EFFECTS OF PERFLUOROOCTANESULFONATE (PFOS) ON THYROID HORMONE STATUS IN RATS

A DISSERTATION
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SHU-CHING CHANG

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MICHAEL J. MURPHY, advisor

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I.

INTRODUCTION
Perfluorooctanesulfonate (PFOS) is an environmentally stable and accumulative compound that has been found to be distributed worldwide in humans and wildlife (Butenhoff et al., 2006; Houde et al., 2006; Lau et al., 2007). It is well-absorbed but poorly eliminated, with an approximated serum elimination half-life of 5.38 years in humans (Olsen et al., 2007). Serum elimination half-lives in animals have not been thoroughly investigated, but are estimated to be in 7.5 days in rats (Johnson and Ober, 1979) and 100-150 days (Seacat et al., 2002) in monkeys. Due to persistence, long half-life, and the fact that laboratory research and human biomonitoring studies have shown that exposure of PFOS can begin very early in life (in utero and via lactation), there have been increased regulatory interests in controlling these materials on an international level (Canadian Government Department of the Environment, 2008; OECD, 2002; USEPA, 2002, 2006).

Alterations in serum thyroid hormone levels (triiodothyronine (T3) and/or thyroxine (T4)) have been reported as a PFOS treatment-related effect in several toxicological studies (Curran et al., 2008; Fuentes et al., 2006; Lau et al., 2003; Luebker et al., 2005b; Martin et al., 2007; Seacat et al., 2002; Thibodeaux et al., 2003; Yu et al., 2009b). These studies have shown reductions in serum total and/or free thyroid hormone levels by immunoassay-based analog methods even though serum samples from two studies (Luebker et al., 2005b; Seacat et al., 2002) also reported normal free thyroid hormone
levels when measured by a direct equilibrium dialysis reference method. Furthermore, the reported alterations in thyroid hormones were not associated with any altered changes in thyroid gland pathology or clinically significant elevations in thyrotropin (thyroid stimulating hormone, TSH). Because the diagnosis of primary hypothyroidism is based on reduced serum free T4 (FT4) and concurrent compensatory elevation of TSH (Ravel, 1995; Sapin, 2001; Sapin and Schlienger, 2003), the observed reduction in serum thyroid hormones following PFOS treatment without a major compensatory increase in TSH does not fit the clinical profile of hypothyroidism. There were also several human biomonitoring studies that evaluated the relationship between serum PFOS concentrations and thyroid hormones (Bloom et al., 2009; Dallaire et al., 2009; Inoue et al., 2004; Olsen et al., 2000; Olsen and Zobel, 2007). The mean thyroid hormone levels in each study population, including the workers from fluorochemical production facilities who had much higher mean serum PFOS levels than general population, were well within the clinical reference range (Olsen et al., 2003a; Olsen and Zobel, 2007). Because thyroid hormones have numerous important roles in growth (Bernal, 2005a, 2005b; Calvo et al., 1992; Oppenheimer et al., 1995) and brain development (Bernal, 2005a, 2005b), the differences between human and laboratory rat thyroid hormone data in the presence of PFOS raise the questions of whether PFOS interferes with the thyroid homeostasis as well as whether laboratory rats serve as an appropriate model to study thyroid biology. Because rodent model is the most commonly used model for risk assessment, to minimize the uncertainty, this thesis investigated the effect of PFOS on thyroid hormone status in rats.
1.1 UNDERSTANDING PFOS FROM A PUBLIC-HEALTH PERSPECTIVE

PFOS is a man-made compound with distinct chemical and physical properties. It was primarily produced by 3M Company since the 1940’s and its manufacturing by 3M Company was discontinued by 2002. Other companies continue to manufacture PFOS for specific use.

1.1.1 Nomenclature, physical characteristics, chemical characteristics, and analytical identification

a. Nomenclature

PFOS is an aliphatic eight-carbon molecule that is fully fluorinated except for the terminal sulfonate group. The molecular formula for PFOS is C<sub>8</sub>F<sub>17</sub>SO<sub>3</sub><sup>-</sup> and its molecular weight is 499 g/mol. The structural formula is CF<sub>3</sub>-CF<sub>2</sub>- CF<sub>2</sub>- CF<sub>2</sub>- CF<sub>2</sub>- CF<sub>2</sub>- CF<sub>2</sub>- CF<sub>2</sub>- CF<sub>2</sub>- CF<sub>2</sub>- CF<sub>2</sub>- SO<sub>3</sub><sup>-</sup>

![Molecular structure of PFOS (C<sub>8</sub>F<sub>17</sub>SO<sub>3</sub><sup>-</sup>)](image)

Figure 1.1: Molecular structure of PFOS (C<sub>8</sub>F<sub>17</sub>SO<sub>3</sub><sup>-</sup>)
The PFOS anion does not have a specific CASRN (Chemical Abstracts Service Registry Number). The acid and salts have the following CASRNs:

<table>
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<tr>
<td>Acid</td>
<td>1763-23-1</td>
</tr>
<tr>
<td>Ammonium ((\text{NH}_4^+)) salt</td>
<td>29081-56-9</td>
</tr>
<tr>
<td>Diethanolamine (DEA) salt</td>
<td>70225-14-8</td>
</tr>
<tr>
<td>Potassium ((\text{K}^+)) salt</td>
<td>2795-39-3</td>
</tr>
<tr>
<td>Lithium ((\text{Li}^+)) salt</td>
<td>29457-72-5</td>
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The IUPAC (International Union of Pure and Applied Chemistry) nomenclature for PFOS acid is \(1,1,2,2,3,3,4,4,5,5,6,6,7,7,8,8,8\)-heptadecafluoro-1-octanesulfonic acid. Other synonyms include 1-perfluorooctanesulfonic acid, heptadecafluoro-1-octanesulfonic acid; perfluoro-\(n\)-octanesulfonic acid, perfluoroctanesulfonic acid, and perfluoroctylsulfonic acid (3M, 2003).

b. Physical characteristics

Although pure PFOS acid is available, it is extremely corrosive and difficult to synthesize. PFOS acid is therefore of little interest as an analytical standard or test compound for toxicological
studies. Instead, PFOS salts have been widely used in industrial and laboratory applications (3M, 2003). Because PFOS potassium salt is the most common salt form, the physical and chemical properties of PFOS potassium salt (K⁺PFOS) are described hereafter.

At room temperature, K⁺PFOS exists as solid white powder. K⁺PFOS has an extremely low vapor pressure (3.31 x 10⁻⁴ Pa at 20°C). Its melting point is greater than 400°C and its air/water partition coefficient is close to 0 (< 2 x 10⁻⁶), which makes it non-volatile (3M, 2003).

PFOS and its salts are amphiphilic. K⁺PFOS has an extremely low water solubility of 600 mg/L in pure water. This solubility decreases significantly to 20 mg/L in a 3.5% NaCl solution and to 12.4 mg/L in natural sea water at 22 – 24°C (3M, 2003).

The octanol water partition coefficient (Kₗₖ) for PFOS cannot be measured, because PFOS forms three immiscible phases when mixed with octanol and water used in the standard octanol/water partition coefficient test. Hence, PFOS is often said to have both oleophobic (oil and fat insoluble) and hydrophobic properties.
K+PFOS salt is soluble in organic solvents such as methanol, ethanol, hexane, acetone, and dimethyl sulfoxide (DMSO). The stability and the unique partitioning property of PFOS make it an excellent surface-active surfactant agent (3M, 2003).

c. Chemical properties

Many of the properties of PFOS and similar molecules can be attributed to the fluorine molecules. Fluorine is the most abundant element in PFOS and the most abundant member of the halogen family. It is one of the most reactive of all elements. It is capable of combining with nearly every other element in the periodic table. Consequently, elemental fluorine is rarely ever found in nature. The van der Waals radius of 1.35 Å for fluorine is comparable to that of oxygen but smaller than that of other halogens. It has an electronegativity of 4.0 on the Pauling scale. An electronegativity of 4.0 is higher than any other element in the periodic table, including all other halides. Ionic fluorine will form weak bonds with electronegative atoms and very strong bonds with electropositive atoms, due in large part to this strong electronegative property (Lehmler, 2005).
Each fluoride ion in PFOS or PFOS salts is bonded to carbon. These carbon-fluorine bonds, have a very high energy of approximately 110 kcal/mol, due in large part to the strong electronegativity of fluorine. The high-energy carbon-fluorine bonds give superb stability to fluorochemicals (3M, 2003).

d. Analytical identification

Analysis of the PFOS anion has presented many analytical challenges over the years. Unique physical properties of PFOS that have made analysis difficult include extremely low volatility, absence of chromophores, resistance to derivitization, and adhesion to suspended particulates and container surfaces. Hence PFOS cannot be quantified via conventional analytical techniques such as UV-VIS or IR (3M, 2003).

The first technique developed and used by researchers in the 1960’s and 1970’s to quantify PFOS semi-quantitatively was total organic fluorine analysis, which measured the organic fluorine (fluorine covalently bonded to carbon) rather than specifically measuring PFOS molecules. Since PFOS is comprised of 65% fluorine by weight, the theoretical amount of PFOS was
consequently calculated based on the amount of organic fluorine detected. This technique, however, was non-specific to PFOS and required hours of sample preparation time and instrument time for a single analysis (3M, 2003).

In the late 1970’s, the development of fluorine NMR allowed the direct speciation and quantification of PFOS. It not only decreased the analytical time, it had also allowed a lower detection limit of 0.5 parts per million (3M, 2003).

The advancement of liquid chromatography and mass spectrometry (Begley et al.) technology in 1990’s enabled more rapid and sensitive analysis of PFOS from small volumes. The detection limit for PFOS in serum was approximately 50 ppb by LC/MS in 1997 (3M, 2003). With continuous efforts in improving the LC/MS-MS instrumentation and methodology, to date, it is possible to quantify serum PFOS at 1 ppb with high degree of precision (Ehresman et al., 2007).

1.1.2 Uses and applications
The predominant commercial application of PFOS has been as a surface-active additive in commercial and consumer product applications. Examples include dust and mist suppression, fire-fighting foam, and hydraulic fuel for aerospace industry (3M, 2003; Lau et al., 2007).

1.1.3 Sources of PFOS: manufacturing, biodegradation, and environmental fate

a. Manufacturing (intentional)

PFOS is a member of the sulfonyl-based perfluoroalkyl substances which had been commercially manufactured via electrochemical fluorination (ECF) for decades. ECF is a process in which anhydrous hydrogen fluoride (HF) is reacted with hydrocarbon feed stock in an electrochemical cell. For many sulfonyl-based fluorochemicals such as PFOS, they were derivatized from perfluorooctanesulfonyl fluoride (POSF, C₈F₁₈SO₂), the basic building block and starting material for sulfonyl-based ECF products. The sulfonyl fluoride moiety on POSF can be modified using conventional hydrocarbon reactions to form N-ethyl perfluorooctanesulfonamido ethanol (N-EtFOSE), N-methyl perfluorooctanesulfonamido ethanol (N-MeFOSE), N-ethyl perfluorooctanesulfonamido acetate (PFOSAA),
perfluorooctanesulfonamido acetate (M556), and PFOS (3M, 2003; Lau et al., 2007; Lehmler, 2005).

b. Biodegradation and environmental fate (unintentional)

It has been shown that chemical or biochemical enzymatic hydrolysis of POSF or POSF-based molecules results in PFOS. PFOS or its salts, however, cannot be further metabolized (3M, 2003; Lau et al., 2007; Xu et al., 2004).

PFOS is not volatile due to its low air/water partition coefficient (vide supra). Initial screening studies on the aqueous photolytic degradation of PFOS demonstrated that PFOS cannot undergo photolysis when exposed to UV for 167 hours, or approximately 7 days (3M, 2003), however, a more recent study by Yamamoto et al. (2007) reported the occurrence of PFOS photolysis when PFOS-containing aqueous medium was exposed to UV for 10 days.

No significant degradation of PFOS was observed with regards to net oxygen consumption, loss of total organic carbon, or loss of PFOS compound in a 28-day Japanese Ministry of International
Trade and Industry (MITI) degradation test (3M, 2003). Therefore, PFOS is the ultimate degradation product of POSF derived fluorochemicals and PFOS will persist in that form in the environment.

1.1.4 PFOS exposures in biota, human, and environment

a. Biota

Invertebrates:
Low concentrations of PFOS in serum or plasma (< 2 ng/mL) were reported for zooplankton, mollusks, and shrimp from the Eastern Canadian Arctic and Asia (So et al., 2006a; Tomy et al., 2004). Elevated concentrations of PFOS (9 – 877 ng/g) were detected in oysters from United States (Kannan et al., 2002), mussels from Portugal (Cunha et al., 2005), and shrimp, starfish, and crabs from the Belgium and Dutch coasts (Van de Vijver et al., 2003).

Fish, amphibians, and reptiles:
PFOS was the predominant compound detected in freshwater and saltwater fishes. The highest PFOS concentration (3250 ng/g) was reported in the liver of the carnivorous and near-bottom feeder
ornate jobfish collected in 2002 from Okinawa, Japan (Taniyasu et al., 2003). The authors did note that the fire-fighting operations from an electric power plant and a military base in Okinawa might have been the source of PFOS. The lowest PFOS concentrations were reported in fish samples collected in the Eastern Canadian Arctic and Faroe Islands (Kallenborn et al., 2004; Tomy et al., 2004). Giesy and Kannan (2001) reported that green frog liver from Michigan had a PFOS concentration of approximately 285 ng/g; and that yellow-bloched map turtles from Mississippi had 190 ng/g PFOS in liver.

**Birds:**

PFOS has also been detected worldwide in seabirds, terrestrial birds, and waterfowl. The highest PFOS concentrations were found in birds from industrialized area. Bird samples from Antarctica and Canada such as polar skua had the lowest plasma PFOS concentration (~ 1 ng/mL) (Giesy and Kannan, 2001). Archived samples of plasma from fish-eating birds obtained in the late 1980’s and 1990’s from U.S. Fish and Wildlife Services were analyzed for PFOS concentrations. Species analyzed included the bald eagle, albatross, and sea eagle. The PFOS levels ranged from
below the limit of detection (1 ppb) to a maximum of 1047 ppb and 2055 ppb in plasma and liver, respectively (3M, 2003).

**Mammals:**

A few biomonitoring studies have been conducted for terrestrial mammals. Wood mice habitating in the area around a fluorochemical plant in Belgium had the highest liver PFOS concentration of 180,000 ng/g (Hoff *et al.*, 2004). In mink, elevated liver PFOS concentrations were found in the Midwestern and northwestern United States (<= 2630 ng/g) (Giesy and Kannan, 2001).

A much greater number of studies have been conducted on marine mammals compared to terrestrial mammals. However, with the exception of bottlenose dolphins, all the other marine mammal samples were obtained when the animals were stranded or dead. Overall, PFOS was detected in the liver and blood from marine mammals located in the U.S. coastal waters (Florida, California, and Alaska), the northern Baltic Sea, the Mediterranean Sea, the Arctic (Spitsbergen), and Sable Island in Canada. The highest PFOS liver concentration were 1520 ng/g and 3100 ng/g in bottlenose dolphin (from Florida) and polar bear (from Sanikiluaq,
Canada) (Martin et al., 2004). The highest PFOS concentrations in blood was 475 ng/mL found in the ringed seals from the northern Baltic Sea (Giesy and Kannan, 2001).

b. Humans:

The mechanisms and pathways leading to the presence of PFOS in human blood have not been fully evaluated but likely are due to environmental exposure to PFOS or its precursor molecules in industrial and commercial products (Butenhoff et al., 2006; Lau et al., 2007). Although organically bound fluorine had been identified in human blood since late 1960’s (Taves, 1968), improvements in analytical methodology in 1990’s allowed for the routine measurement of specific organofluorine molecules such as PFOS in serum from humans with occupational and non-occupational exposures (Hansen et al., 2001).

PFOS has been measured primarily in human serum but some preparations have analyzed plasma or whole blood (Butenhoff et al., 2006). Equivalency of fluorochemical measured in serum and plasma, and their relationship to whole blood had been demonstrated by Ehresman et al. (2007). The ratio between
plasma and serum, when measured for fluorochemicals, is 1:1; and the ratio between plasma and/or serum to whole blood, when analyzed for fluorochemical, is 2:1, suggesting the PFOS is primarily bound to plasma/serum components.

Occupationally-exposed fluorochemical workers at 3M Company (the major manufacturer of PFOS prior to 2002) have measured serum PFOS levels that average 1-2 ppm, with the highest at approximately 13 ppm (Olsen et al., 2003a; Olsen et al., 2003c).

Biomonitoring studies of the general population in the United States have demonstrated that practically all serum samples analyzed contain detectable PFOS. The geometric mean values were approximately 30 - 40 ng/mL between 1999 - 2002 period (Calafat et al., 2006; Calafat et al., 2007a; Olsen et al., 2003b), 20.7 ng/mL between 2003 – 2004 period, and 14.5 ng/mL in 2006 (Olsen et al., 2008). There is no difference in serum PFOS concentration by age within the same sex group.

Children are known to be exposed to PFOS (Calafat et al., 2007b; Olsen et al., 2004), and these exposures can occur from gestational and lactational transfer of PFOS (Apelberg, 2006; Apelberg et al., 2007a; Inoue et al., 2004; Karrman et al., 2007; Midasch et al.,
2007; Monroy et al., 2008; So et al., 2006b; Spliethoff et al., 2008; Tao et al., 2008). As reviewed by Olsen et al. (2009), there are several groups of investigators that have studied the association of PFOS concentrations in human maternal and/or umbilical cord blood to birth outcomes, and one recent study followed developmental landmarks in infants through approximately 18 months of age (Fei et al., 2008a).

Human biomonitoring studies have shown that neonates can be exposed to PFOS effectively through in utero exposure. Inoue et al. (2004) reported that PFOS was detected in all of 15 paired human maternal serum and neonatal cord blood serum samples from Japan obtained in 2003, with neonatal and maternal serum PFOS ranging from 1.6 – 5.3 ng/mL and 4.7 – 17.6 ng/mL, respectively. Midasch et al. (2007) reported detectable PFOS among 11 paired maternal and cord blood plasma samples obtained in 2003 in Germany, with maternal plasma PFOS median concentration of 13.0 and 7.3 ng/mL in cord blood. Monroy et al. (2008) evaluated PFOS concentrations in 101 maternal samples paired with 105 cord blood samples at birth in Canada (includes 4 sets of twins), with mean PFOS concentrations of 16.2 ng/mL and 7.3 ng/mL, respectively. Apelberg et al. (Apelberg et al., 2007b)
found that PFOS concentrations in 293 cord blood sera samples obtained in Baltimore, Maryland, United States, between 2004 - 2005 had a geometric mean of 4.9 ng/mL, indicating that children are exposed to PFOS beginning in utero from placental transfer.

Lactational exposure to PFOS in humans is also possible. Tao et al. (2008) reported a mean milk PFOS concentration of 0.131 ng/mL among 45 samples obtained in 2004 from nursing mothers in Massachusetts, United States. So et al. (2006b) also examined 19 human milk samples in China and reported a mean PFOS concentration of 0.121 ng/mL. Kärrman et al. (2007) found that mean PFOS concentration in milk is 0.201 ng/mL and that was about 1% of the respective maternal serum PFOS concentration (based on 12 samples obtained in Sweden). Wilhelm et al. (2008) reported that PFOS was detected in 99 out of 183 breast milk samples collected in Germany, with the mean PFOS concentration of 0.09 ng/mL.

c. Environment:

PFOS has been found in surface water around the world at low levels ranging from 1 pg/L to 59 ng/L (Boulanger et al., 2004;
Hansen et al., 2002; Taniyasu et al., 2003; Yamashita et al., 2005). It has been found in sediments and sludges (D'eon et al., 2009; Higgins et al., 2005; Higgins et al., 2007; Yu et al., 2009a) and in indoor air and dust (Kubwabo et al., 2005; Shoeib et al., 2004; Shoeib et al., 2005; Strynar and Lindstrom, 2008).

1.2 UNDERSTANDING PFOS FROM A TOXICOLOGICAL PERSPECTIVE

Until 2002, 3M Company was the primary producer of POSF and POSF-related compounds including PFOS for over 40 years (3M, 2003). Due to evidence of widespread presence, persistence, and accumulation of PFOS in the environment, 3M Company announced in 2000 that it would discontinue the manufacture of these materials completely by 2002. The decision to cease the manufacture and importation of POSF-related chemicals to zero was to achieve a corresponding reduction in the type, form, and duration of exposure to this class of chemicals. However, given its physical-chemical and pharmacokinetic properties, PFOS is expected to persist in the environment and therefore in human for many years. The discovery and identification of PFOS in biomonitoring samples of humans and wildlife has led to increased efforts to understand the toxicological properties of PFOS.
1.2.1 Absorption, distribution, metabolism, and elimination (ADME) characteristics

a. Absorption

PFOS is well absorbed following ingestion. Greater than 95% of a single oral dose of $^{14}\text{C}-\text{PFOS}$ (4.2 mg/kg), was absorbed by male Sprague Dawley rats within 24 hours (Johnson and Ober, 1979).

Dermal absorption of PFOS appears to be possible. When PFOS was applied as a single application under occlusion to approximately 10% of the body surface area of male and female New Zealand albino rabbits at 1000 and 5000 mg/kg and left in place for 24 hours with a subsequent 28-day follow up, total blood fluoride analysis indicated some dermal absorption. The serum total fluoride increased from 10.3 ppm on day 1 to 130 ppm on day 28 in males, and 0.9 ppm on day 1 to 128 ppm on day 28 in females (Ebbens, 1981). The study result was concluded as inconclusive because only a small subset of serum samples were analyzed.
No quantitative information is available on the absorption of PFOS from inhalation exposure as PFOS has exceptionally low vapor pressure. Inhalation exposure to vapor would be unlikely.

b. Distribution

Based on studies done in cynomolgus monkeys and Sprague Dawley rats, PFOS distributes mainly in serum and liver, with liver concentrations being potentially several times higher than serum concentrations (Butenhoff et al., 2009; Goldenthal, 1978a, 1979; Johnson and Ober, 1979, 1980; Lau et al., 2003; Luebker et al., 2005b; Seacat et al., 2003; Seacat et al., 2002; Thibodeaux et al., 2003).

A radiolabel study in which adult male Sprague Dawley rats were given a single intravenous dose of 4.2 mg/kg $^{14}$C-labelled K$^+$PFOS $(\text{CF}_3$-$\text{CF}_2)_6^{14}\text{CF}_2$-$\text{SO}_3$K$^+$) demonstrated that the $^{14}$C in liver and plasma represents 25% and 3% of the dose, respectively, after 89 days. During the 89-day post-dose recovery period, the rats excreted a mean of 30.2% of the total $^{14}$C via urine and the mean cumulative fecal excretion was 12.6%. At 89 days, mean tissue concentration of total $^{14}$C expressed as µg $^{14}$C-PFOS equivalents/g
were: liver, 20.6; plasma, 2.2; kidney, 1.1; lung, 1.1; spleen, 0.5; and bone marrow, 0.5. Lower concentrations (<0.5 µg/g) were measured in adrenals, skin, testes, muscle, fat and eye. No radioactivity (<0.05) was detected in brain (Johnson and Ober, 1980).

Monkeys dosed by oral capsule with PFOS (0.02 and 2.0 mg/kg/day) demonstrated a linear ($r^2 > 0.99$) increase in serum concentration throughout the exposure period of 28 days (Thomford, 2002a). There was no apparent sex difference, and the individual slopes of the cumulative PFOS dose versus serum PFOS concentration curves appeared to be virtually identical. At the end of the 28-day dosing period, serum PFOS concentrations from the 0.02 and 2.0 mg/kg/day groups were 3 µg/mL and 300 µg/mL, respectively. These data suggest a volume of distribution of 200 mL/kg for continuous dosing over a two order of magnitude range.

In a 26-week capsule-dosing study in cynomolgus monkeys, a similar pattern of increasing serum concentration with cumulative dose was observed (Seacat et al., 2002). At the lower experimental doses of 0.03 and 0.15 mg/kg/day, serum levels increased in fairly linear fashion. At the end of the dosing period, the mean serum
PFOS concentrations were approximately 15, 75, and 170 µg/mL, respectively, for 0.05, 0.15, and 0.75 mg/kg/day dose groups. The respective liver PFOS concentrations were approximately 20, 65, and 330 µg/g. The mean serum and liver concentrations between males and females appeared similar within each dose group.

PFOS was found to be 99% to 100% bound to rat, monkey, and human plasma over a concentration range of 1 to 500 µg/mL (approximately 2 to 1000 µM) in vitro (Kerstner-Wood et al., 2003). This suggests that major differences in plasma binding capacity for PFOS do not exist between species.

Several laboratory toxicological studies have demonstrated the presence of PFOS in fetus and neonates when the dams were exposed to PFOS during gestation. The concentration of PFOS was generally higher in the liver than in serum of dams, fetus, and neonates (Abbott et al., 2009; Butenhoff et al., 2009; Lau et al., 2003; Luebker et al., 2005a; Thibodeaux et al., 2003).

Thibodeaux et al. (2003) and Lau et al. (2003) treated female Sprague Dawley rats and female CD-1 mice orally with K⁺PFOS up to 10 and 20 mg/kg/day, respectively, from gestation day (GD)
2 – 20 and GD 1 – 17. At term, maternal serum PFOS concentrations in rats from all dose groups up to 5 mg/kg/day ranged from 0.24 to 80 µg/mL while the respective pooled fetal serum ranged from 0.19 to 85 µg/mL. The corresponding maternal and fetal liver concentration ranges were approximated at 0.5 to 300 µg/g and 0.8 to 175 µg/g, respectively. The serum and liver PFOS concentration profiles in mice were very similar to that observed in rats.

Luebker et al. (2005a) conducted a two-generation reproduction and developmental study using Sprague Dawley rats orally treated with K⁺PFOS for up to 3.2 mg/kg/day of K⁺PFOS by oral gavage for 42 days prior to mating, during mating and during gestation, maternal and pooled fetal serum and liver samples were obtained on GD 21. The maternal serum PFOS concentrations ranged from 4.91 to 180 µg/mL while pooled fetal serum PFOS concentrations ranged from 10.5 to 191 µg/mL. The maternal liver PFOS concentrations ranged from 23.4 to 598 µg/g while pooled fetal liver PFOS concentrations ranged from 9.17 to 265 µg/g.

In a developmental neurotoxicity study, Butenhoff et al. (2009) and Chang et al. (2009) treated female Sprague Dawley rats daily
with K`PFOS up to 1 mg/kg/day via oral gavage from GD 0 through postnatal (PND) 20; with offspring being observed up to PND 72. At term on GD 20, maternal serum PFOS concentrations ranged from < LLOQ (lower limit of quantitation, 10 ng/mL for serum) to 26 µg/mL while the respective pooled fetal serum ranged from 0.009 to 31 µg/mL. The corresponding maternal and fetal liver concentration ranges were approximated at < LLOQ to 48 µg/g and < LLOQ to 20 µg/g, respectively.

Evidence of placental transfer of PFOS has also been reported in 129S2/SvImj mice lacking the nuclear receptor peroxisome proliferator activated receptor alpha (PPARα). PPARα belongs to the nuclear receptor super family. It mediates cell proliferation and differentiation and is expressed at various stages of fetal development in several tissues, such as liver, gastrointestinal tract, kidney, heart, fat, CNS, muscle (Abbott, 2009). Abbott et al. (2009) showed that on PND 15, pups from wild type (WT) and PPARα knockout (KO) dams treated with K`PFOS during GD 15 – 18 at up to 10.5 mg/kg/day had serum PFOS levels ranging from 0.007 to 41 µg/mL (WT) and 0.007 to 52 µg/mL (KO). The respective maternal PFOS concentrations on PND 15 were about half of that in pups.
The potential for exposure during lactation has also been reported in a cross-foster study in Sprague Dawley rats with PFOS (Luebker et al., 2005a). Each dam was allowed to deliver naturally, and each resulting litter was immediately placed in the care of another dam for additional 22 lactation days (LD) such that no dam raised her natural litter. The study demonstrated that after 22 days of lactation, lactational transport was evident as pups from control group that were assigned to be raised by PFOS-treated dams had significant PFOS in the serum (22 µg/mL).

All these data demonstrated that PFOS can be transferred from mothers to offspring in utero and via lactation.

c. Metabolism

PFOS is not known to undergo further metabolism or to form conjugates. Data analyses from urine, feces and tissues of rats as well as the inherent stability of perfluorinated anions suggest that PFOS is not metabolized (Johnson et al., 1984). LC/MS-MS analysis of serum and liver samples collected from toxicology studies had not revealed any evidence of metabolism.
d. **Elimination**

Urinary excretion and fecal excretion are the primary routes of PFOS elimination in rats and monkeys. Decreasing serum PFOS levels over time provides indirect evidence of elimination in humans (Olsen *et al.*, 2007). There are species differences in the elimination half-life of PFOS. The serum and whole-body elimination half-life for PFOS in male rats was about 7.5 and 100 days, respectively, after they were given a single intravenous dose of 4.2 mg/kg $^{14}$C-K$^+$PFOS (Johnson and Ober, 1979). Forty-two (42) % of the administered $^{14}$C-K$^+$PFOS dose was eliminated in urine and feces over a period of 89 days. In addition, significant enterohepatic circulation of PFOS in the rat has been reported, as evidenced by the fact that cholestyramine (a cholesterol-lowering drug, administered as 4% by weight in diet) treatment of rats given single intravenous doses of 3.4 mg/kg $^{14}$C –K$^+$PFOS had increased fecal elimination of $^{14}$C that was increased 9.5 times over the control (Johnson *et al.*, 1984). The extent of total elimination of $^{14}$C (urine plus feces) was also higher in the cholestyramine-treated rats.
After three male and three female cynomolgus monkeys were given a single intravenous dose of 2 mg/kg K⁺PFOS, Noker and Gorman (2003) reported mean terminal serum half-lives of 132 days and 110 days in male and female monkeys, respectively. The estimated volumes of distribution ranging from 178 - 220 mL/kg in males and 231 - 327 mL/kg in females.

In a separate study, cynomolgus monkeys (two per sex per dose group) were followed for one year in recovery after treatment with K⁺PFOS for six months with daily oral capsule doses of 0.15 or 0.75 mg/kg/day (Seacat et al., 2002). While the numbers of animals were limited, the serum PFOS values obtained during the recovery period suggested mean serum elimination half-life of ~200 days, in agreement with the monkey intravenous study reported above.

The best estimated of the elimination half-life of PFOS in human serum is 4.8 years based on the geometric mean (range 4.0 – 5.8 years) (Olsen et al., 2007). This approximation was determined using analysis of serum collected from 27 retirees of two fluorochemical manufacturing plants over a 5.5 year period of time (25 males and 2 females).
The serum elimination half-life of PFOS estimated by Olsen et al. (2007) is in agreement with the published general population biomonitoring data in the United States. Prior to the complete PFOS-production phase out in 2002, the reported serum geometric mean for PFOS levels were approximately 30 - 40 ng/mL between 1999 - 2002 period (Calafat et al., 2006; Calafat et al., 2007a; Olsen et al., 2003b). After the phase out, the reported geometric mean of PFOS levels were 20.7 ng/mL between 2003 – 2004 period, and 14.5 ng/mL in 2006 (Calafat et al., 2007a; Olsen et al., 2008). Similar serum half-life estimate has also been reported by Spliethoff et al. (2008) whom evaluated 2640 newborn infant blood samples collected between 1997 – 2007. They reported an estimated serum half-life of 4.4 years for PFOS.

1.2.2 Acute toxicity

a. Acute dermal toxicity:

PFOS did not appear to cause any skin irritation when 0.5 g of K+PFOS was applied to the shaved skin of New Zealand albino rabbits (Biesemeier and Harris, 1974).
b. **Acute ocular toxicity:**

PFOS did cause mild irritation when 0.1 g of K⁺PFOS was applied to the eyes of New Zealand albino rabbits (Biesemeier and Harris, 1974).

c. **Acute inhalation toxicity:**

The 1-hour medium lethal concentration (MLC₅₀) of K⁺PFOS, administered as dust to Sprague Dawley rats, was 5.2 mg/L when administered over a range of 1.89 to 45.97 mg/L (Rusch, 1979). The most common adverse effects observed were decreased body weight and discolored liver at necropsy.

d. **Acute oral toxicity**

The acute oral LD₅₀ in male and female Sprague Dawley rats were 233 and 271 mg/kg, respectively (Dean *et al.*, 1978). The signs of toxicity observed from this study were hyperactivity, decreased limb tone, and ataxia. At necropsy, macroscopic observations
included yellow-stained urogenital region, stomach distention, glandular mucosa irritation, and pulmonary congestion.

1.2.3 Chronic toxicity

a. Ninety-day study in rodents

An oral 90-day dietary study of male and female Sprague Dawley rats (n=5/sex/dose group) was conducted with the dietary administration of K⁺PFOS at 0, 30, 100, 300, 1000, and 3000 ppm. These feed concentrations were calculated to equivalent doses of 0, 2, 6, 18, 60, and 180 K⁺PFOS mg/kg/day. All rats in the 18, 60, and 180 mg/kg/day dose groups and 50% of the rats in the 6 mg/kg/day group died during the study. Time of death was dose-related. At 18 mg/kg/day group, death for males occurred from study day 13 through day 25 and the death for females occurred from study day 18 to study day 28. At 60 mg/kg/day group, death for males and females occurred from study day 8 through study day 14. At 180 mg/kg/day, death for all rats occurred from study day 7 to study day 8 (Goldenthal, 1978a).
Treatment-related gross lesions were observed in all K\(^+\)PFOS-treated rats. These lesions include discoloration and enlargement of the liver, and discoloration of the gastric glandular mucosa.

