CHAPTER I

INTRODUCTION

A number of workers have shown that TSH(thyrotrophin or thyroid stimulating hormone) binds to specific receptor sites on the surface of the thyroid cells[1-5]. The interaction of TSH with its receptor leads to activation of adenyl cyclase and the resulting increase in the level of cyclic adenosine monophosphate (cAMP) mediates the effects of the hormone[6-10]. Binding of polypeptide hormones to specific receptor sites has emerged as a new helpful technology in biology because it offers an opportunity to investigate alteration of the receptors. It was also proved that TSH receptor is an interesting factor associated with a cause of abnormality in thyroid function since investigation of the pathogenesis of Graves' disease[11] suggested that TSH and Graves' immunoglobulins bind to the same receptor molecule. This human thyroid stimulating immunoglobulins (TSI) can displace radiolabelled TSH from human thyroid membrane receptors and stimulate several functions of the thyroid cells.

The purpose of this preliminary study is to compare the numbers of TSH receptors and their binding characteristics for thyroid membranes obtained from different thyroid specimens. It is hoped that

application of this method may be useful in the diagnosis and treatment of thyroid patients, especially for those suffered from thyroid carcinoma and Graves' disease.

Review of Literature

TSH is the major factor that regulates all aspects of the thyroid gland's function[12,13]. It is one of four well-defined glycoprotein hormones produced either by basophilic cells (thyrotrophs) in the par distalis of anterior pituitary[14] or by the placenta[14,15].

A. Chemistry of TSH

The molecular weight of mammalian TSH are in the range of 28,000-30,000 daltons[15]. The molecule, which contains about 15 percent of carbohydrate, is composed of two polypeptide chains or subunits[15,16]. The association of the subunits is by the way of noncovalent forces, and association constant must be very high at physiological pH because the levels of circulating hormone are very low[17]. The subunits dissociate at low pH, and one subunit is found to be common to all four glycoprotein hormones: thyroid stimulating hormone (TSH), luteinizing hormone (LH), follicle stimulating hormone (FSH) and human chorionic gonadotropin (HCG)[16,17]. This has been designated alpha and within a species its amino acid sequence is essentially identical from hormone to hormone. The other subunit, beta, has a different amino acid sequence in each hormone and carries the information that specifics the particular hormonal activity to be

expressed. Isolated subunit of TSH do not show any biological activity but when alpha and beta subunits are recombined to form intact molecules, the reconstitute proteins have biological activity[15,17]. Human TSH (hTSH) is very similar to bovine TSH (bTSH), whose degree of purity has now permitted crystallization[18]. Human TSH differs from bTSH in the relative amounts of two amino acid residues, lysine and leucine, and hTSH was found to be much more sialic acid than bTSH[19].

B. Regulation of the TSH secretion.

Two primary factors participating in the regulation of the rate of TSH secreted by anterior pituitary are hypothalamic-pituitary control and negative feedback control of circulating thyroid hormones.

1. Hypothalamic-pituitary control: Hypothalamic lesions were initially used to identify the regions of the brain involved in pituitary regulation. Subsequent study showed that electrical stimulation of several areas of the hypothalamus, most particularly of the paraventricular area, increases the anterior pituitary secretion of TSH and correspondingly increases the activity of the thyroid gland[20,21]. This control is mediated by a hypothalamic hormone, so-called "thyrotrophin-releasing hormone (TRH)", which is a weakly basic tripeptide consisting of pyroglutamic acid, histidine and prolinamine as shown in Fig.1, page 4 .

The central histidine residue is a critical determinant of TRH activity, as summarized by Wilber[22]. TRH is synthesized within peptidergic neurons of the anterior hypothalamus and transported to the

