THYROGLOBULIN BIOSYNTHESIS - AN ULTRACENFRIFUGAL STUDY.

by

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SUMMARY

This work presents data on the biosynthesis of thyroglobulin. This protein of 19S size constitutes the main thyroid protein in most normal vertebrate thyroid glands; smaller amounts of 3-8S and 27S protein are usually present.

Initially it is shown that in vivo in the rat, as has been previously shown in vitro by other workers, labelled amino acids are incorporated into proteins of 3-8S and 12S size before the label is incorporated into thyroglobulin. The pattern of incorporation is consistent with the 12S and with less certainty the 3-8S proteins being precursor sub-units of the 19S protein. At long time intervals after administration of a pulse of ^{3}H-leucine, the pattern of labelling of the 3-8S fraction could also be consistent with this labelled fraction being a breakdown product of the labelled 19S protein.

These findings with ^{3}H-leucine are in contrast to those seen when ^{125}I is used. In this case ^{125}I is not incorporated into proteins <18S, except for a small percentage transiently incorporated into a 12S protein at very early time intervals after administration of the label. At no time, however,

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was a protein lighter than 18S present as the predominant labelled peak.

The production of a goitrous state in the rat, by means of one of the antithyroid drugs which block at various points the biosynthesis of thyroxine, alters the distribution of the thyroid proteins. Thyroglobulin diminishes in amount whereas the 3-8S proteins increase; the normally present 27S peak is lost and a new OD peak is seen in the 32S region. In these goitrous glands the incorporation of ^{3}H-leucine into the thyroid proteins is enhanced, but a truly 19S protein is not formed. As would be anticipated, the incorporation of ^{125}I is blocked to a varying degree depending on the potency of the goitrogen used.

When a mild iodine deficiency state is induced by feeding a low iodine diet, the incorporation of ^{3}H-leucine or ^{125}I into thyroglobulin is accelerated, presumably due to increased TSH production by the pituitary. When a more severe degree of iodine deficiency is present, an 18S, as opposed to a truly 19S protein, is labelled. There is comparatively little labelling of the 3-8S proteins of iodine deficient glands, as compared to drug-induced goitres.

The/

The administration of T_4 to a goitrous, or normal, animal virtually inhibits the incorporation of ^{3}H-leucine and, to a lesser extent, ^{125}I into the thyroid proteins. A stable 12S protein peak is seen under these conditions, which suggests that this may represent a failure of incorporation of sub-units into 19S thyroglobulin. An ^{125}I labelled 12S protein of high specific activity is found during withdrawal of antithyroid drugs in the rat.

The protein patterns of a series of 100 human thyroid glands were studied. The normal human thyroid gland showed a protein pattern similar to the rat and slices of the tissue incorporated ^{3}H-leucine into the 19S protein and its presumed sub-units in vitro. ^{125}I on the other hand was incorporated only into an 18-19S protein.

In thyrotoxicosis, whether prepared pre-operatively by carbimazole/iodide or by $KClO_4$, the notable feature was the loss of proteins > 19S. This occurred despite differences in distribution of the thyroid proteins in thyroid glands treated pre-operatively with these two different regimes. The glands from patients treated with carbimazole/iodide contained more/

more thyroglobulin and a smaller amount of 3-8S protein than glands treated with $KClO_4$. The lack of a 27S protein could not be correlated with iodine deficiency - the thyrotoxic glands treated by either method of pre-operative preparation having a similar iodine content which was furthermore similar to that found in non-toxic goitre in which a 27S protein was regularly found.

Most non-toxic goitres and thyroid adenomas showed a protein pattern and pattern of incorporation of ^{3}H-leucine and ^{125}I similar to the 'normal' human material. In only 2 of 17 adenomas studied was a 3-8S protein found as the predominant thyroid protein. In both of these glands no thyroglobulin was histologically demonstrable.

In Hashimoto's thyroiditis a relative loss of thyroglobulin with increased 3-8S protein was present. A 32S OD peak was seen in 2 of the 5 glands studied. ^{3}H-leucine was not incorporated into proteins> 3-8S, but ^{125}I was incorporated, not only into thyroglobulin, but also into 12S and 3-8S proteins.

In malignant thyroid glands, as in the other human thyroid glands studied, there was a good correlation between the fraction of the thyroid proteins present as thyroglobulin

and/

and the presence of histologically demonstrable colloid. In poorly differentiated tumours, neither ^{125}I or ^{3}H-leucine were incorporated into the thyroid proteins.

The iodine content of 'normal' human thyroid protein was approximately 3 times that of any group of pathological glands studied. Tumour tissue, as anticipated, contained the least iodine.

In a series of vertebrate thyroid glands studied, the thyroid protein pattern was similar to that of the normal rat or human; the exception being the horse in which no 27S protein was found. The iodine content of the majority of vertebrates was approximately twice that of the normal human, the only exceptions being the guinea pig, which contained less, and the cat and dog, in whom the thyroid proteins were more highly iodinated than in the other animals studied.

Evidence is presented that the 32S OD peak present in the goitrous rat can be demonstrated after a variety of techniques of preparation of the soluble thyroid proteins. Study of this peak showed that it was not of iodoprotein nature, but was consistent with it being composed of RNA.

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PREFACE

This work was started during the tenure of a United States Public Health Service Postdoctoral Research Fellowship which I held during the academic year 1966-1967. In this period I worked at the Harvard Medical School in the laboratory of Dr. Irving H. Goldberg at the Beth Israel Hospital, Boston, Mass., U.S.A.

The initial stages of the project were discussed in broad outline on my arrival in Boston but the day-to-day control of the experiments remained my responsibility. The work performed during this period was published jointly with Dr. Goldberg*.

Following my return to Glasgow the studies have been extended under my direct guidance.

*Thomson, J.A., Goldberg, I.H.
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SECTION 1

INTRODUCTORY

CHAPTER 1

INTRODUCTION

The thyroid gland exists in all but the lowest forms of life and provides the body with a site of manufacture and storage of the active thyroid hormones thyroxine (T_4) and triiodothyronine (T_3). This function of providing a supply of its active hormone sufficient in normal man for some weeks requirements is unique amongst the endocrine organs.

The overall structure of the thyroid gland is shown in fig. 1. It consists of follicles lined in the resting state by flattened cuboidal epithelium; the lumen of the follicles being filled by an amorphous protein material - the colloid.

The synthesis of T_4 and T_3 is achieved by a complex series of metabolic steps. The advent of such techniques as radioisotopes and chromatography and their application to patients with sporadic goitrous cretinism and familial goitre (Stanbury, 1966 and McGirr and Thomson, 1968) coupled with the availability of a variety of antithyroid drugs which can be used experimentally to interupt the process at specific points, has led to the recognition of seven main steps in the synthesis and release of the thyroid hormones. Although the steps are understood in broad outline the

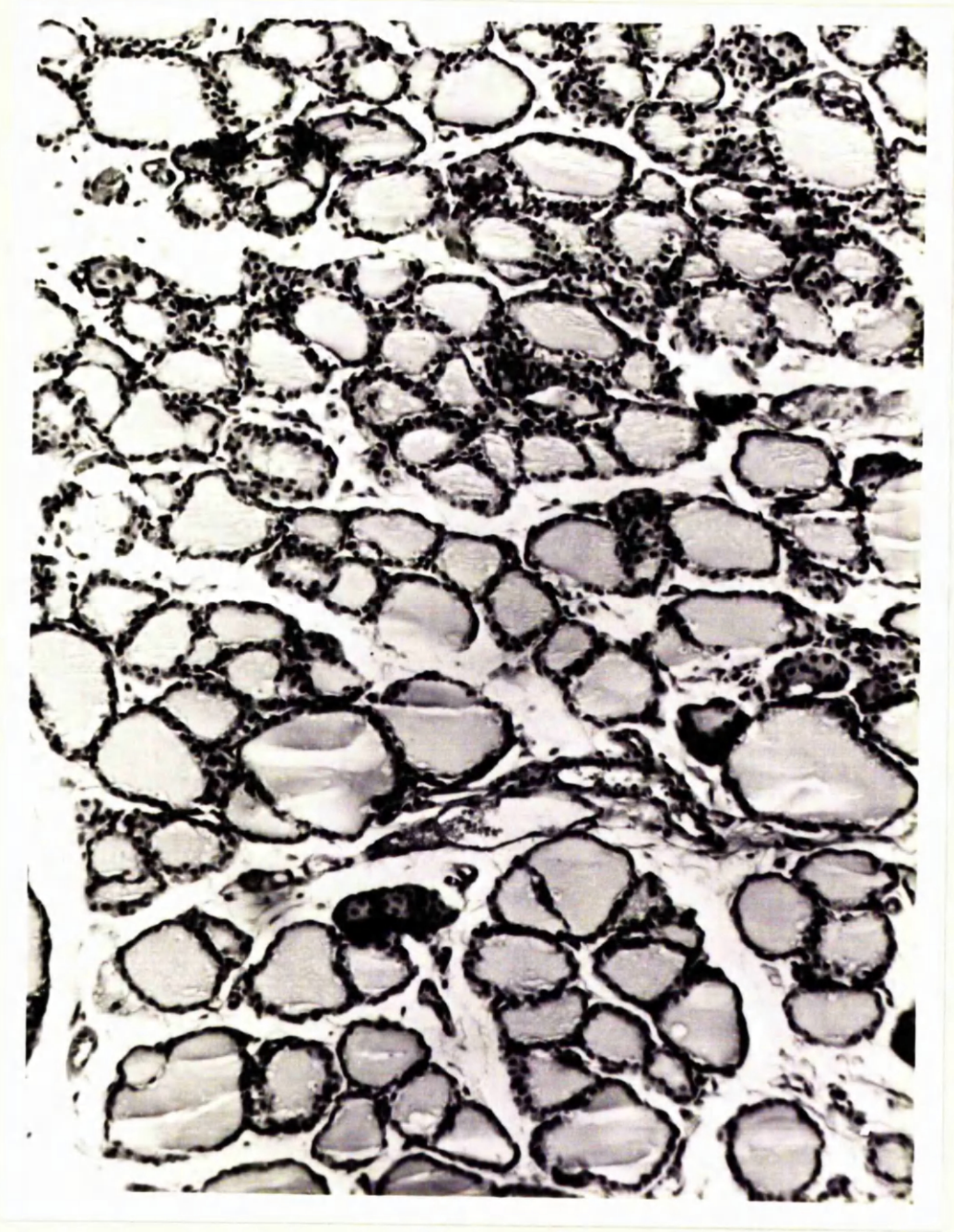

Fig. 1

Histological section of normal rat thyroid. H and E x 190.

detailed biochemical processes by which they are achieved are by no means well defined.

The steps are:

1. The trapping of iodide - this step is blocked by potassium perchlorate ($KClO_4$) and thiocyanate.

2. The conversion of iodide to some active form ?iodine ?iodonium ion - this step is thought to be achieved by a peroxidase enzyme system.

3. The iodination of tyrosine. This step is blocked by antithyroid drugs of the thiocarbamide group such as prophylthiouracil (PTU), carbimazole, methimazole as well as a variety of other drugs such as para-aminosalicylic acid (PAS), phenylbutazone, etc.

4. The coupling of iodotyrosine molecules to form T_4 and T_3 - this can only take place correctly when the following step is satisfied.

5. Synthesis of the peptic structure of thyroglobulin.

6. Breakdown of thyroglobulin by a proteolytic enzyme system.

7. Conservation of iodide released in the form of iodotyrosines during the proteolysis of thyroglobulin - this dehalogenase step involves splitting of the iodine from the

tyrosine residue and the recycling of the iodide in the biosynthetic cycle of the thyroid hormones.

Current thought is that steps 1, 2, 5, and 7 take place in the cell and that the other steps normally take place in the colloid.

The T_4 and T_3 are stored in the colloid of the thyroid follicles as part of the molecules of thyroglobulin. This is a complex iodinated glycopeptide which accounts for some 70-80% of the proteins formed by the thyroid follicular cells and which is characterised by its sedimentation properties in the ultracentrifuge in which it has a sedimentation constant of 19S.

Other proteins are present in the normal thyroid gland. These include both proteins heavier than 19S such as 27S and also lighter than 19S, i.e. 3-8S and occasionally 11-12S. There are certain species differences in the thyroid protein of the various animals examined so far. Guinea pigs, land turtles, dogfish and rabbits have appreciable quantities of a 12S protein normally present (Salvatore et al, 1965a); the predominent protein is however 19S. Recently however Aloj et al (1967) have found in the lamprey a species whose native thyroid protein was predominently a 12S with smaller amounts

of 5S and 17S protein also present.

CHAPTER 2

CHEMISTRY OF THYROGLOBULIN

Most of the recent work on this aspect of the study of thyroglobulin has been performed by Edelhoch and his co-workers. This work has been recently reviewed (Edelhoch and Rall, 1964; Edelhoch, 1965). This present review will concentrate on those aspects of the chemistry of thyroglobulin which are necessary for the understanding of the various proposed biosynthetic mechanisms.

a. Molecular weight of thyroglobulin This has been studied by ultracentrifugation, diffusion and light scattering techniques by Edelhoch (1960a). The results of these various estimations gave a molecular weight of the order of 2/3 million. This estimate is in agreement with that found by other workers.

b. Information derived from the breakdown of the thyroglobulin molecule using

1. pH Edelhoch (1960a) showed that altering the pH of a solution of calf thyroglobulin altered the molecular weight of the protein found. In the pH range 5.9-9.5 some 12S protein was present as well as 19S. At pH 9.5 a 15S and an 8S protein were found although 12S was the predominent

protein until pH 11 was reached. Up to this point the process was reversible on changing the pH back to the neutral region. At pH 12, 8S was the predominent unit and at pH 12.7 all the protein was present in the form of 3-4S units. This worker found a similar pattern of results by altering the ionic strength of the solvent buffer. Certain salts, e.g. KCNS, Hg^{++}, Cu^{++} and Ni^{++}, increased the rate of breakdown in neutral and alkaline solution (Metzger and Edelhoch, 1961).

2. Heat Edelhoch and Metzger (1961) also demonstrated a similar pattern of breakdown of the thyroglobulin molecule by mild heating in that the 19S molecule was broken down into two slower sedimenting components at a point before denaturation was reached.

3. Detergents The effect of sodium dodecylsulphate (SDS) was examined in a series of papers by Edelhoch and co-workers (Edelhoch and Lippoldt, 1960; Edelhoch, 1960b). They showed that low concentrations of this substance (<0.001M) dissociated thyroglobulin into 12S fragments which did not show changes in symmetry or frictional properties from the 19S material. At higher concentrations the thyroglobulin subunits become swollen

and showed changes in frictional properties. These changes were reversible on dialysing out the SDS. In a concentration of 0.015M SDS in 0.01M KHO_3 thyroglobulin was almost completely dissociated into its subunits.

4. Effect of betamercaptoethanol (BME)

de Crombrugghe et al (1965, 1966) investigated the changes in thyroglobulin after the reduction of the disulphide bonds by BME. They were able to demonstrate using this agent that the 12S molecule could be split into two equal sized particles of 6S size. Furthermore they also demonstrated that during prolonged dialysis the disulphide bonds could be reoxidised to reform 12S and 19S protein. This reoxidised thyroglobulin was more resistant to further reduction by BME than the native thyroglobulin and had similar but not identical immunological properties.

5. Proteolytic breakdown This aspect has been comparatively little studied in so far as the production of possible protein subunits as opposed to iodoamino acids has been concerned. The production of subunits in one study (O'Donnell et al, 1958) was found to be dependent on

the absence of salt. However in studies of the tryptic digestion of thyroglobulin (Metzger et al, 1962) although the digestion proceeded faster in the absence of salt, the pattern of results was similar; no components with sedimentation properties between 3S and 19S were found; the 3S components although not in themselves antigenic were able to inhibit the precipitation of native thyroglobulin with rabbit anti-thyroglobulin antibody.

The hydrolysis of thyroglobulin has also been studied using rat thyroid particles sedimenting at 100,000g (Pastan and Almqvist, 1965). The supernatant fraction was not effective. The process could take place at physiological pH but the presence of a salt and BME was needed for maximal activity suggesting that reduction of disulphide bonds was a necessary preliminary to the reaction. This system is different from the thyroid protease studied by other workers which is most active at an acid pH (Pitt-Rivers, 1963) and is stimulated by TSH and antithyroid drugs. Some light on this discrepancy has been shed by the demonstration of proteolytic activity at pH 7.8 in the supernatant and an acid protease in the thyroid particles (Laver and Trikojus, 1955; Weiss, 1953). Using a rat thyroid gland system Alpers et al (1956) were able

to demonstrate only small particles formed during the early stages of hydrolysis without the formation of larger molecules of the 12S size.

c. In vitro formation of thyroglobulin from presumed subunits by non-biological means As mentioned above, when thyroglobulin is broken down the fragments can recombine to form 12S and 19S proteins with some but not all the properties of the native 19S protein providing that the process of degradation stops short of the point of denaturation. Similar results have been shown by Goldberg and Seed (1965) who further demonstrated that the reaction was accelerated by the presence of carrier thyroglobulin and a source of iodine. Toi et al (1963) have also studied the synthesis in vitro of T_4 from thyroglobulin containing labelled mono- and di-iodotyrosine (MIT and DIT) when 4-hydroxy-3, 5 di-iodophenylpyruvic acid (DIHPPA) and an oxygenating system were present.

d. Thyroid proteins other than 19S Normally a 3-8S and a 27S protein are present in the thyroid gland. In certain animals a 12S protein is normally found. On occasion traces of a 13S and a 31-32S protein have been

found (Nunez et al, 1965a). A 16-17S protein is also present under abnormal conditions where iodination is interfered with (Seed and Goldberg, 1966; Nunez et al, 1966). The 3-8S fraction will contain the 6S protein formed in vitro from degradation of 19S protein if the 6S fraction is indeed present physiologically. This fraction however presents considerable difficulties in analysis as it also contains the serum proteins, haemoglobin and structural proteins of the thyroid cell. A 6S protein cannot therefore be isolated in pure form from the thyroid gland and studies on this protein have to be performed on degradation products of 19S protein. Examination of all the different proteins so far studied has shown that their amino acid content is similar (Spiro, 1961).

The protein of this group which has been most studied is the 27S protein (Salvatore et al, 1965b; Vecchio et al, 1966a). These workers have demonstrated common immunological properties of 27S and 19S proteins. The molecular weight of the 27S protein is approximately twice that of 19S and this would fit well with the original thoughts that this represented an aggregate of two 19S units. However Vecchio et al (1966a) have shown that under conditions which

do not break disulphide bonds, namely low ionic strength of buffer, alkaline pH and mild heat that they were able to obtain 19S, 12S and 6S from a pure 27S protein suggesting that it was probable that the 6S units were not in disulphide linkage but rather in a loose aggregation of 19S, 12S and 6S molecules.

e. Differences in iodine content of various thyroid proteins Although as mentioned above the various thyroid proteins show a similar pattern of amino acids there does exist substantial differences in the amount of iodine in each fraction. This was noted early on by Derrien et al (1948) who found that the iodine content of apparently pure thyroglobulin varied. This work has been borne out by more recent studies especially those involving the technique of DEAE cellulose column chromatography where the separation of the iodinated proteins of the thyroid appears to be dependent largely but not exclusively on their iodine content. Studies of this type by Robbins (1963) have demonstrated that beef thyroglobulin can be separated into three fractions by this technique. The fractions differ in their iodine content, the earliest eluting fractions having a lower iodine content than the starting material and the later eluting fractions

having a greater iodine content. This correlated well with the analysis of the iodo-amino acid content in that although the content of MIT was similar in all fractions the DIT and T_4 content increased in the later eluting fractions. The effect of iodination of the starting material in vitro was to cause the early eluting fraction to virtually disappear and the later eluting fraction to increase. Broadly similar results have been obtained by Bouchilloux et al (1964) and Pommier et al (1966) who further demonstrated that a 17S component manufactured in vitro by sheep thyroid slices in the presence of a drug which blocks tyrosine iodination could be artificially iodinated to a 20S component. Goldberg and Seed (1965) and Pommier et al (1966) have both demonstrated that artificially iodinated material is more resistant to the degradative effects of SDS than poorly iodinated material and the latter group have also confirmed that the MIT/DIT ratio is higher for the less iodinated more labile material. Simon et al (1966) have also shown that poorly iodinated thyroglobulin-like proteins are more readily broken down to a 12S protein by freezing and thawing. The 27S component has been found to have a significantly higher iodine content than 19S (Salvatore et al, 1965b) but this was not confirmed by the studies of Lissitzky (1965).

f. Carbohydrate structure of thyroglobulin This has been mainly studied by Spiro and Spiro (1965). Thyroglobulin contains approximately 10% carbohydrate and the above workers have demonstrated that this consists of galactose, mannose, N-acetyl glucosamine, sialic acid and fucose. Thyroglobulin from sheep, pig and calf had a similar constitution. However human thyroglobulin contained more carbohydrate due to increased amounts of mannose and glucosamine. Following pronase digestion it was found that two types of carbohydrate units were present; unit A consisting of 5 residues of mannose to one of N-acetyl glucosamine and having a molecular weight of 1050 and the other unit B, consisting of 3 residues of mannose, 5 of N-acetyl glucosamine, 4 of galactose, 2 of sialic acid and 1 of fucose, had molecular weight of 3200. It was calculated that there were approximately 9 A units and 14 B units in each thyroglobulin molecule. The B chain is thought to consist of several oligosaccharide chains with a terminal sialic acid or fucose residue linked to galactose which is in turn linked to N-acetylglucosamine. Analysis of the aminoacids present in the vicinity of carbohydrate favours the view that the glycopeptide linkages are probably through aspartic acid residues. Murthy et al (1965) found a glycopeptide in a pronase

hydrolysate of sheep thyroglobulin. This contained 60% of the total carbohydrate. The molecular weight was 2400 and it contained 0.5 residue of fucose, 2 of glucosamine, 5 of hexose and 1 of sialic acid. Thyroglobulin isolated on DEAE cellulose column chromatography has given discordant results Robbins (1963) finding that the later eluted fraction from a DEAE cellulose column contained more sialic acid than did the early eluting material and Bouchilloux et al (1964) finding no significant difference in the carbohydrate content of the various fractions. Human thyroglobulins from normal thyroid tissue and from non-toxic goitres were examined by Pierce et al (1965) who found no difference in carbohydrate content.

g. Summary of the chemistry of thyroglobulin The facts presented in the preceding sections although not decisive are consistent with the hypothesis that thyroglobulin (19S) is composed of subunits of 12S and 6S size. A 27S protein which is normally present is twice the molecular weight of 19S but the evidence available favours the view that it represents an aggregate of 19S + 12S + 6S subunits rather than two 19S fractions. Other proteins may be normally present in small amounts. Thyroglobulin contains at least 2 different carbohydrate

subunits linked by aspartic acid residues to the peptide chains. The iodine content of the thyroid protein varies being increased with increasing molecular size. There is considerable evidence that iodination is essential for the stability of the thyroglobulin molecule as well as being needed for the manufacture of thyroxine.

CHAPTER 3

BIOSYNTHESIS OF THYROGLOBULIN

a. Cell free synthesis

1. Incorporation of iodine This has been studied over a period of some years. In 1955 Taurog and colleagues demonstrated that whole thyroid homogenates and the 'mitochondrial' fraction were able to form protein bound MIT from iodine. The 'mitochondrial' fraction was most active and the cell sap was inactive. A note of caution with regard to naming subcellular fractions of thyroid tissue can be appreciated from the work of Ekholm (1961) who attempted to isolate a pure mitochondrial fraction from guinea-pig thyroid and found that in his best preparation there was as much as 50% contamination by microsomes. De Groot and Carvalho (1960) likewise found that sheep 'mitochondrial' and 'microsomal' fractions could form labelled protein bound ^{131}I when ^{131}I was added to the incubation medium. This reaction was heat labile and inhibited by catalase. The $PB^{131}I$ existed as MIT and DIT bound to an albumin-like protein immunologically distinct from thyroglobulin. In contrast to these studies Rappaport et al (1966) found that all cell fractions iodinated 19S thyroglobulin added to the incubating medium. Other

proteins, a 3-8S and a particle bound 10-12S protein were also labelled but the pattern of labelling favoured the view that they were not related to thyroglobulin. They proposed that in vivo thyroglobulin may be the only protein normally iodinated because iodination may occur in a site only accessible to the thyroglobulin molecule.

2. Incorporation of amino acids This has been the subject of a series of papers in the last few years. Singh et al (1964, 1965) showed that 'mitochondrial' fractions incorporated amino acids into protein in an energy dependent reaction which could be inhibited by actinomycin D and puromycin. They further showed that the 'microsomal' fraction shared the same properties but required the presence of cell sap and magnesium. The amino acid incorporation was not sensitive to actinomycin D or deoxyribonuclease suggesting that a stable messenger RNA was present. In these experiments the protein was not characterised. Likewise Nunéz et al (1965a) showed that ^{14}C tyrosine was able to be incorporated into TCA precipitable material in the presence of cell sap and an ATP generating system. After purification of the proteins they found that 3-8S and 19S material was formed. This reaction was inhibited by puromycin.

When the particulate fraction was extracted by digitonin only 3-8S proteins were found. Morais and Goldberg (1967) showed similar findings with the exception that in this instance 80% of the radioactivity was particle bound. The sedimentation pattern of the soluble protein showed that 20-35% of the material sedimented just short of 19S and the rest at 3-8S. Labelling of the thyroglobulin fraction continued after the radioactivity in the particulate fraction reached its peak suggesting that the latter was a precursor of the former. The labelled particulate protein when extracted by digitonin sedimented at 3-8S. Some differences in the incorporation of the various amino acids have been noted by Soffer and Mendelsohn (1966) who showed the arginine was handled in two different ways; one requiring the presence of ribosomes and cell sap; the other needing only cell sap was energy dependent and required a source of S-RNA but did not require magnesium ions and was not inhibited by puromycin. In a recent paper Cartouzou et al (1967) found that sheep thyroid polysomes incorporated ^{14}C leucine into TCA precipitable material when an energy source and cell sap were supplied. The protein formed was however immunologically distinct from thyroglobulin.

3. Incorporation of carbohydrate Cartouzou et al (1967) have further demonstrated the lack of incorporation of ^{14}C glucosamine and ^{14}C mannose by sheep thyroid polysomes.

b. Cell culture techniques Little work appears to have been done by this technique. Pulvertaft et al (1959) using human pathological material showed that iodine was incorporated into MIT in a protein bound form. In one patient T_3 and T_4 appeared to have been formed. In another study Raghupathy et al (1965) showed that sheep thyroid cell monolayers incorporated ^{131}I into a protein with a similar electrophoretic mobility as thyroglobulin, the reaction being enhanced by TSH. They further showed that although the 'mitochondrial' and 'microsomal' fractions contained protein bound iodine this did not seem to be related electrophoretically to thyroglobulin.

c. Thyroid slice techniques

1. Incorporation of iodine Using a slice technique it has been shown by Seed and Goldberg (1963) and Lissitzky et al (1964) that ^{125}I is rapidly incorporated into a thyroglobulin-like protein of approximately 18S size. In the studies of Seed and Goldberg no protein lighter than 18S size was iodinated. However Lissitzky et al found incorporation of ^{125}I into the light weight proteins of 3-8S size at early times of labelling. They furthermore showed that a/

protein of 12S size was also labelled. Seed and Goldberg (1965) showed that in the presence of propylthiouracil (PTU) in concentration of 10^{-3}M, ^{125}I incorporation into thyroglobulin was completely abolished although some incorporation into 3-8S protein persisted. This incorporation of ^{125}I was not inhibited by preincubation with actinomycin. At a similar concentration of PTU Nunez et al (1965c) found iodination of a 17S protein which they called prethyroglobulin. Both they (Nunez et al, 1965d) and Goldberg and Seed (1965) have demonstrated that iodination can result in the formation of a 19S protein from this 17S material.

2. Incorporation of amino acid This has been examined by various groups of workers (Seed and Goldberg, 1963, 1965; Lissitzky et al, 1964; Nunez et al, 1965a). All are agreed that labelled amino acids become incorporated into light weight proteins of 3-8S and 12S size before the label becomes incorporated into the 19S protein which is the pre-existing hormone. Using puromycin iodination can be shown to occur after the cessation of protein synthesis, and using actinomycin it was demonstrated that the RNA template for thyroglobulin synthesis must have a half life of at least 15 hours (Seed and Goldberg, 1963).

3. Incorporation of carbohydrate It has been shown (Spiro and Spiro, 1966) that labelled glucose could be incorporated by calf thyroid slices into a protein with similar electrophoretic and immunological characteristics as thyroglobulin.

d. Review of sites of incorporation of various constituents of thyroglobulin

A. Iodine This has given rise to the greatest degree of controversy as to whether iodination is a function of the colloid, the cell or both. The data favouring iodination taking place in the colloid is based mainly on autoradiographic evidence which indicates in the normal animal at any rate that radioactive iodine is found in the colloid within minutes as opposed to approximately 4 hours using labelled amino acids (Nadler et al, 1964). There is also considerable evidence that iodination takes place at the 17-18S stage i.e. when the polypeptide chain is completed. This was well illustrated by the data of Seed and Goldberg (1965) who showed that puromycin did not block iodination of thyroglobulin. Sellin and Goldberg (1965) found no evidence of ^{125}I incorporation into thyroid cell particles in higher specific activity than in the cell sap. On the other hand there are reports favouring the view that, at

least in certain abnormal conditions iodination is a function of the thyroid epithelial cell. Such suggestions have been made as the result of studies on a rat thyroid tumour in which the thyroid particles accumulated a large percentage of the administered radioiodine (Robbins et al, 1959); from autoradiographic studies in normal rats given stable iodine in addition to radioiodine and in rats who have been hypophysectomised (Pitt-Rivers et al, 1964); and from studies of autoradiographs of human dyshormonogenetic goitre of the iodoprotein and dehalogenase type (Kennedy, 1965). Such autoradiographic studies have been criticised but in the large series of human thyroid material quoted above the appearance of epithelial iodination was only seen in two types of very hyperplastic goitre in which intrafollicular colloid is typically scanty and was not seen in a variety of other thyroidal states. There is also evidence from comparative embryology that the formation of the thyroid hormones precedes the formation of thyroid follicles (Rankin, 1941; Koneff et al, 1949). Pulvertaft et al (1959) have suggested from their experience with isolated thyroid cells which bind iodine that this may be a function which cells possess but which is usually latent except in the absence of colloid. Nunez et al (1965d) have

suggested on the basis of their work that there might be two different sites of iodination, one in the cell and one in the colloid. Recently Benabdeljlil et al (1967) using a technique by which they claim to be able to isolate the apical poles of sheep thyroid cells have provided evidence that these portions had marked iodinating ability and have suggested that this is the physiological site of iodination in vivo.

B. Amino acids There is general agreement that amino acid incorporation into thyroglobulin is a function of the epithelial cells and is associated with the thyroid cell particles (Sellin and Goldberg, 1965). As mentioned previously it is difficult to obtain a pure microsomal fraction which probably accounts for some of the accounts of the activity of the 'mitochondrial' fraction in amino acid incorporation as opposed to the microsomal fraction which would appear more probable on the basis of current theories of molecular biology. Nunez et al (1965a) differ from Sellin and Goldberg (1965) in that the former group found high specific activity of tritiated leucine in the 3-8S and 12S as well as 19S fractions isolated from the thyroid cell particles whereas the latter group found only a highly labelled 19S protein. However,

the techniques used were disimilar in that Nunez et al extracted the particulate proteins with digitonin and deoxycholate and Sellin and Goldberg used an ultrasonic disruption technique.

C. Carbohydrate Spiro and Spiro (1966) have provided data that the incorporation of carbohydrate into thyroglobulin is also particle associated and from studies with puromycin have suggested that the incorporation of carbohydrate occurred after the synthesis of the polypeptide chain and occurred in a stepwise fashion. Bouchilloux and Cheftel (1966) have confirmed that the synthesis of the polypeptide chain occurred before the incorporation of carbohydrate and seemed to be associated with the rough endoplasmic reticulum.

Summary of Thyroglobulin Biosynthesis

From the information thus available it would appear that amino acids are incorporated into thyroglobulin subunits of 3-8S and 12S size before thyroglobulin itself is formed. This incorporation takes place in the thyroid cell particles as does the incorporation of the carbohydrate moiety which appears to be the next stage of carbohydrate synthesis. Iodination of thyroglobulin now follows. The site of this is controversial but the weight of evidence is that this occurs usually at the edge

of the colloid although there is evidence that under certain abnormal conditions iodination may occur in the thyroid epithelial cells, suggesting that the mechanisms of cellular iodination are normally present but perhaps are active only in conditions where there is cell hypertrophy and/or loss of colloid.

Aims of the Present Study

The data presented in this work were designed to extend the study of the *in vitro* biosynthesis of thyroglobulin to the *in vivo* situation in normal and goitrogen treated rats. Some limited studies of biosynthesis of thyroglobulin in different vertebrates were made. A series of human pathological thyroid glands were also examined to see the protein patterns obtained in diseased states and to compare the result of this with the histological examination of the resected material.

SECTION 2

MATERIAL AND METHODS

Chapter 4	Introduction
Chapter 5	Materials
Chapter 6	Dietary regimes
Chapter 7	Experimental techniques

CHAPTER 4

INTRODUCTION

It is proposed in this section to give a general account of the methods used throughout the study. At various times alterations to the standard techniques were used or alternative techniques substituted to try to shed further light on particular aspects of the point under study at that time. These changes to the standard methods will be dealt with in the appropriate section.

CHAPTER 5

MATERIALS

Male Sprague-Dawley rats of approximately 150G weight were used. They were obtained from either the Charles River Corporation, Cambridge, Mass., U.S.A. or from A.J. Tuck and Son, Ltd., Rayleigh, Essex, England.

Propylthiouracil (PTU) was obtained from the Aldrich Chemical Co., or from L. Light and Co. Ltd.; methylthiouracil (MTU) from British Drug Houses Ltd.; methimazole was obtained from the Aldrich Chemical Co.; potassium perchlorate ($KClO_4$) from either Fisher Scientific Co., or from British Drug Houses Ltd.; carbimazole was a gift from Nicholas Laboratories Ltd.; thyroxine (T_4) or triiodothyronine (T_3) were obtained from the Sigma Chemical Co., or from Koch-Light Laboratories Ltd.; cycloheximide was a gift from the Upjohn Co.; L-leucine-4, 5 - ^{3}H (SA 5Ci/mole) was obtained from the New England Nuclear Corporation or in Britain from the Radiochemical Centre, Amersham (SA 250-1000mC/mole); carrier free ^{125}I was obtained from either the New England Nuclear Corporation, or from the Radiochemical Centre, Amersham.

CHAPTER 6

DIETARY REGIMES

The basic diet fed to the rats throughout the study was the low iodine test diet (powdered form) obtained from the Nutritional Biochemical Corporation, Cleveland, Ohio, U.S.A. This diet was supplemented for experimental purposes as follows:

Control rats - basic diet supplemented with 5mg potassium iodide (KI)/k diet.

PTU treated rats - basic diet supplemented with 5mg KI + 0.2G PTU/k diet.

PTU + T_4 treated rats - as for the PTU treated group, but + 2mg T_4/k diet for the last week before sacrifice.

Methimazole treated group - basic diet supplemented with 5mg KI + 1500mg methimazole/k diet.

Carbimazole treated group - basic diet supplemented with 5mg KI and 1500 carbimazole/k diet.

$KClO_4$ treated group - basic diet + 20G $KClO_4$/k diet.

Low iodine diet group - unsupplemented basic diet.

These diets were given for a period of three weeks except for the low iodine diet which was given for times varying from 3-16 weeks.

The low iodine diet and the $KClO_4$ groups received distilled water to drink, the remaining groups received tap water. In some experiments animals received additional iodine supplementation in their drinking water for one week before sacrifice. This was given as KI 0.05% in distilled water unless otherwise stated.

CHAPTER 7

EXPERIMENTAL TECHNIQUES

Rats were injected with ^{3}H-leucine in a dose of 400-500μc/animal for control rats or 100μc/animal for a rat on a goitrogenic regime. The injection was given subcutanesouly into the loose tissues at the back of the neck. When ^{125}I was used a dose of 10-25μc/animal was injected intraperitoneally.

At the appropriate time after the administration of either isotope the animals were killed by exsanguination under ether anaesthesia. The thyroids were rapidly removed and dissected free of adherent fibro-fatty tissue. The thyroids were homogenised in ice cold phosphate buffered saline (PBS) (0.15M sodium chloride in 0.01M potassium phosphate, pH 6.8) using a TRI-R STIR-R fitted with a glass homogenising tube with a teflon pestle. The homogenising tube was kept in crushed ice during the homogenisation process. As few strokes of the pestle were used as was consistent with good disruption of the tissue. As a general rule a goitrous gland was more friable than the thyroid gland from a control rat. The homogenate was spun in a refrigerated centrifuge at 15,000r.p.m.(20,000G) for ten minutes to remove

cellular debris. The supernatant solution was taken to 50% saturation with ammonium sulphate. The mixture was kept on ice for one hour after which it was again centrifuged at 15,000r.p.m. for ten minutes. The supernatant was discarded, the centrifuge tube inverted to drain off as much of the ammonium sulphate solution as possible and the inside of the tube blotted.

When human material was under study a broadly similar programme was followed. Tissue was collected fresh from the operating theatre. It was transported from the operating theatre to the laboratory in a plastic bag on ice inside a vacuum flask. On arrival in the laboratory the specimen was placed in a Petri dish containing a small amount of ice cold PBS. The Petri dish was kept on ice. After dissecting off any fibro-fatty tissue, thin slices of thyroid tissue were made by hand using a razor blade. Some slices were taken immediately for homogenisation and precipitation of the thyroid proteins with ammonium sulphate as described above for the rat thyroid gland. Other slices (approximately 300mg tissue per flask) were incubated in 4ml of a modified Krebs no. 2 buffer (Bisset and Alexander, 1960), pH 7.4 with glucose (1mg/ml) in a small Erlenmeyer flask containing either 20μc

^{3}H-leucine or 20μc ^{125}I. These incubations were performed at 37°C in a Gallenkamp metabolic shaker under an atmosphere of oxygen for a period of four hours as a routine. This time of incubation was selected on the basis of previously published work (Seed and Goldberg, 1965) as being a time when labelled amino acids should be incorporated into thyroglobulin. In certain cases incubation times less than four hours were selected. At the end of incubation the flask contents were homogenised and dealt with as above.

Specimens from animals other than rats and human operative specimens were obtained by sacrifice of other laboratory animals, such as hamsters, rabbits. In these cases the procedures used were similar to that used in the rat. In the case of larger animals, such as sheep, cow, pig, the specimens were collected fresh from the slaughterhouse and transported as in the case of the human operative specimens. Some specimens of thyroid tissue from cat, dog, horse, were obtained at post-mortem examination at the Veterinary Hospital, Garscube, as soon as practicable after death, were transported to the laboratory on ice and dealt with as above.

The proteins precipitated by ammonium sulphate were redissolved in as small a volume of PBS as possible. As far as

possible the specimens were examined immediately by gradient ultracentrifugation. In a few cases this was not practicable and the undissolved specimen was stored in the deep freeze until it could be examined.

The protein solution was applied to a 5-20% sucrose in PBS gradient made using a Beckman gradient former. The specimens were spun in a model L, a model L2-50, or a model L2-65B Beckman ultracentrifuge. The rotor used, the speed and the time of ultracentrifugation used as a routine, were as follows:

a. SW 25.1 rotor at 21,000r.p.m. for 40 hours. This was used for most of the early rat experiments described in Section 3.

b. SW 39 rotor at 24,000r.p.m. for 16 hours.

c. SW 41 rotor at 28,000r.p.m. for 16 hours.

Techniques b. and c. were used for the bulk of the other experiments.

At the end of the period of ultracentrifugation, the pattern of the thyroid proteins was determined by aspiration from the bottom of the ultracentrifuge tube and passage through either a Gilford 2000 or a Beckman DB automatic absorbance recorder at 280μ. Fractions were collected, the number of drops

depending on the size of the gradient, but selected so that approximately 40 samples were collected from each gradient. The samples were normally collected into polyethylene counting vials and counted in a Packard tricarb liquid scintillation counter after the addition of 10ml Bray's solution. In some studies, however, the samples were collected in glass test tubes in order that the contents of various tubes could be pooled for the estimation of the protein content by the method of Lowry et al (1951), or the iodine content either by the method of Farrell and Richmond (1961) or latterly (by courtesy of the Biochemistry Department, Royal Infirmary, Glasgow) by use of the Technicon automated procedure.

