DUPLICATE

WORKSHOP REPORT

EPA/600/R-98/057

Screening Methods for Chemicals That Alter Thyroid Hormone Action, Function and Homeostasis

June 23-25, 1997

Durham, NC

Michael DeVito, Kevin Crofton and Suzanne McMaster

National Health and Environmental Effects Research Laboratory

United States Environmental Protection Agency

WORKSHOP REPORT

EPA/600/R-98/057

Screening Methods for Chemicals That Alter Thyroid Hormone Action, Function and Homeostasis

June 23-25, 1997

Durham, NC

Michael DeVito, Kevin Crofton and Suzanne McMaster

National Health and Environmental Effects Research Laboratory

United States Environmental Protection Agency

Screening Methods for Chemicals That Alter Thyroid Hormone Action, Function and Homeostasis Workshop

Disclaimer

This document has been reviewed in accordance with U.S. Environmental Protection Agency policy and approved for publication. Approval does not signify that the contents necessarily reflect the view and policies of the Agency nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

Workshop On Screening Methods for Chemicals That Alter Thyroid Hormone Action, Function and Homeostasis.

EXECUTIVE SUMMARY

Background

On June 23-25, 1997 the *Workshop On Screening Methods for Chemicals That Alter Thyroid Hormone Homeostasis, Action and Function* was held at the Nicholas School of the Environment at Duke University, Durham, North Carolina. This workshop was attended by 21 scientists from academic, industrial and governmental laboratories. The workshop was the third in a series of workshops sponsored by the Chemical Manufacturers Association, World Wildlife Fund and the US EPA. The meeting was organized by Drs. Ron Miller (CMA), Theo Colborn (WWF), Sue McMaster (EPA), Michael DeVito (EPA), Michael McClain (Hoffman La Roche) and Peter Hauser (VA).

Workshop Objectives:

Convene a workgroup to discuss the technical merits and limitations of currently available or under development *in vivo* and *in vitro* methods for detection of chemicals that interfere with thyroid hormone (TH) action and homeostasis. It is not the purpose of the meeting to recommend a screening battery nor to deal with policy issues pertaining to the use of such screens. The product of the workshop is intended to describe the methods which are currently available or could be developed in the near future for screening and testing.

Workshop Summary

Chemicals that alter thyroid gland function or hormone action can act by altering synthesis, transport, or metabolism of thyroid hormones or by disrupting thyroid hormone

receptor signaling by binding directly to these receptors as either agonists or antagonists or through secondary mechanisms. The workshop examined more than 20 assays in mammalian and non-mammalian systems including *in vivo, ex vivo* and *in vitro* assays. Discussions of these assays focused on their specificity (is the response pathognomonic for alterations in thyroid function), sensitivity (defined as the response of the method to low doses or to weak acting chemicals), test duration, simplicity, and limitations. Confidence in an assay is dependent upon how long the assay has been in use and how widely accepted the method is in pharmacological and toxicological research, hence the historical use of the assay was evaluated.

The consensus of the workshop is that there are assays available which can detect alterations in thyroid gland function and hormone action. These assays include *in vivo, ex vivo* and *in vitro* assays. However, only a few of these assays can be described as potential first tier screens. Many of the assays discussed are either mechanistic in nature or are time consuming and/or too expensive to be considered as screens. Current standard EPA test guidelines do not include screening methods for chemicals that alter thyroid gland function or thyroid hormone action. Several of the methods discussed at the workshop could be added to these guidelines.

Chemicals that affect synthesis, transport or metabolism of thyroid hormones have been well characterized and assays for the detection of these effects are well established. Several of these assays have been used in numerous laboratories examining hundreds of chemicals. *In vivo, ex vivo* and *in vitro* assays have been used to examine these chemicals. Many of the assays discussed are mechanistic based and not necessarily useful as initial screens. These assays may be useful in further testing of the chemicals that are positive in initial screens. The workshop participants concluded that a single screen may not detect weakly goitrogenic chemicals if the studies are not properly designed and evaluated.

ASSAYS FOR THYROID SYSTEM DISRUPTION IN MAMMALS

Serum Hormone Determination and Thyroid Histology:

One possible screen for chemicals that alter thyroid function could be the measurement of serum TH concentrations in experimental animals following treatment with a test compound. Methods for determining serum concentrations of THs are readily available from commercial suppliers and these assays have been in use for many years. The determination of serum thyroid hormone concentrations in animals following chemical exposure provides assessment of thyroid function equivalent to those used clinically in humans. However, due to potential compensatory mechanisms, histological assessment of thyroid gland in conjunction with the measurement of serum TH concentrations can provide a more complete assessment of thyroid function and thyroid hormone action (McClain, 1995; Capen, 1995). Future efforts to determine the most appropriate time point and exposure regimen for examining serum TH concentrations are recommended.

Assays for Chemicals That Alter Synthesis, Secretion, Transport and Catabolism of Thyroid Hormones.

The workshop discussed the peroxidase assay, perchlorate discharge test, TRH challenge test, serum protein binding assays, deiodinase assays, and glucuronidation assays. The assays described in this section are specific for particular mechanisms of action. A combination of these assays could provide predictive information on the availability of intracellular 3,3',5-tri-iodothyronine (T3) concentrations, particularly in the fetus. This information could be useful in assessing the potential adverse effects of chemicals that disrupt thyroid hormone homeostasis and alter tissue TH concentrations. These assays have been used to understand the mechanism of either altered serum concentrations of TH and thyroid stimulating hormone (TSH) or changes in thyroid histopathology (McClain et al., 1995; Atterwell et al., 1993; Poole et al., 1988; Olgivie and Marsden, 1988; Brouwer 1991). These assays are useful tools in understanding the mechanism of action of thyroid hormone disruptors. If these assays were to be used as

initial screens, several of these assays should be performed in order to demonstrate that a chemical does not alter TH concentrations.

Screening for Chemicals That Interact with Thyroid Hormone Receptors or Modulate Thyroid Hormone Receptor Activation.

Chemicals may bind to the thyroid hormone receptors and act as either agonists, antagonists or partial agonists. Evidence of xenobiotics which alter thyroid hormone action by binding to the receptor is limited. While there are QSAR models which predict such interactions, there is no direct evidence that any xenobiotic binds the thyroid hormone receptor (TR). Chemicals can also interact with other proteins and modulate the activation of the TR. There are a number of high throughput screens which can determine if a test chemical binds to the TR or modulates the activation of the receptor. The most frequently performed assays are the receptor binding assays, mammalian cell transfection assays and yeast cell line transformation assay. Also discussed was the GH_3 cell line assay, which examines the proliferation of cells in response to TR agonists. While these assays would have the ability to detect chemicals that bind to TR, there is limited evidence that environmental chemicals bind to these receptors. The development and implementation of screens should reflect known mechanisms of action of thyroid disrupting chemicals

Developmental Assays

Hypothyroidism during development produces profound permanent changes in the auditory system, central nervous system and the male reproductive system. A number of assays or test systems can be used to detect chemicals that produce hypothyroidism. However, most of these assays or tests systems are time consuming and not necessarily specific for hypothyroidism. In addition, pronounced decreases in serum T4 concentrations are required to detect the behavioral or morphological changes. In experimental animals, alterations in serum THs can be detected at lower dose levels than

those required to detect the behavioral and morphological changes in these systems. Because of the greater sensitivity and simplicity, determination of serum TH concentrations is recommended instead of these developmental assays.

Screening for Chemicals That Alter Thyroid Action, Function, and Homeostasis in Non-Mammalian Wildlife.

Thyroid hormones are critical in development for non-mammalian wildlife. There are examples of chemicals that alter thyroid hormones in non-mammalian wildlife and produce developmental toxicities. While there are similarities between mammals and other wildlife species there are some differences. For example, evaluating peripheral processes controlling T3 production and tissue T3 seem more important than serum hormone measurements. Many of the assays discussed for mammalian system can be used for non-mammals provided these assays are sufficiently modified to examine the non-mammalian species of interest. The tadpole metamorphosis assay is potentially useful as a screen but requires further standardization.

CONCLUSION

The workshop participants attempted to address the merits and limitations of numerous assays available as potential screening methods for chemicals that alter thyroid hormone action, function or homeostasis. Not every existing assay was examined due to limitations of time and expertise. Some of the assays evaluated may be useful as screens but most of the assays are more appropriate for mechanistic studies.

