THYROTROPHIC HORMONE METABOLISM IN MAN AND THE RAT

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by
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ABSTRACT

For the assay of thyrotrophic hormone (TSH), a double-antibody radioimmunoassay has been used. For the assay of thyroxine (T_4) and triiodothyronine (T_3) an improved method for the preparation, purification and stabilisation of ^{125}I-T_4 and ^{125}I-T_3 of exceptionally high specific activity has been developed.

To distinguish conditions in which TSH levels are low, where the assay of the hormone in serum has proved inconclusive, an assay in urine has been developed. After comparing three methods of concentration and extraction of the hormone from urine, the dialysis and lyophilisation method, followed by a double-antibody radioimmunoassay has been developed as a standard procedure to measure the urinary excretion of immunoreactive TSH. Normal values were 3.5 - 9.8 μu/hr (mean 5.6±0.3 (s.e.m.)/hr). Values of <1 - 3.5μu/hr (mean 2.6 ± 0.2 μu/hr) were found in hyperthyroid and hypopituitary subjects and of 10.8 - 46.5 μu/hr (mean 25.1 ± 3.3 μu/hr) in hypothyroid subjects. Thus, the measurement of urinary TSH discriminated completely between all these groups.

Gel-filtration studies were carried out on pituitary standard preparations, and sera and urine concentrates from primary hypothyroid subjects. The pituitary and serum materials eluted in one symmetrical peak corresponding to a molecular weight of 34,000. No evidence of "big TSH" emerged, not even after the injection of TSH releasing factor (TRF). In contrast, the urinary TSH was eluted in several broad peaks, the first corresponding to that of the pituitary and serum material. Urinary TSH was therefore found to be largely fragments of the hormone.

As this difference between the elution profiles of the TSH in serum and urine suggested that the kidney is involved in the metabolism

of the hormone, homologous ^{131}I-TSH was injected into rats and its metabolism studied. Most of the ^{131}I-TSH was found in the kidney, and smaller concentrations in the liver, thyroid and the pituitary. Autoradiographic studies on the kidneys of rats injected with ^{125}I-rat TSH at various times provided evidence both of glomerular filtration and of proximal convoluted tubular concentration of the hormone. Renal metabolism of the hormone was suggested by the finding of a small molecular weight peak in the elution profiles of gel-filtration analysis of kidney extracts of rats injected with ^{125}I-TSH at various time intervals. As the TSH peak diminished, this new peak increased with time.

In man, human TSH kinetics were defined by infusing a human pituitary TSH preparation into normal males. The half-life was 77 (70 - 85) mins, the metabolic clearance rate 40 (36 - 46) ml/min, the production rate 99 (87 - 136) mu/day and the distribution space was 4.5 (3.8 - 5.7) litres, the exchangeable TSH in this space was 8.4 (6 - 13) mu. This half-life is considerably longer than previously reported values derived with less stringent criteria. The kinetics of TSH also differ from those of other pituitary glycoprotein hormones, luteinizing hormone (LH)and follicule stimulating hormone (FSH) studied similarly. The biological effects from this intravenously injected human TSH have also been defined. Total serum T_3 and T_4 levels rose but T_3 more and more rapidly. However, the urinary T_3 and T_4 levels, which reflect the serum free levels, rose about equally, suggesting equivalent proportional increases in T_3 and T_4 secretion rates.

The responses of TSH to TRF have been further clarified in various endocrine diseases. When TRF was given simultaneously with gonadotrophin releasing factor (LHRF) and insulin-induced hypoglycaemia, the responses, in serum levels of TSH or of any other pituitary hormones were the same as those reached when the stimuli

were given individually. This now forms the basis of a "combined test" of anterior pituitary function. In a group of 206 euthyroid diabetics, slightly raised serum TSH levels were found in 17%, probably reflecting poor thyroid responsiveness. That these increases were real was confirmed by finding an exagerated serum TSH response to TRF in these subjects. In about 35% of euthyroid untreated acromegalic subjects, a subnormal serum TSH response to TRF was found in spite of concurrently normal serum and urinary thyroid hormone levels. Various studies seeking an explanation for this indicate that excessive growth hormone levels impair the pituitary glands responsiveness to TRF. Also, subjects with pituitary dependent Cushing's syndrome showed a similar unresponsiveness to TRF which recovered after treatment of the Cushing's disease by pituitary ^{198}Au implantation. In Cushing's disease, the prolactin response to TRF was normal, suggesting that TRF mediated TSH and prolactin secretion are mediated through different pathways. However, with prolactin excess, as in prolactin secreting pituitary tumours, TSH response to TRF remained normal.

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ABBREVIATIONS

ABBREVIATION	FULL NAME
ACTH	Corticotrophin
Ag	Silver
CMC	Carboxymethylcellulose
DEAE	Diethylaminoethyl
DS	Distribution space
EDTA	Ethylenediaminetetra-acetic acid
FSH	Follicle stimulating hormone
GH	Growth Hormone
HCG	Human chorionic gonadotrophin
hr	Hour(s)
I	Iodine
LATS	Long acting thyroid stimulator
LH	Luteinizing hormone
LHRF	LH/FSH releasing hormone
MCR	Metabolic clearance rate
MIT	Monoiodotyrosine
min	Minute(s)
PBI	Protein bound iodine
PR	Production rate
SD	Standard deviation
SEM	Standard error of mean
T_2, DIT	Diiodothyronine
T_3	L-Triiodothyronine
T_4	L-Thyroxine
TETRAC	Tetraiodothyroacetic acid
TRIAC	Triiodothyroacetic acid
TRF	TSH releasing hormone
TSH	Thyrotrophic hormone
u	Unit
µg	Microgram
µu	Microunit
µ	Micron

CHAPTER 1

INTRODUCTION

A review of the current knowledge on human thyrotrophic hormone, and aims of the present study .

1. General Historical Introduction.

Thyrotrophic hormone (TSH) is a glycoprotein hormone, produced by the anterior pituitary gland, which acts on the thyroid gland. Its existence was first established by Smith and Smith in 1922, who showed that saline extracts of fresh bovine pituitary glands reactivated the atrophied thyroid glands of hypophysectomized tadpoles.

The next important landmark occurred a decade later when Junkmann and Schoeller (1932) developed their bioassay for TSH in guinea-pigs, this method later giving rise to the Junkmann-Schoeller unit (JSU). Several bioassay methods followed, but only those of D'Angelo, McKenzie, Bottari and Kirkham possessed sufficient sensitivity to detect TSH in neat plasma of normal subjects. The advent of radioimmunoassays for TSH in serum first developed by Odell et al (1965) has revolutionized the assay of hormone because of its simplicity, sensitivity and specificity. The first demonstration of the antigenicity of bovine TSH was by Anderson and Collip (1934) who found that repeated injection of crude TSH preparations into rats produced a subthyroid state refractory to the administration of the hormone, a state which was later shown to be due to the formation of anti-TSH antibodies (Werner 1936 a,b). Later, Werner et al (1960) and Cline et al (1960) showed that antibodies to bovine TSH could be produced by injecting bovine TSH in Freund's adjuvant into rabbits. These observations led Utiger et al (1963) to produce potent antisera to highly purified human and bovine TSH in rabbits. This later led to the development of the radioimmunoassay for serum TSH based on the inhibition of binding technique reported for insulin by Berson and Yalow (1958).

Janssen and Loesser (1931) pioneered the work on the isolation and purification of pituitary TSH but only with moderate success. They were later followed by other workers, notably Fraenkel-Conrat et al (1940) and Ciereszko (1945) who produced bovine TSH preparations of approximately 1 IU/mg in potency. White (1944) and Albert (1949) have reviewed in detail the early attempts to isolate

and purify pituitary TSH. Further advance in the purification and isolation of the hormone came in 1953 when Heidemann introduced the use of the cation exchange resin IRC-50. Condliffe and Bates (1956, 1957) later used another cation exchanger carboxymethylcellulose and an anion exchanger diethylaminoethyl cellulose. The use of gel-filtration in the purification procedures was introduced by Condliffe and Porath (1962). Using this advanced technique, Condliffe has produced human pituitary TSH of 20IU/mg in potency.

Other thyroid stimulating substances which do not originate in the pituitary have been described. Perhaps the best known thyroid stimulating factor of non-pituitary origin is the long acting thyroid stimulator (LATS) discovered by Adams and Purves (1956) and further studied by McKenzie (1958) in the blood of certain hyperthyroid patients. McKenzie (1962a) has shown that LATS, a 7S gamma globulin, does not originate from the pituitary. It also differs from human TSH in chemical, immunological and biological properties (Purves and Adams, 1961; Adams et al, 1962; McKenzie, 1962b). Whether LATS is the sole cause of Graves disease is controversial, because it is not constantly demonstrable in hyperthyroid blood. However, this controversy seems to be resolved now with the discovery of a "LATS protector" which is found in the serum of Graves disease patients without demonstrable LATS (Adams and Kennedy, 1967). This LATS protector also has thyroid stimulating properties (Adams and Kennedy, 1971).

Exophthalmos, a frequent complication of Graves disease, has frequently been attributed to TSH because pituitary extracts which contain TSH often also contain some factor, the exophthalmos producing substance (EPS), which causes proptosis in experimental animals. However, the absence of TSH from sera of patients with exophthalmos would suggest that the hormone is not the causative factor (Kriss et al, 1967). Furthermore, primary hypothyroid patients who have very high

levels of serum TSH do not have exophthalmos. That EPS and TSH are different substances, one probably often contaminating the other, is shown by the work of Dobyns and Steelman (1953), who reported that TSH was separable from EPS by precipitation of EPS with trichloroacetic acid, leaving TSH in the soluble fraction. Brunish (1958) also found that while TSH activity in a given preparation was reduced by treatment with pepsin, EPS was unaffected. Thyrotrophic activity has also been found in transplantable pituitary tumours of mice (Furth, 1955), and in choriocarcinomas, molar TSH (Odell et al, 1963). A TSH, chorionic TSH , has recently been extraced from human placenta (Hershman and Starnes, 1969; Hennen et al, 1969). This substance bears closer immunological resemblance to bovine pituitary TSH than human. It is smaller in size, shorter in duration of action and differs immunologically from molar or pituitary human TSH. The role chorionic TSH plays in the control of thyroid functions in pregnancy is not yet clarified. A bacterial thyrotrophic factor has been purified from Clostridium perfringens by Macchia, Bates and Pastan (1967) and has many actions in common with TSH.

2. Units and Standard Preparations.

The Junkmann-Schoeller Unit (JSU): The first most widely used unit of thyroid stimulating activity was the JSU (Junkmann and Schoeller, 1932) defined as the smallest daily dose of TSH required to produce an histologically detectable response in guinea-pig thyroid. The response was graded as 0-3+. A JSU produces a 2+ response in half the animals injected in three days.

Bovine Standards: The JSU was in wide usage until the USP Thyrotrophin Reference Substance was established in 1952. The USP unit was defined as the activity present in 20mg of the USP reference substance. The USP unit was followed in 1955 by an International Standard (Mussett and Perry, 1955). The International Standard was equipotent with the

with the USP unit, while 10 JSU equalled 1 IU (Hays and Steelman, 1955; Bakke and Lawrence, 1956; Querido and Lameyer, 1956). Both the International Standard and USP reference substances were crude preparations of bovine TSH.

Human Pituitary Standards: To date there is no international reference standard for human pituitary TSH but most laboratories have adopted the human TSH research standard A (MRC 63/14) introduced by the Division of Biological Standards of the National Institute for Medical Research, London, acting on behalf of the WHO. The standard has been given an arbitrary value of 5×10^{-2}u/ampoule because bioassays gave widely varying results for it depending on the method used. The MRC 63/14 is now in short supply and has been replaced by the MRC 68/38 with which it is equipotent. It is necessary to have a human TSH reference preparation for human hormone assays because of the species specificity of the hormone both biologically (Bakke, 1965) and immunologically (Utiger et al, 1963).

3. Assay Methods.

As for most other hormones, e.g. insulin and growth hormone, the earliest methods employed known biological actions of TSH, but these bioassays have been largely replaced by radioimmunoassay, largely because of its simplicity, specificity and sensitivity.

The first bioassay methods for TSH introduced were based on the effects of the hormone on thyroidal histology (Janssen and Loesser, 1931; Junkmann and Schoeller, 1932) but were very laborious and insensitive. It was not surprising that they were largely superceded by a plethora of other methods notably the gravimetric methods which depended on the increases in thyroidal weight following injections of the hormone. Again these were laborious and insensitive and involved injection of large amounts of hormone. A notable exception to this was the method described by Bakke et al (1957) which was very sensitive in comparison

to the other methods. The real advance in the bioassay of the hormone came with the introduction of the isotopic methods employing ^{127}I, ^{131}I or ^{32}P. Of the isotopic methods, the mose widely used is the McKenzie (1958b) method, a modification of the Adams and Purves (1953) assay, by which the latter authors discovered LATS in 1956. The advent of the radioimmunoassay technique in 1965 (Odell et al; Utiger) has revolutionized the assay of the hormone. This technique has made the assay technically easier, such that several samples can be handled at once. It is also specific. However, it has not done much to improve the sensitivity of the assay of TSH since its sensitivity can be matched by that of some bioassays, notably those of D'Angelo, Bakke and Kirkham.

A really great landmark in the sensitivity and specificity of the assay for the hormone may find itself in the cytochemical assay which has already been perfected for corticotrophin (ACTH) by Chayen et al (1971, 1972) and Rees et al (1973) and is being developed for TSH by Bitensky et al (1974). This method involves the introduction of the hormone into sections of the gland and noting the end point histochemically. By this method it is now possible to measure femtogram and picounit levels of ACTH and TSH respectively. Because of the large numbers of assays available for TSH, only those methods which can produce significant estimates of TSH levels in blood or are of historical importance will be discussed.

1) Histometric Methods:-

(a) The Colloid Droplet Method: This was devised by de Robertis and del Conte (1941) and de Robertis (1948). Starved guinea-pigs were injected with 1ml of test substance and killed 30 minutes later. The number of colloid droplets appearing in the acinar cells of the thyroid after histology was counted. The assay was very sensitive, detecting as little as 20μu of TSH.

(b) The "Stasis" Tadpole Method: D'Angelo and Traum (1958) described

a method using starved tadpoles which were injected with 0.2ml of the test solution. The thyroids were removed and stained histologically. A plot of the mean thyroid cell height against the log of the dose in mu gave a straight line. The sensitivity of the assay was fair, (500μu) and they were able to measure TSH in neat plasma.

2) Isotopic Methods:-

These were performed in vivo or in vitro.

(i) In vivo Methods:-

(a) ^{131}I-uptake: by animals pretreated with thyroxine, iodocasein, or by hypophysectomy was widely used. A typical example is the method of Querido et al (1955) who used female mice pretreated with iodocasein to reduce endogenous TSH production. The animals were then injected daily injections of the test solution for 4 days, after which ^{131}I was injected. The sensitivity of the assay was 500μu.

(b) ^{131}I-depletion: The first version of this kind of assay was described by Gilliland and Strudwick (1953) using thyroxine treated baby chicks. This lacked sensitivity (6000μu) and required the injection of large quantities of test plasma. Bates and Cornfield (1957) increased the sensitivity of this method to 500μu/ml by simultaneously injecting propylthiouracil along with thyroxine. However, normal plasma had to be concentrated before assay.

(c) Increase in protein-bound ^{131}I in blood: Adams and Purves (1953, 1955, 1957) introduced a variation of the ^{131}I depletion method by measuring the ^{131}I increase in blood of guinea-pigs, whose thyroid glands had been prelabelled with ^{131}I at 2 hours after the injection of test solution. This method, although requiring long animal preparation and skill, was sensitive and precise. The method is of importance since by its use, LATS was discovered by Adams and Purves in 1956. McKenzie (1958b) described a modification of the Adams and

Purves assay using mice. This modification is one of the most widely used bioassay methods for TSH because of its simplicity. It is also very sensitive, detecting as little as 50μu/ml of plasma.

(d) ^{32}P uptake by the thyroid has been used for bioassay (Borell, 1945, using guinea-pigs; Crooke and Matthews, 1953, Greenspan et al, 1956 using baby chicks; Dedman et al, 1953, Musset and Perry, 1955, using rats.) As shown later, the validity of ^{32}P methods was doubtful.

(ii) In vitro methods:

(a) ^{32}P uptake by cattle thyroid slices has been described by Florsheim et al (1957) as a bioassay method. However, the fact that when the Musset and Perry method was used to assay the International Standard preparation, it gave a potency value differing by a factor of 2 when compared with other methods, has cast some doubt on the validity of ^{32}P methods.

(b) ^{131}I release from pre-labelled thyroid slices: Bottari and Donovan (1958) described a method based on the release of ^{131}I from guinea-pig thyroid slices previously incubated with ^{131}I. The sensitivity and the precision of the assay were very good. El Kabir (1962) and Kirkham (1962, a,b) have further modified this assay by employing methylthiouracil treated guinea-pigs. This modification made this bioassay one of the most sensitive assays known (1.4μu/ml) and it is capable of measuring TSH levels in plasma.

3) Gravimetric Methods:-

Following the observations of Rowland and Parkes (1934) and Smelser (1937) that prolonged injections of TSH will increase thyroid weight, in vivo gain in thyroid weight was used extensively for many years until the development of isotopic methods. However, large amounts of TSH were required, an obvious disadvantage . An in vitro modification by Bakke et al (1957) based on the ability of TSH to prevent the

loss of weight which normally occurs in thyroid slices at 38°C has also been used as a sensitive bioassay method with detection limits at the level of 8μu/ml.

Table 1 shows some of the characteristics of the assay methods for TSH. In general, the in vitro methods are more sensitive than the in vivo methods but their ability to discriminate between concentration levels (i.e. precision) is not good and may in fact be less than the in vivo assays. The radioimmunoassays for TSH are no more sensitive than the in vitro assays such as those of Kirkham (1962) or Bakke (1957) in contrast with the high sensitivity of immunoassays as compared with bioassay for other hormones such as insulin, growth hormone or parathormone.

4) Radioimmunoassay.

The radioimmunoassay technique is now the most widely used assay method for serum TSH. It is less laborious than the bioassay methods and more sensitive than most. Another great advantage of the assay is that many samples can be assayed at once. It is, however, beset with problems. For example, although immunoassays can measure levels in normal blood, most cannot differentiate between normal and the low levels found in hyperthyroidism and hypopituitarism (Hall, 1972). The need for improving assay sensitivity thus is still paramount. Perhaps measurement of the hormone in other biological fluids such as urine, which is easily available in large quantities (as opposed to blood) and the concentration and extraction of the hormones may help distinguish these low levels. Another problem with radioimmunoassay is that it measures the immunogenecity of the hormone, but not its biological activity. The importance of this is underlined by the fact that Hershman and Pittman (1971a) have found that immunoassays consistently gave lower values than bioassays suggesting a dissociation between

Table 1.1*

Characteristics of assay methods for h-TSH

Method	Animal	Injection Volume ml		Smallest detectable amount** μu	Lowest detectable level in plasma** μu/ml	Index of Precision
		Usual.	Max.			
In Vivo:						
"Colloid droplet" (de Robertis, 1948)	Guinea-pig	1.0	-	20	20	-
"Stasis" (d'Angelo and Traum, 1958)	Tadpole Rana pipiens	0.1	0.4	200	500	0.14
"^{131}I uptake" (Querido et al., 1955)	Mouse	2.0	4.0	2000	500	0.15
"^{131}I depletion" (Bates and Cornfield, 1957)	Chick	0.2	6.0	500	80	0.2
"Increase in plasma" ^{131}I (Adams and Purves, 1953, 1955; McKenzie, 1958, 1966)	Guinea-pig	0.1	2.0	100	50	0.2
	Mouse	0.1	0.5	25	50	0.24
		Vols. of medium plus sample				
In Vitro:						
"Weight response in slices" (Bakke et al., 1957)	Bovine	1.0	3.0	8	8	0.28
"^{131}I depletion from slices" (Kirkham, 1962)	Guinea-pig	0.2	-	7	1.4	0.2
"^{131}I discharge from slices" (Bottari and Donovan, 1958; El Kabir, 1962)	Guinea-pig	0.2	0.5	50	10	0.2
"Radio receptor assay" (Smith and Hall, 1974)	Human	0.25	-	100	100	-
"Cytochemical Assay" (Bitensky et al, 1974)	Guinea-pig	12.0	-	400pu	400pu	-
Radioimmunoassay:						
"Inhibition of binding"	-	0.005	0.05	7.5	15	-
(Utiger, 1965; Odell et al., 1965a)	-	0.01	0.4	10	25	-

*Reproduced in a modified form from "Hormones in Blood", Vol.1 page 351 (1967) Gray C.H. and A.L. Bacharach (eds). Academic press London and New York.

** Activities are expressed as international units (u) referred to the bovine reference standard. In terms of the MRC Standard A, the values would be an order lower.

biological and immunological characteristics of the hormone. Perhaps this problem would be resolved by the development of radioreceptor assays which also measure a biological function in addition to being sensitive. This type of assay has been developed for other hormones, notably ACTH (Wolfsen et al, 1972) and luteinizing hormone (LH) (Catt et al, 1972). Radioreceptor assays using thyroid membranes have also been described for TSH by Smith (1972), Manley et al (1974) and Smith and Hall (1974). The sensitivity of the assays, (about100μu/ml) is, however, lower than that presently achieved in the radioimmunoassays.

Radioimmunoassay technique for TSH was first described by Odell et al (1965 a,b) and Utiger (1965), based on the method originally described first for insulin by Yalow and Berson (1960). Briefly, this method involves competitive binding between the hormone in sample under assay and a known amount of hormone labelled with ^{125}I or ^{131}I for an antibody directed at the particular hormone. After incubation of the hormone with the antibody and the labelled hormones two forms of the hormone can then be discerned: the "free" and "bound". Because both forms of the hormone are soluble, various methods have been devised to separate them. For TSH, Utiger (1965 a,b) used chromato-electrophoresis on DEAEC paper strips in an assay similar to that used by Yalow and Berson for insulin in 1960. Odell et al (1965 a,b) used 55% ethanol/5% saline. However, by far the commonest method of separation is the use of a second antibody (double-antibody method) raised against the gamma globulin of the animal in which the anti-TSH was raised to precipitate the bound hormone (Odell et al 1967, Hall et al, 1971). In this method, the amount of radioisotope present in the precipitate is a function of the concentration of the hormone (labelled plus unlabelled) present in the reaction. Thus the higher the radio-activity in the precipitate, the less the unknown in the sample. Because the amount of precipitate is tiny and friable, a carrier protein usually

serum from the nonimmunised animal in which the first antibody is raised is added to produce a large enough precipitate. A standard curve is constructed by plotting the percentage ^{125}I-hormone precipitated against known amounts of hormone. Soeldner and Slone (1965) have critically reviewed the double antibody method and found it to be highly reproducible and less cumbersome than most methods used. An inhibitor in this system (Morgan and Lazarow, 1963; Soeldner and Slone, 1965) has been characterized as complement (Morgan et al, 1964) and they have suggested the use of the chelating agent EDTA to bind calcium and so inhibit complement. Heparin is also used for the same purpose (Soeldner and Slone, 1965).

For labelling of hormones, including TSH, for radioimmunoassay, the chloramine-T method of Hunter and Greenwood (1962) is widely used. Other methods have been described, notably the enzymatic method employing lactoperoxidase (Miyachi et al, 1972) and the electrolytic method (Malani et al, 1974). These latter methods are supposed to be more gentle on the hormone and are particularly advantageous for studies needing the biologically active labelled hormone. Purification of the labelled hormone is usually carried out using Sephadex G75 (Raud and Odell, 1971). Sephadex G50 followed by Sephadex G200 (Franchimont, 1971) and Amberlite and Quso (Hall et al, 1971). Since the sensitivity and the specificity is highly dependent on the purity of the hormone for labelling, highly purified hormones are used for labelling. For TSH, highly purified hormones for labelling are available from the National Pituitary Agency, U.S.A., and the Medical Research Council, London.

Attempts to develop heterologous radioimmunoassay for human TSH using antibodies to the more readily available bovine TSH have not been very successful. This is consequent on the limited cross-reactivity between human and animal antibodies (McKenzie and Fishman, 1960;

Utiger et al, 1963). However, successful radioimmunoassays for human TSH have reported using bovine TSH antibodies (Lemarchand-Beraud and Vanotti, 1965) and porcine TSH antibody (Franchimont, 1972). Franchimont (1972) has, however, shown that for any heterologous system to work, the standard and the labelling material must be human.

4. Physico-Chemical Properties.

Human pituitary TSH is a glycoprotein with a molecular weight of approximately 28,000 (Condliffe and Robbins, 1967; Pierce et al, 1972). It has a sedimentation coefficient of 2.9S (Condliffe and Robbins, 1967). The molecular weight and sedimentation coefficient are similar to those reported for bovine TSH by Fontaine and Condliffe (1963).

TSH, like other glycoprotein hormones of the pituitary, LH and FSH and the human chorionic gonadotrophin (HCG) consists of two subunits alpha and beta (Pierce, 1971; Pierce et al, 1972). Pierce et al (1972) and Hartree et al (1971) have also shown that the alpha subunit of TSH is virtually identical with that of LH, FSH and HCG in aminoacid composition, but the aminoacid composition of the beta subunit is dissimilar in all four hormones. It is the beta subunit structure that confers the immunological and biological specificity on each of the four glycoprotein hormones. Individually, the subunits are essentially hormonally inactive, but recombination of the two restores most of the biological activity. This phenomenon has also been demonstrated for LH by Papkoff et al (1967), for FSH and HCG (Pierce, 1971). Pierce has also shown that recombination of the alpha subunit of one hormone e.g. LH and the beta subunit of another e.g. TSH yielded a hormone showing almost the full biological activity corresponding to the hormone supplying the beta sub-unit, i.e. TSH.

Carbohydrate content:- Another important component of TSH is its

carbohydrate moeity which constitutes about 15% of molecule and is linked to the molecule in a covalent bondage. The sugar residues are mannose, fucose, galactose, glucosamine and galactosamine. The amino sugars are probably N-acetylated (Ward and Coffey, 1964). Human TSH contains some sialic acid in contrast to bovine or ovine TSH. The biological significance of the sialic acid is not well understood, although it is well established for HCG and FSH that it has marked effect on hormonal activity (Van Hall et al, 1971).

Extraction and Purification:-

The concentration of human TSH in the pituitary has been reported to be between 2mu/mg (Bakke et al, 1959 and 1964) and 20mu/mg dry weight (Bates and Condliffe, 1960). The differences in the estimates were probably due to the different bioassays used. The first attempts to isolate TSH (Janssen and Loesser, 1931) used trichloracetic acid to separate the hormone from impurities. The other investigators who followed used salt fractionation techniques as well as fractionation with organic solvents. The most active preparations in these early attempts were only 100 - 300 times as potent as the starting material. Heideman et al (1959) introduced cation exchange resin IRC-50, but Condliffe and Bates (1956) showed that carboxymethyl cellulose (CMC) was about 100 times more effective weight for weight for binding TSH than IRC-50. Following the binding to CMC, TSH was eluted by a linear gradient to 1M NaCl emerging when the conductivity reached $4ohm^{-3}$. Bovine TSH being more basic was not eluted until the gradient reached 8 - 10 $ohms^{-3}$. Using this method Condliffe (1963), starting from Reisfeld's fraction E (Reisfeld et al, 1962) obtained during the purification of human growth hormone, prepared TSH of potency of 20u/mg. TSH of high potency and purity is unstable as shown by the fact that the high potency hormone described above lost 50% of its activity in three

months of storage as a dry powder at 2°C, after its isolation. The TSH as prepared by Condliffe (1963) was free of growth hormone (Utiger et al, 1963), was about 5% contaminated with LH and was low in FSH (Parlow et al, 1965).

Highly purified TSH shows marked polymorphism on disc gel electrophoresis (Condliffe and Robbins, 1967; Pierce, 1971) and on electrofocussing electrophoresis (Schleusener et al, 1972). The components on disc gel electrophoresis are closely related to one another chemically, immunologically and biologically (Shome et al, 1968). However on immunoelectrophoresis (Odell et al, 1967a) double diffusion on Ouchterlony gels (Utiger et al, 1963), TSH appeared homogenous giving only one precipitation line.

TSH in other species: Thyrotrophic activity has been detected in pituitary glands from representative species of all vertebrate classes so far studied (Bates and Condliffe, 1966). Although TSH from all species are similar grossly in that they are glycoprotein hormones having similar physico-chemical properties, there are important differences in their primary structures, conferring the species specificity they show. For example, one preparation of human TSH (Bates and Condliffe, 1966) contained fewer lysine, alanine and valine and more glycine than bovine TSH. Bovine TSH is also more basic than human TSH. Eel TSH also differs considerably in structure from mammalian TSH (Fontaine and Condliffe, 1963). Apart from amino acid composition, the TSH also differs between species in carbohydrate content. For example, although human TSH possesses sialic acid, bovine TSH does not.

TSH preparations from various species are not equally effective in other species, probably because of differences in their structure. Thus, although bovine TSH is biologically highly effective in man (Hays et al, 1961) TSH pituitary extracts exert little or no effect on mammalian thyroids (Fontaine and Fontaine, 1962).

5. Biological Actions.

Effect on the thyroid gland:-

The principal target organ of TSH is the thyroid gland, affecting the gland's blood flow, weight, histology, metabolism and hormone production. Taurog et al (1957, 1958) have shown that hypophysectomy by removing the source of TSH causes great changes in the activity of thyroid which are reversed by exogenous TSH.

Soderberg (1958) has shown that TSH increases the rate of blood flow through the thyroid gland within one minute of injection of the hormone. TSH also increases the weight of thyroid gland *in vitro* (Lawrence and Bakke, 1956) and *in vivo* after 3 - 4 days (Rowland and Parkes, 1934, and Smelser, 1937). The first effect on thyroid weight is probably due to an increase in water and electrolyte transfer into thyroid cells (Solomon, 1961).

TSH stimulates the various steps in the formation and release of thyroid hormones. It promotes the liberation of thyroid hormone from thyroglobulin (Deiss et al, 1966) and the release of iodine from iodotyrosines (Rosenberg et al, 1965). It stimulates the organic binding of iodine (Rosenberg et al, 1965) and the formation of iodotyrosines and iodothyronnines (Shimoda et al, 1966). One of the most important effects of TSH for bioassays, and perhaps the best documented is its effect on release and depletion of iodine from the thyroid (de Robertis, 1949; Nadler et al, 1962), the effect of hormone release is seen histologically as formation of colloid droplets in follicular cells. TSH also stimulates ^{131}I uptake by the thyroid, this effect being adapted as a bioassay method by Querido et al,(1953).

The effect of LATS on the thyroid are similar to those of TSH described above but persist longer (Adams, 1965; Pinchera et al, 1965).

TSH produces increased turnover and secretion rates of thyroid hormones (Taurog et al, 1958; Kondo, 1961 a,b). Larsen (1972) has also shown that intramuscular injection of TSH produces a rise in serum triiodothyronine (T_3) and thyroxine (T_4) reaching a maximum 8 hours after injection. TSH also has a profound effect on the intermediary metabolism of the thyroid gland. The hormone causes an immediate rapid increase in oxygen consumption of thyroid *in vitro* and *in vivo* (Anderson and Alt, 1937; Canzanelli and Rapport, 1938; Freinkel, 1961). Using ^{14}C-glucose, Field and his co-workers (1959, 1960, 1961 and 1963) and Freinkel (1960) have shown that TSH rapidly stimulates glucose oxidation by thyroid slices *in vitro*, an effect not produced by any other pituitary hormone but simulated by other substances such serotonin, acetyl choline, adrenaline and noradrenalin (Pastan and Field,1962). TSH increases phospholipid turnover in the thyroid gland (Freinkel, 1957, 1958) within minutes. It also causes a rapid ^{32}P uptake by thyroid tissue. RNA synthesis is stimulated by the hormone (Matovinoc and Vickery, 1959) which also stimulates the rate of purine and nucleotide biosynthesis (Hall, 1963; Hall and Tubman, 1965).

Mechanism of Action:- Recent studies by Pastan et al (1966) have provided evidence that the first step in the action of TSH is the binding to a specific receptor site on the thyroid cell membrane. Once bound, the hormone sets up a chain of metabolic effects outlined above, mediated probably through the 3'5' cyclic adenosine monophosphate (cyclic AMP) pathway. Evidence that cyclic AMP release is the primary event in the chain of reactions is shown by the fact that cyclic AMP levels are increased in thyroid slices (Gilman and Rall, 1966) and thyroid homogenates (Klainer, 1962). However, although direct attempts to stimulate glucose oxidation by cyclic

AMP have failed (Field et al, 1960), dibutryl cyclic AMP, which penetrates cells more readily, is effective (Posternak et al, 1962). This suggests that both the metabolic effects and the activation of thyroid hormone releasing system by TSH may be due to a common primary event, mediated by cyclic AMP. Macchia and Pastan (1967) have shown that a specific phospholipid in thyroid cell membrane is important for TSH action.

Prostaglandins, particularly PGE_1, reproduce most of the actions of TSH on the thyroid gland (Mashiter and Field, 1974). The fact that PGE_1, like TSH increased cyclic AMP concentration in thyroid slices (Kaneko et al, 1969) has led to the postulate that prostaglandins may act as an intermediary between TSH and cyclic AMP (Yu et al, 1972) but this is unlikely because salicylates and indomethacin which block the effect of prostaglandins on thyroid tissue have no effect on TSH release of cyclic AMP (Mashiter and Field, 1974).

Extrathyroid Effects of TSH:-

TSH acts on rat adipose tissue in vitro to increase oxygen consumption and release of fatty acids (Freinkel, 1961; Rudman et al, 1962). This is the only recognized extrathyroidal effect of the hormone. These effects occur even at very low concentration of the hormone (0.06 - 1mu/ml) in the medium (Jungas and Ball, 1962). The adipose tissue effects are immediate effects.

6. TSH in Biological Fluids.

(1) Plasma:

TSH exists in the plasma in a state indistinguishable by gel-filtration analysis (Utiger, 1965) from highly purified pituitary TSH. Utiger, using Sephadex G200 for analysis, also found that the hormone was more retarded than albumin. Earlier studies placing TSH between beta and gamma globulin (Querido and Lameyer, 1956)

would therefore appear to be wrong. Utiger's work has since been confirmed by many other workers. In blood, TSH is not bound to any other protein for Condliffe et al (1965) have shown that serum TSH from tumour bearing mice was eluted on Sephadex G100 in the 3S zone. The study of the physical properties of TSH produced an important landmark when McKenzie (1962b) demonstrated that while TSH appeared with the 3S peak on Sephadex G200 analysis, LATS appeared with the 7S peak suggesting that the latter had a much higher molecular weight. Plasma TSH, like its pituitary counterpart, appears to be an heterogenous group of closely related molecules on electrophoresis, as has been shown by Schleusener et al (1972), using electrofocussing electrophoresis on starch gel. However, no "big TSH" in plasma or the pituitary has been reported so far.

Circulating levels:- For uniformity and for ease of comparison, the levels given here will be in terms of the old bovine international unit; the values of which are about 10 times higher than those reported using the MRC research standard A. Early data suggested that circulating levels in blood would be of the order of 1mu/ml, but this would appear to be too high for two reasons: (1) Adams and Purves (1955, 1957a,b) using a bioassay of high sensitivity of 50μu/ml could not detect TSH in unprocessed plasma suggesting that the level must be lower. (2) Back-calculations from kinetics of the hormone as shown by Bakke et al (1962) also gave radically different theoretical concentrations. Theoretical considerations put the values as less than 50μu/ml. The work of Kirkham (1966) and other workers using sensitive bioassays have tended to confirm the findings of Adams and Purves. The advent of radioimmunoassays did not improve the sensitivity of the assay beyond those reported by Adams and Purves, Kirkham and others, and the values reported by this assay are of the same order as those

reported by the above workers. Thus Utiger (1965) reported values from undetectable to 50μu/ml while Odell et al (1965a and b) reported values from undetectable to 30μu/ml in euthyroid adults. Two-thirds of Odell's subjects had undetectable levels with the result that normal levels of TSH appear to lie below the sensitivity of even radioimmunoassays, and the problem remains of distinguishing normal from subnormal levels (Hall, 1972).

(2) Urine:

Evidence that TSH exists in normal, hypothyroid or hyperthyroid urine is conflicting. Hertz and Oastler (1936) found TSH activity in urine of myxoedematous patients but not in that of normal or thyrotoxic subjects. However, Cope (1938) using benzoic acid extracts of urine could not demonstrate the hormone in hypothyroid, hyperthyroid or normal subjects. Rawson and Starr (1938) in contrast found the hormone in normal urine and also more of it in hypothyroid urine. Kriss et al (1954) confirmed these findings. Ishigami (1966) has shown that TSH activity is detectable in concentrated (by the Bate's percolation method) normal urine. All the above workers used bioassay methods. There is to date no report of immunoreactive TSH in urine. It is surprising that there has been no such report because the allied glycoproteins, the gonadotrophins, were among the earliest hormones found in urine and have been found easily measurable by the available sensitive immunoassay methods. There might be a use for such a method since the available immunoassay for TSH cannot consistently distinguish low from normal levels.

7. Control of TSH Secretion.

1) The thyroidal feedback system:

TSH comes from the amphophils or transitional basophils of the anterior pituitary (Russfield, 1955; Purves and Griesbach, 1957).

The control of TSH secretion by these cells is dependent on the thyroid feedback system consisting of three main components, the hypothalamus, the pituitary gland and the thyroid gland. These secrete TRF, TSH, and T_3 and T_4 respectively.

(a) Role of the hypothalamus:- Harris (1955) and many other workers have shown that normal functioning of the pituitary-thyroid axis is dependent on the hypothalamus. Thus if the median eminence of the hypothalamus is destroyed, thyroid function is depressed. The median eminence secretes a factor, thyrotrophin releasing factor (TRF) (Reichlin, 1966) which reaches the anterior pituitary through the hypophyseal portal circulation and increases thyroid function in mice and rabbits (Reichlin, 1964; Harris, 1963). The eventual isolation, the characterization of its structure, and the systhesis of TRF were carried out mainly in the laboratories of Guillemin and Schally working independently. The isolation of TRF from many thousands of hypothalami from pigs (Schally et al, 1969) and sheep (Burgus et al, 1970) has led to the elucidation of its structure by Nair et al (1970) and Burgus et al (1970) and its synthesis (Folkers et al, 1969; Baugh et al, 1970). TRF a tripeptide, L-pyroglutamyl-l-histidyl-l-proline (Fig. 1.1) is extremely potent and rapid in action. With rat pituitaries in vitro, concentrations as low as 10pg/ml released TSH (Mittler et al, 1969). In vivo, in man, Bowers et al (1970), Hall et al (1970) and Hershman and Pittman (1971a) have shown that it increases serum TSH levels 2 - 10 mins after injection, reaching a maximum 20 - 30 mins from zero time.

The mechanism of action of TRF is poorly understood. It appears to stimulate the release of preformed TSH followed by increased synthesis of new hormone, a slower process probably secondary to the former (Mittler et al, 1965). TRF in vitro causes the release of

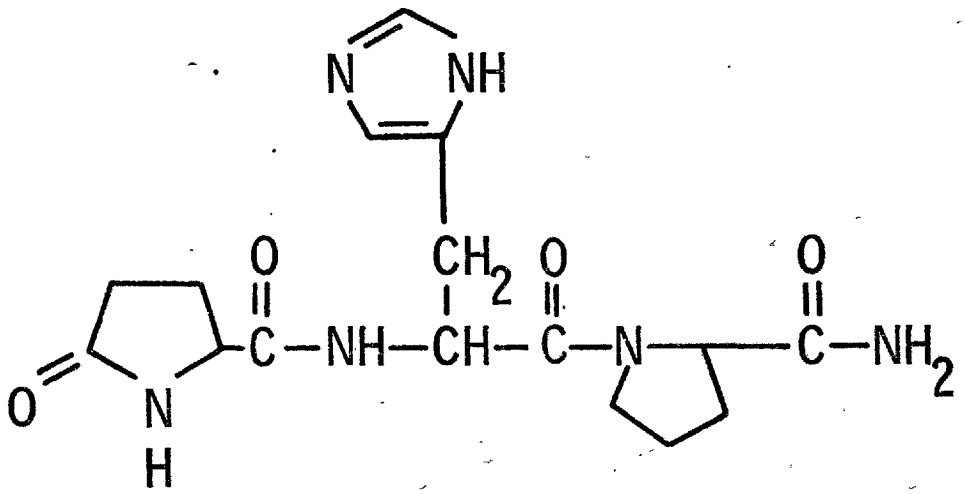

Fig. 1.1 Structure of thyrothrophin releasing hormone (TRF).

cyclic AMP in rat pituitaries (Steiner et al, 1970). Faglia et al (1972) have also shown that theophylline increases the TSH response to TRF in man. These suggest that TRF induced TSH secretion is probably mediated through the adenyl cyclase system. The action of TRF on the pituitary gland is blocked by thyroid hormones provided the hormones are administered about an hour before (Bowers et al, 1967). This blockade, being inhibited by actinomysin D, suggests that these hormones produce their effect by inducing the synthesis of an inhibitor, probably a protein (Bowers et al, 1968). Redding and Schally (1969) have also shown that thyroid hormone may modulate the effect of TRF by modifying its rate of destruction in vivo, thus while normal human plasma inactivated TRF within 15 mins, hypothyroid plasma delayed this inactivation while hyperthyroid plasma accelerated it.

(b) Role of TSH:- There is evidence that TSH may also directly modulate the secretion of TRF (Motta et al, 1969) by the so-called "short feed back loop". It this exists it must be a stimulatory effect since the very high levels of serum TSH seen in hypothyroidism precludes an inhibitory effect.

(c) Role of thyroid hormones:- When hypothyroid patients with high plasma TSH levels are treated with thyroid hormones, the TSH levels fall rapidly (Odell et al, 1965b; Utiger, 1965; Odell et al, 1967a). The levels rise again when the treatment is stopped. This suggests that thyroid and the pituitary gland interact in a negative feed-back mechanism. This feed-back mechanism is modulated by the hypothalamus (Reichlin, 1966) but can however operate autonomously as shown by the work of Furth (1955), and Bates and Garrison (1961) which showed that TSH secretion by mice TSH-secreting tumours can be abolished by the administration of thyroxine.

2) TSH secretion in health:-

Effect of Age: At birth there is a large increase in serum TSH concentrations which probably existed previously in utero because cord-blood TSH levels were higher than maternal blood levels by 2.8 - 5.6μu/ml from the reports of Hershman and Pittman (1971a) and Fisher and Odell (1969). These workers have also shown that the major rise occurred within the first 30 mins of life, this rise falling off rapidly between 30 mins to 4 hours and gradually for 48 hours. By 72 hours, normal levels have been attained. The mechanism of the neonatal rise in serum TSH levels is not clear but transient cooling at the time of delivery has been suggested but not substantiated. However, it would appear that the increased thyroid function that occurs in the neonate is TSH dependent (Van Middlesworth, 1954). Beyond the neonatal period, TSH levels do not appear to increase with age, although Cuttelod et al (1974) have recently reported small increases in elderly subjects.

Effect of Sex: In spite of earlier controversy on the effect of sex on blood TSH levels, the levels do not appear to be sex-dependent. Thus Hershman and Pittman (1971a) and Odell et al (1968) have found no differences, and the reports by Bottari (1958) that women have higher levels than men, and Kirkham (1962a,b) saying the opposite, remain to be confirmed. Perhaps more sensitive assays are required.

Effect of Pregnancy: Serum level of pituitary TSH is normal in pregnancy (Hershman and Pittman, 1971a; Hershman and Starnes, 1969) in spite of the fact that total serum T_4 is raised due to the increase in thyroxine binding globulin levels. However, free thyroid hormone levels in pregnancy are normal, suggesting that the thyroidal feedback system is dependent on the free hormone levels and not the total. The role played by chorionic TSH, described earlier, in pregnancy is not clear.

Effect of Cold: Various reports have shown that exposure to cold produced an elevation of TSH and thyroid function in laboratory animals, a phenomenon probably mediated through cold induced TRF release because appropriately placed lesions in the hypothalamus abolished the response (Reichlin, 1966). In man, Odell et al (1968) have found a small but statistically significant rise of plasma TSH in 12 men exposed to arctic temperatures for seven days. However, Hershman et al (1970) have found no rise in serum levels on exposure of 14 men to 2.8°C for one hour, despite the fall in rectal temperatures, nor did the hypothermia of cardiac surgery cause a rise. In infants, however, cold exposure leads to a marked rise in serum TSH (Wilber and Baun, 1970; Hershman and Pittman, 1971a). Thus, although the human, especially the neonate, is capable of responding to cold with an increased secretion of TSH, the response in adults is blunted in comparison to lower species and is not probably clinically significant.

Effect of serum thyroid hormone: The levels of thyroid hormones in blood have a profound effect on serum TSH levels. Reichlin and Utiger (1967) gave primary hypothyroid patients increasing doses of T_4 and studied the decline of serum TSH levels. They found that serum TSH concentration was related to serum free T_4 levels in a curvilinear fashion, with elevated serum TSH when serum free T_4 was below 2.5ng/100ml and total T_4 below 5µg/100ml. When free T_4 levels rose above 5 - 6ng/100ml or total 10µg/100ml, TSH was undetectable in serum. However, when serum TSH levels of normal persons are plotted against serum T_4 levels, there is considerable scatter and the curvilinear relation is lost. The problem with this kind of study is that the free levels of thyroid hormones are back calculated from total levels. The ideal situation would be direct measurement of the free

hormone in serum. This awaits the advent of very high specific activity labels and very avid antibodies to improve the sensitivity of the assay systems.

3) TSH secretion in disease:-

(a) Thyroid disease:- Clinical primary hypothyroidism is constantly associated with high serum TSH levels. Hershman and Pittman (1971b) found a mean serum TSH level of 110μu/ml (range 23 - 800μu/ml) in 79 hypothyroid patients. Similar findings have been reported by other workers, for example, Utiger (1965), Odell et al (1965 a,b) and Hall et al (1971). This high level is suppressible with thyroid hormones given either acutely or chronically. When large doses (500ug) of T_3 were given acutely to hypothyroid subjects by Odell et al (1967a), the TSH levels fell rapidly. The usual replacement doses of T_3 and T_4 reduced TSH levels to normal range in 3 - 18 days, depending on which was used, T_3 being generally faster than T_4 (Utiger, 1968). The elevated serum TSH levels found in hypothyroidism are a result of both slower metabolic clearance rate (MCR) and increase in the production rate (PR). Odell et al (1967b)found a slower half-life (85min) of TSH in the sera of hypothyroid subjects as opposed to normal (54min). Odell and his co-workers, and Ridgway et al (1974) also found slower MCR and greater PR in hypothyroids than normals, thereby confirming the report of Bakke et al (1962).

There have been suggestions that severe hypothyroidism may impair pituitary function such that TSH secretion is reduced. However, Odell et al (1967a) did not observe transient increases of serum TSH during treatment of hypothyroidism.

In contrast, a raised serum level does not always indicate clinical hypothyroidism. The commonest situation in which this type of situation is found is autoimmune thyroiditis (Hall, 1972; Evered et al, 1973). Greenberg et al (1970) have demonstrated that in this

disease the serum TSH level correlated well with the extent of thyroid damage, but not with the level of T_4 iodine when this is within the normal range. None of their patients was hypothyroid. These findings would suggest that TSH is a more sensitive index of thyroid damage than the level of circulating thyroid hormone, perhaps a higher serum level of TSH is required to achieve euthyroidism. Apart from autoimmune thyroidism, high serum TSH associated with euthyroidism has been reported in iodine deficiency goitre, iodine-induced goitre, subtotal thyroidectomy (Heedley et al, 1971) and after radio-iodine (Slingerland et al, 1970). Treatment of such patients with thyroid hormone usually suppresses the serum TSH levels and results in the decrease in size of the goitre. However, the natural history of this condition has not been followed long enough to determine what percentage of the subjects would develop hypothyroidism.

In regions of endemic goitre serum TSH levels are variably raised. In a survey in New Guinea, Butterfield et al (1968) found a mean value of 18μu/ml, while in South-West Egypt, Coble and Kohler (1970) found 6.9μu/ml, and in Idjwi Island, Delange et al (1971) found 9.6μu/ml. In these surveys, the levels ranged from 2.3 - 123μu/ml, most falling in the normal range, suggesting that enhanced sensitivity to TSH may account for their goitres.

Most workers have reported undetectable levels of TSH in the sera of hyperthyroid subjects, although some values fall in the normal range (Hall, 1972). This underlines the fact that most assays cannot distinguish between normal and subnormal values. This must await the advent of more sensitive assays.

In some euthyroid patients with thyroid cancer, Hargadine et al (1970) have reported high serum TSH levels. The role high levels play in the genesis of these cancers is not well understood.

(b) Pituitary disease:- Hyperthyroidism resulting from excessive TSH from a pituitary tumour is very rare in clinical practice, but there have been some reports (Hamilton et al, 1970). Perhaps with the wider availability of radioimmunoassays, more cases would be discovered. In hypopituitarism from pituitary tumours, TSH is usually the last of the pituitary hormones lost (Rabkin and Franz, 1966; Fraser, 1972). However, loss of TSH and the preservation of other hormones has been reported in several patients (Pittman, 1971).

Perhaps the most versatile use of serum TSH radioimmunoassay is to differentiate pituitary from primary thyroid hypothyroidism, the latter showing very high levels in contrast to the former, where the levels are undetectable.

(c) Hypothalamic disease:- A patient with hypothalamic hypothyroidsim has been described by Pittman et al (1971). This patient had undetectable basal TSH levels which increased after TRF administration. More reports are bound to follow with the increasing use of radioimmunoassays, and TRF as a stimulation test.

4) Tests of Pituitary TSH Reserve:-

As has been discussed earlier, most assays cannot distinguish between normal and subnormal serum TSH levels, with the result that basal serum TSH measurement is not usually helpful in the differential diagnosis of these conditions. Stimulatory or dynamic tests would therefore be preferable.

a. Potential stimuli to TSH release:- A large number of stimuli have been tried for the evaluation of pituitary reserve capacity to secrete TSH, most of them having been established to stimulate other pituitary hormones. Unfortunately most have not been successful. Glucose, exercise, fasting, meals, fever, surgery and arginine do not alter serum TSH levels (Odell et al, 1968b). In laboratory animals

stress or glucocorticoids lower serum TSH levels (Ducommun, 1966). While vasopressin effects the release of TSH in vitro (Krass et al, 1968), it is ineffective in man (Hall, 1972; Read et al, 1969). Neither does electroconvulsive therapy alter serum TSH levels (Hershman and Pittman, 1971a). Antithyroid drugs alone or in combination with iodides given for 2 - 3 weeks failed to cause stimulation of TSH secretion (Sawin et al, 1970; Hall , 1972). Perhaps thyroidal stores of hormone in man are too large to be depleted in such a short time. The failure of adult men to respond to drastic changes in temperature (Odell et al, 1968; Hershman et al, 1970; Hall, 1972) indicates that cold is not a practical method of TSH stimulation. The report by Adams and Maloof (1970) that oestrogens cause increase in serum TSH levels has not been properly substantiated.

b. Synthetic TRF:- Intravenous injection of TRF into normal subjects consistently causes a rise in serum TSH levels within 2 - 5 minutes, reaching a peak in about 20 - 30 minutes, and returning to basal levels in 2 - 3 hours (Bowers et al, 1970; Fleischer et al, 1970; Hall et al, 1970). TRF is effective in a dose as small as 50µg and a log-linear dose response curve is found up to 500µg (Haigler et al, 1971). Increasing the dose beyond 500 - 800µg did not enhance the response. When Hershman and Pittman (1970) infused TRF over 30 mins, they did not elicit a response greater than would occur when the dose was given acutely . Daily intravenous injection of 500µg of TRF for five days led to a progressive decrease in response in some subjects, probably due to a depletion of pituitary TSH (Haigler et al, 1971). Hershman and Pittman (1970) and Ormston et al (1971) have shown that TRF is effective orally in man, its effects being slower and longer.

TRF induced rise in serum TSH causes a release of thyroid hormones. Serum T_3 levels rise earliest, probably within and hour and peak between 3 - 6 hours (Lawton, 1972; Hollander, 1972). PBI and thyroxine rise later, about 2 - 3 hours after TRF and plateau 9 -12 hours later.

Apart from TSH, TRF consistently causes a release of prolactin. Jacobs et al (1971) and L'Hermite et al (1972) demonstrated that intravenous TRF causes a prompt rise in plasma prolactin levels peak 10 - 20 mins after the TRF injection (20 - 40 mins for TSH) in all subjects studied. Occasional release of growth hormone, particularly in acromegaly, has also been reported. 12 of 21 acromegalic subjects of Faglia et al (1973) responded with an increase of serum GH after i.v TRF. Otherwise TRF does not cause a consistent releasing effect on other pituitary hormones (Hall, 1972).

Hypothyroid subjects show an increased sensitivity to TRF while hyperthyroid subjects show no response (Hershman and Pittman, 1971a; Ormston et al, 1971). Effects of other diseases on TSH response to TRF are variable. About 50% of euthyroid acromegalics show an absent or blunted response to TRF (Hall et al, 1972; Tunbridge et al, 1973). Various theories have been propounded for this. These include diminished pituitary TSH reserve, excessive circulating peripheral thyroid hormone causing a negative feedback, inhibitory effect of GH on TSH secretion and so on. However, none of these has been properly documented, and they require further clarification. Administration of 8mg of dexamethasone to hypothyroid patients suppressed basal TSH but did not effect the response to TRF (Wilber and Utiger, 1969) suggesting that corticosteroids may act on the hypothalamus However, recent data by Otsuki et al (1973) have shown that patients on chronic large doses corticosteroid therapy, and those with Cushing's have an impaired response to TRF. This suggests that corticosteriods

may also act on the pituitary. Data on TSH response to TRF in Cushing's disease are few and further studies are required to resolve these controversies.

8. Metabolism of TSH.

Studies of the metabolic fate of TSH in man and other animals have been handicapped by many factors. Firstly, the concentration of the hormone in the blood is very low, and the assays difficult. The advent of the radioimmunoassay technique has gone a long way to solve this problem. Secondly, only bovine TSH was available in such quantities that it could be used for metabolic studies. Therefore, early workers in this field like Bakke and Lawrence(1962) and Bakke et al (1962) used this preparation to study the kinetics and distribution of the hormone in man and the rat. The reliability of these studies is dubious because they assumed that the heterologous material would be metabolised like the homologous hormone. They also used large unphysiological doses of the hormone.With the availability of radioiodine-labelled hormone, which could be used in tracer doses, various reports, for example from Odell et al (1967b),have appeared in the literature. Again the use of labelled hormone would assume that it is metabolised equivalently with the native hormone.

Kassenaar et al (1956, 1959), Levey and Solomon (1957) and Bakke and Lawrence (1962) have studied the distribution of bovine TSH in rabbits and rats at various times after the injection of the unlabelled hormone and measuring by bioassay. They all found significant concentration of the hormone in the kidney (mainly) and the liver. However, the physiological significance of these findings is doubtful, since the exogenous TSH preparations used were from another species (Bates and Condliffe, 1966).

Therefore, on the whole, the studies on the metabolic fate of TSH are unsatisfactory and require further clarification.

9. Aims of the Present Study.

Perhaps one of the most important problems in the study of TSH is the lack of adequate sensitivity of the existing assay systems (both radioimmunoassays and bioassays) e.g. for distinguishing between normal and subnormal levels in blood. This has been emphasised by Hall (1972, 74). This study will investigate the excretion of the hormone in urine, as an alternative to measurements in blood, in health and disease, in an attempt to solve this problem. The nature of the hormone in urine will also be characterised in an attempt to study the role played by the kidney in the metabolism of the hormone.

Recent evidence has shown that the control of TSH secretion is governed more by the free levels as opposed to the total levels of thyroid hormones in blood. Since the free levels of this hormone are extremely low, the present assay systems for thyroid hormones lack sufficient sensitivity to directly measure them in blood. One reason for this is probably the low specific activity of the present commercially available labelled hormones. We have therefore investigated the production and storage of high specific activity labelled thyroid hormones.

Previous workers on the metabolism of TSH in man and experimental animals had been handicapped by the inavailability of homologous hormones in sufficient quantities for use in their studies. These studies, although in many cases elegantly designed, only gave an indirect answer to the problems posed. These homologous materials are now available and have been used in the study of the metabolism of the hormone in man and the rat.

The availability of synthetic TRF has led to its wide application

in the study of the secretion of TSH in health and disease. However, few data are available as to how TRF interacts with the other releasing factors, e.g. gonadotrophin releasing hormone (LHRF) of the hypothalamus. This study aims to elucidate if TSH secretion (or the secretion of the other pituitary hormones) is altered when TRF is in combination with LHRF and insulin-induced hypoglycaemia.

The use of TRF in the study of TSH secretion in euthyroid patients with pituitary tumours has revealed a high incidence of TSH unresponsiveness in acromegalics. These findings remain unexplained and possible mechanisms involved are studied. Few data are available on TSH responsiveness to TRF in pituitary dependent Cushing's disease pre and post treatment. Such data would help resolve the controversy on whether corticosteroid-induced decrease in TSH secretion is at the level of the hypothalamus or the pituitary gland.

We would, thus, attempt to fill some of the gaps of knowledge on the secretion, kinetics and excretion of TSH.

CHAPTER 2

ASSAY METHODS

A. RADIOIMMUNOASSAY OF TSH

As has been alluded to in the introductory chapter, the radioimmunoassay technique is now generally used for the assay of TSH in blood mainly because of its precision, simplicity, sensitivity, and specificity. The method was therefore used for the assays described in this work. Of the variations of the immunoassays available, the double-antibody technique enjoys wide usage, probably because of the relative ease with which it is performed. This laboratory currently measures the peptide hormones, FSH, LH, GH, insulin and prolactin by the double-antibody method and has most experience in this method. Therefore a double-antibody radioimmunoassay based largely on the method described by Hall et al (1971) was used in this study.

a) Production and Assessment of Antisera

1. First Antibody:

a: Production: The first antibody was produced in this laboratory by Drs. W.M.G. Tunbridge and J.C. Marshall. Human TSH for immunization was kindly supplied by Dr. Anne Hartree of Cambridge University. The antibody was produced in young adult guinea-pigs (about 300g weight) by injecting 0.25mg of human TSH (fraction DEAE II) in 1ml of Freund's complete adjuvant intramuscularly into multiple sites (3 - 4) in the inner aspects of the thigh and anterior abdominal wall. The injections were repeated every two weeks for eight weeks after which the guinea-pigs were bled.

b: Assessment: The antisera produced assessed as suggested by Hurn and Landon (1971) and Berson and Yalow (1964), i.e. setting up titration curves and choosing the antibody with the steepest slope, this having the highest affinity for the labelled hormone. The best antiserum, chosen from the above criteria, was then assessed for the

working dilution at which it should be used again by constructing a titration curve using initial dilutions of the guinea-pig antiserum of 1:1000 to 1:512000 and incubating these under assay conditions with 50, 100, and 150pg of ^{131}I labelled human TSH. The result of the titration curves is shown in Fig. 2.1. The working dilution of the antibody defined as that dilution which gives about 60% binding with the label, was found to be between 1:50,000 to 1:70,000 depending on the amount of label added. Addition of various amounts of labelled hormone was assessed because Berson and Yalow (1968) and Greenwood (1969) have suggested that the slope of the titration curve alone is a poor estimate of the affinity of an antibody and that a better approach is to construct titration curves using different amounts of label. An antibody which can distinguish between 50 and 150pg of labelled hormone has a higher affinity than one which cannot. Also the minimum amount of label which will produce a separate curve is a guide to the least amount of antigen which can be detected in the assay. Fig. 2.1 shows that the antibody used here distinguished only slightly between 50 and 100pg, but more so between 50 and 150pg, thus suggesting that the detection limit of this assay was the order of 100pg. This detection limit proved not to be the case as shown later. Perhaps labelled and unlabelled TSH are not immunologically identical.

It must be emphasized that the titration curves above were performed using "TSH-free" serum in the system. Fig 2.2 shows the effect of adding various amounts of serum to the system (the same incubation volumes used for all). The more serum in the system, the more suppressed was the curve. For this and subsequent reasons, "TSH-free" serum was added in the same amount as sample to the standard curve, i.e. such that the final dilution was 1:3.

Details of the specificity of the antiserum are described later.

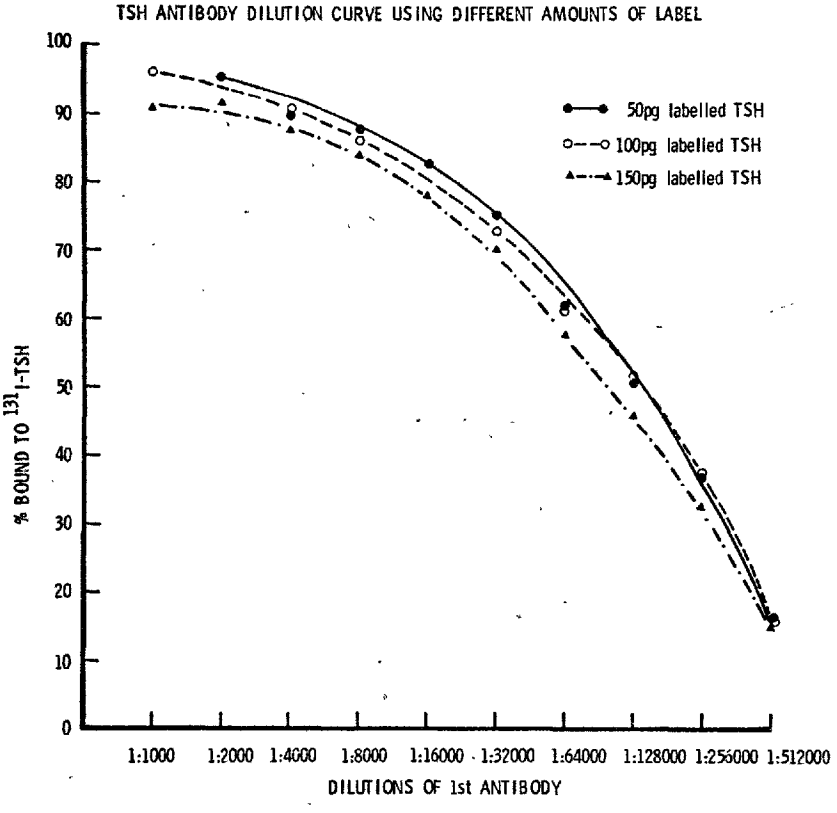

Fig. 2.1. Assessment of Anti-TSH antibody: antibody dilution curves using different amounts of labelled antigen.

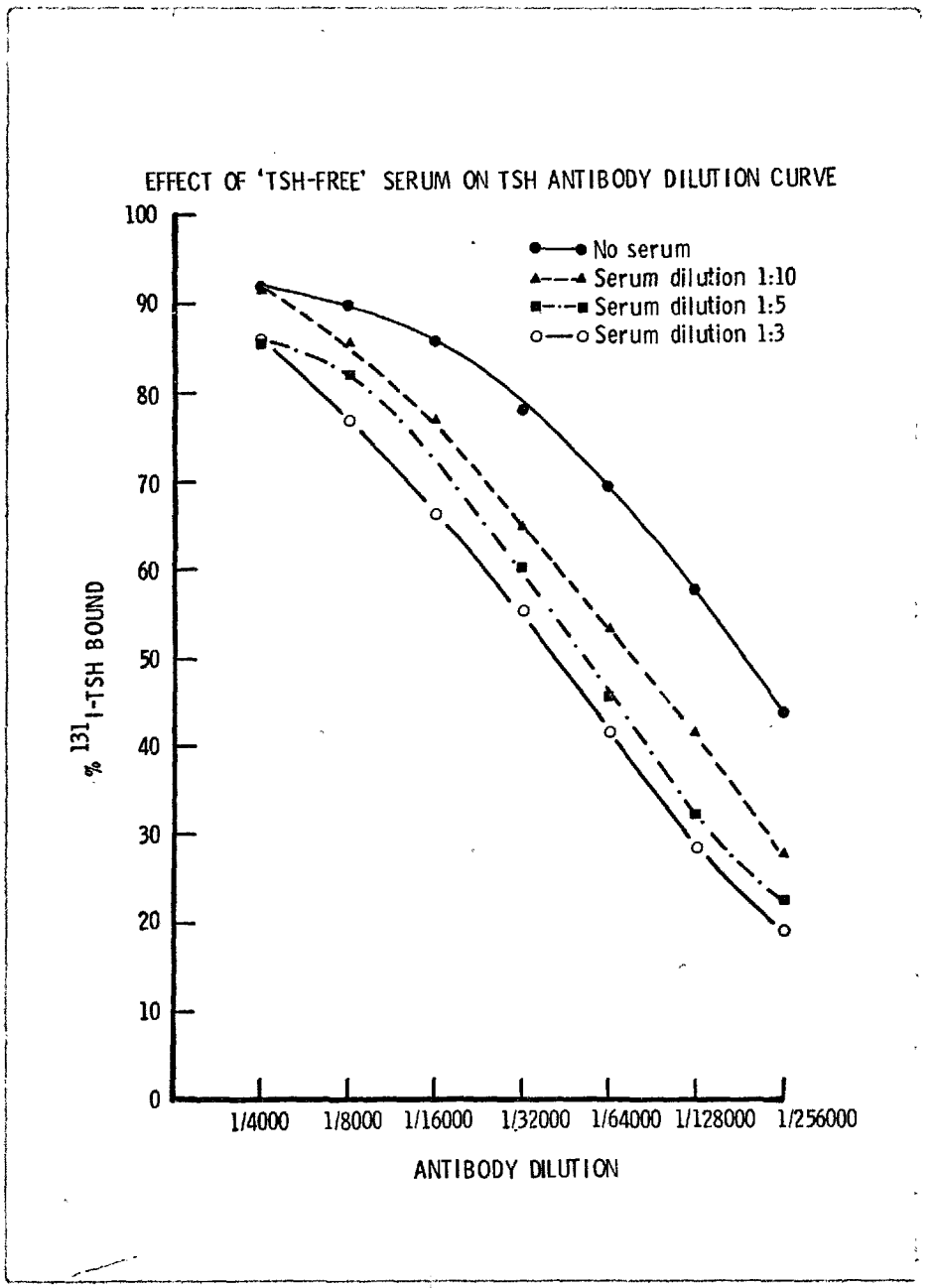

Fig. 2.2 Effect of "TSH-free" serum on the anti-TSH antibody dilution curve.

2. Second precipitating antibody: It is important to assess the completeness of precipitation of the first antibody by the second antibody in the establishment of a radioimmunoassay method using this technique, because incomplete precipitation yields spuriously high values. Spuriously high values can also be due to cross-reaction between the second antibody and human serum , as has been reported by Welborn and Fraser (1965), and by interference from complement (Morgan et al 1964). Also the correct final concentration of carrier serum and second antibody must be decided on. Cross-reaction with various fractions of human serum can be minimized by producing antisera to a purified preparation of guinea-pig gamma globulin , and as the complement reaction depends on the presence of calcium ions, the inclusion of a chelating agent such as ethylene diamine tetra-acetic acid (EDTA) or heparin should overcome this interference.

(a) Production: Antisera to guinea-pig gamma globulin were produced in rabbits as follows. 1mg of the purified guinea-pig gamma globulin (Koch Light) was dissolved in 0·5ml of distilled water and emulsified with 0·5ml Freund's complete adjuvant. The emulsion was then injected into 3-4 sites on the anterior abdominal wall of adult rabbits. Three booster doses were given at monthly intervals . The rabbits were bled from an ear vein and the serum obtained stored at 20°C until used. Further bleeds were taken at monthly intervals, each bleeding being performed one week after booster injection.

