THYROTROPHIC ACTIVITY AND EXOPHTHALMOS PRODUCING SUBSTANCE IN HUMAN PLASMA

Their assay in endocrine disorders, and in an investigation of the tri-iodothyronine suppression test of thyroid function

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To MILLIE

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CONTENTS

CHAPTER 1 : INTRODUCTION

While goitres had been recognised since the earliest times, and Shakespeare could remark in the "Tempest",

> "Who would believe that there were mountaineers
> Dew-lapped like bulls, whose throats had hanging at them
> Wallets of flesh;....."

it was only in 1825 that the first clinical description of hyperthyroidism with exophthalmos was recorded by Caleb H. Parry, who wrote:
"About three months after lying in, while she was suckling her child, a lump about the size of a walnut was perceived in the right side of her neck. This continued to enlarge till the period of my attendance, when it occupied both sides of her neck, so as to have reached an enormous size, projecting forwards before the margin of the lower jaw. The part swelled was the thyroid gland. The carotid artery on each side was greatly distended, the eyes were protruded from their sockets and the countenance exhibited an appearance of agitation and distress, especially in any muscular exertion, which I have rarely seen equalled". (166)

The physiology of the thyroid gland, and the regulation of its secretion, remained speculative until 1851, when Niepce is credited with the first observation of a possible relationship between it and the pituitary gland.(165) He reported anterior pituitary hypertrophy in patients and animals with endemic goitre and cretinism. It was subsequently shown that the pituitary gland in the dog and rabbit enlarged and had an altered microscopic appearance following thyroidectomy.(186)

Conversely, Cushing reported ultimate involution of the thyroid gland with excess colloid formation and a low epithelial lining following hypophysectomy in dogs.(69) This was later noted in humans.(87) An atrophic thyroid was found in all of 34 cases of Simmonds' disease reviewed by Graubuer.(109)

An important link in the understanding of the pituitary-thyroid relationship was forged when Allen demonstrated that the anterior pituitary secreted a thyroid stimulating substance. He reported that the thyroid atrophy in tadpoles following the removal of the anterior pituitary could be completely resolved by anterior-pituitary implants from frogs.(21, 22) Later, Smith and Smith noted similar observations in tadpoles following the injection of bovine anterior pituitary extract.(204) Similar experiments were performed on rats.(202, 23, 203) Atrophy was actually prevented after hypophysectomy by starting treatment immediately.(23) Anterior pituitary extract was shown to stimulate the thyroid morphologically and functionally in the salamander(215) and axolotl larvae(213, 216). This was manifested as an increase in height of the epithelial cells of the thyroid follicle and an acceleration of metamorphosis. Thus the occurrence of histological changes became known for the first time. Since the effect on larval metamorphosis was abolished by thyroidectomy, it was concluded that the anterior lobe of the pituitary gland contained a factor which activated the thyroid gland. Subsequently, hyperplasia of the thyroid induced by the parenteral administration of anterior pituitary extracts was reported in a variety of laboratory animals(139, 25). Thyroid transplants as well as normal glands were found to be stimulated by anterior pituitary administration(148).

From these experimental beginnings, the concept of a thyroid-stimulating hormone arose. Many names have been given to this substance. These include "metamorphic substance" (205), "thyreotropic hormone" (67), "thyreoactivator"(214), thyrotropin, thyrotrophin and thyroid-stimulating hormone (T.S.H.). Throughout this thesis, the name "thyrotrophin" will be used, as it appears to be the correct generic term.

Following the proposal of a thyroid stimulating hormone(67), indirect evidence for its existence was adduced when signs of supposed hyperthyroid activity were reported following anterior pituitary administration. These included basal metabolic rate(194), exophthalmos(191) and tachycardia(189). The utilisation of thyrotrophin by the thyroid gland was suggested when it was administered intravenously and was shown to disappear more quickly from the blood of normal, as opposed to thyroidectomised animals(142), though it did not appear in the urine(193).

The understanding of the thyroid-pituitary relationship was carried further when it was discovered that the resting thyroid gland induced by thyroid substance in the intact animal could be correlated with a reduced pituitary thyrotrophin(55, 2). Subsequently, thyroid hormone was shown to inhibit the histologic response to simultaneously administered thyrotrophin in hypophysectomised(66) and intact animals(140, 137).

From this latter evidence, the concept of a homeostatic control mechanism between the anterior pituitary and thyroid gland was founded (Fig.1). By this mechanism, excess thyroxine is thought to damp down the anterior

pituitary secretion of thyrotrophin, and this would tend to reduce thyroxine output accordingly. When the circulating level of thyroxine is low, the converse occurs.

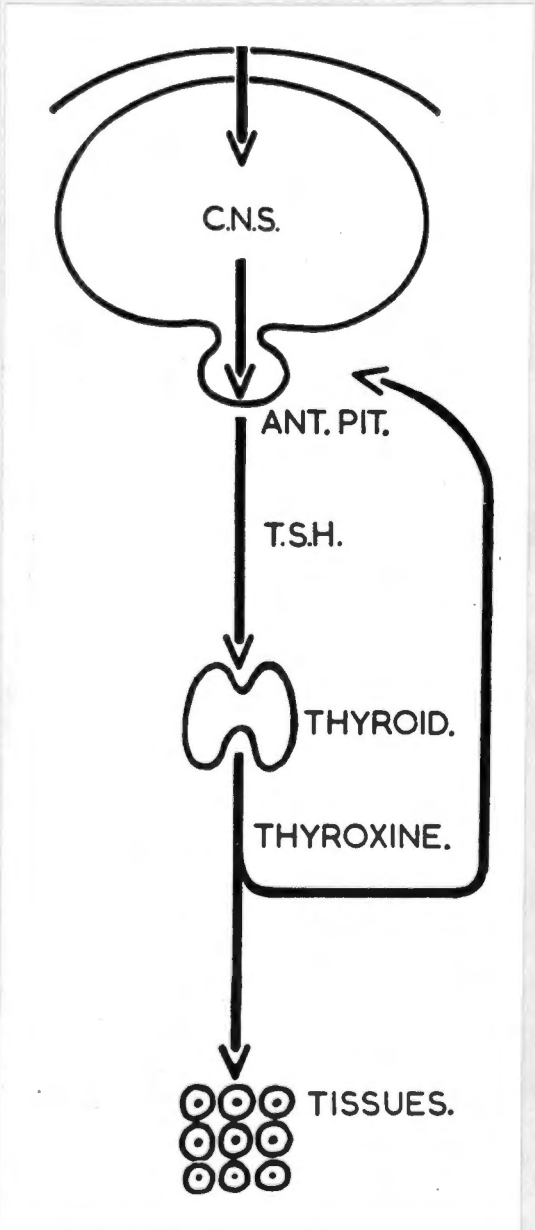


FIGURE 1: Concept of pituitary-thyroid homeostasis.

Soon after 1930, it was reported that exophthalmos could be produced in experimental animals by the injection of anterior pituitary extract rich in thyrotrophin[141, 147, 191]. It was consequently suggested that thyrotrophin might be responsible for this phenomenon despite the rarity of exophthalmos in spontaneous hypothyroidism, where large amounts of circulating thyrotrophin would be expected. This concept, however, gained support when thyroidectomy was found to enhance the exophthalmos-producing effect of anterior pituitary extracts in guinea-pigs[80, 146, 147, 196], presumably by a disturbance of the pituitary-thyroid balance and an enhancement of thyrotrophin secretion. Thyroidectomy alone was claimed to have caused exophthalmos in both animals [78, 108, 220] and man[78]. However, this has been disputed by workers using more refined techniques for measuring exophthalmos in experimental animals[125, 150], especially if allowance is made for weight gain following this procedure[125]. Discrepancies between the levels of circulating thyrotrophin and associated clinical exophthalmos [75, 107] have led to the theory that a separate exophthalmos producing substance (E.P.S.) exists, and this has been given both experimental[36, 37, 84, 126] and clinical backing[85, 150]. The subject will be discussed at greater length in Chapter IV.

The presence of a substance in the blood of some hyperthyroid patients which has been correlated with the presence of associated exophthalmos, has been reported recently[5, 11, 43, 13, 155, 162]. This factor, currently named "long-acting thyroid stimulator" or L.A.T.S.(*), has been shown to have a thyrotrophic action on the thyroid gland[43, 144, 158], but has both a longer circulating half-life than thyrotrophin[6, 156, 160] and a different dose response slope[7].

The association of hyperthyroid and exophthalmic states with assayable levels of this substance have led to the suggestion that it may be the agent responsible for hyperthyroidism[13]. Others feel it to be identical with E.P.S. discussed above[5, 160].

At least two, and possibly three, hormonal factors appear to be involved in the symptom-complex of hyperthyroidism and exophthalmos, but their precise importance is obscure. In addition, the status and interrelationship of these substances in patients with exophthalmos and induced hypothyroid states or pretibial myxoedema, has not been clarified.

Thyrotrophin and exophthalmos producing substance (E.P.S.) appear to be different; but whether E.P.S. is identical with the long acting thyroid stimulator (L.A.T.S.) found in exophthalmic states is not known. It was thought that collaborative biological assays for these three substances in the same aliquot of serum from exophthalmic patients might possibly settle this point.

(*) Terminology informally agreed upon at the Fourth International Goitre Conference, London, July 1960.

The paucity of serial assays of thyrotrophin, L.A.T.S. or E.P.S. in patients receiving therapy for exophthalmic or hyperthyroid states is striking. Only Dobyns[86] and McGill[150] have followed exophthalmic patients and have attempted to correlate clinical change and exophthalmos producing activity, while serial estimations of L.A.T.S. have not been reported. It was felt worthwhile, therefore, to record serial serum levels of all three factors in persons suffering from both hyperthyroid and exophthalmic states and to look for a correlation between clinical change and the hormonal status in these cases.

Only comparatively recently has the assay of thyrotrophic activity in serum been put onto a sound statistical footing. The levels of thyrotrophin have since been estimated in a variety of endocrinopathies with reasonable reliability. However, discrepancies are still noted, for example in hyperthyroidism. In some cases, including hyperthyroid, hypothyroid and hypopituitary states, assay levels are well documented (see Chapter VII); in others, including acromegaly, simple goitre and pretibial myxoedema very few results have been reported. Thyrotrophin has not been assayed in persons suffering from thyroid carcinoma or thyroiditis (both chronic, Hashimoto's, or subacute, De Quervain's). It was considered to be of interest, therefore, to estimate thyrotrophic activity in these states.

The interpretation of the tri-iodothyronine (T3) suppression test of thyroid function has recently been questioned. The concept postulated by Werner[224] that failure of T3 to suppress I^{131} uptake in hyperthyroid

states implied autonomous thyroid activity, has been criticised[100]. As only one very brief report of thyrotrophin levels before and after tri-iodothyronine suppression has appeared[48], it was decided to assay such activity in thyrotoxic and euthyroid subjects with high thyroid uptakes of I^{131} in order to test correlation between suppressibility and serum levels of thyrotrophin and occasionally E.P.S., and possibly to throw light on the mechanism involved in, and the interpretation of, this test of thyroid function.

To summarise, it was hoped by means of collaborative biological assays of thyrotrophin, L.A.T.S. and E.P.S., or by assays of thyrotrophin alone, to investigate the following problems :-

(i) The status and interrelationships of these hormonal substances in the conditions of hyperthyroidism and exophthalmos, and in exophthalmic states associated with induced hypothyroidism, euthyroidism and pretibial myxoedema.

(ii) The single or separate identities of the three substances mentioned, with special regard to L.A.T.S. and E.P.S.

(iii) The correlation between the clinical and hormonal status in patients suffering from hyperthyroidism or exophthalmos during and after therapy.

(iv) The thyrotrophin level in a variety of thyroid disorders, with emphasis on those which have received scant or no attention in published reports.

(v) The mechanism and interpretation of the tri-iodothyronine suppression test of thyroid function.

CHAPTER II

The Biological Assay of Thyrotrophin - A Review

Many years before the concept of a pituitary-thyroid interrelationship had been evaluated, Rénon and Delille reported what was probably the first assay of thyrotrophin when they noted after anterior pituitary injections into rabbits that "la thyroide est le siége de modifications trés remarquables" (183, 184). The first unit of thyrotrophic activity was assayed quantitatively only in 1932 by Junkmann and Schoeller by a subjective grading of histological changes induced in the guinea-pig thyroid following anterior pituitary injections(128). Since then, numerous assay methods have been described(52), based on virtually every known thyrotrophic effect on the thyroid gland, as well as the end organ effects of secondarily released thyroid hormone.

That assay methods have not been directly comparable, has been due not only to the multiplicity of techniques based on different parameters of thyrotrophic effect, but, until recently, to the lack of a standardised reference substance. In 1955, a stock of standard known as U.S.P. Thyrotropin Reference Standard was released; 13.5 mg. being equal to 1 U.S.P. unit(157). The same year, an "International Standard for Thyrotrophin" was established under the World Health Organisation Expert Committee on Biological Standardisation(164). Both U.S.P. and International Standards are equipotent, and the units are identical. Since 1955, it has been possible to compare the sensitivities of all assays in which these reference standards have been used. These comparisons have been further facilitated by the relatively recent application of

statistical analyses to bio-assay results.

Earlier Units of Sensitivity:

Sensitivity of earlier assays was reported in terms of various arbitrary units. As these were based on subjective histological techniques, accurate standardisation was impossible, as was comparison between assays. For this reason, only approximate figures for sensitivity could be reported. The earliest unit to gain popularity was the Junkmann-Schoeller Unit (J.S.U.)[128] defined as the smallest daily dose of anterior pituitary extract which, when injected subcutaneously into guinea-pigs daily for three days, would by the fourth day cause histological evidence of thyroid activity (colloid and follicular epithelial change) in one out of two animals. A slight refinement was the unit of Heyl and Laqueur (H.L.U.)[122] who, in 1934, defined in guinea-pigs six categories of thyroid epithelial response to anterior pituitary extracts injected daily for two successive days. The thyroid gland was examined twenty-four hours after the second injection. An H.L.U. was one-quarter of this total injected dose which yielded a response (the 4th histological category) in two out of three animals. It has been estimated that both J.S. and H.L. units are roughly equivalent[208]. Their relationship to the U.S.P. unit cannot be precisely estimated because of the subjectiveness of these earlier units; but it is generally accepted that one U.S.P. unit equals 10 - 12 J.S.U.[178, 117]. Some comparison (albeit a rather unsatisfactory one) of sensitivities of earlier and more recent assays is thus possible. In this thesis, thyrotrophin levels will be referred to only in terms of international units and milliunits (m.u.).

Confusion was caused by the use of a provisional U.S.P. reference substance in the first standardised assays (47, 177, 179). This was twenty times as potent as the final reference material which was diluted 1 part to 19 parts lactose.

The combination of technical refinements, careful statistical analyses and the use of an international standard material for reference, has led to a degree of uniformity in thyrotrophin assay methods, whereas, until recently, a chaotic state existed.

ASSAY METHODS:

Earlier techniques are of historical interest only and have been reviewed critically and in great detail (19, 52, 133). These will be described briefly but consideration will be given mainly to those methods in which the sensitivity has been of an order to warrant reasonable conclusions regarding thyrotrophic activity in human serum. This review will follow a classification of assay methods based on the various parameters of thyrotrophic effect, both indirect and direct.

INDIRECT METHODS:

Metabolic effects:

Anderson and Collip devised a method based on the response of the basal metabolic rate in hypophysectomised rats to anterior pituitary extracts (24).

Tadpole Method:

D'Angelo and his co-workers[73] showed that the metamorphosis in tadpoles which was inhibited by starving, could be stimulated rapidly by intraperitoneal anterior pituitary extract, with ensuing weight loss, increase in hind limb length, and histological evidence of thyroid gland activity. When these latter two functions were used as combined criteria, a sensitivity of 60 to 120 times that of previous histological methods was claimed. (See page 13). Subsequently, elegant micro-histometric methods for the measurement of acinar cell height added objectivity and a log-dose response curve, suitable for assay purposes, was obtained[71, 74].

DIRECT METHODS:

Gravimetric Methods:

As the hypertrophy of the thyroid induced by thyrotrophin was found to be reflected by weight increase of the gland, this was, for a time, used as an index of thyrotrophic activity in guinea-pigs[188], young chicks[41,65,197] and young ducks[190].

Recently, an in vitro method involving thyroid weight increase has been described[31]. Slices of beef thyroid are incubated in Krebs-Ringer phosphate buffer, thyrotrophin having been added to the media in graded doses. A linear weight increase of the slices related to the logarithm of the dose was recorded. The mechanism of this effect is obscure. Thyrotrophic activity in untreated blood could not be measured by this method; but, when this was separated by means of ion exchange chromatography[118], satisfactory assays were obtained.

General Histological Methods:

The pioneer work concerning histological evidence of thyroid activity and its relationship to anterior pituitary stimulation, has been referred to earlier[122, 128]. These methods and subsequent modifications[127, 151] were abandoned because of insensitivity and great subjectiveness.

Microhistometric method:

The use of an ocular micrometer to measure epithelial cell height directly[88, 181, 182], enabled more objective histological evidence of thyrotrophic activity to be used for assay purposes. One cell was measured in each of a number of follicles and a mean cell height was obtained. The technique was, however, laborious and subjective influences might have governed the selection of cells to be measured. Griesbach and Purves used a modification of this assay in pretreated animals[113]. A similar technique was subsequently used with stasis tadpoles and combined with observations of hind limb length [73, 74]. The sensitivity of this method (0.05 m.u. of the international standard, with an injection volume of 0.25 to 0.35 ml. serum) enabled measurements to be made in untreated blood samples in various endocrine disorders. Other workers have used this method with varying success[27, 195].

Histoquantitative linear measurement methods:

A simpler and more objective method for histological evaluation of thyroid stimulation in guinea-pigs was subsequently described and elaborated[208, 218]. This involved determination of the relative proportions of epithelium, colloid and stroma in the thyroid. Percentage of epithelium (E%) was regarded as the best index of thyroid activity and a direct relationship

between the difference of E% in untreated and treated animals (dE%) and the logarithm of the dose of thyrotrophin was discovered. An assay method was developed with a sensitivity of 1.0 mu.[211] which was used for the assay of thyrotrophin in human serum[210]. A discovery by Ludwig[143] of changes in nuclear volume of thyroid epithelial cells was later adapted for assay purposes[209], but, though sensitive, it was extremely tedious.

Intracellular colloid droplet method:

De Robertis, using guinea-pigs, measured the number of intracellular colloid droplets in the thyroids after intracardiac injection of thyrotrophic extract[185]. He used the ratio between this number and the mean diameter of corresponding follicles to establish a "cytological coefficient". He claimed a sensitivity of 0.02 mu. using a Fellinger[95] extract of blood, with a log-dose response relationship, but this sensitivity has been criticised for a lack of precision[208] and specificity[89, 90].

Measurement of thyroid I_2 depletion:

Following the discovery that thyrotrophin depleted the thyroid of iodine[60, 97], a chemical method[206] for the estimation of blood and thyroid iodine was used to study these effects in animals[70, 207]. A daily injection of pituitary extract for 5 days caused significant depletion of thyroid iodine as early as 24 hours after commencement and, on this basis, an assay method was defined[207] and later elaborated[20, 98, 169]. A linear log-dose response was obtained in non-pretreated chicks, but with poor sensitivity (5 - 20 mu.)[169] However, as chemical estimation of iodine is laborious, the method was abandoned.

In spite of the diversity of the thyrotrophic effects used in assay techniques before radio-isotopes became the tools of choice, the correlation between many of the methods was often remarkably good[20, 209]. The sensitivity was usually poor, the exceptions being the "stasis tadpole" method of D'Angelo[73, 74], the histoquantitative technique of Tala[208] and the *in vitro* assay using thyroid slices, developed by Bakke[118].

Measurement of thyroid P^{32} increase:

Borell reported that the phosphorus content of guinea-pig thyroid increased after thyrotrophic stimulation[45], and he later elaborated a method of assay based on radio-phosphorus uptake[46]. A fairly linear log-dose response to thyrotrophin was obtained over a wide range; but, though his method was easy and objective, the sensitivity was poor. This was later increased, firstly when 2 day-old chicks were used[42, 68] and, later, (but at the expense of the range - 0.5 to 5.0 mu.) by Scandinavian workers [133, 134, 221], who also claimed specificity of this method after noting an excellent correlation with the epithelial percentage (E%) assay[133]. Greenspan and his colleagues noted a similar sensitivity and specificity of response[110]. Assays were attempted in both blood and urine, but subsequent difficulty was encountered[111] and it was felt that non-pituitary factors might have been influencing the assay by stimulating P^{32} uptake. This lack of specificity must weigh very heavily against the usefulness of the method.

The mechanism of this unusual action of thyrotrophin is obscure, but is thought to be a non-specific result of increased metabolism leading to an increasing turnover of phosphorylated compounds[134], as occurs in any growing tissue.

The use of I^{131} in thyrotrophin assays:

There seems clearly a three-fold effect of thyrotrophin on the metabolism of thyroid iodine[58], namely :-

(i) Release of organically bound I_2 from the thyroid.

(ii) Conversion of inorganic I_2 to protein bound compounds.

(iii) Trapping of inorganic I_2 from the blood stream.

These effects were used by various workers as end-points in the development of thyrotrophin assays. The availability of radio-active iodine made these end points both objective and easily definable.

The sequence of these effects has been investigated. Keating and his co-workers[129] showed that the first change to occur in thyrotrophin treated chicks was loss of thyroid I_2 and that this occurred pari passu with histological evidence of activity. An increased I_2 uptake, noted only 24 hours later, was thought to be a passive result of the "iodine containing hormonal purge" induced by thyrotrophin, and not a direct effect at all. This was later confirmed by others[180, 221].

Thus, if radio-iodine is given without carrier, for example, 24 hours before thyrotrophin, release of I^{131} from the thyroid (as P.B.I^{131}) begins after a few hours. This may be measured either by reduction in thyroid radioactivity or its increase in the plasma.

When radio-iodine is given after thyrotrophin stimulation, increased trapping ability is noted in the form of increased I^{131} uptake by the thyroid gland. This continues until iodine stores in the thyroid are loaded, when the trapping ability returns to normal.

Thyroid I^{131} uptake methods:

Hertz and his colleagues[120, 121] first showed an increased I^{131} thyroid uptake following thyrotrophin, and an assay method was later suggested[104] and confirmed[51, 136] in hypophysectomised rats where linear dose responses were obtained. Others repeated this work using tadpoles[53], mice[178] and chicks[171] as test animals. Lack of sensitivity precluded the use of untreated serum in these assays, though Querido and his co-workers[177, 179] were able to concentrate serum and therefore thyrotrophic activity, using the Cohn method[61], and, by this means, were able to record serum thyrotrophin levels. Endogenous thyrotrophic activity was suppressed in the test animal (mouse) by feeding with iodo-casein. Sensitivity of this method was reported as 2 mu.

In vitro preparations of thyroid slices were shown to take up I^{131} when thyrotrophin was added to the incubating medium[30, 47]; but, though used for studies in the rabbit[47], it was found unreliable.

Thyroid I^{131} discharge - measured as depletion of thyroid iodine:

Following the cumbersome chemical estimations of iodine discussed earlier, by which means a linear effect of thyrotrophin in depleting the thyroid of I_2 was elaborated into an assay method, I^{131} was used to measure this effect.

Gilliland and his colleagues used the thyroxine-treated baby chick as test animal[105, 106, 107]. Activity of the thyroid was determined in vivo before and 24 hours after the second of 2 thyrotrophin injections. A drop in thyroid radioactivity conforming to a linear dose response relationship

was found between 2.5 and 40 mu. thyrotrophin.

Bates modified the method by pretreating the chicks with propylthiouracil in addition to thyroxine (38, 63). This was claimed to prevent re-utilization of released I^{131} by the thyroid. However, the sensitivity of the method was little changed in their hands (± 2 mu.). Others, too, have used this method (96, 102).

Thyroid I^{131} discharge - measured as increase in blood iodine:

Amongst the most sensitive of recent methods have been those measuring I^{131} discharge as an increase in blood I^{131} in the test animals. This followed earlier experiments showing the appearance of I^{127} in the blood following injections of anterior pituitary extract in guinea-pigs (60). Later, I^{127} and I^{131} release were correlated (40) and used as an assay procedure (104). A linear dose response relationship was obtained.

The first workers to develop this principle into a method of sensitive assay were Adams and Purves using the guinea-pig as test animal (9, 10, 12). Young animals were pre-treated with thyroxine, and then given carrier-free I^{131} intraperitoneally. 72 hours later, the animals' plasma I^{131} was shown to have been largely cleared by the kidneys and thyroid, and this was adjudged the optimum time for assay. The animals were bled initially and again 3 hours after thyrotrophin injection. The percentage increase in plasma radio-activity 3 hours following thyrotrophin was the recorded response. Linear dose responses were obtained over a range of 0.1 to 0.6 mu.

McKenzie (153,154) used an almost identical technique with thyroxine pretreated mice and improved the sensitivity to 0.025 mu., linearity in the

log-dose response being maintained to 1.6 mu. This method will be considered in greater detail in the following chapter. The great sensitivity makes it an admirable choice for the assay of thyrotrophin in the plasma of man and, for this reason, after slight modifications, it was used in this study. Others have been able to repeat McKenzie's technique and confirm the reproducibility of the method.(4,162,227)

In vitro preparations of guinea-pig thyroid slices were used by Bottari and Donovan in the development of an assay based on I^{131} discharge(49). They obtained a linear log dose response with a remarkable sensitivity (0.001 mu.) and wide range (to 10 mu.). An extensive investigation of thyrotrophic activity in human serum was reported in 1960 using this method(48).

Conclusions:

A brief review of past and current methods used in the biological assay of thyrotrophin has been presented. Results have to be interpreted with great caution and, even in the best hands, are often unpredictable and unsatisfactory. Technique and attention to detail has to be meticulous to avoid discrepancies and, even then, inexplicable biological variations in the responses of the test animals occur. These limit the usefulness of biological methods; but, to date, no chemical techniques for the identification of thyrotrophin have been developed.

Albert(19) and Segaloff(192) have stressed the prerequisites for the ideal bio-assay. The method should be (1) specific, (2) reproducible from laboratory to laboratory, (3) sensitive, (4) statistically sound, (5) simple, (6) rapid, and (7) objective.

The application of these criteria to the more sensitive assay techniques will be considered.

Specificity implies the use of an assay end point, the effect on which must be attributable to thyrotrophin and to no other substance. Thus assays employing indirect effects of thyrotrophin are suspect, as are those in which other substances with similar properties are not assayed for a non-specific "thyrotrophic" effect in control experiments. The assay methods involving I^{131} uptake or discharge, the histoquantitative techniques and the "stasis tadpole" method of D'Angelo are all clearly specific. Doubt has been cast on the colloid droplet method[89, 90], and more recently, the increase in P^{32} uptake which follows thyrotrophin, has been shown to be a non-specific effect of growing tissue and therefore possibly influenced also by non-pituitary factors[111]. The in vitro method employing thyroid weight increase is also clearly non-specific; but this point has not, as yet, been critically explored.

The methods employing P^{32} and I^{131} have all been reproducible to a greater or lesser degree, though minor differences in technique are found in various laboratories. In some, used only by one or two groups of workers, reproducibility can only be assumed. The good correlation of results (for example, the normal level of thyrotrophin in plasma) using these varying methods, makes the above assumption probably valid.

Sensitivity of various methods has already been mentioned. In summary, however, a few techniques only are capable of demonstrating thyrotrophic activity in human serum, and many of these can do so only

in blood which has been treated chemically to concentrate or extract thyrotrophin. While no definite evidence exists that such measures distort or alter thyrotrophic activity, a further source of confusion should be avoided in experimental work already so prone to pitfalls.

For thyrotrophic activity to be detectable in plasma, an assay sensitivity of 1.0 to 2.0 mu. is essential. Only 11 different techniques fall into this range (71 and 74; 118, 208 and 211; 110, 134, 177, 179, 185 and 221 (105, 106 and 107; 38 and 63; 9, 10 and 12; 153 and 154; 49). In a number of these, preliminary extraction of thyrotrophin is necessary (118, 177, 179, 185). The most sensitive assays currently in use are the stasis tadpole technique of D'Angelo (71, 74) (0.05 mu.), the I^{131} discharge method of McKenzie (153, 154) (0.025 mu.) and that of Bottari (49) (0.001 mu.)

One of the great advantages in the use of radio-isotopes as markers in assays, is the great objectivity obtained - an objectivity difficult to claim in methods involving gravimetry and histologic assessment in its various forms.

McKenzie's technique incorporating I^{131} discharge from mouse thyroids (153, 154) fulfils the criteria discussed above, and is, in addition, relatively simple and statistically sound. For these reasons it was developed and used as an assay procedure for this study, and this will be considered in detail in the following chapter.

CHAPTER III

Thyrotrophin assay based on the discharge of radio-iodine from the thyroid gland of the Mouse.

A: MATERIALS AND METHODS

Theoretical basis for the assay:

In the previous chapter, the evolution of a thyrotrophin assay dependent on I^{131} discharge from the thyroid gland was discussed. Following thyrotrophin stimulation, organically bound iodine is released from the thyroid. This appears to be the first and probably the most specific effect of this hormone. If measured as an increase in plasma PBI[•] ^{131}, this occurs within hours of stimulation in rats[(226)], mice[(154)] and guinea-pigs[(10)], and within twenty-four hours if noted as a decrease in thyroid I^{131} [(129, 180, 221)]. Both parameters roughly parallel thyroid hyperplasia[(129, 180, 221)] and this seems strong evidence for a specificity of these end points when used to measure thyrotrophic effect.