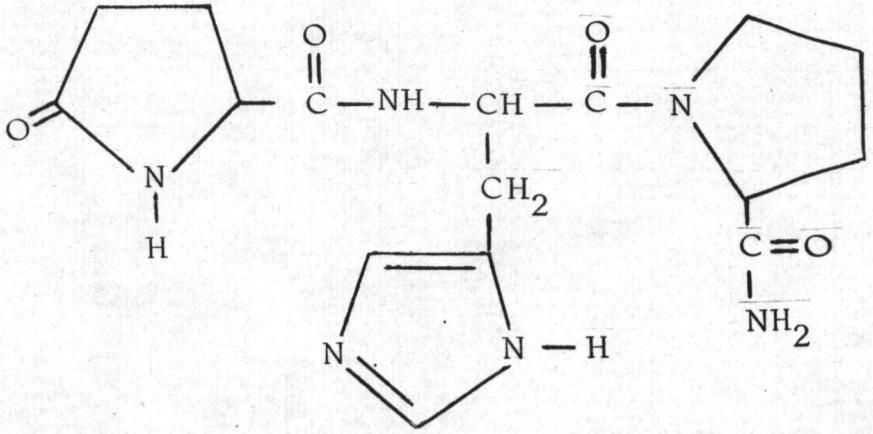

Fig. 1. Structural formula of thyrotrophin-releasing hormone.

median eminence where it is stored[23,24]. From here, it is secreted into the hypophyseal portal venous system and carried to the anterior pituitary gland as illustrated in Fig.2, page 5.

Radioligand binding studies with ^{3}H-labelled TRH have demonstrated that there is a specific binding to anterior pituitary plasma membranes[25-28] and a high degree of specificity of the TRH receptor is suggested by the absence of competitive binding by other hypothalamic peptides and polypeptide hormones[26]. The sequence of events that set into motion by the attachment of TRH to the receptor site appears to involve activation of membrane bound adenyl cyclase and intracellular accumulation of cAMP which could then subserve as the intrapituitary messenger of TRH action and increased the TSH secretion[29,30], as indicated in Fig.3. page 8.

