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Role of Bioactive Peptides
In
Autoimmune Thyroid Disease

Thesis Submitted To The Faculty of Medicine
University of Glasgow
For The Degree of Doctor of Philosophy

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SUMMARY

The thyroid gland is the target of the commonest forms of autoimmune disease. Development of goitre, with an increase in the number of thyrocytes and other cells in the thyroid, is one of manifestation of thyroid autoimmunity in some cases. The enlargement of the gland is not simply the effect of stimulation of the thyrocytes with TSH (Hashimoto's disease), or with thyroid stimulating antibodies (Graves' disease), but involves many other factors, including cell to cell signalling among the various populations of cells in the gland by cytokines or other mediators.

Another consequence of disease is the generation of new blood vessels and infiltration of a variety of mononuclear cells which form aggregates or are diffusely distributed between and within the thyroid follicles. It seems likely that the mononuclear cells migrate from peripheral blood into thyroid tissue where they are retained by interaction with other cells including thyroid epithelial cells by means of cytokines released locally. The interaction of cytokines and growth factors with thyrocytes could also influence their functional pattern.

Finally, the thyroid gland is richly innervated by nerves of different types (adrenergic, cholinergic, and peptidergic) which terminate close to blood vessels and follicular cells. It is likely, therefore, that neurotransmitters released locally may directly influence the activity of the follicular cells.

Previous studies showed that a soluble form of the interleukin-2 receptor (sIL-2R) is present in increased quantities in serum of patients with Graves' disease. Less information is available for the cytokines production among these patients, and the role of neuropeptides in the gland is not clear. The aim of this study was to determine the role of some of these bioactive peptides in Graves' disease.

The concentration of soluble IL-2 receptor was increased in serum of the patients with Graves' disease (7.5 fold compared to healthy controls). Experiments on stimulation of blood cells in vitro showed that part of the increase in production of sIL-2R is due to the elevation of thyroid hormones, while reduction of IL-2 production may be a second factor. In addition, the concentration of sIL-2R depends on other, unknown factors in the plasma, because dilution of whole blood with medium caused an increase in sIL-2R production.

Stimulation of diluted (1:11 diluted with medium) whole blood of patients with untreated Graves' disease with a mixture of LPS/PHA caused a release of IL-2 and IL-4 that was significantly higher than from the reference group, after 72 h. The production of IL-6 was also higher than from the reference group, after 4 h. The production of INF- γ depended on the stimulator applied, but incubation of

unstimulated whole blood of the patients released less INF- γ than control. Finally, TNF- α production of whole blood of the patients was higher than the reference group after 4 h, and was lower than the reference group after 72 h. These results may indicate that cytokine production by immune cells has a different pattern compared with healthy controls.

The effects of methimazole, thyroid hormones, and TSH on cytokines production were also investigated. These compounds did not affect the production of IL-2 and IL-4, while they decreased the production of IL-6, INF- γ , and TNF- α . This shows that high concentrations of TSH (in Hashimoto's disease), and thyroid hormones (in Graves' disease) could influence the production of IL-6, INF- γ , and TNF- α . Furthermore, it also shows that methimazole directly influences the production of these cytokines.

The effects of various growth factors, cytokines, and neuropeptides on iodine uptake and growth of rat FRTL-5 thyroid epithelial cells were investigated.

In the absence of TSH, the iodine uptake of thyrocytes was increased by IGF-1 (in the presence of insulin), TGF, IL-6, INF- γ , neuropeptide Y, low doses of VIP and tachykinins, high doses of iodine, and thyroid hormones. On the other hand, iodine uptake was decreased by FGF, high doses of LPS, VIP, and tachykinins, and SNAP. The growth of cells was increased by insulin, IGF, FGF, low doses of VIP, high doses of thyroid hormones, inorganic iodine, and SNAP. The growth of cells was decreased by high doses of VIP.

In the presence of TSH, the iodine uptake of thyrocytes was increased by insulin, IGF, TGF, endothelin-1, low doses of IL-1 and LPS, IL-6, INF- γ , PACAP, low doses of VIP and tachykinins, high doses of SNAP, and noradrenaline. On the other hand, iodine uptake was decreased by inorganic iodine, thyroid hormones, FGF, high dose of IL-1, LPS, and tachykinins, and low doses of SNAP. The growth of cells was increased by high doses of thyroid hormones, insulin, IGF, FGF, endothelin-1, low doses of LPS, IL-6, and low doses of VIP. The growth of thyrocytes was decreased by IL-4, INF- γ , high doses of VIP, and PACAP.

The pattern of iodine uptake and growth of cells changed when the cells were treated with high concentrations of thyroid hormones and TSH, before and during stimulation with the growth factor, cytokine, or neuropeptide. In most cases the hormones antagonised the action of the peptides. The stimulatory effects of IGF-1, TGF- β , endothelin-1, interferon- γ , PACAP and VIP on iodine uptake were all reduced. In addition, thyroid hormones prevented the inhibitory effects of IL-1 and LPS. However, there was a large potentiation of the increase in iodine uptake caused by noradrenaline.

The actions on growth rate were similar. The stimulatory effects of endothelin-1 and LPS were reduced, as were the inhibitory effects of TGF- β , IL-4

and interferon- γ . The only exceptions were slight growth stimulatory effects in the presence of VIP and neuromedin-U.

Combination of IL-1 with some of the growth factors, cytokines, and neuropeptides revealed that the effects of IL-1 were inhibitory. This may have implications for the situation in autoimmune disease or inflammation, where there is evidence for the presence of IL-1 within the gland. The effects were greater in the presence of TSH, so IL-1 may act to limit the growth and activity of the thyroid in both Hashimoto's and Graves' diseases.

Combination of some of the stimulators with noradrenaline also revealed that noradrenaline is directly involved in the pathogenic mechanisms of hyperthyroidism, and not just in eliciting the symptoms.

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Chapter One

General Introduction

SECTION ONE

THYROID GLAND

1.1.1 Structure

The thyroid is the largest endocrine gland in the human body. In adults, its weight is approximately 20 g (Marshall 1992). The gland synthesises and secretes three hormones: the thyroid hormones thyroxine (T_4) and triiodothyronine (T_3), both of which are iodinated derivatives of tyrosine, and calcitonin, a polypeptide hormone that is functionally unrelated to other thyroid hormones, and is involved in calcium homeostasis.

The thyroid gland is situated in the anterior part of the neck. It consists of two somewhat pear-shaped lobes that lie to the sides of the trachea, and a smaller isthmus joining the medial aspects of the lobes. The lobes are about 5 cm long, 3 cm across at the widest part, and 2 cm thick at the lower pole (McDougall, 1992).

The gland is highly vascular. A euthyroid gland has a blood flow rate of about 100 ml/min. In Graves' hyperthyroidism this can increase to one litre/min and this is clinically apparent by a palpable thrill and a bruit (Halmi, 1986). There are four main arteries supplying blood to the thyroid. The superior thyroid arteries arise as the first branch of the external carotid artery and the inferior arteries arise from a branch of the first part of the subclavian artery. There is a rich anastomosis between superior and inferior vessels as well as between the lobes. The arteries divide into lobar and then lobular branches supplying a lobule consisting of some 20-50 follicles. A plexus of veins on the surface of the thyroid drains into the superior and middle thyroid veins, which in turn drain into internal jugular veins. There is a rich lymphatic supply that follows the veins.

The thyroid encloses a number of nerve fibres, most of which are sympathetic (Halmi, 1986; Åhrén, 1991). The nerves supplying the thyroid itself mostly arise from the middle cervical ganglion and travel with the inferior thyroid artery. Some sympathetic fibres from the superior cervical ganglion also reach the thyroid along with the superior thyroid artery. These nerves are vasoconstrictors. Some contain neuropeptides (Åhrén, 1986 a; and 1991), though few effects on the thyroid have been demonstrated.

The epithelial cells of the thyroid that produce and secrete thyroid hormones are arranged in spherical structures called follicles. The follicular cells are cuboidal with a length of about 10 μm . Under stimulation, they become progressively more

columnar and can be as tall as 20 μm . This appearance is characteristic of Graves' hyperthyroidism that has not been treated; however, a similar appearance is found if the thyroid is lacking iodine and the gland is stimulated to make maximal quantities of hormone by pituitary thyroid stimulating hormone (TSH). A single layer of follicular cells surrounds colloidal material containing stores of hormones incorporated into a protein called thyroglobulin. The dimensions of the follicle, the follicular cells and colloid material vary from 100 to 1000 μm in diameter depending on the availability of iodine, the effect of physiological and pathological stimulators, and the presence of goitrogens. Smaller follicles appear to be more active. When there is excessive prolonged stimulation, the quantity of colloid material decreases reflecting release of stored hormone from thyroglobulin (McDougall, 1992).

Groups of 20-50 follicles form lobules, which have a fine covering of connective tissue that is continuous with the capsule of the gland. Each follicle is surrounded by delicate capillaries, but with standard staining techniques these are not well demonstrated because they collapse. The rich lymphatic system is also difficult to demonstrate in specimens from normal glands, but lymphatics are well seen when carcinoma invades them (McDougall, 1992).

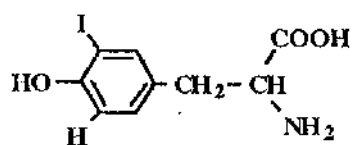
The internal structure of the follicular cells reflects their active involvement in protein formation and secretion. The cytoplasm is rich in mitochondria, rough endoplasmic reticulum, Golgi apparatus and secretory droplets. The nucleus is round or oval and is central in cuboidal cells, but more basal in columnar ones. Numerous microvilli project from the apical end of the cells into the colloid (Halmi, 1986).

Parafollicular cells (C cells) which produce and secrete calcitonin lie between follicles. They are difficult to see in sections of normal thyroid (McDougall, 1992).

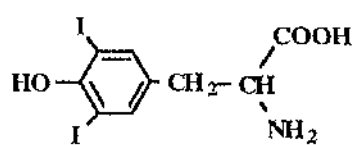
1.1.2 Function

There are two physiologically important thyroid hormones: L-thyroxine, which is more abundant in the circulation and L-triiodothyronine, which is the more important hormone at the cellular level. Thyroxine and triiodothyronine are designated as T_4 and T_3 because they contain 4 and 3 iodine atoms per molecule respectively (figure 1.1).

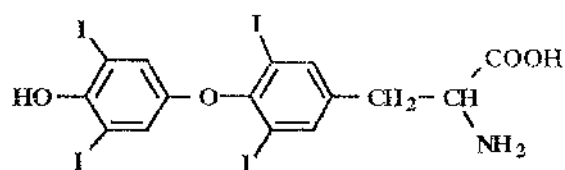
The formation and secretion of T_3 and T_4 are largely controlled by thyrotrophin (TSH). TSH is synthesised in, and secreted from, special cells in the anterior pituitary. Thyrotrophin releasing hormone (TRH) from the hypothalamus stimulates TSH release, and increased concentrations of T_3 or T_4 in the circulation



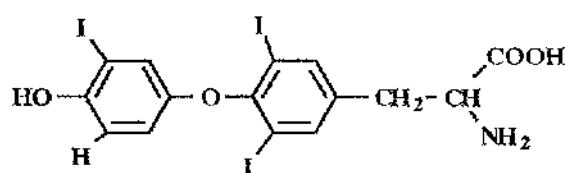
Monoiodotyrosine



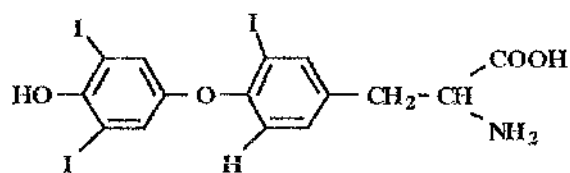
Diiodotyrosine



L-Thyroxine (T₄)



Triiodothyronine (T₃)



Reverse triiodothyronine (rT₃)

Figure 1.1: Structure of monoiodotyrosine (MIT), diiodotyrosine (DIT), triiodothyronine (T₃), thyroxine (T₄), and reverse triiodothyronine (rT₃).

suppress it (Marshall, 1992; Vassart and Dumont, 1992). Several other hormones, neurotransmitters, drugs and clinical conditions modulate this control system (McDougall, 1992), which is summarised in figure 1.2. The hypothalamus is under less well defined control from higher centres. Finally, the thyroid is able to 'auto regulate' all steps of synthesis and release of hormones, although this is much less important than the TSH control mechanism (McDougall, 1992).

1.1.2.1 Thyrotrophin Releasing Hormone

TRH is a tripeptide, L-(pyro)Glu-L-His-L-Prolinamide, with a relative molecular mass of 362 (Guillemin, 1978). TRH is formed in hypothalamic cells as a precursor consisting of five copies of the active molecule; the tripeptide is formed post translationally (Lechan et al, 1986). In addition, TRH is produced in many other sites in the brain, spinal cord, retina, gastrointestinal tract, pancreas and prostate (Griffiths, 1987; Gkonos et al, 1994).

TRH, formed in neurone bodies of hypothalamic neurones, travels in the axons to the primary vascular plexus of the portal system where it gains access to the blood and is transported to the anterior pituitary. There it interacts with high affinity receptors on specific cells, thyrotrophs, which produce TSH. TRH causes a rapid release and a slower formation of TSH from these cells. TRH or TRH-like peptides are believed also to stimulate anterior pituitary cell proliferation (Pawlikowski and Slowinska-Klencka, 1994).

In normal people, injection of TRH causes a brisk rise in serum TSH from its normal basal range of approximately 0.4-5.0 mU/l which reaches a peak at 20-30 min and falls towards normal by 60-90 min. One to 4 h after injection of TRH, there is a rise in serum T_3 of up to 70%, and a 15-20% rise in T_4 (Marshall 1992). This demonstrates the sequence: hypothalamus - pituitary - thyroid.

In patients who are hyperthyroid, irrespective of the cause, injection of TRH fails to produce a surge in serum TSH. Therefore, high levels of T_4 or T_3 , or both, inhibit the effect of TRH on the pituitary. In contrast, in primary hypothyroidism where the thyroid is failing and the levels of T_4 and T_3 are low, TSH is high and TRH causes a dramatic increase in serum TSH which falls to the baseline slowly (Kolesnick and Gershengorn, 1985; McDougall, 1992).

TRH has a half-life of about 5 min in blood. It is degraded by peptidases though some is excreted intact in the urine. When injected intravenously, only about 1-2% crosses the blood-brain barrier (Marshall, 1992).

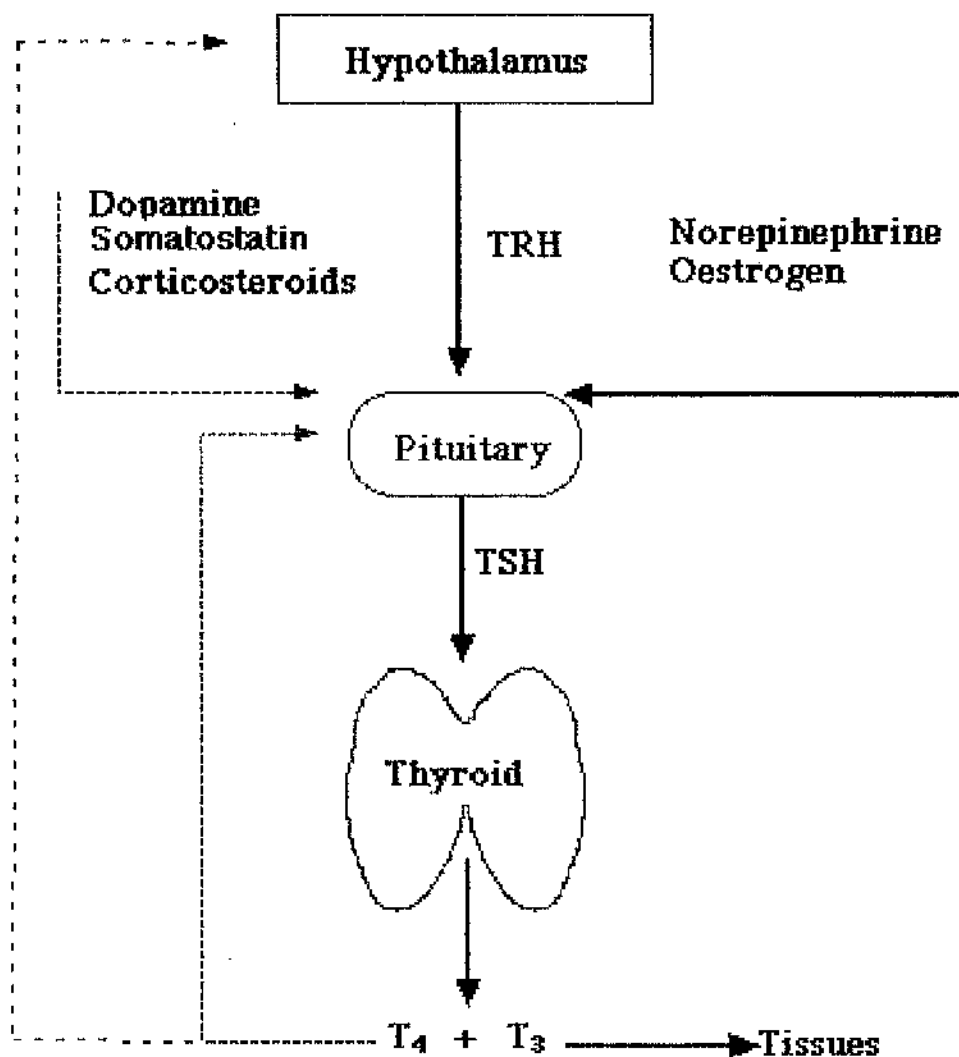


Figure 1.2: Simplified diagram of the control of thyroid function, and additional factors modulating the action of TRH on TSH secretion by the thyrotroph. Positive effects are indicated by solid line, while broken lines indicate negative effects.

1.1.2.2 Thyrotrophin

About 5% of anterior pituitary cells secrete TSH. These thyrotrophs are predominantly located in the anteromedial aspect of the gland. TSH is a glycoprotein of relative molecular mass 29000 which comprises two non-covalently bound polypeptide chains designated alpha and beta. The alpha subunit, of relative molecular mass 14000, is identical to those of luteotrophin (LH), follitrophin (FSH) and chorionic gonadotrophin (HCG) (Pierce and Parsons, 1981). The beta subunit has a relative molecular mass of 15000 with a protein core of 113 amino acids which confers unique biological and immunological properties on each hormone. The gene which encodes the beta subunit is on a different chromosome from that for the alpha chain (Chin et al, 1985). The thyrotroph produces an excess of alpha units and therefore the production of the beta subunit is the rate limiting step. Carbohydrate moieties are added at a post translational step (McDougall, 1992).

The main negative feedback control for production and secretion of TSH is the level of free thyroid hormones in the blood. The thyrotroph is rich in enzymes which deiodinate T_4 to T_3 ($5'$ deiodinase, type II), and about 50% of the T_3 in the thyrotroph is formed in the cell by this mechanism. Therefore, intrathyrotroph T_3 is a more important regulator of TSH synthesis than serum T_3 (De Vito, 1989).

Chin and co-workers (1985) have shown in a mouse model that T_3 in supra-physiological amounts lowers the level of mRNAs which control formation of the TSH subunits, particularly the beta subunit. It appears that T_3 has a direct action at the chromosomal level. High levels of thyroid hormones also reduce the number of TRH receptors on the thyrotroph and T_3 may have a minor inhibitory role on the hypothalamus (De Vito, 1989).

TSH concentrations show diurnal variation, peak levels being from about 12 midnight to 3 or 4 A.M. and the nadir occurring from about 11 A.M. till noon (Adriaanse et al, 1992). The diurnal range is about 1 mU/l. The secretion is pulsatile with peaks every 10 minute which are on average 50% greater than the basal values (Greenspan et al, 1986).

TSH combines with a specific transmembrane receptor in the follicular cells. The receptor has an extracellular region containing 398 amino acids, seven transmembrane segments and an intracellular tail (Vassart et al, 1992). The beta subunit of TSH interacts with the extracellular region of the receptor, while the alpha subunit interacts with the cell membrane through the carboxyl end of the alpha subunit or a sugar residue attached to it.

This interaction produces several important intracellular signals (Takazawa et al, 1995; Corvilain et al, 1994). There is an increase in cAMP, which probably controls iodine uptake into the follicular cell (Chan et al, 1987). There is also an

increase in phosphatidyl inositol degradation and changes in microtubules which result in increased iodide transport into the follicular lumen and increased iodine organification (Corvilain et al, 1994) and iodination of thyroglobulin (Kondo et al, 1989).

1.1.2.3 Formation Of Thyroid Hormones

The functional unit of the thyroid is the follicle. Follicles consist of an outer layer of epithelial cells surrounding the central mass of colloid. The follicular cells are critical for the production and secretion of thyroid hormones, although some of the synthetic steps occur at the interface of the apex of the cell and the colloid. Iodide is transported into the cell and combines with tyrosine to form iodotyrosines. These are coupled to form thyroxine and triiodothyronine hormones which are stored in the colloid, on molecules of thyroglobulin from which they are released as required (Weiss et al, 1984 a; and 1984 b). The synthesis of thyroid hormones takes place in 4 steps.

Iodine in the serum is actively transported into the follicular cell by a mechanism situated at the base of the cell. This is the first and main rate-limiting step in biosynthesis of the thyroid hormones. There is a Na^+/I^- symporter, and probably an I^- channel which concentrates iodine against a chemical and electrical gradient (Carrasco, 1993). The concentration of iodine inside the cell is 20-40 times that in the extracellular fluid, and the ratio can be increased when the thyroid is under TSH stimulation (Marcocci, 1989). Thyroid cells must be intact to trap iodine and the mechanism can be saturated by excess iodine (Many et al, 1992), or inhibited by other anions such as thiocyanate and perchlorate. The transport requires oxidative metabolism and phosphorylation and is increased by high levels of TSH (Carrasco, 1993).

All subsequent steps in formation and storage of thyroid hormones take place at the colloid interface (De Vijlder et al, 1989). Iodine which has been trapped is rapidly transferred to the lumen of the follicle where it combines with tyrosine in thyroglobulin molecules. Iodide must be oxidised to I^0 for this to occur. This is achieved by an enzyme, thyroid peroxidase, in the presence of hydrogen peroxide (H_2O_2) (Carrasco, 1993). This peroxidase is found in microsomes and is the antigen against which antimicrosomal antibodies are directed (McDougall, 1992).

The H_2O_2 necessary for this process is generated via NADH-cytochrome b5 reductase or NADPH-cytochrome C reductase (Hall 1989). The generation of H_2O_2 is controlled through the Ca^{2+} /phospholipase-C pathway, which releases diacylglycerol and inositol 1,4,5-trisphosphate (IP_3) from phosphatidylinositol 4,5-bisphosphate (Takazawa et al, 1995); diacylglycerol activates protein kinase-C,

while IP_3 activates Ca^{2+} release from the endoplasmic reticulum (Björkman and Ekholm, 1992).

The combination of one iodine atom with tyrosine produces monoiodotyrosine (MIT), while binding of two produces diiodotyrosine (DIT). The most important action of anti-thyroid drugs such as propylthiouracil and methimazole are to interfere with this synthetic step, preventing the organification of iodine (Carrasco, 1993).

Two iodotyrosines then combine to form iodothyronine: two DIT molecules produce T_4 and one MIT plus one DIT produces T_3 (figure 1.1). The tertiary structure of thyroglobulin brings the precursor DIT molecules into apposition. Coupling is thought to involve formation of a diphenylether ring, and is catalysed by the same peroxidase enzyme which is involved in oxidation and organification of iodine (Marshall, 1992).

Thyroglobulin makes up the majority of the colloid. It is a large glycoprotein of relative molecular mass 660000, consisting of two dimers of 330000 (Malthiéry and Lissitzky, 1987). It contains approximately 5170 amino acids, of which 120-130 are tyrosine, and about 10% of the total weight are carbohydrate. Iodination is a post translational step which takes place at the interface of the cell and colloid (Chambard et al, 1987). In the normal euthyroid person, when there is adequate iodine in the diet, it has been determined that each thyroglobulin molecule has approximately 7-10 MIT, 5-10 DIT, and 2 T_4 molecules, and there is one T_3 for every 2 or 3 thyroglobulin molecules (Chambard et al, 1987).

Before thyroid hormones are secreted, thyroglobulin has to be taken into the follicular cell and hydrolysed to release the active hormones. Droplets of thyroglobulin are engulfed by pseudopods from the apex of the cell. Colloid droplets enclosed in apical membranes are taken into the cytoplasm of the follicular cell, and lysosomes containing proteolytic enzymes migrate towards them and fuse with them producing phagolysosomes. Migration of these organelles is regulated by intracellular microtubules and microfilaments. Enzymatic degradation of thyroglobulin produces T_4 , T_3 , MIT, DIT and amino acids. The first two are secreted into the circulation, while the iodotyrosines are deiodinated by intracellular deiodinases and the iodine used again for iodination of new thyroglobulin (Marshall, 1992).

1.1.2.4 Transport Of Hormones In Serum

Almost all thyroid hormone in the circulation is reversibly bound to carrier proteins. The small proportion of unbound, or free hormone is metabolically active.

In serum only 0.03 % of T_4 and 0.3 % of T_3 are free, so thyroid hormones are not lost in the urine unless there is severe proteinuria (Marshall, 1992). The protein-bound hormone acts as a buffer, so that sudden changes in levels of hormones in the blood do not cause sudden alterations in thyroid function.

Three plasma proteins are important in transport: thyroid binding globulin (TBG), thyroid binding pre albumin (TBPA), and albumin.

TBG is a single chain glycoprotein produced by the liver. It has a relative molecular mass of 54000 and is present in the lowest concentration of the three, approximately 10-20 mg/l (Tani et al, 1994). The affinity of TBG for T_4 and T_3 is the highest of the transport proteins and although present in the lowest concentration, it carries approximately two-thirds of both hormones. There is one binding site for T_3 and T_4 on each molecule. When TBG is fully saturated, it can carry 150-360 nmoles of T_4 per litre. Since the reference range for T_4 is 56-150 nmol/l, TBG is about one-third saturated. TBG has a half-life of about 5-6 days, and its major site of metabolism is the liver (Robbins and Edelhoch, 1986).

Thyroxine binding pre albumin is present at a concentration of approximately 250 mg/l and has the capacity to bind 300 μ g T_4 . However, it has a lower affinity than TBG for T_4 and normally carries about 10-20% of the circulating hormone. It consists of four identical polypeptide chains which are arranged so there is a central channel containing two T_4 binding sites. Usually only the first site is occupied and only one out of 300 TBPA molecules carries T_4 . TBPA transports very little T_3 . TBPA is produced in the liver, has a half-life of about 1-2 days and it is very sensitive to illness and malnutrition, which cause the level to fall as a direct result of lowered synthesis. It contains no carbohydrate (Robbins and Edelhoch, 1986).

Albumin has the largest capacity for binding thyroid hormones. However, because it has the lowest affinity, it carries only 5-15% of T_4 and about 30-50% of T_3 (Robbins and Edelhoch, 1986).

1.1.2.5 Actions Of Thyroid Hormones

Thyroid hormones in excess or deficiency have dramatic effects on whole animals and on organ systems. In humans, foetal brain and skeletal development are greatly impaired by lack of thyroid hormones. The oxygen consumption of almost all tissues increases with increasing levels of thyroid hormones, the exceptions being the spleen, testis and adult brain (McDougall, 1992).

The actions of thyroid hormones occur mainly in the nucleus and to a lesser extent at the cell membrane and mitochondria. Thyroid hormones enter the cell by diffusion or by binding to a surface receptor and then bind to a nuclear receptor.

The receptor has highest affinity for triac (deaminated T_3), then T_3 , T_4 and reverse T_3 , and this is consistent with the relative metabolic effects of these hormones (Bracco et al, 1993).

There are two genes encoding thyroid receptor proteins, one on chromosome 3, the other on chromosome 17. There are designated c-erb-A beta and c-erb-A alpha respectively (Brent, 1994). Although the two receptors are slightly different structurally, they have the same affinities for thyroid hormones. The receptor has been found in all thyroid hormone responsive cells, and there is good correlation between the amount of receptor in a tissue and the effect of thyroid hormones on that tissue (Brent, 1994). Under normal conditions about 50% of the receptor sites are occupied by thyroid hormones; therefore, the receptor capacity is low. There is also good correlation between the number of receptor sites occupied by T_3 and the thyromimetic effects. Among the sites occupied, about 80% contain T_3 and 10-15% contain T_4 (McDougall, 1992).

T_3 modulates gene expression, usually positively. After addition of T_3 , to responsive cells in vitro, it is possible to demonstrate an increase in RNA polymerase I, ribosomal RNA, RNA polymerase II and messenger RNA. This is followed by synthesis of new protein (Brent, 1994).

Many proteins are known to be produced in increased amounts following increases in thyroid hormone receptor occupancy. These include growth hormone, malic enzyme, acetylcoenzyme A carboxylase, fatty acid synthetase, and glucose-6-phosphate dehydrogenase (Brent, 1994; Weinberger et al, 1986). Somewhat paradoxically, thyroid hormone decreases formation of TSH and prolactin in pituitary cells (Davis et al, 1986).

Thyroid hormone causes an increase in transport of glucose into cells within minutes. The time course is not consistent with a nuclear effect causing translation or transcription of proteins which would facilitate this and provides evidence for effects not involving the classic receptor pathway. In contrast, a similar increase in transport of amino acids is delayed for many hours and probably is due to a nuclear action of T_3 , rather than an action on plasma membranes (McDougall, 1992).

1.1.2.6 Metabolism Of Thyroid Hormones

T_4 is the most abundant thyroid hormone. It is converted to the more active form, T_3 , by removal of an outer ring iodine by the enzyme 5' deiodinase. There are two forms of this enzyme: one, type I, is found in liver, kidney and muscle cells; the other forms, type II, is in pituitary and brown fat cells (Brent, 1994). An alternative breakdown pathway is through formation of reverse T_3 (rT_3) by enzymatic removal of an inner ring iodine (Brent, 1994). This enzyme is a 5 deiodinase (figure 1.3).

The deiodination of T_4 forms about 50% T_3 and 50% rT_3 . The extent of outer ring deiodination is reduced in severe illness, starvation, and by medications such as propylthiouracil, steroids, and propranolol (Beech et al, 1993). These factors inhibit the 5' deiodinase enzyme, type I, which is also involved in deiodination of rT_3 ; therefore, the concentration of T_3 is lower and that of rT_3 is higher under these circumstances. Normally about 80% of T_4 is broken down by deiodination, and the remainder by being conjugated with glucuronide or sulphate in the liver, deaminated, decarboxylated, or subjected to ether cleavage. The net result is formation of metabolically inert compounds, although deaminated T_4 (tetraac) and T_3 (triac) do retain activity. These compounds are also subject to deiodination which removes all activity (McDougall, 1992).

1.1.2.7 Auto-Regulation Of Thyroid Function

The control mechanisms described for the synthesis of thyroid hormones, although exquisitely sensitive to alterations of free thyroid hormone level in blood, do not work instantaneously. In addition, they fail to account for potential changes in thyroid hormone levels brought about by rapid changes in plasma inorganic iodine. The amount of iodine in the diet varies considerably; and there are also sources of iodine such as medications and antiseptics which suddenly provide many orders of magnitude of iodine more than is required for normal function. The thyroid has several well-recognised mechanisms independent of the hypothalamic-pituitary axis which help preserve normality. This was termed auto regulation by Ingbar in 1972 (Nagataki and Ingbar, 1986).

A rise in plasma inorganic iodine causes a reduction in the rate of uptake of iodine into follicular cells. High concentrations of iodide also inhibit both iodination of TG and hormone synthesis (Gruffat et al, 1992). This is called the Wolff-Chaikoff effect (McDougall, 1992). It is transient and in normal subjects usually only lasts for several days. Break-through from this protective mechanism in patients is almost always due to the reduction in the rate of iodine transport. Therefore, there is no longer an excess of intracellular iodine and the thyroid cells can return to the normal rate of hormone production (McDougall, 1992).

When there is a deficiency of iodine, the transport rate of iodine into follicular cells increases. In man the resultant changes are not easy to separate from those caused by increased TSH and, indeed, iodine deficiency probably augments the TSH effects. There is an increase in formation of MIT and T_3 ; therefore, the more active hormone which contains less iodine is produced preferentially (McDougall, 1992).

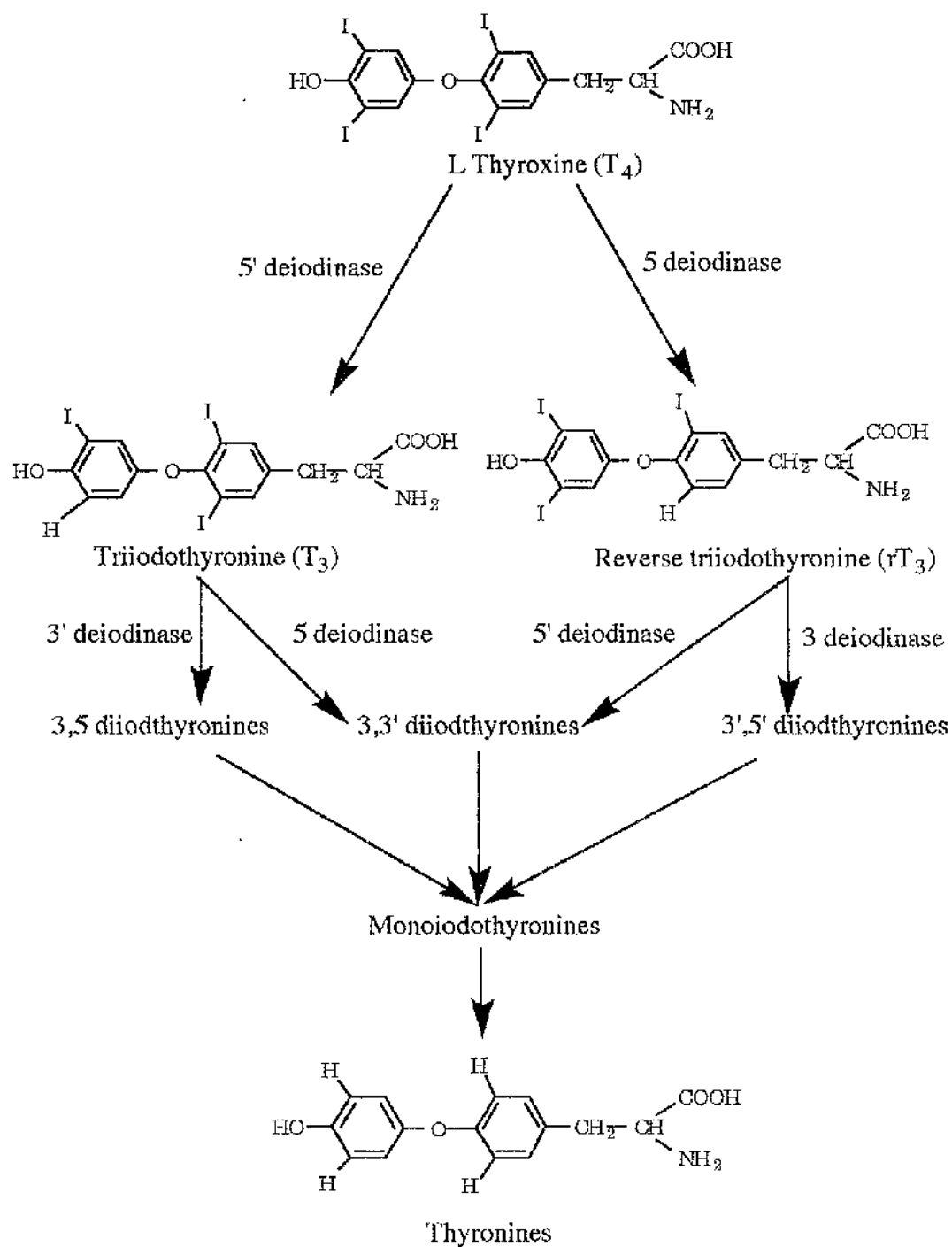


Figure 1.3: Metabolism of thyroid hormones through deiodination of hormones.

1.1.3 Peptides In The Thyroid Gland

Nearly 70% of the cells of the thyroid gland consist of thyrocytes. The remaining cells include endothelial cells, fibroblasts, sympathetic and parasympathetic nerve cells, and parafollicular cells which produce calcitonin (Dumont et al, 1992).

Development of goitre, with an increase in the number of thyrocytes and other cells in the thyroid, is not simply the effect of stimulation of the thyrocytes with TSH, or with TSAb in Graves' disease, but involves many other factors, including cell to cell signalling among the various populations of cells in the gland and environmental factors (Dumont et al, 1991; and 1992). It is known that growth factors such as EGF and FGF are present in the thyroid, and it is likely that growth of the gland is controlled by interactions between the various cell types, co-ordinated by growth factors and other peptides.

Another of the consequences of disease is the generation of new blood vessels and infiltration of a variety of mononuclear cells from peripheral blood which form aggregates or are diffusely distributed between and within the thyroid follicles (Nagataki and Eguchi, 1992). These cells are retained in the gland, by interaction with adhesion molecules, on other cells including thyroid epithelial cells. This interaction is influenced by the presence of cytokines released locally. The interaction of cytokines and growth factors with thyrocytes could also influence their functional pattern.

In addition, the thyroid gland is richly innervated by nerves of different types (adrenergic, cholinergic, and peptidergic) which terminate close to blood vessels and follicular cells (Ahrén, 1986 a; and 1991). It is likely, therefore, that neurotransmitters released locally may directly influence the activity of the follicular cells (Ahrén, 1986 a; and 1991).

Peptides which have been shown to be present in the thyroid gland or are known to affect its function include endothelin, substance P and other tachykinin peptides, VIP, neuropeptide Y, and pituitary adenylyl cyclase activating peptide (PACAP). Substance P, vasoactive intestinal peptide (VIP), and neuropeptide Y (NPY) are found in nerves innervating the thyroid, while endothelin has been detected in thyroid epithelial cells. In addition, it is known that several cytokines associated with the immune response are present in thyroids from patients with autoimmune thyroid disease. These include interleukin-1 (IL-1), IL-6, and tumour necrosis factor α (TNF- α). It is likely that these affect both the growth pattern of the gland and the secretion of thyroid hormones, though only limited studies have been carried out, particularly on the possible effects of the neuropeptides.

SECTION: TWO

IMMUNITY

Normal immune responses are controlled by the reactivity of T lymphocytes to antigens. Since the pattern of responsiveness of these cells is determined by random combinations of T cell receptor genes, there is always a chance that some T cells will react with the body's own proteins. It is therefore necessary to develop tolerance to "self" antigens to allow normal growth, development, and maintenance of existence. When the integrity of self-tolerance is disturbed, an immune response to self may develop. This is termed autoimmunity. While the presence of autoantibodies is a common finding, frank autoimmune disease only develops in a few individuals.

Thyroid autoimmune diseases, the subject of this thesis, are organ specific auto-immune diseases in which there is production of antibodies against different components of thyroid follicular cells including thyroglobulin, microsomes and the TSH receptor (Benjamini and Leskowitz, 1992). Since some knowledge of immunological theory is required to discuss thyroid autoimmunity, a brief review of immunology and autoimmunity will be presented here.

1.2.1 Normal Immune Response

The sequence of events in a normal immune response culminating in the production of antibody can be outlined as: uptake of antigen by antigen presenting cells; its processing and presentation to helper T lymphocytes (a subset of the T lymphocytes which mature in the thymus); stimulation of these T lymphocytes to multiply; and synthesis of antibody by B lymphocytes (which develop in the bone marrow) under the influence of these helper cells (Benjamini and Leskowitz, 1992). Communication between these cells is carried out both by direct cell-cell interaction and by soluble peptide mediators called cytokines. This is a general term for a wide variety of growth factors and other active peptides produced by many different cell types, but the ones active in the immune response are primarily of the interleukin (IL) series. The actions of cytokines which are relevant to the regulation of the immune response will be discussed in this section, while their direct action on thyroid cells will be discussed in chapter 3.

1.2.1.1 Macrophages

Although many cells such as lymphoid dendritic cells, B lymphocytes, and

endothelial cells (Weetman, 1991) are able to present antigen to T cells, macrophages are still regarded as the classical antigen presenting cells.

A soluble foreign antigen may be taken up by phagocytosis or pinocytosis, but for the induction of a productive immune response, the antigen must enter the antigen presenting cell (APC) via a receptor such as those specific for mannose, complement, and the *Fc* fragment of immunoglobulin. This allows foreign antigens to be taken up in preference to potentially competing self antigens (Lorenz et al, 1990).

T lymphocytes only recognise peptide fragments of antigens. These determinants, or epitopes, are around 10-20 amino acids in size, contrasting with the epitopes recognised by B cells which usually comprise conformational determinants depending on the tertiary structure of the intact antigen. The APC must produce these peptide fragments by processing. The exact mechanisms involved in processing have not yet been identified, but lysosomes may be particularly important for phagocytosed antigens, whereas endosomes may contain proteases capable of degrading antigen taken up by receptor-mediated endocytosis (Weetman, 1991). Fusion of endocytic vesicles with others capable of producing degradation is a further possibility (Lanzavecchia, 1990).

Antigenic peptides must be associated with molecules encoded by the major histocompatibility complex (MHC) before their recognition by the T cells. MHC molecules can be divided into two kinds: class I, encoded by the human leukocyte antigen (HLA) A, B, and C regions, and the MHC class II, encoded by the HLA-DR, DQ and DP regions (Benjamini and Leskowitz, 1992). This dichotomy in antigen presenting molecules is reflected by the existence of two subsets of T cells identifiable by their surface markers: CD8 cells, which recognise class I MHC, and CD4, which recognises class II. After uptake and processing, soluble antigens are bound to and presented exclusively by class II MHC molecules which are also called Ia molecules (Weetman, 1991).

One of the key determinants for stimulation of CD4⁺ T cells is the amount of Ia expressed by an APC. A number of factors influence Ia expression by APC. Interferon- γ (INF- γ) derived from stimulated T cells is a key cytokine responsible for such effects (Steeg et al, 1982), though several other modulators of macrophage Ia expression have been identified. In particular, prostaglandins and hydrocortisone (Unanue, 1984) and IL-10 (Quesniaux, 1992) down-regulate Ia expression.

1.2.1.2 T Lymphocytes

T lymphocytes mature in the thymus and then migrate to secondary lymphoid tissues such as lymph nodes. They recognise processed antigenic peptide

combined with an Ia molecule by means of a T cell receptor specific for both antigen and Ia. During their residence in the thymus, cells are selected positively for the ability to recognise peptides only in the presence of Ia and those which recognise self antigens are removed by negative screening.

More than 90% of mature T cells in humans express a clone-specific T cell receptor comprising an α and β glycoprotein chain in association with either CD4 or CD8, while the remaining T cells express a receptor comprising γ and δ chains (Weetman, 1991; Benjamini and Leskowitz, 1992; Janeway and Travers, 1994). The receptor is only triggered by peptide bound to a particular MHC molecule; class II molecules in the case of CD4⁺ T cells, or class I in the case of CD8. Both $\alpha\beta$ and $\gamma\delta$ forms of receptor are non-covalently linked to a complex of peptides with extracellular and intracellular domains termed CD3, which is responsible for signal transduction after receptor-ligand binding. (Weetman, 1991; Benjamini and Leskowitz, 1992; Janeway and Travers, 1994).

The major activities of T cells can be divided into helper functions, cytotoxicity and suppression.

Helper cells (TH) are required for the activation and differentiation of B cells and cytotoxic and suppressor T cells. There are two CD4⁺ subsets described in mice which produce discrete sets of cytokines. TH2 cells (stimulators of antibody production) produce IL-3, IL-4, IL-5 and IL-6, whereas TH1 cells (stimulators of the delayed type hypersensitivity response) produce IL-2, IL-3, interferon γ (INF- γ), lymphotoxin and TNF (Janeway and Travers, 1994). There is regulatory feedback between the two classes of cells, with IL-4, IL-10, and IL-13 from TH2 cells inhibiting the development of TH1 cells. TH1 cells are strongly promoted by IL-12, produced by APCs (Janeway and Travers, 1994).

It is not yet clear whether a similar dichotomy exists in man. Some clones with a similar pattern of cytokine release to the murine TH1 and TH2 classes have been isolated, but many human and murine CD4⁺ T cell clones appear capable of producing a mixed pattern of cytokine release, so-called TH₀ (Janeway and Travers, 1994). One possible explanation for this anomaly is that CD4⁺ T cells begin with one phenotype and function and that some of these mature to become one of several potential CD4⁺ subsets; the density of ligand binding to the T cell receptor and the cytokine and hormonal environment could modulate this development (Weetman, 1991).

As already mentioned, cytotoxic CD8⁺ T cells recognise antigen presented by class I MHC molecules. The prime role of CD8⁺ T cells seems to be reactivity against intracellular antigens such as viruses. The viral antigens are partially degraded to peptides by a proteasome, a multisubunit protease complex which is situated near the endoplasmic reticulum (Janeway and Travers, 1994). The peptides

derived from the foreign protein selectively pass through a complex of transport systems known as "transporters associated with antigen processing" (TAP-1 and TAP-2) to the lumen of the endoplasmic reticulum, where MHC class I is synthesised. Once expressed on the cell surface, this antigen/MHC class I complex is recognised by a specific CD8⁺ T cell and the infected cell then is killed (Benjamini and Leskowitz, 1992; Janeway and Travers, 1994).

The phenomenon of T cell-mediated suppression is controversial, because the specificity and mechanism of action of suppressor T cells is unknown (Janeway and Travers, 1994). Experimentally it has been shown that the activity of CD4⁺ or CD8⁺ lymphocytes can be suppressed by a population of T cells. These cells are necessary for tolerance to self proteins and preventing tissue damage (Janeway and Travers, 1994), because depletion of suppressor T cells lead to aggravated responses to self antigen and grafts. A distinct suppressor cell growth factor derived from monocytes, possibly IL-10 or transforming growth factor β (TGF- β), appears to be necessary for differentiation of human CD8⁺ suppressor cells (Webb et al, 1994), following their initial stimulation by CD4⁺ inducer cells and IL-2.

Antigen-specific CD8⁺ suppressor cell clones have been established from patients with leprosy, capable of inhibiting CD4⁺ T cell proliferation induced by *Mycobacterium leprae*. These clones appear to use the normal $\alpha\beta$ T cell receptor. Furthermore, the suppressor cells appear to recognise specific antigen in a MHC-restricted fashion, but their effect is not necessarily specific or MHC-restricted; they appear to induce inactivation or anergy in the CD4⁺ population by an unknown mechanism (Weetman, 1991). Other explanations for T suppressor cell effects include: (i) release of antigen-specific suppressor factors, (ii) cytotoxicity to helper cells and (iii) reactivity of the suppressor cells against the variable region (idiotype) of the T cell receptor on the target clone, thus behaving as anti-idiotypic cells. It is also apparent that either CD4⁺ or CD8⁺ T cells may become suppressor cells, and that there is considerable leeway for changing function during development of an individual cell, depending on ambient conditions (Weetman, 1991).

1.2.1.3 B Lymphocytes

Each B lymphocyte expresses a surface immunoglobulin or antibody, capable of unique binding to an antigen and generated by a variety of mechanisms to induce diversity. The regulation of B cell development, from a resting state to a plasma cell secreting immunoglobulin, generally depends upon antigen recognition (via surface immunoglobulin) followed by proliferation and differentiation in response to cytokines (Weetman, 1991). Activated human B cells proliferate in

response to IL-4 and this is enhanced by INF- γ ; many other cytokines costimulate this, including IL-1, IL-2, IL-6, TNF, and lymphotoxin. Differentiation seems primarily dependent on IL-6 but this too is costimulated by IL-1, IL-2 and INF- γ (Janeway and Travers, 1994). Transforming growth factor- β inhibits both proliferation and differentiation (Weetman, 1991).

As mentioned earlier, B cells can act as APCs. They have been shown to capture antigen by binding it to their specific immunoglobulin receptors, to internalise and degrade the immunoglobulin-antigen complexes, and then to re-express fragments of the antigen, on their surfaces in association with MHC class II molecules. Additionally, B cells express IL-1 and the membrane protein B7 to serve as second signals (Benjamini and Leskowitz, 1992).

1.2.1.4 Cytokines

The cytokines studied in the current work are IL-1, IL-2, IL-4, IL-6, TNF- α , INF- γ , and TGF- β .

1.2.1.4.1 *Interleukin-1*

Interleukin-1 is synthesised primarily by macrophages, although it is now recognised that most cells can make this molecule (Di Giovine and Duff, 1990). There are at least two species of interleukin-1, IL-1 α and IL-1 β , with only limited sequence homology. They are non-glycosylated peptides with a relative molecular mass of 17500.

IL-1 is central to the activation of resting T cells, while those T cells already activated are dependent on other cytokines like IL-2, IL-4, INF- γ (Janeway and Travers, 1994), and IL-12 (Trinchieri, 1993) for optimal differentiation and proliferation. Other T cell activities, such as cytokine release, may have less requirement for IL-1 (Di Giovine and Duff, 1990).

Macrophage IL-1 synthesis is stimulated by phagocytosis of micro-organisms. Uptake of soluble protein antigens has no direct effect, but their presentation to the T cell leads to release of T cell cytokines such as tumour necrosis factor (TNF), which indirectly stimulate production of IL-1. There is evidence that T cell-APC contact is also stimulatory (Weaver et al, 1989).

Another member of the IL-1 family, known as the IL-1 receptor antagonist (IL-1ra), inhibits the action of IL-1 on its target cells (Svenson et al, 1993; Mandrup-Poulsen et al, 1993; Blakemore et al, 1995). IL-10 (Moore et al, 1993; Bogdan and Nathan, 1993), TGF- β (Bogdan and Nathan 1993; Ruscetti et al, 1993) and IL-4 (Bogdan and Nathan, 1993), inhibit the production of IL-1 by

macrophages.

1.2.1.4.2 *Interleukin-2*

Interleukin-2 (IL-2) is a glycoprotein of relative molecular mass 15500 synthesised and secreted primarily by activated T helper (CD4⁺) lymphocytes (Benjamini and Leskowitz, 1992). At this level, IL-2 is an autocrine factor, driving the expansion of the antigen specific cells (Minami et al, 1993). The synthesis of IL-2 is enhanced by IL-1 produced by activated APC, while INF- α maintains an enhanced level of IL-2 mRNA in the activated cells (Holan et al, 1994).

IL-2 also acts as a paracrine factor. It enhances the proliferation and differentiation of both B cells (Rubin and Nelson, 1990) and cytotoxic T cells (Benjamini and Leskowitz, 1992). IL-2 also enhanced the production of prostaglandin E2 by human monocytes or macrophages activated with lipopolysaccharide (Valitutti et al, 1989).

Defects in IL-2 production have been reported in patients with systemic lupus erythematosus (Horwitz et al, 1994), rheumatoid arthritis (Miyasaka et al, 1984), insulin-dependent diabetes mellitus (Zier et al, 1984), and Graves' disease (Akasu et al, 1991; Eisenstein et al, 1988). The reason for this is unknown, but there are reports suggesting that excessive prostaglandin E2 production by activated APC could be one of the causes (Eisenstein et al, 1994; Hilkens et al, 1995). The production of IL-2 was restored to normal after therapy.

The receptors for IL-2 were originally referred to as high, intermediate, and low affinity receptors. Now it is recognised that the high affinity receptor contains three distinct subunits, IL-2R α , IL-2R β , and IL-2R γ , while the intermediate affinity receptor contains two subunits, IL-2R β and IL-2R γ . The β chain also binds IL-15 (Grabstein et al, 1994), while the γ chain is a common subunit for IL-4, IL-7, and IL-15 (Leonard et al, 1994; Voss et al, 1994).

It is known (Murakami et al, 1995; Murphy et al, 1994; Mariotti et al, 1992 a, and 1994; Balázs and Farid, 1991) that the concentration of a soluble form of the interleukin-2 receptor is elevated in thyrotoxicosis and in many other diseases which are thought to have an immunological component. The soluble form of the receptor which has been described is an α chain. This is a membrane glycoprotein of relative molecular mass 55000, capable of weak binding of IL-2 (Robb and Greene, 1983, Minami et al, 1993).

1.2.1.4.3 *Interleukin-4*

Interleukin-4 was previously known as B cell growth factor-1 (BCGF-1 or

BSF-1) or B cell differentiation factor (BCDF) by virtue of its ability to costimulate B lymphocyte proliferation (Idzerda et al, 1990). It is primarily produced by activated type 2 helper T cells and has diverse functions on a variety of cell types. The primary functions of IL-4 on B cells are activation, growth, induction of MHC class II, and production of immunoglobulins (Weetman, 1991; Benjamini and Leskowitz, 1992; Janeway and Travers, 1994). In the mouse, IL-4 preferentially induces class switching to IgG1 and IgE, while it inhibits IgM, IgG3, and IgG2a production (Weetman 1991; Janeway and Travers, 1994). Its functions on T cells include induction of growth, promotion of survival, down-regulation of cytokine production by cytotoxic T cells (Janeway and Travers, 1994) and suppression of production of IL-2 and the β chain of IL-2R (Llorente et al, 1989; Linqvist et al, 1991). IL-4 also regulates the proliferation and differentiation of CD8 cytotoxic cells synergistically with IL-2 (Hargrove et al, 1993).

IL-4 inhibits macrophage activation by down-regulation of IL-1 production (Bogdan and Nathan, 1993) or down-regulation of c-fos and c-jun mRNA (Dokter et al, 1993), but it induces monocyte-macrophage differentiation and expression of MHC class II (Te Velde et al, 1988).

1.2.1.4.4 *Interleukin-6*

IL-6 is a multi-functional cytokine that is produced by a range of cells, including monocytes or macrophages, T lymphocytes, and activated B cells (Janeway and Travers, 1994). Sources of non-hematopoietic origin include rat testicular cells (Syed et al, 1993), fibroblasts, endothelial cells (Bartalena et al, 1994 a), and thyrocytes in autoimmune thyroid disease (Watson et al, 1994; Zheng et al, 1991) or stimulated in vitro (Kennedy et al, 1992; Iwamoto et al, 1991). It is also produced by many carcinoma cells such as carcinoma of kidney, colon, breast, and pancreas, and malignant melanomas (Gastl et al, 1993).

IL-6 binds to a high affinity receptor complex consisting of two membrane glycoproteins (Baumann et al, 1990; Hibi et al, 1990). This consists of an IL-6 binding subunit (IL-6R or CD 126) and a signal-inducing protein, known as gp130 or CDw 130, that is common for several cytokines, including IL-6 and IL-11 (Yin et al, 1993). The IL-6 receptor has been detected on monocytes, granulocytes, resting and activated CD4⁺ T cells, natural killer cells, and activated, but not resting, B lymphocytes (Wognum et al, 1993).

The effect of IL-6 on B cells is to stimulate differentiation and antibody secretion (Weetman, 1991; Janeway and Travers, 1994). IL-6 exhibits growth factor activity for mature thymic or peripheral T cells and enhances the differentiation of cytotoxic T cells in the presence of IL-2 or INF- γ (Janeway and Travers, 1994). IL-

6 stimulates production of acute phase proteins by hepatocytes, and the activity of hematopoietic stem cells (Weetman, 1991). It stimulates the growth of sarcomas, carcinomas, EBV-transformed B cells, keratinocytes, and mesangial cells (Hirano, 1990; and 1992).

IL-6 and IL-1 help to co-ordinate the body's responses to infection. IL-6 activates hepatocytes to synthesise acute phase proteins such as C-reactive protein (CRP), a process in which IL-1 co-operates by inducing IL-6 production by Kupffer cells (liver macrophages). CRP acts as an opsonin, and this activity is augmented by enhanced recruitment of neutrophils from the bone marrow (Janeway and Travers, 1994). Other acute phase proteins protect the body against proteases (e.g. alpha-1 antitrypsin) or bind toxic products released from cells (e.g. haptoglobin). A major effect of IL-1 and IL-6 is to act on the hypothalamus, altering the body's temperature regulation, and on muscle and fat cells, altering energy mobilisation to increase the body temperature, which may decrease bacterial and viral replication (Janeway and Travers, 1994).

1.2.1.4.5 Tumour Necrosis Factor

Tumour necrosis factor alpha (TNF- α) is predominantly produced by activated macrophages (Tracey and Cerami, 1994; Beyaert and Fiers, 1994), though several other cell types including activated T-cells (CD4⁺ and CD8⁺), B-cells, NK cells, neutrophils, endothelial cells, and smooth muscle cells (Vilcek and Lee, 1991) can produce small amounts of this cytokine. The TNF family includes two structurally and functionally related proteins, TNF- α or cachectin, and TNF- β or lymphotoxin (Tracey and Cerami, 1994; Beyaert and Fiers, 1994; Vilcek and Lee, 1991).

Under denaturing conditions, the relative molecular masses of human TNF- α and TNF- β were approximately 17000 and 25000 respectively, while in native form both TNF- α and TNF- β are in trimers (Vilcek and Lee, 1991).

There are two distinct TNF receptors with relative molecular mass of 55000 and 75000 (Gruss and Dower, 1995; Smith et al, 1994; Tartaglia et al, 1993; Tartaglia and Goeddel, 1992; Chouaib et al, 1991), known as TNF-R1 and TNF-R2 respectively.

Both types of TNF receptors are present on the plasma membranes of virtually all nucleated cells (Tracey and Cerami, 1994; Tartaglia and Goeddel, 1992; Vilcek and Lee, 1991). In addition, cleaved fragments of both receptor types, also known as TNF-binding proteins, have been detected in the urine and serum of patients with a variety of diseases, including cancer, AIDS, and sepsis (Tracey and Cerami, 1994). They may inhibit the activity of TNF by preventing receptor-ligand

interaction, or may delay clearance from serum (Tracey and Cerami, 1994).

Acute exposure to recombinantly derived human TNF caused a syndrome of shock and tissue injury which are virtually indistinguishable from septic shock syndrome (Tracey and Cerami, 1994; Tartaglia et al, 1993; Tartaglia and Goeddel, 1992), including tissue injury, capillary leakage syndrome, hypoxia, pulmonary edema, and multiple organ failure associated with a high mortality rate (Tracey and Cerami, 1994). On the other hand, the net effect of prolonged exposure to TNF- α by any route is the development of cachexia characterised by anorexia, weight loss, dehydration, and depletion of whole body protein and lipid (Tracey and Cerami, 1994). In both cases, some of the effects are directly attributable to the cytokine itself, whereas others are the result of secondary factors triggered by TNF.

The production of TNF- α is increased by LPS (Haziot et al, 1994), and also by other stimuli like viruses and mitogens. Dayer and Burger (1994) considered that the direct contact of monocytes with activated lymphocytes was the most efficient stimuli for TNF- α production. This was caused by interaction with glycoproteins expressed on the surface of activated lymphocytes, particularly CD11 and CD69. Prostaglandins and their precursors, such as dihomogamma linolenic acid, arachidonic acid, and eicosapentaenoic acid, were shown to inhibit T-cell proliferation while increasing their ability to secrete TNF- α (Kumar and Das, 1994).

The most active inhibitors of TNF- α production by activated macrophages are IL-4 (Ranheim and Kipps, 1995), IL-13 (D'Andera et al, 1995), and IL-10 (Ranheim and Kipps, 1995; Marchent et al, 1994). Granulocyte-macrophage colony-stimulating factor (Rhoades et al, 1995), nitric oxide (Eigler et al, 1995), and noradrenaline (Van-Der-Poll et al, 1994) may also have some inhibitory effects.

1.2.1.4.6 *Interferon- γ*

Interferons are produced by leukocytes and fibroblasts in response to viral infection. The family includes three members: INF- α , INF- β , and INF- γ . INF- γ has major effects on the immune system.

INF- γ is produced by activated CD4⁺ TH1 or CD8⁺ cytotoxic cells (Nagataki and Eguchi, 1992). Its receptor, CD119, is expressed by many cells including macrophages, monocytes, B cells (Janeway and Travers, 1994), endothelial cells, connective tissue cells (Evans and Whicher, 1993), and thyrocytes (Mariotti et al, 1992 b).

Interferons trigger the synthesis of several host-cell proteins that contribute to the inhibition of viral replication. One of these is the enzyme oligo-adenylate synthetase, which polymerises ATP into a series of 2'-5'-linked oligomers that

activate an endoribonuclease that then degrades viral RNA. Another protein activated by interferon α and β is a serine/threonine kinase called P1 kinase. This enzyme phosphorylates the eukaryotic protein synthesis factor (eIF2), thereby inhibiting translation of viral proteins. Interferons also increase expression of the MHC class I and TAP transporter proteins, enhancing the ability of virus-infected cells to present viral peptides to CD8 T cells. They also activate NK cells (Janeway and Travers, 1994).

In addition to the above-mentioned functions of interferons, INF- γ can induce the production of both classes of MHC on APCs (Mariotti et al, 1992 b; Janeway and Travers, 1994), and thyrocytes (Toda et al, 1992; Chiovato et al, 1994; Eguchi et al, 1995). MHC class 2 is not present on the surface of normal thyrocytes (Chiovato et al, 1994, Otsubo et al, 1988). It is also reported that INF- γ acts as a macrophage activating factor and synergises with tumor necrosis factor to effect tumor cell lysis and inhibit cell proliferation (Evans and Whicher, 1993). INF- γ also acts on proliferating CD4 T cells (TH0) cells and causes them to differentiate into inflammatory (TH1) CD4⁺ T cells (Janeway and Travers, 1994).

1.2.1.4.7 *Transforming Growth Factor Beta*

Transforming growth factor- β (TGF- β) is a homodimeric protein of relative molecular mass 25000. The TGF- β family is a group of structurally and functionally related peptides that include at least five members, which are known as TGF- β 1 to 5. Three are present in mammals. While specific receptors for these proteins have been found on almost all mammalian cells examined, the effect of the molecule varies depending on the cell type and growth conditions. Generally, TGF- β is stimulatory for cells of mesenchymal origin and inhibitory for cells of epithelial or neuroectodermal origin (Cirafici et al, 1992). TGF- β is released in a latent form, which must be activated proteolytically by enzymes such as plasmin before it can bind to its receptor.

TGF- β has been shown to regulate the immune response. When produced by CD4⁺ helper (TH2) T cells, it inhibited growth of inflammatory CD4 T cells (TH1).

1.2.2 Autoimmunity

Lymphocytes are prevented from reacting with normal tissues by several mechanisms. Those T cells which are capable of reacting with self antigens are deleted in the thymus by a process of negative selection. However, this mechanism is not complete, and self-reactive T lymphocytes may survive. To prevent damage, other mechanisms are active in peripheral tissues.

The phenomenon of anergy occurs when a T cell encounters antigen without a second activating signal such as the presence of specific adhesion molecules (especially B7) and cytokines. The cell becomes incapable of responding to the antigen. This mechanism for T cell tolerance seems well suited for preventing T cell reactivity against peripheral tissues, which will express class I and II MHC antigens in response to inflammatory or cytotoxic cytokines and thus prevent the fortuitous activation of any non-deleted auto-reactive T cells in the vicinity (Weetman, 1991; Janeway and Travers, 1994).

Another mechanism to control thymically untolerised, self-reactive T cells could be the presence of active suppression. The action of T suppressor cells is essentially similar to that of peripheral tolerance, namely blocking a necessary second signal to helper cells (Weetman, 1991) by production of soluble factors, either antigen specific suppressor factors (TSFs) or non-specific inhibitors such as TGF- β .

The development and maturation of B cells take place in bone marrow. There are two mechanisms ensuring the tolerance to self antigens encountered by immature B cells: deletion and anergy. Immature B cells expressing only IgM are eliminated or inactivated if they bind to abundant multivalent ligands in their environment, such as MHC antigens. These B cells are believed to undergo programmed cell death or apoptosis. The second mechanism takes place when immature B cells bind soluble self antigens. The cells are rendered unresponsive or anergic to the antigen. Only immature B cells that do not encounter antigen in the bone marrow at this stage of development can mature, express surface IgD and IgM and migrate from bone marrow to the peripheral lymphoid tissue (Janeway and Travers, 1994).

Autoimmune disease occurs when a specific adaptive immune response is mounted against self, or, in other words, the immune response has failed and reacted with self antigen. It is not known what triggers the autoimmune response, but many factors could be responsible:

- 1) Autoreactive cells fail to be deleted or rendered anergic.
- 2) Neonatally untolerised T cells fail to be kept in check by peripheral tolerance and suppression mechanisms in adult life.
- 3) Cross-reaction with foreign antigens induces a response against a normally 'silent' self antigen (e.g. drug-induced haemolytic anaemia or myocarditis after streptococcal infection in rheumatic fever).

There is evidence for all these possible mechanisms. It is now apparent that intrathymic self tolerance for T cells is generally incomplete. Indeed neonatal thymectomy in mice results in organ-specific autoimmune disease by enhancing this imperfect clonal deletion.

Whichever mechanism proves to be the most important, the production of cytokines by the activated lymphocytes is likely to be a crucial common mechanism. Their release could expand the population of non-tolerised, self-reactive B cells and CD8⁺ cytolytic T cells, and they may directly cause tissue damage.

SECTION: THREE

AUTOIMMUNE THYROID DISEASE

Thyroid autoimmunity occurs mostly in middle aged females. It leads to several different disorders, comprising the commonest autoimmune diseases in the community. The three main disorders, autoimmune thyroidism (Hashimoto's thyroiditis and primary myxoedema), Graves' disease, and postpartum thyroiditis share reactivity against the same thyroid components, albeit to varying extents (Weetman, 1991). Lymphocytes in the gland are thought to be the major site of production of antithyroglobulin, antimicrosomal, and anti-TSH receptor autoantibodies in patients with these diseases (LiVolsi, 1994). No good animal models of thyrotoxic Graves' disease have been developed, but several models of autoimmune thyroid disease are known, and these have provided invaluable insights into the aetiology and pathogenesis of different classes of thyroid autoimmunity. Generally, susceptibility to thyroid autoimmunity is polygenic, and there is a variety of possible endogenous and exogenous factors which modulate the effects of the genetic component (Weetman, 1991). The major diseases and the possible factors that could influence the development of thyroid autoimmunity are reviewed below.

1.3.1 Thyroid Pathology

1.3.1.1 Hashimoto's Thyroiditis

In Hashimoto's disease, there is chronic lymphocytic thyroiditis. The follicles are small and atrophic, and there is a marked lymphocytic infiltrate (LiVolsi, 1994). There may be fibrosis, and patients are usually hypothyroid.

Autoimmune hypothyroidism is common. In an extensive survey of a village in North-East England, the prevalence was at least 1% in women but less than 0.1% in men (Tunbridge et al, 1977). Thyroid auto-reactivity, that is, the presence of thyroid autoantibodies without thyroid dysfunction, was even more common, being found in 10.3 % of women and 2.7% of men, increasing with age in women only

(Tunbridge et al, 1977). This frequency is related to the prevalence of focal thyroiditis, found at routine autopsy in 6% of men and 22% of women; there is a close association between the presence of such lymphocytic infiltration and circulating thyroid antibodies (Yoshida et al, 1978). Thus there is a high prevalence of thyroid auto-reactivity in the community which progresses to overt disease in around 5-10% of cases. The emergence of gland dysfunction is a slow process. For instance, overt hypothyroidism developed at a rate of 5% per year in subjects who had thyroid antibodies and an elevated TSH, but normal T_3 and T_4 at presentation, while the presence of thyroid autoantibodies alone had no impact over a four year period (Weetman, 1991).

Anti-microsomal (thyroid peroxidase, TPO) antibodies are found in about 90% of patients with Hashimoto's thyroiditis or primary myxoedema by haemagglutination assay (Kaufman et al, 1990). It is also possible that patients negative for circulating TPO antibodies could have sufficient intrathyroidal synthesis to mediate pathogenic effects (Baker et al, 1988).

TPO antibodies are important in tissue injury: they bind to thyrocytes *in vivo* and are cytotoxic in the presence of complement *in vitro*. However, in common with many nucleated cells, thyrocytes are relatively resistant to homologous complement. Sub-lethal membrane attack complex formation impairs thyroid cell function *in vitro*: sustained attack may well exhaust the cell metabolically and lead to lysis (Weetman et al, 1990). There is considerable evidence for immune complex formation in Hashimoto's thyroiditis. Elevated levels of terminal complement complexes are present in the circulation and these are also localised around the thyroid follicles (Weetman et al, 1989). In addition to this strong evidence for complement-mediated injury, a proportion of TPO antibodies can inhibit the function of this key enzyme, which could also lead to thyroid dysfunction (Doble et al, 1988).

Thyroid receptor blocking antibodies may contribute separately to hypothyroidism in some patients. It was clear at an early stage that TSH receptor-binding antibodies occurred in up to 14% of patients with autoimmune hypothyroidism (Mukhtar et al, 1975). Subsequent studies have found that blocking antibodies are present in both Hashimoto's thyroiditis and primary myxoedema (Kraiem et al, 1987).

1.3.1.2 Graves' Disease

Graves' disease is caused by thyroid stimulating antibodies (TSAb) which bind to the TSH receptor and activate adenylate cyclase, resulting in thyrotoxicosis. There is a diffuse goitre, with some lymphocytic infiltrate and hyperplastic

epithelium.

There are many features that are shared with autoimmune hypothyroidism, including the presence of thyroglobulin and TPO antibodies and lymphatic infiltration of the thyroid. Hyperthyroidism is common, affecting 1.9% of women and 0.16% of men surveyed in the North-East of England (Weetman, 1991). The majority of these patients have Graves' disease but exact diagnostic criteria have been variable. Others have single or multiple thyroid nodules. Whilst it is assumed that all patients with Graves' disease have TSAbs, the detection of these antibodies depends on assay sensitivity. In 850 European patients with hyperthyroidism, the condition was defined by the presence of TSH receptor-binding antibodies and/or Graves' ophthalmopathy. Of these patients, 59.5% had Graves' disease while 31.3% remained unclassified (Reinwein et al, 1986). In UK the peak age for developing Graves' disease is between 40 and 60, and females dominate males in the ratio of 4-8 to 1 (McDougall, 1992).

Treatment of Graves' patients in UK is initially with anti-thyroid drugs, such as carbimazole or propylthiouracil, for 6-18 months. Propranolol is used for some patients to control acute symptoms. If patients become hypothyroid as a result of drug treatment, they are given thyroxine replacement therapy. Patients who relapse on drug treatment may be offered treatment with radioactive iodine to ablate the gland or, if they are unsuitable for this, may be treated surgically.

1.3.2 Mechanisms Of Thyroid Autoimmunity

1.3.2.1 Genetic Factors

Observations in experimental animals revealed that certain strains of mice were good responders and others poor responders to lesions developing after thyroglobulin immunisation. Hashimoto's disease was detected in half the siblings of good responders (Weetman, 1991). In humans, there is also evidence for genetic factors. Susceptibility to Graves' disease was 60% in monozygotic twins if one twin had the disease, in contrast to 3-9% in dizygotic twins (Weetman, 1991).

A relationship with HLA molecules has been detected in various types of thyroid autoimmunity. Genetic susceptibility to autoimmune hypo or hyperthyroidism is related to HLA-DR3 (Santamaria et al, 1994; Yanagawa et al, 1994), while postpartum thyroiditis is related to HLA-DR4/5 (Weetman, 1991). Genetic susceptibility to Graves' disease is also related to HLA-DQA1*0501 in Caucasian DR3 haplotypes, while Hashimoto's disease is related to DQB1*0301 (Santamaria et al, 1994).

1.3.2.2 Immunological Mechanisms

1.3.2.2.1 *Thyrocytes as Antigen Presenting Cells*

MHC are now known to be essential components in the presentation of antigen, both self and non-self, to the T cell receptors of helper and suppressor T lymphocytes. While MHC class I antigens are widely expressed in many types of cells including thyrocytes (Nagataki and Eguchi, 1992; Davies et al, 1989), the MHC class II antigen has a more restricted degree of constitutive expression, principally within the immune system. It is now known that thyrocytes can express MHC class II molecules on their surface and act as an APC (Chiovato et al, 1994; Asakawa et al, 1992 a; Volpé, 1991). This led to the suggestion that this aberrant class II expression might initiate or perpetuate thyroid autoimmunity (Bottazzo et al, 1983). This was supported by the fact that in Graves' disease, thyroid follicular cells were able to present antigen through HLA class II to autologous T lymphocytes, derived from thyroid gland (Fukazawa et al, 1991; Weetman et al, 1986 a). HLA class II is also present on the surface of thyrocytes in non-immune thyroid diseases such as non-toxic goitre, nodular goitre, and thyroid carcinoma (Grubeck-Loebenstien et al, 1988), but not on normal thyrocytes (Chiovato et al, 1994; Otsubo et al, 1988) and its expression can be induced by INF- γ (Chiovato et al, 1994). The HLA class II molecules on the surface of thyrocytes, from patients with Graves' disease are more efficient in stimulation and proliferation of both allogeneic and autologous peripheral blood mononuclear cells than those in non-toxic goitre (Grubeck-Loebenstien et al, 1988). When thyrocytes from patients with Graves' disease were incubated with autologous peripheral blood T cells the percentage of HLA-DR positive thyrocytes increased. Furthermore, addition of autologous peripheral blood monocytes to the above co-culture increased the cell expression of HLA-DR on both thyrocytes and T-cells (Otsubo et al, 1988). Tumour necrosis factor- α (TNF- α), which is principally produced by monocytes, is capable of enhancing the expression of HLA class I on thyrocytes (Nagataki and Eguchi, 1992), though the major stimulus may be INF- γ produced by TH1 lymphocytes.

The above findings indicate that expression of HLA class II on the surface of thyrocytes is influenced by cytokines that are produced by activated lymphocytes. This is probably, however, a secondary effect of lymphocytic infiltration of the thyroid gland, but not sufficient by itself for the induction of thyroid autoimmunity.

1.3.2.2.2 *Defects in Lymphocytes*

Examination of T cell infiltrates in thyroid tissue from patients with thyroid

autoimmunity has shown a marked accumulation of a variety of specific T cell subsets, in patients with Graves' disease, whereas the infiltrate cells of Hashimoto's disease have reflected the proportions in peripheral blood (Davies et al, 1992). Nagataki and Eguchi (1992) illustrated that the distribution of CD4⁺ and CD8⁺ cells differed in different regions of thyroid tissue in Graves' disease. CD4⁺ cells were mainly found in lymphoid aggregates, whereas CD8⁺ were scattered among the thyroid follicles. Their report showed that activated T cells (HLA-DR⁺, CD25⁺/CD26⁺), helper and memory T cells (CD4⁺CD29⁺) and cytotoxic cells (CD8⁺CD11b⁻) were increased in the intra-thyroid fraction compared to peripheral blood, and that peripheral blood also had higher numbers than normal controls, while what they described as suppressor-inducer T cells, and is now thought to be the naive T cell population (CD4⁺CD45RA⁺), and natural killer cells were reduced.

Increases in the ratio of helper/suppressor cells led to the hypothesis that reduction of the fraction of suppressor T cells could be one of the predisposing factors for thyroid autoimmunity (Volpé, 1991). Unopposed action of helper T cells would cause production of TSA_b, which would directly stimulate the thyroid and also cause increased production of autoantigens.

While this hypothesis is plausible, the question remains as to the identity of the originating antigen. The existence and identification of specific suppressor cells have frequently been called into question (Martin and Davies, 1992), so this mechanism is still very controversial. However, there is no doubt that accumulation of different classes of activated lymphocytes in thyroid tissue leads to the local release of various cytokines. Activated CD4⁺ helper type 1 or CD8⁺ cells are capable of producing IL-2 and INF- γ , while T helper type 2 cells produce IL-4, IL-5, and IL-10, among others (Nagataki and Eguchi, 1992).

Thyroid infiltrating B cells from Graves' patients are able to synthesise anti-thyroidal antibodies in vitro. Most of these antibodies were IgG (Nagataki and Eguchi, 1992).

Whilst it is highly likely that the intrathyroidal T cells are capable of producing the cytokines necessary to stimulate B cell activation and differentiation, an additional source of a key cytokine in this progression, namely IL-6, is the thyrocyte itself (Nagataki and Eguchi, 1992). Thyrocyte production of IL-6 in vitro is enhanced by other cytokines known to be produced by infiltrating mononuclear cells, in particular INF- γ and TNF, as well as by TSH which is elevated in autoimmune hypothyroidism. Thus, the thyroid may contribute to its own destruction by release of IL-6 that stimulates intrathyroidal B and T cell proliferation and differentiation (Weetman, 1991).

1.3.2.2.3 Cytokines

Hypothyroidism and transient thyrotoxicosis with elevated antimicrosomal antibody titres have been described in patients receiving IL-2 and lymphocyte activated killer cell therapy for advanced cancers (Atkins et al, 1988; Sauter et al, 1992). Similar findings have been reported in patients on long-term treatment with INF- α (Gisslinger et al, 1992). It is postulated that the hypothyroidism may have been caused by unmasking sub-clinical autoimmune thyroiditis (Atkins et al, 1988; Gisslinger et al, 1992). These findings are good evidence that disordered production of cytokines could be a mechanism for initiating or maintaining autoimmune thyroid disease.

1.3.2.2.4 Infection

The role of infectious agents as precipitants is unclear. Analysis of 857 British cases of Graves' disease over a 10 year period revealed no clustering of cases in time or space, indicating that infections behaving in an epidemic fashion are unlikely to be precipitating factors (Cox et al, 1989). Antibodies to *Yersinia enterocolitica* are found with increased frequency in Graves' disease and a saturable binding site for TSH was discovered in lysozyme-treated extracts of this organism (Weiss et al, 1983). Such a TSH receptor-like molecule could provoke the formation of cross-reactive TSAbs (Weetman, 1991). Further investigations have suggested that antibodies against a plasmid-encoded protein in enteropathogenic strains of *Yersinia* are strongly associated with various forms of thyroid autoimmunity, although how this can be reconciled with the specific induction of Graves' disease is unclear (Wenzel et al, 1988; and 1990). Similar TSH receptor-binding material has been found in mycoplasma (Sack et al, 1989). An exogenous retrovirus has also been implicated, based on hybridisation of an HIV-I gag probe with thyroidal DNA from Graves' but not control subjects, although it is unclear where the virus is located and what it is (Ciampolillo et al, 1989).

1.3.2.3 Non-Immune Factors

1.3.2.3.1 Hormones

Thyroid disease is commoner in females than in males. Observations of autoimmune thyroiditis in animal models also show that female mice and rats develop more severe thyroid autoimmunity than males. High doses of female hormones increased the risk, while high doses of testosterone could reverse the

effect (Weetman, 1991).

Stimulation of the gland with TSH is immunomodulatory, since this leads to endogenous TG release and increases the expression of adhesion molecules and HLA class I and II on the surface of thyrocytes. High concentrations of T₄ suppress both thyroiditis and formation of TG antibodies, which may be a direct immunological effect of the hormone (Volpé, 1994). Autoimmune thyroid disease also has been reported in individuals with hyperprolactinaemia (Walker et al, 1993).

1.3.2.3.2 Iodine

Dietary iodine is a major environmental determinant in thyroid autoimmunity. Excess iodine tends to enhance disease, whereas a low iodine diet ameliorates it (Volpé, 1994). Several mechanisms might account for this, including direct thyroid cell toxicity with excess iodine. In the Obese strain (OS) chicken it is known that highly iodinated TG is more immunogenic than poorly iodinated forms (Weetman, 1991).

The increasing incidence of Hashimoto's thyroiditis in the mid-western states of the USA between 1920 and 1960 coincided with the introduction of iodine prophylaxis for endemic goitre and a careful 20 year follow-up of thyroidectomy specimens revealed a much higher frequency of lymphocytic infiltration after the introduction of iodisation (Harach et al, 1985). The introduction of iodised oil as prophylaxis on the island of Corfu resulted in the appearance of thyroid autoantibodies in 43% of subjects six months later (Boukis et al, 1983).

Iodine-containing drugs can also be responsible. Iodine in cough medicine was responsible for goitre in asthma patients, half of whom had thyroid autoantibodies (Weetman, 1991).

1.4 BACKGROUND TO CURRENT STUDY

The aetiology of autoimmune thyroid disease remains unknown. Whatever the initial stimulus, however, an immune response involving T and B lymphocytes, macrophages and their soluble products is the result. The nature of the resultant damage is likely to depend on the particular form of the cellular response, such as whether there is primarily a type 1 or type 2 helper T cell reaction, or whether it is mixed. This will alter the pattern of cytokines which is produced and determines whether the predominant lesion is that of destruction or proliferation.

There is some information as to the effect of cytokines on the thyroid, but this is limited and incomplete. In addition, there is little information as to the likely modifying effects of the other peptides and growth factors which are known to be

present in the thyroid gland.

In this study, the presence of soluble interleukin 2 receptors was studied in the blood of patients with Graves' disease. When abnormalities were found, the investigation was extended to study the release of the IL-2 receptor from samples of blood incubated in vitro, and then to the release of several other cytokines from the same preparations.

In parallel with this study, the effects of several cytokines, growth factors and neuropeptides were tested on thyroid cells in culture. The cell line which was used was the FRTL-5 rat thyroid epithelial cell line, which responds to stimulation by TSH and other growth modulators by changes in iodine uptake and growth rate. It is not capable of synthesising thyroid hormones.

The effects of several modulating factors were also studied. Since thyroid cells in patients with Graves' disease are under the combined stimulus of thyroid-stimulating antibodies and thyroid hormones, the experiments were repeated with both TSH and thyroid hormones in the culture medium. It has been suggested that cell-surface protease enzymes may modify the effects of peptide mediators, so attempts were also made to inhibit these enzymes with known inhibitory compounds.

Chapter Two

Production Of Soluble Interleukin-2 Receptor

And Cytokines

By Whole Blood In Graves' Disease

SECTION ONE

INTRODUCTION

2.1.1 Interleukin-2 Receptor

The effects of interleukin-2 are mediated through interaction with specific receptors on the surface of target cells. The receptors for IL-2 were originally referred to as high, intermediate, and low affinity receptors. Now it is recognised that the high affinity receptor contains three distinct subunits, IL-2R α , IL-2R β , and IL-2R γ , while the intermediate affinity receptor contains two subunits, IL-2R β and IL-2R γ . Isolated IL-2R α binds IL-2 with low affinity (Minami et al, 1993).

2.1.1.1 Interleukin-2 Receptor α Chain

The human IL-2R α chain, originally described as the *tac* antigen, was identified as a membrane glycoprotein (p55) capable of binding IL-2 (Robb and Greene, 1983). It is a protein of 251 amino acids, with a signal peptide of 21 amino acids in length. The 219 amino acid residues of the amino-terminal constitute the extracellular region, while the next 19 amino acids form the membrane spanning region, and the cytoplasmic region involves the 13 amino acid residues of the carboxy-terminal (Minami et al, 1993). When the cloned human IL-2R α cDNA was transfected into non lymphoid cells or lymphoid cells lacking IL-2 binding ability, it became evident that IL-2R α constitutes only the low affinity IL-2 binding form, a form that can not cause internalisation of IL-2 or signalling (Greene et al, 1985). However, when the human cDNA was introduced and expressed in murine T cell line, the high-affinity as well as the low-affinity IL-2R were reconstituted on the surface, suggesting a contribution of the α -chain to the formation of the high affinity receptor (Robb, 1986). Mutational analyses have shown that the N-terminal 83 amino acid residues of IL-2R α chain especially residues 1-6 and 35-43 are important for IL-2 binding (Robb et al, 1988).

2.1.1.2 Interleukin-2 Receptor β Chain

The structure of IL-2R β (p75) was elucidated through the expression cloning of the cDNA for human IL-2R β (Hatakeyama et al, 1989). This is a protein of 551 amino acids, with a signal peptide of 26 amino acids in length. The 214 N terminal

amino acids are extracellular, while the next 25 amino acids are membrane-spanning, and the C terminal 286 amino acids constitute the cytoplasmic regions (Hatakeyama et al, 1989). Deletion of the entire cytosolic protein of IL-2R β abrogates all known IL-2 dependent signalling and growth responses. This region is relatively rich in proline and contains two distinctive subdomains that are serine rich and acidic rich. Deletion of the serine rich region blocked IL-2 dependent growth responses, while deletion of the acidic region blocked IL-2 dependent activation of protein tyrosine kinases (Minami et al, 1993). This indicates that some of the signalling functions of IL-2R are encoded by distinct sub-regions within the IL-2R β .

2.1.1.3 Interleukin-2 Receptor γ Chain

The finding that α and β receptor chains were only capable of reconstituting low or intermediate affinity IL-2 receptors, and results obtained from studies of mutants of IL-2R β led to discovery of IL-2R γ (Zurawski et al, 1990). The structure of IL-2R γ (p64) was elucidated by expression cloning (Takeshita et al, 1992). The human IL-2R γ is a protein of 369 amino acids with a signal peptide of 22 amino acids. The N terminal 232 amino acids are extracellular, while the next 29 amino acids are membrane-spanning, and the C terminal 86 amino acids constitute the cytoplasmic region (Takeshita et al, 1992). The presence of the IL-2R γ subunit is required for all IL-2R signalling functions (Takeshita et al, 1992). There is no measurable binding of IL-2 to the individual IL-2R γ subunit, but the presence of IL-2R γ augments the affinity of IL-2R β binding and is necessary for formation of the high affinity IL-2R $\alpha\beta\gamma$ complex (Gaulton and Williamson, 1994). IL-2R γ is also involved in internalisation of the IL-2/IL-2R complex (Voss et al, 1994).

A recent study shows that a mutation in the γ chain of the IL-2R complex is the cause of X-linked severe combined immunodeficiency (Voss et al, 1994; Leonard et al, 1994). The discovery points to a central role of the IL-2R γ chain in development and function of lymphoid cells (Voss et al, 1994).

The IL-2R β and IL-2R γ chains have significant homologies with other cytokine receptors in the length and folding of their extracellular domains, while IL-2R α does not have any homology with other known cytokine receptors (Minami et al, 1993; Cosman, 1993). Furthermore, the γ chain of IL-2R is common to the receptors for IL-2, IL-4, and IL-7 (Leonard et al, 1994; Voss et al, 1994), while the β chain may be shared by the IL-2 and IL-15 receptors (Grabstein et al, 1994). It has been shown that circulating human neutrophils express IL-2R β (Djeu et al, 1993) and IL-2R γ (Liu et al, 1994), but not IL-2R α . Although these two receptor subunits on neutrophils could potentially interact with IL-2 with intermediate affinity and induce

an intracellular signal, the absence of IL-2R α suggests that this receptor may not be specific for IL-2, and may be a receptor for IL-15 (Grabstein et al, 1994). The above findings indicate that the α chain may confer specificity for IL-2 although it does not have any part in intracellular signalling.

IL-2R β and IL-2R γ stimulate at least two intracellular signal transduction pathways that lead to nuclear proto-oncogene induction (Shibuya et al, 1992). One pathway is linked to tyrosine phosphorylation events, mediated by a tyrosine kinase of the *src*-family, which leads to activation of *c-fos*, *c-jun*, and other genes of this family. Another pathway leads to *c-myc* gene induction by an unknown mechanism (Minami et al, 1993). Other signalling events lead to a rapid increase in intracellular pH, as a result of the activation of Na⁺/H⁺ antiport (Gaulton and Williamson, 1994). For further information on this topic see the review of Gaulton and Williamson (1994). The combination of three chains of IL-2R is shown in figure 2.1, while the binding affinity, association time, and signalling functions of each individual subunit of IL-2R and their combinations are summarised in table 2.1.

2.1.2 Soluble Interleukin-2 Receptor

2.1.2.1 Structure

Resting lymphocytes express only intermediate affinity IL-2 receptors. When T cells activated the expression of IL-2R β is increased 5-10 fold, whereas IL-2R γ expression is not changed. However IL-2R α is strongly induced and expressed out of proportion to the amount of IL-2R β and IL-2R γ (Leonard et al, 1994). This leads to release of a soluble form of this chain into the plasma, after enzymatic cleavage from the membrane (Jacques et al, 1990; Robb and Kutny, 1987).

The relative molecular mass of the soluble interleukin-2 receptor (sIL-2R) is 40-45000. It has most of the characteristics of the membrane-bound IL-2R α , including a low affinity for IL-2, but lacks 72 amino acids corresponding to transmembrane and intracytoplasmic regions of the IL-2R α (Rubin et al, 1985). Jacques and colleagues (1990) reported that naturally secreted sIL-2R is a non-covalently bound dimer, suggesting that the cell-bound IL-2R α receptor is also a dimer. This has not yet been confirmed.

Human monocytes and macrophages produce sIL-2R when stimulated with LPS (Kniep et al, 1992) or INF- γ (Valitutti et al, 1989; Espinoza-Delgado et al, 1990); resting monocytes do not express IL-2R α , but do express IL-2R β (Kniep et al, 1992) and IL-2R γ (Epling-Burnette, 1995; Bosco et al, 1994) on their surface. Resting human B cells, natural killer cells, and CD4⁺ and CD8⁺ T cells also express

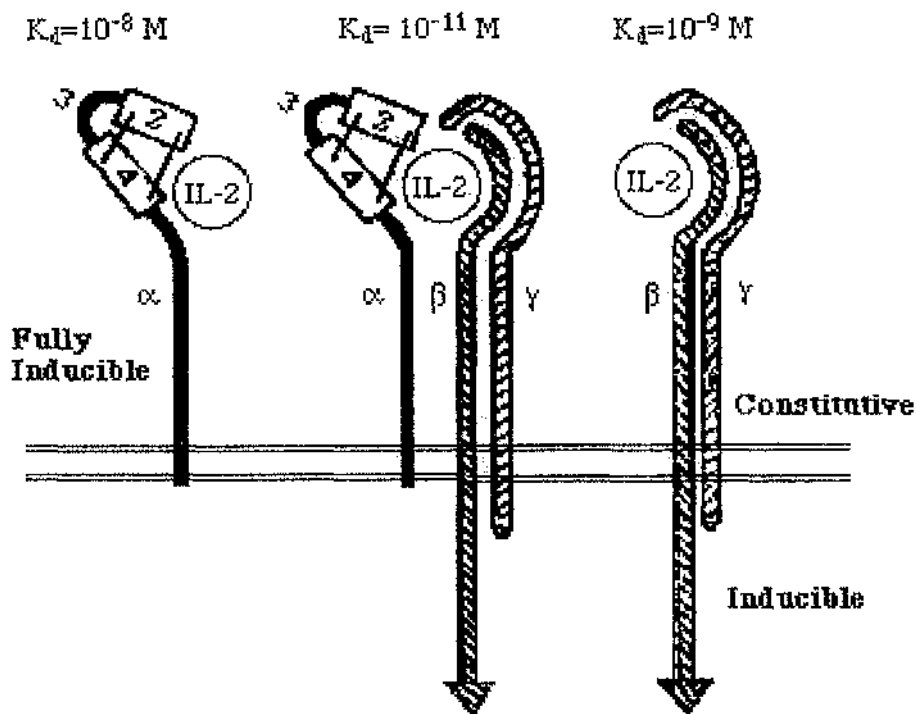


Figure 2.1: Schematic of the human IL-2 receptor. The functional receptor is composed of at least three Polypeptide chains, α , β , and γ . Numbered regions of the IL-2R α chain represent protein domains encoded by exons 2, 3, and 4, respectively (copied from Kaempfer, 1994).

