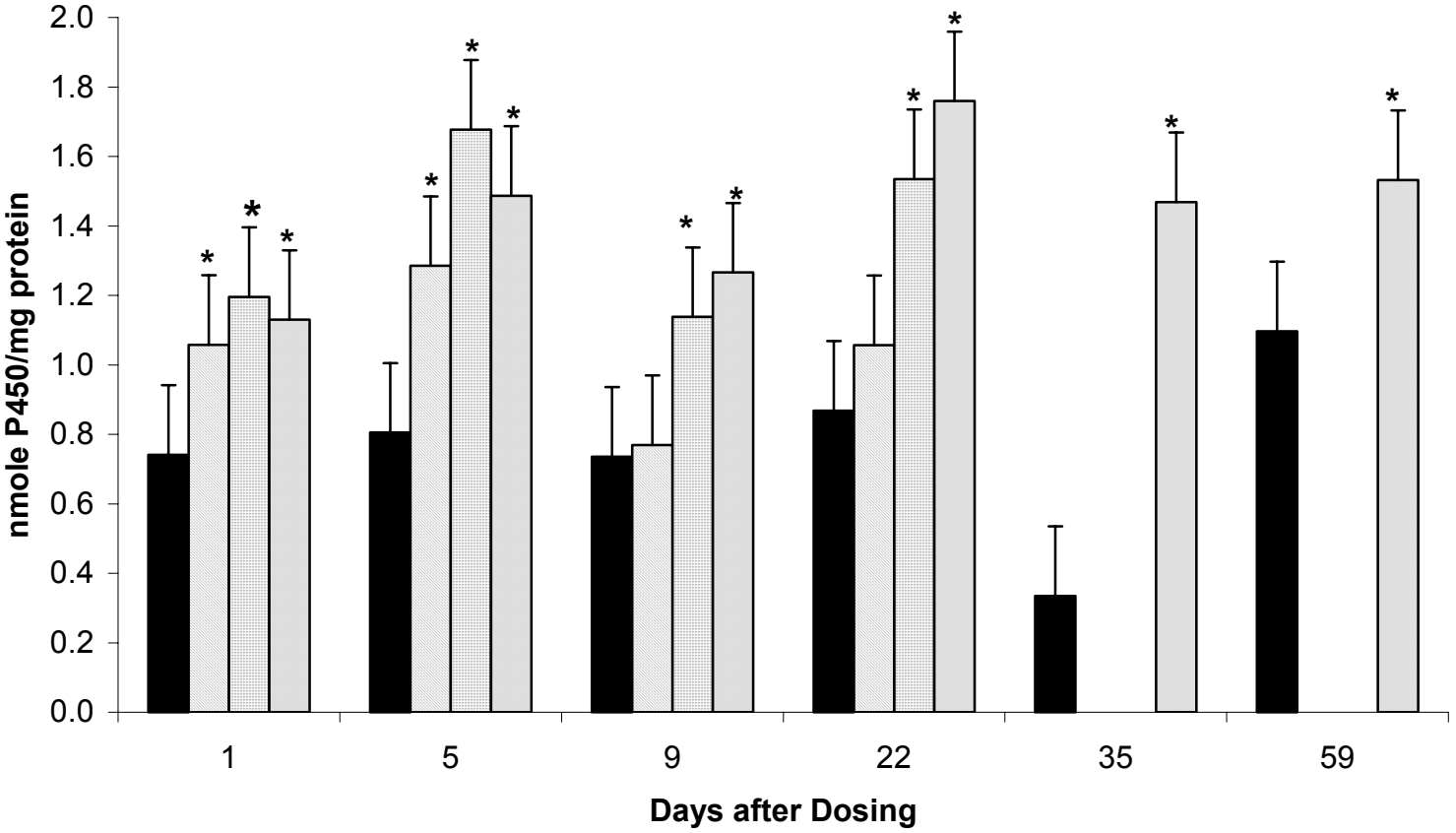
nmole P450/mg protein
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1
5
9
22
35
59
Days after Dosing

Figure 2.2

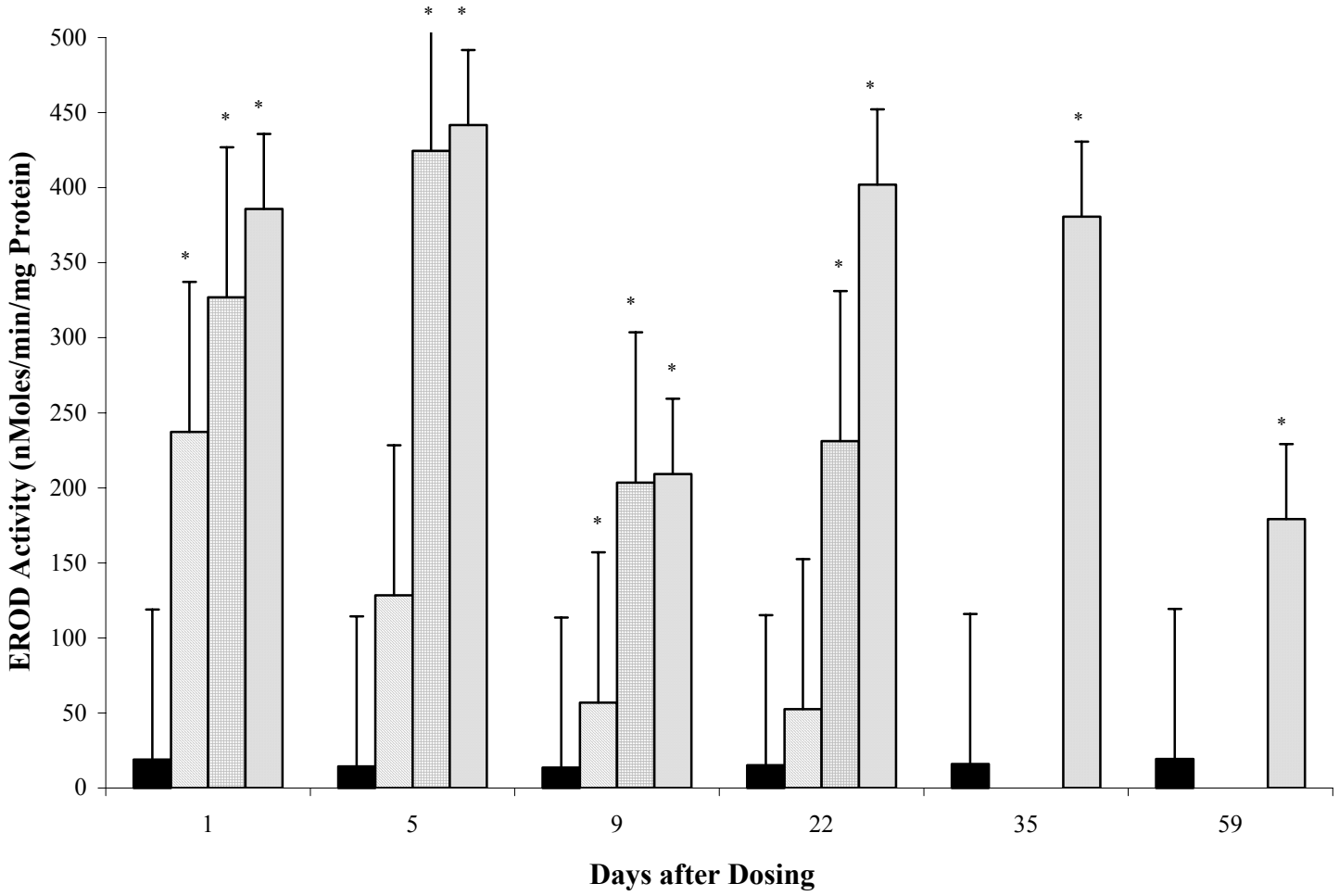
EROD Activity (nMoles/min/mg Protein)
500
450
400
350
300
250
200
150
100
50
0
1
5
9
22
35
59
Days after Dosing