(b) Assessment of the second antibody: This was carried out in two ways:-

1. Excess amounts (1:2000) of the first antibody (anti-TSH guinea-pig antibody) were incubated with labelled TSH and the percentage of the labelled antigen-antibody complex precipitated by the addition of different dilutions of second antibody measured. Fig 2.3a shows

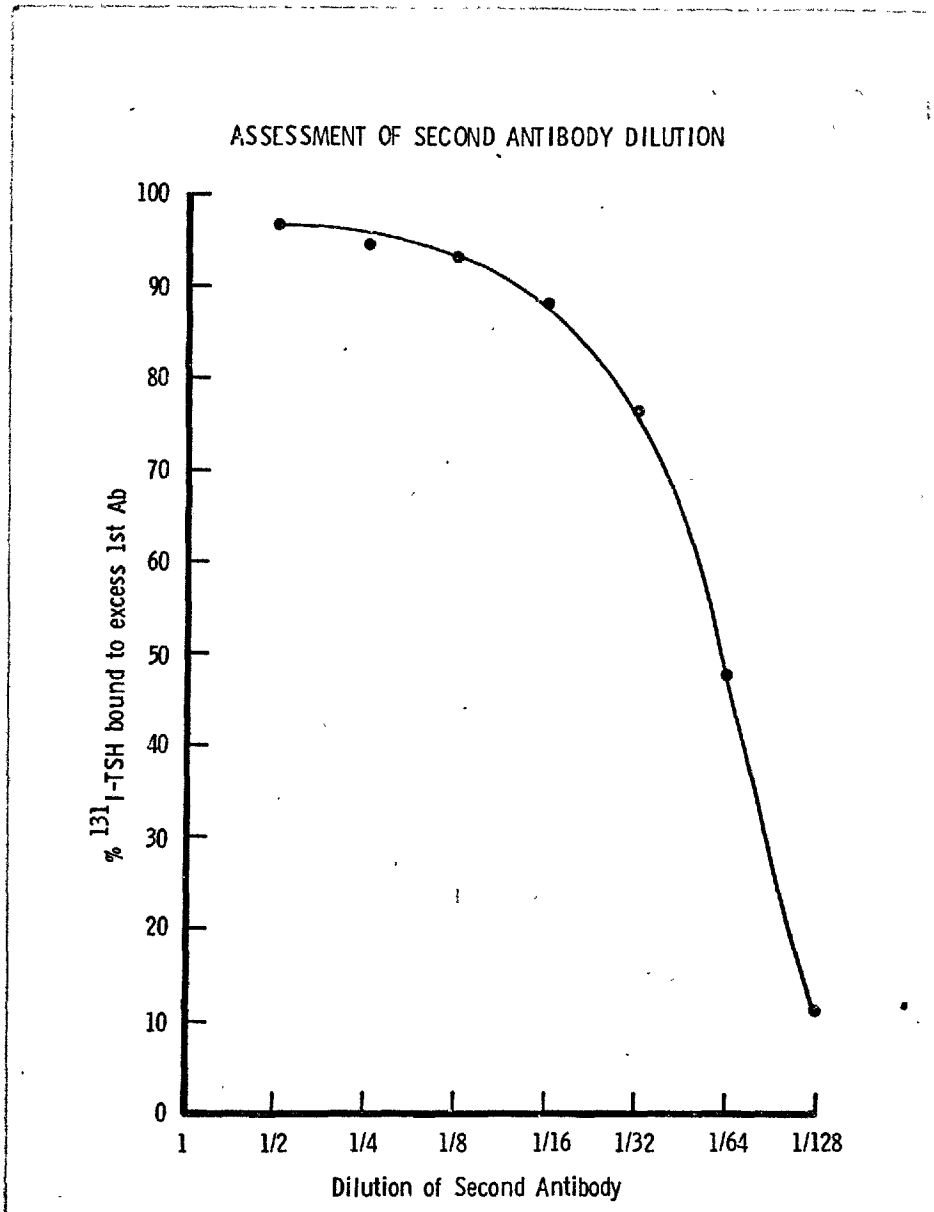

Fig. 2. 3a Assessment of the 2nd antibody: Titration curve of dilutions of second antibody with a constant amount of excess anti-TSH antibody diluted 1:2000.

the result of such an assay. That particular antibody could be used in a dilution of 1:16 or less (initial) because even at this dilution 90% of the label was still precipitated. Dilution beyond 1:16 would not be acceptable since the aim is to have excess antibody precipitation of at least 90% with a good label. It should be noted that this method reproduces the conditions in the normal assay system and so gives a direct assessment of what should be expected. However, relatively high amounts of first antibody are required, an obvious disadvantage to a laboratory in short supply. This method also assumes adequate binding between first antibody and labelled antigen which is never complete.

2. An alternative method to the above is the use of labelled guinea-pig gamma globulin against dilutions of the second antibody and to directly assess the affinity of the second antibody for its highly purified radio labelled antigen, guinea-pig gamma globulin.

i. Preparation of ^{131}I guinea-pig gamma globulin: Highly purified gamma globulin (Cohn fraction II) (Koch Light) was iodinated by the method of Greenwood et al (1963). 5µg gamma globulin was reacted with 1-1·5 mCi of carrier free ^{131}I and 75µg of chloramine-T. The reaction was stopped after 20 secs by the addition of 100µg of sodium metabisulphite and dilution with 0·5mls of 0·015M phosphate buffer. The iodination mixture was then purified on a 15 x 1.2cm column of DEAE Sephadex A-50 column, eluted with 0.015M phosphate buffer. The labelled gamma globulin emerged with the void volume of the column and the completeness of removal of free iodine checked by paper electrophoresis on Whatman 3MC paper.

ii. Assessment of second antibody: 0.1ml of serial dilutions of rabbit antiguinea-pig gamma globulin (1:4, 1:8, 1:10) were added to 0.1ml ^{131}I guinea-pig gamma globulin, 0.1ml of human serum and 0.9ml of borate buffer (pH 8.4 containing 0.1 M EDTA), and 0.1ml of

diluted non-immune guinea-pig carrier serum. Each dilution of the rabbit serum was checked in duplicate against three dilutions of carrier serum (1:50, 1:75, 1:1000). The mixture after shaking was incubated at $4^{\circ}C$ for 24 hours and then centrifuged for 20 mins at 2,000 rpm. The supernatant was then decanted and the precipitate counted. The counts were then expressed as a percentage of the total radioactivity initially added. Rabbit serum was considered suitable for use as second antibody if it precipitated more than 90% of the labelled gamma globulin at a dilution of 1:6 or greater.

Titration of dilutions of non-immune guinea-pig carrier serum showed that dilutions of 1:75 or greater produced maximum precipitation (fig. 2.3b). In general the carrier serum was used in a dilution of 1:75. The carrier serum from the same species as the first antibody was required in the double antibody to produce a visible, firm and adherent precipitate. The concentration of the added carrier serum is important as it contains gamma globulin, and hence, at high concentration, inhibits binding between first and second antibody and produces incomplete precipitation. If however, insufficient carrier serum is added, the precipitate produced is light and wil l not adhere to the tube, making decanting of supernatant impossible. Each batch of second antibody was therefore checked at different dilutions of the carrier serum in addition to the assessment of its titre.

B) Preparation of Labelled TSH

a: Material for iodination: Highly purified TSH preparations for iodination were kindly supplied by the National Pituitary Agency (NPA), U.S.A. and Dr. A.S. Hartree (DE-32-3). Under conditions standardized as detailed later, the NPA material was less easily damaged by the iodination procedure and gave consistently higher "zero" binding with slightly better detection limit that the

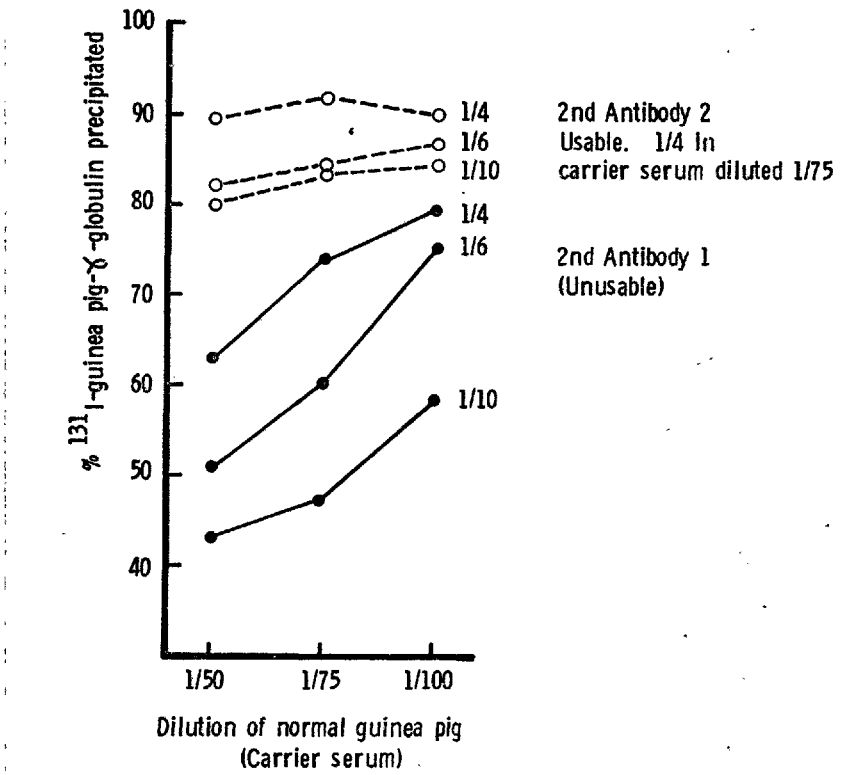

Fig. 2. 3b Assessment of the 2nd antibody: Titration curves of dilutions of second antibody against dilutions of carrier serum (normal guinea-pig serum).

Hartree material (fig 2.4). The NPA material was therefore used for iodination throughout this study. Dr. Hartree's preparation was, however, also satisfactory for use.

Lyophilized material received from the NPA was reconstituted in 0·01 phosphosaline buffer (pH 7·8) and dispensed into small plastic vials in 2·5µg/10µl aliquots and stored at -20°C.

b: Iodination Procedure: The iodination procedure used was a modification of the method described by Greenwood et al (1963). 20µl of 0·5M phosphate buffer was added to the iodination vial, which already contained 2·5µg of TSH using a 25ul microcap pipette (Drummond). The strong buffer is required to buffer the iodine which is alkaline (pH $\bar{c}$ 10). 1-1·5mCi of carrier-free ^{131}I (IBS 30, Radiochemical Centre, Amersham) was then added. 5, 15, or 20µg of chloramine-T (an oxidant, oxidizing iodide to iodine) in 10µl of 0·05M phosphate buffer was then added. Fig.2.5 shows the gamma scans of the electrophoretic strips of iodination mixtures using the various amounts of chloramine-T. It is clear that the more chloramine-T in the system, the greater the damage to the hormone. Routinely 5µg of chloramine-T was therefore used. The reaction was stopped after 10 - 15 seconds by the addition of 60µg (20µl) of sodium metabisulphite and the addition of 1ml of 0·1M barbitone buffer (pH 8·6). The chloramine-T and sodium metabisulphite were added using 25µl microcaps, each addition being followed by gentle bubbling of air to ensure adequate reagent mixing. The iodination mixture was then transferred to a 15 x 1·2cm Sephadex G-75 (fine) column through which 1ml (100mg) solution of bovine serum albumen (BSA) had been eluted. The column was eluted with 0·1M barbitone buffer. Approximately 1ml iodination fractions were collected into universal containers, the emergence of the labelled TSH being monitored by a radiation (NaI crystal) counter

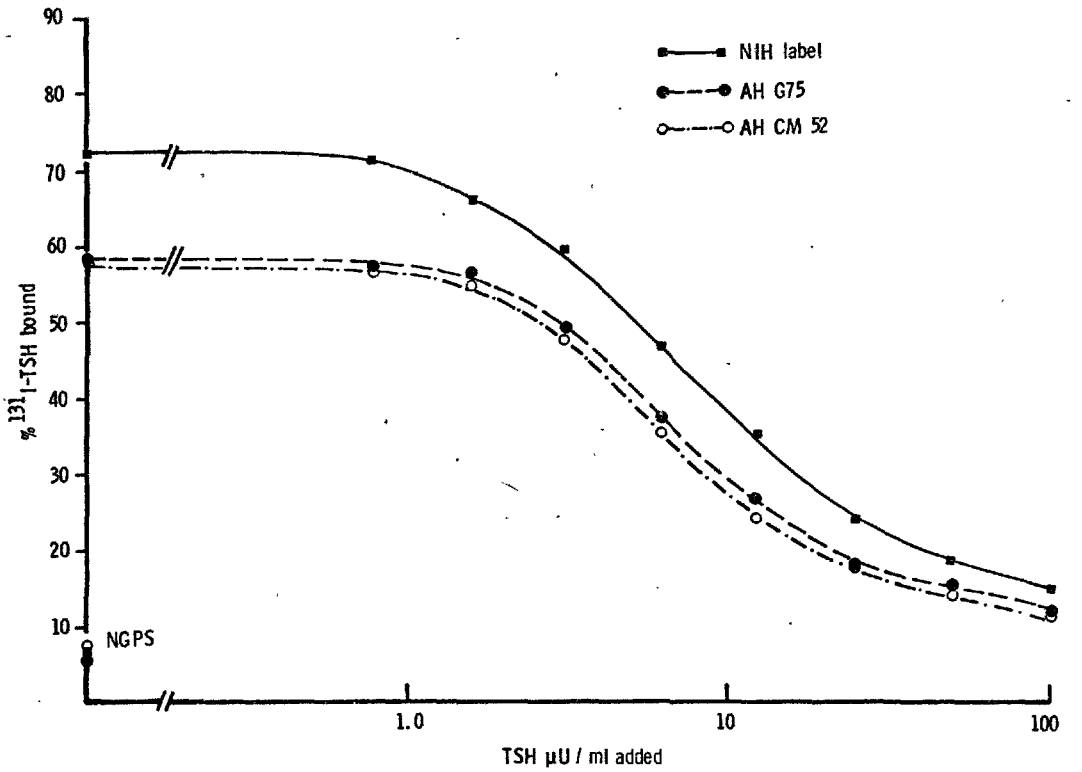

Fig. 2.4 Comparison of the TSH standard curves produced using National Pituitary Agency TSH (marked NIH) and the TSH obtained from Dr. A.S. Hartree (marked AH) for iodination. The effect of purifying the iodinated Hartree TSH on Sephadex G75 (AH, G75) or on carboxymethylcellulose (AH, CM52) is also shown.

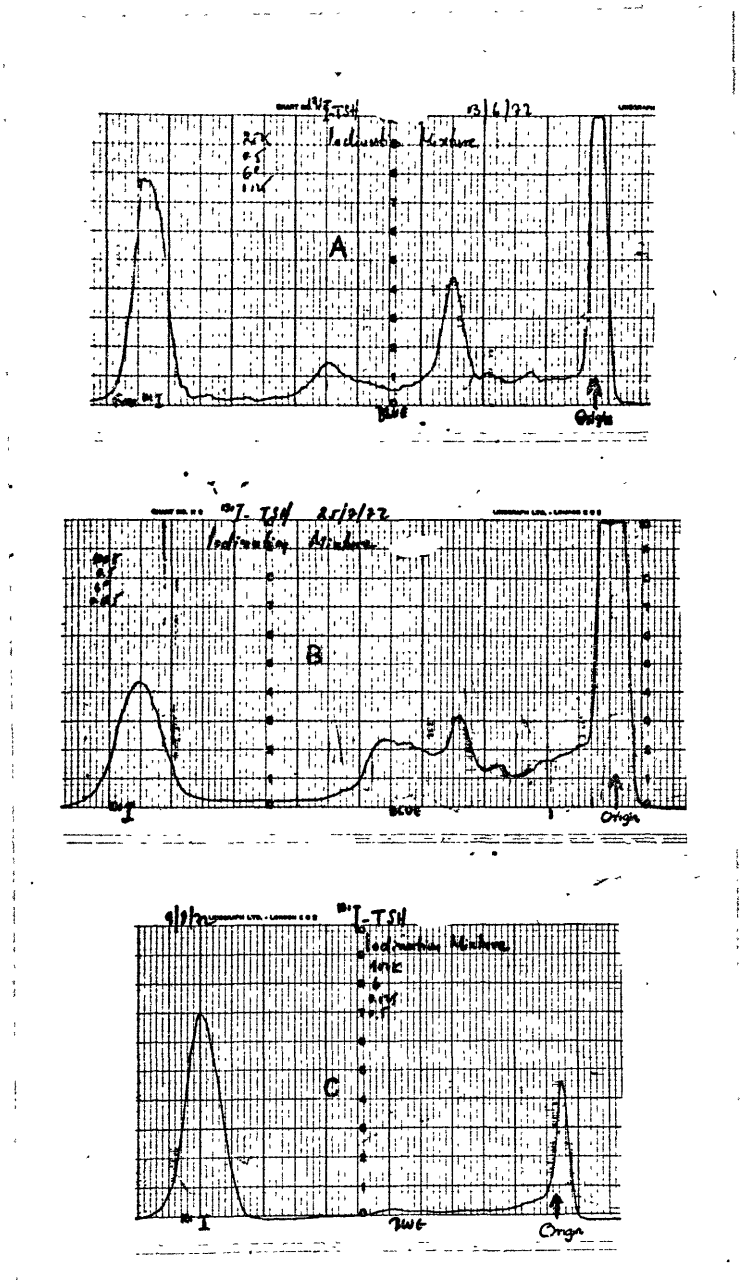

Fig. 2.5 Effect of using various concentrations of chloramine-T in the iodination procedure for TSH.

A = 20µg, B = 15µg, C = 5µg.

connected to a ratemeter and to a proper recorder. Using this system, the radioactivity in each drop from the column could be observed. Each fraction was then counted. A typical elution pattern is shown in Fig. 2.6 which shows a protein peak in fraction 4. 5µl each of this fraction and iodination mixture was then subjected to electrophoresis on Whatman 3MC paper. The electrophoretic strips were then scanned using a "Tracer lab" 4TI gamma scanner. The patterns of radioactivity obtained are shown in Fig. 2.7. From the electrophoresis of the iodination mixture the percentage of radioiodine incorporated in the protein was found and used to calculate the percentage yield and the specific activity of the iodinated TSH produced (Fig. 2.7). Electrophoresis of the fraction from Sephadex G75 was performed to check the completeness of removal of free iodine and any damaged hormone produced during iodination.

The column separation procedure described above was varied in several ways to investigate if it was possible to achieve a better label in terms of purity and assay sensitivity. The following were tested. (a) Gel-filtration of the iodination mixture first through Sephadex G50 (fine) and then the protein peak through G200. (b) Refiltration of the protein peak on another Sephadex G75 column. (c) Gel-filtration of the iodination mixture first through G75 and then by chromatography using a 7cm carboxymethyl cellulose (CM52) equilibrated with 0·08M sodium acetate and the eluted with 0·16M sodium acetate pH 5·0 as described by Franchimont (1972b). None of the above methods proved superior to the single step gel-filtration described above in terms of electrophoretic purity or performance in the assay (Fig.2.4).

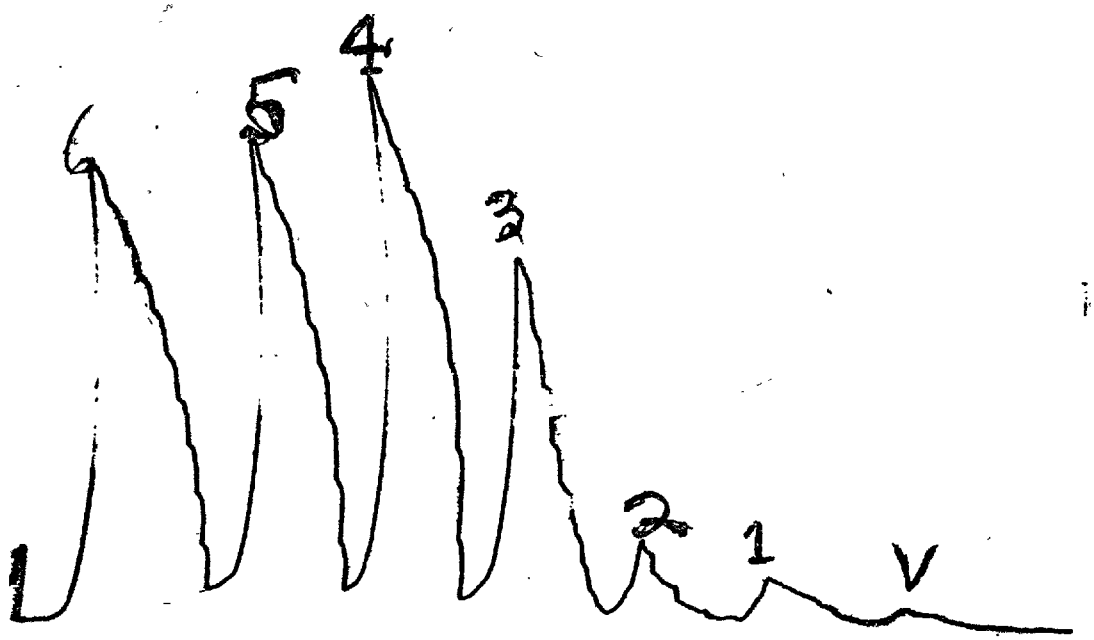

Fig. 2.6 Iodination of TSH: Ratemeter tracing of the 1ml fractions collected during the purification of the iodination mixture using a 1.2 x 15cm Sephadex G75 column. The ratemeter was connected to a NaI crystal radiation counter which monitored the effluent from the Sephadex column. V= void volume.

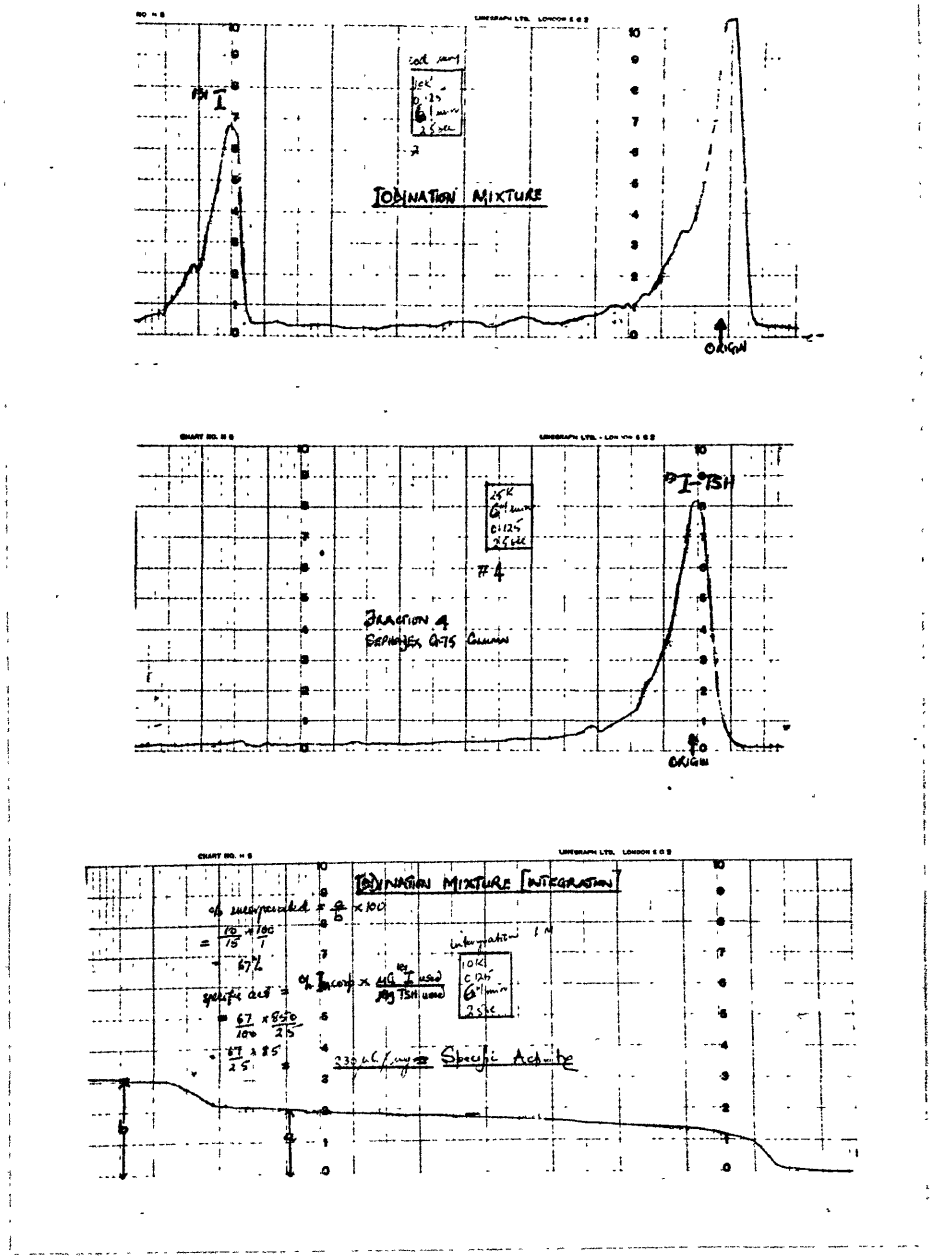

Fig.2.7 Gamma scans (using "Tracerlab 4II" scanner) of electrophoretic strips(on Whatman 3MC paper) of the iodination mixture and the purified labelled TSH (fraction 4 from 15 x 1.2 Sephadex G75 column).

Integration of the iodination mixture scan is shown in the lowest tracing. The specific activity of the labelled hormone was calculated from the product of the % ^{131}I incorporated into the hormone (a/b) and the amounts of ^{131}I used in the iodination process divided by the amount of TSH used. The specific activity achieved in this particular iodination was 230μCi/μg.

c: Selection of Purified Iodinated TSH for Immunoassay: When the various fractions collected in the purification procedure on Sephadex G-75 were tested in the assay system, the protein peak consistently gave the highest "zero" binding and the most sensitive standard curve, and was therefore routinely used in the assays.

d: Assessment of the Labelled TSH: Three methods were used to assess the label:-

(i) Purity on paper electrophoresis, as judged by a single symetrical peak with no areas of damaged hormone nor free iodine. This proved to be rather unreliable because on a number of occasions, labels judged satisfactory by electrophoretic criteria did result in assay failures when other factors could not be held responsible.

(ii) Excess Antibody checks. In the normal assay a duplicate set of tubes containing no hormone but first antibody in excess (1:2000) in addition to other ingredients of the assay were set up. An excess antibody precipitation of 90% and over was regarded as satisfactory. Again, this is not a perfect check on the label. On a number of occasions the excess antibody binding had been 90% and above, whereas the "no hormone" tube gave poor binding at around 30%, in spite of the fact that the first antibody had been properly diluted. The last point was checked by repeating the assay the following week with the same label and very carefully diluting the antibody. The explanation for this might be that the excess antibody used at 1:2000 is too concentrated with the result that it bound most TSH molecules at that concentration (i.e. it is on the far left of the plateau of the antibody dilution curve) (Fig. 2.1) but when the dilution is high such as 1:50,000 and the labelled hormone is partly damaged,

the binding is less avid i.e. the drop on the antibody dilution curve becomes very sharp.

(iii) "Rapid" checks: The best check of the label, eventually, is how it performs under assay conditions. Therefore an assay was set consisting of excess antibody and "zero" tubes each in duplicate. The label was added and the tubes incubated overnight at room temperature. The following morning the second antibody was added and the tubes centrifuged 2 hours later. The precipitates were then counted and the percentage binding (bound/total activity) calculated. A good label usually gave an excess antibody binding of at least 80% and a "zero" binding of at least 40%.

C) Assay Standards

It is fortunate for the TSH radioimmunoassay that one source of standard is used almost universally, unlike the situation for the LH and FSH immunoassays. For this study the MRC research standard A (63/14) was generally used, but later, when this became unavailable, the MRC research standard B (68/38) was used. The standard (MRC A or B) was diluted on receipt with 0·01M phosphosaline buffer pH 7·8 containing 0·5% BSA and EDTA (0·05M) and stored in 1ml aliquots of 100μu/ml at -20°C. For each assay an aliquot was thawed and doubling dilutions were made from 100μu/ml to 0·39μu/ml. More recently several aliquots have been made for each point on the standard curve so that on each assay day an aliquot was taken out for each point. This minimized the inter assay variations due to errors of dilution from assay to assay. There was no difference in the standards stored in this way and those stored at higher concentrations and diluted on the assay day.

D) Other Assay Reagents, and Instruments:

1. "TSH-free"serum:- This was obtained either by bleeding normal volunteers on the tenth day after receiving 120µg L-triiodothyronine daily or from patients who had been completely ablated of their pituitary gland by Yyttrium-90 implants for diabetic retinopathy and were on full thyroid replacement therapy.

2. Buffers:- (a) 0·01M phosphosaline buffer pH 7·8 used for the assays was made as follows:- 3·66g $Na_2HPO_4.2H_2O$ and 17·52g NaCl were dissolved in 1·5 litres of distilled water. 200mg merthiolate and 1g BSA were then added, and the solution made up to 2 litres and pH adjusted to 7·8. The antibodies were diluted in this buffer. The same buffer as above was made 0·05M with respect to EDTA as was used to dilute the standards. This EDTA buffer was used as the general assay buffer.

(b) 0·1M Barbitone Buffer pH 8·6: 35·9g of barbitone sodium was dissolved in 2 litres of distilled water and the pH adjusted with 6N HCl. This was used as the elution buffer in the Sephadex purification of the labelled TSH and as the buffer electrophoresis of the tracer on Whatman's paper.

(c) 0·5M Phosphate Buffer pH 7·4: 16·1g $Na_2HPO_4.12H_2O$ and 0·78g $NaHPO_4.2H_2O$/100ml deionised distilled water was used in the iodination procedure as "strong" buffer. A ten-fold dilution (0·05M) was used in the dilution of the reagents for the iodination procedure. All reagents were "Analar" grade and obtained from "British Drug Houses" (BDH).

3. Instruments:- (a) Counter: An autogamma counter, "Wallac" Decem GTL 500, with a 3" NaI crystal was used.

(b) Pipettes: The samples and standards were dispensed using "Eppendorf" or "Oxford" pipettes. The antisera and tracer were added using "Jencons" repettes.

(c) Test tubes: 2 x ½ in. glass test tubes were used for incubation.

(d) "Mistral" 4L refridgerated centrifuge was used for spinning.

E) Assay Procedure

1. Procedure: The radioimmunoassay procedure, based on that described by Hall et al (1971) is outlined in Table 2.1, which is a flow-chart. The major difference between this assay and that of Hall et al (1971) is the exclusion of HCG from the assay for reasons described later.

As a quality control, known "pool" sera were included in each assay. The incubation volume was 0·6ml giving a final first antibody dilution of 1:300,000, and a sample dilution of 1:3.

2. Derivation of results: Counts in all tubes were expressed as a percentage of the total radioactivity counts to give a bound to total ratio. This figure was used to draw the standard curve. The unknown samples were read directly from the standard curve because both standards and samples were included in the assay in the same final dilution,ie. 1:3. To minimize counting errors, enough counts were added to the tubes to give values about 100 times the background counts, thus allowing the counts at the lower part of the standard curve to be about 10 times background.

3. Assay Sensitivity: A composite standard curve made up from 9 consecutive assays is shown in Fig.2.8. The sensitivity of the assay i.e. the minimum amount of hormone detectable was 0·78uu/ml of MRC standard A. This was the least amount of standard which produced a significant lowering of the percentage of label bound to antibody ($P < 0·02$). The mean difference in binding between the "zero" tubes and the 0·78μu/ml standard

Table 2.1

TSH ASSAY PROCEDURE FLOW CHART

Day 1: Preparation of Standard Curve and Sample Tubes:

(1) To each standard or unknown tube is added:-

- 0•2ml of TSH standard (triplicate) or unknown (duplicate)
- 0•2ml of "TSH-free" serum to the standards or
  0•2ml of phosphosaline buffer to unknown samples
- 0•1ml of first antibody (guinea-pig anti-TSH) diluted 1:50,000 in a 1:75 solution of normal guinea-pig serum made up in phosphosaline buffer.

(2) To "zero" tubes (sextuplicate) :

0•2ml ~~0•4ml~~ phosphosaline buffer

0•2ml of "TSH-free" serum

0•1ml of first antibody diluted as above.

(3) To excess antibody tubes (duplicate):

As for "zero" tubes but first antibody is diluted 1:2000.

(4) No antibody tubes (duplicate):

As for "zero" tubes but substitute 0•1ml normal guinea-pig serum (1:75) for the antibody.

INCUBATE AT $4^{o}C$.

Day 4: Addition of Labelled Tracer:

Add 0•1ml (about 75 - 100pg) of ^{131}I-TSH to all tubes including to 20 tubes containing 0•1ml of ^{131}I-TSH for total radioactivity counting.

INCUBATE AT $4^{o}C$.

Table 2.1 (Continued...)

Day 8: Addition of Second Antibody:

Add 0·1ml rabbit anti-guinea-pig gamma globulin serum at appropriate dilution to all tubes.

INCUBATE AT 4^{o}C.

Day 9: Separation of Precipitate:

Centrifuge all tubes at 2000 rpm for 20 mins at 4^{o}C.

Decant supernatant.

Count tubes containing precipitate.

Count total radioactivity tubes.

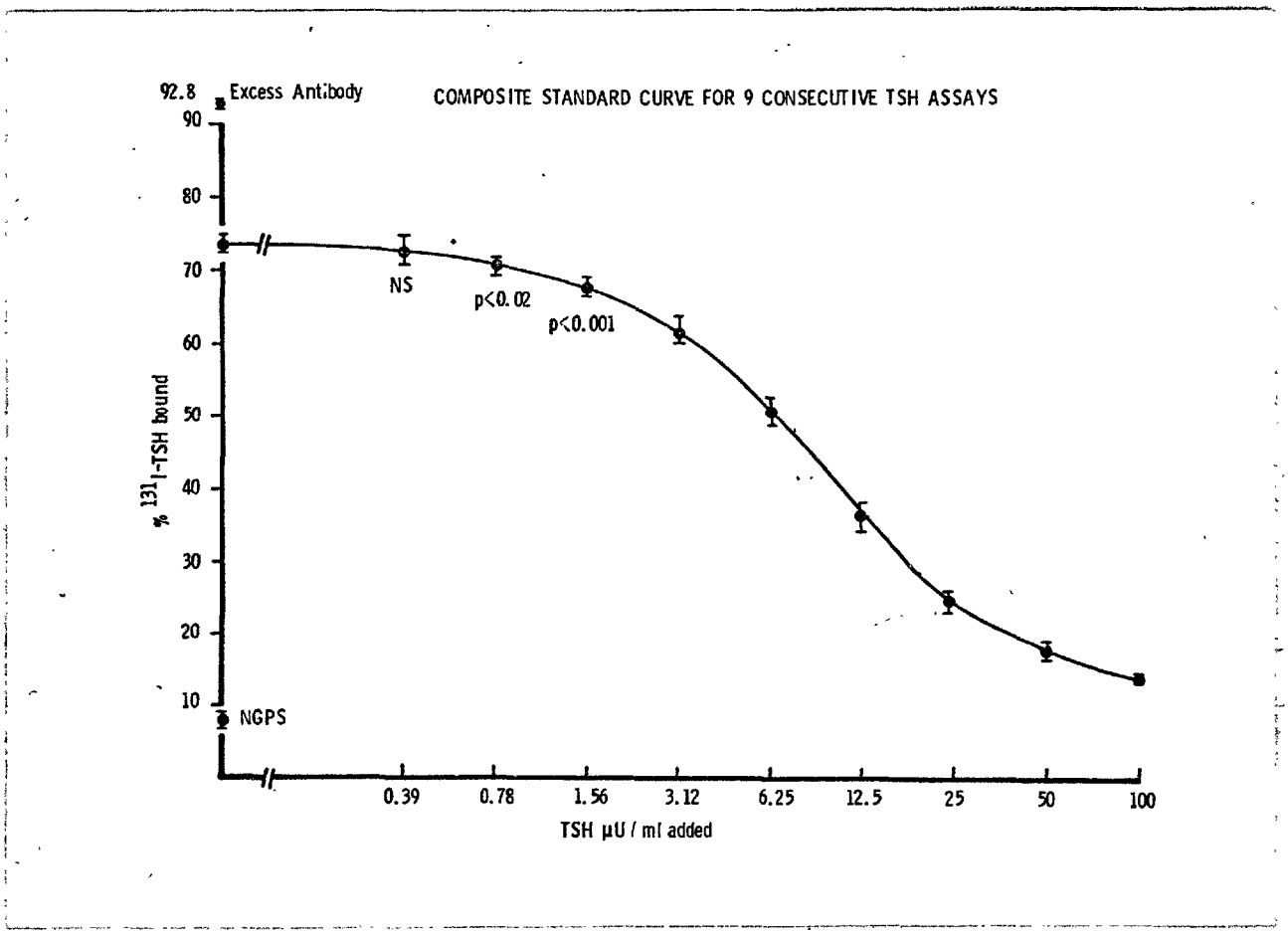

Fig. 2.8 Composite standard curve from 9 consecutive TSH assays. The mean ± SEM of each point is shown and the significance of the difference in binding between the "zero" and the lower concentrations of the standard is indicated.

was 2·6% while that for 1·56μu/ml was 5%. The former value although statistically different from "zero" is in the same range as the coefficient of intraassay variation of the assay. Therefore values reading below 1·0μu/ml have been reported as less than 1·0.

4. Range of measurement and normal values: The amounts of hormone which could be read from the standard curve ranged from 1-50μu/ml. The range extended well above the normal range which in 40 normal adult volunteers and 32 normal surgical patients was <1 - 3·7μu/ml. However, in 20% of the normal subjects the serum levels were <1 (or undetectable). Such a poor sensitivity at the lower range is a disadvantage because such normal subjects could not be differentiated from hyperthyroids, and hypopituitary patients, who would be expected to have low levels on a random serum TSH measurement. The question whether TSH is secreted perhaps at a low key in hyperthyroidism and hypopituitaric (secondary) hypothyroidism can therefore not be solved using a radioimmunoassay on unprocessed serum. Perhaps extraction and concentration of the hormone from serum or urine (more practically) would provide an insight. We have attempted to study this problem using extraction and concentration of the hormone in urine, and the findings are presented in the next chapter.

5. Validation of the assay:

(a) Tests of non-specific factors: The assay of hormones in unextracted serum is subject to interference by many non-specific factors present in the serum, for example, urea, electrolytes, proteins, other related hormones etc. The assay of TSH described here uses serum in the final dilution of 1:3. It is not surprising that the presence of serum in the system

has a profound effect on the binding particularly at the lower hormone concentrations (Fig. 2.9). It is clear from Fig. 2.9 that reading a serum sample off the buffer standard curve would lead to falsely high values. The degree of suppression of binding varies with the amount of serum in the system, as shown in Fig. 2.2.

As will be shown in the next chapter, urea electrolytes and albumin in concentrations at which they are present in serum do not modify the assay to any significant extent.

(b) Parallelism for serum assays: Assay of doubling dilutions of hypothyroid sera with high TSH levels gave bindings parallel to the pituitary standard curve (Fig.2.10), suggesting that the material in the sera suppressing the binding was similar in antigenic properties.

(c) Recovery of added hormone: Results of an experiment to test the recovery of added standard TSH as measured in the assay are shown in table 2.2. The serum had been assayed to find out the endogenous TSH level in it. Appropriate amounts of the TSH standard MRC (68/38) to cover the sensitive part of the standard curve were then added to the samples which were then reassayed after mixing. The recovery of added TSH ranged from 88 - 108% mean being 96·5 $\pm$ 7·1 (SD).

(d) Assay variation and precision: To assess the variation in the measurement of samples within the same assay (intraassay variation) 4 -6 sets of duplicate tubes containing the same sample were assayed. The samples were chosen to fall in different parts of the standard curve. The results are shown in table 2.3. The coefficient of variation for the samples falling about 2μu/ml was 7·2% while for those falling in the range 4 - 7μu/ml was 4·5% and in the range of 17μu/ml was 5·0%. The

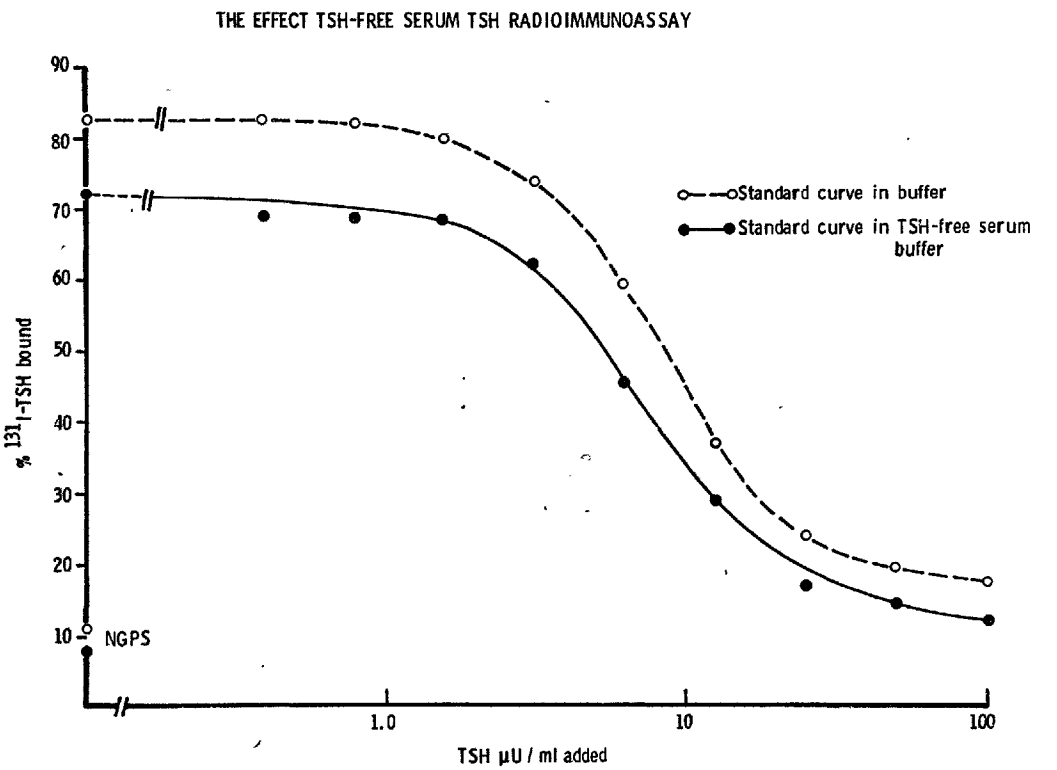

Fig. 2.9 The effect of "TSH-free" serum on the radioimmunoassay of TSH.

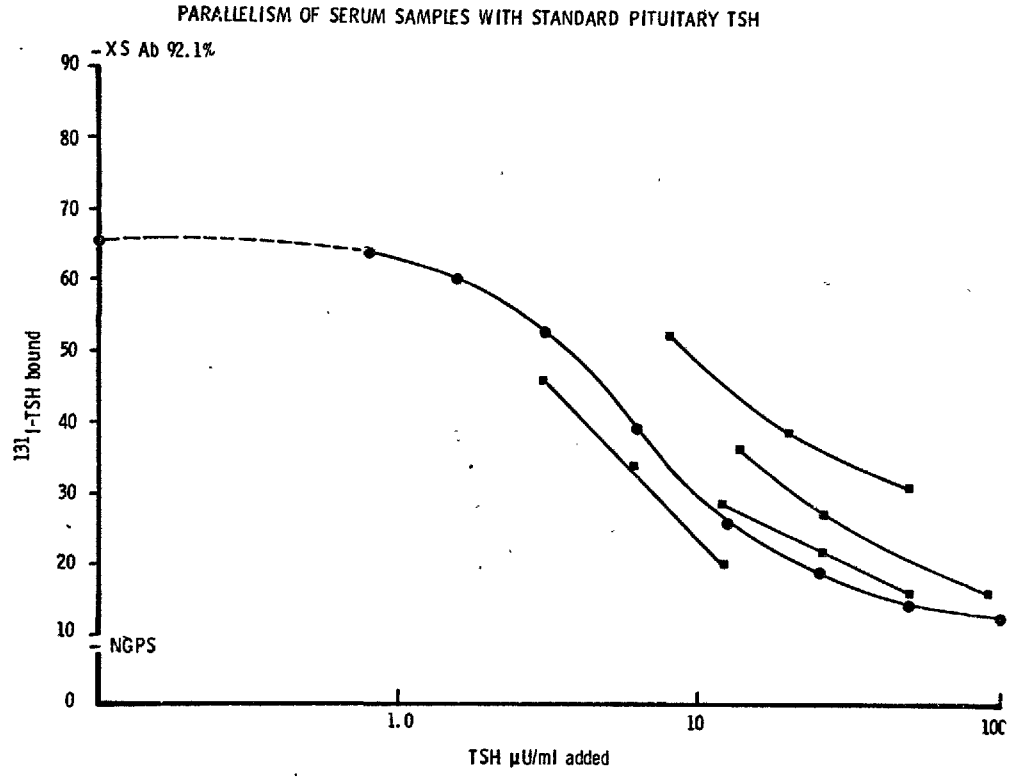

Fig. 2.10 Parallelism of serum samples from 4 hypothyroid subjects with standard pituitary TSH preparation. Each set of 3 points represents the sample assayed undiluted, and diluted 1:2 and 1:4.

Table 2.2

Recovery of added TSH from serum.

Endogenous TSH concentration (a)	Concentration of Standard TSH added µu/ml (b)	Measured a+b=c µu/ml	Recovered c-a = d µu/ml	% Recovered $\frac{d}{b}$ x 100
3·4 µu/ml	2.5	5·6	2·2	88
"	5	9·0	5·4	108
"	10	12·8	9·6	96
"	12·5	15·0	11·6	93
"	20	21·0	18·6	93
"	25	28·8	25·4	101
		Mean Recovery:-		96·5 ± 7·1 (SD)

Range 88 - 108%

	TSH μu/ml measured in			
Sample:- A	B	C	D	E
<1	1•7	4•5	6•5	18•0
<1	1•9	4•5	6•4	16•0
<1	1•9	4•6	7•2	17•0
<1	1•7	4•0	6•2	16•5
Mean ± SD	1•8±0•13	4•4±0•2	6•6±0•3	16•9±0•85
Coefficient of variation	7•2%	4•5%	4•5%	5•0%

Table 2.3: Intra-assay variation of sera each assayed 4 - 6 times in one assay. The mean coefficient of variation (v) was 5.3%.

mean intraassay coefficient of variation was 5·3%.

The interassay coefficient of variation was assessed by including pooled sera, having values which fell in different parts of the standard curve, in each assay. The TSH values measured in four different pools are shown in Fig 2.11. For all the pools the mean coefficient of variation was 14·5% (range 11·9 - 16·8%).

The coefficient of variation (v) was calculated for each pool from the formula:

$$V = \frac{100 \times \text{standard deviation}}{\text{mean value}}$$

(e) Specificity of the antiserum: The specificity of the first antibody used in this study has been assessed by studying the cross-reaction of structurally related and non-related pituitary hormones in the serum TSH immunoassay system. Results are shown in Fig 2.12. Human growth hormone (GH) at a concentration of 1iu/ml did not suppress the binding between labelled TSH and its antibody. This is not surprising since GH and TSH are markedly dissimilar in primary structure. Of the related glycoprotein hormones tested (FSH and LH) only FSH showed much cross-reaction. According to Fig. 2.12, 19·5mU of FSH was equivalent 1μu of TSH. This is either due to true cross-reaction or to contamination of the material with TSH. True cross-reaction is unlikely to account for all the value quoted above because post-menopausal females, having serum FSH values in the range of 60mu/ml, had serum TSH values in the normal range, i.e. $< 3{\cdot}7$μu/ml. Also, as will be shown later, post-menopausal women having urinary FSH in the range of 2U/hr had urinary TSH values in the normal premenopausal or male range i.e. < 10μu/hr. The FSH used for this study was LER-1366 for iodination. The amount of contamination with TSH is not known but is said to be minimal. 1mg of this material is equivalent to 3000iu of FSH. If 19iu of FSH is equivalent on the assay to

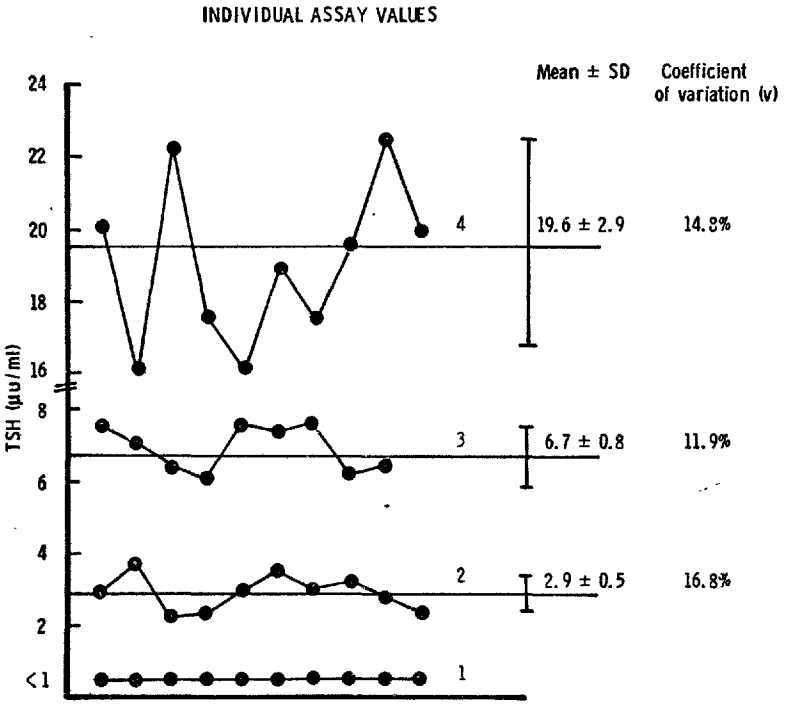

Fig. 2.11 Interassay variation of pool samples. Each point represents the reading obtained for the same pool in a different assay.

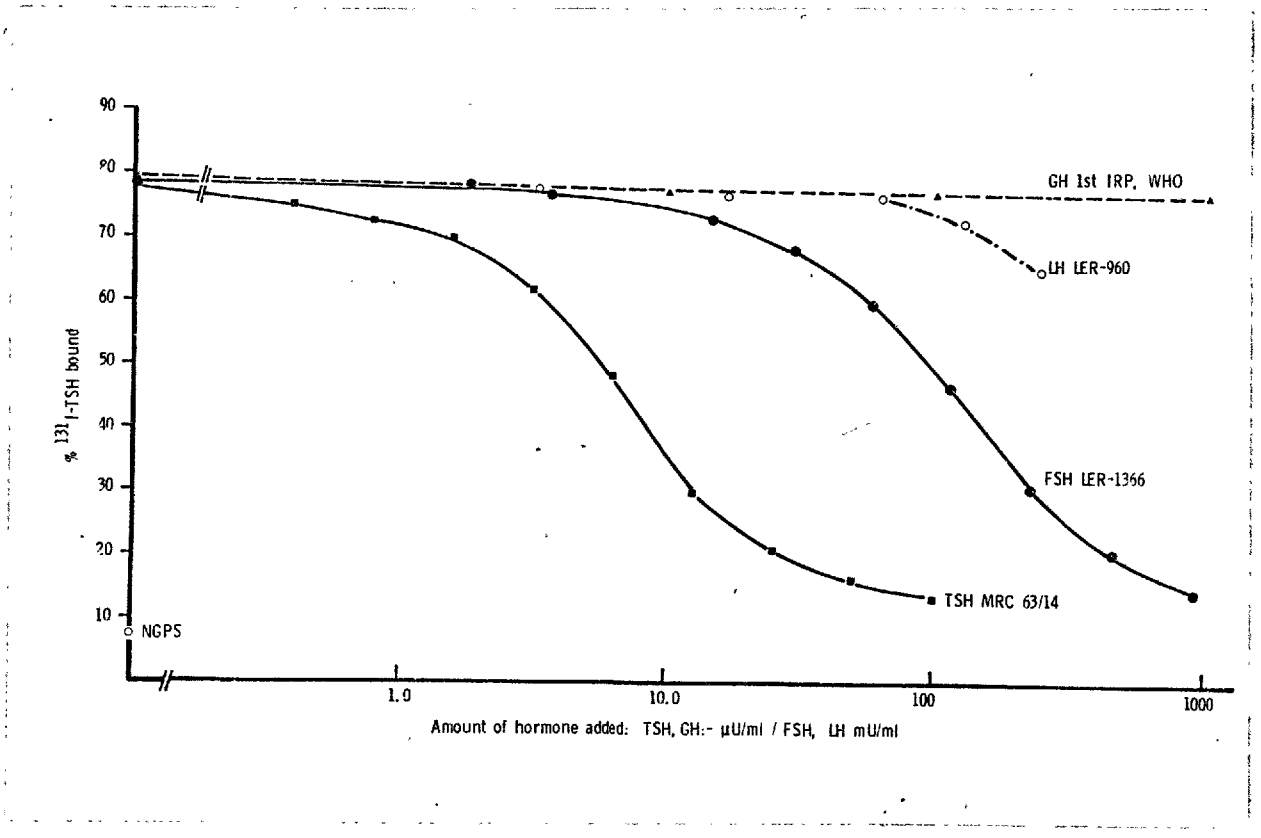

Fig. 2.12 Cross-reaction of human pituitary hormones in the TSH assay.

1u of TSH then this material would have 150u of TSH, a rather high figure. However, the amount of contamination with LH is 213iu. In any case this level of cross-reaction of FSH in the serum assay system is not important for all but the very high levels. LH (LER 960 for iodination) showed a cross-reaction such that 100mu of LH was equivalent to 1μu of TSH. Odell et al (1965) reported partial cross-reaction of their TSH antiserum with LH which was minimized by adding human chorionic gonadotrophin (HCG) to the assay tubes, thereby saturating the binding sites of the antibody for labelled LH. Hunter and Bennie, (1971) later showed that part of this cross-reaction was due to contamination of the TSH used for iodination with LH. In our hands, 10 iu/tube of HCG did not significantly suppress the TSH standard curve (Fig. 2.13), probably due to the specificity of our antibody for TSH or the purity of the tracer. We therefore do not routinely add HCG to our assay tubes.

B. ASSAY OF OTHER HORMONES

1. Thyroid Hormones:

Serum T_3 and T_4 and urinary "free" T_3 and T_4: These were measured by specific radioimmunoassays, developed in this laboratory by Dr. C.W. Burke and Mr. R. Shakespear, using standard T_3 and T_4 purchased from "Sigma". Originally ^{125}I-T_4 and ^{125}I-T_3 used as tracers, were obtained from the"Radiochemical Centre", Amersham. The specific activity (50μCi/μg) of these preparations was low and to achieve satisfactory assay sensitivity for T_3 in particular, high dilutions of the labels were made and counting times increased considerably (5 min per tube). We have now produced ^{125}I-T_3 and ^{125}I-T_4 of high specific activity (1000 - 3000 μCi/μg), and their use in the assays has greatly reduced counting times (100 secs) and have resulted in very high sensitivity assays capable of

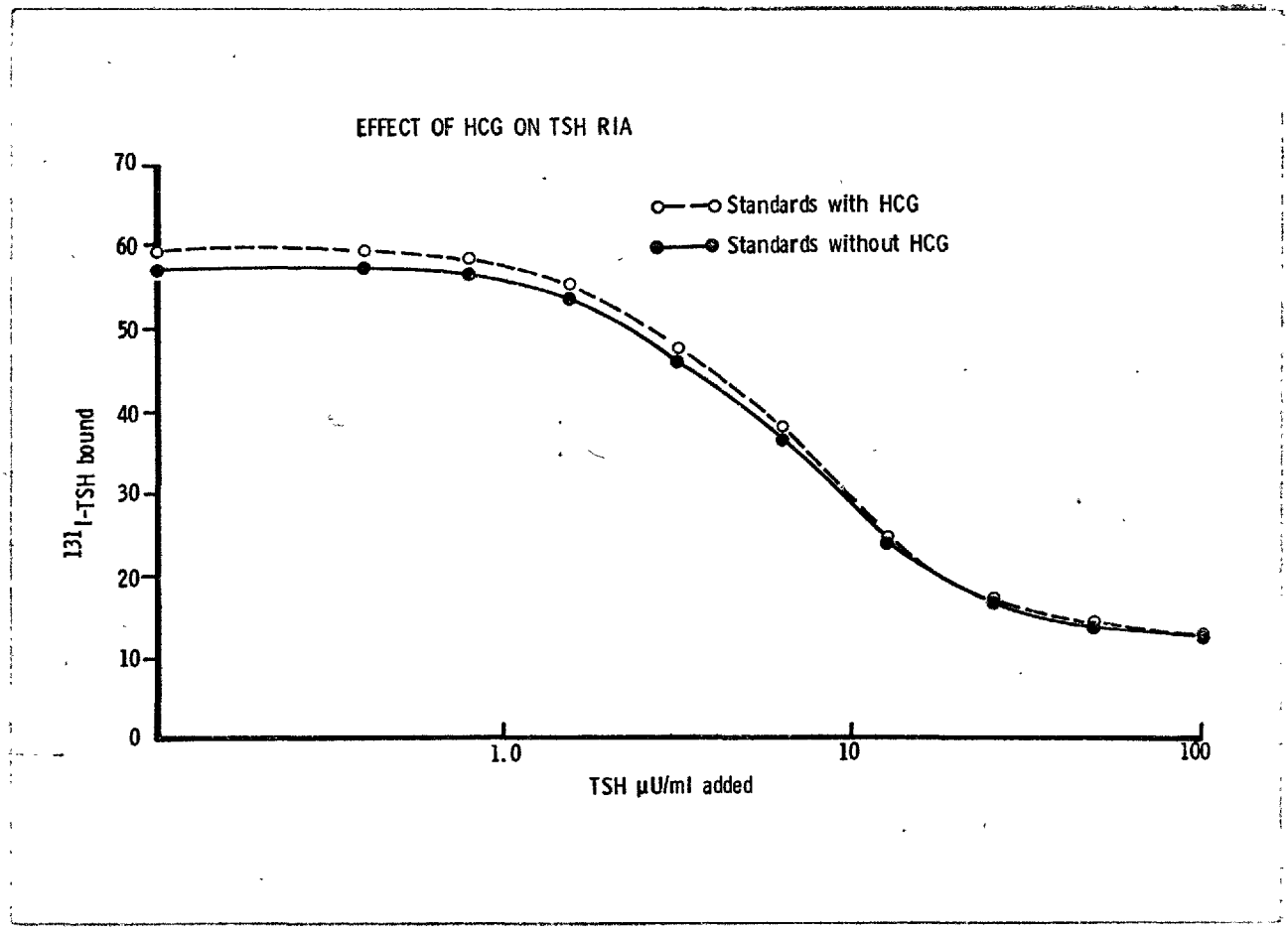

Fig. 2.13 Effect of human chorionic gonadotrophin on the TSH assay.

detecting 1pg of T_3 or T_4/tube. This method is described later. Rabbit anti-T_4 and T_3 antisera used as first antibody were produced by injecting hormone-BSA conjugates into rabbits. To separate free from bound hormone, a second antibody (donkey anti-rabbit gamma globulin from "Burroughs-Wellcome") was used for serum T_4, while for serum T_3 dextran-coated charcoal was used. For urinary T_3 and T_4 assays "Dowex-1" ("Sigma") was used to separate bound from free hormone.

For the serum assays, thyroxine binding globulin (TBG) and other thyroid hormone binding proteins were blocked with anilino-naphthalene sulphonic acid (ANS) ("Sigma"), and merthiolate ("Sigma"). T_4 and T_3 free serum was added to the serum standard tubes.

Flow charts for the assays of the above hormones are shown in appendix 1.

The antibodies to T_3 and T_4 were specific for their respective hormones (Burke, 1973). Cross-reaction of related substances with the L-T_3 antibody was as follows: 2ng d-T_3 = 1ng T_3; 100ng Diiodothyronine (T_2) = 1ng T_3; 8000ng T_4 = 1ng T_3; Monoiodotyrosine (MIT) nil. For the L-T_4 antibody, 1000ng T_3 = 5-10ng T_4; MIT, T_2 and Tricholothyronine did not cross-react in the system.

The detection limit of the assays using the commercial labels was about 50pg/tube (25-100) but with labels produced in this laboratory was < 5pg/tube.

The normal range of the various assays are as follows; Serum T_4; 50-120ng/ml (71-149nmoles/l); urinary T_4; 18-140ng/hr (23-180nmoles/hr); serum T_3; 1-2ng/ml (1·5 - 3nmoles/l); urinary T_3 13-80ng/hr (20-123nmoles/hr).

2. Growth Hormone (GH):

Serum growth hormone was measured by double antibody radioimmunoassay as described by Hartog et al (1964). As standard the first human pituitary international reference preparation, 1st WHO-IRP 66/217 was used. For preparation of ^{131}I-labelled GH, purified human GH supplied by the MRC (69/46) was used. The method of Greenwood et al (1963) was used for labelling while label purification was performed using Sephadex G-50 and then G 200. The first antibody (guinea-pig anti-HGH antiserum) was produced in this laboratory and used in an initial dilution of 1:150,000. The second antibody (rabbit anti-guinea-pig gamma globulin) was also produced in this laboratory.

The sensitivity of the assay was luu/ml. The intraassay variation was 3% while the interassay variation was 12% for values in the range<1 - 30μu/ml. The assay was specific for GH, the other pituitary hormones showing no cross-reaction in the system. The normal range for serum GH was <1-5μu/ml. =

3. Luteinizing Hormone (LH):

LH was measured by the double antibody radioimmunoassay method of Marshall et al (1972). Human pituitary LH (MRC 69/104) was used as standard. As tracer, purified human pituitary LH (IRC-2, kindly supplied by Dr. A. Stockell Hartree) was labelled with ^{131}I and purified as for HGH. The first antibody prepared in this laboratory using human chorionic gonadotrophin ("Pregnyl") was produced in guinea-pigs by Dr. J.C. Marshall and used in a dilution of 1:200,000 (initial). The assay was relatively specific for LH. The assay was unresponsive to lug of either GH or ACTH but began responding at over 88mu/ml, FSH (CPDS-2 from Dr. Butt) and 100μu/ml TSH (MRC 70/292). It however

responded to 4mu/ml of HCG. Most of the cross-reaction to FSH and TSH was probably due to contamination of the test preparations with LH (Marshall, 1973). The interassay coefficient of variation was 13·9% while the intraassay variation was 2·8% in the working part of the standard curve. The normal adult range in serum was 3·6 - 8·2mu/ml.

4. Follicule Stimulation Hormone (FSH):

FSH was measured by the double antibody radioimmunoassay method described by Marshall et al (1973). For standard, human pituitary standard (LER 907) supplied by the National Pituitary Agency (U.S.A.) was used. The first antibody (rabbit anti-FSH antiserum No. 3 preadsorbed with LH) was obtained from the National Pituitary Agency, U.S.A. and used in a final dilution of 1:36,000. The tracer hormone, purified human FSH (kindly supplied by Dr. Butt), was iodinated as for HGH and purified after iodination by column chromatography first on Sephadex G-50 and then on Whatman DE52. The labelled hormone was eluted from the second column, using two concentrations, 0·01 and 0·05mol/l (^{131}I-FSH eluted at this molarity) of K_2HPO_4 buffer (Franchimont, 1968). Donkey anti-rabbit serum ("Burroughs-Wellcome" MR 66) was used as the second precipitating antibody at an initial dilution of 1:20. The lowest serum level of FSH detectable was 1·0mU/ml. The coefficient of variation for replicate samples in the same assay was 3·4% and in different assays 12·8%. The assay was relatively specific for FSH, no cross-reaction with LH being found in concentrations of up to 500ng/ml (IRC-2LH, supplied by Dr. A.S. Hartree). An interference from TSH only occurred at

concentrations of greater than 50ng/ml (National Pituitary Agency TSH for iodination). The normal adult range was 1.6 - 8.6mu/ml expressed in terms of the second IRP-HMG.

Flow charts for the assay of GH, LH and FSH are shown in Appendix 2.

C. SIMULTANEOUS PRODUCTION OF ^{125}I-T_3 AND ^{125}I-T_4 OF HIGH SPECIFIC ACTIVITY AND STABILITY:

1. Introduction:

The present methods available for the production of iodine labelled thyroxine and triiodo-thyronine are time consuming and only yield low specific activity labelled hormones. For T_4, Clayton et al (1950) described a method starting from 3,5 diiodothyronine, ^{131}I and H_2O_2 as oxidizing agent. The yield was only 100µCi/mg. Critchlow and Goldfinch (1954) later described another method starting from T_3, but the yield was lower (10µCi/mg) and the products unstable. Later on Roche et al (1955) described a micro method for producing labelled T_4 of comparatively high specific activity (300µCi/µg). Frieden et al (1948), Taurog et al (1955) and Gleason (1955) have described a method for preparing ^{131}I-T_4 by an exchange reaction involving ^{131}I and cold T_4. The specific activity of the yield, however, again was low.

For T_3, Roche et al (1953) have described production of ^{131}I-T_3 from a controlled iodination of diiodothyroxine similar to that described by Clayton et al (1950) for T_4. An exchange method similar to that of Taurog et al (1955) for T_4 is also available for T_3 (Taurog and Chaikoff, 1970). As for T_4, the labelled T_3 produced by the above methods

were of low specific activity. The methods were also time-consuming.

The highest specific activity of the labelled hormones commercially available is 500μCi/μg, ("Abbot Laboratories") and although these preparations have offered sufficient specific activity to measure total T_3 and T_4 in serum, the T_3 assays really need labels of higher specific activity. In addition, the determination of free T_3 and T_4 levels in blood would require much higher specific activity labels for increased assay sensitivity than currently available.

Recently Weeke and Orskov (1973) described a chloramine-T method for the synthesis of ^{125}I-T_3 and ^{125}I-T_4 of high specific activity in two separate iodinations but their separation technique was poor, and in our own hands, the products were unstable. The chloramine-T method has been widely used for the iodination of peptide hormones, and Shapiro et al (1971) have recently described it for the iodination of DT_4. We now describe a method, also using chloramine-T, for the synthesis of ^{125}I-T_3 and ^{125}I-T_4 simultaneously in one iodination. The stability of the products is also assessed.

2. Material and Methods:-

1) Materials:

(a) Column chromatography: For column chromatography, Sephadex G25 (fine) ("Pharmacea") columns (25 x 0.5cm) were used. The columns were eluted at room temperature with 0.05M K_2HPO_4 pH 11.9 at 40mls/hr using a peristaltic pump. 2mls fractions were collected using an "LKB" fraction collector. The columns were calibrated with ^{125}I-T_3 and

^{125}I-T_4 (Amersham) and ideal separation conditions assessed using columns of different diameters, different lengths, and eluting with buffers of different molarities and pH.

(b) Chemicals for labelling:- $Na^{125}I$ or $Na^{131}I$ (IMS,30, IBS,30) were purchased from the "Radiochemical Centre". Pure T_3, diiodothyronine (T_2) and triiodothyroacetic acid (TRIAC) were obtained from "Sigma". As oxidant chloramine-T ("BDH") was used while sodium metabisulphite ("BDH") was used as a reducing agent. 0·5M phosphate pH 7·4 was used as the neutralizing buffer for the alkaline iodide preparations.

(c) Storage of labelled products:- 50% or 90% ethanol was used. Ideal storage conditions were assessed using propylene glycol, methanol, butanol, glycerol, sodium metabisulphite, arsenite and carbimazole, all purchased from standard commercial sources. All were analar grade as were all reagents used in this study.

2) Labelling Procedure:

2 - 2·5mCi ^{125}I or ^{131}I was added to a vial containing 25µl of 0·5M phosphate buffer pH 7·4. lug (10µl) of T_3 was then added, 1mg T_3 having been dissolved in 10ml 0·05M phosphate buffer adding 2-3 drops 6N NaOH since T_3 is most soluble in alkaline solutions. This was followed by 100µg (10µl) of chloramine-T. The reaction mixture was continuously mixed by bubbling air through it. The reaction was stopped after 20 secs by adding 250µg (100µl) sodium metabisulphite later followed by 0·5ml of 0·05M phosphate buffer.

An identical procedure was carried out starting with T_2 or TRIAC.

The iodination mixture was then subjected to adsorption chromatography on 25 x 0·5cm Sephadex G25 fine columns and eluted as described above. As soon as each rack of the fraction collector was finished, 1 drop of 6N HCl was added, using "Pasteur"pipettes, to bring the pH down to 7·4, since the labelled hormones as will be shown later were highly unstable in alkaline solutions. 50 fractions were collected and 50ul aliquots of these were counted in a "Wallac " auto-gamma counter. The fractions at the hormone peaks and 3 others on either side of them were pooled and stored in 50% ethanol. Lately these pooled fractions have been concentrated further using "Dowex-1" ("Sigma") and stored in 90% ethanol.

3) Results:-

1. Evolution of ideal conditions for Sephadex G25 column chromatographic procedure for the separation of labelled T_3 and T_4 and free iodine:

(a) Effect of column height and diameter: The elution profiles of commercial 125I-T_3 and ^{125}I-T_4 (alone and in combination) on 15 x 1.5cm Sephadex G25 (fine) columns are shown in Fig. 2.14 a,b,c. Fig. 2.14c shows that the labelled hormones when applied in a mixture including ^{131}I were well separated and were eluted in positions as would be expected if they were eluted alone (Fig. 2.15a,b). ^{131}I or ^{125}I were eluted at the void volume of the column while ^{125}I-T_3 and ^{125}I-T_4 were eluted in that order because of their different partition coefficients (T_3 2·35, T_4 5·20, Blasi and de Masi, 1967). The elution volumes of the hormones were large and the peaks broad.

The effect of reducing the diameter of the column from

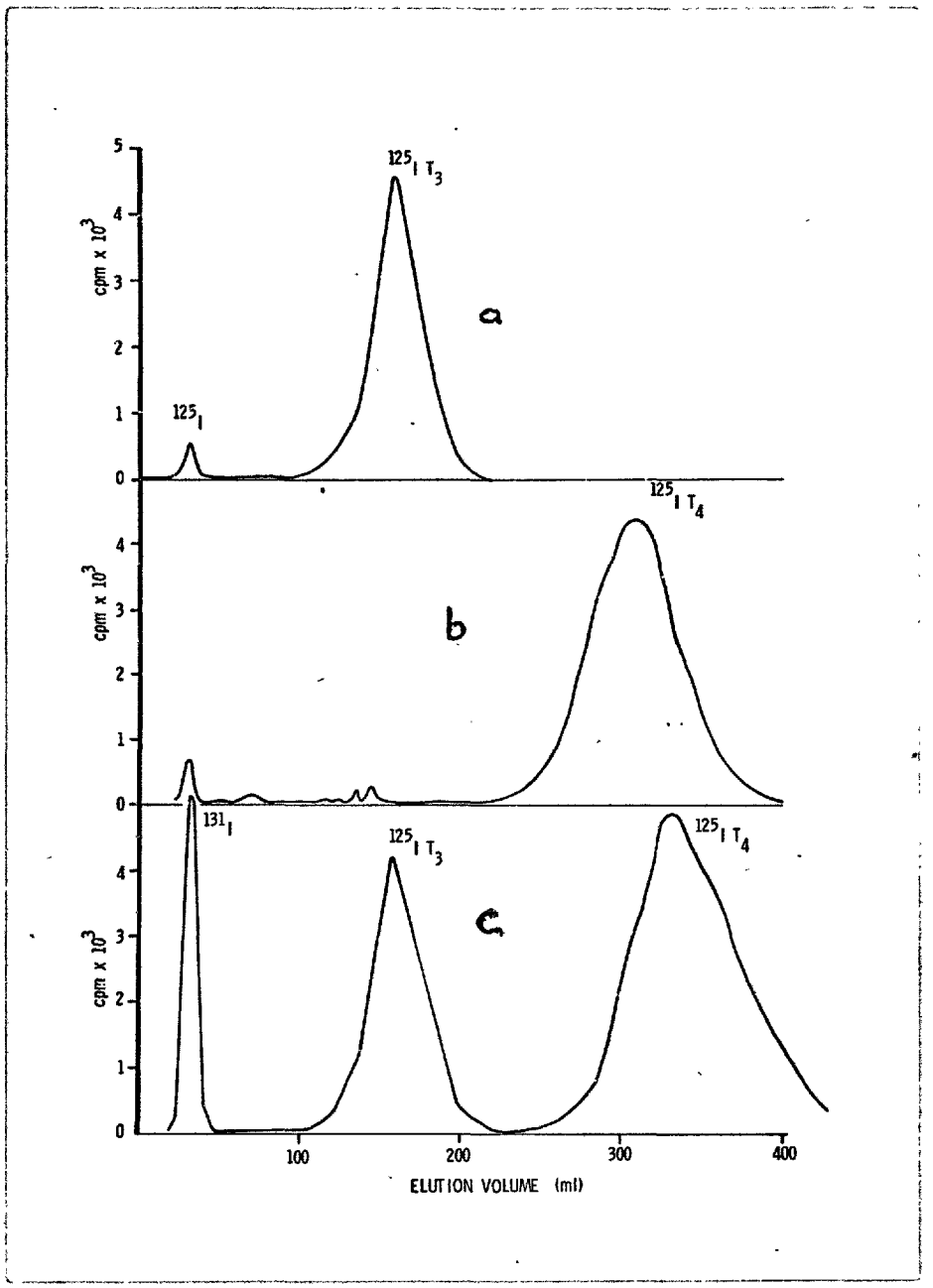

Fig. 2.14 Elution profiles of ^{125}I-T_3 and ^{125}I-T_4 when eluted separately (a and b) and in a mixture with ^{131}I (c) on 15 x 1·5cm Sephadex G25(F) column. Elution was carried out under gravity using 0·05M K_2HPO_4 buffer pH 11·9 at room temperature at rate of 100mls/hr. 4ml fractions were collected. Sample was applied in 5mls of elution buffer.

