Screening Methods for Thyroid Hormone Disruptors

Michael DeVito,[1] Lisa Biegel,[2] Abraham Brouwer,[3] Scott Brown,[4] Franciose Brucker-Davis,[5] Ann Oliver Cheek,[6] Russ Christensen,[7] Theo Colborn,[5] Paul Cooke,[8] James Crissman,[9] Kevin Crofton,[1] Dan Doerge,[10] Earl Gray,[1] Peter Hauser,[11] Pamela Hurley,[12] Michael Kohn,[13] Jozef Lazar,[11] Suzanne McMaster,[1] Michael McClain,[14] Eugene McConnell,[15] Christoph Meier,[16] Ronald Miller,[9] Joseph Tietge,[1] and Rochelle Tyl[17]

[1]National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, NC 27711 USA; [2]Haskell Laboratory for Industrial Medicine, Newark, DE 19714 USA; [3]Department of Toxicology, Wageningen Agricultural University, Wageningen, The Netherlands; [4]National Water Resource Institute, Burlington, Ontario, Canada; [5]World Wildlife Fund, Washington, DC 20037 USA; [6]Center for Bioenvironmental Research, Tulane Medical Center, New Orleans, LA 70112 USA; [7]Bayer Corporation Agriculture Division of Toxicology, Stillwell, KS 66085 USA; [8]College of Veterinary Medicine, University of Illinois, Urbana, IL 61802 USA; [9]The Dow Chemical Company, Midland, MI 48674 USA; [10]National Center for Toxicological Research, Jefferson, AR 72079 USA; [11]Psychiatry Services, Baltimore VA Medical Center, Baltimore, MD 21201 USA; [12]Office of Pesticide Prevention and Toxic Substances, U.S. Environmental Protection Agency, Washington, DC 20460 USA; [13]National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709 USA; [14]Hoffmann-LaRoche Inc., Preclinical Development Administration, Nutley, NJ 07110 USA; [15] Raleigh, NC 27613 USA; [16]Department of Genetics, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115 USA; [17]Research Triangle Institute, Research Triangle Park, NC 27709 USA

The U.S. Congress has passed legislation requiring the EPA to implement screening tests for identifying endocrine-disrupting chemicals. A series of workshops was sponsored by the EPA, the Chemical Manufacturers Association, and the World Wildlife Fund; one workshop focused on screens for chemicals that alter thyroid hormone function and homeostasis. Participants at this meeting identified and examined methods to detect alterations in thyroid hormone synthesis, transport, and catabolism. In addition, some methods to detect chemicals that bind to the thyroid hormone receptors acting as either agonists or antagonists were also identified. Screening methods used in mammals as well as other vertebrate classes were examined. There was a general consensus that all known chemicals which interfere with thyroid hormone function and homeostasis act by either inhibiting synthesis, altering serum transport proteins, or by increasing catabolism of thyroid hormones. There are no direct data to support the assertion that certain environmental chemicals bind and activate the thyroid hormone receptors; further research is indicated. In light of this, screening methods should reflect known mechanisms of action. Most methods examined, albeit useful for mechanistic studies, were thought to be too specific and therefore would not be applicable for broad-based screening. Determination of serum thyroid hormone concentrations following chemical exposure in rodents was thought to be a reasonable initial screen. Concurrent histologic evaluation of the thyroid would strengthen this screen. Similar methods in teleosts may be useful as screens, but would require indicators of tissue production of thyroid hormones. The use of tadpole metamorphosis as a screen may also be useful; however, this method requires validation and standardization prior to use as a broad-based screen. *Key words*: developmental toxicity, endocrine disruptors, neurobehavioral toxicity, screens, thyroid hormone, thyroid hormone receptors. *Environ Health Perspect* 107:407–415 (1999). [Online 8 April 1999]
http://ehpnet1.niehs.nih.gov/docs/1999/107p407-415devito/abstract.html

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 and adversely affects development in both humans and wildlife (*1–3*). Although 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 (*3*). 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) (*4*). A series of workshops sponsored by the Chemical Manufacturers Association, the EPA, and the World Wildlife Fund focused on the development of screens for endocrine-disrupting chemicals for both humans and wildlife (*5,6*). The following report is a consensus from the workshop entitled Screening Methods for Chemicals That Alter Thyroid Hormone Action, Function, and Homeostasis, held at Duke University, Durham, North Carolina, 20–23 June 1997.

The workshop focused on more than 20 assays or test systems that have been used to examine chemicals which alter synthesis, storage, transport, and catabolism of thyroxine (T_4) and 3,5,3′-triiodothyronine (T_3), assays that 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 nonmammalian wildlife models. The workshop focused on chemicals that alter thyroid gland function through pharmacodynamic means and did not include chemicals that were directly cytotoxic to the thyroid gland. The purpose of the workshop was not to recommend a screening battery or to deal with policy issues pertaining to the use of such screens; the product of the workshop was intended to describe and evaluate the methods that are currently available or could be developed in the near future for screening and testing.

Thyroid Function and Regulation

The thyroid gland produces T_4 and T_3. Thyroid hormones (THs) have two predominant functions. The first is a critical role in growth and development. One of the clearest examples of the importance of THs in growth and development is the metamorphosis of amphibians, in particular the metamorphosis of tadpoles into frogs (*7–9*). Other examples of the importance of THs in development are the transformation of salmon from freshwater-dwelling par to seawater-dwelling smolts (*10,11*), flounder metamorphosis (*12*), and development of the central nervous system in humans and other mammals (*13,14*). In humans, severe

Address correspondence to M. DeVito, U.S. EPA (MD-74), Research Triangle Park, NC 27711 USA.
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.
We thank the many people involved in helping conduct the workshop and prepare the report, especially J. Gaye (Duke University). In addition, the authors would like to thank M. Santostefano and B. Slezak for reviewing earlier drafts of this document.
Received 6 November 1998; accepted 28 January 1999.

hypothyroidism during development results in cretinism (*15,16*). The second major function of THs is to maintain metabolic homeostasis in mammals (*17*).

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 T_4. Although a small proportion of thyroid-localized TH is T_3, most T_3 comes from the deiodination of T_4 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 residues within thyroglobulin; 3) coupling of iodotyrosine residues to produce iodothyronines; 4) proteolysis of thyroglobulin and release of T_4 and T_3 into the blood; 5) binding to serum transport proteins; 6) target tissue synthesis of T_3 from T_4; 7) catabolism of T_4 and T_3 in peripheral tissues; and 8) catabolism and biliary elimination of THs in the liver. There are many examples of pharmaceutical, environmental, and naturally occurring chemicals that alter one or more of these processes in mammals; they have been reviewed by Hill et al. (*18*), Atterwill and Aylward (*19*), Brucker-Davis (*20*), and Gaitan (*21*).