Histological lesions were observed in all rats receiving PFOS treatment. Centrilobular to midzonal cytoplasmic hypertrophy and focal necrosis of hepatocytes were observed. Males had a higher incidence and severity of hepatic lesions than females.

Microscopic histological lesions were also reported in the lymphoid organs (thymus, bone marrow, spleen, and mesenteric lymph nodes), stomach, intestines, muscle, and skin. Serum cholesterol values were not measured.

b. Ninety-day study in non-human primates

In a 90-day oral gavage study, male and female rhesus monkeys (n=2/dose group/sex) were treated K\(^+\)PFOS at 0 (control), 10, 30, 100, and 300 mg/kg/day. All monkeys receiving K\(^+\)PFOS treatments died within the first 20 days and the study was terminated (Goldenthal, 1979). Clinical signs of decreased activity, emesis with some diarrhea, body stiffening, general body trembling, twitching, weakness, convulsions, and prostration were observed in all K\(^+\)PFOS-treated animals. Liver discoloration was
observed during gross necropsy. Microscopically, congestion, hemorrhage, and lipid depletion of the adrenal cortex were observed. Serum clinical chemistry measurements were not made.

A follow-up study in monkeys was undertaken using lower K⁺PFOS doses. Rhesus monkeys (n=2/sex/dose group) were administered with 0 (control), 0.5, 1.5 and 4.5 mg/kg/day K⁺PFOS via oral gavage for 90 days (Goldenthal, 1978b). All monkeys in the 4.5 mg/kg/day group died or were euthanized in extremis between weeks 5 and 7 of the study, with similar clinical signs to those observed in the previous study. All monkeys dosed at 0.5 or 1.5 mg/kg/day survived until study termination (90 days). Reported clinical signs included decreased activity, diarrhea and abnormal stools. There was also body weight-loss in both male and female monkeys in the 1.5 mg/kg/day group. There were no K⁺PFOS treatment-related lesions observed macroscopically or microscopically at the lower dose groups. Microscopic lesions in adrenals, pancreas, and salivary gland were observed from 4.5 mg/kg/day monkeys.
For monkeys from the 0.5 mg/kg/day and 1.5 mg/kg/day groups, regardless of sex, there was no statistically significant difference in their mean serum cholesterol values when compared to the respective time- and sex-matched controls after one month or three months of dosing. The male and female control monkeys had a pre-dose mean cholesterol value of 183 and 180 mg/dL, respectively. The respective control values were 198 and 198 mg/dL after one month of dosing, and 162 and 165 mg/dL after three months of dosing. After one month of treatment, the average serum cholesterol values were 87 mg/dL and 112 mg/dL for the male and female monkeys in the 4.5 mg/kg/day dose groups, respectively. There was no serum cholesterol data available from 4.5 mg/kg/day dose group at 3 month necropsy as all the monkeys were dead prior to study termination.

c. Twenty six-week (182-day) study in non-human primates

K⁺PFOS was administered to cynomolgus monkeys by oral capsule at doses of 0, 0.03, 0.15, and 0.75 mg/kg/day for 26 weeks; and the number of monkeys (per sex per dose group) were 6, 4, 6, and 6, respectively (Seacat et al., 2002). At the end of 26 weeks,
two monkeys per sex from 0, 0.15, and 0.75 mg/kg/day group were monitored for additional 6 months.

Two male monkeys from the 0.75 mg/kg/day group did not survive to the scheduled sacrifice. One died during week 23 (day 155) while the other one was euthanized in extremis 3 days prior to the study termination (day 179). Clinical signs noted were low food consumption, excessive salivation, labored respiration, hypoactivity, and dehydration. The cause of death for the first monkey was pulmonary necrosis but was not determined for the second monkey.

Except for the female monkeys from the 0.75 mg/kg/day group, which did not gain any body weight during the 26-weeks of K⁺PFOS treatment period, there was no decrease in body weight-gain seen with the other K⁺PFOS-treated monkeys when compared to the sex-matched controls. The body weight-gain effect in 0.75 mg/kg/day female group was reversed at the end of the recovery period.

Male and female monkeys in the 0.75 mg/kg/day group had lower total cholesterol during the treatment period. The effect was more
pronounced over time. At the termination of the study, mean total cholesterol for males and females in the 0.75 mg/kg/day group were 48 and 82 mg/dL, respectively. The mean control values for male and female were 152 and 160 mg/dL, respectively. The total serum cholesterol effect, however, was resolved to normal within 5 weeks after the cease of PFOS treatment.

Liver effects such as increased absolute liver weight and hepatocellular hypertrophy were noted from the 0.75 mg/kg/day PFOS-treated monkeys, regardless of sex. There did not appear to be a sex difference in PFOS concentrations in serum or liver. At the end of treatment period, approximated mean serum PFOS concentrations were 14, 75, and 173 μg/mL for monkeys in 0.03, 0.15, and 0.75 mg/kg/day dose groups, respectively. The respective mean liver concentrations were 20, 65, and 335 μg/g.

d. Two-year study in rodents

A two-year (104-week) dietary study was conducted in Sprague Dawley rats (n=40 – 70 per sex per dose) with K⁺PFOS at 0, 0.5, 2, 5, and 20 ppm (Thomford, 2002b). The calculated doses would be 0, 0.03, 0.13, 0.3, and 1.3 mg/kg/day. Interim sacrifices were
performed on weeks 4 and 14 for all the dose groups and that portion of the study data was published by Seacat et al. (2003). While interim sacrifice was also performed on week 53 for a subset of rats from control and 20 ppm dose groups, the remaining rats were continuously treated with the respective control or K⁺PFOS diets until the termination of the study (up to one additional year). In addition, a subset of male and female rats that had been exposed with 20 ppm K⁺POFS for 53 weeks were switched to control diet on week 53 and monitored for the remaining the study for up to 106 weeks (stop-dose group) (Thomford, 2002b).

On week 53, mean serum PFOS concentrations were 0.025 and 146 μg/mL in male rats from control and 20 ppm dose groups, respectively. The respective mean serum PFOS concentrations were 0.395 and 220 μg/mL in female rats. On week 105, mean serum PFOS concentrations were 0.018, 1.31, 20.2, 22.5, and 69.3 μg/mL in male rats from 0 (control), 0.5, 2, 5, and 20 ppm dose groups, respectively. The respective mean serum PFOS concentrations in female rats (obtained on week 102) were 0.084, 4.35, 7.60, 75, and 223 μg/mL. The stop-dose group male and
female rats had 2.42 and 9.51 μg/mL as serum PFOS levels on week 106 (3M, 2001).

Compared to controls, both male and female rats receiving 20 ppm K⁺PFOS treatments had significantly lower mean body weights and decreased food consumption during some occasional periods of the study. Across the dose groups, the body burden of serum PFOS levels generally increased proportionally with the K⁺PFOS doses given. Within the dose groups, the levels of PFOS in serum on week 105 were similar or lower than the corresponding serum levels in the groups on week 53 or prior (Seacat et al., 2003), especially in male rats. With the exception of control and 20 ppm stop-dose group rats, all the other rats were continuously treated with K⁺PFOS until sacrifice, therefore, this observation did not fit with the serum PFOS half-life data (at least 7.5 days per Johnson and Ober, 1979)). It appeared that the development of nephropathy in the rats in relation to aging (regardless of treatment groups including control) had attributed to the enhanced elimination of serum components, such as proteins and PFOS. Indeed, increased incidence of nephropathy development is commonly observed in male rat only, a species- and sex-specific phenomenon (Alison et al., 1994).
Microscopically, hepatic hypertrophy was consistently observed in both male and females from the 20 ppm dose group during week 52 sacrifice and terminal sacrifice. This hypertrophic observation was not accompanied by cell proliferation as PCNA and BrDU immunohistochemistry data were comparable to those in controls. There was a positive-sloped trend for hepatocellular adenoma in the 20 ppm dose group male and female rats that were treated with K⁺PFOS for up to 2 years. The degree of incidence was lowered for hepatocellular adenoma in 20 ppm K⁺PFOS-treated rats from the 20 ppm recovery groups. There was a single observation of hepatocellular carcinoma in a 20-ppm female rat. In addition, a negative-sloped trend in mammary fibroadenoma was observed in the 20 ppm dose group female rats.

At the termination of the study, there was no increased incidence of follicular thyroid adenoma in male or female rats receiving 20 K⁺PFOS for up to 2 years. Interestingly, there was an increased incidence of follicular thyroid adenoma noted in the male 20 ppm K⁺PFOS stop-dose group rats.

e. Chronic toxicity – summary
It appears that the mortality dose-response curve for PFOS is very steep for Sprague Dawley rats and rhesus monkeys. All Sprague Dawley rats dosed with 2 mg/kg/day or 0.03 mg/kg/day K⁺PFOS survived 90 days or 2 years of dosing, respectively. All rhesus monkeys dosed at 4.5 mg/kg/day or higher died prior to scheduled study termination (90 days) while those dosed at 1.5 mg/kg/day K⁺PFOS or lower survived the entire study duration. All cynomolgus monkeys treated with K⁺PFOS at 0.15 mg/kg/day or lower survived 26 weeks of dosing and 6 months of recovery.

1.2.4 Mutagenicity

PFOS was tested for its ability to induce mutation in the Ames Salmonella/microsome plate test and in the D4 strain of Saccharomyces cerevisiae. PFOS was also tested in: (1) a Salmonella – Escherichia coli / mammalian-microsome reverse mutation assay; (2) chromosomal aberrations assay \textit{in vitro} in human lymphocytes; and (3) unscheduled DNA synthesis (UDS) in rat primary hepatocyte tissue cultures. It was negative for all assays in which it was tested (3M, 2003). PFOS was also tested in an \textit{in vivo} mouse micronucleus assay, it did not induce
micronuclei in the bone marrow of CD-1 mice and it was negative in the mouse bone marrow micronucleus assay (3M, 2003).

1.2.5 PFOS and thyroid hormone status in laboratory studies

In the same two-year (104-week) dietary study mentioned earlier, Sprague Dawley rats were treated with K⁺PFOS (incorporated in the rat chow) at 0, 0.5, 2, 5, and 20 ppm. For rats from a subset of 20 ppm dose group, K⁺PFOS-containing diet was ceased after one year and control diet was given for the remaining study duration of up to 1 more year (termed “stop-dose group”). There was an increased incidence of thyroid follicular cell adenoma among the stop-dose group rats (whom had received 20 ppm PFOS diet for one year followed by approximately one more year of control diet). In contrast, there was no increased incidence of this tumor type in male and female rats that were continuously treated with 20 ppm K⁺PFOS for up to two years. Serum thyroid hormone measurement was not performed in this study.

In the 26-week cynomolgus monkey toxicity study with K⁺PFOS by Seacat et al. (2002), monkeys received daily oral treatments of K⁺PFOS at 0 (control), 0.03, 0.15, and 0.75 mg/kg/day. As described earlier, the PFOS body burden reached 170 μg/mL from the highest dose group at
0.75 mg/kg/day and this dose was associated with some toxicity such as mortality, decreased serum cholesterol, decreased body weights, and increased liver weights in monkeys. Even so, using the analog method (a radioimmunoassay- or chemiluminometric-based technique), serum TT4, FT4, and FT3 values evaluated from all the K⁺PFOS-treated monkeys were comparable to control. Serum FT4 values were also comparable to control when equilibrium dialysis-radioimmunoassay (ED-RIA, a reference method for free hormone measurement) was used. K⁺PFOS treatment did lead to a dose-dependent decrease in serum TT3 in both males and females at the end of the treatment but it was resolved 61 days post-K⁺PFOS oral treatment. Serum TSH values, mirrored the K⁺PFOS doses given, were slightly increased at the end of treatment but they were all well within reference range. No histological evaluation was done on the thyroid. With regards to serum PFOS distribution, there was not a sex-difference between male and female monkeys. The corresponding serum PFOS concentrations were approximately 170, 75, and 15 μg/mL for the 0.75, 0.15, and 0.03 mg/kg/day dose groups, respectively.

Reduction in serum thyroid hormones without a compensatory increase in TSH was also reported in female rats (pregnant and non-pregnant) and female mice (pregnant) by Thibodeaux et al. (2003). K⁺PFOS solution was given to pregnant rats at 1, 2, 3, 5, 10 mg/kg/day orally during
gestation from GD 2 to GD 20 or 3 and 5 mg/kg/day for 20 days in non-
pregnant rats. K⁺PFOS was also given to pregnant mice at 1, 5, 10, 15,
and 20 mg/kg/day orally from GD 1 to GD 17. At term, the accumulated
body burdens of PFOS in the pregnant rats and mice were approximately
200 - 250 μg/mL from the highest dosed groups. While the maternal
exposure to K⁺PFOS was associated with a dose-dependent suppression of
maternal weight-gain (rats and mice) and hepatomegaly (mice only), it had
also been associated with fetal developmental toxicity at the highest study
doses in both species. Analog radioimmunoassay (RIA) methods were
used to determine serum TT4, FT4, TT3, and TSH levels. Serum TSH
levels were not altered by K⁺PFOS treatments in rats but there were dose-
dependent decreases in serum TT4, FT4, and TT3 (both pregnant and non-
pregnant). A dose-dependent reduction in serum TT4 was also observed
in mice dams on GD 6 and not the later time points during gestation.

Using identical gestational K⁺PFOS doses described in Thibodeaux et al.,
Lau et al. (2003) conducted the postnatal evaluations of the offspring from
K⁺PFOS-exposed rats and mice and reported that high doses of maternal
K⁺PFOS treatments in rats and mice led to decreased postnatal survival
and delayed growth in pups. At term, levels of serum PFOS measured in
neonates were similar to the respective maternal serum PFOS levels. Pup
serum samples obtained throughout postnatal (PND) period (up to PND
were subject to TT4, FT4, TT3, and TSH evaluations with analog methods. Similar to the data reported by Thibodeaux et al. (2003), there were reduced serum TT4 and FT4 in pups from K⁺PFOS-treated rat dams although the respective serum TT3 and TSH were normal. For mouse pups from K⁺PFOS-treated dams, the serum TT4 levels were all within reference range. Further examination of choline acetyltransferase activities (an enzyme that is responsive to changes in thyroid hormone) in rat pups demonstrated that maternal K⁺PFOS treatment only slightly decreased the enzyme activities in the prefrontal cortex but not in hippocampus.

In addition, maternal exposure to K⁺PFOS did not affect learning and memory of the pups using T-maze.

The potential effect of PFOS on neonatal development was further investigated by Luebker et al. (2005b) in rats that received 0.4, 0.8, 1.0, 1.2, 1.6, and 2.0 mg/kg/day K⁺PFOS beginning 6 weeks before mating, during mating, gestation, and lactation. Although K⁺PFOS treatments did not affect mating or fertility parameters, it did cause a significant reduction in gestation length and neonatal viability at doses of 0.8 mg/kg/day or higher. The body burden of PFOS correlated with doses administered to dams and in general, the serum PFOS concentrations in
pups were at least 20 – 50% higher than dams on PND 5. Serum and liver samples obtained on PND 5 from dams and pups were evaluated for thyroid hormone status because hypothyroid condition has been hypothesized in the attribution of the developmental delay observed in a two-generation study with PFOS in rats (Luebker et al., 2005a). Similar to the data reported by Thibodeaux et al. (2003) and Lau et al. (2003), treatment of K+PFOS to female rats prior to mating, during mating, gestation, and lactation was associated with reduced PND 5 serum TT4, TT3, FT3, and FT4, in either dams or pups, when analog methods were used. However, the corresponding serum TSH concentrations were unaltered and when ED-RIA reference methods were used to measure FT3 (pups only) and FT4 (dams and pups) concentrations in these samples, the values of FT3 and FT4 obtained were comparable to the control. Liver malic enzyme activity, an enzyme that is sensitive to the changes in thyroid hormone (Oppenheimer et al., 1977), was not altered in dams or pups on PND 5. There was no effect of maternal K+PFOS exposure on learning or memory in pups.

To evaluate whether prenatal stress can be induced chemically (by PFOS) and potentially interfere with the fetal developmental outcomes, Fuentes et al. (2006) evaluated gestational exposure, with or without restraint, in pregnant mice receiving K+PFOS oral treatment. The K+PFOS doses of
1.5, 3, and 6 mg/kg/day were administered to pregnant mice from GD 6 – GD 18. K⁺PFOS administration or combined with restraining was not associated with any adverse maternal outcome except a dose-dependent increase in liver weight as reported by others (vide supra). Regardless of restrain or not, the levels of stress hormone corticosterone from K⁺PFOS-treated dams were comparable to control dams. The prenatal toxicity included increased mortality in pups from 6 mg/kg/day-dosed dams. Maternal serum thyroid hormones were measured using analog method at term, and relative to control, there were no differences in serum TT4, FT4, TT3, and FT3 concentrations.

In a toxicogenomic analysis study, Martin et al. (2007) treated groups of rats with daily doses of 10 mg/kg K⁺PFOS for 1, 3, or 5 consecutive days. Analysis of liver samples obtained from K⁺PFOS-dosed group rats led to a cluster of cytochrome P450 genes representative of PPARα-, CAR-, and PXR-associated pathways. Furthermore, compared to control, serum samples obtained from K⁺PFOS-treated rats at 24 hours after the last scheduled oral doses reported reductions in serum TT4 and FT4 (as measured by analog methods) regardless of K⁺PFOS-treatment duration. Serum TT3 (determined by analog method) was also reduced, but only from the rats that had received 5 consecutive daily doses of K⁺PFOS.
Using biomarkers to study fatty acid metabolism, Curran et al. (2008) also evaluated serum TT3 and TT4 levels with analog methods because of thyroid hormone’s regulation of lipid metabolism (Larsen et al., 2003; Visser, 1996). After rats received K⁺PFOS at 2, 20, 50, or 100 mg per kg diet for 28 days, similar to what others had reported, Curran et al. also reported hepatomegaly, decreased serum triglyceride and cholesterol, decreased body weight and body weight-gain, and increased liver gene transcript expression for acyl CoA oxidase and Cyp 4A22 in the rats. Overall serum TT3 and TT4 concentrations were reduced relative to the control.

A recent study by Yu et al. (2009b) had investigate the effect of K⁺PFOS in drinking water and potential disturbance in thyroid functions. After 91 days of consecutive ad libitum drinking water exposure with 1.7, 5, and 15 μg/mL K⁺PFOS, the corresponding serum PFOS concentrations were 5, 33.6, and 88.2 μg/mL, respectively. Analog methods were used to measure all serum thyroid hormones. K⁺PFOS treatment did not appear to affect the rat body weights at this concentration range and it did not affect serum TSH levels at all. It caused hepatomegaly and decreased serum TT4 levels relative to control in a dose-dependent manner. There was a transient increase in serum TT3 (at 1.7 μg/mL) and a transient decrease in serum FT4 (at 5.0 μg/mL), however, there was no dose-response trend in
either of these two parameters. Gene transcript analysis on thyroid tissue demonstrated that the expression of sodium iodide symporter (NIS) and TSH receptor (TSHR) were unaltered but deiodinase 1 (D1) expression was increased. The enzyme activity of thyroperoxidase (TPO) in thyroid was unchanged relative to control. Transcript analysis on liver tissue indicated enhanced thyroid hormone turnover with increased UGT1A1 transcript expression.

The observations from these previous repeat-dose toxicological studies suggest that PFOS does not cause a physiological hypothyroid state even though it is capable of reducing serum total thyroid hormones. In the toxicological studies mentioned above, the reductions in TT4, TT3, and FT4 as measured by analog methods were not associated with major compensatory increases in TSH, as would be expected in a hypothyroid condition, or with alterations in thyroid histology or thyroid transporter, receptor, and enzyme gene expressions. Interestingly, when FT4 was assayed using the reference ED-RIA method, there was no apparent change compared to control values (Luebker et al., 2005b; Seacat et al., 2002).
1.2.6 PFOS and thyroid hormone status in humans

In 2000, a medical surveillance program was conducted for 518 employee volunteers of 3M Company whom worked in or near the fluorochemical manufacturing plants in Antwerp, Belgium (N = 255 people) or Decatur, Alabama, United States (N = 263 people) (Olsen et al., 2003c). The geometric mean of serum PFOS concentration among participating employees in the Decatur plant was 0.91 μg/mL (range 0.06 – 10.06 μg/mL). The geometric mean serum for employees in Antwerp plant was 0.44 μg/mL (range 0.38 – 0.51 μg/mL). There were no substantial changes in hematological, lipid, hepatic, urinary, and thyroid parameters (TSH, TT4, FT4, and TT3 as measured by analog methods). The entire sample set was re-analyzed using improved analytical technique for PFOS concentrations but not thyroid hormone measurement (Olsen and Zobel, 2007). Comparable serum PFOS values were reported between the original analyses and latter analyses and there was still no association between serum PFOS and thyroid hormones.

As a comparison, the geometric mean for serum PFOS concentration in the United States was 0.030 μg/mL (range 0.027 – 0.229 μg/mL) from 1999 – 2000 NHANES data (Calafat et al., 2007a). That was approximately 30 times lower than the occupationally exposed human.
Based on the hypothyroxicemia reported in rodent studies, Inoue et al. (Inoue et al., 2004) evaluated 15 paired maternal and cord blood human samples in Japan for PFOS concentration and its potential association with thyroid hormone disturbance. The maternal and fetal serum PFOS concentration ranged from 0.0049 – 0.0176 µg/mL and 0.0016 – 0.0053 µg/mL, respectively. When serum TSH and FT4 were evaluated on the cord blood samples using analog methods, there was no correlation between serum PFOS levels and TSH or FT4.

Because PFOS has been postulated to bioaccumulate in the food chain, especially in the marine species, Dallaire et al. (2009) studied serum PFOS concentrations concurrently with 41 other environmental pollutants to see if they interfere with the thyroid hormone status in 600+ Inuit adults (whose diet mainly consists of seafood) from Nunavik in 2004. The geometric mean of serum PFOS in this group of study subject was 0.01828 µg/mL (range 0.00048 – 0.470 µg/mL), which is similar to the geometric mean in the United States of 0.0207 µg/mL (range 0.0192 – 0.0223 µg/mL) reported by NHANES during 2003 – 2004 period (Calafat et al., 2007b). Serum TSH, FT4, TT3, and TBG (thyroid binding globulin, a carrier protein for transporting thyroid hormones in the blood) were evaluated using analog methods. The mean values for serum TSH,
FT4, TT3, and TBG were all within the reference range but the authors concluded that there was a significant negative association between serum PFOS concentrations and serum TSH, TT4, and a positive association with FT4 levels based on the linear regression model. Further review of the paper revealed that while gender, age, BMI, plasma lipid, cigarette consumption, and education were the confounders adjusted in the model, other key factors that were not included in the model such as frequency of alcohol consumption (in which 50% of the study population consumed alcohol) and medication use (in which 17% of the study population indicated that they did) could bias the regression.

The bioaccumulation potential of PFOS in the food chain also led to Bloom et al. (Bloom et al., 2009) to evaluate the serum PFOS concentration (along with several other perfluoroalkyl acids) and thyroid functions on 31 licensed anglers in New York, whom presumably had frequent contact and consumption of seafood. The geometric mean of serum PFOS concentration was 0.0196 µg/mL (range 0.0163 – 0.0235 µg/mL) and there was no association between serum PFOS concentrations and serum TSH and FT4.
1.2.7 Other studies: reproductive, developmental, and developmental neurotoxicity

Under laboratory study conditions, administration of K⁺PFOS to rats and mice during gestation does not seem to affect the reproductive functions; however, maternal toxicity such as decreased body weight-gains and food consumption can occur (Lau et al., 2003; Grasty et al., 2003; 2005; Luebker et al., 2005a; Thibodeaux et al., 2003). Maternal exposure to K⁺PFOS in rats and mice can cause significant decreases in fetal body weight and postnatal survival, significant increases in external and visceral anomalies, delayed ossification, and skeletal variations (Lau et al., 2004). Structural abnormalities, however, were not present in rabbits (Case et al., 2001).

Grasty et al. (Grasty et al., 2003) evaluated the critical period for neonatal mortality induced by maternal daily exposure to K⁺PFOS in the rats at 25 mg/kg/day during gestation (Gd 2-5, 6-9, 10-13, 14-17, 17-20, or 19-20). K⁺PFOS was also given to time-pregnant rats at 25 mg/kg/day on Gd 19-20. K⁺PFOS treatments during gestation effectively reduced both maternal and fetal body weights but the litter size at birth was unaffected. K⁺PFOS treatment reduced neonatal survival and the extent of neonatal mortality was higher when K⁺PFOS exposure was later during gestation.
Higher maternal serum PFOS level (i.e. > 100 μg/mL) corresponded to higher neonatal mortality. On GD 21, microscopic evaluation on the fetal lungs showed that the fetal lungs from K+PFOS-treated dams were not expanding as much as the fetal lungs from control-treated dams, even though the chemical composition of the lung surfactants and phospholipids were normal (Grasty, 2005).

A two-generation study in rats indicated that treatment of K⁺PFOS to rats, at 6 weeks prior to mating, during gestation and lactation, did not affect mating or fertility (i.e., estrous cycles, primordial follicle counts, sperm count, sperm morphology; mating index and pregnancy rates), however, it reduced F₀ males and F₀ females body weight-gains and food consumption (Luebker et al., 2005a). In the same 2-generation reproduction study mentioned above, parental exposures to K⁺PFOS resulted in decreases in pup body weights at dosages of 0.4 mg/kg/day or higher (Luebker et al., 2005a). It had also resulted in decreases in postnatal survival at dosages of 1.6 mg/kg/day or higher. Assessments on learning, short-term retention, and memory in rat pups beginning on PND 24 (in which they were administered K⁺PFOS beginning on PND 22) using a passive avoidance apparatus were performed, in addition to neuromuscular coordination, swimming ability, learning, and long-term memory using a
water-filled M-maze on PND 70. Exposures to K⁺PFOS did not appear to affect these assessments.

Studies by Lau et al. (2003) and Thibodeaux et al. (2003), as discussed earlier, treated pregnant rats from GD 2 to GD 21 or pregnant mice from GD 1 to GD 17 with K⁺PFOS. Gestational exposure to K⁺PFOS resulted in dose-dependent effects in neonatal body weight (↓), relative liver weights at birth (↑), and postnatal mortality (↓). There were growth delays in pups from dams treated with high K⁺PFOS doses but when evaluated for learning ability in weanling rat pups using a T-maze and delayed alternation, there were no significant alterations in learning and memory behaviors.

Butenhoff et al. (2009) reported decreases in maternal body weight gain and food consumption when rat dams were treated with 1 mg/kg/day K⁺PFOS via oral gavage from GD 0 to PND 20. Even though there were no effects in neonatal body weights or postnatal survival, however, there was an effect on motor activity in the 1.0 mg/kg/day K⁺PFOS maternal dose-group male pups on PND 17. Total and ambulatory activity counts in these males were increased (generally statistically significant; p<0.05) compared to the concurrent control group, and a majority of these males failed to habituate to the test environment on PND 17 (an age when rats
should begin to habituate). The effect on habituation was transient, occurring only on the PND 17 evaluation. It is noteworthy that increased motor activity and decreased habituation were also observed in mice by Fuentes et al. (2007) and Johansson et al. (2008).

Study by Fei et al. (2007) evaluated the relationship between birth outcomes and the corresponding maternal serum PFOS concentration (collected during gestation) in 1400 women participated in the Danish National Birth Cohort program. No association between serum PFOS levels and birth outcomes such as birth weights and growth was reported.
II.

PFOS AND THYROID HORMONES
2.1 PFOS AND THYROID HORMONES

Due to the fact that PFOS is widely present in the environment and human population including children, and exposure of PFOS to neonates and children can begin as early as *in utero*, there has been an increased research interest in evaluating potential health effects with PFOS, as demonstrated by the vast literature review discussed in Chapter I. Particularly, the reported alterations in thyroid hormone levels such as hypothyroxinemia had led to extensive efforts in the evaluation of PFOS on the effects of thyroid hormones and thyroid hormone status, because of their critical roles in growth (Bernal, 2005a, 2005b; Calvo *et al.*, 1992; Oppenheimer *et al.*, 1995) and brain development; in that they control expression of genes involved in myelination, cell differentiation, migration, and signaling (Bernal, 2005a, 2005b).

2.2 THYROID GLAND

2.2.1 Structure and functions

The thyroid gland is a bi-lobed structure connected by an isthmus that extends across the ventral surface of the trachea below the larynx. The thyroid gland is innervated by adrenergic fibers from the cervical ganglia and by cholinergic fibers from the vagus nerve. This autonomic
innervation serves to regulate blood flow. Adrenergic stimuli increases blood flow while cholinergic stimuli decreases it (Larsen et al., 2003).

Histologically, the thyroid gland contains follicles formed by epithelial cells. These follicular cells are cuboidal in a normal gland, columnar in a highly stimulated gland, and squamous in an inactive gland. A clear viscous material is normally present in the lumen of the follicles. This fluid is called colloid. Colloid is primarily comprised of the glycoprotein thyroglobulin and synthesized thyroid hormones.

The thyroid gland has an abundant blood supply. The blood volume of normal humans is about 5 L per minute. This mass moves through the lungs about once a minute, through the kidneys once in five minutes, and through the thyroid approximately once an hour (Larsen et al., 2003). Although the thyroid only represents about 0.4% of body weight, it accounts for 2% of total blood flow with the estimated normal blood flow of 5 mL/min per gram of thyroid tissue. Blood flow through the gland may increase up to 100-fold in cases of thyroid disease.

2.2.2 Thyroid hormones synthesis

The thyroid gland carries out a specific biochemical process in the formation and secretion of thyroid hormones. The two major thyroid
hormones circulating in the serum are L-thyroxine (tetraiodothyronine, T4, M.W. = 777 g/mol) and L-triiodothyronine (T3, M.W. = 651 g/mol).

As illustrated in the figures above, iodine is an essential component of the thyroid hormones. It is 65% and 58% of the total molecular weight of T4 and T3, respectively. Iodine is absorbed through the small intestine and transported into thyrocyte via a Na⁺-dependent, TSH-regulated active transport process mediated by sodium/iodine symporter (NIS) protein found in the basolateral plasma membrane of the thyrocytes (Kogai et al., 1997). I⁻ is transported across the apical membrane by pendrin transporter into follicular lumen. Once inside the lumen, iodine is concentrated, oxidized, and then incorporated into the tyrosyl residues of the thyroglobulin, a polypeptide backbone, to form monoiodotyrosine (MIT),
diiodotyrosine (DIT) in a reaction catalyzed by thyroperoxidase (TPO). MIT and DIT are subsequently coupled for form T3 and T4 in another reaction catalyzed by TPO.

It is worth noting that several key proteins involved in the thyroid hormone synthesis and transport, such as NIS, TPO, and thyroglobulin, are regulated by a cAMP cascade in the thyrocytes, which can be directly stimulated by TSH or β-adrenergic and prostaglandin E receptors (Vassart and Dumont, 1992).

2.3 THYROID HORMONES

2.3.1 Transport of thyroid hormones

Thyroglobulin is the most abundant protein in the thyroid gland, which a concentration ranging from 200 to 300 g/L (Visser, 1990). The main function of thyroglobulin is to serve as a backbone support for the
synthesis and storage of thyroid hormones. When needed, thyroid hormones such as T3 and T4 can be released from thyroglobulin peptide linkage via lysosomal cleavage. Free thyroid hormones can then be transported out of the thyrocytes to enter the circulation. The transport process of T3 and T4 into circulation via receptor-mediated transporters have been suggested (Andersson et al., 1990; Visser, 2007; Visser et al., 2008).

Once in the blood, greater than 99% of the thyroid hormones that had just entered will immediately bind to one of the several serum thyroid hormone binding proteins to maintain stability. In human, serum thyroid hormone binding proteins include thyroxine binding globulin (TBG), transthyretin (TTR, also known as thyroxine-binding prealbumin, TBPA), and albumin (Oppenheimer, 1968). In rats, there is no TBG. TTR exists only for approximately two weeks after birth hence the major thyroid hormone carrier protein in rats is albumin (Young et al., 1988). Despite the fact that the molar concentration of albumin is the highest among the serum thyroid hormone binding proteins (100 times and 2000 times higher than TTR and TBG, respectively), the binding affinity of albumin to T4 (Ka = 1.5 x 10^6/M) is much lower than that of TBG to T4 (Ka = 1.1 x 10^10/M) or TTR to T4 (Ka = 2 x 10^8/M). Similarly, the binding affinity of albumin to T3 (Ka = 2 x 10^5/M) is also lower than that of TBG to T3 (1 x
10⁹/M) but slightly higher than TTR to T3 (1 x 10⁶/M). TBG (~54 kDa), TTR (~55 kDa), and albumin (~67 kDa) have 1, 2, and multiple binding sites for thyroid hormones, respectively (Schussler, 2000).