As a routine a specimen of sheep thyroglobulin was spun in each rotor for comparison with the position of the various protein peaks. The sedimentation constant of the main optical density (OD) peak at 280μ was taken as 19S for reference purposes. In some experiments the position of the protein peak in the thyroglobulin region was checked by spinning some unlabelled thyroid proteins from the test material in the same ultracentrifuge tube as a trace amount of ^{125}I-labelled thyroglobulin from a control animal injected 48 hours before sacrifice at which time it was known to be incorporated into the 19S protein (see Section 3).

The sedimentation values given in this work are those found by either, or both, the above methods. It is appreciated that neither method is exact and that differences of fractions of an S unit (between two individual experiments) are probably not significant. However, the abnormalities reported have been reproduced on enough occasions to make the differences reported significant.

In a representative selection of the animal and human material an attempt was made by planimetry, using an Albrit planimeter, to quantitate the amount of protein in each peak. This proved fairly easy in a non-goitrous gland, but where dealing with a goitrous gland it proved difficult to completely separate the protein peaks from each other and the data present by this method is to be regarded as semi-quantitative only, especially when applied to goitrous material.

SECTION 3

INCORPORATION OF ^{3}H-LEUCINE AND ^{125}I INTO RAT THYROGLOBULIN

CHAPTER 8

INTRODUCTION

The object of this part of the study was to try and demonstrate the incorporation of labelled proteins presumed to be precursors of thyroglobulin into the 19S protein. Conditions of ultracentrifugation were therefore chosen so that the 19S protein was found in the lower half of the gradient at the end of the run. These conditions, however, necessarily result in the crowding together of proteins heavier than 19S. The OD tracings in this section do not, therefore, show the typical three peaks of a normal rat thyroid protein namely 19S, 3-8S and 27S. The distribution of the stable proteins in the rat is dealt with in a subsequent section where different conditions of ultracentrifugation were utilised.

CHAPTER 9

THYROGLOBULIN BIOSYNTHESIS IN CONTROL RATS

^{3}H-leucine incorporation. Control rats were injected subcutaneously with a pulse of ^{3}H-leucine and were sacrificed at intervals of 30 minutes to 72 hours later. The results are shown in fig. 2.

At early time intervals (30 minutes) the leucine was predominately incorporated into proteins lighter than 19S and in fact the predominant peak was a broad band of radioactivity in the 3-8S region. The next largest peak was that in the 12S area. There was, however, a small but well defined peak in the 17S region with a broad tail of radioactivity extending over the thyroglobulin region. At one hour the specific activity (SA) of the 12S peak had fallen, whereas that of the peak running short of the 19S OD peak had increased and had shifted to approximately the 18S position. The SA of the 3-8S remained static. At four hours the labelled peak in the thyroglobulin area was now the predominant one, the SA of the 12S and 3-8S peaks having remained static. At 24 hours the SA of all three peaks had fallen and by 48 hours the 12S peak could no longer be seen. It should be noted that at 48 hours for the first time the labelled peak in the thyroglobulin area corresponded with

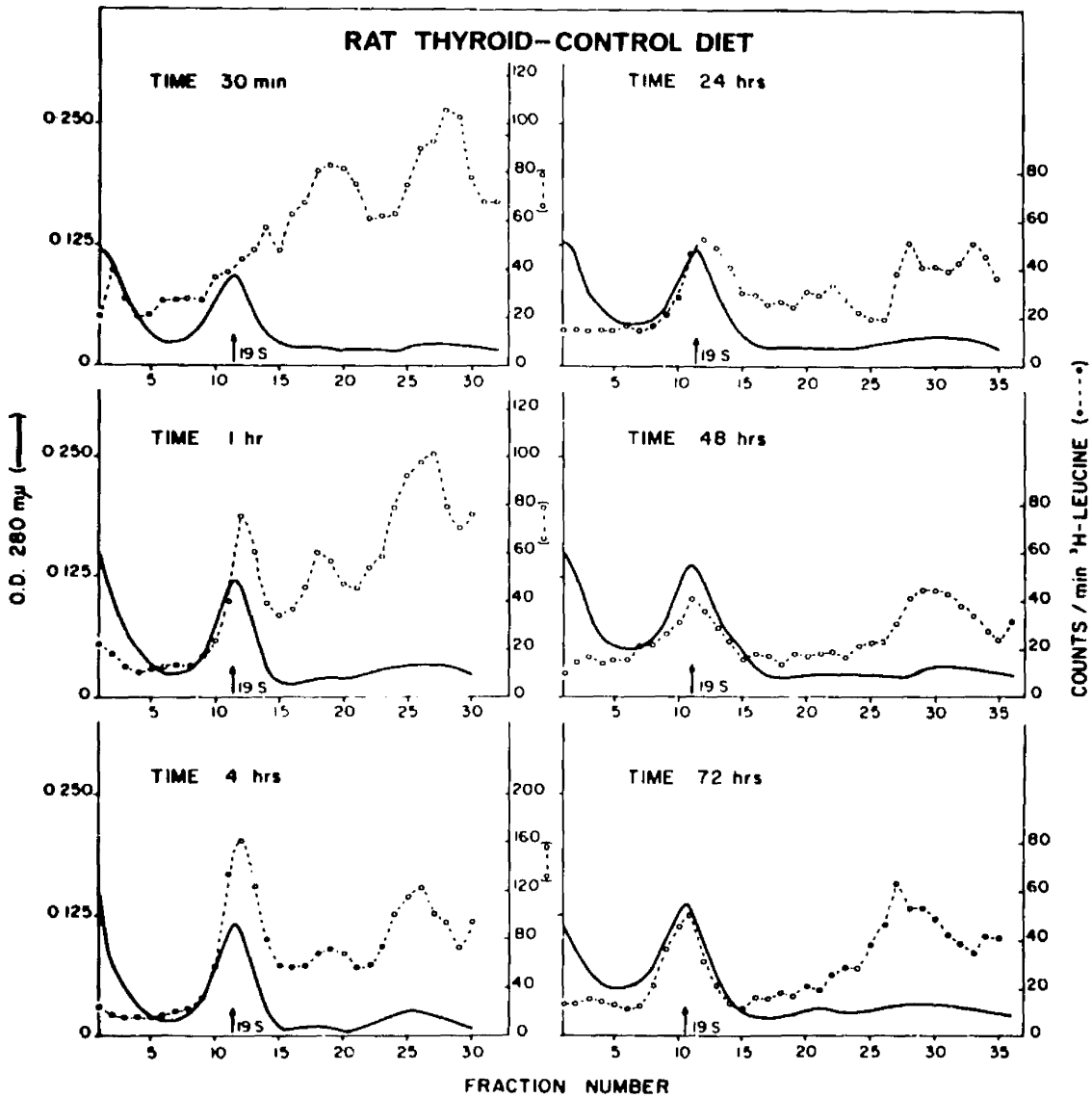

Fig. 2

Pattern of incorporation of ^{3}H-leucine into control rat thyroid. SW 25.1 rotor at 21,000r.p.m. for 40 hours. In this and all subsequent OD tracings the top of the gradient is on the right of the figure and the bottom on the left.

the OD peak of the thyroid proteins. At 72 hours the findings were similar to that at 48 hours with, if anything, a slight increase in the SA of the 3-8S peak.

In another experiment (fig. 3) two of a batch of 6 rats were killed half an hour after all were injected with ^{3}H-leucine to provide a picture of the degree of incorporation of leucine into thyroglobulin and its subunits at this time. In this particular group of rats the leucine was incorporated approximately equally into the 19S, the 12S and the 3-8S proteins at this time. Two of the four remaining rats received intraperitoneally 20mg of cycloheximide dissolved in normal saline at 30 minutes after the injection of the ^{3}H-leucine. This dose of cycloheximide had been shown by previous experiments to block further incorporation of ^{3}H-leucine into the rat thyroid proteins. The two animals injected with cycloheximide were killed along with the two remaining control animals two hours later (i.e. $2\frac{1}{2}$ hours after the start of the experiment). As can be seen in fig. 3 there was a shift of the radioactive peaks from 3-8S and 12S areas to the thyroglobulin area in both groups.

^{125}I incorporation. The pattern of incorporation of ^{125}I in vivo is entirely different from that of ^{3}H-leucine. As shown in fig. 4 at all times the predominant labelled peak is in the thyroglobulin

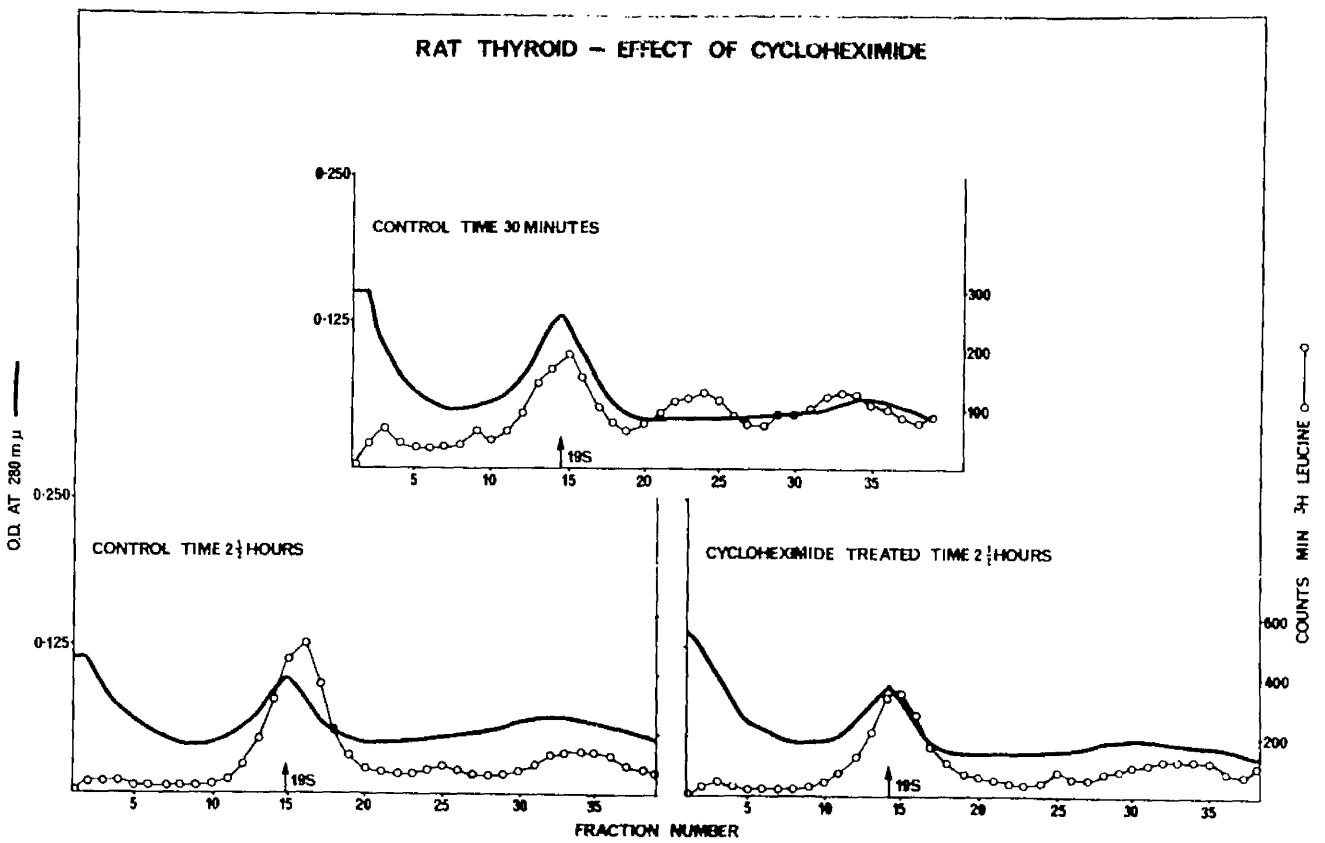

Fig. 3

Pattern of incorporation of ^{3}H-leucine into control rat thyroid - effect of cycloheximide. SW 25.1 rotor at 21,000r.p.m. for 40 hours.

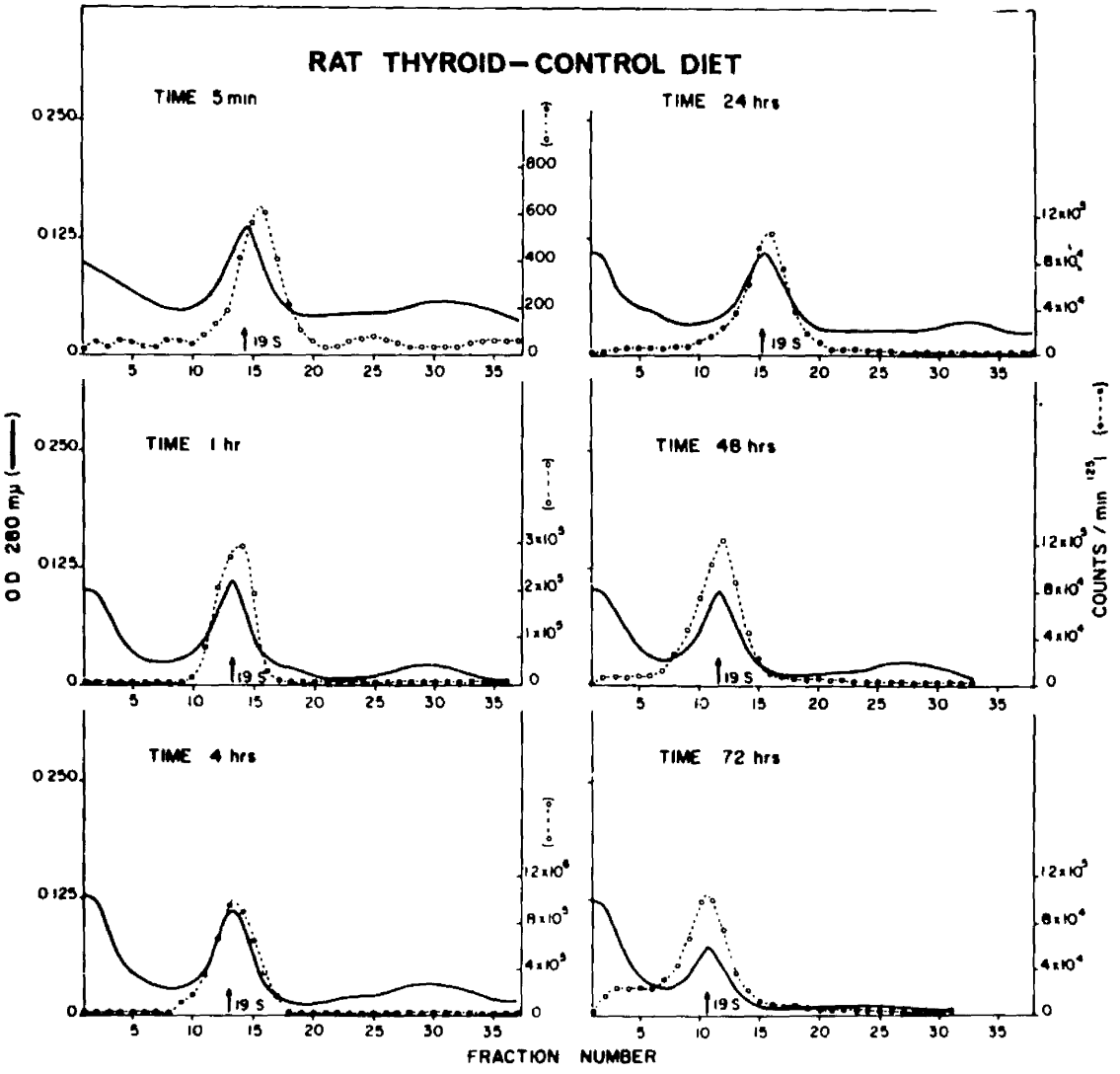

Fig. 4

Pattern of incorporation of ^{125}I into control rat thyroid. SW 25.1 rotor at 21,000r.p.m. for 40 hours.

region although at time intervals of up to 48 hours this peak has an S value of slightly less than 19S. At, and after, 48 hours the OD peak and the radioactive peaks coincide. The SA of the labelled peak in the thyroglobulin region steadily increases to reach a peak at 4 hours and to steadily decline thereafter over the time of the experiment. At very early time intervals after injection of ^{125}I (1-5 minutes) a small amount of labelled 12S protein was found; no ^{125}I was ever found associated with the 3-8S peak. It should, however, be emphasised that the labelled peak in the thyroglobulin region was always the predominant peak and at no time did the radioactivity in the 12S peak appear in more than trace amounts, in contrast to the situation with ^{3}H-leucine.

CHAPTER 10

THYROGLOBULIN BIOSYNTHESIS IN RATS MADE GOITROUS BY ANTITHYROID DRUGS OR LOW IODINE DIET

a. Effect of propylthiouracil (PTU). The administration of PTU and other goitrogenic drugs resulted as anticipated in the formation of a goitre in the rats. Table 1 indicates the size of the resultant glands. Fig. 5 shows the histological appearance of a rat thyroid treated by PTU. This should be contrasted with fig. 1 which shows a normal rat thyroid. (The other goitrogenic regimes gave a very similar histological picture and are omitted to avoid repetition.) The obvious features which are present are that there is in a goitrous gland a marked increase in follicular cell height and a marked loss of colloid as compared to the control animal. The size of the thyroid follicles is also increased.

^{3}H-leucine incorporation (fig. 6). There is a marked change in the OD pattern of the thyroid proteins. This will be discussed in detail in a later section, but for the present it is sufficient to note that there is a striking increase in the protein peak in the 3-8S area - this is now the predominant peak. A definite peak still occurs in the thyroglobulin region, but now falling short of 19S

Table 1:

Rat thyroid
Thyroid weights on various dietary regimes

Type of diet	No. of animals	Thyroid wt. (mg) Mean	Range	S.D.
Control	53	9.8	7-12	±1.5
PTU	30	31.4	25-40	±7.2
PTU + T_4	26	9.4	6-13	±1.8
Methimazole	24	28.9	24-35	±5.4
$KClO_4$	24	25.7	20-33	±5.8
Low iodine (3 weeks)	26	12.2	8-19	±2.3
Low iodine (6 weeks)	36	30.3	23-40	±6.9

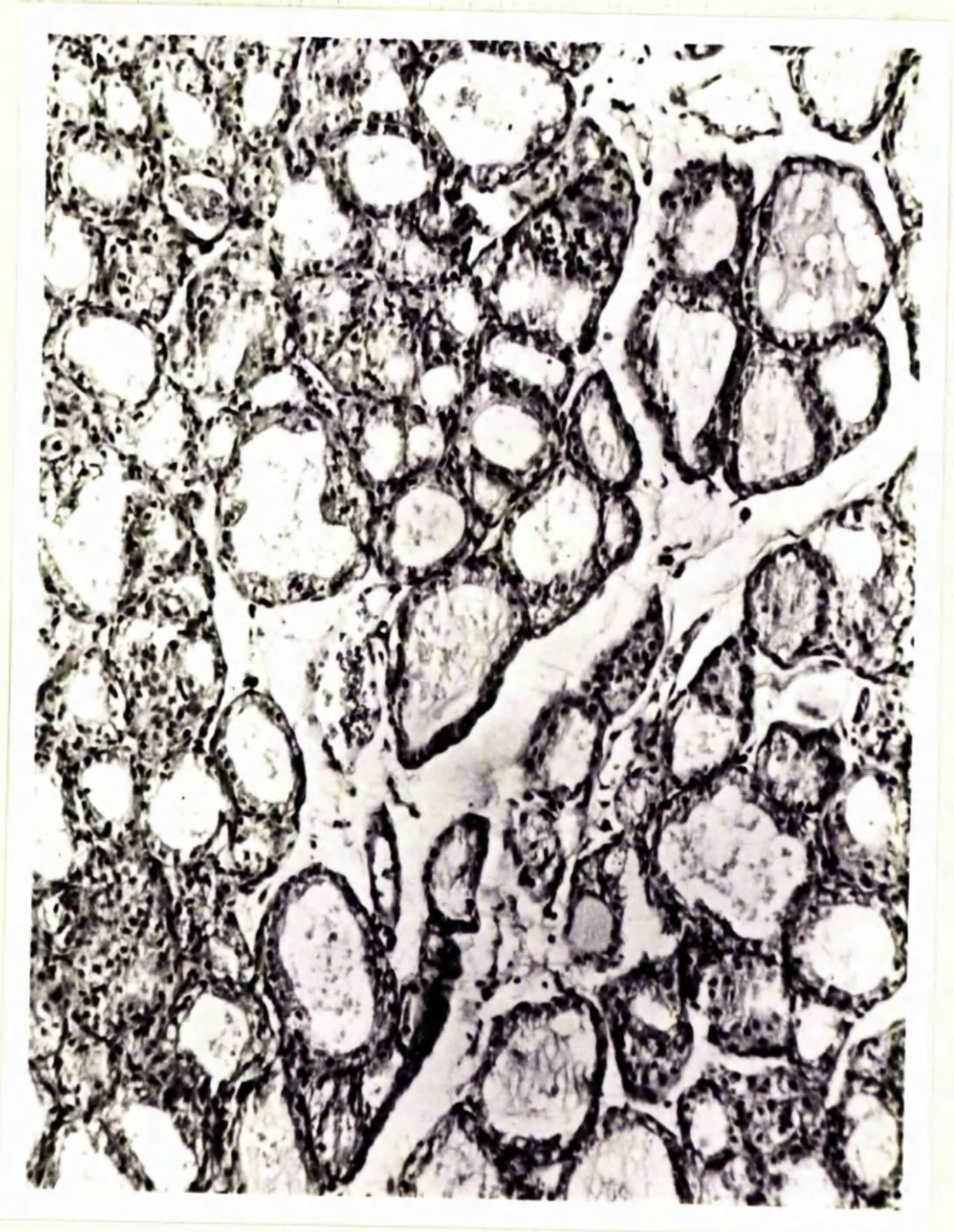

Fig. 5

Histological section of thyroid of PTU treated rat. H and E x 190.

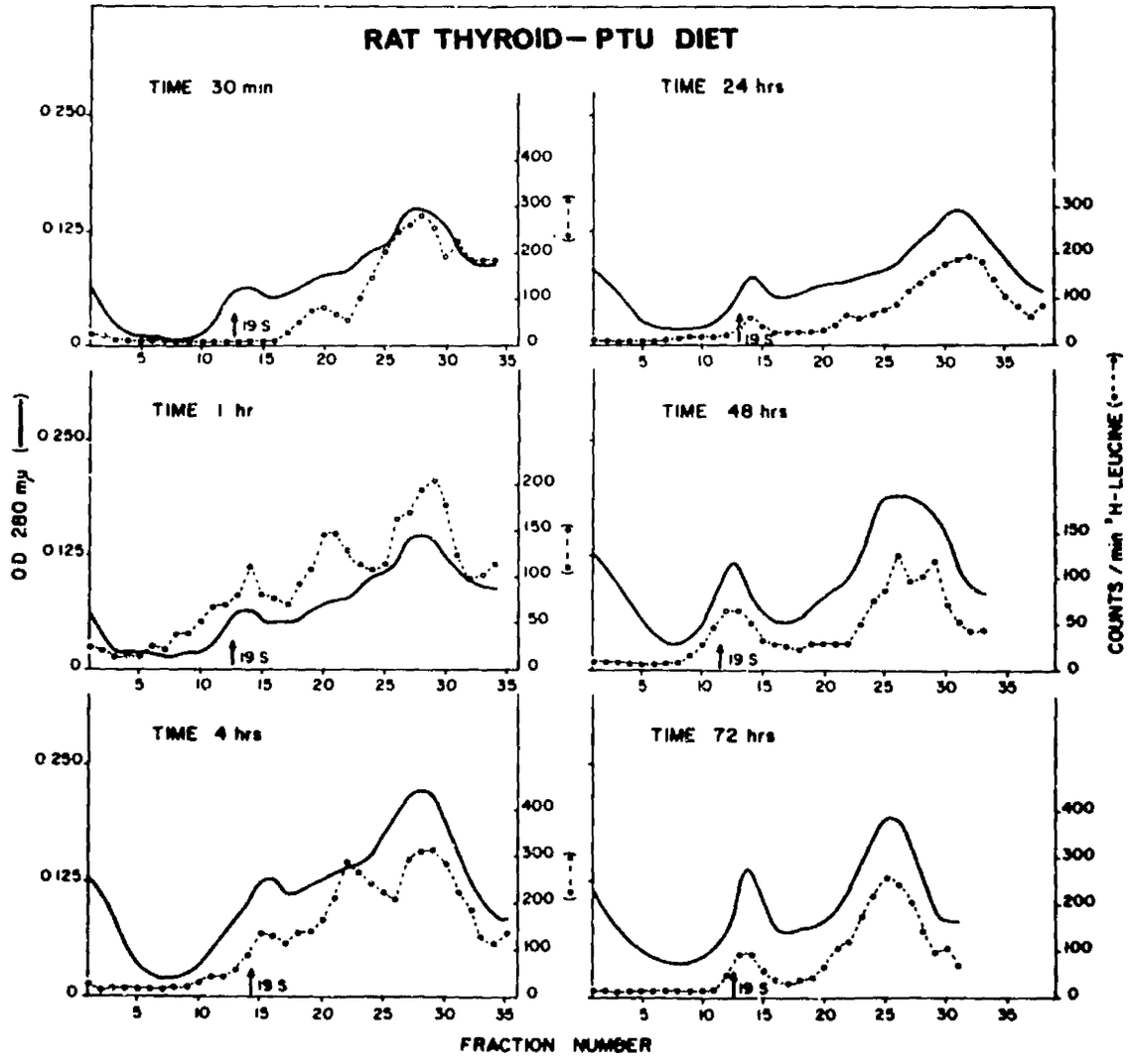

Fig. 6

Pattern of incorporation of ^{3}H-leucine into PTU treated rat thyroid. SW 25.1 rotor at 21,000r.p.m. for 40 hours.

and running in approximately the 18S position. Significant protein was present between the 3-8S peak and the 18S peak and in some of the tracings a definite 12S protein peak could be seen.

The pattern of ^{3}H-leucine incorporation was broadly similar to that found in the control animals. Significant differences were however noted. At 30 minutes the main incorporation of leucine was into the 3-8S peak with a smaller amount incorporated into a 12S protein. In contrast to the control animal there was no incorporation of the label into proteins larger than 12S at this time. At one hour the pattern of incorporation was reminiscent of that of the control animals at 30 minutes; the predominant peak was still the 3-8S peak although the SA of this peak had fallen. The 12S peak had increased relative to the 3-8S peak and its SA had approximately doubled. For the first time a labelled peak in the thyroglobulin region (although running short of the stable peak, which itself was short of 19S) was seen. At 4 hours the 3-8S and 12S peaks were approximately equal in specific activity, the SA of the latter peak in particular having increased markedly. The peak in the thyroglobulin region now corresponded to the OD peak, but it should be noted never truly

reached the 19S position. At 24 hours the SA of all peaks had fallen markedly - the 3-8S peak was again the predominant one; the 12S peak was only just discernible and the peak in the thyroglobulin region was small. At 48 hours the 12S peak could no longer be seen; the SA of the 3-8S peak had fallen further, although at 72 hours this peak did in fact increase in SA.

In summary, the main differences between ^{3}H-leucine incorporation into control and PTU treated animals were that the labelled peak in the thyroglobulin region appeared later in the goitrous animals and never became the predominant peak; this peak never became truly 19S, although it coincided with the OD peak at 4 hours, as opposed to 48 hours in the control animals; the 12S peak was more prominent in the goitrous animals, as was the 3-8S peak, especially at 72 hours after the pulse of leucine.

^{125}I incorporation. In fig. 7 can be seen the pattern of ^{125}I incorporation while on PTU one hour after administration of a pulse of ^{125}I. As could be anticipated, very little of the ^{125}I was specifically incorporated into the thyroglobulin region. Most of the ^{125}I merely appears as a broad band over the top of the gradient almost certainly representing diffusion of

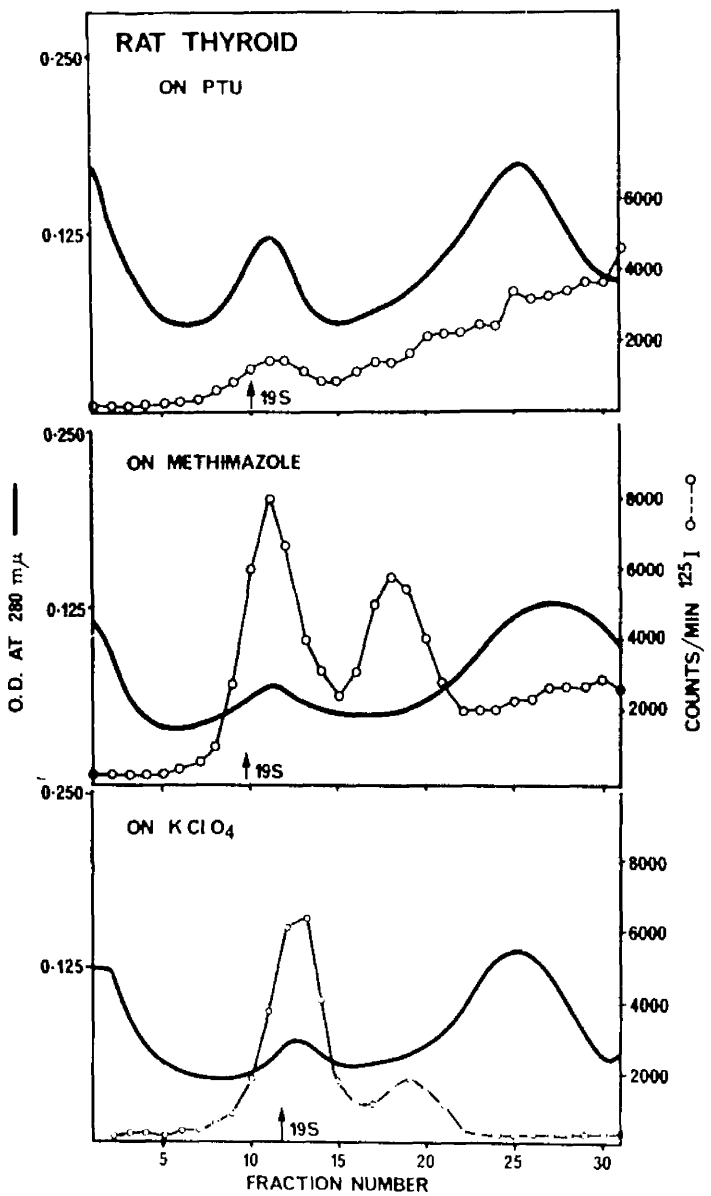

Fig. 7

Pattern of incorporation of ^{125}I into the thyroid proteins of the rat treated by a) top, PTU treated b) middle, methimazole treated c) lower, $KClO_4$ treated. SW 25.1 rotor at 21,000r.p.m. for 40 hours.

iodine and not due to the specific incorporation of the label into any particular protein.

b. Effect of methimazole. This drug has a similar effect to PTU on the thyroid, i.e. it results in the blocking of iodination of tyrosine. The histological changes and the effect on the OD patterns of the thyroid proteins are similar to those found with PTU.

^{3}H-leucine incorporation (fig. 8). In this instance at 30 minutes the 3-8S and the 12S peaks are of similar SA; there is a small labelled peak running short of the OD peak in the thyroglobulin region. By one hour this peak had become the predominant peak and now corresponded to the OD peak; the SA of the 12S and 3-8S peaks had, however, increased in addition. By four hours the peak in the thyroglobulin region was still the predominant one, but the SA of all three peaks had fallen. At 24 hours the SA of the peak in the thyroglobulin region and the 3-8S peaks had fallen further and the 12S peak could no longer be definitely distinguished; the 3-8S peak was now just the predominant one. At 48 hours, although a further fall in SA of both the 3-8S peak and the peak in the thyroglobulin area had resulted, the peak in the 3-8S area was now definitely predominant and

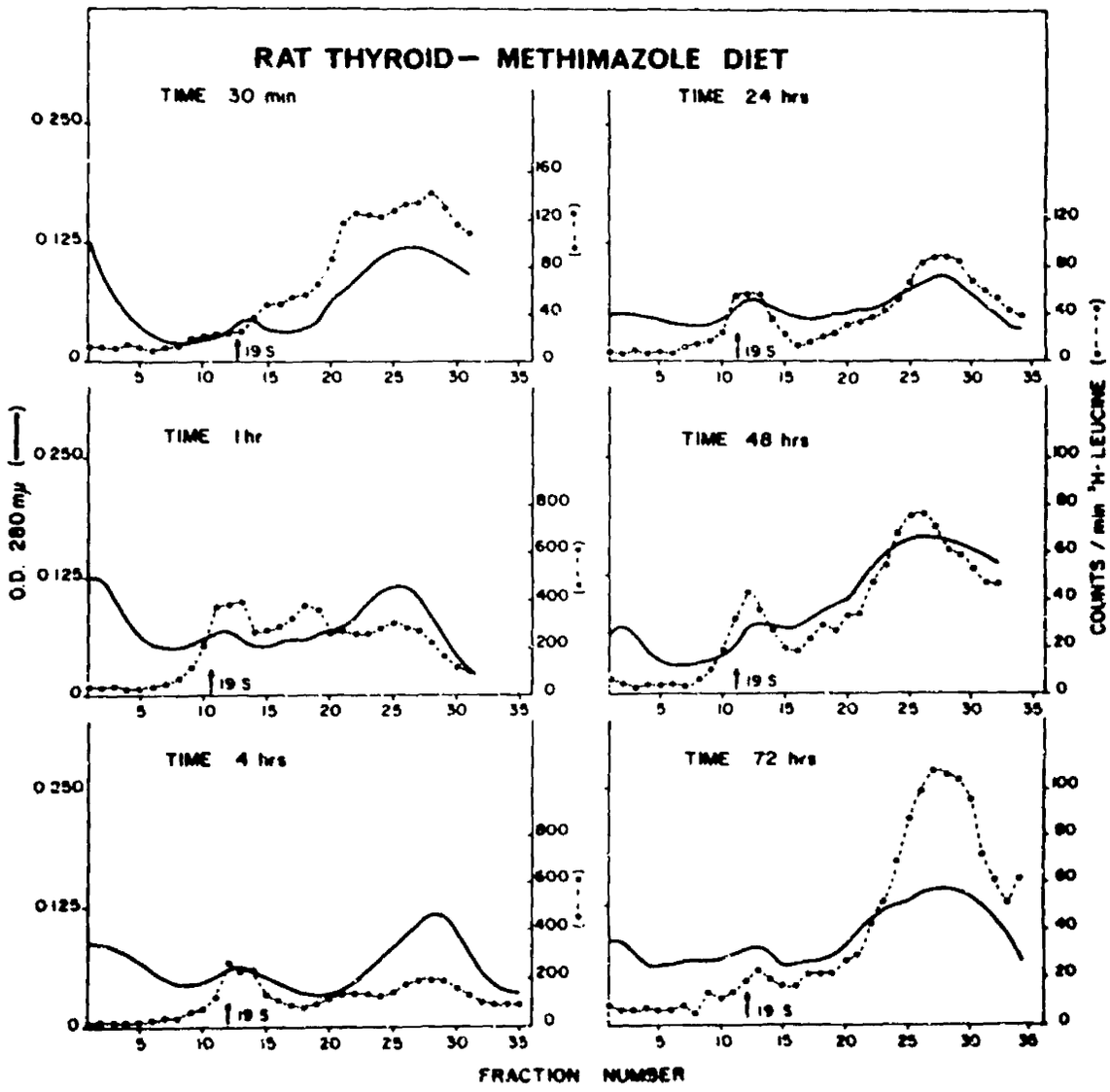

Fig. 8

Pattern of incorporation of ^{3}H-leucine into methimazole treated rat thyroid. SW 25.1 rotor at 21,000r.p.m. for 40 hours.

this became increasingly noticeable at 72 hours.

In summary, therefore, the results from the methimazole treated rats were similar to those of the PTU treated animals, although in the methimazole group the labelled peak in the thyroglobulin region became the predominant one at an early stage, whereas that of the PTU treated rats never became predominant. In this respect the methimazole treated glands tended to resemble the control glands although, like the PTU treated gland, the labelled peak in the 19S area never quite reached the 19S position. In addition, like the PTU treated animals, the 3-8S peak was present in markedly increased amounts relative to the peak in the thyroglobulin region at long time intervals after administration of the pulse of leucine.

^{125}I incorporation. As shown in fig. 7 ^{125}I given one hour before sacrifice was incorporated into the thyroid proteins of the methimazole treated rat. In contrast to a PTU treated animal, the bulk of the isotope present was incorporated into the thyroglobulin region. A significant amount of labelled 12S protein was also present. There was no incorporation of the isotope into 3-8S protein. It should be noted however that the methimazole treated animals have incorporated only approximately 1% of the total counts that the control animals

incorporated into the thyroglobulin region.

c. Effect of $KClO_4$. This drug which acts in a different way to PTU or methimazole, was also studied. The action of $KClO_4$ is to block the trapping of iodine by the thyroid as opposed to the incorporation of iodine into tyrosine, as is the case with the other drugs. The OD pattern of the thyroid proteins and the histological pattern of the thyroid tissue is altered from the control animals in a similar manner to that found when PTU or methimazole is used.

^3H-leucine incorporation. The pattern of incorporation of leucine (fig. 9) is very similar to that found in the methimazole treated animals. At 30 minutes the 3-8S and the 12S peaks are approximately equal in size; there is no incorporation into the thyroglobulin region. At one hour a peak in the thyroglobulin region has appeared and is approximately equal in SA to the 12S and 3-8S peaks which both, however, have increased in SA. At 4 hours all three peaks have shown a marked fall in SA with the 3-8S peak now predominant. At 24 hours the 3-8S peak has maintained its SA, but the peak in the thyroglobulin region is much diminished; the 12S peak can no longer be seen. The pattern at 48 and 72 hours is basically similar to that at 24 hours, with perhaps slight increase in the SA of the 3-8S

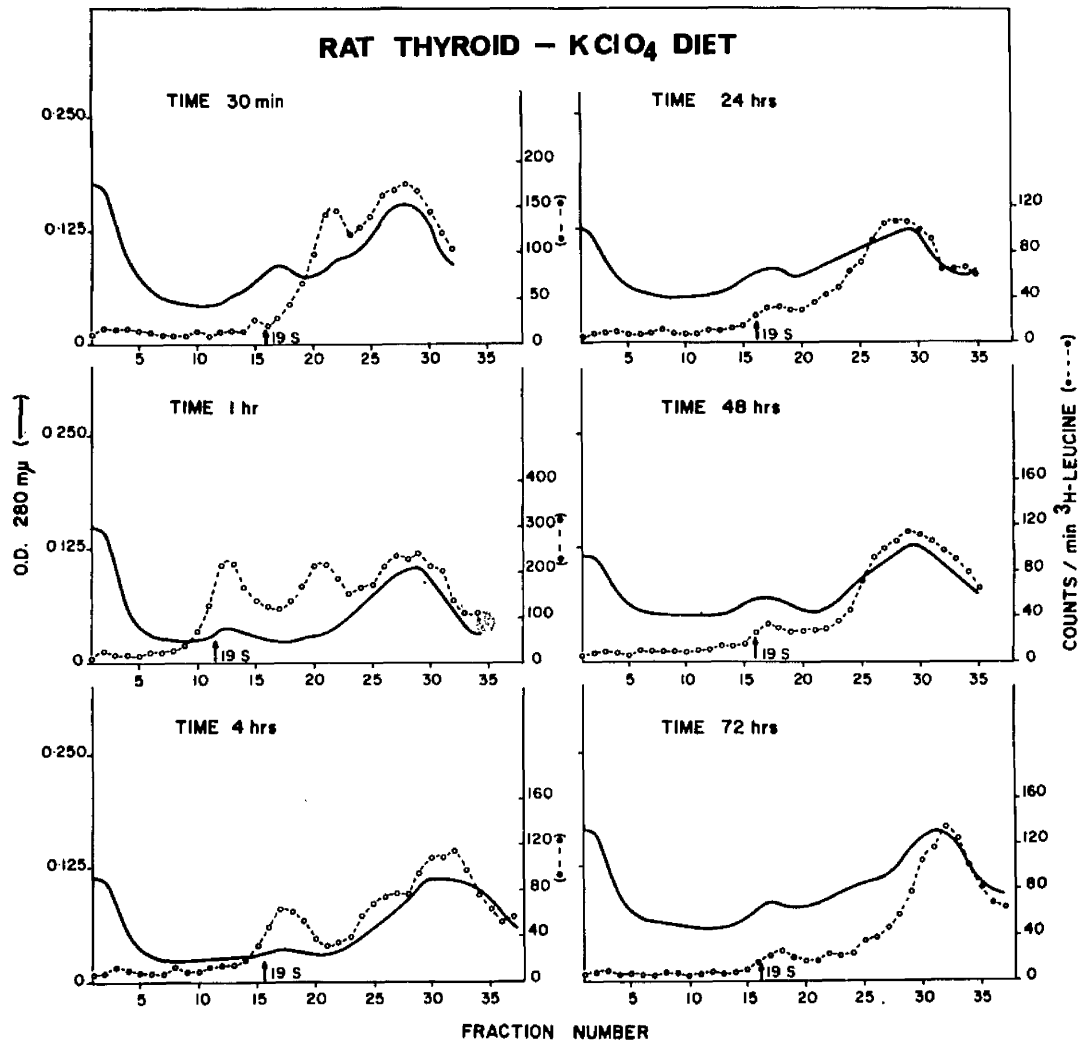

Fig. 9

Pattern of incorporation of ^{3}H-leucine into $KClO_4$ treated rat thyroid. SW 25.1 rotor at 21,000r.p.m. for 40 hours.

peak at 72 hours.

