- S U M M A R Y -

THYROGLOBULIN AND RELATED IODOPROTEINS IN THE THYROID GLAND

The object of this investigation was to study the metabolism of the thyroidal iodoproteins during goitrogenesis in the rat and to examine the physico-chemical properties of the iodoproteins in established goitrous human glands.

Goitrogenesis was induced in rats, following 'equilibrium labelling' of thyroidal iodine, by daily administration of methyl-thiouracil (MTU). After a latent period of 5 days there was a rapid increase in gland weight to 3 times the control value after 10 days on the MTU regime. Throughout goitrogenesis, the total ^{125}I content of the gland decreased logarithmically but the ^{125}I was removed at a faster rate from the 125,000 g supernatant fraction than from the whole gland homogenate. These data suggest that the rate of colloid resorption is greater than the rate of proteolysis or the rate of acquisition of the hydrolytic enzymes by the colloid droplets. In the study of the metabolism of the soluble iodoproteins, the 125,000 g supernatants were fractionated by DEAE cellulose chromatography, sucrose gradient ultracentrifugation and polyacrylamide gel electrophoresis. Analysis of the decrease in ^{125}I content of the iodoprotein fractions, separated by these techniques, showed that the newly iodinated iodoprotein molecules are metabolised at a faster rate than/

than the older more highly iodinated molecules and demonstrated that the 27S iodoprotein is metabolised at a slower rate than the 19S thyroglobulin. In another study, thyroidal protein synthesis during goitrogenesis was examined by ^{3}H-leucine incorporation into thyroidal proteins 'in vivo'. A pronounced stimulation of labelled aminoacid incorporation occurred between the 3rd and 6th days of goitrogenesis and the ^{3}H-leucine was incorporated into a protein with sedimentation constant less than 19S. After 8 days, however, label could also be detected in a heavier protein component corresponding to 24 - 25S. It is suggested that this represents a precursor of the 27S iodoprotein which accumulates when iodide organification is inhibited by MTU and provides additional evidence that iodination is not a prerequisite for subunit aggregation.

In studying the physico-chemical properties of the iodoproteins in established goitrous human glands, the iodoproteins were purified by gel filtration from a number of goitres removed at operation from patients. In general, iodoproteins from non-toxic goitres contained considerably less iodine per mg protein than normal although they resembled thyroglobulin from normal tissue in carbohydrate content, electrophoretic mobility and sedimentation properties. An exception, however, occurred in the iodoproteins from cases of Hashimoto's/

Hashimoto's thyroiditis where the hexose levels were elevated and the iodoprotein had a sedimentation constant of 20 - 21S. Estimation of the degree of heterogeneity of the iodoproteins from goitrous glands by DEAE cellulose revealed that in non-toxic goitres the iodoproteins compared with those from the normal gland in heterogeneity while in all other cases studies the iodoproteins were, in general, considerably more homogeneous. Peptide finger-prints of the S.carboxymethylated iodoproteins (SCM) did not reveal any gross dissimilarities in the primary structures of the proteins although a variability in the presence of certain groups of peptides was found between the native and SCM proteins. In some cases, these variations could be related to the absence of a 27S iodo-protein from these glands and a heterogeneity of the tyrosyl residues in the protein with regard to the extent of iodination. In general, in physical properties studied, the iodoproteins from goitrous glands were indistinguishable from normal thyroglobulin and the dissimilarities which occurred in the chemical properties could, for the most part, be related to the iodination level of the protein.

In two goitrous glands studied, abnormalities were, however, detected in the iodoproteins isolated. In one case, in addition to a normal thyroglobulin component, an iodinated albumin and pre-albumin were isolated. However, a structural similarity between these iodoproteins and normal thyroglobulin was revealed by/

by peptide fingerprinting and it is suggested that they represent iodinated subunits of thyroglobulin. In the other case, a high molecular weight iodoprotein was isolated which, although related to thyroglobulin, had only half the normal carbohydrate content and readily dissociated, under the conditions of isolation, to smaller subunits. Furthermore, an iodoalbumin was also isolated from this gland which produced a peptide fingerprint significantly different from normal thyroglobulin. It is suggested that in this gland an abnormal subunit is synthesised which prevents normal aggregation and stabilisation mechanisms taking place.

THYROGLOBULIN AND RELATED IODOPROTEINS IN THE THYROID GLAND

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ABBREVIATIONS

MTU	-	6 Methyl-5-iodo-2-thiouracil
TSH	-	Thyroid stimulating hormone
MIT	-	Mono-iodo-tyrosine
DIT	-	3,5 di-iodotyrosine
T_3	-	Tri-iodo-thyronine
T_4	-	Thyroxine
SCM	-	S. carboxymethylated
DEAE	-	Diethyl amino ethyl
TRIS	-	2-amino-2hydroxymethylpropane-1,3-diol
t-RNA	-	Transfer ribonucleic acid
PPO	-	2,5 diphenyloxazole
POPOP	-	1,4-bis-2-(4-Methyl-5-phenyloxazolyl)-Benzene
MSH	-	β mercaptoethanol
Molar Ratio	-	Ratio of β mercaptoethanol to potential thyroglobulin sulphydryl groups

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GENERAL INTRODUCTION

HISTORICAL

It was realised towards the end of the 19th Century that the thyroid gland produced a substance essential for health (Schiff, 1884; Hofmeister, 1894; Horsley, 1886; Fox, 1892; Mackenzie, 1892). The search for the postulated active constituent led Baumann (1896 a,b) to the discovery that the thyroid gland contained considerable quantities of iodine confined to a protein fraction which, on hydrolysis, yielded a substance effective in the relief of the signs and symptoms of myxoedema. Purification of the active constituent was not, however, achieved until Kendall (1915, 1919) isolated the crystalline product which contained 65 per cent iodine. Harington (1926 a,b) and Harington and Barger (1927) improved on Kendall's method of isolation and finally characterised and synthesised the compound which Kendall had name thyroxine. Further advances in thyroid biochemistry followed the introduction of newer and more refined analytical techniques. The introduction of radioisotopes of iodine, together with refined chromatographic techniques, led Gross and Pitt-Rivers (1952) to the discovery of a second biologically active constituent of the thyroid gland, namely tri-iodo thyronine.

Parallel with the discovery of thyroxine, another important factor in thyroid function was shown by Smith and Smith (1922). They demonstrated/

demonstrated that the atrophic thyroid gland of the hypophysectomised tadpole could be made hypertrophic by injections of bovine anterior pituitary gland extracts. Thus, the function of the thyroid gland appears to be the synthesis, storage and secretion of its own unique iodine containing hormones - the elaboration of which is stimulated by thyroid stimulating hormone (TSH) from the anterior pituitary. In the following sections an outline of the general biochemistry of the thyroid gland is given, with special attention to the subject under investigation, namely the iodoproteins of the thyroid gland.

MORPHOLOGY AND CYTOLOGY OF THE THYROID GLAND

The unit of thyroid function is the follicle (fig. 1). It consists of a group of cells whose apices border on a spherical mass of colloid. The lateral cell borders are in contact with one another and the bases of the cells rest on the follicular basement membrane which surrounds the group. The normal thyroid follicle, in cross section, appears as a simple ringlet of cuboidal cells surrounding a mass of colloid. In the normal human adult, the follicles vary considerably in size, the average diameter being about 300μ. On stimulation of the thyroid with TSH, the ringlet of cuboidal cells becomes a large irregularly outlined structure composed of tall columnar cells. Functionally the cells of the follicles are the site of hormone synthesis; the homogeneous colloid filling the lumen of the follicle represents the storage form of the hormone - as part of the/