For thyroid hormone measurements, total T4 (TT4) comprised of approximately 0.03% and 99.9% of respective unbound and bound T4 while total T3 (TT3) has approximately 0.3% and 99.5% of unbound and bound T3, respectively. Among the circulating thyroid hormones in serum, about 99% of that is T4 while less than 1% is T3 (Refetoff et al., 1970). In serum, the bound and unbound thyroid hormones exist in equilibrium, and the association / dissociation constants between thyroid hormones and carrier proteins are within seconds (Mendel et al., 1988). Both FT4 and FT3 are considered as the biologically available moiety of the thyroid hormones even though they are small in fraction compared to the bound counterpart (Oppenheimer et al., 1995).

2.3.2 Thyroid hormone functions

FT4 is the prohormone that becomes activated upon its conversion to FT3. FT3 is capable of exerting biological functions via nuclear receptor-mediated gene transcription processes in target cells. FT4 is converted to FT3 via deiodination in the presence of deiodinase 1 (D1) or deiodinse 2
(D2). D1 mainly attributes to plasma T3 production and it is mainly expressed in liver, kidney, and thyroid. D2 is responsible for local peripheral T3 production and it is expressed in brain, pituitary, skeletal, muscle, and heart. Another enzyme, deiodinase 3 (D3), which mediates thyroid hormone degradation by converting FT4 and F3 to inactive forms, has been found in high concentrations in the brain of adults or fetal tissues (Visser, 1990).

Thyroid hormones regulate many physiological processes via thyroid receptor-mediated mechanism. They regulate body’s energy metabolism (i.e., neurological, cardiovascular, musculoskeletal, and reproductive functions) and many other aspects of fetal and neonatal development as thyroid receptors are expressed in virtually all tissues in animals and humans.

Thyroid hormone acts on bone growth via stimulation of growth hormone and insulin-like growth factor I (IFG-I) or by direct effects on the target genes (Mundy et al., 1976). Thyroid hormones can regulate the cardiac functions by interacting with several important target genes such as myosin heavy chain (responsible for cardiac contractility) within the heart (Dillmann, 1990). Thyroid hormones also play an important role in the development and function of brown and white adipose tissues by inducing
tissue differentiation and proliferation via transcription (Flores-Delgado et al., 1987; Grimaldi et al., 1982). Through thyroid receptor regulations, thyroid hormones have multiple effects on liver function including stimulation of enzymes regulating lipogenesis and lipolysis as well as oxidative processes (Oppenheimer et al., 1987). Thyroid hormones also regulate the synthesis and secretion of several important pituitary hormones such as TSH, growth hormones, and sex hormones (Samuels et al., 1988).

Thyroid hormone has major effects on the developing brain in utero and during the neonatal period (Burrow et al., 1994; Larsen, 1988; Morreale de Escobar et al., 1988; Oppenheimer and Schwartz, 1997; Ruiz de Ona et al., 1988; Zoeller et al., 2002). Studies in hypothyroid neonatal rats have shown that the absence of thyroid hormones cause diminished axonal growth and dendritic arborization in the cerebral cortex, visual and auditory cortex, hippocampus, cerebellum, and the Purkinje cells (Anderson et al., 1997; Koibuchi and Chin, 1998; Rabie et al., 1977; Rabie and Legrand, 1973). These developmental effects in neonatal rats can be reversed if thyroid hormone is administered within 2 weeks after birth. In addition, studies have suggested that maternal transfer of thyroid hormones may be important during early fetal development (Calvo et al., 1992; Calvo et al., 1990; Vulsma et al., 1989). In fact, inadequate
production of maternal and fetal thyroid hormones due to severe iodine deficiency during pregnancy is one of the world’s most common preventable causes of mental retardation (endemic cretinism).

c. Thyroid hormone regulations

The control mechanism for serum thyroid hormones is complex in that it belongs to Hypothalamus-Pituitary-Thyroid (H-P-T) axis (Larsen, 1989; Larsen et al., 1981; Oppenheimer et al., 1995). When the free thyroid hormone is low in circulation, the hypothalamus secretes thyrotropin releasing hormone (TRH). The role of TRH is to signal the pituitary gland so that it can release more TSH. The release of TSH stimulates the thyroid gland to synthesize and release more thyroid hormones into circulation. When there are too much free thyroid hormones in the blood, down regulation of TRH, TSH, and thyroid hormone synthesis applies.

The function of the thyroid gland is not only governed by TSH, it also reciprocates a feedback mechanism based on free thyroid hormone levels in the blood that it produces as well (Larsen, 1982). In addition, free thyroid hormones such as T4 and T3 also exert the feedback control at the pituitary and hypothalamic levels. Hence the tightly-maintained interplay
of the H-P-T axis attributes to stable concentration ranges of thyroid hormones and related parameters.

2.3.4 Elimination

As mentioned earlier, FT4 is the prohormone. When the peripheral tissues are in need to thyroid hormones, FT4 then become activated upon its conversion to FT3 via deiodinases (in the peripheral tissues) followed by the subsequent interactions with nuclear receptors. About 33% of FT3 is converted by enzymatic outer ring deiodination (ORD) of FT4 in peripheral tissues (Bianco and Larsen, 2005; Visser, 1996).

Approximately another 33% of FT4 is converted to reverse T3 (rT3), a biologically inactive metabolite, via inner ring deiodination (IRD) while the remainder of the FT4 is metabolized via glucuronidation and sulfation (Visser, 1996).

2.3.5 Thyroid hormone diseases

Euthyroidism is the physiological state characterized by normal thyroid function. Normal serum concentrations of T3 and T4 are derived from euthyroid animals and humans. Two major alterations of euthyroid condition can exist: hypothyroidism and hyperthyroidism. About 20 million Americans currently have some form of thyroid disease, and the
prevalence of thyroid disease does not vary with age or races, but does vary with gender. Women are five to eight times more likely than men to have thyroid diseases (http://www.clevelandclinic.org/health).

Hypothyroidism:

Hypothyroidism is defined as deficiencies in thyroid hormones produced by thyroid gland. Clinical symptoms of hypothyroidism include fatigue, frequent and heavy menstrual periods in women, forgetfulness, weight gain, dry, coarse skin and hair, hoarse voice, and intolerance to temperature changes. Hypothyroidism can be classified as primary (thyroid), secondary (pituitary), or tertiary (hypothalamus) based on the origin of the H-P-T-axis in which abnormal function exists in keeping up with the thyroid hormone economy. Possible causes of hypothyroidism can include: (1) thyroiditis, an inflammation of the thyroid gland which can lower hormone production; (2) Hashimoto’s disease, one form of thyroiditis attributed by the hereditary autoimmune disease condition; (3) postpartum thyroiditis, usually a temporary thyroiditis occurring in 5 to 9 percent of women postpartum; (4) iodine deficiency, with inadequate iodine intake resulting in deficiencies in T4 synthesis; and (5) a non-functioning thyroid gland (Burrow et al., 1994; Drake and Wood, 1998; Larsen et al., 2003; Lind et al., 1998; Shagam, 2001).

Hyperthyroidism:
Hyperthyroidism, opposite of hypothyroidism, is defined as excesses in thyroid hormones produced by thyroid gland. Clinical symptoms of hyperthyroidism include irritability, nervousness, muscle weakness and tremors, infrequent and scant menstrual periods in women, weight loss, sleep disturbances, enlarged thyroid gland, vision problems or eye irritation, and heat sensitivity. Hyperthyroidism can be classified as primary (thyroid), secondary (pituitary), or tertiary (hypothalamus) based on the origin of the H-P-T-axis in which abnormal function exists.

Possible causes of hypothyroidism can include (1) thyroiditis, inflammation of the thyroid gland which can increase hormone production; (2) Grave’s disease, a hereditary autoimmune disease condition in which excess thyroid hormone is being produced; (3) overactive thyroid glands or nodules; and (4) excessive iodine exposure, which may occur from a number of drugs (e.g., Amiodarone and Lugol’s iodine solution) or dietary iodine intake (Larsen et al., 2003; Shagam, 2001).

Treatment:

Clinically, hypothyroidism is treated with drugs such as levothyroxine, a synthetic hormone, to replace and replenish thyroid hormone in the body. Hyperthyroidism, on the other hand, requires more extensive efforts in the normalization of thyroid hormone production. Treatment could involve
drug therapy to block hormone production (e.g., propylthiouracil or methimazole), radioactive iodine treatment that disables the thyroid gland function, or thyroidectomy to remove part or the entire gland (Larsen et al., 2003).

2.4 EVALUATION OF THE THYROID FUNCTIONS

2.4.1 Human vs. rodent models

Rodent is the most common species used for toxicology studies, however, there are significant differences exist in thyroid hormone physiology between laboratory rodents and humans. In humans and monkeys, because circulating T4 is bound primarily to TBG but this high-affinity binding protein is absent in rodents, birds, amphibians or fish (Oppenheimer, 1968), the plasma T4 half-life in rats is considerably shorter (12-24 hours) than in humans (5-9 days) (Capen, 1997). TBG and albumin also are the carrier proteins for T3 in humans, monkeys, and dogs, however, T3 only binds to albumin in rats and mice (Capen, 1997). Because T3 has less binding affinity to binding proteins (vide supra), it has a faster turnover and shorter plasma half-life in most species (e.g., 1.5 days in humans). It has been well demonstrated that, between rats and humans, these differences in plasma half-lives of thyroid hormones and binding affinity to carrier proteins attribute to a greater sensitivity of
rodents in developing thyroid hyperplastic lesions (Capen, 1997; Curran and DeGroot, 1991).

In addition to faster half-lives, rodents are also very susceptible to enhanced thyroid hormone turnover induced by liver cytochrome P450 enzymes, such as induction by phenobarbital, for example (Capen, 1994, 1997). Induction of Cyp2b2 by phenobarbital is a CAR-mediated pathway and in rats, it can lead to increased hepatic uptake of thyroid hormone, increased metabolism, and increased biliary excretion. On the other hand, the human is less prone to such induction.

There are also marked differences in the sensitivity to the inhibition of TPO enzyme, the key protein catalyzing the iodination of tyrosyl residues incorporation with thyroglobulin molecule to synthesize T4 and T3 (Capen, 1997). Studies have shown that the long-term administration of sulfonamide, known inhibitor to TPO in rodents and dogs, can lead to the formation of benign thyroid tumors but not in TPO-insensitive species such as monkeys, guinea pig, chicken, and humans. Furthermore, it is important to realize that TSH levels are usually higher in male rats than in female rats; and in response to the higher TSH level, the follicular cell height and incidence of follicular cell hyperplasia are often higher in male
rats than in female rats as well (Capen, 1997; Capen et al., 1999).

Because of scientific responsibility and regulatory requirements, the use of rodent models to evaluate thyroid functions will remain in practice in the future. Understanding the differences and relevance between animal and human models will help clarify the significance in risk assessments.

2.4.2 Clinical measurements

Clinically, the primary indicator for thyroid function is TSH measurement per its multi-governing functions in the H-P-T axis (Larsen, 1989; Vassart and Dumont, 1992). However, due to their functions as prohormone and bioactive hormone respectively, serum FT4 and FT3, can also be used to delineate thyroid functions as well because they inversely correlate with the TSH levels as part of the feedback mechanism. Serum rT3 and TT3 values are also used in the clinic in helping diagnosing D1 functions as D1 is responsible for converting T4 to either active T3 or inactive rT3. In addition, because the relative serum concentration pools of TT4 is much higher than TT3 (99% to 1%, respectively), FT4 measurement is more frequently performed than FT3. As FT4 is only about 0.03% of the TT4
(Refetoff et al., 1970), high degree of accuracy in FT4 measurement is often required in the clinical setting.

The majority of the medical facilities in the world rely on the automated clinical chemistry analyzers to perform serum chemistry analyses. For serum thyroid hormone such as FT4 and TSH, either ELISA-based or radioimmunoassay-based assays are incorporated in the instruments. These assays utilize the specific binding properties between antigens and antibodies, and the determinants of interest, such as FT4 or TSH, can compete with an analog for binding with the antibody. The antibody or the analog can be conjugated with either radiolabeled or chemiluminometric compounds which can then be quantitated analytically.

2.4.3 Clinical FT4 assay interferences

In serum, even though both bound and unbound thyroid hormones exist in a state of equilibrium, however the association and disassociation of the hormones with their serum carrier proteins are within seconds (vide supra) and there are numerous endogenous and exogenous variables that are known to disrupt the thyroid hormone equilibrium which can also cause incorrect FT4 measurements. TSH does not require carrier protein for
circulation and its measurement appears to be more robust than that in FT4.

One of the most common interference known with FT4 measurements is the presence of a binding competitor in serum. Free fatty acid, for example, generated upon sodium heparin administration, is well-known to displace T4 from its binding proteins (Mendel et al., 1987a). Such displacement will lead to an artificially lowered FT4 value being reported using the analog method because binding competitor not only competes with FT4 but also the analog for binding (onto antibody) in the assay system (Nelson et al., 2004).

The equilibrium state between bound and unbound serum thyroid hormones follows the theory of mass action (Ekins et al., 1984). The theory of mass action states that the concentration of free hormone is proportional to the concentration of protein-bound hormones and inversely proportional to the serum binding capacity. Therefore, factors leading to changes in the amount of protein-bound hormone (e.g., hormone production or binding competition) or serum binding capacity (e.g., binding protein concentration or competition for binding sites) can affect the concentration of free hormone.
In case of serum samples that contain FFA, it is then especially problematic when measuring FT4 using analog methods because (1) FFA reduced the total binding capacity in the serum, and (2) serum proteins present in the assay buffer can also bind with free thyroid hormones in the samples, resulting in a under-represented level of “true” thyroid hormone concentration (a negative bias) (Christofides et al., 1999a, 1999b; Ekins, 1993c; Nelson et al., 1994a; Nelson et al., 1994b; Sapin, 2001)

To measure the free thyroid hormone correctly and to avoid negative bias, equilibrium dialysis is another technique that can be used to measure free hormones. Unlike analog methods, equilibrium dialysis system uses serum-free buffer to dilute the samples. This allows equilibrium to re-establish between free and bound thyroid hormones without the interference of exogenous serum proteins. Equilibrium dialysis is not routinely used in the clinics due to the extensive time and labor resources required, however, it has been considered as the gold standard for measuring free hormones and it is the reference method when developing and validating analog methods (Ekins, 1989a, 1994b).
2.4.4 Other markers

Clinical manifestation of the thyroid can be confirmed by laboratory tests such as TSH for the status of thyroid hormones at the level H-P-T axis, however, subtle changes in thyroid functions are more difficult to identify (Surks et al., 2004).

Contrary to other hormones in which the corresponding receptors reside in or near the specific target organs in which they mediate, the essential role of thyroid hormones in the broad range of biological activities such as metabolism and growth correspond to the wide presence of thyroid hormone receptors in almost all tissues (Oppenheimer, 1985; Oppenheimer et al., 1995). Therefore, from a research perspective, investigation on the thyroid hormone actions at cellular level can yield additional understanding of its mechanism and valuable information in thyroid hormone status evaluation (Oppenheimer, 1985; Oppenheimer et al., 1987).

Microscopically, thyroid status can be evaluated by determining the follicular epithelial cell height and colloid area in histological sections of thyroid gland. The thyroid gland consists of multiple, closely approximated follicles, each of which consists of a central accumulation of
colloid surrounded by a single layer of follicular epithelial cells. The colloid consists of a homogeneous mixture of proteins, including thyroglobulin, iodoproteins, and serum proteins, and serves as a repository of thyroglobulin. Under conditions that require an increase in thyroid function, there is an increase in the rate of colloid resorption, resulting in a decrease in colloid cross-sectional area in histological sections, and an increase in the synthesis activities of follicular epithelial cells, the latter resulting in an increase in the cytoplasmic volume (height) of epithelial cells. Measurement of epithelial cell height and colloid area is easily accomplished from routine histological sections, and provides insight into the level of activity of the thyroid gland.

Thyroid hormones regulate the biological processes through the thyroid hormone receptor-mediated mechanism. Thyroid hormone receptors (TRs) belong to the nuclear receptor super family which also include receptors of glucocorticoid (GR), estrogen (ER), progesterone (PR), androgen (AR), aldosterone or mineralocorticoid (MCR), vitamin D (VDR), retinoic acid (RAR), retinoid X (RXR), and other unknown “orphan” receptors (Aranda and Pascual, 2001; Novac and Heinzel, 2004).

There are two major isoforms of TRs: TRα and TRβ (Oppenheimer et al., 1995). Two proteins are coded by α gene (TRα1 and TRα2) while two
the proteins are coded by β gene (TRβ1 and TRβ2). TRα1, a T3-binding receptor, is widely distributed in all tissues, especially in brain and heart. TRα2 lacks carboxyl terminal amino acid segment necessary for T3 binding and is referred to as a receptor variant. TRβ1 is widely dispersed in all tissues as well but exceptionally high in liver, brain and heart while TRβ2 is restricted to anterior pituitary (Hodin et al., 1990; Hodin et al., 1989; Oppenheimer, 1985; Oppenheimer et al., 1987).

In serum, about 20% of the circulating T3 is directly synthesized and secreted by the thyroid gland and the other 80% is produced by T4 $\rightarrow$ T3 conversion in peripheral tissues where deiodinase exists such as liver, kidneys, and skeletal muscles. The liver, not only is essential in the metabolism of proteins, carbohydrates, lipids, and xenobiotics, it also attributes to the majority of the peripheral T3 conversion (Docter and Krenning, 1990; Larsen et al., 1981). The essential role of thyroid hormones in liver is evident in that liver has high levels of TR expression hence as a research tool, various biochemical markers from liver have been extensively studied to help characterizing thyroid hormone functions.

In liver and thyroid, deiodinase 1 (D1) plays a critical role in the peripheral tissue conversion of FT4 to FT3 (Bianco and Larsen, 2005;
Malic enzyme (ME) is one of the key enzymes involved in lipogenesis and has been frequently used as an index of the thyroid hormone status (Oppenheimer et al., 1995; Towle et al., 1981). A liver protein arbitrarily designated as S14 or thyroid hormone responsive protein (Thrsp), appeared to be unusually sensitive to changes in thyroid hormone and carbohydrate administration (Cai et al., 1996; Oppenheimer et al., 1995; Seelig et al., 1981). Thyroid hormone T3 is also a key determinant of liver cytochrome P450 oioxidoreductase (POR) expression, a microsomal flavoprotein that catalyzes the transfer of electrons from NADPH to other enzymes (both P450-dependent and P450-independent) (Li et al., 2001; Ram and Waxman, 1990, 1991, 1992; Waxman et al., 1989). In addition, UGT family proteins have also been correlated with the glucuronidation and turnover of thyroid hormones (Barter and Klaassen, 1992a, 1994). Thyroid hormones also appear to modulate apolipoprotein A1 (ApoA1) activities positively and negatively, depending how thyroid receptors interact with thyroid response elements (Brent et al., 1991; Hargrove et al., 1999).

Other biochemical markers used for thyroid research include those found in the brain and placental tissues such as deiodinase 3 (D3). D3 catalyzes the inactivation and clearance of T3 and its activity has been widely postulate to protect developing organs against excessive exposure to active
thyroid hormones (Burrow et al., 1994). In neonates, it has been shown that increases in thyroid hormone concentrations in serum and brain appear to be associated with the increase of thyroid hormone receptors $\beta_1$ (TR$\beta_1$) in brain’s Purkinje cells during the first 10 days of life (Strait et al., 1990). Because Purkinje cells maintain the circuitry of cerebellum neuronal function, mRNA expression and/or histological evaluation on the Purkinje cells can also be used to evaluate thyroid hormone status in brain (Anderson et al., 1997; Strait et al., 1991).

2.5 SUMMARY ON THYROID HORMONE STATUS WITH PFOS LABORATORY TOXICOLOGY STUDIES

Table 1 below summarizes laboratory animal data pertaining to the thyroid parameters with PFOS exposures. These studies have been described in detail in Chapter I.
Table 1: Summary of the thyroid hormone data in laboratory studies

N = mean values comparable to controls       -- = Not available or not performed

<table>
<thead>
<tr>
<th>Study</th>
<th>Study Type</th>
<th>Parameters</th>
<th>TSH</th>
<th>TT4</th>
<th>FT4</th>
<th>TT3</th>
<th>FT3</th>
<th>Thyroid Histology</th>
<th>Thyroid morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thomford, 2002b</td>
<td>104-week dietary study in SD rats</td>
<td>5</td>
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<td><em>Increased thyroid follicular cell adenoma in the 20 ppm stop dose group males</em></td>
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<td>Seacat et al., 2002</td>
<td>26-week oral capsule study in Cynomolgus monkeys</td>
<td>170</td>
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<td>ED-RIA</td>
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<td>N</td>
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<tr>
<td>Thibodeaux et al., 2003</td>
<td>Developmental study in rats and mice(GD2-20 in rats, GD1-17 in mice)</td>
<td>200-250 (dams)</td>
<td>N</td>
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<tr>
<td>Lau et al., 2003</td>
<td>Developmental study in rats and mice (GD2-20 in rats, GD1-17 in mice)</td>
<td>100 (PND5)</td>
<td>N</td>
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<tr>
<td>Luebker et al., 2005b</td>
<td>Developmental study in rats (pre-mating to lactation)</td>
<td>150 (PND5)</td>
<td>N</td>
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<td>ED-RIA</td>
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<tr>
<td>Fuentes et al., 2006</td>
<td>Developmental study in mice (GD1-18)</td>
<td>NA</td>
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<tr>
<td>Martin et al., 2007</td>
<td>1, 3 ,or 5 day oral metabolism study in rats</td>
<td>NA</td>
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<tr>
<td>Curran et al., 2008</td>
<td>28-day dietary metabolism study in rats</td>
<td>43</td>
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<tr>
<td>Yu et al., 2009</td>
<td>91-day Drinking water study in rats</td>
<td>88</td>
<td>N</td>
<td>N</td>
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<td></td>
<td>N</td>
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</tr>
</tbody>
</table>
2.6 SUMMARY ON THYROID HORMONE DATA IN HUMAN BIOMONITORING STUDIES

Table 2 below summarizes human biomonitoring data pertaining to the thyroid parameters with PFOS exposures. These studies have also been discussed in detail in Chapter I.

**Table 2: Summary of thyroid hormone data in human studies**

<table>
<thead>
<tr>
<th>Study</th>
<th>Sample Size</th>
<th>Serum [PFOS], geometric mean &amp; range</th>
<th>Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>TSH</td>
</tr>
<tr>
<td>Olsen et al., 2003;</td>
<td>263 (3M US plant workers)</td>
<td>0.91 (0.06 – 10.06)</td>
<td>N</td>
</tr>
<tr>
<td>Olsen and Zobel, 2007</td>
<td>255 (3M Belgium plant workers)</td>
<td>0.44 (0.38 – 0.51)</td>
<td>N</td>
</tr>
<tr>
<td>Inoue et al., 2004</td>
<td>15 (cord blood)</td>
<td>Range = 0.0016 – 0.0052</td>
<td>N</td>
</tr>
<tr>
<td>Dallaire 2009</td>
<td>623 (Inuits)</td>
<td>0.01828 (0.00048 – 0.470)</td>
<td>N</td>
</tr>
<tr>
<td>Bloom et al., 2009</td>
<td>31 (anglers in NY)</td>
<td>0.0196 (0.0163 – 0.0235)</td>
<td>N</td>
</tr>
</tbody>
</table>

N = mean values are normal and within clinical assay’s reference range;
-- = not available or not performed
2.7 KNOWLEDGE GAP

The observations from biomonitoring studies described above (Bloom et al., 2009; Dallaire et al., 2009; Inoue et al., 2004; Olsen et al., 2003a; Olsen and Zobel, 2007) suggest that PFOS does not cause a physiological hypothyroid state in humans even with those that were occupationally-exposed in the fluorochemical production plants (Olsen et al., 2003a; Olsen and Zobel, 2007). In laboratory studies, PFOS exposure has been associated with decreased thyroid hormones without a compensatory elevation of TSH (Curran et al., 2008; Lau et al., 2003; Luebker et al., 2005b; Martin et al., 2007; Seacat et al., 2002; Thibodeaux et al., 2003). Interestingly, data reported on split serum samples when analyzed for free thyroid hormones such as FT4 and/or FT3 by analog method and reference ED-RIA method (Luebker et al., 2005b; Seacat et al., 2002) suggested assay interference with the analog method. The differences between human and laboratory animal data could raise the uncertainty when performing risk assessment but also render the question of whether PFOS can interfere with the thyroid functions and whether the laboratory rodent is a good model to study PFOS effects on thyroid biology.
III.

HYPOTHESES
3.1 RATIONALE

In the toxicological studies with PFOS mentioned above, serum sample obtained from a single rat after K⁺PFOS administration usually would report a statistically significantly lower FT4 value than control using analog method. Interestingly, there was no difference relative to control when the same serum sample was evaluated for FT4 by the reference ED-RIA method (Luebker et al., 2005b; Seacat et al., 2002). The discrepancies noted in these studies with FT4 measurements suggested a negative bias associated with the analog method when measuring PFOS-containing samples similar to that observed in FFA (Ekins, 1983b, 1992, 1993b).

Similar to free fatty acid and its effect on thyroid hormone displacement, the possibility of binding interference of PFOS with thyroid hormone binding proteins is based on PFOS’s resemblance to free fatty acid structurally, and that PFOS can also compete with fatty acid for binding with liver fatty acid binding protein (Luebker et al., 2002). Furthermore, peroxisome proliferator activated receptor alpha (PPARα) agonists has also been shown to effectively compete for thyroid carrier protein binding sites (Hertz et al., 1993). PPARα agonists are typically amphiphilic acids and perfluorinated compounds such as perfluorooctanoic acid (PFOA) and PFOS are PPARα agonists (Berthiaume and Wallace, 2002b; Ikeda et al., 1985; Sohlenius et al., 1993). Other PPARα
agonists also include endogenous fatty acids like FFA, hypolipidemic drugs such as clofibrate, and acetylsalicylic acid like aspirin (Hertz et al., 1993; Klaunig et al., 2003; Larsen, 1972).

It has been demonstrated that the displacement of thyroid hormones from their binding sites by PPARα agonists can lead to increased thyroid hormone-mediated hepatic responses in rats. Gutshall et al. (1989) first reported the displacement of T4 from carrier protein binding sites by perfluorodecanoic acid (PFDA) and the displacement corresponded to increased activities of hepatic ME and glycerol-3-phosphate dehydrogenase (GPD) enzymes, another marker of tissue TH response (Oppenheimer et al., 1995; Oppenheimer et al., 1977; Towle et al., 1981). Harland and Orr (1974) first observed increased GPD and ME activity after treatment with the hypolipidemic drug, clofibrate, a PPARα agonist. An increase in ME in rat liver was also observed after dietary treatment of rats with clofibrate, PFOA, and acetylsalicylic acid (Cai et al., 1996).

The thyromimetic effect observed with these PPARα agonists in these studies may have been due, in part, to increased availability of thyroid hormone as a result of displacement from carrier protein binding sites; and the changes in thyroid hormone equilibrium due to PFOS can directly or indirectly cause the disturbance in metabolism. The alterations reported with thyroid hormones and PFOS have not been fully evaluated hence a study evaluating on the interactions
between PFOS and thyroid hormones using rat model will allow us to understand the effects of PFOS on thyroid hormones functions.

3.2 FOCUS OF THE THESIS

The hypotheses for this thesis postulate that the observation of serum hypothyroxinemia in rats without a major compensatory increase in TSH maybe due to: (1) alterations in thyroid hormone binding capacity, such as displacement reported for PFDA; or (2) disturbances in the central H-P-T axis functions; or (3) changes in tissue uptake and metabolism of thyroid hormone (Curran and DeGroot, 1991; DiStefano and Sapin, 1987; Gutshall et al., 1988, 1989; Larsen, 1989; Oppenheimer, 1968; Schroder-van der Elst et al., 1997).

3.3 UNIFYING HYPOTHESIS

PFOS behaves similarly to an endogenous free fatty acid by competing for binding with circulating serum thyroid hormones which can result in a negative bias in free thyroid hormone measurement (Hypothesis 1). Under certain conditions, the displaced, unbound thyroid hormones may become available to tissues or it may encounter a higher turnover rates (Hypothesis 2). Furthermore, the displaced free TH may consequently increase TH response in TH-responsive tissues (Hypothesis 3).
IV.

PFOS AND NEGATIVE BIAS IN FREE THYROID MEASUREMENT IN RATS
The entire content of this chapter has been published:

Chang et al., 2007, “Negative bias from analog methods used in the analysis of free thyroxine in rat serum containing perfluorooctanesulfonate (PFOS)”, Toxicology, 234, pages 21 – 33.
4.1 INTRODUCTION

Reduction of thyroid hormone levels (triiodothyronine (T3) and/or thyroxine (T4)) in serum, has been reported as a PFOS treatment-related effect in several toxicological studies (Lau et al., 2003; Luebker et al., 2005b; Seacat et al., 2002; Thibodeaux et al., 2003). These studies have shown reductions in serum total and free thyroid hormone levels by analog methods. However, in these studies, serum free thyroid hormone levels were normal when measured by a direct equilibrium dialysis method, and the presence of hypothyroxinemia was not associated with altered thyroid gland pathology or clinically significant elevations in thyrotropin (thyroid stimulating hormone, TSH). Because the diagnosis of primary hypothyroidism is based on reduced serum free T4 (FT4) and consequent compensatory elevation of TSH (Ravel, 1995; Sapin, 2001; Sapin and Schlienger, 2003), the observed reduction in serum thyroid hormones without a major compensatory increase in TSH does not fit the clinical profile of hypothyroidism. Alternatively, these hormonal changes may be due to other factors that could include changes in the hypothalamic-pituitary-thyroid axis, alterations in thyroid hormone binding to serum proteins, and changes in tissue uptake and metabolism of thyroid hormone (Chopra, 1997; Larsen et al., 2002; Ravel, 1995; Seacat et al., 2002).
The association of PFOS in human blood with thyroid hormone status has been studied in individuals with occupational exposure to PFOS (Olsen et al., 2003b) and in a small group of neonates and their mothers (Inoue et al., 2004). These studies did not reveal any alterations in thyroid hormone status associated with PFOS concentrations in maternal, cord, or worker blood. Worker serum PFOS concentrations averaged 1.32 μg/mL (geometric mean = 0.91 μg/mL, range = 0.06 – 10.06 μg/mL).

Because of the observed discrepancies in toxicological studies with PFOS (vide supra) between the analog and reference equilibrium dialysis methods in the amount of FT4 measured, in which analog methods suggested reduced FT4 while equilibrium dialysis suggested normal FT4, it is possible that a negative bias was introduced by the use of analog methods to measure FT4. The protein bound and free thyroid hormones exist in a state of equilibrium based on the law of hormone mass action (Ekins, 1990). This equilibrium can be shifted by changes in serum thyroid hormone binding capacity or inhibitors of thyroid hormone bindings such as free fatty acids (FFA) (Ekins, 1989b, 1993; Ekins et al., 1983; Mendel et al., 1986; Spector, 1975). Analog methods are actually measuring the presence of analog which competes with free thyroid hormone for binding in the assay system as well as in serum. In cases where serum binding capacity for thyroid hormone and analog is lower than normal, either due to reduction in binding protein or available binding sites, analog methods may give artificially lowered values for
free thyroid hormones (Christofides et al., 1999a, 1999b; Ekins, 1993b; Nelson et al., 1994a; Nelson et al., 1994b; Okabayashi et al., 1996; Sapin, 2001; Sapin and d'Herbomez, 2003). It has been demonstrated that increased FFA in serum can produce a negative bias based on competition for binding with T4 (Ekins, 1989b, 1994a; Ekins et al., 1983; Mendel et al., 1986; Nelson et al., 1994a; Nelson et al., 1994b; Spector, 1975). As an example, this can occur when sodium heparin used in catheters increases lipase secretion and FFA production in dialysis patients (Jaume et al., 1996; Laji et al., 2001; Mendel et al., 1987b; Nelson and Weiss, 1985; Stevenson et al., 1998; Wilkins et al., 1982).

In order to avoid potential negative bias in the measurement of FT4 that could be introduced by binding competition, a reference method employing equilibrium dialysis or ultracentrifugation needs to be employed (Chopra, 1997). Direct equilibrium dialysis followed by radio-immunoassay (ED-RIA) method for FT4 is not prone to these negative biases (Nelson et al., 1994a; Nelson et al., 1994b; Sapin, 2001). Due to the lack of bias based on changes in protein bound T4 (PBT4) and serum binding capacity, ED-RIA is considered the reference method for determination of FT4 and is used as a standard for evaluating other methods (Christofides et al., 1999a, 1999b; Ekins, 1993b; Larsen et al., 2002; Nelson et al., 1994a; Nelson et al., 1994b; Ravel, 1995).
Because PFOS resembles a fatty acid in its amphiphilic nature and has been shown to compete for binding with FFA on albumin and liver fatty-acid binding protein (Luebker et al., 2002), the reported reductions in thyroid hormones with PFOS treatment in the previous studies might be due to discrepancies in the thyroid hormone measurement methods. Therefore, we hypothesized that decreased serum free thyroid hormone concentrations measured by analog methods in the presence of PFOS is due to binding interference similar to that reported for FFA. To test this hypothesis, we compared analog methods with ED-RIA in the measurement of serum FT4 in rat sera after short-term in vitro and in vivo exposure and related these measurements to changes in TT4, TSH, and a liver marker for thyroid hormone response, malic enzyme.

