An Examination of Thyroid Function For the Assessment of the Applicability Of Several Assays for use as Screens For Thyroid Function Disruptors

by
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Introduction

On August 3, 1996 the Food Quality Protection Act (FQPA) was signed by President Clinton. Under Title IV of this law are amendments to the Federal Food, Drug, and Cosmetic Act, the purpose of which according to House Report number 104-669(II) are to "modernize the regulation of pesticides" (Bliley, T, 1996 p29.). Within these amendments, under section 405, "Tolerances and Exemptions for Pesticide Chemical Residues," subsection (p), is a congressional mandate for the United States Environmental Protection Agency (EPA) to "develop a screening program, using appropriate validated test systems, and other scientifically relevant information to determine whether certain substances may have an effect in humans similar to an effect produced by a naturally occurring estrogen, or other such endocrine effects as the Administrator may designate" by August 1998 (Food Quality Protection Act). The law states that this testing is to be performed on all pesticide chemicals and "any other substance that may have an effect that is cumulative to an effect of a pesticide chemical if the Administrator determines that a substantial population may be exposed to such substance." This subsection, requiring the screening program, was first offered during Committee mark-up by Representative George E. Brown Jr. of California (Bliley, T, 1996). Representative Brown indicates that the belief that endocrine disrupters "interfere with fundamental biological functions in humans and other organisms" and the suggested link between endocrine disrupters and breast cancer, motivated him in the proposition of this amendment (Brown, G., 1996). This screening program is to be implemented by August 1999 and a year later a progress report must be made to Congress (Food Quality Protection Act).

Under the Federal Advisory Committee Act, the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) was chartered in October 1996, to assist EPA in

establishing the mandated protocols (Office of Prevention Pesticides and Toxic Substances, 1997(a)). EDSTAC is composed of representatives from industry, academia, state and federal government, public health, environmental and labor organizations, and small business (Office of Prevention Pesticides and Toxic Substances. 1997(b)). EDSTAC is addressing a variety of issues in the development of its recommendations to EPA. The endocrine systems on which EDSTAC is focusing are the estrogenic, androgenic and thyroid systems. Other issues include but are not limited to human health and ecological effects as well as the effects of both pure compounds and common mixtures (Office of Prevention Pesticides and Toxic Substances, 1997(b)).

At the EDSTAC February Plenary Session committee members reasserted their consideration of thyroid function as equal to, not secondary to the systems regulating sex steroids (Office of Prevention, Pesticides and Toxic Substances, 1997(c)). Thyroid hormones and factors within the system of thyroid function influence the functions of almost every organ in the body. Thyroxine (T4) and 3,5,3'-triiodothyronine (T3) are the hormones produced by the thyroid gland and they stimulate cellular oxygen consumption, and hence basal metabolism, in a variety of tissues, like heart and skeletal muscle as well as tissue from the liver, kidney, pancreas and pituitary gland. The mechanisms by which these functions are stimulated are uncertain. While thyroid hormone binding sites have been found in mitochondrial plasma membrane in some cell lines, their biological significance is unknown (Davis, P. J., 1991). T3, the more biologically active of the two thyroid hormones, has been demonstrated, in many cases, to act via reaction with nuclear hormone receptors (Jameson, J. L. and DeGroot, L. J., 1995). However a combination of nuclear and extranuclear thyroid hormone activity cannot be ruled out (Davis, P. J., 1991).

Thyroid hormones have been demonstrated to elicit pleiotropic effects in a variety of cell types through both positive and negative transcription regulation (Jameson, J. L. and DeGroot, L. J., 1995). Examining the effects of hyper- or hypothyroidism can illuminate the extent of thyroidal influence over a variety of organ functions. Table 1. Gives a concise overview of these effects in humans. The exact mechanisms in the elicitation of most of these effects have yet to be fully elucidated.

In heart muscle tissue, thyroid hormone has been demonstrated to influence the expression of myosin heavy chain isoforms (Balkman, C., *et al.*, 1992; Izumo, S., *et al.*, 1987; Umeda, P. K., *et al.*, 1987) as well as Na,K-ATPase isoforms (Orlowski, J. and Lingrel, J. B., 1990). There are two isoforms, α and β, of myosin heavy chains(MHC). Their

Table 1.* The Effects of Hypothyroidism and Hyperthyroidism

Body system function	Hypothyroid	Hyperthyroid
Growth and development	Impaired growth	Accelerated growth
Activity and sleep	Decreased activity and increased sleep	Increased activity and decreased sleep
Temperature tolerance	Intolerance to cold	Intolerance to heat
Skin characteristics	Coarse and dry	Smooth
Perspiration	Absent	Excessive
Pulse	Slow	Rapid
Gastrointestinal symptoms	Constipation, decreased appetite, increased weight	Frequent bowel movements, increased appetite, decreased weight
Reflexes	Slow	Rapid
Psychological aspects	Depression and apathy	Nervous and emotional

*adapted from Table 18.11 in S. I. Fox Human Physiology p584.

combination within the structure of myosin protein distinguishes the myosin isozymes, V1 ($\alpha\alpha$), V2 ($\alpha\beta$), and V3 ($\beta\beta$) (Balkman, C., *et al.*, 1992; Izumo, S., *et al.*, 1987; Umeda, P. K., *et al.*,

1987). Thyroid hormones have been shown to stimulate the transcription of α-MHC mRNA and repress the transcription of β-MHC mRNA, this corresponds to an increase in cardiac tissue V1 content and a decrease in V3 (Balkman, C., *et al.*, 1992; Izumo, S., *et al.*, 1987; Umeda, P. K., *et al.*, 1987). α-MHC's contain a higher concentration of ATPase's compared with β-MHC's, imparting greater contractility and shorter contraction periods to muscle tissue high in α-MHC. Na,K-ATPase plays an essential role in the electrical activity muscle, modulating contractility (Orlowski, J. and Lingrel, J. B., 1990). A study by Orlowski and Lingrel (1990) demonstrate thyroid hormone dependent regulation of the expression of the different Na,K-ATPase α and β-subunits. Thyroid hormone regulation of these two proteins probably contributes to the modulation in pulse associated with hypo- or hyperthyroidism, as shown in Table 1. A list of other genes regulated by thyroid hormones can be found in the appendix. Modulation of these and other genes, contributes to the symptoms of thyroid hormone imbalance.

Proper thyroid hormonal balance is crucial for normal growth and development. In amphibians thyroid hormone regulates metamorphosis with a close correlation between metamorphic stages and cellular expression of thyroid hormone receptors (Yaoita, Y. and Brown, D. D., 1990). In mammals severe mental retardation, deaf-mutism, improper skeletal development and organ maturation can all be produced by instances of hypothyroidism at critical stages of development in the affected systems (Schwartz, H., 1983; Thorpe-Beeston J. G. and Nicolaides K. H., 1996). Thyroid hormones appear to play more of a role in cellular differentiation and maturation, as opposed to cellular proliferation. However, thyroid hormone does enhance some activities of growth hormone in many mammals and stimulates its transcription in rat anterior pituitary cells (Schwartz, H., 1983; Yaffe, B. M. and Samuels, H. H., 1984; Ye, Z., *et al*., 1988).

Both intrauterine and postnatal ossification is regulated by thyroid hormone. Hyperthyroid conditions cause accelerated skeletal maturation, leading to stunted growth. A delay in skeletal maturation is observed in hypothyroid rat fetuses and children with acquired hypothyroidism (Schwartz, H., 1983). Normal patterns of ossification return with administration of T4, but only in epiphyses developing subsequent to the administration. Epiphyses developing under hypothyroid conditions remain dysgenic with T4 administration (Schwartz, H., 1983). Thyroid hormone regulation of brain and CNS maturation is also limited by developmental stages (Schwartz, H., 1983; Thorpe-Beeston J. G. and Nicolaides K. H., 1996).