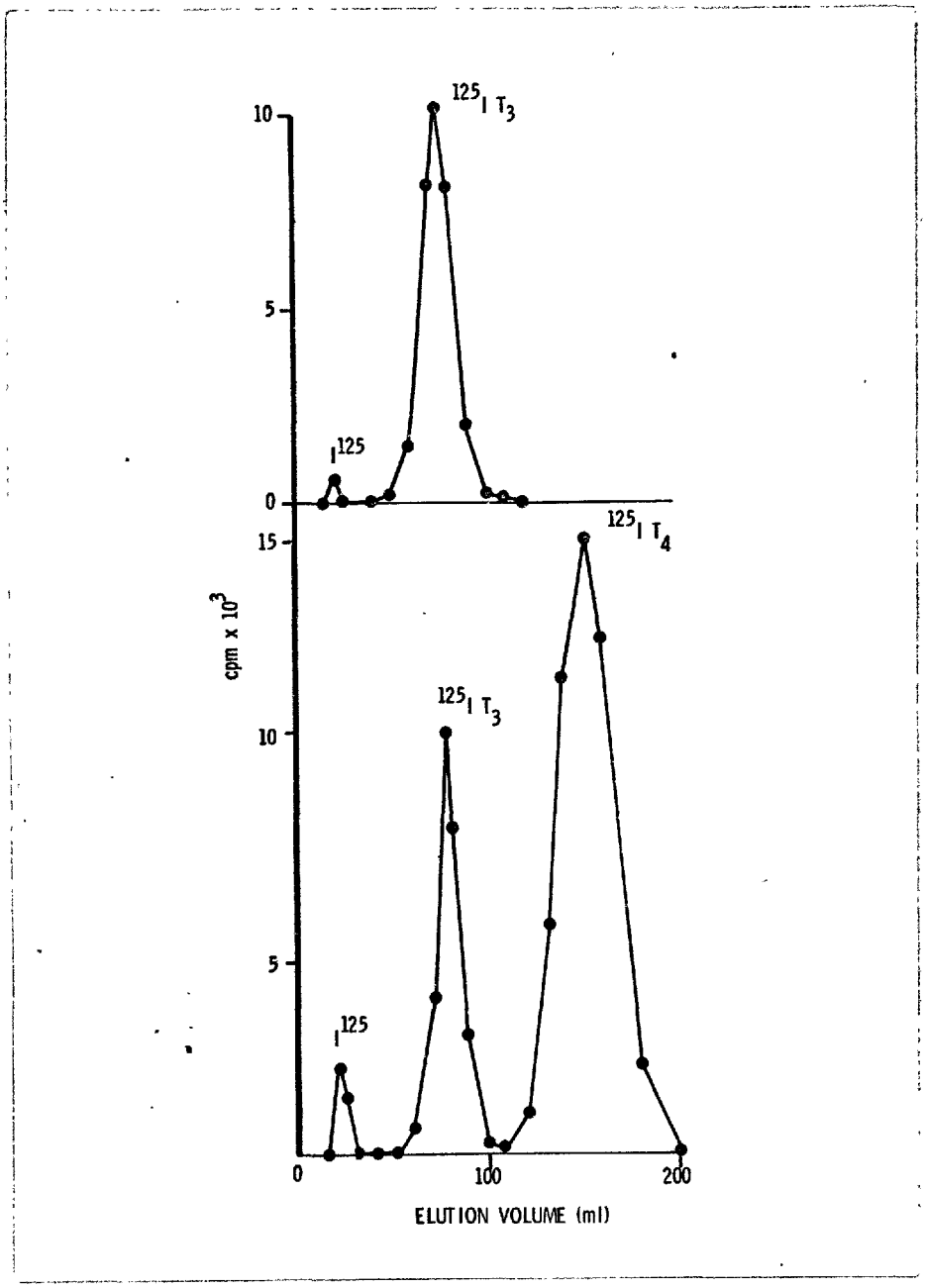

Fig. 2.15 Elution profiles of ^{125}I-T$_3$ (upper curve) and a mixture of ^{125}I-T$_3$ and ^{125}I-T$_4$ (lower curve) on a 15x1·2cm Sephadex G25F column; otherwise procedure as for Fig. 2.14.

1·5 to 1·2cm is shown in Fig. 2.15. The elution volumes were almost halved, the peaks much sharper and the separation just as good. The results of reducing the column diameter to 0·6cm are shown in Fig. 2.16. Although the elution volumes for $^{125}I\text{-}T_4$ and $^{125}I\text{-}T_3$ were further reduced, it was no longer possible to separate the hormones (Fig. 2.16a). When, however, the column length was increased to 22cm, the separation was complete (Fig. 2.16b).

(b) Effect of speed on elution:- The profiles of labelled T_3 and T_4 on 25 x 0·5cm Sephadex G-25 eluted at the rate of 40 ml/hr are shown in Fig. 2.17. When compared with Fig. 2.16b, where the elution speed was 10ml/hr, it is clear that the latter separation was qualitatively better. However, the former was satisfactory as a clear trough between the labelled hormones could be discerned.

(c) Effect of buffer pH:- The effect of using buffers of pH 7·5, 8·4, 9·2, 10·7 and 11·9 on the elution profiles of $^{125}I\text{-}T_3$ and $^{125}I\text{-}T_4$ are shown in Fig. 2.17 and Fig. 2.18. These show that the more alkaline the pH, the smaller the elution volume of the materials and the sharper the peaks. At pH below 10, the separation was poor and unacceptable. These considerations applied no matter the speed of elution since buffer at pH 7·5 gave a poorer profile at a lower speed of elution than pH 11·9 buffer at a high speed (Fig. 2.16 and Fig. 2.17).

(d) Effect of buffer molarity:- Buffer molarity varying from 0·01 - 05M had no effect on the quality of the separation.

(e) Elution profiles of the iodination mixture using ideal separation conditions:- These are shown in Fig. 2.19a (speed 10ml/hr) and Fig. 2.19b (speed 40ml/hr). Although the slow

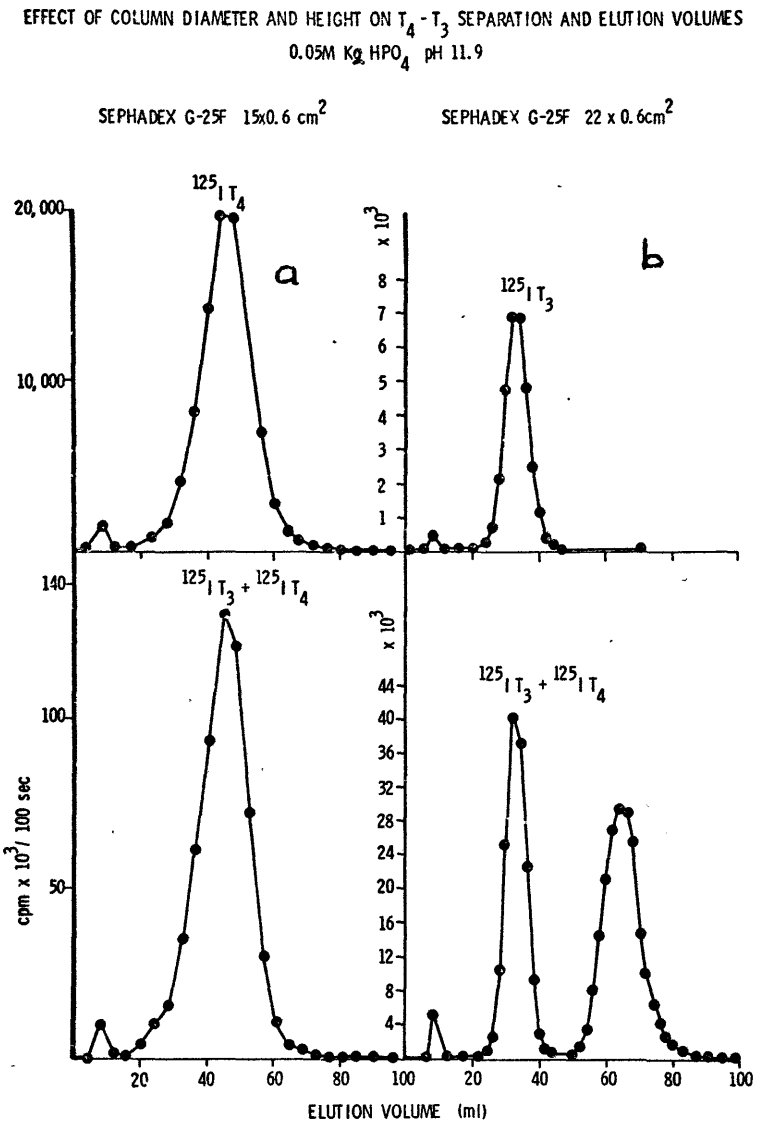

Fig. 2.16 Effect of column diameter and height on the separation of ^{125}I-T_4 and ^{125}I-T_3 in mixture. The upper curves represent either ^{125}I-T_4 or ^{125}I-T_3 alone as marked. Lower curves represent both tracers in a mixture. 2ml fractions were collected at room temperature. Speed of elution 10ml/hr under gravity. Sample application volume = 1ml.

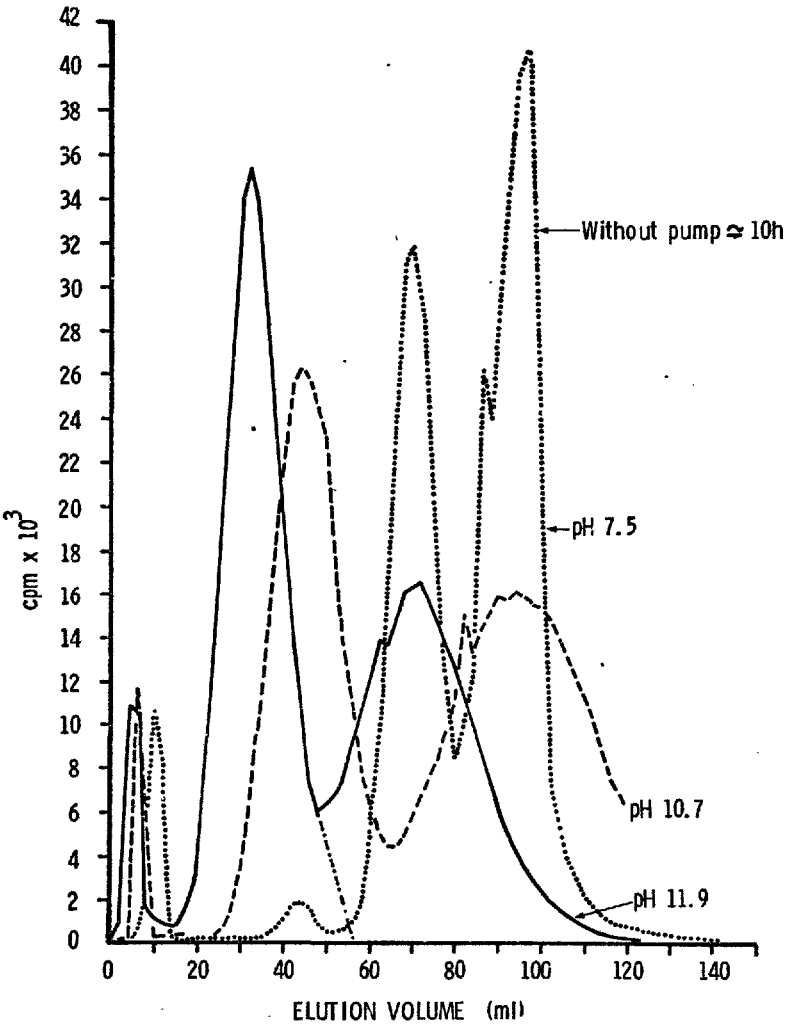

Fig. 2.17 Effect of speed of elution and buffer pH on the chromatographic of separation of ^{125}I-T_3 and ^{125}I-T_4 in a mixture. Elution with 0·05M K_2HPO_4 was carried out using a peristaltic pump at the rate of 40ml/hr, except for the profile marked. 2ml fractions were collected. 1ml of sample was applied to the 25 x 0·5cm Sephadex G25F column.

elution gave qualitatively better separation, the fast elution was satisfactory, and just as acceptable. Rechromatography of each peak gave one single peak suggesting that the separation at the peaks was complete.

2) Specific Activity Measurements:

The specific activities of the $^{125}I\text{-}T_3$ and $^{125}I\text{-}T_4$ were measured by a self displacement technique, that is by calculating the amount of labelled hormone required to cause a definite depression of a point on a standard curve, in this case the "zero" points. At the same time as a standard curve was set up in an assay, quadruplicates of the zero tubes were included. 100 times (for T_4) and 10 times (for T_3) of the tracers used in the standard curve were added to the special "zero" tubes. The difference in the percentage binding in the special "zero" tubes and the "zero" tubes of the standard curve was read off the curve in terms of picograms of T_3 or T_4 since the excess labelled hormone added must have caused the difference in binding by its hormone content. From this value, and the excess radioactivity counts added coupled with the efficiency of the counter, the specific activities of the labelled hormones were calculated.

In six consecutive iodinations using cold T_3 as substrate the specific activity achieved for $^{125}I\text{-}T_3$ ranged from 1000 - 1400 μCi/μg while that for $^{125}I\text{-}T_4$ ranged from 3000 - 4000μCi/μg. When cold T_2 was the substrate, the specific activities for $^{125}I\text{-}T_3$ and $^{125}I\text{-}T_4$ were in the range of 2500μCi/μg.

3) Stability and Storage Tests:

These were performed by rechromatographing the labelled hormones stored in various ways at various time intervals for up to 50 days.

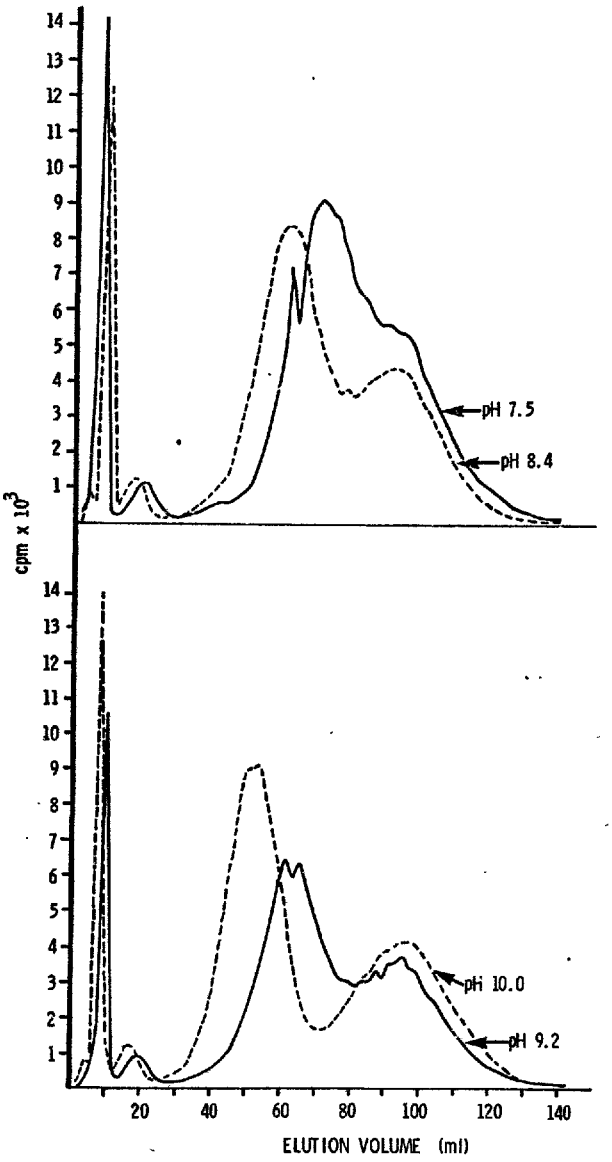

Fig. 2.18 Effect of buffer pH on the separation $^{125}I\text{-}T_3$ and $^{125}I\text{-}T_4$. Chromatographic conditions as for Fig. 2.17.

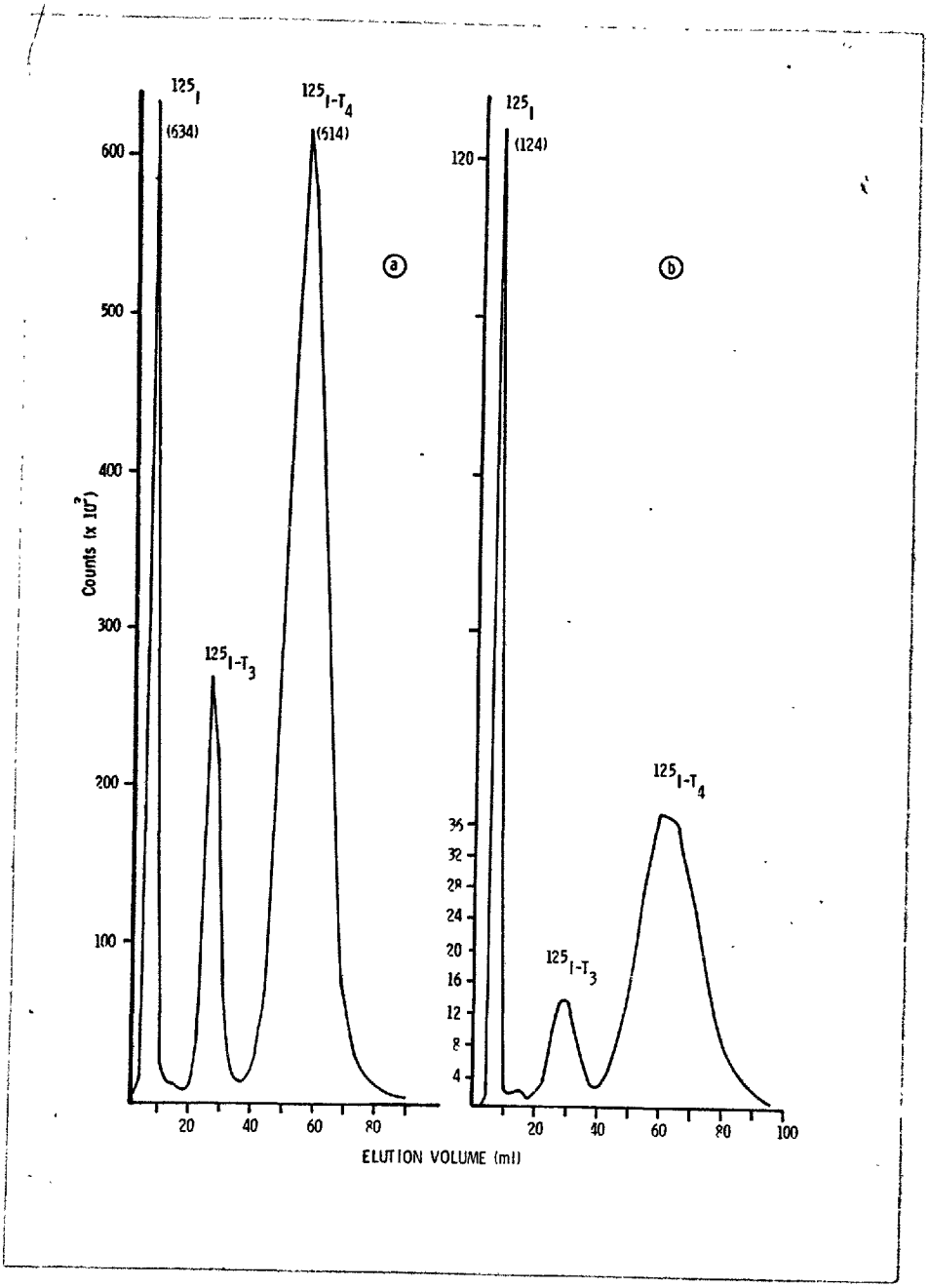

Fig. 2.19 Elution profiles of iodination mixtures (0·05ml) on 25 x 0·5cm Sephadex G25F columns. Elution buffer was 0·05M K_2HPO_4 pH 11·9. Elution was carried out at room temperature using (a) gravity (10ml/hr) and (b) a peristaltic pump (40ml/hr). 2ml fractions were collected. The starting substrate was T_3.

Effect of storage in buffer: When the tracers were stored in the alkaline eluate buffer (pH 11·9) only 10% of the $^{125}I-T_4$ and 50% of $^{125}I-T_3$ were intact after 7 days; the rest was eluted in the iodine zone of the columns. Storage in 0·05M phosphate buffer pH 7·4 improved the stability of the labelled hormones. For T_4 (Fig. 2.20), in 12 days 80% of the elution profile represented the labelled hormone. This diminished in a curvilinear fashion until in 50 days only 50% of the profile was labelled T_4. $^{125}I-T_3$ was considerably more stable at pH 7·4 than $^{125}I-T_4$ (Fig. 2.21). In 11 days 90% of the $^{125}I-T_3$ was intact, while in 40 days 50% of the radioactivity was still in the T_3 region. The $^{125}I-T_4$ breakdown curve was steeper than that of $^{131}I-T_3$.

Effect of storage in organic solvents: These included propylene glycol, methanol, ethanol, propanol and glycerol. Apart from glycerol, all were very effective stabilizing agents for the labelled hormones. Figs. 2.20 and 2.21 show the effect of adding 50% ethanol or 50% propylene glycol to labelled T_4 and T_3 respectively. The breakdown curves became much less steep than the buffer curve. On the whole 50% ethanol was slightly better as a storage medium for $^{125}I-T_4$ than 50% propylene glycol. For $^{125}I-T_3$ however, there was little to choose between them.

The effectiveness of the ethanol in various concentrations, namely 2%, 10% and 90% were also tried. Figures 2.21, 2.22 and 2.23 show that the higher the concentration of ethanol the more stable the labelled hormones.

The effect of 50% methanol or 50% propanol on the stability of $^{125}I-T_4$ is shown in Fig. 2.19. Both were very effective. Although the agents were added to the control tubes on the

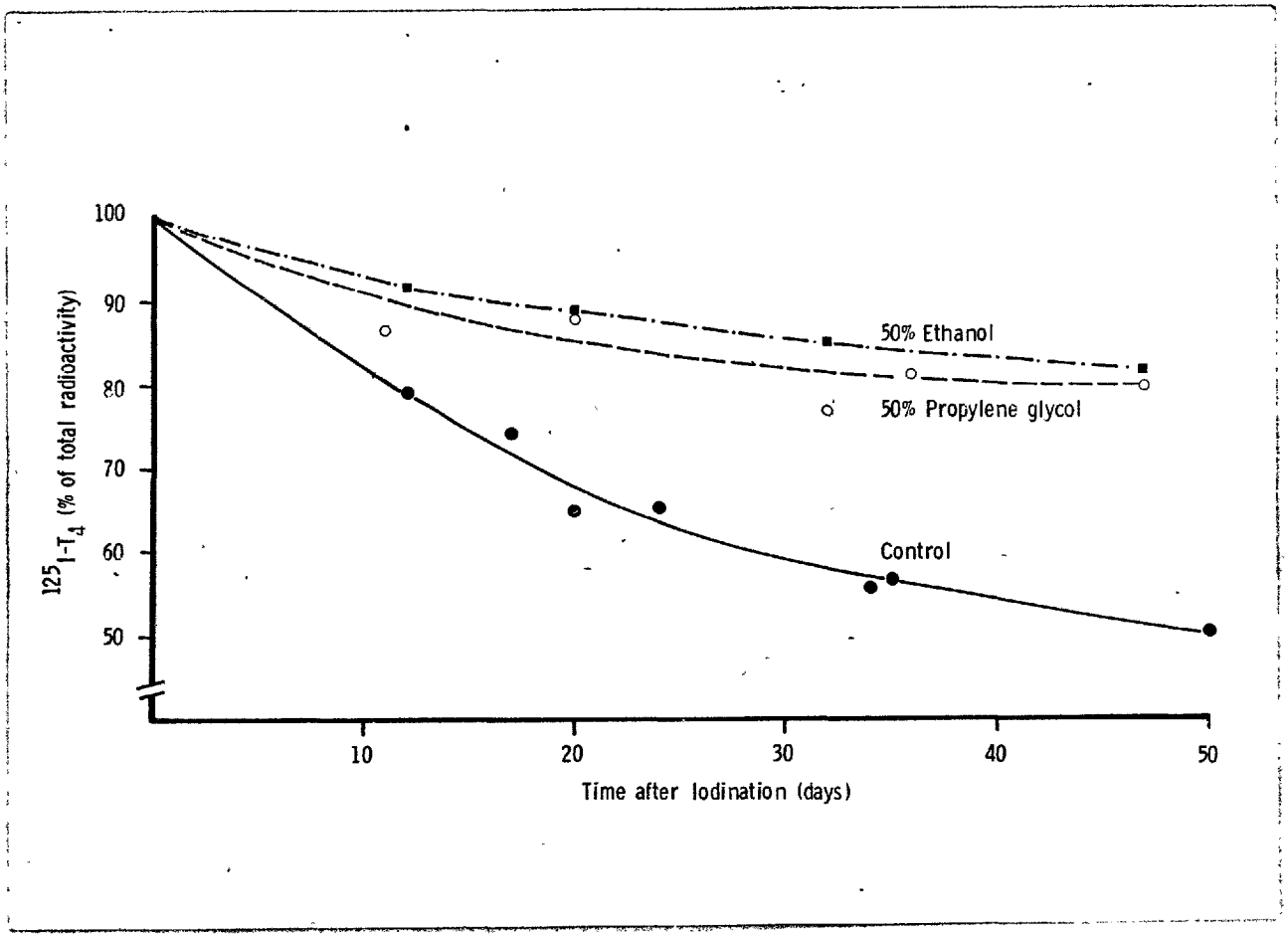

Fig. 2.20 Effect of storage of ^{125}I-T_4 in 0·05M phosphate buffer pH 7·4 (marked control) 50% propylene glycol and 50% ethanol on its stability.

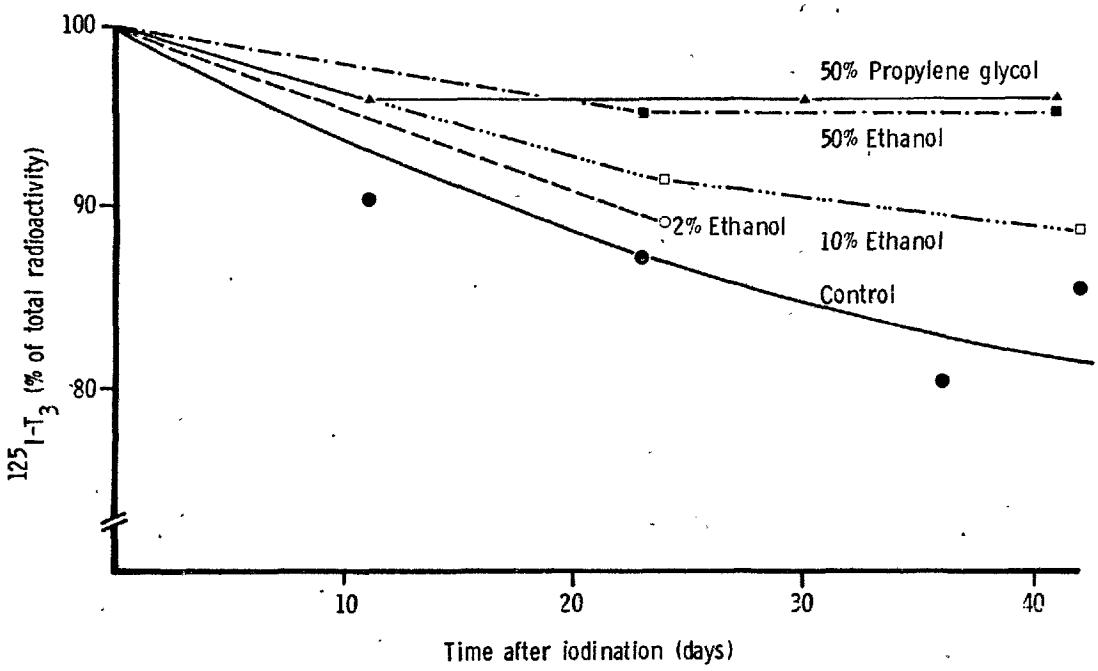

Fig. 2.21 Effect of storage of $^{125}I\text{-}T_3$ in buffer (pH 7·4) 2%, 10% and 50% ethanol and 50% propylene glycol on its stability.

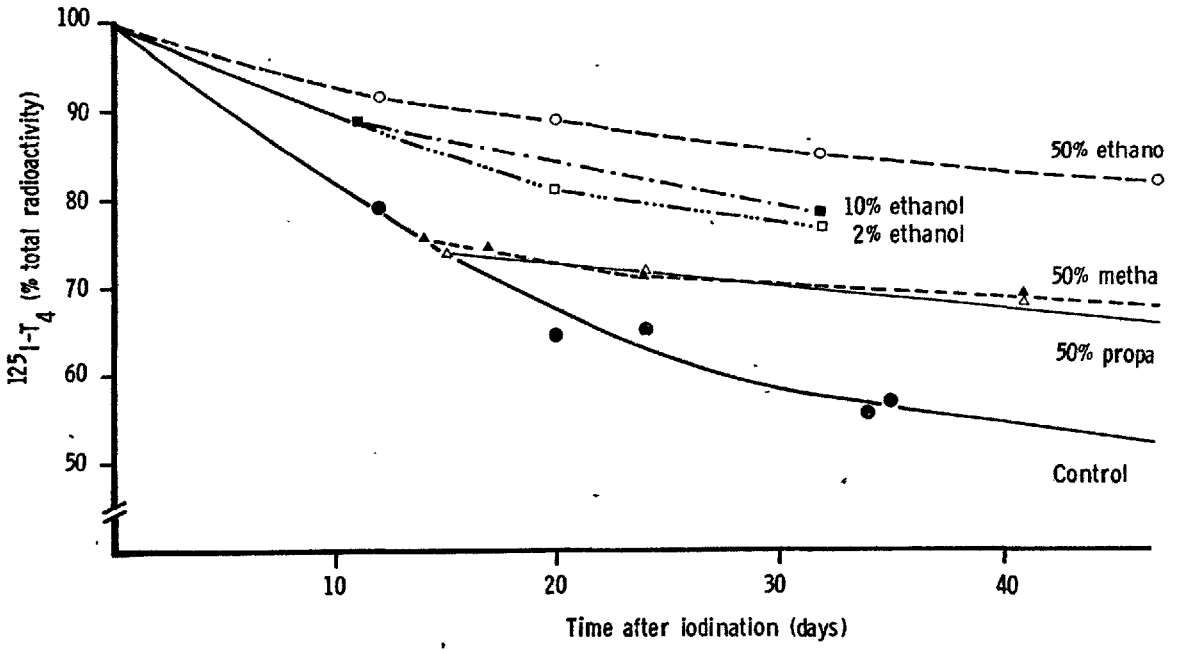

Fig. 2.22 Effect of storage of ^{125}I-T$_4$ in 2,10 and 50% ethanol (ethano), 50% methanol (metha) and 50% propanol (propa) on its stability. The methanol and propanol were added to control sample on the 14th and 15th day respectively after iodination.

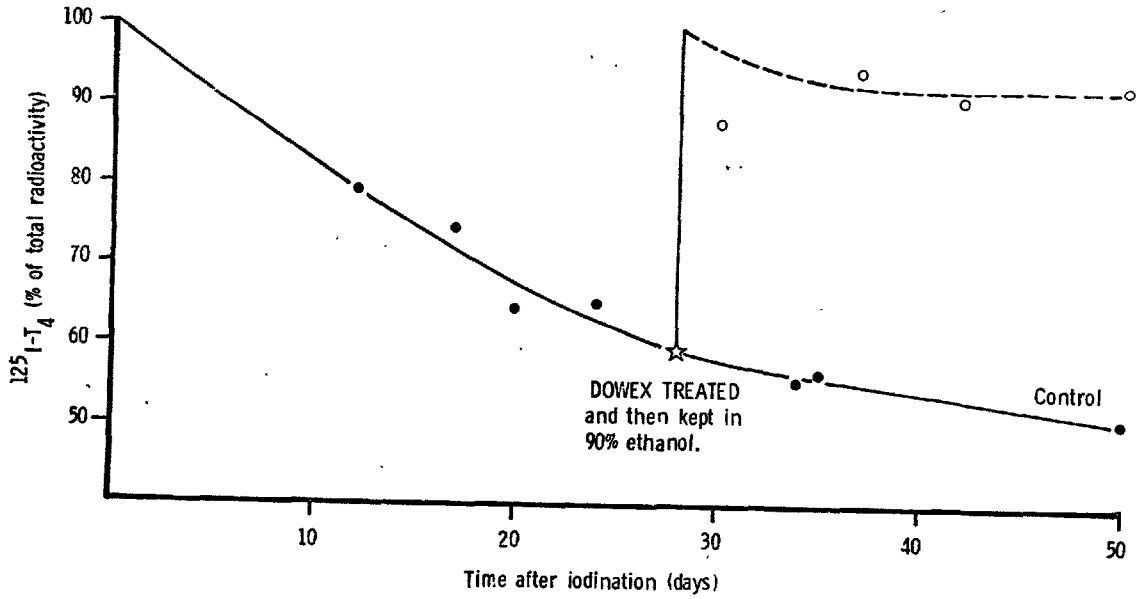

Fig. 2.23 Effect of storage of $^{125}I-T_4$ in 90% ethanol on its stability. On day 28 after the iodination the $^{125}I-T_4$ in buffer was purified using "Dowex-1" and then stored in 90% ethanol.

fourteenth and fifteenth day respectively after iodination, the breakdown rate of the tracer was markedly slowed down and the curves became parallel to the ethanol curves suggesting they were just as effective.

The above results apply to ^{125}I-T_3 and ^{125}I-T_4 produced with cold T_3 as starting material. When T_2 was the starting material, the resulting labelled hormones were very unstable in buffer as shown in Figs. 2.24 and 2.25 but ethanol and propylene glycol were still effective in slowing down the breakdown rate.

Effect of reducing agents on stability: Reducing agents such as sodium metabisulphite, arsenite and carbimazole did not have an effect on the rate of breakdown of the labelled compounds.

4) Iodination of other related materials:-

Starting with cold TRIAC in the iodination procedure, ^{125}I-TRIAC and ^{125}I-tetraiodothyroacetic acid (TETRAC) were produced in very high yields of about 90% (Fig. 2.26). ^{125}I-TRIAC had a slightly greater elution volume than ^{125}I-T_3; similarly ^{125}I-TETRAC was eluted after ^{125}I-T_4 in agreement with the findings of Blasi and de Masi (1967).

4. Discussion:

T_3 and T_4 adsorb to Sephadex with different affinities and therefore possess different partition coefficients on Sephadex. This property has been exploited as a separation technique for the hormones by various workers e.g. Blasi and de Masi (1967) and Horn et al (1972). This method has also been adopted in this study and has proved to be simple, rapid, reliable and efficient.

This study has demonstrated that in order to separate the hormones as sharp small volume peaks (thereby delivering the labelled hormones in concentrated form), a narrow column with

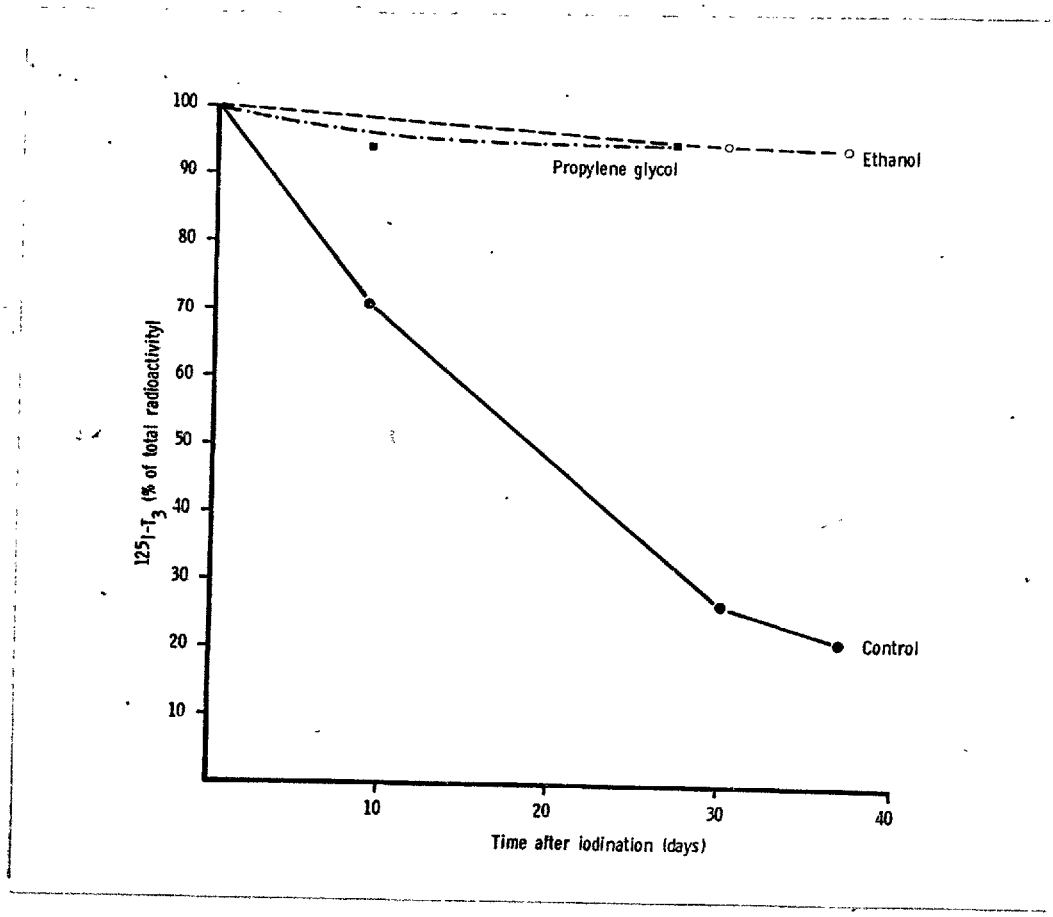

Fig. 2. 24 Effect of storage of ^{125}I-T_3 produced from T_2 in phosphate buffer (pH 7.4) and 50% ethanol and 50% propylene glycol on its stability.

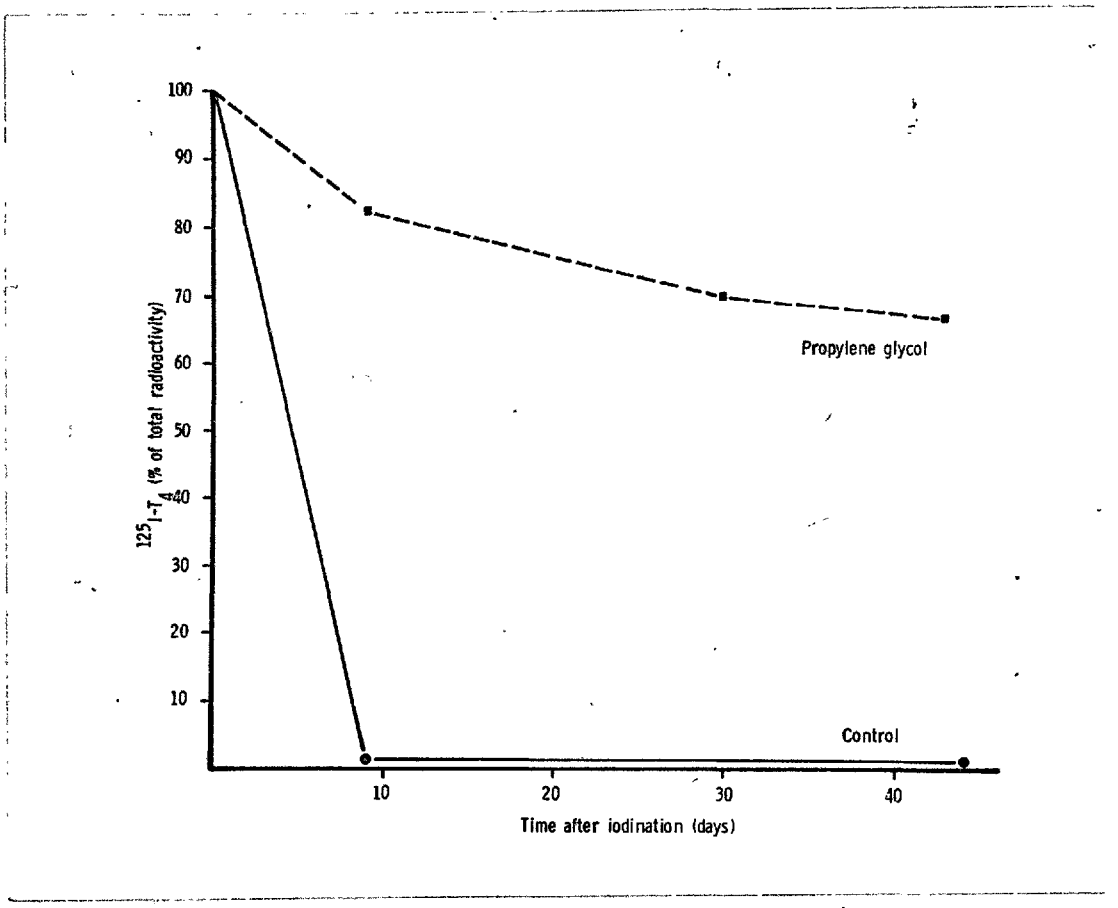

Fig. 2.25 Effect of storage of ^{125}I-T_4 produced from T_2 in phosphate buffer (pH 7·4) and 50% propylene glycol on its stability.

sufficient length and elution with high pH buffer at slow speeds are required. However, the last consideration proved unacceptable because as shown above, the labelled tracers were unstable alkaline solutions, and the earlier the process was finished, and the buffer neutralized, the better. Therefor the speed of 40ml/hr was chosen since it gave satisfactory separation of the peaks which showed no cross-contamination on rechromatography.

In the specific activity measurements we were handicapped by the low avidity of our antibodies when exposed to such high specific activity labels. For the T_4 antibody, it needed the addition of 100 times the amount of the ^{125}I-T_4 to produce a significant displacement of the binding. The T_3 antibody was better since a significant displacement occurred at 10:1 ratio of added labelled hormone. These problems underline the fact that development of highly sensitive assay requires not only a high specific activity tracer but also the production of a highly avid antibody.

The theoretical maximum specific activity for ^{125}I-T_3 is about 3000μCi/μg. This was approached when T_2 was used as starting material but only half of the predicted value was achieved when cold T_3 itself was used as starting material. This suggests that addition of iodine to the molecule takes place more efficiently than exchange of iodine. We infer that the production of ^{125}I-T_3 from cold T_3 was by exchange of labelled for unlabelled iodine since the amount of radioactivity appearing in the T_3 region of the iodination mixture profiles (10 - 20%)could not be accounted for by the amount of impurities e.g. T_2 in the T_3 used for iodination. The T_3 used was quoted by the manufacturers as being about 98% pure.

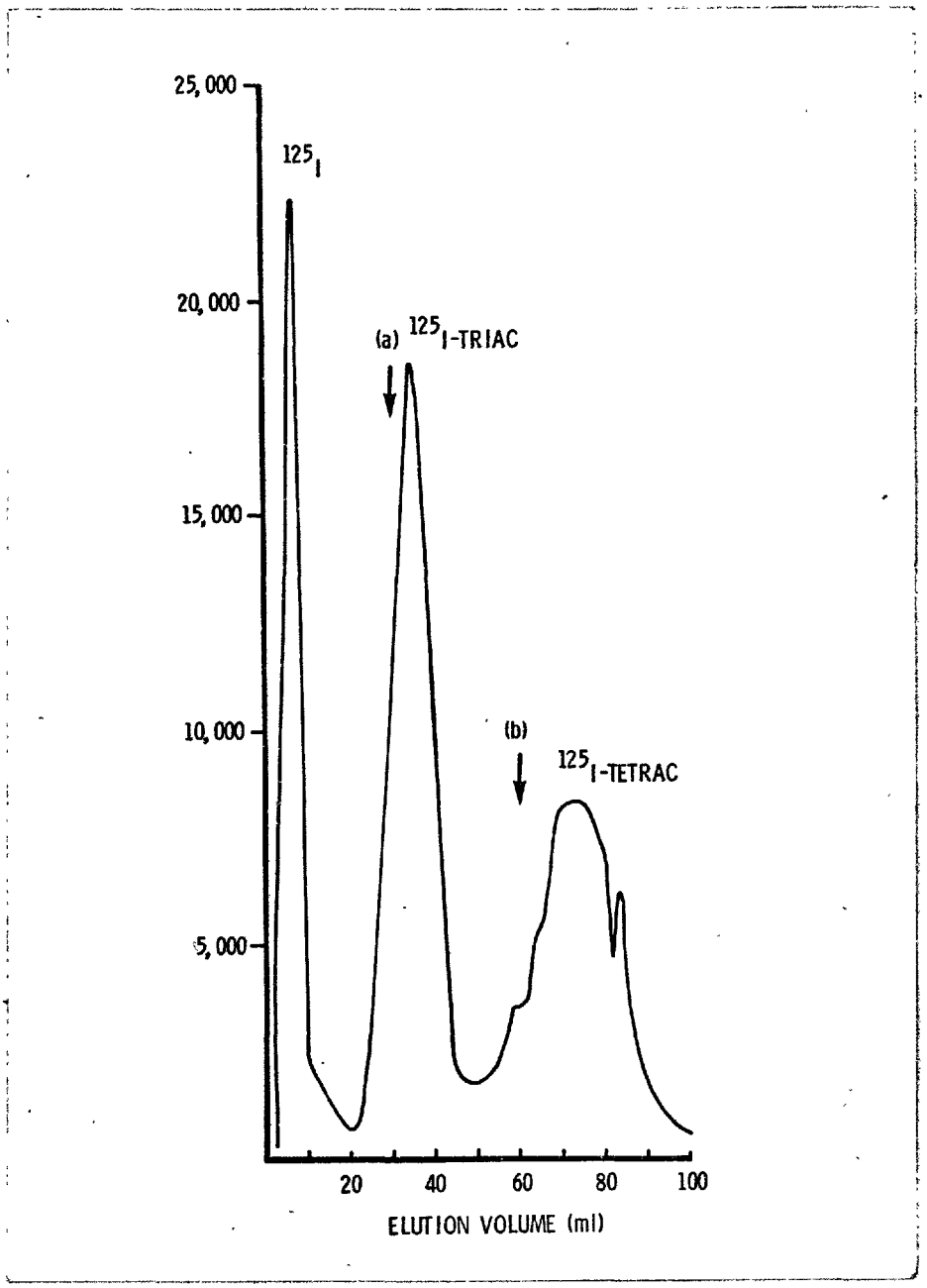

Fig. 2.26 Elution profile of an iodination mixture of ^{125}I, ^{125}I-TRIAC and ^{125}I-TETRAC. Column procedure as for Fig. 2.19. The starting material was cold TRIAC. The elution peaks of ^{125}I-T_3 and ^{125}I-T_4 used to calibrate the column are marked with arrows labelled (a) and (b) respectively.

The theoretical maximum specific activity for mono-labelled ^{125}I-T_4 is about 2500μCi/μg. Since our average specific activity was over 3000uCi/ug it would appear that we were producing some double-labelled T_4 in addition to the mono-labelled hormone starting from cold T_3. All the ^{125}I-T_4 produced from T_2 would, of course, be double-labelled.

Our results show that organic solvents have a profound effect on the stability of the labelled hormones. There was little to choose between ethanol, propylene glycol, methanol and propanol in their stabilizing properties, although ethanol appeared to have an edge. Our results also demonstrate that the higher the concentration of the ethanol, the more stable the compound. We now keep our materials in 90% ethanol.

The ^{125}I-T_3 produced from T_2 was more unstable than that produced from T_3. The reason for this is probably due to the higher specific activity of the former since Hoye (1969) has shown that the rate of decomposition of labelled T_4 increased with specific activity; a similar inference could be made for labelled T_3. In the same way, the greater breakdown rate of ^{125}I-T_4 produced from T_3 as opposed to ^{125}I-T_3 from the same source may be due to its much higher specific activity and perhaps to some degree of double-labelling.

We have also shown that ^{125}I-TRIAC and ^{125}I-TETRAC could be produced using the same system. This is particularly important for the study of T_3 and T_4 metabolism since TRIAC and TETRAC have been recognized as metabolites of T_3 and T_4 metabolism respectively (Braverman et al, 1970). ^{125}I-TETRAC is not commercially available while ^{125}I-TRIAC is commercially very expensive. This technique should therefore prove useful to investigations in this field.

In conclusion we have described a method for the simultaneous

synthesis of ^{125}I-T_3 and ^{125}I-T_4 in one simple, fast, cheap and efficient iodination procedure. With the high specific activities achieved, and an avid antibody, the sensitivity of the assays for T_3 and T_4 could be high enough to measure free hormones in blood and other biological fluids.

CHAPTER 3

IMMUNO-REACTIVE TSH IN HUMAN URINE

1. Introduction

In the assessment of pituitary and thyroid disorders, measurement of serum TSH by radioimmunoassay has now found wide application, but the limited sensitivity of the current immunoassay systems restricts their usefulness. As has been shown earlier, the level of serum TSH in normal subjects crosses the lower limits of sensitivity of the assay, so that the assay often can not distinguish normal subjects from abnormally low TSH levels which occur in hyperthyroid and hypopituitary subjects. This problem could perhaps be solved by concentrating and extracting the serum before assay. Since the amount of serum required for this would be large, it would prove impractical eventually. Urine has been chosen instead for the concentration and extraction process because it is more easily available in large quantities than serum. Measurement of hormones in a timed collection of urine also has the advantage of reflecting the mean serum level over the chosen lapse of time and would be more meaningful than a single measurement in serum.

However, measurement of hormones in urine also has its problems. Urine has a high and variable concentration of urea and salts that may interfere with the assays. When the concentration of the hormone is low, as is the case with TSH, a concentration and extraction step is usually required. There are also the problems of accurate and reliable collections. In renal failure, the measurement of hormones in urine is usually unreliable because of the accompanying nonspecific proteinuria.

Detection of TSH in urine has been claimed by various workers using bioassay methods. Hertz and Oastler (1936) found TSH activity in the urine of myxoedematous patients but not in that of normal or thyrotoxic subjects, while Rawson and Starr (1938) found the hormone in normal urine, also a higher concentration of it in urine from hypothyroid patients. Kriss et al (1954) confirmed these findings.

More recently Ishigami (1966) has reported that TSH activity is detectable in normal urine. However, to date, there has been no report of immunoreactive TSH in urine.

A method is now described for measuring immunoreactive TSH in urine, together with evidence of its specificity and the levels found in hyperthyroidism, hypothyroidism and in normal subjects. We have also carried out investigations on the characterization of the nature of the TSH in urine by Sephadex gel-filtration. These are also reported.

2. Materials and Methods

1) Concentration and Extraction Methods:-

a) Standard procedure for the collection, concentration and extraction of urine by dialysis and lyophilization:-

Overnight 12-hour or 24-hour specimens of urine were collected at room temperature, and either dialysed at once, or stored at -20°C immediately after volume measurement. Urine samples containing protein (assessed with "Albustix") were discarded after volume measurement. For dialysis, 50ml urine were placed in a "Visking" tube and 0.2ml 1% bovine serum albumin was added. Electrophoretically pure ^{131}I labelled TSH (about 1000 c.p.m.) was also added to every tube to check the recovery of the extraction process. Dialysis was carried out against ten volumes of distilled water at 4°C for 24 hours, the distilled water being changed three times during this period. The diffusate was then lyophilized, and finally dissolved in 1ml 0.05M phosphate buffer (pH 7.4) containing 0.05% bovine serum albumin. After 6 - 12 hours at 4°C, the concentrated urine was centrifuged ready for counting and radioimmunoassay. Assay results were corrected for recocery, calculated from the ratio of those counts recovered and the counts added. A "TSH-free" urine pool was obtained from two patients who had been implanted twice with ablative doses of yttrium-90 for diabetic retinopathy and

who satisfied the criteria for hypopituitarism as described later. The subjects were also on full thyroxine replacement therapy.

b) Other procedures investigated:-

i. Alcoholic precipitation:- This was based on the familiar method for LH and HCG in urine as described by Geiger (1971) since TSH is a similar glycoprotein. 70ml of 95% ethanol were added to 10mls of urine in "Quick-fit" tubes. After standing at 4^{o}C for an hour, the tubes were centrifuged at 1000 rpm for 15 minutes and the supernatant discarded. 10ml absolute ethanol were then added to the precipitates, the tubes were mixed and centrifuged again after 30 minutes standing at 4^{o}C. 10mls of diethyl ether were added to the precipitate which was again centrifuged, the ether discarded and the precipitate blown to dryness with nitrogen gas in a fume cupboard. The dry powder was then reconstituted in 1ml 0.05M phosphate buffer pH 7.4 containing 0.05% bovine serum albumin (BSA) to prevent adherence of the TSH to glass. It was kept overnight at 4^{o}C after which it was centrifuged and the supernatant either assayed at once or stored at -20^{o}C until assayed. Recovery of TSH was monitored by adding pure ^{131}I-TSH to each urine sample and the counts recovered after each procedure was checked.

Ultrafiltration:- Ultrafiltration was carried out under vacuum using "Satorius" cellulose nitrate membranes (Satorius, Gottingen, W. Germany) and ultrafiltration apparatus (Jenaer, Glas, Gottingen, W. Germany) set up as shown in Fig. 3.1. The particular membrane selected excluded substances of molecular weight greater than 5,000. The membrane was attached to a glass-tubing and connected to an air-tight, flat-bottomed flask containing some water as shown in the diagram. 10ml of urine were then introduced into the membrane through the glass-tube. This was just enough to fill it and more urine was applied later if necessary as the level of urine in the membrane fell.

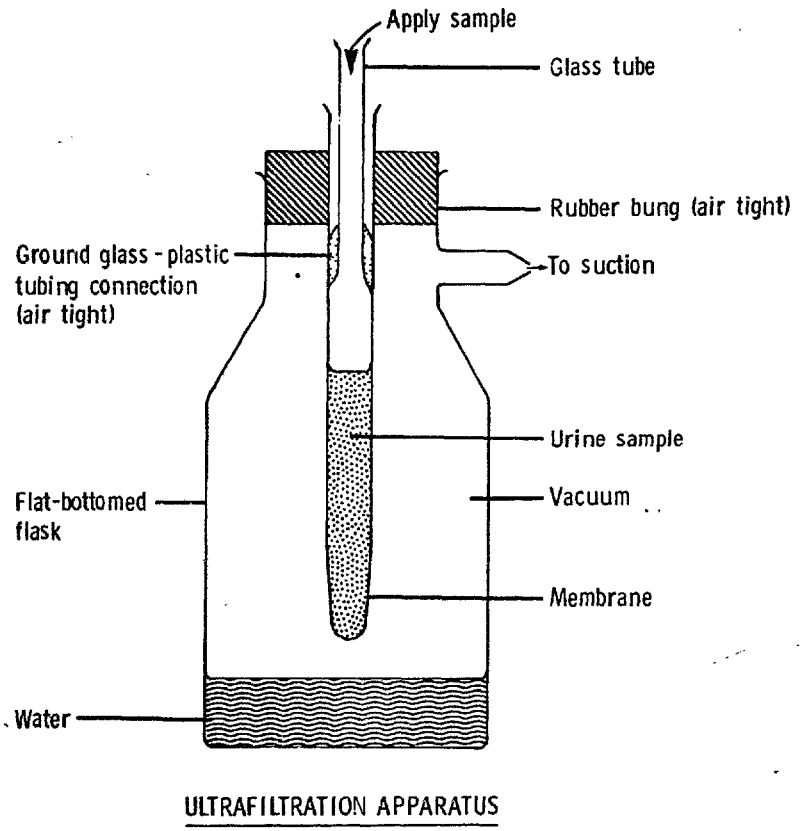

Fig. 3. 1 The ultrafiltration apparatus.

A vacuum was applied to the flat-bottomed flask using a water-tap suction and the process continued until 1ml was left in the membrane (the 1ml mark on the membrane having been predetermined before the experiment). 10mls of urine took 3 - 4 hours to concentrate to 1ml. 10 - 30 mls were concentrated per sample. Recovery of the hormone was checked as described above.

2) Radioimmunoassay procedures:-

Urinary TSH, LH and TSH were measured by radioimmunoassay using the double antibody method described for serum in the previous chapter. The only difference for TSH was that 0.2ml "TSH-free" urine concentrates were added to the standard tubes instead of "TSH-free" serum. The TSH results are expressed in μuMRC human pituitary standard A 63/14 per hour. The results for LH and FSH are expressed in muMRC human pituitary standard for LH and FSH 69/104 per hour, derived from LER-907 (Bangham et al, 1973).

3) Subjects:-

Normal subjects were chosen from members of the medical staff; 18 males aged 25 - 50 years and 12 females aged 22 - 63 years. None had evidence of endocrine or other disease. Samples were studied from 14 subjects with well defined clinical and biochemical evidence of primary hypothyroidism, 14 clinically and biochemically overt cases of hyperthyroidism and 5 patients in chronic renal failure with heavy proteinuria. The hypopituitary group consisted of acromegalics who had undergone pituitary apoplexy and diabetics who had pituitary implants of yttrium-90 for severe proliferative diabetic retinopathy. Not only

did this hypopituitary group have clinical and serum assay evidence of hypothyroidism, hypoadrenalism, hypogonadism, growth hormone deficiency and undetectable levels of serum TSH, but they did not respond to the administration of thyrotrophin releasing factor (TRF) on at least two occasions. Diurnal variation of urinary TSH excretion was assessed in two normal subjects by collecting separately split day and night 12 hr urine specimens for 5 consecutive days. Laboratory data on the hyperthyroid, hypopituitary and the hypothyroid patients are shown in Appendix 3.

4) Infusion of human pituitary TSH into two normal subjects:-

One ampoule of human pituitary standard preparation TSH (MRC 70/9;2U) was injected i.v. into two normal volunteers as follows: 500μu were given as a bolus and the remainder of the dose was then infused at a constant rate of 500μu/hr for 3 hrs in 50ml 0.9% NaCl solution. The subjects were not fasting and remained recumbent during and for 3 hrs after the infusion. Blood samples were taken 15 mins before the injection, during and after the infusion. Urine for TSH assay was collected for the previous 24 hr, during the infusion and then for periods of 3hrs, 6hrs and 12 hrs consecutively after the infusion. The urine was frozen and processed as described above. 150ml (0-3hr and 3-6hr), 250ml (6-12hr) and 400ml (12-24hr) of the urine sample were also concentrated and subjected to downward flow gel-filtration analysis.

Dilutions of the infused pituitary TSH preparations were parallel to the MRC 63/14 human TSH standard curve. Each ampoule gave a value of 2 units in our radioimmunoassay as opposed to 1 unit measured by bioassay by the MRC.

5) Injections of TRF into 4 hypothyroid subjects:-

Four primary hypothyroid subjects with high serum TSH levels were each injected i.v. with 500μg TRF. Venous blood was taken at 0 and 20 mins, the sera separated and 2ml samples subjected to Sephadex

gel-filtration analysis.

6) Gel-filtration studies:-

(a) Materials for analysis: Apart from the urine and serum samples described above, the following samples were also subjected to Sephadex gel-filtration analysis: (1) Human pituitary preparations (MRC 63/14 and MRC 70/9). (2) Sera with raised levels of TSH from 2 hypothyroid subjects. (3) Urine concentrates from 2 primary hypothyroid subjects.

(b) Gel-filtration analysis: Downward flow gel-filtration was performed using a 2 x 50cm column of Sephadex G-100 ("Pharmacea"). Samples (1 - 2ml) were applied to the column and elution was carried out at room temperature at a flow rate of 20ml/hr using 0.1m-borate buffer (pH 8.4); 2.4ml fractions were collected. The column was calibrated using the following substances of known molecular weight: blue Dextran (> 1000000); bovine serum albumin (70000); ovalbumin (45000); alpha chymotrypsin (22500); cytochrome C (12500). All the reagents were obtained from "British Drug Houses". ^{131}I labelled TSH, ^{131}I labelled human GH and ^{125}I labelled insulin were used as radioactive markers. The void volume (Vo) of the column was determined using blue Dextran while total volume (Vt) was determined by carrier free Na ^{131}I. A calibration curve is shown in Fig. 3.2.

3. Results.

1) Concentration methods:-

(i) Alcoholic precipitation: Results of a typical extraction and concentration procedure employing ethanol are shown in Table 3.1. The alcohol precipitated about 80% labelled TSH when the extract was counted as a whole (i.e: final supernatant and precipitate). However, only 70% of the activity appeared in the final supernatant after an overnight incubation. The final recovery ranged from 63 - 80% (mean 71.2%). Considering that the starting volume was 10mls, the concentration

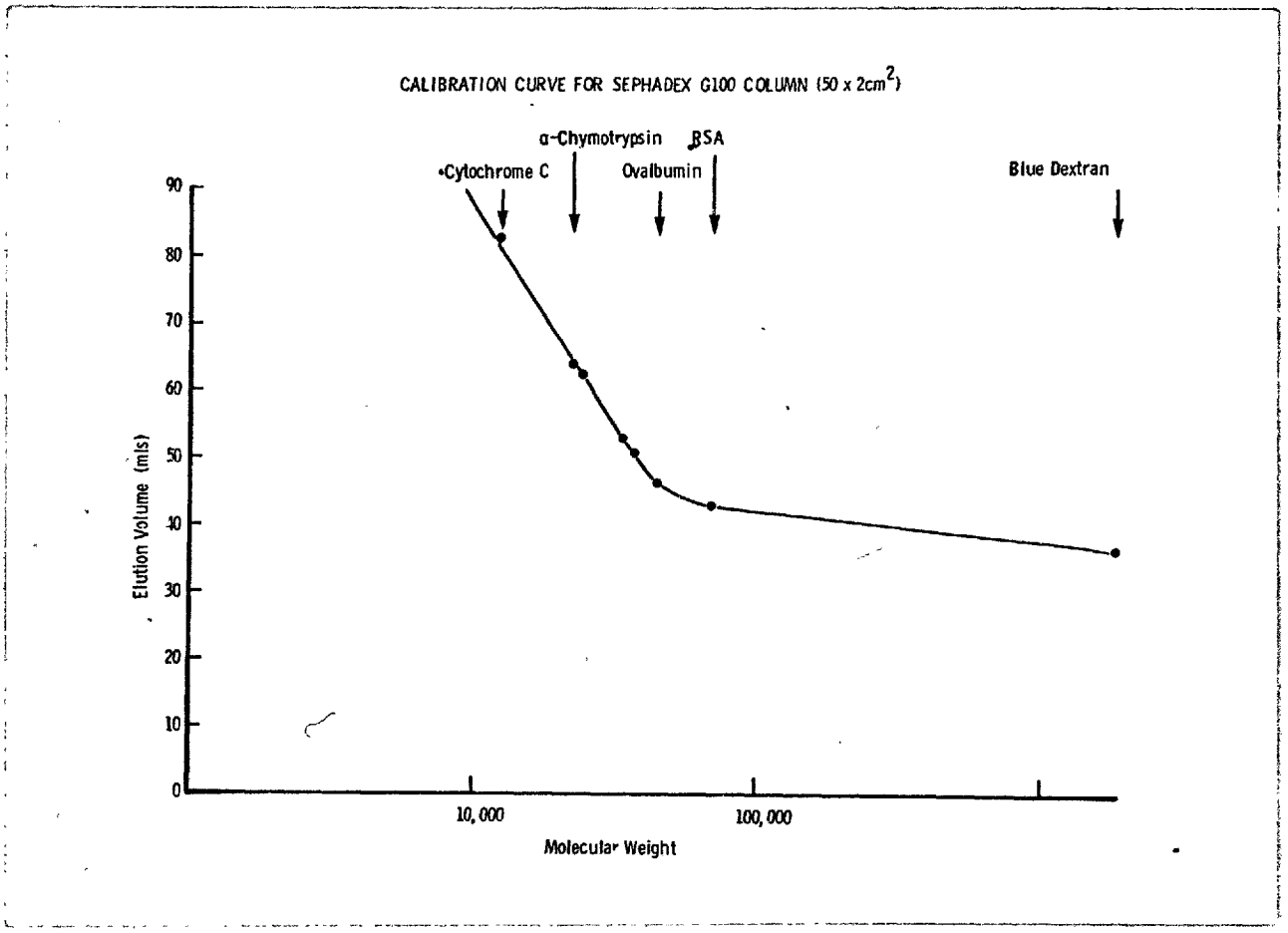

Fig. 3.2 Calibration curve for Sephadex G100 column (50x2cm).

No.	CPM in : 1st Al Wash	2nd Al Wash	Final Supernatant	Ppt	Total	Total % Recovery	Final % Recovery
1	72	66	3098	641	3739	86.6	71.7
2	64	58	3100	544	3644	84.4	71.8
3	77	65	3068	541	3609	83.6	71.1
4	70	72	3161	588	3749	86.8	73.2
5	66	62	3037	513	3540	82.0	70.3
6	-	-	3137	602	3739	86.6	72.7
7	69	70	2767	827	3604	83.5	64.1
8	-	-	2745	680	3425	79.3	63.6
9	64	73	3037	491	3528	81.7	70.4
10	-	-	2898	679	3577	82.8	67.1
11	67	69	3413	292	3705	85.8	79.0
12	-	-	3454	331	3785	87.7	80.0
					Mean:	84.2	71.2
					Range:	79.3-89.7	63.6-80.0

Table 3.1: Extraction and concentration of TSH in urine by alcoholic precipitation. 2ml aliquots of the alcohol were counted after each alcohol step; the counts were barely different from background (mean 60). The total radioactivity added to each tube averaged 4315 cpm. Each pair constitutes a duplicate of a urine sample. The variation of the recovery was seen to be more marked from urine to urine than in duplicates.

Al = Alcohol Ppt = Precipitate

achieved was therefore seven-fold. The recovery of added cold hormone was approximately equal to that of added ^{131}I-TSH (Table 3.2).

(ii) Ultrafiltration: The recovery of labelled TSH was quite variable, ranging from 25 - 90% (Table 3.3). The values also varied from membrane to membrane. Recovery of added cold hormone was similar to that of added hormone as shown in Table 3.2.

(iii) Dialysis and lyophilization: The result of an experiment using this method is shown in Table 3.4. The recovery of labelled TSH was high and constant (77-86%). The recovery of cold TSH was just as good as that of labelled TSH (Table 3.2.).

(iv) Comparison of the 3 methods: When samples processed by the 3 methods were subjected to radioimmunoassay, the values measured were similar whatever the method used, and all distinguished between high and low values (Table 3.5). Comparison of the advantages and disadvantages of the methods is shown in Table 3.6. The dialysis and lyophilization method proved superior to the other two procedures, and was therefore used for this study.

(v) Further tests of the dialysis and lyophilization method:

Recovery experiments: Table 3.7 shows that 76 - 82% of the ^{131}I labelled TSH added to urine before the concentration process was recovered, this is equivalent to 38-41 times the concentration of TSH in urine. When unlabelled TSH in the range of 5 - 100uu was added to unprocessed TSH-free urine, the corrected recovery ranged between 92-120% (Table 3.8). The counts obtained in the ^{131}I labelled TSH check system were used to correct these results. Storage at 4^{o}C for 48 hrs or at room temperature for 24 hrs did not alter the recovery of endogenous or exogenous TSH in urine. Storage at room temperature for 48 hrs however, led to about a 10% loss.

2) Tests of the influence of non-specific factors on the TSH immunoassay:

Bovine serum albumin added to the assay sample over the range of 1-5% did not modify the assay (Fig. 3.3). Similarly, sodium chloride did not

Method	Std. 2.5μu/ml	Std. 5μu/ml
Alcohol	24 (20)	36 (40)
Dialysis & Lyophilization	37 (42.5)	90 (85)
Ultrafiltration	30 (35)	60 (60)

Figures in parentheses represent expected value when corrected with the recovery of added ^{131}I-TSH.

Table 3.2 Comparison of results of known standards extracted by 3 methods.