4.2 MATERIALS and METHODS

All chemicals used in this study were reagent-grade and were purchased from either Sigma-Aldrich (St. Louis, MO) or VWR (West Chester, PA). Potassium perfluorooctanesulfonate (K⁺PFOS, 86.9% pure) was supplied by 3M Specialty Material Division (St. Paul, MN). Pooled rat serum was purchased from Sigma-Aldrich (St. Louis, MO). TT4 and FT4 were determined by two analog methods: RIA (Coat-A-Count® kits, Diagnostic Product Corporation, Los Angeles, CA) or by chemiluminometric immunoassay (Bayer® ADVIA Centaur clinical analyzer, Bayer HealthCare LLC, Tarrytown, NY). FT4 was also measured using
equilibrium dialysis followed by RIA (ED-RIA) with Nichols Institute Diagnostics Free T4 by Equilibrium Dialysis kits (Nichols Institute Diagnostics, San Clemente, CA). Serum TSH values were determined by RIA radioimmunoassay according to methods previously described (Thibodeaux et al., 2003).

Female Sprague-Dawley rats (8-10 weeks old, 200 - 250 g) were purchased from Charles River Laboratory (Portage, MI or Raleigh, NC). All rats were group housed in standard solid bottom cages. Purina Mouse/Rat Chow and tap water were provided to all rats ad libitum throughout the study. Environmental controls for the animal room were set to maintain a temperature of 72 ± 3°F, humidity of 30-70%, a minimum of 10 exchanges of room air per hour and a 12 hour light/dark cycle. In vivo studies were conducted in a facility that is accredited by the International Association for the Accreditation of Laboratory Animal Care. The 3M Institutional Animal Care and Use Committee reviewed procedures involving live rats.

4.2.1 STUDY 1: Effects of PFOS on Serum Thyroid Hormone Measurements

in vivo (3-Daily Repeat Doses)

To evaluate the effects of PFOS on serum thyroid hormone concentrations in vivo, two groups of female Sprague-Dawley rats (N=10 per group) were given three daily doses of either vehicle or 5 mg/kg PFOS suspended in
0.5% Tween 20® in a manner replicating previous work reported by Thibodeaux et al., (2003). Blood samples were taken from abdominal aorta 24 hours after the last dose, and sera were obtained after clotting and centrifugation. Serum samples were immediately split into three batches and flash frozen pending analyses at: (1) Mayo Medical Laboratories (TT4 and FT4 by Bayer® ADVIA Centaur, and FT4 by ED-RIA); (2) EPA-NHEERL (TT4, FT4, and TSH by radioimmunoassay); and (3) 3M Strategic Toxicology Laboratory (serum PFOS analysis). In addition, liver samples were collected for analysis of PFOS concentration and hepatic malic enzyme (ME) mRNA transcript levels and activities.

4.2.2 STUDY 2: FT4 Displacement by PFOS and Oleic Acid in vitro (Concentration-Response)

To test the hypothesis that PFOS in serum may displace FT4 from its binding sites similar to FFA, PFOS was incubated with pooled rat serum in vitro for 0.5 hour and 6 hours at concentrations ranging up to 200 μM. For comparison to a FFA, oleic acid was used as a positive control by similarly incubating it in pooled rat sera for 6 hours at concentrations up to 200 μM. Incubations were followed by measurements of TT4 and FT4, as described below. PFOS and oleic acid were first dissolved in methanol and aliquoted into sterile 15-mL polypropylene conical centrifuge tubes. After methanol was evaporated under nitrogen at room temperature, 2 mL
of pooled rat sera were added to each tube so that the final concentrations of PFOS were 0, 10, 25, 50, 150, and 200 μM for the 0.5 hour incubation. Concentrations of PFOS and oleic acid used for the 6 hour incubation were 0, 12.5, 25, 50, 100, and 200 μM. Triplicate tubes were prepared for each concentration. The tubes were vortexed and incubated for either 0.5 hour or 6 hours at 37°C. At the end of incubation, serum samples were frozen using liquid nitrogen pending analysis for thyroid hormones. TT4 and FT4 were analyzed by analog chemiluminometric immunoassays on the Bayer® ADVIA:Centaur clinical analyzer. FT4 was also determined by the ED-RIA reference method. All thyroid hormone analyses for this part of the study were conducted at Mayo Medical Laboratories (Rochester, MN).

4.2.3 MALIC ENZYME DETERMINATION

a. Malic Enzyme (ME) Activities

Liver cytosolic fractions were isolated and ME activities were determined. Approximately 1 g of liver was homogenized with buffer (0.25M sucrose in 0.05M Tris-HCl, pH 7.4) in a clean polypropylene tube. The ratio between liver and buffer was 1:4 (w/v). Differential centrifugation was performed on the liver homogenates (700 x g, 10 minutes; 10,000 x g, 10 minutes; and 100,000 x g, 1 hour). The supernatant fraction obtained after last
centrifugation was the cytosol. ME activity was determined via spectrophotometry according to the method of (Geer et al., 1980)).

b. **Quantitative RT-PCR**

**RNA Isolation:** Total RNA was isolated from frozen liver samples using RNeasy Mini Kit (Qiagen, Valencia, CA). Frozen tissue was powdered with mortar and pestle under liquid nitrogen and 15 to 25 mg was homogenized in lysis buffer by 10 passes through a 27 gauge needle with an RNase free syringe. Samples were further processed according to manufacturer's instructions including the on-column DNase digestion. Total RNA was eluted in RNase free water and quantitated by spectrophotometer ($A_{260}$). The quality of the samples was assessed using a Bioanalyzer 2000 (Agilent, Palo Alto, CA).

**RT-PCR:** mRNA sequences for malic enzyme (ME) were obtained from the GenBank database ([http://www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) and gene specific primers were designed using Oligo 6 software (ABI, Foster City, CA). Reverse transcription was performed on 1.5 μg of total RNA with the Omniscript RT kit (Qiagen, Valencia, CA) using specific reverse primers for ME and 18s rRNA (housekeeping gene). Quantitative PCR was performed on the
LightCycler (Roche Diagnostics, Indianapolis, IN) using the Sybr Green Master Mix Kit. ME and 18s standards were obtained by pooling several PCR reactions for each transcript and purified with Qiaquick PCR purification kit (Qiagen, Valencia, CA). PCR product was verified by gel electrophoresis and quantitated spectrophotometrically. Copy numbers per microgram of product were approximated based on the dsDNA molecular weight calculated for each PCR product (Ambion Inc., Austin, TX). Samples were amplified using the LightCycler along with serial dilutions of ME and 18s standards. Results were calculated and expressed as copies of specific ME gene per copy of 18s rRNA.

4.2.4 LC-MS/MS ANALYSES FOR PFOS

a. Sample Preparation: Liver

Liver samples from untreated rats (used as blank matrix), PFOS-treated, and vehicle-treated rats were stored at –80°C until ready to begin analysis. Samples were allowed to thaw, and approximately 0.2 g of liver was weighed and homogenized with deionized water in a clean polypropylene tube. The ratio between liver and water was 1:4 (w/w). After the primary homogenization step, the whole homogenate was further digested using 10% (v/v) 1.0 N potassium
hydroxide for a minimum of 16 hours on a mechanical shaker in room temperature. The digested homogenate was centrifuged at 2500 x g for 20 minutes and the 100 µL of supernatant was transferred to a clean polypropylene tube. A dual-labeled $^{18}$O$_2$ analog of PFOS (synthesized by Dr. Herbert Seltzman, Research Triangle Institute, Research Triangle Park, NC) was used as an internal standard by addition to each sample tube. Five-hundred (500) µL of 1.0 N formic acid was added to all tubes followed by 300 µL of water. Samples were vortexed between each addition. Samples were at a pH of approximately 2.5.

b. Sample Preparation: Serum, Serum Retentate, and Dialysate

One hundred (100) µL of serum, dialysis serum retentate, or dialysate samples (obtained after equilibrium dialysis) were aliquoted into clean polypropylene tubes. The dual-labeled $^{18}$O$_2$ analog of PFOS was used as an internal standard by addition to each sample tube. One mL of 1.0 N formic acid was added to all tubes, followed by 100 µL saturated ammonium sulfate. Samples were vortexed between each addition.

c. Sample Preparation: PFOS Standard Curve

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To prepare a LC-MS/MS PFOS standard curve, a series of PFOS solution prepared in methanol was aliquoted volumetrically to clean polypropylene tubes. Methanol was evaporated under a stream of nitrogen air at room temperature. After evaporation, 100 µL of supernatant obtained from blank liver matrix or blank serum was added to each tube to give final concentrations of PFOS of 0, 2.5, 5, 12.5, 25, 50, 75, and 100 µg/mL. Triplicate tubes were prepared for each concentration. Dual-labeled $^{18}$O$_2$ analog of PFOS was added to all tubes followed by formic acid, saturated ammonium sulfate or water treatments (per either liver or serum matrix) as described above.

d. Solid-Phase Extraction (SPE)

All extractions were based on 100 µL of sample matrix and utilized Waters Oasis® hydrophilic-lipophilic balance (HLB) 3 mL columns with column conditioning, column loading, column wash, and column elution performed as described in (Ehresman et al., 2006).

e. LC-MS/MS Conditions
The instrument used for analysis was an API 4000 mass spectrometer (Applied Biosystems / MDS-Sciex Instrument Corporation) configured with Turbo Ion Spray (pneumatically assisted electrospray ionization source) in negative ion mode. A Mac-Mod ACE® C-18, 5 µm, 100 x 2.1 mm i.d. HPLC column with an isocratic flow rate of 0.35 mL/min was used for PFOS analysis. The mobile phase was 51% acetonitrile and 49% 2 mM ammonium acetate. All source parameters were optimized under these conditions according to manufacturer’s guidelines.

Transition ions for PFOS: 499 \rightarrow 80 \text{ atomic mass unit (amu)} \text{ and the transition ions for internal standard dual } ^{18}\text{O-labeled PFOS: 503 } \rightarrow 84 \text{ amu were monitored.}

4.2.5 STATISTICAL ANALYSIS

The Student’s t-test was used to compare data resulting from PFOS treatment to control values using either Microsoft Office Excel or JMP® 5.1 - Windows - Release 5.1.2 (SAS Institute, Inc., Cary, NC). All data are expressed as means \pm standard deviation (SD).

4.3 RESULTS
4.3.1 STUDY 1: Effects of PFOS on Serum Thyroid Hormone Measurements

in vivo (3-Daily Repeat Doses)

Effects of PFOS on serum thyroid hormone measurements in vivo (3-daily repeat doses) are shown in Table 1. Consistent with previous reported in vivo studies (Luebker et al., 2005b; Thibodeaux et al., 2003), serum TSH was unchanged between the control and PFOS-treated groups while serum TT4 and FT4 were significantly lowered with PFOS treatment based on the data obtained from two different analog methods (chemiluminometric immunoassay and radioimmunoassay). Notably, there was no statistically significant difference between control and PFOS-treated serum FT4 as measured by the equilibrium dialysis reference method.

Compared to controls, liver malic enzyme transcript, a biochemical marker sensitive to changes in thyroid hormone (Hertz et al., 1991), was elevated by 20% ($p < 0.05$) with PFOS treatment. There was no significant difference in malic enzyme activity between control and PFOS groups (Table 2).
Table 1. Comparison of serum thyroid hormone measurement methods in sera from adult female rats given either 0 or 5 mg potassium PFOS/kg body weight for three days (serum samples obtained 24 hours after the third dose).

<table>
<thead>
<tr>
<th>Group</th>
<th>Total Thyroxine (TT4)</th>
<th>Free Thyroxine (FT4)</th>
<th>Thyrotropin (TSH)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CL&lt;sup&gt;a&lt;/sup&gt; (μg/dL)</td>
<td>RIA&lt;sup&gt;b&lt;/sup&gt; (μg/dL)</td>
<td>CL (ng/dL)</td>
</tr>
<tr>
<td>Control</td>
<td>3.46 ± 0.90</td>
<td>3.63 ± 0.81</td>
<td>2.17 ± 0.36</td>
</tr>
<tr>
<td>PFOS-Treated</td>
<td>1.55 ± 0.38&lt;sup&gt;*&lt;/sup&gt;</td>
<td>1.79 ± 0.41&lt;sup&gt;*&lt;/sup&gt;</td>
<td>1.28 ± 0.18&lt;sup&gt;★&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Bayer® Advia:Centaur Chemiluminometric Immunoassay

<sup>b</sup> Coat-A-Count® Radioimmunoassay

<sup>c</sup> Nichols Diagnostics Institute Equilibrium Dialysis / Free T4 Assay Kit

<sup>d</sup> Rat TSH Radioimmunoassay OP No. NHEERL-H/RTD/EB/CL/2002-010-000

* Statistically significant compared to control by paired Student’s t (p < 0.05)

# Statistically significant compared to equilibrium dialysis reference method by paired Student’s t (p < 0.05)
Table 2. Liver malic enzyme (ME) activity and transcript expression from adult female rats given either 0 or 5 mg potassium PFOS/kg body weight for three days (liver samples obtained 24 hours after the third dose).

<table>
<thead>
<tr>
<th>Group</th>
<th>Enzyme Activity (μmole/min/g liver)</th>
<th>Transcript Expression (mRNA number per 18s rRNA x 10^5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control^a</td>
<td>0.166 ± 0.049</td>
<td>1.44 ± 0.27</td>
</tr>
<tr>
<td>PFOS-Treated^b</td>
<td>0.164 ± 0.043</td>
<td>1.75 ± 0.35*</td>
</tr>
</tbody>
</table>

^a Female rats (N=10) receiving 3 daily doses of vehicle (0.5% Tween® 20)
^b Female rats (N=10) receiving 3 daily doses of 5 mg/kg PFOS

* Statistically significant compared to control by paired Student’s t (p < 0.05)
PFOS concentrations quantitated via LC-MS/MS for liver, serum, dialysis serum retentate, and dialysate (obtained post equilibrium dialysis) are presented in Table 3. The average liver and serum PFOS concentrations from the PFOS-treated rats were 110.44 μg/g ± 16.36 and 49.33 ± 6.00 μg/mL, respectively. The PFOS concentrations in the dialysis serum retentate and the dialysate buffer were 62.74 ± 9.09 μg/mL and 0.005 ± 0.001 μg/mL, respectively, indicating that very little PFOS diffused across the dialysis membrane (the molecular weight cut-off for the dialysis membrane is 13,000 Dalton).

Table 3. PFOS concentrations in serum and liver samples from adult female rats given either 0 or 5 mg potassium PFOS/kg body weight for three days (serum samples obtained 24 hours after the third dose) and in corresponding dialysis serum retentate and dialysate from the equilibrium dialysis FT4 method.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean (± SD) PFOS Concentration (μg/mL serum, retentate, and dialysate, or μg/g liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control^a</td>
<td>Serum 0.009 ± 0.001 Retentate 0.006 ± 0.002 Dialysate &lt; 0.005^b Liver &lt; 0.005^b</td>
</tr>
<tr>
<td>PFOS-Treated^c</td>
<td>Serum 49.33 ± 6.00 Retentate 62.74 ± 9.09 Dialysate 0.005 ± 0.001 Liver 110.44 ± 16.36</td>
</tr>
</tbody>
</table>

^a Female rats (N=10) receiving 3 daily doses of vehicle (0.5% Tween® 20)
^b All values below the method limit of quantitation (0.005 μg/mL or μg/g)
^c Female rats (N=10) receiving 3 daily doses of 5 mg/kg PFOS
4.3.2 STUDY 2: FT4 Displacement by PFOS and oleic acid in vitro

(Concentration-Response)

Percent increase in FT4 over control sera by treatment at various concentrations of PFOS ranging up to 200 μM PFOS (100 μg/mL) in serum in vitro is shown in Figure 1 for 0.5-hour incubation and in Figure 2 for the 6-hour incubation. Percent increase in FT4 over control sera by treatment at various concentrations of oleic acid ranging up to 200 μM in serum in vitro is also shown in Figure 2. Serum FT4, as measured by both analog (chemiluminometric immunoassay) and ED-RIA methods, increased with increased serum PFOS and oleic acid concentrations (concentration-response). The increase was greater for PFOS at 6 hours than at 0.5 hour. FT4 by ED-RIA gave significantly higher FT4 values as compared to FT4 by the analog method. At identical μM concentrations, PFOS gave higher FT4 measurements than oleic acid. Over the concentration ranges employed (0 – 200 μM), serum TT4 remained unchanged (Figure 3).
Figure 1. Mean serum free thyroxine (FT4) in pooled rat sera *in vitro* incubated at 37°C for 30 minutes with PFOS concentrations ranging from 5 to 200 μMoles/L. Each point represents the mean of triplicate determinations, and error bars represent standard deviations. Solid squares (■) represent FT4 measurement obtained by Nichols Diagnostics Institute Equilibrium Dialysis / Free T4 Assay Kit, and open squares (□) represent FT4 measurements obtained by Bayer® Advia:Centaur chemiluminometric immunoassay. With both assays, determinations were statistically significantly greater than method control mean values (Student’s t, p< 0.05) at PFOS concentrations ≥ 50 μMoles/L.
Figure 2. Mean serum free thyroxine (FT4) in pooled rat sera in vitro incubated at 37°C for 6 hours with PFOS and oleic acid concentrations ranging from 12.5 to 200 μMoles/L. Each point represents the mean of triplicate determinations, and error bars represent standard deviations. Solid squares and circles (■ and ●) represent FT4 measurement obtained by Nichols Diagnostics Institute Equilibrium Dialysis / Free T4 Assay Kit (ED-RIA) for PFOS and oleic acid, respectively. Open squares (□ and ○) represent FT4 measurements obtained by Bayer® Advia:Centaur chemiluminometric immunoassay (CL) for PFOS and oleic acid, respectively. For sera containing PFOS, mean determinations were statistically significantly (Student’s t, p< 0.05) greater than controls at concentrations ≥ 25 μMoles/L when measured by ED-RIA and at concentrations ≥ 100 μMoles/L when measured by CL. In the case of sera treated with oleic acid, only the 200 μMoles/L concentration was statistically significantly different than the control value when measured by ED-RIA.
Figure 3. Mean serum total thyroxine (TT4) as a percent in pooled rat sera \textit{in vitro} incubated at 37°C for 0.5 hour or 6 hours with PFOS oleic acid concentrations ranging up to 200 μMoles/L. Each point represents the mean of triplicate determinations, and error bars represent standard deviations. Closed triangles (▲) represent sera treated with PFOS for 0.5 hour. Open squares and circles (□ and ○) represent sera treated with PFOS or oleic acid for 6 hours, respectively. TT4 measurements were obtained by Bayer® Advia:Centaur chemiluminometric immunoassay. There were no statistically significantly differences (Student’s t, p< 0.05) between treated sera and control sera.
4.4. DISCUSSION

Treatment-related changes in measured serum thyroid hormones have been observed in toxicological studies with PFOS in non-pregnant rats (female only), pregnant rats and their offspring (Lau et al., 2003; Luebker et al., 2005b; Thibodeaux et al., 2003), and monkeys (Seacat et al., 2002). These changes consisted of: (1) decreased serum TT4 and TT3, (2) decreased serum FT4 and FT3 as measured by analog assays (chemiluminometric immunoassay and radioimmunoassay), (3) normal serum FT4 when measured by ED-RIA, and (4) absence of major compensatory increase in TSH. In pregnant mice given daily oral doses of PFOS starting on Day 1 of gestation, there was an apparent decrease in serum TT4 after 5 days of gestation at 10 mg/kg or higher (Thibodeaux et al., 2003); however, the effect was not present on gestation days 12 and 18. In another study in mice, (Fuentes et al., 2006) did not observe significant changes in thyroid hormones in pregnant mice.

With one exception, neither gross nor microscopic treatment-related alterations of the thyroid glands have been observed in repeated-dose toxicological studies with PFOS in rats (Lau et al., 2003; Luebker et al., 2005b; Seacat et al., 2003; Thibodeaux et al., 2003) or monkeys (Seacat et al., 2002). The exception mentioned above was an increased incidence of thyroid follicular cell adenoma reported in a two-year chronic dietary toxicity and cancer bioassay study.
(Thomford, 2002c). In that study, male Sprague-Dawley rats that had received 20 μg/g of potassium PFOS (highest study dose) for one year followed by control diet for the second year experienced an increased incidence of thyroid follicular cell adenoma. In contrast, there was no increased incidence of this tumor type in male and female rats that were continuously treated with 20 μg/g potassium PFOS for up to two years. There is one report suggestive of altered thyroid histology (atrophy of the follicles) in developing *Rana pipiens* tadpoles (Ankley *et al.*, 2004; Inoue *et al.*, 2004); although the authors considered this finding inconclusive.

The observations from previous repeat-dose toxicological studies suggest that PFOS does not cause a physiological hypothyroid state even though it is capable of reducing serum total thyroid hormones. In the toxicological studies mentioned above, the reductions in TT4, TT3, and FT4 as measured by analog methods were not associated with major compensatory increases in TSH, as would be expected in a hypothyroid condition, or with alterations in thyroid histology. When FT4 was assayed using the reference ED-RIA method, there was no apparent change compared to control values.

The differences observed between measurements of FT4 by analog and direct equilibrium dialysis methods led us to consider that PFOS may interfere with FT4 measurement by the analog methods that were employed. Analog methods
commonly used in clinical settings for measuring free thyroid hormones (e.g., FT4 and FT3) are based on the principle that endogenous free thyroid hormone will compete for binding to a specific antibody with a free thyroid hormone analog that is labeled with either a radioactive isotope or a chemiluminescent compound (Burtis and Ashwood, 1994; Ravel, 1995). In most cases, measured analog concentrations are inversely related to actual free hormone concentration due to competition for antibody sorption sites in the assay system (see Figure 4 for a diagrammatic explanation). The labeled analogs may also have some binding affinity for carrier sites in serum, either endogenous serum or serum added to the assay system.

The dynamic nature of thyroid hormone equilibrium is known to be affected by variables such as changes in the concentration or composition of serum proteins, presence of endogenous or exogenous competitors for thyroid hormone carrier protein binding sites, changes in the overall concentration of potential serum binding sites or in protein bound thyroid hormone (Ekins, 1983a, 1987, 1989b, 1993a; Midgley, 2001; Nelson et al., 1994a). Therefore, analog assay systems are potentially affected by not only the equilibrium established between free endogenous thyroid hormone and endogenous thyroid hormone bound to patient serum and serum that may be added to the system, but also by the extent that the analog is bound to carrier proteins in the assay system. If the concentration of potential carrier protein binding sites for the labeled analog is reduced beyond the
normal parameters of the assay, the assay may measure more free analog, leading to a negative bias in the determination of the free hormone due to the inverse relationship between measured analog concentration and free hormone concentration (Figure 4). Both the ADVIA:Centaur® and Coat-a-Count® analog methods have been shown to have a negative bias when used with sera having decreased protein-bound T4 or decreased serum binding capacity (Nelson 1994; Sapin 2001). FFA, which compete for carrier protein binding with T4 (Ekins et al., 1983; Mendel et al., 1986; Spector, 1975), have been shown to induce negative bias in some analog assays, presumably as a result of reduced carrier protein binding by labeled analog (Ekins et al., 1983; Mendel et al., 1986; Spector, 1975).

ED-RIA is one of two reference techniques that can be used to measure free hormones, the other being ultracentrifugation (Ekins, 1993; Ravel, 1995). Unlike analog methods, equilibrium dialysis system uses serum-free buffer to dilute the samples by dialyze serum samples against a serum-free buffer. The molecular weight cut-off for the dialysis membrane used in our studies is 13 KDa. The dialysis process allows the equilibrium to re-establish between free and bound thyroid hormones without the potential interference of exogenous serum proteins or binding of analog to serum proteins. Equilibrium dialysis is not routinely used in the clinics due to the extensive time and labor resources required, however, it is
the reference method for measuring free hormones and in developing and validating analog methods.

Figure 4. Diagrammatic representation of binding interactions in vitro in free thyroxine (FT4) analog assay systems. In these systems, labeled T4 analog binds to assay antibody in inverse proportion to the amount of competing FT4. The amount of assay antibody-bound labeled T4 analog is measured. However, a certain amount of labeled T4 analog may be bound to endogenous and assay serum carrier proteins with varying degrees of affinity, as shown in the left panel, and the assay calibration takes this into account. When a substance that effectively reduces serum carrier protein binding sites through competitive binding is introduced, such as perfluorooctanesulfonate (PFOS) or free fatty acids (FFA) as shown in the right panel, less labeled T4 analog is bound than is normally accounted for in the assay calibration, forcing more labeled T4 analog toward assay antibody binding sites, resulting in an underreporting of FT4. When the PFOS or FFA is added in vitro, T4 is also displaced through competitive binding, increasing the actual FT4 in the assay. However, when PFOS or FFA are added in vivo, it is likely that the excess FT4 is taken up by tissues and metabolized in a rebalancing of thyroid hormone equilibrium in serum. Therefore, the sample taken after in vivo introduction of PFOS or FFA would result in a larger underreporting of FT4 in the analog assay. This is due a larger proportional amount of unbound labeled T4 analog after addition to the assay system as compared to the FT4 present in the serum sample from in vivo treatment.
In our experiments, we tested the hypothesis that PFOS in serum may effectively compete for and reduce the available serum carrier protein binding sites for labeled T4 analog and T4. Moreover, this competition would lead to the apparent negative bias in analog FT4 measurements and effectively increase FT4 in an *in vitro* system. PFOS resembles a FFA (amphiphilic) and it is a strong acid compared to most organic acids. It was previously demonstrated that PFOS was capable of displacing fluorescently-labeled fatty acids from liver fatty-acid binding protein and albumin (Luebker et al., 2002). In addition, it is known that PFOS is highly protein-bound to albumin (Kerstner-Wood et al., 2003) which is also a primary non-specific binding protein for T4 in rats. In our experiment, we compared analog (non-dialysis) FT4 assay methods with the ED-RIA method for FT4 in the estimation of FT4 from PFOS-treated rats, as well as TT4 and TSH. We undertook this study to test this hypothesis using both *in vivo* and *in vitro* models.

*In vivo studies*

A negative bias in analog measurements of FT4 as compared to measurements made with ED-RIA was shown in the *in vivo* study. Our data (Table 1) demonstrated that TT4 and FT4 measured by the chemiluminimetric immunoassay and radioimmunoassay methods were in close agreement, both showing a decrease compared to control. There was no change in TSH. These
observations were in agreement with prior results reported by Thibodeaux et al. (2003). For the FT4 measurement using ED-RIA, the control serum FT4 values were in agreement with control serum FT4 values obtained by both analog assays. By contrast, in serum samples from PFOS-treated rats, the ED-RIA method reported essentially identical results as the control. These results indicate that sera from PFOS-treated rats created a negative bias in the Coat-a-Count® analog and ADVIA: Centaur® methods for FT4 measurements, and that determination of serum FT4 by direct ED-RIA was unchanged by treatment with PFOS under study conditions.

To rule out any interference of PFOS with FT4 measurement during ED-RIA procedures, the concentrations of PFOS were quantitated using LC-MS/MS from serum, dialysis serum retentate, and dialysate. Dialysis serum retentate is the serum sample remaining inside the dialysis membrane cup after dialysis is completed; and the corresponding buffer is referred to as dialysate. As no volume expansion in the retentate was noted post dialysis in our study, the content of retentate is essentially serum. The PFOS concentrations shown in Table 3 for serum, serum dialysis retentate, and dialysate demonstrated that very little free PFOS diffused across the dialysis membrane, suggesting that PFOS is highly protein-bound, a finding that is in agreement with the study results reported by others (Kerstner-Wood et al., 2003; Luebker et al., 2002). Therefore, PFOS is unlikely to affect the analysis of the dialysate for T4 by ED-RIA.
In Vitro Data

As noted above, the in vivo data showed a negative bias in analog measurements of FT4 in serum containing PFOS as compared with ED-RIA and appeared to support the hypothesis that PFOS potentially interferes with certain analog FT4 measurements through competition with endogenous thyroid hormone and labeled analog for binding on carrier proteins. To test this hypothesis, in vitro experiments were conducted in which PFOS and oleic acid were incubated with pooled rat sera over various concentrations for up to 200 μM and analyzed for TT4 (chemiluminometric immunoassay) and FT4 (chemiluminometric immunoassay and ED-RIA). Similar to observations with FFA (Ekins et al., 1983; Mendel et al., 1986), binding displacement of T4 by PFOS in serum would be expected to produce a concentration-dependent increase in FT4 in an in vitro system.

As seen in Figures 1 and 2, increasing PFOS concentrations in pooled rat sera produced proportional increases in FT4 concentrations. When measured using ED-RIA, FT4 concentrations were up to approximately 190% and 260% of control values at the highest PFOS concentrations of 200 μMoles/L for the 0.5-hour (Figure 1) and 6-hour (Figure 2) incubations, respectively. These results and
the fact that PFOS did not alter TT4 provide clear evidence that PFOS can effect
the displacement of bound T4 from rat serum carrier proteins.

In contrast to the results based on the ED-RIA method, the chemiluminometric
immunoassay gave consistently lower results, with a maximum increase in FT4 of
approximately 30% and 20% at 200 μMoles/L for the 0.5-hour and 6-hour
incubations, respectively. The reader should consult Figure 4 to understand the
reason for this increase in FT4 in the analog assay as opposed to the decrease
relative to control sera in analog FT4 measures taken after in vivo treatment of
rats with PFOS. In analog assay systems for FT4, labeled T4 analog binds to
assay antibody in inverse proportion to the amount of competing FT4. The
amount of assay antibody-bound labeled T4 analog is measured. However, a
certain amount of labeled T4 analog may be bound to endogenous and assay
serum carrier proteins with varying degrees of affinity, and the assay calibration
takes this into account. When a substance that effectively reduces serum carrier
protein binding sites through competitive binding, such as PFOS or FFA, is
introduced less labeled T4 analog is bound than is normally accounted for in the
assay calibration, forcing more labeled T4 analog toward assay antibody binding
sites, resulting in an underreporting of FT4. When the PFOS or FFA is added to
the assay system in vitro, T4 is also displaced through competitive binding,
increasing the actual FT4 in the assay. However, when PFOS or FFA are added
in vivo, it is likely that the excess FT4 is taken up by tissues and metabolized in a
rebalancing of thyroid hormone equilibrium in serum. Therefore, the sample
taken after in vivo introduction of PFOS or FFA would result in a larger
underreporting of FT4 in the analog assay than in the in vitro system. This is due
a larger proportional amount of unbound labeled T4 analog after addition to the
assay system as compared to the FT4 present in the serum sample from in vivo
treatment.

Although the oleic acid results did indicate negative bias in the measurement of
FT4 by the chemiluminometric method as compared ED-RIA, the increase in FT4
by ED-RIA with increasing oleic acid concentration only became statistically
significant at 200 µMoles oleic acid/L (Figure 2). These results are consistent
with the known negative bias that can be introduced in analog assays with free
fatty acids (Nelson et al., 1994b).

The activity and expression of hepatic malic enzyme (ME), which is sensitive to
changes in thyroid hormones (Hertz et al., 1991; Hertz et al., 1993), was
evaluated in this study as a secondary measure of concordance of the T4
measurements with physiological response. Although there was no change in ME
activity between control and PFOS-treated livers, the approximately 22 percent
increase in ME transcription after PFOS treatment was statistically significant and
may suggest a slight liver response to the excess circulating free thyroid hormone
in serum (Table 2). In a previous reproduction study, ME activity was measured
in liver from five-day-old rat pups and maternal female rats at five-days post-
partum after treatment of the females for six weeks prior to mating and through
mating, gestation and day four of lactation with 0, 0.4, 1.6, and 2.0 mg K+
PFOS/kg-day (Luebker et al., 2005b). No differences were observed in the
activity of ME between control and treated maternal rats and their respective
pups.

Several previous studies have reported data that suggest that peroxisome
proliferator activated receptor alpha (PPARα) agonists may increase hepatic
responses to thyroid hormone in rats by effective competition for thyroid hormone
carrier protein binding sites. PPARα agonists are typically amphiphilic acids and
include endogenous fatty acids, hypolipidemic drugs, acetylsalicylic acid, and
perfluorinated acids, such as perfluorooctanoic acid (PFOA) and PFOS (Klaunig
et al., 2003; Ikeda et al., 1985; Sohlenius et al., 1993; Berthiaume and Wallace,
2002). In studying the mechanism of thyroid hormone reduction caused by
perfluorodecanoic acid (PFDA), Gutshall et al. (1989) reported displacement of
T4 from carrier protein binding sites by PFDA and increased activities of hepatic
ME and glycerol-3-phosphate dehydrogenase (GPD), another marker of tissue TH
response (Oppenheimer et al., 1977). Harland and Orr (1974) observed increased
GPD and ME activity after treatment with the hypolipidemic drug, clofibrate,
which is also a PPARα agonist. An increase in ME in rat liver was also observed
by Cai et al. (1996) after dietary treatment of rats with clofibrate, PFOA, and
acetylsalicylic acid. The thyromimetic effect observed with other PPARα agonists in these studies may have been due, in part, to increased availability of thyroid hormone as a result of displacement from carrier protein binding sites. However, it should also be noted that there is evidence of a non-thyroid-hormone-receptor mediated signaling pathway for increasing transcription of ME that may involve the PPARα-RXR heterodimer (Hertz et al., 1993; 1996; Ijpenberg et al., 1997).