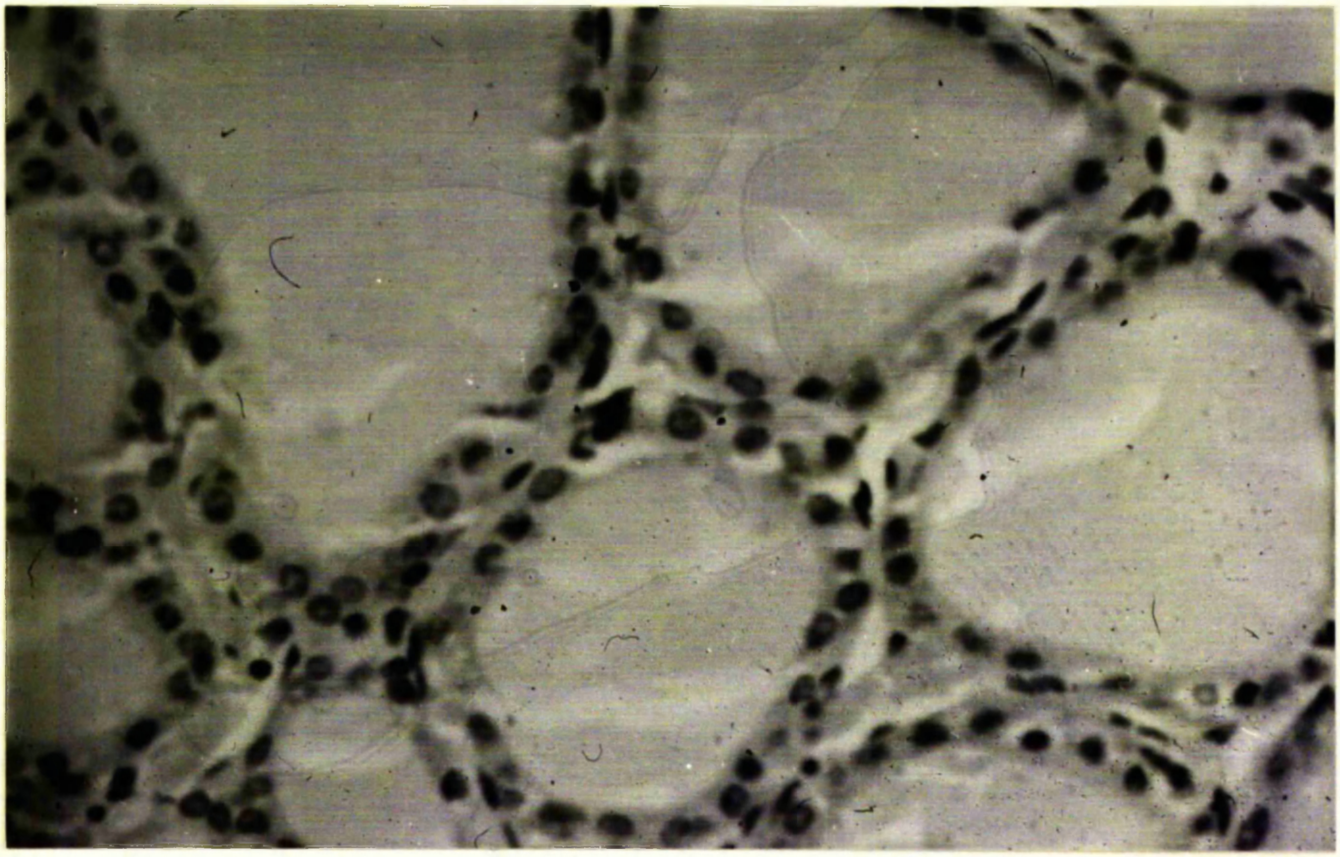

Fig. 1. Section through normal rat thyroid gland showing colloid-filled follicles (x40). Stained with haematoxylin and eosin.

the primary structure of the principal protein of the colloid, thyroglobulin.

The fine structure of the thyroid follicular cell is shown diagrammatically in fig. 2. The electron microscopy studies of Ekholm and Sjostrand (1957) and Wissig (1960) show the colloid as a mass of medium density and faintly granular texture. Thyroid follicular cells appear basically similar to the epithelial cells of exocrine glands engaged in protein excretion. Microvilli occur along the apical surface and the nucleus is centrally placed. The follicle cell is surrounded by a triple layered plasma membrane composed of two osmophilic layers separated by an electron transparent layer. In the apical plasma membrane the three layers are of equal thickness (Seljelid, 1967a) although in the lateral and basal plasma membranes the internal osmophilic layer is twice as thick as the external. The outer surface of the basal membrane is closely applied to the follicular basement membrane which englobes the follicle and helps maintain the integrity of the functional unit.

The cytoplasm of the thyroid cell is a complex structure containing mitochondria, granules and a variety of organelles. The thyroid cell has an extensive ergastoplastic network which is involved in the synthesis and elaboration of thyroglobulin. It also has an extensive Golgi apparatus which consists of smooth surfaced lamellae. The thyroid cell, therefore, appears to be of the merocrine type elaborating thyroglobulin intracellularly and transporting/

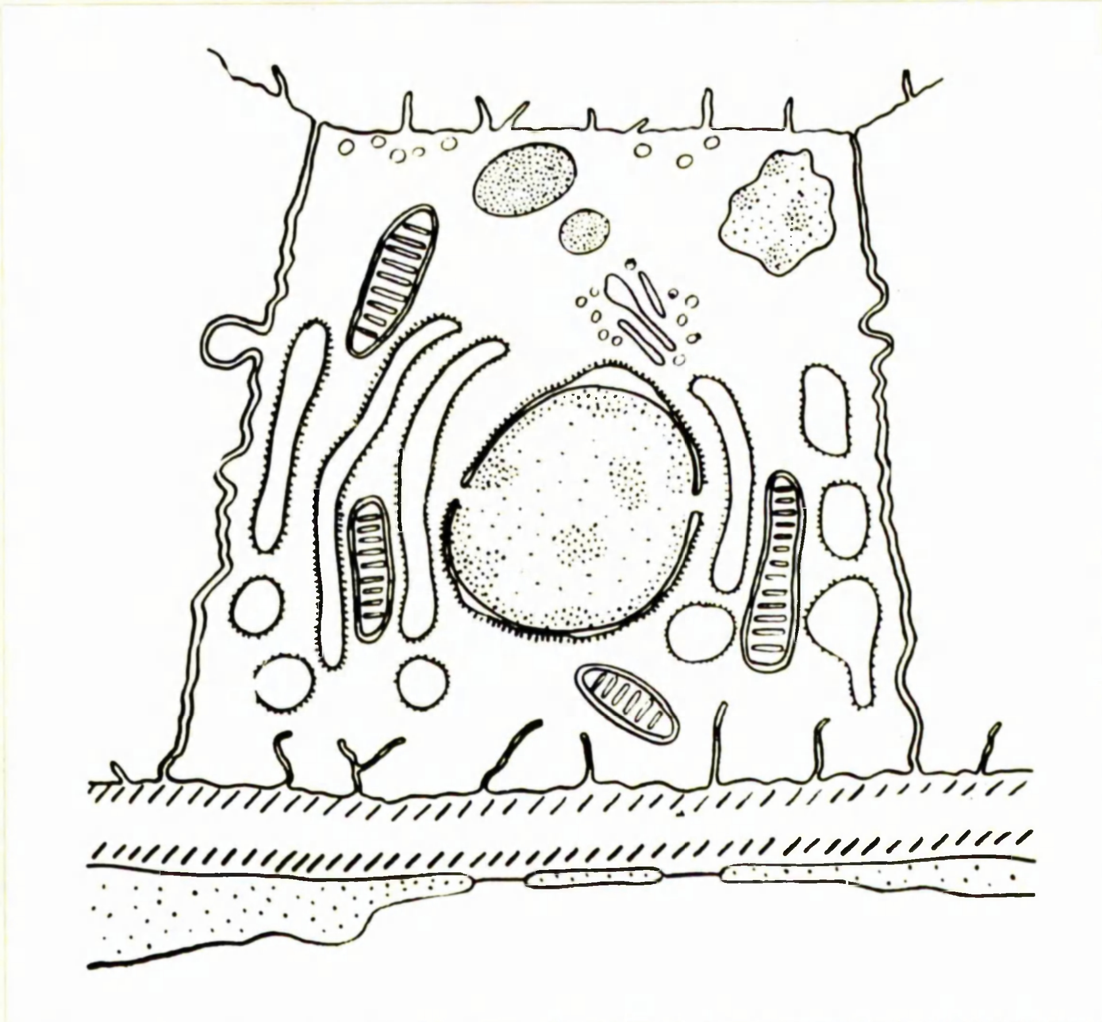

Fig. 2. Schematic representation of a thyroid cell showing the nucleus, mitochondria, ribosome-studded endoplasmic reticulum and Golgi apparatus. Large granules are also shown towards the apex of the cell which borders the colloid. The base of the cell lies in close proximity to a capillary. (reproduced from Ekholm and Sjostrand, 1957).

transporting this material apically to be secreted into the colloid. Unlike exocrine cells, however, they have an additional secretory function since they act as endocrine cells by releasing thyroxine into the perifollicular capillaries. Before the hormones can be released into the circulation, the colloid or thyroglobulin must be resorbed into the follicular cells and hydrolysed. Minute pores in the perifollicular capillaries allow plasma to come into direct contact with the basal membrane permitting diffusion of materials in and out of the acinar cells.

BIOSYNTHESIS AND RELEASE OF THYROID HORMONES

This topic has been reviewed by De Groot (1965). The thyroid gland assimilates inorganic iodide and establishes a concentration gradient between the gland and plasma. The accumulated iodide is initially oxidised and bound to tyrosine residues present in peptide linkage in thyroglobulin. There then occurs a little understood process whereby the iodinated tyrosines mono and di-iodo tyrosines couple to form the iodothyronine molecules, thyroxine and tri-iodo thyronine, which remain within the peptide chains of thyroglobulin. These iodo amino acids are subsequently liberated by the thyroid proteolytic system of enzymes. The iodothyronines are released into the plasma while the iodotyrosines are deiodinated and their iodide reutilised within the gland. The iodothyronines are transported in the plasma by specific serum proteins to their target organs where they exert their metabolic effect and in turn are degraded. Their iodide/

iodide is released to mix with the plasma pool and traverse this cycle once again.

IODIDE CONCENTRATING PROCESS

IODIDE CONCENTRATING PROCESS

This can be arbitrarily divided into two stages:-

i) Iodide transport

ii) Iodide organification.