Std = Standard

No.	Starting Volume (ml)	Final Volume (ml)	Concentration Achieved	% Recovery of ^{131}I-TSH	Concentration Achieved
1	16	0.6	x27	60	x16
2	22	0.7	x31	80	x25
3	16	0.5	x32	70	x22
4	30	0.9	x33	50	x18
5	30	0.9	x33	25	x 9
6	20	1.1	x18	66	x12
7	30	1	x30	25	x 8
8	25	0.9	x27	55	x15
9	20	1.1	x18	70	x13
10	13	0.4	x33	70	x23
11	13	0.5	x26	90	x23

Mean Recovery: 60.1%

Range : 25 - 90%

Table 3.3: Concentration and Extraction of TSH from urine using the ultrafiltration technique: the recovery of ^{131}I-TSH. This was variable. 4 membranes were used for the 11 extractions.

No.	CPM in : Visking Tubes	Precipitate	Supernatant	% Recovery	Concentration
1	56	160	1986	83	x41.5
2	57	138	2086	86	x43
3	67	148	1977	83	x41.5
4	65	206	1949	81	x40.5
5	65	105	2028	85	x42.5
6	50	137	1828	77	x37.5
7	54	112	1979	83	x41.5
			Mean :-	82.6	x41.3
			Range :-	77 - 86	37.5 - 43

Table 3.4: Recovery of ^{131}I-TSH added to urine in the concentration and extraction procedure (dialysis and lyophilization). 50ml of urine was the starting volume. The freeze-dried powder was reconstituted in 1ml of buffer. The counts remaining on the dialysis tubes were not significantly different from background counts (mean 49). The total radioactivity added averaged 2386.

Concentration Method	TSH detected (μu/ml of urine) after		
	Alcohol Precipitation	Ultrafiltration	Dialysis and Lyophilization
1	0.12	0.19	0.24
2	0.18	0.18	0.16
3	0.12	0.12	0.16
4*	0.64	0.55	0.54
5	ND	ND	ND

Table 3.5: Comparison of results of TSH measurements on urines extracted by 3 methods.

* Hypothyroid urine

ND = Undetectable (urine from a hypopituitary subject)

Method	Alcohol	Ultrafiltration	D & L
No. of Samples per week	50	15 - 30	21
^{131}I-TSH Recovery	Reasonably constant	Variable	Reasonably constant
Recovery by RIA	$\simeq$ ^{131} I-TSH	$\simeq$ ^{131} I-TSH	$\simeq$ ^{131}I-TSH
Concentration Achieved	x 7 - 8	x 9 - 30	x 30 - 45
Expense	+++	++	+
Principal Setbacks	Large vols. alcohol and Centrifuge space required	TSH adheres to membrane unpredict-ably	Lypoholizer space required

Table 3.6: Comparison of 3 methods of extraction and concentration of urinary TSH

D & L = Dialysis and lyophilization

RIA = Radioimmunoassay

TABLE 3.7

Recovery of ^{131}I labelled thyroid-stimulating hormone (TSH) added to unprocessed human urine before its concentration by dialysis and lyophilization. Each row gives the mean of one run of tests. The concentration achieved was calculated by multiplying the % recovery of ^{131}I labelled TSH by 50 which is the starting urine volume.

Expt.	No. of Samples	^{131}I Labelled TSH (mean c.p.m.)		Calculated	
		Counts added to 50ml	Counts recovered in final 1ml	% Recovery (Mean of range)	Concentration achieved
1.	10	919	702	76 (72 - 82)	x38.0
2.	14	760	619	82 (72 - 88)	x41.0
3.	14	800	645	81 (77 - 88)	x40.5
4.	14	571	463	81 (71 - 86)	x40.5
5.	7	331	272	82 (71 - 90)	x41.0
6.	7	380	309	81 (70 - 86)	x40.5

TABLE 3.8

Recovery of thyrotrophic hormone (TSH) added to human TSH-free urine before concentration by dialysis and lyophilization procedure and using ^{131}I labelled TSH for correction

TSH added (μu to 50ml sample)	TSH measured in 1 ml concentrate (μu/sample)		Corrected Recovery %
	Uncorrected	Corrected by ^{131}I labelled TSH	
0	<1.0	<1.0	-
5	3.5	3.8	92
10	10.8	9.0	120
25	23.5	20.0	118
50	37.0	42.5	87
60	50.0	45.0	110
100	90.0	82.5	110

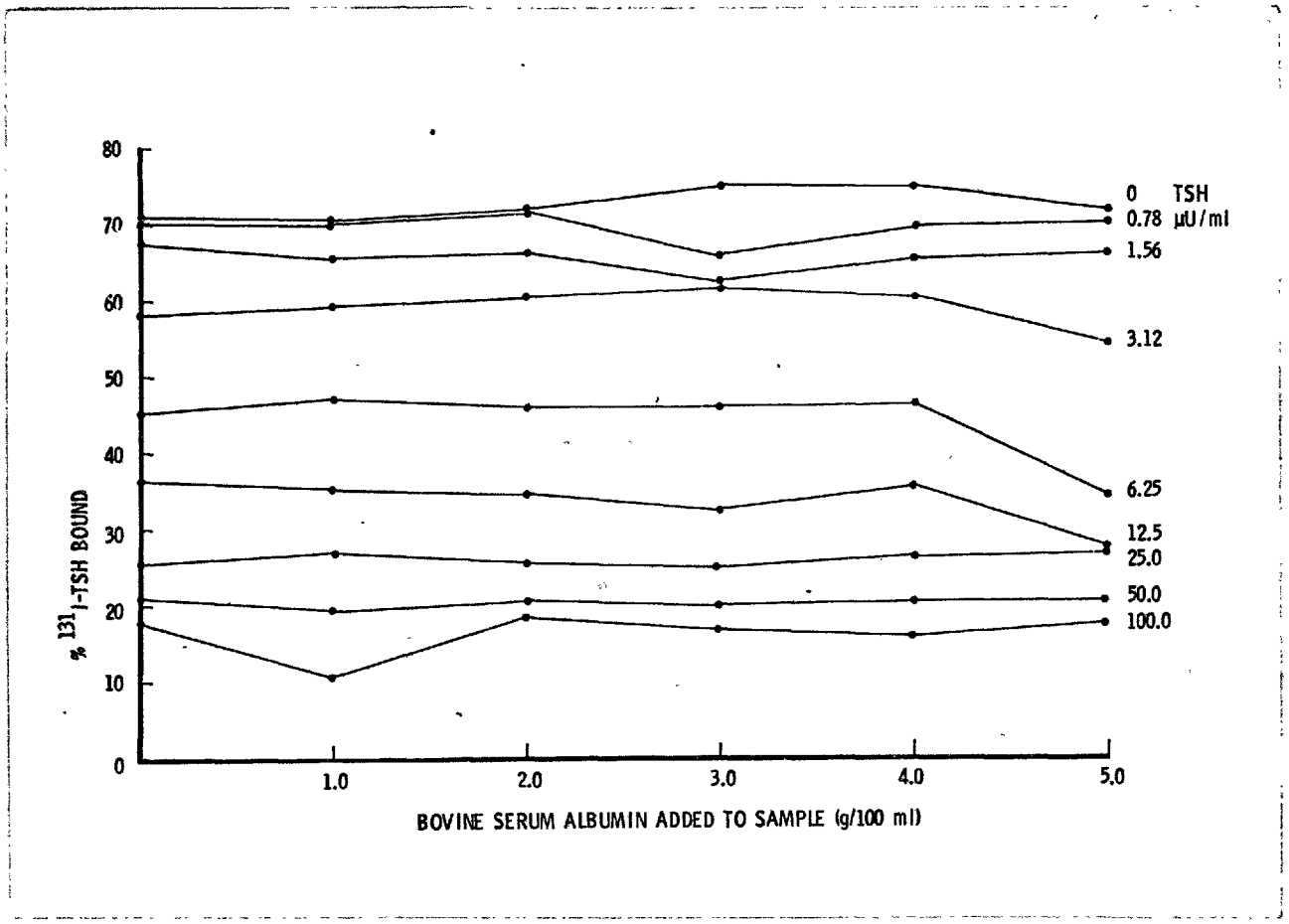

Fig. 3.3 Effect of varying concentration of bovine serum albumin (BSA) in the immunoassay tube on the percentage of ^{131}I-TSH bound in the presence of varying amounts of standard TSH.

modify the assay at concentrations over the range 0.2 - 0.6mol/l, (Fig 3.4) nor did urea over the range 0.5 - 1mol/l (urine being unlikely to exceed 0.4mol/l) (Fig 3.5), although above that concentration the percentage binding of ^{131}I labelled TSH was increasingly depressed. The average molarity of the processed urines was 550mOsmol/l (430-710mOsmol/l), and so molarity was unlikely to effect the assay. We also tested if other constituents of urine would modify the binding. Fig 3.6 compares a curve from standards in buffer with curves from standards in dialysed "TSH-free" urine and from standards in concentrates from "TSH-free" urine. Clearly, both the "TSH-free" urine and more so its concentrate suppress the binding of labelled TSH in the assay. Therefore, for all assays of urinary TSH, the standards were made up in concentrated "TSH-free" urine. Thus this specific radioimmunoassay procedure revealed a factor in urine concentrates which competes with labelled TSH for binding with anti-TSH anitbody. Dilution experiments with urine from three hypothyroid patients revealed a proportionality corresponding to the standard curves, Fig 3.7, suggesting that the addition of "TSH-free" concentrate in the construction of the standard curve had adequately compensated for the residual non-specific inhibition from urine concentrates.

3) Comparison of the rate of urinary TSH excretion with that of LH and FSH including tests for cross-reaction:

Cross-reaction between the hormones TSH, and LH and FSH in our system has been investigated by assaying urine concentrated in this same way not only by our radioimmunoassay for TSH, but also by that for LH and FSH. Table 3.9 shows that normal post-menopausal women, having large amounts of LH and FSH in urine had TSH values in the same range as normal men. Table 3.9 also shows that there is no relationship between the urinary level of FSH or LH and TSH whether in normal, hypothyroid, hypopituitary or hyperthyroid subjects. The excretion rate of TSH in urine whether in units, nanograms or moles, is much less

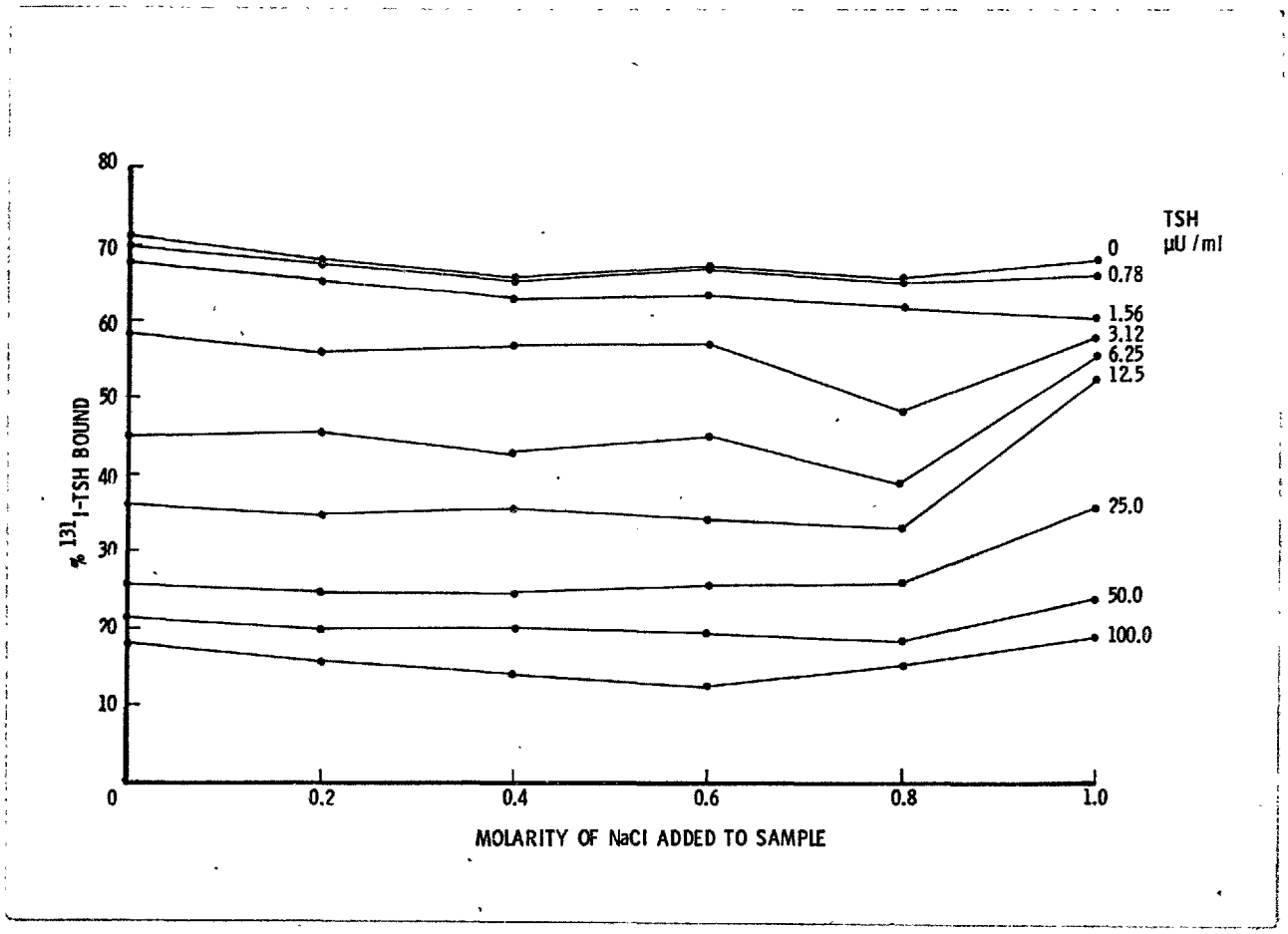

Fig. 3.4 Effect of varying molarity of sodium chloride on the percentage of ^{131}I-TSH bound in the presence of varying amounts of standard TSH.

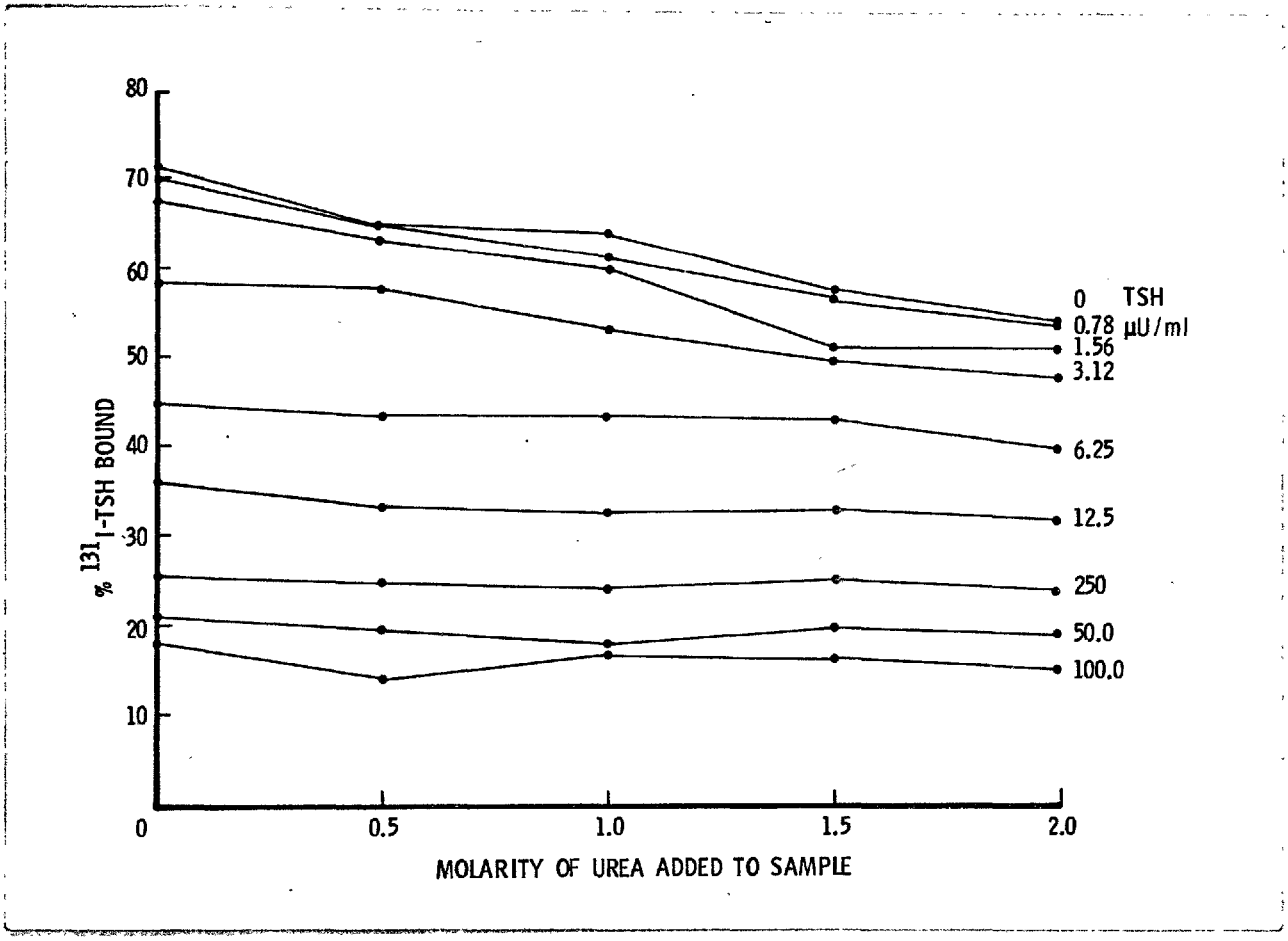

Fig. 3.5 Effect of varying molarity of urea on the percentage of ^{131}I-TSH bound in the presence of varying amounts of standard TSH.

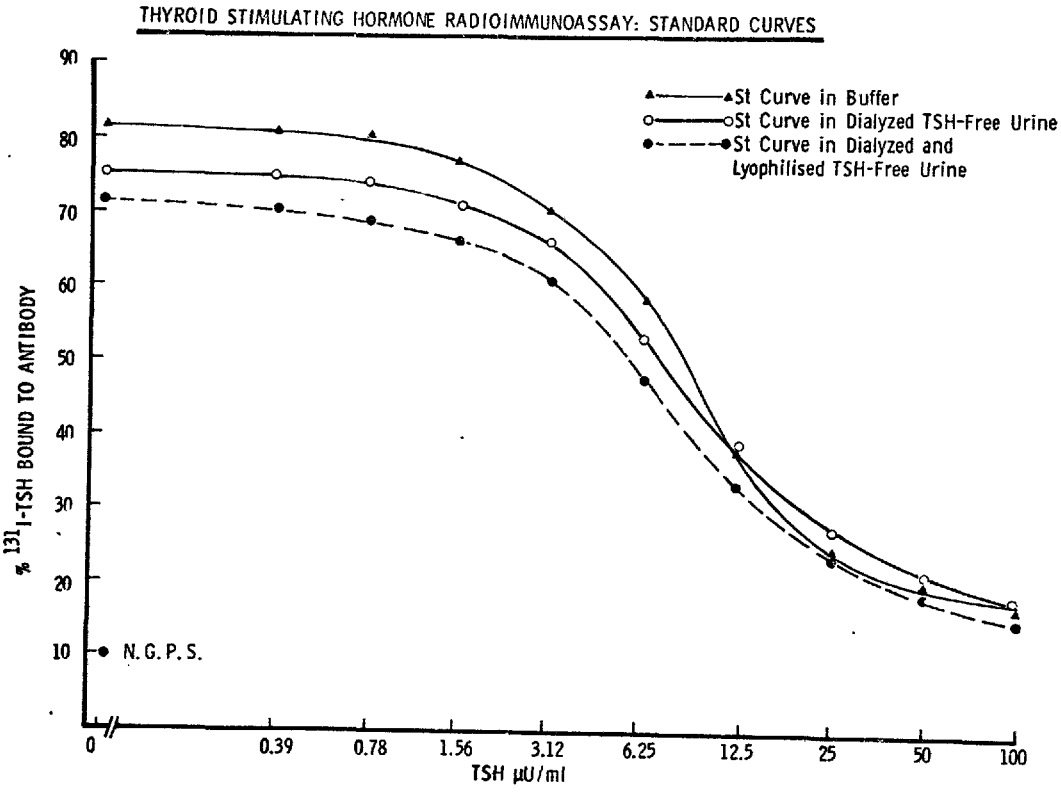

Fig. 3.6 Effect of dialysed only TSH-free urine and TSH-free urine processed in the same way as urinary samples (dialysed and lyophilized) on the TSH standard curve. N.G.P.S.= Normal Guinea-Pig Serum. St. = Standard.

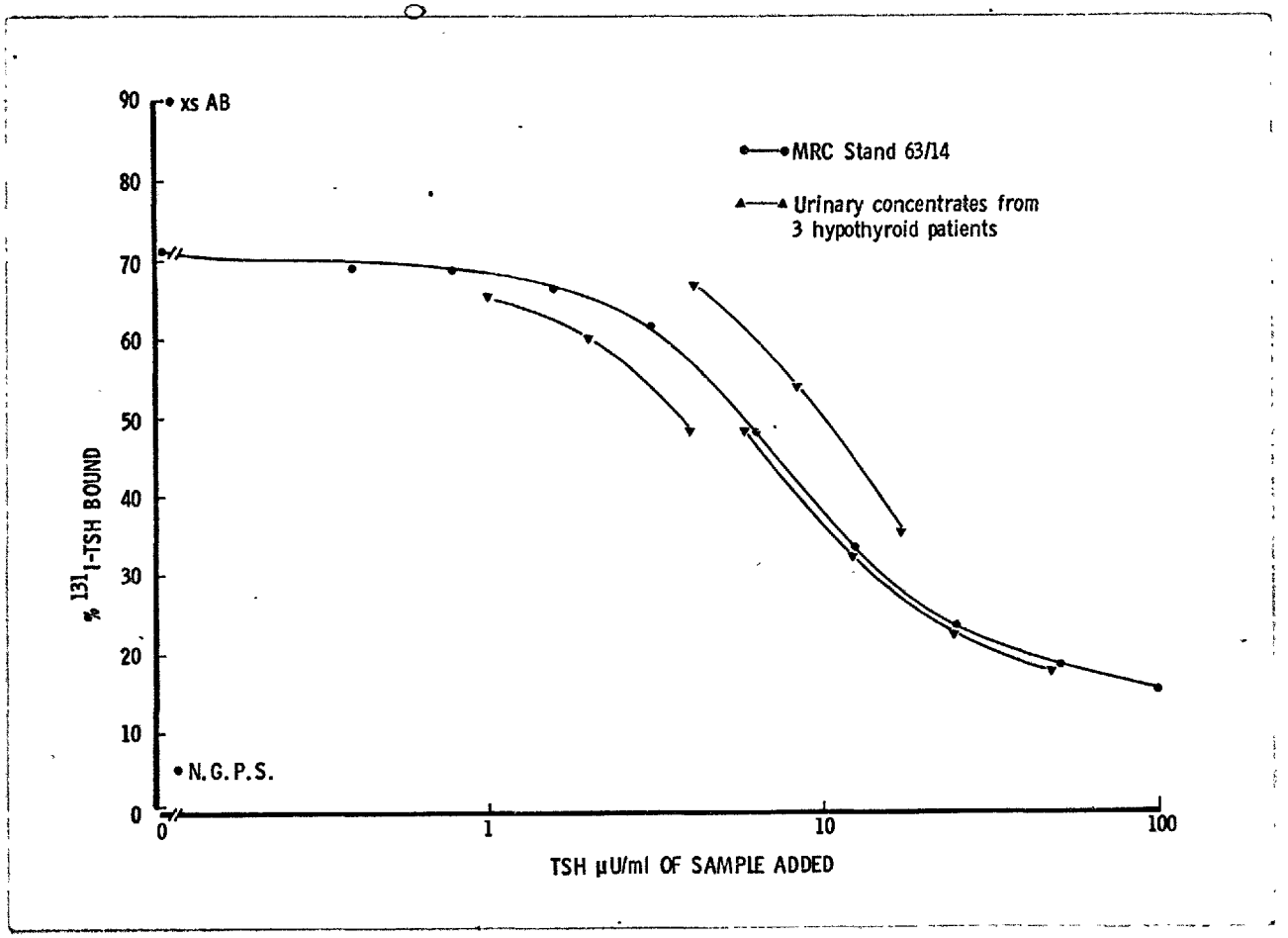

Fig. 3.7. Dilution curves of urinary concentrates from three patients with primary hypothyroidism, showing parallelism to the standard curve. N.G.P.S. = Normal Guinea Pig Serum. XS Ab = Excess antibody.

TABLE 3.9

				TSH/hr			FSH/hr			LH/hr		
Case	Age	Sex	Diagnosis	μu	ng	10^{-15} mol	mu	ng	10^{-15} mol	mu	ng	10^{-15} mol
1	36	M	Normal	3.5	0.17	6.5	185	14.8	493	260	17.4	290
2	29	M	Normal	7.3	0.46	13.5	153	12.2	510	359	21.5	767
3	81	F	Normal P.M.*	7.4	0.37	13.7	1294	64.7	2156	895	53.7	1917
4	62	F	Normal P.M.*	7.2	0.36	13.3	1832	146.6	4886	568	34.1	1218
5	53	F	Normal P.M.*	3.6	0.18	6.6	2118	169.4	5646	604	36.2	1293
6	75	F	Hyperthyroid P.M.*	1.0	-	-	736	58.9	1963	533	32.0	1143
7	44	M	Hyperthyroid	2.7	0.13	5.0	97	7.8	260	129	7.7	275
8	70	F	Hypothyroid P.M.*	16.5	0.82	30.6	1977	158.2	5273	810	48.6	1736
9	22	F	Hypothyroid	45.2	2.26	83.7	176	14.0	466	530	31.8	1135
10	39	F	Hypopituitary	2.8	0.14	5.2	92	7.4	246	57	3.4	121
11	36	M	Hypopituitary	1.5	0.07	2.7	10	0.8	27	5.5	0.3	11.8

Comparison of excretion rates of urinary thyrotrophic hormone, follicle stimulating hormone and luteinizing hormone in various endocrine states chosen to assess any cross-over between the various radioimmunoassays.

The units were converted to nanograms from the highest specific activity of the hormone reported in the literature, (i.e: LH : 17000 i.u./mg, FSH : 12000 i.u./mg, TSH : 20 i.u./mg; Bangham et al, 1972 Wide et al 1973) from which 1 μu TSH = 0.05ng; 1 mu FSH = 0.08ng and 1 mu LH = 0.06ng. Molecular weights:- TSH = 27000, LH = 28000, FSH = 30000 (Pierce, 1971).

M = Male. F = Female. * P.M. = Post Menopausal.

than that of LH or FSH (Table 3.9).

4) Correlation between serum and urinary TSH levels:

The level of urinary TSH correlated well with the clinical status of the patients and also with the serum TSH levels (Fig. 3.8). In one patient the serum and urinary TSH levels were followed monthly for 3 months while on thyroid replacement therapy and both fell in parallel (Fig. 3.9). When another hypothyroid subject was treated with large doses of L-T_3, the rapid fall in serum TSH was also mirrored in the urine (Fig. 3.10). Further evidence for correlation of serum with urinary TSH is shown in Fig. 3.11. This shows the serum and urinary levels achieved during and after the infusion of pituitary TSH in two normal subjects. The highest amount of TSH was obtained during the infusion and fell progressively over the next 24 hours. The overall 24-hr recovery of TSH in urine was 0.2% of the injected dose.

5) Urinary TSH levels in normal subjects and patients with pituitary and thyroid disorders:

The urinary TSH values in normal, hypothyroid, hyperthyroid and hypopituitary subjects are shown in Fig. 3.12. The mean normal level ($\pm$ S.E.M.) was 5.6$\pm$ 0.31μu/hr (range 3.5 - 9.8μu/hr). The mean level (5.4$\pm$ 0.35μu/hr) in normal men was not significantly different from that in normal women(5.9$\pm$ 0.48μu/hr; $P > 0.4$). The hypothyroid subjects showed a mean TSH level of 25.1$\pm$3.3μu/hr (range 10.8 - 46.5μu/hr) i.e. all TSH concentrations were higher than the normal subjects ($P < 0.001$). The values for the hyperthyroid subjects (2.6$\pm$0.2μu/hr, range <1-3.5) were lower than those of normal subjects ($P < 0.001$). The hypopituitary subjects had a mean of 2.5$\pm$0.22μu/hr (range <1 - 3.3) which was in the same range as the hyperthyroid subjects. Values below 2.0μu/hr were below the sensitivity of the assay. The mean day-time excretion of urinary TSH in two subjects was 6.0 and 6.6μu/hr and the nigh-time 6.6 and 4.6μu/hr respectively. These values showed no evidence of

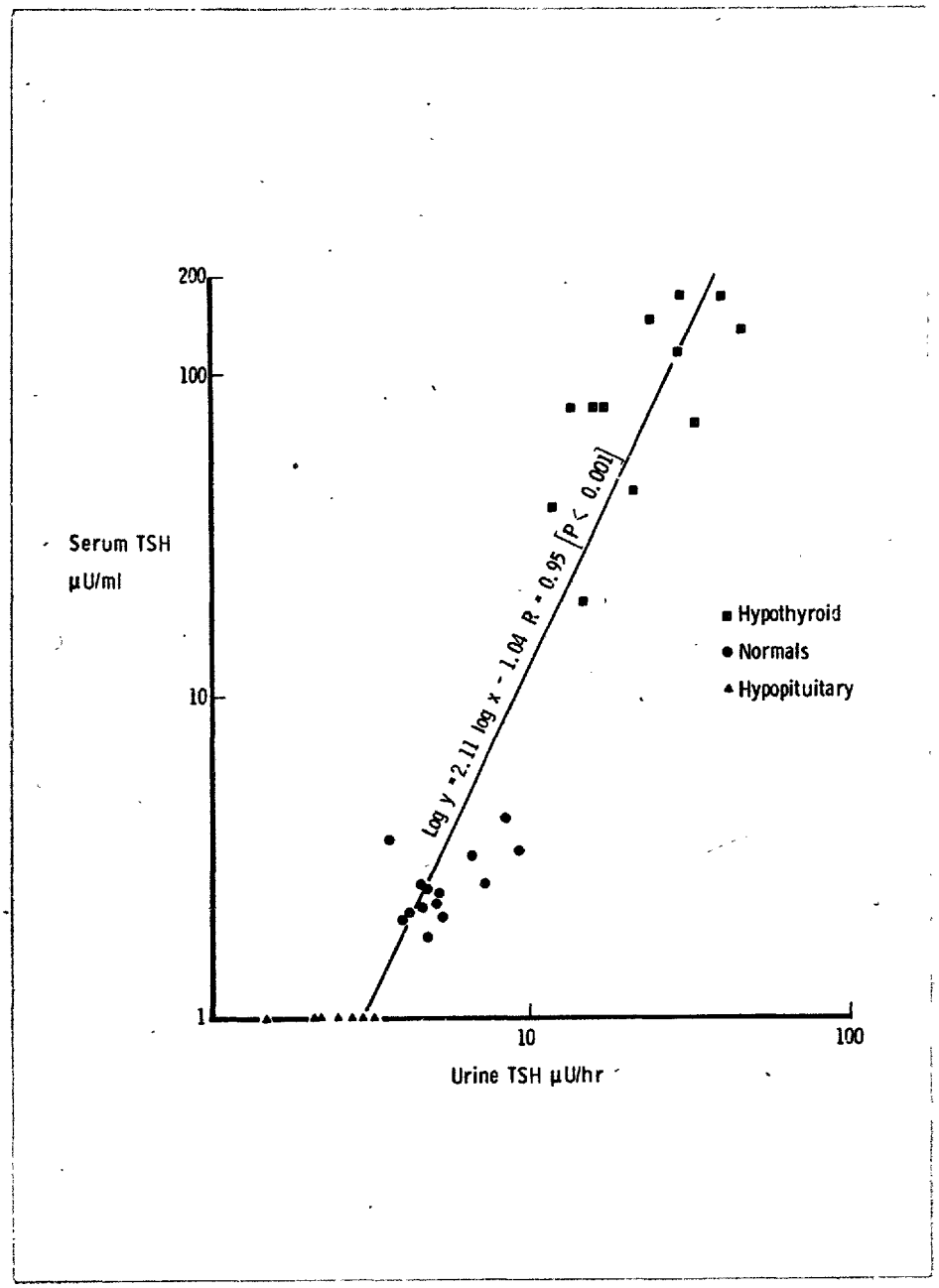

Fig. 3.8 Correlation between serum and urinary TSH levels in normal hypothyroid and hypopituitary subjects.

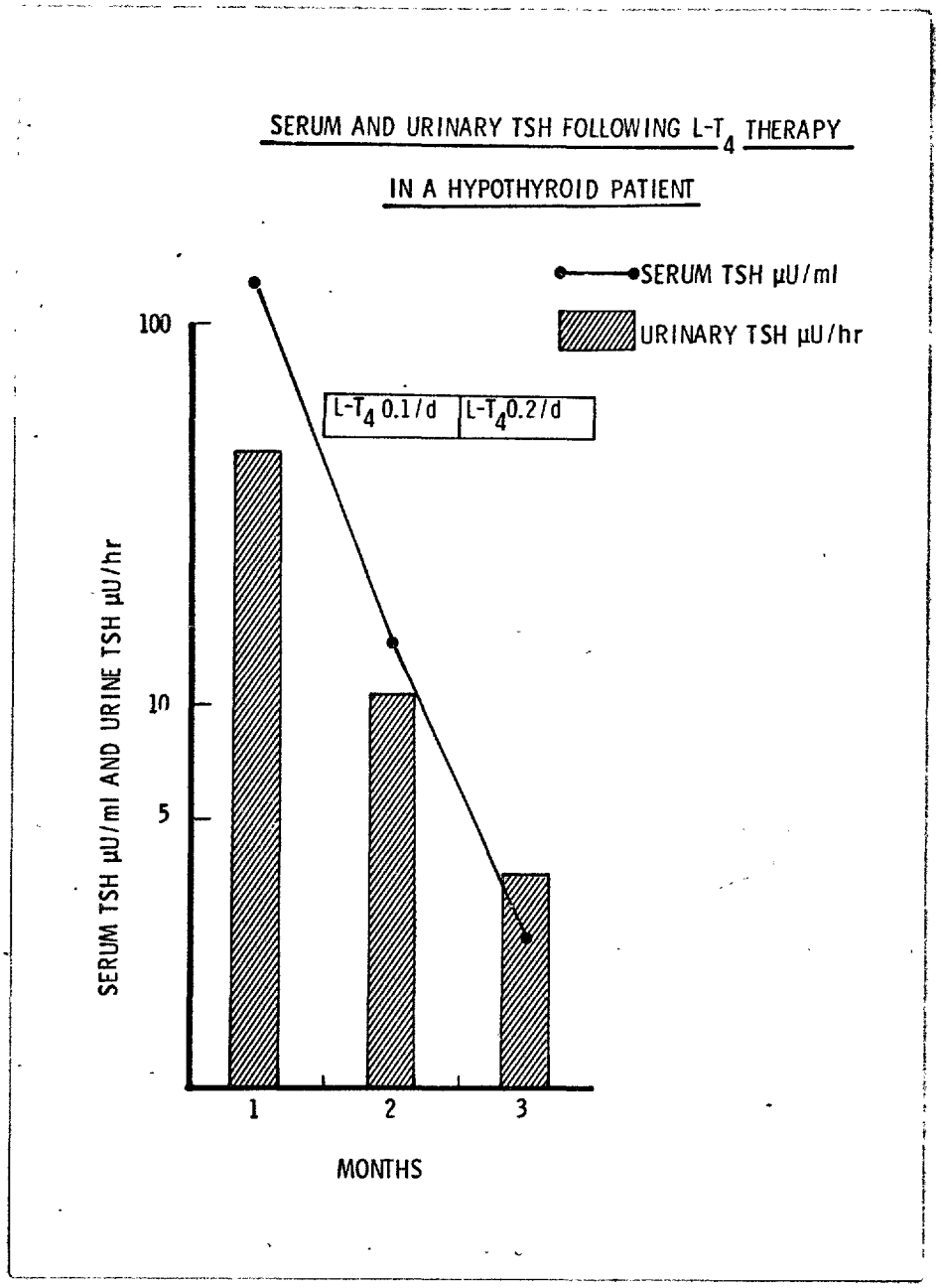

Fig. 3.9 Serum and urinary TSH levels after L-thyroxine ($L-T_4$) therapy in a hypothyroid patient. Serum TSH (uu/ml); urinary TSH uu/hr; $L-T_4$ 0.1/d and $L-T_4$ 0.2/d = L-thyroxine 0.1mg daily for one month followed by 0.2mg daily for the next two months.

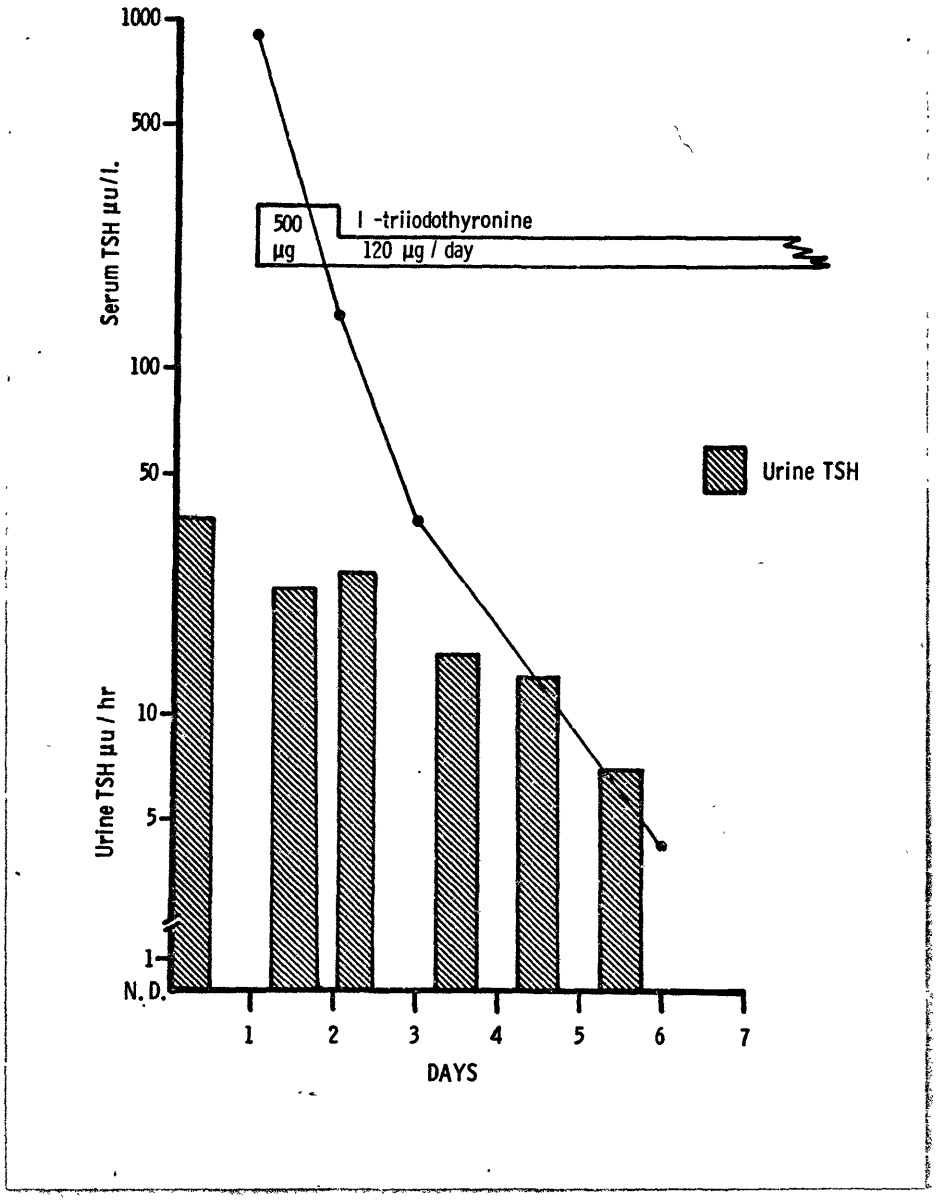

Fig. 3.10 Serum and urinary TSH levels after L-T_3 therapy in a hypothyroid patient. L-T_3 (500µg) was given orally on day 1, followed by 120ug daily for 6 days.•—•Serum TSH(uu/ml).

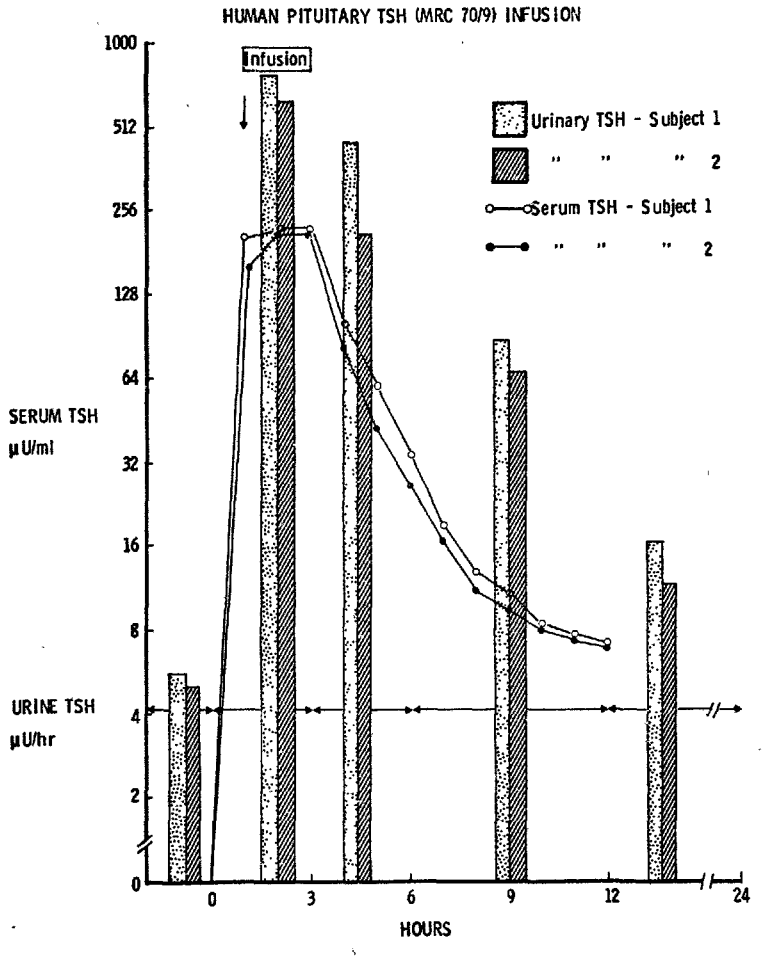

Fig. 3.11 Serum and urine TSH levels before, during and after an intravenous infusion of human pituitary TSH in two normal subjects. Horizontal arrows indicate periods of collection of urine for TSH assay.

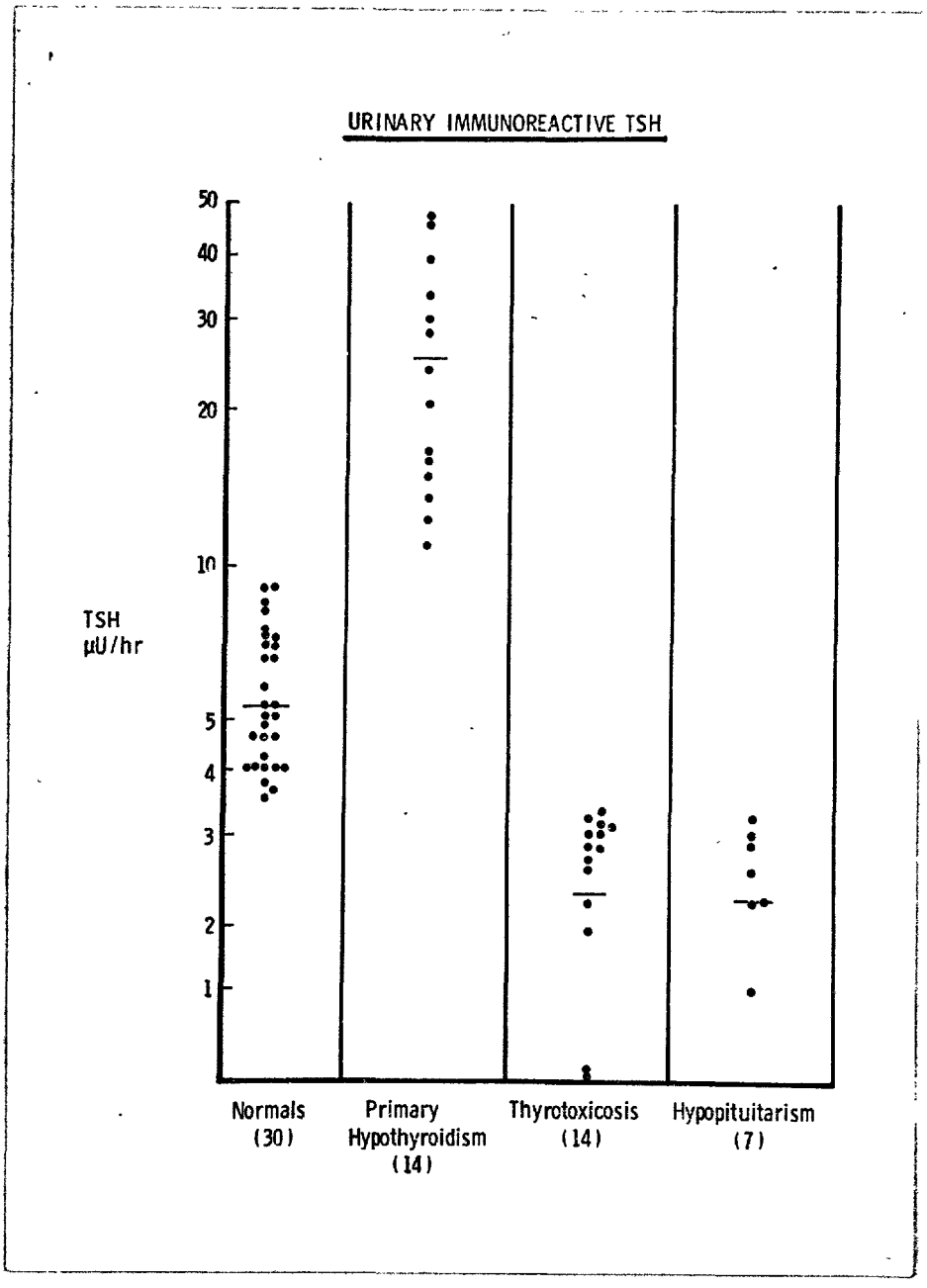

Fig. 3.12 Urinary excretion of TSH in normal subjects and patients with primary hypothyroidism, thyrotoxicosis and hypopituitarism.

diurnal variation ($P > 0.1$).

6) Urinary TSH levels in chronic renal failure:

In all five renal failure subjects studied, urinary TSH excretion per hour was higher than normal (Table 3.10). However, the levels could not be correlated with the degree of proteinuria nor with the blood urea and serum creatinine levels.

7) Gel-filtration analysis:

a) Pituitary TSH preparations, hypothyroid sera and urine concentrates: The elution profiles of pituitary TSH, and hypothyroid sera on Sephadex gel-filtration analysis are shown in Fig. 3.13. This shows that immunoreactive TSH was eluted as a single symmetrical peak with an elution volume (Ve) corresponding to a molecular weight of about 34000. ^{131}I-labelled pituitary TSH was eluted with an identical peak. The pituitary and serum materials showed comparable profiles.

Fig. 3.14 shows the results of gel-filtration analysis of hypothyroid sera pre and post an intravenous injection of 500µg TRF. In all four subjects, the elution profiles were identical whether pre or post stimulation. No evidence of "big" TSH emerged as the materials were eluted at a peak corresponding to M W of 34,000 on the calibration curve (Fig. 3.2). However, in all four subjects, serum TSH levels increased as shown by the higher elution peaks for the 20 minute samples in spite of the fact that the same amount of serum was applied in all cases.

In contrast to the above, elution profiles of basal urinary concentrates showed not only an asymmetrical peak corresponding to the above peak of the pituitary and serum materials, but several minor later peaks. (Fig. 3.13).

b) Urine concentrates after infusion of human pituitary TSH: Gel-filtration analysis of urinary concentrates during and for the three periods after the infusion of human pituitary TSH revealed the elution profiles shown in Fig. 3.15. During the period of infusion, the urinary

Subject	Blood Urea mg/100ml	Serum Creatinine mg/100ml	Urinary Protein g/day	Serum TSH μu/ml	Urinary TSH μu/hr
1	100	9.6	6.2	3.2	93.8
2	134	14.3	2.6	2.3	17.0
3	235	16.0	4.4	2.5	34.6
4	280	16.0	5.6	3.4	13.0
5	300	21.0	7.0	3.0	11.0

Table 3.10: Urinary TSH concentrations in 5 chronic renal failure patients

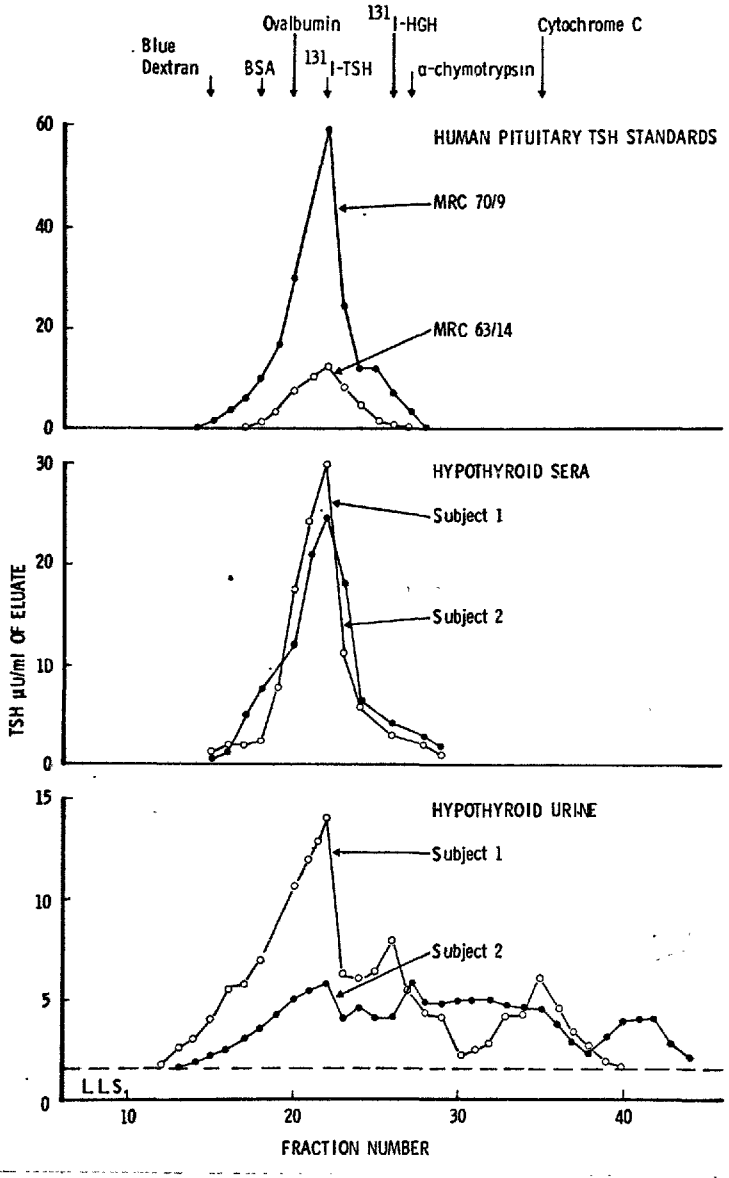

Fig. 3.13 Gel-filtration profiles of (a) human pituitary TSH standards, MRC 70/9; MRC 63/14; (b) sera and (c) urine concentrates from two hypothyroid subjects. Eluate fractions were of 2.4ml each; the arrows above show the positions at the peaks of the standards run to calibrate the column; the 50 x 2cm column of Sephadex G100 was eluted with 0.1M borate buffer (pH 8.4). LLS=lower limit of sensitivity of the radioimmunoassay.

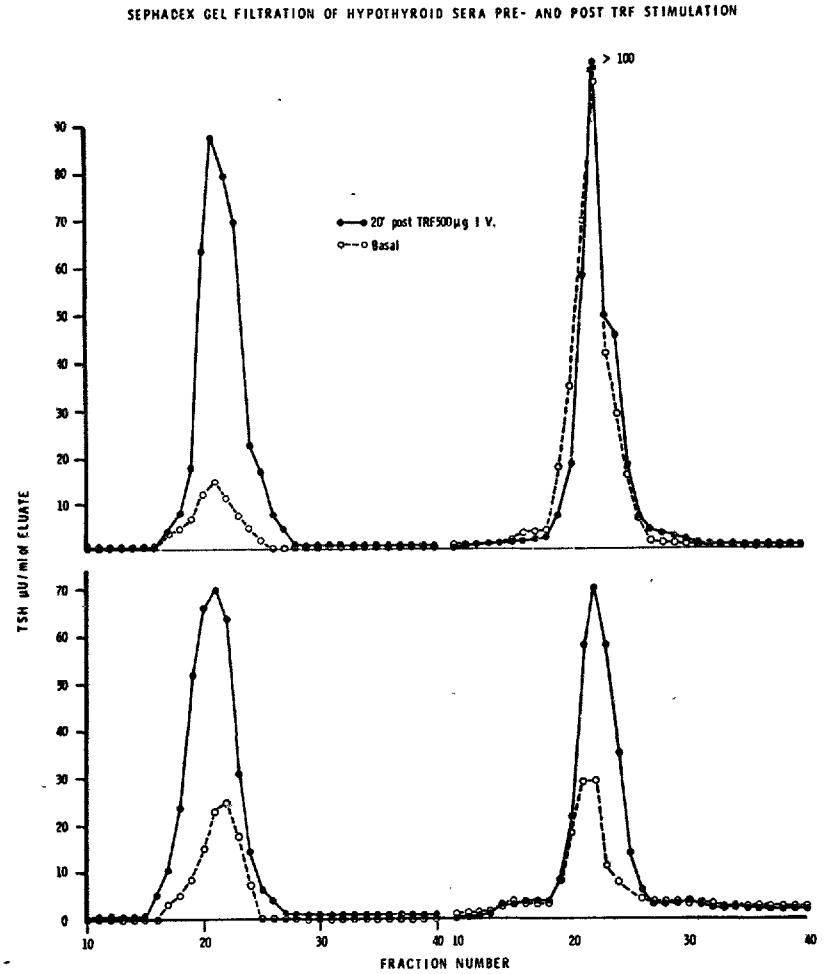

Fig. 3.14 Gel-filtration profiles of immunoreactive TSH in sera of 4 hypothyroid subjects pre and 20 minutes post stimulation with 500ug TRF intravenously. Column procedure as for Fig. 3.13.

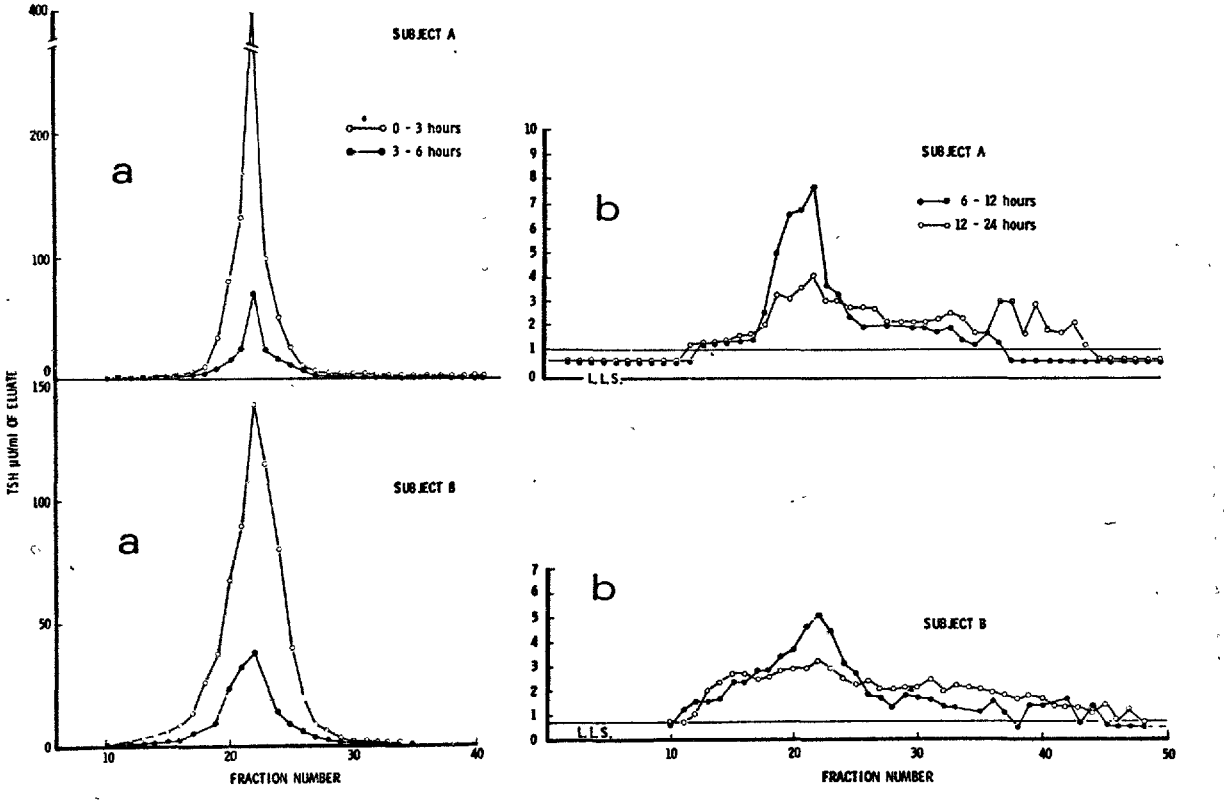

Fig 3.15 Gel filtration profiles of immunoassayable TSH in concentrates of urine (a) during and for 3 - 6 hrs and (b) 6 - 12 hrs and 12 - 24 hrs after i.v. infusion of human TSH (MRC 70/9) into two normal subjects. Column procedure as for Fig. 3.13.

material eluted as one symmetrical peak with an elution volume similar to that of the pituitary material. The elution profile for the period immediately after the infusion was essentially similar to that during the infusion. However, the urinary materials for the period 6-12 hours and 12-24 hours were eluted with a different pattern. The peak corresponding to the pituitary material was broad and asymmetrical while more minor peaks appeared later. (The later small peaks of Fig 3.15b were not visible even on corresponding scale diagrams of Fig.3.15a). The elution profiles of the 12-24hr material were very similar to the profile of the basal hypothyroid urine concentrates shown on Fig. 3.13.

c) Analysis of gel-filtration data: A composite analysis of the above results is shown in Fig. 3.16. For ease of analysis and clarity, the total volume of the column has been divided into segments designated as A, B, C and D based on an assessment of the elution profiles of pituitary TSH standards, a hypothyroid serum and a hypothyroid urine concentrate. A represents material eluted at the void volume of the column, B represents material eluted between fractions 16 and 24, while C represents fractions 24 - 32. Any material eluted after fraction 32 is represented by D. Each block represents a percentage of the entire elution profile. It is apparent that about 90% of the pituitary and serum TSH was eluted in segment B, the remainder mainly in segment C. In contrast, however, only about 50% of the urinary material was eluted in segment B, the remainder eluting in segments C and D, in about equivalent proportions. A significant amount was also seen in the void volume.

In the infusion studies, segment B contributed 90% of the eluted material during the infusion and most of the remainder eluting in segment C. However, with the passage of time, the proportion of eluted material in B fell, while those in A,C and D progressively increased,

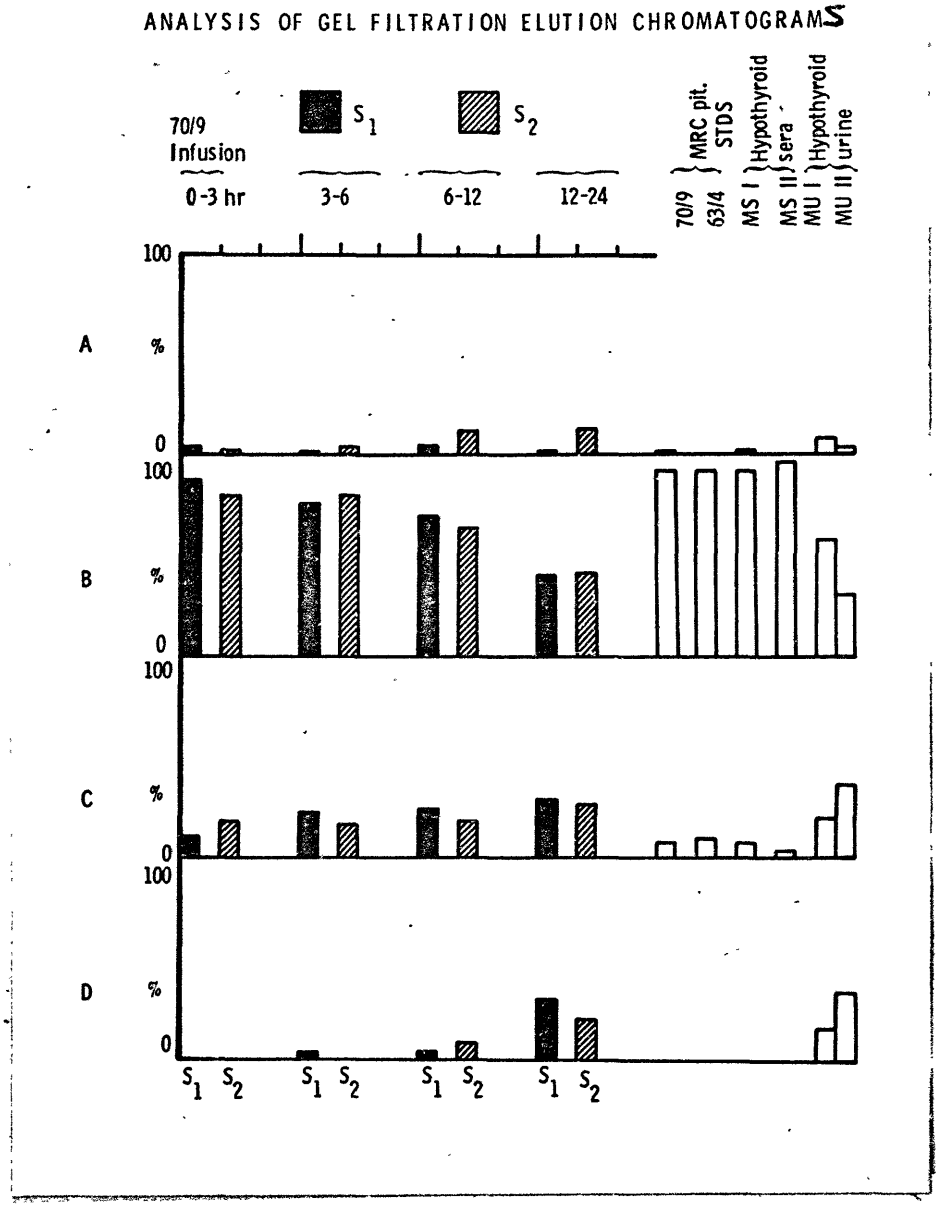

Fig. 3.16 Percentages of TSH in fractions from gel-filtration of pituitary standard preparations sera and urinary concentrates. A, B, C, D represent the percentages of immunoreactive material eluting in the various divisions of the elution profiles as detailed in the text. S1 = Subject 1. S2 = Subject 2, each infused with TSH MRC 70/9.

suggesting the presence of smaller immunoreactive fragments. The segmental distribution of the elution profile of the 12-24hr urinary material was essentially the same as that of the hypothyroid urines. This detailed analysis also showed that the differences in the elution profiles of the urinary material during the earlier part of the infusion and those in the later times could not be accounted for by the difference in the scale of the figures.

4. Discussion.

Of the concentration and extraction techniques investigated in this study, the dialysis and lyophilization method proved the most versatile because of its relative ease, the constancy of recovery and the high concentrations achieved. At the level of concentrations found for this method, the values of the samples on immunoassay were easily measurable, since they fell on the sensitive part of the immunoassay standard curve i.e. 1.5 - 20μu/ml. The major disadvantage of the method was the lyophilizer space required, and with the machine available to us ("Edwards") only seven bottles could be processed at a time. With the process taking about 48 hours, only 21 samples could be processed weekly. The alcoholic precipitation method only gave a seven-fold concentration, with the result that the absolute values on immunoassay fell on the insensitive part of the assay standard curve suggesting that further concentration was required. Yet, the practicability of increasing the concentration to more than 10-fold is severely restricted by the volume of alcohol, centrifuge space and the expense involved. The ultrafiltration procedure, although simple, was handicapped by the length of time required for each sample processing (12hr /30ml). The membranes were also expensive, and the recovery variable. On the whole, the dialysis followed by lyophilization procedure proved superior to the others and was used in this study.

The recovery experiments utilizing labelled and unlabelled hormone show that this is an efficient and reliable procedure. The recovery of unlabelled hormone was generally higher than that of labelled hormone, suggesting that we might be underestimating the true recovery. A possible explanation for this might be that the label contained some dialysable degradation products such as free iodine. This however is unlikely because the label used for the recovery experiments (same as used for immunoassay) was of a high degree of purity using electrophoretic and Sephadex gel-filtration criteria.

The radioimmunoassay system used in this study is specific for TSH, the other glycoprotein hormones LH and FSH which are normally assayed considerably more easily that TSH in urine show little or no cross-reaction with it. Table 3.9 shows that the urinary TSH levels in normal men are in the same range as in post-menopausal women, although the latter excrete large amounts of gonadotrophins as measured by specific radioimmunoassays. The differences found between the various disease states cannot, therefore, be attributed to cross-reaction with FSH and/or LH. Even when the units are converted to nanograms the molar excretion rate is clearly still much less. Perhaps more TSH is broken down to unassayable fragments before urinary excretion or it is not cleared so readily by the renal cells.

Like other workers (Rubenstein et al, 1967; Girard & Greenwood, 1968) , we have found that increasing concentrations of sodium chloride and urea produce increasing non-specific inhibition of the immunoassay system. But while neat "TSH-free" urine produced non-specific inhibition probably largely due to its urea and electrolyte content, most but not all of this inhibition was abolished by dialysis against distilled water. However, the dialysed urinary concentrates still showed some inhibitory effects probably due to non-specific effects

from peptides or other substances concentrated in the dialysed residue since the osmolarity or the concentrate was found to be not very different from that of the buffer. However, to take these residual non-specific effects into account, the standard curves were always made up in similar "TSH-free" urine concentrates prepared in the same way as the samples.

Our data show that urinary TSH measurement separates considerably the normal subjects from the primary hypothyroids on the one hand, and the hyperthyroid and hypopituitary patients on the other, and therefore possesses better discriminatory properties than measurement of TSH in serum in these circumstances. Probably measurement of the hormone in urine has the advantage of reflecting the mean serum level over a chosen lapse of time, thereby a better index of TSH secretory activity than a random serum measurement.

All the patients with proteinuria, had high urinary TSH levels in spite of normal serum TSH concentrations. This was probably due to non-specific leaking of TSH through the damaged glomeruli. The urinary TSH levels did not correlate with the degree of renal failure as shown by blood urea, serum creatinine levels and urinary protein excretion. These findings support the fact that urinary TSH measurement in the face of renal failure is virtually meaningless.

There are several indications that the factor measured in the urinary concentrates very probably was TSH. While dilution of the urine concentrates showed proportionality between the amount of dilution and the inhibition of binding on the assay curve, the dilution curves were also parallel to the standard curve. Furthermore, there was good correlation between serum TSH concentration and the hourly urinary TSH excretion. The fall in the urinary TSH excretion also followed closely that of serum TSH in hypothyroid patients treated with thyroid hormones. However, our data on the correlation between serum and urine TSH in hypopituitary

and thyrotoxic subjects might be interpreted as showing that up to a level of about 2μu/hr in urine may be due to a test blank, but a more sensitive serum assay is needed to chekc this.

When human pituitary TSH was infused for 3hr into normal volunteers the urinary TSH values followed closely the serum values, a further validation of our assay system. It may be noted that only 0.2% of the injected dose was recovered in the urine in 24 hr. This would suggest that TSH is metabolized almost completely in the body, and that very little is excreted into urine in immunoreactive form. Bakke et al (1962), using a bioassay, found that 34% of an injected dose of 10 i.u. bovine TSH was excreted into urine in 24hr. This discrepancy might be due to the fact that bovine TSH being "foreign" is treated differently from the human hormone.

Our study of two normal subjects over a period of 5 days failed to show any significant diurnal variation in urinary TSH excretion. This is not surprising since no consistent diurnal variation has been found in serum TSH levels (Hall, 1972).

Immunoreactive TSH in pituitary preparations and from sera of hypothyroid subjects behaved identically on Sephadex gel filtration under the conditions of buffer concentration, pH and temperature used for this analysis. Thus it may be concluded that no gross differences exist between them. However, subtle differences might emerge if conditions permitting higher resolution were used. Our gel-filtration data did not demonstrate any evidence of "big TSH" in the serum or pituitary preparations as described for some other hormones, for example insulin (Rubenstein et al, 1968), corticotrophin (Yalow & Berson, 1971), growth hormone (Gorden et al, 1973) parathormone (Berson & Yalow, 1968) and gastrin (Yalow & Berson, 1970). Even after TRF injection, "big TSH" did not appear in serum. This is consistent with the findings of Schleusener et al (1972) using electrofocussing electro-

phoresis, who could not demonstrate "big" TSH in serum even after i.v. TRF injection.