INTRODUCTION

Endocrine disruption has emerged as an environmental issue based on the hypothesis that exposure to certain environmental chemicals alters the endocrine system, and increases the incidence of endocrine diseases and disorders in both humans and wildlife (Adams, 1992; Colborn et al., 1992; Kavlock et al., 1996). While research evaluating this hypothesis is ongoing, there are thousands of synthetic and naturally occurring chemicals that must be considered. The development of screening methodology for endocrine disrupting chemicals (EDCs) would enable researchers to narrow the focus of their research efforts (Kavlock et al., 1996). In the United States, screening for EDCs was recently mandated by congressional legislation in the Food Quality Protection Act of 1996 (Public Law 104-170) and the Safe Drinking Water Act of 1996 (Public Law 104-182). A series of workshops sponsored by the Chemical Manufacturers Association, the United States Environmental Protection Agency and the World Wildlife Fund focused on the development of screens for endocrine disrupting chemicals for both humans and wildlife. The following report is a consensus from the workshop entitled *Screening Methods for Chemicals That Alter Thyroid Hormone Action, Function and Homeostasis.*

The workshop focused on over 20 assays or test systems that have been used to examine chemicals that alter synthesis, storage, transport, and catabolism of thyroxine (T4) and 3,5,3'-triiodothyronine (T3), assays which examine ligand binding and activation of the thyroid hormone receptor, and *in vivo* assays that examine the effects of antithyroid agents and thyromimetics in mammalian and non-mammalian wildlife models. The meeting focused on chemicals that alter thyroid gland function through pharmacodynamic means and did not include chemicals that were directly cytotoxic to the thyroid gland. It was not the purpose of the meeting to recommend a screening battery nor to deal with policy issues pertaining to the use of such screens. The product of the workshop is intended to describe the methods which are currently available or could be developed in the near future for screening and testing.

Thyroid Function and Regulation.

The thyroid gland produces T4 and T3. The thyroid hormones (THs) have two predominant functions. The first is a critical role in growth and development. One of the best examples of the importance of thyroid hormones in growth and development is the metamorphosis of amphibians, in particular the metamorphosis of tadpoles into frogs (Kaltenbach, 1996; Dodd et al., 1976; Kollros, 1961). Other examples of the importance of THs in development are the transformation of salmon from freshwater dwelling par to seawater dwelling smolts (Dickhoff and Sullivan, 1987; Specker, 1988), flounder metamorphosis (Inui and Miwa, 1985), and development of the central nervous system in humans and other mammals. In humans, severe hypothyroidism during development results in cretinism (Legrand, 1979; Porterfield, 1994). The second major function of thyroid hormones is to maintain metabolic homeostasis in mammals (Farrell and Braverman, 1995).

The synthesis and storage of TH predominately occurs in the thyroid gland and the synthesis is regulated by the pituitary hormone, thyroid stimulating hormone (TSH). Most of the TH in the thyroid is present as T4. While a small proportion of thyroid localized TH is T3, most T3 comes from the deiodination of T4 by tissue specific deiodinases. The processes involved in the synthesis, storage, release, transport and metabolism of THs are complex and consist of the following: (1) uptake of iodide ion by the thyroid gland, (2) oxidation of iodide and the iodination of tyrosine, (3) coupling of iodotyrosine residues to produce iodothyronines, (4) proteolysis of thyroglobulin and release of T4 and T3 into the blood, (5) binding to serum transport proteins, (6) target tissue synthesis of T3 from T4, (7) catabolism of T4 and T3 in peripheral tissues, (8) catabolism and biliary elimination of THs in the liver. There are many examples of pharmaceutical, environmental and naturally occurring chemicals which alter one or more of these processes in mammals, these have been reviewed by Hill et al. (1989) and Atterwell and Aylward. (1995).

The actions of thyroid hormones are mediated by their interaction with nuclear thyroid hormone receptors (TR). These receptors are part of the steroid receptor super

family (Evans, 1988) and are the cellular homologs of the oncogene c-*erb*-A. There are four known isoforms of the thyroid receptor which are derived from two genes c-*erb*-A beta (TR $beta_{1,2}$) and c-*erb* A alpha (TR $alpha_{1,2}$) (Lazar, 1993). These TRs bind to specific sites on DNA and also form heterodimers with other nuclear receptors such as the retinoid X receptor (RXR). In addition, TRs form complexes with a number of additional modulating and accessory proteins involved in gene transcription. The affinity of the nuclear TRs is 10-20 times greater for T3 than for T4 (Oppenhiemer et al., 1987; Cody, 1991). Unlike the estrogen receptors, there is little evidence of environmental chemicals binding the TR. However, the hypothesis that some environmental chemicals may bind to TR resulting in toxicological responses has not been adequately tested.

There are several sites in the synthesis, transport and metabolism of THs that can be altered by xenobiotics. In addition, it is possible that xenobiotics can alter TH signaling through the TR either by directly binding to TR or indirectly by altering phosphorylation of TR or through interactions with other accessory proteins. Due to the complexity of TH function and regulation, it is unlikely that a single assay will be available to detect chemicals that act on any or all of these pathways. The utility of a screen depends on its specificity (is the response pathognomonic for alterations in thyroid function), sensitivity (defined as the response of the method to low doses or to weak acting chemicals), test duration, simplicity, and limitations. A number of assays or experimental systems were evaluated for their potential use as screens to detect chemicals that disrupt thyroid hormone catabolism and signaling. The workshop participants acknowledged that several of these methods could be used as screening tools.

ASSAYS FOR THYROID SYSTEM DISRUPTION IN MAMMALS

Thyroid hormone concentrations and thyroid gland histology

In humans, alterations in thyroid function are initially diagnosed by either physical examination for enlarged thyroid gland, or by measuring serum hormone concentrations.

Serum hormone concentrations are such good indicators of thyroid function in humans that in the U.S., newborn infants are required to have blood samples collected for TSH and/or T4 determinations prior to leaving the hospital. The American Thyroid Association has recommended determination of serum TSH and free T4 concentrations (Surks et al., 1990) as the standard measure of thyroid function. Total T4 was not recommended as a measure because false positives can be caused by disease states and pharmaceutical agents which alter thyroxine binding globulin (TBG), the main serum binding protein in humans. Changes in TBG alters total serum T4 concentrations, but may not necessarily alter free T4 concentrations. It is thought that free T4 is available to enter the cell and that the concentrations of free T4 are proportional to the tissue concentrations of T3 and T4. The American Thyroid Association considers a diagnosis of primary hypothyroidism confirmed if the patient has decreased free T4 serum concentrations accompanied by increased serum TSH concentrations (Surks et al., 1990). Hyperthyroidism in humans is confirmed if the patient has increased free T4 serum concentrations accompanied by decreases in serum TSH concentrations (Surks et al., 1990).

The synthesis of THs is tightly regulated. Decreases in serum THs as a result of chemical inhibition of TH synthesis or transport or induction of catabolism increases TSH release from the pituitary. The increased TSH causes hypertrophy and hyperplasia of the follicular cells of the thyroid resulting in an increase in thyroid gland weight with a concomitant increase in synthesis and release of thyroid hormones. The stimulation of thyroid hormone synthesis and release by TSH can result in the normalization of serum T4 and T3 concentrations. The importance of these relationships is that there is a compensatory mechanism by which serum TH concentrations are regulated. Early on in chemical exposure, the serum concentration of THs will decrease. The decrease in serum THs results in increased secretion of TSH by the pituitary which may eventually restore normal T4 and T3 serum concentrations. When examining chemical effects on serum TH concentrations at later time points in exposures, serum concentrations may not be affected due to the compensatory mechanisms (McClain et al., 1989; McClain, 1995). In designing

experiments to examine the effects on serum thyroid hormones, this compensatory mechanism must be considered and appropriate temporal relationships examined.

Another difficulty in determining serum concentrations of THs and TSH is their responsiveness to stress and time of day of sampling. For example, transporting animals from one room to another, will increase TSH and T3 by approximately 2 fold over a 1 hour period with initial increases occurring within 5 minutes (Dohler et al., 1979). Circadian rhythms of THs occur in rats with peak serum concentrations occuring at approximately noon (Dohler et al., 1979). In addition, there is some evidence of alterations in TH concentrations associated with stages of the estrous cycle (Dohler et al., 1979). THs also change with age and in male rats increase strikingly from postnatal day 33 to 50 (Dohler et al., 1979). These and several other confounding factors are reviewed by Dohler et al., (1979). The determination of serum concentrations of THs requires careful consideration of these factors particularly for weakly goitrogenic chemicals. Finally, detecting small changes in serum TSH and TH concentrations can be problematic due to the large inter-animal variability and small changes in TSH (20-30%) can have significant impact on thyroid gland function (McClain, 1995).

In humans, free T4 and TSH serum concentrations are used to assess thyroid function. In experimental animals, researchers have measured both free and total T4 and T3 as well as TSH serum concentrations (Bastomsky, 1976; Barter and Klassen, 1992; Brouwer et al., 1991; Brouwer et al., 1998; McClain et al., 1989). Determination of both free and total THs can provide complementary information that would guide further testing of a chemical. For example, free T4 is an indicator of the amount of hormone available for tissue uptake and for fetal transfer.