Figure 2.3

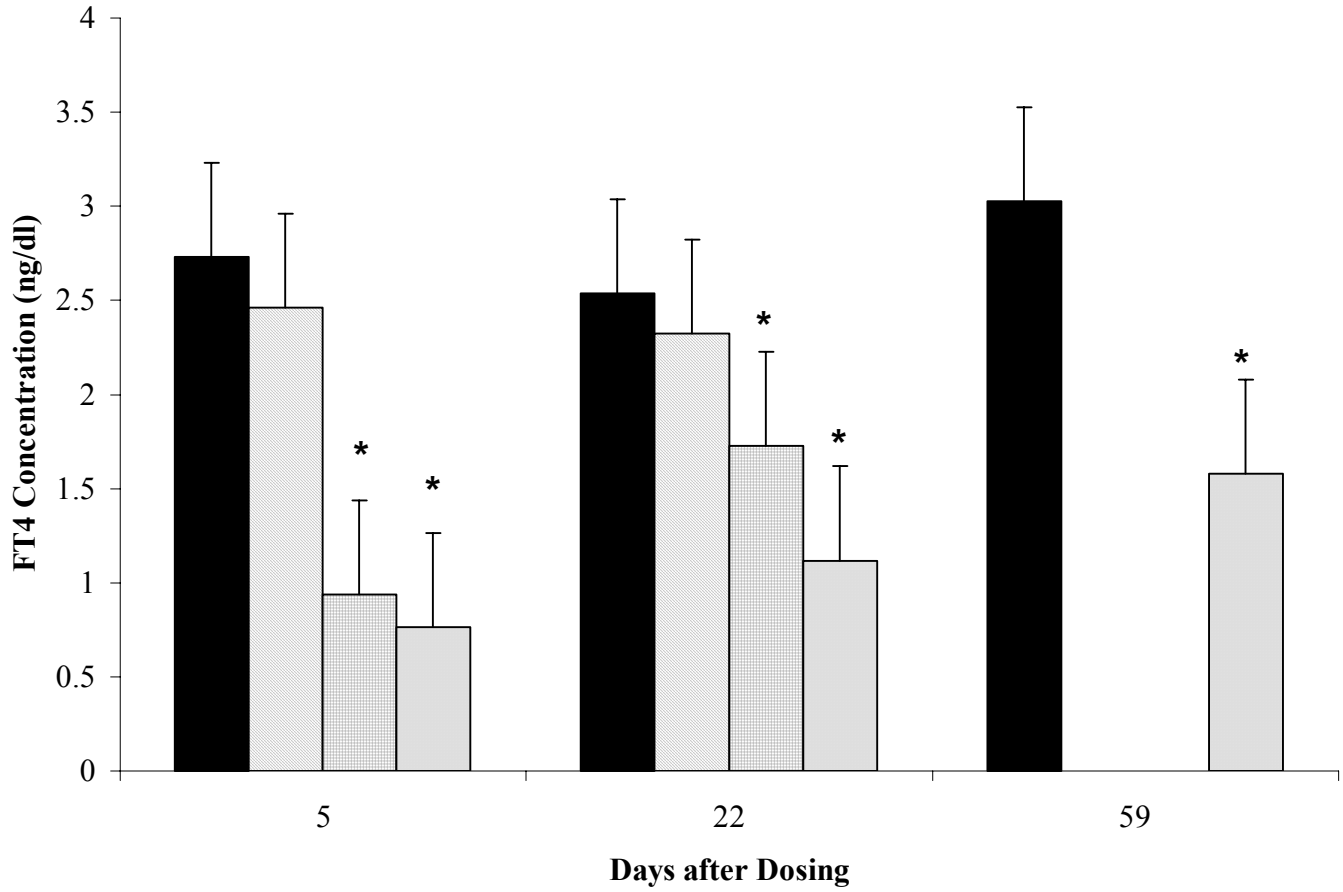
4
3.5
3
2.5
2
1.5
1
0.5
0
FT4 Concentration (ng/dl)
*
*
*
*
*
5
22
59
Days after Dosing

Figure 2.4

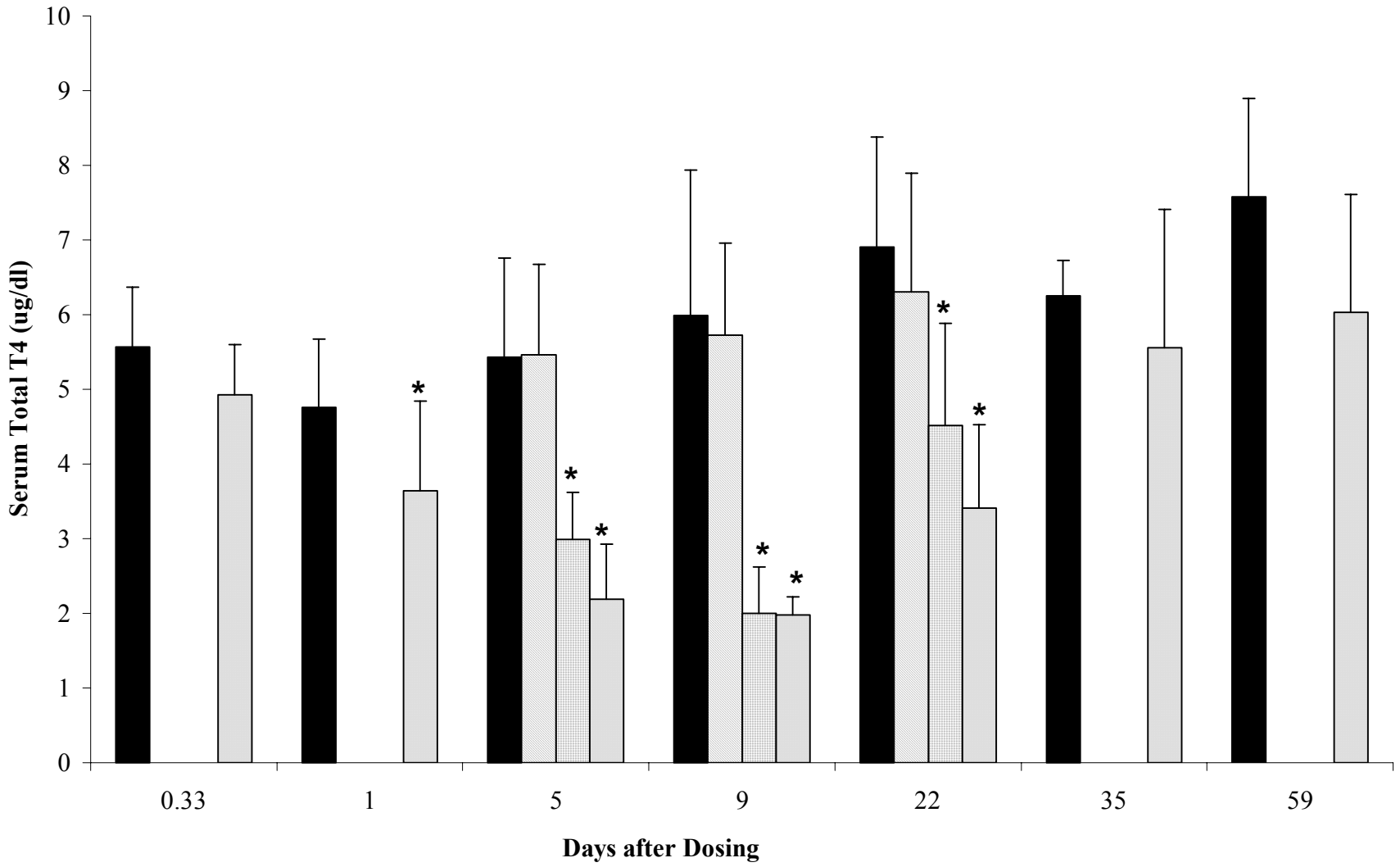
10
9
8
7
6
5
4
3
2
1
0
Serum Total T4 (ug/dl)
*
*
*
*
*
*
0.33
1
5
9
22
35
59
Days after Dosing