The actions of thyroid hormones are mediated by their interaction with nuclear thyroid hormone receptors (TR). There are four known isoforms of TR that are derived from two genes—c-*erb*-A beta (TR $beta_1$ and $beta_2$) and c-*erb*-A alpha (TR $alpha_1$ and $alpha_2$) (*22*). TR $alpha_2$ does not bind TH. These receptors are part of the steroid receptor super family (*23*) and are the cellular homologs of the oncogene c-*erb*-A. These nuclear receptors share a common structure in that they have a ligand-binding domain in the carboxy-terminal region of the receptor protein and a DNA-binding domain in the amino-terminal region (*22,23*). The TRs can modulate the expression of thyroid-responsive genes by binding to specific base pair sequences, known as thyroid hormone response elements (TREs) in the regulatory region of these genes. TRs form homodimers and 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 TRs is 10–20 times greater for T_3 than for T_4 (*24,25*).

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. 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 toxicologic responses, has not been adequately tested. Because of 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 (the response pathognomonic for alterations in thyroid function), sensitivity (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 can be 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 United States, newborn infants are required to have blood samples collected for TSH and/or T_4 determinations prior to leaving the hospital. The American Thyroid Association has recommended determination of serum TSH and free T_4 concentrations (*26*) as the standard measure of thyroid function. Total T_4 was not recommended as a measure because false positives can be caused by conditions and pharmaceutical agents that alter thyroxine-binding globulin (TBG), the main serum-binding protein in humans. Changes in TBG concentrations alter total serum T_4 concentrations, but may not necessarily alter free T_4 concentrations. It is thought that free T_4 is available to enter the cell and that the concentrations of free T_4 are proportional to the tissue concentrations of T_3 and T_4. The American Thyroid Association considers a diagnosis of primary hypothyroidism confirmed if the patient has decreased free T_4 serum concentrations accompanied by increased serum TSH concentrations (*26*). Hyperthyroidism in humans is confirmed if the patient has increased free T_4 serum concentrations accompanied by suppressed serum TSH concentrations (*26*).

The synthesis of THs is tightly regulated. The THs provide the negative feedback on pituitary release of TSH. Therefore, decreases in serum THs due to inhibition of TH synthesis or transport or induction of catabolism leads to increased TSH release from the pituitary. The increased TSH, along with other growth factors, leads to 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 THs. If this compensatory mechanism is adequate, it can result in the normalization of serum T_4 and T_3 concentrations. Early in a chemical exposure, the serum concentration of THs will decrease, but eventually a steady-state is reached. Therefore, when examining chemical effects on serum TH concentrations at later time points in exposures, serum TH concentrations may have returned to normal because of these compensatory mechanisms (*27,28*). When designing experiments to examine the effects on serum THs, we must consider this compensatory mechanism and appropriate temporal relationships must be 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 T_3 by approximately two-fold over a 1-hr period, with initial increases occurring within 5 min (*29*). Circadian rhythms of THs occur in rats with peak serum concentrations occurring at approximately noon (*29*). In addition, there is some evidence of alterations in TH concentrations associated with stages of the estrous cycle (*29*). THs also change with age and increase strikingly in male rats from postnatal day 33 to 50 (*29*). These and several other confounding factors are reviewed by Dohler et al. (*29*). 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 because of the large interanimal variability, whereas small changes in TSH (20–30%) can have significant impact on thyroid gland function (*25,26*). Statistically significant changes in TSH can be detected if adequate sample sizes are used.

In humans, free T_4 and TSH serum concentrations are the preferred assays to assess thyroid function. In experimental animals, researchers have measured both free and total T_4 as well as T_3 and TSH serum concentrations (*27,30–32*). Determination of both free and total THs can provide complementary information that would guide further testing of a chemical. For example,

free T_4 is an indicator of the amount of hormone available for tissue uptake and for fetal transfer. It is T_4, not T_3, that primarily influences thyroid-dependent neurodevelopment. T_4 is the major form of TH that is taken up by the fetal or neonatal central nervous system (*33*).

An area that lacks adequate experimental data is the exact time course of the compensatory mechanism for the different classes of chemicals which alter serum TH concentrations. Time–course data for inducers of uridine diphosphate glucuronyltransferase (UDP-GT) indicate that continued dosing with these chemicals for 7, 14, and 25 days produces alterations in serum TH concentrations (*27,30,31,34*). Histologic changes in the thyroid or changes in thyroid weight occurred following 14 days of dosing (*27,28,30,34*). 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 that alter serum TH concentrations through different mechanisms.

The compensatory increases in TSH result in proliferation of the follicular cells in the thyroid gland. These changes can be detected histologically as increased follicular cell numbers and as increases in thyroid gland weight (*28,35*). These histologic changes appear less sensitive to confounders described previously and may provide a better assessment of thyroid function than serum hormone concentrations. Similar dose–response relationships among decreases in serum hormone concentrations, histologic changes in the thyroid, and increases in thyroid weight were observed in rats administered sulfamethazine for 4 weeks (*27*). 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 1970s, however, because follicular cell hypertrophy indicative of TSH stimulation was not considered pathologic change and may not have been reported. Caution must be used when using histologic changes as a screen; there are examples of chemicals that decrease THs without altering thyroid histology (*30*).

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 TH concentrations in animals following chemical exposure provides assessment of thyroid function equivalent to those used clinically in humans. However, because of potential compensatory mechanisms and the sensitivity of TH and TSH concentrations to stress and other factors, determination of serum TH alone has limitations. Histologic assessment of the thyroid gland should be performed in conjunction with the measurement of serum TH concentrations to provide a more complete assessment of thyroid function and TH action (*23,35*). Future efforts to determine the most appropriate time point and exposure regimen for examining serum TH concentrations are recommended. Again, caution must be used if thyroid histology alone is used as the marker for chemicals that disrupt THs because there is evidence of chemicals altering THs without altering thyroid histology.

Assays for chemicals that alter synthesis, secretion, transport, and catabolism of thyroid hormones. Changes in serum concentrations of THs can be caused by chemicals that inhibit thyroid hormone synthesis, release, and transport, and by chemicals that increase metabolism of THs. If a chemical decreases serum TH 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 that act through specific mechanisms. The assays described examine the synthesis and regulation of serum concentrations of THs.