An area which lacks adequate experimental data is the exact time course of the compensatory mechanism for the different classes of chemicals that alter serum TH concentrations. Time course data for inducers of uridine diphosphate glucuronlytransferase (UDP-GT) indicates that continued dosing with these chemicals for 7, 14, and 21 days produces alterations in serum TH concentrations (Barter and Klaassen,

1992; McClain et al., 1989; Christensen et al., 1994). Histological changes in the thyroid or changes in thyroid weight occurred following 14 days of dosing (Barter and Klassen, 1992; McClain et al., 1989; Christensen et al., 1994). There has been no systematic attempt to determine the time course of the compensatory response to decreases in serum TH concentrations following exposure to different classes of chemicals which alter serum TH concentrations through different mechanisms.

As described above, the compensatory increases in TSH result in proliferation of the follicular cells in the thyroid gland and these changes can be detected histologically as increased follicular cell numbers and as increases in thyroid gland weight (Capen, 1995). These histological changes appear less sensitive to confounders described above and may provide a better assessment of thyroid function than serum hormone concentrations. Similar dose-response relationships between decreases in serum hormone concentrations, histological changes in the thyroid and increases in thyroid weight were observed in rats administered sulfamethazine for 4 weeks (McClain, 1995). Furthermore, the use of thyroid weights and histology may allow for screening chemicals previously tested in subchronic studies. Caution is required for studies examined during the 1970's however, since follicular cell hypertrophy indicative of TSH stimulation were not considered pathological changes and may not have been reported.

Section Summary: One possible screen for chemicals that alter thyroid function could be the measurement of serum TH concentrations in experimental animals following treatment with a test compound. Methods for determining serum concentrations of THs are readily available from commercial suppliers and these assays have been in use for many years. Using determination of serum thyroid hormone concentrations in animals following chemical exposure provides assessment of thyroid function equivalent to those used clinically in humans. However, due to potential compensatory mechanisms, histological assessment of thyroid gland in conjunction with the measurement of serum TH concentrations is desirable in order to provide a more complete assessment of thyroid function and thyroid hormone action (McClain, 1995; Capen, 1995). Future efforts to

determine the most appropriate time point and exposure regimen for examining serum TH concentrations are recommended.

Assays for Chemicals That Alter Synthesis, Secretion, Transport and Catabolism of Thyroid Hormones

Serum concentrations of thyroid hormones can be caused by chemicals that inhibit thyroid hormone synthesis, release and transport and by chemicals that increase metabolism of thyroid hormones. If a chemical decreases serum thyroid hormone concentrations, specific assays can be used to determine the mechanism by which these hormone concentrations are decreased. These assays may be of value in screening for chemicals which act through specific mechanisms. The assays described address steps 1-8 in the synthesis and regulation of serum concentrations of thyroid hormones as described in the introduction.

Peroxidase Assay: Thyroid peroxidases are the key enzymes in the synthesis of thyroid hormones. There are a number of classes of synthetic chemicals that inhibit thyroid peroxidase, e.g. thionamides, such as propylthiouracil, aromatic amines, such as sulfathiazole, and polyhydric phenols such as resorcinol (Hill et al., 1989). In addition, there are a number of naturally occurring chemicals which inhibit thyroid peroxidase such as goitrin found in turnips and other cruciferous vegetables (Capen, 1995) and flavonoids found in other plant products (Divi and Doerge, 1996). Thyroid peroxidases (TPO) have two functions. The first is the iodination of tyrosine residues on thyroglobulin. The second reaction is coupling of specific di- and triiodotyrosyl residues on thyroglobulin. The iodination reaction can be readily determined using bovine serum albumin or tyrosine as substrates (Divi and Doerge, 1996). In addition, the oxidation of guaiacol can be used as an indicator of thyroid peroxidase activity (Divi and Doerge, 1994). All chemicals that inhibit the iodination reaction also inhibit the coupling reaction (Doerge et al., 1993). The coupling reaction can be assayed using either human low iodine thyroglobulin, pre-

iodinated casein or guaiacol as substrates.

A disadvantage of the TPO assay is that purified hog TPO is the only form of TPO commercially available. Purified human TPO is not commercially available, however, there are efforts to develop a recombinant human TPO. Purified lactoperoxidase (LPO) is commercially available. There is a good concordance between inhibitors of TPO and LPO (Divi and Doerge, 1994) and LPO has been used as a model for TPO actions (Divi and Doerge 1994). While TPO can be purified from experimental animals, the size of the gland in rodents is extremely small, and purification of rodent TPO would be impractical as a source of enzymes for a widely used screen.

One of the advantages of the TPO assay is that the sensitivity to chemical inhibition of thyroid peroxidase from human and experimental animals can be directly examined. *In vitro* studies have shown that TPO from monkeys is more resistant to inhibition by PTU and sulfamethazine than is TPO from rodents (Takayama et al., 1986). Comparisons of the relative sensitivity of TPO across species would assist in risk assessment for chemicals that inhibit TPO activity. The iodination and coupling assays are specific for chemicals that inhibit thyroid hormone synthesis and are unlikely to produce false positives. However, these assays examine a specific mechanism of thyroid hormone disruption and chemicals that affect other aspects of thyroid function are not detected. Used alone as a screen, these assays have potential for false negatives (i.e.) chemicals that alter TH concentrations through other mechanisms would not be detected). These assays have been performed for many years, are well established in the scientific literature, and numerous chemicals have been tested using these assays. While there are no published methodologies that can be defined as high throughput screens, modification of this assay into a high through put screen is under development in several laboratories.

Perchlorate Discharge Test: This assay examines the ability of the thyroid gland to organify iodide into thyroid hormones (Baschieri, 1963; Atterwell et al., 1987; Christenson et al 1996). This assay has been used for decades in both animals and humans (Baschieri

et al., 1983; Morgan and Trotter, 1957; Atterwell et al., 1987; Christenson et al 1996). In this assay, animals are exposed to a test chemical and then administered $Na^{125}I$ followed later with perchlorate. Accumulation of ^{125}I in the thyroid is then determined. When administered after a dose of radioactive iodine, perchorlate promotes release of iodine that has not been incorporated into thyroglobulin. If a chemical inhibits or deactivates thyroid peroxidase or blocks iodide uptake into the thyroid, there would be a decreased accumulation of ^{125}I in the thyroid gland. This assay is specific for chemicals which inhibit or deactivate thyroid peroxidase or block iodine uptake. This assay has potential for providing mechanistic information on the actions of chemicals that alter thyroid function but does not necessarily meet the requirements of a screen.

TRH Challenge Test: This assay examines the functional integrity of the hypothalamus-pituitary-thyroid axis (Christensen et al. 1996). Briefly, this assay measures TSH concentrations before and after challenge with thyrotropin releasing hormone (TRH). Challenge with TRH should increase serum concentrations of TSH. Differences in TSH concentrations in animals treated with test compounds compared to controls may suggest a pituitary site of action for the chemical. This assay has been used both clinically (Sarne and Refetoff, 1995) and experimentally (Christensen et al., 1996). The TRH challenge can be used to distinguish between pituitary and hypothalamic causes of hypothyroidism (Sarne and Refetoff, 1995). However, the underlying assumption prior to performing this test is that the chemical of interest produces hypothyroidism. While the TRH challenge has potential for providing mechanistic information on the actions of chemicals that alter thyroid function the assay may not be a useful screen due to the limited number of chemicals which may act through this mechanism.

Serum Protein Binding Assays: In mammalian systems, the serum binding proteins for thyroid hormones are thyroid binding globulin (TBG), transthyretin (TTR), and albumin. TBG and TTR are specific for THs and T4 has a greater affinity for these serum

binding proteins than T3 (Dohler et al., 1979). TBG is present in humans and primates and appears responsible for the much longer half-life of T4 and T3 in humans compared to other species (Dohler et al., 1979). TTR is present in humans, rodents and non-human primates. In humans, TBG is the predominant binding protein while in rodents TTR is the predominant carrier of THs. In humans, the main function of TTR appears to be the transport of T4 into the cerebral spinal fluid (Hebert et al., 1986). In addition, TTR transports T4 into the fetus. There are a number of reports of chemicals which displace T4 from TTR and TBG. The research on environmentally relevant chemicals has focused mainly on the polyhalogenated dibenzo-p-dioxins, biphenyls and diphenylethers (Brouwer, et al., 1990; Brouwer, 1991; Brouwer et al., 1998; McKinney et al., 1985; McKinney et al., 1987; McKinney and Waller, 1994). The displacement of T4 from these serum binding proteins is hypothesized to increase the clearance of T4 and decrease serum T4 concentrations. It has also been suggested that TTR binding is predictive of interactions with other TH binding proteins such as the deiodinases and sulfotransferases as well as chemicals with high fetal accumulation (Brouwer, et al., 1998).

