HORMONAL IMMUNOASSAY STUDIES OF THYROID FUNCTION IN HEALTHY ADULTS AND PREGNANT SUBJECTS IN DAR-ES-SALAAM, TANZANIA.

EDWARD ANGELO RUGABERA KABYEMELA.

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Abstract

Iodine deficiency disorders (IDD) are a major public health problem in many parts of the world, including Tanzania. The consequences of iodine deficiency include thyroid dysfunction abnormalities, endemic goitre and cretinism, endemic mental retardation, decreased fertility rate, increased perinatal death and infant mortality. Assays of thyroid hormones and thyrotropin are essential for the diagnosis and treatment of IDD. Until now, the Department of Clinical Biochemistry at the Muhimbili Medical Centre, Dar-es-Salaam has no thyroidology service.

This study was undertaken to evaluate thyroid function in healthy pregnant and non-pregnant Tanzanian subjects from iodine replete areas in order to establish baseline information for projected studies on effects of IDD on human reproduction. Furthermore the analytical performance of Serozyme immunoassays for the measurement of T_4, T_3, TSH and T_3Uptake as a possible basis for a future thyroidology service were evaluated. This system was proposed on the basis of pilot studies comparing the results of Serozyme assays and the methods routinely used in the Laboratory for Endocrinology and Reproduction, Academic Hospital Nijmegen, St Radboud, The Netherlands. Moreover Serozyme assays are relatively easy to perform and require inexpensive instrumentation which is easy to operate.

Serum was obtained from clinically euthyroid pregnant and non- pregnant adult subjects living in Dar-es-Salaam, Tanzania.

Results from the analysis of these samples using routine assays confirmed to a large extent that levels of thyroid hormones and thyrotropin were comparable to those published for other euthyroid populations, except that Tanzanian males had higher T_3 levels than females and Tanzanian adults had higher T_4 and T_3 levels than Dutch

adults. Thyroid function in clinically euthyroid pregnant Tanzanian women was also found to be in accordance with findings from similar studies. Assessed by free T_4 and TSH levels almost all (99%) of pregnant Tanzanian women were found to maintain euthyroidism.

Serozyme assays for T_4, T_3 and TSH generally showed a reasonable agreement with routine assays. Yet, the precision of the Serozyme T_4 assay was not satisfactory. Moreover it was noted that in a relatively large proportion of the Tanzanian samples (about 20%) the Serozyme T_3 assay failed to detect T_3 whereas it was detectable by Amersham T_3 RIA. Also, levels assessed using the Serozyme TSH assay were spuriously elevated in about 20% of the samples. It is suggested that heterophilic antibodies present in the serum of Tanzanian subjects may cause this interference. In this study, several approaches were investigated to circumvent this interference. Serozyme T_3 Uptake did not distinguish between pregnant and non- pregnant groups, so that Serozyme free T_4 index did not reflect the euthyroid status of normal pregnancy: about half of the subjects had their free thyroxine index falling within the non-pregnant range.

It is concluded that, although initially Serozyme assays seemed to have many advantages, they are not the preferential choice for the introduction of diagnostic services in Tanzania unless measures can be taken to prevent interference. It is recommended that further studies should be done to conclusively identify the interferant in the T_3 and TSH assays. Taking into account the costs and poor analytical performance of the Serozyme kits, the possibilities of developing in-house immunoassays which can be run on the Serozyme 1 analyzer should be explored.

Declaration

I declare that this thesis has not been submitted for a diploma or degree in any other University.

Date 8th October 1997

E.A.R. Kabyemela.

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The completion of this thesis marks a product of hard work and sacrifices from many personalities and mentioning each and every name will result yet into another book. I particularly wish to heartily thank my supervisors, Prof. Dr. Th. J. Benraad and Dr. L.M.M. Chuwa who have guided me throughout the two years of my study and enabled me to produce this thesis. Most of the work was done in the Department of Experimental and Chemical Endocrinology of the Catholic University of Nijmegen. I am grateful to Prof. Benraad for according me this opportunity and putting to my disposal all the necessary inputs. From the Department I secured all the funding of my research work in Nijmegen and most of the costs for field work in Tanzania. I thank the University of Nijmegen for according me a fellowship which paid for the costs of travelling and keeping me in Nijmegen for two years. I am grateful to all the staff members of the laboratories of Endocrinology and Reproduction at the St. Radboud University Hospital-Nijmegen. From them I received all the possible assistance I needed for this work. I particularly wish to thank Dr. L.M.J.W. Swinkels who worked hand in hand with me. He introduced me to this field of immunoassays in diagnostic endocrinology. He coordinated all my work with all other people. From him I have learnt the little I know in laboratory medicine. My sincere thanks are due to Dr. H.A. Ross. He constantly reviewed my work. I am grateful for his helpful ideas and suggestions which have contributed a lot in this thesis. Dr. W.M.V. Dolmans (of the Institute of International Health at the Catholic University of Nijmegen) physically coordinated most of the preparations before I could officially start my work. He has followed this work with interest and made useful reviews from time to time. I heartily thank him. I acknowledge with appreciation the skilful computer assistance I received

from Drs. H. van Hoof and Mrs I. Swinkels in finalising the layout of this thesis.

The end product of this work will be marked by the introduction of enzyme immunoassay services in diagnostic endocrinology at Muhimbili Medical Centre-Dar-es-salaam. I am grateful to the Department of Experimental and Chemical Endocrinology at the St. Radboud Hospital. The Department has funded the costs of obtaining the Serozyme 1 analyzer and will continue to support our services by buying for us the needed kits.

I sincerely wish to express my appreciation for the services to me by the International Relations Centre of the Catholic University of Nijmegen.

My thanks are due to all the subjects from whom I obtained the samples assayed and reported in this thesis. They have laid a foundation for the much needed services to their brothers and sisters in Tanzania. Last but not least I wish to thank all the other people not mentioned by name here who in one way or another have been of help to me during my research and preparation of this thesis.

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LIST OF ABBREVIATIONS.

BEI	Butanol extractable iodine.
BMR	Basal metabolic rate.
CIA	Chemi-immunoluminometric-assay.
CV	Coefficient of variation.
DIT	Diiodothyronine.
EIA	Enzymeimmunoassay.
ER	Endoplasmatic reticulum.
FDH	Familiar dysalbuminemic hyperthyroxinaemia.
FIA	Fluoroimmunoassay.
FT_4	Free thyroxine.
fT_4%	Free fraction of thyroxine.
FT_3I	Free triiodothyronine index.
FT_4I	Free thyroxine index.
hCG	human chorionic gonadotropin.
ICMA	Immunochemiluminometric assay.
IDD	Iodine deficiency disorders.
IEMA	Immunoenzymometric assay.
IFMA	Immunofluorometric assay.
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IMA	Immunometric assay.
IRMA	Imrnunoradiometric assay.
MIT	Monoiodothyronine.
mRNA	messenger-ribose nucleic acid.

NTI	Non-thyroidal illness.
PBI	Protein bound iodine.
RIA	Radioimmunoassay.
rRNA	ribosomal-ribose nucleic acid.
SEM	Standard error of the mean.
T_3	Triiodothyronine.
T_4	Thyroxine.
TBG	Thyroxine binding globulin.
TBPA	Thyroxine binding pre-albumin.
Tg	Thyroglobulin.
TGBI	Thyroid Growth Blocking Immunoglobulin
TGSAb	Thyroid Growth Stimulating Antibody
TRH	Thyrotropin releasing hormone.
TSAb	Thyroid Stimulating Antibody
TSH	Thyroid stimulating hormone.
WHO	World health organisation.

Chapter One

General Introduction and Scope of the Thesis.

Part 1

1.1 The thyroid gland: functional anatomy and histology

In human embryos the thyroid can be recognized from the first month after conception. It is derived from the midline endoderm of the pharyngeal floor. In adults the thyroid gland, situated in the anterior aspect of the lower neck, is one of the largest of the endocrine organs and weighs approximately 20 grams. Macroscopically the gland is made up of two lobes joined by a thin band of tissue called the isthmus. Occasionally a pyramidal lobe may be seen as a fingerlike projection directed upward from the isthmus and this occurs especially when the rest of the gland is enlarged. With light microscopy, the gland is seen to be made up of closely packed sacs called follicles. These follicles, also called acini, are the functional and structural units of the thyroid gland and are richly supplied with blood capillaries. The follicle is made up of a single layer of cuboidal epithelium whose interior is filled with a proteinaceous colloid which normally is the major constituent of total thyroid mass. The height of the follicular epithelium varies with the activity of the gland becoming columnar when stimulated and flat when resting. About 20-40 follicles are demarcated by connective tissue septa and form a lobule which is supplied with a single artery. By electron microscopy it can be seen that from the apical part of the follicular cell microvilli project in the lumen of the follicle.

The main constituent of the colloid is thyroglobulin (Tg), a glycoprotein (MW 660.000) which is synthesized in the follicular cell before being extruded into the follicular lumen by exocytosis.

1.2 Thyroid hormones: physiology and metabolism.

As pointed out above, the functional unit of the thyroid gland is the follicle. From the apical portion of the follicular cell many of the processes of thyroid hormone synthesis and secretion take place.

The main hormones secreted by the thyroid gland are Thyroxine (T_4) and Triiodothyronine (T_3) (figure 1.1). The synthesis of these hormones requires iodine which constitutes 66% and 58% of the molecular weights of T_4 and T_3 respectively. Iodine in the diet is derived from sea food, milk, eggs and iodised salt. The optimum daily requirement has been suggested to be about 150 μg in adults (Delange F 1993).

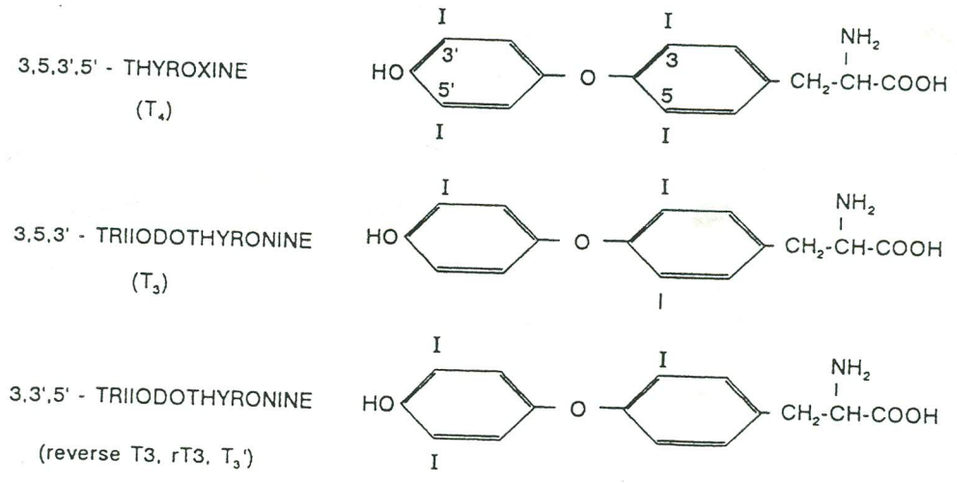


Figure 1.1 Chemical structures of Thyroxine (T_4), Triiodothyronine (T_3) and reverse T_3 (rT_3).

In healthy subjects about 90 μg of T_4 and 30 μg of T_3 are secreted daily (Himsworth RL 1992). The steps in the synthesis and secretion of T_4 and T_3 are outlined below and presented diagrammatically in figure 1.2.

a) *Iodide trapping.* Following the absorption of iodide in the small intestine, thyroid cells actively transport 20% of this circulating iodide through the basement membranes into their cytoplasm. Under normal intake conditions about 80% of circulating iodide is cleared by the kidneys.

b) *Organification.* Inside the cell's apical membrane the absorbed iodide is oxidised and incorporated into tyrosyl residues present in the thyroglobulin (Tg) molecules which are themselves synthesized in the endoplasmic reticulum (ER) of the cells and stored in the colloid. This organification process requires the enzyme peroxidase and results into formation of monoiodotyrosines (MIT) and diiodotyrosines (DIT).

c) *Coupling.* Thyroxine is formed when two diiodothyronines are joined together in a process referred to as coupling. This process is also catalysed by peroxidase. Coupling of a diiodothyronine and a monoiodothyronine results into T_3.

d) *Storage and secretion.* The formed hormones are stored in the colloid. Secretion of the hormones involves the formation of pseudopods of the apical membrane to enclose the colloid within the central space of the cell forming a colloid droplet. The colloid droplet is joined by the lysosome, forming a phagosome. The acid proteases and peptidases from the lysosome digest thyroglobulin molecules. T_4 and T_3 are liberated and secreted into capillaries at the base of the cell. Amino acids and non-hormone iodine are recovered in the cell for recycling.

The thyroid production of T_4 and T_3 is regulated from one end by circulating thyroid stimulating hormone (TSH) from the anterior pituitary gland and the circulating levels

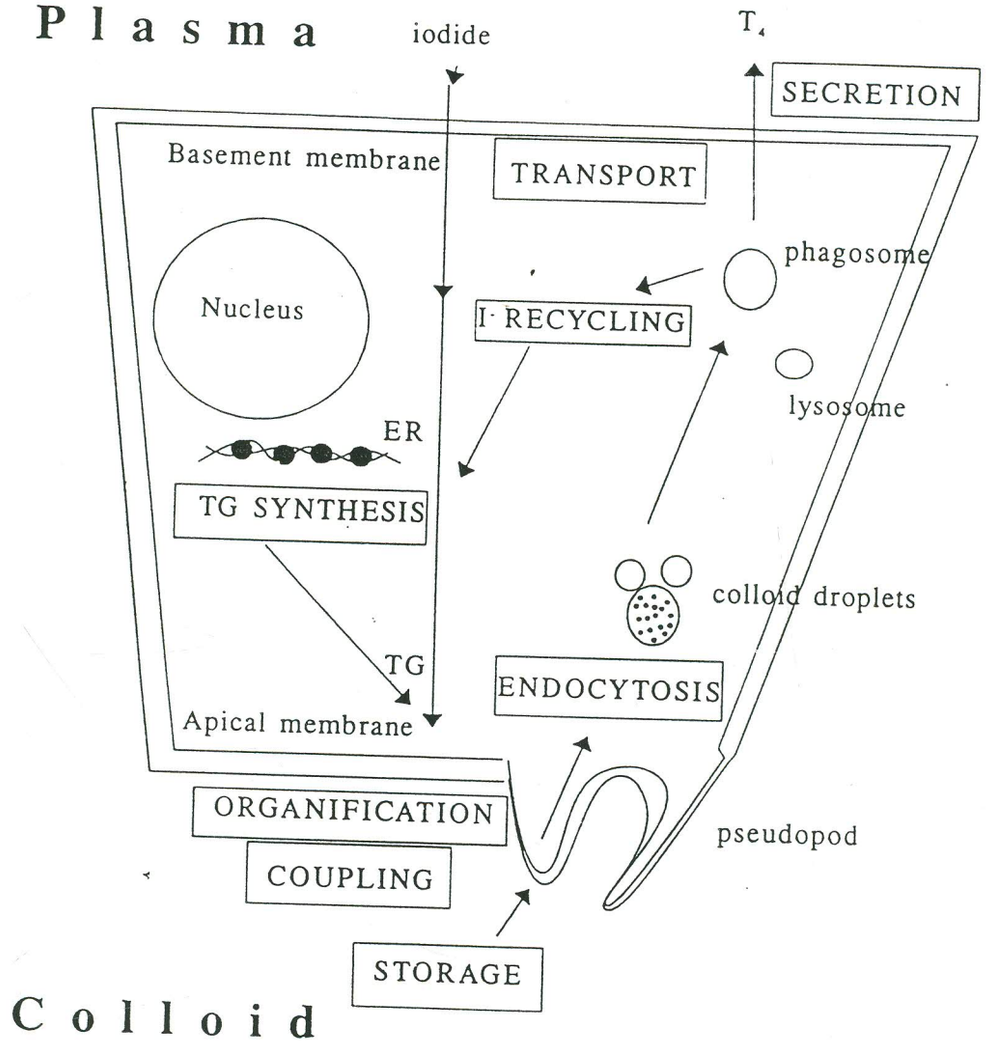


Figure 1.2 Thyroid hormone synthesis and secretion.

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of free T_4 and free T_3 from the other end. This integrated system of the hypothalamic-pituitary-thyroid axis is presented in figure 1.3.

Thyroid stimulating hormone (TSH) binds to specific membrane receptors on the surface of thyroid cells. This activates intracellular adenylate cyclase, and through increased cAMP, activates protein kinases. The kinases stimulate the synthesis of thyroid hormones.

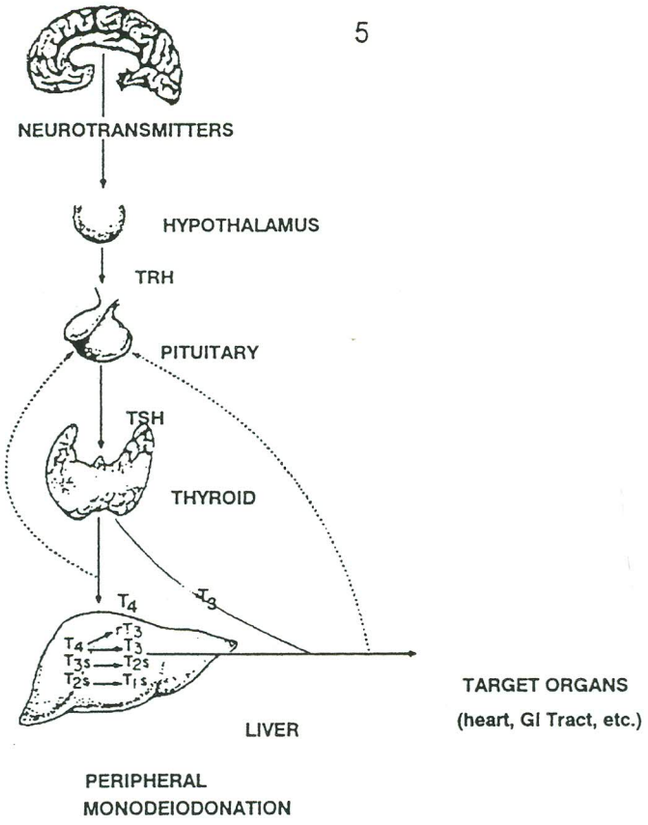


Figure 1.3. The hypothalamic-pituitary-thyroid axis and the regulation of thyroid function.

TSH synthesis and secretion are stimulated by thyrotropin releasing hormone (TRH) from the hypothalamus and inhibited by the negative feedback from the circulating free T_4 and free T_3.

Under normal conditions over two thirds of the T_3 which is in circulation derives from peripheral T_4 deiodination (Larsen PR 1972). There is considerably more T_4 than T_3 in human plasma, the molar ratio of T_4 to T_3 is about 50:1 (Abuid J and Larsen PR 1974). T_3 has three to four times higher biological activity compared to T_4. Indeed it has been advanced that most if not all of the biologic activity of T_4 is attributed to its conversion to T_3 and that this may be an obligatory step in the expression of

maximum biologic activity of T_4 (Obregon MJ *et al* 1979).

While in circulation, both hormones are reversibly bound to Thyroxine Binding Globulin (TBG), Thyroxine Binding Pre-Albumin (TBPA); also referred to as transthyretin, and albumin with decreasing affinity but increasing capacity in that order (Oppenheimer JH 1968). Thyroxine is about 99.97% bound and T_3 is about 99.7% bound to these proteins. Only about 0.03% of T_4 and 0.3% of T_3 respectively circulate in the free form. The free hormone concentration (as measured in vitro) is believed to be the essential determinant of the physiological activity of the hormone. The basic notion of this so-called free hormone hypothesis lies in the observation, that the biological effect of a hormone is better reflected by the free than the total or protein-bound concentration. Superficially, this may be explained by assuming that only the free hormone is capable of entering the cell. However, the factor most relevant to the biological effect is the rate at which the hormone is delivered to target tissues. Originally, two opposing views existed concerning this question. In one view it is assumed that bound (steroid) hormones do not dissociate from their specific binding proteins during capillary transit (Tait JF and Burstein S 1964), so that the free fraction that is available for transport into the cells is not replenished during passage. Therefore, capillary blood flow should be rate-limiting for tissue uptake. Investigators adhering to this model have difficulty explaining how amounts exceeding the free fraction can be taken up by tissue (Pardridge WM 1981; Pardridge WM and Landaw EM 1984). These investigators developed the so called "enhanced dissociation" theory in order to accommodate a seemingly discrepancy between labelled hormone taken up by an organ from albumin containing and supposedly "protein-free" media. The opposite view, which corresponds with the

original Robbins and Rall model (Robbins J and Rall JE 1957) states that during capillary transit bound hormone dissociation is very rapid, so that the equilibrium free hormone concentration is maintained, implying that the free hormone concentration determines hormone uptake. The latter model is consistent with a single euthyroid range for free T_4, applying independent of binding protein concentration. It is still doubtful whether this is the case, e.g. a number of investigators have reported reduction in equilibrium free T_4 in pregnancy (Price *et al* 1989). The free hormone concentration even may fall during passage as the total hormone is gradually depleted in the absence of longitudinal mixing. The latter model adequately explains experiments by Mendel *et al* (Mendel *et al* 1987; Mendel *et al* 1988) who showed that the presence of binding proteins results in uniform hepatic distribution of labelled T_4, whereas in the absence of binding proteins all tracer is taken up directly in the region of injection. Physicochemical refinements of the model (Ekins RP 1990) open the possibility that under certain circumstances the bound hormone concentration may become rate-limiting. This would occur when hormone is taken up at a high rate from small capillaries. A steady state may arise with the free hormone below its equilibrium concentration because bound hormone does not dissociate rapid enough from its binding protein so that dissociation, which is proportional to the bound hormone concentration will also be rate-controlling. Additionally, free hormone depletion may occur at the interface of the capillary circulation and the targer cells, which must be replenished by transversal diffusion. As diffusion transport predominantly takes place by the bound hormone, again a possibility for control of uptake rate by bound hormone is created. Thus the presence of high concentrations of bound hormone may be required under certain circumstances possibly in

pregnancy (Ekins RP 1985, Sinha AK *et al* 1992) to provide adequate hormone supply, while the free concentration may be subnormal.

The hormone action at the cellular level follows a sequence of steps including; hormone entry into the cytoplasm, movement into the nucleus, nuclear receptor binding and finally cellular responses, which are RNA-directed changes in protein synthesis leading to specific cellular effects as shown in figure 1.4.

The thyroid hormones L-thyroxine (T_4) and L-triiodothyronine (T_3) affect a wide range of physiological and biochemical processes. The hormones are most vitally involved in those functions which are part of the growth and development of the organism. In the mammalian fetus, differentiation and organisation of the central nervous system require the presence of these hormones. In the adult, hypersecretion of thyroid hormones lead to a wide variety of clinical symptoms including weight loss, tachycardia, increased cardiac output and mental agitation. In the absence of the hormone, weight gain, muscle weakness and fatigue, cold intolerance and slowed mentation can be noted.

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The peripheral disposal of thyroid hormones is achieved through three main pathways.

i) *Sequential deiodination.* This process is catalysed by an enzyme deiodinase type I which is predominantly found in the liver and kidney and is sensitive to an untethered drug propylthiouracil (PTU). This process accounts for 85% of peripheral disposal of thyroid hormones. The 5' deiodination i.e. removal of iodine from the outer ring (the 3' or 5' position) gives 3,5,3'-T_3 and is referred to as activation

pathway. Tyrosyl ring deiodination of T_4 (i.e. removal of iodine from the inner ring 3- or 5- positions) gives a metabolically inactive reverse 3,3',5'T_3 (rT_3) the reaction being catalysed by 5-deiodinase. Further deiodination results into the inactive diiodothyronines, monoiodothyronines and thyronine as shown in figure 1.5.

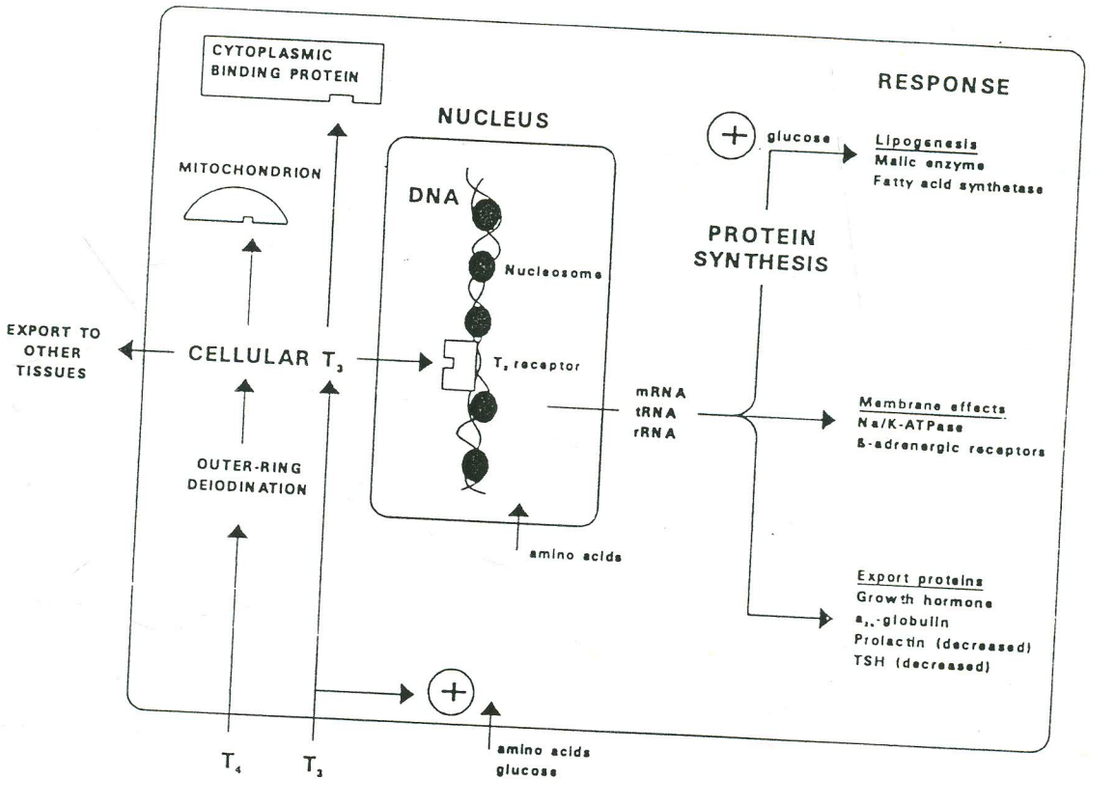


Figure 1.4. Cellular actions of thyroid hormones. The diagram depicts entry into the cell, mitochondrial and cytoplasmic binding, entry and binding to nuclear T_3 binding receptors and mRNA, tRNA and rRNA initiated changes in protein synthesis (Adopted from Pangaro LN 1991).