Peroxidase assay. Thyroid peroxidases (TPOs) are the key enzymes in the synthesis of THs. 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 (*18–21*). In addition, there are a number of naturally occurring chemicals that inhibit thyroid peroxidase, such as goitrin, which is found in turnips and other cruciferous vegetables (*35*), and flavonoids, which are found in other plant products (*36*). TPOs have two functions: first is the iodination of tyrosine residues on thyroglobulin, and second is the coupling of specific di- and triiodotyrosyl residues on thyroglobulin. The iodination reaction can be readily determined using bovine serum albumin or tyrosine as substrates (*36*). In addition, the oxidation of guaiacol can be used as an indicator of thyroid peroxidase activity (*37*). All chemicals that inhibit the iodination reaction also inhibit the coupling reaction (*38*). The coupling reaction can be assayed using either human low iodine thyroglobulin, preiodinated casein, or guaiacol as substrates.

A disadvantage of the TPO assay is that purified hog TPO is the only form 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 (*37*) and LPO has been used as a model for TPO actions (*37*). Although TPO can be purified from experimental animals, the size of the gland is extremely small in rodents, 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 propylthiouracil (PTU) and sulfamethazine than is TPO from rodents (*39*). 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 TH synthesis and are unlikely to produce false positives. However, used alone as a screen, these assays have high potential for false negatives, as 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. Although there are no published methodologies that can be defined as high through-put screens, modification of this assay into a high through-put screen is under development in several laboratories.

Perchlorate discharge test. Perchlorate competes with iodide for thyroid uptake and could also promote the efflux of iodide from the follicular cells (*34,40,41*). A perchlorate discharge test has been used for decades in both animals and humans to detect iodide organification defects (*34,40–43*). In this assay, animals are exposed to a test chemical and then administered $Na^{125}I$ followed by perchlorate. Accumulation of ^{125}I in the thyroid is determined before and after administration of perchlorate. Perchlorate promotes the release of iodine that has not been incorporated into thyroglobulin. If a chemical inhibits or deactivates thyroid peroxidase, there would be a brisk decrease in the accumulation of ^{125}I in the thyroid gland. This assay has the potential for providing mechanistic information on the actions of chemicals that alter thyroid function, but it does not necessarily meet the requirements of a screen.

Thyrotropin-releasing hormone (TRH) challenge test. This assay examines the functional integrity of the hypothalamus–pituitary–thyroid axis (*34*). Briefly,

this assay measures TSH concentrations before and after challenge with TRH. Challenge with TRH should increase serum concentrations of TSH. A hyperreactive response is observed in the case of hypothyroidism, occurring at the level of the thyroid while a decreased response is observed in hypothyroidism of central origin. This assay has been used both clinically (*44*) and experimentally (*34*). The TRH challenge can also help to distinguish between pituitary and hypothalamic causes of hypothyroidism (*44*). Although 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 because of the limited number of chemicals which may act through this mechanism.

Serum protein-binding assays. In mammalian systems, the serum-binding proteins for THs are thyroid-binding globulin (TBG), transthyretin (TTR), and albumin. TBG and TTR are specific for THs and T_4 has a greater affinity for these serum-binding proteins than T_3 (*25*). TBG is present in humans and primates but is not present in rodents. It appears that TBG is responsible for the much longer half-life of T_4 and T_3 in humans as compared to other species, such as rodents (*25*). TTR is present in humans, rodents, and nonhuman primates. In humans, TBG is the predominant binding protein, whereas in rodents TTR is the predominant carrier of THs. TTR is also secreted by the choroid plexus and is present in the cerebral spinal fluid, suggesting a role in the transport of T_4 into the cerebral spinal fluid and eventually to periventricular areas of the brain (*45*). In addition, TTR transports T_4 into the fetus. There are a number of reports of chemicals that displace T_4 from TTR. The research on environmentally relevant chemicals has focused mainly on the polyhalogenated dibenzo-*p*-dioxins, biphenyls, and diphenylethers (*32,33,46–48*). The displacement of T_4 from serum binding proteins is hypothesized to increase the clearance of T_4 and decrease serum T_4 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 potential for high fetal accumulation (*33*).

These assays have been performed in several laboratories examining xenobiotics for several decades (*46,49,50*). Although these assays can be modified for high through-put screening they are specific for chemicals that compete with THs 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 nonmammalian 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 in this capacity

Deiodinase assays. In mammals, approximately 80% of the T_4 secreted by the thyroid gland is deiodinated in target tissues into either T_3, the most active form of the THs, or reverse T_3 (rT_3), an inactive iodothyronine (*51*). There are several enzymes involved in the deiodination of T_4, T_3, and their metabolites, and the expression of these proteins is tissue specific. Type I deiodinase catalyzes the 5′-deiodination of T_4, rT_3, and the sulfated metabolites of T_4 and T_3 (*52*). Type I deiodinase is sensitive to PTU inhibition and is found in liver, lung, kidney, pituitary, and thyroid (*53,54*). Type II deiodinase is present in the central nervous system, brown adipose tissue, anterior pituitary, and the placenta (*55–57*). Type II deiodinase is insensitive to PTU. In the brain, type II deiodinase converts T_4 into T_3 and ensures adequate brain concentrations of T_3 during critical periods of development (*57,58*) and during hypothyroidism (*59*). Type III deiodinase is resistant to PTU and catalyzes the conversion of T_3 and T_4 into 3,3′-diiodothyronine and rT_3, respectively, in brain, skin, placenta, and fetal tissues (*60–62*).

The deiodinase enzymes are critical in regulation of serum and tissue concentrations of THs. Decreases in serum concentrations of T_4 alter expression of the tissue deiodinases. For example, prenatal exposure to Aroclor 1254 increases brain type II deiodinase in rats with decreased serum T_4 (*63*). There are also tissue-specific and isoform-specific changes in deiodinases following thyroidectomy and T_4 and T_3 replacement in rats (*59*). Deiodinase assays have been used for decades to understand the metabolism of THs. Because the activity of these enzymes is dependent on the serum concentrations of these hormones, these assays would be sensitive toward chemicals that alter serum TH concentrations. However, alterations in deiodinase activity also alter serum TH concentrations. The relationship between serum TH concentrations and deiodinase activity may decrease the usefulness 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 T_4 is one of the major pathways of deactivation of T_4. In humans there is evidence of sulfation of T_4 as well. In mammals, there are at least three isoforms of UDP-GT that glucuronidate T_4 (*64*). Several classes of chemicals induce UDP-GTs responsible for the glucuronidation of T_4 (*17,23,31,32*). Induction of T_4 glucuronidation increases clearance and decreases serum concentrations of T_4. Induction of T_4 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 T_4 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 use as high through-put screening tests and at present are laborious. Additionally, although these assays provide data useful in understanding the mechanisms of action, not all chemicals that effect the thyroid produce alterations in T_4 glucuronidation.

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 T_3 concentrations, particularly in the fetus. This information could be useful in assessing the potential adverse effects of chemicals that disrupt TH homeostasis and tissue concentrations. These assays have been used to understand the mechanism of chemically induced alterations in serum concentrations of TH and TSH or changes in thyroid histopathology (*17,23,32, 50,65*). If these assays were to be used as initial screens, all of them would have to be performed to demonstrate that a chemical does not alter TH concentrations.

