IMMUNOLOGICAL ASPECTS OF THYROTOXICOSIS

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STATEMENT

This thesis contains no material which has been accepted for any other degree or diploma in any University and does not contain any material previously published or written by another person, except where due reference is made to such material in the text.

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SUMMARY

The thesis was introduced by reviewing the development of knowledge of the pathogenesis of thyrotoxicosis. The significance of the long acting thyroid stimulator (L.A.T.S.) in the development of the main clinical features of Graves' disease, namely hyperthyroidism, ophthalmopathy and dermopathy was then examined. Next, the criteria by which an autoimmune disease can be defined were discussed in relation to thyrotoxicosis. Finally, the scope of the present studies was outlined.

In the methodology section, the clinical and laboratory methods used in these studies were described. The methods used were L.A.T.S. concentration and bioassay, antithyroglobulin estimation, the immunofluorescent antibody technique and estimation of immunoglobulins using the radial diffusion method. In particular, the limitations and problems associated with the L.A.T.S. bioassay and the definition of positive L.A.T.S. responses used in these studies were discussed.

In the clinical studies, 135 patients with thyrotoxicosis were studied. Several patients with other autoimmune diseases and 43 euthyroid relatives from four families with a high prevalence of thyrotoxicosis were also studied. Finally, patients with other medical diseases and normal people were investigated.

In the experimental studies animals were immunized with whole thyroid extracts or the microsomal fraction prepared from autopsy specimens. Thyroid function was assessed by measuring protein bound iodine and ^{131}I uptake. L.A.T.S. bioassays were carried out on the concentrated IgG fraction from the immunized animals and the antithyroglobulin titre was estimated using sheep red blood cells coated with human thyroglobulin.

Peripheral lymphocytes from thyrotoxic patients were cultured for six days in the presence of the non specific lymphocyte stimulant phytohaemagglutinin (PHA). Lymphocytes from normal people were cultured in the same way. A total of 11 cultures from 7 thyrotoxic patients were carried out.

The main findings regarding L.A.T.S. were as follows. Firstly, the prevalence of detectable L.A.T.S. was found to correlate best with the number of chief clinical features present. Patients with severe Graves' disease, who had the highest L.A.T.S. levels, tended also to have the highest prevalence of other antibodies and of raised immunoglobulin levels.

Secondly, all ten patients with dermopathy had detectable L.A.T.S. and usually in high levels. L.A.T.S. levels correlated closely with the clinical state of the dermopathy in six patients with this lesion in whom serial assays were carried out. Worsening of the dermopathy was associated with a rise in L.A.T.S. levels, whilst remission

was in all but one case, associated with a decrease or disappearance of L.A.T.S.

Thirdly, there was not a particularly close association of L.A.T.S. with ophthalmopathy. Whilst most patients with severe ophthalmopathy had detectable L.A.T.S. the occurrence of severe ophthalmopathy without L.A.T.S. and of high L.A.T.S. levels with only mild eye signs suggested that L.A.T.S. was not the direct cause of the eye lesion. However, the association with increased levels of immunoglobulins and significant antibody titres and the good response to immunosuppressive agents, which was noted in many cases, made it likely that the ophthalmopathy was also due to an immunological abnormality.

In a genetic study of four families with a high prevalence of thyrotoxicosis L.A.T.S. was detected in the concentrated IgG fraction from eight of 43 euthyroid relatives. L.A.T.S. was also found in four patients with other autoimmune diseases as well as in one patient with panhypopituitarism, in another with thyroid malignancy and in one normal person. The presence of L.A.T.S. may indicate a genetic predisposition to thyrotoxicosis.

The results of the experimental studies showed that L.A.T.S.-like activity was present in rabbits immunized with whole thyroid extract and thyroidal microsomes. Although none of the animals was overtly toxic several had

elevated levels of protein bound iodine and increased ^{131}I uptakes whilst one developed gross unilateral exophthalmos.

Finally, L.A.T.S. was produced by lymphocytes from thyrotoxic patients when cultured in the presence of the nonspecific stimulant phytohaemagglutinin (PHA). The antibody was completely neutralised on one occasion by anti-IgG serum.

The discussion concerned the concept of thyrotoxicosis as an autoimmune disease, in which a range of antibodies including L.A.T.S. were produced. It was concluded that thyrotoxicosis was likely to be an autoimmune disease, but that whilst L.A.T.S. might be the cause of the hyperthyroidism other antibodies were probably more closely related to the ophthalmopathy and dermopathy.

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CHAPTER I

INTRODUCTION

(A) HISTORICAL REVIEW

(1) Introduction

Graves' disease or thyrotoxicosis, is a syndrome characterized by one or more of the following clinical features, each one pathognomonic in itself:

i. Diffuse hyperplasia of the thyroid gland, usually with goitre and hyperthyroidism;

ii. Ophthalmopathy;

iii. Dermopathy. Rarely there may also be some acropachy (McKenzie, 1968, Solomon *et al*, 1968, Hetzel, 1970).

Most patients with thyrotoxicosis have hyperthyroidism. The classical signs and symptoms are due to the excess of circulating thyroid hormones. Over-activity of the sympathetic nervous system is frequently present producing spastic lid retraction and lag, tachycardia, increased sweating and warm flushed skin (Solomon *et al*, 1968).

The prevalence of severe ophthalmopathy and dermopathy is much less (Graciansky *et al*, 1957). However, because of the risk of blindness or gross deformity of the legs and feet, these features are of the greatest significance (Williams, 1962).

Mild exophthalmos, together with lid retraction and lag, is a common feature of Graves' disease (Hall et al, 1967) and does not in itself indicate serious eye involvement. On the other hand, gross exophthalmos, orbital infiltration and ophthalmoplegia which may lead to ulceration, eyeball destruction and blindness, although occurring in only about 10% of patients with thyrotoxicosis, requires vigorous treatment (Williams, 1962, Pequegnat et al, 1967).

Whereas mild eye signs tend to disappear following treatment of the hyperthyroidism (Eden and Trotter, 1942a) severe ophthalmopathy is often unaltered by treatment and may worsen, particularly after treatment with radio-iodine (Hamilton et al, 1967) or if the patient becomes hypothyroid (Hales and Rundle, 1960). Frequently, severe eye signs only appear after treatment (Kriss et al, 1967).

In this thesis, the term ophthalmopathy will be used as a general term to include all eye signs and symptoms. The classification of ophthalmopathy (Hamilton et al, 1967, Werner, 1969) will be discussed in a later chapter.

Pretibial myxoedema is a specific nodular or plaque-like indurated lesion of the skin in the pretibial or pedal position. There may be massive involvement with gross deformity. The lesion is firm and does not tend to ulcerate although occasionally it may pit and may resemble elephantiasis (Gilmette, 1964). Histologically, there is

disorganisation of the dermis with an excess of mucoid ground substance (Trotter and Eden, 1942, Beierwaltes and Bollett, 1959). However, the lesion can occur occasionally in sites other than the pretibial region of the legs. Because of this, and because of the confusing reference to myxoedema, the term thyrotoxic dermopathy, as recommended by Solomon et al (1968) will be used in this thesis.

The features of thyroid acropachy are clubbing of the fingers and toes, swelling of the subcutaneous tissues and periosteal new bone formation in the extremities. Acropachy usually develops after treatment, in patients with severe ophthalmopathy and dermopathy (Gilmette, 1960).

It is well known that in severe thyrotoxicosis hyperthyroidism, severe ophthalmopathy and dermopathy are all present. On the other hand in mild cases only hyperthyroidism is present (Lipman et al, 1967). In this thesis, thyrotoxicosis has been studied in reference to the number of clinical features present. The classification into mild, moderate and severe grades will be discussed in a later chapter.

(2) Pathogenesis of Thyrotoxicosis

The first account of Graves' disease was given by Parry, who in 1786, described a case of goitre with exophthalmos. He observed several other cases in the following years, all of whom had goitre and palpitations,

but no exophthalmos. In 1835, Graves described three cases of hyperthyroidism with goitre and palpitations. One of these patients had exophthalmos. In 1840, Von Basedow described a further four cases with exophthalmos, goitre and palpitations.

Since these first classical descriptions of thyrotoxicosis, many theories of the pathogenesis of the disease have been suggested, and it was not until the end of the 19th Century that the thyroid gland itself was considered the cause of the disorder (Rehn, 1884, Baumann, 1896a,b). Before that time thyrotoxicosis was considered to be a cardiac neurosis, because of the presence of palpitations and the association with stress, anxiety and tremor (Charcot, 1856).

Towards the end of the 19th Century, with the demonstration by Cannon of the effect of adrenaline on the sympathetic nervous system, it was thought that thyrotoxicosis was due to a disorder of the nervous system (Osler, 1898).

Following the discovery that there was a disorder of iodine metabolism in thyrotoxicosis, the thyroid gland was thought to be the cause of the disease (Baumann, 1896a,b). Then Magnus-Levy in 1895 showed that the basal metabolic rate was elevated and he suggested that the signs and symptoms of the disease were due to the increase in metabolism.

The recognition of pituitary control of thyroid function (Aron et al, 1931) and the preparation of potent thyroid stimulatory pituitary extracts, led to the hypothesis that thyrotoxicosis was due to an excess of thyroid stimulating hormone (TSH) or to a failure of feedback control (Loeb et al, 1930, Drouet, 1934). Marine and Rosen in 1934 were able to produce exophthalmos and hyperthyroidism in experimental animals with pituitary extracts and they thought that the disease was due to hyperpituitarism. When, however, it was finally found that plasma TSH was not increased in Graves' disease (D'Angelo, 1963, Utiger, 1965, Pinchera et al, 1965) and that the pituitary-thyroid feedback system was normal with suppression of TSH by exogenous thyroid hormone (Astwood, 1949, McKenzie, 1963, Adams, 1965), it became clear that the pituitary was not directly involved. Normal amounts of TSH have also been found in the pituitaries of patients with Graves' disease (McKenzie, 1968, unpublished). Finally, the failure of the thyroid to be suppressed by large doses of thyroid hormone suggested again that the thyroid gland itself was at fault, and not the pituitary (Werner, 1955).

(3) Thyrotropin Assays

The long acting thyroid stimulator (L.A.T.S.) was discovered when serum from thyrotoxic patients was tested in the thyrotropin (TSH) bioassay system, hence a review of the methods used for assay of TSH is presented.

Most of the methods used over the past twenty years have involved the use of radio-isotopes. The amount of labelled thyroid hormones released from the test animal's thyroid gland after an injection of test serum is proportional to the amount of TSH injected (Adams and Purves, 1955). This relatively sensitive bioassay system has been modified and is now used routinely for the detection and estimation of both TSH and L.A.T.S.

The first assay for TSH was described in 1908 by Rénon and De Lille who injected pituitary extracts into rabbits and noted thyroid stimulation. The first quantitative assay was described in 1932 by Junkmann and Schoeller when the thyrotropic effect of pituitary extracts on histological change in the thyroid of guinea pigs, injected with TSH, was graded. Since then many assay methods have been described detecting a variety of thyroid responses. It was not until a standard reference TSH became available however, that a direct comparison of these methods was possible.

The thyroid uptake of ^{131}I was first used as a measure of TSH activity by Henry in 1951. Techniques were subsequently described using a variety of animals (Querido et al, 1953, Bloche-Michel and Henry, 1955, Henry and Bloche-Michel, 1955), but because of the insensitivity of these assays they were replaced by assays which measured

discharge of ^{131}I from the stimulated thyroid gland (Gilliland and Strudwick, 1956).

Finally, the increase in labelled thyroid hormones in the plasma of the test animal was measured. This principle was first used by Adams and Purves in 1955 in measuring thyrotropin in human blood. A more sensitive method was developed by McKenzie, in 1958, in which 0.025 mU of TSH could be detected in mice labelled with ^{131}I and whose pituitary-thyroid axis had been suppressed by exogenous thyroxine.

Other assays used included histometric techniques in which changes in thyroid histology were measured (DeRobertis, 1948, D'Angelo and Sunderman, 1959), and methods in which increase in weight of beef thyroid slices was determined (Bakke et al, 1957). Finally, the most sensitive of all the ^{131}I discharge methods in which release of ^{131}I-labelled hormones by thyroid slices was determined, was developed by Bottari and Donovan in 1958.

Recently, a radio-immunoassay for TSH has been described (Utiger, 1965, Odell et al, 1967). Because of animal variation and the variable sensitivity of the bioassay and the time involved in using it, it is likely that an immunoassay will eventually replace the mouse bioassay.

Concentration of thyrotropic activity has been carried out in order to increase the sensitivity of the assay

(Purves and Adams, 1960, Bates, 1963, Adams and Kennedy, 1968). It is not yet certain which fraction TSH is associated with, although it has been claimed to reside in the beta-globulin fraction (Cohn, 1945a,b) with albumen (Roberts, 1957), Cohn Fractions, II, III and IV-4 (Querido and Lameijer, 1956), and gamma globulin (McKenzie, 1968). It is likely however that TSH, a low molecular weight glycoprotein, binds nonspecifically with several fractions.

(4) Long Acting Thyroid Stimulator

(a) Introduction

The long acting thyroid stimulator (L.A.T.S.) was discovered in 1956 by Adams and Purves. They found that serum from two thyrotoxic patients and one patient with exophthalmos gave an abnormal response in the guinea pig TSH bioassay. Whilst TSH gave a peak increase in ^{131}I-labelled thyroid hormones at 3 - 5 hours the abnormal substance gave a peak increase at 16 - 20 hours, and the response at 24 hours was greater than the response at 3 hours. This was confirmed by McKenzie in 1958 and Munro in 1959 using McKenzie's modification of the TSH assay method with mice instead of guinea pigs. This substance, when injected into rats, had a half life of about $7\frac{1}{2}$ hours compared with 10 - 20 minutes for TSH (McKenzie, 1959, Adams, 1960).

The action of L.A.T.S. on the thyroid gland of experimental animals was the same as that of TSH. Both stimulators caused an increase in colloid droplet formation, ^{131}I uptake, protein bound ^{131}I and acinar cell height (Major and Munro, 1960, McKenzie, 1960, Shishiba et al, 1967). L.A.T.S., when injected into hypophysectomised mice, produced highly significant responses. This showed that the site of action of the stimulator was not the pituitary (Munro, 1959, Adams et al, 1961).

(b) L.A.T.S. - An Immunoglobulin

i. Properties of Immunoglobulins

Most antibodies reside in the globulin fraction which has gamma mobility by electrophoresis. These are called immunoglobulins. Immunoglobulin molecules consist of polypeptide chains joined together by disulphide bonds. The large chains, called heavy chains, have a molecular weight of about 55,000 whereas the smaller chains, called light chains have a molecular weight of about 22,000 (Cohen and Porter, 1964, Cohen, 1965).

Several classes of immunoglobulins have been identified depending on the structure of the heavy chain. A different structural genetic locus is involved in coding for the heavy chains of proteins of that class. Each polypeptide chain type is determined by a separate gene locus (Cohen and Milstein, 1967, Warner, 1969).

The most important classes are immunoglobulin G (IgG) which makes up about 85% of the total plasma immunoglobulin level (Stiehm and Fudenberg, 1966), immunoglobulin M (IgM) which makes up about 5% of the total and immunoglobulin A (IgA) which makes up about 10% of the total (Warner, 1969). Most antibodies belong to the IgG class (Warner, 1969). This classification, recommended by the World Health Organisation (Ceppelini et al, 1964) is now used universally.

Plasma proteins can be separated in many ways (Cohn, 1945a,b). When separated by ultracentrifugation the rate of sedimentation, which is related directly to the molecular weight, is quantitated in terms of the sedimentation coefficient. The unit is the Svedberg unit S (Harper, 1969). Thus, when the immunoglobulins are separated in this way molecules with a molecular weight of about 150,000 have a coefficient of 7S whilst molecules with a molecular weight of about 800,000 sediment more rapidly and have a coefficient of 19-20 S. IgG and IgA are 7S globulins whilst IgM is a 19 S globulin (Warner, 1969).

When plasma proteins are separated by chromatography on a Sephadex G-200 column three protein peaks are eluted out (Flodin and Killander, 1962). Peak I consists of macroglobulins including IgM molecules, peak II consists of 7S beta and gamma globulins, whilst peak III consists largely of albumen with a flotation rate of 4S. This latter procedure is commonly used to isolate gamma globulins (Kriss et al. 1964. Dorrington et al. 1965).

ii. Antibody Nature of L.A.T.S.

Immunological differences between TSH and L.A.T.S. have been demonstrated. Anti-TSH serum neutralised TSH but not L.A.T.S. activity (Werner et al, 1960, Adams et al, 1962). On the other hand, antiserum to 7S gamma globulins completely neutralised L.A.T.S. activity (Kriss et al, 1964, Dorrington and Munro, 1965).

Using gel filtration with Sephadex G-200 which separated serum proteins into 19S globulins, 7S globulins and albumen, L.A.T.S. activity was found mainly in the 7S globulin fraction (Kriss et al, 1964, McKenzie, 1967, Hoffmann et al, 1967). These findings raised the possibility that L.A.T.S. might be an immunoglobulin.

Using procedures known to separate light and heavy chains of immunoglobulins (Cohen and Porter, 1964) it was shown that the biological activity resided in the molecule itself and was not bound to the gamma globulins (Dorrington et al, 1965). After treatment with mercapto-ethanol the L.A.T.S. activity remained with the heavy chain (McKenzie, 1967) but after cleavage with papain, which gave two slow fragments and one fast fragment, the activity, which remained with the slow fragments, was short acting, consistent with a smaller molecular weight and more rapid renal clearance (Meek et al, 1964, Dorrington et al,

1965). The slow fragment is known to contain the antigen binding sites (Porter, 1959). This led to the hypothesis that L.A.T.S., a 7S globulin, was an auto-antibody produced as a result of antigenic stimulation (Dorrington and Munro, 1966, Benhamou-Glynn et al, 1967, McKenzie, 1968).

Kriss et al, in 1964 showed that L.A.T.S. activity could be neutralised by incubating L.A.T.S. serum with thyroid slices and Beall and Solomon in 1966 subsequently were able to remove L.A.T.S. activity from serum by incubating the serum with a thyroidal microsomal fraction. They then eluted the L.A.T.S. from the microsomes under conditions appropriate for dissociation of antibody-antigen complexes. This was confirmed by Benhamou-Glynn et al, (1969), who eluted 23% of L.A.T.S.-IgG but only 1% of normal IgG, and by Wong and Litman who found, in 1969, that eluted L.A.T.S. had a high degree of affinity and specificity for thyroidal microsomes. They suggested that microsomal neutralisation could be used as an in vitro detection system for L.A.T.S. Dorrington et al; in 1966 showed that unfractionated thyroid homogenate completely absorbed L.A.T.S.-IgG. The microsomal fraction was also fully effective in absorbing L.A.T.S. at the same concentration, whereas the nuclear and mitochondrial fractions were less effective and only gave significant absorption at a high concentration. Endoplasmic reticulum

was equally as effective as the whole microsomal fraction.

On the other hand, Berumen et al, in 1967 and Smith in 1969 who fractionated thyroid proteins on Sephadex G-200 found that soluble fractions were the most active L.A.T.S. inhibitors. The former group found that the inhibitory activity could not be separated from the 19S fraction and suggested that thyroglobulin itself may be important in L.A.T.S. absorption. Benhamou-Glynn et al (1969) and Smith (1969) however showed that the L.A.T.S. inhibitory activity was not associated with thyroglobulin.

Gel filtration on Sephadex G-200 of the soluble fraction yields three protein fractions (Salvatore et al, 1964). It is likely that the thyroid specific antigens are associated with the 4S fraction (Shulman et al, 1967, Mates and Shulman, 1969, Smith, 1969).

In the condition of neonatal thyrotoxicosis, a transient form of hyperthyroidism often associated with exophthalmos which occurred occasionally in newborn babies of mothers with past or present Graves' disease (Keynes, 1952, Rosenberg et al, 1963, McKenzie, 1964) both mother and baby had circulating L.A.T.S. (Hoffmann et al, 1966). The subsequent disappearance and spontaneous recovery of the babies was in accordance with a half life of about two weeks for L.A.T.S. IgG is the only protein able to pass the placental barrier (Kohler and Farr, 1966).

Further evidence to support the hypothesis that L.A.T.S. is an antibody was provided in 1967 when it was shown that lymphocytes from thyrotoxic patients, cultured in the presence of the nonspecific lymphocyte stimulant, phytohaemagglutinin (PHA) produced L.A.T.S. (McKenzie and Gordon, 1965). This was confirmed by Miyai et al, in 1967 who showed that the L.A.T.S. activity was neutralised by anti-IgG serum.

Finally, since L.A.T.S. was thought to be an antibody directed against a thyroid tissue component, workers immunized animals with human thyroid fractions and tested the serum for L.A.T.S. activity. L.A.T.S. activity, although differing in its assay response from human L.A.T.S., was consistently found in rabbits immunized with thyroidal microsomes, but not in animals which had received liver microsomes (Beall and Solomon, 1968a). This was confirmed by Burke (1968). McKenzie however was unable to detect L.A.T.S. activity in rabbits immunized with microsomes but found the antibody in animals given whole thyroid extract (McKenzie, 1967).

Although there is thus much evidence that L.A.T.S. is an antibody Burke (1967), using a variety of sensitive immunological tests, was unable to demonstrate antibody-antigen union between L.A.T.S.-IgG and thyroid fractions. Neither was he able to demonstrate selective

thyroidal absorption of parenterally administered ^{131}I-labelled L.A.T.S.-IgG over that obtained by ^{131}I-labelled normal IgG (Burke, 1967).

(5) Relationship of L.A.T.S. to the Clinical Features of Thyrotoxicosis

(a) Introduction

The long acting thyroid stimulator can be detected in the plasma of approximately 40% of patients with thyrotoxicosis. This is increased to about 80% when the IgG fraction, which contains the L.A.T.S. activity, is separated and assayed for L.A.T.S. activity (Munro et al, 1960, McKenzie, 1961, Hoffmann and Hetzel, 1966, Carneiro et al, 1966b). Higher L.A.T.S. levels have been noted in patients with visible goitre and in patients with a recurrence of thyrotoxicosis (Hoffmann and Hetzel, 1966).

(b) Hyperthyroidism

Although the long acting thyroid stimulator acts on the thyroid gland in the mouse (Major and Munro, 1962), guinea pig (Adams, 1958), rabbit (Adams, 1965) and rat (Purves and Adams, 1960), the evidence that it does so in humans is still circumstantial. However, a close correlation has been found between the PBI^{131} divided by the gland mass and plasma L.A.T.S. levels (Carneiro et al, 1966a) and thyroid stimulation was observed, in one study, in human volunteers infused with L.A.T.S.-plasma (Arnaud et al, 1965).

The most impressive evidence that L.A.T.S. stimulates the human thyroid gland lies in the rare occurrence of transient hyperthyroidism in some babies whose mothers had circulating L.A.T.S. at the end of pregnancy (Hoffmann et al, 1966). These findings have made it likely that L.A.T.S. is the cause of the hyperthyroidism of Graves' disease.

(c) Ophthalmopathy

The relationship between L.A.T.S. and the other chief features of Graves' disease however is less certain. Many patients with severe ophthalmopathy have high plasma L.A.T.S. levels (Hoffmann and Hetzel, 1966, Lipman et al, 1967) but the occurrence of severe eye involvement in the absence of detectable L.A.T.S. and the presence of high L.A.T.S. levels with only mild ophthalmopathy makes a direct relationship unlikely (McKenzie and McCullagh, 1968). Some of the series published reported a close association between severe ophthalmopathy and L.A.T.S. (Adams, 1958, Hoffmann and Hetzel, 1966, Kriss et al, 1967). In larger series however, the prevalence of L.A.T.S., whilst being greater in patients in the presence of severe ophthalmopathy was still quite high in patients without eye involvement (McKenzie, 1961, Major and Munro, 1962, Noguchi et al, 1964, Pinchera et al, 1965, Lipman et al, 1967, Kurihara et al, 1967).

(d) Dermopathy

Most patients with thyrotoxic dermopathy have detectable plasma L.A.T.S. and usually in high concentrations (Kriss et al, 1964, Pinchera et al, 1965, Hoffmann and Hetzel, 1966). Although L.A.T.S. has not been observed attached to the skin by methods such as immunofluorescence which labels gamma globulin in situ, it is likely, because of the close association of the skin lesion with plasma L.A.T.S., that L.A.T.S. is involved in the pathogenesis of the dermopathy (Kriss et al, 1964, Hetzel, 1970).

Analysis of the association of L.A.T.S. with the chief manifestations of Graves' disease has shown a close relationship between the presence of L.A.T.S. and the number of features. Patients with mild thyrotoxicosis with only hyperthyroidism, had the lowest prevalence of L.A.T.S. whilst patients with all three features had a very high prevalence of L.A.T.S. (Lipman et al, 1967).

(6) Genetic Aspects of Thyrotoxicosis

Thyrotoxicosis tends to run in families (Bartels, 1941). Monozygous co-twins of thyrotoxic probands have a greater prevalence of the disease than dizygous twins (Ingbar et al, 1956, Vogel, 1959). Thyrotoxicosis has been reported in four sets of monozygous twins (Hassan et al, 1966). These people were also

concordant for thyroid antibodies in significant titres. Thyroid diseases in general tend to run in the same family (Heinmann, 1966). In particular, a familial relationship between thyrotoxicosis and Hashimoto's disease has been observed (Means et al, 1963). A pair of monozygous co-twins, one of whom had thyrotoxicosis and the other who had Hashimoto's disease were followed for several years by Doniach and her colleagues. Both of these patients had detectable L.A.T.S. (Doniach et al, 1967).

The relatives of thyrotoxic patients have an increased prevalence of thyroid antibodies as compared to normal people (Saxena, 1965, Evans et al, 1967). Some of the relatives have an increased iodine turnover (Ingbar et al, 1956) but it is not known whether a latent thyrotoxic state exists in predisposed people as occurs for example in relatives of diabetic patients.

(B) ROLE OF AUTOIMMUNITY IN THYROTOXICOSIS

(1) Immunological Abnormalities

When L.A.T.S. was characterized as an immunoglobulin and found to have properties of an antibody it was postulated that Graves' disease may be an autoimmune disease (Roitt and Doniach, 1958, Adams, 1965, Hetzel, 1968) in which the main clinical features were due to a disorder of immunological tolerance. The disease might

result from the immunological combination of L.A.T.S. and its corresponding antigen, thought to reside in the microsomal fraction of the thyroid cell, and possibly with a similar antigen in the skin and orbital tissues (Kriss et al, 1964).

Patients with thyrotoxicosis had an increased prevalence of other thyroid antibodies (Goudie et al, 1957, Roitt and Doniach, 1958). The presence of these antibodies however did not generally correlate with the presence of L.A.T.S. (Ochi and De Groot, 1968, Beall and Solomon, 1968b) although Pinchera et al, in 1967 found a correlation between L.A.T.S. and antithyroglobulin.

Although an increase in gamma globulin levels has not usually been found in thyrotoxic patients (Lamberg and Grasbeck, 1955) in one study a large proportion of patients had elevated levels of IgG (Yamakido et al, 1969).

The accumulation of lymphocytes and plasma cells in involved tissues was well recognised and in some cases the histological picture in the thyroid resembled Hashimoto's thyroiditis (Levitt, 1951, Levitt, 1952). Steroids and immunosuppressive agents brought about improvement in the dermopathy and ophthalmopathy (Kriss et al, 1964, Lipman et al, 1967) associated with a fall in plasma L.A.T.S. levels (Kriss et al, 1964). There was also some evidence of improvement of the hyperthyroidism in patients treated with steroids (Werner and Platman, 1965).

(2) Relationship to Other Autoimmune Diseases

Thyrotoxicosis is associated with several diseases of possible autoimmune etiology. The tendency for relatives of thyrotoxic patients to develop Hashimoto's disease has been mentioned (Anderson et al, 1964, Jayson et al, 1967). Hashimoto's disease has been considered to be a classical autoimmune disease and its close relationship to Graves' disease cited as evidence that the latter disease is also autoimmune (Hetzel, 1968).

There is a clinical association between thyrotoxicosis and idiopathic Addison's disease (Blizzard and Kyle, 1963, Irvine, 1964, Burke and Feldman, 1965). The tendency for thyrotoxicosis and pernicious anaemia to occur together has also been recognised (McNicol, 1961, Doniach et al, 1963). In one study, 6-7% of patients with Graves' disease were found to have intrinsic factor antibody, and 33% to have gastric parietal cell antibody (Doniach et al, 1963). Thyrotoxicosis is also associated with Sjogren's disease. Patients with Sjögren's disease have an increased prevalence of thyroid antibodies as compared to normals (Bertram and Halberg, 1965).

(3) Thyrotoxicosis as an Autoimmune Disease

Milgrom and Witebsky have proposed certain criteria as requirements for a disease to be considered autoimmune. Firstly, free circulating antibodies active

at body temperature should be demonstrable in all cases. Secondly, the specific antigen should be recognised in the tissues involved in the disease. Thirdly, it should be possible to produce the same antibodies against the antigen in experimental animals and that pathological changes in the corresponding tissues of the animals should occur and be basically similar to those of the human disease. Finally, the disease should be transferable by antibody containing serum or by immunologically competent lymphoid cells (Milgrom and Witebsky, 1962).

In this thesis, the question as to whether thyrotoxicosis can be regarded as an autoimmune disease has been investigated with particular reference to these criteria.

(C) SCOPE OF PRESENT STUDY

Clinical and experimental studies were carried out to test the hypothesis that Graves' disease may be an autoimmune disease.

In the clinical studies assays for the long acting thyroid stimulator (L.A.T.S.) were carried out on the plasma and IgG fraction of patients with thyrotoxicosis. Euthyroid relatives, patients with other autoimmune diseases as well as patients with non autoimmune diseases and normal people were also studied. Other antibodies and immunoglobulin levels were estimated in these patients and family

history of autoimmune diseases or of other thyroid diseases noted.

In Chapter III the relationship between the presence of detectable L.A.T.S. and the number of main clinical features is examined. The prevalence of L.A.T.S. in patients with newly diagnosed thyrotoxicosis or with a recurrence of the disease was compared for plasma and concentrated IgG. One hundred and thirty-five patients were studied over the three year period of the investigation.

The role of L.A.T.S. in the pathogenesis of dermopathy is discussed in Chapter IV. The relationship between plasma L.A.T.S., other antibodies, immunoglobulin levels and the skin lesion was assessed in ten patients. Serial L.A.T.S. measurements were made in six of these patients.

The relationship between the severity of the eye changes and the immunological parameters were studied in all patients with Graves' disease including 12 patients with euthyroid ophthalmopathy. The results of this study are reported in Chapter V.

L.A.T.S. assays were carried out on the IgG fraction of relatives of thyrotoxic patients in four families with a high prevalence of the disease to determine whether L.A.T.S. was present in euthyroid people with a genetically determined tendency to develop thyrotoxicosis. The results of this study are reported in Chapter VI.

Assays for L.A.T.S. were carried out on 17 patients with autoimmune diseases such as disseminated lupus erythematosus, rheumatoid arthritis, and Hashimoto's disease as well as on 17 patients with diseases of possible autoimmune etiology such as Addison's disease, Sjögren's disease and pernicious anaemia, which occur in combination with Graves' disease in a small proportion of cases.

L.A.T.S. assays were also carried out on the IgG fraction from normal people and from patients with other medical diseases to examine the possibility that the antibody may be present in some normal people, and in people with non autoimmune diseases. These studies are reported in Chapter VII.

In the experimental studies, animals were immunized with thyroid antigens to determine whether thyroid stimulating antibody could be produced in the animals, and whether clinical or pathological changes, similar to those of human thyrotoxicosis, occurred in animals with circulating antibody. These are reported in Chapter VIII.

In Chapter IX, experiments in which cultures of peripheral lymphocytes from thyrotoxic patients were set up in the presence of the nonspecific lymphocyte stimulant phytohaemagglutinin, in an attempt to produce L.A.T.S. under conditions appropriate for antibody production, are described.

In the concluding chapter all the evidence is gathered together and discussed in reference to the criteria of Milgrom and Witebsky and the pathological markers of MacKay and Burnet, as to whether thyrotoxicosis can be regarded as an autoimmune disease.

CHAPTER II

METHODOLOGY

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- (b) Classification
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(C) BIOASSAY FOR LONG ACTING THYROID STIMULATOR

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(D) SEPARATION AND CONCENTRATION OF 7S GLOBULINS

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- (2) Preparation of D.E.A.E. Sephadex A-50
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  - (a) Sucrose Concentration
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CHAPTER II Cont'd

(F) IMMUNOGLOBULIN ESTIMATIONS

(G) ESTIMATION OF OTHER ANTIBODIES

(1) Antithyroglobulin
(2) Immunofluorescent Technique

(H) OTHER METHODS

CHAPTER II

METHODOLOGY

(A) CLINICAL DIAGNOSIS OF THYROID DISORDERS

Most of the patients studied were seen at The Queen Elizabeth Hospital, Adelaide, either in the general medical wards or at the special Endocrine Outpatients Clinic. The other patients studied were from other parts of Australia and were not seen personally. In order to standardise the information obtained comprehensive proforma were sent to the referring Medical Officers who gave details of the patient's history, examination findings, laboratory investigations and treatment.

(1) Thyrotoxicosis

(a) Clinical Features

The diagnosis of thyrotoxicosis (Graves' disease) was made on the history of weight loss with excessive appetite, fatigue, diarrhoea, intolerance of hot weather, nervousness, tremor and palpitations. The symptoms varied with age. Younger patients complained of these symptoms, whereas older patients often complained of the symptoms of cardiac failure such as shortness of breath and ankle swelling.

The classical signs of hyperthyroidism including a smooth goitre, hot moist skin, tachycardia,

tremor of outstretched hands and hyperkinesia were found in most cases. Many patients also had eye signs, whilst a few had dermopathy or acropachy. Mild exophthalmos and lid lag with stare were common findings in thyrotoxic patients and did not have the sinister significance of severe exophthalmos with orbital infiltration and ophthalmoplegia.

(b) Classification

The severity of thyrotoxicosis has been classified for the purpose of these studies according to the number of the main clinical features present. These are hyperthyroidism, ophthalmopathy, dermopathy and acropachy.

In mild thyrotoxicosis only one of the features was present. This was usually hyperthyroidism, but in a few patients ophthalmopathy was the only feature. Dermopathy was not seen in the absence of either hyperthyroidism or ophthalmopathy.

Moderate thyrotoxicosis consisted of two of the chief features, usually hyperthyroidism and ophthalmopathy. Finally, in severe thyrotoxicosis all three of the chief clinical features were present. Acropachy was occasionally present in patients with severe Graves' disease.

(c) Other Modes of Presentation

Patients with thyrotoxicosis occasionally presented with other signs and symptoms, less obviously related to over-activity of the thyroid gland. Some of the more common ones included atrial fibrillation with congestive cardiac failure, a severe proximal myopathy or a malabsorption syndrome. Rarely, patients presented with liver disease or a myasthenic syndrome. Two patients had thyroid crises, one of whom died.

(d) Euthyroid Graves' Disease

Several patients with ophthalmopathy and apparently normal thyroid function were studied. Despite being clinically euthyroid however, half have non-suppressible thyroid ^{131}I uptake, and about one third have detectable plasma L.A.T.S. The diagnosis of Graves' ophthalmopathy in this series was often made only by excluding other causes of ophthalmopathy such as orbital tumours and cavernous sinus thrombosis.

(e) Ophthalmopathy

The eye changes of Graves' disease have recently been classified and the previously confusing nomenclature standardised (Werner, 1969). The American Thyroid Association has recommended that this classification be used universally. An abridged version of this classification, which has been used for these studies, is shown in Table 1.

TABLE I

ABRIDGED CLASSIFICATION OF EYE CHANGES

	Class	Eye Changes
MILD	0	No signs or symptoms
	1	Upper lid retraction and stare
SEVERE	2	Soft tissue involvement
	3	Proptosis
	4	Extraocular muscle involvement
	5	Corneal involvement
	6	Optic nerve involvement

Classes 0 and 1 represent mild or non-infiltrative ophthalmopathy with only mild exophthalmos, lid lag and stare. Classes 2 to 6 represent eye changes formerly called "severe", "progressive", or "malignant", with a potentially serious prognosis. The eye changes can be characterized as active or inactive and subgraded according to severity. This classification will be discussed more fully in a later chapter.

(f) Dermopathy

The diagnosis of thyrotoxic dermopathy was based on the presence of bilateral indurated plaque-like or nodular lesions in the pretibial or dermal region of the lower limbs. The diagnosis was confirmed in most cases by the demonstration of inflammatory changes and excess mucoid substance in a biopsy sample of the affected tissues.

(g) Laboratory Investigations

The clinical diagnosis of thyrotoxicosis was confirmed biochemically by finding one or more parameters of increased thyroid function including elevated protein bound iodine (PBI), T_3 resin uptake, total thyroxine or an increased ^{131}I uptake by the thyroid gland at 3 and 24 hours. In some cases, additional investigations were carried out including thyroid scintigraphy with iodine - ^{131}I or pertechnetate - ^{99m}Tc and T_3 suppression test. The most useful tests were found to be PBI and ^{131}I uptake.

(h) Follow up

After treatment, patients were followed at the Endocrine Clinic for variable periods of time. Some patients with severe ophthalmopathy and dermopathy have been followed for several years. At each visit an assessment was made of thyroid function and where present, the severity and extent of the dermopathy and ophthalmopathy. Blood was taken for thyroid function studies, L.A.T.S. estimation and measurement of other antibodies and gamma globulin levels. In some cases photographs were taken to show changes in the ophthalmopathy or dermopathy.

(2) Other Thyroid Disorders

(a) Myxoedema

The diagnosis of myxoedema was made on the classical history of progressive lassitude and weakness associated with apathy, weight gain and intolerance of cold weather. The patients, usually women, became constipated, lost their appetite and developed a hoarse voice, thick skin and brittle hair. Occasionally, if untreated, the patient became comatose.

Patients with myxoedema were usually overweight, with cold scaly skin, a hoarse voice, a slow pulse and sluggish peripheral reflexes with a slow relaxation phase. They often had swelling of the eyelids and face and peripheral oedema. An example of the characteristic facies is shown in Figure I. Occasionally patients

FIGURE 1

MYXOEDEMA

CHARACTERISTIC FACIES

presented with a peripheral neuropathy, pericardial effusion with a myocardopathy or menorrhagia (Macgregor, 1964). Although the usual mental state was one of apathy with slow mentation, drowsiness and poor memory, patients sometimes were nervous and apprehensive. Occasionally they were frankly psychotic.

Although the diagnosis was usually obvious from the history and examination, confirmation was obtained by finding a low PBI, low basal metabolic rate (BMR) increased serum cholesterol level and a low thyroid ^{131}I uptake. Increased levels of plasma TSH were often detected in patients with myxoedema.

(b) Hashimoto's Thyroiditis

Most cases of Hashimoto's disease (autoimmune thyroiditis) are not diagnosed clinically and many doubtless progressed to myxoedema. Quite commonly, the disease was asymptomatic apart from the development of thyroid enlargement. Other patients experienced mild neck pain, pressure manifestations from the goitre and rarely, fever and malaise. On examination, patients with Hashimoto's disease had a firm symmetrical goitre with a smooth or lobulated surface. They were euthyroid.

The diagnosis was confirmed by finding a high titre of antithyroglobulin and thyroid cytoplasmic antibody and an elevated gamma globulin level. The PBI was normal or low with characteristically a PBI - BEI

(Butanol extractable iodine) difference of greater than 2 μg/100 ml. The ^{131}I uptake was normal or high and the BMR usually low. Biopsy of the thyroid showed infiltration with lymphoid tissue, thyroid destruction and fibrosis.

(c) Non-Toxic Goitre

Simple, non-toxic goitres are commonly found in areas of the world where there is a low iodine content in the water and soil, such as the highlands of New Guinea and Tasmania (Buttfield et al, 1966, Buttfield and Hetzel, 1967). Generally, the goitre was large and multi-nodular, occasionally extending retrosternally and obstructing the superior vena cava.

Although most patients with non-toxic nodular goitres in this series came from endemic areas, a few women were studied in whom a physiological goitre, which had developed at puberty or during pregnancy, had persisted and become multinodular.

(d) Toxic Nodular Goitre

Patients with toxic nodular goitre (Plummer's disease) have had a multinodular goitre for many years. One nodule becomes autonomous and its secretion suppresses the rest of the gland. Clinically, the patients had features of hyperthyroidism but not ophthalmopathy or dermopathy (Sheline and McCormach, 1960, Hamburger et al, 1965). The diagnosis was confirmed by finding a single active area

("hot nodule") in the thyroid scintigram with the rest of the gland being underactive (Demeester-Mirkine and Ermans, 1967). The PBI and ^{131}I uptake were elevated but, in contrast to thyrotoxicosis, L.A.T.S. was not detected in the plasma or IgG fraction. Toxic nodules were especially common in elderly women who had had a goitre for many years. Frequently, they presented with atrial fibrillation and congestive cardiac failure.

(B) DIAGNOSIS OF OTHER DISEASES

(1) Diseases Occurring with Thyrotoxicosis

The diseases which occur with an increased prevalence in patients with thyrotoxicosis are Addison's disease, Sjögren's disease and pernicious anaemia. The diagnosis of these diseases was made by clinical assessment and confirmed by appropriate biochemical and immunological investigations. Although organ-specific antibodies have been found in each of these diseases the role of auto-immunity is uncertain.

(2) Other Autoimmune Diseases

Diseases which may have an autoimmune mechanism include rheumatoid arthritis, scleroderma, disseminated lupus erythematosus, chronic active hepatitis and poly-glandular syndrome. In the latter condition, in which several glandular deficiencies occur, particularly diabetes, pernicious anaemia, hypothyroidism and hypoadrenalism, a range of organ-specific antibodies are found.

The diagnosis of autoimmune disorders was made by clinical assessment and laboratory tests of immunological function. Both humoral and cellular aspects were investigated. Auto-antibodies, including antinuclear factor (ANF) thyroid antibodies, smooth muscle antibody and mitochondrial ("M") antibody were estimated, using the indirect immunofluorescent technique (Weller and Coons, 1954). Antithyroglobulin was estimated using the tanned red cell agglutination method (Boyden, 1951). The rheumatoid factor was detected by the Rose agglutination method or by a complement fixation test. Other diagnostic tests used included the lupus erythematosus (LE) cell test, estimation of immunoglobulins, lymphocyte transformation with PHA and serum protein electrophoresis (Forbes, 1970). In many cases, the involved tissues were biopsied and examined for the characteristic lymphocyte and plasma cell infiltration which is associated with autoimmune disease (MacKay and Burnet, 1963).

(3) Other Diseases

Patients with diseases in which autoimmune mechanisms did not play a significant role were also studied. Firstly, patients with other endocrine disorders including panhypopituitarism, acromegaly, diabetes and Cushing's disease were investigated. These diseases were diagnosed clinically by the characteristic features

associated with hormone excess or deficiency, and confirmed by serum and urine estimations of the hormones or their metabolites and by demonstrating the abnormality in provocative tests. Secondly, patients with a variety of diseases including infectious mononucleosis, Hodgkin's disease, disseminated sclerosis and sarcoidosis were investigated.

(C) BIOASSAY FOR LONG ACTING THYROID STIMULATOR

(1) Introduction

The L.A.T.S. bioassay used in this laboratory is based on the method of McKenzie (1958b). The first bioassay was described in 1955 by Adams and Purves. They measured the increase in ^{131}I-labelled thyroid hormones in guinea pigs after injection of test serum containing L.A.T.S. or TSH.

(2) McKenzie Bioassay

In the McKenzie method, albino mice are used instead of guinea pigs. The mice were bred in the laboratory and weaned at four weeks of age and then fed on a dog biscuit diet low in iodine content (100 μg/Kgm). The mice were used in the assay at eight weeks of age.

Each mouse received 6 μCi of ^{131}I intraperitoneally. Endogenous secretion of thyrotropin was suppressed by the subcutaneous injection of 10 μg of L-thyroxine, immediately after the radio-iodine injection,

and by the addition of dessicated thyroid extract (0.1% w/v) to the drinking water for the period of the assay.

The mice were used in the assay procedure three days later. They were numbered using a binary system (Hoffmann, 1965) by placing dots on the head, legs and back with a dye and randomly distributed into groups of five. Groups were allotted the chosen treatments at random. Each mouse of a group received 0.5 ml of the test plasma or IgG solution.

On the day of the assay a pretreatment blood sample was obtained by puncture of the retro-orbital sinus with an 0.1 ml constriction pipette (Figure 2). The blood was plated onto aluminium planchettes for counting. The mice were then injected with 0.5 ml of the test solution intraperitoneally. The control mice received 1% or 5% human serum albumen or Normal saline. The mice were bled at 3, 7, and 24 hours after injection. The increase in radio-activity in the 0.1 ml sample was measured using a gas-flow end-window proportional counter (Nuclear- Chicago, Model D-47), with an automatic sample changer and print-out timer (Figure 3). The time to register 1,000 counts was recorded for each sample, measuring to a constant probable error of counting of 3%. This value was converted to counts per 300 seconds and the background count subtracted.

FIGURE 2

L.A.T.S. BIOASSAY

ORBITAL SINUS PUNCTURE TECHNIQUE

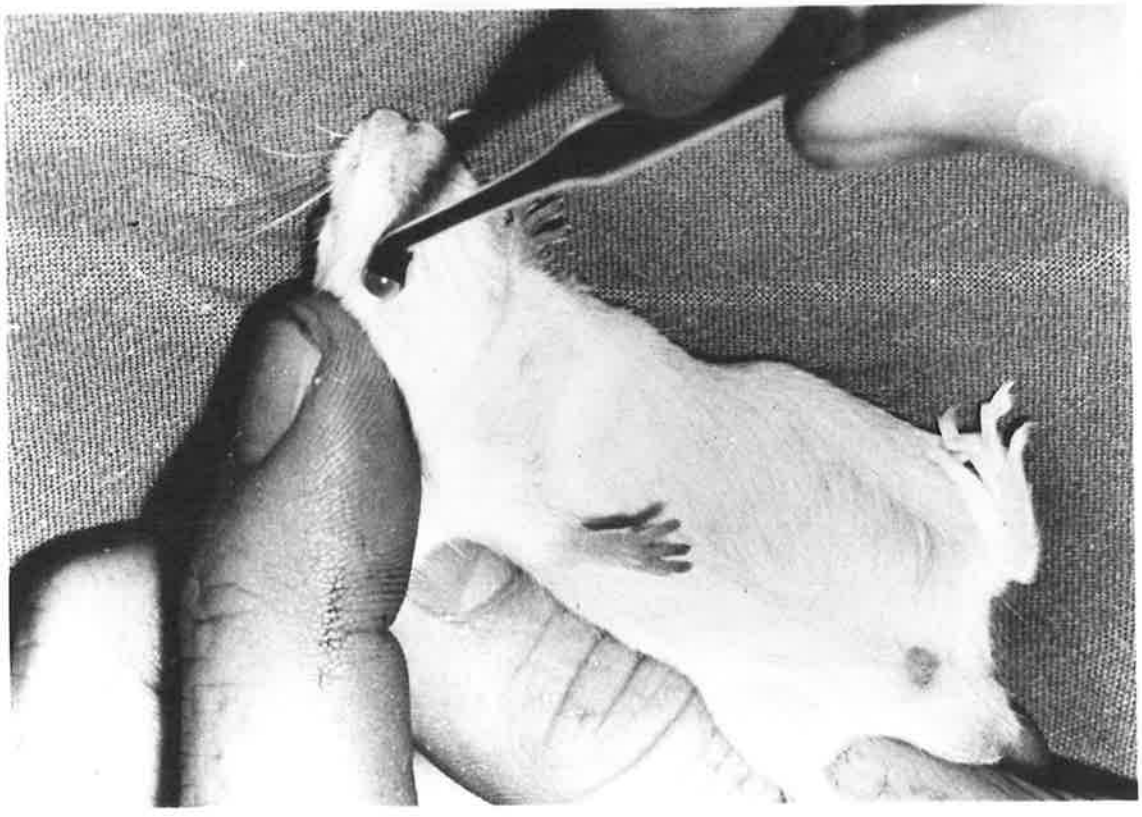
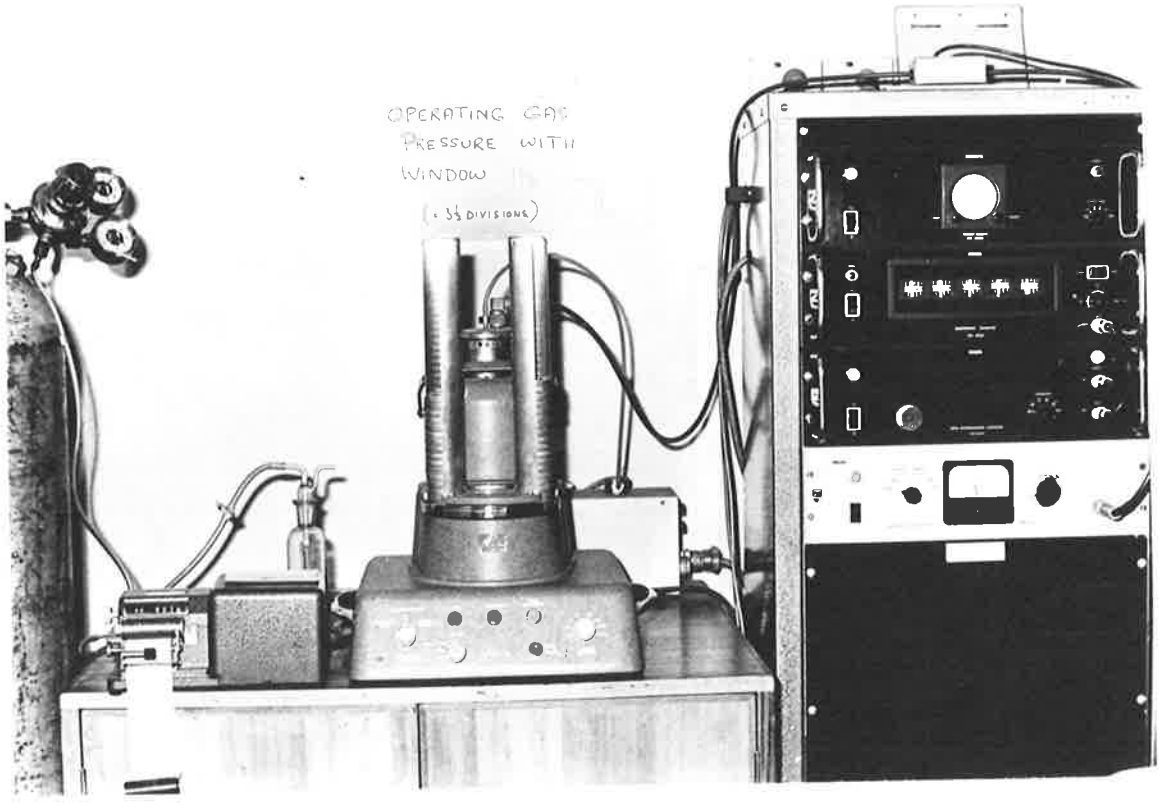


FIGURE 3

L.A.T.S. BIOASSAY

GAS FLOW COUNTER AND AUTOMATIC PRINT OUT TIMER



The percentage increase in radio-activity at 3, 7, and 24 hours over the pretreatment level was calculated and the results analysed by "Student's" test. The results of a typical assay showing raw counts and the calculated percentage increase in radio-activity at 3 and 24 hours are shown. Analysis of the results has been carried out with "Student's" test (Table 2).