Figure 2.5

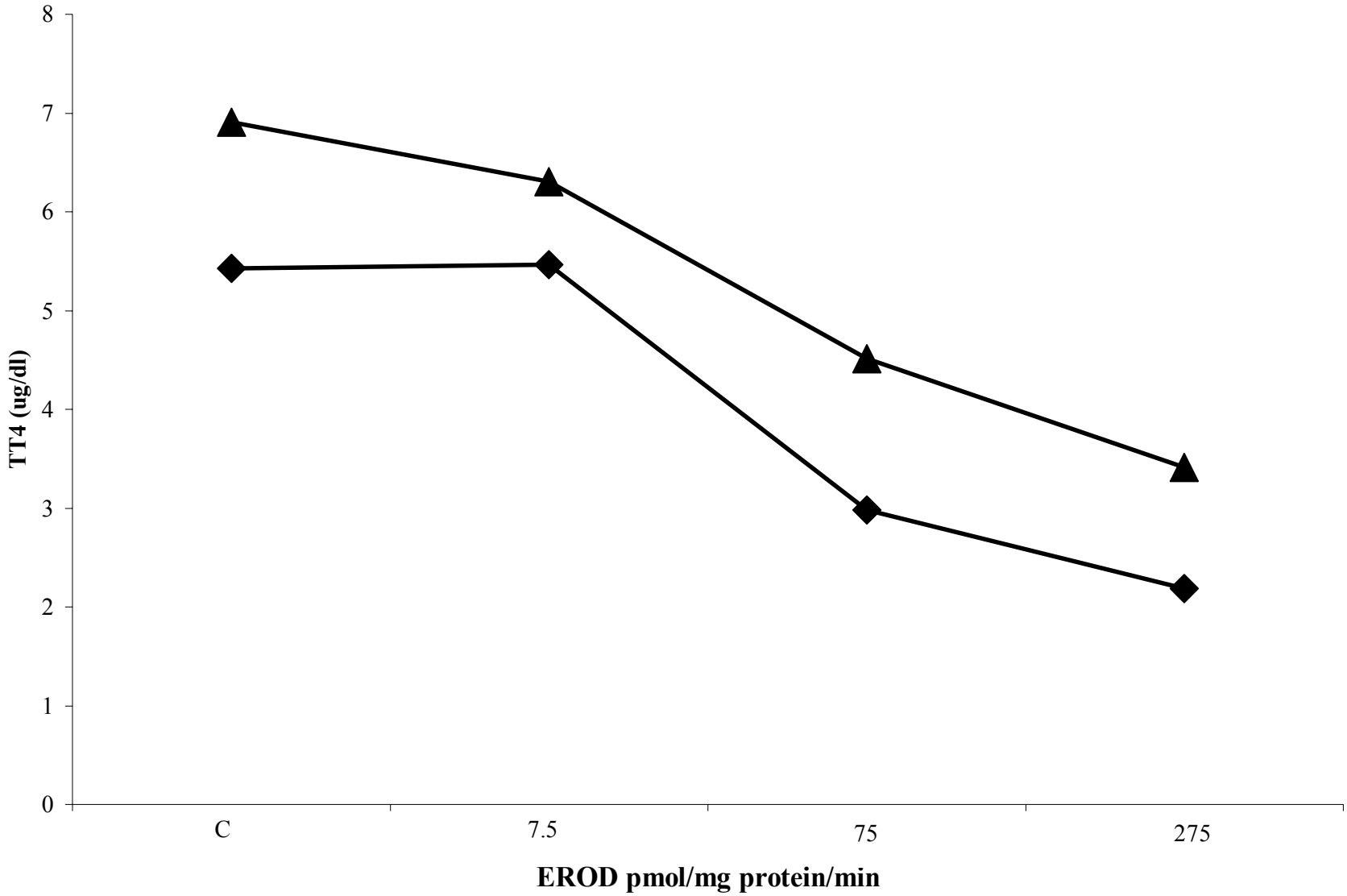

Figure 2.6a

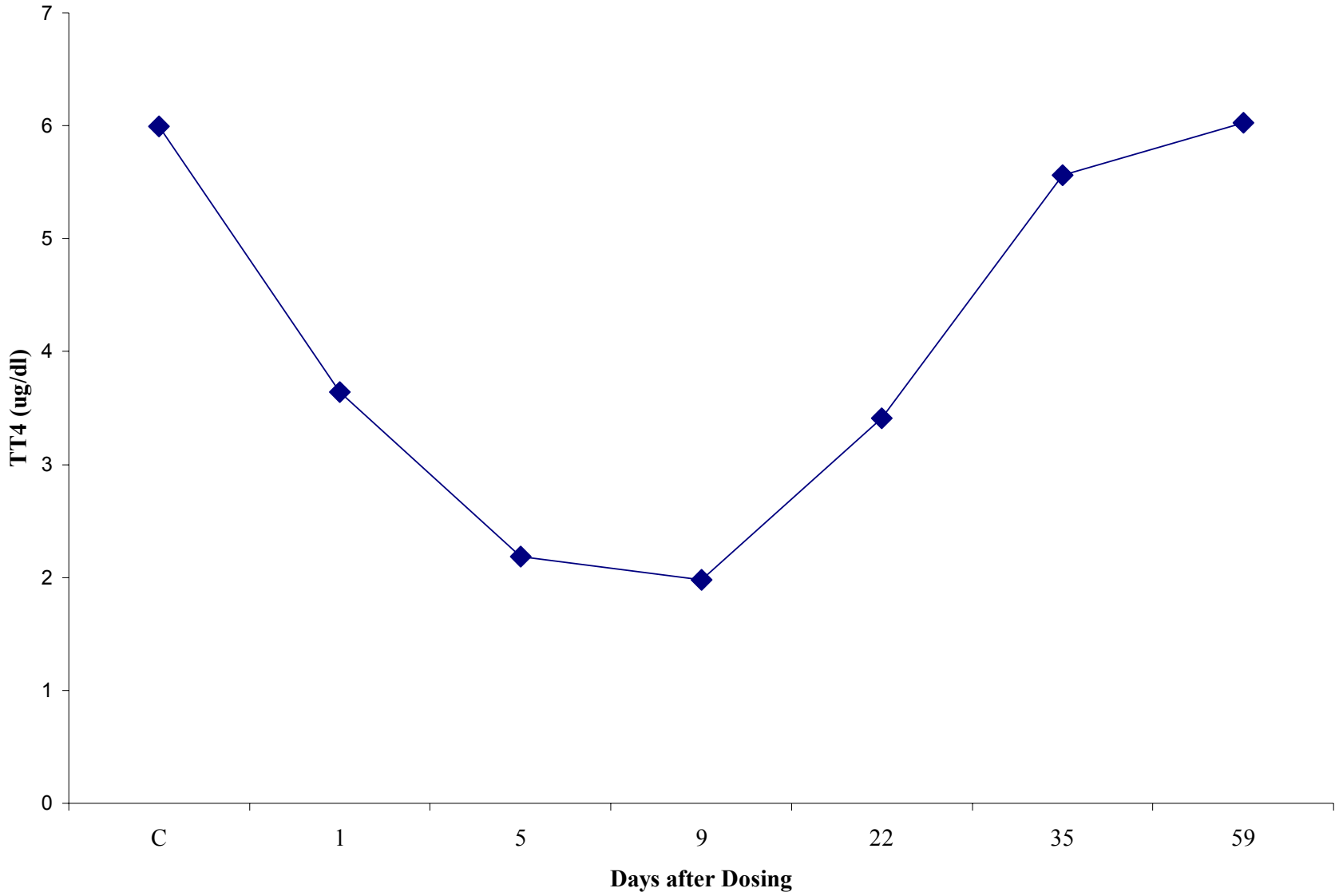

Figure 2.6b

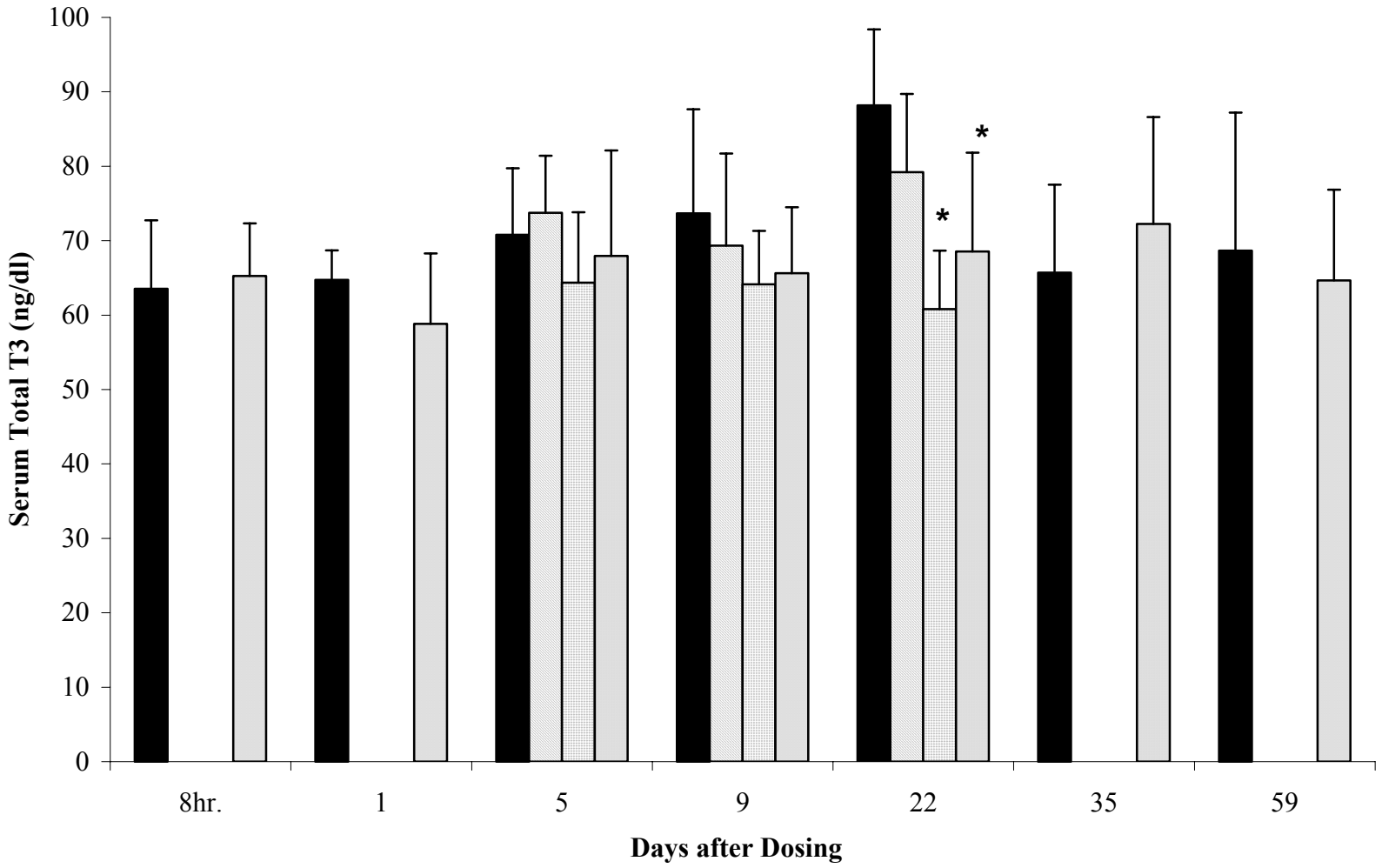
100
90
80
70
60
50
40
30
20
10
0
Serum Total T3 (ng/dl)