Thyroid hormone receptor binding and activation. Chemicals can alter thyroid hormone action by binding to TR. There are several isoforms of the receptors that have tissue specific localization (*19*). The structure–activity relationships for binding to the nuclear thyroid hormone receptor have been determined using crude nuclear homogenates (*20,21*) as well as various TR isoforms expressed in *Escherichia coli* or translated *in vitro* (*66,69*). These binding studies have focused on T_3 analogs and not on environmentally relevant chemicals (*24,25,66–69*). Several environmentally relevant classes of chemicals have been proposed to bind to the nuclear T_3 receptors, such as the polyhalogenated dibenzo-*p*-dioxin,

dibenzofurans, biphenyls, and diphenyl ethers (*47,48*). 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 chemicals that have been proposed to bind to TRs also decrease serum TH concentrations in experimental animals and are known thyroid hormone disruptors (*30,31,70*).

***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 (*66*). More recent studies have used various TR isoforms expressed in *E. coli* or translated *in vitro* (*66–69*). These assays require separating bound from free hormones using either filtering or chromatographic methods. Either separation method is cumbersome and time consuming. More recent advances have used solid-state binding assays using specific isoforms of TRs. The solid-state binding assays developed allow for high through-put screening. In the solid-state binding assays, the TR 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 ligands without the use of either filtering or chromatographic methods. Only three of the four TR isoforms have ligand-binding capability and two of these (TR $beta_1$ and TR $beta_2$) have identical ligand-binding domains. Binding assays are expected to have a 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 that 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 luciferase, beta-galactosidase, or choline acetyl transferase (*70*). Transformed yeast cell lines containing TR gene constructs have also been developed. In these systems, T_3 or other ligands to TR bind and activate the receptor, which then interacts with specific response elements upstream from the reporter gene and enhances 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 T_3 to determine agonist or antagonist properties. Similar systems have been used to examine the interactions of TR with different response elements (*71*), different cofactors (*72*), and with phosphorylation of TR (*73*). Although these systems have not been used for screening for environmental chemicals that are TR ligands, similar screens have been developed for estrogens and androgen agonists and antagonists (*4*).

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 among the assays used for estrogen, androgen, and thyroid hormone receptors. There are currently only two recognized mammalian estrogen receptors and a single androgen receptor, in contrast to the four isoforms of TR. TRs act predominately as heterodimers with RXR (*74–76*), whereas the estrogen and androgen receptors are active as homodimers. Both TR and the peroxisome proliferator-activated receptor (PPAR) form heterodimers with RXR and agonists of PPAR can alter TR mediated gene expression by binding and competing for RXR (*71*). Hence, chemicals might alter TR activation by altering RXR or PPAR pathways. TR activation is also regulated by phosphorylation (*72,73*), similar to the estrogen and androgen receptors. In designing a screen for TR ligands, chemicals may have different effects depending on the TR transfected, the response element used, and their interactions with PPAR and RXR. Because of the complexity of this system, several 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 polychlorinated biphenyls (PCBs) and the dioxins. The transformation assays in yeast have additional drawbacks in that for many chemicals entry into the yeast is limited because of the cell wall.

GH_3 cell assay for thyroid hormone action. An *in vitro* bioassay has been designed that can detect TR agonists (*77*). This assay uses the rat pituitary tumor cell line GH_3. The growth of these cells are dependent on TH when plated at low-density in serum-free medium (*77*). 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 monotetrazolium (MTT) tetraxolium salt into MTT fromazan by mitochondrial enzymes (*77*). This assay is performed on microwell plates and can be considered a high through-put screen. Although this assay is relatively new, it has the potential to provide information as a screen for chemicals that activate TR.

Section summary. Although there are no known environmental chemicals that act as either TR agonists or antagonists, there are clear examples of environmental chemicals, both synthetic and naturally occurring, that bind to the estrogen receptor and act as agonists, antagonists, or partial agonists (*3*). In addition, there are several chemicals found in the environment that act as antiandrogens (*78*). Although the hypothesis that environmental chemicals bind TR has been proposed, it has not been adequately tested. Recent methodological developments resulting in high through-put assays could be performed on a limited number of chemicals to test this hypothesis. However, broad-based screening should reflect known biologic mechanisms and at this time there is no evidence of any xenobiotic binding to the thyroid receptor.

Developmental assays. The role of THs 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 on thyroid hormones for control of neuronal proliferation, initiation of neuronal differentiation, formation and development of neuronal processes, and timely myelinization of the neurons (*14*). 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. Congenital hypothyroidism also results in cognitive impairment and growth delay. In addition, maternal hypothyroidism during pregnancy results in an increased incidence of neurologic and behavioral disorders in the offspring. In rodents maternal hypothyroidism

produces a variety of behavioral and morphologic changes in the brain similar to those observed in humans.

Morphologic and biochemical assays in developing brains. Morphologic 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 (*79,80*). Perinatal hypothyroidism also results in morphologic abnormalities in the organ of Corti (*81–83*). Biochemical changes observed in hypothyroid animals include decreases in myelin basic protein and alterations in neurotransmitter concentrations, among others (*14*). The morphologic and biochemical changes induced by hypothyroidism are detectable when maternal, fetal, or neonatal serum T_4 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 (*84*), reflex development (*84*) and weaning (*85*) in rodents. Decreased motor activity has also been demonstrated following developmental hypothyroidism (*86*). Exposure to PTU in drinking water from gestational day 18 to postnatal day 25 produces delays in eye opening, reduced body weights, decreased or delayed preweaning motor activity, and increased postweaning motor activity (*87*). Similar to humans, developmental hypothyroidism in rodents permanently alters auditory function (*87,88*). These behavioral assays can be used to detect hypothyroidism; however, most of these behavioral changes may not be specific to hypothyroidism and have the potential for a high rate of false negatives. More importantly, these behavioral changes occur only when there are significant decreases in serum T_4 concentrations (*87,88*).

Male reproductive system development. **Testes weight and sperm counts.** Hypothyroidism in humans during the juvenile stage 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 weight and sperm counts in rats when the animals reach maturity (*89–91*). Similar findings have been reported in mice, hamsters, and roosters. Conversely, neonatal hyperthyroidism results in decreased testes weights and lower sperm counts (*92*). Hence, testes weights 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. The 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 end points. In response to a thyrotoxic agent, these responses are observed only when there are significant decreases in serum TH concentrations (*89–91*). Additionally, testes weights and sperm counts can also be affected by other types of agents, such as estrogenic antiandrogenic compounds, as well as chemicals that have a direct effect on the testis.

