REVIEW
THYROID GLAND & ITS REGULATIONS
HISTORY OF THYROID DISCOVERIES

The relationship between the thyroid and various body functions was first studied by experimental thyroidectomy (Cooper 1836). The concept of internal secretory function of thyroid gland was hypothesized by King (1836). The relationship of iodine with the functioning of the thyroid gland was noted by Baumann (1895) who discovered a particularly high concentration of iodine within the gland. Intensive persuasion of Kendall (1915) was to isolate the active component of the thyroid gland, resulted in isolation of thyroxine (T4). Harrington and Barger (1927) improved Kendall’s method of isolation and determined the chemical structure of thyroxine (3,5,3',5' tetraiodothyronine). Gross and Pitt–Rivers (1952) isolated and identified another hormone namely 3,5,3' triiodothyronine (T3) from thyroid and plasma. They also assumed that T4 is the form in which thyroid hormone is secreted whereas T3 is the form in which thyroid hormone is effective in target tissues.

PHYLOGENY OF THE THYROID GLAND

The primary event in the phylogeny of the thyroid was the development of the capability of collecting iodide ion in living forms and binding it to protein. These activities have been observed widely among plants and in the invertebrate members of the animal kingdom. Drechsel (1896) recognized in 1896 that sponges and corals contain large quantities of iodine as iodotyrosines. Iodohistidine and bromotyrosine have also been detected. Monoiodotyrosine (MIT) and diiodotyrosine (DIT) have been found in starfish, mollusks, annelids, crustacea, and insects (Berg et al. 1959). Tong and Chaikoff (1955) found a marine alga that concentrates iodide and binds to tyrosine forming MIT and DIT. The first evidence of an organ related to the thyroid is found in the protochordates, intermediate forms between vertebrates and invertebrates. The most primitive vertebrate in which a thyroid anlage can be definitely demonstrated is the ammocoete, the larva of the lamprey, a cyclostome. An open tubular structure in the hypopharynx collects iodide and binds it to a protein, apparently related to thyroglobulin (Tg) forming T4 and T3 (Suzuki and Kondo 1971). A thyroid capable of forming iodotyrosines and iodothyronines is present in all extant vertebrates. Its level of function varies widely from species to species and season to season. With the exceptions thyroid
activity in the poikilotherms is very low. Seasonal changes in thyroid activity have been found in both warm and cold blooded animals. In the adult lamprey and in bony fish, the gland is not encapsulated whereas the thyroid is encapsulated in cartilaginous fish. In the higher vertebrate forms, the thyroid is a one or two lobed encapsulated structure. A most striking effect of thyroid hormone is the induction of metamorphosis in certain amphibians, first reported by Gundematsch (Gundematsch 1912). T₄ is apparently involved in the metamorphosis of most amphibians.

**DEVELOPMENT OF THYROID GLAND IN RODENT**

The thyroid gland is derived from the fusion of a medial outpouching from the floor of the primitive pharynx, the precursor of the thyroxine (T₄) producing follicular cells and bilateral evaginations of the fourth pharyngeal pouch, which give rise to the parafollicular, or calcitonin (C) secreting cells (Missro et al. 1998). The growth and descent of the thyroid anlage into the neck results from the coordinate action of a number of recently cloned transcription factors. These include the thyroid transcription factors TTF-1 and TTF-2 and Pax 8, all of which are expressed in the rat prior to or just following the first appearance of the thyroid diverticulum on fetal day 9.5-10 (Zannini et al. 1997).

**Table 1. Approximate timing of molecular events controlling thyroid gland development in the rat (Zannini et al. 1997)**

<table>
<thead>
<tr>
<th>Fetal age (Days)</th>
<th>Molecular event</th>
<th>Development concomitant</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.5-9.5</td>
<td>HOX-A3 and HOX-B3 genes expressed in primitive pharynx</td>
<td>---</td>
</tr>
<tr>
<td>9.5-10</td>
<td>TTF-1, TTF-2 and PAX-8 genes expressed</td>
<td>Appearance of thyroid diverticulum</td>
</tr>
<tr>
<td>14-16</td>
<td>TTF-2 expression decreases and disappears</td>
<td>---</td>
</tr>
<tr>
<td>14.5-15</td>
<td>---</td>
<td>Parathyroid glands join thyroid. Migration completed.</td>
</tr>
<tr>
<td>15-15.5</td>
<td>Tg, TPO, TSH receptor expressed</td>
<td>---</td>
</tr>
<tr>
<td>17-18</td>
<td>Tg, TPO, TSH receptor expression unregulated</td>
<td>Increased TSH in circulation. First appearance of follicles, iodide fixation, thyroid hormonogenesis</td>
</tr>
<tr>
<td>21</td>
<td>---</td>
<td>Delivery</td>
</tr>
</tbody>
</table>

Tg, thyroglobulin; TPO, thyroid peroxidase; TSH, thyrotropin.
DEVELOPMENT OF THYROID GLAND IN HUMAN

The main anlage of the thyroid gland develops as a median endodermal down growth from the tongue. It can be seen in the human embryo before the end of the third week. It is located near the primordium of the heart and as the heart is pulled caudally, the thyroid anlage follows. At about the 30th day it has developed into a hollow bilobed structure, and by the 40th day, the original hollow stalk connecting it to the pharyngeal floor atrophies and then breaks. Shortly thereafter the lateral extensions of the median anlage make contact with the ultimobranchial bodies developing from the 4th pharyngeal pouches, the so-called lateral anlage of the thyroid. The ultimobranchial cells are the origin of calcitonin secreting C cells in the thyroid gland and probably contribute to the formation of follicular cells as well (Soyama 1973). By the 8th week the cells have a tubular arrangement, and cell clusters are apparent. Two weeks later when the embryo is approximately 80 mm long follicles are present. Shortly after this time the follicles contain colloid, and the thyroid accumulates and binds iodide by the 11th-12th week (Burrow et al. 1994). Secondary follicles arise by budding from the primary follicles; they increase in number until the embryo reaches a length of about 160 mm. After this time the follicles increase in size, but the number remains the same. Under intense stimulation, the adult thyroid can form new follicles. Thyroid hormones are detectable in fetal serum by gestational age 12 weeks with both thyroxine (T₄) and triiodothyronine (T₃) being measurable (Thorpe-Beeston et al. 1992).

ANATOMY

Thyroid is often called as the "shield gland" and the English name, derived from the Greek, means the same thing. The gland as seen from the front is more nearly the shape of a butterfly. It wraps itself about and becomes firmly fixed by fibrous tissue to the anterior and lateral parts of the larynx and trachea. Anteriorly, its surface is convex; posteriorly, it is concave. The isthmus lies across the trachea anteriorly just below the level of the cricoid cartilage (Ekholm 1995). The lateral lobes extend along either side of the larynx as roughly conical projections reaching the level of the middle of the thyroid cartilage. Their upper extremities are known as the upper poles of the gland. Similarly, the lower extremities of the lateral lobes are spoken of as the lower poles, although they make no such prominent projections (Wollman 1980).
The weight of the thyroid of the normal non-goitrous adult human is 10-20 gm depending on body size and iodine supply. Their breadth is 15-20 mm and their thickness is 20-39 mm. The thyroid is enveloped by a thin, fibrous, nonstripping capsule that sends septa into the gland substance to produce an irregular, incomplete lobulation. The lateral lobes lie in a bed between the trachea and the larynx medially and between the carotid sheath and the sternomastoid muscles laterally. The pyramidal lobe is a narrow projection of thyroid tissue extending upward from the isthmus and lying on the surface of the thyroid cartilage, to the right or left of the prominence of that structure. It is a vestige of the embryonic thyroglossal tract (Fujita 1988). The thyroid has an abundant blood supply. The nerve supply seems to be primarily from the autonomic system. The lymphatics of the gland connect with the other lymphatics of the neck and the mediastinum (Wollman 1980).

The thyroid is made up of multiple acini (follicles). Each spherical follicle is surrounded by a single layer of cells and filled with pink staining proteinaceous material called colloid. When the gland is inactive the colloid is abundant, the follicles are large and the cells lining them are flat. When the gland is active the follicles are small the cells are cuboid and columnar and the edge of the colloid is scalloped forming many small 'reabsorption lacunae' (Wollman 1980).

CONTROL OF THYROID FUNCTION

The principal physiologic control of thyroid function is mediated through thyroid stimulating hormone (TSH) impinging upon the cell. TSH activates cell membrane adenylate cyclase, through its interaction with the TSH receptor located on the plasma membrane (Vassart and Dumont 1992). Specific sites on the extra-cellular domain of the TSH receptor have been reported as critical for TSH-binding and signaling. Interaction of TSH with its receptor increases intracellular cAMP by transduction coupled to a Gi, a G protein which increases oxidation of glucose, synthesis of protein, formation of RNA, uptake of I and formation and secretion of thyroid hormone (Vassart and Dumont 1992). cAMP is presumed to act as an intracellular "second messenger" in most of these activities. It has been recently found that TSH may also act via the phosphatidylinositol pathway. This pathway accounts for the stimulation of \( \text{H}_2\text{O}_2 \) generation and thyroid hormone synthesis (Jacquemin 1991).
SYNTHESIS OF THYROID HORMONES

Iodine enters the thyroid follicular cells as inorganic iodide and transformed through a series of metabolic steps into thyroid hormones. The metabolic events involved in the transformation are:

1) The active transport of iodide
2) Iodination of tyrosyl residues in thyroglobulin (Tg)
3) Coupling of iodotyrosine within the Tg molecule to form thyroid hormones
4) Proteolysis of Tg with the release of thyronines and iodotyrosines, release of iodine from the iodotyrosines and reutilization of the liberated iodide within the thyroid

Iodide transport

The first step in the synthesis of thyroid hormones is concentrating iodide from the extra cellular fluid. This is referred to as iodide concentrating mechanism (Wolff 1964; Halmi 1961). Iodide transported into the thyroid follicular cell against both concentration and electrochemical gradient, it rapidly diffuses into the follicular lumen (Wolff 1964). Iodide is actively transported by the iodide Na+/T symporter (NIS) against the electrical gradient at the basal membrane of the thyrocyte and diffuses by a specialized channel (Scott et al. 1999) from the cell to the lumen at the apical membrane. The opposite fluxes of iodide, from the lumen to the cell and from the cell to the outside, are generally considered to be passive and nonspecific. Iodide transport occurs at the basal membrane of the follicular cells. It is an energy dependent saturable process, requiring oxidative metabolism and phosphorylation (Wolff 1964). The transport mechanism is not specific for iodide alone. Ions of similar size, shapes are also transported and are thus competitive inhibitors of iodide transport. Thyrotropin is an important stimulator of thyroid iodide transport (Halmi 1961).

Thyroglobulin synthesis

Iodide transported into the thyroid gland is incorporated into the tyrosyl residues of the Tg molecule. The iodination and coupling of tyrosyl residues to form iodothyronines takes place within the matrix of Tg molecule. Tg is a glycoprotein constituting about 75% of the protein content of thyroid gland and is almost entirely
found in the follicular lumen (Van Herle et al. 1979). Molecular weight of Tg is 660 kDa and is composed of 2 identical non-covalently linked subunits (Salvatore and Edelhoc 1973). It contains about 10% of carbohydrate. Tg is the most abundant protein in the thyroid gland. Its main function is to provide the polypeptide backbone for synthesis and storage of thyroid hormones (Dunn and Dunn 2000). It also offers a convenient depot for iodine storage and retrieval when external iodine availability is scarce or uneven. Neosynthesised Tg polypeptide chains entering the lumen of the rough endoplasmic reticulum (rER) are subjected to core glycosylation, dimerises and are transferred to the golgi where they undergo terminal glycosylation. Iodination and hormone formation of Tg occur at the apical plasma membrane - lumen boundary and the mature hormone-containing molecules are stored in the follicular lumen where they make up the bulk of the thyroid follicle colloid content (Dunn and Dunn 2000).