Processes of neuronal development including synaptogenesis (Nicholson, J. L. and Altman, J., 1972), myelination, and cellular differentiation and migration are under thyroid hormone control (Schwartz, H., 1983). In humans the critical stage for these events is the period between mid-gestation and two years, postnatal (Porterfield, S. P., 1994). Abnormal neural morphology has been observed in neonates with congenital hypothyroidism (Schwartz, H., 1983). The resulting mental retardation can be diminished with early T4 replacement therapy, but some researchers have reported residual behavioral and cognitive problems increasing with the length of delay before T4 treatment (Schwartz, H., 1983). Similar effects have been observed in rats, but the studies have been indicative of a lesser role for fetal thyroid hormone in rat fetal development (Morreale de Escobar, G., *et al.*, 1993; Schwartz, H., 1983). At birth, the developmental stage of the rat is considered to be equivalent to that of a human fetus entering third trimester, this could contribute to the differences in the role of fetal thyroid function observed between the two species (Schwartz, H., 1983).

Several environmental contaminants have been demonstrated to interrupt thyroid function. A decrease in serum levels of thyroid hormone can increase serum levels of

thyrotropin, as explained later in this paper. The increased levels of thyrotropin can stimulate hyperplasia within thyroid tissue. The enlargement of the thyroid gland, due to this hyperplasia is referred to as a goiter. Many environmental contaminants are goitrogens, included among these are resorcinol, amitrole and ethylenebis[dithiocarbimate] (EBDC), through the action of its metabolite ethylenethiourea (ETU) (Hill *et al.*, 1989). Resorcinol is usually associated with plants, humic substances and coal. It has been identified in water supplies receiving effluent from coal processing plants, from areas with endemic goiter (Cooksey, R.C., *et al.*, 1985; Jolley, R.L., *et al.*, 1986; Lindsay, R.H., *et al.*, 1986; Lindsay, R.H., *et al.*, 1992,). Ethylenebis[dithiocarbimate] (EBDC) is a class of fungicides, which were applied to over one third of the fruit and vegetable crops in the U. S. in 1990 (Doerge D. R., and Takazawa, R. S., 1990). Amitrole is an herbicide. As will be discussed later, all of these compounds are known to interrupt thyroid gland hormonogenesis in at a common point in the system of thyroid function (Divi, R. L. and Doerge, D. R., 1994; Doerge, D. R. and Niemczura, W. P., 1989; Doerge D. R., and Takazawa, R. S., 1990). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), mixtures of polychlorinated biphenyls (PCB) and mixtures of polybrominated biphenyls (PBB) have also been implicated in the modulation of thyroid function (Bahn, A. K., *et al.*, 1980; Barter, .R. A. and Klaassen, C. D., 1992; Barter, .R. A. and Klaassen, C. D., 1994; Goldey, E. S., *et al.*, 1995; Gray, L. E., *et al.*, 1993; Juarez de Ku, L. M., *et al.*, 1994; Koopman-Esseboom, C., *et al.*, 1994; Morse, D. C., *et al.*, 1996; Ness, D. K., *et al.*, 1993; Sewall, C. H., *et al.*, 1995).

In the laboratory, rats treated with PCB's, in particular aroclor 1254, show significant reductions in serum T4 and this effect appears to be more pronounced in perinatally exposed offspring compared with adults(Barter, .R. A. and Klaassen, C. D., 1992; Goldey, E. S., *et al.*, 1995; Gray, L. E., *et al.*, 1993; Morse, D. C., *et al.*, 1996). But the data from an

epidemiological study in a PCB contaminated area do not demonstrate such dramatic effects on thyroid status (Koopman-Esseboom, C., *et al.*, 1994). This study is part of a larger study termed the Dutch PCB/Dioxin study examining the possible adverse effects of these pollutants on human beings. The Koopman-Esseboom study (1994) examined T3, T4 and thyrotropin levels in serum, and PCB congeners in breast milk, from 78 mother-infant pairs in a PCB contaminated area near Rotterdam, the Netherlands. All but one mother-infant pair showed thyroid hormone and thyrotropin serum levels within the normal range for their respective age group (Koopman-Esseboom, C., *et al.*, 1994). However, when mother-infant pairs were placed into groups according to the toxic equivalency (TE) of the PCB congeners identified in the breast milk, there was an inverse relationship between the TE of the PCB congeners and the levels of serum T4 in the offspring (Koopman-Esseboom, C., *et al.*, 1994).

A later study from this same Dutch PCB/Dioxin study examined the effect of perinatal exposure to PCB's on neonatal neurological development (Huisman, M., *et al*., 1995). Mother-infant pairs from the same area of the Netherlands were divided into groups in which infants were either breast-fed or bottle-fed. The premise of this separation being that the breast-fed infants would receive a higher dose of PCB's through the breast milk, compared with the bottle-fed infants (Huisman, M., *et al*., 1995). Data from this study demonstrate a slight decrease in muscle tone and neurological development in the breast-fed infants compared with the bottle-fed infants. In this study the effect of PCB on neurological development is somewhat similar to the decrease in neurological development seen in congenitally hypothyroid children (Frost G. J. and Parkin, J. M., 1986). Another epidemiological study examined children from a region in Taiwan where a large percentage of the population had been exposed to high levels of PCB's from consumption of PCB contaminated cooking oil in 1978 through 1979 (Chen, Y. J., et al., 1992).

The children were all born after the PCB poisoning incident and all displayed poorer cognitive development compared with matched controls (Chen, Y. J., et al., 1992). The level of cognitive development of these children is similar to that observed in hypothyroid children (Frost G. J. and Parkin, J. M., 1986), but there are no corresponding measures of serum thyroid hormone for the children in the Chen (1992) study. The connection between PCB, thyroid status and the effects associated with PCB poisoning is not very clear from the available epidemiological studies, more compelling evidence can be found from laboratory studies.

Two separate studies with rats demonstrate a correlation between a PCB dose-dependent drop in serum T4 levels and a change in T4-dependent development. Development of the organ of Corti in the middle ear has been shown to be dependent on thyroid hormone (Meyerhoff, W. L., 1976) and as mentioned above, hearing loss and deaf-mutism are associated with congenital hypothyroidism. A study by Goldey (1995 a) demonstrates that pre- and postnatal exposure to araclor 1254 produces not only a PCB dose-dependent drop in serum T4 in developing rats, PCB dose-dependent hearing loss was also demonstrated. The effect was similar to the results obtained in a companion study by the same group in which hearing loss was observed in developing rats made hypothyroid by 6-n-propyl-2-thiouracil (PTU) administration (Goldey, E. S., *et al*., 1995 b). PTU is commonly used in laboratories to create hypothyroid conditions in test animals. Juarez de Ku (1994) presents a stronger demonstration of PCB action through interruption of thyroid function.

In the Juarez de Ku (1994) study, rats were exposed both pre and postnatally to PCB's. From postnatal days 4-14, some of the developing rats exposed to PCB's received replacement doses of either T3 or T4. The exposed rats, which did not receive replacement thyroid hormones, the activity of choline acetyltransferase (ChAT) was greatly reduced in the

hippocampus and forebrain. The authors indicate that the development of these regions is important in learning and memory (Juarez de Ku , L. M., et al., 1994). Replacement of T4 but not T3 produced an increase in ChAT activity to 86% of the control group, indicating that the PCB induced drop in ChAT could be due in part to the PCB dependent drop in serum T4 (Juarez de Ku, L. M., et al., 1994).