In contrast, however, immunoreactive TSH from urine showed gross differences from the above preparations in that a substantial amount of it exists in a range of molecular weight smaller than pituitary and serum TSH. This confirms the work of Ishigami (1966) who found that urinary TSH measured by bioassay was more retarded on Sephadex than serum TSH. These low molecular weight substances, we believe, represent metabolized fragments among which are fractions which could correspond to α and β subunits of the hormone, and also other smaller fragments.

Concentrates from the urine during and for 3 hr after the infusion of exogenous TSH showed essentially identical profiles, on gel-filtration, which were also similar to those of the infused material. Thus, during or immediately after the infusion only a small proportion of the urinary elution profile was due to low molecular weight fragments. However, as the basal state is approached, i.e. during the later hours after infusion, a greater proportion of the elution profiles represents smaller molecular weight substances, probably fragments. These findings would suggest that TSH appears in urine partly as an intact molecule cleared from the serum, but also partly as smaller fragments presumably produced from metabolism of the hormone. The urinary concentration of the molecule rises immediately with the infusion but the increased excretion of the fragments is delayed, presumably till after their metabolism by the kidney. In the basal state most of the immunoassayable TSH in urine is in the form of these smaller fragments.

Our findings are consistent with reported data on the behaviour of related hormones on Sephadex gel-filtration. Our own data from this laboratory (Young et al, 1974) show that urinary LH includes profiles more retarded on gel-filtration than the pituitary or serum hormones. Similar findings have been reported for HCG (Matthias and Diczfalusy,

1971; Franchimont et al, 1972). Talas et al, (1973) have however shown that urinary FSH shows gel-filtration profiles essentially identical with that of the pituitary hormone.

On the whole, the differences between gel-filtration profiles of serum and urine would suggest that the kidney is an important site of degradation of TSH which confirms the findings of other workers. Altschuler et al, (1968) have shown that radio-iodinated TSH when injected into rats is largely localized in the kidney, therefore supporting our concept. This is not surprising because kidney has been shown to be the major site of localization of many peptide hormones: GH (Collip et al, 1966); corticotrophin (Richards & Sayers, 1951); prolactin (Sonnenberg et al, 1951); TSH (Kassenaaret al, 1959) and insulin (Elgee et al, 1954). The mechanism by which the kidney metabolizes TSH requires further investigation but it is likely that it would be a proximal tubular phenomenon from the studies of Collip et al (1966) and Rabkin et al (1973) who by autoradiographic techniques showed localization of GH in renal proximal tubules. These speculations on the role of the kidney on the metabolism of TSH have been investigated and are presented in the next chapter.

CHAPTER 4

TISSUE DISTRIBUTION AND RENAL HANDLING OF INJECTED HOMOLOGOUS TSH IN THE RAT.

1. Introduction.

The distribution of bovine TSH, after its intravenous injection into the rat,has been studied by several groups. In the earliest reports, relatively large doses of TSH were given. Sonnenberg et al (1952) used ^{35}S labelled TSH which had a low biological activity and was contaminated with gonadotrophins. Fluorescein-labelled TSH was administered by Mancini et al (1961) and localization was found in various tissues, including the thyroid gland. Kassenaar et al (1956), Levey and Solomon (1957) and Bakke and Lawrence (1962) have measured by bioassay the concentrations of TSH in various organs of the rat, especially the kidney at various times after intravenous injections of unlabelled TSH. In 1968, Altschuller et al reported distribution studies in the rat using physiological tracer doses of ^{131}I-labelled bovine TSH.

In general, the concencus was that significant localization (up to 30% of the injected dose) of the hormone occurred mainly in the kidney, some in the liver, and very little in the thyroid gland. Altschuller et al (1968) who reported that up to 2% of the injected ^{131}I-TSH given to rats was recovered in the thyroid gland, did not block the thyroid gland with iodide and so they probably were measuring free ^{131}I released after the metabolism of the hormone.

The major role of the kidney in the metabolism of TSH was first suggested by Kassenaar et al (1956) who recovered 10 - 30% of 3000mU of bovine TSH injected into rats five minutes after the injection. Querido and Kassenaar (1959) later showed that ligation of the kidney hilus slowed the disappearance rate of injected TSH, a finding similar to that reported earlier by Levey (1958) who demonstrated that the disappearance rate of injected TSH from the plasma of nephrectomised rats was markedly slowed down.

In spite of the fact that the kidney appears to be the main organ of TSH localization *in vivo*, early attempts to demonstrate inactivation of the hormone by renal tissue *in vitro* have failed (Kassenaar and Kerkhofs, 1955; Levey, 1958; Bakke and Lawrence, 1962). It remains to be shown if the kidney actually catabolizes the hormone. In this study we have attempted to resolve this problem.

All the above distribution studies have utilized TSH preparations from another species, and the tissue distribution observed may have little physiological significance (Bates and Condliffe, 1966). We have therefore tried to solve the problem using physiological amounts of injected homologous ^{131}I-TSH in rats.

The high uptake of heterologous TSH by the kidney described in the above reports, if proved using homologous material, also raises the question of the precise anatomical localization of the hormone in kidney tissue, and whether it is metabolized there. These have been investigated by sequential autoradiography following intravenous injections of ^{125}I-rat TSH (rTSH) into rats, and Sephadex gel-filtration analysis of kidney extracts at various times after the injection of ^{125}I-rTSH into the rats. Localization of ^{125}I-rTSH by autoradiography in the rat thyroid gland is also reported.

2. Materials and Methods.

(a) Preparation of low specific activity ^{131}I-rTSH and ^{125}I-rTSH:- The rat TSH, a gift from the National Pituitary Agency, U.S.A., was highly purified with little or no contamination with other hormones (Kieffer et al, 1974). It was iodinated by a modification of the procedure described for human TSH in Chapter 2 to produce lower specific activity labelled hormone. 5µg of rTSH was used instead of

2.5µg for hTSH. While the same amount of chloramine-T was used, the reaction time was shorter, usually 6 - 8 seconds. Purity of the labelled hormone was checked by electrophoresis and all tracers containing more than 2% "damaged" hormone on electrophoresis were discarded; all tracers were free of unreacted iodide. Specific activities, calculated as shown in Fig. 2.7, achieved, ranged between 80 and 110µCi/µg. At this level of specific activity, Utiger et al (1963) have shown that the hormone retained most of its biological activity.

The labelled hormone was stored in phosphate buffer (0.2% BSA) pH 7.4 and used within 24 hours of production.

(b) Rats:- Male Wistar rats, weighing 280 - 320g, were used in this study. They were fed normally with ad libitum pellet diet and tap water. 18 - 24 hours before the injection of labelledrTSH, the rats were given potasium iodide, KI, (240µg/ml) in their drinking water to block thyroidal radio-iodine uptake. This was 10 - 20 times their daily iodine requirement. One group of control rats studied in the distribution experiments were not pretreated with KI.

(c) Procedures:-

i) Tissue distribution studies:- The rats were lightly anaesthetized with diethyl ether ("BDH") and 1µCi of ^{131}I-rTSH in 0.5ml of normal saline was injected via a tail vein. With a specific activity of about 100µCi/µg, this dose was equivalent to 10ng. In a 300g rat with a plasma volume of 3% of body weight, measured by ^{125}I-albumin as shown later, this would be distributed in 9ml of plasma, giving a level of about 1ng/ml. This is in the range of TSH concentration in rat plasma which was found to be 2.06$\pm$ 0.47ng/ml (Kieffer et al, 1974) and is therefore in physiological quantities.

At 2, 10, 30, 60 and 180 minutes after the injection, the rats, two at a time, were sacrificed by decapitation after light anaesthesia.

Pieces of kidney, liver, psoas muscle, fat, thyroid,pituitary and hypothalamus and 1ml of blood were removed and weighed. The tissues were homogenized in 5ml of 10% trichloroacetic acid (TCA). The homogenate was centrifuged, the supernatant discarded and TCA-precipitable activity counted in well-type gamma scintillation counter.

Control studies were performed to exclude the possibility of non-specific localization in the various organs studied by injecting normal rats of the same weight with 1uCi of ^{125}I-human serum albumin (HSA), obtained from the "Radiochemical Centre", Amersham. The animals were sacrificed at 10, 60 and 180 mins and pieces of kidney, liver and thyroid, and 1ml of blood processed as described above.

ii) Autoradiographic studies:- ^{125}I was used for labelling in preference to ^{131}I for these studies because of its shorter path track and therefore better resolving power.

After light anaesthesia with ether, each of six rats, one per time interval, was injected with 50µCi of ^{125}I-rTSH in a volume of 0.5ml normal saline, in the tail vein. The dose was equivalent to 500ng of TSH (specific activity of material = 100µCi/µg). A rat was sacrificed by decapitation at each of the following times: 2, 5, 10, 30, 60 and 180 minutes after the injection of tracer. Both kidneys were removed within a minute of decapitation and bisected longitudinally. One half of each kidney was immediately frozen by immersing in n-hexane at -60^{o}C for 45 seconds and later used for autoradiography. The other halves were immediately homogenized in normal saline, the homogenate centrifuged at 2000rpm at 4^{0}C for 20 minutes, and the supernatant kept at -20^{o}C for Sephadex gel-filtration analysis. Thyroid glands were also excised from two rats sacrificed at 60 and 180 mins, frozen in n-hexane at -60^{o}C and autoradiographed.

Autoradiography:-

Gelatin-subbed slides for autoradiography were prepared as follows: Chance microscopic slides (Low ^{40}K content) were cleaned with chromic acid and coated with a solution containing gelatin (5 g/l) and chrome alum 0.5g/l in distilled water. The slides were then dried vertically in a hot air oven at approximately 120°C.

The stripping technique was used for autoradiography. Two variations of tissue preparation were performed: (a) Formalin fixation of tissue which allowed water-soluble substances in tissue to leach out. (b) the fixation of material by using cryostatic sections and keeping all materials at sub-zero temperatures to keep tissue frozen. This technique was first described by Appleton (1969). This preserved all materials including those which are water-soluble.

Histological sections, 5μ and 20μ thick were cut at -30°C in a refrigerated microtome for light microscopic autoradiography and gross autoradiography respectively.

A set of 5μ sections were picked up on gelatine-subbed microscope cover glass and formalin-sapour fixed at ambient temperatures overnight. Afterwards A.R.10 stripping films (Kodak Ltd., London) were wet-mounted over the histological sections according to the method outlined by the manufacturer and air-dried. Another set of 5μ thick sections were picked up directly on pre-cooled A.R.10 stripping film already mounted on microscopic cover glass as described by Appleton (1969). Both sets of wet and dry mounted films were packed in light-tight boxes, also containing some silica gel and soda-lime. The films were exposed at -20°C for five weeks (low temperatures were used to minimize the background clouding of the films). The films were then developed as follows. The developer, D19, was prepared fresh from the following formula: Elon 2.2g, crystalline sodium sulphite 144.0g, Hydroquinone 8.8g, crystalline sodium carbonate 130g, potasium bromide 4.0g.

750ml of distilled water was heated to 40°C and the chemicals added in the above order. When all the chemicals were completely dissolved, the solution was made up to 1 litre with distilled water, filtered and used in the dark room as follows: The slides were transferred from light-tight exposure boxes into developing dishes. The developer already cooled to about 17°C was then poured into the dishes. After 5 minutes the developer was poured from the dish and the slides washed in running tap water briefly. The slides were then fixed in Johnson's FixSol (100ml distilled water and 20ml Johnson's FixSol) for 5 minutes. The FixSol was poured off, the slides washed well in running water for 30 minutes and stained with fresh Ehrlich's haematoxilin for 12 mins. They were washed again in tap water for 5 minutes, then differentiated rapidly in 1% acid alcohol, blued in running tap water for 20 minutes and counterstained with 1% Eosin for 1 minute. They were then dehydrated, cleaned and mounted. The processed autoradiographs were analysed under light microscope and phase contrast microscope to study the silver grain distribution (Ag) in relation to cell types.

Thick histological sections of the kidney (20μ) were formalin vapour fixed as described above and placed on "Kodirex" films (Kodak Ltd., London) with an inter-layer of 5μ thick "Melinex" (ICI Ltd.) between the histological section and film. The films were exposed for 5 weeks at 0°C to minimize tissue decomposition.

iii) Sephadex gel-filtration analysis:- 1 - 3ml of saline extracts of kidney homogenates at 5, 30, 60 and 180 minutes after i.v. ^{125}I-rTSH injections described under (ii) were subjected to analysis on 50x2 cm Sephadex G100 column calibrated as described in Chapter 3. Elution was carried out at room temperature using 0.1M barbitone buffer pH 8.6 and collecting 3ml fractions. The fractions were counted in a well-type autogamma counter. All gel-filtration studies were completed

within 48 hours of injection of the tracer.

3. Results.

(i) Tissue distribution studies:- The disappearance curve of TCA-precipitable ^{131}I-rTSH from the blood is shown in Fig. 4.1. The counts recovered in 1ml of blood were expressed as a percentage of the total dose of radioactivity administered. The ^{131}I-rTSH administered was 97% TCA-precipitable. The hormone disappeared from the blood with a half-life of 13 minutes. The decline was linear for 30 minutes after the injection, by which time about 80% of the radioactivity had disappeared from the blood. Later the decline was much slower, probably due to the appearance of metabolites. The ^{125}I-HSA decay curve in blood represented a control and showed a decline commensurate with a longer half-life.

When both curves on Fig. 4.1 were extrapolated to zero time, they cut the Y axis at different points: the rTSH curve at a higher point suggesting that the hormone was distributed in a space greater than plasma volume (represented approximately by the point at which the HSA curve cut the Y-axis at zero time). The distribution space of ^{131}I-rTSH was 1.1 times the plasma volume, which was calculated to be 9mls or 3% of body weight.

The findings described above were obtained from KI pretreated rats; the results in non KI treated rats were similar.

Fig 4.2a shows the tissue concentrations of TCA-precipitable radioactivity, expressed as percent dose/g of tissue, at various times after the injection of ^{131}I-rTSH. The highest concentration of activity was found in the kidney. The kidney radioactivity increased with time, reaching a peak at 30 minutes, and then falling away. At 30 minutes about 12% of the injected dose of ^{131}I-rTSH was present in 1g of kidney tissue. That the kidney concentrated the tracer against

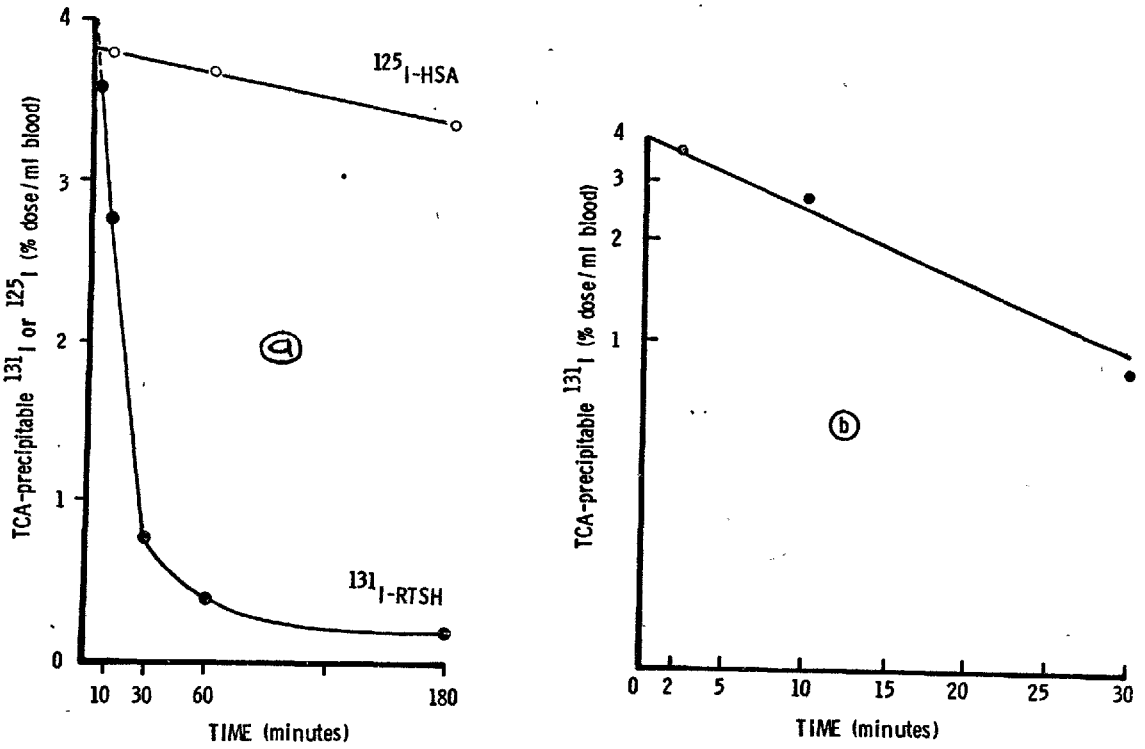

Fig. 4.1 (a) Decline in blood of ^{131}I-rTSH and ^{125}I-HSA after their intravenous injection into rats.

(b) Semilogarithmic plot of the linear part of the ^{131}I-rTSH curve shown in (a).

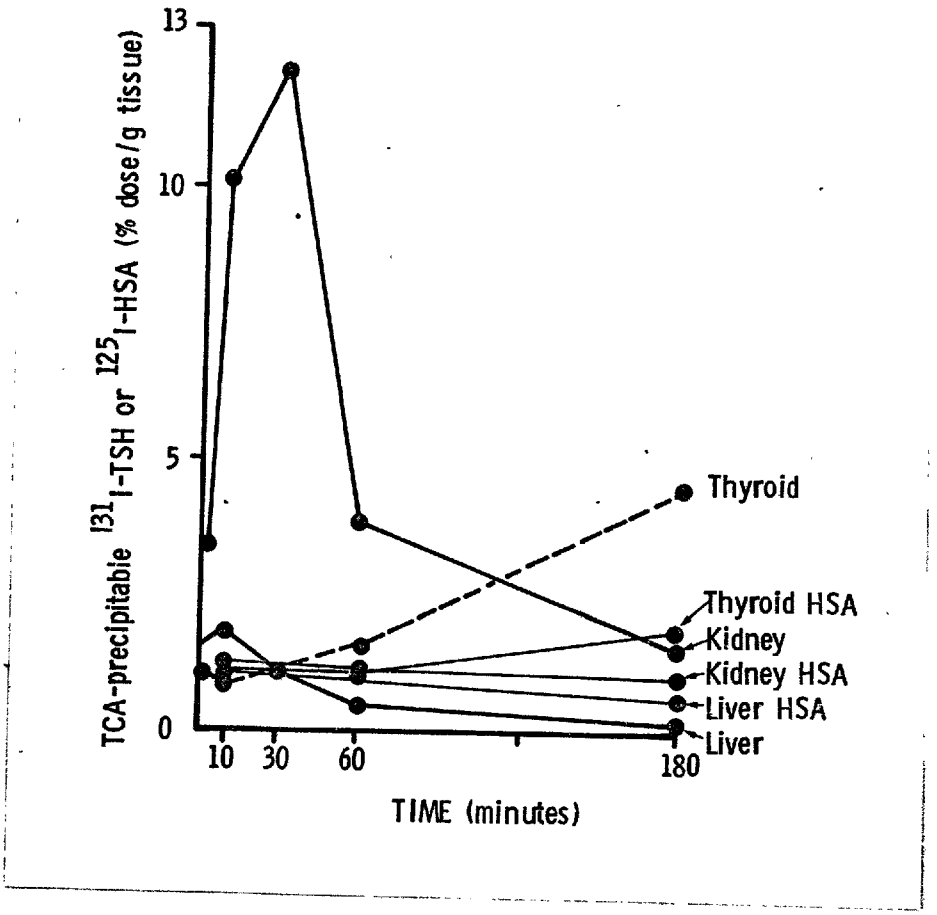

Fig. 4.2a Concentrations of ^{131}I-rTSH or ^{125}I-HSA found in various organs after their intravenous injection into rats.

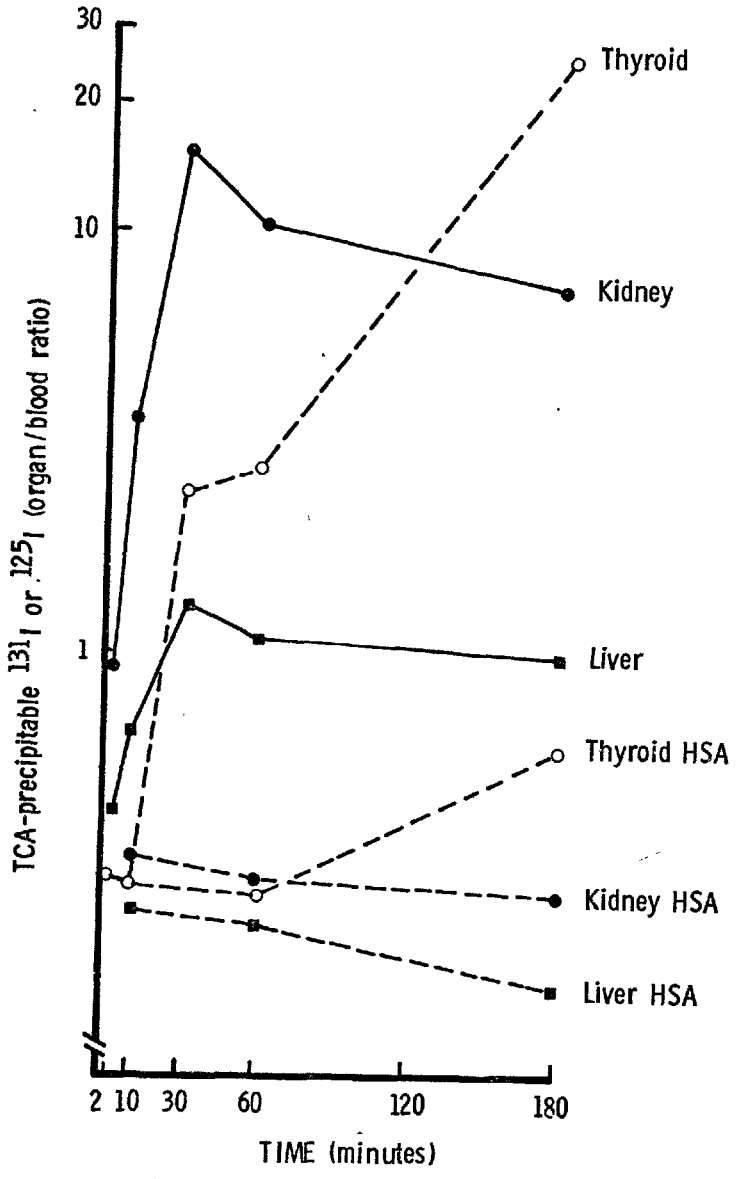

Fig. 4.2b Concentrations of ^{131}I-rTSH or ^{125}I-HSA found in various organs after their intravenous injection into rats.

a gradient in the blood is shown in Fig.4.2b which is a plot of radioactivity in 1g of tissue as a ratio of radioactivity in 1ml of blood as a function of time. At 30mins, the kidney/blood ratio of radioactivity was about 16.

Relatively less localization of TCA-precipitable radioactivity was found in the liver all through the period of study (Fig.4.2a,b).

Again pretreatment of the rats with KI did not make any difference to the amount of radioactivity found in the kidney or liver.

The TCA-precipitable radioactivity in the thyroid increased progressively with time (Fig.4.2a,b). There was a considerable difference between the results obtained from KI pretreated and the non-KI treated rats, the radioactivity in the thyroid in the former being very much less than in the latter (Fig.4.3).

Table 4.1 shows the distribution of TCA-precipitable radioactivity in the pituitary, muscle, fat, hypothalamus and testis after ^{131}I-rTSH injection. Apart from the pituitary, the radioactivity found in these tissues was low.

When TCA-precipitable radioactivity was studied for 180min in the kidney, liver and thyroid after the injection of ^{125}I-HSA, it was found that the radioactivity over these organs whether expressed as percentage dose or organ/blood ratio was low and constant all through the period of study (Fig. 2.a,b).

(ii) Autoradiographic studies:-

a) Gross Autoradiography:- Autoradiographic imprints of 20μ kidney sections were visually examined and the gross localization of ^{125}I radioactivity studied. Kidney radioactivity was found to localize overwhelmingly in the cortex of the kidney (Fig. 4.4). Radioactivity in the cortical zone was present in high amounts, 2 minutes after the injection, and the high levels of radioactivity were also present in the cortex of the kidney taken at 5, 30 and 60 minutes after injection.

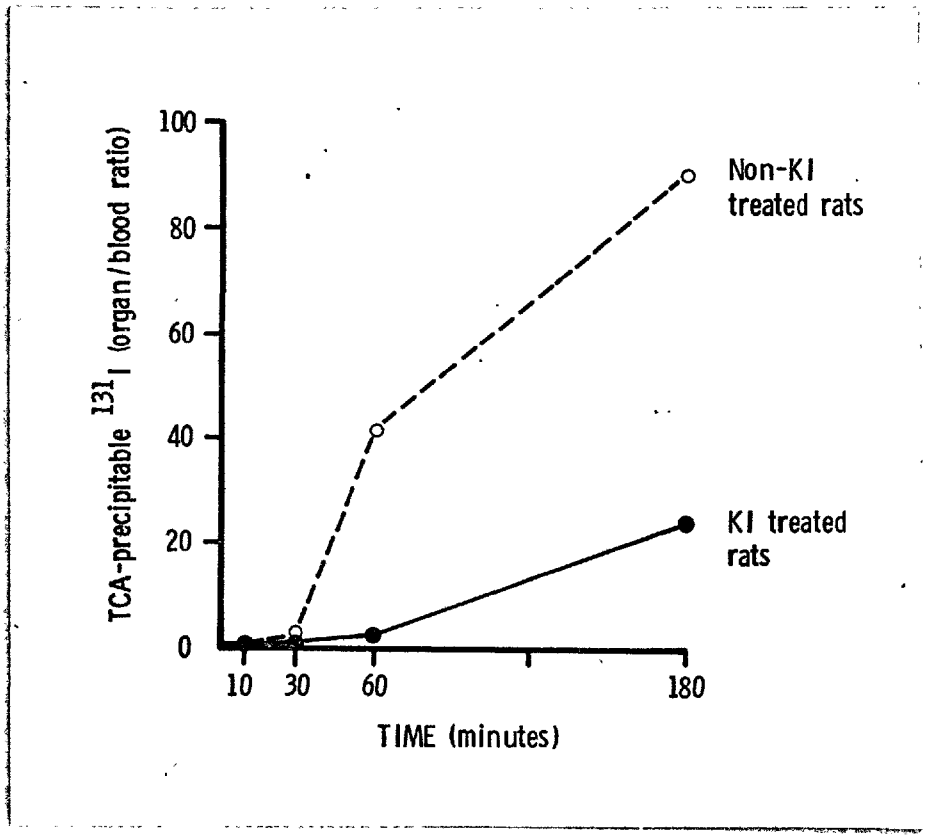

Fig. 4.3 Comparison of the organ/blood concentration ratios of TCA-precipitable ^{131}I in the thyroid glands of KI treated and non-KI treated rats at various times after an intravenous injection of ^{131}I-rTSH.

Time (min)	Muscle		Fat		Testis		Hypothalamus		Pituitary	
	% Dose/g Tissue	Organ/ Blood	% Dose/g Tissue	Organ/ Blood	% Dose/g Tissue	Organ/ Blood	% Dose/g Tissue	Organ/ Blood	% Dose/g Tissue	Organ/ Blood
10	0.07	0.03	0.02	0.01	0.06	0.02	0.08	0.03	1.2	0.42
30	0.03	0.04	0.01	0.02	0.05	0.07	0.02	0.03	0.68	0.87
60	0.05	0.01	0.01	0.05	0.05	0.1	0.04	0.1	1.0	4.0
180	0.02	0.09	0.01	0.02	0.03	0.1	0.03	0.08	0.35	1.5

Table 4.1 Tissue distribution of TCA-precipitable radioactivity after ^{131}I-rTSH injection in the rat.

Organ/Blood = counts/g tissue ÷ counts/ml of blood.

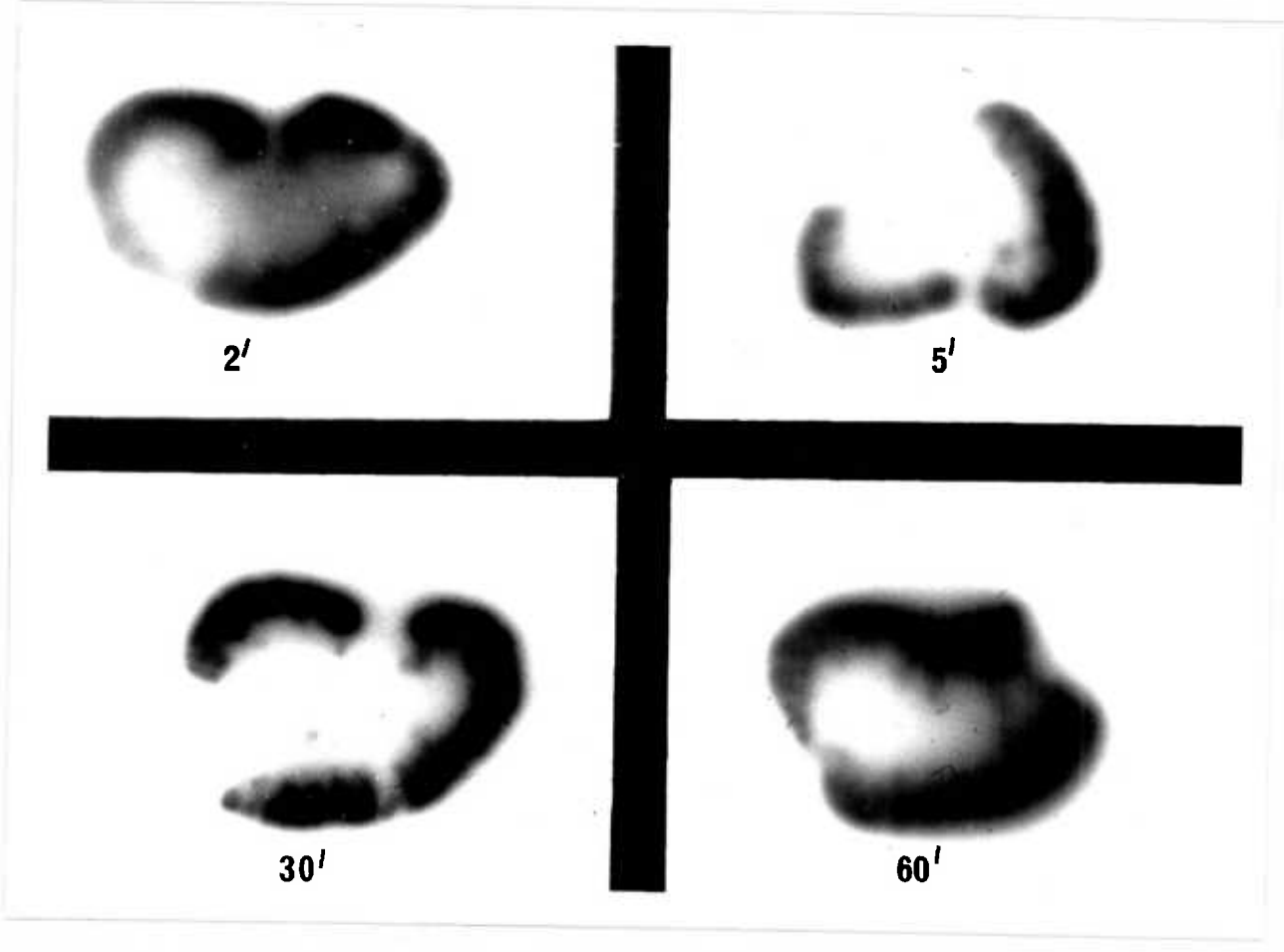

Fig. 4.4 Gross autoradiogram showing selective concentration of ^{125}I-rTSH in the cortex of thin sections of rat kidney. Histological sections (20μ) at 2, 5, 30 min. were obtained in a mid-vertical plane and included the hilus. The 60 min. section was obtained in a sagittal plane near the convexity. The slight radioactivity seen in the medulla in the 2 min. section could be accounted for by the high levels of radioactivity in the blood at this time.

Very low levels of radioactivity were present in the medullary zone and the hilus at all times studied.

b) Light microscopic autoradiography:- Autoradiographs of the 5μ kidney sections picked up directly on the AR10 films using the Appleton technique when viewed at a low magnification showed localized clusters of Ag grains over the cortical zone, 2 minutes after injection of ^{125}I-rTSH. These localized clusters were present predominantly over the periglomerular mass of renal tubules (Fig. 4.5a,b). At higher magnifications in these sections, the Ag grain density over the blood vessels was found to be relatively low. In the longitudinally cut nephrons with intact vascular and renal poles, a very low Ag grain density was noticed over the afferent and efferent arterioles and the glomerular tuft. But the majority of Ag grains were found to localize over the base of the Bowman's capsule, near the renal pole and over the origin of the proximal tubule (Fig. 4.6a,b). The profusely radioactive periglomerular renal tubules were identified with Dr. David Evans (kidney histopathologist, Hammersmith Hospital, London) as proximal convoluted tubules. In the distal tubules only a very low Ag grain density was found. Autoradiographs at 2 min also showed only low levels of radioactivity over the thick descending limbs of the proximal tubules. Over the proximal convoluted tubule, Ag grains localized discretely over the tubular lumen and the luminar end of the tubular epithelium (Fig. 4.7). Over those proximal tubules cut obliquely, excluding the tubular lumen, Ag grain density was low. In the kidney sections obtained at later times, Ag grain distribution was similar to that seen at 2 minutes, but the grain density over proximal tubules tended to decrease with time (Fig. 4.8). In some nephrons, Ag grains were also found to localize at the base of the Bowman's capsule and at the origin of the proximal tubule for up to 30 minutes, but this localization was not observed at later times

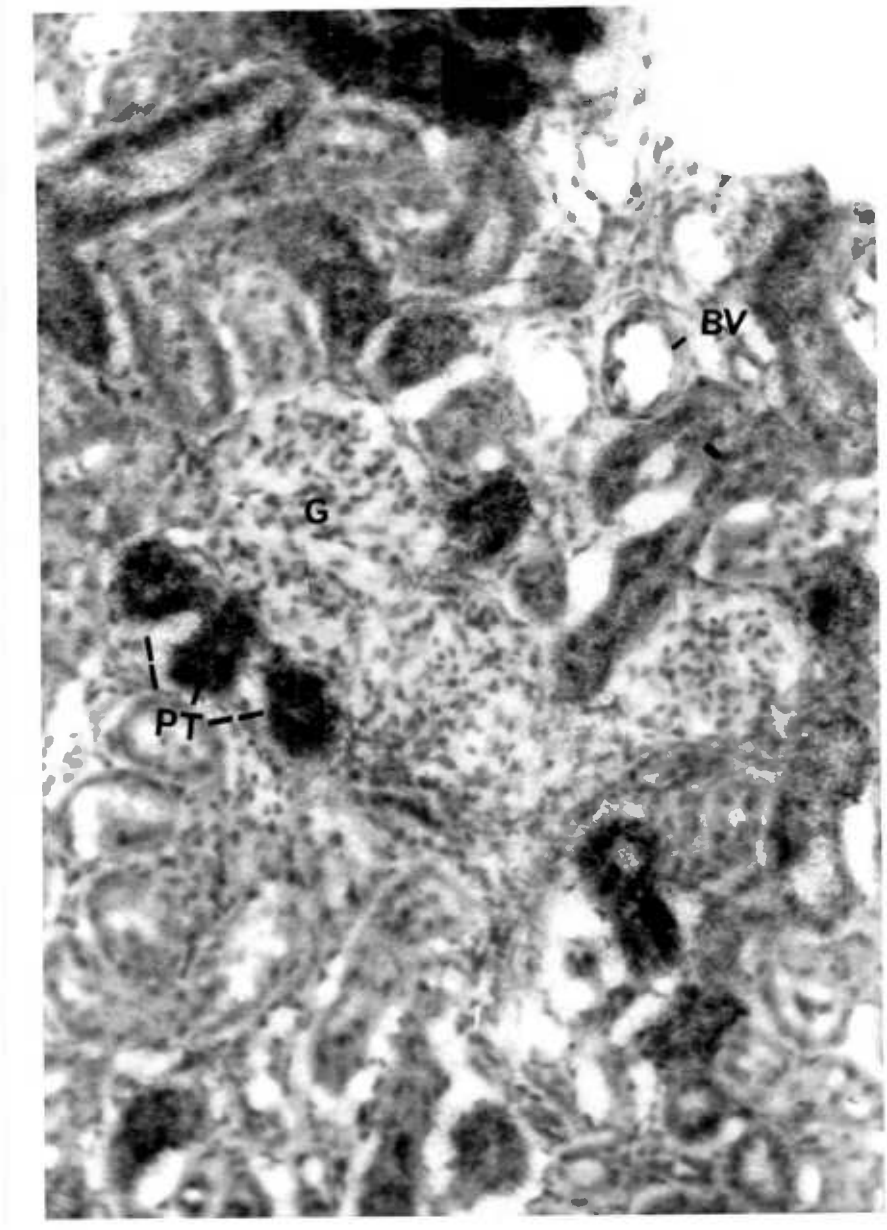

Fig. 4.5a Autoradiogram of rat kidney 2 min. after i.v. injection of ^{125}I-rTSH showing heavy localization of radioactivity in periglomerular renal tubules. G = glomerulus; BV = Blood vessels; PT = proximal tubules. Magnification x 50.

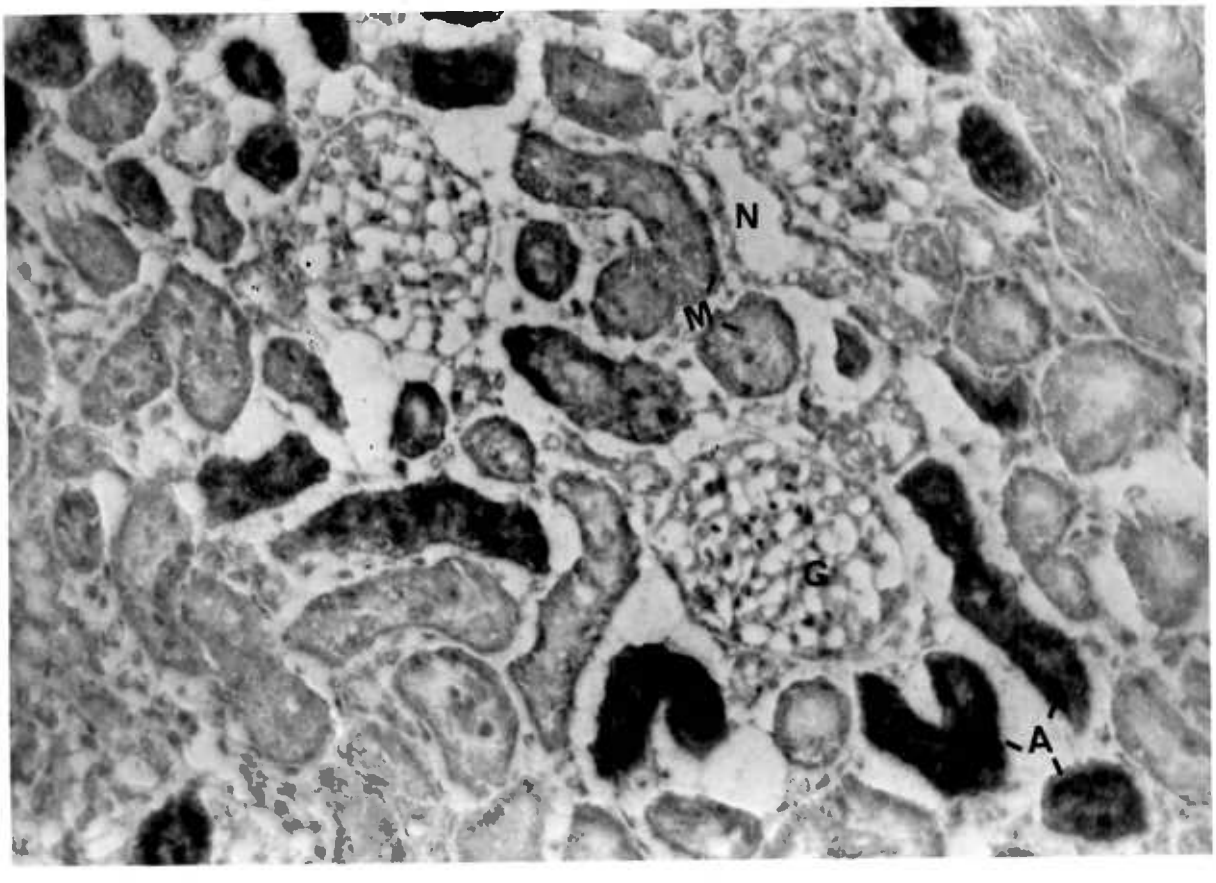

Fig. 4.5b Autoradiogram of rat kidney showing ^{125}I-rTSH localization in periglomerular renal tubules 5 min. after i.v. injection of ^{125}I-rTSH. G = glomerulus; A = Heavily radioactive proximal tubule ; M = Mildly radioactive proximal tubule; N = Non-radioactive distal tubule. Phase contrast photomicrograph; magnification x 50.

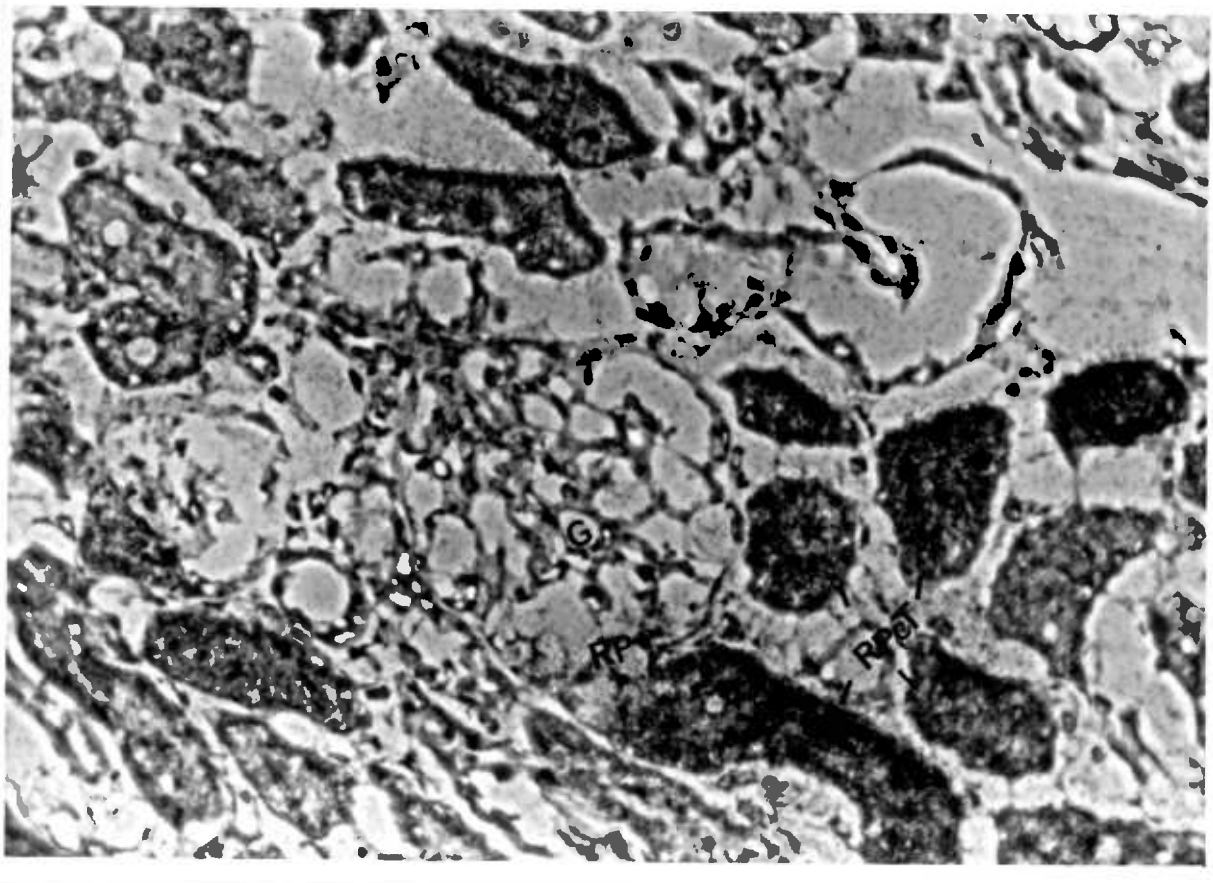

Fig. 4. 6a Autoradiographic demonstration of ^{125}I-rTSH glomerular filtration. G = glomerulus; RPcT = radioactive proximal convoluted renal tubule; RP = renal pole. Note the presence of ^{125}I radioactivity at the base of the Bowman's capsule and the origin of the proximal tubule. Phase contrast photomicrography, magnification x 80. Time after injection, 5 min.

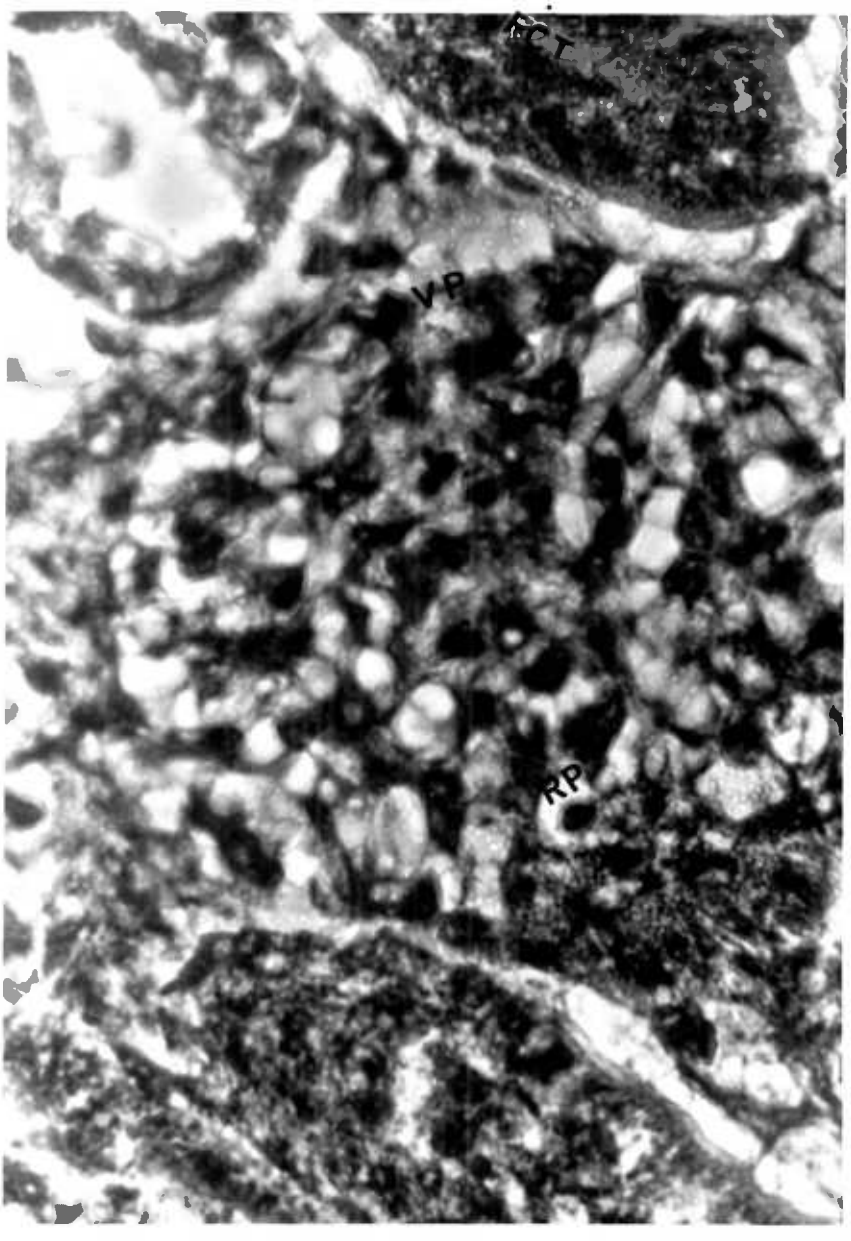

Fig. 4. 6b Autoradiographic demonstration of ^{125}I-rTSH accumulation at the renal pole of the glomerulus. VP = vascular pole; RP = renal pole; PCT = proximal convoluted tubule. Time after injection, 5 min. Phase contrast photomicrograph, magnification x 600.

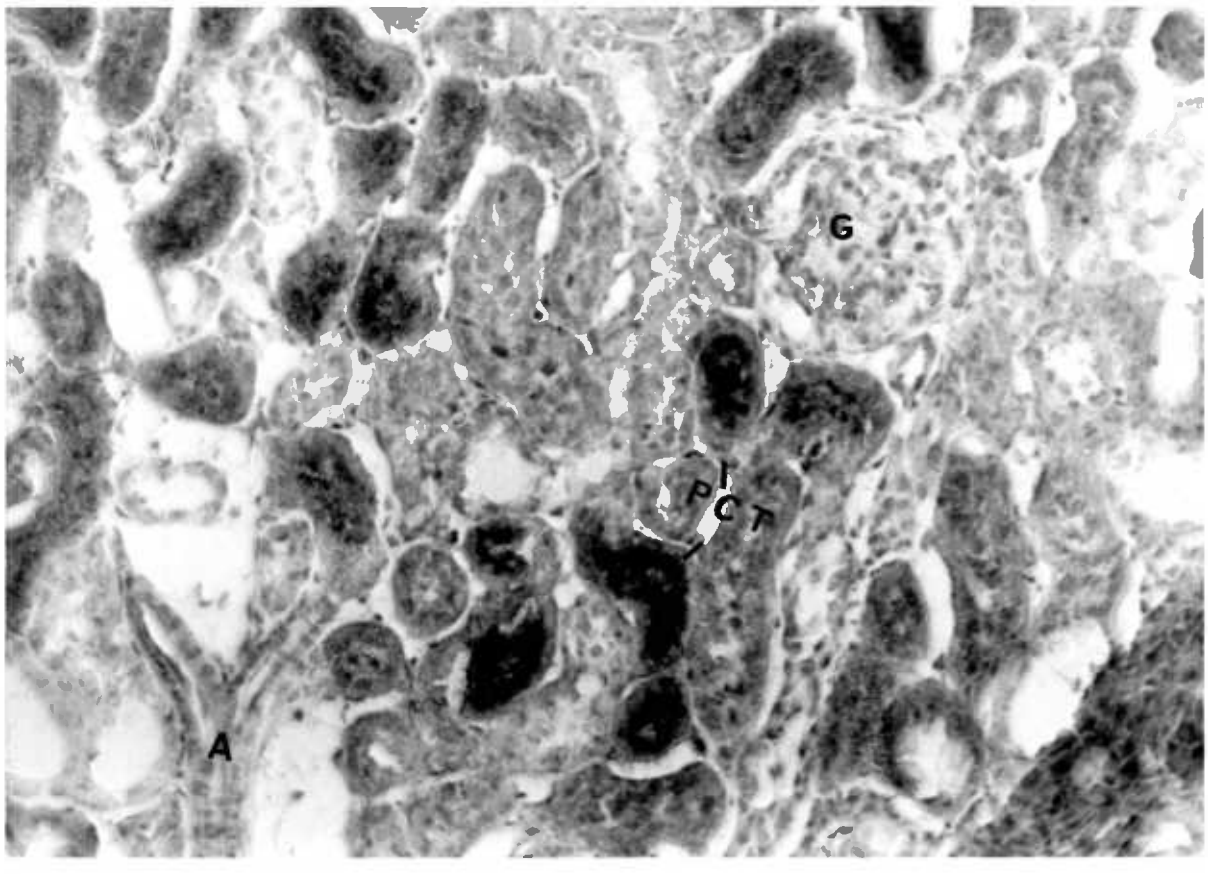

Fig. 4.7 Autoradiogram showing ^{125}I-rTSH concentration in proximal tubular epithelium and lumen. A = arteriole; G = glomerulus; PCT = proximal convoluted tubule. Magnification x 50. Time after injection 5 min.

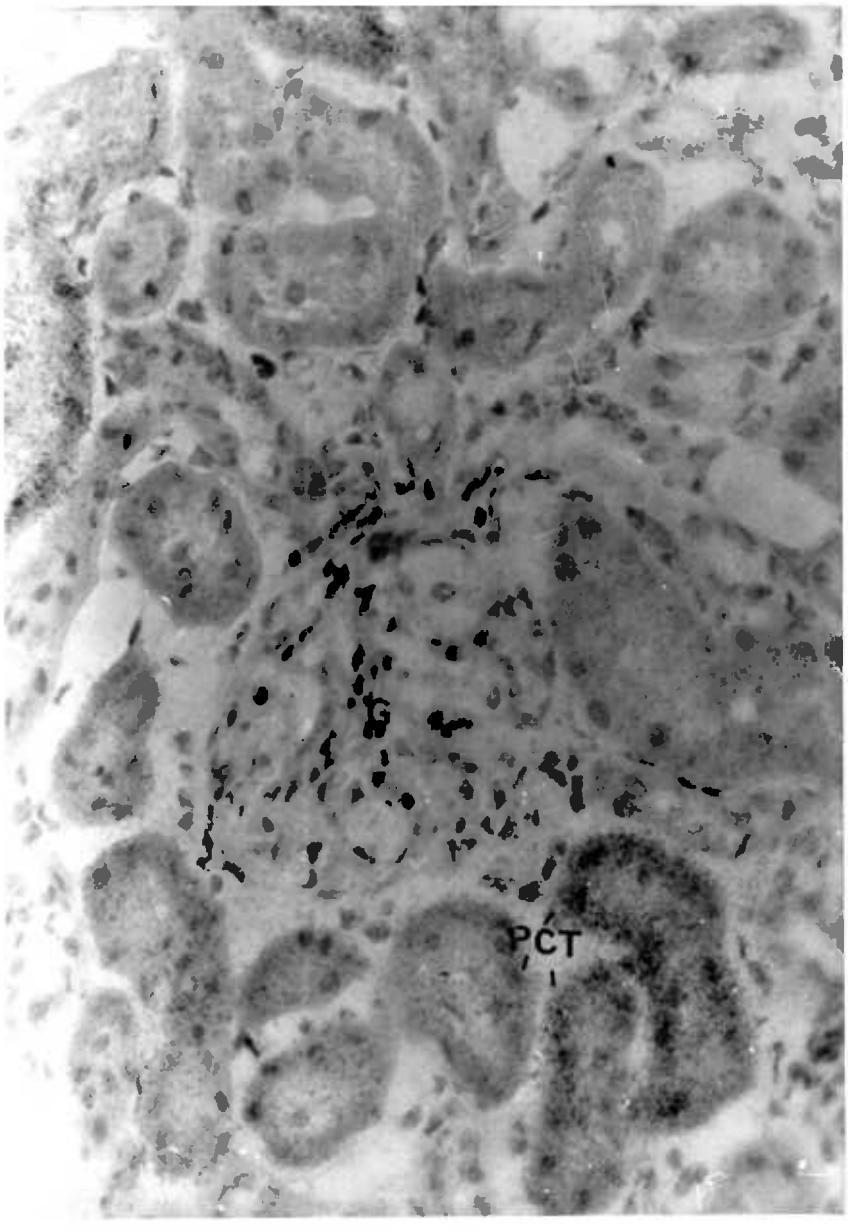

Fig. 4.8 Autoradiogram of rat kidney 60 min. after i.v. injection of ^{125}I-rTSH showing concentration of radioactivity mainly in proximal tubular epithelium. Note also that the silver grain density is less than at earlier times shown in previous autoradiographs. G = glomerulus; PCT = proximal convoluted tubule. Magnification x 320.

(60 and 180 mins.). At later times, in contrast to the earlier times, Ag grains were present over the thick descending limb (straight portion of the proximal tubule)(Fig. 4.9). At later times also Ag grains were found to localize increasingly over the tubular epithelium with little appearing in the tubular lumen (Fig. 4.8). In the collecting ducts and interstitium no signigicant levels of the radioactivity were present all through the period of study.

Autoradiographs obtained on wet-mounted films (formalin fixed tissues) showed a diffuse Ag grain pattern over the kidney cortex (Fig. 4.10). The Ag grains were found mainly over the proximal tubules but less sharply localized, probably due to diffusion of water soluble substances, and less easily interpretable in comparison to the dry-mounted preparations.

Thyroid autoradiographs at 60 and 180 mins showed a diffuse Ag grain pattern, a great majority over follicular epithelium but some over the follicular lumen (Fig. 4.11).

iii) Sephadex gel-filtration analysis:-

Fig 4.12 shows the gel-filtration elution profiles of the injected ^{125}I-rTSH of the saline extracts of the kidney homogenates at 5, 30, 60 and 180 mins after ^{125}I-rTSH injections. The injected ^{125}I-rTSH was eluted in two peaks (Fig.4.12a) at a ratio of 1 : 3 (Peak 1/Peak 2). The first peak corresponds to the peak of blue dextran and thus the void volume and probably represents an aggregate of the molecule. The second peak corresponds to a MW of 30,000 from the calibration curve shown in Fig. 3.2 and is compatible with TSH monomer. Similar Sephadex gel-filtration profile of labelled rTSH from the same source has been reported by Kieffer et al (1974).

The elution profiles of the extracts showed, in contrast, 3 distinct peaks. The first 2 peaks corresponding to those of the injected ^{125}I-rTSH. The third peak corresponds to the salt peak of

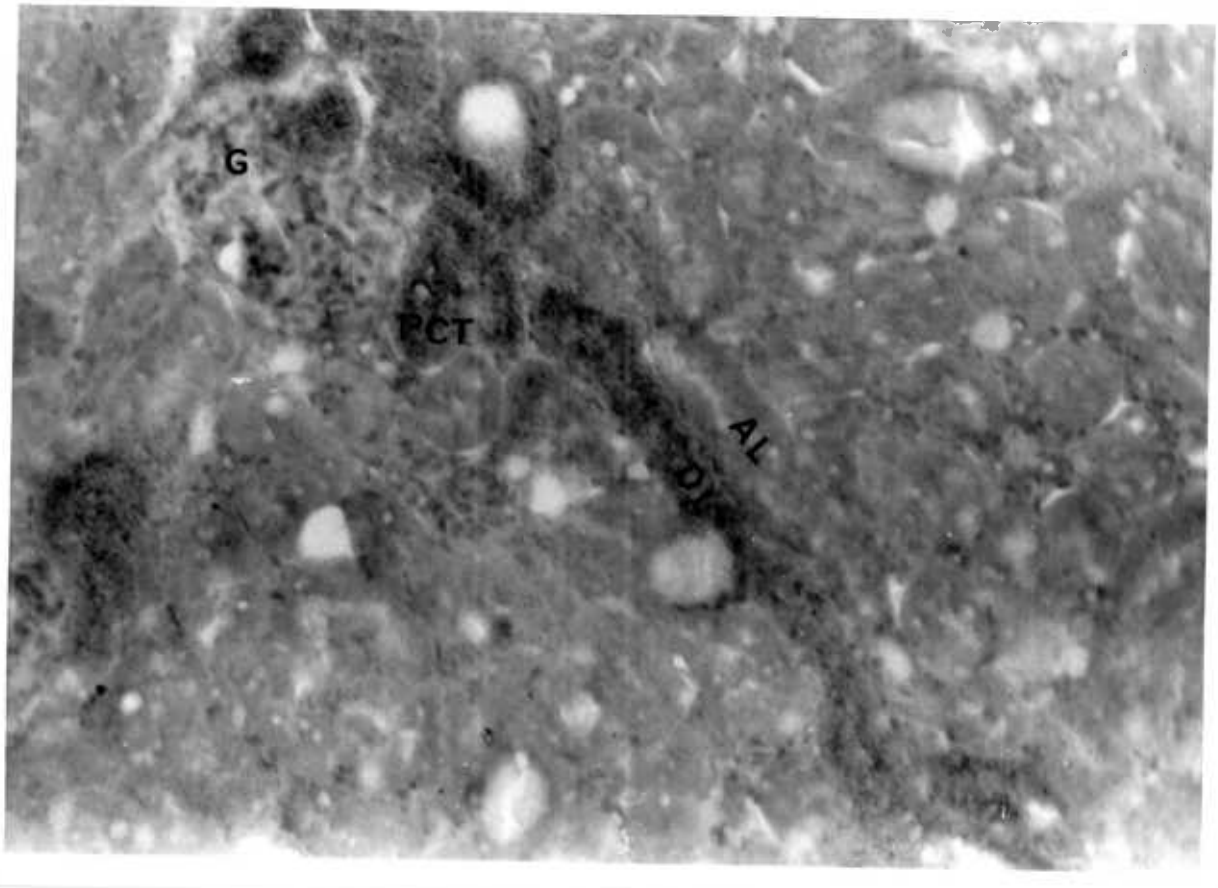

Fig. 4.9 Autoradiogram of rat kidney showing localization of radioactivity in the thick descending limb of the proximal renal tubule 60 min. after ^{125}I-rTSH injection. G = glomerulus; PCT = proximal convoluted tubule; DL = descending limb; AL = ascending limb. Magnification x 50. Note the absence of radioactivity in the ascending limb.

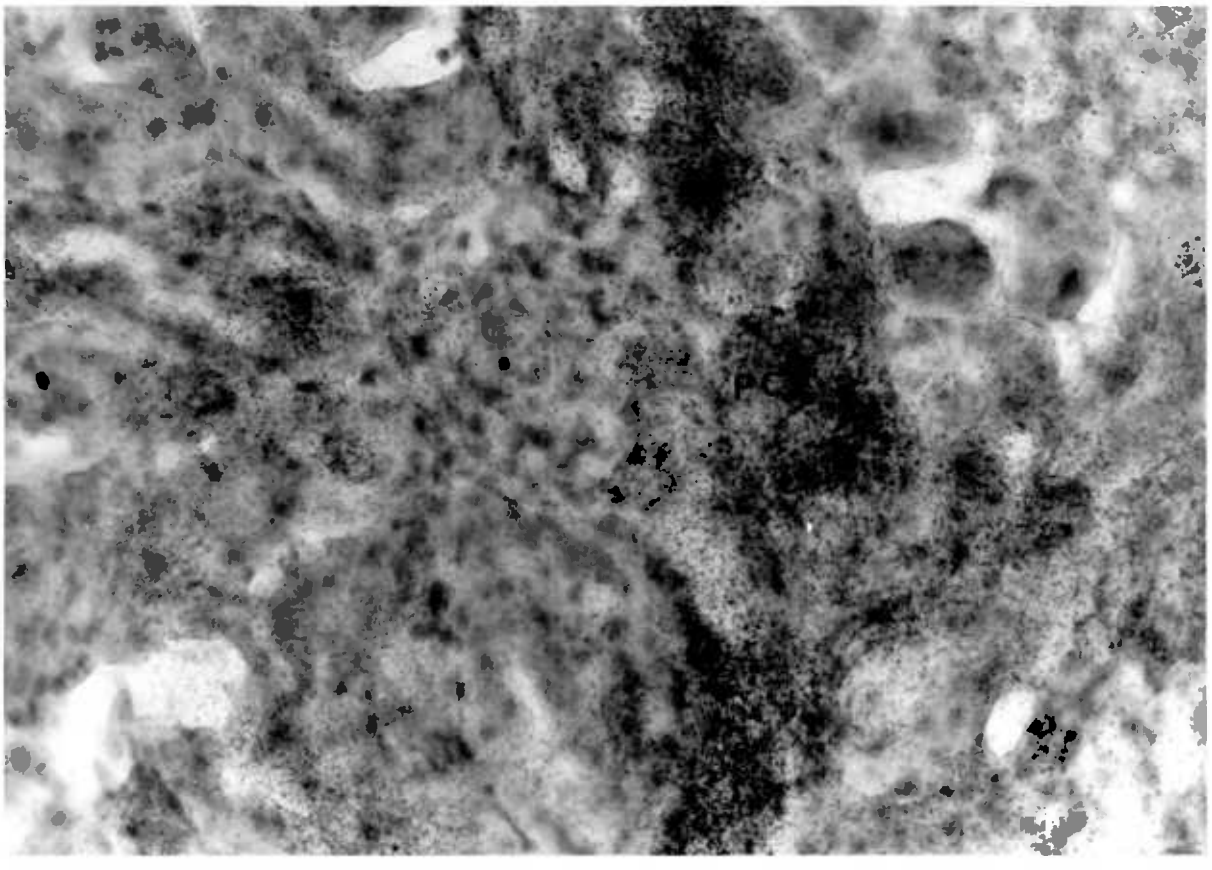

Fig. 4.10 Autoradiogram prepared from formalin fixed paraffin sections of rat kidney. Note the diffuse Ag grain pattern due to solvent action. Magnification x 80. G = glomerulus; PCT = proximal convoluted tubule.

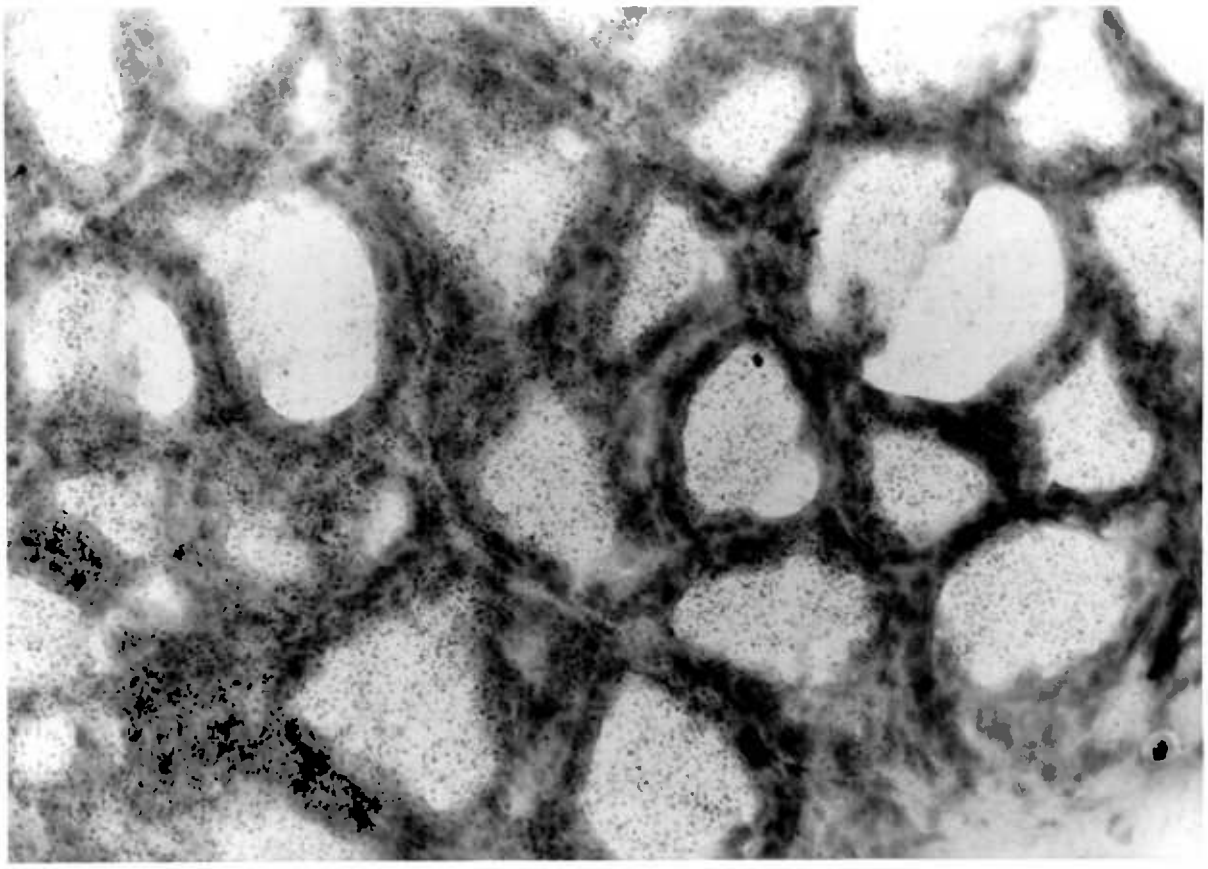

Fig. 4.11 Autoradiogram of rat thyroid, 60 min. after i.v. injection of ^{125}I-rTSH, showing localization of radioactivity in follicular epithelium (mainly) and lumen. Magnification x 80.

the column and may represent ^{125}I and/or ^{125}I-labelled monoiodotyrosine which would not be resolved by this system. Fig. 4.13 shows that peaks 1 and 2 diminished while peak 3 increased with time. By 180 mins most of the radioactivity (78%) was in peak 3. It is also shown that peak 2 diminished faster than peak 1.

4. Discussion.

These results obtained using homologous hormone in physiological doses demonstrate that with these doses, the kidney is the major site of localization of injected ^{125}I-rTSH since the concentration gradient in the kidney was far in excess of any other tissue. This study has also removed the doubt about the physiological significance of other reports on the metabolism of the hormone using large unphysiological dose of heterologous hormone.

The uptake of TSH by renal tissue is not peculair to that hormone only, since the kidney also appears to play a similar quantitatively major role in the fixation of other peptide hormones e.g. insulin, glucagon, prolactin, ACTH (Kassenaar et al, 1959) and GH (Collip et al, 1966). However, the renal uptake of the hormones appears specific for these biologically active substances since uptake patterns were quite different from that observed when HSA was injected.