IL-2R	α	β	γ	$\alpha\beta$	$\beta\gamma$	$\alpha\beta\gamma$
Binding affinity (mol/l)	10^{-8}	10^{-7}	?	10^{-10}	10^{-9}	10^{-11}
$t_{1/2}$ Dissociation	35 s	1.6 min	?	18.5 min	255 min	255 min
Internalisation	No	No	?	?	Yes	Yes
Signalling functions	No	?	No	?	Yes	Yes

Table 2.1: Binding affinity for IL-2, time of association, and function of subunit of interleukin-2 receptor (IL-2R).

IL-2R β on their surface, but not IL-2R α . After activation, newly synthesised IL-2R α appears on the surface of these cells (Ohashi et al, 1989). Recent studies indicate that the up regulation and expression of IL-2R α on stimulated B cells is enhanced by IL-10 (Itoh et al, 1994), and retinoic acid (Bhatti and Sidell, 1994). There is a report that indicates the expression of IL-2R α on CD4 and CD8 T cells are decreased in HIV infected patients, while expression of IL-2R β did not change compared to control (Vanham et al, 1994). This may indicate that the α chain confers specificity for IL-2 and is therefore regulated independently of the other chains of the receptor. Moreover, the high levels of sIL-2R found in many instances where IL-2 dependent function is impaired suggests that this molecule may act as an antagonist of IL-2 mediated cell responses (Rubin and Nelson, 1990).

2.1.2.2 Clinical Applications

Increased concentrations of sIL-2R have been reported in a wide variety of diseases. Some of the recent publications are described here in brief but only thyroid disease will be characterised in detail.

2.1.2.2.1 Malignant Disease

Serial measurement of sIL-2R levels was shown to be of clinical importance in adult T-cell leukaemia, because changes in the level of sIL-2R correlated with disease progression, especially in the early phase of adult T-cell leukaemia, where it is more useful than LDH (Kamihira et al, 1994).

The plasma sIL-2R was found to be significantly elevated in patients with primary myelofibrosis compared to polycythaemia vera, while that of patients with polycythaemia vera was significantly higher than control. This may be secondary to T-cell activation resulting from autoimmune phenomena and myeloblast activation. In primary myelofibrosis, plasma sIL-2R concentrations were also found to be correlated to survival, circulating blast cell count and thrombocytopenia, but not to white blood cell counts, LDH levels, degree of marrow fibrosis or degree of splenomegaly (Wang and Wang, 1994).

Serum concentration of sIL-2R was increased in females with advanced ovarian cancer (Gadducci et al, 1994); the increase was also detected in their ascitic fluid (Hurteau et al, 1994).

The level of serum sIL-2R in patients with gastric cancer with lymph node metastases were higher than those patients that did not have lymph node metastases (Murakami et al, 1994).

2.1.2.2.2 Autoimmune Disease

An association of high concentrations of serum sIL-2R with rheumatoid arthritis was shown by Polisson and co-workers (1994). The level decreased significantly during treatment, but there was no correlation of the sIL-2R concentrations with joint pain or tenderness (Ward et al, 1994). Cyclosporin A reduced the level of sIL-2R in patients with rheumatoid arthritis (Crilly et al, 1995).

Serum concentration of sIL-R was significantly raised in patients with systemic lupus erythematosus, coeliac disease and Crohn's disease (Srivatava et al, 1995).

2.1.2.2.3 Infection

Serum concentrations of sIL-R were significantly elevated in patients with pulmonary tuberculosis; the sIL-2R concentrations were still higher than control after treatment though they had decreased significantly, suggesting a delayed resolution of the inflammation in these patients (Chan et al, 1995).

Both neopterin (released from activated monocytes and macrophages) and sIL-2R were identified as significant predictors of a state of shock caused by gram negative sepsis, but neither of them contributed any additional significant predictive information to standard measurements (Delogu et al, 1995).

2.1.2.2.4 Transplantation

During rejection of renal transplants, serum and urine sIL-2R concentrations are affected by both rejection episodes and infection. While Bock and co-workers (1994) claimed that serial measurement of sIL-2R excretion may be a useful adjunct to the diagnosis of rejection, Montagnino and colleagues (1995) did not find that measurement of sIL-2R concentration was superior to clinical diagnosis.

2.1.2.2.5 Thyroid Diseases

The serum concentration of sIL-2R of patients previously treated for differentiated thyroid carcinoma by total thyroidectomy while they were off treatment with L-thyroxine was nearly half that in the control group (Mariotti et al, 1994). In contrast, highly variable circulating sIL-2R concentrations were found in 49 patients

with untreated Hashimoto's thyroiditis and 22 patients with idiopathic myxoedema (Mariotti et al, 1992 a).

All publications agree that the serum or plasma concentrations of sIL-2R are significantly increased in untreated patients with non-autoimmune hyperthyroid patients (single toxic adenoma) (Mariotti et al, 1992 a) or Graves' disease (Murakami et al, 1995; Murphy et al, 1994; Mariotti et al, 1992 a, and 1992 b; Balazs and Farid, 1991), but conflicting data have been published on production of sIL-2R in treated patients.

Balazs and Farid (1991), who studied the sIL-2R of 20 patients with untreated Graves' disease, showed a strong correlation between sIL-2R and TSAb ($r=0.94$). Weryha and co-workers (1991) have shown that untreated patients with Graves' disease had a strong correlation between T_3 and sIL-2R ($r=0.93$), but a lower correlation between TSAb and sIL-2R ($r=0.74$), while Mariotti's group (1992 a, 1994) and Murakami's group (1995) could not detect any correlation between thyroid TSAb and sIL-2R. It is not clear why the reported correlation is so variable or why there is only a correlation between TSAb and sIL-2R, not microsomal or thyroglobulin antibodies.

Mariotti and co-workers (1992 a, 1994) have shown that treatment of both hypothyroid and hyperthyroid patients returned the concentration of sIL-2R to normal. They believed that the normalisation of thyroid hormones was responsible for this rather than any effect on the immune process itself. This statement is consistent with the results of Balazs and Farid (1991), but it is only based on indirect evidence, and requires confirmation.

Murphy and co-workers (1994) showed that sIL-2R concentrations decreased in patients treated with propylthiouracil, and did not return to normal when euthyroidism was established, so it was in their opinion unlikely that anti-thyroid drugs could be responsible for the observed reduction of sIL-2R concentrations. Volpé (1994) also stated that anti-thyroid drugs could not mediate any immunosuppressive effect and suggested that the actions of anti-thyroid drugs were mediated only through modifying the function of thyrocytes. Balazs and Farid (1991) have shown the concentrations of sIL-2R normalised earlier than other immunological and hormonal parameters, and believed that this was the effect of methimazole treatment, because after cessation of drug the concentration of sIL-2R increased. There is no direct evidence whether or not anti-thyroid drugs could affect the production of sIL-2R, and this needs further investigation.

2.1.3 Soluble IL-2 Receptor And Cytokines In Blood

In the current study, the concentration of sIL-2 receptor in serum was measured in a group of patients with Graves' disease before and during treatment. In addition, various experiments were carried out to investigate whether the observed increases could be accounted for by a direct effect of thyroid hormones and drug treatment on blood cells. To do this, samples of blood from normal volunteers were incubated with thyroid hormones, TSH and methimazole in the presence of standard stimulators of peripheral blood cells, and the concentrations of sIL-2R which were released into the medium were measured. This study was then extended to cover measurement of several other cytokines which could be of importance in the immune process of Graves' disease. These were IL-2, IL-4, IL-6, TNF- α , and INF- γ .

In addition, similar stimulation experiments were carried out using blood from untreated patients with Graves' disease, without exogenous thyroid hormones, in order to compare the response of blood cells with the reference group.

Most groups who have studied the release of cytokines from blood cells have used various populations of isolated cells (monocytes, T or B lymphocytes), and stimulated them with mitogens or cytokines. It was decided to use peripheral whole blood (WB) as the test medium. The reasons for this decision are discussed below.

The separation of cells requires density centrifugation of blood and adherence of cells to tissue culture plastic to isolate monocytes, however, under these conditions of isolation, at least three variables potentially affect the separated cells. Firstly, the adherence step of monocyte purification is known to induce mRNA for TNF (Desch et al, 1989), and so it presumably also affects other cytokines which it is desirable to study. Secondly, it is difficult to avoid contamination of reagents and tissue culture medium by low levels of endotoxin. As monocytes are sensitive to picogram quantities of LPS, this can have a marked effect on results. Thirdly, the presence of heterologous serum may alter the effect of stimulators on cultivated cells.

Furthermore, removal of cells from their physiological environment (plasma) also uncouples cell to cell interactions and production of cytokines, both known and unknown, that orchestrate interactions between the different cells in vivo. There is evidence, for instance, that granulocyte macrophage colony stimulating factor (GM-CSF) could enhance the plasma level of sIL-2R of cancer patients treated with this cytokine (Crispino et al, 1993). In vitro stimulation of whole blood (diluted 1:1 or 1:10 with medium) with LPS caused a significant release of TNF within the first 4-8 hours of stimulation, while the same number of isolated monocytes only produced

one fifth of the quantity of this cytokine after 24 hours (Strieter et al, 1990; De Groote et al, 1992).

Similar observations were made for other cytokines. The production of IL-1 β was 8 times higher when whole blood was used as substrate and stimulated with LPS than when isolated cells were used (Allen et al, 1992). Expression of sIL-2R was minimally induced on surface of CD8 bright cells when whole blood was stimulated with CD3 monoclonal antibody, while all CD8 bright cells expressed sIL-2R when isolated mononuclear cells were used (Janssen et al, 1994). Also, production of IL-6 and IL-2 were lower in whole blood than isolated peripheral mononuclear cells (De Groote et al, 1992). It seems likely, therefore, that isolation of cells significantly alters their responsiveness to stimulation in an unpredictable manner, and it may be preferable to use whole blood cultures in clinical situations.

SECTION TWO

METHODS

2.2.1 Blood Samples

2.2.1.1 Blood Samples for Study of Serum sIL-2R

The required amounts (4-8 ml) of peripheral blood were collected by venepuncture from 17 healthy adults (11 females and 5 males) aged between 17 to 57 years or from patients with Graves' disease into plain Vacutainer tubes, and serum was separated within 30 min by centrifuging the sample at 800 g. The sera were transferred into another tube and stored at -20° C until assay for sIL-2R. Specimens for thyroid function testing were collected from patients at the same time. Follow up samples were collected from these patients on their review visits to hospital.

2.2.1.2 Samples For Study Of Release Of sIL-2R From Whole Blood

The required amounts (30 ml) of peripheral blood were collected by venepuncture from healthy adults aged between 25-40 years, into 3 plain Vacutainer tubes, and 500 µl of concentrated heparin solution (1000 U/ml) was injected into each tube immediately after collection. The experiment was set up within one hour after collection. Samples of 10 ml were collected using the same protocol from patients with untreated Graves' disease.

2.2.1.3 Samples For Study Of Release Of Cytokines From Whole Blood

Peripheral blood samples (4 ml x 4 tubes) were collected by venepuncture from healthy adults aged between 25-50 years, or from patients with untreated Graves' disease, into endotoxin free heparinized vacuum blood collection tubes (Endotubes).

2.2.2 Soluble IL-2 Receptor Production By Whole Blood In Vitro

Heparinized blood samples were processed using two different protocols. All experiments were carried out in triplicate.

A portion of the sample was centrifuged at 800 g for 5 min. Plasma was stored at -20° C until assay of sIL-2R. This plasma served as a pre-control.

2.2.2.1 Protocol 1 (undiluted)

Various amounts of concentrated LPS and PHA solution, and combinations of these, were mixed with medium immediately before use. 450 μ l of whole blood (WB) were transferred into each well of a 12-well plate. 50 μ l of medium were added to triplicate control wells. To other sets of triplicate wells, 50 μ l of medium containing LPS, PHA, or a mixture of LPS and PHA were added. The plate was incubated in a 37° C incubator with 95 % air and 5 % CO₂, for various periods.

After incubation, the plate was removed from the incubator, and blood from each well was transferred to tubes which were centrifuged at 500 g for 5 min. Plasmas were separated and saved at -20° C, until assay of sIL-2R.

2.2.2.2 Protocol 2 (1:2 dilution)

0.5 ml of whole blood was transferred into each well of a 12-well plate. 0.5 ml of medium with or without stimulators was added to triplicate wells as described above. The plate was incubated in a 37° C incubator with 95 % air and 5 % CO₂, for various periods. The remaining steps were the same as protocol 1.

2.2.3 Determination of Soluble IL-2 Receptor

To determine the amount of sIL-2R in plasma, and cell culture supernatant, a kit manufactured by T Cell Diagnostics Inc. was used. The kit contained the following reagents and materials:

Anti-Human IL-2R Coated Microtitre plate: Each plate consists of twelve 8-well strips precoated with murine monoclonal antibody to human IL-2R.

Soluble IL-2R conjugate: Each vial contains horseradish peroxidase (HRP)-conjugated murine monoclonal antibody to human sIL-2R in a buffered solution with bovine serum protein and thimerosal. This solution should be protected from light.

IL-2R Specimen Diluent: Each vial contains equine-derived serum proteins and thimerosal.

IL-2R standards: Each vial contains recombinant human IL-2R in a buffered solution with bovine derived serum proteins and thimerosal at the following concentrations: 0, 200, 800, 1600, and 3200 U/ml, a unit being defined by the

manufacturer from the content of a standard prepared from PHA-stimulated lymphocytes.

IL-2R controls: Each vial contains recombinant human IL-2R in a buffered solution with bovine derived serum proteins and thimerosal. The concentrations of each control differ from batch to batch. Usually they are approximately 500 and 1200 U/ml.

Chromogen tablets: Contain O-Phenylenediamine and fillers.

Substrate: A buffered solution containing 5.3 mmol/l urea peroxide and thimerosal.

PBS salt: Each packet contains pre-measured salts sufficient to make one litre of buffer.

Surfactant solution: Each vial contains a 50 % solution of surfactant.

Plastic plate sealers.

All reagents were stored at 2-8° C: One hour before use, they were brought out to room temperature.

Wash buffer salt was dissolved in one litre of distilled water, and one ml of surfactant solution was added to it. The shelf life of this solution is 30 days.

Chromogen substrate solution **must be** prepared only 10 min before use. For its preparation 4 chromogen tablets were transferred to 20 ml of substrate solution. They dissolved in 1-2 min if left undisturbed. The solution was mixed by swirling before use. The resultant solution **must be** colourless to pale yellow. A yellow-orange colour indicates chromogen deterioration and solution should be discarded.

Stop solution, which is 1 mol/l (2 normal) H₂SO₄, is not provided in the kit. For preparation of this solution and other acid preparations, the following formula was used throughout this study.

$$\text{Volume of acid (ml) required to make one litre of Normal solution} = \frac{\text{Molecular Mass} \times 100}{\text{Specific gravity} \times \text{Valency} \times \text{purity}}$$

The assay was performed according to the manufacturer's instructions. Briefly, standards and samples (50 µl) were added to duplicate wells of a microtitre plate coated with murine monoclonal antibody to human SIIL-2R, followed immediately by addition of 100 µl horseradish peroxidase conjugated murine monoclonal antibody to human IL-2 R, leaving the first two wells empty as a blank. The plate was sealed and shaken on a plate shaker for 3 h.

Ten min before the end of the incubation, chromogen solution was prepared. The sealer was removed and discarded and the solution in each well was aspirated. Each well was washed at least 3 times with 350 µl of wash buffer. Excess of wash solution was removed from the wells by inverting the plate over tissue paper.

Immediately after washing, 100 μ l of a chromogen solution was added to all wells including blanks. The reaction was terminated by addition of 50 μ l of stopping solution (2 Normal solution of H_2SO_4) to each well, and the plate was read at 490 nm. All the tests were carried out in duplicate. Figure 2.1 shows an example of a standard curve for measurement of sIL-2R.

2.2.4 Cytokine Production by Samples of Whole Blood in Vitro

Three sets of triplicate sterile polypropylene tubes was labelled as control, LPS/PHA, and Ca/PMA. To all tubes of the second set, 25 μ l of working solution of LPS/PHA, and to the third set 25 μ l of working Ca ionophore (8 μ g/ml), and 25 μ l of working PMA (0.24 μ g/ml) solution were added. To all tubes, 2 ml of pre-warmed medium were added, followed by addition of 0.2 ml of well-mixed heparinized whole blood from either patients or reference controls. The tubes were capped and mixed. The caps were loosened, and the tubes were incubated in a 37° C incubator in 95 % air and 5% CO_2 , for periods of 4, 24, and 72 h.

At the end of each incubation time, one tube from each set was taken out of the incubator and centrifuged at 500 g for 5 min. Medium from each tube was transferred to appropriate labelled tubes and stored at -20° C, till shipment to Medgenix Diagnostics (Fleurus, Belgium) for analysis of cytokines.

A portion of the original blood was centrifuged at 800 g for 5 min, and plasma was removed and transferred to a new tube. This tube was capped, and stored at -20° C till shipment of specimens. These plasmas served as untreated control. Soluble IL-2 receptors were also assayed by the T-Cell Diagnostics kit, but only on the samples incubated for 72 h.

For each reference sample, an additional three groups of 9 sterile polypropylene tubes were treated with thyroid hormones, TSH or an anti-thyroid drug (methimazole) in addition to the stimulators mentioned above. Either 23 μ l of Methimazole solution (100 mmol/l), 23 μ l of TSH solution (5 U/l), or a mixture of 23 μ l of T_4 solution (100 μ mol/l) and 23 μ l of T_3 solution (1 μ mol/l) were added. To all tubes, 2 ml of pre-warmed medium were added, followed by 0.2 ml of well-mixed heparinized whole blood. Tubes were capped and mixed. The caps were loosened, and the tubes were incubated in a 37° C incubator in 95 % air and 5% CO_2 , for periods of 4, 24, and 72 h.

At the end of each incubation time, the samples were centrifuged and stored as above until they were sent to Medgenix Diagnostics for analysis. Soluble IL-2 receptor assays were also carried out on the samples in this experiment using the T-Cell Diagnostics kit.

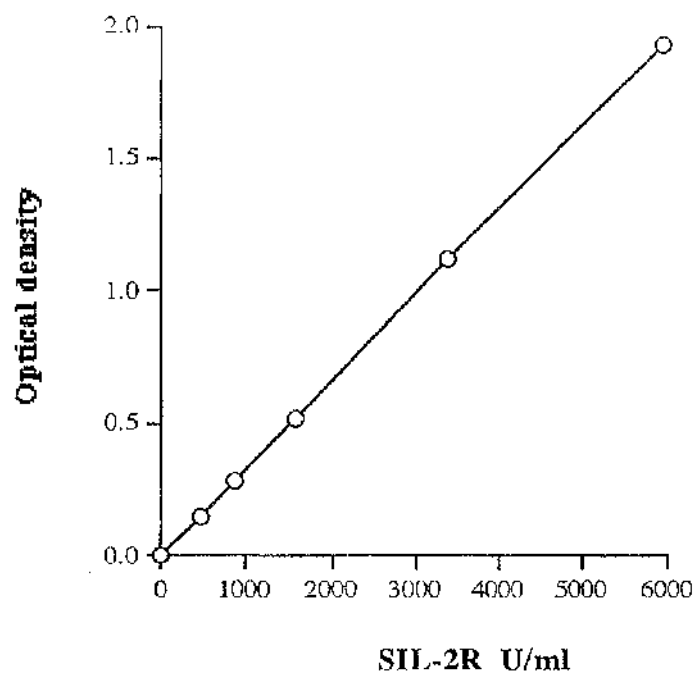


Figure 2.2: Standard curve used for measurement of sIL-2R, in serum and cell culture supernatant.

2.2.5 Measurement of Thyroid Function Tests

Total T_4 was measured using the Abbott IMx instrument. Free T_4 was measured using the Amerlex MAb kit and total T_3 was measured using the Amerlex M kit manufactured by Amersham International. Thyroid receptor antibody (TRAb) was measured using the kit manufactured by RSR Ltd, Cardiff, UK. Thyroid microsomal and thyroglobulin antibodies were measured using the Thymune-M and Thymune T kits manufactured by Murex Diagnostic Limited, Dartford, England.

2.2.6 Statistical Analysis

Statistical analysis was carried out using the StatView computer package. Comparison of patients with controls at the same time point was carried out using unpaired t-tests, while analyses of the time course of stimulation and the effect of thyroid hormones, TSH, or methimazole were performed by analysis of variance (ANOVA), using the Bonferroni/Dunn criterion of significance.

The differences were defined as significant when the calculated critical difference as determined by the Bonferroni/Dunn criteria (5% significance level) was less than the mean difference ($P < 0.05$), while they are defined as highly significant when the calculated critical difference is less than half of mean difference ($P < 0.001$).

SECTION THREE

RESULTS

2.3.1 Serum Soluble IL-2 Receptor in Graves' Disease

During the study period we received 24 samples from patients with untreated Graves' disease. Of these, 18 were females, and 6 were males. The average age of these patients was 37.37 years (range of 17-77 years). Figure 2.3 shows the age distribution of these patients. The histogram illustrates that the highest incidence of disease was between 20 and 50 years.

The mean serum concentration and standard deviation of soluble Interleukin-2 receptor (sIL-R) for these patients were 2465 U/ml \pm 833.

The thyroid function of the patients was evaluated by measurements of total T₄, T₃, TSH, and TSH receptor antibody (TRAb). Free T₄, titre of thyroid microsomal antibodies (M) and titre of thyroglobulin antibodies (T) were determined for some cases. The results of these tests were extracted from the case files of these patients.

23 out of 24 cases had increases in the serum level of T₄, ranging from 113 to 453 nmol/l (reference range in our laboratory is 55-145 nmol/l). The level of T₃ was determined for 23 cases, ranging from 2.5 to 12.1 nmol/l (reference range in our laboratory is 0.9-2.7 nmol/l). TSH was undetectable in almost all cases (<0.1 mU/l). The highest value was 0.16 mU/l (reference range 0.4-5.0 mU/l). Thyroid receptor antibody (TRAb) was determined for 22 cases. Two of these cases were within the normal range (<10 %), a further two cases were borderline (10-20 %), and the remainder had increased level of thyroid antibody. Free T₄ was determined for 11 cases, and all of them also had increased values for this parameter (reference range 11-24 pmol/l). The titre of thyroid microsomal antibodies (M) was determined for 19 cases. Titres for six cases were undetectable or normal (at or below 1/100), and the remaining cases had increased titres ranging from 1/400 to 1/102400. Finally the thyroglobulin antibody titre (T) was determined for 19 cases. Titres for 14 cases were normal (<1/10), and remaining cases had increased titres ranging from 1/80 to 1/5120. The summary of these data is shown in table 2.2. For the calculations, values of the titres of thyroid microsomal antibodies and of thyroid antibody were expressed as real numbers. The cases that had their titre of thyroid microsomal antibodies (M) below 1/100 were designated as 80, and cases that had their titre of thyroglobulin antibodies (T) below 1/10 were designated as 8.

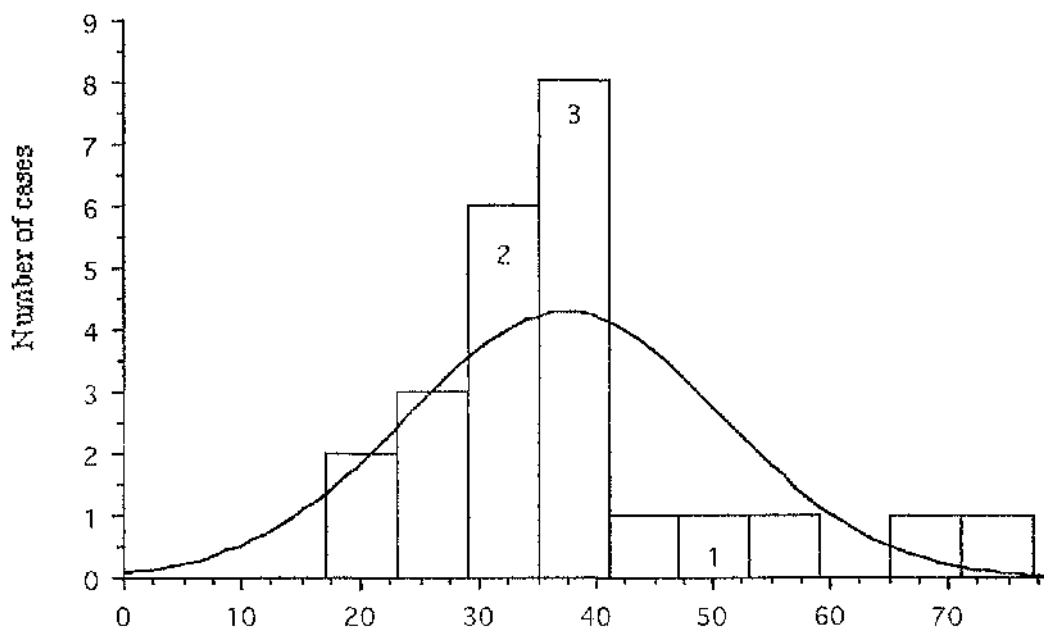


Figure 2.3: Age distribution histogram of patients with untreated Graves' disease. Numbers in the bars represent male cases.

Variable	Mean	Std. Dev.	Count	Minimum	Maximum
Age	37.4	13.4	24	17	77
sIL-2R	2465	833	24	930	4250
T ₄ nmol/l	236.9	85.0	24	113	453
T ₃ nmol/l	6.6	2.3	23	2.5	12.1
FT ₄ pmol/l	61.2	22.1	11	36.1	110.2
TSH mU/l	0.103	0.103	22	0.1	0.16
TR Ab %	35.7	21.9	22	6	80
M	8513	23498	19	<100	102400
T	325.9	1170.1	19	<10	5120

Table 2.2: Results of investigations carried out on untreated patients with Graves' disease. M and T stand for thyroid microsomal antibody titre and thyroglobulin antibody titre. For calculation these titres were shown as real numbers.

Treatment for most patients was started by prescribing anti-thyroid drugs (20 mg carbimazole, twice a day, or propylthiouracil). All patients with elevated T_4 or T_3 were also prescribed 40 mg propranolol, twice per day.

Follow up samples were collected on their review visits to hospital. Two patients did not have follow up samples, and five patients had only one follow up sample after one or two months of treatment. The remaining patients had at least two to four follow up samples. All together we received 49 further samples. An analysis of these samples is given in table 2.3. The results of thyroid function tests and the other related parameters that were carried out on the same date were extracted from patient files.

Generally, after first month of treatment, T_4 and T_3 concentrations decreased to within the reference range, as did the free T_4 concentration, but TSH was not brought into the reference range in 87 % of the patients. Titres of thyroid antibodies (TRAb, M, and T) were still above normal level. The concentration of sIL-2R was reduced compared to initial levels, but still above normal.

After two months of treatment, serum concentrations of T_4 and T_3 (T_4 53 ± 34 , $n=10$; T_3 1.5 ± 0.63 , $n=9$) decreased to below the reference range. Patients were then started on L-thyroxine (0.1 mg per day). TSH was still abnormal, and thyroid antibodies (TRAb, M, and T) levels were starting to decrease. The level of serum sIL-2R was reduced again, but not to normal levels.

In the third month post treatment, the level of serum sIL-2R tended to increase again as the concentrations of thyroid hormones also rose with hormone replacement, although improvements were seen in TSH and thyroid antibodies levels. The concentration of serum sIL-2R continued to fluctuate till eight months post treatment (figure 2.4).

The fluctuations in serum sIL-2R levels till eight months post treatment suggest that the concentration of this receptor in serum not only depends on the immune state of the patient, but also on the concentrations of thyroid hormones. This could be tested statistically by calculation of correlation coefficients for each hormone against sIL-2R. However, the numbers of post treatment samples after the first 2 months were insufficient to carry out calculations for individual time points. Therefore all post treatment samples were combined for statistical purposes. A summary of the descriptive statistics for these data is given in table 2.4.

Statistical analysis of the values for serum sIL-2R of patients before and after treatment revealed a highly significant difference between groups. The serum concentration of sIL-2R in patients with Graves' disease, before treatment, is also significantly different from the normal subjects (327 ± 88 U/ml). The summaries of the statistical analysis are presented in figure 2.5.

Period of treatment	Number of cases	Percentage of total
1 month	15	30.31
2 months	12	24.49
3 months	3	6.12
4 months	6	12.24
5 months	3	6.12
6 months	2	4.81
7 months	4	8.16
8 months	2	4.81
9 months	1	2.04
10 months	1	2.04
Total	49	100

Table 2.3: Number of follow up samples received after corresponding period of treatment.

Variable	Mean	Std. Dev.	Count	Minimum	Maximum
Age	37.7	13.7	49	17	77
sIL-2R	1040	412	49	260	2050
T ₄ nmol/l	87.4	36.7	43	8	157
T ₃ nmol/l	1.82	0.55	37	0.6	3.1
FT ₄ pmol/l	12.45	7.20	12	1.3	27.4
TSH mU/l	3.17	6.1	48	0.1	26.2
TR Ab %	34.25	27.2	36	1	84
M	6444.5	18939.7	31	<100	102400
T	62.1	136.1	31	<10	640

Table 2.4: Results of investigations carried out on treated patients with Graves' disease. M and T stand for thyroid microsomal antibody titre and thyroglobulin antibody titre. For calculation these titres were shown as real numbers.

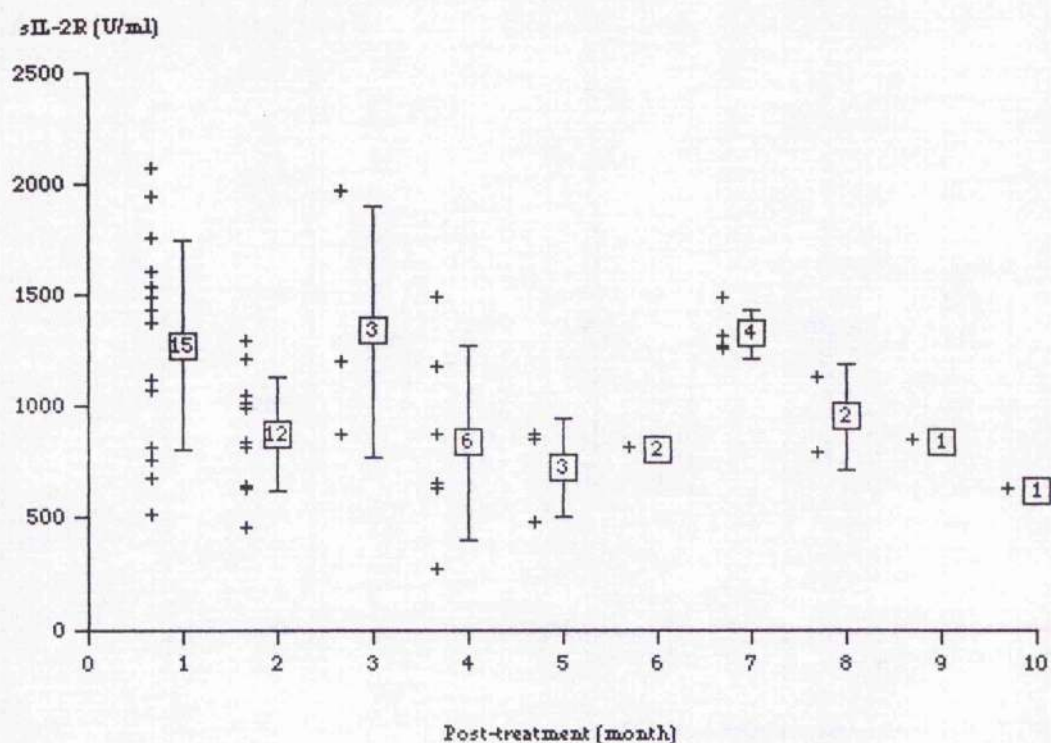


Figure 2.4: Serum level of sIL-2R after different periods of treatment. The bar represents the standard deviation, while numbers in the boxes (mean) represent the number of cases.

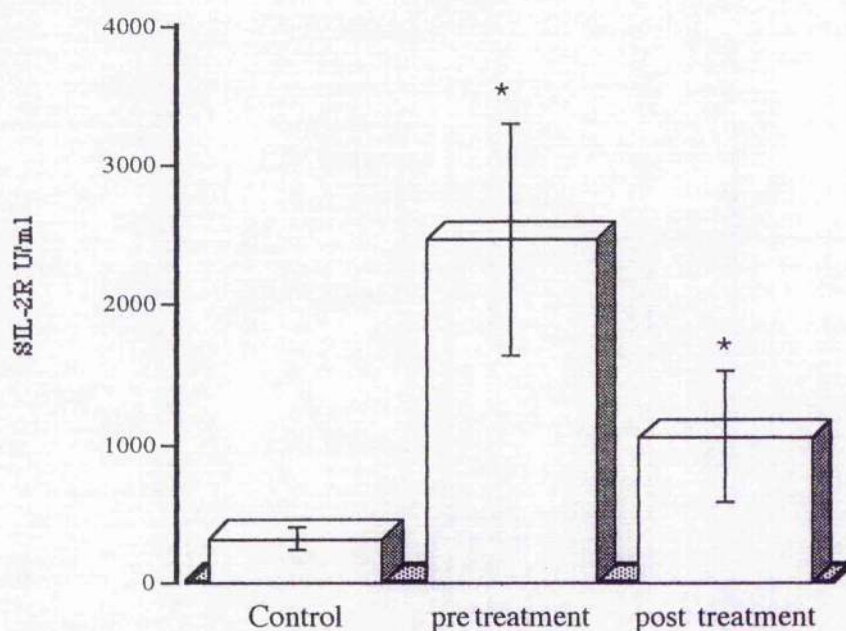


Figure 2.5: Serum level of sIL-2R in patients with Graves' disease before and after treatment. Error bars represent one standard deviation, while the (*) shows significant differences compared to control subjects.

After treatment the serum level of sIL-2R decreased significantly to 1040 ± 412 U/ml. This could be a direct effect of drug treatment on the disease process or could be related to the decreases in thyroid hormones that took place during the same time interval. However, the serum level of sIL-2R was still significantly different from the control group.

Regression analysis was carried out against sIL-2R for all measured parameters before and after treatment. There was a highly significant correlation between T_4 and sIL-2R before treatment ($r=0.684$, $P<0.001$). The correlation between T_3 and sIL-2R before treatment was also highly significant ($r=0.695$, $P<0.001$). The correlation between free T_4 and corresponding sIL-2R in untreated Graves' disease was lower ($r=0.54$) and not significant. This may be partly because of the lower number of cases. There were no significant correlation between the titres of thyroid microsomal antibodies, thyroglobulin antibody, or thyroid receptor antibody with sIL-2R. Summaries of the regression analyses and their plots are presented in figure 2.6.

Among 49 cases post treatment, 43 patients had results available for T_4 . There was no correlation between T_4 and sIL-2R post treatment ($r=0.216$, $P>0.05$). Only 37 post treatment cases had results for T_3 . The correlation between T_3 and sIL-2R was low but significant ($r=0.507$, $P<0.01$). Only 12 results were obtained for free T_4 post treatment. There was no significant correlation between this parameter and sIL-2R ($r=8.96 \times 10^{-4}$, $P>0.05$). Thyroid receptor antibody (TRAb) values were obtained for 36 cases post treatment. There was a significant correlation between TRAb and sIL-2R ($r=0.469$, $P<0.01$).

TSH concentration of serum increased by the end of study in most cases. There were 29 cases below reference range, 10 cases within the range and 9 cases above the range. The mean value was 3.17 mU/l ± 6.1 . The correlation between TSH and sIL-2R was 0.350 , $p<0.05$, after treatment.

Thyroid microsomal antibodies were measured on 31 occasions post treatment. Out of these, 11 cases had a normal titre, and the 20 increased values reported were scattered in a wide range, from 1/400 to 1/102400; there was no correlation between this parameter and sIL-2R. From 31 results for thyroglobulin antibody titre, only 8 cases had increased titre ranging from 1/80 to 1/640. There was no correlation between these parameters and sIL-2R in patients post treatment. Summaries of the regression analyses and their plots are presented in figure 2.7.

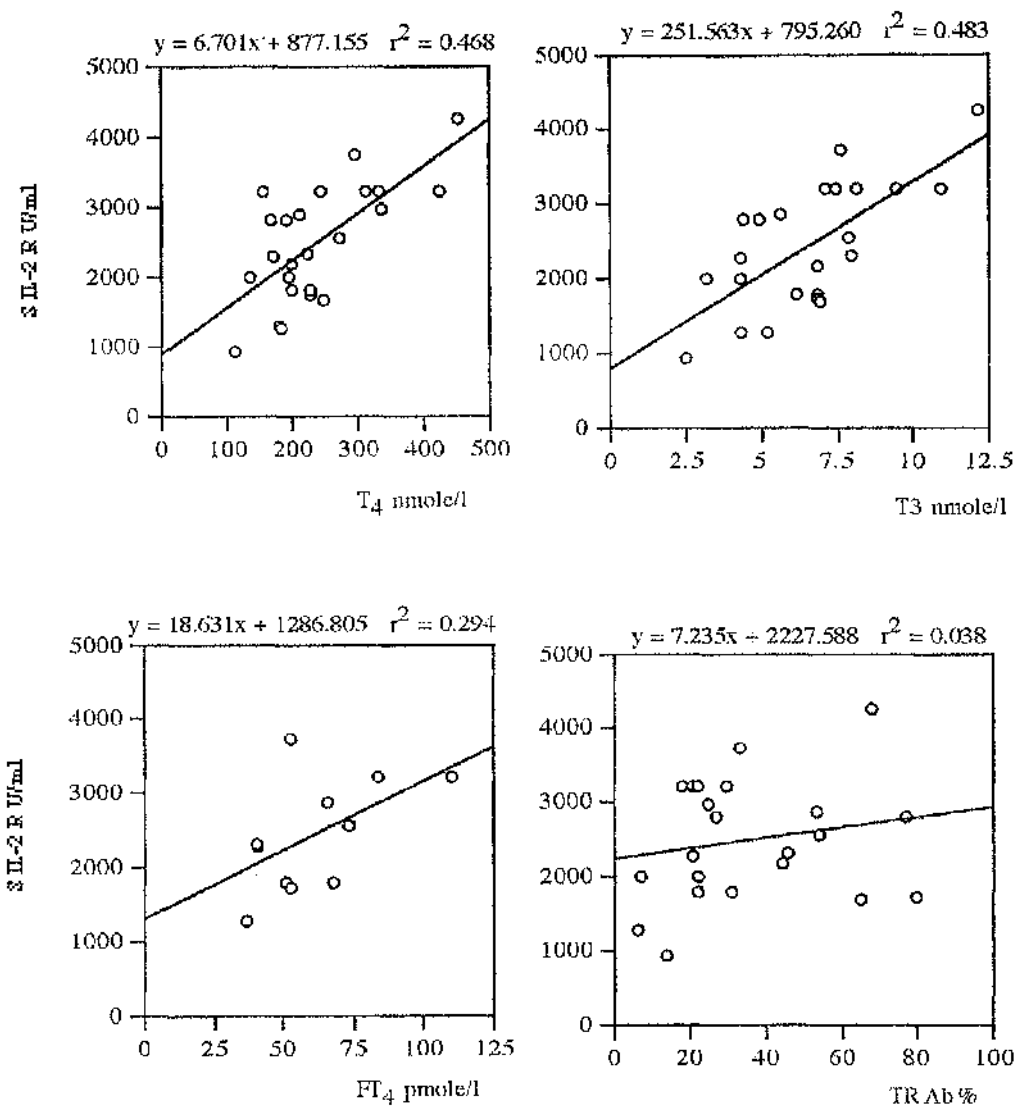


Figure 2.6: Regression plots for serum concentrations of T_4 , T_3 , FT_4 , and TSH receptor antibody versus sIL-2R in untreated patients with Graves' disease.

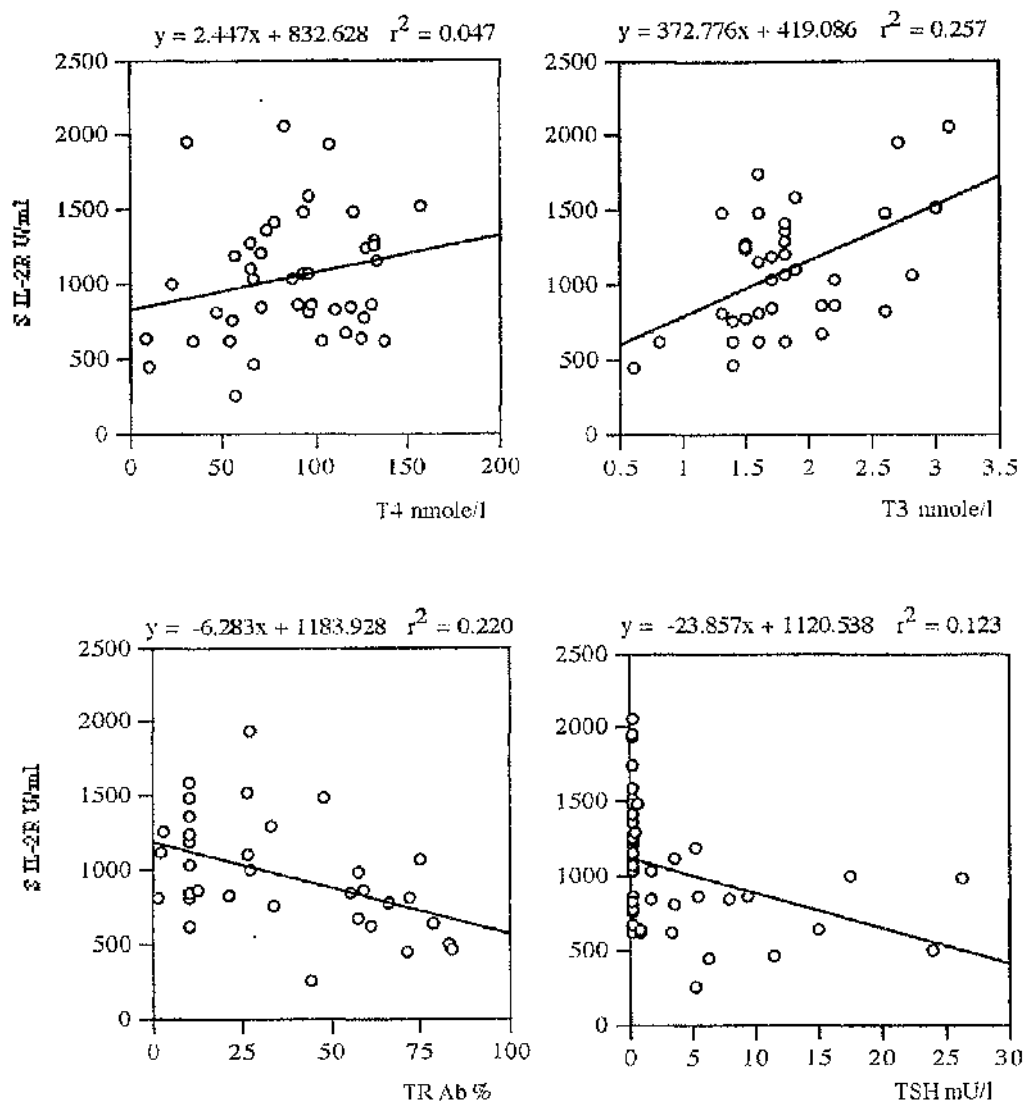


Figure 2.7: Regression plots for serum concentrations of T₄, T₃, TSH receptor antibody, and TSH versus sIL-2R in treated patients with Graves' disease.

2.3.2 Production Of sIL-2R by Whole Blood

2.3.2.1 Preliminary Experiments

Blood was collected from healthy volunteers and prepared as described in section 2.2.1.2. Initial experiments were set up using whole blood (WB) diluted 9:10 with RPMI medium containing stimulators. It was stimulated with 10 $\mu\text{g/ml}$ of LPS, 10 $\mu\text{g/ml}$ of PHA, or a mixture of them (10 $\mu\text{g/ml}$ of each one), for 24 h. Control wells contained medium in place of stimulator. Plasma separated from the blood immediately after collection served as pre-control.

In all incubated wells, the plasma level of sIL-2R increased significantly compared to pre-control ($401 \text{ U/ml} \pm 3.0$). The level of sIL-2R in the control group increased to $521 \text{ U/ml} \pm 12.2$, while that in the group treated with LPS was $830 \text{ U/ml} \pm 16.8$. The level of sIL-2R for PHA stimulated cells was $724 \pm 29.1 \text{ U/ml}$, while that for LPS/PHA mixture was $1247 \text{ U/ml} \pm 50.5$.

The above data shows that the sum of the stimulatory effects of LPS and PHA is approximately the same as that for stimulation with the mixture of LPS and PHA. This suggests that either there are different receptors on the same cell for each of the stimulators used, or different stimulators act on a different population of cells.

This experiment was repeated on three different occasions and similar results were obtained.

2.3.2.2 Time Course Study

The optimal time course was investigated for each stimulator used. Three 12-well plates were set up as for the first experiment, using the same concentrations of stimulators. All plates were incubated as before. Every 24 h, one plate was removed from the incubator, and plasma from each well was separated as described in section 2.2.3.1.

The concentration of sIL-2R in control wells containing no stimulant was itself elevated after 24, 48 and 72 h compared to the pre control at 0 time. The concentration at 0 time was $583 \text{ U/ml} \pm 14.0$, and at 24, 48 and 72 h the concentrations were $713 \text{ U/ml} \pm 20.4$, $913 \text{ U/ml} \pm 41.0$ and $963 \text{ U/ml} \pm 35.5$ respectively. There was a statistically significant difference between the concentrations at 24 and 48 h, but not between 48 and 72 h.

The concentrations of sIL-2R in wells stimulated with LPS were $948 \text{ U/ml} \pm 23.4$ at 24 h, $1422 \text{ U/ml} \pm 40.0$ at 48 h and $1612 \text{ U/ml} \pm 30.6$ at 72 h. These were highly significantly increased ($p < 0.0001$) compared to the control cells at the same

time points. There were statistically significant differences between the concentrations at 24 and 48 h and between 48 and 72 h.

The concentrations produced by cells stimulated by PHA were $1167 \text{ U/ml} \pm 29.6$, $2389 \text{ U/ml} \pm 23.0$ and $2820 \text{ U/ml} \pm 106.7$ at 24, 48 and 72 h. These were again highly significantly increased over control cells incubated for the same time. There were highly significant differences between the concentrations at 24 and 48 h and between 48 and 72 h.

Cells stimulated with $10 \mu\text{g/ml}$ of PHA produced more sIL-2R than those stimulated with the same concentration of LPS. This difference was highly significant ($p < 0.0001$) at all time points.

The concentrations of sIL-2R produced by cells stimulated by the mixture of LPS and PHA were $1520 \text{ U/ml} \pm 31.1$, $3011 \text{ U/ml} \pm 54.2$ and $4392 \text{ U/ml} \pm 46.7$ at the three time points. These were highly significantly increased ($p < 0.0001$) over control cells. The concentrations at 48 h were significantly higher than those at 24 h and those at 72 h were higher than at 48 h ($p < 0.0001$).

2.3.2.3 Dose-Response Curves

To determine the optimal dose for each stimulator, three 12-well plates were set up by dispensing $450 \mu\text{l}$ whole blood into each well. To triplicate sets of wells in the first plate, $50 \mu\text{l}$ of medium containing 0, 1, 5 or $10 \mu\text{g/ml}$ of LPS were added. The stimulator used for the second plate was a series of identical concentrations of PHA, whereas the third plate was stimulated with a mixture of LPS and PHA. The plates were incubated for 72 h.

As had been found in the previous experiment, cells incubated without stimulators still produced a certain amount of sIL-2R. The concentration in the combined control wells of the three plates was $907 \text{ U/ml} \pm 21.3$, compared to $588 \text{ U/ml} \pm 17.2$ in the pre-control.

There were no significant differences between the amounts of sIL-2R produced by cells stimulated by 1, 5, or $10 \mu\text{g/ml}$ of LPS, these being 1807 ± 69.29 , 1891 ± 48.52 and $1889 \pm 52.88 \text{ U/ml}$ respectively (figure 2.8). All were, however, significantly different from control cells ($907 \pm 11.02 \text{ U/ml}$).

There was a significant dose-response curve for PHA. The concentrations at 1, 5, and $10 \mu\text{g/ml}$ were 906 ± 14.73 , 1743 ± 22.28 and $2901 \pm 48.14 \text{ U/ml}$ respectively. The concentrations produced by 5 and $10 \mu\text{g/ml}$ of PHA were significantly different from control ($903 \pm 40.53 \text{ U/ml}$).

Once more, the concentrations of sIL-2R produced under the influence of a mixture of LPS and PHA were equivalent to the sum of the individual stimulators. Summaries of these results are presented in figure 2.9.

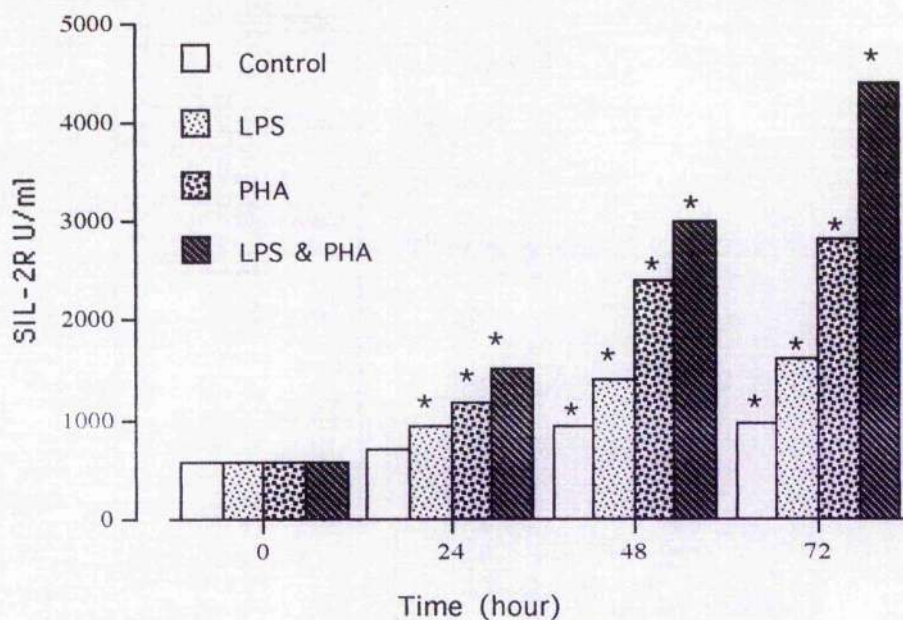


Figure 2.8: Soluble IL-2R produced by whole blood, when incubated with 10 $\mu\text{g/ml}$ of LPS, PHA, or a mixture of LPS and PHA (10 $\mu\text{g/ml}$ of each) for various times. Stars show significant differences between value obtained for each stimulator at each time point, and value obtained for unstimulated cells (zero time point).

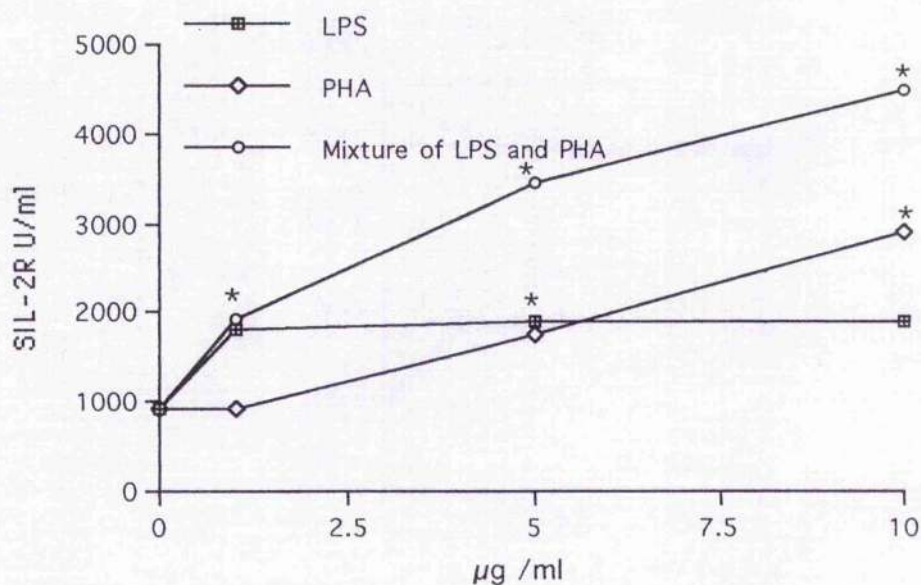


Figure 2.9: Dose-response curve of sIL-2R produced by whole blood, when incubated with LPS, PHA, or a mixture of them for 72 hours. Stars show significant differences between value obtained for each stimulator and the value of unstimulated cells (zero concentration).

2.3.2.4 Effect Of Dilution Of The Sample

In initial experiments, the minimal volume of stimulator was added to avoid dilution of whole blood in culture. However we always detected an increase in the level of sIL-2R for control sets. Some of this increase could be due to the activation of monocytes and macrophages by adhesion to the plate, or lack of movement for different groups of cells. It is also possible that part of this increase could be caused by dilution of blood by the medium. To investigate the effect of dilution in our protocol, we set up a new series of experiments. For this experiment the whole blood was diluted two fold with media as given in section 2.2.3.2. The final concentration for LPS was selected as 1 $\mu\text{g/ml}$, because in the previous experiment it was shown to be the lowest effective dose. The final concentration of PHA was selected as 10 $\mu\text{g/ml}$. Three identical plates were set up using the above concentrations of LPS, PHA, and the combination. The plates were incubated as before. Every 24 h, one plate was removed from the incubator, and supernatant from each well was separated. To compare the result of the two protocols, all values were expressed as units per ml of whole blood rather than concentration in the wells.

As before, the control samples produced sIL-2R, and the amounts were comparable to those in undiluted blood. The pre-control was 727 ± 37.0 U/ml, and the concentrations at 24, 48 and 72 h were 1039 ± 42.8 , 1144 ± 62.4 and 1240 ± 32.4 U/ml. The concentration at 72 h was significantly greater than that at 24, but not 48, h. The concentration produced by cells stimulated with LPS was 2327 ± 77.3 U/ml at 24 h, 2588 ± 25.3 U/ml at 48 h and 3253 ± 109.1 U/ml at 72 h. These figures are markedly greater than the amounts secreted by undiluted cultures (see figure 2.10). The amounts produced by cells stimulated by PHA or by the mixture were also approximately doubled when cells were diluted 1:2. The concentrations produced under PHA stimulation were 2674 ± 267.0 , 4523 ± 55.1 and 6047 ± 198.7 U/ml and for the mixture were 4658 ± 349.1 , 7064 ± 198.5 and 8991 ± 244.8 U/ml respectively.

Similar experiments were carried out with whole blood that was collected from healthy volunteers. The effect of Graves' disease on whole blood release of sIL-2R was investigated in a pilot experiment. For this, blood was collected from three patients with untreated Graves' disease. Samples were prepared as before, and experiments were set up within one hour after collection. Two 12-well plates were set up for each patient. The first plate was set up undiluted and the second plate was set up using samples diluted 1:2. The final concentration of LPS for both protocols was 1 $\mu\text{g/ml}$, and that of PHA was 10 $\mu\text{g/ml}$. The final concentrations of mixed

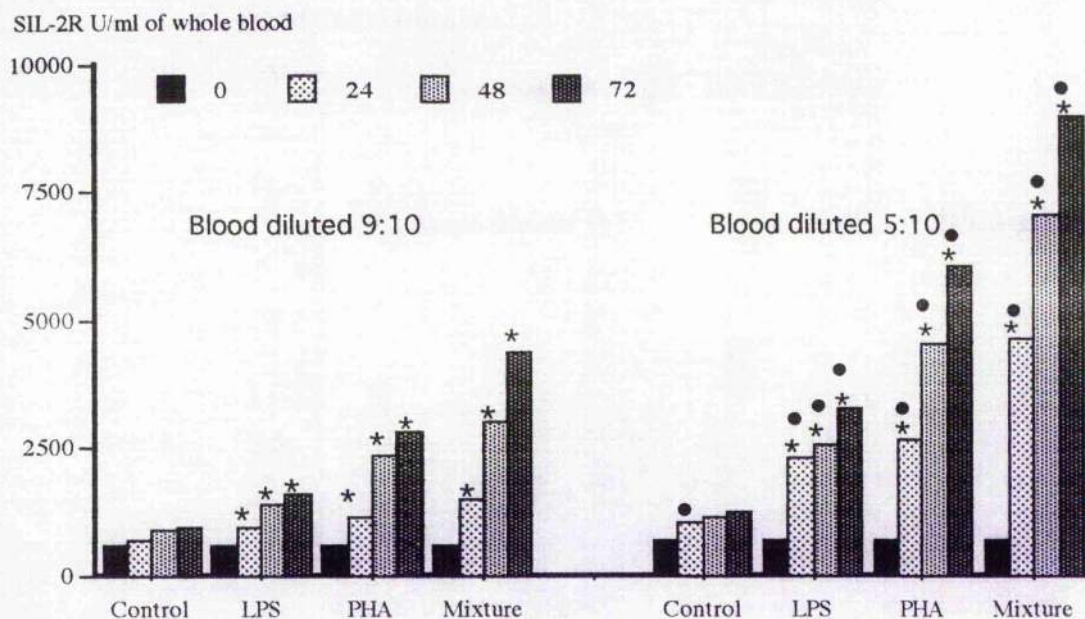


Figure 2.10: Effect of dilution of whole blood on production of sIL-2R. Whole blood was stimulated under identical conditions, for various times, with different dilution factors. The final concentration of LPS was 1 $\mu\text{g/l}$, while that of PHA was 10 $\mu\text{g/l}$. Stars show significant differences between value obtained for each stimulator at each time point, and value obtained for unstimulated cells (control), while (•) show significant differences between corresponding values in each set that were caused by change of dilution factor.

stimulators were 1 $\mu\text{g/ml}$ of LPS and 10 $\mu\text{g/ml}$ of PHA. The incubation time for both plates was 72 h.

The concentration of sIL-2R in the pre-control sample of the patients was 2040 ± 58.5 U/ml, considerably greater than that in normal subjects. In unstimulated wells, the concentration of sIL-2R increased to 4319 ± 100.8 U/ml (undiluted samples) and 4400 ± 91.6 U/ml (diluted 1:2). The absolute magnitude of the increase and percentage change were also greater than those for healthy controls (112% compared to 65% for undiluted samples, and 116% compared to 70% for diluted samples). The results of this experiment are shown in figures 2.11 and 2.12.

The concentration of sIL-2R produced by cells stimulated by LPS was 6135 ± 56.9 U/ml (undiluted) and 6393 ± 123.4 U/ml (diluted). While these are not different from each other, in contrast to the results found with normal blood, they are clearly much greater than the levels produced by normal cells. The absolute magnitude of the increase over control was also greater, but only in the undiluted cultures (1816 compared to 650 U/ml). The increase was similar in both groups of subjects in the diluted cultures (1993 compared to 2013) and the percentage increase was actually considerably less in patients than in healthy controls because of the elevated initial concentrations (undiluted: 42.0% versus 67.5%; diluted: 45.2% versus 162.3%).

Similar results were obtained in cultures stimulated with PHA or the mixture of PHA and LPS. The undiluted PHA cultures produced 7544 ± 125.8 U/ml in patients compared to 2820 ± 106.7 U/ml in healthy controls, while in diluted cultures, the values were 10923 ± 245.8 and 6047 ± 198.7 U/ml. In this case, there was a difference between patients and healthy controls in both culture systems. The absolute magnitudes of the increases over control were greater for patients than controls, but again the percentage increases were lower (undiluted: 74.6% versus 192.8%; diluted: 148.2% versus 387.6%).

When both stimulators were used, the patients produced 9320 ± 30.5 U/ml (undiluted) and 17640 ± 244.3 U/ml (diluted). Again, the absolute magnitude of the increase compared to control cells was greater in patients, but the percentage increase was less (undiluted: 115.7% versus 356.07%; diluted: 300.9% versus 625.0%).

Consistently, then, the response of blood cells from patients with Graves' disease was greater than that of normal control individuals. The difference in response was seen even in control cultures, though there was also a difference in the response to stimulation. This was particularly marked in the diluted cultures exposed to the combined stimulus (figures 2.11 and 2.12).

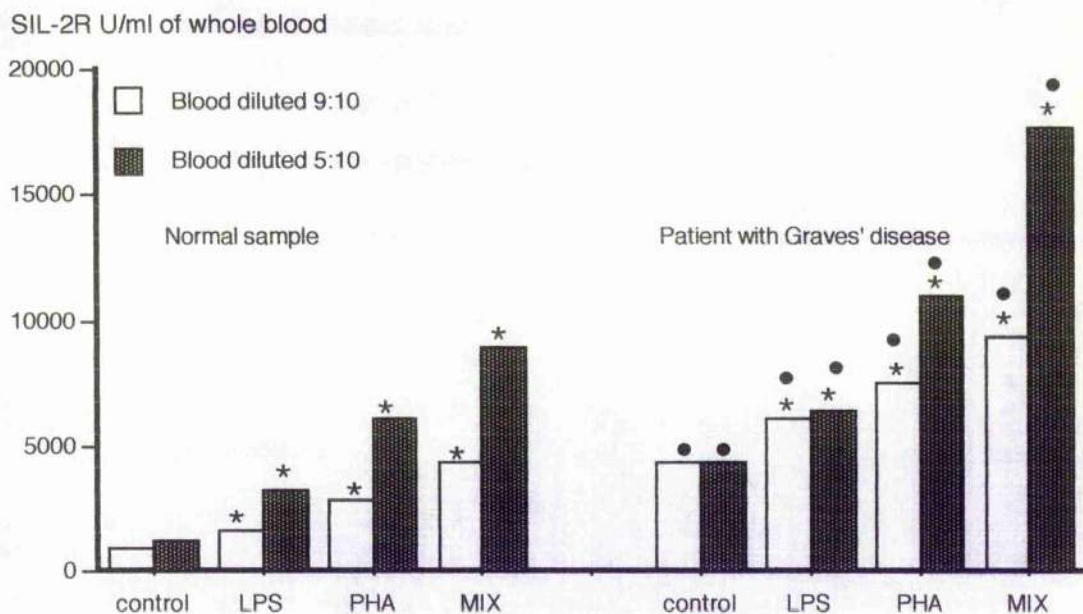


Figure 2.11: Effect of dilution of normal and patient blood on production of sIL-2R, when stimulated by 1 $\mu\text{g/ml}$ of LPS, 10 $\mu\text{g/ml}$ of PHA, and mixture of them for 72 hours. Stars show significant differences between value obtained for each stimulator and value of unstimulated cells (control) while (•) shows significant differences between corresponding values obtained for the reference and patient samples.

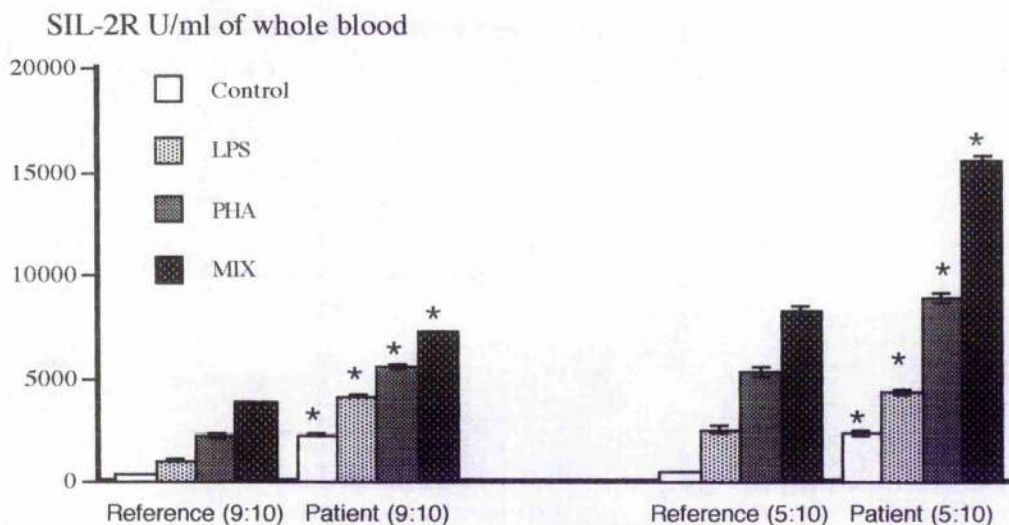


Figure 2.12: Increment in production of sIL-2R by whole blood of reference and patient samples after 72 hours stimulation with 1 $\mu\text{g/ml}$ of LPS, 10 $\mu\text{g/ml}$ PHA, or a mixture of them. All values were corrected by subtracting the values of pre-control from the results obtained for each set. Stars show significant differences between value obtained for each stimulator or control for the patients and corresponding values obtained for the reference group.

2.3.3 Production of sIL-2R and Cytokines by Whole Blood

For a further set of experiments on the release of cytokines and sIL-2R by cultures of whole blood, we adopted a protocol provided by Medgenix Diagnostics. This protocol was used for the following reasons:

This company agreed to carry out measurement of various cytokines on our samples if we followed a method which had been validated by De Groote and co-workers (1992). With this method whole blood was diluted 1:11 with the medium, which would allow us to examine the effect of higher dilution factors on sIL-2R release. An advantage of this protocol was that polypropylene tubes are used instead of plates, and cells were less liable to stick to these tubes than to the flat-well plates used previously.

Whole blood samples from reference group and patients with Graves' disease were obtained as described in section 2.2, and stimulated with a mixture of LPS/PHA or a mixture of Ca ionophore A23187 and PMA for 4, 24, or 72 h as described in section 2.2.5. Because the values of cytokine production were highly skewed and the variances between the groups were greatly different, mean and standard deviation are mentioned in the text and graphs.

2.3.3.1 Soluble IL-2 Receptor

Soluble IL-2R was determined in plasmas separated before incubation and in supernatants from LPS/PHA stimulated WB at 72 h only, since the preliminary experiments had indicated that production would be maximal at this time. Since the same volume of whole blood was used throughout this set of experiments, all values determined for supernatants are expressed in unit per ml of supernatant.

2.3.3.1.1 *Reference range for plasma*

A new reference range was determined for sIL-2R in heparinized plasma (449 ± 134.35 U/ml). There was a significant difference ($p=0.02$) between this range and the previous reference range established for serum (326 ± 88.20 U/ml). Although the number of cases is small ($n=6$ for plasma, and $n=16$ for serum), this suggests that the plasma level of sIL-2R may be higher than the corresponding value for serum.

The plasma level of sIL-2R for patients with Graves' disease had a mean value of 2106 ± 983.46 U/ml. This was significantly different ($p<0.001$) from the reference range. This result agreed with the findings for untreated Graves' patients that were reported in the previous section.

2.3.3.1.2 Production from blood in vitro

When peripheral bloods of the reference group ($n=6$) were incubated without stimulation for 72 h, the amount of sIL-2R produced was 871 ± 36 U/ml (mean \pm SD). Stimulation of the whole blood with a mixture of LPS and PHA increased the production of sIL-2R to 1575 ± 25 U/ml, significantly different ($P<0.0001$) from the unstimulated group.

When peripheral bloods of the patients' group ($n=16$) were incubated unstimulated for 72 h, the production of sIL-2R was 881 ± 33 U/ml. Statistically there was no significant difference between the values obtained for supernatant separated from the unstimulated set of reference samples (871 ± 36 U/ml), and the patient samples.

The production of sIL-2R by blood samples of patients had a mean value of 3200 ± 173 U/ml after stimulation with LPS and PHA, whereas the corresponding value for the reference samples was 1575 ± 25 U/ml. Statistically the difference was highly significant ($P<0.0001$). Furthermore, there was a significant difference between the values obtained for the stimulated cells of the patient group and its unstimulated control ($P<0.0001$). This finding also matched our previous experiments. Summaries of above results are presented in figure 2.13.

To determine the actual production of sIL-2R during the course of the experiment, the value of the pre-control was subtracted from values obtained for control and stimulated cells after incubation (after correction of values for dilution factor of 11) to determine the response of the cells to dilution and stimulation.

The mean production of sIL-2R by the WB cells of the reference group due to the effect of dilution alone (unstimulated wells) was 9136 ± 352 U/ml of whole blood, and the corresponding value for patient samples was 7591 ± 398 U/ml of whole blood. This indicates that dilution and incubation itself caused a significant increase of sIL-2R for both groups, when these values were compared to the pre-control. Production of sIL-2R by the reference group was significantly higher than that of the patient samples ($p<0.05$).

The recalculated results for stimulated whole blood (LPS/PHA) indicate that the production of sIL-2R by the cells of both groups was increased significantly when compared to their unstimulated controls. The mean value was 16883 ± 270 U/ml of WB for the reference group, and the corresponding value for patient samples was 33098 ± 1881 U/ml of WB. There was a highly significant difference ($p<0.0001$) between these increases. This shows that the cells of patient group are more sensitive to stimulation than the cells of reference group. Summaries of recalculated results are presented in figure 2.13.

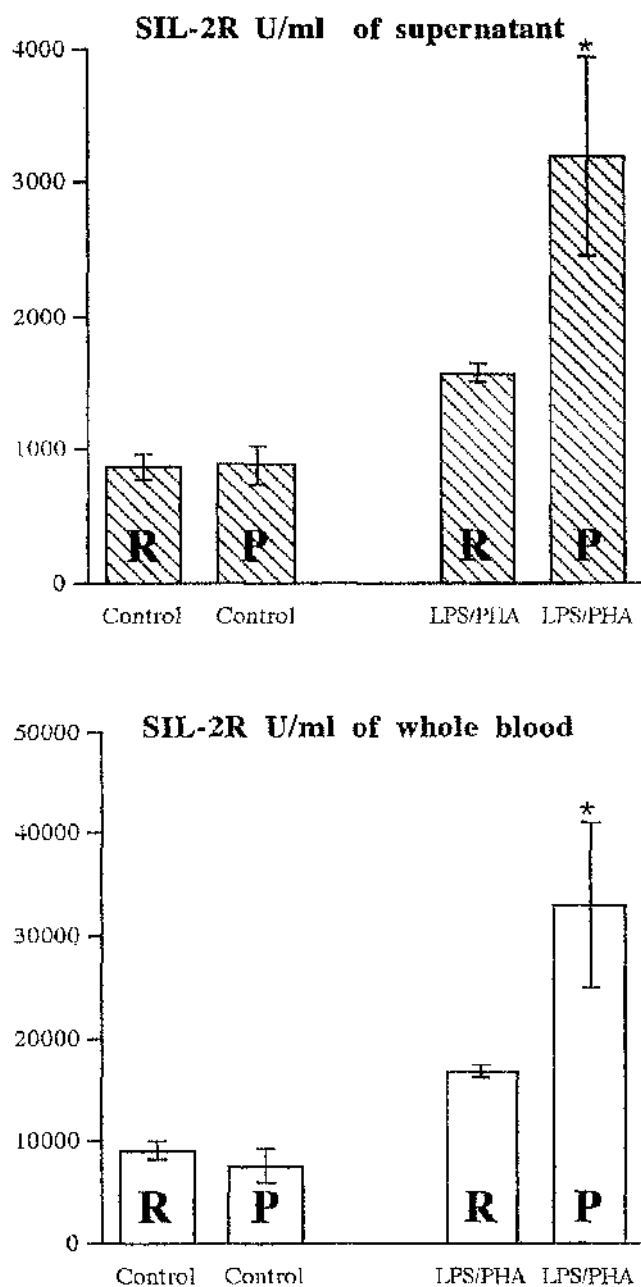


Figure 2.13: Effect of dilution (1:11; Medgenix protocol) and stimulation of whole blood of the reference (R, n=6) and patient samples (P, n=16) on production of sIL-2R. Upper graph: the mean and standard deviation of sIL-2R obtained in supernatants of the cells when incubated for 72 hours. Lower graph: same values after recalculating the values as per ml of whole blood, and subtracting the values of pre-control from each sample.

2.3.3.2 Interleukin-2

2.3.3.2.1 *Unstimulated*

When peripheral bloods of the reference group (n=6) were incubated without addition of stimulators, the production of IL-2 was undetectable in 4 cases and reached a maximum level of 54 pg/ml in the remainder after 72 h. In the patients with Graves' disease, production of IL-2 without stimulation was also undetectable for 4 cases throughout the study. The mean production of IL-2 was 72 ± 38 pg/ml at 4 h, 157 ± 87 pg/ml at 24 h, and 201 ± 117 pg/ml at 72 h. The values obtained for each time point were not significantly different from each other. Furthermore, there were no significant difference between the values obtained for the reference group and the patient group at any of the three time points.

2.3.3.2.2 *LPS/PHA*

Stimulation of whole blood from control subjects with a mixture of LPS and PHA increased the production of IL-2. The mean values were 64 ± 59 pg/ml (mean \pm SD) after 4 h, 370 ± 59 pg/ml after 24 h, and 149 ± 38 pg/ml after 72 h. Statistically, the release of IL-2 at 24 h and 72 h was significantly different from the unstimulated controls ($p < 0.01$ and < 0.05). The production reached its highest level after 24 h. The value at this time point was significantly higher than at 4 h ($P = 0.001$), and declined by 72 h to a level significantly lower ($P < 0.01$) than at 24 h, while not significantly different from the value obtained for 4 h.

Stimulation of whole blood of the patients with a mixture of LPS and PHA increased the production of IL-2 to 120 ± 50 pg/ml after 4 h, 762 ± 141 pg/ml after 24 h, and 986 ± 166 pg/ml after 72 h. The value obtained at 4 h was not different from the unstimulated control at the same time point, whereas the values obtained at 24 h and 72 h were significantly higher than their controls ($p < 0.01$). Furthermore, there were significant differences between the value obtained for 4 h and the other time points ($p < 0.001$ between 4 and 24 h and < 0.0001 between 4 and 72 h).

The pattern of IL-2 production by the patient group was different from that of the reference group. In the reference group, the production of this cytokine reached its highest level at 24 h and declined after this time, whereas in the patients' group its production continued after 24 h, and the highest level was detected after 72 h stimulation. There was a significant difference between patients and controls after 72 h ($p < 0.01$).

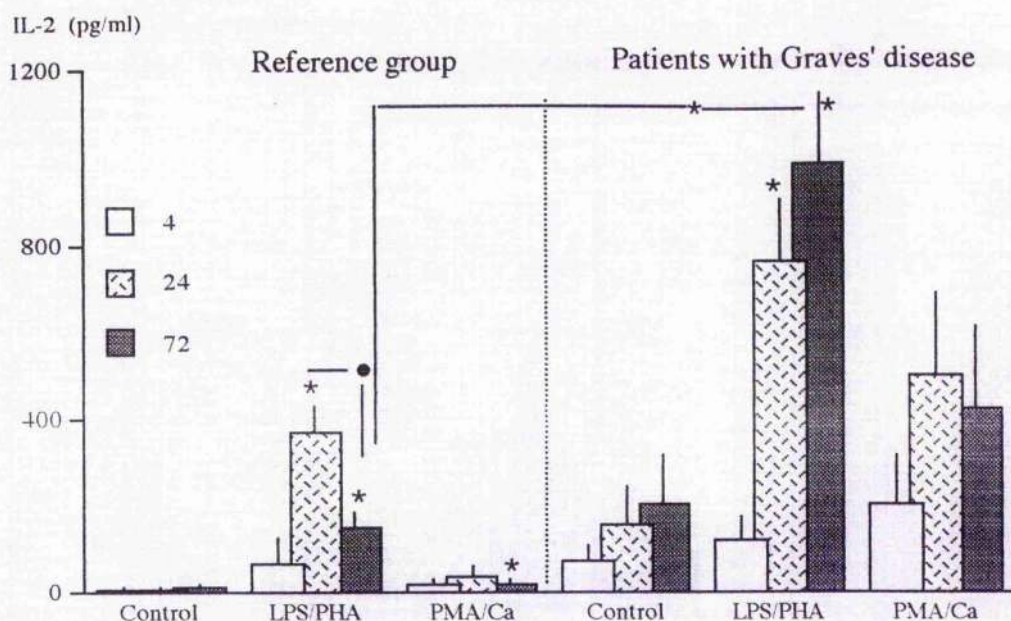


Figure 2.14: Increases in production of IL-2 by whole blood of reference and patient samples after stimulation with a mixture of LPS and PHA, or a mixture of PMA and Ca ionophore, for different periods. The final concentrations were 22.5 mg/l for LPS, 4.5 mg/l for PHA, 89 μ g/l for Ca ionophore, and 2.7 μ g/l for PMA. The (*) shows significant increases compared to the self-control, and (•) shows significant differences.

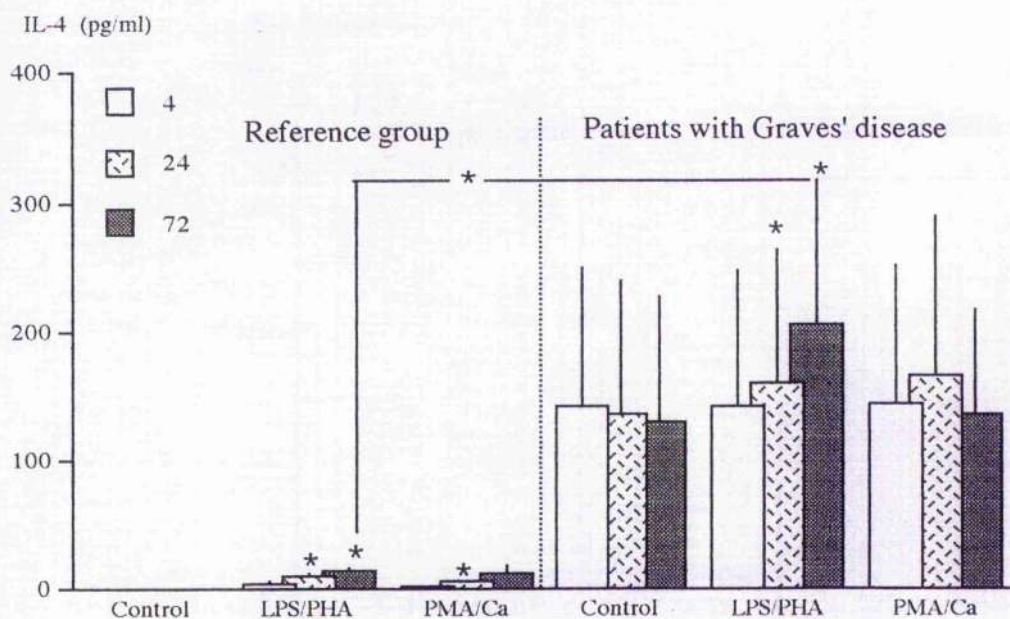


Figure 2.15: Increases in production of IL-4 by whole blood of reference and patient samples after stimulation with a mixture of LPS and PHA, or a mixture of PMA and Ca ionophore, for different periods. The final concentrations were 22.5 mg/l for LPS, 4.5 mg/l for PHA, 89 μ g/l for Ca ionophore, and 2.7 μ g/l for PMA. The (*) shows significant increases, compared to self-control.

2.3.3.2.3 Ca/PMA

Stimulation of whole blood of controls with a mixture of Ca ionophore and PMA also increased the production of IL-2. The mean values were 15 ± 15 pg/ml after 4 h, 37 ± 20 pg/ml after 24 h, and 18 ± 11 pg/ml after 72 h. The value at 72 h was significantly different from its unstimulated control ($P < 0.05$).

Stimulation of whole blood of the patients with a mixture of Ca ionophore and PMA increased the production of IL-2 to 201 ± 117 pg/ml after 4 h, 503 ± 186 pg/ml after 24 h, and 420 ± 196 pg/ml after 72 h. The production of IL-2 at 4, 24, and 72 h was not different from the unstimulated controls at the same time point. There were no significant differences between the reference group and the patient group (figure 2.14).

2.3.3.3 Interleukin-4

2.3.3.3.1 Unstimulated

When peripheral bloods of the reference group ($n=6$) were incubated without stimulation no production of IL-4 was detectable at all three time points. Peripheral blood samples of the patients with Graves' disease ($n=18$) tended to release more IL-4 than the control group. The production of IL-4 was undetectable for 11 cases after 4 h incubation. The mean value obtained for this set was 141 ± 109 pg/ml. The mean value obtained for the unstimulated cells after 24 h incubation was 136 ± 104 pg/ml. After 72 h, the value was 130 ± 98 pg/ml. Statistically, the values obtained at each time point were not significantly different from each other. Furthermore, there were no significant differences between the values obtained for the reference group and the patient group at any of the three time points.

2.3.3.3.2 LPS/PHA

Stimulation with a mixture of LPS and PHA increased the production of IL-4 from control cells slightly. The mean values were 3 ± 3 pg/ml after 4 h, 10 ± 2 pg/ml after 24 h, and 13 ± 3 pg/ml after 72 h. Statistically, the values obtained at 24 h and 72 h were significantly higher than their controls ($p < 0.05$). Stimulation of the whole blood of the patients with a mixture of LPS and PHA increased the production of IL-4 to 141 ± 107 pg/ml after 4 h, 160 ± 104 pg/ml after 24 h, and 206 ± 111 pg/ml after 72 h. Statistically, the values obtained at 24 and 72 h were significantly higher than the unstimulated controls ($p < 0.05$). There was a

significantly greater production of IL-4 from patients' samples than from the reference group at 72 h ($p < 0.05$).

2.3.3.3.3 *Ca/PMA*

Stimulation with a mixture of Ca ionophore and PMA also increased the production of IL-4 slightly. The mean values were 1 ± 0.4 pg/ml after 4 h, 5 ± 1 pg/ml after 24 h, and 11 ± 5 pg/ml after 72 h. The value obtained at 24 h was significantly different from its unstimulated control ($p < 0.05$). The values obtained at each time point were not significantly different from each other.

Stimulation of the whole blood of the patients with a mixture of Ca ionophore and PMA increased the production of IL-4 to 143 ± 109 pg/ml after 4 h, 166 ± 124 pg/ml after 24 h, and 135 ± 82 pg/ml after 72 h. Statistically, the values obtained for 4, 24, and 72 h were not different from their unstimulated controls. There were also no significant differences between patients and the reference group (figure 2.15).

2.3.3.4 Interleukin-6

2.3.3.4.1 *Unstimulated*

The production of IL-6 in the reference group ($n=6$) was either undetectable ($n=5$) or reached a maximum level of 9 pg/ml ($n=1$) after 72 h. Samples from patients with Graves' disease ($n=18$) released more IL-6 than the control group. The production of IL-6 from the unstimulated cells was undetectable for only 1 case after 4 h incubation. The mean value obtained for this group was 185 ± 105 pg/ml. The mean value obtained after 24 h incubation was 233 ± 111 pg/ml, and after 72 h, it was 229 ± 128 pg/ml. There were no statistically significant differences between these values. There was a significantly greater production of IL-6 from patients' samples than from the reference group at 4 h ($p < 0.05$), but not at 24 h or 72 h.

2.3.3.4.2 *LPS/PHA*

Stimulation of control cells with a mixture of LPS and PHA increased the production of IL-6 to 667 ± 212 pg/ml after 4 h, 7269 ± 2531 pg/ml after 24 h, and 23623 ± 4564 pg/ml after 72 h. Statistically, the values obtained for all three time points were significantly different from their unstimulated controls at the same time point ($p < 0.05$ at 4 and 24 h, < 0.01 at 72 h). The value at 72 h was significantly higher than at 4 h ($p < 0.001$) or 24 h ($p < 0.01$).

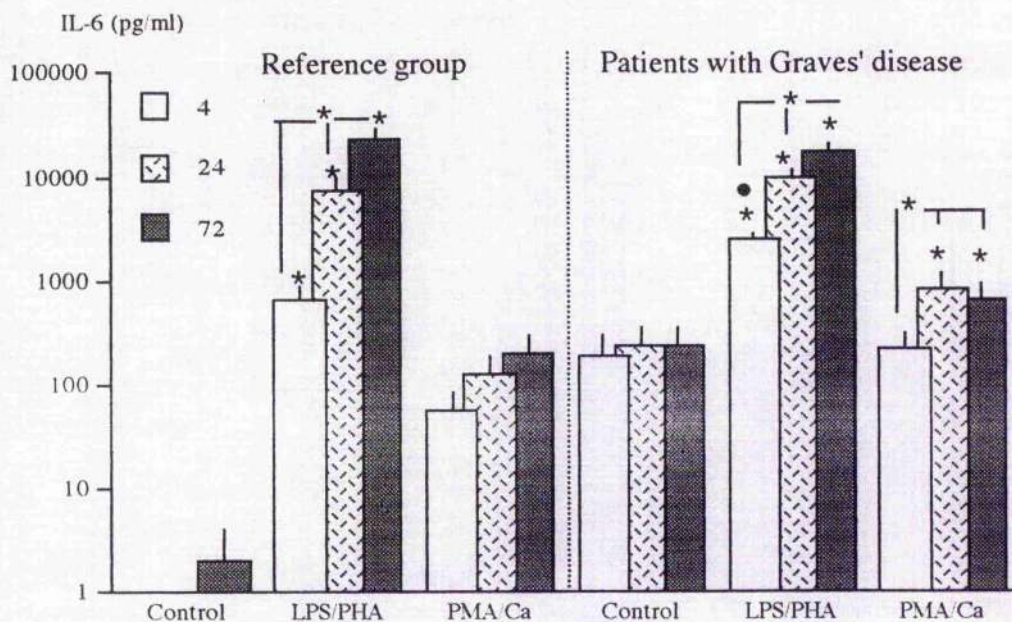


Figure 2.16: Increases in production of IL-6 by whole blood of reference and patient samples after stimulation with a mixture of LPS and PHA, or a mixture of PMA and Ca ionophore, for different periods. The final concentrations were 22.5 mg/l for LPS, 4.5 mg/l for PHA, 89 μ g/l for Ca ionophore, and 2.7 μ g/l for PMA. The (*) shows significant increase, compared to self-control, while (•) shows significant increases, compared to reference group.

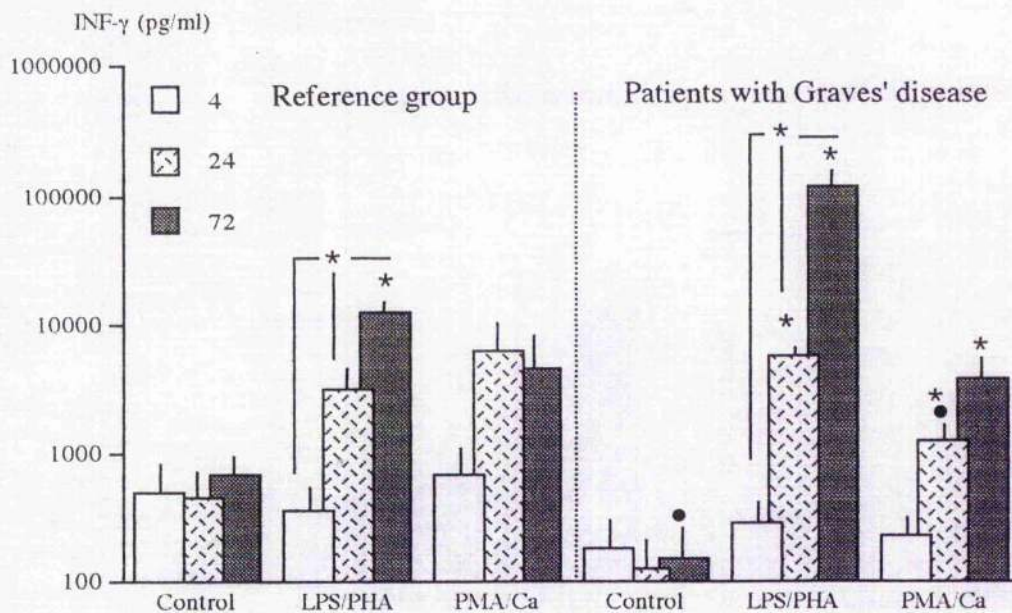


Figure 2.17: Increases in production of INF- γ by whole blood of reference and patient samples after stimulation with a mixture of LPS and PHA, or a mixture of PMA and Ca ionophore, for different periods. The final concentrations were 22.5 mg/l for LPS, 4.5 mg/l for PHA, 89 μ g/l for Ca ionophore, and 2.7 μ g/l for PMA. * shows significant increase, compared to self-control, while (•) indicates significant decreases, compared to the reference group. The (*) shows significant increase, compared to reference group.

Stimulation of blood from patients with a mixture of LPS and PHA also increased the production of IL-6. The mean values were 2472 ± 293 pg/ml after 4 h, 9564 ± 2197 pg/ml after 24 h, and 17231 ± 2837 pg/ml after 72 h. Statistically, the values obtained for all three time points were significantly higher than their unstimulated controls ($p < 0.0001$ at 4 h, < 0.0005 at 24 h and < 0.0001 at 72 h). Additionally, the value obtained for 72 h was significantly higher than those obtained for 4 h ($p < 0.0001$), and 24 h ($p < 0.05$). The patient group produced significantly more IL-6 than the reference group at 4 h ($p < 0.0001$) but not at later times.

2.3.3.4.3 *Ca/PMA*

Stimulation of the whole blood from controls with a mixture of Ca ionophore and PMA increased the production of IL-6 to 55 ± 30 pg/ml after 4 h, 123 ± 61 pg/ml after 24 h, and 199 ± 90 pg/ml after 72 h. The values obtained for 4, 24, and 72 h were not different from their unstimulated controls at the same time point. Furthermore, there were no significant differences between the values obtained at each time point, although there was a trend towards a time-dependent increase.

Stimulation of patient samples with a mixture of Ca ionophore and PMA did not cause further production of IL-6. The mean values obtained were 226 ± 92 pg/ml after 4 h, 848 ± 293 pg/ml after 24 h, and 675 ± 235 pg/ml after 72 h. Statistically, the values obtained for 4, 24, and 72 h were not significantly different from their unstimulated controls at the same time point. There were also no significant differences between corresponding values obtained for the reference group and the patient group. The summary of above data is presented in figure 2.16.