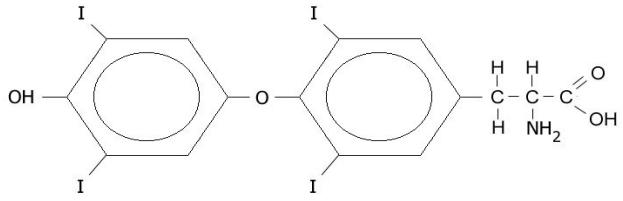
The effect of many environmental compounds on thyroid function is clearly established in the laboratory setting, and can be seen in the field as with cases of resorcinol induced goiter. However, the elicitation of toxic effects by these compounds through interruption of thyroid function has not yet been clearly established in epidemiological studies. More research may uncover these types of mechanisms in the field. Laboratory studies indicate that there is an interaction of environmental contaminants and thyroid function in the elicitation of several toxic effects. The wording of the FQPA indicates that the screening and testing program should apply to estrogenic compounds, but left the ultimate determination of the program focus to the Director of the EPA. Thyroid function was chosen as a foci of the EDSTAC in the development of the mandated screening and testing program because of its importance in growth and development (Tyl, R., 1997), and because as indicated by the EDSTAC chair, Lynn Goldman, "…panel members believed there is a sufficient research base upon which to build a program. And that there is sufficient evidence of documented effects to warrant it." (Goldman, L., 1997)

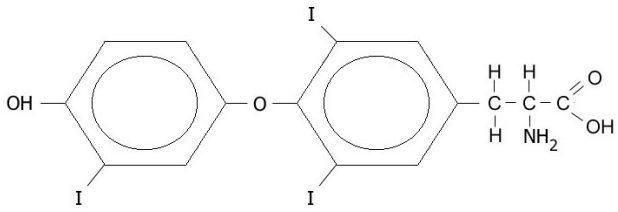
Though not expressly related to the activities of EDSTAC, a three day workshop "on screening methods for endocrine disruptors in the thyroid" was convened at the Nicholas School of the Environment in June of 1997 (McMaster, S., 1997). One of the organizers is an EDSTAC member (McMaster, S., 1997; Office of Prevention, Pesticides and Toxic Substances, 1997(d)) and several of the attendant panelists are members of the Screening and Testing Workgroup

within EDSTAC (McMaster, S., 1997; Office of Prevention, Pesticides and Toxic Substances, 1997(e)). The purpose of the workshop was not the recommendation of a screening battery, but rather discussion of methods currently available which may be adapted for use as tier 1 screens for disruption of thyroid function (McMaster, S., 1997). The workshop panel discussed many assays and currently used procedures, which showed promise for use as screens. To shed light on a small fraction of the knowledge required to implement the program mandated by Congress, this paper presents an overview of thyroid function, showing the different areas of thyroid function that can be affected by endocrine disruptors. Within the context of thyroid function, several of the assays and procedures discussed at the workshop will be examined for their applicability as potential screens for disruptors of thyroid function.

Thyroid gland production and release of T4 and T3

Thyroxine (T4)





3',3,5,-Triiodothyronone (T3)

T4 and T3 are produced and released from the thyroid follicles. These follicles consist of a lumen, which is filled with a protein rich fluid known as colloid, and lined with principal cells (Fox, S.I., 1987). Iodide (I^-) is actively transported from extracellular fluid to the lumen by these principal cells (Taurog, A, 1991). Crossing into the lumen, the I^- is oxidized by an apical membrane bound protein, thyroid peroxidase (TPO), in the presence of hydrogen peroxide. TPO

then catalyzes the iodination of tyrosyl residues as well as the coupling of iodinated tyrosyl residues within a colloid protein, thyroglobulin, in the synthesis of the thyroid hormones (Magnusson, R.P., et al, 1984; Taurog, A, 1991.; Taurog, A, et al, 1996.)

Thyroglobulin, the matrix on which T4 and T3 are synthesized, is a dimeric, 660 kd glycoprotein (Dunn, J.T., 1991). In initial synthesis, the monomers which make-up thyroglobulin are identical, but after glycosylation and iodination, thyroglobulin is a heterodimer (Dunn, J.T., 1991). Thyrotropin or thyroid stimulating hormone (TSH), an enzyme released from the pituitary gland, can influence the structure of thyroglobulin. Iodination stabilizes thyroglobulin dimerization (Dunn, J.T., 1991). Though human thyroglobulin has about 134 tyrosyl residues, not all are available for iodination and hormonogenesis. This trend appears common for other species studied (Dunn, J.T., 1991). Only two to four molecules of thyroid hormone are produced from each molecule of thyroglobulin (Taurog, A, 1991).

The first step in the proposed mechanism of hormonogenesis is a two electron oxidation of TPO by peroxide to form compound I (Magnusson, R.P., et al, 1984; Nakamura M., *et al.*, 1984; Taurog, A, 1991; Taurog, A, et al, 1996.). There are two forms of compound I. Initially one electron is removed from the iron (FeIII) within the TPO heme structure, forming an oxoferryl group (FeIV). A porphyrin π-cation radical is formed when the second electron is withdrawn from the porphyrin ring. *In vitro* this form can then spontaneously isomerize to a protein radical, the most stable form of compound I, in which an electron is withdrawn from a nearby amino acid residue to replace the one withdrawn from the porphyrin ring (Doerge, D.R. and Divi, R.L., 1995; Taurog, A, et al, 1996.). The exact mechanism of hormonogenesis is unknown, though it is recognized that TPO catalyzes both iodination and coupling. It has been proposed that the porphyrin π-cation radical isomer is involved in iodination while the protein

radical form catalyzed coupling (Taurog, A, 1991), while other researchers have proposed that the porphyrin π-cation radical isomer is involved in both processes (Taurog, A., *et al.* 1996).

Iodination occurs in a sequential fashion (Dunn, J.T., 1991; Taurog, A, 1991; Turner, C.D., *et al.*, 1983; Xiao, S., *et al.*, 1996.). The porphyrin π-cation radical oxidizes iodide ions. An enzyme bound hypoiodite species is formed which iodinates thyroglobulin tyrosyl residues (Taurog, A, 1991; Taurog, A., *et al.* 1996). The first sites of iodination are hormonogenic sites. The sites of iodination and the sequence of iodination appear to be determined by the structure of the thyroglobulin molecule itself, rather than interactions between thyroglobulin and TPO (Turner, C.D., *et al.*, 1983; Xiao, S., *et al.*, 1996.). Experiments which have utilized both chemical and TPO catalyzed iodination have shown that the same thyroglobulin tyrosyl residues are iodinated with either catalyst (Turner, C.D., *et al.*, 1983; Xiao, S., *et al.*, 1996.). There is, however, an increase in diiodotyrosine (DIT) production over monoiodotyrosine (MIT) with TPO catalyzed iodination compared with the chemical catalyst (Xiao, S., *et al.*, 1996.).

Coupling of iodinated tyrosyl residues does not occur until there is a sufficient concentration of DIT within the thyroglobulin molecule. Hormonogenesis is an intramolecular event, although it is not yet been determined whether coupling occurs between or within the two polypeptide chains of thyroglobulin (Taurog, A, 1991). The proposed coupling mechanism begins with the generation of DIT radicals within the thyroglobulin matrix, catalyzed by TPO, in the presence of peroxide (Taurog, A, 1991). Two DIT radicals then couple to form a quinol ether intermediate within the matrix. In the case of T3 formation, coupling would take place between a DIT radical and a MIT radical, which would be contributing the outer phenol ring. Non-enzymatically, the quinol ether intermediate splits, leaving a dehydroalanine residue at the

site of phenol contribution (Taurog, A, 1991). The newly formed iodothyronines are stored within the thyroglobulin matrix.

As mentioned above, resorcinol, amitrole and ethylenethiourea are environmentally relevant chemicals known to disrupt thyroid function in this area of hormonogenesis (Hill *et al.*, 1989). The herbicide, amitrole, and resorcinol are referred to as suicide inhibitors as both types of these compounds have been shown to irreversibly inhibit the enzymatic activity of TPO (Divi, R. L. and Doerge, D. R., 1994; Doerge, D. R. and Niemczura, W. P., 1989). Ethylenethiourea, the fungicide metabolite, inhibits hormonogenesis by acting as an alternate substrate in the catalytic action of TPO (Doerge D. R., and Takazawa, R. S., 1990).