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 test systems are time consuming and not necessarily specific for hypothyroidism. In addition, pronounced decreases in serum T_4 concentrations are required to detect the behavioral or morphologic changes. Alterations in serum THs can be detected at lower dose levels than those required to detect the behavioral and morphologic 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, whereas it may not be quantitatively predictive of dose or response in fetal tissue.

Screening for chemicals that alter thyroid function and homeostasis in nonmammalian wildlife. Similar to mammalian systems, the thyroid and THs are critical in the development of amphibians, birds, fish, and reptiles (*7–9*). Although there are similarities in the basic structure and function of the thyroid system among vertebrate species (*93,94*), there are also differences that must be considered when recommending tests of thyroid function. TRs have been cloned in one species of teleosts (*95*), in two species of frogs (*96*), and in chickens (*19*). TRs from all species examined show similar structure-binding activity relationships with regard to T_4, T_3, and their analogs (*97–100*). However, there are some differences in the regulation of THs by nonmammalian wildlife. In teleosts a negligible amount of T_3 is synthesized and secreted (*93*) from the thyroid gland. The plasma proteins involved in transport of THs in teleosts bind T_3 preferentially in contrast to the mammalian plasma proteins that bind T_4 preferentially (*93*). The serum TH binding proteins in teleosts do not appear to be structurally related to TTR (*101*). Although the serum-binding protein found in the bullfrog tadpole is a homolog of TTR, it preferentially binds T_3 (*102*). In addition, there are seasonal changes in THs in teleosts that are not apparent in mammalian systems.

One important difference among mammals, fish, and amphibians is the hypothalamic control of TSH from the pituitary. In teleosts, the hypothalamus negatively controls the release of TSH, whereas 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 appears to play a role in osmoregulation by regulating prolactin release from the pituitary (*103*). This suggests that assays routinely used in rodents, such as the TRH challenge and the TTR binding assays, may not be uniformly applicable to nonmammalian species. Some of the assays used to assess thyroid function in rodents must be viewed cautiously when applied to nonmammalian 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 TH function or homeostasis (*93*). An example of chemicals that demonstrate significant differences in species sensitivity are the *mono*-ortho substituted PCBs, which are efficacious in decreasing plasma or serum T_4 in rodents but have little effects on plasma TH in fish. Many of the assays described previously 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 (*93*), similar to the mammalian system. However, it should be noted that the thyroid gland in most teleosts is not encapsulated and consists of diffuse scattered follicles, making metrics like thyroid weights more difficult to obtain. This anatomical difference also makes histologic evaluation difficult, particularly for weak goitrogens. In fish, there appears to be considerable control of the thyroid system via the mechanisms controlling peripheral T_3 production (*93*). Consequently, measures of deiodinase activities in conjunction with peripheral T_4 assessments are required to thoroughly evaluate T_3 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. THs are required for metamorphosis (*7–9*) but TH action is modulated by other hormones

(*104,105*). In conjunction with T_3, corticosterone accelerates metamorphosis at later stages of development (*106*). Circulating prolactin concentrations increase toward the end of metamorphosis (*7*) and prolactin down regulates TR expression, apparently modulating the stimulatory action of T_3 (*107*). 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 (*108*). The tadpole metamorphosis assay may be a valuable tool for screening chemicals that alter TH signaling pathways either directly or indirectly.

One disadvantage of the tadpole assay is that it has not been validated and several questions need to be answered prior to use of this assay as a screen. There are a number of different classes of chemicals that alter TH synthesis, transport, and catabolism. Few of these chemicals have been examined in this assay. It is not clear whether the tadpole will respond to different inducers of UDP-GT in a manner similar to mammals or even to other aquatic species. Clearly this assay requires further validation and standardization prior to use as a screen.

Section summary. THs are critical in development for nonmammalian wildlife and there are examples of chemicals that alter THs and produce alterations in nonmammalian wildlife. Many of the assays discussed for mammalian system can be used for nonmammals provided these assays are sufficiently modified to examine the nonmammalian species of interest. The tadpole metamorphosis assay is potentially useful as a screen but requires further validation and 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 because of limitations of time and expertise. Some combinations of the assays evaluated may be useful as screens. Chemicals appear to alter the thyroid system 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.

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 chemical's thyrotoxic potency and efficacy. However, these assays are not necessarily screens, and they 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 chemicals 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 T_3 production are also required to evaluate thyroid status and should be included when determining the effects of chemicals on teleosts.