(3) Good and Stenhouse Modification

The McKenzie method has been modified by Good and Stenhouse (1966) and applied for the estimation of L.A.T.S. by Mason et al (1967). The design of the assay and the statistical analysis employed permitted elimination of the factors of animal and day variation and the residual effects of the test doses, so that a pure estimate of the response to treatment was obtained (Shishiba and Solomon, 1969).

On day 1 each mouse in a group received the same treatment. On day 2 the treatments were randomised so that the mice of each group received different treatments. The mice were bled at 7 and 24 hours on each day and the counts registered in 300 seconds corrected for background, was recorded for each sample. Analysis of variance of the treatment effects was carried out to test for a significant difference between the means and between the slopes of the dose response curves.

TABLE 2

TYPICAL L.A.T.S. BIOASSAY RESULTS SHOWING RAW COUNTS AND PERCENTAGE INCREASES

Treatment	0 HOURS			3 HOURS				24 HOURS			
	Time(s) for 1000c.	Counts per 300 s.	•Corrected count	Time(s) for 1000c.	Counts per 300 s.	Corrected count	% increase	Time(s) for 1000c.	Counts per 300 s.	Corrected count	% increase
1% H.S.A.	758	396	336	954	314	254	76	1162	258	198	56
	739	406	346	816	368	308	89	812	369	309	89
	856	350	290	747	402	342	118	726	413	353	122
	734	409	349	753	398	338	97	749	401	341	98
	1118	268	208	1170	256	196	94	1410	213	153	74
						Mean	95%		N.S.	Mean	88%
TSH 0.4mU	1541	195	135	784	383	323	239	1223	245	185	137
	939	319	259	430	698	638	246	860	349	289	112
	1059	283	223	529	567	507	227	1141	263	203	91
	1597	188	128	728	412	352	275	1301	231	171	134
	771	389	329	337	890	830	252	633	474	414	126
						Mean	248%			Mean	120%
M.C. Plasma	506	593	533	126	2381	2321	440	44	6818	6758	2900
	1361	220	160	199	1508	1448	905	83	3614	3554	2221
	1011	297	237	158	1899	1839	776	43	6977	6917	2919
	728	412	352	121	2479	2419	687	39	7692	7632	2168
	1361	220	160	239	1255	1195	747	72	4167	4107	2567
						Mean	711%			Mean	*2555%
E.T. Plasma	519	578	518	408	735	675	130	200	1456	1396	269
	961	312	252	881	341	281	112	484	620	560	222
	786	382	322	563	533	473	147	334	898	838	260
	966	311	251	988	304	244	97	577	520	460	183
	812	369	309	854	351	291	94	358	838	778	252
						Mean	121%			Mean	+237%

*=P<0.001: +=P<0.025: NS=Not Significant: •= Background count subtracted

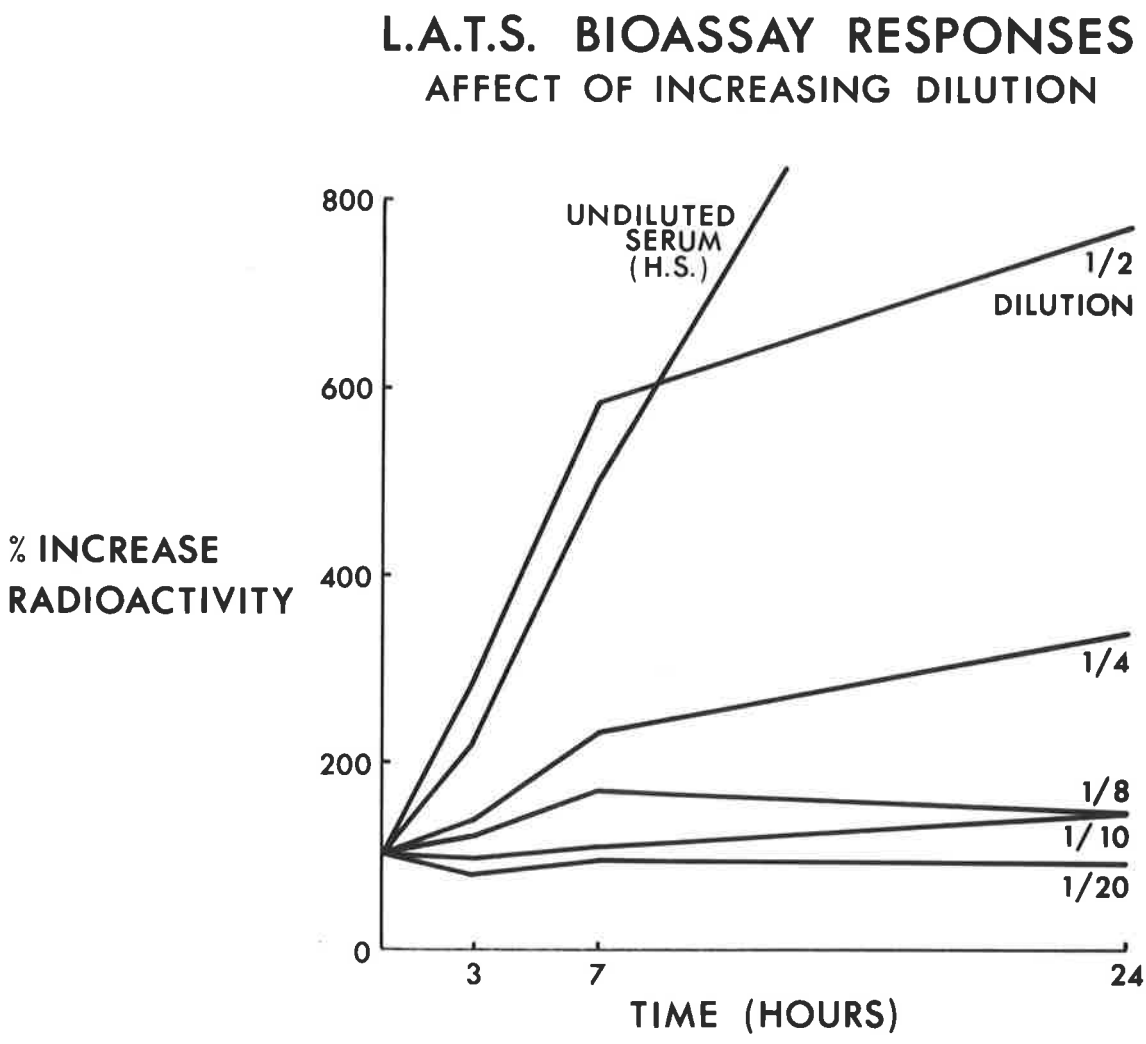
(4) Definition of Positive L.A.T.S. Responses

When L.A.T.S. is present in the test plasma there is a delayed and prolonged stimulation of the mouse thyroid gland with a peak increase in blood radio-activity at 7 or 24 hours, compared with TSH, which gives a peak at 3 hours (Adams and Purves, 1955, McKenzie, 1958a). If the 3-7 hour or 3-24 hour difference is significant (as determined by "Student's" test) or if the 24 hour level is greater than 250%, the response is positive for L.A.T.S. It is usually found that with a 24 hour increase of greater than 200% the 3-24 hour difference is significant. A borderline response has been defined, for the purpose of these studies, as a 24 hour increase of between 200% and 250%.

In these studies "Student's" test was applied to all borderline results and whenever possible, in order to make the results comparable, the 24 hour point was used to decide whether L.A.T.S. was present in the test plasma or IgG concentrate.

With increasing concentrations of L.A.T.S. the assay response curve alters (Figure 4). Low levels of L.A.T.S. give a peak increase in blood radio-activity at 7 hours, and by 24 hours the level is back to control values (100%). As the L.A.T.S. concentration increases the 24 hour reading increases, and at a given concentration it becomes higher than the 7 hour point. With very high

FIGURE 4


L.A.T.S. BIOASSAY RESPONSES
AFFECT OF INCREASING DILUTION
% INCREASE RADIOACTIVITY
800
600
400
200
0
UNDILUTED SERUM (H.S.)
1/2 DILUTION
1/4
1/8
1/10
1/20
3
7
24
TIME (HOURS)

L.A.T.S. concentrations the curve approaches linearity with the peak increase in radio-activity being beyond 24 hours. In the example shown, the change from a 7 hour peak to a 24 hour peak occurred at a 1/6 dilution of a potent L.A.T.S. plasma (Patient H.S.). The 24 hour L.A.T.S. level of the undiluted plasma was 2,000% whereas at a 1/8 dilution L.A.T.S. was not detected.

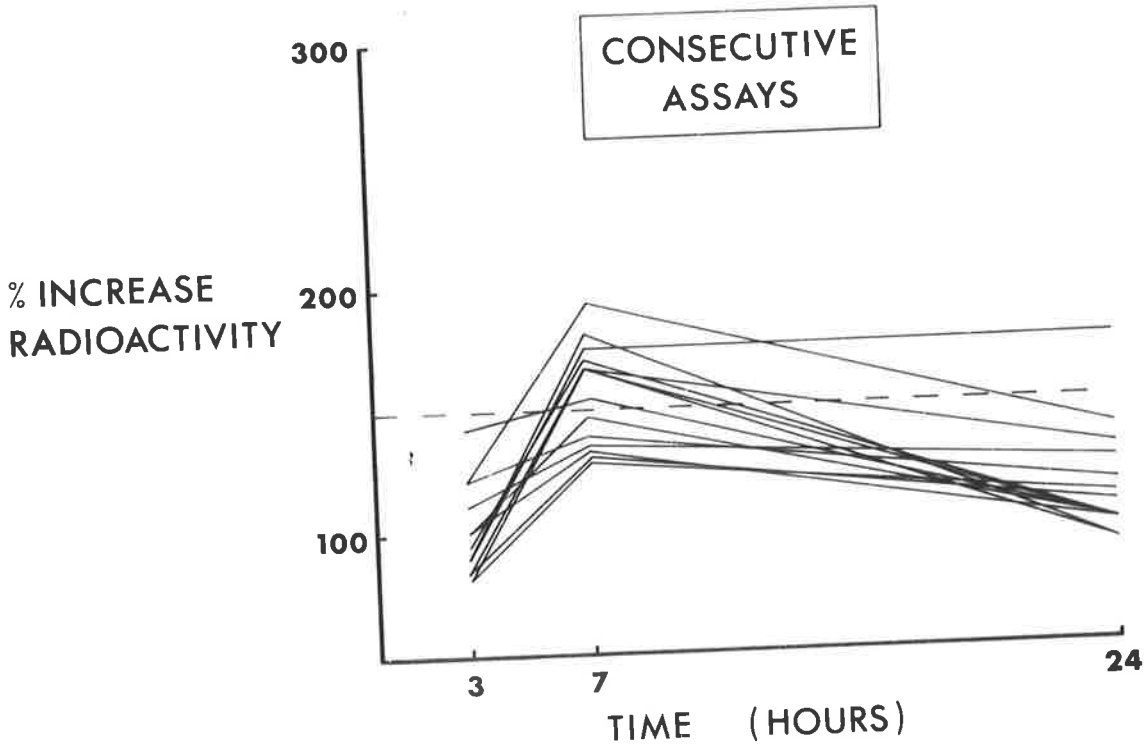
(5) Nonspecific Responses

Nonspecific bioassay responses are given by substances, other than L.A.T.S., which produce a delayed and prolonged stimulation of the test animal thyroid gland such that the 3-7 hour difference or the 3-24 hour difference is statistically significant. Nonspecific responses are given by several substances including normal serum, 5% human serum albumen, dextran-saline, ACTH, and angiotensin (Yamazaki et al, 1961, Major and Munro, 1962, Adams, 1965, Adams et al, 1966, Carneiro et al, 1966b).

Substances which produce nonspecific responses rarely cause significant stimulation at 24 hours so that if a 24 hour increase in radio-activity of greater than 250% is used to indicate L.A.T.S. nonspecific responses will by and large be excluded (Figure 5). On the other hand low levels of L.A.T.S., with a significant 3-7 hour difference but with a 24 hour level of less than 250%, cannot be distinguished from nonspecific responses by statistical analysis.

FIGURE 5

NON SPECIFIC RESPONSES- 5% HUMAN SERUM ALBUMEN



Incubation with anti-IgG serum, which neutralises L.A.T.S. activity but has no effect on nonspecific substances, allows differentiation. Furthermore, concentration of the IgG fraction increases the response given by L.A.T.S., but has no effect on nonspecific responses which are probably caused by substances other than gamma globulins (Adams, 1965, Adams et al, 1966, Carneiro et al, 1966b, Dorrington and Munro, 1966).

(D) SEPARATION AND CONCENTRATION OF 7S GLOBULINS

(1) Introduction

Gamma globulins can be separated from the other plasma proteins by several methods, including differential ammonium sulphate precipitation (Derrien, 1952), sucrose gradient centrifugation (Martin and Ames, 1961), cold ethanol precipitation (Adams and Kennedy, 1962) and by chromatography on diethyl amino ethyl (D.E.A.E.) cellulose (Baumstock et al, 1964).

Separation of the 7S globulins by chromatography on the anion exchanger D.E.A.E. Sephadex A-50 has been used successfully by several groups to purify and concentrate L.A.T.S. (Kriss et al, 1964, Miyai and Werner, 1966, Hoffmann et al, 1967).

Using this method the 7S globulins can be separated from the other plasma proteins in a simple batchwise procedure. The method used in this laboratory is based on the procedure of Baumstock et al (1964).

(2) Preparation of D.E.A.E. Sephadex A-50

Ten g. of dried D.E.A.E. Sephadex A-50 (Pharmacia Chemicals, Uppsala) was weighed out and mixed with 1600 ml of deionized water. This was left for 10 minutes. The supernatant was discarded and the process repeated. The Sephadex solution was then placed in a Buchner funnel and washed with 1 litre of 0.5N NAOH. The residual alkali was removed with about 3 litres of deionized water. This step was repeated with 0.5N HCL.

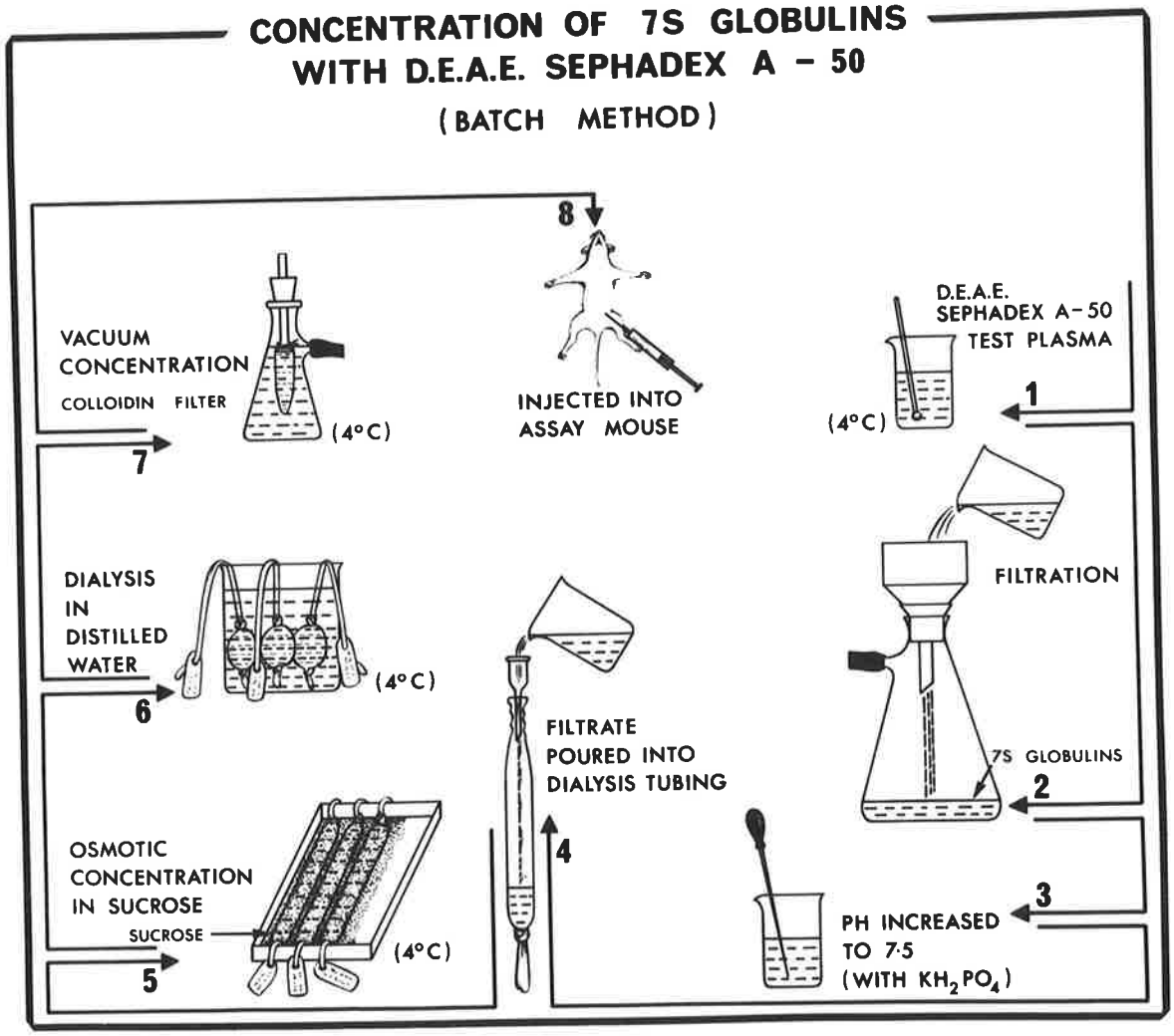
The gel was then suspended in 1600 ml of deionized water and the pH adjusted to 6.5 with 4 ml of 1N NAOH. The Sephadex was allowed to settle and the supernatant was decanted. One litre of phosphate buffer (pH 6.5) was added to the gel which was allowed to stand for 15 minutes. The Sephadex was filtered and washed slowly with 1 litre of buffer. The excess buffer was drawn off and the moist gel removed from the funnel and placed in a covered container. The Sephadex gel was kept at 4°C until use (Baumstock et al, 1964, Perper and Okimoto, 1967).

(3) Separation of 7S Globulins

Approximately 100 gm of moist Sephadex gel was mixed with each 50 ml of test plasma in a beaker, together with an equal volume of phosphate buffer. The mixture was left at 4°C for 3 hours (Figure 6, Step 1).

The plasma-Sephadex mixture was then filtered in a Buchner funnel and washed with phosphate buffer (Step 2).

FIGURE 6


CONCENTRATION OF 7S GLOBULINS
WITH D.E.A.E. SEPHADEX A - 50
(BATCH METHOD)
8
INJECTED INTO
ASSAY MOUSE
D.E.A.E.
SEPHADEX A-50
TEST PLASMA
(4°C)
1
VACUUM
CONCENTRATION
COLLOIDIN FILTER
(4°C)
7
FILTRATION
DIALYSIS
IN
DISTILLED
WATER
(4°C)
6
FILTRATE
POURED INTO
DIALYSIS TUBING
7S GLOBULINS
2
4
OSMOTIC
CONCENTRATION
IN SUCROSE
SUCROSE
(4°C)
5
3
PH INCREASED
TO 7·5
(WITH KH_2PO_4)

This separates the 7S globulins from the other plasma proteins which remain attached to the Sephadex gel.

Finally, the pH of the filtrate was adjusted to 7.5 with $KH_2 PO_4$ (Step 3).

(4) Concentration of 7S Globulins

(a) Sucrose Concentration

The filtrate was poured into one inch dialysis tubing, covered by sucrose (Step 4), and left at 4°C overnight. During this period the volume reduced to about 10% of the original volume (approximately 30 ml) by dialysis across a gradient produced by the hyperosmolar sucrose (Step 5). The 7S globulin solution was washed to the bottom of the tubing, which was tied off. The balloon so formed was suspended in 3 litres of deionized distilled water for about 18 hours (Step 6). Excess sucrose, salts and toxic peptides diffused from the solution into the water, leaving a relatively non-toxic solution.

(b) Vacuum Dialysis

The 7S globulins were further concentrated by the process of vacuum dialysis. The solution was removed from the balloon and placed in a colloidin bag (Sartorius Membranfiltergesellshaft, Gottingen, Germany) attached to a Buchner flask (Step 7), which was in turn connected to a pump which produced a vacuum in the distilled water surrounding the bag. Water was drawn out of the

colloidin bag, reducing the volume of the filtrate to 3 ml in about 18 hours. From 6-8 samples could be concentrated in this way (Figure 7).

(c) Lyopholisation

The more commonly used method for concentrating 7S globulins however is freeze drying (Lyopholisation). In this procedure, which has been used by most workers for the concentration of L.A.T.S., the filtrate is shell frozen and concentrated under a vacuum (Kriss et al, 1964, Baumstock et al, 1964, Werner and Miyai, 1965).

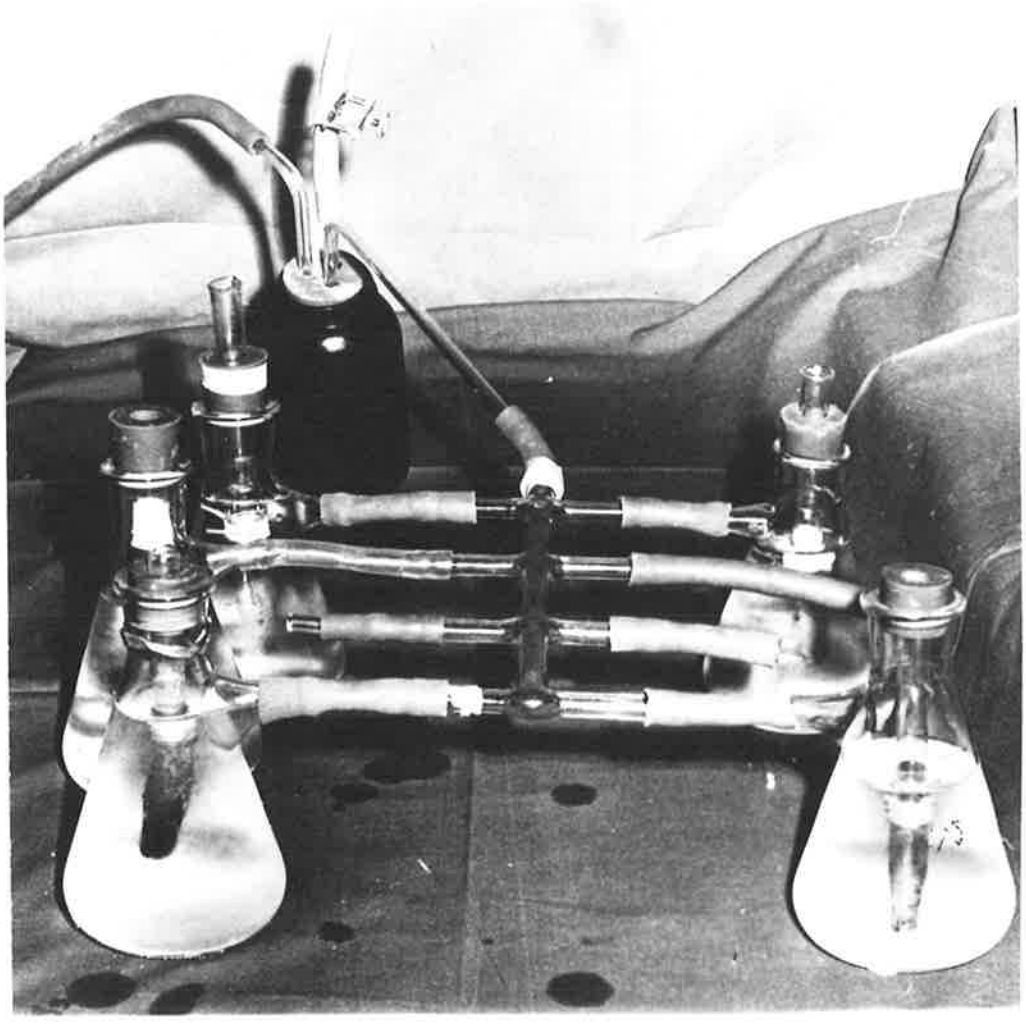
Because of previous experience from this laboratory when it was found that as much as 60% of the theoretical gamma globulin yield was lost during lyopholisation and because of the technical difficulties associated with this procedure an alternative method for concentration of L.A.T.S. was sought. Concentration by dialysis against sucrose followed by vacuum dialysis across a colloidin filter (as described above) was clearly a simple and relatively gentle process, particularly as the whole procedure could be carried out at $4^{o}C$.

A comparison of vacuum dialysis and lyopholisation was subsequently made by measuring the yield of gamma globulins and the L.A.T.S. assay responses after concentration of a L.A.T.S. positive serum by the two methods.

FIGURE 7

NEGATIVE PRESSURE CONCENTRATION

(VACUUM DIALYSIS)



A five ml aliquot of plasma was removed for assay of plasma L.A.T.S. The remainder was divided into two equal aliquots. Each half was treated in the way described above until the last concentration step. One aliquot was then concentrated by vacuum dialysis whilst the other was lyopholised.

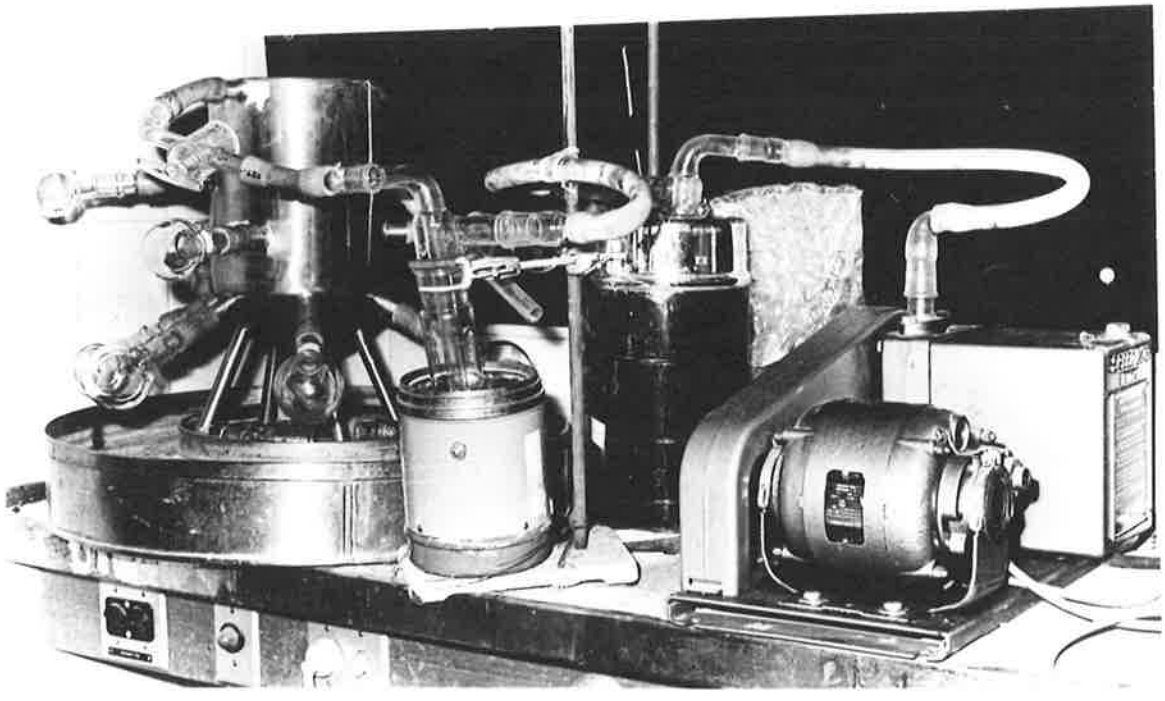
In the latter procedure the filtrate was frozen onto the surface of a round bottomed flask by rotating the flask in absolute alcohol to which was added dry ice. The flask was attached to an electrically operated vacuum pump, cooled by an alcohol - dry ice mixture (Figure 8). The frozen 7S globulin solution gradually receded down the inside of the flask, leaving, after about 8 hours, a small amount of dry powder at the bottom of the flask. This was dissolved in 3 ml of isotonic saline for injection into the assay mice.

The plasma and the two concentrated samples were assayed for L.A.T.S. activity using the McKenzie method. The protein concentrations of the unconcentrated plasma, the 7S globulin filtrate and the two aliquots after concentration were determined using the spectro-photometric method (to be described). From these results the recoveries of 7S globulins after concentration could be calculated.

FIGURE 8

CONCENTRATION OF 7S GLOBULINS

FREEZE DRYING APPARATUS



Results

The recovery of 7S globulins after concentration was 70.2% when freeze drying was used for the final concentration and 82.4% when vacuum dialysis was used. The corresponding 24 hour L.A.T.S. levels were 2678% and 3379%, and the 3-7 hour differences 173% and 928% respectively (Table 3).

These results indicated that freeze drying was more destructive of L.A.T.S.-IgG than vacuum dialysis.

(5) T.S.H. Concentration

The assay response given by TSH is characteristically short lived with a peak at 3 hours. Repeated injections of TSH however, can give a L.A.T.S.-like response (Hoffmann et al, 1967).

It is not known exactly how TSH, a low molecular weight glycoprotein, is carried in the plasma, but it is probably attached nonspecifically to several fractions. Thus TSH may also be attached to gamma globulins (McKenzie, 1968).

An experiment was designed to determine whether TSH attached to the 7S globulins sufficiently firmly to concentrate with this fraction in the procedure outlined above. If it could be demonstrated that TSH did not concentrate with the 7S globulins, TSH-like assay responses could not have been due to TSH itself.

TABLE 3

CONCENTRATION OF 7S GLOBULINS

Comparison of % recoveries and L.A.T.S. levels after vacuum dialysis and lyopholisation as the final concentration procedure.

Final Concentration Procedure	*L.A.T.S. Levels (% increase)			% Recovery of 7S Globulins after concentration
	3 hours	7 hours	24 hours	
Vacuum Dialysis	663	1591	3379	82.4%
Lyopholisation	324	497	2678	70.2%

* PLASMA L.A.T.S. LEVEL = 330-1130-1820

Four mU of standard TSH was added to 40 ml of normal plasma and incubated at 37°C for 5 hours, and then at 4°C overnight. Forty ml of plasma, but without TSH, was incubated for the same period. Each aliquot was then mixed in a beaker with D.E.A.E. Sephadex A-50 gel and 40 ml of phosphate buffer. The 7S globulins were filtered and concentrated by sucrose concentration and vacuum dialysis.

Each IgG concentrate was made up to a volume of 3 ml and assayed for TSH-activity (Table 4). The results indicated that no detectable TSH was present in either of the concentrated IgG fractions. Since the smallest amount of TSH that can be detected in the bioassay in this laboratory is approximately 0.1 mU, less than 10% of the added 4mU had been concentrated with the 7S globulins.

(E) PROTEIN DETERMINATIONS

During the separation and concentration of the 7S globulins small aliquots of the plasma, filtrate and final concentrate were removed for protein determinations. The protein concentrations were calculated by the spectrophotometric method utilising Behr's Law, which states that "the amount of protein in a test sample is proportional to the optical density (O.D.) for a greater than 1/1,000 dilution of a normal serum sample." At 210 mλ wavelength

TABLE 4

CONCENTRATION OF 7S GLOBULINS
- TSH ADDED TO PLASMA

Treatment	% Increase Radioactivity		
	3 hour	7 hour	24 hour
Normal Plasma	86	103	72
Concentrated IgG	140	218	144
Concentrated IgG + 4mU TSH initially	121	97	103
0.1 mU TSH	293	253	114

most of the optical density reading is due to gamma globulins (Tombs et al, 1959).

A standard curve was drawn by reading the optical density of 3 dilutions (1/60, 1/30 and 1/15) of a known protein solution (Armour Pharmaceutical Co., 625 g protein). The total plasma protein concentration before separation of the 7S globulins was calculated by reading off the protein concentration corresponding to the O.D. of a 1/2,000 dilution of the test plasma.

The protein concentration of the filtrate (consisting largely of 7S globulins), was estimated by reading the optical density of a 1/50 dilution of filtrate. From these values the percentage recovery of 7S globulins from plasma before concentration was calculated.

Similarly, by calculating the amount of protein present after concentration of the filtrate the percentage recovery of 7S globulins from the filtrate after concentration and the overall concentration factor could be determined.

The total plasma protein concentration, recovery of gamma globulin before and after concentration of the filtrate, and the concentration factor are shown for 20 patients in whom separation and concentration of the 7S globulin fraction was carried out (Table 5). The mean recovery of 7S globulins after concentration was 64.4% and the mean concentration factor was 6.5.

TABLE 5

PROTEIN DETERMINATIONS OF PLASMA, FILTRATE AND 7S γ-GLOBULIN CONCENTRATE

Plasma Protein (g%)	Filtrate Protein (mg%)	% recovery γ-globulin before concentration	% recovery after concentration	Concentration Factor
6.7	454	19.5	57.5	6.8
7.2	302	16.6	69.9	5.8
6.7	235	16.7	71.1	6.0
8.5	155	11.4	61.3	3.3
6.9	135	10.8	54.8	3.2
8.3	320	14.9	55.0	4.7
7.1	244	14.4	84.4	6.7
7.0	387	12.1	63.0	9.5
6.2	200	9.8	68.0	7.7
7.1	380	14.1	63.9	8.2
7.5	286	16.5	74.5	5.8
4.5	250	18.5	48.0	4.8
6.0	365	15.2	74.0	9.6
5.7	181	12.1	77.3	6.6
5.3	153	9.4	72.5	7.6
8.9	230	7.5	53.9	6.2
6.9	340	15.4	58.8	6.2
8.9	426	15.8	63.4	6.3
6.3	445	20.7	51.0	5.8
7.1	304	10.6	66.1	8.9

(F) IMMUNOGLOBULIN ESTIMATIONS

Introduction

The three main classes of immunoglobulins are immunoglobulin G (IgG), immunoglobulin M (IgM) and immunoglobulin A (IgA). These were measured by the radial diffusion method (Ouchterlony, 1949, Mancini et al, 1965) using commercial "Partigen plates" (Behringwerke AG, Marburg).

When test serum containing antigen is placed in a cylindrical well cut in agar mixed with a known concentration of the antibody, the antigen diffuses into the surrounding agar and reacts with the antibody forming a precipitation ring. The concentration of the antigen in the serum is proportional to the radius of the precipitation ring, and can be calculated by constructing a standard curve with dilutions of a standard serum containing a known concentration of the antigen.

Method

Two μl of diluted test serum containing an unknown amount of IgG, IgM or IgA was placed in one of the wells in the agar gel. About 30 unknowns could be tested on each plate. Three dilutions of stabilized standard human serum (Behringwerke AG, Marburg) were placed in adjacent wells and the plate was left at room temperature for 48 hours. The diameters of the rings were then

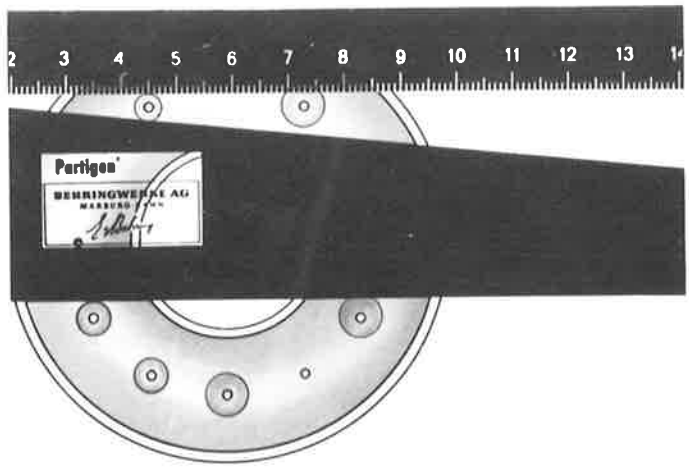
measured in two planes with a vernier rule. A standard curve was constructed from the diameters of the control rings and the corresponding concentrations of antigen. Over a wide range of immunoglobulin concentrations the amount of antigen is proportional to the diameter of the ring (Mancini et al, 1965). The concentration of the antigen in the unknown serum could be calculated by reading off the concentration of antigen corresponding to the diameter of the precipitation ring.

The method is shown diagrammatically (Figure 9). In order to estimate the concentration of IgG in an unknown serum, three dilutions, 1/10, 1/30 and 1/50 of standard serum were placed in the first three wells of the anti-IgG plate. A 1/40 dilution of the unknown serum was placed in the fourth well. The diameters of the standard rings were 8.3, 6.4 and 4.7 mm respectively, after 48 hours. Standard serum contains 740 mg% of IgG, so the concentration of IgG at these dilutions was 74.0, 24.7 and 148 mg% respectively. The diameter of the unknown ring was 5.9 mm. Reading from the standard curve, the concentration of IgG in the diluted serum added to the well was 31 mg%. The concentration of IgG in the patient's serum was thus, 1240 mg% (31 x 40). The concentration of IgA and IgM could be calculated in the same way using the corresponding antiserum and appropriate dilutions of the standard serum.

FIGURE 9

IMMUNOGLOBULIN ESTIMATION RADIAL DIFFUSION METHOD

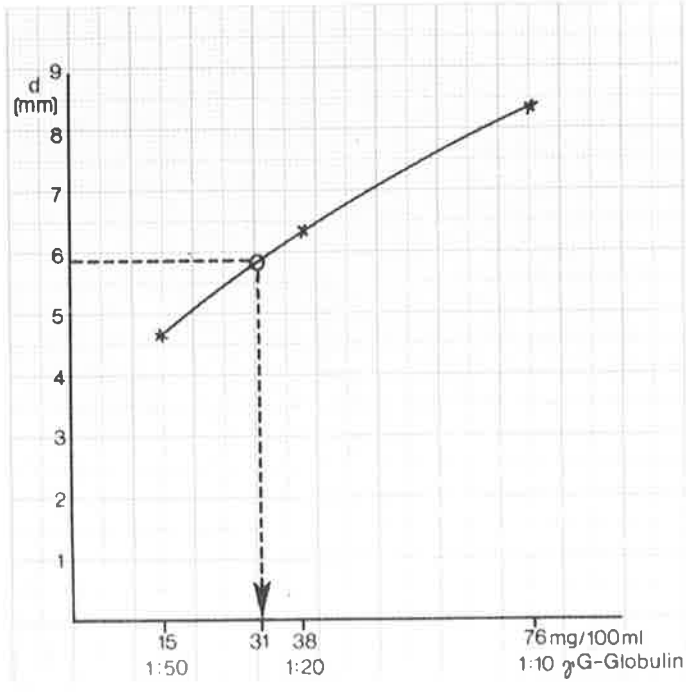
a) Measure the diameter (d) of the precipitate rings from the lower surface of the plate, using a measuring grid



b) Construction of the reference curve

Dilutions of standard serum	γG-globulin concentration	measured diameter (d)
1:50	15 mg/100 ml	e. g. 4,7 mm
1:20	38 mg/100 ml	6,4 mm
1:10	76 mg/100 ml	8,3 mm

Reference curve (example):



Read off the concentration of γG-globulin in the patient's serum by reference to the standard curve

Example: the diameter of the precipitate ring formed from the 1:40 dilution of the patient's serum is 5.9 mm

5.9 mm ≙ 31 mg/100 ml
31 × 40 = 1240 mg/100 ml

The normal ranges established in this laboratory for IgG, IgM and IgA were:

IgG - 830.7 ± 234.8 (Mean ± 2 S.D.)

IgM - 95.8 ± 51.3

IgA - 216.6 ± 94.2

(G) ESTIMATION OF OTHER ANTIBODIES

(1) Antithyroglobulin

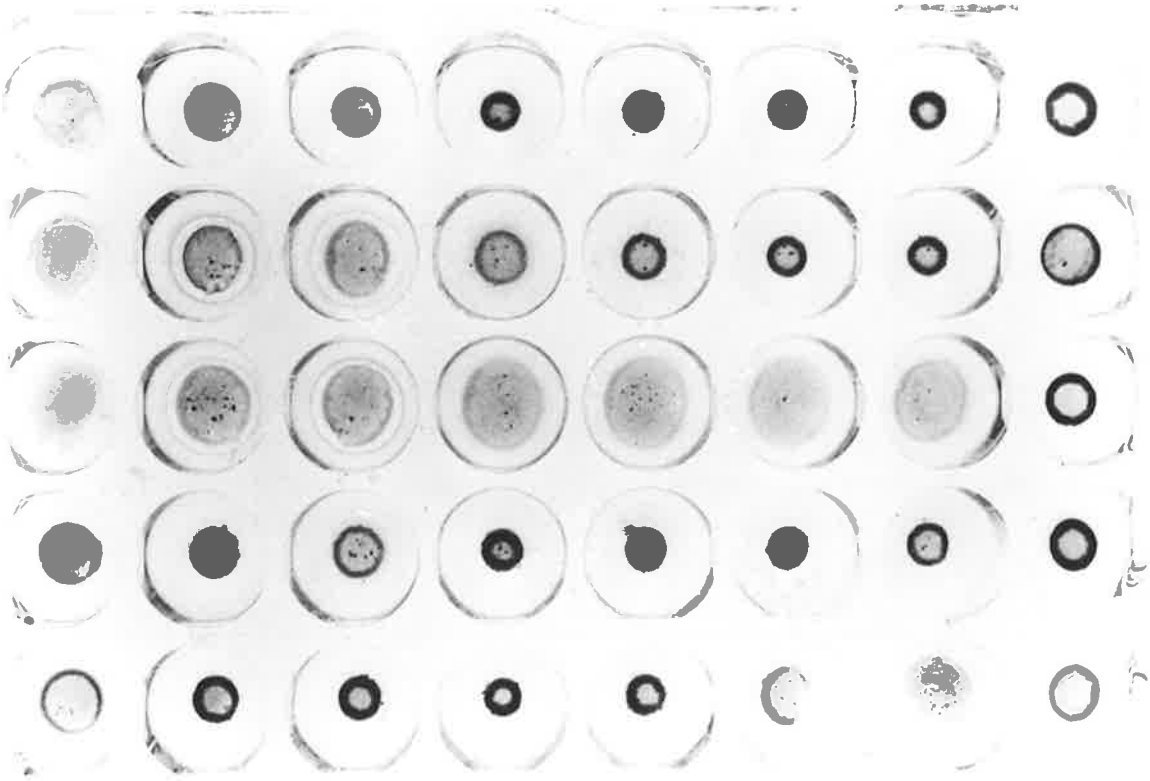
Antithyroglobulin was measured by the sensitive tanned red blood cell method of Boyden (1951). Sheep red cells tanned and sensitized with human thyroglobulin (Burroughs Wellcome, England) were added to serial dilutions of the test serum in a perspex agglutination tray. Tenfold dilutions were used, ranging from 1/25 up to 1 in 2.5 million. Sensitized cells (0.1 ml) were added to 0.1 ml of diluted serum in each well. The same volume of a control (unsensitized) suspension of cells was added to the final well.

In the presence of the antibody the red cells agglutinated, forming a diffuse film on the bottom of the well. If there was no antibody present the cells settled to the bottom of the well as a solid button or discreet ring (Figure 10). The titre represented the greatest dilution of test serum which gave 3+ or greater agglutination, as determined by an arbitrary scale, namely:

FIGURE 10

ANTITHYROGLOBULIN ESTIMATION

TANNED RED CELL AGGLUTINATION METHOD



0 = no agglutination (discreet button or ring)

\+ = slight agglutination

++ = definite, diffuse agglutination

+++ = moderate agglutination

++++ = peripheral clumping of cells only - agglutination almost complete

+++++ = complete agglutination

For example, the agglutination pattern given by serum 2 in Figure 10 was 5, 4, 4, 2, 0, 0, 0, 0. The titre was thus 1/250. In these studies a titre of less than 1/250 was not considered significant since many otherwise normal people had low titres of the antibody. In a series of 43 blood donors, unselected for age and sex, 7% had significant titres of antithyroglobulin whilst a further 20% had titres of less than 1/250.

(2) Immunofluorescent Technique

Thyroid cytoplasmic antibody, gastric parietal cell antibody and the smooth muscle antibody were detected by the immunofluorescent method (Weller and Coons, 1954).

In this method, the attachment of serum antibody to a tissue antigen is rendered visible by counterstaining with anti-human globulin conjugated with fluorescein isothiocyanate.

Frozen sections of human group O gastric mucosa and thyrotoxic thyroid gland, 6 microns thick, were placed

close together on a glass slide and air dried for 30 minutes. A small amount of 1/4 dilution of the serum to be tested was placed on the sections and the slides were incubated for 30 minutes at 37°C. During this period antibody attached to the corresponding tissue antigen.

After washing off the excess serum with Coon's buffer, fluorescein labelled anti-gamma globulin was placed on the sections, which were incubated for a further 30 minutes at 37°C.

The slides were washed again, dried, and mounted in a mixture of glycerine and Coon's buffer. The sections were examined under a Leitz ultra-violet microscope with a HBO, 200 lamp as the light source, a UG 1 exciting filter and K 430 absorption filter. The presence of antibody in the test serum was evident as bright fluorescence limited to the distribution of the corresponding antigen in the tissue.