The reasons for the choice of an assay method involving increase in plasma PBI ^{131} have been alluded to in the previous chapter. To these may be added the convenience of an early demonstrable thyrotrophic effect and the clear specificity of the mechanism involved, as outlined above.

The assay animal:

A variety of test animals has been used in thyrotrophin assays. Earlier workers used the guinea-pig and chick. These were shown to have

[•] PBI refers to protein-bound iodine.

the lowest endogenous thyrotrophin secretion[1, 91, 138] and therefore were ideally suitable for accurate estimations. However, really sensitive methods are not available using these animals. Conversely, large amounts of thyrotrophin have been demonstrated in the anterior pituitaries of rats and mice[1, 91, 138] and the use of these animals is precluded unless measures are taken to suppress endogenous thyrotrophin secretion, which would spontaneously release the administered radio-iodine from the thyroid and negate the assay. Such measures include hypophysectomy[51, 136] and pretreatment with either iodo-casein[177, 178, 179], thyroxine or dry thyroid powder[10, 153, 154]. Because of their ready availability, inexpensiveness and the efficacy of pretreatment, mice are particularly suited as test animals and have been used in the method to be discussed.

It has been shown in chicks that differences in strain may lead to large discrepancies when the same techniques are used in different, or even the same, laboratories[20, 133]. Similarly, sex differences may influence the response in chicks[41], and mice[208]. In addition, variations in temperature[34, 217] and light[173] may influence thyroid function in test animals.

The state of nutrition may be important. D'Angelo[71] demonstrated a low thyrotrophin level in the blood of starved male rats and mice, while the effects of goitrogenic diets in increasing anterior pituitary thyrotrophin are well known. Finally, a seasonal variation in the functional activity of guinea-pig thyroid has been shown[208].

The existence of so many variables makes careful choice and control of the test animal imperative. For the assay in this study, weanling mice of a single strain, inbred in the animal house, University of Cape Town Medical School, were consistently used. They were all pretrested to suppress endogenous thyrotrophin. All were in the same weight range (13 - 19 G); were of the same sex (female) and were kept under strict basal conditions, at a thermostatically controlled temperature in an animal room adjoining the laboratory. Variations in light were reduced to a minimum. The diet was consistent and mice were never starved prior to assay.

Development of the Method:

Pretreatment:

The reasonable suggestion[10, 154] that a period on a low iodine intake would increase the avidity of the thyroid of the test animal for radio-iodine, and thus lead to a more significant response to injected thyrotrophin, was followed. All mice used in this assay were kept on powdered mouse-biscuit (Vereeniging Milling Co., Cape Town) of a low iodine content (70 µg./kg.) and distilled water for ten days.

Suppression of endogenous thyrotrophin secretion is imperative in mice. McKenzie[154] investigated the efficacy of exogenous thyroid hormone in this respect by means of thyroidal I^{131} uptake tests. He found optimal suppression of I^{131} uptake with a single subcutaneous injection of 10 µg. L-thyroxine coupled with thyroid powder added to the food at 0.066% concentration. He also investigated the maximal permissible thyroid content of

I^{131} and found 2 µc. to be the critical level above which radionecrosis occurred. He concluded that, as the I^{131} uptake averaged 20% after one week on a low iodine diet, a suitable dose would be 8 µc. I^{131}, which would lead to a thyroid content of 1.6 µc. This was given intraperitoneally. It was also shown that subcutaneous thyroxine, given immediately after I^{131}, did not significantly affect the uptake.

These recommendations were carried out in the present assay and, on the eleventh day of low iodine intake, 8 µc. of carrier-free I^{131} in 0.2 ml. of normal saline (obtained from Amersham, U.K.) was injected intraperitoneally under light ether anaesthesia. This was followed immediately by 10 µg. L-thyroxine in 0.2 ml. saline, injected subcutaneously into the dorsal neck region, and thyroid sicca (Burroughs-Wellcome) powder was added to the diet - 1 gr. (66 mg.) per 100 G. The thyroxine powder was dissolved in a small volume ($\pm$ 1 ml.) of 0.1 NaOH, before being diluted with saline. The solution was freshly prepared before each assay.

Four days were allowed for the adequate excretion of I^{131} not taken up by the thyroid. On the fifth day, the mice were considered ready for assay, with an optimum amount of radio-iodine in their thyroid glands and a minimum of circulating radioactivity.

With this scheme of pretreatment, unsatisfactory responses to thyrotrophin were obtained; possibly a result of inadequate suppression of endogenous thyrotrophin. Accordingly, a second dose of 10 µg. L-thyroxine was administered subcutaneously two days after the initial injection and thyroid sicca was replaced by thyroxine powder B.P., which is synthetic and regarded as a much more stable preparation.

On this regimen, blood radioactivity dropped to consistent levels and the responses to administered thyrotrophin became regular and predictable.

Summary of pretreatment:

Day 1 - day 10	:	Low iodine diet, including distilled water.
Day 11	:	8 µc. carrier-free I^{131} by intraperitoneal injection, followed by 10 µg. L.-thyroxine by subcutaneous injection and 0.1 mg. thyroxinepowder per 100 G. food.
Day 13	:	10 µg. L-thyroxine by subcutaneous injection.
Day 15	:	Mice ready for assay.

This scheme was followed in all assays included in this thesis.

Bleeding of Mice:

0.1 ml. blood was obtained from the cavernous sinuses of the mice under light ether anaesthesia by means of an angled glass pipette, suitably graduated. (Fig. 2).

FIGURE 2: Technique of bleeding from cavernous sinus of the mouse.

The pipette was cleaned with normal saline and acetone and was primed with heparin to prevent coagulation of the aspirated blood. This routine was repeated after each bleeding. Aspiration from the cavernous sinus was achieved largely by capillarity; but more definitive control was obtained by attaching the pipette by smugly-fitting thick pressure tubing to a closed plastic cylindrical case, compressed by the jaws of a micrometer screw. By manipulating the screw and thus increasing or decreasing the column of the plastic cylinder, blood was either sucked into or expelled from the pipette with accurate control. (Fig. 3).

On completion of the procedure, the eyeball resumed its place in its orbit and effectively sealed off further bleeding. The mice emerged none the worse for this experience and only very occasional deaths were encountered, as a result of the anaesthetic and not the bleeding procedure.

Exactly 0.1 ml. of blood was expelled into a test tube containing 1.9 ml. of tap water. Immediate haemolysis occurred and therefore an even dispersion of radioactivity was obtained.

FIGURE 3: Apparatus for bleeding from cavernous sinus.

Intravenous injections:

The mouse has five conveniently situated veins, accessible for intravenous injection, running longitudinally down the tail. Adams and Purves[10] showed that intravenously administered thyrotrophin resulted in a sensitivity five to ten times that of thyrotrophin given subcutaneously or intraperitoneally, and all substances to be tested were therefore given intravenously.

Mice were immobilized in a perspex container and, after preliminary immersion of the tails in warm water, a 26 gauge needle was easily inserted into a dilated tail vein and the injection given slowly over two to three minutes. (Figs. 4 and 5).

FIGURE 4: Mouse immobilized in perspex cylinder.

FIGURE 5: Technique of intravenous injection.

Substances injected intravenously:

Three classes of material were injected intravenously to elicit an I^{131} discharge effect:-

(i) Normal saline.

(ii) U.S.P. Thyrotropin Reference Standard.

(iii) The plasma or tissue homogenate assayed for thyrotrophic activity.

Volume, nature and dilutions of injected material:

The volume injected throughout all experiments was 0.5 ml. This allowed an injectable range of 0.2 to 0.5 ml. plasma, essential for testing parallelism with standard reference substance. (See(iii) below).

(i) Normal (0.9%) saline was the obvious choice as a control solution to test non-specific responses and haemo dilution effect.

(ii) U.S.P. Thyrotropin Reference Standard [1] was used as reference substance. 13.5 mg. of the powdered standard is equal to 1 U.S.P. and International unit of thyrotrophin(157) and, by simple weighing, standard solutions in normal saline were prepared freshly before assay. The dilutions were made to correspond to concentrations of 0.025, 0.05, 0.15 and 0.45 milliunits (mu.) in 0.5 ml. For the most part, 0.05, 0.15 and 0.45 were used as the three dose levels in any one experiment; but on various occasions, lower concentrations were used so that the sensitivity of the assay could be explored.

(iii) Plasma was occasionally injected undiluted, but, in the majority of assays, 0.3 ml. was found convenient, in which case 0.2 ml. of normal saline was added to make up the bulk and the necessary 0.5 ml. Plasma was always freshly obtained by venipuncture on the morning of the assay, heparinized, separated and used within a few hours of bleeding.

(1) Kindly donated by Armour Laboratories, Ltd.

Radioactive counting:

Mouse blood I^{131} was assayed in a well-type scintillation counter (ECHO Electronics) using a thallium-activated sodium iodide crystal(93). Two counters were used, but both initial and final blood samples in each animal were assayed on the same counter. Counts were recorded over 300 seconds.

On the morning of each assay, a 3,000 second background count was performed. These ranged between 4.2 - 4.4 counts per second (cps.) and 4.9 - 5.1 cps. on the two counters used. Blood radio-activity was recorded in cps. above background. Initial counts below 1.28 cps. above background were regarded as not significant and discarded, as were those over 7.36 cps. which fell above the statistical limits set for the range of initial counts.

Total observed counts (x)	Standard deviation $\sqrt{x}$	Random Error % (68.3% confidence)
100	10	10
1,000	31.6	3.2
10,000	100	1.0

TABLE I: Statistics of Counting:

(As $\sqrt{x}$ is usually small compared to x, the error involved in assuming that standard deviation = $\sqrt{x}$, instead of the square root of the mean count, is usually negligible, and the "true" count is therefore $x \pm \sqrt{x}$ with one chance in three that these limits of error will be exceeded).

It can be deduced from Table I that the random error (R.E.) when activity between 1.28 and 7.36 cps. is counted for 300 seconds, is 2 - 5% at one standard deviation.

Measurement of the assay response:

At a fixed time after injection of thyrotrophin standard, saline or the unknown plasma, the mice were re-bled in exactly the same way as initially.

McKenzie[154] suggested that the increase in blood I^{131} be noted in two ways; (i) as an absolute increase in counts per minute/0.1 ml. blood over the initial count and (ii) as a percentage increase on initial activity, the latter being regarded as 100%. He compared the dose response relationship in four ways, by plotting (i) and (ii) against the dose of thyrotrophin on both a logarithmic and arithmetic scale.

Initially, both methods of measuring response were plotted; but it was soon found that the percentage increase in activity was much more satisfactory and consistent, and it was adopted as the parameter of choice, being plotted against the logarithm of the dose.

Maximal thyrotrophic effect; optimal bleeding time:

McKenzie[154] also reported a gradual increase of circulating plasma radioactivity following intravenous (I.V.) thyrotrophin in his mice, reaching a maximum at two hours, with a slow decline. He felt that the optimal bleeding time was therefore two hours after the injection.

This was confirmed in an experiment (Figure 6.) which shows a levelling off of activity two hours after I.V. thyrotrophin, and this time was found convenient and adopted routinely for the second bleeding except in assays for the "long-acting thyroid stimulator" substance (See Chapter VI).

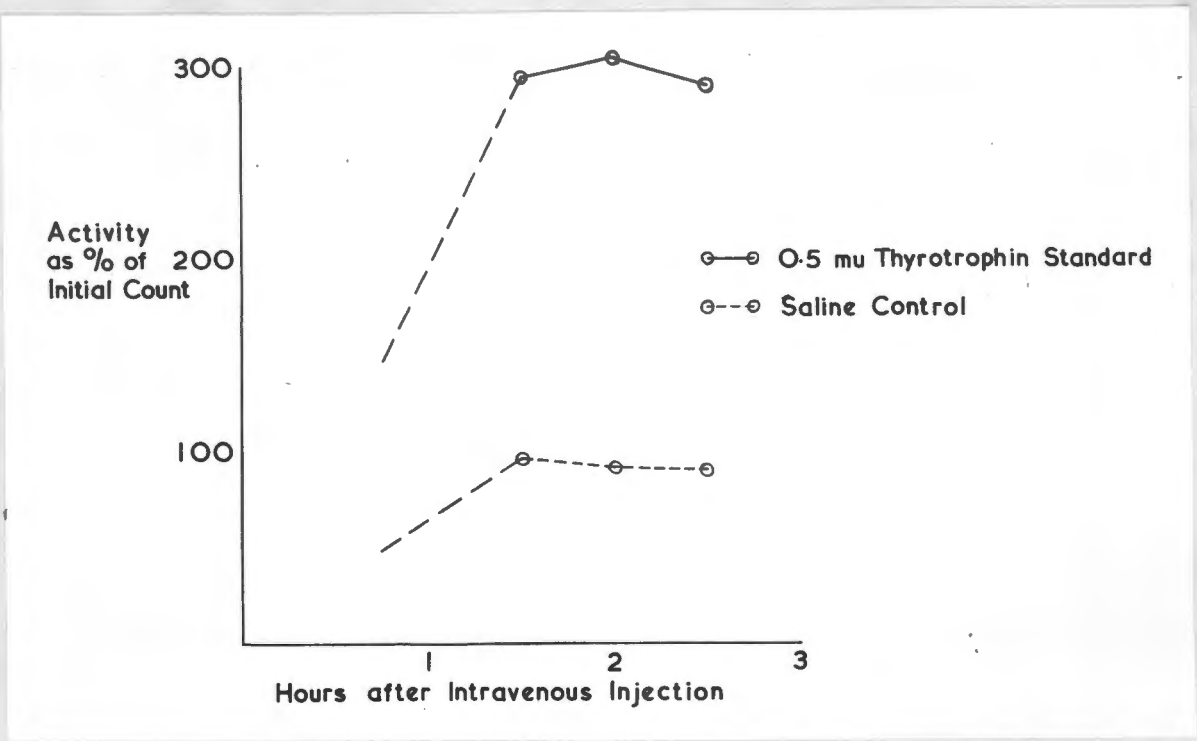


FIGURE 6: The release of I^{131} after intravenous thyrotrophin reaches a maximum at $1\frac{1}{2}$ to $2\frac{1}{2}$ hours. Each point represents the mean of seven assay responses.

Specificity of I^{131} discharge in this assay:

The theoretical arguments favouring this reaction as being a highly specific response to thyrotrophin have already been noted (page 22). This supposition was put to the test and other substances were injected and assayed for possible thyrotrophin-like activity.

(1) Saline: Physiological saline was injected in a volume of 0.5 ml. on 184 occasions. The mean response was 87% (a drop in radioactivity occurred) with a standard deviation (S.D.) of 28% and a standard error (S.E.) of 2.06%. Thus no effect from saline was observed, confirming McKenzie's observations(154). The consistent fall is probably due to haemodilution, as smaller volumes of saline caused a significantly smaller drop in radioactivity (see page 41).

This is not surprising, as the blood volume of a 15 - 20 G. mouse is in the neighbourhood of 1.5 to 2.0 ml. This is deduced by analogy with the rat, in which the average blood volume has been found to be 4.0 to 5.3 ml. per 100 G; the smaller animals having larger blood volumes relative to their body weight[114]. At these levels, the rapid addition of 0.5 ml. could alter the blood volume appreciably and lead to haemodilution.

(2) Hydrocortisone: McKenzie[154] and others[227] have investigated the effect of stressful handling of mice by injecting hydrocortisone and adrenalin, and reported no change in plasma radioactivity. In the present study, 0.01 mg. hydrocortisone hemisuccinate, diluted in saline caused a response not significantly different from saline alone. (Table 2). This, and the lack of correlation between the "toxic" effects to mice of certain plasma samples and subsequent I^{131} release, suggests that the effect of stress on the specificity of this assay is negligible.

(3) Gelatin: To exclude the possibility of non-specific responses to protein material, a 5% solution of gelatin in normal saline was injected into six mice. The results, again not significantly different from saline (Table 2) suggest that an innocuous protein will have no false-positive "thyrotrophic" effect. This confirmed Wahlberg's observations in chicks[221] and those of McKenzie in mice under similar experimental conditions[160]

SALINE	HYDROCORTISONE	GELATIN
92 ± 6	91 ± 6	94 ± 5

TABLE 2: Percentage change in radioactivity two hours after administration of saline, 0.01 mg. hydrocortisone in saline and 5% gelatin in saline. Mean, ± S.E. of six responses are shown in each case.

(4) Plasma with no detectable thyrotrophic activity: In a number of plasma samples no detectable thyrotrophic activity was noted, the radioactivity dropping to saline levels. One was from a patient with proven hypopituitary function (See Chapter IX) and this is probably the strongest evidence that the assay method is specific in detecting thyrotrophic effect.

(5) "Recovery" experiments: Yamazaki and his colleagues[227] added a known amount of thyrotrophin to pooled serum from euthyroid and hyperthyroid patients and assayed the mixture using an almost identical technique to that used in this study. A recovery of 115% and from 81 - 98% was noted in the euthyroid and hyperthyroid sera respectively.

The validity of the method, therefore, seems clearly established.

Difficulties encountered during the development of the Assay method used in this study:

The bleeding and injection techniques used in this assay called for a degree of proficiency which came only after much practice. In the early stages of this work, much difficulty was encountered because of this.

It has been recommended [154] that both the thyroxine used in the pretreatment and the thyrotrophin reference standard be prepared freshly before each assay, as denaturation of the proteins occurs in solution with loss of effectiveness. This was confirmed in this study when a solution of reference standard was inadvertently used five days after preparation. A loss of potency of about 50% was noted.

Occasionally, saline or thyrotrophin standard was injected too rapidly. The mice became cyanosed and obviously dyspnoeic and died almost immediately. These effects seemed due to pulmonary oedema following acute circulatory overload, though autopsies were not performed to confirm this. This effect was avoided completely when more time (one minute) was taken over this procedure.

Finally, a marked "toxic" effect of certain plasma samples was encountered in some assays. The mice appeared well immediately following the injection, but 5 - 10 minutes later became hyperexcitable, developed twitchings and died rapidly in a "decerebrate" posture with hyperextension of all limbs and arching of the back. Autopsies revealed moderate pulmonary oedema only, and no evidence of intracranial or other haemorrhage, or a state of anaphylaxis. (Autopsies were kindly performed and interpreted by Dr. C. Campbell, Senior Lecturer, Dept. Pathology, University of Cape Town). The precise mechanism remains obscure. Similar "toxicity" of intravenously injected serum has been noted by Adams [8, 12] in guinea-pigs and McKenzie [159] in mice. Adams felt that toxicity was due to haemolysis and broncho-constriction and suggested serotonin release as the cause. A possible

underlying genetic predisposition was also mooted[8]. He had the impression that toxicity was removed by diluting with saline and then reconcentrating by dialysis against a dextran-saline mixture. McKenzie[159] passed "toxic" serum down a short column of Sephadex G-25 (a dextran complex marketed by Pharmacia of Uppsala, Sweden) in distilled water. The effluent serum was found acceptable to animals.

These measures were not found necessary in this study. Greater caution with intravenous injections (given over three minutes), coupled with a maximum volume of 0.3 ml. (with 0.2 ml. saline to complete the required bulk) in all instances where reactions occurred, led to the reduction of animal deaths to a minimum. When these did occur, extra mice were utilised to bring the complement up to the required number for assay purposes. The impression was gained that mice appeared to tolerate plasma better, irrespective of speed or volume of injection, in the summer months. Strain susceptibility seems most unlikely as <u>all</u> mice used were a single inbred strain obtained from the Animal Laboratories, Medical School, University of Cape Town.

<u>Final experimental design:</u>

Bliss[44] suggests that biological assays based on log-dose response relationships should adhere to a symmetrical design with a group of animals being used for each assay point and with equal numbers of animals in each group. He adds that statistical analysis is facilitated by using dose levels at equal log-intervals apart.

The following experimental design was therefore adhered to :-

Groups of six mice were used to determine each assay point. Each group was placed in a separate cage, distinctively lettered. In each cage, the mice were marked with Indian ink to enable easy individual identification.

In each experiment, six mice received saline and six received each of 3 dose levels of thyrotrophin reference standard. Usually, 0.05, 0.15 and 0.45 mu. in 0.5 ml. were injected; but, on occasions, 0.025 mu. was given to test the sensitivity of the assay. Thus twenty-four mice were used in each assay to construct a log-dose response line. As a definite and consistent drop was noted after saline administration, the percentage increase in radioactivity following the 3 dose levels of reference standard was plotted as a function of the saline response, which was regarded as 100%.

All the individual results at each dose level were collated and a mean log-dose response line was constructed for the entire study. In 95% of all experiments, two or all three points corresponded closely to the final mean result and lay within the fiducial limits decided on (one standard deviation). If many individual readings fell outside the limits, the experiment was declared invalid and not used.

In those experiments in which only two of the three points of the log-dose line were acceptable, the result was still considered to be valid if the resultant slope corresponded to the final mean slope. The few assays in which only one point fell into the required limits were discarded, and were used only to construct the final mean figure.

Tests for Parallelism:

Plasma at one dose level, either 0.3 or 0.5 ml., but always in a volume of 0.5 ml., was injected into each of six mice, grouped as described. Three plasma samples were injected at two dose levels into similar groups and the slopes were compared to those obtained with the three point line using standard reference substance.

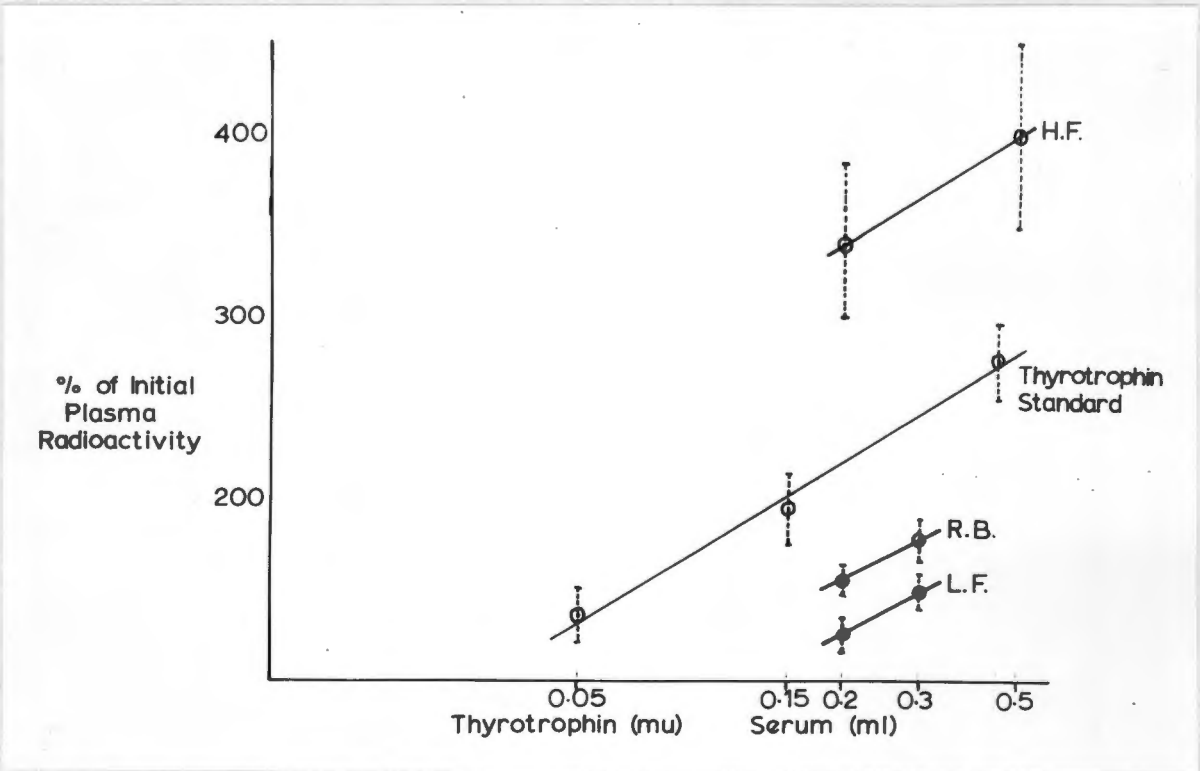


FIGURE 7: The response to three plasma samples assayed at two dose levels, compared to a three point dose response to thyrotrophin standard. Each point represents the mean of six readings. Two standard errors are shown.

The results show a good degree of parallelism. The important question of parallelism between plasma and standard has been more extensively investigated and confirmed by McKenzie, using the same method(154). It was therefore considered that plasma had comparable thyrotrophic effects to the standard, and that it would be valid to assay plasma at one dose level only. A surprising degree of consistency was noted and those samples which produced marked variations in activity from mouse to mouse were not used in this study.

The mean (± S.E.) of plasma responses was plotted in relation to the standard and, by extrapolation, values were obtained in mu./0.3 ml. or mu./0.5 ml. plasma. These values were finally expressed as mu. thyrotrophin/100 ml. plasma. A range which included the 95% confidence limits of the result was calculated in each case.

Batches of 54 or 60 mice were used in each assay experiment and it was thus possible on each occasion to assay 5 or 6 unknown samples at one dose level, thyrotrophin standard at three levels and a saline control.

B RESULTS

Dose Response Relationship:

(1) The initial blood radioactivity:

The mean of the initial blood radioactivity in all experiments included in this study was found to be 3.61 cps. above background, with a S.D. of 1.80 cps and S.E. of 0.048 cps. This was based on a total of 1,381 observations. In order to reduce marked fluctuations, and exclude the occasional count which differed greatly from the mean, those more than

2 S.D. on either side of the mean were discarded. This limit however would include counts in the lower range statistically insignificant above background. Final limits of 1.25 S.D. below the mean to 2 S.D. above, were used, and counts which fall outside this range were discarded.

(2) Saline responses:

Initially responses following the injection of 0.2 ml. saline intravenously were calculated. 35 such responses were obtained, with a mean result of 96% (that is, a slight drop in radioactivity) and S.D. of 20%, S.E. of 3.3%. This matches McKenzie's findings closely[154].

During subsequent assays, volumes of plasma up to 0.5 ml. were injected, and control volumes of saline were correspondingly increased. Following 0.5 ml. saline, a decrease in radioactivity to 87% was noted (S.D. 28.0%, S.E. 2.06%) for 184 observations. This is significantly lower than the result following 0.2 ml. ($t = 2.308$; $0.05 < p < 0.02$) and must be attributed to a haemodilution effect associated with the rapid injection of a large quantity of fluid relative to the very small blood volume.

All readings resulting from injection volumes of 0.5 ml. were then corrected for the haemodilution factor by expressing them as a function of the saline response, where such a response was regarded as 100%.

McKenzie[152, 153, 154] makes no mention of the effects of haemodilution. He uses volumes ranging from 0.2 to 0.5 ml., and it is not clear whether he keeps these volumes constant for any one experiment. Comparison of responses is not valid when they result from different volumes of injected material.

(3) Responses to thyrotrophin reference standard:

The responses to doses of thyrotrophin ranging from 0.025 to 0.45 mu. are tabulated below. The bulk of the assays were done at 0.05, 0.15, 0.45 mu. dose levels.

DOSE LEVEL (mu.)	No. observations	MEAN ± S.E.
Saline	184	100 ± 2.06
0.025	28	122 ± 6.2
0.05	161	151 ± 5.5
0.15	133	227 ± 7.8
0.45	135	280 ± 9.9

From these results, the following overall response line, plotted against the logarithm of the dose, was derived.

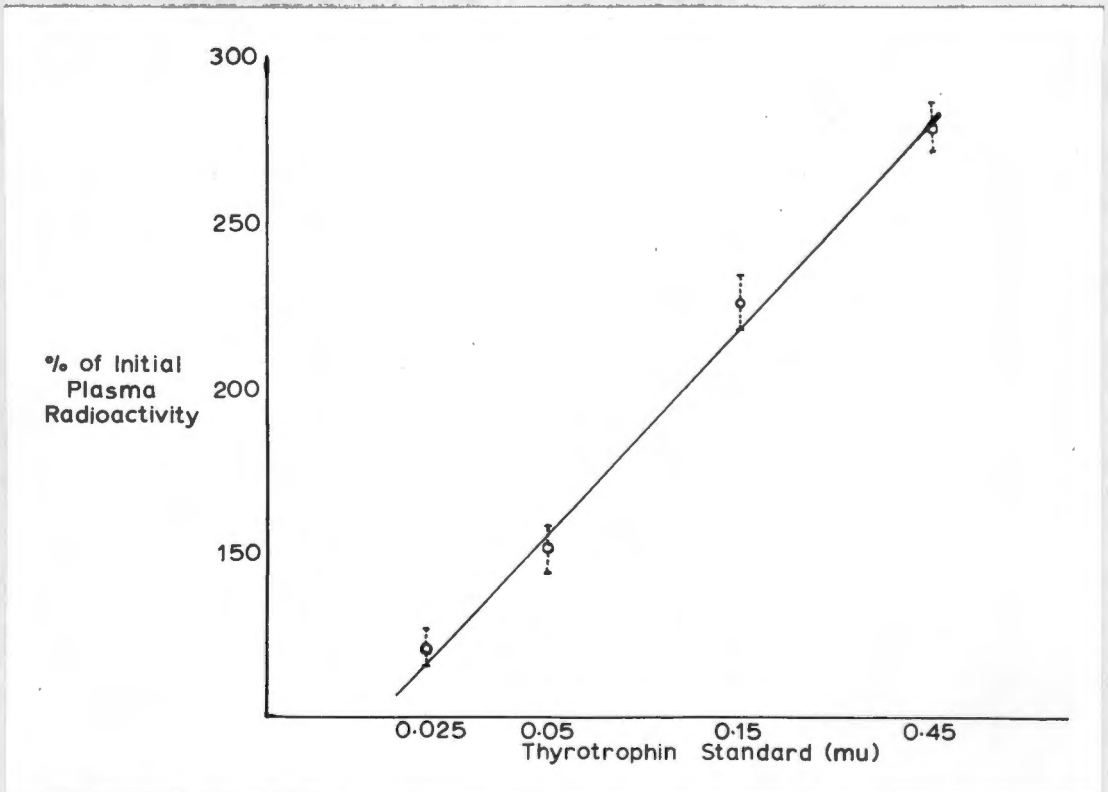


FIGURE 8: Thyroidal I^{131} release expressed as a linear response to the logarithm of graded doses of thyrotrophin.