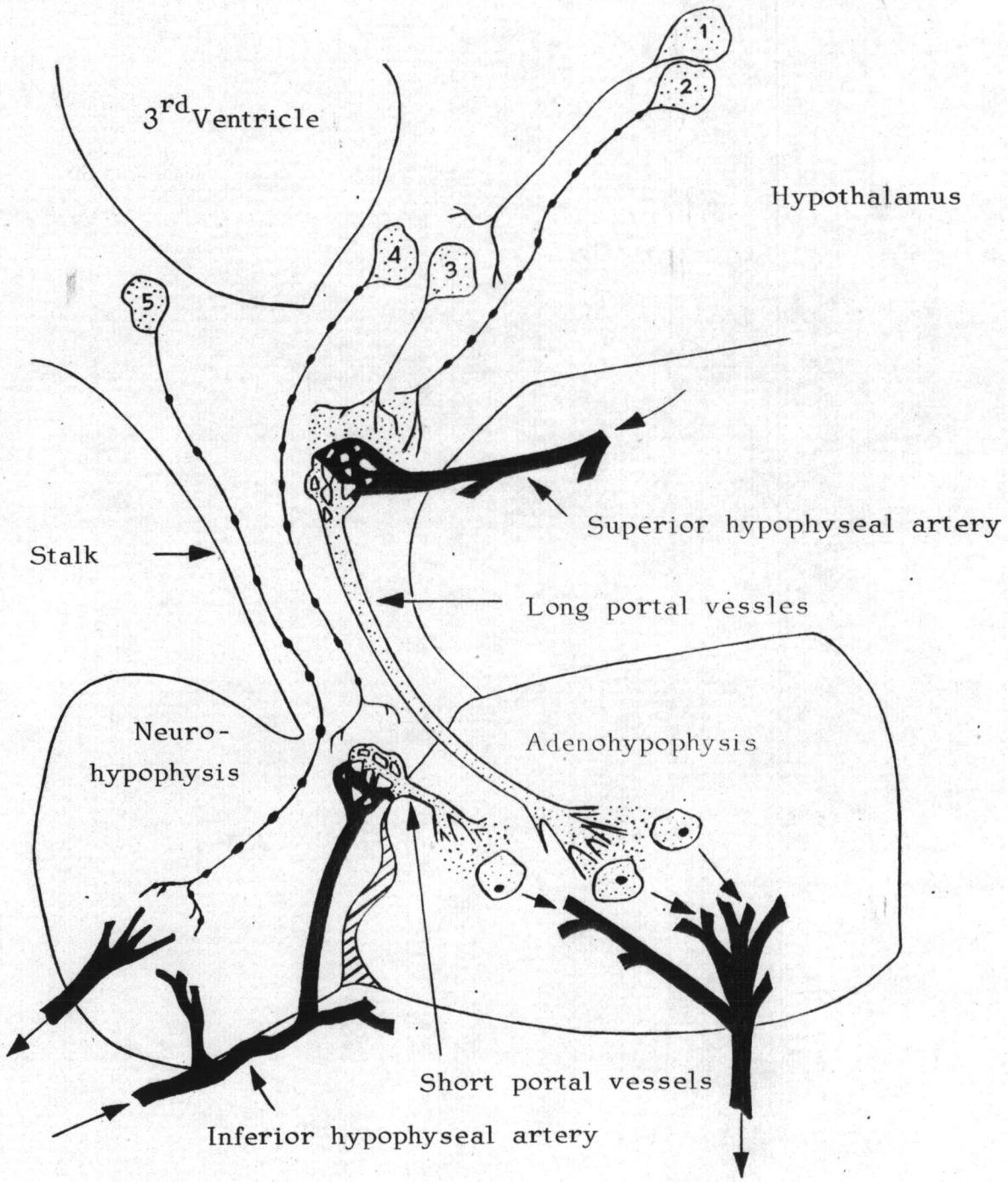

Fig. 2. Hypothalamic-pituitary unit.

Fig 2. Hypothalamic-pitutitary unit. This diagram outlines two general types of neurons which are involved in anterior pituitary regulation. One type, the peptidergic neuron, forms the releasing hormones (No.3, ending in the median eminence, and No.4, ending in the pituitary stalk), both in relation to the capillary plexus of the hypophyseal-portal vessels. These neurons combine the function of excitable electrical tissue and secretory function and serve as "neuro endocrine transducers" to convert neural information to hormonal information. The second type, the monaminergic neuron, is believed to end on the cell-body of the peptidergic neuron in a conventional manner (No.1), or on the axon terminus of the peptidergic neuron (No.2) in a manner termed by Schneider and McCann "axoaxonic" (Following Gay, V. "The Hypothalamus" Physiology and Clinical Use of Releasing Factors". Fertil. Steril. 23 1972-63).

In addition to stimulation of TSH secretion, TRH also stimulates TSH synthesis[31]. A biphasic pattern of TSH secretion is seen after prolonged intravenous infusion of TRH in man[32], and the early phase may well reflect the release of a readily releasable pool of stored TSH within the thyrotrophs, whereas the later phase could be due to release of newly synthesized TSH produced under the influence of increased TRH drive.

2. Negative feedback control by thyroid hormones: The feedback control by thyroid hormones at the pituitary level has been recently demonstrated in man. Small doses of liothyronine ($L\text{-}T_3$ 15 μg)

and levothyroxine sodium (L-T_4 60 μg) daily for 3-4 weeks, amounts insufficient to raise plasma throid hormones level significantly, resulted in the 76 percent reduction of TSH response to TRH[33]

As a complementary finding minimal decrease in plasma thyroid hormone level produced by short-term administration of pharmacological doses of iodide is sufficient to elevate basal and TRH-stimulated TSH levels[34,35]. These observations can be interpreted that the fine adjustment of TSH secretion is mediated at the pituitary level by the feedback effect of the thyroid hormones, as presented in Fig.4, page 9. Application of T_3 and T_4 to rat's anterior pituitary cell cultures suggested that T_4 itself had no influence on TRH responsiveness and that its feedback action resulted solely from its monodeiodinated to T_3[36]. Recent evidence suggests that suppression of TSH secretion in thyroidectomized (hypothyroid) rats occurs by interaction of T_3 with the nuclear receptor of the thyrotroph (Fig.3, page 8), and after T_4 injection, the T_3 found in the nucleus is derived from rapid intrapituitary monodeiodination[37]. When the conversion of T_4 to T_3 is blocked by administration of propylthiouracil to patients, the pituitary becomes more responsive to TRH[38]. This effect may reflect peripheral changes as well as changes at the pituitary level, indicating that T_3 is more potent than T_4 in inhibiting TSH secretion.