Thyroid cytoplasmic antibody combines with an antigen in the thyroid microsomes and is seen as diffuse cytoplasmic fluorescence with a clear, unstained central nucleus (Figure 11). Similarly, gastric parietal cell antibody reacts with a cytoplasmic antigen in the parietal cells of the gastric mucosa and is seen as a diffuse cytoplasmic fluorescence (Figure 12). The smooth muscle antibody produced a linear pattern in the mucosa of the

FIGURE 11

IMMUNOFLUORESCENT TECHNIQUE
THYROID CYTOPLASMIC ANTIBODY

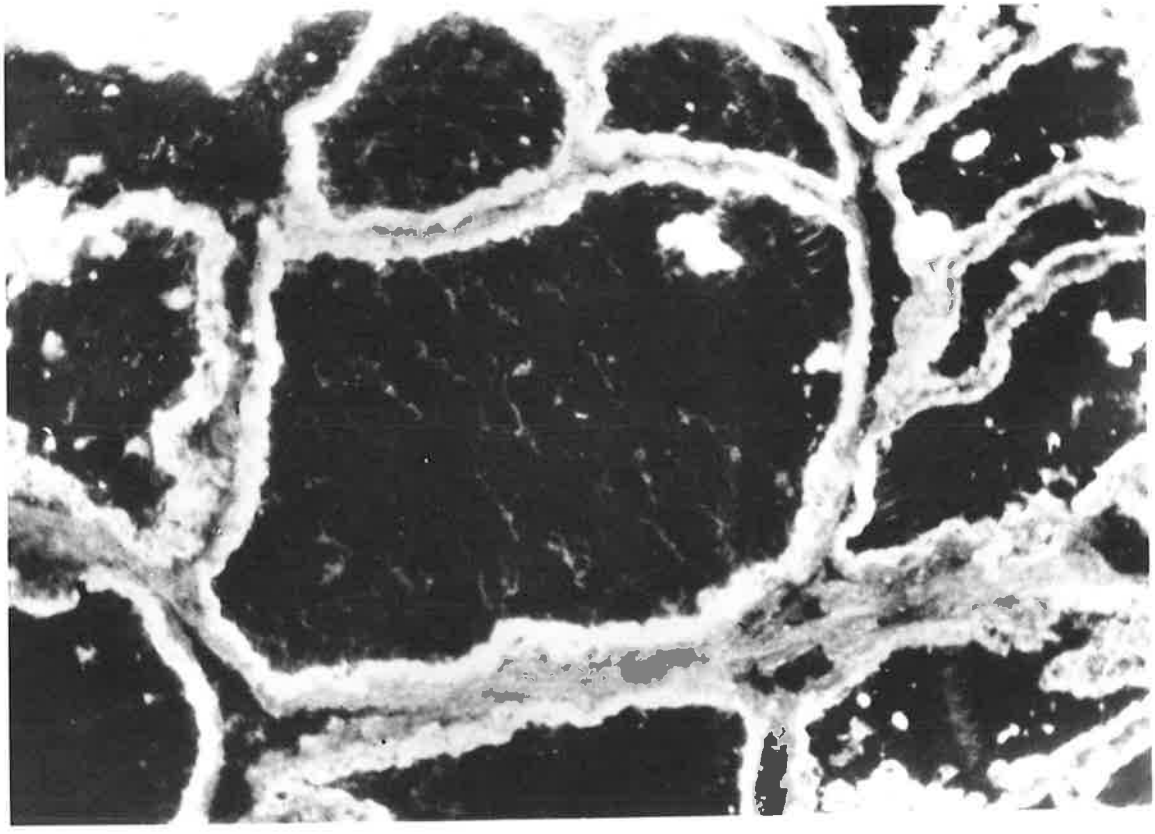
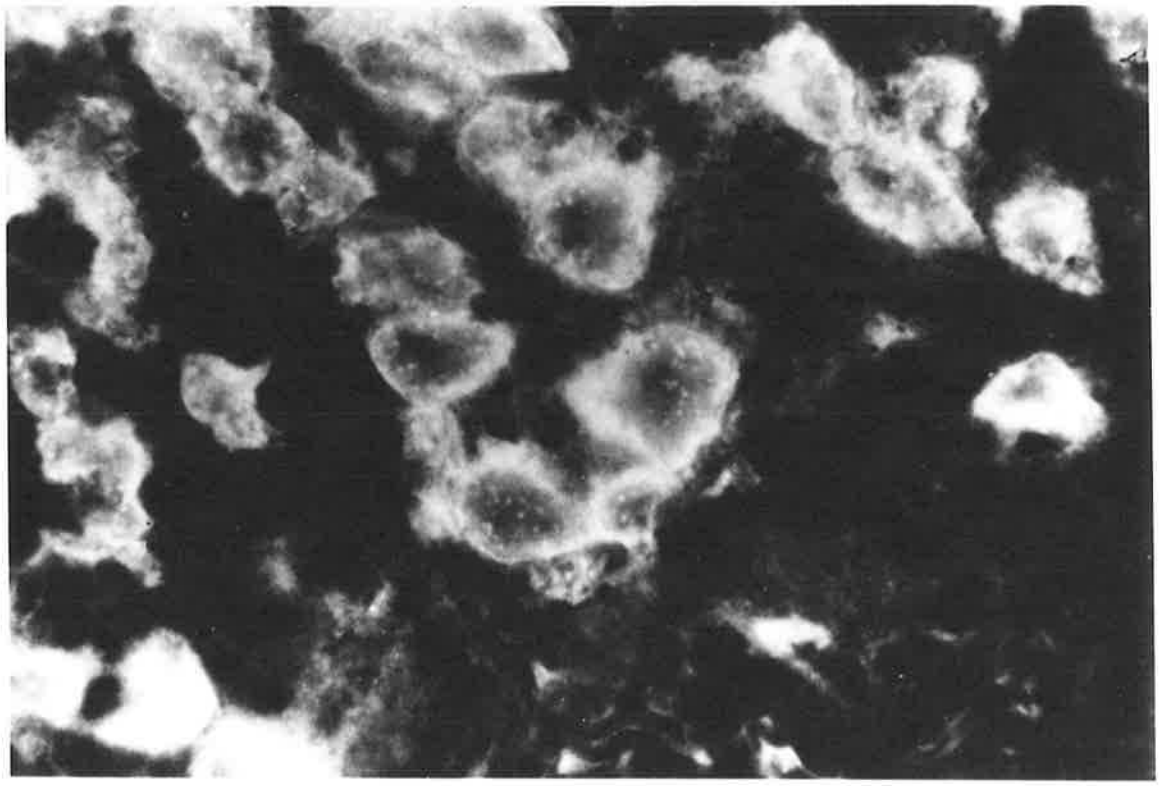


FIGURE 12

IMMUNOFLUORESCENT TECHNIQUE
GASTRIC PARIETAL CELL ANTIBODY



stomach, as well as in the muscular coat of the small arteries. Occasionally, antinuclear factor may be present in sufficiently high titres to be demonstrated in gastric or thyroid tissue. It produced a nuclear fluorescence in an otherwise unstained cell.

The intensity of fluorescence was graded on an arbitrary scale. Borderline fluorescence was considered negative, definite fluorescence was recorded as + and marked fluorescence as ++. Rarely, the fluorescence was extremely marked and classified +++.

(H) OTHER METHODS

The other methods used in the experimental studies will be described in detail in the subsequent chapters. These methods include the setting up of lymphocyte cultures, immunization procedures for experimental animals and the histological classification of pathological changes in the thyroid gland after immunization.

CHAPTER III

THYROTOXICOSIS

(A) INTRODUCTION

(B) CLINICAL SUBJECTS

(C) METHODS AND MATERIALS

(D) RESULTS

(1) Mild Thyrotoxicosis
- (a) Clinical Findings
- (b) L.A.T.S. Assays
- (c) Antibody Titres
- (d) Immunoglobulin Levels

(2) Moderate Thyrotoxicosis
- (a) Clinical Findings
- (b) L.A.T.S. Assays
- (c) Antibody Titres
- (d) Immunoglobulin Levels

(3) Severe Thyrotoxicosis
- (a) Clinical Findings
- (b) L.A.T.S. Assays
- (c) Antibody Titres
- (d) Immunoglobulin Levels

(4) Recurrence of Thyrotoxicosis
- (a) Clinical Findings
- (b) L.A.T.S. Assays
- (c) Antibody Titres
- (d) Immunoglobulin Levels

(E) DISCUSSION

(F) SUMMARY AND CONCLUSIONS

CHAPTER III

THYROTOXICOSIS

(A) INTRODUCTION

Thyrotoxicosis consists of several main clinical features. These are goitre, usually with hyperthyroidism, ophthalmopathy, dermopathy and rarely, acropachy. They may occur singly or in combination. Most patients have hyperthyroidism which produces many of the characteristic signs and symptoms of the disease. Ophthalmopathy is the next most common feature. Many patients have mild eye signs but only about 10% have severe ophthalmopathy. About 3% of patients develop infiltrative dermopathy (Solomon et al, 1968). Acropachy is even less common (Lipman et al, 1967, Hetzel, 1968, McKenzie, 1968).

It has long been recognised that the more severe cases have, as well as hyperthyroidism, severe ophthalmopathy and dermopathy (Kriss et al, 1964, Lipman et al, 1967). These patients, who usually have high plasma L.A.T.S. levels, require vigorous treatment to prevent progression of the eye lesion which sometimes leads to blindness. With the availability of satisfactory treatment for hyperthyroidism, the chief morbidity of the disease is in fact now due to eye damage.

The severity of thyrotoxicosis has been classified for these studies according to the number of clinical features present. In mild cases only hyperthyroidism or ophthalmopathy is present. Patients with moderate thyrotoxicosis have two of the main features, usually hyperthyroidism and ophthalmopathy but occasionally hyperthyroidism and dermopathy. In severe thyrotoxicosis all three features are present and sometimes acropachy as well (Lipman et al, 1967, Solomon et al, 1968).

(B) CLINICAL SUBJECTS

One hundred and thirty-five patients with Graves' disease were studied over a three year period. Most of these were new cases, whilst 16 had relapsed, following previous treatment. Many of these patients were seen at the special Endocrine Clinic at The Queen Elizabeth Hospital. Other patients were treated elsewhere, plasma being sent to this laboratory for L.A.T.S. and antibody estimation. Clinical details were obtained from the referring physician, so that full assessment of the severity and course of the disease was possible. On some occasions, a second sample of blood was obtained after treatment to compare L.A.T.S. levels and antibody titres before and after treatment.

In this chapter, thyrotoxicosis will be discussed with particular reference to the prevalence of L.A.T.S., other

antibodies and increased levels of immunoglobulins, and the relationship of these to the severity of the disease and to treatment. In the next two chapters, dermopathy and ophthalmopathy will be discussed individually.

The clinical diagnosis of thyrotoxicosis has been discussed in Chapter II. At the initial examination assessment was made of the thyroid status and eye involvement, and the legs were examined for the presence of dermopathy. The patient was questioned about previous illnesses, particularly thyroid disorders and other autoimmune diseases and for family history of thyroid or autoimmune diseases.

The diagnosis of hyperthyroidism was confirmed biochemically. Whenever possible 100 ml of blood was drawn for IgG concentration and L.A.T.S. assay.

Patients were followed at the Endocrine Clinic until a satisfactory response to treatment was obtained. With the development of severe ophthalmopathy or dermopathy patients were reassessed, often in consultation with an ophthalmologist, and further treatment undertaken. Several patients who developed severe ophthalmopathy or dermopathy have been followed at the Clinic for many years.

In most cases, particularly where hyperthyroidism alone was present, definitive treatment with ^{131}I or subtotal thyroidectomy was successful. These patients were discharged

after euthyroid status had been achieved. Patients with high L.A.T.S. levels, because of the risk of development of severe ophthalmopathy, were followed for several months after treatment. Some of the successfully treated patients however would eventually become hypothyroid and a few would have a recurrence of their thyrotoxicosis.

Many of the patients referred from other centres for L.A.T.S. assay had severe ophthalmopathy or dermopathy. Because of this, the series is biased toward patients with severe disease.

Finally, a group of 13 patients was studied in whom thyrotoxicosis occurred in combination with other diseases. In each case the thyrotoxicosis was mild and responded to treatment. These diseases, many of which may have an autoimmune mechanism, are listed in Table 6.

Euthyroid Graves' Disease

Patients with so-called euthyroid Graves' disease have ophthalmopathy as the only overt manifestation of the disease (Werner, 1955, Liddle et al, 1965). In this study they have been classified as having mild thyrotoxicosis even though, in many cases, the ophthalmopathy was severe.

The diagnosis of euthyroid ophthalmopathy depended on the presence of the characteristic eye changes with, in about 50% of cases, failure of suppression of ^{131}I uptake after exogenous thyroid hormone administration. Because T_3

TABLE 6

DISEASES OCCURRING IN COMBINATION WITH THYROTOXICOSIS

Disease	Number of Cases
Acromegaly	2
Ulcerative Colitis	3
Diabetes	4
Zollinger Ellison Syndrome	1
Sjögren's Disease	1
Myasthenia Gravis	1
Rheumatoid Arthritis	1

suppression tests were not always done on these patients however, and because only a small number had detectable L.A.T.S. the diagnosis was largely dependent on the exclusion of other causes of ophthalmopathy, particularly in mild cases with only slight proptosis or mild infiltrative changes.

(C) MATERIALS AND METHODS

Assays for L.A.T.S. were carried out, whenever possible, on the concentrated IgG fraction as well as on the plasma. This enabled a more accurate assessment of the prevalence of L.A.T.S. in the various clinical situations. The results of L.A.T.S. assays, antibody tests and gamma globulin estimations will be discussed separately for each class of thyrotoxicosis. Two groups of patients in each class were studied. These were, firstly, patients on whom the initial investigations were carried out before treatment of the hyperthyroidism, and secondly, patients on whom the initial investigations were not carried out until after treatment. The immunological parameters, before and after treatment, could then be correlated with the severity of the disease.

The prevalence of L.A.T.S., other antibodies and increased immunoglobulin levels was also investigated in patients with a recurrence of disease, and in patients with a combination of thyrotoxicosis and other diseases.

The antithyroglobulin titre was determined by the tanned red cell agglutination method (Boyden, 1951). The other antibodies were determined using the immunofluorescent technique (Weller and Coons, 1954) whilst immunoglobulins were estimated using the radial diffusion method (Mancini et al, 1965). These methods have been described in Chapter II.

In some cases photographs were taken, particularly when a significant change had occurred in the ophthalmopathy or dermopathy.

(D) RESULTS

One hundred and thirty-five patients with thyrotoxicosis were investigated for L.A.T.S. The overall prevalence of detectable plasma L.A.T.S. in this laboratory was 27%. Of the L.A.T.S. negative patients, 42% had detectable L.A.T.S. after concentration.

(1) Mild Thyrotoxicosis

(a) Clinical Findings

Eighty-three patients with mild thyrotoxicosis were studied. Seventy-one of these had hyperthyroidism as the only clinical feature. The other 12 patients, in whom ophthalmopathy was the only feature, will be discussed more fully in a later chapter. Two patients had a family history of thyrotoxicosis whilst several others had a family history of other autoimmune diseases or other thyroid disorders.

(b) L.A.T.S. Assays

Plasma L.A.T.S. was detected in only six out of the 44 patients (14%) who were tested before treatment. One out of a further ten patients (10%) had detectable L.A.T.S. only after concentration whilst two others had borderline levels (Figure 13).

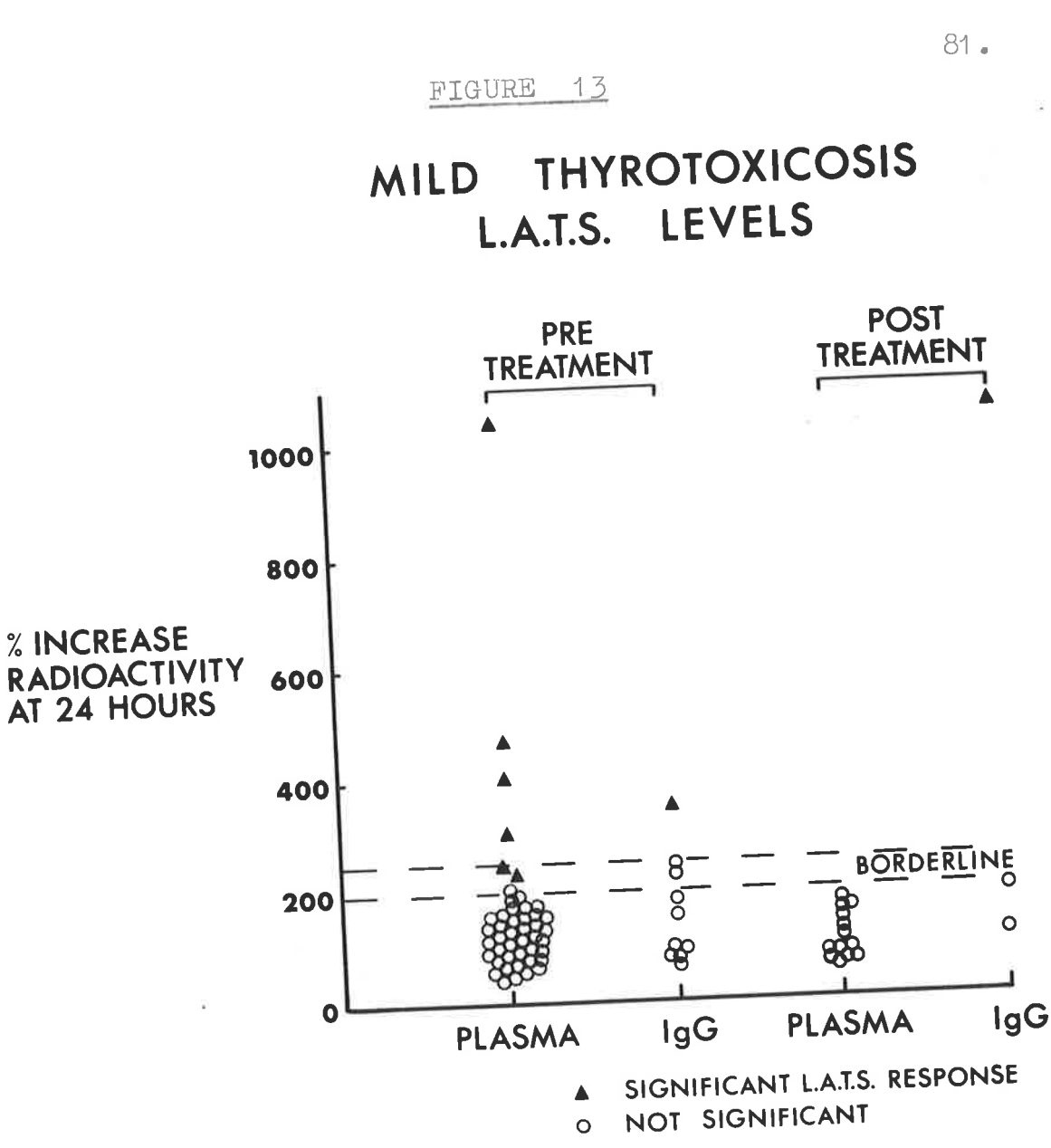
Sixteen patients were investigated for L.A.T.S. after treatment of the hyperthyroidism. Of the 13 who had plasma assays none had detectable L.A.T.S. L.A.T.S. was detected on one of three occasions in which concentration was carried out before assay.

One of four patients (25%) with mild thyrotoxicosis and other diseases, investigated before treatment, had detectable plasma L.A.T.S. IgG concentration was carried out on a further four patients of this group of whom none had detectable L.A.T.S. None of the five patients tested after treatment had detectable L.A.T.S.

(c) Antibody Titres

The prevalence of other antibodies in patients with only one feature was also low. Only 16% of the pretreatment group had significant titres of anti-thyroglobulin whilst 29% and 15% had significant titres of cytoplasmic antibody and gastric parietal cell antibody respectively. The prevalence of antibodies in the group tested after treatment was similar, as was the prevalence

FIGURE 13


MILD THYROTOXICOSIS
L.A.T.S. LEVELS
PRE TREATMENT
POST TREATMENT
% INCREASE RADIOACTIVITY AT 24 HOURS
1000
800
600
400
200
0
BORDERLINE
PLASMA
IgG
PLASMA
IgG
SIGNIFICANT L.A.T.S. RESPONSE
NOT SIGNIFICANT

of antibodies in patients with other diseases as well as thyrotoxicosis (Table 7).

(d) Immunoglobulin Levels

Approximately one third of patients with mild thyrotoxicosis tested before treatment had elevated levels of one or more of the three classes of immunoglobulins. Whilst the prevalence of increased IgG was higher (67%) in patients with a combination of diseases there was no difference in prevalence of increased IgM or IgA levels.

A greater prevalence of increased immunoglobulin levels was found in the group tested after treatment (Table 8).

(2) Moderate Thyrotoxicosis

(a) Clinical Findings

Twenty-four patients with two features were studied, none of whom had a family history of thyrotoxicosis or other autoimmune diseases.

(b) L.A.T.S. Assays

The results of L.A.T.S. assays for this group are summarized in Figure 14. Six of nine patients (67%) tested before treatment had detectable plasma L.A.T.S. One other patient had detectable L.A.T.S. after concentration.

TABLE 7

MILD THYROTOXICOSIS

Prevalence of Significant Antibody Titres

	Anti-Thyro-Globulin	Thyroid Cytoplasmic Antibody	Gastric Parietal Cell Antibody	Smooth Muscle Antibody
Pre-treatment	8/49*(16%)	13/45(29%)	9/46(19%)	0/45(0%)
Post-treatment	3/15 (20%)	5/15(33%)	1/13 (8%)	0/13(0%)
Thyrotoxicosis and Other Diseases	2/9 (22%)	1/7(14%)	2/7 (29%)	0/7 (0%)

* A few patients, in whom L.A.T.S. assays were not carried out, were investigated for antibodies and increased levels of immunoglobulins.

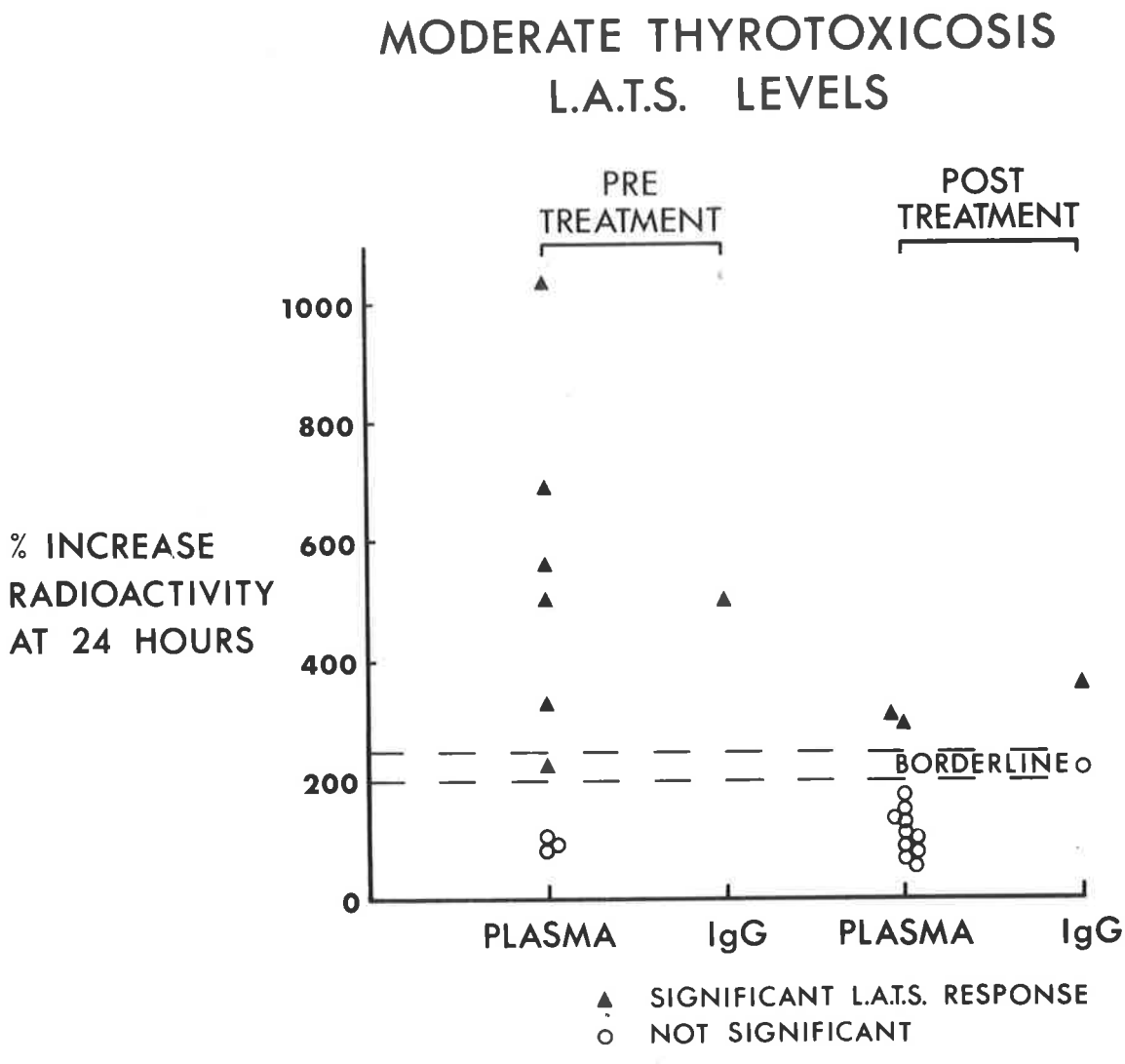
TABLE 8

MILD THYROTOXICOSIS

Prevalence of Elevated Immunoglobulin Levels

	IgG	IgM	IgA
Pre-treatment	17/46(37%)	13/45(29%)	12/46(26%)
Post-treatment	6/13(46%)	9/14(64%)	8/13(61%)
Thyrotoxicosis and Other Diseases	6/9 (67%)	3/8 (37%)	1/8 (12%)

FIGURE 14


MODERATE THYROTOXICOSIS
L.A.T.S. LEVELS
PRE TREATMENT
POST TREATMENT
% INCREASE RADIOACTIVITY AT 24 HOURS
1000
800
600
400
200
0
BORDERLINE
PLASMA
IgG
PLASMA
IgG
SIGNIFICANT L.A.T.S. RESPONSE
NOT SIGNIFICANT

Two out of 12 patients (17%) tested after treatment had detectable L.A.T.S. Of the two patients tested after concentration one had detectable L.A.T.S. whilst the other had a borderline level.

(c) Antibody Titres

The prevalence of significant titres of antibodies was similar to that in patients with one feature. A higher proportion of patients however had antithyroglobulin but less had the cytoplasmic antibody. The prevalence of antibodies in the group tested after treatment was greater than for the pre-treatment group (Table 9).

(d) Immunoglobulin Levels

Approximately 40% of patients with two clinical features had elevated levels of IgG, IgM or IgA. The prevalence of elevated IgG was the highest. There was a slightly lower prevalence of elevated levels of IgG and IgM (but not IgA) in patients investigated after treatment of the hyperthyroidism (Table 10).

(3) Severe Thyrotoxicosis

(a) Clinical Findings

Twelve patients with all three major clinical features were studied. Three of these were new cases, whilst nine had been followed for several years at the Clinic. These patients were in remission, but in all cases,

TABLE 9

MODERATE THYROTOXICOSIS

Prevalence of Significant Antibody Titres

	Anti-Thyro-Globulin	Thyroid Cytoplasmic Antibody	Gastric Parietal Cell Antibody	Smooth Muscle Antibody
Pre-treatment	2/7 (29%)	1/7 (14%)	1/7 (14%)	0/7 (0%)
Post-treatment	5/15(33%)	6/15(40%)	4/15(27%)	0/15(0%)

TABLE 10

MODERATE THYROTOXICOSIS

Prevalence of Elevated Immunoglobulin Levels

	IgG	IgM	IgA
Pre-treatment	3/6 (50%)	2/6 (33%)	2/6 (33%)
Post-treatment	6/15(40%)	4/15(27%)	6/15(40%)

had residual eye damage or dermopathy. None of the patients in this group had a family history of Graves' disease but two had a family history of other autoimmune diseases including one patient with a strong family history of pernicious anaemia.

(b) L.A.T.S. Assays

The highest prevalence of detectable L.A.T.S. was found in patients with severe thyrotoxicosis (Figure 15). Two of the three patients tested before treatment had detectable plasma L.A.T.S. whilst the other had a moderately high L.A.T.S. level only after concentration.

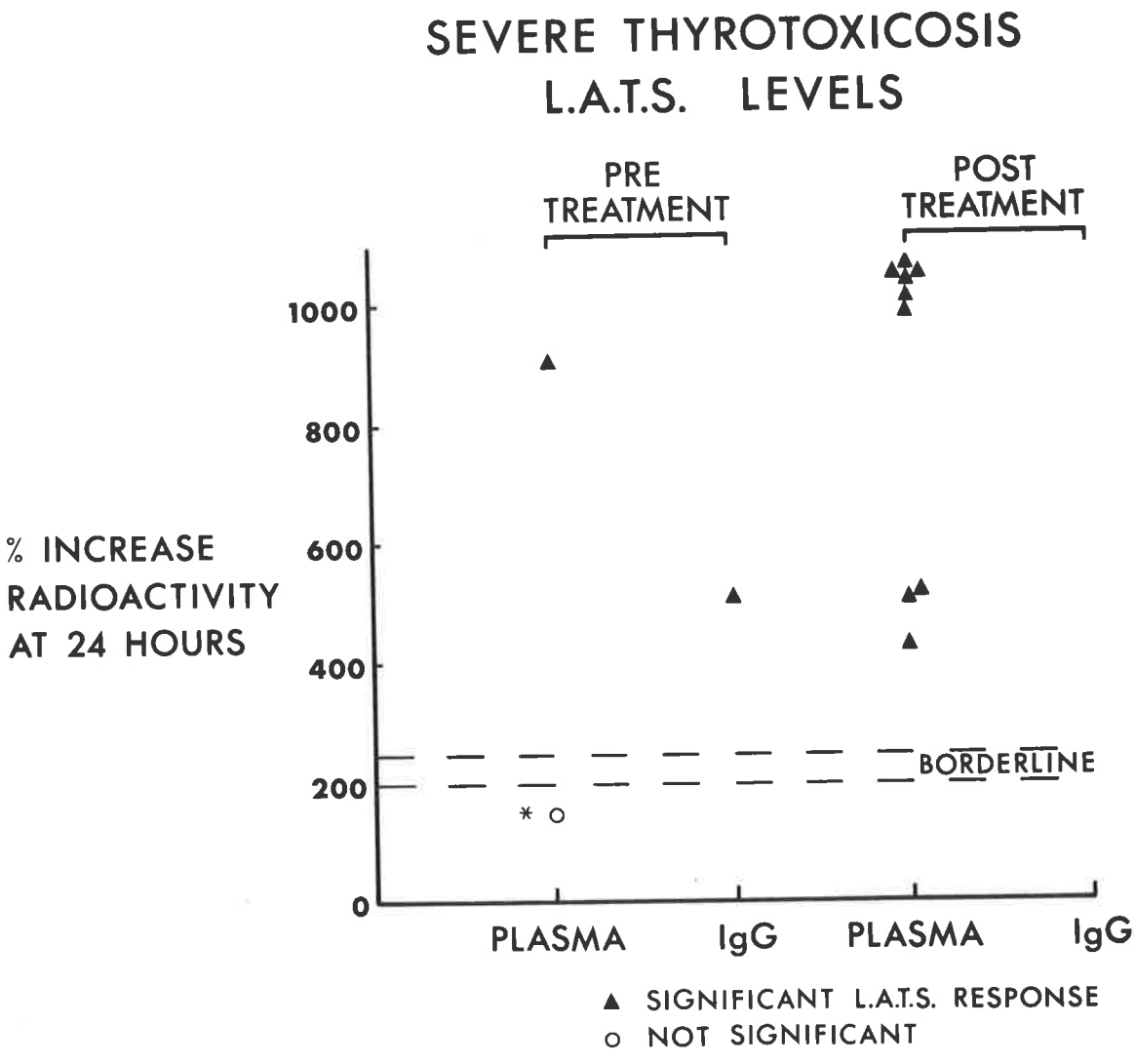
All of the patients tested after treatment had detectable plasma L.A.T.S. As can be seen from the Figure, the 24 hour levels were much higher in this group than in the others; several patients had levels of over 1,000%. Patients with severe thyrotoxicosis and high L.A.T.S. levels thus had persistently high levels even after the eye and skin lesions had become quiescent.

No patient with severe thyrotoxicosis in this small series had features of other diseases.

(c) Antibody Titres

Only a small number of patients with all three clinical features were studied, hence it was not possible to assess accurately the prevalence of antibodies in relation to treatment of the hyperthyroidism in this group.

FIGURE 15



* L.A.T.S. detected after concentration

Furthermore, since many of the patients who were tested after treatment for the first time had been euthyroid for several years, whilst others were being treated with cortico-steroids or immunosuppressive agents for severe ophthalmopathy or dermopathy, the low prevalence of antibodies probably did not reflect the true immunological status of these patients (Table 11).

(d) Immunoglobulin Levels

Again, because of the small number of patients tested in this group the prevalence of elevated immunoglobulin levels could not be accurately assessed (Table 12).

(4) Recurrence of Thyrotoxicosis

(a) Clinical Findings

Sixteen patients were studied in whom relapse of thyrotoxicosis had occurred following treatment. The initial episode was from six months to 40 years before. Patients treated with drugs only were not included. Two patients gave a family history of thyrotoxicosis.

Ten of the patients had hyperthyroidism only whilst six had hyperthyroidism and ophthalmopathy. No patient in this series had dermopathy. Severe ophthalmopathy developed unusually frequently when the hyperthyroidism recurred, as shown by the high prevalence (38%) of patients with this feature.

TABLE 11

SEVERE THYROTOXICOSIS

Prevalence of Significant Antibody Titres

	Anti-Thyro-Globulin	Thyroid Cytoplasmic Antibody	Gastric Parietal Cell Antibody	Smooth Muscle Antibody
Pre-treatment	1/3	1/3	1/3	1/3
Post-treatment	1/7 (14%)	1/6 (17%)	0/5	0/6

TABLE 12

SEVERE THYROTOXICOSIS

Prevalence of Elevated Immunoglobulin Levels

	IgG	IgM	IgA
Pre-treatment	2/3	1/3	1/3
Post-treatment	4/7 (57%)	3/7 (43%)	3/7 (43%)

(b) L.A.T.S. Assays

Plasma and IgG L.A.T.S. levels for the two groups (mild and moderate disease) before and after treatment are shown in Figure 16. In this series two of four patients with mild disease and two of three patients with moderate disease had detectable plasma L.A.T.S. before treatment. None had plasma L.A.T.S. after treatment. Because of the small size of the series however the differences are not significant. None of four patients with mild disease had detectable L.A.T.S. after concentration, whereas all three patients with moderate thyrotoxicosis had L.A.T.S. Two of these had high levels.

(c) Antibody Levels

The prevalence of significant titres of antibodies is shown in Table 13. The prevalence of antibodies in patients with two clinical features was greater than in those with only one faature, but because of the small size of the series, the difference was not significant. The prevalence was similar to that in the corresponding groups of patients with newly diagnosed Graves' disease.

(d) Immunoglobulin Levels

Of the patients with one feature, five (63%) had elevated IgG levels, six (75%) had elevated IgM levels whilst three (38%) had elevated IgA levels. The prevalence

FIGURE 16

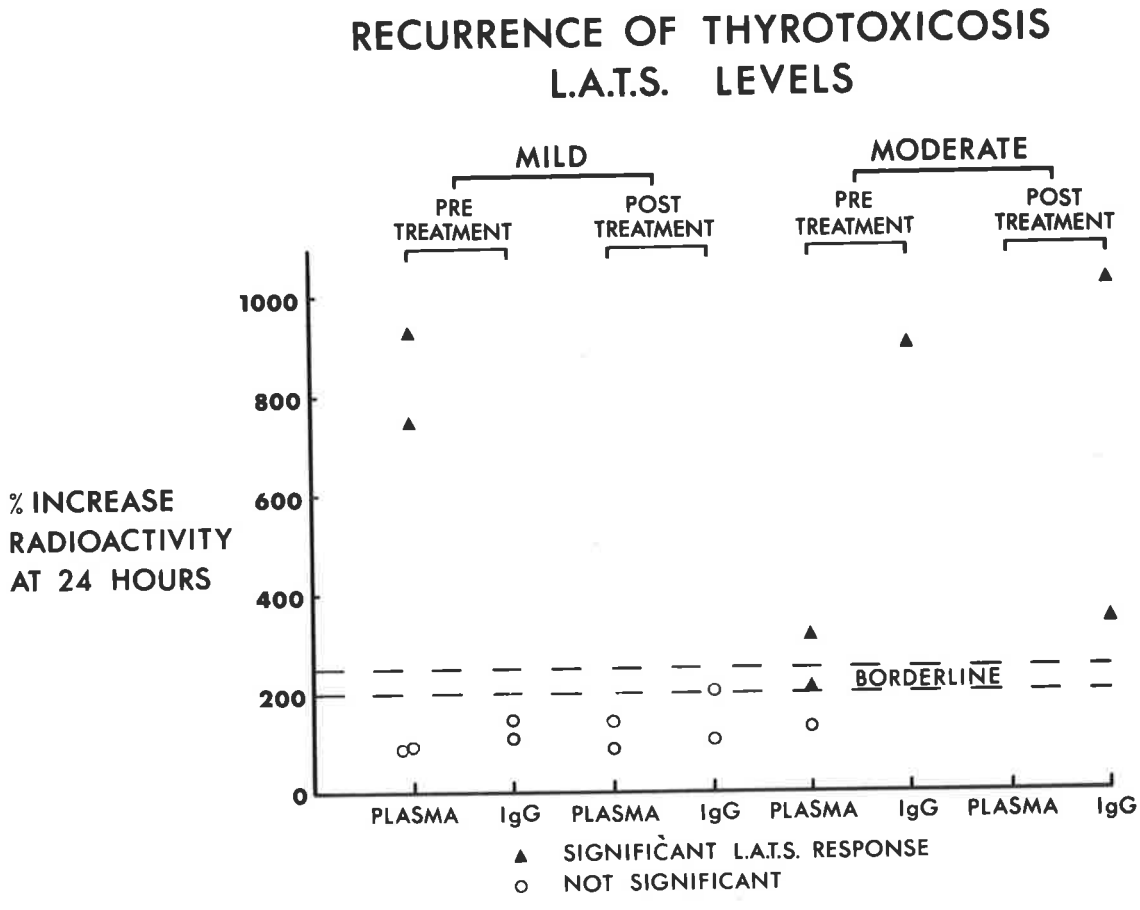

RECURRENCE OF THYROTOXICOSIS
L.A.T.S. LEVELS
MILD
MODERATE
PRE TREATMENT
POST TREATMENT
PRE TREATMENT
POST TREATMENT
% INCREASE RADIOACTIVITY AT 24 HOURS
1000
800
600
400
200
0
BORDERLINE
PLASMA
IgG
PLASMA
IgG
PLASMA
IgG
PLASMA
IgG
▲ SIGNIFICANT L.A.T.S. RESPONSE
o NOT SIGNIFICANT

TABLE 13

RECURRENCE OF THYROTOXICOSIS

Prevalence of Significant Antibody Titres

	Anti-Thyro-Globulin	Thyroid Cytoplasmic Antibody	Gastric Parietal Cell Antibody	Smooth Muscle Antibody
Mild Thyrotoxicosis	1/9 (11%)	1/6 (17%)	0/6 (0%)	1/6 (17%)
Moderate Thyrotoxicosis	2/5 (40%)	3/5 (60%)	1/5 (20%)	0/5 (0%)

The relationship of the time of testing to treatment was disregarded for this study.

was greater, for each class of immunoglobulin, than that found in patients with first occurrence of mild Graves' disease.

Too few patients with two clinical features were investigated to comment on the prevalence of increased levels of immunoglobulins in this group (Table 14).

(E) DISCUSSION

If L.A.T.S. is the antibody which is the cause of thyrotoxicosis it should be present in every case (Milgrom and Witebsky, 1962). Although it is likely that L.A.T.S. would become fixed to the target organ a certain amount would presumably still be circulating. However, since L.A.T.S. can only be detected by a relatively insensitive bioassay it may in fact be present in all cases but sometimes in amounts too small to be detected (Hetzel, 1968, Foldes et al, 1969). That L.A.T.S. is detected in a greater proportion of cases when the concentrated IgG fraction, which contains L.A.T.S. activity, is assayed supports this hypothesis (Carneiro et al, 1966b). Another reason for the apparent absence of L.A.T.S. in some cases of thyrotoxicosis could be due to a poor fit of human L.A.T.S. with the antibody-binding site of the assay animal thyroid (Adams, 1970).

It is likely that tissue-bound L.A.T.S., rather than the circulating antibody, is more significant in the

TABLE 14

RECURRENCE OF THYROTOXICOSIS

Prevalence of Elevated Immunoglobulin Levels

	IgG	IgM	IgA
Mild Thyrotoxicosis	5/8 (63%)	6/8 (75%)	3/8 (38%)
Moderate Thyrotoxicosis	2/3 (67%)	1/3 (33%)	2/3 (67%)

The relationship of the time of testing to treatment was disregarded for this study.

pathogenesis of the disease. Sensitized immunocompetent cells may also play a part. Because of the tendency for patients with dermopathy and severe ophthalmopathy to have high circulating L.A.T.S. levels however (Kriss et al, 1964, Lipman et al, 1967) the plasma levels probably reflect the intensity of the immunological process in the target tissues.

In this series, plasma L.A.T.S. was detected in 27% of all thyrotoxic patients and in 42% of the plasma-negative patients when the concentrated IgG fraction was assayed. These figures are lower than for most other series (Munro et al, 1960, Noguchi et al, 1964). This may be due to the exclusion, in these studies, of low grade levels which cannot be differentiated from nonspecific responses. The much larger prevalence of L.A.T.S. after concentration of the IgG fraction suggests that with more gentle concentration procedures and a more sensitive test for L.A.T.S., the prevalence of the stimulator may approach 100%.

Thyrotoxicosis is associated with several immunological abnormalities. For example, thyrotoxic patients have a high prevalence of thyroid antibodies (Goudie et al, 1957, Trotter et al, 1957, Roitt and Doniach, 1960) and the disease occurs occasionally with other diseases of possible autoimmune etiology such as Sjögren's disease, Addison's disease and pernicious anaemia (Doniach et al, 1963,

Blizzard and Kyle, 1963). The relatives of thyrotoxic patients also have an increased prevalence of thyrotoxicosis and other autoimmune diseases, particularly Hashimoto's disease (Means et al, 1963, Anderson et al, 1964). Other markers of autoimmune diseases are also present in patients with Graves' disease, including increased levels of IgG (Yamakido et al, 1969) and lymphocytic infiltration of the target tissues (Rose and Witebsky, 1968).

In this study there was a high prevalence of thyroid antibodies and gastric parietal cell antibody but there was not a close relationship between these parameters and the severity of the disease. Furthermore, the occurrence of the antibodies did not appear to be influenced by treatment.

There was also a high prevalence of elevated levels of IgG, IgM and IgA in this series. More patients had elevated IgG than the other immunoglobulins but this was not related to the presence of L.A.T.S., supporting the suggestion that L.A.T.S. must comprise only a small percentage of the total IgG (Yamakido et al, 1969). Again, there was not a close correlation between the immunoglobulin levels and the severity of the thyrotoxicosis and the levels, generally, were not lower after treatment, although this was not assessed in individual patients.

The relationship of L.A.T.S. to the clinical features of Graves' disease is uncertain. L.A.T.S., which stimulates thyroid metabolism in animals (Purves and Adams, 1960, El Kabir, 1964) and in human volunteers (Björkman et al, 1961, Arnaud et al, 1965), has until recently, been detected almost exclusively in patients with thyrotoxicosis.

Furthermore, plasma L.A.T.S. levels correlate with estimates of thyroid gland mass. Patients with larger glands tend to have higher plasma L.A.T.S. levels (Carneiro et al, 1966a). Clinically, plasma L.A.T.S. correlates with the presence of visible goitre (Hoffmann and Hetzel, 1966). The best correlation was between plasma L.A.T.S. and the protein bound iodine divided by the estimated gland mass. This fraction was a measure of the rate of production of ^{131}I-labelled hormone per unit weight of thyroid tissue (Carneiro et al, 1966a). For these reasons it is likely that L.A.T.S. causes the hyperthyroidism. This may involve an immunological reaction with an antigen in the thyroid cell.

The occurrence of L.A.T.S. in euthyroid people (Major and Munro, 1962, Liddle et al, 1965) however, indicates that although the antibody stimulates the thyroid of experimental animals and presumably in babies of thyrotoxic mothers in the rare condition of neonatal thyrotoxicosis (McKenzie, 1964) it can be present without causing overt

hyperthyroidism. It is possible that another immunological reaction initiates the disease with the release of antigen and production of L.A.T.S. leading to thyroid stimulation. This other hypothetical antibody may act on the presumptive inhibitor of thyroid mitosis (Chalone, Garry and Hall, 1970) allowing an unbalanced stimulation by naturally occurring L.A.T.S. This could account for the presence of low levels of the stimulator in normal people where thyroid stimulation may be subclinical and in patients with euthyroid ophthalmopathy (Liddle et al, 1965, Werner, 1955).

Thus, although the relationship between L.A.T.S. and thyroid function is close, it is not yet certain whether the production of L.A.T.S. and subsequent thyroid stimulation is the chief mechanism of hyperthyroidism or the result of another process. This question will be raised again in the final chapter in relation to possible mechanisms of thyrotoxicosis.

The relationship of L.A.T.S. to the other features of Graves' disease however is even less understood. It is possible that L.A.T.S. causes these lesions by cross reacting with similar antigens in the orbital tissues and skin (Kriss et al, 1964, Hetzel, 1968). The evidence for this however is largely circumstantial. This is based on the observation that L.A.T.S. is more often found in patients with these features, and often to higher levels, than when

only hyperthyroidism is present (Lipman et al, 1967, Foldes et al, 1969). But, as will be seen later, the relationship is not absolute because some patients with high L.A.T.S. levels do not have ophthalmopathy or dermopathy and conversely, patients with these lesions do not always have detectable L.A.T.S. It is more likely that, as a result of the initiating immunological reaction, several antibodies, including L.A.T.S. are produced some of which may cause the ophthalmopathy and dermopathy. This too will be discussed in the concluding chapter.

The highest prevalence, in this study, of detectable L.A.T.S. was found in the group of patients with all three clinical features. Dermopathy in particular was closely related to L.A.T.S. The lowest prevalence was found in patients with only one clinical feature. This is in agreement with the findings of Lipman et al (1967) who found that L.A.T.S. correlated best with the number of manifestations present rather than with any individual manifestation. They found that the presence or absence of hyperthyroidism was statistically associated with L.A.T.S. only when it was the sole feature of the disease. Dermopathy on the other hand, was the only feature to show a statistically significant association with L.A.T.S. regardless of the presence or absence of other features (Lipman et al, 1967).

Patients with a recurrence of thyrotoxicosis generally have a higher prevalence of L.A.T.S., although this was not demonstrated in this series. The presence of L.A.T.S. initially, and its persistence after treatment, probably indicates a tendency to relapse (Hoffmann and Hetzel, 1966, Lipman et al, 1967). In this series, raised immunoglobulin levels were also more often demonstrated in patients who relapsed.

(F) SUMMARY AND CONCLUSIONS

L.A.T.S. was detected in the plasma of 27% of patients with thyrotoxicosis and in 42% of the plasma-negative patients after concentration.

Thyrotoxic patients had a high prevalence of other thyroid antibodies and elevated gamma globulin levels as well as an increased prevalence of other autoimmune diseases.

The presence of L.A.T.S. correlated closely with the number of clinical features present, in that patients with severe thyrotoxicosis had the highest prevalence and levels of L.A.T.S. whilst patients with only hyperthyroidism or ophthalmopathy had the lowest prevalence of L.A.T.S.

The relationship of L.A.T.S. to the features of Graves' disease was discussed in general terms. Although it is likely that L.A.T.S. causes the hyperthyroidism, the mechanism is not known. L.A.T.S. may be released as a result of another immunological reaction and cause thyroid overactivity as a secondary phenomenon.

CHAPTER IV

DERMOPATHY

(A) INTRODUCTION

(B) CLINICAL SUBJECTS

(C) CASE HISTORIES AND L.A.T.S. RESULTS

(D) OTHER RESULTS

(E) DISCUSSION

(F) SUMMARY AND CONCLUSIONS

CHAPTER IV

DERMOPATHY

(A) INTRODUCTION

Dermopathy has been found to occur in about 3% of patients with thyrotoxicosis and often in association with high L.A.T.S. levels (Kriss et al, 1964, Hoffmann and Hetzel, 1966, Carneiro et al, 1966a). Although the antibody has not been observed attached to the tissues of the skin, it has been suggested that combination of L.A.T.S. and a cross reacting antigen in the skin may cause an inflammatory reaction with eventual deposition of excess mucoid ground substance rich in hyaluronic acid (Kriss et al, 1964, Benoit and Greenspan, 1967, McKenzie, 1968). In this study the relationship between dermopathy and plasma L.A.T.S. levels and other immunological factors has been evaluated in ten patients. In six of these, serial L.A.T.S. levels were correlated with changes in the clinical state of the skin lesion.