Protein portion of Tg is synthesized on rough endoplasmic reticulum. Initial glycosylation occurs before release of the synthesized protein from the rER. The glycosylation is completed after the protein portion is transported to golgi apparatus. From golgi apparatus the glycosyalated protein is transported to the luminal colloid. At the apical surface of acinar cell iodine is added to the glycosylated protein. The fully iodinated Tg is then stored in colloid (Dunn and Dunn 2000).

**Thyroid peroxidase (TPO)**

Thyroid peroxidase (TPO) oxidizes iodide in the presence of hydrogen peroxide ($\text{H}_2\text{O}_2$). The enzyme activity is dependent on the association with a heme, the ferriprotoporphyrin IX or a closely related porphyrin (Ohtaki et al. 1982). Chemical removal of the prosthetic group inactivates the enzyme, and recombination with the heme protein restores activity (Krinsky and Alexander 1971). TPO synthesized on polysomes is inserted in the membrane of the endoplasmic reticulum and undergoes core glycosylation. TPO is then transported to the golgi where it is subjected to terminal glycosylation and packaged into transport vesicles along with Tg (Ericson et al. 1990). These vesicles fuse with the apical plasma membrane in a process stimulated by TSH. TPO delivered at the apical pole of thyrocytes exposes its catalytic site with the attached heme in the thyroid follicular lumen (Yokoyama and Taurog 1988). TPO activity is restricted to the apical membrane, but most of the thyroid TPO is intracellular, being located in the perinuclear part of the endoplasmic reticulum (Fayadat et al. 1998).
H$_2$O$_2$ generating system

By definition, a peroxidase requires H$_2$O$_2$ for its oxidative function. H$_2$O$_2$ is produced at the apical plasma membrane by an enzyme that requires both calcium and NADPH (Dupuy et al. 1990). The enzyme named thyroid NADPH oxidase, probably composed of several molecular species, has not yet been completely characterized. Complementary DNAs corresponding to two members of the NADPH oxidase family of 1,551 (ThOX1) and 1,548 (ThOX2) amino acids have been cloned. The two proteins share 83% homology; in their N-terminal part, they present 43% similarity with TPO (de Deken et al. 2000). Both ThOX proteins contain intracellular consensus sequence for FAD and NADPH binding sites and two EF-hand motives as calcium binding sites (de Deken et al. 2000). The current model assigns seven transmembrane domains to ThOX1 and ThOX2. The two proteins are glycoproteins, their apparent molecular mass ranges from 170-180 kDa. Immunolabelling experiments revealed the presence of ThOX inside the cells and at the apical plasma membrane (de Deken et al. 2000). ThOX proteins are components of the H$_2$O$_2$ generating system but additional polypeptide chains are required to get the complete thyroid H$_2$O$_2$ generating system (de Deken et al. 2002). One scheme for H$_2$O$_2$ generation by NADPH oxidase proposes the reduction of O$_2$ into superoxide ion (O$_2^-$) and the conversion to H$_2$O$_2$ by superoxide dismutase (Nakamura et al. 1989). Another scheme involves the direct formation of H$_2$O$_2$ from O$_2$ (Dupuy et al. 1991). TSH, via the cAMP cascade, activates the expression of ThOX1 and ThOX2 (de Deken et al. 2000). TSH stimulates NADPH oxidase activity and H$_2$O$_2$ production and this effect is mediated through cAMP. H$_2$O$_2$ generation in in vitro systems appears to be regulated by iodide. At low concentrations and short-term incubation, iodide increases H$_2$O$_2$ production whereas at high iodide concentrations H$_2$O$_2$ generation is inhibited (Corvilain et al. 2000).

Thyroglobulin iodination

Iodide that enters thyroid gland must first be oxidized to a higher oxidative state before it can participate in intra-thyroidal iodination reactions. Hydrogen peroxide and oxygen are two known biological oxidizing agents that potentially oxidize iodine (Taurog 1964). Hydrogen peroxide is probably generated by NADPH cytochrome C reductase system in the thyroid gland (Virion et al. 1984). Hydrogen peroxide must be produced before iodide oxidation or organification can take place. These reactions occur
in exocytotic vesicles fused with the apical cell membrane within the follicular lumen. Both oxidation and organification are catalysed by thyroid peroxidase. Thus, in the thyroid iodide is very rapidly oxidized and then covalently bound to tyrosyl residues of Tg (Pommier et al. 1973).

First, iodide must be oxidized to an iodinating form. An extensive literature has sought to identify the iodinating species, but the issue is still not resolved. One scheme proposes that oxidation produces free radicals of iodine and tyrosine, while both are bound to TPO to form MIT that then separates from the enzyme. Further reaction between free radicals of iodine and MIT gives DIT. Experimental studies by Taurog (2000) and others suggest that the TPO reduction occurs directly in a two electron reaction. A second proposal, based on studies of rapid spectral absorption changes (Ohtaki et al. 1982; Taurog 2000) is that TPO-I⁺ is the iodination intermediate and that the preferred route is oxidation of TPO by H₂O₂ followed by two electron oxidation of I⁻ to I⁻, which then reacts within a tyrosine. As a third possibility, Taurog (2000) proposed a reaction between oxidized TPO and I⁻ to produce hypoiode (OI⁻) that also involves a two electron reaction. It is clear that iodide is oxidized by H₂O₂ and TPO and transferred to the tyrosyl groups of Tg. All tyrosine residues of Tg are not equally accessible to iodination. The molecule has about 132 tyrosyl residues among its two identical chains; at most only about 1/3 of the tyrosyls are iodinated.

**Coupling reaction**

Iodothyronines are produced as a result of coupling reactions between iodoxyrosyl residues within the Tg. Coupling takes place while both acceptor and donor iodoxyrosyl are in peptide linkage within the Tg molecule. The reaction is catalyzed by TPO, required H₂O₂ (Cahmann et al. 1977) and is stringently dependent on Tg structure (Lamas et al. 1974). The generation of the iodoxytronine residue involves the formation of an ether bond between the iodophenol part of a donor tyrosyl and the hydroxyl group of the acceptor tyrosyl. After the cleavage reaction that gives the iodophenol, the alanine side chain of the donor tyrosyl remains in the Tg polypeptide chain as dehydroalanine (Gavaret et al. 1981). The coupling reaction is concerted succession of three steps: oxidation of two iodoxyrosyl residues to an activated form, nonoxidative formation of a covalent bond linking them and nonoxidative decomposition of the coupling product, leading to the formation of equimolar amounts of hormone and dehydroalanine residues.
with the polypeptide chain of Tg (Gavaret et al. 1981). Correct alignment of two iodotyrosines is a prerequisite for coupling (Cahnmann et al. 1977). Two molecules of DIT may couple to form T₄ and an MIT may couple to form T₃. The coupling of two DIT residues may occur either through a free radical or an ionic mechanism. The latter probably prevails in nonenzymatic conditions, while it is not clear whether enzymatic coupling occurs by means of free radical formation (Nunez 1984). The radical mechanism requires the formation of two DIT radicals by the one-electron oxidation of two iodotyrosines. The ionic mechanism requires the two electron oxidation of one of the two iodotyrosyl residues. In either case, a charge transfer complex is formed consisting of a Zwitter ion – biradical resonance hybrid. This is followed by the formation of a quinol ether intermediate and by its splitting at the side chain of one of the two former DIT residues, with the formation of a T₄ residue at the site of the acceptor iodotyrosine, and of a dehydroalanine residue at the site of the donor iodotyrosine (Gavaret et al. 1981). The formation of T₃ occurs similarly via the coupling between MIT that contributes the outer ring and DIT that contribute the inner ring (Pommier et al. 1973).

![Possible coupling reaction sequence](image)

**Fig 1.** Possible coupling reaction sequence. Oxidation of iodotyrosines may produce iodotyrosyl radicals. The free radicals could combine to generate the iodothyronine residue (at the tyrosine acceptor site) and a dehydroalanine residue (at the tyrosine donor site), which in the presence of H₂O converts into a serine.

Coupling reaction occurs in one or more limited domains of the Tg that have unique amino acid sequence. The primary T₄ acceptor is the 5ᵗʰ tyrosine residue and
forms about 50% of the $T_4$ in Tg (Dunn et al. 1987). The two other acceptor tyrosine residues are at the location near the COOH terminus. More than 50% of the $T_3$ is found at the third tyrosine residues from the COOH terminus of Tg. This Tg containing iodinated tyrosine residues is stored in the colloid of follicular lumen. Molecular weight of $T_3$ and $T_4$ are 650 and 777 d respectively. Observations both in vivo and in vitro show an appreciable delay in coupling after initial formation of iodotyrosines. A typical distribution for a Tg containing 0.5% iodine (a normal amount for iodine-sufficient individuals) is 5 residues MIT, 5 of DIT, 2.5 of $T_4$ and 0.7 of $T_3$ (Dunn and Dunn 2000). More iodine increases the ratios of DIT / MIT and $T_4 / T_3$, while iodine deficiency decreases them.

Fig 2. Structural formula of thyroid hormones and precursor compounds

HORMONE STORAGE

Tg molecules vectorially delivered to the follicle lumen by exocytosis accumulates to reach uncommon concentrations. The mechanism operating such a "packaging" is unknown. Water and ion extraction from the follicle lumen might represent an active process leading to Tg concentration. As the follicle lumen is a site of $Ca^{++}$ accumulation (Fonlupt et al. 1997) the high degree of compaction of luminal Tg might depend on electrostatic interactions between $Ca^{++}$ and anionic residues of Tg, which is an acidic protein. Stored Tg molecules undergo iodination and hormone
formation reactions at the apical plasma membrane-lumen boundary (Wollman and Ekholm 1981), where peroxidase and \( \text{H}_2\text{O}_2 \) generating system reside. The mature Tg molecules containing MIT, DIT, \( \text{T}_4 \) and \( \text{T}_3 \) remain extra cellular in the lumen of thyroid follicles.

Tg as usually isolated from the thyroid is chiefly the 19S, 660 kDa dimer that has been glycosylated and iodinated. Iodination and hormone formation of Tg is more complex than generally thought because of the slow diffusion of molecules that are in a colloidal state in the follicle lumen. It has been reported that TSH alters the hydrodynamic properties of intrafollicular Tg molecules (Gerber et al. 1985). There is evidence for the presence of insoluble Tg. Insoluble Tg has many internal crosslinks through disulfide bonds, dityrosine, and glutamyl-lysine bonds, the latter generated by transglutaminase (Saber-Lichtenberg et al. 2000).

**Proteolysis of thyroglobulin**

To be useful, thyroid hormones must be released from Tg and delivered to the circulation for action at their distant target tissues. Depending on numerous factors including - the supply of iodide as substrate, the activity of enzymes catalyzing hormone formation, the concentration and physicochemical state of Tg - the hormone content of lumenal Tg molecules varies to a rather large extent (Ericson 1981).

The way the thyroid follicle proceeds to generate free hormones from stored hormone containing Tg molecules has been known for a long time. Tg molecules are first taken up by polarized thyrocytes and then conveyed to lysosomal compartments for proteolytic cleavage that release \( \text{T}_4 \) and \( \text{T}_3 \) from their peptide linkages. The first step represents the limiting point in the thyroid hormone secretory pathway. It has been considered that it could proceed via a mechanism different from phagocytosis, also named macropinocytosis, evidenced in rats under acute TSH stimulation (Ericson 1981). There is now a number of experimental data indicating that in the thyroid of different species under physiological circumstances, internalization of Tg, mainly if not exclusively, occurs via vesicle-mediated endocytosis or micropinocytosis (Marino and McCluskey 2000).