Significant amounts of the hormone were also taken up by the liver. Allowing for the relatively larger mass of the liver, the amount of ^{131}I-rTSH appearing in it would equal that appearing in the kidney. The kidney, however, would appear to be far more important than the liver in the metabolism of the hormone because the organ/blood ratio, a measure of how actively the organ was concentrating the hormone, was much higher for the kidney than the liver. Most reports e.g. by Sonenberg et al (1952), D'Angelo (1956), Levey and Solomon (1957) and Altschuller (1968), have tended to overestimate the role of the liver

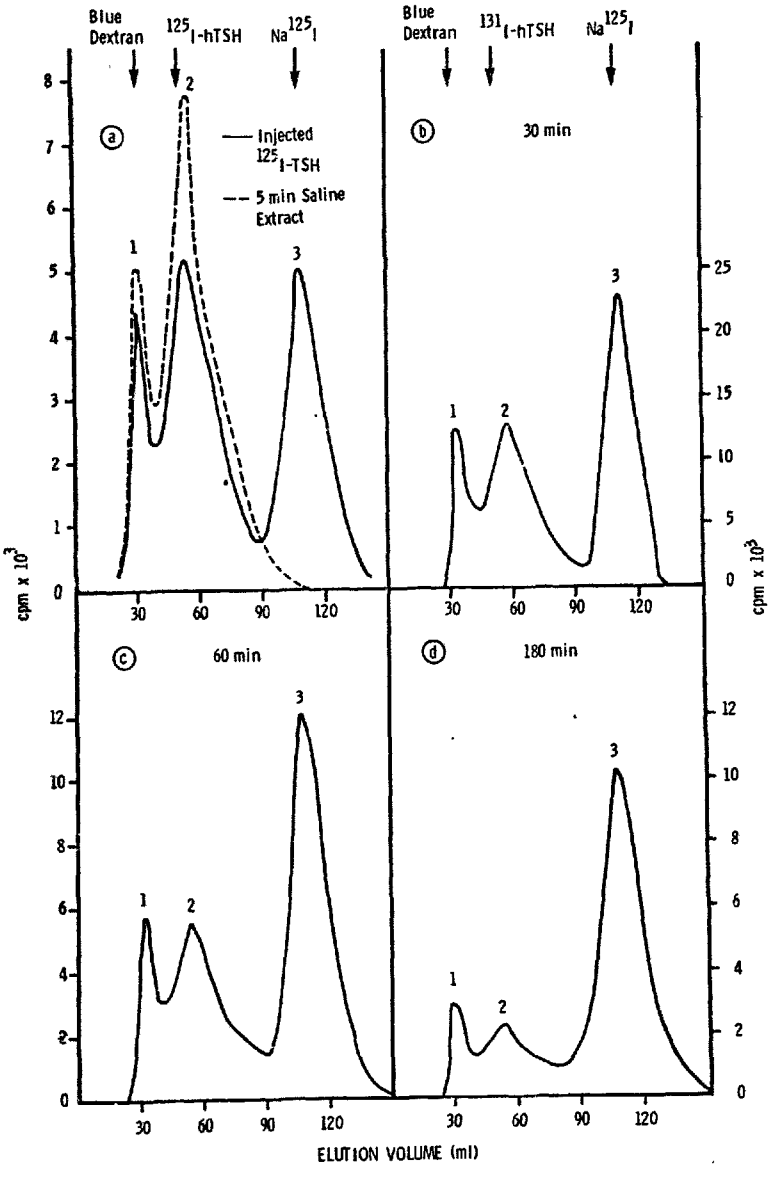

Fig. 4.12 Gel-filtration elution profiles of saline extracts of kidney homogenates of rats sacrificed at 5 (a), 30 (b), 60 (c) and 180 (d) mins after an intravenous injection of ^{125}I-rTSH. A profile of the ^{125}I-rTSH injected is also shown in (a). The 50 x 2cm Sephadex G100 column, calibrated as described in Chapter 3, was eluted with 0.1M barbitone buffer pH 8.6 at room temperature. 3ml fractions were collected. The peaks are marked 1,2 and 3. Peak 2 is slightly beyond the ^{131}I-human TSH (hTSH) marker, suggesting that rat-TSH monomer has a lower molecular weight than the human.

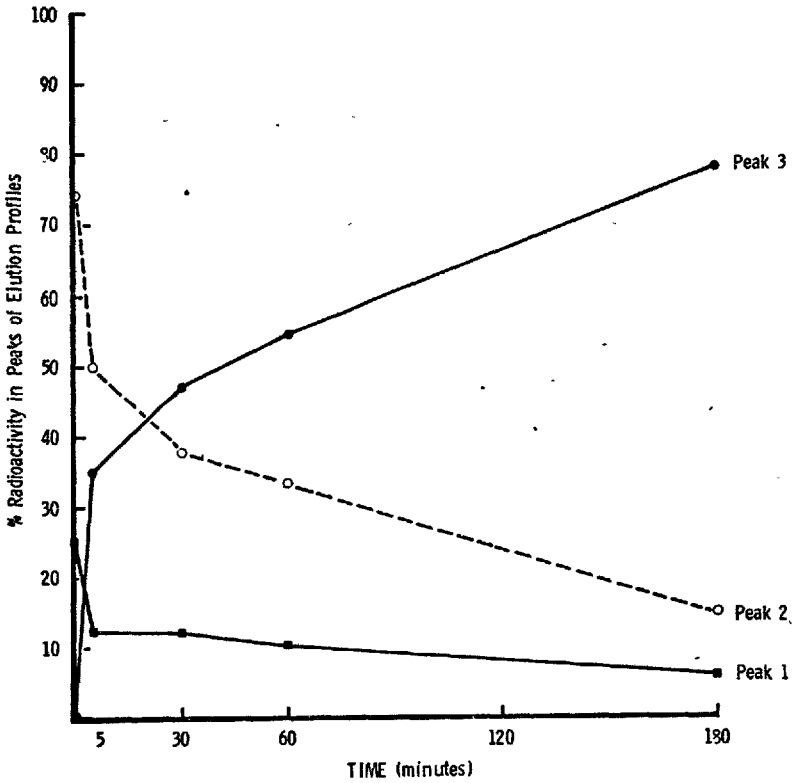

Fig. 4.13 A composite analysis of Fig. 4.12 showing the evolution of radioactivity in the various peaks with time. Each point represents the percentage of the total area of the profile, for each time, represented in each peak.

by considering it as a whole organ rather than in terms of its ability gram for gram to concentrate the hormone from the blood. A possible hepatic role in the inactivation of the hormone was, however, suggested by the report of Levey and Solomon (1957) who found that during a 5 hour perfusion of an isolated rabbit liver, there was a 29% loss of the hormone from the perfusion fluid.

A significant amount of TCA-precipitable radioactivity was localized in the thyroid gland, and this increased with time. The amount of radioactivity in the glands of the control (non-KI treated) rats was far in excess of that in the KI treated rats, suggesting that most of this radioactivity was ^{131}I taken up by the gland as it was released from the metabolism of the ^{131}I-rTSH in the periphery. That the ^{131}I was TCA-precipitable was probably due to the fact that it was already organified and bound to thyroglobulin. When our results are compared with those of others, notable differences emerge. Altschuller et al (1968) also used radioiodine labelled TSH and found significant localization of radioactivity in the thyroid. In fact their figures were similar to those we obtained for our non-KI pretreated rats (they showed total counts present in the thyroid glands at 180 mins as about 2% of the injected dose. This corrected as percent dose/g of thyroid would be perhaps higher than our figures). Clearly these workers measured released ^{131}I rather than intact ^{131}I-TSH bound to thyroid tissue. Other workers, notably Kassenaar et al (1956) could not demonstrate significant amount of TSH in thyroid tissue. Bakke and Lawrence (1962) found measurable amounts of TSH only in goitrous glands. However, Sonenberg et al (1952) found ^{32}S-TSH localization in the thyroid. The KI-pretreated rats also showed localization of activity in the thyroid gland. However, although the rats were given 10 - 20 times their daily requirements of iodine, we could not be sure that their glands were completely blocked, and what proportion of the

radioactivity found was recycled iodine.

Increased TCA-precipitable radioactivity was demonstrated in the pituitary gland, the highest concentration being found 10 mins after the injection, but the highest organ/blood ratio occurring at 60 mins. These findings agree with those of Bakke and Lawrence (1962) who found their highest concentration occurring 9 mins after the injection. However, Altschuller et al (1968) did not find a significant localization of ^{131}I-TSH in rat pituitary glands. The localization of ^{131}I-TSH in pituitary glands found in this study may suggest that the TSH stored in the gland may be bound in a reversible and dynamic equilibrium awaiting elution by the factors governing its release. Holub et al (1959) and Kitay et al (1959) described an analogous situation in which pituitary corticotrophin content rose following corticotrophin injection in normal or adrenalectomised rats. The localization of ^{125}I-TSH in the pituitary gland supports the hypothesis by Kitay et al (1959) that a trophic hormone may have a direct effect on the pituitary that may contribute to its regulation. Motta et al (1969) have already reported the presence of a "short feed-back loop" involving the hypothalamus, the pituitary and TSH in the regulation of TSH secretion.

The localization of radioactivity in the testis was minimal, thus confirming that the material used in this study was not contaminated with gonadotrophins.

No significant localization of TCA-precipitable radioactivity in the hypothalamus was found in this study. This disagrees with the findings of Bakke and Lawrence (1962) who measured TSH in the hypothalamus of injected rats. Neither did the muscle nor fat contain any significant amount of radioactivity. The insignificant localization of TSH in adipose tissue is of interest because the only known extrathyroidal effect of TSH is its lipolytic activity (Freinkel,1961). However, only minute quantities of the hormone are required for this (Jungas and Ball, 1962).

The half-life (13 mins) of ^{131}I-rTSH found in this study was similar to that found by Bakke and Lawrence (1962) (14 mins) who measure the disappearance of bovine and a crude preparation rat TSH from rat serum by bioassay. However, D'Angelo (1951, 1955) found much shorter half-lives of only 2 - 5 mins for bovine TSH in rats. These differences may reflect the differences in assay methods, and the larger doses of hormone used in the latter study. However, the disappearance from the blood of a bolus injection of material may be a function of the following factors. (a) Initial mixing within the intravascular compartment (b) distribution in extravascular fluids (c) uptake by various tissues (d) excretion and (e) re-entry into the circulation. In view of all these possible factors, single bolus injections for metabolic studies can only be interpretated with caution. Most of the above could be eliminated by carrying out a constant infusion of the hormone designed to saturate these various compartments (Tait, 1963). In the study of the metabolism of TSH in man various precautions were taken in the light of the above and these are described in the next chapter.

The kidney is partly concerned with the metabolism of some compounds and also with the reabsorption or elimination of the intact compounds or their metabolites in water soluble form. Therefore the experimental methods selected in the investigation of these metabolic processes must be very carefully selected. Of the two methods of tissue preparation for autoradiography tested, the procedure outlined by Appleton (1969) for the study of cellular uptake and localization of water soluble compounds was found more satisfactory. The wet-mounting method, even though the tissues were fixed in formalin vapour, caused movement of radioactivity when the tissues were submerged in water and during the period of drying.

The results suggest that it is possible to trace the major pathway

in the rat for the removal of ^{125}I labelled rTSH from the blood and its metabolism in the rat following intravenous administration. Within the kidney ^{125}I-rTSH localized in the cortex and the radioactivity remained attached to the cortical tissue during the course of this study. The absence of ^{125}I radioactivity in the medulla and hilus as indicated in the gross autoradiographs established that ^{125}I- TSH was not eliminated by renal excretion in significant amounts during the period of study.

The radioactivity present in the cortex localized exclusively in the proximal convoluted tubules initially, with some subsequently reaching the straight portion of proximal tubules. The radioactivity in the proximal tubules could originate by glomerular filtration or by transport from peritubular blood vessels. Evidence for glomerular filtration as the major pathway was obtained from the analysis of light microscopic autoradiographs. Although the presence of radioactivity within the glomerular tuft was minimal, the presence of radioactivity within the base of the Bowman's capsule, perhaps due to a collapse of capsular cavity, and the presence of radioactivity over the proximal tubular lumen 2 - 5 mins after injection in addition to the initial absence of radioactivity at the basal region of the tubular epithelium suggested that intravenously injected ^{125}I- TSH was filtered very rapidly by the glomerulus. Also, the presence of radioactivity at the luminar end of the tubular epithelial cells at early times and the gradual build up of radioactivity within the epithelial cell at later times indicated readsorption of radioactivity from the proximal tubular lumen, by the epithelium. This evidence as well as the absence of high levels of radioactivity in the loop of Henle, the distal tubules and collecting tubules support the evidence presented for almost complete reabsorption of hormone and minimal excretion into urine. That TSH is excreted into human urine in very small amounts has already been shown

in Chapter 3 and these findings lend support to it.

Proximal tubule localization has been demonstrated by autoradiography for other protein hormones e.g. growth hormone (Collip et al, 1966; Rabkin et al, 1973), insulin and glucagon (Nahara et al, 1958). Maunsbach (1966 a,b) has shown that the proximal tubules also reabsorb other non-hormonal proteins such as albumin and ferritin. Therefore the proximal tubule would appear to be a common pathway for the reabsorption of proteins in general.

The radioactivity demonstrated over the thyroid gland by autoradiography was found both over the follicular epithelium and in the follicular lumen. Since TSH is believed to act on cell membranes and not to cross them the presence of radioactivity in the lumen of the follicule is probably not due to ^{125}I-rTSH. The most likely source of the radioactivity would be free ^{125}I, liberated during the metabolism of hormone peripherally and trapped in the gland. Our distribution studies showed that appearances of radioactivity in the thyroid increased with time and the radioactivity over the gland could be considerably reduced by blocking the thyroidal iodine uptake using KI pretreatment. The fact that radioactivity was still found in the follicular lumen would suggest that even at the level of KI given (10 - 20 times daily requirements) the glandular uptake of iodine was not completely blocked.

The results of the Sephadex gel-filtration experiments show that the kidney catabolised ^{125}I- TSH progressively with time. The appearance of a third peak which progressively increased in height while the others diminished would support this concept. The third peak corresponds to the total volume of the column as measured with ^{125}I. ^{125}I monoiodotyrosine is also eluted in about the same peak. It would therefore appear that the kidney metabolises ^{125}I- TSH either by de-iodination or by complete breakdown to aminoacids and peptides, with perhaps the tyrosine being then deiodinated. The latter theory would appear to be

the case for the following reasons: as shown in Chapter 3 Sephadex gel-filtration analysis of urinary TSH showed that it was largely in fragmentary form and only 0.2% of injected human TSH appeared in urine. Odell et al (1967b) in their studies of ^{131}I-TSH metabolism in man recovered all injected radioactivity in 12 - 24 hours, but not in TCA-precipitable form, suggesting it was mainly ^{131}I they recovered.

CHAPTER 5

HUMAN TSH KINETICS AND BIOLOGICAL EFFECTS IN EUTHYROID MALES

1. Introduction.

The metabolism of TSH in man has been studied using bovine TSH (Bakke et al, 1962) and ^{131}I labelled human TSH (Odell et al, 1967b; Beckers et al, 1971; Ridgway et al, 1974; Cuttelod et al, 1974). The use of bovine TSH would assume that the heterologous hormone was metabolised in the same way as the human hormone, while using the labelled hormone also assumes that labelled and unlabelled hormones are metabolised identically. Bolus injection studies as used by Odell et al (1967b) must assume that the hormone is instantly distributed through its volume of distribution and also that a steady state exists during the period of study. Thus there is need for data on TSH metabolism not dependent on these assumptions.

Human TSH suitable for intravenous infusion recently became available from the Medical Research Council, London, and, using this material and a steady state intravenous infusion as described by Tait (1963), we have studied the kinetics of infused human TSH, and the effect on the serum and urine levels of T_4 and T_3 in normal males.

To compare the kinetics of TSH and those of the other pituitary glycoprotein hormones (LH and FSH) and because data on these latter hormones using unlabelled human hormones are few, the kinetics of these hormones have also been studied in a similar way to that of TSH.

2. Subjects and Methods.

(a) Subjects:- Subjects were normal euthyroid and eugonad male volunteers, aged 29 - 35 years, who were members of staff of the Hammersmith Hospital, London.

(b) Infusion Procedures:-

(i) Human TSH:- 2 units of human pituitary TSH (MRC 70/9) were

infused intravenously to each of three subjects as detailed in Chapter 3. Serial blood samples were taken from 15 minutes immediately before the injection, during and up to 9 hours after the infusion, as shown in Fig. 5.1, for TSH, T_3 and T_4 estimations. Urine for TSH, T_3 and T_4 estimation was collected for 12 hours before, during and at 3, 6 and 12 hours after the infusion. There were no untoward reactions during the TSH infusion.

(ii) TRF:- 2 volunteers were also given a bolus injection of 500μg TRF intravenously at zero time and then infused with 500μg TRF for 30 mins, half an hour after the bolus injection. Blood was taken at frequent intervals for TSH determinations as shown in Fig. 5.2.

(iii) Human LH and FSH:- 505 units and 280 units of human pituitary LH and FSH respectively (both measured by our radioimmunoassay) were injected together into 2 eugonad males as follows:- For LH, one quarter of the dose was given intravenously at zero time, followed by infusion of the rest at a rate of 1.6u/min. 40% of the FSH dose was injected intravenously at zero time, and the rest infused at a rate of 0.7 unit/min. Both hormones were infused together in 50ml normal saline for 4 hours using an infusion pump. Serial blood samples were taken before, during and after the infusion as shown in Fig. 5.3 for LH and FSH determinations.

(c) Calculation of Kinetic data:-

The half-lives of serum TSH, LH and FSH were derived from a semilogarithmic plot of the serum levels of the hormones expressed as a percentage of the mean hormone value during the equilibrium phase of the infusion of TSH, TRF, LH or FSH. A regression line was fitted through all the points from the time of the last equilibrium point to 300 min (TSH), 125 min (TRF), 480 min (LH) or 720 min (FSH), using a method of least squares. An example of such a method is shown for TSH in Fig. 5.4.

The metabolic clearance rate (MCR) was estimated from the formula (Tait, 1963):

$$MCR = \frac{\text{Infusion rate of hormone per minute}}{\text{Serum hormone concentration at equilibrium}}$$

The production rate (PR) was calculated from:- PR = MCR x i, where "i" is the basal serum concentration of endogenous hormone.
The distribution space (DS) = MCR/K where "K" is the disappearance constant = 0.693/half-life in minutes (Zilversmit, 1960). From the DS, the hormone pool was estimated from the product of the endogenous hormone level and the DS, i.e. DS x i.

(d) Assay Procedures:- Serum TSH, LH, FSH, T_3 and T_4 and urinary "free" T_3 and T_4 were measured by specific radioimmunoassays already described. Protein bound iodine (PBI) was measured by the "Technicon" autoanalyser method.

3. Results.

(a) TSH:- The levels of serum TSH achieved after the infusion of human TSH into normal subjects are shown in Fig. 5.1. The serum TSH levels reached a plateau after 90 mins of infusion and the levels persisted for 5 - 15 mins after the infusion was stopped. Decline of serum TSH concentrations then followed a single exponential for 5 hr in all 3 subjects. The individual results for the kinetic data are shown in Table 5.1. The mean half-life (measured starting from the last equilibrium point) was 76.7 mins while the MCR was 40.3ml/min. The PR averaged 98.8mU/day. The mean DS was 4.5 litres. The exchangeable TSH in this space was 8.4mU.

In the two subjects infused with TRF, an equilibrated level of serum TSH was also achieved and the half-lives were 70 and 90 min (mean 80 min) (Fig. 5.2).

The mean urinary clearance of TSH during the period of exogenous

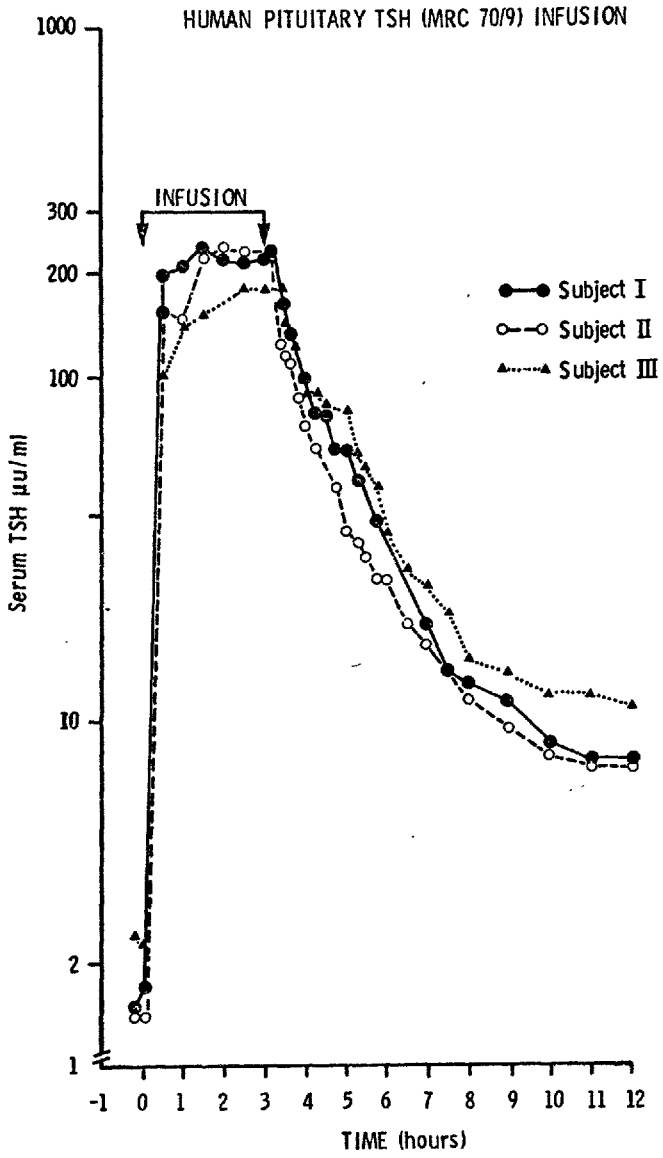

Fig. 5.1 Serum TSH levels before, during and after a three hour intravenous infusion of human thyrotrophic hormone in three normal male subjects.

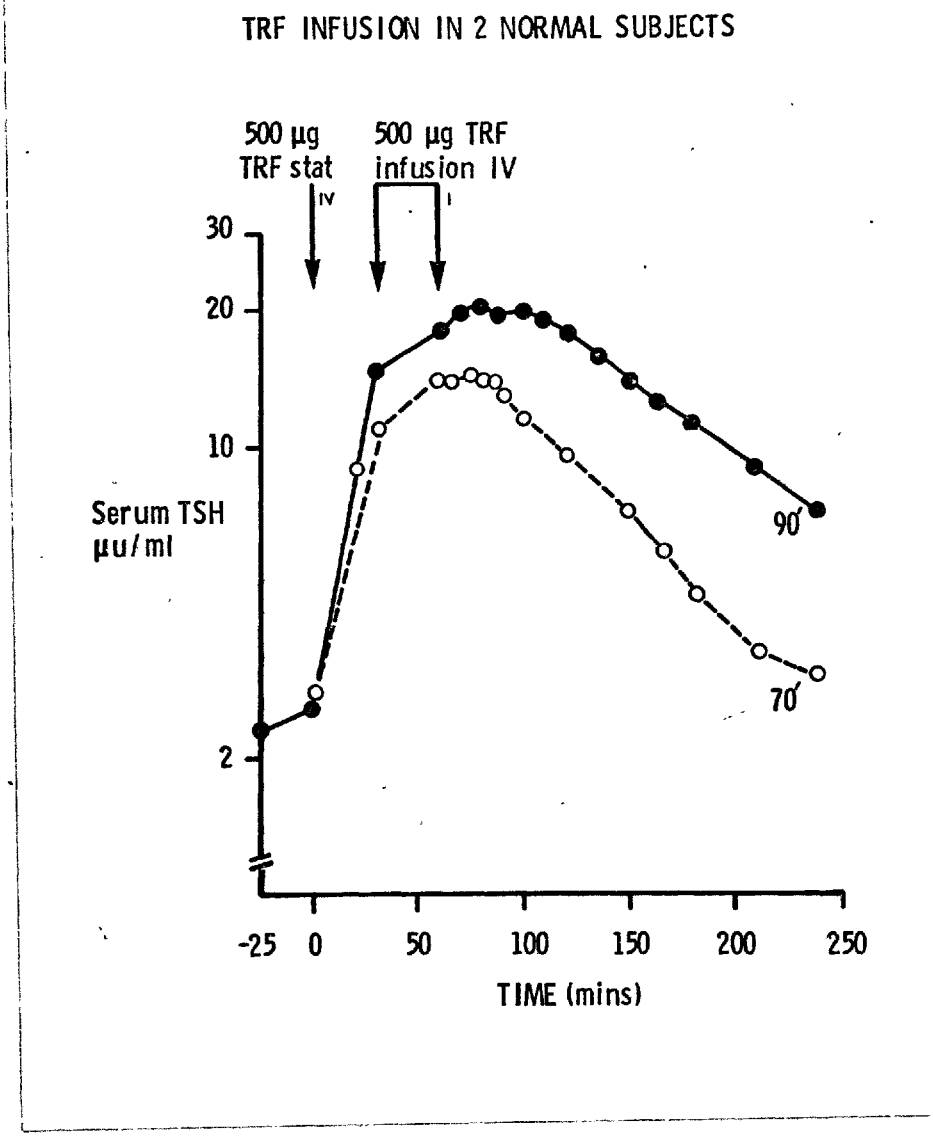

Fig. 5.2 Serum TSH levels before, during and after an intravenous infusion of thyrotrophin releasing factor (TRF) in two normal subjects. 500µg of TRF was given at zero time, followed 30 minutes later by an infusion of 500µg of TRF over 30 minutes. 90' and 70' represent the half-life (in minutes) of TSH achieved in each subject.

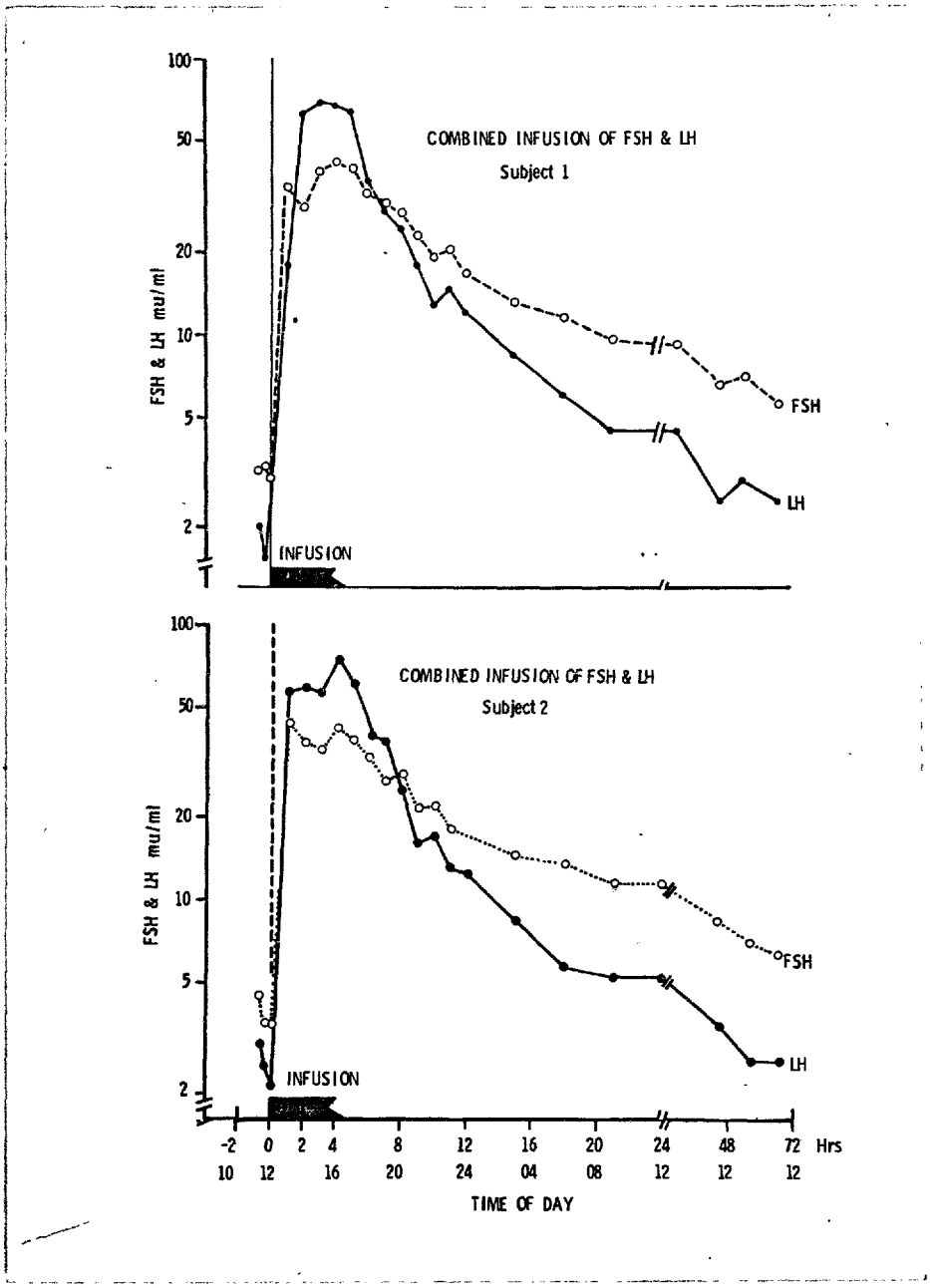

Fig. 5.3 Serum LH and FSH levels before during and after a combined intravenous infusion of human LH and FSH in two normal male subjects.

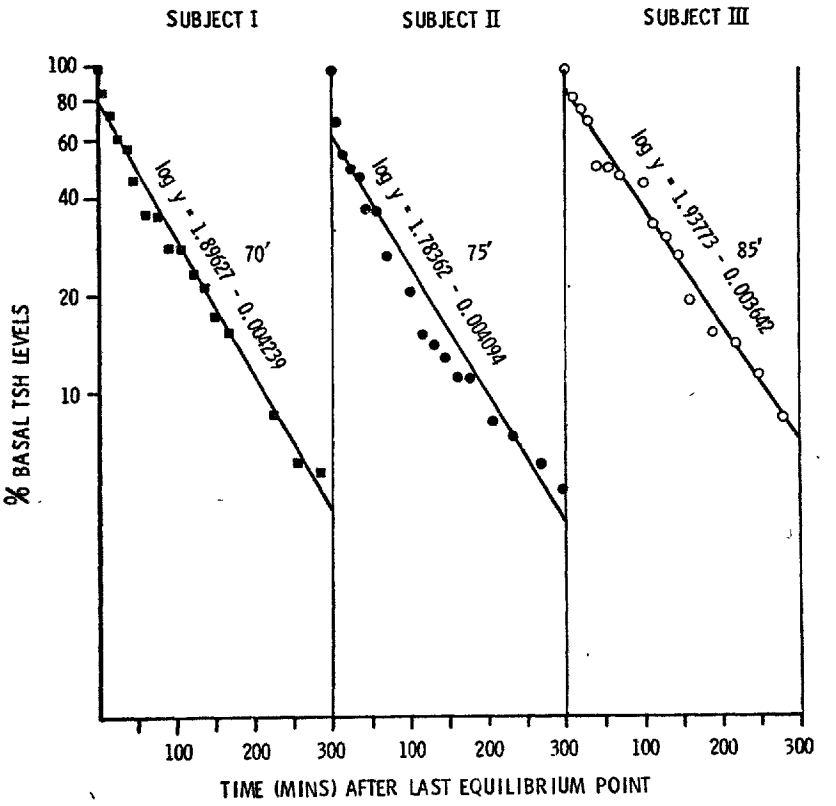

Fig. 5.4 Serum half-life of injected TSH in three normal male subjects. TSH levels, in logarithmic scale, are expressed as a percentage of the baseline value (mean TSH level during the equilibrium phase of the infusion). The time scale indicates the time after the last point in the equilibrium phase. This point varied from 5 - 15 minutes after the end of the infusion (Fig. 5.1). 70', 75' and 85' represent the half-life in minutes of TSH in subject 1, 2 and 3 respectively.

TABLE 5.1

Subject	Basal TSH μu/ml	$t_{\frac{1}{2}}$ (min)	MCR (ml/min)	PR (mu/d)	DS (ml)	TSH pool (mu)	Urine clearance (ml/min)
1	1.6	70	38.0	87.6	3,834	6.1	0.05
2	1.4	75	36.3	73.0	3,945	5.5	0.05
3	2.4	85	46.6	135.8	5,721	13.7	0.025
Mean	1.4	76.7	40.3	98.8	4,504	8.4	0.04

Thyrotrophic hormone kinetics in three normal male subjects.

MCR (ml/min) = Metabolic clearance rate; PR (mu/d) = production rate; DS (ml) = distribution space;

$t_{\frac{1}{2}}$ = half-life in minutes

	TSH	LH	TSH
BL	1.8	2.3 (1.9,2.6)	3.5 (3.2,3.8)
$T\frac{1}{2}$	77	180 (175,185)	398 (377,419)
MCR	40	27.0 (26, 28)	18.5 (18, 19)
PR	99	88.0 (71, 105)	65.0 (59, 71)
DS	4.5	6.9 (7.4,6.4)	10.5 (10, 11)

Table 5.2: Comparison of TSH, LH and FSH Kinetics.

BL = Basal serum hormone levels in µu/ml (TSH) and mu/ml (LH and FSH)

$T\frac{1}{2}$ = half-life in minutes

MCR = metabolic clearance rate in ml/min

PR = production rate in mu/day (TSH) and U/day (LH and FSH). From Table 3.9 1mu TSH $\underline{\sim}$ 1u of LH or FSH in terms of weight.

DS = distribution space in litres. For LH and FSH individual values are shown in brackets.

hormone infusion was 0.04ml/min (Table 5.1).

(b) LH and FSH:- The levels of serum LH and FSH achieved after a combined infusion into male volunteers are shown in Fig. 5.3. Stable LH and FSH levels were achieved in both subjects during the infusion. For LH, the decline followed a single exponential for 8 hrs while for FSH it lasted for 12 hrs. A comparison of the kinetics of TSH, LH and FSH is shown in Table 5.2. The half-life of LH averaged 180 mins which was shorter than the 398 mins for FSH, but longer than the 77 mins for TSH. The MCR was smallest for FSH (16.6ml/min) and largest for TSH (40.3ml/min) with the LH intermediate 27.2ml/min). The PR and DS followed the same order as for MCR, i.e: FSH, LH, TSH.

(c) Effects on T_3 and T_4 levels:- Fig. 5.5 shows the levels of serum T_3 and T_4 achieved in two subjects during the human TSH infusion. The first effect on serum T_3 in both subjects and on serum T_4 in one subject was evident within the first hour of infusion, but the peak levels of these hormones did not occur until 3 - 4 hours after the end of the infusion, when the serum TSH levels had returned to near normal levels. Thereafter, there was a decline of serum levels but at 21 hr after the injection, these levels were still slightly higher than basal. The maximal rise of serum T_3 was proportionately greater, being approximately 3 times the basal level compared with serum T_4, whose maximal rise was 50 - 100% above basal levels.

The urinary free T_3 and T_4 levels, which index the free serum hormone levels, (Burke et al,1972) increased in parallel to approximately double the basal levels, the peak changes being attained 6 - 12 hours after starting the infusion, this coinciding with the peak serum levels (Fig. 5.5).

PBI measured at 0 and 6 hr later gave the following results: 5.0 and 6.0; 4.8 and 5.9 and 5.5 and 6.3µg/100ml for each subject, a mean rise of 0.97µg/100ml (19%).

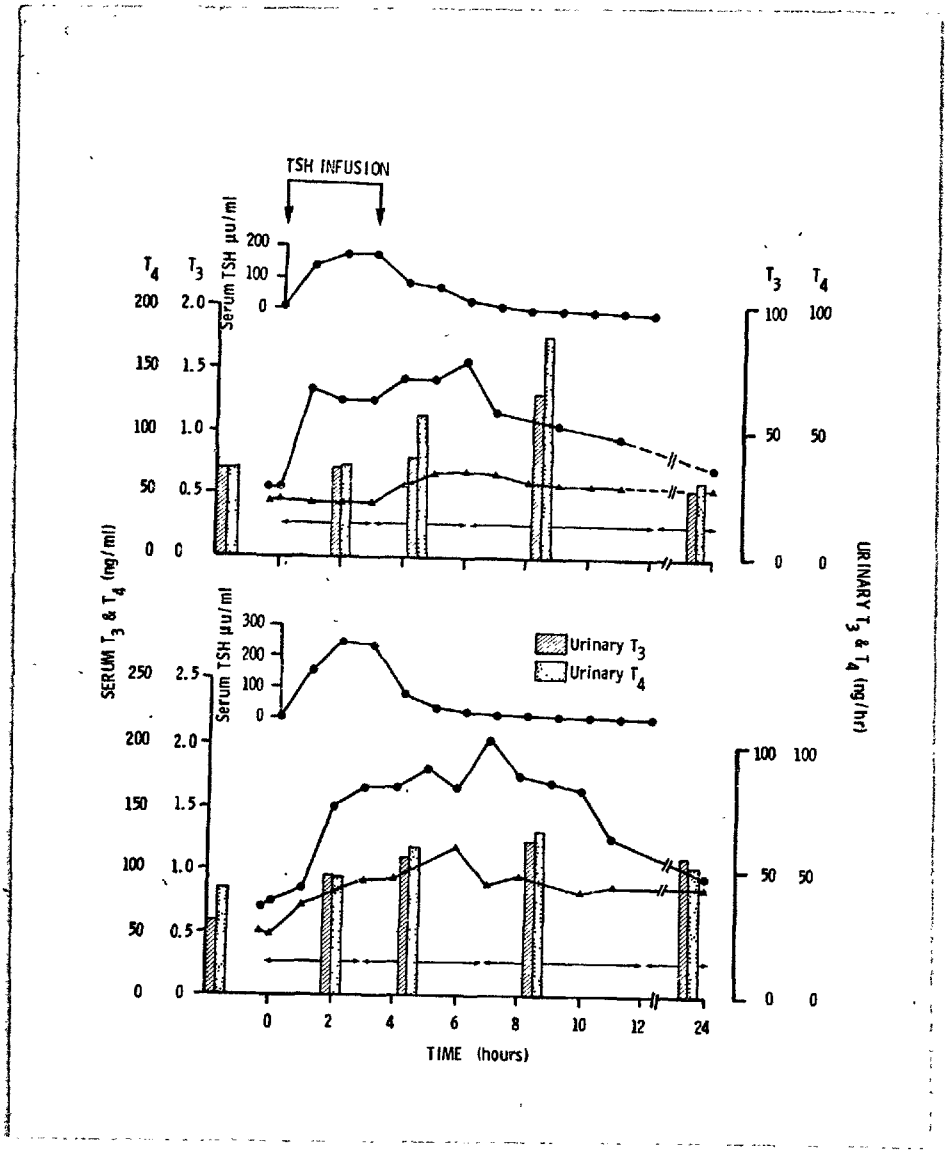

Fig. 5.5 Serum and urinary "free" levels of thyroxine (T_4) and triiodothyronine (T_3) in two of the three subjects shown in Fig. 5.1 before, during and after the infusion of human thyrotrophic hormone.

●——● Serum T_3; ▲——▲ Serum T_4.

4. Discussion.

There are several reasons for suggesting that these estimates of human TSH kinetics in man should be more valid than previously published data. It is rather doubtful that heterologous TSH (Bakke et al, 1962) and labelled TSH (Odell et al, 1967b, Beckers et al, 1971; Ridgway et al, 1974; Cuttelod et al, 1974) would be metabolised as a precise tracer for human TSH. Furthermore, by infusing the hormone until an equilibrium concentration is achieved in serum, its disposal rate, following the stopping of the infusion, approximates to the metabolic disposal rate, for the hormone should have equilibrated with all the pools draining the plasma (Tait, 1963). However, it is still questionable whether the clearance of physiological concentrations of TSH is different from that measured after such large loading infusions as we have used. But observations on other polypeptide hormones (insulin: Stern et al, 1968; GH: Taylor et al, 1969 and FSH: Coble et al, 1969) suggest that this can be valid. Furthermore, the finding that the rate of fall of serum TSH which followed the cessation of a prolonged infusion of TRF was similar to that observed after the human TSH infusion suggests that exogenous and endogenous TSH at the levels achieved were metabolised similarly.

The mean half-life of 77 mins found in this study was 40% higher than that reported by Odell et al (1967b) and Cuttelod et al, (1974) using a bolus injection of ^{131}I-TSH and twice as long as that reported by Bakke et al (1962) using bovine TSH and measuring by bioassay. In our studies with TRF, the infusion was continued until a constant level of serum TSH was achieved, and we found the half-life of endogenous TSH of 80 mins in agreement with the exogenous TSH half-life. Hershman and Pittman (1971a) found the half-life of TRF-stimulated serum TSH to be 58 and 76 mins in 2 normal subjects. A closer look at their results shows that a serum TSH equilibrium was

achieved in the subject who had a serum TSH half-life of 76 mins, which is similar to our findings. The other subject in whom an equilibrium was not achieved had a much shorter half-life of TSH (58 mins).

An MCR for human TSH of 40.3ml/min found in this study was similar to that reported by Odell et al (1967) (42.5ml/min) but is smaller than the figures of 56.2ml/min and 50.7ml/min reported by Beckers et al, (1971) and Ridgway et al (1974) respectively. Our mean PR of 99mu/day is similar to that reported by Ridgway et al (1974) but lower than Odell's value of 165mu/day. The values we report for DS and TSH pool are similar to those found by Odell et al (1967). A summary of TSH kinetics reported in the literature is shown in Table 5.3.

The urinary clearance of the infused material (0.04ml/min) was low when compared with that of other glycoprotein hormones. After infusing human pituitary LH, Marshall et al (1973) found a clearance of 3.4ml/min. Keller (1966) reported values of 0.1ml/min for LH and 0.58ml/min for FSH. For HCG, Loraine (1950) reported values of 0.95ml/min. We have previously reported lower urinary excretion rates of TSH in comparison with LH and FSH in Chapter 3.

For TSH, with an MCR of 40ml/min and a urinary clearance of 0.04 ml/min, urinary excretion cannot account for more than 0.1% of the MCR showing that the hormone is almost completely metabolised in the body. The major organ for this has been shown in the previous chapter as the kidney.

In the kinetic studies of LH and FSH, the hormones were given simultaneously intravenously on the assumption that the metabolism of one hormone would not effect that of the other. Coble et al (1969) have shown that this assumption is valid. They found that the MCR of labelled LH or FSH was the same, whether the hormones were injected

TABLE 5.3

Reference	Hormone	Injection Method	$t_{\frac{1}{2}}$ min	PR mu/day	MCR ml/min	DS litres	Pool mu
Bakke et al (1962)	b TSH	Bolus	35	109	-	4.0	-
Odell et al (1967b)	^{131}I-hTSH	Bolus	54	165	43	3.3	8.7
Beckers et al (1971)	^{131}I-hTSH	Bolus	-	419	56	-	-
Hershman and Pittman (1971a)	TRF	Bolus	67	-	-	-	-
Cuttelod et al (1974)	^{131}I-hTSH	Bolus	56	179	31	2.4	9.9
Ridway et al (1974)	^{131}I-hTSH	Infusion	-	104	51	-	-
Present study	TRF	Infusion	80	-	-	-	-
" "	hTSH	Infusion	77	99	40	4.5	8.4

TSH kinetics reported by various workers in man compared with those found in the present study.

b = bovine. h = human. $t_{\frac{1}{2}}$ = half-life. PR = production rate. MCR = metabolic clearance rate. DS = distribution space.

singly or simultaneously. The LH half-life of 180mins is of the same order as that reported by Marshall et al (1973) from this laboratory, using identical conditions as we have used here, but is considerably longer than the values of between 30 and 80 mins reported by Parlow (1965), Schalch et al (1968) and Kohler et al (1968), using labelled LH in a single i.v. injection. In the same way, the half-life of 398 mins reported here for FSH is considerably longer than the 180 mins reported by Parlow (1965) again using a bolus injection of iodine labelled FSH. These findings for LH and FSH as well as for TSH, suggest that studies of kinetics following bolus injections of labelled hormones may underestimate the half-life of the hormone and may therefore be invalid.

The differences found in the half-lives of TSH, LH andFSH may be due to differences in sialic acid content of the hormones which is roughly proportional to their half-lives. Van Hall et al (1971) showed that progressive desialylation of HCG markedly reduced its half-life in plasma. Also, the half-life (35 mins) of bovine TSH, which contains no sialic acid, in human plasma (Bakke et al, 1962) is much shorter than the 54 mins reported for human TSH by Odell et al (1967) both using bolus injection techniques.

The MCR for LH of 27.2 ml/min is of the same order as the 21.3ml/min and the 22.9ml/min reported by Marshall et al (1973) and Kohler et al (1968) respectively. The MCR for FSH of 17ml/min is slightly higher than the 11ml/min reported by Coble et al (1969). As is to be expected, the MCR and PR for TSH, LH and FSH were in inverse relationship to their half-lives.

We have also confirmed the biological potency of the human TSH preparation in man by observing rises in serum and urinary T_3 and T_4, and also of PBI in blood. The proportional rise in serum T_3 levels was greater and more rapid than that of T_4. This is in agreement

with the reports of Larsen (1972) who used bovine TSH, and Hollander et al (1972) and Lawton (1972) using TRF. Shimoda and Greer (1966) have also shown preferential TSH stimulation of *in vitro* iodine incorporation in T_3 as opposed to T_4 in rats. However, the approximately parallel rise in the urinary "free" T_3 and T_4 levels suggests that the increased secretion may have effected free serum T_3 and T_4 levels equivalently. The serum thyroxine binding globulin which binds mainly T_4 may have minimized the proportional effect on serum T_4. More subjects need to be studied to be sure of this.

CHAPTER 6

TSH RESPONSIVENESS TO TRF IN NON-THYROIDAL ENDOCRINE DISEASE

1. Introduction.

The need for a "combined" test of anterior pituitary function.

TSH secretion after synthetic TRF has been extensively studied both in normal subjects and in patients with thyroid and hypothalamo-pituitary disease (Fleischer et al, 1970; Ormston et al, 1971; Hershman and Pittman, 1971a; Anderson et al, 1971, Haigler et al, 1971; Hall et al, 1972, Ormston, 1972; Gual et al, 1972). Similarly LH and FSH responses to LHRF have been reported both in normal subjects and in some patients with abnormalities of hypothalamo-pituitary function (Marshall et al, 1972; Kastin et al, 1972; Besser et al, 1972; Yen et al, 1972; Nillius and Wide, 1972).

Most workers have found that the releasing hormones are specific, secretion of other pituitary hormones being unchanged after TRF and LHRF (Fleischer et al, 1970; Ormston et al, 1971; Kastin et al, 1971; Besser et al, 1972). Increased GH levels after TRF have however been noted in some subjects (Anderson et al, 1971; Faglia et al, 1973; Samaan et al, 1974) and both increase and decrease in plasma cortisol has been reported (Karlberg et al, 1971; Rotherbuchner et al, 1971). Both ACTH and GH are effected by stress, however, so it remains uncertain as to whether these changes relate to specific effect of the releasing hormones.

Assessment of pituitary secretion of GH and ACTH is also commonly performed by GH and cortisol measurement during insulin-induced hypoglycaemia. This in addition to the use of the releasing factors now allows the evaluation of "pituitary reserve" for these trophic hormones.

There is little data on the inter-actions of these different stimuli in man. Besser et al (1971) reported that TSH responses to TRF were not affected by coexistent insulin hypoglycaemia. Similarly, GH responses to insulin were unchanged by addition of TRF but cortisol responses were slightly greater after TRF and insulin than after insulin alone. The present study is designed to establish whether all three

tests could be combined into a single procedure, without any significant alteration of the hormone responses, compared to those produced by each agent individually.

Evaluation of pituitary function has usually been based on the performance of each provocative test seriatim, involving the patient in considerable amount of time, and often admission to hospital. A single test would thus be of benefit to the patient who would only be required to attend the hospital for a few hours, and to the hospital, which would be spared the need to provide in-patient care.

TSH responsiveness in pituitary disease associated with excessive pituitary hormone production

Most reports on the serum TSH response to an intravenous injection of TRF in euthyroid acromegalic patients agree that as many as half of the patients have an absent or impaired response to the releasing hormone. Of the 9 untreated euthyroid acromegalics studied by Hall et al (1972) only 4 showed a normal response, while one had an exaggerated response, and in 4 the response was impaired. In their treated patients only 3 of 16 euthyroid patients had a normal response. Faglia et al (1973) studied 21 euthyroid acromegalics. Only 12 patients respondend normally, while 4 showed an impaired response and the other 4 had an absent response. Tunbridge et al (1973) showed that of 18 euthyroid patients with acromegaly, as many as 10 (56%) had subnormal TSH responses before treatment, while 67% of euthyroid acromegalics had subnormal responses post treatment. However, Schalch et al (1972) in their study of 19 euthyroid acromegalics (13 untreated) and 20 normal controls could not find a significant difference in the responses between the two groups.

In contrast, the incidence of TSH unresponsiveness in euthyroid patients with other pituitary tumours is much lower at about 25% (Hall et al, 1972; Tunbridge et al, 1973). Various postulates could be put forward in an attempt to explain thehigh incidence of TSH unresponsiveness in acromegaly. By definition, the impaired response of TSH

would imply a diminished pituitary TSH reserve capacity. Rotherbuchner (1972) has shown that the pituitary can be stimulated to produce TSH for long periods of time (about 3 hours) by a constant infusion of TRF. It would be of interest to investigate if non-responders could be made to secrete TSH to the normal range by an infusion of TRF. If so, it would imply that the pituitary reserve is normal, but that control of hormone secretion is faulty.

Since 6 of the 19 euthyroid non-responders in the report of Hall et al (1972) had autonomous nodular goitres, they have postulated that the hormones secreted from these glands may suppress TSH response to TRF in a way analogous to the situation seen in hyperthyroidism (Ormston, 1972). However, it remains to be proved that the peripheral thyroid hormone levels of the non-responders are greater than those of the responders. The free (unbound) levels of thyroid hormone if higher in the group of non-responders could be the explanation. This needs testing.

Could high circulating levels of growth hormone inhibit the response of the thyrotrophs to TRF stimulation? Root et al (1970) have shown that short-term administration of GH was followed by a decline in 24hr ^{131}I neck uptake in 8 of 15 children. They also found that plasma TSH levels decreased by 20 - 40% in 2 boys with primary untreated hypothyroidism after GH administration. Macleod et al (1966, 1968) have demonstrated decreased thyroid function and low pituitary TSH content in rats bearing GH-prolactin secreting tumours suggesting that either GH or prolactin may inhibit TSH secretion. Recently Root et al (1973) have shown that in 9 children with short stature on acute GH trial, serum TSH response to TRF was significantly less on the ninth day after daily GH administration, compared with baseline values. All the above data would suggest the GH may inhibit TSH secretion. It is not known whether prolactin excess would inhibit TSH secretion in man.

This study is designed to investigate the above postulates in an

attempt to clarify the mechanisms involved in the high incidence of TSH unresponsiveness in acromegaly, and to investigate the effect of high circulating levels of prolactin on the secretion of TSH in man.

Although TRF is well established as producing a rise in the levels of both serum TSH (Fleischer et al, 1970) and prolactin (Jacobs et al, 1971, 1973; L'Hermite et al, 1972), the regulatory mechanisms involved in the control of these TRF effects now appear to be different and dissociable. Rappaport et al (1973) have shown that T_3 suppressed the serum TSH response to TRF in hypothyroid patients but not in the serum prolactin response. Similarly the prolactin response was the same in euthyroid subjects before and after treatment with T_4 (Synder et al, 1973). In thyrotoxicosis where no response of serum TSH to TRF is the usual finding (Hershman and Pittman, 1971a; Ormston et al, 1971) the prolactin response remains, although slightly lower than normal (Toft et al, 1974).

While glucocorticoids, in large doses, have been reported to increase prolactin secretion <u>in vitro</u> (Johnson et al, 1955; Ben-David et al, 1964), recent evidence shows that they depress basal serum TSH levels in normal and hypothyroid subjects. Wilber and Utiger (1969) found that, after 2 - 8mg of dexamethasone, the basal serum TSH levels fell by 23 - 96% in hypothyroid patients, and 18 - 47% in normal subjects at 24 - 48 hours. Nicoloff et al (1970) also found suppression of basal serum TSH levels in euthyroid subjects given 60mg prednisolone daily for three days. Haigler et al (1971) reported that administration of 8mg dexamethasone for two days in four hypothyroid subjects suppressed their basal TSH levels without affecting their TSH response to TRF; a finding similar to that reported by Wilber and Utiger (1969) with rats. Acute corticosteroid-induced inhibition of TSH secretion at the hypothalamic level was therefore suggested. However, Otsuki et al (1973) have reported that in patients on long term corticosteroid replacement therapy and in three patients with Cushing's syndrome due to adrenocortical adenoma, serum TSH responses to

TRF were subnormal. This would suggest that prolonged corticosteroid suppression of TSH is mediated through the pituitary gland. But other data are few on TSH responsiveness in untreated Cushing's syndrome whether pituitary or adrenal in origin. The one patient studied by Hall et al (1972) did not show a serum TSH response while Hershman and Pittman (1971a) found that their one Cushing's disease subject responded normally. In this study, we report measurements of TSH and prolactin responses to TRF in Cushing's disease before and TSH responses after its treatment by pituitary implant with ^{198}Au.

TSH responsiveness in euthyroid diabetics with moderately elevated serum TSH levels

In surveys of apparently normal population a small incidence of subjects showing either positive thyroid antibodies or raised TSH levels or both, usually without other evidence of abnormal thyroid function, has been found (Dingle et al, 1966; Bastenie et al, 1967; Fowler et al, 1970; Evered et al, 1973). Raised serum TSH level in the face of euthyroidism has been called subclinical hypothyroidism (Evered et al, 1973) but insufficient long follow-up data are available to assess whether these are always pointers of more serious thyroid disease. The incidence of this abnormality rises with age and among diabetics (Hunton et al, 1965; Bastenie et al, 1967).

During a survey of mild euthyroid diabetics carried out in this hospital (Kaufman et al, 1974) mildly raised serum TSH levels were found in 35 of the 206 (16.9%) patients studied. We have confirmed the reality of these mildly elevated serum TSH levels by TRF stimulation tests.

Therefore we have planned in this study to (1) evolve new tests of anterior pituitary function, namely the "combined" test and the long TRF test, (2) use the long TRF and the well-established short TRF test to ellucidate (a) the TSH responsiveness in euthyroid pituitary disease

associated with excessive serum levels of pituitary hormones, namely GH, prolactin and ACTH and (b) the nature and mechanisms involved in the secretion of TSH and prolactin mediated through one releasing hormone, TRF.

2. Methods and Subjects.

(A) Test Procedures:-

(i) The combined test:- The combined test was begun between 09.00 and 10.00 hr. after an overnight fast. The subjects were recumbent and an indwelling forearm venous cannula was inserted 30-60 mins before the basal blood samples were taken. Soluble insulin (0.05 - 0.3 units/ kg body weight) was injected through the cannula and followed immediately by a mixture of 200µg of TRF and 100µg of LHRF in 5mls sterile distilled water. Blood samples were taken at 30, 60, 90, 120 mins for determination of glucose, GH and cortisol, and at 20 and 60 mins for TSH, FSH and LH.

For the insulin hypoglycaemia test the same pre-test procedure was followed and samples taken at 30, 60, 90 and 120 mins. for glucose, GH and cortisol. After both the combined and insulin hypoglycaemia tests a carbohydrate rich meal was given to the patients after the last blood sample. Out-patients were observed for 1 - 2 hours and if believed to be panhypopituitary they were also given 5mg prednisone before being allowed to go home.

The separate LHRF and/or TRF tests were performed on non-fasted patients. The releasing hormones dissolved in sterile water were given by rapid intravenous injection. Samples for the appropriate hormone estimations were taken immediately before and 20 and 60 mins. after the injection. In each subject the same doses of releasing hormones or insulin were used in the individual tests and in the combined test.

(ii) Short TRF test (i.e. the TRF test incorporated in the combined test) This test was performed on non-fasting ambulant subjects with 200µg of

synthetic TRF. At zero time blood was taken for TSH, T_4 and/or PBI, and prolactin (untreated Cushing's disease patients) and 200µg TRF dissolved in 1ml sterile distilled water injected rapidly intravenously. Blood was taken for TSH and prolactin (untreated Cushing's disease patients) 20 and 60 mins. later.

(iii) Long TRF tests (i.e. the short TRF test followed by a TRF infusion):- The non-fasting subjects were recumbent and an indwelling forearm venous cannula was inserted. Basal blood samples were withdrawn for TSH, PBI and T_4 determinations. 200µg TRF was then rapidly injected intravenously and blood subsequently withdrawn at 20 and 60 mins. At 60 mins. 500µg TRF in 20ml normal saline was then infused at a constant rate for 30 mins using an "LKB" infusion pump. Further blood samples for TSH were withdrawn at 90, 120, 180 and 240 mins. from zero time.

With the long TRF tests urinary studies were also carried out on all acromegalic subjects and on 4 normal subjects. Urine was collected basally (12hr prior to test) and also during the test (4hr) and for 6hr after the test for TSH and T_3 determinations.

(iv) Acute growth hormone tests:- 10 units of MRC human pituitary GH (HWP8) clinical grade was given intramuscularly daily for 6 days. On day 1, a short TRF test was performed prior to the GH injections. A second test was performed 2hr after the last GH injection. Venous blood was withdrawn for TSH determination at standard times (0, 20, 60 mins).

(v) Side effects:- All the subjects given insulin experienced symptoms of hypoglycaemia and 50% of those given TRF noted some nausea or flushing and a desire to micturate. No other side effects were noted.

(B) Subjects:-

(i) The "combined" test:- 4 normal men and 20 patients with hypothalamic or pituitary disease were studied. The diagnoses of the patients based on clinical, radiological and biochemical evidence are shown in Table 6.1. 4 normal subjects and 7 patients had both combined test

Diagnosis		Number
Acromegaly, untreated	(o)	5
Acromegaly, post ^{90}Y implantation	(•)	3
Chromophobe adenoma, untreated	(△)	4
Chromophobe adenoma, post ^{90}Y implantation	(▲)	1
Diabetic retinopathy, post ^{90}Y implantation	(*)	3
Isolated gonadotrophin deficiency	(■)	2
Hand-Schüller-Christian, diabetes insipidus	(x)	1
Functional amenorrhoea	(▼)	1
Normal males		4

Table 6.1: Subjects studied in the "Combined" Test.

The patient with functional amenorrhoea had had episodes of depressive illness, and no hypothalamic-pituitary abnormality was demonstrated.

The symbols refer to the same subjects as in Figs. 6.3 and 6.4.

and also individual tests performed separately in random order on different days. The interval between the separate tests was between 2 and 7 days. The combined test was paired with simultaneous administration of LHRF and TRF in 10 patients with LHRF alone in 2, and with TRF alone in one.

(ii) Short TRF tests: 20 normal subjects (30 tests) were studied to establish the normal range of response of TSH in serum to TRF. All the subjects were members of staff of the Hammersmith Hospital, London, and aged between 20 - 40 years.

24 euthyroid untreated acromegalics were also studied. The diagnosis of acromegaly was based on clinical, radiological (enlarged pituitary fossae) and biochemical (failure of high basal GH levels to suppress to $<5\mu u/ml$ following an oral load of glucose) evidence of the disease.

10 patients with prolactin-secreting pituitary tumours were also studied.

8 patients with clinical and biochemical evidence of Cushing's disease were studied. Laboratory diagnosis of Cushing's disease was based on a high urinary free cortisol which was suppressed to at least 50% of basal values when the patients were given 8mg dexamethasone daily for 3 days, and urinary 17-oxogenic steroids which rose to at least double basal levels after metyropone given 750mg orally 4 hourly for 6 doses. All the patients were clinically and biochemically euthyroid. 4 of these subjects were studied again 6 - 12 months after treatment with pituitary implant of ^{198}Au when urinary free cortisol levels had returned to normal. 2 other such subjects were also studied only after successful treatment of their Cushing's disease.

Finally, 14 euthyroid mildly diabetic patients with moderately elevated serum TSH levels were also studied.

(iii) Long TRF tests:- For these tests 8 normal subjects were studied.

11 consecutive euthyroid untreated acromegalics were also studied, as were 2 euthyroid patients with pituitary tumours, and 2 Cushing's disease patients.

(iv) Acute GH tests:- 3 male subjects were studied. 2 were dwarfs, aged 10 and 18 years, with isolated growth hormone deficiency. The third subject was a normal adult aged 30 years.

(C) Glucose, PBI and Hormone Assays:-

Serum TSH, FSH, LH and GH, and urinary TSH and free T_3 were measured as described in Chapters 2 and 3.

Serum prolactin was measured by a specific double antibody homologous radioimmunoassay (Nader et al, 1974). Urinary free cortisol was measured by a competitive protein binding assay, (Beardwell et al, 1968), while plasma cortisol was measured by a modification of the urinary method for plasma (Marshall et al, 1972b).

Blood glucose and PBI were measured using the "Technicon" auto-analyser methods (ferricyanide for glucose and wet-digest for PBI) in the Department of Chemical Pathology, Royal Postgraduate Medical School; they also kindly measured urinary 17-oxogenic steroid by the Zimmerman colorimetric method.

3. Results.

(i) Standardization of TRF tests.

(a) Normal serum TSH responses in the short TRF test:- Table 6.2 shows a summary of the results of 30 short TRF tests performed on 20 normal subjects. The mean increment was defined as the mean of the 20 and 60 minute value less the basal value. From these normal values, a response less than 5.1μu/ml at 20 mins was graded as impaired, as also was a mean increment less than 4.5μu/ml. A mean increment less than 1μu was graded as "nil" or absent.

TABLE 6.2

	Absolute levels µu/ml			Mean Increment µu/ml
	Basal	20 min	60 min	(Mean of 20 + 60 min) - Basal levels
Mean ± SD	1.8±0.8	12.2±5.0	9.6±3.9	9.8 ± 4.2
Range	<1-3.7	5.1-25.0	3.8-21.5	4.5 - 20.4

Serum TSH response to the TRF (short tests) in 20 normal subjects (30 tests).

(b) Normal serum TSH responses in the long TRF test:- The response of serum TSH to long TRF tests in 8 euthyroid subjects is shown in Fig. 6.1. The first 60 min. of the test being analogous to the short TRF test, shows the same pattern with the latter. At the end of the infusion, the levels of TSH between 90 and 120 min. were higher than the peak response at 20 min. by 50 - 300%. In one subject, the peak value occurred at the end of the infusion, i.e. 90 min., while in 4 others the peak value did not occur until 30 min. after, i.e. 120 min. In the other 3, the values at 90 and 120 min. were about equal.

The increment in serum TSH levels was defined as the mean of the values between 20 - 120 min. less the basal value. The increment values are shown in Table 6.3. The mean increment was 13.2 $\pm$ 7.0 (SD) range 5.2 - 27.3uu/ml.

(c) Normal urinary TSH and free T_3 responses in the long TRF test:- The responses of urinary TSH and free T_3 during the long TRF test in 4 euthyroid subjects are shown in Table 6.3. The peak values of TSH occurred during the test, i.e. when the serum TSH values were highest and reached values 50 - 200% above basal values. 3 of the 4 normal subjects showed a rise in urinary free T_3 which was used here as in indirect measure of the serum free T_3, which it reflects (Burke et al, 1972). The peak response of urinary T_3 occurred in the third urine collection in 2 of the 3 responders. For TSH, the increment was defined as the level during the test less the basal value; this averaged 6.9 $\pm$ 3.7 (SD) uu/hr (range 3.6 - 12.2 uu/hr). For T_3, the mean increment was calculated from the mean of the levels during (0 - 4hr) and the last collection of urine (4 - 10hr) less the basal level. This averaged 99 $\pm$ 130.3 (SD) ng/hr (range 20 - 294 ng/hr).

(ii) Practicability of the combined test of anterior pituitary function:

All the subjects given insulin experienced adequate hypoglycaemia (blood glucose values less than 40 mg% and less than 50% of fasting value).

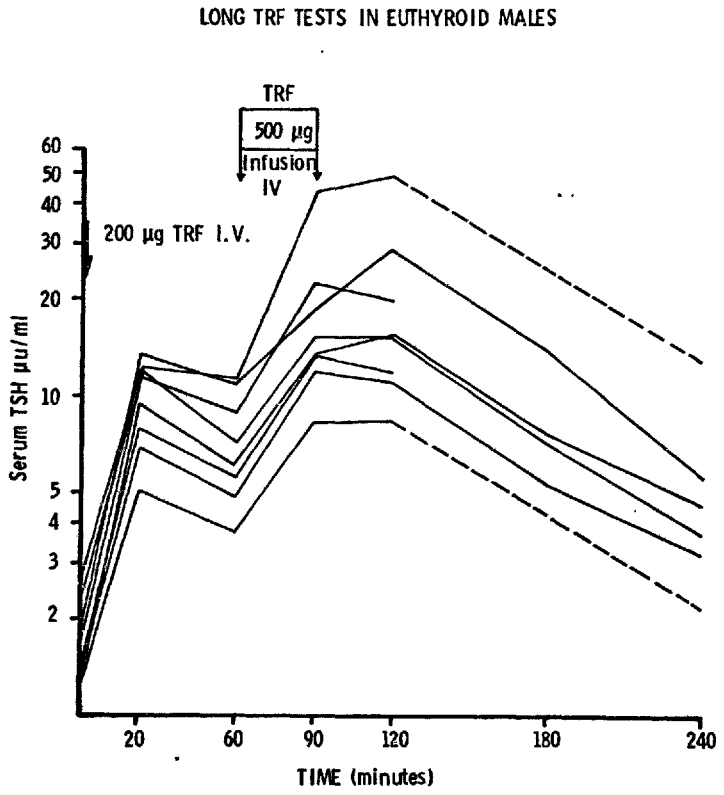

Fig. 6.1 Serum TSH responses in 8 euthyroid males during a long TRF test. 200µg TRF was injected at zero time followed at 60 min. with a 30 minute infusion of 500µg TRF.

TABLE 6.3

Subjects	Serum TSH μu/ml		Urine TSH μu/hr		Urine T_3 ng/hr		PBI ug/100ml	T_4 ng/100ml	GH uu/ml	Goitre
Acromegalics	Basal	Mean Increment	Basal	Increment	Basal	Mean Increment				
1	1.2	0.3	2.0	0.5	67	2	6.8	81	23	+*
2	1.6	1.0	2.5	0.5	51	3	5.8	113	35	-
3	1.4	1.7	6.6	3.2	20	29	5.6	105	500	+*
4	1.6	3.0	3.3	1.0	38	0	5.5	110	91	-
5	<1	6.7	8.5	5.8	72	17	-	100	152	-
6	<1	9.3	5.5	2.8	53	4	-	120	81	-
7	3.6	10.9	4.6	5.1	86	29	7.4	80	125	-
8	<1	11.8	4.9	11.5	41	108	5.8	-	210	+
9	1.8	15.1	6.3	4.9	50	100	5.5	70	152	-
10	2.9	16.6	6.7	5.9	71	94	6.7	80	108	-
11	1.3	17.2	8.0	4.7	52	21	-	115	195	-
Normals										
1	1.3	5.2	-	-	-	-	6.0			
2	1.6	6.2	6.1	5.0	55	46	6.4			
3	1.0	10.2	7.5	6.9	33	294	7.5			
4	1.7	10.7	7.3	3.6	39	37	7.0			
5	2.3	13.5	-	-	-	-	6.3			
6	1.8	15.2	-	-	-	-	6.1			
7	1.2	16.9	4.8	12.2	63	20	5.0			
8	2.2	27.3	-	-	-	-	5.6			

Serum TSH and urinary TSH and T_3 increments in euthyroid untreated acromegalics and normal subjects in response to a long TRF test. Basal values also shown for serum TSH, T_4, GH, PBI and urinary TSH and T_3. The mean serum TSH increment was derived from the mean TSH level from 20-120min less the basal level. The urinary TSH increment = concentration in urine during the test (4hr) less the basal value. While the mean T_3 increment was defined as the mean of the level in the 4hr and 6hr urinary collections less the basal. The GH level is mean of the serum levels found at 60,90 and 120 mins during an oral glucose tolerance test. +* automous goitre.

The mean hormone responses during the combined and single tests in normal subjects are shown in Fig. 6.2. The ranges of normal response shown were obtained previously in 22 normal subjects aged between 20 and 40 years. There was no significant difference in the degree of any of the responses, whether induced by combined or individual administration of the stimuli, and similarly the pattern of response was unchanged.

The results in patients who had both combined and single tests, or a combined test with later LHRF and/or TRF, are shown for those with untreated pituitary tumours in Fig. 6.3 , for those with other untreated hypothalamic-pituitary disorders in Fig. 6.4. Results in patients with pituitary tumours or diabetic retinopathy previously treated by pituitary implantation of Yttrium 90 are shown in Fig. 6.5. No change in the pattern of response was seen in any patient, and to allow direct comparison of the combined and separate stimulation procedures the sum of the serum levels at 20 and 60 min. for LH, FSH and TSH, and the sum at 60 and 90 min for GH and cortisol are shown. In the majority of patients there was little difference in the summated levels seen during the two tests, and analysis by paired t-test showed that responses were not significantly different. (Tables 6.4, 6.5.)

In some patients small differences in responses were seen, but in the case of the glycoprotein hormones this occurred when the hormone levels were low and nearing the limits of detection of the assays. GH responses were the most variable, but no consistent trend nor significant difference was found. One patient with treated acromegaly (Fig. 6.5) was postmenopausal, and this explained the elevated FSH levels seen.

No instances of a gross difference in response were seen, i.e. unresponsive to combined test and responsive in single test, or vice-versa.