These assays have been performed in several laboratories examining xenobiotics for several decades (Brouwer, et al., 1990; Ogilvie and Ramsden, 1988). While these assays can be modified for high through-put screening they are specific for chemicals that compete with thyroid hormones for serum binding proteins and will not detect chemicals that act through other mechanisms. In addition, the use of either TBG or TTR may not be relevant for non-mammalian species such as teleosts. However, one of the strengths of this assay is that it may be predictive of chemicals that alter fetal concentrations of TH and may provide for a useful screen.

Deiodinase Assays: In mammals, approximately 80% of the T4 secreted by the thyroid gland is deiodinated in target tissues into either T3, the most active form of the THs or into reverse T3, an inactive iodothyronine (Engler and Burger, 1984). There are several enzymes involved in the deiodination of T4, T3 and their metabolites and the expression

of these proteins is tissue specific. Type I deiodinase catalyzes the 5'-deiodination of T4, rT3 and the sulfated metabolites of T4 and T3 (Visser, 1988). Type I deiodinase is sensitive to PTU inhibition and is found in liver, lung, kidney, pituitary and thyroid (Chopra, 1977; Green, 1978). Type II deiodinase is present in the CNS, brown adipose tissue, anterior pituitary and the placenta (Silva et al., 1982; Visser et al., 1983; de Ona et al., 1991). Type II deiodinase is insensitive to PTU. In the brain type II deiodinase converts T4 into T3 and ensures adequate brain concentrations of T3 during critical periods of development (de Ona et al., 1991; Calvo et al., 1990; Obregon, et al., 1991) and during hypothyroidism (Escobar-Morreale et al., 1997). Type III deiodinase is resistant to PTU and catalyzes the conversion of T3 and T4 into 3,3'diiodothyronine and rT3, respectively in brain, skin, placenta and fetal tissues (Kaplan et al., 1983; Huang et al., 1985; Huang et al., 1988).

The deiodinases are critical in regulation of serum and tissue concentrations of THs. Decreases in serum concentrations of T4 alters expression of the different tissue deiodinases. For example, prenatal exposure to Aroclor 1254 increases brain type II deiodinase in rats with decreased serum T4 (Morse et al., 1996). There are also tissue-specific and isoform-specific changes in deiodinases following thyroidectomy and T4 and T3 replacement in rats (Escobar-Morreale, 1997). Some chemicals, such as PTU, also inhibit deiodinase activity. Deiodinase assays have been used for decades to understand the metabolism of THs. Because the activity of these enzymes are dependent upon serum concentrations of these hormones, these assays would be sensitive towards chemicals that alter serum TH concentrations. However, alterations in deiodinase activity also alters serum TH concentrations. This dose response relationship decreases the utility of this assay as a screen. If serum TH concentrations are changed by deiodinase inhibitors, it may be easier to measure serum TH concentrations than it is to determine deiodinase activity. Similar to many of the assays described above, these assays have greater utility in understanding the mechanism of action of a chemical rather than as an initial screen.

Glucuronidation Assays: Glucuronidation followed by biliary elimination of T4 is one of the major pathways of deactivation of T4. In humans there is evidence of sulfation of T4 as well. In mammals, there are at least 4 isoforms of uridine diphosphate glucuronosyltransferases (UDP-GT) which glucuronidate T4. Several classes of chemicals induce UDP-GTs responsible for the glucuronidation of T4 (Barter and Klassen, 1992; McClain, 1995; Capen and Martin, 1989; Atterwill and Aylward 1995; Brouwer, 1991). Induction of thyroxine glucuronidation increases clearance and decreases serum concentrations of T4. Induction of T4 glucuronidation is typically determined in hepatic microsomes from animals treated with test chemicals. These assays have been performed for decades in numerous laboratories throughout the world. These *ex vivo* assays require several days of dosing of the test chemical. The advantage of this type of assay is that it is responsive to metabolic activation of the test chemical because exposure occurs *in vivo*. The activity of hepatic microsomal thyroxine glucuronidation is not as sensitive to stress and circadian rhythms as is measurements of serum TH concentrations. The disadvantage is that these assays are not developed for high throughput screening and at present are laborious.

Section Summary: The assays described in this section are specific for particular mechanisms of action. A combination of these assays could provide predictive information on the availability of intracellular T3 concentrations, particularly in the fetus. This information could be useful in assessing the potential adverse effects of chemicals that disrupt thyroid hormone homeostasis and tissue concentrations. These assays have been used to understand the mechanism of either altered serum concentrations of TH and TSH or changes in thyroid histopathology (McClain et al., 1995; Atterwell and Aylward, 1995; Poole et al., 1988; Olgivie and Marsden, 1988; Brouwer 1991). If these assays were to be used as initial screens, all of them would have to be performed in order to demonstrate that a chemical does not alter TH concentrations.

TR Binding and Activation.

Chemicals can alter thyroid hormone action by binding to the T3 specific nuclear receptors. These receptors are part of the steroid receptor superfamily (Evans, 1988) and are the cellular homologs of the c-erbA oncogenes. There are several isoforms of the receptors which have tissue specific localization (Lazar, 1993). The structure-activity relationships for binding to the nuclear thyroid hormone receptor have been determined using crude nuclear homogenates (Oppenheimer et al., 1987; Cody, 1992) as well as various TR isoforms expressed in *E. Coli.* or translated *in vitro* (Cheng et al., 1994; Schueler et al., 1990). These binding studies have focused on T3 analogs and not on environmentally relevant chemicals (Oppenheimer et al., 1987; Cody, 1992; Cheng et al., 1994; Schueler et al., 1990). Several environmentally relevant classes of chemicals have been proposed to bind to the nuclear T3 receptors, such as the polyhalogenated dibenzo-p-dioxins, dibenzofurans, biphenyls and diphenyl ethers (McKinney, et al., 1987; McKinney and Waller, 1994). However, this hypothesis has not been adequately tested. At present there is a lack of evidence that environmentally relevant chemicals bind to TRs, which should not be confused with the presence of negative evidence. It should be noted that the chemical that have been proposed to bind to TRs also decreases serum TH concentrations in experimental animals and are known thyroid hormone disruptors (Bastomsky, 1976; Barter and Klaassen, 1992; Brouwer, 1991; van Birgelen et al., 1994).

In Vitro Binding Assays. In vitro binding assays can be used as potential screens for chemicals that bind to TRs. The classical binding assays have used nuclear extracts from a variety of tissues and cell lines expressing TRs (Cheng et al., 1994). More recent studies have used various TR isoforms expressed in E. Coli or translated in vitro (Cheng et al., 1994; Schueler et al., 1990). These assays require separating bound from free hormone using either filtering or chromatographic methods. Either separation method is cumbersome and time consuming. More recent advances have employed solid-state

binding assays using specific isoforms of TRs. The solid-state binding assays developed allow for high throughput screening. In the solid state binding assays, the thyroid hormone receptor is coupled to either a multiwell plate or to beads. Coupling of the receptors to plates or beads readily enables the separation of free and bound ligand without the use of either filtering or chromatographic methods. Only 3 of the 4 TR isoforms have ligand binding capability and of two of these (TR $beta_1$ and TR $beta_2$) have identical ligand binding domains. Binding assays are expected to have a very low rate of false positives. False negatives can occur if the chemical requires metabolic activation or if solubility problems are encountered.

Transfection and Transformation Assays. One of the problems with TR binding assays is that they cannot differentiate between agonists and antagonists. Alternative assays which would examine receptor binding and differentiate between agonists and antagonists are systems in which a specific TR is transfected into a mammalian cell line along with a reporter gene, typically coding for either luciferase or beta-galactosidase (Allegreto and Hayman, 1992). Transformed yeast cell lines containing TR gene constructs have also been developed. In these systems T3, or other ligands to TR, bind and activate the receptor which then interacts with specific response elements upstream from the reporter gene and enhance its transcription. The increased transcription is determined by increased enzymatic activity of the reporter gene product, e.g. luciferase. Chemicals can be tested alone or in combination with T3 to determine agonist or antagonist properties. Similar systems have been used to examine the interactions of TR with different response elements (Lazar et al., 1994), different cofactors (Katz et al., 1993) and with phosphorylation of TR (Boat, et al., 1994). While these systems have not been used for screening for environmental chemicals which are TR ligands, similar screens have been developed for estrogens and androgen agonists and antagonists (Allegreto and Heyman, 1996).

Transformed yeast cell and transfected mammalian cell lines have been used to

study several of the steroid hormone receptor super family members. There are differences between the assays used for estrogen, androgen and thyroid hormone receptors. There are only two estrogen receptors and a single androgen receptor in contrast to the four isoforms of TR. TRs form heterodimers with RXR (Berrodin et al., 1992; Reginato, et al., 1996; Meier et al., 1993) while the estrogen and androgen receptors form homodimers. Both TR and peroxisome proliferator-activated receptor (PPAR) form heterodimers with RXR and agonists of PPAR can alter TR mediated gene expression by binding competing for RXR (Bhat et al., 1996). Hence, chemicals can alter TR activation by altering RXR or PPAR pathways. TR activation is also regulated by phosphorylation (Bhat et al., 1994), similar to the estrogen and androgen receptors. In designing a screen for TR ligands, chemicals may have different effects depending upon the TR transfected, the response element used and their interactions with PPAR and RXR. Because of the complexity of this system, several different screens would have to be incorporated to account for the multiplicity of interactions of the different TR isoforms. An advantage of the transfection assays is that chemicals that alter TR activation through mechanisms not involving direct binding to TR would be detected in these assays. Another advantage of these assays is that they are readily adapted to high through put screens.