Approximately 30% to 35% of T_4 is mono de-iodinated to T_3 and about 40% to rT_3. In the pituitary intracellular conversion of T_4 to T_3 occurs (Larsen PR *et al* 1981) and this is catalysed by a different enzyme-type II deiodinase which is insensitive to propylthiouracil (PTU).

ii) *Conjugation*. T_4 is conjugated to glucuronides and T_3 to sulphates and excreted in bile. While a small percentage of this is reabsorbed in the enterohepatic circulation, most of it is lost in faeces.

iii) *Oxidation*. The alanine side chain of T_4 or T_3 may be oxidised to form acetic acid derivatives, tetraiodoacetic acid (tetrac) and triiodoacetic acid (triac).

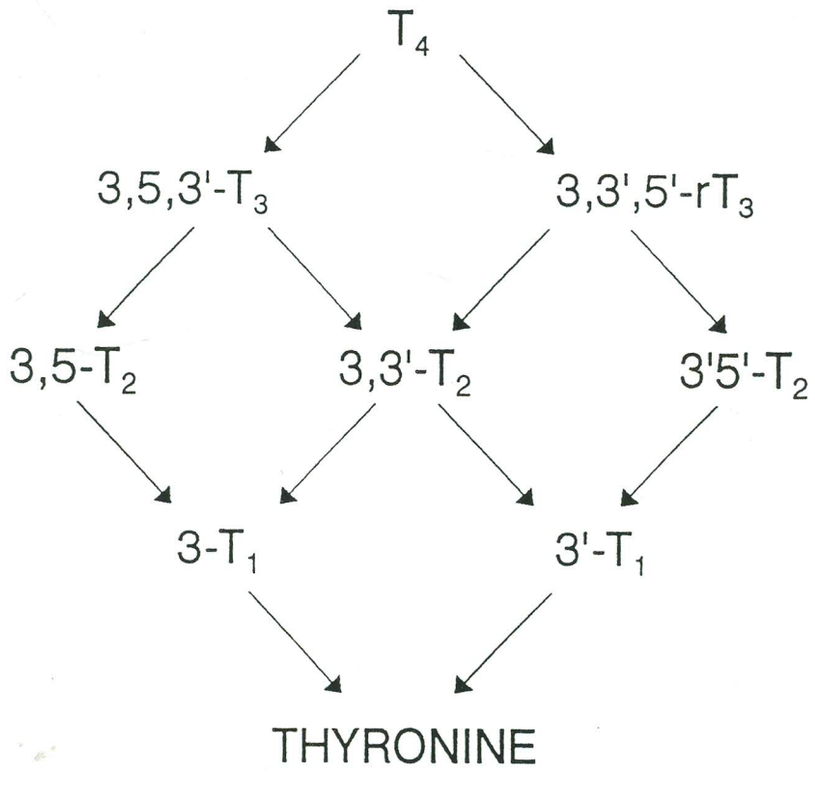


Figure 1.5 Sequential thyroid hormone deiodination.

1.3 Thyroid hormones and thyrotropin: Pathophysiology in functional thyroid diseases.

1.3.1 Clinical utility of T_4 and T_3 measurements in hyperthyroidism and hypothyroidism.

Most cases of hyperthyroidism are the result of Graves' disease, toxic multinodular goitre or toxic adenoma. In most cases of hyperthyroidism both T_4 and T_3 are elevated. Greater increases occur in T_3 (with an average increase of 4.5 times the normal mean) than in T_4 levels (with an average increase of 2.5 times the normal mean) (Ishii H *et al* 1981). However atypical forms of thyrotoxicosis, T_3 toxicosis and T_4 toxicosis may also be encountered. T_3 toxicosis (Sterling K *et al* 1970) prevalence has been variously reported as being 4% in North America and up to 25% in England. Higher incidence rates are associated with environmental iodine deficiency (Hollander CS *et al* 1972). T_4 toxicosis (raised T_4 and normal T_3) has been reported in patients with thyrotoxicosis and an intercurrent illness (Caplan RH *et al* 1980), probably as a result of an inhibition of peripheral T_4 to T_3 conversion in association with a systemic illness.

Primary thyroid failure as a result of autoimmune processes, therapeutic ablation (surgery or radioiodine) and sporadic cretinism (resulting from developmental defects of the thyroid) are the major causes of hypothyroidism in iodine replete areas. In other areas hypothyroidism is mainly a result of environmental iodine deficiency. Both serum T_4 and T_3 are reduced in hypothyroidism. However smaller decreases are seen in the secondary forms. In primary hypothyroidism low T_4, raised TSH and

normal T_3 may be seen in as much as 20-30% of cases (Larsen PR 1986). This combination of laboratory findings seem to be a stage through which the failing thyroid gland passes implying that serum T_3 is not a very useful marker of hypothyroidism. Only in patients with severe hypothyroidism this hormone is regularly reduced in concentration.

1.3.2 Limitations of serum total T_4 and total T_3 in thyroid testing.

Alterations in the capacity and affinity of serum binding proteins may alter the concentrations of total thyroid hormones in blood in the absence of thyroid disease thus limiting the usefulness of total thyroid hormonal assays in the diagnosis of thyroid dysfunction. Raised total T_4 and T_3 occur due to increases in serum TBG concentrations as in pregnancy (Franklyn JA 1983), in infectious hepatitis, chronic active hepatitis, congenital TBG excess (prevalence of 3:100,000), familial dysalbuminaemic hyperthyroxinaemia-FDH (Docter R *et al* 1981) and due to increased avidity of TBPA for T_4 (Moses CA and Lawlor J 1982).

In non-thyroidal illnesses multiple alterations in production, distribution and metabolism of thyroid hormones do occur. In the euthyroid sick syndrome with an incidence of 37-70% in hospitalised patients (Rajatanavin R and Braverman LE 1983) characteristic changes include increase in rT_3 and a decrease in T_3 due to reduced peripheral conversion of T_4 to T_3 and rT_3 to 3,5-diiodothyronine. T_4 may be increased or decreased. Also a high prevalence (13-33%) of the high total T_4 state of illness occurring in patients with psychiatric disorders has been reported (Spratt DI *et al* 1982).

Alterations in serum thyroid hormones without thyroid dysfunction may derive from

the influence of drugs on thyroid function parameters (Wenzel KW 1981). Drugs can compete with thyroid hormones for binding proteins (salicylates), induce enzymes involved in thyroid hormone metabolism (diphenylhydantoin) or inhibit T_4 to T_3 conversion and many other mechanisms. Amiadarone, an iodine containing drug is used frequently in the treatment of cardiac arrhythmias and angina pectoris. The drug has many effects on the metabolism of thyroid hormones including iodine induced hypothyroidism or hyperthyroidism in patients chronically treated with it. However, this drug through decreasing the production of T_3 and decreasing the clearance of T_4 and rT_3 may induce an elevated serum thyroxine and rT_3 and reduced T_3 levels in the absence of intrinsic thyroid dysfunction.

A familial thyroid hormone resistance syndrome was first described in 1967 (Reffetoff S *et al* 1967). At least 200 cases in 50 families were reported until 1991 (Franklyn JA 1991). The pattern of genetic transmission has been described as either autosomal dominant or autosomal recessive (Cooper DS and Landenson WP 1982). In the generalised form high serum total thyroid hormones are seen in the face of euthyroidism.

1.3.3 Free thyroid hormones in the diagnosis of thyroid disorders.

The measurement of free hormones (FT_4 and FT_3) in the diagnosis of thyroid function derives from the concept of free hormone hypothesis as discussed earlier. Observations of maintenance of euthyroidism in the face of greatly changed total hormone levels in some conditions e.g. in pregnancy formed the experimental basis of the hypothesis. Measurement of free hormones is currently one of the principal tests in thyroid function testing.

1.3.4 TSH in the diagnosis of thyroid dysfunction

The place of TSH assays in the diagnosis of thyroid dysfunction has been extensively reviewed (Spencer CA and Nicollof JT 1990). Measurement of TSH presently serves as the most sensitive and specific indicator of the biologic effects of circulating thyroid hormones. All varieties of thyrotoxicosis and hypothyroidism are associated with changes in the thyroregulatory control mechanism. Raised TSH levels are characteristic of primary hypothyroidism. In trophoprivic hypothyroidism clinical diagnosis is associated with below normal basal TSH concentration. To distinguish between the pituitary or hypothalamic origin of secondary hypothyroidism other tests assessing the pituitary function and testing the TSH response to TRH stimulation are needed.

Except in the rare cases of hyperthyroidism due to hypersecretion of TSH, the TSH secretory mechanism is suppressed and TSH concentrations are subnormal or undetectable (depending on the sensitivity of the assay used) in all patients with thyrotoxicosis regardless of its cause.

The ability of the TSH measurement to appropriately assess the thyroid status is, by definition, dependent on the functional and structural integrity of the hypothalamic-pituitary thyroid axis (see figure 1.5). Therefore from the clinical point of view, the finding of TSH levels inconsistent with the clinical presentation of the patient is possible e.g. hypothalamic or pituitary lesions may lead to secretion of TSH that has reduced or absent bioactivity but normal immunoreactivity. In such cases, paradoxically normal TSH values may be seen in the face of substantial reductions in circulating FT_4 levels and overt hypothyroidism. At present TSH measurement is optimally performed in ambulatory, otherwise well, patient populations in whom the

pituitary-hypothalamic axis is functionally and structurally intact. TSH measurement is used for monitoring thyroid hormone replacement therapy, monitoring thyroid hormone suppressive therapy and for exclusion of thyroid disease in patients suspected of harbouring primary thyroid disease on clinical grounds.

Thyrotropin releasing hormone (TRH) whether given orally, intramuscularly or intravenously, provides a supraphysiological challenge to the TSH secretory mechanism within the thyrotrophic cell, and makes it possible to determine the intrinsic TSH reserve. Over a broad range, the extent of increase in the serum TSH concentration induced by TRH is closely correlated with the basal serum TSH concentration. Although the advent of sensitive TSH assays has largely replaced TRH testing in the diagnosis of thyroid dysfunction, the test retains its usefulness in some clinical settings. In trophoprivic hypothyroidism (without an increase in basal serum TSH concentration), in patients whose disease arises in the pituitary TSH displays a subnormal or no response to TRH while in the hypothalamic form TSH displays a normal but retarded response. The test also discriminates between sick euthyroid patients and sick thyrotoxic patients (both these patient categories have suppressed serum TSH levels suggesting thyrotoxicosis). Sick euthyroid patients will generally display a detectable TSH response to TRH while the thyrotoxic patient will not. In subclinical hypothyroidism (for example in patients treated with radioactive iodine for hyperthyroidism and when the thyroid is beginning to fail), both the clinical picture and basal TSH levels may be inconclusive. In these patients accentuated TSH response to TRH is an additional evidence of hypothyroidism.

1.4. Thyroid diseases in Tanzania.

The extent of different thyroid disorders in the Tanzanian community is largely unknown. For more than three decades now it is known that some parts of the country are afflicted by iodine deficiency disorders (IDD). The earliest report of thyroid diseases in Tanzanian appeared in 1965 (Latham MC 1965), when a survey revealed a high prevalence of endemic goiter in the highlands of the southern part of the country. About 40% of the population is at risk of IDD because of living in iodine deficient areas. Community goitre surveys in 34 districts of Tanzania were done until 1985. From the results of these surveys it was estimated that IDD could be responsible for 30% of perinatal mortality in the country. It was also estimated that there were about 600,000 cases of cretins and cretinoids in Tanzania (Van der Haal *et al* 1988). At present it is estimated that IDD affect 25% of the entire Tanzanian population and that IDD affect 40% of the children, and 52% of the pregnant and lactating mothers (Kavishe FP and Yambi O 1991). The results of goitre surveys as per 1988 are presented in figure 1.6.

At the Muhimbili Medical Centre requests for laboratory investigation of suspected thyroid dysfunction rank second in all suspected endocrine diseases (Kabyemela EA, Chuwa LMM - unpublished data).

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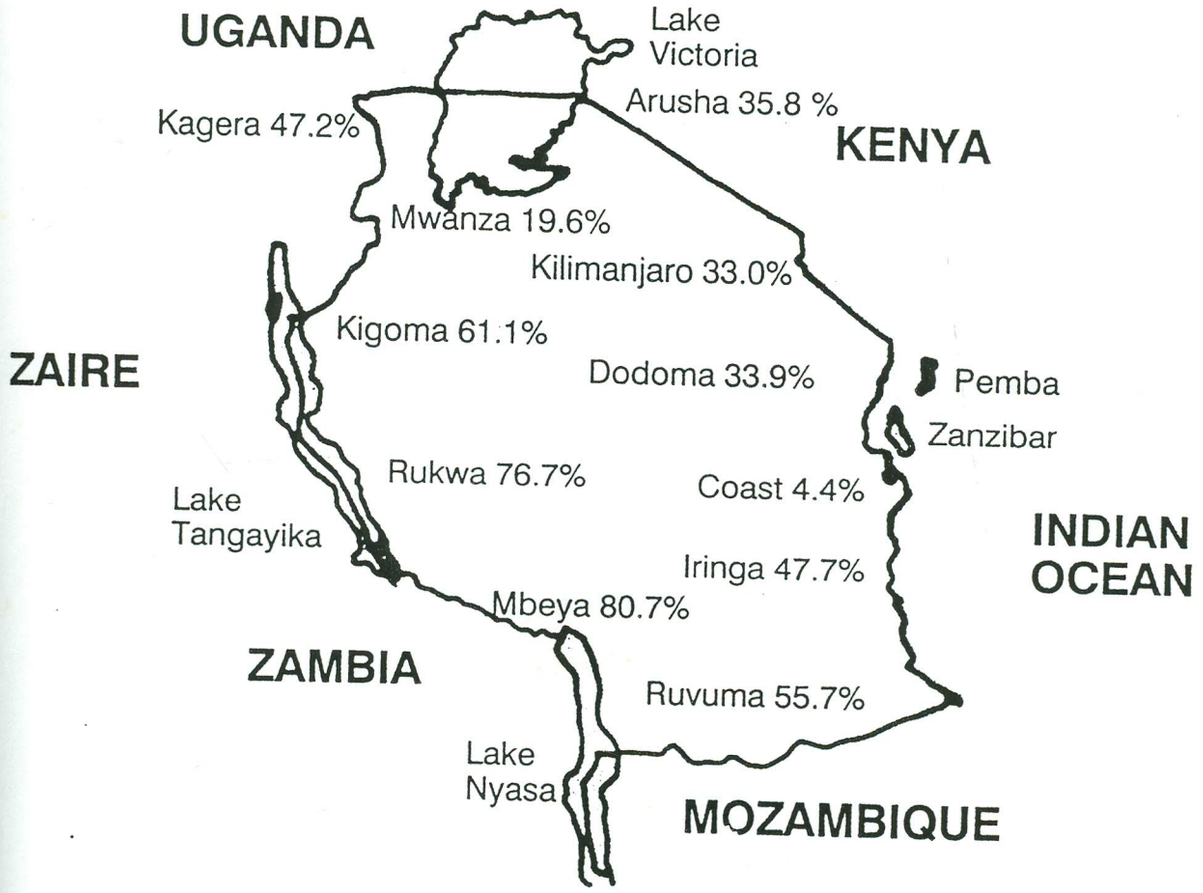


Figure 1.6. Map of Tanzania showing the areas highly affected by Iodine Deficiency Disorders (reconstructed from Kavishe FP and Yambi O 1991) The respective percentages show the prevalence of all grades of goitre at the time of survey 1981-1988.

1.5 Iodine Deficiency Disorders: Etiology, pathophysiology, and complications.

Iodine is a trace element present in the human body in minute amounts (15-20 mg, i.e. $0.02*10^{-3}$ % of body weight), and is an essential element for the synthesis of thyroid hormones. The daily requirements of iodine is at least equal to the amount of hormonal iodine that is degraded and unrecovered daily by the thyroid gland. The classical recommended dietary allowances of iodine is 100 μg/day for adolescents and adults (150 μg/day in pregnant and lactating women). It is 60-100 μg/day for children aged 1-10 years, 40 μg/day for infants aged 6-12 months, and 35 μg/day for infants aged 6 months or younger (Recommended Dietary Allowances 1986).

When the physiological requirements of iodine are not met in a given population, a series of functional and developmental abnormalities occur as summarised in table 1.1 including thyroid dysfunction abnormalities, and when the deficiency is severe, endemic goiter and cretinism, endemic mental retardation, decreased fertility rate, increased perinatal deaths and infant mortality. These complications which hinder the development of affected communities are grouped together under the general heading of Iodine Deficiency Disorders (IDD). Iodine deficiency disorders constitute a major public health problem and one of the most common preventable cause of mental deficiency in the world today (Marberly GF 1994).

Large populations are at risk of IDD because they live in an iodine deficient environment. This iodine deficient environment is characterised by soil from which iodine has been leached by glaciation, high rainfall or floods. This occurs most often in the mountainous areas of the Himalayan region, the Andean region and the vast mountain ranges in China. However, low lying areas subject to flooding, as in the

Ganges Valley in India and Bangladesh, are also severely iodine deficient. Iodine deficiency disorders are also a public health problem in scattered areas in Africa, especially the hilly, mountainous regions and elevated plateau of southern Africa including Tanzania (Hetzel BS 1989).

Table 1.1. The manifestations of IDD in different age groups (adopted from Hetzel BS 1987).

Phase of life	Disorders
Unborn fetus	Abortions, still birth, deformities during development.
Neonates and infants	Neonatal goitre, neonatal hypothyroidism, increased perinatal mortality, increased infant mortality, neurological cretinism (mental deficiency, deaf mutism, spastic diplegia.squint), myxedematous cretinism (dwarfism, mental deficiency) and psychomotor defects.
Children	Goitre, juvenile hypothyroidism, impaired mental function (e.g.low learning capacity) retarded physical development.
Adults	Goitre, hypothyroidism, impaired mental function (e.g.lack of initiative, socialisation and stamina).

1.5.1. Endemic goitre: (Mal)Adapation to iodine deficiency.

According to the Pan American Health Organisation (PAHO) a thyroid gland is considered goitrous if its lateral lobes have a volume greater than the terminal phalanges of the thumbs of the person examined, and an area is defined as endemic with respect to goitre if more than 10% of the population or of children aged 6-12 years are found to be goitrous (Delange *et al* 1986).

The role of insufficient dietary supply of iodine as the main cause of endemic goiter and cretinism has been confirmed by numerous epidemiological, clinical and experimental studies. However, reports of endemic goitre in regions without iodine deficiency (Peltola P and Virtanen A 1954; Clements FW and Wishart JW 1956; Costa A and Cottino F 1963), and conversely, absence of endemic goitre in areas with iodine deficiency (Roche M *et al* 1961) are available. The conclusion drawn following the above observations was that goitrogenic factors in the diet or environment other than iodine deficiency could play a role in the etiology of endemic goitre.

Indeed other natural goitrogens have been demonstrated e.g. in vegetables of the genus *Brassica* (the Cruciferae family). Their untethered action is related to the presence of thioglucosides, which, after ingestion, release thiocyanate and isothiocyanate (Gmelin R and Virtanen A 1960; Langer P and Greer MA 1977; Ermans AM and Bourdoux P 1989). Cyanoglucosides is another group of naturally occurring goitrogens and have been found in cassava, maize, bamboo shoots, sweet potatoes and lima beans (Delange *et al* 1982). After ingestion these glucosides are converted to cyanide which is detoxified by conversion to thiocyanate. Thiocyanate is a powerful goitrogen by acutely inhibiting iodide uptake by the thyroid and at higher doses by competing with iodide in the organification process (Woolman SH 1962; Wolff J 1964; Ermans AM *et al* 1980).

Review of studies done extensively in Africa (Thilly CH *et al* 1992), have confirmed the role of cassava in goitrogenesis. Cassava is cultivated extensively in developing countries and represents an essential source of calories for more than 200 million people living in the tropics. It has been shown that the determining factor involved

in the goitrogenic action of cassava is the balance between dietary supply of iodine and thiocyanate. Goitre develops when the urinary iodine/thiocyanate ratio, used as an index of this balance, decreases below a critical threshold of about 3μg iodine per mg thiocyanate.

Classically, endemic goitre has been conceived as an adaptive disease in response to an insufficient supply of dietary iodine (Studer H *et al* 1974; Beckers C and Delange F 1980; Matovinovic J 1983). Currently however, it is considered that large goitres as usually seen in iodine deficient endemias indicate maladaptation to iodine deficiency. Because of their large size, these goitres contain insufficiently iodinated thyroglobulin. The goiters also show increased leakage of iodide which aggravates the iodine deficiency (Dumont JE *et al* 1995).

Iodine deficiency causes depletion of thyroid iodine stores with reduced daily production of thyroid hormone (T_4). A fall in the blood levels of T_4 triggers the secretion of increased amounts of pituitary thyroid stimulating hormone (TSH) which increases thyroid activity with hyperplasia of the thyroid.

Increased thyroidal uptake of iodide is a fundamental mechanism by which the gland adapts to an insufficient iodine supply resulting in accumulation in the gland of larger percentage of the ingested exogenous iodide and a more efficient re-use of iodide directly released by the thyroid or generated by the degradation of thyroid hormones (Stanbury JB 1954). These processes are also triggered and maintained by increased secretion of TSH. Elevated levels of serum TSH have been reported repeatedly in humans with chronic iodine deficiency (Buttfield IH *et al* 1966; Delange F *et al* 1971; Chopra IJ *et al* 1975; Thilly CH *et al* 1993). However, within a given area, large and striking variations in serum TSH levels are observed in adults

independently of the presence or absence of goitre (Delange F *et al* 1971; Chopra IJ *et al* 1975). Differences in the duration of elevated TSH levels or in thyroid responsiveness as well as other factors may determine wether goitre develops or not (Studer H *et al* 1989; Brabant G *et al* 1992). The possible role of thyroid autoimmunity in the pathogenesis of endemic goitre remains largely unknown although one study (Vitti P *et al* 1992) failed to confirm any role of autoimmunity.

The goitrous gland shows characteristic morphological changes. In the young subjects diffuse enlargement of the gland is seen. Histologically, the parenchyma is abundant, follicular epithelium is high with papillary infolding, and colloid is rare. A later stage is the formation of small nodules that dissect the entire gland into nodules of different size and consistency. Histologically, the major part of the gland is occupied by extremely distended vesicles with a flattened epithelium filled with colloid. A few patches of thyroid follicles, on the contrary show a typical pattern of stimulation (Dumont JE *et al* 1995).

The consequences of modified intrathyroidal metabolism of iodine lead to alterations in the pattern of circulating thyroid hormones. Studies in the rat show that thyroid hyperplasia induced by iodine deficiency is associated with an altered pattern of tracer distribution in the gland characterised by increased poorly iodinated compounds; monoiodothyronine (MIT) and triiodothyronine (T_3), and a decrease in diiodothyronine and thyroxine (Lamas L and Morreale de Escobar G 1972). These severely iodine deficient animals have a low serum concentration of T_4 and a high serum concentration of T_3. Similar findings have been found in people in areas with severe iodine deficiency (Delange F *et al* 1972; Chopra IJ *et al* 1975). Several mechanisms are advanced as being responsible for this pattern and they include

secretion of T_4 and T_3 in the same proportion in which they exist in the thyroid gland, preferential secretion of T_3 or an increase in the peripheral conversion of T_4 to T_3. The shift to T_3 secretion and the increased serum T_3:T_4 ratios play an important role in the adaptation to iodine deficiency, since T_3 possesses about four times the metabolic potency of T_4 but requires only 75% as much iodine for synthesis (figure 1.1). In severe endemic goitre, there is an inverse relationship between serum T_4 and TSH but this correlation is not found for T_3, which is the most active thyroid hormone. This paradoxical finding is explained by the fact that the direct effect of T_4 on TSH suppression results from intrapituitary T_4 to T_3 conversion and the subsequent T_3 nuclear binding to the thyrotropes; in other tissues, the largest part of intracellular T_3 originates from circulating T_3 (Larsen PR *et al* 1981; Larsen PR 1989). These findings account for the observation in endemic goitre that normal serum T_3 levels enable a patient to maintain overall euthyroid status but pituitary stimulation persists as long as the serum T_4 levels are depressed. In less severe endemias serum T_4 and T_3 are only slightly modified or may remain even normal, TSH is slightly elevated and the TSH response to TRH is often exaggerated indicating subclinical hypothyroidism (Evered DC *et al* 1973).

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1.5.2. Iodine Deficiency Disorders in infancy and childhood.

In children iodine deficiency is characteristically associated with goitre. Goitre rate increases with age so that it reaches a maximum with adolescence. Girls have a higher prevalence than boys. Recent studies in school children living in iodine deficient areas from a number of countries indicate impaired school performance and lower IQs in comparison with matched controls from noniodine deficient areas. In

Papua New Guinea, children from the controlled trial in the Western Highlands have been tested periodically. Differences in bimanual dexterity were revealed by threading beads or putting pegs into a pegboard in relation to wether the mother was severely iodine-deficient or not. These differences were significantly related to the level of maternal T_4 at the time of pregnancy (Connoly KJ *et al* 1979; Pharoah POD *et al* 1981; Pharoah POD *et al* 1984). Using a wide range of psychological tests, it has been shown (Bleichrodt N *et al* 1987) that the mental development of children from iodine deficient areas lags behind that of children from noniodine deficient areas. The differences in psychomotor development became apparent only after the age of 2.5 years. Similarly inferior performance is reported in children with goitre when compared with those without goitre even when all live in the same area (Muzzo S *et al* 1986). The determinant of brain (and pituitary) T_3 is serum T_4 and not T_3. Low level of brain T_3 have been demonstrated in the iodine deficient rat in association with reduced levels of serum T_4 and these have been restored to normal with correction of iodine deficiency. These findings would provide a rationale for sub-optimal brain function in subjects with endemic goitre and lowered serum T_4 levels and its improvement following correction of iodine deficiency. Indeed controlled trials of iodine supplementation show that the supplementation in children reduces goitre size, leads to better scholastic achievement, improve mental performance, and raise the levels of circulating thyroid hormones (the latter indicating a mild degree of hypothyroidism which was not apparent before supplementation.