Two patients with unusual features are included in the series. One of these patients had transient dermopathy and hyperthyroidism, which remitted spontaneously. The other patient had a skin lesion, which although characteristic clinically, when biopsied was shown to contain no excess mucoid material and she did not have detectable plasma L.A.T.S.

(B) CLINICAL SUBJECTS

The relationship between thyrotoxic dermopathy and plasma L.A.T.S. levels has been evaluated in ten thyrotoxic patients, one male and nine females, ranging in age from 34 to 62 years, who had dermopathy either as a presenting feature or who developed the skin lesion after treatment. The diagnosis of the dermopathy was based on the presence of a bilateral erythematous, painless, nodular or diffuse lesion in the characteristic pretibial or pedal position. The diagnosis was confirmed in most cases by biopsy and histological examination (Palitz and Brunner, 1950, Windrum, 1958, Lever, 1961).

Full case histories of the development and subsequent remission or exacerbation of the dermopathy in six of the patients and clinical summaries for the other patients are given below. L.A.T.S. assays were carried out at the time of onset of the dermopathy in all but two patients. Assays carried out periodically on six of these patients during the course of the illness are shown in the figures. L.A.T.S. activity is expressed as the percentage increase in radio-activity at 24 hours over the initial level. A summary of the clinical details of all patients is shown in Table 15.

Photographs were taken on several occasions to show changes in the skin condition. Thyroid and other antibodies and immunoglobulin levels were measured in most cases at

TABLE 15

CLINICAL DETAILS OF PATIENTS WITH DERMOPATHY

Case	Age at Onset	Sex	Thyroid Status at Onset	Treatment of Hyper-thyroidism	Appearance of Dermopathy	Treatment of Dermopathy	Severe Ophthal-mopathy
1. H.S.	41	F	Hypothyroid	Subtotal thyroidectomy	6 weeks after treatment	Azathiaprine	Yes
2. H.P.	34	F	Hyperthyroid	Subtotal thyroidectomy	Before treatment	Local Steroids	Yes
3. P.D.	47	F	Hyperthyroid	^{131}I (14 mCi)	Before treatment	-	Yes
4. K.K.	46	M	Euthyroid	^{131}I (6 mCi)	2 months after treatment	-	No
5. V.R.	61	F	Hyperthyroid	^{131}I (28.5mCi)	Before treatment	Systemic Steroids	No
6. I.P.	62	F	Hyperthyroid	^{131}I (14 mCi)	Before treatment	Local Steroids	Yes
7. R.Mc.	60	F	Euthyroid	Subtotal thyroidectomy	After treatment	Orbital irradiation	Yes
8. T.W.	53	F	Hyperthyroid	^{131}I (6 mCi)	6 months after treatment	-	Yes
9. L.Mc.	35	F	Hyperthyroid	Carbimazole	Before treatment	-	No
10. P.A.	47	F	Euthyroid	^{131}I (2 doses)	12 months after treatment	-	Yes

the time of onset of the dermopathy, and at various other times during the illness.

(C) CASE HISTORIES AND L.A.T.S. RESULTS

Case 1: Mrs. H.S.

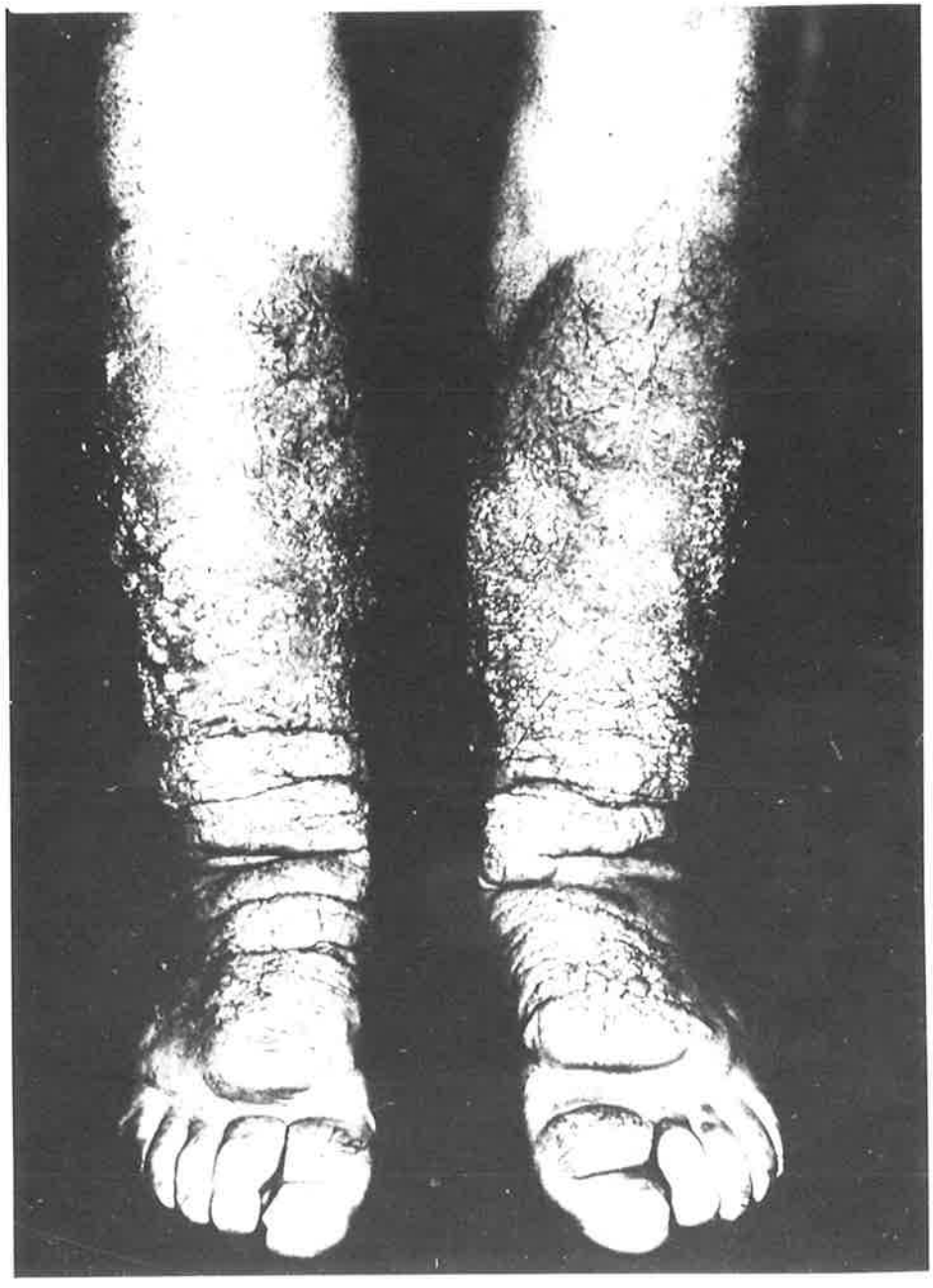
A 51 year old housewife presented 17 years ago with a history of excessive tiredness and irritability, increased appetite, weight loss and excessive sweating during the previous six months. On examination she had a smooth goitre, tremor of outstretched hands, hot moist skin, tachycardia and finger clubbing, and only mild exophthalmos. The clinical diagnosis of thyrotoxicosis was confirmed by the demonstration of elevated protein bound iodine (18.1 μgm%). A subtotal thyroidectomy was performed. Several weeks post-operatively, hypothyroidism, severe progressive ophthalmopathy and dermopathy developed.

The dermopathy gradually progressed over the next eight years by which time the initial plasma assay for L.A.T.S. showed a moderately high level. Over the next four years the dermopathy gradually became more nodular and extensive and spread over the dorsa of her feet, which became very uncomfortable (Figure 17). She was treated with Azathiaprine (Imuran) 150 mg daily for six months, with a fall in plasma L.A.T.S. Her dermopathy improved slightly in that it became less nodular and indurated but did not lessen in its extent. When Azathiaprine therapy

FIGURE 17

DERMOPATHY

GROSS LESION (H.S.)



was stopped due to toxic response in the bone marrow her skin lesion remained stationary.

Several months later a piece of grossly nodular tissue was removed from the patient's left foot to enable her to wear shoes without discomfort, and a split skin graft was applied from her left thigh. About three months later the donor site became irritated and indurated, and nine months after the operation the occurrence of typical dermopathy was observed in the donor site, limited exactly by the outline of the skin removed (Figure 18).

It can be seen in Figure 19, which gives the L.A.T.S. assay results over the long course of her illness, that during the four year period when the dermopathy was progressing plasma L.A.T.S. values steadily increased to reach a peak of 1800% just before Azathiaprine was commenced. During the six months therapy with the immunosuppressive drug, when there was some subjective and objective improvement in her skin lesion, plasma L.A.T.S. levels decreased. Over the next two years, during which time the dermopathy remained stationary, L.A.T.S. levels, although fluctuating from week to week, remained relatively constant. The next rise in L.A.T.S. levels occurred following the skin graft, reaching a peak two weeks before the dermopathy had fully developed in the skin graft donor site on her thigh. Since then L.A.T.S. levels have remained steady and the dermopathy has also remained quiescent.

FIGURE 18

DERMOPATHY
LESION IN SKIN GRAFT DONOR SITE

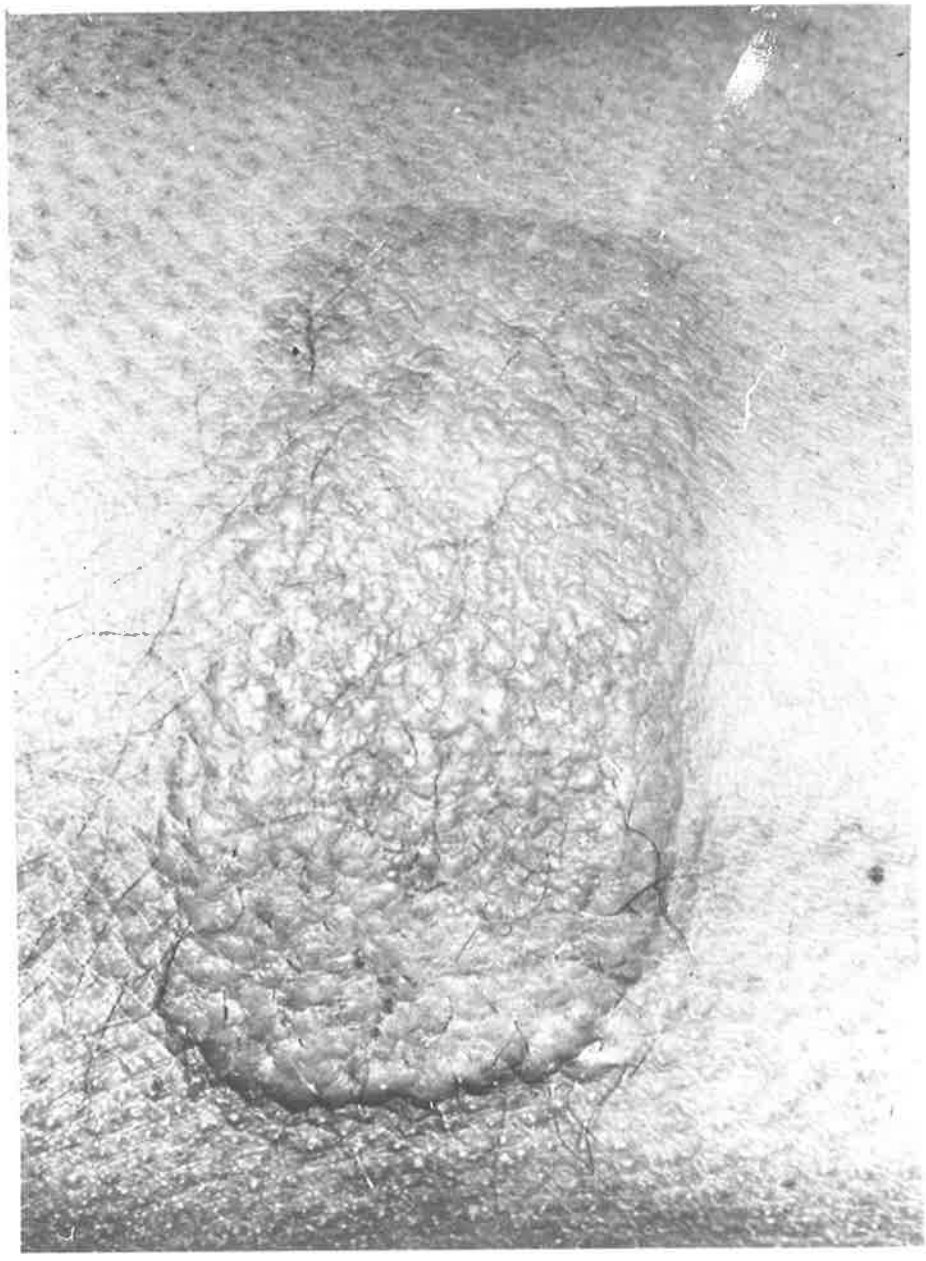
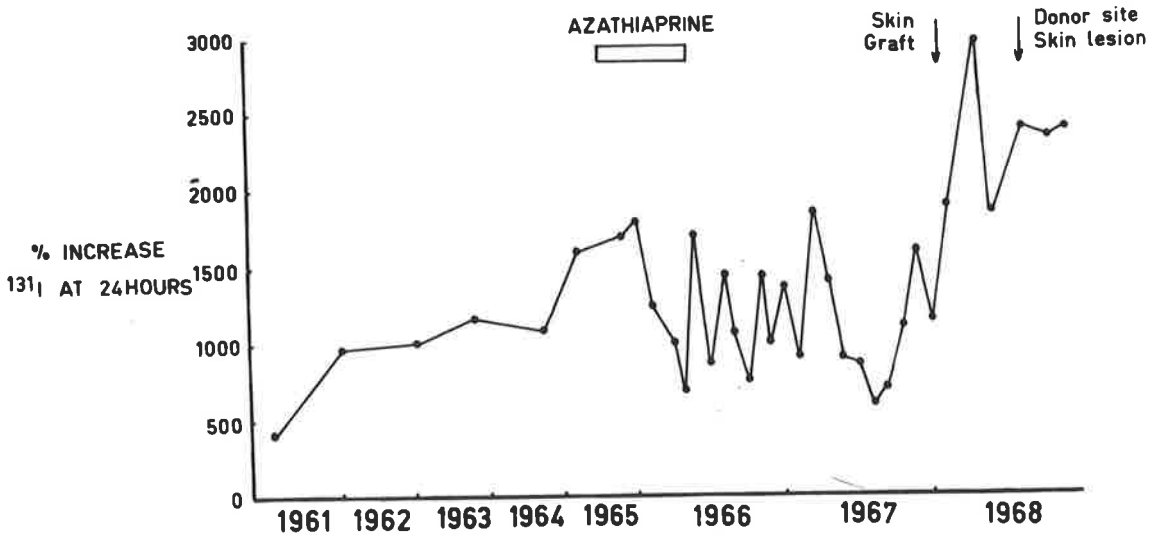


FIGURE 19

SERIAL L.A.T.S. LEVELS

H.S.



Case 2: Mrs. H.P.

A 34 year old housewife presented 11 years ago with a six month history of weight loss, increased appetite, emotional lability, excess sweating and prominence of her eyes. Examination revealed the classical features of thyrotoxicosis including ophthalmopathy and dermopathy.

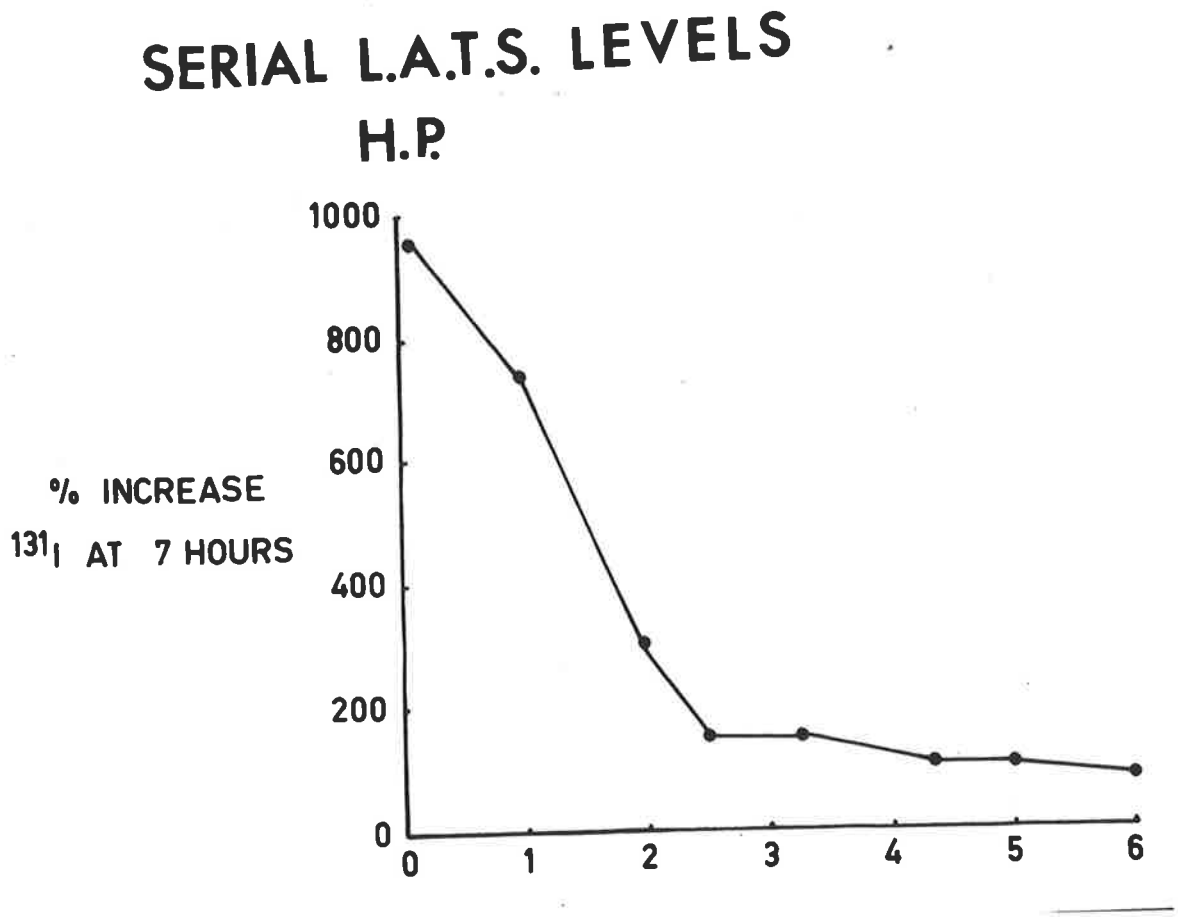
Following biochemical confirmation of the diagnosis she also underwent subtotal thyroidectomy. Her dermopathy remained stationary for several months after the operation. On becoming pregnant thyrotoxicosis returned, and the dermopathy worsened. After the delivery of a macerated foetus however the dermopathy improved, and she quickly became euthyroid with antithyroid drug treatment. She has now only mild residual dermopathy in one leg.

The first L.A.T.S. assay was carried out on Mrs. H.P. after the delivery of the macerated foetus, when the dermopathy was beginning to improve. Subsequent estimations showed that remission of the patient's dermopathy was associated with a steady fall in L.A.T.S. values and that after two years L.A.T.S. had disappeared from the plasma (Figure 20).

Case 3: Mrs. P.D.

This patient, a 45 year old housewife, presented in 1964 with classical thyrotoxicosis, including severe

FIGURE 20


SERIAL L.A.T.S. LEVELS
H.P.
% INCREASE
131I AT 7 HOURS
1000
800
600
400
200
0
0
1
2
3
4
5
6

ophthalmopathy and dermopathy. She was treated with 7 mCi of ^{131}I, but relapsed four months later and required a further dose of ^{131}I. Following the second dose of ^{131}I the dermopathy (as well as the ophthalmopathy) began to regress. Results of L.A.T.S. assays carried out on the plasma of this patient soon after the second treatment with ^{131}I and subsequently, are shown in Figure 21. It can be seen that the levels fell as the patient's dermopathy became less extensive.

Case 4: Mr. K.K.

The patient, a 46 year old former P.O.W. developed the classical features of thyrotoxicosis in 1966, and was given 6 mCi of ^{131}I with control of the hyperthyroidism. However, two months later dermopathy was evident, but over the subsequent 12 months his dermopathy began to improve without further treatment.

Figure 22 shows the results of L.A.T.S. estimations for Mr. K.K. As in the previous two cases, L.A.T.S. levels fell over the period when the patient's dermopathy was undergoing improvement.

Case 5: Mrs. V.R.

A 64 year old woman with a strong family history of pernicious anaemia showed many of the features described for Case 1. She presented four years ago with a history of insomnia, fatigue, palpitations and weight loss over

FIGURE 21

SERIAL L.A.T.S. LEVELS

P.D.

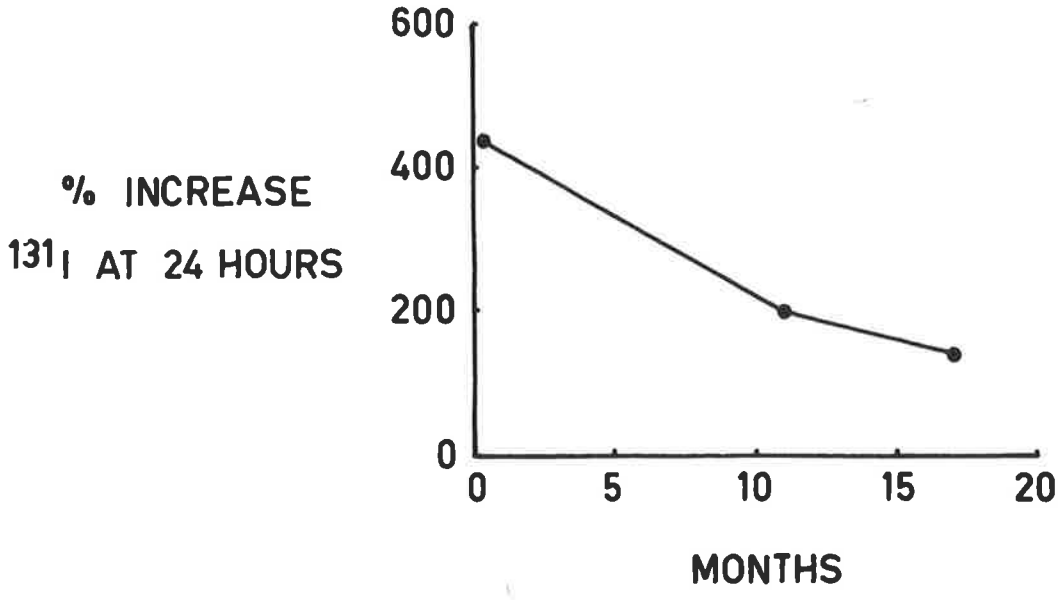
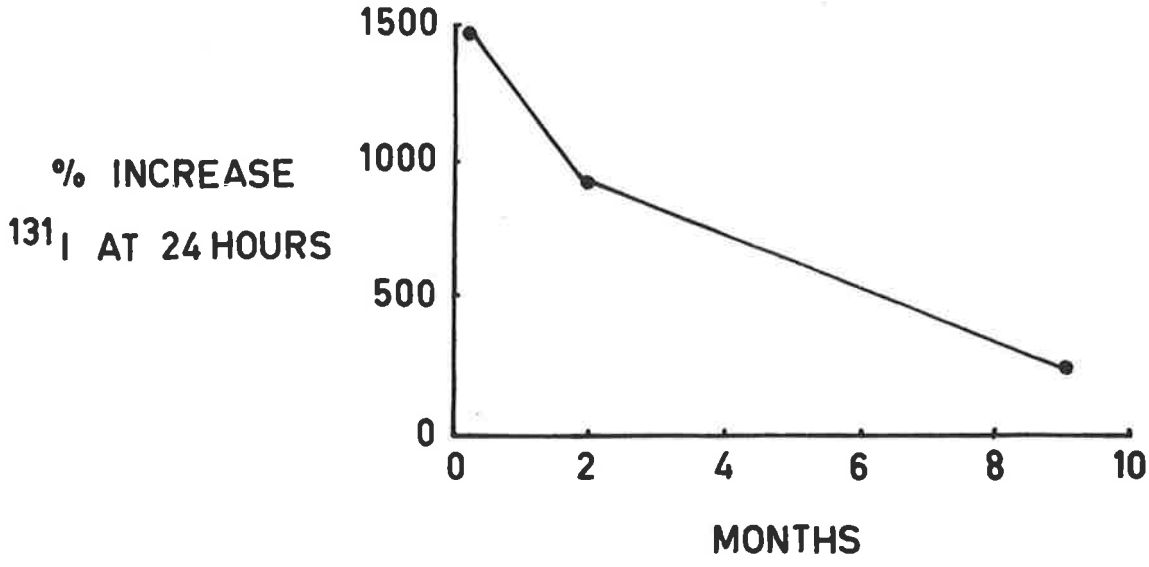


FIGURE 22

SERIAL L.A.T.S. LEVELS

K.K.



the previous 12 months. She was found to have thyrotoxicosis with dermopathy but ophthalmopathy was not present. She was treated with ^{131}I but relapsed soon afterwards and a second dose of ^{131}I was administered. She relapsed again and was given a third dose. Following the initial treatment with ^{131}I this patient's dermopathy worsened, becoming more nodular and irritating. There was no change in the dermopathy following the second dose of ^{131}I. About three months after the last dose of ^{131}I the dermopathy progressed for the second time to extend over the dorsa of both feet, before becoming quiescent again.

L.A.T.S. levels again corresponded with changes in the patient's dermopathy in that the initial worsening of the dermopathy was associated with a rise in L.A.T.S. and when her dermopathy was stationary the L.A.T.S. levels were fairly constant. The second exacerbation of the skin lesion was associated with a further rise in L.A.T.S., whilst subsequent L.A.T.S. levels have been steady and the dermopathy has remained quiescent.

Case 6: Mrs. I.P.

A 60 year old housewife presented nine months before with a short history of weight loss, excessive appetite, nervousness, prominence of her eyes with severe irritation and blinking and thickening and itchiness of the skin of her legs.

When examined she had the features of severe Graves' disease with hyperthyroidism, severe ophthalmopathy and dermopathy. The diagnosis of Graves' disease was confirmed biochemically. Following treatment with ^{131}I the patient rapidly became euthyroid and her ophthalmopathy regressed. At the same time her dermopathy underwent almost complete resolution leaving only mild discoloration of her legs.

When first seen this patient had a high plasma L.A.T.S. level. Five months later, when her dermopathy had undergone almost complete regression, she had a low level (Figure 23). At this time the patient had become hypothyroid and required replacement thyroxine therapy. Since that time however she has had persistently high plasma L.A.T.S. levels, even though the dermopathy has remained quiescent.

Case 7: Mrs. L.Mc.

This patient presented with a six month history of increased appetite, intolerance of hot weather and blurring of vision with diplopia. On examination, she had the classical features of Graves' disease including severe ophthalmopathy and mild dermopathy which was confirmed histologically (Figure 24). Biochemical investigations confirmed the diagnosis of hyperthyroidism, and the patient was given Carbimazole in preparation for definitive treatment with ^{131}I.

FIGURE 23

SERIAL L.A.T.S. LEVELS

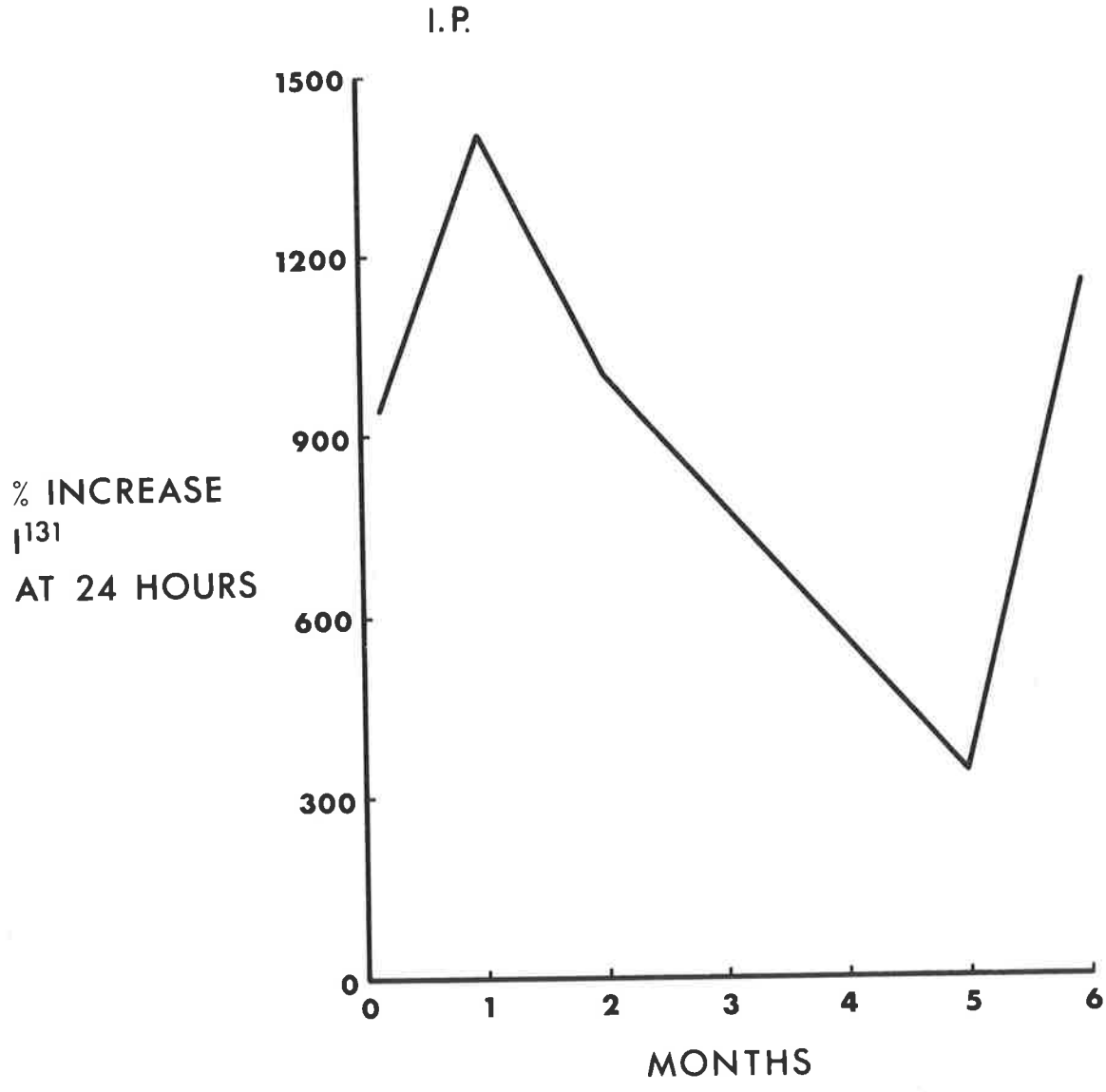
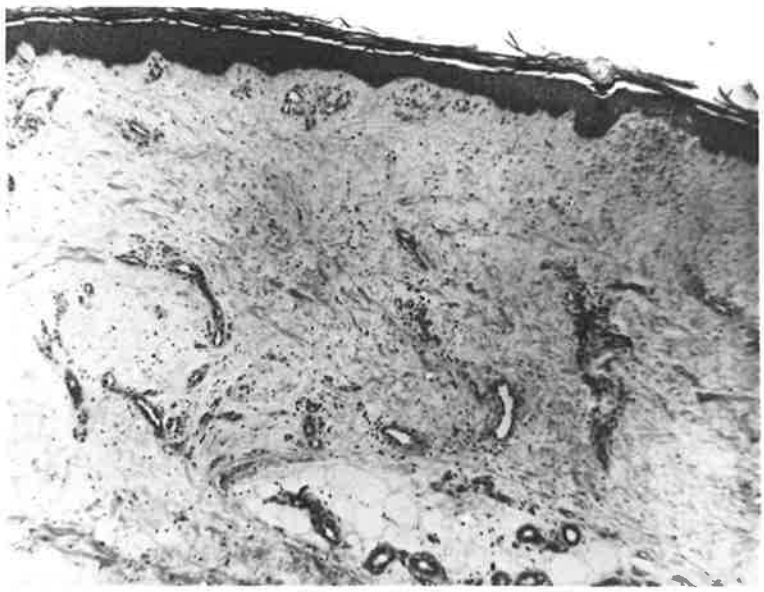
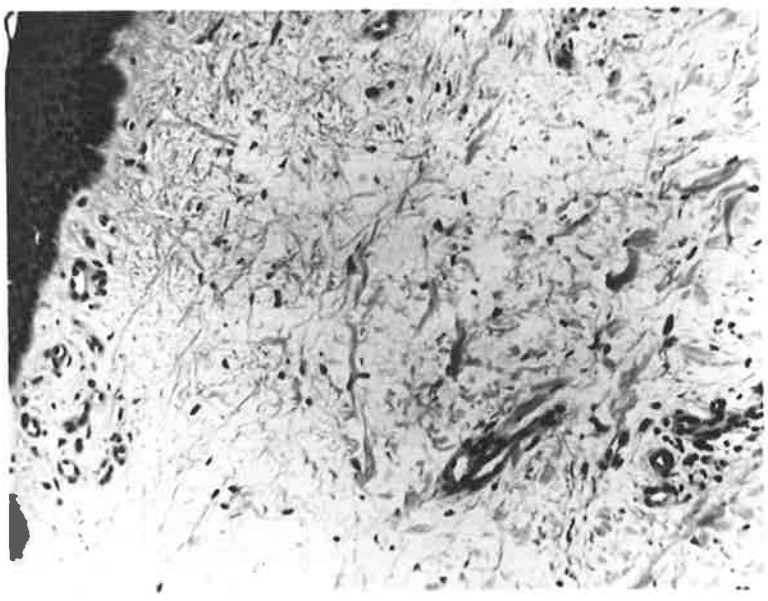


FIGURE 24

DERMOPATHY — HISTOLOGY



LOW POWER



HIGH POWER

A few days later however the patient began to improve rapidly. She became euthyroid and the ophthalmopathy and dermopathy disappeared.

L.A.T.S. activity was not detected in the initial plasma sample but the concentrated IgG fraction was shown to contain a moderately high level of L.A.T.S. (510%). Following remission of the dermopathy however, L.A.T.S. was not detected in either the plasma or the concentrated IgG fraction.

Case 8: Mrs. E.B.

This 47 year old lady presented with a short history of weight loss, excessive appetite, intolerance of hot weather, and nervousness. On examination she had features of thyrotoxicosis including moderate ophthalmopathy and a diffuse nodular skin lesion with the appearance of thyrotoxic dermopathy. The diagnosis of hyperthyroidism was confirmed biochemically and a biopsy of the skin lesion taken. Histological examination of the skin however showed only nonspecific inflammatory tissue with no excess mucoid material. L.A.T.S. was not detected in the plasma. Following treatment with ^{131}I the patient became euthyroid and the ophthalmopathy improved, but there was no change in the skin lesion. L.A.T.S. was not detected in subsequent plasma samples.

Cases 9. 10 and 11.

Three other patients with dermopathy have been included in this series. L.A.T.S. estimations were made soon after the appearance of the skin lesion. Two of these patients developed dermopathy after treatment of the hyperthyroidism, whilst the third had dermopathy before treatment. At the time of onset of their skin lesion all had high L.A.T.S. levels (Figure 25).

(D) OTHER RESULTS

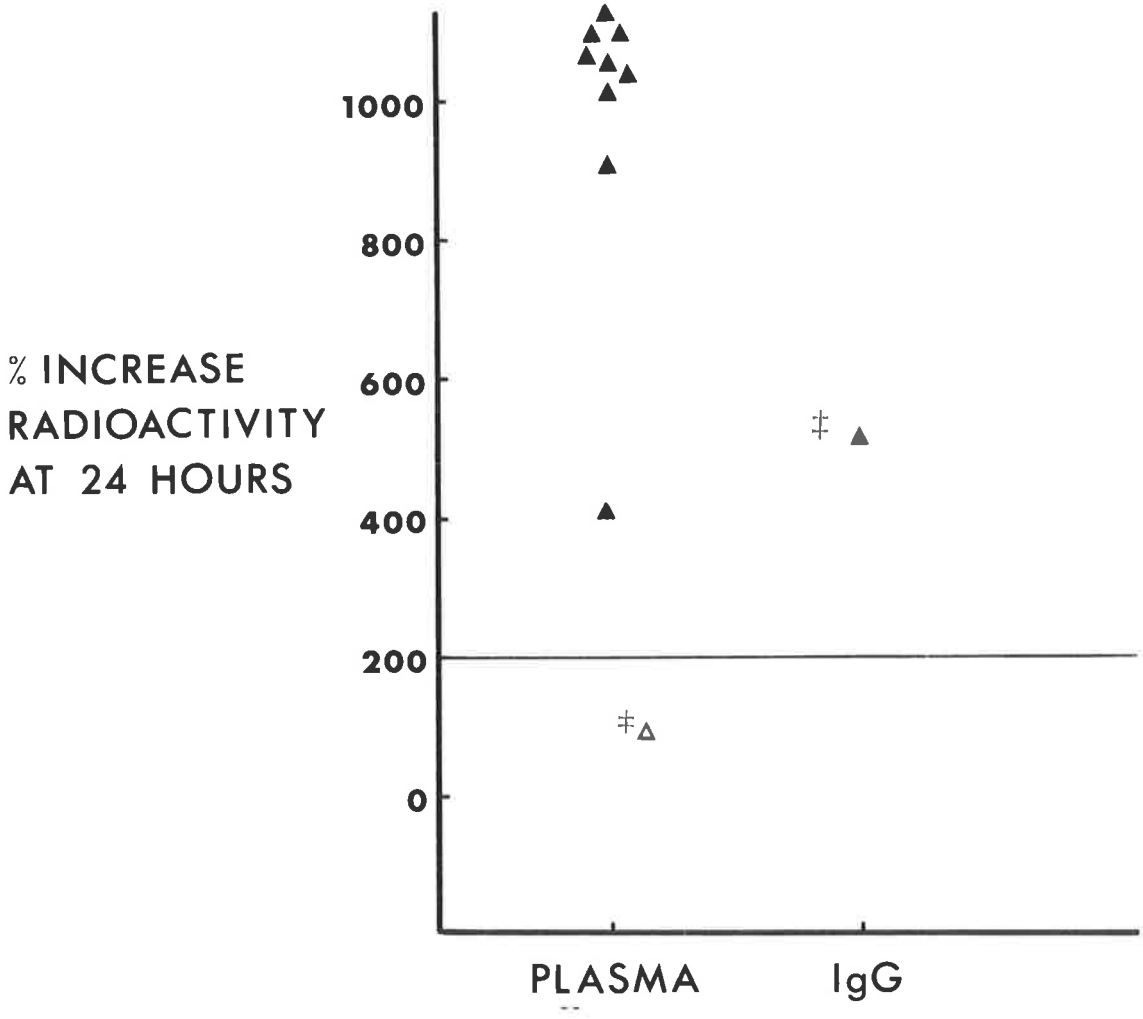
Antithyroglobulin, thyroid cytoplasmic antibody, gastric parietal cell antibody and smooth muscle antibody were estimated for most patients soon after the onset of the skin lesion, but in three cases not until the dermopathy was quiescent. IgG, IgM and IgA levels were also estimated at this time (Table 16).

Because of the small size of the series and because antibodies and immunoglobulins were not estimated, in many cases, until after treatment of the hyperthyroidism or when the dermopathy was quiescent it was not possible to determine whether progression of the skin lesion was associated with higher antibody titres or immunoglobulin levels than during regression or in quiescence.

Estimations were carried out on only four patients during progression of the dermopathy. Of these, one had significant antibody titres, two had elevated IgG

FIGURE 25

THYROTOXIC DERMOPATHY PLASMA L.A.T.S. RESPONSES



‡ = PLASMA AND IgG LEVELS OF PATIENT L.Mc.

TABLE 16

IMMUNOLOGICAL FINDINGS OF PATIENTS WITH DERMOPATHY

Case	24 hour Plasma L.A.T.S. Level	Anti-Thyro-Globulin Titre	Thyroid Cyto-plasmic Antibody	Gastric Parietal Cell Antibody	Smooth Muscle Antibody	IgG	IgM	IgA
H.S.*	576	+1/25	-	-	-	1300	100	173
H.P.	980	-ve	o	o	o	ǂ		
P.D.*	420	o	o	o	o	1180	110	220
K.K.	500	o	o	o	o	o	o	o
V.R.*	417	+1/25	-	-	-	1750	72	115
I.P.	912	+1/250,000	++	+	-	940	200	480
R.Mc.	4131	o	o	o	o	o	o	o
T.W.	1392	+1/5	-	-	-	900	135	320
L.Mc.	$^{146}_{x512}$	-ve	-	-	-	3200	95	176
P.A.	1087	+1/5	-	-	-	1720	115	312

o = Not Done
x = IgG level
* = Antibodies and gamma globulins estimated only when dermopathy quiescent
ǂ = Total gamma globulin level normal

levels and three had elevated IgA. Three patients were investigated for antibodies and increased immunoglobulin levels when the skin lesion was quiescent. Although none of these had significant antibody titres all had elevated levels of IgG.

Thus, in this series, there was no obvious relationship between the clinical state of the dermopathy and the presence of other antibodies and of elevated immunoglobulin levels. The actual titres of antibodies and levels of immunoglobulins did not correlate with the L.A.T.S. levels, although fall in L.A.T.S. levels after regression of the dermopathy was associated with fall in IgG levels in all three cases tested during the active stage and again during remission. There was no change in IgM or IgA levels however.

(E) DISCUSSION

In this series nine out of ten patients with dermopathy had detectable L.A.T.S. whilst the other patient had L.A.T.S. detectable by concentration of the IgG fraction. Most of these patients had high L.A.T.S. levels before treatment. Although the prevalence of significant antibody titres was low at the time of testing, which was often during remission of the skin lesion, several patients had increased immunoglobulin levels.

Serial L.A.T.S. assays carried out on six patients showed a close relationship between the skin lesion and L.A.T.S. levels. When the dermopathy was progressing L.A.T.S. levels were increasing, and when the skin lesion was quiescent, L.A.T.S. levels were fairly constant, and finally, when the lesion regressed plasma L.A.T.S. fell, although only transiently in one patient (I.P.)

In three of the patients (P.D., H.P. and L.Mc.) marked regression of the dermopathy was associated with the disappearance of L.A.T.S. For patient K.K., although plasma L.A.T.S. fell to a very low level, only moderate improvement of his dermopathy occurred.

In cases V.R. and H.S., with more chronic skin lesions, the changes in the dermopathy and plasma L.A.T.S. levels were not so prominent. Because of the development of dense fibrous tissue in the legs, complete regression of the dermopathy did not occur, but decreased L.A.T.S. levels were associated with slight improvement, and further progression of the skin lesion with rise in plasma L.A.T.S.

The decline in L.A.T.S. levels after treatment of the hyperthyroidism may have been due to the removal of the thyroid "antigen mass". This would reduce the stimulus to the production of L.A.T.S. (and possibly other antibodies) by the antibody forming tissues with, subsequently, less antibody to react with antigen in the skin. Fall in

L.A.T.S. levels would therefore be followed by reduced cellular infiltration and inflammatory change.

Pimstone et al, (1963) claimed to have extracted L.A.T.S. from the skin lesion but as yet this has not been confirmed (Pinchera et al, 1965). Furthermore, L.A.T.S. has not been detected attached to the skin tissues in biopsy specimens from patients with dermopathy. With recent methods involving the use of fluorescein labelled anti-gamma globulin however, L.A.T.S. has been detected in thyroid cell cytoplasm (Greenspan and Hargadine, 1964, Blum et al, 1967) though this has not yet been confirmed. It may be possible to localise L.A.T.S. in the skin using this method.

The association of elevated immunoglobulin levels and significant titres of thyroid and other antibodies in patients with dermopathy (Foldes et al, 1969) supports the hypothesis (Kriss et al, 1964, Hetzel, 1968) that this lesion is due to an immunological abnormality. Five out of seven patients in this series had elevated levels of IgG but only one (I.P.) had significant titres of thyroid antibodies.

It has been suggested that the requirements for dermopathy in thyrotoxicosis include antibody production together with a local factor operating in the legs or elsewhere (Kriss et al, 1964, McKenzie, 1968). Such

precipitating factors may be oedema, trauma, venous stasis or chronic irritation. The common occurrence of these factors in the legs would explain the almost exclusive pretibial and pedal distribution of the lesion. Surgical trauma in case H.S. provided the necessary stimulus to the development of dermopathy in the skin graft donor site. A similar occurrence was reported by Patterson in 1958. These local factors may stimulate immunocompetent cells to accumulate and produce L.A.T.S. and possibly other antibodies (Kriss et al, 1964).

An unusual feature of this series was that five of the ten patients presented with dermopathy as a feature of thyrotoxicosis before treatment, rather than after treatment as has usually been reported (Kriss et al, 1964, Pinchera et al, 1965). Because of the intimate relationship of L.A.T.S. to the development of the lesion however, this is not surprising. In all cases L.A.T.S. was present at the time of progression of the lesion and it is likely that patients can develop dermopathy regardless of their thyroid status.

It has frequently been observed that destruction of the thyroid by ^{131}I may lead to the development of dermopathy or ophthalmopathy or to the worsening of pre-existing lesions (Webster et al, 1963, Kriss et al, 1967, Pinchera et al, 1967). This was thought to be due to a

transient lympholysis and rise in the level of circulating L.A.T.S. with relocalisation of the antibody in the skin or in the orbital tissues. This was observed on only one occasion in this series however.

The relationship between the dermopathy and plasma L.A.T.S. levels is thus clearly very close. The occurrence of the skin lesion without detectable L.A.T.S. is uncommon (Lipman et al, 1967, McKenzie, 1968), and in these cases inability to detect L.A.T.S. may be due to insensitivity of the assay or to lack of cross reactivity with the assay animal thyroid (Adams, 1970). The only patient in this series without detectable plasma or IgG L.A.T.S. had what may be called "pseudo-dermopathy". Although her skin lesion looked like classical thyrotoxic dermopathy the biopsy revealed that there was no excess of mucoid material and a diagnosis of "chronic nonspecific inflammation" was made. It is possible therefore that "skin-reactive" auto-antibodies are associated specifically with excess ground substance deposition, and that in the absence of these antibodies, as reflected by the absence of L.A.T.S., a similar lesion, but without mucoid material can develop as a response to trauma, oedema or other local factors.

The inter-relationship between plasma L.A.T.S. and dermopathy shown in this study thus provides further, if

circumstantial, evidence that autoimmune factors involving interaction of L.A.T.S. and possibly other antibodies with tissue antigens, are significant in the pathogenesis of this feature of thyrotoxicosis.

(F) SUMMARY AND CONCLUSIONS

The relationship between plasma L.A.T.S. levels and thyrotoxic dermopathy was investigated in ten patients. Serial assays for L.A.T.S. were carried out in six cases. In all cases where serial levels were estimated, it was found that progression or exacerbation of the skin lesion was associated with a rise in L.A.T.S. and that remission was associated with a fall in L.A.T.S. levels (although only transiently in one patient). Nine out of ten patients in the series had high plasma L.A.T.S. levels when first tested. The other patient had a moderately high level when concentrated IgG was assayed for L.A.T.S. activity.

The development of typical dermopathy in a skin graft donor site of one patient was described. It was suggested that local trauma may have initiated the inflammatory reaction associated with mucoid deposition and high L.A.T.S. levels. The frequency of trauma, oedema and stasis in the legs may account for the usual occurrence of the lesion in this site.

Two further cases were described. One of these patients had transient dermopathy associated with transient hyperthyroidism. The other had a chronic indurated skin lesion which looked like classical dermopathy but which did not contain excess mucoid material when examined histologically. L.A.T.S. was not detected in this patient.