The mechanism of resorcinol TPO inactivation proposed by Divi and Doerge involves the oxidation of resorcinol by either isomer of TPO compound I to generate a resorcinol radical and either an oxoferryl or protein radical compound II. The oxoferryl compound II has been shown to isomerize to the protein radical form (Deme, D., *et al.*, 1985). Inactivation becomes irreversible when the resorcinol radical covalently binds to the protein radical compound II so that the synthesis of more TPO is necessary for hormonogenesis. Enzymatic activity could not be restored to recorcinol inactivated TPO or lactoperoxidase by dialysis or chromatocentrifugation (Divi, R. L. and Doerge, D. R., 1994). The exact binding of the resorcinol intermediate on the compound II isomer is unknown, however, changes in the visible spectrum of the inactivated enzyme indicate a change in the prostetic heme structure. The binding ratio appears to be 10 mol of resorcinol to 1 enzyme equivalent (Divi, R. L. and Doerge, D. R., 1994).

Amitrole is a known goitrogen and has also been shown to covalently bind to peroxidase. In this experiment lactoperoxidase (LPO) was used instead of TPO, but the two peroxidases are

similar in structure and activity (Doerge, D. R. and Niemczura, W. P., 1989). LPO is commonly used in experiments examining model TPO systems. As in the incidence of recorcinol inactivation, a radical amitrole intermediate is involved in covalent binding to the enzyme. However, in the case of amitrole inactivation, there is no change in the visible spectrum of inactivated LPO. This is an indication that the binding of amitrole and LPO does not involve the prostetic heme active site of the enzyme. More evidence supporting amitrole binding to the protein moitey of the enzyme is the observed binding of amitrole to cyano-inactivated LPO, in which there should be no involvement of the heme group with amitrole binding (Doerge, D. R. and Niemczura, W. P., 1989).

As mentioned above, ETU acts as a competitive substrate for an intermediate in the TPO catalyzed iodination of thyroglobulin tyrosyl residues (Doerge D. R., and Takazawa, R. S., 1990). The enzyme bound hypoiodite species (EOI), involved in iodination, will bind ETU thereby inhibiting thyroid hormonogenesis at the level of thyroglobulin tyrosyl residue iodination. Unlike the aforementioned chemically induced inhibitions, ETU inhibition is reversible. In experiments with ETU and TPO, ETU appears to be consumed in a catalytic process with EOI, in the presence of peroxide and iodide ion (Doerge D. R., and Takazawa, R. S., 1990). In this same set of experiments, tyrosyl iodination resumed after total consumption of ETU, supporting the assertion that ETU inhibition is reversible.

Barring inhibition from the aforementioned or similar compounds, thyroid hormone is released from thyroglobulin upon thyroidal thyrotropin (TSH) stimulation. Hormone release from the thyroglobulin matrix occurs in the follicular cells, but the mechanism of thyroglobulin transfer, from the lumen, is as yet uncertain. Two mechanisms have been observed, macropinocytosis and micropinocytosis (Dunn, A. D., 1996.). The occurrence of

either or both of these mechanisms appears to be species dependent, and can be affected by various physiological changes (Bernier-Valentin, F., *et al.*, 1991; Dunn, A. D., 1996).

TSH induced macropinocytosis in rat thyroid tissue was reported by Wetzel, Spicer and Wollman in 1965 (Wetzel, B., 1965). TSH was administered to rats, which were killed at different time intervals after TSH administration. The thyroid lobes were immediately removed and fixed *post mortem*. Five minutes after TSH administration, there was the development of apical follicular membrane pseudopod activity into the follicular lumen. Within the newly forming pseudopodia there were rare appearances of small droplets of luminal colloid. At 10 minutes post-administration, these droplets were more numerous in the pseudopodia and there were several droplets appearing in the apical cytoplasm. Pairs of adjacent pseudopodia were observed to have joined, engulfing fairly large volumes of colloid (Wetzel, B., 1965). Once formed, the colloid droplets appeared to exhibit basipetal migration. A transition, from relatively large, less dense, homogenous colloid droplets to smaller, denser, heterogeneous granules appeared to occur during this migration. Some of the large colloid droplets were observed to be associated with dense granules. Acid phosphatase activity within the droplets was observed within 15 minutes after TSH administration. The amount of a reaction product, indicating acid phosphatase activity, was seen to increase in the droplets as they became more dense and basally located (Wetzel, B., 1965).

Their observation of the bulk retrieval of colloid via pseudopodia engulfment and subsequent proteolysis of thyroglobulin within the colloid droplets led this research group to propose that this mechanism plays a major role in the release of thyroid hormones from the thyroglobulin matrix (Wetzel, B., 1965). However, it has been asserted that observations of thyroid pseudopodia and colloid droplets are rare except in rodents, and this process has been

documented only in thyroid glands stimulated by supraphysiological concentrations of TSH (Bernier-Valentin, F., *et al.*, 1991; Dunn, A., 1996). So the physiological importance of this process in the release of thyroid hormones in other species is questionable. An alternative mechanism for hormone release is the process of micropinocytosis (Bernier-Valentin, F., *et al.*, 1991; Dunn, A. D., 1996; Seljelid, R., *et al.*, 1970).

Micropinocytosis, the formation of vesicles by invagination of the cell membrane, has been observed in rodent thyroid tissue (Seljelid, R., *et al.*, 1970), as well as porcine thyroid tissue (Bernier-Valentin, F., *et al.*, 1990; Bernier-Valentin, F., *et al.*, 1991). In these studies, the colloid containing endocytotic vesicles were observed in the presence or absence of TSH stimulation, however, their formation was increased with TSH stimulation (Bernier-Valentin, F., *et al.*, 1990; Bernier-Valentin, F., *et al.*, 1991; Seljelid, R., *et al.*, 1970). Of special significance is the formation of these vesicles in TSH stimulated porcine thyroid tissue, in the absence of pseudopod or colloid droplet formation, which further emphasizes the questionable physiological significance of macropinocytosis in non-rodent species. (Bernier-Valentin, F., *et al.*, 1990; Bernier-Valentin, F., *et al.*, 1991).

A study by Kostrouch (1991) reports observations of the transition of thyroglobulin containing follicular coated vesicles from early endosomes, with strong staining for mannose-6-phosphate receptors (MPR), to late endosomes which stained for both MPR and arylsulfatase-A (ArS-A), a lysosomal enzyme. The early endosomes were found predominately in the apical regions of the cells, while late endosomes were more basally localized. Thyroglobulin concentrations within the endosomes, measured via gold complexed thyroglobulin, decreased with the transition from early to late endosome phases. Follicular lysosomes, which stained strongly for ArS-A, but had lost MPR staining, were also characterized in this study, but did not

show the presence of thyroglobulin (Kostrouch, Z. et. al, 1991). The morphology of the vesicles and the progression of enzymatic activity observed in this study led the authors of this paper to compare the endocytotic processing of thyroglobulin to endosomal transport and proteolysis studied extensively in other tissues like baby hamster kidneys (Kostrouch, Z. et. al, 1991).

Most endocytotic processes begin with the binding of the ligand, which is transported by the endosome, to a receptor on or within the plasma membrane (Blum, J. S., *et al.*, 1993). It has been observed that the highly iodinated thyroglobulin is concentrated in the follicle compared with the more heterogeneous mixture of iodinated thyroglobulin in the lumen (Dunn A. D., 1996; Seljelid, R., *et al.*, 1970). A receptor for iodinated thyroglobulin was implicated in this observation (Dunn, A. D., 1996; Kostrouch, Z. et. al, 1993; Lemansky, P. and Herzog, V., 1992). The mannose-6-phosphate receptor, found in follicular endosomes and in the apical membrane surface was shown to have no influence in thyroglobulin micropinocytosis, and no thyroglobulin specific receptor has been found on the apical membrane (Dunn, A. D., 1996; Kostrouch, Z. et. al, 1993; Lemansky, P. and Herzog, V., 1992).

A study of the thyroglobulin endocytotic pathway, demonstrates a lack of selectivity in endosome formation (Kostrouch, Z. *et al*., 1993). Early endosomes in this study contained a heterogeneous mixture of both the gold-complexed thyroglobulin (G-thyroglobulin) as well as the gold-complexed bovine serum albumin (G-BSA), microinjected into the follicular lumen of reconstructed thyroid follicles. This finding supports a mechanism like fluid-phase endocytosis (Blum, J. S., *et al.*, 1993; Kostrouch, Z. et. al, 1993). Late endosomes showed signs of protein sorting, as many were homogenous for either G-BSA or G-thyroglobulin, and G-thyroglobulin was also shown to be transported back to the lumen after endocytosis and sorting. Kostrouch, Z. et. al propose that this sorting is a mechanism through which highly iodinated thyroglobulin is

concentrated in the follicular cells, while newly formed thyroglobulin can be returned to the lumen for iodination.