Iodide transport

This has been reviewed by Wolff (1964) and Halmi (1964). Entry of iodide into the follicular cell occurs by two processes; a) by diffusion and b) transport. Diffusion is, however, an almost negligible pathway of iodide entry into the thyroid gland at physiological iodide concentrations in the plasma. Active transport is the more important mechanism by which the gland assimilates the anion. Administration of ^{131}I after goitrogen treatment (which prevents organic binding of iodide) leads to accumulation of iodide in the follicular lumina (Pitt-Rivers and Trotter, 1953). Although this might suggest that the site of active transport is confined to the apical margin of the cell, transferring iodide into the colloid space, data are available which locate the mechanism at the cell base. The most direct evidence for this is the concentration of free iodide by isolated thyroid cells (Tong, Kerkoff and Chaikoff, 1962). The exact mechanism of iodide transport is unknown, although several reports have provided evidence for the existence of a specific iodide carrier in the cell membrane (Berson and Yalow, 1955/

1955; Wollman, 1956; Wollman and Reed, 1962; Wyngaarden, Stanbury and Rapp, 1953). The form of the proposed iodide carrier is unknown although Vilkki (1962) suggested that thyroidal lecithin was involved. The kinetics of iodide binding to thyroidal lecithins has been studied by Schneider and Wolff (1965). Furthermore, it has been shown that the mechanism is energy dependent (Freinkel and Ingbar, 1955), K^+ - dependent (Wolff and Maurey, 1958) and related to the ATPase activity of the follicular cell membrane (Turkington, 1962). Seljelid (1967c) using cytochemical procedures has shown that ATPase activity is present in the basal plasma membrane - the proposed site of active transport - but not in the apical plasma membrane.

Iodide organification

The actual site of iodide organification is still largely speculative although it is universally accepted that organification involves the binding of iodine to tyrosine moieties in the primary structure of thyroglobulin and not to free tyrosine molecules which are subsequently incorporated into protein. The evidence for this sequence of events is the complete absence of specific iodotyrosine-activating enzymes or receptor t-RNA in the thyroid gland (Alexander, 1964; Cartouzou, Aquaron and Lissitsky, 1964), and the inhibition of amino acid incorporation into thyroglobulin by puromycin without affecting the organification of iodide (Soodak, Maloof and Sato, 1964; Seed and Goldberg, 1965). One of the major controversies/

controversies is whether the site of organification is confined to the colloid, the follicular cell or occurs in both. In this respect, radioautographic studies have been generally conflicting. Protein bound ^{131}I has been demonstrated completely confined to the follicular colloid even a few seconds after administration of tracer isotope (Wollman and Wodinsky, 1955; Pitt-Rivers, Niven and Young, 1964). However, at very early times after administration of the isotope, the radioactivity was detected, at first as rings around the periphery of the luminal space, later diffusing to give uniform blackening of the colloid. It seems therefore that iodination initially occurs at or near the colloid-cell interface in close proximity to the microvilli (Stein and Gross, 1964; Williams and Vickery, 1965). In support of this, Benabdeljlil, Michel-Bechet and Lissitsky (1967) have produced evidence that the apical poles of the follicular cells are the sole sites of iodination. However, radioautographs of foetal thyroids have shown that prior to the development of colloid-filled follicles, protein bound ^{131}I is located within the follicular cell (Rankin, 1941; Koneff, Nichols, Wolff and Chaikoff, 1949). On the other hand, from studies with surviving sheep thyroid slices, Nunez, Mauchamp, Macchia and Roche (1965) have postulated two independent sites of organification, one intracellular involving newly synthesised thyroglobulin and the other colloidal where the older thyroglobulin molecules undergo further iodination.

FORMATION OF IODOTYROSINES AND IODOTHYRONINES

Iodotyrosines

From in vivo studies, it has been shown that mono-iodotyrosine is the precursor of di-iodotyrosine (Taurog, Tong and Chaikoff, 1958; Pitt-Rivers, 1962). However, observations by de Groot and Davis (1961) have suggested that this is not a simple precursor product relationship. Mayberry, Rall, Bertoli and Berman (1964) and Roche, Lissitsky, Michel and Michel (1951), studying in vitro model systems of tyrosine iodination, showed that the formation of mono-iodotyrosine from tyrosine goes through a maximum with increasing iodination before the di-iodo form is produced. In contrast, Edelhoch (1962) and Van Zyl and Edelhoch (1967) showed that on iodination, in vitro, of a preparation of thyroglobulin containing 110 moles tyrosine, addition of 55 moles iodine produced only a small increase in mono iodotyrosine content with no further increase occurring up to 350 moles iodine added. The di-iodotyrosine content, on the other hand, increased almost linearly from 0-110 moles iodine before levelling off. When, however, the organisation of the protein was altered with 8M urea, iodination approached that expected from the model system. It has been proposed (Van Zyl and Edelhoch, 1967) that the globular form of the protein functions to prevent the extensive synthesis of mono-iodotyrosine and promote the synthesis of di-iodotyrosine thereby conserving iodide for iodothyronine synthesis.

The/

The mechanism of cellular synthesis of mono and di-iodotyrosines has been studied by de Groot and co-workers (de Groot and Carvalho, 1960; de Groot and Davis, 1961; de Groot, Thomson and Dunn, 1965) in subcellularparticles from sheep and calf thyroid tissue. Both tissues contain an enzyme capable of binding ^{131}I to soluble tyrosine (de Groot and Davis, 1962; Mahoney and Igo, 1966). These reactions are augmented by H_2O_2 producing systems and are inhibited by SH compounds, catalase and especially anti thyroid drugs. The available evidence suggests that either peroxide per se or some other biological oxidation system oxidises the iodide to a more reactive state for iodination of the tyrosine. Fischer, Schulz and Oliner (1966) have suggested a role for mono amine oxidase of mitochondrial and microsomal fractions of thyroid homogenates in the production of thyroidal peroxide. The thyroidal microsomal enzyme is present in greater amounts than in any other tissue and differs in substrate specificity from extrathyroidal enzyme. Although there is no evidence for the existence of a specific tyrosine iodinase, there is evidence that a single enzyme catalyses both the oxidation of iodide and the iodination of tyrosine (Klebanoff, Yip and Kessler, 1962; Taurog and Howells, 1966). Further evidence that a specific enzyme is not necessary has been produced (de Crombrugghe, Beckers and de Visscher, 1967) by studying in vitro iodination of human thyroglobulin.

Iodothyronines/

Iodothyronines

The iodothyronines, thyroxine and tri-iodothyronine, are formed by the coupling of two iodotyrosine residues with the splitting out of the residue of one alanine side chain (Johnson and Tewkesbury, 1942; Plaskett and Barnaby, 1964). Pitt-Rivers (1962) produced evidence that, in the rat, the distribution of ^{131}I in di-iodotyrosine and thyroxine suggested a precursor product relationship. Edelhoch (1962) and Van Zyl and Edelhoch (1967), from studies on in vitro iodination of native thyroglobulin showed that thyroxine was only formed after di-iodotyrosine synthesis was largely completed. This contrasts with thyroglobulin extracted from thyroid glands where the iodine content is relatively low. In this instance, about one-third of the iodine is in the thyronyl derivatives (Robbins and Rall, 1960) implying that, in vivo, there is a much higher efficiency of utilisation of incorporated iodine for hormone synthesis. Furthermore, Edelhoch (1962) showed that the native structure of thyroglobulin was not a pre-requisite for thyroxine synthesis in vitro, since synthesis was similar when iodination was carried out in either water or 8M urea. However, polypeptide chains containing only a single tyrosyl residue were not effective on thyroxine synthesis. De Crombrugghe, Beckers and de Visscher (1967) provided evidence that coupling occurs between di-iodotyrosines in the polypeptide chains.

Another/

Another mechanism for thyronine synthesis has emerged from studies with model systems involving the coupling of di-iodotyrosine residues in peptide linkage with a free molecule of di hydroxy-phenyl pyruvic acid (DIHPPA). Toi, Salvatore and Cahnmann (1963, 1965) could find no increase in thyroxine levels of thyroglobulin which had been iodinated in vitro. However, when the iodinated protein was reacted with DIHPPA in the presence of O_2, analysis of the protein revealed labelled thyroxine and tri iodothyronine. It has been suggested that the coupling reaction involves formation of a free radicle of DIHPPA (Matsuura, Kon and Cahnmann, 1964; Blasi, 1966 a and b). Recently Surtes (1968) has shown the presence of DIHPPA in extracts of rat thyroid glands. Fischer, Schulz, and Oliner (1965) have shown that a bovine microsomal fraction contains an enzyme which catalyses thyronine synthesis from mono iodohydroxy phenyl pyruvic acid and mono iodotyrosine in the presence of pyridoxal phosphate and Mn^{2+} ions. It is suggested that a pyridoxal phosphate - iodotyrosine chelate of the manganic ion may function as the initiator of the free radicle which has been suggested is involved in the coupling reaction.

THYROIDAL IODOPROTEINS

Thyroglobulin

Thyroglobulin serves as the matrix within which iodination takes place to form the iodotyrosines and the coupling of iodotyrosines occurs to form the iodothyronines. In addition, it represents the form in which the hormones are stored within the follicular colloid. Release of the thyroid hormones depends on resorption of thyroglobulin from the colloid followed by proteolysis within the follicular cell. The structure and biosynthesis of thyroglobulin are therefore of prime importance in the understanding of thyroid function.