The close relationship between L.A.T.S. and dermopathy demonstrated in this study suggests that this feature of Graves' disease is closely related to L.A.T.S. L.A.T.S., or another antibody associated with the stimulator, may cause the lesion by reacting with an antigen in the skin.

CHAPTER V

EYE CHANGES

(A) INTRODUCTION

(B) CLINICAL SUBJECTS AND METHODS

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- (2) Classification and Description of Eye Changes
  - (a) Classification
  - (b) Exophthalmos
  - (c) Orbital Infiltration
  - (d) Ophthalmoplegia
- (3) L.A.T.S. Assays
- (4) Antibody Titres and Immunoglobulin Levels

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- (2) Relationship of L.A.T.S. to the Course of the Eye Changes
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(D) EUTHYROID OPHTHALMOPATHY

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CHAPTER V

EYE CHANGES

(A) INTRODUCTION

Most patients with Graves' disease have lid lag and stare (Hoffmann, 1965, Hall et al, 1967) and many have mild exophthalmos (Rundle, 1945). These signs usually disappear after treatment of the hyperthyroidism (Hales and Rundle, 1960, Lipman et al, 1967). About 10% of patients however, develop a severe progressive ophthalmopathy, which can lead to blindness. Apart from severe exophthalmos, these patients have conjunctival injection, chemosis and ophthalmoplegia (Aranow and Day, 1965, Lipman et al, 1967). Eventually the eyes become fixed and protruding with, finally, corneal ulceration or panophthalmitis which may lead to destruction of the eye-ball. These changes may occur following treatment of the hyperthyroidism (Kriss et al, 1966, Hamilton et al, 1967), but more often severe ophthalmopathy develops as part of the presenting clinical picture and the two features run separate courses (Solomon et al, 1968).

Most patients with severe ophthalmopathy have detectable plasma L.A.T.S. and often in high concentration (Pimstone et al, 1963, Hoffmann and Hetzel, 1966). Treatment with cortico-steroids or immunosuppressive agents often results in marked improvement in the eye lesion and fall in plasma

L.A.T.S. levels (Snyder et al, 1964, Lipman et al, 1967, Werner, 1967). Thus it is likely that immunological factors are involved in the pathogenesis of the severe grades of ophthalmopathy (Hoffmann et al, 1966, McKenzie and McCullagh, 1968).

In this study, the severity of the ophthalmopathy has been correlated with L.A.T.S. levels, antibody titres and immunoglobulin levels in patients with thyrotoxicosis. The degree of eye involvement was classified according to a system recommended by a committee of the American Thyroid Association (Werner, 1969). The prevalence of detectable L.A.T.S., significant titres of other antibodies and raised immunoglobulin levels was investigated for each class.

Finally, the response to the various forms of treatment used for severe ophthalmopathy was studied. Changes in L.A.T.S. levels, antibody titres and immunoglobulin levels were correlated with the clinical course of the eye lesion after treatment with cortico-steroids, immunosuppressive agents or orbital irradiation which were used when conventional anti-thyroid treatment failed. Because autoimmune mechanisms may be involved in the pathogenesis of ophthalmopathy (Hales et al, 1961, McGill and Asper, 1962), the use of Azathiaprine was of particular interest.

(B) CLINICAL SUBJECTS AND METHODS

(1) Clinical Subjects

One hundred and twenty-two patients with Graves' disease were studied including three patients with severe ophthalmopathy in whom progression had occurred several years before. The eye lesion was quiescent in each of these patients at the time of study. Twelve patients with euthyroid Graves' disease were also included in the series.

The degree of eye involvement was classified at the initial diagnosis of Graves' disease, and at the time of progression of eye changes in patients who subsequently developed severe ophthalmopathy. The assessment was made retrospectively to the time of progression in the three patients mentioned above.

(2) Classification and Description of Eye Changes

(a) Classification

Because of the confusion relating to nomenclature of the eye changes of Graves' disease, the terminology has been standardised by a committee of the American Thyroid Association (Werner, 1969). A classification which summarizes the situation after the diagnosis of Graves' ophthalmopathy has been recommended (Table 17).

Class 0 represents absence of eye changes.

Class 1 represents the changes formerly called "mild or non infiltrative" with an excellent prognosis.

TABLE 17

EYE CHANGES OF GRAVES' DISEASE

Classification of Signs and Symptoms

MNEMONIC	N	O	S	P	E	C	S
Class	0	1	2	3	4	5	6
Stare		+					
Lid Lag and/or Proptosis		+					
Sandy sensation			+				
Lacrimation			+				
Photophobia			+				
Conjunctival Injection			+				
Chemosis			+				
Lid Fullness			+				
Lagophthalmos			+			+	
Proptosis (> 20mm)				+			
Diplopia					+		
Extraocular Muscle Involvement					+		
Corneal Involvement						+	
Optic Nerve Involvement							+

Classes 0 and 1 represent mild ophthalmopathy
Classes 2 to 6 represent severe ophthalmopathy

Classes 2 - 6 represent the severe eye changes previously called "severe", "malignant" or "progressive". Each class is subgraded to indicate absent, minimal, moderate or marked. The eye changes can be additionally characterized as active or inactive (Werner, 1969). An abridged version of the classification in shown in Table 18.

This classification has been used in this study. The number of patients investigated in each class is shown in Table 19.

(b) Exophthalmos

Exophthalmos (proptosis) is the most characteristic of the eye changes of Graves' disease. As far as the patient is concerned this is also the most disturbing. Certainly the patient with bulging eyes, lid retraction and stare presents an arresting picture. In most cases however, these signs disappear following treatment (Rundle, 1964, McKenzie, 1968), but in a small percentage of cases the exophthalmos progresses and the orbital tissues become oedematous. Lid swelling and chemosis may occur. Eventually the extraocular muscles become splinted by oedema and inflammatory tissue and the eyeballs become fixed. The fixed, congested, protruding eyeball is prone to corneal ulceration and infection and the oedematous constricted optic nerve is liable to ischemic damage which may lead to loss of sight. Although the natural course

TABLE 18

EYE CHANGES OF GRAVES' DISEASE

Abridged Classification

Class	Eye Changes
0	No signs or symptoms
1	Upper lid retraction and stare, with or without lid lag and proptosis
2	Soft tissue involvement (symptoms and signs)
3	Proptosis
4	Extraocular muscle involvement
5	Corneal Involvement
6	Optic nerve involvement (with loss of sight)

TABLE 19

EYE CHANGES OF GRAVES' DISEASE

Number of Cases Studied

Class	Number
0	41
1	33
2	2
3	20
4	24
5	2
6	-
Total	122

is for these changes to "burn themselves out" (Mann, 1948, Rundle, 1964), eyeball destruction and blindness may result. Even with specific treatment there may be residual damage to the eyes consisting of gross protrusion with congestion, double vision and some loss of sight (Figure 26) (Werner, 1961, Lipman et al, 1967).

The degree of exophthalmos was measured with a Hertel exophthalmometer. Proptosis of one or both eyes of more than 20 mm was considered significant. Changes in the degree of eyeball protrusion, when measured carefully by the same observer, provided a sensitive parameter of worsening or improvement in the eye lesion.

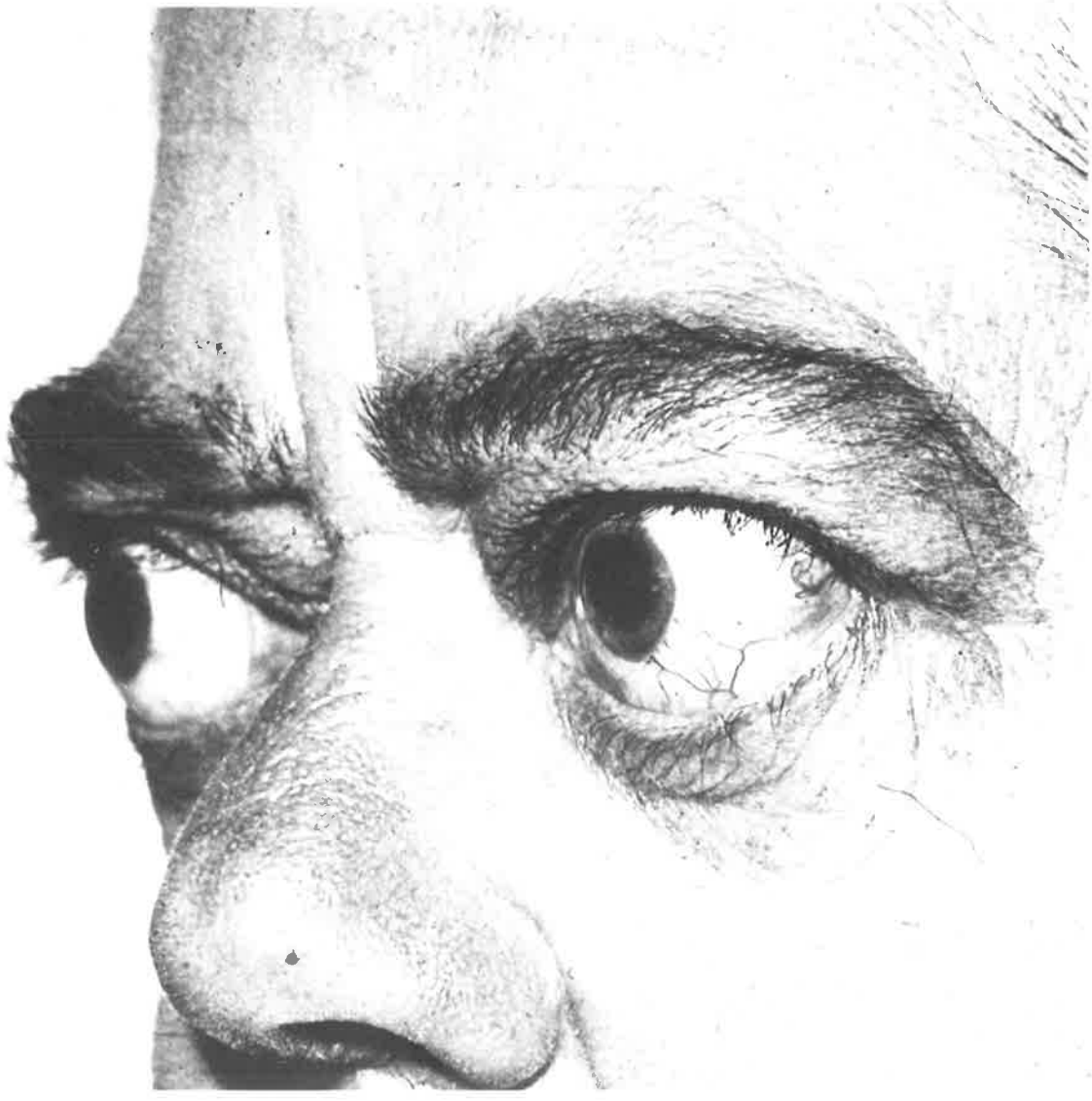
(c) Orbital Infiltration

Swelling of the lids, congestion of the conjunctiva and chemosis are characteristic signs of severe ophthalmopathy (Figure 26) (Rundle, 1964, Aranow and Day, 1965). The orbital contents increase, largely due to deposition of adipose tissue and fluid, particularly in the orbital muscles which are enlarged to three times normal size (Burch, 1929, Rundle and Pochin, 1944). Apart from proptosis, and rarely subluxation due to pressure behind the eyeball, other results of orbital swelling are epiphora and photophobia, heaping up of the chemotic conjunctiva preventing apposition of the lids, and occasionally, papilloedema. The appearance of papilloedema with loss of

FIGURE 26

OPHTHALMOPATHY

—CLASS 4



visual acuity represents an emergency requiring immediate orbital decompression to prevent blindness (Rundle, 1964).

(d) Ophthalmoplegia

Involvement of the extraocular muscles with oedema and inflammatory tissue leads to weakness in eyeball movement. Upward gaze is affected first (Rundle, 1964). This leads to diplopia when the patient looks upwards. Although protrusion and congestion may indicate severe eye involvement, the development of extraocular muscle weakness is one of the most important signs of serious disease. Vigorous treatment is required at this stage to prevent permanent eye damage (Rundle, 1957).

(3) L.A.T.S. Assays

Assays for L.A.T.S. were carried out on all thyrotoxic patients at diagnosis or on progression of the eye changes. Serial estimations were made in several patients with severe ophthalmopathy to assess the relationship between L.A.T.S. levels and the degree of eye involvement.

(4) Antibody Titres and Immunoglobulin Levels

Antibody titres and immunoglobulin levels were measured in conjunction with L.A.T.S. at the time of diagnosis of Graves' disease or on progression of the eye signs. Serial estimations were made in some cases.

(C) RESULTS

(1) L.A.T.S. Assays

The 24 hour plasma L.A.T.S. levels are shown in Figure 27 and the findings are summarized in Table 20.

The prevalence of detectable plasma L.A.T.S. rose from 10% in Class 0 to 59% in Class 4. Overall, 57% of patients with severe ophthalmopathy (Classes 2-6) had L.A.T.S. whereas only 9% of patients with mild ophthalmopathy (classes 0 and 1) had detectable L.A.T.S. Patients with the most severe eye disease tended to have the highest levels.

Although a relationship between L.A.T.S. and the severity of the ophthalmopathy was apparent, this was not close since severe ophthalmopathy occurred in the absence of L.A.T.S. and conversely, some patients with high L.A.T.S. levels had only mild eye involvement.

Of the patients with the most severe grades of ophthalmopathy (Classes 4-6) progression occurred before treatment of the hyperthyroidism in one-third of patients and after treatment in two-thirds. Therefore, in this series, the development of severe ophthalmopathy was associated with high L.A.T.S. levels and tended to occur after treatment, despite the euthyroid status of most patients at this time.

FIGURE 27

EYE CHANGES OF GRAVES' DISEASE
PLASMA L.A.T.S. LEVELS

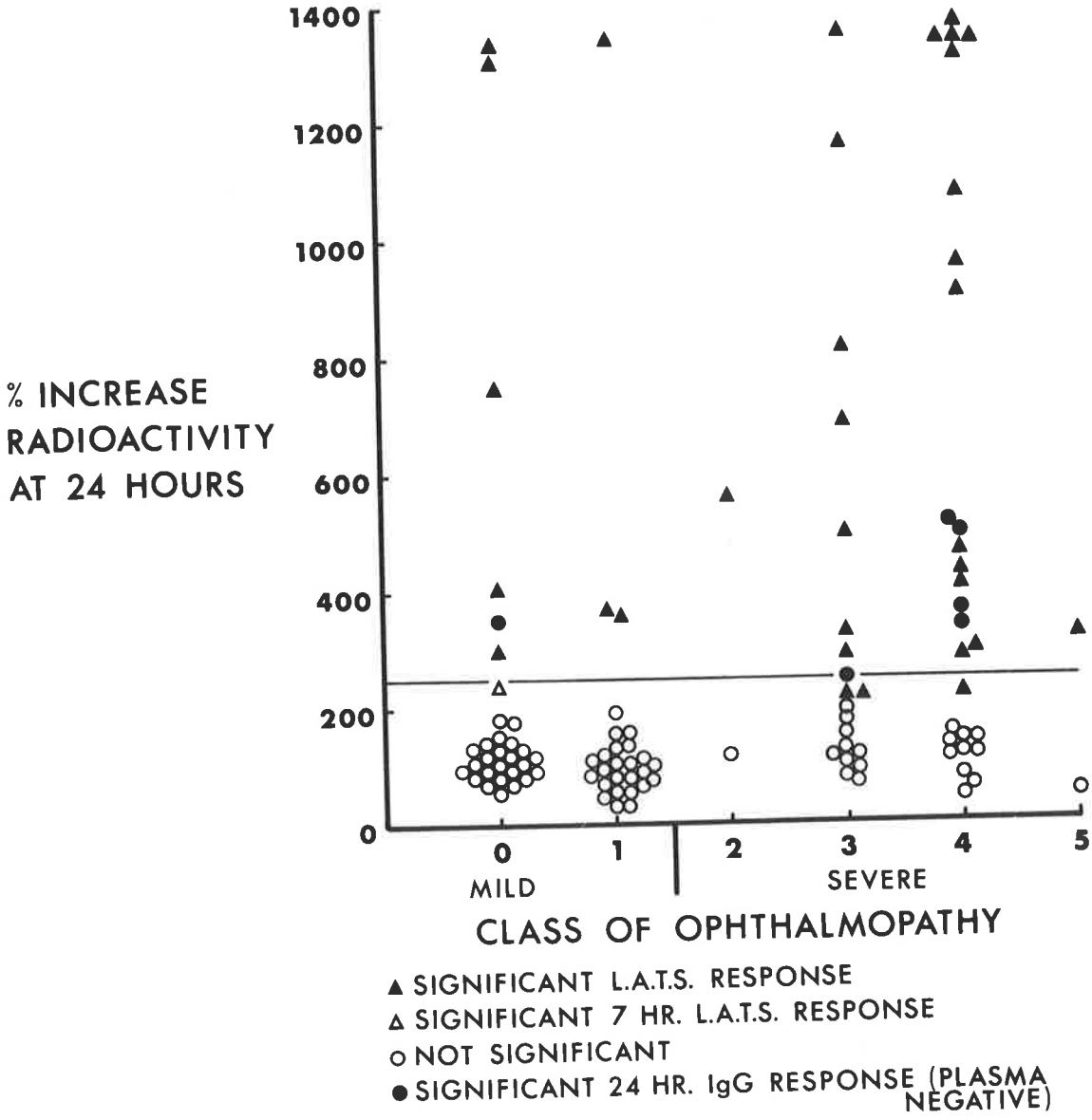


TABLE 20

EYE CHANGES OF GRAVES' DISEASE

Prevalence of Detectable Plasma L.A.T.S.

Class	L.A.T.S.
0	4/41 (10%)
1	3/33 (9%)
2	1/2 (50%)
3	9/20 (45%)
4	14/24 (59%)
5	1/2 (50%)
6	-

(2) Relationship of L.A.T.S. to the Course of the Eye Changes

Changes in L.A.T.S. levels were correlated with changes in the degree of eye involvement in the 18 patients with severe ophthalmopathy in whom serial assays were carried out (Table 21). L.A.T.S. estimations were made as soon as possible after diagnosis and subsequently with change in the eye lesion. On three occasions however the initial assay was not carried out until after diagnosis.

In ten of the 18 patients severe ophthalmopathy developed after treatment of the hyperthyroidism, whilst in five cases severe changes developed before treatment. The other three patients in the series had euthyroid ophthalmopathy and were treated with immunosuppressive agents (two cases) or by orbital irradiation (one case).

Improvement in the eye lesion following treatment of the hyperthyroidism occurred in four of the five patients in whom severe ophthalmopathy developed as part of the presenting picture. L.A.T.S. levels fell in two of these patients and remained high in the other two. In the fifth patient progression of the eye changes followed treatment, in association with persistently high L.A.T.S. levels.

Of the ten patients who developed severe ophthalmopathy after treatment five were treated with immunosuppressive agents or by orbital irradiation with improvement in four.

TABLE 21

EYE CHANGES OF GRAVES' DISEASE

Relationship of L.A.T.S. Levels to the Clinical Course of the Eye Lesion

Case	Initial Plasma L.A.T.S. Level	L.A.T.S. at onset of Ophthal-mopathy	Relation of onset of Ophthalmo-pathy to treatment	Additional Treatment	Change in Ophthal-mopathy after treatment	Subsequent L.A.T.S. Level	Correlation between L.A.T.S. and Ophthal-mopathy
I.P.	912	Yes	Before	-	Much better	1000	No
D.U.	500	Yes	Before	-	Worse	309	No
M.P.	125NS	∅	After	Azathiaprine Irradiation	Slightly better	‡491	No
E.N.	1726	Yes	After	Azathiaprine Steroids	Remission	185NS	Yes
N.E.	76NS	No	After	-	No change	∅213	Yes
E.F.	112NS	Yes	After	-	No change	207	Yes
A.N.	1400	Yes	After	Azathiaprine Irradiation	Remission	178NS	Yes
H.S.	400	Yes	After	Azathiaprine	Worse then stationary	1107	Yes
*M.G.	469	Yes	-	Steroids	Remission	105NS	Yes
P.A.	1087	∅	After	-	Slightly better	1107	No
K.K.	1500	Yes	After	-	Remission	300	Yes
H.P.	900	Yes	Before	-	Remission	150 NS	Yes
P.D.	430	Yes	Before	-	Better	180NS	Yes
*O.C.	∅284	Yes	-	Azathiaprine	Remission	∅195	No

/contd.

TABLE 21 (contd)

Case	Initial Plasma L.A.T.S. Level	L.A.T.S. at onset of Ophthal-mopathy	Relation of onset of Ophthalmo-pathy to treatment	Additional Treatment	Change in Ophthal-mopathy after treatment	Subsequent L.A.T.S. level	Correlation between L.A.T.S. and Ophthal-mopathy
*B.T.	403	ø	-	Azathiaprine Irradiation	Remission	192NS	Yes
M.R.	800	Yes	After	-	Better	300	Yes
D.P.	134NS	No	After	Azathiaprine Steroids	Better	142NS	No
V.R.	1500	Yes	Before	-	Better	2000	No

ø = 7 hr (IgG) response

‡ = Plasma 7 hr response

NS = Not significant

ø = Initial assay not done until after onset of severe eye changes

* = Euthyroid Graves' Disease

Of these, L.A.T.S. levels fell in two cases, rose in one and remained fairly constant in the other. The other five patients did not require additional treatment since the eye lesion improved spontaneously in three and remained quiescent (but still severe) in the other two patients.

As can be seen from Table 21 there was a positive correlation between change in L.A.T.S. levels and change in the clinical state of the eye lesion in 11 of the 18 cases. Worsening of the ophthalmopathy was associated with rise in L.A.T.S. levels in three patients, while improvement was associated with fall in L.A.T.S. levels in eight cases.

The case history of one of these patients (O.C.) in whom remarkable improvement in the severity of the ophthalmopathy occurred following treatment with Azathiaprine is presented in more detail.

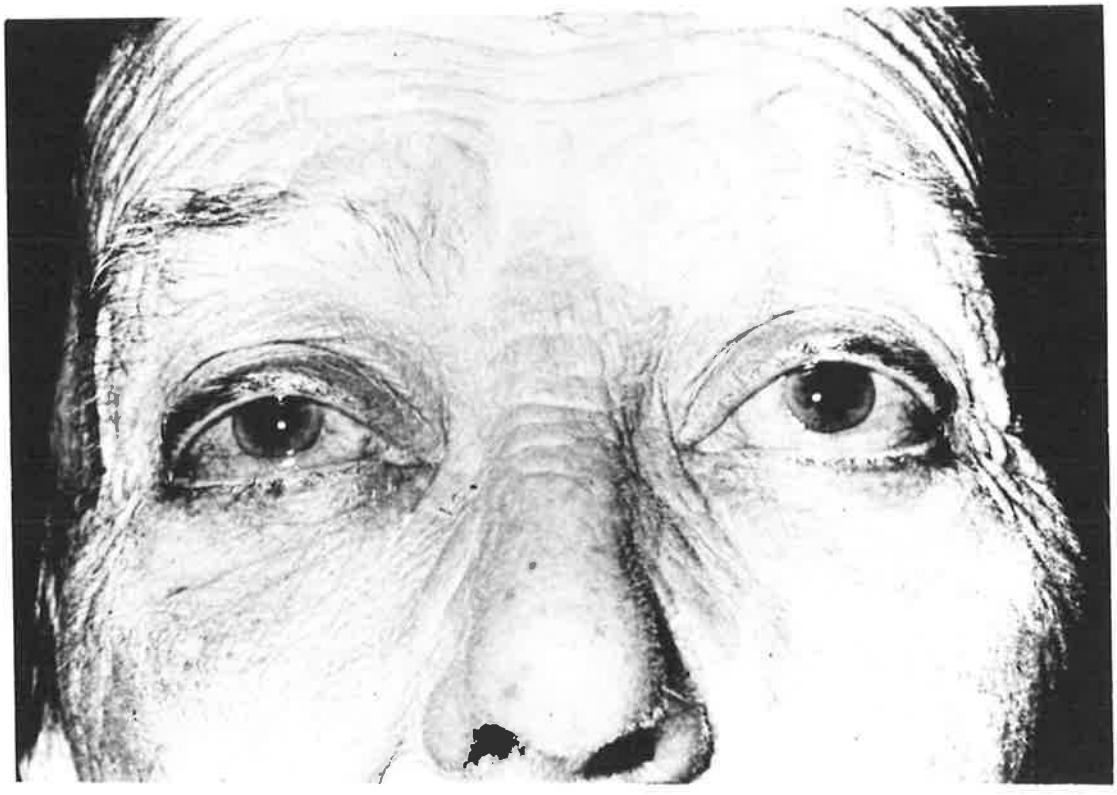
Case O.C.:

This elderly lady who had euthyroid ophthalmopathy was treated with Azathiaprine (150 mg per day). At the height of her illness she had severe exophthalmos, gross orbital infiltration, marked ophthalmoplegia and significant loss of visual acuity (Figure 28). After six weeks of treatment with the immunosuppressive agent however her eyes had become almost

FIGURE 28

RESPONSE TO AZATHIAPRINE —BEFORE TREATMENT

RESTRICTED UPWARD GAZE



normal (Figure 29). There was no exophthalmos or congestion and she had full movement of her eyes and normal visual acuity.

Despite the excellent response to Azathiaprine there was no change in L.A.T.S. levels or antibody titres, although her IgG level fell sharply with treatment.

(3) Antibody Titres

The prevalence of other antibodies was not greater in patients with severe ophthalmopathy than in those with only mild eye signs (Table 22). The titres were those at diagnosis of Graves' disease or on progression of the ophthalmopathy. Significant titres of antithyroglobulin antibodies were present in 23% of patients with severe ophthalmopathy and in 18% of those with mild involvement. Thyroid cytoplasmic antibody was present in 31% of mild cases and in 30% of severe cases, while gastric parietal cell antibody occurred in 20% and 22% of cases respectively.

(4) Immunoglobulin Levels

A higher prevalence of increased levels of IgG was found in patients with severe grades of ophthalmopathy (58%) compared to those with only mild disease (44%). Only about 30% of patients in each class, however, had elevated levels of IgM or IgA and the prevalence of

FIGURE 29

—AFTER TREATMENT

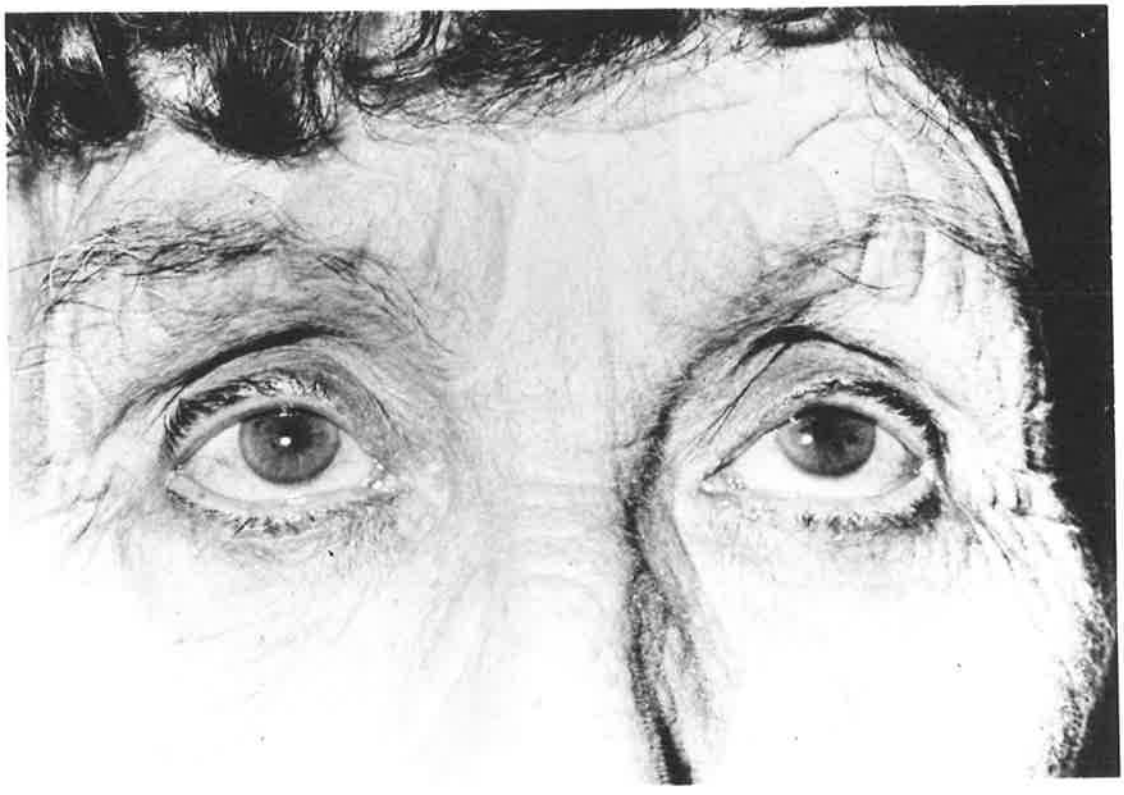


TABLE 22

EYE CHANGES OF GRAVES' DISEASE

Prevalence of Significant Antibody Titres

Class	Anti-Thyro-Globulin	Thyroid Cytoplasmic Antibody	Gastric Parietal Cell Antibody	Smooth Muscle Antibody
0	7/38(18%)	11/39(28%)	9/39(23%)	1/39 (2%)
1	5/30(17%)	9/25(36%)	4/25(16%)	0/28 (0%)
2	0/1	-	-	-
3	3/18(17%)	6/19(32%)	6/19(32%)	0/19 (0%)
4	5/19(26%)	4/17(23%)	2/17(12%)	0/17 (0%)
5	1/1	1/1	0/1	0/1
6	-	-	-	-

increased levels did not correlate with the severity of the eye lesion (Table 23).

(D) EUTHYROID OPHTHALMOPATHY

(1) Clinical Subjects

Twelve patients with severe ophthalmopathy as the only feature of Graves' disease were studied. The diagnosis was made on the presence of the classical features of Graves' ophthalmopathy, namely exophthalmos, congestive changes and ophthalmoplegia, when other causes had been excluded (Werner, 1955, Liddle et al, 1965). T_3 suppression tests were carried out in only a few of these patients with a failure of suppression of ^{131}I uptake after exogenous thyroid hormone administration in about half.

(2) Results

(a) L.A.T.S. Assays

Only one patient had detectable plasma L.A.T.S., whilst two others had borderline levels (200-250%). The prevalence (8%) was thus comparable to that found in patients with hyperthyroidism as the only feature.

(b) Antibody Titres

Only two of these patients had significant titres of antithyroglobulin. Three had thyroid cytoplasmic antibody, two had gastric parietal cell antibody and none had smooth muscle antibody (Table 24).

TABLE 23

EYE CHANGES OF GRAVES' DISEASE

Prevalence of Increased Immunoglobulin Levels

Class	IgG	IgM	IgA
0	21/38(55%)	14/38(37%)	16/38(42%)
1	8/28(29%)	9/28(32%)	4/28(14%)
2	1/1	1/1	0/1
3	11/18(61%)	6/19(32%)	5/18(28%)
4	11/22(50%)	10/22(41%)	8/22(36%)
5	2/2	0/2	0/2
6	-	-	-

TABLE 24

EUTHYROID GRAVES' DISEASE

Prevalence of Significant Antibody Titres

Anti-Thyro-Globulin	Thyroid Cytoplasmic Antibody	Gastric Parietal Cell Antibody	Smooth Muscle Antibody
2/12(17%)	3/12(25%)	2/12(17%)	0/12

(c) Immunoglobulin Levels

The prevalence of increased levels of immunoglobulins was also low in this group of patients. Only three patients had elevated IgG whilst one and two patients respectively had increased levels of IgM and IgA (Table 25).

(E) DISCUSSION

In the present study the prevalence of positive L.A.T.S. responses correlated with the severity of the eye changes. Whilst only 9% of patients with mild ophthalmopathy had detectable L.A.T.S. the stimulator was detected in 57% of patients with severe ophthalmopathy. Furthermore, patients with severe disease generally had higher L.A.T.S. levels than those with only mild disease. Although there was not a close relationship between the degree of ophthalmopathy and the prevalence of significant titres of antibodies, there was a greater prevalence of increased IgG levels in patients with severe disease compared to those with mild ophthalmopathy.

However, rise or fall in L.A.T.S. levels correlated with worsening or improvement in the severity of the eye lesion in only 11 of the 18 patients in whom serial assays were carried out. Of these, worsening of the ophthalmopathy was associated with increase in L.A.T.S. levels in three patients whilst improvement was associated with

TABLE 25

EUTHYROID GRAVES' DISEASE

Prevalence of Increased Immunoglobulin Levels

IgG	IgM	IgA
3/12 (25%)	1/12 (8%)	2/12 (17%)

fall in L.A.T.S. in eight patients. In one other patient, whilst remarkable improvement in the eye lesion occurred during treatment with the immunosuppressive drug Azathiaprine, the L.A.T.S. levels did not fall. Furthermore, a few patients with high L.A.T.S. levels had only mild eye signs whilst others, with severe ophthalmopathy, had no detectable L.A.T.S., and in seven of the 18 patients studied serially, L.A.T.S. levels and the changes in the severity of the eye lesion ran divergent courses.

Thus although there was a tendency for severe ophthalmopathy to be associated with an increased prevalence of detectable L.A.T.S. and higher levels the relationship was not close.

A close relationship between L.A.T.S. and the presence of severe ophthalmopathy has been claimed by many groups (Adams, 1958, Major and Munro, 1962, Hoffmann and Hetzel, 1966, Kurihara et al, 1967). Inmost series however, although the prevalence of positive assays was greater in the presence of severe ophthalmopathy the prevalence in its absence was usually not much less (McKenzie and McCullagh, 1968). However, other workers have not found such a close relationship (Lipman et al, 1967, McKenzie and McCullagh, 1968). The latter group found that neither the concentration, nor even the presence of L.A.T.S. correlated well with the presence or degree of ophthalmopathy.

Despite this, it cannot be doubted that patients with severe eye disease tend to have higher L.A.T.S. levels than those with only mild disease. Failure to find L.A.T.S. in all patients with severe ophthalmopathy may reflect insensitivity of the assay or an absence of circulating L.A.T.S. after the stimulator has become attached to the target tissue, although retro-orbital tissue failed to neutralise the biological activity of L.A.T.S. in a study reported by Shillinglaw and Utiger (1968). On the other hand, it is likely that some other factor is the direct cause of the ophthalmopathy. This factor may be another antibody which is produced under similar circumstances to L.A.T.S., in which case L.A.T.S. may be only a "marker" of another immunological process (MacKay, 1969).

Although the role of L.A.T.S. in the pathogenesis of ophthalmopathy is uncertain there is considerable evidence that the lesion is due to an immunological abnormality (McGill and Asper, 1962). Patients with severe ophthalmopathy generally have a greater prevalence of significant titres of antithyroglobulin than those with only mild signs (Hales et al, 1961). The association with high L.A.T.S. levels (Adams, 1958, Hoffmann and Hetzel, 1966, Kurihara et al, 1967) has been mentioned. High dosage cortico-steroid therapy often alleviates severe ophthalmopathy and is associated with fall in L.A.T.S. levels (Brown et al, 1963, Werner, 1966). Marked

improvement in the eye lesion after treatment with Azathiaprine was observed in one patient in the present series, but without associated fall in L.A.T.S. levels or thyroid antibody titres.

On the other hand, immunofluorescence tests with orbital tissue and patients' serum *in vitro* has failed to identify an antigen-antibody combination (McGill and Asper, 1962) and as McKenzie (1968) has pointed out, the non-specific anti-inflammatory properties of cortico-steroids (and also Azathiaprine) could have accounted for the improvement, as well as the fall in L.A.T.S. levels and antibody titres (Snyder *et al*, 1964, Kriss *et al*, 1964).

The prevalence of detectable plasma L.A.T.S. in patients with euthyroid Graves' disease was, in this series, low. Other groups have also reported a low prevalence of L.A.T.S. in patients with only ophthalmopathy as a feature of their disease (Pinchera *et al*, 1965, Lipman *et al*, 1967).

As stated in a previous chapter the prevalence of detectable L.A.T.S. is probably most closely related to the number of clinical features present (Lipman *et al*, 1967). Thus patients with severe ophthalmopathy, who also tend to have hyperthyroidism and dermopathy, have the highest prevalence of L.A.T.S., whilst patients with only one feature have the lowest prevalence of L.A.T.S.

To conclude then, it is apparent that because of the occurrence of high levels of plasma L.A.T.S. in the absence of significant eye signs and because of the presence of severe ophthalmopathy without detectable plasma L.A.T.S., even after concentration, it is unlikely that L.A.T.S. itself is the direct cause of the eye changes of Graves' disease.

However, in view of the association of severe ophthalmopathy with other immunological abnormalities, and the response to immunosuppressive agents, it is likely that other antibodies are involved in the pathogenesis of this feature. Perhaps delayed hypersensitivity plays a role as well. These and other factors may be closely related to the presence of L.A.T.S. which may be a "marker" of a more complex immunological process.

(F) SUMMARY AND CONCLUSIONS

The relationship between L.A.T.S. and the ophthalmopathy of Graves' disease was studied in 122 patients with thyrotoxicosis including 12 patients with euthyroid Graves' disease. The prevalence of plasma L.A.T.S., other antibodies and increased immunoglobulin levels was correlated with the severity of the eye changes.

A higher prevalence of L.A.T.S. was found in patients with severe ophthalmopathy than in those with mild disease. Patients with severe eye involvement also had higher levels than those with only mild involvement.

L.A.T.S. levels fell in most patients after treatment, and on several occasions correlated with clinical improvement in the ophthalmopathy. However, because high L.A.T.S. levels were found in some patients with only mild disease and conversely, because severe ophthalmopathy occurred without detectable L.A.T.S., the relationship did not appear to be close.

It was postulated that other immunological factors may be involved in the pathogenesis of severe ophthalmopathy. It was considered likely that L.A.T.S. was closely associated with these factors, if only as a "marker".

CHAPTER VI

GENETIC STUDY

(A) INTRODUCTION

(B) FAMILIES AND METHODS

(1) Families
(2) Methods

(C) RESULTS

(1) Clinical Findings
(2) L.A.T.S. Assays
(3) Antibody Titres
(4) Immunoglobulin Levels
(5) Relationship of Clinical Findings to the Immunological Abnormalities
(6) Follow up Study

(D) DISCUSSION

(E) SUMMARY AND CONCLUSIONS

CHAPTER VI

GENETIC STUDY

(A) INTRODUCTION

The tendency for thyrotoxicosis to run in families is well known (Bartels, 1941, Saxena et al, 1964, Evans et al, 1967). Relatives of thyrotoxic patients have an increased prevalence of thyroid and other antibodies as compared to normals, matched for age and sex (Roitt et al, 1956, Hall et al, 1960, Saxena, 1965, Solomon et al, 1968). These findings have indicated that a genetically determined disturbance of immunological tolerance exists in the families of thyrotoxic patients, with the tendency to develop a range of auto-antibodies as well as the specific tendency to develop thyrotoxicosis (McKenzie, 1968, Hetzel, 1968).

In this study assays for L.A.T.S. were carried out on the concentrated IgG fraction from euthyroid relatives of thyrotoxic patients. Other antibodies and immunoglobulin levels were also estimated in order to provide an assessment of the immunological status of the relatives. These results were compared with control series of thyrotoxic patients and normal people.

(B) FAMILIES AND METHODS

(1) Families

Four families with an unusually high prevalence of thyrotoxicosis were studied. The pedigrees are shown in Figures 30 and 31. Over 50 euthyroid relatives were examined for signs or symptoms of thyrotoxicosis, other thyroid diseases, or other diseases of possible autoimmune etiology.

(2) Methods

One hundred ml of blood was drawn from forty-three of the relatives, children under ten years of age being excluded from the study. L.A.T.S. assays were carried out on the plasma and concentrated IgG fraction. Protein bound iodine was measured by the Biochemistry Department using the method of Acland (1951). Anti-thyroglobulin titre was estimated by the tanned red cell agglutination method (Boyden, 1951), and thyroid cyto-plasmic, gastric parietal cell and smooth muscle antibodies by the immunofluorescent technique (Weller and Coons, 1954). Finally, IgG, IgM and IgA levels were estimated using the single radial immunodiffusion method (Mancini et al, 1965). These methods have been described in Chapter II.

(C) RESULTS

(1) Clinical Findings

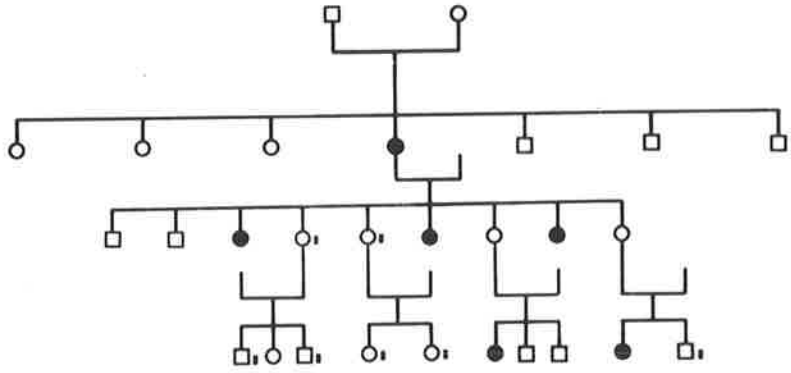
All but one of the relatives examined were clinically euthyroid, the exception being an obese girl

FIGURE 30

THYROXICOSIS GENETIC STUDY

PEDIGREES

O'CONNELL FAMILY



● affected ♀ ○ normal ♀

■ affected ♂ □ normal ♂

, L.A.T.S. CONCENTRATION

HOBBY FAMILY

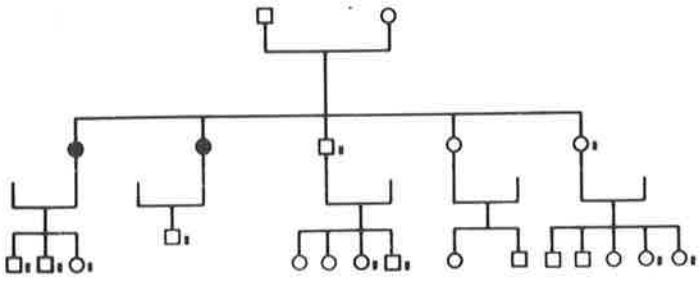
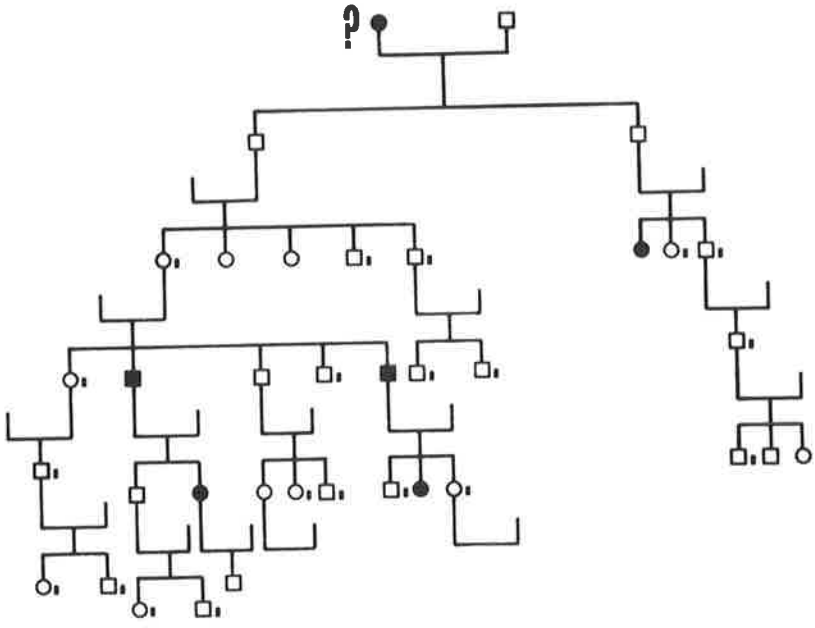


FIGURE 31

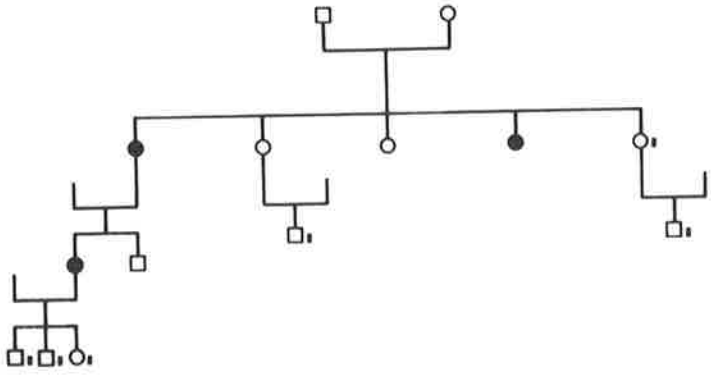
THYROXICOSIS GENETIC STUDY

PEDIGREES

MARSHMAN FAMILY



SINCLAIR FAMILY



who was mildly hypothyroid with a borderline PBI of 2.9 µg%. Although none of the other relatives were clinically toxic two, one of whom was subsequently shown to have a borderline level of L.A.T.S. had elevated PBI (8.1 and 8.9 µg%). Amongst the relatives studied however, there were several findings which could indicate a latent thyrotoxic tendency. Two relatives had mild exophthalmos with lid lag and stare whilst three others had a stare and lid lag alone. Another of the relatives had intolerance of hot weather with excessive sweating, whilst three women had oligomenorrhoea. There was not an increased prevalence of other thyroid diseases or other autoimmune diseases amongst the relatives.

(2) L.A.T.S. Assays

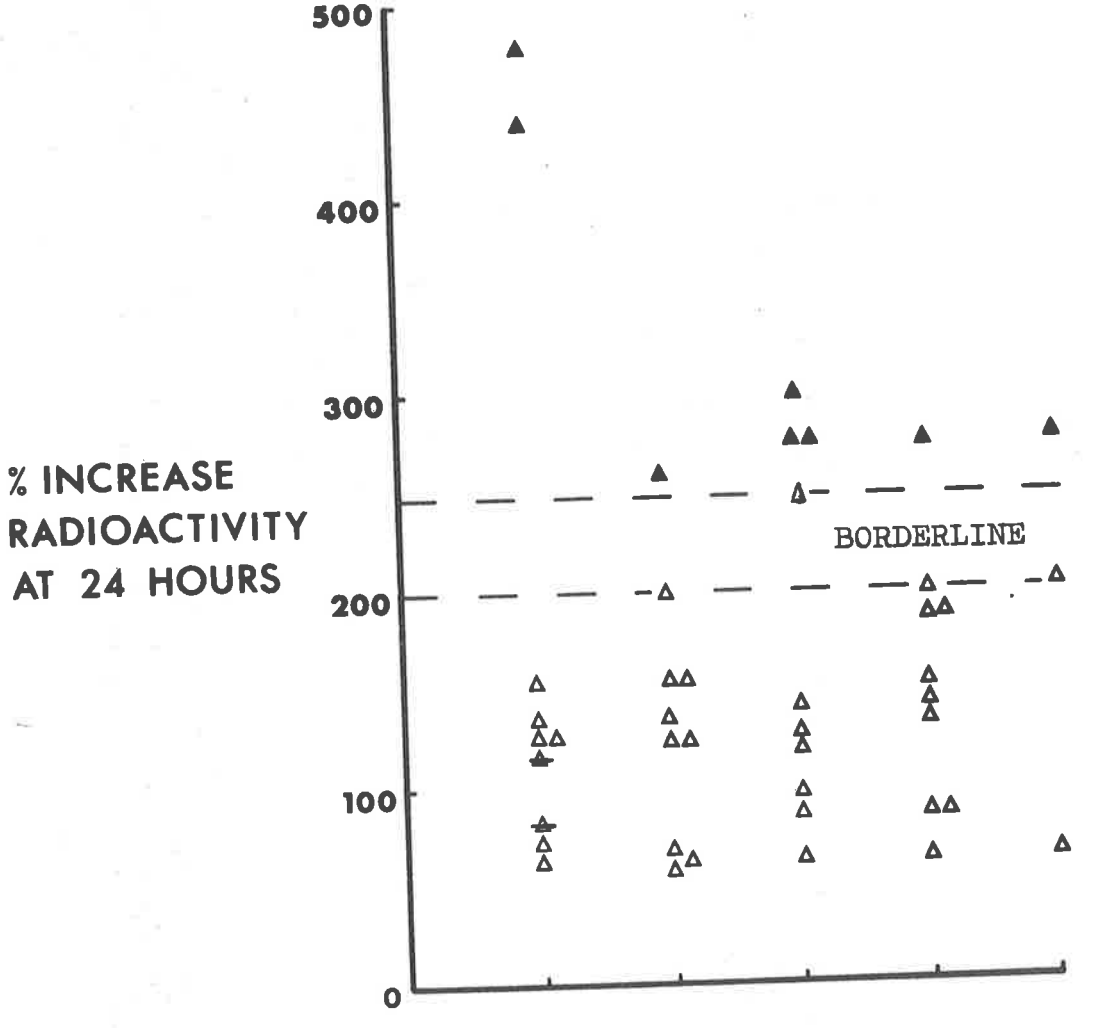
L.A.T.S. was detected in eight (19%) of the 43 relatives when plasma and the concentrated IgG fraction was assayed for L.A.T.S. activity (Figure 32). L.A.T.S. was detected only after concentration in all but one case. As can be seen from the figure, the levels generally were low, with 24 hour values of between 250% and 300% but two of the relatives had moderately high levels of over 400%.