1.5.3. Neonatal hypothyroidism.

The importance of the state of the thyroid function in the neonate relates to the fact

that at birth the brain of the human infant has only reached about one third of its full size and continues to grow rapidly until the end of the second year. The thyroid hormone, dependent on the adequate supply of iodine is essential for the normal brain development as is now well confirmed by several studies as reviewed by Bernal J and Nunez J (1995).

In iodine deficient areas, there is a high prevalence of neonatal hypothyroidism (Kochupillai N *et al* 1986). Neonatal hypothyroidism may be transient in some infants but remains unchanged in others. The abnormalities of neonatal thyroid function are prevented by correcting iodine deficiency in mothers before or during pregnancy. The incidence of neonatal hypothyroidism is related to the severity of the iodine deficiency as indicated by the prevalence of goitre and cretinism and the level of excretion of iodine in urine. Alterations of thyroid function in neonates have been subsequently reported from other less severe endemias (higher TSH levels and low T_4) if the deficiency is not corrected it may result into retardation of physical and mental development. These observations suggest a much greater risk of mental defect in severely iodine deficient populations than is indicated by obvious presentation of endemic cretinism.

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1.5.4. Endemic cretinism.

The greatest morbidity of severe iodine deficiency arises, not by its primary consequences on thyroid gland enlargement, but by its secondary effects on thyroid hormone homeostasis acting on fetal and neonatal brain development. In severe endemic goitre areas, an abnormally high number of patients exhibit irreversible anomalies of intellectual and physical development grouped under the general

heading of endemic cretinism. A prevalence of cretinism from 3-15% is commonly found in severely affected rural populations in many endemias around the world (Halpern JP *et al* 1991). It is by far the most serious complication of endemic goitre and represents a veritable scourge, both medically and socially. Endemic cretinism is defined on epidemiological grounds based on three features (Delange F *et al* 1986).

A. It is associated with endemic goitre and severe iodine deficiency.

B. Clinical features: These comprise mental deficiency, together with either:

1). A predominant neurological syndrome including defects of hearing and speech and characteristic disorders of stance and gait of varying degrees or;

2). Predominant hypothyroidism and stunted growth.

Although in some regions, one of the two types may predominate, in other areas a mixture of the two syndromes will occur.

C. Prevention. In areas in which iodine deficiency has been adequately corrected, endemic cretinism has been prevented.

In the subsequent sections, the clinical presentation, and the etiopathology of the two syndromes of endemic cretinism (as classically perceived) are presented.

Neurological cretinism. The endemic neurological cretins are extremely mentally retarded and most of them are reduced to a vegetative existence. Almost all are deaf-mutes and are afflicted with the following neurological defects:

(a) Impaired voluntary motor activity, usually involving paresis or paralysis of pyramidal origin, chiefly in the lower limb, with hypertonia, clonus, and plantar cutaneous reflexes in extension. Extrapyramidal signs are occasional.

(b) Spastic or ataxic gait. In the severe cases walking or even standing is impossible.

(c) Strabismus.

Computerised tomographic scans of cretins from Ecuador demonstrated widespread atrophy including the cortex, subcortical structures and the brainstem, with corresponding enlargement of the basal cisterns, the lateral ventricles, and the sulci over the surface of the cerebral cortex (Ramirez I *et al* 1983). The cretins are usually clinically euthyroid and the prevalence of goitre in neurological cretins is as high as the non-cretin population of the area. Thyroid function is usually normal (Boyages SC *et al* 1988) but can indicate subclinical hypothyroidism with elevated basal TSH or exaggerated TSH response to TRH (Zhu XY 1983).

Myxedematous cretinism. The typical myxedematous cretins show less mental retardation than the neurological cretins. They exhibit major clinical symptoms of long standing hypothyroidism namely dwarfism, myxedema, dry skin, sparseness of hair, retarded sexual development, and retarded maturation of body proportions. The prevalence of goitre in the myxedematous cretins is much lower than in the non-cretin population. Many of them have nonpalpable thyroid tissue although thyroid scintigrams show small residues of thyroid tissue located in normal position (Delange F *et al* 1972), precluding thyroid dysgenesis (agenesis or ectopic thyroid) as a cause of hypothyroidism. Biochemically, hypothyroidism is revealed by very low serum concentrations of T_4 and T_3 and high levels of TSH. These cretins also show retarded bone maturation and epiphyseal dysgenesis indicating hypothyroidism of perinatal onset.

The distribution of the two types of cretinism in different endemias differ, and it is agreed that between the two extremes there are a number of mixed forms characterised by predominant neurological disorders or predominant hypothyroidism

in the same individual (Lagasse R *et al* 1989). In severe endemic goitre, neuromotor and intellectual deficits are also frequently observed in individuals who do not show any of the other signs of endemic cretinism (Delange F 1991).

Etiology of endemic cretinism.

Iodine deficiency: The etiological role of iodine deficiency in endemic cretinism is evidenced by the observations that on the one hand there is correlation between the frequency of endemic cretinism and the severity of iodine deficiency (Querido A 1972) and on the other hand that iodine prophylaxis has been shown to reduce the incidence of endemic cretinism (Thilly CH *et al* 1973; Hetzel BS *et al* 1990). Equally important, was the observation that endemic cretinism surfaced in an area following the emergence of iodine deficiency (Pharoah POD and Hornabrook RW 1974). Animal studies also support the etiology of iodine deficiency in endemic cretinism (Smith RM 1981).

Naturally occurring goitrogens: Thiocyanate overload, has been considered as playing an additional role in the etiology of myxedematous endemic cretinism by aggravating iodine deficiency and thus resulting into fetal hypothyroidism (Thilly CH *et al* 1992). Thiocyanate crosses the placenta (Braverman LE 1989), and in severely iodine deficient mothers, increased urinary thiocyanate excretion is accompanied by a further increase in serum TSH and a fall in T_4 in cord serum of the new born (Delange F *et al* 1982). Thiocyanate interferes with iodide trapping by the placenta and fetal thyroid, leading to reduction in build up of iodine stores within the thyroid gland during the fetal and early postnatal life. Indeed very low iodine stores have been reported in the thyroid glands of myxedematous cretins. More recently,

thiocyanate overload aggravating the iodine deficiency has been adopted as an explanation of juvenile hypothyroidism developing after weaning in some iodine deficient areas (Vanderpas JB *et al* 1984; Moreno-Reyes R *et al* 1993).

Thyroid autoimmunity: The role of thyroid autoimmunity in the etiopathogenesis of goitre and cretinism is variously reported in literature. Vitti *et al* (1992) reported studies for thyroid adenylate cyclase stimulating antibody (TSAb; cAMP increase over basal production) and thyroid growth stimulating antibody (TGSAb; percent ^{3}H-thymidine incorporation and DNA content in FRTL-5 cells) in patients with endemic goitre and endemic cretinism. Immunoglobulin-G (IgGs) preparations from these subjects were tested. IgGs from goitrous patients were devoid of TSAb and TGSAb activities, while in the same conditions IgGs from patients with Graves' disease had demonstrable positive activities indicating that TSAb and TGSAb have no direct role in the pathogenesis of IDD. On the other hand, thyroid growth blocking immunoglobulins (TGBI) have been found in myxedematous cretins with atrophic thyroids (Boyages SC *et al* 1989). However these immunoglobulins were not found in neurologic cretins or euthyroid subjects from the same area (Boyages SC *et al* 1989). TGBI have been reported present (Boyages SC *et al* 1989) but absent (Chiovato L *et al* 1989; Brown RS 1990) in sporadic congenital hypothyroidism.

Trace elements: Recently, selenium deficiency has been reported in areas where the prevalence of myxedematous cretinism is very high and it has been proposed that selenium deficiency may play a role as a co-factor to iodine deficiency to thyroid destruction (Goyens P *et al* 1987; Vanderpas JB *et al* 1990). Indeed, most of the identified selenoproteins (e.g. glutathione peroxidase-GPX and phospholipid hydroperoxidase-PhGPX) are involved in antioxidant defences through their ability

to reduce either H_2O_2 or other H_2O_2-derived radicals and lipid peroxidase. When investigating the effects of possibly impaired cellular defence mechanisms associated with selenium deficiency on thyroid necrosis and repair, it was found (Contempre JE *et al* 1995) that selenium deficiency coupled to iodine deficiency increased necrosis, induced fibrosis and impeded compensatory epithelial cell proliferation. These results are compatible with histological and functional descriptions of thyroid tissue from myxedematous cretins.

Selenium is found in the thyroid in high concentrations (0.72ug/g) (Aaseth J *et al* 1990). It is also a component of type 1, 5'-deiodinase. Selenium deficiency in the pathogenesis of cretinism has of recent been used to advance an hypothesis on the development and differential occurrence of the two "types" of cretinism. It is conceptualised that iodine deficiency leads to TSH hyperstimulation of the thyroid with increased production of H_2O_2. Selenium deficiency results in glutathione-peroxidase deficiency and the accumulation of H_2O_2 which could induce thyroid cell destruction and finally fibrosis resulting in myxedematous cretinism. On the other hand deficiency of the seleno-enzyme 5'deiodinase in the pregnant subject affected by both iodine and selenium deficiencies could result into less T_4 to T_3 conversion, allowing increased amounts of maternal T_4 being available to the developing fetus and its brain thus sparing it from neurological cretinism.

Pathogenesis of endemic cretinism.

Both fetal and maternal hypothyroidism are important events in the etiology of endemic cretinism.

Fetal hypothyroidism: Myxematous endemic cretinism is now thought to results

from severe thyroid failure occurring during fetal or early postnatal life. Several observations from IDD endemias and endemic cretinism are cited in an attempt to substantiate proposed pathogenesis. Thyroid failure at birth has been evidenced in several endemic areas with myxedematous endemic cretinism (Connoly KJ *et al* 1979; Chaouki ML *et al* 1986). In some affected areas (Kochupillai N *et al* 1986) about 10% of infants are clinically hypothyroid, some with delayed bone maturation which directly correlates with serum TSH and inversely with T_4. Epiphyseal dysgenesis in adult myxedematous endemic cretins with clinical and radiological signs of longstanding hypothyroidism suggest that hypothyroidism was present before or around birth (Wilkins L 1941; Handersen HJ 1961). With the observation of a direct correlation between mental retardation, height and bone maturation in myxematous endemic cretins it is suggested that hypothyroidism present in early life would account for the mental retardation of these patients (Lagasse R *et al* 1989).

Maternal hypothyroidism: Maternal hypothyroxinaemia is very rare in non-endemic areas. It can result in impaired neurointellectual development in the offspring (Man EB *et al* 1991). Different from non-endemic areas, maternal hypothyroxinaemia is extremely frequent in endemic areas (Stanbury JB 1972; Vermiglio F *et al* 1995). It is associated with increased mortality and morbidity in the offspring (Thilly CH *et al* 1980) and increased incidence of neonatal hypothyroidism. Maternal hypothyroxinaemia due to iodine deficiency (in rats) results in lack of thyroid hormones in fetal tissues during early pregnancy, even before the onset of fetal thyroid function, and also later on, during fetal development (Morreale de Escobar G *et al* 1985; Escobar del Rey F *et al* 1986; Morreale de Escobar G *et al* 1993). These data indicate that in rats, there is substantial transfer of thyroid hormones from

mother to fetus during early gestation, which plays a role in fetal development (Woods RJ *et al* 1984), especially of the brain. In humans, studies in infants born without thyroid glands (thyroid agenesis) or with non-functioning thyroids (dyshormogenesis) show that these infants still have detectable levels of thyroid hormones at birth (cord blood) which rapidly decrease thereafter (Vulsma T *et al* 1989). These findings defeat old concepts that there is no or only minimal transfer of thyroid hormones across the human placenta.

The clinical dilemma that has puzzled researchers in this field for many years is to explain the variable clinical pattern of endemic cretinism. It is now accepted that the two broad categorisation (neurologic *vs* myxedematous cretinism) are not exclusive entities and that in both "types" features of neurological damage and features of perinatal hypothyroidism are found.

Recently, Boyages and Halpern (Boyages and Halpern 1993) have tried a unifying hypothesis on the "types" of cretinism. The hypothesis is that the clinical picture of endemic cretinism results from the product of two pathophysiological events, both resulting from iodine deficiency: First is maternal and/or fetal hypothyroidism. This, they say occurs in all cretins and results in the neurological features of endemic cretinism (from their studies these authors show that features of neurological damage occur in both "types" of cretinism). The second event is postnatal hypothyroidism which they say does not occur in all cretins. It is hypothesised that postnatal hypothyroidism is a result of failure of an individual's thyroid gland functional capacity postnatally. Therefore those who will be affected by postnatal hypothyroidism will present with growth, sexual and mental retardation and other features as observed more predominantly in the "myxedematous" form of cretinism (it must be appreciated

that in their hypothesis the authors show that "myxedematous" features of cretinism are also observed in the so called "neurologic" cretins.

Part 2

1.6 The laboratory diagnosis of thyroid diseases.

1.6.1 The earliest methods.

Measurement of Basal Metabolic Rate (BMR) is the first documented test of thyroid function (Gruhn JG 1987). The search for better means of chemical laboratory evaluation of thyroid function has been going on. The earliest chemical methods were based on the estimation of Protein Bound Iodine (PBI) (Backer BS *et al* 1951) or Butanol-Extractable Iodine (BEI) in plasma (Man EB *et al* 1951). All these methods were useful because iodine in T_4 normally accounts for 80-90% of all iodine in serum. However the tests were severely hampered by interference by a large number of exogenous iodinated compounds used for diagnostic and therapeutic purposes. Tests reflecting the peripheral metabolic effects of thyroid function e.g. serum cholesterol, aspartate aminotransferase, creatine phosphokinase, and lactic dehydrogenase (Fleisher GA *et al* 1965) were therefore employed along with PBI or BEI tests in the diagnosis of thyroid function. Further developments aimed at searching for a more specific and simple method for routine laboratory use.

Bioassays for the measurement of TSH in human plasma were developed as early as 1960's (Bates W *et al* 1960). Because of the complex methodology, limited sensitivity, specificity and reproducibility such assays were not used routinely by clinical laboratories.

Nowadays both T_4, T_3 and TSH are measured by immunoassays.

1.6.2 Basic principles of immunoassays; assay characteristics and alternatives to radioactive labels.

Immunoassay technique as an analytical method derives from the development of a thyroxine assay based on "saturation analysis" technique by Ekins (Ekins RP 1960), and the independent development of a radioimmunoassay (RIA) for insulin by Yalow and Berson (Yalow RS and Berson SA 1960).

Immunoassays are those techniques which rely on an antibody as a specific analytical reagent. The basis of all immunoassay techniques is the reaction between the analyte and the specific antibody. The amount of the analyte (e.g. T_4) is then deduced from the extent of that reaction. The basic reaction equation in any immunoassay system (which is fundamental to the understanding of the basis of immunoassay technique) is given according to the Law of Mass Action:

$$Ag + Ab \underset{k_2}{\overset{k_1}{\rightleftarrows}} AgAb$$

Where;

Ag = antigen (analyte); Ab = antibody; AgAb = antigen-antibody complex

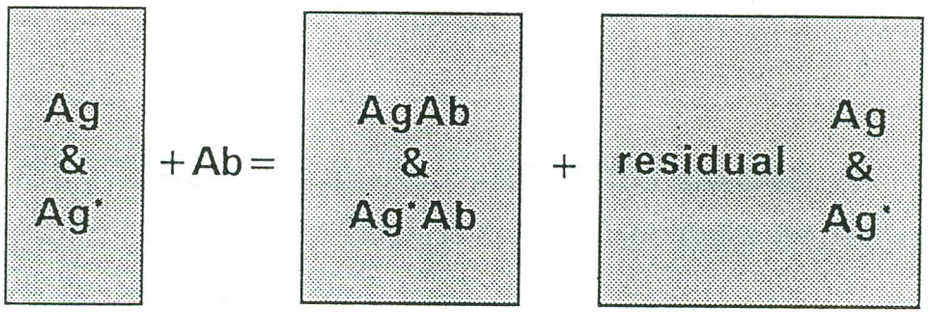
k_1 = association rate constant and k_2 = dissociation rate constant.

At equilibrium the rates of complex formation and dissociation are equal and the equilibrium constant of the reaction is expressed as follows:

$$K = [AgAb]/[Ag]\,[Ab]$$

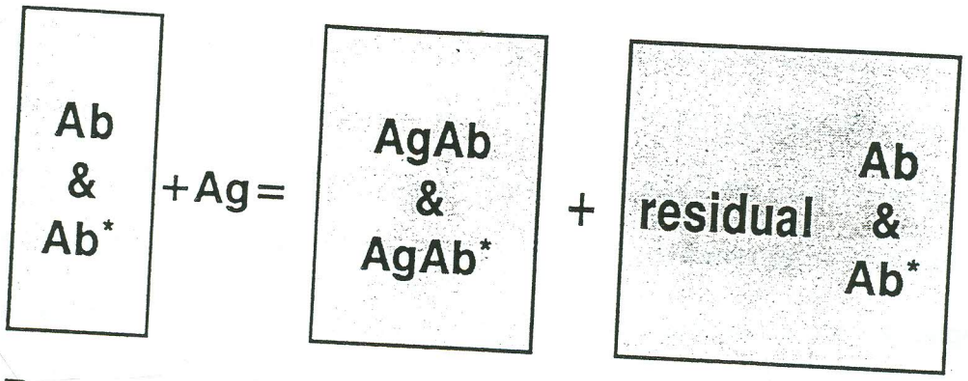
There are two fundamental designs of immunoassays (figures 1.7 and 1.8). The distribution of the analyte or antibody (either of which may be labelled) between the bound and the free form following reaction may be used as an index of analyte

concentration. In the case of labelled analyte competitive assays the analyte concentration is inferred from the ***relative*** amounts of the labelled analyte-antibody complex *vs* native analyte-antibody complex. This is the original radioimmunoassay (RIA) design. The signal in this type of assay i.e. antibody bound radioactivity is therefore ***inversely*** related to the (native) analyte concentration in the test sample. On the contrary in the non-competitive assays the analyte concentration is inferred from the ***absolute*** amount of analyte-labelled antibody complex. This type of assay is referred to as immunometric assay (IMA). The assay signal e.g. labelled antibody radioactivity is ***directly proportional*** (within the working range of the assay) with the analyte concentration in the test sample. Competitive assays based on labelled antibodies have also been designed (see *e.g.* Serozyme assays in chapter four of this thesis). The first immunoassay systems (inclusive of assays for T_4 and T_3) were based on labelling the analyte. The use of antibodies as labelled reagents were introduced in the late 1960's (Wide L *et al* 1967; Miles LEM and Hales CN 1968). In this thesis the terms EIA, LIA and FIA will be used for assays designed according to the former (competitive) type and IEMA, ILMA and IFMA for assays designed according to the non-competitive type. Others refer to 'immunometric' assay for all types of assay in which the antibody is labelled even if it is competitive in nature (Gosling JP 1990).



Response variable: Final distribution of labelled Ag* between complex and residuum e.g. RIA, EIA.

Figure 1.7. The basic principle underlying techniques which rely on observation of the extent of reaction of an analyte and specific reagent. These techniques are described as "labelled analyte", "saturation ", "competitive (protein) binding " or "limited reagent" assays (Adopted from Jackson TM and Ekins RP 1986).



Response variable: Final distribution of labelled Ab* between complex and residuum e.g. IRMA, IEMA.

Figure 1.8. The basic principle underlying techniques relying on observation of the extent of reaction of specific reagent with the analyte. They are also referred to as "labelled reagent", "non-competitive " or "reagent excess" assays (Adopted from Jackson TM and Ekins RP 1986).

Competitive assays (figure 1.7) essentially depend on the notion of "saturation" of antibody binding sites by an analyte. In designing these assays therefore very low concentrations of antibody are used (thus the term ***limited reagent*** assay). Because of using very low concentration of antibody, both the rate of analyte-antibody complex formation and (at thermodynamic equilibrium) the concentration of the complex itself are also low. Two characteristics follow from this type of design of immunoassay. First, maximal sensitivity is achieved when the antibody concentration approaches zero. Secondly, this type of assay (competitive assay) has a longer turnaround time when compared to non-competitive ones.

On the contrary, the design of non-competitive assays (fig 1.8) uses high concentrations of antibody which tend to drive the reaction towards complex formation. Two characteristics of this assay design (compared with competitive assays) are that maximal sensitivity is attained using an amount of antibody approaching infinity and also these assays have shorter turnaround times when compared to competitive ones.

Assay characteristics

Two important concepts used in judging analytical performance of all analytical methods (including immunoassays) are ***analytical accuracy*** and ***analytical precision.*** These two wider notions encompass specificity and sensitivity. Analytical accuracy refers to an agreement between the best estimate of a quantity and its "true" value. Analytical precision refers to the agreement between replicates. A method will be highly ***accurate*** when it is properly standardised and specific. A method is said to be ***precise*** if replicate concentration measurements show little

random variation, most conveniently expressed in terms of standard deviation or coefficient of variation. The magnitude of this variation equals the quotient of the random error in the response signal and the slope of the dose/response relation at that concentration. It is always these two factors that determine assay precision, and -when speaking of the zero concentration 'point- sensitivity. Sensitivity can be expressed in terms of the detection limit, which is the upper confidence limit (e.g. at $p<0.05$) of measurement results obtained in samples that do not contain any analyte. Specificity of an analytical method is explained as "the ability of an assay to determine solely the substance for which it was designed.

Non-specificity of an assay (which is a negative attribute of an assay) may be a consequence of (a) "specific non-specificity"- refering to interference by identifiable materials which are physicochemically similar to ligand and thus may react directly with the binder (cross-reaction) or of (b) "non-specific non-specificity"-reffering to the interference by materials which do not directly react with the binder, but can nevertheless affect the primary binder-ligand reaction-for example, acid conditions which produce partial or total inhibition of an antigen-antibody reaction.

In competitive assays the magnitude of the observed cross reaction depends on the level of the analyte while in noncompetitive assays the use of two different (monoclonal) antibodies in sandwich assays may lead to very high specificity. The eventual significance of a cross-reacting material lies in the extent to which it is likely to interfere with the practical operation of an assay. For example, a RIA for T_4 may show a substantial cross reaction say 10% with T_3. However, as the circulating levels of T_3 are at least two orders of magnitude lower than those of T_4 this cross reaction is unimportant in practice. At the other extreme if a radioimmunoassay for T_3 shows

a cross reaction of 1% for T_4, then with such an assay circulating T_4 would make a substantial contribution to the result.

Imprecision of an assay results from random errors which are part of any measurement procedure. These errors arise for example as a result of random variations in the pipetting of reagents, separation of bound and free fractions, and radioactive sample counting. The eventual effect is that there occur random errors in the estimation of the dose and in the estimation of the response. In immunoassay the precision profile of an assay is used to characterise an assay. The precision profile relates to the within-run or between-run s.d. or c.v. to the dose level. It is an important aid in assay optimisation. By defining the acceptable precision in the assay procedure (represented by CVs) the working range of that particular assay can be defined. When the precision of the method is specifically considered at the low levels of the analyte of interest, then the concept of the assay sensitivity comes in. Good sensitivity is considered a hallmark of a good assay. In some situations sensitivity of an assay is of paramount practical significance. For example one of the breakthroughs in the assays for TSH was the development of a RIA which was sensitive enough for determining not only the raised levels associated with primary hypothyroidism but also the normal concentrations of TSH (Odell WD *et al* 1965) or suppressed levels associated with hyperthyroidism (Wide L and Dahlberg PA 1980). There are some situations however in which sensitivity is of less importance since the levels of an analyte of interest in biological fluids are orders of magnitude above the detection limit of most assay methodologies employed, including assays for total T_4 and total T_3.

Alternative labels in immunoassays

Both the assay design and the type of label used in immunoassay do influence the potential sensitivity that can be achieved (Ekins RP 1981; Jackson TM and Ekins RP 1986). Higher sensitivity in competitive assays results form the use of antibody with high affinity constant and a reduction in the relative error in the measurement of the bound analyte. In non-competitive assays, the sensitivity of the system is ultimately depends on the use of a label with high "specific activity" (i.e. the number of observable events per unit time) and by minimising the background "noise" that may be present.

Quantitation of radioactivity and fluorescence is more precise than end point detection in enzyme immunoassay, the latter requiring stringent attention to such factors as pH, temperature and timing of enzymatic reaction. Because of these differences in precision of end point detection, signal measurement error in immunoassay is conceivably influenced by the type of the label used.

Background noise is not a common problem with assays based on radioisotopes since background radioactivity (i.e. radioactivity inherent in a patient's sample) can only be seen in patients who have received radioisotopes either for treatment (e.g. radioactive iodine for the treatment of thyroid carcinoma) or for diagnostic purposes. On the other hand background noise is common with various non-isotopic label-based assays. For instance, biological fluids contain natural enzymes which can be increased in various pathological or physiological conditions and interfere with the assay. Examples of these is the increased activity of alkaline phosphatase in Paget's disease of the bone (Krane SM 1978). There are a number of substances which exhibit fluorescence. In fluoroimmunoassays or immunofluorometric assays the

problem of background noise is encountered because of the naturally occurring fluorophores e.g. bilirubin and sometimes the fluorescence from plastic tubes used in some assay procedures.

Compared to radioisotopes, non-isotopic labels may possess higher specific activities. When measures are taken to reduce high background noise, highly sensitive non-isotopic immunometric assays can be designed. In immunoenzymometric assays, high specific activity derive from the fact that conversion of many moiecules of substrate by a single molecule of enzyme implies an amplification effect-increasing the detectability of enzyme labelled antibody molecules. Highly sensitive IEMA have been reported which rely on fluorogenic or radioactive substrates (essentially to improve the relative sensitivity of the detection system) or an additional enzyme amplification cycles (Harris CC *et al* 1979; Shalev A *et al* 1980; Johhanson A *et al* 1986).

Immunofluorometric assays of high sensitivity also reflect in part, the high specific activity of the fluorophore. In fluorimetry excitation/emission cycle can be repeated and this lead to emission of many photons from each fluorescent label in a short time (somewhat similar to the enzyme amplification effect).

Highly sensitive IFMA are possible with pulsed light and time-resolved fluorescent measurement in which "bleaching" effects and high background noise are circumvented (Hemilla I 1986).