Configurational properties of thyroglobulin

This has been reviewed by Edelhoch and Rall (1964) and Edelhoch (1965). It has been well documented that thyroglobulin is an iodoprotein of molecular weight close to 650,000 and axial ratio 10 (Derrien, Michel, Pederson and Roche, 1949; Edelhoch, 1960; O'Donnell, Baldwin and Williams, 1958; Shulman, Rose and Witebsky, 1955).

Subunit structure of thyroglobulin

1) Effect of pH and ionic strength on native thyroglobulin

Edelhoch (1960), using purified 19S calf thyroglobulin, studied the effect of variation of pH on the ultracentrifugal pattern of the protein. In 0.15M KNO_3, increasing the pH to 9.5 led to the appearance/

appearance of a slower sedimenting 12S component which amounted to 30 per cent of the total protein at pH 9.5. Further increases in pH were accompanied by increased 12S component and the appearance of a 17S component which Steiner and Edelhoch (1961) suggested had the same molecular weight as the 19S component arising by association of two 12S components at new surfaces exposed during unfolding of the protein. These transformations were almost completely reversible. At pH values above pH 11, 19S component gave rise to an additional component with sedimentation constant 8 (Edelhoch and Metzger, 1961). At pH 12, a 3S component was also found to be present. It was suggested that these latter transformations may arise by rupture of labile intramolecular covalent bonds under the alkaline conditions.

Similar transformations of the 19S component to the 12S component were produced by lowering the ionic strength of the solution (Lundgren and Williams, 1939; O'Donnell, Baldwin and Williams, 1958). Furthermore, sensitivity of the 19S component to alkali was enhanced by lowering the ionic strength of the solution (Edelhoch, 1960). Edelhoch and Metzger (1961) showed that the rate of dissociation could also be increased by the presence of certain divalent cations Hg^{2+}, Ni^{2+} and Cu^{2+}.

ii) Effect of heating on native thyroglobulin

Denaturation of a protein is always initiated by raising the temperature/

temperature of the solution to levels depending on the protein, pH and ionic strength of the solution. Thyroglobulin undergoes denaturation at measurable rates at room temperature and pH 11.3 (Metzger and Edelhoch, 1961a). At neutral pH, elevated temperatures are required and the presence of neutral salts increase the rate of denaturation at both neutral and alkaline pH.

Edelhoch and Metzger (1961) also showed that at pH 9.5 and 53°C, 19S thyroglobulin dissociated to produce 12S and 17S components. These species were denatured since they had lost solubility at pH5. However, at pH 9.5 and temperatures below 53°C, the same two products resulted but were soluble at pH5. From viscosity and optical rotation measurements of the two solutions, it was concluded that the loss of solubility in the former instance was due to minor configurational changes (Metzger and Edelhoch, 1961b).

iii) Effect of protein denaturants on native thyroglobulin

Protein denaturants such as sodium dodecyl sulphate (SDS), guanidine and urea are effective in rupturing both intermolecular and intramolecular non-covalent bonds in proteins. At low concentrations SDS (<0.001M), a 12S component was produced from native 19S thyroglobulin without accompanying unfolding of either form (Edelhoch and Lippoldt, 1960). Increasing the concentration of SDS led to progressive unfolding of both the 19S and 12S components to forms which behaved as random chain polymers.

The/

The effect of urea was studied by Edelhoch and Lippoldt (1964). At concentrations less than 2M urea, the 12S component only was formed. At higher concentrations the results were similar to those found in aqueous alkaline solutions; three new molecular species with sedimentation coefficient between 12S and 19S were formed. At 9M urea, unfolding of both 19S and 12S components occurred to produce new sedimenting boundaries at 10.0S and 6.2S respectively. At no concentration of either SDS or urea was transformation of 19S into the 12S subunits complete and it was suggested that, at neutral pH, there exists a fraction of native 19S thyroglobulin which is resistant to dissociation.

iv) Reduction of disulphide linkages in native thyroglobulin

Disulphide linkages in proteins can be either intrachain or interchain; reduction of the former produces changes in shape while reduction of the latter can result, predominantly, in changes in molecular size. De Crombrugghe, Pitt-Rivers and Edelhoch (1966) studied the effect of variation of the molar ratio of MSH (i.e. the ratio, [number of moles MSH]:[number of moles thyroglobulin x 202]) on 19S thyroglobulin. They showed that the extent of reduction was a function of pH, concentration of reductant, the solvent, and the time of reduction. The degree of reduction of disulphide bonds was considerably less in water than in 8M urea at corresponding pH values.

Employing/

Employing low molar ratios, De Crombrugghe, Pitt-Rivers and Edelhoch (1966) studied the molecular products of reduction in the analytical ultracentrifuge. The conditions were so adjusted that predominantly interchain disulphide linkages would be reduced with the minimum intrachain reductions. In 2M urea, pH 8.1, the unreduced protein showed as three boundaries corresponding to 19S, 17S and 12S. Reduction at molar ratios of 0.6 or less resulted in the appearance of a new component with sedimentation coefficient 6S. Increasing the molar ratio produced a second new component at 8S. At molar ratio 25 the latter species predominated, the 6S component almost disappeared and a much slower sedimenting 3S component was present. Viscosity measurements showed that these new components were formed by dissociation of the parent molecule and not from an increase in frictional coefficients. Similar results were obtained at pH 9.0 and 1.0M urea although at pH 10.1 and 1M urea, the 6S and 8S components were formed simultaneously. It has been suggested that the 6S component is formed from the 12S component and from the viscosity data has a molecular weight of 165,000. The 8S component is presumed to be an associated from of the 6S species.

From this and other data it has been suggested that the 19S thyroglobulin is composed of two 12S components which are linked by primarily non-covalent bonds. The 12S components, in turn, are themselves/

themselves composed of two polypeptide chains of similar molecular weight folded into globular forms. The disulphide bonds in thyroglobulin are predominantly intrachain with only a few serving to link the two chains comprising the 12S molecule.

Edelhoch and de Crombrugghe (1966) studied the structure of reduced and alkylated thyroglobulin. Complete reduction of 19S thyroglobulin with MSH in 8M urea followed by transfer to an aqueous medium showed that reduced thyroglobulin is a two chain molecule with a rigidity comparable to the native molecule although highly permeated with water. That there still existed considerable tertiary structure suggested that the pairing of the disulphide linkages is not a random process in thyroglobulin but a function of the primary structure of the protein and therefore the expression of the genetic mechanism. Furthermore, reoxidation of reduced thyroglobulin (Edelhoch and de Crombrugghe, 1966b))produced components with sedimentation coefficients of 19 and 12. This reoxidised protein had a partial identity to native thyroglobulin immunologically but differed chemically in that it was only partially reduced under conditions which normally completely reduce native thyroglobulin.

(v) Effect of in vitro iodination on native thyroglobulin

Edelhoch and Lippoldt (1962) studied the configurational changes occurring in native thyroglobulin iodinated in vitro to different levels. They showed that the configurational properties remained/

remained unaltered until approximately 40 per cent of the tyrosyl residues had been iodinated. At low levels of iodination, however, the molecule becomes more prone to thermal and alkaline pH treatment. This instability increases as the level of iodination increases, resulting in enhanced formation of 12S and 17S components. Similar results were obtained by Robbins (1963) on the iodoprotein fractions of native thyroglobulin isolated by DEAE cellulose. At high levels of iodination thyroglobulin is denatured and the properties of the denatured protein closely resemble those of the thermally denatured protein (Edelhoch and Lippoldt, 1962; Edelhoch and Metzger, 1961). Furthermore, the rate of tryptic hydrolysis, which is extremely sensitive to minor alterations in protein structure, was unaltered at low levels of iodination but increased at higher levels.

Nunez, Mauchamp, Pommier, Cirkovic and Roche (1966) using an enzymic method of iodination showed that the introduction of an additional 10 atoms I/molecule did not affect the sedimentation coefficient of thyroglobulin which already contained 20-30 atoms per molecule. However, molecules with 100 atoms I per molecule had sedimentation coefficients approaching 20S. This transition occurred between 20-30 atoms I per molecule. Furthermore, ease of dissociation increased the lower the iodination level of the molecule.

THYROIDAL IODOPROTEINS OTHER THAN 19S THYROGLOBULIN

Normally, analytical ultracentrifugation of thyroid gland extracts show/

show boundaries at 27S, 3-8S as well as at 19S. In certain species e.g. guinea pig, dormouse and poikilotherms, a native 12S component has also been reported (Lachiver, Fontaine and Martin, 1965). Nunez, Jaquemin, Brun and Roche (1965) have also solubilised a 32S protein from particles of sheep thyroid slices. Furthermore, Shulman, Mates and Bronson (1967) have recently isolated from human thyroid glands two additional proteins which occur in the 3-8S fraction.