A further four of the relatives had borderline levels of L.A.T.S. with 24 hour values of between 200% and 250%.

FIGURE 32

GENETIC STUDY

IgG L.A.T.S. LEVELS



= Plasma Levels - IgG toxic to mice

= Significant Response

= Not Significant

(3) Other Antibodies

A number of the relatives had thyroid and other antibodies, although mostly in low titres (Table 26). The prevalence of significant titres however was greater than in a similar series of normal relatives of probands with myxoedema, acromegaly and Sjögren's disease respectively but less than in a similar series of thyrotoxic patients.

(4) Immunoglobulin Levels

IgG, IgM and IgA levels were elevated in about 40% of the relatives. The prevalence of increased levels was greater than in the control series but less than in the series of thyrotoxic patients (Table 27).

(5) Relationship of Clinical Findings to the Immunological Abnormalities

The clinical findings amongst the relatives were found equally in those with detectable L.A.T.S. (including borderline levels) and in those without L.A.T.S. For example although two of the former group had eye signs, one patient without detectable L.A.T.S. had mild exophthalmos while another had lid lag and stare.

In this series also, there was not a close relationship between the presence of L.A.T.S. and significant titres of other antibodies or increased immunoglobulin levels, in that the L.A.T.S.-positive relatives

TABLE 26

PREVALENCE OF SIGNIFICANT ANTIBODY TITRES

	Anti-Thyro-Globulin	Thyroid Cytoplasmic Antibody	Gastric Parietal Cell Antibody	Smooth Muscle Antibody
Relatives This series (43)	12/43(28%)	5/43(12%)	5/43 (12%)	2/43(4.5%)
Thyrotoxics (43)	18/43(42%)	16/43(37%)	9/43 (21%)	0/43(0%)
Controls (43)	3/43(7%)	3/43(7%)	2/43 (5%)	0/43(0%)

TABLE 27

PREVALENCE OF INCREASED IMMUNOGLOBULIN LEVELS

	IgG	IgM	IgA
Relatives This series (43)	18/43(42%)	15/43(35%)	17/43(40%)
Thyrotoxics (43)	23/43(53%)	16/43(37%)	18/43(42%)
Controls (43)	12/43 (27%)	5/43(12%)	10/43(23%)

did not have a greater prevalence of significant titres of antibodies or elevated immunoglobulin levels than the L.A.T.S.-negative relatives. They did tend however to have higher PBIs than the L.A.T.S. negative relatives, the means for the two groups being 6.5 µg% and 5.6 µg% respectively.

(6) Follow up Study

L.A.T.S. estimations were repeated twelve months later in five of the relatives, three previously L.A.T.S.-positive, one with a borderline level and the other previously L.A.T.S.-negative. Three of the relatives who had L.A.T.S. initially had significant or borderline levels on this occasion (Table 28) whilst L.O., who did not have detectable L.A.T.S. previously, had a borderline level. None of these people had become clinically thyrotoxic.

(D) DISCUSSION

Thyroid disease generally is known to run in families and clinically different thyroid syndromes may arise within the same family (Rolleston, 1936, Rundle, 1941, Kitchin and Evans, 1960). The tendency for thyrotoxicosis itself to run in families has been mentioned. In a retrospective study Harvald and Hauge (1956) found thyrotoxicosis in 12 of 41 thyrotoxic probands of monozygotic pairs compared with only two affected of 59 dizygotic pairs of

TABLE 28

IgG L.A.T.S. LEVELS AFTER TWELVE MONTHS

Relative	24 hour L.A.T.S. levels	
	1969	1970
J.O'C.	•220	162
F.M.	445	•217
A.N.	291	*244-125
M.H.	310	546
L.O'C.	ǂ63	•221

ǂ Plasma Level

* 7 hour Level

• Borderline Level

the same sex. Furthermore, Ingbar et al (1956) reported more rapid turnover of thyroxine and a higher uptake of radio-iodine in relatives of patients with thyrotoxicosis who were euthyroid.

Although the exact mode of inheritance is not known statistical analysis has indicated that a single recessive factor may be involved (Martin and Fisher, 1945).

Several studies have shown that the relatives of thyrotoxic patients have an increased prevalence of thyroid antibodies as compared to normals (Roitt et al, 1956, Hall et al, 1960) as well as an increased tendency to develop other thyroid diseases, particularly those with an autoimmune mechanism, and other autoimmune diseases. For example, there is an increased prevalence of Hashimoto's disease in the relatives of thyrotoxics and to a lesser extent myxoedema and simple goitre (Means et al, 1963, Anderson et al, 1964, Heinmann, 1966).

There is also a relationship between thyrotoxicosis and Addison's disease, pernicious anaemia, and Sjögren's disease, as these diseases occasionally occur in combination with thyrotoxicosis (Blizzard and Kyle, 1963, Doniach et al, 1963). The common factor is probably autoimmunity, even though it has not been proven that these diseases are autoimmune.

The presence of L.A.T.S. in the normal relatives of

thyrotoxic patients is thus not unexpected and may indicate a genetic predisposition to develop the disease. With the appropriate stimulus such as infection or emotional disturbance (Hetzel, 1960, Brown and Hetzel, 1963, Alexander et al, 1968) those people with L.A.T.S. may develop overt hyperthyroidism.

It is possible that the thyroid diseases which are most likely to have an autoimmune pathogenesis, namely thyrotoxicosis, Hashimoto's disease and myxoedema all start as a nonspecific thyroiditis, caused by many factors, including viral infection, trauma or iodine deficiency. The resultant disease would depend on the antibodies produced, so that if there was an excess of antithyroglobulin and the cytoplasmic antibody thyroid destruction and Hashimoto's thyroiditis may result (Witebsky and Rose, 1956, Irvine, 1964), progressing to myxoedema, but if excess L.A.T.S. was produced, hyperthyroidism may result (Lipman et al, 1967).

This tendency to react to antigenic stimuli with the formation of a range of antibodies is probably genetically determined as indicated by the strong familial tendency for the production of these diseases and the corresponding antibodies. Thus, patients with a genetically determined abnormality of the antibody forming system may react excessively to thyroid antigenic stimuli

with release of antibodies and appearance of clinical features of the appropriate disease. Some people, on the other hand, may have circulating antibodies but do not have overt disease. With the presence of other critical factors however, such as stress, iodine deficiency or an unusually strong antigenic stimulus, as, for example following infection, the reaction between the antibodies and the target tissues may initiate a self-perpetuating disease process.

In order to test this hypothesis it is intended to follow the relatives who had detectable L.A.T.S. to determine the frequency of subsequent clinical manifestations of thyrotoxicosis.

(E) SUMMARY AND CONCLUSIONS

Bioassays for L.A.T.S. were carried out on the plasma and IgG fraction of euthyroid relatives of thyrotoxic patients in four families with an unusually high prevalence of the disease. The relatives were examined for signs of thyroid disease or other autoimmune diseases and blood was taken for L.A.T.S. assay, as well as for the estimation of protein bound iodine, antibody titres and IgG, IgM and IgA levels.

L.A.T.S. was detected in 8 (19%) of 43 euthyroid relatives from the four families. Four others had borderline levels. The relatives also had an increased

prevalence of other antibodies and increased immunoglobulin levels, but these parameters did not correlate with the presence of detectable L.A.T.S.

The presence of L.A.T.S. in otherwise normal relatives may indicate a predisposition to develop thyrotoxicosis. As other antibodies are increased in the relatives, the abnormality may be in the antibody forming system with the tendency for production of several antibodies and the corresponding autoimmune diseases.

Since L.A.T.S. was detected in euthyroid relatives it is now apparent that the stimulator can be present without causing overt hyperthyroidism. This will be discussed more fully in the final chapter of this thesis.

CHAPTER VII

L.A.T.S. IN OTHER DISEASES

(A) INTRODUCTION

(B) CLINICAL SUBJECTS AND METHODS

(1) Clinical Subjects
(2) Methods

(C) RESULTS

(1) Clinical Findings
(2) L.A.T.S. Assays
(3) Antibody Titres
(4) Immunoglobulin Levels
(5) Correlations

(D) DISCUSSION

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CHAPTER VII

L.A.T.S. IN OTHER DISEASES

(A) INTRODUCTION

MacKay and Burnet (1963) have defined an autoimmune disease as a "condition in which structural or functional damage is produced by the action of immunologically competent cells or antibodies against normal constituents of the body".

Autoimmune diseases occur when there is a breakdown of immunological tolerance to the body's own antigens. Auto-antibodies are produced which react with the corresponding tissue antigens. This process of antibody-antigen combination is associated with infiltration of the target organs with antibody forming cells (lymphocytes and plasma cells) and progressive destruction and replacement by fibrous tissue, with characteristic clinical features. Although other processes, such as delayed hypersensitivity, macrophage transport of antigen and chemical modification of tissue antigens are involved, the production of auto-antibodies appears to be fundamental in the pathogenesis of autoimmune diseases (Milgrom and Witebsky, 1962, Humphrey and White, 1963).

The "markers" which characterise autoimmune diseases include deposition of gamma globulin at certain sites,

elevated serum gamma globulin level (above 1.5 gm/100 ml), accumulation of antibody forming cells in damaged tissues and benefit from cortico-steroid drugs or immunosuppressive agents (MacKay and Burnet, 1963, Hetzel, 1970).

Autoimmune disease may involve more than one organ and patients may have features of two or more distinct diseases. For example, Hashimoto's disease may occur with a Coomb's positive hemolytic anaemia (Burnet, 1959), and myxoedema and pernicious anaemia frequently coexist. Furthermore, localised disease involving organ specific antibodies may develop into a multi-system disease such as disseminated lupus erythematosus in which both organ specific and non organ specific antibodies (such as anti-nuclear factor, rheumatoid factor and the Wasserman anti-body), are found (Kunkel and Tan, 1964).

Finally, members of the same family may have different autoimmune diseases (Hassan et al, 1966). The tendency for thyrotoxicosis and Hashimoto's disease to occur in the same family is striking. In these families there is also an increased prevalence of thyroid antibodies, other antibodies such as gastric parietal cell antibody (Hall et al, 1960) and other autoimmune diseases (Hassan et al, 1966).

The principle aim of this study was to determine whether the long acting thyroid stimulator could be detected

in patients with other autoimmune diseases or with diseases having a possible autoimmune mechanism, but without thyrotoxicosis.

L.A.T.S. concentration studies were made of firstly, patients with diseases of probable autoimmune etiology such as rheumatoid arthritis, Hashimoto's disease and disseminated lupus erythematosus, secondly, of patients with diseases of possible autoimmune etiology such as Addison's disease, pernicious anaemia and Sjögren's disease, and finally, of patients with a variety of non autoimmune diseases and from normal people, including normal relatives of patients with autoimmune diseases. Other antibodies and immunoglobulin levels were also estimated in these studies.

(B) CLINICAL SUBJECTS AND METHODS

(1) Clinical Subjects

Seventeen patients with autoimmune diseases and a similar number of patients with diseases of possible autoimmune mechanism were studied (Table 29). The diagnosis of these diseases was made by clinical assessment and laboratory investigations including tests for auto-antibodies and estimation of immunoglobulin levels. In most cases special tests of immunological function were carried out in which both humoral and cellular aspects were investigated (Forbes et al, 1970).

TABLE 29

DISEASES INVESTIGATED FOR L.A.T.S.

	Disease	Number of Cases Studied
Autoimmune Diseases	Systemic Lupus Erythematosus	3
	Rheumatoid Arthritis	5
	Hashimoto's Disease	2
	Sjögren's Disease	3
	Multiple-system Autoimmunity	3
	Chronic Active Hepatitis	1 (17)
Possible Autoimmune Diseases	Myxoedema	5
	Pernicious Anaemia	1
	Polyglandular Syndrome	6
	Scleroderma	2
	Addison's Disease	3 (17)
Non Autoimmune Diseases	Hyperparathyroidism	1
	Pan Hypopituitarism	1
	Infectious Mononucleosis	1
	Hodgkin's Disease	2
	Non-toxic Goitre	8
	Others	4 (17)
Others	Normal People	13
	Normal Relatives of Patients with Autoimmune Diseases	34 (47)

Thirty-four normal relatives of patients with possible autoimmune diseases (Sjögren's disease, myxoedema and disseminated sclerosis) were investigated for L.A.T.S. Seventeen patients with a variety of diseases, including Hodgkin's disease, sarcoidosis and infectious mononucleosis and 13 normal people were also studied. Included amongst the non autoimmune group were patients with other endocrinopathies including endemic goitre, pan hypopituitarism and hyperparathyroidism.

(2) Methods

The patients were assessed clinically for features of thyrotoxicosis and questioned for family history of thyroid diseases or other autoimmune diseases. Confirmation of the thyroid status was made by estimating serum protein bound iodine and, whenever possible, 100 ml of blood was drawn for L.A.T.S. concentration as well as for estimation of antibody titres and immunoglobulin levels.

(C) **RESULTS**

(1) Clinical Findings

Of particular interest were patients with thyroid disorders. Only one patient, a woman with proven Hashimoto's disease, was clinically thyrotoxic. Apart from six patients with myxoedema most patients with polyglandular syndrome had hypothyroidism as one of the features.

All of the patients with endemic goitres were clinically and biochemically euthyroid despite the very low levels of iodine in the water and soil.

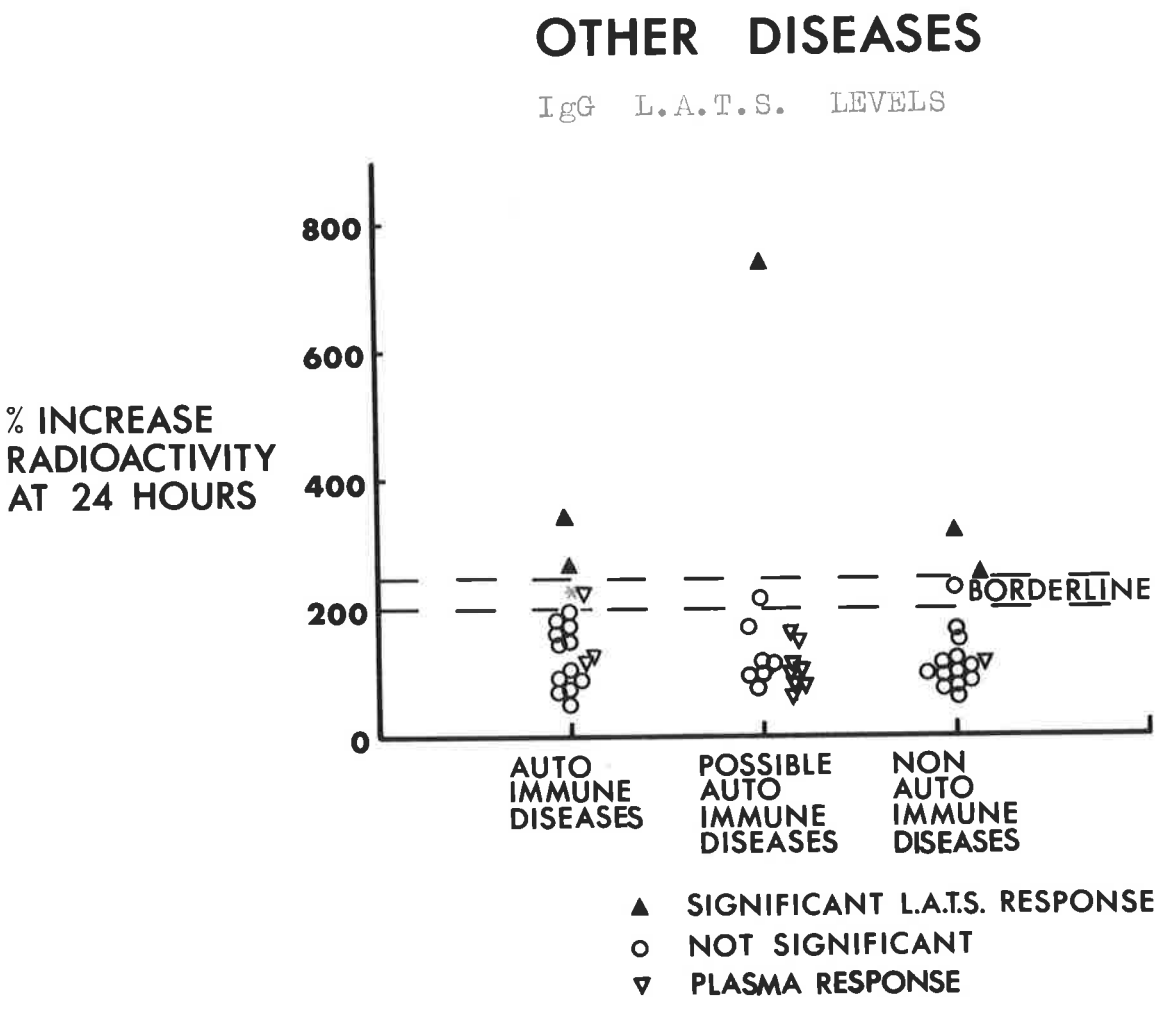
Only two patients with proven Hashimoto's disease were investigated, both of whom had very high titres of thyroid antibodies. At the time of the study one was hypothyroid whilst the other had features of hyperthyroidism. Since Hashimoto's disease is often insidious however, the diagnosis was probably missed, patients presenting some time later with myxoedema and low antibody titres.

(2) L.A.T.S. Assay

The results of L.A.T.S. assays are summarised in Figures 33 and 34. Significant levels of L.A.T.S. were detected in one case of rheumatoid arthritis, in one case of Hashimoto's disease, in one case of pan hypopituitarism, in one case of polyglandular syndrome and in one case of thyroid malignancy. L.A.T.S. was detected in the plasma of two of these patients but in the concentrated 7S globulins only from the other three. L.A.T.S. was also detected in one normal person (Figure 34).

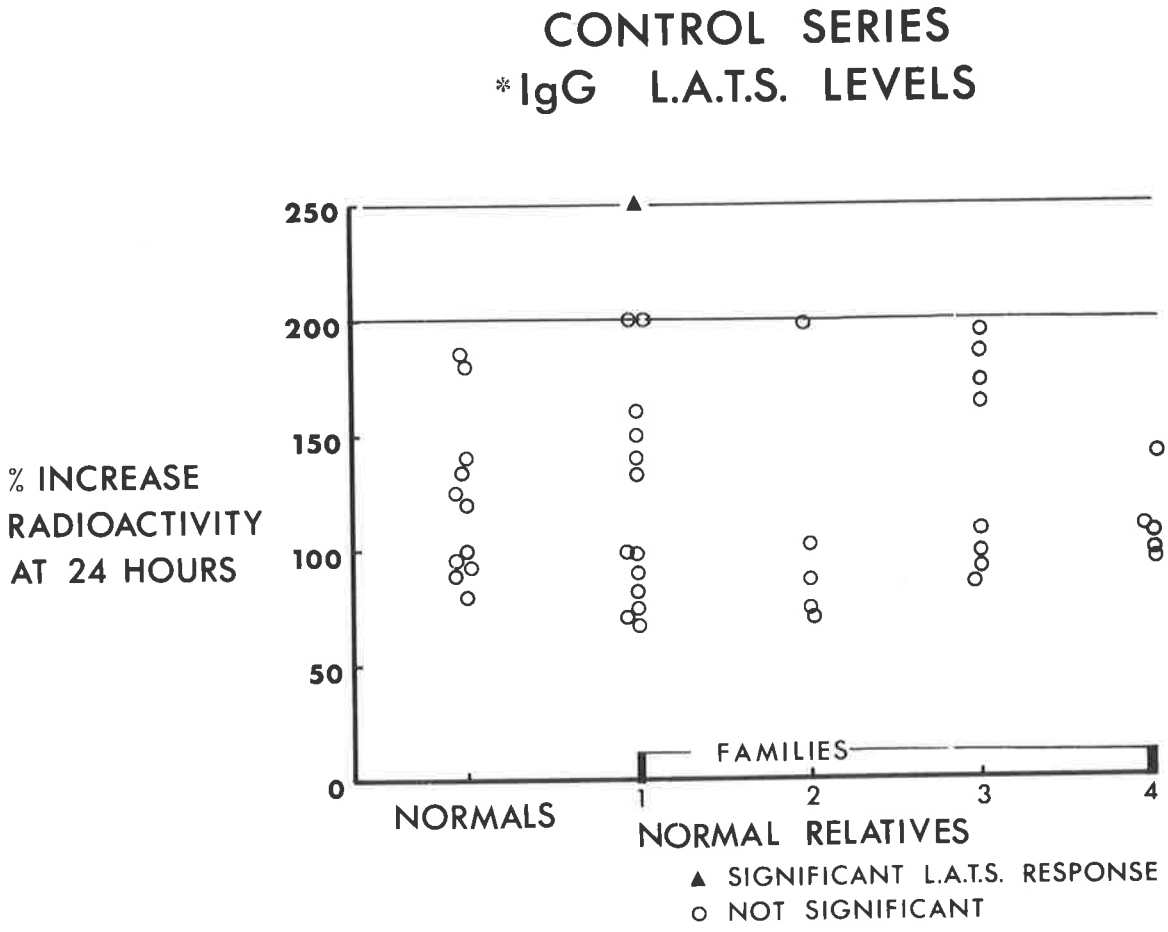
Two other patients, one with polyglandular syndrome and one with bronchiectasis and three normal relatives had borderline levels of L.A.T.S. One patient with rheumatoid arthritis had a borderline plasma level.

FIGURE 33



*▽Borderline plasma L.A.T.S. - concentration not carried out

FIGURE 34



* PLASMA LEVELS ONLY ON FOUR OCCASIONS BECAUSE OF MOUSE TOXICITY (NOT SHOWN)

(3) Antibody Titres

The prevalence of significant titres of other antibodies in patients with autoimmune diseases, was, in this series, low thus only about 20% of these patients had significant titres (Table 30). The prevalence in patients with non autoimmune diseases and in normals was similar. The only correlation was with gastric parietal cell antibody which was present in 30% of patients with autoimmune diseases but in only 14% of patients with other diseases and in 14% of normals (Table 30).

At the time of the study however, most patients with autoimmune diseases were being treated with corticosteroids or immunosuppressive agents. This may have accounted for the rather low prevalence of antibodies in this group.

(4) Immunoglobulin Levels

Elevated levels of IgG and IgA were found in a large proportion of patients with autoimmune diseases. The prevalence of increased IgM levels however was much less (Table 31).

A similar proportion of patients with non autoimmune diseases had elevated levels of immunoglobulins but several of these had disorders of antibody forming tissues such as Hodgkin's disease, sarcoidosis and infectious mononucleosis in which increased immunoglobulins

TABLE 30

PREVALENCE OF SIGNIFICANT ANTIBODY TITRES

	Anti-Thyro-Globulin	Thyroid Cytoplasmic Antibody	Gastric Parietal Cell Antibody	Smooth Muscle Antibody
Autoimmune Diseases	3/12 (25%)	2/13 (15%)	3/13 (23%)	0/13 (0%)
Possible Autoimmune Diseases	5/17 (29%)	2/17 (12%)	6/17 (35%)	1/17 (6%)
Non Autoimmune Diseases	2/14 (14%)	3/14 (21%)	2/14 (14%)	1/14 (7%)
Normal People and Relatives	4/34 (12%)	6/28 (21%)	4/28 (14%)	0/28 (0%)

TABLE 31

PREVALENCE OF INCREASED IMMUNOGLOBULIN LEVELS

	IgG	IgM	IgA
Autoimmune Diseases	8/15(53%)	5/15(33%)	10/15(67%)
Possible Autoimmune Diseases	10/16(63%)	5/16(31%)	8/16(50%)
Non Autoimmune Diseases	7/13(54%)	7/13(54%)	7/13(54%)
Normal People and Relatives	9/31(29%)	8/31(26%)	13/31(42%)

were often found. On the other hand the prevalence in normal people and normal relatives of patients with autoimmune diseases was lower.

(5) Correlations

There was no obvious correlation, in this series, between the presence of detectable L.A.T.S. and significant antibody titres or elevated immunoglobulin levels.

(D) DISCUSSION

In this study L.A.T.S. was detected in three (9%) patients with autoimmune diseases, in two (12%) patients with non autoimmune diseases and in one normal person. About 30% of patients with autoimmune diseases had significant antibody titres and 50% had elevated levels of immunoglobulins. The prevalence of significant antibody titres and elevated levels of IgG, IgM or IgA in patients with non autoimmune diseases was less, but greater than for the control group.

The process whereby an antigenic component of the body's own tissues calls forth the production of antibody or results in specific sensitization to this antigen is called auto-immunization or auto-sensitization (Humphrey and White, 1963). Several possible mechanisms have been suggested. There may be an anomaly either on the side of the antigenic stimulus or on the side of the

immune response (MacKay, 1969). Firstly, there may be escape of previously "sequestered" tissue antigens, which is now thought to be unlikely. Secondly, by an immunizing effect of micro-organisms, antigens which are cross-reactive with "self" antigens may be released. Thirdly, mild tissue injury could alter "self" antigens sufficiently to allow these to break tolerance. Finally, there may be failure of an enzyme system to remove potentially immunogenic components of tissue released during tissue injury (Lamoureux et al, 1967).

Anomalies on the side of the immuno-response itself, due to a genetically determined disorder of the lympho-reticular system, results in normal tissue components being treated as foreign or extrinsic antigens (Burnet, 1959, MacKay, 1969).

The most characteristic feature of autoimmune diseases is the presence of circulating auto-antibodies. For example, systemic lupus erythematosus is associated with antinuclear antibodies (MacKay, 1969), autoimmune thyroiditis with anti-thyroid antibodies (Roitt and Doniach, 1958) and pernicious anaemia with anti-parietal cell and anti-intrinsic factor antibodies (Blizzard and Kyle, 1963).

Although autoimmune serological reactions may be important in recognition of autoimmune diseases, the

appearance of auto-antibodies may be only a "marker" of a basic immunological disturbance (MacKay, 1969). Antibodies frequently appear after tissue injury (Weir, 1964), for example, the high prevalence of thyroid antibodies in subacute thyroiditis (Doniach and Roitt, 1958) and in patients with colloid goitre (Philip et al, 1962) may be due to tissue damage or release of "sequestered" antigens.

As regards thyroid autoimmunity perhaps the most significant feature is the marked familial association of immunological abnormalities. Thus certain families have a tendency to develop thyroid autoimmune diseases (Means et al, 1963, Anderson et al, 1964), the clinical features depending on the antibodies produced. Whilst some members may have an excess of thyroid aggressive antibodies and develop Hashimoto's thyroiditis, others may have L.A.T.S. or closely related antibodies and develop thyrotoxicosis. On the other hand, features of both diseases may occur in the same patient (Doniach et al, 1967).

As further evidence of the considerable clinical and serological overlap in autoimmune diseases, it has been reported that patients with Sjögren's disease, pernicious anaemia and Addison's disease have an increased prevalence of thyroid antibodies (Doniach et al, 1963, Bloch and Bunim, 1963, Blizzard and Kyle, 1963, Anderson et al, 1964),

whilst other patients with features of several autoimmune diseases and a variety of auto-antibodies may develop a "multisystem" disease such as disseminated lupus erythematosus. The occasional occurrence of Graves' disease with Addison's disease, Sjögren's disease and pernicious anaemia is thus likely to have an autoimmune mechanism since organ specific antibodies have been found in all of these diseases (Goudie et al, 1957, Doniach et al, 1963, Anderson et al, 1964).

Thyrotoxicosis can be compared with another disease in which an autoimmune mechanism is generally accepted, namely rheumatoid arthritis (Hetzel, 1968, Hetzel, 1970). Rheumatoid arthritis, a disease often precipitated by stress or infection is characterized by the presence in the serum of "rheumatoid factor", which is detectable in about 98% of patients with active disease, but in only 70% one year after the onset. The antibody is present in approximately 7% of patients with other forms of arthritis and in 6% of random patients. It is also present in 20% of close relatives (Kellgren and Ball, 1959).

Similarly, plasma L.A.T.S. was found in these studies in 100% of patients with severe thyrotoxicosis but in only 14% of patients with one feature. The stimulator was detected in 19% of euthyroid relatives of probands after concentration of the 7S globulins, in 9% of patients with autoimmune diseases and in one normal person.

It seems likely therefore that the relationship between L.A.T.S. and thyrotoxicosis is similar to that between the "rheumatoid factor" and rheumatoid arthritis (Hetzel, 1970), a finding, however, which does not necessarily indicate cause and effect.

In conclusion, one may postulate that people with circulating L.A.T.S., because of a genetically determined abnormality of the immune system, may be predisposed to develop thyrotoxicosis. However, as proposed in Chapter VI, whilst L.A.T.S. itself probably causes the hyperthyroidism, it may be only a "marker" of a more complex immunological disturbance which causes the other features of thyrotoxicosis.

(E) SUMMARY AND CONCLUSIONS

Assays for L.A.T.S. were carried out on the plasma and concentrated 7S globulin fraction from patients with autoimmune diseases. Patients with non autoimmune diseases and normal people were also investigated for L.A.T.S.

L.A.T.S. was detected in three (9%) patients with autoimmune diseases, in two (12%) patients with non autoimmune diseases and in one of 13 normals. Although the prevalence of other antibodies was not significantly greater in patients with autoimmune diseases, increased immunoglobulin levels were more often found in this group.

The possible mechanisms of autoimmune diseases and the significance of circulating auto-antibodies were discussed in regard to thyrotoxicosis.

It was concluded that euthyroid persons with circulating L.A.T.S. were predisposed to develop thyrotoxicosis, but that while L.A.T.S. itself probably caused the hyperthyroidism it was likely to be only a "marker" of a more complex immunological disturbance which caused the other features of thyrotoxicosis.

CHAPTER VIII

EXPERIMENTAL PRODUCTION OF THYROID STIMULATING ANTIBODY

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    Thyroid Function
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    Pathological Changes
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    Thyroid Function
      i. Protein Bound Iodine
      ii. ^{131}I Uptake
    Thyroid Stimulating Antibody
    Antithyroglobulin Titres
    Pathological Changes

CHAPTER VIII

EXPERIMENTAL PRODUCTION OF THYROID STIMULATING ANTIBODY

(A) INTRODUCTION

As outlined in Chapter I, in order to test the hypothesis that L.A.T.S. is an auto-antibody against a thyroidal microsomal antigen, it is proposed to immunize experimental animals with thyroidal microsomes (Milgrom and Witebsky, 1962). The concentrated IgG fraction will be assayed for L.A.T.S.-like activity. If, in the animals with circulating thyroid stimulating antibody, there were changes indicative of thyrotoxicosis similar to human thyrotoxicosis, it would be likely that the presence of the stimulator was closely related to the development of these clinical features.

In this study, rabbits and rats were immunized with whole thyroid extract or the microsomal fraction. Thyroid function was studied in the animals by determining the serum protein bound iodine and 24 hour ^{131}I uptake. Anti-thyroglobulin titres were also measured.

Two experiments in which rabbits were immunized with whole thyroid extract and the microsomal fraction respectively, and two experiments in which rats were immunized with these antigens will be described.

(B) MATERIALS AND METHODS

(1) Experimental Animals

Several varieties of adult male and female rabbits, weighing between two and four kgm were used. They were housed individually in pens in the animal house, which was kept at 70±5°F., and fed standard rabbit cubes supplimented with green feed and carrots.

Inbred chocolate brown adult rats weighing between 150 and 250 gm were used. They were housed under similar conditions in large pens and fed standard rat cubes. Males and females were kept in separate pens during the course of the experiments.

(2) Preparation of Antigens

(a) Whole Extract

Thyroid tissue was obtained at post mortem as soon as possible after death from subjects without a previous history of thyroid disease. A fresh extract was prepared for each injection and kept at 4°C during preparation to minimise proteolysis.

Thyroid tissue was trimmed of excess fibrous tissue, cut into fine pieces with scissors and then homogenised in a Vertis blender with phosphate buffer (pH 6.5). The mixture was filtered through gauze and concentrated by placing in dialysis tubing and leaving in sucrose at 4°C overnight (McKenzie, 1966). The concentrated extract was divided into equal aliquots for injection. To each aliquot

was added 0.05 mg of sodium azide as preservative and an equal volume of complete Freund's adjuvant (Doebbler and Rose, 1961). The mixture was emulsified and injected into the animals. Each animal received one gm equivalent of original tissue per injection.

Liver extract, which was given to control animals, was prepared in the same way from normal liver obtained at post mortem and emulsified with an equal volume of complete Freund's adjuvant.

(b) Microsomal Fraction

Microsomal subcellular fractions were prepared from tissue extracts by differential centrifugation in an M.S.E. ultracentrifuge.

The cellular debris was removed by centrifuging the extract at low speed (2,000 rpm) for 20 minutes. Next, the mitochondria were removed by centrifuging the supernatant at 8,500 g for ten minutes. Finally, the microsomal fraction was separated by centrifuging at 32,000g (16,000 rpm) for 30 minutes. The microsomal pellet was washed in 2.5 M sucrose and re-centrifuged at 32,000 g, and suspended in isotonic saline containing calcium and magnesium ions. Liver microsomes were prepared in the same way from fresh liver extract. Aliquots of the microsomal suspension , each containing one gm equivalent of the original tissue, were emulsified

with an equal volume of complete Freund's adjuvant for injection into the experimental animals. An electron microscope photograph of a thyroid microsomal suspension is shown in Figure 35.

(3) Immunization of Experimental Animals

(a) Rabbits

Each rabbit received a course of five, weekly, intramuscular injections of one gm equivalent of antigen mixed with an equal volume of complete Freund's adjuvant and then monthly subcutaneous boosters.

(b) Rats

In the first experiment, groups of rats received an intradermal injection of antigen emulsified with complete Freund's adjuvant, into the root of the tail followed by a series of booster injections. In the second experiment half of the animals received one intradermal booster injection, a few weeks after the first injection. Control rats were immunized with the corresponding liver antigen.

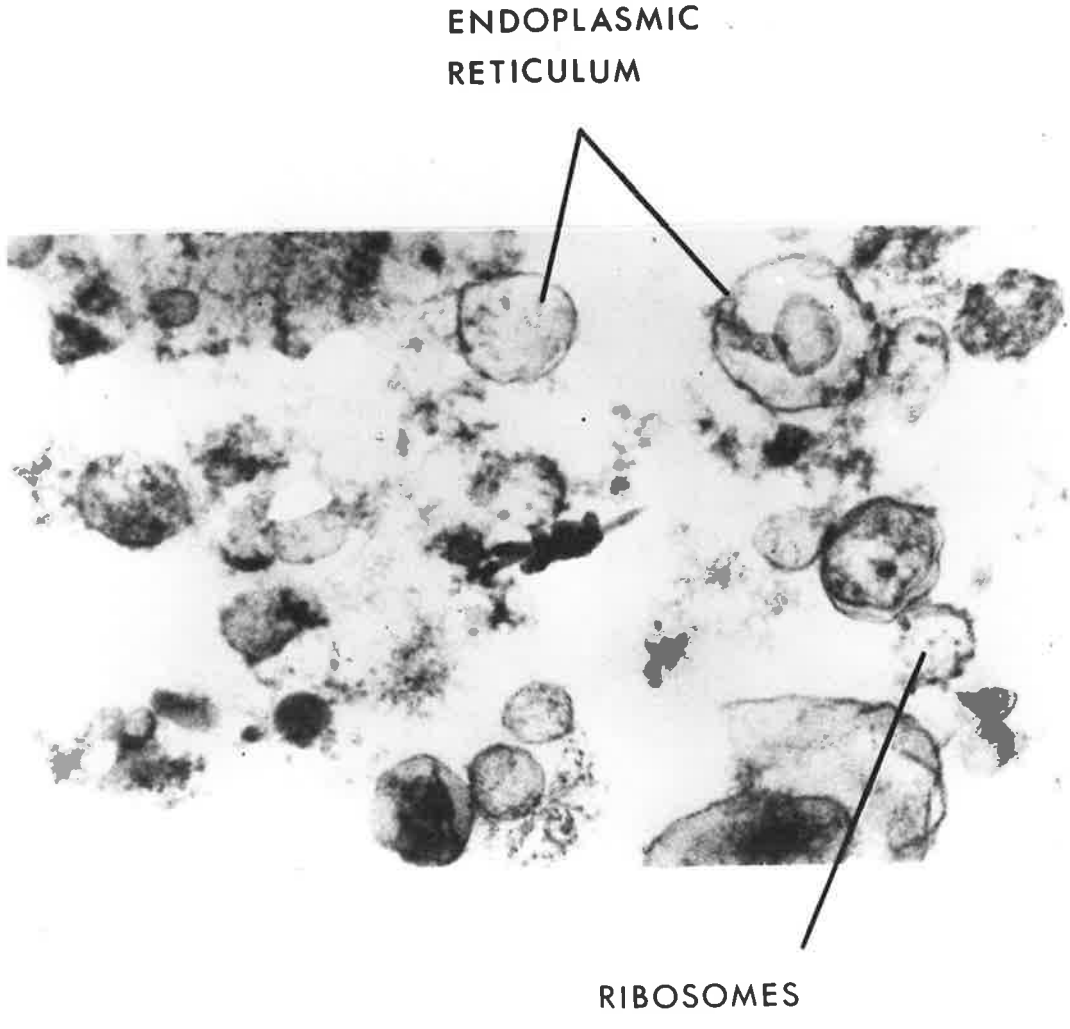
(4) Bleeding Techniques

(a) Rabbits

The rabbits were bled by cutting the marginal ear vein with a sterile scapel blade. The ear was previously shaved, and xyol was gently rubbed onto the vessel to dilate it. Twenty ml of blood was obtained at each venesection.

FIGURE 35

ELECTRON MICROSCOPE PHOTOGRAPH OF A THYROID MICROSOMAL PREPARATION



(b) Rats

Because of the smaller volume of blood obtained from a rat, blood from several rats was pooled for concentration. The rats were anaesthetized with intraperitoneal barbiturate and bled to death from the cannulated inferior vena cava. Ten ml of blood was obtained in this way. Of each group of ten rats, half were immunized with thyroid antigen and half with the corresponding liver antigen so that two pools of blood were obtained at each venesection.

(5) Clinical Assessment

The immunized animals were assessed clinically by observing weight change, feeding habits and general activity. The eyes were examined for development of ophthalmopathy and other signs indicative of thyrotoxicosis noted.

(6) Thyroid Function Studies

Thyroid function was assessed in the experimental animals by measuring 24 hour ^{131}I uptake and serum protein bound iodine (PBI).

(a) ^{131}I Uptake

The 24 hour ^{131}I uptake was measured by comparing the radio-activity over the thyroid gland 24 hours after a tracer dose of ^{131}I with the radio-activity of a standard cotton wool mock thyroid infiltrated with the same

amount of ^{131}I. The radio-activity was counted with a portable collumated scintillation detector (Nuclear Enterprises G.B., Model 5011)(Figure 36).

Rabbits were given 20μCi of ^{131}I intraperitoneally and rats 10μCi. At 24 hours the animal was placed on its back in a wooden cradle (Figure 37), with its chest covered by a lead plate and its neck extended so that the thyroid lay under the centre of the probe. The time taken for 10,000 counts (1,000 counts for rats) was recorded. The mock thyroid was placed at the same distance from the probe and the time for 10,000 counts measured. The percentage ^{131}I uptake was calculated from the number of counts recorded in 100 seconds, compared to the counts emitted by the standard in the same time. Thus:

$$\% \text{ 24 hour } {}^{131}I \text{ uptake} = \frac{\text{Counts / 100 secs (R)} - \text{background}}{\text{Counts / 100 secs (S)} - \text{background}} \times 100\%$$

where

(R) = Rabbit or Rat thyroid

(S) = Standard (mock) thyroid

In order to determine the time for the 24 hour ^{131}I uptake of rabbits to return to background levels after a single injection of radio-iodine three normal rabbits were

FIGURE 36

^{131}I UPTAKE APPARATUS

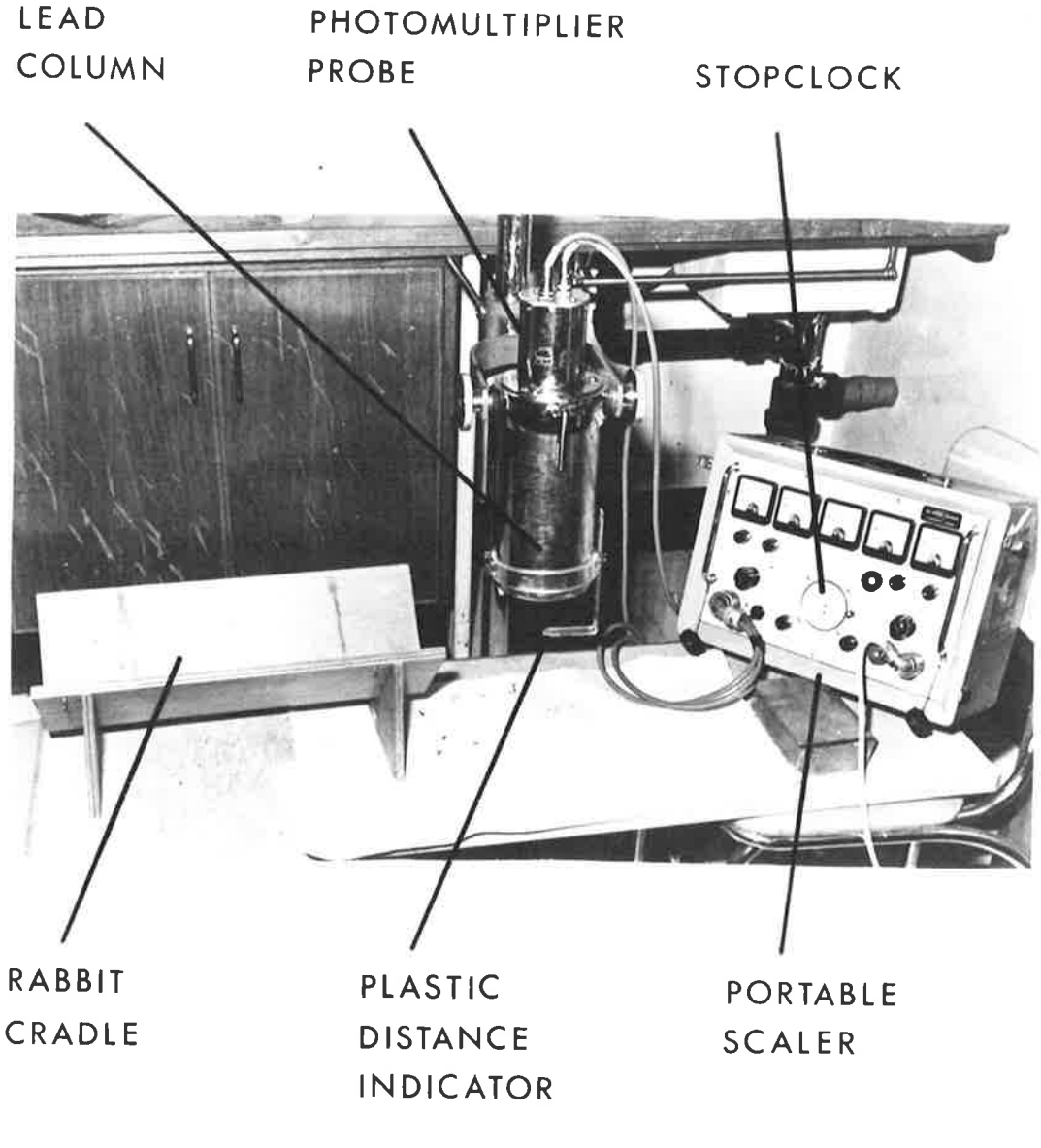


FIGURE 37

^{131}I UPTAKE - RABBIT IN POSITION

given 20 μCi of ^{131}I and the uptake measured every 48 hours for several weeks.

It took four weeks for the uptake to return to background levels. This was thus the minimum time interval in which successive measurements could be made on the same animal.

The normal range of 24 hour ^{131}I uptakes was 10-24% and 15-30% for rabbits and rats respectively.

(b) Protein Bound Iodine

The method for determination of the serum protein bound iodine was based on the alkaline ash method of Acland (1957). The mean PBI for normal rabbits and rats was 2.7 μgm% (S.D. 0.045) and 2.3 μgm% (0.04) respectively.

A more accurate measurement of thyroid function in these studies, where increase in PBI may be due to binding of thyroid hormones by circulating antibodies (McKenzie and Haibach, 1967, Burke, 1968), would have been the free thyroxine level. Because of the technical difficulties in this measurement however it was decided to use PBI despite this limitation.

(7) Assay for Thyroid Stimulating Antibody

The 7S globulin fraction of experimental animals was concentrated and assayed for L.A.T.S.-like activity. Rabbits were bled before immunization, one week after the

initial course of injections and then one week after each booster injection. Blood from groups of rats was pooled and concentrated before, and at various times after, immunization.

The assay procedure was modified slightly for these studies in that the assay mice were bled before and at 2, 5, 9, and 24 hours after injection of the test IgG solution, instead of at 3, 7, and 24 hours as in the assay for clinical samples, to enable a wider range of assay responses to be detected. A positive L.A.T.S. response was therefore defined as a significant 2-9 hour difference or a 24 hour level which was greater than 250%.

(8) Antithyroglobulin Estimation

Antithyroglobulin titres were estimated using the tanned red cell agglutination method (Boyden, 1951) with human thyroglobulin. The antibodies measured were thus antibodies to human thyroglobulin (heteroantibodies) rather than auto-antibodies. It is known however (Witebsky and Rose, 1959, Terplan et al, 1960) that a series of antibody molecules are produced after immunization with heterologous antigens, some of which are true auto-antibodies (Witebsky and Rose, 1956, Rose and Witebsky, 1956). Thus estimation of anti-human thyroglobulin titres in experimental animals provided an indication of the immunological response to the thyroid antigens.

(9) Pathological Changes

The classical pathological features of experimental thyroiditis include, in the early stages, a mild increase in lymphoid tissue, progressing in the more severe cases, to infiltration and destruction of the colloid material and follicle cells with, finally, massive fibrous replacement of the gland (Rose and Witebsky, 1956, Lindsay, 1964).

(C) EXPERIMENTS

Experimental animals were immunized with whole human thyroid extract or the microsomal fraction, emulsified with complete Freund's adjuvant. Control animals were immunized with the corresponding liver antigen or with saline or Freund's adjuvant alone.

Baseline measurements of PBI, ^{131}I uptake, thyroid stimulating activity and antithyroglobulin titres were made and the animals were weighed and observed under constant environmental conditions for several weeks before immunization.

At death, post mortem examination of the animals was carried out, the thyroid, liver and spleen in particular being examined macroscopically and biopsied.

The immunization procedure and experimental design for each experiment is summarized below.

Experiment	Animals	Antigens		Total Number of Animals	Primary Immunization Course	Booster Injections
		Thyroid	Control			
1	Rabbits	Whole Extract (4)	Liver Extract (3)	7	5 x weekly injections (I.M.)	Monthly
2	Rabbits	Microsomes (4)	Liver Microsomes(2) Freund's (2)	8	5 x weekly injections (I.M.)	Monthly
3	Rats	Whole Extract (25)	Liver Extract (25)	50	One injection (I.D.)	All, after each venesection
4	Rats	Microsomes (20)	Liver Microsomes (20)	40	One injection (I.D.)	Half, after ten days only

(D) RESULTS

(1) Rabbit Immunization

(a) Immunization with Whole Thyroid Extract

Clinical Assessment

None of the rabbits that received whole thyroid extract developed signs of thyrotoxicosis and there was no change, when compared to the control animals, in general behaviour, feeding habits or weight.