In summary, the pattern of leucine incorporation when $KClO_4$ has been given most closely resembles methimazole with the early appearance of a prominent peak corresponding to the OD peak in the thyroglobulin region, but always remaining short of 19S. The pattern of a predominant 3-8S peak, especially at long time intervals after administration of the leucine was similar to that found with other goitrogenic regimes.

^{125}I incorporation (fig. 7). In contrast to the situation found in the PTU treated animals, but similar to that found in the methimazole treated animals, there is incorporation of ^{125}I, given one hour before sacrifice, into the thyroglobulin region to the extent of approximately 2% of that found in the control animals. A 12S labelled peak was also present, but in approximately half the amount present in the methimazole treated animals. This incorporation of ^{125}I at one hour into a 12S protein is quite different to the results obtained in the control animals, where a small amount of label was present in the 12S protein only within a few minutes of its administration.

d. Studies of the thyroid protein patterns and the pattern of ^{125}I incorporation during induction of a goitrous state and during withdrawal of the goitrogenic agent. In fig. 10 can be seen the alteration in the thyroid protein pattern and ^{125}I incorporation during induction of the goitrous state. At early time intervals (two days) some 12S labelling appeared, although no alteration in OD had yet become manifest; at day 6 the 3-8S protein peak increased in size and the stable peak in the thyroglobulin region shifted to 18S. At day 13 on the diet the typical pattern of a drug induced goitre appeared.

During withdrawal of PTU (fig. 11) definite alterations in the protein pattern and in the pattern of ^{125}I labelling occurred. The pattern of ^{125}I labelling while on the drug has already been discussed. As the drug is withdrawn there is a progressive relative increase in incorporation of ^{125}I into thyroglobulin. At one hour off the drug a small amount of the label is incorporated into a 12S protein. The proportion of the label incorporated into the 12S protein relative to the 19S protein steadily increases with time until at 24 hours off the drug, the size of the labelled 12S peak actually exceeds the labelled 19S peak. At this time a stable 12S peak can be faintly discerned. At 48 hours off the

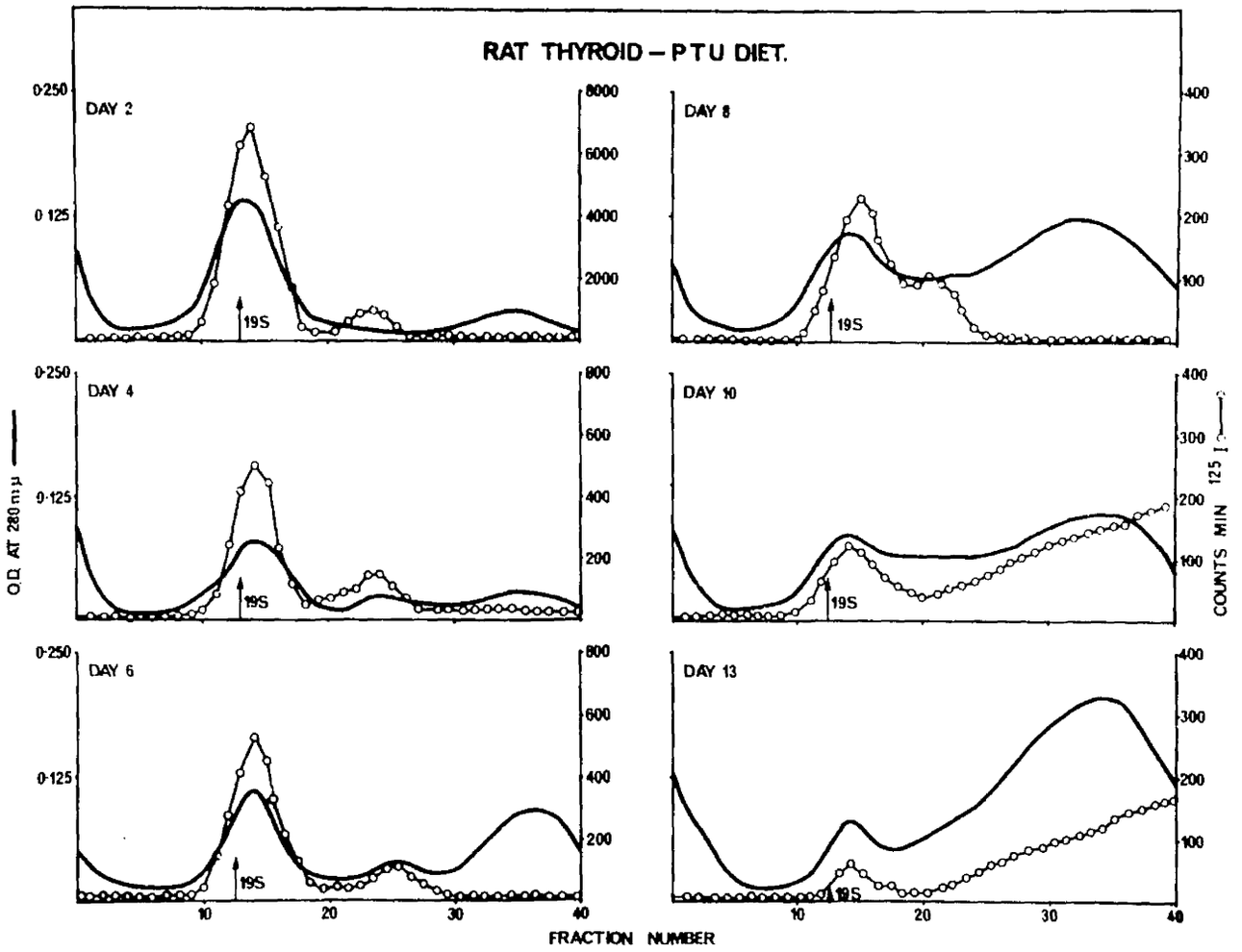

Fig. 10

Alteration of rat thyroid proteins during induction of goitre by PTU treatment. Effect on ^{125}I incorporation. SW 25.1 rotor at 21,000r.p.m. for 40 hours.

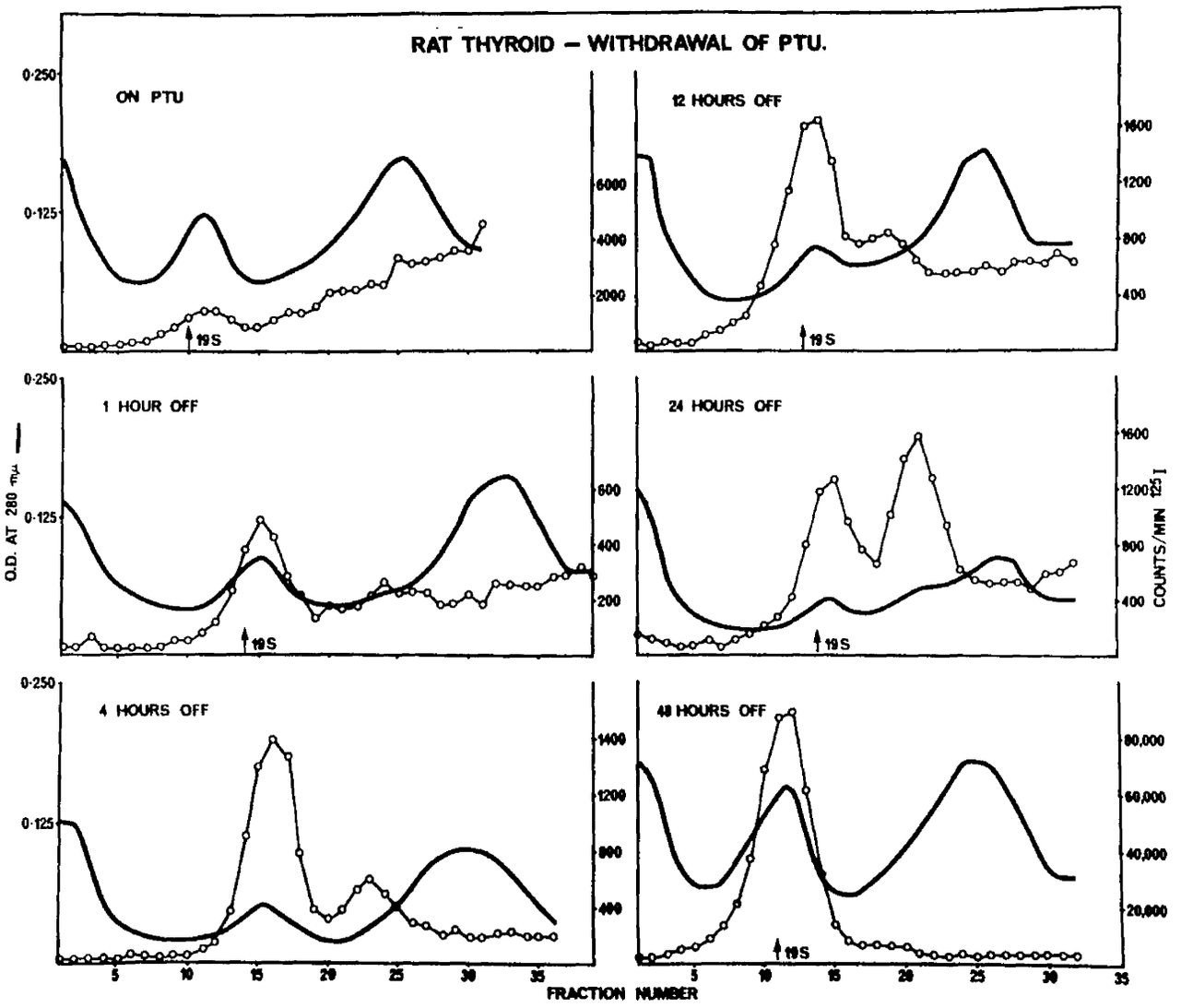

Fig. 11

Alteration of rat thyroid proteins during withdrawal of PTU treatment. Effect on ^{125}I incorporation. SW 25.1 rotor at 21,000r.p.m. for 40 hours.

drug the ^{125}I is virtually all incorporated into the thyroglobulin region; a trace amount only being seen in the 12S position. At this time there was a striking increase in the percentage of the administered dose of ^{125}I incorporated into the thyroid protein.

Similar studies were performed during the withdrawal of methimazole (fig. 12). As already pointed out, when the rats were treated with methimazole there was significant labelling of protein in the thyroglobulin region and of 12S protein while the drug was still being administered. At one hour off the drug the labelled 12S protein increased proportionally to the labelled protein in the thyroglobulin region. However, at 4 hours the proportion of labelled 12S protein had strikingly decreased relative to the labelled peak in the thyroglobulin area. At this time the percentage of the administered dose of ^{125}I incorporated into protein had increased. At 12 hours off the drug the SA of the 12S peak had increased once more, but rapidly declined thereafter, to be not detectable at 48 hours off the drug.

During withdrawal of $KClO_4$ (fig. 13) a similar series of events took place. As in the case of methimazole, there was significant labelling of protein in the thyroglobulin region and

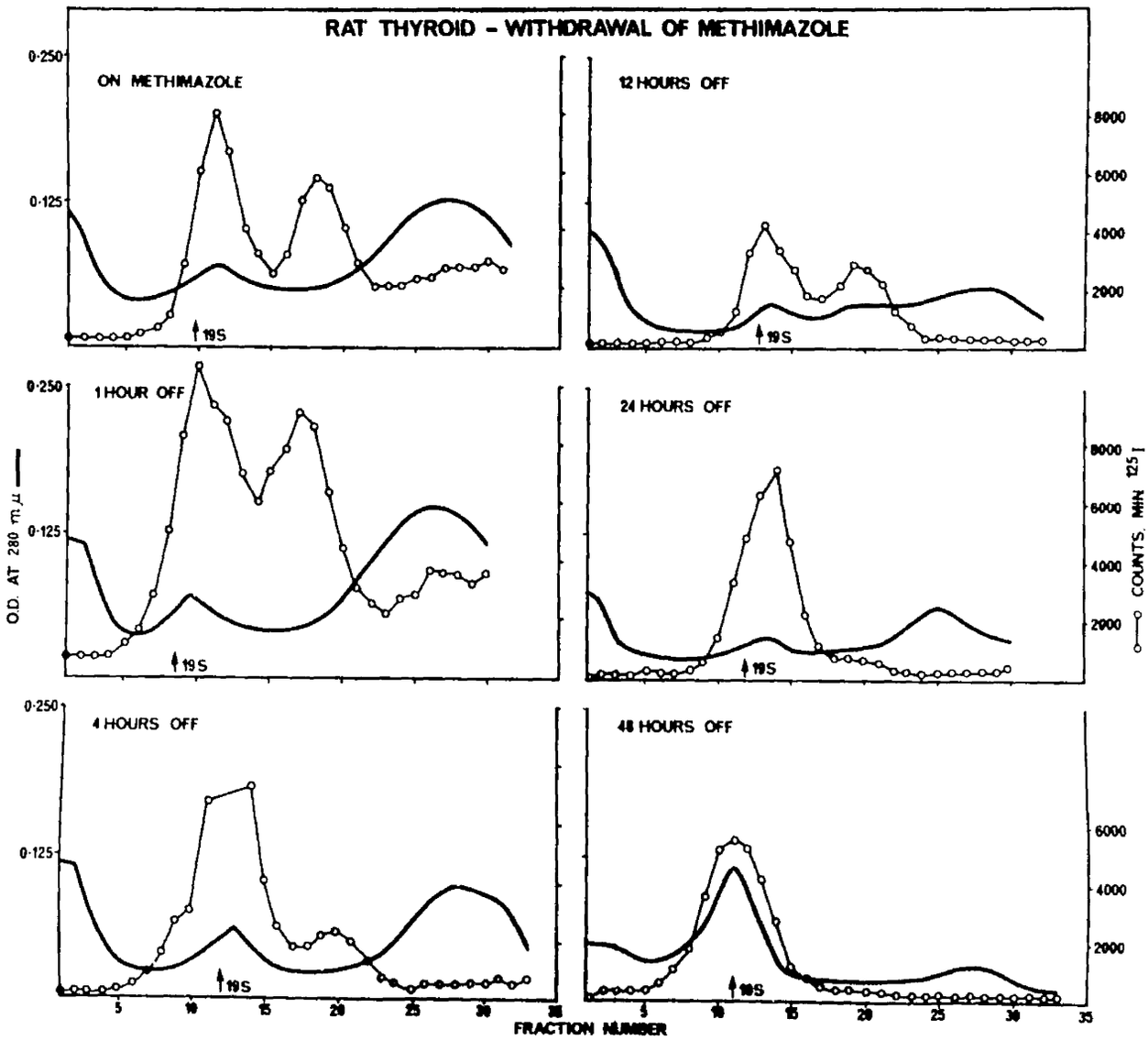

Fig. 12

Alteration of rat thyroid proteins during withdrawal of methimazole treatment. Effect on ^{125}I incorporation. SW 25.1 rotor at 21,000r.p.m. for 40 hours.

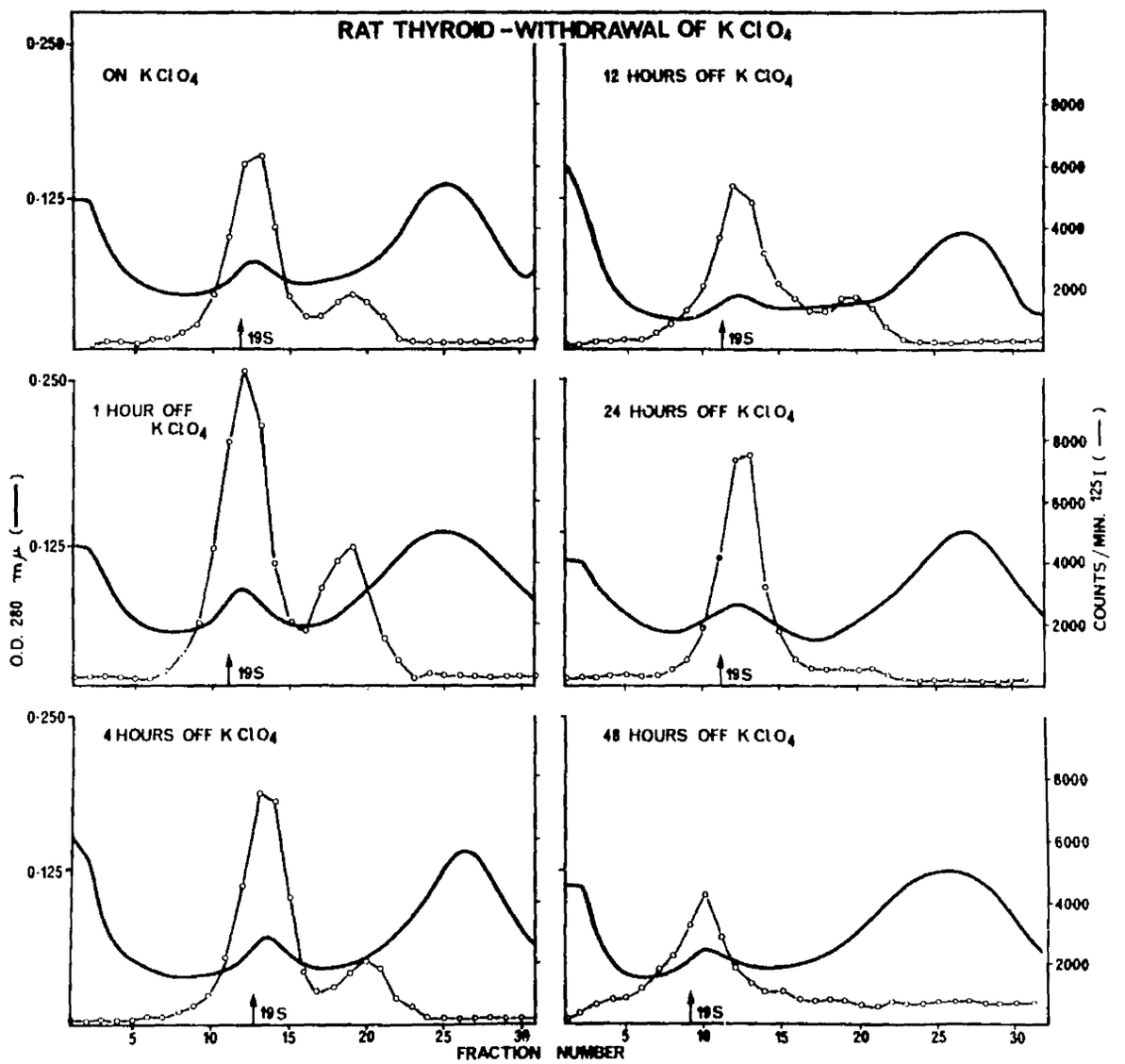

Fig. 13

Alteration of rat thyroid proteins during withdrawal of $KClO_4$ treatment. Effect on ^{125}I incorporation. SW 25.1 rotor at 21,000r.p.m. for 40 hours.

in the 12S region during administration of the drug. At one hour off the drug the proportion of labelled 12S protein showed a relative increase, but by 4 hours off the drug the 12S labelled peak had decreased, but it did however persist until 24 hours off the drug, when it could no longer be distinguished.

e. Effect of a low iodine diet

^{3}H-leucine incorporation. A similar series of studies were conducted after induction of a goitre in the rats by placing them on a low iodine diet. The initial studies were done 3 weeks after starting the diet. At this time no goitre had yet resulted (Table 1) but histological changes of increased thyroid stimulation namely increased follicular cell height and increased numbers of vesicles at the periphery of the colloid had appeared. The OD pattern of the thyroid proteins (fig. 14) was similar to that of the control animals. However in the low iodine diet group the ^{3}H-leucine was incorporated into thyroglobulin at very early time intervals after subcutaneous injection of the isotope so that by 15 minutes the labelled peak in the thyroglobulin area was already the predominant one. At 30 minutes the 12S labelled peak had increased in SA as had the 3-8S peak. At one hour the SA of the thyroglobulin peak had shown a striking increase whereas

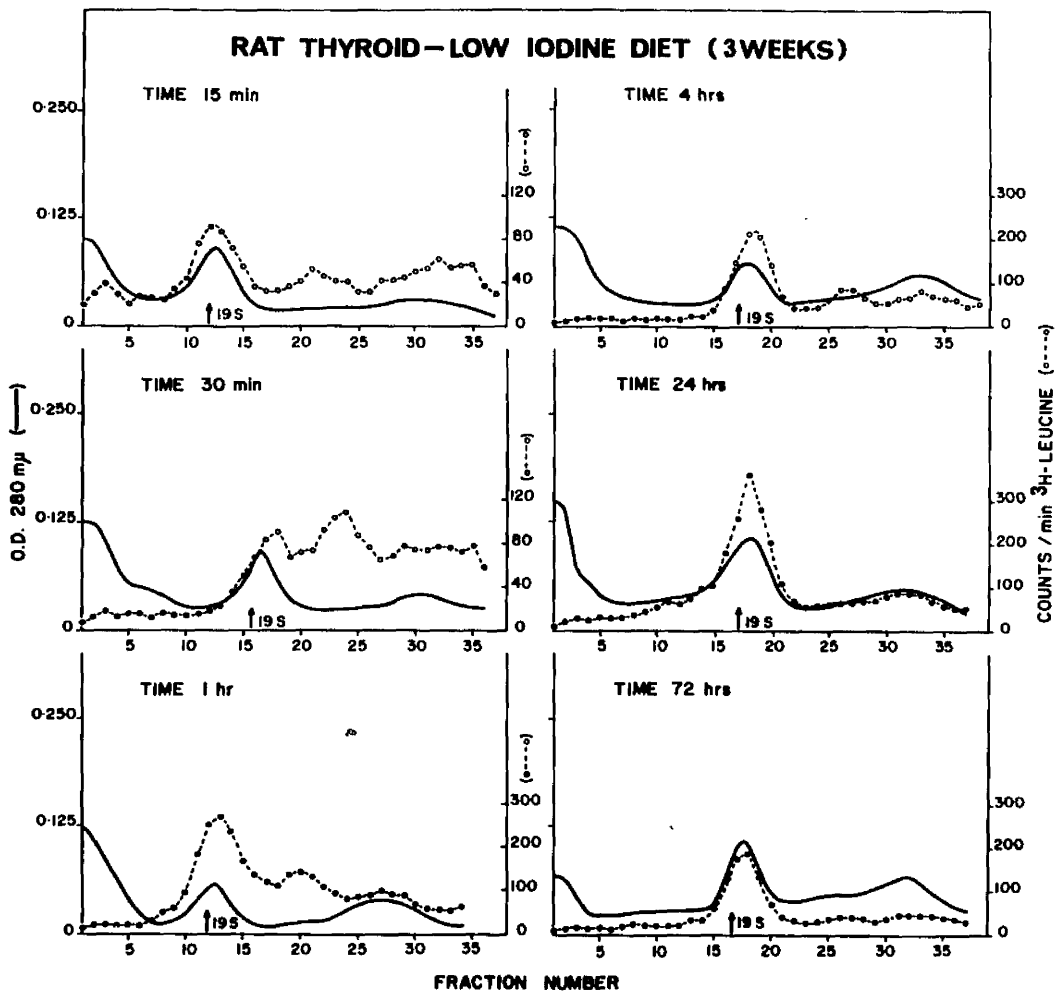

Fig. 14

Pattern of incorporation of ^{3}H-leucine in the rat after 3 weeks on low iodine diet. SW 25.1 rotor at 21,000r.p.m. for 40 hours.

the SA of the 12S and 3-8S peaks did not significantly change. At 4 hours there was a slight diminution in SA of all three peaks. At 24 hours the 12S peak had virtually disappeared, the SA of the thyroglobulin peak had increased whereas that of the 3-8S peak had remained static. At 72 hours the 3-8S peak was no longer significantly labelled and the thyroglobulin peak was the only one containing a significant amount of radioactivity.

Subsequent studies were carried out at 6 weeks on the diet at which time significant thyroid enlargement had occurred. The pattern of results can be seen in fig. 15. The changes in OD pattern were similar to those obtained in other goitrogenic regimes, except that although the expected increase in 3-8S protein occurred the OD peak in the thyroglobulin region remained at 19S. The pattern of ^{3}H-leucine incorporation was broadly similar to that obtained with the drug induced goitres, but with certain important differences. Even as early as 30 minutes after administration of the leucine the isotope had appeared in the thyroglobulin region and in fact corresponded exactly to the OD peak at 19S. At this time, however, the labelled 12S peak had the highest SA and the 3-8S peak was also labelled. At times of one hour and later after

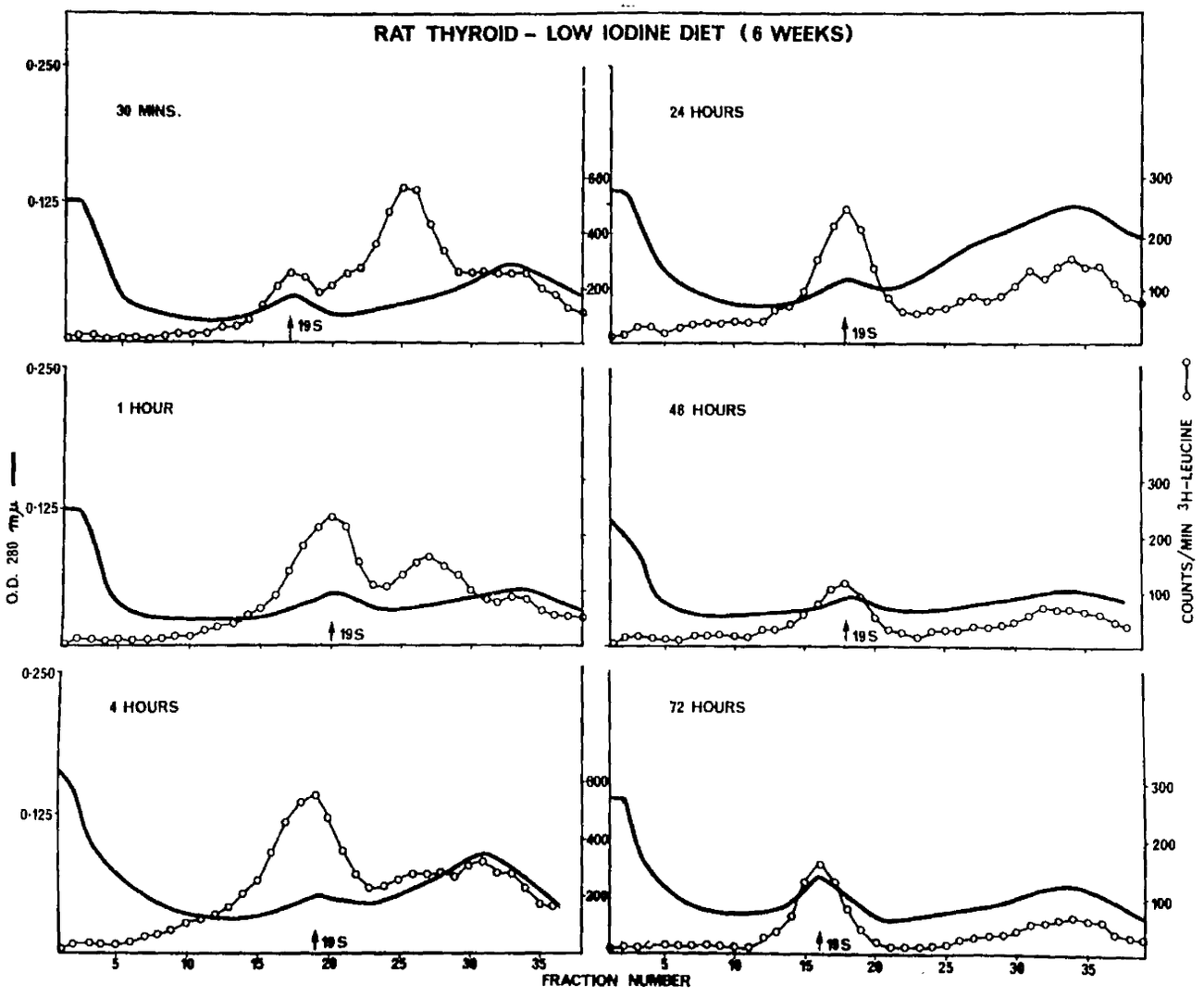

Fig. 15

Pattern of incorporation of ^{3}H-leucine in the rat after 6 weeks on low iodine diet. SW 25.1 rotor at 21,000r.p.m. for 40 hours.

administration of the label the 19S peak was the predominant labelled peak present, but the 12S labelled peak could be seen until 24 hours, after which it could nolonger be visualised. The SA of the 19S peak was highest at 4 hours after administration of the leucine, after which it fell. It was noticeable that neither at early nor at late time intervals after administration of the label was the 3-8S peak of the highest specific activity, this being in striking contrast to that found in all the other goitrous states.

^{125}I incorporation. Using ^{125}I a similar early incorporation of the label into the 19S protein was found (fig. 16). It should be noted that as early as one minute after the intraperitoneal injection of the label it appeared in the thyroglobulin region corresponding exactly to the OD peak. A trace amount of labelled 12S protein was also found.

Because of subsequent other publications on the incorporation of ^{3}H-leucine and ^{125}I into the thyroid gland of iodine deficient rats, which suggested that in iodine deficient glands a protein much less than 18-19S was labelled with these isotopes (see Discussion), the degree of iodine deficiency was increased in later studies by increasing the period on the low iodine diet up to 16 weeks. Table 2 shows that a significant

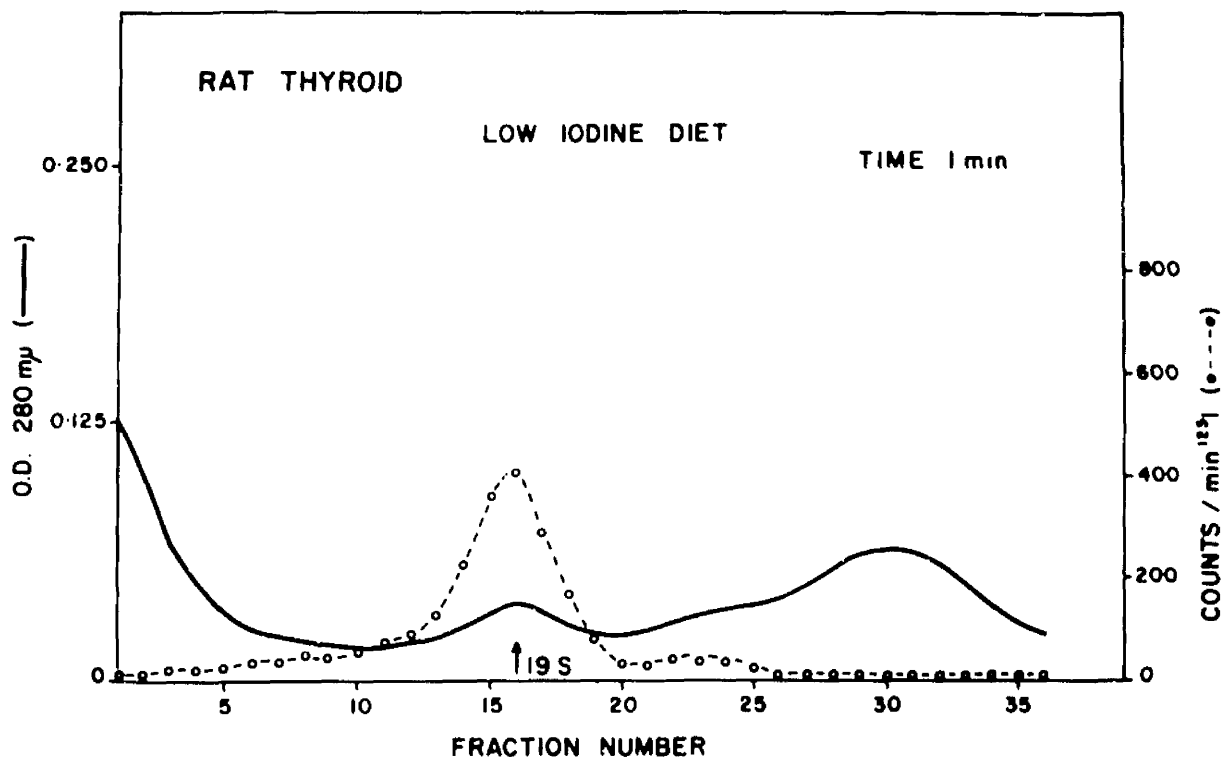

Fig. 16

Pattern of incorporation of ^{125}I into the thyroid proteins of the rat after 6 weeks on low iodine diet. Time 1 minute after i.p. injection. SW 25.1 rotor at 21,000r.p.m. for 40 hours.

Table 2:

Relationship of iodine content of thyroid proteins to duration of low-iodine diet in the rat

Duration of diet (weeks)	Iodine content of thyroid proteins (µg iodine/mg protein)
0	3.460
6	1.646
10	0.185
12	0.183
14	0.077
16	0.056

increase in goitre size and decrease in iodine content of the thyroid protein was achieved by this means. Despite the increased degree of goitre formation, the OD peak remained in the thyroglobulin region, although shifting from 19S to approximately 18-18.5S (mean of 9 experiments 18.5S, range 18.1-18.8S). ^{125}I was incorporated into the 18S region at short time intervals (5 minutes) after injection of a pulse of label (fig. 17). A slight degree of shouldering of the ^{125}I labelled peak consistent with the presence of a small amount of labelled material of S value less than 18S. Likewise, ^{3}H-leucine was incorporated into the 18S protein (fig. 18) 2 hours after the subcutaneous injection of the labelled aminoacid.

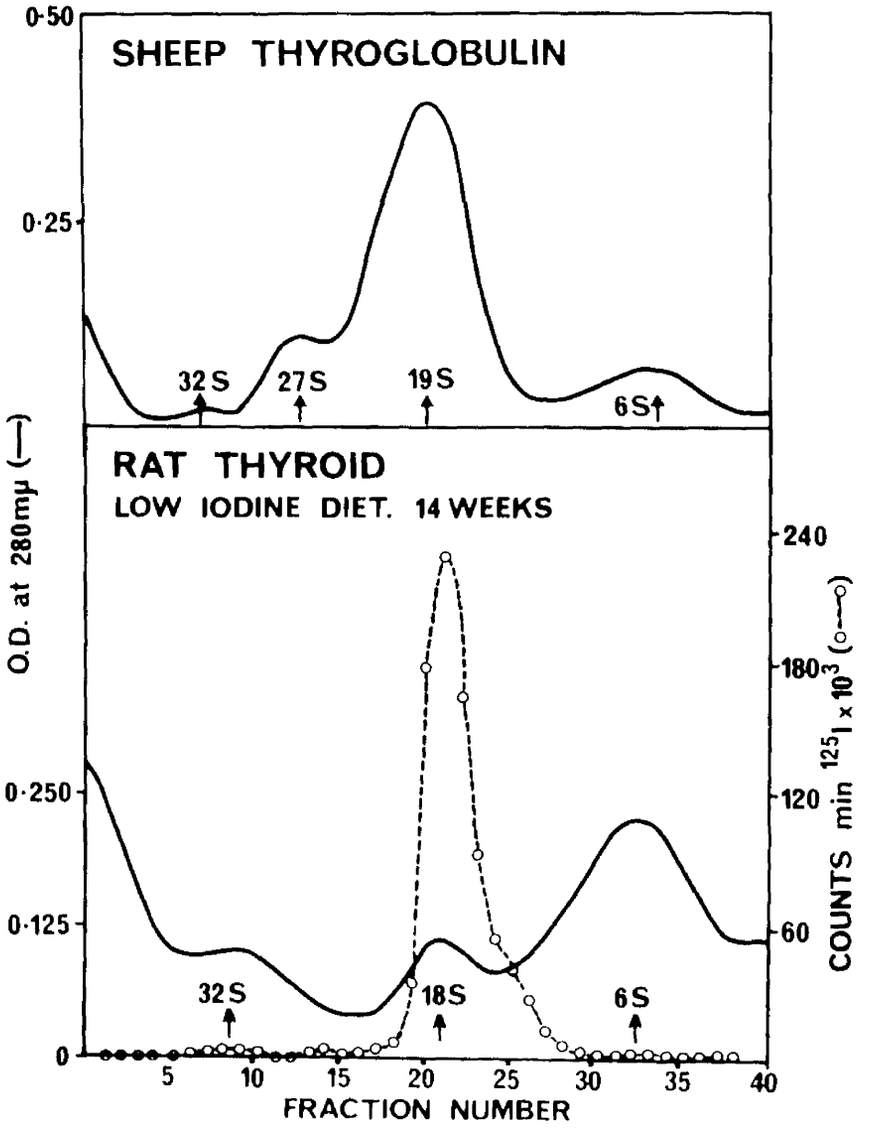

Fig. 17

Pattern of incorporation of ^{125}I into the thyroid proteins of the rat after 14 weeks on low iodine diet. Time 5 minutes after i.p. injection. SW 25.1 rotor at 21,000r.p.m. for 40 hours.

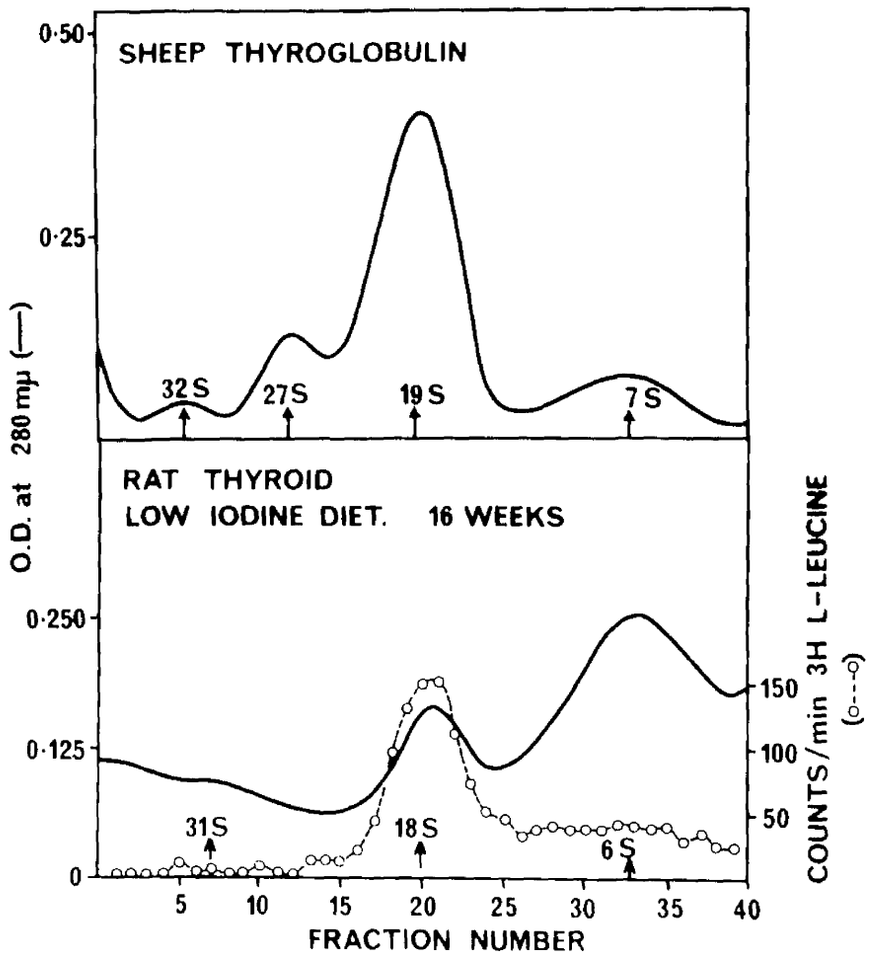

Fig. 18

Pattern of incorporation of ^{3}H-leucine into the thyroid proteins of the rat after 16 weeks on low iodine diet. Time 2 hours after subcutaneous injection. SW 25.1 rotor at 21,000r.p.m. for 40 hours.