2.3.3.5 Interferon- γ

2.3.3.5.1 *Unstimulated*

The mean production from control samples was 488 ± 336 , 452 ± 271 , and 674 ± 268 pg/ml after 4, 24, and 72 h. The mean production of INF- γ from the patients with Graves' disease ($n=18$) was 183 ± 112 pg/ml at 4 h, 126 ± 86 pg/ml after 24 h, and 151 ± 112 pg/ml at 72 h. The values obtained for the patient group were significantly lower than the corresponding values obtained for the reference group at 72 h ($p < 0.05$), while there were no significant differences at the other two time points.

2.3.3.5.2 LPS/PHA

Stimulation of the whole blood of controls with a mixture of LPS and PHA increased the production of INF- γ at 24 h and 72 h. The mean values obtained for the stimulated cells were 365 ± 182 pg/ml after 4 h, 3142 ± 1473 pg/ml after 24 h, and 12466 ± 2725 pg/ml after 72 h. Only the value obtained at 72 h was significantly different from its unstimulated control ($p < 0.01$). The increase in production of INF- γ was time dependent, because the values obtained for 72 h were significantly different from the value obtained for 4 h ($p < 0.001$), or 24 h ($p < 0.01$).

Stimulation of the whole blood of the patients with a mixture of LPS and PHA increased the production of INF- γ to 284 ± 136 pg/ml after 4 h, 5682 ± 898 pg/ml after 24 h, and 117733 ± 36092 pg/ml after 72 h. Statistically, the value obtained at 4 h was not different from its unstimulated control, while values obtained for 24 h and 72 h were significantly higher than their unstimulated controls ($p < 0.001$, < 0.01). Additionally, the value obtained for 72 h was significantly higher than the values obtained for 4 h ($p < 0.01$), and 24 h ($p < 0.01$). Statistically, the patients with Graves' disease secreted more INF- γ than control subjects after 72 h ($p < 0.05$).

2.3.3.5.3 Ca/PMA

Stimulation of the whole blood of controls with a mixture of Ca ionophore and PMA also affected the production of INF- γ . The mean values obtained were 674 ± 408 pg/ml after 4 h, 6195 ± 3948 pg/ml after 24 h, and 4547 ± 3576 pg/ml after 72 h. Statistically, the values obtained for 4, 24, and 72 h were not different from their unstimulated controls at the same time points. Furthermore, there were no significant differences among the values obtained at each time point.

Stimulation of whole blood of the patients with a mixture of Ca ionophore and PMA caused release of 230 ± 83 pg/ml after 4 h, 1258 ± 399 pg/ml after 24 h, and 3784 ± 1585 pg/ml after 72 h. The values obtained for 24, and 72 h were significantly different from their unstimulated controls ($p < 0.05$). The production of INF- γ after 72 h was significantly higher than the value obtained for 4 h ($p < 0.05$). There was a significant difference between release of INF- γ between patients and controls after 24 h ($p < 0.05$). The summary of above data is presented in figure 2.17.

2.3.3.6 Tumor Necrosis Factor- α

2.3.3.6.1 *Unstimulated*

Incubation of unstimulated peripheral bloods from the reference group ($n=6$) did not induce production of TNF. In 5 cases the level of TNF was undetectable throughout the study, and only one case had a detectable concentration of TNF, after 4 h incubation.

The mean production of TNF from unstimulated patient samples ($n=18$) was 294 ± 168 pg/ml at 4 h, 218 ± 114 pg/ml after 24 h incubation and 249 ± 143 pg/ml at 72 h. There were no significant differences between these values. The values obtained for the patient group were not significantly different from those obtained for the reference group.

2.3.3.6.2 *LPS/PHA*

Stimulation of the whole blood of controls with a mixture of LPS and PHA increased the production of TNF to 339 ± 94 pg/ml after 4 h, 3790 ± 493 pg/ml after 24 h, and 7986 ± 1283 pg/ml after 72 h. These were significantly higher than their unstimulated controls at the same time points ($p<0.05$, <0.001 , <0.01). Furthermore, the increase in production of TNF was time dependent, because the values obtained for 72 h were significantly different from the value obtained for 4 h ($p<0.0001$), or 24 h ($p<0.01$).

Stimulation of whole blood of the patients with a mixture of LPS and PHA increased the production of TNF to 1668 ± 237 pg/ml after 4 h, 2488 ± 343 pg/ml after 24 h, and 2731 ± 379 pg/ml after 72 h. Statistically, the values obtained for all three time points were significantly higher than their unstimulated controls ($p<0.001$, <0.0001 , <0.0001).

At 4 h, the production of TNF by the patient group was significantly higher than the reference group ($p<0.0001$). At 24 h and 72 h, patients released less TNF than controls. This decrease just failed to reach significance at 24 h ($p=0.06$), but was significant at 72 h ($p<0.0001$).

2.3.3.6.3 *Ca/PMA*

Stimulation of the whole blood with a mixture of Ca ionophore and PMA also increased the production of TNF. The mean values were 19 ± 15 pg/ml after 4

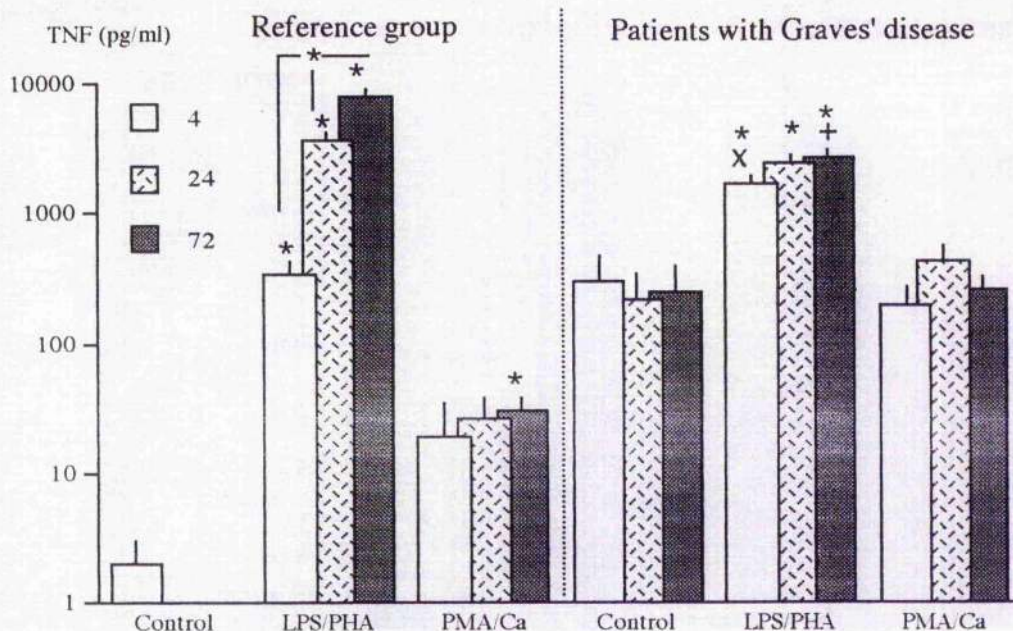


Figure 2.18: Increases in production of TNF- α by whole blood of reference and patient samples after stimulation with a mixture of LPS and PHA, or a mixture of PMA and Ca ionophore, for different periods. The final concentrations were 22.5 mg/l for LPS, 4.5 mg/l for PHA, 89 μ g/l for Ca ionophore, and 2.7 μ g/l for PMA. The (*) shows significant increase, compared to self-control, while x shows significant increases, and + indicates significant decreases, compared to reference group.

SIL-2R U/ml of supernatant

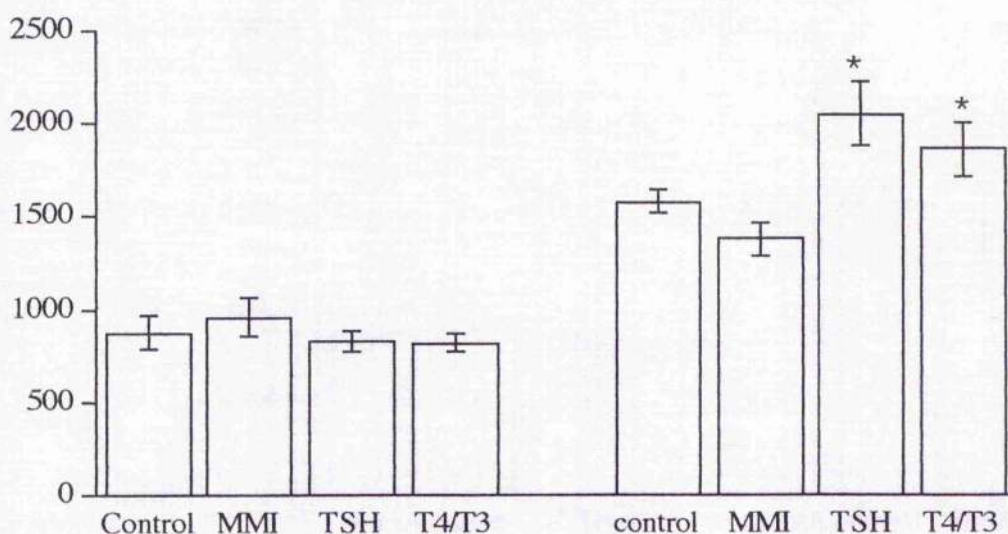


Figure 2.19: Effect of Methimazole, TSH, and thyroid hormones on whole blood production of sIL-2R, in presence (right side) and absence (left side) of a mixture of LPS and PHA for 72 hours. The (*) shows significant differences compared to self-control.

h, 26 ± 12 pg/ml after 24 h, and 30 ± 7 pg/ml after 72 h. The value obtained for 72 h was significantly higher than its unstimulated control.

Stimulation of the whole blood of the patients with a mixture of Ca ionophore and PMA caused production of TNF of 194 ± 72 pg/ml after 4 h, 424 ± 135 pg/ml after 24 h, and 261 ± 67 pg/ml after 72 h. Statistically, the values obtained for 4, 24, and 72 h were not significantly different from their unstimulated controls at the same time point, or from each other. The production of TNF was not significantly greater in patients than controls, at any time point. The summary of the above data is presented in figure 2.18.

2.3.4 Effects of Thyroid Hormones, TSH and Methimazole on Cytokine Production

As the concentration of thyroid hormones in blood altered during the treatment of the patients with Graves' disease studied in section 2.3.1, the concentration of sIL-2R appeared to change in parallel. It seemed possible that the thyroid hormones themselves might affect the concentration of cytokines. To test this hypothesis in vitro, blood from normal subjects was incubated with thyroid hormones, TSH, or carbimazole, the most commonly used anti-thyroid drug in this country, as described in section 2.2.5, and the production of cytokines was measured.

Only the 72 h specimen was assayed for sIL-2R. A significant effect of treatment was seen for both the unstimulated cells and for the cells stimulated with LPS/PHA. In the unstimulated cells, this was caused by an increase in the cells treated with methimazole (957 ± 40.6 U/ml). While this just failed to reach significance when compared to control (871.4 ± 35.7 , $p=0.06$), the difference between methimazole and the cells treated with either thyroid hormones (820 ± 20.7 U/ml) or TSH (825 ± 24.1 U/ml) was significant at the level of $p < 0.01$.

When the cells were stimulated with LPS/PHA, the control value was 1575 ± 25.3 U/ml. There was a decrease in the cells stimulated with methimazole (1378 ± 37.3 U/ml) which again just failed to reach significance when tested by the Bonferroni-Dunn criterion ($p=0.014$, critical value $p<0.008$), but there was a significant increase in those treated with TSH to 2046 ± 71.3 U/ml and in those treated with thyroid hormones to 1859 ± 61.5 U/ml (figure 2.19).

The production of cytokines by the cells that were stimulated only with thyroid hormones, TSH, or methimazole were not significant compared to the control cells (unstimulated cells). These results were shown in figure 2.20 to 2.23. The cytokine production by the cells that were stimulated with thyroid hormones, TSH, or methimazole, in the presence of a mixture of Ca ionophore and PMA, was

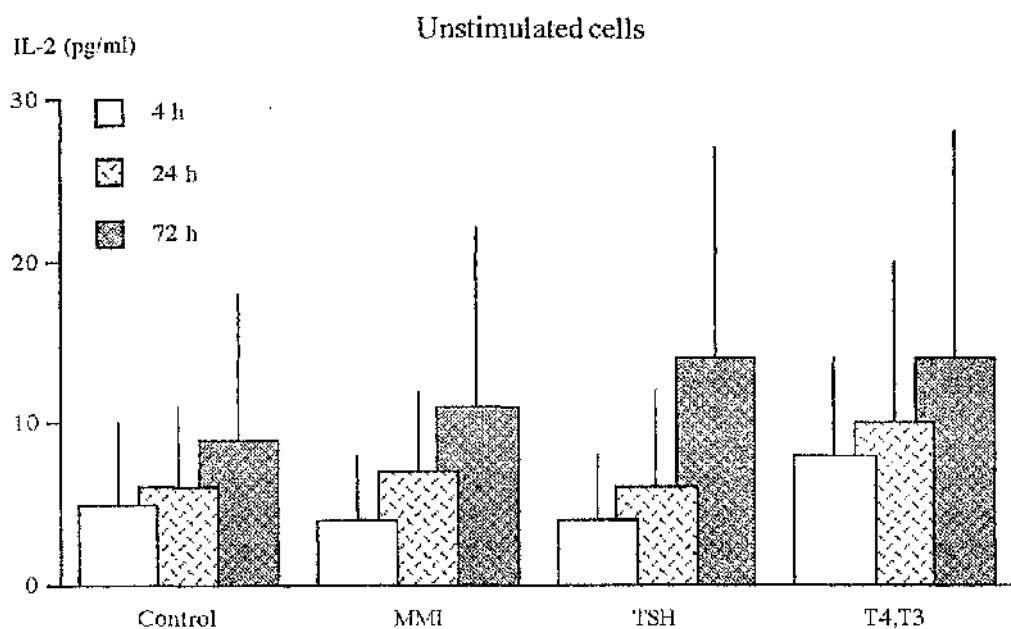


Figure 2.20: Production of IL-2 by whole blood (reference group) diluted 1:11 with the medium, and stimulated with methimazole (1m mol/l), TSH (50 mU/l) or a mixture of T₄ (1 μ mol/l) and T₃ (10 nmol/l), for different periods.

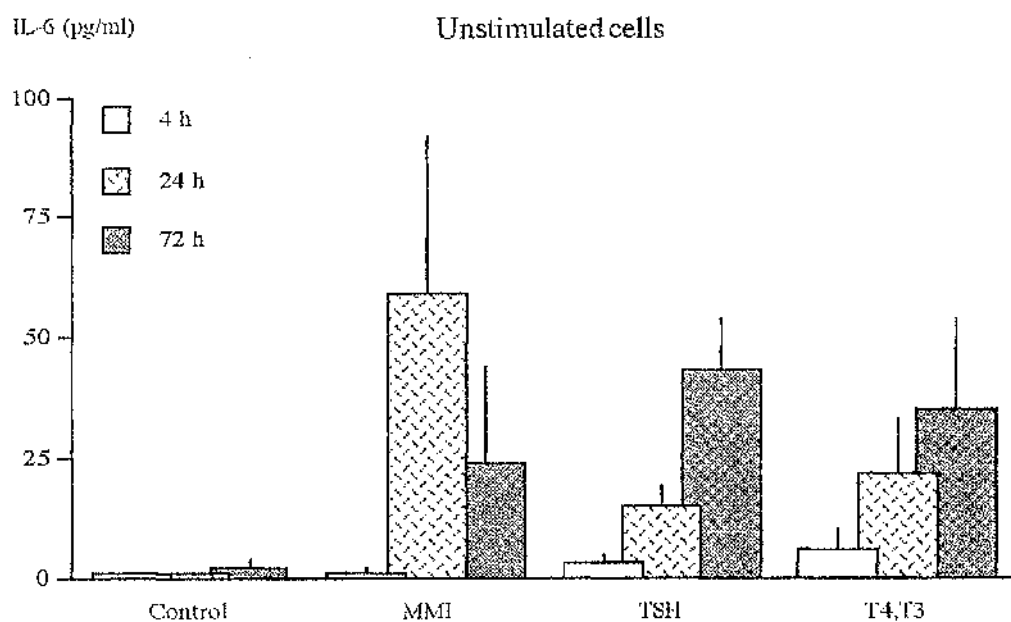


Figure 2.21: Production of IL-6 by whole blood (reference group) diluted 1:11 with the medium, and stimulated with methimazole (1m mol/l), TSH (50 mU/l) or a mixture of T₄ (1 μ mol/l) and T₃ (10 nmol/l), for different periods.

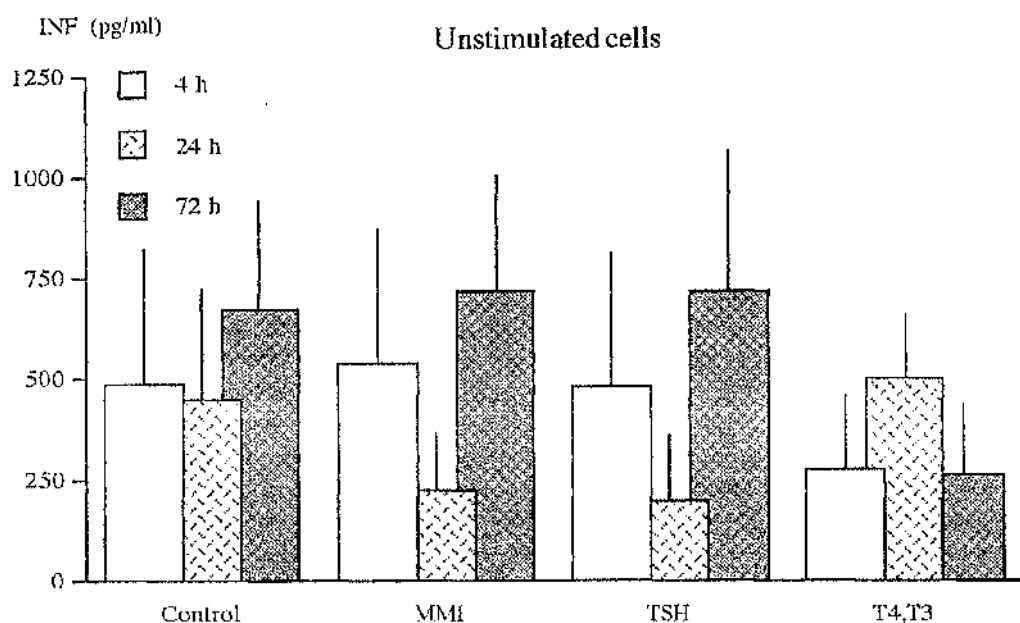


Figure 2.22: Production of INF- γ by whole blood (reference group) diluted 1:11 with the medium, and stimulated with methimazole (1m mol/l), TSH (50 mU/l) or a mixture of T₄ (1 μ mol/l) and T₃ (10 nmol/l), for different periods.

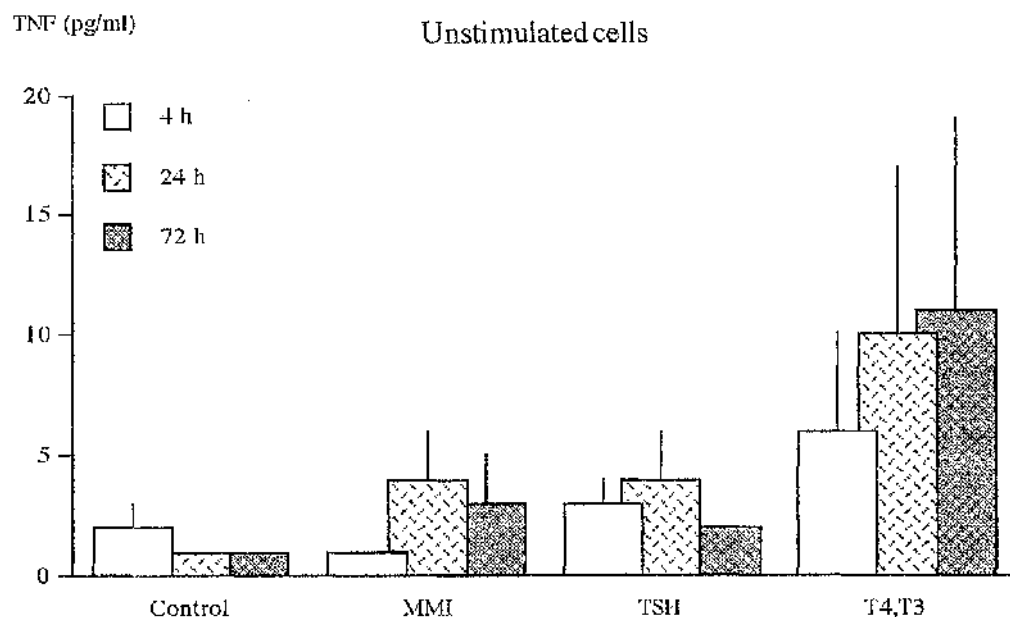


Figure 2.23: Production of TNF- α by whole blood (reference group) diluted 1:11 with the medium, and stimulated with methimazole (1m mol/l), TSH (50 mU/l) or a mixture of T₄ (1 μ mol/l) and T₃ (10 nmol/l), for different periods.

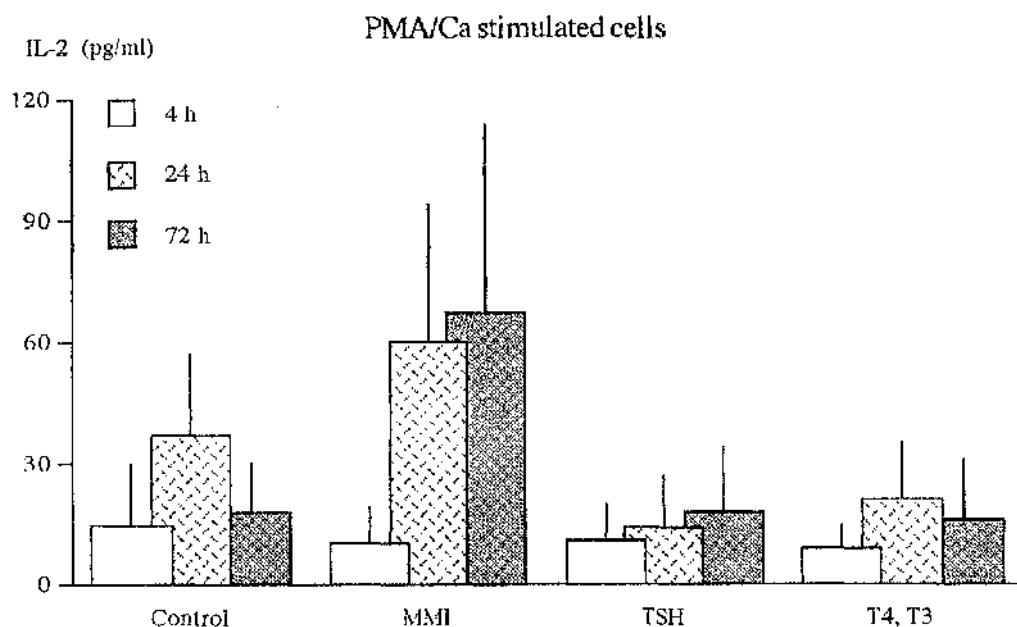


Figure 2.24: Production of IL-2 by whole blood (reference group) diluted (1:11) with the medium containing a mixture of PMA ($2.7 \mu\text{g/l}$) and Ca ionophore ($89 \mu\text{g/l}$), and stimulated with methimazole (1m mol/l), TSH (50mU/l) or a mixture of T_4 ($1 \mu\text{mol/l}$) and T_3 (10nmol/l), for different periods.

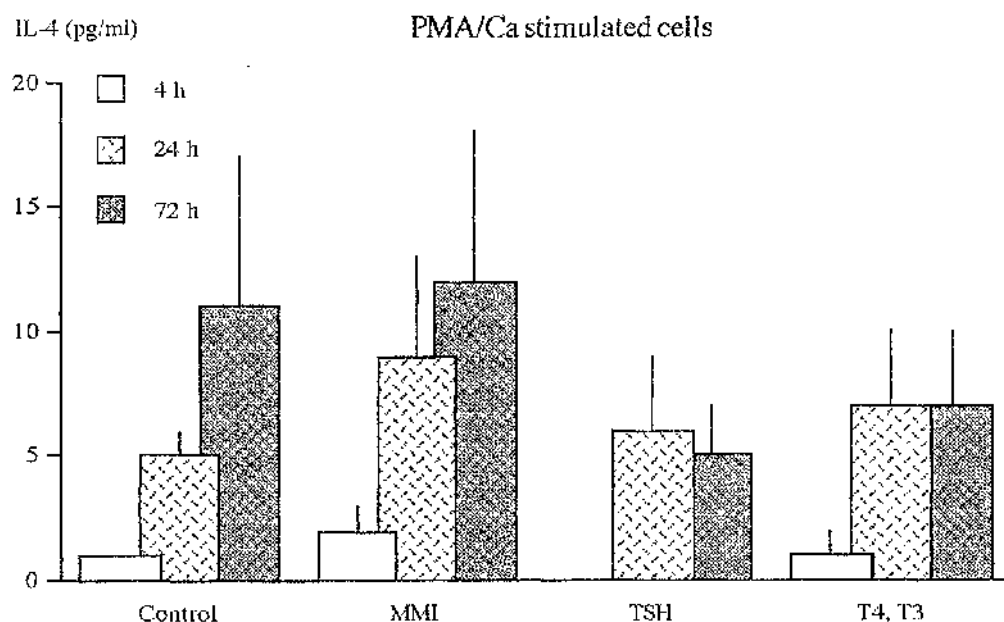


Figure 2.25: Production of IL-4 by whole blood (reference group) diluted (1:11) with the medium containing a mixture of PMA ($2.7 \mu\text{g/l}$) and Ca ionophore ($89 \mu\text{g/l}$), and stimulated with methimazole (1m mol/l), TSH (50mU/l) or a mixture of T_4 ($1 \mu\text{mol/l}$) and T_3 (10nmol/l), for different periods.

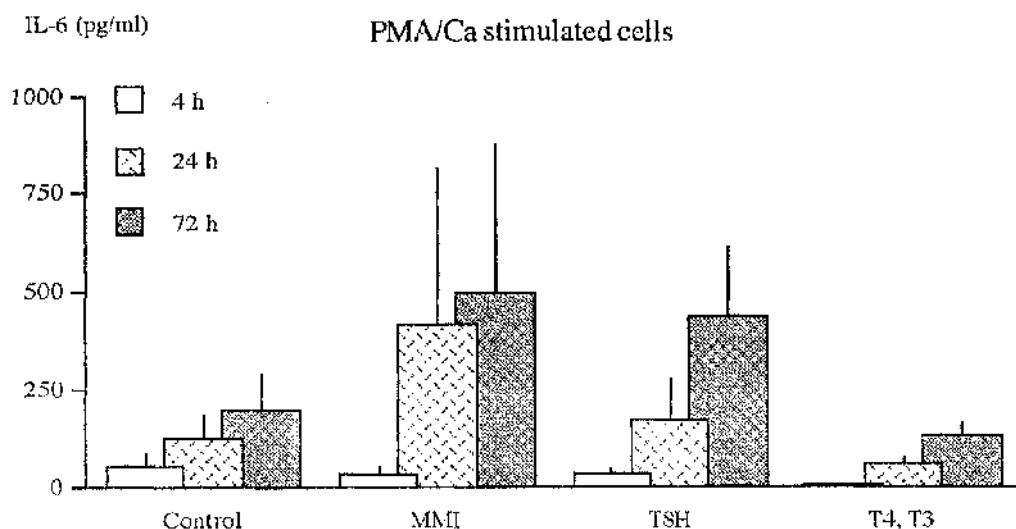


Figure 2.26: Production of IL-6 by whole blood (reference group) diluted (1:11) with the medium containing a mixture of PMA ($2.7 \mu\text{g/l}$) and Ca ionophore ($89 \mu\text{g/l}$), and stimulated with methimazole (1m mol/l), TSH (50mU/l) or a mixture of T_4 ($1 \mu\text{mol/l}$) and T_3 (10nmol/l), for different periods.

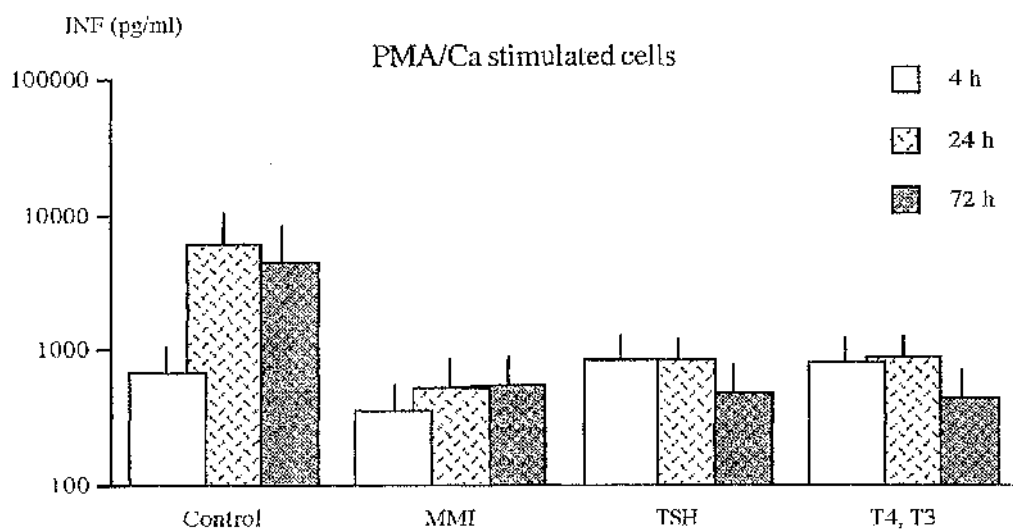


Figure 2.27: Production of INF- γ by whole blood (reference group) diluted (1:11) with the medium containing a mixture of PMA ($2.7 \mu\text{g/l}$) and Ca ionophore ($89 \mu\text{g/l}$), and stimulated with methimazole (1m mol/l), TSH (50mU/l) or a mixture of T_4 ($1 \mu\text{mol/l}$) and T_3 (10nmol/l), for different periods.

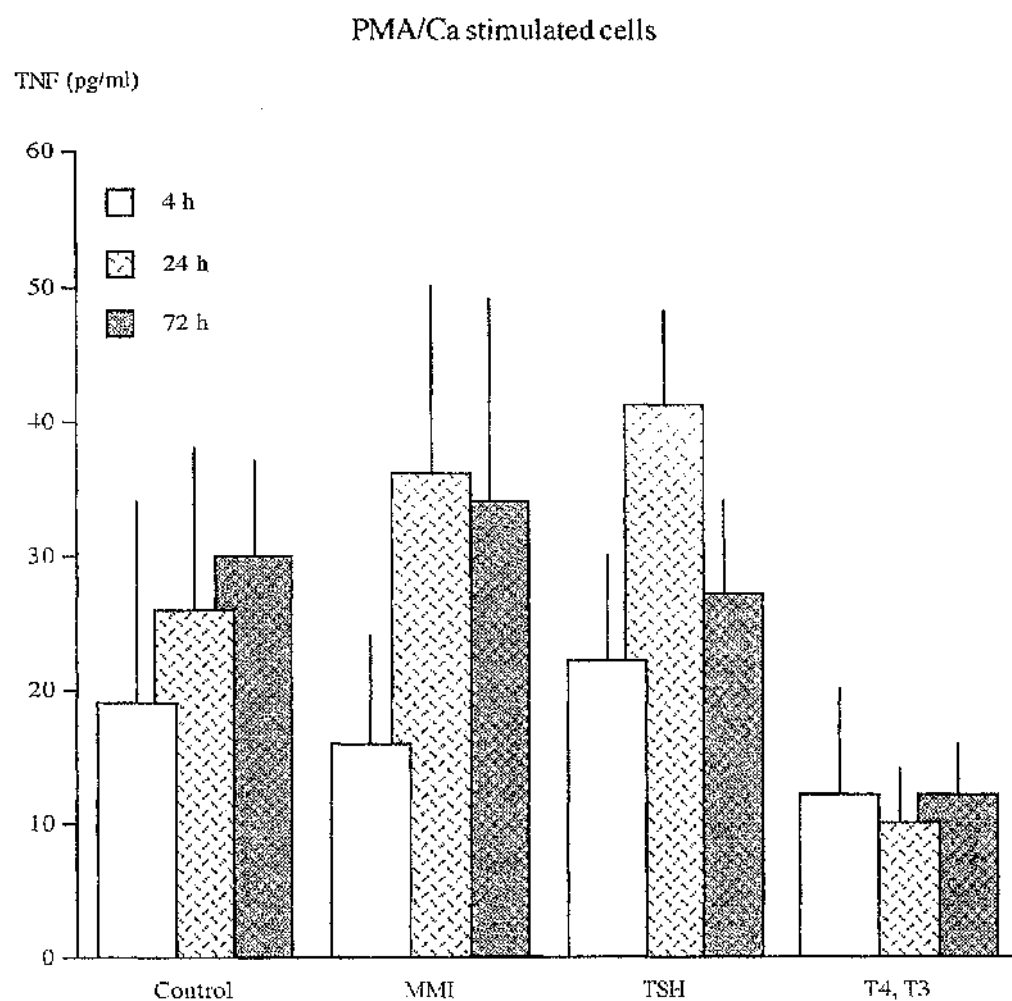


Figure 2.28: Production of TNF- α by whole blood (reference group) diluted (1:11) with the medium containing a mixture of PMA (2.7 $\mu\text{g/l}$) and Ca ionophore (89 $\mu\text{g/l}$), and stimulated with methimazole (1m mol/l), TSH (50 mU/l) or a mixture of T₄ (1 $\mu\text{mol/l}$) and T₃ (10 nmol/l), for different periods.

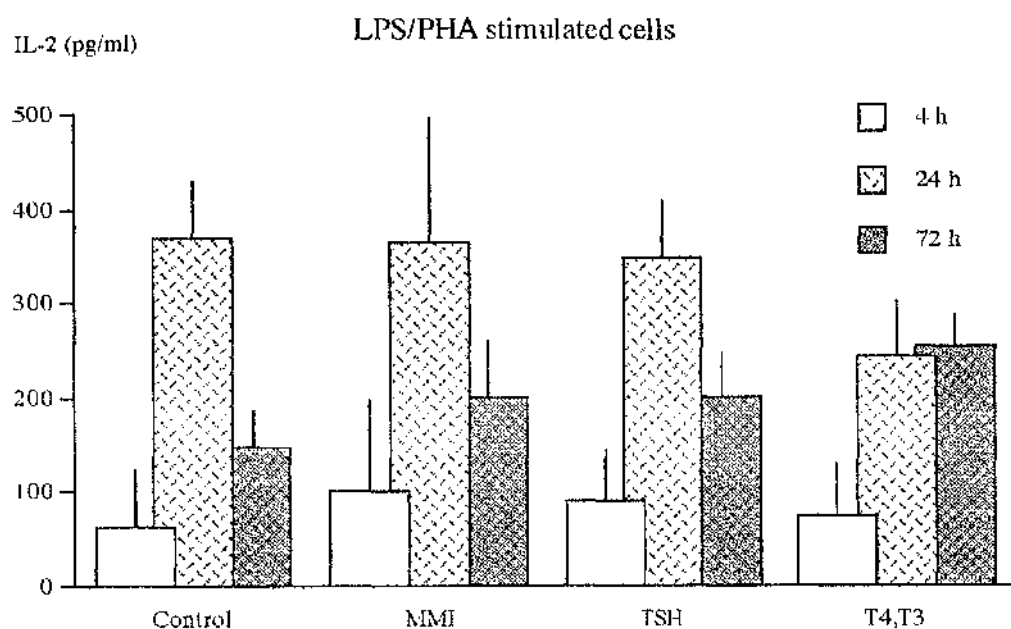


Figure 2.29: Production of IL-2 by whole blood (reference group) diluted (1:11) with the medium containing a mixture of LPS (22.5 mg/l) and PHA (4.5 mg/l), and stimulated with methimazole (1m mol/l), TSH (50 mU/l) or a mixture of T₄ (1 μ mol/l) and T₃ (10 nmol/l), for different periods.

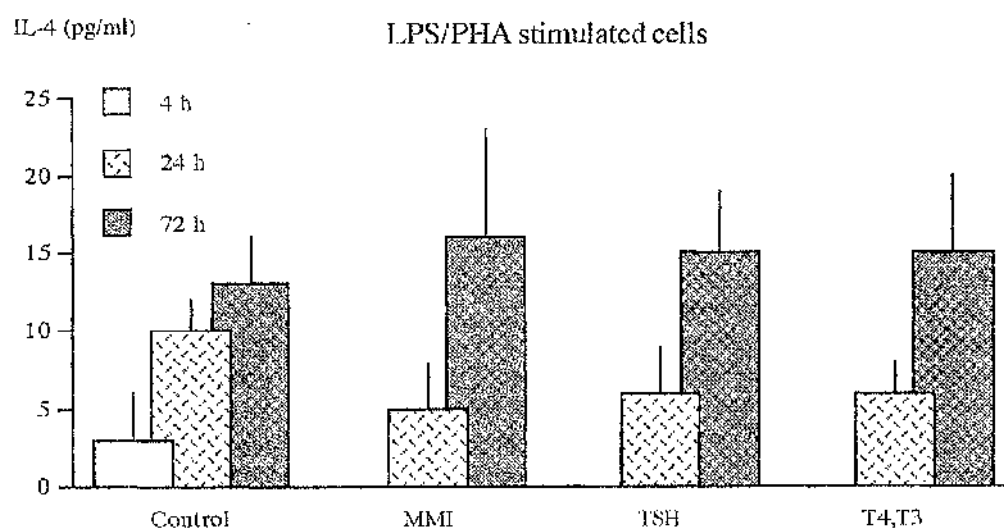


Figure 2.30: Production of IL-4 by whole blood (reference group) diluted (1:11) with the medium containing a mixture of LPS (22.5 mg/l) and PHA (4.5 mg/l), and stimulated with methimazole (1m mol/l), TSH (50 mU/l) or a mixture of T₄ (1 μ mol/l) and T₃ (10 nmol/l), for different periods.

also not significantly different from control, when tested by the Bonferroni/Dunn criterion (figure 2.24 to 2.28).

For the cytokines, significant effects of thyroid hormones, TSH, or methimazole were only seen in those samples which were stimulated with a mixture of LPS and PHA. There were no significant changes in the production of IL-2 (figure 2.29) and IL-4 (figure 2.30).

After 4 h, methimazole, TSH and thyroid hormones decreased production of TNF from 339.3 ± 94 pg/ml to 48.8 ± 23.8 pg/ml, 129.8 ± 31.8 pg/ml and 47.1 ± 19.6 pg/ml respectively. The changes caused by methimazole and thyroid hormones were significant, while that caused by TSH just failed to reach significance by the Bonferroni-Dunn criterion (figure 2.33).

After 24 h, there were significant changes in both TNF and IL-6 production. TNF (figure 2.33) release was reduced from 3789 ± 493 pg/ml to 156 ± 35 pg/ml, 254 ± 40 pg/ml and 124 ± 12 pg/ml respectively. IL-6 production was reduced from 7269 ± 2530 pg/ml to 282 ± 82 pg/ml, 653 ± 360 pg/ml and 607 ± 354 pg/ml (figure 2.31). All these changes were significant.

Finally, at 72 h, INF- γ was also affected (figure 2.32). It was reduced from 12466 ± 2724 ng/ml to 415 ± 318 ng/ml, 559 ± 318 ng/ml, and 1005 ± 445 ng/ml. TNF was reduced from 7986 ± 1282 pg/ml to 89.5 ± 16.2 pg/ml, 174 ± 40.7 pg/ml and 147.6 ± 18 pg/ml. IL-6 was reduced from 23623 ± 4563 ng/ml to 529 ± 209 ng/ml, 936 ± 326 ng/ml, and 1180 ± 355 ng/ml. All of these alterations were significant.

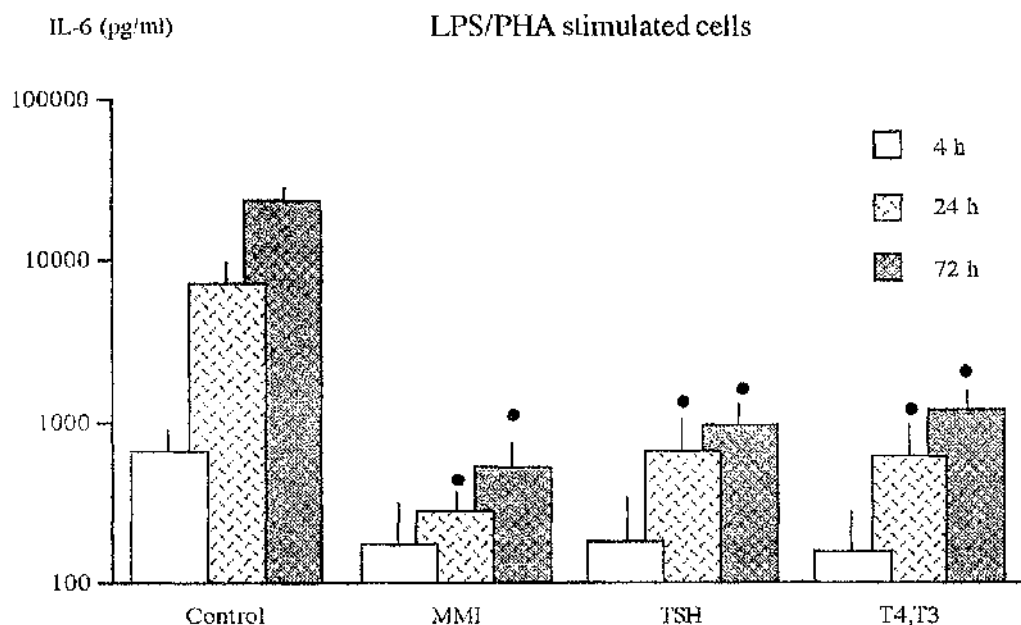


Figure 2.31: Production of IL-6 by whole blood (reference group) diluted (1:11) with the medium containing a mixture of LPS (22.5 mg/l) and PHA (4.5 mg/l), and stimulated with methimazole (1m mol/l), TSH (50 mU/l) or a mixture of T₄ (1 μ mol/l) and T₃ (10 nmol/l), for different periods. The (*) shows significant decreases, compared to the control.

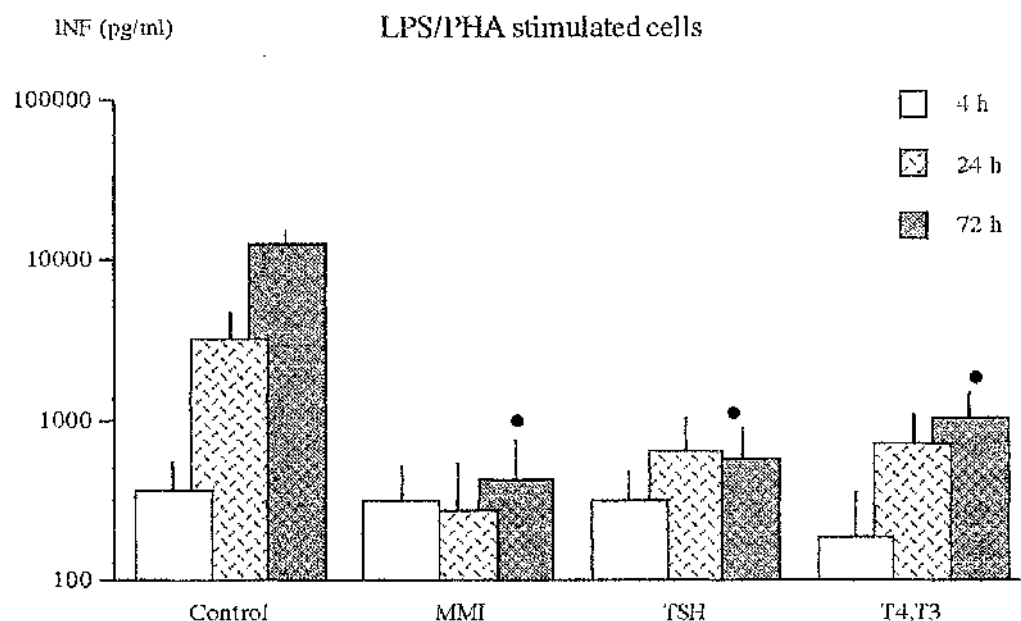


Figure 2.32: Production of INF- γ by whole blood (reference group) diluted (1:11) with the medium containing a mixture of LPS (22.5 mg/l) and PHA (4.5 mg/l), and stimulated with methimazole (1m mol/l), TSH (50 mU/l) or a mixture of T₄ (1 μ mol/l) and T₃ (10 nmol/l), for different periods. The (*) shows significant decreases, compared to the control.

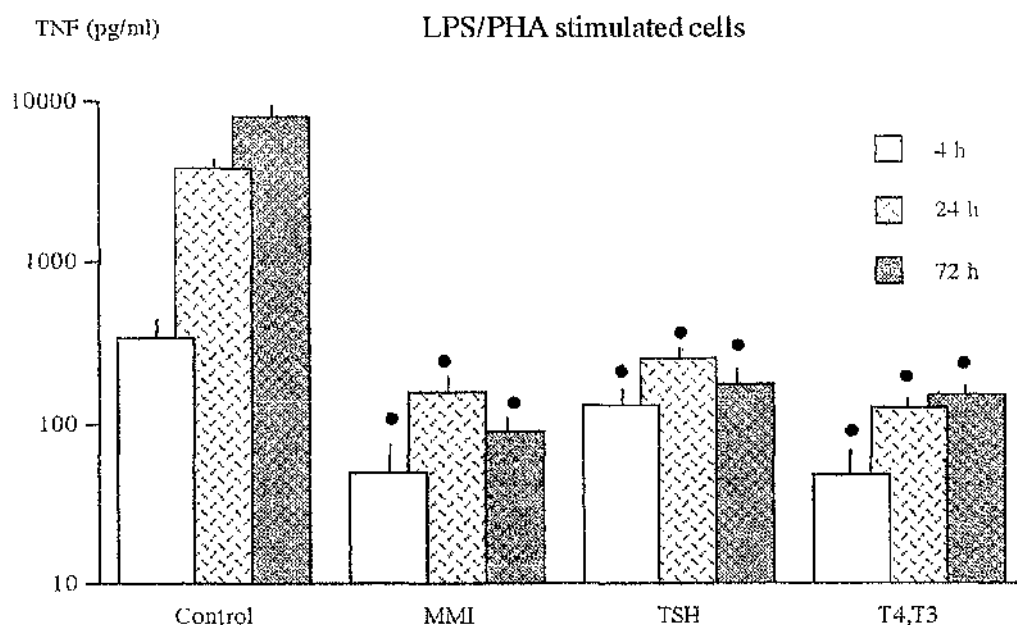


Figure 2.33: Production of TNF- α by whole blood (reference group) diluted (1:11) with the medium containing a mixture of LPS (22.5 mg/l) and PHA (4.5 mg/l), and stimulated with methimazole (1m mol/l), TSH (50 mU/l) or a mixture of T₄ (1 μ mol/l) and T₃ (10 nmol/l), for different periods. The (*) shows the significant decreases, compared to control.

SECTION FOUR

DISCUSSION

2.4.1 Soluble IL-2 Receptor In Serum

Soluble interleukin-2 receptor is a protein with a molecular mass of 40-45000. It is an enzymatic cleavage product of the α chain of the IL-2 receptor, and is present in plasma in healthy subjects. The basal plasma concentration of sIL-2R does not vary with age or sex (Giannitsis et al, 1991), but has a circadian rhythm with a peak value at 12:29 hour and a trough at 4:14 hour (Lemmer et al, 1992). The plasma level of this protein increases in many diseases including Graves' disease.

In the present study, the mean serum concentration of sIL-2R among the untreated patients was 7.5 fold higher than the value obtained for the healthy subjects who served as controls. This increase has also been reported by other investigators (Murakami et al, 1995; Murphy and Kallio, 1994; Mariotti et al, 1992 a; Weryha et al, 1991; Balázs and Farid, 1991), but the reasons for the increase in Graves' disease or other diseases listed in the introduction (section 2.1.5) are unknown.

In the present study, among untreated patients, the sIL-2R concentration in serum correlated with serum concentrations of T_4 , T_3 and FT_4 , but not with any of the thyroid antibodies. Significant correlations between the serum level of sIL-2R and either T_4 or FT_4 in Graves' disease have been reported previously (Koukkou et al, 1991). A correlation between T_3 and sIL-2R was also reported by other groups (Mariotti et al, 1992 a; Weryha et al, 1991; Koukkou et al, 1991), in agreement with the current results.

It is possible that part of the increase in the level of sIL-2R could be caused directly by the high concentration of thyroid hormones. This possibility is supported by the studies of Mariotti and colleagues (1994) who showed that the serum level of sIL-2R was decreased in patients with thyroid carcinoma who were treated with total thyroidectomy. The concentration was normalised by L-thyroxine therapy. In addition, serum IL-2R is also increased in patients with non-immune causes of hyperthyroidism, such as adenomas.

If thyroid hormones do induce sIL-2R, it is not clear whether T_4 or T_3 would be primarily responsible. Although T_4 acts primarily as a prohormone for cells that contain the 5'-deiodinase enzyme which converts it to T_3 (Brent, 1994), the thyroid receptors also have affinity for T_4 and are usually occupied by 10-15% T_4 (McDougall, 1992). It is not clear whether the immune cells that synthesise sIL-2R

contain 5'-deiodinase, leading to the local production of T_3 , which may be an inducer of sIL-2R. This needs further investigation.

Two different groups (Balázs and Farid, 1991; Weryha et al, 1991) detected a highly significant correlation between the serum level of TRAb and sIL-2R, while recent reports (Mariotti et al, 1992 a; and 1994; Murakami et al, 1995) showed no correlation between these two parameters in untreated patients with Graves' disease. Moreover, none of these groups detected a correlation between the sIL-2R and the titre of thyroglobulin or microsomal antibodies. There was no correlation between sIL-2R and the titre of TRAb or other antibodies (T and M) in the present study, suggesting that the increase in sIL-2R is not directly related to that aspect of the immune process.

Among the 49 samples collected after initiation of therapy, 30.3% were collected after one month, and 24.5% after two months of treatment. The remainder were collected between the third month and tenth month after initiation of treatment. The treatment of patients with carbimazole reduced the serum level of sIL-2R significantly, but it remained significantly higher than the control group. At this stage of the study, it could not be determined whether this decrease was a direct or indirect effect of the anti-thyroid drug on the immune system.

After initiation of treatment, there was no significant correlation between T_4 ($n=43$) or FT_4 ($n=12$) and the level of sIL-2R, while there was a significant correlation between sIL-2R and T_3 ($n=37$). There were significant negative correlations between sIL-2R and serum level of TSH and TRAb. The absence of correlation between T_4 or FT_4 and sIL-2R may be caused by the fact that T_4 returned to normal values, while the presence of a significant correlation between T_3 and sIL-2R supports the suggestion that it can modulate the serum concentration of sIL-2R (figure 2.6).

The negative correlation between TSH and sIL-2R in treated patients may be simply a reflection of the correction of the thyroid function abnormalities, or methimazole may have a direct effect on IL-2R.

The negative correlation between sIL-2R and the titre of TRAb may also indirectly reflect a suppressive effect of methimazole on the immune system. Furthermore, it shows that the serum level of sIL-2R normalised faster in the patients with higher titre of TR Ab rather than in patients with negative or low titre.

2.4.2 Study Of sIL-2R Release From Whole Blood

To explore further the possible mechanisms of the increased concentration of sIL-2R in serum, it was decided to investigate whether it was possible to stimulate blood cells in vitro to release sIL-2R. To ensure that all relevant cell-cell interactions

were preserved and to minimise disturbances to the system, unseparated whole blood was used.

For establishing the method heparinized whole blood of healthy volunteers was stimulated with LPS, PHA, or a mixture of them. Initial experiments indicated that sIL-2R production occurs not only from the stimulated whole blood cells (WB), but also from control WB that were treated with medium instead of stimulator. The 29.7% increase that occurred after 24 h in the control set could be partly due to activation of monocytes or macrophages by attachment to the plastic plate. However, there was a marked response to both LPS and PHA stimulation.

There was continued release of sIL-2R up to at least 72 hours. The data obtained from the time course experiment indicates that at least two distinct populations of cells responded to stimulation, because at all time points the response to LPS and PHA was additive. The dose-response study confirmed this finding (figure 2.8).

The population of cells that responds to LPS may consist of monocytes, which are known to produce IL-2 receptors when stimulated by LPS (Valitutti et al, 1989; Kniep et al, 1992), while the dominant cells of the set responding to PHA may be T lymphocytes (Rieckmann et al, 1995; Kniep et al, 1992; Ohashi et al, 1989; Rubin et al, 1985). There is a possibility that other cells such as B-lymphocytes and natural killer cells also contribute to sIL-2R production, stimulated by cytokines released by activated macrophages or T-lymphocytes. It has been shown that WB cells released different sets of cytokines under the influence of LPS and PHA. Stimulation by PHA induced production of IL-6, TNF- α , IL-2, INF- γ , and GM-CSF (De Groote et al, 1992), while stimulation by LPS caused production of IL-1 β , TNF- α (De Groote et al, 1992; Allen et al, 1992), and IL-6 (De Groote et al, 1992).

The data obtained from the dose study experiments also revealed that the lowest dose of LPS which was tested (1 μ g/ml) was sufficient to produce the maximum effect on release of sIL-R, whereas greater than 1 μ g/ml of PHA was required to have any effect. This suggests that the production of IL-2R from macrophages is as sensitive to LPS as that of IL-1 or TNF.

2.4.2.1 Effect of dilution of whole blood

The emphasis in studies of the use of whole blood as a substrate has been to show that the method is reliable and to compare it to the use of separated cell populations. The critical variables of the methods have not been systematically studied. Various dilution factors were used by different investigators. Some used undiluted whole blood with only the minor dilution caused by addition of stimulator (Desch et al, 1989; Allen et al, 1992; Van-Deuren et al, 1993), while others used

blood diluted in ratios of 1:1 (Strieter et al, 1990), 1:5 (Lyte, 1987), and 1:10 (Ellaure et al, 1991; De Groote et al, 1992; Elsässer-Beile et al, 1994; Hornef et al, 1995).

The release of sIL-2R from whole blood has not previously been studied, so no protocols were available. For the initial series of experiments in this study, the dilution factor was 9:10, the minimum practical. When the time-course experiment was repeated with a dilution factor of 5:10, the production of sIL-2R by the same volume of whole blood when stimulated was doubled compared to undiluted cells. The effect was less in the unstimulated controls.

The reasons for this increase are not certain. There may be inhibitory components of blood which are diluted out, such as cytokines produced by the cells, hormones, or other components of plasma.

When the experiments were repeated with both dilution factors (9:10 and 1:2) using WB samples collected from the patients with untreated Graves' disease (n=3), the pattern of results obtained was the same, but the release of sIL-2R into the medium was greater. Since it was necessary to compare this set of results with the previous results obtained from WB of the healthy reference sample, the values of pre-controls were subtracted from both the sets to allow assessment of the increase due to stimulation. The amount of sIL-2R released by the unstimulated cells from patients was higher than that produced by the reference samples. This indicates that immune cells *in vivo* are under the control of an endogenous stimulator, whose effect is not altered by dilution. Cells further stimulated with LPS and PHA also showed an increased production compared to controls.

In addition, the effect of dilution was not detected with LPS stimulated cells, while it remained with the cells stimulated with PHA or the mixture of LPS and PHA. This may show that macrophages are insensitive to whichever inhibitor is present in serum, whereas the cells, presumably lymphocytes, which are sensitive to PHA are affected by it.

The increase in sIL-2R production is consistent with the findings of Nagataki and Eguchi (1992) who have shown in Graves' disease that the number of activated T cells (helper, memory and cytotoxic cells) is increased in peripheral blood compared to normal controls, while suppressor-inducer T cells and natural killer cells were reduced.

2.4.3 Production Of Cytokines By Whole Blood In Vitro

The experiments using whole blood to investigate the release of sIL-2R from cells was extended to include the measurement of several other cytokines. The protocol

was slightly different, with a higher dilution of blood and the use of a stimulation with PMA and calcium ionophore as well as LPS and PHA.

2.4.3.1 Interleukin-2

Incubation of diluted (1:11) whole blood of reference group did not cause a noticeable effect on IL-2 production. On the other hand, incubation of whole blood of the patients increased the production of IL-2, in an apparently time dependent fashion. In these sets of experiments, polypropylene tubes were used instead of plates, and for this reason the auto-activation of cells is less likely. It seems the unknown stimulator which was postulated to stimulate sIL-2R release also induced the production of IL-2 in the patients with Graves' disease. This effect was more conspicuous when the blood was stimulated with a mixture of LPS and PHA, or a mixture of PMA and Ca ionophore (figure 2.12).

Many factors could be responsible for the induction of IL-2 production. The synthesis of IL-2 is under complex regulation. It is enhanced after interaction of B7, on the surface of APC, with its counterpart on the surface of T-cells, CD28 (Paul and Seder, 1994), and by IL-1 produced by activated macrophages, in response to stimulation by LPS. INF- α maintains an enhanced level of IL-2 mRNA in the activated cells (Holan et al, 1994). In addition, there is interaction between the populations of helper T cells. Activated CD4⁺ TH1 cells are capable of producing IL-2, INF- γ , and TNF- α , while TH2 cells produce IL-4, IL-5, IL-10, GM-CSF, and IL-13 (Paul and Seder, 1994). It is now clear that cytokines produced by either of the sets of helper cells influence the production of cytokines by the other group (Paul and Seder, 1994; Seder and Paul, 1994). It is clear that the number of activated immune cells in peripheral blood is increased as detected by Nagataki and Eguchi (1992).

Defects in IL-2 production have been reported in patients with Graves' disease (Eisenstein et al, 1988; Akasu et al, 1991). The reason for this reduction is unknown, but there are reports suggesting that excessive prostaglandin E2 production by activated APC could be one of causes (Eisenstein et al, 1994; Hikens et al, 1995). The production of IL-2 was restored to normal after proper therapy. In the present study, the production of IL-2 was higher among these patients, when the whole blood was diluted 1:11. This could be due to dilution of the elevated thyroid hormones presented in the WB of untreated patients with Graves' disease, or cytokines produced by other cells. This needs further investigation.

2.4.3.2 Interleukin-4

Incubation of diluted whole blood of reference group did not cause a detectable release of IL-4. On the other hand, the level of IL-4 in the incubated whole blood of the patients was apparently higher than in the reference group at the 4 h time point, followed by an apparently time dependent diminution. Furthermore, stimulation of the whole blood of both groups (reference as well as patients) with a mixture of LPS and PHA significantly increased the production of IL-4, which seemed to be time dependent. The mean value obtained for the patients' group was higher (nearly 1.5 fold) than the corresponding value obtained for the control group (figure 2.15). However due to the high degree of variability of the data, there were no significant differences between the values obtained for the patients and reference group after 4 or 24 hours. The patients' IL-4 release became significantly higher than the reference group after 72 h. This finding agrees with the report published by Hirooka et al, (1993), who demonstrated that peripheral blood mononuclear cells of patients with Graves' disease produced more IL-4 than the normal controls when stimulated with PHA.

The quantity of IL-4 produced was low and variable. This may reflect the fact that primary stimulation of resting T cells produces mostly IL-2 rather than IL-4 (Seder and Paul, 1994). Other cytokines like IL-4, TNF- β , INF- γ , IL-10, and IL-12 are required for differentiation of the resting lymphocytes to either TH1 or TH2 cells (Seder and Paul, 1994). This process may take several days, and those patients who produce high levels of IL-4 at the start may go on to produce more IL-4 throughout the stimulation period.

Interleukin-4 is primarily produced by activated T helper type 2 cells, along with IL-5, IL-10 (Nagataki and Eguchi, 1992), GM-CSF, and IL-13 (Paul and Seder, 1994). It suppresses the production of IL-2 and the β chain of IL-2R (Llorente et al, 1989; Lindqvist et al, 1991; Seder and Paul, 1994). This could be one of the causes, of the increase in the plasma level of sIL-R α , as it is shed from the cells, in the absence of the other chains of the IL-2 receptor, and the decrease in IL-2 production which has been reported in patients with Graves' disease.

Helper cells which could produce IL-4 are present in the infiltrating mononuclear cells, in the thyroid gland of patients with autoimmune disease (Nagataki and Eguchi, 1992; Mariotti et al, 1992 b). Since it has been shown that IL-4 together with INF- γ enhances the expression of MHC class II on endothelial cells (Masinovsky et al, 1990) and facilitates the migration of immune cells to the neighbouring tissue, elevated levels in the thyroid may contribute to the accumulation of cells within the gland.

2.4.3.3 Interleukin-6

Incubation of diluted whole blood of the reference group for different periods did not cause a noticeable release of IL-6. On the other hand, incubation of whole blood of the patients with Graves' disease released more IL-6 into the medium than the reference group after 4 h, but not after 24 or 72 h. This could indicate an increase in the number of activated immune cells, in the peripheral blood of the patients with Graves' disease. The concentration of IL-6 may also be higher in the plasma of these patients, as detected before (Hirano, 1992). Some of the IL-6 in blood could come from activated immune cells, including monocytes or macrophages, T lymphocytes, and activated B cells (Janeway and Travers, 1994), or from thyroid follicular cells (Kennedy et al, 1991; Zheng et al, 1991; Watson et al, 1994). However, the apparently time dependent increase in the production of IL-6 suggests that the primary source is the activated cells in blood.

Stimulation of whole blood of the reference group, with a mixture of LPS and PHA caused a time dependent increase in the production of IL-6. This result corresponds with those reported before (Eggesbo et al, 1994; De Groote et al, 1992), and could be due to a combination of IL-6 produced by monocytes or macrophages that respond to LPS, and IL-6 produced by lymphocytes that respond to PHA.

The production of this cytokine by stimulated (LPS/PHA) whole blood of the patients with Graves' disease had a different pattern. In the first 24 h, production was higher than that of the reference group, while after 72 h, the production of this cytokines tended to be lower than from the reference group, although this did not reach significance.

There are many possible reasons for this change in the pattern of production. One could be the presence of high levels of thyroid hormones in the plasma of these patients, because it has been shown that high levels of thyroid hormones and other iodinated compounds enhance the ability of the monocytes to mature into cytologically and functionally characteristic veiled/dendritic cells (Mooij et al, 1994). This may lead to changes in the pattern of IL-6 production. The higher level of thyroid hormone also suppressed the production of this cytokine as detected in this study. Other factors in the serum of patients, such as the presence of high levels of immunoglobulins (especially antibodies to TSH receptor, thyroglobulin, and thyroid peroxidase), may antagonise the induction of IL-6 by stimulators, and the increased value detected after 4 h may reflect the initial elevated level of this cytokine *in vivo*. Alternatively, the cultures may produce inhibitory cytokines after the first few hours *in vitro*.

Another possibility is that the activated immune cells become exhausted and can not respond to the stimulator, as happens in septic patients (Ertel et al, 1995). For the confirmation of these possibilities, further investigations are necessary.

2.4.3.4 INF- γ

Production of INF- γ by diluted whole blood of the reference group (unstimulated) was higher than the whole blood of the patients with Graves' disease. This reduction was also observed by Akasu and co-workers (1991) in patients with Graves' or Hashimoto's disease. They suggested this event takes place when there is an abnormal reactivity of the stimulator/inducer subset, and T cells proliferate in response to non-T cells. Such auto-activation of lymphocytes is referred to as the autologous mixed lymphocyte reaction, and has been detected in several other human autoimmune diseases (Akasu et al, 1991). In addition, elevated levels of IL-4 can suppress the production of INF- γ by activated CD4⁺ cells (Seder and Paul, 1994). As mentioned before, the level of IL-4 was apparently higher at all three time points (4, 24, and 72 h) among the patients with Graves' disease.

Stimulation by LPS/PHA or PMA/Ca ionophore of diluted whole blood of both groups (patients and reference) caused significant and time dependent increases in INF- γ production. When whole blood of the patients with Graves' disease was stimulated with a mixture of LPS and PHA, the production of INF- γ was higher than the reference group after 72 h. On the other hand, when whole blood of Graves' patients was stimulated with a mixture of PMA and Ca ionophore, INF- γ production was significantly lower than the corresponding value obtained for the reference group (at 24 h time point). These findings may indicate that the production of INF- γ depends on the stimulator used, as suggested by Seder and Paul (1994). PMA and Ca ionophore are protein kinase agonists (Hershman and Pang, 1993), while LPS can activate macrophages, and PHA activates naive T cells.

INF- γ is produced by activated CD4⁺ TH1 or CD8⁺ cytotoxic cells (Nagataki and Eguchi, 1992), and blocks the growth of TH2 cells (Janeway and Travers, 1994). On the other hand, it is also known that undifferentiated T cells (TH0) can produce IL-2, IL-3, IL-4, and INF- γ (Seder and Paul, 1994) after primary activation. The finding of both IL-4 and INF- γ in supernatants of stimulated whole blood cells may indicate that both sub-types of lymphocytes, or undifferentiated TH0 cells, are present among the activated immune cells, in the peripheral blood of the patients with Graves' disease.

2.4.3.5 TNF- α

The level of TNF- α , in the supernatant of unstimulated whole blood of the reference group was undetectable for the first 72 h. On the other hand, the level of TNF- α was variably detectable in the supernatant recovered from the unstimulated

whole blood of the patients with Graves' disease, without noticeable differences between the three time points.

Factors responsible for this increase in TNF production could include the presence of high levels of thyroid hormones or other cytokines in blood, and the increase in the number of activated cells *in vivo* (Nagataki and Eguchi, 1992). It is, however, unlikely that thyroid hormones could be involved directly, since their effect was to suppress release of TNF *in vitro*. There are no data on the level of this cytokine in the plasma of untreated patients with Graves' disease, though there are reports (Mooradian et al, 1990; Pang et al, 1989 a; Van-Der-Poll et al, 1990) showing a negative correlation between the plasma level of TNF- α and thyroid hormones (especially T₃).

Stimulation of whole blood with a mixture of LPS and PHA caused a time dependent increase in the production of TNF- α . The increase in production of TNF- α was significantly higher in patients than controls after 4 h. However, there was no significant difference between the values obtained for the reference group and the patients' group after 24 h, and by 72 h, TNF- α release was significantly lower than from the reference group.

The elevation of TNF- α production after 4 h shows that this cytokine is one of the group of cytokines that is synthesised and released early in response to stimulation. Tumor necrosis factor is predominantly produced by activated macrophages (Tracey and Cerami, 1994; Beyaert and Fiers, 1994), though several other blood cells including activated T-cells (CD4⁺, and CD8⁺), B-cells, natural killer cells, and neutrophils (Vilcek and Lee, 1991) can produce small amounts. The higher early release of this cytokine detected in the patients with Graves' disease could be a reflection of the increase in the number of activated immune cells of these patients. The failure to continue the initial rate of increase could be due to production of other down-regulatory cytokines, to inhibitory substances in plasma or to cell exhaustion as suggested for IL-6.

Another possibility is that the release of cleaved fragments of the TNF receptors (also known as TNF-binding proteins) may be increased in the supernatants recovered from the stimulated cells of the patients after 72 h and may interfere with the immunoassay which was used. The assay is, however, claimed to be free of such interference.

2.4.4 Effects Of Methimazole, Thyroid Hormones, And TSH On Cytokine Production

In the study of sIL-2R in serum, it was observed that there was a positive correlation between the serum concentration of sIL-2R and thyroid hormones. In addition, there was a negative correlation between sIL-2R and TSH or TRAb. Weetman (1986 b) reported that methimazole enhances IL-2 production by peripheral blood mononuclear cells in vitro. In the present study, the effects of these compounds on cytokine release from blood cells were determined, using WB of the reference group, stimulated with these compounds with or without a mixture of LPS and PHA, or Ca ionophore and PMA.

Stimulation of WB with thyroid hormones, TSH, or methimazole in the absence of any stimulator did not affect cytokine production. In addition, in the presence of a mixture of Ca ionophore and PMA, these compounds also failed to alter cytokine production significantly. However, in the presence of a mixture of LPS and PHA, they did effect the pattern of production of sIL-2R, IL-6, INF- γ , and TNF- α .

Both TSH and thyroid hormones caused an increase in sIL-2R production, while there was a tendency to a decrease in the presence of methimazole. These changes are in agreement with the increase seen in vivo and the prompt decrease on treatment. The increase in the presence of thyroid hormones alone may also explain the increase in serum sIL-2R seen in cases of adenomas, which are presumed to have little or no immune component.

There was a tendency for the changes in IL-2 release to be reciprocal to those of the receptor, though they did not reach significance. This may point to a role for IL-2, in control of the synthesis of its own receptor, but the evidence is weak. Weetman (1986 b) reported that methimazole enhanced IL-2 production by peripheral blood mononuclear cells in vitro, but Eisenstein and colleagues (1988) did not see any effect of anti-thyroid drugs. In addition Volpé (1994), in a review of the actions of anti-thyroid drugs, denied that anti-thyroid drugs could mediate any immunosuppressive effects and claimed that their action was mediated only through modifying the function of thyrocytes.

Against this, the present results indicate that methimazole itself can suppress the cytokine production of immune cells, especially IL-6, INF- γ , and TNF- α . This confirms the observation of Weetman (1986 b). The mechanism is not known, but methimazole is known to effect thyroid function through inhibition of thyroid peroxidase. It may also affect oxidative functions in monocytes or lymphocytes. This needs further investigation.

The present study is not the first report on the effect of TSH on immune system. Komorowski et al, (1993) have shown that stimulation of lymphocytes with

TSH (maximum dose used 25 mU/l), in the presence of PHA (10 μ g/ml) could increase the production of IL-2. The negative results obtained in the present study could be due to differences in the concentration of PHA, or the use of whole blood. The influence of TSH on the immune system, and the detection of the TSH beta subunit gene in human lymphocytes (Pecle et al, 1993) and cloned lymphocyte cell lines (Harbour et al, 1989) indicate that TSH can modulate the activity of immune cells. To determine the role of TSH in the immune system, further study is necessary.

IL-1 (Van-Haasteren et al, 1994; Mandrup-Poulsen et al, 1996), IL-6 (Stouthard et al, 1994; Bartalena et al, 1994 c; Van-Haasteren et al, 1994), and TNF- α (Pang et al, 1989 a; Van-Der-Poll et al, 1990; Mandrup-Poulsen et al, 1996) are known to suppress the production of TSH. Infusion of large amounts of IL-6 in humans (Stouthard et al, 1994; Bartalena et al, 1994 b; Hashimoto et al, 1994) and rats (Bartalena et al, 1994 c; Van-Haasteren et al, 1994) were associated with a decrease in thyroid hormones and TSH. It has been shown here that high levels of thyroid hormones and TSH suppress the production of IL-6, INF- γ , and TNF- α from blood cells. This suggests that a feedback system may operate *in vivo*. It remains to be proved whether the action of thyroid hormones is through direct action on the cells that produce these cytokines, or through the induction of inhibitory cytokines like IL-10 and IL-13.

In Graves' disease, it is therefore likely that the combined increase in thyroid hormones and TRAb, simulating TSH, will cause a functional defect in the production of IL-6, INF- γ , and TNF- α . The early increases seen *in vitro* in the patients in this study may then not simply be due to activation of cells within the patients, but be caused by release of inhibition in culture when the blood is diluted.

Chapter Three

Bioactive Peptides And Thyroid Function

SECTION ONE

INTRODUCTION

About 70% of the cells in the thyroid gland are follicular epithelial cells. Among the rest are stromal cells, vascular endothelial cells and nerve cells (Dumont et al, 1992). All these are capable of releasing factors which may affect the function and growth patterns of the gland in health and disease. In this section, the effects on the thyroid gland are discussed of the growth factors, cytokines, and other peptides that may be released locally during autoimmune thyroid diseases by infiltrating cells or by other cells in the gland and which were studied in the current work.

3.1.1 Growth Factors

3.1.1.1 Insulin-Like Growth Factor

Insulin-like growth factors IGF-1 and IGF-II (previously known as somatomedin-C and Multiplication Stimulating Activity) are nonglycosylated, single chain peptides that are structurally related to insulin (Saji et al, 1987).

IGF-1 plays a central role in development. It is primarily synthesised by the liver under the control of growth hormone (Delafontaine and Lou, 1993). IGF-1 is also expressed in a variety of tissues and by different cell types including thyroid follicular cells (Dumont et al, 1991; and 1992). The production of IGFs are considered to have major autocrine/paracrine effects on growth and protein metabolism (Delafontaine and Lou, 1993).

The expression of IGF-II is high in most fetal tissues and declines after birth, except in neural tissue (Gluckman and Ambler, 1993). Thyroid epithelial cells can produce IGF-I when stimulated with TSH or growth hormone (Bachrach et al, 1988), while they can synthesise IGF-II when stimulated with IGF-1 (Dumont et al, 1991; and 1992).

The IGFs in blood and other biological fluids occur are complexed with specific binding proteins (Yang et al, 1989). The six identified IGF binding proteins (IGFBPs) transport the IGFs in plasma and across capillary membranes, and control the interaction of the IGF with receptors. It has been shown that IGFBP-1 is present in the normal and carcinomatous thyroid gland, while IGFBP-2 and IGFBP-3 were detected only in medullary carcinomas (Van-Der-Laan et al, 1995).

IGF-1 produces its effect through the IGF type-1 receptor, which is related to the insulin receptor, while insulin can use the same receptor, with lower affinity (Saji

et al, 1987); IGF-II has its own receptor (type 2), which is the same as the mannose-6-phosphate receptor (Gluckman and Ambler, 1993). Both types of receptors are present on the surface of thyrocytes (Bachrach et al, 1988) and their expression can be increased by administration of growth hormone (Näntö-Salonen et al, 1993; Bachrach et al, 1988), while TSH down-regulates the receptor for IGF-1 (Tramontano et al, 1988 a).

Both insulin (Smith et al, 1986; Bachrach et al, 1988; Takahashi et al, 1990) and IGF-1 (Tramontano et al, 1986; Isozaki et al, 1987; Santisteban et al, 1987; Maciel et al, 1988; Pang et al, 1990 a; Takasu et al, 1989; Takada et al, 1990) can increase the growth of animal thyroid cells when combined with TSH. Both are also required for synthesis of prostaglandin E₂ by FRTL-5 cells (Tahara et al, 1991). The transport of amino acids is increased in presence of IGF-1 (Villone et al, 1993). IGF-1 can also induce production of thyroglobulin mRNA by FRTL-5 cells (Isozaki et al, 1987; Santisteban et al, 1987).

Iodine uptake by thyroid cells is believed to be controlled by intracellular cAMP, which itself is increased by TSH. Tramontano and colleagues (1988 a) showed a slight increase in cAMP when FRTL-5 cells were stimulated with a combination of TSH and IGF-1, but Pang and co-workers (1990 a) have shown a decrease in iodine uptake of the same cell line. Although it is plausible that excessive concentrations of IGF-1 could be implicated in goitre formation, there is sufficient conflict in the experimental results for further experimentation to be necessary. In particular, none of the studies was carried out in the presence of thyroid hormones, which might be expected to have an influence on hyperthyroid goitres.

3.1.1.2 Transforming Growth Factor- β

TGF- β is produced by both normal and diseased thyroid glands (Grubeck-Loebenstein et al, 1989; Cowin and Bidey, 1994; and 1995), and its synthesis is regulated by intra-thyroid iodine concentration (Yuasa et al, 1992; Cowin and Bidey, 1994; and 1995). The concentration of TGF- β mRNA was found to be lower in thyrocytes of patients with iodine-deficient non toxic goitre than in Graves' disease (Grubeck-Loebenstein et al, 1989).

Reduction in growth of human (Widder et al, 1991; Taton et al, 1993) or animal (Morris III et al, 1988; Tsushima et al, 1988; Pang et al, 1992; Cirafici et al, 1992) thyroid cells under the influence of TGF- β is well documented. TGF- β inhibited the growth of medullary thyroid carcinoma (Khosla et al, 1994), several thyroid cancer cell lines derived from the primary tumour and lymph node and lung metastases of a single patient (Hölting et al, 1994), and a papillary carcinoma cell line (Usa et al, 1994).

Besides this inhibitory effect, the presence of TGF- β influenced the morphological changes induced by TSH on thyroid cells. Addition of TGF- β to the culture medium of thyrocytes caused cells to enlarge and stick to the plate more firmly. This was shown to be caused by a striking effect of TGF- β on actin microfilaments, which counteracted the actions of TSH (Garbi and Nitsch, 1989). It also inhibited the migration of carcinoma cell lines significantly by enhancing the adhesion of cells to collagen (Hölting et al, 1994).

A down-regulatory effect of TGF- β was also shown on thyroid peroxidase production (Widder et al, 1991; Taton et al, 1993) without effecting cAMP induced by TSH (Taton et al, 1993). TGF- β was shown to decrease the iodine uptake induced by TSH in porcine thyroid cells (Tsushima et al, 1988), and rat FRTL-5 cells (Pang et al, 1992). On the other hand, Morris and colleagues (1988) demonstrated that in the presence of TSH, TGF- β had a stimulatory effect on iodine uptake of FRTL-5 cells.

3.1.1.3 Fibroblast Growth Factor

Fibroblast growth factors are a family of polypeptides that play a crucial role in normal development, in the maintenance of tissues and in wound healing and repair (Gospodarowicz et al, 1987). They act on cells of meso-, ecto- and endoderm origin, and cause changes in migration, morphology and function, as well as proliferation (Isozaki et al, 1992). The family includes nine members which show 30-50% sequence identity at the amino acid level and conservation of the positions of two cysteine residues. The factors are designated FGF-1 through 9, though the names FGF acidic and basic are used for FGF-1 and FGF-2, respectively.

Acidic and basic FGF, the prototypical members of this family, were named for their differing isoelectric points. They have similar molecular masses and biological activities, and show approximately 55% amino acid identity but with different genes located on chromosome 4 (acidic FGF) and 5 (basic FGF) (Gospodarowicz et al, 1987). FGFs bind to heparin, and most of extracellular FGF is sequestered by binding to heparan sulphate in the extra-cellular matrix, where it can be released by heparin-like molecules and by heparan sulphate-degrading enzymes. Their binding to heparin causes a conformational change that protects FGF from proteolysis and denaturation.

The receptor for bFGF is detected on many cells and many cells produce bFGF, in contrast to aFGF, which has a more restricted cellular distribution. FGF is thus a fundamental regulatory molecule, acting by both autocrine and paracrine mechanisms (Logan et al, 1992).

Normal human thyrocytes (Taylor et al, 1993) and those of normal animals (Bechtner et al, 1993; Logan et al, 1992), and human carcinoma cell lines (Eggo et al, 1995; Matsuo et al, 1993; Daa et al, 1993) can synthesise basic FGF, although another potential source could be the endothelial cells in the gland. The amount produced was shown to correlate with the degree of malignancy in human thyroid cancer (Shingu et al, 1994) and hyperplasia of thyroid in rat (Becks et al, 1994).

It has been shown that both the acidic (De Vito et al, 1992; Chanoine et al, 1992) and basic (Emoto et al, 1991; Black et al, 1990) forms of FGF increase the growth or DNA synthesis of thyroid cells. The increase in growth is accompanied after a delay by an alteration in some of the functions of thyrocytes, including a decrease in iodine uptake induced by TSH (Hill et al, 1994; Emoto et al, 1991). Furthermore, down-regulation of 5' deiodinase is also reported to be an effect of FGF (Tang et al, 1994).

The enlargement of the gland during goitre formation is always accompanied by angiogenesis. FGFs are potent angiogenic factors, so they may be involved in this process in Graves' disease.

3.1.2 Cytokines

3.1.2.1 Interleukin 1

In several animal models it has been demonstrated that interleukin 1 (IL-1) can affect thyroid function in vivo. Daily injection of mice with recombinant IL-1 α (1-15 μ g) for seven days decreased the serum concentration of T₄ to undetectable levels (Enomoto et al, 1990), primarily by blocking release of T₄ by the thyroid. Daily injection of rats with 2-4 μ g/kg of recombinant IL-1 β also resulted in the development of lymphocytic thyroiditis and hypothyroidism (Vertrees et al, 1991; Hermus et al, 1992). IL-1 α also affected the hypothalamic-hypophyseal-thyroid axis. Continuous infusion of IL-1 α decreased hypothalamic pro-TRH mRNA by 73%, and reduced hypophyseal mRNA for the β -chain of TSH by 62% (Van-Haasteren et al, 1994). The reduction in the hypophyseal TSH β chain mRNA occurred before the reduction in the mRNA for hypothalamic pro-TRH.

It is not clear whether reduction of TSH or direct interaction of IL-1 with thyrocytes is more important for development of hypothyroidism in these animals. The serum concentration of IL-1 and the period of the experiment could potentially both influence results. In a recent report it was shown rats given two injections of IL-1 α or IL-1 β in a single day did not develop hypothyroidism, but the growth of thyrocytes increased when IL-1 was infused (Zerek-Melen et al, 1994).

In vitro experiments are more contradictory. Zeki and co-workers (1993) demonstrated that the growth of a human thyroid carcinoma cell line (NIM 1) increased when the cells were incubated with 10 $\mu\text{g/L}$ of IL-1 α or IL-1 β . They showed that this cell line produced IL-1 which acted as an autocrine growth factor. On the other hand, Kimura and co-workers (1992) and Yip and colleagues (1995) reported that both IL-1 α and IL-1 β suppressed DNA synthesis in at least some thyroid carcinoma cell lines.

It was shown that both IL-1 α and IL-1 β inhibited the incorporation of radioactive iodine and iodothyronine release in human thyrocytes (Sato et al, 1990). It was also shown that IL-1 β (10 U/L) inhibited basal and TSH-stimulated iodine uptake and organification in porcine thyroid cells, while it did not influence their growth (Nolte et al, 1994). A low dose of IL-1 α has, however, been reported to increase iodine uptake of porcine thyroid cells, though this changed to inhibition if cells were preincubated with IL-1 for 42 hours (Westermarck et al, 1990).

IL-1 α or IL-1 β on their own did not affect the morphology of rat thyroid follicles cultured as semi-organs, but they reduced the effects of TSH on morphological changes (elongation of microvilli and formation of reabsorbed colloid droplets). This action depended on the dose and time of exposure to IL-1 (Asakawa et al, 1991 a). In the absence of TSH, the growth and cAMP production of rat thyroid cells (FRTL-5) which were incubated with IL-1 α or IL-1 β was increased, while in the presence of TSH both growth and cAMP production were decreased (Zeki et al, 1991; Mine et al, 1987). On the other hand Rasmussen and co-workers (1989; 1990) have shown that in the presence of IL-1 β , the growth of FRTL-5 cells decreased, while cAMP production was not affected. Pang and colleagues (1990 b) showed that IL-1 increased growth of this cell line, while decreasing iodine uptake, but there was no effect on production of cAMP. They claimed that the site of IL-1 action is distal to the production of cAMP. Since the conditions of the experiments were different in these studies, it is difficult to reach a conclusion, but it is clear that IL-1 affects the function of thyroid cells.

3.1.2.2 Interleukin 4

Helper cells are present among the infiltrating mononuclear cells in the thyroid gland of patients with autoimmune disease (Nagataki and Eguchi, 1992; Mariotti et al, 1992 b). Interleukin 4 (IL-4) not only induced the expression of adhesion molecules on endothelial cells but markedly enhanced expression stimulated by IL-1 β or INF- γ (Masinovsky et al, 1990). Adhesion of lymphocytes to these cells is the requisite first element in the multi-step process of transmigration from blood across the post capillary venules, so IL-4 may affect the accumulation of immune cells in the thyroid.

Hirooka and colleagues (1993) demonstrated that when peripheral blood mononuclear cells of patients with Graves' disease were stimulated with PHA, IL-4 production was significantly higher than in normal controls. A recent study shows that mouse spleen cells pre-treated with murine thyroglobulin (mTG) and IL-4 exhibit slightly decreased proliferative responses and increased cytotoxic responses toward mTG-pulsed macrophages compared with mTG-stimulated cells cultured in the absence of cytokine. The injection of mTG-activated spleen cells, cultured in the presence of IL-4, into irradiated CBA/J mice reduces circulating anti mTG antibody significantly (Godefroy et al, 1995). These findings suggest that there is release of IL-4 during the development of autoimmune thyroid disease and that IL-4 could potentially modify the immune response, but whether this cytokine can affect functions of thyrocytes is not known.

3.1.2.3 Interleukin 6

Increased serum interleukin 6 (IL-6) concentrations have been reported in many diseases, including autoimmune diseases of thyroid gland (Hirano 1992) and sub-acute thyroiditis (Bartalena et al, 1994 a).

Increases in serum concentration of IL-6 among patients with non-thyroidal illness may also influence thyroid function. Rats injected with rIL-6 for different periods developed hypothyroidism with low T_4 and T_3 (Bartalena et al, 1994 c; Van-Haasteren et al, 1994). A reduction of TSH was reported after a week, though without detectable change in mRNA, while Van-Haasteren co-workers (1994) did not detect any changes in serum TSH after one day. Administration of rIL-6 in patients with renal cancer caused a significant decrease in serum TSH and T_3 , while it induced an increase in reverse T_3 (r T_3), but did not affect the total or free T_4 concentration (Stouthard et al, 1994). These findings are similar to those reported in patients with non-thyroidal illness.

In vitro experiments showed that IL-6 inhibited TSH-induced mRNA expression for thyroid peroxidase in human thyrocytes dispersed from Graves' thyroid tissue, while in the absence of TSH the cytokine did not affect expression (Tominaga et al, 1991). It was also demonstrated that the combination of TSH and IL-6 stimulated DNA synthesis in FRTL-5 cells (Nishiyama et al, 1993; and 1994) while it did not modulate cAMP accumulation in the presence or absence of TSH (Nishiyama et al, 1993). The in vitro studies are limited, and further experiments are required to determine the role of IL-6 in autoimmune thyroid diseases.

3.1.2.4 Tumour Necrosis Factor

In vivo experiments showed that the serum concentration of TSH, T_4 , T_3 , free T_4 , and hypothalamic TRH decreased significantly after injection of rats with $TNF-\alpha$ (Pang et al, 1989 a). The same group also reported that the decrease in TSH was due to a reduction of TRH production, as well as a reduction in mRNA for the β -subunit of TSH. The injection of $TNF-\alpha$ into humans also caused the same effect (Van-Der-Poll et al, 1990). The low level of thyroid hormones and TSH resembles the condition of sick euthyroid syndrome. However, there is no significant difference between the plasma level of $TNF-\alpha$ of healthy controls and patients with non-thyroidal illness, hypothyroidism, or hyperthyroidism (Chopra et al, 1991).

At the same time, different groups have shown that thyroid epithelial cells possess the receptors for TNF (Pang et al, 1989 b; Buscema et al, 1989; Deuss et al, 1992). Pang and co-workers (1989) showed a 3 fold increase in the number of TNF receptors, after treatment of the cells with TSH. The group of Buscema (1989) also showed that the number of the receptors for $INF-\gamma$ increased when the human thyroid cells were exposed to $TNF-\alpha$.

While low doses of $TNF-\alpha$ increased the growth of human and rat thyrocytes, high doses were less effective (Deuss et al, 1992; Parwardhan and Lombardi, 1991; Pang 1990 b; Zakarija and McKenzie, 1989 b). In addition, the TSH-stimulated iodine uptake of both human and rat thyrocytes was decreased by $TNF-\alpha$ (Pang 1990 b; Zakarija and McKenzie, 1989 b; Sato et al, 1990). Synthesis of thyroglobulin (Tang et al, 1995; Rasmussen et al, 1994), thyroid peroxidase (Tang et al, 1995), and 5'-deiodinase (Ongphiphadhanakul 1994) were also reduced by $TNF-\alpha$. All of this data clearly indicates that $TNF-\alpha$ can directly alter various functions of thyroid follicular cells.

3.1.2.5 Interferon- γ

$INF-\gamma$ has been recognised to possess diverse non-immunological effects on epithelial cells such as alterations in growth and differentiation state. In vitro, besides the expression of HLA-DR on the surface of thyrocytes, other effects of $INF-\gamma$ on human or animal thyrocytes are usually inhibitory. $INF-\gamma$ inhibits TSH induced morphological changes such as elongation of microvilli and appearance of pseudopods in human (Asakawa et al, 1991 b; Kung et al, 1992) and mouse thyrocytes (Asakawa et al, 1990). Addition of $INF-\gamma$ to the medium of human thyrocytes resulted in marked degeneration with shrinkage of the cell membrane, vacuolation of cytoplasm, swollen mitochondria and presence of lysosomal granules

(Kung et al, 1992). It also reduced the number of actin filaments in mouse thyrocytes (Asakawa et al, 1990).

INF- γ decreased the growth of human thyroid cells as measured by ^3H thymidine incorporation into cellular DNA and cell counts (Kraiem et al, 1990; Huber and Davies, 1990; Yip et al, 1995). In the presence of TSH, the growth of FRTL-5 cells was also reduced (Zakarija and McKenzie, 1989 b; Misaki et al, 1988).

INF- γ caused reduction of TG production in presence of TSH by human (Rasmussen et al, 1994; Kung et al, 1992; Kung and Lau, 1990; Huber and Davies, 1990) and FRTL-5 thyrocytes (Misaki et al, 1988; Zakarija and McKenzie 1989 b; Graves et al, 1989; Tang et al, 1995), and inhibited TG gene transcription in the absence of TSH (Kung et al, 1992). It also reduced TPO production by human (Asakawa et al, 1992 b; Chiovato et al, 1994) and rat (Tang et al, 1995) thyrocytes.

INF- γ has also been reported to influence iodine uptake and cAMP production in human and rat thyrocytes, but the results are conflicting. Iodine uptake has been reported to be decreased in human thyrocytes in the presence of TSH (Kraiem et al, 1990; Sato, 1990), but increased in FRTL-5 cells (Misaki et al, 1988; Zakarija and McKenzie, 1989 b). Production of cAMP by human thyrocytes was reduced (Rasmussen et al, 1994), while it was increased in FRTL-5 cells (Zakarija and McKenzie, 1989 b).