In addition to its direct inhibitory effects on TSH synthesis and secretion, thyroid hormones may have a physiological role in regulating TRH receptor density on the thyrotroph cell. Studies in vitro[28] have demonstrated a two-fold increase in TRH binding in hypothyroid

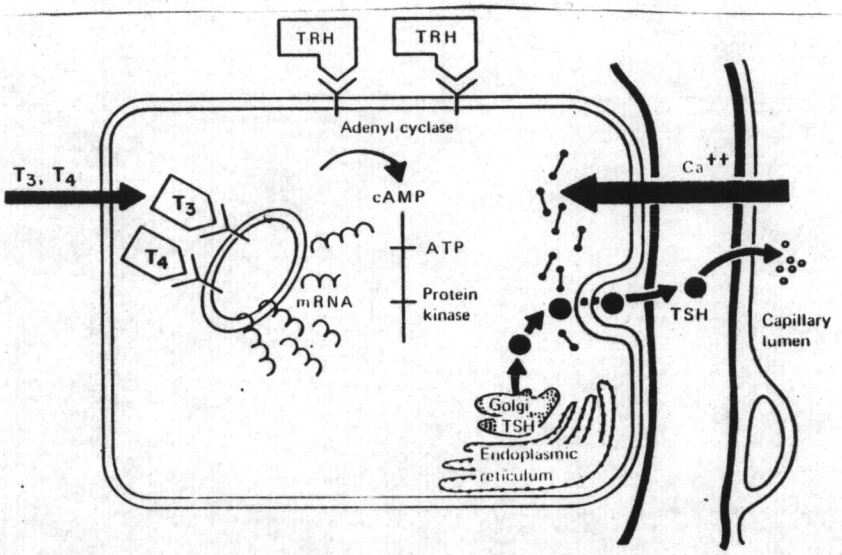

Fig. 3. Postulated mechanisms by which thyroid hormone and TRH act for the control of TSH secretion. TSH synthesis takes place in the endoplasmic reticulum on ribosomes under the influence of messenger RNA. The hormone is concentrated in the Golgi apparatus, formed into granules and then maintained either as the storage granule, released to the exterior of the cell by the process of reverse pinocytosis. When the granule has entered the perivascular space it is lysed, and the soluble hormone is then free to diffuse into the capillary lumen. TRH binds to thyrotrope cell membranes and activates the adenyl cyclase cyclic AMP-protein kinase second messenger cascade. In the presence of $Ca^{2}+$, active release of TSH-containing granules takes place. Over time, new TSH is synthesized T_4 and T_3 bind specifically to nuclear receptors and stimulate the formation of a messenger RNA (mRNA) that directs the synthesis of protein, which in turn inhibits both the release of TSH from the pituitary and the formation of new TSH.