In spite of low quantum yield, chemiluminescent labels potentially yield higher sensitivity because luminescence can be triggered at will and the signal contributions of all molecules can be collected in a much shorter time than in the case of ^{125}I (Weeks I, 1984).

Finally, it should be noted that various labels are endowed with particular characteristics which need to be taken into account especially when appraising methodologies which may be more suited to particular logistic and other problems faced in particular countries.

Reports of escape of an appreciable amounts of gaseous radioactive iodine from immunoassay tubes were available in the mid-seventies (Bogdanove EM and Strash AM 1975). The elimination of the radiation hazards of radioisotopes was above all else, the principal reason for the development of alternative labels. This has been a motive behind severe legislative restrictions on the use of radioisotopes. However it has been urged that the radiation hazard for RIA is very small. In assays employing ^{125}I it is believed that the amount of ^{125}I released in the course of the assay is less than 1% of the total (the total being a few microcuries). However, care is essential in performing radioiodination of the tracer, since several mCi of for example ^{125}I are commonly employed. RIA are traditionally subject to stringent measures of monitoring the possible hazards to immunoassayists and that is why some countries have so framed their legislation as to prohibit all but a few centres from performing RIA. On the other hand it is urged that some nonisotopic labels may pose new health hazards for example some of the substrates used in EIA (e.g. o-phenylene diamine) are powerful mutagens (Bos RP *et al* 1981).

Isotopic tracers using ^{125}I are inherently unstable and have a shelf-life ranging from 3 weeks to three months this reflecting a short half life of 60 days. Non-isotopic tracers potentially have indefinite stability. In reality however, many enzyme labelled antibody preparations have proved remarkably unstable, great care has to be taken to ensure shelf-life of twelve months. This advantage of longer shelf-life has greatest potential to consumers who are not equipped enough to set up inhouse-assays.

Instrumentation of hormone assay laboratory depends to a large extent on the label

used (some laboratory equipments e.g. refrigerators, water purifiers, water baths being universal in their use). Radiation counters, fluorometers and filter-or spectrophotometers are needed for isotopes, fluorophores and enzymes as labels respectively. Relatively inexpensive and efficient (over 70% efficiency for ^{125}I) manual and automatic gamma-counters are available, which are simple to use, involving only introduction of the tube into the well of the sodium iodide crystal. Counting precision is satisfactory and sufficient counts are usually generated in 60 seconds or less. Counting many tubes in short time is made possible with the multihead counters. Fully automated systems for assays based on radioisotopes have been developed, both of the continuous-flow and discrete type. Filter photometers or spectrophotometers suffice in assays based on enzymes as labels.

The large, relatively expensive spectrophotofluorometers employ a monochromator to enable maximum versatility in wavelength selection. Filter fluorometers employ filters of the absorbence or interference type and are less expensive, simpler to employ and, provided the filters used are well-matched and of good quality, are as sensitive as spectrofluorometers.

Also because of unavailability or of unreliable power supply in most of the developing countries, instruments which can be driven by a battery are more suitable to such areas. Therefore, the initial cost of instrumentation (for end-point detection) depend on the label and the work load of the laboratory (with few samples per month simple, less expensive-manual systems are sufficient).

1.6.3 Assays for T_4, T_3 and TSH

The successful production of antibodies against T_4 and T_3 by immunising rabbits against thyroglobulin and T_4- and T_3-bovine albumin conjugates (Chopra IJ *et al* 1971) enabled the development of radioimmunoassays of Thyroxine (T_4) (Chopra IJ

1972) and Triiodothyronine (T_3) (Gharib H *et al* 1971). These were followed by wide spread introduction of radioimmunoassay services in many clinical laboratories. Apart from radioisotopes, a number of alternative labels have been and continue to be developed. Non-isotopic T_4 and T_3 immunoassays and immunoradiometric assays based on enzymes (Blake C *et al* 1982), fluorescents (Soini E and Kajola H 1983) and luminescents (Sturgess ML and Weeks I 1986) have been developed.

Radioimmunoassays of TSH were also developed (Wide L, Dahlberg PA 1980). From the mid-1980's a second generation of more sensitive TSH immunometric assays; immunoradiometric-IRMA (Helenius T and Tikanoja S 1986), Immunoenzymometric-IEMA (Watenabe Y *et al* 1984), immunofluorometric-IFMA (Kaihola HL *et al* 1985) and immunochemiluminometric-ICMA (Weeks I and Sturgess M 1984) have been developed.

The concept of "functional sensitivity" of an assay has been advanced (Spencer CA 1990). The functional sensitivity of an assay depends on the interassay performance because it is the precision of the assay week to week and month to month that determines diagnostic potential in the patient care. Functional sensitivity is derived from the interassay precision profile, the latter representing a period in time covering the use of different batches of reagents and different technicians. A 20% coefficient of variation read from the interassay precision profile curve provides a clinically relevant (i.e. ability of the assay to distinguish normal from hyperthyroid TSH concentrations) estimate of assay functional sensitivity (Hay ID *et al* 1991). The concept of functional sensitivity of an assay has led to a generational approach to TSH assay nomenclature dividing them into first, second and third generation assays with functional sensitivities of 1-2 mIU/L, 0.1-0.2 mIU/L and 0.01-0.02 mIU/L

respectively. In a particular laboratory a functional sensitivity of a TSH assay can be obtained by repetitive TSH measurement of serum based pools having subnormal TSH values measured over a period of time, with different batches of reagents and technical personnel.

1.6.4 Assays of free T_4.

The earliest methods for free T_4 estimation involved equilibrium dialysis, employing isotopically labelled T_4 (Sterling K and Brenner MA 1966) in which the free fraction of T_4 was determined and then multiplied by total T_4 to derive free T_4 concentration. Since the mid-seventies a multitude of immunoassay FT_4 kits have been developed and these are the most widely used routine methods.

In the following sections the conceptual basis, relative merits and disadvantages of available approaches for the measurement of free thyroid hormones are presented.

Classification of free hormone assay techniques.

According to Ekins (Ekins RP 1990) the methods for free hormone assay can be classified on the basis of two criteria; "Direct" versus "indirect", and "absolute" versus "comparative".

Direct methods: Two steps are involved. First is to sequester the free hormone from the protein bound moiety followed by direct measurement of the amount sequestered. The measurement can be done for example by a radioimmunoassay as reported in one method (Ekins RP and Ellis SM 1976).

Indirect methods: In these methods two quantities are measured. First the total hormone is measured. Secondly the free hormone fraction is measured for example

by equilibrium dialysis (e.g. Sterling 1966). Alternatively the free hormone fraction may be inferred from a measured quantity reflecting the free hormone fraction (e.g. fractional hormone uptake by a solid adsorbent or a solid phase antibody).

Depending on the standards that are used to calibrate an assay, a second classification of free assay techniques divides them between "absolute" and "comparative" methods.

Absolute methods: These rely on standard hormone solutions in buffer as calibrants. The amounts of hormones dissolved in buffer standards are established for example gravimetrically.

Comparative methods: In these methods standards are made from normal serum in which levels of totai hormone have been adjusted. The corresponding free hormone concentration in these standards is established by using an "absolute" method.



Dialysis methods for the measurement of free hormones.

These are divided into equilibrium (which can be either direct or indirect) and symmetric dialysis techniques.

Equilibrium dialysis: This technique relies on bringing serum (the "dialysand") into contact with a buffer (the "dialysate"), the two being separated by a membrane permeable only to hormone (and to other small molecules) and not to the serum proteins responsible for hormone binding. The free hormone molecules pass through the membrane into the dialysate, resulting into net dissociation of the protein bound hormone in the dialysand and restoration of the thermodynamic equilibrium

throughout the entire system. When equilibrium is reached the concentration of the free hormone on both sides of the membrane is identical. The free hormone in the dialysate may then be measured either directly by e.g. immunoassay or indirectly. In the indirect approach a radioactively labelled hormone (assumed to behave identically to the endogenous hormone) is initially introduced in the test sample and is assumed to distribute itself between the bound and the free form in identical manner with the endogenous hormone. At equilibrium, the free hormone concentration in the dialysate is essentially identical to that originally present in the sample. Therefore the fraction of the total radioactivity which appears in the dialysate is taken to reflect the original free hormone fraction in the test sample.

Symmetric dialysis: In this approach (Ross HA 1980) a serum sample, to which a tracer hormone has been added, is dialysed against an identical sample (without a tracer), so that a symmetric arrangement results. The tracer distributes itself between the two compartments until equilibrium is reached. The rate at which the tracer migrates is assessed as a measure of the free hormone fraction.

Ultrafiltration methods for the measurement of free hormones:

This may likewise be subdivided into direct and indirect methods, depending on wether labelled hormone is added to serum before ultrafiltration or the free hormone in the ultrafiltrate is measured directly.

Gel-filtration and adsorption methods.

These depend on Sephadex gel separation of bound and free hormone fractions, (Romelli PB *et al* 1979) and may be of both "direct" and "indirect" approach. They

rely on the penetration of free hormone into, and/or adsorption by Sephadex particles, serum binding proteins being excluded. The direct approach of free thyroid hormone measurement relies on adsorption of free hormone onto a short Sephadex column, from which the hormone is subsequently eluted and measured by RIA. This technique is theoretically analogue to equilibrium dialysis

Artifactual errors in dialysis and ultrafiltration techniques.

Radioactive impurities: Impurities (e.g. radioiodine) are a major source of error in indirect techniques. They may be present in the tracer hormone before use (as a product of labelling procedure), or arise during the fractionation process. Depending on their amount relative to the labelled hormone that appears in the dialysate or ultrafiltrate they may lead to gross overestimation of the free hormone fraction and hence the free hormone concentration. Purification techniques (Cooper E and Burke CW 1978) may be employed.

Serum dilution: Dialysis techniques involve a measure of serum dilution because the dialysate in itself constitute a serum diluent. For example if the dialysate volume is four times the sample, the effective dilution is 1:5. This dilution leads to possible distortion of the free hormone concentration. Serum dilution causes dissociation of the bound hormone in accordance with the mass action laws, the free hormone concentration although falling, being thus maintained at a level closely comparable to that in the undiluted sample. The extent to which the free hormone concentration is reduced, depends on the fraction of the free hormone in the undiluted sample, the greater the fraction, the greater the fall caused by dilution. Since this effect is well recognised by most investigators and can be predicted by mass action laws

considerations it does not constitute a major problem in free thyroid hormone assays by these techniques.

In normal serum there may be present some substances which may be competing with thyroid hormones for binding to serum proteins. Dilution of these substances in the course of an assay may cause serious distortion of the free hormone fraction and therefore the free hormone concentration. These competitors are exemplified by drugs such as salicylates, and endogenous competitors such as free (non-esterified) fatty acids. Serum dilution causes alterations in the competitive effects of such substances. The effects of dilution of binding competitors can not be predicted and that is why this may lead to erroneous determination of free hormone concentration in samples containing such substances.

Dialysis and ultrafiltration techniques are usually time consuming and require more than average expertise in their performance, and therefore are primarily reserved for research purposes. For routine laboratories a number of simpler techniques are used.

The free thyroxine index; principle and limitations.

The "free thyroxine index" provides an approximate indication of the free T_4 level. The index is derived by combining the result of a total T_4 measurement with that of a T_4 or T_3 "Uptake test". The uptake test is considered to reflect the concentration of thyroid hormone binding proteins in test serum sample. The free thyroxine index is expected to correlate closely with the serum FT_4 concentration.

Principle of the T_3-Uptake test.

The basic principle of the T_3U test may be explained in different ways. Usually it is

pictured as a competition between empty binding sites on T_3- (and T_4-) binding serum proteins and some separable binding or adsorbing agent such as a resin or a solid phase-anti-T_3 antibody for labelled T_3. Both elevated T_4 such as in hyperthyroidism and reduced binding protein (e.g. TBG deficiency, usually a euthyroid state) lead to a reduction in unoccupied binding sites which result in increased T_3 tracer (labelled T_3) uptake by the adsorbent. Multiplication of the percent uptake with elevated total T_4 in the first instance results in an elevated free T_4 index (FT_4I). In the second instance T_3 uptake is multiplied by a strongly or moderately reduced total T_4 to yield a "normal" value of free T_4 index. On the other hand, both hypothyroidism and pregnancy lead to an increased level of unoccupied binding sites and a reduced T_3 Uptake. The latter results in a more pronounced low FT_4I in hypothyroidism and a proper normalisation of the elevated total T_4 levels of pregnancy. Predicted directional changes in FT_4I in various clinical situations is presented in table 1.2

Table 1.2. Diagnostic utility of the Free Thyroxine Index values.

Clinical situation.	Total T_4	T_3 Uptake	Free Thyroxine Index (FT_4)
Euthyroid	Normal	Normal	Normal
Hyperthyroid	High	High	High
Hypothyroid	Low	Low	Low
Increased TBG	High	Low	Normal
Decreased TBG	Low	High	Normal

Generally, whenever total T_4 and T_3 Uptake values diverge and the FT_4I is normal, binding protein concentration of TBG are probably abnormal. In these settings, there is an excellent correlation between FT_4I and free T_4 determined by equilibrium

dialysis. Also free T_3 index calculated as a product of total T_3 and T_3 uptake provides a reasonable estimate of FT_3. A related approach relies on combining the results of a total T_4 measurement and a specific thyroxine binding globulin (TBG) determination, the combination likewise yielding a quantity correlating reasonably closely with serum FT_4 (Glinoer D *et al* 1978), except in the complete absence of TBG.

Diagnostic limitations of T_3U and FT_4I.

For a better understanding of the limitations of T_3U, the following description is advanced:

When labelled T_3 is added to a serum sample, it is distributed over free and protein bound fractions equal to the endogenous hormone. After addition of an adsorbent or any other substance that has a high binding capacity for T_3, T_3 is taken up by that substance at a rate that is ***proportional to the free concentration.*** Therefore, the uptake rate of labelled T_3 will necessarily be proportional to the ***fraction or percentage of free T_3.*** As the latter parameter correlates (at a high order of magnitude) with the ***percent of free T_4*** it becomes clear that the product of uptake rate and total T_4 must somehow reflect the ***free T_4 concentration.*** The said proportionality however pertains to the ***initial uptake rate,*** rather than to the net uptake that is observed after a significant proportion of labelled T_3 has been taken up. Here may be one of the reasons why many versions of this test perform poorly at the extremes of TBG concentrations. One other reason may be that when a nonspecific adsorbent, such as an ion exchange resin is employed, also T_4 is stripped from serum, which would result in a lower free T_4 fraction (or larger number

of unoccupied sites) during the process-leading to artifactually lower T_3 uptake. This would constitute a reason to prefer anti-T_3 as an agent to sequester labelled T_3 to an unselective adsorbent. The use of antiT_3 may present another drawback, however. As the capacity of the antibody is usually limited, it may partially behave like a free T_3 immunoassay. As the absolute value of the percent uptake depends on the amount and avidity of the sequestering agent, an attempt has been made to normalise the uptake by dividing the percent uptake of unknowns by the percent uptake of an average, euthyroid sample. Thus the average of the normalised T_3Uptake tend to be unity. Nevertheless, the free T_4 index obtained thus cannot be directly compared with reference ranges of total T_4. Deviation from non-initial rate conditions may be partially corrected for using reciprocal or logarithmic transformations of T_3U. This has been successful in the past in certain cases.

Free thyroxine index methods therefore have been attended by results in certain patient groups which are considered inappropriate to the clinical situations. FT_4I methods may give inappropriately high values at TBG extremes (Helenius T and Liewendahl K 1983), and in pregnancy (Tutlebee JW 1982). FT_4I is also high in FDH (Stockigt JR *et al* 1981; Ruiz M *et al* 1982). In the low total T_4 state of NTI, FT_4I methods give low values that are indistinguishable from hypothyroidism (Wood DG *et al* 1980; Kaptein EM *et al* 1981; Slag MF *et al* 1981). The use of TT_4 and TBG ratio offers no improvement in most of these problem states. For example TT_4/TBG ratio give low values in high TBG states (Leculeuil M *et al* 1978) pregnancy (Wand JM *et al* 1983) and low total T_4 states of nonthyroidal illnesses (Wenzel KW 1985). The ratio gives high values in low TBG states (Kono N *et al* 1983) and in FDH (Horn K 1985).

This misclassification of patients requires costly follow-up tests, leads to patient inconvenience and may, if unrecognised, result in inappropriate patient treatment. An additional disadvantage of FT_4I methods is, the additional cost and probability of error implicit in the conduct of two separate measurements. For these reasons there has been an inevitable dissatisfaction with FT_4I methodology, giving impetus for the development of new FT_4 immunoassays which are discussed in the following section of this thesis.

Free hormone immunoassay techniques; merits and limitations.

The principle behind free hormone immunoassay can be explained as follows. When a small amount of antibody is introduced into a sample containing free and bound hormone the antibody binding sites are occupied by the hormone to the extent that is only determined by the free hormone concentration in the sample. The amount of the antibody is such that its sequestration of hormone causes negligible disturbance to the existing free hormone concentration in the sample, that is, only a small fraction of the total hormone in the sample (generally accepted as below approximately 1-5%) is bound to the antibody. This results in the dissociation of bound hormone and restoration of the preexisting equilibrium state, the ambient free hormone concentration thus falling by not more than 1-5%. The cardinal rule governing all valid free immunoassays (Ekins RP 1993) is that neither the reagents used nor the conditions in which the assay is conducted should significantly disturb the pre-existing equilibrium between the free and bound hormone in both standards and unknown (test) sera. In other words, the ambient free hormone concentration should not be significantly altered by assay procedure. Antibody occupancy can be

determined in various ways.

These techniques can also be subdivided into "direct" and "indirect" types and four approaches are available as follows (Ekins RP 1990).

1. "Indirect" free hormone immunoassay: "the labelled hormone, antibody uptake" approach.

2. "Direct" free hormone immunoassay: "the labelled hormone, back titration" ("two step") approach.

3. "Direct" free hormone immunoassay: "the unbound labelled hormone-analog" ("single step") approach.

4. "Direct" free hormone immunoassay: "the labelled antibody -solid phase unlabelled analog" (single step) approach.

The labelled hormone antibody uptake approach.

This approach for free T_4 involves two steps (i) The measurement of total hormone in the sample and (ii) The measurement of the free fraction of the hormone.

A known tracer amount of labelled hormone is added to the sample. This results into the endogenous hormone pool being radiolabelled at a certain specific activity. After incubation with a standard amount of (solid phase) antibody against the hormone, radioactivity bound to antibody is determined. With the knowledge of its specific activity, the occupancy of antibody sites can be inferred. The hormone occupancy of the antibody reflects the ambient free hormone in the sample. Meanwhile by following the same procedure with serum standards of known free hormone content (previously established using a dialysis or ultrafiltration procedure) a dose response curve can be constructed relating antibody occupancy to free hormone concentration,

thus providing the basis for free hormone estimation in the unknowns.

The labelled hormone back titration approach.

This approach (Ekins RP *et al* 1980) is described as follows. In the first stage, the test serum is incubated with solid phase antibody against the hormone. The antibody preparation is washed and subsequently exposed to labelled hormone for a further period of incubation. During the second stage of incubation, labelled hormone binds to residual unoccupied antibody binding sites to the extent essentially proportional to the latter's concentration. Thus, the greater the free hormone concentration in the test sample, the lower the concentration of residual antibody binding sites at the end of first incubation, and the greater the amount of labelled hormone ultimately bound to the solid phased antibody. With this approach many reports showed close agreement with equilibrium dialysis in many clinical states. For example normal values were reported at TBG extremes (Helenius T and Liewendahl K 1983) and in FDH (Rajatanavin R *et al* 1982). In pregnancy normal values were reported (Tutlebee JW 1982). Normal values were also reported for studies on low T_4 NTI (Slag MF *et al* 1981). One general disadvantage associated with this approach is that of sequential incubations which may lead to imprecision and assay drift.

The labelled hormone-analog approach.

The principal governing this approach is that the hormone analog is totally unreactive with serum proteins but retains the ability to cross react against native hormone for antibody binding sites. A solid phase antibody is incubated with a test serum and the

labelled hormone analog. The free hormone in the test serum competes with the labelled hormone analog for binding sites on the antibody. At the end of the incubation the serum is removed and after washing the antibody bound activity is measured and is inversely proportional to the free hormone concentration originally present in the test sample. This type of assay has been one of the thoroughly analysed in terms of expected and real performance characteristics. Errors generated in the analog based free T_4 assay kits and the physicochemical basis thereof have been extensively discussed (Ekins RP 1993). Until now, no analog that completely conforms to the physicochemical basis of the technique has been found. Analogs do bind to serum proteins especially albumin and TBPA. In addition some of the reagents commonly added to components of these assays may interfere with the prevailing equilibrium between free and bound hormone moieties and also distort the effects of binding competitors. The consequence of these effects are that results on specific patient categories have been variable. However normal levels of FT_4 were reported in high TBG states (Ashwell K *et al* 1983). Assays based on this technique have been shown to give inappropriate results whenever there is abnormal albumin (in terms of concentration or affinity characteristics) or in situations when there are other compounds in test sera which may compete with the analog for albumin binding. For example, inappropriately high FT_4 values have been reported in FDH (Rajatanavin R *et al* 1982; Stockigt JR *et al* 1981; DeNayer P *et al* 1984) and low TT_4-NTI state (Liewendahl K *et al* 1984). The analog methods were also shown to give low FT_4 values in the second and third trimesters of pregnancy (Helenius T and Liewendahl K 1983; Wand JM *et al* 1983). This may in part reflect a physiological lowering of FT_4 levels suggested by some studies using reliable methods (Price A

et al 1989) but also may reflect methodological interference due to decreased albumin (Amino N *et al* 1983) and non-esterified fatty acids (NEFA) elevation associated with late pregnancy (NEFA competes with the analog for binding to albumin).

The labelled antibody approach.

Labelled antibody techniques of free hormone assay as reported (Christophides ND *et al* 1992), rely on the same basic principle as that underlying the labelled analog immunoassay methods described above. In this particular radioassay, free T_4 in the sample competes with a gross molar excess (over antibody) of a cross reactant (T_3) chemically coupled to magnetisable polymer particles, for binding to avid ^{125}I labelled monoclonal antithyroxine antibodies. The ^{125}I counts bound to the solid phase (T_3-magnetisable particles) are inversely proportional to the sample FT_4 concentration. If the solid phase analog will bind to (endogenous) serum proteins, then the same distortions as they occur in the conventional labelled analog approach may be expected.

In conclusion, no FT_4 methodology exists that can claim to accurately measure FT_4 in all clinical situations without a minimum probability of failure (either due to known reasons or unforeseen circumstances). Therefore various methods for the measurement of free thyroid hormones present varied merits and limitations.

1.7 Thyroid assessment strategy: TSH and/or free T_4

Currently it is widely accepted that the assessment of thyroid function should include

the measurement of free T_4 and TSH. The measurement of the two hormones and the directional changes expected in hypo- or hyperthyroidism are well documented as a result of the well known TSH and free T_4 relationship.

The pituitary TSH secretion is very sensitive to the ambient FT_4 concentration; the serum TSH concentration will become abnormal with mild T_4 deficiency or excess, even before FT_4 concentrations have changed perceptively. Studies in mono- and dizygotic twins suggest that individuals have genetically determined setpoint for the TSH-FT_4 relationship. Serum TSH becomes abnormal in earliest hypo or hyperthyroidism because the pituitary senses mild FT_4 deficiency or excess relative to the patients set-point even though serum FT_4 concentrations remain within normal limits for the population as a whole. This fine sensitivity of the pituitary to the ambient FT_4 concentration reflects the physiological relationship between serum TSH and FT_4 Thus, the serum TSH concentration is the most sensitive indicator of mild (subclinical) degrees of hypo or hyperthyroidism. The classic reciprocal log-linear relationship between serum TSH and FT_4 predicts that in hypothyroidism when FT_4 is low, serum TSH will be raised and in hyperthyroidism when FT_4 is raised serum TSH will be depressed. However, discordance between FT_4 and TSH may arise in various clinical conditions. The discordance may be methodological, physiological or pathological. In this kind of situations TSH or FT_4 may be diagnostically accurate and the other parameter being wrong. Correct understanding of the possible cause of the discordance is essential in order to avoid diagnostic misinterpretation of the laboratory result.

Serum TSH is diagnostically correct but discordant with FT_4 in a number of clinical

situations of which some examples are given :

(a). Subclinical thyroid disease.

Subclinical thyroid disease is biochemically defined by a persistent TSH abnormality, either low or high, associated with a normal FT_4 value. The TSH abnormality reflects the pituitary sensitivity to a mild deficiency or excess in the ambient FT_4 concentration relative to the patient's set point. These patients usually have other indicators of autoimmune thyroid disease such as anti-thyroid peroxidase (anti-TPO). The importance of identifying these patients is the fact that thyroid diseases have insidious onset (with nonspecific symptomatology) and that these patients with subclinical disease usually will progress to overt disease with time unless there is intervention (Cooper DS 1984; Cooper DS 1987; Rosenthal MJ 1987).

(b). Binding protein abnormalities.

In a number of situations, serum TSH is normal and clinically the patient is euthyroid. Serum free T_4 when measured by equilibrium dialysis is normal but with most other methods of free T_4 estimation the results of FT_4 are abnormal because of methodological failure (Rajatanavin R *et al* 1982).

(c). Non-neoplastic thyroid hormone resistance.

Thyroid hormone resistance syndrome (Franklyn JA 1991) can be either generalised, partial, or of the selective pituitary resistance type. In the former, the discordance is such that elevated serum TSH concentration is accompanied by elevated FT_4 concentration although (this is clinically appropriate because elevated FT_4 is needed to maintain euthyroidism in the face of thyroid hormone resistance).

In a number of other clinical situations discordance between serum TSH and free T_4

may occur when the later is diagnostically correct. The following are examples of these situations:

(a). Acute changes in thyroid status.

The pituitary is slow in registering an acute change in the thyroid status. It takes weeks for the restoration of an appropriate TSH-FT_4 relationship after acutely changing thyroid status. For example, after initiation of antithyroid treatment for hyperthyroidism, TSH may remain subnormal for 2-3 months even when FT_4 have fallen to normal or hypothyroid range. Similarly, after beginning T_4 replacement therapy for primary hypothyroidism, serum TSH may remain elevated for six to eight weeks despite normalisation of FT_4 concentrations.

(b). Pituitary or hypothalamic disease.