CHAPTER 11

THYROGLOBULIN BIOSYNTHESIS DURING THE ADMINISTRATION OF T_4 OR T_3 TO CONTROL AND GOITROUS RATS

A series of experiments was conducted in which following the induction of a goitre by a goitrogenic drug, such as PTU, the diet was supplemented by T_4. This resulted, after a period of two days, in the appearance of a small protein peak in the 12S region (fig. 19) which steadily increased in quantity as the T_4 suppressed the thyroid, as shown by the decreasing gland weight (Table 3). During this time the peak in the thyroglobulin area increased and that in the 3-8S area decreased. At 7 days on T_4 in addition to PTU the histological appearance of the thyroid gland was as shown in fig. 21. Comparison with fig. 1 and 6 will show that the histological pattern of the rat thyroid now resembles very closely the pattern of the control rat. The effect of T_4 in suppressing TSH production by the pituitary has resulted in a diminution of follicular cell height and a re-accumulation of colloid in the lumen of the thyroid follicles. At this time the OD pattern of the thyroid proteins was as shown in the upper part of fig. 20. There was a large peak

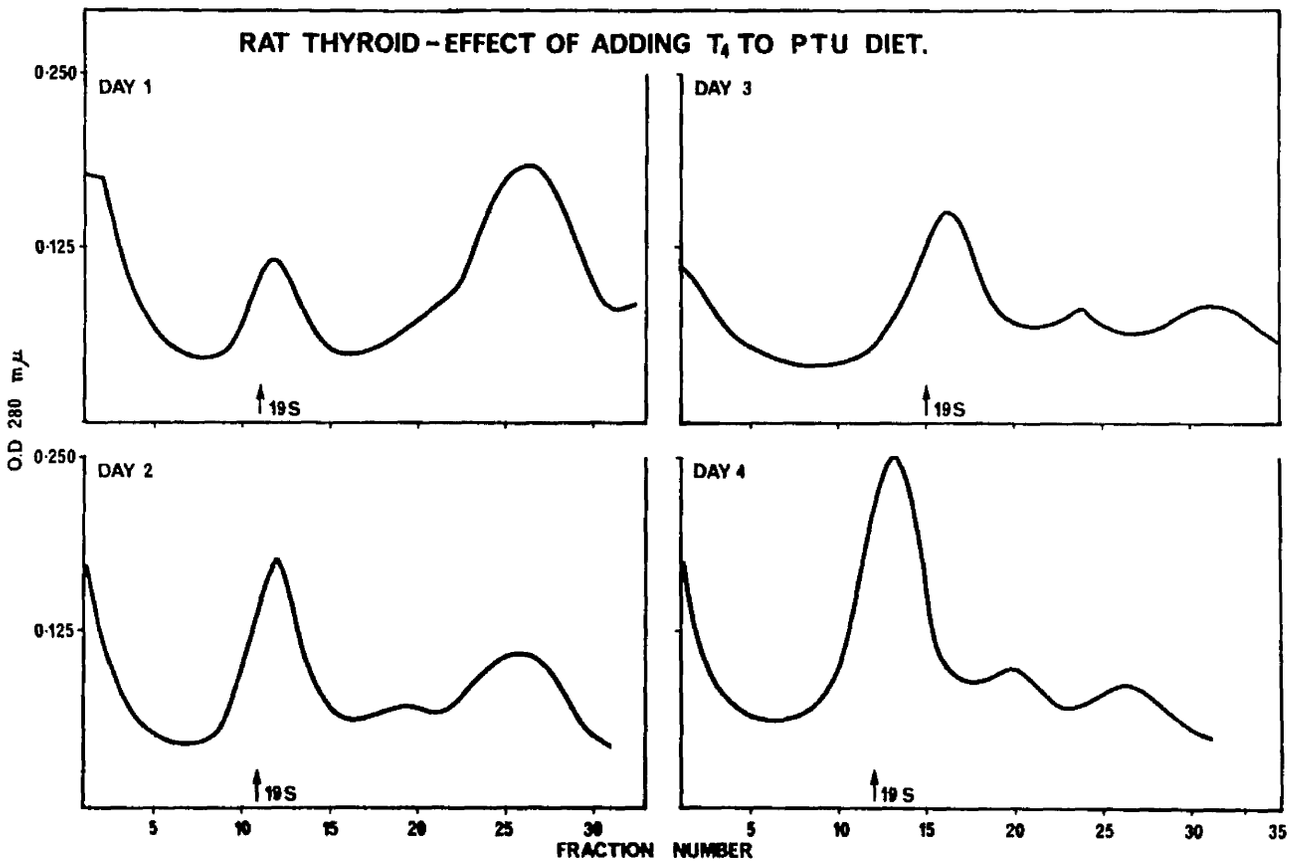

Fig. 19

Effect of T_4 on the thyroid proteins of PTU treated rats.
SW 25.1 rotor at 21,000r.p.m. for 40 hours.

Table 3:

Relationship of thyroid weight to the duration of thyroxine suppression of PTU treated rat thyroid gland

Duration of thyroxine treatment	Gland weight (mg) (mean of 2 animals)
1 day	38.5
2 days	24.5
3 days	22.5
4 days	19.5
5 days	18.5
6 days	16.0
7 days	10.5

in the thyroglobulin region, a definite peak in the 12S region, with a small 3-8S peak. The area between the thyroglobulin peak and the 12S peak was shouldered suggesting the possibility of a protein of S value between 19S and 12S being present. Both the thyroglobulin peak and the 12S peaks could be labelled with iodine in vivo roughly in proportion to their concentration, but the specific activity of labelling was understandably low in view of the suppression of TSH by thyroxine. A very similar OD pattern of thyroid proteins was produced by the administration of T_3 in equivalent dosage, either parenterally or orally.

Attempts were made to label PTU + T_4 treated glands with ^{3}H-leucine. There was uniform lack of incorporation of the label into the thyroid proteins of rats treated this way.

The effect of administration of T_4 or T_3 to control rats. A similar experiment to the above of administering T_4 or T_3 to the diet of a control animal altered the OD tracing of the thyroid proteins as shown in the lower part of fig. 20. Although the predominant protein remained 19S, a small but definite 12S peak was seen in addition to the 3-8S peak. Both the 19S and the 12S peaks were labelled with ^{125}I in proportion to the stable proteins present.

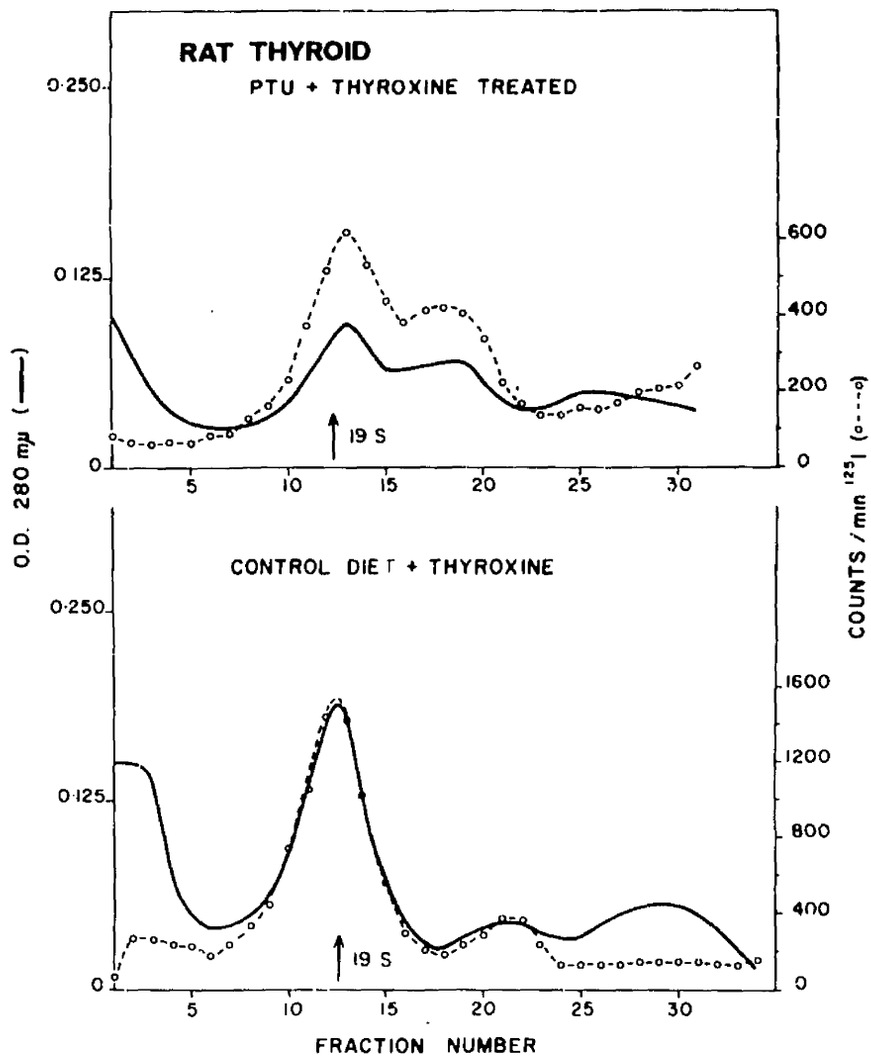

Fig. 20

Thyroid protein patterns of a) upper, PTU + T_4 treated rat b) lower, control diet + T_4 treated rat. SW 25.1 rotor at 21,000r.p.m. for 40 hours.

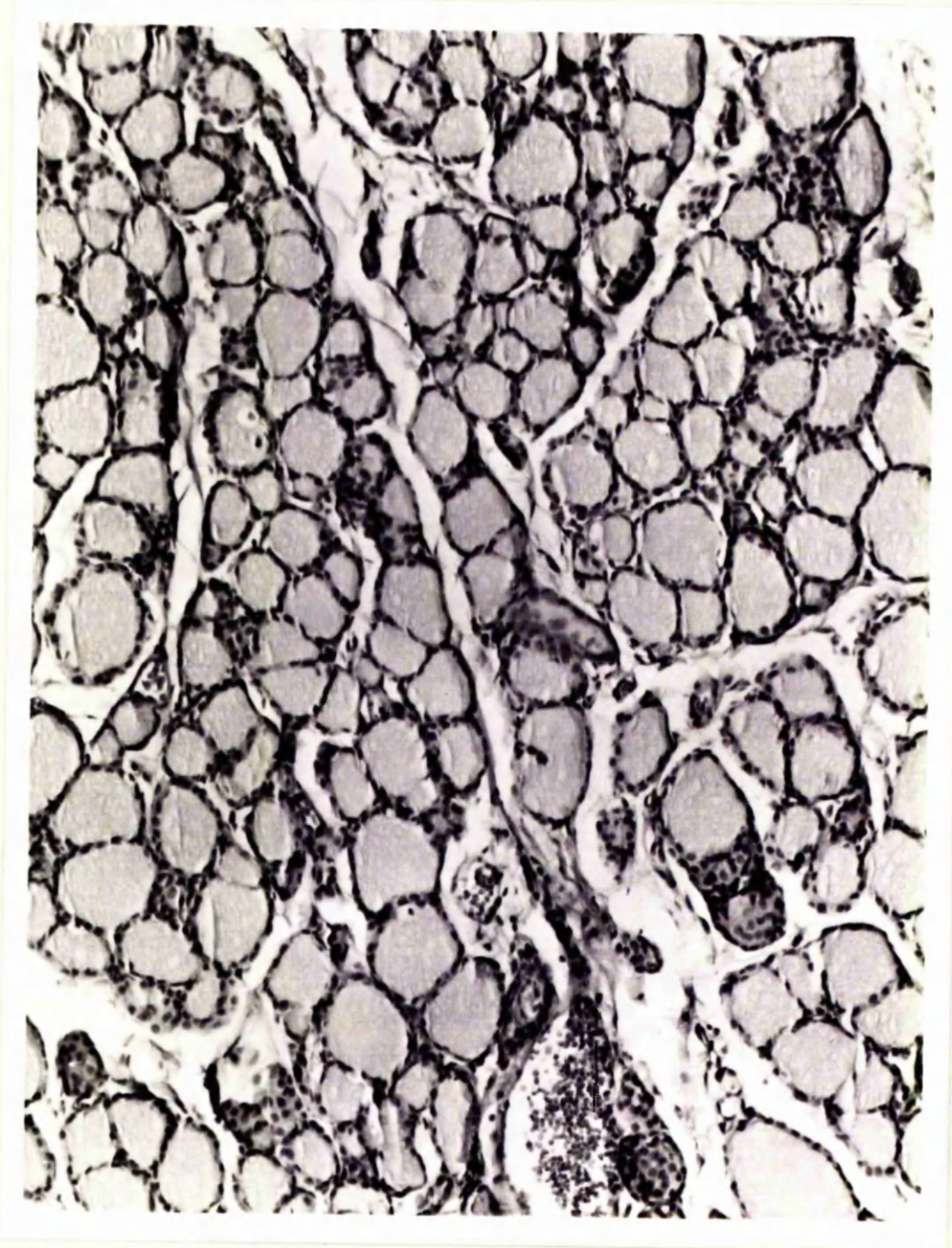

Fig. 21

Histological section of PTU + T_4 treated rat thyroid.
H and E x 190.

CHAPTER 12

SUMMARY OF SECTION 3

The studies presented in this Section confirm that *in vivo* as *in vitro* that ^{3}H leucine was incorporated in the normal rat into proteins lighter than 19S and that the pattern of incorporation was consistent with the 12S and possibly the 3-8S proteins being precursors of the 19S protein.

In contrast to the findings with ^{3}H-leucine, ^{125}I was incorporated rapidly into thyroglobulin and only transiently and minimally into the 12S protein and not at all into the 3-8S protein.

Goitrogenic drugs, although they alter the OD pattern of the thyroid proteins giving a larger amount of the 3-8S protein relative to thyroglobulin, do not fundamentally alter the pattern of ^{3}H-leucine incorporation. These drugs, however, prevent either a stable or a labelled protein which is truly 19S being formed. ^{125}I labelling of the thyroid proteins was markedly diminished while an antithyroid drug was being administered. Some differences existed amongst the individual drugs in this respect, the PTU regime being more effective in blocking iodination than either methimazole or $KClO_4$.

During withdrawal of a goitrogenic drug, a ^{125}I labelled 12S protein can be demonstrated in increased amount.

When a low iodine diet is given for such a time to produce a modest degree of iodine deficiency, there was an accelerated incorporation of both ^{3}H-leucine and ^{125}I into the 19S protein. When the degree of iodine deficiency was more severe, a protein of 18-18.5S size was labelled instead of a truly 19S protein.

The addition of either T_4 or T_3 to both a goitrogenic regime or to control animals, resulted in increased quantities of a stable 12S protein being formed. When a goitrous gland was so treated, it furthermore showed a diminished stable 3-8S peak and an increased peak in the thyroglobulin region.

SECTION 4

PROTEIN PATTERNS OF HUMAN THYROID GLANDS

CHAPTER 13

'NORMAL' HUMAN THYROID GLANDS (Case 1-8)

'Normal' human thyroid tissue was obtained from 8 patients, 6 of whom were undergoing neck exploration for parathyroid adenomata and 2 of whom were having neck operations for non-endocrine conditions. In all 8 the thyroid appeared normal on naked eye examination and part of the biopsy was submitted to histological examination and was shown to be of normal appearance. Fig. 22 shows the thyroid from one of the patients. The tissue is composed of large well filled vesicles with abundant colloid lined by a flattened cuboidal epithelium. Fig. 23 shows the OD pattern of the corresponding thyroid gland. The predominant OD peak is in the thyroglobulin region with smaller, but definite, peaks in the 27S and 3-8S region. In one of the 8 patients studied a small peak in the 32S region was noted.

In the group of 8 patients (Table 4) the 19S protein made up between 61.0 and 86.5% (mean 73.3, SD $\pm$ 9.2) of the total protein present. Between 5.7 and 22.7% of the protein was present in the 27S component (mean 13.0%, SD $\pm$ 6.8) and between 5.5 and 23.2% (mean 13.7, SD $\pm$ 6.6) in the 3-8S region. Fig. 24 shows the incorporation of ^{125}I and ^{3}H-leucine into slices of a 'normal'

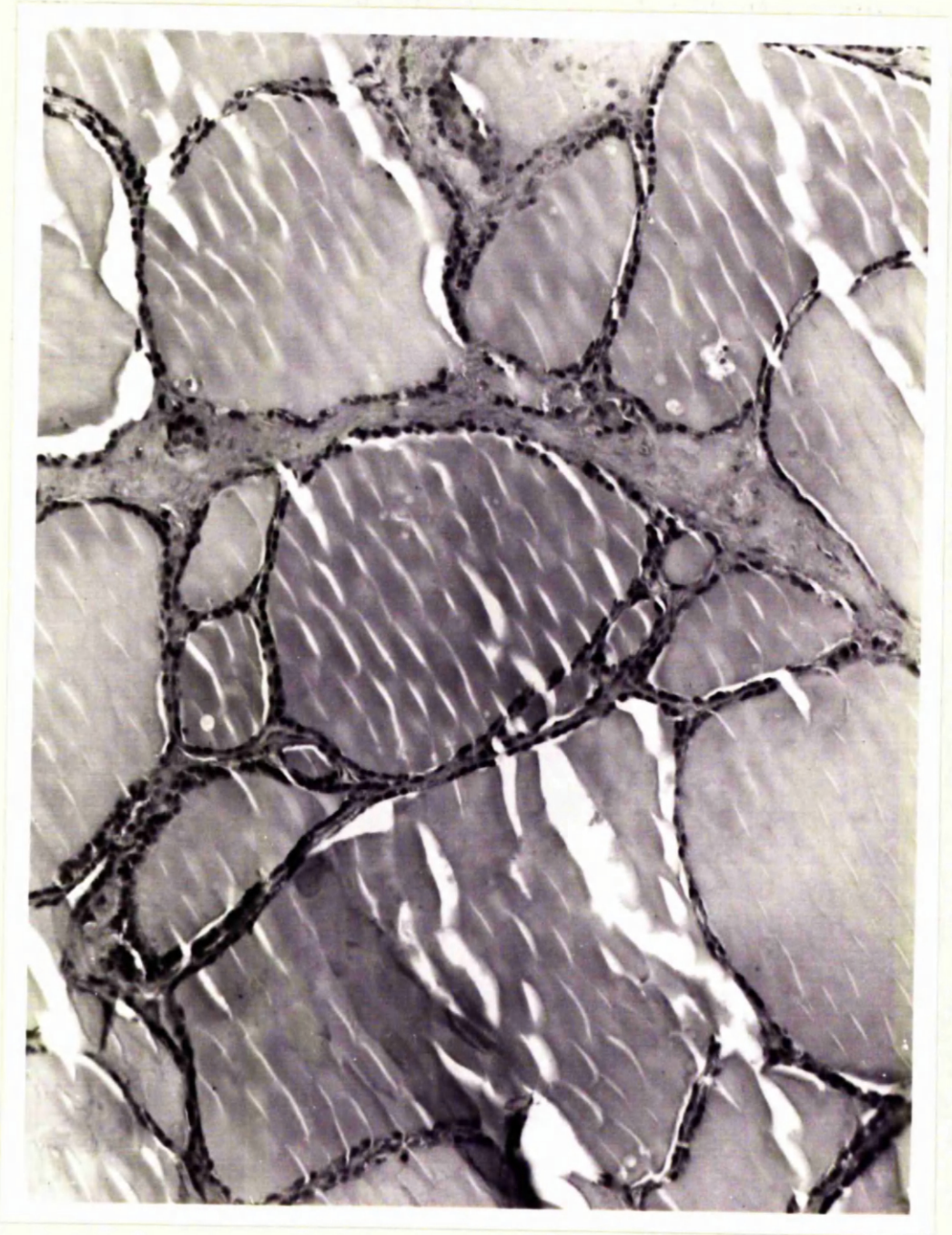

Fig. 22

Histological section of 'normal' human thyroid. H and E x 190.

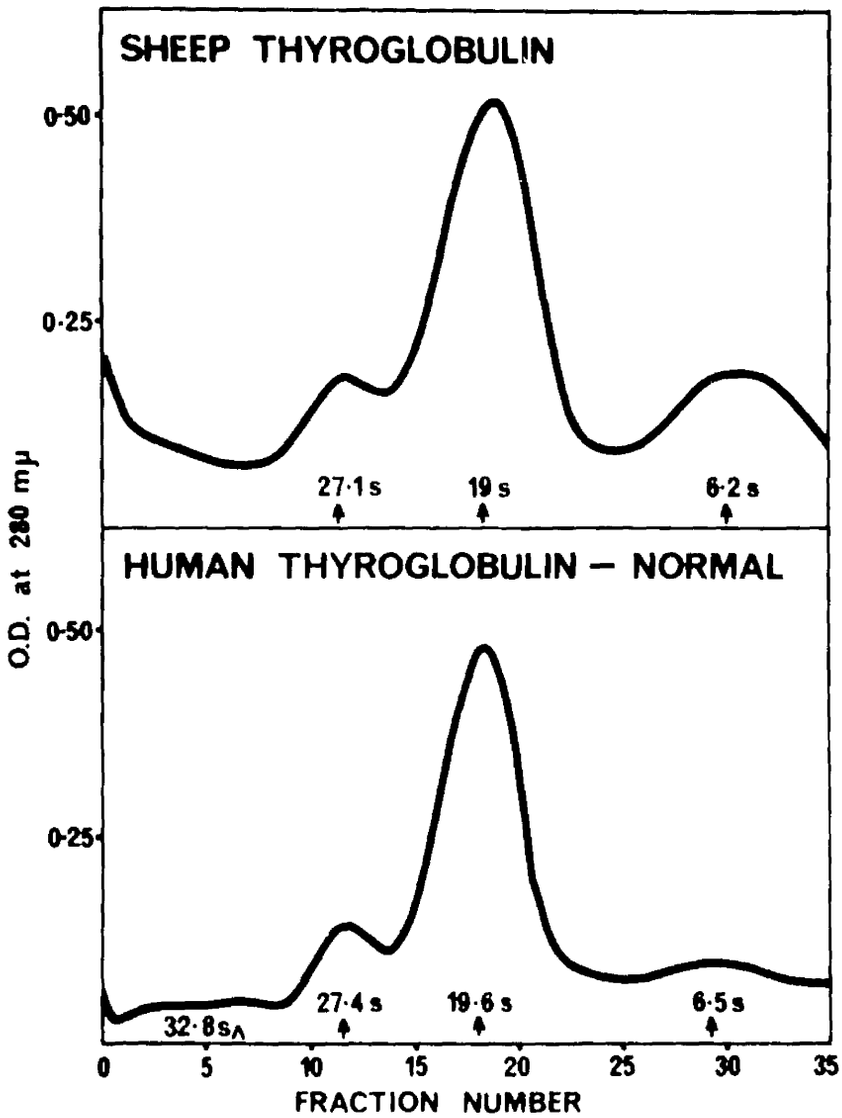

Fig. 23

Pattern of thyroid proteins of 'normal' human thyroid. SW 39 rotor at 24,000r.p.m. for 16 hours.

Table 4:

'Normal' human thyroid glands
% of thyroid proteins

Case no.	27S	19S	3-8S
1	16.0	74.0	10.0
2	22.7	68.1	9.2
3	6.0	86.5	7.5
4	14.8	62.0	23.2
5	11.5	83.0	5.5
6	21.2	61.0	17.8
7	5.7	73.5	20.8
8	6.1	78.2	15.7
	Mean 13.0 S.D. ±6.8	Mean 73.3 S.D. ±9.2	Mean 13.7 S.D. ±6.6

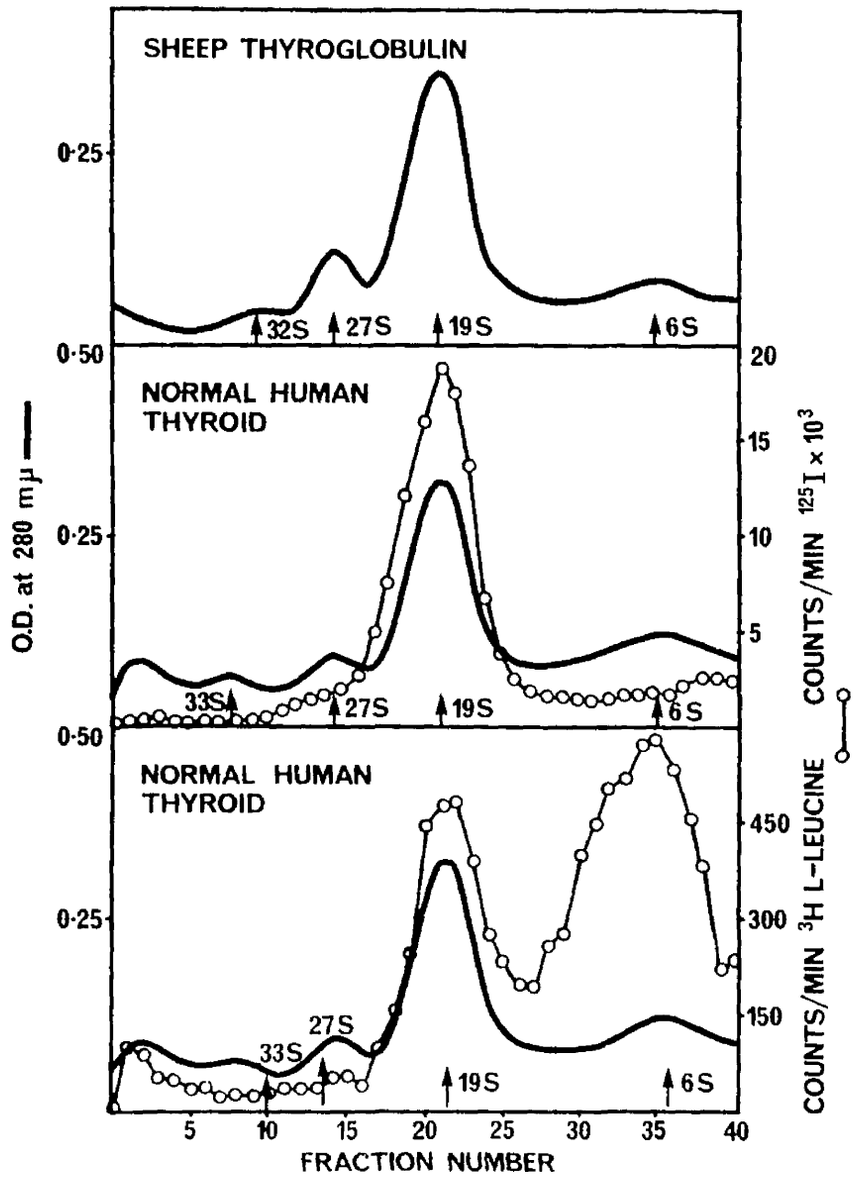

Fig. 24

Pattern of incorporation of ^{125}I and ^{3}H-leucine into thyroid slices of 'normal' human thyroid gland after 4 hours incubation. SW 39 rotor at 24,000r.p.m. for 16 hours.

human thyroid gland. ^{125}I is incorporated almost exclusively into the 19S protein whereas ^{3}H-leucine in addition to being incorporated into the thyroglobulin region just short of the main OD peak, is also incorporated into lighter proteins - at this time of incubation (4 hours) mainly into the 3-8S fraction.

CHAPTER 14

THYROTOXIC HUMAN THYROID GLANDS (Cases 9-43)

Material was obtained at operation from a series of 35 human thyrotoxic glands. The patients were prepared for surgery in two ways. (1) By control of the thyrotoxicosis by carbimazole which acts by blocking the iodination of tyrosine, followed by 7-10 days pre-operative treatment with iodide, either in the form of KI 30mg t.i.d. or Lugol's iodine 0.3ml t.i.d. (Lugol's iodine contains 5%W/V of iodine and 10% KI in water) (Cases 9-38); or, (2) by pre-operative control of the thyrotoxicosis by $KClO_4$, which acts by blocking the uptake of iodine (Cases 39-43). In these patients iodine is not given pre-operatively in case the drug induced block in iodine uptake is overcome by diffusion of the iodide into the thyroid.

Pre-treatment with carbimazole/iodide. Fig. 25 shows the typical OD pattern obtained from such a thyroid. The full details of protein composition are presented in Table 5. It will be noted that the predominant protein peak is in the thyroglobulin region (mean 68.8%, SD ± 11.4), with a well marked 3-8S OD peak also present (mean 29.2%, SD ± 11.3). No peak heavier than 19S was seen in the majority of the thyroids and this was

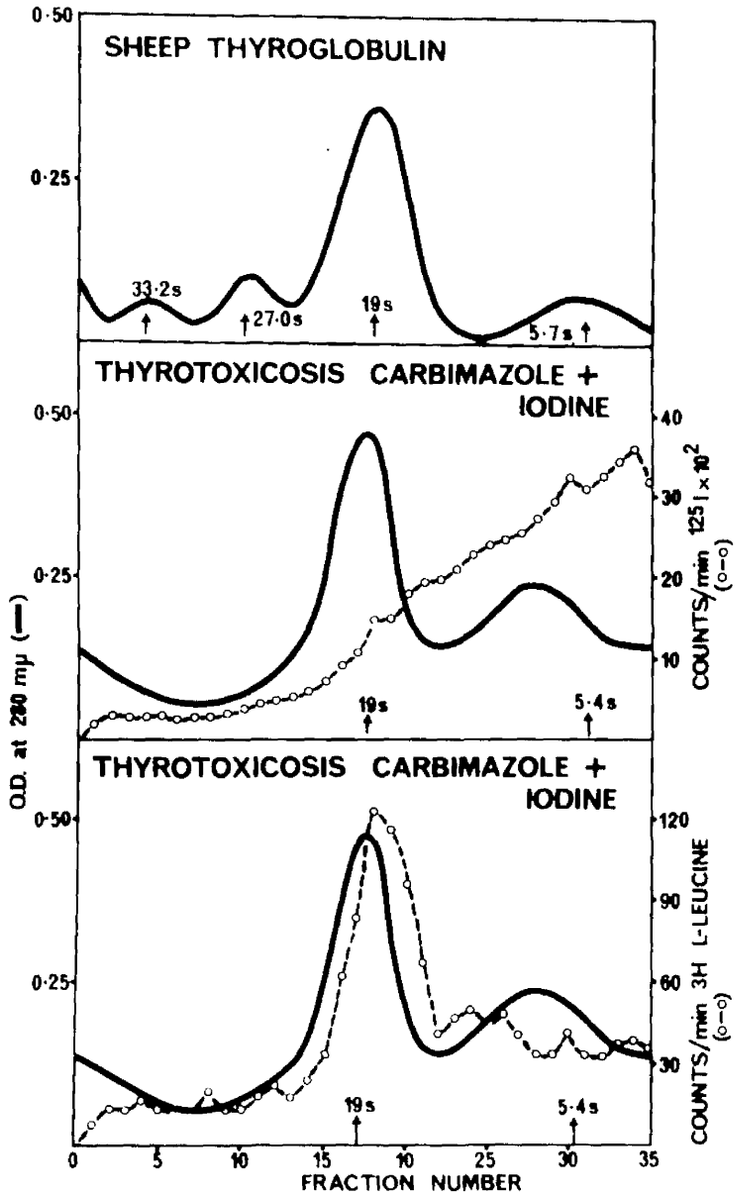

Fig. 25

Pattern of thyroid proteins from a thyrotoxic human thyroid treated with carbimazole/iodide. Incorporation of ^{125}I and ^{3}H-leucine into thyroid slices after 4 hours incubation. SW 39 rotor at 24,000r.p.m. for 16 hours.

Table 5:

Human thyrotoxic glands - controlled before operation with carbimazole/iodide treatment
% of thyroid proteins

Case no.	27S	19S	12S	3-8S
9	-	67.5	-	32.5
10	-	69.0	-	31.0
11	-	86.0	-	14.0
12	-	69.1	-	30.9
13	-	77.6	-	22.4
14	1.3	57.7	-	41.0
15	-	72.9	-	27.1
16	2.4	78.1	-	19.5
17	-	71.2	-	28.8
18	-	65.0	-	35.0
19	-	52.9	-	47.1
20	-	64.3	15.7	20.0
21	-	77.0	-	23.0
22	-	84.4	-	15.6
23	-	74.8	-	25.2
24	-	81.5	-	18.5
25	-	71.8	-	28.2
26	-	62.8	-	37.2
27	-	63.4	-	36.6
28	-	62.5	-	37.5
29	-	65.3	-	34.7
30	1.9	74.5	-	23.6
31	-	62.6	-	37.4
32	-	53.8	-	46.2
33	3.6	69.6	-	26.8
34	-	73.2	-	26.8
35	-	57.0	18.6	24.4
36	Trace	95.1	-	4.9
37	-	73.4	-	26.6
*38	-	2.3	-	97.7
		Mean 68.8		Mean 29.2
		S.D. ±11.4		S.D. ±11.3

*Excluded from statistical analysis, case description in text

confirmed in a further study (fig. 26) where the proteins were spun at a lower speed to give better separation of proteins >19S. As will be seen from Table 5, in 5 of the 30 patients a trace amount (up to 3.6%) of the total proteins was present in the 27S area. In 2 patients (Cases 20 and 35) a significant amount of 12S protein was present. Both were in patients whose pre-operative control was achieved with considerable difficulty. In addition, in both cases the thyroid proteins precipitated by 50% saturation with ammonium sulphate had been stored in the frozen state for 7 and 23 days respectively before study. In these cases it is therefore impossible to exclude some breakdown of the 19S protein (see Discussion).

As will be seen in fig. 25, ^{3}H-leucine was well incorporated into the thyroglobulin region, although slightly short of the OD peak; ^{125}I was not significantly incorporated into the proteins of the thyroid slices, instead it merely appeared as a broad band across the upper half of the gradient, almost certainly representing diffusion of non-organically bound ^{125}I and not specific incorporation of the isotope into any protein bound fraction.

Fig. 27 shows the histological appearance of a thyrotoxic gland treated pre-operatively with carbimazole/iodide. Although

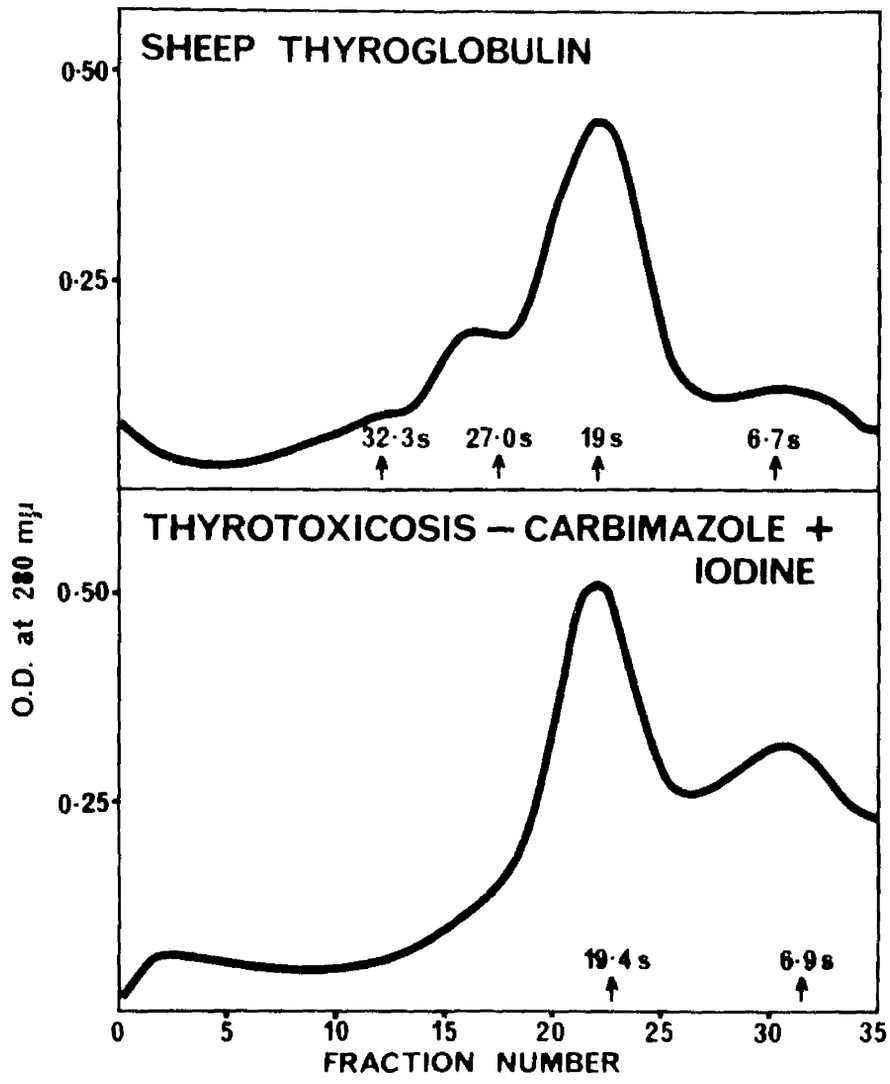

Fig. 26

Pattern of thyroid proteins from a thyrotoxic human thyroid treated with carbimazole/iodide. SW 39 rotor at 21,000r.p.m. for 16 hours.

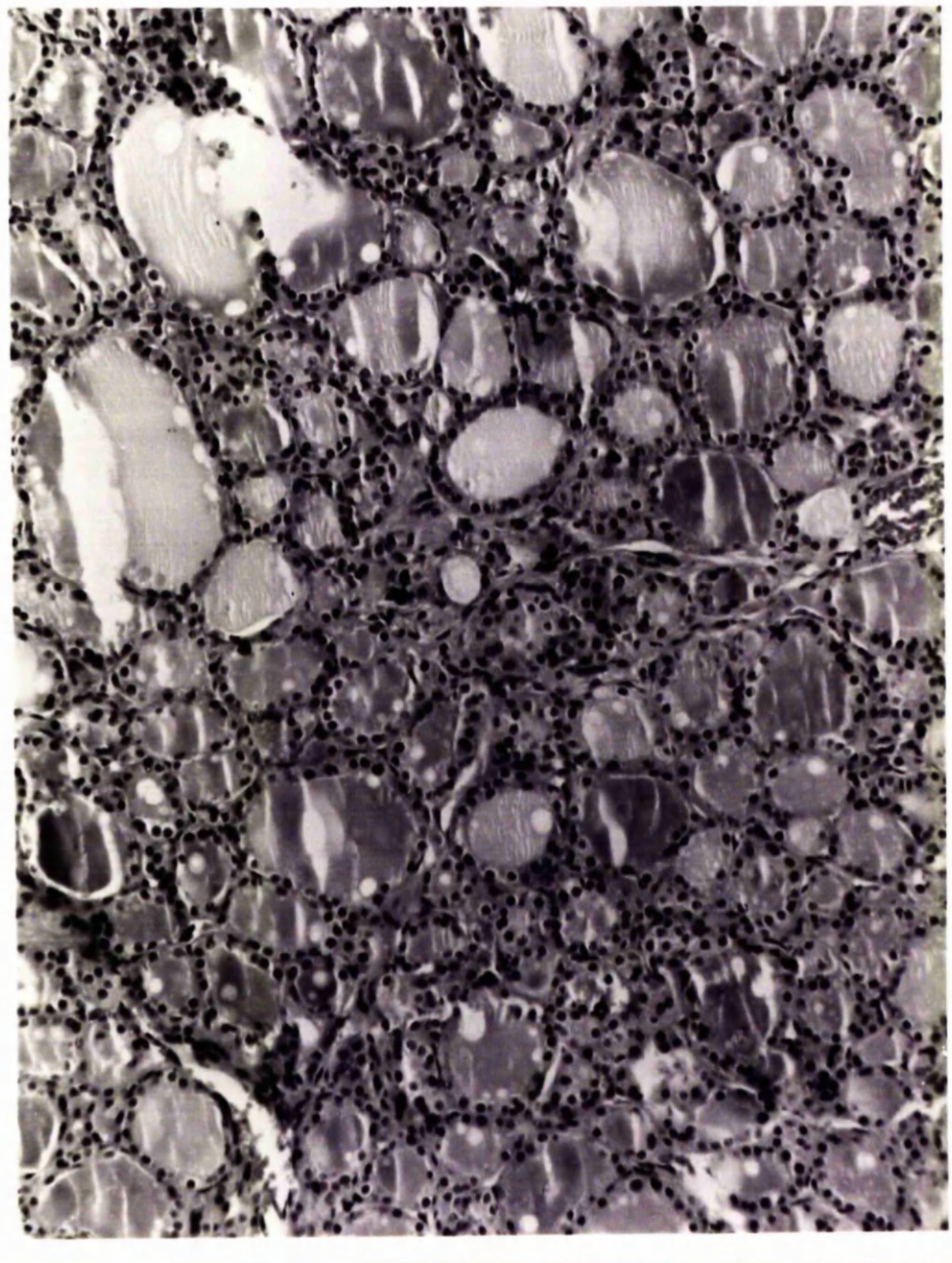

Fig. 27

Histological section of thyrotoxic human thyroid gland treated with carbimazole/iodide. H and E x 190.

the follicular epithelium is more cuboidal than in the normal gland (fig. 22), there is abundant colloid present which correlates with the predominant thyroglobulin peak.