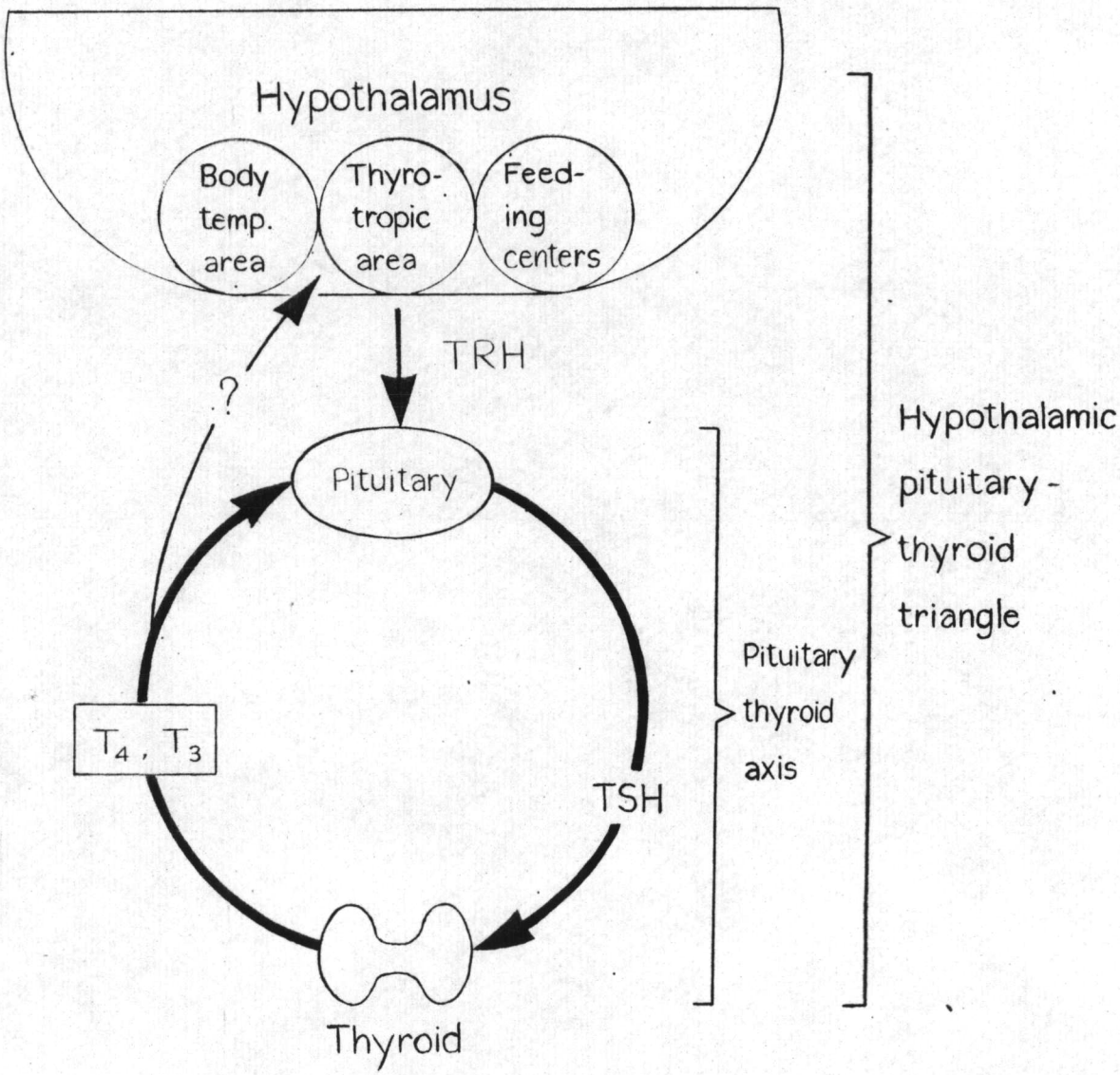

Fig.4. Regulation of the secretion of TSH and thyroid hormones.

animals, which can be reduced by thyroid hormone replacement. This inhibitory effect occurs even when the anterior pituitary has been completely separated from the hypothalamus, although the effect is greater if the hypothalamus and hypothalamic hypophyseal portal system are intact.

Therefore, thyroid hormones exert a powerful, dose-related negative feedback over TSH secretion in two different ways:

a. By a direct effect on the anterior pituitary, and

b. By an indirect effect acting through the hypothalamus.

Within the physiological range, low levels of thyroid hormones stimulate and higher levels inhibit TSH production. Thyroid hormones control the overall metabolic activity of the body so it is tempting to believe that the factor controlled at a nearly constant rate by the TSH-thyroid hormone control system in same aspect of cell metabolism, perhaps the cellular metaboiic rate itself. Thus, if the cellular metabolic rate should become too low, the TSH-thyroid system would become activated until enough thyroid hormone became available to increase the metabolic activities back to normal. Conversely, if these metabolic activities should become too great, the feedback system would become inactivated until the thyroid hormone level fell low enough to allow normal metabolic activities once again.

C. Effects of cold and other neurogenic stimuli on TSH secretion.

1. Effect of cold: Many studies in laboratory animals showed significant elevation of serum TSH and thyroid function with exposure to cold or local cooling of the hypothalamus[39-42]. TRH activity was

higher after 2 and 24 hours of cold exposure in the median eminence and after 8 hours in the anterior hypothalamus-preoptic area[43]. Changes of TRH activity in the median eminence coincided with the elevated serum TSH, they are assumed to reflect increased TRH production and secretion[43]. TSH levels are also increased in infants[44,45]. There is a slightly but significant initial increases in serum TSH response to cold stress in adult man[46,47].

2. Effect of dopamine: The initial studies of Besses and co-workers[48] demonstrated that dopamine infusion intravenously inhibited the TSH response to TRH and this has now been confirmed by Burrow et.al.[49]. Furthermore, dopamine infusion lowers basal TSH levels in both euthyroid and primary hypothyroid subjects[50,51]

3. Effect of somatostatin: Studies in vitro[52] and in vivo[53] suggest that somatostatin (growth hormone inhibitory factor) may also be a physiological inhibitor of TSH release. However, there is no such evidence in healthy man, although somatostatin infusion lowers the elevated basal TSH levels in patients with primary thyroid failure[54] and suppresses the TSH secretion induced by TRH[55].