(iii) <u>TSH responsiveness in euthyroid subjects with "active" pituitary disease</u>:

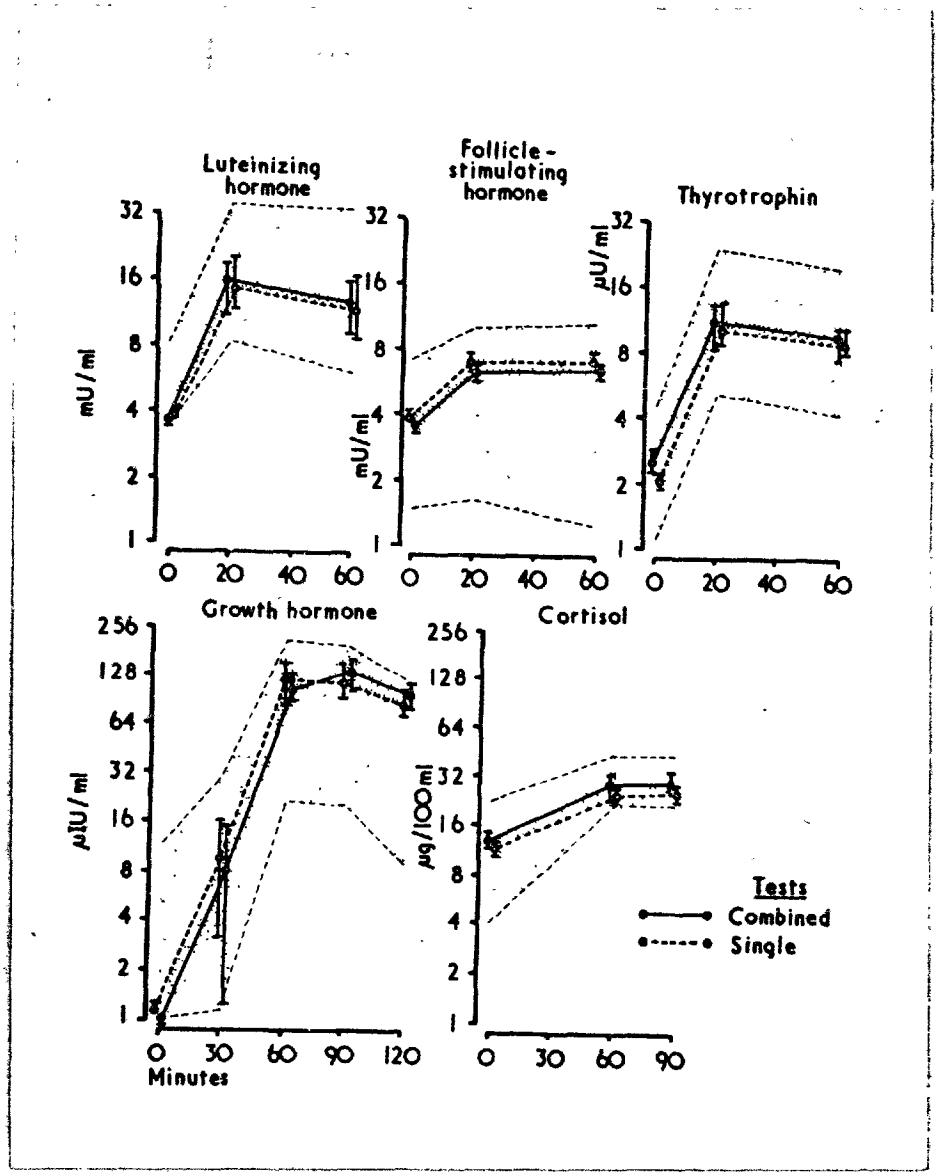

Fig. 6.2 Hormone responses during combined and single tests in four normal subjects. Mean values ± SEM are shown, and the shaded area represents the range of response seen in normal subjects.

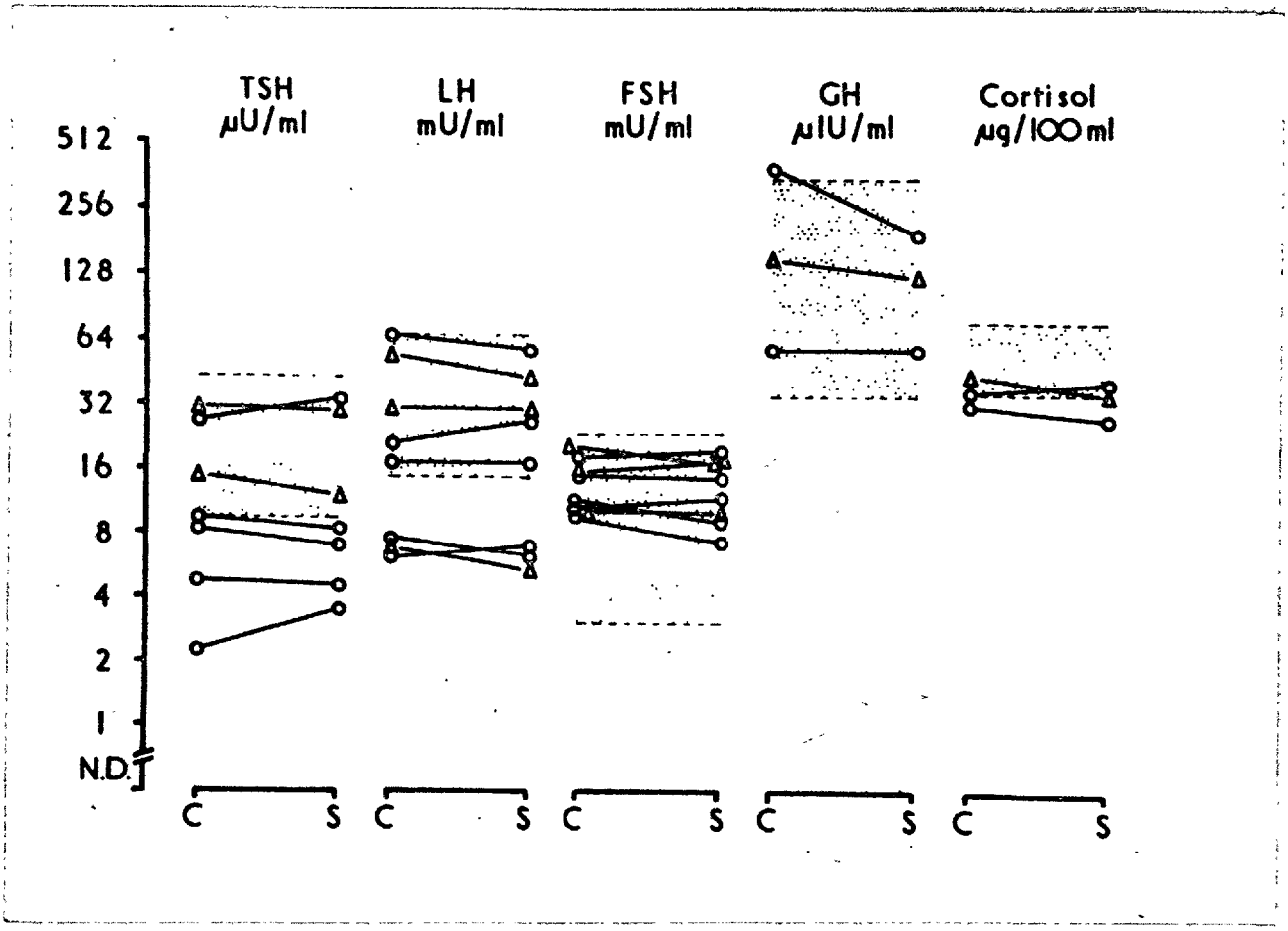

Fig. 6.3 Comparison of the hormone responses in patients with untreated pituitary tumours. Each point represents the sum of the hormone levels at 20 and 60 min. for TSH, LH and FSH, and at 60 and 90 min. for GH and cortisol. The hatched areas represent the ranges found in normal subjects expressed in this form. The values in the left hand columns are from the combined test (c), and those on the right from single tests or LH-RH plus TRH (s). The symbols refer to the diagnoses shown in Table 6.1.

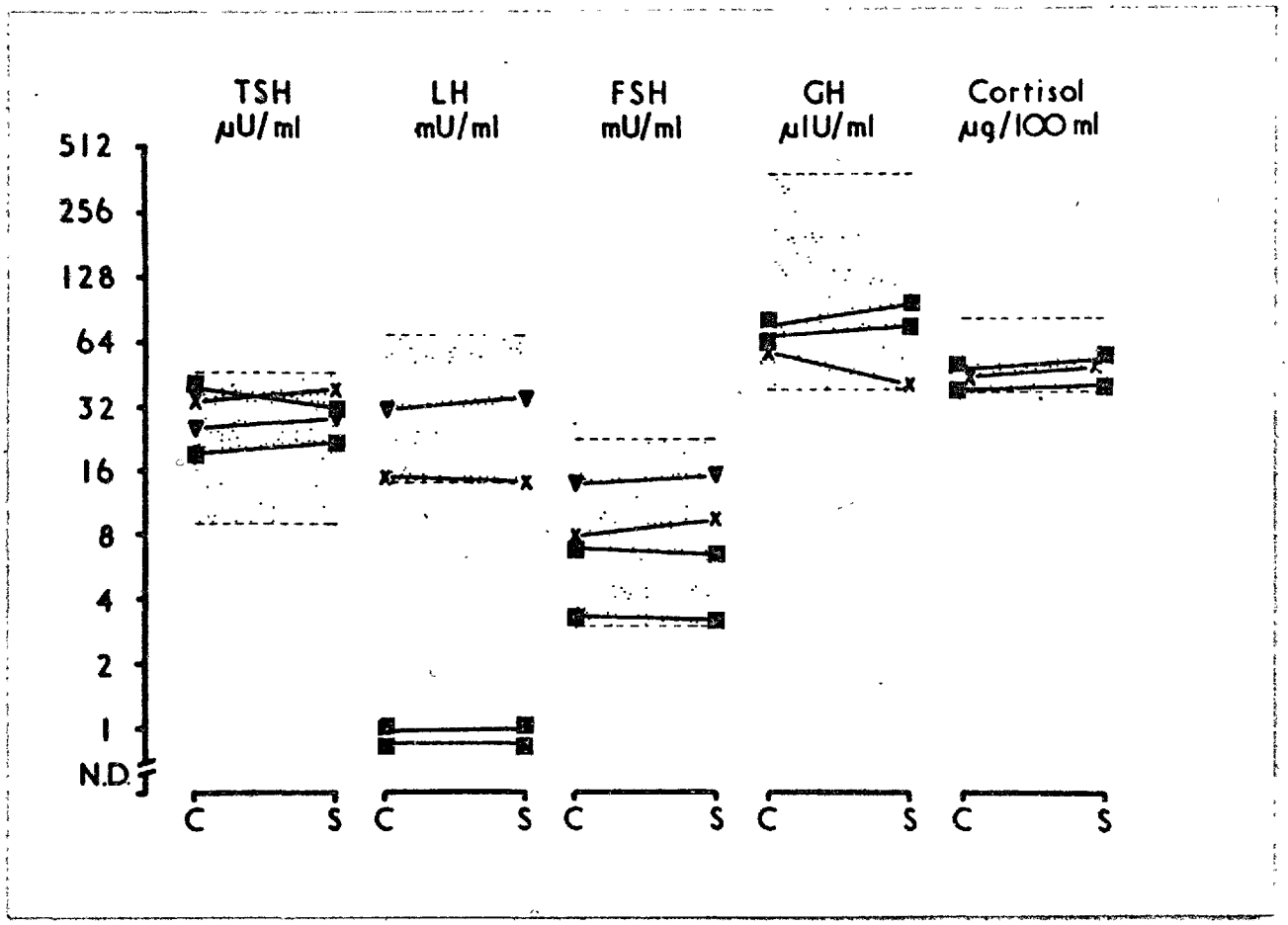

Fig. 6.4 Comparison of the hormone responses in patients with other untreated hypothalamic-pituitary disorders. The same format is used as in Fig. 6.3. The symbols refer to the diagnoses shown in Table 6.1.

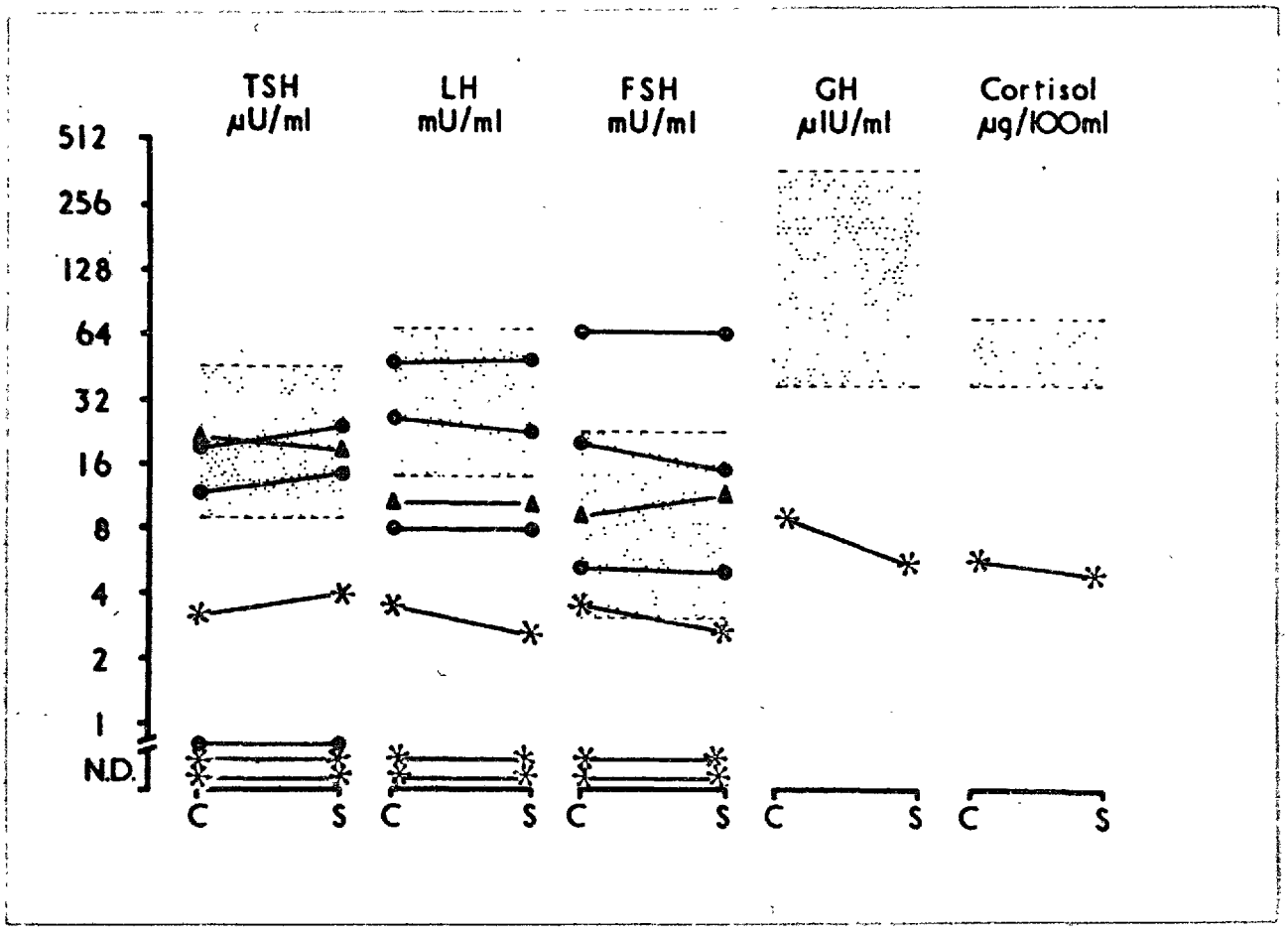

Fig. 6.5 Comparison of the hormone responses in patients previously treated by pituitary implantation of Yttrium 90. The same format is used as in Figs. 6.3 and 6.4. The symbols refer to the diagnoses shown in Table 6.1.

Hormone	No. Tests	Hormone Response		|A - B|
		Combined Test	Single Test	
TSH/µu/ml	9	23.5 ± 3.5	24.5 ± 3.5	0.97 (P ≥ 0.46)
LH mu/ml	11	27.9 ± 6.2	27.0 ± 5.7	0.90 (P ≥ 0.56)
FSH mu/ml	11	27.0 ± 14.9	29.3 ± 17.1	2.3 (P ≥ 0.32)
GH µu/ml	10	249.1 ± 51.0	172.3 ± 34.3	76.8 (P ≥ 0.08)
Cortisol µg/100ml	9	48.7 ± 4.5	47.2 ± 3.3	1.5 (P ≥ 0.58)

Table 6.4: Comparison of hormone responses in 11 subjects who had a combined adminstration of ITT, LH/FSH-RF, TRF (A) and the same tests separately on consecutive days (B).

The figure represents the mean ± SEM of the sum of the responses in 20 and 60 minutes for TSH, LH and FSH, in 60, 90, 120 minutes for GH and 60 and 90 for cortisol.

|A - B| represents the mean difference of the two tests.

P = probability on a paired t-test shows that |A - B| highly insignificant.

Hormone	No. Tests	Hormone Responses		|A - C|
		Combined Test (A)	Double Test (C)	
TSH μu/ml	12	11.8 ± 3.2	11.9 ± 3.2	0.05 P ≥ 0.96
LH mu/ml	12	17.7 ± 4.3	17.6 ± 4.7	0.07 P ≥ 0.93
FSH mu/ml	12	15.5 ± 5.0	15.0 ± 5.0	0.55 P ≥ 0.53

Table 6.5: Comparison of hormone responses in 13 subjects who had a combined administration of ITT, LHRF, TRF (A) and the LH/FSH-RF, TRF tests done together another day. The figures represent the mean ± SEM of the sum of the responses in 20 and 60 minutes (A - C) is the mean difference of the two tests.

P is the probability in a paired t test and shows that |A - C| is highly insignificant.

A) Acromegaly

i) TSH responses in the short TRF test:- Table 6.6 shows the individual values found in 24 untreated acromegalics. Abnormally low responses were found in 8/24 (33%) of the patients: 4 impaired, 4 absent.

The incidence of a goitre in the normal responders was 4/16 (25%) and in the patients with an impaired response was 3/8 (38%). As shown in Table 6.6, in all patients the serum T_4 and/or PBI levels were in the normal range.

ii) TSH responses in the long TRF test:- Serum TSH responses in 11 acromegalics are shown in Fig. 6.6. 4 of the 11 tests (36%) fell below the normal range of response. TSH increment values are shown in Table 6.3, again the same 4 subjects fell below the normal range.

iii) Investigation of other probable factors involved in the genesis of the high incidence of TSH unresponsiveness in acromegaly:-

a. Urinary TSH and free T_3 responses in the long TRF tests: These are shown in Table 6.3. The same 4 subjects who showed subnormal serum TSH responses also showed impaired urinary TSH responses. When increment values were considered, a fifth subject also qualified as having an impaired response.

4 of the 5 patients who did not show normal urinary TSH increment did not also show normal urinary free T_3 increment.

b. Serum PBI, T_4, GH levels in patients studied in the long TRF tests: Individual values of serum PBI, T_4 and GH are shown in Table 6.3. The GH values were derived from the mean of the serum GH values found at 60, 90, and 120 min. during an oral glucose tolerance test. The PBI and T_4 levels were in the normal range. The GH values were compatible with the diagnosis of acromegaly.

c. Thyroid size and T_3 suppression tests:- 3 of the 11 acromegalics tested with the long TRF test had a small goitre. Of these, two were amongst the non-responders. T_3 suppression tests, using 120ug of hT_3

Subject	Sex	TSH μu/ml				T_4 ng/ml	PBI μg/100ml	GH μu/ml	Goitre
		0'	20'	60'	Mean Increment				
1	F	<1	<1	<1	0	105	-		+
2	F	4.7	4.1	2.6	0	-	4.7	27	+
3	M	1.1	1.6	1.4	0.4	-	6.7	33	+
4	F	1.2	1.9	2.0	0.7	-	6.0	90	-
5	F	1.7	2.2	2.6	0.7	-	5.8	35	-
6	F	<1.0	2.5	2.2	1.3	-	7.6	87	-
7	F	1.6	3.8	3.1	1.8	-	5.5	31	-
8	M	1.5	5.1	3.6	2.8	-	5.4	28	-
9	M	2.2	8.4	5.7	4.8	-	6.7	20	-
10	M	<1	7.9	4.0	4.9	-	6.0	415	-
11	M	1.6	8.4	6.4	5.8	95	7.4	81	-
12	F	<1	8.0	6.2	6.1	-	6.8	266	+
13	F	2.1	10.0	6.5	6.1	-	4.1	500	-
14	F	1.2	7.7	7.6	6.4	60	6.6	36	+
15	F	3.6	10.8	9.6	6.6	80	7.4	20	-
16	M	2.6	9.7	9.0	6.7	115	-	108	-
17	F	2.8	15.0	8.8	9.1	-	4.8	253	-
18	F	3.6	25.0	2.8	10.3	57	-	68	+
19	M	2.9	16.6	11.0	10.9	80	6.7	108	-
20	M	2.0	18.5	11.0	12.7	-	4.5	13	-
21	M	2.7	22.0	12.0	14.3	-	5.1	11	-
22	F	3.3	19.0	20.5	16.4	66	4.4	28	-
23	M	2.6	22.0	17.0	16.9	70	5.5	94	-
24	F	2.9	27.0	27.0	24.1	108	5.5	305	+
Normal Range: Refer Table 6.2						55-115	4.0-8.0	<5	

Table 6.6: Serum TSH responses to TRF in 24 euthyroid untreated acromegalics. Mean increment = mean of 20 + 60 min values less basal value. GH values derived from mean serum levels at 60, 90 and 120 min during an oral glucose tolerance test.

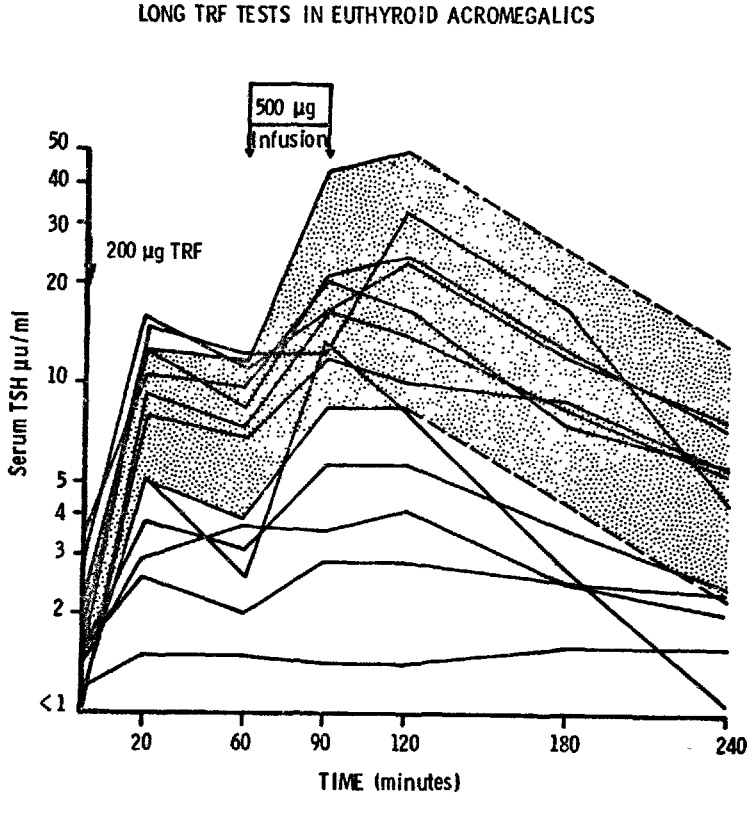

Fig. 6.6 Serum TSH responses during a long TRF test in 11 euthyroid untreated acromegalics. The shaded area represents the range of response in 8 normal males shown in Fig. 6.1.

daily orally for 7 days, done on these 2 non-responders gave the following results for 24hr ^{131}I neck uptakes: 39% before and 32% after; 22% before and 32% after. Both subjects therefore showed negative T_3 suppression tests.

iv) Analysis of the results of the long TRF tests:

a) Results of the normal subjects and acromegalics pooled:- These are shown in Table 6.7 which shows that serum TSH increments correlated well with the urinary TSH increments ($p < 0.04$) just as urinary TSH increment correlated with the urinary T_3 increment ($p < 0.01$). However, there were no correlations between basal urinary T_3 levels and serum or urinary TSH increment, nor were there any correlations between basal serum T_4 levels and the increment of TSH in serum and urine. Serum TSH increments did not correlate with the GH levels in the acromegalics.

b) Results of the acromegalics with impaired TSH responses compared with those with normal responses and with those of normal subjects:- These are shown in Table 6.8. There was no difference in the basal serum TSH levels in the 3 groups. However, the basal urinary TSH levels were significantly lower amongst the acromegalic non-responders when compared with the acromegalic responders and the normal subjects, suggesting the urinary TSH measurement is a better discriminant in conditions where TSH secretion is low. There was no difference between the basal urinary free T_3, and serum PBI and T_4 levels in all the 3 groups; nor was there a difference in the serum GH levels among the responding and non-responding acromegalics.

v) Analysis of the results of the short TRF tests:- These gave similar results to above, i.e. although there was a significant difference between the serum TSH increments of the subjects with impaired responses and those with normal responses ($p \leq 0.03$) there was no correlation between the serum TSH increments and the serum levels of T_4 ($p > 0.3$), PBI ($p > 0.4$) or GH ($p > 0.5$).

vi) Acute GH tests:- Results of the serum TSH response to TRF before and

Correlations Tested				R	P
1	STSH I	Vs	UTSH I	0.65	<0.04*
2	UTSH I	Vs	UT_3 I	0.77	<0.01*
3	Ba UT_3	Vs	STSH I	-0.45	>0.1
4	UTSH I	Vs	Basal UT_3	0.06	>0.8
5	ST_4	Vs	STSH I	-0.25	>0.4
6	ST_4	Vs	UTSH I	0.12	>0.7
7	GH	Vs	STSH I	0.09	>0.7

Table 6.7: Statistical analysis of the parameters investigated in the long TRF tests performed on euthyroid acromegalics and normal subjects. All the data (both from normal subjects and patients) were pooled for the purposes of this analysis.

U = Urine S = Serum I = Increment

R = Correlation coefficient P = Probability

Only those parameters marked * reached statistical significance.

TABLE 6.8

Parameter	Mean ± SEM			p values	
	Normal (a)	AR (b)	ANR (c)	aVs c	bVs c
Basal STSH μu/ml	1.6 ± 0.2	1.6 ± 0.05	1.5 ± 0.1	>0.4	>0.8
Basal UTSH μu/hr	6.4 ± 0.6	6.3 ± 0.6	3.6 ± 1.0	<0.05*	<0.03*
Basal UT_3 ng/hr	47.5 ± 6.9	70.1 ± 4.9	44.3 ± 10.2	>0.7	>0.2
UT_3 I ng/hr	99.3 ± 65.1	53.3 ± 17.1	8.6 ± 6.8	>0.2	>0.08
PBI ng/100ml	6.3 ± 0.3	6.3 ± 0.4	5.9 ± 0.3	>0.4	>0.4
SGH μu/ml	-	146 ± 17	162 ± 113	-	>0.8

Student's t test analysis of the means of the parameters investigated in the long TRF test. The acromegalic non-responders (ANR) were compared with the acromegalic responders (AR) and normal subjects.

S = Serum U = Urine * Statistically significant.

after human growth hormone injections in 3 subjects are shown in Table 6.9. In one subject, there was no TSH response to TRF after the GH injections. In another, the response, as judged by the increment in the second test, was reduced by 30%. In the third subject, there was no difference in the 2 responses.

vii) Further investigations on the role of GH:- In order to investigate further the role GH plays in the inhibition of TSH secretion, the results of all the short TRF tests performed on euthyroid acromegalics at the Hammersmith Hospital since the introduction of TRF were analysed in relationship to the completeness of treatment as judged by a fall in serum GH levels to below 50μu/ml. The results are shown in Table 6.10a which demonstrates that 3/9 (33%) tests before treatment were impaired while 10/16 (62%) tests post treatment were impaired, showing that treatment tended to worsen the response. When the results in Table 6.10a were reanalysed on the basis of the serum GH levels after treatment, the following emerged (Table 6.10b): only 4/9 (44%) patients with GH levels 10μu/ml had an impaired response, while all 4 (100%) of those with levels between 11 and 20μu/ml and 2/3 (67%) of those with levels between 21 and 50μu/ml had an impaired response. These findings suggest that GH may suppress TRF mediated TSH secretion.

Further indirect evidence that GH may suppress TSH secretion is shown in Table 6.11 which is an analysis of the response of euthyroid acromegalic patients before and after treatment. Again the number of impaired TSH responses became greater after treatment (62% as opposed to 36% before treatment). Similarly the incidence of gonadotrophin and ACTH failure became higher after treatment, suggesting that the treatment itself was responsible for the pituitary failure. Table 6.11 also shows that in the patients, before treatment, there was an even distribution of gonadotrophin failure among the patients with normal or impaired TSH responses, suggesting that pressure of the tumour on the pituitary cells

TABLE 6.9

Subject	Age (years) (Sex)	Diagnosis	TSH Responses (μu/ml)							
			Before				After			
			0	20′	60′	I	0	20′	60′	I
1*	10 (M)	Craniopharygioma	3.7	8.7	9.2	4.3	<1	<1	<1	0
2	18 (M)	Isolated growth hormone deficiency	1.5	6.5	6.5	5.0	3.4	10.5	7.8	5.7
3*	30 (M)	Normal	<1	15	11	13.0	1.5	12.0	9.0	9.0

Serum TSH responses to TRF before and after 6 daily injections of 10 iu human growth hormone in 3 subjects.

* Significantly different responses

I = Mean increment

Subject	GH μu/ml		TSH response μu/ml (Mean Increment)	
	Before Rx	After Rx	Before Rx	After Rx
1	32.0	<1	-	5.0
2	48.1	1.0	-	2.3*
3	75.0	2.7	~~9.0~~ -	7.3
4	24.8	3.0	5.1	<1*
5	57.0	7.0	-	<1*
6	108	7.1	9.8	5.0
7	184	7.3	6.1	3.1*
8	8.0	8.0	14.3	13.0
9	192	14.0	5.5	3.5*
10	38	16.0	-	<1*
11	90	16.0	<1*	-
12	111	18.0	-	<1*
13	142	24.0	-	7.6
14	352	34.0	<1*	<1*
15	190	39.0	<1*	<1*
16	275	44.0	7.1	2.3*

Table 6.10a: The relationship of TSH responsiveness to TRF, in euthyroid acromegalics and serum growth hormone levels. The patients were judged to have had a good response to treatment by a fall of serum GH level to <50 μu/ml. The general tendency is to more cases of ~~improvement~~ impaired responses (10/16, 62%) after treatment than before (3/9, 33%)

* = impaired response

Rx = treatment

	Response to TRF		
Serum GH μu/ml	Impaired (I)	Normal	I/Total
<10	4	~~5~~ 4	4/~~8~~ (~~44%~~) ~~75~~ 50%
11 - 20	4	-	~~4/4~~ (100%) 3 3
21 - 50	3	~~2~~ 1	3/~~3~~ (~~66%~~) 4 75

Table 6.10b: Relationships between the serum GH level and TSH responsiveness in the treated acromegalics shown in Table 6.10a. The incidence of impaired responsiveness is least in those in whom serum GH had dropped to <10 uu/ml.

Sub-groups	TSH Response			
	Before Rx		After Rx	
	Normal	Impaired	Normal	Impaired
Hypogonad; on Cortisol	-	1	-	6
Hypogonad; not on Cortisol	12	5	8	11
Other pituitary function normal	11	6	6	8
Total:	23	12	14	25
Grand Total:	35		39	

Table 6.11: Pituitary function in treated and untreated euthyroid acromegalics in relationship to the incidence of TSH unresponsiveness to TRF in these patients. There is no correlation between the incidence of TSH unresponsiveness and the degree of pituitary function failure.

could not be held solely responsible for the TSH unresponsiveness thus leaving GH as the most likely factor responsible.

B) Prolactin-secreting pituitary tumours

9 of the 10 euthyroid subjects studied had a normal serum TSH response to the short TRF test (Table 6.12). There was no correlation between the prolactin levels and the mean serum TSH increments ($p > 0.2$).

C) "Functionless" pituitary tumours

The responses of serum TSH to the long TRF tests in 2 euthyroid subjects with functionless pituitary tumours are shown in Fig. 6.7. One subject had a response in the normal range. The other subject did not respond earlier on in the test, but with the infusion his response became normal, suggesting that the long TRF test might pick out the occasional non-responder with a normal pituitary TSH reserve capacity.

D) Cushing's disease

Among the untreated subjects, the basal serum TSH values were probably low, all 8 values lying under the detection limit of the assay (1.5μu/ml) compared with the high normal values in 2/6 treated subjects (Fig. 6.8). Serum TSH levels found in the short TRF tests are shown in Fig. 6.8. In all 8 untreated patients, the responses were subnormal (the average mean increment being 1.5 ± 1.2 (SD) μu/ml, range < 1 - 4.1 μu/ml). In the treated group, all the responses were in the normal range (average mean increment being 6.3 ± 1.3 (SD) μu/ml, range 4.5 - 8.5μu/ml). The two untreated patients tested with the long TRF procedure showed no response (Fig.6.7).

The serum prolactin responses in 5 untreated patients are shown in Fig. 6.9. In all patients the response was in the normal range (average mean increment being 21.5 ± 11.0 (SD) ng/ml, range 16.0-33.7ng/ml compared with the normal average mean increment of 19.6 ± 19.0 (SD) ng/ml, range 8.0 - 61ng/ml.

In confirmation of the diagnosis of Cushing's syndrome, the urinary

Subject	Sex	Serum TSH μu/ml				Serum Prolaction ng/ml
		0'	30'	60'	Mean Increment	
1	F	<1	<1	<1	<1	490
2	F	2.8	9.8	5.1	4.8	132
3	F	<1	7.2	4.0	5.8	600
4	F	<1	16.3	10.7	13.5	205
5	F	1.9	19.5	11.5	14.0	158
6	F	3.0	17.0	14.2	14.5	243
7	F	<1	19.0	12.0	15.5	150
8	F	2.4	20.0	12.0	15.8	170
9	F	3.2	19.0	16.0	15.9	1450
10	F	2.6	25.0	17.0	19.5	500

Table 6.12: Serum TSH responses to TRF in patients with prolactin secreting pituitary tumours.

' = minutes

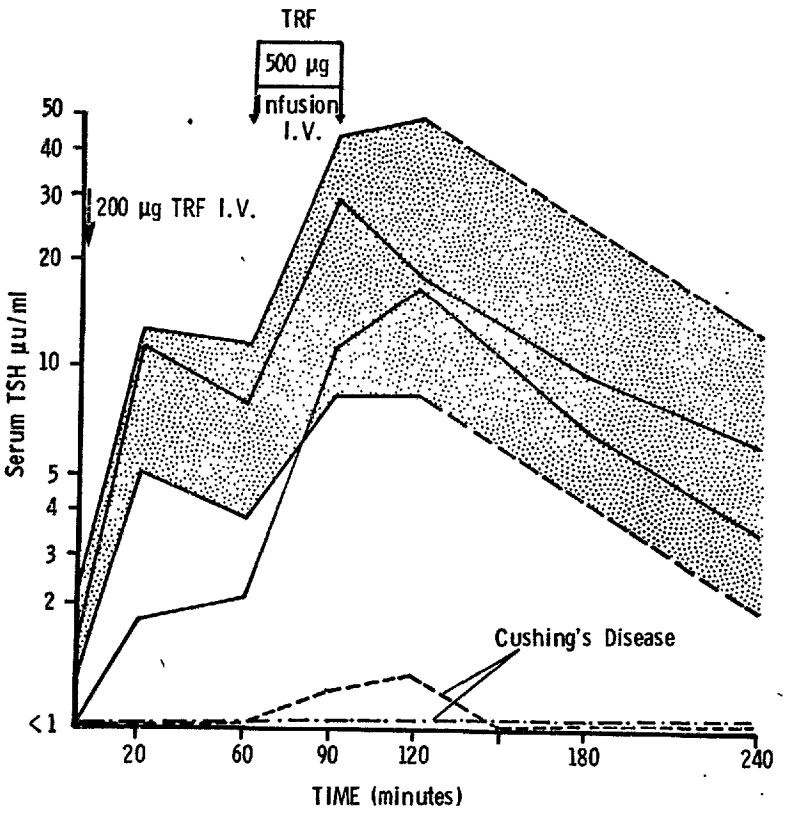

Fig. 6.7 Serum TSH response in (a) 2 patients with pituitary tumour (b) 2 patients with pituitary dependent Cushing's disease during a long TRF test. The shaded area represents the range of response found in normal subjects (Fig. 6.1).

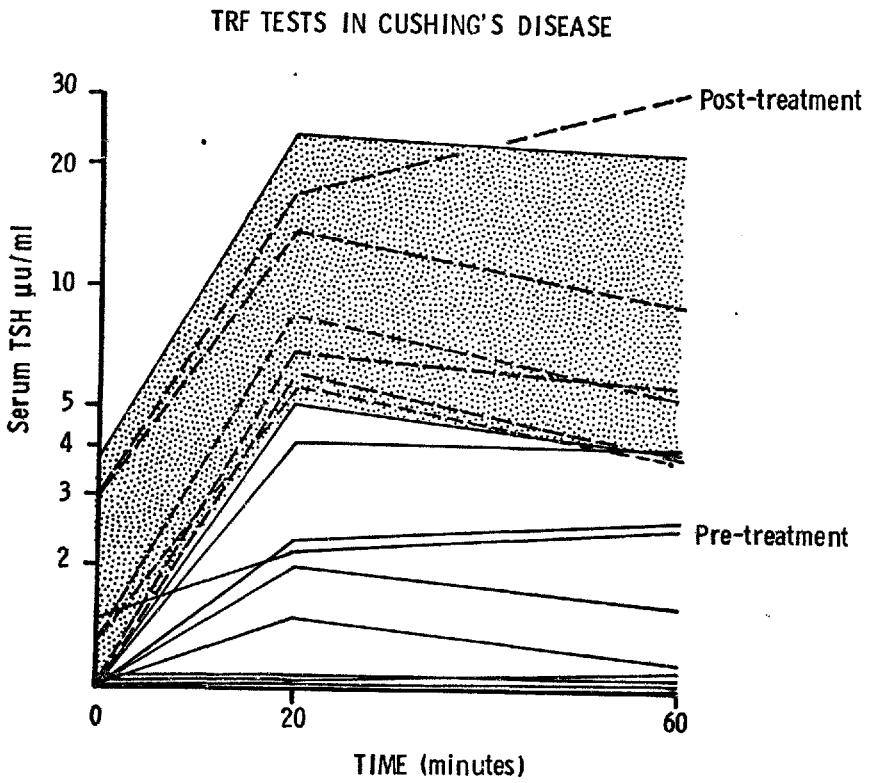

Fig. 6.8 Serum TSH responses to an intravenous injection of TRF in Cushing's disease patients before and after treatment with ^{198}Au. The shaded area represents the range of response found in 20 normal subjects.

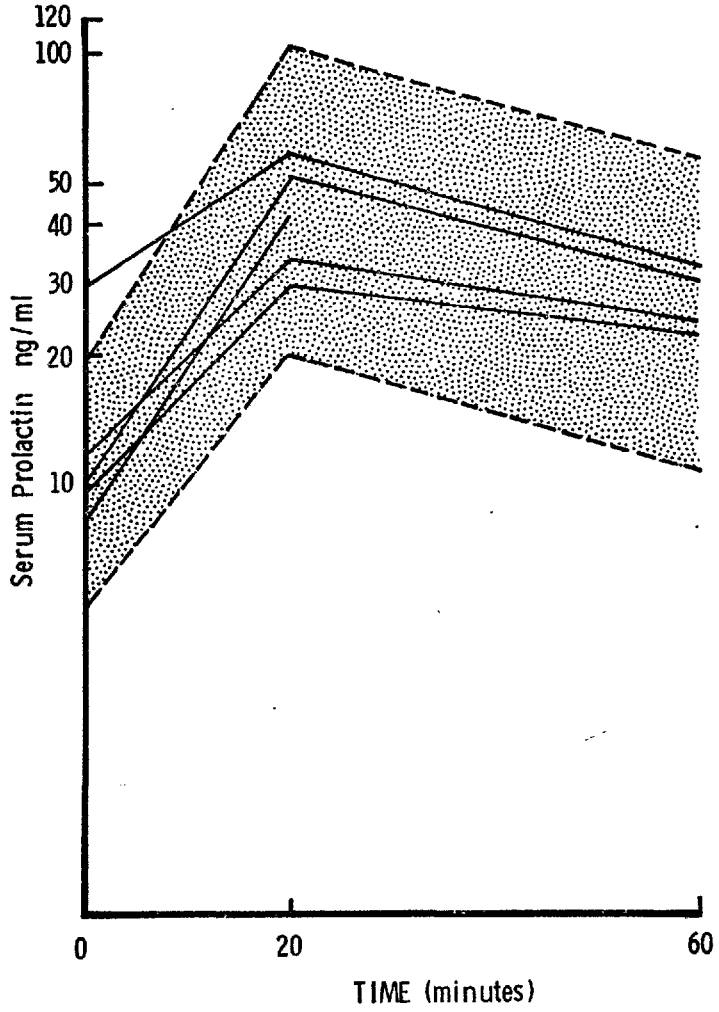

Fig. 6.9 Serum prolactin responses to an intravenous injection of TRF in untreated Cushing's disease patients. The shaded area represents the range of response seen in 7 normal subjects.

free cortisol levels were raised in all subjects tested pre-treatment, and all those tested after treatment had normal levels (Table 6.13). Further circulating thyroid hormone levels were normal pre and post-treatment .

(iv) TSH responsiveness in mildly diabetic patients with elevated basal serum TSH levels:

Fig. 6.10 shows the serum TSH of the 14 patients during the short TRF test. Their high basal serum TSH level (9.3 ± 11.3 (SD) μu/ml) was confirmed. 6/14 patients had excessive and sustained response while the 8 responses fell in the upper normal range.

Basal serum T_4 and PBI levels in these patients are shown in Table 6.14. All patients had normal PBI levels. 9 patients were in the lower range of normal, while 2 had low T_4 levels.

4. Discussion.

A: New Tests of Anterior Pituitary Function:-

a) The "Combined Test":- The results of the study demonstrate that the releasing factors (TRF and LHRF) and insulin can be given together without significantly changing individual hormone responses. Furthermore, in patients with disorders of the hypothalamo-pituitary axis, the same classification of "pituitary reserve", i.e. normal, borderline or impaired, was obtained with the combined test as was found after the single stimulatory tests performed separately. Thus the assessment of pituitary status was not changed by the use of the combined procedure.

The only differences in responses were seen with GH, which is known to be rapidly labile in response to any stress. However repeat insulin hypoglycaemia tests performed in this hospital on different occasions in the same subject have shown up to two-fold differences in peak GH levels after comparable degrees of hypoglycaemia and when the subject was clearly basal. The differences seen in GH responses in the present study fell

Patient	Before Treatment		After Treatment	
	Urinary Free cortisol µg/24hr	Serum T_4 ng/ml	Urinary Free cortisol µg/24hr	Serum T_4 ng/ml
1	338	55	28	60
2	305	88	<10	74
3	213	68	<10	80
4	492	80	<10	75
5	243	68	-	-
6	299	88	-	-
7	279	82	-	-
8	326	82	-	-
9	-	-	12	88
10	-	-	32	75
Normal Values	<100	55-115		

Table 6.13: Urinary free cortisol and serum T_4 levels before and after treatment in Cushing disease patients.

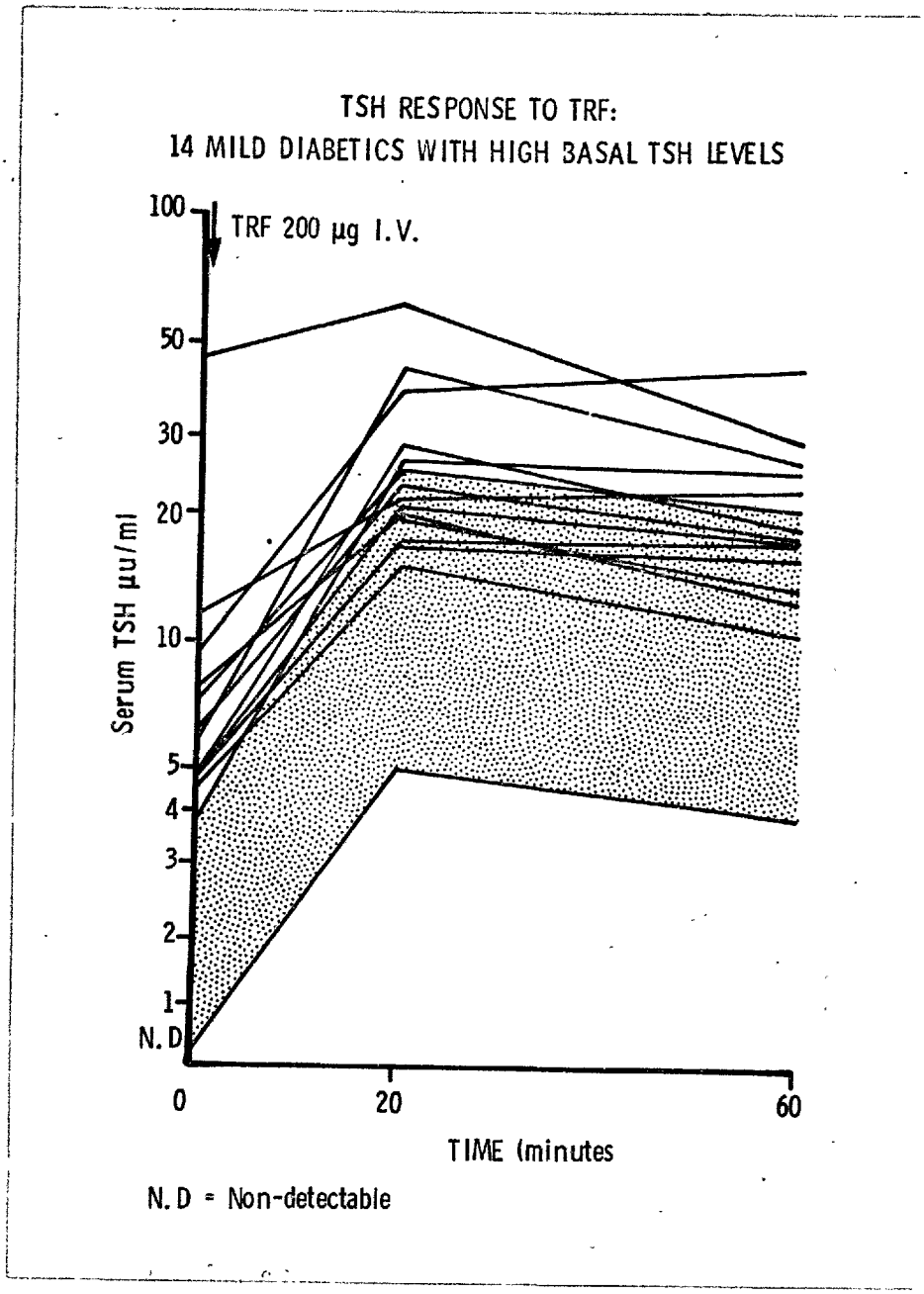

Fig. 6.10 Serum TSH responses to an i.v. injection of TRF in 14 mild diabetics with elevated basal TSH levels. The normal range of response is shown by the shaded area.

Subject	Serum TSH μu/ml	PBI μg/100ml	T_4 ng/ml
1	4.5	7.4	90
2	4.8	-	50
3	4.9	6.6	68
4	4.9	5.6	69
5	4.9	6.1	68
6	5.1	5.0	59
7	5.8	6.1	80
8	5.8	5.0	84
9	6.1	5.0	45
10	7.3	4.4	64
11	7.9	5.0	66
12	9.5	5.6	55
13	11.5	4.8	86
14	48.0	6.1	60
Mean ± SD	9.3 ± 11.3	5.0 ± 1.8	65.5 ± 12.1
Normal Range	<1 - 3.7	4.0 - 8.0	55 - 115

Table 6.14: Serum TSH, T_4 and PBI in 14 mild euthyroid diabetics.

well within this degree of variation. Although TRF has been shown to release GH in acromegalics (Faglia et al, 1973, Samaan et al, 1974) the GH responses to TRF and insulin hypoglycaemia were not consistently higher than the response to insulin hypoglycaemia alone in our acromegalic subjects, suggesting that the TRF effect may not be constant.

Prolactin was not measured in these subjects, but the combined test may also prove useful for the assessment of pituitary prolactin secretion, for serum levels of this hormone are known to be increased by both TRF and insulin-induced hypoglycaemia (Bowers et al, 1971; Jacobs et al, 1971; L'Hermite et al, 1972; Mortimer et al, 1973).

Recently, Mortimer et al (1973) have also demonstrated that in normal individuals, TRF, LHRF and insulin hypoglycaemia did not interfere with each other in releasing their specific hormones when given in combination. Our findings are therefore in agreement with theirs.

It is therefore proposed that anterior pituitary function can be assessed in a single combined test which is convenient and time-saving and can be performed on an out-patient basis. This is now established practice at the Hammersmith Hospital, London.

b) <u>The Long TRF Test</u>:- The results of the long TRF test in "functionless" pituitary tumour patients shown in Fig. 6.7 support the postulate put forward earlier that euthyroid subjects who do not respond to the short TRF test may have normal pituitary TSH reserve capacity which may be released on persistent stimulation with TRF. Thus one patient who had impaired response in the part of the test corresponding to the short TRF test, showed a response in the normal range during the infusion of TRF.

The long TRF test incorporates the short TRF test as the first part of the test, with the added advantage that the patient can be assessed in the standard way at the same time.

Since the urinary TSH and free T_3 responses during the test correl-

ated well with serum TSH responses (Table 6.7), the former responses could also be used to monitor the effect of the long TRF stimulation in subjects with normal renal function.

B: TSH Secretion in Euthyroid "Active" Pituitary Disease:-

i) Acromegaly: Our short TRF test results agree with other reports (Hall et al, 1972; Tunbridge et al, 1973; Faglia et al, 1973) that the incidence of an impaired response of serum TSH to TRF stimulation in euthyroid acromegalics is high. However, the incidence of 35% (12/35) we found in this study is slightly lower than the 40 - 56% reported by the above workers.

The long TRF tests confirm the above findings in the short TRF tests, and in none of the acromegalics with impaired response in the short TRF test part of the procedure did the TRF infusion stimulate TSH secretion into the normal range. This suggests that the non-responding patients had a low pituitary TSH reserve capacity. Another explanation is that perhaps the persistent stimulation during the TRF infusion was not enough to overcome the factor or factors antagonizing the TRF action on the thyrotrophics. The results of the tests in acromegalics and normal subjects were therefore further analysed to see if such antagonists do exist.

When the normal and acromegalic subjects were analysed as a whole in the long TRF test, there was no correlation between the basal urinary free T_3 levels and the serum TSH response. Nor was there any difference between the basal urinary free T_3 levels of the responders and non-responders. Since urinary T_3 levels reflect the serum free T_3 concentrations (Burke et al, 1972) and the secretion of TSH by the pituitary is modulated by free thyroid hormone levels in the blood (Hershman and Pittman, 1971b) it would appear that excessive peripheral levels of T_3 is probably not responsible for the lack of response of serum TSH to TRF seen in these subjects. This is further supported by the fact that in both the long and short TRF tests, there was no correlation between the basal serum T_4

levels and the serum TSH increments.

Among all the 35 euthyroid untreated acromegalics studied, 10 had nodular goitre, of which 5 were in the impaired TSH response group, therefore goitres occurred almost twice as frequently in this group as in the group of the normal responders. When 2 of the 5 non-responders with goitres were tested for thyroid autonomy with T_3 suppression tests both patients were found to have autonomous thyroid glands. These findings are similar to those of Hall et al (1972) who found that 6 of their 19 euthyroid acromegalics with absent or impaired responses had nodular goitres with autonomous function. Hall et al (1972) postulated that thyroid hormones secreted by such autonomous glands may suppress the TSH response to TRF in a way analogous to the situation found in hyperthyroidism. However, in hyperthyroidism the level of one or both of the thyroid hormones is elevated above the normal range, and this would adequately explain the negative feedback on the hypothalamo-pituitary axis. In none of the acromegalics studied were the thyroid hormones elevated either in the serum or urine, nor was there a correlation between the thyroid hormone levels and the TSH response. On this evidence it is doubtful if excessive thyroid hormone secretion in these subjects would explain their lack of response. Perhaps these subjects maintained their euthyroid state in the face of low pituitary TSH reserve capacity and low pituitary TSH output as judged by the low basal urinary TSH excretion because of their autonomous thyroid function. Hall et al (1972) have reported that in patients with pituitary tumours other than acromegaly and who showed impaired TSH response to TRF had a higher incidence of hypothyroidism than their acromegalic counterparts. None of the patients studied here was hypothyroid. Perhaps acromegalics are able to maintain an autonomous thyroid function in the face of low TSH secretion by the pituitary for yet unexplained reasons .

From the study of the role played by GH in the high incidence of

TSH unresponsiveness in acromegaly certain facts emerged: there was no correlation between the serum GH levels and the serum TSH increment after the long or short TRF test, nor was there a difference between the mean serum GH levels of responders and non-responders (Table 6.7, 6.8). Tunbridge et al (1973) reported similar findings. These would suggest that GH had little or no part to play in the genesis of the TSH unresponsiveness in some acromegalics. The situation is probably more complex; perhaps all that is required is a threshold level of raised serum GH. The evidence currently available from the reports of Macleod et al (1966, 1968) and Root et al (1970, 1973) would suggest that GH may inhibit TSH secretion by the pituitary. The findings in this study that in 2 out of 3 subjects the serum TSH response to TRF was impaired after 6 days of growth hormone injections would support the findings of the above workers.

In treated euthyroid acromegalics in whom serum GH has been judged to be satisfactorily lowered, the general tendency is for the serum TSH responsiveness to get worse, in agreement with the findings of Hall et al (1972) and Tunbridge et al (1973). This might be construed to mean that lowering the GH levels did not improve the response in these subjects. However, since the secretion of other pituitary hormones in these patients also tends to be worse after treatment, it would appear that the treatment per se may be responsible for the worsening of the response. Table 6.10b presents evidence that the incidence of non-response of serum TSH is least in those patients in whom the growth hormone response has been lowered to $< 10\mu u/ml$. This suggests that high GH may play an important role in TRF mediated TSH secretion.

The question also arises whether the high incidence of TSH unresponsiveness in acromegaly is due to selective pressure of the growth hormone secreting tumour on pituitary thyrotrophs. As shown in Table

6.11, there was an even distribution of gonadotrophin failure, before treatment, amongst the responders and non-responders, suggesting that pressure effects from the tumour could not be held solely responsible, leaving excessive growth hormone levels as most likely causative factor, particularly since TSH secretion tends to be the last to fail in non-growth hormone secreting tumours of the pituitary gland (Fraser, 1970).

In conclusion this study agrees with published data on the increased incidence of TSH unresponsiveness to TRF stimulation in acromegalics. Acromegalics, with impaired TSH response to TRF probably maintain their euthyroidism by a fortunate coincidence of autonomous thyroid function, the genesis of which may or may not be related to the disease. This requires further study. The cause of the high incidence of TSH unresponsiveness in this disease remains an enigma, but it would appear that excessive GH levels are responsible until proved otherwise. More subjects are required for this, but the very rarity of the disease precludes this in the present study.

It is also of interest that although basal serum TSH measurements did not distinguish between the acromegalic non-responders and the responders, basal urinary TSH levels clearly did so (Table 6.8). This is further evidence that urinary TSH measurements are superior to basal measurements in serum in conditions where TSH secretion is low. Also, urinary TSH measurement could probably be used instead for TRF stimulation tests where facilities for the use of the latter are not available.

(ii) Patients with prolactin-secreting pituitary tumours:-

Our results show that only one out of ten patients studied showed an impaired serum TSH response to TRF suggesting that excessive prolactin levels in serum do not impair TSH secretion in man. Macleod et al (1966, 1968) however found that prolactin secreting tumours in the rat diminished pituitary TSH content, suggesting that control of TSH secretion may differ

in man and the rat.

The reports of Hall et al (1972) and Tunbridge et al (1973) of the low incidence of TSH unresponsiveness in chromophobe or "function-less" pituitary tumours are similar to ours, although no prolactin measurements were reported in their studies. The so-called "function-less" pituitary tumours, with the recent availability of prolactin radioimmunoassays, are now increasingly being recognized as prolactin secreting tumours.

(iii) Cushing's Disease:

This study shows that serum TSH response to TRF is impaired in active Cushing's disease, and that the impairment is reversed on adequate treatment of the disease. The effect of chronic cortisol excess is therefore evidently active at the level of the pituitary gland in agreement with similar observations reported by Otsuki et al (1973). Other data (Wilber and Utiger, 1969; Haigler et al, 1971) have suggested that glucocorticoids, in the short term, may also be active as well at the hypothalamus for within a few days of large doses of these hormones basal serum TSH level is low, its response to TRF normal.

These effects on TSH would appear to be analogous to the suppression of serum GH response to insulin hypoglycaemia also seen in Cushing's disease, or chronic glucocorticoid administration and which also restores normal after treatment of the Cushing's syndrome (Hartog et al, 1964a).

Since low basal serum TSH values but normal TSH responsiveness to TRF are seen within a few days of the administration of large doses of glucocorticoids (Haigler et al, 1971), the initial suppressive effect may be mainly at the hypothalamus while full suppressive effect involving loss of pituitary responsiveness may only be evident after chronic glucocorticoid excess. This probably involves suppressive effects at both sites.

Among the untreated patients, the basal TSH values were probably

low, all 8 values lying under 1.5μu/ml (the detection limit of the assay) as compared with the levels in the treated subjects where 2/6 values were in the high normal range. However, the TSH and radioimmunoassay is not yet sensitive enough to distinguish between normal and subnormal values.

We have also demonstrated that the TRF effects on TSH and prolactin secretion can be dissociated since all our untreated Cushing's disease patients who did not respond to TRF by a rise in serum TSH levels showed a rise in serum prolactin levels. This is further evidence that the control of TSH and prolactin secretion is independent even if the secretion of each hormone is stimulated by the single releasing hormone, TRF. Evidence pointing to this has already been obtained using T_4 (Synder et al, 1973), T_3 (Rappaport et al, 1973) and oestrogens (Carlson et al, 1973.).

C. Diabetic patients:

Our results show that 6/14 patients with the mildly elevated serum TSH levels had a serum TSH above the normal range, the other 8 having responses in the upper normal range. This implies that our patients had an increased pituitary TSH reserve capacity. Although most of the subjects had a normal serum T_4 and/or PBI level, the majority of them had serum T_4 in the lower normal range. Since serum TSH level is a better index of thyroid failure than serum thyroid hormone level (Greenberg et al, 1970), it would appear that our patients had a certain degree of thyroid failure and are compensating with an increased TSH drive. This condition has been described by Evered et al (1973) as subclinical hypothyroidism.

TRF tests may therefore prove useful in the diagnosis of mild degrees of thyroid failure.

CHAPTER 7

CONCLUSIONS.

Immuno-reactive TSH has been demonstrated in urine, and evidence has been adduced to show that the concentrations found correlated well with the clinical state of the subject. Our measurements in urine completely discriminated between normal and subnormal TSH levels and therefore proved to be superior to measurements in serum.

The method developed for the urinary TSH assay, dialysis and lyophilization followed by double-antibody radioimmunoassay, is efficient and reliable. The extraction procedure can also be used for other allied peptide hormones since we were able to quantitate LH and FSH levels in the urine concentrates and our measurements correlated well with the gonadotrophic state of the subjects. The urinary TSH excretion was also shown to be quantitatively much less than that of LH or FSH.

Since a concentration and extraction step is required in this urinary assay method, it is of necessity laborious and time-consuming, and its practicability on the large scale,and so its use in the diagnosis of hypothyrotrophin states, limited. In hypopituitarism, however, urinary TSH measurement should prove a good diagnostic tool, particularly where TRF stimulation tests are not available, since we have shown that in acromegalics and normal subjects, basal urinary TSH levels correlated well with serum TSH response to TRF, as opposed to the basal serum TSH levels which did not. Urinary TSH measurement therefore probably has equivalent potential usefulness as a predictor of pituitary TSH reserve to the TRF test. Because hyperthyroidism is best diagnosed using peripheral thyroid hormone levels, particularly T_3 (Larsen, 1972a) urinary TSH measurements can only act as an adjunct to diagnosis. Also, urinary TSH measurements should not be performed when proteinuria is present.

A method has also been described for producing iodine labelled

thyroid hormones of very high specific activity in one iodination procedure. The specific activities achieved for these hormones are some of the highest reported. The fact that we could harness both labelled T_3 and T_4 in one iodination procedure, largely because of our separation techniques, makes the method economical, easy and efficient. It was also shown that the method could be used to produce radioiodine labelled TRIAC and TETRAC, both important metabolites of T_3 and T_4 respectively. With the high specific activity thyroid hormones, sufficient sensitivity of the radioimmunoassays could be achieved to measure free levels of these hormones in blood and in vitro. The labelled metabolites, not commercially available in this country, would benefit investigators in this field.

Urinary TSH measurement has also enabled us to further clarify the metabolism of the hormone. We have shown that the hormone is excreted in urine only in very small amounts, even after the large loading doses that we used in the infusion of hTSH. This suggests that the hormone is almost completely metabolized in the body before excretion. With an MCR of 40ml/min and a urinary clearance of 0.04ml/min, urinary excretion could only account for 0.1% of the metabolism of the hormone.

The gel-filtration studies of urinary TSH showed that, in urine, the hormone is largely in fragment form in contrast to the serum and pituitary hormones which were largely intact. This is further indirect evidence that the kidney probably plays a significant role in the metabolism of the hormone in man. Similar conclusions, but derived from evidence of a different nature, have been reached by Bakke et al (1962), Beckers et al (1971) and Cuttelod et al (1974), who demonstrated that the half-life of the hormone in patients with chronic renal failure was longer and the metabolic clearance rate lower than normal.

We have demonstrated, using homologous hormone in physiological quantities, that the kidney is the major organ of localization of injected TSH in rats. This confirms previous reports using heterologous hormone largely in unphysiological doses and whose validity was open to doubt. This study has removed the doubt on the physiological significance of such reports.

We have also shown that the precise anatomical localization of TSH in the kidney, hitherto unknown, is the proximal tubular epithelium using autoradiographic techniques. Therefore TSH should be added to the list of hormonal and non-hormonal proteins such as insulin, GH, ferritin and albumin filtered by the glomerulus and reabsorbed by the proximal tubules. It was also demonstrated by the gel-filtration studies on kidney extracts, that the kidney catabolised the hormone in vivo, and that it is not a passive reabsorptive organ. Hitherto all attempts to obtain direct evidence of renal breakdown of TSH have used in vitro methods, and have failed.

Our studies on human TSH kinetics in man revealed that previous reports using bolus injection of ^{131}I-hTSH or bovine TSH may be grossly underestimating the half-life of the hormone in serum since our values were 140 - 200% of those previously reported. The fact that we used unlabelled homologous TSH given as a constant infusion overcame the flaws in the previous studies and therefore probably makes our report more valid. Corroborative evidence was also obtained from studies on LH and FSH kinetics using human LH and FSH infusion and where data using such stringent criteria are few. The half-lives of LH and FSH in serum were considerably longer than those reported previously, and derived from bolus injection of labelled hormones. Our findings that the half-life of FSH was longer than that of LH which was longer than TSH half-life, we believe, is probably due to the differences in their sialic acid content. The biological significance of the differences in

their half-lives may also be related to their various functions and the half-lives of their target hormones. Thus, the thyroid hormones with their long half-lives would require a trophic hormone with a short half-life, and conversely for the sex-hormones. These speculations require further investigation.

The hTSH preparation used in this study has been shown to be biologically effective, causing a prompt and sustained rise in serum T_3 and T_4. The temporal patterns of the increases in T_3 and T_4 in serum are similar to those described after the injection of bovine TSH and TRF i.e. the proportional increases in T_3 were greater and more rapid. However, we found that, in urine where the measurement reflects the serum unbound thyroid hormone levels, both hormones rose in parallel and maintained the same ratios in which they are found in normal basal urine. This suggested that the TSH stimulated the hormones from the thyroid gland in about equal proportions and that the proportional effect on serum T_4 was probably "dampened" by thyroxine binding globulin which is more avid for T_4 than T_3. Therefore the suggestion that T_3 is the acute hormone of the thyroid (Larsen, 1972a) still remains to be substantiated.

TSH secretion in response to TRF in various non-thyroid endocrine diseases has also been clarified. We have shown that in both normal subjects and in patients with hypothalamo-pituitary disorders, whether TRF, LHRF or insulin-induced hypoglycaemia was given alone or in combination, no difference was found between the hormone responses (TSH, LH, FSH GH and cortisol) seen to these stimuli. A combined administration of insulin and the hypothalamic releasing hormones can therefore be used as a single test for the assessment of anterior pituitary function. The test is convenient and time-saving for the patient, the doctor and the hospital and, with care, can be performed in outpatients.

Our findings that the incidence of TSH unresponsiveness to TRF in untreated euthyroid acromegalics is high is in accordance with published reports, though the incidence in our series is about 10% lower. The TSH unresponsiveness could not be correlated with the amount of pituitary damage, as evidenced by the failure of other pituitary hormones, so local effect of the tumours could not be held responsible. The TSH unresponsiveness could also not be correlated with the peripheral thyroid hormone levels which were normal in all subjects. Our analysis of the role of growth hormone suggested that this hormone may play some part in the genesis of these findings. This requires further investigation.

If excessive serum levels of GH impairs serum TSH response to TRF, excessive prolactin does not appear to do so in man, in contrast to findings in experimental animals.

We have also shown that serum TSH response to TRF in pituitary-dependent Cushing's syndrome is invariably impaired or absent before treatment and that this is reversed after adequate treatment. The consistency of this finding would suggest that the TRF stimulation test may be a useful adjunct in the investigation and follow up of Cushing's disease in a way analogous to its advocated use in hyperthyroidism. Our results also suggest that chronic excessive plasma levels of cortisol inhibits TSH secretion at the pituitary level in contrast to the finding of other workers who showed that administration of corticosteroids inhibits TSH secretion at the hypothalamic level; but the steroids were only given for a brief period. The untreated Cushing's disease patients, although they had an impaired serum TSH response, had a normal serum prolactin response to TRF. This dissociation between the TSH and prolactin response to TRF provides further evidence that the release of both hormones by TRF is governed by independent mechanisms.

Our diabetic patients with mildly elevated basal serum TSH levels also had exagerrated serum TSH responses to TRF, suggesting increased pituitary TSH reserve capacity. Since the patients were euthyroid by clinical and biochemical criteria, they may be in the compensated phase of mild thyroid failure, the so-called "sub-clinical hypothyroidism". TSH responsiveness to TRF may therefore be a useful tool in the diagnosis of mild degrees of thyroid failure.

A P P E N D I X

APPENDIX 1

RADIOIMMUNOASSAY OF SERUM AND URINARY TRI-IODOTHYRONINE (T3) AND (T4)

REAGENTS:

1a) STOCK PHOSPHATE BUFFER

M/15 K_2HPO_4/KH_2PO_4 pH 7.4
3.6 gms KH_2PO_4
18.6 gms K_2HPO_4
Distilled water to 2 litres

1b) WORKING BUFFER

Stock phosphate buffer
+ 0.01% Merthiolate
+ 0.1% gelatin

2a) STOCK T3 AND T4 STANDARDS (Sigma)

100µg/ml in purified ethanol
5mg T3 or T4 washed into 50ml volumetric flask with 5 changes of ethanol each containing 2 drops 0.88 (Sp.gr.) Ammonia and made up to volume.

Keep at 4°C.

2b) WORKING T3 AND T4 STANDARDS

(a) Serum T4 12.5 - 400ng/ml
(b) Serum T3 0.25 - 8ng/ml
(c) Urine T3 and T4 0.1 - 2ng/ml

These are made up in working phosphate buffer to which has been added 0.01% Merthiolate + 0.1% Gelatin (which act as preservatives) and can be kept at 4°C for up to three months.

3) BLOCKERS (for serum assays only)

ANS 500µg/ml + Merthiolate 5mg/ml in stock phosphate buffer.

4a) STOCK ^{125}I-T3, ^{125}I-T4

Stored in 50% ethanol at 4°C.

4b) WORKING ^{125}I-T3 and ^{125}I-T4

DILUTE in blockers accordingly to have approximately 10,000 counts/100 secs. in 0.2ml.

5a) STOCK ANTIBODY (Kindly donated by Dr. Burke)

T3: Rabbit Anti-T3 from Rabbit ARCHIE (8.8.73)
Aliquots of 1ml/100 in working phosphate buffer kept at -20°C.
T4: Rabbit Anti-T4 (ERNEST) 1:50 in phosphate buffer kept at -20°C.

5b) WORKING ANTIBODY

T3 (1:3500, serum; urine 1:650 initial dilution
T4 (1:1000, serum; urine 1:1000 initial dilution

6) T4, T3-FREE SERUM (for serum assays only)

Wash approximately 20gms AMBERLITE resin CG-400(Cl) in distilled water 5 times or until water is clear.
Take 150mls (approx) Pooled serum to pH 10.6 (use pH meter)
(Usual procedure is to add 3ml 2N TRIS which will take pH to approximately 9.0 and then to add 5N NaOH (2 - 5 drops) to take pH to 10.6)

6) Cont.

Add ^{125}I-T4 to serum in a quantity which will give 5000cpm/ml - leave for 15 minutes. In Universal containers add 1ml Amberlite slurry to 10ml treated serum. Shake gently for one hour on "Rotator Mixer".
Centrifuge for 10 minutes at 2,500 rpm at 4°C.
Remove serum supernatant and take pH back to between 8 & 9 with 3N HCl.
Supernatant must not contain more than 1% of the initial counts.
Store 1ml aliquots at -20°C.