The hypothyroidism of pituitary origin (deficient TSH) or hypothalamic origin (deficient TRH) typically presents with discordant FT_4 and TSH values in that there is low TSH and low FT_4 concentrations in the same individual (in fact, inappropriately low TSH relative to hypothyroxinaemia and clinical hypothyroidism are the hallmarks of the disease). It should be noted however that TSH secreted by the damaged or hypostimulated pituitary may differ in glycosylation (Taylor T 1988) and biological potency (Beck-Peccoz P 1985) from normal TSH. This biologically inactive TSH is however, immunologically detected by immunoassays and may give rise to a paradoxically normal or high TSH value and low FT_4 value in central hypothyroidism. Pituitary tumours that secret biologically active TSH and produce clinical hyperthyroidism are rare (Gesundheit N 1989). They however present an inappropriate TSH secretion in which a discordant situation between serum TSH and free T_4 occur (high TSH accompanied with high FT_4).

(c). Heterophilic antibody interference

Heterophilic antibody interference in immunometric assays (Bascato LM and Stuart MC 1988) presents another situation in which a discordance between TSH (spuriously high) and a normal FT_4 value may be encountered as a result of (TSH) methodological failure. This combination of values (i.e. high TSH and normal FT_4) is also seen in subclinical hypothyroidism. Anti-TPO antibody measurement and TRH stimulation testing may aid the differential diagnosis. Very often, the abnormality is not found with a different set of reagents.

1.8 Statement of the problem, objectives of the study and the scope of the thesis.

While the complete picture of thyroid diseases in the Tanzanian community is not available, it is established that IDD are a public health problem in many parts of the country. As reviewed from studies in other areas, Iodine Deficiency Disorders affect the whole community with pregnant women (and therefore the outcome of pregnancy) and children constituting the most vulnerable groups. The most serious consequences of IDD can be tackled if intervention is directed towards pregnant women and children.

The clinical laboratory plays a major role in the diagnosis and treatment follow up of thyroid diseases by providing assays of thyroid hormones and thyrotropin which are needed for objective assessment of thyroid function. As reviewed above, numerous approaches of assaying thyroid hormones and thyrotropin have been developed and the relative merits and disadvantages of each are now widely documented.

To date, health institutions in Tanzania have no established thyroidology services. In Tanzania no original studies of thyroid function in healthy people have ever been performed and what is expected as normal is adopted from the medical literature. Taking into consideration the economic constraints, and the existing infrastructure of the health care system it was earlier decided to adopt the commercially available Serozyme enzyme immunoassay for diagnostic thyroidology at Muhimbili Medical Centre in Dar-Es-Salaam. This study was initiated as a preparatory step for the establishment of thyroid diagnostic services at Muhimbili Medical Centre and therefore its objectives which are answered in specific chapters of this thesis were

as follows:

1. To study thyroid function in healthy adult Tanzanians in order to ascertain whether thyroid function in these subjects is in accordance with the published literature. In this respect thyroid function in Tanzanians was studied by using methods with established analytical and clinical utility in thyroidology (Chapter two).

2. To study thyroid function during normal pregnancy in Tanzanians so as to obtain information regarding what changes do occur in thyroid function. This group was particularly studied in order to lay the foundation for projected future studies on the effects of IDD on human reproduction in Tanzanians (Chapter three).

3. To evaluate the analytical performance of Serozyme enzyme immunoassays for the measurement of T_4, T_3, TSH and FT_4I and establish their reference ranges in healthy adults and pregnant Tanzanians. This study was done following the earlier decision to adopt the commercially available Serozyme enzyme immunoassays for thyroidology services at Muhimbili Medical Centre (Chapter four).

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Chapter Two

Thyroid Function in Adult Tanzanians.

2.1. Introduction.

As it is the case with many other biological indices of endocrine function, what is expected as the physiologically normal level and pattern of circulating thyroid hormones and thyrotropin in the healthy Tanzanian population, is adopted from medical literature primarily originating from research done elsewhere and especially in the developed world. While this information may be useful, the potential non-transferability of such information should not be ruled out by *a priori* assumption. In healthy subjects the measured levels of circulating thyroid hormones and thyrotropin are dependent on the analytical method used to assay them (Dofour 1990). However levels and patterns of T_4, T_3 and TSH may also be affected by environmental (such as climate, altitude, diet including (in)sufficiency of certain micronutrients) and genetic factors.

Environmental influence is exemplified by the findings that clinically euthyroid residents of areas with moderate to severe iodine deficiency usually have lower serum T_4, elevated TSH and normal or supranormal T_3 levels when compared to values obtained in residents of iodine replete environments as a result of altered T_4:T_3 ratio in the gland, preferential secretion of T_3 or increased peripheral conversion of T_4 to T_3 in iodine deficiency states (Delange F *et al.* 1972; Patel YC *et al.* 1973; Lazarus JH *et al.* 1992).

In the Andes iodine deficiency had relatively less goitrogenic effect than in the low altitude regions. This was attributed to increased T_4 sensitivity at high altitude and thus a decreased requirement for T_4 (Fierro BR 1965). However, Mordes *et al.*

(1983), when studying the effects of high altitude on thyroid function found that acclimatisation to high altitude was accompanied with elevated total and FT_4, elevated TSH and increased TSH response to TRH in euthyroid subjects. This, they concluded, was suggestive of an alteration in the pituitary negative feedback set point for secretion of TSH at high altitude, which perhaps was due to impaired pituitary conversion of T_4 to T_3.

Regarding the influence of climate it has been shown that the uptake and release of ^{131}I by the thyroid, as well as peripheral use of thyroid hormones are depressed during exposure to a high environmental temperature (Collins KJ and Weiner JS 1968). On the other hand increased serum T_3 with nonchanged T_4 and basal TSH levels were reported in Japanese subjects during winter when they were kept in temperature controlled (-6 to -3°C) rooms (Nagata H *et al.* 1978). Studies in rats (Schammell JG *et al.* 1981) have also indicated that cold enhances the peripheral conversion of T_4 to T_3. Smals *et al.* (1977) while studying seasonal variations in serum T_4 and T_3 concentrations in normal subjects in a temperate climate found that the values for both hormones appeared to vary inversely with environmental temperature and were lowest in the summer.

Alterations in nutritional state, wether short term or chronic, and wether the result of underfeeding, overfeeding or merely a change in the diet, affect various aspects of thyroid economy, especially peripheral hormone metabolism . Studies in mono- and dizygotic twins (Meikle AW *et al.* 1988) revealed strong genetic influence on the interindividual variation of T_4 and FT_4 but not T_3 and basal TSH in plasma.

Therefore, the environmental (climate, diet) and genetic differences between populations in different parts of the world may be associated with particular patterns

of circulating thyroid hormones in health with potential nontransferability of such data from one geographical area to another.

The present study aims at elucidating the levels and patterns of serum thyroid hormones and thyrotropin in clinically euthyroid Tanzanian adults. By using assay methods whose analytical performances have been optimised inhouse, thyroid hormones and thyrotropin were assayed in samples from clinically euthyroid Tanzanian and Dutch adults and the results compared.

2.2. Subjects, materials and methods.

The Tanzanian samples were obtained from 95 clinically euthyroid adults, living in Dar-es-Salaam; a tropical city on the shores of the Indian Ocean. Euthyroidism was defined according to the criteria indicated in the questionnaires appearring in appendix II. They include 65 males and 30 nonpregnant females aged 18-54 years (mean 30.8 years). None of the female subjects used oral contraceptive pills. The Dutch samples were obtained from 48 adult healthy blood donors, 26 males and 22 females respectively. They aged 20-64 years (mean 41 years). From each Tanzanian subject ten milliliters of venous blood was collected in an empty sterile bottle. Clotting was allowed for two hours at room temperature. Samples were centrifuged for 10 minutes at 3000g. The serum was aliquoted and stored frozen at -20°C, transported to Nijmegen-The Netherlands and thawed on the day of analysis.

T_4 and T_3 were assayed using commercial RIA kits (Amerlite Diagnostics, Buckinghamshire, England). The interassay CV's were 3.7%, 2.4%, and 3.5% at T_4 levels of 65.4, 103.5 and 152.1 nmol/L respectively. For T_3 the interaassay CV's were 3.0% (at 1.06 nmol/L), 3.3% (at 1.80 nmol/L) and 3.6% (at 3.20) nmol/L.

FT_4 was determined using a labelled hormone antibody uptake approach (RIA kit-SPAC fT_4% from Byk-Sangtec Diagnostica, Dietzenbach, Germany) as previously described (Ross HA and Benraad Th J 1984).

TSH was assayed using an IFMA-Delfia kit (Wallac Oy, Turku Finland) which is standardised against WHO 2nd IRP(80/558) (see chapter four). The interassay CV's were 1.6% (at 0.55 mIU/L), 2.3% (at 1.88 mIU/L) and 2.2% at TSH level of 6.04 mI-U/L. The analytical sensitivity of TSH assay was 0.02 mIU/L. For more detailed

description of these assays see chapter four.

The data was analysed by a statistical package SOLO (Dr J.L.Hintze (1987) Kaysville, Utah 84037). Reference ranges for T_4, T_3, and FT_4 were derived as the central 95% distribution of the data after ascertaining normality of the distribution. The reference ranges of TSH were derived as the central 95% of the distribution after logarithmic transformation of the data to normalise the originally non-gaussian distribution. Group means were compared by unpaired student t-tests.

2.3. Results.

Serum T_4, T_3, FT_4, TSH levels and T_4:T_3 molar ratios in Tanzanian and Dutch adults are shown in table 2.1. Their comparative frequency distributions are presented in figures 2.1 through 2.5 respectively.

Tanzanians had significantly higher serum T_4 and T_3 concentrations than the Dutch subjects. Moreover the T_4:T_3 molar ratios were significantly lower in Tanzanians than in Dutch subjects. The differences in serum TSH and FT_4 between Tanzanian and Dutch subjects were not significant.

These findings also hold when comparing male Tanzanian with male Dutch subjects or female Tanzanian with female Dutch subjects with the exception of the molar ratio of T_4:T_3, which is not significantly different between females of the two populations. There were no significant sex differences in the levels of circulating T_4, FT_4 and TSH in both Tanzanian and Dutch groups. In the Tanzanian (but not Dutch) group, males had significantly higher levels of T_3 than females with mean (SD) of 2.10 (0.36) vs 1.84 (0.24) nmol/L ($p < 0.001$). The molar ratio of T_4:T_3 was significantly higher in Tanzanian (but not in Dutch) females than in their male counterparts. Figures 2.6 and 2.7 show individual scatter (and mean) values of T_3 and T_4:T_3 molar ratios in Tanzanian and Dutch subjects by sex.

In both populations, there was no correlation between the age of subjects and the levels of T_4, T_3, FT_4 and TSH. In both Tanzanian and Dutch subjects the percent free T_4 (fT_4%) and T_4 levels were significantly inversely correlated (in both groups $r = -0.44$ $p < 0.001$). In the Tanzanian subjects (but not in Dutch subjects) a significant inverse correlation was found between TSH and FT_4 ($r=0.30$ $p<0.01$) . TSH in the

Tanzanian subjects was also correlated with total T_3 (r=0.28, p<0.01). Analysis of Tanzanian data by sex revealed a significant correlation between TSH and FT_4 in both the male (r=-0.31 p<0.01) and the female (r=-0.29 p<0.01) subgroup.

Table 2.1. Mean (SD) of serum T_4, T_3, FT_4, TSH and T_4:T_3 ratio in Tanzanian and Dutch subjects. The range was calculated as mean ± 2SD. The reference ranges according to the kit's manufacterer are also indicated.

		T4 nmol/L	T3 nmol/L	FT4 pmol/L	TSH mIU/L	T4:T3 ratio
Tanzania	All	100.6 (15.4)	2.03 (0.35)	11.47 (2.01)	1.23	50.4 (8.2)
	Males	100.4 (16.5)^{ns}	2.10 (0.36)^{a}	11.86 (2.09)^{ns}	1.23^{ns}	48.2 (6.8)^{b}
	Females	101.2 (12.6)	1.84 (0.24)	11.46 (1.82)	1.22	55.6 (9.1)
	Range	69.8-131.4	1.32-2.74	7.45-15.50	0.47-3.17	34.0-66.8
Holland	All	91.3(14.9)	1.73 (0.30)	11.37 (1.89)	1.45	53.4(8.1)
	Males	92.7(14.9)^{ns}	1.79 (0.31)^{ns}	11.63 (2.01)^{ns}	1.39^{ns}	52.6(8.5)^{ns}
	Females	89.7(15.1)	1.67(0.30)	11.06 (1.74)	1.52	54.4(7.7)
	Range	61.5-121.1	1.13-2.33	7.59-15.15	0.50-4.24	37.2-69.6
	Range (kit)	62-165	0.8-2.7	7.7-23.2	0.3-3.5	-
p-values	all	0.001	0.001	ns	ns	0.05
Tanzania	males	0.01	0.001	ns	ns	0.001
vs Holland	females	0.01	0.01	ns	ns	ns

$^{a}p < 0.001$ Male versus female value within the population (unpaired Student t test)

$^{b}p < 0.01$ Male versus female value within the population (unpaired Student t test)

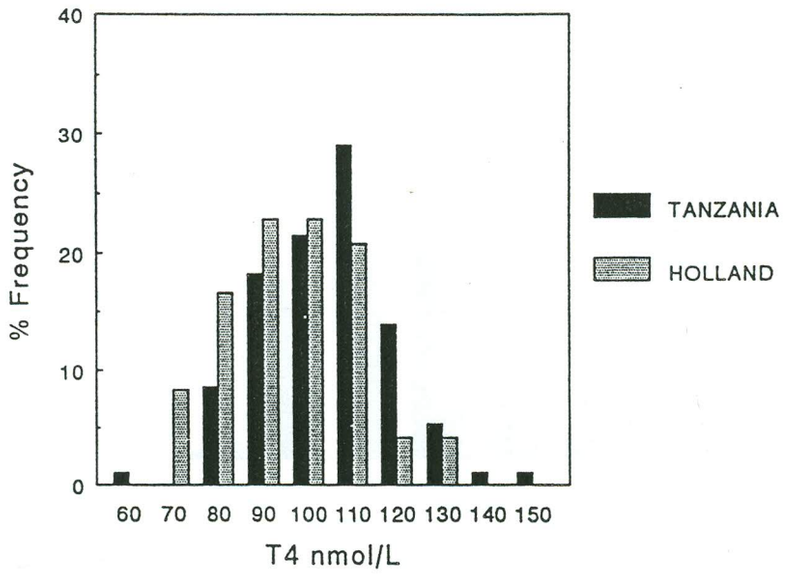


Figure 2.1. Comparative frequency distribution of serum T_4 in adult Tanzanian and Dutch populations.

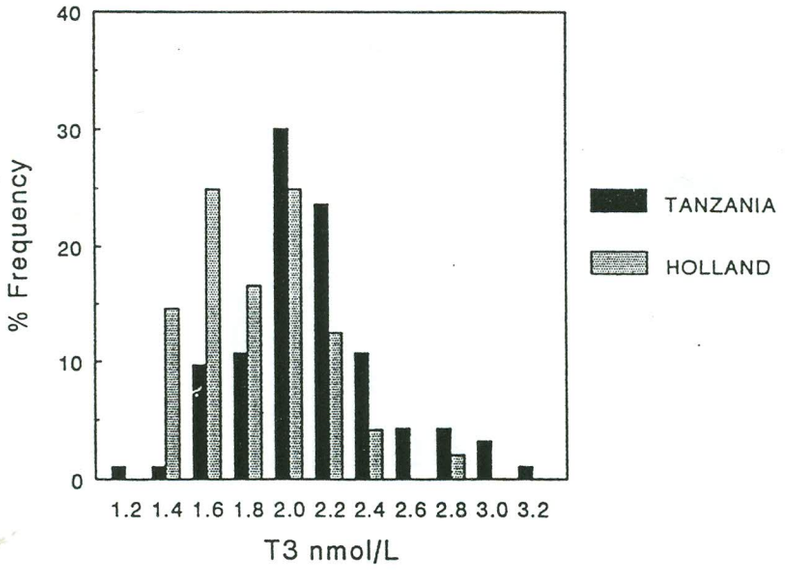


Figure 2.2. Comparative frequency distribution of serum T_3 in Tanzanian and Dutch populations.

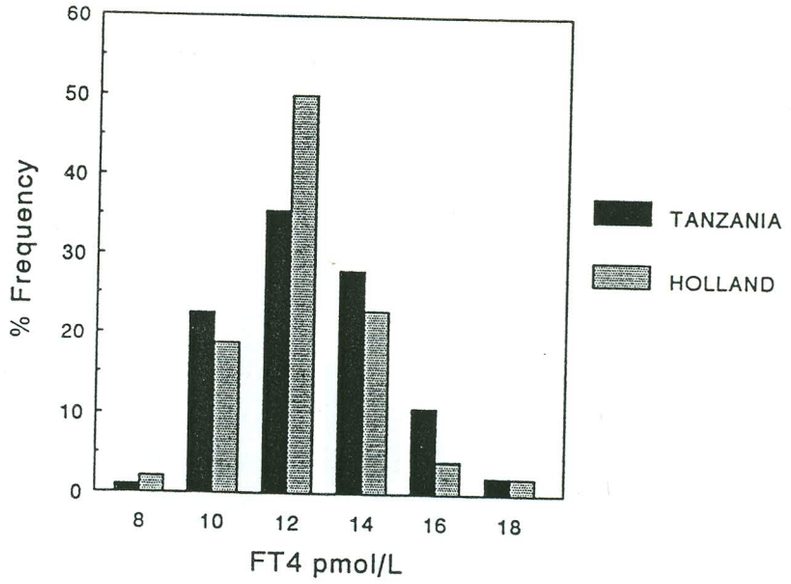


Figure 2.3 Comparative frequency distribution of FT_4 in Tanzanian and Dutch populations.

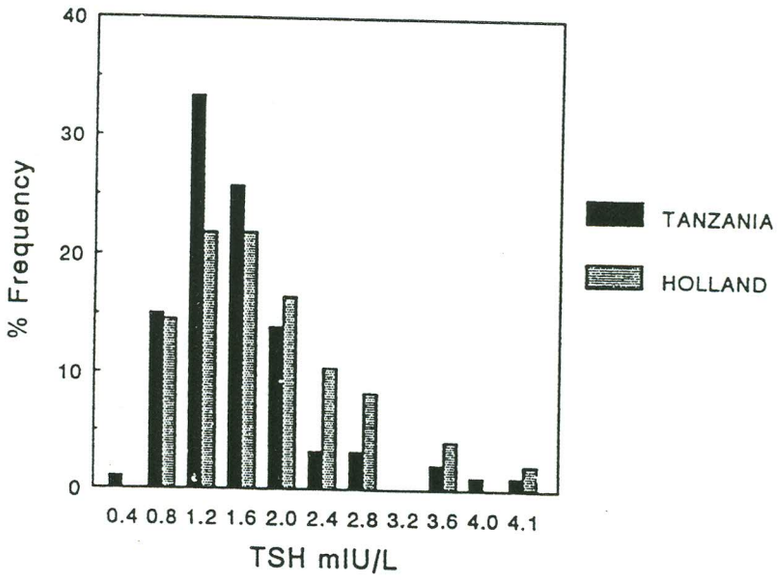


Figure 2.4 Comparative frequency distribution of TSH in Tanzanian and Dutch populations.

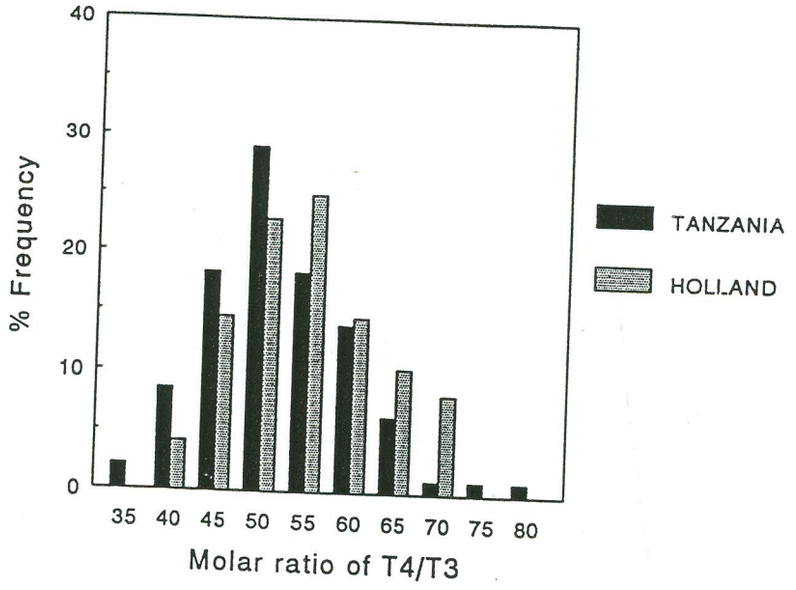


Figure 2.5 Comparative frequency distribution of the T_4:T_3 molar ratio in Tanzanian and Dutch populations.

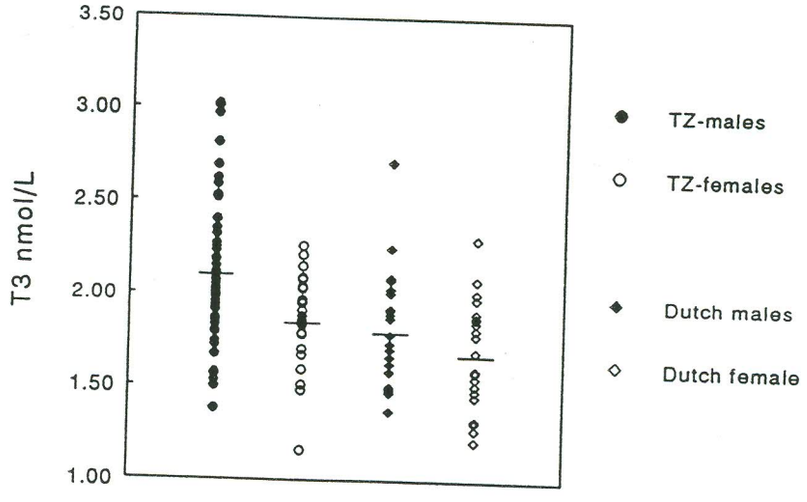


Figure 2.6. Scatter of T_3 values in Tanzanian and Dutch subjects by sex.

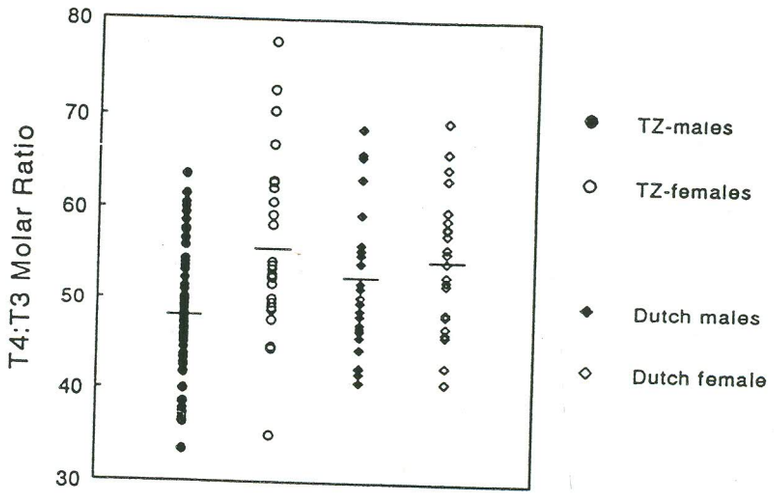


Figure 2.7. Scatter of T_4:T_3 molar ratios in Tanzanian and Dutch subjects by sex.

2.4. Discussion.

In the present study significantly higher serum T_4 and T_3 levels were found in Tanzanian than in Dutch subjects. The difference was more marked in T_3 levels than in T_4 levels. This resulted in a significantly lower mean molar ratio of T_4:T_3 in the Tanzanian when compared to the Dutch population. Higher T_3 levels and lower molar ratios of T_4:T_3 are characteristics of populations which live in iodine deficient areas. Iodine deficiency in the Tanzanian subjects is unexpected because the sampling of the Tanzanian subjects was done in Dar-es-Salaam, an area considered to be iodine replete. Nutritional differences between the two studied populations other than iodine intake, may be responsible for higher T_3 levels in Tanzanian than in the Dutch subjects. Unlike in the Netherlands, carbohydrate is the principal caloric source in Tanzania. A carbohydrate-rich diet has been shown to increase T_3 production rate and serum T_3 concentrations (Danforth E *et al.* 1979).

Sex differences exist in concentrations of TBG (lower in men) and TBPA (higher in men) (Braverman LE *et al.* 1967; Bigazzi M *et al.* 1980; Franklyn JA *et al.* 1985). The higher level of TBG is attributed to the effect of oestrogens on its synthesis (Dowling JT *et al.* 1965). In the present study Tanzanian females had significantly lower T_3 than their male counterparts which is in agreement with other studies (Wilke JT 1983; Franklyn JA *et al.* 1985) which reported lower total T_3 (in addition to low T_4:TBG ratio and free T_4) in healthy females than in males. However in the Dutch group no sex differences were detected. The influence of sex on the levels of thyroid hormones is difficult to explain because the higher TBG levels in females are predictably expected to be associated with higher total T_4 and T_3 in females than in males in contrast to

what has been found from the present and other cited studies. It is known that caloric deprivation is associated with decreased T_3 levels (Portnay GI *et al.* 1974; Chopra IJ *et al.* 1975).

From the present study no influence of age (in both populations) on the levels of serum thyroid hormones and TSH was found in agreement with other reports (Oddie TH and Fisher DA 1967; Olsen T *et al.* 1978; Franklyn JA *et al.* 1985). Some authors (Synder PJ and Utiger RD 1972; Hermann J *et al.* 1974; Wilke JT 1983) observed a fall in the levels of total T_4, total T_3 and free T_4 with age. These findings are thought to have been due to the high prevalence of diseases in the old aged subjects in the studies.

Studies on serum TSH and FT_4 concentrations show that individuals have a genetically determined setpoint for the TSH-FT_4 relationship and therefore in euthyroid populations no correlation between TSH and FT_4 would be expected. From the present study the significant inverse relationship between TSH and FT_4 and between TSH and T_3 in Tanzanians suggests that probably the sampling included subjects with subclinical hypothyroidism characterized by relatively elevated levels of TSH and decreased FT_4 relative to their euthyroid set points.