References and Notes

1. Adams NR. Organizational and activational effects of phytoestrogens on the reproductive tract of the ewe. Proc Soc Exper Biol Med 208:87–91 (1995).
2. Colborn T, Clement C. Chemically induced alterations in sexual and functional development: the wildlife/human connection. In: Advances in Modern Environmental Toxicology, Vol XXI. Princeton, NJ:Princeton Scientific Publishing, 1992;401.
3. Kavlock RJ, Daston GP, DeRosa C, Fenner-Crisp P, Gray LE, Kaattari S, Lucier G, Luster M, Mac MJ, Maczka C, et al. 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 Perspect 104:715–740 (1996).
4. Thomas Legislative Information on the Internet. Thomas bill text, 104th Congress, 1995–1996. Available: http://thomas.loc.gov/home/c104query.html [cited 22 March 1999].
5. Gray LE, Kelce WR, Wiese T, Tyl R, Gaido K, Cook J, Klinefelter G, Desaulniers D, Wilson E, Zacharewski T, et al. Endocrine screening methods workshop report: detection of estrogen and androgenic hormonal and antihormonal activity for chemicals that act via receptor or steroidogenic enzyme mechanisms. Reprod Toxicol 5:719–750 (1997).
6. Ankley G, Mihaich E, Stahl R, Tillitt D, Colborn T, McMaster S, Miller R, Bantle J, Campbell P, Denslow N, et al. Overview of a workshop on screening methods for detecting potential (anti-) estrogenic/androgenic chemicals in wildlife. Environ Toxicol Chem 17:68–87 (1997).
7. Kaltenbach JC. Endocrinology of amphibian metamorphosis. In: Metamorphosis: Postembryonic Reprogramming of Gene Expression in Amphibian and Insect Cells (Gilbert LI, Tata JR, Atkinson BG, eds). New York:Academic Press, 1966;403–431.
8. Dodd MHL, Dodd JM. The biology of metamorphosis. In: Physiology of the Amphibia (Lofts B, ed). New York:Academic Press, 1976;467–599.
9. Kollros JJ. Mechanisms of amphibian metamorphosis: hormones. Am Zool 1:107–114 (1961).
10. Dickhoff WW, Sullivan CV. Involvement of the thyroid gland in smoltification, with special reference to metabolic and developmental processes. Am Fish Soc Symp 1:197–210 (1987).
11. Specker JL. Preadaptive role of thyroid hormones in larval and juvenile salmon: growth, the gut and evolutionary considerations. Am Zool 28:337–349 (1988).
12. Inui T, Miwa K. Thyroid hormone induces metamorphosis in flounder larvae. Gen Comp Endocrinol 60:450–454 (1985).
13. Bernal J, Nunez J. Thyroid hormones and brain development. Eur J Endocrinol 133:390–398 (1995).
14. Dussault JH, Ruel J. Thyroid hormones and brain development. Annu Rev Physiol 49:321–334 (1987).
15. Legrand J. Morphogenic actions of thyroid hormones. Trends Neurosci 2:234–236 (1979).
16. Porterfield SP. Vulnerability of developing brain to thyroid abnormalities: environmental insults to the thyroid system. Environ Health Perspect 102:125–130 (1994).
17. Farrell AP, Braverman LE. Thyroid and antithyroid drugs. In: Goodman and Gilman's The Pharmacological Basis of Therapeutics (Hardmann JG, Limbird LE, Molinoff PB, Ruddon RW, Gilman AG, eds). New York:McGraw Hill, 1995;1383–1410.
18. Hill RC, Erdreich LS, Paynter OE, Roberts PA, Rosenthal SL, Wilkinson CF. Thyroid follicular cell carcinogenesis. Fundam Appl Toxicol 12:629–697 (1989).
19. Atterwill CK, Aylward SP. Endocrine toxicology of the thyroid for industrial compounds. In: Toxicology of Industrial Compounds (Thomas H, Hess R, Waechter F, eds). New York:Taylor and Francis Publishers, 1995;257–280.
20. Brucker-Davis F. Effects of environmental synthetic chemicals on thyroid function. Thyroid 8:827–856 (1998).
21. Gaitan E. Environmental goitrogens. In: The Thyroid Gland: Practical Clinical Treatise (Van Middlesworth L, ed). Chicago, IL:Year Book Medical Publ., 1986;263–280.
22. Evans RM. The steroid and thyroid hormone receptor super family. Science 240:889–895 (1988).
23. Lazar MA. Thyroid hormone receptors: multiple forms, multiple possibilities. Endocrinol Rev 14(2):184–193 (1993).
24. Oppenheimer JH, Schwartz HL, Mariash CN, Kinlaw WB, Wrong NCW, Freake HC. Advances in our understanding of thyroid hormone action at the cellular level. Endocrinol Rev 8:288–308 (1987).
25. Cody V. Thyroid hormone structure–function relationships. In: Werner and Ingbar's The Thyroid (Braverman LE, Utiger RD, eds). Philadelphia, PA:JB Lippincott Co., 1991;887–919.
26. Surks IJC, Mariash CN, Nicoloff JT, Solomon DH. American Thyroid Association guidelines for use of laboratory tests in thyroid disorders. JAMA 263(11):1529–1532 (1990).
27. McClain RM, Levin AA, Posch R, Downing JC. The effect of phenobarbital on the metabolism and excretion of thyroxine in rats. Toxicol Appl Pharmacol 99:216–228 (1989).
28. McClain RM. Mechanistic considerations for the relevance of animal data on thyroid neoplasia to human risk assessment. Mutat Res 333:131–148 (1995).
29. Dohler K-D, Wong CC, von zur Muhlen A. The rat as model for the study of drug effects on thyroid function: consideration of methodological problems. Pharmacol Ther 5:305–318 (1979).
30. Barter R, Klaassen C. UDP-glucuronosyltransferase inducers reduce thyroid hormone levels in rats by an extra thyroidal mechanism. Toxicol Appl Pharmacol 111:36–42 (1992).
31. Brouwer A. The role of biotransformation in PCB-induced alterations in vitamin A and thyroid hormone metabolism in laboratory and wildlife species. Biochem Soc Trans 19:731–737 (1991).
32. Brouwer A, Morse DC, Lans MC, Gerlienke Schuur A, Murk AJ, Klasson-Wehler E, Bergman A, Visser TJ. Interactions of persistent environmental organohalogens with the thyroid hormone system: Mechanisms and possible consequences for animal and human health. Toxicol Ind Health 14:59–84 (1998).
33. Calvo R, Obergon MJ, Ruiz de Ona C, Escobar del Ray F, Morreale de Escobar G. 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 (1990).
34. Christenson WR, Becker BD, Wahle BS, Moore KD, Das PD, Lake SG, Van Boethem DL, Stuart BP, Sangha GK, Thyssen JH. Evidence of chemical stimulation of hepatic metabolism by an experimental acetanilide (FOE 5043) in directly mediating reductions in circulating thyroid hormone levels in the male rat. Toxicol Appl Pharmacol 29:251–259 (1996).
35. Capen CC. Toxic responses of the endocrine system. In: Casarett and Doull's Toxicology: The Basic Science of Poisons (Klaassen CD, ed). New York:McGraw-Hill, 1995;617–640.