*
*
8hr.
1
5
9
22
35
59
Days after Dosing

Figure 2.7

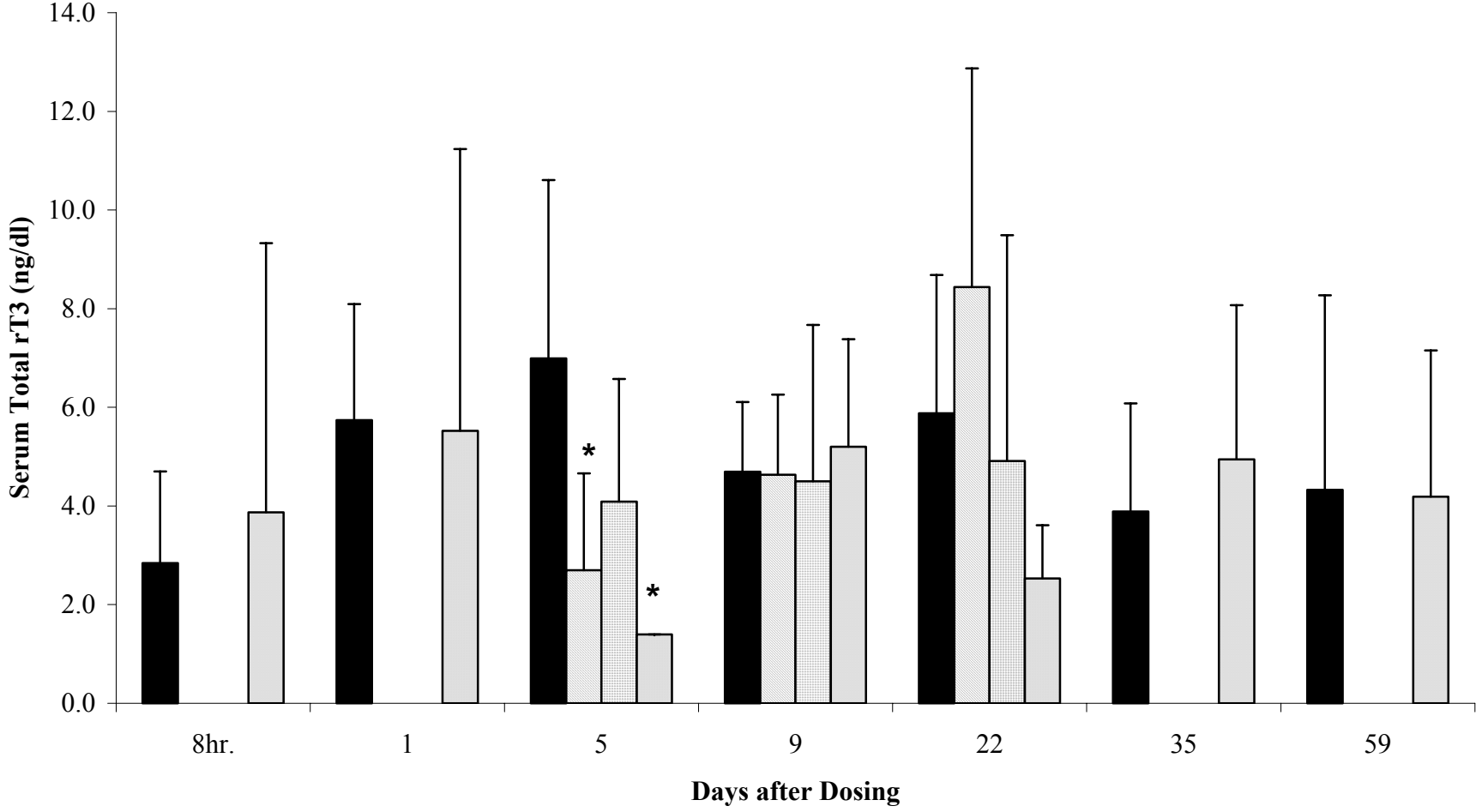
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
Serum Total rT3 (ng/dl)
*
*
8hr.
1
5
9
22
35
59
Days after Dosing