Regarding the clinical usefulness of kit insert, no agreement was found between the kit's suggested reference ranges of T_4, T_3, FT_4 and TSH and the ranges found in the present study (in both populations). Indeed this demonstrates that it is necessary to study any "new" method locally. Reference ranges for the normal population should be established using the population the method intends to serve.

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Chapter Three

Thyroid Function in Pregnant Tanzanian Women.

3.1. Introduction.

The pregnant woman's thyroid function is subjected to two challenging factors, *i.e.* increased maternal thyroid secretory activity, and decreased availability of iodine to the maternal thyroid gland. The maternal thyroid shows increased secretory activity in response to a) increased levels of TBG and b) thyrotropic activity of human chorionic gonadotropin. The concentration of TBG increases during pregnancy as a result of placental estrogen thereby greatly increasing the serum thyroid hormone binding capacity (Burr WA *et al* 1977) and consequently total serum thyroid hormones. The free hormone fraction decreases permanently while the free T_4 concentration decreases transiently due to these increased levels of TBG. The hypothalamic-pituitary-thyroid axis feedback mechanism stimulates TSH release in order to increase hormonal output and re-establish the free hormone levels. In addition, T_4 disposal will temporarily decrease as a consequence of lower FT_4.

Large quantities of human chorionic gonadotropin (hCG) are produced by the placenta. hCG has thyrotropic activity (Carayon P *et al* 1980; Ballabio M *et al* 1987; Hershman JM *et al* 1988; Glinoer D *et al* 1990) and stimulates the maternal thyroid. This stimulation takes place early in pregnancy and presents an extra load on the thyroid gland which needs rapid adjustment. An adequate adjustment requires the integrity of the thyroid functional capacity. If this functional capacity is in any way compromised the pregnant woman's thyroid will not be able to cope

with the challenge. For example hypothyroid pregnant patients on T_4 replacement therapy were shown to require in an average a 40-50% increase in their daily dosages in order to maintain euthyroidism (Kaplan MM 1992). Female patients with anti-thyroid antibodies (who have reduced functional capacity of their thyroid parenchyma because of the autoimmune disorder) were shown to carry a risk of developing subclinical hypothyroidism during gestation (Glinoer D *et al* 1994).

In pregnancy, there is an increased iodine loss through the increased renal clearance of iodide (Aboul-Khair SA and Crooks J 1965). This loss is aggravated during the second half of gestation because a fraction of the maternal inorganic iodide pool is diverted towards the feto-placental unit. The fetal thyroid activity (whose functional maturation acquires the capacity to concentrate iodine and synthesise iodothyronines by 10-12 weeks of gestation) depends entirely upon the availability of iodine transferred from the maternal circulation.

The importance of maternal euthyroidism for normal fetal brain and nervous system development has been indicated in chapter one. Moreover maternal hyperthyroxinaemia has been associated with schizophrenia in the offsprings (Mac Sweeney D and Timms P 1978).

The consequences of iodine deficiency to fetal development were initially thought to be a direct effect of elemental iodine deficiency (Hetzel BS and Querido A 1980) to the fetus; thyroid hormones were assumed not to cross the placental barrier. However, transplacental transfer of thyroid hormones has been demonstrated (Vulsma T *et al* 1989). Maternal hypothyroidism has been shown to result into deficiency in T_4 and T_3 in embryonic tissues (Woods RJ *et al* 1984; Morreale de Escobar G *et al* 1985; Escobar del Rey F *et al* 1986; Morreale de

Escobar G *et al* 1993). Even in areas where dietary supply of iodine is suboptimal but not overtly deficient, pregnancy (in otherwise healthy subjects) results in relative iodine deficiency state and hypothyroxinaemia characterised by TBG desaturation, free T_4 near the lower limit or below normal, preferential T_3 secretion, high TSH levels and high serum thyroglobulin (Tg) levels (Glinoer D *et al* 1990). The newborns of those mothers present with evidence of extreme stress to their thyroid glands characterised by very high cord serum TSH and Tg levels (Glinoer D *et al* 1992). All these effects are ameliorated by iodine supplementation during pregnancy (Pedersen KM *et al* 1993; Glinoer D *et al* 1995).

The prevalence of maternal goitre and indeed the increase in thyroid volume (delineated by modern ultrasonographic techniques) increases with the degree of environmental iodine deficiency (Crooks J *et al* 1967; Levy RP *et al* 1980; Bauch K *et al* 1986, Brander A and Kivisaari L 1989; Smyth PPA and Hetherton AM 1991).

The study of thyroid function in relation to reproduction therefore forms an important component in the efforts to combat the effects of iodine deficiency on reproduction in affected communities. Tanzania is among those countries affected by iodine deficiency disorders. Although no precise figures exist regarding the effects of iodine deficiency on reproduction in the country, based on results of goitre surveys (Kavishe and Maletnlema 1987) it is estimated that due to IDD there are about 160,000 cretins, 450,000 cretinoids and that 30% of perinatal mortality in the country would be attributable to iodine deficiency (Van der Haal *et al* 1988). The present study aims at assessing thyroid function in normal pregnant

Tanzanians residing in an iodine sufficient area and forms a basis for evaluating thyroid function in pregnant women from iodine deficient areas in the process of assessing the effects of IDD on human reproduction in the country.

3.2. Subjects, materials and methods.

The study group comprised 93 clinically euthyroid pregnant women attending the routine antenatal clinic at Muhimbili Medical Centre, Dar-es-Salaam, Tanzania, during the months of August and September 1992. They aged 15 to 49 years (mean 26.1), had parity of up to 7 (mean 1.8) and gravidity of 1 to 9 (mean 3). Gestational age was calculated from the last normal menstrual period. A sample of 30 clinically euthyroid females volunteers, aged 19 to 54 years (mean 33) formed the control (nonpregnant) group. They were all apparently healthy with no signs or symptoms of any other nonthyroidal illness, none used oral contraceptive pills or any long term drug therapy known to affect thyroid function. Analytical methods used to assay T_4, T_3, FT_4 and TSH (and the performance characteristics of these assays) were as explained in the materials and methods section (2.2) of chapter two.

3.3. Results.

The mean (SD) of serum levels of T_4, T_3, fT_4%, FT_4, and TSH in pregnant and control subjects are shown in table 3.1 and their distribution in figures 3.1 through 3.4. Serum T_4 and T_3 in pregnant subjects are significantly higher than in nonpregnant controls. Eightyfour percent of pregnant subjects had serum T_4 values above the upper reference limit for the nonpregnant range. For T_3 this was

observed in 87% of pregnant subjects. Both the free fraction of T_4 (fT_4%) and concentration of free T_4 (FT_4) were significantly lower in pregnant subjects when compared to nonpregnant controls. Serum TSH was significantly higher in pregnant subjects than in controls.

The small number of subjects in the first trimester (n = 5), does not allow for sound statistical analysis *i.e.* comparison to pregnant subjects in other trimesters or to nonpregnant controls. Nevertheless, a number of observations are worth noting. Pregnant subjects in each trimesters of pregnancy had higher mean T_4 and T_3 values, and lower mean fT_4% and FT_4 levels when compared to controls. Subjects in the first trimester appeared to have lower TSH but higher FT_4 levels when compared to controls. Subjects in the second and third trimester had significantly higher TSH but lower FT_4 levels when compared to controls.

Table 3.1 Mean (SD) of Serum T_4, T_3, fT_4%, FT_4 and TSH in pregnant and nonpregnant Tanzanian subjects.

Parameter	Control n = 30	Pregnant All n = 93	Trimester 1 n = 5	Trimester 2 n = 25	Trimester 3 n = 63
T_4 nmol/L	101.4 (12.4)	163.3[a] (39.2)	177.0	152.0(26.6)[b]	166(41.3)[b]
T_3 nmol/L	1.84 (0.24)	3.10[a] (0.72)	2.88	3.2(0.61)[b]	3.0(0.75)[b]
fT_4%	0.0117 (0.0017)	0.0064[a](0.0014)	0.0075	0.0064(0.0009)[b]	0.0063(0.0012)[b]
FT_4 pmol/L	11.46 (1.82)	10.42[a] (2.19)	11.12	9.72(2.23)[b]	10.46(2.15)[b]
TSH mIU/L	1.22	1.56[a]	0.28	1.74[b]	1.67[b]

Pregnancy values (generally and in the second and third trimesters) are compared to nonpregnant control values. Trimester 1 values were not tested.

[a]$p < 0.05$; Pregnant value significantly different from nonpregnant (control) value.

[b]$p < 0.05$; Trimester value significantly different from nonpregnant (control) value.

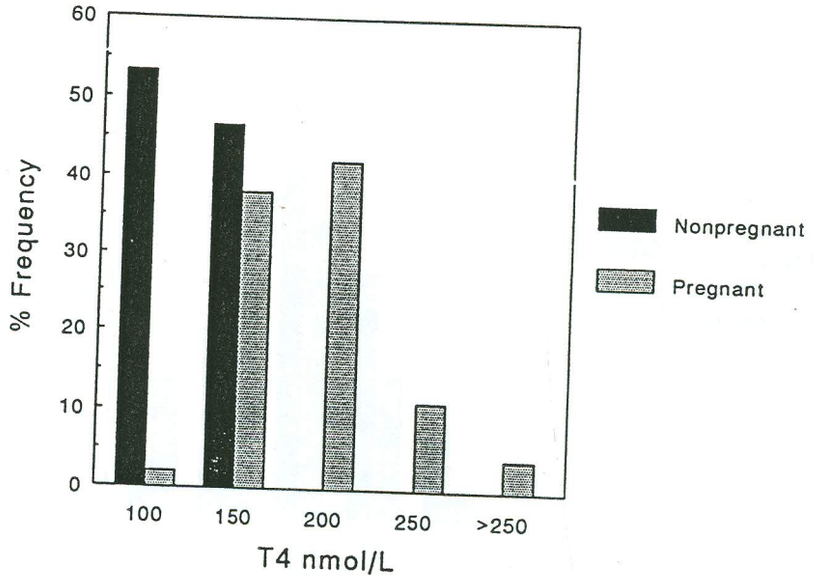


Figure 3.1. Percentage frequency distribution of serum T_4 values in pregnant and nonpregnant subjects.

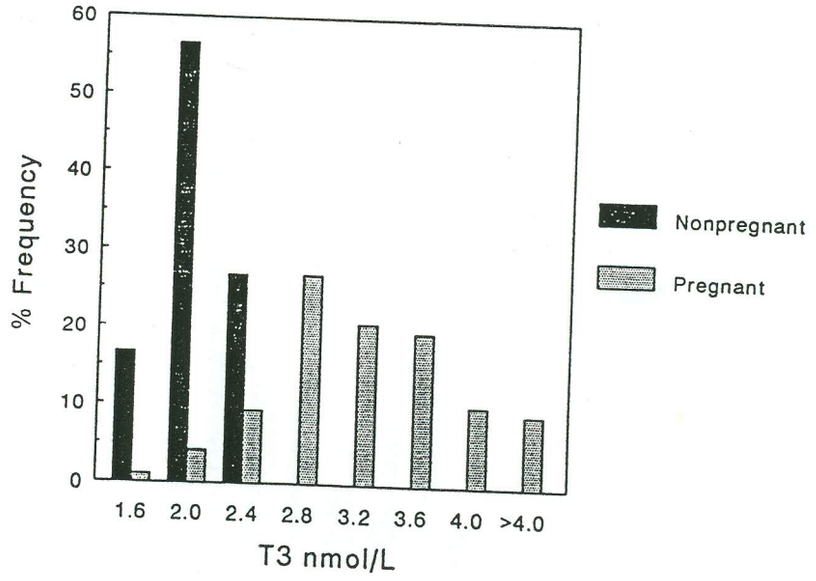


Figure 3.2. Percentage frequency distribution of serum T_3 in pregnant and nonpregnant subjects.

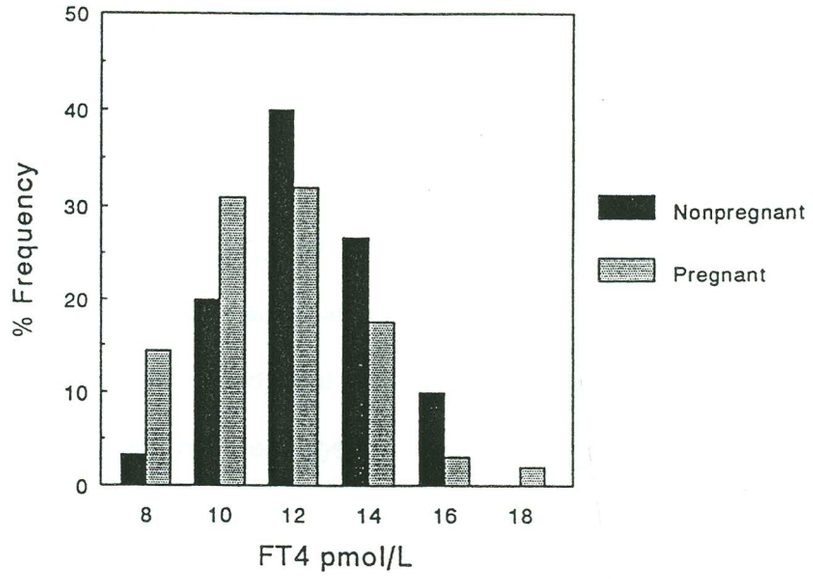


Figure 3.3. Percentage frequency distribution of serum FT_4 values in pregnant and nonpregnant subjects.

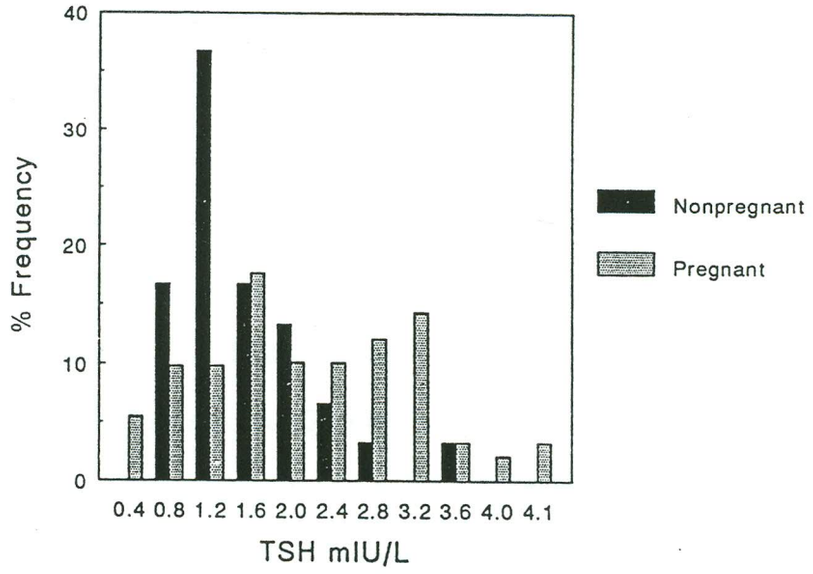


Figure 3.4. Percentage frequency distributuion of serum TSH values in pregnant and nonpregnant subjects.

3.4. Discussion

In this chapter the biochemical parameters of thyroid function in healthy pregnant, clinically euthyroid, Tanzanian women residing in an iodine replete environment have been elucidated. The best way to define the changes in thyroid function as a result of pregnancy is through a longitudinal prospective study of pregnant subjects with their preconception values as controls. This kind of study design is difficult to achieve. The present study is a cross-sectional case control one. However, interesting findings have emerged.

High serum total T_4 and T_3 in pregnant subjets is a finding that has been known for many years and is attributable to raised levels of TBG (Glinoer *D et al* 1990). High TBG levels are the result of production (under the influence of placental estrogens) of a TBG variant which is highly sialyated and therefore cleared less rapidly than normal from the circulation (Ain KB *et al* 1987). Mirror opposite concepts concerning the effects of marked TBG rise in pregnancy are known i.e. from the early concept that TBG prevent maternal T_4 form traversing the placenta to the fetal circulation (Oscorio C and Myant NB 1960), to the current concept that TBG rise during pregnancy may serve to redistribute thyroid hormones and specifically that it may enhance transplacental transfer of maternal T_4 to the developing fetus (Ekins RP 1985; Sinha AK *et al* 1992).

From a number of longitudinal studies (Glinoer D *et al* 1990; Ballabio M *et al* 1991) of thyroid function it was shown that the rise in total T_4 concentration is relatively smaller than that of TBG concentrations. The rise in TBG relative to the rise in T_4 is such, that when the concentration and affinity of the other binding proteins remains the same, lower FT_4 results. In the present study 84% of

subjects had serum total T_4 values above the reference limit comparable to 90% reported by Whitworth *et al* (1982). Glinoer *et al* (1990) found 50% of their subjects in the third trimester with T_4 levels above the nonpregnant reference range, the lower percentage was attributed to mild iodine deficiency in the area of the study.

The levels of serum concentations of TSH and free T_4 (FT_4) in pregnancy have been variably reported. Many recent studies indicate a rise in the serum TSH concentration during pregnancy. Pacchiarroti *et al* (1986) reported a small but significant increase in serum TSH in the second and third trimester. Serum TSH concentrations was also reported to be higher in late pregnancy by Harada A *et al* (1979), Week J *et al* (1982) Guillaume J et al (1985) Price A *et al* (1989) and Ballabio M *et al* (1991). Lower (Ballabio M *et al* 1991) and higher TSH values have been reported in the first trimester of pregnancy.

In the present study pregnant subjects in the first trimester appeared to have greatly decreased TSH levels when compared to controls. On the other hand subjects in the second and third trimesters had significantly raised TSH levels when compared to controls.

The fall in TSH early in pregnancy was shown to coincide with a peak rise in serum hCG and the two were found to be negatively correlated with each other (Pekonen F *et al* 1988; Ballabio M *et al* 1991). The high levels of circulating hCG observed in early pregnancy were also found to be positively correlated with FT_3 and FT_4 (Pekonen F *et al* 1988). Many studies have investigated the possible thyrostimulatory activity of hCG (Carayon P *et al* 1980; Ballabio M *et al* 1987; Hershman JM *et al* 1988; Pekonen F *et al* 1988; Ballabio M *et al* 1991). By

demonstrating the lack of correlation between hCG and either total T_4 (which was raised) or free T_4 (which was within the normal range) accompanied by significantly decreased TSH in the first trimester Ballabio M *et al* (1991) concluded that the thyroid may not be exclusively TSH driven in early pregnancy. This further supports the thyrostimulatory activity of hCG.

In the present study correlations between TSH, FT_4 and hCG were not investigated of the small number of subjects in the first trimester and the absence of hCG assay. In accordance with the pituitary-thyroid negative feedback mechanism, depressed TSH levels in the first trimester indicate maternal pituitary hyperthyroxinaemia. Summarising, it may be hypothesised, that the additional requirement of thyroid stimulation throughout pregnancy is provided by a combination of hCG and TSH in the first trimester and later by elevated TSH.

In the present study, lower FT_4 levels were found in the second and third trimester pregnant subjects when compared to controls. Studies elsewhere, both in iodine sufficient and marginally deficient environments demonstrated a decline in FT_4 and FT_3 (Kurtz A *et al* 1979; Glinoer D *et al* 1990; Ballabio M *et al* 1991) in the late period of gestation but another study reported increased FT_4 levels in second and third trimester (Yamamoto T *et al* 1979).

The combination of low FT_4, TBG desaturation and raised TSH (features of late pregnancy) suggest a primary decline in thyroid secretion during the later period of pregnancy. The fall of serum FT_4 in pregnancy in otherwise euthyroid subjects has been a challenge to the validity of free hormone hypothesis. Conventional concepts of hormone transport (based on the free hormone hypothesis) imply a reduced supply of T_4 to maternal tissues in these circumstances. However the

analysis of thyroid hormone transport kinetics (Ekins RP 1990) indicates that free hormone concentrations as measured under equilibrium conditions *in vitro* may not accurately reflect the efflux rate from target tissue capillaries *in vivo.* This is because hormone delivery may be partially controlled by the protein-bound fraction, so that adequate T_4 supply may be ensured by the increased TBG. Transplacental transfer of thyroid hormones has been constantly contested by (Fisher DA 1981). Transplacental transfer of maternal T_4 has been demonstrated in rats (Woods RJ *et al* 1985, Morreale de Escobar G *et al* 1985, Hubank M *et al* 1986, Pickard MR *et al* 1987). Later, similar transfer was demonstrated in late gestation in humans especially when the fetus has severe congenital hypothyoidism (Vulsma T *et al* 1989). These observations point to a crucial T_4 requiremnt for the foetus' neurological system during the phase that its own thyroid still does not function. The importance of late transfer in euthyroid neonates is not known but animal studies suggest that late gestation maternal thyroid hypofunction (as might be concluded from the findings of decreased FT_4 and raised TSH levels) is not detrimental to the fetus because the latter's thyroid is by then functional (Bonet B and Herrera E 1988).

From the present study it is concluded that the alterations in serum thyroid hormones and thyrotropin in pregnant Tanzanian subjects residing in iodine sufficient environment are similar to those reported form other areas not affected by iodine deficiency. The present findings form a basis upon which thyroid function in pregnant women from iodine deficient areas in Tanzania can be evaluated.

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Chapter Four.

Evaluation of Serozyme Enzyme Immunoassays for T_4, T_3, TSH and Free Thyroxine Index.

4.1 Introduction.

Endocrine diagnostic services in the Muhimbili Medical Centre have been provided sporadically by the department of biochemistry, at the Faculty of Medicine. This fact prompted us to investigate analytical systems which would enable the introduction of such services in a more reliable and sustainable way.

The Serozyme system (Serono Diagnostic SA, Coinsins, Switzerland) was chosen for this purpose because of the simple instrumentation involved, which requires minimal maintenance. Separation of bound and free fractions in the Serozyme immunoassays is achieved through magnetizable particles, so that a large laboratory centrifuge is not needed. The Serozyme assays are based on the use of a non-isotopic enzyme label, thus circumventing problems associated with the use of radioisotopes (disposal of radioactive waste, monitoring of contamination) and ensuring a relatively long shelf-life of the kits. Finally, a broad spectrum of Serozyme assays is available, including assays in the field of fertility (LH, FSH, prolactin, progesterone, oestradiol) thyroidology (T_4, T_3, TSH, FT_4I) tumor markers (hCG, AFP, CEA) and anemia (ferritin). Following the decision to adopt Serozyme assays, the objective of this chapter was to evaluate their performance characteristics and to document their merits and shortcomings. This evaluation was confined to thyroid

hormones and thyrotropin. The Serozyme assays were compared with methods routinely used in the laboratory for Endocrinology and Reproduction, Academic Hospital Nijmegen, St Radboud, Nijmegen, The Netherlands for the determination of T_4, T_3 (Amersham) TSH (Delfia) and free T_4 (Spac-FT_4).

4.2 Methods

4.2.1 Amersham and Serozyme T_4 and T_3 assays.

Table 4.1 summarises the major features and protocols of these assays.

The Amersham T_4 and T_3 assays are ***labelled analyte (competitive)*** radioimmunoassays. These assay methods are based on the competition between thyroxine or triiodothyronine in the serum and ^{125}I-T_4 or ^{125}I-T_3 for a limited number of binding sites on T_4 or T_3 specific antibodies. The Amerlex-M antibody suspension contains antibody that is bound to magnetisable polymer particles. Separation of the antibody bound fraction of the label is achieved by magnetic precipitation of the Amerlex M suspension followed by decantation of the supernatant. By measuring the proportion of the radioactivity that is bound in the presence of reference standards, the concentration of T_4 or T_3 present in the unknown samples can be read off a standard curve. The standard curve is generated by inclusion in each assay run six standards at different analyte levels. Interference by binding proteins in serum is minimised by a combination of an appropriate buffer and a TBG blocking agent (8-Anilino-1-naphthalene sulfonic acid; ANS). This agent prevents binding of T_4 to TBG.

Serozyme T_4 and T_3 assays are ***labelled antibody (competitive)*** enzyme immunoassays (figure 4.1). T_4 or T_3 in a sample, control, or standard competes with a fixed amount of fluorescein-labelled T_4 or fluorescein-labelled T_3 for binding to a limited amount of T_4 or T_3

immunoassays (figure 4.1). T_4 or T_3 in a sample, control, or standard competes with a fixed amount of fluorescein-labelled T_4 or fluorescein-labelled T_3 for binding to a limited amount of T_4 or T_3 monoclonal antibody conjugated to an enzyme (alkaline phosphatase) (fig.4.1:B). 8-Anilino-1-naphthalene sulfonic acid (ANS) is used to displace T_4 or T_3 from binding plasma proteins. The amount of fluorescein-labelled T_4 or fluorescein-labelled T_3 (i.e. T_4 or T_3 derivative respectively) bound by the antibody is ***inversely proportional*** to the endogenous concentration of T_4 or T_3 in the sample. At the end of 15 minutes incubation at 37°C antifluorescein coupled to a magnetic solid phase is added in excess (fig.4.1:C). This rapidly and specifically binds to the fluorescein-T_4- or fluorescein-T_3- antibody complex and is sedimented in a magnetic field obviating the need for centrifugation (fig 4.1:D). After decanting and washing the sediment the tubes are incubated with an enzyme substrate solution [phenolphthalein monophosphate (PMP)]. (fig.4.1E). After 15 minutes of incubation at 37°C the enzyme reaction is ended by addition of the stop solution and the intensity of the colour developed is measured photometrically (fig.4.1:F). The intensity of the colour formed by the enzyme reaction is inversely proportional (within the working range of the assay) to the concentration of T_4 or T_3 in the sample. The concentration of T_4 or T_3 in a test sample or control can then be read off the standard curve.