The internalization process starts with the organization of micro-domains at the apical plasma membrane of thyrocytes; these micro-domains or pits, resulting from the
recruitment and assembly of proteins on the cytoplasmic side of the membrane, invaginate to finally generate coated vesicles after membrane fission. Lumenal Tg molecules, either free or associated to membrane proteins acting as Tg receptors, enter the pits and are then sequestrated into the newly formed vesicles (Bernier-Valentin et al. 1991). The vesicles lose their coat and, through a complex fusion process, deliver their content into a first type of endocytic compartments, the early apical endosomes (Kostrouch et al. 1991).

Fig 3. Schematic representation of the two modes of internalization of Tg; Micropinocytosis (on the right) and Macropinocytosis or phagocytosis (on the left). Intralumenal Tg stores potentially subjected to endocytosis are composed of (recently secreted) non-iodinated Tg, iodinated Tg (Tg-I) and iodinated Tg containing iodothyronine residues (Tg-Ith). Abbreviations are: CV, Coated Vesicle; EE, Early Endosome; LE, Late Endosome; L, Lysosome; Pp, Pseudopod; CD, Colloid Droplet; PL, Phagolysosome. The scheme on the right indicates the three possible routes of transport of internalized Tg molecules reaching the EE: transport to LE, recycling towards the follicle lumen and transcytosis i.e. transport towards the basolateral plasma membrane.

In these compartments, Tg molecules probably undergo sorting on the basis of recognition of different physicochemical parameters. It has been shown that internalized Tg molecules can follow different intracellular pathways. Part of Tg molecules are conveyed via a vesicle transport system to the second type of endocytic compartments, late endosomes or prelysosomes. This route ending to lysosomes corresponds to the Tg degradation pathway for the generation of free thyroid hormones. The other Tg
molecules with no or a low hormone content, present in early apical endosomes, enter either of the two following routes; they are recycled back into the follicle lumen through a direct vesicular transport towards the apical plasma membrane (Kostrouch et al. 1993) or via a two-step vesicular transport to the golgi apparatus and then to the apical plasma membrane (Miquelis et al. 1993). Alternately, Tg molecules are transported and released at the basolateral membrane domain of thyrocytes via transcytotic vesicles; a process accounting for the presence of Tg in plasma (Romagnoli and Herzog 1991).

**Intra-thyroidal deiodination**

Iodotyrosines liberated as a result of Tg hydrolysis are rapidly deiodinated by an NADPH dependent iodotyrosine deiodinase, and the released iodine is recycled. The $T_4$ release is stimulated by TSH, as may be the 5'-monodeiodination of $T_4$ to $T_3$ by the types I and II iodothyronine deiodinases (Erickson et al. 1982). The ratio of $T_4$ to $T_3$ in Tg is about 15:1 and the ratio of $T_4$ to $T_3$ in secreted hormones is about 10:1. This difference is due to the fact that in addition to coupling reaction, some intra-thyroidal $T_3$ is formed from the 5'-deiodination of $T_4$ (Chanoine et al. 1992). Thyroid hormones thus formed by coupling and deiodination diffuse into the circulation.

**TRANSPORT OF THYROID HORMONES**

Thyroid hormone effect is dependent on the quantity of the hormone that reaches the tissues and the availability of unaltered thyroid hormone receptors in the cells nuclei. Since intracellular hormone is in equilibrium with the free thyroid hormone in serum, the concentration of the latter rather than that of total hormone is usually a more accurate indicator of the activity level of thyroid hormone-dependent processes (Oppenheimer 1968). Under normal conditions, changes in free hormone level are corrected by appropriate stimulation or suppression of hormone secretion and disposal. Total hormone concentration in serum is normally kept at a level proportional to the concentration of carrier proteins, and appropriate to maintain a constant free hormone level. Most carrier protein dependent alterations in total hormone concentration in serum are due to quantitative changes in the hormone-binding proteins and less commonly to changes in affinity for the hormone (Refetoff 1989).
Thyroxine-binding globulin (TBG)

TBG is a 54 kDa acidic glycoprotein. The term thyroxine-binding globulin is a misnomer since the molecule also binds T₃ and r T₃. It was first recognized to serve as the major thyroid hormone transport protein in serum (Robbins 1992). Since TBG binds 75% of serum T₄ and T₃, quantitative and qualitative abnormalities of this protein have most profound effects on the total iodothyronine levels in serum. TBG is synthesized in the liver as single polypeptide chain of 415 amino acids. The mature molecule is composed of 395 amino acids (44 kDa) and four heterosaccharide units with 5 to 9 terminal sialic acids. The TBG molecule has a single iodothyronine-binding site with affinity slightly higher for T₄ than for T₃ (Hocman 1981). Optimal binding activity requires the presence of the L-alanine side chain, an unsubstituted 4'-hydroxyl group, a diphenyl ether bridge, and halogen (I or Br) constituents at the 3,5,3' and 5' positions (Cody 1980).

Transthyretin (TTR)

TTR is a 55 kDa tetramer which is highly acidic although it contains no carbohydrate. Formerly known as thyroxine-binding prealbumin (TBPA), for its electrophoretic mobility anodal to albumin, was first recognized to bind T₄ (Ingbar 1958). TTR circulates in blood as a stable tetramer of identical subunits, each containing 127 amino acids with two iodothyronine binding sites (Tsuzuki et al. 1985). TTR usually binds only one T₄ molecule because the binding affinity of the second site is greatly reduced through a negative cooperative effect (Irace and Edelhoch 1978). Despite the 20 fold higher concentration of TTR in serum relative to that of TBG, it plays a lesser role in iodothyronine transport. In the presence of normal levels of TBG, wide fluctuations in TTR concentration or its removal from serum by specific antibodies has little influence on the concentration of free T₄ (Woeber and Ingbar 1968).

Human serum albumin (HSA)

HSA is a 66.5 kDa protein synthesized by the liver. It is composed of 585 amino acids with high content of cystines and charged amino acids but no carbohydrate (Peters 1985). HSA associates with a wide variety of substances including hormones and drugs possessing a hydrophobic region and thus the association of thyroid hormone to HSA can be viewed as nonspecific. Of the several iodothyronine-binding sites on the HSA
molecule, only one has a relatively high affinity for T₄ and T₃, yet these are 10,000 fold inferior to those of TBG. Because of the low affinity and despite the high capacity of HSA for iodothyronines, its contribution to thyroid hormone transport is relatively minor (Beeken 1962).

Lipoproteins

Lipoproteins bind T₄ and to some extent T₃ (Benvenga and Robbins 1993). The affinity for T₄ - binding is similar to that of TTR. These proteins are estimated to transport roughly 3% of the total T₄ and perhaps as much as 6% of the total T₃ in serum. The binding site of apolipoprotein A1 is a region of the molecule that is distinct from that portion which binds to the cellular lipoprotein receptors and the physiological role of such binding is still unclear.

PERIPHERAL CONVERSION OF THYROID HORMONES

The most important metabolic pathway of T₄ is the monodeiodination of its outer ring (β ring) in peripheral tissues like liver, kidney etc. to form the active thyroid hormone T₃ (Larsen et al. 1981). Inner ring (α ring) monodeiodination of T₄ generates inactive reverse T₃ (3, 3',5'- triiodothyronine) (Larsen and Berry 1995). Only 10-30% of the circulating T₃ is derived directly from the thyroid gland while 70-90% of the circulating T₃ arises from the peripheral monodeiodination of T₄. Two types of outer ring monodeiodinase (MDI) have been identified. Type I 5'-deiodinase predominantly found in liver and kidney is a high kinetic activity (km) enzyme partially inhibited (20%) by propyl thiouracil (Saberi et al. 1975). Its activity is increased in hyperthyroidism and also decreased in hypothyroidism (Larsen and Berry 1995). Type II 5'-deiodinase is primarily present in pituitary, central nervous system, brown fat, placenta and also in skeletal, cardiac and thyroid tissues. It is a low kinetic activity enzyme and resistant to inhibition by propyl thiouracil or gold (Larsen and Berry 1995). Type III 5'-deiodinase has also been identified which is responsible for inner ring monodeiodination of T₄ to produce rT₃. Type III also inactivates T₃ by converting it to 3, 3'- diiodothyronine. These three deiodinases contain selenocysteine in the active centre. Selenium deficiency reduces type I deiodinase activity. When T₄ synthesis is impaired deiodination by type I deiodinase regulates the level of T₄ in the circulation. Since T₄ is required for the generation of much of the T₃ in the central nervous system via the action of type I...
deiodinase, selenium deficiency protects the brain from T₃ deficiency (Larsen et al. 1981).

**METABOLISM OF THYROID HORMONES**

In addition to deiodination, iodothyronines are metabolized by conjugation of the phenolic hydroxyl group with sulfate or glucuronic acid (Visser 1996).

![Pathways of Thyroid Hormone Metabolism](image)

**Fig 4. Pathways of thyroid hormone metabolism**

Sulfation and glucuronidation are so-called phase II detoxication reactions, the general purpose of which is to increase the water-solubility of the substrates and thus to facilitate their biliary and/or urinary clearance (Visser 1996). Small amounts of T₄ and its metabolites undergo deaminations and decarboxylations. All of the metabolites can be conjugated with glucuronate and to a lesser extent with sulphate. The products and small amounts of free hormone are secreted into bile and some of the molecules are lost to the faeces (Martin 1985).

**MECHANISM OF ACTION OF THYROID HORMONES**

It is assumed that biochemical mechanisms through which thyroid hormones exert their developmental and tissue specific effects are mediated by a number of cell components. Many theories have been proposed to explain its mechanism of hormone
action. The proposed models have included uncoupling oxidative phosphorylation, stimulation of energy expenditure by the activation of Na\(^+\)-K\(^+\) ATPase activity, and direct modulation of thyroid hormone transporters and enzymes in the plasma membrane and mitochondria (Davis and Davis 1996). Although there is increasing evidence for non-genomic actions, the major effects of thyroid hormone occur via nuclear receptors that mediate changes in gene expression.

**Nuclear receptors**

Nuclear thyroid hormone receptors were cloned in 1986 by several laboratories (Weinberger et al. 1986; Sap et al. 1986). They were discovered to be the cellular homologs of an avian retroviral oncoprotein, denoted c-erbA. The thyroid hormone receptors are derived from two genes, c-erbA\(\alpha\) (TR\(\alpha\)) and c-erbA\(\beta\) (TR\(\beta\)), with multiple isoforms identified (Lazar 1993). TR\(\alpha\)1 and TR\(\beta\)1 are found in virtually all tissues that respond to thyroid hormone, where as the other isoforms exhibit a more tissue specific distribution. TR\(\beta\)2 is expressed solely in the anterior pituitary. c-erbA\(\alpha\)2, an isoform that binds to the thyroid response element (TRE) but does not bind thyroid hormone, is the most abundant isoform in brain (Strait et al. 1990).

Receptors specifically bind T\(_3\). Under physiological conditions, 90% of receptors bound hormone is T\(_3\); the affinity of nuclear receptors for T\(_3\) is 10 fold more than that of T\(_4\) (Samuels and Tsai 1973). These receptors bind to the specific TRE sites on DNA in the absence of T\(_3\) unlike the case with the steroid hormone receptors. The TREs are located near, generally upstream with respect to the start of transcription, to the promoters where transcription of specific thyroid hormone responsive genes is initiated. T\(_3\) binding to the receptors result in stimulation or in case inhibition of the transcription of these genes with consequent changes in the levels of mRNAs transcribed from them. The changes in mRNA levels alter the level of the protein product of these genes. These proteins then mediate the thyroid hormone response (Greenspan 1994).

**Extra nuclear binding sites**

In addition to the nuclear receptor mediated actions, there are several well characterized non genomic actions of thyroid hormones, including these occurring at the level of the plasma membrane (Davis et al. 1989) and on the cellular cytoarchitecture (Siegrist-Kaiser et al. 1990). In addition, there are well characterized thyroid hormone
binding sites on the mitochondria (Sterling 1989). In several of these processes T₄ is the hormone that produces the response. The overall contribution of the extra nuclear sites to cellular regulation by thyroid hormone is likely to be minor.