A clear linearity in response is demonstrated. The correct regression line was determined by the method of "least squares".

Precision of the Assay:

Using the method described by Gaddum[103], both McKenzie[154] and Yamazaki[227] have investigated fully the average index of precision of a virtually identical assay to that used in this study. As their results matched almost exactly, the preision of this technique seemed established and it was not considered necessary to repeat their work.

Sensitivity and Range of the Assay:

A highly significant increase in radioactivity was noted at a dose level of thyrotrophin of 0.025 mu. ($t = 3.4$ $p < 0.01$). As the amount of human plasma injected ranged from 0.3 to 0.5 ml. (the total volume was always made up to 0.5 ml.), thyrotrophin levels of 0.05 to 0.10 mu./ml. are detectable using this assay.

No more than two plasma samples assayed in this study elicited thyrotrophic activity in excess of 0.45 mu., so that linearity was not explored beyond this limit. McKenzie has, however, reported linearity of response to 1.6 mu. using an identical method[154]

Although responses greatly in excess of these were obtained from plasma with L.A.T.S. activity, these results are not directly comparable with Thyrotrophin Reference Standard (Chapter VI).

CONCLUSION

An assay technique, outlined in some detail, has been used for the bio-assay of thyrotrophin in human plasma. It must be stressed, however, that what in fact has been assayed and called thyrotrophin, is merely a substance which discharges I^{131} from the thyroid gland two hours after injection. Though this is probably the most important parameter of thyrotrophin function, other types of thyrotrophin may be active, and antihormones or antagonists may block this action and negate a particular assay. What is being measured, then, is the sum of positive and negative effects - effects better termed thyrotrophin-like activity. All results must be interpreted in this light.

CHAPTER IV

Exophthalmos Producing Substance (E.P.S.) : Its nature and biological assay.

Exophthalmos producing substance of the anterior pituitary:

In 1931, exophthalmos was induced in ducks[191] and guinea-pigs[80, 141, 146, 147] by the administration of anterior pituitary extracts. Histologic evidence of associated thyroid hyperplasia was noted[191] and a pituitary thyrotrophic factor was incriminated as the causal agent of both effects. This concept gained further support when thyroidectomy was found to enhance the exophthalmos producing effects of anterior pituitary extracts in guinea-pigs[80, 146, 147, 196], by a presumed disturbance in pituitary-thyroid balance and resultant increase in thyrotrophin secretion. Thyroidectomy alone was reported to have caused exophthalmos in rabbits[108], a puppy[108], foxes[220] and guinea-pigs[78], while Dobyns made serial examinations of eye protrusion in 233 patients before and after thyroidectomy and noted an increase in prominence in 95% of cases[78]. Finally, when anterior pituitary extracts richest in thyrotrophin were found to contain most exophthalmos producing activity[16, 17, 18, 80, 167], the single identity of thyrotrophin and the exophthalmos producing factor seemed certain.

This view, however, could not account for the rarity of exophthalmos in spontaneous myxoedema, where large amounts of circulating thyrotrophin would be expected. Subsequently, Jefferies, using a more refined method of measurement, failed to confirm the exophthalmos stimulating effect of

thyroidectomy alone, or following anterior pituitary extract in guinea-pigs[125]. Patients suffering from acromegaly and myxoedema, in whose blood a high level of thyrotrophin had been assayed, showed no clinical evidence of exophthalmos[75]. Conversely, the serum of only two out of eleven severely exophthalmic patients contained an increased thyrotrophin level[75, 107]. Recently, McGill found no exophthalmos producing substance (E.P.S.) in the serum of three patients after thyroidectomy for hyperthyroidism and three cases of myxoedema, where high levels of thyrotrophin are usually present[150].

Jefferies, by iodination at pH 4.2, was able to destroy thyrotrophic activity and retain the exophthalmic factor in anterior pituitary extracts[126]; while Dobyns and Steelman noticed that some commercial thyrotrophin preparations had marked thyrotrophic and little exophthalmos producing activity when each substance was separately assayed[84]. The discrepancy was brought out by the differing solubility of these two factors in 8% trichloracetic acid, the exophthalmic factor being precipitated. Brunish showed that the activity of thyrotrophic pituitary extracts decreased to between one fifth and one tenth of the original after peptic digestion, whereas the exophthalmos producing activity remained[54]. Bates and Condliffe recently assayed both thyrotrophin and exophthalmost producing substance (E.P.S.) in transplantable thyrotrophin producing tumours in mice and found enormous levels of thyrotrophin (1,500 mu./mg.) but no exophthalmos producing factor, even after the administration of 4,500 mu. to the test animal.[36, 37] When

they ran tumour extracts through a carboxymethylcellulose column at pH 6, E.P.S. was found in the unadsorbed fraction, whereas thyrotrophin was adsorbed on the column[37].

Unequivocal evidence of the separate identity of thyrotrophin and a factor possibly responsible for exophthalmos, therefore exists.

The nature of the exophthalmos producing substance:

McGill, with fish as test animals, assayed exophthalmos producing activity in serum under a variety of experimental conditions[150]. He noted that if serum was filtered through a collodion membrane, no E.P.S. was present in the protein-free filtrate, but all occurred in the protein-rich material. He concluded that E.P.S. has a protein structure, like all hormones. Its presence in anterior pituitary extracts has been mentioned and it has not been detected in sera of persons with hypopituitary function[150] E.P.S. levels also dropped sharply in a patient with severe exophthalmos, following anterior pituitary irradiation[150]. Therefore, the anterior pituitary appears to be the site of origin of this hormone.

E.P.S. is rapidly destroyed when plasma is stored at 37°C. but at 4°C. potency is relatively stable[150] and at -22°C. markedly so[86]. Others have noted a maintainance of potency for about a week at room temperature[85].

The action of E.P.S.

The action of E.P.S. is widespread, affecting tissues throughout the body. Smelser first demonstrated the orbital fibro-fatty oedematous infiltration in exophthalmos[198, 199, 201]. Albert confirmed the presence

of orbital lymphocyte-laden fat, infiltrating and stretching the local muscles[18]. He noted a curious gel-like substance in addition and assayed E.P.S. from this fluid, and also showed that exophthalmos was purely the mechanical effect of accumulation of material in the retrobulbar area. Sympathectomy and hypophysectomy had no effect. Dobyns indicated the more generalized nature of the fatty disturbance following anterior pituitary injections in animals, demonstrating fatty, polymorphonuclear and round-cell infiltration in skeletal and cardiac muscle, connective tissue, liver and kidney[79]. An increase in plasma fat and acetone was mentioned and circulating leucocytes were found to contain fat globules[81]. These findings were later confirmed by others[26]. They showed an increase in mast cells and also demonstrated a replacement of the retrobulbar fatty infiltration by a muco-polysaccharide. The presence of mast cells was related to hyaluronic acid production.

Following current understanding, it appears that in patients who are clinically exophthalmic, an exophthalmos producing substance is secreted by the anterior pituitary. This may only be one of several hormones secreted in excess in this state. E.P.S. appears to cause a disturbance of fat metabolism with generalized fibro-fatty infiltration. This process then localizes in the orbit for obscure reasons, but can occur elsewhere, as in "pretibial myxoedema". A muco-polysaccharide replacement of the local fibro-fatty change then ensues, and exophthalmos develops for mechanical reasons. ACTH and cortisone may have an enhancing effect on this process[28, 57].

The biological assay of E.P.S.

The assay methods fall into two groups:-

(i) Those using direct measurement of ocular protrusion in test animals, following injection of material with exophthalmos producing activity.

(ii) That based on the sensitivity of the Harderian gland to the exophthalmos producing substance.

(i) Assays based on measurement of ocular protrusion:

The experimental production of exophthalmos in test animals has already been discussed. For assay purposes, guinea-pigs were preferred by earlier workers because of their ease of handling and ready availability[78, 80, 125, 201]. Albert reported exophthalmos in the Atlantic Minnow (Fundulus heteroclitus, Linn.) following the intracoelomic injection of anterior pituitary extracts[18]. He used twenty fish to assay each dose level of pituitary extract and measured the percentage of fish at each dose level which developed "significant" exophthalmos at six hours (the time of maximal response). He was able to construct a dose response curve by this means and detected exophthalmos producing activity in as little as 12.5 mg. equivalents of anterior pituitary. He also noted that the degree of exophthalmos in individual fish increased directly with the number of positive results. He finally defined a unit of exophthalmic activity as that amount of a substance causing exophthalmos in 25% of fish, six hours after intraperitoneal injection. Fish were chosen as a suitable test animal because the lateral position of their eyes enabled the measurement of intercorneal

distance, rather than that of each eye, to be used as a criterion of exophthalmos. Subsequent to these experiments, fish have been exclusively used for the bioassay of E.P.S.[54, 57, 84, 85, 86, 130, 150].

The method was improved by Dobyns and his associates who, for the first time, demonstrated exophthalmos producing activity in the sera of patients with progressive exophthalmos[84, 85]. They also used Fundulus as test animals in groups of eight to twelve to assess responses at each dose level. An elaborate apparatus with a vernier slide rule allowed intercorneal distance to be measured in duplicate to 0.1 mm. A 3% error was noted. 0.25 ml. material, often repeated twice, was injected into the coelomic cavity via the cloaca. Percentage increase in the intercorneal distance (I.C.D.) over saline controls was used as an index of exophthalmos producing activity. A maximal effect was noted within two days following anterior pituitary extracts and seven days after the injection of serum from exophthalmic patients. They therefore correlated E.P.S. with quantitative measurement of I.C. distance, rather than with a percentage of fish showing a significant response, as had Albert. However, they reported then, and since, a considerable variation in the responses of individual fish to a single dose level, so that an accurate dose response line could not be produced. Nevertheless, an acceptable dose response trend was shown[86]

Cañadell and Barraquer confirmed the exophthalmos producing capacity of sera from patients with endocrine exophthalmos, using goldfish (Carassius auratus)[56], while more recently, McGill[150] and der Kinderen and his co-workers[130] used a virtually identical assay technique with Shubunkins

(Carassius auratus, Linn.) and carp respectively. Both obtained linear log-dose responses and the latter workers demonstrated parallelism between the saline pituitary extract responses and those of human serum. This has not been repeated by others. Brunish, using the same method in Fundulus, produced a curved dose response line[54].

Maximal exophthalmic effect; optimal time for measurement:

The time of maximal exophthalmic effect in response to saline extracts of beef anterior pituitary and serum has varied considerably in the hands of various workers. In the minnow, pituitary extract was found to cause maximal exophthalmos at 4 - 24 hours depending on the dose[18, 86]; in the carp this was noted at 48 hours[130], and in a variety of goldfish at 18 hours[150].

Following the injection of human serum, maximal exophthalmos was present at 4 - 10 days in the minnow[86], 72 hours in the carp[130], and at 3 hours in the goldfish[150]. Dobyns commented on the prolonged length of time ocular protrusion persisted[85], while McGill found it to return to normal in 24 hours[150]. A mixture of anterior pituitary extract and serum retarded the effect slightly in the carp, although it was still maximal at 72 hours[130]; while a 3 hour peak was reported in goldfish[150]. The differences appear to relate to the species of fish used, and claims for the presence of a serum accelerator substance[150] or a serum factor inhibiting E.P.S.[85] have no real validity.

Specificity:

The question of the specificity of exophthalmos as an index of E.P.S.

activity has to be answered. Albert, in his original investigations, tested various endocrine extracts, including sheep pituitary extracts in various degrees of purification, in the minnow[18]. Exophthalmos producing activity was found only in those extracts rich in thyrotrophin. Subsequently, Dobyns and Steelman confirmed these findings, but reported a great fluctuation in E.P.S. activity in thyrotrophin extracts, related to the use of trichloracetic acid in the preparation thereof[84]. They were then able to separate E.P.S. and thyrotrophin by their differential solubility in trichloracetic acid, and showed them to be different substances, even though often present in the same extract and possibly related. It would thus appear that other hormonal substances, including purified thyrotrophin, do not cause protrusion in test fish, and that this response is specific to an unknown exophthalmos producing factor. Aterman has claimed that cortisone alone will precipitate exophthalmos in rats[29], and will increase exophthalmos induced by thyrotrophin in guinea-pigs[28]. This effect was not noted however, when a group of eight rats was given cortisone 1.5 mg. a day subcutaneously for 4 - 6 weeks in this laboratory.

As pooled[85, 86] and individual sera[130, 150] from normal persons have generally failed to elicit a significant proptotic response, a non-specific action of serum proteins in this respect may be ruled out. This was confirmed in this study. (See Chapter VII).

Further evidence supporting specificity of the reaction may be drawn from the correlation between proptotic responses of fish to serum from

patients suffering from exophthalmos, and the degree and progression of the disease in those patients[86, 130, 150].

Normal saline injections have failed to elicit an exophthalmic response in fish[85, 150], although recently a slight increase in I.C.D. over four days in the minnow has been reported[86]. Hypotonic saline has led to weight gain, but no exophthalmos[150]. This confirms that the pathogenesis is not related to simple fluid retention and weight gain, but to the more specific effect of an injected hormone.

It may be concluded, therefore, that the increase in ocular protrusion in goldfish following injections of serum or anterior pituitary extract, is a specific effect of a substance associated with, if not partly responsible for, clinical exophthalmos, and this may be used as a valid method of biological assay for this substance

The various assay methods differ in minor technicalities which include the volume of injected material, number of injections, the standard preparation, method of measuring I.C.D., optimum time for such measurement and species of fish used. This fact precludes strict comparison of results which, in addition, is made difficult by the crudeness of the methods, poor sensitivity, lack of uniformity of response and inability to apply statistical analyses to the results. Finally, as no reference standard of E.P.S. activity exists, either for saline pituitary extracts or serum (which appears to behave differently), quantitative estimations are not possible.

The limitations of such results must be clearly recognised. The assay does, however, serve as a crude index of exophthalmos producing activity and has been shown to correlate fairly well with the degree and clinical course of exophthalmos[86, 130, 150].

With minor modifications, this technique has been adopted in this study.

(ii) Assay based on Harderian gland sensitivity:

This assay is currently being investigated, but has not yet been applied to the detection of exophthalmos producing activity in serum.

Early investigators noted an increase in weight of the Harderian gland in experimental animals after the administration of anterior pituitary extract rich in thyrotrophin[170, 200]. It was also claimed that thyroidectomy alone caused hypertrophy of this gland in the guinea-pig and that this procedure enhanced the hypertrophy induced by anterior pituitary extracts[200].

A recent report showed that thyrotrophic pituitary preparations significantly increased the uptake of S^{35} labelled sulphate by the Harderian gland and retrobulbar connective tissue within 24 hours of hormonal stimulation[135]. S^{35} autoradiography of the Harderian and ventral lachrymal glands has shown the radioactivity to be in the parenchymal cells of both glands but in the secretions of the latter glands only[222].

Following this work, Wegelius and his colleagues developed an assay method for the detection of "ophthalmotrophic" activity in thyrotrophin preparations[223]. Using guinea-pigs, they injected varying doses of thyrotrophin and saline controls simultaneously with 150 μc.S^{35}-labelled $Na_2 SO_4$

and carrier. 24 hours later, the animals were sacrificed, the Harderian and ventral lachrymal glands dissected out, weighed, ashed and finally counted in an end-window Geiger-Müller tube. Radioactivity was expressed as counts per minute per 100 mg. wet weight for Harderian gland, and as counts per minute per mg. wet weight for ventral lachrymal gland. Data were statistically analysed (but no details were included in the paper) by the analysis of variance technique. An increase in S^{35} uptake was noted following thyrotrophin. That this effect was due to E.P.S. was suggested by its enhancement following peptic digestion of the pituitary extract, which has been shown to destroy thyrotrophin but has no effect on E.P.S.(54).

This assay has detected E.P.S. in 3.0 mg. and 5.0 mg. thyrotrophin (1.62 and 2.7 U.S.P. units); but no details of dose response and range are available. The sensitivity is probably less than the bioassay using fish and, until it is carefully investigated as regards specificity and dose responsiveness, must remain an interesting experimental observation only.

Conclusions:

That the present concepts regarding the aetiology and nature of endocrine exophthalmos are somewhat vague, and often contradictory, is to an extent due to a lack of a suitable assay for the detection of exophthalmos producing substance or substances in human serum. Such methods as are available have been discussed. Great caution must be exercised in the interpretation of the results of these methods which are lacking in many of the fundamental prerequisites for a valid biological assay. At the most, only

a crude assessment of E.P.S. activity can be made, and attempts to quantitate such levels are impossible because of the difficulties mentioned and the lack of a reference standard.

The only available assay relies on the estimation of proptosis in fish following the administration of the test substances. An alternative technique, based on the effects of E.P.S. on the S^{35} uptake by the Harderian gland, is as yet experimental.

As a crude but definite correlation has been found between the clinical degree and progression of exophthalmos and the "level" of E.P.S. as defined in this chapter and as assayed in fish, this technique was used for such estimations in this study.

CHAPTER V

Assay of E.P.S. based on the increase in intercorneal distance of the goldfish (Carassius auratus).

A: MATERIALS AND METHODS

The Assay Animal:

Species: The Atlantic Minnow (Fundulus) has been used by American workers in this field[18, 54, 84, 85, 86] but marked seasonal fluctuations have been reported with this species[86]. Better responses were obtained during the winter months, as opposed to the responses of the carp[130] and goldfish[150], where a more consistent result was noted throughout the year. In addition, goldfish did not become refractory to E.P.S. following repeated assays[150]. For these reasons, and because of their inexpensiveness and availability, goldfish were used as the test animal in this study.

Batches of 50 - 100 fish were periodically procured from the Jonkershoek Hatcheries. The weight was kept as constant as possible and fish between 4.0 and 8.0 G. only were used.

Care: As susceptibility to exophthalmos has been shown to vary directly with the environmental temperature[150], this was kept constant at 25°C. by means of thermostatically controlled electric heaters. Fish were kept in batches of 40 in three large glass storage tanks (± 20 gallon capacity), adequately aerated by motor pump. On the day of each experiment, groups of six fish were transferred to each of seven glass assay tanks, 25 cm. in diameter, where they remained until the assay was completed.

In the earlier experiments, fish could be individually identified by means of coloured cottons threaded through the mouth, or by their natural markings. Later this was not pursued.

The diet was constant throughout the study, a locally manufactured goldfish food being used with the occasional addition of live daphnia. The fish were starved for eighteen hours before each assay.

Fish were handled only during injecting and then, in thoroughly wet hands. They were caught in a small fish net and otherwise manipulated by a specially constructed perspex holder with sponge-rubber lining. Care was thus taken to avoid the introduction of superficial fungal infection, an important cause of fish morbidity.

The anaesthetic:

Adequate anaesthesia, essential for the required manipulations, was accomplished by the immersion of the fish in a 1 : 3,500 solution of MS 222 SANDOZ(x), an isomer of benzocaine. No adverse effects have been reported in this concentration.

Fish became anaesthetised in two to three minutes and remained so from two to ten minutes, during which time, the necessary procedures were carried out. Morbidity attributable to the anaesthetic was low.

Measurement of the intercorneal distance:

The anaesthetised fish were steadied in a perspex holder and intercorneal distance was measured by two independent observers using a micrometer screw. The head of the fish was placed between the limbs of the apparatus

(x) MS 222 - Information bulletin, published by Sandoz, Ltd., Basle, Switzerland.

which were carefully screwed together until each just touched the laterally placed corneae, without damaging them in any way. (Figure 9). Readings to the nearest 1/1000 inch (0.025 mm.) were possible and the two observers were found to correlate with a great degree of accuracy, usually to within 3/1000 to 5/1000 inch (0.1 to 0.15 mm.) The mean of the two observed readings was taken as the intercorneal distance (I.C.D.). This very simple procedure was found to be eminently satisfactory. As the usual I.C.D. was about 0.450 inch, an error of about 1% was present. The use of duplicate readings greatly increased the objectivity of the technique.

FIGURE 9: Measurement of intercorneal distance.

Injecting the substances to be assayed:

Technique: A 26 gauge needle attached to a tuberculin syringe was inserted into the cloaca. The barrel of the syringe was swung caudally and the needle pushed forward 5 mm. to pierce the gut and enter the coelomic cavity into which the contents were injected. During the procedure, which took about 30 seconds, the anaesthetised fish was held in the wet hand, the ventral aspect facing the injector.

FIGURE 10: Technique of intracoelomic injection.

The volume of any material injected was 0.5 ml. Fish varied a great deal in their tolerance to such a volume and, on occasions, when the injections were more hurried, a considerable mortality was noted about 48 - 72 hours afterwards. Injections were therefore performed very slowly in the smaller fish. McGill, using a radioactive tracer, has shown that excellent retention of the injected material occurs using this technique[150]. When this is not retained, the leak is quite obvious.

Nature of injected substances: Fish were divided into groups of six. In each experiment, a group received (i) physiological saline as a control, (ii) "Thytropar" (x), a beef anterior pituitary extract rich in E.P.S. in a fixed dose of 0.2 u. thyrotrophin, or 50 mg. equivalents of beef anterior pituitary and (iii) the "unknown" undiluted plasma or tissue homogenate assayed. Up to seven groups of fish were used in any one experiment, in which the assay of five unknown substances was therefore possible. After each experiment, the fish were not used again for two weeks.

Solutions of "Thytropar" were made, just prior to assay, in normal saline. Plasma or homogenates were stored at -15°C. for up to two months before assay, although the majority of estimations were done within four weeks of storage. This lengthy storage does not affect assay results, as Dobyns has shown that E.P.S. does not lose its potency when stored at -22°C. for up to twenty months[86], and at room temperature for up to one week[85]. McGill confirmed the relative stability at 4°C., but reported rapid decay over 24 hours at 37°C.[150]

(x) Thyrotrophin "Armour".

Measurement of the assay response:

I.C.D. was measured before and at an optimal time after the injection. The percentage increase was then calculated individually for each fish and the mean of the six results in each group was taken as the overall response to each substance injected. In later experiments, the intercorneal distances were measured for each group, without special note being made of the individual increases of each fish, as it became clear that great fluctuations within a group prevented any great statistical accuracy. Therefore, the assay response to each injected substance was the mean I.C.D. following intracoelomic injection of that substance into six fish.

FIGURE 11: Exophthalmos in goldfish following the injection of plasma from an exophthalmic subject. A fish injected with saline serves as a control.

Maximal exophthalmic effect: Optimal time for measurement:

The tremendous variability reported in the time responses to the various materials injected has been discussed. These must be attributed to differences in species and the relationships were therefore investigated in this study.

(i) Anterior pituitary extract: "Thytropar", 1 unit (250 mg. equivalent anterior pituitary) was injected into the coelomic cavities of twelve goldfish. The result (Figure 12) confirms McGill's observations[150]. A maximal response was noted between 3 and 21 hours while at 27 hours, the response was declining.

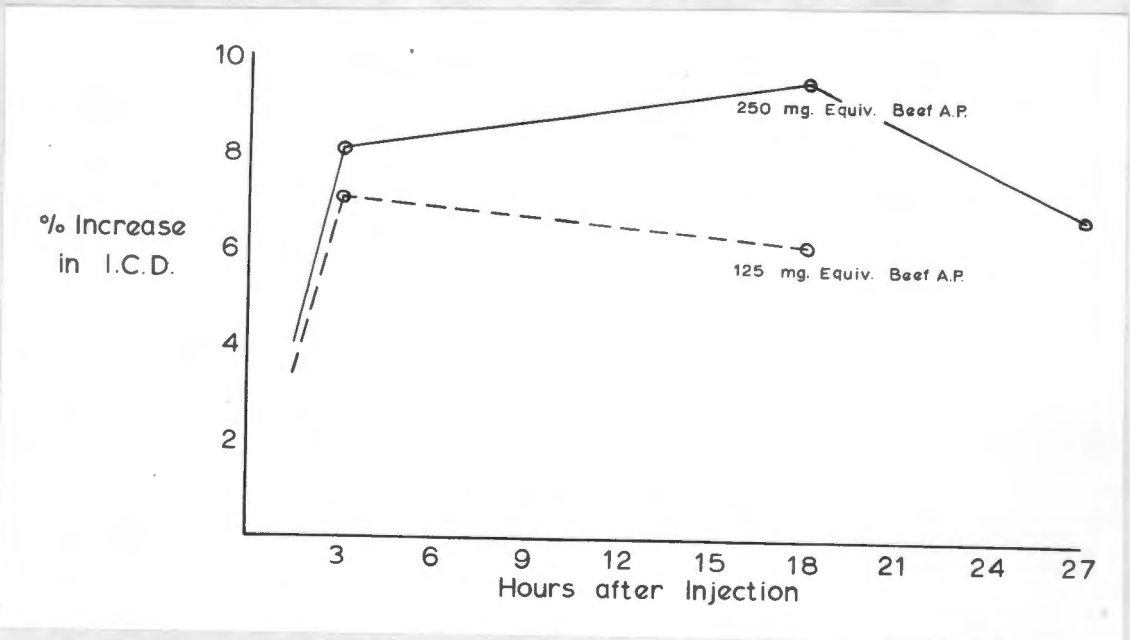


FIGURE 12: Time relationships in the exophthalmic responses to two doses of "Thytropar". Each point represents the mean of six responses.

In addition, the responses appeared to vary according to the injected dose, those in the lower dose range declining more rapidly. This confirms Dobyns' statement that the peak of response to anterior pituitary extracts occurs later and is sustained longer if the dose level is increased[86].

(ii) Plasma: 0.5 ml. undiluted plasma was administered in a single injection, to each of six fish. Three typical results are shown below. A peak effect at three hours was found with every plasma in which E.P.S. was assayed, confirming McGill's report with the same species of fish[150].

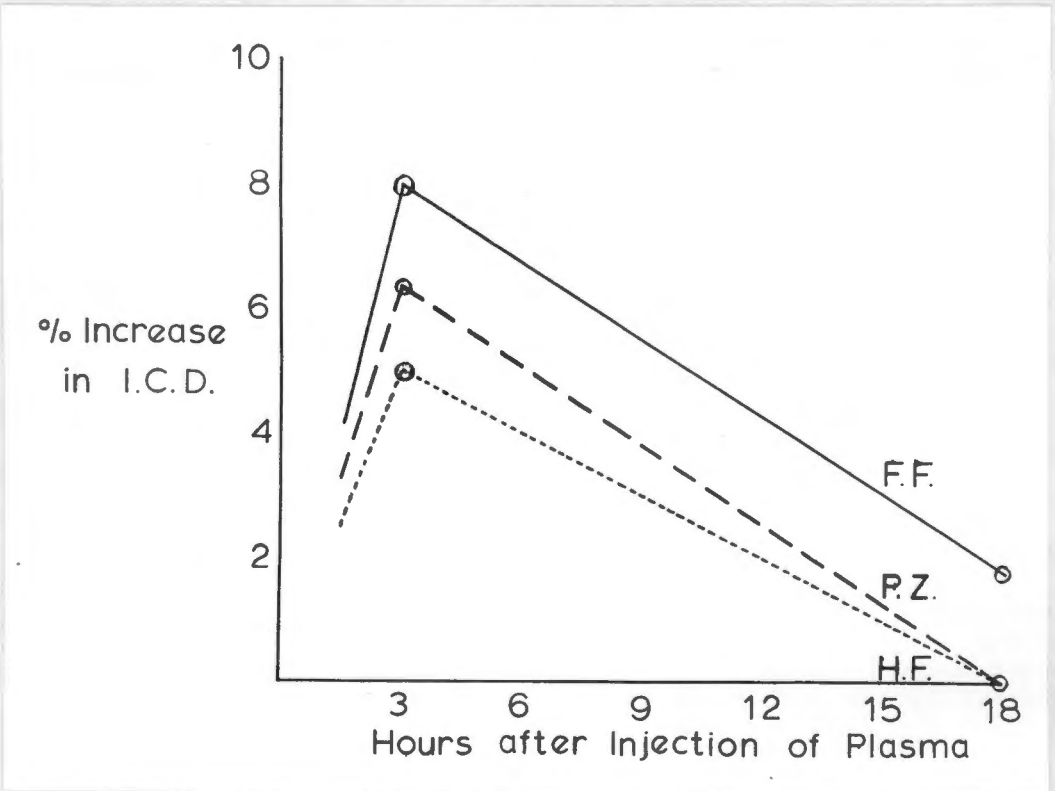


FIGURE 13: Time relationships in the exophthalmic responses to three plasma samples. Each point represents the mean of six responses.

For convenience, the 3 hour reading was chosen as optimal for both the plasma and the anterior pituitary extract used as a standard. This represents the time of peak response to plasma and, probably, the near-peak response to the standard extract in the dose used in this study.

Difficulties encountered during the development of the technique:

The method is technically very simple. No difficulties were encountered with the intracoelomic injections and the excellent correlation between the independent observations of the I.C.D. has been mentioned. The morbidity immediately following the procedures was usually very slight.