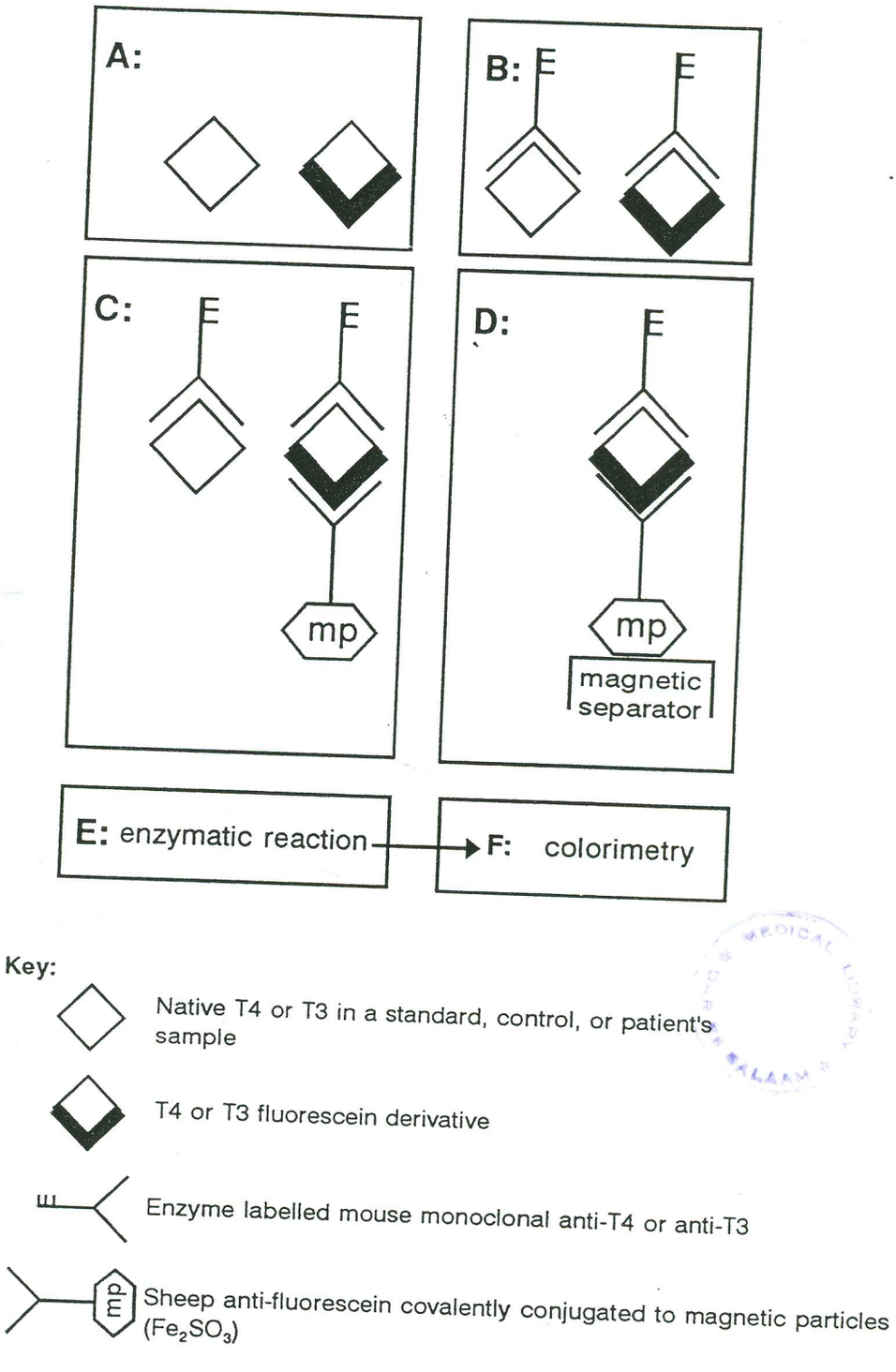


Figure 4.1. Diagrammatic representation of the Serozyme T_4 and T_3 enzyme immunoassay.

Table 4.1. Major features and protocols of Serozyme and Amersham T_4 assays.

PARAMETER	SEROZYME	AMERSHAM
Type	Competitive labelled antibody enzyme immunoassay	Competitive labelled analyte radioimmunoassay
Label	Alkaline phosphatase	^{125}I
Antibody	Mouse monoclonal	Not indicated.
Separation	Magnetic particles	Magnetic particles
Standards	6/liquid	6/liquid
Sample volume	50 μl	25 μl
Protocol	Add sample/T_4-fluorescein derivative/anti-T_4. anti-T_4	Add sample/^{125}I-T_4/
	Incubate at 37 ^{o}C;	Incubate at room
	15 minutes.	temperature;45minutes
	Add antifluorescein	Separate for 15
	Incubate at 37 ^{o}C;10 minutes	Decant and drain
	Separate/decant.	Count.
	Wash/decant.	Calculate results.
	Add substrate/incubate	
	at 37 ^{o}C for 15 minutes	
	Add stop solution/separate/Readresults.	

Table 4.2. Major features and protocols of Serozyme and Amersham T_3 assays.

PARAMETER	SEROZYME.	AMERSHAM.
Type	Competitive labelled antibody enzyme-immunoassay	Competitive labelled analyte radioimmunoassay
Label	Alkaline phosphatase	^{125}I
Antibody	Mouse monoclonal	Not indicated
Separation	Magnetic particles	Magnetic particles
Standards	6/liquid	6/liquid
Sample volume	100 μl	50 μl
Assay Protocol	Add sample/T_3-fluorescein derivative/anti-T_3 Incubate at 37°C;15 minutes Add anti-fluorescein Incubate at 37°C; 10 minutes. Separate; 2 minutes. Wash and decant. Add substrate and incubate at 37°C for 15 minutes. Add stop solution, separate and read the results.	Add sample/^{125}I-T_3/ anti-T_3. Incubate at room temperature; 45 minutes Separate for 15 minutes. Decant, drain and count Calculate the results.

4.2.2 Delfia and Serozyme TSH assays.

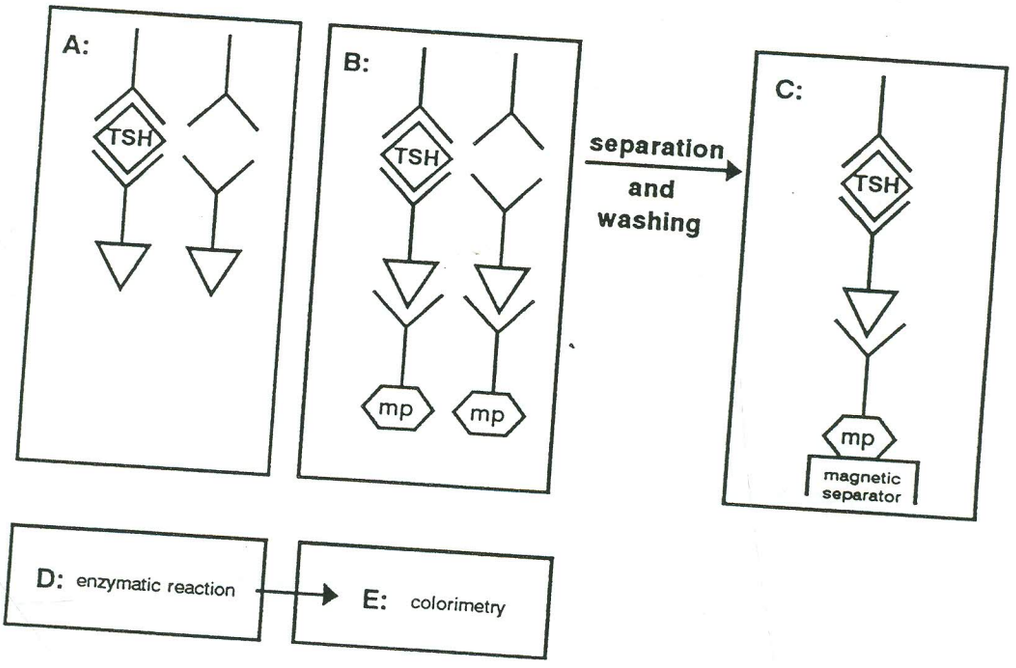
The Delfia® (dissociation-enhanced lanthanide fluoroimmunoassay) TSH assay is a solid phase two site immunofluorometric assay (IFMA) based on the sandwich technique in which two mouse monoclonal antibodies are directed against two different antigenic determinants on the TSH molecule.

The detecting antibody in this system is labelled with an europium chelate. After the immunoreaction the europium is released and coupled to other chelating reagents in a protective micell. Thus fluorescence quenching by water is prevented, resulting in intense fluorescence. These fluorescent compounds are characterized by a large Stokes' shift and a long decay time (500 μs). A fast light pulse is used to excite the europium chelate. The advantage of this approach is that non-specific fluorescence, with a decay time of less than 0.01 μs is eliminated by using a gated detection system. The fluorescence is measured typically 400 - 800 μs after light excitation with a cycle time of 1 ms.

Standards, controls, and patient samples containing TSH are reacted simultaneously with immobilised (on walls of microtitre plates) monoclonal antibodies against a specific antigenic site on the beta subunit as well as Europium-labelled monoclonal antibodies directed against a different specific antigenic site located partly on the beta subunit and partly on the alfa subunit. After a thorough wash to remove the unbound Europium labelled antibody the "enhancement" solution is added, to dissociate Europium ions from the labelled antibody into solution where the ions form highly fluorescent chelates with components of the enhancement solution. The fluorescence is then measured in a time resolved fluorimeter (Arcus 1230, LKB-Wallac, Turku, Finland) with a counting time of one second per sample.

The Serozyme TSH assay is also a "sandwich" type assay. It incorporates two high affinity monoclonal antibodies directed against two different antigenic sites on the TSH molecule (figure 4.2). Standards, controls and patient samples are reacted in test tubes with a mixture of monoclonal antibodies to TSH. One antibody to which alkaline phosphatase is conjugated

attaches to a discrete site on the TSH molecule. A second monoclonal antibody linked to fluorescein binds at a second site on the TSH molecule forming a sandwich (fig. 4.2:A). At the end of 30 minutes incubation at 37°C, antifluorescein coupled to a magnetic solid phase is added in excess (fig. 4.2:B). This rapidly and specifically binds to the TSH-fluorescein labelled monoclonal complex and is sedimented in a magnetic field (fig. 4.2:C). After decanting and washing the sedimented complex, each tube is incubated with an enzyme substrate solution (fig. 4.2:D). After 60 minutes of incubation at 37°C the enzyme substrate reaction (similar to T_4 and T_3 assays) is stopped by addition of the stop solution and the intensity of the colour developed is measured photometrically (fig. 4.2:E). The intensity of the colour formed by the enzyme reaction increases (within the working range of the assay) with the concentration of TSH in the sample. The concentration of TSH in the patient sample or control can then be determined directly by reading from the standard curve.



Key:



TSH in a standard, control, or patient's sample



Enzyme labelled mouse monoclonal anti-TSH



Fluorescein labelled mouse monoclonal anti-TSH



Sheep anti-fluorescein covalently conjugated to magnetic particles (Fe_2SO_3)

Figure 4.2 Diagrammatic presentation of the Serozyme TSH assay

Table 4.3. Major features and protocols of Serozyme and Delfia TSH assays.

PARAMETER	SEROZYME.	DELFIA.
Type	Immunoenzymometric assay	Immunofluorometric assay
Label	Alkaline phosphatase	Europium
Anti-TSH	i. Mouse monoclonal labelled with fluorescein. ii. Mouse monoclonal labelled with an enzyme. *(Subunit specificity not indicated).*	i. Mouse monoclonal labelled with europium and directed partly against α- and partly against β-subunit. ii. Mouse monoclonal immobilised on microtitre plates. Directed against β-subunit.
Standardisation (number and form)	WHO 2nd IRP (80/558) 6/liquid.	WHO 2nd IRP (80/558). 6/liquid.
Sample volume	200 μl.	25 μl
Assay Protocol	Add sample, Add anti-TSH (both antibodies) Incubate at 37°C for 30 minutes Add anti-fluorescein. Incubate at 37°C for 5 minutes. Separate, decant. Wash twice Add substrate/incubate at 37°C;60 minutes. Add stop solution. Read the results.	Wash the plates. Add sample. Add Eu-labelled anti-TSH. Shake the plate for 2 min. Incubate at 4°C for 12 hrs. Shake at room temperature for 60 minutes Aspirate the plate. Wash the plate. Add the enhancement solution. Shake at room temperature for 5 minutes. Read fluorescence/calculate results.

4.2.3 Serozyme T_3 Uptake (T_3U) assay and the Free Thyroxine Index (FT_4I).

In the Serozyme T_3 uptake assay, samples, calibrator or controls are reacted in test tubes with fluorescein labelled monoclonal antibody to T_3, enzyme (Alkaline phosphatase E.C 3.1.3.1) labelled T_3 and unlabelled T_3. A proportion of this exogenous unlabelled T_3 will be taken up by the serum proteins in the sample, calibrator or control. Enzyme labelled T_3 does not react with the serum proteins. The residual unlabelled T_3 competes with enzyme labelled T_3 for anti T_3 antibody. The residual unlabelled T_3 will be lower, the higher the concentration of unoccupied binding sites on serum binding proteins, and hence will compete less strongly for the antibody binding sites with the labelled T_3. Thus, a positive relation is expected between the amount of tracer bound and the binding capacity of serum proteins. Unbound T_3 is removed by washing followed by enzymatic incubation to quantitate the amount of enzyme labelled T_3 which is antibody bound. The T_3 uptake in a patient sample is determined by comparison of the color produced to that of the calibrator comprising pooled human sera and assigned a nominal T_3 Uptake value of 100. Free thyroxine index is then calculated as

FT_4I = Total T_4/T_3Uptake *100.

4.2.4 Spac fT_4% assay and the calculation of FT_4I.

This free T_4 assay procedure is a labelled hormone antibody uptake approach as described by Ross HA and Benraad ThJ (1984). In summary, the serum sample is incubated with ^{125}I labelled T_4 in tubes coated with high binding capacity, low affinity T_4 monoclonal antibody. By this feature, the amount of T_4 bound to the antibody is almost directly proportional to the free T_4 concentration at equilibrium, as the number

of free antibody binding sites hardly changes. Therefore, the ratio antibody-bound to non-antibody-bound T_4 tracer is directly proportional to the free T_4 *fraction*. This is because the non-antibody-bound tracer is proportional to total serum T_4. Assessment of the fraction of free T_4 has an important advantage over assaying free T_4 concentration because the former parameter is much less affected by sequestration of a significant proportion of total T_4. For example, sequestering 5% of total T_4 leads roughly to the same percent fall in the free T_4 concentration, resulting in a free fraction that only marginally falls as a consequence of TBG desaturation.

The method is standardised using a reference serum (fT_4 fraction standard) of which the fT_4 fraction was determined by direct equilibrium dialysis. A proportionality factor is obtained and is used to calculate the free fraction of T_4 in the test sample. The calculation also involves a small correction, using the antibody binding capacity, for the fact that in the higher FT_4 range some discernible antibody saturation occurs, which would lead to underestimation of the free T_4 fraction. The test adequately fulfils the requirement of independence of FT_4 estimations on dilution in buffer.

4.2.5 The Serozyme 1 Analyser.

The Serozyme 1 analyser is a discrete wavelength, trichromatic photometer which measures the differential absorbance of any type of solution and converts those readings into concentration values by use of an on board microprocessor using a four parameter logistic procedure to fit the standard curve. The machine is a single module system comprising a 20-key pad which allows the operator an access to a 128 Kb microprocessor, a photometer, a liquid crystal display and a thermal printer. The discrete wavelength photometer allows for analysis of results obtained from

numerous types of procedures which are read at selected wavelength between 340 to 600 nm. 12 mm diameter clear polystyrene test tubes are required for photometric reading. Memory backup for the main program of the Serozyme 1 enables all stored protocols, control data etc to be stored for the life of the instrument. Power requirements are easily met by a selection of appropriate power transformer and line cords according to local power voltage supplies. The machine can be operated in an environmental temperature range of 5 °C to 35 °C and 10 to 85 percent humidity.

4.2.6 Studies on interferences in Serozyme T_3 and TSH assays.

In the course of Serozyme T_3 and TSH assays two observations were made.

a) Non-detectability of T_3 by Serozyme assay.

This was noted in about 20% of Tanzanian specimens. Upon analysis these specimens showed optical densities above those of the zero standard in the Serozyme assay. Upon repeating the assays by both methods (table 1-Appendix I) these results were reproducible (i.e. T_3 detectable by Amersham assay but not by Serozyme assay). This interference in the T_3 Serozyme assay was investigated as follows:

(i) A 1:1 dilution of the affected samples in the kit's zero standard before the assay resulted into variable effects (table 2-Appendix I). The values of T_3 recorded in the Serozyme assay ranged between 15-51% of the expected values (i.e. values obtained by Amersham assay). This procedure did not alter the T_3 values in the Tanzanian samples initially showing T_3 comparable by the two methods.

(ii) Sheep and mouse IgGs were added to the affected samples at the concentrations of 0.5 mg/ml and 5 mg/ml. Neither of these succeeded in removing the interference

in this assay (table 3-Appendix I).

(iii) Addition of 25 μl of native bovine or mouse serum to 200 μl of the sample showed variable effects (table 4-Appendix I).

(iv) Modification of the Serozyme T_3 assay was done by the exclusion of the addition of T_3-fluorescein derivative in the assay protocol. Cross-linking of the sheep antifluorescein and enzyme labelled mouse monoclonal anti-T_3 was demonstrated in the "affected" samples. These samples showed greater optical densities than the Serozyme-T_3 zero standard (table 5-Appendix I).

(v) Rheumatoid factors were assayed in seven of the affected specimens. All the results were within the normal reference ranges (table 8-Appendix I).

(b) False high TSH by Serozyme assay.

The first indication that the TSH Serozyme assay was yielding spuriously high values was an observation that in 28 of 94 samples, Serozyme TSH values were above the upper reference limit of 6.5 mU/L as suggested by the kit insert. This provisional conclusion was based on two reasons. First, these Tanzanian samples had been obtained from clinically euthyroid subjects. Secondly, the same samples had been assayed earlier by Delfia TSH assay and the TSH values of these samples were within the normal reference range. Some TSH values by the Serozyme assay were very high when compared with results by Delfia assay. In trying to correlate the two sets of data a very poor correlation between the two methods was obtained.

TSH Serozyme = 5.3 mU/L + 0.3 * TSH Delfia.

$r = 0.03$ ($p = 0.7$) $n = 94$.

When all values with Serozyme TSH values above 6.5 mU/L were removed from

correlation study, this greatly improved the comparability of the two methods as follows.

TSH IEMA = 1.2 mU/L + 1.02 * TSH IFMA.

r = 0.56 ($p < 0.0001$); n = 66

In pursuit of this interference a number of studies were performed as follows:

(i) Twenty five samples were reassayed by both methods. Initial findings were reproduced in all samples (table 6-Appendix I).

(ii) Seven samples with Serozyme TSH values higher than expected were diluted with the kit's zero standard and reassayed (table 7-Appendix I). This procedure resulted in lowering of TSH values to within the expected values which were then very highly correlated with Delfia TSH values as follows:

Serozyme TSH = 0.2 mU/L + 1.2 * Delfia TSH.

r = 0.97 ($p < 0.0001$); n = 7

(iii) Five sample with spurious TSH values by Serozyme assay were diluted (1:1) with phosphate buffered saline. This did not succeed to reduce the high Serozyme TSH values (recalculated after correction for dilution).

(iv) Bovine serum was added to the "affected" samples at a concentration of 10% This procedure was variably effective in lowering the spuriously raised Serozyme TSH levels.

(v) Mouse serum at a concentration of 10% of the sample was added to "affected" samples. This procedure was not effective in lowering Serozyme TSH value in affected samples.

(vi) Combined bovine and mouse serum each at the concentration of 6% w/v also showed variable effects.

4.2.7 Statistical analysis of the results

The intraassay precision of the assay was evaluated as follows:

The whole ranges of T_4, T_3 and TSH values by each method were divided into concentration intervals with an equal number of observations. For each interval, the arithmetic mean concentration of the hormone was calculated. For each class interval a mean CV was calculated as the root of the mean of squares of the CV's i.e. quadratic averaging of the CV's within an interval as follows:

$$\sqrt{\sum_{n}^{i} \frac{CV^2{}_i + CV^2{}_{ii} + \ldots + CV^2{}_n}{n}}$$

Where cv_i = cv of i^{th} observation, and

n = number of observations (i.e. 10) in the T_4, T_3 or TSH concentration interval.

Also the CVs along the standard curves of the assays were calculated by the same method as above. Duplicate variation of standards from five assay runs for each hormone (and for each method) were used.

The mean values and standard deviations of pooled human serum samples with low, medium and high T_4, T_3 and TSH concentrations, determined in five consecutive runs were used to calculate the interassay coefficient of variation (CV%). The samples were measured in duplicate.

Only those samples that were measured for T_4 by both methods are reported.

In total 179 results were used for T_4 correlation and derivation of reference ranges. Regarding T_3, all the samples with undetectable T_3 by Serozyme assay were excluded from further analysis. A total of 161 results remained for correlation studies

and derivation of reference ranges.

Some samples showed suspiciously high Serozyme TSH values. A ratio of SerozymeTSH:Delfia TSH was calculated for each sample. All those with a ratio of more than 3 were considered to be showing spurious result by Serozyme. In total 172 results are used for TSH assay correlation studies and derivation of reference ranges.

Considering the fact that neither of the assays is free of residual error, the non-parametric regression analysis method by Passing and Bablok was used. In this method no assumption is made concerning residual errors or proportions of residual errors.

The distribution of Serozyme T_4 and T_3 values in both adults and pregnant subjects were gaussian. The ranges were derived as central 95% of the distribution. Serozyme TSH values in both groups were not normally distributed. The ranges were derived after logarithmic transformation of the TSH data.

4.3 Results of comparative studies.

4.3.1 Precision

The Serozyme T_4 assay showed inferior precision when compared to the Amersham T_4 assay (Figure 4.3). Intraassay precision of the Serozyme T_4 assay lies in the range of 7-15%, irrespective of the T_4 concentration level. The average CV of 12% is higher than that of the Amersham T_4 assay which is about 4%. The highest CVs in the Serozyme T_4 are recorded within the expected normal range of T_4 in healthy subjects i.e. between 80-120 nmol/L. On the other hand low CV's are noted at the extremes, *i.e.* below 75 nmol/L and above 150 nmol/L. The Amersham T_4 intraassay precision is more or less uniform along the whole concentration range of T_4. The same conclusion with respect to Serozyme and Amersham T_4 intra-assay CV can be drawn when the intraassay precision is calculated along the standard curves (table 4.4). The inter-assay reproducibility is also better with the Amersham T_4 when compared to Serozyme T_4 assay (table 4.5).

Also the Amersham T_3 assay has better precision (both within assay and between assay) than the Serozyme T_3 assay (figure 4.4 and tables 4.6 and 4.7). In both assays the intraassay CV is highest at low and high levels of T_3.

The Delfia TSH assay (Figure 4.5 and tables 4.8 and 4.9) and Serozyme TSH assay (figure 4.8 and tables 4.7 and 4.9) showed very comparable within and between assay precisions.

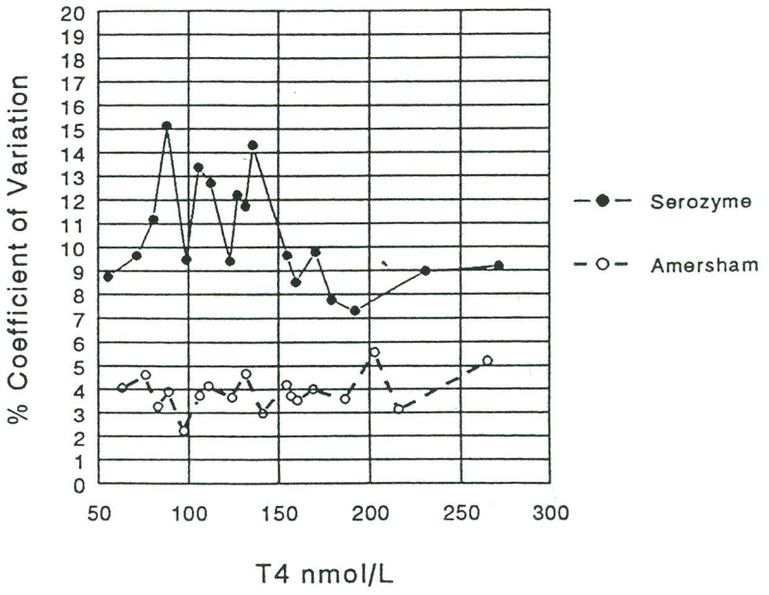


Figure 4.3 Intraassay precision profiles of T_4 assays

Table 4.4 Intraassay precision of T_4 assays along the standard curve.

Serozyme T_4 nmol/L	%CV	Amersham T_4 nmol/L	%CV
25.7	7.4	22.5	4.9
51.5	10.4	45.0	3.2
103.0	7.1	90.0	2.9
193.0	8.4	180.0	3.4
386.0	4.9	360.0	5.1

Table 4.5 Interassay precision of T_4 assays.

	Serozyme (n = 5)			Amersham RIA (n = 5)		
Description	Low	Medium	High	Low	Medium	High
Mean nmol/l	57.9	102.2	142.5	65.4	103.5	152.1
CV	11.6%	9.3%	12.4%	3.7%	2.4%	3.5%

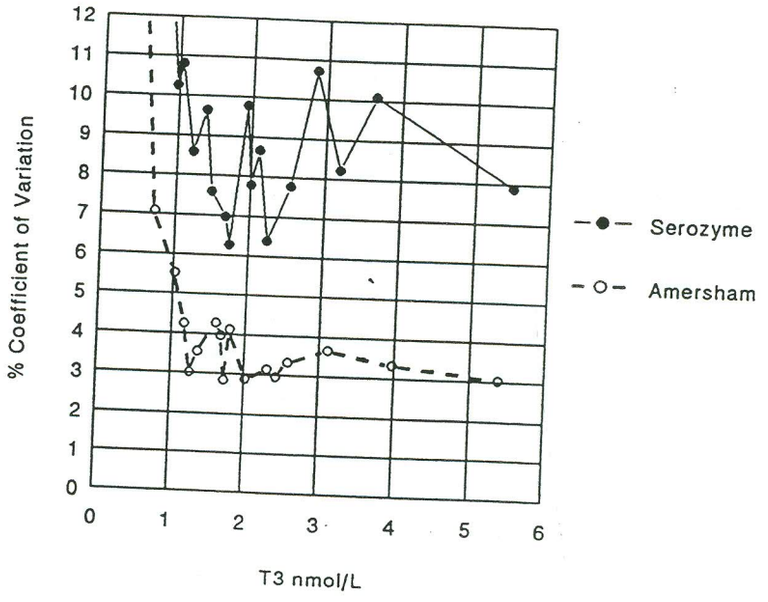


Fig 4.4 Intraassay precision profiles of T_3 assays.