Figure 2.8

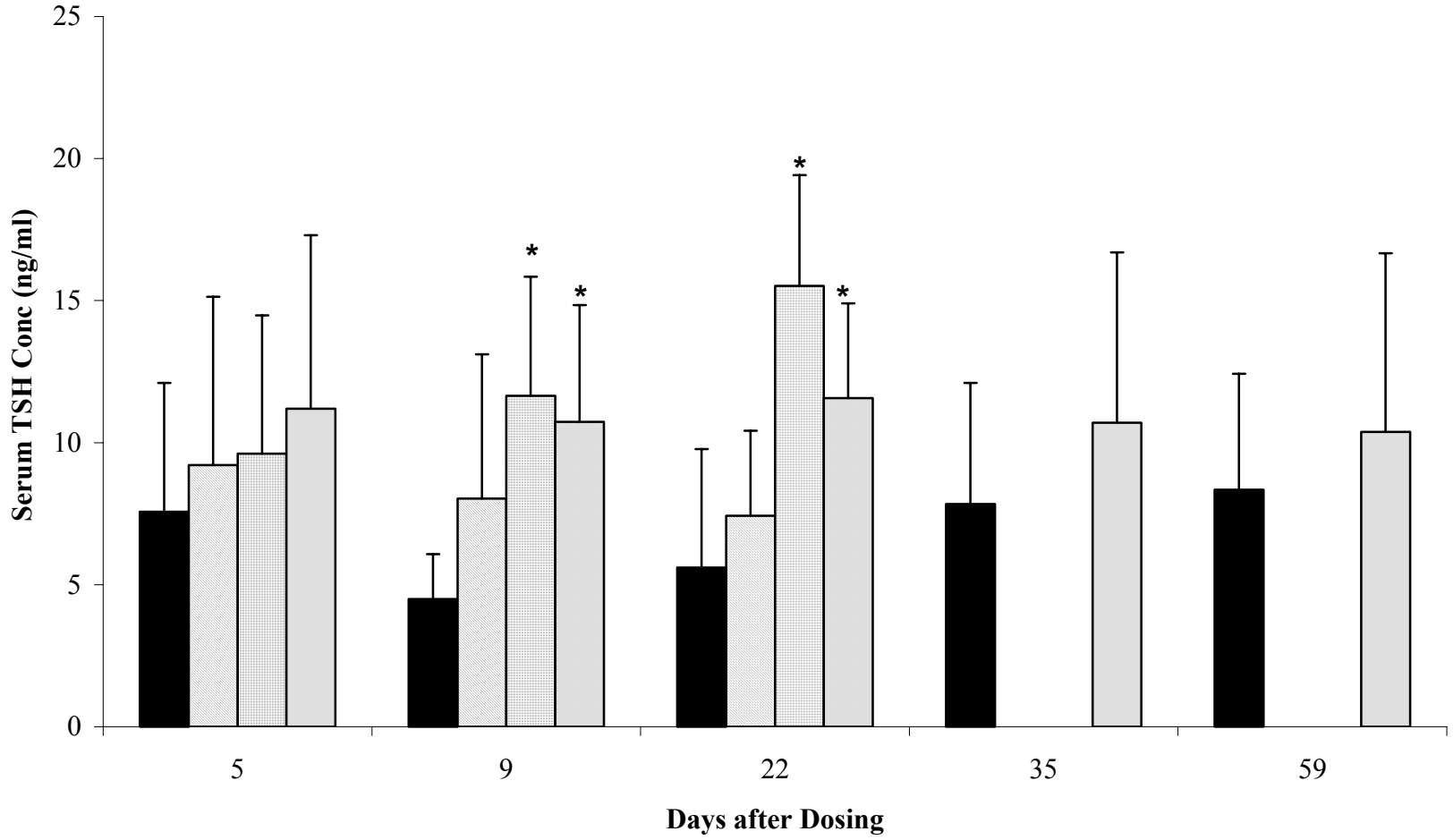

Figure 2.9

Post Gavage Day	Dose	Thyroid Follicle Colloid/Epithelium Volume Ratios (± SD)
22	**0**	**0.89 (0.27)**
22	**7.5ug/kg***	**0.84 (0.23)**
22	**75ug/kg***	**0.55 (0.14)**
22	**275ug/kg***	**0.51(0.10)**
35	**0**	**1.11 (0.26)**
35	**275ug/kg***	**0.66 (0.19)**
59	**0**	**1.17 (0.44)**
59	**275ug/kg***	**0.77 (0.24)**

Table 1. Thyroid follicle colloid epithelium volume ratios from rats dosed with PCB 126 were significantly increased for all dose groups on day 22, and for the highest dose group on day 35 and 59.

Post Gavage Day	Dose (ug/kg)	Type I 5'-deiodinase Activity (fmol I- produced/min/mg protein)
9	**0**	**646 +/- 55**
9	**7.5**	**476 +/- 165**
9	**75**	**345 +/- 66**
9	**275**	**242* +/- 228**
35	**0**	**375 +/- 49**
35	**275**	**347 +/- 104**
59	**0**	**339 +/- 76**
59	**275**	**193** +/- 82**

Table 2. Type 1 5'-deiodinase activity in the liver from rats dosed with PCB 126 was significantly decreased at 275ug/kg dosage on day 10, and on day 59 at $p < 0.0636$**.

Study	Time Point Sacrifice	Dose	Terminal Body Weight	EROD	UDPGT	TT4	FT4	TT3
		(Cumulative dose)	% of control	fold above control	% of control			
	(Day)	ug/kg/day	grams	nMoles/min/mg protein	pmol/min/mg protein	ug/dl	ng/dl	ng/dl
Craft (2002)	**4**	0.03 (0.12)						
	4	0.1(0.4)		0.95	78.9	111		
	4	0.3 (1.2)						
	4	1(4)		7.7	115.8	92.5		
	4	3 (12)						
	4	10 (40)		16.4	363.2	74		
	4	30 (120)						
	4	100 (400)		11.9	742.1	50.7		
Desaulniers (1999)	**2**	6.25 (13)	104.8	45		88		109
	2	25 (50)	110.8	93		70		93
	2	100 (200)	75	136		63		76
	2	400 (800)	-42.2	127		32		59
Van Birgelen (1994)	**91**	7 (11)	92.2	32		91.6	104.3	
	91	50 (73)	825.9	48		57.5	61.4	
	91	180 (231)	77.3	49		68.7	46.6	
Martin (2002)	**7**	2.5 (17.5)	100.6	32.9		80.1	76.9	83.5
	7	5 (35)	105.1	47.9		74.9	66.4	73.6
	7	10 (70)	95.3	36.5		64.9	53.8	85.8
	7	20 (140)	98.9	37.5		74.4	65.2	81.5
	7	40 (280)	95.5	52.9	789	32.3	36.9	88.1
Almekinder (2004)	**8 Hour**	275				88.5		102.8
	1	7.5	108.3	12.5				
	1	75	80.5	17.2				
	1	275	56.9	20.3		76.6		90.9
	5	7.5	110	8.9		100.5	90.1	104.2
	5	75	105.9	29.5		54.9	34.4	90.9
	5	275	115.8	30.7		40.1	28.2	95.9
	9	7.5	113.1	4.2		95.7		94.1
	9	75	89.6	14.9		33.4		87.1
	9	275	88.6	15.4		33.1		89.1
	22	7.5	96.2	3.4		91.3	91.3	89.8
	22	75	86.6	15.1		65.2	68.1	68.9
	22	275	81.9	76.4		49.4	44.1	77.7
	35	275	78.2	23.8		88.9		109.9
	59	275	96.7	9.2		79.6	52.1	84.2

Table 3. Previous and present data on effects of PCB 126 on liver enzymes and the thyroid axis.

* All measurements evaluated as % of control (EROD measurements expressed in fold)

CHAPTER 3

CONCLUSION

CONCLUSIONS

The present study is the first to evaluate a single oral bolus dose for PCB 126 in rats to evaluate on thyroid homeostasis. Although exposure to one specific congener of PCB is not likely, understanding the effects and potential thyroid implications of the most toxic PCB congener can help in assessing the upper bounds of toxicity.

The effects of exposure to PCB 126 were assessed by measuring the dose and time related changes in liver metabolizing enzymes and serum hormone levels and TSH in adult male rats. Our results demonstrated that circulating T4, FT4 and TSH levels are altered by PCB 126 exposure. PCB 126 treatment decreased serum T4 and FT4 (Fig. 2.4, Fig. 2.5, Fig 2.6b) and increased serum TSH (Fig. 2.9). Some of our findings are unique compared to previous studies (Table 3) of similar doses. Decreased serum T4 concentrations levels were comparable to other studies, however significantly increased serum TSH levels were unique to the present study (Desaulniers et al., 1999; Martin, 2002). Other studies have shown total T4 concentration beginning to significantly decline at cumulative doses of PCB 126 at 35, 40, and 50 ug/kg (Desaulniers et al., 1999; Craft et al., 2002; Martin 2002), yet TSH levels were not significantly increased (Desaulniers et al., 1999; Martin 2002). Studying the endocrine events through time following the single dose exposure on day 0, trends were found in the recovery effects of T4 by day 35 (Fig 2.6b). This could be explained by the pituitary's detection of low free TH concentrations in the serum, and the feedback mechanism of the hypothalamic-pituitary –thyroid axis to turn on the production of TSH to stimulate TH production in the thyroid. The response of T3 and rT3 serum concentration levels (Fig 2.7, Fig 2.8) were minimal yet consistent with other studies indicating that changes are less dramatic than T4 reductions (Martin 2002). A study by

Desaulniers et al. (1999) showed dose response reductions in TT3 levels and regarded these reductions significant at a cumulative dose of 200ug/kg.