Intermittently, a heavy mortality was noted. Two "epidemics" of an unknown infection occurred and these spread rapidly to all three storage tanks with a heavy toll. The causal agent was never discovered; autopsies proving non-informative with no obvious fungal infection present. On rare occasions, up to one-third of fish used in a particular experiment would succumb 48 to 72 hours later. This mortality seemed to be related to the speed of, and the care taken over, the injections. Any trauma or bleeding related to this procedure was badly tolerated.

Final experimental design:

As no accurate quantitation of E.P.S. activity was possible, a dose response line was not attempted in every assay. Once a reasonable dose response relationship had been established, a single dose level of the standard was used for comparison in each assay. Groups of fish, of equal number[44], were used for each unknown or standard.

In summary, the following experimental design was followed:-

Groups of six fish were used. Each group was kept in a separate tank and in the earlier experiments fish were individually marked.

In each experiment, one group received 0.5 ml. normal saline, one, 50 mg. equivalent of beef anterior pituitary (0.2 u. "Thytropar") in 0.5 ml. saline and the remaining two to five groups received 0.5 ml. of the undiluted plasma or tissue homogenate to be assayed, so that a total of 24 to 42 fish were used.

At the beginning of the study, the contents of ten vials of "Thytropar" were pooled and used as a standard throughout. "Thytropar", in common with every other known E.P.S.-rich pituitary extract, has not been standardized for this substance. It was felt that a common "pool" of the standard powder would minimize possible unknown fluctuations occurring from batch to batch and provide a degree of consistency in the level of E.P.S. used as a standard for comparison.

The I.C.D. was measured in each fish before and 3 hours after each preparation injected and the mean of the six readings on each group was calculated and used to find the increase in I.C.D. The responses to saline were subtracted from those following the anterior pituitary standard and the unknown substances to provide an absolute value. The three hour response following the unknown was then expressed as a percentage of that response following the standard - thus, $\frac{\text{mean increase I.C.D. (unknown)}}{\text{mean increase I.C.D. (standard)}} \times 100$

An attempt at standardization was therefore made to counter possible seasonal

and other fluctuations. Dobyns has recently published a similar scheme of expressing results[86].

A crude system of quantitation was adopted, the responses being graded as follows:-

0	:	No increase in I.C.D.
±	:	Increase in I.C.D. 0-25% of standard
+	:	Increase in I.C.D. 25-50% of standard
++	:	" " " 50-100% " "
+++	:	" " " 100-200% " "
++++	:	" " " over 200% " "

B: RESULTS:

(1) The basal intercorneal distance: The mean of all the measurements of basal I.C.D. used in this study was 0.452 inch with a standard deviation of 0.020 inch. 500 measurements were used to compile these figures. As the range of these basal readings is narrow, it was not found necessary to discard any.

(2) The saline response: A total of 140 fish received 0.5 ml. normal saline, six in each experiment. A three hour increase in I.C.D. of 1.45% was noted (S.D. 0.71%; S.E. 0.06%). On occasions when the fish were found to be exceptionally responsive, increases as much as 3.4% and 3.6% were recorded. During these experiments, this responsiveness was mirrored by a correspondingly larger increase in response to the anterior pituitary standard.

(3) Response to anterior pituitary standard: An arbitrary fixed dose of 50 mg. equivalent of beef anterior pituitary was used as a standard in each experiment. A mean response of 6.5% increase in I.C.D. was found in 20 experiments (S.D. 1.8%; S.E. 0.40%). This represents a fair degree of consistency, but occasional unpredictable fluctuations occurred, which did not appear to be a seasonal effect, although this has been reported(86).

(4) Dose response relationship: Three groups of six fish were injected with "Thytropar", in doses of 125, 250 and 375 mg. equivalents of been anterior pituitary. A linear log dose response was obtained.

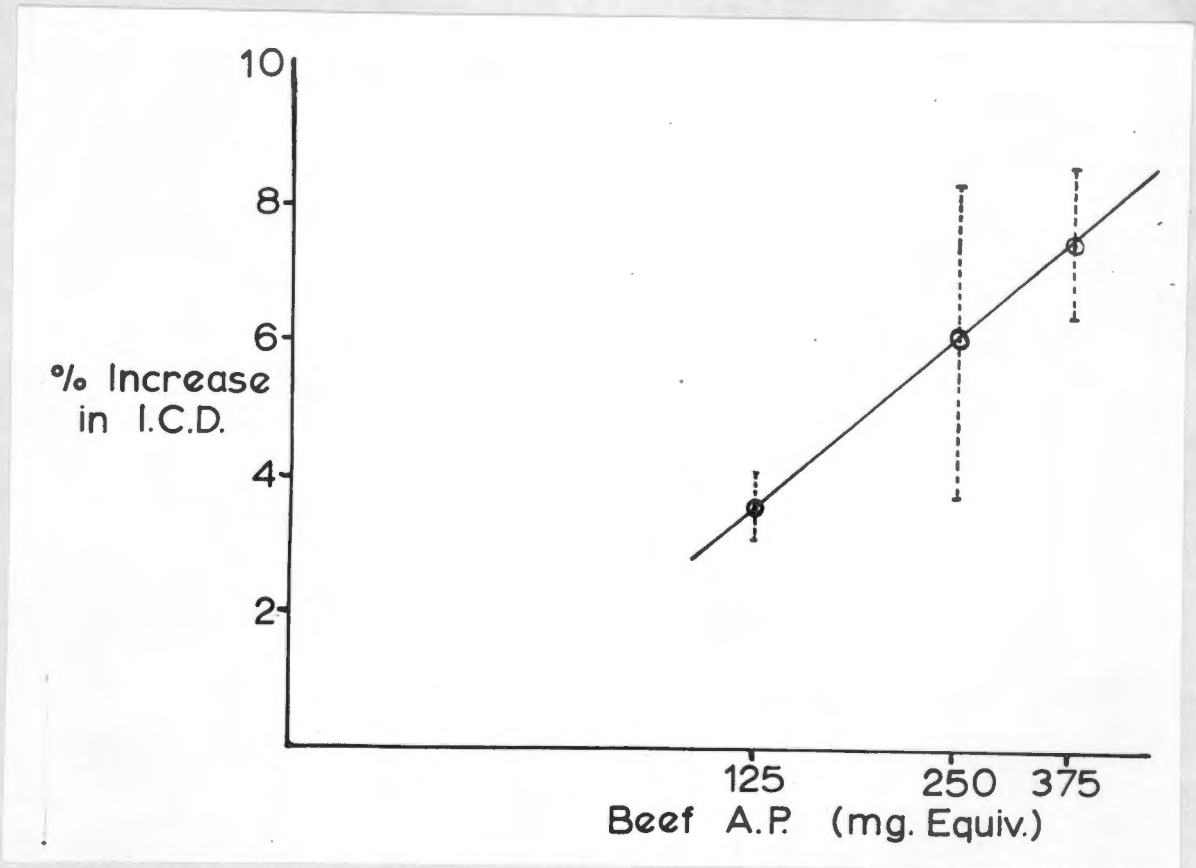


FIGURE 14: Increase in I.C.D. as a function of the log-dose of "Thytropar" standard. Each point represents the mean and standard error in six fish. I.C.D. was measured at three hours.

At the time this experiment was recorded, the fish were passing through a phase of very poor responsiveness and the readings are much lower than those usually obtained. Note the very large standard error of the response to 250 mg. equivalent of an anterior pituitary which must cast some doubt on the validity of the line. Dobyns had similar difficulty in obtaining good linearity of the log-dose responses; although, as in this study, an undoubted dose response trend was apparent[86].

Sensitivity and range of the Assay:

As no fixed assay technique exists for the estimation of E.P.S. activity, sensitivity cannot be compared to that noted by other workers. In addition, there appeared to be fluctuations in the responsiveness of the fish for reasons at present obscure. Seasonal factors could not be incriminated.

During periods when the fish were reacting well, as little as 25 mg. equivalent of beef anterior pituitary caused a three hour increase in I.C.D. of 4.8% over saline (S.D. 1.2%; S.E. 0.5%). This response is highly significant ($t = 6.2264$ $p < 0.01$).

The upper limits of responsiveness were not explored as plasma samples with such gross E.P.S. activity were not found.

CHAPTER VI

The Long Acting Thyroid Stimulator - Its Nature and Assay.

The nature of the long-acting thyroid stimulator:

In 1956, Adams noted abnormal responses in his guinea-pig thyrotrophin assay while testing various human sera for thyrotrophic activity[3, 11]. Instead of the usual maximal discharge of I^{131} three hours following thyrotrophin injection, a peak was found sixteen hours after injection in certain cases of hyperthyroidism. This abnormal thyrotrophic response was confirmed in a similar assay using mice as test animals, when the usual two hour peak was displaced to seven to twelve hours after thyrotrophin administration[4, 155, 162]. Once again it was noted that this response was virtually confined to serum from hyperthyroid subjects and especially those with significant exophthalmos, although blood from euthyroid or hypothyroid exophthalmic patients was found to elicit this abnormal reaction[155, 162].

These effects have recently been tested on human volunteers[43]. Blood from eight exophthalmic patients and two controls was transfused into normal recipients. In other subjects, thyrotrophin was injected intravenously at two dose levels. The protein bound iodine (P.B.I.) was estimated before and twelve hourly after the transfusion for 72 hours. A maximal increase in P.B.I. was found 30 hours after thyrotrophin, and 40 to 65 hours after blood transfusion from six out of eight exophthalmic patients.

The discharge of I^{131} is specific and appears to represent actual stimulation of thyroid function in animals[144, 158] and man[43]. Serum

from appropriate subjects has been shown to cause elevation of the P.B.I.[43,158] and to stimulate an increased thyroidal uptake of I^{131} with histologic evidence of thyroid hyperplasia in thyroxine treated mice[144, 158]. This substance then, has thyrotrophic properties. It has been given a number of names including "Abnormal thyroid stimulating hormone"[5], "Abnormal thyroid stimulator"[5] and "Thyroid activator"[156, 158]. It is by agreement currently termed "Long-acting thyroid stimulator" or L.A.T.S.[@]

L.A.T.S. appears to act directly on the thyroid gland as typical "delayed" responses are still obtainable in hypophysectomised mice[15, 162]. The more sustained and delayed effects are probably due to a longer half life in the circulating blood, compared to normal thyrotrophin. This difference has been clearly shown in mice[160] and rats[6] after intravenous injection of appropriate sera.

Further differences between L.A.T.S. and normal thyrotrophin have been reported. Thyrotrophic activity in the sera of patients with myxoedema is associated with the gamma-globulin fraction on a starch-block electrophoresis[152, 160, 162]. Using the same procedure, L.A.T.S. was not found in association with any specific fraction of the serum proteins[160, 162]. When serum was fractionated by means of chromatography using a column of diethylaminoethyl cellulose (DEAE)[160], a similar lack of specific association with a serum protein was noted, as opposed to normal thyrotrophin which again was found in the gamma-globulin fraction.

L.A.T.S. is poorly recovered after exposure to acetone as opposed to thyrotrophin[7] and, following Bates' alcohol percolation method[37], McKenzie

@ Terminology informally agreed upon at the Fourth International Goitre Conference, London, July 1960.

found very little of the original L.A.T.S. activity in the four fractions assayed, whereas thyrotrophic activity was easily concentrated[160]. Adams has investigated the dose response relationship of L.A.T.S. and found a steeper slope and a higher maximal linear response[7].

Heat has been shown to destroy thyrotrophin much more readily than L.A.T.S.[162] which is, in addition, very stable when stored at 4°C., no change in activity being noted after 176 days[162]. Thyrotrophin loses its potency very rapidly, even if frozen. (Figure 15).

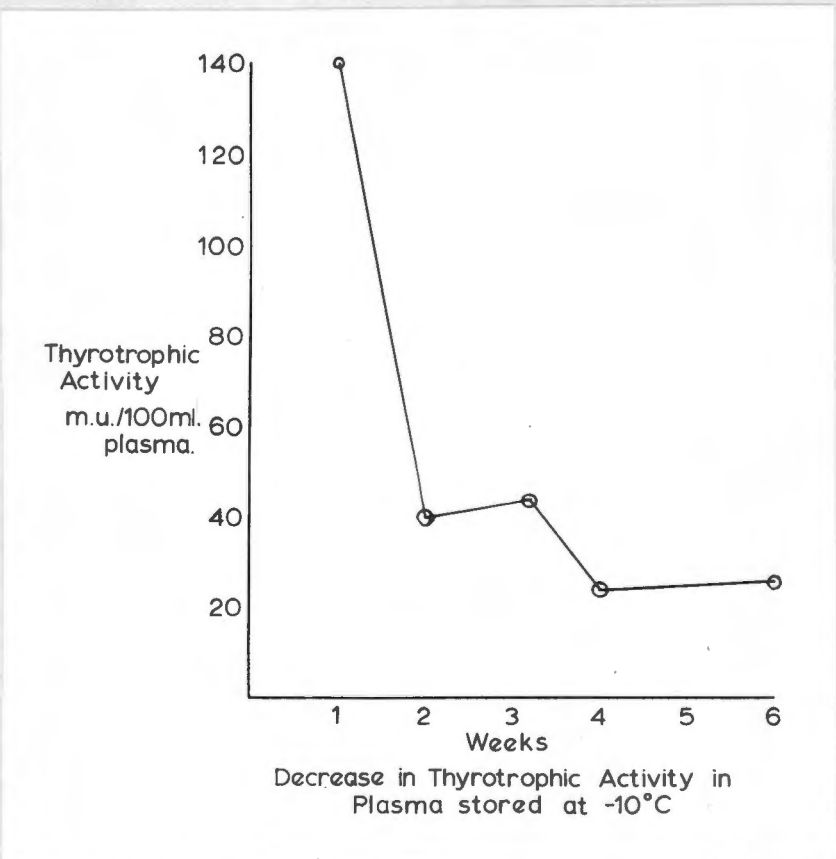


FIGURE 15: Rapid decline in thyrotrophic activity in plasma stored at -10°C. Weekly assays were done on a single large aliquot of plasma from a myxoedematous subject. Each point represents the mean of six observations.

Finally, L.A.T.S. does not appear to originate from the anterior pituitary. Four patients who developed Graves' disease after surgical ablation of the pituitary for various reasons, had serum L.A.T.S. activity[160]. Similarly, the anterior pituitary glands removed at necropsy from three cases of active or recent hyperthyroidism showed no L.A.T.S.[160, 163], although this had been present in the blood of one patient during life[160].

It is clearly apparent therefore that at least two substances with thyrotrophic activity appear to exist.

The association between levels of L.A.T.S. and the clinical states mentioned above has naturally led to speculation as to whether this is the causal agent in Graves' disease[13] and a remarkable case has been cited in this context[144]. An exophthalmic person, euthyroid following thyroidectomy for hyperthyroidism seven years previously, was found to have enormous levels of L.A.T.S. She gave birth to two live infants who were considered to be suffering from congenital thyrotoxicosis. The aetiological connection between maternal L.A.T.S. and the hyperthyroid state of the offspring has been seriously considered.

Other authors[5, 160] have suggested that L.A.T.S. may be the E.P.S. assayed in fish by earlier workers; again, because of similar clinical associations. Like L.A.T.S., E.P.S. is stable for long periods at 4°C.[150] or -22°C.[86]

<u>The site of origin and mode of action of L.A.T.S.</u> remains speculative. It does not appear to originate in the anterior pituitary, nor is the action

mediated via that gland[15, 162]. It has been found in equal concentration in the internal jugular and ante-cubital veins of hyperthyroid subjects[163].

L.A.T.S. may represent normal thyrotrophin modified by an unknown factor present in patients with the hyperthyroid-exophthalmos complex, as the incubation of thyrotrophin with thyrotoxic serum appears to alter the normal character of thyrotrophin in the direction of L.A.T.S.[163]

Assay of L.A.T.S.

L.A.T.S. was initially reported, and is still conveniently assayed, using I^{131} discharge as a criterion of activity[4, 155, 162], the increase in plasma radioactivity being noted seven to twelve hours after the serum injection, instead of the usual two to three hours.

In this study L.A.T.S. was assayed using precisely the technique for normal thyrotrophin assay as outlined in Chapter III. In addition to the two hour bleeding, aspiration of 0.1 ml. of blood was carried out seven hours after injection of plasma with suspected L.A.T.S. activity and after occasional controls. Batches of six pretreated mice were used and radioactive counting was performed as described earlier. Once again, the assay response was recorded as the percentage of the initial blood radioactivity.

As opposed to thyrotrophin assays, no L.A.T.S. reference standard exists, so that no attempt has been made to quantitate this substance in terms of any form of unit. All values, therefore, have been expressed directly in terms of the percentages referred to above, at two and seven hours.

Results:

Figure 16 illustrates a typical delayed response elicited from an appropriate plasma. This is contrasted with the responses both from plasma of an acromegalic subject, and from 0.05 and 0.15 mu. of thyrotrophin reference standard.

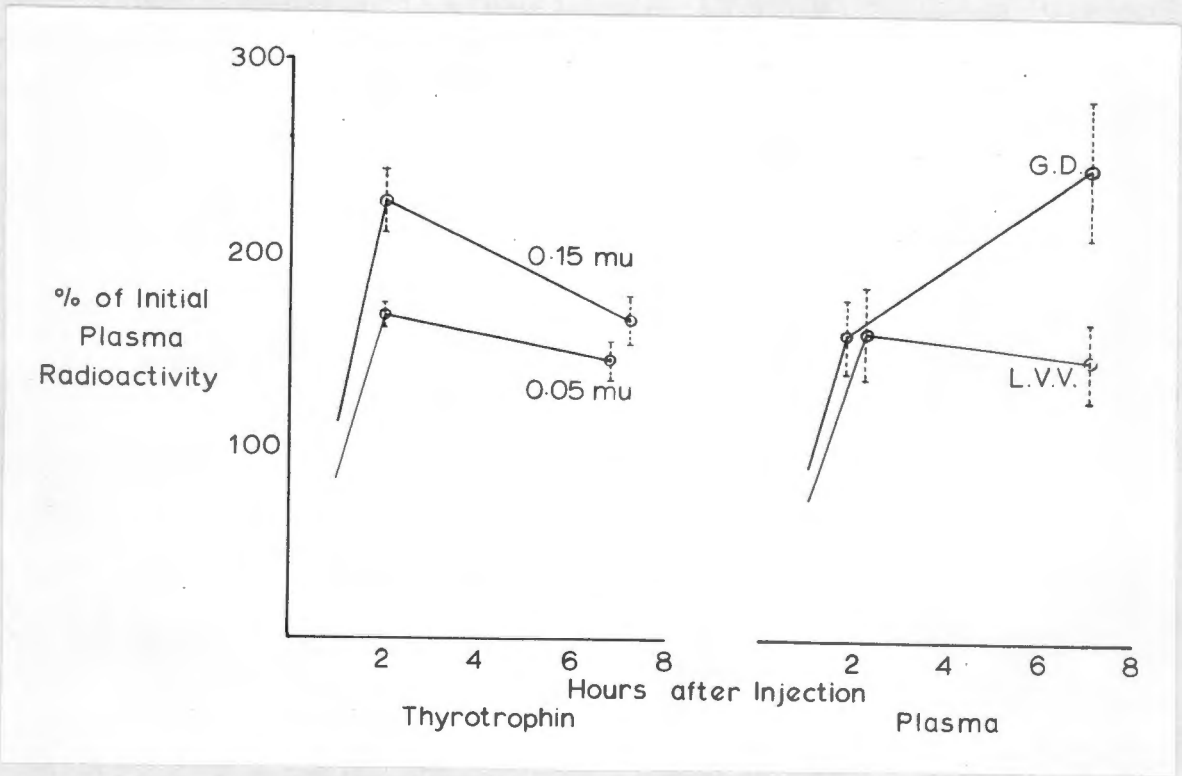


FIGURE 16: Two and seven hour release of I^{131} from the thyroid glands of mice following the injection of thyrotrophin standard and plasma from both an acromegalic subject and a patient with severe exophthalmos and pretibial myxoedema.

This assay, in conjunction with that described for E.P.S. was performed on plasma of patients suffering from exophthalmos and other endocrinopathies. The results of such assays will be described in subsequent chapters.

CHAPTER VII

Activity of Thyrotrophin and Exophthalmos Producing Substance in the Serum and Plasma of normal subjects.

(1) THYROTROPHIC ACTIVITY IN THE SERUM OF NORMAL SUBJECTS:

Although a few of the earlier workers had reported arbitrary levels of thyrotrophic activity in treated[64, 95] and untreated serum[24, 119], the techniques used were insensitive, usually lacking in statistical precision and were seldom reproducible. As recently as 1949, Albert expressed doubts as to whether thyrotrophin had been convincingly assayed in fractionated or unfractionated serum and he stressed the complete lack of uniformity of results at that time[19].

Assay methods, though still subject to much criticism, have improved to the extent of having provided a reasonably consistent overall picture of the thyrotrophin level in normal serum, although very recent work has reopenedcontroversy[32].

A range of 5 to 20 mu./100 ml. has been noted by some workers[74, 75, 107, 179, 227], although the majority report levels somewhat higher, averaging between 20 and 50 mu./100 ml.[12, 35, 37, 39, 48, 64, 77, 154, 185]

The most extensive survey of thyrotrophic activity in serum yet reported is that of Bottari[48]. He found a range of 7 - 80 mu./100 ml. (mean 25 mu.) in 110 normal males; 30 - 100 mu./100 ml. (mean 60 mu.) in 36 pre-menopausal females; 8 - 45 mu./100 ml. (mean 23 mu.) in 28 post-

menopausal females and levels of from 0 - 8 mu. (with occasional exceptions) in 20 persons of both sexes over 60. The general range of results corresponds with those reported above, though significantly higher values occur in pre-menopausal females and a definite decline with age is noted, as has been reported in the rat[157].

In 1961, Bakke and his colleagues reduced 1,000 ml. pooled fresh plasma to a 50 ml. clear sample by an extraction method and on assaying this fraction using thyroid slices in vitro, found a thyrotrophin concentration of 0.2 mu./100 ml.[32] This was repeated once, with the same result. Bates is quoted as having revised his previously reported levels down fifty-fold to 1.0 mu./100 ml.[35] This work remains to be repeated and confirmed by others.

Thyrotrophic activity as assayed in the present study:

Thyrotrophin levels were assayed (as outlined in Chapter III) in plasma from 15 normal subjects, all of whom were clinically euthyroid and had no evidence of goitre. Thyroid function was not otherwise tested. Results are summarised in Table III:-

Patient	Age	Sex	Thyrotrophic Activity mu./100 ml.
1. R.H.	39	M	26 (13 - 47)
2. R.C.	24	M	25 (17 - 38)
3. E.B.	29	F	31 (16 - 55)
4. G.B.	25	F	13 (8 - 21)
5. R.K.	65	F	20 (13 - 31)
6. G.E.	48	F	37 (30 - 48)
7. B.P.	28	M	20 (17 - 25)
8. B.L.	26	M	40 (31 - 54)
9. E.G.W.	22	F	17 (12 - 25)
10. R.G.	40	F	17 (11 - 27)
11. M.F.	21	F	10 (8 - 14)
12. W.G.	25	M	0 (0 - 7)
13. R.M.	37	F	28 (20 - 41)
14. J.L.	35	F	36 (24 - 55)
15. M.N.	40	M	21 (18 - 26)

TABLE III. Plasma thyrotrophic activity in 15 normal subjects. Each value represents the mean of six assay responses. 95% confidence limits are in parenthesis.

Comment: The mean activity of 22 mu./100 ml. plasma in the six males and 24 mu./100 ml. in the nine females is in close agreement with many current reports[12,35,37,39,48,64,77,154,185], and especially with that of McKenzie who uses a similar method[154]. It is difficult to understand the failure of Munro to find significant levels of thyrotrophin in 8 of 9 normal sera, using the same technique[162].

The much higher mean level (60 mu./100 ml.) reported in pre-menopausal females by Bottari[48] has not been confirmed by other workers, nor do the data derived from the 7 pre-menopausal females in this small study lend support to his report.

Serial estimations of thyrotrophic activity:

These were performed on a normal subject (R.H.) to study the fluctuation of thyrotrophic activity in a single individual. The results are shown below:

R.H. aged 39

Date	Thyrotrophic Activity (mu./100 ml.)
3. 7.61	26 (13 - 47)
12. 7.61	22 (17 - 30)
31. 7.61	22 (16 - 32)
23. 8.61	32 (24 - 45)
25.10.61	27 (22 - 35)

TABLE IV.

Serial assays of thyrotrophic activity in a normal subject. Each value represents the mean of six assays. 95% confidence limits are in parenthesis.

Comment: (1) Only minor fluctuations occurred in this normal subject over the three months during which the assays were performed.

(2) The consistency in results during this period confirms the reproducibility of the method.

A similar study in which the same results were obtained has recently been reported on four healthy subjects over a five day period[227].

(2) THE LEVEL OF E.P.S. IN THE SERUM OF NORMAL SUBJECTS:

Exophthalmos producing activity has not been unequivocally demonstrated in the serum of normal subjects. McGill[150] reported "slight" exophthalmos in fish three hours after injection of serum from 60 normal persons, but it is doubtful whether the 1 - 2% increase in intercorneal distance he noted is significant. Others have failed to record significant activity[85, 86, 130], except in occasional serum samples. Dobyns found pooled normal serum to give consistently negative results[86].

E.P.S. activity as assayed in the present study:

Plasma from 10 normal subjects was assayed for exophthalmos producing activity as described in Chapter V. Results are graded from 0 to ++++ and summarised below:-

	Patient	Age	Sex	E.P. Activity
1.	B.P.	28	M	±
2.	M.R.	19	F	0
3.	W.M.	46	F	+
4.	D.P.	62	F	±
5.	L.W.	35	F	0
6.	E.J.	39	F	0
7.	D.M.	30	F	0
8.	J.G.	39	M	±
9	R.H.	39	M	0
10.	J.E.	48	F	0

TABLE V. E.P.S. ACTIVITY IN PLASMA OF 10 NORMAL SUBJECTS

Comment: Exophthalmos producing activity has been found in the plasma of one out of ten normal subjects, confirming other reports. If E.P.S. is present in plasma in health, existing assay methods are obviously far too insensitive to detect it.

CHAPTER VIII

Activity of Thyrotrophin, L.A.T.S. and E.P.S. in the plasma of patients with hypothyroid and hyperthyroid function.

(1) THYROTROPHIC ACTIVITY IN HYPOTHYROID STATES:

In 1936, Hertz and Oastler reported the qualitative determination of thyrotrophic activity from the sera of nine myxoedematous subjects[119] and others subsequently confirmed this[62, 92, 175, 182, 195]. It was noted that sera from hypothyroid persons gave positive assay responses far more commonly than those from patients suffering from other thyroid disorders.

When quantitative estimations became possible, thyrotrophin levels were found to vary a great deal. De Robertis reported very high levels (one over 5 u./100 ml.) in two cases, and negligible amounts in two others[185]. D'Angelo noted elevated amounts (20, 30, 40 mu./100 ml.), normal levels (7, 8 mu./100 ml.) and absent activity (5 cases) in ten sera[75]. Gilliland and Strudwick had the same experience; 72 - 126 mu./100 ml. in eight out of ten sera and no activity in two others. Sera from two cretins were found to have levels of 160 and 282 mu./100 ml.[107]. Bowers and his colleagues reported increased activity in three cretins (37, 74, 407 mu./100 ml.)[50], as did Adams in four cases (100 - 250 mu./100 ml.)[5]; while Di George and his co-workers reported variable levels (0, 80, 120, 360 mu./100 ml.)[77]

Others have confirmed elevated thyrotrophin levels in myxoedema, including McKenzie (34 - 70 mu./100 ml.)[154] and Bakke (1 - 7 mu./100 ml., which is ten times the normal value with this assay)[32], while Bottari

noted either very high (200 - 450 mu./100 ml.) or negligible amounts (0 - 4 mu./100 ml.) in his series[48].

In general, thyrotrophin activity may either be grossly elevated or trivial in hypothyroid states, though normal activity is occasionally reported[75]. The most elevated levels appear to be associated with induced myxoedema [107], or cretinism [50, 77, 107].

Thyrotrophic activity investigated in the present study:

Plasma from eight patients was assayed. Two patients were suffering from spontaneous myxoedema, three from juvenile hypothyroidism, two had had total thyroidectomies (for thyroid carcinoma) followed by I^{131} therapy and one, a partial thyroidectomy for a single nodule. All patients were definitely hypothyroid clinically and subsequently responded to thyroxine. The results are summarised in Table VI.

Patient	Age (years)	Sex	Thyrotrophin level (mu./100 ml.)	Remarks
1. L.W.	76	F	5 (0 - 20)	I^{131} uptakes 12,17%. PBI 2.1 µg.%. Spontaneous myxoedema.
2. S.B.	60	F	150 (70 - 250)	I^{131} uptakes 6,4%. RBC uptakes 8.4%. Spontaneous myxoedema.
3. A.A.	49	F	0 (0 - 6)	I^{131} uptakes 14,16%. RBC uptakes 9.0%. Hypothyroid following partial thyroidectomy for thyroid nodule.
4. P.Z.	32	F	310 (200 - 460)	I^{131} uptakes 1.5%; 48 Hour urines - 90% of dose. Total thyroidectomy for carcinoma 6 months previously. Followed by 134 mc.I131
5. M.K.	36	F	21 (14 - 32)	I^{131} uptakes 6%. Total thyroidectomy for carcinoma 3 years previously. Thyroxine stopped 3 weeks before assay.
6. HduP.	15	F	72 (52 - 105)	I^{131} uptake 5,2%. RBC uptake 10%. PBI 4.0 µg.%. Juvenile hypothyroid.
7. B.C.	5/12	F	135 (90 - 204)	I^{131} uptake not done. Cretin.
8. B.F.	7	M	68 (40 - 114)	I^{131} uptakes 70%. KSCN discharge negative. PBI 1.5 µg.%. Goitrous cretin.