4. Effect of fasting: Investigations of the effect of fasting in man found that it leads to impaired peripheral conversion of T_4 into T_3, resulting in low total and free T_3, normal total T_4 and normal or slightly elevated free T_4 levels[56,57]. The initial fall of TSH and subsequent failure of the response to TRH in the face of low levels of T_3 suggests inhibition of TSH release[58].

5. Circadian variation: Daily fluctuation in plasma concentration of TSH using twenty hours continuous blood-sampling technique showed significant circadian variation of TSH secretion with a maximum between 2.00 a.m. and 4.00 a.m. and a minimum between 6.00 p.m. and 8.00 p.m.[59]. Extreme states of excitement and anxiety conditions that greatly stimulate the sympathetic nervous system cause acute decrease in secretion of TSH, perhaps because these states increase the metabolic rate and body heat.

D. Mechanism of action of TSH

The initial physiological action of TSH involves binding of the hormone to specific receptor site on the thyroid plasma membrane[60]. Such binding was first indicated by the indirect studies of Pastan et al.[61] but now has been directly demonstrated in isolated thyroid cells, thyroid slices, thyroid homogenates and purified thyroid plasma membranes[1-5,8,10,62]. This reaction leads to activation of membrane-bound adenyl cyclase[63-70] and an increase of intracellular cAMP[63,64,71,72] which dissociates an inactive enzyme, protein kinase, into two subunits, a regulatory and a catalytic subunits[73]. The latter the mediates phosphorylation in a number of subtrates, which are responsible for the action of TSH on the thyroid gland. These specific effects increase in : a) activity of iodide pump, b) exocytosis of thyroglobulin into the follicular lumen, c) thyroid hormogenesis, d) size and secretory activity of the thyroid cells and e) number of thyroid cells, as shown in Fig.5 and 6, page 13.