Induction of hepatic microsomal enzymes, specifically EROD (a marker for CYP1A1) and UDPGTs has been consistently found together with decreased serum T4 concentrations (Fig 2.6a) (Craft et al. 2002; Martin, 2002). The effect of PCB 126 on P450 enzymes, mainly CYP1A1, (Fig 2.2; Fig 2.3) was clearly evident starting at the 7.5ug/kg dose by day 1 and still significant by day 59 at the 275ug/kg dose. The induction of CYP1A1 through the end of the study suggests that PCB 126 is still initiating its effect and further analyses of thyroid hormones would be useful in obtaining a more detailed overview of endocrine effects over time. Induction of the CYP1A1 enzyme was expected and consistent with other findings following PCB 126 exposure (Martin, 2002; Craft et al., 2002; Desaulniers et al., 1999). The decrease in concentration levels of T4 in circulation, compared to those in control rat, suggest that PCB 126 alters thyroid function through an increase in Phase II biometabolic enzymes in the liver (McCleary, 2001), specifically UDPGTs. Previous studies showing significant increases in T4 UGT activity have associated such inductions with increased biliary excretion of T4 from circulation (Martin 2002; Craft et al., 2002). It has been shown that UDP-GT is a primary mechanism responsible for instituting a pathway for excretion of steroid and thyroid hormones that are relatively lipophilic and the rapid clearance of T4, leading to hypothyroidism in the rat. This study indirectly supports this mechanism of action and further analysis will be carried out to quantify UDP-GT. The persistently low FT4 concentrations (Fig 2.4) with no serum reduction in T3 or rT3 concentrations are highly suggestive that non-deiodinative thyroxine metabolism is enhanced.

In the present study quantitative histopathologic volumetry results from thyroid gland tissue found overall significant decrease in all doses versus control in thyroid/colloid epithelium ratios (Table 1). A study by Desaulniers et al. (1999) analyzed the histology of the thyroid glands revealed that the follicular colloid is less dense in PCB 126 treated animals than in control. Important changes in thyroid histology are usually associated with significant changes in TSH levels, but also recognizing that T4 is the most sensitive endocrine indicator of PCB exposure (Desaulniers et al., 1999). The present study is a therefore consistent in the findings the decrease in FT4 concentrations (fig 2.4) on days 5 and 22 at the 75 and 275ug/kg doses indicate a direct thyrotoxic effect which can be corroborated by the reduced thyroid colloid/ follicle epithelium ratios at these time points.

In addition to thyroid production of serum T3 concentrations, T3 is also derived from Outer-Ring deiodination (ORD) of T4. Increased deiodination is a potential physiological mechanism that may play a role in maintaining serum T3 concentration in rats treated with microsomal enzyme inducers (Hood and Klaassen 2000). It has been shown that TSH stimulated type-I ORD activity in the thyroid (Wu et al., 1985) A previous study that analyzed the effects of Aroclor 1254 (a commercial PCB mixture), and recognized that in order to maintain levels of T3 following exposure to microsomal enzyme inducers, Type I deiodinase activity should be induced. However, the previous and present studies both show that Type I deiodinase activity in the liver were reduced in a dose-related manner (Table 2). Since ORD activities in the liver are regulated by thyroid hormones, it is possible that the reduced ORD activities are a physiological response to the hypothyroid state. (Hood and Klaassen 2000).

Since few statistically significant T3 serum concentrations dose related differences were noticed, we speculate that adaptive responses occurred to maintain T3 levels such as an increase

in 5' Type II deiodinase activity in the pituitary or increased production of T3 by the thyroid (Schurr et al., 1997). Type II 5' deiodinase activity has been previously measured in rat tissue (Hood and Klaassen 2000), and showed that when TH concentrations are low, brain Type II 5'deiodination activity increases. Alteration of sulfation could also be corroborated with maintenance of serum T3 concentrations.

The direct dialysis measurement was utilized at first thaw of the serum samples and to our knowledge this was one of the first studies to apply the technique of FT4 measurements by equilibrium dialysis to studies of PCB effects on thyroid hormone metabolism. Although most studies show a severe depression of FT4 concentrations also, it is unfortunate that most of the studies have examined FT4 by a non-dialysis procedure. Non-dialysis procedures are generally not accurate in non-primate species because of the lower amount of high affinity TBG protein in these species. Also, PCBs have been shown to be inhibitors of TTR**.** This effect may result in an underestimation of the FT4 measurements.

In the present study, we hypothesized that with an estimated half-life of 30 days (Yoshimura et al. 1985) for PCB 126, those doses as low as 7.5ug/kg would result in alterations and dose-dependant disruptions of thyroid hormone homeostasis and increased elimination of TH from circulation. It would be more informative if all doses were carried out for 59 days. Also, the half-life estimations and a sub-chronic study may offer a more complete view of thyroid hormone homeostasis effects. EROD levels still being induced by day 59 at 275ug/kg dose (Fig 2.3), and the recovery effect of T4 (Fig 2.6b) paired with slight induction in TSH, this data leads to the assumption that PCB 126 is still initiating its effect in the body.

Other studies that would lead to a greater understanding of PCBs and their impact on endocrine disruptions would be to conduct studies with mixtures of PCBs. Since PCBs are

persistent in the environment in congener mixtures, further evaluation of potential environmental pollutants mat assess a more realistic evaluation of thyroid homeostasis in the body. PCB 126 has also been implicated with reproduction and developmental impacts, therefore a study regarding pregnant animals may be considered along with additional assessments of the other hormones located in the pituitary: Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH).

Ongoing research into PCB effects on the body remain important for assessing endocrine toxicity of persistent and bio-accumulative compounds with the potential for initiating human health effects through dietary exposures.

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APPENDICES

Measurement of Cytochrome P-450 in Microsomes:

As adapted by Omura and Sato Method, 1964

Procedure:

1. Label glass tubes in duplicated, one set if 12x75mm for protein analysis and another 13x100mm for p450 to be assayed.
2. Dilute the microsomal suspension with 0.1 M PO_4 buffer to a total volume of 7 ml. By taking 350ul suspension and 6.65 ml of buffer in a 13x100mm tube. Mix by inversion. This can be done easily. By pipetting 7 ml of buffer and taking out 350ul off and be replaced with the microsomal suspension.
3. Pipette approximately 0.02 ml of each diluted sample to a set of tubes for Lowry's protein determination (12x75mm).
4. Pour into 3.0 ml disposable or quartz cuvette (two cuvettes per sample), add about 50 mg of fresh Sodium Dithionite to reduce the Cytochrome P-450; Mix well by inversion with a piece if parafilm.
5. Put 6 cuvettes into cuvette holder. Pass Carbon Monoxide into every other cuvette (2, 4, 6) very slowly, making sure that the solution does not bubble over, for approximately 30 sec. The cuvettes, which does not get the CO, is the reference.
6. Put the cuvette holder back into the response unit and read the absorbance or scan the samples from 500nm through 400nm using wavelength scan from the library of programs
7. Get a tabular printout of the absorbance readings over the scanning wavelength.
8. Subtract the absorbance at 490nm form the absorbance at 450nm; divide by 0.091 to give the result as nmole Cyt.P-450/mg protein. (0.091 is the Extinction Coefficient for P-450)
9. After measuring the concentration of the protein using Lowry's method, the values can be corrected for actual protein present in the diluted sample.
10. nmol P-450
    (-------- X ml dilution)/mg protein = nmol Cyt.P450/mg protein

Protein Determination in Microsomes:

As adapted by Lowry et al., 1951, J. Biol. Chem. (193:265-275)

Reagents:
2% Na_2CO_3 in 0.1 N NaOH (A_1)
1% $CuSO_4$ (B_2)
2% K-Na Tartrate (B_1)
1 N Folin Reagent (Comes as 2 N Solution, dilute 1:1 with distilled water)
0.5 mg/ml or 1 mg/ml Bovine Serum Albumin (BSA)

Procedure:

1. Set up a standard curve ranging from 10ug to 100ug using the BSA solution. You may have to pipet either 20,40,60,80, & 100ul or 10,20,30,40, or 50ul into sets of tubes (12x75mm), depending in which standard solution is being used. Q.S. the standards to 200ul with distilled water. Add a pair of tubes for blank, which contains only water or the volume if buffer in the sample being analyzed.
2. To a row of tubes (12x75mm) labeled in duplicate, add 180ul of distilled water
3. Dilute serum/microsomal/urine samples to 1:10 with water (1 part sample to 9 parts water). <u>If you have already diluted the samples for P450 skip this step.</u>
4. Pipet out 20 ul of diluted sample to the tubes labeled in duplicate.
5. Prepare reagents:
    a. Pipet out (prepare fresh) 0.5 ml of B_1 and 0.5 ml of B_2; Add 49 ml of A_1. Mix well. This is your working solution, you may label it C.
6. Add 1 ml of this working solution (C) to all tubes including the standards. Vortex. Let Stand.
7. After exactly 10 min., add 0.1 ml (100ul) of Folin reagents to all tubes, in the same order, as the assay was set up. Let stand for 30 min.
8. Read color/absorbance at 660nm, using either Gilford Response or Gilford 260 model spectrophotometer. Follow machine SOP for set up.
9. Calculate the slope, intercept and r-value for the standard curve. R-value tells you how linear your standard curve is.
10. Using Computer or a calculator, calculates the protein concentration of your samples, with the following formula as a guide.

$$\text{mg/ml} = \frac{\text{ug}}{\text{sample vol}} \times \text{dilution}$$

11. Solutions a, B1, B2 and Folin Reagent can be kept for long periods of time unmixed, (Aromatic Amino Acid) Protein forms a colorless complex in the presence of Sodium-Potassium Tartrate. This in turn forms a blue-green color complex with Folin's (phenol) Reagent.

Assay for Ethoxy Resorufin O-Deethylase (EROD/PROD) or Cytochrome p 1A1/1B1:

Chemicals and solutions needed:

0.1M PO_4 Buffer

A. 45.65 g K_2HPO_4 q.s. to 1 liter with distilled water
B. 27.22 g KH_2PO_2 q.s. to 1 liter with distilled water
C. 500 ml A + ~110 ml B (enough to adjust buffer to approx. pH 7.4
D. 500 ml C q.s. to 1 liter with distilled water = 0.1M KPO_4

Assay Buffer:

A. Add 2 mg Bovine Serum Albumin per ml of KPO_4 buffer needed for the assay.

Ethoxyresorufin/Pentoxyresorufin (substrates)

A. Add 1mg Ethoxyresorufin plus 5.5 ml DMSO=0.75mM, frozen in 500ul aliquots.
B. Add 1.17mg Pentoxyresorufin plus 5.5 ml DMSO=0.75mM, frozen in 500ml aliquots.

Resorufin Stock Solution:
Add 4.7mg Resorufin plus 50ml Methanol = 0.4 mM Resorufin

NADPH: (prepare fresh and on ice)
Make 5 mg/ml of 0.1 $KPO_{4\ buffer}$ used for the assay

Resorufin Standard Curve:

2000 nm	0.125ml of Resorufin stock q.s. to 25ml	
Concentration	Stock Solution	0.1M KPO_4 Buffer (ml)
0nM	0 ml	5.0 ml
5nM	0.25 ml of 100nM	4.75 ml
10nM	0.5 ml of 100nM	4.5 ml
20nM	1 ml of 100nM	4.0 ml
40nM	2 ml of 100nM	3.0 ml
60nM	3 ml of 100nM	2.0 ml
80nM	4 ml of 100nM	1.0 ml
100nM	1 ml of 2000nM	19 ml
140nM	0.25 ml of 2000nM	4.65 ml
200nM	0.5 ml of 2000nM	4.5 ml

Set the Fluorimeter conditions as follows:

Excitation Wavelength: 550nM
Emission Wavelength: 581nM
Scan Speed: Slow; Response: Auto
Bandwidth (nm): EX 1.5
EM 10
Sensitivity: High/Low (depending on previous experience)

Assay Procedure:

1. To a duplicate set of 13x100 glass tubes, add 2.3 ml of working 0.1 PO_4 buffer spike with BSA (at 2mg/ml).
2. Add 25 ul of microsomal solution (no need to dilute unless the activity is very high).
3. Add 6 ul of the substrate (ER or PR).
4. Vortex and incubate in a staggered manner, in a 37ºC water bath for 4 min.
5. At the end of the 4 min, add 100ul of NADPH and quickly vortex for 5-10 sec.
6. Pour the mixture into a fluorimeter cuvette (clear on all sides) and place in the fluorimeter, allow 20 sec before starting the run.
7. Record activity for 1.5 – 2 minutes.

Calculation:

A. Calculate the fluorescence activity by subtracting the value at 90 sec and 30 sec of reading.
B. Calculate the protein used in the assay (from previous protein assay for p450).

Activity (nmole or pmole/mg protein) =
Amount of resorufin (from standard curve)/Total Protein)
*(*total volume used for the assay)* **2.3 ml** for liver microsomes

Total T4 Assay

Chemicals and Reagents needed:

PBS/BSA Buffer: Use 0.25% solution; Add 0.5 g BSA to 200 ml PBS
Add stir bar and mix very gently to prevent foaming

Tracer: Use T_4-^{125}I, PBS/BSA +ANS (2mg ANS/1ml PBS/BSA)
1 uCi = 2.2 x 10^6 cpm;

Total Volume: Need 50ul tracer/tube x number of tubes (say, 100) = 500ul total

Total I^{125}: Need 10,000 – 20, 000 counts per tube (av. 15,000).
Example calculation:
100 tubes x 15,000 cpm/tube = $\frac{1,500,000}{18,480}$ = 81 ul of tracer needed
(cpm/ul stock)

First Antibody: Use Anti-T_4 1:50 dilution with 0.25% BSA, 0.01 M PO_4,
0.15 M NaCl, 0.05 M EDTA.

0.05 M EDTA: (0.005 moles in 100ml) (MW of EDTA = 292.3) = 1.461 grams of EDTA added to 100ml PBS/BSA. Add NaOH whole mixing gently on stir plate to bring pH to 7.5; liquid should become clear.

To dessicated antiserum (T_4-15 Endocrine Science) add 1.1 ml of dH_2O. Shake. Add this to 54 ml of 0.05 M EDTA solution.

Second Antibody: Use Second Antibody Suspension (GAR), spinning at room temp.

PBS: (Make up 2 liters)
0.15 M NaCl = (0.15 moles/liter) (58.44 g/mole)
= (8.766) (2 Liters)
= 17.52 g NaCl needed for 2 L deionized H_2O
0.01 M NaH_2PO_4 = (0.01 moles)(137.99 g/mole)
(monobasic) = 2.76 g NaH_2PO_4 needed for 2 L dH_2O
*pH to 7.5 with NaOH

Dilutions:
Number 10 tubes
Tube #1: 4.9 ml PBS/BSA + 0.1 ml of T_4 Working Standard (2.5 ug/ml L-T_4)
Tube #2: Add 1ml PBS/BSA, then transfer 1 ml of Tube #1 to Tube #2 (1:1 Dilution), vortex vigorously; transfer 1 ml of Tube #2 to Tube #3, vortex, etc…

Counting:
Samples were counted on a gamma counter for 60s each and average readings were recorded.

Basic Procedure:

1. Add materials to tubes in order given below.
2. Incubate overnight at 4ºC.
3. On day 2 add second antibody and incubate 15 min at room temp;
4. Count ten tubes for total counts per tube.
5. Add PBS and centrifuge at 3000rpm for 10 min at 4ºC.
6. Immediately aspirate supernatant.
7. Count.

Total T3 Assay

Chemicals:

PBS, pH 7.5 = 17.52g NaCl + 2.76 NaH2PO4 (monobasic), q.s. 2L

PBS/BSA BUFFER:

Use 0.25% solution.
Add 0.5g of BSA to 200mls PBS
Add Stir bar and mix very gently to prevent foaming

PBS/BSA/ANS

20mg ANS into 10mls PBS/ANS (2mg/ml) –
-the amount made will depend on amount needed for assay

Standard Dilutions:

1. Number 10 tubes plus 2 extra. Use 10ul T3 Standard Stock (50ug/ml) + 6.25 ml PBS/BSA. 100ul concentration = 8ng.
2. Dilute 1:2 from 8ng :4:2:1, etc
3. OMIT TUBES WITH CONCENTRATIONS OF 4 AND 2.