TABLE VI. Thyrotrophic activity in hypothyroidism.

I^{131} uptake figures refer to 6 and 24 hour thyroidal uptakes following 25 µc. I^{131} given orally.

RBC uptake refers to the percentage uptakes of I^{131} by the red cells after incubation for two hours with I^{131} labelled triiodothyronine.

TABLE VI (contd.)

P.B.I.(X) is the protein bound iodine estimation and measures the amount of circulating organic iodine loosely bound to the serum proteins, i.e. the iodine of circulating thyroid hormone.
Each assay result represents the mean of six observations, the 95% confidence limits of which are in parenthesis.

These explanatory remarks apply to all subsequent tables.

Comments: All three patients suffering from juvenile hypothyroidism or cretinism show plasma thyrotrophic activity greatly elevated, three to five times the normal value obtained by this assay. This confirms the general experience in this form of hypothyroidism.

One of the two subjects studied with spontaneous myxoedema shows activity six times the normal value, while the other shows trivial levels. This, too, accords with comments in the literature.

Finally, of the two patients after total thyroidectomy, one has tremendous values (twelve to thirteen times normal) and one, normal levels, while no activity was noted in the plasma of one patient after partial thyroidectomy. The patient in whom normal results are obtained, had only been off thyroxine therapy for three weeks and this may have modified the final thyrotrophin level.

(X) Estimated by Dr. A. van Zyl, Department of Physiology.

The interpretation of an increased thyrotrophic activity is easily made according to the concept of a pituitary-thyroid homeostatic state outlined in earlier chapters. The failure of thyroxine production, whatever the mechanism concerned, is attended by a reciprocal increase in thyrotrophic output by the anterior pituitary, presumably in an attempt to stimulate the thyroid to produce thyroxine and restore the balance. As secretion cannot be "stepped-up", homeostasis is never reached and the thyrotrophin levels remain permanently high.

Those cases in which diminished thyrotrophic activity has been demonstrated remain puzzling. D'Angelo[75], Di George[77] and Gilliland and Strudwick[107] have reported such cases in which thyrotrophin levels were subsequently raised considerably (50 mu./100 ml. 200 mu./100 ml. and 99 mu./100 ml. respectively) after treatment with thyroxine. These results may be interpreted as indicating that the myxoedematous process may affect the pituitary gland resulting in a diminished metabolic and secretory capacity[99]. This may concern not only the output of thyrotrophin but other hormones as well, as urinary gonadotrophin has been shown in one case to rise after thyroxine[99].

(2) THYROTROPHIC AND L.A.T.S. ACTIVITY IN HYPERTHYROID STATES:

Hertz and Oastler were unable to detect thyrotrophic activity in the blood of eight thyrotoxic patients, using an assay which detected activity in myxoedema[119]. This was noted by other workers[64, 175, 185], who found detectable levels after treatment of the condition[175, 185].

D'Angelo and his colleagues reported a wide range in ten cases[75]. In three, no activity was found; in six, activity was normal (6 - 10 mu./100 ml.) and in one case, the thyrotrophin level was a little raised (20 mu./100 ml.). Others have also reported variable results[27, 179, 195]. Adams and Purves reviewed this subject in 1957[13]. In 17 of 41 cases they collected from the literature, thyrotrophin levels were found to be raised, while in the rest, the levels were normal or diminished. Recently, Bottari reported a series of 45 cases, in which thyrotrophic activity was normal in nine, moderately elevated in 23 (100 - 800 mu./100 ml.) and grossly raised in thirteen (over 1 u./100 ml.) [48]. No association was found between the severity of hyperthyroidism and the level of thyrotrophin.

Gilliland and Strudwick attempted to correlate the wide range of results with the degree of associated eye signs[107]. They found increased activity in ten of fourteen patients, with the greatest increases confined to those cases with severe eye signs.

This great discrepancy in results has been a source of discussion and speculation. On the basis of the pituitary-thyroid homeostatic concept, high levels of circulating thyroxine should lead to <u>low</u> or absent thyrotrophic activity in all cases. It has been suggested that high values may be due to some artifact, which is noxious to thyroid tissue and causes iodine release in the assay experiments employing this end-point[48]. Another view is that the hyperplastic thyroid inactivates thyrotrophin and this accounts for low levels[82]; but this has not been shown to occur <u>in vivo</u>[72].

The long acting thyroid stimulator: The presence of inexplicably high values for thyrotrophin in hyperthyroidism may be more effectively explained in terms of L.A.T.S. The nature of this substance and the marked correlation between its presence and hyperthyroid states associated with severe exophthalmos has been fully discussed. It is not unreasonable to postulate that the activity attributed to thyrotrophin in the assays quoted, resulted in fact from the thyrotrophin-like properties of L.A.T.S. These earlier assays failed to distinguish between this substance and normal thyrotrophin.

Adams, using an assay employing thyroid I^{131} discharge reported delayed activity suggestive of a L.A.T.S. effect in three of five untreated thyrotoxic patients with exophthalmos and none of three patients without exophthalmos(5). McKenzie found delayed activity in the sera of thirteen of fifteen thyrotoxic subjects, two euthyroid exophthalmic patients and in one normal person(155,158); while Munro noted L.A.T.S. in six of eleven hyperthyroid patients, two of three euthyroid persons with exophthalmos and one of nine normal sera tested(162). Recently, Yamazaki reported L.A.T.S. activity in three of nine sera from thyrotoxic patients(227) and Bjorkman noted this in six of eight cases, using human volunteers as test subjects(43).

Thyrotrophic and L.A.T.S. activity investigated in the present study:

Initially thyrotrophic activity was estimated in ten hyperthyroid subjects. All patients had definite clinical and laboratory evidence of this state and responded to subsequent antithyroid therapy. The results are shown in Table VII.

Patient	Sex	Age (years)	Thyrotrophin level (mu./100 ml.)	Remarks
9. F.F.	F	37	5 (0 - 17)	Slight exophthalmos. I^{131} uptakes 69,69 → 97,77% after T3. RBC uptake 23.5%
10. G.D.	F	42	40 (27 - 61)	Marked exophthalmos. I^{131} uptakes 70,69 → 81,68% after T3. RBC uptake 23.8%. PBI 9.0 µg.%
11. R.B.	F	44	27 (17 - 43)	No exophthalmos. I^{131} uptakes 86,83 → 78,71% after T3. RBC uptake 21.5%. PBI 9.5 µg.%
12. J.H.	F	36	43 (37 - 53)	Mild exophthalmos. I^{131} uptakes 82,55 → 85,68% after T3. RBC uptake 22.8%
13. J.T.	F	25	33 (18 - 57)	No exophthalmos. I^{131} uptakes 76,60 → 71,61% after T3. RBC uptake 21.7%
14. M.G.	F	44	27 (20 - 38)	No exophthalmos. I^{131} uptakes 75,57 → 79,58% after T3. RBC uptake 17.6%
15. P.O'K.	M	48	8 (0 - 15)	No exophthalmos. I^{131} uptakes 80,78%. RBC uptake 31.8%. PBI 13.7 µg.%
16. M.N.	F	49	20 (4 - 46)	No exophthalmos. I^{131} uptakes 76,68 → 71,79% after T3.
17. L.G.	F	31	18 (11 - 29)	Slight exophthalmos. I^{131} uptakes 73,80 → 73,85% after T3. RBC uptake 22.6%
18. D.M.	F	54	10 (0 - 29)	No exophthalmos. I^{131} uptakes 81, 80 → 79,81% after T3. RBC uptake 25.3%. PBI 14 µg.%

TABLE VII. Thyrotrophic activity in Hyperthyroidism.

In this, and in subsequent tables, T3 refers to tri-iodothyronine, which is given at a dose of 120 µg./day for seven days, in an attempt to suppress thyroidal I^{131} uptake.

Comment: The mean thyrotrophin level of 23 mu./100 ml. is almost identical with the levels in normal subjects reported in the previous chapter. One patient with severe exophthalmos had a level of 40 mu./100 ml. A range from 5 mu. to 43 mu./100 ml. is present and this corresponds to that reported in the literature.

These results, however, may have a limited significance as they possibly represent part of L.A.T.S. activity, which in many cases appears to produce a response as early as two hours after the injection of plasma.

L.A.T.S. activity was then investigated in nine clinically thyrotoxic patients. The results are shown in Table VIII.

Patient	Age	Sex	L.A.T.S. % of initial radioactivity 2 Hours	L.A.T.S. % of initial radioactivity 7 Hours	Response-Ratio (7 Hr/2 Hr)	Result	Remarks
13. J.T.	25	F	145 ± 13	149 ± 13	1.03	Absent	No exophthalmos. RBC uptake: 21.7% I^{131} uptakes 76,60 → 71,61% after T3.
17. L.G.	31	F	117 ± 11	161 ± 15	1.38	Significant ($t = 2.3158$ $p < 0.05$)	Slight exophthalmos. RBC uptake 22.6% I^{131} uptakes 73,80 → 73,85% after T3
16. M.N.	49	F	109 ± 16	167 ± 22	1.53	? Significant ($t = 2.1482$ $0.05 < p < 0.1$)	No exophthalmos. Marked lid lag and retraction with periorbital oedema. I^{131} uptakes 76,68 → 71,79% after T3
19. L.A.	36	F	126 ± 9	154 ± 14	1.21	Suggestive ($t = 1.6250$ $0.1 < p < 0.2$)	No exophthalmos. Marked lid lag and retraction. RBC uptake 27.6%. I^{131} uptakes 75,87 → 92,94% after T3.
10. G.D.	42	F	158 ± 19	245 ± 36	1.55	? Significant ($t = 2.1220$ $0.05 < p < 0.10$	Marked exophthalmos. RBC uptake 23.8% I^{131} uptakes 70,69 → 81,70% after T3. PBI 9.0 µg.%.
20. J.P.	26	F	102 ± 16	140 ± 6	1.37	Significant ($t = 2.2353$ $p < 0.05$)	No exophthalmos. Marked lid lag and retraction. RBC uptake 23%. I^{131} uptakes 91,96 → 100,89% after T3.
21. M.M.	51	F	189 ± 13	195 ± 13	1.03	Absent	No exophthalmos. Marked lid lag and retraction. RBC uptake 22.3%. I^{131} uptakes 94,76 → 86,71% after T3.
c22. J.B.	63	M	151 ± 15	190 ± 19	1.26	Suggestive ($t = 1.625$ $0.1 < p < 0.2$)	No exophthalmos. RBC uptake 36.3%. I^{131} uptakes 76,80%.
23. E.O.	27	F	185 ± 25	266 ± 36	1.44	Suggestive ($t = 1.841$ $0.05 < p < 0.1$)	Slight exophthalmos. RBC uptake 27.0%. I^{131} uptakes 85,79%

TABLE VIII. L.A.T.S. Activity in Hyperthyroidism.

TABLE VIII. L.A.T.S. Activity in Hyperthyroidism.

Each recorded result is the mean of six observations ± the standard error. When the 7 hour reading is significantly increased as compared to that at 2 hours, the assay is regarded as conclusive for L.A.T.S. Where the increase is definitely present, but not statistically significant, L.A.T.S. activity is "suggestive"; and where a trivial increase or decrease, occurs, activity is regarded as being absent. A "response ratio" was calculated, being the ratio of the 7 hour to the 2 hour response. This scheme will be followed in all subsequent relevant tables.

The evaluation of the significance of a 7 hour response is difficult. As has been mentioned, this has been calculated as compared to the 2 hour reading, but as this reading is already extremely elevated in some cases, this does not seem a rational method. Unfortunately, no other standard for comparison exists. It does not seem logical to compare the plasma 7 hour response with the response to saline at that time, though it may be valid to compare it with a standard thyrotrophin solution or a plasma sample with a high "normal" thyrotrophin content, as did Adams[5].

It was finally decided to determine the significance of the 7 hour response compared to that at 2 hours as outlined above. It should be noted however, that though a response ratio of 1.10 or over may be insignificant statistically, L.A.T.S. activity may still be present (as a negative ratio is virtually the rule with "normal thyrotrophin). The term "suggestive" was therefore used to cover this rather indefinite intermediate group.

Comment: The highest L.A.T.S. level is present in the only patient clinically "hyperophthalmopathic", which accords with current reports. Four of the nine patients had significant activity, while a further three had a distinct increase at seven hours, but of doubtful significance. A significant rise in mouse plasma radioactivity at two hours was present in seven cases.

This small study suggests the presence of L.A.T.S. in a high proportion of thyrotoxic patients, whether true exophthalmos is associated or not.

(3) THE ACTIVITY OF E.P.S. IN HYPOTHYROID AND HYPERTHYROID STATES:

The nature and significance of the exophthalmos producing substance has been fully discussed. In summary, significant levels of this substance are associated almost exclusively with marked degrees of clinical exophthalmos, irrespective of thyroid function(86, 150), Thyrotoxic patients without exophthalmos usually show no E.P.S. activity(86,150), though Dobyns has noted three cases with significant levels which have antedated the development of exophthalmos by weeks or months(86). McGill found that serum from three hypothyroid subjects and from three patients after thyroidectomy for hyperthyroidism failed to show E.P.S. activity(150), but no other studies on similar patients have been reported.

E.P.S. activity investigated in the present study:

E.P.S. was assayed in two patients with spontaneous myxoedema, two cretins, two patients following total thyroidectomy for carcinoma of the thyroid and eleven hyperthyroid subjects.

The results are tabulated in Table IX.

Patient	Age	Sex	Diagnosis	EPS Activity	Thyrotrophic or L.A.T.S. Activity
1. L.W.	76	F	Spontaneous myxoedema	0	T. 5 mu./100 ml.
2. S.B.	60	F	Spontaneous myxoedema	0	T. 150 mu./100 ml.
4. P.Z.	32	F	Total thyroidectomy for CA.	++	T. 310 mu./100 ml.
5. M.K.	36	F	Total thyroidectomy for CA.	±	T. 21 mu./100 ml.
6.HduP.	15	F	Juvenile hypothyroidism	0	T. 70 mu./100 ml.
7. B.C.	5/12	F	Cretin	+	T. 135 mu./100 ml.
9. F.F.	37	F	Hyperthyroid. Slight exophthalmos.	+++	T. 5 mu./100 ml.
10. G.D.	42	F	Hyperthyroid. Severe exophthalmos.	++++	T. 40 mu./100 ml. L. 245% R.R. 1.55
11. R.B.	44	F	Hyperthyroid. No exophthalmos.	++	T. 27 mu./100 ml.
12. J.H.	36	F	Hyperthyroid. Mild exophthalmos.	0	T. 43 mu./100 ml.
13. J.T.	25	F	Hyperthyroid. No exophthalmos	0	T. 33 mu./100 ml. L. 149% R.R. 1.03
14. M.G.	44	F	Hyperthyroid. No exophthalmos	±	T. 27 mu./100 ml.
17. L.G.	31	F	Hyperthyroid. Slight exophthalmos.	0	T. 18 mu./100 ml. L. 161% R.R. 1.38
16. M.N.	49	F	Hyperthyroid. No exophthalmos. Periorbital oedema.	++	T. 20 mu./100 ml. L. 167% R.R. 1.53
19. L.A.	36	F	Hyperthyroid. No exophthalmos.	++	T. 9 mu./100 ml. L. 152% R.R. 1.21
20. J.P.	26	F	Hyperthyroid. Marked lid lag, and retraction.	+++	T. 0 L. 140% R.R. 1.37
21. M.M.	51	F	Hyperthyroid. Marked lid lag, and retraction.	±	T. 40 mu./100 ml. L. 195% R.R. 1.03

TABLE IX. E.P.S. Activity in Hypothyroid and Hyperthyroid States.

T., L. and R.R. refer to thyrotrophin, L.A.T.S. and response-ratio respectively. Relevant laboratory data has already been included in Tables VI, VII and VIII.

Comment: (a) E.P.S. activity: In hypothyroidism, trivial or no E.P.S. activity was found, the single exception being a patient assayed after total thyroidectomy. The other thyroidectomised subject had elicitable, though slight, activity. Neither patient had exophthalmos. Though the results in general accord with those published by others, E.P.S. has not been reported in serum from patients after total thyroidectomy.

The significance of these observations is obscure, but they may bear a relationship to the early reports of exophthalmos following total thyroidectomy in animals[78,108,220] and man[78]. Such a complication is extremely rare in hypothyroidism arising spontaneously nor is E.P.S. present[150]. The mechanism of this discrepancy is puzzling, and it is possible that in myxoedema the thyroid, though failing in its output of thyroxine, may still be producing a substance controlling E.P.S. secretion by the anterior pituitary gland and preserving homeostasis. After total thyroidectomy, both this factor and thyroxine would be absent, and accelerated release of E.P.S., in addition to thyrotrophin, might ensue. This speculation can only be clarified by the collection of data from more cases similar to the ones studied.

Though McGill reports an absence of E.P.S. in three patients following thyroidectomy for hyperthyroidism[150], these operations are likely to have been sub-total however, and the above suggestion is not invalidated.

In hyperthyroidism, E.P.S. was assayed more often and at higher levels, as would be expected. The greatest response was associated with exophthalmos, and this has been the experience of others[86,150]; but four patients in whom this complication was not present had increased activity. Two of these had

marked lid lag and retraction, with periorbital oedema in one instance. This has been reported by Dobyns[86], who found that such cases ultimately tended to develop exophthalmos.

(b) <u>The non-identity of thyrotrophin and E.P.S.</u> In spite of greatly increased levels of thyrotrophin in the plasma of cases 2 and 6, no E.P.S. activity was assayed. This strongly favours the different identities of these two substances and confirms the other evidence for this discussed in Chapter IV.

Figure 17 shows the results of E.P.S. on thyrotrophin assays discussed in this and the preceding Chapter.

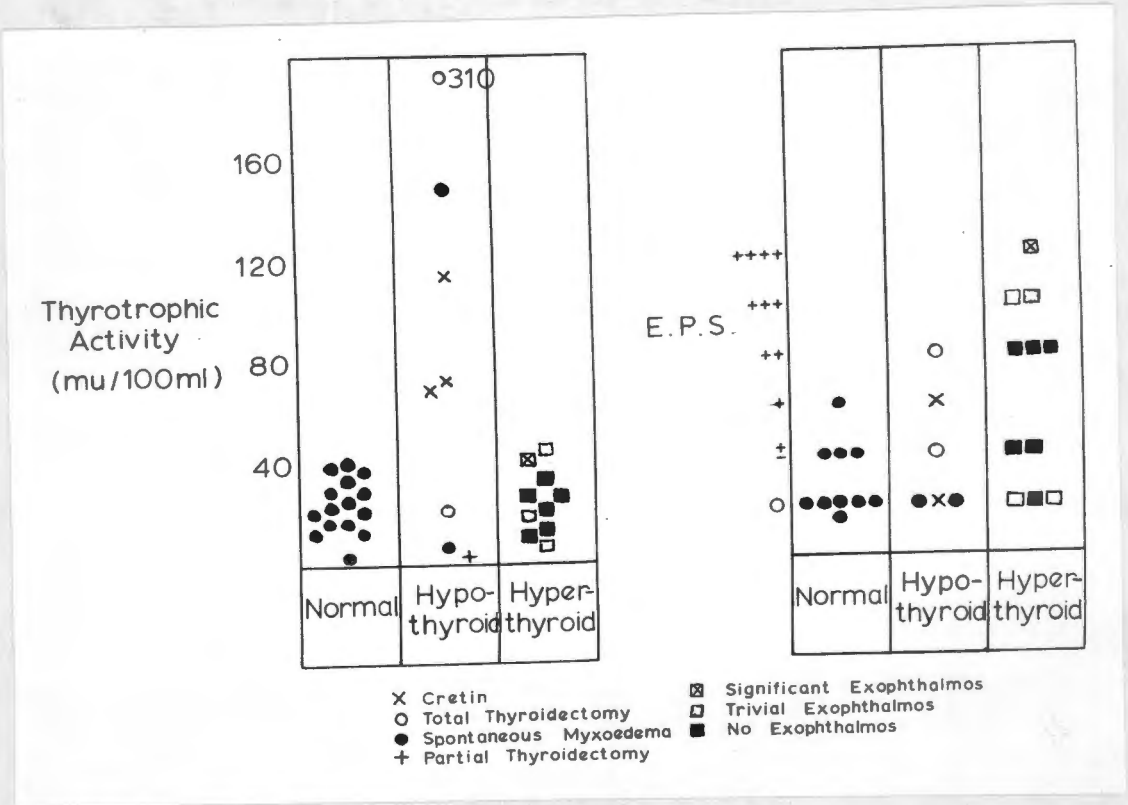


FIGURE 17: Activity of thyrotrophin and E.P.S. in plasma from normal subjects and those suffering from hypothyroid and hyperthyroid states.

CHAPTER IX

Activity of Thyrotrophin and E.P.S. in the Plasma of Patients with various Endocrine disorders

(1) ACTIVITY OF THYROTROPHIN AND E.P.S. IN EUTHYROID PATIENTS WITH GOITRE

The assay of thyrotrophic and E.P.S. activity in patients with goitre has not been the subject of many reports. Munro found no significant thyrotrophin levels in three cases of simple goitre[162], and Fregola and Ferrara reported no activity in cases of endemic goitre although the sensitivity of their method was not stated[101].

As the mechanism underlying the development of non-toxic goitre remains obscure, thyrotrophic activity was assayed in this study in 14 persons with diffuse goitre, 6 with multinodular goitre, one with a solitary nodule and one with a huge goitre, possibly associated with an enzymic block. All subjects were euthyroid as assessed clinically and by the usual laboratory investigations. The results are tabulated in Table X.

Patient	Sex	Age	Goitre	Thyrotrophin (mu./100 ml.)	E.P.S.	Remarks
1. C.G.	F	41	Diffuse	56 (45 - 72)		I^{131} uptakes 32, 51%. RBC uptake 19.6%
2. N.C.	F	30	Diffuse	28 (23 - 36)	0	I^{131} uptakes 36, 50%. RBC uptake 13.4%
3. J.M.	F	39	Diffuse	18 (14 - 26)		I^{131} uptakes 41, 61%. RBC uptake 18.9%
4. L.S.	F	17	Diffuse	34 (30 - 41)	±	I^{131} uptakes 60, 64 ⟶ 21, 25% after T3 RBC uptake 12.5%
5. E.F.	F	38	Diffuse	17 (12 - 25)	+++	I^{131} uptakes 58, 36 ⟶ 25, 28% after T3 RBC uptake 13.7%
6. S.J.	F	39	Diffuse	34 (28 - 44)		I^{131} uptakes 34, 44%. RBC uptake 20.1%
7. D.M.	F	30	Diffuse	17 (13 - 24)	0	I^{131} uptakes 57, 68 ⟶ 28, 34% after T3 RBC uptake 13.4%
8. D.G.	F	34	Diffuse	0		I^{131} uptakes 58, 72 ⟶ 33, 40% after T3 RBC uptake 15.8%
9. P.P.	F	27	Diffuse	16 (11 - 24)		I^{131} uptakes 41, 52 ⟶ 16, 14% after T3 RBC 14.3%
10. M.A.	F	29	Diffuse	25 (20 - 34)		I^{131} uptakes 26, 42%. RBC uptake 17.5%
11. J.A.	F	27	Diffuse	13 (10 - 18)		I^{131} uptakes 19, 21%. RBC uptake 21.7%
12. L.V.	M	32	Diffuse	20 (15 - 27)		I^{131} uptakes 50, 60 ⟶ 15, 24% RBC uptake 17.1%
13. J.K.	F	31	Diffuse	25 (21 - 32)		I^{131} uptakes 39, 57 ⟶ 20% after T3 RBC uptake 16.3%
14. J.E.	F	33	Diffuse	18 (16 - 23)		I^{131} uptakes 27, 38%. RBC uptake 14.9%
15. E.Z.	F	29	Multinod.	31 (27 - 37)	±	I^{131} uptakes 40, 50%. Clinically euthyroid
16. R.H.	F	59	Multinod.	20 (17 - 25)	0	I^{131} uptakes 33, 43%
17. C.C.	F	73	Multinod.	23 (20 - 29)		I^{131} uptakes 19, 31% Retrosternal extension
18. A.C.	F	42	Multinod.	14 (12 - 18)		I^{131} uptakes 43, 56 ⟶ 23, 23% after T3 RBC uptake 17.3%
19. E.P.	F	61	Multinod.	16 (11 - 23)		I^{131} uptakes 37, 56%. RBC uptake 10.2% Retrosternal extension
20. S.L.	F	48	Multinod	21 (17 - 29)		I^{131} uptakes 37, 41%. RBC uptake 15.0%
21. M.F.	F	40	Single nodule	8 (0 - 21)		I^{131} uptakes 18, 32%. RBC uptake 14.6%
22. D.H.	M	16	? Enzymic defect	16 (11 - 24)		I^{131} uptakes 67, 78 ⟶ 30, 45% after T3 RBC uptake 14.4%. PBI 1.5 µg./100 ml. Alb.4.4G. Glob.3.1G. Liver function tests: normal.

TABLE X. Thyrotrophin activity in euthyroid subjects with goitre.

Comment: In 14 subjects with diffuse thyroid enlargement, the mean thyrotrophin level is 23 mu./100 ml. and only one shows thyrotrophic activity outside the normal range (56 mu./100 ml.). One has greatly increased E.P.S. activity, while in three others, E.P.S. is insignificantly increased or absent.

The mean thyrotrophin level in six patients with multinodular goitre is 21 mu./100 ml. All readings are within the normal range. Plasma from two cases has been assayed for E.P.S., which is not present at significant levels.

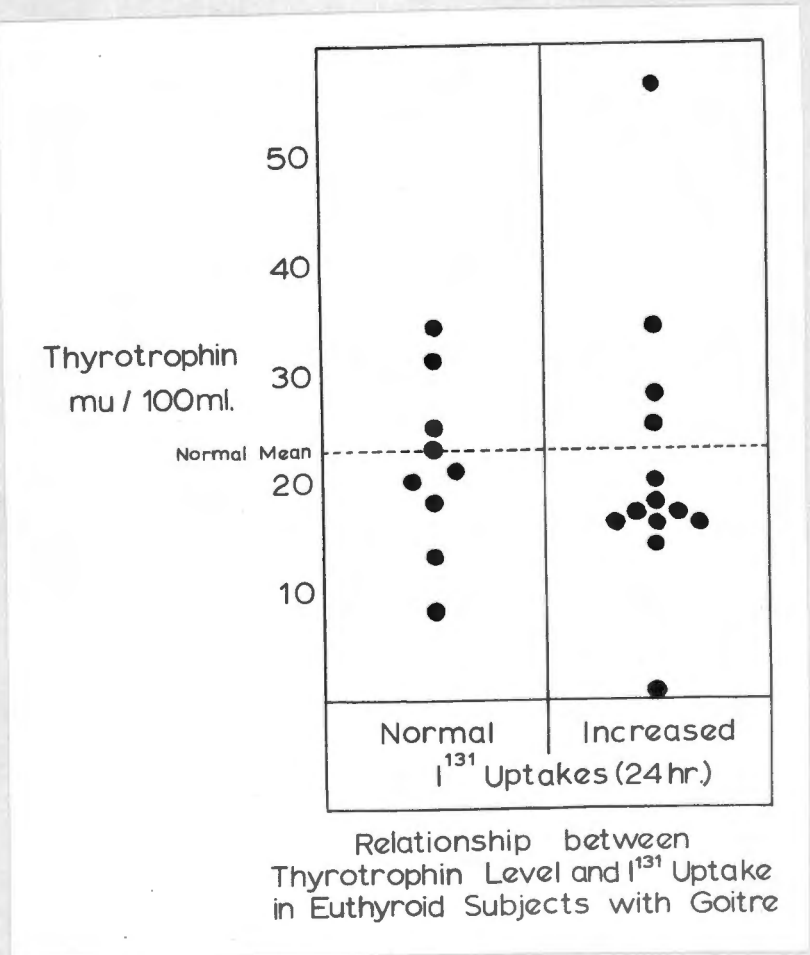
Both the patient with a single thyroid nodule and the one with a probable thyroid enzymic defect have normal levels of thyrotrophin.

Euthyroid goitrous states do not appear to be associated with increased thyrotrophic activity therefore and this suggests that an increased secretion of thyrotrophin is unlikely to be directly linked in the pathogenesis of these conditions. It must be stressed once more that what has been assayed is thyrotrophin-like activity, and that the presence of antagonists or anti-hormones in the plasma of such subjects cannot be excluded.

The presence of E.P.S. activity in one euthyroid patient with a diffuse goitre is not lightly explained. That this may be the first indication of the impending development of hyperthyroidism and exophthalmos, is very remote. Dobyns, in his extensive experience, has occasionally noted exophthalmic activity in the individual serum from a normal subject, and feels that this may represent a lack of specificity on the part of the assay as pooled sera invariably failed to elicit such responses[86]. The latter reason is more acceptable to explain the E.P.S. activity found in the single case mentioned above.