36. Divi RL, Doerge DR. Inhibition of thyroid peroxidase by dietary flavonoids. Chem Res Toxicol 9(1):16–23 (1996).
37. Divi RL, Doerge DR. Mechanism-based inactivation of lactoperoxidase and thyroid peroxidase by resorcinol derivatives. Biochemistry 33(32):9668–9674 (1994).
38. Doerge DR, Decker CJ, Takazawa RS. Chemical and enzymatic oxidation of benzimidazoline-2-thiones: a dichotomy in the mechanism of peroxidase inhibition. Biochemistry 32(1):58–65 (1993).
39. Takayama S, Aihara K, Onodera T, Akimoto T. Antithyroid effects of propylthiouracil and sulfamonomethoxine in rats and monkeys. Toxicol Appl Pharmacol 82:191–199 (1986).
40. Baschieri L, Benedetti G, deLuca F, Negri M. Evaluation and limitations of the perchlorate test in the study of thyroid function. J Clin Endcrinol Metab 23:786–791 (1963).
41. Atterwill CK, Collins P, Brown CG, Harland RF. The perchlorate discharge test for examining thyroid function in rats. J Pharmacol Methods 18:199–203 (1987).
42. Morgans ME, Trotter WR. Defective organic binding of iodine by the thyroid in Hashimoto's thyroiditis. Lancet 272:553–555 (1955).
43. Wolff J. Perchlorate and the thyroid gland. Pharmacol Rev 50:89–105 (1998).
44. Sarne DH, Refetoff S. Thyroid function tests. In: Endocrinology (DeGroot LJ, ed). Philadelphia, PA:WB Saunders Co, 1995;617–664.
45. Hebert J, Wilcox JN, Pham KT, Fremeau RT Jr, Zeviani M, Dwork A, Soprano DR, Makover A, Goodman DS, Simmerman EA. Transthyretin: a choroid plexus-specific transport protein in human brain. The 1986 S. Weir Mitchell award. Neurology 36:900–911 (1986).
46. Brouwer A, Klasson-Wehler E, Bokdam M, Morse DC, Traag WA. Competitive inhibition of thyroxine binding to transthyretin by monohydroxy metabolites of 3,4,3′,4′-tetrachlorobiphenyl. Chemosphere 20:1257–1262 (1990).
47. Van den Berg KJ, van Raaji JAGM, Bragt PC, Notten WRF. Interactions of halogenated industrial chemicals with thransthyretin and effects on thyroid hormone levels *in vivo*. Arch Toxicol 65:15–19 (1991).
48. McKinney JD, Waller CL. Polychlorinated biphenyls as hormonally active structural analogues. Environ Health Perspect 102:290–297 (1994).
49. Lans MC, Spiertx C, Brouwer A, Koeman JH. Different competition of thyroxine binding to transthyretin and thyroxine-binding globulin by hydroxy-PCBs, PCDDs, and PCDFs. Eur J Pharmacol 170:129–136 (1994).
50. Ogilvie LM, Ramsden DB. Ioxynil and 3,5,3′-triiodothyronine: comparison of binding to human plasma proteins. Toxicol Lett 44:281–287 (1988).
51. Engler D, Burger AG. The deiodination of the iodothyronines and their derivatives in man. Endocrinol Rev 5:151–184 (1988).
52. Visser TJ. Metabolism of thyroid hormones. In: Hormones And Their Action, Vol 1 (Cooke BA, King RJB, van der Molen HJ, eds). New York:Elsevier, 1988;81–103.
53. Chopra IJ. A study of extra thyroidal conversion of thyroxine to 3,3′,5-triiodothyronine *in vitro*. Endocrinology 101:453–463 (1977).
54. Green WL. Metabolism of thyroid hormones by the rat thyroid tissue *in vitro*. Endocrinology 103:826–837 (1978).
55. Silva JE, Leonard JL, Crantz FR, Larsen PR. Evidence for two tissue-specific pathways for *in vivo* thyroxine 5′-deiodination in the rat. J Clin Invest 69:1176–1184 (1982).
56. Visser TJ, Kaplan MM, Leonard JL and Larsen PR. 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 (1983).
57. Ruiz de Ona C, Morreale de Escobar G, Calvo R, Escobar del Rey F, Obregon MJ. Thyroid hormones and 5′-deiodinase in the rat fetus late in gestation: effects of maternal hypothyroidism. Endocrinology 128:422–432 (1991).
58. Obregon MJ, Ruiz de Ona C, Calvo R, Escobar del Rey F, Morreale de Escobar G. Outer ring iodothyronine deiodinases and thyroid hormone economy: responses to iodine deficiency in rat fetus and neonate. Endocrinology 129:2663–2673 (1991).
59. Escobar-Morreale HF, Obregon MJ, Hernandez, A, Escobar del Ray F, Morreale de Escobar G. Regulation of iodothyronine deiodinase activity as studied in thyroidectomized rats infused with thyroxine or triiodothyronine. Endocrinology 138:2559–2568 (1997).
60. Kaplan MM, Visser TJ, Yaskoski KA, Leonard JL. Characteristics of iodothyronine tyrosil ring deiodination by rat cerebral cortical microsomes. Endocrinology 112:35–42 (1983).
61. Huang T, Chopra IJ, Beredo A, Solomon DH, Chua Teco GN. Skin is an active site of inner ring monodeiodination of thyroxine to 3,3′,5-triiodothyronine. Endocrinology 117:2106–2113 (1985).
62. Huang T, Chopra IJ, Boado R, Solomon DH, Chua Teco GN. Thyroxine inner ring monodeiodinating activity in fetal rat tissues. Pediatr Res 23:196–199 (1988).
63. Morse DC, Klasson-Wehler E, Wesseling W, Koeman JH, Brouwer A. Alterations in rat brain thyroid hormone status following pre- and postnatal exposure to polychlorinated biphenyls (Aroclor 1254). Toxicol Appl Pharmacol 136:269–279 (1996).
64. Visser TJ, Kaptein E, van Toor H, van Raaij JAGM, van den Berg KJ, Tjin CT, van Engelen JGM, Brouwer A. Glucuronidation of thyroid hormone in rat liver: effects of *in vivo* treatment with microsomal enzyme inducers and *in vitro* assay conditions. Endocrinology 133:2177–2186 (1993).
65. Poole A, Pritchard D, Jones RB, Catto L, Leonard T. *In vivo* biliary excretion and *in vitro* cellular accumulation of thyroxine by rats or cultured rat hepatocytes treated with a novel histamine H1-receptor antagonist. Arch Toxicol 64:474–481 (1990).
66. Cheng SY, Gansom SC, McPhie P, Bhat MK, Mixson AJ, Wintraub B. Analysis of the binding of 3,3′,5-triiodo-L-thyronine and its analogues to mutant human beta 1 thyroid hormone receptors: a model of the hormone binding site. Biochemistry 33:4319–4326 (1994).
67. Schueler PA, Schwartz HL, Strait KA, Mariash CN, Oppenheimer JH. Binding of 3,5,3′-triiodothyronine (T3) and its analogs to the *in vitro* translational products of c-*erb*A protooncogenes: differences in the affinity of the alpha- and beta-forms for the acetic acid analog and failure of the human testis and kidney alpha-2 products to bind T3. Mol Endocrinol 4:227–234 (1990).
68. Van Beeren HC, Bakker O, Wiersinga WM. Structure–function relationship of the inhibition of the 3,5,3′-triiodothyronine binding to the alpha(1)- and beta(1)-thyroid hormone receptor by amiodarone analogs. Endocrinology 137:2807–2814 (1996).
69. Van Birgelen APJM, Smit EA, Kampen IM, Groeneveld CN, Fase KM, Van der Kolk J, Poiger H, Van den Berg M, Koeman JH, Brouwer A. Subchronic effects of 2,3,7,8-TCDD or PCBs on thyroid hormone metabolism: Use in risk assessment. Eur J Pharmacol 293:77–85 (1995).
70. Allegreto EA, Heyman RA. Intracellular receptor characterization and ligand screening by transactivation and hormone-binding assays. Methods Mol Genet 8:405–420 (1996).
71. Yen PM, Chin WW. New advances in understanding the molecular mechanisms of thyroid action. Trends Endocrinol Metab 5:65–72 (1994).
72. Katz D, Reginato MJ, Lazar MA. Functional regulation of thyroid hormone receptor variant TR alpha 2 by phosphorylation. Mol Cell Biol 15(5):2341–2348 (1995).
73. Bhat MK, Ashizawa K, Cheng SY. Phosphorylation enhances the target gene sequence-dependent dimerization of thyroid hormone receptor with retinoid X receptor. Proc Natl Acad Sci USA 91:7927–7931 (1994).
74. Berrodin TJ, Marks MS, Ozato K, Linney E, Lazar MA. 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):1468–1478 (1992).
75. Reginato MJ, Zhang J, Lazar MA. 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): 28199–28205 (1996).
76. Meier CA, Parkison C, Chen A, Ashizawa K, Meier-Heusler SC, Muchmore P, Cheng SY, Weintraub BD. 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):1986–1993 (1993).
77. Hohenwarter O, Waltenberger A, Katinger H. An *in vitro* system for thyroid hormone action. Anal Biochem 234:56–59 (1994).
78. Gray LE, Kelce WR. Latent effects of pesticides and toxic substances on sexual differentiation of rodents. Toxicol Ind Health 12:515–531 (1996).
79. Balaza R, Kovacs S, Teichgraber P, Cocks WA, Eayrs JT. Biochemical effects of thyroid deficiency on the developing brain. J Neurochem 15:1335–1349 (1968).
80. Behnam-Rassoli M, Hebert LC, Howard V, Pharoah POD, Stanisstreet M. Effect of propylthiouracil treatment during prenatal and early postnatal development on the neocortex of rat pups. Neuroendrocrinology 53:321–327 (1991).
81. Doel MS. An experimental approach to understanding and treatment of hereditary syndromes with congenital deafness and hypothyroidism. J Med Genet 10:2039–2045 (1973).
82. Uziel A, Gabrion J, Ohresser M, Legrand C. Effects of hypothyroidism on the structural development of the organ of Corti in the rat. Acta Otolaryngol 92:469–480 (1981).
83. Uziel A, Legrand C, Ohresser M, Marot M. Maturational and degenerative processes in the organ of Corti after neonatal hypothyroidism. Hearing Res 11:203–218 (1983).
84. Coner CP, Norton S. Effects of perinatal methimazole exposure on a developmental test battery for neurobehavioral toxicity in rats. Toxicol Appl Pharmacol 63:133–141 (1982).
85 Blake HH, Hennings SJ. Effect of propylthiouracil dose on serum thyroxine, growth and weaning in young rats. Am J Physiol 248:R524–R530 (1985).
86. Rastogi RB, Lapierre Y, Singhal RL. Evidence for the role of brain biogenic amines in depressed motor activity seen in chemically thyroidectomized rats. J Neurochem 26:443–449 (1976).
87. Goldey ES, Rehnberg GL, Crofton KM. Effects of developmental hypothyroidism on auditory and motor function in the rat. Toxicol Appl Pharmacol 135: 67–77 (1996).
88. Goldey ES, Lau C, Rehnberg GL, Crofton KM. Developmental exposure to polychlorinated biphenyls (Aroclor 1254) reduces circulating thyroid hormone concentrations and causes hearing deficits in rats. Toxicol Appl Pharmacol 135:77–88 (1996).
89. Kirby JD, Jetton AE, Cooke PS, Hess RA, Bunick D, Ackland JF, Turek FW, Schwartz NB. Developmental hormonal profiles accompanying the neonatal hypothyroidism-induced increase in adult testicular size and sperm production in the rat. Endocrinology 131(2): 559–565 (1992).
90. Cooke PS, Kirby JD, Porcelli J. 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 (1993).
91. Cooke PS, Zhao Y-D, Hansen LD. Neonatal polychlorinated biphenyl (PCB) treatment increases adult testis size and sperm production in the rat. Toxicol Appl Pharmacol 136:112–117 (1996).
92. Van Haaster LH, de Jong FH, Docter R, de Rooji DG. 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 (1993).
93. Eales JG, Brown SB. Measurement and regulation of thyroidal status in teleost fish. Rev Fish Biol Fisheries 3:299–347(1993).
94. Gorbaman A, Dickhoff WW, Vigna SR, Clark CL, Ralph CL. Comparative Endocrinology. New York:John Wiley & Sons, 1983.