3.1.3 Neuropeptides

3.1.3.1 Pituitary Adenylate Cyclase Activating Polypeptide

Pituitary adenylate cyclase activating polypeptide (PACAP), originally isolated from ovine hypothalamus, is a neuropeptide considered to be a member of the family of peptides including vasoactive intestinal peptide (VIP), glucagon, secretin, and growth hormone-releasing hormone (Kononen et al, 1994; Journot et al, 1994). Two forms of PACAP, PACAP38 and PACAP27, which have 38 and 27 amino acids respectively, have been demonstrated in the rat hypothalamus (Dow et al, 1994; Journot et al, 1994). Hannibal and co-workers (1995) recently detected a third member of the PACAP family in the rat hypothalamus called PACAP-related peptide (PRP), but the function of this new member is not known. PACAP27 corresponds to the 27 N-terminal amino acids of PACAP38, and it is also 68% identical to VIP (Tatsuno et al, 1992). Although certain bioactivities of PACAP are similar to those of VIP, there are considerable qualitative and quantitative differences (Tatsuno et al, 1992; Arimura, 1992).

PACAPs are present in the central nervous system as well as in peripheral organs and tissues such as pituitary, lung, liver, gastro-intestinal tract, adrenal medulla, and testes (Journot et al, 1994). PACAP38 is present in higher concentrations in nervous tissue than PACAP27. In the pituitary, PACAP was shown to induce or to potentiate release of GH, prolactin, ACTH, LH, FSH, and α -MSH, and to stimulate accumulation of cAMP (Arimura, 1992; Dow et al, 1994).

There are two different receptors for PACAPs. The type I PACAP receptor has a much higher affinity for both PACAP27 and PACAP38 than VIP (Arimura, 1992), whereas the type II receptor does not discriminate efficiently between these two peptides (Kononen et al, 1994). The type I receptor predominates in the central nervous system, while the type II receptor is higher in other tissues like liver, lung, digestive tract, prostate gland, and testis (Arimura, 1992; Journot et al, 1994). Both types of receptors have been detected on the rat medullary thyroid carcinoma cell line 6/23 (Vertongen et al, 1994).

Since PACAP and VIP are neuropeptides and not circulating hormones, they may directly influence thyroid function only if they are locally released from nerve endings or if they act as autocrine or paracrine regulators. Nerves containing PACAP have not yet been described in the thyroid gland, but PACAP was found to increase T_4 production in porcine thyroid cells in vitro (Chen et al, 1993). It was also shown that presence of PACAP could inhibit the binding of labelled TSH to thyroid membranes, while increasing cAMP production (Chen et al, 1993).

3.1.3.2 Neuropeptide Y

Neuropeptide-Y (NPY) is synthesised and released by adrenergic nerves (Ahrén, 1991). NPY is widely distributed in central and peripheral neurones including peripheral sympathetic nerve fibres, where it co-exists with noradrenaline (Grundemar and Håkanson, 1994). The NPY family includes the hormones peptide YY and pancreatic polypeptide.

NPY produces a multitude of biological effects in the brain and periphery. NPY and peptide YY are among the most powerful stimulants of eating known. NPY also controls mood, and regulates central autonomic functions. In the periphery, sympathetic NPY plays a role as a vasopressor and vasoconstrictor (Grundemar and Håkanson, 1994). NPY from the hypothalamus enhances the FSH and LH secretory responses to LH releasing hormone in vivo (Freeman, 1993).

Adrenergic nerve fibres containing NPY have been detected in the thyroid gland of mouse, rat, guinea pig, cat, dog, sheep, pig, calf, and man (Grunditz et al, 1984). In the anterior pituitary gland of the rat, it seems that the concentration of T_4 controls the release of NPY. Moderate and low concentrations of T_4 induced its

release from nerve fibres (Jones et al, 1994), though higher concentrations failed to have an effect. In vivo, it has been shown that a combination of TSH or VIP with NPY caused increased release of thyroid hormones in mice compared to the use of single hormones (Grunditz et al, 1984). In another in vivo study, it was shown that intravenous administration of antiserum against NPY in normal rats increased thyroid blood flow after one hour without affecting thyroid hormone or TSH concentrations (Michalkiewicz et al, 1993). The effect on NPY on the function of isolated thyroid cells has not been studied.

3.1.3.3 Vasoactive intestinal peptide

Vasoactive intestinal peptide (VIP) is a 28 amino acid peptide that functions as a neurotransmitter in the brain and peripheral nervous system (Metwali et al, 1993). Since the concentration of VIP in various brain areas changes markedly with development, it has been suggested that this neuropeptide is involved in maturation, growth, and maintenance of neurones (Gozes and Brenneman, 1993). VIP is also a mediator of water and anion secretion by pulmonary, intestinal and pancreatic epithelia, and causes relaxation of gastrointestinal, bronchial and uterine smooth muscle, and vasodilatation of cerebral, peripheral and pulmonary blood vessels (Sreedharan et al, 1993). During investigation of the vasodilatory actions of VIP, it was shown that VIP was an inducer of NO synthesis in gastric muscle cells (Murthy et al, 1993), rat pinealocytes (Spessert 1993) and many other cells like smooth muscle (Grider et al, 1992) and blood vessels (Murthy et al, 1993).

VIP belongs to a family of regulatory peptides including secretin, growth hormone-releasing hormone (GHRH), glucagon, gastric inhibitory polypeptide and PACAP. It has been reported that the secretion of VIP in the adrenal gland is associated with catecholamines (Wakade et al, 1991).

Apart from the well-described cross reaction with PACAP type I and II receptors, VIP has two specific receptors (Usdin et al, 1994; Lutz et al, 1993). These receptors are present in liver, lung, and intestine as well as in several regions of the brain (Lutz et al, 1993).

The first neuropeptide demonstrated in thyroid nerves was VIP (Ahrén, 1986 a). The VIP-containing nerves were shown to exist around and close to the follicular cells, and subsequent experiments with denervation and retrograde tracing have shown that the intrathyroid VIP nerves are intrinsic, originating in a thyroid ganglion (Ahrén, 1991). It has also been extracted from human parathyroid glands along with other peptides like substance P, gastrin-releasing peptide, and calcitonin (Weber et al, 1991).

Intracellular cAMP increased when the cells of normal and hyperplastic human thyroids from patients with Graves' disease (Toccafondi et al, 1984; Siperstein et al, 1988) or guinea-pig thyroid sections (Ealey et al, 1985) were incubated with VIP, but the cAMP concentration was lower than that produced by TSH (Siperstein et al, 1988).

There is a negative correlation between the serum concentration of T_4 and intrathyroid VIP concentration in rats (Jones et al, 1994). It has also been shown that while VIP alone did not affect the 3H thymidine uptake of rat thyroid lobes, it increased the uptake produced by TSH (Karbownik et al, 1995). The activity of thymidine kinase, an enzyme involved in the supply of precursors for DNA synthesis, was increased in homogenates of rat thyroid lobes incubated in vitro in the presence of low concentrations of VIP (10^{-15} - 10^{-9} mol/l), while high concentrations of VIP (10^{-7} - 10^{-5} mol/l) had the reverse effect (Karbownik et al, 1995). On the other hand, when anti-VIP antibodies were administered to normal and iodine deficient rats, there was no alteration in blood flow or thyroid hormone production (Michalkiewicz et al, 1993).

3.1.3.4 Neuromedin U

Neuromedin U (NmU), a peptide originally isolated from porcine spinal cord, stimulates uterine smooth muscle contraction and causes selective vasoconstriction (Lo et al, 1992). This neuropeptide family includes two members, NmU 8 and NmU 25. NmU 25 has 25 amino acids, having an identical C-terminus to NmU 8 (Lo et al, 1992). NmU has been found to have a widespread distribution extending throughout the mammalian central nervous system and in the gastrointestinal tract, particularly in the ileum, genitourinary tract, hypothalamus, spinal cord, thyroid, and the endocrine cells of pituitary gland (Bockman et al, 1989; Domin 1990; Lo et al, 1992). Although the NmU 25 separated from rat is two amino acids shorter and contains nine substitutions compared with porcine NmU 25, the five amino acids at the C-terminal of the peptide are totally conserved among different species (Lo et al, 1992). The receptor for NmU has not been cloned yet. Nandha and colleagues (1993) showed that a specific receptor for these peptides is present in the rat uterus, and suggested that the receptor is coupled to a G-protein.

NmU immunoreactivity occurs in corticotrophs of all species including human (Steel et al, 1988; Cimini et al, 1993). NmU immunoreactivity was significantly increased when rats were subjected to adrenalectomy (Cimini et al, 1993). NmU is also detected in some thyrotroph cells (Domin et al, 1989; Cimini et al, 1993), and oral administration of TRH to a euthyroid animal evoked a five fold increase in peptide content of anterior pituitary (Domin et al, 1989). The effect was caused by

changes in circulating levels of thyroid hormone, since administration of TRH to a thyroidectomized animal failed to show a similar effect.

Domin et al, (1990) showed that there is a significant amount (331 ± 67 fmole/gland) of NmU in the thyroid gland of the rat, and treatment of rats for two weeks with anti thyroid drugs caused a significant decrease in its content; thyrotoxicosis induced by exogenous T_4 failed to alter the thyroid content of this peptide. No studies have been published on its effect on isolated thyroid cells.

3.1.3.5 Tachykinins

Substance P (SP) is a member of the tachykinin family of peptides. These are peptides with a common C-terminal sequence that were isolated from equine brain and gut in 1931 by Von-Euler and Gaddum, but only sequenced 40 years later (Maggi et al, 1993). The mammalian members of this tachykinin family include substance P, neurokinin A (also called substance K, neurokinin α , or neuromedin L), and neurokinin B (also called neurokinin β , or neuromedin K) (Maggi et al, 1993; Mussap et al, 1993). Other members of the tachykinin family are synthesised from alternative splicing of mammalian tachykinin mRNAs, followed by differential post transcriptional processing, including N-terminally extended forms of neurokinin A. These peptides are neurokinin A (3-10), neuropeptide K, and neuropeptide γ (Helke et al, 1990; Mussap et al, 1993). The non-mammalian members of the tachykinin family include physalaemin, eledoisin and kassinin, isolated from amphibian skin (Regoli and Nantel, 1991; Maggi et al, 1993).

The term tachykinin was introduced to describe members of this peptide family because of their relatively rapid initiation of smooth muscle contraction compared with the slower acting bradykinin. Tachykinins possess the common C-terminal sequence Phe-X-Gly-Leu-Met-NH₂, where X represents either Phe, Tyr, Ile, or Val (Mussap et al, 1993; Maggi et al, 1993). Among the tachykinin peptides, substance P, neurokinin A, neurokinin A (3-10), and neuropeptide γ are produced from a single preprotachykinin gene as a result of differential RNA splicing and posttranslational processing, while neurokinin B is produced from a separate preprotachykinin gene (Krause et al, 1989; Helke et al, 1990; Regoli and Nantel 1991; Mussap et al, 1993; Maggi et al, 1993).

Substance P and neurokinins were found to be present in both the central nervous system and in peripheral organs (Mussap et al, 1993). Neurones are the major source of tachykinins both in the central nervous system and in peripheral organs, but tachykinin-like immunoreactivity has also been found in other cell types, such as certain endocrine cells of the gut, principal parenchymal cells in the carotid body, chromaffin cells in the adrenal gland, cells of the anterior pituitary,

eosinophils, cells in pig skin, and vascular endothelial cells. There is no firm evidence that their synthesis occurs in these non-neuronal elements (Maggi et al, 1993). Intrinsic neurons of the gut and peripheral endings of primary afferent neurons are the primary peripheral source of tachykinins (Maggi et al, 1993).

Substance P and other members of the tachykinin family interact with one (or more) of three receptors, which are known as neurokinin (NK) receptors (Krause et al, 1989; Helke et al, 1990; Regoli and Nantel 1991; Mussap et al, 1993; Maggi et al, 1993). Since there are three receptors and cross reactivity of several members of the tachykinin family with more than one receptor has been detected; it was necessary to develop specific agonists or antagonists for each receptor. There are many synthetic agonists and antagonists of these peptides now available that recognise one of the three receptors (Maggi et al, 1993, Mussap et al, 1993).

Substance P and other members of the tachykinin family produce different effects in different organs because of the presence of different proportions of the three receptors. Results of various experiments indicate that NK1, NK2 and NK3 receptors are present in the central nervous system, but the number of NK2 receptors is less than that of the other two (Maggi et al, 1993). All three receptors also have been detected in peripheral tissues with NK2 dominating the other two (Helke et al, 1990; Maggi et al, 1993). The affinity for NK1 is in the order substance P > neuropeptide γ \approx neurokinin A = neuropeptide K > neurokinin B, while for NK2 it is neuropeptide K = neuropeptide γ \approx neurokinin A > neurokinin B > substance P, and for NK3 it is neurokinin B > neurokinin A > substance P (Helke et al, 1990).

Tachykinins produce numerous actions, including contraction of urinary, respiratory, and gastrointestinal smooth muscle, increased salivary, respiratory, and gastrointestinal secretion, both contraction and endothelial-dependent relaxation of vascular smooth muscle, plasma extravasation and histamine release (Mussap et al, 1993).

Substance P (Ahrén et al, 1983) and neurokinin A (Grunditz et al, 1987) have been detected in the thyroid gland by immunoreactivity. There is a report that mRNA for substance P is expressed by the thyroid carcinoma cell line 6/23, which is a C cell line (Cremins et al, 1992). It has been reported that in the dog substance P stimulated thyroid hormone secretion, whereas in the mouse it had no effect (Ahrén, 1991). It has also been reported that the concentration of substance P in the rat anterior pituitary gland is inversely proportional to the concentration of serum T₄ (Jones et al, 1994). Although substance P has been reported to be present around the follicular cells and blood vessels (Ahrén, 1991) its direct influence on thyroid cells has not been studied. In this study, along with other neuropeptides, the effects of substance P and specific agonists for the NK1, NK2, and NK3 receptors on the iodine uptake and growth of thyroid cells were investigated.

3.1.3.6 Endothelin

Endothelins are a family of three bicyclic 21-amino acid peptides, which are constrictors of vascular smooth muscle (Inoue et al, 1989). The most potent member of the family, endothelin-1 (ET-1), was originally isolated from the supernatant of porcine aortic endothelial cells (Kennedy et al, 1993). Endothelin-2 (ET-2) has two and Endothelin-3 (ET-3) has six (Haynes and Webb, 1993; Cody and Doherty, 1995) amino acid differences from ET-1. They also have structural resemblance with sarafotoxins isolated from the venom of the Israeli burrowing asp (Haynes and Webb, 1993).

Immunoreactive ET-1, or expression of mRNA of preproendothelin-1 can be detected in a wide variety of tissues including blood vessels, lungs, pancreas, spleen, kidney (Haynes and Webb, 1993), rat parathyroid epithelial cells (Fujii et al, 1991), and human (Tseng et al, 1993), rat (Colin et al, 1992; and 1994) and porcine (Colin et al, 1992; Isozaki et al, 1993) thyroid follicular cells. The ET concentration in the plasma is between 0.25 and 20 ng/l, which is below the concentration generally associated with biological effects, and mainly consists of ET-1 and ET-3 (Kennedy et al, 1993; Haynes and Webb, 1993). ET-1 and ET-2 are predominately produced in endothelial cells and act in an autocrine or paracrine manner, whereas ET-3 predominates in the central nervous system and anterior pituitary (Haynes and Webb, 1993).

Two endothelin receptor subtypes have been cloned (Cody and Doherty, 1995). These are termed ET_A, which is selective for ET-1 and ET-2 over ET-3, and ET_B, which is non selective for the ET isopeptides. An ET_C receptor subtype, selective for ET-3, has been reported, but has not been fully characterised (Kennedy et al, 1993; Cody and Doherty, 1995). The endothelin receptor subtypes are widely distributed in several tissues and appear to vary in function in different species (Cody and Doherty, 1995). Receptor for ET has been detected in human thyroid cells (Jackson et al, 1992; Tseng et al, 1993), and there is indirect evidence for its presence on the surface of thyroid cells from other species like pig (Isozaki et al, 1993) and rat (Miyakawa et al, 1992).

There are many reports that ET-1 may influence the growth or the functions of thyroid cells. It was shown by Jackson and co-workers (1992) that ET-1 decreased the release of TG by human thyroid cells without affecting growth or cAMP production, while Eguchi and colleagues (1993) showed an increase in growth of human thyrocytes measured by ³H thymidine incorporation into DNA as well as by cell count assessed by the trypan blue method. On the other hand, it was shown by

Miyakawa and colleagues (1992) that stimulation of FRTL-5 cells with ET-1 alone increased c-fos mRNA expression without affecting growth, while in the presence of TSH or IGF it caused a decrease or an increase in the growth of cells respectively. ET-1 also decreased the iodine uptake of porcine thyroid cells without affecting cAMP production (Tsushima et al, 1994).

Goitre formation is characterised not only by hypertrophy and hyperplasia of thyrocytes, but also by rapid and organ-specific increase in blood flow, and endothelial cell proliferation (Colin et al, 1994). The increase in the number of small capillaries is partly due to local release of ET-1 which is synthesised by thyrocytes and endothelial cells in the thyroid gland.

It was shown by Yoshizumi and colleagues (1990) that IL-1 increased the production of ET-1 by endothelial cells. Furthermore sodium iodide (NaI) increases the production of ET-1 mRNA by porcine thyroid cells (Isozaki et al, 1993), while other investigators have shown that human thyroid cells stimulated with TGF- β could synthesise a significant amount of ET-1 and release it into the medium (Tseng et al, 1993). IL-1, iodine, and TGF- β are known to be involved in thyroid autoimmunity, so it is likely that ET is also of importance.

3.1.4 Model Systems Of Thyrocyte Function

In vitro studies of thyroid function have either used primary cell cultures derived from humans or from a wide variety of animals, or used established cell lines like the rat FRTL-5 cell line or other cell lines developed from thyroid carcinomas.

There are many problems associated with the use of human thyrocytes. It is not common for thyroids to be removed in most centres, since Graves' disease can be controlled medically. The tissue which is available is not strictly speaking normal, and it is difficult to purify the epithelial cells in the presence of large numbers of other cells, particularly fibroblasts, which grow actively in culture.

Normal animal thyroid glands are easily collectable from a slaughterhouse or from laboratory animals, but other problems like bacterial or fungal contamination and the presence of other cells beside thyrocytes are still serious. Moreover, neither human nor animal cells separated as mentioned above grow well under laboratory conditions. Furthermore, to carry out many experiments as was intended for this study, many different batches of cells would be needed. This might cause unreproducible results due to treatments carried out on different patients, including anti thyroid therapy or radiation of the gland, before removal of the gland by surgery. If small animals such as rats were used, the low number of cells available in each gland would be limiting.

A very useful model for the functional study of thyroid cells in vitro is the FRTL-5 cell line, a line of normal differentiated rat thyroid cells. This cell line was originally derived from thyroid follicular cells of the Fisher rat by Ambesi-Impiombate, Parks and Coon in 1980 and named FRTL. The original cell line had a doubling time of 7-10 days, but subsequent cloning gave rise to the FRTL-5 cell line, which grows in the presence of 5% serum and a mixture of six hormones with a doubling time of only 31 hours (Bidey et al, 1988; Tramontano, 1990). The only draw-back of this cell line is that though the cells are capable of transport of iodine and production of cAMP, they incorporate iodine very inefficiently into thyroglobulin and do not produce thyroid hormones. In spite of this, they are comparatively easy to use and give reproducible results, at least within individual laboratories.

3.1.5 Aim Of Study

The aim of this section of the study was to investigate the effects of cytokines, growth factors and neuropeptides on the function of thyroid cells. The FRTL-5 cell line was used as the experimental model, and iodine uptake and growth rate were measured.

The effect of certain combinations of mediators which are likely to occur in vivo was also studied. Since the disease of interest, Graves' disease, causes an excess of thyroid hormone in the blood, the effect of addition of thyroid hormones was investigated. Finally, since it is known that cell-surface proteases can modify the effect of peptides in some investigative systems, inhibitors of these enzymes were included in some experiments.

SECTION TWO: METHODS

3.2.1 Growth Of FRTL-5

FRTL-5 cells were grown in Coon's modified Ham's F-12 medium supplemented with 5% heat inactivated foetal calf serum and six hormone mixture (6H).

2×10^6 cells were added to an 80 cm² flask, and 20 ml of freshly filtered 6H medium were added. Every 3 days the medium was changed till the cells had grown to confluence. Usually this time was about two weeks. The growing area of the flask was never fully covered by cells, because the cells tended to form follicles and grew over each other (Figure 3.1 and 3.2).

3.2.1.1 Cell Passage

After the cells had grown to confluence, they were passaged. The medium of the flask was removed and the cells were washed with 10 ml of the HBSS without calcium and magnesium. Then the HBSS was removed and replaced by 5 ml of freshly filtered CTC medium. The flask was incubated at 37° C in 95% air and 5% CO₂ for 10 min. After this time the cells had separated from the flask, but were in clumps. To separate them, the medium was sucked into a 10 ml pipette and blown out into the flask several times, and then 5 ml of freshly filtered stopping medium was added to the flask and incubated at 37° C for a further 15 min. After this second incubation, the cell suspension was removed and transferred to a 50 ml sterile conical tube. The flask was washed with 5 ml of freshly filtered 6H medium, and the wash was transferred to the same tube. The tube was centrifuged at 120 g for 5 min, after which the supernatant was discarded and the cells were immediately resuspended in 10 ml of growth (6H) medium. Cells were separated from each other by sucking in and out of an 18 gauge needle and syringe. At this stage a small aliquot (50 µl) of cell suspension was transferred to an Eppendorf tube, followed by addition of the same volume of trypan blue solution (4 g/l in the PBS). The numbers of live cells per ml of cell suspension were determined visually in an improved Neubauer counting chamber. The concentration of the cells was adjusted according to the requirement for the particular experiment.

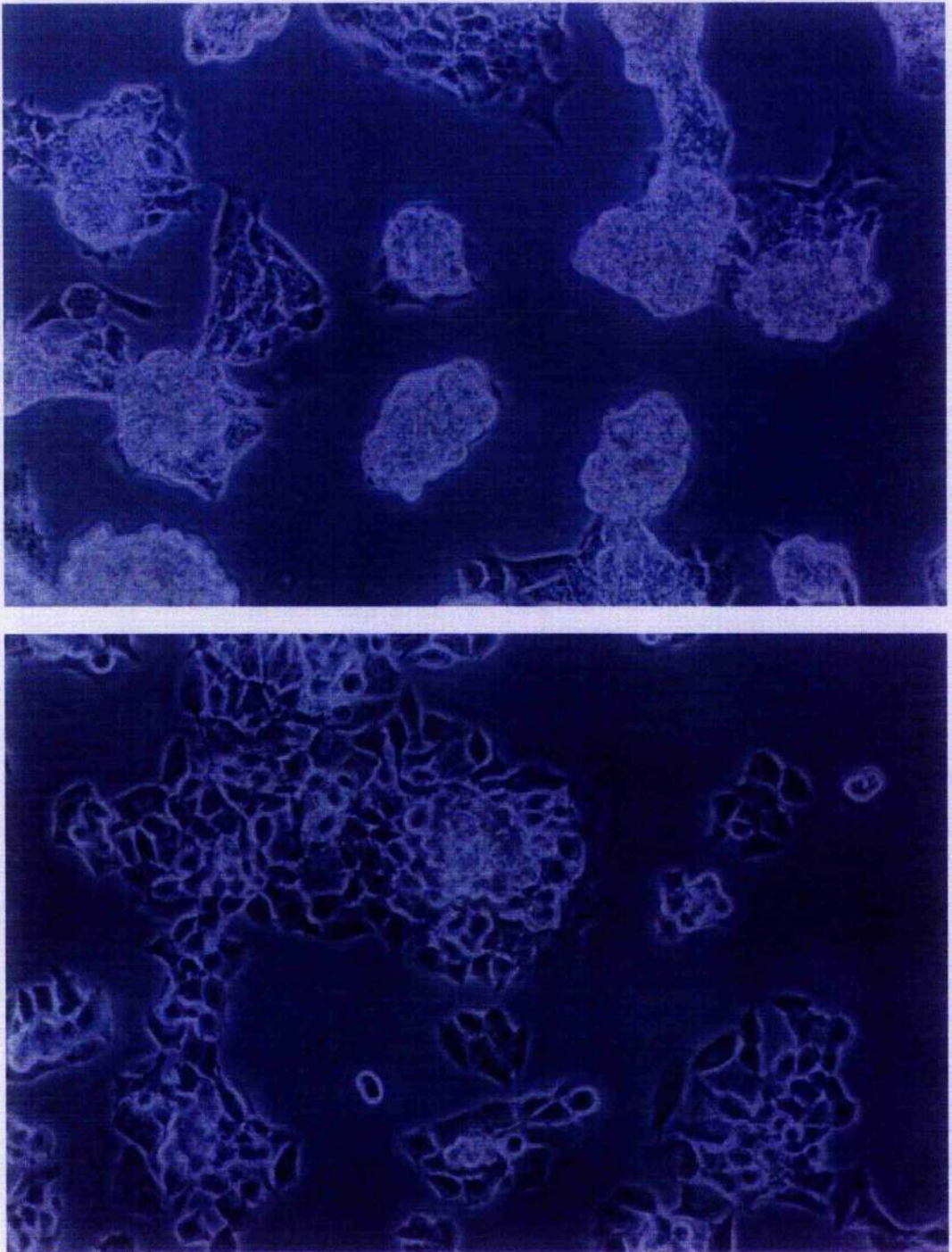


Figure 3.1: FRTL-5 cells cultured in 6H medium. Top picture shows over-confluent cells in the 6H medium forming follicles and growing over each other after 10 days. Lower picture indicates initiation of follicle formation after one week in 6H medium.

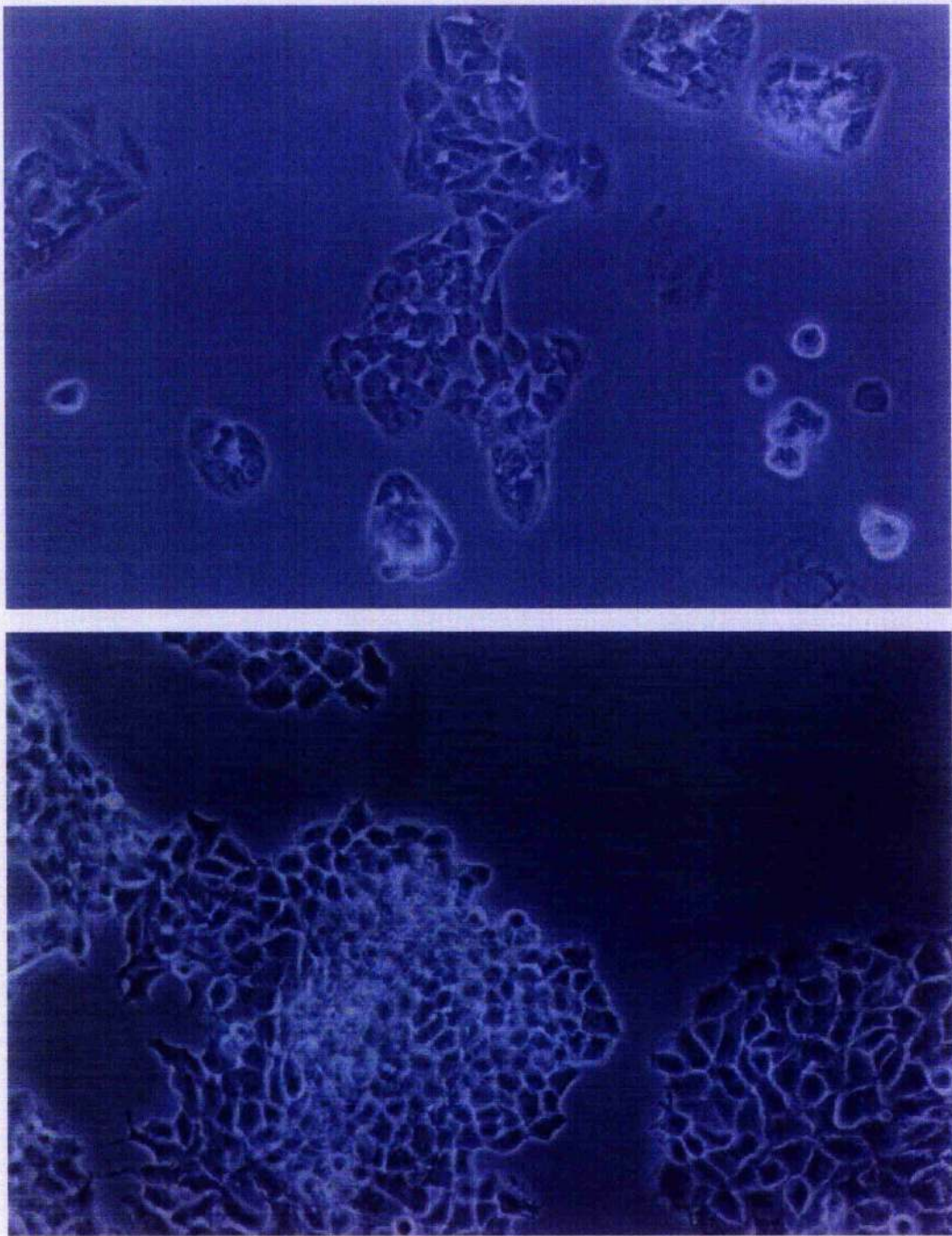


Figure 3.2: FRTL-5 cells grown for 5 days in 5H (top picture) or 6H (lower picture) media, after initial growth in 6H medium for 3 days.

3.2.1.2 Recovery of Frozen Cells

To recover cells from liquid nitrogen, the vial was washed in a beaker of 70% ethanol, and quickly thawed at 37° C. The vial then was transferred to a laminar flow cabinet and the ethanol was allowed to evaporate. The vial was mixed slowly and opened carefully. The cells were removed by pipette, care being taken not to touch the edge of the tube, and then transferred to a 16 ml sterile tube, followed by addition of 10 ml of 6H medium. The cell suspension was centrifuged at 120 g for 5 min. The supernatant was discarded and cells were suspended in 5 ml of fresh 6H medium. The suspension was transferred to a 80 ml flask with a pipette, and another 15 ml of 6H medium was added. The flask was incubated at 37° C in an incubator under an atmosphere of 95% air and 5% CO₂. It was left for 2 days undisturbed, to allow cells to attach to the flask.

3.2.1.3 Preservation of Cells

To preserve stocks of cells, cells were suspended in freezing medium at 2×10^6 cells/ml and 1 ml of cell suspension was added to each Cryotube (Nunc). The tubes were sealed and transferred to a polystyrene foam box. The box was sealed and kept at -20° C for 3-4 h. After this period the box was transferred to a -70° C freezer for 24 h. This procedure is necessary for cooling the cells at 1-3 degree per min. After 24 h at -70° C, tubes were taken out of the box and immediately transferred to liquid nitrogen.

3.2.1.4 Experimental Procedure

To study the effects of different cytokines or growth factors, the cells were grown in 12 or 24-well plates. For a 24-well plate, $0.5-0.8 \times 10^5$, and for a 12-well plate, 1×10^5 cells were dispensed in each well, followed by addition of 0.5 or 1 ml of medium (6H) respectively. The plate was incubated at 37° C for 3 days, to allow the cells to attach to the plate and start growing. This time is sufficient for at least a two fold increase in cell number. After the first incubation, the medium was removed, the cells were washed with 2 ml of the HBSS, and 0.5 or 1 ml of 5H medium with 5% serum was added. The plate was incubated at 37° C for at least five days, with one change of medium after 3 days.

The cells were now ready for the experiment. At this stage, the cells were washed, the concentrated solution of stimulator was added to the required volume of freshly filtered 5H or 6H medium containing additional amino acids and 0.5% FCS, and this was added to appropriate wells.

3.2.2 Protocol For Iodine Uptake

After stimulation of cells for the required time, the medium containing stimulator was aspirated from the wells, and the cells were washed twice with the HBSS.

For a 12-well plate one ml of iodine uptake medium and for a 24-well plate 0.5 ml was added to each well. The plate was incubated at 37°C for 20 min. After the incubation period, the medium was aspirated from the well and discarded. Each well was given a quick wash (**5-10 sec**) with ice cold HBSS and the plate was dried over tissue paper. The cells were overlaid with 1 ml of ice cold ethanol and the plate was incubated at -20° C for 30 min. The ethanol was removed from each well and transferred to a tube for counting the activity with a γ counter.

3.2.3 Measurement Of Cell Growth Using The MTT Assay

3.2.3.1 Principle Of Assay

The cleavage of the tetrazolium salt MTT into a blue or purple coloured product (formazan) by the mitochondrial enzyme succinate-dehydrogenase (Slater et al, 1963) is used for assaying cell survival and proliferation. The conversion takes place only in living cells and the amount of formazan produced is proportional to the number of cells present. The fact that this enzyme is not present in serum (Landegren, 1984) allowed the development of a rapid and simple assay by Mosmann (1983). Denizot and Lang (1986) and Carmichael and co-workers (1987) modified the original method. Companies such as Sigma and Bio-Rad have also produced kits based on these modified methods. However, the method required further modification to be applicable as a tool for measurement of cell growth under the conditions of these experiments.

3.2.3.2 Protocol For MTT Assay

After stimulation, and without disturbing the cells, 100 μ l (for 24-well plate) or 150 μ l (for 12-well plate) of MTT stock solution were added to each well. The plate was incubated at 37° C in an atmosphere of 95% air and 5% CO₂ for one h. The yellow colour of the MTT dye changed to black spots around the cells. The plate was centrifuged at 800 g for 10 min, to sediment any suspended formazan. The medium was carefully aspirated, and any remaining medium was removed from the wells by inverting the plate over tissue paper. To dissolve the formazan formed, 1 ml of

DMSO was added to each well of a 12-well plate (500 μ l for a 24-well plate). To assist quick and complete dissolution, the plate was agitated on a plate shaker for 5 min. A 100 μ l aliquot of the pink solution which developed was transferred to a 96 well plate in duplicate and the absorbance was measured with a plate reader at a wavelength of 450 nm and a background correction at 780 nm.

3.2.4 Protein Assay

Iodine uptake was corrected for the number of cells present by measuring the protein contents of each well, and results were calculated as cpm per μ g of protein.

The protein assay was carried out using a kit manufactured by Bio-Rad. This method is a dye binding assay based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. It requires a single reagent and an incubation time of 5 min.

The protocol for protein measurement was modified to obey Beer's Law over a range of 0 to 100 μ g/ml.

For a standard solution, a protein solution of 400 μ g/ml was used. This solution was diluted with physiological saline to give protein concentrations of 20, 40, 60, 80, and 100 μ g/ml for construction of a standard curve. Figure 3.3 shows an example of a standard curve for protein measurement.

To measure the protein concentration in each well, the cell remnants that were still adherent to the plate after iodine uptake were solubilised in 0.5 ml (24-well plate) or 1.0 ml (12-well plate) of 0.2 mol/l NaOH. The plates were sealed and incubated at room temperature overnight. Next day, the solution was resuspended by shaking the plate on a plate shaker. Then, the contents of each well were mixed thoroughly at least 4 times, using a one ml automatic pipette. Aliquots of 20 μ l of samples and standards were transferred to a 96-well plate in duplicate, followed by addition of 200 μ l of stock dye reagent, using the first two wells as blank. The plate was mixed on a plate shaker for 5 min. The absorbance of the solution was determined using a plate reader, with a filter of 595 nm, against a solution 20 μ l of distilled water and 200 μ l of reagent as reagent blank.

3.2.5 Extraction Of cAMP From FRTL-5 Cells

Into each well of a 6-well plate, 10×10^5 cells were seeded, followed by addition of 2.5 ml of 6H medium containing 10 U/l of TSH. Two different protocols were investigated for extraction of cAMP using separate plates.

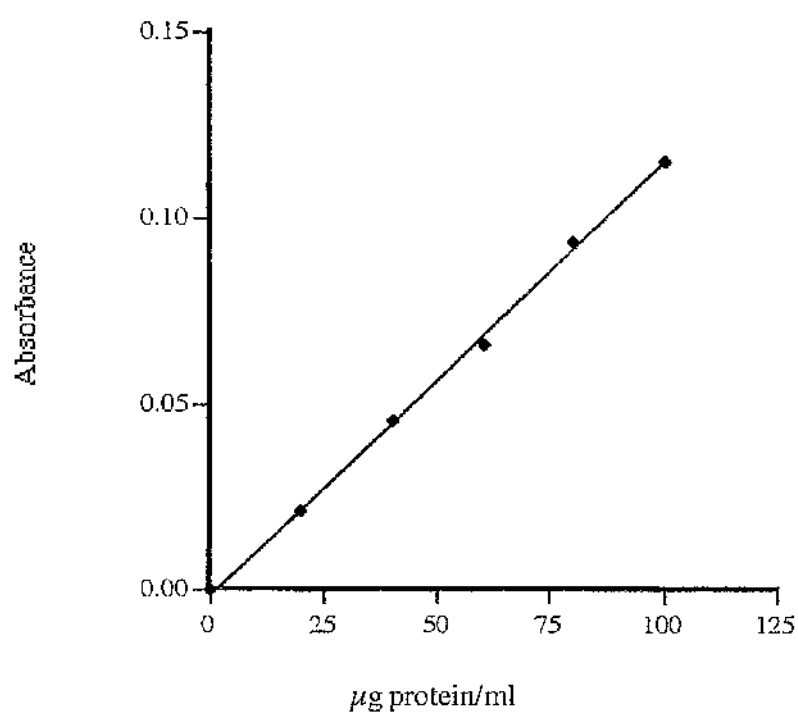


Figure 3.3: Standard curve used for measurement of cellular protein.

Plates were incubated at 37° C in an atmosphere of 95% air and 5% CO₂ for 3 days. After this time, the medium was removed from each well, and cells were washed twice with the HBSS. To each well, 2.5 ml of freshly filtered SH medium were added, and plates were incubated for a further 5 days (medium was changed once after three days). After this second incubation, the medium of all wells was removed, and the cells were washed using the HBSS. One plate was used for each extraction protocol.

3.2.5.1 Protocol 1

After washing the cells, 2.5 ml of HBSS containing 2 g/l BSA and 0.5 mmol/l IBMX (3-isobutyl-methyl xanthine) were added to all wells. Three wells served as control and to another 3 wells a concentrated TSH solution was added to give a final concentration of 10 U/l. The plate was incubated at 37° C. After 3 h incubation, media from all wells were aspirated and discarded. To each well 2.5 ml of ice-cold absolute ethanol were added, and the plate was incubated at -20° C overnight. The alcohol from each well was removed and transferred to appropriate labelled tubes, and lyophilised. Immediately before cAMP assay, 2.5 ml of cAMP buffer were added to each control tube, and 5 ml to tubes containing the extract of stimulated cells, to reconstitute the samples for assay.

3.2.5.2 Protocol 2

The buffer used for this protocol was low sodium salt (composition of this solution is given in table 3.1). This buffer causes diffusion of cAMP out of the cells. To each well of the second plate, 2.5 ml of low sodium buffer containing 2 g/l BSA and 0.5 mmol/l IBMX were added. Three wells served as control and to another 3 wells a concentrated solution of TSH was added to give a final concentration of 10 U/l. The plate was incubated at 37° C. After 3 h incubation, media from all wells were aspirated and transferred to appropriate labelled tubes. Tubes were kept at -20° C until they were assayed. To tubes corresponding to stimulated wells 2.5 ml of assay buffer were added.

To dissolve the cells remaining in the wells of both plates, 3 ml of 0.2 mol/l NaOH were added to each well, and plates were incubated at room temperature overnight. Protein assay was carried out as described above.

Ingredient	mmol/l	M.W.	g/l
KCl	5.00	74.55	0.375
CaCl₂.2H₂O	1.30	147.0	0.191
MgSO₄	0.40	120.5	0.048
Na₂HPO₄	0.34	142.0	0.048
KH₂PO₄	0.44	136.1	0.060
Glucose	5.55	180.2	1.000
HEPES	10.0	238.3	2.383
BSA	-	-	1.000

Table 3.1: Ingredients of buffer solution with low sodium, pH 7.4.

3.2.6 Determination of cAMP

3.2.6.1 Principles of the Assay

To determine the amount of cAMP in cell culture supernatants, a commercial kit (Amersham Life Sciences) was used. This kit method is based upon competition between unlabelled cAMP and a fixed quantity of peroxidase-labelled cAMP for a limited number of binding sites on a cAMP specific antibody. With fixed amounts of antibody and peroxidase-labelled cAMP, the amount of peroxidase-labelled ligand bound by the antibody will be inversely proportional to the concentration of added unlabelled ligand.

The rabbit anti-cAMP antibody is immobilised on to polystyrene microtitre wells precoated with second antibody (Donkey anti rabbit IgG). Thus any unbound ligand can be removed from the well by washing.

The amount of peroxidase labelled cAMP bound to the antibody is determined by addition of a tetramethylbenzidine (TMB)/hydrogen peroxidase substrate. The reaction is stopped by addition of acid, and the resultant colour read at 450 nm in a microtitre plate spectrophotometer.

The kit contained the reagents and materials listed below.

Microtitre plate: Plate containing 8 x12-well strips coated with donkey anti-rabbit IgG, ready for use.

Peroxidase conjugate: 11 ml diluted assay buffer were added to the contents of a vial of lyophilised cAMP-horseradish peroxidase.

Standard: To one vial of lyophilised cAMP standard containing 64 pmol cAMP, 2 ml distilled water were added. The vial after reconstitution contained 32 nmol/l of cAMP in 0.05 mol/l acetate buffer and 0.01 % (w/v) thimerosal.

Eight working standards were prepared by serial dilution of the stock standard of 32 nmol/l with the assay buffer. The final concentrations ranged from 0.125 to 16 nmol/l.

Antiserum: 11 ml distilled water were added to the contents of a vial of rabbit anti cAMP. The solution contained anti-cAMP serum in 0.05 mol/l acetate buffer pH 5.8, 0.5 % (w/v) bovine serum albumin and 0.01 % (w/v) thimerosal.

TMB Substrate: One bottle of enzyme substrate was provided, containing 22 ml solution of 3,3',5,5'-tetramethylbenzidine (TMB)/hydrogen peroxide, ready for use.

Assay buffer: One bottle was provided, containing 10 ml. This was made up to 500 ml with distilled water. The diluted buffer contained 0.05 mol/l sodium acetate buffer pH 5.8, 0.02 % (w/v) bovine serum albumin and 0.005 % (w/v) thimerosal.

Wash buffer: One bottle was provided, containing 12.5 ml. This was made up to 500 ml with distilled water. The diluted buffer contained 10 mmol/l phosphate buffer pH 7.5, and 0.05 % (w/v) thimerosal.

3.2.6.2 Protocol for Measurement of cAMP

Before assay all reagents and samples were left to equilibrate to room temperature. All steps of the protocol were performed according to the manufacturer's instructions.

The required numbers of microtitre strips were fixed on the frame provided. Two wells were used as blank and another two as non-specific binding (NSB), and 200 μ l of assay buffer were added to these in place of standard or samples. To another two wells (B_0), 100 μ l of assay buffer were added. From each working standard or sample, 100 μ l were transferred into the appropriate duplicate wells of the microtitre plate. To all wells except blank and NSB wells, 100 μ l of antiserum were added. The plate was sealed, gently mixed, and incubated for exactly 2 h over crushed ice.

After incubation, the plate sealer was removed, and to all wells except the blank, 50 μ l of cAMP-peroxidase conjugate were added. The plate was sealed, gently mixed, and incubated over crushed ice for another hour.

After the second incubation, the plate sealer was removed, and the wells were decanted by inverting the plate. The wells were washed 5 times with 400 μ l of wash buffer and dried by placing the inverted plate over tissue paper. Immediately after washing, 150 μ l of enzyme substrate were dispensed into all wells. The plate was sealed again and mixed on a microtitre plate shaker for one h at room temperature. The reaction was stopped with 100 μ l of 1 mol/l H_2SO_4 and absorbance was measured at 450 nm. The final colour was stable for 30 min.

To construct the standard curve and determine results, the average optical density of each standard or sample was converted to percentage of bound labelled cAMP, using following formula:

$$\% \frac{B}{B_0} = \frac{\text{Absorbance of standard or samples} - \text{Absorbance of NSB}}{\text{Absorbance of } B_0 - \text{Absorbance of NSB}} \times 100$$

An example of standard curve for cAMP assay is presented in figure 3.4.

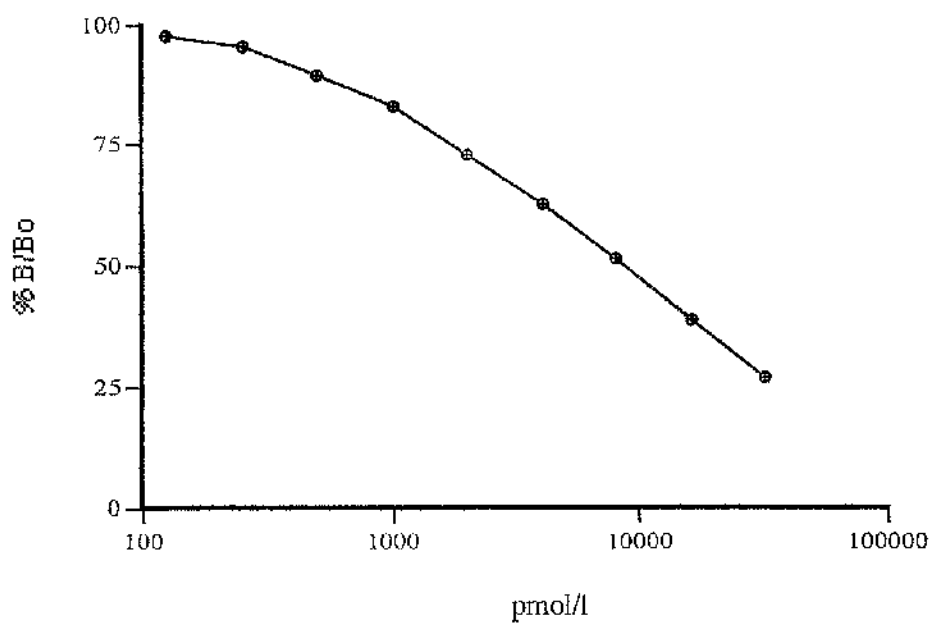


Figure 3.4: Standard curve used for measurement of cAMP.

3.2.7 Acrylamide Gel Preparation And Electrophoresis

For polyacrylamide gel preparation, and electrophoresis an electrophoresis set (Protean II Slab Cell) manufactured by Bio-Rad was used. Electrophoresis was carried out as stated in the manufacturer's instructions.

The required volume of stock acrylamide/Bis for preparation of 50 ml of solution was calculated. This is equal to 12.5 ml for a 7.5 % gel. This was transferred to a beaker that contained 24.25 ml of distilled water, followed by addition of 12.5 ml of 1.5 mol/l Tris-HCl buffer (pH 8.8). The solution was mixed thoroughly and was transferred to a filtration unit (Corning, Vacuum Filtration System), connected to a vacuum pump. The pump was started, and the solution was filtered. After filtration, the pump was run for a further 20 min to remove air trapped in the solution.

While the reagents were being filtered, the apparatus was set up as described in the Bio-Rad manual.

After de-aerating the monomer solution, 500 μ l of freshly prepared 10% solution of ammonium persulphate were added, followed by rapid addition of 50 μ l of TEMED (N,N,N',N'-tetramethylethylenediamine) to initiate polymerisation. The solution was mixed quickly with a pipette. By using a 30 ml syringe and a needle, 22 ml of monomer solution were transferred to the gel sandwich assembly, between the glass plates. Care was taken not to trap any air bubbles. Immediately, the monomer solution was overlaid with 1 ml of water. The assembly was left undisturbed for 1 h to allow gel formation to take place.

During polymerisation of the separating gel the stacking gel solution was prepared. 10 ml of 4% stacking monomer solution was prepared by addition of 1.3 ml of stock acrylamide/Bis solution (30% T, 2.67% C), and 2.5 ml of 0.5 mol/l Tris-HCl buffer, pH 6.8, to 6.2 ml of distilled water in a beaker. This was filtered and de-aerated, as described above.

When polymerisation was complete, the liquid was removed from the top of the gel and the surface of the gel was dried with filter paper. Then, a 10-toothed well comb was placed between the glass plates in such a way as to make an angle of 15-20° with the surface of the running gel. This prevents air being trapped under the comb teeth while pouring the monomer solution. The assembly was then ready for introduction of the stacking gel.

The stacking gel solution was prepared by addition of 50 μ l of 10% ammonium persulphate solution to the stacking monomer solution, followed immediately by 50 μ l of TEMED. This was poured with a 10 ml syringe and needle. When all teeth had been covered by the solution, the comb was pushed down and aligned. Then more solution was added to fill the sandwich completely. It was left

undisturbed for 1 h for the gel to polymerise. Gently, the comb was removed by pulling it straight up. The sandwich assembly was attached to the cooling system of the tank. An acrylic dam was used to form the upper buffer chamber. The lower buffer chamber was filled with the required volume of diluted running buffer (1.2 l). Then the cooling system and attached gel were put in place and the cooling system was connected to a water tap. The upper buffer chamber was filled with running buffer (approximately 350 ml). The lid of the tank was put in place, and the apparatus leads were connected to the power supply. The system was run without sample at a constant current of 15 mA for 0.5 h. Then the power supply was disconnected.

During the above period, the samples for electrophoresis were prepared by addition of 60 μ l of sample buffer to 20 μ l of sample in an Eppendorf tube. The contents of the tube were mixed by vortexing. Using an automatic pipette with a narrow tip, 30 μ l of this mixture were transferred to each well. The lid of the tank was replaced, and the run was carried out at constant current of 18 mA for 2 h. When the dye had entered the separating gel, the current was reduced to 5 mA and the system was run overnight. When the dye (BPB) reached the end of gel, the electric supply was disconnected.

The gel was removed carefully from the glass plates and was immediately transferred to the staining tray, which contained 100 ml of stain solution, and left for 30 min, with occasional shaking. The stain was discarded from the tray and was replaced by 50 ml of destaining solution. The gel was washed thoroughly in this solution. The solution was removed and discarded, and was replaced by another 100 ml of destaining solution. This changing of destaining solution was carried out every 30 min, with shaking every 5 min until the gel was clear.

3.2.8 Statistical Analysis

The FRTL-5 cells used for this study were between the 14th and 19th passage. The experiments for this part of the work were repeated at least twice, and in some cases up to five times. The results presented for growth are for the measurement of cellular protein, and are the mean \pm standard deviation of triplicate results obtained in one set of experiments. Statistical analysis was carried out using the StatView computer package. Data were analysed by factorial analysis of variance (ANOVA). The Bonferroni/Dunn criterion was used to determine significant differences in multiple comparisons.

The P value is mentioned in the text wherever necessary. Significant differences are shown in the graphs by (*), while in text they are defined as significant or highly significant.

The differences were defined as significant when the calculated critical difference as determined by the Bonferroni/Dunn criteria (5% significance level) was less than the mean difference ($P < 0.05$), while they are defined as highly significant when the calculated critical difference is less than half of mean difference ($P < 0.001$).

SECTION THREE: RESULTS

3.3.1 Characterisation of FRTL-5 Cells

The ability of the FRTL-5 cells to respond to TSH was confirmed by determination of cAMP production, iodine uptake and growth.

3.3.1.1 cAMP Production

For extraction of cAMP from the cells, two different protocols were quoted in the literature, using either ethanol or hypotonic saline solution. To compare these, two parallel 6-well plates were set up as given in methods' sections (3.2.1.4). The mean value of cAMP extracted by ethanol in the control group was 15.71 fmol/ μ g protein, compared to 549.8 fmol/ μ g protein when cells were stimulated by 10 U/ml of TSH for 3 h. When hypotonic saline solution was used, the mean value obtained for control wells was 15.70 fmol/ μ g protein, and for stimulated cells was 528.4 fmol/ μ g protein. Statistically there were no differences between the two extraction methods for either controls or stimulated cells. These results confirmed that the cells were capable of responding to TSH by increasing cAMP production and showed that there was no difference in efficiency between the extraction methods.

3.3.1.2 Growth Measurement

The MTT assay has not been used previously for growth measurement of FRTL-5 cells, so its suitability was tested. Two 24-well plates were seeded with 1×10^4 to 1×10^6 FRTL-5 cells in 0.5 ml of medium, either without TSH or containing 10 U/l of TSH. Plates were incubated at 37° C for 24 h, and the MTT assay was then carried out as described in section 3.2.3.

The graph of absorbance versus number of cells (figure 3.5), was a straight line passing through the origin. This shows that the MTT assay is a reliable method for measurement of growth for this cell line. The mean value of the ratio of absorbance of stimulated cells versus unstimulated cells at 24 h was 1.626 ± 0.043 .

3.3.1.3 Iodine Uptake

Since several protocols for uptake of iodine into FRTL-5 cells have been published, optimal conditions were investigated.

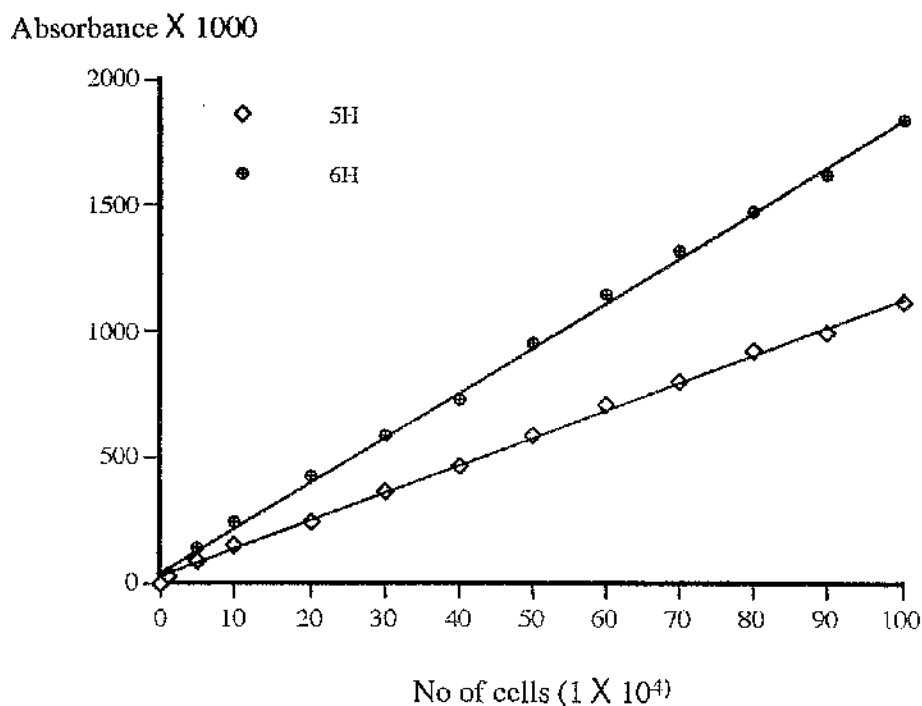


Figure 3.5: Growth of FRTL-5 cells as assessed by the MTT assay. Different numbers of FRTL-5 cells were seeded in 5H and 6H media for 24 h, and MTT assay was performed.

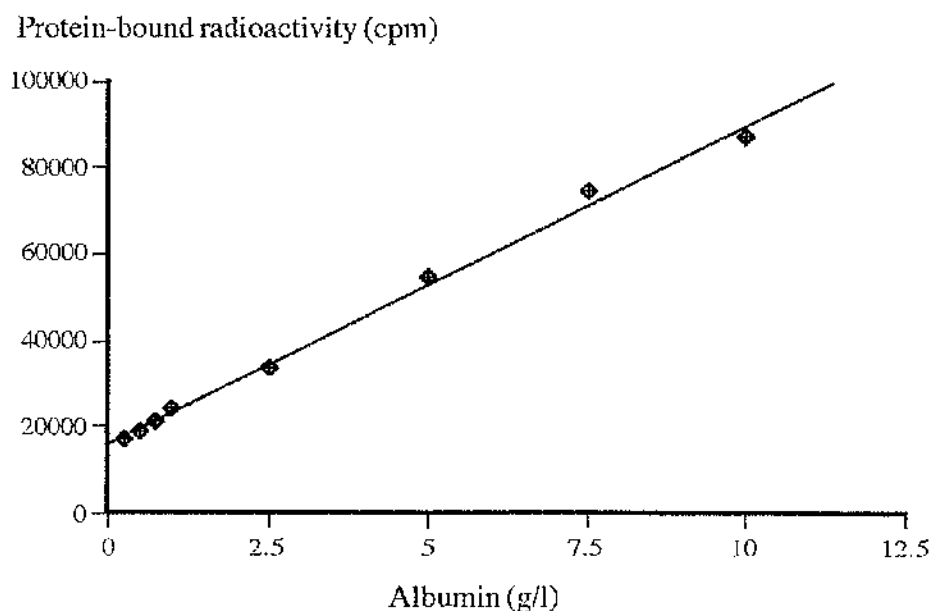


Figure 3.6: Association of radioactive iodine with albumin. A dose of $0.5 \mu\text{Ci}$ of ^{125}I in 10 nmol/l KI was incubated with albumin at pH 7.35. Protein was precipitated with TCA and radioactivity was counted.

3.3.1.3.1 *Effect of pH*

Salt solutions with and without additional buffering in the form of HEPES were compared as iodine uptake media. Two 12-well plates were seeded with approximately 1×10^5 cells per well. The cells were incubated for 3 days in 6H (TSH 2 U/l), followed by 5 days in 5H medium. The medium of 6 wells from each plate was changed to 5H and the other 6 wells to 6H (TSH 500 mU/l). The plates were incubated for a further 3 days. In one plate the HBSS containing bicarbonate buffer was used (pH 7.4), and in the second plate the HBSS containing 10 mmol/l of HEPES, pH 7.4. To these buffers, KI and radioactive iodine were added to final concentrations of 1 μ mol/l and 0.2 μ Ci/ml respectively. Iodine uptakes were performed for 30 min at 37° C. The pH of the first medium changed to 6.3-6.4 during this incubation, as assessed by the colour change of the phenol red indicator.

The uptake of iodine into TSH-deprived cells incubated in HEPES was 494.42 ± 48.17 cpm/ μ g protein, and for TSH-stimulated cells was 631.46 ± 39.64 . The stimulatory effect of TSH was significant ($P < 0.01$). The values obtained for iodine uptake of the cells incubated in medium not containing HEPES was 716.73 ± 24.23 cpm/ μ g protein when unstimulated, and 735.85 ± 46.43 cpm/ μ g protein when stimulated by TSH. There is no significant difference between these values. This shows that adequate buffering of the medium is essential for the cells to respond to TSH.

3.3.1.3.2 *Effect of albumin*

Incubation media described in the literature sometimes, but not always, contain albumin. It is possible that albumin may bind to iodine and act as a carrier, thus modifying the uptake characteristics of the cells. Experiments were carried out to assess the extent of uptake of radioactive iodine by albumin in solution, in the absence of cells. Different concentrations of albumin ranging from 0 to 10 g/l were prepared in HBSS with 10 mmol/l of HEPES (pH 7.35). A mixture of radioactive iodine (0.5 μ Ci/ml) and non-radioactive iodine (10 nmol/l potassium iodide) was added to the solutions. They were incubated in a 37° C water bath for 30 min. For each concentration of albumin four replicate tubes were prepared. One ml from each solution was transferred to a tube containing 1.0 ml of 10% TCA. After incubation for 10 min in ice, the tubes were centrifuged at 1500 g, the supernatants were discarded, and the precipitates were washed once with TCA, before measurement of their radioactivity in a γ counter.

The mean value obtained for four tubes without albumin was considered to be non-specific binding and was subtracted from the remaining values. The results are

presented in figure 3.6. There is a direct relation between concentration of albumin and bound iodine.

To investigate whether the association of albumin and iodine was saturable, the experiment was repeated with a fixed concentration of albumin (5 g/l) and radioactive iodine (0.5 $\mu\text{Ci/ml}$) and various concentrations of KI ranging from 10 nmol/l to 10 $\mu\text{mol/l}$. Increasing the concentration of KI caused a decrease in association of albumin and radioactive iodine, implying competition for a saturable binding site (figure 3.7).

To investigate the time course for association to albumin, the above experiment was repeated using a fixed concentration of albumin (20 g/l) and iodine (10 nmol/l of KI and 0.5 $\mu\text{Ci/ml}$ of radioactive iodine), and various incubation times ranging from 15 seconds to 60 min. There were no differences between values obtained for each time point. This suggests that the association of iodine and albumin was instantaneous.

To identify the carrier of iodine in serum, blood was taken from 8 healthy adults (4 males, 4 females). After separation of serum, 10 μCi radioactive iodine (1 μl) was added to 50 μl of each sample, and the sera were prepared for polyacrylamide gel electrophoresis as described in section 3.2.7. After the destaining step, the gel was cut into strips, one for each sample. Each strip was further cut into 8 segments. The radioactivity of each segment was counted in a γ counter.

Approximately 15 % of the total count of the radioactive iodine was attached to the pre-albumin band, 37.5 % to albumin, and 12.5 % to the last band in the gamma globulin region, although some was also found in other bands (figure 3.8).

The results from the above experiments suggest that inorganic iodide is bound to proteins, especially pre-albumin and albumin, by a strong bond that is not disrupted by protein precipitation. Both pre-albumin and albumin are known to carry thyroid hormones, and this evidence suggests that the same proteins may also be carriers for inorganic iodine.

3.3.1.3.3 *Optimisation Of Iodine Uptake Buffer*

1×10^5 FRTL-5 cells were seeded in each well of four 12-well plates, and incubated as described in section 3.2.1.4 (3 days in 6H, 5 days in 5H, and 2 days in 6H). For the iodine uptake step, two sets of solutions of HBSS buffer with 10 mmol/l HEPES were prepared. Both contained concentrations of KI ranging from 0.1 $\mu\text{mol/l}$ to 200 $\mu\text{mol/l}$, keeping the ratio of radioactive iodine to KI constant (50 mCi/mmol of KI). The second buffer also contained 5 g/l of albumin. Iodine uptake was carried out for 5 min according to the protocol given in section 3.2.2, using one concentration of KI for each set of triplicate wells.

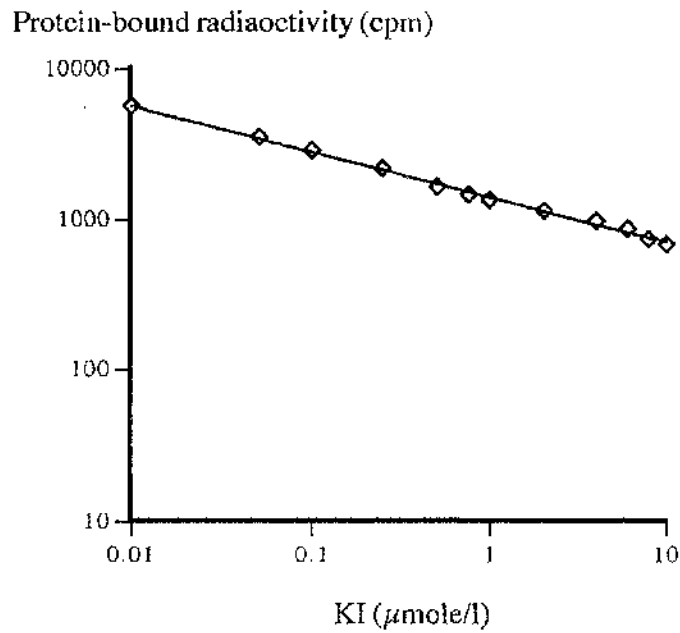


Figure 3.7: Effect of KI on association of fixed amount of radioactive iodine ($0.5 \mu\text{Ci}$) to protein in a solution of 5 g/l of albumin.

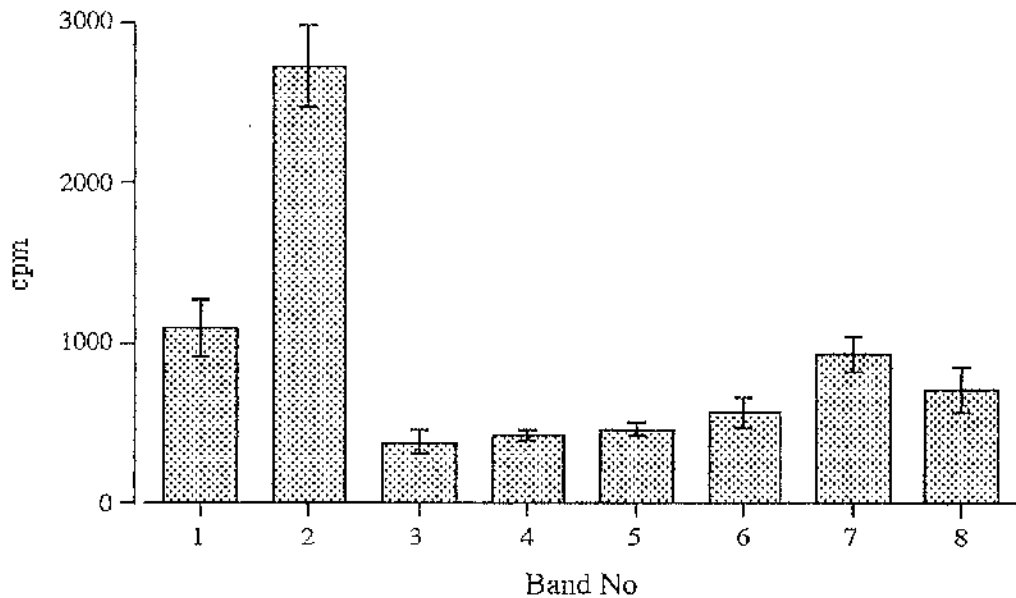


Figure 3.8: Binding of radioactive iodine to serum proteins. The bands extended from pre-albumin (1) through albumin (2), and gamma globulin (7), to the material that was excluded from the gel (8). Serum was separated on a non-denaturing polyacrylamide gel (7.5%)

The result of this experiment is presented in figure 3.9. At all concentrations of iodine, the iodine uptake was greater in the presence of albumin than its absence. The curves for both media demonstrated saturable kinetics.

The data were used to determine K_m and V_{max} of iodine uptake in the presence and absence of 5 g/l albumin by plotting as a Lineweaver-Burk plot (figure 3.10). The K_m was 52.6 $\mu\text{mol/l}$ in the absence of albumin, and was 27.0 $\mu\text{mol/l}$ when albumin was present in the iodine uptake buffer. The corresponding values of V_{max} were 3.57 and 2.70 $\text{pmol}/\mu\text{g protein}/\text{min}$. This indicates that the presence of albumin in the iodine uptake medium caused a decrease of 50 % in the value of K_m , and decreased the value of V_{max} by 0.87 $\text{pmol}/\mu\text{g protein}/\text{min}$.

To ensure that measurements were made in the approximately linear section of the response curve, a concentration of KI of 10 $\mu\text{mol/l}$ was used. Albumin (5 g/l) was also included in the buffer.

To determine the optimal incubation time, 1×10^5 FRTL-5 cells were seeded per well of eight 12-well plates, and incubated as given in methods' section 3.2.1.4. For iodine uptake two different HBSS buffers with 10 mmol/l HEPES (pH 7.38) were prepared. Both contained 10 $\mu\text{mol/l}$ of KI and 0.5 $\mu\text{Ci/ml}$ of radioactive iodine, (50 mCi/mmol of KI). The second buffer contained 5 g/l of albumin in addition. For iodine uptake, 6 wells from each plate were filled with one ml of buffer alone, and the other 6 wells were filled with buffer that contained albumin. Each plate was incubated for a different period ranging from 7 to 56 min.

At all time points the amount of iodine taken up by cells in the presence of albumin was higher than when it was absent. When albumin was absent from the buffer, iodine uptakes increased significantly with time up to 35 min, but after 35 min there were small, non significant increases up to 56 min (figure 3.11). The iodine uptakes at successive time points in the presence of albumin were significantly different from each other up to 21 min. After this time, there were small and non significant increases up to 56 min. An incubation time of 20 min was chosen.

3.3.1.3.4 Optimal Concentrations Of TSH And Serum

The recommended concentration of TSH in the original paper (Kohn and Valente, 1989) was 10 U/l in 6H medium. Many other investigators used different concentrations of TSH in the range of mU/l to U/l. With high concentrations of TSH (10 U/l), small effects of other stimulators may be difficult to detect. Zakarija and McKenzie (1989 a) showed that the concentration of serum or its replacement with albumin could also change the pattern of iodine uptake and growth of cells. In the present study, FRTL-5 cells did not grow (nearly 10 % of the cells survived) when serum was replaced by albumin or if the concentration of serum was less than 0.5%.

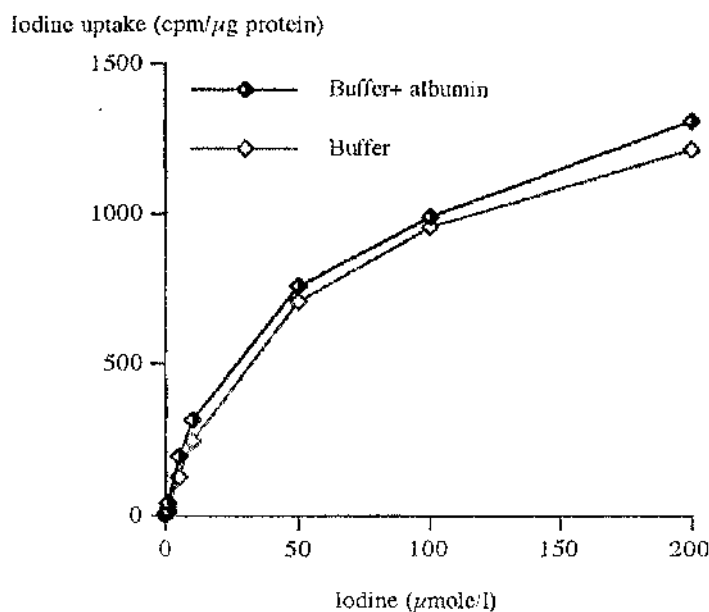


Figure 3.9: Iodine uptake of FRTL-5 cells. Cells were incubated with various concentrations of iodine in presence and absence of albumin for 5 min. The specific radio-activity of iodine was 50 mCi radioactive iodine/mmmole KI.

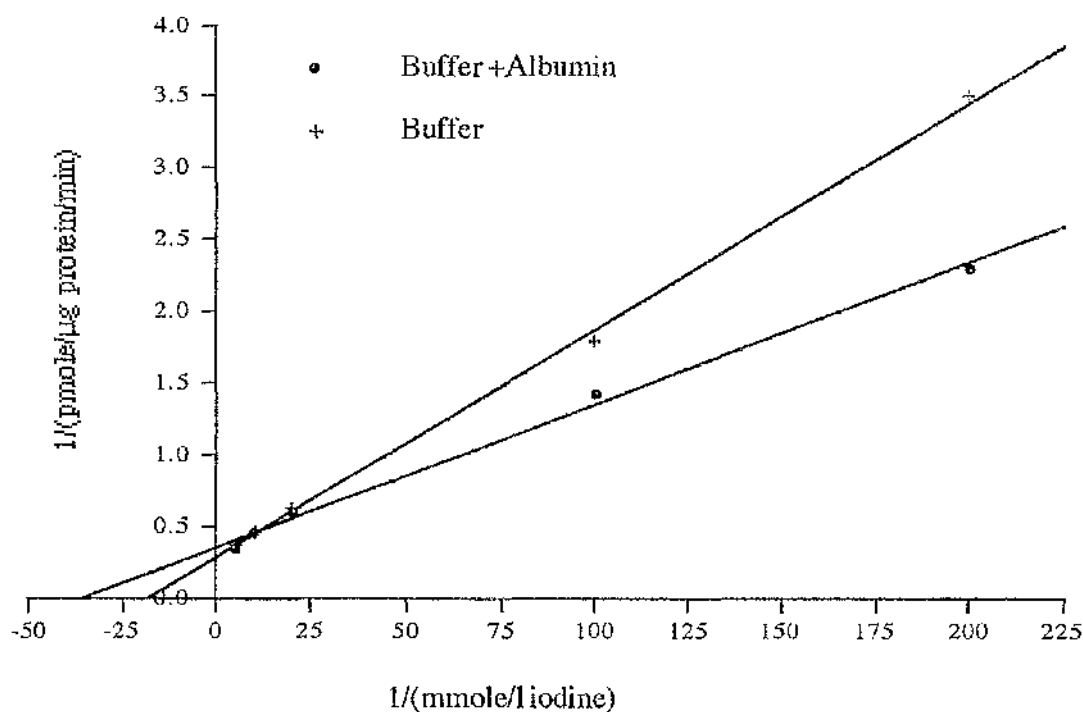


Figure 3.10: Lineweaver-Burk plot of iodine uptake of FRTL-5 cells. The specific activity of iodine was 50 mCi/mmmole in the presence and absence of 5 g/l of albumin. The time of exposure of cells to iodination medium was 5 min.

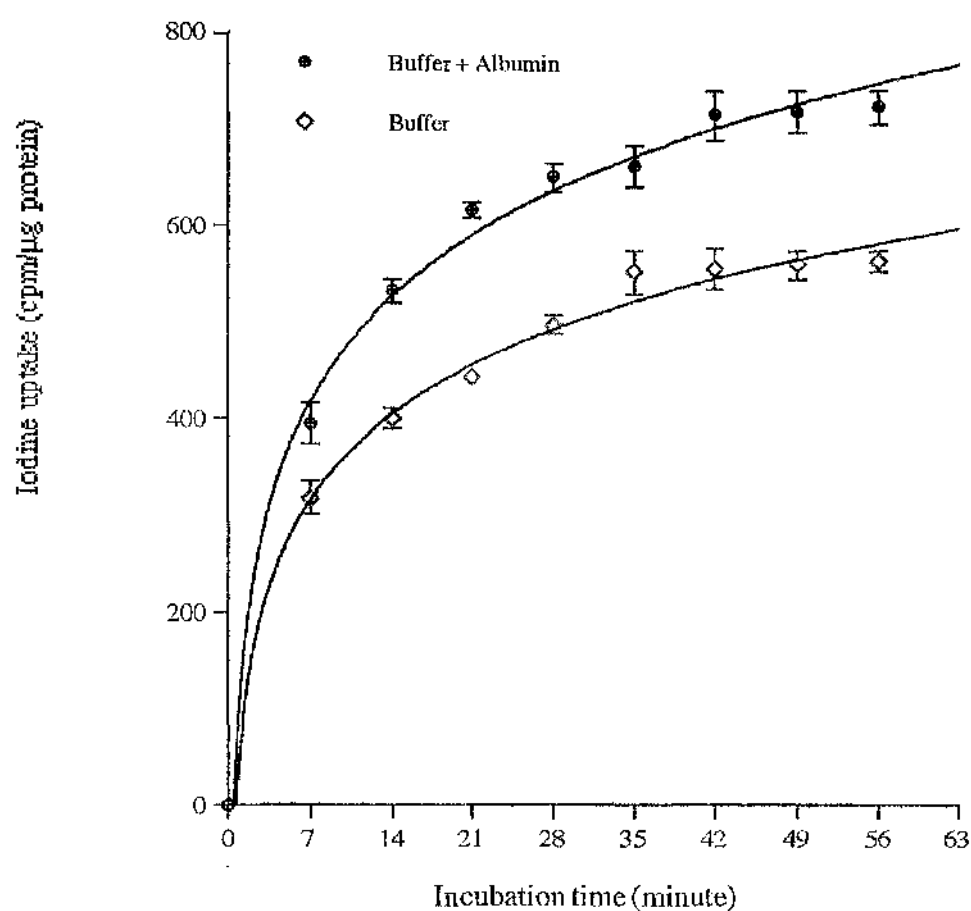


Figure 3.11: Time course of iodine uptake of FRTL-5 cells in presence and absence of albumin. The concentration of iodine was $10 \mu\text{mol/l}$ and the specific activity was 50 mCi/mmol .

To determine the most suitable concentrations of TSH and serum for iodine uptake and growth, two 24-well plates were seeded with approximately 1×10^5 cells per well. The plates were incubated as given in section 3.2.1.4. For stimulation, media containing different concentrations of serum (0.5 to 5 %) and TSH (0 to 2 U/l) were used. Each set of triplicate wells had a different combination of serum and TSH. The plates were again incubated for a further 3 days. The iodine uptake assay and protein measurements were carried out as given in sections 3.2.2 and 3.2.4.

The iodine uptake of cells incubated in the absence of TSH (5H) did not change when the serum concentration was changed from 0.5 to 5%. When the cells were stimulated with a constant concentration of TSH (0.5, 1, or 2 U/l), and serum concentration was changed from 0.5 to 5 %, the iodine uptake decreased. Statistically, the uptake obtained at a serum concentration of 2% was significantly decreased compared to values at 0.5 %. The decrease was highly significant when 5 % serum was used (figure 3.12). The cells were most sensitive to changes in TSH concentration when the serum content in the medium was 0.5 % and least sensitive when the serum content of the medium was 5 %. At a serum content of 5%, there was no significant response to changes in TSH concentrations between 0.5 and 2.0 U/l.

Since the highest iodine uptake was seen at 2 U/l of TSH and serum content of 0.5 %, these concentrations were used in further experiments.

Cell growth in the absence of TSH did not change significantly when the serum content was changed from 0.5 to 5 %. At a constant concentration of TSH, growth increased as serum concentration was changed from 0.5 to 5% (figure 3.13). Statistically, values obtained for 2 and 5% were significantly greater than those at 0.5%.

Since optimal growth required high serum concentrations, but optimal response of iodine uptake to TSH required low concentrations, additional amino acids were added to the medium to provide the requirements for growth. To do this, 10 ml of 50 x concentrated MEM amino acids solution with L-glutamine (Sigma), and 10 ml of 100 x concentrated MEM nonessential amino acid solution (Sigma) were added per litre of 5H medium containing 0.5% heat inactivated FCS.

3.3.2 Thyroglobulin Production By FRTL-5 Cells

The activation of the thyroglobulin gene of FRTL-5 cells, in response to thyrotropin stimulation have been reported (Graves and Davies, 1993; Kamikubo et al, 1990; Bone et al, 1986 b; Van-Heuverswyn et al, 1984), but there is less information on the production of iodinated protein.

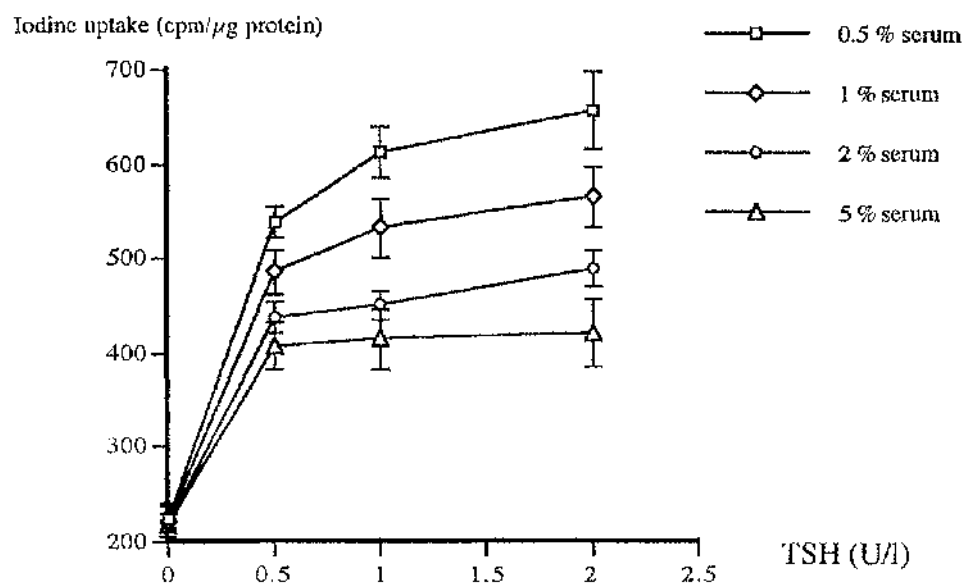


Figure 3.12: Effect of serum content of medium and concentration of TSH on iodine uptake of FRTL-5 cells after 72 h.

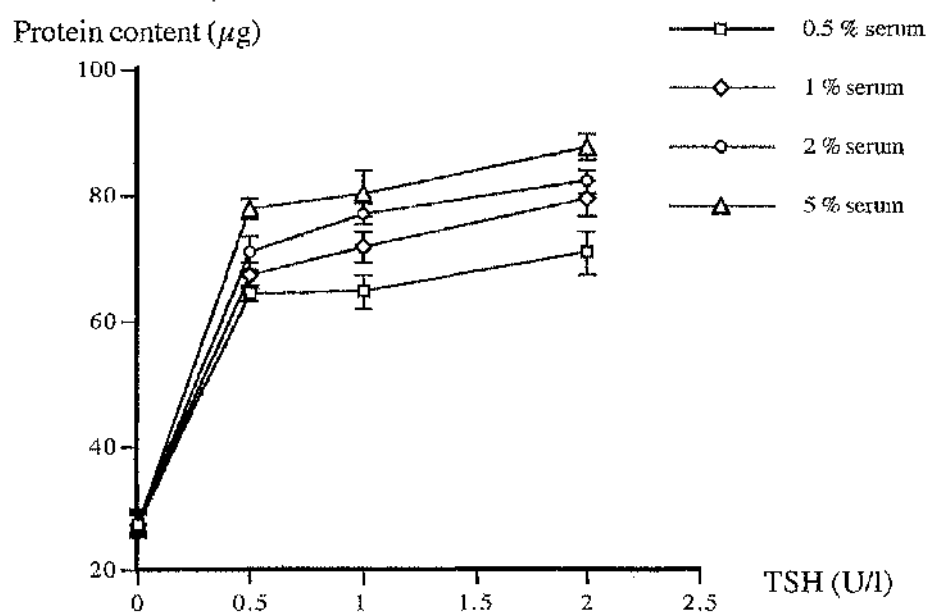


Figure 3.13: Effect of serum content of medium and concentration of TSH on growth of FRTL-5 cells after 72 h.

To determine whether the FRTL-5 cells were capable of producing thyroglobulin, one 6-well plate was seeded with approximately 5×10^5 cells per well. The plate was incubated for 3 days with 6H medium and 5 days with 5H medium. At this stage, the medium was changed to either fresh 5H medium or 6H medium (2 U/l TSH) containing 0.5 % FCS, additional amino acids, $0.5 \mu\text{mol/l}$ KI, and 0.5 mCi/l radioactive iodine. The plate was incubated for a further 72 h.

After the final incubation, the medium from each well was transferred to appropriate labelled tubes, the cells were washed twice with ice-cold HBSS, and their iodine content was extracted with 1 ml of ethanol and counted with a γ counter. The protein content of each well was measured as given in section 3.2.4. The protein in the media was precipitated by 2.5 ml of 10 % TCA for 10 min, followed by centrifuging at 1500 g. The precipitates were washed twice, each with 1 ml of TCA, and counted.

The activity of radioactive iodine bound to protein in the medium of unstimulated cells (5H medium) was 143811 ± 9374 cpm, whereas the value for 6H medium was 245083 ± 22565 cpm, a 70.4 % increase. When those data were corrected for number of cells, the activity of radioactive iodine bound to proteins in the 5H medium was 1168.60 ± 47.80 cpm/ μg cell protein, while the value for 6H medium was 1400.13 ± 77.18 cpm/ μg cell protein. There is a significant difference between these values, which represents either newly synthesised protein or binding of iodine to serum components.

To determine whether there was synthesis of protein at the expected molecular mass of thyroglobulin, the distribution of iodine bound to protein was studied by electrophoresis. The experiment was repeated, under similar conditions. At the last stage the media were concentrated, using Centriflo membrane filter cones (CF-25). The concentrated proteins were diluted twice with 2 ml of the HBSS and concentrated again, to remove any unbound radioactive iodine. The final volumes of the concentrated protein solutions were adjusted to 50 μl . To each tube 50 μl of sample buffer was added, and all 100 μl was used for non-denaturing polyacrylamide electrophoresis.

After electrophoresis of the media, most of the radioactivity was shown to be bound to serum proteins, and only a small fraction was present in the high molecular mass band that was assumed to contain thyroglobulin. The average activity in this band for the media from unstimulated cells was 413.00 ± 87.07 cpm (corrected for background), whereas the corresponding value for stimulated cells was 1145.67 ± 114.73 cpm. The values corrected for protein content of the media were 3.35 ± 0.63 cpm/ μg protein for 5H and 6.54 ± 0.32 cpm/ μg protein for 6H. Statistically, the difference between these values was significant ($p=0.0054$). This indicates that the

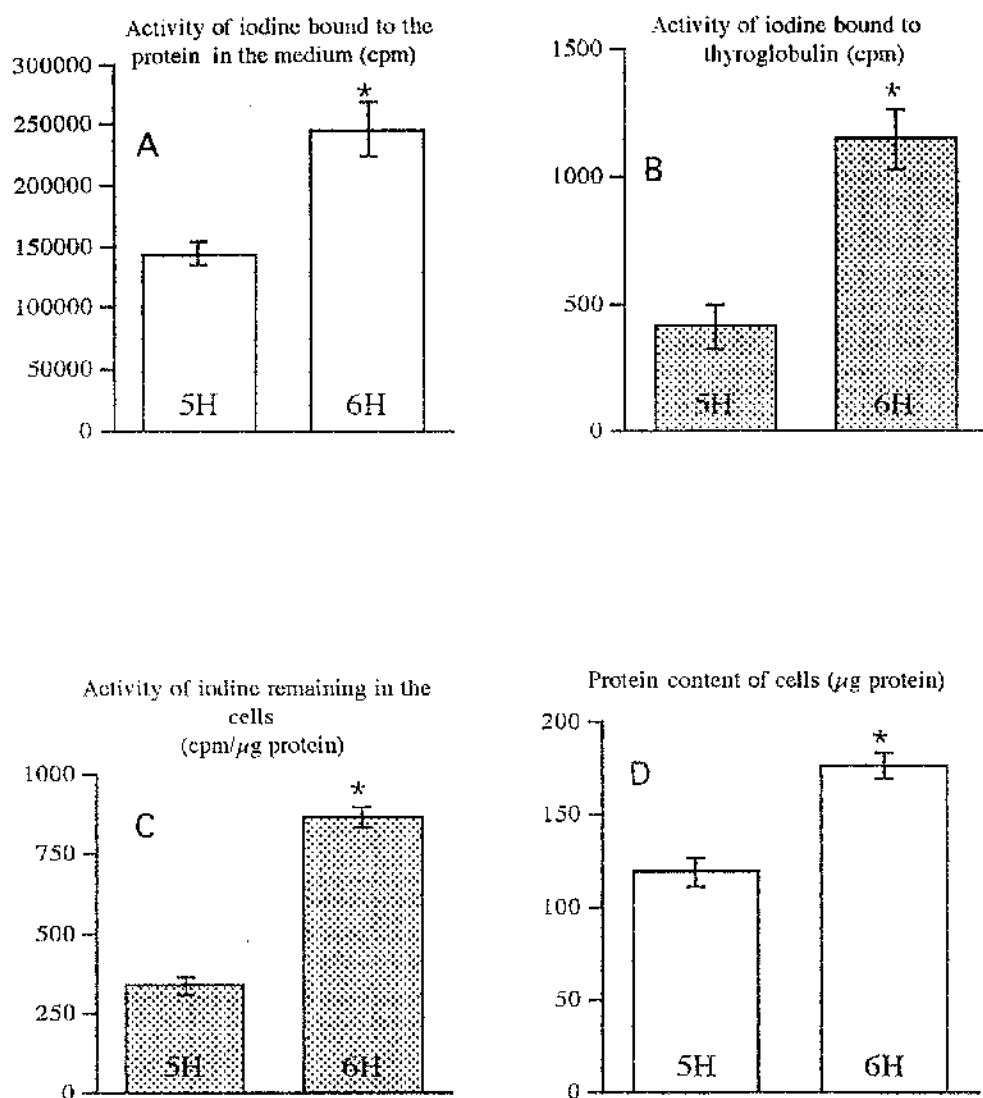


Figure 3.14: Thyroglobulin production by FRTL-5 cells. Cells were incubated for 72 h in media that contained 0.5 % FCS, additional amino acids, 0.5 μ mol/l KI and 0.5 mCi/l radioactive iodine. A: Activity of iodine bound to the protein in the medium. B: Activity of iodine bound to the thyroglobulin that was released to the medium (measured by electrophoresis). C: Activity of iodine remaining in the cells. D: Growth of cells.

FRTL-5 cells were capable of responding to stimulation with TSH by increasing the production of a high molecular mass iodinated protein, presumably thyroglobulin. The absolute amounts were, however, small. A summary of this experiment is presented in figure 3.14.

3.3.3 Effect Of Inorganic Iodide On Growth

Endemic goitre is an adaptive disease that develops in response to an insufficient supply of dietary iodine (Studer and Gebel, 1986), and administration of iodine is known to affect the size of the goitre of Graves' disease acutely. It is therefore possible that iodine concentration could effect the growth of thyroid cells in vitro. To investigate the effect of inorganic iodine on growth of FRTL-5 cells, approximately 1×10^5 cells were seeded in each well of four 12-well plates. The plates were incubated as above. When preparation of the cells was completed, the medium in triplicate wells of two plates was replaced by 5H medium containing 5 % FCS and concentrations of KI ranging from 0.01 to 1000 $\mu\text{mol/l}$. The medium of the other two plates was changed to 6H medium (2 U/l of TSH) containing similar concentrations of KI and FCS. The plates were incubated for a further 72 h. After the final incubation, the growth of cells was measured using the MTT assay.

The results of this experiment are presented in figure 3.15. In the presence of TSH, growth decreased as the concentration of KI present in the medium increased above 1 $\mu\text{mol/l}$. The growth in the presence of 1 $\mu\text{mol/l}$ of KI was 8.43 % less than the value obtained for zero concentration. The growth in 10 $\mu\text{mol/l}$ of KI was 14.7 % less than zero concentration, and a greater decrease in growth was caused by 1000 $\mu\text{mol/l}$ of KI. These results indicate that optimal growth of FRTL-5 cells requires the absence of inorganic iodine in the medium.

In the absence of TSH, the FRTL-5 cells do not grow detectably, so there was no apparent effect of KI. However, the highest concentration of 1 mmol/l was toxic, and caused a 13.08 % decrease in the number of live cells.

3.3.4 Thyroid Hormones

Thyroid hormones may affect the function of thyroid cells directly (Akiguchi et al, 1992). To investigate the effect of these hormones on FRTL-5 cells, approximately 1×10^5 cells were seeded per well of four 12-well plates. The cells were prepared for stimulation as given in section 3.2.1.4. Stimulation was carried out with 5H or 6H (2 U/L of TSH) medium that contained 0.5 % FCS, additional amino acids and different concentrations of thyroid hormones as given in table 3.2 for 72 h. Cells with only 5H or 6H medium served as control.