TRACER:

Use T3-125I, PBS/BSA+ ANS (2mg/1ml PBS/BSA). 1uCi = 2.2 X 10^6cpm;
Total Volume: need 50ul tracer/tube X # of tubes (i.e.100) = 500ul total
Total 125I :
Example: Need 10,000-20,000 counts per tube (avg. 15,000)
Therefore 100 tubes X 15,000cpm/tube = 1,500,000/18480(cpm/ul stock) = 81ul tracer needed

1st antibody: (Anti-T3) 1:188 dilution with 0.8 dH20 X 188 = 150.4 – 0.8 = 149.6 final volume of 0.25% BSA. O.01 M PO4, 0.15 M NaCl, 0.05 M EDTA
0.05 M EDTA = (0.005 moles in 100ml) x (MW of PBS/BSA)
Add NaOH while mixing gently on stir plate to bring pH to 7.5
Liquid should be clear

2nd antibody:

Use Sigma GAR-agarose 1:100 (50% suspension diluted 1:50)

PBS/PEG:

35g PEG(MW 8000) into 140ml PBS

PBS/BGG:

0.2g BGG into 20mls PBS

Counting:

Samples were counted on a gamma counter for 60s and average readings were recorded.

Procedure:

1. Add Material to tubes as shown in the following chart
2. Incubate overnight at 4ºC
3. On day 2 add 2nd antibody and incubate 2hr at 37ºC. Mix occasionally.
4. Count 10 tubes for total counts per tube.
5. Add PBS and centrifuge at 3,000rpm for 10min at 4ºC
6. Immediately aspirate supernatant and count 60s.

Tube #	Blank Serum	Std.Dilution (100ul) Tube#/Conc. ug=ng	PBS/BSA	Tracer	1st Anti-body	2nd Anti-body	BGG/PEG
1,15	100ul	1 8000ng	0	50ul	75ul	150ul	100ul/1ml
2,16		2 1000ng	0	50	75	150	100ul/1ml
3,17		3 500	0	50	75	150	100ul/1ml
4,18		4 250	0	50	75	150	100ul/1ml
5,19		5 125	0	50	75	150	100ul/1ml
6,20		6 62.5	0	50	75	150	100ul/1ml
7,21		7 31.2	0	50	75	150	100ul/1ml
8,22		8 15.6	0	50	75	150	100ul/1ml
9,23		9 7.8	0	50	75	150	100ul/1ml
10,24		10 3.9	0	50	75	150	100ul/1ml
11,25		0	100ul	50	75	150	100ul/1ml
12,26		0	100	50	75	150	100ul/1ml
13,27	Normal pool	0	100	50	75	150	100ul/1ml
14,28	100ul	0	100	50	75	150	100ul/1ml

Incubate overnight at 4ºC

2 hr Incubation 37ºC

Reverse T3 Assay

Chemicals:

PBS/BSA/ANS (2mg/ml)

Example: 10.2mg ANS into 5.1 ml PBS/BSA (different depending on assay size)

1st Antibody (IgG)

100ul anti-rT3 serum + 75 ml PBS/BSA/EDTA
+ 1.415 ml of rabbit IgG solution
IgG = 10ug/50ul

Tracer:

8ul/ml of 125 I –rT3 in 1.0ml PBS/BSA/ANS
Total counts ~ 17,000cpm (100ul)

PBS/BGG:

0.8g into 80mls PBS

PEG/PBS:

200g into 800ml PBS

Procedure continued on next page.

Procedure:

1. Add Material to tubes as shown in the following chart
2. Incubate overnight at 4ºC
3. On day 2 add 2nd antibody and incubate 2hr at 37ºC. Mix occasionally.
4. Count 10 tubes for total counts per tube.
5. Add PBS and centrifuge at 3,000rpm for 10min at 4ºC
6. Immediately aspirate supernatant and count 60s.

Tube #	Blank Serum	Std.Dilution (100ul) Tube#/Conc. ug=ng	PBS/BSA	Tracer	1st Anti-body	2nd Anti-body	BGG/PEG
1,15	100ul	1 8000ng	0	50ul	75ul	150ul	100ul/1ml
2,16		2 1000ng	0	50	75	150	100ul/1ml
3,17		3 500	0	50	75	150	100ul/1ml
4,18		4 250	0	50	75	150	100ul/1ml
5,19		5 125	0	50	75	150	100ul/1ml
6,20		6 62.5	0	50	75	150	100ul/1ml
7,21		7 31.2	0	50	75	150	100ul/1ml
8,22		8 15.6	0	50	75	150	100ul/1ml
9,23		9 7.8	0	50	75	150	100ul/1ml
10,24		10 3.9	0	50	75	150	100ul/1ml
11,25		0	100ul	50	75	150	100ul/1ml
12,26		0	100	50	75	150	100ul/1ml
13,27	Normal pool	0	100	50	75	150	100ul/1ml
14,28	100ul	0	100	50	75	150	100ul/1ml

Incubate overnight at 4ºC

2 hr Incubation 37ºC

Liver Microsome Preparation

Chemicals:

0.02 M Tris-HCl

0.15 M KCl (pH 7.4)

0.25 M Sucrose

10mM EDTA

1.15% KCl (pH 7.4)

Procedure:

1. Take 5 grams of fresh liver tissue
2. Homogenize in 0.02 M Tris-HCl containing 0.15 M KCl (pH 7.4)
3. Centrifuge at 10,000g for 30 min at 4ºC.
4. Wash and resuspend pellet in 0.25 M sucrose containing 10 mM EDTA and 1.15% KCl (pH 7.4)
5. Store at –80ºC until further use.

Homogenate Protocol

Chemicals:

100mmol/L potassium phosphate, pH 7.0

1mmol/L Ethylenediamine tetraacetate (EDTA)

1mmol/L dithiothreitol (DTT)

Procedure:

For Liver

1. Homogenize 5 grams of liver in 5mls of 100mmol/L potassium phosphate, pH 7.0, containing 1mmol/L Ethylenediamine tetraacetate (EDTA), 1mmol/L dithiothreitol (DTT).

For Pituitary

1. Pooled 4 pituitary's per tube and homogenized in 5x the pituitary weight volume of 100mmol/L potassium phosphate, pH 7.0, containing 1mmol/L EDTA, and 20mmol/L DTT.

TYPE I 5'Deiodinase

Reagents:

EDTA: 100mmol/L potassium phosphate, pH 7.0 containing 1 mmol/L ethylenediamine tetraacetate

DTT: 2mmol/L dithiothreitol

PTU: 1mmol/L 6-propyl-2-thiouracil

125 I-rT3

rT3 cold

FBS: (fetal bovine serum)

10% TCA

Protein Homogenate

PTU buffer (100 mmol/L potassium phosphate, pH 7.0, containing 1mmol/L Ethylenediamine tetraacetate (EDTA), 2mmol/L dithiothreitol(DTT), 1mmol/L 6-propyl-2-thiouracil, 3ul (depending on radioactivity)/ml 125 I-rT3, and 5.0ug/ml rT3 cold)

Non-PTU buffer (100 mmol/L potassium phosphate, pH 7.0, containing 1mmol/L Ethylenediamine tetraacetate (EDTA), 2mmol/L dithiothreitol(DTT), 3ul(depending on radioactivity)/ml 125 I-rT3, and 5.0ug/ml rT3 cold) buffers

Protocol:

1. Measure 10ug / 20ul liver homogenate samples in 12x75mm glass tubes in triplicate
2. Place tubes in a 37°C water bath
3. Start reaction with the addition of 100ul of PTU in triplicate per sample and non-PTU in triplicate per sample:

4. Set reaction time for 2 minutes and 12 minutes each for a total of 12 tubes per sample.
5. Terminate the reaction with the addition of 500ul of fetal bovine serum (FBS)
6. Immediately incubated on ice for 30 minutes.
7. After incubation, add 500ul of 10% TCA to each tube,
8. Vortex
9. Centrifuge at 3,000 rpm for 15minutes at 4°C
10. Take 500ul was from supernatant and place in a fresh tube.
11. Count pellet and supernatant for radioactivity with a gamma counter for 60 seconds.