Thyroid Function

i. Protein Bound Iodine

The PBI results are shown for each rabbit throughout the course of the experiment (Table 32). Of the four rabbits which received thyroid extract, the PBI rose in three and did not change in one. The PBI of the control animals remained steady throughout the experimental period.

ii. ^{131}I Uptake

The ^{131}I uptake fell in all rabbits that received thyroid extract. There was no change in any of the control animals (Table 33).

Thyroid Stimulating Antibody

The L.A.T.S. assay responses are summarized in Table 34. The thyroid stimulating activity is expressed as the percentage increase in mouse blood radio-activity at 9 and 24 hours after injection of the test IgG.

Thyroid stimulating antibody was present, at some stage, in all rabbits that received thyroid extract. In the control animals a significant response was found on one occasion but the activity was lower than was found in any of the thyroid extract group. There was no tendency for the levels to increase with boosters over the course of the experiment.

TABLE 32

PROTEIN BOUND IODINE LEVELS OF RABBITS IMMUNIZED WITH WHOLE THYROID EXTRACTS

Rabbit	Antigen	Time (months) after Primary Immunization							
		0	1	2	3	4	5	6	7
17	T	2.1	8.0	6.2	7.3	3.5	+		
22	T	2.3	2.5	5.6	5.4	5.5	-	+	
23	T	1.7	2.3	8.4	6.2	5.9	6.5	8.1	-
25	T	-	0.7	-	3.3	3.1	2.3	2.9	2.1
19	L	2.1	2.5	3.7	2.5	2.4	1.8	2.0	2.5
20	L	2.3	-	2.9	3.1	2.3	-	-	+
21	L	2.7	2.1	0.7	+				

T = Thyroid Extract
L = Liver Extract
+ = Died during experiment

TABLE 33

^{131}I UPTAKE LEVELS OF RABBITS IMMUNIZED WITH WHOLE THYROID EXTRACTS

abbit	Antigen	Time (months) after Primary Immunization							
		0	1	2	3	4	5	6	7
17	T	16.8	9.2	12.7	6.0	5.9	+		
22	T	19.1	17.3	14.5	2.8	14.2	10.0	+	
23	T	15.2	8.6	6.9	8.4	21.5	9.8	9.0	-
25	T	23.7	11.7	-	15.2	31.0	14.0	-	-
19	L	13.0	15.3	17.0	11.9	14.6	16.0	-	-
20	L	15.0	14.4	15.5	-	11.5	9.2	-	+
21	L	12.5	15.0	25.0	+				

T = Thyroid Extract

L = Liver Extract

\+ = Died during experiment

TABLE 34

THYROID STIMULATING ACTIVITY IN THE 7S GLOBULINS OF RABBITS IMMUNIZED WITH WHOLE EXTRACTS

Rabbit	Antigen	Time (months) After Primary Immunization							
		0	1	2	3	4	5	6	7
17	T	128-106 NS	113-α NS	□180-100	244-173 NS	*202- 77	+		
18	T	166-120 NS	170-α NS	*259-156	+				
22	T	121- 79 NS	120- 85 NS	-	148- 71 NS	*190-163	*286-202	+	
23	T	133-169 NS	□160- 52	127- 38 NS	200-117 NS	96-136 NS	240- 42 NS	*315-278	311-*282
25	T	111-122 NS	177-*268	100-112 NS	165-*266	143-122 NS	□221-154	175- 86 NS	-
19	L	168- 92 NS	150- 91 NS	148- 67 NS	90-100 NS	292-197 NS	□148- 96	§132-138	176-123 NS
20	L	120- 90 NS	-	114-α NS	159- 76 NS	-	116- 76 NS	182-102 NS	+
21	L	130-123 NS	-	148- 87 NS	+				

T=Thyroid extract: L=Liver extract: +=Died during experiment: NS=Not Significant
*=P<0.05: ‡=P<0.02: □=P<0.01: §=P<0.001: α =Mice died

Antithyroglobulin Titres

High titres of antithyroglobulin were present in the four rabbits that received thyroid extract whilst control rabbits had only low titres (Table 35). There was no relationship however between antithyroglobulin titres and thyroid stimulating antibody levels.

Pathological Changes

At death, there was no macroscopic abnormality of the thyroid gland in any of the rabbits and the liver, spleen and other organs appeared normal. In several rabbits however, there were large fibrous nodules in the injection sites and in some cases these contained purulent material.

There was no microscopic evidence of hyperactivity of the thyroid gland. Although the amount of lymphoid tissue was increased slightly in some of the animals, there was no infiltration, tissue destruction or other signs of thyroiditis. None of the control animals had microscopic abnormalities in the organs examined, in particular the liver was normal in the rabbits that were immunized with liver extract.

(b) Immunization with Thyroidal Microsomes

Clinical Assessment

None of the rabbits that received the thyroid microsomal fraction developed features of overt thyrotoxicosis. There was no difference in weight change,

TABLE 35

ANTITHYROGLOBULIN TITRES OF RABBITS IMMUNIZED WITH WHOLE EXTRACTS

Rabbit	Antigen	Time (months) After Primary Immunization							
		0	1	2	3	4	5	6	7
17	T	-ve	*250,000	25	250,000	25,000	+		
22	T	-ve	2,500	25	2,500	250,000	250,000	+	
23	T	-ve	2,500	250	2,500,000	25,000	25,000	2,500,000	2,500,000
25	T	-ve	25	2,500	2,500,000	2,500,000	250,000	25,000	25
19	L	-ve	2,500	2,500	250	25	25	-ve	250
20	L	-ve	-ve	-ve	25	-ve	250	5	+
21	L	-ve	5	5	+				

T = Thyroid extract
L = Liver extract
\+ = Died during experiment
* = Titre = reciprocal of serum dilution

feeding habits or general activity as compared to the control rabbits.

One of the rabbits however developed prominent unilateral exophthalmos which remitted spontaneously six weeks later (Figure 38). Although the animal developed conjunctivitis in the affected eye, which resolved during penicillin treatment, it was considered unlikely that the exophthalmos was due to orbital infection. At death, some weeks later, the eye appeared normal and there was no sign of brain tumour. Unfortunately, histological examination was not carried out. This animal had circulating thyroid stimulating antibody at the time of appearance of the exophthalmos.

Thyroid Function

i. Protein Bound Iodine

In contrast to rabbits that were immunized with whole extract, the PBI of animals that received the microsomal fraction rose after the primary course of injections in all but one animal, reaching a peak at about four months, before falling (Table 36).

Mean PBI values for thyroid and control rabbits were plotted for the course of the experiment (Figure 39). As can be seen, the mean PBI for the thyroid group was greater than for the control group throughout the experiment. There was no change in PBI levels in the rabbits that received liver microsomes or Freund's adjuvant alone.

FIGURE 38

UNILATERAL EXOPHTHALMOS

TABLE 36

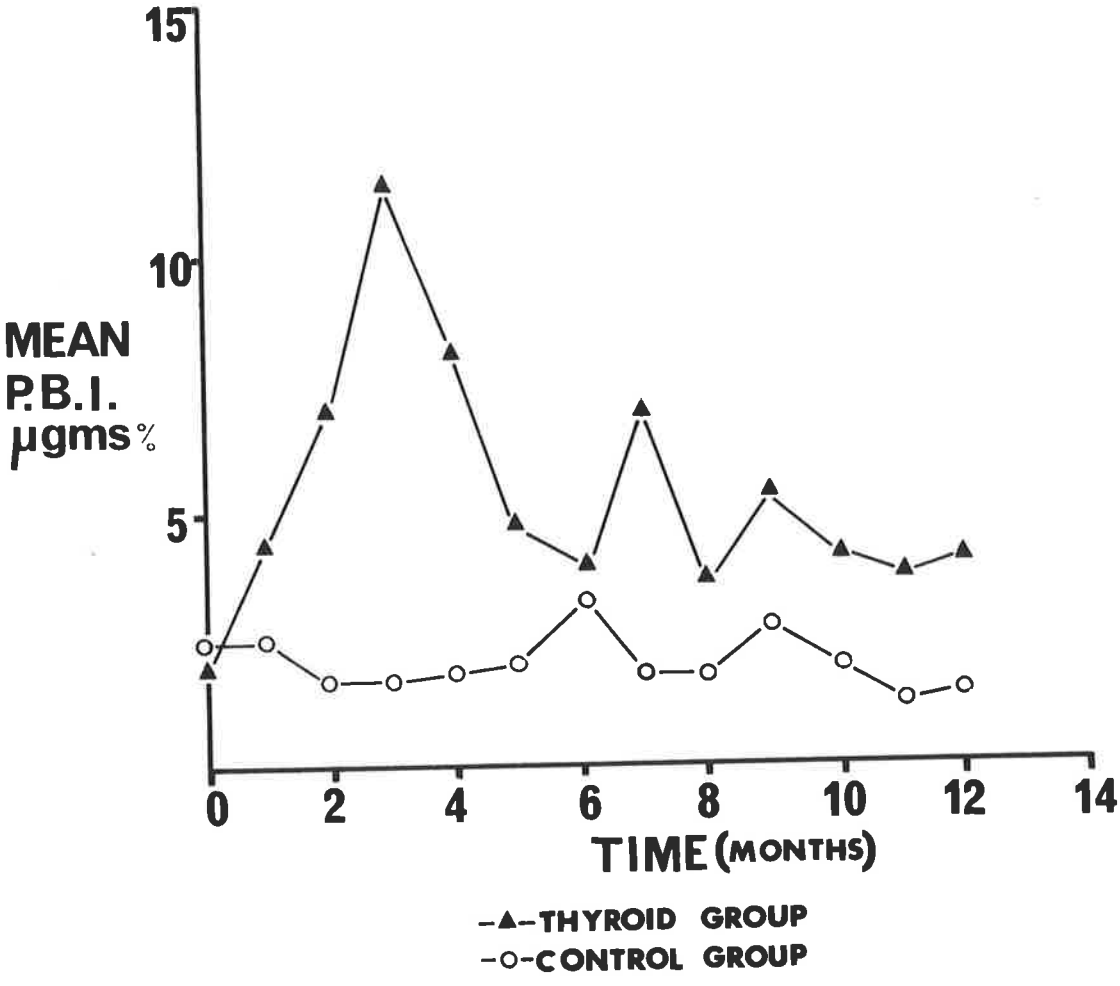
PROTEIN BOUND IODINE LEVELS OF RABBITS IMMUNIZED WITH MICROSOMES

Rabbit	Antigen	Time (months) after Primary Immunization														
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
9	T	1.1	3.2	8.9	7.3	5.2	3.5	6.3	9.4	1.1	+					
10	T	2.2	9.5	4.1	2.7	2.2	1.9	2.1	1.3	2.6	1.7	2.2	2.3	2.3	2.5	1.9
12	T	2.3	4.1	11.8	30.0	17.8	8.4	2.1	11.2	3.2	5.5	5.1	3.0	7.0	1.0	+
14	T	2.6	0.9	3.2	5.6	6.9	6.0	5.3	6.0	6.8	10.3	4.9	5.6	4.1	3.2	3.9
11	F	1.8	1.3	0.9	0.6	‡	1.8	4.1	1.1	1.0	0.9	1.2	1.1	1.6	1.1	1.3
13	L	1.3	3.9	1.2	2.3	2.2	‡	2.5	2.1	2.3	6.0	2.2	1.2	1.9	2.1	2.1
15	L	4.3	3.8	3.1	1.5	0.9	1.9	1.6	2.7	1.9	1.6	1.9	1.7	1.7	1.2	1.6
16	F	2.8	1.3	0.8	+											

T = Thyroidal Microsomes
L = Liver Microsomes
F = Freund's Adjuvant Only
\+ = Died during experiment

FIGURE 39

MEAN P.B.I. VALUES OF RABBITS IMMUNIZED WITH MICROSOMES



ii. ^{131}I Uptake

The mean ^{131}I uptake increased in the animals that were injected with thyroidal microsomes as compared to control animals, reaching a peak at five months (Figure 40). The uptake for individual rabbits however was variable and rose in only one.

Thyroid Stimulating Antibody

The L.A.T.S. assay results are summarized in Table 37. All rabbits that received thyroidal microsomes had thyroid stimulating activity in the concentrated IgG fraction. In most cases, the peak increase was at 9 hours, but on a few occasions there was peak activity at 5 or 24 hours. The thyroid stimulating antibody levels, which were higher and more persistent than in the first experiment, reached a peak, in all cases, at about five months and then fell so that at the end of the experiment the stimulator was detected in only two of the rabbits. Significant responses were occasionally found when the IgG fraction from control rabbits was assayed, but the levels were low. The mean thyroid stimulating antibody levels for the two groups are shown in Figure 41 where it can be seen that the mean for the thyroid group was greater than for the control group throughout most of the experiment.

Antithyroglobulin Titres

All but one of the rabbits immunized with thyroidal microsomes had significant titres of circulating

FIGURE 40

MEAN ^{131}I UPTAKE VALUES OF RABBITS IMMUNIZED WITH MICROSOMES

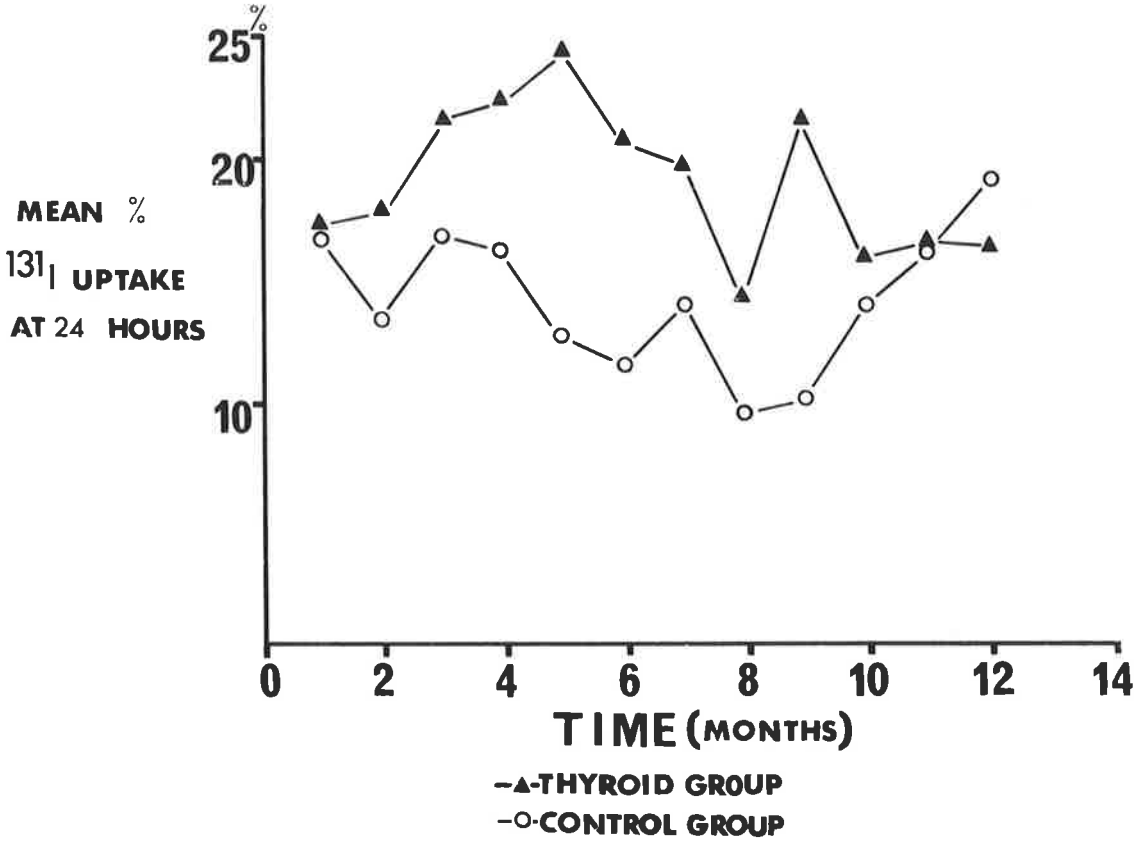


TABLE 37

THYROID STIMULATING ACTIVITY IN THE 7S GLOBULINS OF RABBITS IMMUNIZED WITH MICROSOMES

Rabbit	Antigen	Time (Months) After Primary Immunisation						
		0	1	2	3	4	5	6
9	T	69-61 NS	222- 96 NS	§296-122	173-117 NS	§172-111	279-□258	§232-118
10	T	148-64 NS	105- 82 NS	‡180-120	*200-103	*215- 94	*310-360	242-214 NS
12	T	83-59 NS	105- 84 NS	§374-148	‡237-114	§236-142	□334-184	202-142 NS
14	T	110-58 NS	163- 88 NS	§135- 74	209-118 NS	149-110 NS	§187-103	281-142
11	F	103-64 NS	118- 71 NS	125- 85 NS	149- 99 NS	§176-196	147- 76 NS	155- 96 NS
13	L	95-68 NS	280-190 NS	204- 90 NS	166- 92 NS	164-100 NS	133- 84 NS	143- 88 NS
15	L	105-66 NS	151- 84 NS	-	131-115 NS	138- 83 NS	*165-112	254-328 NS
16	F	90-54 NS	119- 81 NS	153-125 NS	+			

/contd.

Rabbit	Antigen	Time (Months) After Primary Immunisation							
		7	8	9	10	11	12	13	14
9	T	§213-132	+						
10	T	*190-218	85- 76 NS	130-*242	176-137 NS	-	226-*252	186-158 NS	169-81 NS
12	T	178-146 NS	148-113 NS	□222-160	146-137 NS	117-130 NS	242-145 NS	+	
14	T	‡166- 98	212-199 NS	122-106 NS	108- 99 NS	103-103 NS	207-182 NS	§204-118	⊠
11	F	153- 80 NS	97- 62 NS	146- 88 NS	121- 98 NS	103-103 NS	169-⊠ NS	135- 73 NS	128-⊠
13	L	130-126 NS	90- 93 NS	129- 85 NS	101-119 NS	89- 97 NS	119-189 NS	142-127 NS	⊠
15	L	80- 80 NS	80- 86 NS	139-132 NS	115- 88 NS	93- 92 NS	201-166 NS	200- 91 NS	117- 86 NS
16	F								

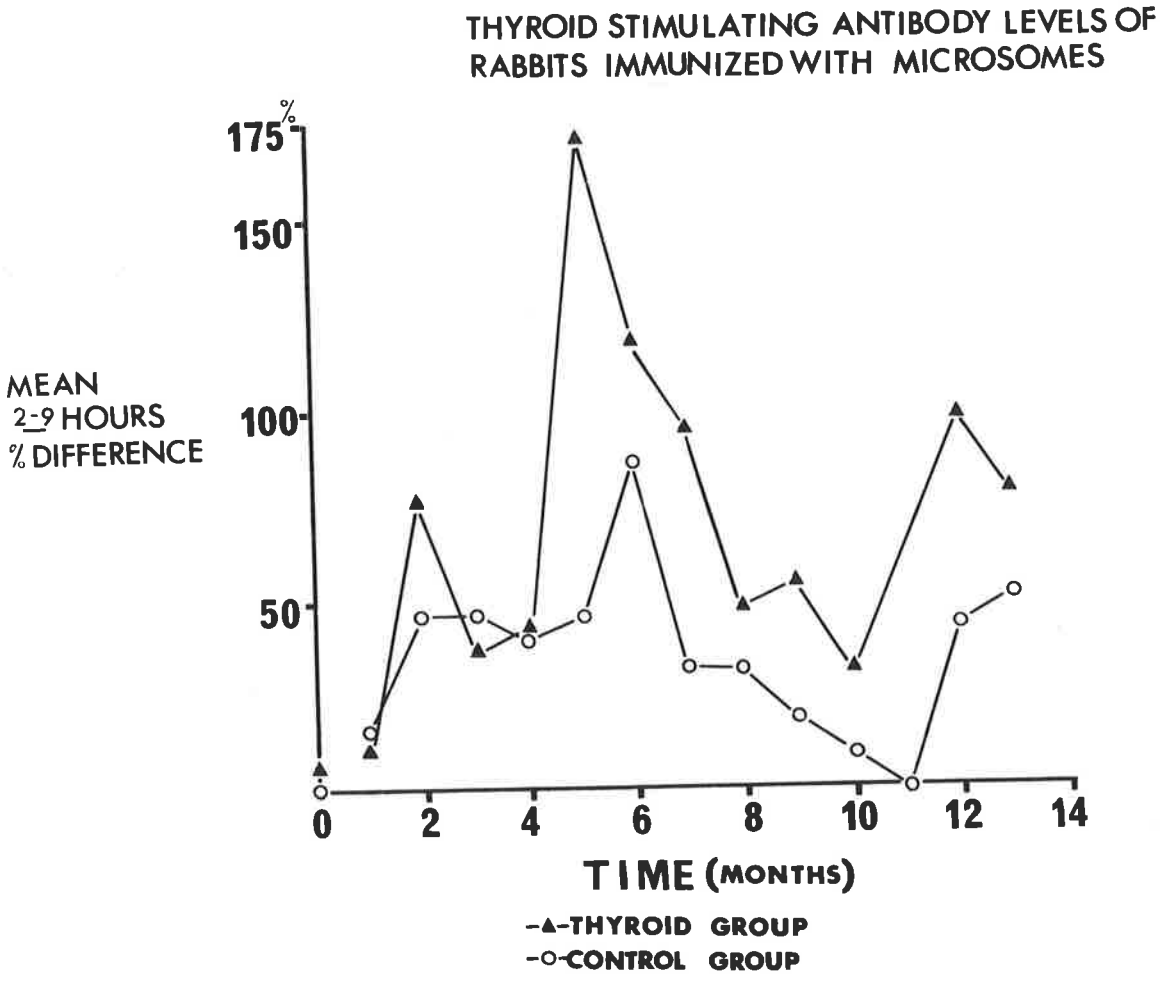
T = Thyroid microsomes L = Liver microsomes

F = Freund's adjuvant only + = died during experiment

NS = Not significant ⊠ = Mice died

* $P<0.02$ ‡ $P<0.01$ § $P<0.05$ □ $P<0.001$

FIGURE 41


THYROID STIMULATING ANTIBODY LEVELS OF RABBITS IMMUNIZED WITH MICROSOMES
%
175
150
100
50
MEAN
2-9 HOURS
% DIFFERENCE
0
2
4
6
8
10
12
14
TIME (MONTHS)
-▲-THYROID GROUP
-o-CONTROL GROUP

antibody after the primary course of injections (Table 38). Control animals had low levels of antibody. The pattern for individual rabbits was variable. The titres reached a peak at five months and then fell in two, remained at very high levels in one and fluctuated in the other.

The titres corresponded with the levels of thyroid stimulating antibody in those animals with the highest titres of antithyroglobulin.

Pathological Changes

The thyroid was macroscopically normal in all rabbits whilst histologically there was no evidence of hyperthyroidism or thyroiditis. Two representative sections are shown (Figure 42) comparing a normal rabbit thyroid with the thyroid from a rabbit immunized with thyroidal microsomes. Apart from a slight increase in lymphoid tissue in the latter there has been no change. The liver and spleen were normal in all rabbits.

(2) Rat Immunization

(a) Whole Thyroid Extract

Clinical appraisal revealed that none of the rats was thyrotoxic. At post mortem examination the thyroid glands appeared normal and there was no histological evidence of thyroiditis or hyperthyroidism. There were no clinical or pathological changes in any of the control rats.

ANTITHYROGLOBULIN TITRES OF RABBITS IMMUNIZED WITH MICROSOMES

Rabbit	Antigen	Time (Months) After Primary Immunization														
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
9	T	-ve	5	25	2.5×10^5	2.5×10^5	2.5×10^3	2,500	2.5×10^4	-	+					
10	T	-ve	5	25	2.5×10^5	2.5×10^5	2.5×10^6	250	2,500	-	-	2.5×10^4	25	250	250	2.5×10^6
12	T	-ve	5	2.5×10^4	2.5×10^6	2.5×10^5	2.5×10^5	25	2.5×10^4	2.5×10^4	2.5×10^5	2.5×10^4	25	-	-	+
14	T	5	5	2.5×10^4	2.5×10^4	-	2.5×10^5	2.5×10^5	2.5×10^5	2.5×10^4	2.5×10^6	2.5×10^6	2.5×10^6	2.5×10^6	2.5×10^6	2.5×10^6
16	F	-ve	25	25	+											
11	F	-ve	-ve	-ve	-ve	25	-ve	-ve	5	5	5	25	5	-ve	-ve	-ve
13	L	-ve	-ve	-ve	-ve	-ve	-ve	-ve	250	250	25	250	5	-ve	-ve	-ve
15	L	5	-ve	5	5	25	250	-ve	5	5	5	5	-ve	-ve	250	+

T = Thyroidal microsomes

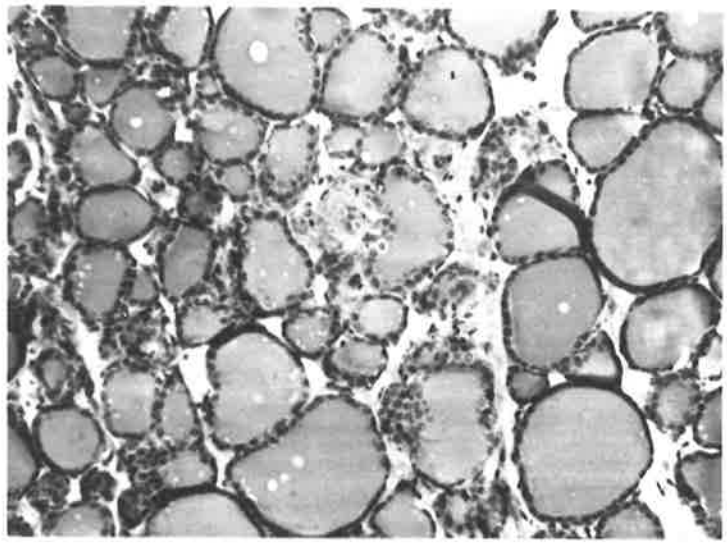
L = Liver microsomes

F = Freund's adjuvant only

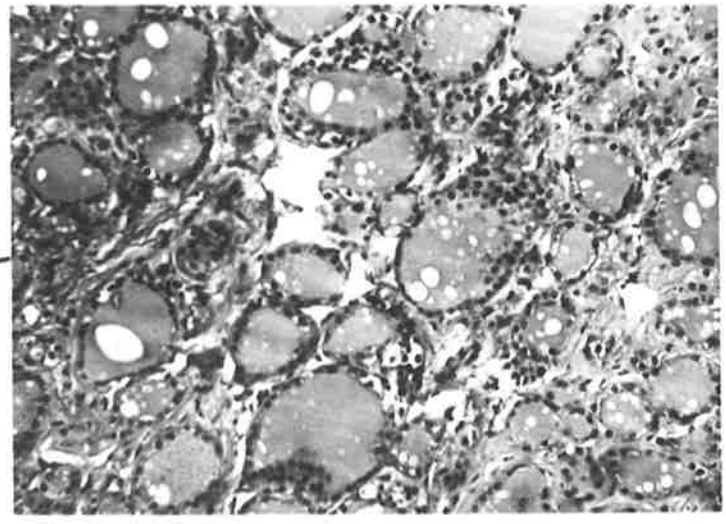
\+ = died during experiment

FIGURE 42

POST MORTEM THYROID HISTOLOGY



THYROID TISSUE AFTER IMMUNISATION
WITH LIVER MICROSOMES



THYROID TISSUE AFTER IMMUNISATION
WITH THYROID MICROSOMES

The ^{131}I uptake of rats immunized with thyroid tissue increased, reaching maximal levels at about three weeks. The levels then tended to fall, so that by the end of the experiment there was no difference between the mean uptake values of the thyroid and control groups. On the other hand, the PBI remained within the normal range or fell. There was no change in ^{131}I uptake or PBI in any of the control rats.

Rats that were immunized with thyroid extract had, generally, high titres of antithyroglobulin which persisted until the end of the experiment when the last animals were sacrificed.

Finally, low levels of thyroid stimulating antibody were present in two of the thyroid pools at two and 16 weeks respectively after the first injection (Table 39).

(b) Thyroidal Microsomes

None of the rats that received thyroidal microsomes had clinical features of thyrotoxicosis and there was no histological evidence of thyroiditis or hyperthyroidism. The PBI at death was in the normal range in all but two animals which had moderately elevated PBI levels. There was no change in the ^{131}I uptake in these rats; in particular the rise in uptake seen in the first experiment did not occur.

TABLE 39

THYROID STIMULATING ACTIVITY IN 7S GLOBULINS OF RATS IMMUNIZED WITH WHOLE EXTRACTS

Pool	Antigen	Time (weeks) After Primary Immunization					
		0	2	5	8	13	16
Thyroid	Whole Human Thyroid Extract	114-106 NS	*151- 94	124- 86 NS	120-176 NS	95-146 NS	ǂ110-78
Control	Whole Human Liver Extract	114-106 NS	114-112 NS	193-116 NS	187- 84 NS	159-165 NS	113-77 NS

ǂ = $P<0.02$

* = $P<0.01$

NS = Not significant

Most of the rats that were immunized with thyroidal microsomes had circulating antithyroglobulin but the titres were generally lower than found in rats immunized with whole extract.

In none of the control rats was there a change in PBI or ^{131}I uptake and histological examination of the thyroid revealed no abnormality. Antithyroglobulin was not detected in any of these rats.

In this experiment low levels of thyroid stimulating antibody were detected in three of the control pools, and in one of the thyroid pools (Table 40).

(E) DISCUSSION

In these studies thyroid stimulating antibody was detected in all rabbits immunized with whole human thyroid tissue or the microsomal fraction. Low levels were found occasionally in control rabbits immunized with the corresponding liver antigen. Although significant activity was also found in some of the rat pools the levels were low and hence could not be distinguished from nonspecific responses. Although none of the rabbits with circulating thyroid stimulating antibody were clinically toxic, the development of exophthalmos in one animal and the tendency for the PBI and ^{131}I uptake to rise in many of these animals, suggested that there was a close relationship between the presence of the stimulator, increased thyroid function and the development of exophthalmos.

TABLE 40

THYROID STIMULATING ACTIVITY IN 7S GLOBULINS OF RATS IMMUNIZED WITH MICROSOMES

Pool	Antigen	Time (weeks) After Primary Immunization				
		0	2	6	8	10
Thyroid	Thyroidal Microsomes	147-113 NS	143-77 NS	126-91 NS	*165-74	128-52 NS
Control	Liver Microsomes	147-113 NS	‡167-91	*157-100	*180-74	71-66 NS

‡ = $P < 0.02$

* = $P < 0.05$

NS = Not Significant

In the experiments with rabbits, higher and more persistent levels of the stimulator were obtained after immunization with microsomes than with whole extract. There is evidence that the antigen which stimulates human L.A.T.S. production resides in the microsomal fraction (Beall and Solomon, 1966, McKenzie, 1967). Immunization with microsomes should therefore be more specific. On the other hand, the adjuvant effect of foreign proteins in human thyroid extract may be significant (Beall and Solomon, 1968a) possibly explaining why McKenzie was unable to demonstrate thyroid stimulating antibody in rabbits immunized with thyroidal microsomes but did so when he used whole extract (McKenzie, 1967).

There was not a close relationship between the levels of thyroid stimulating antibody and the antithyroglobulin titres although when microsomes were injected, high levels of the stimulator tended to be associated with high titres of antithyroglobulin, confirming the findings of Beall et al (1969) in baboons. In these rabbits also the peak increase in PBI and ^{131}I uptake occurred at the same time as the maximal increase in thyroid stimulating antibody levels. In rabbits that received whole thyroid extract however, there was no relationship between antibody levels and thyroid function.

Although significant L.A.T.S.-like activity was demonstrated in rats immunized with thyroid antigens the

prevalence amongst the control animals was as great and the levels were low. The only increase in thyroid function was found in rats that had received whole extract in whom the ^{131}I uptake tended to increase for the first few weeks after immunization and then to fall again, although this was not repeated in later experiments. None of the rats appeared clinically thyrotoxic and on no occasion was there histological evidence of hyperthyroidism. Furthermore there was no relationship between antibody levels (antithyroglobulin and thyroid stimulating antibody) and thyroid function in any of the rats.

There are several possible explanations for the failure to produce the experimental equivalent of human thyrotoxicosis. Firstly, thyrotoxicosis may have in fact been produced in rabbits but not recognised clinically. Secondly, lack of specificity in the immunization procedures used may have mitigated against maximal antibody production. Although it is clear that thyroid extracts and the microsomal fraction were strongly antigenic, because of the high titres of antithyroglobulin demonstrated, recognition of the specific antigen which stimulates production of L.A.T.S. (or its experimental equivalent) would allow titration for optimal antibody production. Thirdly, since striking differences in susceptibility to experimental allergic diseases occurs between species (Beall et al, 1969) host

factors may be of primary importance in determining which antibodies are produced and what clinical picture develops. Thus Beall et al (1969) reported differences in the immunological responses between rabbits and baboons immunized with thyroid antigens. Whereas rabbits developed L.A.T.S.-like activity, baboons developed activity with all of the characteristics of TSH.

Although McKenzie (1967) was unable to demonstrate thyroid stimulating antibody in rabbits immunized with the microsomal fraction Beall and Solomon (1968a) found thyroid stimulating activity in 24 of 32 rabbits immunized with human thyroidal microsomes, the levels increasing with booster injections. This was confirmed by Burke (1968) who found that whilst thyroid stimulating activity was consistently present in rabbits immunized with thyroidal microsomes the levels fell despite booster injections and despite rising titres of antithyroglobulin. This suggested to Burke that as an alternative hypothesis to the thyroid stimulating activity being due to an antibody against a thyroid component, the activity may have been due to binding of the assay mouse ^{131}I-labelled thyroid hormones rather than to thyroid stimulation.

In the studies reported by McKenzie (1968) and Beall and Solomon (1968a) elevated levels of protein bound iodine and total thyroxine were found in rabbits immunized with

thyroid antigens, and McKenzie reported increased 24 hour ^{131}I uptake compared to control rabbits. Furthermore, ^{131}I release in two rabbits was significantly faster than in all controls and was not suppressed by the administration of thyroxine for two weeks. Beall and Solomon however did not find an increased ^{131}I uptake in their rabbits and there was no change in PBI.

Thus although the experimental counterpart to human thyrotoxicosis has not been convincingly produced, the development of exophthalmos in one of the rabbits with detectable thyroid stimulating antibody and the correlation between increase in PBI and ^{131}I uptake and thyroid stimulating antibody levels in rabbits immunized with thyroidal microsomes in this study suggests that with recognition of the specific antigen (and a better understanding of host factors) a state similar to human thyrotoxicosis may be produced in experimental animals even though, as will be discussed in the concluding chapter, this is probably an artificial situation.

(F) SUMMARY AND CONCLUSIONS

Rabbits and rats were immunized with whole human thyroid extract or the microsomal fraction. An equal number of controls were immunized with the corresponding liver material. Assays for L.A.T.S. activity were carried out on the concentrated IgG fraction, and thyroid function was assessed

by protein bound iodine and ^{131}I uptake measurements. The animals were examined clinically for signs of thyrotoxicosis.

Detectable thyroid stimulating antibody was present in rabbits immunized with whole thyroid extract or microsomes, the levels being higher and more persistent in the latter group. Although no animal was clinically toxic and there was no histological evidence of hyperthyroidism, one rabbit developed marked unilateral exophthalmos.

The PBI and ^{131}I uptake tended to be elevated in rabbits which were immunized with thyroidal microsomes, the levels correlating with the levels of thyroid stimulating antibody. Although a few of the control rabbits had detectable L.A.T.S.-like activity the levels were low and there were no changes in thyroid function.

Thyroid stimulating antibody was detected in rats immunized with whole extract or the microsomal fraction, but significant responses were also found in some of the control rats and in all cases the levels were low and could not be distinguished from nonspecific responses. None of the rats had clinical, biochemical or histological evidence of thyrotoxicosis.

The tendency for thyroid function to increase in some animals with circulating thyroid stimulating antibody and the development of exophthalmos in one rabbit, suggests that with recognition of the specific antigen and a better under-

standing of host factors it may be possible to produce the experimental equivalent of human thyrotoxicosis.

CHAPTER IX

PRODUCTION OF L.A.T.S. BY PERIPHERAL LYMPHOCYTES

(A) INTRODUCTION

(B) CLINICAL SUBJECTS

(C) MATERIALS AND METHODS

(1) Preparation of Lymphocyte Cultures
- (a) Introduction
- (b) Harvesting of Lymphocytes
- (c) Culture Medium
- (d) Setting Up of Cultures
- (e) Control Cultures
  - i. Normal Lymphocytes
  - ii. Medium Controls
  - iii. Dead Cell Control

(2) Neutralisation of L.A.T.S. Activity with Anti-IgG Serum
- (a) Introduction
- (b) Determination of Anti-IgG Titre
- (c) Neutralisation of L.A.T.S.-IgG

(3) L.A.T.S. Bioassay

(D) RESULTS

- (1) Lymphocyte Cultures from Thyrotoxic Patients
- (2) Control Cultures
- (3) Neutralisation of L.A.T.S.-IgG

(E) DISCUSSION

(F) SUMMARY AND CONCLUSIONS

CHAPTER IX

PRODUCTION OF L.A.T.S. BY PERIPHERAL LYMPHOCYTES

(A) INTRODUCTION

Peripheral lymphocytes undergo blastoid transformation when cultured in the presence of the kidney bean extract phytohaemagglutinin (PHA) (Hungerford et al, 1959, Nowell, 1960, Hirschhorn et al, 1963, Elves et al, 1963). Several workers have reported their findings suggesting a possible antibody synthesis by lymphocytes in vitro (Forbes, 1965, Forbes and Turner, 1965, Van Furth et al, 1966, Forbes and Smith, 1969).

Since L.A.T.S. is almost certainly an antibody the stimulator may be produced by lymphocytes from patients with thyrotoxicosis when cultured with PHA under similar conditions.

In this study then it is proposed to culture peripheral lymphocytes from patients previously exposed to the specific antigen. Lymphocytes from normal people will be cultured under the same conditions for control experiments. The concentrated culture medium and suspensions of the destroyed cells will be assayed for L.A.T.S. activity. Detection of L.A.T.S. in this system would provide further evidence that L.A.T.S. originates from antibody forming tissues in Graves' disease.

(B) CLINICAL SUBJECTS

Peripheral lymphocytes were harvested from seven patients with Graves' disease. The clinical details of the patients at venesection are summarized in Table 41.

Five of the patients had severe ophthalmopathy whilst three had dermopathy. In all cases, the skin and eye lesions were quiescent at the time of venesection. Only one patient (I.P.) was hyperthyroid.

Plasma L.A.T.S. levels, antibody titres and immunoglobulin levels at the time of venesection are shown in Table 42. Only four of the patients had significant antibody titres whilst four had increased IgG levels, three had increased IgM and four had elevated levels of IgA. Most patients had high L.A.T.S. levels but two had low levels at the time of the study.

(C) MATERIALS AND METHODS

(1) Preparation of Lymphocyte Cultures

(a) Introduction

Peripheral lymphocytes were harvested and cultured following the methods used in this laboratory (Turner and Forbes, 1966, Smith et al, 1967). All glassware was sterilised and all operations were carried out with aseptic tissue culture techniques.

(b) Harvesting of Lymphocytes

Four hundred ml of blood was collected by venesection into a sterile bottle containing 5,000 I.U. of

TABLE 41

CLINICAL DETAILS OF PATIENTS AT VENESECTION

Patient	Age	Sex	Severe Ophthal-mopathy	Dermopathy	Thyroid Status	Present Treatment
E.N.	50	M	Yes	No	Euthyroid	Azathiaprine (150 mg)
V.R.	64	F	Yes	Yes	Euthyroid	-
H.S.	51	F	Yes	Yes	Euthyroid	Azathiaprine (150 mg) Thyroxine (0.3 mg)
D.P.	41	F	No	No	Euthyroid	-
H.N.	67	M	Yes	No	Euthyroid	-
E.O.	61	M	No	No	Euthyroid	Thyroxine (0.3 mg)
I.P.	60	F	Yes	Yes	Hyper-thyroid	Carbimazole (40 mg)

TABLE 42

IMMUNOLOGICAL DATA OF PATIENTS AT TIME OF VENESECTION

Patient	Plasma L.A.T.S. Levels	Antibody Titres		Immunoglobulin Levels		
		Anti-Thyro-Globulin	Thyroid Cytoplasmic Antibody	IgG	IgM	IgA
E.N.	153- 295- 190	25	-ve	380	200	600
V.R.	799-1602-1791	250	-ve	1750	72	115
H.G.	542-1073-2093	25	-ve	1360	100	173
D.P.	577-1378-1471	25	-ve	1240	72	208
E.O.	200- 450- 561	2,500	-ve	1920	230	520
A.N.	97- 239- 178	-ve	+	608	88	540
I.P.	207- 458- 928	25	++	940	200	480

heparin (Commonwealth Serum Laboratories, Parkville, Victoria) and dextran (5% final concentration of a 6% dextran solution in saline, Glaxo, Greenford, England). The erythrocytes were allowed to sediment for one hour at 37°C (Figure 43).

The supernatant was collected and centrifuged at 1,500 rpm for ten minutes. The lymphocyte-rich suspension was removed and resuspended in about 50 ml of autologous plasma. This suspension was introduced into a sterile glass column (2 x 30 cm) loosely packed with cotton wool and incubated at 37°C for 40 minutes. During incubation most of the polymorphs adsorbed to the cotton wool.

The cells were eluted from the column with 100 ml of autologous plasma and centrifuged. The remaining erythrocytes were destroyed with 10 ml of lysing fluid (1 gm of sodium citrate and 7 ml of saturated sodium chloride in distilled water). After 30 seconds an equal volume of neutralising fluid (7 gm of sodium citrate and 77 ml of sodium chloride in distilled water) was added to prevent lysis of the lymphocytes.

The cells were then washed twice with Hank's balanced salt solution (B.S.S.) by centrifugation at 1,500 rpm for 15 minutes and suspended in culture medium.

(c) Culture Medium

Medium 199 (Commonwealth Serum Laboratories, Parkville, Victoria) containing 20 u/ml of Neomycin and 10 u/ml

FIGURE 43

PREPARATION OF LYMPHOCYTE CULTURES

(A) HARVESTING OF LYMPHOCYTES

400 mls. BLOOD
37°C 60 mins.

↓

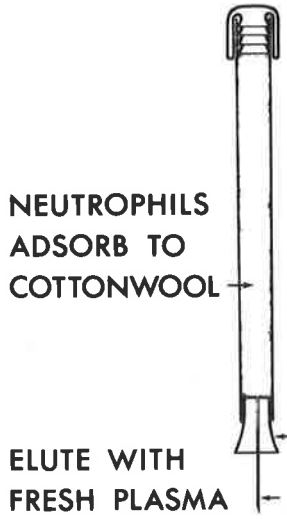
200 mls. SUPERNATANT
1500 R.P.M.

↓

40 mls. LEUCOCYTE ENRICHED PLASMA

↓

COLUMN



ELUATE
(LYMPHOCYTE ENRICHED PLASMA)

↓

LYSIS OF REMAINING RED BLOOD CELLS

↓

WASH PRECIPITATED LYMPHOCYTES IN HANK'S SOLUTION x 2

↓

COLLECT CELLS IN CULTURE MEDIUM

of polymyxin B was used in all cultures. Foetal human serum was added to a final concentration of 10%.

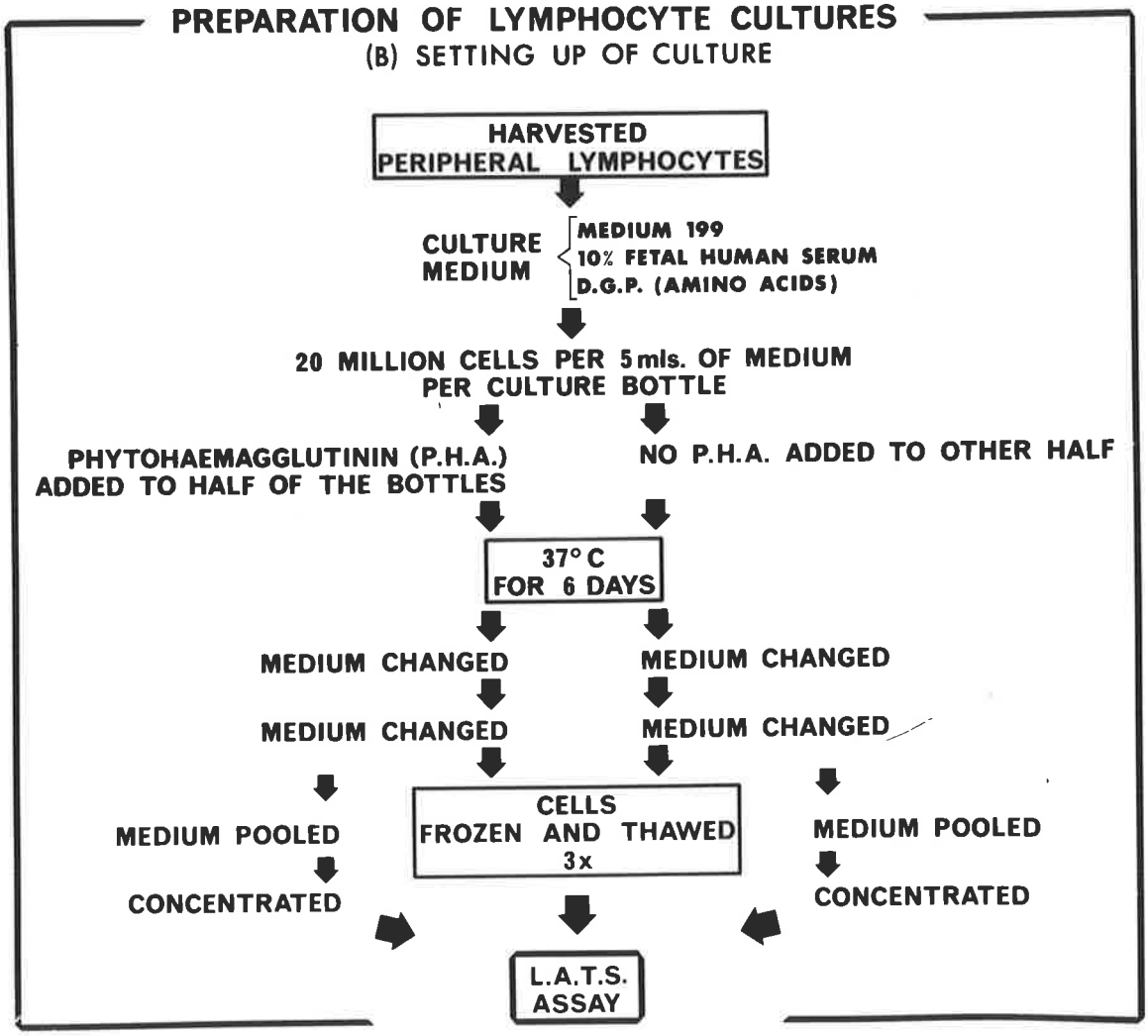
(d) Setting Up of Cultures

Lymphocytes were suspended in culture medium in a concentration of 4 million cells per one ml of medium, and 0.1 ml of phytohaemagglutinin (PHA, Burroughs Wellcome, England) added to half of the cultures (Figure 44). The cultures were incubated for six days at 37°C in an atmosphere of 95% air and 5% carbon dioxide. Generally, six or eight culture bottles were prepared in this way in each experiment.

Most of the medium was removed after 48 hours and replaced with fresh medium. This procedure was repeated on the fourth day of culture. At the end of six days the cultures were centrifuged at 1,500 rpm for 15 minutes and the supernatant removed and pooled with medium removed on days 2 and 4. The culture supernatant was concentrated by vacuum dialysis in a colloidin bag (Membranfiltergesellschaft, Gottingen, Germany) against distilled water at 4°C to a final volume of 3 ml. Supernatant pools, with and without PHA, were concentrated in this way and assayed for L.A.T.S. activity.

The cells were destroyed by freezing and thawing three times and suspended in 3 ml of saline for L.A.T.S. assay (Figure 44). Eleven cultures from seven patients with thyrotoxicosis were carried out.