Medium	T ₄ nmol/l	T ₃ nmol/l
control	0.0	0.0
M1	50	0.5
M2	100	1.0
M3	250	2.5
M4	500	5.0
M5	750	7.5
M6	1000	10.0

Table 3.2: Concentrations of T₄ and T₃ that were added to 5H or 6H medium to study the effect of thyroid hormones on iodine uptake and growth of FRTL-5 cells.

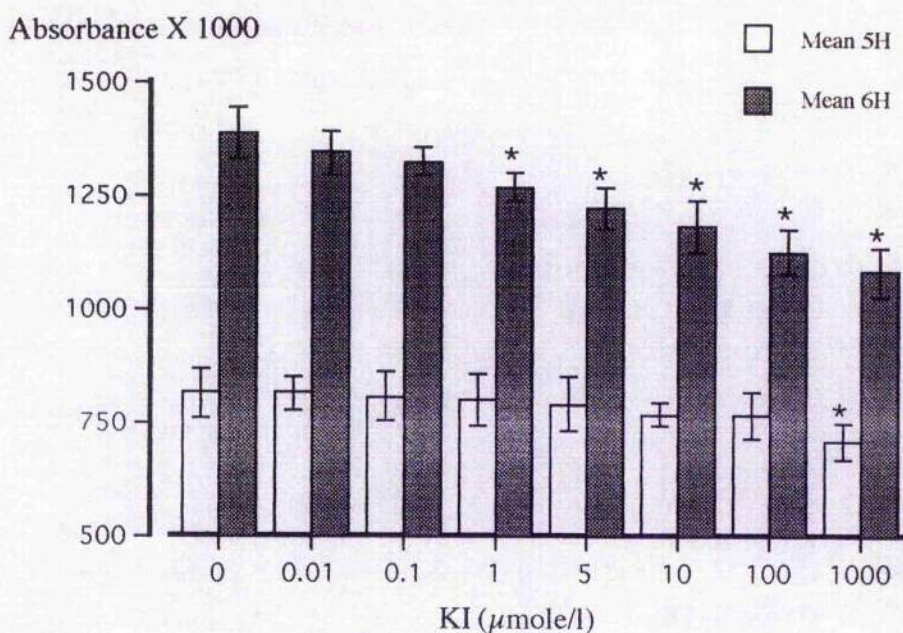


Figure 3.15: Effect of different concentrations of KI on growth of FRTL-5. MTT assay was performed after incubation for 72 h, in presence and absence of 2 U/l of TSH.

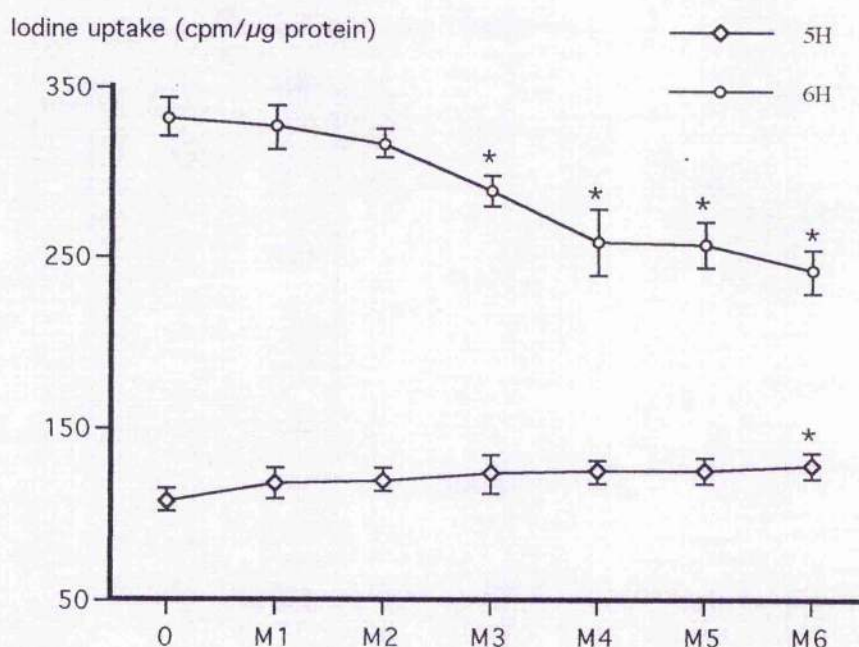


Figure 3.16: Effect of different concentrations of thyroid hormones on iodine uptake of FRTL-5 cells. The concentrations of T_4 and T_3 represented by M1-M6 are presented in table 3.3. Cells were incubated in the presence or absence of 2U/l of TSH for 72 h.

The mean iodine uptake of the cells incubated in 5H without thyroid hormones was 108.36 ± 6.27 cpm/ μ g protein. The addition of thyroid hormones caused a significant increase in iodine uptake of 19.83 % at the highest dose ($1 \mu\text{mol/l}$ T_4 and 10 nmol/l T_3) only.

In the presence of 2 U/l of TSH (6H), the iodine uptake of cells incubated without thyroid hormones was 331.95 ± 11.36 cpm/ μ g protein. Thyroid hormones caused significant decreases in iodine uptake of cells, at concentrations of M3 (250 nmol/l T_4 and 2.5 nmol/l T_3) and above. The maximum decrease was 27.01 % at a concentration of $1 \mu\text{mol/l}$ T_4 and 10 nmol/l T_3 . These results are summarised in figure 3.16.

In the absence of thyroid hormones and TSH, the protein content of cells was $30.97 \pm 2.29 \mu\text{g protein/well}$. There was a significant increase in the growth of cells (17.1 %) when the concentrations of T_4 and T_3 in the medium were $1 \mu\text{mol/l}$ and 10 nmol/l respectively.

In the presence of 2 U/l of TSH (6H), the protein content of control cells was $63.50 \pm 1.46 \mu\text{g protein/well}$. Here also the highest dose of thyroid hormones produced a significant increase in growth of cells (9.89%). These results are presented in figure 3.17.

3.3.5 Insulin Like Growth Factor-1 And Insulin

Insulin like growth factor-1 (IGF-1) is known to affect iodine uptake and growth of FRTL-5 cells (Pang and Hershman, 1990 a; Saji et al, 1987). Therefore IGF-1 was used to verify our protocols. Since insulin and IGF-1 may bind to the same receptor, the two were compared directly. For this purpose approximately 1×10^5 cells were seeded per well of four 12-well plates. The plates were incubated for 3 days with 6H and for 5 days with 4H (no TSH or insulin) containing 5 % FCS. After this, the medium of one set of triplicate wells was changed to 4H containing 0.5 % FCS (control). The medium of the other wells of two plates was changed to 4H containing 100 ng/ml of IGF-1, $10 \mu\text{g/ml}$ of insulin or a mixture of the two. The medium of the other two plates was changed to 4H containing TSH (2 U/l of TSH) and similar concentrations of other stimulators. The plates were incubated for a further 72 h. The iodine uptake and protein measurement were carried out on one plate from each set as given in the methods' sections 3.2.2, and 3.2.4. The growth of cells was measured on the remaining two plates using the MTT assay.

The mean value obtained for iodine uptake of the control set was 320 ± 14.07 cpm/ μ g protein. When the cells were stimulated with insulin, the iodine uptake was increased by 5.64 %, whereas IGF-1 produced an increase of 7.71 %. Neither of these increases was significant. When the cells were stimulated with the mixture of

insulin and IGF-1 there was an increase of 16.95 %. This was significant when compared with the control set, but not significant when compared to values obtained for Insulin or IGF-1.

The mean value obtained for iodine uptake of cells that were stimulated with TSH in 4H medium was 571.15 ± 27.93 cpm/ μ g protein. This was 78.33 % higher than unstimulated cells and was highly significant ($p < 0.001$).

The effect of insulin on TSH-stimulated cells was not significant, but that of IGF-1 and the mixture of IGF-1 and insulin was (increases of 15.81% and 20.96% respectively). The effect of the combination of IGF-1 and insulin was significantly greater than that of insulin alone, but not than that of IGF-1 (figure 3.18).

The pattern of results as measured for growth as assessed by the MTT assay was similar to that assessed by protein measurement on the cells remaining after iodine uptake (figure 3.19). Since results obtained from both protocols were the same, the protein content of the cells was used in the remaining experiments.

The protein content of cells after incubation in 4H medium was 26.20 ± 0.70 μ g protein/well. Insulin alone significantly increased the protein content of cells to 29.84 ± 0.73 μ g protein/well, 13.89 % higher than the control. IGF-1 alone also significantly increased protein by 42.82 %. The mixture of insulin and IGF-1 caused an increase in growth of 80.31 %, more than the sum of the effects of individual stimulators.

TSH caused a highly significant increase in protein content to 58.51 ± 0.73 μ g protein/well, 123.32 % higher than the control (4H medium). Addition of insulin caused a further significant increase of 26.58 %, as did IGF-1, of 47.58 %. When insulin and IGF-1 were combined, the protein content increased by 53.94 % (figure 3.19).

3.3.6 Stimulation Of FRTL-5 Cells By Bioactive Peptides

A series of experiments was carried out to investigate the effect of selected cytokines, growth factors and neuropeptides on the function of FRTL-5 cells. Iodine uptake and growth rate, as assessed by protein content of the cells, were measured. Stimulators were used singly and in a limited number of combinations.

First, a dose response curve was measured for each stimulator. An effective dose was chosen from this, to investigate the effect of protease inhibitors or thyroid hormones.

Bestatin is an inhibitor of neutral endopeptidase (CD10/NEP) and aminopeptidase N (CD13/APN), while DL-thiorphan is an inhibitor of aminopeptidase A (BP-1/6C3/APA). These glycoprotein enzymes are type II integral membrane proteins that are associated with peptide hormone receptors on the surface

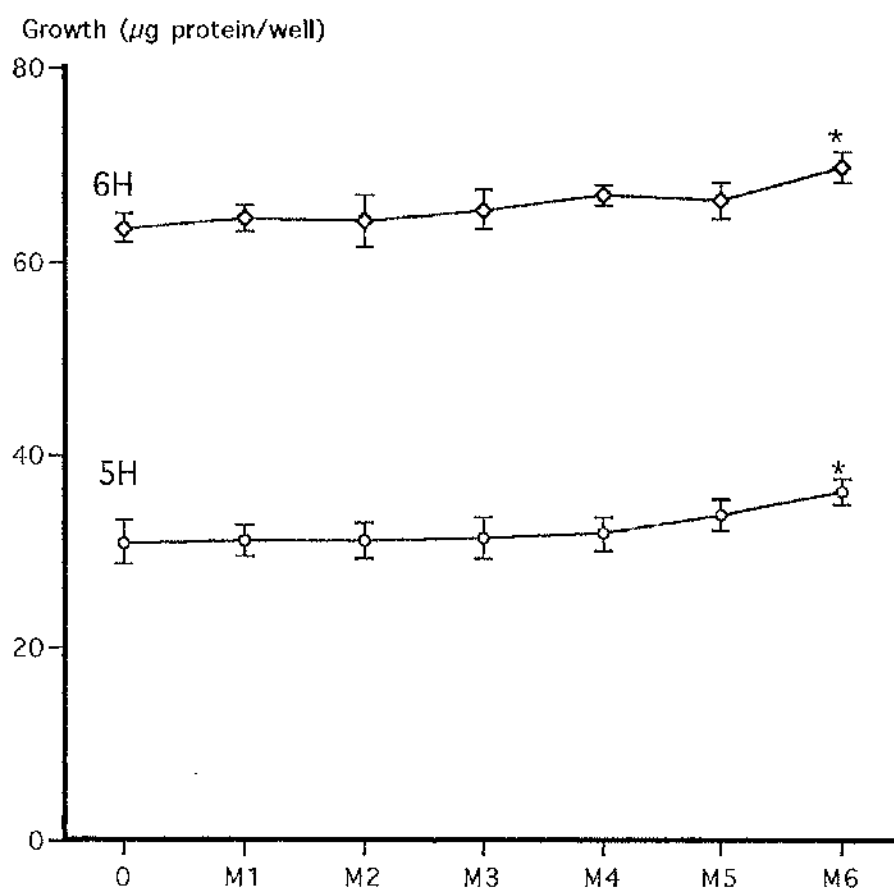


Figure 3.17: Effect of different concentrations of thyroid hormones on growth of FRTL-5 cells. Cells were incubated as described in figure 3.16.

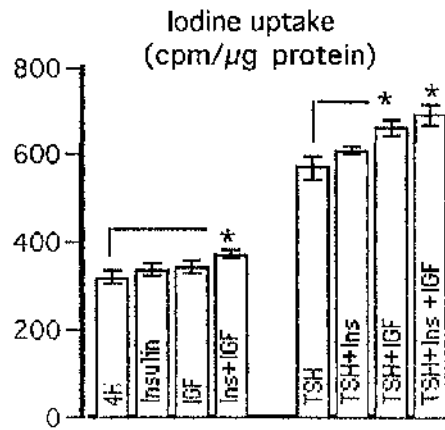


Figure 3.18: Iodine uptake of FRTL-5 cells in 4H medium stimulated with insulin, IGF-1, TSH, or combination of them. Cells were incubated for 72 h in 4H medium containing 0.5 % FCS, additional amino acids, and TSH, insulin, IGF-1 or combinations of them. The final concentrations of the stimulators used were 2 U/l of TSH, 10 mg/l of Insulin, and 100μg/l of IGF-1.

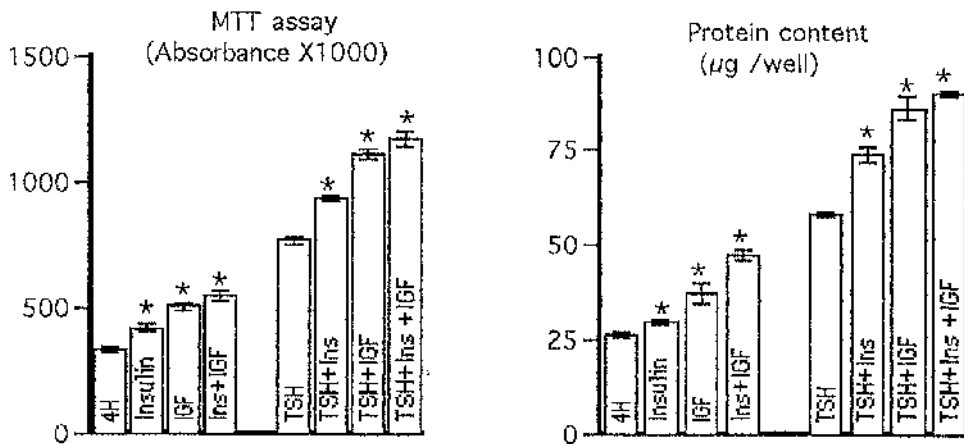


Figure 3.19: Comparison of estimation of growth rate by MTT assay and protein content. Cells were incubated as described in figure 3.18. Significant differences from control without growth factors are denoted by (*).

of lymphoid progenitor cells, monocytes, granulocytes, renal proximal tubular epithelial cells, small intestinal epithelium, biliary canaliculae, and certain solid tumour cell lines. In some experimental systems, they down regulate cellular responses to the peptide substrates (Shipp and Look, 1993). The inhibitors were used to test whether these proteases are present on the thyrocytes or not, and if present, inhibitions of them have a significant effect on the responses of FRTL-5 cells.

In Graves' disease, thyroid cells are under the influence of both thyroid hormones and thyroid stimulating antibodies, but few studies have tested the effects of thyroid hormones on stimulation of thyroid cells in vitro. Experiments were therefore planned to test stimulators in the presence of thyroid hormones.

The protocol was similar for all the experiments to be described below. Cells were incubated in 12-well plates for 3 days in medium containing TSH and then deprived of TSH for 5 days. They were incubated for 48 h in stimulation medium containing 0.5 % FCS and amino acids, with or without 2 U/l of TSH and thyroid hormones. After third incubation, cells were stimulated for 48 h with mediators, in stimulation medium containing 0.5 % FCS, additional amino acids, TSH (2 U/l), with or without thyroid hormones (1 $\mu\text{mol/l}$ T_4 and 10 nmol/l T_3).

Although it had been shown that IGF-1 was capable of stimulating the cells in the absence of insulin, insulin was included in the medium at a concentration of 10 mg/l. Wells without stimulator were used as controls. Iodine uptake and protein content were assayed as described in sections 3.2.2 and 3.2.4.

Experiments using Protease inhibitors and thyroid hormones used a single dose of stimulator. Protease inhibitors (1 $\mu\text{mol/l}$ each of bestatin and thiorphan) were included only at the stimulation stage, while thyroid hormones (1 $\mu\text{mol/l}$ T_4 , 10 nmol/l T_3) were preincubated with TSH-stimulated cells for 48 h, before stimulation with a mixture of thyroid hormones and TSH.

3.3.6.1 Insulin Growth Factor-1

3.3.6.1.1 Dose study

Doses of 1 to 1000 $\mu\text{g/ml}$ were used. A dose of 500 $\mu\text{g/ml}$ (highest significant effective dose, in dose study experiment) was used for studies of inhibitors and thyroid hormones.

Doses of 50 $\mu\text{g/l}$ of IGF-1 and above increased iodine uptake significantly in the absence of TSH, to a maximum of 18.5 % more than control at a concentration of 1000 $\mu\text{g/ml}$. There were no significant differences between successive doses from 10 to 1000 $\mu\text{g/l}$ of IGF-1. Doses above 5 $\mu\text{g/ml}$ also caused a significant increase in

growth rate, to a maximum of 24.8 % more than control at the highest dose (figure 3.20).

In the presence of 2 U/l of TSH, doses of IGF-1 of 50 $\mu\text{g/l}$ and above increased iodine uptake significantly, to a maximum of 38.7 % more than control at 1000 $\mu\text{g/ml}$. All doses above 5 $\mu\text{g/l}$ also caused a significant increase in growth of cells. The highest increase of 16.1 % was caused by 500 $\mu\text{g/l}$ of IGF-1. There were no significant differences among increases in growth caused by successive doses above 10 $\mu\text{g/l}$.

3.3.6.1.2 *Protease Inhibitors*

The inclusion of bestatin and thiorphan alone or with TSH failed to effect iodine uptake or growth of cells. The effects of these inhibitors on iodine uptake and growth of FRTL-5 cells were repeated on at least 35 different occasions during the stimulation experiments, and similar results were obtained.

The protease inhibitors did not alter the effect of IGF-1 on either iodine uptake or growth, either in the presence or the absence of TSH. The summaries of these results are presented in figure 3.21.

3.3.6.1.3 *Thyroid Hormones*

In the presence of T_4 and T_3 , IGF-1 failed to increase iodine uptake significantly. In the absence of thyroid hormones the same dose of IGF-1 produced an increase of 37.29 % in the rate of iodine uptake (figure 3.22). On the other hand, there was no significant change in the stimulation of growth caused by IGF-1 when thyroid hormones were added (17.33 % increase compared to 16.10 % when thyroid hormones were absent). This indicates that the thyroid hormones partially antagonised the effect of IGF-1.

3.3.6.2 Transforming Growth Factor- β (TGF)

3.3.6.2.1 *Dose Study*

Doses of 1 to 100 $\mu\text{g/l}$ were used. A dose of 50 $\mu\text{g/l}$ was chosen for further study.

TGF- β increased iodine uptake at all doses in the absence of TSH when compared with control. The increase associated with 2.5 $\mu\text{g/l}$ of TGF- β was 18.2 %

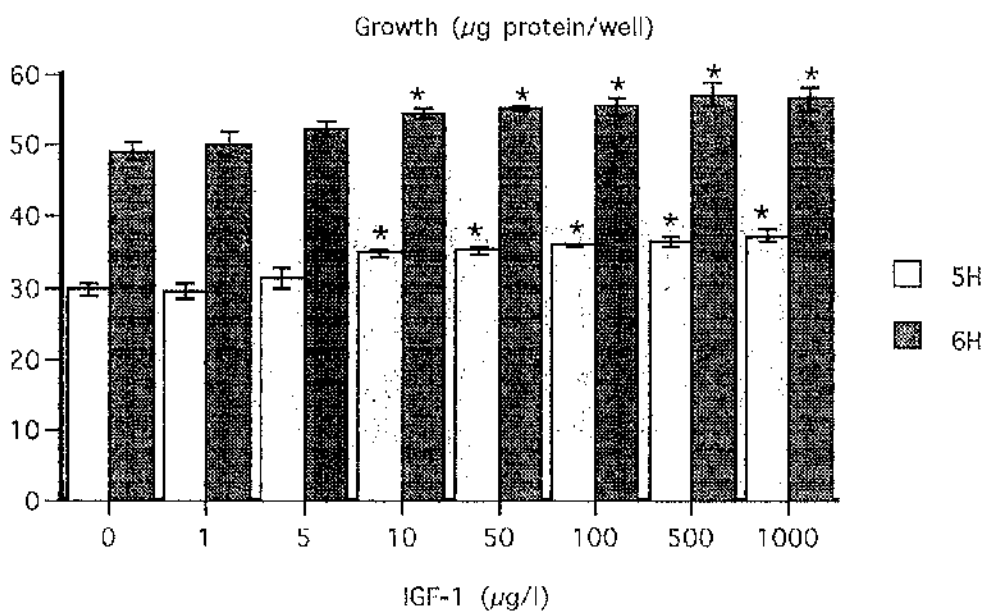
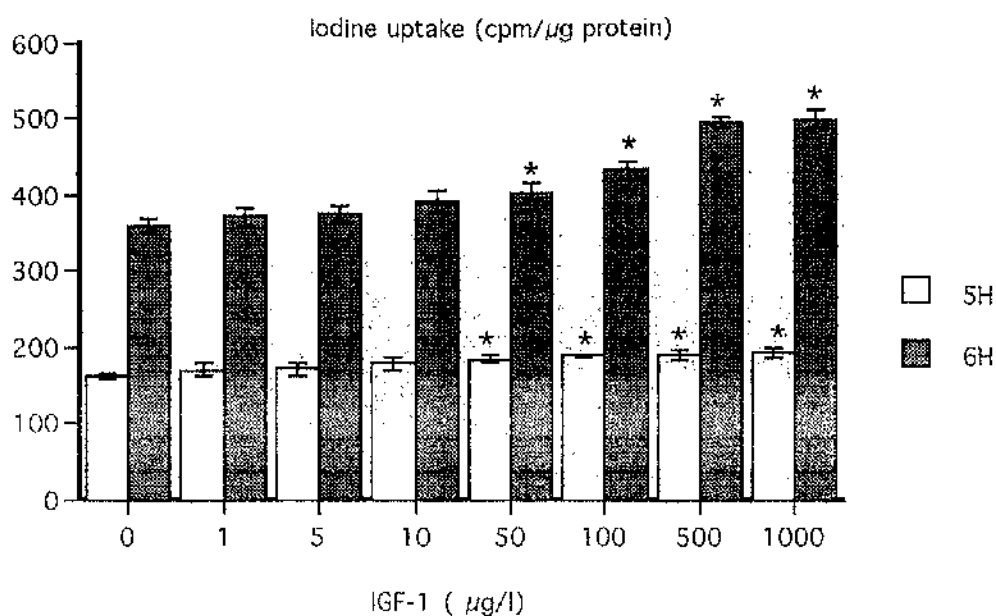


Figure 3.20: Effect of IGF-1 on iodine uptake and growth of FRTL-5 cells. Cells were incubated in 5H or 6H (2U/l of TSH) medium containing 0.5 % FCS and additional amino acids for 48 h. Significant differences between stimulated and unstimulated cells (control or zero concentration) are shown by *.

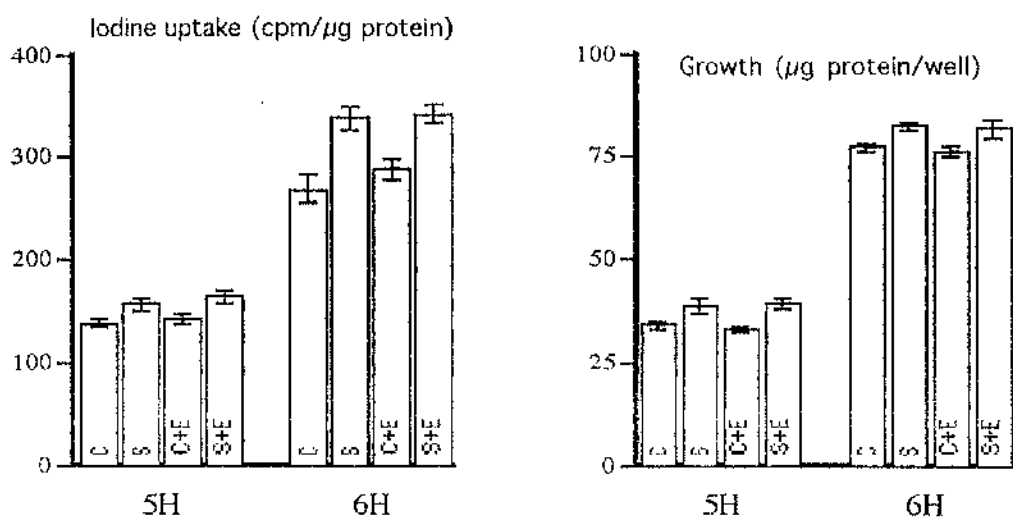


Figure 3.21: Effect of protease inhibitors on stimulation of FRTL-5 cells with IGF-1. Cells were incubated as before, without IGF-1 (C), with 500 $\mu\text{g/l}$ of IGF-1 (S), or with a mixture of 1 $\mu\text{mol/l}$ of bestatin and 1 $\mu\text{mol/l}$ of thiorphan in the absence (E) or the presence of IGF-1 (S+E).

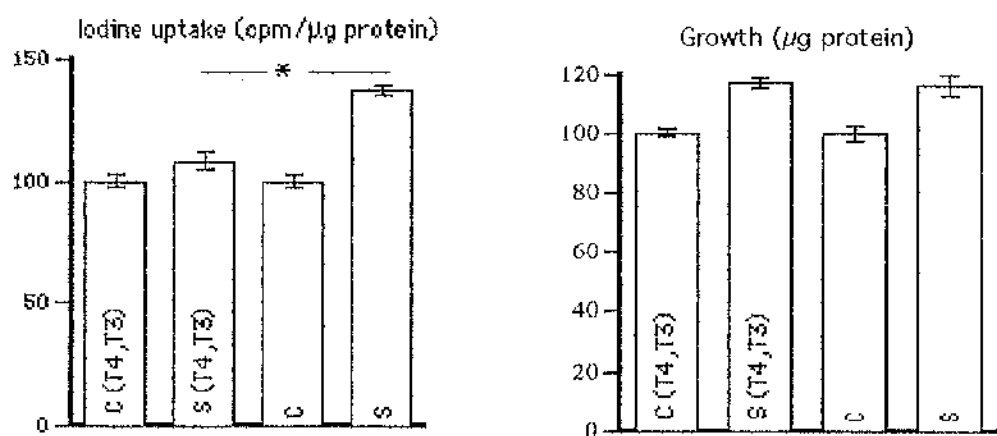


Figure 3.22: Effect of thyroid hormones on stimulation of FRTL-5 cells with IGF-1 and TSH. Cells were stimulated in 6H medium (2 U/l of TSH) as before, without IGF-1 (C), with 500 $\mu\text{g/l}$ of IGF-1 (S), or stimulated in the 6H medium containing 0.5 % FCS, additional amino acids, 1 $\mu\text{mol/l}$ of T₄ and 10 nmol/l of T₃, 48 h before and 48 h after addition of IGF-1 [S (T₄, T₃)]. The cells that were stimulated only with thyroid hormones for 96 h served as control [C (T₄, T₃)] for this set. Significant differences between presence and absence of thyroid hormones are shown by *.

higher than control, while the maximum increase was seen with 50 $\mu\text{g/l}$ of TGF- β , 28.0 % more than control. However, a clear dose-response curve could not be demonstrated. TGF- β had no effect on growth rate (figure 3.23).

In the presence of TSH, all doses of TGF- β caused a significant increase in iodine uptake. At 2.5 $\mu\text{g/l}$ of TGF- β , the increase was 18.5 %, while the maximum increase of 64.4 % was associated with 50 $\mu\text{g/l}$. All doses of TGF- β also decreased the growth of cells. At 5 $\mu\text{g/l}$ of TGF- β , the decrease was 11.4 %. The maximum decrease in growth of 27.7 % was associated with 50 $\mu\text{g/l}$ of TGF- β .

3.3.6.2.2 *Protease Inhibitors*

The inclusion of bestatin and thiorphan did not alter the effect of TGF- β on iodine uptake by the cells in the absence of TSH and did not alter the effects on growth rate either in the absence or the presence of TSH (figure 3.24). However, bestatin and thiorphan caused a significant ($p < 0.05$) reduction in the effect of TGF- β on iodine uptake (40.0 % decrease compared to 59.4 %).

3.3.6.2.3 *Thyroid Hormones*

Thyroid hormones significantly ($p < 0.01$) suppressed the increase in iodine uptake in the presence of TGF- β . The effect was reduced from an increase of 64.4 % to only 27.5 %. The effect on growth was also antagonised (figure 3.25).

3.3.6.3 Basic Fibroblast Growth Factor (bFGF)

3.3.6.3.1 *Dose Study*

Doses of bFGF from 1 to 100 $\mu\text{g/l}$ were used. A dose of 10 $\mu\text{g/l}$ was chosen for further study.

Doses of bFGF from 2.5 $\mu\text{g/l}$ significantly decreased the iodine uptake compared to unstimulated cells in a dose dependent manner. The decrease caused by 2.5 $\mu\text{g/l}$ of bFGF was 27.3 %. The maximum decrease was 45.4 % lower than the control, at 25 $\mu\text{g/l}$ of bFGF, and there were no further decreases with higher doses. Addition of bFGF also caused an increase in growth of cells. A dose of 2.5 $\mu\text{g/l}$ caused an increase of 29.6 %, and the maximum increase in growth was observed with a concentration of 10 $\mu\text{g/l}$, 53.9 % more than control. At higher concentrations, the effect was less than at 10 $\mu\text{g/l}$, but still significant.

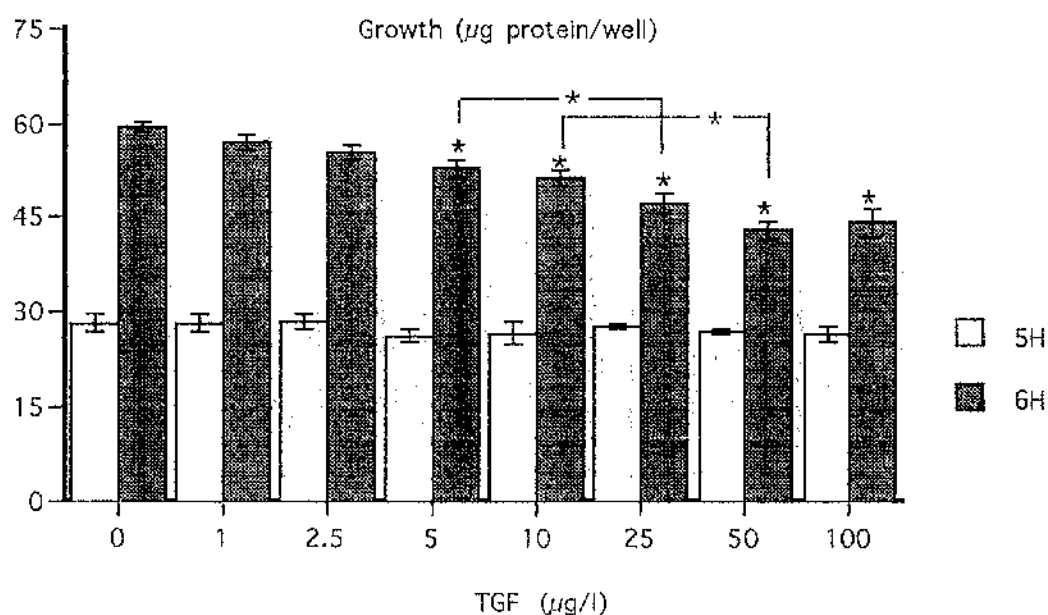
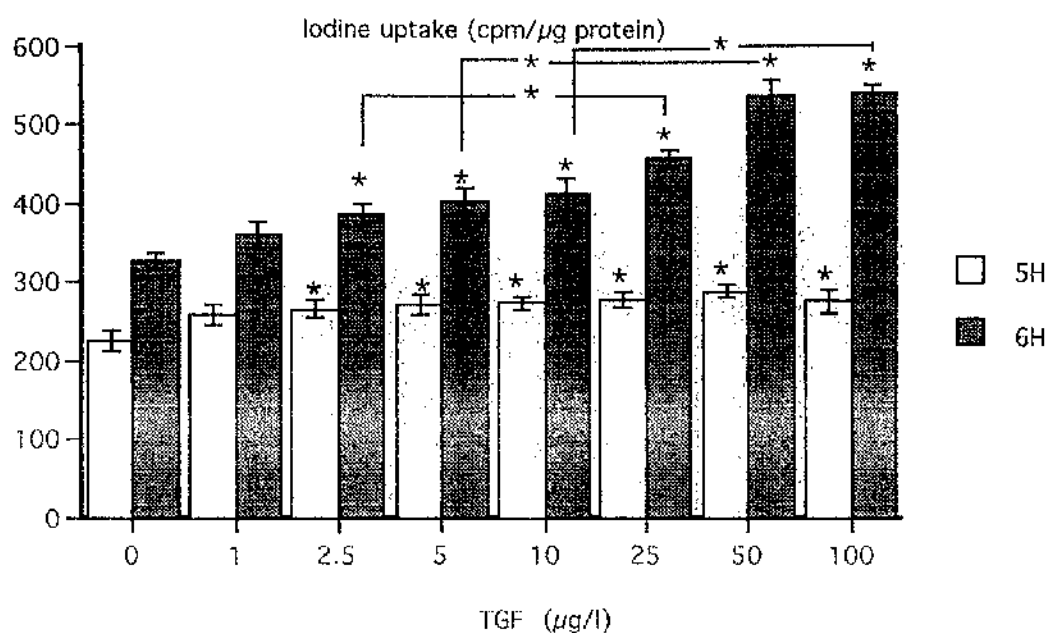


Figure 3.23: Effect of TGF- β on iodine uptake and growth of FRTL-5 cells. Cells were incubated in 5H or 6H (2U/l of TSH) medium containing 0.5 % FCS and additional amino acids for 48 h. Significant differences are shown by *.

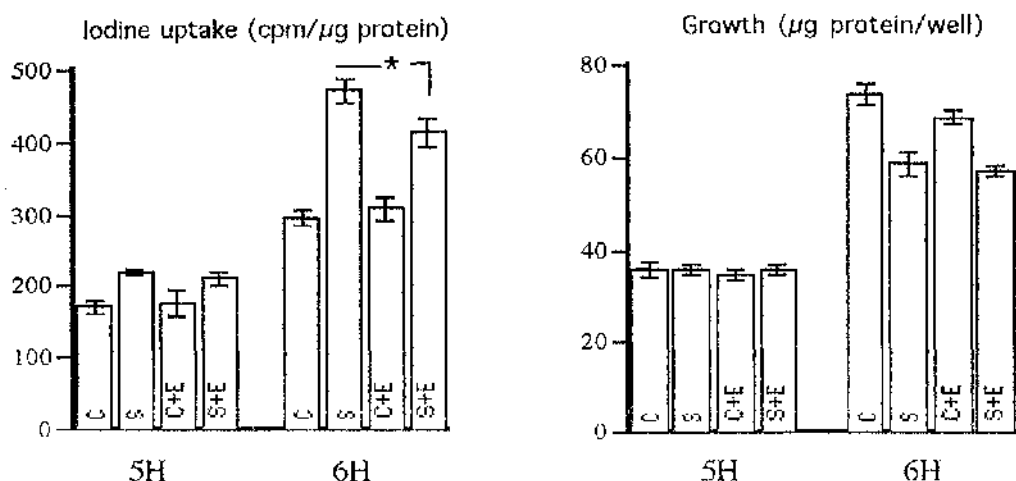


Figure 3.24: Effect of protease inhibitors on stimulation of FRTL-5 cells with TGF- β . Cells were incubated as before, without TGF- β (C), with 50 $\mu\text{g/l}$ of TGF- β (S), or with a mixture of 1 $\mu\text{mol/l}$ of bestatin and 1 $\mu\text{mol/l}$ of thiorphan in the absence (E) or the presence of TGF- β (S+E). Significant differences caused by inhibitors are represented by (*).

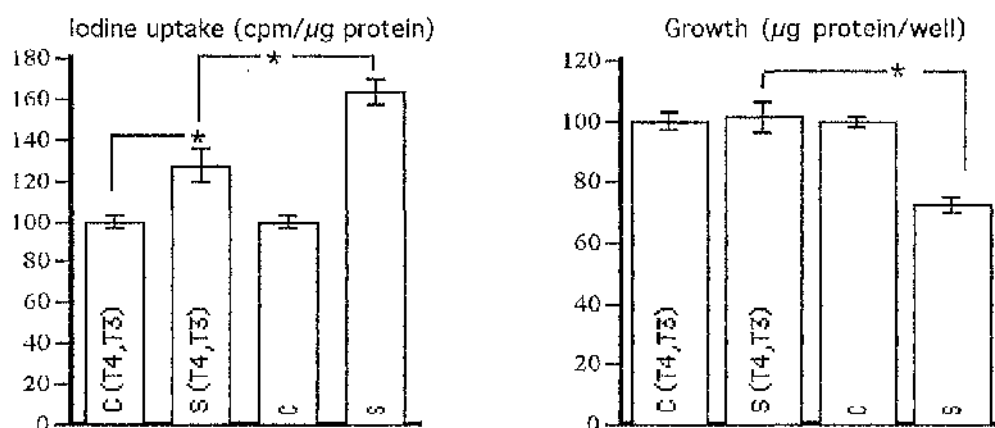


Figure 3.25: Effect of thyroid hormones, on stimulation of FRTL-5 cells with TGF- β and TSH. Cells were stimulated in 6H medium (2 U/l of TSH) as before, without TGF- β (C), with 50 $\mu\text{g/l}$ of TGF- β (S), or stimulated in the 6H medium containing 0.5 % FCS, additional amino acids, 1 $\mu\text{mol/l}$ of T₄ and 10 nmol/l of T₃, 48 h before and 48 h after addition of TGF- β [S (T₄, T₃)]. The cells that were stimulated only with thyroid hormones for 96 h served as control [C (T₄, T₃)] for this set. Significant differences between the presence and the absence of thyroid hormones are shown by (*).

In the presence of TSH, all doses greater than 1 $\mu\text{g/l}$ caused a significant decrease in iodine uptake. The maximum decrease of 39.1 % was caused by 10 $\mu\text{g/l}$ of bFGF (figure 3.26). All doses of bFGF increased the growth of cells, to a maximum of 18.5 % at 100 $\mu\text{g/l}$. Statistically there were no differences between the growth rates at 2.5 $\mu\text{g/l}$ of bFGF and above.

3.3.6.3.2 *Protease Inhibitors*

The inclusion of bestatin and thiorphan in 5H or 6H medium did not alter the effect of bFGF on iodine uptake of cells (figure 3.27). However, they antagonised the effects of FGF on growth of the cells unstimulated with TSH. The growth stimulation was only 19.5 % compared to 51.6 % in the absence of inhibitors. In the presence of TSH, the antagonistic effect failed to reach significance (7.7 % increase compared to 16.3 %).

3.3.6.3.3 *Thyroid Hormones*

Thyroid hormones did not significantly alter the effects of bFGF on either iodine uptake or growth rate of the cells (figure 3.28).

3.3.6.4 Endothelin-1 (ET-1)

3.3.6.4.1 *Dose Study*

The final concentrations used ranged from 0.5 to 100 nmol/l. A dose of 50 nmol/l was chosen for further study.

Addition of Endothelin-1 to medium without TSH did not change iodine uptake or growth rate of cells at any concentration (figure 3.29).

In the presence of 2 U/l of TSH, concentrations of 50 nmol/l and above caused a significant increase in the iodine uptake of stimulated cells. The maximum increase of 15.07 % above control occurred at a concentration of 100 nmol/l. There were no significant differences among the values obtained for doses over 25 nmol/l.

Endothelin-1 also caused a significant increase in growth at concentrations of 25 nmol/l and above. The maximum increase of 9.78 % over control occurred at a concentration of 100 nmol/l. There were no significant differences between values obtained for doses of 25, 50, and 100 nmol/l.

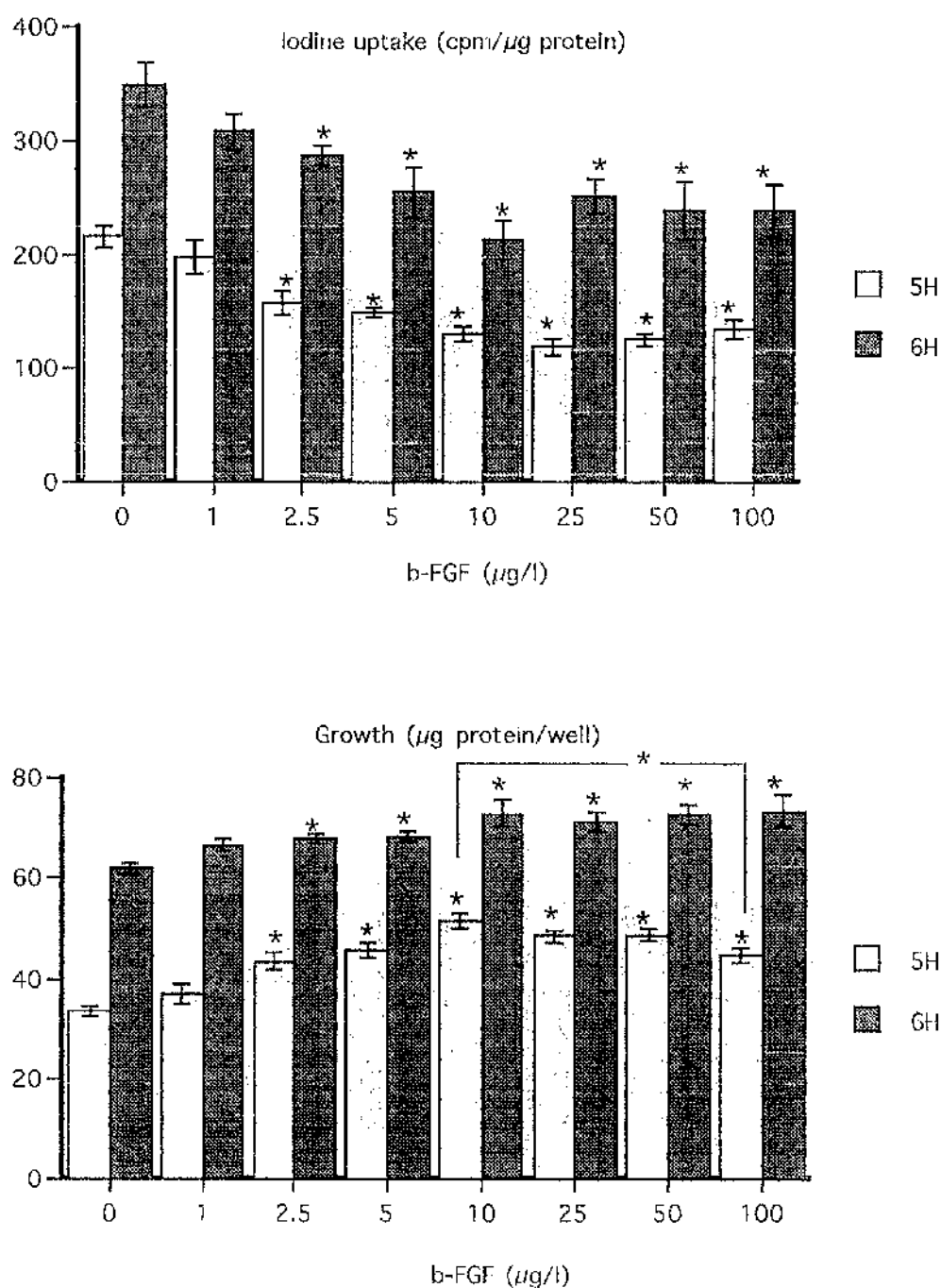


Figure 3.26: Effect of basic FGF on iodine uptake and growth of FRTL-5 cells. Cells were incubated in 5H or 6H (2U/l of TSH) medium containing 0.5 % FCS and additional amino acids for 48 h. Significant differences are shown by *.

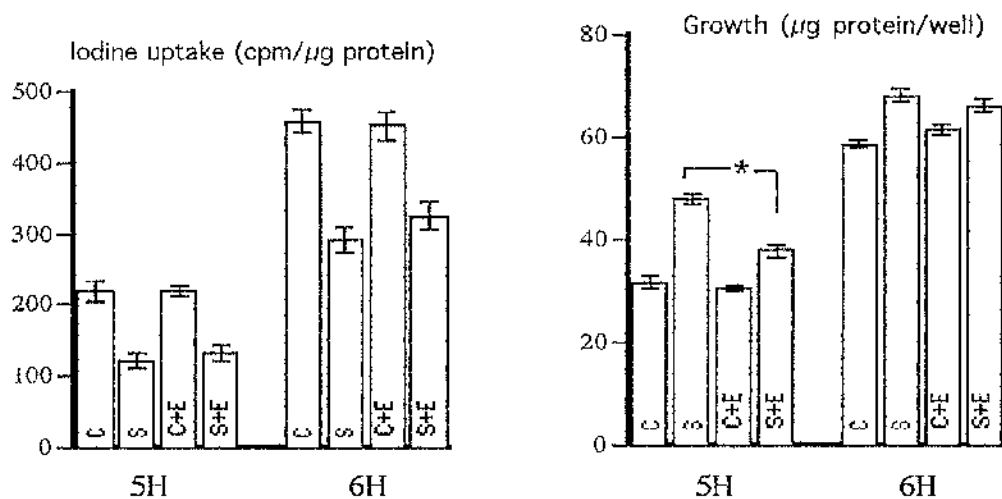


Figure 3.27: Effect of protease inhibitors, on stimulation of FRTL-5 cells with bFGF. Cells were incubated as before, without bFGF (C), with 10 $\mu\text{g/l}$ of bFGF (S), or with a mixture of 1 $\mu\text{mol/l}$ of bestatin and 1 $\mu\text{mol/l}$ of thiorphan in the absence (E) or the presence of bFGF (S+E). Significant differences caused by inhibitors are represented by (*).

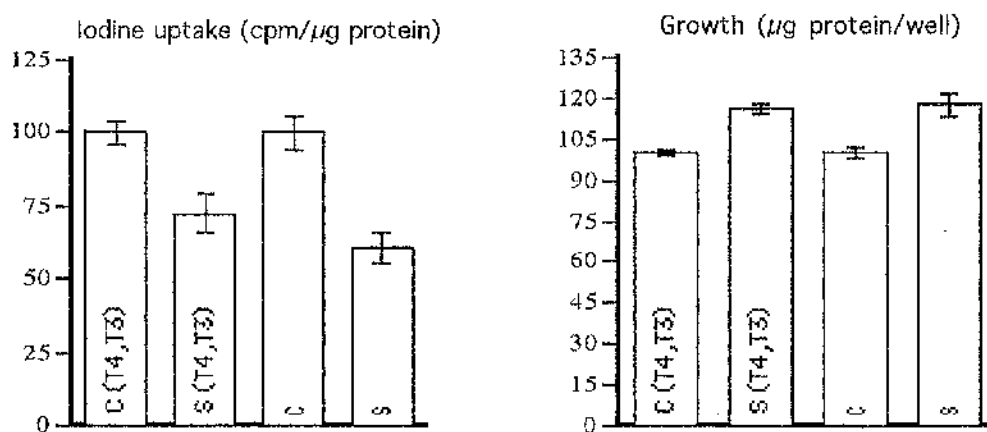


Figure 3.28: Effect of thyroid hormones, on stimulation of FRTL-5 cells with bFGF and TSH. Cells were stimulated in 6H medium (2 U/l of TSH) as before, without bFGF (C), with 10 $\mu\text{g/l}$ of bFGF (S), or stimulated in the 6H medium containing 0.5 % FCS, additional amino acids, 1 $\mu\text{mol/l}$ of T₄ and 10 nmol/l of T₃, 48 h before and 48 h after addition of bFGF [S (T₄, T₃)]. The cells that were stimulated only with thyroid hormones for 96 h served as control [C (T₄, T₃)] for this set.

3.3.6.4.2 *Protease Inhibitors*

Inclusion of bestatin and thiorphan in 5H medium did not alter the effect of Endothelin-1 on iodine uptake or growth rate of the cells.

In the presence of 2 U/l of TSH (6H), bestatin and thiorphan completely suppressed the stimulatory effect of endothelin-1 on the iodine uptake of cells (figure 3.30) but did not affect growth.

3.3.6.4.3 *Thyroid Hormones*

Thyroid hormones significantly ($p < 0.05$) antagonised the effect of Endothelin-1 on iodine uptake. The rate of uptake was decreased slightly (4.0 %) compared to an increase of 15.1 % in the absence of thyroid hormones. Thyroid hormones also antagonised the effect of Endothelin on growth (figure 3.31). There was a 3.5 % decrease in protein content compared to an increase of 9.2 %.

3.3.6.5 Interleukin-1 (IL-1 β)

3.3.6.5.1 *Dose Study*

The final concentrations of IL-1 used ranged from 0.5 to 20 $\mu\text{g/l}$. Concentrations of 1 $\mu\text{g/l}$ and 20 $\mu\text{g/l}$ were chosen for further study.

IL-1 did not alter the iodine uptake or growth rate of cells incubated in the absence of TSH at any dose.

In the presence of TSH, all doses of IL-1 above 2.5 $\mu\text{g/l}$ decreased iodine uptake significantly ($p < 0.001$). The maximum decrease, at 20 $\mu\text{g/l}$ of IL-1, was 29.84 %. This decrease was highly significant (figure 3.32). IL-1 did not affect growth rate.

3.3.6.5.2 *Protease Inhibitors*

The inclusion of bestatin and thiorphan did not alter the iodine uptake or growth of cells incubated in 5H or 6H media containing 1 or 20 $\mu\text{g/l}$ of IL-1 (figure 3.33).

3.3.6.5.3 *Thyroid Hormones*

Thyroid hormones caused a significant ($p < 0.01$) increase (23.6 %) in the rate of iodine uptake in the presence of 1 $\mu\text{g/l}$ of IL-1, compared to a non-significant

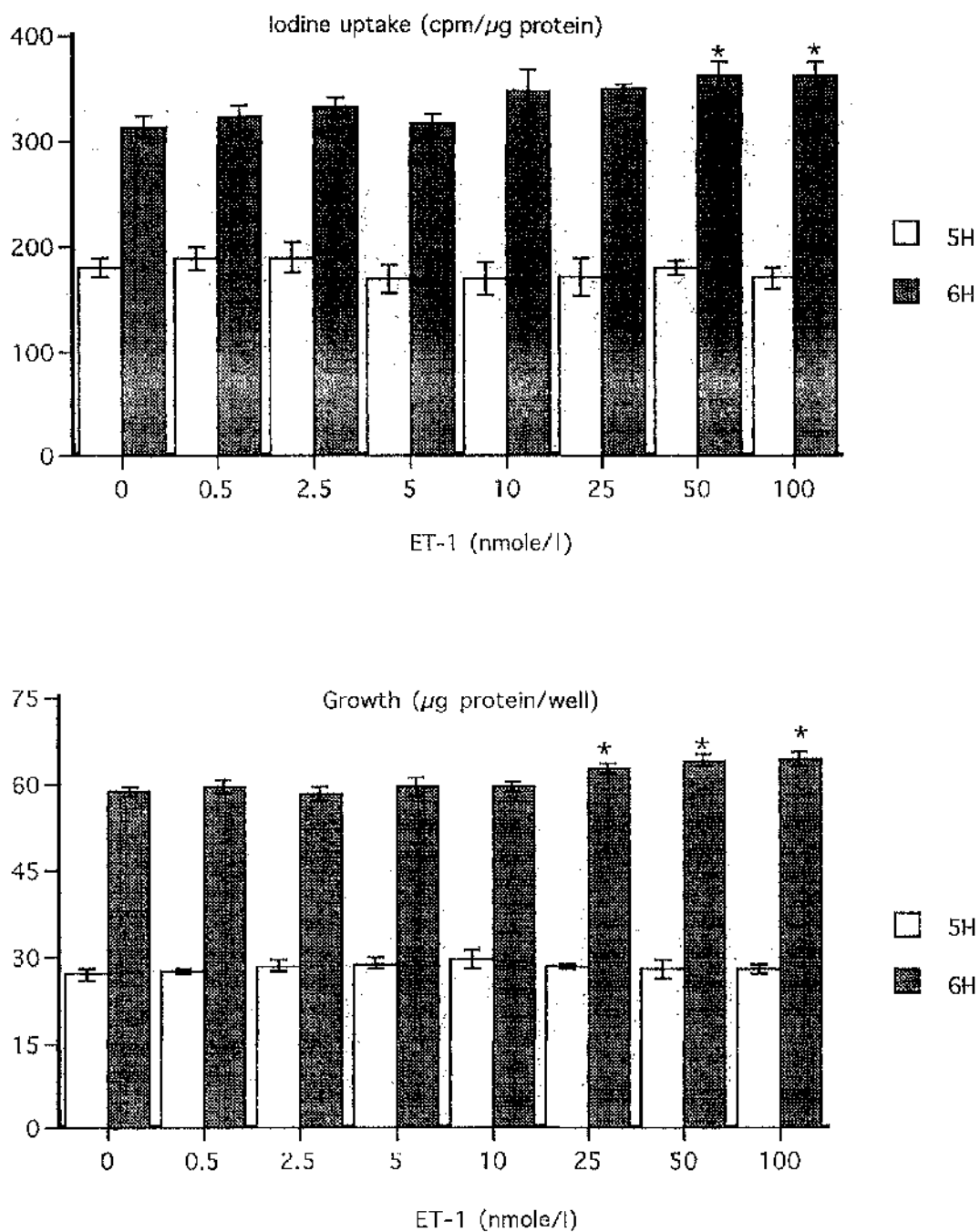


Figure 3.29: Effect of Endothelin-1 on iodine uptake and growth of FRTL-5 cells. Cells were incubated in 5H or 6H (2U/l of TSH) medium containing 0.5 % FCS and additional amino acids for 48 h. Significant differences between stimulated and unstimulated cells (control or zero concentration) are shown by *.

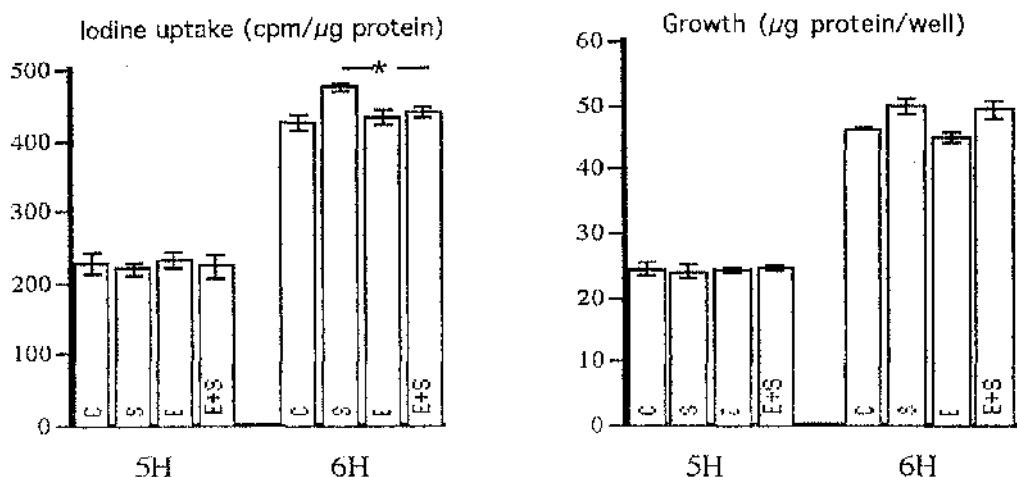


Figure 3.30: Effect of protease inhibitors, on stimulation of FRTL-5 cells with Endothelin-1. Cells were incubated as before, without Endothelin-1 (C), with 50 nmol/l of Endothelin-1 (S), or with a mixture of 1 μ mol/l of bestatin and 1 μ mol/l of thiorphan in the absence (E) or the presence of Endothelin-1 (S+E). Significant differences caused by inhibitors are represented by *.

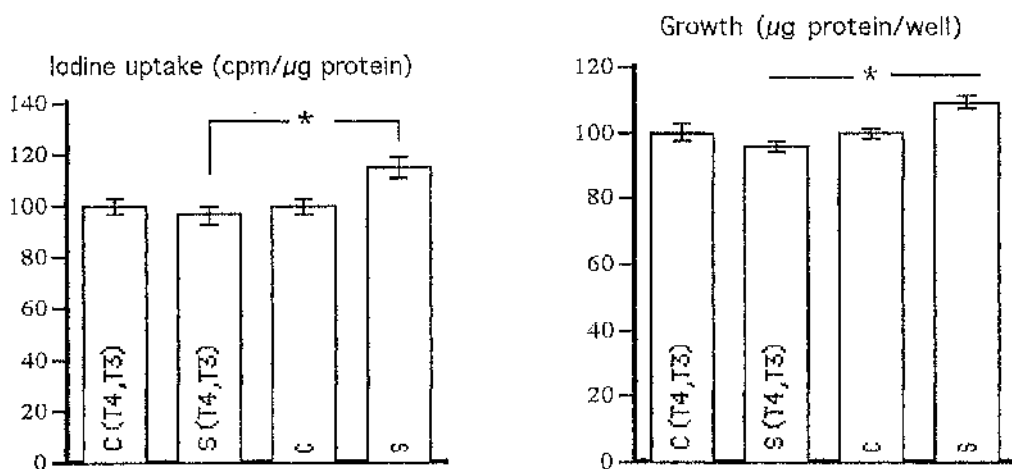


Figure 3.31: Effect of thyroid hormones, on stimulation of FRTL-5 cells with Endothelin-1 and TSH. Cells were stimulated in 6H medium (2 U/l of TSH) as before, without Endothelin-1 (C), with 50 nmol/l of Endothelin-1 (S), or stimulated in the 6H medium containing 0.5 % FCS, additional amino acids, 1 μ mol/l of T₄ and 10 nmol/l of T₃, 48 h before and 48 h after addition of Endothelin-1 [S (T₄, T₃)]. The cells that were stimulated only with thyroid hormones for 96 h served as control [C (T₄, T₃)] for this set. Significant differences that occurred in the presence and the absence of thyroid hormones are shown by *.

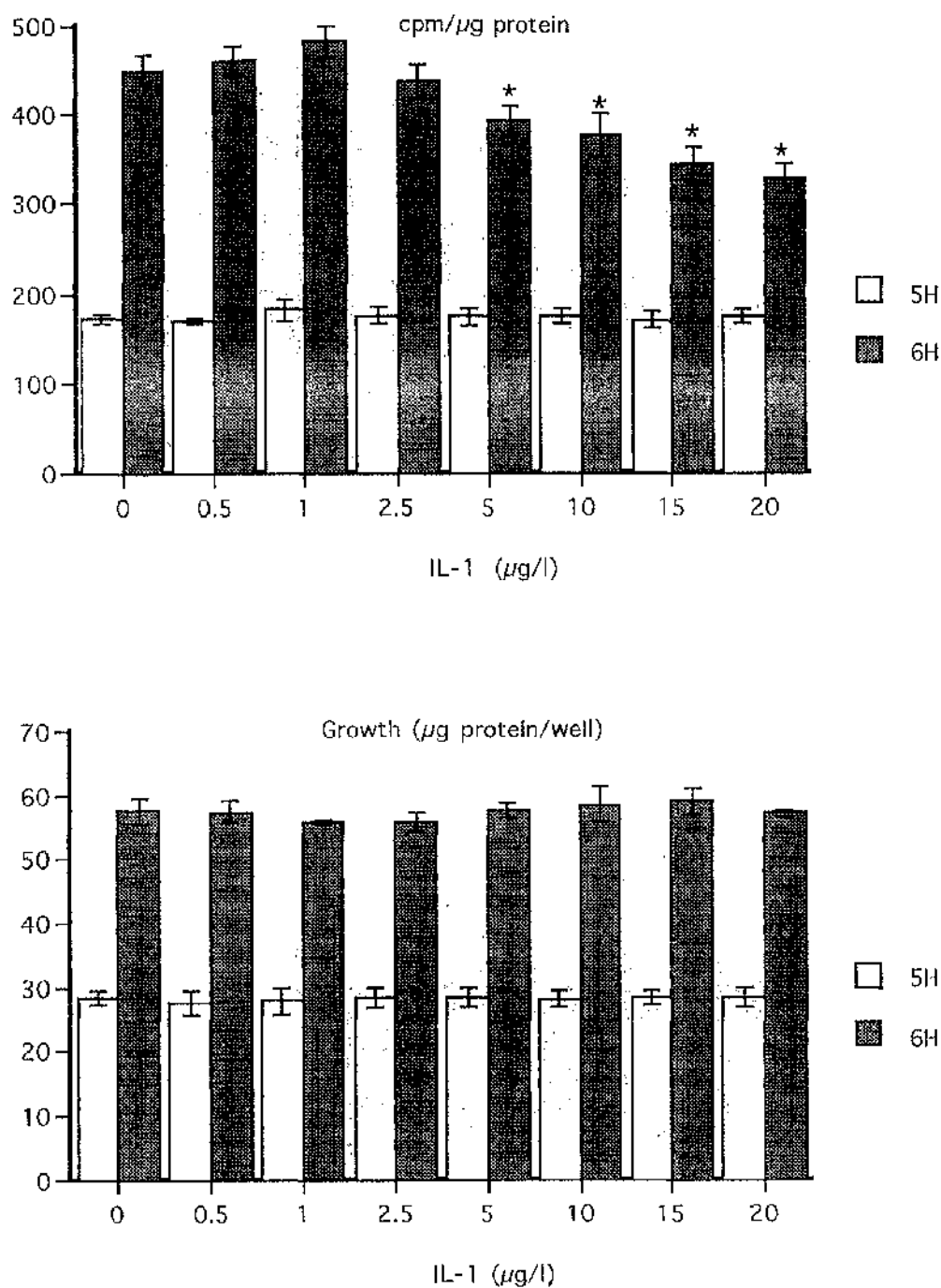


Figure 3.32: Effect of IL-1 on iodine uptake and growth of FRTL-5 cells. Cells were incubated in 5H or 6H (2U/l of TSH) medium containing 0.5 % FCS and additional amino acids for 48 h. Significant differences between stimulated and unstimulated cells (control or zero concentration) are shown by *.

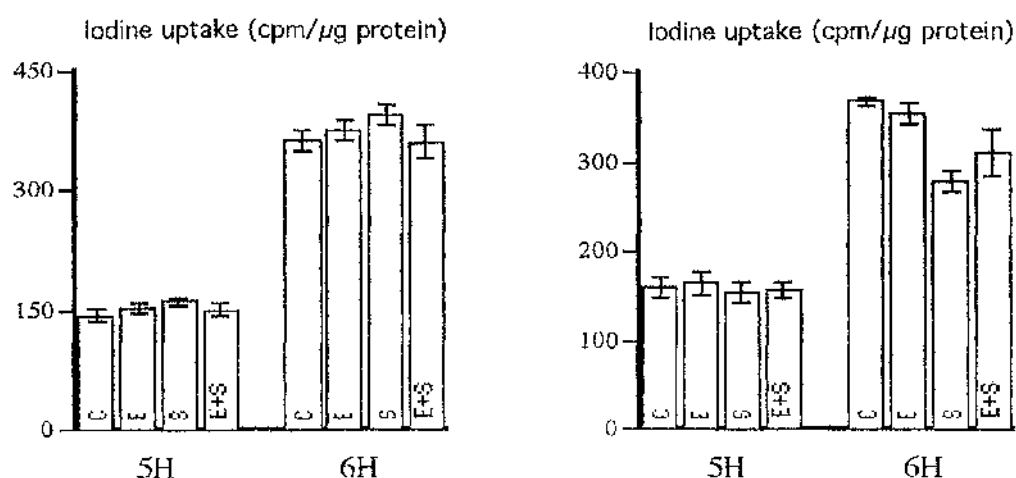


Figure 3.33: Effect of protease inhibitors, on stimulation of FRTL-5 cells with IL-1. Cells were incubated as before, without IL-1 (C), with 1 (left) or 20 (right) $\mu\text{g/l}$ of IL-1 (S), or with a mixture of 1 $\mu\text{mol/l}$ of bestatin and 1 $\mu\text{mol/l}$ of thiorphan in the absence (E) or the presence of IL-1 (S+E).

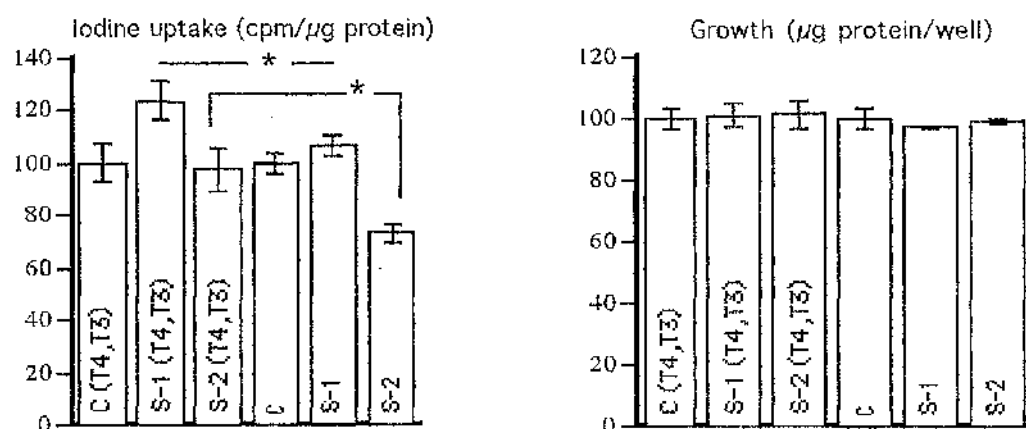


Figure 3.34: Effect of thyroid hormones, on stimulation of FRTL-5 cells with IL-1 and TSH. Cells were stimulated in 6H medium (2 U/l of TSH) as before, without IL-1 (C), with 1 (S-1) or 20 (S-2) $\mu\text{g/l}$ of IL-1, or stimulated in the 6H medium containing 0.5 % FCS, additional amino acids, 1 $\mu\text{mol/l}$ of T₄ and 10 nmol/l of T₃, 48 h before and 48 h after addition of IL-1 [S-1 (T₄, T₃), and S-2 (T₄, T₃) respectively]. The cells that were stimulated only with thyroid hormones for 96 h served as control [C (T₄, T₃)] for these sets. Significant differences that occurred in presence and absence of thyroid hormones are shown by *.

increase of 6.75% in the absence of thyroid hormones. IL-1 at 20 μ g/l did not affect iodine uptake in the presence of hormone, but it produced a significant decrease of 26.49 % in their absence. Therefore, in the presence of thyroid hormones, the iodine uptake of cells was higher (figure 3.34). Thyroid hormones did not affect the growth of cells.

3.3.6.6 Lipopolysaccharide (LPS)

3.3.6.6.1 Dose Study

The final concentrations of LPS used ranged from 0.1 to 10 mg/l. Concentrations of 0.1 and 10 mg/l were chosen for further study.

LPS caused significant decreases in iodine uptake of cells in the absence of TSH at concentrations of 2.5 mg/l and above. The maximum decrease of 25.1 % was obtained at a concentration of 10 mg/l. There were no significant differences among the doses of 2.5 to 10 mg/l. LPS did not affect growth rate.

In the presence of TSH, 0.1 mg/l of LPS caused a significant increase of 19.15 % in iodine uptake. On the other hand, significant decreases in iodine uptake occurred at concentration of 7.5 mg/l and 10 mg/l. The maximum decrease of 24.62 % occurred at a concentration of 10 mg/l (figure 3.35). Concentrations of 2.5 mg/l of LPS and above increased the growth rate significantly ($p < 0.05$). The maximum significant increase of 19.1 %, occurred at a concentration of 10 mg/l. There were no significant differences between the growth rates of cells stimulated with 5, 7.5 or 10 mg/l of LPS.

3.3.6.6.2 Protease Inhibitors

The inclusion of bestatin and thiorphan did not alter the effect of LPS on either iodine uptake or growth, either in the presence or the absence of TSH (figure 3.36).

3.3.6.6.3 Thyroid Hormones

Thyroid hormones did not significantly change the effects of LPS on iodine uptake, but completely suppressed the effect of 10 mg/l on growth (figure 3.37).

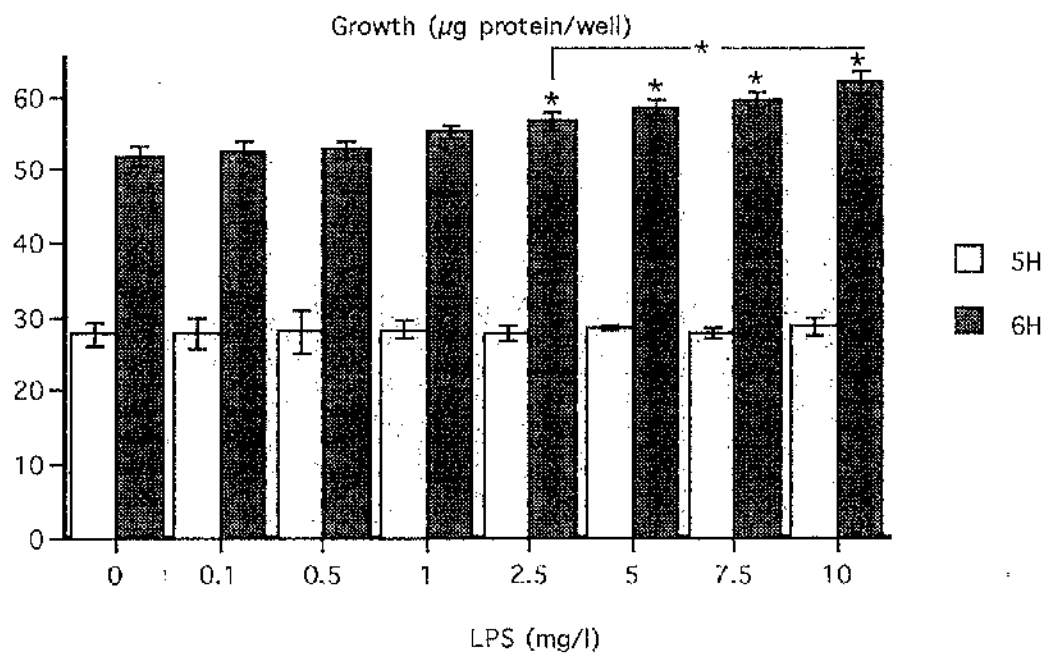
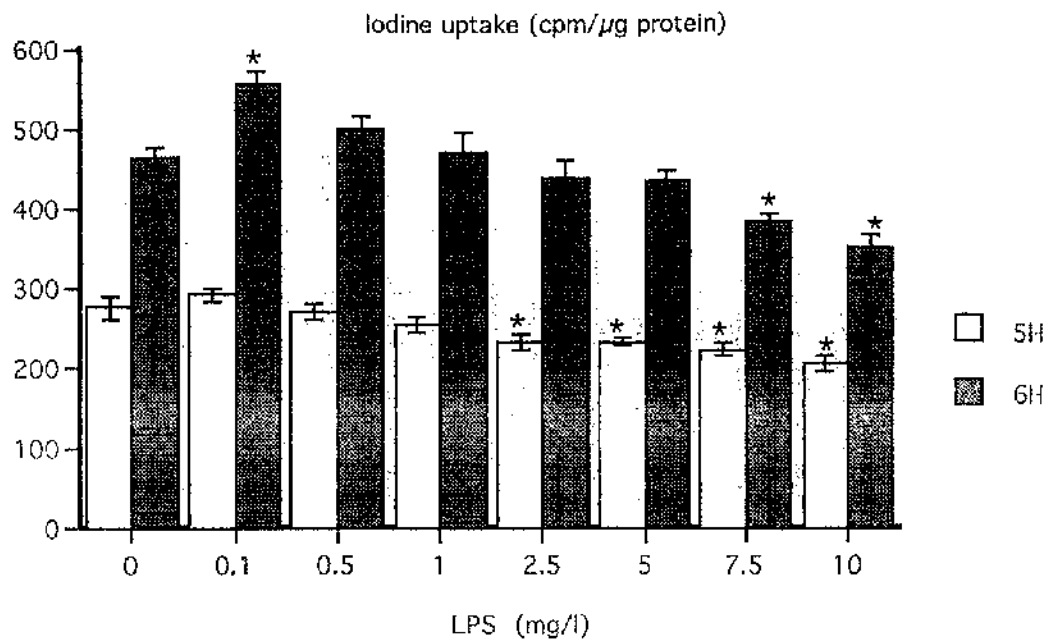


Figure 3.35: Effect of LPS on iodine uptake and growth of FRTL-5 cells. Cells were incubated in 5H or 6H (2U/l of TSH) medium containing 0.5 % FCS and additional amino acids for 48 h. Significant differences between stimulated and unstimulated cells (control or zero concentration) are shown by *.

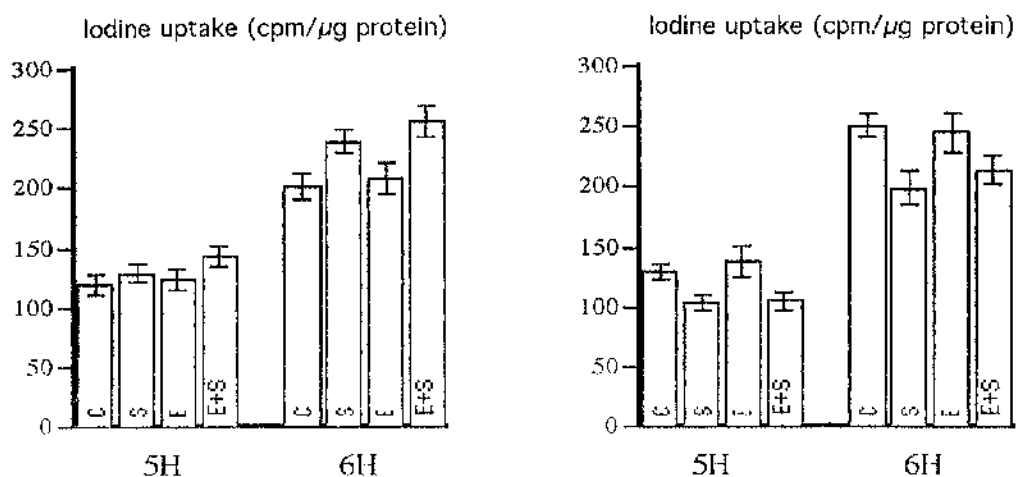


Figure 3.36: Effect of protease inhibitors, on stimulation of FRTL-5 cells with LPS. Cells were incubated as before, without LPS (C), with 0.1 (left) or 10 (right) mg/l of LPS (S), or with a mixture of 1 μ mol/l of bestatin and 1 μ mol/l of thiorphan in the absence (E) or the presence of LPS (S+E).

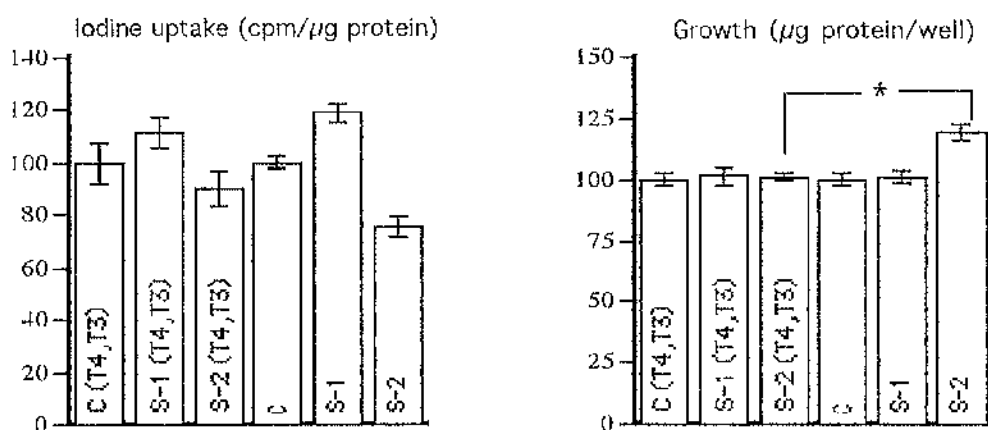


Figure 3.37: Effect of thyroid hormones, on stimulation of FRTL-5 cells with LPS and TSH. Cells were stimulated in 6H medium (2 U/l of TSH) as before, without LPS (C), with 0.1 (S-1) or 10 (S-2) mg/l of LPS, or stimulated in the 6H medium containing 0.5 % FCS, additional amino acids, 1 μ mol/l of T₄ and 10 nmol/l of T₃, 48 h before and 48 h after addition of LPS [S-1 (T₄, T₃), and S-2 (T₄, T₃) respectively]. The cells that were stimulated only with thyroid hormones for 96 h served as control [C (T₄, T₃)] for these sets. Significant differences between the presence and absence of thyroid hormones is shown by *.

3.3.6.7 Interleukin-4 (IL-4)

3.3.6.7.1 Dose Study

The final concentrations of IL-4 used ranged from 0.5 to 20 $\mu\text{g/l}$. A concentration of 20 $\mu\text{g/l}$ was chosen for further study.

In the presence or absence of TSH, the iodine uptake of cells did not change when IL-4 was added. IL-4 also had no effect on growth in the absence of TSH, but did cause decreases in growth in the presence of TSH. The maximum decrease was 23.6 %, at 20 $\mu\text{g/l}$ (figure 3.38).

3.3.6.7.2 Protease Inhibitors

The inclusion of bestatin and thiorphan did not alter the effects of IL-4 on either iodine uptake or growth, either in the presence or the absence of TSH (figure 3.39).

3.3.6.7.3 Thyroid Hormones

Exposure of cells to thyroid hormones did not change the rate of iodine uptake, but prevented IL-4 from decreasing the growth rate (figure 3.40).

3.3.6.8 Interleukin-6 (IL-6)

3.3.6.8.1 Dose Study

The final concentrations of IL-6 used ranged from 0.5 to 20 $\mu\text{g/l}$. A concentration of 20 $\mu\text{g/l}$ was chosen for further study.

In the absence of TSH, iodine uptake and growth rate of cells increased significantly only at 20 $\mu\text{g/l}$ of IL-6. These increases were 17.0 % and 26.6 % respectively.

In the presence of TSH, the iodine uptake of stimulated cells increased significantly ($p < 0.01$) with the doses of IL-6 above 10 $\mu\text{g/l}$. The highest increase was caused by 20 $\mu\text{g/l}$ of IL-6, that was 33.4 % higher than the control. There were no significant differences between the values obtained for iodine uptake of doses above 10 $\mu\text{g/l}$ when they were compared with each other (figure 3.41). IL-6 did not produce any changes in the growth of cells.

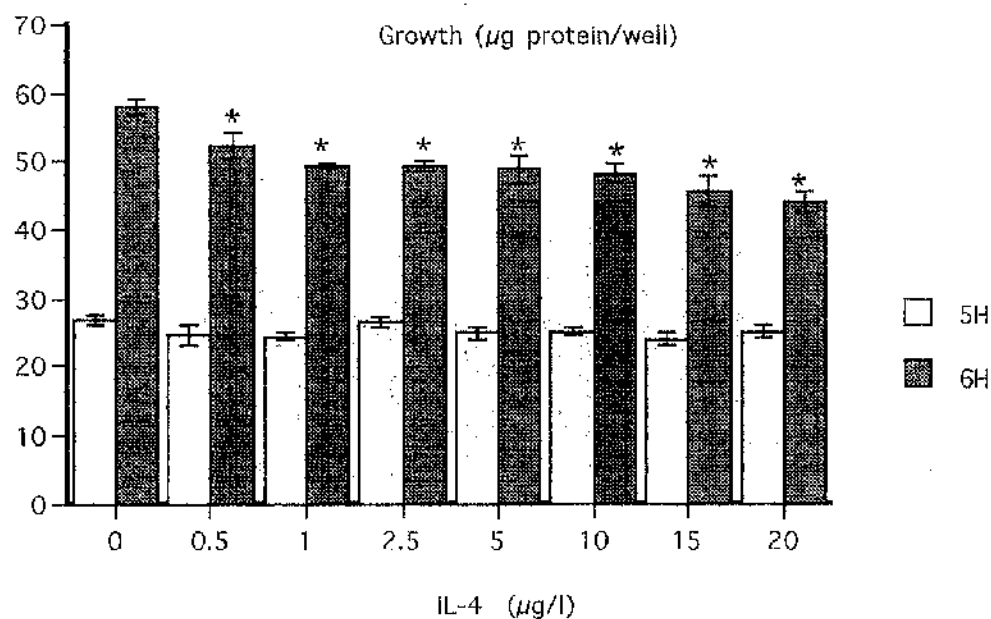
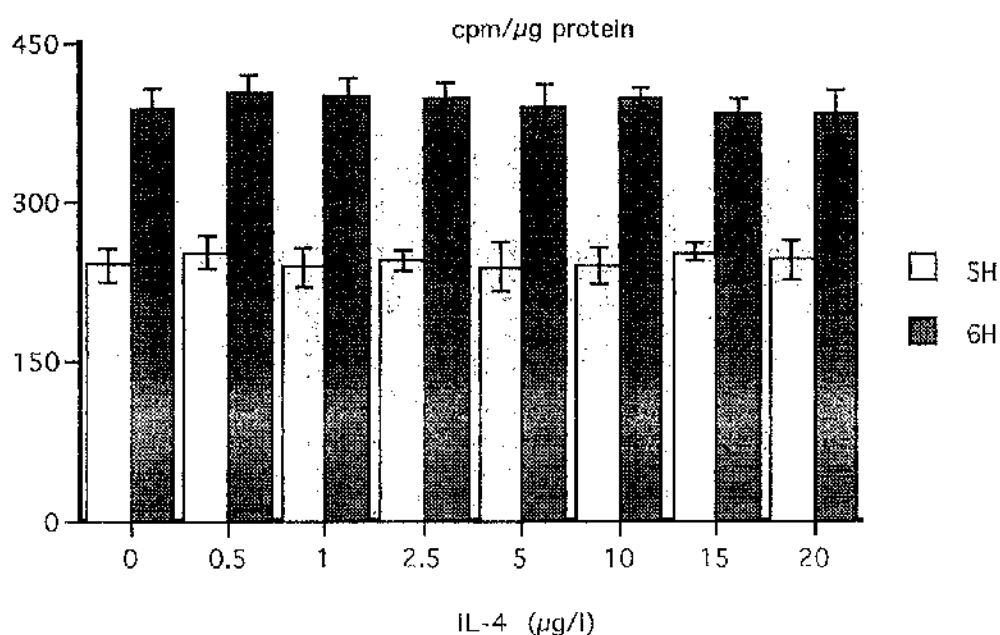


Figure 3.38: Effect of IL-4 on iodine uptake and growth of FRTL-5 cells. Cells were incubated in 5H or 6H (2U/l of TSH) medium containing 0.5 % FCS and additional amino acids for 48 h. Significant differences between stimulated and unstimulated cells (control or zero concentration) are shown by *.

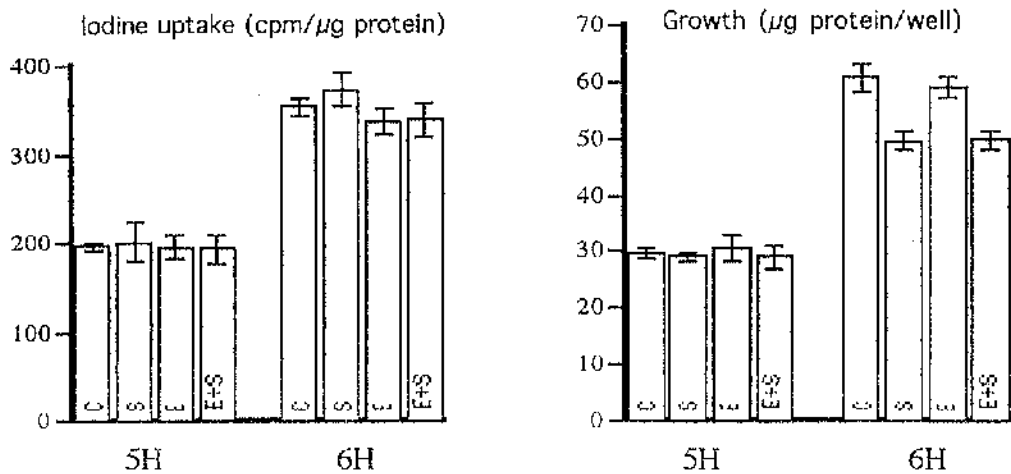


Figure 3.39: Effect of protease inhibitors, on stimulation of FRTL-5 cells with IL-4. Cells were incubated as before, without IL-4 (C), with 20 $\mu\text{g/l}$ of IL-4 (S), or with a mixture of 1 $\mu\text{mol/l}$ of bestatin and 1 $\mu\text{mol/l}$ of thiorphan in the absence (E) or the presence of IL-4 (S+E).

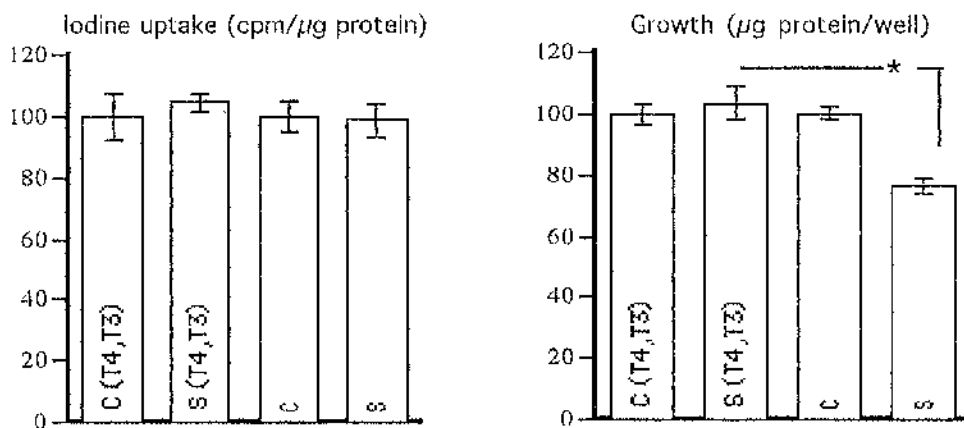


Figure 3.40: Effect of thyroid hormones, on stimulation of FRTL-5 cells with IL-4 and TSH. Cells were stimulated in 6H medium (2 U/l of TSH) as before, without IL-4 (C), with 20 $\mu\text{g/l}$ of IL-4 (S), or stimulated in the 6H medium containing 0.5 % FCS, additional amino acids, 1 $\mu\text{mol/l}$ of T₄ and 10 nmol/l of T₃, 48 h before and 48 h after addition of IL-4 [S (T₄, T₃)]. The cells that were stimulated only with thyroid hormones for 96 h served as control [C (T₄, T₃)] for this set. Significant differences between the presence and absence of thyroid hormones is shown by *.

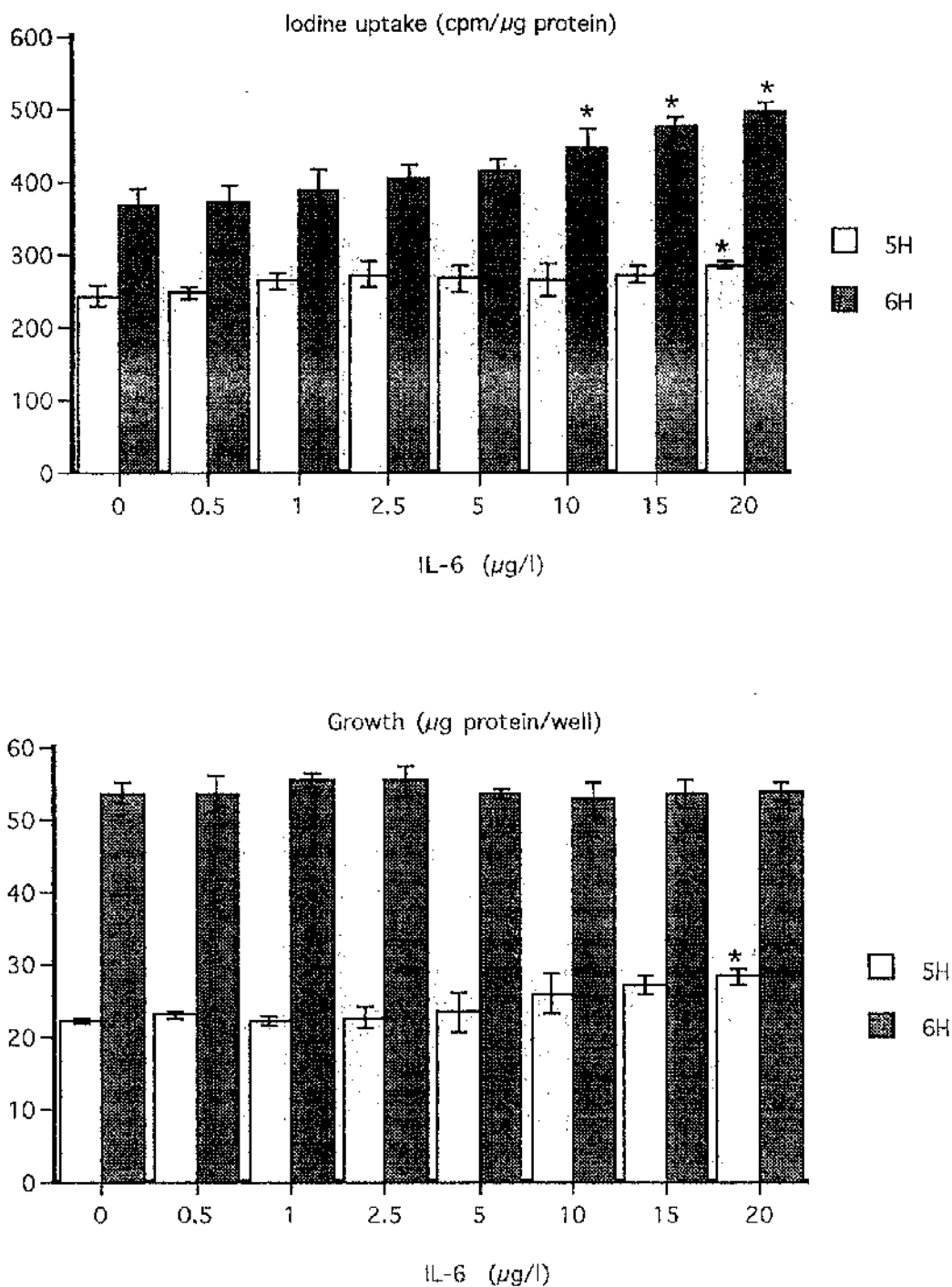


Figure 3.41: Effect of IL-6 on iodine uptake and growth of FRTL-5 cells. Cells were incubated in 5H or 6H (2U/l of TSH) medium containing 0.5 % FCS and additional amino acids for 48 h. Significant differences between stimulated and unstimulated cells (control or zero concentration) are shown by *.