The controversy over the mechanism of thyroglobulin uptake and release has yet to be settled. Many researchers believe that micropinocytosis is probably the major mechanism of thyroid hormone release for larger animals, like humans and pigs, under physiological conditions (Bernier-Valentin, F., *et al.*, 1990; Bernier-Valentin, F., *et al.*, 1991; Dunn, A. D., 1996; Kostrouch, Z. et. al, 1991; Seljelid, R., *et al.*, 1970). Once thyroid hormones are released into the follicular cells, after either method of thyroglobulin transport and proteolysis, they rapidly enter the serum. The actual mechanism of hormone secretion is unknown (Dunn, A. D., 1996).

Hypothalamus-Pituitary-Thyroid axis

The major regulator of thyroid hormone production and release appears to be TSH. Processes from thyroidal iodide transport, to the lysosomal enzyme activity, involved in thyroglobulin processing, are regulated by TSH (Taurog, A., 1991; Dunn, A. D., 1984; Perrild, H., *et al.*, 1989). The absence of TSH, created *in vivo* via hypophysectomy, or in culture medium, causes a marked decrease in the intracellular/extracellular [I^-] ratio, which is restored by TSH administration (Taurog, A., 1991). TSH stimulates transcription of the thyroglobulin gene and thyroglobulin glycosylation appears to be affected by TSH (Dunn, J. T., 1991). As mentioned above, TSH also stimulates follicular transport of iodinated thyroglobulin from the lumen. TSH, in some way, affects almost every aspect of thyroid hormone production.

TSH is produced in thyrotropes located in the anterior pituitary gland or adenohypophysis. It is composed of α and β subunits. The α subunit is a common subunit sharing structural similarities with the α subunits of other hormones like luteinizing hormone or chorionic gonadotropin, while

biological specificity is imparted by the β subunit (Wondisford, F. E., *et al.*, 1995). Thyrotropin-releasing hormone (TRH) and T3 regulate TSH production and secretion.

TRH stimulates the production and release of TSH by several mechanisms reliant on calcium and protein kinase C (PKC) (Geras, E. J., and Gershengorn M. C., 1982; Murakami, M., *et al.*, 1991; Shupnik, M. A., *et al.*, 1996). TRH stimulation of TSH secretion appears to be biphasic, reflecting, perhaps, an initial release of stored TSH pools and the subsequent production of TSH (Scanlon, M. F., 1991). Using mouse thyrotrophic tumor cells, Geras, *et al.* (1982) demonstrated that TRH induces both calcium influx by these cells and calcium efflux with the subsequent secretion of TSH. Calcium influx occurred with simultaneous addition of calcium and TRH to cell cultures or with the addition of only TRH to cells which had reached a steady-state level of calcium uptake prior to TRH addition. Calcium efflux, with subsequent TSH secretion, was observed to occur regardless of extracellular calcium concentrations. The signal transduction, initiated by the binding of TRH to its receptor in the thyrotroph plasma membrane, involves the stimulation of phospholipase C. Phospholipase C, in turn, hydrolyzes phosphatidylinositol bisphosphate into inositol triphosphate (IP_3) and diacylglycerol (DAG) (Scanlon, M. F., 1991). The IP_3 stimulates the release of intracellular calcium pools, while DAG activates PKC. This is a common route for signal transduction.

In addition to stimulation of TSH secretion, TRH has been demonstrated to stimulate an increase in thyrotrophic mRNA coding for the α and β subunits of thyrotropin (Murakami, M., *et al.*, 1991; Shupnik, M. A., *et al.*, 1996). Both the calcium and PKC signal transduction routes appear to play a role in this effect (Scanlon, M. F., 1991). The influx of extracellular calcium appears to be important as well, at least in the transcription of the β-subunit (Shupnik, M. A., *et al.*, 1996). DNA binding of a TRH-receptor complex is not apparent in the transcription of these

subunits, but the binding of a T3-receptor complex inhibits transcription of the β-subunit (Steinfelder, H. J., *et al.*, 1991).

There are several levels on which T3 is thought to inhibit the action of TRH. It has been demonstrated, in TSH secreting systems, that T3 decreases TSH α and β- subunit mRNA, and that T3 regulation is proportional to T3 nuclear receptor occupancy (Shupnik, M. A. and Ridgeway, E. C., 1985; Shupnik, M. A., *et al.*, 1986; Shupnik, M. A. and Ridgeway, E. C., 1987). Steinfelder *et al.* (1991) demonstrated that deletion of the DNA sequences, which are responsible for the T3 responsiveness of TSH β-subunit transcription, significantly reduces the transcription of this subunit. The sequences corresponding to the T3 response element, appear to be located near the common start site for both the α and β- subunits (Steinfelder, H. J., *et al.*, 1991). These experiments were run with human *TSHB* constructs in GH_3 cells, somatomammotrophs instead of thyrotropes, but these are also pituitary cells which have cell-surface TRH receptors, and the production of hormones within these cells appears to be sensitive to TRH stimulation (Scanlon, M. F., and Hall, R., 1995). Steinfelder *et al.* (1991) indicate that Pit-1/GHF-1 is a transcription factor common to both cell types, somatomammotrophs and thyrotropes. DNase I footprints from a human *TSHB* DNA fragment show that the Pit-1/GHF-1 binding site may be close to the binding site of the T3 receptor complex (Steinfelder, H. J., *et al.*, 1991). Pit-1/GHF-1 was shown to eliminate DNase I footprints yielded from an assay with the extracts from the nuclei of a TSH secreting cell line, by Steinfelder *et al.* (1991). The common sequences eliminated by Pit-1/GHF-1 are close to sequences attributed to the T3 response element (Steinfelder, H. J., *et al.*, 1991). Steinfelder *et al.* (1991) indicate that T3 may inhibit TRH sensitive, TSH transcription by inhibition of Pit-1/GHF-1 binding when the *TSHB* T3-response element is occupied.

T3 has also been proposed to inhibit the TRH stimulation of TSH secretion by decreasing the number of TRH cell-surface receptors on TSH secreting cells (Scanlon, M. F., and Hall, R., 1995). Some studies indicate that administration of T3 to anterior pituitary cell lines causes a decrease in the level of TRH binding (De Lean, A., *et al.*, 1977; Hinkle, P. M, Perrone, M. H., and Schonbrunn, A., 1981; Perrone, M. H., and Hinkle, P. M., 1978). Many of these studies were performed with somatomammotrophs (GH_3) and or pituitary tumor cells (GH_4C_1). The authors indicate that the T3-dependent TRH receptor reduction displayed by GH_3 and GH_4C_1 is suggestive of a similar role for the T3-dependent reduction in serum TSH (De Lean, A., *et al.*, 1977; Hinkle, P. M, Perrone, M. H., and Schonbrunn, A., 1981; Perrone, M. H., and Hinkle, P. M., 1978). Beyond this level of T3 control of TSH production and secretion, recent evidence implicates T3 as a negative regulator of TRH transcription (Scanlon, M. F., and Hall, R., 1995).

An important factor in the T3 feedback mechanisms in the anterior pituitary is the role of type I and II 5'-deiodinases within the tissues of this gland (Leonard, J. L. and Koehrle, 1996). Though T3 is the active hormone in the aforementioned processes, the major source of T3 for this gland is the local conversion of T4 via deiodination (Larsen, P. R., et al., 1981). So the levels of serum TSH correlate more with serum concentrations of T4 as opposed to T3.