REGULATION OF THYROID FUNCTION

The growth and function of the thyroid gland are controlled by a number of mechanisms. The thyroid gland takes part in the feedback control along with the hypothalamus and the pituitary. On the other hand, there is an inverse relationship between the glandular organic iodine level and the rate of hormone synthesis in spite of fluctuations in the availability of iodine (Wolff 1969). Finally the hypothalamic pituitary feedback mechanism senses variations in the availability of thyroid hormones and corrects the abnormalities in the concentration of thyroid hormones in blood (Wilber and Yamada 1990).

The hypothalamic-pituitary-thyroid axis

The activity of the thyroid gland is predominantly regulated by the concentration of the pituitary glycoprotein hormone, thyroid stimulating hormone (TSH).

Fig 5. Basic elements in the regulation of thyroid function. TRH is a necessary tonic stimulus to TSH synthesis and release. TRH synthesis is regulated directly by thyroid hormones. T₄ is the predominant secretory product of the thyroid gland, with peripheral deiodination of T₄ to T₃ in the liver and kidney supplying roughly 80% of the circulating T₃. Both circulating T₃ and T₄ directly inhibit TSH synthesis and release independently; T₄ via its rapid conversion to T₃. SRIH = somatostatin.

Regulation of thyroid function in normal individuals is to a large extent determined by the factors that regulate the synthesis and secretion of TSH. The hypothalamic-pituitary-
thyroid axis consists principally of thyrotropin releasing hormone (TRH) and the feedback effects of circulating thyroid hormones at the hypothalamic and pituitary levels. The consequence of the dynamic interplay of these two dominant influences on TSH secretion, the positive effect of TRH on the one hand and the negative effects of thyroid hormones on the other, result in a remarkably stable concentration of TSH in the circulation and consequently little alteration in the level of circulating thyroid hormones from day to day and year to year (Wilber and Yamada 1990).

**Thyroid stimulating hormone (TSH)**

TSH is the major regulator of the morphological and functional status of the thyroid gland. It is secreted from the thyrotropes in the anteromedial portion of the adenohypophysis. TSH is a heterodimer consisting of α and β subunits tightly, but non-covalently, bound (Grossmann et al. 1997). The α subunit is common to TSH, follicle stimulating hormone (FSH), luteinizing hormone (LH) and chorionic gonadotropin (CG). The β subunit confers specificity to the molecule since it interacts with the thyroid cell TSH receptor and is rate-limiting in the formation of the mature heterodimeric protein. However, the free β subunit is inactive and requires noncovalent combination with the α subunit to express hormonal bioactivity. The linear sequence of human α subunit is represented by 92 amino acids including 10 half-cystine residues, all of which exist in disulfide linkage (Grossmann et al. 1997). The human α subunit gene is located on chromosome 6 and the TSH β gene on chromosome 1 (Naylor et al. 1983). The structure of α and β subunit gene has been determined and isolated in several animal species (Gordon et al. 1988). The genes of each species are approximately of the same size and similarly organized in four exons and three introns. In contrast to the α subunit, the organization of the TSH β gene is somewhat variable between the different species. Proper TSH glycosylation is also necessary to attain normal bioactivity (Amir et al. 1987), a process that requires the interaction of the thyrotropin releasing hormone (TRH), with its receptor on the thyrotroph (Taylor and Weintraub 1989). Specific amino acid sequences in the common α and TSH β subunits are critical for the heterodimerization, secretion and bioactivity of mature TSH. These sequences include highly conserved segments that are essential for TSH receptor binding and biological activity (Grossmann et al. 1997).
TRH increases and dopamine suppresses the TSH synthesis. The TSH level is increased in hypothyroidism and reduced in hyperthyroidism (Sarne and Refetoff 1995). TRH stimulates the synthesis of TSH, while thyroid hormones inhibit this function. Thus thyroid hormones mediate the feedback regulation of TSH secretion and TRH determines its set point. The acute inhibition of TSH release by physiological quantities of T4 is mediated by the T3 produced in the pituitary gland (Silva and Larsen 1977). The cytokines interleukin-1β (IL-1β), IL-6 and tumour necrosis factor α (TNFα) inhibit TSH release (Larsen et al. 1998). TSH enhances essentially all processes leading to the synthesis and secretion of thyroid hormones. TSH controls iodide transport system of the thyroid gland. In dispersed bovine thyroid cells TSH elicits a biphasic response of iodide transport; an acute depression due to an increase of the iodide efflux followed by a slow increase of the iodide efflux above the initial value. Besides TSH regulates intrathyroidal conversion of T4 to T3 (Erickson et al. 1982).

**Thyrotropin releasing hormone (TRH)**

TRH is the primary regulator of both the synthesis and secretion of pituitary TSH, a modified tripeptide, is derived from a large prepro-TRH molecule (Wilber and Yamada 1990). The parvocellular region of the paraventricular nuclei of the hypothalamus is the source of the hypothalamic portal TRH. It regulates TSH secretion. T3 suppresses the levels of prepro-TRH mRNA in the hypothalamus (Segerson et al. 1987) but this feedback requires a combination of T4 and T3 in the circulation, T4 giving rise to T3 via direct local synthesis in the central nervous system (Kakucska et al. 1992). The thyroid hormones also block the capacity of TRH release from the pituitary thyrotropes (Koenig et al. 1984).

![Fig 6. Structure of TRH](Pyro-J-Glu-His-Pro(NH2))

Fig 6. Structure of TRH

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Importance of TRH is that it not only activates TSH biosynthesis and TSH secretion but also modulates post-translational glycosylation of the TSH molecule, such that TRH participates in stimulating full glycosylation of the TSH molecule required for optimal biological activity (Wilber and Yamada 1990).

**Autoregulation**

In contrast to feedback control via TSH, intrinsic autoregulatory mechanisms maintain the constancy of thyroid hormones stores and help the thyroid gland to overcome factors that impair thyroid hormone synthesis.

**Iodine deficiency**

The responses to vertebrates to iodine deficiency conserve this limited resource and adjust at the hypothalamic pituitary, thyroid and peripheral levels. Removal of iodine from the diet causes a rapid decrease in serum T₄ concentrations and an increase in serum TSH. Circulating T₃ concentrations are maintained in spite of marked fall in T₄ level but eventually T₃ level fall if restriction is continued (Larsen et al. 1998). Thyroidal T₄ is gradually depleted while T₃ synthesis is maintained in the enlarging thyroid. The increase in TSH and decrease in iodine availability cause changes in Tg namely an increase in the ratio of MIT to DIT and in the ratio of T₃ to T₄ owing to a decrease in T₄ synthesis. On the other hand, iodine uptake and organification are enhanced owing to the increased TSH stimulation, a decrease in the autoregulatory suppression of thyroid gland by iodine and an increase in the efficiency of intra-thyroidal conversion of iodine (Reisco et al. 1977).

**Iodine excess**

Autoregulatory mechanism that enhances TSH mediated adaptations to reduced iodine intake also protects against excessive iodine ingestion so that plasma thyroid hormone concentrations remains normal. An excess of iodine in the medium of dispersed thyroid cells suppresses iodide uptake and cause a concomitant increase in the Km of the iodide pump i.e. a decrease in its affinity for I⁻ ions. These effects are mediated by a mechanism that requires protein synthesis (Sherwin and Price 1986). In response to large quantities of iodine there is a sharp decrease in organification (Wolff and Chaikoff 1948). Excessive iodine intake can also block thyroid hormone synthesis by inhibiting
the enzyme involved in the synthetic process (Fisher 1996). The decrease in
organification depends on a high concentration of iodide within the gland and results in
decreased formation of thyroid hormones. If excessive iodine ingestion continues, the
thyroid gland adapts by decreasing active transport of iodide into thyroid cell
(Braverman and Ingbar 1963).

Other factors

There are a number of other regulatory factors that may influence thyroid
function. Apart from the well known effects of iodine in this respect, neuropeptides
belonging to the adrenergic, cholinergic and peptidergic nervous system may be
stimulatory or inhibitory on thyroid function in some species. Also peptides derived from
the C cells, atrial natriuretic factor, growth factors and cytokines may be relevant to
thyroid function in physiological and pathological conditions (Dumont et al. 1992).

Besides that several other endogenous and exogenous factors are involved in the
regulation of thyroid function viz. pregnancy, age, sex steroids, growth hormone,
environmental temperature, nutrition, calmodulin etc. Pregnancy affects all aspects of
thyroid hormone economy. Total serum $T_4$ and $T_3$ concentrations raise to levels twice
those of non-pregnant women (Guilaume et al. 1985). Thyroid hormone production
rates are higher per unit body weight in neonatal infants and children than in adults.
Thyroid disorders are more common in women than in men and goitre commonly
develops during puberty and pregnancy (Glinoer and Leome 1992). Growth hormone
cause an increase in serum free $T_3$ and a decrease in free $T_4$ in both $T_4$ treated and normal
individuals suggesting increased conversion of $T_4$ to $T_3$ (Jorgensen et al. 1989). Repeated
exposure to extreme cold for several months causes a decrease in the total serum $T_4$
concentration but no change in free $T_4$ (Smals et al. 1977). Chronic malnutrition as in
protein calorie malnutrition and anorexia nervosa is also associated with a decreased
serum $T_3$ concentration, serum $T_4$ levels also tends to be slightly decreased but serum
TSH concentrations and their response to exogenous TRH are usually normal (Danforth
et al. 1979).

FACTORS REGULATING THYROID GLAND FUNCTION

A large number of agents in the environment, both naturally occurring and
anthropogenic, as well as some medications, are known to interfere with thyroid gland
morphology and function posing the danger of thyroid disease. Agents that cause thyroid enlargement are known as environmental goitrogens which may cause the goitrous condition by acting directly on the thyroid gland but also indirectly by altering the regulatory mechanisms of the thyroid gland and the peripheral metabolism and excretion of thyroid hormones (Gaitan 1988; Gaitan 1989; Gaitan 1990). However, the mechanism that induces the trophic changes leading to goitre formation is not well understood, because besides thyrotropin other humoral, paracrine and autocrine growth factors may be involved in the process (Gaitan 1990).

Anti-thyroid compounds may enter into the food, water and air exposure pathways, becoming an important environmental goitrogenic factor (Gaitan 1990). Their effect may be additive to those of iodine deficiency (ID) making the intensity of the manifestations of goitre and iodine deficiency disorders (IDD) more severe. In iodine sufficient areas, these compounds may be responsible for the development of sporadic goitre or the persistence of the goitre endemia with its associated disorders (Ermans and Bourdoux 1989).

Fig 7. Sites of action of environmental goitrogens
Environmental agents acting through the foodstuff or water exposure along with bacterial contamination, malnutrition with chemicals and drugs responsible for the thyroid disorders are summarized.

**Anti-thyroid Drugs**

According to their principal mode of action on thyroidal iodine metabolism, anti-thyroid drugs are divided into two categories:

1. The monovalent anions which inhibit iodide transport into the thyroid gland viz. thiocyanate, perchlorate and nitrate ions (SCN\(^-\), Cl\(^{O_4}\), NO\(^{3}\)) inhibit transport of iodide into the thyroid gland and thereby depress iodide uptake and hormone formation (Ermans and Goossens 1961; Chow *et al.* 1969). Thiocyanate stimulates efflux of iodide from the thyroid and also inhibits iodide binding and probably coupling (Scranton *et al.* 1969). These ions have a molecular volume and charge similar to those of iodide, and may compete with iodide for transport (Anbar *et al.* 1960).

2. A large number of compounds that act through inhibition of thyroidal iodide binding and iodotyrosine coupling. The most important representatives of this category of compounds are the group of thionamides. They may be competitive substrates for thyroid iodide peroxidase, preventing the peroxidation of iodide by this enzyme (Yamazaki *et al.* 1960).