FIGURE 44


PREPARATION OF LYMPHOCYTE CULTURES
(B) SETTING UP OF CULTURE
HARVESTED PERIPHERAL LYMPHOCYTES
CULTURE MEDIUM
MEDIUM 199
10% FETAL HUMAN SERUM
D.G.P. (AMINO ACIDS)
20 MILLION CELLS PER 5 mls. OF MEDIUM PER CULTURE BOTTLE
PHYTOHAEMAGGLUTININ (P.H.A.) ADDED TO HALF OF THE BOTTLES
NO P.H.A. ADDED TO OTHER HALF
37° C FOR 6 DAYS
MEDIUM CHANGED
MEDIUM CHANGED
MEDIUM CHANGED
MEDIUM CHANGED
MEDIUM POOLED
CONCENTRATED
CELLS FROZEN AND THAWED 3x
MEDIUM POOLED
CONCENTRATED
L.A.T.S. ASSAY

(e) Control Cultures

i. Normal Lymphocytes

Peripheral lymphocytes from three normal blood donors were harvested and cultured as control experiments.

ii. Medium Controls

Culture medium, with and without PHA, was incubated in the absence of lymphocytes on several occasions as medium controls.

iii. Dead Cell Control

In order to determine whether L.A.T.S. had been produced by lymphocytes *in vitro* or produced *in vivo* and released on lysis of the cells during incubation, an experiment was designed in which half of the cell yield was destroyed by freezing at -20°C, whilst the other half was incubated with PHA at 37°C for six days.

On days 2 and 4 the frozen preparation was thawed and centrifuged, the supernatant drawn off and the cells resuspended in fresh medium. The cell suspension was then refrozen.

At the end of the culture the supernatant pools were concentrated and assayed for L.A.T.S. activity.

(2) Neutralisation of L.A.T.S. Activity with Anti-IgG Serum

(a) Introduction

In order to demonstrate that the L.A.T.S. activity in concentrated supernatant is due to a specific immunoglobulin it is necessary to demonstrate loss of L.A.T.S. activity after neutralisation of the IgG content of the medium with specific antiserum.

In one experiment lymphocytes from a thyrotoxic patient were cultured with PHA and the IgG content of half of the pooled culture medium was neutralized with anti-IgG serum.

(b) Determination of Anti-IgG Titre

The IgG concentration of the culture medium was calculated using the radial diffusion method (Mancini et al, 1965).

Anti-IgG serum (Behringwerke AG, Marburg, Germany) was dissolved in molten agar to a final concentration of 5%, and poured into a petri dish to a depth of 2 mm. Wells, 2 mm diameter, were punched in the solidified agar. Twelve μl of three dilutions of standard human serum (Behringwerke AG, Marburg, Germany) with a known concentration of IgG (740 mg%) was placed in adjacent wells. The diameters of the precipitation rings were measured at 48 hours. A standard curve was plotted, and from this the slope was obtained.

The titre (T) of the antiserum could be calculated from the equation (Becker, 1969):

$$T = \frac{4 \cdot V_{Ag}}{P \cdot \pi \cdot h \cdot k}$$

where -

V_{Ag} = volume (ml) of a standard antigen solution

P = amount of antiserum (ml) in 100 ml of antiserum containing gel.

h = thickness of gel layer (mm)

k = slope of a straight line, obtained by plotting the squared diameters D^2 (mm^2) of circular precipitates as a function of concentration (C Ag) of the standard antigen solution. This slope was determined graphically.

(c) Neutralization of L.A.T.S.-IgG

The titre gave the amount of IgG reacting with the anti-IgG antibody content of 1 ml of the antiserum. Thus, the amount of antiserum required to neutralize all the IgG in half of the medium could be calculated. This was added to one aliquot and the mixture was incubated at 37°C for two hours with constant stirring, and then left at 4°C overnight. The other aliquot was also incubated at 37°C but without antiserum. The two aliquots were then concentrated by vacuum dialysis to a final volume of 3 ml and assayed for L.A.T.S. activity.

(3) L.A.T.S. Bioassay

The routine McKenzie bioassay was used. Prepared assay mice were bled before and at 3, 7, and 24 hours after injection of concentrated culture medium or lymphocyte suspension. For most culture experiments four samples, namely the supernatant pools with and without PHA and the corresponding cell preparations, were assayed for L.A.T.S.

The other components of the culture system, namely concentrated culture medium with or without PHA, PHA alone and 10% foetal human serum alone were included as assay controls.

(D) RESULTS

(1) Lymphocyte Cultures from Thyrotoxic Patients

The results of L.A.T.S. assays carried out on the concentrated culture medium and destroyed cell preparations from thyrotoxic patients are summarized in Table 43 and Figure 45. L.A.T.S.-like activity was detected in culture medium in seven out of 11 cultures with PHA, but in only two out of nine cultures without PHA. L.A.T.S. was detected in the destroyed cell suspension on only one out of seven occasions.

(2) Control Cultures

L.A.T.S. activity was not detected in any of the controls, namely medium in which normal lymphocytes were cultured and the corresponding cell preparations, medium

TABLE 43

L.A.T.S. ACTIVITY IN LYMPHOCYTE CULTURE FROM THYROTOXIC PATIENTS

Patient	L.A.T.S. Response at 7 and 24 hours			
	Concentrated Culture Medium		Destroyed Cell Suspension	
	PHA (+)	PHA (-)	PHA (+)	PHA (-)
H.G.	‡168-113	175-134(NS)	99- 84(NS)	99- 90(NS)
D.P.	170-158(NS)	‡245-146	99-121(NS)	77- 65(NS)
A.N.	*164-145	220-*432	-	-
I.P.	*180-249	129-188(NS)	94- 75(NS)	121- 96(NS)
H.S.	130- 89(NS)	116- 88(NS)	136- 93(NS)	110-125(NS)
I.P.	144- 95(NS)	111- 97(NS)	68- 70(NS)	90- 93(NS)
V.R.	131- 166(NS)	84- 67(NS)	-	-
E.O.	‡254- 98	-	-	-
E.N.	‡171- 97	92- 94(NS)	‡144- 78	102- 81(NS)
V.R.	*183-138	113- 98(NS)	122-102(NS)	113- 91(NS)
I.P.	ʢ241- 65	-	-	-

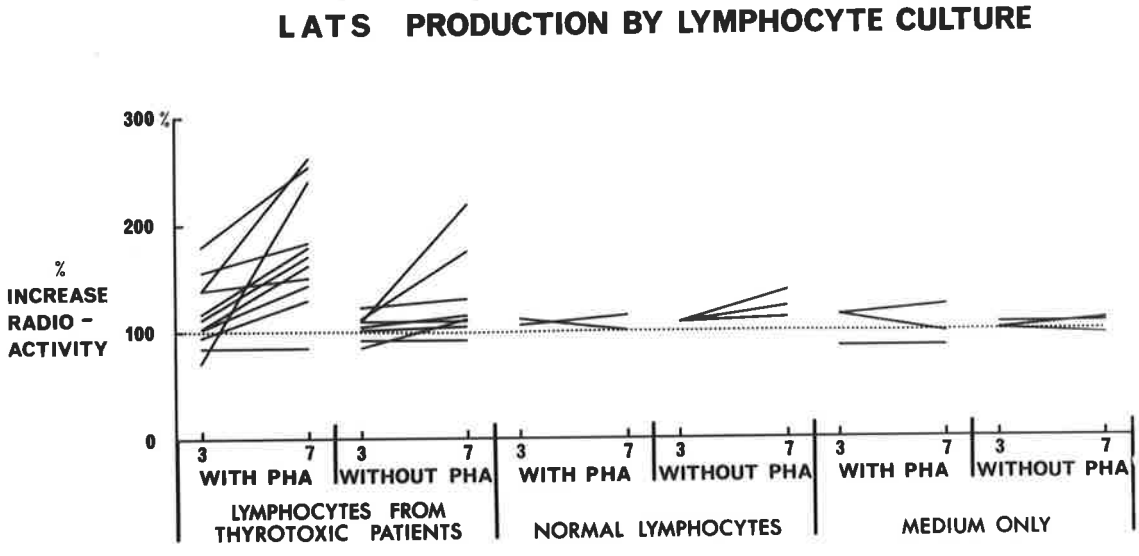
‡ = P<0.02

* = P<0.01

ʢ = P<0.001

NS = Not Significant

FIGURE 45


LATS PRODUCTION BY LYMPHOCYTE CULTURE
% INCREASE RADIO - ACTIVITY
300 %
200
100
0
3
7
WITH PHA
WITHOUT PHA
LYMPHOCYTES FROM THYROTOXIC PATIENTS
NORMAL LYMPHOCYTES
MEDIUM ONLY

incubated in the absence of lymphocytes with or without PHA, 10% foetal human serum alone or PHA alone (Table 44, Figure 45).

Dead Cell Control Culture

Figure 46 summarizes the L.A.T.S. responses given by medium in which destroyed cells had been suspended and frozen at -20°C (dead cell culture) and medium in which live cells from the same patient were cultured at 37°C (live cell culture).

Whilst L.A.T.S. activity was not detected in the dead cell culture supernatant, a L.A.T.S. response was given by the medium from the live cell culture.

(3) Neutralisation of L.A.T.S.-IgG

The L.A.T.S. responses given by the two aliquots, with and without antiserum, are summarized in Figure 47. The L.A.T.S. activity which was detected in the concentrated medium was completely neutralized by anti-IgG serum.

(E) DISCUSSION

L.A.T.S.-like activity was detected in culture medium when peripheral lymphocytes from thyrotoxic patients were cultured with phytohaemagglutinin (PHA) a nonspecific lymphocyte stimulant which causes blastoid transformation of antibody forming cells. The L.A.T.S.-like activity was completely neutralised by anti-IgG serum, showing that the activity resided in the IgG fraction. On two occasions,

TABLE 44

L.A.T.S. ACTIVITY IN CONTROL CULTURES

Culture	L.A.T.S. Response at 7 and 24 hours			
	Concentrated Culture Medium		Destroyed Cell Suspension	
	PHA (+)	PHA (-)	PHA (+)	PHA (-)
Normal Subjects	269-104(NS) 116-104(NS) 88-153(NS)	111- 80(NS) 124-113(NS) 95-109(NS)	79- 51(NS) 138-112(NS) 81-90 (NS)	108- 88(NS) 188-188(NS) 104-120(NS)
Medium Without Cells	86- 91(NS) 124-110(NS) 101-113(NS) 98-106(NS)	111- 87(NS) 96- 70(NS) 105-101(NS) 108- 81(NS)	- - - -	- - - -
PHA Only	64- 72(NS)	-	-	-
10% Foetal Serum	89-102(NS) 130-112(NS)	-	-	-

NS = Not Significant

FIGURE 46

L.A.T.S. PRODUCTION BY LYMPHOCYTES

ABSENCE OF L.A.T.S. ACTIVITY IN DEAD CELL CULTURE

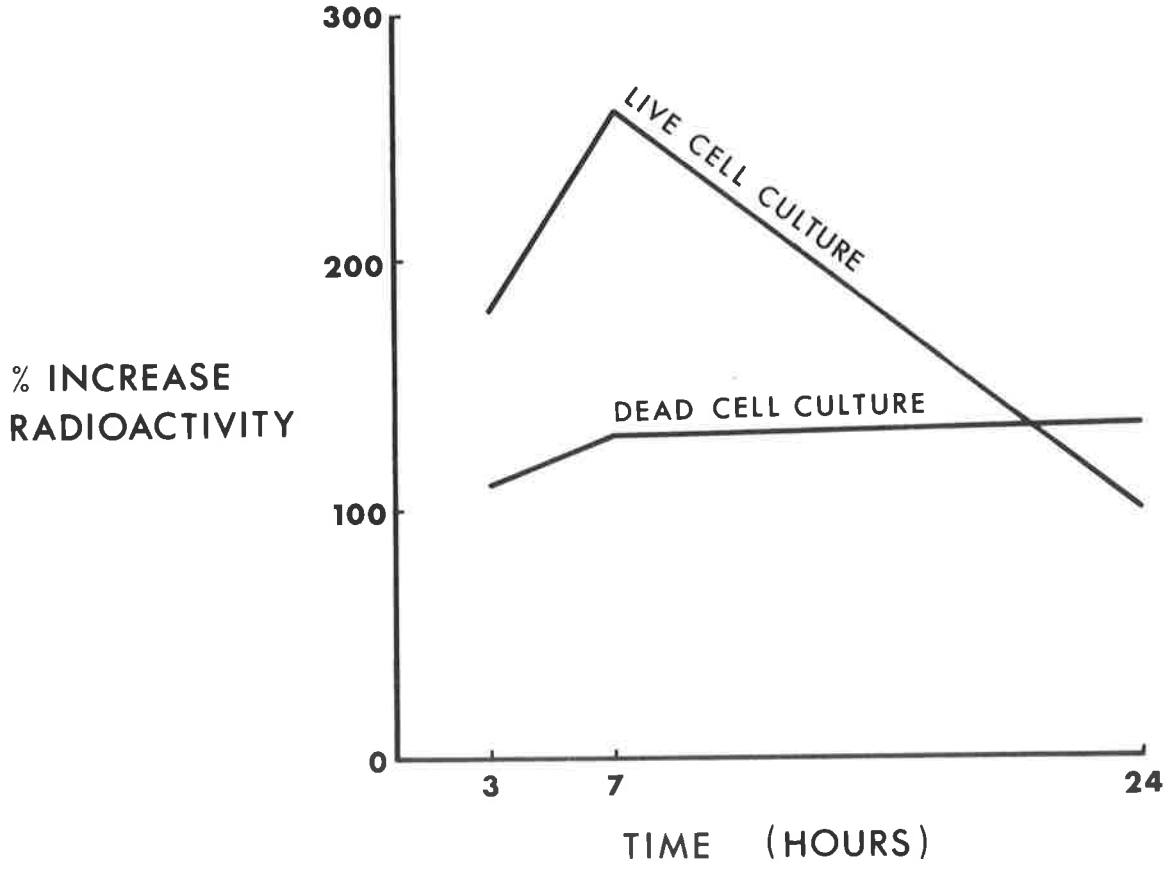
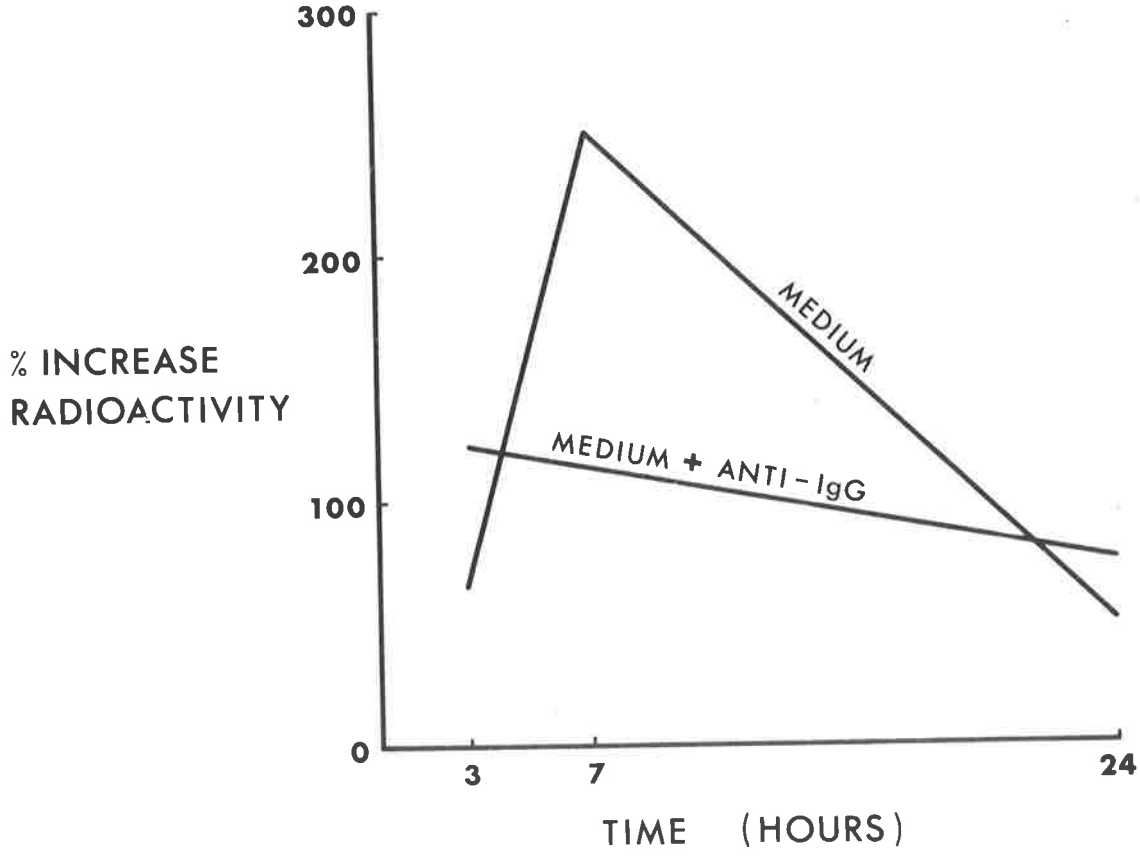


FIGURE 47

L.A.T.S. PRODUCTION BY LYMPHOCYTES

NEUTRALISATION OF L.A.T.S.-LIKE ACTIVITY BY ANTI-IgG SERUM



significant L.A.T.S. activity was also detected in the medium in which cells had been cultured without PHA. Thus, although PHA stimulated gamma globulin production L.A.T.S., in this study, was also produced under appropriate culture conditions by unstimulated lymphocytes.

L.A.T.S. activity was detected in a suspension of the destroyed lymphocytes on only one occasion. Since L.A.T.S. was also detected in the medium in which these cells had been cultured it is likely that lymphocytes which are producing immunoglobulins contain residual antibody at the end of the culture period, which is subsequently released when the cells are disrupted.

Absence of L.A.T.S. in medium containing destroyed cells whilst significant activity was detected in the medium in which an equal number of cells were cultured at 37°C with PHA, indicated that the L.A.T.S. detected in the medium was produced during incubation rather than preformed in vivo and released on destruction of the cells.

Finally, L.A.T.S. was not detected in the cultures from normal blood donors or in medium incubated without cells.

The question as to whether PHA stimulates antibody synthesis by lymphocytes has caused much controversy. In some instances PHA increased the number of antibody producing cells and the levels of antibody in vivo (Gamble, 1966, Hege and Cole, 1967) whereas in other cases PHA depressed antibody

production (Gamble, 1966, Gengozian and Hubner, 1967). Harris and Littleton (1966) were unable to demonstrate increase in the number of antibody forming cells when suspensions of these cells were incubated with PHA during a secondary response *in vitro*.

Reports on the effects of PHA on immunoglobulin and specific antibody synthesis *in vitro* has also been conflicting (Graves and Roitt, 1968, review) however more recent evidence has strongly suggested that the incorporation of PHA into the medium resulted in a shortened induction period of a secondary immune response and increased production of specific antibodies (Forbes, 1965, Ripps and Hirschhorn, 1967, Svehag *et al*, 1968, Forbes and Smith, 1969).

PHA and certain other nonspecific agents cause blastogenesis of peripheral lymphocytes *in vitro* (Nowell, 1960, Svehag *et al*, 1968, Forbes and Smith, 1969). More than 80% of cells cultured with PHA undergo blastoid transformation (Miyai *et al*, 1968). Stimulation of autologous lymphocyte transformation has also been induced by deoxyribonucleic acid in cells from patients with systemic lupus erythematosus (Patrucco *et al*, 1967) and by a liver tissue antigen in some cases of hepatitis (Tobias *et al*, 1967).

The results reported in this study of L.A.T.S. production in lymphocyte culture confirm the findings of McKenzie and Gordon (1968) who demonstrated L.A.T.S. activity

in culture medium when cells from a patient with very high plasma L.A.T.S. levels were cultured with PHA. L.A.T.S. was not detected in culture without PHA or when cells from normal people were cultured with or without PHA.

Miyai et al, (1968) demonstrated L.A.T.S. activity in medium in which cells from patients with much lower L.A.T.S. levels were cultured with PHA. The L.A.T.S.-like activity was neutralised by anti-IgG serum.

Although PHA nonspecifically stimulated L.A.T.S. production in this study, and in those reported by McKenzie and Gordon and Miyai et al, human thyroid microsomal fraction and human thyroid supernatant were unable to stimulate blastoid transformation or protein production in a study reported by De Groot and Jaksina (1969). There are several possible explanations for this. Firstly, the antigen may not have been sufficiently soluble to enter the antibody forming cells. Secondly, L.A.T.S. may be produced by thyroidal lymphoid tissue in vivo, rather than by circulating lymphocytes. Thirdly, it is likely that any L.A.T.S. produced in tissue culture would be neutralised by the antigen and therefore not detectable in the supernatant.

The production of L.A.T.S. by lymphocyte cultures with PHA and its subsequent neutralisation by anti-IgG serum supports the hypothesis that L.A.T.S. is an immunoglobulin

produced by antibody forming tissue. Specific stimulation by the antigen however would provide more conclusive evidence that autoimmune mechanisms operate in Graves' disease, although this may not be possible using this system.

(F) SUMMARY AND CONCLUSIONS

Peripheral lymphocytes from thyrotoxic patients with detectable plasma L.A.T.S. were cultured for six days with phytohaemagglutinin (PHA) a nonspecific lymphocyte stimulant. L.A.T.S. was detected in seven of 11 cultures with PHA, but on only two occasions without PHA. The L.A.T.S. activity was neutralised by anti-IgG serum.

L.A.T.S. was not detected in the medium in which cells from normal people were cultured or when medium with or without PHA was incubated in the absence of cells. Significant L.A.T.S. activity was detected in a suspension of the destroyed cells on only one occasion.

That the L.A.T.S. activity demonstrated was produced by the cells during culture rather than being due to release of antibody preformed *in vivo*, was confirmed by failure to demonstrate L.A.T.S. in medium in which cells were killed by freezing, whilst L.A.T.S. activity was detected in medium in which cells had been cultured at 37°C.

These findings provide further evidence that L.A.T.S. is an immunoglobulin produced by antibody forming tissue

and thereby support the hypothesis that thyrotoxicosis is an autoimmune disease, in which L.A.T.S., and possibly other antibodies, are produced *in vivo* as a result of specific antigenic stimulation.

CHAPTER X

DISCUSSION OF RESULTS AND CONCLUSIONS

(A) INTRODUCTION

(B) SUMMARY OF RESULTS

(C) THE LONG ACTING THYROID STIMULATOR (L.A.T.S.)

(D) EVIDENCE THAT THYROTOXICOSIS IS AN AUTOIMMUNE DISEASE

- (1) Introduction
- (2) Criteria of Milgrom and Witebsky
  - (a) Circulating Antibodies
  - (b) Recognition of the Specific Antigen
  - (c) Production of the Antibody in Experimental Animals
  - (d) Pathological Changes in the Target Tissues
  - (e) Transfer of Disease by Serum or Sensitized Cells
- (3) Pathological Markers of MacKay and Burnet
  - (a) Elevated Levels of Immunoglobulins
  - (b) Relationship to Other Autoimmune Diseases
  - (c) Infiltration of Target Organs with Lymphoid Tissue
  - (d) Significant Benefit from Immunosuppressive Agents

(E) RELATIONSHIP OF L.A.T.S. TO CLINICAL FEATURES

- (1) Introduction
- (2) Hyperthyroidism
- (3) Dermopathy
- (4) Ophthalmopathy

(F) PATHOGENESIS OF THYROTOXICOSIS

- (1) Immunological Factors
- (2) Genetic Factors
- (3) Precipitating Factors

(G) SCOPE FOR FURTHER RESEARCH AND CONCLUSIONS

CHAPTER X

DISCUSSION OF RESULTS AND CONCLUSIONS

(A) INTRODUCTION

The clinical and experimental studies which formed the basis of this thesis have been carried out to test the hypothesis, proposed by several workers including Stuart and Allan (1958), Roitt and Doniach (1958), Buchanan et al (1961), and Hetzel (1968), that thyrotoxicosis is an autoimmune disease. Their object has been to examine Witebsky's and Milgrom's criteria of autoimmunity, and certain markers which represent the clinical and pathological manifestations of the autoimmune process (MacKay and Burnet, 1963).

In the clinical studies, 135 patients with thyrotoxicosis were investigated for the presence of L.A.T.S. Sixteen of these patients had recurrence of disease whilst 12 had ophthalmopathy without hyperthyroidism (euthyroid Graves' disease). Whenever possible L.A.T.S. assays were carried out on the concentrated IgG fraction as well as on the plasma.

L.A.T.S. concentration studies of 43 euthyroid relatives from four families with a high prevalence of thyrotoxicosis and of patients with autoimmune diseases and other diseases were reported. L.A.T.S. assays were also carried out on several normal people and on normal relatives of patients with autoimmune diseases.

In the experimental studies, rabbits and rats were immunized with thyroid antigens and investigated for the presence of L.A.T.S.-like activity and for features of the experimental equivalent of thyrotoxicosis. Thyroid function was assessed in the animals by measuring protein bound iodine and ^{131}I uptake.

Finally, lymphocytes from thyrotoxic patients were cultured in the presence of phytohaemagglutinin (PHA), a nonspecific lymphocyte stimulant, to determine whether L.A.T.S. could be produced *in vitro* by antibody forming tissues.

(B) SUMMARY OF RESULTS

The main findings as regards L.A.T.S. were firstly, the prevalence of L.A.T.S. was related to the number of features present. Patients with all three features had the highest prevalence (100%) of L.A.T.S., whilst patients with only hyperthyroidism or ophthalmopathy had a very low prevalence (14%). Secondly, a close relationship was found between the clinical course of the dermopathy and plasma L.A.T.S. levels in six patients in whom serial assays were carried out. All ten patients with dermopathy had high levels of L.A.T.S.

Thirdly, although 57% of patients with severe ophthalmopathy had detectable L.A.T.S., as compared to 9% with mild eye signs, the relationship was not as close as for

dermopathy since several patients with severe ophthalmopathy did not have detectable L.A.T.S. whilst others, with high L.A.T.S. levels, had only mild eye signs. Furthermore, in seven out of 18 patients who developed severe ophthalmopathy, changes in the eye lesion did not correlate with changes in L.A.T.S. levels.

Finally, in the clinical studies, L.A.T.S. was detected in eight out of 43 euthyroid relatives from the four families studied, and in four out of 34 patients with diseases likely to have an autoimmune mechanism. L.A.T.S. was also detected in one patient with panhypopituitarism, in another with thyroid malignancy and in one normal person.

In the experimental studies thyroid stimulating activity was found more often in rabbits immunized with thyroid extracts or the microsomal fraction than in animals immunized with the corresponding liver antigen. Although none of the rabbits was overtly thyrotoxic several had elevated levels of PBI and ^{131}I uptake and one developed unilateral exophthalmos.

Finally, L.A.T.S. was produced by peripheral lymphocytes from thyrotoxic patients, cultured with phytohaemagglutinin, but not by lymphocytes from normal persons. The L.A.T.S.-activity was neutralised by anti-IgG serum.

(C) THE LONG ACTING THYROID STIMULATOR (L.A.T.S.)

The long acting thyroid stimulator is closely related to the pathogenesis of thyrotoxicosis. L.A.T.S., which has

many but certainly not all of the features of an antibody, may be produced, in genetically predisposed persons, as a result of a specific antigenic stimulus, and may be the cause of one or more of the clinical features of thyrotoxicosis.

Because L.A.T.S. is detected almost exclusively in patients with past or present hyperthyroidism (Pinchera et al, 1965, McKenzie, 1966), and because experimental evidence links L.A.T.S. with thyroid stimulation (Adams, 1965, Carneiro et al, 1966a) it has been postulated (McKenzie, 1961, Major and Munro, 1962, Adams and Kennedy, 1965, Hetzel, 1968) that L.A.T.S. is the direct cause of the hyperthyroidism of Graves' disease.

The results reported in these studies confirm the relationship noted by others (Purves and Adams, 1960, Kriss et al, 1964, Solomon et al, 1964, Hoffmann and Hetzel, 1966, Lipman et al, 1967) between L.A.T.S. and the other main features, namely ophthalmopathy and dermopathy, in that patients with these features tend to have a higher prevalence of the stimulator than those with only hyperthyroidism. In order for a single antibody to be the direct cause of all three features it would be necessary to postulate that L.A.T.S. cross reacts with similar antigens in the skin and in the tissues of the orbit (Kriss et al, 1964, Benoit and Greenspan, 1967, Hetzel, 1968).

It is apparent however that L.A.T.S. cannot be implicated equally in all of the clinical features of Graves' disease. Whilst it is likely that L.A.T.S. causes hyperthyroidism (McKenzie, 1968), the relationship with ophthalmopathy and dermopathy is not so obvious. The low prevalence of L.A.T.S. in patients with only ophthalmopathy and the occurrence of severe eye disease in some patients without detectable L.A.T.S., reported in the present study, makes it unlikely that L.A.T.S. is the direct cause of these features. Certainly it was found, in a study reported in Chapter IV, that dermopathy was closely associated with L.A.T.S. levels in patients with this feature, but since all of these patients had ophthalmopathy and hyperthyroidism the presence and levels of L.A.T.S. may be most closely related to the number of features present rather than to any single one (Lipman et al, 1967). Furthermore, correlation between change in L.A.T.S. levels and the clinical course does not necessarily imply cause and effect (McKenzie, 1968).

(D) EVIDENCE THAT THYROTOXICOSIS IS AN AUTOIMMUNE DISEASE

(1) Introduction

In the following sections the criteria for autoimmunity (Milgrom and Witebsky, 1962) and the pathological markers which characterize the process (MacKay and Burnet, 1963) will be discussed. The evidence will be examined in respect to the hypothesis that thyrotoxicosis is an autoimmune disease and

particularly as to the role of L.A.T.S. in the pathogenesis of the clinical features of the disease. The possible mechanisms of autoimmunity in thyrotoxicosis will be discussed and the importance of precipitating factors evaluated. Finally, some of the more important questions raised by these investigations will be mentioned with reference to scope for further research.

(2) Criteria of Milgrom and Witebsky.

(a) Circulating Antibodies

For a disease to be considered autoimmune free circulating or cell-bound antibodies that are active at body temperature should be demonstrated in all cases. These antibodies must be directed against the patients own tissues (Milgrom and Witebsky, 1962).

Antibodies against thyroglobulin and thyroid cytoplasm are detected in the serum of many patients with Graves' disease, but these are also found in other thyroid diseases particularly those with autoimmune mechanisms (Doniach and Roitt, 1957, Roitt and Doniach, 1958, MacKay and Perry, 1960, Hales et al, 1961, Irvine, 1962, Anderson et al, 1964). L.A.T.S., on the other hand, which is almost unique to Graves' disease may be the common mechanism in cases of hyperthyroidism, although other antibodies are likely to be involved in the pathogenesis of the ophthalmopathy and dermopathy. Whilst the incidence of

thyroid cytoplasmic antibody and antithyroglobulin is high in patients with Graves' disease neither antibody has been found, generally, to correlate with the presence of L.A.T.S. (Seif, 1967, Beall and Solomon, 1968b, Ochi and De Groot, 1968, Beall et al, 1969). Thus some patients have L.A.T.S. but not the other antibodies whilst others have antithyroglobulin and the thyroid cytoplasmic antibody but not L.A.T.S. Pinchera et al (1967) on the other hand, found an association of L.A.T.S. with antithyroglobulin but not with the cytoplasmic antibody.

L.A.T.S. was detected, in this series, in the plasma from only 27% of patients with thyrotoxicosis and in 43% of the L.A.T.S.-negative patients after concentration. There are at least four possible explanations for the failure to detect L.A.T.S. in all patients with thyrotoxicosis. Firstly, L.A.T.S. may be unrelated to the disease or produced as an incidental side effect in some patients with thyrotoxicosis. Secondly, L.A.T.S. may be present, but in amounts too small to be detected by the relatively insensitive bioassay. Thirdly, there may be a "poor fit" of human L.A.T.S. onto the antigenic sites of the mouse thyroid (Adams, 1970) and finally, inhibitors of L.A.T.S. may be present (Burke, 1968).

(b) Recognition of the Specific Antigen

With the identification of autoantibodies in patients with a disease suspected of being autoimmune, the specific antigen against which the antibody is directed should be recognised.

Although the microsomal fraction of the thyroid is thought generally to contain the antigen with which L.A.T.S. reacts the evidence for this is not conclusive. Thus although Beall and Solomon (1966) were able to show that incubation of L.A.T.S. serum with thyroidal microsomes, but not with microsomes from other tissues, gave neutralisation of L.A.T.S. activity they and others (Dorrington et al, 1966, Burke, 1967) have demonstrated significant neutralisation by other thyroid fractions. Furthermore, in other studies the soluble fraction gave the best neutralisation (Berumen et al, 1967, Smith, 1969). Smith and others found that the L.A.T.S. absorbing activity was associated with the 4S protein peak obtained by gel filtration on Sephadex G-200 of the soluble fraction from thyroid homogenates (Salvatore et al, 1964, Shulman et al, 1967, Smith, 1969). No attempt was made to identify the antigen in the present studies although immunization of rabbits with thyroid microsomes led to greater and more persistent levels of thyroid stimulating antibody than with whole thyroid extract.

(c) Production of the Antibody in Experimental Animals

With recognition of the antigen, it should be possible to produce a similar antibody in experimental animals by immunizing them with the antigen.

L.A.T.S.-like activity was detected, in these studies, in rabbits immunized with whole thyroid extract or the microsomal fraction, higher and more persistent levels being produced in rabbits immunized with microsomes. However, low levels of antibody were occasionally detected in control rabbits that had received the corresponding liver fraction.

(d) Pathological Changes in the Target Tissues

With the production of autoantibodies, pathological changes, basically similar to those in the human disease, should appear in the corresponding tissues of the sensitized experimental animals.

A whole range of antibodies is produced as a result of immunization with heterologous tissue (Witebsky and Rose, 1956, Twarog and Rose, 1968). Thus immunization of rabbits with human thyroid tissue results in hetero-antibod production as well as the production of antibodies which react with the homologous antigen or with antigens in the animals own tissues (autoantibodies). The autoanti-bodies may produce disease by initiating a self-perpetuating process.

In the experimental studies described in Chapter VIII many of the rabbits that had detectable thyroid stimulating activity had elevated protein bound iodine and radio-iodine uptake whilst one animal developed marked unilateral exophthalmos. On the other hand, no rabbit was overtly thyrotoxic and histological examination revealed essentially normal thyroid tissue in all animals. Thus, although the tendency to develop thyrotoxicosis probably existed in animals immunized with thyroid antigens, significant clinical and pathological changes were not observed.

(e) Transfer of disease by Serum or Sensitized Cells

The disease should be transferable by antibody or by sensitized cells.

The one baby with neonatal thyrotoxicosis in the present study had low levels of L.A.T.S., whilst the mother, who had been treated for thyrotoxicosis previously, also had detectable L.A.T.S.

IgG is the only immunoglobulin able to pass the placental barrier (Kohler and Farr, 1966). Thus, the appearance of L.A.T.S. in the baby and its disappearance at a rate consistent with the known half life of IgG antibodies, which correlated well with the observed duration of the clinical syndrome, suggests that the disease can indeed be transferred (McKenzie, 1964, Sunshine et al, 1965, Kriss, 1968).

(3) Pathological Markers of MacKay and Burnet

(a) Elevated Levels of Immunoglobulins

IgG was elevated in 47% of patients with untreated Graves' disease whilst IgA and IgM were elevated in 27% and 36% of patients respectively. Hence a significant proportion of patients with thyrotoxicosis in this series had increased levels of immunoglobulins. The prevalence of hypergammaglobulinaemia in most other series however has been low (Lamberg and Grasbeck, 1955) although Yamakido et al (1969) reported elevated levels of IgG in 12 out of 29 patients with thyrotoxicosis and levels at the upper limit of normal in a further 11 patients.

(b) Relationship to Other Autoimmune Diseases

As stated in Chapter VII, thyrotoxicosis occurs occasionally with Addison's disease, pernicious anaemia and Sjögren's disease, all of which may be autoimmune diseases (Doniach et al, 1963, Blizzard and Kyle, 1963, Anderson et al, 1964, Irvine, 1963). In this series of 135 patients, thyrotoxicosis was associated with acromegaly (two patients) diabetes and ulcerative colitis (two), Sjögren's disease (one), diabetes alone (two), rheumatoid arthritis (one case), and myasthenia gravis (one case), some of which may be autoimmune diseases.

Several patients with thyrotoxicosis had a past or family history of other autoimmune diseases,

particularly rheumatoid arthritis and Hashimoto's disease whilst the patients, and their relatives, also had a higher prevalence of gastric parietal cell antibody than normal subjects. Furthermore, 34 patients with autoimmune diseases had a 12% prevalence of detectable L.A.T.S. when the concentrated 7S globulins were assayed for L.A.T.S. activity.

(c) Infiltration of Target Organs with Lymphoid Tissue

An increase in lymphoid tissue is commonly found in the thyroid gland of patients with thyrotoxicosis. This may be extensive and the histological picture can resemble that of Hashimoto's disease (Levitt, 1951, Greene, 1953, Lindsay, 1964).

On many occasions in this study, subtotal thyroidectomy was carried out as the definite treatment of the hyperthyroidism. Lymphoid tissue was frequently present in excessive amounts.

(d) Significant Benefit from Immunosuppressive Agents

Therapy with cortico-steroids and immuno-suppressive agents is frequently associated with decrease in ophthalmopathy and dermopathy (Snyder et al, 1964, Kriss et al, 1964). Rapid remission in her ophthalmopathy was seen in one patient in this series treated with Azathiaprine.

(E) RELATIONSHIP OF L.A.T.S. TO CLINICAL FEATURES

(1) Introduction

In these studies, the overall prevalence of L.A.T.S. was only 27% when plasma from thyrotoxic patients was assayed for L.A.T.S. activity. However, the prevalence was much higher in patients with severe grades of ophthalmopathy and in patients with dermopathy. This is probably because the prevalence of L.A.T.S. increases with the number of features present. Patients with only hyperthyroidism or ophthalmopathy have the lowest prevalence, whilst patients with all three features have a very high prevalence (Lipman et al, 1967). Furthermore, patients with all three features usually have high levels of L.A.T.S.

(2) Hyperthyroidism

Because L.A.T.S. causes thyroid hyperplasia and stimulates protein synthesis (Ochi and De Groot, 1968), ^{131}I uptake and thyroid cell height (Adams and Purves, 1956, Major and Munro, 1962) in experimental animals and in human volunteers (Arnaud et al, 1965) it would seem likely that L.A.T.S. causes the hyperthyroidism of Graves' disease (McKenzie, 1968).

The presence of L.A.T.S. has been equated with non-suppressibility of thyroid ^{131}I uptake by exogenous thyroxine or T_3 (Werner et al, 1955, Liddle et al, 1965 McLarty and McGill, 1967). Thus the abnormal thyroid

stimulator was thought to cause autonomous thyroid activity which was unaffected by TSH (Adams et al, 1969) or by excess circulating thyroid hormone. Following treatment, return of suppressibility was associated with disappearance of circulating L.A.T.S. (Alexander and Harden, 1967).

Quite recently however the relationship of L.A.T.S. to thyroid overactivity has been questioned (Lancet Editorial, 1970), since L.A.T.S. has been found in euthyroid relatives of thyrotoxic patients and in patients with other autoimmune diseases in studies reported in this thesis, in patients with normal suppressibility of thyroidal ^{131}I uptake (Chopra et al, 1970) and in subjects with no clinical signs of hyperthyroidism (Adams, 1961, Adams, 1965, Lipman et al, 1967). Furthermore, some patients with non-suppressible thyroid function do not have detectable L.A.T.S. (Chopra et al, 1970, Hall et al, 1970) and in one study, lymphocytes sensitized to thyroglobulin and to the thyroid component with which L.A.T.S. combines, were found not only in each of 19 patients with thyrotoxicosis but also in 19 of 20 normal subjects (Field et al, 1970).

Thus L.A.T.S. can be present without causing thyroid overactivity. This means either that L.A.T.S. does not cause the hyperthyroidism or that other factors, apart from thyroid stimulation, are important in the pathogenesis of the disease. Even if L.A.T.S. is a by-product of the

thyroid disorder rather than its direct cause the latter is the more likely explanation. It is well known for example that infection or stress may precipitate hyperthyroidism in susceptible people (Conrad, 1934, Brown and Hetzel, 1963, Kriss, 1968). These people may have circulating L.A.T.S.

(3) Dermopathy

All patients with dermopathy in this series had high levels of L.A.T.S. and the levels correlated closely with the clinical course of the skin lesion in that rises in L.A.T.S. levels were associated with worsening of the dermopathy whilst falling levels were associated with remission. In other series, almost all patients with dermopathy had high plasma L.A.T.S. levels (Kriss et al, 1964, Solomon et al, 1964, Pinchera et al, 1965). On the other hand, as already stated, because patients with this feature usually also have hyperthyroidism and ophthalmopathy the presence of L.A.T.S. is probably related to the severity of the disease rather than to the dermopathy itself.

Because of the association with high L.A.T.S. levels, significant titres of thyroid antibodies, and increased immunoglobulin levels, and because of the response to cortico-steroids and immunosuppressive agents, it is likely that immunological processes are also involved in the pathogenesis of this feature (Kriss et al, 1964).

However, since L.A.T.S. has not been observed attached to the tissues of the skin lesion, and since the antibody has not been convincingly extracted from the affected skin (Pimstone et al, 1964, Solomon et al, 1964, Pinchera et al, 1965), other antibodies may be more directly involved (McKenzie, 1968).

(4) Ophthalmopathy

The relationship of L.A.T.S. to ophthalmopathy is even more uncertain. Fifty-seven per cent of patients with severe grades of ophthalmopathy in this series had detectable L.A.T.S. compared to only 9% with mild ophthalmopathy. On the other hand, because some patients with high L.A.T.S. levels had only mild ophthalmopathy whilst others with severe eye lesions had no detectable L.A.T.S. the stimulator probably has no direct role in the pathogenesis of ophthalmopathy. Thus, in a study reported by Shillinglaw and Utiger (1968), retro-orbital tissues failed to neutralise L.A.T.S. activity whereas even small amounts of thyroid protein produced complete inactivation of L.A.T.S. Immunological processes are likely to be involved in the severe eye lesions however, because treatment with immunosuppressive agents often brought about improvement in the eye lesion and fall in L.A.T.S. levels and thyroid antibody titres (Snyder et al, 1964, Lipman et al, 1967, Werner, 1967).

(F) PATHOGENESIS OF THYROTOXICOSIS

(1) Immunological Factors

The role of L.A.T.S. in the pathogenesis of thyrotoxicosis is uncertain, but there are several possible mechanisms. Firstly, L.A.T.S. may be produced as a result of a specific antigenic stimulus and itself cause the main features of the disease by reacting with the antigen in the thyroid and with similar antigens in the orbits and skin. This hypothesis must be considered unlikely in view of the evidence outlined above. Secondly, as a result of nonspecific thyroid damage, antibodies may be produced which react with tissue antigens, and indirectly lead to L.A.T.S. production. L.A.T.S. may then cause the hyperthyroidism, other antibodies producing the lesions in the orbits and skin. Thus L.A.T.S. may be a "marker" for the dermopathy and ophthalmopathy which may be due to a more complex defect in immunological homeostasis.

Thirdly, a naturally occurring L.A.T.S. may combine in an antibody-antigen reaction with the presumptive thyroid mitotic inhibitor (Chalone) and allow an unbalanced thyroid stimulation to occur, leading to thyroid enlargement and hyperthyroidism (Garry and Hall, 1970).

Finally, L.A.T.S. may be produced without prior antigenic stimulation, by a "forbidden clone" (Burnet, 1959) of immunologically competent cells, produced as a result of a

genetically determined mutation of lymphocytes (Kriss, 1968, Volpé et al, 1969). Because of the associated immunological abnormalities in thyrotoxicosis one must postulate several such "forbidden clones". This latter hypothesis, because of its broad concept, is the most acceptable.

(2) Genetic Factors

There is a marked familial prevalence of Graves' disease (Bartels, 1941, Martin and Fisher, 1945) and genetic factors are thus likely to be of importance in the pathogenesis of the disease. In the present study L.A.T.S. was detected in 19% of normal relatives in four families with a high prevalence of thyrotoxicosis. Its presence in these people supports the hypothesis that Graves' disease is due to a genetically determined abnormality of the antibody forming tissues.

Hashimoto's disease, which may have a common genetic mechanism with thyrotoxicosis (Mason and Walsh, 1963, Doniach et al, 1967) may, on rare occasions, undergo transition into classical Graves' disease (Jason et al, 1967). Both diseases may develop from primary viral thyroiditis, the end result depending on the antibodies produced. To support this, there is evidence that viral infection may induce autoimmune disease in sheep (Parry, 1962) and in man (Stanley and Walters, 1966).

(3) Precipitating Factors

Other factors, apart from antibody formation, are apparently involved in the pathogenesis of thyrotoxicosis (Volpé et al, 1967) since, as shown in this and other studies, patients with circulating L.A.T.S. do not necessarily have features of thyrotoxicosis (Major and Munro, 1962, Lipman et al, 1967, Greenwald, 1966, Alexander et al, 1968). Emotional stress (McKenzie and Solomon, 1967), bacterial and viral infection, or iodine deficiency (Stewart et al, 1970), may precipitate signs and symptoms in people with circulating antibodies or with the genetic predisposition to produce antibodies. Such nonspecific thyroid damage may lead to a state of heightened immunological reaction with excessive antibody production. Viral infection of the thyroid in particular, because of the intimate relationship of the infective particles to host DNA with subsequent changes in the genetic material of the host, may initiate thyrotoxicosis. Subacute thyroiditis which sometimes follows mumps (Eylan et al, 1957) and other viral illnesses (Skillern, 1964a) however is not associated with L.A.T.S. or thyrotoxicosis, but Hashimoto's thyroiditis, which is likely to be autoimmune (Hall, 1962), sometimes starts as an acute inflammation with a clinical picture resembling subacute thyroiditis (Skillern, 1964b).

(G) SCOPE FOR FURTHER RESEARCH AND CONCLUSIONS

Whilst the hypothesis that thyrotoxicosis is an auto-immune disease is not yet proven the evidence is strong.

One of the most confusing concepts of this hypothesis has been that the disease is associated with a specific antibody (L.A.T.S.) whose role is not clear. L.A.T.S. may cause the hyperthyroidism but almost certainly not the other features. In fact there has also been some doubt expressed quite recently as to whether L.A.T.S. is the direct cause of the thyroid stimulation or merely produced as a result of another immunological process. It must be accepted however that the antibodies detected in auto-immune diseases may be the result rather than the cause of the disease.

Although the other features, namely ophthalmopathy and dermopathy, are not so closely related to L.A.T.S. they are also likely to be due to immunological abnormalities, for which L.A.T.S. may be a "marker".

The relationship of the disease to other autoimmune disorders and the tendency for certain families to have an increased prevalence of thyrotoxicosis and other auto-immune diseases is important evidence. On the other hand, the response to cortico-steroids and immunosuppressive agents, which is frequently cited as evidence of autoimmunity may only represent the nonspecific anti-inflammatory action of these agents.

In order to understand more fully the pathogenesis of the disease, and particularly the role of L.A.T.S., a more sensitive and specific *in vitro* assay for the detection of L.A.T.S. must be introduced. This will depend on identification of the specific antigen against which L.A.T.S. is directed.

As regards scope for further research, it is clearly important for the relatives of thyrotoxic patients to be investigated more fully. A full range of thyroid function studies including T_3 suppression tests, free T_4 and free T_3 measurements and thyroid scanning, may demonstrate a "latent" thyrotoxic state. Follow-up of the euthyroid relatives with detectable L.A.T.S., probably for many years, to determine the frequency of development of overt thyrotoxicosis will be carried out from this laboratory.

Once the specific antigen is identified titration of the amount necessary for maximal antibody response in experimental animals will be possible. If a disease similar to human thyrotoxicosis can be convincingly produced in animals immunized with the antigen conclusive evidence that thyrotoxicosis is an autoimmune disease would be provided. However, as pointed out by Adams (1970), the production of the experimental equivalent is not a sine qua non for an autoimmune disease since the factors which are involved in the artificial process of immunization

with heterologous or even autologous antigens may not be related to the pathogenesis of the disease in man. For example, if there is a genetically determined mutation in lymphoid cells with development of "forbidden clones" it is unlikely that this would occur experimentally.

Finally, the production of L.A.T.S. and other antibodies which may be shown to be significant in the pathogenesis of the disease, by lymphocytes from thyrotoxic patients when cultured in the presence of the specific antigen, would also be strong evidence that autoimmune mechanisms are involved.

In conclusion then it is apparent that whilst Graves' disease has the characteristics of an autoimmune disorder there are many questions yet to be answered. It may be many more years before the mechanisms of the disease are less of a controversy by which time the phenomenon of autoimmunity itself, and the relationship of autoimmunity and disease, may be understood.

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