27S iodoprotein has been isolated in a pure form by Salvatore, Vecchio, Salvatore, Cahnmann and Robbins (1965). It has a molecular weight of 1.2×10^{6}, the same amino acid composition and immunochemical properties as 19S thyroglobulin although the iodine content is much higher. Employing conditions which rupture only non-cavalent linkages, Vecchio, Edelhoch, Robbins and Weathers (1966) have shown that the 27S iodoprotein may not simply be a dimer of 19S molecules as previously postulated since the data could equally well fit a molecule with equimolar amounts of 19S, 12S and 6S sub-units. However, the matter is not fully resolved since experiments with rats produced data which was best explained by the dimer hypothesis. One additional feature of the 27S iodoprotein is that there was a complete lack of reversibility in the dissociation experiments.

Recently Salvatore, Aloj, Salvatore and Edelhoch (1967) isolated the native 12S protein from guinea pig thyroid glands. It differed in/

in iodine content from the 19S thyroglobulin prepared from the same extract. It was neither derived from nor did it dimerise to 19S thyroglobulin although it could form hybrid molecules with 12S components prepared by dissociation of 19S thyroglobulin. They have suggested that thyroglobulin consists of two different 12S subunits one of which is equivalent to the native 12S molecule. Salvatore, Aloj, Salvatore and Roche (1967) have also isolated a native 12S protein from lamprey thyroid gland. This had a sedimentation constant of 11.7S and a molecular weight of 331,000. Pulse labelling of the lamprey thyroid also indicated the presence of iodinated 17S and 5S proteins. It is suggested that in the lamprey the rate of aggregation is much slower than the rate of iodination resulting in the iodination of subunits.

A 4S thyroid protein has been reported by various workers in both normal and abnormal thyroid glands from different animal species (Witebsky, Rose and Shulman, 1956; Roche, Michel, Michel, Deltour and Lissitsky, 1950; Robbins, Wolff and Rall, 1959; Stanley, 1963; Hales, Lane, Richards and Stanley, 1965; Ramogopal, Spiro and Stanbury, 1965; Roitt, Jones and Mills, 1965). Shulman proposed the name thyralbumin for the 4S protein isolated from hog thyroid. The exact nature of the 4S proteins from the different species is as yet unknown. Ramogopal et al (1965) showed that the 4S iodo-protein from the abnormal human glands was immunologically and electro-phoretically similar to human serum albumin although Tata, Rall and Rawson/

Rawson (1956) could show no immunologic similarity to serum albumin in their preparation. Beckers and de Visscher (1961, 1963) showed that non-iodinated proteins occurred in certain human thyroid extracts which were similar to 4S protein from hog thyroid extracts. Recently, Shulman, Mates and Bronson (1967) iodinated the 4S protein from human thyroid extracts and showed that it could be resolved electrophoretically into two components, both of which had antigenic properties related to 19S thyroglobulin. Furthermore, an additional 7S component was also isolated which could be resolved into two components electrophoretically; only one of these, however, bore an antigenic relationship to thyroglobulin. The precise role of these lightweight thyroidal proteins in the normal functioning thyroid gland and the reason for their increase in pathological glands is still largely speculative and awaits clarification.

<u>HETEROGENEITY OF THYROGLOBULIN PREPARATIONS</u>

(i) <u>Iodine and Iodoamino acid content</u>

It has been known for some time that the iodine content of thyroglobulin can vary considerably from one preparation to another although the amino acid compositions remained identical (Derrien, Michel, Pederson and Roche, 1949; Robbins and Rall, 1960). However, from studies on DEAE celluose column chromatography of thyroglobulin preparations it has been found that the protein did not behave homogeneously. Ingbar, Askonas and Work (1959) using a gradient elution technique/

technique, showed that sheep thyroglobulin was eluted as a single assymmetric peak. Differences in iodine content as determined with ^{131}I were observed between the early and late eluting fractions. Robbins (1961) using a stepwise elution gradient fractionated beef thyroglobulin into three distinct fractions which, when rechromatographed were eluted in their original positions. Ui, Tarutani, Kondo and Tamura (1961) by a similar procedure fractionated hog thyroglobulin into six arbitrarily selected fractions and showed that the fractions eluted at higher ionic strength contained higher ^{127}I to nitrogen ratios than the early fractions. Shulman and Stanley (1961) using a gradient elution showed that hog thyroglobulin sub-fractions differed in iodo amino acid content. Robbins (1963) studied further the three sub-fractions obtained previously showed that the fraction eluted at the highest ionic strength had more iodine than the starting material while that eluted at the lowest ionic strength had less. All three fractions had identical mono-iodotyrosine contents but differed in di-iodotyrosine content and to a lesser degree thyroxine. Iodination of the starting material prior to chromatography resulted in the disappearance of the early eluting fraction and an increased, higher iodinated, later eluting fraction. These results showed that the behaviour of thyroglobulin on DEAE cellulose could be related, at least partly, to the iodine content of the molecules. Iodination of tyrosyl residues results in a lowering of the pK value of the phenolic hydroxyl group and since chromatography was performed at pH 6.9, where only diiodinated tyrosines and thyronines are ionized, the higher the

di/

diiodotyrosine content of the molecule, the more firmly it will be bound to the DEAE cellulose. However, while this is undoubtedly a major factor, the influence of other factors such as sialic acid content of the thyroglobulin molecules cannot be excluded (Robbins, 1963). Similar results were obtained by Bouchilloux, Rolland, Torresani, Roques and Lissitsky (1964) who fractionated sheep thyroglobulin on DEAE cellulose using a stepwise elution gradient. However, they could find no difference in sialic acid content between the fractions obtained. They showed that the amino acid composition and peptide maps after tryptic digestion were identical in all thyroglobulin fractions. Thus, it would appear that thyroglobulin represents a mixture of a large number of molecules which differ only in the relative proportions of the constituent iodo amino acids and in these properties reflecting this factor.

ii) <u>Ultracentrifugal heterogeneity of thyroglobulin</u>

Robbins, Salvatore, Vecchio and Ui (1966) showed that 19S thyroglobulin could be separated, by sucrose gradient centrifugation, into two fractions differing in iodine content. These results correlated with the behaviour of the 19S component on DEAE cellulose; the faster sedimenting components were the more strongly retained. This heterogeneity appears to be related to molecular weight, density and shape of the protein molecule. Ui (1962) showed that the higher the iodine content of thyroglobulin, the greater is the density, as shown/

shown by $CsCl_2$ density centrifugation. Nunez, Mauchamp, Macchia and Roche (1965) and Seed and Goldberg (1965) both showed that non-iodinated thyroglobulin has a sedimentation coefficient of 17 and sediments in the ascending limb of the normal thyroglobulin peak on sucrose density gradient centri fugation.

iii) <u>Heterogeneity after 'pulse-labelling' of thyroglobulin</u>

In addition to the above aspects, thyroglobulin has also been found to display a heterogeneity after pulse labelling with radioisotope. DEAE cellulose chromatography of thyroglobulin, pulse labelled with radioiodine, resulted in the elution of fractions which differed in specific activity. There was a decrease in the specific activity of the fractions eluted with increasing ionic strength of the eluting buffer (Robbins, 1963; Ingbar, Askonas and Work, 1959; Bouchilloux, Rolland, Torresani, Roques and Lissitsky, 1964; Nunez, Mauchamp, Pommier, Cirkovic and Roche, 1966). The latter authors have also shown that on incubation of thyroid slices with ^{3}H-tyrosine, the non-iodinated ^{3}H-thyroglobulin was eluted at a lower ionic strength than the halogenated molecules. Furthermore, the distribution of radioiodine in the thyroglobulin peak, on density gradient centrifugation, varied with the interval between pulse labelling and sampling. At short intervals, the highest specific activities were recorded in the slowest sedimenting fractions, whereas after longer intervals the specific activities were highest in the faster sedimenting fractions (Robbins, Salvatore, Vecchio and Ui, 1966). Similarly Nunez et al (1966), using thyroid/

thyroid slices incubated in vitro, showed that ^{125}I was incorporated into 17S and 18.6 S components which could be separated by DEAE cellulose chromatography. The in vitro labelled protein is more labile to SDS than preformed thyroglobulin (Sellin and Goldberg, 1965) and has a higher MIT/DIT ratio (Nunez et al, 1966). Similar results have been obtained with iodoproteins labelled in vivo in the rat (Lissitsky, Roques, Torresani and Simon, 1964; Lissitsky , Roques, Torresani and Simon, 1965; Lissitsky, Simon, Roques and Torresani, 1966). Vecchio, Edelhoch, Robbins and Weathers (1966) have also indicated the existence of a degree of heterogeneity among the molecules of 27S iodoprotein.

In conclusion, it would appear that the evidence at present indicates that thyroglobulin represents a complete spectrum of molecular species which have the same primary structure but differ in iodine content, possibly carbohydrate content, negative charge at neutral pH, density and symmetry. All these factors have been related to the maturity of the molecule and are probable all manifestations of the different iodine contents of the individual molecules.