The relationship of the thyrotrophin level to I^{131} uptake in euthyroid goitrous subjects:

Amongst the 22 cases summarised in Table X are 9 in which an increased thyroidal uptake of I^{131} was found. In each case, this was suppressed by tri-iodothyronine. The comparison between thyrotrophin levels in the high and normal I^{131} uptake groups is shown below:-



Relationship between Thyrotrophin Level and I^{131} Uptake in Euthyroid Subjects with Goitre

FIGURE 18: The relationship of the thyrotrophin level to I^{131} uptake in euthyroid subjects with goitre.

Comment: In both groups, results adhere fairly closely to the mean normal level. The tendency of the results to be slightly lower in the group with raised I^{131} uptakes is not significant. The presence of a raised I^{131} uptake in certain euthyroid patients with goitre does not appear to be associated with an increased thyrotrophic activity as measured by this assay.

(2) ACTIVITY OF THYROTROPHIN AND E.P.S. IN PATIENTS WITH HYPOPITUITARY FUNCTION:

Gilliland and Strudwick[107] and D'Angelo[75] have reported negative assays for thyrotrophin in hypopituitrism, as would be expected. Two such cases were assayed in this study and another assay was performed on the plasma from a patient after partial hypophysectomy for a chromophobe adenoma.

Results are summarised in Table XI

Patient	Sex	Age	Thyrotrophin (mu./100 ml.)	Remarks
1. J.J.	F	70	0	Classical hypopituitarism following post-partum haemorrhage years before. Grossly myxoedematous. Death, from intercurrent pneumonia, before I^{131} studies performed. Autopsy: Atrophied pituitary and fibrosed thyroid.
2. J.L.	F	50	0	Hypopituitarism following post-partum haemorrhage. Classical clinical features. I^{131} uptakes 6, 4%. RBC uptake 12%.
3. E.W.	F	38	17 (3-40)	Chromophobe adenoma removed six weeks before assay. Considered to be hypopituitary clinically but thyroid and adrenal function normal. I^{131} uptakes 34, 28%. RBC uptake 15.2%

TABLE XI. Thyrotrophic activity in patients with definite and suspected hypopituitary function.

Comment: In the two cases with undoubted hypopituitary function, no thyrotrophin was assayed, in keeping with the clinical findings. The third patient was very doubtfully hypopituitary, as normal I^{131} uptakes, urinary ketosteroid, hydroxycorticosteroid and gonadotrophin excretion were found. It is therefore not surprising that a normal thyrotrophin level was assayed from the plasma.

(3) ACTIVITY OF THYROTROPHIN, L.A.T.S. AND E.P.S. IN ACROMEGALY:

Only one report on thyrotrophic activity in acromegaly has appeared in the literature. D'Angelo found levels up to eight times the normal value in three untreated cases[75]. It is surprising that these results have never been confirmed, as the association between acromegaly and goitre is well documented[76], while cases of hyperthyroidism co-existing with acromegaly are known[76, 149].

For this reason, plasma from three untreated cases of acromegaly was assayed for thyrotrophin, L.A.T.S. and E.P.S. The results are tabulated below:-

Patient	Age	Sex	Mean % Increase Radioactivity 2 Hr.	7 Hr.	Response Ratio	E.P.S. Assay	Thyrotrophin Level (mu./100 ml. at 2 Hr.	Remarks
1. K.B.	35	M	135±16	156±25	1.16 Inconclusive t = 0.70 p not significant	0	16(0 - 42)	Classical acromegaly. I^{131} uptake 26%. RBC uptake 11.5%
2. D.A.	17	M	174±10	197±12	1.13 Suggestive ($t = 1.497$ $0.1 < p < 0.2$)	+	20(14 - 30)	Gigantism. I^{131} uptakes 35, 61% RBC uptake 22.2%
3. KvV.	39	M	160±24	146±22	0.91 Absent	0	17(6 - 35)	Active acromegaly. I^{131} uptakes 17, 33%

TABLE XII. E.P.S., L.A.T.S. and Thyrotrophin in Acromegaly.

Comment: As would be expected, no E.P.S. is demonstrable in two cases nor are the thyrotrophin levels excessive. However, L.A.T.S. and E.P.S. are probably present in one case (D.A.) This very interesting finding suggests that L.A.T.S. activity (and not thyrotrophin) may have been assayed in this condition by D'Angelo.

A possible aetiological connection between L.A.T.S. and Graves' disease has already been discussed. It is tempting to invoke that theory to link this result and the clinical association of acromegaly and hyperthyroidism just mentioned. Patient D.A. fits rather neatly into the concept, as the I^{131} uptakes are at high normal levels, the RBC uptake of I^{131} is distinctly raised (22.2%), and E.P.S. is present. The thyrotrophin-like action of L.A.T.S. may be responsible for the raised I^{131} uptake in this case. L.A.T.S. activity is unlikely to be a non-specific effect of growth hormone, as crude extracts of human, rat and bovine pituitaries containing this hormone have not been shown to give rise to the "delayed" response characteristic of L.A.T.S.[5].

The source of the L.A.T.S. in acromegaly is as obscure as in hyperthyroidism. It is logical to incriminate an anterior pituitary adenoma in this respect, but L.A.T.S. has never been isolated from beef pituitary or that of the human in hyperthyroidism, even when found in the blood at high levels[160,163].

(4) THYROTROPHIC ACTIVITY IN THYROID CARCINOMA AND SUBACUTE THYROIDITIS:

No reports have appeared in the literature regarding thyrotrophin levels in thyroid carcinoma. As metastasising thyroid tumours in rats have been found to be thyrotrophin-dependent occasionally[174,176] and a therapeutic

effect has been reported from the administration of thyroid hormone to patients with thyroid carcinoma[14,33,145,212], information regarding thyrotrophic activity would be of great interest. Two patients with untreated thyroid carcinoma were seen during the period of this study and assays were undertaken.

Acute thyroiditis has also received no mention in the literature. One such case was studied; and the result, together with those found in the patients with carcinoma, is shown in the following table.

Patient	Age	Sex	Thyrotrophin (mu./100 ml.)	Remarks
1. J.H.	62	F	20 (8 - 39)	Hurthle cell tumour with cervical metastases. I^{131} uptakes 9, 17%
2. R.H.	63	M	17 (12 - 25)	Adenocarcinoma. No metastases. I^{131} uptakes 31, 27%
3. C.W.	48	M	75 (60 - 99)	Acute thyroiditis, responding well to corticosteriods subsequent to the assay. I^{131} uptakes 39, 46%. RBC uptake 19.8%

TABLE XIII. Thyrotrophic activity in thyroid carcinoma and thyroiditis.

Comment: (1) In cases 1 and 2, thyrotrophic activity is in the normal range and a quantitative disturbance of thyrotrophin secretion has not been demonstrated in the two patients with thyroid carcinoma.

(2) Thyrotrophic activity is increased in case 3. Thyroiditis was acute and systemic symptoms were present at the time of assay. Naturally, no conclusion can be drawn from one case. Circulating antibodies which are known to occur in the disease may have interfered in some way with the assay and the result may not be a true reflection of the serum thyrotrophin level.

(5) THYROTROPHIC AND E.P.S. ACTIVITY IN HASHIMOTO'S DISEASE:

No data is available regarding the activity of these two substances in Hashimoto's disease. These were assayed in three patients and the results follow in Table XIV:

Patient	Sex	Age	Thyrotrophic Activity (mu./100 ml.)	E.P.S. Assay	Remarks
1. J.S.	F	47	14 (10 - 21)	0	Thyroid clinically typical of Hashimoto's disease. PBI 5.0 µg.% I^{131} uptakes 54,92 → 15,19% after T3. Normal flocculation tests and serum proteins.
2. L.M.	F	36	0	0	Clinical diagnosis only. PBI 8.0 µg.% I^{131} uptakes 44,58 → 38,44% after T3. RBC uptake 24% Normal flocculation tests and serum proteins.
3. W.N.	M	63	0 (0 - 3)	0	Diagnosis confirmed histologically. I^{131} uptakes 9.0, 12.9% RBC uptake 16.6%

TABLE XIV. Thyrotrophic activity in Hashimoto's disease.

Comment: Two patients have no thyrotrophic activity, and the remaining patient shows low normal levels of thyrotrophin. None of the three patients was clinically hypothyroid, although the I^{131} uptakes were low in case 3.

The results are difficult to interpret, especially as the series of cases is very small. These may just be chance findings. Alternatively, they may be related in some way to the auto-immune process which occurs in this state.

Apart from the presence of thyroid auto-antibodies, a serum thyroid cytotoxic factor has been demonstrated[172, 187]. This substance may be interfering with the assay by causing necrosis of the mouse thyroid gland resulting in haphazard and perhaps rapid release of radio-iodine which may be excreted before the two hour blood sampling.

No E.P.S. activity was found in the three cases.

(6) ACTIVITY OF L.A.T.S. AND E.P.S. IN PROBABLE EUTHYROID PATIENTS WITH EYE SIGNS OF THYROTOXICOSIS:

Three patients were seen at the Endocrine Clinic, Groote Schuur Hospital, with symptoms slightly suggestive of hyperthyroidism. All had marked lid-lag and lid retraction and slight exophthalmos, but investigations, follow up or therapeutic trial failed to confirm the suspected hyperthyroid state. E.P.S. and L.A.T.S. were assayed and the results are tabulated in Table XV.

Patient	Sex	Age	Mean % Increase in Radioactivity 2 hr.	7 hr.	Response Ratio	E.P.S. Assay	Remarks
1. E.R.	M	38	125±11	175±21	1.40 ? Significant ($t = 2.08$, $0.05 < p < 0.1$)	0	No heat intolerance. Cool, dry palms. Pulse 100/min. Bilateral exophthalmos. Marked L.L., L.R.(x) I^{131} uptakes 60, 68 → 57, 65% RBC uptake 16%. PBI 6 µg.%. Followed up for two years during which time adequate methimazole therapy for 3 months failed.
2. S.S.	F	16	134±20	165±23	1.23 Inconclusive (p not significant.)	+	Nervous, sweaty. Pulse 82/min. Small diffuse goitre. Slight proptosis. L.L., L.R. marked on right side. I^{131} uptakes 18, 36%. RBC uptake 19.6%.
3. S.F.	F	30	160±22	186±31	1.11 Inconclusive (p not significant.)	not done.	2 pregnancies appeared hyperkinetic with marked L.L., L.R., some exophthalmos. Following pregnancy this subsided. I^{131} uptakes 87,97 → 51,74% after T3. RBC uptake 14.7%. Followed up 5-6 years and although marked L.L. and L.R. present, definitely no hyperthyroidism has developed.

TABLE XV. L.A.T.S. and E.P.S. activity in euthyroid subjects with eye sign of thyrotoxicosis.

(x) L.L., L.R. refer to lid-lag and lid retraction respectively.

Comment: All 3 patients had a positive response-ratio, although only Case 1 elicited a 7 hour response significantly different to the 2 hour reading.

The status of these patients is uncertain. They have the eye signs of hyperthyroidism but other clinical features only partly suggest the diagnosis. Laboratory tests were not confirmatory and Cases 1 and 3, followed up over years, did not develop this state, nor did Case 1 respond to an adequate course of methimazole.

Evidence of L.A.T.S. activity is interesting in view of the prominence of the eye signs and lends support to the theory that a closer relationship exists between this substance and exophthalmos than between it and hyperthyroidism. Alternatively, this could be evidence for an aetiological connection with Graves' disease, either anteceding the onset of the illness by months or years or not. Such a concept has been put forward by Werner[224b] who cited ten similar cases. Only prolonged follow up of these patients will confirm or refute this interesting possibility.

(7) ACTIVITY OF L.A.T.S. AND E.P.S. IN THE SYNDROME OF PRETIBIAL MYXOEDEMA AND EXOPHTHALMOS:

L.A.T.S. has been found consistently in sera of euthyroid patients with malignant exophthalmos[5, 160, 162], even without antecedent hyperthyroidism [160, 162]. This is a possible explanation for the high levels of "thyrotrophin" assayed in this condition by earlier workers[75, 107, 175, 185]. In pretibial myxoedema, greatly increased thyrotrophic activity has been found in serum[185] and urine[219], but no reports of L.A.T.S. assays have appeared. As L.A.T.S. activity seemed likely, assays were undertaken on plasma samples from three subjects for this study. E.P.S. was concurrently estimated.

Albert found E.P.S. activity in the muco-polysaccharide substance which accumulates in the retro-orbital space in exophthalmos[18]. In view of this, L.A.T.S. and E.P.S. activity was assayed in homogenised samples of pretibial myxoedematous tissue.

Such tissue was biopsied, washed clean of blood, dried as much as was possible, weighed and homogenised in saline by a hand homogeniser, to give a final concentration of 5 mg. tissue / ml. saline. Homogenates were centrifuged and the supernatant assayed. The results were a response to 0.5 ml. of the supernatant fluid i.e. 2.5 mg. tissue.

After the responses of the first case, it was realised that control homogenates should be used. This was done subsequently with subcutaneous fat removed from a site distant from the pretibial myxoedema and homogenised and prepared in the same way. The results are tabulated in Table XVI.

Patient	Age	Sex	Mean % Increase in Radioactivity	2 hr.	7 hr.	Response Ratio	E.P.S. Assay	Remarks
1. J.S.	42	M	Plasma	283±24	329±27	1.16 ? Absent ($t = 1.279$ p not significant.)	0	No evidence, past or present, of hyperthyroidism. Active progressive exophthalmos. Warm, tender pretibial myxoedema. Acropachy. I^{131} uptakes 23, 43%. RBC uptake 17%. Pretibial myxoedema confirmed histologically.
			Pretib. Myx.	158±13	213±25	1.29 ? Significant ($t = 1.964$ $0.05 < p < 0.1$)	±	
2. H.F.	40	F	Plasma	810±108	1904±164	2.35 Significant ($t = 5.582$ $p < 0.01$)	0	No exophthalmos. Questionable hyperthyroidism one year previously. Pretibial myxoedema; L.L., L.R. Acropachy. Firm thyroid. (Histology Hashimoto's disease). Abnormal flocculation tests. Serum precipitins negative. I^{131} uptakes 97,75 → 92,56% after T3. PBI 2.75 µg.%. On thyroxine 0.1 mg. bd.
			Pretib. Myx.	131±15	243±34	1.86 Significant ($t = 3.027$ $p < 0.02$)	+	
			Fat	116± 7	150±21	1.30 Suggestive ($t = 1.545$ $0.1 < p < 0.2$)	0	
3. E.J.	46	F	Plasma	504±40	1093±203	2.17 Significant ($t = 2.845$ $p < 0.02$)	+++	Thyrotoxicosis 10 years previously. Exophthalmos followed thyroidectomy one year later. Pretibial myxoedema 18 months. Mildly myxoedematous. Off thyroxine 2 weeks before assay
			Pretib. Myx.	315±52	364±95	1.16 ? Absent ($t = 0.4537$ p not significant.)	+	
			Fat	94±11	76±11	0.81 Absent	++	

TABLE XVI. L.A.T.S. and E.P.S. in pretibial myxoedema.

Comment: Enormous levels of L.A.T.S. are present in the plasma of two of the three patients, and some late activity is possibly present in the third. It appears that concentrations of L.A.T.S. of this degree are associated strongly with the development of pretibial myxoedema.

Late activity was likewise found in two of these samples of pretibial myxoedema, but to a considerably lesser degree than in the plasma. A positive response, but of no significance, was obtained in the third case. In view of the very high plasma figures, the possibility of trapped plasma must be considered as a cause of this high response, but the significantly lesser response from the apparently equally vascular subcutaneous fat would invalidate this suggestion.

The finding of such L.A.T.S. activity in pretibial myxoedema raises interesting speculation as to its aetiology.

E.P.S. levels were inconsistent and in the first two cases did not parallel L.A.T.S., though some activity was present in the pretibial myxoedematous tissue in each case. Case 3 had +++ activity in the serum, and somewhat less in the tissues, but its presence in the otherwise inert fat raises a possibility of a false positive reaction.

(8) ASSAY OF L.A.T.S. AND E.P.S. IN MALIGNANT EXOPHTHALMOS FOLLOWING THYROIDECTOMY:

The presence of large amounts of L.A.T.S. in malignant exophthalmos is well documented[5, 160, 162]. The majority of patients either suffered from a mild degree of hyperthyroidism or had been treated for this condition, although occasional cases without such a preceding history have been reported[160, 162].

E.P.S., too, has been assayed in exophthalmic patients with great frequency, irrespective of thyroid function[86, 150].

L.A.T.S. and E.P.S. assays were performed on two euthyroid patients each of whom had undergone two thyroidectomies for recurrent thyrotoxicosis during the preceding 15 years. The results are shown in Table XVII.

Patient	Sex	Age	Mean % Increase in Radioactivity 2 hr.	7 hr.	Response Ratio	E.P.S. Assay	Remarks
1. J.M.	F	53	195±23	278±32	1.43 ? Significant ($t = 2.1298$ $0.05 < p < 0.1$)	++	Thyroidectomy twice in 16 years for recurrent hyperthyroidism. Gross exophthalmos with visual impairment, static for 6 years.
2. A.L.	F	54	146±20	185±25	1.27 ? Absent ($t = 1.279$ p not significant.)	0	Thyroidectomy twice in 10 years for recurrent hyperthyroidism. Euthyroid at present, with severe exophthalmos. I^{131} uptakes 25, 30%. RBC uptake 22.0%.

TABLE XVII. E.P.S. and L.A.T.S. in malignant exophthalmos.

Comment: In both cases, L.A.T.S. activity is demonstrated although this may not be significant in Case 2, and this conforms to the reported incidence. The type of clinical background present would appear to provide the ideal setting for the development of exophthalmos, and certainly the highest levels of L.A.T.S. appear to have been found under these conditions[5, 160, 163]. E.P.S. was found in the subject with the greatest L.A.T.S. activity only.

THE RELATIONSHIP BETWEEN L.A.T.S. AND E.P.S.

The association between the presence of L.A.T.S. and clinical exophthalmos already discussed and shown in this study (Chapters VIII and IX) has led to speculation as to whether L.A.T.S. may be identical with E.P.S., and whether two entirely different assay techniques are, in fact, measuring the same substance. Unlike thyrotrophin, both L.A.T.S. and E.P.S. are stable if stored in a frozen state[86, 150, 162].

Undoubted differences exist however. E.P.S. is always found in the thyrotrophin fraction of anterior pituitary extracts[16,17,18,80,167], whereas the anterior pituitary glands of three patients with the hyperthyroidism-exophthalmos complex have been found to contain no L.A.T.S.[160,163], although this had been present in the serum of one patient during life[160]. Furthermore, four patients who developed Graves' disease after surgical ablation of the pituitary for various reasons had L.A.T.S. activity in their serum[160]. The site of origin of these two substances is therefore dissimilar.

To settle this issue, collaborative bioassays for both these substances, performed on a single aliquot of plasma and reported in this and the previous chapter, are reviewed in Table XVIII.

Patient	Age	Sex	L.A.T.S. % increase radioactivity 2 hr.	7 hr.	Response ratio	E.P.S.	Remarks
1. G.D.	42	F	158 ± 19	245 ± 36	1.55	++++	Hyperthyroid. Severe exophthalmos.
2. J.T.	25	F	145 ± 13	149 ± 13	1.03	0	Hyperthyroid. No exophthalmos.
3. L.G.	31	F	117 ± 11	161 ± 15	1.38	0	Hyperthyroid. Slight exophthalmos.
4. K.B.	35	M	135 ± 16	156 ± 25	1.16	0	Acromegaly, untreated.
5. E.R.	38	M	125 ± 11	175 ± 21	1.40	0	Euthyroid - eye signs.
6. H.F.	40	F	plasma 810 ± 108	1904 ± 164	2.35	0	Pretibial myxoedema with exophthalmos.
			myx. 131 ± 15	243 ± 34	1.86	+	Euthyroid.
			fat 116 ± 7	150 ± 21	1.30	0	
7. J.S.	42	M	plasma 283 ± 24	329 ± 27	1.16	0	Pretibial myxoedema with exophthalmos
			myx. 158 ± 13	213 ± 25	1.29	±	Euthyroid.
8. E.J.	46	F	plasma 504 ± 40	1093 ± 203	2.17	+++	Pretibial myxoedema with exophthalmos
			myx. 315 ± 52	364 ± 95	1.16	+	Slightly hypothyroid.
			fat 94 ± 11	76 ± 11	0.81	++	
9. J.M.	53	F	195 ± 23	278 ± 32	1.43	++	Malignant exophthalmos. Euthyroid. Recurrent hyperthyroidism - partial thyroidectomy twice.
10. A.L.	54	F	146 ± 20	185 ± 25	1.27	0	Malignant exophthalmos, following 2 thyroidectomies for recurrent hyperthyroidism. Euthyroid when tested.
11. M.N.	49	F	109 ± 16	167 ± 22	1.53	++	Hyperthyroid. Slight exophthalmos. Marked L.L., L.R.
12. J.P.	26	F	102 ± 16	140 ± 6	1.37	+++	Hyperthyroid. No exophthalmos.
13. L.A.	36	F	126 ± 9	152 ± 14	1.21	++	Hyperthyroid. No exophthalmos.
14. D.A.	17	M	174 ± 10	197 ± 12	1.13	+	Gigantism, untreated.
15. S.S.	16	F	134 ± 20	165 ± 23	1.23	+	Euthyroid - eye signs.
16. M.M.	54	F	189 ± 13	195 ± 13	1.03	±	Hyperthyroid. No exophthalmos.

TABLE XVIII. The results of 21 collaborative bioassays for E.P.S. and L.A.T.S. on samples of plasma or tissue homogenates.

Comment: On 21 occasions, collaborative bioassays were performed. All except one of the eleven plasma or homogenate specimens which yielded a positive (+ to ++++) E.P.S. assay had significant L.A.T.S. activity. In fact, higher levels of E.P.S. tended to correlate with increased amounts of L.A.T.S. (cases 1, 9, 11 and plasma of Case 8). Of the remaining ten specimens without significant E.P.S. activity, seven actually contained significant L.A.T.S. levels (Cases 3, 5, 10, plasma and fat homogenate of Case 6 and plasma and homogenate of pretibial myxoedema of Case 7). The plasma of H.F. (Case 6) which had tremendous L.A.T.S. activity was strikingly lacking in E.P.S.

Three samples contained neither L.A.T.S. nor E.P.S., while an homogenate of fat of Case 8, used as a control, showed no L.A.T.S. or thyrotrophic activity, yet had ++ E.P.S. As has been mentioned, this last observation is of doubtful significance as occasional false positive E.P.S. assays are reported.

Six patients were severely exophthalmic. The plasma of all contained greatly increased amounts of L.A.T.S. (in five instances, readings of $\pm$ 250% or higher are present - Cases 1, 6, 7, 8 and 9). In only three of these was E.P.S. found, but on each of these occasions, at high levels.

At times, therefore, a correlation between E.P.S. and L.A.T.S. appears to exist; but at others, a marked discrepancy was demonstrated (at a time when the fish used in the E.P.S. assays were responding well). This suggests that though both factors are related in their associations, this is not absolute, and they are probably different substances. As a generalisation, L.A.T.S. was found to parallel the clinical state more closely than E.P.S. levels (this will be further shown in Chapter X), and this may be the more important factor in the genesis of the puzzling symptom complex of endocrine exophthalmos.

On the other hand, the relative failure to correlate E.P.S. with the clinical status of the patients in this study may reflect on the accuracy and sensitivity of the method, rather than on its actual presence in the plasma. It is possible that anterior pituitary E.P.S. levels may be more pertinent to the clinical picture as it is not unreasonable to postulate that it may mediate its effects by another humoral mechanism, possibly even L.A.T.S.

SUMMARY OF L.A.T.S. ACTIVITY IN ENDOCRINE DISORDERS:

The assays for the presence of L.A.T.S. performed during this study are graphically represented below. The highest results are seen in pretibial myxoedema with exophthalmos, malignant exophthalmos in euthyroid subjects and "hyperophthalmopathic" Graves' disease. Similarly elevated response ratios are seen in these states. This suggests a very strong association with exophthalmos.

Two dose levels of thyrotrophin standard, as well as plasma from an acromegalic and a myxoedematous subject showed a negative response ratio suggestive of "early" or true thyrotrophic activity.

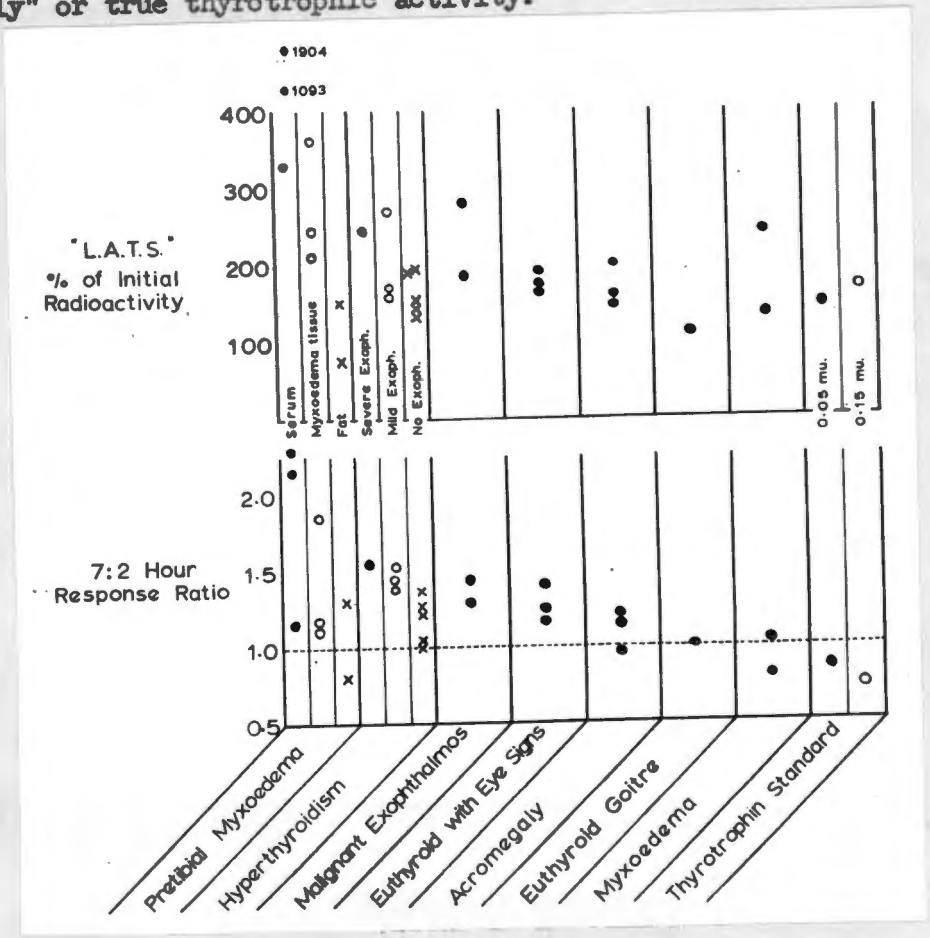


FIGURE 19: L.A.T.S. assays in endocrine disorders, normal subjects and thyrotrophin standard.

SUMMARY OF E.P.S. ACTIVITY IN ENDOCRINE DISORDERS:

Assays for E.P.S. performed in this study are shown in Figure 20. Those results found in hyperthyroidism and myxoedema have been excluded (see Fig. 17). Significant levels were found in pretibial myxoedema and malignant exophthalmos in euthyroid subjects, though with much less consistency compared to L.A.T.S. The other endocrinopathies yielded insignificant results apart from the single positive assay in an acromegalic. This has been commented on already.

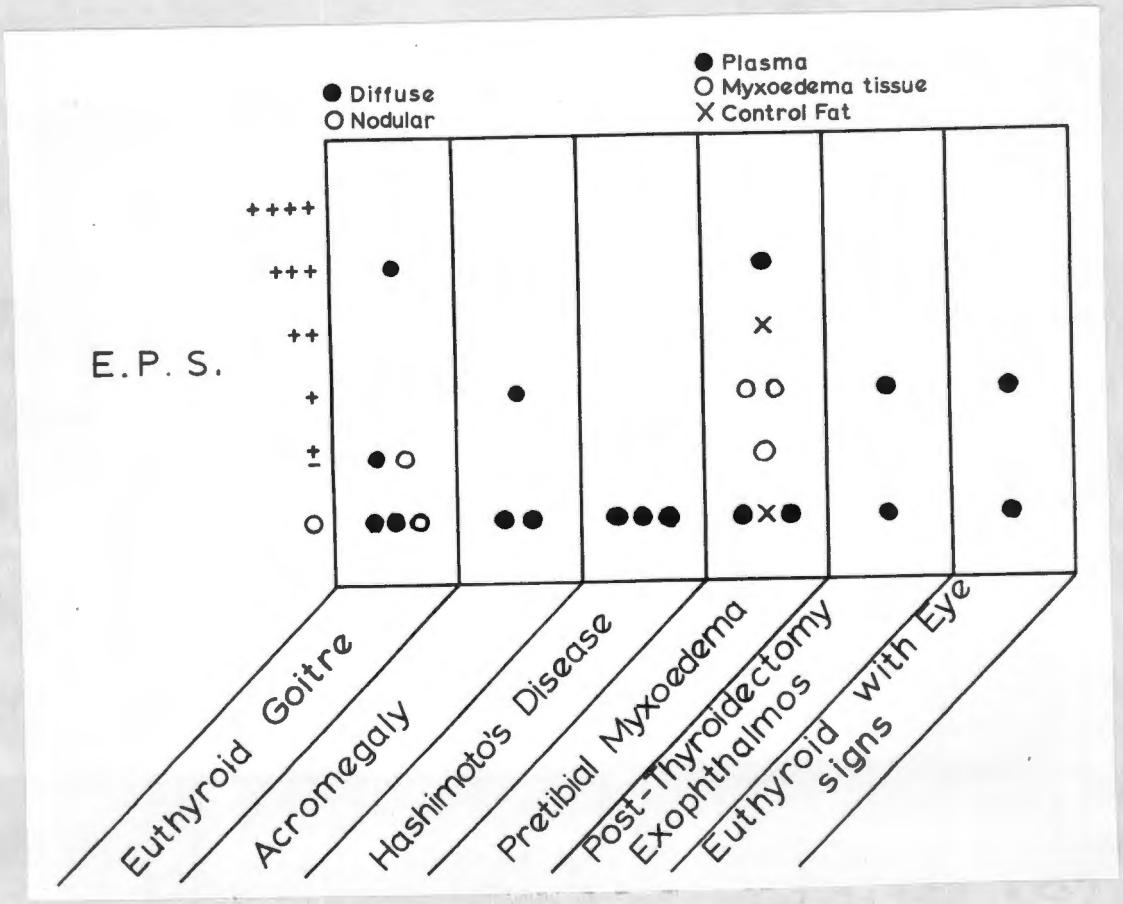


FIGURE 20: E.P.S. assays in endocrine disorders.