A major disadvantage of these *in vitro* screens is the lack of metabolic capability of the cells or assays. It is possible that the metabolites of some chemicals would produce these effects and not the parent compound. The cell lines typically used in these assays have limited ability to metabolize the test compounds, particularly persistent organic pollutants such as the PCBs and the dioxins. The transformation assays in the yeast have additional drawbacks in that for many chemicals entry into the yeast is limited due to the cell wall.

GH_3 Cell Assay for Thyroid Hormone Action: An in vitro bioassay has been designed which can detect TR agonists (Hohenwarter et al., 1996). This assay uses the rat pituitary tumor cell line GH3. The growth of these cells are dependent upon thyroid hormones when plated at low-density in serum-free medium (Hohenwarter et al., 1996).

In addition, the morphology of these cells is also altered by THs in a dose-dependent manner. One form of the assay measures cell proliferation in response to TR agonists by the determination of the transformation of MTT tetraxolium salt into MTT fromazan by mitochondrial enzymes (Hohenwarter et al., 1996). This assay is performed on micro-well plates and can be considered a high through-put screen. Although this assay is relatively new, it has the potential to provide valuable information as a screen for chemicals that activate TR.

Section Summary: There are clear examples of environmental estrogens, both synthetic and naturally occurring, that bind to the estrogen receptor and act as agonists, antagonists or partial agonists (Gray et al., 1997). In addition, there are several chemicals found in the environment that act as anti-androgens (Gray and Kelce, 1996). There are no know environmental chemicals that act as either TR agonists or antagonists. While the hypothesis that environmental chemicals bind TR has been proposed, this hypothesis has not been adequately tested. Recent methodological developments resulting in high through-put screens could be performed on a limited number of chemicals to test this hypothesis. However, broad-based screening should reflect known biological mechanisms.

Developmental Assays.

The role of thyroid hormones in developing humans and other animals is well documented. Hypothyroidism during development leads to permanent alterations in a number of organ systems including the central nervous system and the male reproductive system. The sensitivity of developing animals may provide models for testing and screening chemicals that alter thyroid hormone catabolism or interfere with thyroid hormone signaling.

Neurodevelopmental Assays

The development of the central nervous system is dependent upon thyroid hormones for control of neuronal proliferation, initiation of neuronal differentiation, formation and development of neuronal processes and timely myelinization of the neurons (Porterfield, 1994). In humans, hypothyroidism induced by iodine deficiency results in neurologic endemic cretinism. This disorder is characterized by a high incidence of severe mental retardation, deaf-mutism, and problems with gross and fine motor coordination. Maternal hypothyroidism during pregnancy results in a increased incidence of neurologic and behavioral disorders in the offspring. In rodents maternal hypothyroidism produces a variety of behavioral and morphological changes in the brain similar to those observed in humans.

Morphological and Biochemical Assays in Developing Brains: Morphological and biochemical changes in the developing brain have been observed in animals exposed to agents that decrease thyroid hormone concentrations, such as PTU. For example, decreased brain weight occurs in rodents with marked decreases in serum THs during perinatal development (Balaza et al., 1968; Behnam-Rassoli et al., 1991). Perinatal hypothyroidism also results in morphological abnormalities in the organ of Corti (Deol, 1973; Uziel et al., 1981, 1983). Biochemical changes observed in hypothyroid animals include decreases in myelin basic protein and alterations in neurotransmitter concentrations among others (Porterfield, 1994). The morphological and biochemical changes induced by hypothyroidism are detectable when maternal, fetal or neonatal serum T4 concentrations are significantly decreased.

Behavioral Testing: Numerous behavioral assays have examined the effects of goitrogens or iodine deficiency in developing mammals. Hypothyroidism during development delays eye opening (Coner and Norton, 1982), reflex development (Coner and Norton, 1982) and weaning (Blake and Henning, 1985) in rodents. Depressed motor activity has also been demonstrated following developmental hypothyroidism (Rastogi et al., 1976). Exposure to PTU in the drinking water from gestational day 18 to postnatal day

21 produces delays in eye opening, reduced body weights, decreased or delayed preweaning motor activity, and increased postweaning motor activity (Goldey et al., 1995).

Similar to humans, developmental hypothyroidism in rodents permanently alters auditory function (Goldey et al., 1995a; Goldey et al., 1995b). These behavioral assays can be used to detect hypothyroidism, however, most of these behavioral changes may not be specific to hypothyroidism and have potential for a high rate of false negatives. More importantly, these behavioral changes occur only when there are significant decreases in serum T4 concentrations (Goldey et al., 1995a; Goldey et al., 1995b).

Male Reproductive System Development

Testes Size and Sperm Counts: Thyroid hormones are critical for developing humans and animals. The effects of hypothyroidism in infants can be severe and are permanent. Hypothyroidism in humans during the juvenile stage in boys is associated with megalotestis and high sperm counts. Maternal iodine deficiency or repeated exposure to goitrogens, such as PTU or PCBs during lactation increases testes size and sperm counts in rats when the animals reach maturity (Kirby et al., 1992; Cooke et al., 1993; Cooke et al., 1996). Similar findings have been reported in mice, hamsters and roosters. Conversely, neonatal hyperthyroidism results in the decreased testis size and lower sperm counts (van Haaster et al., 1993). Hence, testis weight and sperm counts can be used as measures of thyroid status in developing animals. An advantage of these measurements is their ease. Testes weights are simple to determine and methods to measure sperm counts have been developed over decades and are readily performed. A disadvantage of this assay is that it requires repeated dosing of the animals during lactation and a waiting period of several weeks prior to measuring the endpoints. In addition, these responses are observed only when there are significant decreases in serum thyroid hormones concentrations (Kirby et al. 1992; Cooke et al., 1993).

Section Summary: Hypothyroidism during development produces profound permanent change in the auditory system, central nervous system and the male reproductive system. A number of assays or test systems can be used to detect chemicals

that produce hypothyroidism. However, most of these assays or tests systems are time consuming and not necessarily specific for hypothyroidism. In addition, pronounced decreases in serum T4 concentrations are required to detect the behavioral or morphological changes. Alterations in serum THs can be detected at lower dose levels than those required to detect the behavioral and morphological changes in these systems. Because of the greater sensitivity and simplicity, determination of serum TH concentrations is recommended instead of these developmental assays. It should be remembered that using adult, pubescent or prepubescent animals may be qualitatively predictive of fetal response, it may not be quantitatively predictive of dose or response in fetal tissue.

Screening for Chemicals That Alter Thyroid Function, and Homeostasis in Non-Mammalian Wildlife.

Similar to mammalian systems, the thyroid and THs are critical in the development of amphibians, birds, fish and reptiles (Kaltenbach, 1996;McNabb, 1992; Eales and Brown, 1993). While there are similarities in the basic structure and function of the thyroid system among vertebrate species (Gorbman et al., 1993; Eales and Brown, 1993), there are also differences that must be considered when recommending tests of thyroid function. TRs have been cloned in one species of teleosts (Yamano and Inui, 1995), in two species of frogs (Tata, 1996), and in chickens (Lazar, 1993). TRs from all species examined show similar structure-binding activity relationships with regard to T4, T3 and their analogs (Galton, 1980; Darling et al., 1982; Bres and Eales, 1986; Sullivan, 1987). However, there are some differences in the regulation of THs by non-mammalian wildlife. In teleosts negligible amounts of T3 is synthesized and secreted (Eales and Brown, 1993) from the thyroid gland. The plasma proteins involved in transport of THs in teleosts bind T3 preferentially in contrast to the mammalian plasma proteins which bind T4 preferentially (Eales and Brown, 1993). The serum TH binding proteins in teleosts does not appear to be structurally related to transthyretin. While the serum binding protein

found in the bullfrog tadpole is a homolog of transthyretin, it preferentially binds T3 (Yamauchi et al., 1993) In addition, there are seasonal changes in thyroid hormones in teleosts that are not apparent in mammalian systems.

One important difference between mammals and fish and amphibians is the hypothalamic control of TSH from the pituitary. In teleosts, the hypothalamus negatively controls release of TSH, while in mammals it is positively controlled. In developing tadpoles, the hypothalamus positively controls TSH release via corticotropin releasing factor rather than TRH. TRH in tadpoles and adult frogs appear to play a role in osmoregulation by regulating prolactin release from the pituitary (Denver, 1996). This suggests that tests routinely used in rodents, such as the TRH challenge and the TTR binding assays, may not be uniformly applicable in non-mammalian species. Some of the assays used to assess thyroid function in rodents must be viewed cautiously when applied to non-mammalian systems.