CARBOHYDRATE STRUCTURE OF THYROGLOBULIN

Spiro and Spiro (1965) have shown that thyroglobulin contains approximately ten per cent carbohydrate in the form of galactose, mannose, fucose, N acetyl glucosamine and sialic acid. Similar proportions of these sugars have been found in sheep, pig and calf thyroglobulins although human thyroglobulin differed, in that it contained/

contained relatively more mannose and glucosamine. These carbohydrates are contained in two distinct glycopeptides: glycopeptide A with molecular weight 1050 which consisted of 5 residues mannose to 1 residue N.acetyl glucosamine and glycopeptide B (mol.wt. 3,200) containing mannose, N.acetyl glucosamine, galactose, fucose and sialic acid in the ratio 3:5:4:1:2. Thyroglobulin was shown to contain 9 moles A and 14 moles B per mole protein. Amino acid analysis of the glycopeptides suggested that, as in ovalbumin, aspartic acid is probably the amino acid involved in the glycopeptide linkage. Furthermore, from the ease of removal of sialic acid by neuramidase, sialic acid is also probably in a terminal position in the glycopeptide. Similar results have been obtained by Narishimha Murthy, Raghupathy and Chaikoff (1965).

BIOSYNTHESIS OF THE CARBOHYDRATE MOIETY OF THYROGLOBULIN

By the thyroid slice technique Spiro and Spiro (1966) showed that ^{14}C-glucose was incorporated into particle bound protein which, on solubilisation, behaves immuno-chemically and electrophoretically like thyroglobulin. Using a cell free system, similar incorporation of both ^{14}C-leucine and ^{14}C-glucose was obtained and the label was incorporated into both glycopeptide units. Using puromycin they showed that the carbohydrate moieties are added after completion of the polypeptide and probably in a stepwise fashion. Similar results have been obtained by Cartouzou,Greiff, Dipieds and Lissitsky (1967) and Bouchilloux and Cheftel (1966).

AMINO ACID COMPOSITION OF THYROGLOBULIN

Amino acid compositions of thyroglobulin from various animal species have been reported by Bouchilloux, Rolland, Torresani, Roques and Lissitsky (1964); Edelhoch and Rall (1964); Pierce, Rawitz, Brown and Stanley (1965). A statistical analysis of the composition of thyroglobulin from 7 mammals and 1 bird was carried out by Rolland, Bismuth, Fondarai and Lissitsky (1966). They showed that hog, beef and horse had identical amino acid compositions although significant variation occurred among the other species. One constant factor, however, was the content of valine, leucine and cystine as well as the sum of the amino acids with hydrophobic side chains which suggested a great similarity in secondary and tertiary structure of the thyroglobulins studied. Recently Pitt-Rivers and Schwartz (1968) have shown that in thyroglobulin the cysteine residues are not all present in disulphide linkage, but are present as free sulphdryl groups.

BIOSYNTHESIS OF THYROGLOBULIN

1. In vitro thyroid slice technique

Seed and Goldberg (1963, 1965a) and Lissitsky, Roques, Torresani and Simon (1964) demonstrated the rapid incorporation of ^{125}I into a protein with sedimentation coefficient 18.5 S. At very early times, the label was also incorporated into light weight components (3-8S) and also a 12S component. The origin of these smaller labelled units isolated from thyroid slices has not been

fully/

fully resolved; either they are formed by iodination of the lighter precursor proteins or result from the dissociation of an extremely labile thyroglobulin. Evidence that newly iodinated thyroglobulin is very labile, at least in comparison with preformed thyroglobulin, has been produced by Nunez, Mauchamp, Pommier, Circovic and Roche (1966); Sellin and Goldberg (1965); Lissitsky, Simon, Roques, Torresani, (1966).

Many workers have shown that iodination occurs after the formation of the polypeptide backbone of thyroglobulin (Goldberg, Seed, Schneider and Sellin, 1964; Taurog and Howells, 1964; Soodak, Maloof and Sato, 1964). In addition Seed and Goldberg (1963, 1965a), Lissitsky et al (1964), Nunez, Mauchamp, Macchia and Roche (1965) showed that labelled amino acids were incorporated into a 3-8S and 12S protein before appearing in a 17S-18S protein. The hypothesis that the lighter weight proteins represent subunits of thyroglobulin and possibly precursors was supported by kinetic, physical and immunological studies (Seed and Goldberg, 1963, 1965; Lissitsky et al, 1964; Sellin and Goldberg, 1965). Furthermore, since iodination of the amino acid labelled 17-18S protein produced 19S thyroglobulin (Nunez et al, 1966; Goldberg and Seed, 1965) and since ^{125}I-labelled thyroglobulin was intermediate in ease of dissociation between amino acid labelled pre-thyroglobulin and preformed thyroglobulin (Nunez et al, 1966; Lissitsky et al, 1966; Sellin and Goldberg, 1965) it

has/

has been suggested that iodination is not a pre-requisite for subunit aggregation but is essential for maturation, i.e. increased stability of the protein. Further proof of this hypothesis has been provided by Seed and Goldberg (1965b).

2. <u>In vivo biosynthesis</u>

At very short times after injection of ^{125}I, the label was incorporated into an 18S protein - incorporation into 19S protein only occurred at much later times up to 48 hours (Thomson and Goldberg, 1968). Since at no time was ^{125}I detected in either 3-8S or 12S proteins this would suggest that the appearance of these labelled proteins in the in vitro studies was indeed artifactual. However, the same authors have demonstrated a labelled 12S protein in rats recently withdrawn from a propylthiouracil regime. It is not precisely known whether , in this case, a true iodination of a subunit has occurred or whether this is, as before, a dissociation products of a labile 18-19S iodinated protein.

Using labelled amino acids, Thomson and Goldberg (1967) confirmed the in vitro studied in the rat demonstrating incorporation of the label into 3-8S and 12S protein prior to incorporation into a 19S protein after 48 hours. On the other hand, Vecchio, Salvatore and Salvatore (1966) could not demonstrate a labelled 12S protein in the rat although they did find this species in the guinea pig. Recently, Claar, Carlamagno and Vecchio (1968) have isolated a labelled 6S protein/

protein from the 3-8S fraction and shown a precursor product relationship between this species and 19S thyroglobulin. They have suggested that the 6S protein represents one quarter of the 19S molecule.

Site of Protein Synthesis

There is general agreement that protein synthesis is the property of the epithelial cell occurring in the particulate fraction of tissue homogenates. From autoradiographic studies Nadler, Young, Le blond and Mitmaker (1964) showed that within 10 minutes of injection of ^{3}H-leucine the label could be detected over the endoplasmic reticulum. Over the next hour the label travelled from the ribosomes to the lumen of the cisternaë of the endoplasmic reticulum but did not appear in the colloid until 4 hours later. Singh, Raghupathy and Chaikoff (1964, 1965) and Ekholm and Strandberg (1966) demonstrated that the microsomal fractions of tissue homogenates incorporated amino acids into protein in an energy requiring reaction. The latter authors also demonstrated the transfer of the label from the microsomal fraction to the soluble supernatant. Characterisation of the protein synthesised by the thyroidal microsomal system has been performed by Nunez, Mauchamp, Macchia and Jerusalmi (1965) and Morrais and Goldberg (1966). The labelled amino acid was incorporated into a 3-8S protein and a 19S protein although digitonin extraction of the particles yielded 3-8S protein only. Recently Cartouzou, Greiff, Dipieds and Lissitksy (1967) and Morrais and Goldberg (1967) have successfully shown the incorporation of/

of labelled amino acids into protein by isolated polyribosomal fractions. The protein synthesised was, however, immunologically different from thyroglobulin. Morrais and Goldberg (1967) have suggested that membranous structures are essential in the synthesis of true thyroglobulin precursor protein.

RELEASE OF THYROID HORMONE

The function of thyroglobulin is the synthesis and storage of the thyroid hormones, release of which requires the action of proteolytic enzymes. Several proteases exist in the thyroid gland which are capable of hydrolysing thyroglobulin (Litonjua, 1960; McQuillan, Mathews and Trikojus, 1961; Poffenbarger, Powell and Deiss, 1963; Lundbland, Bernback and Widemann, 1966). The association of this proteolytic activity with a thyroidal particulate fraction has been demonstrated by Pastan and Alinquist (1965) and Deiss, Balasubramaniam, Peake, Starret and Powell (1966). However, the exact nature of the enzyme-containing particles is not known. Nevertheless, the presence in many cells of organelles called lysosomes (Applemans, Wattiaux and de Duve, 1955) which contain a variety of proteolytic enzymes has led to the proposition that these organelles may be involved in the proteolysis of thyroglobulin. Colloid droplets have been detected in the cytoplasm of the follicular cells after TSH stimulation (Wollman, Spicer and Burstone, 1964; Wetzel, Spicer and Wollman, 1965; Seljelid, 1967d). Acid phosphatase and esterase activity were located in these particles/

particles and also in cytosomes which were found in and around the Golgi apparatus. These hydrolytic enzymes are characteristic of lysosomes (de Duve, 1959). Seljelid (1967d) has demonstrated the fusion of the cytosomes containing hydrolytic activity with colloid droplets. Thus it appears that the follicular cells contain preformed hydrolytic enzymes associated with cytosomes or lysosomes. Biochemical evidence for this was provided by Balasubramaniam, Deiss, Tan and Powell (1965). Dog thyroids were prelabelled with ^{131}I and the animals killed after TSH treatment. Differential centrifugation of the homogenised glands showed the highest labelling of the $PB^{131}I$ in that fraction where lysosomes sediment.