Case 38 was excluded from the statistical analysis because the pattern of the thyroid proteins was obviously different from that of the other cases studied. Although this patient presented the classical clinical picture of thyrotoxicosis and showed appropriate clinical improvement on treatment with antithyroid drugs, histological examination of the resected specimen (fig. 28) showed the presence of severe generalised thyroiditis, indistinguishable from that of Hashimoto's disease (auto-immune thyroiditis). As can be seen, very little colloid was present in this gland.

Pre-treatment with $KClO_4$. Fig. 29 shows the OD pattern of the thyroid proteins usually present in a thyrotoxic gland controlled with this drug. As shown in Table 6, in Cases 39-42 inclusive, there was more protein in the 3-8S peak (mean 62.6%) than in the 19S peak (mean 37.4%). No OD peak >19S was seen in any of these glands.

Both ^{125}I and ^{3}H-leucine were incorporated into the thyroglobulin region, although the SA of ^{125}I labelling was very low. ^{3}H-leucine was also incorporated into the 3-8S region.

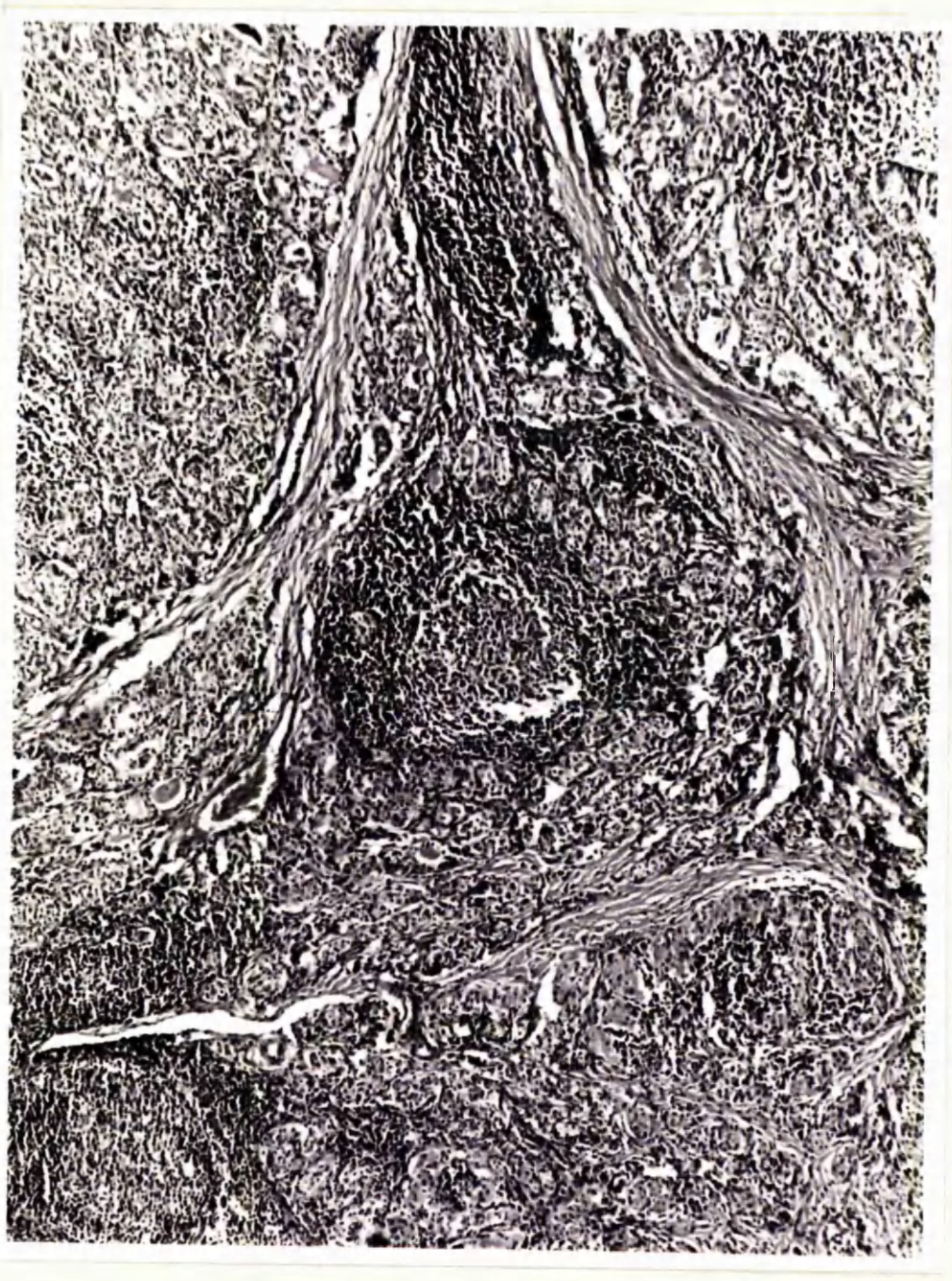

Fig. 28

Histological section of the thyroid gland of Case 38. H and E x 75.

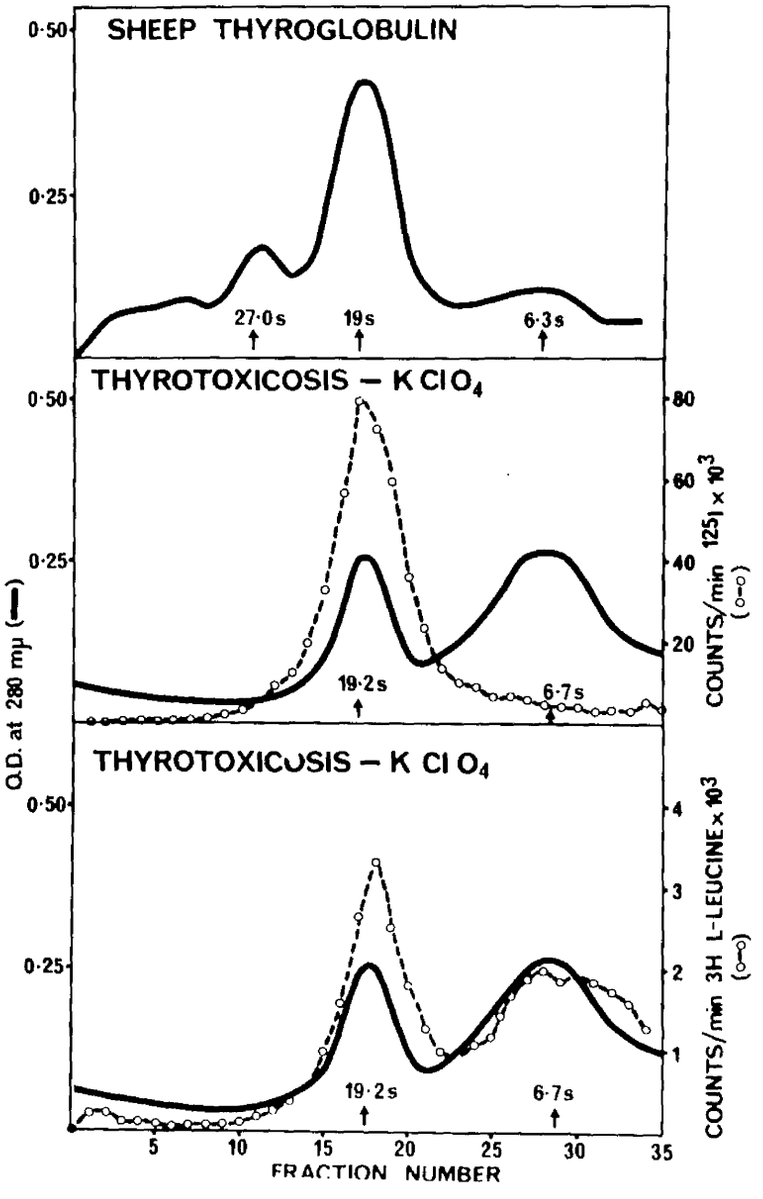

Fig. 29

Pattern of thyroid proteins from a thyrotoxic human thyroid treated with $KClO_4$. Incorporation of ^{125}I and ^{3}H-leucine into thyroid slices after 4 hours incubation. SW 39 rotor at 21,000r.p.m. for 16 hours.

Table 6:

Human thyrotoxic glands - controlled before operation with $KClO_4$ treatment

Case no.	27S	19S	3-8S
39	-	37.9	62.1
40	-	45.2	54.8
41	-	38.5	61.5
42	-	28.1	71.9
*43	-	75.5	24.5
		Mean 37.4	Mean 62.6

*Excluded from calculation of mean values, case description in text

Case 43 showed a protein pattern at variance with those of the other patients in this group. Further investigation of this patient's pre-operative drug history showed that he had inadvertantly received two doses of 0.3ml Lugol's iodine three days before operation.

The typical histological picture of a thyrotoxic gland treated with $KClO_4$ is shown in fig. 30. It will be noted that there is intense cellular hyperplasia. The lumina of the thyroid follicles are reduced to slits and only very little colloid is present.

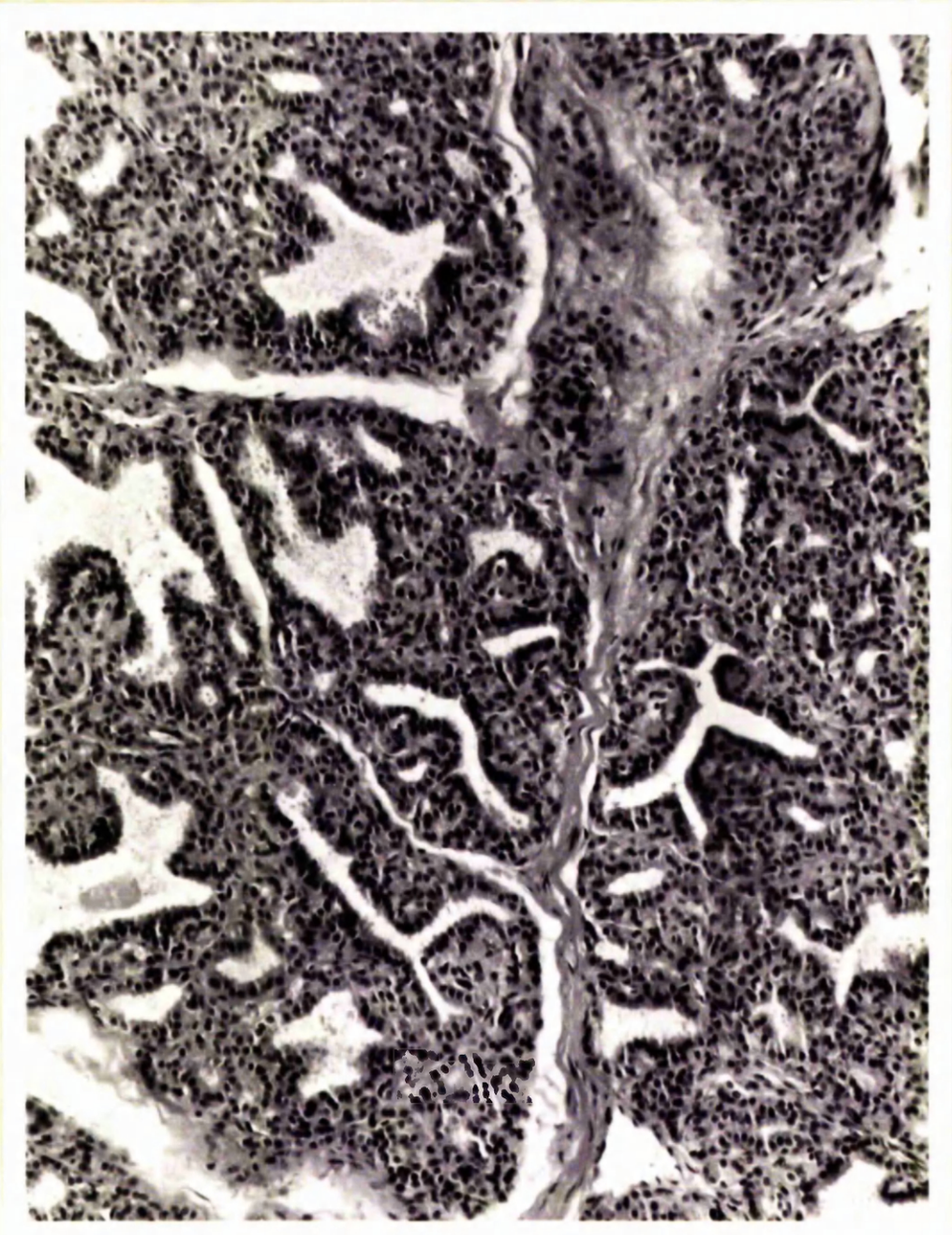

Fig. 30

Histological section of a thyrotoxic human thyroid gland treated with $KClO_4$. H and E x 190.

NON-TOXIC GOITRES (Cases 44-89)

These were dealt with in two groups. (1) Those patients with a diffuse or multinodular non-toxic goitre (Cases 44-72). (2) Those with a clinically single thyroid nodule (Cases 73-89).

In both groups, however, the OD patterns were basically similar (fig. 31); the predominant peak was in the thyroglobulin region (mean 74.4%, SD ± 7.7) for diffuse and multinodular goitres (Table 7) and 66.0% (SD ± 12.2) for thyroid adenomas (Table 8). In diffuse or multinodular goitres 8.6% (SD ± 4.4%) of the total proteins were in the 27S area; in the case of the thyroid nodules 6.6% (SD ± 4.3%). One diffusely enlarged thyroid gland and two adenomas showed no demonstrable 27S protein. Two of these, the diffusely enlarged gland and one nodule, were studied early on in the series and on review of the OD patterns, it is impossible to exclude slight overloading of the sucrose gradients which would obscure protein >19S. Other two patients (Cases 88 and 89) will be separately discussed.

The 3-8S protein constituted 17.6% (SD ± 6.1) of the protein in the diffuse or multinodular non-toxic goitre group and 27.3% (SD ± 12.5) in the case of the solitary nodules.

An attempt was made to see whether the thyroid protein

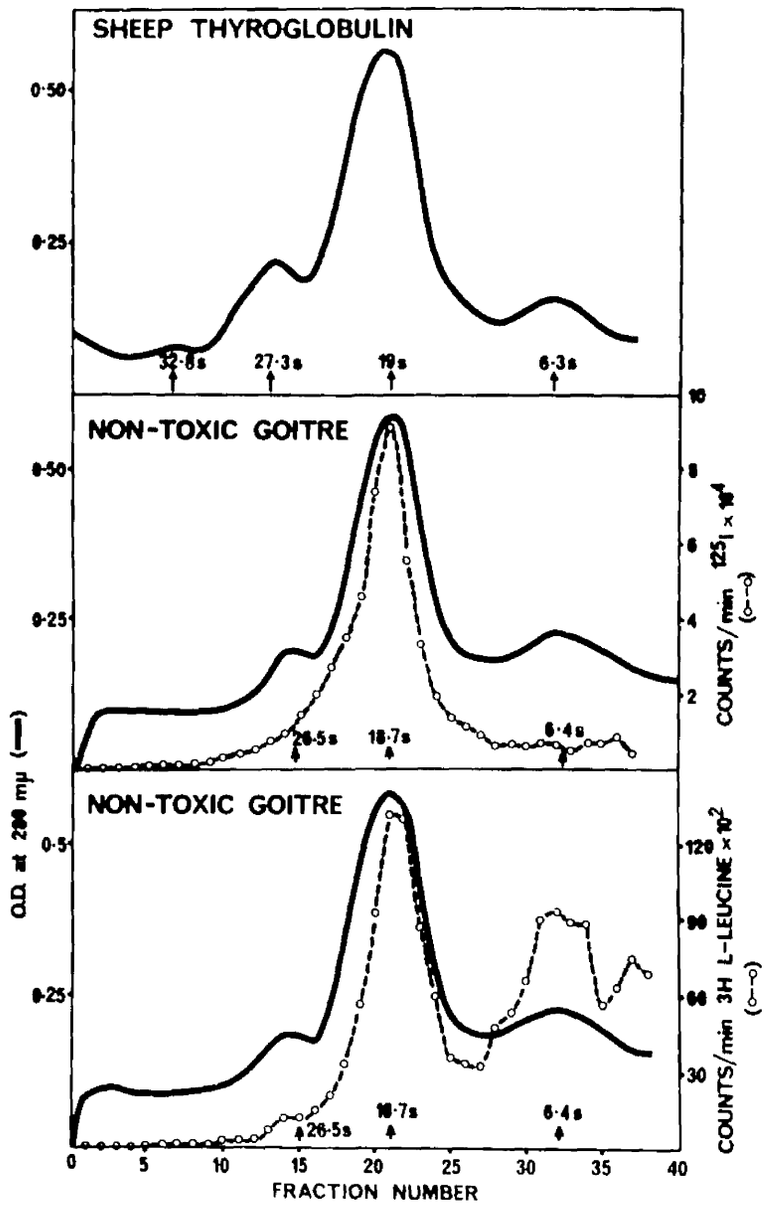

Fig. 31

Pattern of thyroid proteins from a diffuse non-toxic human goitre. Incorporation of ^{125}I and ^{3}H-leucine into thyroid slices after 4 hours incubation. SW 39 rotor at 24,000r.p.m. for 16 hours.

Table 7:

Human thyroid glands - diffuse and multinodular non-toxic goitres
% of thyroid proteins

Case no.	27S	19S	3-8S
44	-	77.5	22.5
45	6.1	74.2	19.7
46	13.6	65.1	21.3
47	5.1	85.2	9.7
48	6.3	73.4	20.3
49	11.0	65.5	23.5
50	9.6	70.4	20.0
51	3.4	71.0	25.6
52	5.7	77.3	17.0
53	11.2	64.5	24.3
54	6.2	82.0	11.8
55	11.3	68.5	20.2
56	18.1	69.0	12.9
57	5.7	84.8	9.5
58	14.7	64.8	20.5
59	4.0	76.0	20.0
60	11.0	83.6	5.4
61	4.5	86.2	9.3
62	9.2	75.0	15.8
63	7.0	62.0	31.0
64	13.1	77.0	9.9
65	7.2	69.8	23.0
66	11.5	76.5	12.0
67	2.4	89.0	8.6
68	16.2	63.2	20.6
69	8.4	70.6	21.0
70	14.7	65.6	19.7
71	7.2	72.1	20.7
72	4.9	79.1	16.0
	Mean 8.6	Mean 74.4	Mean 17.6
	S.D. ±4.4	S.D. ±7.7	S.D. ±6.1

Table 8:

Human thyroid glands -
clinically solitary thyroid nodules
% of thyroid proteins

Case no.	27S	19S	3-8S
73	-	76.5	23.5
74	5.6	51.8	42.6
75	12.5	79.2	8.3
76	2.5	81.0	16.5
77	6.0	61.5	32.5
78	10.7	62.0	27.3
79	3.9	54.4	41.7
80	-	52.2	47.8
81	8.6	84.0	7.4
82	12.0	66.3	21.7
83	11.8	45.4	42.8
84	6.1	65.1	28.8
85	2.4	76.3	21.3
86	10.1	59.0	30.9
87	7.1	76.6	16.3
*88	-	-	100
*89	-	5.0	95.0
	Mean 6.6	Mean 66.0	Mean 27.3
	S.D. ±4.3	S.D. ±12.2	S.D. ±12.5

*Excluded from statistical analysis;
case description in text

pattern of solitary nodules showed any correlation with the pre-operative thyroid scan. There was no significant difference in the OD patterns of a nodule which had been 'hot' on pre-operative scanning and one which had been 'cold'.

In all cases of diffuse or multinodular goitre, the main protein present was thyroglobulin. This correlated well with the histological picture of the thyroid glands from these patients as illustrated in fig. 32 which shows large follicles filled with colloid and lined with a flattened epithelium.

With two exceptions (Cases 88 and 89) all the nodules studied also contained similar well filled follicles with abundant colloid present.

Case 88 showed almost entirely a 3-8S peak with only a small peak present in the thyroglobulin region. The nodule in this patient had appeared some years after a therapeutic dose of ^{131}I had been given to the patient for treatment of thyrotoxicosis. Because of the time of appearance of the nodule and its firm consistency, it was decided that the possibility of malignant change in this irradiated remnant could not be excluded and the nodule was accordingly excised. Histological examination showed it to be composed of necrotic material. The OD pattern of the gland

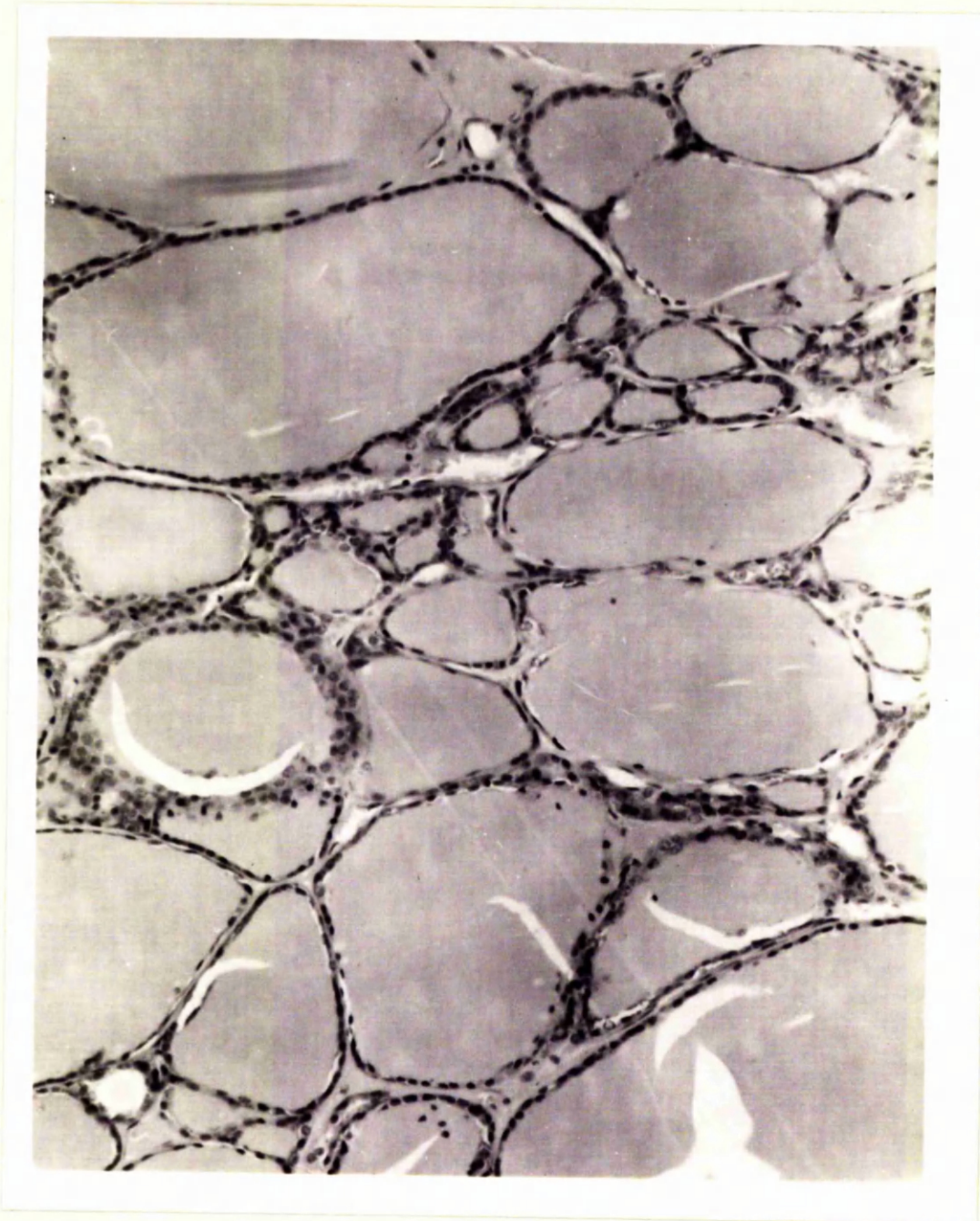

Fig. 32

Histological section of diffuse non-toxic human goitre. H and E x 190.

showed it to be entirely composed of light weight protein without any thyroglobulin present. ^{125}I and ^{3}H-leucine were not incorporated into protein >3-8S.

The remaining patient (Case 89) in this group was submitted to thyroidectomy because of a rapidly growing firm nodule in the right lobe of the thyroid gland. This likewise contained very little protein in the thyroglobulin region (approximately 5%), with the greatest amount of protein being present in the 3-8S region. ^{125}I and ^{3}H-leucine was not incorporated into protein larger than 3-8S. Histological examination of the nodule (fig. 33) shows this to be a solid adenoma composed of eosinophilic cells - a Hurtle cell adenoma. Very little colloid was present which correlates well with the OD pattern.

Fig. 33

Histological section of thyroid nodule (Case 89) showing Hurtle cell adenoma. H and E x 190.

CHAPTER 16

HASHIMOTO'S THYROIDITIS (Cases 90-94)

In all 5 patients with this condition were studied. Four of the patients presented the classical histological picture of this condition, as shown in fig. 34, but the remaining patient (Case 92) was histologically atypical, as shown in fig. 35, but was eventually judged by the pathologist as falling into this group of conditions.

A variety of OD patterns were obtained. In all, however, the obvious feature was a relative decrease in thyroglobulin and increase in 3-8S protein as compared with the normal gland (fig. 23) and Table 9. Indeed, in Case 92, only a light weight peak was present. In none of the 5 glands studied was a protein peak running in the 27S position seen. In two glands, however, as illustrated in fig. 36, a peak in the 32S region was noted. There was no histological difference between those with a 32S protein present or absent. The typical pattern of incorporation of ^{125}I and ^{3}H-leucine can be seen in fig. 36. ^{125}I was regularly incorporated in the thyroglobulin region and also into protein in the 12S and 3-8S stage. This pattern of ^{125}I incorporation was seen even in Case 92 in whom there was no thyroglobulin demonstrable on the thyroid protein scan. (Fig. 37).

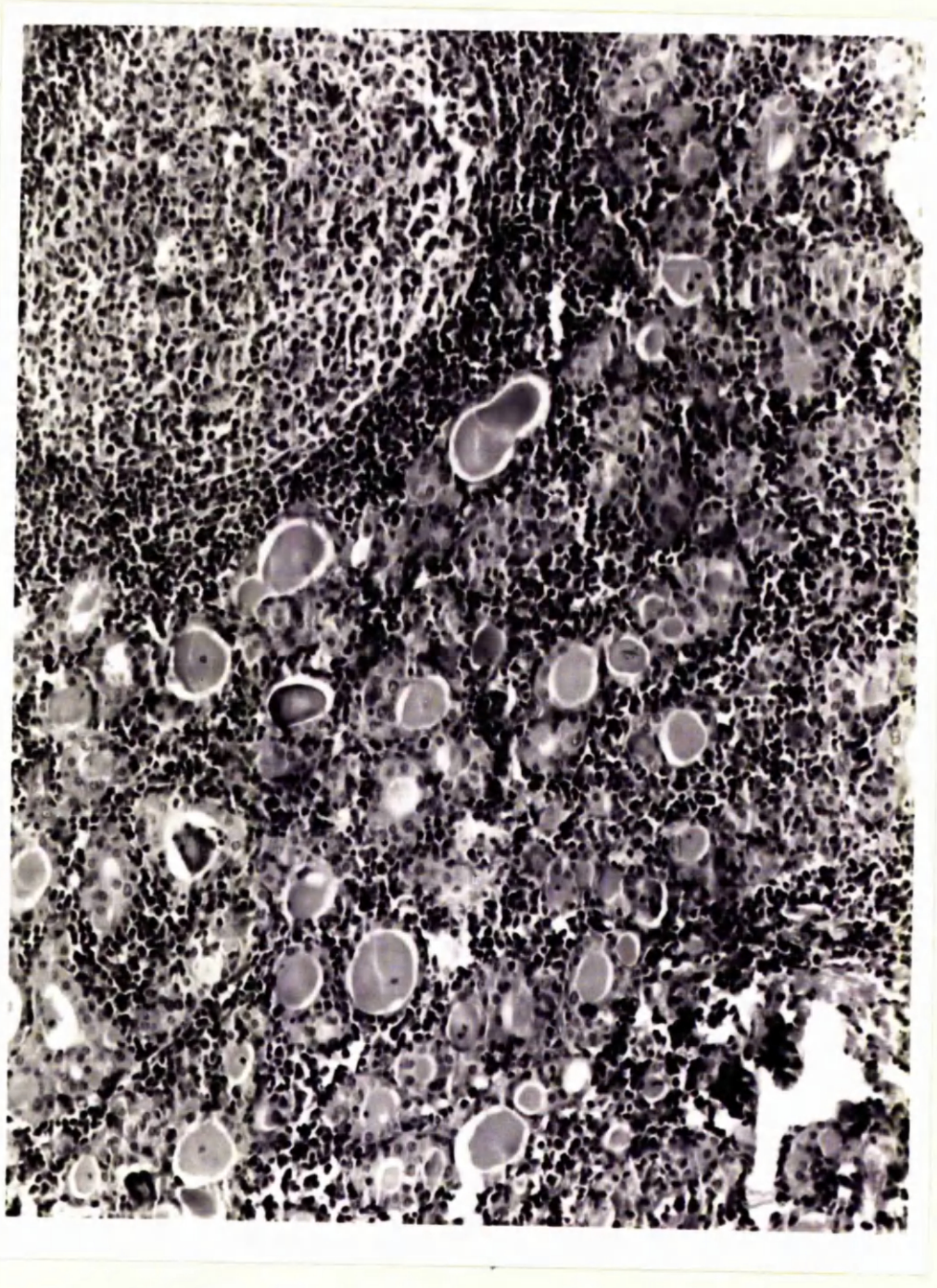

Fig. 34

Histological section of human thyroid showing appearances of Hashimoto's thyroiditis. H and E x 190.

Fig. 35

Histological section of thyroid gland of Case 92. H and E x 190.

Table 9:

Human thyroid glands from patients
with Hashimoto's thyroiditis
% of thyroid proteins

Case no.	32S	27S	19S	3-8S
90	-	-	38.0	62.0
91	10.4	-	32.0	57.6
92	-	-	-	100
93	5.8	-	-	94.2
94	-	-	53.5	46.5

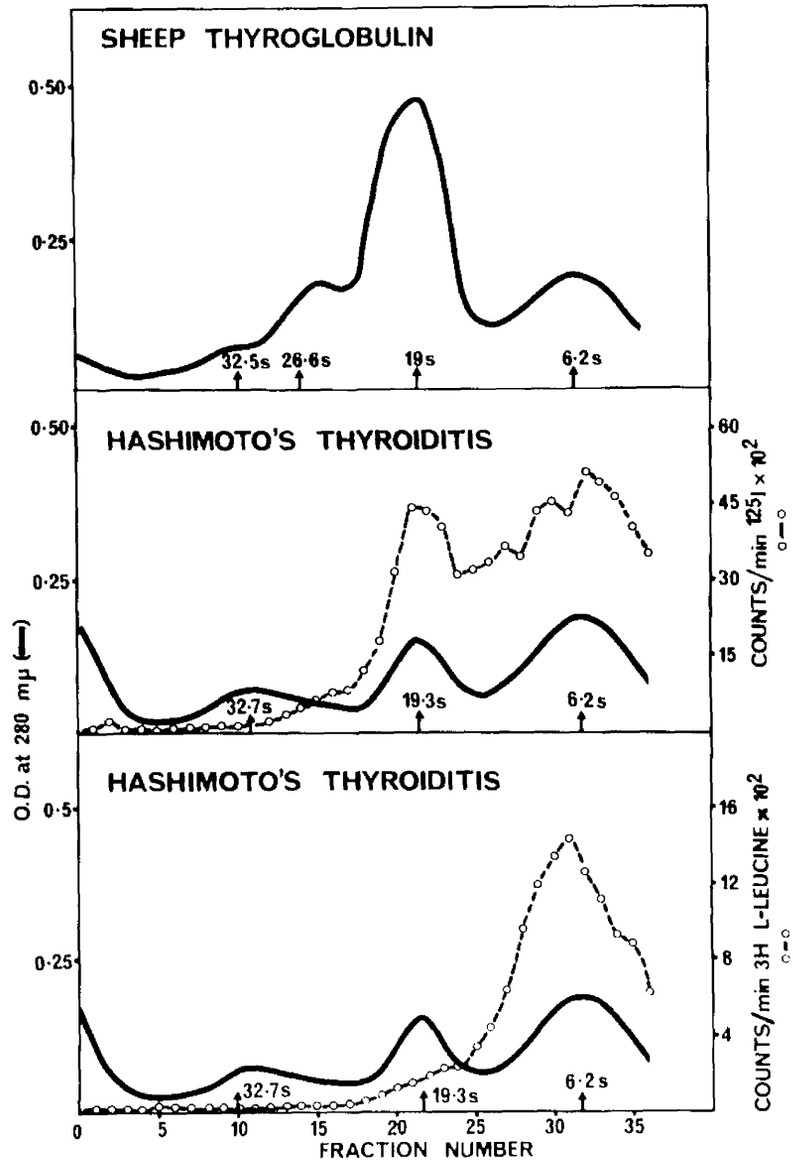

Fig. 36

Pattern of thyroid proteins from Hashimoto's thyroiditis (Case 91). Incorporation of ^{125}I and ^{3}H-leucine into thyroid slices after 4 hours incubation. SW 39 rotor at 24,000r.p.m. for 16 hours.

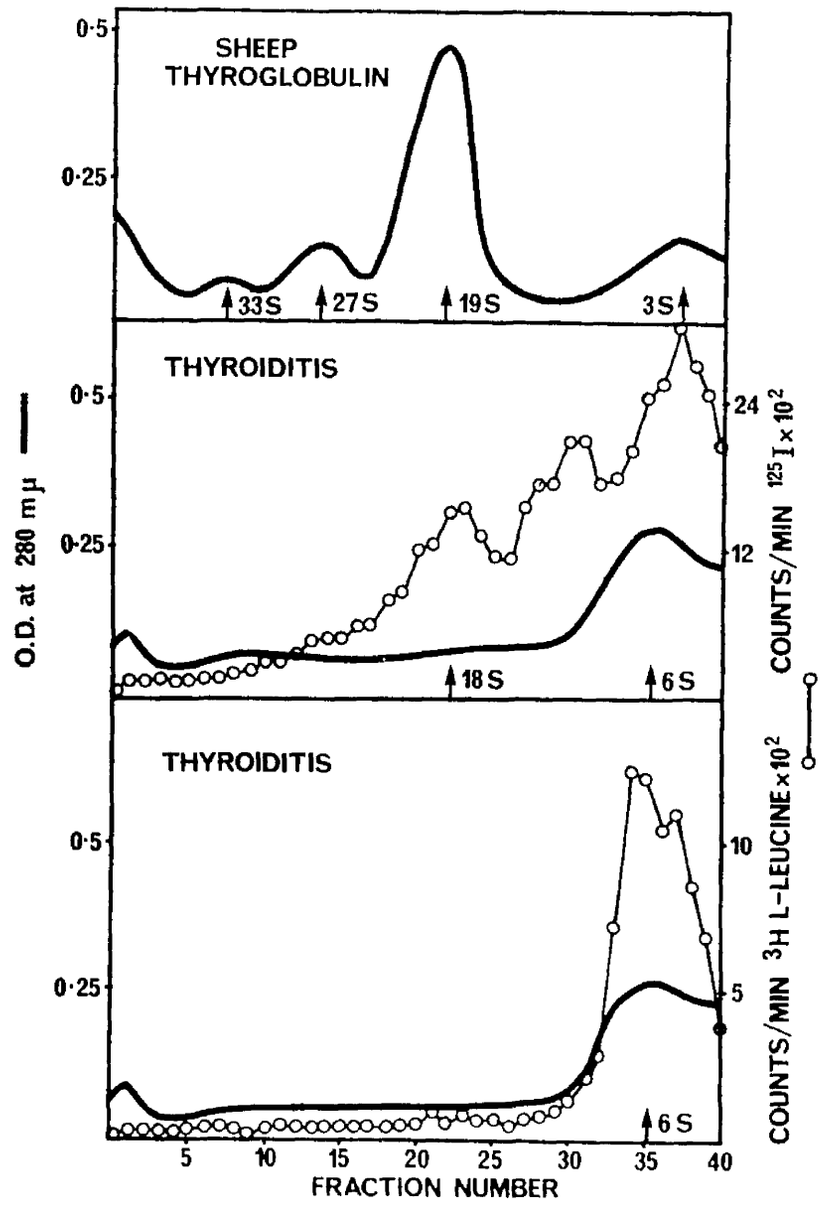

Fig. 37

Pattern of thyroid proteins and incorporation of ^{125}I and ^{3}H-leucine into the thyroid proteins after 4 hours incubation (Case 92). SW 39 rotor at 24,000r.p.m. for 16 hours.

CHAPTER 17

MALIGNANT THYROID GLANDS (Cases 95-99) (Table 10)

During the course of this study the opportunity arose to study material from 5 patients with thyroid neoplasia.

In 3 an anaplastic carcinoma was the histological diagnosis. In 2 of these the OD pattern was as shown in the middle portion of fig. 38. As can be seen, there is virtually no discernible OD peak in the thyroglobulin region; the protein consists almost entirely of a broad 3-8S peak. This correlates well with the histological picture of these tumours, as shown in fig. 39. It will be seen that the tissue is composed entirely of rather necrotic cells with no obvious colloid present in the sections.

In the remaining patient (Case 97) with an anaplastic tumour, a significant amount of protein was present in the thyroglobulin region as well as a peak in the 36S position. Histological examination of this tumour (fig. 40) showed that despite the diffuse replacement of the follicular tissue by malignant cells, there were surviving islets of follicles in the gland.

As might be anticipated, ^{125}I and ^{3}H-leucine were incorporated into the thyroglobulin region only in this last patient (Case 97). The predominant protein iodinated, however, was the

Table 10:

Malignant human thyroid glands

Case no.	Type of carcinoma	27S	19S	3-8S
95	Anaplastic	-	10.0	90.0
96	Anaplastic	-	-	100
97	Anaplastic	16.3	50.5	33.2
98	Papillary	12.3	77.8	9.9
99	Follicular	-	5.0	95.0

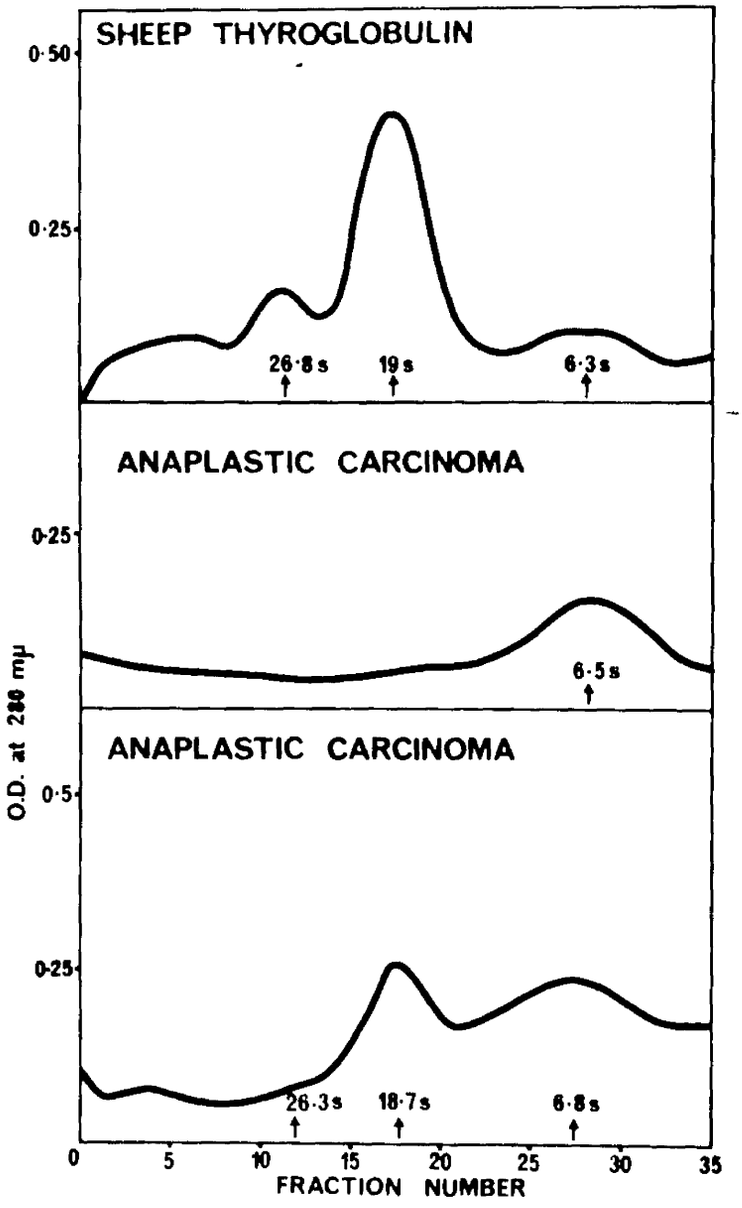

Fig. 38

Patterns of thyroid proteins in anaplastic carcinoma of the human thyroid. SW 39 rotor at 24,000r.p.m. for 16 hours.