Despite some of the species differences in TH regulation, there is a concordance between mammals and fish in response to many chemicals that alter thyroid hormone function or homeostasis (Eales and Brown, 1993). Chemicals which demonstrate significant differences in species sensitivity are the mono-ortho substituted PCBs which are extremely efficacious in decreasing plasma or serum T4 in rodents but have little effects on plasma TH in fish. Also several metals such as Hg and Cd alter TH concentrations in fish but not in mammals (Bleau et al., 1996; Hontella et al., 1996; Kirubagaran and Joy, 1995). Many of the assays described in previous sections could be used to examine chemical effects on TH function and homeostasis in fish and other wildlife if appropriately adapted for the species of interest. Alterations in thyroid function can be examined histologically in teleosts (Eales and Brown, 1993), similar to the mammalian system. However, it should be noted that the thyroid gland in teleosts is not encapsulated and consists of diffuse, scattered follicles making metrics like thyroid weights difficult to obtain. Because the thyroid consists of diffuse and scattered follicles, histological evaluation can be difficult, particularly for weak goitrogens. In fish, there

appear to be considerable control of the thyroid system via the mechanisms controlling peripheral T3 production (Eales and Brown, 1993). Consequently measures of deiodinase activities in conjunction with peripheral T4 assessments are required to thoroughly evaluate T3 availability to target tissues.

Tadpole Metamorphosis Assay: The development of tadpoles into frogs occurs in multiple stages with different organ systems developing at different times. Thyroid hormones are required for metamorphosis (Kaltenbach, 1996; Dodd et al., 1976; Kollros, 1961) but TH action is modulated by other hormones (Wright et al., 1994; Iwamuro and Tata, 1995) . In conjunction with T3, corticosterone accelerates metamorphosis at later stages of development (Galton, 1990).. Circulating prolactin concentrations increase toward the end of metamorphosis (Kaltenbach, 1996) and prolactin down regulates TR expression, apparently modulating the stimulatory action of T3 (Tata, 1996). Chemicals that alter tadpole development may not interact directly with TRs or directly alter TH concentrations, but may act indirectly by altering other endocrine pathways. In addition, chemicals that alter calcium homeostasis such as calmodulin antagonists also alter metamorphosis (Kumar et al., 1993) The tadpole metamorphosis assay may be a valuable tool for screening chemicals that alter TH signaling pathways either directly or indirectly. However, this assay requires further validation and standardization prior to use as a screen.

Section Summary: Thyroid hormones are critical in development for non-mammalian wildlife and there are examples of chemicals that alter thyroid hormones and produce alterations in non-mammalian wildlife. Many of the assays discussed for mammalian system can be used for non-mammals provided these assays are sufficiently modified to examine the non-mammalian species of interest. The tadpole metamorphosis assay is potentially useful as a screen but requires further validation and standardization.

Conclusions:

The workshop participants attempted to address the merits and limitations of numerous assays available as potential screening methods for chemicals that alter thyroid hormone action, function or homeostasis. Not every existing assay was examined due to limitations of time and expertise. Some combinations of the assays evaluated may be useful as screens. Chemicals appear to alter thyroid hormone action by either inhibiting synthesis of THs, altering serum binding to transport proteins or by increasing TH metabolism. Few if any environmentally relevant chemicals have been demonstrated to act as either TR agonists or antagonists. The development and implementation of screens should reflect the known mechanism of action of thyrotoxic chemicals.

Screening for chemicals using either thyroid histology or serum TH concentrations in mammals should provide tests that would produce few false negatives or false positives Subchronic studies in mammals examining thyroid histology provide the most useful measure of a chemicals thyrotoxic potency and efficacy. However, these assays are not necessarily screens, and require dosing animals for at least 2-6 weeks to observe consistent responses. Determination of serum TH concentrations in short-term tests may provide an adequate initial screen for these chemical in mammals. The exact dosing regimen and time course for these responses have not been adequately examined in the published literature. Determination of serum TH concentrations and thyroid histology may also be of value in teleosts however, indices of peripheral T3 production are better markers of thyroid status and should be included when determining the effects of chemicals on teleosts.

REFERENCES

Adams, NR. (1995). Organizational and activational effects of phytoestrogens on the reproductive tract of the ewe. Proc. Soc. Exper. Biol. Med. 208:87-91.

Atterwill, CK, and Aylward, SP (1995). Endocrine toxicology of the thyroid for industrial compounds. In "Toxicology of Industrial Compounds. H Thomas, R. Hess and F. Waechter eds. Taylor and Francis Publishers, pp257-280.

Atterwill, CK, Collins, P, Brown, CG, and Harland, RF (1987). The perchlorate discharge test for examining thyroid function in rats. J. Pharmacol. Methods. 18:199-203.

Barter, R and Klassen, C. (1992). UDP-glucuronosyltransferase inducers reduce thyroid hormone levels in rats by an extra thyroidal mechanism. Toxicol. Appl. Pharmacol. 111:36-42.

Bastomsky, CH (1977). Enhanced thyroxine metabolism and high uptake goiters in rats after a single dose of 2,3,7,8-tetrachlorodibenzo-p-dioxin. Endocrinology 101:292-296.

Berrodin, T. J., M. S. Marks, et al. (1992). Heterodimerization among thyroid hormone receptor, retinoic acid receptor, retinoid X receptor, chicken ovalbumin upstream promoter transcription factor, and an endogenous liver protein. Mol Endocrinol 6(9): 1468-78.

Balaza, R, Kovacs, S, Teichgraber, P, Cocks, WA, and Eayrs, JT (1968). Biochemical effects of thyroid deficiency on the developing brain. J. Neurochem. 15:1335-1349.

Behnam-Rassoli, M, Hebert, LC, Howard, V, Pharoah POD, and Stanisstreet, M (1991). Effect of propylthiouracil treatment during prenatal and early postnatal development on the neocortex of rat pups. Neuorendocrinology 53:321-327.

Blake, HH, and Hennings, SJ (1985). Effect of propylthiouracil dose on serum thyroxine, growth and weaning in young rats. Am J Physiol. 248:R524-R530.

Bres, O., and Eales, J.G. (1986). Thyroid hormone binding to isolated trout (Salmo gairdneri) liver nuclei in vitro; binding affinity, capacity and chemical specificity. Gen Comp. Endocrinol. 69:71-79.

Brouwer, A. (1991). The role of biotransformation in PCB-induced alterations in vitamin A and thyroid hormone metabolism in laboratory and wildlife species. Biochem. Soc. Trans. 19:731-7.

Brouwer, A, Klasson-Wehler, E, Bokdam, M, Morse, DC, and Traag, WA (1990). Competitive inhibition of thyroxine binding to transthyretin by monohydroxy metabolites of 3,4,3',4'-tetrachlorobiphenyl. Chemosphere 20:1257-1262.

Brouwer, A., Morse, DC, Lans, MC, Gerlienke Schuur, A., Murk, AJ, Klasson-Wehler, E., Bergman, A., and Visser, TJ (1998). Interactions of persistent environmental organohalogens with the thyroid hormone system: Mechanisms and possible consequences for animal and human health. Toxicol. Indust. Health 14:59-84.

Calvo. R, Obergon, MJ,. Ruiz de Ona, C, Escobar del Ray, F, Morreale de Escobar, G (1990). Congenital hypothyroidism, as studied in rats. Crucial role of maternal thyroxine but not of 3,5,3'-triiodothyronine in the protection of the fetal brain. J. Clin. Invest. 86:889-899.

Chopra, IJ (1977). A study of extra thyroidal conversion of thyroxine to 3,3',5-triiodothyronine in vitro. Endocrinology 101:453-463.

Christenson, WR, Becker, BD, Wahle, BS, Moore, KD, Das, PD, Lake, SG, Van Boethem, DL, Stuart, BP, Sangha, GK, and Thyssen, JH. (1996). Evidence of chemical stimulation of hepatic metabolism by an experimental acetanilide (FOE 5043) indirectly mediating reductions in circulating thyroid hormone levels in the male rat. Toxicol. Appl. Pharmacol. 29:251-259.

Cody, V. (1991). Thyroid hormone structure-function relationships. In, Werner and Ingbar's The Thyroid (Braverman, LE and Utiger, RD, eds) JB Lippincott Co., Philidelphia, pp. 887-919.

Colborn, T and Clement, C (eds). Chemically induced alterations in sexual and functional development: the wildlife/human connection. Advances in Modern environmental Toxicology, Vol XXI. Princeton, NJ:Princeton Scientific Publishing, 1992;401pp.