We did not specifically validate the Coat-a-Count® serum total thyroxine, Coat-a-Count® serum free thyroxine, ADVIA Centaur® serum total thyroxine, ADVIA Centaur® serum free thyroxine, or Nichols Diagnostics free thyroxine by ED-RIA assays for use with rat sera. The Coat-A-Count® total T4 kit has been evaluated and made available for veterinary diagnostic use with rat serum (Diagnostic Products Corporation, 1993). For female Sprague Dawley rat control serum total thyroxine, both the Coat-a-Count® and ADVIA Centaur® methods gave results that were essentially equivalent and were within the reported normal range (Diagnostic Products Corporation, 1993; Loeb and Quimby, 1999). In the total thyroxine methods, proteins are degraded to release bound thyroxine, and these methods should not result in protein-based interferences. For female Sprague Dawley rat control serum free thyroxine, the ADVIA Centaur® method gave results that were within the reported normal range; however, the Coat-a-Count® method gave results that were less than the low end of the reported range. Human
analog free thyroxine methods are dependent on high-affinity thyroid binding globulin interactions, which are lacking in rats, and, in veterinary practice, the use of direct free thyroxine measurement by dialysis ED-RIA using the Nichols kit has been advocated (Reimers, 1999). The use of the latter method results in a protein-free dialysate that gives a more accurate direct determination of serum free thyroxine that obviates potential interferences based on protein content. The TSH assay is specific to the rat.

The data obtained from this study supported the hypothesis that FT4 and labeled FT4 analogs can be displaced from the serum binding proteins in the presence of PFOS. The presence of PFOS in serum resulted in a negative bias in chemiluminometric immunoassay and radioimmunoassay analog FT4 measurements, as has been observed with dietary free fatty acids in serum (e.g., oleic acid). It is of interest to note that a recent paper by Fisher et al. (2006) made similar conclusions with regard to the value of ED-RIA in measuring FT4 in rat sera from rats treated with polychlorinated biphenyls. Therefore, analog methods did not accurately measure FT4 in serum in the presence of PFOS, and a reference method such as ED-RIA should be used for determination of FT4 when experimental animals are dosed with PFOS.

We are continuing to investigate the causative factors and potential physiological responses related to the decreases in serum TT4 that have been observed in this
study as well as previous toxicological studies. In preliminary studies, we have observed a decrease in serum TT4, a transient increase in serum FT4, a transient decrease in TSH, an increase in liver ME transcripts and activities, an increase in UDP-GT transcripts, and increased excretion of $^{125}$I from labeled $^{125}$I-T4 over the course of 24 hours following a single dose of PFOS.

In conclusion, our data demonstrated that PFOS did not reduce TSH, FT4 by ED-RIA, or liver ME response to thyroid hormones. These observations suggest that prior reports of reduced free thyroid hormone in the presence of PFOS were artifacts of the analog methods.
V.

THYROID HORMONE STATUS IN RATS WITH
SHORT-TERM PFOS ADMINISTRATIONS
The entire content of this chapter has been published:

Chang et al., 2008, “Thyroid hormone status and pituitary function in adult rats given oral doses of perfluorooctanesulfonate (PFOS)”, Toxicology, 243, pages 330 – 339.
5.1. INTRODUCTION

As reported in Chapter IV, measurements of free thyroxine (FT4) in serum containing PFOS by analog methods indeed are prone to negative bias due to displacement of bound T4 from carrier proteins in serum by PFOS. The bias is attributed by the fact that analog system uses labeled thyroxine (T4) analog to bind with assay antibody in inverse proportion to the amount of competing FT4; and the amount of antibody-bound labeled T4 analog is measured. However, a certain amount of labeled T4 analog may be bound to endogenous assay serum carrier proteins with varying degrees of affinity, even though the assay calibration process takes this into account. When a substance that effectively reduces serum carrier protein binding sites through competitive binding is introduced, such as PFOS or free fatty acids, less labeled T4 analog is bound than is normally accounted for in the assay calibration, forcing more labeled T4 analog toward assay antibody binding sites, resulting in an underreporting of FT4. The bias can be minimized with a reference method, equilibrium dialysis followed by radioimmunoassay, or ED-RIA. ED-RIA uses buffers that do not contain serum proteins so that binding interferences are greatly reduced.

When PFOS was added to rat sera in vitro at concentrations ranging up to 200 μM, FT4 was shown to be increased up to 260% over paired control using ED-RIA but only 30% using analog method. Furthermore, total thyroxine (TT4)
remained unchanged. A negative bias in the analog method for measurement of serum FT4 after PFOS exposures was also demonstrated in vivo. Twenty-four hours following the last of three consecutive daily oral doses of either vehicle or 5 mg potassium PFOS/kg body weight to adult female Sprague-Dawley rats (Chang et al., 2007), we observed that, compared to controls: 1) FT4 (by ED-RIA) and TSH in serum were not altered by PFOS; 2) serum TT4 was decreased by almost 50%; and 3) mRNA transcripts for hepatic malic enzyme (ME), which responds to changes in thyroid hormones (Bogazzi et al., 1997; Hertz et al., 1991; Oppenheimer et al., 1977), were increased by approximately 20% (p < 0.05). However, hepatic ME activity was unchanged. Based on these preliminary data, the lack of change in FT4 (by ED-RIA), TSH, and ME activity suggested the PFOS-treated rats were in a euthyroid state. However, it remained unclear as why there was a reduction of serum TT4. We considered the plausibility that putative increased FT4 in serum by PFOS displacement might result in increased FT4 uptake, utilization, and metabolism by peripheral tissues, which in turn led to a net loss of TT4 (Menjo et al., 1999).

The goal of this study was to further investigate the short-term thyroid hormone status in rats treated with PFOS. We hypothesized that: 1) PFOS competes (directly or indirectly) with T4 for serum carrier protein-binding sites, and the thyroid hormones displacement results in a transient elevation of FT4 after an oral dose of PFOS; 2) this transient elevation in FT4 leads to increased turnover and
elimination of T4; and 3) PFOS would not have an effect on the ability of the pituitary to release TSH or to respond to hypothalamic thyrotropin releasing hormone (TRH).

To test these hypotheses, serum thyroid hormones (TT4, TT3, TSH, total triiodothyronine (TT3), and reverse triiodothyronine (rT3)) were measured after PFOS exposures. Changes of these measurements were related to biochemical markers ME and UDP-glucuronosyltransferase 1A (UGT1A) in the liver. Furthermore, urinary and fecal excretion of $^{125}$I from $^{125}$I-labelled T4 after a single oral dose of PFOS in rats was evaluated and pituitary function with respect to TSH release in response to TRH were investigated.

5.2. MATERIALS AND METHODS

5.2.1. Materials

All chemicals used in this study were reagent-grade and were purchased from Sigma-Aldrich (St. Louis, MO), VWR (West Chester, PA), or Perkin-Elmer (Boston, MA). Perfluorooctanesulfonate (PFOS, 86.9% pure) was supplied by 3M Specialty Material Division (St. Paul, MN). TT4, TT3, and rT3 were determined by chemiluminometric analog method (ADVIA Centaur® clinical analyzer, Bayer HealthCare LLC, Tarrytown, NY). FT4 was also measured using equilibrium dialysis followed by radioimmunoassay (ED-RIA) with Nichols Institute
Diagnostics Free T4 by Equilibrium Dialysis kits (Nichols Institute Diagnostics, San Clemente, CA). TSH values were determined via RIA (Rat TSH Radioimmunoassay OP No. NHEERL-H/RTD/EB/CL/2002-010-000, EPA, RTP, NC or Rat TSH Radioimmunoassay, National Hormone and Pituitary Program – UCLA Harbor Medical Center, Torrance, CA).

5.2.2. Animals and Husbandry

Male and female Sprague-Dawley (SD) rats (8-10 weeks old, 200 – 250 g) were purchased from Charles River Laboratory (Portage, MI or Raleigh, NC). All rats were group housed in standard solid bottom cages. Purina Mouse/Rat Chow and tap water were provided to all rats ad libitum throughout the study. Environmental controls for the animal room was set to maintain a temperature of 72 ± 3°F, humidity of 30-70%, a minimum of 10 exchanges of room air per hour and a 12 hour light/dark cycle. Studies were performed in laboratories accredited by International Association for the Accreditation of Laboratory Animal Care. All procedures involving rats were reviewed and approved by Institutional Animal Care and Use Committees. Animals care and procedures followed the US Department of Health and Human Services guide for the care and the use of laboratory animal guideline (Institute of Laboratory Animal Resources, 1996).
5.2.3. Study 1: Effects of Time and PFOS on Serum Thyroid Hormones *in vivo*

To evaluate the acute effects of PFOS on serum thyroid hormone concentrations *in vivo* within 24 hours of treatment, groups of female Sprague Dawley rats (N = 5 – 15 per group) were given either a single dose of vehicle (0.5% Tween 20® in distilled water, 3 groups) or 15 mg potassium PFOS/kg body weight suspended in vehicle (3 groups) at a dose volume of 1.0 mL/kg. The 15 mg/kg potassium PFOS dose was chosen to achieve a target concentration of PFOS in serum of approximately 50-75 μg/mL, similar to the serum PFOS concentration reached after three daily 5 mg/kg-d doses in our previous study (Chang et al., 2007).

Control and PFOS-treated rats were sacrificed by CO2 asphyxiation at 2, 6, and 24 hours post dosing. Blood samples were taken via abdominal aorta at 2, 6, and 24 hours post-treatment. After clotting, serum samples were separated after centrifugation (2000 x g for 15 minutes) and were flash-frozen using liquid nitrogen, pending analysis for PFOS concentration and thyroid hormones. Serum PFOS concentrations were analyzed by LC-MS/MS in the 3M Medical Department Bioanalytical Laboratory (St. Paul, MN, USA) as described in Chang et al. (2007). For this study, serum thyroid hormones were determined by Bayer ADVIA Centaur® chemiluminometric analog assays (for TT4, TT3, and rT3 measurements) and by reference equilibrium dialysis method (for FT4
measurements) (Mayo Medical Laboratories, Rochester, MN, USA).
Serum TSH was measured at 6 and 24 hours via rat TSH
radioimmunoassay (National Hormone and Pituitary Program – ULCA
Harbor Medical Center, Torrance, CA, USA). In addition, liver tissues
were collected at necropsy and flash-frozen in liquid nitrogen pending
analysis of PFOS concentration (via LC-MS/MS) and assessments of
hepatic biochemical markers ME and UGT1A mRNA transcript levels and
ME activities by methods described in Chang et al. (2007).

5.2.4. Study 2: Effects of PFOS on $^{125}$I elimination in vivo

Because our previous study observed that treatment with PFOS caused a
reduction of serum TT4 in vivo but not in vitro, the objective of this study
was to investigate if the PFOS-mediated reduction of serum TT4 in vivo
might be the result of increased turnover of TT4. Male and female SD rats
were injected with either 11 $\mu$Ci (male rats, n=4/group) or 9.3 $\mu$Ci (female
rats, n=5/group) of $^{125}$I-T4 (specific activity = 1250 $\mu$Ci/$\mu$g) followed by a
subsequent single oral dose of either vehicle (control) or 15 mg potassium
PFOS/kg body weight. To determine the elimination of $^{125}$I, urine and
feces were collected over a 24-hour period after PFOS administration.
Serum and liver were harvested at the end of 24 hours. Serum, liver,
urine, and feces were measured for $^{125}$I radioactivities with a gamma
counter (PerkinElmer® 1470 Wizard Automatic Gamma Counter,
Waltham, MA, USA). Serum TT4 concentration was analyzed by the Bayer ADVIA Centaur® chemiluminometric method (Mayo Medical Laboratories, Rochester, MN, USA).

5.2.5. Study 3: Pituitary Function Studies In Vivo / Ex Vivo (8-Day Repeat Dose)

The synthesis and the release of thyroid hormones from thyroid gland is primarily stimulated by TSH, which in turn, is secreted by pituitary. As pituitary is controlled by thyrotropin-releasing hormone (TRH), which is released from hypothalamus, Propylthiouracil (PTU) was used to inhibit thyroid hormone synthesis (in thyroid) which leads to increased pituitary secretion of TSH. The goal of this study is to evaluate the effects of PFOS only, PTU only, and combined treatment of PFOS and PTU, on TRH-mediated pituitary release of TSH. The rationale for combined treatment of PFOS and PTU was to further ascertain whether the presence of PFOS could influence the release of TSH while PTU was applied.

Adult male SD rats (4 groups of 6 rats) were given either (1) vehicle, 0.5% Tween® 20 (oral gavage, 1 mL/kg); (2) 3 mg potassium PFOS/ kg body weight per day suspended in 0.5% Tween 20® (oral gavage); (3) 10 μg/mL (10 ppm) propylthiouracil (PTU) in drinking water; or (4) 10 ppm
PTU + 3 mg potassium PFOS/kg body weight per day (drinking water and oral gavage) for 7 consecutive days.

Both terminal serum samples (obtained from decapitation on day 8, 24 hours after the last treatment) and interim serum samples obtained by tail-bleeding on days 1, 3, and 7 were analyzed for TT4 and TT3 by Coat-a-Count® RIA kits (Diagnostic Products Corporation, Los Angeles, CA, USA) and TSH by rat radioimmunoassay. The pituitary was removed on Day 8 and evaluated for TSH release *ex vivo* in static tissue culture per method described by Goldman and Cooper (1993). Specifically, the anterior pituitary was hemisected along the midline and both halves were weighed. A hemisected fragment of pituitary was cut into blocks of ~1 mm$^3$ and placed in 0.6 mL of Medium 199 (Medium 199 supplemented with 0.2% BSA, 13 mM sodium bicarbonate, 10 mM HEPES, and 50 µM bacitracin, pH 7.4) in a 24-well flat bottom plate. The plate was kept on ice until all pituitaries were in place. The plate was incubated in a shaking water bath at 37°C for 30 minutes to allow for the initial traumatic release of TSH. The medium was collected by carefully aspirating with a pipette, and the pituitary tissues were transferred to a new plate containing 0.6 mL of Medium 199 in each well. The plate was incubated at 37°C, and the medium was changed out every hour and frozen for future TSH analysis. At 4 hours and 7 hours, the pituitaries were incubated for 1 hour in
Medium 199 containing 50 nM of TRH (Bachem, King of Prussia, PA, USA). The addition of TRH was to stimulate the secretion of TSH (Askew and Ramsden, 1984). At 11 hours of incubation, the pituitaries were incubated for 15 minutes in Medium 199 containing 60 mM KCl to release all TSH. After 12 hours of incubation, the tissue was removed from the plate and sonicated and frozen in 0.6 mL of Medium 199 for determination of post-incubation hormone concentrations. The collected media were stored frozen at -20°C pending TSH analysis via rat-specific RIA as described.

5.2.6 Statistics

The Student’s t-test was used to compare data resulting from PFOS treatment to control values using either Microsoft Office Excel or JMP® 5.1 - Windows - Release 5.1.2 (SAS Institute, Inc., Cary, NC, USA). All data are expressed as means ± standard errors.

5.3. RESULTS

5.3.1. Study 1: Effects of Time and PFOS on Serum Thyroid Hormones in vivo

Effects of time and PFOS on serum thyroid hormone measurements are shown in Table 1. Consistent with what we observed previously (Chang et al., 2007), serum TT4 decreased significantly within 24 hours with PFOS treatments. The decrease in serum TT4 was statistically significant
at all time points. Compared to control, there was no change in TT3 and rT3 at 2 hours and 6 hours. However, both TT3 and rT3 were reduced at 24 hours. FT4 was increased significantly at 2 and 6 hours (68% and 90%, respectively, over control) and it returned to a level comparable to the control by 24 hours. The transient increase in rat serum FT4 observed at 2 and 6 hours after PFOS treatment, as described above, was associated with a transient decrease of serum TSH at 6 hours which returned to control levels by 24 hours.

Serum and liver PFOS concentrations are also listed in Table 1. After PFOS administration, serum PFOS concentrations appeared to plateau at 6 hours; this is similar to a previous study by Johnson et al. (1979) which reported that in rats, T_{max} equals to 6 – 8 hours after receiving PFOS.

Liver transcript data for ME and UGT1A, as well as enzyme activity data for ME are also presented in Table 1. Compared to controls, there were
Table 1. Comparison of serum thyroid hormone concentrations, serum and liver PFOS concentrations, and liver thyroid hormone sensitive enzyme transcripts and activities from adult female rats given either 0 or 15 mg potassium PFOS/kg body weight (serum and liver samples obtained at 2, 6, and 24 hours post-dose).

<table>
<thead>
<tr>
<th>Time</th>
<th>Dose Group</th>
<th>TT4 (μg/dL)</th>
<th>TT3 (ng/dL)</th>
<th>rT3 (nmol/L)</th>
<th>dFT4 (ng/dL)</th>
<th>TSH (IU/L)</th>
<th>Serum PFOS (μg/mL)</th>
<th>Liver PFOS (μg/g)</th>
<th>ME transcript activity (x 10^6)</th>
<th>ME transcript</th>
<th>UGT1A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Controld</td>
<td>3.18</td>
<td>66.00</td>
<td>0.18</td>
<td>2.20</td>
<td>N/A</td>
<td>&lt; LLOQf</td>
<td>&lt; LLOQf</td>
<td>7.14</td>
<td>2.19</td>
<td>39.37</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>0.21</td>
<td>2.74</td>
<td>0.01</td>
<td>0.23</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>1.05</td>
<td>0.21</td>
<td>2.20</td>
</tr>
<tr>
<td>2 Hours</td>
<td>PFOSi</td>
<td>2.42*</td>
<td>60.00</td>
<td>0.17</td>
<td>3.70*</td>
<td>N/A</td>
<td>37.28*</td>
<td>30.60*</td>
<td>11.30*</td>
<td>2.27</td>
<td>54.46*</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>0.20</td>
<td>3.45</td>
<td>0.02</td>
<td>0.57</td>
<td>8.49</td>
<td>5.72</td>
<td>1.28</td>
<td>0.13</td>
<td>1.28</td>
<td>5.72</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>3.12</td>
<td>64.25</td>
<td>0.17</td>
<td>2.63</td>
<td>2.03</td>
<td>&lt; LLOQ</td>
<td>&lt; LLOQ</td>
<td>9.41</td>
<td>2.09</td>
<td>35.49</td>
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<tr>
<td></td>
<td>SE</td>
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<td>3.64</td>
<td>0.04</td>
<td>0.19</td>
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<td>N/A</td>
<td>1.99</td>
<td>0.35</td>
<td>2.70</td>
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<tr>
<td>6 Hours</td>
<td>PFOS</td>
<td>1.94*</td>
<td>57.40</td>
<td>0.21</td>
<td>3.55</td>
<td>1.53</td>
<td>66.90</td>
<td>44.84</td>
<td>8.76</td>
<td>2.35</td>
<td>86.31</td>
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<td>0.20</td>
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<td>1.92</td>
<td>2.60</td>
<td>&lt; LLOQ</td>
<td>&lt; LLOQ</td>
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<td>2.08</td>
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</tr>
<tr>
<td></td>
<td>SE</td>
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<td>1.88</td>
<td>0.01</td>
<td>0.08</td>
<td>0.46</td>
<td>N/A</td>
<td>N/A</td>
<td>2.88</td>
<td>0.28</td>
<td>3.56</td>
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### 24 Hours

<table>
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<tr>
<th></th>
<th>PFOS</th>
<th>SE</th>
<th>24 Hours PFOS</th>
<th>SE</th>
<th>24 Hours SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.47*</td>
<td>0.16</td>
<td>44.60*</td>
<td>2.75</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>0.13*</td>
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<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>2.26</td>
<td>0.20</td>
<td>61.58*</td>
<td>8.81</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>45.00*</td>
<td>5.42</td>
<td>11.94</td>
<td>1.80</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>2.97*</td>
<td>6.38</td>
<td>36.22</td>
<td>6.38</td>
<td>6.38</td>
</tr>
</tbody>
</table>

- **a**: transcript, with unit = ME transcript per 18s rRNA copies
- **b**: Unit = μmole/min/g liver
- **c**: transcript, with unit = UGT1A transcript per 18s rRNA copies
- **d**: Female rats receiving single dose of vehicle (0.5% Tween® 20)
- **e**: N/A: not available due to limited sample quantity
- **f**: < LOQ = less than lower limit of quantitation in serum = 10 μg/mL
- **g**: < LOQ = less than lower limit of quantitation in liver = 50 μg/mL
- **h**: SE = standard error
- **i**: Female rats receiving a single dose of 15 mg/kg K+ PFOS
- *****: Statistically significant compared to control by paired Student’s t (p < 0.05)
statistically significant increases in ME transcripts 2 hours after PFOS treatment. In addition, ME activity was increased by 4, 12, and 43% over paired control at 2, 6, and 24 hours, respectively, and it was statistically significant at 24 hours. Liver UGT1A mRNA transcripts from PFOS-treated rats were elevated over control values at 2 and 6 hours post-treatment, and returned to control levels by 24 hours.

5.3.2 Study 2: Effects of PFOS on $^{125}$I elimination in vivo

Serum TT4 measurements, and $^{125}$I radioactivities for serum, liver, urine, and feces, are shown in Figure 1 as the percent of control values. Compared to controls, serum TT4 concentrations from PFOS-treated rats (both males and females) were reduced by 55% at the end of 24-hour PFOS treatment. The decrease in serum TT4 correlated with a decrease in serum $^{125}$I radioactivity as well. While liver $^{125}$I radioactivity were reduced by approximately 40% and 30% in males and females, respectively, urine and fecal $^{125}$I radioactivities were markedly increased in samples obtained from PFOS-treated rats, indicating an increased turnover and a loss of thyroid hormones.
Figure 1. Serum TT4 concentrations (measured by Bayer ADVIA Centaur® chemiluminometric immunoassay) and ¹²⁵I activities in serum, liver, urine, and feces (measured using PerkinElmer® Wizard 1470 Gamma Counter), represented as % over or under control, from rats receiving a ¹²⁵I-T4 injection (11 μCi and 9.3 μCi for male and female rats, respectively) followed by a subsequent oral dose of either vehicle (0.5% Tween® 20) or 15 mg potassium PFOS/kg body weight. Open bars (□) represent male rats while solid bars (■) represent female rats. Asterisk (*) denotes significant difference from control (p < 0.05).
5.3.3. Study 3: Pituitary Function Studies *In Vivo / Ex Vivo* (8-Day Repeat Dose)

Treatment of rats with PTU (with or without concurrent PFOS treatment) significantly increased serum TSH levels and decreased TT4 (Figure 2) and TT3 (Figure 3) over the 8-day treatment period. TSH concentrations in sera from PFOS-only treated rats did not differ from the control group (Figure 4), even though TT4 and TT3 were significantly reduced (Figures 2 and 3). Results from PTU+PFOS group did not differ significantly from PTU-treated group. In excised pituitaries from these rats, there was no effect of PFOS on the TRH-mediated release of TSH in static tissue culture (Figure 5).
Figure 2. Mean serum total thyroxine (TT4) concentrations in adult male rats treated with: (1) vehicle, 0.5% Tween® 20 (oral gavage); (2) 3 mg/kg-d PFOS suspended in vehicle (oral gavage); (3) 10 μg/mL (10 ppm) PTU in drinking water; or (4) 10 ppm PTU + 3 mg/kg-d PFOS (drinking water and oral gavage) for 8 consecutive days (6 rats per dose group). Interim serum samples were obtained on days 1, 3, 7, and 8 to measure serum TT4 by RIA (Diagnostic Products Corporation, Los Angeles, CA). Each point represents the mean serum TT4 determinations and error bars represent standard errors. Solid circle (●) represent serum TT4 values obtained from vehicle-treated rats and solid triangle (▲) represent serum TT4 values obtained from PFOS-treated rats. Solid square (■) represent serum TT4 values obtained from PTU-treated rats and solid diamond (♦) represent serum TT4 valued obtained from (PTU+PFOS)-treated rats. Serum TT4 values obtained from rats receiving PFOS or PTU or (PFOS+PTU) were statistically significantly lower than control rats at all time points (Student’s t, p< 0.05).
Figure 3. Mean serum total triiodothyronine (TT3) concentrations in adult male rats treated with:
(1) vehicle, 0.5% Tween® 20 (oral gavage); (2) 3 mg/kg-d PFOS suspended in vehicle (oral gavage);
(3) 10 μg/mL (10 ppm) in drinking water; or (4) 10 ppm PTU + 3 mg/kg-d PFOS (drinking water and
oral gavage) for 8 consecutive days (6 rats per dose group). Interim serum samples were obtained
on days 1, 3, 7, and 8 to measure serum TT3 by RIA (Diagnostic Products Corporation, Los Angeles,
CA). Each point represents the mean serum TT3 determinations and error bars represent standard
errors. Solid circle (●) represent serum TT3 values obtained from vehicle-treated rats and solid
triangle (▲) represent serum TT3 values obtained from PFOS-treated rats. Solid square (■)
represent serum TT3 values obtained from PTU-treated rats and solid diamond (♦) represent serum
TT3 valued obtained from (PTU+PFOS)-treated rats. Serum TT3 values obtained from rats receiving
PFOS or PTU or (PFOS+PTU) were statistically significantly lower than control rats at all time
points (Student’s t, p< 0.05).
Figure 4. Mean serum thyrotropin (TSH) concentrations in adult male rats treated with: (1) vehicle, 0.5% Tween® 20 (oral gavage); (2) 3 mg/kg-d PFOS suspended in vehicle (oral gavage); (3) 10 μg/mL (10 ppm) PTU in drinking water; or (4) 10 ppm PTU + 3 mg/kg-d PFOS (drinking water and oral gavage) for 8 consecutive days (6 rats per dose group). Interim serum samples were obtained on days 1, 3, 7, and 8 to measure serum TSH by rat TSH RIA (EPA-NHEERL TSH rat radioimmunoassay based on materials supplied by the National Hormone and Pituitary Program, Torrance, CA). Each point represents the mean serum TSH determinations and error bars represent standard errors. Solid circle (●) represent serum TSH values obtained from vehicle-treated rats and solid triangle (▲) represent serum TSH values obtained from PFOS-treated rats. Solid square (■) represent serum TSH values obtained from PTU-treated rats and solid diamond (♦) represent serum TSH values obtained from (PTU+PFOS)-treated rats. Serum TSH values obtained from rats receiving PFOS were comparable to control values. Serum TSH values from rats treated with PTU or (PFOS+PTU) were statistically significantly higher than control rats at all time points (Student’s t, p< 0.05).
Figure 5. Thyrotropin (TSH) concentrations released from tissue culture medium containing anterior pituitary excised from vehicle-treated (open bars, ), PFOS-treated (left-striped bars, ), PTU-treated (right-striped bars, ), or (PFOS+PTU)-treated rats (crisscrossed bars, ). After plating (6 rats per dose group), cells were allowed to reach equilibrium for 4 hours and the TSH readings at 4 hours were the baselines. TSH was measured via RIA (EPA-NHEERL TSH rat radioimmunoassay based on materials supplied by the National Hormone and Pituitary Program, Torrance, CA). Each bar represents the mean serum TSH determinations and error bars represent standard errors.

For the PFOS-treatment group, the net TSH released in response to the first and second TRH stimulations was comparable to the control. While the net TSH released from the PTU and (PFOS+PTU) groups were similar to the control during the first TRH challenge, they were significantly lower upon the second TRH challenge. Asterisk (*) denotes significant difference from control (p < 0.05).
DISCUSSION

The series of experiments described herein was undertaken to develop a better understanding of the potential effects of PFOS on serum thyroid hormones and physiological thyroid homeostasis in rats. Even though reduced thyroid hormone levels in serum (hypothyroxinemia) have been reported as a PFOS treatment-related effect in several toxicological studies (Butenhoff et al., 2002; Lau et al., 2003; Luebker et al., 2005b; Seacat et al., 2002; Thibodeaux et al., 2003); however, in these studies, reductions in serum thyroid hormones (T3 and/or T4) occurred in the absence of changes in thyroid gland histology or clinically-significant elevations of TSH. These findings therefore raised the question of whether the animals were rendered hypothyroid by PFOS.

Production and release of metabolically active thyroid hormones by the thyroid gland is regulated by pituitary secretion of TSH and hypothalamic section of TRH. This regulatory process for controlling circulating thyroid hormone concentrations constitutes what is commonly referred to as the hypothalamic-pituitary-thyroid axis (Menjo et al., 1999). In this system, serum free (unbound) thyroxine and free triiodothyronine (FT4 and FT3, respectively) function as primary or secondary feedback signals, acting on the hypothalamus and/or pituitary when there is an imbalance in their circulating concentrations. Although
measurement of free thyroid hormones in serum can provide information on thyroid hormone status, the diagnosis of primary hypothyroidism is based mainly on a substantial elevation of TSH in response to reduced free thyroid hormones (Larsen et al., 2003; Ravel, 1995; Sapin, 2001; Sapin and Schlienger, 2003).

In a previous study, Chang et al. (2007) demonstrated that measurement of FT4 by analog methods in serum containing PFOS is prone to negative bias in that PFOS competes with FT4 for binding to serum proteins. Therefore, to measure FT4 correctly with serum containing PFOS, an equilibrium dialysis reference method (ED-RIA) should be used. Under this condition, PFOS was found to increase the free fraction of T4 in concentration-dependent manner in rat serum in vitro while maintaining TT4 at a constant level. Twenty-four hours after PFOS exposure, sera FT4 and TSH were not different between PFOS-treated and control rats; however, mean TT4 concentration in PFOS-treated rats was reduced to less than half the control value. This led us to hypothesize that a PFOS-induced increase in FT4 via competitive displacement from serum binding sites may occur after oral dosing with PFOS and can lead to increased tissue uptake and turnover of T4 which would result in a lowering of serum TT4 while rebalancing the equilibrium between bound and free T4. When released by the thyroid gland into serum, thyroid hormones such as thyroxine are bound to serum carrier proteins for circulation. For T4, approximately 99.97% are bound to carrier proteins while a very small fraction circulates as unbound (free) fraction (approximately 0.03%).
Since only FT4 is capable of exerting biological activity, FT4 can be converted from (inactive) protein-bound T4 as needed. Under the conditions in which there is a lowering of serum TT4, the large concentration of protein-bound thyroxine is able to act as a reservoir to maintain a constant concentration of FT4.

In the experiments that we report here, a transient increase in serum FT4 and a corresponding transient decrease in serum TSH were observed over the first six hours following a single oral dose of PFOS. By 24 hours post-dose, the FT4 and TSH concentrations had returned to control levels. However, TT4 in serum was reduced in a time-dependent manner.

In addition to the increase in FT4, the increased liver UGT1A family mRNA transcripts observed at 2 and 6 hours may have been representative of induction of increased glucuronidation and turnover of T4 (Barter and Klaassen, 1992b). It is unclear whether PFOS played a role in this induction process or whether increases in tissue-active thyroid hormone contributed to this effect (Haberkorn et al., 2003). The fact that TT3 was not as greatly reduced in our time-course experiment compared to TT4 (23% and 55% reductions, respectively compared to control values at 24 hours post-dose) may represent a more prominent induction of T4-specific UGT isoforms, UGT1A1 and/or UGT1A6, as opposed to the T3-specific UGT isoform, UGT2A2 (Vansell and Klaassen, 2002a, 2002b). This would also pose a possible explanation for the lack of an elevation of TSH in our
experiments, as it has been found that treatment of rats with inducers of T4-specific UGT1A family isoforms in rat liver does not result in clinically-significant increases in TSH and hypertrophy/hyperplasia of the rat thyroid based on studies of Klaassen et al. (2001) and Vansell et al. (2004).

The observation that liver ME mRNA transcripts were increased two hours following a dose of PFOS and that liver ME activity was increased at 24 hours also suggests a potential increased tissue response to thyroid hormone. However, PFOS is an agonist for the nuclear receptor, PPARα (Berthiaume and Wallace, 2002a; Shipley et al., 2004; Sohlenius et al., 1994; Takacs and Abbott, 2007; Vanden Heuvel et al., 2006), and PPAR-α agonists, including perfluorooctanoate (PFOA), a carboxylated analog of PFOS, have been shown to have thyromimetic effects in rat liver believed to be due to transcriptional activation of thyroid-hormone-dependent genes such as liver ME, mitochondrial glycerol-3-phosphate dehydrogenase (αG3PD), glucose-6-phosphate dehydrogenase (G6PD), and S14 (Cai et al., 1996; Hertz et al., 1991; Hertz et al., 1993; Hertz et al., 1996). The activation of ME is believed to result from binding of PPARα/RXR (Retinoid X Receptor) heterodimer to a 5’-flanking enhancer of the malic enzyme promoter and is distinct from the action of thyroid hormone (Hertz et al., 1996; IJpenberg et al., 1997). Therefore, the investigation of additional markers of liver thyroid hormone response would confirm this interpretation.
Additional support for increased turnover of T4 comes from the $^{125}$I-labelled T4 elimination study. When rats were pre-treated with $^{125}$I-radiolabelled T4 followed by a single oral dose of PFOS, a decrease in $^{125}$I activity in serum and liver and a corresponding increase in $^{125}$I activity in urine and feces was observed in PFOS-treated rats compared to their controls. This corresponded with decreased TT4 in PFOS-treated rats, and provided evidence to support the hypothesis that the reduction in TT4 is due to increased turnover and elimination.