CHAPTER X

Serial Assays of E.P.S. and L.A.T.S. in relation to the therapy of hyperthyroidism and exophthalmos

As the correlation of the course and progression of exophthalmos with E.P.S. and, more especially, L.A.T.S. levels is a close one, serial assays of these substances would be valuable in the investigation of the effects of the various drugs used in the treatment of hyperthyroidism and exophthalmos. Antithyroid drugs are reported to precipitate or aggravate exophthalmos when used in the treatment of thyrotoxicosis[83], but this remains a clinical impression, rather than a scientific observation.

The status of medical treatment of severe exophthalmos is likewise arbitrary. Claims have been made in favour of oestrogens[219] and for[123,132] and against[161] corticosteroids, while thyroxine remains the traditional treatment; but adequately controlled trials are sparsely reported, and assessment in individual cases is notoriously difficult, because of the great tendency for the disease to fluctuate spontaneously.

As a correlation between the clinical results of treatment and serial levels of L.A.T.S. and E.P.S. would make assessment of these effects more objective and less of a "clinical impression", such estimations were carried out under a variety of conditions, in an attempt to confirm or refute the therapeutic claims indicated above.

Assays were serially performed under four sets of conditions:-

(1) E.P.S. was assayed during the conventional treatment of a severely thyrotoxic patient with propylthiouracil and thyroxine.

(2) A patient suffering from acute pretibial myxoedema and severe progressive exophthalmos was given massive doses of dexamethasone. The progress was followed both clinically and by serial bioassays of L.A.T.S. and E.P.S.

(3) L.A.T.S. and E.P.S. were assayed during the treatment of two thyrotoxic patients with methimazole alone.

(4) A thyrotoxic patient receiving methimazole treatment and a euthyroid subject with eye signs of thyrotoxicosis, both of whom had significant L.A.T.S. levels were given thyroxine and followed serially.

The results are reported below:-

(1) <u>Effect of antithyroid drugs and thyroxine on E.P.S. activity in hyperthyroidism.</u>

A 42 year old female suffering from a severe hyperthyroid state with a marked degree of exophthalmos was treated with propylthiouracil 200 mg.tds., and thyroxine 0.1 mg. bd. E.P.S. activity was estimated weekly during a seven week course of treatment, and the results are indicated in Figure 21.

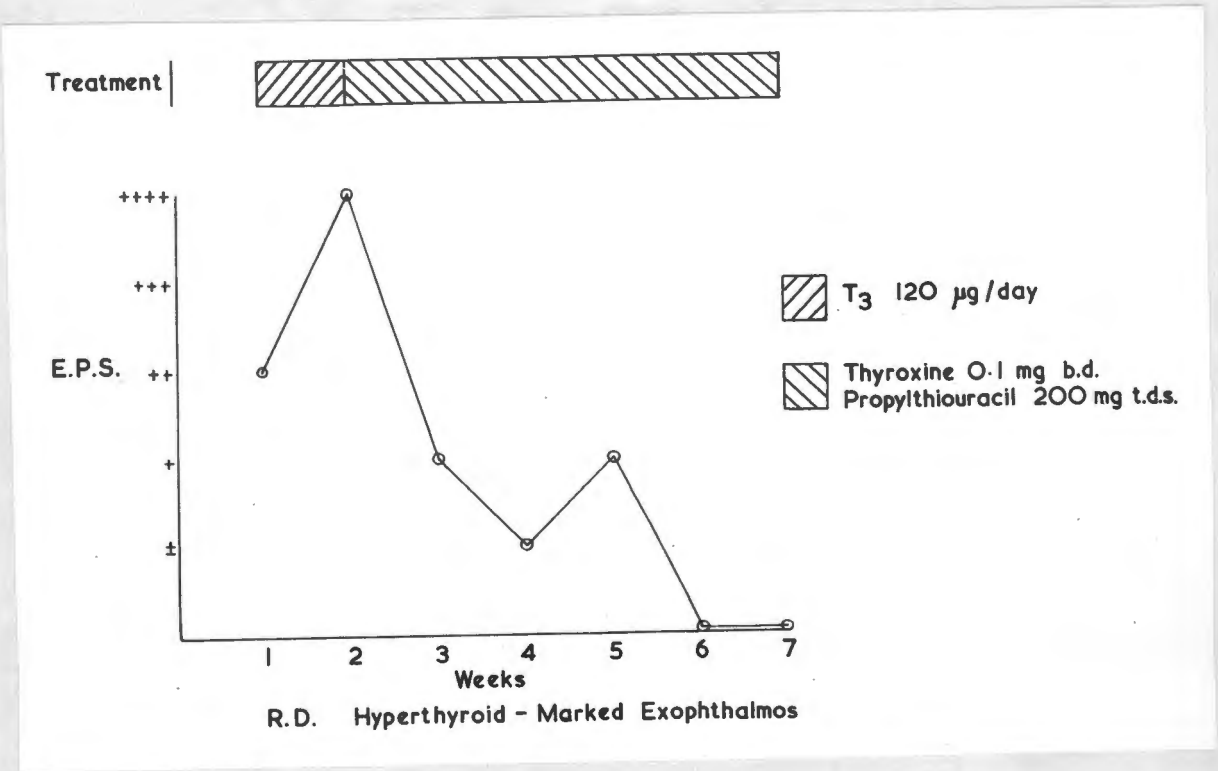


FIGURE 21: E.P.S. assays during therapy for hyperthyroidism.

Comment: An E.P.S. level, which was extremely high, dropped progressively during this treatment until finally no activity was found. Unfortunately exophthalmometry was not performed so that the impression of clinical improvement cannot be assessed objectively. However, the hyperthyroidism had considerably lessened, and at the end of the recorded period, all initial symptoms had abated, while a weight gain, drop in pulse rate and disappearance of the tremor were noted.

This response is somewhat difficult to assess. It is accepted by some that the exophthalmos of the usual case of Graves' disease improves as the thyroid overactivity is brought under control, while in malignant exophthalmos with mild or no hyperthyroidism, eye signs may worsen if treated by antithyroid drugs[123]. In this instance, the diminution of E.P.S. activity which paralleled improvement in "toxicity" suggests that the case falls into the former category. A diminution in E.P.S. activity may possibly be a prognostic pointer as to the direction in which a particular case of Graves' disease is moving.

Although the explanation offered seems most likely in the case reported above, opinion must be guarded in the light of a single set of observations, and more especially as the patient received not only antithyroid drugs but, in addition, thyroxine.

(2) Effect of large doses of corticosteroids in progressive exophthalmos:

A 42 year old male presented with a six week history of progressive swelling of both eyes. Examination revealed gross exophthalmos. (Right and left eyes 24 mm. as measured by a modification of the Luedde exophthalmometer), warm, slightly tender pretibial myxoedema and classical acropachy, with

radiological evidence of periostial reaction. Thyroid and red cell uptakes were in the euthyroid range (23, 43% and 17% respectively), and the pretibial myxoedema was confirmed histologically.

After a single baseline plasma assay of E.P.S. and L.A.T.S., a heavy dose of dexamethasone was commenced. Serial assays were performed during two weeks of therapy, and the results are graphically shown:-

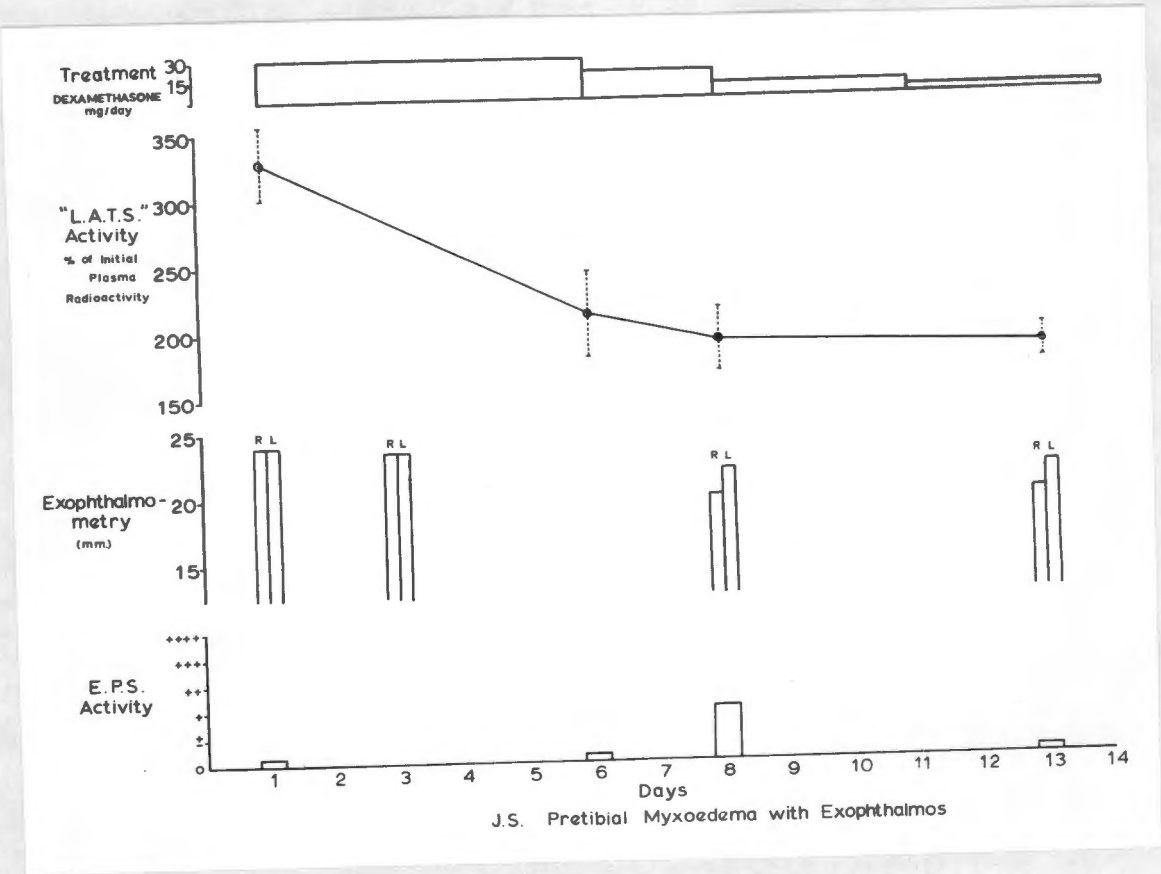


FIGURE 22: Treatment of severe exophthalmos with corticosteroids.

Comment: The response to dexamethosone is striking. Objectively, great reduction in exophthalmos was coupled with a marked subjective improvement, and an associated drop in plasma L.A.T.S. activity.

The correlation between L.A.T.S. levels and exophthalmos has been notable, whereas E.P.S. has shown an inconsistent relationship. This confirms the impression recorded in the previous chapter, and suggests that L.A.T.S. assay may be a useful laboratory confirmation of the clinical trend in an exophthalmic patient.

Of greater interest has been the response to corticosteroids. The reported results have been diverse[59,123,131,132,161], but some workers have felt that failure to elicit an improvement reflects on the inadequacy of the dose rather than that of the drug[123].

Although one must be cautious in interpreting the results of a single case, this appears to support the reports favouring corticosteroids in large doses as treatment of exophthalmos[123,131] and would suggest further controlled trials of this drug.

(3) Effect of methimazole in L.A.T.S. and E.P.S. activity:

Two patients with classical hyperthyroidism and mild exophthalmos, and who had significant levels of L.A,T.S. activity, received methimazole 20 mg. tds. therapeutically, and serial assays for E.P.S. and L.A.T.S. activities were conducted weekly. There was subjective improvement in the general condition and degree of hyperthyroidism in response to treatment in both cases.

One patient, L.G., aged 35, had thyroid I^{131} uptakes of 73, 80% which did not suppress after T3, and red cell uptake of 22.6%. The second subject, L.A., aged 36, had similarly raised thyroid uptakes of 75, 87%, which also did not diminish after T3, with red cell uptake of 27.6%.

The results of the serial assays are shown in the following two figures:-

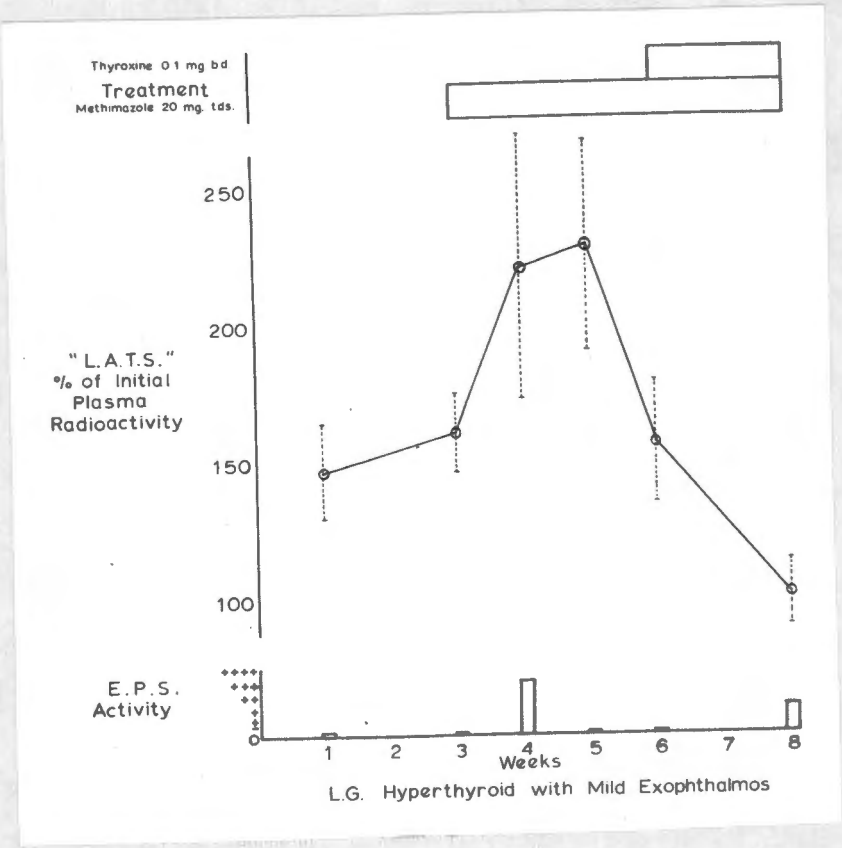


FIGURE 23: Effect of methimazole on L.A.T.S. and E.P.S. activity in hyperthyroidism.

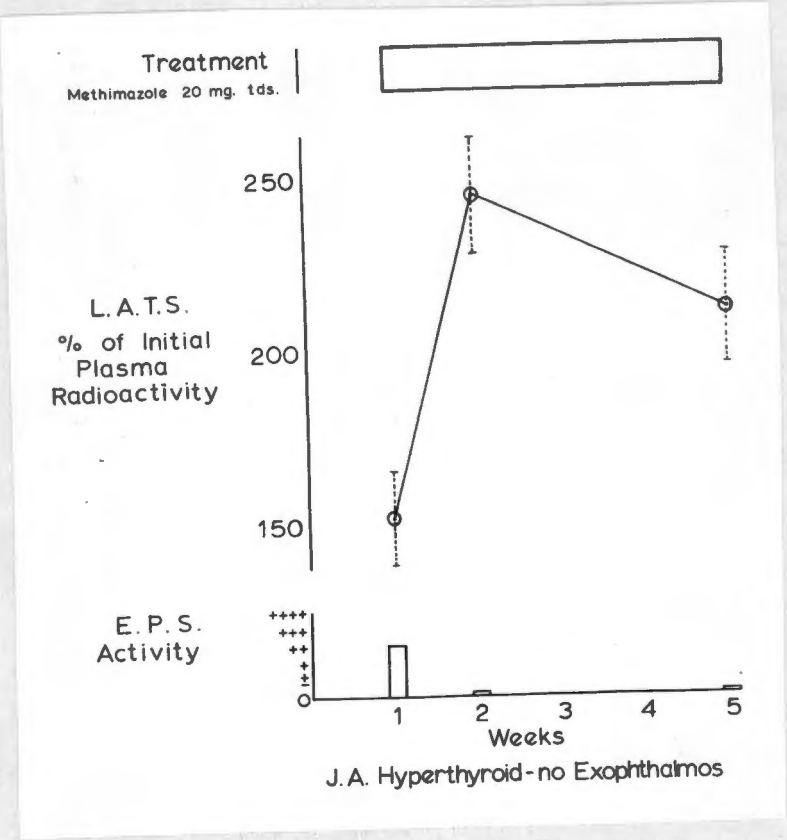


FIGURE 24: Effect of methimazole on L.A.T.S. and E.P.S. activity in hyperthyroidism.

Comment: In both cases, a striking rise in the level of L.A.T.S. is evident. Routine exophthalmometry was not carried out, but no subjective or objective evidence of exacerbation of exophthalmos was noted.

The implications, on the basis of two cases, must be cautiously interpreted. The clinical impression that antithyroid drugs exacerbate exophthalmos has long been held[83], but has never been put onto a scientific footing. The observation that L.A.T.S. levels are increased by these agents, suggest that some basis may exist for the above view. The effects may, however, be transient

only, as in Case 1, L.A.T.S. levels were beginning to drop sharply before the therapy had been altered and Case 2 appears to be following the same course. During these periods of increased L.A.T.S. activity, no obvious deterioration in exophthalmos was evident.

Once again, E.P.S. assays yielded results which did not parallel L.A.T.S. activity and is further evidence for their independent identities. However, at the point of peak L.A.T.S. activity, E.P.S. was present in the plasma of Case 1, but its presence was confined to that single occasion. Conversely, E.P.S. was maximal at the commencement of therapy in Case 2, and disappeared subsequently. This is very similar to the first case reported in this chapter.

(4) Effect of thyroxine on L.A.T.S. and E.P.S.

In L.G., the thyrotoxic patient on methimazole just described, and in another subject, E.R., considered to be euthyroid but with the eye signs of hyperthyroidism, thyroxine was given in an attempt to assess the effect of this drug on the high levels of L.A.T.S. present in these two cases. In addition, E.P.S. was assayed serially.

The results in L.G. have been indicated in Figure 23, while those in the second subject are shown graphically in Figure 25:

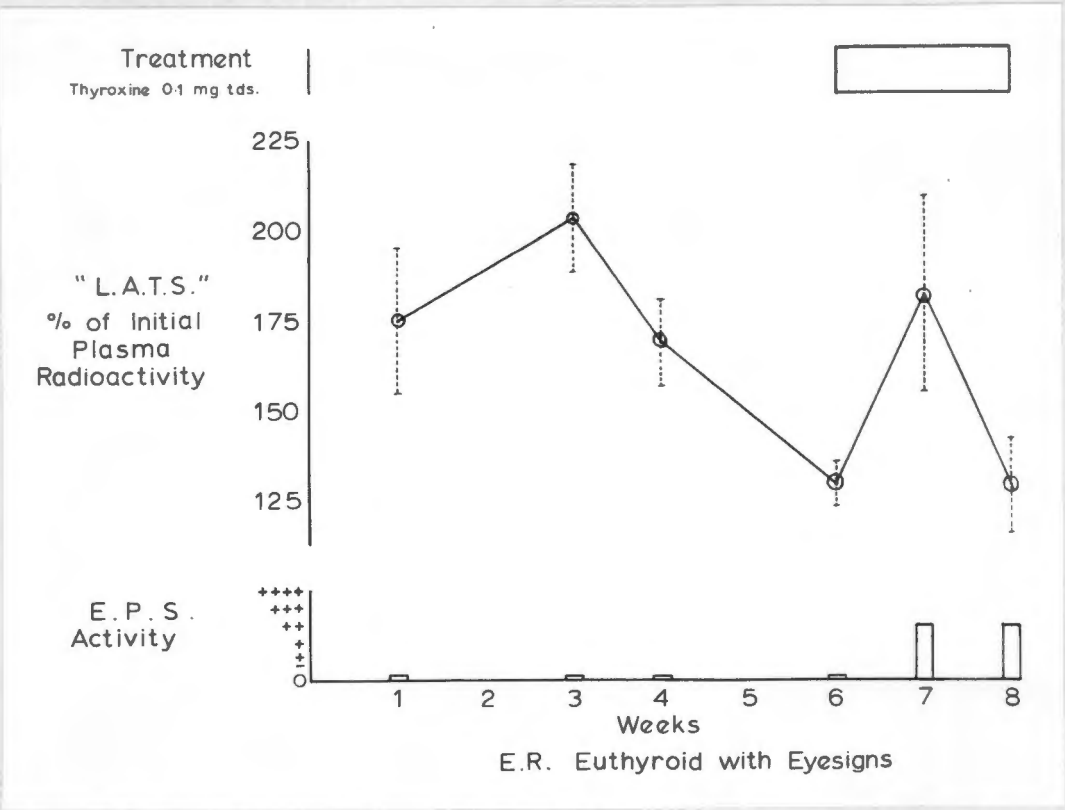


FIGURE 25: L.A.T.S. assays in a euthyroid subject with severe eye signs of hyperthyroidism, treated with thyroxine.

Comment: The effect of thyroxine administration in the two cases studied is impossible to assess. L.A.T.S. activity was already beginning to drop in the plasma of L.G. while she was on methimazole only, and the subsequent disappearance of this activity may have reflected the natural course of the treated hyperthyroidism, rather than a specific effect of thyroxine added at a late stage.

Wide fluctuations in L.A.T.S. activity occurred in the case of E.R., and the initial rise, and subsequent drop in L.A.T.S. levels can only be interpreted in the light of such fluctuations.

In actual fact, no definite trend was found in either case following thyroxine, though lower levels of L.A.T.S. tended to occur. Although adequate explanations can be found to account for this phenomenon, further studies are in progress to clarify this important point.

High values for L.A.T.S. have been reported in patients on thyroxine therapy[160] and L.A.T.S. has actually been shown to rise when assayed in a thyrotoxic patient before and after thyroxine[5]. In addition, the highest assay values in this study were found in the plasma of a patient with pretibial myxoedema on maintainance thyroxine therapy.

It would appear, therefore, that thyroxine in the dosage employed, has not been conclusively shown to affect the levels of L.A.T.S. It would be important in the assessment of this drug to use it in higher dosage in the treatment of rapidly progressive exophthalmos and to follow such cases with serial assays of the hormone. Until this is done, judgement on the status of thyroxine must remain reserved.

CHAPTER XI

The effects of tri-iodothyronine (T3) on thyrotrophic, L.A.T.S. and E.P.S. activity in human plasma

An investigation of the mechanism of the T3 suppression test of thyroid function

The demonstration of an increased thyroidal uptake of radioactive iodine was a useful collaborative laboratory test in the diagnosis of Graves' disease, but confusion arose as euthyroid patients with goitre were not infrequently found to have high uptakes. The introduction of thyroid hormone, and, later, tri-iodothyronine (T3) solved this difficulty as these hormones suppressed I^{131} uptakes in euthyroid states but failed to do so in Graves' disease[112, 225].

The pathways by which this suppression normally occurs are well documented[168] but the reason why suppression is not demonstrable in Graves' disease is unknown. Werner[224] postulated the concept of autonomous thyroid activity to explain this. Suppressibility has been shown to return with the euthyroid state, whether induced by radio-iodine, thyroidectomy or antithyroid drugs[116], even though the abnormal metabolism of thyroxine[124] and T3[115] persists. This, too, has been taken to suggest that the resistance to suppression in Graves' disease may be directly related to the hyperfunctioning thyroid gland, as suppressibility has returned after treatment which has cured the hyperthyroid state by apparently exerting an effect only on the thyroid gland and its production of hormone[116]. Werner's concept has been criticised by Russell Fraser[100] who feels that resistance to suppression means an abnormality of

the pituitary-thyroid homeostatic mechanism and not of necessity primarily a thyroid dysfunction.

The assay of thyrotrophin and L.A.T.S. provides an excellent opportunity for testing these hypotheses in euthyroid subjects with high 24 hour thyroidal I^{131} uptakes in whom suppression is induced by T3, and hyperthyroid patients with high uptakes in whom this does not occur. This is of particular interest as L.A.T.S. has been postulated as the aetiological factor responsible for Graves' disease, mediating its effect presumably via its thyrotrophin-like action. It is even possible that the high thyroidal I^{131} uptakes in Graves' disease are a result of the action of this substance.

For these reasons thyrotrophic and L.A.T.S. activities were assayed before and after a T3 suppression test in euthyroid and hyperthyroid subjects. All had high 6 and 24 hour I^{131} uptakes following 25 μc. oral I^{131} and received 120 μg. T3 daily for 7 days, when the 6 and 24 hour uptakes were repeated. On the day of both uptakes, blood was sampled and thyrotrophin or L.A.T.S. was assayed.

(1) <u>Effect of tri-iodothyronine administration on thyrotrophic activity in euthyroid subjects with high thyroidal I^{131} uptakes</u>.

Seven persons presented with diffuse goitre but no symptoms or signs of hyperthyroidism. All had high thyroidal I^{131} uptakes which were suppressed by T3. Thyrotrophic activity was estimated before and after T3 suppression as indicated above. Results are tabulated and graphically shown in Table XIX and Figure 26 respectively.

PATIENT	Age	Sex	6, 24 hour I^{131} uptakes		Uptake: Post T3/Pre T3 %	Thyrotrophin (mu/100 ml.)		Remarks
			Pre T3	Post T3		Pre T3	Post T3	
1. L.S.	17	F	60,64	21,25	40%	34 (30-41)	0	Diffuse goitre. RBC uptake 12.5%.
2. P.P.	27	F	41,52	16,14	28%	16 (11-24)	0	Diffuse goitre. RBC uptake 14.3%.
3. J.K.	31	F	39,57	20,21	37%	25 (21-32)	0	Diffuse goitre. RBC uptake 16.3%.
4. L.V.	32	M	50,60	15,24	40%	20 (15-27)	0	Diffuse goitre. RBC uptake 17.1%.
5. D.H.	16	M	67,78	30,45	58%	16 (11-24)	13 (9-20) ?	Huge lobulated goitre. Enzyme defect.PBI 1.5 µg.% RBC uptake 14.4%.
6. E.F.	38	F	58,46	25,28	61%	17 (12-25)	21 (16-29)	Diffuse goitre. RBC uptake 13.7%.
7. D.M.	30	F	57,68	28,39	57%	17 (13-24)	27 (20-38)	Diffuse goitre. RBC uptake 13.4%.

TABLE XIX. Thyrotrophic activity before and after tri-iodothyronine in seven euthyroid subjects. The 24 hour uptake figures after T3 is expressed as a percentage of the initial 24 hour reading in this and subsequent tables.

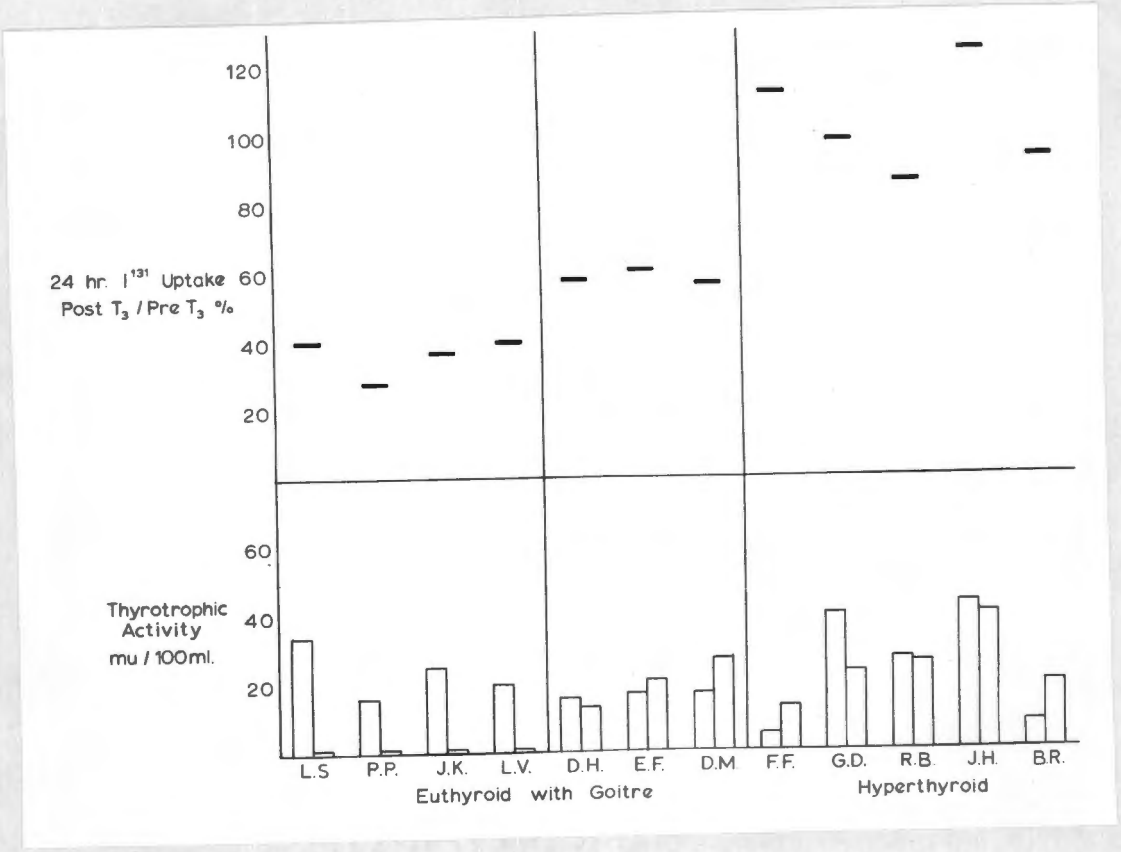


FIGURE 26: Thyrotrophic activity in euthyroid and hyperthyroid subjects before and after tri-iodothyronine. This is compared to the 24 hour uptake $\frac{\text{Post T3}}{\text{Pre T3}}$, expressed as a percentage.