Coner, CP, and Norton, S. (1982). Effects of perinatal methimazole exposure on a developmental test battery for neurobehavioral toxicity in rats. Toxicol. Appl. Pharmacol. 63:133-141.

Cooke, PS, Kirby, JD, and Porcelli, J. (1993). Increased testis growth and sperm production in adult rats following transient neonatal goitrogen treatment: Optimization of the propylthiouracil dose and effects of methimazole. J. Reprod. Fertil. 97:493-499.

Cooke, PS, Zhao, Y-D, and Hansen, LD (1996). Neonatal polychlorinated biphenyl (PCB) treatement increases adult testis size and sperm production in the rat. Toxicol. Appl. Pharmacol. 136:112-117.

Darling, D.S., Dickhoff, W.W., and Borbman, A. (1982). Comparisons of thyroid hormone binding to hepatic nuclei of the rat and a teleost (Oncorhynchus kisutch). Endocrinology 111:1936-42.

Davis, K. D. and M. A. Lazar (1992). Selective antagonism of thyroid hormone action by retinoic acid. J Biol Chem 267(5): 3185-9.

Denver, RJ. (1996). Neuroendocrine control of amphibian metamorphosis. In L.I. Gilbert, J.R. Tata, and B.G. Atkinson, eds., *Metamorphosis: Postembryonic reprogramming of gene expression in amphibian and insect cells.* Academic Press, New York, New York USA, pp 433-464.

Dickhoff, W.W. and Sullivan, C.V. (1987). Involvement of the thyroid gland in smoltification , with special reference to metabolic and developmental processes. American Fisheries Society Symposium, 1:197-210.

Divi, R. L. and D. R. Doerge (1994). Mechanism-based inactivation of lactoperoxidase and thyroid peroxidase by resorcinol derivatives. Biochemistry 33(32): 9668-74.

Divi, R. L. and D. R. Doerge (1996). Inhibition of thyroid peroxidase by dietary flavonoids. Chem Res Toxicol 9(1): 16-23.

Dodd, MHL, and Dodd, JM (1976). The biology of metamorphosis. In: Lofts B (ed) Physiology of the Amphibia. Academic Press, New York, pp 467-599.

Doel, MS (1973). An experimental approach to understanding and treatment of hereditary syndromes with congenital deafness and hypothyroidism. J. Med. Genet. 10:2039-2045.

Doerge, D. R., C. J. Decker, et al. (1993). Chemical and enzymatic oxidation of benzimidazoline-2-thiones: a dichotomy in the mechanism of peroxidase inhibition. Biochemistry 32(1): 58-65.

Dohler, K-D, Wrong, CC, and von Ur. Muhlen, A (1979). The rat as model for the study of drug effects on thyroid function: consideration of methodological problems. Pharmac. Ther. 5:305-318.

Eales, JG, and Brown, SB (1993). Measurement and regulation of thyroidal status in teleost fish. Rev Fish Biol. Fisheries. 3:299-347.

Engler, D, and Burger, AG (1984). The deiodination of the iodothyronines and their derivatives in man. Endocr. Rev. 5:151-184.

Galton, V.A., (1980). Binding of thyroid hormones in vivo by hepatic nuclei of Rana catesbeiana tadpoles. Endocrinology, 106:859-870.

Galton, V. A. (1990). Mechanisms underlying the acceleration of thyroid hormone-induced tadpole metamorphosis by corticosterone. Endocrinology 127(6): 2997-3002.

Goldey, E.S., Rehnberg, GL, and Crofton, KM (1995a). Effects of Developmental hypothyroidism on auditory and motor function in the rat. Toxicol and Appl Pharmacol 135: 67-77.

Goldey, ES., Lau, C, Rehnberg, GL, and Crofton, KM (1995b). Developmental exposure to polychlorinated biphenyls (Aroclor 1254) reduces circulating thyroid hormone concentrations and causes hearing deficits in rats. Toxicol and Appl Pharmacol 135: 77-88.

Gorbaman, A., Dickhoff, W.W, Vigna, S.R., Clark, C.L., and Ralph, C.L. (1983). Comparative endocrinology. John Wiley and Sons, New York, New York.

Gray LE and Kelce, WR (1996). Latent effects of pesticides and toxic substances on sexual differentiation of rodents. Toxicol. Indust. Health. 12:515-531.

Gray LE et al., (1997). Endocrine Screening methods workshop report: Detection of estrogen and androgenic hormonal and antihormonal activity for chemicals that act via receptor or steroidogenic enzyme mechanisms. Reproductive Toxicology 5:719-750.

Green, WL (1978). Metabolism of thyroid hormones by the rat thyroid tissue in vitro. Endocrinology, 103:826-837.

Hill, RC, Erdreich, LS, Paynter, OE, Roberts, PA, Rosenthal, SL, and Wilkinson, CF (1989). Thyroid follicular cell carcinogenesis. Fund. Appl. Toxicol. 12:629-697.

Hohenwarter, O., Waltenberger, A., and Katinger, H. (1996). An in vitro system for thyroid hormone action. Anal. Biochem. 234:56-59.

Huang, T., Chopra, IJ, Beredo, A., Solomon, DH, Chua Teco, GN (1985). Skin is an active site of inner ring monodeiodination of thyroxine to 3,3',5-triiodothyronine. Endocrinology, 117:20106-2113.

Huang, T., Chopra, IJ, Boado, R., Solomon, DH, Chua Teco, GN (1988). Thyroxine inner ring monodeiodinating activity in fetal rat tissues. Pediatr. Res. 23:196-199.

Iwamuro, S. and J. R. Tata (1995). Contrasting patterns of expression of thyroid hormone and retinoid X receptor genes during hormonal manipulation of Xenopus tadpole tail regression in culture. Mol Cell Endocrinol 113(2): 235-43.

Juge Aubry, C. E., A. Gorla Bajszczak, et al. (1995). Peroxisome proliferator-activated receptor mediates cross-talk with thyroid hormone receptor by competition for retinoid X receptor. Possible role of a leucine zipper-like heptad repeat. J Biol Chem 270(30): 18117-22.

Kaltenbach, J.C. (1996). Endocrinology of amphibian metamorphosis. In L.I. Gilbert, J.R. Tata, and B.G. Atkinson, eds., *Metamorphosis: Postembryonic reprogramming of gene expression inamphibian and insect cells.* Academic Press, New York, New York USA, pp 403-431.

Kaplan, MM, Visser, TJ, Yaskoski, KA, and Leonard, JL (1983). Characteristics of iodothyronine tyrosil ring deiodination by rat cerebral cortical nicrosomes. Endocrinology 112:35-42.

Katz, D., M. J. Reginato, et al. (1995). Functional regulation of thyroid hormone receptor variant TR alpha 2 by phosphorylation. Mol Cell Biol 15(5): 2341-8.

Kavlock, RJ, Daston, GP, DeRosa, C., Fenner-Crisp, P (1996). Research needs for the risk assessment of health and environmental effects of endocrine disruptors: A report of the U.S. EPA-sponsored Workshop. Environ. Health Persp. 104 (4):715-740

Kirby, J. D., A. E. Jetton, et al. (1992). Developmental hormonal profiles accompanying the neonatal hypothyroidism-induced increase in adult testicular size and sperm production in the rat. Endocrinology 131(2): 559-65.

Kollros, JJ (1961). Mechanisms of amphibian metamorphosis: Hormones. Am. Zol. 1:107-114.

Kumar, B. A., K. R. Vinod, et al. (1993). Effect of calcium and calmodulin antagonists on metamorphosis in the anuran tadpole, Rana tigerina. Funct Dev Morphol 3(4): 237-42.

Lazar, M. A. (1993). Thyroid hormone receptors: multiple forms, multiple possibilities. Endocr Rev 14(2): 184-93.

Legrand, J (1979). Morphogenic actions of thyroid hormones. Trends Neurosci. 2:234-236.

McClain, R.M., Levin, A.A., Posch, R., and Downing, J.C. (1989). The effect of phenobarbital on the metabolism and excretion of thyroxine in rats. Toxicol. Appl. Pharmacol. 99:216-228.

McClain, R. M. (1995). Mechanistic considerations for the relevance of animal data on thyroid neoplasia to human risk assessment. Mutat Res 333(1-2): 131-42.

McKinney, JD, Fannin, R., Jordan, S, Chae, K., Rickenbacher, U., and Pedersen L (1987). Polychlorinated biphenyls and related compound interactions with specific binding sites for thyroxine in rat liver nuclear extracts. N. Med. Chem. 30:79-86.

McKinney JD, and Waller CL (1994). Polychlorinated biphenyls as hormonally active structural analogues. Environ. Health Persp. 102:290-297.

Meier, C. A., C. Parkison, et al. (1993). Interaction of human beta 1 thyroid hormone receptor and its mutants with DNA and retinoid X receptor beta. T3 response element-dependent dominant negative potency. J Clin Invest 92(4): 1986-93.