A number of other drugs, including the aminoheterocyclic compounds and substituted phenols, act as goitrogens principally by impairing Tg iodination. They are in general far less potent in their goitrogenic effect than the thionamides (Milne and Greer 1962).

The effect of the drugs in the first category is counteracted by exposure to excess iodine, whereas iodine has no effects and at times even potentiates the action of drugs in the second category. Other drugs inhibit thyroid hormone secretion or act through yet unknown mechanism.

**Nutrition**

Since thyroid hormone plays a central role in the regulation of total body metabolism, various nutritional factors may profoundly alter the regulation, supply, and disposal of this thermogenic hormone. Although many dietary changes can affect the
thyroid economy, the most striking and important effects are related to alterations in total caloric intake and the supply of iodine. The changes associated with caloric deprivation appear homeostatic in nature, producing alterations in thyroid hormones that would conserve energy through a reduction in catabolic expenditure (Gaitan 1990).

**Protein Calorie Malnutrition (PCM)**

Protein intake might be a determining factor in the malnourished individual and in experimental animals while on a poor protein diet. PCM is associated with a low serum $T_3$ concentration and increased $rT_3$ levels, probably due to similar changes in iodothyronine monodeiodination (Pimstone *et al.* 1973). The experimental model of protein malnutrition in the rat yielded different results from those observed in humans. A low protein diet in rats impairs the thyroidal transport of iodine, decreases iodine concentration in the thyroid and is accompanied by an enlargement of the thyroid. Serum $T_4$ and $T_3$ levels were found to be both elevated (Tulp *et al.* 1979). Under these circumstances the goitrogenic effect of anti-thyroid substances are enhanced. The administration of protein reverses these alterations and decreases the action of such goitrogenic agents (Gaitan *et al.* 1986).

**Iodine**

Of the many minerals that may affect thyroid function, iodine is the most important. It is an essential substrate for thyroid hormone synthesis and also interacts with the function of the thyroid gland at several levels.

Acute administration of increasing doses of iodide enhances total hormone synthesis until a critical level of intrathyroidal iodide is reached. Beyond this level, iodide organification and hormone synthesis are blocked (the acute Wolff-Chaikoff block). Chronic or repeated administration of moderate to large doses of iodine causes a decrease in iodide transport resulting in a decrease in its intrathyroidal concentration. The latter relieves the Wolff-Chaikoff block and is known as the *escape or adaptation phenomenon*. The mechanism through which iodide acts is via desensitization of the thyroid gland to TSH (Robison *et al.* 1998). In TSH stimulated glands, iodine rapidly reduces the level of the mRNA for thyroid peroxidase (TPO) and the Na$^+/T$ symporter (NIS) but not for Tg or the TSH receptor (TSHr). Iodine also antagonizes TSH stimulated thyrocyte proliferation (Uyttersprot *et al.* 1995).
Another effect of large doses of iodine, apparently independent of TSH and hormone synthesis, is the prompt inhibition of hormone release. In normal persons, the inhibitory effect of large doses of iodine on thyroid hormone release produces a transient decrease in the serum concentration of $T_4$ and $T_3$. It causes, in turn, a compensatory increase in serum TSH, which stimulates hormone secretion and thus counteracts the effect of iodine (Vagenakis et al. 1973). The most intriguing effects of iodine are the involution of hyperplasia and the decrease in vascularity that occur when the ion is administered to patients with diffuse toxic goitre. Iodine may be able to induce apoptosis in thyroid cells (Burikhanov and Matsuzaki 2000). Under different circumstances, iodide may intensify the hyperplasia and produce a goitre (Fisher 1996).

Iodine deficiency used to be the leading cause of goitre in the world and still remains so in certain regions. When severe, it can cause hypothyroidism and cretinism (Gaitan 1990). Excess iodine can be responsible for the development of goitre, hypothyroidism, and thyrotoxicosis. More rarely, ingestion of excess iodide may cause thyrotoxicosis (Stanbury et al. 1998).

Lithium

Lithium ion is goitrogenic when used in the treatment of manic-depressive psychosis and can induce myxedema. Experimentally, lithium increases thyroid weight and slows thyroid iodine release. Inhibition of thyroid hormone release may be the dominant effect of the ion (Lazarus 1998). One well documented phenomenon is a potentialisation of an iodide-induced block of binding and hormone release perhaps because lithium is concentrated by the thyroid and increases the intrathyroidal iodide concentration (Spaulding et al. 1972). In rat brain, lithium administration decreased both the levels of the TypeI 5' Deiodinase and the TypeIII 5' Deiodinase (Baumgartner et al. 1997).

Hardness of water

In 1931 Slott suggested that goitre in India was directly related to the high calcium content in drinking water (Slott 1931). Murray et al. concluded on the basis of their studies that even where the iodine levels were similar, there was a greater incidence of visible thyroid glands in people in areas with hard water as in England, than in areas with soft water, as in Scotland (Murray et al. 1948). However, it has also been mentioned
that goitre could take place with soft water. High mineral content, particularly of magnesium and calcium salt have been implicated as goitrogenic factor in water (Langer 1960; Koutras 1980).

**Bacterial contamination**

Bacteriological studies in western Colombia showed significantly higher goitre prevalence associated with overall concentration of bacteria in the pipeline system and lower prevalence associated with *Klebsiella pneumoniae* in water source. These results represent natural examples of bacterial intermediation in the process of biomagnification and biodegradation of the organic pollutants that produce goitre (Gaitan *et al.* 1980). Moreover evidence of immunologic cross reactivity between the human thyroid plasma membrane and antigenic determinants in *E. coli* and *Yersinia entercolitica* raise the interesting possibility that bacterial antibodies exert a growth promoting effects on thyroid, thus playing a role in the pathogenesis of goitre in endemic areas (Weiss *et al.* 1983).

**Humic substances / organic matter**

Studies on the physical state of organic goitrogens in water indicate that the active compounds form dissociable complexes and that they are part of larger organic molecule, possibly humic substances (HS) (Gaitan *et al.* 1983). HS, high molecular weight complex polymeric compounds are the principal organic component of soils and water. More than 90% of total matter in water consists of HS, which are also present in coals and shales (Cooksey *et al.* 1985). Fulvic and humic acid, two major fractions of HS, inhibit TPO. In contrast to lignin derived HS, up to 70% of flavonoids HS may be made up of phenolic and carboxylic benzene ring. Decaying organic matter becomes the substrate of lignin and flavonoid type HS during the process of fossilization (Gaitan 1990). Epidemiologic evidences demonstrate that the geologic composition of aquifiers and watersheds is significantly associated with goitre prevalence rates (Gaitan 1983).

**Water-borne goitrogens**

Water-borne goitrogens may be the most important factor underlying the goitre endemia of regions. Coal is a source of a large variety of anti-thyroid and goitrogenic compounds viz. phenol, dihydroxyphenols (resorcinol), substituted dihydroxybenzenes,
phthalic acid, pyridines, halogenated and polycyclic aromatic hydrocarbons (PAH) (Gaitan 1988). Most of these compounds have been identified in drinking water from the iodine-sufficient goitrous areas of Kentucky and Columbia (Gaitan 1986a).

**Polyhydroxy phenol and phenol derivatives**

Phenolics are the major organic pollutants in wastewater effluents from various types of coal treatment processes (Gaitan 1988). Resorcinol, substituted resorcinol and other anti-thyroid phenolic pollutants are present in cola derived effluents. Up to 8% shale bitumen are composed of phenols. Resorcinol and substituted resorcinol dihydroxyacetophenone have also been isolated from water supplies of goitre endemic areas (Gaitan 1983). Resorcinol, substituted resorcinol inhibits both thyroidal uptake of I\(^{125}\) and its organification. Resorcinol is antithyroid and goitrogenic both in humans and experimental animals (Gaitan 1988).

**Phthalic esters and metabolites**

Phthalic acid esters or phthalates are ubiquitous in their distribution and have been frequently identified as water pollutants (Peakall 1975; Gaitan 1989b). Dibutyl (DBP) and dioctyl phthalates (DCP) have been identified from goitre endemic areas of western Columbia and eastern Kentucky (Gaitan 1988). Phthalate esters are mostly found as industrial pollutants. They are commonly used as plasticizers to give flexibility to plastics, particularly polyvinyl chloride (PVC) polymers that have wide variety of biomedical and other uses (Peakall 1975). Although phthalate esters and phthalic acid do not possess intrinsic anti-thyroid activity but they undergo degradation by gram negative bacteria to form dihydroxybenzoic acid (DHBA). DHBA are known to inhibit *in vitro* TPO activity and incorporation of iodide into thyroid hormones (Gaitan 1988). Marked ultra structural changes in thyroid gland have been observed in rats treated with phthalic acid esters (Hinton *et al.* 1986).

**Poly chlorinated and poly brominated biphenyls (PCB and PBB)**

These are aromatic compounds containing two benzene rings with two or more substituent chlorine or bromine atoms. They have a wide variety of industrial application, including electric transformers, capacitors and heat transformers (Buckley 1982). In addition to their occurrence in surface water like river lakes etc., PCBs have
also been detected in drinking water. The most significant human exposures are limited to individuals consuming fresh water fish from contaminated streams and lakes, and to occupational exposure of industrial workers (Gaitan 1988). PCBs are potent hepatic microsomal enzyme inducers (Barsano 1989). Rats exposed to PCBs exhibit an enhanced biliary excretion of $T_4$ (Gaitan 1988; Barsano 1989). The enhanced peripheral metabolism and reduced binding of $T_4$ to serum proteins in PCB treated animals result in markedly decreased serum $T_4$ concentrations. These low levels result ultimately in goitre formation (Gaitan 1988). PBB acts similarly like PCB. There are some indications that they may also interfere directly with the process of hormonal synthesis in the thyroid gland (Gaitan 1988).

**Polycyclic aromatic hydrocarbons (PAH)**

Polycyclic aromatic hydrocarbons (PAH) have been found in food and domestic water supplies and in industrial and municipal waste effluents (Barsano 1989). They occur naturally in coal, soils, ground water and surface water and in sediments and biota. The PAH carcinogens, BaP (3,4-benzpyrene) and 3-methylcolanthrene (MCA) by enhancement of hepatic UDP glucuronyltransferase and glucuronidation, accelerate $T_4$ metabolism and excretion of $T_4$ glucuronide resulting in decreased serum $T_4$ concentrations, activation of pituitary-thyrotropin-thyroid axis and eventually in goitre formation (Barsano 1989). There is also indication that MCA interferes directly with the process of hormonal synthesis in the thyroid gland.

**Pyridines**

Hydroxypyridines also occur in aqueous effluents from coal conversion processes, as well as cigarette smoke (Gaitan 1988). Dihydroxypyridines and 3-hydroxypyridines are potent inhibitor of thyroid peroxidase producing effects comparable to or greater than those of PTU (Lindsay et al. 1992). After ingestion, mimosine, a naturally occurring amino acid in the seeds and foliage of the tropical legume, *Leucaena leucocephala*, is metabolised to 3, 4-dihydroxy pyridine, a potent anti-thyroid agent that produces goitre (Christie et al. 1979).
Other organochlorines

DDT (2, 2 bis-[p-chlorophenyl]-1,1,1-trichloroethane) is polychlorinated and non-degradable. It is insoluble in water and resistant to destruction by light and oxidation. Its stability has created difficulties in residue removal from water, soil and foodstuffs (Gaitan 1990). The dominant degradative reaction of DDT is dehydrochlorination to form DDE. Another breakdown product of DDT is DDD. Dieldrin is one of the cyclodiene insecticides. DDT is known to cause goitre and increased hepatobiliary excretion of thyroid hormones in rats (Barsano 1989). All these compounds (DDT, DDE, DDD and dieldrin) induce microsomal enzyme activity that may affect thyroid hormone metabolism in a similar way to that of polyhalogenated biphenyls and polycyclic aromatic hydrocarbons (PAH) (Barsano 1989).