TRH production has been observed in a variety of tissues (Scanlon, M. F., and Hall, R., 1995). However, the majority of blood flow entering the pituitary gland comes through the hypophyseal portal from the hypothalamus (Riskind, P. N. and Martin, J. B., 1995), so the majority of hypophysiotropic TRH probably originates in the hypothalamus. The parvocellular region of the hypothalamic paraventricular nucleus is the most likely origin of hypophysiotropic TRH within the hypothalamus (Riskind, P. N. and Martin, J. B., 1995). Several studies have shown an inverse relationship between the concentration of circulating thyroid hormones and the

amount of TRH and TRH prohormone (proTRH) in the hypothalamic paraventricular nucleus (Dahl, G. F., *et al.*, 1994; Scanlon, M. F., and Hall, R., 1995; Segerson, T. P., *et al.*, 1987).

Several isoforms of thyroid hormone receptors (TR's) have been found within cells of the paraventricular nucleus (Lechan, R. M., *et al.*, 1994), and binding sites for these receptors have been identified in murine and human TRH promoters (Hollenberg, A. N., *et al.*, 1995; Satoh, T., 1996). T3, in the absence of TR's has no effect on TRH promoter activity. The binding of unliganded TR's has been shown to stimulate TRH promoter activity, while the presence of T3 and the TR's, or deletion of some of these TR binding sites, has an inhibitory effect on promoter activity (Hollenberg, A. N., *et al.*, 1995; Satoh, T., 1996). T3 responses in the hypothalamus are not as dependent on local T3 to T4 conversion as in the anterior pituitary (Leonard, J. L. and Koehrle, 1996), so this feedback mechanism is more closely related to serum T3 levels.

Another level of T3 control over levels of TRH involves TRH degradation. Studies from Bauer (1988) and Suen and Wilk (1989) have shown that T3 stimulates pyroglutamyl peptidase II in the anterior pituitary. Pyroglutamyl peptidase II is a membrane bound peptidase that appears to be specific for TRH (Bauer, 1988; Suen and Wilk, 1989). Interestingly, Bauer (1988) found that the effect of T3 on this peptidase was decreased in female rats compared with male rats. The Suen and Wilk (1989), study only used male rats. Ovariectomy of female rats increased the T3 effect to the level of male rats, while estradiol benzoate administration to these ovariectomized females antagonized the effects of T3 on the peptidase (Bauer, 1988).

Iodothyronine deiodination

T4 is the main secretory product of the thyroid. In humans it is secreted in amounts that exceed T3 secretion by at least 20-fold (Jameson, J. L. and DeGroot, L. J., 1995). About 80% of

human T3 is derived from deiodination of T4 in peripheral tissues (Larsen P. R., *et al.*, 1981). The majority of circulating T3 results from T4 deiodination in the liver (Leonard, J. L. and Koehrle, 1996). Three types of deiodinases have been characterized, type I 5'-deiodinase, type II 5'-deiodinase, and 5-deiodinase (Leonard, J. L. and Koehrle, 1996). Deiodination can lead to generation of T3 from T4 or inactivation and degradation of the hormones. rT3, largely generated from 5-deiodination of T4 and other lesser iodinated iodothyronines are rapidly metabolized by many cell types releasing iodine back to bloodstream (Leonard, J. L. and Koehrle, 1996).

5-deiodinase catalyzes both 5- and 5'deiodination, but its 5-deiodination activity is around an order of magnitude greater than its 5'-deiodination activity, so this membrane bound enzyme is mainly involved in processes of thyroid hormone inactivation and degradation (Leonard, J. L. and Koehrle, 1996). 5-deiodination is observed in almost every tissue except anterior pituitary. Some of the enzymes associated with this activity appear to have a selenocysteine active site, like type I 5'-deiodinase, and can be inhibited by PTU similarly to type I 5'-deiodinase. However, the dose of PTU required for inactivation of these enzymes are an order of magnitude higher compared with type I 5'-deiodinase inhibition (Leonard, J. L. and Koehrle, 1996).

Type I 5'-deiodinase is found in most tissues in rats and humans. This membrane bound isozyme is important in local T3 generation as well as thyroid hormone clearance by catalyzing both 5'-deiodination and 5-deiodination of iodothyronines (Leonard, J. L. and Koehrle, 1996). Its substrate preference in decreasing order is: r-T3, T4, T3 (Leonard, J. L. and Koehrle, 1996). 4'-sulfation of type I 5'-deiodinase substrates inhibits 5'-deiodonation, yet increases the 5-deiodination of T3 and 3,3'-diiodothyronine, catalyzed this isozyme (Leonard, J. L. and Koehrle,

1996). As mentioned above, the activity of this enzyme is blocked by PTU. Since the active site of this isozyme contains selenium, selenium deficiency results in decreased deiodination (Leonard, J. L. and Koehrle, 1996).

Type II 5'-deiodinase is not inhibited by PTU (Leonard, J. L. and Koehrle, 1996). This isozyme has a more limited tissue distribution compared with type I 5'-deiodinase. In the rat, the activity of this enzyme has been demonstrated in the CNS, anterior pituitary, brown adipose tissue (BAT) and placenta (Leonard, J. L. and Koehrle, 1996). A high percentage of the nuclear T3 in these tissues is generated locally via T4 5'-deiodination. Iodothyronine 5'-deiodination is the most reported activity associated with type II 5'-deiodinase. Iodothyronine 5-deiodination is not reported for this isozyme, so, unlike the type I isozyme, this isozyme does not participate in thyroid hormone clearance via r-T3 formation. However the action of type II 5'-deiodinase is crucial to the function of affected organs (Leonard, J. L. and Koehrle, 1996).

A study by Bianco and Silva (1987) demonstrated the effect of hypothyroxemia, compared with hypothyroidism, on the initiation of "cold response" in rodent BAT. The activity of malic enzyme, glucose–6-phosphate dehydrogenase (G-6-PD), acetyl coenzyme A (CoA) carboxylase, and mitochondrial α-glycerolphosphate dehydrogenase (α-GPD) should increase in these animals in response to exposure to cold (Bianco, A. C. and Silva, J. E., 1987). The activity of these proteins was measured in the tissues of different of organs from euthyroid and hypothyroid rodents exposed to cold. Under hypothyroid conditions, the response of these proteins to cold was greatly diminished. Administration of high levels of T3 for 5-7 days, inducing hyperthyroid conditions, were required to return the response of these proteins to control levels in BAT. Replacement of T4 for 2 days was sufficient to restore normal responses in this tissue (Bianco, A. C. and Silva, J. E., 1987). However, in the liver, which has a high

concentration of the type I isozyme and an extremely low type II concentration, less T3 and more T4, were required to restore normality, compared with responses in BAT (Bianco, A. C. and Silva, J. E., 1987).

The aforementioned serum T4 sensitivity has been observed in other tissues high in type II 5'-deiodinases. T4 sensitivity in the brain and developing fetus have been the subject of numerous studies (Larsen, P. R., *et al*., 1981; Morreale De Escobar, G., *et al*., 1990). Several compounds have been demonstrated to inhibit the activity of type I- or type I- and type II-5'-deiodinase (Leonard, J. L. and Koehrle, 1996), not much is known about compounds which inhibit only the activity of type II-5'-deiodinase. However, given the observed iodothyronine specificity of responses to serum iodothyronine concentrations in different tissues and the differential response of the 5'-deiodinase isozymes to known chemical inhibitors, the effects of compounds on the deiodinase system should be an important consideration in the development of a screening battery for thyroid function disruptors.

Serum iodothyronines

Once in the serum the majority of thyroid hormones are bound to a variety of proteins. In humans 70% of circulating thyroid hormones are bound to T4-binding globulin, 10% is carried by transthyretin, and 15-20% is bound to serum albumin. A small fraction is carried by other lipoproteins (Robbins, J., 1991). The three major carrier proteins are produced in the liver, though transthyretin is also produced in the epithelium of the choroid plexus and the pancreatic islet cells. Transthyretin is the major carrier of thyroid hormones to the brain and along the central nervous system, in cerebrospinal fluid. Its production in the choroid plexus contributes to its high concentration in this region (Robbins, J., 1991). Aside from serum storage of thyroid

hormones, no other role in thyroid function has been determined for these proteins. There is still debate over the mechanism of thyroid hormone entry into the cell. Some authors have indicated a role for plasma thyroid hormone-binding proteins in facilitating hormone entry into cells (Robbins, J., 1991). Though cell surface receptors for free T4 and T3 have been reported, most authors consider diffusion of free thyroid hormones across the plasma membrane as the main physiological route of thyroid hormone cell entry (Jameson J. L. and DeGroot, L. J., 1995).