In conclusion, the available evidence suggests that thyroglobulin is resorbed from the colloid by a process of endocytosis to form colloid droplets. A fusion process then occurs between the colloid droplets and the enzyme-containing lysosomes. Thyroglobulin is subsequently hydrolysed within these vacuoles with release of the hormones into the perifollicular capillary network.

NATURE OF THE PRESENT INVESTIGATIONS

The present investigations were designed to study the metabolism of the thyroidal iodoproteins during goitrogenesis and to examine the physicochemical properties of the iodoproteins in established goitres.

Since a number of clinically defined goitres have been described in humans, goitres removed from patients at operation were used as the/

the source of iodoproteins from established goitres. The following parameters of the iodoproteins from the goitrous glands have been studied and compared with thyroglobulin isolated from a normal gland, electrophoretic and sedimentation properties, iodine and carbohydrate content, heterogeneity on DEAE cellulose and peptide fingerprints.

In the study of the thyroidal iodoprotein metabolism during goitrogenesis, it was impossible for obvious reasons to use human thyroid glands. Goitres were therefore induced in rats by the administration of methylthiouracil. The iodoproteins, at intervals throughout goitrogenesis, were fractionated on DEAE cellulose, sucrose gradients and polyacrylamide electrophoresis, to examine the relationship between the heterogeneity exhibited by the iodoproteins in those systems and thyroid function. Furthermore, during the goitrogen regime, the iodo amino acid content of the iodoproteins was estimated to demonstrate whether or not the iodoproteins with the slowest turnover rates had also the highest iodothyronine content. Finally, the effect of the goitrogen regime on thyroidal iodoprotein synthesis was studied in vivo by the administration of ^{3}H-leucine to the rats throughout goitrogenesis.

SECTION 1

METABOLISM OF THYROIDAL IODO PROTEINS IN THE RAT DURING GOITRE INDUCTION.

INTRODUCTION

The evidence for the heterogeneity of the soluble iodoproteins has been outlined in the general introduction. In addition to this physical and chemical heterogeneity, several workers have reported evidence for the existence of a functional heterogeneity. Triantaphylidis (1958 a, b) showed, in perchlorate treated rats, following a single injection of ^{131}I, that the thyroid glands lost a greater per cent of the ^{131}I than the ^{127}I. In the absence of goitrogen, she also demonstrated that radioiodine accumulated in the gland over a period of time was lost more slowly than ^{131}I taken up from a single injection. She concluded that newly accumulated iodine is metabolised in a pool comprising approximately one-third of the total thyroidal iodoproteins and that this small pool is the source of the secreted hormone. Similarly, Schneider (1964) in a double-labelling experiment, was able to distinguish between the release of old iodine and newly accumulated iodine. From the results, he formulated an hypothesis whereby the thyroid gland uses recently formed organic iodine before the older, in a "last come, first served" manner. These results were confirmed by Rosenberg, La Roche and Ehlert (1966).

The existence of a physioo-chemical heterogeneity among thyroglobulin molecules was originally demonstrated by techniques such as DEAE cellulose chromatography and sucrose gradient ultra-centrifugation. In addition it was possible to differentiate, by these/

these techniques, the newly iodinated from the older more highly iodinated molecules. It followed, therefore, that it should be possible to demonstrate the functional heterogeneity of thyroglobulin by fractionation of the iodoproteins with these techniques.

The following section deals with this and other aspects of thyroidal protein metabolism during goitrogenesis.

MATERIALS AND METHODS

1. Materials

Male rats were obtained from A. Tuck and Sons Ltd., Essex. Vims dog biscuits were manufactured by the Molassine Co. Ltd., Greenwich. ^{125}I and L.Leucine-4,5-^{3}H were obtained from the Radiochemical Centre, Amersham, Bucks. Amylopectin (pure), Carbowax (polyethylene glycol M.W.6000) and Pronase were supplied by Koch-Light Laboratories, Bucks. Silica gel was obtained from Merck as Keiselgel G containing $CaSO_4$ as binder. Methylthiouracil (MTU) and Cyanogum were supplied by B.D.H. Ltd., Poole.

2. Treatment of Animals

Male rats of approximately 350 g weight were maintained for 2-3 weeks on a low iodine diet consisting of Vims dog biscuits and de-ionised glass distilled water. A physically decaying solution of ^{125}I-labelled KI was then added to the drinking water; sp.act. at/

at the onset was 0.1 μC. ^{125}I per μg ^{127}I at a concentration of 0.1 mg per cent KI. The rats remained on this diet for 6-9 weeks, after which time it was considered that they had sufficiently approached the state of 'isotopic equilibrium'. ^{125}I-labelled KI was withdrawn from the diet and the rats were given daily, by gavage, 40 mg MTU dissolved in a slightly alkaline solution.

In ^{3}H-leucine labelling experiments the procedure was similar with the exception that ^{125}I was omitted from the diet. During the MTU regime 50 μC ^{3}H-leucine was given daily by intraperitoneal injection.

In one other experiment not involving 'equilibrium labelling' rats on a control diet were given, by gavage, a single dose of 10 mg or 40 mg MTU. They were then given a single injection of 50 μC ^{125}I intraperitoneally one hour before sacrifice over a period of 24 hours.

3. Preparation of thyroidal iodoproteins

At the appropriate time the rats were killed by ether anaesthesia. The thyroid glands were removed, dissected free from fibro-fatty tissue and carefully minced by hand at 4°C. The minced tissue was transferred to ice-cold phosphate buffered saline (P.B.S., 0.15M NaCl in 0.01M potassium phosphate buffer pH 6.8) and homogenised using a loosely fitting Teflon pestle (12 strokes at setting 4.5 on a Tri-R, Stir-R tissue homogeniser, Jamaica, New York). The homogenate was then centrifuged at 125,000 g for 30 minutes in the Spinco model L ultracentrifuge/

ultracentrifuge at $4^{\circ}C$. The supernatant was dialysed for 24 hours at $4^{\circ}C$ against 0.9 per cent NaCl and finally concentrated by Carbowax to give a final protein concentration of 1.5 mg per 0.1 ml. Protein estimations were performed prior to concentration by the method of Lowry, Rosebrough, Farr and Randall (1951).

In experiments involving tissue fractionation, the minced thyroid glands were homogenised in ice-cold 0.44M sucrose and the homogenate centrifuged as before at 125,000 g for 2 hours. The pellet was resuspended in 1.0 ml 0.44M sucrose and gently rehomogenised (4 strokes at setting 3.5) and the suspension re-centrifuged. The final pellet was washed once with the ice-cold sucrose solution and the supernatants and washings pooled. Aliquots of the original homogenate, 125,000 g supernatant and the resuspended particulate fraction were taken for radioactivity measurements.

4. Analytical procedures

A. Fractionation of thyroidal iodoproteins

i) DEAE cellulose chromatography

Whatman DEAE cellulose was pre-cycled by washing with 0.5 N HCl followed by deionised water to pH 4-5. The cellulose was then washed with 0.5 N NaOH and finally deionised water until the washings were pH 7. The treated cellulose was freed from 'fines' and stored in 2M NaCl.

The column was half-filled with 2M NaCl solution and the DEAE cellulose/

cellulose slurry poured into the column. The excess fluid was allowed to run out of the bottom of the column leaving sufficient fluid above to permit air bubbles to escape while the slurry was being added. The column was packed to the desired volume (26 cms x 1cm) by applying pressure with a small handpump. The addition of the DEAE cellulose slurry in saline solution prevents the tendency to mat and gives a uniform bed. The column was then washed free from chloride with deionised water. Presence of chloride was detected by adding a few drops of a 10 per cent Ag NO_3 solution to a sample of the column effluent acidified with conc. nitric acid. The column was finally equilibrated with the starting buffer - 0.025M potassium phosphate, pH 6.8.

The 125,000 g supernatant from three rat thyroid glands was applied to the column and washed on with 1 column volume of the starting buffer. The column was eluted by applying a stepwise gradient of NaCl in 0.025M phosphate buffer pH 6.8. The protein eluted at 0.15M, 0.2M, 0.25M, 0.30M and 2.0M NaCl was collected. The column effluent was collected in 3 ml fractions. The flow rate was approximately 15 ml per hour. The total iodoprotein eluted in each of the steps was obtained by measuring the ^{125}I content of the fractions eluted.

ii) Sucrose gradient ultracentrifugation

Approximately 0.1 - 0.2 ml of the 125,000 g supernatants containing 1-2 mg protein was applied to a 5ml, 5-20 per cent linear sucrose density gradient in PBS. Gradients were centrifuged for 6 hours at/

at 38,000 rpm in a Spinco Model L. ultracentrifuge (S.W.39 rotor). The optical density at 280 mμ (O.D. at 280 mμ) of the gradient was recorded with an automatic absorbance recorder attached to the Beckman DB spectrophotometer; 10 drop fractions were collected.