Goitrogens from foodstuffs

Cyanogenic glucosides

Cyanogenic glucosides are present in more than 2000 different plant species, including ferns, monocots and dicots. Upon disruption of plant tissue containing cyanogenic glucosides, these are hydrolyzed with concomitant generation of glucose, an aldehyde or ketone and hydrogen cyanide (HCN). In some species, including the agriculturally important tropical crop cassava, they are present in high amounts (Conn 1980). Cyanogenic glucosides release cyanide after ingestion or during emergency. The cyanide is detoxified to thiocyanate and the thiocyanate may cause goitre by displacement of iodide from the thyroid and by blocking organic binding of iodine (Greer et al. 1966; Hershman et al. 1985).

Sulfurated organics

Sulfurated organics mainly include thiocyanate and thiocyanate derivatives and disulfides.

Glucosinolates, thiocyanate, isothiocyanate and thio-oxazolidone (goitrin)

Glucosinolates are thioethers. They generally consist of a sugar entity, b-D-thioglucose, with an ester bond to an organic aglycone that is an alkyl group yielding isothiocyanate, nitrile, thiocyanate or a similar compound upon hydrolysis. These compounds often
contribute a bitter, "hot" taste to condiments (mustard, horseradish) and may exhibit goitrogenic or anti-thyroid activity (Mithen et al. 1987).

**Isothiocyanates** - are irritating to mucous membranes and not readily consumed in sufficient quantities to be toxic. However, if they are consumed as glucosinolates and then hydrolyzed to isothiocyanates in the gut, they can have powerful anti-thyroid effects and interfere with the synthesis of necessary thyroid hormones (Ermans and Bourdoux 1989).

**Oxazolidine-2-thiones** - they are produced by the conversion of the glucosinolates progoitrin in rapeseed meal to goitrin. Oxazolidine-2-thiones depress growth and increase the incidence of goitres. They inhibit thyroid function by blocking the incorporation of iodine into thyroxine precursors and by suppressing thyroxine secretion from the thyroid (Ermans and Bourdoux 1989).

**Nitriles** - depress growth, cause liver and kidney lesions, and in severe cases liver necrosis, bile duct hyperplasia, and megalocytosis of tubular epithelium in the kidney (Ermans and Bourdoux 1989).

**Thiocyanate** - inhibit iodine uptake by the thyroid, leading to reduced iodination of tyrosine and resulting in decreased production of the important thyroid hormone thyroxine (Ermans and Bourdoux 1989).

Thiocyanate and isothiocyanate present in Cruciferae plants have goitrogenic activity while anti-thyroid compound goitrin have been isolated from yellow turnip and from Brassica seeds. After ingestion cyanogenic glucosides are converted to thiocyanate mainly by tissue enzymes (Langer and Greer 1977; Ermans and Bourdoux 1989). Thiocyanate is also present in high concentration in waste water effluents of coal conversion process and in body fluid as a metabolite of hydrogen cyanide gas consumed while smoking (Gaitan 1986a).

**Disulphides**

The small aliphatic disulphides, the major components of onion and garlic, exert marked thiourea like anti-thyroid activity in the rats (Gaitan 1988). n-propyl disulphide also suppresses the radioactive iodine uptake by the thyroid in rats on a low iodine diet.
These disulphides inhibit in vitro thyroid peroxidase enzyme, but the fraction with sulphur-bearing organic compounds possibly aliphatic disulphides from the goitrogenic well supplying a Colombian district with endemic goitre, inhibited in vitro organification (Gaitan et al. 1969). Disulphides are also present in high concentration in aqueous effluents from coal conversion processes and they have also been identified as water contaminants (Ermans and Bourdoux 1989).

**Flavonoids**

Flavonoids are important stable organic constituents of a wide variety of plants. They are present in high concentrations in polymeric (tannins) and oligomeric (pigments) forms in various staple foods viz. millet, sorghum, beans, ground nut etc. (Cody 1989). Flavonoids exert a thiourea like anti-thyroid effects (Gaitan et al. 1989). After ingestion by mammals, flavonoid glycosides are hydrolysed by intestinal microbial glycosidases to flavonoid aglycones. These may be absorbed and undergo metabolism by mammalian tissues or be further metabolized by intestinal microorganisms to undergo B-ring hydroxylation and middle ring fusion with production of various metabolic compounds, including phenolic acids, phloroglucinol and gallic acid. Each metabolic step is characterized by marked increase in anti-thyroid effects. Flavonoids not only inhibit the thyroid peroxidase but acting on iodothyronine deiodinase enzyme, also inhibit the peripheral metabolism of thyroid hormones (Cody 1989).

**FUNCTIONS OF THYROID HORMONES**

T<sub>3</sub> and T<sub>4</sub> regulate growth and development, especially critical for the fetus, with requirements lasting past the time of birth. T<sub>3</sub> and T<sub>4</sub> impact upon numerous systems including muscle, bones, central nervous system development, myelination, dendritic formation and synapse formation (Harrison et al. 1996; Poterfield and Hendrich 1993).

T<sub>3</sub> and T<sub>4</sub> act as metabolic stimulants (calorigenic effects) to increase the rate of oxygen consumption by the heart, skeletal muscle, liver, and kidney. In adults, brain, spleen, and gonad metabolism is less susceptible to the effects of T<sub>3</sub> and T<sub>4</sub>. CNS input through the hypothalamus (TRH) in cold temperatures increases T<sub>3</sub> and T<sub>4</sub> secretion to increase metabolic rates (Hennemann et al. 1988; Welle and Campbell 1986).
\[ T_4 \] and \[ T_3 \] have cardiovascular effects, increasing the heart rate and force of contraction to achieve increased cardiac output. Excess \[ T_4 \] and \[ T_3 \] can increase heart stroke volume and heart rate by increasing its sensitivity to catecholamines. In hyperthyroidism, the number of \( \beta \)-adrenergic receptors in the heart is increased (Bahouth et al. 1997).

Thyroid deficiency in childhood causes mental retardation. In adults, \[ T_4 \] and \[ T_3 \] deficiency causes lethargy and blunting of intellect. \[ T_4 \] and \[ T_3 \] excess causes restlessness and hypersensitivity (Hetzel 1987). Besides that a number of disorders develop in iodine deficient condition affecting thyroid functions largely. These are collectively called iodine deficiency disorders (IDD) and associated disorders.

**IODINE DEFICIENCY DISORDERS (IDD)**

Iodine deficiency is a world wide problem that leads to goitre and syndrome of endemic cretinism along with clinical and subclinical defects of motor and cognitive functions. These spectrums of defects have been collectively labeled as iodine deficiency disorders (IDD) (Hetzel 1987).

**Iodine deficiency in animals**

Iodine deficiency in animals is, as in human, a cause of goitre and malfunction of the thyroid, the major morphological and functional abnormalities. Beckers and Delange (1980) have pointed out that animals subjected to chronic iodine deficiency with alternating periods of normal iodine intake have similar thyroidal changes to humans that may be summarized as

i) A low cellular iodine content compared to large total iodine storage

ii) A heterogeneous activity of the thyroid gland as shown by the iodine kinetic studies

iii) A qualitative change in the thyroid secretion with greater synthesis of \( T_3 \)

iv) A reduced iodination of thyroglobulin associated with impaired proteolysis and a decreased iodinating activity of iodotyrosines and

v) In the goitrous gland a significant escape of nonhormonal iodine.

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Experimental studies on the effect of iodine deficiency in animals have confirmed the morphological and biochemical modifications as seen in the hyperplastic goitre of man. It was reported that serum $T_4$ levels but not $T_3$ were reduced after 6 months exposure to the diet and maternal iodine deficiency in the rat has also been shown to reduce the number of viable embryos and their individual body weight (Hetzel et al. 1990).

**Iodine deficiency in human**

The best known effect of iodine deficiency is endemic goitre. Goitre is a swelling of thyroid gland, well known from the ancient period and has continued to excite interest over the centuries (Langer 1960). Iodine deficiency is the major primary etiological factor in endemic goitre. Goitre also arises from causes other than iodine deficiency due to a variety of agents known as goitrogens. Goitrogens in general are of secondary importance to iodine deficiency as etiological factors in endemic goitre. Apart from goitre itself, more recent work on the effects of iodine deficiency in man has revealed a great variety of effects on human growth and development. These IDDs are mostly seen in different phases of life (Hetzel et al. 1990).

Iodine deficiency of the foetus is the result of iodine deficiency in the mother. This condition is associated with a greater incidence of still birth, abortions and congenital abnormalities. Another major effect of foetal iodine deficiency is the condition of endemic cretinism. It is characterized by mental deficiency, deaf mutism and spastic diplegia squint, which is referred to as the nervous or neurological type in contrast to the less common myxedematous type characterized by dwarfism and mental deficiency (Hetzel et al. 1990). Continuing presence of severe iodine deficiency affects neonatal thyroid function and hampers the early brain development. The incidence of neonatal hypothyroidism was related to the severity of iodine deficiency as evidenced by the prevalence of goitre and cretinism and the level of excretion of iodine in urine. Iodine deficiency in children is characteristically associated with goitre. The goitre rate increases with age so that it reaches a maximum with adolescence. Girls have higher prevalence than boys. It is generally accompanied by impaired mental function with retarded physical development. The common effect of iodine deficiency in adults is goitre. Characteristically there is an absence of classical clinical hypothyroidism with
reduced T₄ levels often accompanied by normal T₃ levels and raised TSH levels in adults (Hetzel et al. 1990).

Table 2. The Spectrum of IDD (Hetzel et al. 1990)

<table>
<thead>
<tr>
<th>Stage</th>
<th>Abnormalities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foetus</td>
<td>Abortion, Still birth, Congenital abnormalities, Increased perinatal mortality, Increased infant mortality, Neurological cretinism, Myxedematous cretinism, Psychomotor defects</td>
</tr>
<tr>
<td>Neonate</td>
<td>Neonatal goitre, Neonatal hypothyroidism</td>
</tr>
<tr>
<td>Child &amp; adolescent</td>
<td>Goitre, Juvenile hypothyroidism, Impaired mental function, Retarded physical development</td>
</tr>
<tr>
<td>Adult</td>
<td>Goitre with its complications, Hypothyroidism, Impaired mental function, Iodine-induced hyperthyroidism</td>
</tr>
</tbody>
</table>

Iodine administration in the form of iodised salt, iodised bread or iodised oil have been demonstrated to be effective in the prevention of goitre in adults. Incidence of hyperthyroidism has been reported from various countries after iodine supplementation through salt, bread etc. (Hetzel et al. 1990)

The review shows that the morphological and functional status of thyroid gland is interfered by a number of natural and anthropogenic factors other than the iodine deficiency that enter mainly through food and water. In addition these factors are region specific. To prevent the iodine deficiency disorders that considered as a major public health problem, further studies are necessary to evaluate the goitrogenic / anti-thyroid potentiality of the suspected fodds where endemic goitre is prevalent in post salt iodization phase. Studies on anti-thyroid potentiality of commonly consumed cyanogenic plant foods of Indian origin are scanty. Therefore in the present study attempt has been made to evaluate the goitrogenic / anti-thyroid potency of commonly used selective Brassica plants.
AIMS AND OBJECTIVES

Cabbage (*Brassica oleracea* var. *capitata*), cauliflower (*Brassica oleracea* var. *botrytis*), mustard (*Brassica juncea*) and radish (*Raphanus sativus*) are the common cyanogenic plants of Brassica family that are used as vegetables both in fresh / raw and also after cooking and thus the goitrogen content and the anti-thyroid potential of those plants of Indian origin were studied in both the fresh / uncooked and after cooking the edible parts of those plants. The present study undertaken with the following objectives

i) Chemical analysis of selective goitrogenic foods of cyanogenic origin for the determination of goitrogen content viz. cyanogenic glucosides, glucosinolates and thiocyanate

ii) Determination of *in vitro* inhibitory effect of selected plants on thyroid peroxidase activity and their relative anti-thyroid potency using human thyroid tissue

iii) Evaluation of morphological and functional status of thyroid gland under the influence of the selective dietary goitrogens in albino rats through

- Evaluation of morphological status by thyroid gland weight and histology
- Evaluation of iodine nutritional status by excretion pattern of iodine
- Evaluation of consumption level of goitrogens by excretion pattern of thiocyanate
- Evaluation of functional status by assay of thyroid peroxidase activity and thyroid hormone profiles
MATERIALS & METHODS

Selection of plants

Cyanogenic plant foods generally used as vegetables viz. cabbage (Brassica oleracea var. capitata), cauliflower (Brassica oleracea var. botrytis), mustard seeds (Brassica juncea) and radish (Rapanus sativus) were selected for the present study. Fresh samples of those plants were collected at random from different areas of West Bengal for the measurement of goitrogenic constituents, assay of \textit{in vitro} thyroid peroxidase activity and also for feeding the experimental animals to evaluate morphological and functional status of thyroid gland.