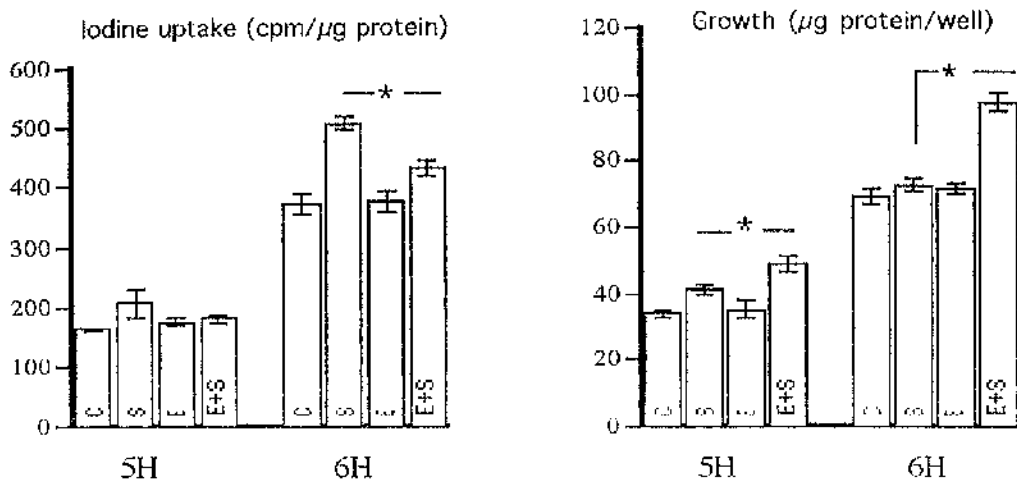


Figure 3.42: Effect of protease inhibitors, on stimulation of FRTL-5 cells with IL-6. Cells were incubated as before, without IL-6 (C), with 20 $\mu\text{g/l}$ of IL-6 (S), or with a mixture of 1 $\mu\text{mol/l}$ of bestatin and 1 $\mu\text{mol/l}$ of thiorphan in the absence (E) or the presence of IL-6 (S+E). Significant differences caused by inhibitors are represented by *.

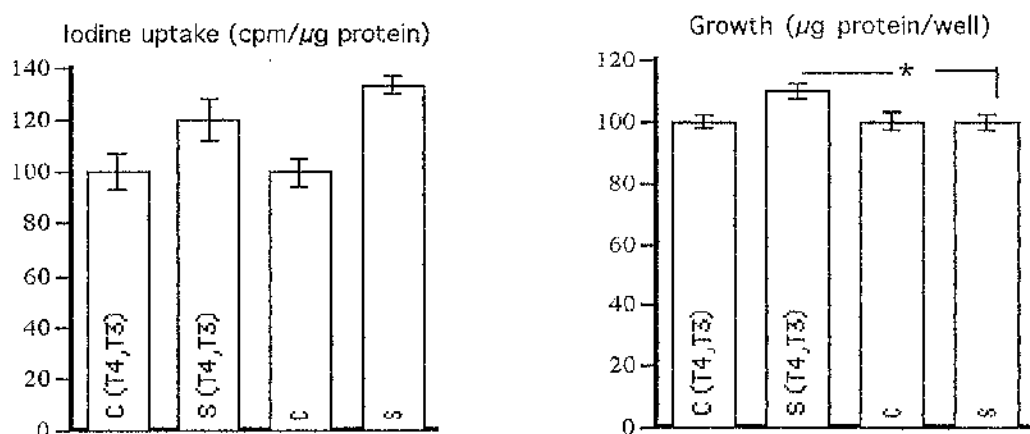


Figure 3.43: Effect of thyroid hormones, on stimulation of FRTL-5 cells with IL-6 and TSH. Cells were stimulated in 6H medium (2 U/l of TSH) as before, without IL-6 (C), with 20 $\mu\text{g/l}$ of IL-6 (S), or stimulated in the 6H medium containing 0.5 % FCS, additional amino acids, 1 $\mu\text{mol/l}$ of T₄ and 10 nmol/l of T₃, 48 h before and 48 h after addition of IL-6 [S (T₄, T₃)]. The cells that were stimulated only with thyroid hormones for 96 h served as control [C (T₄, T₃)] for this set. Significant differences between the presence and the absence of thyroid hormones is shown by *.

3.3.6.8.2 *Protease Inhibitors*

Enzyme inhibitors had no significant effect on the iodine uptake of cells incubated without TSH, but amplified the growth effect of IL-6. The stimulation was 41.0 % compared to 20.7 % (figure 3.42).

In the presence of TSH, the inhibitors suppressed the effect of IL-6 on iodine uptake. The increase was only 16.6 % compared to 36.4 %. In the presence of inhibitors, the growth rate increased significantly by 37.3 % in the presence of IL-6, an effect which was not present in their absence.

3.3.6.8.3 *Thyroid Hormones*

Thyroid hormones did not alter the effect of IL-6 on iodine uptake of cells significantly. They did, however, stimulate growth of cells by 9.9 %, whereas the same concentration of IL-6 had no effect in their absence (figure 3.43).

3.3.6.9 Interferon Gamma (INF- γ)

3.3.6.9.1 *Dose Study*

The final concentrations of INF- γ used ranged from 1 to 1000 $\mu\text{g/l}$. A concentration of 100 $\mu\text{g/l}$ was chosen for further study.

The addition of INF- γ to cells in the absence of TSH caused a significant increase in iodine uptake at concentrations of 50 $\mu\text{g/l}$ and above. The maximum increase of 21.23 % over control occurred at a concentration of 1000 $\mu\text{g/l}$. INF- γ had no effect on growth rate.

In the presence of TSH, INF- γ caused a significant increase in iodine uptake at concentrations of 10 $\mu\text{g/l}$ and above. The maximum increase was 75.65 % at 1000 $\mu\text{g/l}$ (figure 3.44). INF- γ also caused decreases in growth rate at concentrations of 1 $\mu\text{g/l}$ and above, with the maximum effect at 1000 $\mu\text{g/l}$ (36.0 %)

3.3.6.9.2 *Protease Inhibitors*

The inclusion of bestatin and thiorphan did not alter the effects of INF- γ on either iodine uptake or growth, in the presence or absence of TSH (figure 3.45).

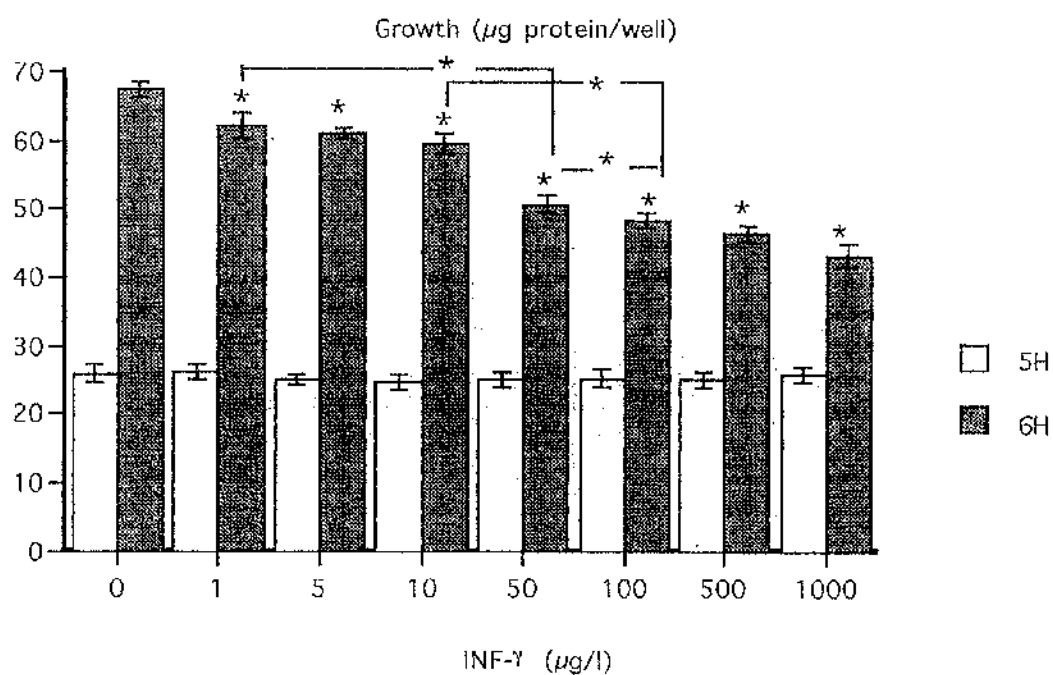
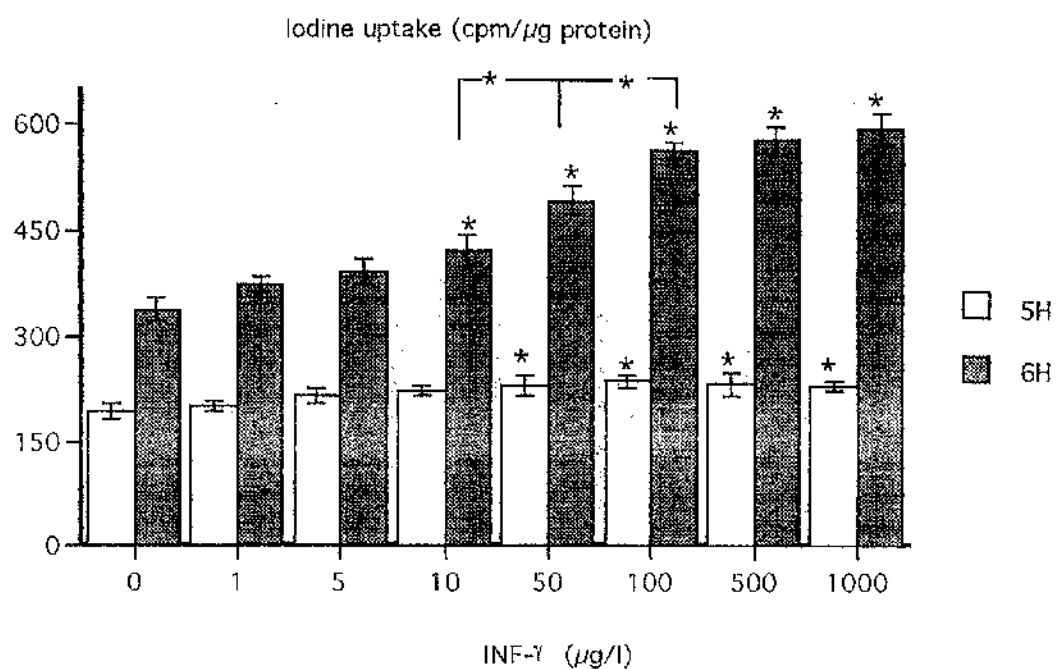


Figure 3.44: Effect of INF- γ on iodine uptake and growth of FRTL-5 cells. Cells were incubated in 5H or 6H (20U/l of TSH) medium containing 0.5 % FCS and additional amino acids for 48 h. Significant differences are shown by *.

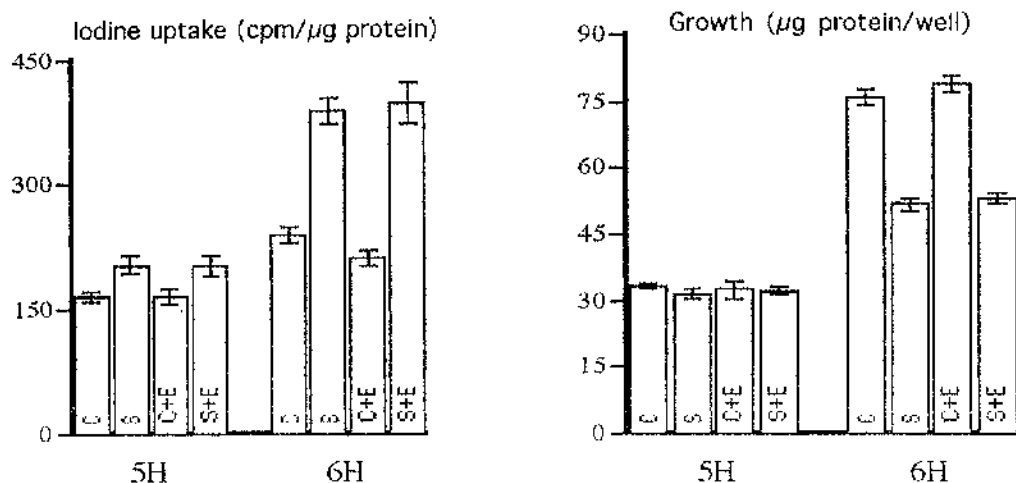


Figure 3.45: Effect of protease inhibitors, on stimulation of FRTL-5 cells with INF- γ . Cells were incubated as before, without INF- γ (C), with 100 $\mu\text{g/l}$ of INF- γ (S), or with a mixture of 1 $\mu\text{mol/l}$ of bestatin and 1 $\mu\text{mol/l}$ of thiorphan in the absence (E) or the presence of INF- γ (S+E).

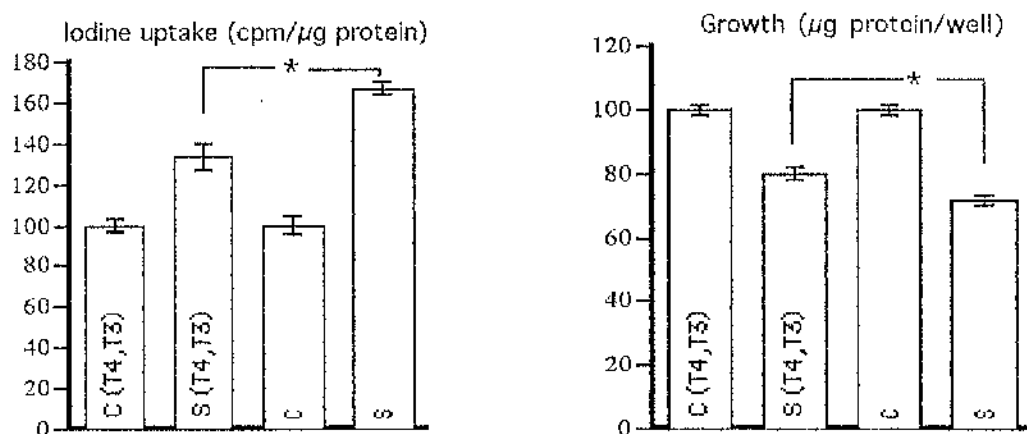


Figure 3.46: Effect of thyroid hormones, on stimulation of FRTL-5 cells with INF- γ and TSH. Cells were stimulated in 6H medium (2 U/l of TSH) as before, without INF- γ (C), with 100 $\mu\text{g/l}$ of INF- γ (S), or stimulated in the 6H medium containing 0.5 % FCS, additional amino acids, 1 $\mu\text{mol/l}$ of T₄ and 10 nmol/l of T₃, 48 h before and 48 h after addition of INF- γ [S (T₄, T₃)]. The cells that were stimulated only with thyroid hormones for 96 h served as control [C (T₄, T₃)] for this set. Significant differences between the presence and absence of thyroid hormones are shown by *.

3.3.6.9.3 *Thyroid Hormones*

In the presence of thyroid hormones, the increase in iodine uptake caused by INF- γ was 33.35 %. In the absence of thyroid hormones this increase was significantly less, 66.70 %. Thyroid hormones also partially antagonised the effect of INF- γ on growth. The decrease in growth rate was 19.9 % compared to 28.5 %.

3.3.6.10 Pituitary Adenylate Cyclase Activating Polypeptide (PACAP)

3.3.6.10.1 *Dose Study*

The final concentrations of PACAP used ranged from 1 to 500 nmol/l. A concentration of 250 nmol/l was used for further studies.

Inclusion of PACAP in 5H medium did not change the rate of iodine uptake or growth.

In the presence of TSH, all doses of PACAP caused an increase in iodine uptake, up to a concentration of 250 nmol/l, which caused the maximum increase of 28.5 %. At a concentration of 500 nmol/l, iodine uptake was less than that at 250 nmol/l (figure 3.47). PACAP had no effect on growth.

3.3.6.10.2 *Protease Inhibitors*

The inclusion of bestatin and thiorphan did not alter the effect of PACAP on either iodine uptake or growth, either in the presence or the absence of TSH (figure 3.48).

3.3.6.10.3 *Thyroid Hormones*

Thyroid hormones antagonised the effect of PACAP on iodine uptake (figure 3.49). The increase in the presence of hormones was 11.3 %, compared to 28.5 % in their absence. There were small, non-significant changes in growth rate.

3.3.6.11 Neuropeptide Y (NPY)

3.3.6.11.1 *Dose Study*

The final concentrations of NPY used ranged from 10 to 2000 nmol/l. A concentration of 500 nmol/l was chosen for further study.

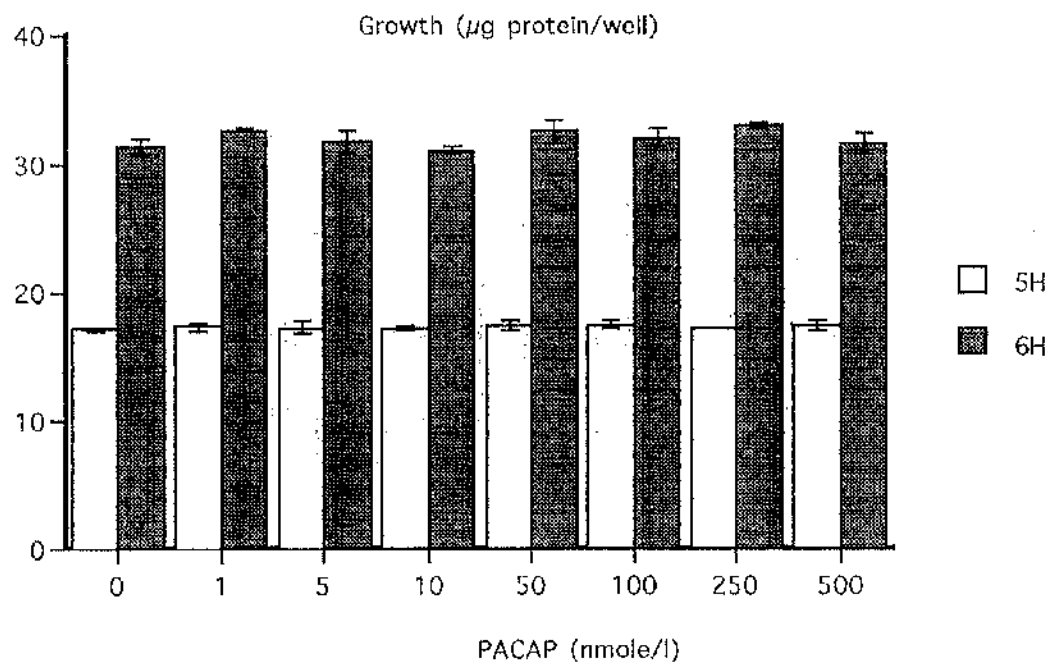
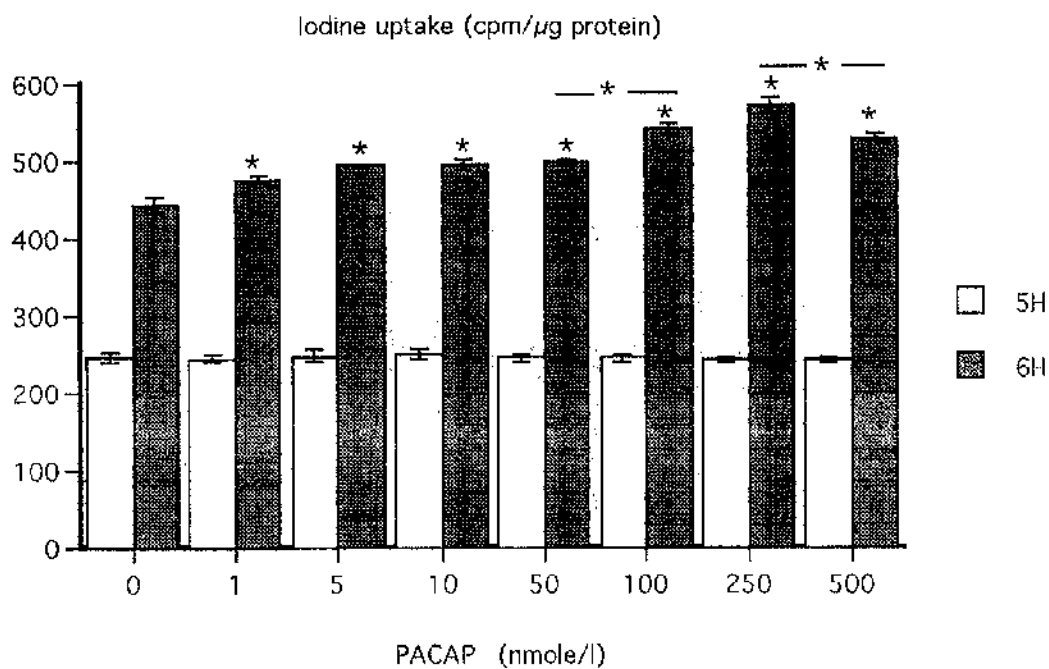


Figure 3.47: Effect of PACAP on iodine uptake and growth of FRTL-5 cells. Cells were incubated in 5H or 6H (2U/l of TSH) medium containing 0.5 % FCS and additional amino acids for 48 h. Significant differences between stimulated and unstimulated cells (control or zero concentration) are shown by *.

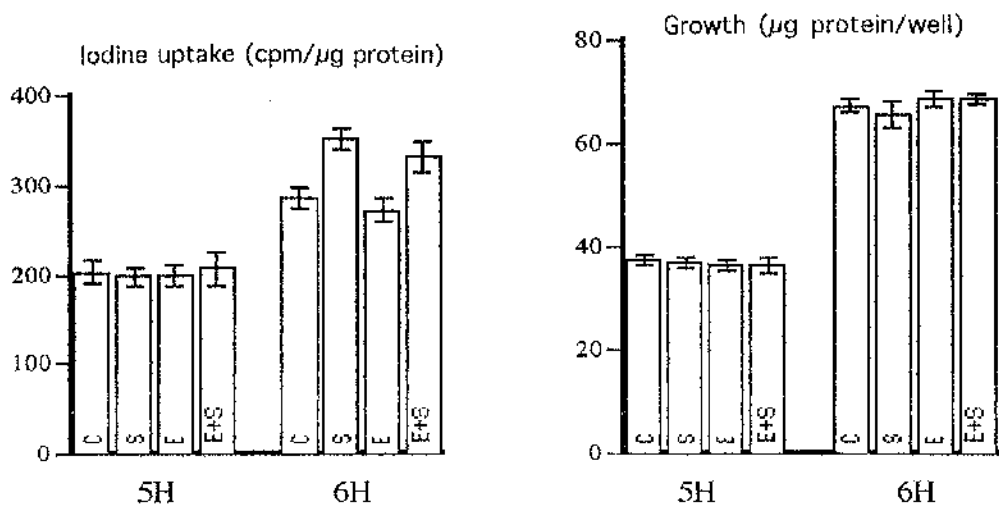


Figure 3.48: Effect of protease inhibitors, on stimulation of FRTL-5 cells with PACAP. Cells were incubated as before, without PACAP (C), with 250 nmol/l of PACAP (S), or with a mixture of 1 μ mol/l of bestatin and 1 μ mol/l of thiorphan in the absence (E) or the presence of PACAP (S+E).

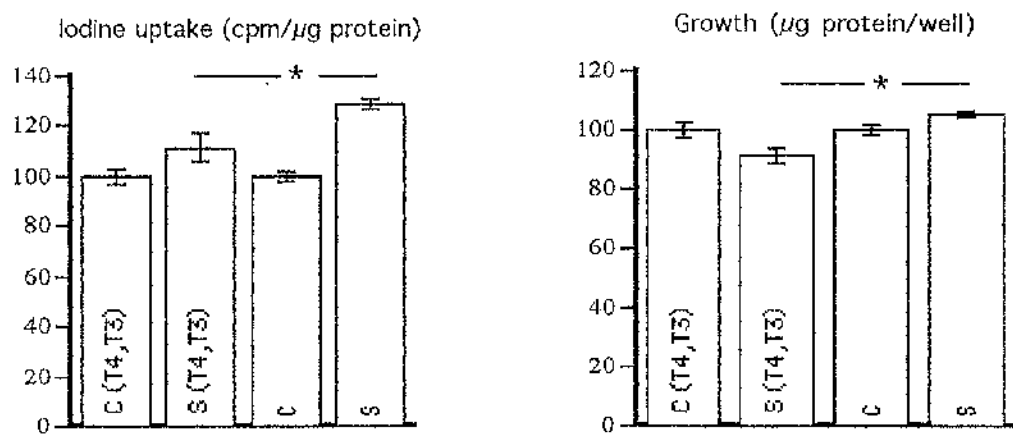


Figure 3.49: Effect of thyroid hormones, on stimulation of FRTL-5 cells with PACAP and TSH. Cells were stimulated in 6H medium (2 U/l of TSH) as before, without PACAP (C), with 250 nmol/l of PACAP (S), or stimulated in the 6H medium containing 0.5 % FCS, additional amino acids, 1 μ mol/l of T₄ and 10 nmol/l of T₃, 48 h before and 48 h after addition of PACAP [S (T₄, T₃)]. The cells that were stimulated only with thyroid hormones for 96 h served as control [C (T₄, T₃)] for this set. Significant differences that occurred in presence and absence of thyroid hormones are shown by *.

Iodine uptake of cells increased significantly ($p < 0.05$) at concentrations of 500 nmol/l and above in the absence of TSH. The maximum increase of 12.30 % occurred at a concentration of 2 μ mol/l. There were no significant differences among values obtained for iodine uptake of doses above 500 nmol/l (figure 3.50).

In the presence of TSH, the iodine uptake of stimulated cells increased significantly ($p < 0.05$) with doses of NPY above 250 nmol/l. The maximum increase was caused by a concentration of 2 μ mol/l, 11.59 % higher than the control. There were no significant differences between the value obtained for iodine uptake of doses above 250 nmol/l and above when their values were compared with each other.

The inclusion of NPY in 5H or 6H medium did not alter the growth of cells significantly at any concentration.

3.3.6.11.2 *Protease Inhibitors*

Bestatin and thiorphan did not alter the effect of 500 nmol/l of NPY on iodine uptake or growth of cells in the absence of TSH (figure 3.51).

Addition of bestatin and thiorphan to the medium containing TSH prevented the stimulatory effect of 500 nmol/l of NPY on iodine uptake but did not affect growth.

3.3.6.11.3 *Thyroid Hormones*

Thyroid hormones did not alter the effects of NPY (figure 3.52).

3.3.6.12 Vasoactive Intestinal Peptide (VIP)

3.3.6.12.1 *Dose Study*

The final concentrations of VIP used ranged from 10 to 2000 nmol/l. A concentration of 500 nmol/l was chosen for further study.

VIP increased the iodine uptake in the absence of TSH significantly at concentrations of 500 nmol/l and 1 μ mol/l, while the value obtained for 2 μ mol/l was not different from control. The maximum increase of 14.2 % over control occurred at a concentration of 1 μ mol/l (figure 3.53). VIP had no effect on growth rate.

In the presence of TSH, the iodine uptake of stimulated cells increased significantly ($p < 0.05$) at concentrations of 10 nmol/l and above. The maximum increase of 48.9 % above control was obtained for 500 nmol/l. The values obtained for iodine uptake at concentrations of 1 and 2 μ mol/l were significantly lower than the corresponding value for 500 nmol/l, although both values were significantly higher

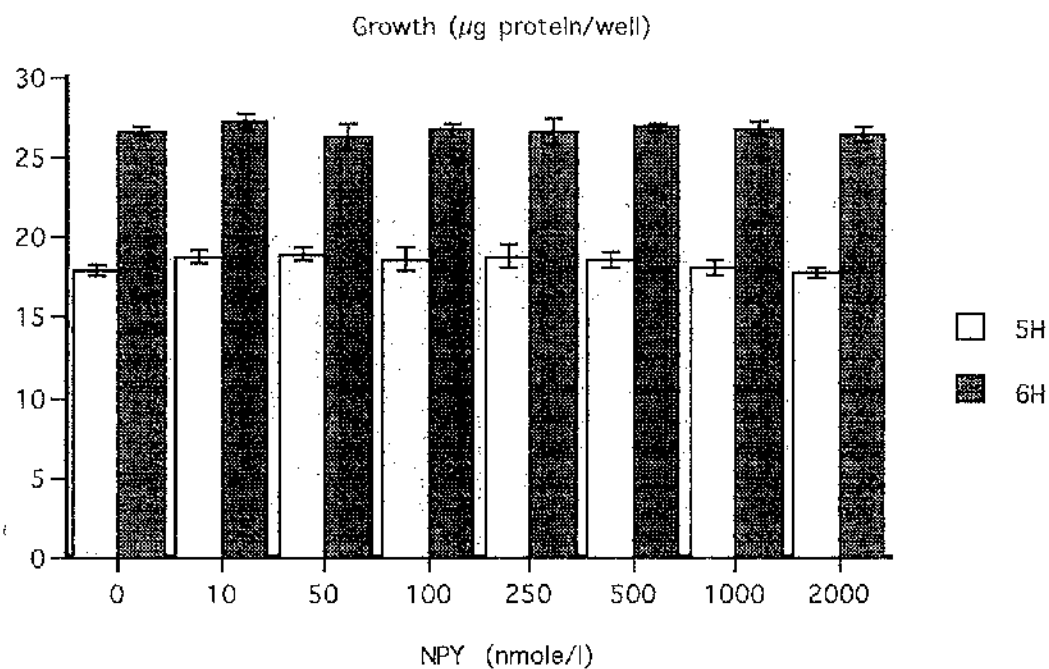
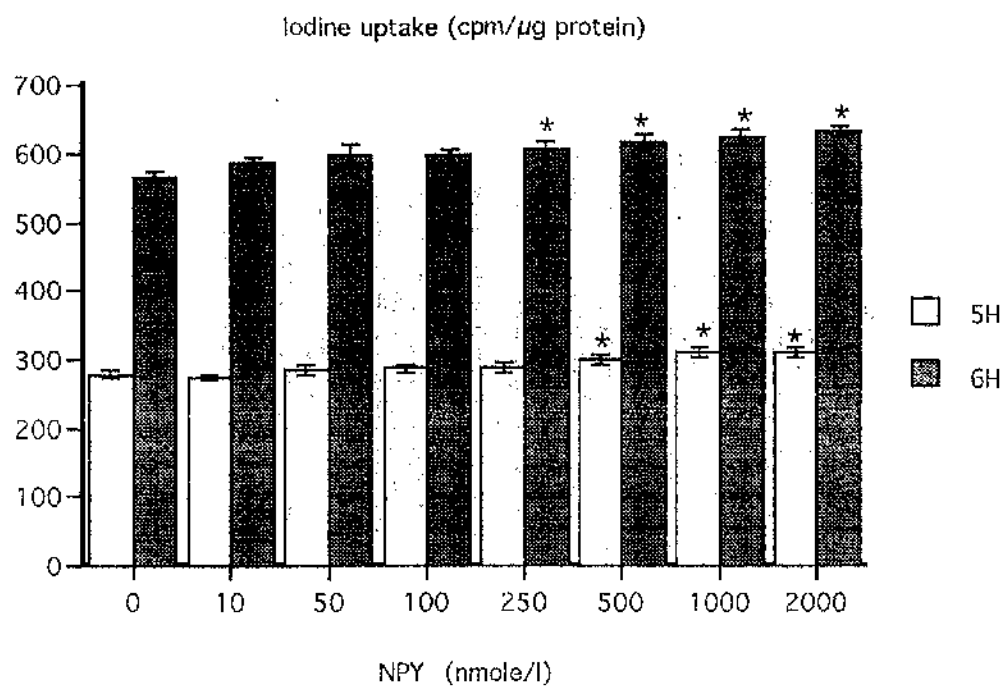


Figure 3.50: Effect of NPY on iodine uptake and growth of FRTL-5 cells. Cells were incubated in 5H or 6H (2U/l of TSH) medium containing 0.5 % FCS and additional amino acids for 48 h. Significant differences between stimulated and unstimulated cells (control or zero concentration) are shown by *.

than control. VIP caused significant increases in growth at concentrations of 10 and 50 nmol/l, while it caused a significant decrease at a concentration of 2 μ mol/l (figure 3.53).

3.3.6.12.2 *Protease Inhibitors*

Inclusion of bestatin and thiorphan in 5H medium did not alter the effect of VIP on iodine uptake or growth of the cells.

In the presence of 2 U/l of TSH (6H), the inhibitors significantly ($p < 0.05$) reduced the effect of VIP on iodine uptake from 30.1 % to only 19.9 %, but did not affect growth (figure 3.54).

3.3.6.12.3 *Thyroid Hormones*

Thyroid hormones antagonised the effect of VIP on iodine uptake. The increase of 48.9 % in their absence was reduced to 2.0 % (figure 3.55). Thyroid hormones also slightly increased the growth of cells. The increase was 6.6 % in their presence, compared to a decrease of 3.7 % in their absence.

3.3.6.13 Neuromedin U-25

3.3.6.13.1 *Dose Study*

The final concentrations used ranged from 5 to 1000 nmol/l. A concentration of 250 nmol/l was chosen for further study.

In the presence or absence of TSH, the iodine uptake and growth rate of cells did not change when cells were incubated with Neuromedin U (figure 3.56).

3.3.6.13.2 *Protease Inhibitors*

The inclusion of bestatin and thiorphan did not alter the effects of Neuromedin on either iodine uptake or growth, either in the presence or the absence of TSH (figure 3.57).

3.3.6.13.3 *Thyroid Hormones*

The only effect of including thyroid hormones in the medium was a slight increase in growth rate of 14.1 % (figure 3.58).

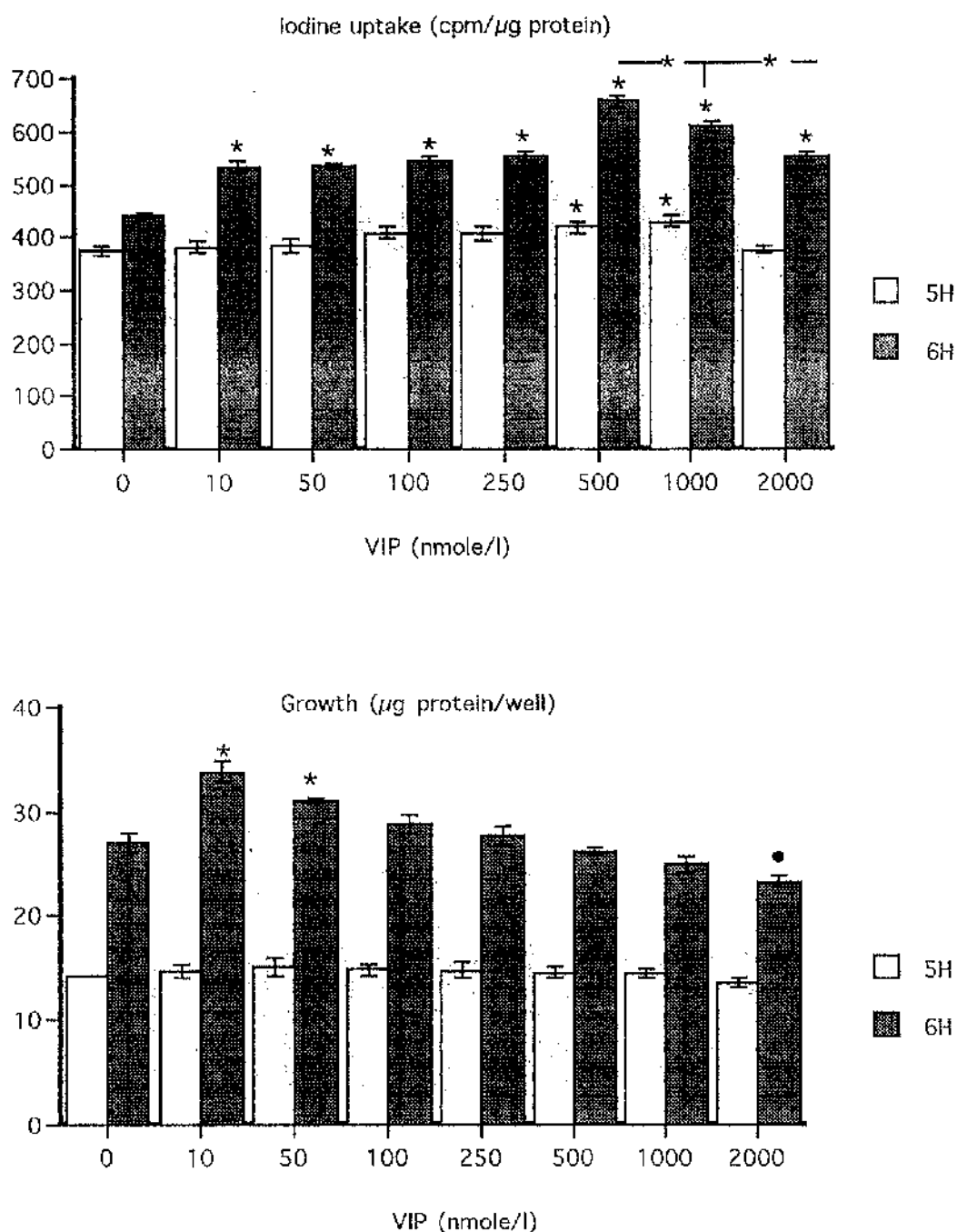


Figure 3.53: Effect of VIP on iodine uptake and growth of FRTL-5 cells. Cells were incubated in 5H or 6H (2U/l of TSH) medium containing 0.5 % FCS and additional amino acids for 48 h. Significant differences between stimulated and unstimulated cells (control or zero concentration) are shown by *, while • shows significant decrease compared to control.

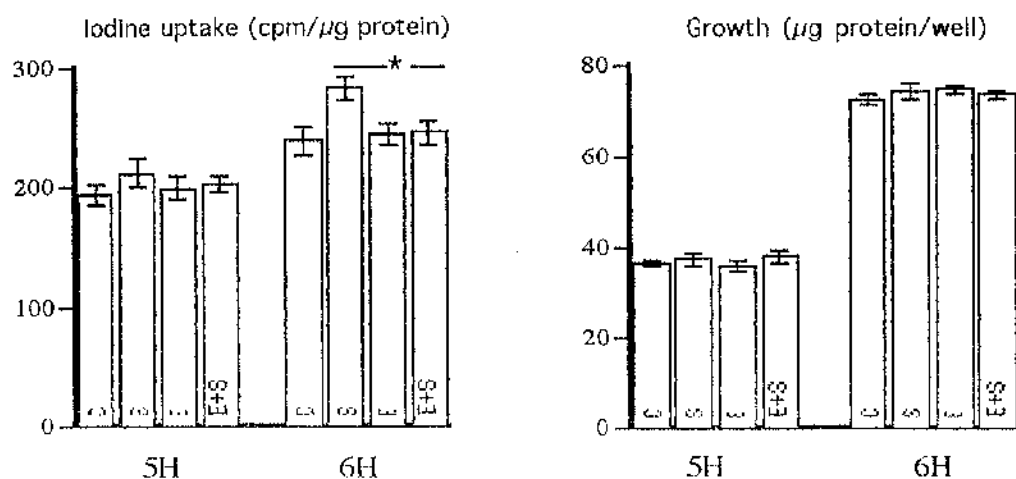


Figure 3.51: Effect of protease inhibitors, on stimulation of FRTL-5 cells with NPY. Cells were incubated as before, without NPY (C), with 500 nmol/l of NPY (S), or with a mixture of 1 μ mol/l of bestatin and 1 μ mol/l of thiorphan in the absence (E) or the presence of NPY (S+E). Significant differences caused by inhibitors are represented by *.

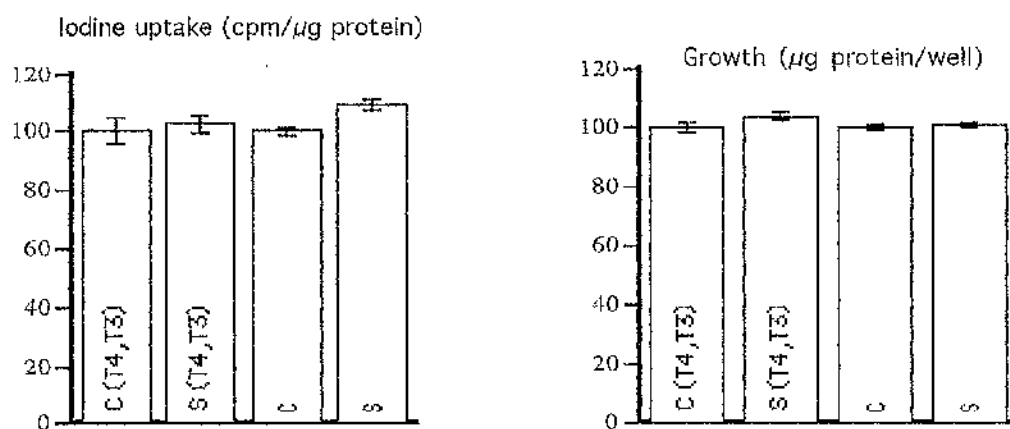


Figure 3.52: Effect of thyroid hormones, on stimulation of FRTL-5 cells with NPY and TSH. Cells were stimulated in 6H medium (2 U/l of TSH) as before, without NPY (C), with 500 nmol/l of NPY (S), or stimulated in the 6H medium containing 0.5 % FCS, additional amino acids, 1 μ mol/l of T₄ and 10 nmol/l of T₃, 48 h before and 48 h after addition of NPY [S (T₄, T₃)]. The cells that were stimulated only with thyroid hormones for 96 h served as control [C (T₄, T₃)] for this set.

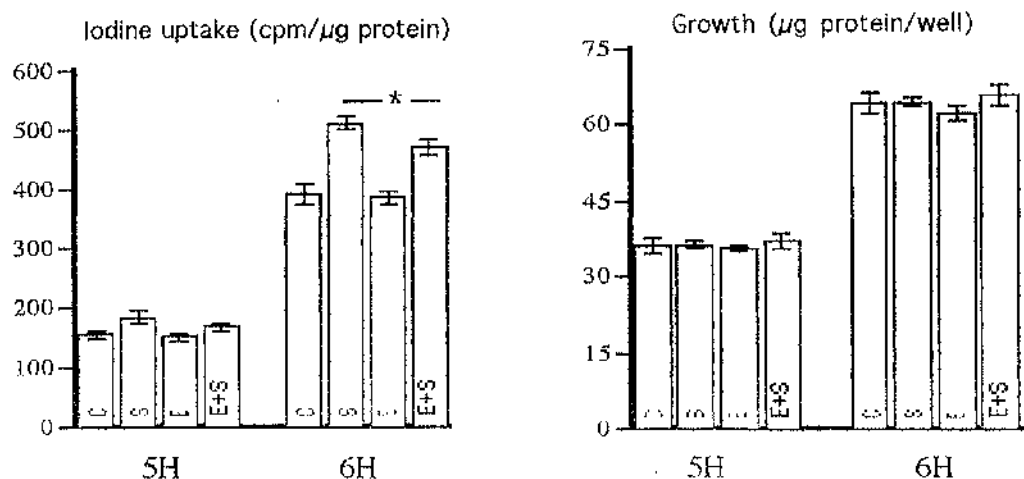


Figure 3.54: Effect of protease inhibitors, on stimulation of FRTL-5 cells with VIP. Cells were incubated as before, without VIP (C), with 500 nmol/l of VIP (S), or with a mixture of 1 μ mol/l of bestatin and 1 μ mol/l of thiorphan in the absence (E) or the presence of VIP (S+E). Significant differences caused by inhibitors are represented by *.

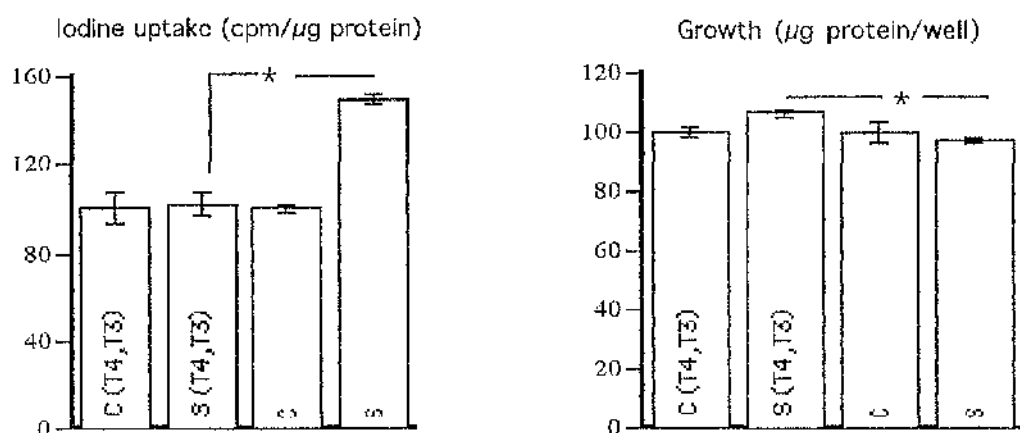


Figure 3.55: Effect of thyroid hormones, on stimulation of FRTL-5 cells with VIP and TSH. Cells were stimulated in 6H medium (2 U/l of TSH) as before, without VIP (C), with 500 nmol/l of VIP (S), or stimulated in the 6H medium containing 0.5 % FCS, additional amino acids, 1 μ mol/l of T₄ and 10 nmol/l of T₃, 48 h before and 48 h after addition of VIP [S (T₄, T₃)]. The cells that were stimulated only with thyroid hormones for 96 h served as control [C (T₄, T₃)] for this set. Significant differences between the presence and absence of thyroid hormones are shown by (*).

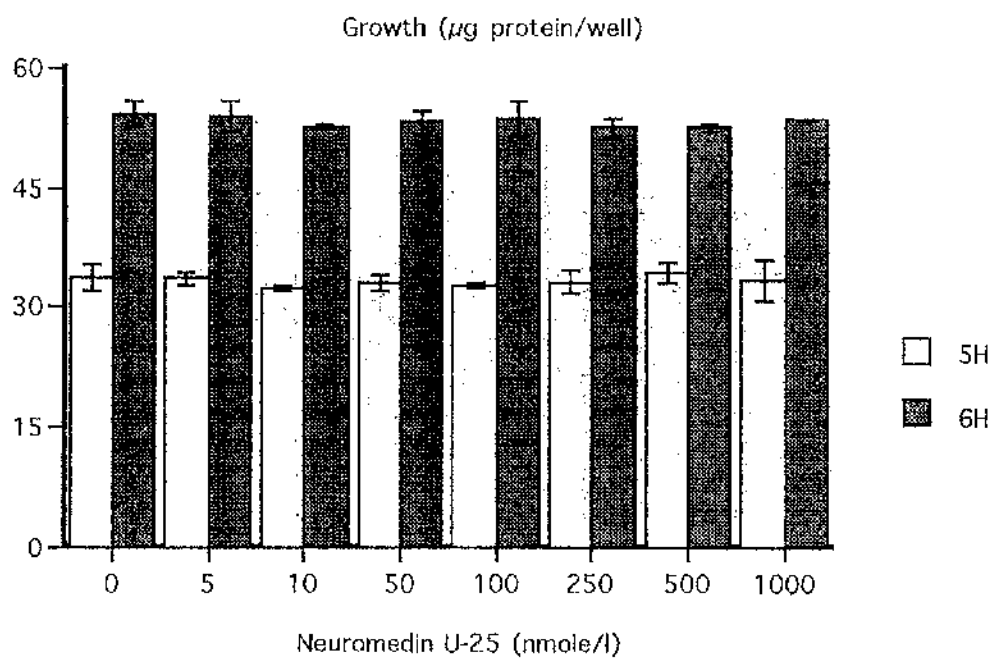
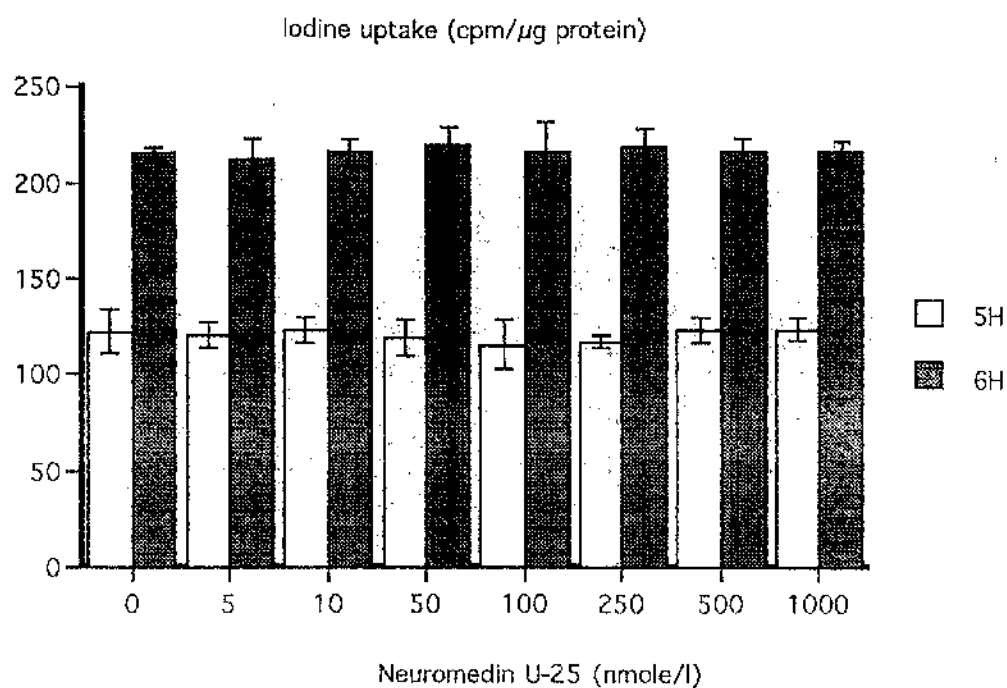


Figure 3.56: Effect of Neuromedin-U on iodine uptake and growth of FRTL-5 cells. Cells were incubated in 5H or 6H (2U/l of TSH) medium containing 0.5 % FCS and additional amino acids for 48 h.

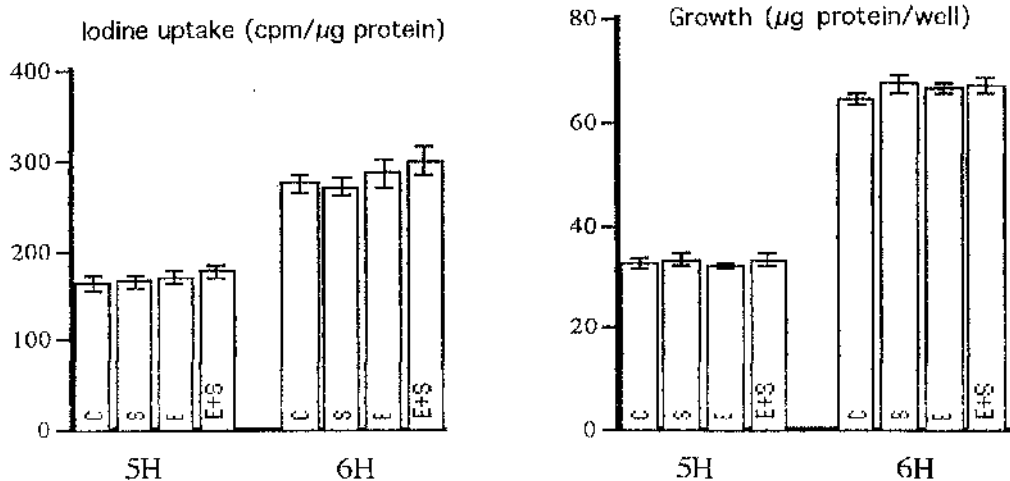


Figure 3.57: Effect of protease inhibitors, on stimulation of FRTL-5 cells with Neuromedin-U. Cells were incubated as before, without Neuromedin-U (C), with 250 nmol/l of Neuromedin-U (S), or with a mixture of 1 μ mol/l of bestatin and 1 μ mol/l of thiorphan in the absence (E) or the presence of Neuromedin-U (S+E).

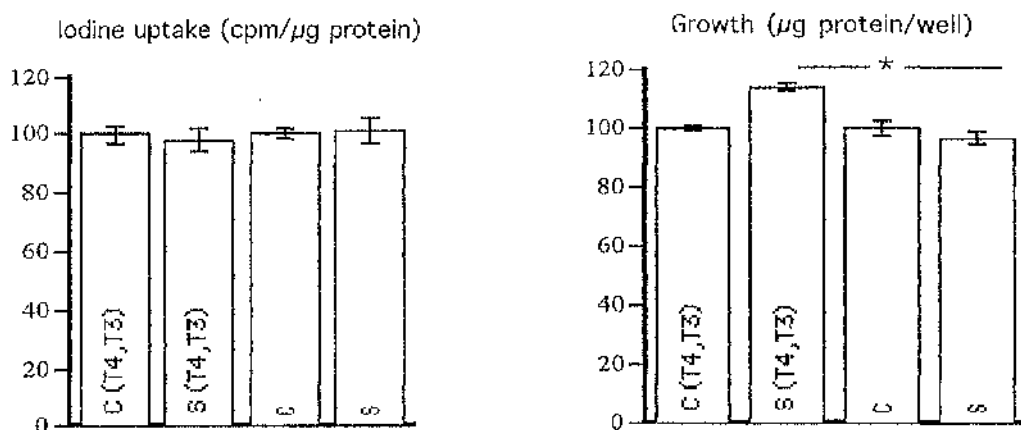


Figure 3.58: Effect of thyroid hormones, on stimulation of FRTL-5 cells with Neuromedin-U and TSH. Cells were stimulated in 6H medium (2 U/l of TSH) as before, without Neuromedin-U (C), with 250 nmol/l of Neuromedin-U (S), or stimulated in the 6H medium containing 0.5 % FCS, additional amino acids, 1 μ mol/l of T₄ and 10 nmol/l of T₃, 48 h before and 48 h after addition of Neuromedin-U [S (T₄, T₃)]. The cells that were stimulated only with thyroid hormones for 96 h served as control [C (T₄, T₃)] for this set. Significant differences between the presence and absence of thyroid hormones is shown by *.

3.3.6.14 Substance P (SP)

3.3.6.14.1 Dose Study

The final concentrations of substance P used ranged from 10 to 2000 nmol/l. A concentration of 250 nmol/l was chosen for further study.

SP increased the iodine uptake of cells at concentrations of 100 and 250 nmol/l in the absence of TSH. The maximum increase of 26.2 % above control occurred at 250 nmol/l. There were no significant increases at higher doses. SP did not affect growth (figure 3.59).

In the presence of TSH, the iodine uptake of cells increased significantly ($p < 0.05$) at concentrations of 100 nmol/l and above. The maximum increase was associated with 250 nmol/l of SP; this was 14.55 % higher than control. SP did not affect growth.

3.3.6.14.2 Protease Inhibitors

Protease inhibitors prevented SP from stimulating iodine uptake, both in the absence and presence of TSH (figure 3.60). The increase in the presence of inhibitors was only 7.3 % in 5H medium compared to 32.8 %, and 7.1 % compared to 17.1 % in 6H medium. The inhibitors had no effect on growth rate.

3.3.6.14.3 Thyroid Hormones

Neither iodine uptake nor growth of the cells was affected by thyroid hormones (figure 3.61).

The above experiments indicate that the iodine uptake of cells increases when stimulated with SP. Since SP binds to more than one of the neurokinin receptors, the experiments were repeated using a specific agonist for each of the three known receptors.

3.3.6.15 [Sar⁹, Met(O₂)¹¹]-Substance P (SMO-SP)

3.3.6.15.1 Dose Study

[Sar⁹, Met(O₂)¹¹]-Substance P (SMO-SP) is a specific agonist for the Neurokinin 1 receptor. Its final concentrations ranged from 10 to 2000 nmol/l. A concentration of 500 nmol/l was chosen for further study.

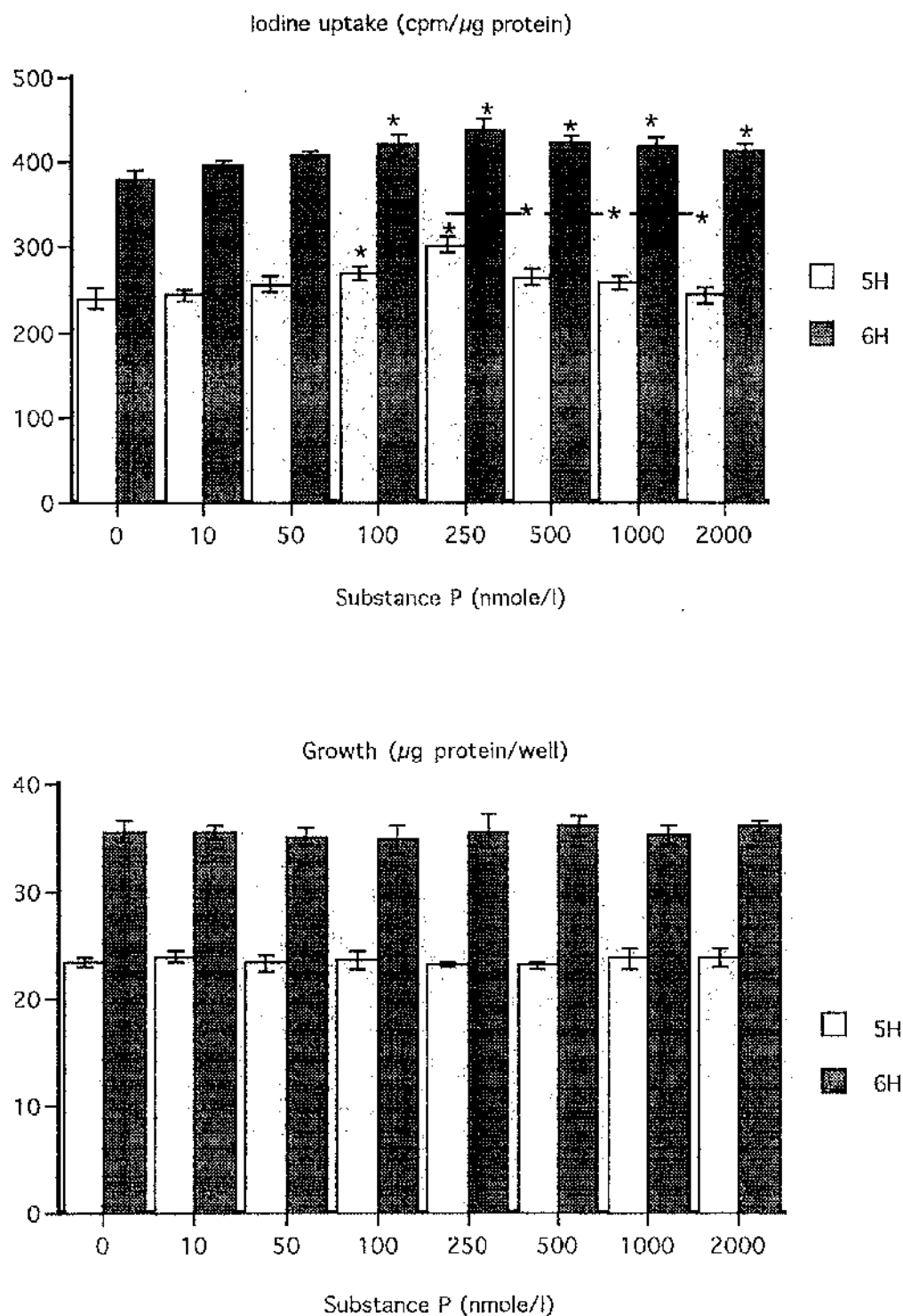


Figure 3.59: Effect of substance P on iodine uptake and growth of FRTL-5 cells. Cells were incubated in 5H or 6H (2U/l of TSH) medium containing 0.5 % FCS and additional amino acids for 48 h. Significant differences between stimulated and unstimulated cells (control or zero concentration) are shown by *.

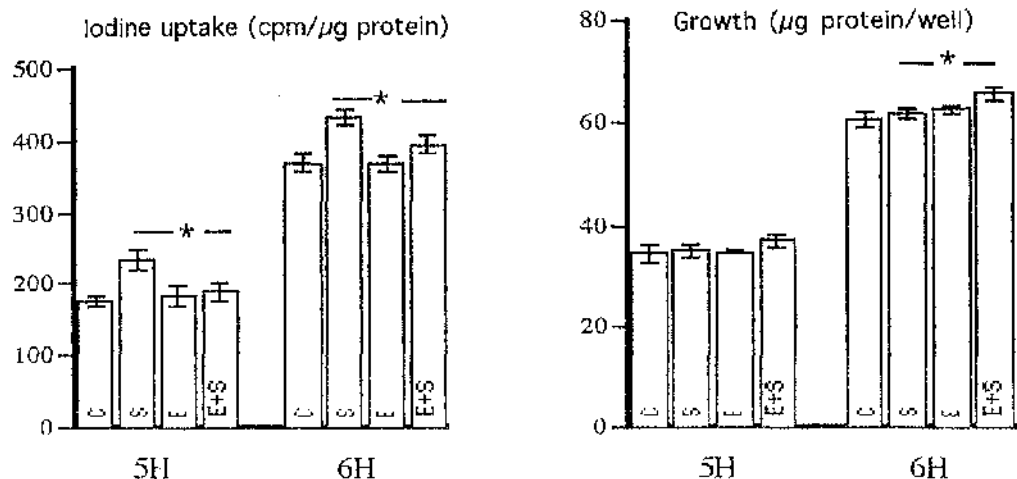


Figure 3.60: Effect of protease inhibitors, on stimulation of FRTL-5 cells with substance P. Cells were incubated as before, without substance P (C), with 250 nmol/l of substance P (S), or with a mixture of 1 μ mol/l of bestatin and 1 μ mol/l of thiorphan in the absence (E) or the presence of substance P (S+E). Significant differences caused by inhibitors are represented by *.

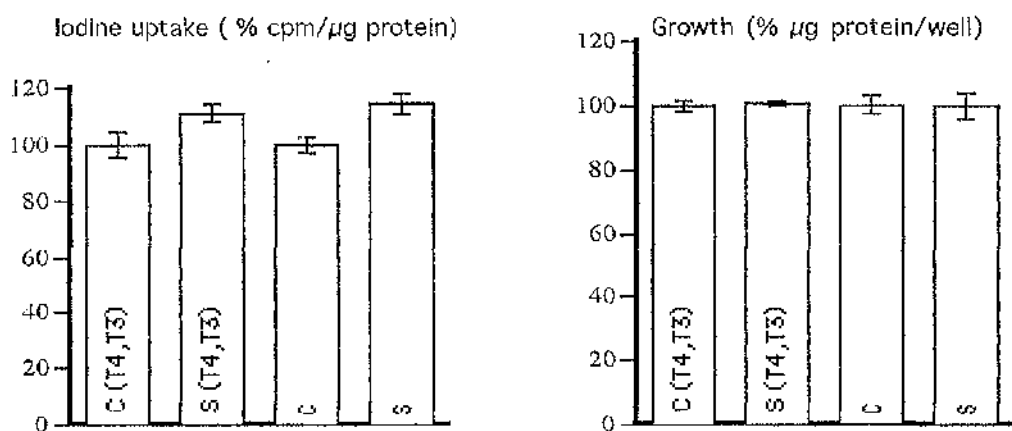


Figure 3.61: Effect of thyroid hormones, on stimulation of FRTL-5 cells with substance P and TSH. Cells were stimulated in 6H medium (2 U/l of TSH) as before, without substance P (C), with 250 nmol/l of substance P (S), or stimulated in the 6H medium containing 0.5 % FCS, additional amino acids, 1 μ mol/l of T₄ and 10 nmol/l of T₃, 48 h before and 48 h after addition of substance P [S (T₄,T₃)]. The cells that were stimulated only with thyroid hormones for 96 h served as control [C (T₄,T₃)] for this set.

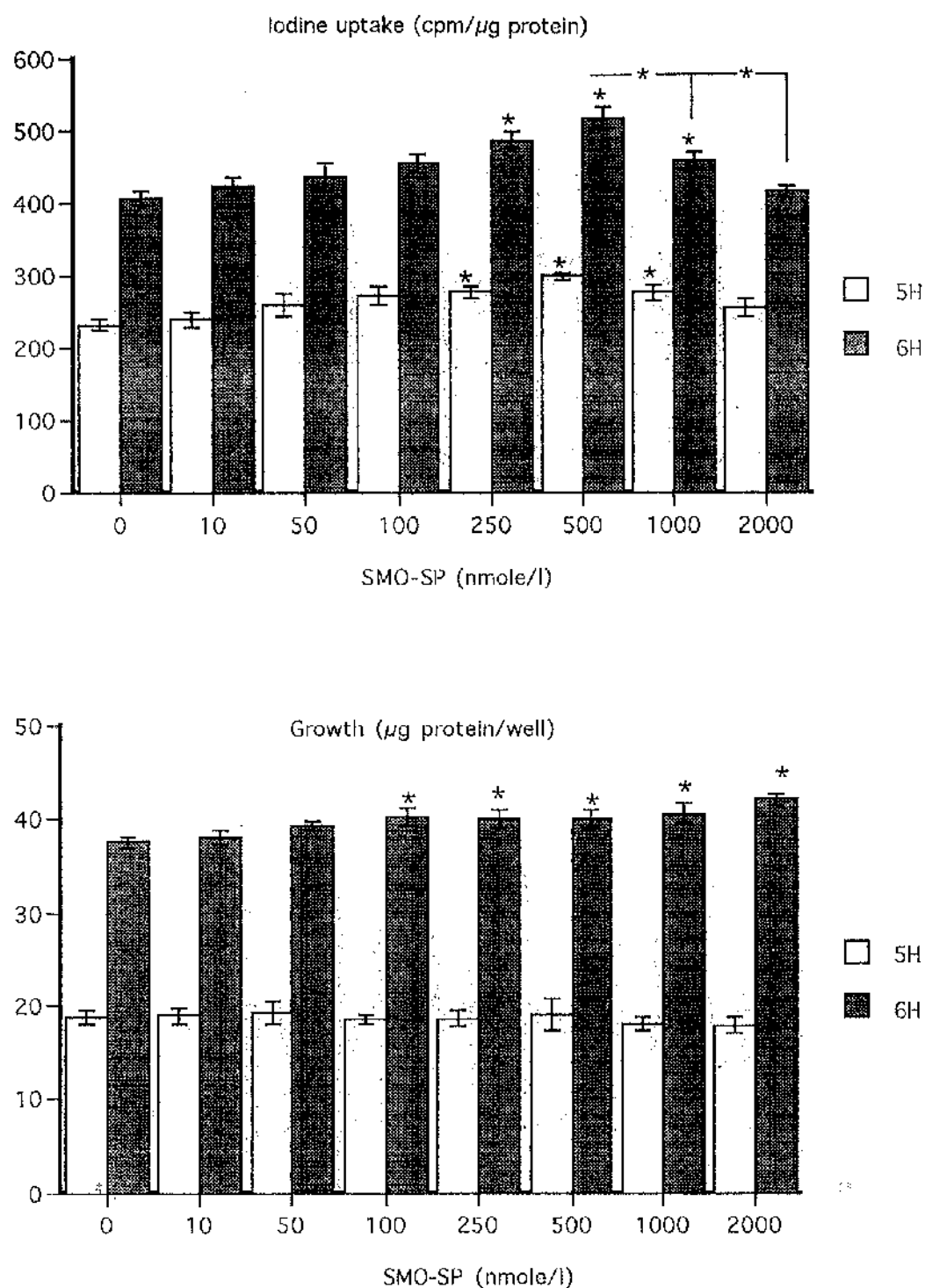


Figure 3.62: Effect of SMO-SP (agonist for NK-1 receptor) on iodine uptake and growth of FRTL-5 cells. Cells were incubated in 5H or 6H (2U/l of TSH) medium containing 0.5 % FCS and additional amino acids for 48 h. Significant differences between stimulated and unstimulated cells (control or zero concentration) are shown by *.

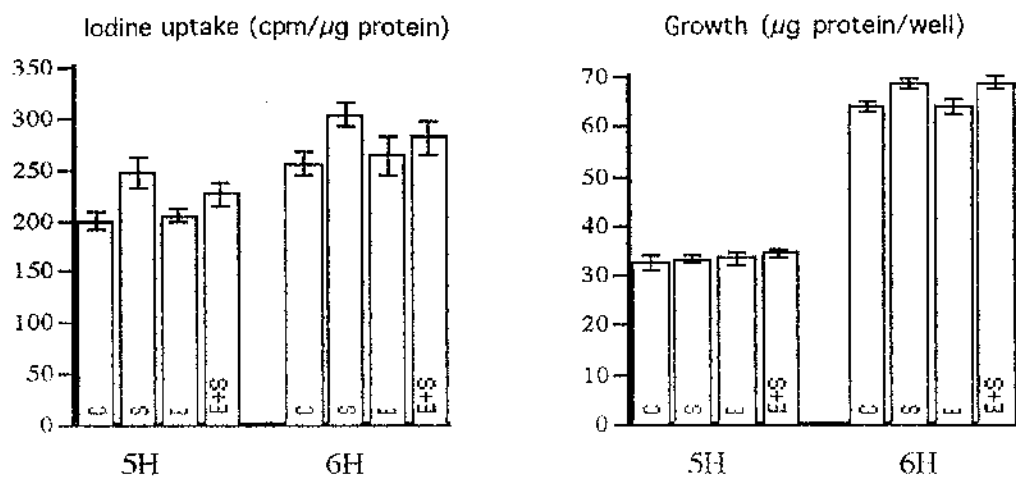


Figure 3.63: Effect of protease inhibitors, on stimulation of FRTL-5 cells with SMO-SP. Cells were incubated as before, without SMO-SP (C), with 500 nmol/l of SMO-SP (S), or with a mixture of 1 μ mol/l of bestatin and 1 μ mol/l of thiorphan in the absence (E) or the presence of SMO-SP (S+E).

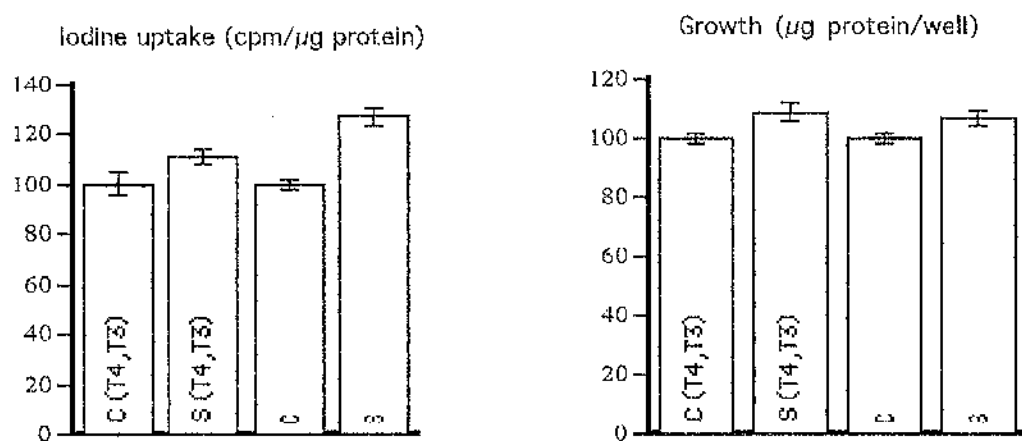


Figure 3.64: Effect of thyroid hormones, on stimulation of FRTL-5 cells with SMO-SP and TSH. Cells were stimulated in 6H medium (2 U/l of TSH) as before, without SMO-SP (C), with 500 nmol/l of SMO-SP (S), or stimulated in the 6H medium containing 0.5 % FCS, additional amino acids, 1 μ mol/l of T₄ and 10 nmol/l of T₃, 48 h before and 48 h after addition of SMO-SP [S (T₄, T₃)]. The cells that were stimulated only with thyroid hormones for 96 h served as control [C (T₄, T₃)] for this set.

Concentrations of 250, 500 and 1000 nmol/l caused significant increases in iodine uptake of the cells incubated in 5H medium. The maximum increase of 29.1 % above control was detected at a concentration of 500 nmol/l (figure 3.62). SMO-SP did not affect the growth of the cells at any concentration.

In the presence of TSH, SMO-SP at concentrations of up to 500 nmol/l increased the iodine uptake of cells. The maximum increase was 27.0 %. The iodine uptake at concentrations of 1000 and 2000 nmol/l was significantly less than at 500 nmol/l. A significant increase of growth rate of 12.3 % occurred at a concentration of 2 μ mol/l.

3.3.6.15.2 *Protease Inhibitors*

Protease inhibitors reduced the stimulatory effect of SMO-SP from 23.3 % to 9.8 % in the absence of TSH, and from 19.0 % to 9.8 % in the presence of TSH (figure 3.63). They had no effect on growth.

3.3.6.15.3 *Thyroid Hormones*

Thyroid hormones reduced the stimulatory effect of SMO-SP from 26.7 % to 10.9 %, but did not affect growth rate (figure 3.64).

3.3.6.16 α -Neurokinin Fragment 4-10 (NK)

3.3.6.16.1 *Dose Study*

α -Neurokinin fragment 4-10 (NK) is a specific agonist for the Neurokinin 2 receptor. Its final concentrations ranged from 10 to 2000 nmol/l. A concentration of 250 nmol/l was chosen for further study.

NK significantly ($p < 0.05$) increased iodine uptake in the absence of TSH at concentrations of 100 and 250 nmol/l. The maximum increase of 21.16 % was detected at a concentration of 250 nmol/l. The value obtained for iodine uptake was significantly lower at a concentration of 2000 nmol/l than at 250 nmol/l. In the presence of TSH, NK increased the iodine uptake of cells significantly at all concentrations. The maximum increase was caused by 250 nmol/l of NK, 26.25 % higher than control (figure 3.65).

Addition of NK did not affect the growth of the cells at any concentration, either in the presence or the absence of TSH.

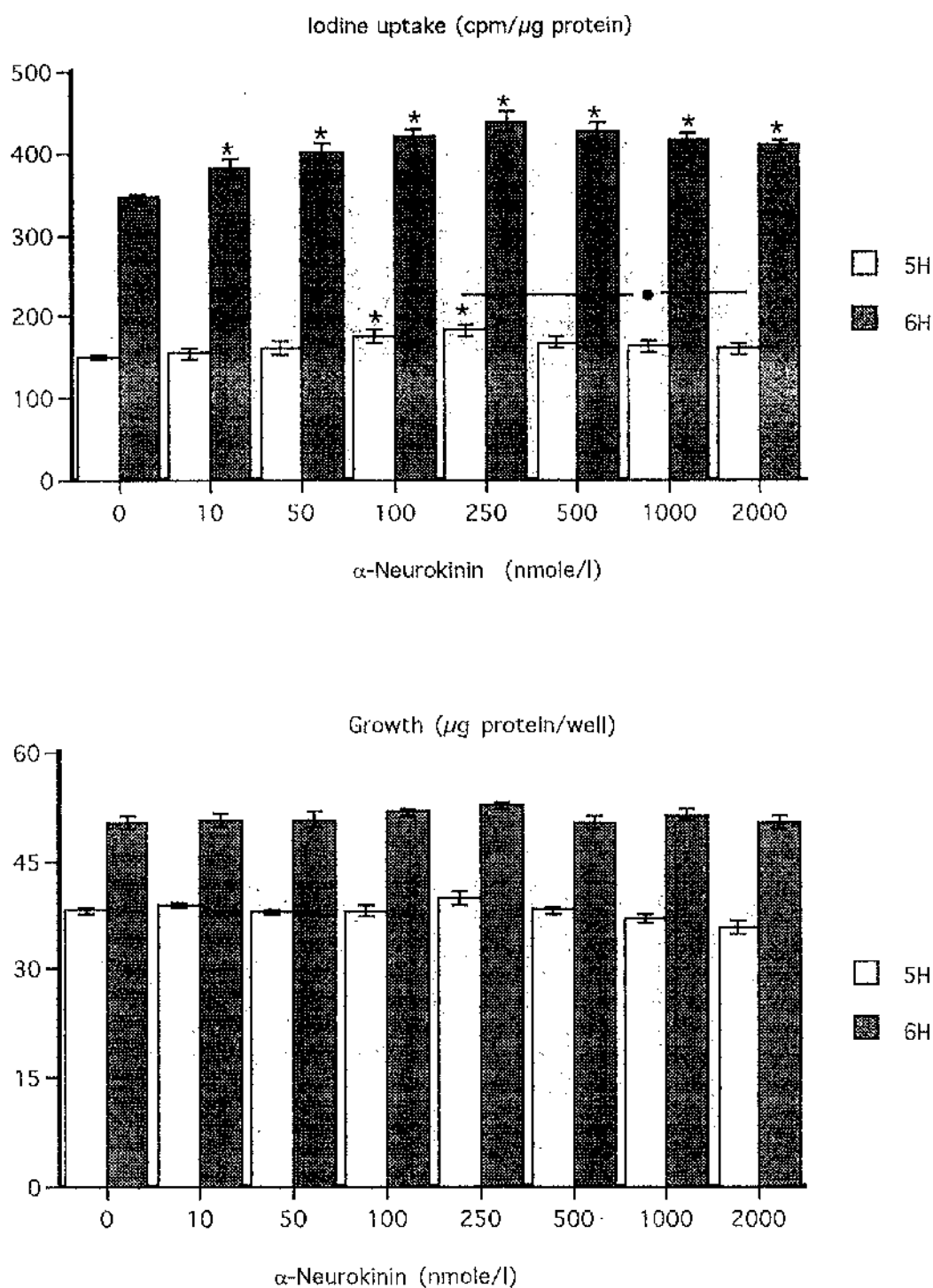


Figure 3.65: Effect of NK (agonist for NK-2 receptor) on iodine uptake and growth of FRTL-5 cells. Cells were incubated in 5H or 6H (2U/l of TSH) medium containing 0.5 % FCS and additional amino acids for 48 h. Significant differences between stimulated and unstimulated cells (control or zero concentration) are shown by *, while • shows a significant decrease.

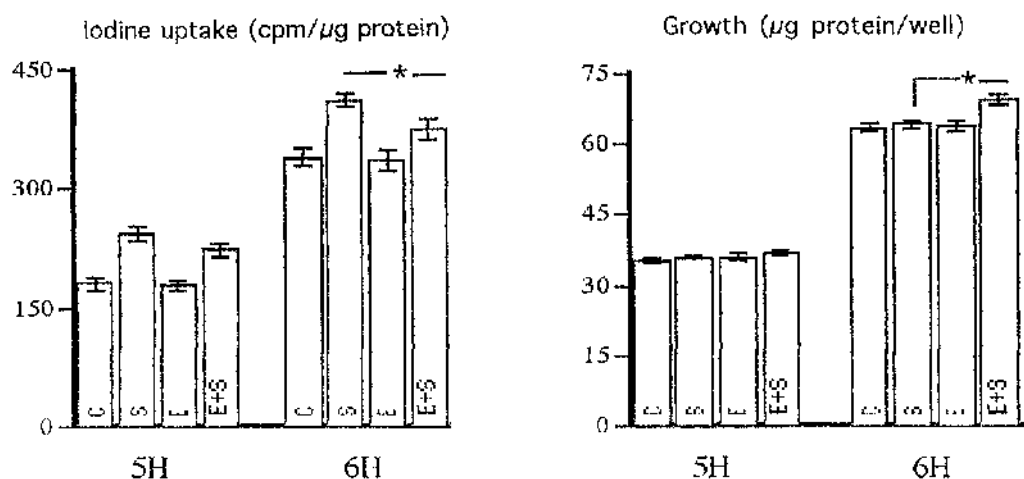


Figure 3.66: Effect of protease inhibitors, on stimulation of FRTL-5 cells with NK. Cells were incubated as before, without NK (C), with 250 nmol/l of NK (S), or with a mixture of 1 μ mol/l of bestatin and 1 μ mol/l of thiorphan in the absence (E) or the presence of NK (S+E). Significant differences caused by inhibitors are represented by (*).

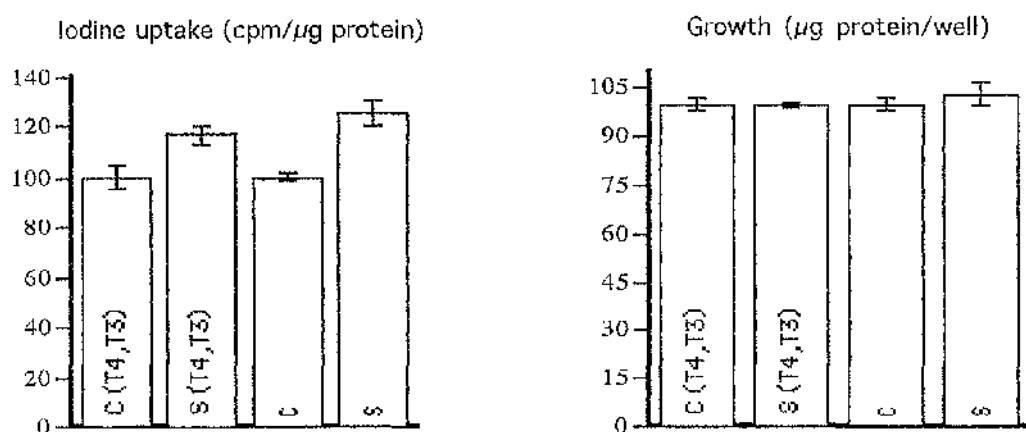


Figure 3.67: Effect of thyroid hormones, on stimulation of FRTL-5 cells with NK and TSH. Cells were stimulated in 6H medium (2 U/l of TSH) as before, without NK (C), with 250 nmol/l of NK (S), or stimulated in the 6H medium containing 0.5 % FCS, additional amino acids, 1 μ mol/l of T₄ and 10 nmol/l of T₃, 48 h before and 48 h after addition of NK [S (T₄,T₃)]. The cells that were stimulated only with thyroid hormones for 96 h served as control [C (T₄,T₃)] for this set.

3.3.6.16.2 *Protease Inhibitors*

Protease inhibitors did not alter the effect of NK on iodine uptake in the absence of TSH, but reduced its stimulatory effect in the presence of TSH from 21.5 % to 11.4 % (figure 3.66). They did not alter the effect of NK on growth of cells incubated in 5H medium, but caused a slight but significant increase in growth rate of 8.7 % in the presence of TSH.

3.3.6.16.3 *Thyroid Hormones*

Thyroid hormones moderately suppressed the effect of NK on iodine uptake (figure 3.67). The increase was 16.5 % compared to 25.1 %. Thyroid hormones did not alter the growth of cells stimulated with NK.

3.3.6.17 Succinyl-[Asp⁶, N-Me-Phe⁸]-Substance P (S-SP)

3.3.6.17.1 *Dose Study*

Succinyl-[Asp⁶, N-Me-Phe⁸]-Substance P is a specific agonist for the Neurokinin 3 receptor. Its final concentrations ranged from 10 to 2000 nmol/l. A concentration of 250 nmol/l was chosen for further studies.

S-SP increased the iodine uptake significantly at concentrations of 250 and 500 nmol/l in the absence of TSH. The maximum increase of 12.68 % was detected at a concentration of 250 nmol/l. S-SP did not affect the growth of cells.

In the presence of TSH, S-SP increased the iodine uptake of cells at concentrations of 10 to 500 nmol/l. The maximum increase was associated with 250 nmol/l of S-SP, 16.29 % higher than control (figure 3.68). At concentrations between 250 and 1000 nmol/l, S-SP increased growth rate, by a maximum of 7.8 % at 500 nmol/l.

3.3.6.17.2 *Protease Inhibitors*

Protease inhibitors inhibited the effect of S-SP on iodine uptake significantly in the absence of TSH. The increase was only 6.2 % compared to 21.4 % (figure 3.69).

In the presence of TSH, the inhibitors also inhibited the effect of S-SP on iodine uptake. The uptake was increased by 7.1 % compared to 19.9 %.

Addition of bestatin and thiorphan did not alter the effect of S-SP on growth of cells incubated in 5H or 6H medium.

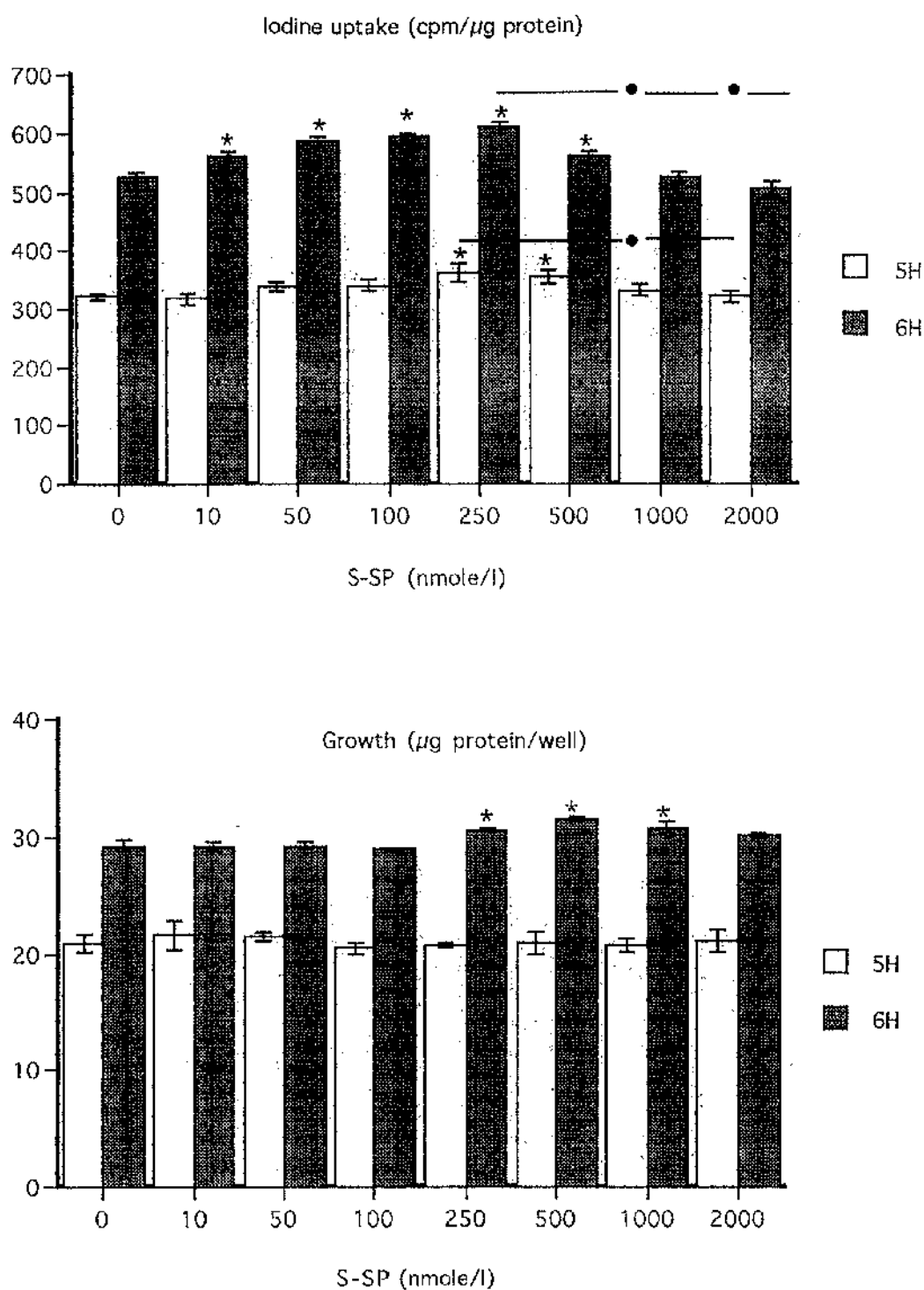


Figure 3.68: Effect of S-SP (agonist for NK-3 receptor) on iodine uptake and growth of FRTL-5 cells. Cells were incubated in 5H or 6H (2U/l of TSH) medium containing 0.5 % FCS and additional amino acids for 48 h. Significant differences between stimulated and unstimulated cells (control or zero concentration) are shown by *, while • indicates significant decreases.

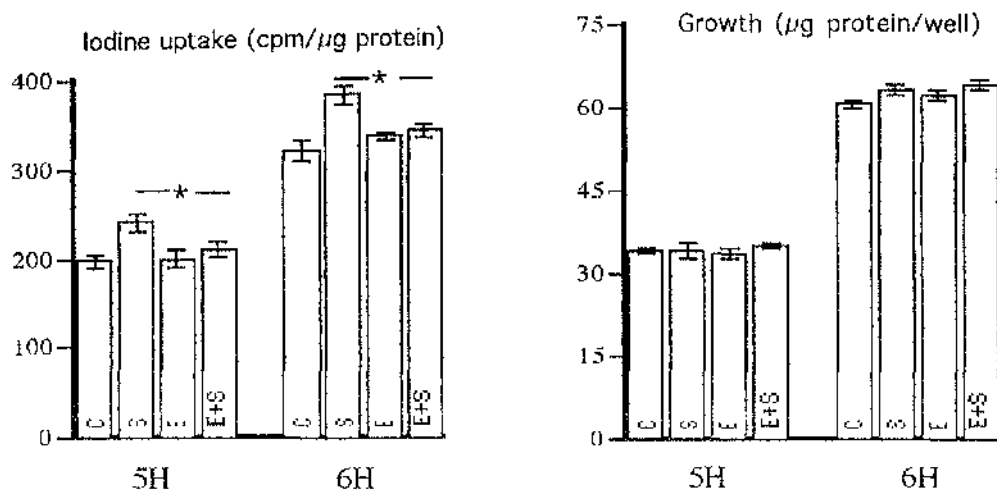


Figure 3.69: Effect of protease inhibitors, on stimulation of FRTL-5 cells with S-SP. Cells were incubated as before, without S-SP (C), with 250 nmol/l of S-SP (S), or with a mixture of 1 μ mol/l of bestatin and 1 μ mol/l of thiorphan in the absence (E) or the presence of S-SP (S+E). Significant differences caused by inhibitors are represented by *.