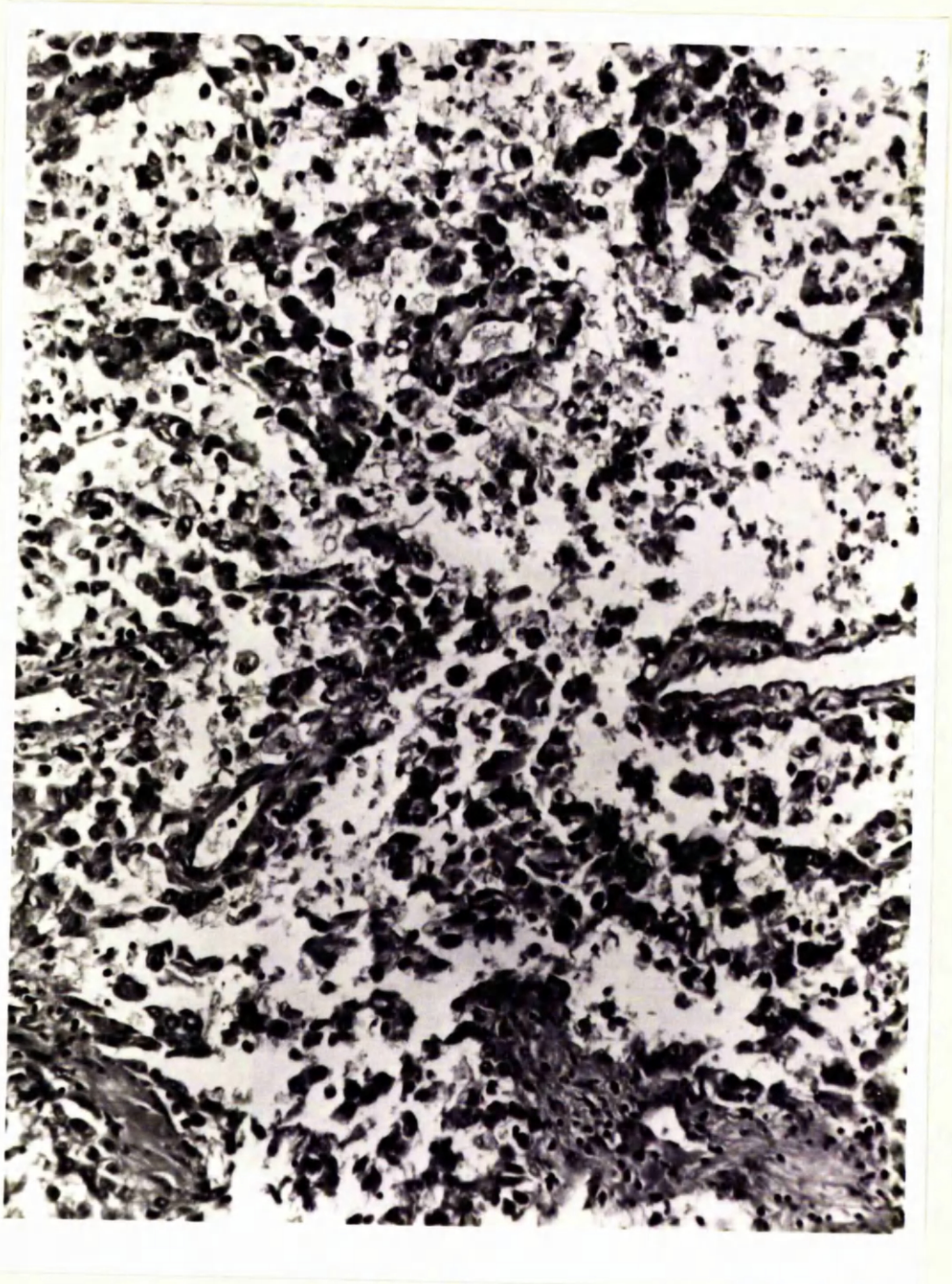

Fig. 39

Histological section of anaplastic thyroid carcinoma (Case 95). H and E x 190.

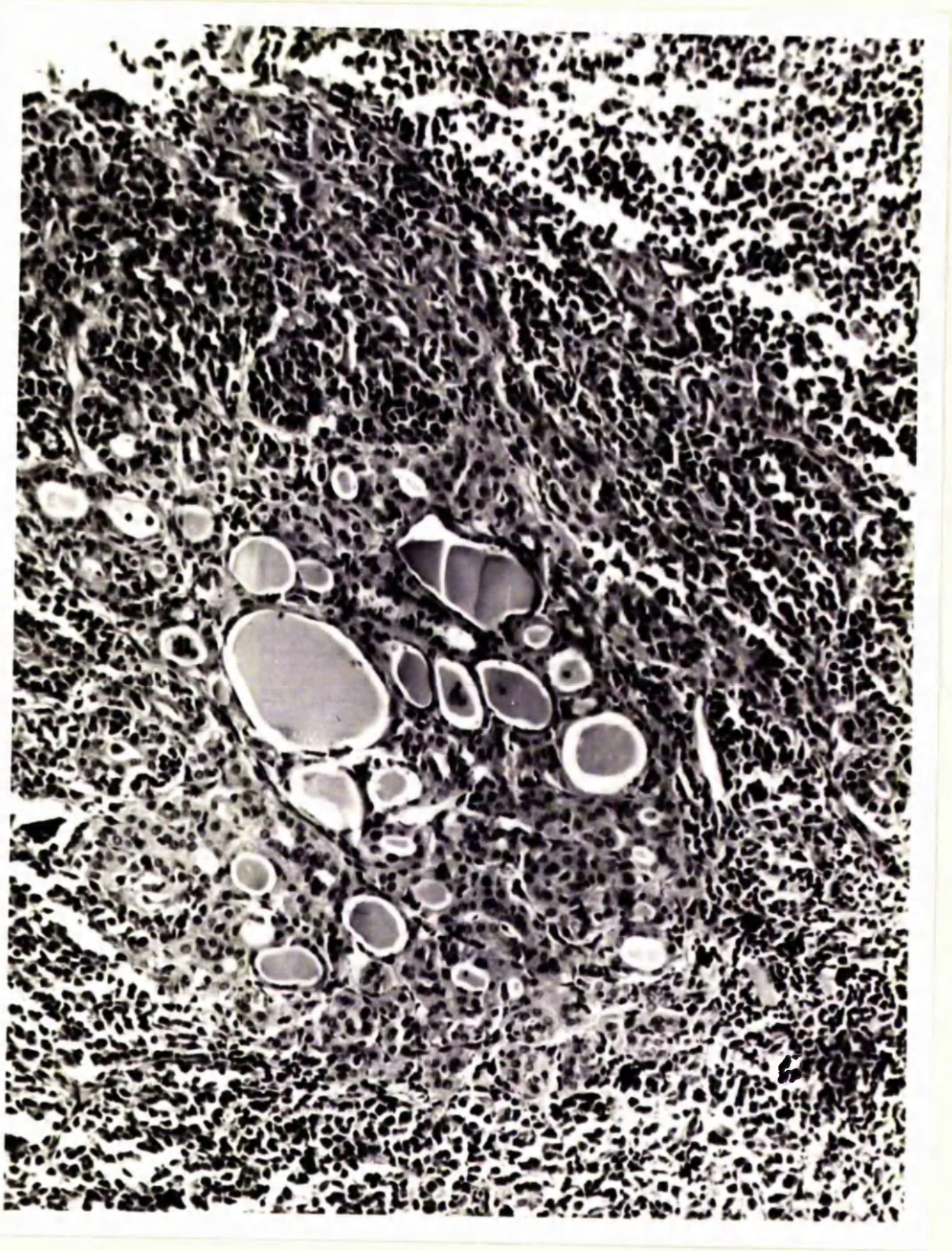

Fig. 40

Histological section of anaplastic carcinoma (Case 97). H and E x 190.

broad 3-8S peak. In the others ^{125}I was not incorporated and ^{3}H-leucine was incorporated only into the light-weight proteins.

One patient (Case 98) with a recurrent papillary tumour of the thyroid was seen. The OD pattern was very similar to that of a non-toxic goitre (fig. 31). Histological examination of the recurrent tumour showed that the papillary elements in fact made up a small proportion of the goitre (fig. 41).

One patient was seen (Case 99) in whom the diagnosis of a low grade follicular carcinoma of the thyroid was made. This patient presented with a large non-toxic goitre present for many years. At thyroidectomy, performed because of pressure symptoms, a 500G thyroid was removed. Histological examination of most of the resected tissue showed the appearance of a simple non-toxic goitre, and the OD pattern of the thyroid proteins was similar to fig. 31. In one area of the gland, however, a tumour invading the gland capsule was found and this showed appearances consistent with follicular carcinoma of the thyroid, containing very little thyroglobulin (fig. 42). The OD pattern of the thyroid protein from this part of the gland was consistent with this.

Fig. 41

Histological section of recurrent papillary carcinoma of human thyroid (Case 98). H and E x 190.

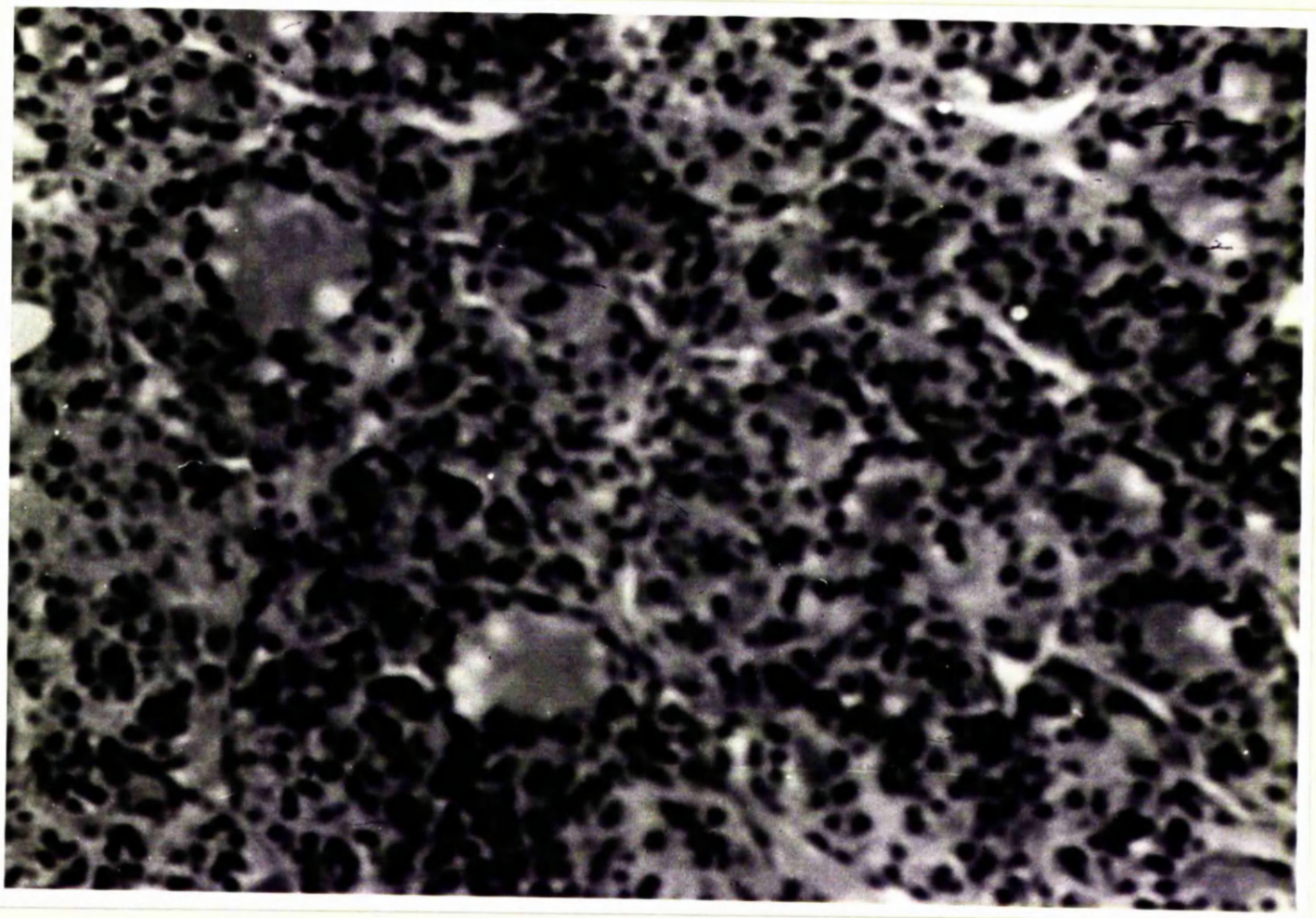

Fig. 42

Histological section of follicular carcinoma of thyroid (Case 99). H and E x 190.

CHAPTER 18

A POSSIBLE DYSHORMONOGENETIC GOITRE (Case 100)

This patient, a female aged 28, presented initially to the Thyroid Clinic at the Royal Infirmary, Glasgow in 1962. At that time she gave a history of a goitre of 6 months duration. For the preceding two years she had been taking large amounts of iodide in the form of 'elixir Sibec' a proprietary cough mixture which contains 3.588% W/V sodium iodide. At that time her uptake of radioiodine was extremely low and a diagnosis of iodide induced goitre was made. Following withdrawal of the iodide-containing medication her goitre became smaller.

In 1969 she was again referred because of increasing thyroid enlargement. She had taken no iodine-containing medication for at least two years. At this time she was clinically hypothyroid. This was confirmed by a T_3 resin sponge test result of 23.3% (normal 25-35%). The thyroid gland was of a firm consistency. A precipitin test for the presence of anti-thyroglobulin antibodies was negative. The ^{131}I tests carried out at this time showed an initially normal uptake pattern (gland uptake at 2 hours, 14% dose and at 4 hours, 30% of dose) but

which later fell at 24 hours to 14% of dose; the total plasma ^{131}I ($TP^{131}I$) at 48 hours was 0.38% dose/litre of plasma and the protein bound ^{131}I ($PB^{131}I$) was 0.01% dose/litre of plasma. A perchlorate discharge test shows no discharge of accumulated ^{131}I suggesting that there was no defect in tyrosine iodination. In view of the doubt about the diagnosis, and the history of recent enlargement, thyroidectomy was carried out.

Histological examination of the resected tissue (fig. 43) shows marked thyroid hyperplasia and no evidence of thyroiditis.

The OD pattern of the thyroid protein and the pattern of incorporation of ^{125}I and ^{3}H-leucine is shown in fig. 44. It will be noted that there is a relative loss of thyroglobulin and an increased 3-8S protein peak. There is no protein peak >19S. ^{125}I and ^{3}H-leucine are both incorporated into the 19S protein; ^{125}I is incorporated to a slight extent into the 12S protein. ^{3}H-leucine is incorporated into the light weight (3-8S) peak. No evidence of an abnormal iodinated 3-8S protein was obtained. In vitro studies using ^{125}I-labelled MIT showed that the gland deiodinated normally.

It is difficult to precisely pinpoint the defect in this gland,

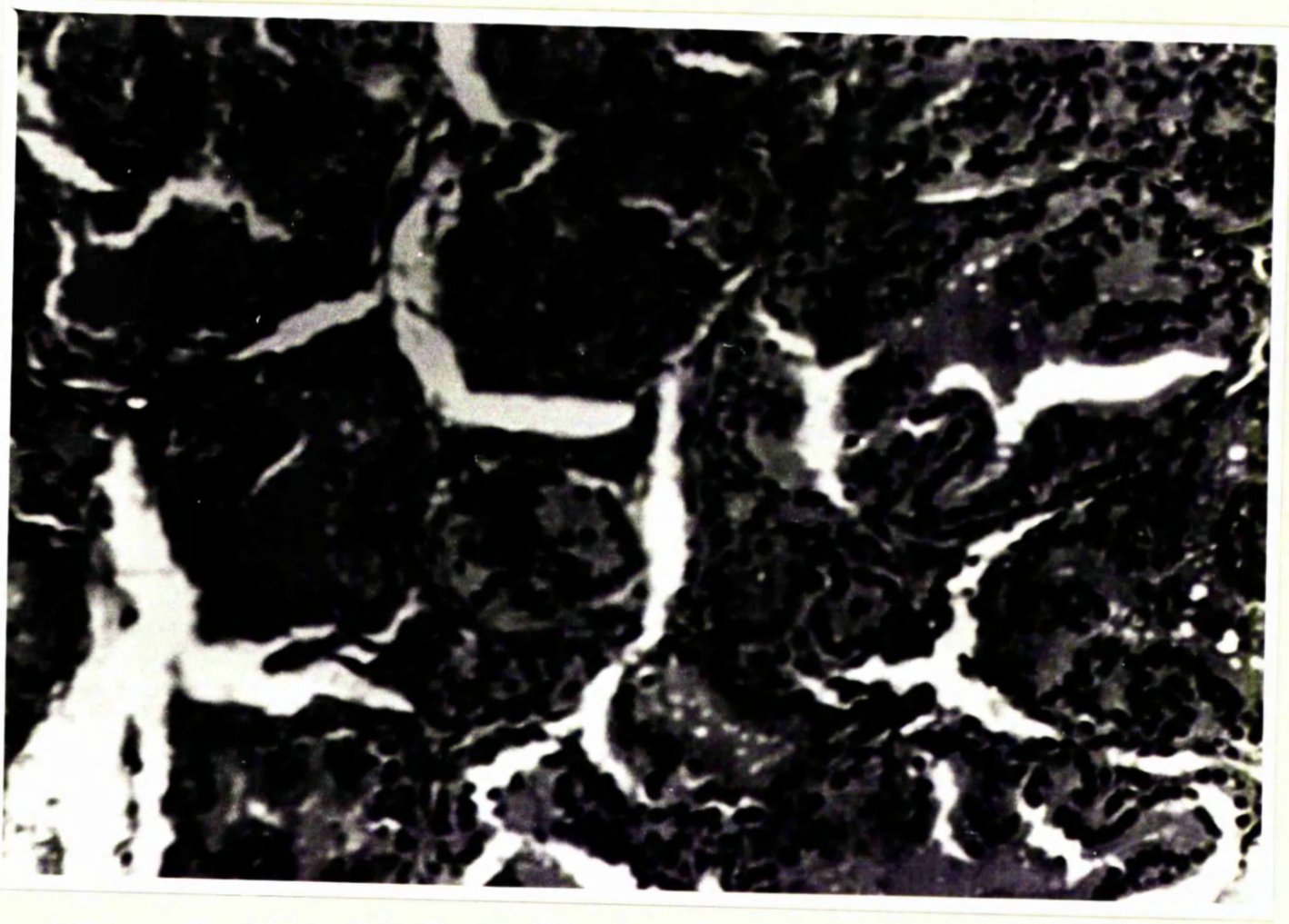

Fig. 43

Histological section of thyroid gland of Case 100. H and E x 190.

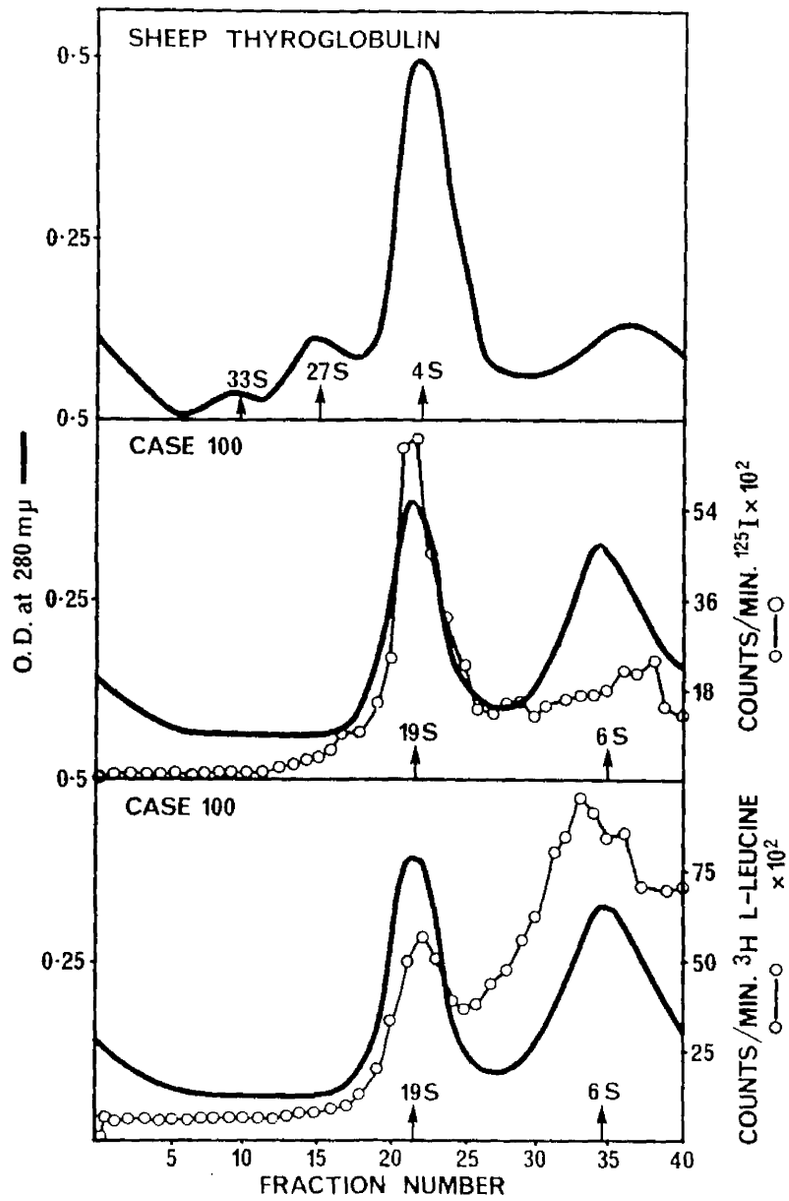

Fig. 44

Pattern of thyroid proteins from Case 100. Incorporation of ^{125}I and ^{3}H-leucine into thyroid slices after 4 hours incubation. SW 41 rotor at 28,000r.p.m. for 16 hours.

but the hyperplastic gland obviously under marked TSH stimulation and without evidence of thyroiditis, is suggestive of some defect in the pathway of thyroxine synthesis.

CORRELATION OF IODINE CONTENT OF THYROID PROTEINS WITH THYROID PATHOLOGY

The degree of iodination of the thyroid proteins was estimated by determining iodine/protein ratio of the soluble thyroidal proteins precipitated by 50% ammonium sulphate. This was done in all the 'normal' glands, all the thyrotoxic glands from patients treated with $KClO_4$, all Hashimoto's thyroiditis and all thyroid tumours. The same procedures were also carried out in randomly selected groups of 10 thyrotoxic patients treated with carbimazole and iodine (Case 38 being excluded), and 10 patients with either diffuse or multinodular non-toxic goitre or thyroid adenoma (Cases 88 and 89 being excluded). The results are shown in Table 11.

In the group of 8 normal glands, the mean iodine content of the thyroid proteins was 2.81μG iodine/mg protein (range 1.60-3.68). In thyrotoxic patients pre-treated with carbimazole and iodine, the mean result was 0.87μG iodine/mg protein (range 0.42-1.74). This is significantly different from the result obtained in the 'normal' group ($p<0.001$), but is not significantly different from the group of thyroxic patients treated before operation with $KClO_4$ (mean 0.73μG iodine/mg

Table 11:

Correlation of iodine content of thyroid proteins with thyroid pathology

Histological diagnosis	No. in group	Iodine content of thyroid proteins (µG iodine/mg protein)
'Normal'	8	2.81 (range 1.60-3.68)
Thyrotoxicosis (carbimazole/iodide treatment)	10	0,87 (range 0.42-1.74)
Thyrotoxicosis ($KClO_4$ treatment)	5	0.73 (range 0.51-1.12)
Non-toxic goitre (including thyroid adenoma)	10	0.83 (range 0.13-1.40)
Hashimoto's thyroiditis	5	0.81 (range 0.16-1.95)
Thyroid carcinoma	5	0,25 (range 0.02-0.42)

protein - range 0.51-1.12). The number in this last group is too small to permit formal statistical analysis.

The group of non-toxic goitre (including thyroid adenoma) was also not significantly different in iodine content (mean 0.83μG iodine/mg protein - range 0.13-1.40) from the thyrotoxic patients. The mean of this group is, however, significantly different from the 'normal' group ($p<0.001$).

The mean value for the group of patients with Hashimoto's disease was 0.81μG iodine/mg protein (range 0.16-1.95). This falls into the same range as the results of the patients with thyrotoxicosis or non-toxic goitre.

The small group of patients with thyroid carcinoma had, as anticipated, the lowest value for iodination of the thyroid protein (mean value 0.25μG iodine/mg protein - range 0.02-0.42).

It is noteworthy that only one pathological gland had an iodine content whose value overlapped the lowest value from the group of 'normal' glands. This was from a thyrotoxic patient treated with carbimazole and iodine pre-operatively.

CHAPTER 20

SUMMARY OF SECTION 4

In this section the changes in the thyroid protein patterns in human thyroids - both 'normal' and from a variety of diseased states - are described and the results of *in vitro* incubation of thyroid slices from these glands with ^{125}I and ^{3}H-leucine documented.

In the 'normal' human thyroid the main protein was thyroglobulin and both ^{125}I and ^{3}H-leucine were well incorporated into this protein. A 27S and 3-8S protein peak were also seen.

In thyrotoxicosis the amount of thyroglobulin found varied with the pre-operative drug treatment, but the striking feature was a lack of thyroid proteins >19S. ^{3}H-leucine was well incorporated into thyroglobin but ^{125}I was poorly incorporated due to the pre-operative drug treatment.

In non-toxic goitre and thyroid adenomas (with occasional exceptions) a normal protein pattern was found and ^{125}I and ^{3}H-leucine was normally incorporated into thyroglobulin.

In Hashimoto's thyroiditis a variety of thyroid protein patterns were found, but there was a constant diminution in the percentage of the thyroid proteins present as thyroglobulin.

In 2 of 5 glands a 32S peak was noted. ^{125}I was incorporated not only into thyroglobulin, but also into lighter proteins; ^{3}H-leucine was not incorporated *in vitro* into thyroglobulin.

In malignant thyroids there was a diminution in the thyroglobulin content of the thyroids and an increased light-weight protein. ^{125}I and ^{3}H-leucine were poorly incorporated into the thyroid proteins, the degree of incorporation reflecting the degree of differentiation of the thyroid tumour.

In general there was a good correlation between the protein pattern and the histological appearance of the thyroid. Where little colloid was seen there was a relative lack of 19S protein and a reciprocal increase in 3-8S protein.

The mean iodine content of the thyroid proteins (2.81μG/mg protein) was approximately three times greater in the 'normal' thyroids than in any of the other pathological groups. The other groups (thyrotoxicosis, non-toxic goitre, Hashimoto's thyroiditis) all had a remarkably similar iodine content per mg protein. Thyroid carcinomas contained on average approximately 10% of the 'normal' iodine content per mg protein.

SECTION 5

THYROID PROTEINS IN VERTEBRATES

CHAPTER 21

THYROID PROTEINS OF NORMAL VERTEBRATES

A limited study was made of the distribution of the thyroid proteins in different vertebrates. The results are summarised in Table 12. The patterns found in 'normal' human thyroid material has already been detailed in Table 4.

Most of the vertebrates studied had an OD pattern of thyroid proteins similar to that of the normal human, as shown in fig. 23. Thyroglobulin constituted the main protein in all glands studied from normal animals, ranging from a mean value of 68.4% of the total thyroid protein in the rat to 88.1% in the cow.

In all a well marked 3-8S peak was present. This ranged in amount from 6.5% in the cow to 25.3% in the rat.

In all animals studied, except the horse, a definite 27S peak was seen. This ranged in amount from 5.4% in the cow to 15.0% in the guinea pig. There was no constant alteration in distribution of the thyroid proteins with body size.

In the two examples of horse thyroid studied the pattern was that shown in fig. 45. At the most only the merest trace of 27S protein was present.

In sheep thyroid preparations a 32S peak was commonly seen.

Table 12:

Distribution of thyroidal proteins in different vertebrates

Animal	No. in group	27S	19S	3-8S
Mouse	6	5.3	80.7	14.0
Rat	10	6.3	68.4	25.3
Hamster	3	10.1	70.3	19.6
Guinea pig	3	15.0	78.2	6.8
Rabbit	2	8.1	75.4	16.5
Cat	2	7.5	75.7	16.8
Dog	2	5.8	78.5	15.7
Sheep	10	12.6	77.3	10.1
Cow	2	5.4	88.1	6.5
Horse	2	-	87.4	12.6

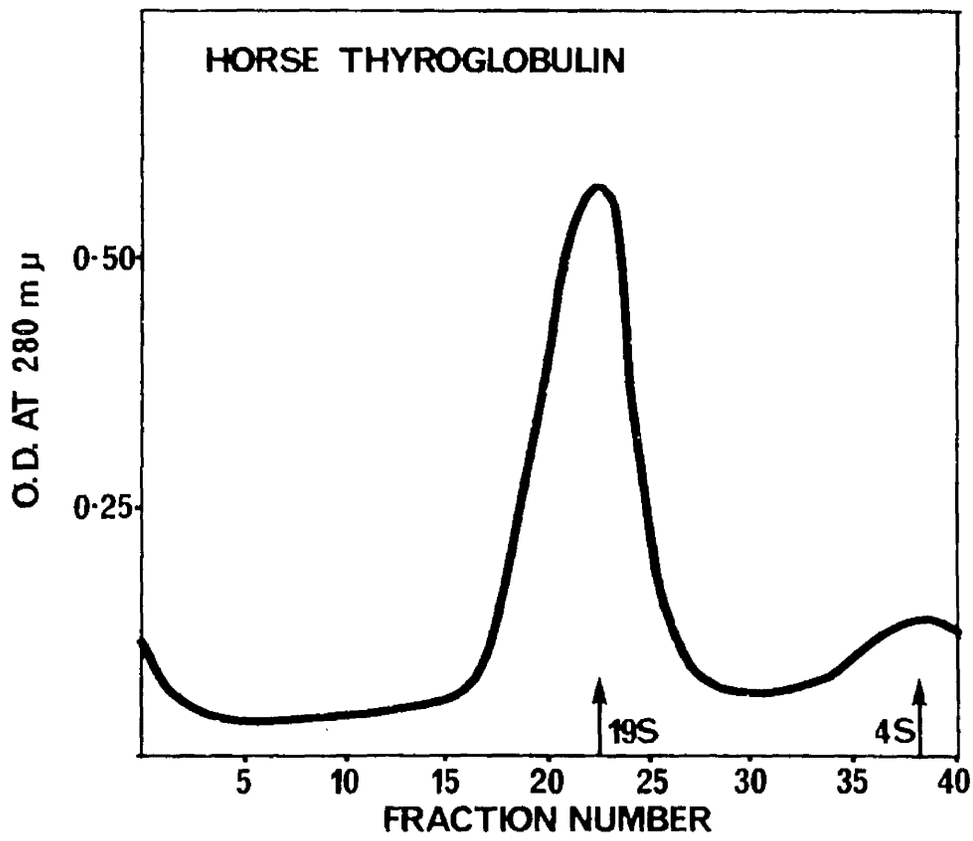

Fig. 45

Pattern of thyroid proteins from horse thyroid gland.
SW 41 rotor at 28,000r.p.m. for 16 hours.

Histological examination of the thyroid glands of the vertebrates studied showed a similar pattern to that of the normal rat and human, shown in fig. 1 and 22 respectively, and are, to avoid repetition, not illustrated. The exception was the horse in which the histological picture was as shown in fig. 46. There was in both animals studied evidence of increased TSH activity, as shown by the appearance of many absorption vesicles at the periphery of the colloid.

Iodine content of normal vertebrate glands

The results are detailed in Table 13. It will be seen that most of the animals studied had more iodine/mg protein than did the 'normal' human studied (mean 2.81μG iodine/mg protein; range 1.60-3.68). The majority of animals fell in the range of approximately 4.0-6.0μG iodine/mg protein. The only samples which fell outside this range were the cat and dog - perhaps due to the iodine content as fish supplements of proprietary pet foods - and the guinea pig which was lower than the rest at 1.99μG iodine/mg protein.

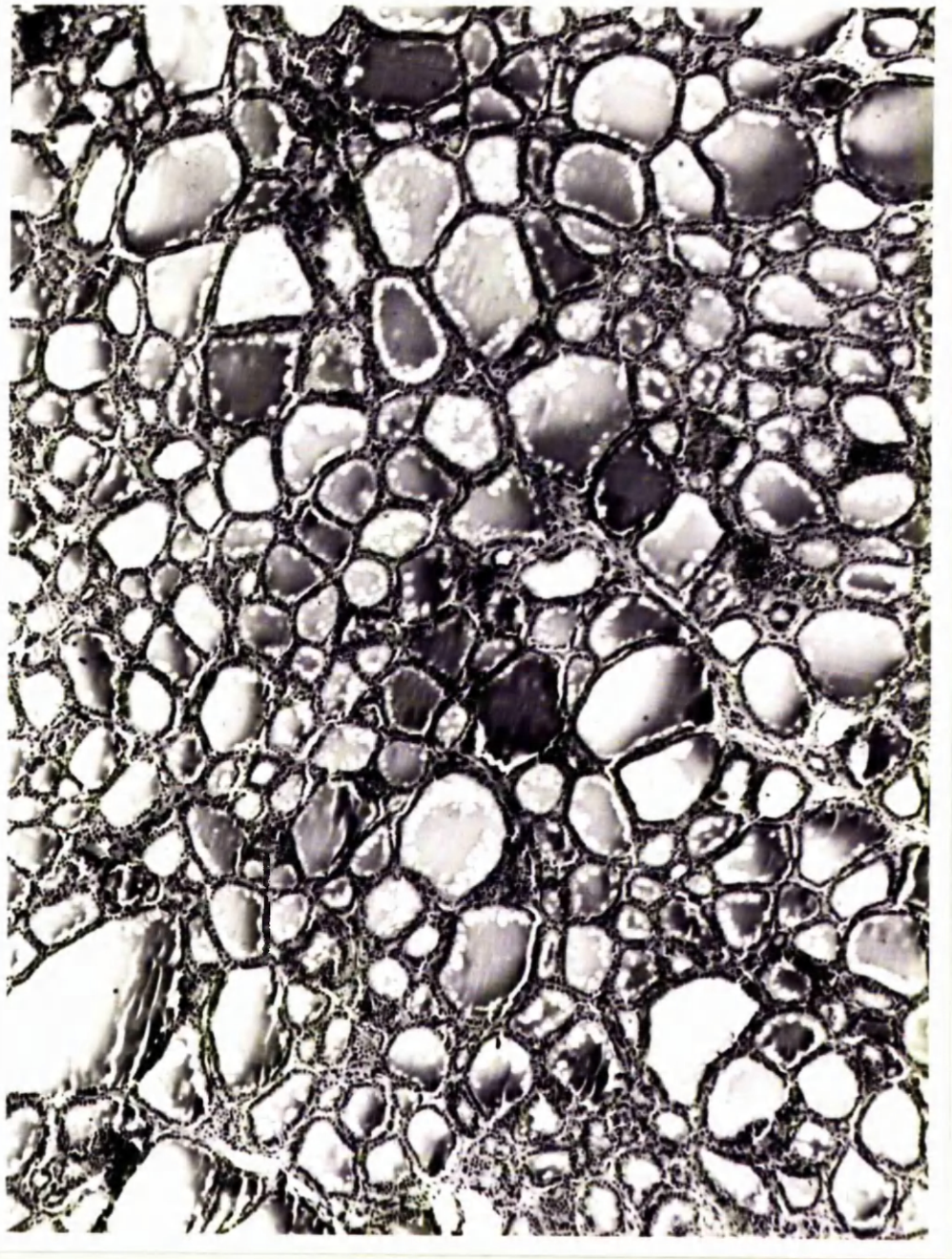

Fig. 46

Histological section of thyroid tissue of horse. H and E x 75.

Table 13:

Iodine content of thyroid proteins of different vertebrates

Animal	No. in group	Iodine content of thyroid protein (µG iodine/mg protein)
Mouse	6	5.15
Rat	10	5.40
Hamster	3	4.80
Guinea pig	3	1.99
Rabbit	2	6.15
Cat	2	8.16
Dog	2	8.11
Sheep	10	5.15
Cow	2	5.97
Horse	2	4.06

CHAPTER 22

THYROID PROTEINS OF ANIMAL GOITRES

During the course of the study the opportunity arose to study spontaneously occurring goitre in two animals.

The first was a thyrotoxic boxer dog, which was studied in Boston. In this animal the thyrotoxic state was due to a hyperfunctioning thyroid adenoma. Tissue was obtained at thyroidectomy performed without preliminary antithyroid medication, the peripheral manifestations of thyrotoxicosis being controlled with propranolol during anaesthesia. At operation a necrotic cystic nodule was removed. Portions were incubated in vitro as described in Section 2.

The OD pattern of the thyroid protein was as shown in fig. 47. At this time alterations in thyroid protein >19S were not appreciated and no conclusions can be drawn about these. It will be noted that a small amount of thyroid protein runs in the thyroglobulin region although short of 19S. The main peak is in the 3-8S position. ^{125}I is poorly incorporated into the thyroid proteins, but does appear to a slight extent in the 18S protein. ^{3}H-leucine is well incorporated into the 18S protein and also into the 12S and 3-8S proteins.

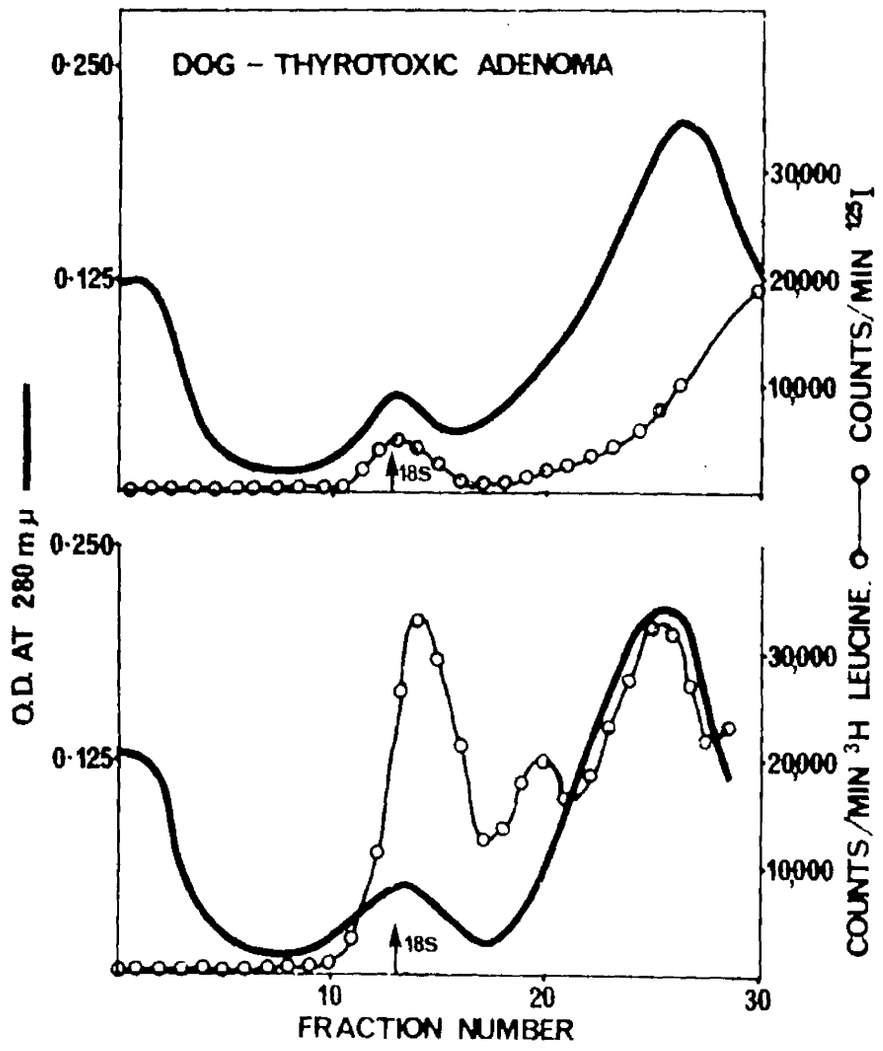

Fig. 47

Pattern of thyroid proteins from thyrotoxic boxer dog.
Incorporation of ^{125}I and ^{3}H-leucine into thyroid slices after 4 hours incubation. SW 25.1 rotor at 21,000r.p.m. for 40 hours.