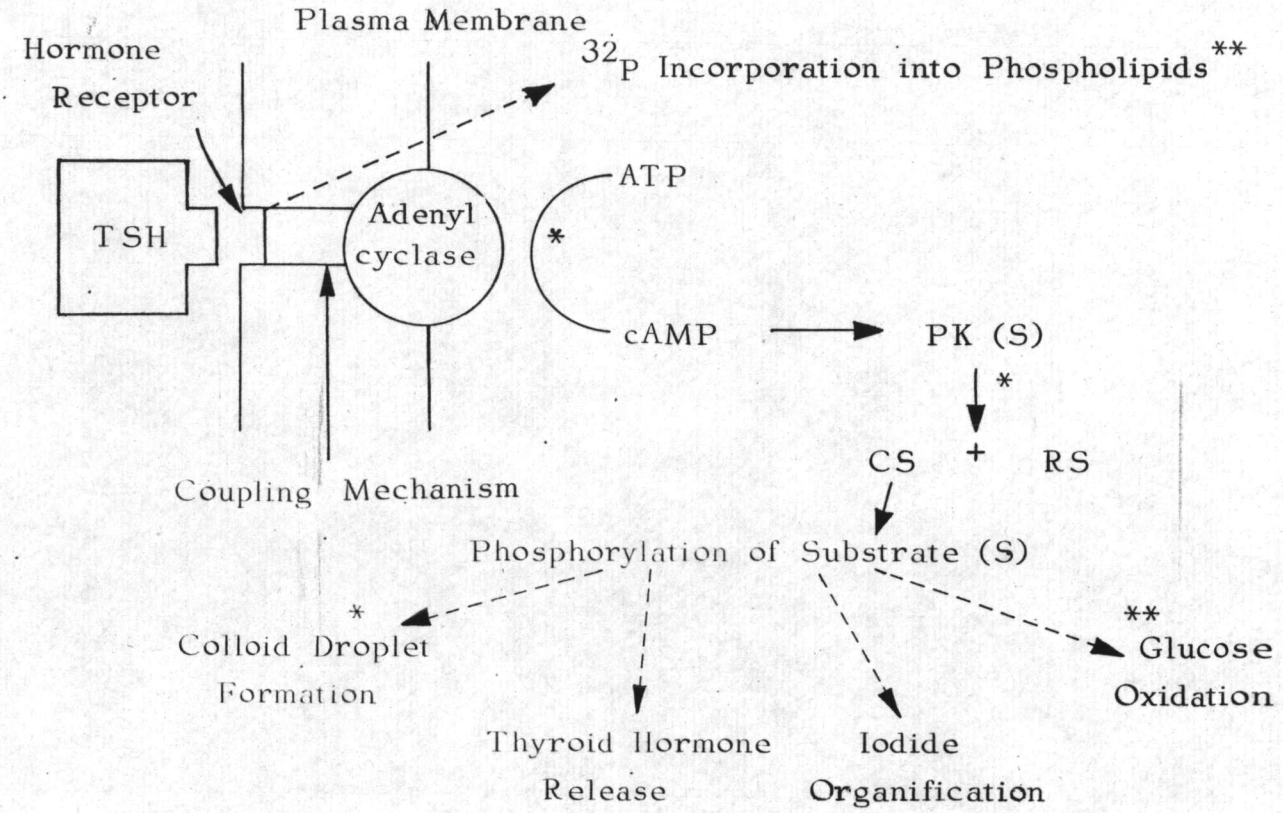

Fig. 5. Representation of mechanism of action of TSH. Phospholipids are an essestial component of either the hormone receptor or the coupling mechanism. * indicates that Ca^{2+} is not required for this action and ** indicates that the effect is Ca^{2+} dependent.

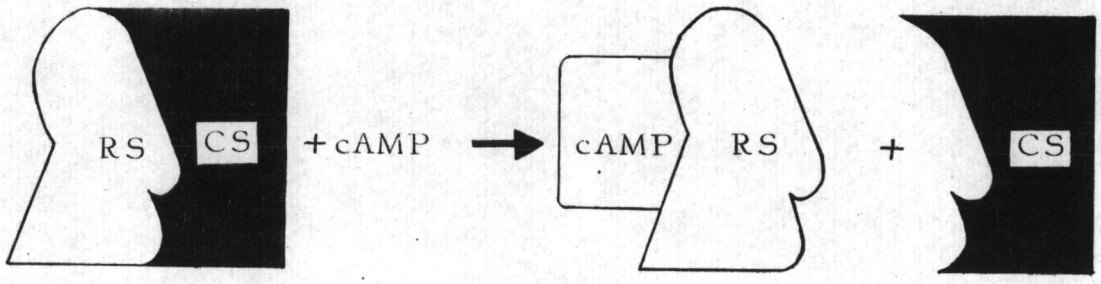

Fig. 6. Activation of protein kinase by cAMP, RS : Regulatory (inhibitory) subunit and CS : Catalytic subunit.

E. The TSH receptors.

There is general agreement that the term "receptor" refers to a molecule (or molecular complex) which is capable of recognizing and selectively interacting with the hormone or neurotransmitter, and which, after binding it, is capable of generating some signal that initiates the chain of events leading to the biological response.

1. Chemical nature of hormone receptors: The TSH receptor is generally large, asymmatric glycoproteins with estimated molecular weights of 280,000 daltons[74]. Experiments with detergents suggest that the TSH receptor is a molecule with two different regions a hydrophobic and a hydrophilic[2,74,75]. The hydrophilic region is in contact with the external environment of the cell and appears to contain the TSH-binding site. The hydrophobic region, however, is embeded in the hydrophobic interior of the cell membrane[76] and may provide a contact with such components as adenyl cyclase which is located on the inner surface of the cell membrane. It has been suggested that gangliosides form an integral part of the TSH receptor[77] but further works and confirmatory studies are required before this can be generally accepted. Preliminary studies with detergent extracts of human thyroid membranes suggest that the human TSH receptor molecule has an isoelectric point of about pH 4 and is associated with a 50,000 molecular-weight protein fraction[78].

2. Characteristics of hormone receptor: Hormone binding to its receptor is largely dependent on time, temperature and the concentrations of hormone and receptor. Using physiological concentrations

of hormone, the binding may reach a steady state within a few minutes at 37°C, while at lower temperature it may not be reached for hours[79]. Kinetic studies with ^{125}I - labelled TSH indicate that contact between TSH and its receptor is transient; both association reactions being fairly rapid[80]. The hormone dissociated from the receptor appears to be identical with the native hormone with respect to its physical properties, interaction with antibodies or other membrane receptors, and biological activity. Addition of excess unlabelled hormone, dilution of the reaction mixture or changing pH results in a rapid dissociation of the bound tracer.