95. Yamano K, Inui Y. cDNA cloning of the thyroid hormone receptor beta for Japanese flounder. Gen Comp Endocrinol 99:197–203 (1995).
96. Tata JR. Hormonal regulation of programmed cell death during amphibian metamorphosis. Biochem Cell Biol 72(11–12):581–588 (1994).
97. Galton VA. Binding of thyroid hormones *in vivo* by hepatic nuclei of *Rana catesbeiana* tadpoles. Endocrinology 106:859–870 (1980).
98. Darling DS, Dickhoff WW, Borbman A. Comparisons of thyroid hormone binding to hepatic nuclei of the rat and a teleost (*Oncorhynchus kisutch*). Endocrinology 111:1936–1942 (1982).
99. Bres O, Eales JG. Thyroid hormone binding to isolated trout (*Salmo gairdneri*) liver nuclei *in vitro*; binding affinity, capacity and chemical specificity. Gen Comp Endocrinol 69:71–79 (1986).
100. Sullivan CV, Darling DS, Dickhoff WW. Nuclear receptors for L-triiodothyronine in trout erythrocytes. Gen Comp Endocrinol 65:149–160 (1987).
101. Babin PJ. Binding of thyroxine and 3,5,4′-triiodothyronine to trout plasma proteins. Am J Physiol 262(Endocrinol Metabol 25):E710–E720 (1992).
102. Yamauchi K, Kasahara T, Hayashi I, Horiochi R. 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):2254–2261 (1993).
103. Denver RJ. Neuroendocrine control of amphibian metamorphosis. In: Metamorphosis: Postembryonic Reprogramming of Gene Expression in Amphibian and Insect Cells (Gilbert LI, Tata JR, Atkinson BG, eds). New York:Academic Press, 1996;433–464.
104. Wright ML, Cykowski LJ, Lundrigan L, Hemond KL, Kochran DM, Faszewski EE, Anuszewski CM. 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):175–188 (1994).
105. Iwamuro S, Tata JR. 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):235–243 (1995).
106. Galton VA. Mechanisms underlying the acceleration of thyroid hormone-induced tadpole metamorphosis by corticosterone. Endocrinology 127(6):2997–3002 (1990).
107. Tata JR. Hormonal interplay and thyroid hormone receptor expression during amphibian metamorphosis. In: Metamorphosis: Postembryonic Reprogramming of Gene Expression in Amphibian and Insect Cells (Gilbert LI, Tata JR, Atkinson BG, eds). New York:Academic Press, 1996;466–503.
108. Kumar BA, Vinod KR, Paul VF, Pilo B. Effect of calcium and calmodulin antagonists on metamorphosis in the anuran tadpole, *Rana tigerina*. Funct Dev Morphol 3(4): 237–242 (1992).