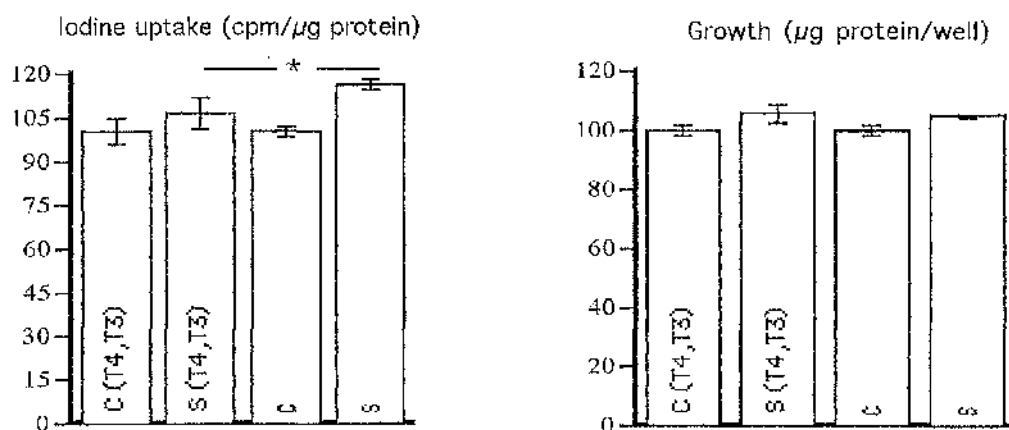


Figure 3.70: Effect of thyroid hormones, on stimulation of FRTL-5 cells with S-SP and TSH. Cells were stimulated in 6H medium (2 U/l of TSH) as before, without S-SP (C), with 250 nmol/l of S-SP (S), or stimulated in the 6H medium containing 0.5 % FCS, additional amino acids, 1 μ mol/l of T₄ and 10 nmol/l of T₃, 48 h before and 48 h after addition of S-SP [S (T₄,T₃)]. The cells that were stimulated only with thyroid hormones for 96 h served as control [C (T₄,T₃)] for this set. Significant differences between the presence and the absence of thyroid hormones is shown by (*).

3.3.6.17.3 *Thyroid Hormones*

Thyroid hormones reduced the effect of S-SP on iodine uptake from an increase of 16.3 % to 6.5 % (figure 3.70). Thyroid hormones did not alter the growth of cells stimulated with S-SP.

3.3.6.18 Noradrenaline (NA)

3.3.6.18.1 *Dose Study*

The final concentrations of \pm noradrenaline ranged from 0.05 to 20 $\mu\text{mol/l}$. A concentration of 20 $\mu\text{mol/l}$ was chosen for further study.

Inclusion of noradrenaline in 5H medium did not change the rate of iodine uptake or growth.

In the presence of TSH, the iodine uptake of stimulated cells increased significantly with all doses of noradrenaline. The highest increase was associated with 20 $\mu\text{mol/l}$ of noradrenaline, 93.9 % higher than the value obtained for the control (figure 3.71). Growth was unaltered.

3.3.6.18.2 *Protease Inhibitors*

The inclusion of bestatin and thiorphan did not alter the effect of noradrenaline on either iodine uptake or growth, either in the presence or the absence of TSH (figure 3.72).

3.3.6.18.3 *Thyroid Hormones*

Thyroid hormones increased the effect of noradrenaline on iodine uptake but not growth (figure 3.73). The increase in uptake was 121.1 % compared to 93.9 %.

3.3.6.19 S-Nitroso-N-Acetyl Penicillamine (SNAP)

3.3.6.19.1 *Dose Study*

SNAP is a stable donor of nitric oxide and releases it into the medium during the incubation. The final concentrations of SNAP used ranged from 1 to 10 $\mu\text{mol/l}$.

SNAP decreased the iodine uptake of cells significantly at concentrations of 5 and 10 $\mu\text{mol/l}$ in the absence of TSH. The decreases were 12.6 and 30.4 % lower than control. The difference between values obtained for iodine uptake of cells at

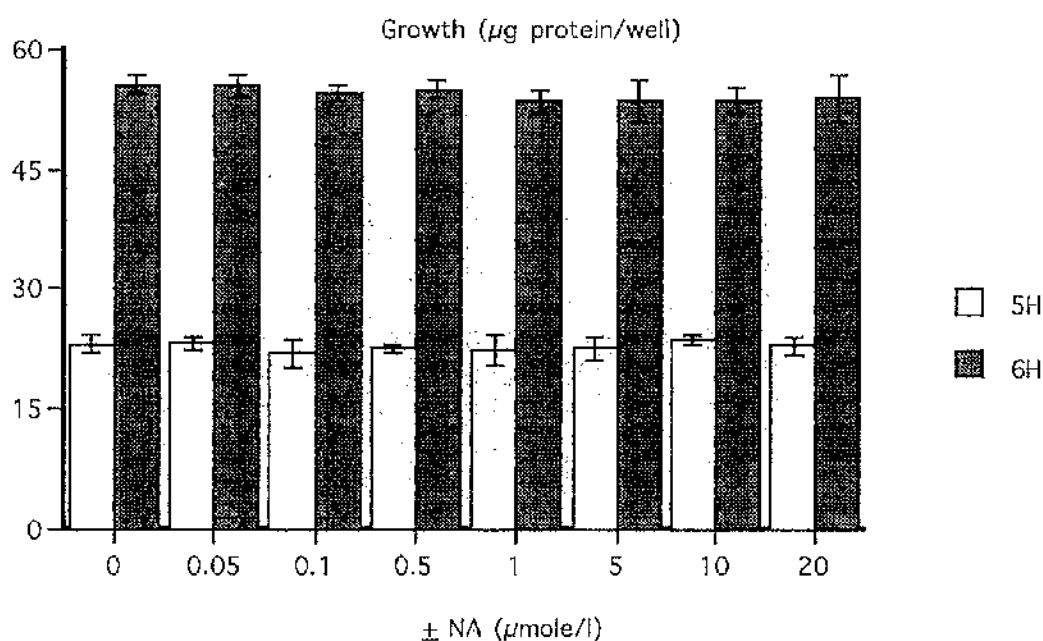
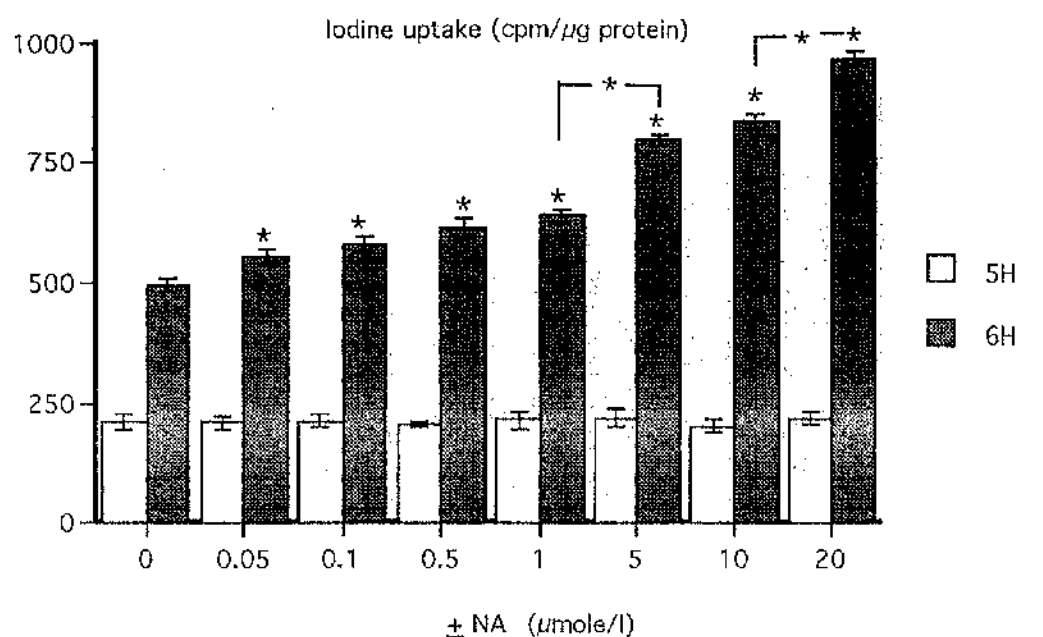


Figure 3.71: Effect of \pm noradrenaline on iodine uptake and growth of FRTL-5 cells. Cells were incubated in 5H or 6H (2U/l of TSH) medium containing 0.5 % FCS and additional amino acids for 48 h. Significant differences between stimulated and unstimulated cells (control or zero concentration) are shown by *.

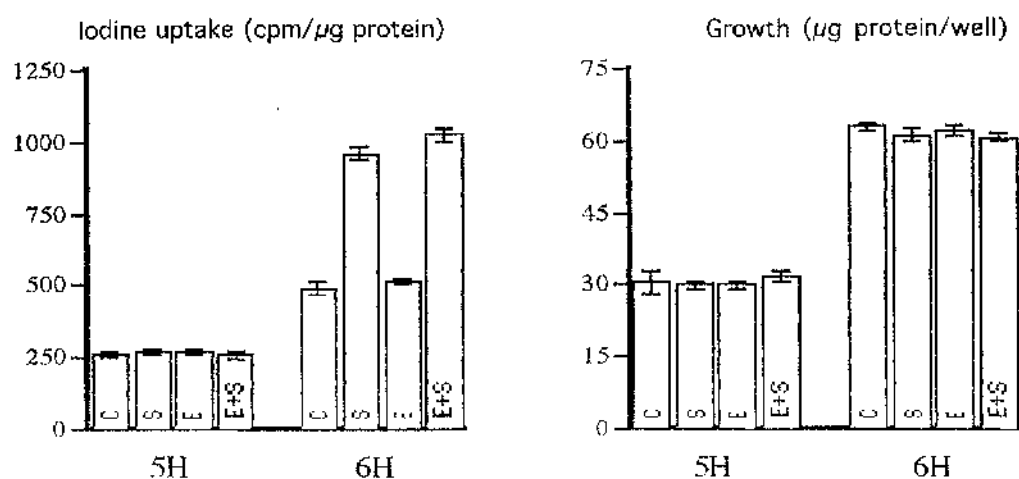


Figure 3.72: Effect of protease inhibitors, on stimulation of FRTL-5 cells with \pm noradrenaline. Cells were incubated as before, without \pm noradrenaline (C), with 20 $\mu\text{mol/l}$ of \pm noradrenaline (S), or with a mixture of 1 $\mu\text{mol/l}$ of bestatin and 1 $\mu\text{mol/l}$ of thiorphan in the absence (E) or the presence of \pm noradrenaline (S+E).

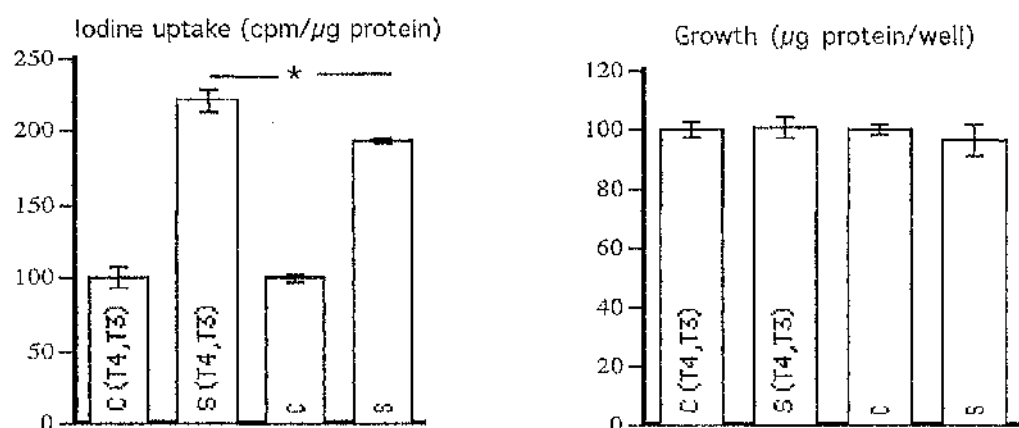


Figure 3.53: Effect of thyroid hormones, on stimulation of FRTL-5 cells with \pm noradrenaline and TSH. Cells were stimulated in 6H medium (2 U/l of TSH) as before, without \pm noradrenaline (C), with 20 $\mu\text{mol/l}$ of \pm noradrenaline (S), or stimulated in the 6H medium containing 0.5 % FCS, additional amino acids, 1 $\mu\text{mol/l}$ of T₄ and 10 nmol/l of T₃, 48 h before and 48 h after addition of \pm noradrenaline [S (T₄, T₃)]. The cells that were stimulated only with thyroid hormones for 96 h served as control [C (T₄, T₃)] for this set. Significant differences between the presence and the absence of thyroid hormones is shown by *.

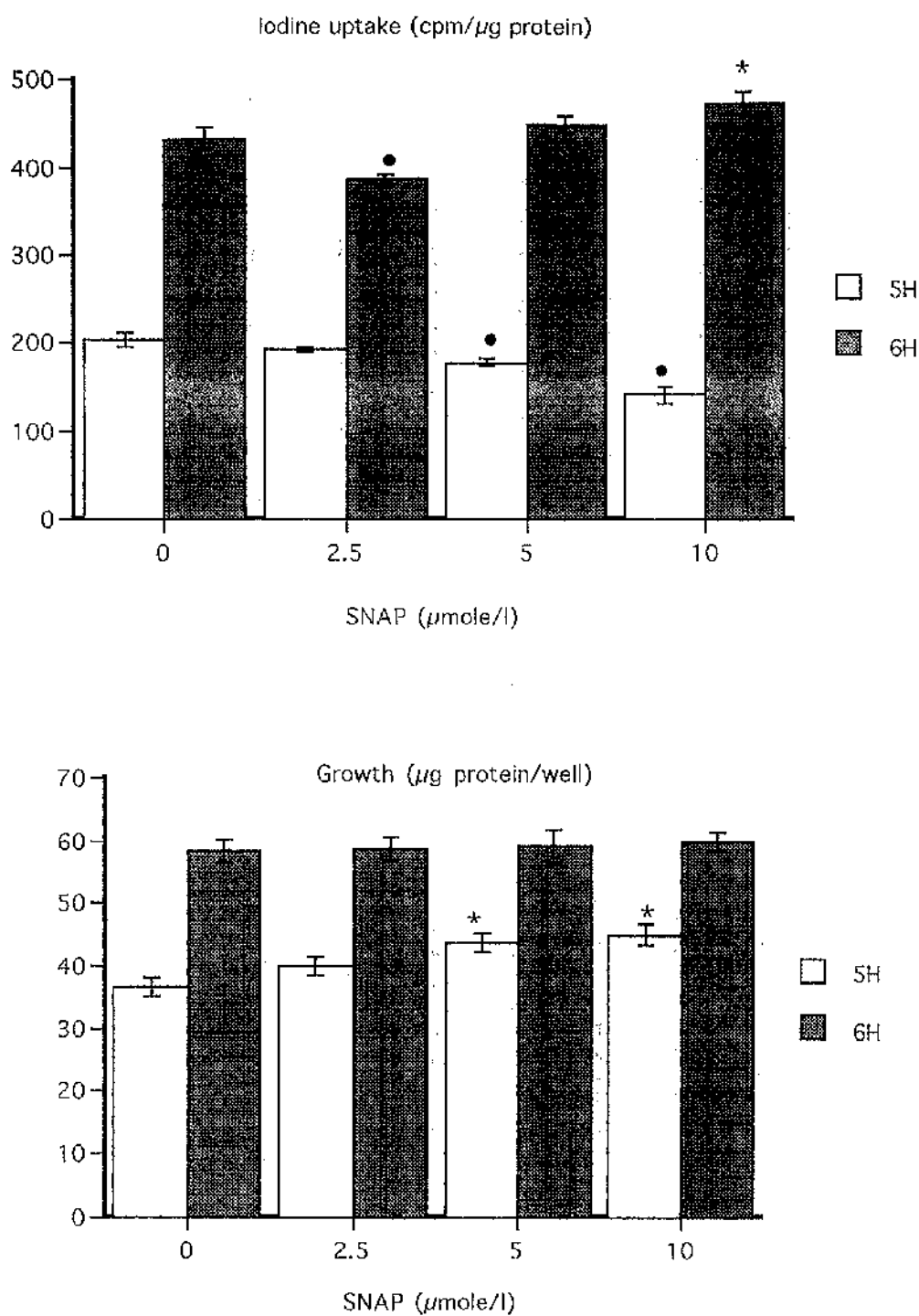


Figure 3.74: Effect of SNAP on iodine uptake and growth of FRTL-5 cells. Cells were incubated in 5H or 6H (2U/l of TSH) medium containing 0.5 % FCS and additional amino acids for 48 h. Significant differences between stimulated and unstimulated cells (control or zero concentration) are shown by *, while • shows significant decreases compared to control.

concentrations of 5 and 10 $\mu\text{mol/l}$ was significant (figure 3.74). SNAP increased growth significantly at concentrations of 5 $\mu\text{mol/l}$ and above, to a maximum of 22.82 % at a concentration of 10 $\mu\text{mol/l}$.

In the presence of 2 U/l of TSH (6H), SNAP decreased the iodine uptake of cells significantly at a concentration of 2.5 $\mu\text{mol/l}$, while it increased uptake significantly at a concentration of 10 $\mu\text{mol/l}$. The decrease was 11.1 %, and the increase was 9.1 %. SNAP did not change the growth of cells at any concentration.

3.3.6.20 Interleukin-1 And Insulin Like Growth Factor-1

The final concentration of IL-1 was 1 or 20 $\mu\text{g/l}$, while that of IGF-1 was 500 $\mu\text{g/l}$.

In 5H medium, addition of IL-1 did not alter the effect of IGF-1 on iodine uptake. A concentration of 20 $\mu\text{g/l}$ of IL-1 did reduce the growth effect of IGF-1 from 22.4 % to 10.2 %, but 1 $\mu\text{g/l}$ had no effect (figure 3.75).

In the presence of TSH, IL-1 at a concentration of 1 $\mu\text{g/l}$ did not alter the effect of IGF-1 on iodine uptake. At a concentration of 20 $\mu\text{g/l}$ of IL-1 the effects of IGF-1 and IL-1 were additive, IGF-1 causing an increase of 38.6 %, IL-1 causing a decrease of 23.8 %, and the combination resulting in a 7.8 % increase. Both 1 $\mu\text{g/l}$ and 20 $\mu\text{g/l}$ of IL-1 reduced the effect of IGF-1 on growth. The increase was reduced from 18.6 % to 11.0 % by the lower and to 8.6 % by the higher dose.

3.3.6.21 Interleukin-1 And Interferon- γ

The final concentration of IL-1 was 20 $\mu\text{g/l}$, while that of INF- γ was 100 $\mu\text{g/l}$.

In 5H medium, IL-1 did not alter the increase in iodine uptake of 27.1 % caused by INF- γ (figure 3.76), and neither altered growth rate.

In the presence of TSH, the effects of IL-1 and INF- γ on iodine uptake were additive. IL-1 decreased it by 23.77 %, INF- γ caused an increase of 73.08 %, and the mixture of IL-1 and INF- γ caused an increase of 31.35 % in iodine uptake. IL-1 did not significantly change the decrease of 31.9 % in growth rate caused by INF- γ .

3.3.6.22 Interleukin-1 And Neuropeptide Y

The final concentration of IL-1 was 1 or 20 $\mu\text{g/l}$, while that of NPY was 500 nmol/l.

In 5H medium, neither IL-1 at concentrations of 1 or 20 $\mu\text{g/l}$ nor NPY affected iodine uptake of the cells significantly in isolation, but the mixture of 1 $\mu\text{g/l}$

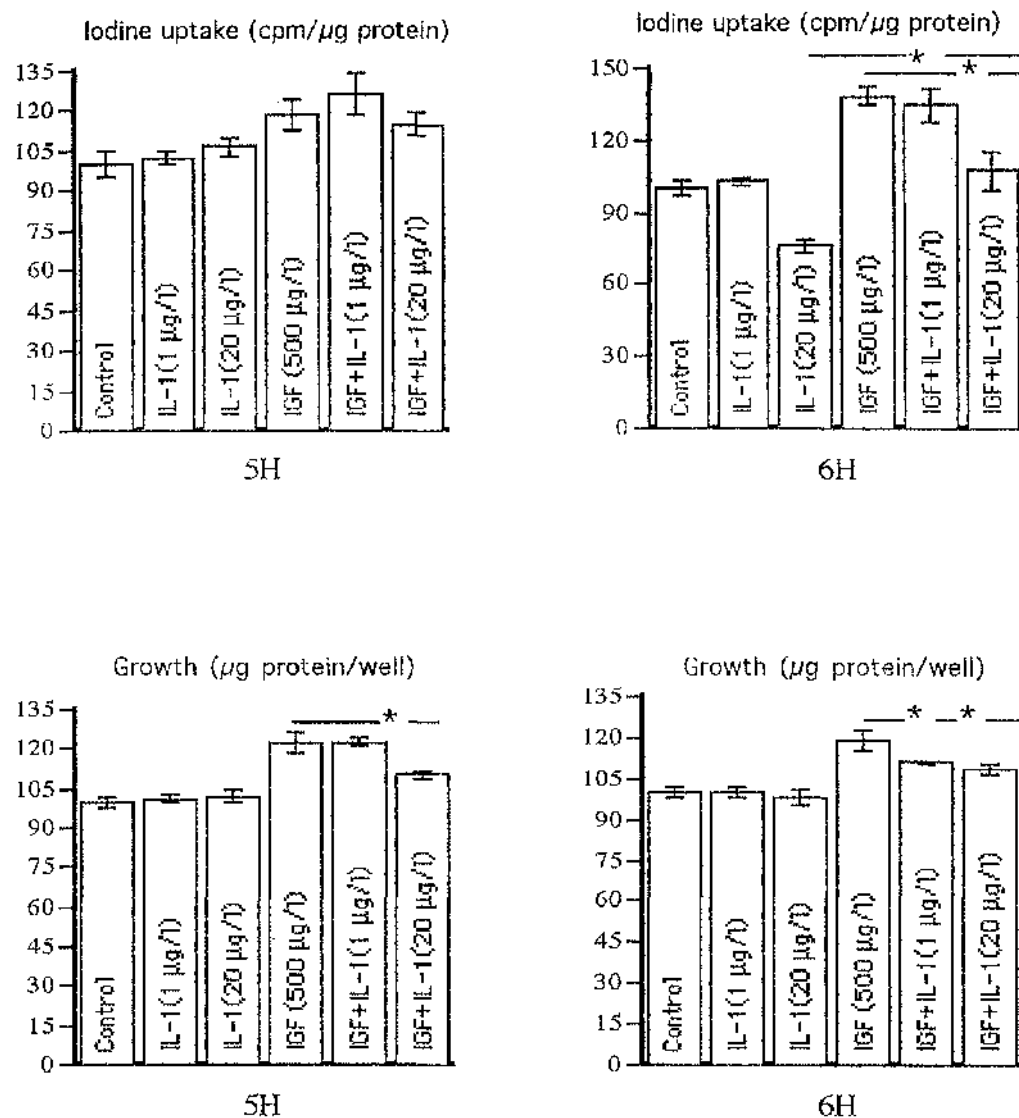


Figure 3.75: Effect of combining IL-1 and IGF-1 on FRTL-5 cells. Cells were stimulated for 48 h as before, with single stimulator or combination of them as labelled in the columns. Unstimulated cells served as control. The symbol (*) shows significant differences compared with individual stimulator.

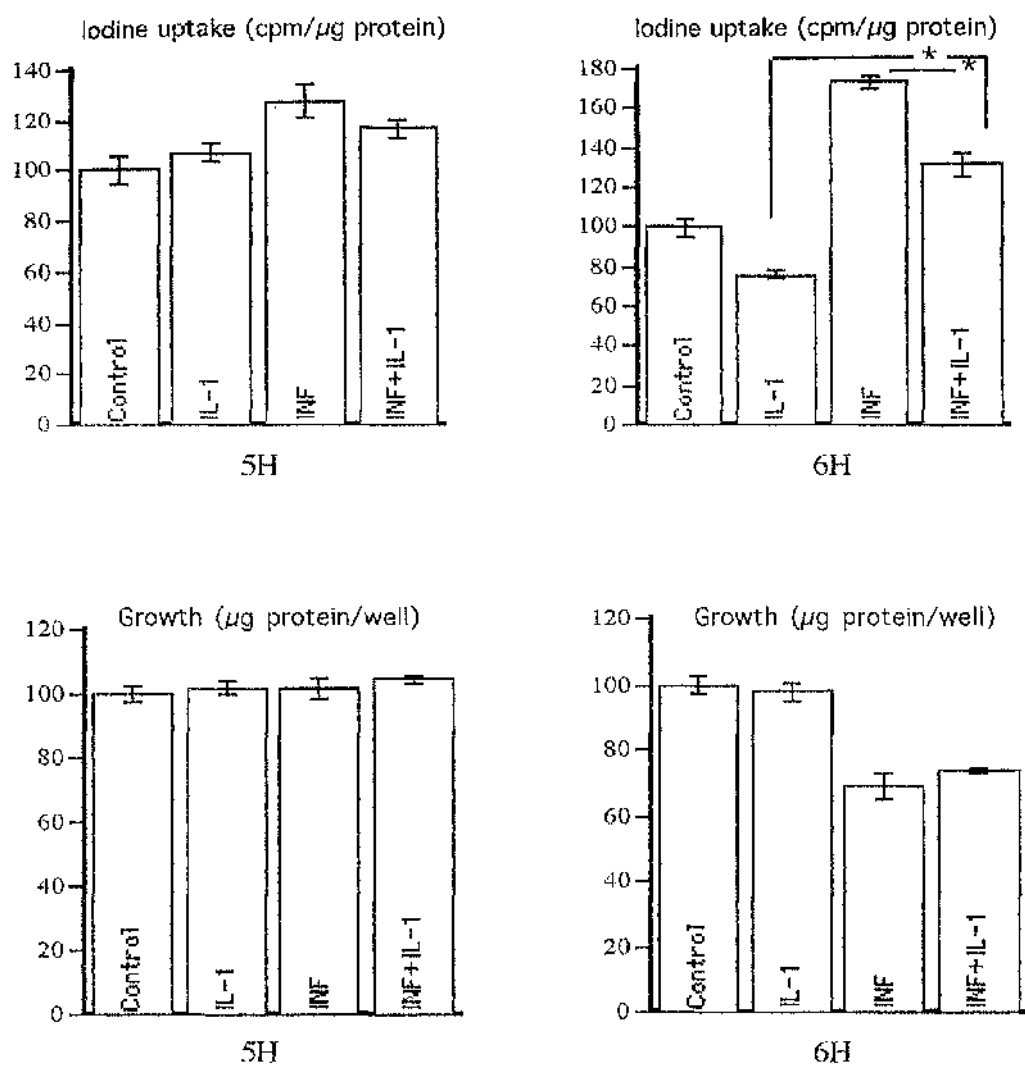


Figure 3.76: Effect of combining IL-1 and INF- γ on FRTL-5 cells. Cells were stimulated for 48 h as before, with single stimulator or combination of them as labelled in the columns. Unstimulated cells served as control. The final concentrations were 2 U/l of TSH, 20 μ g/l of IL-1, and 100 μ g/l of INF- γ . The symbol (*) shows significant differences compared with individual stimulator.

of IL-1 and NPY caused a significant increase of 16.49 %. A non-significant decrease of 7.63 % occurred in iodine uptake of cells stimulated with a mixture of 20 $\mu\text{g/l}$ of IL-1 and NPY (figure 3.77).

In the presence of TSH, a mixture of 1 $\mu\text{g/l}$ of IL-1 and NPY caused an increase of 14.08 % in iodine uptake, greater than that for NPY alone (9.8 %). The mixture of 20 $\mu\text{g/l}$ of IL-1 and NPY caused a significant decrease of 27.82 %, similar to that obtained with 20 $\mu\text{g/l}$ of IL-1 alone. In the presence or the absence of TSH, neither IL-1 nor NPY nor the mixture affected the growth of cells.

3.3.6.23 Interleukin-1 And Noradrenaline

The final concentration of IL-1 was 1 or 20 $\mu\text{g/l}$, while that of \pm noradrenaline (NA) was 20 $\mu\text{mol/l}$.

In 5H medium, neither IL-1 at a concentration of 1 or 20 $\mu\text{g/l}$, NA alone, nor their combination affected iodine uptake of the cells (figure 3.78).

In the presence of TSH (6H), NA caused a significant increase of 64.8 %. The mixture of 1 $\mu\text{g/l}$ of IL-1 and NA caused an increase of significantly lower magnitude, 53.1 %. The mixture of 20 $\mu\text{g/l}$ of IL-1 and NA also caused a smaller increase, 42.10 %, than NA alone. This indicates that IL-1 antagonised the effect of NA on iodine uptake. In the presence or the absence of TSH, neither the individual stimulators nor their combination affected the growth of cells.

3.3.6.24 Interleukin-1 And Vasoactive Intestinal Peptide

The final concentration of IL-1 was 1 or 20 $\mu\text{g/l}$, while that of VIP was 500 nmol/l.

In 5H medium, IL-1 at concentrations of 1 or 20 $\mu\text{g/l}$ did not alter the increase in iodine uptake caused by VIP (figure 3.79). Stimulation of the cells with 1 or 20 $\mu\text{g/l}$ of IL-1 did not alter the effect of VIP on growth.

In the presence of TSH (6H), IL-1 a concentration of 1 $\mu\text{g/l}$ did not affect the iodine uptake caused by VIP, but a concentration of 20 $\mu\text{g/l}$ antagonised the increase. The increase caused by VIP alone was 42.3 %, while that in the presence of IL-1 was 11.2 %. Neither of the stimulators IL-1 nor VIP, nor their combination, affected the growth of cells.

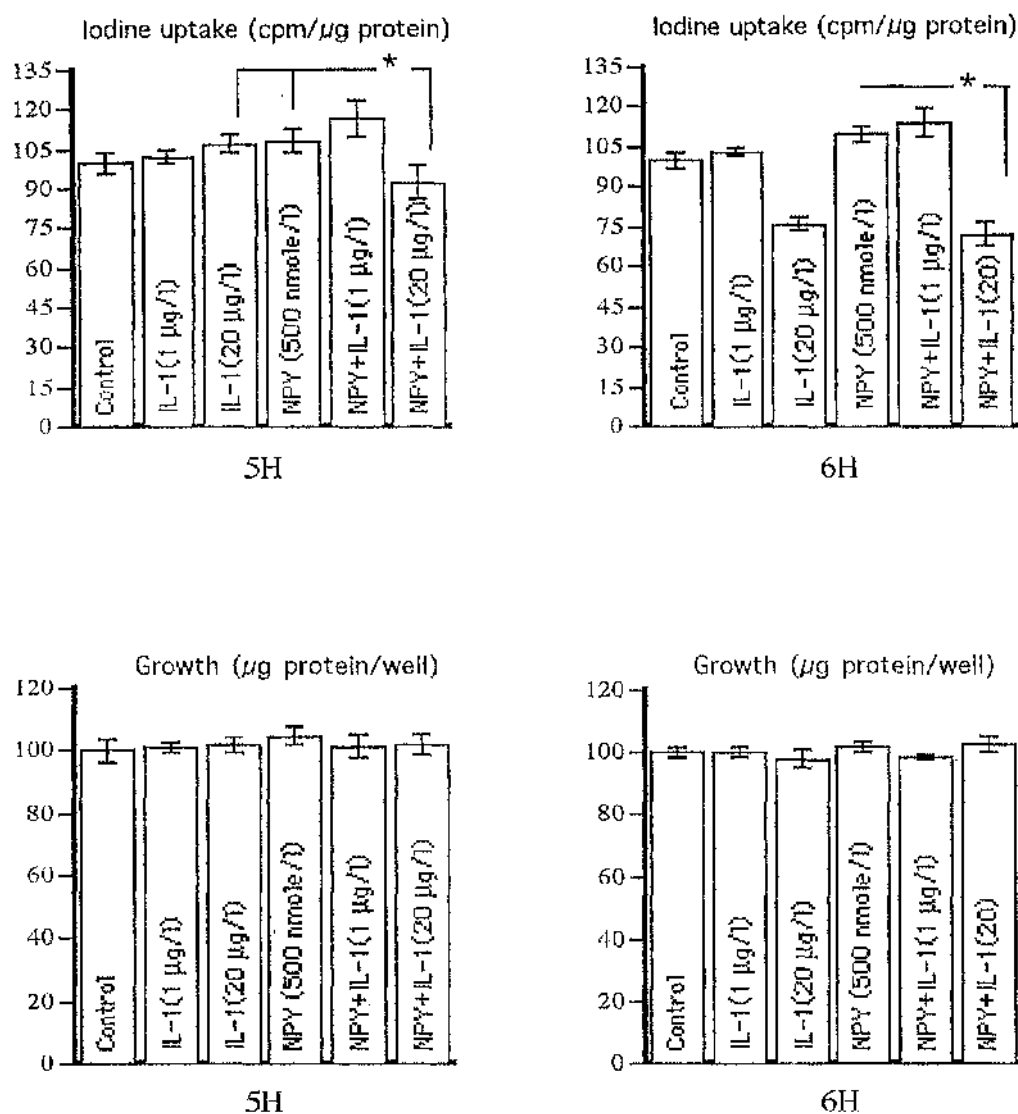


Figure 3.77: Effect of combining IL-1 and NPY on FRTL-5 cells. Cells were stimulated for 48 h as before, with single stimulator or combination of them as labelled in the columns. Unstimulated cells served as control. The symbol (*) shows significant differences compared with individual stimulator.

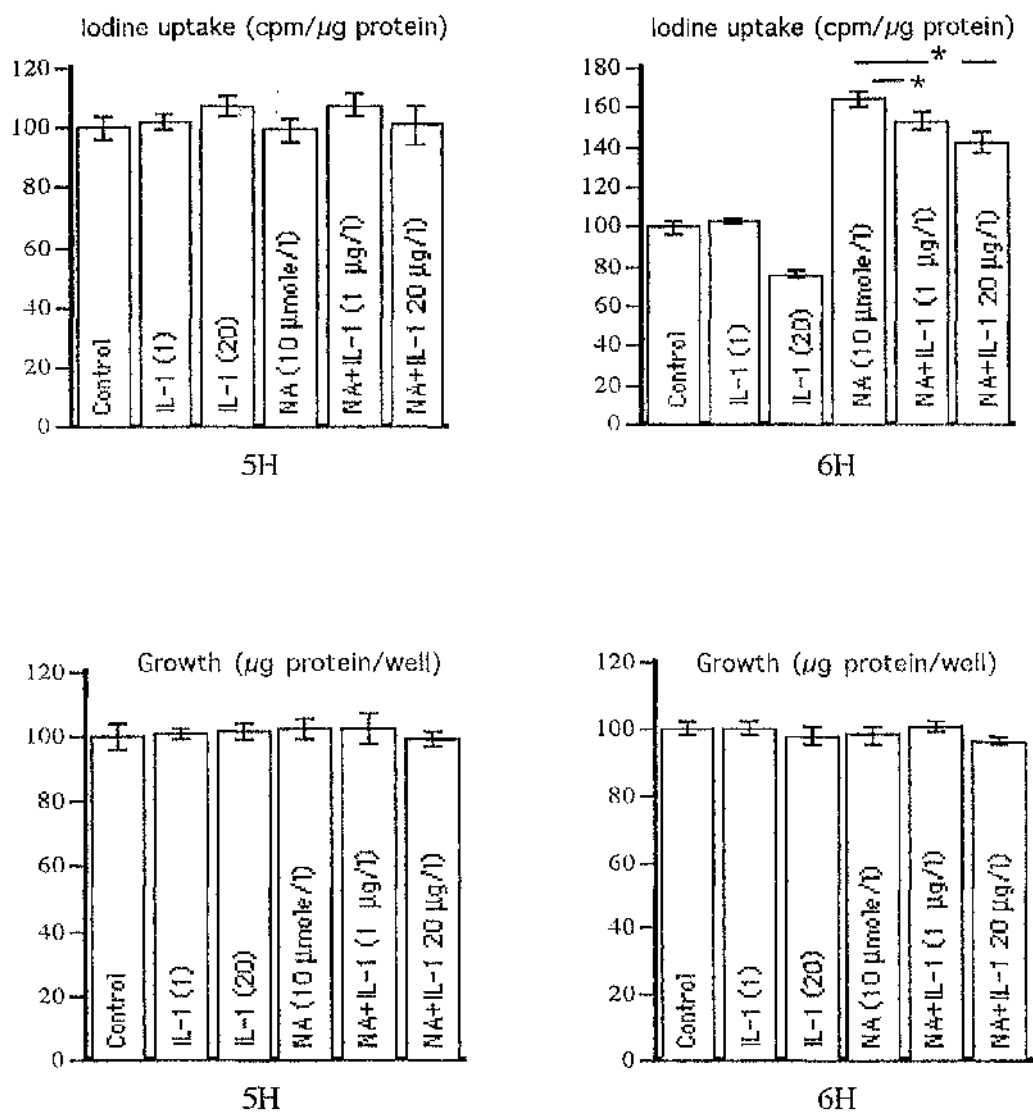


Figure 3.78: Effect of combining IL-1 and \pm noradrenaline on FRTL-5 cells. Cells were stimulated for 48 h as before, with single stimulator or combination of them as labelled in the columns. Unstimulated cells served as control. The symbol (*) shows significant differences compared with individual stimulator.

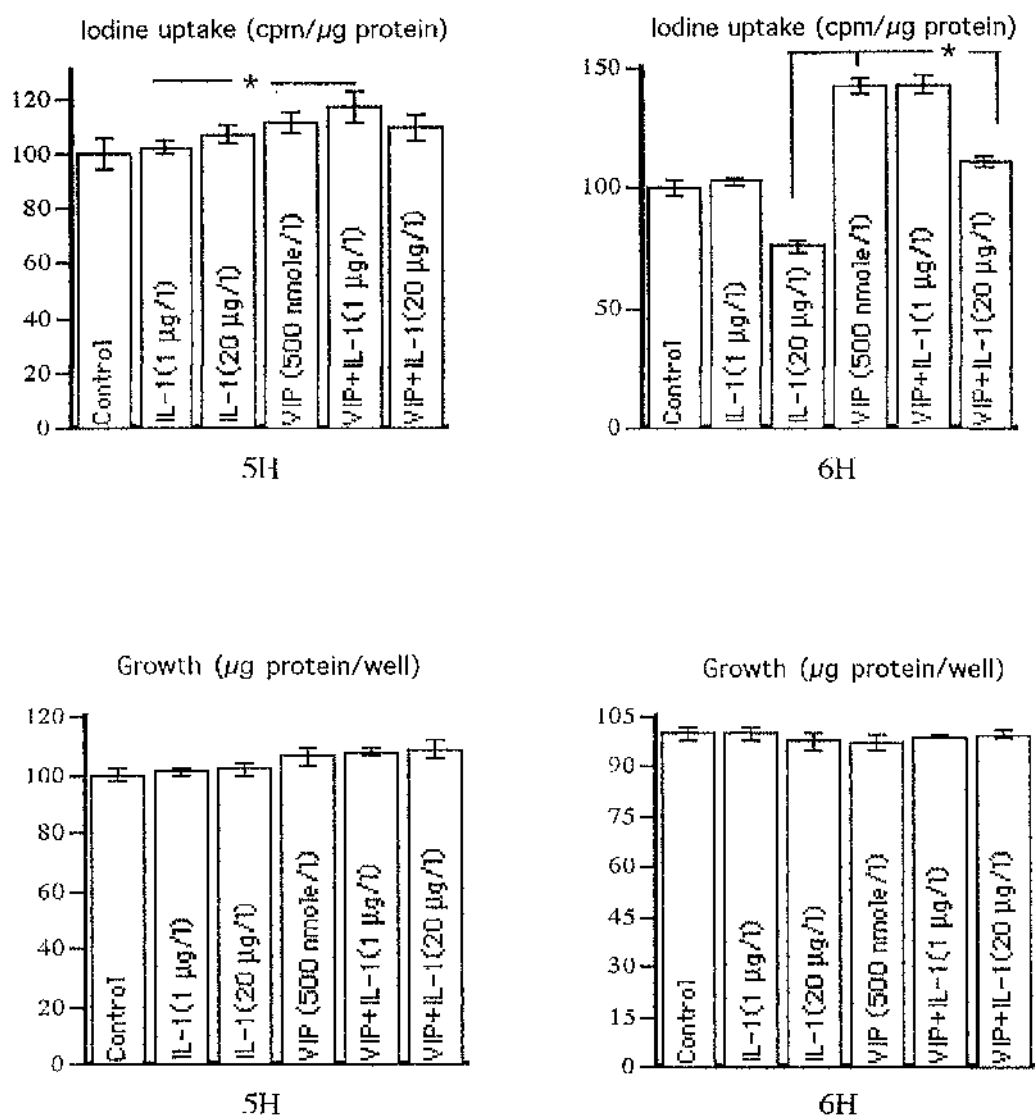


Figure 3.79: Effect of combining IL-1 and VIP on FRTL-5 cells. Cells were stimulated for 48 h as before, with single stimulator or combination of them as labelled in the columns. Unstimulated cells served as control. The symbol (*) shows significant differences compared with individual stimulator.

3.3.6.25 Interleukin-1 And [Sar⁹, Met(O₂)¹¹]-Substance P

The final concentration of IL-1 was 1 µg/l, while that of [Sar⁹, Met(O₂)¹¹]-Substance P (SMO-SP) was 250 nmol/l.

In 5H medium, the increase caused by the mixture of IL-1 and SMO-SP was 30.4 %, significantly higher than the corresponding values obtained for IL-1 and SMO-SP alone (figure 3.80). In the presence of TSH, IL-1 had no effect on the increase of 19.6 % caused by SMO-SP on iodine uptake. In the presence or absence of TSH, neither of the stimulators nor their combination affected the growth of cells.

3.3.6.26 Interleukin-1 And α-Neurokinin

The final concentration of IL-1 was 1 µg/l, while that of α-Neurokinin (NK) was 250 nmol/l.

IL-1 did not alter the effects of NK on iodine uptake, either in the presence or absence of TSH (figure 3.81). Neither IL-1 nor NK nor their combination affected the growth of cells.

3.3.6.27 Interleukin-1 And Succinyl-[Asp⁶, N-Me-Phe⁸]-Substance P

The final concentration of IL-1 was 1 µg/l, while that of Succinyl-[Asp⁶, N-Me-Phe⁸]-Substance P (S-SP) was 250 nmol/l.

IL-1 did not significantly affect the increase in iodine uptake caused by S-SP alone either in the presence or absence of TSH (figure 3.82). Neither IL-1 nor S-SP nor their combination affected the growth of cells.

3.3.6.28 Selective Agonists Of Neurokinin Receptors

The final concentration of each agonist was 250 nmol/l.

The iodine uptake of the cells stimulated with 5H medium containing any one of the agonists was significantly increased. The increases above control were 18.8 % for SMO-SP, 20.0 % for NK, and 11.9 for S-SP. The increases above control when two agonists were added simultaneously were 26.4 % for a mixture of SMO-SP and NK, 29.3 % for a mixture of SMO-SP and S-SP, and 27.2 % for a mixture of NK and S-SP. Stimulation of the cells with medium containing all 3 agonists increased the rate of iodine uptake by even more, 34.0 %.

None of the increases caused by combinations of two agonists was significant when compared with the value obtained for each individual stimulator, while the

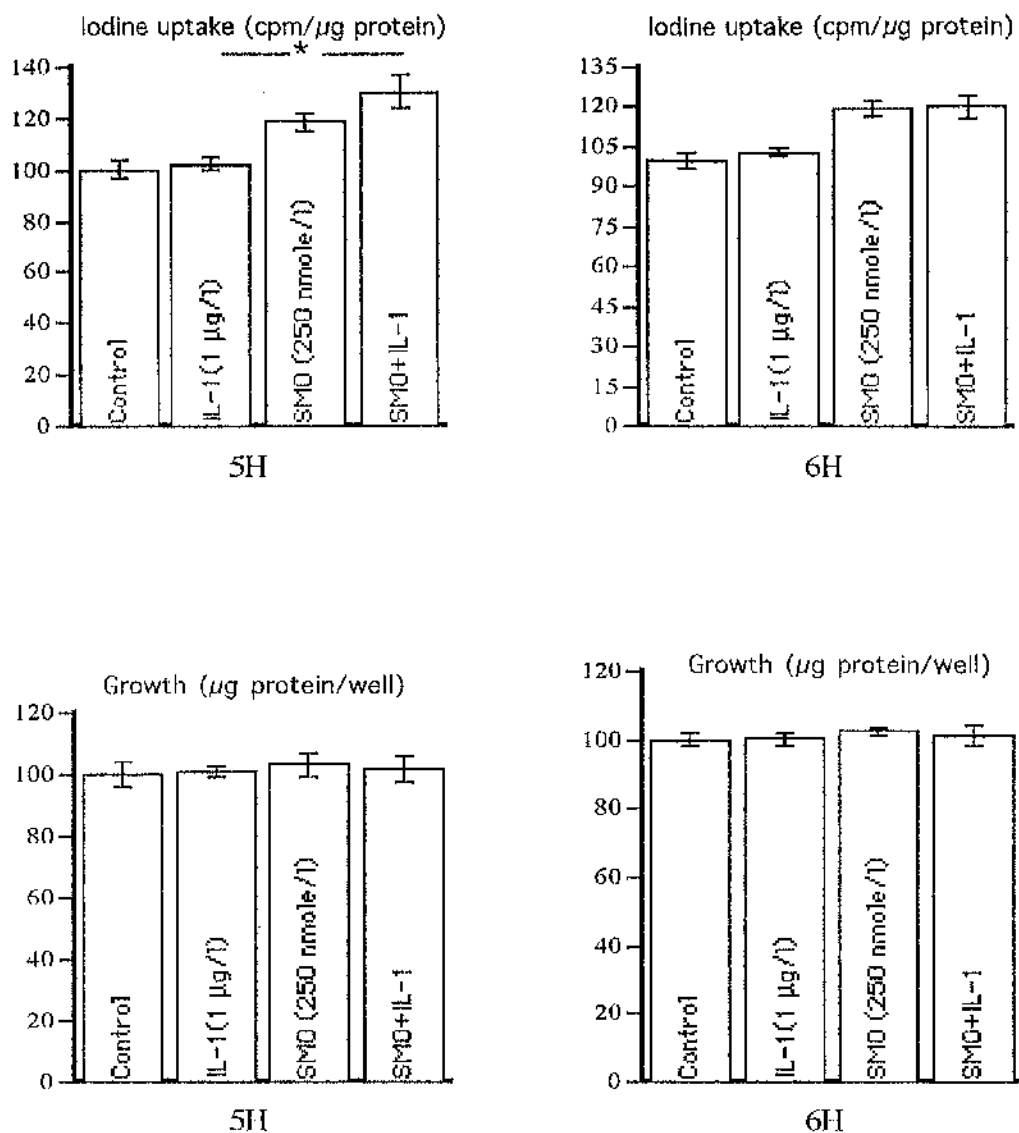


Figure 3.80: Effect of combining IL-1 and SMO-SP on FRTL-5 cells. Cells were stimulated for 48 h as before, with single stimulator or combination of them as labelled in the columns. Unstimulated cells served as control. The symbol (*) shows significant differences compared with individual stimulator.

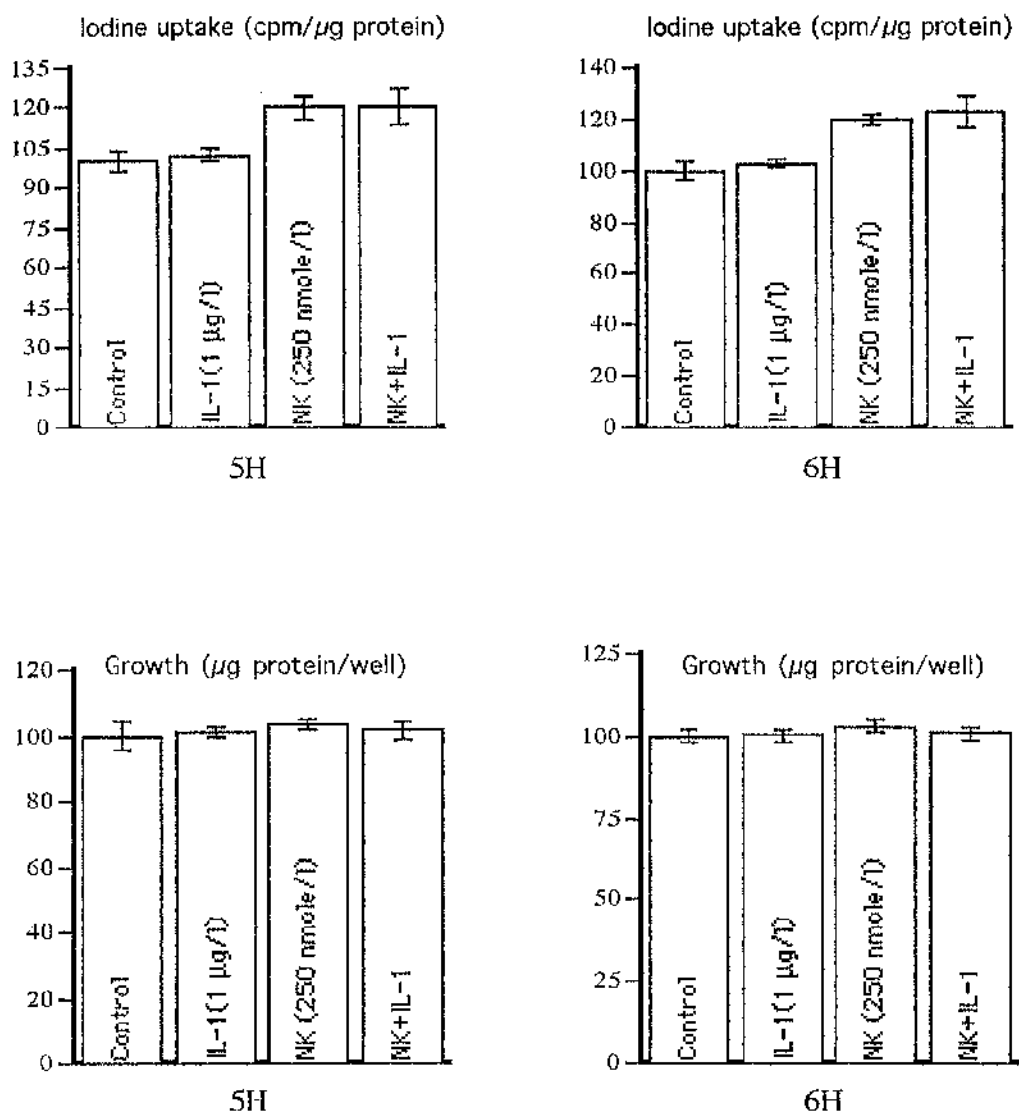


Figure 3.81: Effect of combining IL-1 and NK on FRTL-5 cells. Cells were stimulated for 48 h as before, with single stimulator or combination of them as labelled in the columns. Unstimulated cells served as control.

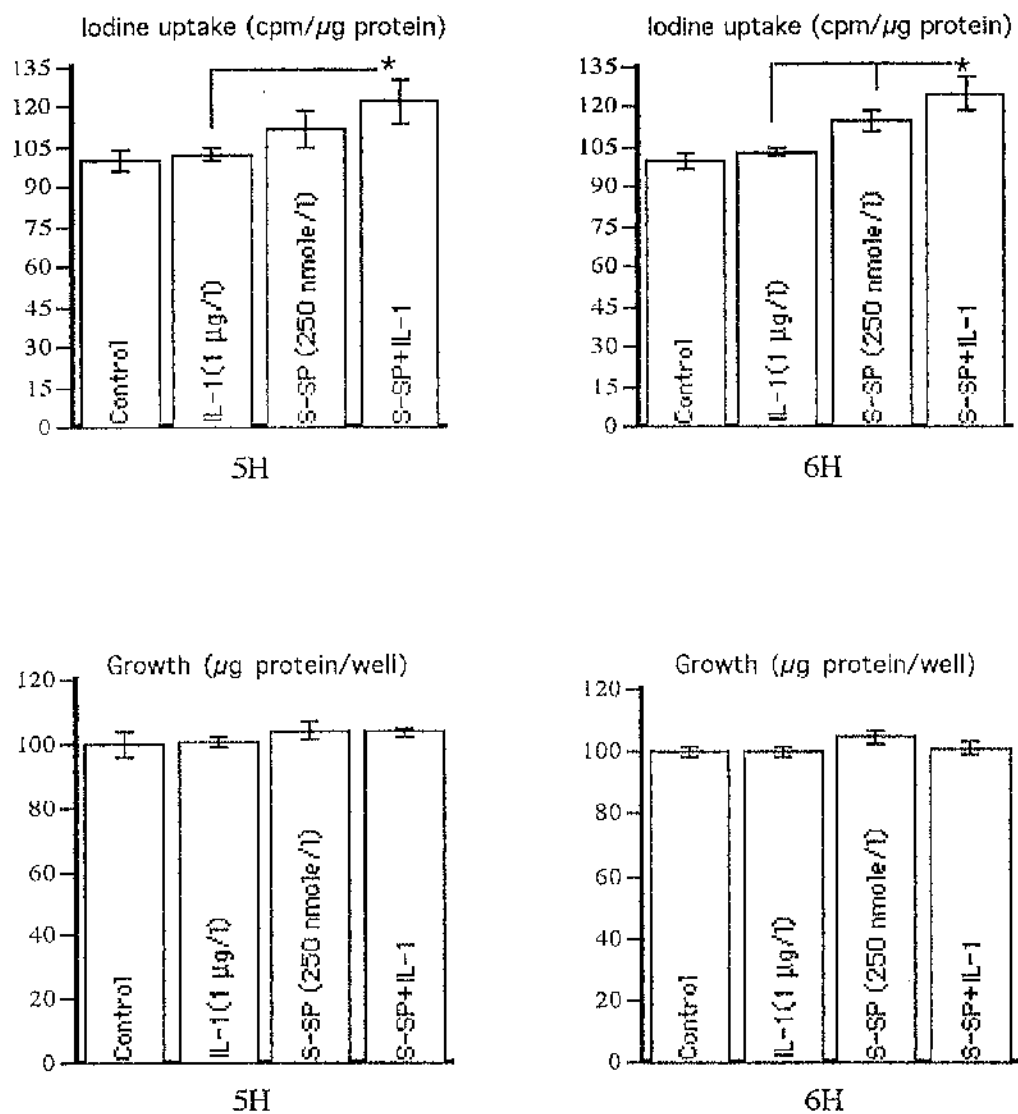


Figure 3.82: Effect of combining IL-1 and S-SP on FRTL-5 cells. Cells were stimulated for 48 h as before, with single stimulator or combination of them as labelled in the columns. Unstimulated cells served as control. The symbol (*) shows significant differences compared with individual stimulator.

value obtained for the mixture containing 3 agonists was significantly higher than the iodine uptake of each individual agonist (figure 3.83).

The iodine uptake of the cells stimulated with TSH containing any one of the agonists was significantly increased. The increases above control were 19.6 % for SMO-SP, 20.1 % for NK, and 14.9 for S-SP. Stimulation of cells with 6H medium containing two agonists also increased the rate of iodine uptake significantly. The increases above control were 36.9 % for a mixture of SMO-SP and NK, 30.7 % for a mixture of SMO-SP and S-SP, and 37.7 % for a mixture of NK and S-SP.

Stimulation of the cells with 6H medium containing all 3 agonists increased the rate of iodine uptake by 37.0 %.

All of the increases caused by combinations of two agonists were significant when compared with the value obtained for each individual stimulator, but the value obtained for the mixture containing 3 agonists was not different from the result obtained for the mixture containing two agonists (figure 3.83).

In the presence or absence of TSH, none of the stimulators affected the growth of cells, alone or in combination.

3.3.6.29 Noradrenaline And Insulin Like Growth Factor-1

The final concentration of \pm noradrenaline (NA) was 10 $\mu\text{mol/l}$, while that of Insulin like growth factor-1 was 500 $\mu\text{g/l}$.

NA did not alter the effect of IGF-1 on iodine uptake or growth rate in 5H medium (figure 3.84).

In the presence of TSH, the mixture of IGF and NA caused an increase of 96.9 % in iodine uptake. NA alone caused an increase of 64.8 %, while IGF caused an increase of 38.6 %. The effects of the stimulators were additive. NA did not alter the increase in growth rate caused by IGF.

3.3.6.30 Noradrenaline And Vasoactive Intestinal Peptide

The final concentration of \pm noradrenaline (NA) was 10 $\mu\text{mol/l}$, while that of Vasoactive Intestinal Peptide (VIP) was 500 nmol/l.

In the absence of TSH, NA did not alter the significant increase in iodine uptake caused by VIP (figure 3.85). However, in the presence of TSH, the mixture of 500 nmol/l of VIP and NA caused an increase of 94.5 %, compared to 64.8 % and 42.4 % for the single stimulators.

In the absence of TSH, NA did not affect the increase in growth caused by VIP. In the presence of TSH, 10 $\mu\text{mol/l}$ of NA or 500 nmol/l of VIP for 48 h did not

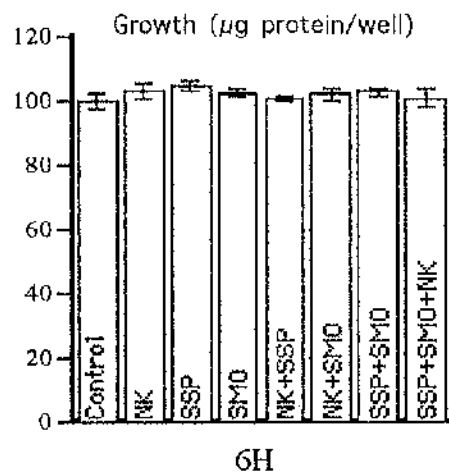
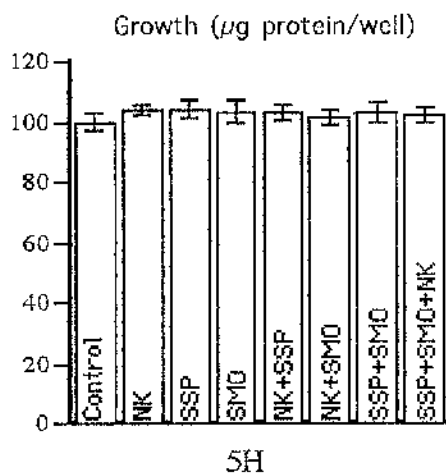
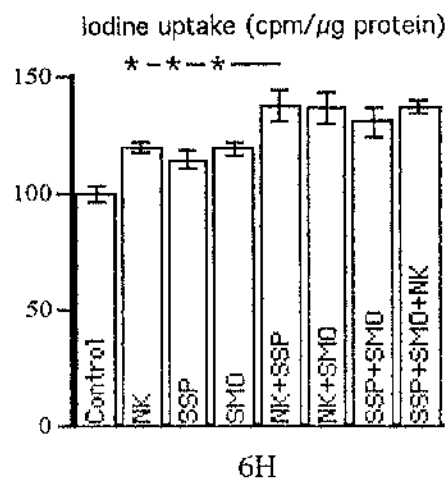
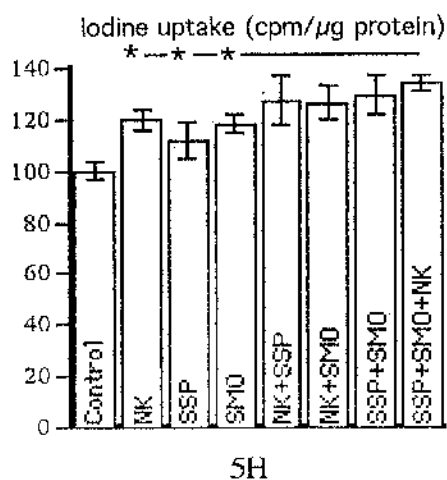


Figure 3.83: Effect of combining agonists of tachykinin receptors on FRTL-5 cells. Cells were stimulated for 48 h as before, with single stimulator or combination of them as labelled in the columns. Unstimulated cells served as control. The symbol (*) shows significant differences compared with individual stimulator.

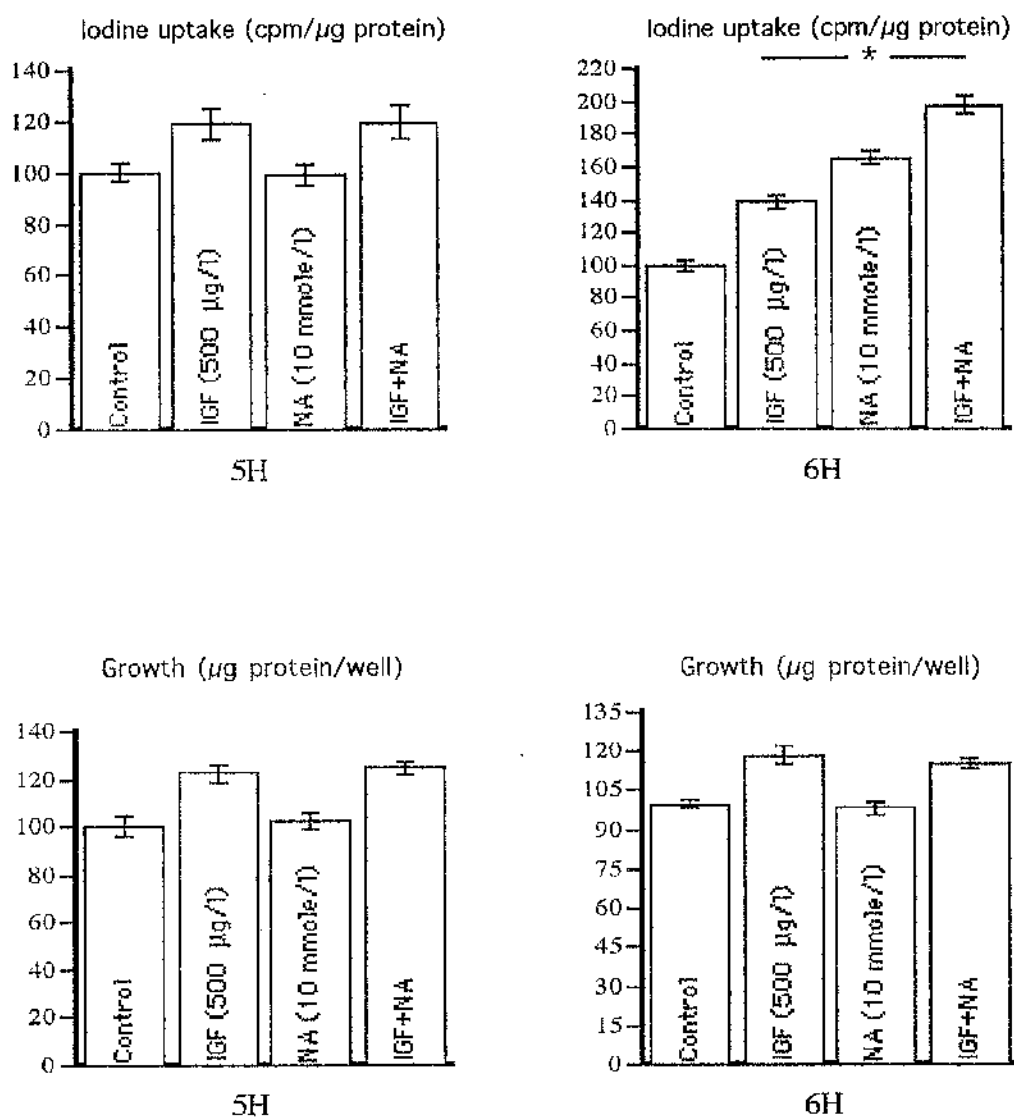


Figure 3.84: Effect of combining \pm noradrenaline and IGF-1 on FRTL-5 cells. Cells were stimulated for 48 h as before, with single stimulator or combination of them as labelled in the columns. Unstimulated cells served as control. The symbol (*) shows significant differences compared with individual stimulator.

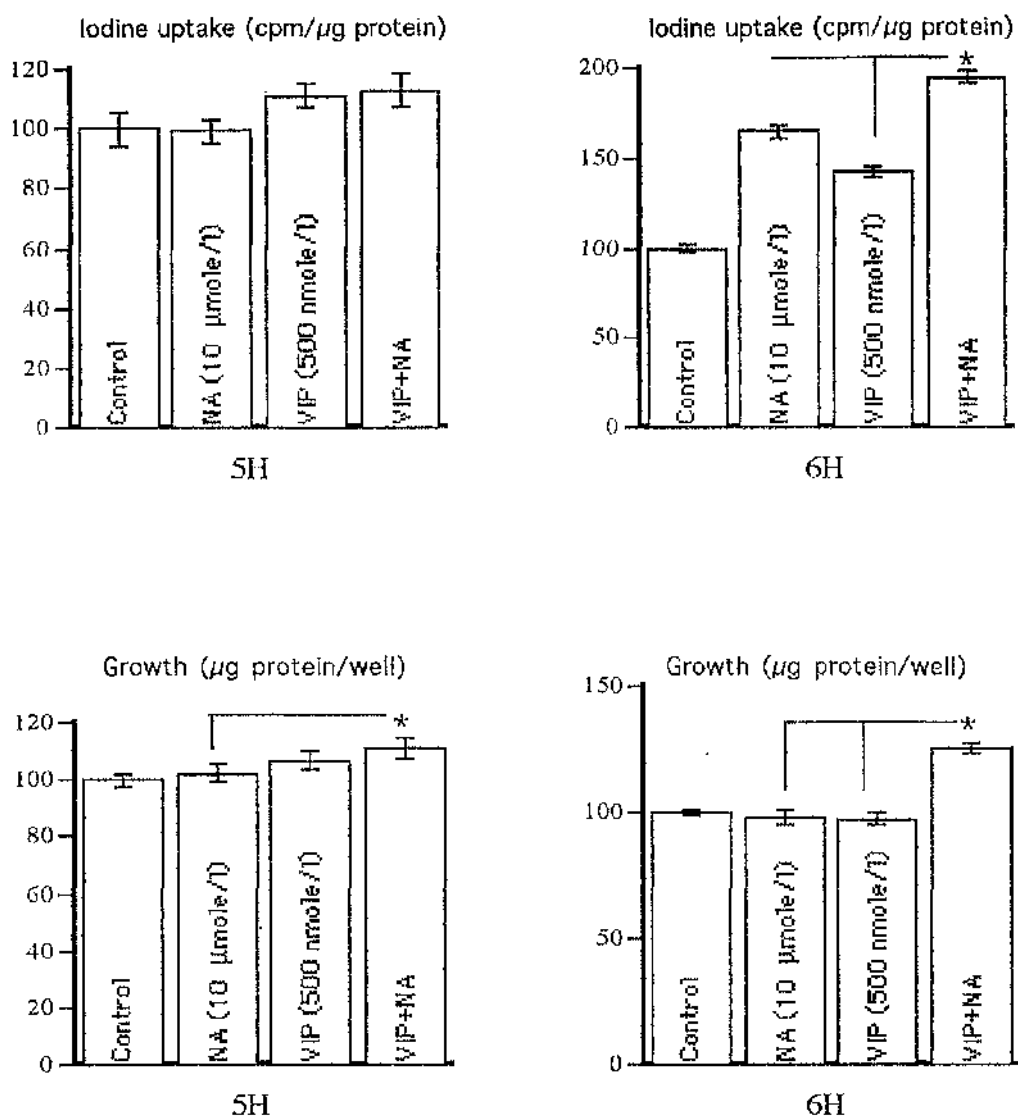


Figure 3.85: Effect of combining \pm noradrenaline and VIP on FRTL-5 cells. Cells were stimulated for 48 h as before, with single stimulator or combination of them as labelled in the columns. Unstimulated cells served as control. The symbol (*) shows significant differences compared with individual stimulator.

affect growth, while stimulation of the cells with a mixture of VIP and NA caused a significant increase of 25.4 %.

3.3.6.31 Noradrenaline And Neuropeptide-Y

The final concentration of \pm noradrenaline (NA) was 10 $\mu\text{mol/l}$, while that of Neuropeptide-Y (NPY) was 500 nmol/l.

In 5H medium, 10 $\mu\text{mol/l}$ of NA or 500 nmol/l of NPY did not affect iodine uptake individually, while the iodine uptake of the cells stimulated with their combination increased significantly, by 15.0 % above control (figure 3.86).

In the presence of TSH, the mixture of 500 nmol/l of NPY and NA caused an increase of 76.0 % in iodine uptake, compared to 9.8 % and 64.8 % for the individual stimulators. This indicates that NPY and NA act independently (figure 3.86).

In the absence of TSH neither of the stimulators affected the growth of cells individually, but in combination, the increase in growth was 12.88 % above control, a significant increase. Neither the individual stimulators nor their combination affected growth in the presence of TSH.

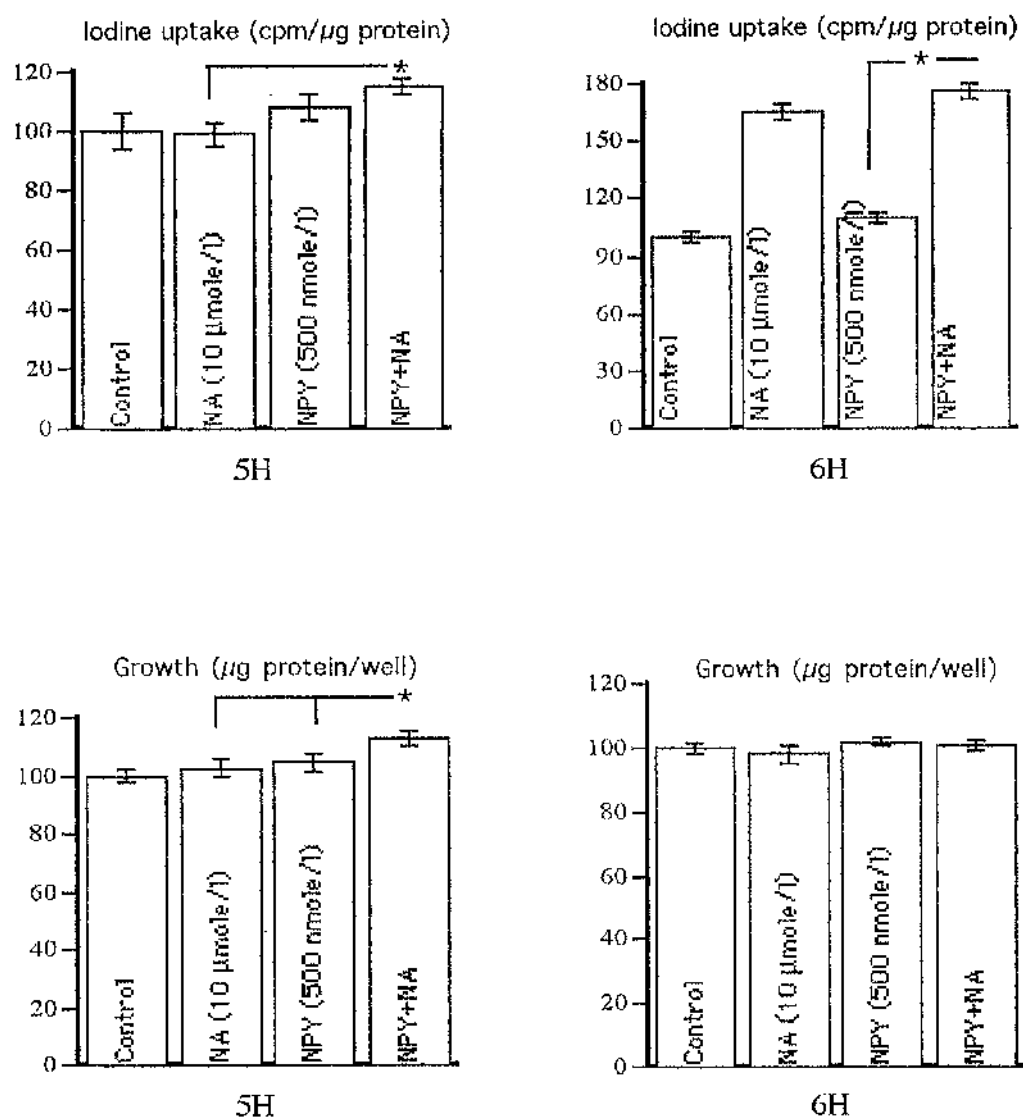


Figure 3.86: Effect of combining \pm noradrenaline and NPY on FRTL-5 cells. Cells were stimulated for 48 h as before, with single stimulator or combination of them as labelled in the columns. Unstimulated cells served as control. The symbol (*) shows significant differences compared with individual stimulator.

SECTION FOUR: DISCUSSION

3.4.1 Characteristics Of FRTL-5 Thyroid Follicular Cell

The FRTL-5 cell line is an established, continuously growing line that responds to TSH in culture medium. In our hands, exposure of the cells to TSH for two or three days after a period of deprivation of five days caused an increase of nearly two fold in growth and in iodine uptake. Confluency was not achieved because the cells grow over each other to form follicle-like structures (figure 3.1). The production of cAMP by the cells also increased 33 fold following exposure to TSH for 3 h after a period of five days deprivation. These observations are in agreement with the previously reported characteristics for this cell line (Bidey et al, 1988; Kohn and Valente, 1989; Tramontano, 1990).

Deprivation of TSH for 5 days caused the cells to swell, and re-exposure to TSH reversed this process (figure 3.2). TSH has a trophic effect on thyroid follicular cells. TSH deprivation in vivo such as that caused by hypophysectomy or T_3 and T_4 treatment induces several changes in follicular cell morphology (Nitsch et al, 1989). In medium deprived of TSH, FRTL-5 cells do not grow (Kohn and Valente, 1989; Tramontano, 1990) and undergo a dramatic modification in cell shape (Nitsch et al, 1989). Nitsch and co-workers (1989) reported that incubation of FRTL-5 cells in SH medium caused actin filaments to reassemble in thick bundles, and the cells lost rough endoplasmic reticulum after 7 days. The morphological changes detected during the present study could be due to these changes.

3.4.2 Iodine Influx

It was found that adequate buffering was essential for there to be a response of iodine uptake to TSH. In the presence of phosphate and HEPES buffers, there was an increase of nearly two fold in iodine uptake, while in phosphate/bicarbonate buffer, there was no response and the uptake rate was high even in the absence of TSH. The pH in this buffer fell to 6.2-6.4 during the time of the uptake experiment, which may have had a direct effect on the permeability of the cells or on the transporter.

In support of this suggestion, Weiss and co-workers (1984 b) reported an increase at pH 6.5 in both iodine influx measured at 5 min and efflux measured at 40 min. The present result was obtained in a 30 min incubation and may include a smaller component of the efflux process. This result suggests that iodine uptake requires a pH difference between the extra-cellular and cytoplasmic spaces. It is known that activation of these cells is accompanied by an increase in cytoplasmic pH

which could be caused by an electroneutral, amiloride-sensitive Na^+/H^+ exchanger that is present on the surface (Bidey et al, 1988). This may then be responsible for iodine accumulation through an active Na^+/I^- symporter and an iodine channel (Carrasco, 1993).

3.4.3 Plasma Carrier For Iodine

There is disagreement in the literature whether it is necessary to include protein in the uptake medium as a carrier for iodine or whether it is adequate to use carrier-free solutions. There is very little published work describing the presence of carrier proteins for inorganic iodine in serum, most workers having concentrated on the carriers for thyroid hormones (Underwood, 1977; Parsad, 1978). We investigated whether inorganic iodine bound to serum protein to a significant extent and whether any binding which might be present could affect the rate of iodine uptake.

The present results indicate that iodine bound to albumin immediately at 37°C , and this association is dose dependent and saturable (figure 3.6 and 3.7). Electrophoresis of serum incubated with radioactive iodine showed that it was mostly carried by pre-albumin and albumin, with a certain amount carried by other proteins. The iodine associated with pre-albumin amounted to 15 % of the total counts and that associated with albumin was 37.6 %. The concentration of pre albumin in the plasma is approximately 160 times lower (250 mg/l) than the concentration of albumin (40 g/l), so pre-albumin carries the highest amount of inorganic iodine per molecule. Thrall and co-workers (1992) showed in rats that 63% of injected radioactive iodine (I_2) or iodide (I^-) was associated with albumin, globulin, and lipids. They did not measure the iodine associated with pre-albumin, but showed that the largest amount was associated with lipids (43%). Our present work has also identified two bands (figure 3.8, bands 7 and 8) at the extreme end of gel with could be accounted for by lipoproteins which did not enter the gel. The sum of the activity associated with these two bands (22.5%) was less than that found by Thrall and co-workers. These results suggest that there is a carrier system for inorganic iodine in vivo.

3.4.4 Effect Of Albumin In Iodine Uptake Medium

The effect of including albumin as a carrier of iodine was examined. The rate of iodine uptake was measured at 5 min to avoid the effect of the efflux pathway, and Michaelis-Menten parameters were calculated using a Lineweaver-Burk plot. The K_m was found to be $52.6 \mu\text{mol/l}$ and the V_{max} of iodine uptake was $3.57 \text{ pmol}/\mu\text{g}$ protein/min. The value of K_m found in the present study is higher than the study of

Weiss and co-workers (1984), in which the K_m for iodine uptake of this cell line was reported to be $31 \mu\text{mol/l}$. Values for V_{max} have not been reported previously.

Inclusion of 5 g/l of albumin as an iodine carrier in the iodine uptake medium caused decreases in both K_m and V_{max} of iodine influx. The K_m was nearly halved ($27.0 \mu\text{mol/l}$), while V_{max} decreased by $0.87 \text{ pmol}/\mu\text{g protein/min}$. This value for K_m was much closer to previously reported values.

Since there is both influx and efflux of iodine in thyroid cells, transport eventually reaches steady state when these are equal. It has been previously reported that iodine transport reached steady state by 40 min when the iodine uptake medium included $10 \mu\text{mol/l}$ of iodide, but not albumin (Weiss et al, 1984; Kohn and Valente, 1989). In the present study steady state was reached between 35 and 42 min in carrier-free medium. Inclusion of 5 g/l of albumin in the medium reduced the time to steady state to 21 min when the concentration of iodine was $10 \mu\text{mol/l}$. This is presumably a complex effect of the observed changes in initial uptake rate and in efflux rate, which was not measured directly in these studies.

3.4.5 Effect Of Serum In Medium

In the presence of a constant concentration of TSH, an increase in serum concentration from 0.5 to 5% in the medium of growing cells caused a significant decrease in iodine uptake, while it produced a significant increase in growth. The results presented here correspond with previous results obtained for human (Roger et al, 1988; Kraiem et al, 1991) or FRTL-5 (Di Marino et al, 1989; Zakarija and McKenzie, 1989 a) thyroid cells, although their experimental conditions were different from the present study. The interpretation of each group was different and corresponds to the experimental condition applied.

Roger and co-workers (1988) compared the effects of TSH on human thyroid cells in the presence and absence of serum and claimed that treatment of cells with medium containing 1% serum induced TSH effects which were responsible for morphological changes and growth increases, while inhibiting the action of TSH on iodine uptake. The group of Kraiem (1991), who grew cells on collagen gel in the absence or presence of 10% serum, showed that higher amounts of iodine uptake, organification and T_3 synthesis were obtained when cells were stimulated with TSH in the absence of serum. Di Marino and co-workers (1989) reported that serum itself did not have any growth activity on FRTL-5 cells and only prevented other growth factors from sticking to plastic substrates. They believed that the IGF-1 present in serum was responsible for the increases in growth which they obtained with different concentrations of serum at a constant level of TSH. On the other hand, Zakarija and

McKenzie (1989 a) claimed that serum did not contain a sufficient concentration of IGF-1 to induce growth.

In the present study, some of the cells died (nearly 90 %), when serum content of 5H was replaced with 2 g/l bovine serum albumin, while 0.5 % of serum was sufficient to keep the cells healthy. This indicates that the albumin content of serum alone is not sufficient to maintain the cells, because the quantity of albumin which was used was much higher than the protein content of 0.5% serum, approximately 400 mg/l. Other components of serum must be necessary for growth of thyroid follicular cells.

One possible factor in serum which might cause these effects is inorganic iodine. Two different groups (Heldin et al, 1987; Kasai et al, 1992) have provided evidence for effects of iodine on thyroid cells. Heldin and co-workers (1987) showed that iodine increased c-myc mRNA and incorporation of ^3H thymidine 30 to 36 h after its addition to the medium of porcine thyroid cells, while Kasai and colleagues (1992) shown that addition of NaI to the medium increased T_3 formation.

The receptor for thyroid hormones is present in FRTL-5 cells (Akiguchi et al, 1992). T_3 alone increased ^3H thymidine incorporation at concentrations below 1 nmol/l, while in combination with TSH or IGF-1 the increase was 1.5-2.2 fold higher. T_3 also induced 5'-deiodinase in FRTL-5 cells (Toyoda et al, 1992), thus providing a mechanism for increasing its own levels in the presence of T_4 . These observations clearly show that T_3 and T_4 in serum may also cause growth effects.

The synthesis of cell membranes requires the synthesis of new lipids, including cholesterol. The low density lipoprotein receptor is present on the surface of FRTL-5 cells (Aloj, 1989). Although thyrocytes are able to synthesise cholesterol and other lipids which are necessary for the synthesis of new cell membranes, the presence of this receptor indicates a need for an external source of lipids. Obviously the external source in vitro is serum, and this could be another factor responsible for the growth effect of serum in the presence of stimulators such as TSH or IGF-1.

It is also possible that there are peptide growth factors in serum in addition to IGF-1 which may be necessary for growth. FGF, for example is known to have a powerful effect on growth of thyroid cells while simultaneously reducing differentiated functions such as iodine uptake, exactly the effect of serum which was seen in these experiments.

3.4.6 Thyroglobulin Production By FRTL-5

Most previous reports measured the increase in mRNA of thyroglobulin induced by TSH, or insulin or IGF-1 (Bone et al, 1986 b; Santisteban et al, 1987; Kamikubo et al, 1990; Graves and Davies, 1993), or used radioactive labelling

(Chambard et al, 1987) of the protein synthesised. Two different groups measured the actual protein production using porcine thyroid cells on porous bottom chambers (Grufft et al, 1992), and FRTL-5 cells in monolayers (Ossendorp et al, 1989). In the present study, iodinated thyroglobulin that was released to the medium was measured using 7.5% polyacrylamide gel electrophoresis as described in section 3.3.4.

The results obtained correspond to previous reports, and showed that FRTL-5 cells are able to synthesise and iodinate at least some thyroglobulin in the presence of adequate ($0.5 \mu\text{mol/l}$) iodine in the medium. In the absence of TSH for 8 days, the production was half of that when the cells were stimulated with 2 U/L of TSH for 72 h. Part of the basal production in the absence of TSH could be due to the influence of insulin (Bone et al, 1986 b; Santisteban et al, 1987; Isozaki et al, 1989; Kamikubo et al, 1990; Graves and Davies, 1993) or other hormones present in the medium, while some might be induced by iodine uptake of the cells.

Graves and Davies (1993) reported that there are two genes for thyroglobulin in rats, which they named rTg-1 and rTg-2. The rTg-1 gene is independent of TSH but is insulin and IGF-1 dependent. However, cells deprived of insulin, TSH, and serum still showed a background thyroglobulin mRNA production (Bone et al, 1986 b; Ossendorp et al, 1989). On the other hand, the presence of thyroglobulin protein does not guarantee iodination. The production of H_2O_2 is partially impaired (Bidey et al, 1988) in FRTL-5 cells, so that organification does not take place or is at a minimum level. This defect may be partly due to the absence of iodine in the culture medium, because the present results indirectly show that newly synthesised TG molecules are iodinated when adequate inorganic iodine is present in the medium. This suggestion is supported by a recent study of Corvilain and co-workers (1994), who have shown that a low level of iodine induces production of H_2O_2 by thyroid cells.

3.4.7 Effect Of Inorganic Iodine On Growth

In the present study, it was shown that inclusion of concentrations of inorganic iodine above $1 \mu\text{mol/l}$ to the medium of growing cells reduced their growth dose dependently, while 1 mmol/l of iodine started to cause cell damage in 5H medium containing 5% serum. These results correspond with previously reported results (Saji et al, 1988; Many et al, 1992), but using a wider range of concentrations and different methods.

An excess of iodine in vitro causes a chain of events which are possibly related to the Wolff-Chaikoff effect observed in vivo. They take place in successive order. The excess iodine results in more iodine entering the cell than normal. This causes a reduction in cAMP (Saji et al, 1988), followed by reduction in H_2O_2 , a

necessary substrate for organification of iodine (Corvilain et al, 1994). This is accompanied by reduction of thyroglobulin synthesis (Gruffat et al, 1992) and finally by reduction in hormone synthesis (Gruffat et al, 1992). These processes are transient in the presence of moderate doses ($1 \mu\text{mol/l}$ - $10 \mu\text{mol/l}$) of iodine and in normal subjects usually only last for several days. Break-through from this mechanism in patients is usually due to a protective mechanism reducing the amount of iodine entering the cell. Therefore, there is no longer an excess of intracellular iodine and the iodination can return to the normal rate (McDougall 1992). Excess inorganic iodine (1 mmol/l and above) has a direct acute toxic effect on thyroid follicular cells, inducing a necrosis of the cells followed by deletion or apoptosis, a physiological cell death (Many et al, 1992).

If the thyroid is under negative regulation when iodine concentrations are normal, these findings could explain the increased growth seen in endemic goitre caused by iodine deficiency. On the other hand, autoimmune thyroid diseases are more common among inhabitants of south east Asian countries who eat large quantities of sea food, a rich source of inorganic iodine. The amount of iodine in the diet varies in western countries, but there are other sources of iodine such as medications and antiseptics which suddenly provide many orders of magnitude of iodine more than is required for normal function. The development of thyroid autoimmune disease among people with high iodine intake is more common (Harach et al, 1985; Boukis et al, 1983; Weetman, 1991), consistent with the idea that fluctuation in iodine supply causing episodes of thyroid damage may initiate thyroid autoimmunity.

3.4.8 Effect Of Thyroid Hormones On Iodine Uptake And Growth

The present study showed a dose dependent decrease in iodine uptake of cells stimulated with 2 U/l of TSH and a mixture of T_4 and T_3 at concentrations which are higher than those circulating in normal subjects. This result confirmed previous reports (Akiguchi et al, 1992) with an extended dose range. Furthermore, the present work also showed for the first time apparent increases in iodine uptake of cells that had been deprived of TSH for 8 days, which became significant (18.9 % above control) at a dose of $1 \mu\text{mol/l}$ of T_4 and 10 nmol/l of T_3 . These effects could form an autoregulatory loop in Graves' disease. Thyroid cells in this disease would be expected to accumulate large quantities of iodine under the continuous stimulation of TSH-receptor antibodies. The elevated concentrations of thyroid hormones could then limit further iodine uptake and prevent thyroid function from completely escaping from hormonal control.

In the present study, there were an apparent increase in growth rate of cells that were stimulated with thyroid hormones, which became significant at the highest dose only (1 $\mu\text{mol/l}$ of T_4 , and 10 nmol/l of T_3). The increases at this concentration were 17.1 % for 5H and 9.9 % for 6H medium. These results contrast with the results reported by Akiguchi et al, (1992) who detected an increase at a concentration of 0.1 nmol/l of T_3 . The difference could be in their methodology, because they measured ^3H thymidine incorporation, while in the present study growth was assessed by protein measurement and by MTT assay. Furthermore, their medium did not contain any additives, whereas medium used for present work contained additional amino acids and 0.5 % FCS. Kamikubo and co-workers (1992) showed that mRNA for the alpha thyroid hormone receptor was increased by withdrawal of TSH, insulin, and serum from the medium without affecting mRNA for the beta receptor. Addition of TSH reduced the numbers of receptors to its previous level after 24 h, while insulin acted at a slower rate. We found that the growth stimulation in the absence of TSH was higher than in its presence, which is consistent with a negative regulatory effect of TSH on receptors.

These results may indicate that part of the abnormal growth of cells in patients with toxic goitres may be caused by the elevation in thyroid hormones themselves, though TSH may modify the effects.

3.4.9 Insulin

Insulin is included in both 5H and 6H media. To investigate the effect of IGF-1 on functions of thyroid follicular cells most investigators have omitted insulin from the culture medium, because they believed that insulin would cross-react with the type I receptor for IGF-1 (Smith et al, 1986; Saji et al, 1987; Bachrach et al, 1988; Becks et al, 1992). This may be true, but it does not reflect the situation in vivo, because a basal concentration of insulin is present in the plasma of all healthy individuals, and omission of insulin from culture medium could lead to reduction of glucose entry along with other unknown consequences. Furthermore, it has been shown that the synthesis of prostaglandin by thyroid cells needs the presence of TSH and either insulin or IGF-1 in the medium (Tahara et al, 1991). In this work, the effects of IGF-1 were studied both in the presence and absence of insulin.

The results indicate that both in the presence or the absence of TSH, the effects of insulin and IGF-1 on iodine uptake of cells were apparently independent of each other, since the cells that were stimulated with a mixture of both hormones demonstrated an increase in iodine uptake that was approximately equal to the sum of the increases elicited by the individual hormones. This increase in iodine uptake was consistent with the results reported by Becks and co-workers (1992), while it

contrasts with the results reported by Pang and colleagues (1990 a), who measured the iodine uptake at steady state. Tramontano and co-workers (1988 a) detected a slight increase in cAMP production by cells stimulated with IGF-1, which could account for the small increases in iodine uptake detected in the current study. Moreover, both IGF-1 and insulin can increase intra-cellular Ca^{2+} concentration (Takada et al, 1990). Influx of extracellular Ca^{2+} and amino acids (Takasu et al, 1989; Villone et al, 1993) may lead to the alkalisation of cytoplasmic pH or acidification of extra-cellular medium, both of which can contribute to an increase in iodine uptake.