Table 4.6 Intraassay precision of T_3 assays along the standard curve.

Serozyme T_3 nmol/L	%CV	Amersham T_3 nmol/L	%CV
0.77	9.8	0.63	4.2
1.54	3.3	1.62	2.6
3.85	2.8	3.15	3.4
7.70	3.8	6.01	4.9
15.40	4.5	12.78	7.4

Table 4.7 Interassay precision of T_3 assays.

	Serozyme (n = 5)			Amersham (n = 5)		
Description	Low	Medium	High	Low	Medium	High
Mean T_3 nmol/L	0.97	1.62	2.59	1.06	1.80	3.2
CV%	16.6%	12.3%	9.7%	3.0%	3.3%	3.6%

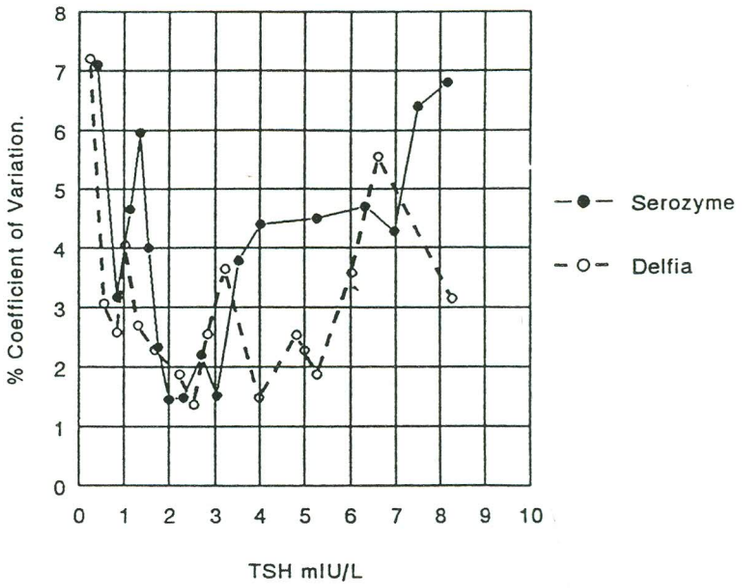


Figure 4.5 Intraassay precision profile of TSH assays

Table 4.8 Intraassay precision of TSH assays along the standard curve.

Serozyme TSH mIU/L	%CV	Delfia TSH mIU/L	%CV
0.15	4.0	0.03	5.0
0.50	5.0	0.1	2.8
1.50	1.3	1.0	1.7
5.00	3.1	10.0	1.0
50.00	2.9	100.0	1.8

Table 4.9 Interassay precision of TSH assays.

Description	Serozyme (n = 5)			Delfia (n = 5)		
	Low	Medium	High	Low	Medium	High
Mean TSH mU/l.	1.58	4.44	15.32	0.55	1.88	6.04
CV	5.2%	2.8%	4.4%	1.6%	2.3%	2.2%

4.3.2 Accuracy

Serozyme and Amersham T_4 and T_3 assays and Delfia and Serozyme TSH assays are compared in figures 4.6, 4.7 and 4.8 respectively.

There was only a moderate but significant correlation ($r=0.770$, $p<0.001$) between Serozyme and Amersham T_4 results. There was no bias between the two methods compared [slope: 1.279 (95% confidence interval: 1.116 to 1.452), intercept -29.9 nmol/L (95% confidence interval -50.5 to -13.3 nmol/L)].

With the paired student t-test there was no significant difference between the values obtained by the two compaired methods [mean (SEM) difference was 1.56 (2.38) nmol/L] (fig 4.6).

A poor comparability ($r=0.570$, $p<0.001$) of T_3 values by Serozyme and Amersham methods was found. Moreover the Serozyme assay gave significantly lower T_3 values when compared to results obtained with the Amersham kit [slope: 0.840 (95% confidence interval: 0.709 to 1.010), intercept: -0.05 nmol/L (95% confidence interval -0.44 to -0.27 nmol/L)]. The mean (SEM) difference was 0.5 (0.05) nmol/L ($p <0.001$; paired student t-test) (fig. 4.7).

There was a good correlation between both TSH assays ($r=0.896$ $p<0.001$). The Serozyme assay gave significantly higher values than the Delfia method ($p < 0.001$, Wilcoxon Signed Ranks Test) [slope: 1.304 (95% confidence interval: 1.216 to 1.417), intercept 0.16 nmol/L (95% confidence interval 0.03 to 0.26 nmol/L)]. (fig 4.8).

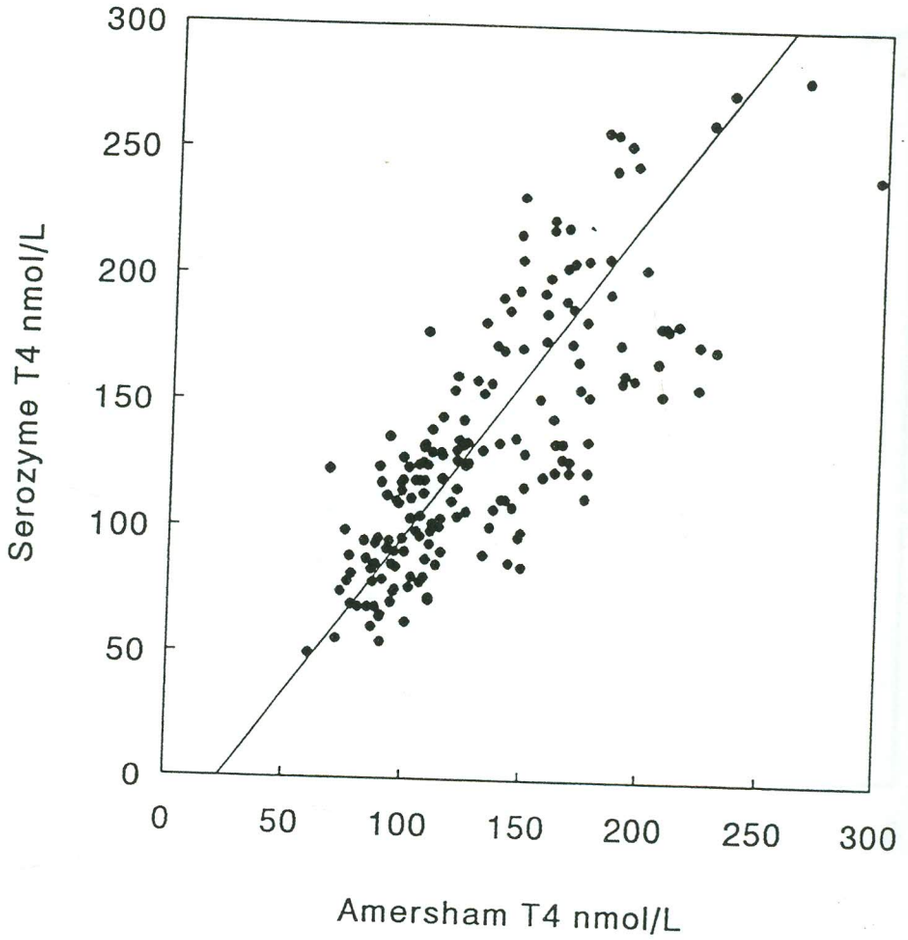


Figure 4.6 Relation between T_4 values obtained by Amersham (x-axis) and Serozyme (y-axis) assays.

Serozyme T_4 nmol/L = - 29.85 nmol/L + 1.28 * T_4 Amersham

n=179, r=0.770, p<0.001.

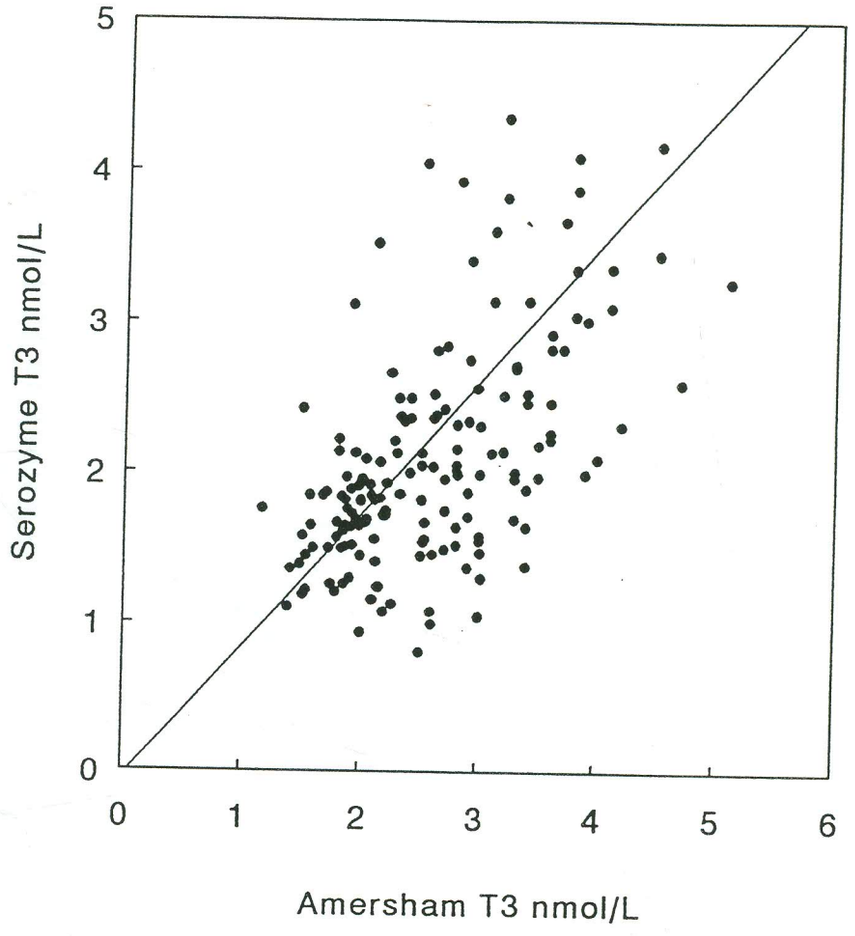


Figure 4.7 Relation between T_3 values obatined by Amersham (x-axis) and Serozyme assays (y-axis).

Serozyme T_3 nmol/L = -0.05 nmol/L + 0.84 * Amersham T_3

n=161, r=0.570, p<0.001

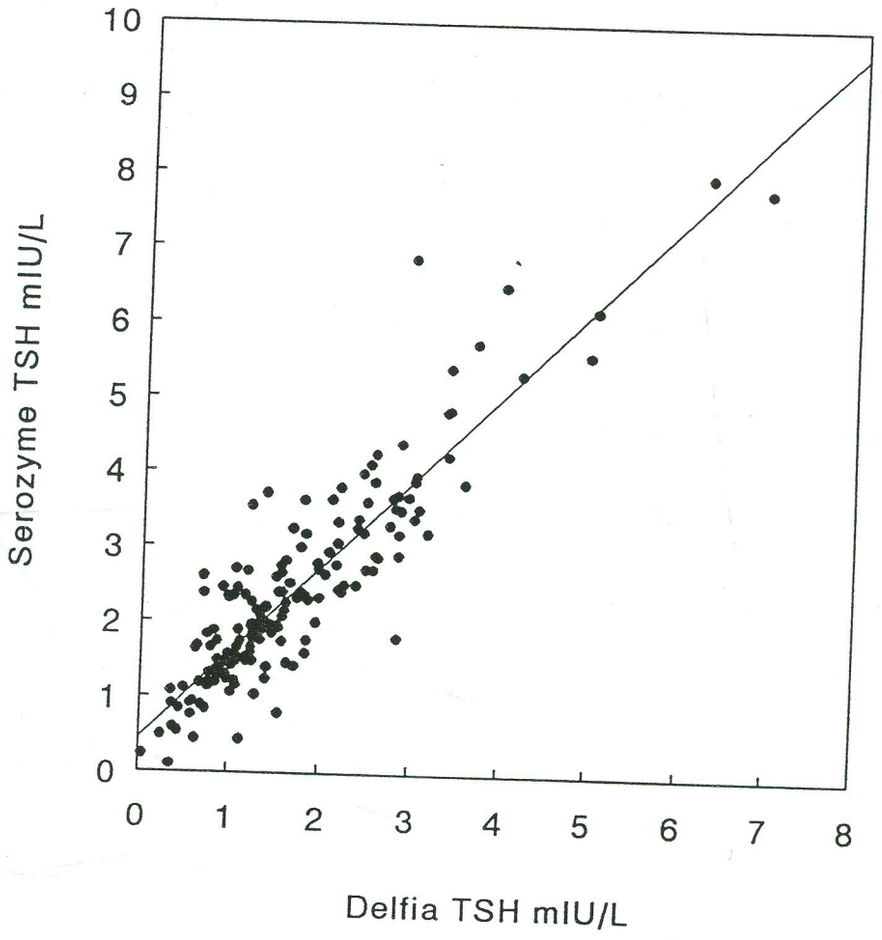


Figure 4.8 Relation between TSH values obtained by Delfia (x-axis) and Serozyme (y-axis) assays.

Serozyme TSH mIU/L = 0.16 mIU/L + 1.30 * Delfia TSH

n=172, r=0.890, p<0.001

4.4 Ranges of Serozyme T_4, T_3 and TSH in Tanzanian subjects.

Table 4.10 Mean, SD and ranges of Serozyme T_4 assay in euthyroid adults and pregnant Tanzanian subjects.

Description	Adults (n = 88)	Pregnant subjects (n = 91)
Mean T_4 nmol/L	98.9	173.6
SD	22.6	52.2
Range nmol/L	54 - 144 nmol/L	69 - 279 nmol/L

Table 4.11 Mean, SD and ranges of Serozyme T_3 assay in euthyroid adults and pregnant Tanzanian subjects.

Description	Adults (n = 78)	Pregnant subjects (n = 83)
Mean T_3 nmol/L	1.73	2.32
SD	0.37	0.85
Range nmol/L.	0.98 - 2.5	0.6-4.0

Table 4.12 Mean, SD and ranges of Serozyme TSH assay in euthyroid adults and pregnant Tanzanian subjects.

Description	Adults (n = 85)	Pregnant subjects (n = 87)
Mean TSH mIU/L	1.87	3.58
Range mIU/L	0.7-5.2	1.1 - 10.8

4.5 Serozyme T_3Uptake and Free Thyroxine Index in nonpregnant and pregnant Tanzanian subjects.

T_3Uptake and the calculated Free Thyroxine Index in Tanzanian subjects are presented in table 4.13.

Table 4.13 Mean values and the ranges of T_3 Uptake and the FT_4I in adults and pregnant Tanzanians.

Serozyme T_3 Uptake	Males	Females	Pregnant subjects
Mean (SD)	106 (5.5)[a]	114 (15.5)	122 (10.8)
Reference range	94-117	84-144	100-144
Serozyme FT_4I	Males	Females	Pregnant subjects
Mean (SD)	97 (23.0)[a]	87 (18.0)	142 (41.9)[b]
Reference range.	51-143	51-123	58-226.

[a] *$p < 0.05$ Male value significantly different from female value.*

[b] *$p < 0.001$ Pregnant value significantly different from nonpregnant female value.*

T_3 Uptake values were significantly higher in females than males with mean (sd) 114.2 (15.1) vs 106 (7.5). But the calculated FT_4I was significantly higher in males than in females with mean(sd) of 97 (23.0) versus 87 (18.0).

Pregnant subjects had slightly higher T_3 Uptake than nonpregnant female subjects but the difference was not significant. However FT_4I in pregnancy was higher (and highly significantly so) when compared to nonpregnant female subjects value.

Low correlation coefficients were found between Spac fT_4% and T_3U (Serozyme) ($r = -0.41$) and between Serozyme FT_4I and Spac FT_4 ($r = 0.43$).

Table 4.14 Summary of pregnancy induced changes in the parameters determining Serozyme FT_4I and Spac FT_4.

Parameter mean value	Serozyme T_4 nmol/L	Serozyme T_3U	Amersham T_4 nmol/L	Spac fT_4%
Nonpregnant females	94.0	114.0	100.6	0.0117
Pregnant females	173.6	122.0	163.3	0.0064
Percentage Change due to pregnancy	Up by 84%	Up by 7%	Up by 62%	Down by 45%

4.6 Discussion.

In this chapter the performance of Serozyme immunoassays was compared with that of established routine immunoassays for T_4 and T_3 and TSH is use in the laboratory for Endocrinology and Reproduction, Academic Hospital Nijmegen, The Netherlands. The Serozyme assays have an additional step of enzymatic incubation following the immunological reaction. However, the turn-around time of compared T4 and T3 assays is on the average three hours. The Serozyme assay has the advantage of having the final results calculated immediately on reading the absorbances, thus obviating the need of an additional computing equipment. The color of the Serozyme assay enzymatic product was found to be stable for at least 2 hours (data not shown), as indicated by the kit manufacturer.

In the evaluation of these assays, two types of errors have been investigated, *i.e.* those which are systematic and those which are random. Systematic errors manifest themselves as bias and random errors manifest themselves as imprecision.

The Serozyme assays showed lower precision (both intra and interassay) when compared to the other three studied assays. However the precision of both T_3 and TSH Serozyme assays were within acceptable limits. The low correlation coefficients between the compared methods are likely to be due to multiple factors identified with the Serozyme assays. The probable reasons for the lower precision of the Serozyme assays in this study include:

(1) Loss of magnetic particles during the separation step. This was observed during the assays despite strict adherence to the assay instructions.

(2) The inherent nature of the enzyme assays in which end point detection is less precise when compared to radioisotopes or fluorophores.

(3) In general there are more steps in the Serozyme assays when compared with the Amersham assays (for T_4 and T_3) and Delfia assay (for TSH). Each step introduces a random error which is inevitably of different magnitude in each tube.

The Serozyme T_3 and TSH assays were found to be subject to interference. This phenomenon was not linked *i.e.* samples which were subject to interference in the T_3 assay not necessarily showed falsely elevated TSH levels. Additional studies suggested that cross-linking reagents, e.g heterophilic antibodies, may be responsible for this interference. The common feature of the Serozyme T_3 and TSH assays is the labelled antibody in these systems. While the T_3 assay is of the competitive type, TSH assay is a non-competitive one. However in the final phase of all these assays, a sandwich of an analyte between a solid phase and detection antibody (see figure 4.1 and 4.2) is formed (this being a result of "second antibody" separation technique in the T_3 assay). In all cases therefore crosslinking leads to an increased signal. In the present study, the effect manifested itself as undetectable T_3 and very high TSH values in some of the analysed samples.

According to the T_3 kit insert this phenomenon had been observed in one sample. The reasons for this were not indicated. Although the interference was not apparent in the Serozyme T_4 assay, the fact that this assay relies on exactly the same principle as the T_3 assay raises the possibility that similar interference could be operating. The observed effect, however, seems to be "all or nothing" rather than gradual, since fig 4.7 shows that after exclusion of the "negative" results, no value is below 0.8 nmol/l. Also, no T_4 value below 50 nmol/l is observed. Therefore, it also

must be considered as less likely that such an effect is responsible for the poor correlations.

Non-analyte linking of the capture and signal antibodies by heterophilic antibodies has frequently been reported in immunometric assays (Hedenborg G et al 1975; Cusick CF et al 1987; Clark MSP 1987). This interference lead to erroneous quantification of the assayed analyte in the affected sample(s). Apart from the deliberate design of the so called "interference assays" in order to study the incidence of such antibodies in various populations (Boscato LM and Stuart MC 1986; Thompson RJ et al 1986), most of the reported cases have been of sporadic nature. The developers of commercial and inhouse assay reagents recognise this potential source of error, and it has been a practice to include non-immune serum or immunoglobulins of species from which the reagent antibodies are raised from in order to block this potential interference (Skovsted L and Hansen JM 1988; Kahn BB et al 1988; Boerman OC et al 1990). However, cases of escape from this blockade have been reported (Harvey RD et al 1988; Zweig MH et al 1988).

Several workers have reported heterophilic antibody interferences restricted to immunoenzymometric assays using monoclonal antibodies but not immunoradiometric assays using monoclonal antibodies or immunoenzymometric assays and immunoradiometric assays using polyclonal antibodies (Bock JL et al 1985; Clark MSP and Price CP 1987; MacCarthy RC et al 1988). In one of these reports (MacCarthy RC et al 1988) an evaluation of different types of immunoenzymometric assays showed that this interference was positive in 9 of 10 of the assay formulations. In all the three reports, the interference was shown to be caused by an IgM type of antibody.

Two basic requirements to block this kind of interference are that, the non-immune serum or immunoglobulin added to the assay reagents should possess the antigenicity which is complementary to the heterophile and should be of an appropriate amount.

The failure of mouse and/or sheep IgG to block the interference in the Serozyme T_3 assay could have been due different antigenicities when compared to the ones which provoked the production of the heterophiles.

The Serozyme TSH assay standards are made in bovine serum and also the anti-TSH reagent is supplemented with sheep, horse and bovine serum proteins. The anti-T_3 reagent and T_3 derivative reagent are also supplemented with sheep and bovine serum proteins.

In Serozyme TSH but not in T_3 assay, dilution of the affected specimen in the zero assay standard was effective in blocking the interference. In both assays addition of bovine serum to the affected specimens before the assay succeeded in blocking the interference. It should be noted however, that no bovine derived antibodies are used in the assays' reagents!

From the present study the most enlightening nature of this interference is provided by the results from the "modified" Serozyme T_3 assay in which it was demonstrated that there was linking of the antibody conjugated to the magnetic particles and the enzyme labelled antibody in the absence of T_3-fluorescein derivative. This suggests that the interference is due to an antibody of multiple specificity capable of linking mouse and sheep antibodies.

In trying to explain the selective interference of heterophilic antibodies in immunoenzymometric assays (MacCarthy RC at al 1988) a model has been

proposed in which the interfering antibody is of necessity an IgM class antibody. It is also indicated that this effect is due to the macromolecular nature of the indicator in which the enzyme label is linked to multiple tracer antibodies.

In the present study the nature of the proposed antibody was not confirmed.

If the proposed nature of interference is real, then this represents a high incidence of interference in the Tanzanian samples. This high incidence could be explained by the fact that this community is constantly exposed to immunological stimulation (e.g. by protozoal and bacterial infections). It is known that the production of heterophilic antibodies is part of normal process by which antibodies develop monospecific diversity toward an untold number of antigens (Levinson SS 1992).

In evaluating the Serozyme FT_4I, the euthyroid status of normal pregnancy was assessed by two approaches and the results compared (i.e. Serozyme FT_4I and Spac FT_4). Pregnancy provides a model for studying the ability of Serozyme T_3 Uptake (and FT_4I) method to correct for raised T_4 values (in otherwise euthyroid subjects) due to increased TBG concentration. Serozyme free thyroxine index values in pregnant subjects in the present study are far above the normal ranges of nonpregnant subjects. This implies that the Serozyme FT_4I has failed to reflect the euthyroid status of normal pregnancy. In only 52% of the pregnant subjects were FT_4I values within the reference normal range of nonpregnant population. It may be recalled that when assessed by Spac FT_4 almost all (99%) of pregnant subjects had their FT_4 values falling within the normal nonpregnant range. It was found that whereas the pregnancy induced increase in total T_4 by the two methods is comparable, T_3 uptake in pregnancy did not differ significantly from nonpregnant values. These results made further subdivision of the data according to trimester

useless. The free fraction of T_4 in pregnancy was found to be reduced by 45% when compared to nonpregnant value.

The principle and limitations of T_3Uptake methodology were reviewed in chapter one of this thesis. The Serozyme T_3Uptake assay does not conform to the conventional principle of this technique. In the conventional technique of T_3U the labelled T_3 introduced into the sample binds to both the sequestering agent (e.g. solid phase antibody) as well as the serum binding proteins the amount bound to the antibody representing the fractional uptake of T_3 which is inversely proportional to the concentration of free binding sites in the sample. In the Serozyme assay the enzyme labelled T_3 is assumed not bind to serum proteins. The exogenously introduced T_3 is assumed to saturate the free binding sites and the amount remaining to compete with labelled T_3 for the T_3 antibody. Therefore in the Serozyme T_3Uptake assay it is predicted that the fractional uptake of labelled T_3 to the sequestering agent (i.e. anti T_3) will be proportional to the free binding sites in the sample. From this analysis it follows that a number of factors may play a part in invalidating the results of Serozyme T_3 Uptake assay.

The system resembles a free T_3 estimation by labelled analog methodology. It differs from such an assay by the fact that exogenous T_3 has been added in such quantities, that the total T_3 concentration will be almost the same in all samples. Consequently, among the samples the concentration of T_3 that is not bound to serum proteins, will be proportional to the free T_3 *fraction*, which, as stated before, in its turn is proportional to the free T_4 fraction. It is this concentration that determines what proportion of the tracer is bound, roughly in an inversely proportional manner. Since the relation is so indirect, it is unlikely that T_3 Uptake will parallel fT_4 and the FT_4-

index calculated in the above way will accurately parallel the free T_4 concentration. This test probably operates at or near equilibrium, so that considerations in chapter one that refer to conventional T_3Uptake test concerning the difference between initial uptake rate and net uptake rate are not relevant here. However, the total failure of this test to reflect elevated TBG cannot be explained.

All in all the lack of correlation between T_3Uptake and fT_4% and between Serozyme FT_4I and Spac FT_4 shows that the principle on which Serozyme T_3Uptake is based is not sound. The highly increased binding sites in pregnancy (due to increased TBG levels) are not reflected in increased T_3Uptake values. These results are not contradictory towards the manufacterers' specifications. These also show almost complete overlap of T_3 Uptake values for pregnant and non-pregnant women. For FT_4I the second trimester median is 28% higher than for non-pregnant women.

In conclusion, the Serozyme assays are easy to perform, requiring an inexpensive reader which is easy to operate. Although there is a general agreement between the results of Serozyme assays and Amersham T_3 and T_4 and the Delfia TSH method, especially the precision of the Serozyme T_4 assay is unsatisfactory. Moreover, in a relatively large proportion of the samples interference in the Serozyme T_3 and TSH assays, probably caused by heterophilic antibodies, was detected.

The Serozyme T_3 Uptake test fails to discriminate even between group means of pregnant and non-pregnant subjects. Incorporated in a FT_4 index it fails to compensate for elevated T_4 during pregnancy, and therefore the T_3Uptake test should be considered as useless. Therefore, the use of pregnancy specific reference ranges of total T_4 must be preferred.

4.8 References.

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