In order to investigate whether the lack of HPT-response was not due to an uncompromised function of the pituitary, the ability of the pituitary to respond to TRH stimulation was evaluated and the results were compared to the responses induced by a classical goitrogen, PTU. Following the last dose, the pituitaries were removed and cultured to monitor TSH concentrations over a 12-hour incubation period during which TRH-stimulation of TSH was tested twice. Compared to control, serum TSH secretions (during dosing) and TRH-mediated TSH releases (during static culture) were reduced by either PTU treatment alone or PTU and PFOS combined treatment. On the other hand, there was no effect of PFOS on either serum TSH during dosing or TRH-mediated TSH release from the pituitary during static culture. These observations provide evidence that PFOS does not lower TT4 through disruption of the hypothalamic-pituitary-thyroid axis.
In summary, findings from our study suggest that PFOS does not induce a hypothyroid state in rats under the conditions of dosing. Neither does PFOS treatment appear to alter the function of the hypothalamic-pituitary-thyroid axis.

5.5. Acknowledgement

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VI.

CHANGES IN SERUM THYROID HORMONES IN RELATION TO INCREASING AND DECREASING SERUM PERFLUOROOCTANESULFONATE (PFOS) LEVELS IN RATS DURING AND AFTER SUBCHRONIC EXPOSURE TO PFOS
6.1. INTRODUCTION

The goal of this study was to further investigate thyroid hormone status in rats treated daily with K⁺PFOS for 28 days, both during and for 10 weeks after treatment. Previous study results described in Chapter IV and Chapter V had shown that binding of PFOS to serum proteins reduces T4 bound to carrier proteins and causes the overall TT4 concentrations to remain decreased without appearing to affect thyroid hormone homeostasis. The large concentration of bound T4 relative to FT4 allows a sufficient capacity for the body to maintain an adequate concentration of FT4 for thyroid hormone homeostasis. It is conceivable that once the physical binding of PFOS to thyroid hormone carrier proteins is removed, the extent of thyroxine displacement by PFOS will be diminished and that TT4 will return to normal range. The aims in this study were to relate serum concentrations of PFOS to concentrations of TT4 and TSH in rat serum during an extended dosing period (28 days) followed by a 10-week recovery period. This study was to test the hypotheses that: (1) serum TSH values would be similar between control and K⁺PFOS-treated rats; (2) serum TT4 would decrease as PFOS concentration increased on repeat dosing over the 28-day period; and (3) serum TT4 would increase as PFOS concentration decreased on the 10-week recovery period. Several genes in liver representative of thyroid hormone responses, proliferation, and glucuronidation processes were also
evaluated via quantitative RT-PCR; along with characterization of elimination profiles of PFOS in urine and feces during the recovery period.

6.2 Materials and methods

6.2.1 Materials

All chemicals used in this study were reagent-grade and were purchased from Sigma-Aldrich (St. Louis, MO), VWR (West Chester, PA), Bachem (King of Prussia, PA), or Perkin-Elmer (Boston, MN). Perfluorooctanesulfonate potassium salt (K⁺PFOS, 86.9% pure) was supplied by 3M Company (St. Paul, MN).

6.2.2 Animals and husbandry

Male and female Sprague Dawley rats (8-10 weeks old, 200-250 g) were purchased from Charles River Laboratory (Portage, MI). All rats were group housed in standard solid bottom cages except during the weekly urine and feces collection in the recovery period, in which rats were single housed in wired bottom metabolism cages for 24 hours at specified times. Purina mouse/rat chow and tap water were provided to all rats ad libitum throughout the study. Environmental controls for the animal room were
set to maintain a temperature of 72 ± 3°F, humidity of 30-70%, a
minimum of 10 exchanges of room air per hour, and a 12-hour light/dark
cycle. All procedures were approved by the Institutional Animal Care and
Use Committee of 3M Company, an Association for Assessment and
Accreditation of Laboratory Animal Care International accredited facility.
Animal care and procedures conformed to the Guide for the Care and Use
of Laboratory Animals Guidelines (ILAR, 1996).

6.2.3 Experimental design

To evaluate the serum thyroid hormone status in rats under repeated
treatment condition, male and female Sprague Dawley rats
(n=10/sex/treatment group) received daily oral doses of either vehicle
control (0.5% Tween 20® in distilled water) or 1 mg/kg K⁺PFOS
suspended in vehicle for 28 days. The first day of dosing was designated
as study day 1 (SD 1), and the dose volumes (1 mL per kg body weight)
were calculated based on the weekly body weights. Dosing period
consisted of SD 1 - 28 and recovery period consisted of SD 29 – 99 and
SD 29 – 98 for males and females, respectively.

6.2.4 Treatment (dosing) phase and end-of-treatment necropsy
During the treatment period, rats were orally dosed at approximately the same time each day for 28 days with weekly body weight measurements. In addition, weekly blood samples (via tail vein) were obtained and processed to serum prior to the scheduled daily dosing from all rats on SD 2, 8, 15, 22, and 28. The blood samples were allowed to clot at room temperature followed by centrifugation at 2000 x g for 15 minutes to obtained serum. On SD 29 (24 hours post-last dose), 5 randomly-selected rats per sex per treatment group were sacrificed via CO₂ asphyxiation and gross necropsy was performed. Serum processed from blood (after clotting at room temperature and centrifugation at 2000 x g for 15 minutes) and majority of liver sections were flash-frozen using liquid nitrogen. A small section of liver (approximately 1 gram) was preserved in RNALater (Applied Biosystems/Ambion®, Austin, TX) for transcript profiling.

6.2.5 Recovery phase and end-of-recovery necropsy

On SD 29, the remaining rats (5/sex/treatment group) that were not sacrificed were allowed to recover for additional 10 weeks. Similar to treatment phase, body weight data and blood samples were collected on a weekly basis. Specifically, serum (processed from blood after clotting at room temperature and centrifugation at 2000 x g for 15 minutes) were
obtained on SD 29, 35, 42, 50, 56, 63, 70, 84, 92, and 99 for male rats; and on SD 29, 34, 41, 49, 55, 62, 69, 78, 91, and 98 for female rats. In addition, 24 hours prior to the schedule weekly blood sampling, all rats were individually placed into metabolic chambers for a 24-hour urine and feces collection. On SD 99 and 98 for males and females, respectively, all rats were sacrificed via CO2 asphyxiation and gross necropsy was performed. Serum processed from blood (after clotting at room temperature and centrifugation at 2000 x g for 15 minutes) and majority of liver sections were flash-frozen using liquid nitrogen. A small section of liver (approximately 1 gram) was preserved in RNALater for transcript profiling.

6.2.6 Thyroid hormone measurement

Aliquots of serum samples collected in this study were evaluated for TSH and TT4 concentrations with rat-specific TSH radioimmunoassay and TT4 radioimmunoassay, respectively, (National Hormone and Pituitary Program – UCLA Harbor Medical Center, Torrance, CA).

6.2.7 LC-MS/MS analyses for PFOS
Serum and liver PFOS concentrations were analyzed by LC-MS/MS in the 3M Medical Department Bioanalytical Laboratory (St. Paul, MN) as described in Chang et al. (2007). Furthermore, urine and feces samples collected during the recovery period were also evaluated for PFOS concentration by LC-MS/MS.

To prepare urine sample for analyses, urine from untreated rats (used as blank matrix), K⁺PFOS-treated, and vehicle-treated rats were stored at –80°C prior to analysis. One hundred (100) μL of urine samples were aliquoted into clean polypropylene tubes. The dual-labeled “18O₂ analog of PFOS was used as an internal standard by addition to each sample tube. One mL of 1.0 N formic acid was added to all tubes, followed by 100 μL saturated ammonium sulfate. Samples were vortexed between each addition.

To prepare fecal samples for analyses, feces from untreated rats (used as blank matrix), K⁺PFOS-treated, and vehicle-treated rats were stored at –80°C prior to analysis. Samples were allowed to thaw, all feces collected per rat was weighed and homogenized with deionized water in a clean polypropylene tube. The ratio between feces and water was 1:3 (w/w). After the primary homogenization step, the whole homogenate was sonicated for 30 minutes followed by centrifugation (2500 x g, 20
minutes). The supernatant was collected to a clean polypropylene tube. One hundred (100) μL of supernatant was mixed with the addition of a dual-labeled \(^{18}\rm{O}_2\) analog of PFOS as an internal standard, followed by 1 mL of 1.0 N formic acid and 100 μL saturated ammonium sulfate. Five hundred (500) μL of 1.0 N formic acid was added to all tubes followed by 300 μL of water. Samples were vortexed between each addition. Sample matrixes had a pH of approximately 2.5. Similar to serum and liver, urine and fecal extracts were analyzed for PFOS as described in Chang et al. (2007).

6.2.8 Quantitative RT-PCR

Total RNA were isolated from all liver samples followed by quantitative RT-PCR using the method described previously (Chang et al., 2009; Chang et al., 2007). The transcripts evaluated are listed in Table 1. Results were calculated and expressed as copies of gene per copy of 18s rRNA.
6.2.9 Statistical analysis

The Student’s t-test was used to compare data resulting from $K^+PFOS$ treatment to control values using Microsoft Office Excel. All data are expressed as means ± standard error (SE).
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<th>Lower Primer</th>
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6.3 RESULTS

All rats survived to the scheduled necropsy. No K⁺PFOS treatment-related clinical findings were noted during the study period. Mean body weights (BW), body weight-gain (BWG), liver weights (LW), and liver weight to body weight percent (LW/BW) data are summarized in Table 2. There were no statistical differences in mean body weight or mean body weight-gains, either at the end of treatment or recovery, from K⁺PFOS-treated rats with respect to sex-matched control. Under the study conditions, K⁺PFOS treatments did not appear to affect liver weights in female rats; however, absolute liver weights were statistically significantly higher than controls from the K⁺PFOS-treated males at the end of treatment period. Absolute liver weights in males were similar to control at the end of the 10-week recovery. Body weight data collected during the entire study duration are also illustrated in Figure 1.
Table 2: Mean body weights (BW), body-weight gain (BWG), liver weights (LW), % liver weight to body weight ratio (LW/BW) data (± standard error, SE) in male (M) and female (F) rats during treatment and recovery periods.

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<td>BWG&lt;sup&gt;d&lt;/sup&gt; (g)</td>
<td>LW (g)</td>
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<tr>
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<td>Control</td>
<td>215.9 ± 2.3</td>
<td>406.2 ± 4.8</td>
<td>177.0 ± 6.8</td>
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<td>PFOS</td>
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<td>424.7 ± 7.3</td>
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<td>Control</td>
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<td>274.6 ± 3.9</td>
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<td>206.3 ± 2.3</td>
<td>259.1 ± 2.9</td>
<td>56.6 ± 2.7</td>
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<sup>a</sup> Prior to the start of dosing
<sup>b</sup> After 28-day oral treatments
<sup>c</sup> 10 weeks after last oral treatment
<sup>d</sup> Body weight difference between weight data obtained at the end of treatment and weight data obtained pre-dose.
<sup>e</sup> Body weight difference between weight data obtained at the end of recovery and weight data obtained pre-dose.
<sup>*</sup> Values are statistically significant different from controls (p < 0.05)
Figure 1. Mean weekly body weight data (± standard error) for male (M) rats during study day (SD) 1 – 99 and female (F) rats during SD 1 – 98. Rats received 28 daily treatments (n=10/sex/group) of either vehicle control (0.5% Tween 20, open square and open circle for M and F rats, respectively) or 1 mg/kg-d of KPFOS (solid square and solid circle for M and F rats, respectively) via oral gavage. After scheduled necropsy on SD 29 with 5 randomly-selected rats per treatment group, the remaining 5 rats per treatment group were allowed to recover for 10 weeks.
Mean serum PFOS concentrations in both male and female rats during the 28-day K\(^+\)PFOS oral treatment and 10-week recovery phases are illustrated in Figure 2 and summarized in Tables 3a (males) and 3b (females). Also included in Tables 3a and 3b are mean liver PFOS concentrations as well. Serum PFOS concentrations in both male and female rats appeared to increase linearly during the 28-day treatment period and gradually decreased during the 10-week recovery period. The body burden appeared to be higher in female rats than male rats.

Figure 2. Mean serum PFOS concentrations (± standard error) in male and female rats received 28 daily oral doses of 1 mg/kg-d K\(^+\)PFOS during dosing period (n=10/sex/group) followed by 10-week recovery period (n=5/sex/group). Solid squares denote male serum data and solid circles represent female serum data. Linear regression model was performed on both male and female serum PFOS data, shown as solid lines, during the dosing period. It suggested that serum PFOS concentration increased linearly under the study condition. No regressions were performed for the serum PFOS data during recovery period.
Table 3a: Mean serum PFOS, serum thyrotropin (TSH), serum total thyroxine (TT4), and liver PFOS concentrations in male rats during treatment and recovery periods. Rats received either control (C) or 1 mg/kg-d K⁺PFOS (P). Values presented as mean ± standard error (SE).

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<td>[PFOS] P</td>
<td>2.79 9.64 20.00 35.45 50.62 44.40 30.66 26.16 21.64 17.18 24.94 20.22 15.90 18.48</td>
<td>(±0.26)</td>
<td>(±0.51)</td>
<td>(±2.33)</td>
<td>(±4.07)</td>
<td>(±3.55)</td>
<td>(±2.67)</td>
<td>(±3.17)</td>
<td>(±0.99)</td>
<td>(±0.74)</td>
<td>(±0.78)</td>
<td>(±1.40)</td>
<td>(±0.76)</td>
<td>(±0.85)</td>
<td>(±1.41)</td>
</tr>
<tr>
<td>Serum C</td>
<td>4.10 4.62 3.91 5.04 5.22 3.82 4.38 3.68 4.75 4.05 3.53 4.18 3.93 3.43 4.03 3.65</td>
<td>(±0.46)</td>
<td>(±0.48)</td>
<td>(±0.77)</td>
<td>(±0.44)</td>
<td>(±0.88)</td>
<td>(±1.27)</td>
<td>(±0.86)</td>
<td>(±1.11)</td>
<td>(±0.80)</td>
<td>(±0.56)</td>
<td>(±0.71)</td>
<td></td>
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</tr>
<tr>
<td>Serum C</td>
<td>3.48 3.33* 3.79 3.84 5.39 4.00 3.68 4.04 3.34 3.96 3.12 4.24 3.62 4.04 4.44 3.76</td>
<td>(±0.36)</td>
<td>(±0.30)</td>
<td>(±0.35)</td>
<td>(±0.46)</td>
<td>(±0.70)</td>
<td>(±0.42)</td>
<td>(±0.48)</td>
<td>(±0.51)</td>
<td>(±0.71)</td>
<td>(±0.80)</td>
<td>(±0.35)</td>
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<td>(±0.58)</td>
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<td>5.56</td>
<td>5.65</td>
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<td>5.97</td>
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<td>(±0.22)</td>
<td>(±0.20)</td>
<td>(±0.14)</td>
<td>(±0.19)</td>
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<td>(±0.07)</td>
<td>(±0.25)</td>
<td>(±0.09)</td>
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<td>(±0.17)</td>
<td>(±0.35)</td>
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<tr>
<td>(µg/dL)</td>
<td>P</td>
<td>6.24</td>
<td>4.15*</td>
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<td>2.37*</td>
<td>1.78*</td>
<td>1.23*</td>
<td>2.49*</td>
<td>2.96*</td>
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<td>(±0.22)</td>
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<td>(µg/g)</td>
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<td>--</td>
<td>--</td>
<td>--</td>
<td>46.40</td>
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<td>(±1.68)</td>
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<td>--</td>
<td>--</td>
<td>(±5.96)</td>
</tr>
</tbody>
</table>

*a* The first day of dosing was designated as SD 1.

*b* Treatment phase consisted of 28 daily oral treatments consecutively (SD 1 – SD 28).

*c* Recovery phase was 10 weeks after last oral treatment.

*d* Control rats received 0.5% Tween 20 in water orally every day for 28 days.

*e* Limit of quantitation (LOQ) for serum is 0.010 µg/mL.

*f* K⁺PFOS-treated rats received 1 mg/kg-d K⁺PFOS orally every day for 28 days.

*g* Not available.

*h* Limit of quantitation (LOQ) for liver is 0.050 µg/g.

* Statistically significantly different than mean control values (*p < 0.05*).
Table 3b: Mean serum PFOS, serum thyrotropin (TSH), serum total thyroxine (TT4), and liver PFOS concentrations in female rats during treatment and recovery periods. Rats received either control (C) or 1 mg/kg-d K⁺PFOS (P). Values presented as mean ± standard error (SE).

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<th>Study Days (SD)</th>
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<th>Recovery Phase</th>
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<tr>
<td>Parameter</td>
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<tr>
<td>Serum C [PFOS]</td>
<td>&lt;LOQ &lt;LOQ &lt;LOQ &lt;LOQ &lt;LOQ &lt;LOQ &lt;LOQ &lt;LOQ &lt;LOQ &lt;LOQ &lt;LOQ &lt;LOQ &lt;LOQ &lt;LOQ &lt;LOQ</td>
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</tr>
<tr>
<td>(μg/mL) P</td>
<td>3.20 16.31 28.97 48.01 69.43 75.12 68.02 55.68 53.04 53.94 46.24 38.94 39.66 32.90 27.24 19.86</td>
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</tr>
<tr>
<td>(±0.18) (±1.07) (±1.80) (±2.59) (±4.10) (±1.63) (±3.22) (±2.53) (±3.55) (±1.63) (±2.24) (±2.72) (±2.42) (±2.26) (±3.09)</td>
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<td></td>
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<tr>
<td>Serum C [TSH]</td>
<td>1.94 2.23 1.84 2.62 2.15 2.10 1.82 1.82 1.62 2.04 2.02 1.96 1.70 1.98 1.98 3.12</td>
<td></td>
</tr>
<tr>
<td>(μg/mL) P</td>
<td>(±0.21) (±0.24) (±0.24) (±0.38) (±0.22) (±0.23) (±0.34) (±0.17) (±0.17) (±0.37) (±0.38) (±0.38) (±0.22) (±0.25) (±0.19) (±0.51)</td>
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<tr>
<td>(±0.34) (±0.24) (±0.25) (±0.33) (±0.17) (±0.20) (±0.29) (±0.25) (±0.21) (±0.32) (±0.37) (±0.48) (±0.33) (±0.25) (±0.30)</td>
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166
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<tr>
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<th>C</th>
<th>4.96</th>
<th>4.73</th>
<th>4.30</th>
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<th>4.79</th>
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<td>(±0.46)</td>
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<tr>
<td></td>
<td>(μg/dL) P</td>
<td>3.49*</td>
<td>2.75*</td>
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<td>(±0.23)</td>
<td>(±0.17)</td>
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<th>--</th>
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<td>--</td>
<td>55.22</td>
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<td>126.20</td>
</tr>
<tr>
<td></td>
<td>(μg/g) P</td>
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<td>--</td>
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<td>--</td>
<td>--</td>
<td>--</td>
<td>(±3.28)</td>
<td>--</td>
<td>--</td>
<td>(±1.53)</td>
</tr>
</tbody>
</table>

---

*a* The first day of dosing was designated as SD 1.

*b* Treatment phase consisted of 28 daily oral treatments consecutively (SD 1 – SD 28).

*c* Recovery phase was 10 weeks after last oral treatment.

*d* Control rats received 0.5% Tween 20 in water orally every day for 28 days.

*e* Limit of quantitation (LOQ) for serum is 0.010 µg/mL.

*f* K⁺PFOS-treated rats received 1 mg/kg-d K⁺PFOS orally every day for 28 days.

*g* Not available.

*h* Limit of quantitation (LOQ) for liver is 0.050 µg/g.

* Statistically significantly different than mean control values ($p < 0.05$).
Mean male and female serum TSH and TT4 values obtained during the treatment phase and recovery phase are also presented in Tables 3A and 3B. Regardless of sex or study phases (either during treatment or recovery), serum TSH values in rats receiving K⁺PFOS treatments were similar to their respective controls (see illustration in Figure 3). Mean male and female serum TT4 values, in conjunction with the corresponding serum PFOS concentrations, are illustrated in Figure 4 and Figure 5, respectively. While serum TT4 concentrations remained unchanged throughout the study in male and female controls, similar to what we observed previously (Chang et al., 2008; Chang et al., 2007), serum TT4 were decreased significantly in both K⁺PFOS-treated male and female rats compared to the respective controls. In both cases, serum PFOS concentrations were inversely correlated with serum TT4 concentrations.

The mean % of administered PFOS excreted in urine and feces during the weekly 24-hour interval collection from K⁺PFOS-treated male and female rats are illustrated in Figure 6. It appeared that PFOS was eliminated slowly in both urine and feces as less than 1% of the total administered PFOS were found in both matrices at each given interval. Urinary excretion of PFOS appeared to be more dominate than fecal excretion.

The mRNA transcript data are summarized as mean ± SE in Table 4. Due to freezer malfunction, only the treatment phase liver samples retained good RNA
quality upon isolation and the corresponding results are reported here. Compared to control, mean Cyp2b2 level was increased with statistical significance in both sexes while mean Apo level was decreased with statistical significance in both sexes. The mean Dio1 level was statistically significantly decreased among K⁺PFOS-treated female rats at the end of the treatment phase, Ugt1a1 expression was statistically significantly increased in male rats only. There were no other statistically significant differences among other transcripts evaluated.
Figure 3. Mean serum TSH concentrations (± standard error) in male (M) and female (F) rats received 28 daily oral treatments (n=10/sex/group) of either vehicle control (0.5% Tween 20, open square and open circle for M and F rats, respectively) or 1 mg/kg-d K⁺PFOS (solid square and solid circle for M and F rats, respectively). After scheduled necropsy on study day 29 (24 hours after last oral dose), 5 randomly-selected rats per treatment group were euthanized via CO₂ asphyxiation while the remaining rats (n=5/sex/group) were allowed to recover for 10 weeks. There were no significant differences in TSH between control and K⁺PFOS-treated rats during dosing or recovery periods.
Figure 4. Mean serum PFOS concentrations (± standard error) in 1 mg/kg-d K⁺PFOS-treated male rats (represented as solid diamonds) with the corresponding mean serum TT4 concentrations (± standard error) are denoted as solid squares. The open squares represented here in Figure 4 are mean serum TT4 levels from vehicle control-treated male rats. While the corresponding serum TT4 concentration remained constant throughout the study in male rats receiving vehicle control, the increase in serum PFOS concentration was closely correlated with decreased serum TT4 during dosing; and during the recovery phase, the decrease in serum PFOS was correlated with increasing serum TT4.
Figure 5. Mean serum PFOS concentrations (± standard error) in 1 mg/kg-d K⁺PFOS-treated female rats (represented as solid triangles) with the corresponding mean serum TT4 concentrations (± standard error) are denoted as solid circles. The open circles represented here in Figure 5 are mean serum TT4 levels from vehicle control-treated female rats. Similar to what we observed in male rats, while the corresponding serum TT4 concentration remained constant throughout the study in female rats receiving vehicle control, the increase in serum PFOS concentration was closely correlated with decreased serum TT4 during dosing; and during the recovery phase, the decrease in serum PFOS was correlated with increasing serum TT4.

Figure 6. Mean % PFOS excreted in urine (open and dotted bars) or feces (solid and checkered bars) during the 24-hour weekly collections in recovery period in relation to the total cumulative PFOS administered (6.82 ± 0.25 mg and 5.20 ± 0.18 mg in male and female rats, respectively). PFOS was eliminated slowly in both urine and feces because less than 1% of the administered PFOS was eliminated at any given 24-hour interval. Urinary elimination appeared to be slightly dominant than fecal elimination.
Table 4: Mean mRNA transcript levels (expressed as mean number of mRNA copies per 18s rRNA ± standard error) for liver samples obtained at the end of treatment.

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<th>Group</th>
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<th>Female Rats</th>
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\(^a\) Represents rats receiving vehicle control solution, 0.5% Tween 20 in water

\(^b\) Represents rats receiving 1 mg/kg-d of K⁺PFOS

\(^*\) Values are statistically significant from control (p < 0.05).
This study was undertaken to evaluate the potential effects of PFOS treatment on serum thyroid hormone status in rats after 28 repeated oral doses. Prior to 2005, hypothyroxinemia has been observed in toxicological studies of rats treated with K⁺PFOS (Butenhoff et al., 2002; Lau et al., 2003, Luebker et al., 2005; Seacat et al., 2002; Thibodeaux et al., 2003) but none had reported changes in thyroid histology or significant elevations of TSH.

We designed a series of experiments to determine whether animals were rendered hypothyroid by PFOS treatments in vivo. Our previous studies had shown that even though PFOS can lead to increased tissue uptake and turnover of T4 which would result in a lowering of serum TT4, the thyroid homeostasis can still be maintained as it was driven by FT4 balances. When released by the thyroid gland into serum, thyroid hormones such as T4 are bound to serum carrier proteins for circulation. For T4, approximately 99.97% are bound to carrier proteins while a very small fraction circulates as unbound (free) fraction (approximately 0.03%). Since only FT4 is capable of exerting biological activity, FT4 can be converted from (inactive) protein-bound T4 as needed. Under the conditions in which there is a lowering of serum TT4 due to fast turnover, the large concentration of protein-bound thyroxine is able to act as a reservoir to maintain a constant concentration of FT4.
Furthermore, short-term *in vivo* treatment of K⁺PFOS in rats did not appear to interfere with central H-P-T axis (hypothalamus, pituitary, and thyroid) as the ability of the thyroid to maintain thyroid hormone equilibrium and the ability of pituitary to release TSH were not impaired in response to propylthiouracil (an antithyroid drug) depletion of thyroid hormones *in vivo* in conjunction of a 8-day K⁺PFOS treatment period. In addition, the pituitary cultures harvested after the 8-day K⁺PFOS treatment were still responsive to TRH stimulation (Chang et al., 2008). Because the biological functions of the thyroid and thyroid hormones did not appear to be rendered in rats even when the body burden reached 50 μg/mL or higher, it led us to hypothesize that the attribution of PFOS in serum and hypothyroxinemia observed is a binding phenomenon, and that a PFOS-induced reduction in TT4 (due to higher turnover) could be reversed once the effect of PFOS body burden is attenuated.

The results obtained from this current study concurred with our previously published data (Chang et al., 2008; Chang et al., 2007). Compared to the sex-matched controls, in both male and female rats, repeated oral treatments of K⁺PFOS for 28 consecutive days did not affect the serum TSH values, the primary clinical diagnostic indicator of thyroid function. Compared to time-matched and sex-matched controls, the only statistically significant change observed with regards to serum mean TSH values was on SD2 with K⁺PFOS-treated male rats.
This was likely due to one exceptional high TSH value from an individual rat in the control group. Mean serum TSH values were not statistically significantly different than controls at other time periods in either male or female rats.

As expected, serum TT4 levels from K⁺PFOS-treated rats in both sexes were statistically significantly lower than control throughout the study period (both treatment phase and recovery phase). On SD 29 (24 hours after last oral treatment), K⁺PFOS-treated male and female rats had serum PFOS concentrations of 50.62 ± 3.55 and 75.12 ± 4.10 μg/mL, respectively, and the respective serum PFOS concentrations after 10 weeks of recovery were 18.48 ± 1.41 and 19.86 ± 3.09 μg/mL. As illustrated in Figures 3 and 4, it was apparent that when serum PFOS concentration decreased over time during recovery phase, serum TT4 levels were gradually increasing with an upward trend in both male and female rats.

The serum PFOS concentrations obtained throughout this study suggested a serum elimination half-life greater than 7 days in rats, which was different than that estimated by Johnson and Ober (1979). It is worth noting that although serum elimination of PFOS appeared to be following a linear kinetics overall, the serum concentration pattern over time suggested a more complex model, however. During recovery period, serum PFOS concentrations decreased in a “stair-stepping” pattern which can suggest a possible resorption process modeled by Andersen et al. (2006).
The mean theoretical cumulative amount of PFOS (adjusted for potassium salt and purity) administered to male rats and female rats at the end of treatment phase were 6.82 ± 0.25 and 5.20 ± 0.18 mg, respectively. During the recovery period, both urine and fecal samples were collected for 24 hours immediately prior to the weekly blood draw. Despite the fact that the corresponding serum PFOS concentrations right after urine and feces collection were greater than 15 \( \mu \text{g/mL} \) during all phases of recovery period, less than 1% of the PFOS was found in the respective urine and feces, indicating that PFOS was eliminated slowly in both urine and feces, with urinary elimination appeared to dominate.

Several previous studies have reported data that suggest that peroxisome proliferator activated receptor alpha (PPAR\(\alpha\)) agonists may increase hepatic responses to thyroid hormone in rats by effective competition for thyroid hormone carrier protein binding sites. PPAR\(\alpha\) agonists are typically amphiphilic acids and include endogenous fatty acids, hypolipidemic drugs, acetylsalicylic acid, and perfluorinated acids, such as perfluorooctanoic acid (PFOA) and PFOS (Berthiaume and Wallace, 2002b; Ikeda et al., 1985; Klaunig et al., 2003; Sohlenius et al., 1993). The thyromimetic effects by PPAR\(\alpha\) agonists in rat liver is believed to be due to transcriptional activation of genes regulated by thyroid hormone, either directly or indirectly, such as liver malic enzyme (ME), deiodinase 1 (Dio1), apolipoprotein A1 (ApoA1); P450 oxdireductase (Por),
thyroid hormone response protein (Thrsp), and UGT family (Cai et al., 1996; Ellis et al., 1998; Hertz et al., 1991; Hertz et al., 1993; Hertz et al., 1996; Hylemon et al., 1992; Ness et al., 1990; Pandak et al., 1997). The mRNA transcript levels of these enzymes were evaluated in this study as a secondary measure of concordance of the thyroid hormone responses. The statistical significant increase in Ugt1a1 expression in males (but not females) suggested that livers were responding to the turnover of T4, which supported the hypothesis that increased metabolism of T4 may have occurred. In K+PFOS-treated females (but not males), there was a statistical significant decrease in Dio1 transcript level, the major enzyme for converting bioactive thyroid hormone to its inactive form. Interestingly, Dio1 enzyme is up-regulated when there are excess bioavailable thyroid hormones such as T4 and T3 but can also be down-regulated when there are excess inactive thyroid hormone such as rT3 (St Germain, 1988). In addition, alteration in serum lipid metabolism with K+PFOS treatment, characterized by decreased serum cholesterol and triglycerides, have also been observed in the laboratory animal studies (Luebker et al., 2005b; Seacat et al., 2003; Seacat et al., 2002). Because of limited serum quantity, serum lipid chemistry was not performed in this study. In our study, we did observe a decrease in liver ApoA1 mRNA transcript, the major serum protein component of high-density lipoprotein particles, or HDL, at the end of treatment in both male and female rats. Even though thyroid hormones have been reported to up-regulate ApoA1 gene activity in rodents upon binding with thyroid receptors (TR) and then thyroid response
element (TRE), however, thyroid hormones can also directly bind to TRE without prior dimerization with TR and suppresses ApoA1 transcription (Brent et al., 1991; Hargrove et al., 1999). Besides, since HDL particles are synthesized by both liver and small intestine in the body (Smith et al., 1978), decreased ApoA1 activity observed in this study warrants further investigation before establishing a conclusion.

Hepatomegaly has been associated with PFOS treatments in rodents and monkeys (Lau et al., 2003; Luebker et al., 2005b; Seacat et al., 2003; Seacat et al., 2002; Thibodeaux et al., 2003). In rodents, it could be attributed by the induction of the nuclear hormone receptors such as PPARα, pregnane X receptor (PXR), or constitutive androstane receptor (CAR). Chemical such as Wyeth 14,643, dexamethasone, and phenobarbital are the “model” chemicals used to study activations of PPARα, PXR, and CAR receptors, respectively. In rodents, Wy14,643 induced hepatomegaly that was characterized by peroxisome proliferation and selective induction of Cyp4a1 (Gbaguidi and Agellon, 2004). Dexamethasone produces hepatomegaly as a consequence of extensive periportal fat accumulation and induction of Cyp3a1 (Micuda et al., 2007). In contrast, phenobarbital induces hepatomegaly by simulating cell proliferation and the induction of Cyp2b2 and other cell proliferation markers such as acyl CoA oxidase (ACoA) (Micuda et al., 2007). Compared to the respective controls at the end of the treatment phase, liver Cyp2b2, Cyb3a1, and Cyb4a1 from K⁺PFOS-
treated male rats were higher by approximately 350%, 40%, and 50%, respectively, while liver Cyp2b2, Cyb3a1, and Cyb4a1 expression from K⁺PFOS-treated female rats were higher by approximately 270%, 50%, and 20%, respectively. The increase in Cyp2b2 was with statistical significance in both male and female K⁺PFOS-treated rats. ACoA expressions were comparable between control and K⁺PFOS-treated rats regardless of sex. The profile from these transcripts suggests that the mechanisms of hepatomegaly by PFOS in rodents involve PPAR, PXR, and CAR. In this study, it appears that CAR-mediated metabolic process was more dominant and hepatomegaly was apparent with increased % liver weight relative to body weight in both males and females. There were no other statistically significant differences among other transcripts evaluated.