The results obtained for growth in the presence of insulin and IGF-1 also seemed to be additive both in presence or the absence of TSH, and confirmed the report of Tramontano (1989). In the presence of TSH the growth responses produced by each hormone were greater than when TSH was absent. These results are in accordance with earlier reports (Isozaki et al, 1987; Saji et al, 1987; Bachrach et al, 1988; Tramontano et al, 1988 a; Pang et al, 1990 a; Takahashi et al, 1990; Becks et al, 1992; Villone et al, 1993). Since the present results showed that the presence of insulin does not interfere with the effect of IGF-1, other experiments were carried out in the presence of the standard concentrations of insulin in the medium normally required for optimal growth of the cells.

3.4.10 Insulin Like Growth Factor-1

IGF-1 stimulated both the iodine uptake and growth of cells when it was applied for 48 h in either 5H or 6H medium. The minimum effective dose for iodine uptake was 50 $\mu\text{g/l}$, while that for growth was 10 $\mu\text{g/l}$. The highest dose tested was 500 $\mu\text{g/l}$. Inclusion of bestatin and thiorphan, which are inhibitors of cell surface proteinases (Shipp and Look, 1993), did not alter the effects of IGF-1 on either iodine uptake or growth, whereas treatment of cells before and during stimulation with thyroid hormones (1 $\mu\text{mol/l}$ of T_4 , and 10 nmol/l of T_3) reduced the iodine uptake, without affecting the increase in growth. This is the first time the effect of IGF-1 has been reported in the presence of insulin or thyroid hormones.

The present data shows that the growth effect of IGF-1 in the absence of TSH is greater than when TSH is present. These results agree with the results reported by Isozaki and co-workers (1987), who used a single dose of IGF-1 (100 $\mu\text{g/l}$) and with the results reported by Tramontano and colleagues (1988 b) who have shown that TSH down-regulates the receptors for IGF-1. On the other hand, they are in contrast with work that was carried out in the absence of insulin and showed that the effect of TSH and IGF-1 together was much greater than the sum of individual responses (Tramontano et al, 1986 and 1988 a; Takahashi et al, 1990).

IGF-1 is mainly produced by liver (Delafontaine and Lou, 1993), but thyrocytes can also synthesise and secrete it (Dumont, 1991; and 1992). It acts as an autocrine factor for growth of thyroid cells or a paracrine factor to induce the growth of endothelial or fibroblast cells. Thyroid epithelial cells can produce IGF-I when stimulated with TSH or growth hormone (Bachrach et al, 1988), and IGF-II when stimulated with IGF-1 (Dumont, 1991; and 1992), or TSH in the absence of IGF-1 (Maciel et al, 1988). Recently it was shown that thyroid hormones could induce IGF-1 mRNA in an osteoblast cell line (MC3T3-E1) (Varga et al, 1994), and increase the synthesis of growth hormone, the inducer of IGF-1 production in many cells (Nântö-Salonen 1993).

In Graves' disease, thyroid stimulating antibodies, which act like TSH (Gafny et al, 1992; Leedman et al, 1992; Shoda et al, 1993), may induce the production of IGF-1 by thyroid cells, or the excess of thyroid hormones may induce IGF-1 production by other cells. The increase in IGF-1 could partly contribute to the growth of the thyroid gland and development of goitre. On the other hand, continued stimulation of thyrocytes with TRAb and IGF-1 will down-regulate the receptors for both TSH and IGF-1 and prevent further growth of the gland. Furthermore, the presence of a high level of thyroid hormones will tend to suppress iodine uptake and prevent further formation of thyroid hormones in such patients.

3.4.11 Transforming Growth Factor- β

Transforming growth factors have diverse effects on growth and differentiation of the thyroid follicular cells of human and other species. TGF- α has a growth promoting effect on thyrocytes (Hölting et al, 1995; Arai et al, 1995), while TGF- β has a negative effect. In the present study, TGF- β produced an increase in iodine uptake both in the presence or absence of TSH, while it reduced the growth induced by TSH dose dependently.

Previous studies have reported various results for the iodine uptake of thyrocytes when stimulated with TGF- β . A decrease in iodine uptake was reported for human (Taton et al, 1993) and porcine (Tsushima et al, 1988) thyroid cells. An increase was reported by Morris and co-workers (1988) in FRTL-5 cells, while Pang and colleagues (1992) showed a significant decrease.

Several factors may have caused such contrasting results. The concentration of TSH used may be one. The concentration of TSH used in the experiments of the group of Morris (1988) and the present study, who both found an increase in uptake, was higher (10 U/l and 2 U/l respectively), than the concentrations used by others who detected a decrease [Pang et al, (1992) 11.1 mU/l; Tsushima et al, (1988) 1 U/l; and Taton et al, (1993) 250 mU/l]. However, this is unlikely fully to explain the

results. In the pilot experiments carried out for the present work TGF- β caused a decrease of 6.2% in the presence of 10 U/l of TSH when cells were grown in a medium containing 5% serum, and stimulated with 10 μ g/l of TGF- β for 72 h.

A second factor could be the serum concentration in the medium, which could provide the cells with both iodine and growth stimulators. This factor may be less important for freshly isolated cells, which are exposed to serum in the culture medium for a shorter time.

In this study it was shown that TGF- β was a potent growth inhibitor for TSH-stimulated rat thyroid cells. Down-regulatory effects of TGF- β on growth of thyroid follicular cells have been previously reported for human (Taton et al, 1993), porcine (Tsushima et al, 1988) and FRTL-5 cells (Morris et al, 1988; Pang et al, 1992), using different methodologies. It has also been shown that TGF- β is involved in induction of apoptosis in certain epithelial cells (Bursch et al, 1993). In the present study, neither reduction in growth nor cell death occurred, even in the presence of a high dose of TGF- β (100 μ g/l), when cells were grown in 5H medium. TGF- β could only reduce the growth induced by other growth factors such as TSH, IGF-1, and FGF (Pang et al, 1992), and does not decrease the number of quiescent cells in short term culture.

3.4.12 Fibroblast Growth Factor

Basic FGF decreased the iodine influx, while it increased the growth of FRTL-5 cells. These effects were independent of TSH. Minimum effects were detected at a concentration of 2.5 μ g/l, while the maximum effects were at a concentration of 10 μ g/l. The effects of concentrations greater than 10 mg/l were lower, though still significant.

The reduction in iodine influx of the cells reported in this study confirmed a previous report (Isozaki et al, 1992) for the FRTL-5 cell line. Similar results were also obtained by other groups in porcine (Emoto et al, 1991) or sheep (Hill et al, 1994) thyroid cells. A wider range of concentrations was used in this study, showing that doses above 10 μ g/l are less effective.

Basic FGF increased the growth of the cells. In the present study, growth of the cells was measured as the protein content of the cells. The present results are also in agreement with previous reports for FRTL-5 cells (Black et al, 1990; Isozaki et al, 1992) and other thyroid cells, such as porcine (Emoto et al, 1991), sheep (Hill et al, 1994), or human (Taylor et al, 1993) thyroid cells.

3.4.13 Endothelin-1

Immunoreactive ET-1 or expression of mRNA of preproendothelin-1 has been detected in a wide variety of tissues including human (Tseng et al, 1993), rat (Colin et al, 1992; and 1994) and porcine (Colin et al, 1992; Isozaki et al, 1993) thyroid follicular cells, though it is predominately produced in endothelial cells and used locally in an autocrine or paracrine manner (Haynes and Webb, 1993). Receptor for ET has been detected in human thyroid cells (Jackson et al, 1992; Tseng et al, 1993), and there is indirect evidence that at least one form of receptor is present on the surface of thyroid cells (Isozaki et al, 1993; Miyakawa et al, 1992).

In the presence of TSH (2U/l), 50 and 100 nmol/l of endothelin-1 increased iodine influx, but it had no effect in the absence of TSH. This result contrasts with the work of Tsushima and co-workers (1994) who detected an inhibition of the iodine uptake induced by TSH when they stimulated porcine thyrocytes with doses as low as 100 pmol/l. The difference could be due to the species difference or to the differences between cell line and primary cultures.

ET-1 at a dose of 25 nmol/l significantly increased the growth rate of cells, but only if they were stimulated with TSH. The effect plateaued above this. These results correspond with the results obtained by Eguchi and co-workers (1993) who detected an increase in growth of human thyrocytes by measuring ³H thymidine incorporation into DNA as well as by cell count assessed by trypan blue, but not with the results of Miyakawa' group (1992) on FRTL-5 cells. They showed that ET-1 alone increased c-fos mRNA expression without affecting growth, while in the presence of TSH or IGF it caused a decrease or an increase in the growth of cells respectively. The difference might be due to the concentration of TSH used, since the concentration of TSH in the present study was 2 U/l, while Miyakawa' group used 10 mU/l TSH. It is also surprising that the combination of ET with IGF-1 and TSH should give opposite effects, since all three act through the inositol phosphate/calcium pathway, causing increases in c-fos and c-jun (Kennedy et al, 1993; Corvilain et al, 1994; Takazawa et al, 1995).

3.4.14 Interleukin-1 β

Interleukin-1 is synthesised and secreted by many cells including monocytes, macrophages, B cells, dendritic cells (Weetman, 1991; Benjamini and Leskowitz, 1992; Janeway and Travers, 1994), endothelial cells (Miyazaki et al, 1989; Asakawa et al, 1991 a; Rasmussen et al, 1993), and thyrocytes (Zheng et al, 1991; Nagataki and Eguchi, 1992; Zeki et al, 1993; Watson et al, 1994). The presence of so many possible sources of production within the gland suggest that this cytokine may

influence the functions of thyrocytes in an autocrine or paracrine manner during inflammatory diseases.

IL-1 had no effect on iodine uptake or growth in the absence of TSH. TSH-stimulated cells demonstrated a significant decrease in uptake at high doses, 5 to 20 $\mu\text{g/l}$. There was a suggestion of an increase in uptake at a concentration of 1 $\mu\text{g/l}$, but this did not reach significance.

The current results are in agreement with those of Rasmussen and co-workers (1989), who found no change in the cAMP production of FRTL-5 cells stimulated with IL-1 in the absence of TSH. On the other hand, Pang and colleagues (1990 b) demonstrated a decrease in uptake in FRTL-5 cells, and Zeki and co-workers (1991) demonstrated an increase in cAMP production when cells were stimulated with IL-1. It is difficult to explain these differences, because variables such as the stimulation time and cell line were the same. The only clear differences in protocol were the concentration of serum in the medium and the duration of deprivation of cells of TSH. The studies of Rasmussen and Zeki both used 5 % serum in the medium and four days of deprivation, while Pang used medium containing 0.1 % serum and 0.5 % albumin and 24 h deprivation. In the present study, the medium used contained 0.5 % serum and additional amino acids, while cells were deprived of TSH for 5 days. It is unclear whether any of these *in vitro* findings actually corresponds with the *in vivo* condition. There is evidence (Hermus et al, 1992; Van-Haasteren et al, 1994) indicates that continuous infusion of rats with IL- β for one week decreased the plasma concentration of TSH and thyroid hormones. These results indicate that the finding of no effect or a decrease in iodine uptake is more likely to reflect the *in vivo* situation than a stimulation of activity.

The decrease in iodine uptake observed in TSH-stimulated cells is more consistently in agreement with results obtained for human (Sato et al, 1990), porcine (Westermarck et al, 1990; Nolte et al, 1994), and FRTL-5 cells (Pang et al, 1990 b). The tendency towards an increase that occurred at a concentration of 1 $\mu\text{g/l}$ was also found by Rasmussen and co-workers (1994). This shows that IL-1 released by infiltrating cells or by cells within the thyroid could suppress the function of thyroid epithelial cells in inflammatory diseases, possibly contributing to the damage occurring in autoimmune thyroiditis or sub-acute thyroiditis.

In the present study, it was found that IL-1 β did not affect the growth of cells either in the presence or the absence of TSH. These results agree with the results obtained by Nolte and co-workers (1994) in porcine thyroid cells. However, other workers have shown an increase in the growth of FRTL-5 cells stimulated in 5H medium (Pang et al, 1990 b; Zeki et al, 1991), and a decrease in growth of cells in 6H medium (Pang et al, 1990 b; Zeki et al, 1991; Rasmussen et al, 1989; and 1990). In these studies, growth was measured by ^3H thymidine incorporation into DNA.

This method is more sensitive than protein measurement, but is indirect and subject to interference from factors such as DNA repair synthesis which could be taking place within the cells. Details of experimental protocols were also variable between the various groups.

3.4.15 Lipopolysaccharide (LPS)

LPS is a major constituent of the cell wall of gram negative bacteria that can elicit a wide range of immune responses, including stimulation of release of cytokine such as IL-1 from macrophages. In the present study, stimulation of thyroid follicular cells with LPS increased iodine uptake at a low dose (100 μ g/l in 6H medium), while it decreased iodine uptake at concentrations above 2.5 mg/l (in 5H medium), or 7.5 mg/l (in 6H medium). Furthermore, LPS increased the growth of cells in 6H medium at doses above 2.5 mg/l. The increases were dose dependent.

The pattern of decreases in iodine uptake that occurred in 6H medium resemble the results obtained for IL-1, while the decreases seen in 5H medium did not. Moreover, the LPS produced a dose dependent increase in the growth of cells that did not occur with IL-1. These findings indicate that the changes in the proliferation and iodine influx are caused by LPS directly and not by an autocrine action of IL-1 that may be induced by LPS. This suggestion is supported by a recent study carried out by Van-Der-Poll and co-workers (1995). They have shown that the injection of endotoxin into humans was associated with significant decreases in the plasma concentrations of T₄, free T₄, T₃, TSH and a rise in reverse T₃. They also showed that these changes are independent of IL-1.

This is the first report that shows effects of LPS on the proliferation and iodine influx of thyroid epithelial cells. The reduction in iodine influx and increase in growth may contribute to the *in vivo* changes (reduction in thyroid hormones with no change in TSH concentration) that occur in patients suffering from the euthyroid sick syndrome.

3.4.16 Interleukin-4

In the present study, stimulation of the FRTL-5 cells with various doses of IL-4 (0.5 to 20 μ g/l) did not affect iodine influx, while it caused a dose dependent decrease in the growth of cells induced by TSH.

During the development of autoimmune thyroid diseases, immune cells migrate into the thyroid gland, so it is possible that IL-4 may be produced in the gland by TH2 helper T cells.

Inhibitory effects of IL-4 on megakaryocytes (Sonoda et al, 1993), myeloblastic leukaemic cell lines (Imai et al, 1991), or growth of monocytes (Dokter et al, 1993) induced by LPS have been reported previously. Although there are reports (Lowenthal et al, 1988; Idzerda et al, 1990; Beckmann et al, 1992) that indicate that epithelial cells do have receptor for IL-4, there is no previous report to show the effect of this cytokine on proliferation of non-hematopoietic cells. These results may indicate that high concentrations of IL-4 may down-regulate the growth of thyroid follicular cells in patients with hypothyroidism and high concentrations of TSH.

3.4.17 Interleukin-6

There are many recent reports (Stouthard et al, 1994; Hashimoto et al, 1994; Bartalena et al, 1994 a, b and c; Bartalena et al, 1995) indicating that elevated IL-6 may be responsible for the alterations in thyroid function in patients with non-thyroidal illness. Rats injected with rIL-6 for different periods developed a low serum T₄ and T₃ (Bartalena et al, 1994 c; Van-Haasteren et al, 1994). A reduction of TSH was reported by Bartalena et al (1994 c), and Van-Haasteren et al (1994) after a week, though without detectable changes in mRNA. These *in vivo* findings may explain the reduction of thyroid hormones in patients with non-thyroidal illness, but are insufficient to show a direct action of cytokine on thyroid follicular cells.

In the present study, it was found that stimulation of cells that were deprived of TSH with IL-6 increased iodine influx at a concentration of 20 µg/l (17.0% above control). Furthermore, the iodine influx increased significantly at doses above 10 µg/l when the cells were stimulated in 6H medium. Tominaga and co-workers (1991) found that IL-6 inhibited thyroid peroxidase gene expression induced by TSH (5U/l) in human thyrocytes. This inhibition was dose dependent. These findings result in a pattern of increased iodine uptake and reduced thyroid hormone formation which has resemblances to the Wolff-Chaikoff effect, the suppression of thyroid hormone release by excessive iodine. They are also consistent with the involvement of IL-6 in the suppression of thyroid hormone synthesis in non-thyroidal illness or subacute thyroiditis. IL-6 could be produced by the thyroid cells in an autocrine manner (Kennedy et al, 1992; Zheng et al, 1991; Watson et al, 1994), by other cell types within the gland or could arise from increases in the serum concentration.

The growth of thyroid follicular cells was also slightly effected by IL-6. IL-6 increased the growth rate of the cells in 5H medium, but only at the highest concentration tested. In contrast, Nishiyama and co-workers (1993) have shown a dose dependent increase in the growth of FRTL-5 cells that were stimulated with TSH (10 mU/l) and IL-6. The difference between the present results and the one

obtained by this group may be explained by the medium used by this group, which did not include the standard 5 hormone supplement or serum, and the very low concentration of TSH which was used.

3.4.18 Interferon- γ

Interferon- γ (INF- γ) is synthesised and secreted by activated CD4⁺ helper cells and CD8⁺ cytotoxic cells (Nagataki and Eguchi, 1992). During the development of thyroid autoimmunity, these cells migrate into the thyroid gland. The local release of INF- γ contributes to morphological changes in thyroid follicular cells that include production of class 1 and 2 of MHC on the surface of cells (Toda et al, 1992; Chiovato et al, 1994; Eguchi et al, 1995). Additionally, INF- γ also inhibits TSH induced morphological changes such as elongation of microvilli and appearance of pseudopods in human as well as animal thyrocytes (Asakawa et al, 1990; and 1991b; Kung et al, 1992).

In the present study, it was found that stimulation of FRTL-5 cells with doses above 50 $\mu\text{g/l}$ (5H) or 10 $\mu\text{g/l}$ (6H) of INF- γ increases the iodine influx. The increases detected in 5H medium were apparently dose dependent, while in the 6H medium (2U/l of TSH) the increases detected for doses of 10, 50, and 100 $\mu\text{g/l}$ were not significantly different for each other. On the other hand, stimulation of the cells in 5H medium did not affect the growth at any concentration, while it decreased the growth induced by TSH at all concentrations (1-1000 $\mu\text{g/l}$). These decreases were also dose dependent.

The results obtained for the iodine uptake in this study correspond with previous results obtained for the FRTL-5 (Misaki et al, 1988; Zakarija and McKenzie 1989 b) while they oppose the results obtained for human thyroid cells (Kraiem et al, 1990; Sato et al, 1990). Furthermore, the decrease obtained for the growth in present study corresponds with the previous reports for the FRTL-5 cell line (Misaki et al, 1988; Zakarija et al, 1989 b) as well as for human thyroid follicular cells (Huber and Davies, 1990; Kraiem et al, 1990; Yip et al, 1995).

The opposite results for iodine uptake in human and rat may be due to species differences or to the fact that human cells are usually isolated from glands of patients who have already been treated with drugs affecting the thyroid.

INF- γ suppresses other thyroid functions like TSH induced thyroid peroxidase synthesis (Asakawa et al, 1992 a; Chiovato et al, 1994; Tang et al, 1995) and thyroglobulin production (Misaki et al, 1988; Graves et al, 1989; Huber and Davies, 1990; Kung and Lau, 1990; and 1992; Graves and Davies, 1993; Rasmussen et al, 1994; Tang et al, 1995) in both human and rat, leading to the reduction of thyroid hormone synthesis (Kraiem et al, 1990). In a similar way to IL-6, the action

of INF- γ on iodine uptake of the thyroid cells could be compared to the Wolff-Chaikoff effect. The increase in the intracellular concentration of iodine may lead to depression of thyroid peroxidase, thyroglobulin synthesis, and thyroid hormones production.

3.4.19 Pituitary Adenylate Cyclase Activating Polypeptide

Pituitary Adenylate Cyclase Activating Polypeptide (PACAP) is a novel hypothalamic peptide that is widely distributed in neurones (Portbury et al, 1995), but there are no reports of its presence in the thyroid gland. On the other hand, both types of PACAP receptors were detected on the surface of rat medullary carcinoma of the thyroid cell line 6/23 (Vertongen et al, 1994). Furthermore, there is a report (Chen et al, 1993) indicating that both PACAP-27 and PACAP-38 increased cAMP production by porcine thyroid cells. In the present study in the absence of TSH, stimulation of the FRTL-5 with PACAP-27 (1-500 nmol/l) did not affect the iodine influx or the growth of cells; whereas in the presence of 2U/l of TSH, stimulation of the cells with PACAP-27 did increase the iodine influx without affecting the growth. This data is the first report that shows a direct effect of PACAP on iodine influx of the FRTL-5 cell line.

Two different receptors for PACAP has been identified (Arimura 1992; Journot et al, 1994). The type I receptor predominates in the central nervous system, while type II receptors are distributed in other organs including liver, lung, digestive tract, prostate gland, and testis (Arimura 1992; Journot et al, 1994). The interaction of PACAP with the type I receptor increases intracellular cAMP and phospholipase C, while the type II receptor causes increases of only cAMP (Kononen et al, 1994). Since the growth of FRTL-5 cells is stimulated through activation of the phosphatidylinositol- Ca^{2+} pathway (Dumont et al, 1991; Corvilain et al, 1994; Takazawa et al, 1995), the absence of growth in the present study favours the presence of type II receptors. This would be consistent with the results of Chen and co-workers (1993) who detected an increase in cAMP of porcine thyroid follicular cells, or increase in the serum T_4 after injection of rat with PACAP.

3.4.20 Neuropeptide Y

Adrenergic nerve fibres containing Neuropeptide Y (NPY) have been detected in the thyroid gland of several mammals, including man (Grunditz et al, 1984; Ahrén, 1986 a; and 1991). These fibres occur around blood vessels and follicles in the gland

(Grunditz et al, 1984), and T₄ can induce the production and release of NPY from them (Jones et al, 1994).

In the present study, stimulation of cells with NPY produced an increase in iodine influx without affecting growth. The increases in iodine influx were also TSH independent. The $10 \pm 4\%$ increase in iodine influx detected at a concentration of 500 nmol/l was consistently found in repeated experiments.

These minor increases in the iodine influx suggest that this neuropeptide is not expressly involved in thyroid functions, and local release of this neuropeptide may regulate the blood flow of the thyroid as observed by Michalkiewicz and colleagues (1993).

3.4.21 Vasoactive Intestinal Peptide

Vasoactive intestinal peptide (VIP) was the first neuropeptide demonstrated in thyroid gland (Ahrén, 1986 a). The VIP-containing nerves were shown to exist around and close to the follicular cells, and subsequent experiments with denervation and retrograde tracing have shown that they are intrinsic, originating in a thyroid ganglion (Ahrén, 1991). Apart from the well-described cross reaction with PACAP type I and II receptors, VIP has two specific receptors, named VIP₁ and VIP₂ (Usdin et al, 1994; Lutz et al, 1993). These receptors are present in liver, lung, and intestine as well as in several regions of the brain like cerebral cortex and hippocampus (Lutz et al, 1993). The interaction of VIP with its receptors stimulated cAMP production (Lutz et al, 1993; Sreedharan et al, 1993; Usdin 1994; Fatatis et al, 1994). It has also been shown that VIP increases intracellular calcium (Spessert, 1993; Murthy et al, 1993; Fatatis et al, 1994).

In the present study, VIP increased the iodine influx of cells that were deprived of TSH for 5 days, at concentrations of 500 and 1000 nmol/l. In the presence of TSH, VIP increased the iodine influx at concentrations from 10 nmol/l to 2000 nmol/l, but the increases detected at concentrations of 1000 and 2000 nmol/l were significantly lower than the value obtained for 500 nmol/l. These results may be related to the increase in the intracellular concentration of cAMP of human (Toccafondi et al, 1984; Siperstein et al, 1988) or mouse thyrocytes (Ahrén, 1986 a; and 1991) caused by VIP. Ealey and co-workers (1985) also showed that stimulation of guinea-pig thyroid section with VIP increases β -naphthylamidase activity in the thyroid cells. Toccafondi and colleagues (1984) have shown that stimulation of human thyroid cells with VIP increases the production of T₄. On the other hand, Jones and colleagues (1994) have shown that intrathyroid release of VIP is inversely correlated with the serum concentration of T₄. In vivo, Michalkiewicz and co-workers (1994) did not detect any changes in blood flow or production of thyroid

hormone after immunisation of rats against VIP. This difference may be due to an insufficient dose of anti-VIP serum used by this group or may show that the influence of VIP is minor *in vivo*.

Stimulation of cells that were deprived of TSH for 5 days with VIP did not affect their growth. On the other hand, stimulation of cells in the 6H medium (2U/l of TSH) with low doses of VIP (10 and 50 nmol/l) increased growth, whereas the doses of 100 to 1000 nmol/l did not alter it. Furthermore, growth of the cells decreased at a concentration of 2000 nmol/l. These data correspond with the results obtained by Karbownik' group (1994 and 1995) who used rat thyroid lobes, but used different methodology. The growth of thyroid cells is controlled through the Ca^{2+} /protein kinase C pathway (Dumont et al, 1991; Corvilain et al, 1994; Takazawa et al, 1995), which is consistent with the known actions of VIP on calcium transport (Spessert 1993; Murthy et al, 1993; Fatatis et al, 1994). All these findings suggest that intrathyroidal VIP nerves have a stimulatory function on thyroid hormone secretion and growth, because the intrathyroidal concentration of VIP may never reach 2 $\mu\text{mol/l}$ which was associated with a decrease in growth *in vitro*. Local release of VIP could also contribute to the stimulation of thyroid function pathologically in the presence of TSH or thyroid stimulating antibodies.

3.4.22 Neuromedin U-25

Neuromedin U (NmU), a peptide originally isolated from porcine spinal cord (Lo et al, 1992), and has been found to have a widespread distribution extending throughout the mammalian central nervous system, or in the gastrointestinal tract, particularly in the ileum, genitourinary tract, hypothalamus, spinal cord, thyroid, and the endocrine cells of pituitary gland (Bockman et al, 1989; Domin 1990; Lo et al, 1992).

A large number of regulatory peptides initially identified in the brain and gut have subsequently been localised in the thyroid gland where they have been proposed to act as local transmitters or paracrine regulators. In the present study, stimulation of the FRTL-5 in 5H or 6H medium with different doses of NmU-25 (5 to 1000 nmol/l) did not affect the iodine influx or the growth of cells.

3.4.23 Tachykinins

Tachykinins or neurokinins comprise a family of closely related peptides that participate in the regulation of diverse biological processes. The tachykinin peptides substance P, neurokinin-A, neurokinin-A (3-10), neuropeptide K, and neuropeptide γ are produced from a single preprotachykinin gene as a result of differential RNA

splicing and posttranslational processing. Another tachykinin, neurokinin B, is produced from a separate preprotachykinin gene (Krause et al, 1989; Helke et al, 1990; Regoli and Nantel, 1991; Mussap et al, 1993; Maggi et al, 1993).

Substance P and neurokinins were found to be present in both the central nervous system and in peripheral organs (Mussap et al, 1993) including thyroid gland (Ahrén, 1991). Neurones are the major source of tachykinins both in the central nervous system and in peripheral organs, but tachykinin-like immunoreactivity has also been found in other cell types, such as certain endocrine cells including the medullary thyroid carcinoma cell line 6/23 (Cremins et al, 1992), principal parenchymal cells in the carotid body, chromaffin cells in the adrenal gland, cells of the anterior pituitary, eosinophils, cells in pig skin, and vascular endothelial cells (Maggi et al, 1993).

Substance P and other members of the tachykinin family produce their actions through interaction with one (or more) of three receptors, which are known as neurokinin or NK receptors (Krause et al, 1989; Helke et al, 1990; Regoli and Nantel, 1991; Mussap et al, 1993; Maggi et al, 1993). Since there are three receptors, and cross-reactivity of members of the tachykinin family with more than one receptor has been detected, it is necessary to use different specific agonists to identify the presence of each receptor. [Sar⁹, Met(O₂)¹¹]-Substance P (**SMO-SP**) is a specific agonist for the NK1 receptor, α -Neurokinin Fragment 4-10 (**NK 4-10**) is a specific agonist for the NK2 receptor, and succinyl-[Asp⁶, N-Me-Phe⁸]-Substance P (**S-SP**) is a specific agonist for the NK3 receptor, although they all do cross-react with other receptors with lower affinity (Maggi et al, 1993; Mussap et al, 1993).

Substance P (Ahrén et al, 1983) and neurokinin-A (Grunditz et al, 1987) have been detected in the thyroid gland when assessed by immunoreactivity. It is also reported that in the dog substance-P stimulated thyroid hormone secretion, whereas in the mouse it had no effect (Ahrén, 1991). Results of various experiments indicate that NK1, NK2 and NK3 receptors are present in the central nervous system, but the number of NK2 receptors is less than that of the other two (Maggi et al, 1993). All three receptors also have been detected in peripheral tissues with NK2 dominating the other two (Helke et al, 1990; Maggi et al, 1993).

In this study, stimulation of the FRTL-5 cells with different doses of substance P in the absence of TSH increased the iodine influx at concentrations of 100 and 250 nmol/l, without affecting growth. When this experiment was repeated using specific agonists for each receptor as stimulators, the same pattern was obtained. These data indicate that any member of the tachykinin family can increase the iodine influx of this cell line at concentrations ranging between 100 to 250 nmol/l, and it is possible that all three receptors are present on the surface of this cell line.

However, it is not clear whether the concentrations of any of the peptides *in vivo* reach those required to show biological effects.

The increase in the iodine influx may lead to an increase in the production of thyroid hormones, as detected in the dog (Ahrén, 1991). Additionally, among the patients with Hashimoto's disease, the reduced concentration of T₄ may increase the local release of substance P as it could do in the anterior pituitary gland (Jones et al, 1994). This increase would lead to an increase in the iodine influx, followed by an increase in the production of thyroid hormones. Furthermore, the present study also indicates that local release of other member of the tachykinin family (neurokinin A, neuropeptide K, and neuropeptide γ or neurokinin-B) could also increase the iodine influx or increase the production of thyroid hormones.

Stimulation of the cells in 6H medium (2U/l of TSH) with substance P caused increases in the iodine influx, without affecting growth. The increases in iodine influx occurred at concentrations ranging from 100 to 2000 nmol/l. Stimulation with SMO-SP caused a bell-shaped response-curve, with increases detected at concentrations ranging from 250 to 1000 nmol/l, while there was no change in the iodine influx of the cells stimulated with 2 μ mol/l. Furthermore, there was an apparent increase in the growth of the cells. On the other hand, stimulation of the cells with α -neurokinin (NK 4-10), the specific agonist for the NK2 receptor, caused increases at all concentrations ranging from 10 to 2000 nmol/l, without affecting the growth. Finally, stimulation of cells with the NK3 receptor agonist succinyl-[Asp⁶, N-Me-Phe⁸]-Substance P (S-SP) produced increases at concentrations ranging from 10 to 500 nmol/l, and no effect at concentrations above 500 nmol/l. Beside the increases in the iodine influx, S-SP also increased the growth of cells at concentrations ranging from 250 to 1000 nmol/l.

The increases in the iodine influxes obtained for the low doses of NK 4-10 and S-SP suggest strongly that NK2 and NK3 receptors are present on the surface of the FRTL-5 cells, and local releases of neuropeptide γ , neurokinin-A, neuropeptide K, or neurokinin-B may increase the synthesis of thyroid hormones. On the other hand, the NK1 receptor may not be present, because neither substance P nor SMO-SP could increase the iodine influx at low concentration (10-100 nmol/l), and the increases in the iodine influxes obtained at higher concentrations could be caused by the cross-reaction of these peptides with other receptors (NK2 and NK3). Other experiments carried out in the present study showed that stimulation of the cells with a mixture of three agonists did not increase iodine uptake to a higher level than stimulation with two, though this effect could also be caused by desensitisation of the receptors or by the concentrations reaching the descending portion of the dose-response curve.

3.4.24 Noradrenaline

The presence of adrenergic nerves in the thyroid gland has been known for more than 20 years (Ahrén, 1986 b). Electron auto-radiography has demonstrated, both in human and rodent tissue, that adrenergic nerve terminals occur in close connection with the follicular cells (Ahrén, 1991). A species variation exists with regard to the abundance of adrenergic innervation of the thyroid gland: human, mice, and sheep have a rich supply of adrenergic nerves within the thyroid in contrast to rats, dogs, and pig; and it seems that the density of adrenergic terminals in thyroid gland declines with age (Ahrén, 1986 b).

Noradrenaline is the classical adrenergic neurotransmitter, and the $\alpha 1$ adrenergic receptor mediates its action. There is evidence that both synthesis and appearance of the $\alpha 1$ adrenergic receptor on the surface of thyrocytes are regulated by TSH through a cAMP/protein kinase-A dependent mechanism (Bone et al, 1986 a; Corda et al, 1989; Meucci et al, 1994).

In the present study, it was found that thyroid follicular cells that were deprived of TSH for five days did not respond to noradrenaline (50 nmol/l to 20 μ mol/l of \pm Arterenol), although there was a tendency to an increase in iodine influx. This may indicate that the number of $\alpha 1$ adrenergic receptors on the cell membranes was reduced by deprivation of TSH. On the other hand, stimulation of the cells in the presence of TSH increased iodine influx, without affecting the growth. These increases were dose dependent.

There are reports indicating that noradrenaline increases the synthesis of thyroid hormones by increasing iodine influx (Marcocci et al, 1987) and its incorporation into thyroglobulin (Rani and Field, 1988). On the other hand, there are contrasting reports that show noradrenaline does not change (Taguchi and Field, 1988; Oda et al, 1993), or reduces (Cochaux et al, 1982; Ahrén et al, 1986 b) the intracellular concentration of cAMP induced by TSH and the production of thyroid hormones.

The changes of iodine influx caused by noradrenaline could be caused by the activation of phospholipase A2 (Marcocci et al, 1987) consequent to increases in the free cytosolic concentration of calcium and inositol phosphates (Philp and Grollman, 1986; Marcocci et al, 1987; Grollman et al, 1989; Bizzarri and Corda, 1994; Meucci et al, 1993; 1994; and 1995). The increase in the intracellular concentration of calcium may also increase the intracellular pH and induce the influx of iodine through iodine channel.

3.4.25 S-Nitroso-N-Acetyl Penicillamine

S-Nitroso-N-acetyl penicillamine (SNAP) is a compound that slowly releases nitric oxide (NO) into solution. In the present study, this compound was used to investigate the effect of NO on the iodine influx and growth of FRTL-5 cells. The iodine influx of cells that were deprived of TSH for five days decreased after incubation for 48 h in medium containing 5 or 10 $\mu\text{mol/l}$ of SNAP, while there were increases in growth. On the other hand, in the presence of TSH, iodine influx decreased at a concentration of 2.5 $\mu\text{mol/l}$. At 5 $\mu\text{mol/l}$ there was no change, and there was an increase at a concentration of 10 $\mu\text{mol/l}$. In the presence of TSH, growth of the cells was not affected by any concentration of SNAP.

These results are the first report that shows a direct effect of nitric oxide on the iodine influx and growth of thyroid follicular cells. An action of NO on thyroid follicular cells of humans (Millatt et al, 1993), and thyroid slices from the dog (Esteves et al, 1992) has been shown indirectly in the presence of sodium nitroprusside, which breaks down to NO and stimulates cGMP production. However, stimulation of thyrocytes for 48 h with sodium nitroprusside had no effect on the release of T_3 . Esteves and co-workers (1992) also showed that the increase in cGMP is caused by activation of the PIP₂/calcium cascade.

These reports show that NO can influence the intercellular signalling of thyroid follicular cells. The increases in the cytosolic concentrations of calcium, PIP₂, and cGMP may lead to the decreases in the iodine influxes and increases in growth detected in the absence of TSH. The reasons for the biphasic response in the presence of TSH are not clear, and require further investigation.

One of the sources for local release of NO is the endothelial cell. Since the thyroid gland is extensively vascularised, NO released by these cells may influence the iodine uptake of the thyroid follicular cells. Additionally, the macrophages infiltrating the gland in autoimmune thyroid diseases could be a second source of local release of NO. VIP is an inducer of NO synthesis in gastric muscle cells (Murthy et al, 1993), rat pinealocytes (Spessert, 1993) and many other cells such as smooth muscle (Grider et al, 1992), intestinal, sphincteric, visceral, corpora cavernosa, and cerebral vessels (Murthy et al, 1993). There is a possibility that the VIP released in the gland also induces the synthesis of NO by thyroid follicular cells.

3.4.26 Multiple Stimulators

3.4.26.1 Combination with Interleukin-1

Since IL-1 is an important cytokine released by antigen presenting cells after activation and during development of autoimmune diseases, this cytokine was used in combination with other stimulators.

The addition of IL-1 at a concentration of 1 $\mu\text{g/l}$ had little effect on other stimulators. At a concentration of 20 $\mu\text{g/l}$ in the presence of TSH, the effects of a mixture of IGF-1 and IL-1 were approximately additive, IL-1 significantly reducing the effect of IGF-1. Similarly, IL-1 significantly decreased the growth effect of IGF-1, particularly in the presence of TSH.

IL-1 at 20 $\mu\text{g/l}$ also inhibited the increase in iodine uptake caused by INF- γ . The effects of INF- γ and IL-1 in the presence of TSH were additive. IL-1 itself had no effect on growth rate, and did not modify the inhibitory effect of INF- γ .

IL-1 decreased the effects of NPY, VIP and noradrenaline on iodine uptake. Only the lower concentration was tested in combination with the tachykinins, and this had no effect, as would be expected.

In general, therefore, the effects of IL-1 were inhibitory. This may have implications for the situation in autoimmune disease or inflammation, where there is evidence for the presence of IL-1 within the gland. The effects were greater in the presence of TSH, so IL-1 may act to limit the growth and activity of the thyroid in both Hashimoto's and Graves' diseases.

3.4.26.2 Combination with Noradrenaline

Noradrenaline is known to be present within the thyroid gland and is thought to be responsible for many of the symptoms of hyperthyroidism. It has also been shown to share neurones with other mediators such as NPY.

NA significantly increased the stimulatory effects of IGF-1, VIP and NPY on iodine uptake in the presence of TSH. Surprisingly, there were also growth-stimulatory effects of NA in the presence of VIP and NPY, even when the individual compounds had no effect. This implies that noradrenaline may be directly involved in the pathogenic mechanisms of hyperthyroidism, and not just in causing the symptoms.

3.4.27 Protease Inhibitors

The cell-surface protease inhibitors bestatin and thiorphan had no effect on either unstimulated or TSH-stimulated iodine uptake or growth rate. They did, however, partially inhibit the stimulation of iodine uptake caused by TGF- β , endothelin, IL-6, NPY, VIP and the tachykinins, particularly in the presence of TSH. This effect was surprising. In other systems, it has been generally assumed that the effect of the proteases is to degrade the peptides, and the inhibitors were added in order to protect them and potentiate their actions. From these results, it seems more likely that activation of iodine uptake itself involves the cleavage of a protein, possibly to activate the transporter or associated molecules, and that this process was inhibited by bestatin or thiorphan. Clearly, more work is required before the mechanism of action can be clarified.

There was also a stimulatory effect on growth, but this was seen only in the cases of IL-6 and the tachykinins. This effect would correspond more closely to what might be expected if the proteases were involved in degradation of these particular peptides.

3.4.28 Thyroid Hormones

The addition of thyroid hormones to medium containing TSH considerably modified the activity of the stimulators. In most cases the hormones antagonised the action of the peptides. The stimulatory effects of IGF-1, TGF- β , endothelin-1, interferon- γ , PACAP and VIP on iodine uptake were all reduced. In addition, thyroid hormones prevented the inhibitory effects of IL-1 and LPS. However, there was a large potentiation of the increase in iodine uptake caused by noradrenaline.

The actions on growth rate were similar. The stimulatory effects of endothelin-1 and LPS were reduced, as were the inhibitory effects of TGF- β , IL-4 and interferon- γ . The only exceptions were slight growth stimulatory effects in the presence of VIP and neuromedin-U.

These results call into question the role of growth factors and peptides in hyperthyroidism. Only FGF and the tachykinins were completely unaffected by thyroid hormones. The effects of the others were greatly reduced to an extent that they may be of little importance *in vivo*. In autoimmune hypothyroidism, however, the absence of thyroid hormones may allow the peptides to act fully and they may contribute to goitre formation under those circumstances.

The most striking finding was the increase in iodine uptake in the presence of noradrenaline. Coupled with the fact that noradrenaline increased the effect of IGF-1, NPY and VIP on iodine uptake and also caused growth-stimulatory effects, it seems

that it is the single most important influence on the metabolism of thyroid cells in the hyperthyroid state.

Chapter Four

Conclusion

CHAPTER FOUR

CONCLUSION

The initiating cause of autoimmune hyperthyroidism or Graves' disease is unknown. However, the result is an immune response involving macrophages, T and B lymphocytes, and their soluble products, leading to the development of goitre. Local release of cytokines by infiltrating immune cells, growth factors by thyrocytes or other cells in the gland, and neuropeptides released by nerves could influence the functions of the thyroid follicular cells.

The purpose of this study was to investigate the relationship between thyroid disease and cytokine production. Initially, the level of sIL-2R was investigated in samples of serum collected from patients with Graves' disease before and after treatment. The study was extended by stimulating whole blood samples from untreated patients with Graves' disease, to assess the production of sIL-2R and other cytokines *in vitro*, and the results were compared with those obtained from blood collected from healthy control subjects. Then, FRTL-5 rat thyroid epithelial cells were used as a model to investigate the effects of these cytokines and various other peptides that would be expected to be present in the thyroid and influence its activity. Finally, the effects of these bioactive peptides were investigated in the presence of TSH and thyroid hormones, that could resemble the *in vivo* circumstances of Graves' disease.

4.1 SOLUBLE IL-2 RECEPTOR AND CYTOKINE PRODUCTION IN GRAVES' DISEASE

The present study confirmed that there are high levels of sIL-2R in serum of patients with untreated Graves' disease. The reasons for this abnormality are not clear. Increases in serum sIL-2R have been previously ascribed to a general activation of the immune system. There have also been suggestions that the concentrations of thyroid hormones could directly affect its production, because release correlated with thyroxine use in patients with total thyroidectomy, and increases are also found in patients with non-immune thyrotoxicosis. The effect of thyroid hormones and anti-thyroid drugs (methimazole) were directly studied on preparations of blood cells. Effects were detected, though only in stimulated cells. The production of sIL-2R was increased by thyroid hormones and TSH, while that of IL-6, TNF- α and INF- γ was markedly reduced by both the hormones and methimazole. This gives some support to the suggestion that thyroid hormones and the drugs used in the treatment of thyroid

disease can directly affect the production of sIL-2R and several cytokines from blood cells. This could imply an aberrant inflammatory response in both untreated and treated patients with thyroid disease.

The source of the sIL-2R could not be identified in this study, since the decision was taken to use unseparated blood cells to avoid possible artefacts caused by the procedures used to separate the cells. However, the fact that the cells responded to both LPS and PHA suggests that there are contributions from monocytes and lymphocytes, as would be expected from previous work.

The pattern of production of cytokines by whole blood of the patients' group was also different from that obtained for the control group. The abnormalities in the release of other cytokines did not appear to fit the hypothesis of separate sets of TH1 and TH2 cells. Both INF- γ and IL-4 were released, though the amount of INF- γ was much greater. PMA and Ca ionophore were much weaker stimuli than LPS and PHA, possibly because of direct effects on monocytes and release of stimulatory cytokines. Overall, the pattern suggested that there were both TH1 and TH2 cells present. However, the amount of IL-4 released in patients was more than in controls, and the amount of INF- γ was less. This may imply a degree of switching towards a TH2 phenotype, but it is clearly not complete. The marked effect of varying the dilution of blood also suggests that care must be taken to avoid the effect of interfering substances in blood plasma.

Recent reports (Achiron et al, 1994; Wolf et al, 1994; Yentis et al 1994) have also shown that elevated levels of immunoglobulin (IgG and IgA) could down-regulate the release of inflammatory cytokines. The presence of antibodies against TSH receptor, thyroglobulin or TPO among the patients with autoimmune thyroid disease could be one of the reasons for change in the pattern of cytokines production detected in the presence study.

Whether the abnormalities seen in blood cells reflect the activities of the cells in the thyroid itself is open to question. It is possible that blood may be depleted of certain populations of lymphocytes which are retained in the thyroid. However, it is known that there is an increased number of activated blood cells, in the blood of patients with Graves' disease, so this is unlikely. It is not known whether this represents an epiphenomenon or is part of the pathogenesis of the disease. The facts that patients treated with IL-2 and INF- γ tend to develop autoimmune phenomena would, however, tend to suggest that abnormalities in cytokine production are connected with the presence of disease.

Stimulation of whole blood of the reference group with a mixture of LPS and PHA, in presence of high concentrations of TSH, thyroid hormones, or methimazole significantly reduced the production of IL-6, INF- γ , and TNF- α . These findings indicate that the change in the pattern of cytokine production detected among the

patients with Graves' disease could be the consequence of elevated thyroid hormones or TRAb, while among the patients with Hashimoto's disease, high levels of TSH could change the pattern of cytokine production. Since this is the first report that shows that these compounds could influence the cytokines production, it is too early to reach a conclusion, and further experiments are necessary. It may suggest that the increase in the number of TH2 and decrease in the number of TH1 or suppressor T cells reported previously could be due to the influence of elevated thyroid hormones or TRAb. In addition, these results also indicate that methimazole has an immunological effect, but do not show whether this effect is a direct effect on the synthesis of cytokines, or an indirect effect through induction of other cytokines. The answers to these questions need further investigation.

4.2 CARRIER PROTEINS FOR INORGANIC IODINE IN SERUM

Because of disagreements in the literature whether it is necessary to include protein in the uptake medium as a carrier for iodine or whether it is adequate to use carrier-free solution, it was decided to investigate whether there was a carrier for inorganic iodine in serum. In this study, it was shown that iodine bound to albumin immediately at 37° C, and this association is dose dependent and saturable (figure 3.6 and 3.7). Follow up experiments showed that more than 50 % of inorganic iodine was carried by pre-albumin and albumin, while the remainder was carried by other proteins especially lipoproteins.

Inclusion of albumin as a carrier for iodine decreased both the K_m and the V_{max} of iodine uptake of FRTL-5 cells. These results suggest that there is a carrier system for inorganic iodine in vivo, as is true for other trace elements. The thyroid gland has a reservoir of thyroid hormones in the form of colloid inside the follicles, and there are carrier proteins, including pre-albumin and albumin, that carry 15-30 % of thyroid hormones in the serum. Since the same proteins carry both iodine and thyroid hormones, the question arises is whether excess of inorganic iodine in the serum could compete with thyroid hormones, and release them from their binders. If so, an excess of iodine could change the normal pattern of cytokine production, and be an initial step in development of autoimmune thyroid disease. The answer to this question needs further investigation, though there are reports (Harach et al, 1985; Boukis et al 1983; Weetman 1991) indicating that high iodine intake could lead to thyroid autoimmune diseases.

4.3 THYROID HORMONES AND THYROID FUNCTION

In this study, it was shown that thyroid hormones also could control the function of thyroid follicular cells. The stimulation of FRTL-5 cells with high concentrations of thyroid hormones and TSH increased the growth of thyroid follicular cells, while it reduced the iodine influx. These effects could form an auto-regulatory loop in Graves' disease. Thyroid cells in this disease would be expected to accumulate large quantities of iodine under the continuous stimulation of TSH-receptor antibodies. The elevated concentrations of thyroid hormones could then limit further iodine uptake and prevent thyroid function from completely escaping from hormonal control.

On the other hand, in the absence of TSH, stimulation of the cells with high concentration of thyroid hormones increased growth as well as iodine uptake. These results may indicate that part of the abnormal growth of cells in patients with toxic goitres may be caused by the elevation in thyroid hormones themselves, though TSH may modify the effects.

4.4 BIOACTIVE PEPTIDES AND THYROID FUNCTIONS

In this study, effect of some bioactive peptides on iodine uptake and growth of thyroid follicular cells were investigated. Some of these peptides could be synthesised and released by infiltrating immune cells, or cells in the gland, and act in a paracrine manner, while others such as IGF-1 and IL-6 may be synthesised and released by the thyroid follicular cells themselves and act in an autocrine manner. In addition, the effects of these peptides were investigated in the presence of protease inhibitors to found out the presence of cell-surface proteases might affect the action of mediators. Finally, for the first time, the effects of these peptides on the thyroid follicular cells were investigated in conditions which resemble that found in Graves' disease (TSH and high concentration of thyroid hormones), to determine the effect of these peptides in such patients. The summary of the results obtained in this study is presented in table 4.1.

The effects of IGF-1, TGF- β , bFGF, IL-1, INF- γ , and noradrenaline were mostly in agreement with previous reports, as was discussed in chapter three. The results obtained for the remaining stimulators are reported in this study for the first time. Most of the peptides did have some effect on the cells, though often weak.

The addition of thyroid hormones to medium containing TSH considerably modified the activity of the stimulators. In most cases the hormones antagonised the action of the peptides. The stimulatory effects of IGF-1, TGF- β , endothelin-1, interferon- γ , PACAP and VIP on iodine uptake were all reduced. In addition, thyroid

Condition	5H		6H		5H+P.I		6H+P.I		Graves' disease	
Peptide	I	G	I	G	I	G	I	G	I	G
IGF-1	NE	+	+	+	NC	NC	NC	NC	NE	+
TGF- β	+	NE	+	-	NC	NC	-	NC	+	NE
bFGF	-	+	-	+	NC	-	NC	NC	-	+
ET-1	NE	+	NE	+	NC	NC	-	NC	NE	NE
IL-1: high dose	NE	NE	-	NE	NC	NC	NC	NC	NE	NE
IL-1: low dose	NE	NE	NE	NE	NC	NC	NC	NC	+	NE
IL-4	NE	NE	NE	-	NC	NC	NC	NC	NE	NE
IL-6	+	+	+	NE	NC	+	-	+	+	+
INF- γ	+	NE	+	-	NC	NC	NC	NC	+	-
LPS: high dose	-	NE	-	+	NC	NC	NC	NC	NE	NE
LPS: low dose	NE	NE	+	NE	NC	NC	NC	NC	NE	NE
PACAP	NE	NE	+	NE	NC	NC	NC	NC	+	-
NPY	+	+	NE	NE	NC	NC	-	NC	NE	NE
VIP: high dose	+	NE	+	-	NC	NC	-	NC	NE	+
VIP; low dose	NE	NE	+	+	ND	ND	ND	ND	ND	ND
Neuromedin	NE	NE	NE	NE	NC	NC	NC	NC	NE	+
SP	+	NE	+	NE	-	NC	-	-	+	NE
SMO-SP	+	NE	+	+	NC	NC	NC	NC	+	+
NK	+	NE	+	NE	NC	NC	-	+	+	NE
S-SP	+	NE	+	+	-	NC	-	NC	NE	+
NA	NE	NE	+	NE	NC	NC	NC	NC	+	NE
SNAP	-	+	±	NE	ND	ND	ND	ND	ND	ND

Table 4.1: Effect of different bioactive peptides on iodine uptake (I) and growth (G) of FRTL-5 cells, in different experimental conditions. The media containing protease inhibitors (bestatin and thiorphan) are shown by P.I., while stimulation with 6H medium containing thyroid hormones is referred to as Graves' disease. NE stands for not affected, and ND means not determined. In the column dealing with the effect of protease inhibitors, the + or - sign shows significant increases or decrease respectively compared to controls without protease inhibitors. NC indicates no changes compared with the corresponding response in the absence of protease inhibitor.

hormones prevented the inhibitory effects of IL-1 and LPS. However, there was a large potentiation of the increase in iodine uptake caused by noradrenaline.

The actions on growth rate were similar. The stimulatory effects of endothelin-1 and LPS were reduced, as were the inhibitory effects of TGF- β , IL-4 and interferon- γ . The only exceptions were slight growth stimulatory effects in the presence of VIP and neuromedin-U.

These results call into question the role of growth factors and peptides in autoimmune hyperthyroidism. Only FGF and the tachykinins were completely unaffected by thyroid hormones. The effects of the others were greatly reduced to an extent that they may be of little importance *in vivo*. In autoimmune hypothyroidism, however, the absence of thyroid hormones may allow the peptides to act fully and they may contribute to goitre formation under those circumstances.

The most striking finding was the increase in iodine uptake in the presence of noradrenaline. Coupled with the fact that noradrenaline increased the effect of IGF-1, NPY and VIP on iodine uptake and also caused growth-stimulatory effects, it seems that it is the single most important influence on the metabolism of thyroid cells in the hyperthyroid state.

The results obtained in this study may show that in Graves' disease, when the thyroid follicular cells are under continuous stimulation by TRAb and high concentration of thyroid hormones, local release of TGF- β , IL-1, IL-6, INF- γ , PACAP, noradrenaline, and tachykinins, or the presence of LPS could increase the iodine uptake of the cells, while bFGF could decrease the iodine uptake. The concentration of noradrenaline in the glands of these patients is another factor that can influence the outcome. Because most of these peptides could increase the iodine uptake, which may increase the intracellular concentration of this element, this may lead to inhibition of thyroid hormone synthesis through the Wolff-Chaikoff effect. On the other hand, noradrenaline not only increases the iodine uptake but also increases the efflux of iodine, that may lead to an increase in the production of thyroid hormones.

Goitre formation in Graves' disease could be the consequence of the local release of IGF-1, bFGF, IL-6, VIP, neuromedin-U, and tachykinins, while down-regulation of growth could be the result of local release of INF- γ and PACAP. There is a possibility that a down-regulatory effect could be produced by a combination of TGF- β and IL-4. This possibility could be a starting point for follow up experiments.

The cell-surface protease inhibitors bestaatin and thiorphan had no effect on either unstimulated or TSH-stimulated iodine uptake or growth rate. They did, however, partially inhibit the stimulation of iodine uptake caused by TGF- β , endothelin, IL-6, NPY, VIP and the tachykinins, particularly in the presence of TSH. This effect was surprising. It seems more likely that activation of iodine uptake itself

involves the cleavage of a protein, possibly to activate the transporter or associated molecules, and this process was inhibited by bestatin or thiorphan. Clearly, more work is required before the mechanism of action can be clarified.

There was also a stimulatory effect on growth, but this was seen only in the cases of IL-6 and the tachykinins. This effect would correspond more closely to what might be expected if the proteases were involved in degradation of these particular peptides.

Since IL-1 is an important cytokine released by antigen presenting cells after activation and during development of autoimmune diseases, this cytokine was used in combination with some of the stimulators. The results obtained indicated that the effects of IL-1 were inhibitory. This may have implications for the situation in autoimmune disease or inflammation, where there is evidence for the presence of IL-1 within the gland. The effects were greater in the presence of TSH, so IL-1 may act to limit the growth and activity of the thyroid in both Hashimoto's and Graves' diseases.

Noradrenaline is known to be present within the thyroid gland and is thought to be responsible for many of the symptoms of hyperthyroidism. It has also been shown to share neurones with other mediators such as NPY.

NA significantly increased the stimulatory effects of IGF-1, VIP and NPY on iodine uptake in the presence of TSH. Surprisingly, there were also growth-stimulatory effects of NA in the presence of VIP and NPY, even when the individual compounds had no effect. This implies that noradrenaline is directly involved in the pathogenic mechanisms of hyperthyroidism, and not just in eliciting the symptoms.

These studies have shown that several peptides have direct effects on thyroid cells. Abnormalities have been shown in production of the same peptides in thyroid disease. This gives strong support to the proposal that abnormalities in cytokine production are central to the abnormalities found in autoimmune disease. Much more work is needed in patients, however, to prove which of these peptides are important *in vivo* and what their role is in human disease.

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Appendices

APPENDIX 1

LIST OF ABBREVIATIONS

4H:	4 hormone medium
5H:	5 hormone medium
6H:	6 hormone medium
MSH:	Melanocyte stimulating hormone
ACTH:	Adrenocorticotrophic hormone
APA:	Aminopeptidase A, also called BP-1 or 6C3
APC:	Antigen presenting cell
ATP:	Adenosine triphosphate
BCGF-1:	B cell growth factor-1, an older term used for IL-4
Bis:	N,N'-methylene-bis-acrylamide
BSA:	Bovine Serum Albumin
BSF-1:	B cell stimulatory factor-1, an older term used for IL-4
BSF-2:	B cell stimulatory factor-2, an older term used for IL-6
Ca:	Calcium
C cells:	Parafollicular cells
CD:	Cluster of Differentiation
CD3:	T cell receptor complex
CD4:	Surface protein characterising helper T cells
CD8:	Surface protein characterising cytotoxic T cells
CD10:	Neutral endopeptidase (NEP)
CD11:	α subunit of integrin LFA-1
CD13:	Aminopeptidase N (APN)
CD18:	Equivalent term for LFA-1 or CD11a
CD19:	Co-receptor for B cells that forms complex with CR2
CD21:	Receptor for complement component or CR2
CD25:	Soluble interleukin-2 receptor, and α chain of IL-2 R
CD27:	Equivalent term for member of tumour necrosis growth factor super-family on the surface of T cells
CD28:	Adhesion molecules, on the surface of T cells
CD40:	Equivalent term for member of tumour necrosis growth factor super-family on the surface of B cells, and monocytes
CD44:	Binds hyaluronic acid, mediates adhesion of leukocytes
CD45:	Equivalent term for leukocyte common antigen
CD50:	Adhesion molecules, equivalent term for ICAM-3

CD54:	Equivalent term for ICAM-1
CD69:	Activation inducer molecule on the surface of activated lymphocytes, and monocytes
CD80:	Adhesion molecules, on the surface of APC, equivalent term for B7-1
CD86:	Adhesion molecules, equivalent term for B7-2
CD102:	Adhesion molecules, equivalent term for ICAM-2
CD118:	Receptor for interferon α and β
CD119:	Receptor for interferon- γ
CD126:	One of the subunits of IL-6 receptor
CDw 130:	One of the subunits of IL-6 receptor also called gp 130
cAMP:	Cyclic adenosine monophosphate
CRP:	C-reactive protein
CTC:	Chicken serum-trypsin-collagenase solution
DIT:	Diiodotyrosine
DMSO:	Dimethyl Sulfoxide
EDTA:	Ethylenediamine-tetra-acetate
eIF2:	Eukaryotic protein synthesis factor-2
ELISA:	Enzyme linked immunosorbent assay
ET-1:	Endothelin-1
ET_A:	Endothelin receptor
ET_B:	Endothelin receptor
Fc:	Fragment of antibody without antigen sites
FCS:	Foetal calf serum
FGF:	Fibroblast growth factor
FRTL-5:	A line of normal differentiated Fisher rat thyroid cells
FSH:	Follitrophin
GH:	Growth hormone
GHRH:	Growth hormone-releasing hormone
GM-CSF:	Macrophage colony stimulating factor
GMP-140:	Granule membrane adhesion protein 140
HBSS:	Hanks' Balanced Salt Solution
HCG:	Human chorionic gonadotrophin
HEPES:	N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]
HLA:	Human leukocyte antigen (equivalent term for animal MHC)
HSF:	Hepatocyte stimulating factor, an older term used for IL-6
Ia:	Immune response associated proteins
IBMX:	3-isobutyl-methyl xanthine
IgD:	One of the classes of immunoglobulin
IgE:	One of the classes of immunoglobulin

IgG:	One of the classes of immunoglobulin
IgM:	One of the classes of immunoglobulin
IGF-1:	Insulin-like growth factor 1
IGF-II:	Insulin-like growth factor 2
Ii:	Invariant chain (associated with MHC II, in the endoplasmic reticulum)
IL-1α:	Interleukin 1 α
IL-1β:	Interleukin 1 β
IL-1ra:	IL-1 receptor antagonist
IL-2:	Interleukin 2
IL-2R:	IL-2 receptor
IL-2Rα:	One of the subunits of IL-2 receptor or CD25
IL-2Rβ:	One of the subunits of IL-2 receptor
IL-2Rγ:	One of the subunits of IL-2 receptor
IL-3:	Interleukin 3
IL-4:	Interleukin 4
IL-5:	Interleukin 5
IL-6:	Interleukin 6
IL-7:	Interleukin 7
IL-10:	Interleukin 10
IL-12:	Interleukin 12
IL-13:	Interleukin 13
INF-β:	Interferon- β
INF-β2:	Interferon β 2, an older term used for IL-6
INF-γ:	Interferon- γ
IP3:	Inositol 1,4,5-trisphosphate
LAK:	Lymphocyte-activated killer cells
LH:	Luteotrophin
LPS:	Lipopolysaccharide
Mg:	Magnesium
MHC:	Major histocompatibility complex
MIT:	Monoiodotyrosine
MTT:	[3-(4,5-Dimethylthiazol)-2-yl]-2,5-Diphenyltetrazolium Bromide; Thiazolyl Blue
NA:	Noradrenaline
NIM 1:	Human thyroid carcinoma cell line
NK:	Neurokinin
NK 4-10:	α -Neurokinin Fragment 4-10, a specific agonist for the NK2 receptor
NK1:	Neurokinin receptor

NK2:	Neurokinin receptor
NK3:	Neurokinin receptor
NmU:	Neuromedin U-25
NPY:	Neuropeptide-Y
NSB:	Non-specific binding
PACAP:	Pituitary adenylate cyclase activating polypeptide
PBS:	Phosphate buffered saline
PHA:	Lectin, or Phytohemagglutinin
PMA:	Phorbol 12-myristate, 13-acetate
rT3:	Reverse triiodothyronine
S-SP:	Succinyl-[Asp ⁶ , N-Mc-Phe ⁸]-Substance P, a specific agonist for the NK3 receptor
SCF:	Stem-cell factor
sIL-2R:	Soluble interleukin-2 receptor
SMO-SP:	[Sar ⁹ , Met(O ₂) ¹¹]-Substance P, a specific agonist for the NK1 receptor
SNAP:	S-Nitroso-N-acetyl Penicillamine, a stable donor of nitric oxide
SP:	Substance P
T₃:	L-triiodothyronine
T₄:	L-thyroxine
TAP-1:	Transporters associated with antigen processing-1
TAP-2:	Transporters associated with antigen processing-2
TBG:	Thyroid binding globulin
TBPA:	Thyroid binding pre-albumin
TCR:	T cell receptor
TEMED:	N,N,N',N'-tetramethylethylenediamine
TG:	Thyroglobulin
TGF-β:	Transforming growth factor-β
TIAb:	TSH receptor inhibitory antibody
TMB:	3,3',5,5'-tetramethylbenzidine
TNF:	Tumour necrosis factor
TNF-R1:	TNF receptor
TNF-R2:	TNF receptor
TPO:	Thyroid peroxidase
TRH:	Thyrotrophin releasing hormone
TSAb:	TSH receptor stimulating antibody
TSFs:	Antigen specific suppressor factors
TSH:	Thyrotrophin or thyroid stimulating hormone
VIP:	Vasoactive intestinal peptide

VIP₁: Specific receptor for VIP
VIP₂: Specific receptor for VIP

APPENDIX 2

LIST OF MATERIALS

The sources and catalogue numbers of consumables and chemicals used for this study are given below.

Amersham Life Sciences, Little Chalfont, Bucks, UK

Iodine-125, Carrier-Free, Cat No. IMS 30.

cAMP Elisa Kit, Cat No. RPN 225

Amicon Ltd, Stonehouse, Glos, UK

Centriflo Membrane Filter Cones, CF-25

BDH, Merck Ltd, Poole, Dorset, UK

Acetic Acid (Glacial) Cat No. 27014

Acrylamide: Cat No. 15212

Ammonium Persulphate: Cat No. 10032

Disodium Hydrogen Phosphate, Anhydrous: Cat No. 10249

Ethanol (Absolute 99.7-100 %): Cat No. 14007

Glycerol: Cat No. 10118

Hydrochloric Acid (Analar): Cat No. 10307

Methanol (Dried Analar): Cat No. 10473

N'N'-Methylene-Bis-Acrylamide (Bis): Cat No. 15213

Sodium Chloride: Cat No. 10241

Sodium Dihydrogen Phosphate: Cat No. 10245

Sulphuric Acid (Analar): Cat No. 10276

Tris [Hydroxymethyl] Amino Methane: Cat No. 10315

Becton Dickinson Europe, UK:

18 Gauge Needles

Plastipack Syringes 1 ml, 5 ml, 10 ml, 20 ml, and 30 ml

Sterile 6 ml Polypropylene Tubes with Cap, 12 X 75 mm (Falcon Cat No.2054, 2063)

Vacutainer Tubes

Bibby Sterilin Ltd. UK.

1 ml Sterile Plastic Pipettes

5 ml Sterile Plastic Pipettes

10 ml Sterile Plastic Pipettes

25 ml Sterile Plastic Pipettes

Bio-Rad Laboratories Ltd, Hemel Hempstead, Herts, UK

Bio-Rad Protein Assay Kit 11, Cat No. 500-0002

British Bio-Technology Products Ltd, Abingdon, UK (Now R&D Systems)

Basic Fibroblast Growth Factor (Basic FGF) Human Sequence,

Recombinant: Cat No. BDP 37

Transforming Growth Factor β -1 (TGF β -1) Human Sequence,

Recombinant: Cat No. 240-B-002

Calbiochem-Novabiochem UK Ltd, Nottingham, UK

Ca Ionophore A23187: Cat No. 100105

Phorbol 12-myristate, 13 acetate: Cat No. 524400

Cambridge Bioscience, Cambridge, UK

S-Nitroso-N-Acetyl Penicillamine: Cat No. N-7892

European Collection Of Animal Cell Cultures, Porton Down, UK

FRTL-5 Rat Thyroid Cells

Gelman Sciences, Northampton, UK

Sterile Acrodisc Syringe Filters: Cat No. 6224192.

Gibco BRL, Life Technologies, Inc. Paisley, Scotland

1.8 ml Cryotubes: Nunc Cat No. 3-68638k

12 ml Sterile Conical Centrifuge Tubes: Nunc Cat No. 3-43141a

15 ml Sterile Conical Centrifuge Tubes: Nunc Cat No. 3-74632a

50 ml Sterile Centrifuge Tubes: Nunc Cat No. 3-73660a

6 Well Plates: Nunc Cat No. 152795 A

24 Well Plates: Nunc Cat No. 143982 A

96 Well Plates: Nunc Cat No. 167008 A

25 Cm² Tissue Culture Flask: Nunc Cat No. 1-63371 A

25 Cm² Tissue Culture Flask, Filter Cap: Nunc Cat No. 1-36196 A

80 Cm² Tissue Culture Flask: Nunc Cat No. 1-53732 A

80 Cm² Tissue Culture Flask, Filter Cap: Nunc Cat No. 1-78905 A

Chicken Serum: Cat No. 16110-033

Foetal Calf Serum: Cat No. 10091-064

L-Glutamine: Cat No. 25030-032

Hanks' Balanced Salt Solution Without Sodium Bicarbonate (10x): Cat No. 14060-040

Interleukin-1 β (Murine, Recombinant): Cat No. 3321ss

Interleukin-4 (Murine, Recombinant): Cat No. 3323ss

Interleukin-6 (Human, Recombinant): Cat No. 3240ss

Penicillin/Streptomycin Solution: Cat No. 15140-031

Phosphate Buffered Saline: Cat No. 21300-017

RPMI-1640 Without L-Glutamine: Cat No. 31870-017

RPMI-1640: Cat No. 041-01875

Trypsin 1:250: Cat No. 27250-042

Leo Laboratories, UK

Heparin

Precision Research Biochemicals, London, UK

Recombinant Human Insulin Like Growth Factor-1 (IGF-1): Cat No. 100-11

Recombinant Human Interferon Gamma (INF- γ): Cat No. 300-02

Quadrantech, Epsom, Surrey, UK

Endotoxin-free heparinised vacuum blood collection tubes (Endotube Et)

Sigma Chemical Company Ltd. Dorset, UK

6 Well Plate: Corning Cat No. 25810-6

12 Well Plate: Corning Cat No. 25815-12

24 Well Plate: Corning Cat No. 25820-24

96 Well Plate: Corning Cat No. 25860-96

Sterile 50 ml Centrifuge Tubes: Corning Cat No. 25330-50

Vacuum Filtration System (500 Ml): Corning Cat No. F 9893

Vacuum Filtration System (1000 Ml): Corning Cat No. F1145

\pm Arterenol (\pm Nor Epinephrine) Bitartrate Salt: Cat No. A 0937

3-Isobutyl Methyl Xanthine (IBMX): Cat No. I 5879

Adenylate Cyclase Activating Polypeptide-27: Cat No. A 9808

Albumin Fraction V: Cat No. A 2153

Amphotericin B: Cat No. A 9528

Bestatin Hydrochloride; Synthetic: Cat No. B8385

Bromophenol Blue (Free Acid): Cat No. B 6896

Coomassie Brilliant Blue (R): Cat No. B 0630

Dimethyl Sulfoxide (DMSO): Cat No. D 8779

DL Thiorphan: Cat No. T 6031

EDTA tetra sodium salt: Cat. No. E 5391

Endothelin-1 Synthetic: Cat No. E 7764

F-12 Coon's Modification with L-Glutamine and 0.863 mg Zinc Sulphate per litre, without Sodium Bicarbonate: Cat No. F 6636

Gentamicin Solution (50 Mg/Ml): Cat No. G 1397

Glycyl-Histidyl-Lysine Acetate: Cat No. G 7383

Hanks' Balanced Salt Solution with Sodium Bicarbonate: Cat No. H2513

Hanks' Balanced Salt Solution with Sodium Bicarbonate, without Calcium Chloride and Magnesium Sulphate: Cat No. H 8389

Hepes (N-[2-Hydroxyethyl]Piperazine-N'-[2-Ethanesulfonic Acid]): Cat No.

H 7523

Hydrocortisone: Cat No. H 0888
 Insulin: Cat No. I 5500
 Lectin (Phytohemagglutinin From Phaseolus Vulgaris): Cat No. L 9132
 Lipopolysaccharide From Salmonella Enteritidis (LPS): Cat No. L 7770
 MEM Amino Acids Solution Without L-Glutamine (50x): Cat No. M 7020
 MEM Nonessential Amino Acid Solution (100x): Cat No. M 7145
 MTT { [3-(4,5-Dimethylthiazol)-2-Yl] -2,5- Diphenyltetrazolium Bromide;
 Thiazolyl Blue}: Cat No. M 2128
 Neurokinin Fragment 4-10: Cat No. N 5141
 Neuromedin U-25: Cat No. N 8138
 Neuropeptide Y: Cat No. N 3266
 Potassium Iodide: Cat No. P 8256
 Protein Standard (Bovine Serum Albumin) P 7656
 [Sar⁹, Met (O₂)¹¹]-Substance P: Cat No. S 2150
 Sodium Bicarbonate: Cat No. S 8875
 Sodium Bicarbonate Solution (7.5 %): Cat No. S 5761
 Sodium Hydroxide: Cat No. S 5881
 Somatostatin: Cat No. S 1763
 Substance P Acetate Salt: Cat No. S 6883
 Succinyl-[Asp⁶,N-Me-Phe⁸]-Substance P Fragment 6-11: Cat No. S 6772
 T₃ (3,3',5-Triiodo-L-Thyronine)Sodium Salt: Cat No. T 5516
 T₄ (L-Thyroxine) {3-[4-(4-Hydroxy-3,5-Diiodophenoxy)-3,5
 Diiodophenyl]-L-Alanine} Sodium Salt, Pentahydrate. Crystalline: Cat No. T
 0397
 TEMED (N,N,N',N'-Tetramethyl-Ethylenediamine): Cat No. T 8133
 Thyrotropin (TSH) 10U/Vial: Cat No. T 3538
 Transferrin: Cat No. T 7786
 Trypan Blue: Cat No. T 6146
 Vasoactive Intestinal Peptide : Cat No. V 3628
**T Cell Diagnostics Inc, supplied by Laboratory Impex, Teddington,
 UK**
 CELLFREE Interleukin-2 Receptor test kit: Cat No CK1020

APPENDIX 3

REAGENT PREPARATION

REAGENTS FOR STUDY OF sIL-2R RELEASE FROM WHOLE BLOOD

Phosphate Buffered Saline With Bovine Albumin

To 100 ml of sterile phosphate buffered saline (PBS) solution pH 7.4, 200 mg of bovine albumin was added. The albumin was dissolved by standing at room temperature for approximately 30 min. The solution was sterilised again by filtering through an Acrodisc syringe filter into a sterile bottle. The bottle was stored at 4° C. This solution is named PBS-A.

Lipopolysaccharide

The contents of one vial, 1 mg of Lipopolysaccharide from *Salmonella enteritidis* (LPS), were dissolved in one ml of sterile phosphate buffered saline with albumin (PBS-A), to give a final concentration of 1 mg/ml. Portions of 25 μ l were prepared in sterile Eppendorf tubes, and stored at -20° C. When required, portions were allowed to thaw at room temperature. Then the tubes were centrifuged at 1000 g for 2-3 min, to sediment liquid sticking to the internal wall of the tube.

Phytohaemagglutinin

The contents of one vial, 5 mg of Phytohaemagglutinin from *Phaseolus Vulgaris* (PHA), were dissolved in 5 ml of sterile PBS-A, to give a final concentration of 1 mg/ml. Portions of 25 μ l were prepared in sterile Eppendorf tubes, and stored at -20° C.

Penicillin/Streptomycin Solution

Portions of 5 ml were made from a concentrated solution of penicillin/streptomycin containing 10000 U/ml and 10 mg/ml respectively, and kept at -20° C.

Medium

RPM1-1640 medium with 3 g/l L-Glutamine and 2 g/l of NaHCO_3 was used for this section of study. To 500 ml of medium, 5 ml of 100x concentrated solution of penicillin/streptomycin was added to give a final concentration of 100 U/ml of Penicillin and 100 $\mu\text{g/ml}$ of Streptomycin. This medium was kept at 4° C. Immediately before use, the required volume was transferred to a sterile container and warmed up to 37° C.

REAGENTS FOR STUDY OF CYTOKINE RELEASE FROM WHOLE BLOOD

Mixture of LPS and PHA

A combined solution of 2 mg/ml LPS from *Salmonella enteritidis* and 0.4 mg/ml PHA was provided by Medgenix Diagnostics. Portions of 80 μl were dispensed into sterile Eppendorf tubes, which were kept at -20° C.

Phorbol 12-Myristate, 13-Acetate Solution

The contents of a vial (5 mg) of Phorbol 12-myristate, 13-acetate (PMA) were dissolved in 200 μl of 100% ethanol, to prepare a stock solution of 25 mg/ml. Portions of 5 μl were made from this solution, and kept at -20° C.

Before an experiment, 2.4 μl from an aliquot was diluted to 500 μl with medium, to produce an intermediate concentration of 120 $\mu\text{g/ml}$. From this intermediate solution, 4 μl was added to 2 ml of fresh medium, to produce the working solution. The concentration of this solution was 240 ng/ml.

Ca Ionophore A23187 Solution

The contents of a vial (10 mg) were dissolved in 500 μl of DMSO to prepare a stock solution of 20 mg/ml. Portions of 10 μl were made from this solution, and kept at -20° C.

Before an experiment, 2 μl from an aliquot were diluted to 400 μl with medium, to produce an intermediate concentration of 100 $\mu\text{g/ml}$. To 920 μl of fresh medium, 80 μl of intermediate solution was added to produce the working solution of Ca ionophore. The concentration of this solution was 8 $\mu\text{g/ml}$.

Methimazole Solution

One gram of Methimazole, relative molecular mass of 114.2, was dissolved in 87.57 ml of distilled water, to produce a stock solution of 100 mmol/l. The solution was filtered through a 0.22 μ m sterile syringe filter, into a sterile container. Portions of 250 μ l were made from this solution and stored at -20° C.

Thyrotropin Solution

The contents of one vial (10 U) were dissolved in 10 ml of HBSS containing 0.2 g Bovine Serum Albumin. This gives a solution of TSH containing 1 U/ml. Portions of 100 μ l were made in sterile Eppendorf tubes, and kept at -20° C, for preparation of working TSH solution.

The contents of one aliquot were further diluted to 20 ml with medium, to have a solution of 5 mU/ml. Portions of 250 μ l were made in sterile Eppendorf tubes, and kept at -20° C.

T₄ Solution

The contents of one vial (1 mg), of crystalline L-thyroxine sodium salt, pentahydrate (T₄), relative molecular mass of 888.9, were dissolved in 11.25 ml of HBSS, containing 0.25 ml of 1 mol/l NaOH. This gives a solution of 100 μ mol/l. Portions of 250 μ l were made from this solution, and stored at -20° C.

T₃ Solution

The contents of one vial (1 mg), of crystalline 3,3' 5-Triiodo-L-Thyronine sodium salt (T₃), relative molecular mass 673, were dissolved in 13.86 ml of HBSS, and one ml of 1 mol/l NaOH. This gives an intermediate solution of 100 μ mol/l. From this intermediate solution, 50 μ l were further diluted with HBSS to 5 ml. The concentration of this solution is 1 μ mol/l. Portions of 250 μ l were made from this solution, and stored at -20° C.

Medium

RPM1-1640 medium without L-Glutamine, with 2 g/l of NaHCO₃, was used for this study. To 500 ml of medium, 5 ml of a concentrated solution of 200 mmol/l L-glutamine was added, followed by 5 ml of 100 x concentrated solution of penicillin/streptomycin. The final concentrations of added ingredients were 2 mmol/l

of L-glutamine, 100 U/ml of penicillin, and 100 μ g/ml of streptomycin.

The medium was kept in a refrigerator (4-8° C). Immediately before use, the required volume was transferred to a sterile container and warmed to 37° C.

REAGENTS FOR GROWTH OF FRTL-5 CELLS

Heat Inactivated Foetal Calf Serum

A bottle of 100 ml of frozen serum was thawed at room temperature. When thawed, serum was mixed by gently inverting the bottle several times, avoiding foam formation. The bottle was transferred to a pre-heated 56° C water bath and heated for one hour. The bottle was cooled at room temperature, washed externally with 75% methylated spirit, and transferred to a laminar flow cabinet. Aseptically, the serum was divided into 25 or 50 ml aliquots in 50 ml sterile conical tubes and stored at -20° C.

Insulin Solution, 10 g/l

The contents of a vial (500 mg) were dissolved in 50 ml of Hanks' Balanced Salt Solution (HBSS) containing 1 g/l Bovine Serum Albumin (BSA) and 750 μ l of 1 mol/l sodium hydroxide, and stored at -20° C.

Transferrin Solution, 10 g/l

The contents of a vial (100 mg) were dissolved in 10 ml HBSS containing 1 g/l BSA. This solution was made as required.

Somatostatin Solution, 500 mg/l

The contents of a vial (1 mg) were dissolved in 2 ml of the HBSS containing 1 g/l BSA. Aliquots of 0.4 ml were made in sterile Eppendorf tubes, and stored at -20° C.

Glycyl-Histidyl-Lysine Acetate Solution, 50 μ g/ml

The contents of a vial (0.5 mg) were dissolved in 10 ml of the HBSS containing 1 g/l Bovine Serum Albumin. Aliquots of 4 ml were made in sterile tubes, and stored at -20° C.

Hydrocortisone Solution, 0.5 mmol/l

The contents of a vial (1 mg) were dissolved in 165.3 ml of Ethanol. This solution was kept at -20°C . 1.5 ml was further diluted to 50 ml with the HBSS to give a solution of 0.5 mmol/l. Aliquots of 0.4 ml were made in sterile Eppendorf tubes, and stored at -20°C .

Thyrotropin (TSH) Solution, 2 U/ml

The contents of vial (10 U) were dissolved in 5 ml of the HBSS containing 1 g/l BSA. Aliquots of one ml were made in sterile tubes, and stored at -20°C .

Five Hormones' Mixture

To prepare 200 ml of a concentrated mixture of five hormones, 20 ml of insulin solution (10 g/l), 10 ml of transferrin solution (10 g/l), 0.4 ml of somatostatin solution (500 mg/l), 4 ml of Gly-His-Lysine solution (50 mg/l), and 0.4 ml of hydrocortisone solution (0.5 mmol/l) were added to 165.2 ml of HBSS. The final concentrations were 1.0 mg/ml insulin, 0.5 mg/ml transferrin, 1.0 $\mu\text{g/ml}$ somatostatin, 1.0 $\mu\text{g/ml}$ Gly-His-Lys, and 1.0 $\mu\text{mol/l}$ hydrocortisone. Aliquots of 10 ml were prepared and stored at -20°C till the time of use.

Four Hormones' Mixture

This mixture was prepared by omitting insulin from the above five hormones' mixture and replacing its volume with the HBSS. Since the amount of concentrated four hormone solution needed during the study was limited, all the volumes were divided by 10 for preparation of 20 ml of this solution.

Five Hormones' Medium

For preparation of five hormones' medium (5H), one vial of Coon's modified F-12 medium containing ascorbic acid and glutamine was dissolved in approximately 900 ml of sterile distilled water. The container was washed with 40 ml of sterile distilled water, and the washings were added to the original medium. When the powder had dissolved completely, 35.7 ml of 75 g/l NaHCO_3 was added, followed by 10 ml penicillin streptomycin (10^5 U , $10^5\text{ }\mu\text{g/ml}$), 1 ml of Gentamicin (50 mg/ml), 10 ml of concentrated five hormones' mixture, and 50 ml of heat inactivated Foetal

Calf Serum (FCS). The volume was made up to 1050 ml by addition of sterile distilled water. The final concentrations were 10 µg/ml insulin, 5 µg/ml transferrin, 10 ng/ml somatostatin, 10 ng/ml Gly-His-Lys, 10 pmol/l hydrocortisone, 100 U/ml penicillin, 100 µg/ml streptomycin, 30 µg/ml gentamycin, and 5% serum. After all constituents had been added to the medium, it was filtered into a sterile bottle supplied with a 0.22 µm filter attached (Sigma).

Six Hormones' Medium

To prepare six hormones' medium (6H) with TSH concentration of 2 U/l, 1 ml of a stock solution of TSH (2 U/ml) was added to one litre of 5 hormones' medium before filtration. After mixing, the medium was filtered through a 0.2 micron filter.

Stimulation Medium

For stimulation of cells with added mediators, the 5 or 6 hormone medium used for propagation and preparation of the cells was replaced by medium containing 0.5% serum and additional amino acids. For preparation of this medium the standard protocol was followed, except for the fact that 10 ml of 50 x concentrated essential amino acid solution in minimum essential medium (MEM) without L-glutamine, and 10 ml of 100 x concentrated nonessential amino acid solution in MEM were added to the medium. The volume of FCS was reduced to 5 ml/l.

Chicken Serum-Trypsin-Collagenase Solution

To separate the FRTL-5 cells from the plate or flask, chicken serum-trypsin-collagenase solution (CTC) was used.

To 450 ml of the HBSS without calcium and magnesium, 10 ml of heat inactivated chicken serum was added, followed by addition of 745 mg of EDTA (sodium salt), 375 mg of trypsin, and 2.5 ml of collagenase (5000 units /ml). After all components had dissolved, the volume was adjusted to 500 ml with the HBSS without calcium and magnesium. The pH was adjusted to 9.0. The solution was kept in a refrigerator for 4 h, then filtered through a 0.2 micron filter, and stored at -20° C. The final concentrations were 750 mg/l trypsin, 250 U/l collagenase, 2 mmol/l EDTA, and 2% chicken serum.

Stopping Solution

To stop the action of trypsin, Coon's modified Ham's F-12 medium containing 5% serum and 2 mmol/l EDTA (1.49 g/l) was used.

Freezing Medium

To store cells in liquid nitrogen, a freezing medium consisting of 90 % foetal calf serum and 10 % DMSO was used.

REAGENTS FOR IODINE UPTAKE STUDY

Potassium Iodide

For preparation of a stock solution of potassium iodide (10 mmol/l), 166 mg of KI were dissolved in 100 ml distilled water.

Radioactive Iodine

One mCi was supplied in 10 μ l of NaOH. To prepare a stock solution of 10 mCi/ml, 90 μ l of 0.5 mol/l PBS was added to the vial.

Hanks' Balanced Salt Solution with 10 mmol/l HEPES

To a one litre bottle of HBSS, 2.383 grams of HEPES (N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]) was added, and dissolved by shaking the bottle for 2-3 min.

Iodine Uptake Medium

On the day of the experiment, the required amount of albumin was added to the HBSS containing 10 mmol/l HEPES, to give a final concentration of 5 g/l. The required amount of stock solution of potassium iodide (10 mmol/l) was then added to give a final concentration of 10 μ mol/l and the pH of the buffer was adjusted to 7.35 with 2.5 mol/l NaOH solution. At this stage, the buffer was heated to 37° C. Immediately before use, the required volume of radioactive iodine was added to the buffer to give a concentration of 0.5 μ Ci/ml, at a specific activity of 50 mCi/mmol.

Substance	Amount per vial	Volume (ml)	Concentration of stock
α -NK	1 mg	5.470	200 μ mol/l
Bestatin	5 mg	13.77	1 mmol/l
basic FGF	25 μ g	0.500	50 μ g/ml
Endothelin-1	10 μ g	0.800	4 μ mol/l
IBMX	56 mg	10	25.2 mmol/l
IGF-1	100 μ g	1.000	100 μ g/ml
IL-1	0.5 μ g	0.100	5 μ g/ml
IL-4	0.5 μ g	0.100	5 μ g/ml
IL-6	1.0 μ g	0.200	5 μ g/ml
INF- γ	100 μ g	1.000	100 μ g/ml
LPS	1.0 mg	1.000	1.0 mg/ml
Neuromedin U	100 μ g	0.626	50 μ mol/l
Noradrenaline	6.39 mg	10.00	20 mmol/l
NPY	100 μ g	0.372	50 μ mol/l
PACAP	100 μ g	0.222	100 μ mol/l
SMO-SP	1.0 mg	0.508	1.0 mmol/l
SNAP	25 mg	1.135	100 mmol/l
Substance P	500 μ g	3.550	83 μ mol/l
S-SP	1.0 mg	1.188	1.0 mmol/l
T ₃	1.0 mg	59.50	25 μ mol/l
T ₄	1.0 mg	50.00	22.5 μ mol/l
TGF β -1	2 μ g	0.500	4 μ g/ml
Thiorphan	5 mg	19.74	1 mmol/l
TSH	10 U	10.00	1 U/ml
VIP	250 mg	1.31	50 μ mol/l

Table A.3: Composition of stock solutions of stimulators. Most were dissolved in PBS containing 2 g/l BSA, except α -NK (0.5 mol/l acetic acid with 2 g/l BSA), succinyl-(Asp⁶, N-Me-Phe⁸)-substance P (0.05 mol/l acetic acid with BSA), T₃ (HBSS with 1 ml of 1M NaOH), T₄ (HBSS), thiorphan (100% ethanol), TSH (HBSS with BSA), TGF β -1 (4 mmol/l HCl with 5 g/l BSA), IBMX (methanol) and SNAP (DMSO). All were aliquoted and stored at -20° C.

PREPARATION OF STOCK SOLUTIONS OF STIMULATORS

The procedure for preparation of stock solutions of stimulators is described in the table 3A.1. All solutions were stored at -20° C.

REAGENTS FOR POLYACRYLAMIDE GEL ELECTROPHORESIS

Acrylamide/Bis (30% T, 2.67% C)*

On the day of gel preparation, 14.6 g of Acrylamide were dissolved in 40 ml of distilled water, followed by 400 mg of N,N'-methylene-bis-acrylamide (Bis). When the powders had dissolved completely, the volume was adjusted to 50 ml. As the maximum shelf life of this solution in a dark container at refrigerator temperature is 30 days, a small quantity of this reagent was made freshly on the day of gel preparation.

* T = Total monomer concentration. The pore size of the polyacrylamide gel is defined by percentage of T, and calculated by the formula below.

$$\% T = \frac{\text{grams of acrylamide} + \text{grams of Bis}}{\text{Total Volume}} \times 100$$

* C = Cross linking monomer concentration. In diluting a stock solution, the cross linking monomer concentration (%C) is independent of the final total monomer concentration. The percentage of C is calculated by the following formula.

$$\% C = \frac{\text{grams of Bis}}{\text{grams of acrylamide} + \text{grams of Bis}} \times 100$$

Tris-HCl buffer, 1.5 mol/l, pH 8.8

For preparation of this buffer, 54.45 g of Tris base{Tris [hydroxymethyl] amino methane} was dissolved in 200 ml of distilled water, and the pH of the solution was adjusted by addition of 2 mol/l HCl (approximately 80 ml). The final volume was adjusted to 300 ml. The buffer was stored at 4° C.

Tris-HCl Buffer, 0.5 mol/l, pH 6.8

6 g of Tris base were dissolved in 60 ml of distilled water, and the pH of the solution was adjusted by addition of 2 mol/l HCl (approximately 25 ml). The final volume was adjusted to 100 ml. The buffer was stored at 4° C.

Ammonium Persulphate Solution, 10%

One gram of ammonium persulphate was dissolved in 10 ml of distilled water. As the solution is unstable, it was made on the day when gel was prepared.

Bromophenol Blue Solution, 1%

100 mg of bromophenol blue were dissolved in 10 ml of Tris-HCl buffer, pH 6.8.

Sample Buffer

1 ml of Glycerol (99%) was added to 8.5 ml of Tris-HCl buffer (pH 6.8), followed by addition of 0.5 ml of bromophenol blue (BPB) solution. The concentration of glycerol was 10 %, and that of BPB was 0.05%. This solution was stable at 4° C for several months.

Concentrated Electrode Buffer

For preparation of this buffer, 45 g of Tris base were dissolved in 2 l of distilled water, followed by addition of 216 g of glycine. When the powders had dissolved completely, the final volume was adjusted to 3 l with distilled water. For preparation of working electrode or running buffer solution, 300 ml of this buffer were diluted to 1400 ml with distilled water, and the pH was adjusted to 8.3 with 1 mol/l HCl or NaOH. The final volume was adjusted to 1.5 l.

Staining Solution

Staining and fixation were carried out simultaneously with this solution. For preparation of one litre, 100 ml of concentrated acetic acid solution were added to 500 ml of distilled water, followed by addition of 400 ml of 98 % methanol and 1 g of Coomassie blue dye (R-250). The solution was mixed until the dye had dissolved completely and filtered immediately before use. The concentration of Coomassie blue

was 0.1% in 10% acetic acid and 40% methanol.

Destaining Solution

This solution contained the same concentrations of acetic acid and methanol as the staining solution, but did not contain any dye.

DYE PREPARATION FOR MTT PROTOCOL

A stock solution of MTT (2 g/l) was prepared by dissolving 20 mg of MTT in 10 ml of the PBS, pH 7.3. The solution was sterilised by filtration (0.22 μ m), and stored at 4° C, in the dark. This solution was stable for several weeks.

Note: It should be mentioned that the reagents that were common to more than one study are not repeated under different headings.