7) CHARCOAL/DEXTRAN MIXTURE

2.6gms Charcoal
0.26gms Dextran T-70
0.1gms Merthiolate
Made up to 400ml with stock phosphate buffer.

8) WORKING SECOND ANTIBODY

Burroughs-Wellcome Anti-rabbit gamma globulin (1:20)

9) "DOWEX -1" (SIGMA) (Urine Only)

Wash x4 in phosphate buffer, dry on "Buchner" with 50% ethanol and desicate.

10)
a) ASSAY PROCEDURE FOR SERUM T3

"Oxford Pipette", "Compu-Pet" or "Repettes" may be used.
Always include one Standard Curve in each batch of 72 tubes (1 centrifuge load) to monitor variations in charcoal addition.

1) Standards:

Into LP3 tubes 0.1ml Std + 0.1ml T3-Free serum.

Unknowns:

Into LP3 tubes 0.1ml Unknown and 0.1ml working phosphate buffer.
Include No Antibody tubes with each batch.
These contain 0.1ml T3-Free serum + 0.1ml working phosphate buffer.
All in duplicate.

2) Add 0.1ml working antibody to each tube except TRA tubes and No Antibody tubes. Add a further 0.1ml buffer to No Antibody tubes.

3) Add 0.2ml working label in blockers (see Reagents 3 & 4b) TRA tubes have 0.2ml label in blockers alone in ten tubes.

4) Vortex mix all tubes.

5) Incubate 24 hours at 4°C.

6) Charcoal and centrifuge in batches of 72 (1 centrifuge load) at a time. Add 0.8ml Charcoal/Dextran mixture to each tube. Start by adding charcoal to one of the duplicates of each standard. Continue to add charcoal to the unknown tubes and finish the batch by adding charcoal to the other tube of the duplicate standard. This minimizes errors due to variation in timing of charcoal addition. Vortex mix each tube and after finishing mixing last tube in batch start stopclock. Incubate at 4°C exactly 15 minutes.

10) Contd.

7) Centrifuge for 15 minutes at 4°C at 2000 rpm.

8) Remove supernatant carefully.
Count precipitate (free).

9) Calculate % Free T3 and ultimately % bound T3 and plot against ng/ml T3.
Read off unknowns from their % bound.
(Alternately supernatant can be counted and % bound calculated directly from the counts.)

b) ASSAY PROCEDURE FOR SERUM T4

1) Using Compu-Pet:

Standards: Into LP3 tubes 10ul Std + 100ul phosphate buffer.
Then..... 10ul T4-Free serum + 100ul phosphate buffer.

Unknowns: Into LP3 tubes 10ul Unknown + 100ul phosphate buffer.
Then..... 110ul phosphate buffer.

No Antibody Tubes:
Into LP3 tubes 10ul T4-Free serum + 100ul buffer.
Then..... 110ul buffer.

All in duplicate.

2) Using Compu-Pet:

Add 0.2ml working label in blockers (see Reagents 3b & 4).
TRA tubes have 0.2ml label in blockers alone in 10 tubes.

3) Using Repette:

Add 0.1ml working 1st Antibody to each tube except TRA TUBES AND NO ANTIBODY TUBES.
Add a further 0.1ml buffer to No Antibody tubes.

4) Mix all tubes well.

5) Incubate 24 - 48 hours at 4°C.

6) Using Repette:

Add 0.1ml 2nd antibody to each tube except TRA tubes.
Mix.

7) Incubate all tubes overnight at 4°C.

8) Centrifuge for 20 minutes at 2000 rpm at 4°C.
Discard the supernatant.
Count the precipitate.

9) Calculate % bound and plot against ng/ml T4.
Read off unknowns from their % bound.

APPENDIX 2

GH, LH & FSH ASSAY PROCEDURE:- FLOW CHART

DAY 1:- Preparation of Standard Curve and Sample Tubes

Added to Each Tube:-

- 0.1ml standard or sample
- 0.9ml buffer:- borate pH 8.4, 0.2% BSA (LH & FSH), barbitone - acetate-saline pH 8.6, 0.2% BSA (GH)
- 0.1ml first antibody diluted in carrier serum 1/75:- 1/200,000 (LH); 1:150,000 (GH); 1:3000(FSH)

To "Zero"Tubes:-

- 1.0ml buffer
- 0.1ml first antibody

To Excess Antibody Tubes:-

- 0.1ml first antibody:- 1:10,000 (LH); 1:5000 (GH) 1:500 (FSH)
- 1.0ml buffer

To No Antibody Tubes:-

- 0.1ml carrier serum 1:75
- 1.0ml buffer

INCUBATE FOR 24 HOURS AT $4^{o}C$

DAY 2:- Addition of Labelled Tracer

0.1m (approx. 75 - 100pg) of tracer to all tubes including 20 total radioactivity tubes (TRA)
INCUBATE FOR 72 HOURS AT $4^{o}C$

DAY 5:- Addition of Second Antibody:-

To all tubes:-

0.1ml second antibody
INCUBATE FOR 24 HOURS AT $4^{o}C$

DAY 6:- Separation of Precipitate:

Centrifuge all tubes at 2000 rpm for 20 mins at $4^{o}C$.
Decant supernatant.
Count tubes containing precipitates, and TRA tubes.

Patient	Age (years)	Sex	Urinary TSH μu/hr	Serum			
				TSH μu/ml	PBI μg/100ml	T_4 ng/ml	Cholesterol mg/100ml
1	46	F	10.8	110	1.4	22	242
2	63	F	12.2	39.0	3.8	-	261
3	50	F	13.4	78	2.5	30	300
4	14	M	14.8	20	3.8	47	240
5	49	M	16.0	78	0.8	ND	400
6	65	F	16.5	78	2.4	30	276
7	63	F	20.6	43	1.2	24	610
8	33	M	24.0	149	1.9	-	280
9	77	F	28.6	115	2.4	13	490
10	70	M	30.1	70	0.8	ND	310
11	70	F	33.3	70	1.9	ND	330
12	65	F	39.6	170	1.6	-	350
13	55	F	45.2	132	1.2	-	320
14	20	F	46.5	920	1.8	-	425
Normal Range:			3.5-9.8	<1-3.7	4.0-8.0	55-115	140-240

Appendix 3.1: Laboratory data on Hypothyroid patients.

ND = Undetectable

Patient	Age (Years)	Sex	Urinary TSH (μu/hr)	Serum TSH (μu/ml)	PBI μg/100ml	Serum T_4 ng/ml
1	40	F	<1	<1	14.0	192
2	78	F	<1	2.7	13.0	156
3	36	M	1.9	1.0	10.5	194
4	28	F	2.4	<1	10.0	170
5	47	F	2.7	<1	11.0	137
6	45	M	2.7	1.5	11.0	150
7	56	F	2.8	<1	12.0	206
8	44	M	2.9	<1	11.5	280
9	68	F	3.0	1.3	12.5	216
10	22	F	3.1	1.2	14.0	250
11	47	M	3.1	<1	13.6	210
12	21	F	3.2	<1	14.5	274
13	60	F	3.3	<1	12.0	173
14	54	F	3.3	<1	13.5	220
Normal Range:			3.5-9.8	<1-3.7	4.0-8.0	55-115

Appendix 3.2: Laboratory data on Hyperthyroid patients.

APPENDIX 3.3

Patient	Age (years)	Sex	Diagnosis		Urinary TSH μu/hr	Increments in Serum			
						TSH μu/ml	LH mu/ml	GH μu/ml	Cortisol μg/100ml
1	46	M	Diabetes	PI	<1	<1	<1	<1	<1
2	28	M	Acromegaly	PI	2.2	<1	<1	<1	1.5
3	60	F	Acromegaly	PI	2.2	<1	<1	5.0	<1
4	27	M	Pituitary Tumour	PI	2.6	<1	1.0	1.2	3.0
5	39	F	Diabetes	PI	2.8	<1	<1	<1	<1
6	30	M	Diabetes	PI	3.0	<1	<1	<1	<1
7	21	F	Acromegaly	PA	3.3	1.7	<1	20	2.5
Normal Range:					3.5 - 9.8	4.5 - 20.4	2.6 - 33.6	>20	>15

Laboratory data on Hypopituitary patients

PI = post implant with ^{90}Y. PA. = pituitary apoplexy. The diabetic patients had pituitary ^{90}Y implants for proliferative retinopathy. Serum increment values derived from mean 20 + 60 min value less basal level during TRF (TSH) or LHRF (LH) tests. For GH andcortisol values derived from mean 60 + 90 level less basal value during an insulin tolerance test.

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PAPERS SUBMITTED IN SUPPORT OF THESIS

(1) Kuku, S.F., Harsoulis, P., Young, J.L. and T.R. Fraser: Concentrations of immunoreactive thyrotrophic hormone in urine of normal subjects, patients with thyroid disorders and hypopituitarism, and after infusion of human thyrotrophic hormone. J. Endocr. 62:645

(2) Kuku, S.F., Harsoulis, P., Young, J.L. Que, N.D. and T.R. Fraser (1974). Sephadex gel-filtration analysis of immunoreactive thyrotrophic hormone in urine. J. Endocr. 62:657

(3) Harsoulis, P., Marshall, J.C., Kuku, S.F., Burke, C.W., London, D.R. and T.R. Fraser: Combined test for assessment of anterior pituitary function. B. Med. J. 4 :326

(4) Kuku, S.F., Harsoulis, P., Kjeld, M., and T.R. Fraser (1974). Human thyrotrophic hormone kinetics and effects in euthyroid males. Horm. Metab. Res. (Accepted for publication).

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CONCENTRATIONS OF IMMUNOREACTIVE THYROTROPHIC HORMONE IN URINE OF NORMAL SUBJECTS, PATIENTS WITH THYROID DISORDERS AND HYPOPITUITARISM, AND AFTER INFUSION OF HUMAN THYROTROPHIC HORMONE

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SUMMARY

The urinary excretion of thyroid-stimulating hormone (TSH) has been measured by double antibody radioimmunoassay after concentration by dialysis followed by lyophilization. Among 30 normal subjects, the excretion was $5{\cdot}6 \pm 0{\cdot}31$ (S.E.M.) μu./h. No diurnal variation nor differences between sexes were discerned. In 14 primary hypothyroid subjects the urinary excretion was raised ($P < 0{\cdot}001$) to $25{\cdot}1 \pm 3{\cdot}3$ μu./h. In 14 hyperthyroid and 7 hypopituitary subjects subnormal levels of $2{\cdot}6 \pm 0{\cdot}2$ and $2{\cdot}5 \pm 0{\cdot}22$ μu./h ($P < 0{\cdot}001$) respectively, were found. Serum and urinary TSH concentrations were measured before, during and after an infusion of human pituitary TSH (MRC 70/9) in two subjects and showed a correlation.

Urinary TSH measurement is thus a good discriminant between normal and hyperthyroid or hypopituitary patients.

INTRODUCTION

Detection of thyrotrophic hormone (TSH) in urine has been claimed by various workers using bioassay methods. Hertz & Oastler (1936) found TSH activity in the urine of myxoedematous patients but not in that of normal or thyrotoxic subjects, while Rawson & Starr (1938) found the hormone in normal urine and also a higher concentration of it in urine from hypothyroid patients. Kriss, Greenspan & Lew (1954) confirmed these findings. More recently Ishigami (1966) has reported that TSH activity is detectable in concentrated normal urine. However, no report of immunoreactive TSH in urine has previously been reported.

We now report a method for measuring immunoreactive TSH in urine together with some evidence of its specificity and the levels found in hyperthyroidism, hypopituitarism, hypothyroidism and in normal subjects.

MATERIALS AND METHODS

Collection, concentration and extraction of urine

Overnight 12-h or 24-h specimens of urine were collected at room temperature, and either dialysed at once, or stored at −20 °C immediately after volume measurement. For dialysis, 50 ml urine were placed in a 'Visking' tube and 0·2 ml 1% bovine serum albumin was added. Electrophoretically pure ^{131}I-labelled TSH (about 1000 c.p.m.) was also added to every tube to check the recovery of the extraction process. Dialysis was carried out against ten volumes of distilled water at 4 °C for 24 h, the distilled water being changed three times during this period. The diffusate was then lyophilized, and finally dissolved in 1 ml 0·05 M-phosphate buffer (pH 7·4) containing 0·05% bovine serum albumin. After 6–12 h at 4 °C, the concentrated urine was centrifuged ready for counting and radioimmunoassay. Assay results were corrected for recovery, calculated from the ratio of those counts recovered and the counts added. A 'TSH-free' urine pool was obtained from two patients who had been implanted twice with ablative doses of yttrium-90 for diabetic retinopathy and who satisfied the criteria for hypopotuitarism as described later. The subjects were also on full thyroxine replacement therapy.

Radioimmunoassay procedures

Urinary TSH was measured by radioimmunoassay using a double antibody method described for serum TSH by Tunbridge, Jackson, Iniguez & Fraser (1973). Purified human TSH, supplied by the National Pituitary Agency, U.S.A., was iodinated by the method of Greenwood, Hunter & Glover (1963). As standard, the MRC research standard A (63/14) was used. The guinea-pig anti-human TSH and the rabbit anti-guinea-pig gamma globulin were prepared in our laboratory. The assay has a sensitivity of 1·5 μu./ml of added sample and its upper limit for normal serum TSH is 3·7 μu./ml. The coefficient of variation for ten determinations of a single sample within one assay was 2·5% and between the assays 14% for levels in the range 1–10 μu./ml. For urine assays, 0·2 ml concentrate was assayed in a final incubation volume of 0·6 ml, in duplicate, and 'TSH-free' urine concentrates were added to the standard tubes. For the luteinizing hormone (LH) and follicle-stimulating hormone (FSH) assays carried out on some samples the double antibody method described by Marshall, Anderson, Burke, Galvao Teles & Fraser (1972) and Marshall, Anderson, Fraser & Harsoulis (1973) was used. Results are expressed in mu. MRC human pituitary standard for FSH and LH 69/104/h, derived from LER-907 (Bangham, Berryman, Burger, Cotes, Furnival, Hunter, Midgely, Musset, Reichert, Rosenberg, Ryan & Wide, 1973).

Subjects

Normal subjects were chosen from members of the medical staff; 18 men aged between 25 and 50 years and 12 women aged between 22 and 63 years. None had evidence of endocrine or other disease. Samples were studied from 14 subjects with well defined clinical and biochemical evidence of primary hypothyroidism, and 14 clinically and biochemically overt cases of hyperthyroidism. The hypopituitary group consisted of acromegalics who had undergone pituitary apoplexy and diabetics

who had pituitary implants of yttrium-90 for severe proliferative diabetic retinopathy. Not only did this hypopoituitary group have clinical and serum assay evidence of hypothyroidism, hypodrenalism, hypogonadism, growth hormone deficiency and undetectable levels of serum TSH but they did not respond to the administration of thyrotrophin releasing factor on at least two occasions. Diurnal variation of urinary TSH excretion was assessed in two normal subjects by collecting separately split day and night 12 h urine specimens for 5 consecutive days.

Infusion of human pituitary TSH in normal subjects

One ampoule of human pituitary standard preparation TSH (MRC 70/9; 1 i.u.) was injected i.v. into two normal volunteers as follows: 250 μu. were given as a bolus and the remainder of the dose was then infused at a constant rate of 250 μu./h for 3 h in 50 ml 0·9% NaCl solution. The subjects were not fasting and remained recumbent during and for 3 h after the infusion. Blood samples were taken 15 min before the injection, during and after the infusion. Urine for TSH assay was collected for the previous 24 h, during the infusion and then for periods of 3, 6 and 12 h consecutively after the infusion. The urine was frozen and processed as described above.

RESULTS

Tests of the method

Recovery experiments

Table 1 shows that 76–82% of the ^{131}I-labelled TSH added to urine before the concentration process was recovered, this is equivalent to 38 to 41 times the concentration of TSH in urine. When unlabelled TSH in the range of 5–100 μu. was added to unprocessed TSH-free urine, the corrected recovery ranged between 92 and 120% (Table 2). The counts obtained in the ^{131}I-labelled TSH check system were used to correct these results. Storage at 4 °C for 48 h or at room temperature for 24 h did not alter the recovery of endogenous or exogenous TSH in urine. Storage at room temperature for 48 h however, led to about a 10% loss of recovery.

Table 1. *Recovery of ^{131}I-labelled thyroid-stimulating hormone (TSH) added to unprocessed human urine before its concentration*

(Each row gives the mean of one run of tests. The concentration achieved was calculated by multiplying the % recovery of ^{131}I-labelled TSH by 50 which was the starting urine volume.)

		^{131}I-Labelled TSH (mean c.p.m.)		Calculated	
Expt no.	No. of samples	Counts added to 50 ml	Counts recovered in final 1 ml	% Recovery (mean of range)	Concentration achieved
1	10	919	702	76 (72–82)	×38·0
2	14	760	619	82 (72–88)	×41·0
3	14	800	645	81 (77–88)	×40·5
4	14	571	463	81 (71–86)	×40·5
5	7	331	272	82 (71–90)	×41·0
6	7	380	309	81 (70–86)	×40·5

Table 2. *Recovery of thyrotrophic hormone (TSH) added to human TSH-free urine before the concentration procedure and using ^{131}I-labelled TSH for correction*

TSH added to 50 ml sample (μu.)	TSH measured in 1 ml concentrate (μu./sample)		Corrected recovery %
	Uncorrected	Corrected by ^{131}I-labelled TSH	
0	< 1·0	< 1·0	—
5	3·5	3·8	92
10	10·8	9·0	120
25	23·5	20·0	118
50	37·0	42·5	87
60	50·0	45·0	110
100	90·0	82·5	110

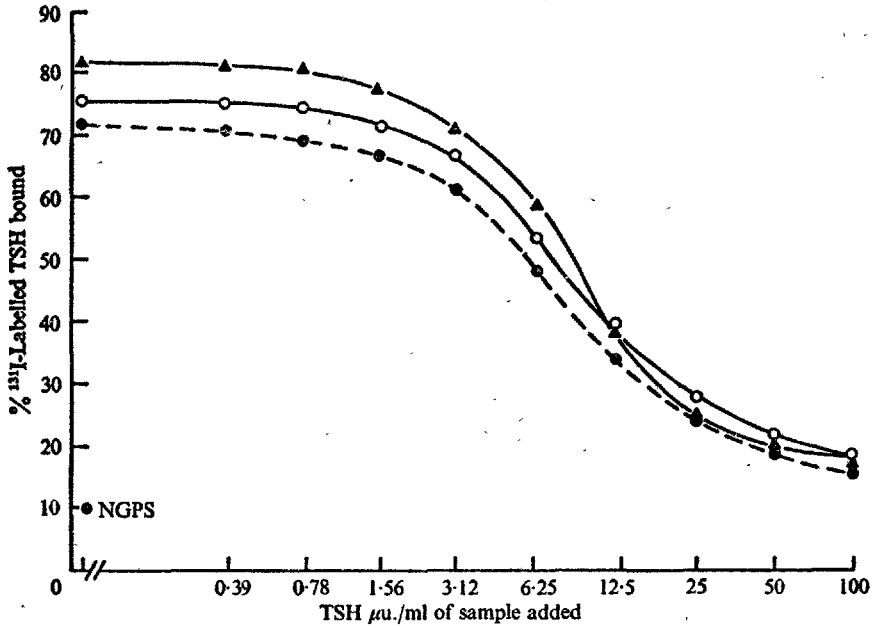

Fig. 1. Effect of dialysed thyrotrophic hormone (TSH)-free urine and TSH-free urine processed in the same way as urinary samples (dialysed and lyophilized) on the TSH standard curve. NGPS = Normal guinea-pig serum; ▲, standard curve in buffer; ○, standard curve in dialysed TSH-free urine; ●, standard curve in dialysed and lyophilized TSH-free urine.

Tests of the influence of non-specific factors on the TSH immunoassay

Bovine serum albumin added to the assay sample over the range 1–5% did not modify the assay. Similarly, sodium chloride did not modify the assay at concentrations over the range 0·2–0·6 mol/l nor did urea over the range 0·5–1 mol/l (urea being unlikely to exceed 0·4 mol/l), although above that concentration the percentage binding of ^{131}I-labelled TSH was increasingly depressed. The average molarity of the processed urines was 550 mosmol/l (430-710 mosmol/l), and so molarity was unlikely to affect the assay. We also tested if other constituents of urine would modify the binding. Figure 1 compares a curve from standards in buffer with curves from standards in dialysed 'TSH-free' urine and from standards in concentrates from 'TSH-

Table 3. *Comparison of excretion rates of urinary thyrotrophic hormone (TSH), follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in various endocrine states chosen to assess any cross-reaction between the various radioimmunoassays*

				TSH/h			FSH/h			LH/h		
Case	Age	Sex	Diagnosis	μu.	ng	10^{-15} mol	mu.	ng	10^{-15} mol	mu.	ng	10^{-15} mol
1	36	M	Normal	3·5	0·17	6·5	185	14·8	493	260	17·4	290
2	29	M	Normal	7·3	0·36	13·5	153	12·2	510	359	21·5	767
3	81	F	Normal P.M.	7·4	0·37	13·7	1294	64·7	2156	895	53·7	1917
4	62	F	Normal P.M.	7·2	0·36	13·3	1832	146·6	4886	568	34·1	1218
5	53	F	Normal P.M.	3·6	0·18	6·6	2118	169·4	5646	604	36·2	1293
6	75	F	Hyperthyroid P.M.	< 1·0	—	—	736	58·9	1963	533	32·0	1143
7	44	M	Hyperthyroid	2·7	0·13	5·0	97	7·8	260	129	7·7	275
8	70	F	Hypothyroid P.M.	16·5	0·82	30·6	1977	158·2	5273	810	48·6	1736
9	22	F	Hypothyroid	45·2	2·26	83·7	176	14·0	466	530	31·8	1135
10	39	F	Hypopituitary	2·8	0·14	5·2	92	7·4	246	57	3·4	121
11	36	M	Hypopituitary	1·5	0·07	2·7	10	0·8	27	5·5	0·3	11·8

P.M., Post menopausal; M, male; F, female. The units were converted to nanograms from the highest sp. act. of the hormone reported in the literature (i.e. LH, 17000 i.u./mg; FSH, 12000 i.u./mg; TSH, 20 i.u./mg; Bangham, Butt, Stockell Hartree, Lunenfield, Reichart, Ross & Ryan, 1972; Wide, Nillius, Gemzell & Roos, 1973) from which 1 μu. TSH = 0·05 ng; 1 mu. FSH = 0·08 ng and 1 mu. LH = 0·06 ng. We used the following molecular weight values: TSH = 27000. LH = 28000, FSH = 30000 (Pierce, 1971).

free' urine. Clearly, both the 'TSH-free' urine and more so its concentrate suppress the binding of labelled TSH in the assay. Therefore, for all assays of urinary TSH, the standards were made up in concentrated 'TSH-free' urine. Thus this specific radioimmunoassay procedure revealed a factor in urine concentrates which competes with labelled TSH for binding with anti-TSH antibody. Dilution experiments with urine from three hypothyroid patients revealed a proportionality corresponding to the standard curves, suggesting that the addition of 'TSH-free' concentrate in the construction of the standard curve had adequately compensated for the residual non-specific inhibition from urine concentrates.

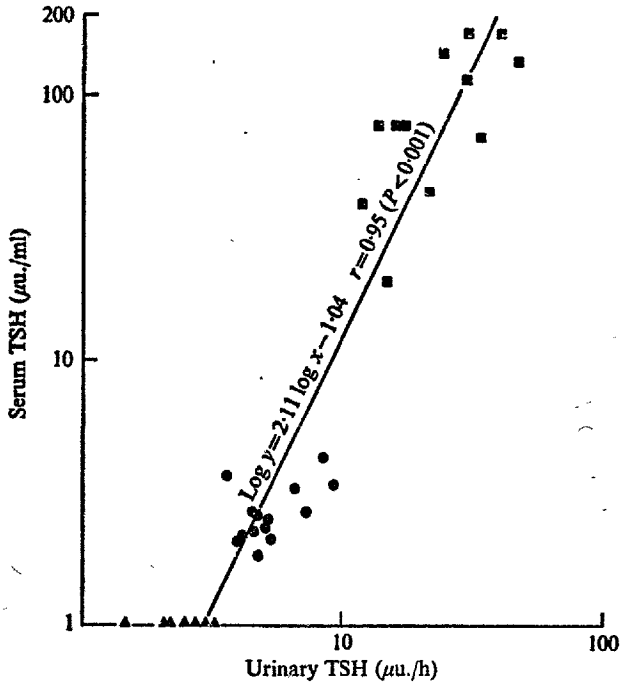

Fig. 2. Correlation between serum and urinary thyrotrophic hormone levels in normal, hypothyroid and hypopituitary subjects. ■, Hypothyroid; ●, normal; ▲, hypopituitary.

Comparison of the rate of urinary TSH excretion with that of LH and FSH, including tests for cross-reaction

Cross-reaction between the hormones TSH, and LH and FSH in our system has been investigated by assaying urine concentrated in this same way not only by our radioimmunoassay for TSH but also by that for LH and FSH. Table 3 shows that normal post-menopausal women, having large amounts of LH and FSH in urine, had TSH values in the same range as normal men. Table 3 also shows that there is no relationship between the urinary level of FSH or LH and TSH whether in normal, hypothyroid, hypopituitary or hyperthyroid subjects. The excretion rate of TSH in urine whether in units, nanograms or moles is much less than that of LH or FSH (Table 3).

Correlation between serum and urinary TSH levels

The level of urinary TSH correlated well with the clinical status of the patients and also with the serum TSH levels (Fig. 2). In one patient the serum and urinary TSH levels were followed monthly for 3 months while on thyroid replacement therapy and both fell in parallel (Fig. 3). Further evidence for correlation of serum with urinary TSH is shown in Fig. 4. This shows the serum and urinary levels achieved during and after the infusion of pituitary TSH in two normal subjects. The highest amount of TSH was obtained during the infusion and fell progressively over the next 24 h. The overall 24-h recovery of TSH in urine was 0·4% of the injected dose.

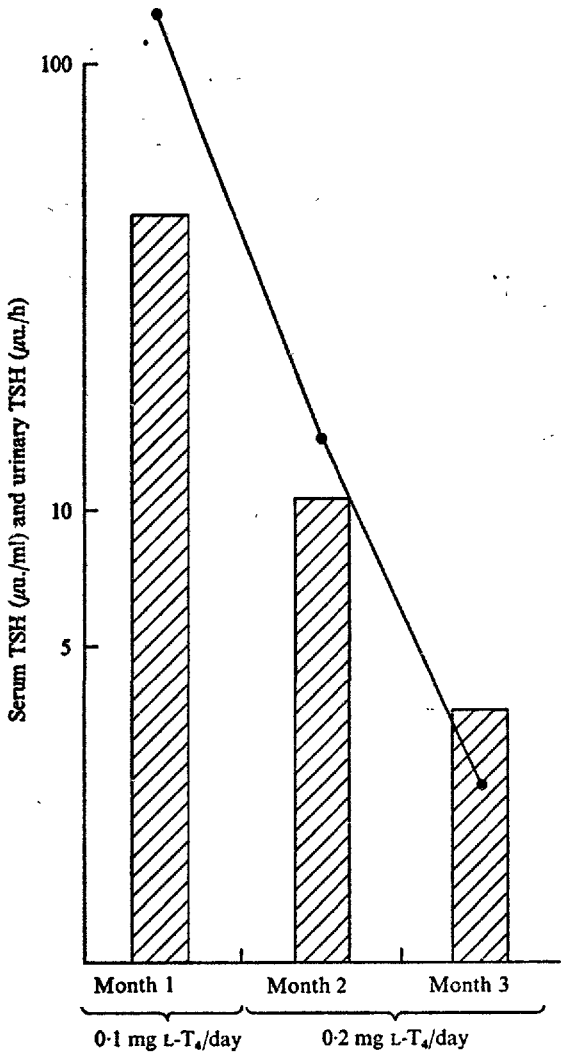

Fig. 3. Serum and urinary thyrotrophic hormone (TSH) levels after L-thyroxine (L-T_4) therapy in a hypothyroid patient. ●, Serum TSH (μu./ml); ▨, urinary TSH (μu./h). A volume of 0·1 mg L-T_4 was given daily for 1 month followed by 0·2 mg daily for the next 2 months.

Urinary TSH levels in normal subjects and patients with pituitary and thyroid disorders

The urinary TSH values in normal, hypothyroid, hyperthyroid and hypopituitary subjects are shown in Fig. 5. The mean level ($\pm$ S.E.M.) was $5{\cdot}6 \pm 0{\cdot}31$ μu./h (range 3·5–9·8 μu./h). The mean level ($5{\cdot}4 \pm 0{\cdot}35$ μu./h) in men was not significantly different

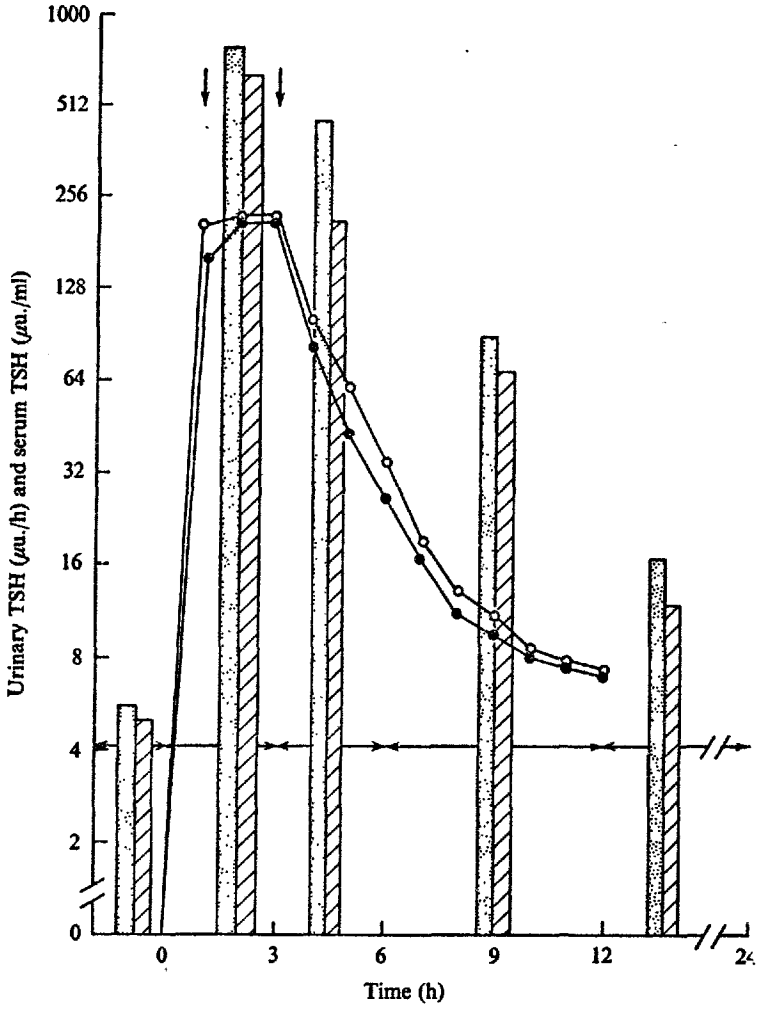

Fig. 4. Serum and urine thyrotrophic hormone (TSH) levels before, during and after an i.v. infusion of human pituitary TSH in two normal subjects. ▦, Urinary TSH, subject 1; ▨, urinary TSH, subject 2; ○, serum TSH, subject 1; ●, serum TSH, subject 2. Horizontal arrows indicate periods of collection of urine for TSH assay. Vertical arrows indicate period of infusion.

from that in women ($5{\cdot}9 \pm 0{\cdot}48$ μu./h; $P > 0{\cdot}4$). The thyroid subjects showed a mean TSH level of $25{\cdot}1 \pm 3{\cdot}3$ μu./h (range 10·8–46·5 μu./h), i.e. all TSH concentrations were higher than those of the normal subjects ($P < 0{\cdot}001$). The values for the hyperthyroid subjects ($2{\cdot}6 \pm 0{\cdot}2$ μu./h, range < 1–3·5) were lower than those of normal subjects ($P < 0{\cdot}001$). The hypopituitary subjects had a mean of $2{\cdot}5 \pm 0{\cdot}22$ μu./h (range < 1–3·3) which was in the same range as the hyperthyroid subjects. Values

below 2·0 μu./h were below the sensitivity of the assay. The mean daytime excretion of urinary TSH in two subjects was 6·0 and 6·6 μu./h and during the night 6·6 and 4·6 μu./h respectively. These values showed no evidence of diurnal variation ($P > 0·1$).

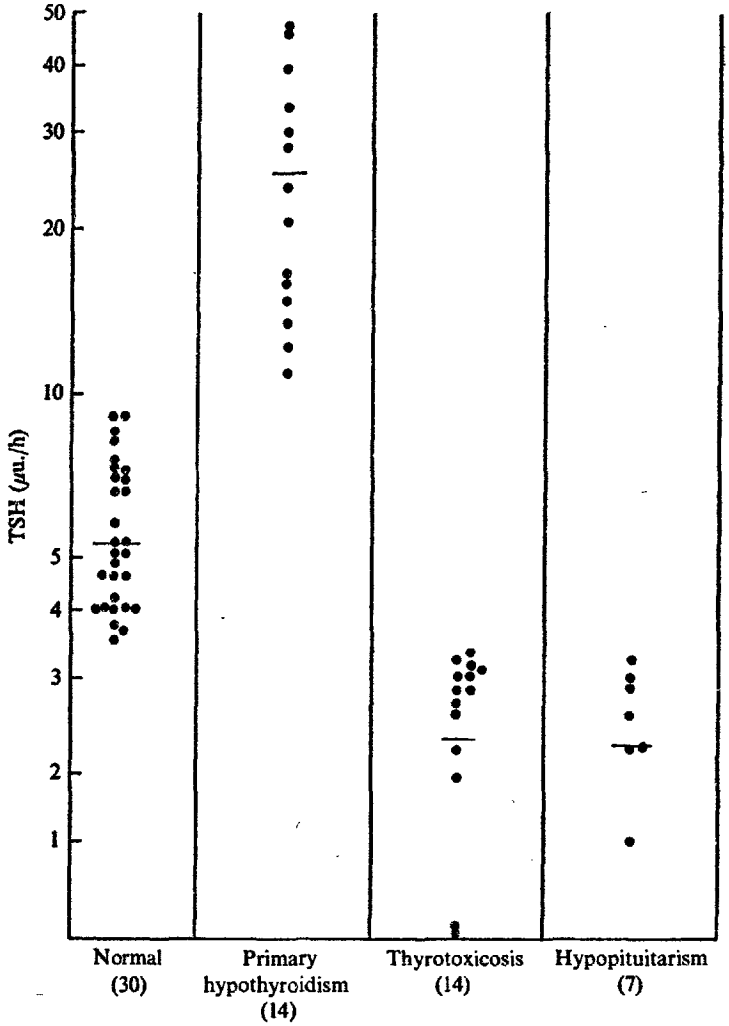

Fig. 5. Urinary excretion of thyrotrophic hormone in normal subjects and patients with primary hypothyroidism, thyrotoxicosis and hypopituitarism.

DISCUSSION

In the assessment of pituitary and thyroid disorders measurement of serum TSH by radioimmunoassay has now found wide application but its limited sensitivity restricts its usefulness somewhat. The level of serum TSH in normal subjects crosses the lower limits of the sensitivity of the assay, so that the assay often cannot distinguish normal subjects from abnormally low TSH levels which occur in hyperthyroid and hypopituitary subjects. We have tried to solve this problem by measuring instead the urinary TSH output/h. Such a measurement has the advantage of reflecting the mean serum level over the chosen lapse of time and our results show that

it is a good index of TSH secretory activity for there is considerable separation between the amounts of TSH secreted into urine by the various groups.

Because TSH is excreted into urine in very small amounts, a concentration procedure involving dialysis and lyophilization, has been used. The recovery experiments utilizing labelled and unlabelled hormone show that this is an efficient and reliable procedure. The recovery of unlabelled hormone was generally higher than that of labelled hormone suggesting that we might be underestimating the true recovery. A possible explanation for this might be that the label contained some dialysable degradation products such as free iodine. This however is unlikely because the label used for the recovery experiments (same as used for immunoassay) was of a high degree of purity using electrophoretic and Sephadex gel-filtration criteria.

The radioimmunoassay system used in this study is specific for TSH, the other glycoprotein hormones LH and FSH which are normally assayed considerably more easily than TSH in urine show little or no cross-reaction with it. Table 3 shows that the urinary TSH levels in normal men are in the same range as in post-menopausal women, although the latter excrete large amounts of gonadotrophins as measured by specific radioimmunoassays. The differences found between the various disease states cannot, therefore, be attributed to cross-reaction with FSH and/or LH. It may be noted also that although these hormones differ little in molecular weight, nevertheless much less TSH is found in urine than FSH and LH. Even when the units are converted to nanograms (Table 3) the molar excretion rate is clearly still much less. Perhaps more TSH is broken down to unassayable fragments before urinary excretion or it is not cleared so readily by the renal cells.

Like other workers (Rubenstein, Lowy, Welborn & Fraser, 1967; Girard & Greenwood, 1968), we have found that increasing concentrations of sodium chloride and urea produce increasing non-specific inhibition of the immunoassay system. But while neat 'TSH-free' urine produced non-specific inhibition probably largely due to its urea and electrolyte content, most but not all of this inhibition was abolished by dialysis against distilled water. However, the dialysed urinary concentrates still showed some inhibitory effects probably due to non-specific effects from peptides or other substances concentrated in the dialysed residue since the osmolarity of the concentrate was found to be not very different from that of the buffer. However, to take these residual non-specific effects into account, the standard curves were always made up in similar 'TSH-free' urine concentrates prepared in the same way as the samples.

There are several indications that the factor measured in the urinary concentrates very probably was TSH. While dilution of the uurine concentrates showed proportionality between the amount of dilution and the inhibition of binding on the assay curve, the dilution curves were also parallel to the standard curve. Furthermore, there was good correlation between serum TSH concentration and the hourly urinary TSH excretion (Fig. 2). The fall in the urinary TSH excretion also followed closely that of serum TSH in hypothyroid patients treated with thyroxine. However, our data on the correlation between serum and urine TSH in hypopituitary and thyrotoxic subjects might be interpreted as showing that TSH up to a level of about 2 μu./h in urine may be due to a test blank, but a more sensitive serum assay is needed to check this.

When human pituitary TSH was infused for 3 h into normal volunteers the urinary TSH values followed closely the serum values, a further validation of our assay system. It may be noted that only 0·4% of the injected dose was recovered in the urine in 24 h. This would suggest that TSH is metabolized almost completely in the body and that very little is excreted into urine in immunoreactive form. Bakke, Lawrence & Roy (1962), using a bioassay, found that 34% of an injected dose of 10 i.u. bovine TSH was excreted into urine in 24 h. This discrepancy might be due to the fact that bovine TSH being 'foreign' is treated differently from the human hormone.

Our study of two normal subjects over a period of 5 days failed to shoJ any significant diurnal variation in urinary TSH excretion. This is not surprising sinc no consistent diurnal variation has been found in serum TSH levels (Hall, 1972).

We have carried out investigations on the characterization/of the nature of the TSH in urine by Sephadex gel-filtration. These are presented in the accompanying paper.

We are grateful to the National Institute of Biological Standards, Mill Hill, London and the National Pituitary Agency for supplies of hormones used in this study, and to Miss P. Heyma and Miss M. Tooley for skilled technical assistance.

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SEPHADEX GEL FILTRATION ANALYSIS OF IMMUNOREACTIVE THYROTROPHIC HORMONE IN HUMAN URINE

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SUMMARY

To assess whether urinary immunoassayable thyroid-stimulating hormone (TSH) differed from pituitary and serum TSH, urinary concentrates from two hypothyroid subjects were analysed by Sephadex G-100 gel filtration.

The elution profiles, measured by radioimmunoassay, were then compared with those of neat sera from hypothyroid patients and human pituitary TSH preparations. The pituitary preparations and the hypothyroid serum were eluted as a comparable single symmetrical peak corresponding to that obtained from a highly purified radio-iodinated human TSH of pituitary origin; no evidence of 'big' TSH emerged. In contrast, however, the material eluted from the hypothyroid urine concentrates not only revealed an asymmetrical peak corresponding to that described above but several other minor peaks eluting later and probably corresponding to fragments of TSH.

When human pituitary TSH was infused into two normal subjects, gel filtration analysis of concentrates from urinary samples obtained during and at fixed periods after the infusion revealed a single peak during the infusion but more peaks appeared with the later samples.

INTRODUCTION

Gel filtration analysis in combination with measurement of eluate fractions by radioimmunoassay has been widely used in the characterization of peptide hormones in urine. Urinary growth hormone (GH) (Chakmakjian & Langston, 1972) and follicle-stimulating hormone (Talas, Midgley & Jaffe, 1973) have been reported to show gel chromatographic profiles essentially identical with those of the pituitary hormones. However, urinary human chorionic gonadotrophin (Matthias & Diczfalusy, 1971; Franchimont, Gaspard, Reuter & Heyman, 1972) and luteinizing hormone (J. L. Young, P. Harsoulis, S. F. Kuku, J. C. Marshall & T. R. Fraser, unpublished data) include profiles more retarded on Sephadex gel filtration than the pituitary hormones. Using a bioassay technique, Ishigami (1966) has found thyrotrophic hormone (TSH) in urine, and that this hormone also includes profiles more retarded on Sephadex than the serum hormone. Elsewhere we have presented evidence that immuno-

reactive TSH is present in urine in amounts correlating with the serum levels, which vary in pituitary and thyroid disease (Kuku, Harsoulis, Young & Fraser, 1974). We present here data showing the Sephadex G-100 gel filtration characteristics of urinary TSH.

MATERIALS AND METHODS

Gel filtration analysis

Downward flow gel filtration was performed using a 2 × 50 cm column of Sephadex G-100. Samples (1–2 ml) were applied to the column and elution was carried out at room temperature at a flow rate of 20 ml/h, using 0·1 m-borate buffer (pH 8·4); 2·4-ml fractions were collected. The column was calibrated using the following substances of known molecular weight: blue dextran (> 1000000); bovine serum albumin (70000), ovalbumin (45000), α-chymotrypsin (22500), cytochrome C (12500). All the reagents were obtained from standard commercial sources. ^{131}I-labelled TSH, ^{131}I-labelled human GH and ^{125}I-labelled insulin were used as radioactive markers. The void volume (V_o) of the column was determined using blue dextran while total volume (V_t) was determined by carrier-free Na ^{131}I.

Materials

The following samples were subjected to gel filtration analysis: (1) human pituitary TSH preparations (MRC Standard A 63/14 and MRC 70/9) obtained from the National Institute of Biological Standards, Mill Hill, London. (2) Sera with raised levels of TSH from two primary hypothyroid subjects. (3) Urine concentrates from two primary hypothyroid subjects. The concentrates were prepared from timed urine collections from the subjects and processed by dialysis and lyophilization as described previously (Kuku *et al.* 1974).

Infusion of human pituitary TSH into two normal subjects

To study renal handling of exogenous TSH and to characterize urinary TSH under these circumstances, one ampoule (2 i.u.) human pituitary TSH MRC 70/9 was infused into each of two normal volunteers as previously described (Kuku *et al.* 1974) Urine samples were collected for 24 h before, 0–3 h during and for periods 3–6, 6–12, and 12–24 h after the infusion. Samples of 150 ml for 0–3 and 3–6 h, 250 ml for 6–12 h and 400 ml for 12–24 h were concentrated and subjected to downward flow gel filtration analysis.

Radioimmunoassay of TSH

Thyrotrophic hormone levels in the eluted fractions from gel filtration analysis were measured by a double antibody radioimmunoassay technique (Tunbridge, Jackson, Iniguez & Fraser, 1973) with the slight modification of omitting 'TS-free' serum from the standard curve which was produced using buffer.

RESULTS

The elution profiles of pituitary TSH preparations and hypothyroid sera on gel filtration analysis are shown in Fig. 1. This shows that immunoreactive TSH is eluted as a single symmetrical peak with an elution volume (V_e) corresponding to a

molecular weight of about 34000. ^{131}I-labelled pituitary TSH was eluted with an identical peak. The pituitary and serum material showed comparable profiles. In contrast, however, elution profiles of basal urinary concentrates showed not only an asymmetrical peak corresponding to the above peak but several minor later peaks.

Gel filtration analysis of urinary concentrates during and for the three periods after the infusion of human pituitary TSH revealed the elution profiles shown in Fig. 2. During the period of infusion, the urinary material eluted as one symmetrical peak with an elution volume similar to that of the pituitary material. The elution

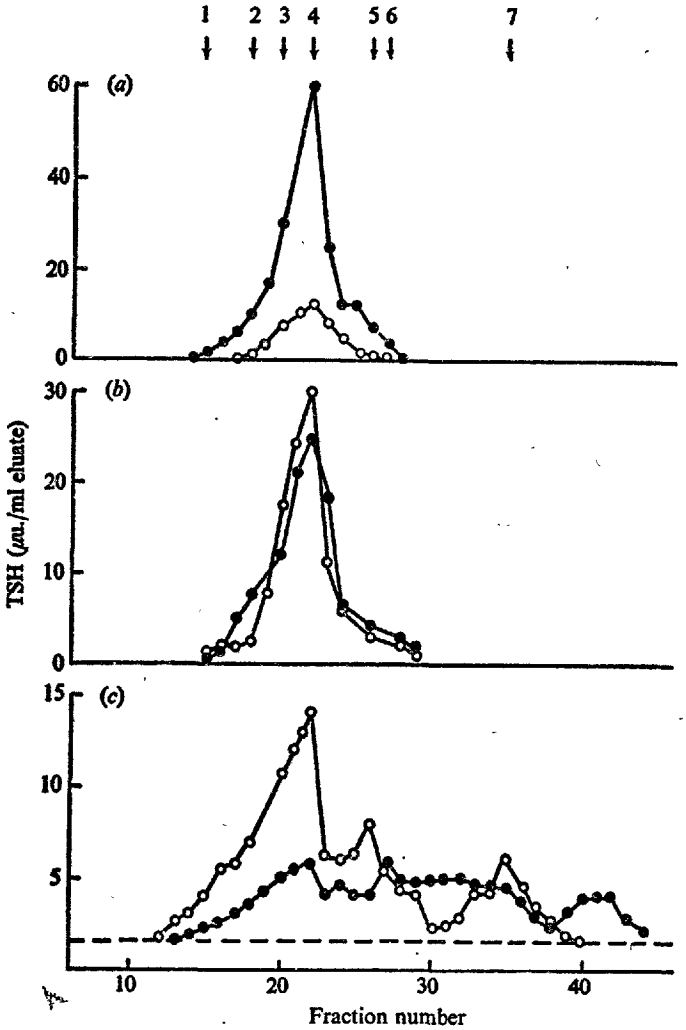

Fig. 1. Gel-filtration profiles of thyrotrophin (TSH) of (*a*) human pituitary TSH standards (●, MRC 70/9; ○, MRC 63/14), (*b*) sera and (*c*) urine concentrates from two hypothyroid subjects (○, subject 1; ●, subject 2). Eluate fractions were of 2·4 ml each; the numbered arrows at the top show the positions of the peaks of the various standards run to calibrate the column; (1) blue dextran, (2) bovine serum albumin, (3) ovalbumin, (4) ^{131}I-labelled TSH, (5) ^{131}I-labelled human growth hormone, (6) α-chymotrypsin, (7) cytochrome C. The 50 × 2 cm² column of Sephadex G-100 was eluated with 0·1 M-borate buffer (pH 8·4). LLS, Lower limit of sensitivity of the radioimmunoassay.

profile for the period immediately after the infusion was essentially similar to that during the infusion. However, the urinary materials for the period 6–12 h and 12–24 h were eluted with a different pattern (Fig. 2). The peak corresponding to the pituitary material was broad and asymmetrical while more minor peaks appeared later. (The later small peaks of Fig. 2*b* were not visible even on corresponding scale diagrams of Fig. 2*a*.) The elution profiles of the 12–24 h material are very similar to the profile of the basal hypothyroid urine concentrates shown on Fig. 1.

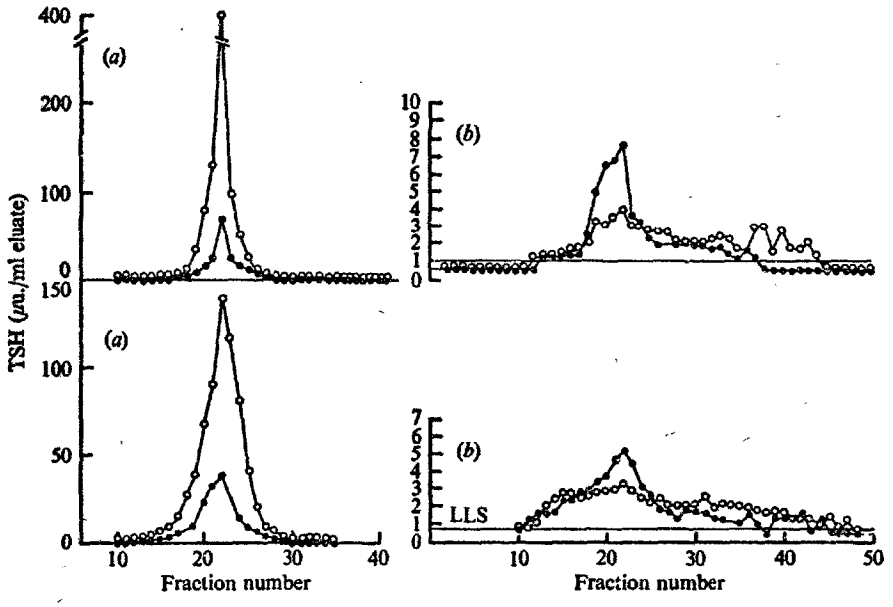

Fig. 2. Gel-filtration profiles of immunoassayable thyrotrophin (TSH) in concentrates of urine (*a*) during (○) and 3–6 (●), and (*b*) 6–12 (●), and 12–24 h (○) after i.v. infusion of human TSH (MRC 70/9) into two normal subjects. Column procedure was as described in legend to Fig. 1. LLS, Lower limit of sensitivity of the radioimmunoassay.

DISCUSSION

Immunoreactive TSH in pituitary preparations and from sera of hypothyroid subjects behaved identically on Sephadex gel filtration under the conditions of buffer concentration, pH and temperature used for this analysis. Thus it may be concluded that no gross differences exist between them. However, subtle differences might emerge if conditions permitting higher resolution were used. Our gel filtration data did not demonstrate any evidence of 'big TSH' in the serum or pituitary preparations as described for some other hormones, for example insulin (Rubenstein, Cho & Steiner, 1968) corticotrophin (Yalow & Berson, 1971), growth hormone (Gorden, Hendricks & Roth 1973), Para-thor-mone (Berson & Yalow, 1968) and gastrin (Yalow & Berson, 1970). In contrast, however, immunoreactive TSH from urine showed gross differences from the above preparations in that a substantial amount of it exists in a range of molecular weight smaller than pituitary and serum TSH. This confirms the work of Ishigami (1966) who found that urinary TSH measured by bioassay was more retarded on Sephadex than serum TSH. These low molecular weight substances, we believe, represent metabolized fragments among

which are fractions which could correspond to α and β subunits of the hormone, and also other smaller fragments.

Concentrates from the urine during and for 3 h after the infusion of exogenous TSH showed essentially identical profiles on gel filtration, which were also similar to those of the infused material. Thus, during or immediately after the infusion only a small proportion of the urinary elution profile was due to low molecular weight fragments. However, as the basal state is approached, i.e. during the later hours after infusion, a greater proportion of the elution profiles represents smaller molecular weight substances, probably fragments. These findings would suggest that TSH appears in urine partly as an intact molecule cleared from the serum, but also partly as smaller fragments presumably produced from metabolism of the hormone. The urinary concentration of the molecule rises immediately with the infusion but the increased excretion of the fragments is delayed, presumably till after their metabolism by the kidney. In the basal state most of the immunoassayable TSH in urine is in the form of these smaller fragments.

On the whole, the differences between gel filtration profiles of serum and urine would suggest that the kidney is an important site of degradation of TSH which confirms the findings of other workers. Altschuler, Pisarev, Mitta & Orlando (1967) have shown that radio-iodinated TSH when injected into rats is mainly localized in the kidney, therefore supporting our concept. This is not surprising because kidney has been shown to be the major site of metabolism of many peptide hormones: HGH (Collip, Patrick, Goodheart & Kaplan 1966), corticotrophin (Richards & Sayers, 1951), prolactin (Sonnenberg, Money, Keston, Fitzgerald & Goodwin, 1951), TSH (Kassenaar, Kerkhofs & Querido, 1959) and insulin (Elgee, Williams & Ince, 1954). The mechanism by which the kidney metabolizes TSH requires further investigation but it could be speculated that it would be a proximal tubular phenomenon from the studies of Collip *et al.* (1966) and Rabkin, Pimstone & Eales (1973) who by autoradiographic techniques showed localization of HGH in renal proximal tubules.

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Combined Test for Assessment of Anterior Pituitary Function

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Summary

A combined test consisting of the simultaneous administration of insulin, thyrotrophin-releasing hormone (TRH), and luteinizing hormone and follicle stimulating hormone-releasing hormone (LH/FSH-RH) was performed in 24 people. Eleven of these also had the three individual tests performed separately, and the remaining 13 had a separate test of either LH/FSH-RH and TRH together or singly at a later date. In both normal people and patients, whether the tests were performed alone or in combination, no difference was found between the hormone responses (growth hormone, cortisol, LH, FSH, thyroid-stimulating hormone) seen to these stimuli.

It is proposed that combined administration of insulin and the hypothalamic releasing hormones may be used as a single test for the assessment of anterior pituitary function. The test is convenient and time saving, and with care can be performed in outpatients.

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Introduction

Measurement of growth hormone (GH) and cortisol during insulin-induced hypoglycaemia are commonly used to assess pituitary secretion of GH and adrenocorticotrophin (ACTH) (Greenwood *et al.*, 1966). Stimulation by the recently introduced hypothalamic releasing hormones, thyrotrophin-releasing hormone (TRH) and luteinizing hormone/follicle stimulating hormone-releasing hormone (LH/FSH-RH), now allows "pituitary reserve" for these trophic hormones to be evaluated.

Thyroid-stimulating hormone (TSH) secretion after synthetic TRH has been extensively studied both in normal people and in patients with hypothalamic-pituitary disease (Fleischer *et al.*, 1970; Anderson *et al.*, 1971; Haigler *et al.*, 1971; Ormston *et al.*, 1971; Gual *et al.*, 1972; Hall *et al.*, 1972). Similarly LH and FSH responses to synthetic LH/FSH-RH have been

reported both in normal people (Besser *et al.*, 1972; Nillius and Wide, 1972; Yen *et al.*, 1972) and in some patients with abnormalities of hypothalamic-pituitary function (Kastin *et al.*, 1972; Marshall *et al.*, 1972 a).

Most workers have found that the releasing hormones are specific, secretion of other pituitary hormones being unchanged after TRH and LH/FSH-RH (Fleischer *et al.*, 1970; Kastin *et al.*, 1971; Ormston *et al.*, 1971; Besser *et al.*, 1972). Increased GH levels after TRH have, however, been noted in some subjects (Anderson *et al.*, 1971), and both increase and decrease of plasma cortisol has been reported (Karlberg *et al.*, 1971; Rothenbuchner *et al.*, 1971). Both GH and ACTH are affected by stress, however, so it remains uncertain as to whether these changes relate to a specific effect of the releasing hormones.

There is little data on the interactions of these different stimuli in man. In one study (Besser *et al.*, 1971) TSH responses to TRH alone were not affected by coexistent insulin hypoglycaemia. Similarly GH responses to insulin were unchanged by addition of TRH, but cortisol responses were slightly greater after TRH and insulin than after insulin alone. The present study aimed to establish whether all three tests could be combined into a single procedure without any significant alteration of the hormone responses compared to those produced by each agent individually.

Hitherto pituitary function has usually been evaluated on the performance of each provocative test seriatim, involving the patient in a considerable amount of time, and often admission to hospital. A single test would thus benefit the patient, who would be required to attend the hospital for only a few hours, and the hospital, which would be spared the need to provide inpatient care.

Subjects and Methods

Four normal men and 20 patients with hypothalamic or pituitary disease were studied. The diagnoses of the patients, based on clinical, radiological, and biochemical evidence, are shown in the table. The four normals and seven patients had a combined test (insulin, LH/FSH-RH, and TRH) and the individual tests, performed separately in random order on different days. The interval between the separate tests was between two and seven days. The combined test was paired with simultaneous administration of LH/FSH-RH and TRH in 10 patients, with LH/FSH-RH alone in two, and with TRH alone in one. Informed consent was obtained from both patients and normal subjects.

Disorders Found in the 24 Subjects Studied

Diagnosis	No. of Patients
Acromegaly, untreated	5
Acromegaly, after ^{90}Y implantation	3
Chromophobe adenoma, untreated	4
Chromophobe adenoma, after ^{90}Y implantation	1
Diabetic retinopathy, after ^{90}Y implantation	3
Isolated gonadotrophin deficiency	2
Hand-Schuller-Christian, diabetes insipidus	1
Functional amenorrhoea	1
Normal males	4

The patient with functional amenorrhoea had had episodes of depressive illness, and no hypothalamic-pituitary abnormality was found.

Test Procedures.—The combined test was begun between 09.00 and 10.00 hours after an overnight fast. The subjects were recumbent, and an indwelling forearm venous cannula was inserted 30-60 min before the basal blood samples were taken. Soluble insulin (0·05-0·3 units/kg body weight) was injected through the cannula, and followed immediately by a mixture of 200 or 500 μg of TRH and 100 μg of LH/FSH-RH in 5 ml sterile water. Blood samples were taken at 30, 60, 90, and 120 min for determination of glucose, growth hormone, and cortisol, and at 20 and 60 min for LH, FSH, and TSH. For the insulin hypoglycaemia test the same pretest procedure was followed and samples were taken at 30, 60, 90, and 120 min for glucose, growth hormone, and cortisol determinations. After both the combined and insulin hypoglycaemia tests a carbohydrate-rich meal was given to the patients after the last blood sample. Outpatients were observed for 1 to 2 hours and if believed to be panhypopituitary they were also given 5 mg prednisone before being allowed to go home. The separate LH/FSH-RH or TRH tests or a combination of both were performed on non-fasted patients. The releasing hormones dissolved in sterile water were given by rapid intravenous injection. Samples for the appropriate hormone estimations were taken immediately before and 20 and 60 min after the injection. In each subject the same doses of releasing hormones or insulin were used in the individual tests and in the combined test. All the subjects given insulin experienced symptoms of hypoglycaemia, and seven of those given TRH noted some nausea or flushing and a desire to micturate. No other side effects were noted.

Hormone Assays.—Serum LH, FSH, and human growth hormone were measured by double antibody immunoassays as previously described (Hartog *et al.*, 1964; Marshall *et al.*, 1972 b; Marshall *et al.*, 1973). Results of LH and FSH are expressed as mU/ml of M.R.C. Human Pituitary Standard for LH and FSH (69/104) and GH as μIU/ml of World Health Organization International Reference Preparation. Serum TSH was also measured in a double antibody immunoassay. Purified human TSH (National Pituitary Agency) was used for iodination, and M.R.C. human TSH, Research Standard A, as the standard preparation. The antihuman TSH serum was prepared in guinea-pigs and used at a final dilution of 1/300,000. The least amount of TSH detectable in serum is 1·5 μU/ml, and the upper limit of the normal range 4·2 μU/ml. The coefficient of variation in the same assay is 2·5% and between assays 14·4%. LH and FSH do not cross-react in the assay below a level of 100 mU/ml. Plasma cortisol was measured by a competitive binding assay (Beardwell *et al.*, 1968), slightly modified for use with plasma (Marshall *et al.*, 1972 b). All samples from an individual subject were measured in the same assay.

Results

All the subjects given insulin experienced adequate hypoglycaemia (blood glucose values less than 40 mg/100 ml and less than 50% of fasting value). The mean hormone responses during the combined and single tests in normal subjects are shown in fig. 1. The ranges of responses shown were obtained previously in 21 normal subjects aged between 22 and 40 years. There was no significant difference in the degree of any of the responses, whether induced by combined or individual administration of the stimuli, and similarly the pattern of response was unchanged.

The results in patients who had both combined and single tests, or a combined test with later LH/FSH-RH or TRH or both, are shown for those with untreated pituitary tumours in fig. 2, and for those with other untreated hypothalamic-pituitary disorders in fig. 3. Results in patients with pituitary tumours or diabetic retinopathy previously treated by pituitary implantation of ^{90}-Y are shown in fig. 4. No change in the pattern of response was seen in any patient, and to allow direct comparison of the combined and separate stimulation procedures the sum of the serum levels at 20 and 60 min for LH, FSH, and TSH and the sum at 60 and 90 min for GH and cortisol are shown. In most patients there was little difference in the summated levels seen during the two tests, and analysis by paired *t*-test showed that responses were not significantly different.

In some patients small differences in responses were seen, but in the case of the glycoprotein hormones this occurred when the hormone levels were low and nearing the limits of detection of the assays. GH responses were the most variable, but no consistent trend or significant difference was found. One patient with treated acromegaly (fig. 4) was postmenopausal,

and this explained the raised FSH levels seen. No instances of a gross difference in response were seen—that is, unresponsive to combined test and responsive in single test, or vice-versa.

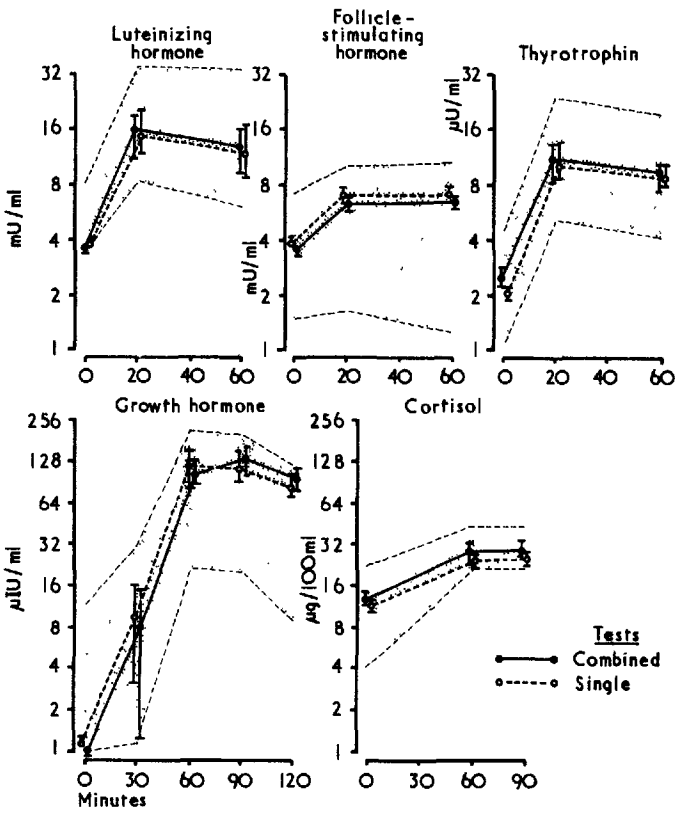

FIG. 1—Hormone responses during combined and single tests in four normal subjects. Mean values ± S.E. of mean are shown. Shaded area represents range of response seen in normal subjects.

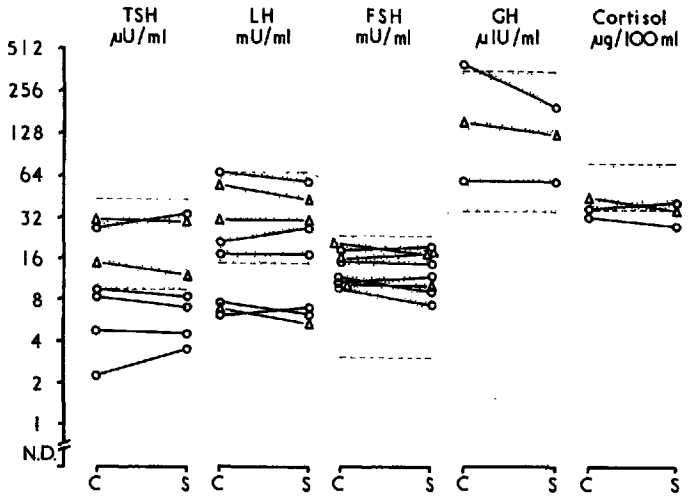

FIG. 2—Comparison of hormone responses in patients with untreated pituitary tumours. Each point represents sum of hormone levels at 20 and 60 min for TSH, LH, and FSH, and at 60 and 90 min for GH and cortisol. Hatched areas represent ranges found in normal subjects expressed in this form. Values in left hand columns are from combined test (c), and those on right from single tests or LH/FSH-RH plus TRH (s). O = Acromegaly, untreated. △ = Chromophobe adenoma, untreated. N.D. = Not detectable.

Discussion

Insulin hypoglycaemia and TRH are now well established at tests of anterior pituitary function, and the early results suggest

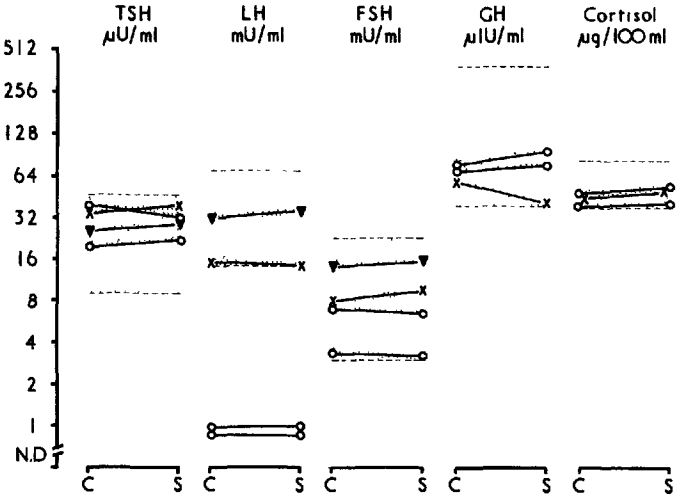

FIG. 3—Comparison of hormone responses in patients with other untreated hypothalamic-pituitary disorders. Same format is used as in fig. 2. ▼ = Functional amenorrhoea. × = Hand-Schüller-Christian, diabetes insipidus. O = Isolated gonadotrophin deficiency.

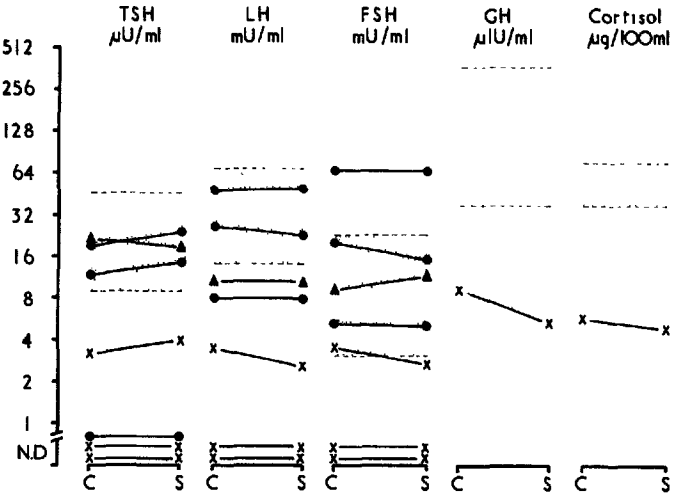

FIG. 4—Comparison of hormone responses in patients previously treated by pituitary implantation of ^{90}Y. Same format is used as in figs. 2 and 3. ● = Acromegaly, after ^{90}Y. ▲ = Chromophobe, after ^{90}Y. × = Diabetic retinopathy, after ^{90}Y.

that LH/FSH-RH can be used to assess pituitary gonadotrophin reserve. At present these stimuli are used separately, but the data of Besser *et al.* (1971) suggested that the simultaneous administration of insulin and TRH did not appreciably affect the various hormone responses. Our results, showing that both releasing hormones and insulin can be given together without significantly changing individual hormone responses, agree with their findings. Furthermore, in patients with disorders of the hypothalamic-pituitary axis, the same classification of "pituitary reserve"—that is, normal, borderline, or impaired—was obtained with the combined test as was found after the single stimulation tests used separately. Thus the assessment of pituitary status was not changed by the use of the combined procedure.

The only differences in response were seen with GH, which is known to be definitely labile. We have, however, performed repeat insulin hypoglycaemia tests on different occasions in the same subject and have shown two-fold differences in peak GH levels after comparable degrees of hypoglycaemia. The differences seen in GH responses in the present study fell well within this degree of variation.

A 20-min sample was included in the combined test to allow comparison with the releasing hormone test procedure as previously published (Besser *et al.*, 1972; Hall *et al.*, 1972). The data for TSH responses to TRH (Anderson *et al.*, 1971) and LH responses to LH/FSH-RH (Besser *et al.*, 1972), however, show that peak levels of these hormones persist up to 40 min after injection. Thus a sample at 30 min would show the peak equally well, and the 20-min sample could be omitted for routine use of the combined test. Prolactin was not measured in these subjects, but the combined test may also prove useful for assessment of pituitary prolactin secretion, for serum levels of this hormone are known to be increased by TRH (Bowers *et al.*, 1971; Jacobs *et al.*, 1971; L'Hermite *et al.*, 1972).

It is proposed that anterior pituitary function can be assessed in a single combined test, which is convenient and time saving and can be performed on outpatients.

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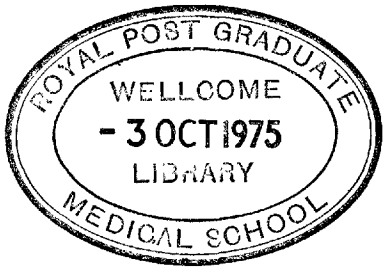

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