Comments: In Cases 1 - 4, thyroidal I^{131} uptakes were effectively suppressed by tri-iodothyronine in the dose stated earlier. In each case, this appears clearly to be related to an effective suppression of plasma thyrotrophic activity. This confirms results obtained in a different type of study, where the effects of exogenous thyrotrophin were suppressed by administered thyroid hormone(168) with resultant lowering of thyroidal I^{131} uptake. The mechanism of this test in euthyroid individuals seems clear, therefore. Thyrotrophic activity was within normal limits initially and it is unlikely to have been directly responsible for the increased I^{131} uptakes. This phenomenon is more probably related to the status of the thyroid gland in these goitrous subjects.

Cases 5 - 7 showed increased I^{131} uptakes, but only partial suppression by T3 administration was achieved. This, too, was paralleled by the absence of the usual drop in thyrotrophic activity, which appears to confirm the close relationship between thyrotrophin levels and thyroidal I^{131} uptake in the euthyroid subject. The evidence is based on a very small number of cases however, and until a larger series is completed, the results must remain an interesting, though probably correct, speculation. Bottari has reported similar results in a few cases he studied, but no details have been quoted(48)

(2) Effect of tri-iodothyronine administration on thyrotrophic activity in hyperthyroid subjects:

Thyrotrophic activity was estimated in five subjects with classical Graves' disease who had the typical high thyroidal I^{131} uptakes, not suppressed by T3. Assays were done at the commencement and the end of T3 administration, as indicated earlier. Results are shown in Figure 26, and Table XX:-

Patient	Age	Sex	6, 24 hour I^{131} uptakes		Thyrotrophin (mu./100.ml.)		Uptake: Post T3% Pre T3	Remarks
			Pre T3	Post T3	Pre T3	Post T3		
1. F.F.	37	F	69, 69	97, 77	5(0-17)	13(6-24)	112%	RBC uptake 23.5%. Slight exophthalmos.
2. G.D.	42	F	70, 69	81, 68	40(27-61)	23(15-36)	98%	RBC uptake 23.8%. PBI 9.0 µg.%. Marked exophthalmos.
3. R.B.	44	F	86, 83	78, 71	27(17-43)	26(16-42)	86%	RBC uptakes 21.5%. PBI 9.5 µg.%.
4. J.H.	36	F	82, 55	85, 68	43(37-53)	40(24-66)	124%	RBC uptake 22.8%. Mild exophthalmos.
5. B.R.	47	F	71,70	54, 65	8(0 - 20)	20(11-34)	93%	RBC uptake 26.8%. No exophthalmos.

TABLE XX. Thyrotrophic activity before and after tri-iodothyronine in five hyperthyroid subjects.

Comment: It is quite clear from these results that thyrotrophic activity does not alter significantly or consistently in any one direction following the administration of T3. This is paralleled by a lack of suppression of the high thyroidal I^{131} uptakes.

It was pointed out earlier that assays for "thyrotrophin" in hyperthyroidism must have limited, if any, significance when carried out by the methods used in this study. Such activity possibly represents L.A.T.S. which can exert part of its effect as early as two hours after administration. The great majority of the cases with high levels of L.A.T.S. assayed in this study had significant activity at that time.

As these results have a very limited meaning, and as they might be taken as evidence that L.A.T.S., rather than thyrotrophin, was the active substance unresponsive to T3, this substance was assayed in three cases of Graves' disease before and after T3 administration in the usual "suppressive" dosage.

(3) Effect of tri-iodothyronine administration on L.A.T.S. activity in hyperthyroid subjects:

The results of L.A.T.S. assays in three subjects are shown in the following table and graph.

Patient	Age	Sex	6, 24 hr. I^{131} uptakes		Uptake: Post T3 / Pre T3 %	L.A.T.S. (% initial activity)				Remarks
			Pre T3	Post T3		Pre T3		Post T3		
						2 hr.	7 hr.	2 hr.	7 hr.	
1. M.N.	49	F	76, 68	71, 79	116%	109±16	167±22	228±47	280±39	No exophthalmos.
2. J.P.	26	F	91, 96	100,89	93%	102±16	140± 6	148±11	203±36	Slight exophthalmos. L.L., L.R.
3. M.M.	54	F	94, 76	86, 71	93%	189±13	195±13	151±20	211±30	No exophthalmos. L.L., L.R.

TABLE XXI. L.A.T.S. activity before and after tri-iodothyronine.

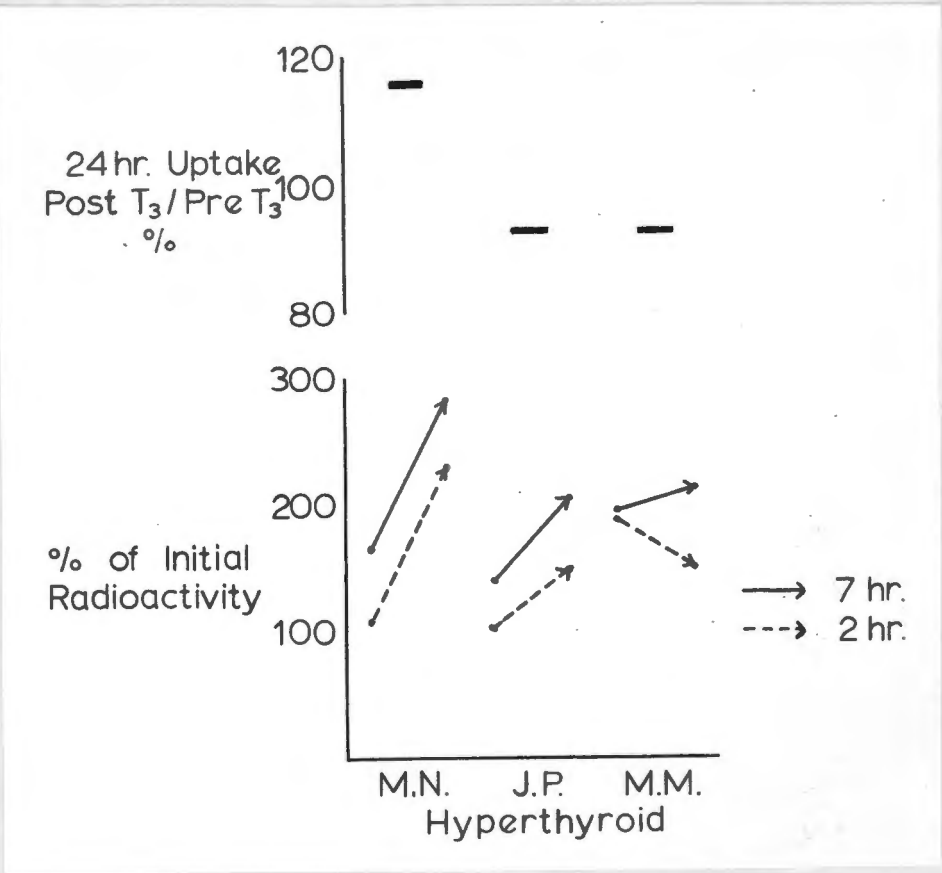


FIGURE 27: L.A.T.S. activity in hyperthyroid subjects before and after tri-iodothyronine. This is compared to thyroidal 24 hour uptakes post T3/pre T3, expressed as a percentage.

Comment: A delayed rise in activity and positive response ratio characteristic of L.A.T.S. occurred in every case, even though the responses at 7 hours were not very significantly increased over those at 2 hours. In all three instances, these values rose after the administration of T3, which in this small series of cases appears to have no suppressive effect either on L.A.T.S. or the thyroidal I^{131} uptake in hyperthyroidism.

This is not a surprising finding, as McKenzie in fact obtained many of his positive assay results in patients either taking thyroid hormone, or undergoing a suppression test(160).

As the results seem to show a definite trend, it is tempting to speculate on the implications of these findings.

L.A.T.S. is found in the great majority of patients suffering from Graves' disease[5,155,160,162]. It has a thyrotrophin-like action in that it stimulates not only the discharge of I^{131} (used as an assay method), but increases thyroidal uptake of I^{131} with histologic evidence of thyroid hyperplasia[144,158]. Its mode of effect therefore is compatible with it being the agent responsible for maintaining the high I^{131} uptake levels in hyperthyroidism.

Werner's suggestion that all forms of endocrine exophthalmos, whether in hyperthyroid subjects or not, are associated with a raised thyroidal I^{131} uptake which T3 does not suppress[224(a)], may be linked with the observation, mentioned earlier, that L.A.T.S. levels correlate well with exophthalmos, even when it is not necessarily part of Graves' disease.

Thus L.A.T.S. and raised thyroidal I^{131} uptakes have a common denominator both as regards their broad clinical association with exophthalmos, and also the absence of a suppressive effect of T3, as suggested by this small study. The implication that L.A.T.S. is in fact the agent responsible for the pathologically raised I^{131} uptakes in the hyperthyroidism-exophthalmos complex is very tempting, but further studies will be needed to confirm this point.

The non-response of L.A.T.S. levels to T3 administration is not supprising as its origin is not in the pituitary[160,163] and it has probably no direct bearing on the anterior pituitary-thyroid homeostatic system in health. As such, it could hardly be expected to depress after T3 which exerts its effects, as shown earlier, by depressing thyrotrophin. If L.A.T.S. is indeed the agent responsible for the high I^{131} uptake in hyperthyroidism, the reason for the absence of suppression by T3 becomes self-evident.

There are, however, two main objections to this hypothesis. Firstly, not all thyrotoxic patients have a raised L.A.T.S. level, yet failure to suppress I^{131} uptakes by T3 is a constant finding. It is possible that every thyrotoxic patient may, in fact, have increased amounts of L.A.T.S., but that the assay methods available may be too crude for its detection at lower, and yet abnormal, levels.

The second objection concerns the observation that suppressibility by T3 returns after successful therapy of Graves' disease[116], therapy which is directed at the thyroid gland and its excessive production of hormone, rather than the primary, but unknown, cause. The inference that the agent responsible for the resistance to T3 suppression lies in the thyroid gland, is difficult to refute. The site of origin of L.A.T.S. is unknown. It is, perhaps, the agent concerned.

CHAPTER XII

Summary and Conclusions.

In the presentation of this work, speculation on and discussion of individual results has, to a large extent, followed immediately on their recording and has been confined to the relevant chapters. As numbers of topics, not directly related, were studied, it was thought that such a system would make for easier and more direct appraisal of findings. However, to conclude the thesis, some sort of integration is necessary. To achieve this, the study will be reviewed with the emphasis on facts, rather than implication or discussion, and with the exclusion of references to the literature unless they have not been mentioned in the earlier text. Such a review now follows.

The development of an understanding of thyroid physiology and the regulation of its hormonal secretion was traced until the concept of an anterior pituitary-thyroid homeostatic balance had been founded and the existence of a thyrotrophic hormone proven by crude biological assay.

A review of such assay methods was presented, with special emphasis on the more recent techniques involving the use of radio-isotopes. The limitations of biological assays were stressed and more essential prerequisites for the ideal assay, including objectivity, sensitivity, reproducibility, specificity and statistical validity were discussed in relation to newer methods.

An assay based on the discharge of radio-iodine from the thyroid glands of mice was adapted very slightly from that developed by McKenzie(154) and was used in this study. It appeared to fulfil the prerequisites outlined above.

Weanling female mice received 8 μc. I^{131} intraperitoneally, followed by thyroxine subcutaneously to suppress endogenous thyrotrophin secretion. Four days later, they were used for the assay experiment when whole blood was counted for radioactivity before and two hours after the intravenous injection of three dose levels of thyrotrophin standard, a saline control and the "unknown" plasma samples or tissue homogenates. The increase in radioactivity of the "unknown" substances, plotted in relation to the standard dose response line, was translated into an absolute value for thyrotrophin and finally expressed in milliunits (mμ.)/ 100 ml. plasma. The technique enabled detection of 0.05 to 0.08 mu. thyrotrophin/ 100 ml. plasma, a sensitivity enough to detect normal thyrotrophic activity. Although this assay end point constitutes what is probably the most important parameter of thyrotrophin function, the quantitative measurement of such function does not necessarily imply an equivalent quantitation of thyrotrophin. Such biological methods of determination cannot, for instance, distinguish negative effects due to antihormones or other circulating antagonists. What was measured then, was thyrotrophin-like activity, and all results are best considered in this light.

Evidence for the existence of a circulating exophthalmos producing substance (E.P.S.) as distinct from thyrotrophin, was reviewed. Its nature and mode of action were discussed and the existing assays used to determine its presence in the serum of exophthalmic patients were outlined. The only currently

used assay depends on the induction of measurable degrees of proptosis in fish, either by anterior pituitary extracts or sera and such a technique was consequently employed in this study, with the goldfish as test animal. The intercorneal distance was measured before and three hours after the intracoelomic injection of plasma, anterior pituitary extract or saline. No standard reference substance exists for E.P.S. assays, but an anterior pituitary extract with high E.P.S. activity was used consistently at a single dose level throughout this study. An attempt was made to standardise an otherwise crude procedure and compensate for seasonal and other fluctuations by expressing the increase in intercorneal distance (I.C.D.) invoked by the unknown substance as a percentage of that invoked by the fixed dose of standard. Such percentages were graded 0 - ++++, in which terms E.P.S. activity was ultimately expressed. An adequate dose response relationship was established though great fluctuations from fish to fish made it difficult to evaluate results statistically, and this was not attempted. In spite of this, reported assay results have tended to parallel the degree and progression of exophthalmos.

The association of a circulating substance with both Graves' disease and exophthalmic states has been reported recently. This substance has a slow and very sustained thyrotrophin-like action and has been named "long-acting thyroid stimulator" or L.A.T.S. This property has enabled its assay to be undertaken by the same method used for "true" thyrotrophin, but as the release of radioiodine occurs much later, mouse blood radioactivity is counted seven to twelve hours after injection. The statistical interpretation of such "late" activity is not easy as no valid standard for comparison exists. As "true" thyrotrophic

activity had almost invariably dropped at seven hours, any increase as compared to the two hour reading was considered suggestive and a statistically significant rise was regarded as conclusive. A "response ratio" was evaluated, being the ratio of the seven hour to the two hour response in each unknown assay.

Using the techniques outlined, assays for the presence of these substances, either singly or as a collaborative assay for all three, were performed on aliquots of plasma.

Thyrotrophic and E.P.S. activity was investigated in plasma from normal volunteers. Mean thyrotrophin levels of 22 mu. and 24 mu./100 ml. were found in males and females respectively, while E.P.S. activity was detected in only one of the subjects. The recent suggestion that serum thyrotrophin levels may be considerably lower[32] has to be interpreted cautiously in view of the complicated extraction procedures used before such levels could be convincingly shown.

Serial estimations of thyrotrophin levels in a normal subject revealed virtually no fluctuations from week to week.

In hypothyroid states great extremes of thyrotrophic activity were assayed ranging from 0 to 310 mu./100 ml. in eight patients. With the anticipated disturbance in pituitary-thyroid balance, thyrotrophin values are usually raised, often considerably. Low levels have been explained by a secondarily induced hypopituitary function as a result of subnormal cellular metabolism in the myxoedematous state. E.P.S. activity was assayed and found generally to be absent, in spite of these high levels, confirming the different identities of E.P.S. and thyrotrophin. However, two patients who had undergone total

thyroidectomies for carcinoma had detectable E.P.S. A hypothetical E.P.S. inhibitor, originating in the thyroid gland, was postulated to account for the unusual findings, though a technical false positive effect was more likely.

In hyperthyroid states, thyrotrophin estimationa adhered closely to the normal distribution range. As these readings may have represented merely a facet of L.A.T.S. activity, their significance is doubtful. L.A.T.S. assays were performed on nine thyrotoxic patients, in only two of whom was activity unequivocally absent. It was highest in the single subject who could be termed hyperophthalmopathic. E.P.S. was assayed more consistently than in hypothyroidism, and at higher levels. Though the greatest response was in the severely exophthalmic patient, E.P.S. was found in subjects with mild eye signs. It has been suggested that such patients may ultimately develop exophthalmos.

In 22 euthyroid subjects with various types of goitre thyrotrophic activity was generally normal. The thyrotrophin level appeared to bear no relationship to the thyroidal I^{131} uptakes, as the high and low uptake groups had very similar activity. The cause of raised thyroidal I^{131} uptakes in goitre remains obscure, therefore, but appears to be independent of raised plasma thyrotrophin levels. E.P.S. activity was not increased in the few patients on whom assays were performed.

In proven hypopituitarism no thyrotrophic activity was detected, while in two cases of thyroid carcinoma, it was within the normal range. Levels of thyrotrophin, two to three times those of normal, were found in a single case of subacute thyroiditis. The significance of this single value is doubtful. Of three patients with Hashimoto's disease, two had absent thyrotrophic activity.

The importance of such results in a small number of cases when normal blood may on occasion have no detectable thyrotrophic activity, is doubtful. The result may, however, be related to the presence of a cytotoxic factor in the sera of many patients with this disease. E.P.S. was absent in all three cases.

In three cases of untreated acromegaly, thyrotrophin was normal, but one case had L.A.T.S. activity which was probably significant. This patient also had increased levels of E.P.S. and rather high thyroidal and RBC I^{131} uptakes. This is interesting in view of the association between acromegaly and both hyperthyroidism and goitre.

Three euthyroid patients with marked lid lag, lid retraction and slight exophthalmos were shown to have L.A.T.S. activity. This syndrome has been considered to occupy one end of the spectrum of hyperthyroidism(224b), and the results reported may strengthen this concept. E.P.S. was present in one of two such cases tested.

Two patients presented with malignant exophthalmos following repeated thyroidectomies. One had definite, and the other suggestive, L.A.T.S. activity. Again, E.P.S. assays yielded inconsistent values, being raised in the one case only.

Striking results were obtained when plasma of three subjects with pretibial myxoedema and exophthalmos was assayed. In two, enormous amounts of L.A.T.S. were present. Homogenates of the pretibial myxoedematous tissue appeared to have L.A.T.S. activity too, this being significantly increased as compared to control suspensions of subcutaneous fat. The significance of these findings is obscure, but the presence of L.A.T.S. in very high concentration in

plasma and pretibial tissues of such patients may point to a direct aetiological association between L.A.T.S. and this very interesting syndrome.

The results of all the collaborative assays for E.P.S. and L.A.T.S. were correlated and though both substances were found to parallel exophthalmos to a certain extent, L.A.T.S. was shown to be much more consistent in this respect. Furthermore, plasma with extremely high L.A.T.S. activity failed to elicit an exophthalmic response in the test animals. If one excludes the possibility of false negative assays due to non-responsiveness of the fish, it would appear that L.A.T.S. and E.P.S. are different substances although both are clearly related to the pathogenesis of exophthalmos. Pituitary E.P.S. levels rather than plasma values, may be more important in this respect, and it is not impossible that E.P.S. mediates its action via other substances, possibly even L.A.T.S.

In summary, the highest responses in the L.A.T.S. assays performed in this study were obtained in the pretibial myxoedema syndrome, malignant exophthalmos in euthyroid subjects and "hyperophthalmopathic" Graves' disease. This suggests a very strong relationship to exophthalmos. Thyrotrophin standard, and plasma from myxoedematous and acromegalic subjects showed a considerably reduced "late" response, that is, no L.A.T.S. was found.

E.P.S. was assayed most consistently and at highest levels in patients with hyperthyroidism but activity was also found in the plasma of euthyroid patients both with malignant exophthalmos and with the eye signs of hyperthyroidism and those with pretibial myxoedema. The occasional false positive result was noted with this assay.

Serial assays for E.P.S. and L.A.T.S. were performed on a number of patients to investigate the effects of various forms of therapy in hyperthyroidism or exophthalmos.

In one thyrotoxic patient with exophthalmos, an elevated E.P.S. value diminished progressively during therapy with propylthiouracil and thyroxine and this result may represent the counterpart to the clinical response of exophthalmos to antithyroid therapy which is claimed to occur in some cases of hyperthyroidism.

The progression of exophthalmos in a patient with pretibial myxoedema was dramatically reversed by the exhibition of large doses of dexamethasone. This effect was followed by exophthalmometry and a concomitant drop in L.A.T.S. levels was demonstrated. E.P.S. appeared inconsistently in the plasma however, and did not parallel the clinical state at any time. The close link between L.A.T.S. levels and exophthalmos was well illustrated as was the striking therapeutic response to dexamethasone in large doses.

In each of two cases studied, methimazole appeared to elevate plasma L.A.T.S. levels markedly, but the effect may have been a transient one. No conclusions are warranted on such meagre results, but if this is confirmed in a larger series, a basis may have been provided for the reputed adverse effects of antithyroid drugs on the exophthalmos in some patients with Graves' disease. However, in one of these cases, E.P.S. actually disappeared from the plasma during therapy. Again, E.P.S. and L.A.T.S. values have been found to be contradictory.

The effect of thyroxine on L.A.T.S. was inconclusive in the two cases studied. One patient had levels which fluctuated greatly, and L.A.T.S. appeared to be subsiding before thyroxine administration in the second. No clear-cut effect was noted, but it was thought that this would be unlikely in view of the high amounts of L.A.T.S. reported in sera from thyrotoxic patients on thyroxine or during a tri-iodothyronine suppression test.

The suppressive effect of tri-iodothyronine (T3) on the high thyroidal I^{131} uptakes in euthyroid subjects with goitre was shown to be due probably to a depression of endogenous thyrotrophin secretion, as plasma thyrotrophic activity disappeared in four such patients after the administration of T3. In three, in whom partial suppression of I^{131} uptakes occurred, depression of thyrotrophic activity was not recorded.

In hyperthyroidism, lack of a suppressive effect of T3 on the elevated I^{131} uptakes correlated with the persistence of thyrotrophic activity in five cases studied. As thyrotrophin estimations are of doubtful significance in the presence of L.A.T.S., the latter substance was assayed in three cases, before and after T3. No effect was noted. This result is not surprising, as L.A.T.S. does not appear to originate in the anterior pituitary gland and would not be expected to participate in the homeostatic effects of thyroxine. L.A.T.S. has been shown to stimulate thyroidal I^{131} uptake, and it is speculated that the presence of this substance in the plasma of thyrotoxic patients is responsible for both the elevated thyroidal I^{131} uptakes and the lack of suppression by T3, so typical of that condition. That the presence of L.A.T.S. is by no means

as universal in hyperthyroidism as the abnormal I^{131} uptakes, must constitute a major objection to this attractive theory. It remains to be assessed whether the return to normal of the I^{131} uptakes with the euthyroid state runs pari passu with a diminution of L.A.T.S. activity. Such studies are in progress.

CHAPTER XIII

Recommendations for future work.

> ".....One cannot fly through the air on a broomstick. It must at least have a machine on it. But as yet there is no such machine. Perhaps there will never be, for man is too heavy. But, of course, one cannot tell. We don't know nearly enough, Giuseppe. We are really only at the beginning."
>
> Bertolt Brecht. - The Life of Galileo.

When new or uncommon techniques are developed, they may either be used to study a limited project extensively, or be employed in a probing fashion to sound out a number of problems, as has been done in this work. The resulting thesis may be circumscribed and complete or discursive and provocative. Discursive methods often raise questions rather than answer them, and in this concluding chapter, some of these questions will be considered, and the attempts being made to pursue them will be outlined.

(1) One of the most important facets of this study concerned the suggestion that L.A.T.S. may be the agent responsible for the elevated thyroidal I^{131} uptakes and the associated resistance to T3 suppression so characteristic of hyperthyroidism. While it was shown that T3 did not suppress L.A.T.S. activity in a small series of thyrotoxic patients, the rest of the theory was supplied by analogy and supposition, but it may well be correct. It is being pursued in two ways:-

(a) the number of thyrotoxic subjects who are being subjected to L.A.T.S. assays during a T3 suppression test, is being increased. (b) Patients are being followed up, and the return of suppressibility following T3 which is said to parallel clinical improvement, will be compared to serial L.A.T.S. levels. If a close correlation is shown to exist, the circumstantial evidence for the above assumption will be strong.

(2) The abnormal thyrotrophin levels found in Hashimoto's disease and subacute thyroiditis, and the L.A.T.S. assayed in acromegaly, all await confirmation in larger series of cases. These studies will continue until enough material is obtained to warrant a more conclusive statement.

(3) Collaborative assays have led to the suggestion that E.P.S. and L.A.T.S. are different substances. This finding will be further investigated by fractionating plasma and assaying each fraction for both substances. Should they differ in their mobility either on electrophoresis or on a DEAE column, their non-identity will be conclusively proven. L.A.T.S. however, apparently migrates with no specific serum fraction when eluted from a starch block after electrophoresis[160,162], or when fractionated on a DEAE column[160]. The establishment of such differential mobility may therefore be difficult, or even impossible.

(4) L.A.T.S. has been found in truly remarkable amounts in the plasma of a patient suffering from pretibial myxoedema and exophthalmos. The presence of

this substance in such high concentration may allow its chemical extraction and perhaps even purification. Such possibilities are at present under investigation.

(5) Serial plasma assays for E.P.S. and L.A.T.S. in thyrotoxic and exophthalmic patients under therapy are being continued. Of particular importance using these techniques, is the evaluation of the possible effect of antithyroid drugs in raising the L.A.T.S. level and the clinical parallel of this effect with exophthalmos.

The effect of thyroxine on L.A.T.S. and exophthalmos is likewise under further scrutiny, both at conventional and at much higher dosages.

The field of both endocrine exophthalmos and hyperthyroidism has always been an open one. Exciting recent researches, while shedding new light, have sometimes obscured the old. It was an attempt to explore some of the shadows which led to this study. That much darkness has been dispelled cannot be claimed; perhaps more has been added. Fundamental difficulties remain, and until more refined tools are available for hormone research and assay, many problems will stay unanswered.

APPENDIX

STATISTICAL METHODS USED IN THIS STUDY.

(1) Mean value $\bar{x} = \frac{\Sigma x}{n}$ where x = single observation and n = number of observations .

(2) Standard deviation $s = \sqrt{\frac{\Sigma (x - \bar{x})^2}{n - 1}}$

or more conveniently expressed when large numbers of observations are used ,

$$s = \sqrt{\frac{\Sigma (x)^2 - \frac{(\Sigma x)^2}{n}}{n - 1}} \quad .$$

(3) Standard error of the mean $e = \frac{s}{\sqrt{n}}$.

(4) Limits for the variation of $\bar{x}$ were decided on the distribution of t for the corresponding numbers of degrees of freedom $(n - 1 = f)$. At a "probability" value of 0·05 (95°/₀ confidence limits) , the variation of $\bar{x}$ would be calculated as $\bar{x} \pm e.t_{0.05}$.

(5) Significance of difference between x and y was estimated by the students "t" test , where

$$t = \frac{\bar{x} - \bar{y}}{\sqrt{e_x^2 + e_y^2}}$$

e_x and e_y represent the standard errors of $\bar{x}$ and $\bar{y}$.

Using statistical tables , significance was read in "probability" in the corresponding number of degrees of

freedom ($n_x + n_y - 2 = f$) . A difference was regarded as significant if $p < 0{\cdot}05$. The statistical difficulties regarding the significance of L.A.T.S. assays have been alluded to. In these instances, the result was regarded as probably significant if $p < 0{\cdot}1$.

(6) The slope of the log.-dose response line was calculated by <u>regression analysis</u> , made by the method of least squares. The <u>regression line</u> in a given range was regarded as straight , and corresponded to the formula $y = mx + c$, where x and y are variables (the logarithm of the dose and of the response respectively) , and m and c are constants .

$$m = \frac{\Sigma (x-\bar{x})(y-\bar{y})}{\Sigma (x-\bar{x})^2} \quad \text{or} \quad \frac{\Sigma xy - \frac{\Sigma x \Sigma y}{n}}{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}$$

$\bar{y}$ = mean of y variables

$\bar{x}$ = mean of x variables

c was derived by substituting $\bar{x}$, $\bar{y}$ and m in the equation $y = mx + c$, and is the point the response line crosses the y axis .

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