The quantitative aspects of hormone-receptor interaction are complex[81]. Binding has been considered to be the sum of at least two processes, one of which is saturable and has been termed "specific" bindings, and the other is nonsaturable over the range of concentrations studied and has been termed "nonspecific". The latter is composed of low affinity binding to the membrane, as well as nonspecific trapping and adsorption to the glass or plastic incubation tube. For TSH receptor the binding appears to be composed of more than one component, with a high-affinity, low capacity site and a low-affinity, high-capacity site[82]. In the physiological range, most binding occurs to the specific receptor sites, which by definition are finite in number. The actual number of specific receptor sites for TSH is about 500 sites per cell[10].

A variety of mathematical methods have been applied to the

analysis of steady-state binding data in an attempt to derive affinity constants for the hormone receptor interaction[81,83]. In most of these analyses, the hormone receptor interaction is depicted as a simple reversible bimolecular equilibrium,

$$[H] + [R] \underset{k_d}{\overset{k_a}{\rightleftharpoons}} [HR] \quad \text{---(1)}$$

and

$$\frac{k_a}{k_d} = \frac{[HR]}{[H][R]} = K \quad \text{---(2)}$$

where [H] is the concentration of free hormone, [R] is the concentration of unoccupied receptors, [HR] is the concentration of hormone-receptor complexes, k_a and k_d are the association and dissociation rate constants, and K is the affinity or equilibrium constant for the reaction. But in the case of TSH receptor, the reaction appears to be more complex because there are two orders of binding sites which differ in affinity as described above.

DeMeyts and his co-workers in 1976[83,84] suggested that the complex binding isotherms in many hormone-receptor interaction may be the result of site-site interactions or cooperativity among receptors. The reaction is considered positive cooperativity if the binding of the first ligand increases the affinity of the receptor for the second and negative cooperativity if the binding decreases the affinity[83]. TSH receptor has been suggested to have negative cooperativity and the decrease in receptor affinity appears to be due to an increase in the rate of dissociation of the hormone-receptor complex[74].

The major characteristics and essence of the functional definition of the hormone receptor are that, there are specific binding sites for the hormone which can be related to its biological effects A single hormone receptor binds only to one type of hormone and this binding can be correlated with biological activity of the hormone. In practice, these properties are usually demonstrated by showing that there is a preference in competition for binding of labelled TSH to the TSH-receptor by unlabelled TSH as compared with that of others unlabelled hormone such as hFSH, hLH and hCG[74,85]

Introduction to Experimental Work.

The relationship between TSH and its receptors has been investigated using human thyroid membranes. Receptor binding was studied by use of ^{125}I-labelled bTSH, which had been purified by adsorption to and elution from human thyroid membranes. The assay procedures were modified from that of Kermode et al.(1981)[86].

Before starting the assay of TSH receptors, it is necessary to perform some preliminary investigations to establish the optimum methods for thyroid membrane preparations, iodination of bTSH with Na^{125}I, receptor purification of ^{125}I-labelled TSH and optimum conditions of the assay system (e.g. pH, reaction time and temperature etc.). In order to enable accurate assessments of radioreceptor assay, it is essential for the success of this type of investigation that the quality of labelled TSH is very important to affect the sensitivity and specificity of assay results. For this reason, several methods of iodination and of

the assay procedures were tested and compared in this thesis.

Principle of Radioreceptor Assay.

The ability to measure directly and quantitatively the interaction of a hormone with its biologically significant receptor has given new insight into the basic mechanism of hormonal action. In addition, hormone receptor studies may be used to study the composition and structure of the hormone or the nature of the receptor. Because the hormone receptor has a high affinity and specificity for a hormone, it has been used to develop "radioreceptor" assays which work much like the radioimmunoassay and other competitive protein binding assays. A radioactivity labelled hormone (hormone*) is incubated with suitable receptor preparation, usually isolated cells or cell membrane in the absence or presence of various amount of unlabelled hormone, as shown:

$$\text{HORMONE}^{*} + \text{RECEPTOR} \rightleftharpoons (\text{HORMONE}^{*}\text{-RECEPTOR})$$

$$+$$

$$\text{HORMONE} \quad \downarrow\uparrow$$

$$(\text{HORMONE-RECEPTOR})$$

After the appropriate amount of time the hormone bound to the receptor is separated from the free hormone* by centrifugation, filtration or precipitation and the amount of radioactive hormone bound to the receptors is ascertained.