Morse, DC, Klasson-Wehler, E, Wesseling, W, Koeman, JH, and Brouwer, A. (1996). Alterations in rat brain thyroid hormone status following pre- and postnatal exposure to polychlorinated biphenyls (Aroclor 1254). Toxicol Appl. Pharmacol. 136:269-279.

Obregon, MJ, Ruiz de Ona, C., Calvo, R., Escobar del Rey, F., and Morreale de Escobar G. (1990). Outer ring iodothyronine deiodnases and thyroid hormone economy: responses to iodine deficiency in rat fetus and neonate. Endocrinology 129-2663-2673.

Olgivie, LM and Ramsden, DB (1988). Ioxynil and 3,5,3'-triiodothyronine: comparison of binding to human plasma proteins. Toxicol. Lett. 44:281-287.

Oppenheimer, JH, Schwartz, HL, Mariash, CN, Kinlaw, WB, Wrong, NCW, and Freake, HC. (1987). Advances in our understanding of thyroid hormone action at the cellular level. Endocr. Rev. 8:288-308.

Poole, A., Pritchard, D., Jones, R.B., Catto, L., and Leonard, T. (1990). In vivo biliary excretion and in vitro cellular accumulation of thyroxine by rats or cultured tat hepatocytes treated with a novel histamine H_1-receptor antagonist. Arch Toxicol. 64:474-481.

Porterfield, SP (1994). Vulnerability of developing brain to thyroid abnormalities: Environmental insults to the thyroid system. Environ Health Persp. 102(2):125-130.

Rastogi, RB, Lapierre, Y, and Singhal, RL (1976). Evidence for the role of brain biogenic amines in depressed motor activity seen in chemically thyroidectomized rats. J. Neurochem. 26:443-449.

Reginato, M. J., J. Zhang, et al. (1996). DNA-independent and DNA-dependent mechanisms regulate the differential heterodimerization of the isoforms of the thyroid hormone receptor with retinoid X receptor. J Biol Chem 271(45): 28199-205.

Ruiz de Ona, C., Morreale de Escobar, G., Calvo, R., Escobar del Rey, F, and Obregon, MJ (1991). Thyriod hormones and 5'-deiodinase in the rat fetus late in gestation: effects of maternal hypothyroidism. Endocrinology 128:422-432.

Silva, JE, Leonard, JL, Crantz, FR, and Larsen, PR. (1982). Evidence for two tissue-specific pathways for in vivo thyroxine 5'-deiodination in the rat. J. Clin. Invest. 69:1176-1184.

Specker, J.L. (1988). Preadaptive role of thyroid hormones in larval and juvenile salmon: Growth, the gut and evolutionary considerations. Am. Zool. 28:337-349.

Sullivan, CV, Darling, DS, and Dickhoff, WW (1987). Nuclear receptors for L-triiodothyronine in trout erythrocytes. Gen. Comp. Endocrinol. 65:149-160.

Surks, I. J. C., Cary N., Mariash, John T. Nicoloff, David H. Solomon (1990). American Thyroid Association guidelines for use of laboratory tests in thyroid disorders. Journal American Medical Association 263(11): 1529-1532.

Tata, J. R. (1994). Hormonal regulation of programmed cell death during amphibian metamorphosis. Biochem Cell Biol 72(11-12): 581-8.

Tata, J.R. (1996). Hormonal interplay and thyroid hormone receptor expression during amphibian metamorphosis. In L.I. Gilbert, J.R. Tata, and B.G. Atkinson, eds., *Metamorphosis: Postembryonic reprogramming of gene expression inamphibian and insect cells.* Academic Press, New York, New York USA, pp 466-503.

Uziel, A, Gabrion, J, Ohresser, M., and Legrand, C (1981). Effects of hypothyroidism on the structural develpoment of the organ of Corti in the rat. Acta Otolaryngol. 92:469-480.

Uziel, A., Legrand, C., Ohresser, M., and Marot, M. (1983). Maturational and degenerative processes in the organ of Corti after neonatal hypothyroidism. Hearing Res. 11:203-218.

Van Haaster, LH, de Jong, FH, Docter, R and de Rooji, DG (1993). High neonatal triiodothyronine levels reduce the period of Sertoli cell proliferation and accelerate tubular lumen formation in the rat testis, and increase serum inhibin levels. Endocrinology, 133:755-760.

Visser, TJ (1988) Metabolism of thyroid hormones. In Hormones and their action (BA Cooke, RJB King, and HJ van der Molen, eds) Elsevier, New York, vol. 1 pp. 81-103.

Visser, TJ, Kaplan, MM, Leonard, JL and Larsen, PR (1983). Evidence for two pathways of iodothyronine 5'-deiodination in rat pituitary that differ in kinetics propylthiouracil sensitivity and response to hypothyroidism. J. Clin. Invest. 71:992-1002.

Wright, M. L., L. J. Cykowski, et al. (1994). Anterior pituitary and adrenal cortical hormones accelerate or inhibit tadpole hind limb growth and development depending on stage of spontaneous development or thyroxine concentration in induced metamorphosis. J Exp Zool 270(2): 175-88.

Yamano, K. and Inui, Y. (1995). cDNA cloning of the thyroid hormone receptor beta for Japanese flounder. Gen. Comp. Endocrinol. 99:197-203.

Yamauchi, K., T. Kasahara, et al. (1993). Purification and characterization of a 3,5,3'-L-triiodothyronine-specific binding protein from bullfrog tadpole plasma: a homolog of mammalian transthyretin. Endocrinology 132(5): 2254-61.

Appendix 1

LIST OF ABBREVIATIONS

EDCs - Endocrine Disrupting Chemicals
Lactoperoxidase - LPO
PPAR - Proxisomal Proliferator-Activating Receptor
PTU - Propylthiouracil
rT3 - reverse T3
RXR - Retinoid X receptor
T3 - triiodothyronine
T4 - thyroxine
TBG - Thyroid Binding Globulin
TH - thyroid hormone
TPO - thyroid peroxidase
TR - thyroid receptor
TRH - Thyrotropin Releasing Hormone
TSH - Thyroid Stimulating Hormone
TTR - Transthyretin
UDP-GT - Uridine Diphosphate Glucuronlytransferase

Appendix 2

PARTICIPANTS

Lisa Biegel
Haskell Laboratory for Industrial Medicine
Molecular Carcinogenesis & Mutagenesis
PO Box 50 Elkton Rd.
Newark DE, 19714

Abraham Brouwer
Wageningen Agricultural University
Department of Toxicology
Tuinlaan 5
Wangeningen 6307
Netherlands

Scott Brown
National Water Resource Institute
Box 5050
867 Lake Shore Road
Burlington Ont L7R 4A6
Canada

Ann Olive Cheek
Tulane Medical Center
Center for Bioenvironmental Research
1430 Tulane Ave. SL3
New Orleans LA 70112

Francoise Brucker-Davis
World Wildlife Fund
1250 24th St, NW
6th Floor
Washington DC 20037

Russ Christensen
Bayer Corporation
Agriculture Division of Toxicology
17745 South Metcalfe
Stilwell Kansas 66085-9104

Paul Cooke
University of Illinois
College of Veterinary Medicine
2001 south Lincoln Ave
Urbana Ill, 61802

James Crissman
The Dow Chemical Co.
Health & Environmental Research Laboratory
1803 Building
Midland, MI 48674

Kevin Crofton
USEPA
Neurotoxicology Division
NHEERL (MD74-b)
RTP, NC 27711

Michael DeVito (Chair)
USEPA
Experimental Toxicology Division
NHEERL (MD-74)
RTP, NC 27711

Dan Doerge
Dept. Of Health and Human Services
Division of Chemistry
3900 NCTR Lane
Jefferson AR 72079-9502

Earl Gray
USEPA
Reproductive Toxicology Division
NHEERL (MD-72).
RTP, NC 27711

Peter Hauser
Psychiatry Services (116A)
VA Mdical Center
10 North Green Street
Baltimore MD 21201

Pamela Hurley
USEPA
Crystal Mall 2
1921 Jefferson Davis Highway
Arlington VA 22202

Michael Kohn
NIEHS
PO Box 12233
MD-A3-06
RTP, NC 27709

Jozef Lazar
Psychiatry Services (116A)
VA Mdical Center
10 North Green Street
Baltimore MD 21201

Michael McClain
Hoffmann-LaRoche Inc
Preclinical Development Administration
340 Kingsland Street
Nutley NJ 07110

Eugene McConnell
3028 Ethan Lane
Lawndale Est
Raleigh NC 27613

Suzanne McMaster
USEPA
NHEERL (MD-51A)
RTP, NC 27711

Joseph Tietge
USEPA
NHEERL/MED
6201 Cogdon Blvd.
Duluth, MN 55804

Shelly Tyl
Research Triangle Institute
Herman Laboratory Building
Room 45
PO Box 12194
RTP, NC 27711