Preparation / Processing of plant foods

i) Uncooked plant foods were prepared by coarsely chopping in a cutter (except mustard seeds which was prepared by coarsely grinded in hammer) and then incubated in an oven at 30-40°C for 4 hours following the method of de Groot \textit{et al.} (1991) to enhance the degradation of glucosinolates by the tissue enzyme myrosinase, and then mixed with the other ingredients of the diet.

ii) Cooked plant foods were obtained by boiling in an equal weight of tap water for 15 minutes. The cooked material was drained and immediately used for preparing diets, measuring goitrogenic constituents and in \textit{in vitro} studies following the method of de Groot \textit{et al.} (1991).

All the chemicals and solvents used were of analytical grade.

Measurement of dietary goitrogens in plant foods

\textit{Estimation of Cyanogenic glucosides}

\textbf{Principle} : Edible parts of fresh / uncooked and cooked plants were hydrolysed by the enzyme glucosidase and the hydrocyanic acid thus liberated was trapped in sodium hydroxide. Cyanide content of trapped hydrocyanic acid was then determined quantitatively. Cyanogenic glycoside was measured following the method of Lambert \textit{et al.} (1975).
Reagents:

i) Standard sodium cyanide (NaCN) solution - 0.2 mM NaCN solution in 0.1 M NaOH was made by diluting 1ml $10^{-2}$ NaCN in 1 ml NaOH to 50 ml 0.1 M NaOH. For standard curve construction, 100-500 µl solutions were taken and each made up to final volume of 1ml with 0.1 M NaOH.

ii) 1M Acetic acid

iii) Succinimide and N-chloro succinimide - A solution of 2.5 gm succinimide and 0.25 gm N-chloro succinimide in 1 L double distilled water was employed in the experiment.

iv) Barbituric acid and Pyridine mixture - 24 gm barbituric acid and 120 ml pyridine were taken and made up to 400 ml with double distilled water.

Procedure:

1. Foods were rapidly homogenised without adding liquid.

2. An aliquot of the homogenate was weighed and transferred into a 25 ml Erlenmeyer flask containing 5 ml of acetate buffer (0.1 M; pH 5.5) with the enzyme glucosidase and 0.5 ml 1 M NaOH was added in the centre well.

3. The flask was closed hermetically and incubated at 37°C for 20 hours with agitation.

4. The hydrocyanic acid liberated by autolysis was trapped in the centre well.

5. Then it was assayed against standards as follows: the standards or the samples from the centre wells were mixed with 0.9 ml 0.1 M NaOH, 0.5 ml 1.0 M acetic acid, 5 ml succinimide / N-chlorosuccinimide reagent (succinimide 0.25% and N-chlorosuccinimide 0.025%), and 1 ml barbituric acid / pyridine reagent (barbituric acid 6% and pyridine-water, 30:70 v/v).

6. After 10 minutes, optical density was measured against blank at 580 nm.
Estimation of glucosinolates

Principle: The enzyme myrosinase (thioglucosidase) reacts with thioglucosides present in plant foods to generate thiocyanate. Following this principle glucosinolates (thioglucosides) was measured by the procedure of Gmelin and Virtanen (1960). Thiocyanate thus formed by the action of myrosinase was estimated following the method of Aldridge (1945) as modified by Michajlovskij and Langer (1958).

Reagents:

i) Methanol
ii) 70% Methanol
iii) 20% Lead acetate solution - 20 gm lead acetate was dissolved in 100 ml of double distilled water.
iv) Hydrogen sulphide
v) Myrosinase
vi) Phosphate buffer (pH 7.0)
vii) 15% Trichloro acetic acid (TCA) - 15 gm of TCA was dissolved in 100 ml double distilled water.
viii) Saturated Bromine water - Bromine was added into some amount of double distilled water and mixed. It was ensured that there were some drops of undissolved bromine at the bottom of the flask.
ix) 4% Arsenious tri oxide (As₂O₃) in 2% NaOH - 2 gm of NaOH was dissolved in 100 ml double distilled water. 4 gm of As₂O₃ was mixed with some amount of 2% NaOH in a hot plate during heating and then the total volume of NaOH was added.
x) Mixture of Benzidine hydrochloride and Pyridine - Benzidine hydrochloride and pyridine were mixed immediately before use. 1.6 ml of benzidine hydrochloride and 2 ml of pyridine was mixed for a sample.
xi) Stock thiocyanate solution - 500 mg of potassium thiocyanate (KSCN) was weighed instantly and dissolved in 100 ml double distilled water. To make SCN solution of 100 mg/dl strength from the stock solution a calculated amount of KSCN solution was taken into 100 ml volumetric flask and the volume was made 100 ml with double distilled water.

xii) Working thiocyanate solution - Stock solution was diluted with double distilled water to make working standards of 0.1 mg/dl, 0.25 mg/dl, 0.5 mg/dl, 1.0 mg/dl and 1.5 mg/dl.

Procedure:

1. 10 gm of fresh edible part of respective fresh / uncooked and cooked plant was immersed in adequate amount of absolute methanol.

2. The methanol solution was decanted and the material was thoroughly ground in mortar and was once more extracted boiling in 50 ml of 70% methanol.

3. Both the extracts were evaporated in vacuo.

4. The residue was taken in double distilled water and the solution was treated with 0.6 ml of 20% lead acetate solution.

5. Excess lead ions in the filtrate were precipitated by hydrogen sulphide.

6. The filtrate was concentrated in vacuo to about 20 ml and was brought to 25 ml.

7. Ten ml of this solution, 1 ml of myrosinase (thioglucosidase obtained from Sigma Chemicals Co.) and 1 ml phosphate buffer of pH 7.0 were incubated for 2 hours at 37°C.

8. 0.5 ml of test solution was mixed with 2 ml of 15% trichloroacetic acid and centrifuged for 10 minutes.

9. Aliquot of the supernatant (1.5 ml) was transferred to a test tube.

10. Standards were prepared by mixing 0.5 ml KSCN solutions (0.1, 0.25, 0.5, 1.0, and 1.25 mg SCN/dl) with 2 ml of 15% trichloroacetic acid.
11. The following reagents were then added subsequently to each of the sample and standard: 0.2 ml saturated bromine water, 0.2 ml 4% arsenous trioxide in 2% NaOH and 3.6 ml benzidine- pyridine mixture (2 ml of pyridine and 1.6 ml of 1% benzedine hydrochloride acidified with HCl).

12. After 30 minutes, optical density was measured against blank at 525 nm using spectrophotometer.

**Estimation of thiocyanate**

**Principle**: Following the crushing of the plant food thiocyanate is released and thiocyanate content of the plant food was then measured by the method of Aldridge (1945) as modified by Michajlovskij and Langer (1958).

**Reagents:**

i) 15% Trichloro acetic acid (TCA) - 15 gm of TCA was dissolved in 100ml double distilled water.

ii) Saturated Bromine water - Bromine was added into some amount of double distilled water and mixed. It was ensured that there were some drops of undissolved bromine at the bottom of the flask.

iii) 4% Arsenious tri oxide (As$_2$O$_3$) in 2% sodium hydroxide (NaOH) - 2 gm of NaOH was dissolved in 100 ml double distilled water. 4 gm of As$_2$O$_3$ was mixed with some amount of 2% NaOH in a hot plate during heating and then the total volume of NaOH was added.

iv) Mixture of Benzidine hydrochloride and Pyridine - Benzidine hydrochloride and pyridine were mixed immediately before use; 1.6 ml of benzidine hydrochloride and 2 ml of pyridine were mixed for each sample.

v) Stock thiocyanate solution - 500 mg of potassium thiocyanate (KSCN) was weighed instantly and dissolved in 100 ml double distilled water. To make SCN solution of 100 mg/dl strength from it a calculated amount of KSCN solution was taken into 100 ml volumetric flask and the volume was made 100 ml with double distilled water.
vi) Working thiocyanate solution - Stock solution was diluted with double distilled water to make working standards of 0.1 mg/dl, 0.25 mg/dl, 0.5 mg/dl, 1.0 mg/dl and 1.5 mg/dl.

Procedure:

1. The fresh/uncooked and cooked plant foods (10 gm) were crushed with clean sand and then extracted with about 25 ml double distilled water and refluxed for 20 minutes subsequently in a conical flask.

2. The substance was then cooled and filtered.

3. The residue in the filter paper was washed repeatedly with double distilled water and the volume of the filtrate was made 100 ml.

4. 0.5 ml of this extract containing thiocyanate was treated with trichloroacetic acid followed by saturated bromine water and arsenous trioxide and allowed to react with pyridine-benzidine hydrochloride mixture. The intensity of colour thus formed was measured using spectrophotometer (UV-1240 Shimadzu).

Animal maintenance and treatment

Ten growing female wistar rats weighing 80 ± 5 gm were allocated to one control and each experimental group of each selected plant food. Animals were caged in unheated well-ventilated stainless steel cages and maintained on laboratory standardised normal diet (20% protein) which consisted of 70% wheat, 20% Bengal gram, 5% fish meal powder, 4% dry yeast powder, 0.75% refined til oil and 0.25% shark liver oil and water ad libitum with dietary supplementation in experimental group. Control rats were fed normal laboratory diet (Mukhopadhyay et al. 2004). Cabbage, cauliflower, mustard seeds and radish fed group of rats were fed normal laboratory diet replacing one-third portion of the diet with fresh/uncooked and cooked plant foods respectively. The animals were maintained on above dietary regimen for 60 days.

Feed consumption, corrected for feed wasted and body weight were measured every seven days. In the last week of the treatment each group of animals were kept in metabolic cages for 24 hours to collect the urine over xylene for the analysis of urinary iodine and thiocyanate. At the end of the experimental period body weights of the rats were recorded and the animals were sacrificed following ethical procedure. After
overnight fasting just before sacrifice, blood samples were collected from each rat from the portal vein under ether anaesthesia and the serum were separated for the assay of $T_4$ and $T_3$ and kept at $-20^\circ$C till analysis. Just after sacrifice, thyroid glands were weighed after removing connective tissues and preserved for assay of thyroid peroxidase activity (TPO) and also for histological studies.

**Morphological and histological study of the thyroid gland**

After completion of the experimental period thyroid glands of rats in each group were dissected out. The weight of the dissected thyroid gland was noted and then fixed in formal for subsequent histological examination and then embedded in paraffin bath after usual processing. The sections were cut at 5-6 micron thickness and stained with haematoxyline and eosin. The stained sections of each group were then micro photographed and compared thereafter.