6.5 CONCLUSION

The decrease in serum TT4 observed during 28 days of dosing correlated with increasing serum PFOS concentrations and resulted in hypothyroxinemia which resolved during recovery as serum PFOS concentrations diminished. We did not observe the development of hypothyroidism in rats after 28 daily doses of K⁺PFOS treatments based on serum TSH concentrations as a diagnostic indicator.
VII.

THYROID HORMONE STATUS IN RATS FROM A DEVELOPMENTAL NEUROTOXICITY STUDY WITH PFOS
The entire content of this chapter has been published:

Chang et al., 2009, “Gestational and lactational exposure to potassium perfluorooctanesulphonate (K+PFOS) in rats: toxicokinetics, thyroid hormone status, and related gene expression”, Reproductive Toxicology, 27, pages 387 – 399.
7.1 INTRODUCTION

Children are known to be exposed to PFOS (Calafat et al., 2007b; Olsen et al., 2004), and these exposures can occur from gestational and lactational transfer of PFOS (Apelberg, 2006; Apelberg et al., 2007a; Inoue et al., 2004; Karrman et al., 2007; Midasch et al., 2007; Monroy et al., 2008; So et al., 2006b; Tao et al., 2008). As reviewed by Olsen et al. (Olsen et al., Submitted 2008), there are several groups of investigators that have studied the association of PFOS concentrations in human maternal and/or umbilical cord blood to birth outcomes, and one recent study followed developmental landmarks in infants through approximately 18 months of age (Fei et al., 2008a).

The developmental toxicity of PFOS has been studied extensively in laboratory rats and mice (Abbott et al., 2008; Butenhoff et al., 2008; Case et al., 2001; Fuentes et al., 2007; Grasty et al., 2005, 2006; Johansson et al., 2008; Lau et al., 2004; Lau et al., 2003; Luebker et al., 2005a; Luebker et al., 2005b; Thibodeaux et al., 2003, 2004), and one developmental study in rabbits has been reported (Case et al., 2001). In the developmental and reproductive studies with rats and mice, it has been demonstrated that neonates can be exposed to PFOS from in utero and lactational exposures (Lau et al., 2003; Luebker et al., 2005a). Effects on gestation length, birth weight, postnatal growth and developmental delays, and neonatal survival have been noted (Abbott et al., 2008; Grasty et al., 2005, 2006;
Neonatal mortality and delayed postnatal growth in rat and mouse pups exposed to PFOS in utero are pronounced effects in these laboratory species that occur at doses not affecting the maternal rodents in an obvious manner. In exploring the potential etiology of these effects in laboratory rats and mice, several investigations have focused on lung development (Grasty et al., 2005, 2006; Luebker et al., 2005a), cholesterol metabolism (Luebker et al., 2005b), the potential role of activation of the nuclear receptor peroxisome proliferator activated receptor α (Abbott et al., 2008), and thyroid hormone status (Butenhoff et al., 2008; Lau et al., 2003; Luebker et al., 2005b; Thibodeaux et al., 2003). At the present time, neonatal mortality resulting from in utero exposure of rats to PFOS is hypothesized to result from alterations in lung function at birth, perhaps
through a direct interaction of PFOS with components of pulmonary surfactant (Grasty et al., 2005; Xie et al., 2007). Alterations in cholesterol metabolism did not appear to relate to altered developmental outcomes (Luebker et al., 2005b). Recent studies suggest that PFOS, unlike the eight-carbon perfluorinated carboxylate, perfluorooctanoate (PFOA), may not act via PPARα activation to produce the majority of observed developmental effects (Abbott et al., 2008; Abbott et al., 2007).

Thyroid hormones have numerous important roles in development (Bernal, 2005a, 2005b; Calvo et al., 1992; Oppenheimer et al., 1995) and are critical for mammalian brain development and maturation in that they control expression of genes involved in myelination, cell differentiation, migration, and signaling (Bernal, 2005a, 2005b). PFOS exposure has been associated with maternal and offspring hypothyroxinemia without a compensatory elevation of TSH in laboratory rats (Lau et al., 2003; Luebker et al., 2005b; Thibodeaux et al., 2003). Although hypothyroxinemia was noted in a dose-dependent manner in pregnant mice on GD 6 by Thibodeaux et al. (Thibodeaux et al., 2003), it was not present on GD 12 or 18 in their study or in pregnant mice on GD 18 in a study reported by Fuentes et al. (Fuentes et al., 2006). Neither was hypothyroxinemia observed in neonatal mice from PFOS-treated dams (Lau et al., 2003). The ability of the pituitary to respond to hypothalamic thyrotropin-releasing hormone to release TSH in response to decreased thyroid hormone production after treatment with
propylthiouracil was not altered in rats by co-treatment with PFOS (Chang et al., 2008). The activity of choline acetyltransferase, an enzyme sensitive to thyroid hormone status, was marginally but statistically-significantly reduced in rat pups from PFOS-treated dams; however, activity in the hippocampus was unaffected (Lau et al., 2003). PFOS-exposed rats appear to maintain a euthyroid state despite significant reductions in serum total thyroid hormones, likely due to competition for binding sites between PFOS and thyroid hormones in rat serum, leading to an adequate supply of free hormone while reducing the concentration of hormone carried on serum binding proteins (Chang et al., 2008; Chang et al., 2007; Larsen et al., 2003).

The PFOS-induced hypothyroxinemia reported in previous developmental studies (Lau et al., 2003; Luebker et al., 2005b; Thibodeaux et al., 2003) led us to evaluate thyroid hormone status and histomorphological factors associated with thyroid follicles during the course of a developmental neurotoxicological study in rats. The maternal, birth outcome, litter, and developmental neurotoxicity endpoints have been reported separately in a companion article to this (Butenhoff et al., 2008). Herein, we report the results of thyroid parameters, in addition to concentrations of PFOS in samples of serum, liver, and brain taken at various times during the study as well as and the results of quantitative evaluation of a select set of liver mRNA transcripts associated with liver hypertrophic modes of action, thyroid hormone and cholesterol metabolism, and liver cell proliferation.
7.2 METHODS

7.2.1 Study Design

The work presented in this article represents the analysis of biological samples collected during the course of a developmental neurotoxicity study involving gestational and lactational exposure to potassium PFOS (K\(^+\)PFOS, CASRN 2795-39-3, Lot number 217 (86.9% pure) 3M Company, St. Paul, Minnesota) in rats. The details of treatment as well as results of birth outcomes, litter parameters, and neurological investigations have been reported in a companion article (Butenhoff et al., 2008).

Briefly, groups of 25 pregnant Sprague Dawley rats (maternal rats) were given daily oral doses of either vehicle control (0.5% Tween 20 in water) or K\(^+\)PFOS (suspended in 0.5% Tween 20 in water due to limited water solubility of K\(^+\)PFOS) at 0.1, 0.3, and 1.0 mg/kg-d from gestation day (GD) 0 (day positive for mating) through postnatal day (PND) 20. An additional 10 pregnant females per treatment group were treated through GD 19 and sacrificed on GD 20 in order to obtain maternal and fetal serum and tissue samples at the end of gestation. Pups were allowed to nurse until PND 21 and evaluated for developmental neurotoxicity landmarks specified per protocol throughout PND 72. Rats were euthanized by carbon dioxide asphyxiation. There was no evidence of treatment-related effect on postnatal growth and survival.
Aliquots of the dosing solutions were analyzed for concentration, stability, and homogeneity. The LC-MS/MS analyses indicated that the dosing solution aliquots were 97 – 100% of the target concentration and all were stable and homogeneous.

All experiments involving live animals were performed in laboratory accredited by Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and all procedures were reviewed and approved by facility’s Institutional Animal Care and Use Committees (IACUC). Animal care and procedures were followed according to the US Department of Health and Human Services guide for the care and the use of laboratory animal guideline (ILAR, 1996).

7.2.2 Determination of PFOS concentrations in Serum, Liver, and Brain
Serum samples for PFOS analysis were obtained from dams on GD 20, PND 4, and PND 21, fetuses (pooled by litter) on GD 20, pups (pooled by litter) on PND 4, and individual male and female offspring on PND 21 and 72. Samples of livers for PFOS analysis were obtained from dams on GD 20, fetuses and pups (pooled by litter) on GD 20 and PND 4, respectively, and individual offspring on PND 21 and 72. Samples of brains for PFOS analysis were obtained from dams on GD 20, fetuses and pups (pooled by litter) on GD 20 and PND 4, respectively, and individual offspring on
PND 21. Samples of serum, liver, and brain were snap frozen and remained frozen at approximately -80º C until processing for analysis.

New Zealand newborn calf serum (Invitrogen, Carlsbad, CA) and liver and brain homogenates obtained from naïve Sprague Dawley rats (male and female, 10-12 weeks old) were used as the blank matrices to prepare the appropriate matrix-matched PFOS standard curves.

To analyze for PFOS concentrations, serum and liver samples were obtained from GD 20 maternal rats and fetuses, PND 4 maternal rats (serum only) and pups, PND 21 maternal rats (serum only) and male and female pups. Due to limited sample volume and size, serum and liver samples from GD 20 fetus and PND 4 pups were pooled by litter. PFOS concentrations were determined by LC-MS/MS as described in Chang et al. (Chang et al., 2007). Briefly, liver samples were homogenized in water (1 part liver and 4 parts water, w/w) with IKA® WERKE Ultra-Turrax T25 homogenizer at 20,000 rpm for ~ 1.5 min followed by sonication in a water bath sonicator (30 min). After adding $^{18}$O$_2$-PFOS internal standard, 100 μL serum samples and/or liver homogenate aliquots were further treated with 1 mL of 1.0N formic acid, 100 μL of saturated ammonium sulfate (serum samples only), and 300 μL of water followed by solid phase extraction (SPE).
For the determination of PFOS concentrations in brain collected on GD 20 (maternal rats and their pooled fetuses), PND 4 (pups, pooled by litter), and PND 21 (male and female pups), approximately 0.2 g of brain was weighed and homogenized with deionized water in a clean polypropylene tube. The ratio between brain and water was 1:4 (w/w). After homogenization, 100 μL of the whole homogenate was further digested with 100 μL 1.0 N KOH. The mixture was vortexed for 2 h at room temperature, followed by the addition of 1 mL 1.0 N formic acid, 400 μL of saturated ammonium sulfate, and 5 mL of acetonitrile. The solution was mixed using a mechanical shaker for 30 min at room temperature followed by centrifugation (2,500 x g, 5 min). The organic layer was transferred to a clean polypropylene tube and evaporated. One mL of 1.0 N formic acid and 100 μL saturated ammonium sulfate were added to the remaining aqueous solution followed by vortex and SPE.

SPE extractions were based on 100 μL of sample matrix and utilized Waters Oasis® hydrophilic-lipophilic balance (HLB) 3 mL columns with column conditioning, column loading, column wash, and column elution performed as described in Ehresman et al. (Ehresman et al., 2007). The instruments used for analysis of PFOS were API 4000 mass spectrometer (for serum and liver samples) and API 5000 mass spectrometer (for brain
samples). Both instruments were from Applied Biosystems / MDS-Sciex Instrument Corporation and were configured with Turbo Ion Spray (pneumatically assisted electrospray ionization source) in negative-ion mode. A Mac-Mod ACE® C-18, 5 µm, 75 x 2.1 mm i.d. HPLC column with a flow rate of 0.25 mL/min was used for PFOS analysis. The gradient condition for the mobile phase started with 30% acetonitrile and 70% 2 mM ammonium acetate and ramped to 90% acetonitrile and 10% 2 mM ammonium acetate in 5.5 minutes. All source parameters were optimized under these conditions according to manufacturer’s guidelines. Mass spectroscopy transition ions (in atomic mass units, or amu) monitored were 499 → 80 for PFOS and 503 → 84 for the internal standard, 18O2-labeled PFOS.

7.2.3 Serum TSH Measurements

Serum samples collected on GD 20 (maternal rats and fetuses (pooled by litter), PND 4 (maternal rats and pups (pooled by litter)), and PND 21 (maternal rats and male and female pups) were analyzed for TSH levels using the Biotrak™ rat thyroid stimulating hormone 125I assay system (Amersham Pharmacia Biotech, Piscataway, NJ). The assay is based on the competition between unlabelled TSH (present in the serum samples) and a fixed quantity of 125I-TSH for a limited number of TSH antibody binding sites. With fixed amounts of antibody and radioactive ligand, the
amount of radioactive ligand bound by the antibody, measured by gamma counter, was inversely proportional to the concentration of TSH present in the serum.

7.2.4 Thyroid Histology & Morphometry

On PND 4 and PND 21, thyroids from 1 pup/sex/litter from 10 randomly selected litters in each treatment group were collected and preserved in 10% neutral-buffered formalin. The thyroids were then embedded in paraffin, processed via standard histological procedures, sectioned, and stained with hematoxylin and eosin (H&E). Offspring thyroids obtained from the control and highest maternal dose (1.0 mg/kg-d) group were evaluated microscopically first. Because there were no significant toxicological findings in the offspring thyroids obtained from the highest maternal dose group compared to controls, further evaluation of the thyroids from the lower maternal dose groups was not performed. The histopathological examination of H&E-stained thyroid sections also included a simple morphometric analysis, consisting of measurement of thyroid follicular epithelial cell height and dimensions of colloid contained within thyroid follicles.

For thyroid follicular epithelial cell height measurements, digital images of thyroid glands were captured using an Olympus BX-51 microscope.
equipped with an Olympus DP-70 digital camera. Linear measurements of epithelial cell height were made using Image Pro® Plus version 5.1 software (Media Cybernetics, Silver Spring, MD). For each thyroid slide, 5 follicles were chosen for the follicular epithelial cell height measurement; and in each follicle, 3 cells (located approximately at 12, 4, and 8 o’clock positions) were measured for the cell heights. Measurements extended from the deep aspect of nuclear membranes up to the luminal surface of selected follicular epithelial cells. The mean and standard deviation were calculated for all 15 measurements on each thyroid slide, and the group mean and standard deviation was calculated for each treatment group.

For thyroid follicular area measurements, digital images for each thyroid slide were captured as described above and Image Pro® Plus software was used for measurements. Long and short axis measurements were performed on the colloid mass of the 10 largest follicles that appeared in each digital image. The mean and standard error were calculated for all 10 measurements on each thyroid slide, and the group mean and standard error was calculated for each treatment group.

7.2.5 Thyroid Proliferation Assay

Ki-67 immunohistochemical staining was performed on thyroid glands obtained from GD 20 fetuses for the evaluation of any PFOS-related
alterations in cellular proliferation. GD 20 control male and female fetal thyroids (n = 6 and 7, respectively) and 1.0 mg/kg-d dose group male and female fetal thyroids (n = 6 and 5, respectively) were preserved in 10% neutral-buffered formalin and embedded in paraffin followed by sectioning. The sections were then evaluated for cellular proliferation via Ki-67 immunohistochemical staining using monoclonal mouse anti-rat Ki-67 antigen (DAKO Corporation, Carpinteria, CA) and a standard avidin-biotin (ABC) staining procedure. All procedures were performed according to the manufacturer’s guidelines.

7.2.6 Quantitative RT-PCR
To evaluate whether PFOS treatment elicited any changes in hepatic gene expression, liver samples from dams and fetuses (pooled by litter) on GD 20 and male pups on PND 21 were analyzed by quantitative real-time PCR for selected mRNA transcripts. Genes associated with various nuclear receptors, thyroid hormones, and hepatic conjugation enzymes were examined (Table 1). The samples that underwent RT-PCR transcript profiling on selective genes were livers (preserved in RNALater®, Applied Biosystems) from control and highest-dose group (1.0 mg/kg-d) obtained on GD 20 (maternal rats and fetuses) and PND 21 (male pups only).
To isolate mRNA, approximately 25 mg liver tissue was homogenized in 600 μL of lysis buffer using a ground glass on glass Duall® tissue grinder. Samples were further homogenized by passing 5 times through a 27 gauge needle using a sterile 1 mL syringe. The liver homogenate was loaded onto Qiagen RNeasy® spin columns followed by DNAse digestion according to the manufactures protocol using Qiagen RNAse free DNAse set. The RNA was eluted in 35 μL of RNase free water and quantified by measuring the absorbance at 260 nm using the Nanodrop™ ND1000 spectrophotometer.

**RT-PCR Instrumentation:** mRNA sequences for target genes were obtained from the GenBank database (http://www.ncbi.nlm.nih.gov) and gene specific primers were designed using IDT PrimerQuestSM (www.idtdna.com). DNA standards for RT-PCR quantitation were made by PCR of a reverse transcription reaction using random primers, specific primers, or oligo dT primers with Qiagen’s HotStarTaq Master Mix (Qiagen, Valencia, CA). After verification by gel electrophoresis, the standards were further purified using Qiagen’s QIAquick™ chromatography columns. The standards were quantitated by measuring absorbance at 260 nm. The identities and the corresponding primer sequences used in this study are listed Table 1. Reverse transcription with the Qiagen’s Omniscript RT kit was performed using random nonamer primers and 1.5 μg of total RNA according to the manufacturer’s protocol. Quantitative PCR was performed with the LightCycler® using the Sybr® Green Master
Mix Kit (Roche, Indianapolis, IN). PCR was carried out on samples, along with a 10-fold serial dilution of a target specific DNA standard, for selected transcripts using target sequence specific primers. Target specific mRNA expression was normalized against 18s rRNA, and the results were reported as transcript specific mRNA copies per 18s rRNA copies.
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<td>GCC TTC CCA GTG TTA GTG ATT</td>
<td>CTT GGT TTG GGA ACA CAA TAG</td>
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<td>U20551</td>
</tr>
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<td>Ugt1a1</td>
<td>AGG AAG TAC CCT GTG CCA TCC CAA</td>
<td>TCT GGA TCA AAG ACA CTC CGC CCA</td>
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<td>AAC TGG CAG CAA AGT GGT TGT T</td>
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<td>Ugt2b</td>
<td>GTG CAC TGG AGG AAG TCA TAG ACA</td>
<td>TAG GCT GGT CAT GGT GAA TCC TTG</td>
<td>81</td>
<td>XM_001062335</td>
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7.2.7 Statistical Analysis

Following ANOVA, Dunnett’s tests were used to compare data resulting from PFOS treatment to control values using SAS or JMP® 5.1 - Windows - Release 5.1.2 (SAS Institute, Inc., Cary, NC). All data are expressed as means ± standard error (SE). Individual means were calculated for all endpoints with the exception of GD 20 and PND 4 fetal serum, liver, and brains samples, which were pooled by litter and are presented as litter means.

7.3 RESULTS

7.3.1 PFOS concentrations in Serum, Liver, and Brain

Presented in Table 2 are mean PFOS concentrations (± standard error, SE) for serum, liver, and brains. The mean percent ratio of liver and brain PFOS concentrations to serum PFOS concentration for each dose group are presented in Table 3.

From GD 20 through PND 21, the PFOS concentrations in maternal rat serum, liver, and brain correlated well with the daily K⁺PFOS doses given to the maternal rats. While the maternal liver-to-serum PFOS concentration ratios ranged from 1.8 to 4.9, the corresponding maternal brain-to-serum PFOS concentration ratios ranged from 0.04 to 0.09.
Table 2: Mean PFOS Concentrations (± standard error) in serum, liver, and brain in dams (Dam), fetus (Fetus), pups (Pup, male = M, female = F). None of the offspring directly received K⁺PFOS doses.

<table>
<thead>
<tr>
<th>Time</th>
<th>Dose Group</th>
<th>Serum [PFOS], µg/mL</th>
<th>Liver&lt;sup&gt;a&lt;/sup&gt; [PFOS], µg/g</th>
<th>Brain&lt;sup&gt;a&lt;/sup&gt; [PFOS], µg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Dam</td>
<td>Fetus&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Dam</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GD 20</td>
<td>Control&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&lt; LLOQ&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.009 ± 0.001</td>
<td>&lt; LLOQ&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>0.1 mg/kg-d&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.722 ± 0.068</td>
<td>3.906 ± 0.096</td>
<td>8.349 ± 0.344</td>
</tr>
<tr>
<td></td>
<td>0.3 mg/kg-d&lt;sup&gt;d&lt;/sup&gt;</td>
<td>6.245 ± 0.901</td>
<td>10.446 ± 0.291</td>
<td>21.725 ± 0.721</td>
</tr>
<tr>
<td></td>
<td>1.0 mg/kg-d&lt;sup&gt;d&lt;/sup&gt;</td>
<td>26.630 ± 3.943</td>
<td>31.463 ± 1.032</td>
<td>48.875 ± 72.733</td>
</tr>
<tr>
<td>PND 4</td>
<td>Control</td>
<td>0.008 ± 0.000</td>
<td>&lt; LLOQ</td>
<td>NS&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
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<td>0.1 mg/kg-d</td>
<td>3.307 ± 0.080</td>
<td>2.236 ± 0.070</td>
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<tr>
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<td>0.3 mg/kg-d</td>
<td>10.449 ± 0.234</td>
<td>6.960 ± 0.163</td>
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</tr>
<tr>
<td></td>
<td>1.0 mg/kg-d</td>
<td>34.320 ± 31.154</td>
<td>22.440 ± 0.723</td>
<td>NS</td>
</tr>
<tr>
<td>Time</td>
<td>Dose Group</td>
<td>Serum [PFOS], µg/mL</td>
<td>Liver&lt;sup&gt;a&lt;/sup&gt; [PFOS], µg/g</td>
<td>Brain&lt;sup&gt;a&lt;/sup&gt; [PFOS], µg/g</td>
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<tr>
<td>-------</td>
<td>------------</td>
<td>---------------------</td>
<td>-------------------------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dam M Pup F Pup</td>
<td>Dam M Pup F Pup</td>
<td>Dam M Pup F Pup</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>0.007 ± 0.000 &lt;LLOQ</td>
<td>&lt;LLOQ NS &lt;LLOQ &lt;LLOQ</td>
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<tr>
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<td>0.1 mg/kg-d</td>
<td>3.159 ± 0.081 1.729 ± 0.079 1.771 ± 0.076 NS 5.980 ± 0.614 5.278 ± 0.174 NS 0.220 ± 0.014 0.229 ± 0.011</td>
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<td></td>
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<tr>
<td></td>
<td>0.3 mg/kg-d</td>
<td>8.981 ± 0.275 5.048 ± 0.108 5.246 ± 0.138 NS 14.780 ± 0.832 13.550 ± 0.298 NS 0.649 ± 0.053 0.735 ± 0.039</td>
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<tr>
<td></td>
<td>1.0 mg/kg-d</td>
<td>30.480 ± 1.294 18.611 ± 1.011 18.010 ± 0.744 NS 44.890 ± 2.637 41.230 ± 2.295 NS 2.619 ± 0.165 2.700 ± 0.187</td>
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<td></td>
</tr>
<tr>
<td>PND 72</td>
<td>Control</td>
<td>NA&lt;sup&gt;b&lt;/sup&gt; &lt; LLOQ &lt; LLOQ</td>
<td>NA &lt; LLOQ &lt; LLOQ</td>
<td>NA NS NS</td>
</tr>
<tr>
<td></td>
<td>0.1 mg/kg-d</td>
<td>NA 0.042 ± 0.004 0.207 ± 0.042 NA 0.981 ± 0.091 0.801 ± 0.082 NA NS NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.3 mg/kg-d</td>
<td>NA 0.120 ± 0.009 0.556 ± 0.062 NA 2.464 ± 0.073 2.252 ± 0.095 NA NS NS</td>
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</tr>
<tr>
<td></td>
<td>1.0 mg/kg-d</td>
<td>NA 0.560 ± 0.105 1.993 ± 0.293 NA 7.170 ± 0.382 7.204 ± 0.414 NA NS NS</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Non-perfused samples

<sup>b</sup> Samples pooled by litters

<sup>c</sup> Represents dams receiving vehicle control solution, 0.5% Tween 20 in water

<sup>d</sup> Lower limit of quantitation (LLOQ) for serum is 0.010 µg/mL

<sup>e</sup> Lower limit of quantitation (LLOQ) for liver is 0.050 µg/g

<sup>f</sup> Lower limit of quantitation (LLOQ) for brain is 0.025 µg/g
$g$ Represents dams receiving 0.1 mg of potassium PFOS per kg body weight per day
$h$ Represents dams receiving 0.3 mg of potassium PFOS per kg body weight per day
$i$ Represents dams receiving 1.0 mg of potassium PFOS per kg body weight per day
$j$ NS = no sample collected
$k$ NA = not applicable; all dams were sacrificed on PND 21
Table 3: Mean liver [PFOS]-to-serum [PFOS] and brain [PFOS]-to-serum [PFOS] ratios in dams (Dam), fetuses (Fetus, pooled by litter), pups (Pup, male = M, female = F). None of the offspring directly received K\textsuperscript+PFOS doses.

<table>
<thead>
<tr>
<th>Time</th>
<th>Dose Group</th>
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<th>[PFOS] brain[PFOS] serum</th>
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<td>Dam</td>
<td>Fetus\textsuperscript{a}</td>
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<tr>
<td>GD 20</td>
<td>Control\textsuperscript{b}</td>
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<td>NA</td>
</tr>
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<td>0.1 mg/kg-d\textsuperscript{d}</td>
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<td>0.3 mg/kg-d\textsuperscript{e}</td>
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<td>1.0 mg/kg-d\textsuperscript{f}</td>
<td>1.84</td>
<td>0.64</td>
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<table>
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<tr>
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<th>Pup\textsuperscript{a}</th>
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<table>
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<th>[PFOS] brain[PFOS] serum</th>
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</tr>
<tr>
<td></td>
<td>Dam</td>
<td>M Pup</td>
<td>F Pup</td>
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<td><strong>PND 72</strong></td>
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</tr>
<tr>
<td>Control</td>
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<td>NA</td>
</tr>
<tr>
<td>0.1 mg/kg-d</td>
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<td>3.88</td>
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<td>20.53</td>
<td>4.05</td>
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<tr>
<td>1.0 mg/kg-d</td>
<td>--</td>
<td>12.79</td>
<td>3.61</td>
</tr>
</tbody>
</table>

- **a** Samples pooled by litters
- **b** Represents dams receiving vehicle control solution, 0.5% Tween 20 in water
- **c** NA = not applicable; ratio cannot be calculated as PFOS concentrations in serum and liver were below Lower limit of quantitation (LLOQ)
- **d** Represents dams receiving 0.1 mg of potassium PFOS per kg body weight per day
- **e** Represents dams receiving 0.3 mg of potassium PFOS per kg body weight per day
- **f** Represents dams rats receiving 1.0 mg of potassium PFOS per kg body weight per day
- **g** “--” means no sample collected hence no ratio can be calculated
From GD 20 to PND 72, the PFOS concentrations in fetal and pup serum, liver, and brain correlated well with the daily litter-matched maternal K⁺PFOS doses. Even though none of the rat fetuses and pups were dosed directly with K⁺PFOS, *in utero* exposure to PFOS was evident in fetal litters from K⁺PFOS-treated dams. Because cross-fostering of neonates from control dams with K⁺PFOS-treated dams was not incorporated into our design, it is not possible to comment quantitatively on the extent to which lactation contributed to exposure after birth in this study. Mean fetal serum PFOS concentrations were higher than those of dams on GD 20. In both maternal rats and their offspring, liver PFOS concentrations were higher than the respective serum PFOS concentrations at all times, and brain PFOS concentrations were always lower than time-matched serum concentrations.

There did not appear to be a sex difference in serum, liver, or brain PFOS concentrations between male and female offspring through PND 21. On PND 72, liver PFOS concentrations remained comparable between male and female offspring by maternal K⁺PFOS-treatment group; however, female offspring had higher serum PFOS concentrations than respective males.

7.3.2 Serum TSH Measurements
Mean maternal serum TSH concentration data are presented in Figure 1, and fetal and pup TSH concentration data are presented in Figure 2. Mean serum TSH values of maternal rats and offspring from K⁺PFOS-treated groups were not significantly different from controls on GD 20, PND4, and PND 21.

![Figure 1](image_url)

Figure 1: Mean serum TSH concentrations in dams obtained on GD 20, PND 4, and PND 21. Dams were treated with either vehicle control (0.5% Tween® 20, dotted bars), 0.1 mg/kg-d K⁺PFOS (left-striped bars), 0.3 mg/kg-d K⁺PFOS (checkered bars), or 1.0 mg/kg-d K⁺PFOS (solid bars). TSH was measured via Biotrak™ rat thyroid stimulating hormone assay system (Amersham Pharmacia Biotech, Piscataway, NJ). Each bar represents the mean serum TSH determinations and error bars represent standard errors. There were no differences in maternal serum TSH levels between PFOS-treatment groups and the control.
Figure 2: Mean serum TSH concentrations in fetal rats obtained on GD 20 (pooled by litters), PND 4 (pooled by litters), and PND 21. Rats were the offspring from dams treated with either vehicle control (0.5% Tween® 20, dotted bars), 0.1 mg/kg·d K⁺PFOS (left-striped bars), 0.3 mg/kg·d K⁺PFOS (checkered bars), or 1.0 mg/kg·d K⁺PFOS (solid bars). None of the offspring received K⁺PFOS treatments directly. TSH was measured via Biotrak™ rat thyroid stimulating hormone ¹²⁵I assay system (Amersham Pharmacia Biotech, Piscataway, NJ). Each bar represents the mean serum TSH determinations and error bars represent standard errors. There were no differences in offspring serum TSH levels between PFOS-treatment groups and the control.
7.3.3 Thyroid Histology and Morphometry

Thyroid histology findings in the offspring are summarized in Table 4. Only control and 1.0 mg/kg-d maternal dose group fetal and pup thyroids obtained from GD 20, PND 4, and PND 21 were examined. Subjective histopathological evaluation revealed no PFOS treatment-related histological changes, including the number of follicles present and the distribution of follicle sizes, in the H&E-stained thyroid sections obtained from GD 20 fetuses, PND 4 pups, and PND 21 pups in the 1.0 mg/kg-d maternal dose-group when compared to controls.

Mean thyroid follicular epithelial cell heights and thyroid follicular colloid area morphometric data for pups are presented in Figure 3 and Figure 4. There were no K⁺PFOS-treatment related alterations in thyroid follicular colloid area on PND 4 and PND 21. Follicular epithelial cell height was similar in all groups on PND 4. On PND 21, mean thyroid follicular epithelial cell height from the 1.0 mg/kg-d maternal dose group male pups was significantly (p<0.01) higher than that of the control group. This difference was suspected to be spurious due to the extraordinarily low value in the male control group compared to the female control group on PND 21.
Table 4: Thyroid histology in fetuses and pups from control and 1.0 mg/kg-d K⁺PFOS maternal dose groups only. None of the pups directly received K⁺PFOS doses.

<table>
<thead>
<tr>
<th></th>
<th>Control&lt;sup&gt;a&lt;/sup&gt;</th>
<th>0.1 mg/kg-d&lt;sup&gt;b&lt;/sup&gt;</th>
<th>0.3 mg/kg-d&lt;sup&gt;c&lt;/sup&gt;</th>
<th>1.0 mg/kg-d&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
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<tr>
<td><strong>GD 20</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total # of thyroid examined</td>
<td>10</td>
<td>0&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0&lt;sup&gt;e&lt;/sup&gt;</td>
<td>6</td>
</tr>
<tr>
<td>Decreased colloid</td>
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<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td>Severe</td>
<td>5</td>
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<td>Minimal</td>
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<td>--</td>
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<tr>
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<sup>a</sup> Values are not available for control group.
<sup>b</sup> Values are not available for 0.1 mg/kg-d group.
<sup>c</sup> Values are not available for 0.3 mg/kg-d group.
<sup>d</sup> Values are not available for 1.0 mg/kg-d group.
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### Females

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<th>0.3 mg/kg-d</th>
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### PND 4

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<tr>
<td>Decreased colloid</td>
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<td>Minimal</td>
<td>2</td>
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<td>Mitotic figures</td>
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<td>Minimal</td>
<td>7</td>
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<td>3</td>
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<td>Mild</td>
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### PND 21

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<tbody>
<tr>
<td>Total # of thyroid examined</td>
<td>10</td>
<td>--</td>
<td>--</td>
<td>10</td>
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<tr>
<td>Cellular luminal debris</td>
<td></td>
<td></td>
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<tr>
<td>Minimal</td>
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<td>4</td>
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</tbody>
</table>

210
| Mitotic figures | Minimal | 5 | -- | -- | 5 |

\(a\) Represents dams receiving vehicle control solution, 0.5% Tween 20 in water.  
\(b\) Represents dams receiving 0.1 mg of potassium PFOS per kg body weight per day  
\(c\) Represents dams receiving 0.3 mg of potassium PFOS per kg body weight per day  
\(d\) Represents dams receiving 1.0 mg of potassium PFOS per kg body weight per day  
\(e\) The thyroids were collected and processed, but not evaluated microscopically.  
\(f\) "--" means no histology evaluation available.
Figure 3: Mean thyroid follicular epithelial cell heights in offspring on PND 4 and PND 21. Only control and 1.0 mg/kg-d K⁺PFOS dose groups were evaluated. Control group is represented with dotted bars (■) while 1.0 mg/kg-d K⁺PFOS group is represented with solid bars (▲). Each bar represents the mean and error bars represent standard errors. Asterisk (*) denotes significant difference from control (p < 0.05). Follicular epithelial cell height was similar in all groups on PND 4. On PND 21, mean thyroid follicular epithelial cell height from the 1.0 mg/kg-d maternal dose group male pups was significantly higher than that of the control group. This difference was suspected to be spurious due to the extraordinarily low value in the concurrent control group compared to other groups on PND 21 in this study.
Figure 4: Mean thyroid follicular colloid area in offspring on PND 4 and PND 21. Only control and 1.0 mg/kg-d K⁺PFOS dose groups were evaluated. Control group is represented with dotted bars ( ) while 1.0 mg/kg-d K⁺PFOS group is represented with solid bars ( ). Each bar represents the mean and error bars represent standard errors. There were no K⁺PFOS-treatment related alterations in thyroid follicular colloid area on PND 4 and PND 21.
7.3.4 Thyroid Proliferation Assay

Ki-67 proliferation assay data for GD 20 fetal thyroids are presented in Table 5. The mean number (± SE) of Ki-67-positive thyroid follicular epithelial cells in female fetal thyroids from the 1.0 mg/kg-d dose group was 2.1-fold higher than the control group and was statistically significant. The range of values in the female controls was quite wide (4 – 113, N = 7) compared to the range in female fetal thyroids from the 1.0 mg/kg-d dose group (64 – 116, N = 5).
Table 5: Individual and mean (± standard error) thyroid follicular epithelial cell counts based on positive staining for Ki-67 as evidence of cell proliferation on GD 20 male and female fetal rats.