Assays Indicative of Thyroid Hormone Imbalance

One series of test discussed at the workshop measures serum levels of several hormones involved in thyroid function: T4, T3, rT3 and TSH (McMaster, S., 1997). Measurement of serum thyroid hormones is a common procedure in both the clinical and laboratory setting. There are a variety of companies that sell radioimmunoassay (RIA) and enzyme-linked immunoassay (ELISA) kits for the measurement of all of these hormones in serum. Used singly, these tests may not provide a lot of information, but used together their results can show interruption in many of the aforementioned areas involved in thyroid production and release.

As indicated above, T4 is the major secretory product of the thyroid gland. Though T4 serum levels can vary with nonthyroidal illness, serum levels of T4 will change more readily from the effects of weakly goitrogenic compounds compared with T3 serum levels. In cases of mild hypothyroidism and in areas of endemic goiter, usually from iodine deficiency, T3 serum levels are observed to be within the normal range, while there is a drop in serum T4 accompanied by an increase in serum TSH (Larsen, P. R., *et al*., 1981).

A similar effect is observed with polyhalogenated aryl hydrocarbon stimulation of hepatic T4 glucuronidation (Schuur, A. G., *et al.*, 1997; Sewall, C. H., *et al.*, 1995). In humans,

about 10% of total T4 is cleared from the body via hepatic T4 glucuronidation, with subsequent biliary excretion of the T4-glucuronide (Chopra, I. J., 1991). Chronic administration of TCDD to Sprague-Dawley rats resulted in decreased serum levels of T4 with increased serum levels of TSH (Sewall, C. H., *et al.*, 1995). Acute exposure resulted in decreased serum T4, with no affect on TSH (Schuur, A. G., *et al.*, 1997). In both studies the decrease in serum T4 was determined to result from TCDD stimulation of hepatic T4 glucuronidation (Schuur, A. G., *et al.*, 1997; Sewall, C. H., *et al.*, 1995). In both studies, serum levels of T3 were not significantly affected by TCDD treatment (Schuur, A. G., *et al.*, 1997; Sewall, C. H., *et al.*, 1995).

Serum levels of T3 fall under conditions of clinical hypothyroidism. Decreased T4 and rT3 as well as increased TSH accompany this fall in T3. Hyperthyroid conditions, however, are usually marked with a disproportionate increase in serum T3 compared with T4, so serum T3 has been found to be a better indicator of these conditions, though these conditions usually produce increased serum T4 and rT3, with decreased TSH (Sarne, D. H. and Refetoff, S., 1995). Since, as indicated above, the majority of serum T3 is generated by peripheral tissue deiodination, changes in serum T3, concomitant with changes in the other hormones could also be indicative of interference in deiodination processes. As discussed in section on deiodination, PTU inhibits type I 5'-deiodinase. Administration of PTU to rats produces decreased serum T3 and increased TSH, with no change in serum T4 (Larsen, P. R., *et al*., 1981). Changes in serum rT3, relative to the levels of the other hormones, may also be indicative of interference with deiodination.

Interference in the function of the thyroid gland itself, the hypothalamic-pituitary-thyroid axis, the deiodination processes and T4-glucuronide formation can all be assessed with the proper use of the combination of these tests. An advantage to the use of tests like these is that the possible effects of a compound's metabolites or metabolic inactivation of a compound can be

observed with these *in vivo* tests. More specific tests were discussed at the workshop, such as an assay measuring the ability of a compound to inhibit lactoperoxidase, and the TRH-challenge test (McMaster, S., 1997), performed mostly in the clinical setting to measure the anterior pituitary response to TRH (Sarne, D. H. and Refetoff, S., 1995). These test however, are only indicative of single steps within the system of thyroid function and may be of better use as tier 2 tests. Histopathological examination of the thyroid gland after compound exposure was also discussed to pick up affects on the thyroid gland that may not be evident from serum hormone levels (McMaster, S., 1997). These tests, however, will not be indicative of interference of thyroid hormone action at target tissue.

T3 in the nucleus

As discussed above, T3 regulation in target tissue appears to involve the binding of T3 to nuclear T3 receptors (TR) and the binding of these receptors to thyroid response elements upstream of the promoter of T3 responsive genes. There are two families of TR isoforms, α and β, and the number of isoforms within each family appears to be species specific as three isoforms have been identified in humans, $\alpha 1$, $\alpha 2$ and β (Rentoumis, A., et al., 1990); at least four have been identified in rats, $\alpha 1$, $\alpha 2$ and $\beta 1$, $\beta 2$ (Murray, M. B., *et al*., 1988; Hodin, R. A., *et al*., 1989; Lazar, M. A., *et al.*, 1988), and a large number have been identified in the isoform families of amphibians (Jameson, J. L. and DeGroot, L. J., 1995). The structure of the TR isoform places TR's within the superfamily of receptors including the steroid hormone receptors, retinoic acid receptors and receptors for vitamin D3 among others (Evans, R. M., 1988). Most of the receptors in this superfamily require ligand association for chromatin binding; however, TR chromatin binding has been demonstrated to independently of T3 association (Spindler, B. J., *et*

al., 1975), while TR mediated stimulation or repression of transcription is shown to be T3 dependent.

Since both α and β TR isoforms participate in positive and negative transcription regulation, the nature of their regulation appears to be dependent on the thyroid hormone response elements (TRE's) to which they are bound (Brent, G. A., *et al*., 1992; Carr, F. E. and Wong, N. C. W., 1994; Rentoumis, A., *et al*., 1990; Umesono, K., et al., 1991). The consensus sequence within various TRE's thus far studied is (A/G)GGT(C/A)A, though some degradation appears to be tolerated. TRE's, located upstream of the promoter region of a variety of T3-positively regulated genes, contain this sequence in either palindromes, direct repeats, or a combination of the two (Brent, G. A., *et al*., 1992; Rentoumis, A., *et al*., 1990; Umesono, K., et al., 1991).

A study by Brent (1992) demonstrates that the arrangement of these hexameric binding-motifs within the promoter regions examined is conducive to dimeric receptor binding and, T3 induction is proportional to TR dimer binding. As shown in Table 2., the arrangement of these hexamers within the rat promoter region of the α MHC utilized by Brent (1992) contains two direct repeats followed by an inverted copy, termed $^{3'}$ A, B and C' $^{5'}$ in this study. Mutation of one of the conserved G residues within either A, B or C resulted in a reduction of TR binding to 37%, 3% and 37% of wild type binding respectively. As shown by binding assays, mutation of the conserved G residue in the A hexamer decreased dimer binding to the promoter region, mutation of this residue in the B region prevented dimer binding, the C' region mutation was least effective in dimer inhibition (Brent, G. A., *et al*., 1992). The use of these mutated promoter regions showed that the reduction in T3 induction was proportional to the decrease in dimer binding for each mutation (Brent, G. A., *et al*., 1992). Other studies have shown the propensity

of dimeric TR binding and positive T3 regulation (Beebe, J. S., et al., 1991; Glass, C. K., et al., 1989; Graupner, G., et al., 1989; Meier, C. A., et al., 1993; Rentoumis, A., *et al*., 1990; Umesono, K., et al., 1991).

Table 2. Sequences of Selected Positive TRE's

Gene	TRE's	Sources
pTRE	**AGGTCATGACCT**	Umesono (1991)
rGH	A**AGGTAA**GATC**AGGGAC**G**TGACCG**C	Brent (1992)
r α MHC	G**AGGTGA**CAGG**AGGACA**G**CAGCCT**	Brent (1992)
rME	**AGGACG**TTG**GGGTTA**GGGG**AGGACA**G TG	Brent (1992)

pTRE-palindromic TRE rGH-rat growth hormone r a MHC-rat a myosin heavy chain rME-rat malic enzyme

Bold type denotes hexamers.