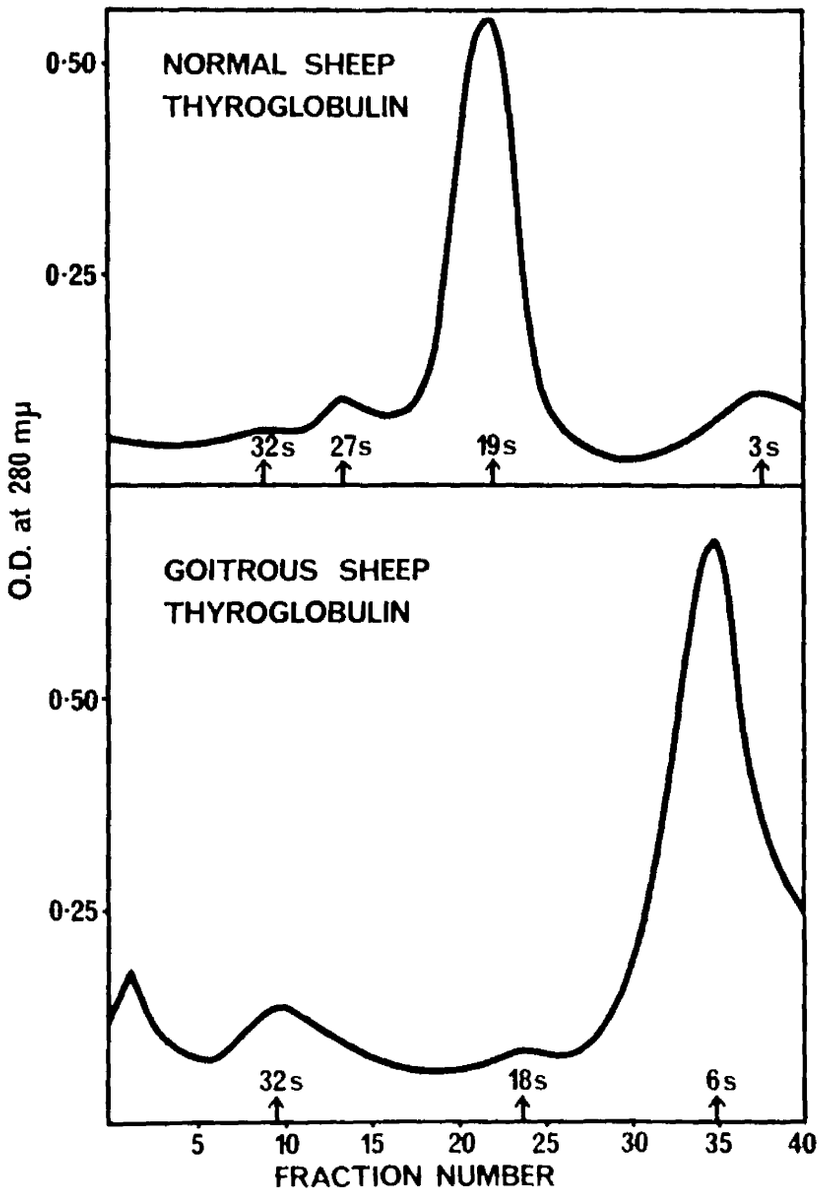

Fig. 48

Pattern of thyroid proteins from goitrous merino sheep. SW 41 rotor at 28,000r.p.m. for 16 hours.

The second goitrous animal studied was a thyroid gland from one of the goitrous merino sheep described in detail by Falconer (1966). In these animals the present evidence points to there being a congenital goitre associated with the production of an abnormal iodinated thyroid protein. Thyroid tissue removed from a goitrous sheep was transported in a frozen state by air from Adelaide to Glasgow. Because of the time of transportation no incubation studies were performed. The OD pattern is shown in fig. 48. It will be seen that very little protein is present in the thyroglobulin region. A large 3-8S peak is present as is a well defined 32S peak. No 27S protein is present. The iodine content of the thyroid proteins from this animal was very low at 0.4μG iodine/mg protein.

CHAPTER 23

SUMMARY OF SECTION 5

From the limited studies carried out it would appear that most of the vertebrates studied have a very similar pattern of thyroid proteins. The horse, however, is unusual in having virtually no 27S protein.

Likewise, the iodine content of the thyroid proteins of the vertebrates studied fall into a very narrow range. In some - the cat and the dog - there are probably adequate dietary reasons to explain the higher values found. The guinea pig has a lower iodine content than the other animals studied.

In the two animals studied with spontaneous goitres, the OD pattern of the thyroid proteins was similar to that obtained in experimentally induced goitres in the rat.

SECTION 6

THYROID PROTEINS >19S

CHAPTER 24

INTRODUCTION

It has already been noted in the preceding Section that in most vertebrates a 27S protein peak is present in addition to the 19S and 3-8S peaks. However, as detailed in Section 4, in thyrotoxic human thyroid glands usually no protein >19S was present, whereas in Hashimoto's thyroiditis a 32S OD peak was seen on occasions. A small 32S peak was also frequently seen in preparations of normal sheep thyroid proteins and on rare occasions in preparations of 'normal' human thyroid proteins.

In this Section it is intended to explore the situations in which alterations in the OD peaks >19S occur.

CHAPTER 25

THYROID PROTEIN >19S IN THE GOITROUS RAT

The OD tracings of the thyroid proteins from normal rats and from rats made goitrous by PTU administration are shown in fig. 49. For this study conditions of ultracentrifugation were chosen so that proteins >19S were well separated from the bottom of the gradient. It will be noted that although in the normal rat the previously described pattern of predominant 19S peak with smaller 27S and 3-8S peaks is seen, in the goitrous rat the 3-8S protein peak is now the predominant one, with the peak in the thyroglobulin area being markedly diminished relative to the 3-8S peak. No definite 27S peak can be seen in the goitrous gland, but a well marked OD peak in approximately the 32S area is seen.

This OD pattern of thyroid proteins was found in the goitrous rat whether the goitre had been caused by the administration of a goitrogenic drug such as PTU, MTU (methylthiouracil), carbimazole, methimazole, $KClO_4$, or by the prolonged administration of a low iodine diet.

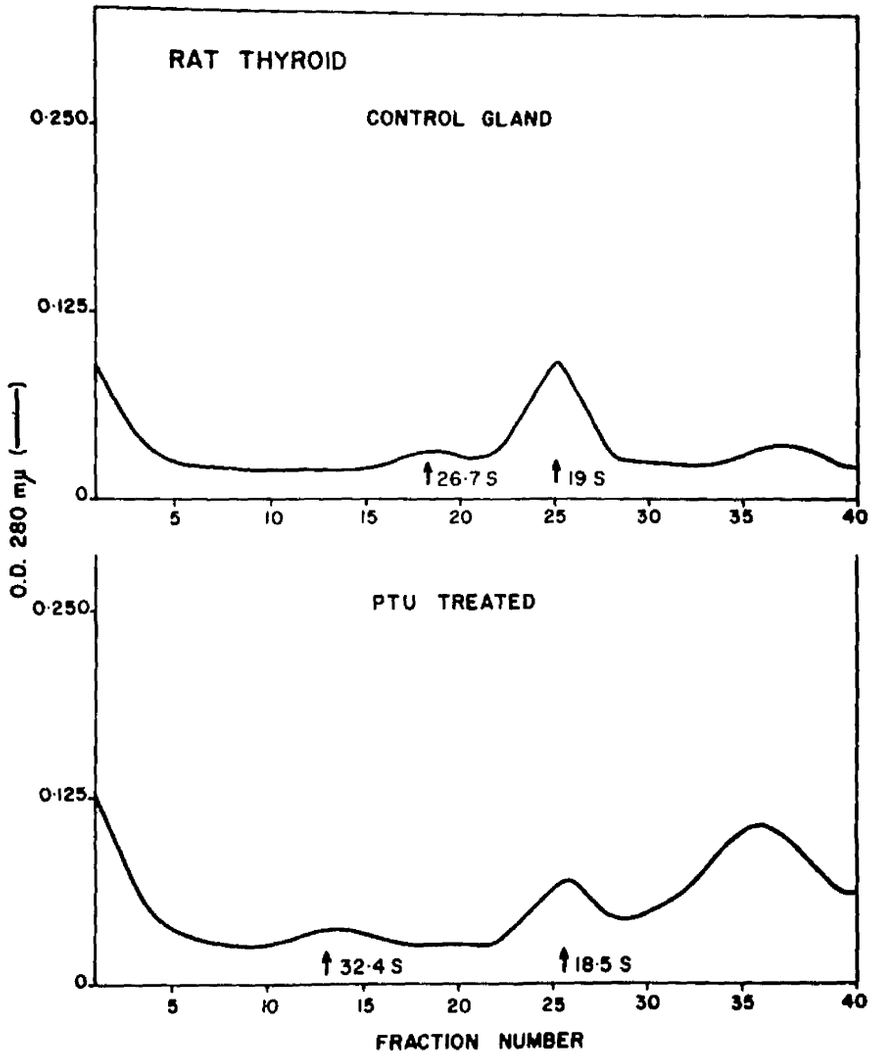

Fig. 49

Pattern of thyroid proteins from normal and PTU treated rats. SW 25.1 rotor at 21,000r.p.m. for 40 hours.

Effect of iodine supplementation and thyroxine administration on the thyroid proteins of the goitrous rat

When a PTU-treated rat was given 0.05% KI in the drinking water for one week before sacrifice, the change in OD pattern from the unsupplemented animal was as shown in fig. 50. It will be noted that in the animal receiving iodine supplementation there is a relative increase in thyroglobulin and a reciprocal decrease in 3-8S protein. The 32S peak is no longer visible. It is doubtful whether any 27S peak is present in the iodine treated animal. A very similar pattern of results was obtained in carbimazole-treated rats supplemented with iodine. Once again the 32S peak disappeared, but no convincing 27S peak could be seen.

In contrast to this, in the case of rats rendered goitrous by $KClO_4$ treatment, in which the same iodide supplementation was given before sacrifice, a different pattern was obtained. In these animals, although the initial OD tracing was very similar to the PTU-treated animals, following iodine supplementation disappearance of the 32S OD peak was associated with the return of a 27S peak in addition to the expected changes in the thyroglobulin and 3-8S peaks (fig. 51).

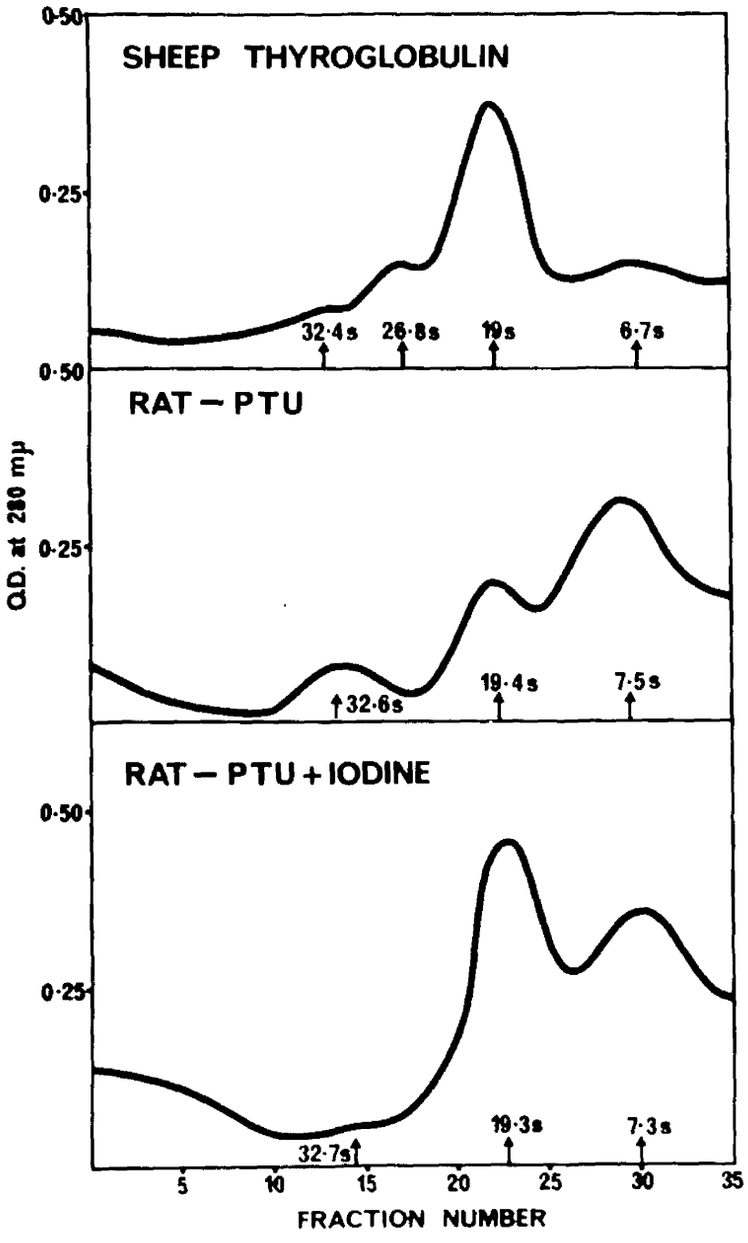

Fig. 50

Pattern of thyroid proteins from PTU treated rats without and with iodine supplements. SW 39 rotor at 24,000r.p.m. for 16 hours.

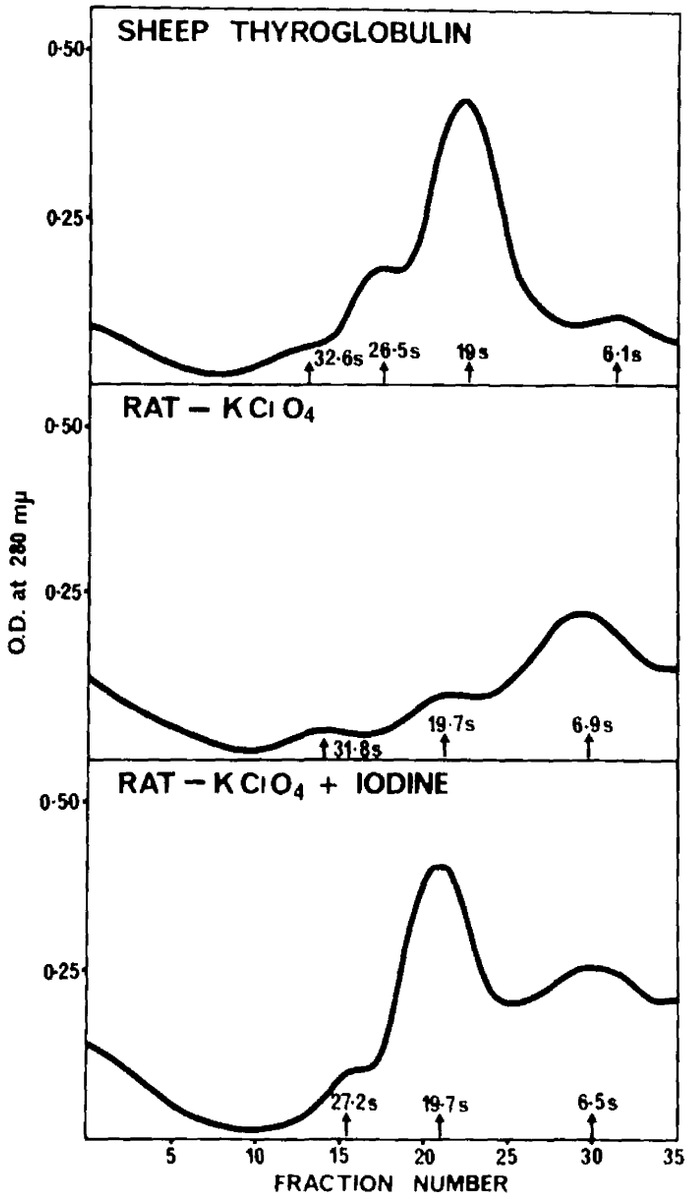

Fig. 51

Pattern of thyroid proteins from $KClO_4$ treated rats without and with iodine supplements. SW 39 rotor at 24,000r.p.m. for 16 hours.

A similar pattern to the $KClO_4$-treated rats was obtained from rats rendered goitrous by a low iodine diet and subsequently supplemented by iodide (fig. 52).

As will be seen from the lower part of fig. 52, the administration of T_4 in the diet of this last group of animals, in the dosage of 2mg T_4/Kg diet for the last week before sacrifice, resulted in changes very similar to those produced by iodine supplementation. The thyroglobulin peak increased as compared to the animals treated by the goitrogenic diet alone; the 3-8S peak diminished relative to the thyroglobulin peak and a definite 27S peak was seen as the only OD peak >19S.

The effect of T_4 supplementation of a goitrogen-treated animal (for example PTU or carbimazole treated) resulted in a similar disappearance of the 32S OD peak and the formation of a definite 27S peak.

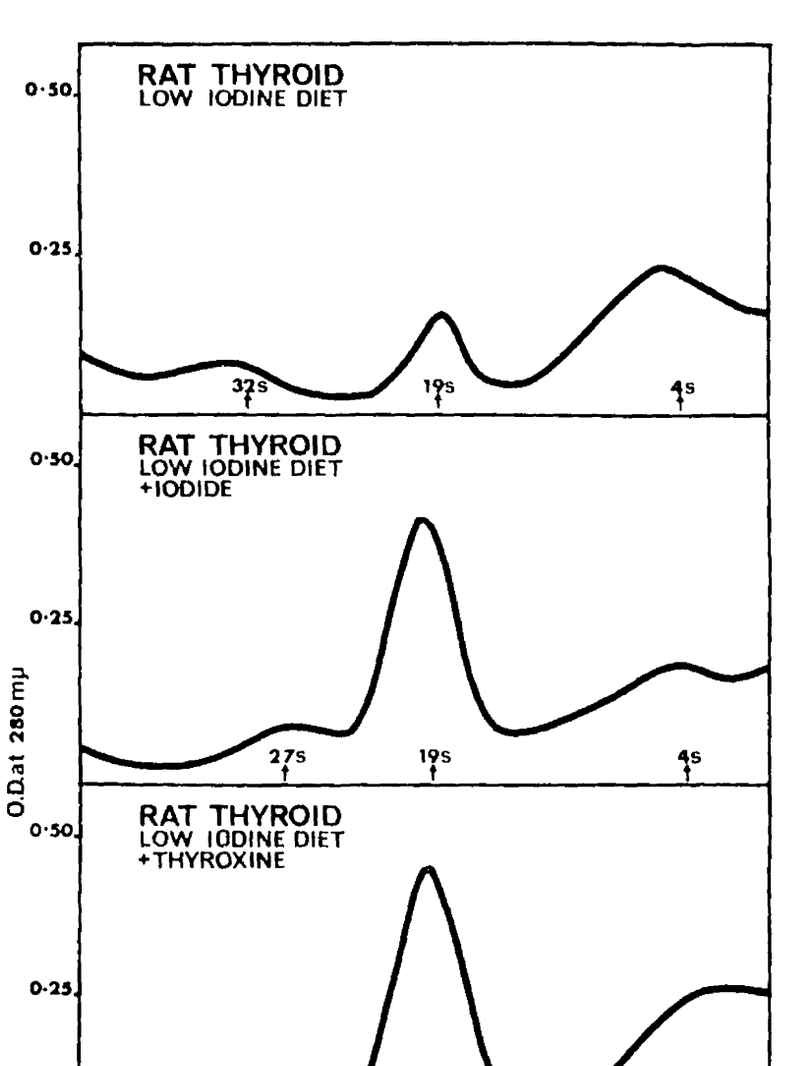

Fig. 52

Pattern of thyroid proteins from rats on low iodine diet - effect of iodine supplements and thyroxine treatment. SW 39 rotor at 24,000r.p.m. for 16 hours.

CHAPTER 26

EFFECT OF DIFFERENT TECHNIQUES OF PREPARATION OF THE SOLUBLE THYROID PROTEINS ON THE PRESENCE OF THE 32S OD PEAK IN THE GOITROUS RAT THYROID

In an attempt to ensure that the 32S OD peak was not a technical artefact in preparation of the soluble thyroid proteins, a variety of standard techniques of preparation of the soluble thyroid proteins were examined. For control purposes the results of the standard methods used are reproduced in fig. 53. This method has been described in detail in Section 2. Briefly, it consists of a homogenisation step followed by an initial centrifugation at 20,000g for 10 minutes to remove cellular debris. The OD peak of the resultant supernatant is shown in the upper part of fig. 53. It will be noted that much the largest OD peak is in the 3-8S region. A smaller peak in the thyroglobulin region can be seen as can a small peak in the 32S region. This initial step is followed by precipitation of the soluble thyroid proteins by 50% saturation with ammonium sulphate with subsequent recentrifugation at 20,000g for 10 minutes. The OD pattern of this material - the standard preparation used in this work - is shown in the lower part of fig. 53. It will be seen

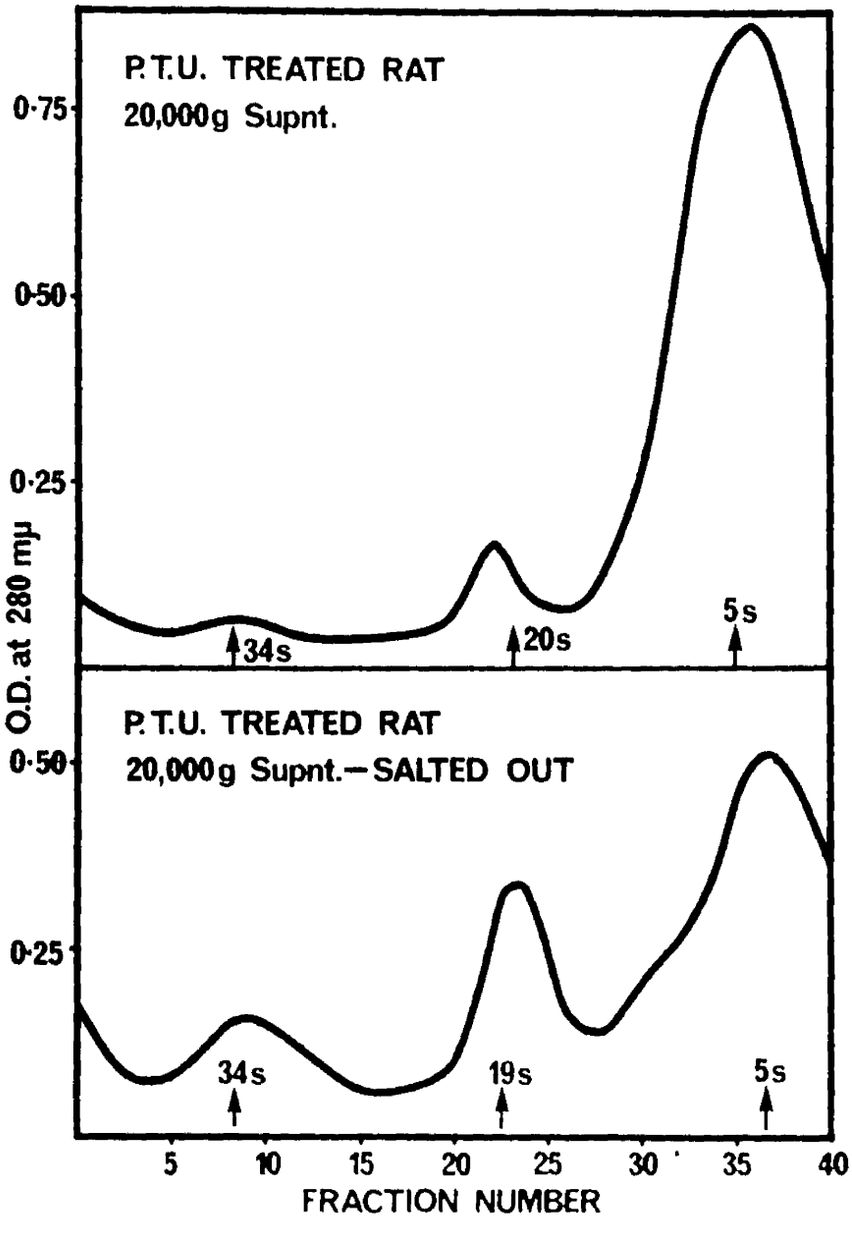

Fig. 53

Pattern of thyroid proteins from PTU treated rat. Effect of 50% saturation by ammonium sulphate on the 20,000g supernatant. SW 41 rotor at 28,000r.p.m. for 16 hours.

that the step of ammonium sulphate fractionation has caused a relative diminution in the 3-8S peak, with a relative increase in the peak in the thyroglobulin region and of the 32S OD peak.

In fig. 54 is illustrated the results of the more complex method of preparation of the soluble thyroid protein used by Derrien et al (1948). This method consists essentially of a series of fractionation steps using ammonium sulphate and the final preparation consists of the soluble thyroid proteins precipitating between 35% and 41% saturation with ammonium sulphate. As can be seen, the pattern of the thyroid proteins obtained is similar to that obtained with the standard technique used in this thesis, i.e. a predominant 3-8S peak is present, as is the rather smaller peak in the thyroglobulin region. A 32S peak is also present.

Lastly a standard technique for preparation of soluble cellular proteins was used. Following homogenisation of the thyroid tissue the preparation was spun in a type 50 rotor at 105,000g for one hour. The supernatant from this step was then submitted to sucrose density ultracentrifugation as shown in the upper part of fig. 55. It will be noted that the 3-8S peak is very large indeed; the thyroglobulin peak is relatively small

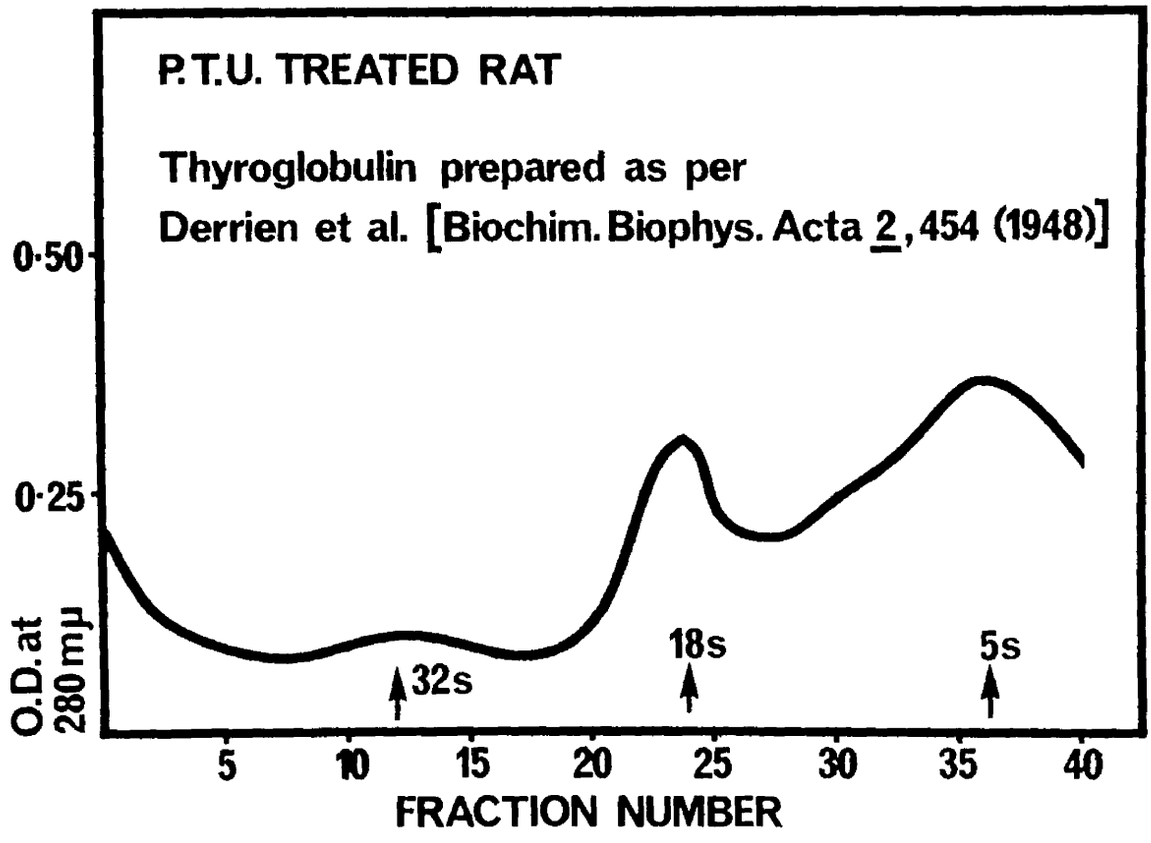

Fig. 54

Pattern of thyroid proteins from PTU treated rat prepared by the technique of Derrien et al (1948). SW 41 rotor at 28,000r.p.m. for 16 hours.

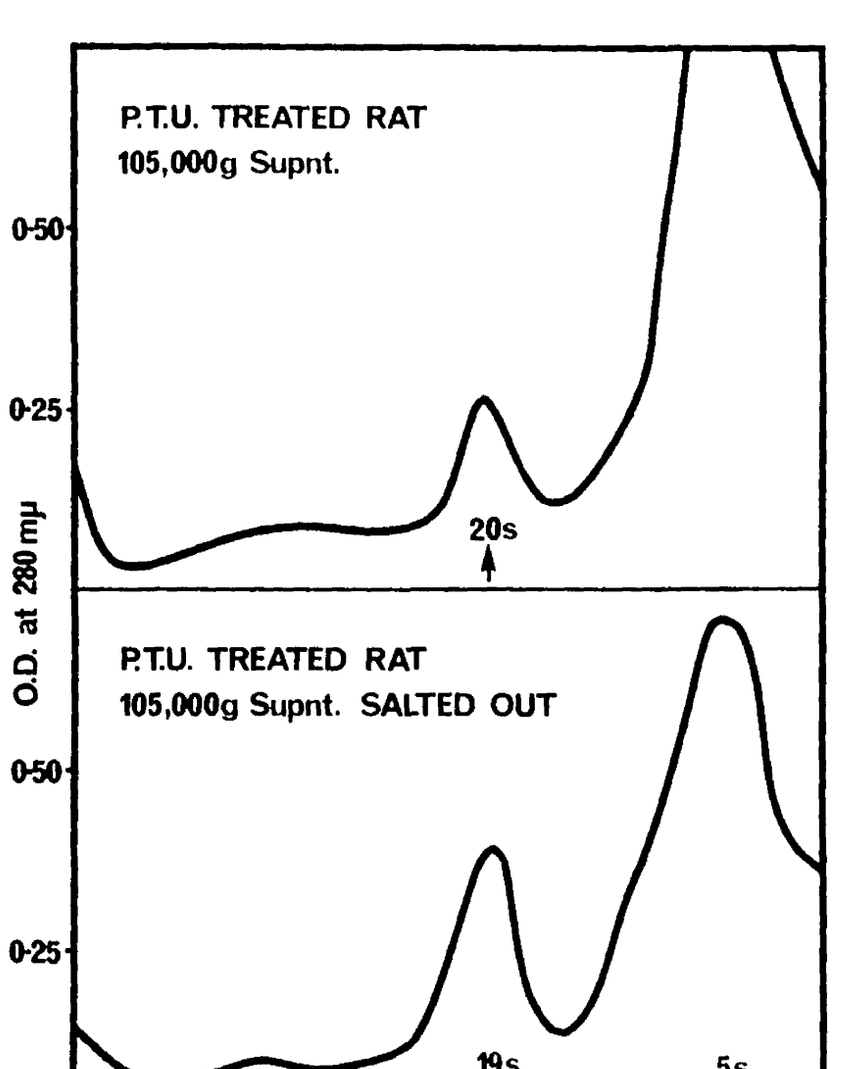

Fig. 55

Pattern of thyroid proteins from PTU treated rat. Effect of 50% saturation by ammonium sulphate on the 105,000g supernatant. SW 41 rotor at 28,000r.p.m. for 16 hours.

and no definite peak >19S can be seen. If, however, the 105,000g supernatant is taken to 50% saturation with ammonium sulphate, the protein pattern of the resultant precipitate is shown in the lower part of fig. 55. The 3-8S peak is diminished in size; the peak in the thyroglobulin region is relatively increased and a small but definite 32S OD peak can now be seen.

It therefore appeared reasonable to conclude that the 32S OD peak is not merely a technical artefact of one method of preparation of the soluble thyroid proteins, but is a valid finding in the goitrous rat thyroid.

CHAPTER 27

EVIDENCE OF THE NATURE OF THE 32S OD PEAK MATERIAL

Incorporation of ^{125}I and ^{3}H-leucine

Attempts were made to study the incorporation of ^{125}I into the 32S OD peak in the iodine deficient rat thyroid. The technique used was to approach equilibrium labelling of the rat thyroid proteins by taking rats which had been 11 weeks on a low iodine diet and supplementing the drinking water for 5 weeks before sacrifice with a constant concentration of carrier-free ^{125}I. The results obtained can be seen in fig. 56. The OD tracing is typical of the thyroid proteins of a goitrous rat. The ^{125}I is well incorporated into the thyroglobulin region, but not at all into the 32S OD region. It should be noted in passing that even with equilibrium labelling the large 3-8S peak is not labelled with ^{125}I.

Similar attempts were made to study the incorporation of ^{3}H-leucine into the 32S OD peak. The results are shown in fig. 57. The study was performed at two times. In the upper part of the figure is seen the pattern of incorporation of ^{3}H-leucine 15 minutes after a subcutaneous injection of the labelled amino acid.

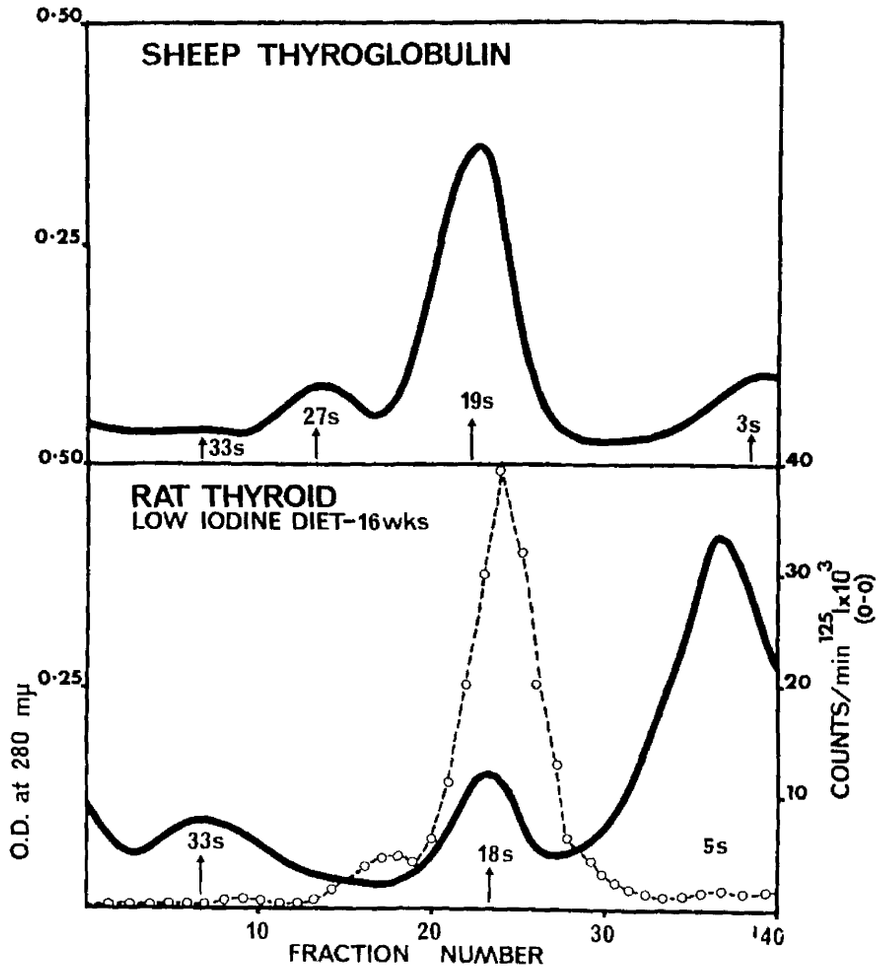

Fig. 56

Pattern of thyroid proteins and incorporation of ^{125}I by equilibrium labelling of the thyroid glands of rats on a low iodine diet. SW 41 rotor at 28,000r.p.m. for 16 hours.

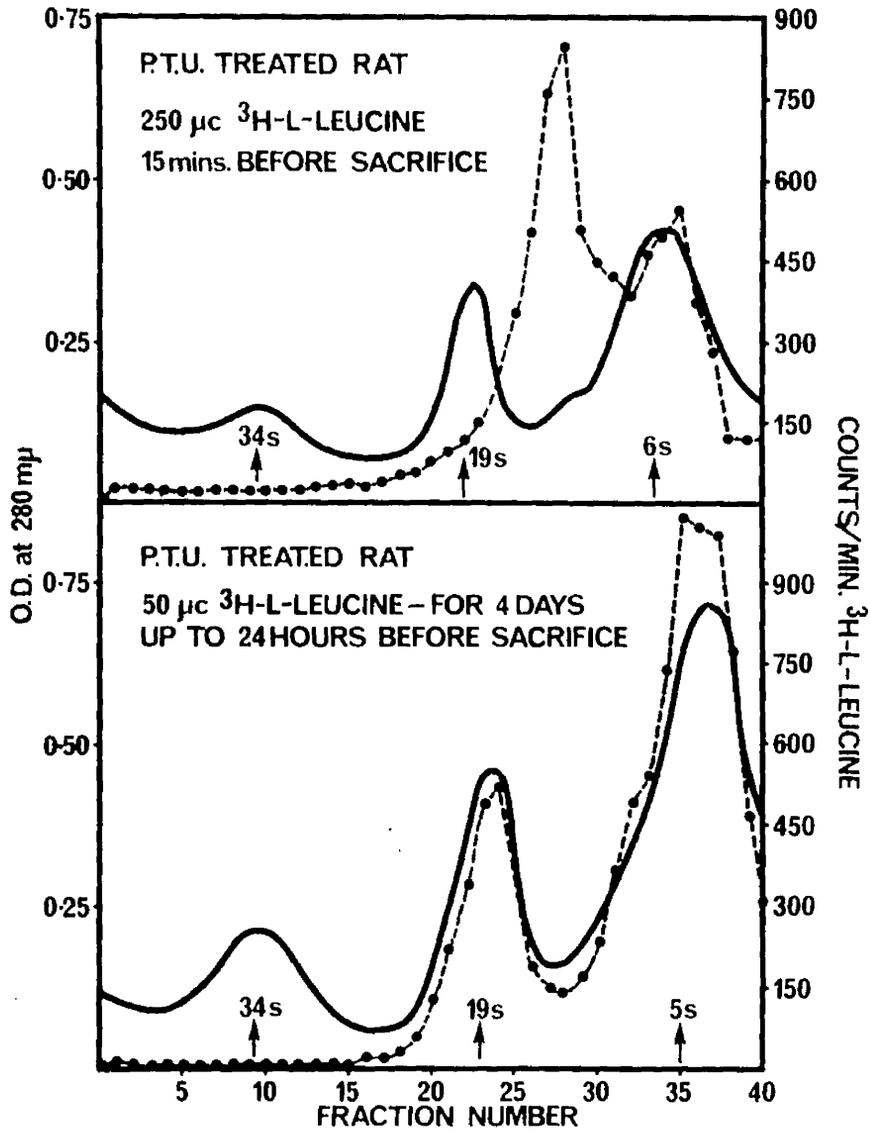

Fig. 57

Pattern of thyroid proteins and incorporation of ^{3}H-leucine in rats on a low iodine diet. a) 15 minutes after injection of ^{3}H-leucine b) after 4 injections of ^{3}H-leucine at intervals of 24 hours, the last injection being 24 hours before sacrifice. SW 41 rotor at 28,000r.p.m. for 16 hours.

It will be seen at this early time interval that the label is incorporated into the 12S and 3-8S protein, but not into the 19S or 32S area.

A further group of rats received a subcutaneous injection of ^{3}H-leucine daily for 4 days - the last occasion being 24 hours before sacrifice. The results are shown in the lower part of fig. 57. At this stage it can be seen that the ^{3}H-leucine is well incorporated into the 3-8S proteins and into the thyroglobulin region, but once more not into the 32S region.