<table>
<thead>
<tr>
<th></th>
<th>Males</th>
<th>Females</th>
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<tr>
<td></td>
<td>Control(^a) 1 mg/kg-d(^b)</td>
<td>Control 1.0 mg/kg-d</td>
</tr>
<tr>
<td>Individual Counts</td>
<td>18 10</td>
<td>4 64</td>
</tr>
<tr>
<td></td>
<td>22 14</td>
<td>22 83</td>
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<tr>
<td></td>
<td>25 24</td>
<td>23 88</td>
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<td></td>
<td>36 29</td>
<td>34 89</td>
</tr>
<tr>
<td></td>
<td>70 36</td>
<td>39 116</td>
</tr>
<tr>
<td></td>
<td>70 80</td>
<td>57 113</td>
</tr>
<tr>
<td>Number of thyroids</td>
<td>6 6</td>
<td>7 5</td>
</tr>
<tr>
<td>Mean Ki-67-positive Cells</td>
<td>40 ± 10</td>
<td>42 ± 13</td>
</tr>
<tr>
<td></td>
<td>32 ± 10</td>
<td>88 ± 8*</td>
</tr>
</tbody>
</table>

\(^a\) Fetal rats from dams receiving vehicle control solution (0.5% Tween 20 in water).

\(^b\) Fetal rats from dams receiving 1 mg of potassium PFOS per kg body weight per day

* Values are statistically significant from control (p < 0.05).
7.3.5 Quantitative RT-PCR

The mRNA transcript data for the control and 1.0 mg/kg-d dose groups are summarized as mean ± SE in Table 6 for the GD 20 dams, GD 20 fetuses, and PND 21 male pups. Mean Cyp2b2 levels for dams and their male pups in the 1.0 mg/kg-d maternal dose-group were higher than the control group values on GD 20 and PND 21. Additionally, on PND 21, mean ACoA and Cyp4a1 levels for male pups in the 1.0 mg/kg-d maternal dose group were higher than the control group, while the mean Cyp7a1 level was lower than the control group. There were no other statistically significant differences among other transcripts evaluated.
Table 6: Mean mRNA transcript levels (expressed as mean number of mRNA copies per 18s rRNA ± standard error from n = 10 liver samples per group) in dams and fetus obtained on GD 20, as well as male pups on PND 21 from control and 1.0 mg/kg-d K⁺PFOS maternal dose groups. Dam liver samples were not collected on PND 21. None of the offspring directly received K⁺PFOS doses.

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Dose Group</th>
<th>GD 20</th>
<th>PND 21</th>
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<tr>
<td></td>
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<td>Dam</td>
<td>Fetus</td>
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<tr>
<td>ACoA</td>
<td>Controla</td>
<td>5.55E-05 ± 1.08E-05</td>
<td>1.51E-05 ± 1.53E-06</td>
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<td></td>
<td>1.0 mg/kg-db</td>
<td>6.41E-05 ± 5.57E-06</td>
<td>1.65E-05 ± 5.10E-06*</td>
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<tr>
<td>PCNA</td>
<td>Control</td>
<td>2.00E-06 ± 1.37E-07</td>
<td>4.25E-05 ± 4.12E-06</td>
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<tr>
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<td>1.0 mg/kg-d</td>
<td>1.97E-06 ± 1.12E-07</td>
<td>3.80E-05 ± 3.96E-06</td>
</tr>
<tr>
<td>ME</td>
<td>Control</td>
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<td>6.68E-07 ± 5.59E-08</td>
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<td></td>
<td>1.0 mg/kg-d</td>
<td>5.22E-06 ± 9.02E-07</td>
<td>6.71E-07 ± 1.28E-07</td>
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<td></td>
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<td>Diol 1</td>
<td>Por</td>
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<td></td>
<td></td>
<td>Control</td>
<td>Control</td>
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<tr>
<td></td>
<td></td>
<td>4.18E-06 ± 1.35E-06</td>
<td>3.32E-06 ± 6.08E-07</td>
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<td>0.00E00 ±00</td>
<td>2.85E-06 ± 2.88E-07</td>
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<td>8.52E-06 ± 7.31E-07</td>
<td>1.09E-05 ± 1.28E-06</td>
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<td></td>
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<td>Por</td>
<td>ApoA1</td>
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<tr>
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<td>1.0 mg/kg-d</td>
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<td>3.47E-06 ± 4.39E-07</td>
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<td>0.00E00 ±00</td>
<td>2.62E-06 ± 1.87E-07</td>
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<td>8.62E-06 ± 6.60E-07</td>
<td>1.49E-05 ± 2.90E-06</td>
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<td>Por</td>
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<td>3.89E-06 ± 7.95E-07</td>
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<td>8.62E-06 ± 6.60E-07</td>
<td>1.49E-05 ± 2.90E-06</td>
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<tr>
<td>Gene</td>
<td>Condition</td>
<td>Cyp7a1 Control</td>
<td>Cyp7a1 1.0 mg/kg-d</td>
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<td>5.82E-06 ± 1.02E-06</td>
<td>4.60E-06 ± 9.97E-07</td>
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<td>5.88E-06 ± 1.67E-06</td>
<td>4.45E-06 ± 1.22E-06</td>
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<td>9.55E-07 ± 8.54E-08</td>
<td>4.41E-06 ± 8.52E-07</td>
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<td>2.37E-01 ± 3.31E-02</td>
<td>1.55E-01 ± 4.43E-02</td>
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<td>2.97E-06 ± 9.69E-07</td>
<td>6.47E-05 ± 7.34E-06</td>
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<td></td>
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<td>1.40E-04 ± 2.46E-05</td>
<td>2.20E-05 ± 2.43E-06</td>
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</table>
\(a\) Represents dams receiving vehicle control solution, 0.5\% Tween 20 in water

\(b\) Represents dams receiving 1.0 mg of potassium PFOS per kg body weight per day

\(c\) Represents values that were too low for quantitation

\(\ast\) Values are statistically significant from control (p < 0.05).
7.4 DISCUSSION

Because of the evidence for exposure of children to PFOS beginning as early as in utero, there has been an extensive research effort in evaluating potential developmental effects in laboratory animals and humans (Apelberg et al., 2007a; Apelberg et al., 2007b; Fei et al., 2008a; Fei et al., 2007, 2008b; Lau et al., 2004; Lau et al., 2003; Luebker et al., 2005a). However, there have been limited data available on the potential association of PFOS exposure with developmental neurotoxicity. In a companion paper to this, Butenhoff et al. (Butenhoff et al., 2008) reported the results of a developmental neurotoxicity study with PFOS per current test guidelines. Maternal rats were given K⁺PFOS by oral gavage through gestation and lactation, and pups were evaluated for various indices of neurological development through PND 72. Outcomes of the companion study included slight but statistically-significant effects on maternal food consumption and weight gain in the highest maternal dose group (1.0 mg/kg-d) and a transient decrease in habituation in male offspring from the same maternal dose group on PND 17. During the conduct of that study, samples were obtained on GD 20, PND 4, PND 21, and PND 72 in order to study: 1) the concentrations of PFOS in serum, liver, and brain during the study; 2) thyroid hormone status and thyroid tissue histology; and 3) quantitative expression of a selected liver mRNA transcripts in dams and their fetuses on GD 20 and male pups on PND 21. The results of analyses of those samples have been the focus of this article.
Human biomonitoring studies have shown that neonates can be exposed to PFOS effectively through *in utero* exposure. Inoue et al. (Inoue *et al.*, 2004) reported that PFOS was detected in all of 15 paired human maternal serum and neonatal cord blood serum samples from Japan obtained in 2003, with neonatal and maternal serum PFOS ranging from 1.6 – 5.3 ng/mL and 4.7 – 17.6 ng/mL, respectively. Midasch et al. (Midasch *et al.*, 2007) reported detectable PFOS among 11 paired maternal and cord blood plasma samples obtained in 2003 in Germany, with maternal plasma PFOS median concentration of 13.0 and 7.3 ng/mL in cord blood. Monroy et al. (Monroy *et al.*, 2008) evaluated PFOS concentrations in 101 maternal samples paired with 105 cord blood at birth (includes 4 sets of twins), with mean PFOS concentrations of 16.2 ng/mL and 7.3 ng/mL, respectively. Apelberg et al. (Apelberg *et al.*, 2007b) found that PFOS concentrations in 293 cord blood sera samples obtained in Baltimore, Maryland in 2004 - 2005 had a geometric mean of 4.9 ng/mL, indicating that children are exposed to PFOS beginning *in utero* from placental transfer.

Lactational exposure to PFOS in humans is also possible. Tao et al. (Tao *et al.*, 2008) reported a mean milk PFOS concentration of 0.131 ng/mL among the 45 samples obtained from nursing mothers in Massachusetts in 2004. So et al. (So *et al.*, 2006b) also examined 19 human milk samples in China and reported a mean PFOS concentration of 0.121 ng/mL. Kärrman et al. (Karrman *et al.*, 2007) found that mean PFOS concentration in milk is 0.201 ng/mL and that was about 1% of
the respective maternal serum PFOS concentration (based on 12 samples obtained in Sweden). All together, the comparative data between laboratory studies and human biomonitoring reports should be taken into consideration carefully when performing risk assessment, as the difference in body burden also delineates the outcome.

Concentrations of PFOS measured in samples of serum, liver, and brain from rat dams, fetuses, and pups across maternal K⁺PFOS treatment levels were generally in proportion to the relative differences between maternal dose levels. Thus, internal dose was in proportion to administered maternal dose.

Based on paired maternal and fetal samples obtained on GD 20, significant placental transfer of PFOS from rat dams to their developing fetuses occurred. Fetal serum PFOS concentrations were 1.2 to 2.3 times greater than maternal serum PFOS on GD 20, and fetal brain PFOS concentrations were approximately 10 times maternal brain PFOS. The latter observation likely reflects the fact that the "blood-brain barrier" in GD 20 fetal rats has not been established. However, maternal liver PFOS concentrations were 2.4 to 3.7 times greater than those of GD 20 fetuses.

Compared to GD 20 fetal serum PFOS concentrations, pup serum PFOS concentrations on PND 4 were lesser by about one-third to one-half. On PND 21,
pup serum PFOS concentrations had decreased slightly from PND 4 levels, even though pups had gained approximately eight times their PND 4 weight. When body weight and corresponding volume expansion increases are considered, this observation suggests a significant contribution from lactational transfer.

Although the present study did not incorporate a cross-fostering design, Luebker et al. (Luebker et al., 2005a) have provided evidence of lactation exposure in pups born to control rats fostered by dams treated with 1.6 mg/kg-d K⁺PFOS, and subsequent analysis of two paired maternal serum and milk samples from K⁺PFOS-treated dams from the Luebker et al. study by Kuklenyik et al. (Kuklenyik et al., 2004) showed that milk PFOS concentrations were 11% and 51% of the respective maternal serum PFOS concentrations.

Based on data from Table 4 of the companion article (Butenhoff et al., 2008), on PND 21, compared to controls, there was no difference in mean body weights of pups by sex in any of the treatment groups when compared to sex-specific controls, and we found no significant within-group difference between male and female pups in PND 21 serum, liver, and brain PFOS concentrations that are reported herein. Mean serum PFOS on PND 72 was less than on PND 21 in males and females in all K⁺PFOS-treated groups. However, on PND 72 male serum PFOS was approximately a fifth to a third that of female serum PFOS, and female body weights were approximately two-thirds of male body weights. These observations suggest that a sex difference in serum PFOS elimination occurred
between PND 21 and PND 72, perhaps associated with sexual maturation during this period, with males eliminating PFOS from serum at a somewhat higher rate.

PFOS appeared to concentrate in the livers of dams and pups, with liver PFOS concentrations at least twice serum PFOS concentrations in pups. However, liver concentrations were 60 to 80% of serum concentrations in fetuses on GD 20. PND 4 pup liver PFOS concentrations by maternal dose group were approximately 2 to 3 times greater than respective GD 20 fetal liver PFOS concentrations, yet serum PFOS concentrations in pups on PND 4 were less by 30 to 40% as compared to GD 20 fetal serum PFOS concentrations in the same respective dose groups. The increase in liver concentration in pups on PND 4 may represent redistribution from serum to liver after birth, perhaps as a result of enterohepatic recirculation (Johnson et al., 1984). Despite the marked difference between males and females in serum PFOS concentrations on PND 72, liver concentrations were essentially the same in both sexes, having decreased by approximately 85% from PND 21 values.

The observations from the pharmacokinetic data described above suggest that differences in urinary elimination may develop between males and females during sexual maturation and that enterohepatic recirculation and fecal elimination may be similar between genders after sexual maturation. Johnson et al. (Johnson et al., 1984) provided evidence that enterohepatic recirculation and fecal elimination of
PFOS in rats were factors in PFOS pharmacokinetics. This study may be the first observation of a sex difference in serum PFOS elimination in rats, with male rats apparently eliminating PFOS from serum at a greater rate than females. It is interesting to note that the eight-carbon perfluorinated carboxylate, perfluorooctanoate (PFOA), exhibits a sex difference in renal elimination in rats (Kudo et al., 2002; Kudo and Kawashima, 2003); however, in the case of PFOA, the elimination rate in females is much greater than in males. This sex difference in PFOA elimination appears to develop during sexual maturation (Hinderliter et al., 2006), likely the result of differences in the expression of organic anion transporters (Katakura et al., 2007).

The concentration of PFOS in non-perfused brain tissue was generally lower than in serum. On GD 20, the average PFOS concentrations for the non-perfused brain tissues of dams were between 4 to 9% of the corresponding serum PFOS concentrations. Fetal brain concentrations at GD 20 were higher than those in respective dose group dams by a factor of approximately 10, and were 30 to 41% of the respective fetal serum PFOS concentrations. Although it is not possible to know how much of the PFOS in brain tissue was associated with blood versus that amount associated with tissue uptake, these data suggest that brain uptake may have been higher in fetal rats, perhaps due to the undeveloped state of the “blood-brain barrier” on GD 20.
The "blood-brain barrier" represents the complex tight junctions between endothelial cells of brain capillaries. It is physiologically defined by low permeability for small hydrophilic molecules (Schulze and Firth, 1992). In rats, the "blood-brain barrier" is not fully developed until PND 24, hence embryonic and early neonatal brain capillaries are more permeable for substances (Kniesel et al., 1996). This could be why highest levels of PFOS were detected in neonatal brains on GD 20. It is conceivable that the volume expansion (i.e., brain growth) could contribute to the lowering of brain PFOS concentrations seen postnatally (even with lactational exposure). Brain PFOS concentrations were less than corresponding serum PFOS concentrations in all dose groups. Compared to liver-to-serum ratio (which increased during the postnatal period), the corresponding brain-to-serum ratio were all less than 1. The only other published data on brain concentrations in PFOS-exposed rats comes from a study by Austin et al. (Austin et al., 2003) in which female rats were given daily i.p. injections of 1 or 10 mg/kg-d PFOS for 14 days. Brain region concentrations were analyzed for PFOS concentration (it was not noted whether or not the brains were perfused). At the 1 mg/kg-d dose, concentrations of PFOS in the various brain regions were a fraction (approximately 2 to 4%) of the serum concentration. Therefore, PFOS does not appear to concentrate in brain tissue relative to serum concentrations.

Thyroid hormones have numerous important roles in development (Bernal, 2005a, 2005b; Calvo et al., 1992; Oppenheimer et al., 1995). Although PFOS-induced
hypothyroxinemia has not been observed in pregnant and neonatal mice (Fuentes et al., 2006; Lau et al., 2003; Thibodeaux et al., 2003) (with the exception of the 20 mg/kg dose on GD 6 (not GD 12 and 18) in Thibodeaux et al. (Thibodeaux et al., 2003)), PFOS exposure has been associated with maternal and offspring hypothyroxinemia without a compensatory elevation of TSH in laboratory rats (Lau et al., 2003; Luebker et al., 2005b; Thibodeaux et al., 2003). The observed hypothyroxinemia is characterized by decreased serum total thyroid hormones (thyroxine and triiodothyronine) without a major compensatory increase in TSH (Chang et al., 2008; Lau et al., 2003; Luebker et al., 2005b; Thibodeaux et al., 2003). Although significant reductions in serum free thyroxine were reported by Thibodeaux et al. and Lau et al. (Lau et al., 2003; Thibodeaux et al., 2003) using analog methods, PFOS-induced reductions in serum free thyroxine and free triiodothyronine hormones were not observed when measured by equilibrium dialysis (Chang et al., 2008; Chang et al., 2007; Luebker et al., 2005b). PFOS-exposed adult and neonatal rats appear to maintain an euthyroid state despite significant reductions in serum total thyroid hormones (Chang et al., 2008; Luebker et al., 2005b). This is believed to be due to competition for binding sites between PFOS and thyroid hormones in rat serum, leading to an adequate supply of free hormone while reducing the concentration of hormone carried on serum binding proteins (Chang et al., 2007). Lau et al. (Lau et al., 2003) measured choline acetyltransferase (ChAT) activity, an enzyme sensitive to thyroid hormone status, in prefrontal cortex and hippocampus of rat pups exposed in
uterine at maternal doses of 3 mg/kg-d PFOS given orally from GD 2 to GD 21. At this dose, marked hypothyroxinemia occurred without a compensatory change in TSH. Free thyroxine was decreased; however, an indicated above, an analog assay was used that has since been shown to be prone to negative bias (Chang et al., 2007). These investigators also evaluated learning and memory using a T-maze. Hippocampal activity of ChAT and T-maze performance were unaltered when compared to controls. ChAT activity in the pre-frontal cortex was slightly (but with statistical significance) decreased. Luebker et al. (Luebker et al., 2005a) included an evaluation of learning and memory using a modified M-maze in rat pups whose mothers were given daily oral doses of 0.1 and 0.4 mg/kg-d K⁺PFOS beginning 6 weeks before mating, and during mating, gestation, and lactation. A second study in which the same dosing protocol was used (Luebker et al., 2005b) produced marked hypothyroxinemia in PND 5 pups without a clinically meaningful change in free thyroxine when measured by equilibrium dialysis or TSH (based on data from higher doses). There was no effect on learning or memory. In the companion paper to this, there were also no effects on learning or memory (Butenhoff et al., 2008).

Production and release of metabolically active thyroid hormones by the thyroid gland is regulated by pituitary secretion of TSH and hypothalamic secretion of TRH. This regulatory process for controlling circulating thyroid hormone concentrations constitutes what is commonly referred to as the hypothalamic-
pituitary-thyroid (H-P-T) axis. In this system, serum free thyroxine and free triiodothyronine function as primary or secondary feedback signals, acting on the hypothalamus and/or pituitary when there is an imbalance in their circulating concentrations. Although measurement of free thyroid hormones in serum can provide information on thyroid status, the clinical diagnosis of primary hypothyroidism is based mainly on a substantial elevation of TSH in response to reduced free thyroid hormones (Larsen et al., 2003). In a study designed to evaluate the potential effect of PFOS on the H-P-T axis (Chang et al., 2008), the goiterogen, propylthiouracil (PTU), was administered to rats with and without concurrent PFOS treatment. Rats treated with only PFOS experienced hypothyroxinemia without a change in TSH as compared to controls. PTU reduced serum thyroid hormones to a greater extent than PFOS in rats given only PTU, and serum TSH was increased significantly over control concentrations. PFOS given in conjunction with PTU did not alter the PTU-induced response to TSH. In addition, the TRH-induced release of TSH in excised, cultured pituitaries from rats exposed to PFOS or PFOS and PTU in combination was not affected by PFOS treatment.

In addition to TSH measurement, thyroid hormone status can also be evaluated by determining the follicular epithelial cell height and colloid area in histological sections of thyroid gland. The thyroid gland consists of multiple, closely approximated follicles, each of which consists of a central accumulation of colloid
surrounded by a single layer of follicular epithelial cells. The colloid consists of a homogeneous mixture of proteins, including thyroglobulin, iodoproteins, and serum proteins, and serves as a repository of thyroglobulin. Under conditions that require an increase in thyroid function, there is an increase in the rate of colloid resorption, resulting in a decrease in colloid cross-sectional area in histological sections, and an increase in the synthesis activities of follicular epithelial cells, the latter resulting in an increase in the cytoplasmic volume (height) of epithelial cells. Measurement of epithelial cell height and colloid area is easily accomplished from routine histological sections, and provides insight into the level of activity of the thyroid gland.

This study reported herein revealed no evidence of PFOS treatment-related alterations in TSH values for maternal and their offspring. PFOS treatment did not elicit any histological alterations of the thyroid glands in fetuses (GD 20) and pups (PND 21). Morphometric analysis on the thyroid revealed no PFOS treatment-related changes in follicular colloid area in pups on PND 4 and PND 21 when compared to control. There was a higher mean thyroid follicular epithelial cell height in male pups from the 1.0 mg/kg-d maternal dose group on PND 21, but this was suspected to be a spurious observation, as an unusually low value in the concurrent control group was obtained in this study when compared to the laboratory's historical control values.
Evaluation of cell proliferation in GD 20 fetal thyroid follicular epithelial cells from fetuses of the control and high-dose groups by Ki-67 immunohistochemistry produced a statistically-significant 2.1-fold increase in high-dose group female fetuses. The range of values in the female controls was quite wide (range 4 – 113, N = 7), and the highest value for the control females approximated the high end of the range in female thyroids from the 1.0 mg/kg-d dose group (range 64 – 116, N = 5). In GD 20 fetuses from dams treated with K+PFOS, TSH was not increased, and, in PND 4 and PND 21 female pups, morphometric analysis of thyroid follicles did not reveal effects due to gestational and lactational exposure to K+PFOS. Furthermore, adult females given 20 ppm K+PFOS in their diet for two years (equivalent to approximately 1.5 mg/kg-d) did not exhibit an increase in proliferative lesions of the thyroid (Thomford, 2002c). Therefore, interpretation of the toxicological significance of fetal cell proliferation data is problematic without further study of fetal rats due to the range of control values and limited number of samples analyzed.

Several previous studies have reported data that suggest that peroxisome proliferator activated receptor alpha (PPARα) agonists increase hepatic responses to thyroid hormone in rats by effective competition for thyroid hormone carrier protein binding sites. PPARα agonists are typically amphiphilic acids and include endogenous fatty acids, hypolipidemic drugs, acetylsalicylic acid, and perfluorinated acids, such as perfluorooctanoic acid (PFOA) and PFOS.
(Berthiaume and Wallace, 2002b; Ikeda et al., 1985; Klaunig et al., 2003; Sohlenius et al., 1993). The thyromimetic effects by PPARα agonists in rat liver is believed to be due to transcriptional activation of genes regulated by thyroid hormone, either directly or indirectly, such as liver malic enzyme (ME), deiodinase 1 (Dio1), apolipoprotein A1 (Apoa1); P450 oxidoreductase (Por), and the UDP glucuronyl transferases (Ugt) family of proteins (Cai et al., 1996; Hertz et al., 1991; Hertz et al., 1993; Ness et al., 1993; O'Leary et al., 1997).

For the control and 1.0 mg/kg-d dose group, the mRNA transcript levels of these enzymes were evaluated in this study as a secondary measure of concordance of the thyroid hormone responses in both dams (GD20) and their male offspring (GD 20 and PND 21). While both maternal and fetal tissues were examined on GD 20, only male pups from PND 21 were included because in a two-year dietary feeding study, there was an increased incidence of thyroid follicular cell adenoma observed in male rats fed with K⁺PFOS diet for one year followed by control diet for another year (Thomford, 2002c). Oral administration of K⁺PFOS to pregnant rats through gestation did not appear to elicit any changes in the hepatic genes mediated by thyroid hormones.

PFOS has been shown to induce hepatomegaly in rodents and monkeys (Abbott et al., 2008; Seacat et al., 2003; Seacat et al., 2002). The liver enlargement in rodents seen following the PFOS administration can be attributed by the induction
of the nuclear hormone receptors such as PPARα, pregnane X receptor (PXR), or constitutive androstane receptor (CAR). Chemicals such as Wyeth 14,643, dexamethasone, and phenobarbital are prototypical agonists used to study activation of PPARα, PXR, and CAR receptors, respectively. Wy14,643 induces hepatomegaly that was characterized by peroxisome proliferation and selective induction of Cyp4a1 and Cyp7a1 (Bars et al., 1993; Gbaguidi and Agellon, 2004; Moody et al., 1991). Dexamethasone produces hepatomegaly as a consequence of extensive periportal fat accumulation and induction of Cyp3a1 (Kliewer et al., 1998; Micuda et al., 2007). Phenobarbital induces hepatomegaly by simulating cell proliferation and the induction of Cyp2b2 (Connor et al., 1995; Larsen and Jefcoate, 1995) and other cell proliferation markers such as proliferating cell nuclear antigen (PCNA).

Compared to control, the RT-PCR analyses on all the transcripts evaluated suggest that: (1) induction of hepatic CAR in the dam was suggested by the 1.0 mg/kg-d maternal rats on GD 20 based on a 2.8-fold increase in Cyp2b2; (2) there was no statistically-significant difference in gene expression in 1.0 mg/kg-d maternal dose group on GD 20; and (3) male pups from the 1.0 mg/kg-d maternal dose group on PND 21 had an increased expression for the PPARα-regulated genes, ACoA (increased by 1.5 fold) and Cyp4a1 (increased by 2.1 fold), and the CAR regulated gene Cyp2b2 (increased by 1.8 fold). Interestingly, decreased expression of Cyp7a1 (decreased by 0.3 fold) was observed in PND 21 male pups,
even though ACoA and Cyp4A1 were increased. Transcripts with a potential relationship to thyroid status (ME, Por, Dio1, Ugt1A family, and ApoA1) were unaffected in offspring following maternal K⁺PFOS treatment.

7.5 CONCLUSION

Data reported herein demonstrate that K⁺PFOS administration to maternal rats during gestation and lactation: 1) leads to significant placental transfer and potentially significant lactational transfer; and 2) has no clear adverse effect on thyroid status (morphology, hormone homeostasis, proliferation, and liver gene expression). In addition, sex difference in serum elimination of PFOS (but not liver or brain elimination) appears to develop in association with sexual maturation, with males eliminating PFOS from serum at a somewhat greater rate than females.

Samples reported on herein were obtained during the course of a developmental neurotoxicity study (reported as a companion article (Butenhoff et al., 2008)) in which the only developmental effect observed were increased motor activity and decreased habituation in males from the highest maternal dose group (1.0 mg/kg-d) on PND 17. At this dose, there was also a slight but statistically-significant effect on maternal weight and food consumption. Maternal and fetal serum concentrations in the 1.0 mg/kg-d dose group proximal to birth (GD 20) were

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approximately 27 and 31 µg/mL. No adverse maternal or developmental outcomes were noted at 0.3 mg/kg-d, and maternal and fetal serum concentrations on GD 20 were approximately 6 and 10 µg/mL, respectively. Based on the most recent (2003-2004 sampling period) report on PFOS concentrations in human serum of a representative sample of females across the United States, geometric mean serum PFOS was 0.0184 µg/mL and 95th percentile was 0.0457 µg/mL. Thus, when serum PFOS in this sample of United States females is compared to those in GD 20 dams from no observed adverse effect level of the study, margins of exposures of 333 and 130 exist at the geometric mean and 95th percentile for United States females, respectively.

7.6 ACKNOWLEDGEMENTS

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VIII.

OVERALL CONCLUSION
PFOS exposure has been associated with hypothyroxinemia without a compensatory elevation of TSH in laboratory rats (Chang et al., 2009; Chang et al., 2008; Chang et al., 2007; Lau et al., 2003; Luebker et al., 2005b; Thibodeaux et al., 2003). Rats receiving PFOS appeared to maintain a euthyroid state despite significant reductions in serum total thyroid hormones, likely due to competition for binding sites between PFOS and thyroid hormones in rat serum as postulated by Hypothesis 1 stated in Chapter III. Experimental data from Chapter IV demonstrated that PFOS can indeed cause a transient surplus of FT4 in the circulation and systematically introduces a negative bias when conventional analog methods were used to determine serum FT4 levels. The ED-RIA reference method, also described in Chapter IV, was not prone to such bias.

Presented in Chapter V were data showing enhanced thyroid hormone excretion as $^{125}$I activity in urine and feces in rats (after a single intravenous injection of $^{125}$I-T4 with a subsequent single oral K$^+$PFOS administration). There were also increases in gene transcript expression and enzyme activity in liver malic enzyme, which was sensitive to changes in thyroid hormones (vide supra), after K$^+$PFSO administration. Together with data indicating increased thyroid hormone turnover such as gene transcripts for liver glucuronidases, these experimental findings supported enhanced thyroid hormone turnover as postulated in Hypothesis 2 as well as increased responses in thyroid hormone-sensitive tissues such as liver (Hypothesis 3). In addition, the binding competition between PFOS and thyroid hormones did not appear to interfere with the central H-P-T axis because the ability of the pituitary to respond to hypothalamic thyrotropin-releasing
hormone to release TSH in response to decreased thyroid hormone production after treatment with propylthiouracil was not altered in rats by co-treatment with PFOS (Chapter V). This finding was in agreement with the data reported by Yu et al. (2008), whom showed that treatment of PFOS to rats did cause not cause any perturbation in TSH levels nor did it have any effect on protein functions involved in thyroid hormone synthesis such as TPO, NIS, and TSH receptor.

Under a prolonged PFOS treatment regime, daily oral administration of PFOS to rats for 28 consecutive days led to hypothyroxinemia as anticipated; however, the overall thyroid hormone status did not appear to be compromised, because the serum TSH levels from PFOS-treated rats were similar to those in control rats throughout the treatment period as well as during the 10-week recovery period (Chapter VI). The changes in thyroid in the presence of PFOS appears to be a simple physical phenomenon, because the gradual increases in serum TT4 in the recovery period were accompanied by diminishing serum PFOS concentrations. Similarly, increased liver expression of mRNA transcript such as Ugt1a1 obtained at the end of PFOS treatment indicated enhanced thyroid hormone turnover, again supporting the displacement theory.

Because (1) PFOS is widely present in the environment, (2) PFOS exposure can be begin very early in life by means of in utero and/or lactational transfer, and (2) thyroid hormone is essential for the normal function of brain development and growth in the early childhood, PFOS-induced hypothyroxinemia reported in previous developmental studies
(Lau et al., 2003; Luebker et al., 2005b; Thibodeaux et al., 2003) led us to evaluate thyroid status and histomorphological factors associated with thyroid follicles during the course of a developmental neurotoxicological study with PFOS in rats. The only adverse effect observed was decreased habituation in male pups from the highest maternal dose group (1 mg/kg/day) on PND 17 but not any other days (Butenhoff et al., 2009). PFOS administration to maternal rats during gestation and lactation has no clear adverse effect with regards to thyroid morphology, thyroid hormone status, thyroid cell proliferation, and liver gene expression in offsprings (Chapter VII).

From a health risk communication perspective, margins of exposure of PFOS in female humans with respect to pregnancy and thyroid functions can be calculated based on the data based on the following. From the most recent NHANES data published which including 2003-2004 blood sampling period, the geometric mean PFOS concentrations in human serum of a representative sample of females across the United States was 0.0184 µg/mL and the 95th percentile was 0.0457 µg/mL (Calafat et al., 2007b). The mean serum PFOS concentrations in the female rats with no observed adverse effect level (NOAEL) reported for thyroid function was 6 µg/mL from pregnant dams receiving 0.3 mg/kg/day K⁺PFOS during gestation (Butenhoff et al., 2009). The pups from this group of maternal rats had an average of 10 µg/mL PFOS in the serum at birth (Chapter VII).

In a conservative estimate, when serum PFOS in this sample of United States NHANES females (0.0184 µg/mL and 0.0457 µg/mL for geometric mean and the 95th percentile,
respectively) is compared to the serum PFOS concentrations of the pregnant female rats at NOAEL of 6 µg/mL, margins of exposure are 333 and 130 at the geometric mean and 95th percentile for United States females, respectively.

In conclusion, PFOS treatment does not appear to suppress the physiological thyroid hormone status in rats.
IX.

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