TR dimers can be homologous or heterogeneous, consisting of different TR isoforms (Rentoumis, A., *et al*., 1990), TR retinoic acid receptor (RAR) combinations (Glass, C. K., et al., 1989; Umesono, K., et al., 1991), TR retinoid X receptor (RXR) combinations (Meier, C. A., et al., 1993), and some studies indicate dimerization between TR's and uncharacterized protein components within nuclear extracts (Beebe, J. S., et al., 1991). The proteins within nuclear extracts termed TR auxiliary proteins (TRAP) appear to enhance TR binding (Beebe, J. S., et al., 1991), and nuclear extracts have been used in many studies with TR/TRE binding assays. *In vitro* RAR's have been shown to bind the palindromic TRE's (pTRE) (Graupner, G., et al., 1989). Addition of retinoic acid (RA) to incubation medium with RAR's has been shown to

activate RAR-RA mediated regulation of genes with these pTRE's in the promoter region and the combination of RAR's and TR's produces enhanced TR regulation through these pTRE's (Glass, C. K., et al., 1989; Graupner, G., et al., 1989; Umesono, K., et al., 1991). While addition of RAR's to systems containing the direct repeat/palindrome TRE motif, has been shown to repress basal expression of these genes and RAR added to these systems with TR's decreases TR regulation (Glass, C. K., et al., 1989; Umesono, K., et al., 1991). hTR α2, which can bind TRE's but not T3, has this same inhibitory effect on the actions of hTR α1 and hTR β (Rentoumis, A., *et al*., 1990).

Monomeric TR binding has been associated with T3 negative regulation (Carr, F. E. and Wong, N. C. W., 1994; Rentoumis, A., *et al*., 1990). Negative TRE's have not been well characterized, but several studies have examined the TRE's within the genes for the α and β TSH subunits and one study has examined T3 negative regulation of human growth hormone (hGH). An examination of the α and β TSH subunit promoters by Chatterjee (1989) showed that unlike positive TRE's, which are usually located upstream of the promoter, the TRE's for these subunits are proximal to the promoter, between the TATA box and the transcription start site. The TRE within the rat TSHβ–subunit was examined by Carr and Wong (1994). Though the sequence determined for the TRE contains a palindrome as well as a single hexamer, this study demonstrated that T3 negative regulation only requires the single hexamer, allowing monomeric, but not dimeric binding of the TR (Carr, F. E. and Wong, N. C. W., 1994). Interestingly, the Carr (1994) study demonstrates that TR binding to this TRE, in absence of T3, enhances basal transcription, this is contrasted by the observations of Rentoumis (1990) which indicated that the binding of TR's to positive TRE's repressed basal transcription in absence of the ligand. The addition of RXRβ to the system blunted or abolished T3 negative regulation of rTSHβ, while the

same study showed that RXRβ enhanced T3-TR stimulation of a construct containing the pTRE (Carr, F. E. and Wong, N. C. W., 1994). Interference of competing receptors was not observed by Rentoumis (1990) for the negative T3 regulation of TSHα, but the competing receptor used in this study was TRα2. The addition of TRAP's to the TSHβ system in the Beebe (1991) study had no effect on T3 negative regulation. Another negative TRE, examined by Zhang (1992), appears consists of several direct repeats, but is localized in a novel position with respect to other positive or negative TRE's.

The Zhang (1992) study characterized the negative TRE from hGH. Binding assays in this study demonstrated the binding of TR's, not within the promoter region, but within the 3' untranslated/flanking region of the gene just upstream of the poly A region (Zhang, W., et al., 1992). Placed in the same position on constructs with a chorionic sommatomammotropin promoter, normally stimulated by TR/T3, the hGH TRE region confers T3 negative regulation to this construct; however, placed upstream of the hGH promoter region, the same TRE confers T3 positive regulation to the construct (Zhang, W., et al., 1992). No other TRE's have been found in a similar location.

T3 positive and negative regulation via the two TR isoform families, provides a great deal of information to consider when designing a screen for the interruption of this activity. The workshop group discussed the use of solid state binding assays in which the TR's would be bound to either a multiwell plate or beads (McMaster, 1997). These types of assays can be used to test large numbers of compounds, quickly and efficiently conferring the quality of high-throughput to these assays. The choice of the TR isoform may be problematic and due to the variable tissue specificity of the isoforms and the dependence of their expression on developmental stages it may be prudent to utilize at one isoform from both the α and β families.

In vitro transcription assays in mammalian cell lines were discussed as well (McMaster, 1997). These types of assays are commonly utilized in studies examining the mechanisms of thyroid hormone regulation. The systems utilized can be well manipulated to include other factors such as RAR's and RXR's, known to affect TR binding. Nuclear components such as TRAP's and other cellular components responsible for phosphorylation and activation of TR binding are present in these cell lines so the effects of compounds that may hamper these components of T3 nuclear regulation could be observed with this type of assay. Representatives of both positive and negative regulation should be utilized due the specificity of these respective mechanisms. As with the above assay, this assay can be used as a high-through-put screen. The major drawback for both of these assay types is the lack of information concerning compound metabolites.

Conclusion

Given the complexity of the biological system through which thyroid function is enacted, no single screen will be sufficient to detect disruptors of this system. This paper provides a brief overview of some of the mechanisms involved in thyroid function and demonstrates some of the numerous levels at which thyroid function can be disrupted. From the information provided in this paper, the use of a combination of assays measuring the serum levels of several of the hormones involved in thyroid function, should provide a reasonably sound indication of any major disruption of the processes responsible for thyroid hormone production and tissue availability. The mechanisms underlying these disruptions will not be addressed by these assays, but as they are to be used simply as tier 1 screens, the exact mechanisms of disruption are not at issue. Tier 2 testing may address the mechanisms of disruption. The positive and negative

regulation exerted by T3, as well as the tissue and developmental stage specificity of the receptor isoforms through which T3 exerts it effects, necessitate the use of several assays to screen for disruption of T3 nuclear regulation. Both receptor isoform families should be represented in the screens. Both types of T3 regulation should be represented.

The time limitation provided by Congress for the design and implementation of the screening and testing program may limit the development of new techniques to be used as screens. Existing assays can provide a reasonable assessment of a compound's disruptive effects on thyroid function in mammals. Hopefully, as knowledge increases concerning the exact mechanisms through which thyroid function is enacted, and technological advances improve the efficiency of assay techniques, the screens and test can be improved upon to provide an even greater level of safety and assurrance.

Appendix A

Genes positively regulated by thyroid hormone*

Rat growth hormone
Myosin heavy chain α
Malic enzyme
Spot 14 lipogenic enzyme
Oxytocin
Phosphoenolpyruvate carboxykinase
Moloney leukemia virus enhancer
Lysozyme silencer
Glucokinase
Acetyl CoA carboxylase
HMG-CoA reductase
Apo A1
Pyruvate kinase M1
Fatty acid synthase
6-Phosphofructo-2-kinase
6-Phosphogluconate dehydrogenase
Ca^{2+}-ATPase
Mitochondrial β F1 ATPase
Cytochrome C oxidase
Na^{+},K^{+}-ATPase
B1 Adrenergic receptor
Uncoupling protein
Glucose transporter
Erythrocyte anion transporter (band 3)
Atrial natriuretic protein
Angiotensinogen
Preprotachykinin-A
Fibronectin
Chorionic somatomammotropin
Nerve growth factor
Epidermal growth factor
Brain RC3 glycoprotein
Purkinje PCP2

Genes negatively regulated by thyroid hormone*

TSHα
TSHβ
EGF receptor
Human growth hormone
TRH
Myosin heavy chain β
G protein β
Neural cell adhesion molecule
Peptidylglycine a-amidating monooxygenase
Thyroid hormone receptor β2

*These lists are presented as tables 37-3 and 37-4 respectively in Jameson and DeGroot, "Mechanisms of Thyroid Hormone Action" in Endocrinology (1995) on p.587.

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