**Analysis of urine**

**Estimation of iodine**

**Principle**: Iodine in urine generally occurs as the iodide ion. Most of the available methods for urinary iodine determination include an initial stage in which the urine is either digested in strong acid or ashed at high temperature. Following the step, iodine is measure by its catalytic action on the reduction of the ceric ion ($Ce^{4+}$) to cerous ion ($Ce^{3+}$) coupled to the oxidation of arsenite $As^{3+}$ to $As^{5+}$. This reaction is called Sandal-Kolthof reaction (Sandell and Kolthof 1937). The ceric ion ($Ce^{4+}$) has a yellow colour while the cerous ion ($Ce^{3+}$) is colourless. Thus the course of reaction can be followed by disappearance of yellow colour as the ceric ion is reduced. With the other reactants held stable, the speed of this colour disappearance is directly proportional to the amount of iodide catalysing it. It was measured by dry ashing following the method of Karmarkar et al. (1986).

**Reagent**:

i) Potassium carbonate ($K_2CO_3$) 2.5 N - 17.5 gm of anhydrous $K_2CO_3$ was dissolved in small amount of double distilled water and diluted to 100 ml. It was stored in a polyethylene bottle.
ii) Ceric ammonium sulphate \([\text{Ce (NH}_4\text{)}_2\text{(SO}_4\text{)\_2\cdot 2H}_2\text{O})\text{ 0.005N - 3.17 gm of ceric ammonium sulphate was dissolved in 500 ml of double distilled water and 57 ml concentrated H}_2\text{SO}_4\text{. It was diluted to 1 L and stored in dark bottle away from light at room temperature.}\\

iii) Sodium meta arsenite \((\text{NaAsO}_3\text{) 0.03 N - 2.27 gm of sodium meta arsenite was dissolved in 500 ml of double distilled water. 46 ml of concentrated H}_2\text{SO}_4\text{ was added to this and the volume was made up to 1 L by adding double distilled water.}\\

iv) Stock iodine solution \((1\text{mg iodine/ml})\\

v) Working iodine standard - Stock iodine solution was diluted with double distilled water to make working standards of 5 μg/dl, 10 μg/dl, 15 μg/dl, 20 μg/dl and 25 μg/dl and stored in dark bottle.

Procedure:

1. Urine sample was mixed to evenly suspend any sediment.
2. 100 μl of each urine sample or diluted urine sample was taken and 100 μl of double distilled water was taken as blank.
3. 100 μl of each working standards was also taken.
4. 300 μl of K$_2$CO$_3$ solution was added to each tube and mixed.
5. The samples were placed in a test tube rack and kept in the oven for drying at a temperature of 80-100°C over night.
6. The dried samples were placed in the muffle furnace for ashing at 600°C for two hours.
7. The furnace was switched off, the temperature was allowed to come down and the rack was removed from the furnace, usually when ashing is complete no black residue is present.
8. When the tubes had cooled to room temperature, 3 ml of sodium meta arsenite solution was added to each tube. If the ash was not completely soluble, the tubes were centrifuged at 2500 g for 10 minutes and then the supernatant was transferred to another sets of tubes.
9. The tubes were incubated with the supernatant for 5 minutes in a water bath at 56°C.

10. 3 ml of ceric ammonium sulphate was added at 30 seconds intervals to each tube and mixed and placed in water bath.

11. Twenty minutes after the addition of ceric ammonium sulphate, its transmittance was read at 420 nm in a colorimeter against water blank. A stopwatch was used to keep a constant interval i.e. 30 seconds, between additions of two successive tubes.

12. Then the concentration of iodine was determined by interpolation from the standard curve using the blank corrected transmittance. The obtained result was expressed as µg/dl and moles/L.

**Estimation of thiocyanate**

**Principle**: The thiocyanate in urine is oxidised by bromine. The oxidised form of thiocyanate then reacts with the chromophore benzidine hydrochloride to form a coloured complex. The intensity of the colour formed is estimated at 525 nm. Urinary thiocyanate (SCN) concentration was measured in the same urine samples using the method of Aldridge (1945) and modified by Michajlovskij and Langer (1958).

**Reagents**: As already mentioned

**Procedure**:

Trichloroacetic acid was added to 0.5 ml of urine sample, mixed and centrifuged. To the supernatant saturated bromine water was added and 4% arsenic trioxide (As$_2$O$_3$) was then added to oxidise all bromine present in the sample. After that benzidine hydrochloride and pyridine mixture were added and the colour developed gradually. After 30 minutes optical density was measured at the wavelength of 525 nm.

**Assay of thyroid peroxidase activity**

**Principle**: Peroxidase catalyses the formation of I$_2$ and pericdide formation is then instantaneous when there is an excess of iodide substrate. Nearly all of the enzymatically generated I$_2$ is bound as I$_2^-$. Periodide can be spectrophotometrically determined at its
absorption peaks at 353 nm and the yield of I$_3$ is directly proportional to peroxidase concentration in reaction mixtures containing approximate amounts of hydrogen peroxide (H$_2$O$_2$) and enzyme. Thyroid peroxidase activity was measured following I$_3$' from iodide in presence of H$_2$O$_2$ in the assay medium by the method of Alexander (1962). The tissue protein level was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard. The results are expressed as AOD /min/mg protein.

**Reagents:**

i) Phosphate buffer (pH 7.2, 100 mM)

ii) Sucrose solution (500 mM)

iii) Acetate buffer (pH 5.2, 50 mM)

iv) Potassium iodide (1.7 mM)

v) Hydrogen peroxide (0.3 mM)

**Procedure:**

1. A 10% homogenate was prepared using thyroid tissues collected from the sacrificed animals; in phosphate buffer (pH 7.2, 100 mM) and sucrose solution (500 mM) at 4°C. Homogenisation was carried out in a glass homogeniser (Potter-Elvehjem) for 45-60 sec at 4400 g and about 15 strokes/min.

2. The homogenate was centrifuged at 1000 g for 10 min.

3. This low speed supernatant was further centrifuged at 10,000 g for 10 min at 4°C to get the mitochondrial fraction.

4. The microsomal fraction containing most of the peroxidase activity was obtained by centrifuging the post mitochondrial supernatant at 1,05,000 g for one hour.

5. Immediately after centrifugation the precipitate was solubilized in phosphate buffer (pH 7.2).
6. Thyroid peroxidase activity was measured in a 1 ml cuvette containing 0.9 ml acetate buffer (pH 5.2, 50 mM), 10 µl potassium iodide (1.7 mM), 20 µl microsomal fraction of thyroid tissue and freshly prepared 20 µl hydrogen peroxide (0.3 mM) was added lastly to start the reaction for assaying the TPO activity (ΔOD/min/mg protein) in spectrophotometer (UV-1240 Shimadzu) at 353 nm.

**Estimation of tissue protein**

**Principle**: When protein is placed in an alkaline solution containing Cu$^{+2}$, a weak coloured complex can form between the peptide bonds in the protein and the copper atom. It is thought that this binding involves the reduction of Cu$^{+2}$ to Cu$^{+1}$. This "Biuret" reaction has been used for several decades to estimate the quantity of protein in samples. The reaction is not very sensitive and large quantities of protein have to be used to get accurate results. Lowry *et al.* (1951) added Folin's reagent to the Biuret assay, greatly improving its sensitivity. The Lowry method combines the biuret reagent with another reagent (the Folin-Ciocalteaus phenol reagent) that reacts with tyrosine and tryptophan residues in proteins. This gives a bluish colour which can be read somewhere between 500 - 750 nm depending on the sensitivity required.

**Reagents**:

i) Bovine Serum albumin (BSA) – 10 mg BSA in 10 ml 0.9% sodium chloride (NaCl) solution is prepared one day prior to measurement.

ii) 0.9% NaCl solution

iii) Sodium carbonate (Na$_2$CO$_3$) – Na$_2$CO$_3$ solution was prepared with 10 gm of Na$_2$CO$_3$ dissolved in 100ml of double distilled water, 50 ml of 0.1 N NaOH is added to the solution and then the volume was made up to 500 ml.

iv) 1% Sodium potassium tartrate

v) 0.5% Copper sulphate

vi) Alkaline copper reagent was prepared freshly by mixing 1% Sodium potassium tartrate and 0.5% copper sulphate in 1:1 proportion and then 1 ml of above solution was mixed with 50 ml of Na$_2$CO$_3$ solution.
Folin ciocalteu reagent was prepared in 1:1 dilution with double distilled water.

**Procedure:**

1. From BSA solution 25 µl, 50 µl, 100 µl, 150 µl and 200 µl standards were taken and mixed with 0.975 ml, 0.950 ml, 0.900 ml, 0.850 ml and 0.800 ml of NaCl solution and in blank 1 ml of NaCl solution was taken and for the measurement of tissue protein 25 µl of sample was taken and mixed with 0.975 ml of NaCl solution.

2. 5 ml of freshly prepared alkaline copper reagent was added to each sample, standards and blank, mixed well in vortex and kept for 15 minutes.

3. 0.5 ml of 1:1 freshly prepared Folin ciocalteu reagent was added to each sample, standard and blank, mixed well in vortex and kept for 30 minutes.

4. After 30 minutes optical density was measured at 660 nm in a spectrophotometer

**Assay of anti-TPO activity of plant foods**

To evaluate *in vitro* anti-TPO activity of plants, human thyroid tissue was collected from ENT Department, S.S.K.M. Hospital, Kolkata.

Edible parts of each fresh plant (fresh and cooked respectively) was homogenised in assay buffer (5 mg plant tissue in 5 ml phosphate buffer - pH 7.2, 100 mM) and then centrifuged at 700 g for 10 min. After centrifugation, fixed amount of aliquot of the supernatant of fresh and cooked plants were added separately in a 1ml cuvette containing acetate buffer (pH 5.2, 50 mM), potassium iodide (1.7 mM), microsomal fraction of thyroid tissue and freshly prepared hydrogen peroxide (0.3 mM) was added lastly to start the reaction to assay the thyroid peroxidase activity (ΔOD/min/mg protein) under the influence of respective plant following the procedure of Gaitan *et al.* (1989).

**Assay of (IC₅₀) and PTU equivalence**

The activity of each uncooked and cooked plant extracts also evaluated in terms of the concentration necessary to produce 50% inhibition (IC₅₀) of thyroid peroxidase activity to evaluate their relative antithyroidal potency. The effects of fresh / uncooked
and cooked plant extracts were also studied at different concentrations ranging from 10 μg to 100 μg original fresh and cooked plants to determine the concentration required to produce IC\(_{50}\) of thyroid peroxidase activity. The TPO activity under the influence of a plant at a particular concentration, as a percentage of inhibition of the control value was plotted against the concentration of the original plant extract and the concentration at which the 50% inhibition occurred (IC\(_{50}\)) was determined from the plot following Gaitan \textit{et al.} (1989). The IC\(_{50}\) value of a plant is given as mean ± SD of 6 observations. To compare the relative anti TPO activity of the studied plant against a known antagonist, IC\(_{50}\) of PTU, 6-n-propyl-2-thiouracil (obtained from Sigma Chemical Co.) was determined and presented as PTU equivalence.

**Enzyme linked immunosorbant assay of total circulating thyroxine and triiodothyronine**

Circulating thyroxine level was measured using Monobind, Inc. total T\(_4\) kit [kit no. MBI 32718/083001]. In this method serum sample (25 μl) was taken in a microplate well and enzyme - T\(_4\) conjugate was added, then the reactants were mixed. After the completion of the required incubation period, the antibody bound enzyme thyroxine conjugate was separated from the unbound enzyme thyroxine conjugate by decantation. The activity of the enzyme present on the surface of the well was quantitated by the reaction with tetra methyl benzidine (TMB) substrate solution with 15 minutes incubation and finally by adding 1N HCl as stop solution and absorbance was read against 450 nm wavelength in ELISA Reader [Merck]. Circulating T\(_3\) level was measured by using Monobind, Inc. total T\(_3\) kit [kit no. MBI 33091/31902] with 50 μl serum sample following the above mentioned method.

**Statistical analysis**

All data were statistically analysed by performing Single Factor ANOVA. Multiple comparison 't' test was applied to determine the significance of difference between the two related groups when F ratios reached significance (p<0.05). The data presented in the Table as mean ± SD. The p<0.05 was considered statistically significant.