Evidence that the 32S OD peak contains ribonucleic acid (RNA)

The results of scanning of the ultracentrifugal pattern of the soluble thyroid proteins precipitable by 50% ammonium sulphate at an OD of 260mμ instead of the usual 280mμ is shown in fig. 58. In the upper part of the figure which shows the pattern of the material obtained from normal rat thyroid, it can be seen that the peak in the thyroglobulin region is still the predominant one. The 3-8S peak is also seen, but the 27S peak is no longer visible. In contrast to this in the lower part of the figure is seen the pattern obtained from rats treated with PTU. The peak in the thyroglobulin region is now very small, and the 3-8S OD peak probably increased. Much the most striking

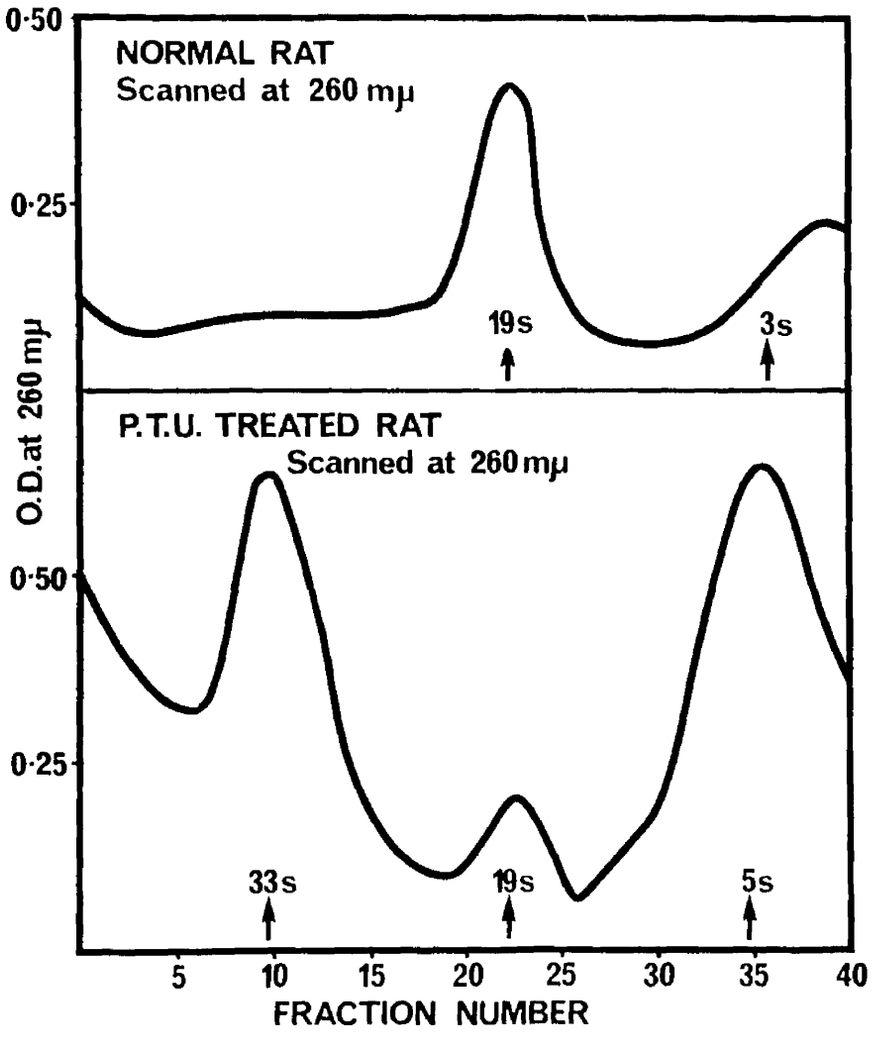

Fig. 58

Pattern of thyroid proteins from normal and PTU treated rats. Scanned at 260mμ. SW 41 rotor at 28,000r.p.m. for 16 hours.

feature, however, was the striking increase in the peak in 32S region.

The fact that the 32S material gave a maximal absorbance at 260mμ was confirmed by collecting the 32S OD peak and the 19S OD peak by fractionation of sucrose density gradients from PTU-treated animals. After an overnight dialysis against 0.9% sodium chloride the material from these OD peaks was scanned on a Unicam SP800 spectrophotometer. It will be noted (fig. 59) that the maximal absorbance of the 32S material, which shows a peak at 260mμ characteristic of RNA, is quite different from the characteristic protein pattern of the 19S material with a maximal absorbance at 280mμ.

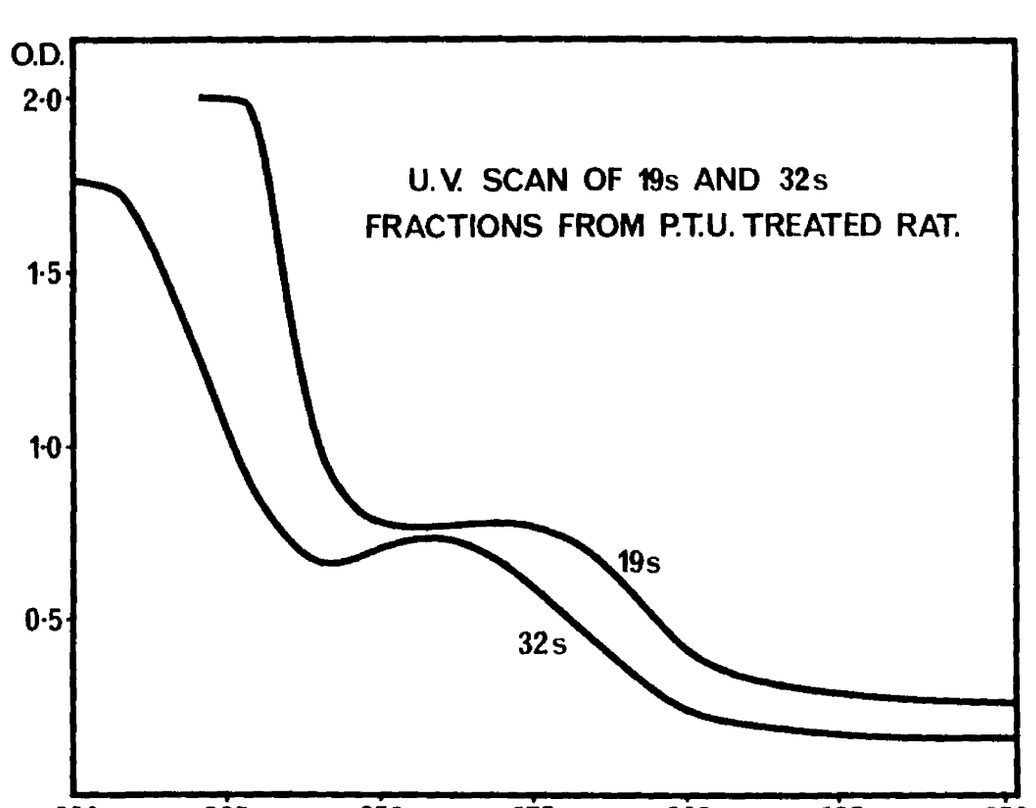

Fig. 59

UV scan by Unicam SP800 of 19S and 32S OD peaks from a PTU treated rat.

CHAPTER 28

SUMMARY OF SECTION 6

In this Section the conditions which result in alteration of the OD pattern of the material running in the ultracentrifuge with an 'S' value of >19S have been explored.

In the goitrous rat the 27S OD peak constantly disappears and is replaced by a 32S OD peak. This is reversible by T_4 treatment and in certain types of goitrogen-treated animals by iodine supplementation. In goitre caused by a drug blocking tyrosine iodination such as PTU or carbimazole, however, although the 32S OD peak disappears, the 27S peak is not reformed.

Evidence is presented that the 32S peak is not a technical artefact of one method of preparation of the soluble thyroid proteins, but is a constant finding.

The fact that neither ^{125}I or ^{3}H-leucine are incorporated into the 32S OD peak and also the demonstration that the maximal absorbance of this material is at 260mμ indicate that this material is not of an iodoprotein nature and favours the view that this material consists of RNA.

SECTION 7

GENERAL DISCUSSION

GENERAL DISCUSSION

In Section 3 the data on the biosynthesis of thyroglobulin in the normal and goitrous rat is presented. The results of the incorporation of ^{3}H-leucine obtained are comparable to those of Cavalieri and Searle (1967a), who found a similar pattern of in vivo incorporation of ^{14}C-leucine in the rat to that obtained in the present study, i.e. incorporation of the isotope into the 3-8S and 12S protein before incorporation into the 19S protein occurred. However, the time sequence of incorporation in their studies was different than in the present work. They found incorporation into the 3-8S and 12S proteins at one hour, as compared with 15-30 minutes in the present studies; into a 16S protein at 4 hours and into an 18S protein at 20 hours, compared with one hour in the present work. Forty-eight hours was required in both studies before a truly 19S protein was labelled.

A pattern of ^{3}H-leucine incorporation similar to that obtained in the rat in the present study was found in the guinea pig and the rat by Ekholm and Strandberg (1967a and b). However, Vecchio et al (1966b) found that although the labelled amino acid was incorporated into the 12S protein of the guinea pig, this was not the case in the rat.

Further studies by Ekholm and Strandberg (1967a) and by Cavalieri and Searle (1967b) have provided part at least of the explanation of the different time sequences of *in vivo* labelling of the thyroid protein by amino acids by demonstrating that *in vivo* administration of thyroid stimulating hormone (TSH) accelerated the incorporation of the labelled amino acid into the 19S protein. In this context the difference between the studies of Cavalieri and Searle (1967a) and the present work is surprising, as the former authors utilised a low iodine diet which should, theoretically at least, have resulted in increased TSH levels in their animals. In the control animals used in the present study, as can be seen from the histology of the thyroid gland, there is no evidence of undue TSH stimulation. TSH increases not only the speed of incorporation of amino acids into the thyroid protein and their presumed sub-units, but also the degree of incorporation. Administration of T_4, which diminishes TSH secretion by the pituitary by the well recognised negative feedback mechanism, results in almost complete abolition of uptake of *in vivo* administered labelled amino acids by either the normal or goitrous thyroid.

Although no studies of TSH effect on the incorporation of ^{3}H-leucine were made in the present experiments, the data produced from rats on a low iodine diet where there was incorporation of ^{3}H-leucine into the 19S protein within 15 minutes of its in vivo administration, is consistent with this being a TSH induced effect.

The first question to be settled is the possible relationship of the 12S and 3-8S proteins to the 19S material. The findings of Edelhoch and his collaborators described in the Introduction, make it tempting to assume that the 12S and 3-8S labelled material found during in vivo or in vitro biosynthesis experiments correspond to the 12S and 6S proteins found on chemical fractionation of purified 19S material. Is this justified?

The possibility which has been previously raised (Thomson and Goldberg, 1968) that the 12S material labelled by ^{3}H-leucine represents the breakdown of an especially labile larger protein of 17-19S size cannot be completely excluded. In favour of this hypothesis is the fact that it has previously shown that newly formed thyroglobulin is more liable to dissociation into the 12S sub-unit than is material which has been formed for a longer time (Sellin and Goldberg, 1965).

However, the weight of evidence from the present studies and from the studies of the in vivo incorporation reported by the other authors cited above, is that the pattern of labelling of the 12S material is consistent with this being a precursor unit of the 19S protein.

The fact that the administration of cycloheximide in a dose sufficient to block the further uptake in vivo of ^{3}H-leucine by the rat thyroid did not prevent the formation of the 19S protein, also argues strongly in favour of the 12S (and 3-8S) proteins being precursor units of the 19S protein.

It has been noted that when T_4 is administered to a normal and especially to a goitrous animal 12S protein accumulates to such an extent as to produce a visible protein peak in the OD tracing. When T_4 is given and TSH release by the pituitary is cut off, both the synthesis and breakdown of thyroglobulin are halted. It would seem then reasonable to postulate that the accumulation of 12S protein in this situation is due to the accumulation of 12S units which cannot be further incorporated into the 19S protein because of the lack of TSH. The alternative explanation, i.e. that the accumulation of 12S proteins represents the increased breakdown of thyroglobulin when T_4 is given, would

seem unlikely. The rate of thyroglobulin degradation in the normal gland is already slow and would be further slowed by the action of T_4 damping down TSH production, and thus the production of a stable 12S OD peak when T_4 is given to normal animals is strong evidence in favour of this being an accumulation of sub-units due to lack of synthesis of 19S thyroglobulin as opposed to degradation. The histological findings in the rats on the PTU + T_4 diet, where no evidence of TSH effect on the thyroid was seen is also compatible with this hypothesis.

It has been shown (Goldberg and Seed, 1965; Pommier et al, 1966) that in vitro the ease of degradation of thyroglobulin depended on the degree of iodination of the protein. Simon et al (1966) have shown that poorly iodinated thyroglobulin from rats treated with PTU is more liable to breakdown as the result of freezing and thawing. This work has been recently extended by Inoue and Taurog (1968b) who showed the effect of freezing on the breakdown of thyroglobulin was most marked in the presence of 0.05% methyl-mercaptoimidazole. In the present study some 12S protein was probably present during the administration of antithyroid drugs although no definite OD peak could be seen due to the confluence of the large 3-8S and thyroglobulin

peaks. ^{125}I labelled 12S material was present in maximal amounts during withdrawal of antithyroid drugs. This increase in 12S protein labelled during withdrawal of the goitrogenic regime is against this effect being simply a dose-related effect of the antithyroid drug causing breakdown of thyroglobulin, as one would in this circumstance have expected the breakdown to be maximal during the administration of the drug. It may be that at some critical concentration there is a maximum effect on blocking of incorporation of thyroglobulin sub-units into the 19S protein.

The relationship of the 3-8S material to the 19S protein is less clear. The main problem is that this is an extremely heterogenous collection of proteins, some of which, like haemoglobin and the serum proteins, have no relevance to thyroglobulin. Unfortunately when goitres are induced experimentally, these proteins are also increased in amount due to the increased vascularity of the thyroid gland.

The pattern of incorporation of labelled amino acids into the 3-8S is consistent with these being precursor units of the 12S and 19S proteins. This is consistent with previous in vitro work (Seed and Goldberg, 1965) as well as the in vivo results in

the present work and to those alluded to above. There are, however, some discrepancies which make it impossible to accept that the only role of incorporation of labelled amino acids into the 3-8S moiety is as precursor sub-units of the 19S material. Seed and Goldberg (1965) showed with in vitro incubation studies using a thyroid slice system that prolonged incubation resulted in increased labelled 3-8S protein being formed. This corresponded in time to leaching of colloid from the thyroid slices into the medium as demonstrated by standard histological techniques. They suggested that this might represent either a block in sub-unit aggregation at long time intervals of incubation or a leaching off of thyroglobulin sub-units from the subcellular particles.

Similarly, in the present in vivo studies, especially when a goitrous state had been achieved by use of one of the goitrogenic drugs, there was an increased proportion of the label in the 3-8S fraction at late time intervals after administration of a pulse of ^{3}H-leucine. The pattern of labelling found would therefore be consistent with the labelled 3-8S fraction being not only a precursor unit of the 19S protein, but also perhaps a breakdown product. A similar late labelling of 3-8S protein in high specific activity has been found by Ekholm and Strandberg (1967a).

The results obtained in the groups of rats on a low iodine diet show curiously little incorporation of the labelled amino acid into the 3-8S fraction at either early or late times after injection. The reason for this discrepancy is not clear. Obviously the effects of an antithyroid drug are not strictly comparable to goitre produced solely as a result of iodine deficiency, even when a similar alteration in the stable thyroid proteins occurs. This finding in a hyperplastic vascular goitre induced by a low iodine diet is at least reassuring in that the incorporation of ^{3}H-leucine into the 3-8S proteins of goitrous glands does not seem to represent the incorporation of the label to any extent into non-thyroidal proteins such as the serum proteins.

In contrast to the situation when labelled amino acids are used, iodine is not incorporated to any extent *in vivo* into 12S and 3-8S proteins in the normal animal. Instead it is incorporated into a protein of approximately 18S size initially followed after an interval of time (48 hours in the present work) before a truly 19S protein is formed. This is consistent with previous *in vitro* work (Seed and Goldberg, 1963). Lissitzky et al (1964) also found *in vitro* that thyroid slices incorporated ^{125}I into the 12S and 3-8S fractions. This was not found in the present *in vivo* work,

except for a small degree of labelling of the 12S protein by ^{125}I at early time intervals after administration of the label. At all times, however, the ^{125}I labelled peak in the thyroglobulin area was always the major radioactive component. As will be discussed later, only under very abnormal circumstances in the in vitro studies reported in this work, was there labelling by ^{125}I of proteins other than 19S thyroglobulin.

In the case of rats on a low iodine diet where presumably TSH was increased, the time taken for incorporation of ^{125}I into a truly 19S protein was greatly diminished. This is similar to the effect on ^{3}H-leucine incorporation noted above.

The relationship of the 12S and 3-8S protein to thyroglobulin has been studied by other workers by immunological means (Goldberg et al, 1964; Sellin and Goldberg, 1965; Morais and Goldberg, 1967). There is general agreement that the 12S material is related immunologically to the 19S thyroglobulin, but the situation with regard to the 3-8S material is much less certain. At the most, it has been found that only a fraction of the 3-8S protein is precipitable with anti-19S thyroglobulin antibodies. It is, however, impossible to say that the remainder of the 3-8S protein is not related to the 19S protein. Because of its known

heterogeneous nature, one would not anticipate that all this fraction of the soluble thyroid proteins would be related to thyroglobulin. There may however be thyroglobulin sub-units in this fraction which lack the complete antigenic construction, for instance due to lack of correct spatial aligment of antigenic groups, or due to lack of incorporation of the carbohydrate moiety of thyroglobulin at this stage, which render them non-precipitable and therefore non-identifiable by these techniques.

The alterations of the stable thyroid proteins found when a goitrogenic regime is employed is similar to, but not identical with, the effects found by other authors (Perelmutter et al, 1965). In both studies a decrease in 19S protein and an increase in light-weight (3-8S) protein were found; in both an increase of protein in the 11-12S region was found during induction of goitre but this was not apparent once the goitrous state had been achieved. The apparent loss of 11-12S protein in goitrous states was probably due merely to the rapidly increasing 3-8S peak obscuring a discrete 12S protein peak.

The administration of a goitrogenic drug, or low iodine diet, enhanced the percentage incorporation of ^{3}H-leucine into the thyroid proteins. As pointed out in Section 2, there were slight

differences found with the different goitrogenic regimes employed, PTU tending to diminish the relative proportion of the label in the thyroglobulin region as compared to methimazole and $KClO_4$. In all the protein in the thyroglobulin region failed to reach 19S size due to the blocking of the iodination process stopping maturation of the protein to its fully iodinated state.

The dissociation of the process of incorporation of amino acids from ^{125}I as shown in the above experiments is similar to the in vitro findings of Seed and Goldberg (1963) and the in vivo findings of Maloof et al (1964) who both demonstrated that iodination could be blocked by thiourea or PTU without affecting amino acid incorporation and conversely that amino acid incorporation could be prevented by actinomycin without, in the short term, affecting radioiodine incorporation into the thyroid proteins.

The various goitrogenic drugs used in the present study showed a variable effectiveness in blocking ^{125}I incorporation. All were reasonably effective, but the most complete effect was obtained with PTU and the least with $KClO_4$. Likewise during withdrawal of the antithyroid drugs, the effect of PTU

was apparent for a longer time than with the other drugs, $KClO_4$ being the shortest acting. Recently Alexander et al (1969) have presented some preliminary data demonstrating marked differences in the duration of effect of antithyroid drugs. Curiously in their studies in man, ^{35}S-labelled PTU had a shorter biological half-life than ^{35}S methimazole. Obviously much more work remains to be done in the area of metabolism of antithyroid drugs in different vertebrates.

Using a low iodine diet, the results obtained in the present studies differ in certain respects from those reported recently by Inoue and Taurog (1968a) who showed in rats on a low iodine diet that no stable protein peak could be visualised in the thyroglobulin area and that ^{125}I and ^{14}C-labelled amino acids were incorporated into a protein significantly lighter than thyroglobulin, this effect being reversible with the acute administration of stable iodine. Because of this discrepancy the degree of iodine deficiency used in the present work was extended to achieve a degree of goitre formation similar to that used by the above workers. However, in the present studies ^{125}I and ^{3}H-leucine were not incorporated into a protein in the thyroglobulin region of less than 18S size. It is impossible to exclude some breakdown of the

poorly iodinated protein in the studies of Inoue and Taurog. The finding of a 19S protein when stable iodine was administered could be consistent with the known effect of iodination in rendering poorly iodinated thyroglobulin less liable to dissociation.

The results of the study of a group of 100 human thyroid glands was presented in Section 4. The 'normal' human thyroid contained, as expected from previously published work on vertebrate thyroid proteins (Salvatore et al, 1965a), three protein peaks - a predominant 19S peak (thyroglobulin) with smaller 27S and 3-8S peaks.

In thyrotoxic glands, prepared with carbimazole and iodine pre-operative medication, usually no 27S protein was present. This is similar to the experience of others (Stanley, 1964; Ramagopal et al, 1965). Neither of these authors gave details of the pre-operative drug treatment and curiously no comment was made about the lack of formation of protein >19S in most thyrotoxic glands studied. This lack of 27S protein was also true of thyrotoxic glands treated pre-operatively with $KClO_4$. The 27S protein with its ratio of iodine/protein of approximately 2/1, as compared to the 19S protein in the normal gland, has

been postulated as a slowly metabolised store of highly iodinated protein. The lack of the 27S protein in thyrotoxic glands may just then merely reflect the increased stimulation to which these glands have been exposed, resulting in the utilisation of these stores. This would certainly be the simplest explanation. However, the amount of thyroglobulin which can be demonstrated in a carbimazole/iodide treated gland and in a $KClO_4$ treated gland either by study of the ultracentrifugal pattern of the thyroid proteins, or by examination of histological sections of the thyroid gland, are very different - yet the same lack of 27S protein is present in both cases (with a few exceptions in the carbimazole/iodide treated group). It may be that the iodide had been given for insufficient time to allow the 27S protein to reaccumulate. In this context the data on the goitrogen treated rats receiving iodine supplementation before sacrifice to mimic the human thyrotoxic situation is of interest (Section 6). In these rats a goitrogenic drug such as PTU or carbimazole, which acts by blocking tyrosine iodination, had a different effect than $KClO_4$ (or a low iodine diet alone). In the former case, no 27S protein was reformed after iodine supplementation, whereas in the latter groups a 27S peak was reformed.

This difference can either be attributed to the greater potency or duration of action of the thiouracil group of drugs in blocking iodination of the thyroid proteins, as compared to $KClO_4$. Alternatively there may be some as yet unknown effect of the drugs which inhibit the formation of a 27S protein from thyroglobulin and its subunits.

In two thyrotoxic thyroid glands (Case 20 and 38) a significant amount of 12S protein was present. As mentioned in Section 4 both these specimens had been stored for a period of time before analysis. Stanley (1964) found 12S protein in the thyrotoxic glands and other goitres, both simple and malignant, studied by him. This was not found in the studies of Ramagopal et al (1965). The technique used by Stanley involved a step of freeze drying of the thyroid proteins and transportation by air from Australia to the U.S.A. where the specimens were reconstituted and analysed. It has been shown by Inoue and Taurog (1968b) that freezing induced breakdown of the thyroid proteins of iodine deficient rats. In the experience of our laboratory, the storage of thyroid proteins from goitrous glands in a frozen state has resulted in the loss of thyroglobulin-like proteins with the formation of a stable 12S thyroid protein peak. This did not, however, occur in preparations of normal sheep proteins.

Surprisingly, considering the doses of iodine given, the degree of iodination of the thyroid proteins from thyrotoxic glands treated by carbimazole/iodide, as compared to $KClO_4$ is very similar (0.87 as compared to 0.73μG iodine/mg protein) - especially when it is remembered that the iodine content was estimated on the total soluble thyroid proteins precipitable by 50% ammonium sulphate and not on a purified thyroglobulin fraction. Thus, in the $KClO_4$ treated glands with their proportionally greater quantity of 3-8S protein, the degree of iodination of thyroglobulin must be at least as high as the carbimazole/iodide treated group and both are comparable to the group of non-toxic goitre. This would strongly suggest that the lack of the 27S protein in thyrotoxic glands is not simply due to a lack of iodination.

It would obviously have been of great interest to study the OD tracing and iodine content of the thyroid proteins from untreated thyrotoxic thyroid glands. Ethical considerations make this impossible to achieve in the human. It is possible, merely as a hypothesis, that in a thyrotoxic gland, or in someone predisposed to develop the disease, some subtle abnormality of the thyroid protein exists which acts against the formation of a slowly turning over store of highly iodinated protein and that therefore the thyroid proteins are more liable to the normal degradative

process with resultant undue release of T_4 and T_3 into the circulation, with consequent production of clinical thyrotoxicosis. This, however, at present remains purely conjectural and further studies are required to confirm or refute this possibility.

What is the situation in non-toxic goitre? Can the production of this disease be in any way attributed to abnormalities of the thyroid proteins by the techniques used in the study? The OD pattern of the thyroid proteins were almost universally normal in diffuse or multinodular non-toxic goitre and the incorporation in vitro of ^{125}I and ^{3}H-leucine into slices from these glands was also normal. The degree of iodination of the thyroid proteins was certainly lower than normal, but of course this gives no indication of the total iodine content of the thyroid gland. On the present evidence, it must be concluded that non-toxic goitre is not produced by abnormalities in formation of the thyroid proteins unless this is achieved by means so subtle as to be not recognisable by current techniques. Such a possible defect could be some minor abnormality in the synthesis of the polypeptide backbone of thyroglobulin which rendered its tyrosyl groupings less accessible for iodination, with resultant deficient T_4 and T_3 production thus producing a goitre by compensatory TSH

stimulation of thyroid growth by the pituitary gland. In this connection it is of interest that in a recent small series Bismuth et al (1966) showed no difference in amino acid composition of the 19S thyroglobulin in normal human thyroids, thyrotoxicosis, familial goitre and non-toxic goitre.

The results of the thyroid protein analysis and degree of iodination of the thyroid proteins from cases of solitary thyroid nodules gave results broadly similar to those obtained in the non-toxic goitre. These nodules obtained abundant colloid on histological examination. Only two glands (Case 88 and 89) gave results of thyroid protein analysis which were different from the rest. In both of these the histological examination of the resected specimen gave ample confirmation of the virtual absence of thyroglobulin. Similar results have been obtained in certain thyroid nodules by Ramagopal et al (1965) and by Stanley (1964).

In Hashimoto's thyroiditis, as has been shown, a variety of protein patterns were obtained. In all, a relative loss of thyroglobulin was found which correlates well with a lack of thyroglobulin on histological examination of the thyroid. An increased 3-8S protein peak was seen in all. It is difficult to know how much of this increase is due to thyroglobulin sub-units and how much is due to the great increase in cellular content of the gland affected

by Hashimoto's thyroiditis, as compared to the normal. The majority of this cellular increase is of course due to non-thyroid components, for example, lymphocyte and plasma cell infiltration. The thyroid epithelium which is present is also abnormal - the Askanazy cell epithelium. A 32S OD peak was seen in two of five patients studied.

In certain interesting ways the thyroid proteins in this condition were different from all other thyroid glands studied. This disease was the only one in which in vitro incorporation of ^{125}I into 12S and 3-8S proteins in addition to the 19S protein was regularly present. Abnormalities in the iodinated proteins found in Hashimoto's disease have been previously reported (Murray and McGirr, 1960; De Groot et al, 1962; Stanley, 1964; Ramagopal et al, 1965). These have been demonstrated by the low butanol extractable ^{131}I level in the plasma after tracer doses of ^{131}I; by electrophoresis and autoradiography of the thyroid proteins and by ultracentrifugal techniques.

In addition, a constant and curious difference in the incorporation of ^{125}I and ^{3}H-leucine was seen. ^{3}H-leucine was never incorporated into thyroglobulin in vitro even when this was shown histologically to be present in the gland. None of the patients

studied was clinically hypothyroid and obviously were synthesising some thyroglobulin. Perhaps in this condition the thyroid epithelium which is left is so diseased that its ability to incorporate amino acids into the polypeptide backbone of thyroglobulin is severely curtailed. Results similar to those of the present series have been recently reported by Etling et al (1968).

Only a small number of malignant thyroids were studied. The 3 cases of anaplastic tumour gave results in keeping with the much larger series of Valenta et al (1968). As stated in that paper the results of ultracentrifugation of the thyroid proteins and the incorporation of radioiodine depend on how much relatively normal thyroid tissue is present in the part under study. Stanley (1964) has shown similar results in anaplastic tumours.

In the one case of papillary carcinoma studied, the normal results obtained were undoubtedly due to the low ratio of the papillary elements in the thyroid gland in relation to the surrounding relatively normal tissue. Similarly, the other differentiated thyroid tumour studied - a follicular carcinoma - although containing comparatively little thyroglobulin, did

incorporate ^{3}H-leucine and ^{125}I into the thyroglobulin region of the tumour.

Case 100, in whom a presumptive diagnosis of dyshormonogenesis (type unknown) was made, showed no protein >19S. This gland was hyperplastic, although the patient was clinically hypothyroid. This patient would provide further evidence for one mechanism at least for the loss of the 27S protein being due to thyroid stimulation. It must be admitted, however, that some more subtle abnormality in thyroid protein synthesis in this particular gland cannot be excluded. The only other dyshormonogenetic gland which was studied was the goitrous merino sheep in which an OD pattern of a greatly diminished thyroglobulin peak and increased 3-8S peak were found. A marked 32S OD peak was also seen. Falconer (1966) and Rac et al (1968) have described in detail the findings in these animals. The evidence is that the animals suffer from congenital goitrous cretinism associated with the production of an abnormal iodinated protein. Such abnormalities have been previously described in humans (De Groot et al, 1958; McGirr et al, 1960). The usual abnormality found is a protein with the electrophoretic characteristics of an iodinated albumin present both in the thyroid gland and in the

peripheral circulation. One major problem has been to decide what, if anything, has this abnormal material to do with the production of goitrous hypothyroidism. Did this clinical state result from the iodination of this non-biologically active protein as the primary defect, or, as has been recently claimed (Lissitzky et al, 1968), is the production of this abnormal compound consequent on the presence of the goitre? Lissitzky et al (1968) have recently produced evidence of such an abnormal iodinated albumin in the thyroid gland of a young girl whose basic defect appeared to be lack of the dehalogenase (deiodinase) enzyme system. This is contrary to the experience of the thyroid laboratory of the University Department of Medicine, Glasgow Royal Infirmary. However, support for the proposal that the abnormal iodinated protein was the consequence of the goitrous process and not its cause, comes from the description of such a compound from a wide variety of thyroid diseases, such as thyroid cancer (Robbins et al, 1955; Tata et al, 1956), thyrotoxicosis (Stanbury et al, 1962), autonomous thyroid nodules (Kahn et al, 1962), as well as in Hashimoto's thyroiditis and congenital goitrous hypothyroidism.

Lissitzky et al (1968) suggest that the abnormal iodinated albumin is in fact iodinated serum albumin which has been casually iodinated during passage of the blood through a gland rendered hyperplastic by some other primary disease process. Theoretical support for such a possibility comes from the work of Shimoaka and Thompson (1965), who showed that bovine albumin injected in the rat can become iodinated in vivo if radioiodine is subsequently injected. Torresani et al (1968) have further shown an iodo-albumin to be normally present in the rat. This contained MIT, DIT, T_3 and T_4. This work is yet however to be confirmed by other laboratories.

To further complicate the issue, a congenital goitre associated with the production of an abnormal iodinated protein has been described in South African cattle (Robbins et al, 1966b). In these animals the abnormal iodinated protein appeared to be heterogeneous. Large quantities of circulating iodinated albumin were present, but these were only present in slight degree in the thyroid gland. One of the abnormal iodoproteins in the thyroid showed immunological characteristics which suggested that it might be an abnormal iodinated immunoglobulin. A similar iodinated light-weight protein with immunological characteristics

suggesting that it was an iodinated gamma globulin has recently been described in differentiated human thyroid cancers (Lupulescu et al, 1968). Obviously a great deal of work remains to be done in this area before any dogmatic statements of the role of abnormal iodinated proteins in the pathogenesis of goitre can be made.

Medeiros-Neto et al (1968) have described their findings in two patients with Pendred's syndrome - congenital goitre associated with perceptive deafness. In this condition the basic problem in the thyroid gland is a lack of iodination of tyrosine in thyroglobulin which is presumed to be due to lack of the peroxidase enzyme system in the thyroid, although a recent case description by Ljunggren and Vecchio (1969) has cast doubt on this hypothesis. Medeiros-Neto et al (1968) showed, as expected, that these hyperplastic glands contained little thyroglobulin (about 15% of the total thyroid proteins), the main protein present being in the 3-8S region. They further noted an increased amount of radioiodine associated with the particulate fraction of the thyroid cells. This abnormality has also been described in a transplantable rat thyroid tumour (Robbins et al, 1959), but its relationship to thyroglobulin is not clear.

Most of the vertebrate thyroid glands gave a similar pattern of thyroid proteins to the rat and human. Previous studies by Salvatore et al (1965a) have given broadly similar results, although in the present work an appreciable amount of 12S protein in the guinea pig and rabbit as described by these workers was not found. It should be noted, however, that in their studies the thyroid tissue was frozen before analysis and it may be, especially in the guinea pig with its poorly iodinated thyroglobulin, that this has resulted in the breakdown of some of the 19S protein.

The horse has no protein >19S, confirming the results of Salvatore et al (1965a). Histological examination of the horse thyroid gland suggested that it was under excessive TSH stimulation as compared to the other animals studied. Not enough is known about thyroid metabolism in the horse to say if this is a physiological state or not. Certainly, the lack of 27S protein could not be attributed to lack of iodination since the values obtained in the horse fell into the same range for iodination of thyroid protein as for most of the other vertebrates studied, except the dog and cat - presumably due to fish-containing foods - and the guinea pig - presumably due to its vegetarian habits.

In Section 6 proteins larger than thyroglobulin are considered. The loss of the 27S protein when a goitre is produced in the rat and its recovery when TSH activity is diminished by T_4 medication, has been already discussed, as has the difference in response of the drug-treated goitres to iodine supplementation, depending on the particular antithyroid drug used.

Evidence is presented that the 32S material found in the goitrous glands is not of iodopeptide nature, but is composed of RNA. Its virtual absence from normal glands (except for a trace amount in the sheep, and very occasionally in the human) and its constant finding in the goitrous rat, would fit in well with its origin in hyperplasia of the thyroid cell. Evidence in support of this theory of origin comes from the work of Goldberg et al (1968), who showed that in thyrotoxicosis, and especially in Hashimoto's thyroiditis, the RNA content of the thyroid gland was increased, as was the DNA content. The results obtained in thyroid adenoma did not differ from normal thyroid tissue. In the present study, in addition to artificially induced goitres in the rat, a 32S OD peak was seen in Hashimoto's thyroiditis and in a goitrous sheep thyroid. If the mechanism of its production

is simply thyroid cell hyperplasia, it was surprising that it was not found in thyrotoxic glands, especially in ones prepared for surgery by $KClO_4$ in which the greatest degree of thyroid hyperplasia seen in the present work was found. Perhaps in some way the antithyroid drug interferes with its production in the human, but not in the rat. No obvious explanation for this discrepancy is available and this too must await further experimentation before it can be settled.

Previous work by others (Nunez et al, 1965a and Robbins et al, 1966a) has indicated the presence of a small amount of 31-33S iodoprotein material from non-stimulated rat thyroid glands. The evidence presented for its iodoprotein nature is based on the fact that radioiodine was incorporated into this fraction both in equilibrium labelling experiments in vivo and in slice incubation experiments in vitro. This was not found in the present work and if an iodoprotein compound of this S value is confirmed by other workers, it still remains to be fully characterised and its relationship to 19S thyroglobulin and its role in thyroid metabolism remains to be defined.

SECTION 8

SUMMARY

SUMMARY

This work presents data on the biosynthesis of thyroglobulin. This protein of 19S size constitutes the main thyroid protein in most normal vertebrate thyroid glands; smaller amounts of 3-8S and 27S protein are usually present.

Initially it is shown that in vivo in the rat, as has been previously shown in vitro by other workers, labelled amino acids are incorporated into proteins of 3-8S and 12S size before the label is incorporated into thyroglobulin. The pattern of incorporation is consistent with the 12S and with less certainty with the 3-8S proteins being precursor subunits of the 19S protein. At long time intervals after administration of a pulse of ^{3}H-leucine, the pattern of labelling of the 3-8S fraction could also be consistent with this labelled fraction being a breakdown product of the labelled 19S protein.

These findings with ^{3}H-leucine are in contrast to those seen when ^{125}I is used. In this case ^{125}I is not incorporated into proteins <18S, except for a small percentage transiently incorporated into a 12S protein at very early time intervals after administration of the label. At no time, however, was a protein lighter than 18S present as the predominant labelled peak.

The production of a goitrous state in the rat, by means of one of the antithyroid drugs which block at various points the biosynthesis of thyroxine, alters the distribution of the thyroid proteins. Thyroglobulin diminishes in amount whereas the 3-8S proteins increase; the normally present 27S peak is lost and a new OD peak is seen in the 32S region. In these goitrous glands the incorporation of ^{3}H-leucine into the thyroid proteins is enhanced, but a truly 19S protein is not formed. As would be anticipated, the incorporation of ^{125}I is blocked to a varying degree depending on the potency of the goitrogen used.

When a mild iodine deficiency state is induced by feeding a low iodine diet, the incorporation of ^{3}H-leucine or ^{125}I into thyroglobulin is accelerated, presumably due to increased TSH production by the pituitary. When a more severe degree of iodine deficiency is present, an 18S, as opposed to a truly 19S protein, is labelled. There is comparatively little labelling of the 3-8S proteins of iodine deficient glands by ^{3}H-leucine, as compared to drug-induced goitres.

The administration of T_4 to a goitrous, or normal, animal virtually inhibits the incorporation of ^{3}H-leucine and, to a lesser extent, ^{125}I into the thyroid proteins. A stable 12S

protein peak is seen under these conditions, which suggests that this may represent a failure of incorporation of sub-units into 19S thyroglobulin. An ^{125}I labelled 12S protein of high specific activity is found during withdrawal of antithyroid drugs in the rat.

The protein patterns of a series of 100 human thyroid glands were studied. The 'normal' human thyroid gland showed a protein pattern similar to the rat and slices of the tissue incorporated ^{3}H-leucine into the 19S protein and its presumed sub-units in vitro. ^{125}I on the other hand was incorporated only into an 18-19S protein.

In thyrotoxicosis, whether prepared pre-operatively by carbimazole/iodide or by $KClO_4$, the notable feature was the loss of proteins >19S. This occurred despite differences in distribution of the thyroid proteins in thyroid glands treated pre-operatively with these two different regimes. The glands from patients treated with carbimazole/iodide contained more thyroglobulin and a smaller amount of 3-8S protein than glands treated with $KClO_4$. The lack of a 27S protein could not be correlated with iodine deficiency - the thyrotoxic glands treated by either method of pre-operative preparation having a similar iodine

content which was furthermore similar to that found in non-toxic goitre in which a 27S protein was regularly found.

Most non-toxic goitres and thyroid adenomas showed a protein pattern and pattern of incorporation of ^{3}H-leucine and ^{125}I similar to the 'normal' human material. In only two of 17 adenomas studied was a 3-8S protein found as the predominant thyroid protein. In both of these glands no thyroglobulin was histologically demonstrable.

In Hashimoto's thyroiditis a relative loss of thyroglobulin with increased 3-8S protein was present. A 32S OD peak was seen in two of the 5 glands studied. ^{3}H-leucine was not incorporated into proteins >3-8S, but ^{125}I was incorporated, not only into thyroglobulin, but also into 12S and 3-8S proteins.

In malignant thyroid glands, as in the other human thyroid glands studied, there was a good correlation between the fraction of the thyroid proteins present as thyroglobulin and the presence of histologically demonstrable colloid. In poorly differentiated tumours, neither ^{125}I or ^{3}H-leucine were incorporated into the thyroid proteins.

The iodine content of 'normal' human thyroid protein was approximately 3 times that of any group of pathological glands

studied. Tumour tissue, as anticipated, contained the least iodine.

In a series of vertebrate thyroid glands studied, the thyroid protein pattern was similar to that of the normal rat or human; the exception being the horse in which no 27S protein was found. The iodine content of the majority of vertebrates was approximately twice that of the normal human, the only exceptions being the guinea pig, which contained less, and the cat and dog, in whom the thyroid proteins were more highly iodinated than in the other animals studied.

Evidence is presented that the 32S OD peak present in the goitrous rat can be demonstrated after a variety of techniques of preparation of the soluble thyroid proteins. Study of this peak showed that it was not of iodoprotein nature, but was consistent with it being composed of RNA.

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