A specimen of sheep thyroglobulin was always spun in each rotor as a control. The sedimentation constant of the main OD peak at 280 mμ in the control was taken as 19S for reference purposes.

iii) Polyacrylamide gel electrophoresis

Electrophoresis was carried out on 5 per cent gels prepared as follows. 50 ml 25 per cent Cyanogum were added to 25 ml 0.35M TRIS 0.05M sodium citrate buffer pH 8.5 and diluted with 175 ml distilled water. The solution was mixed and de-aereated in vacuo. 0.5 ml 25 per cent (W/V) ammonium persulphate and 0.25 ml N, N, N^1, N^1-tetramethylethylenediamine were then added and the solution mixed by gently rotating the flask. The contents of the flaks were rapidly poured into a perspex mould (dimensions 17 x 12 x 0.8 cm) and covered with a lid fitted with projections to provide sample slots in the gel. Fifteen-thirty minutes were allowed for polymerisation.

0.1 ml samples of the 125,000 g supernatants were pipetted into the sample slots in the gel and electrophoresis was carried out at 250 v for three hours at $4^{o}C$. The buffer in the electrophoresis tank was prepared from 18.5 g boric acid and 20 g NaOH dissolved in 1 litre distilled water and adjusted to pH 8.5.

After/

After electrophoresis a 2-3 mm slice was removed from the upper surface of the gel, using the Shandon gel slicing apparatus. The remaining portion was stained with 1 per cent amido black solution in 5 per cent glacial acetic acid for 2-3 minutes. The background stain was removed from the gel by continuous washing with 5 per cent glacial acetic acid. This process was greatly facilitated by washing at 37°C.

To determine the distribution of ^{125}I, the stained gel was sliced into 1 mm sections using an ordinary tissue slicer (H. Mickle, Surrey). The individual slices were placed in test tubes containing 2 ml water for radio-activity measurements.

B. Estimation of iodoamino acid content of thyroidal iodoproteins

i) Pronase digestion

125,000 g supernatants from three rat thyroids were incubated with pronase under the conditions of Robbins, Salvatore, Vecchio and Ui (1966).

ii) Paper chromatography

Descending chromatography was carried out with Whatman 3 MM paper at room temperature using n.butanol-acetic acid-water (78:5:17) as solvent. The iodoamino acids were identified by comparison with known markers run on every chromatogram.

iii) Thin layer chromatograph (T.L.C.)

TLC was performed by a modification of the method of West, Wayne and Chavre (1965). The silica gel was washed with 1.2 N HCl in 90 per cent aqueous methanol until the eluates were free from iron by the/

the dipyridyl test, followed by absolute methanol until alkaline to Congo red. The washed gel was dried at 90°C.

Thin layers (0.25 mm) of silica gel were prepared on clean glass plates (5 x 20 cm) using the Shandon applicator. The gel was obtained by mixing thoroughly 28.5 g washed Kieselgel, G., 1.5 g amylopectin and 65 ml water, (Ballieux, Sebens and Mul, 1967). The plates were dried in a desiccator and used within 48 hours. The addition of amylopectin prevented the thin layer crumbling after chromatography.

5-10 µl aliquots of the pronase digests were applied to the thin layers and dried in a stream of cold air. The thin layer plate was developed in a single dimension with n-butanol saturated with 2N NH_4OH to a solvent front 15 cm from the origin. The iodoamino acids were identified by comparison with known markers run on every plate.

C. Measurement of radioactivity

Measurement of ^{125}I was carried out on a smaple changer type N695 Ecko Electronics, Essex. Radioiodine quantitation of iodoamino acids separated by paper chromatography was performed on segments cut from the paper strips. Similarly, with TLC this was performed on portions of the gel which had been scraped from the glass plate.

^3H-leucine was measured in a Nuclear Chicago liquid scintillation spectrometer. Fractions from sucrose gradients were washed into liquid scintillation vials with 10 ml Bray's solution (720 g Napthalene, 48 g PPO, 24 g POPOP, 1.2 litres methanol and 240 ml ethylene glycol made up to 12 litres with p-dioxane).

RESULTS

i) Effect of MTU dose on the organification of ^{125}I by the rat thyroid gland

The incorporation of ^{125}I into the 125,000 g supernatant fraction of rat thyroid homogenates, which had been previously dialysed to remove inorganic iodide, was taken as an index of iodide organification. The results are shown in figure 3. Both MTU doses effect maximum inhibition of iodine organification within 5 hours of administration although in neither instance is inhibition complete. At the level of 10 mg MTU, between 8 and 24 hours there is a reversal of the inhibition and at 24 hours the level of organification has returned to 78 per cent of the control value. At 40 mg MTU on the other hand there is only a very small reversal of the inhibition over this period and at 24 hours the level of organification is still only 3 per cent of the control.

The purpose of this experiment was the determination of a MTU dose which would reduce iodine organification to a minimum over a period of 24 hours so that follicular iodine metabolism could be studied without the complication of the utilisation of extra thyroidal iodine or re-cycled iodine. The results show that at 10 mg MTU the detoxication reactions of rat tissues have reduced the effectiveness of the goitrogen considerably over the 24 hour period. Therefore, the dose level of 40 mg MTU was chosen as that which when administered daily would fulfil the requirements outlined above.

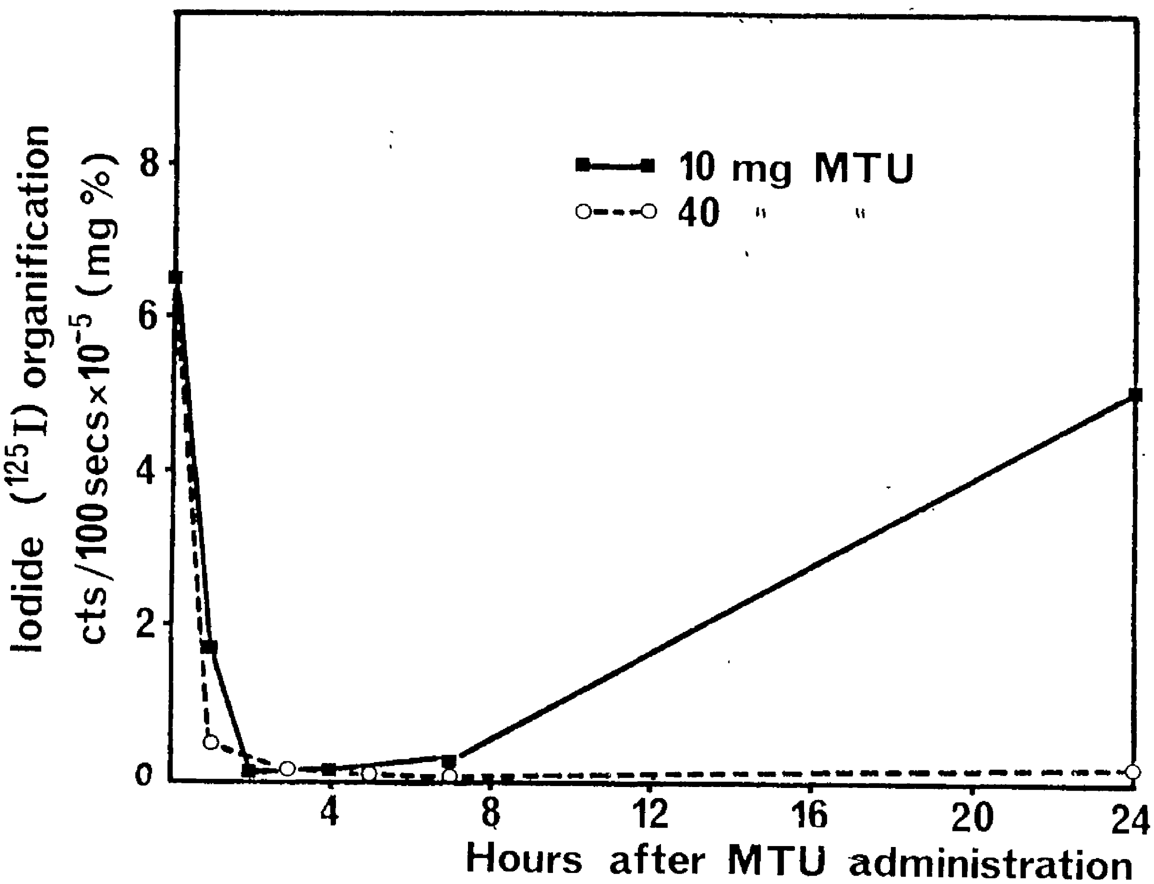

Fig. 3. Male rats were given, by gavage, a single dose of 10mg or 40 mg M.T.U. followed by a single injection of 50μC ^{125}I, intraperitoneally, 1 hr. before sacrifice. The organification of iodide is expressed as the cts/100 secs. ^{125}I incorporated into the dialysed 125,000g supernatant fraction X the thyroid weight per 100g body weight. Each point represents the mean of two experiments involving 2 rats per estimation.

ii) Response of the rat thyroid gland to MTU

The effect of daily administration of 40 mg MTU on the thyroid gland weight is shown in figure 4 and table 1. A typical response in the gland weight as a function of the duration of goitrogen treatment was obtained. The mean gland weights ranged from 4.4 mg per cent body weight for untreated animals to a maximum of 14 mg per cent for rats treated with MTU for 14 days. There was no significant increase in gland size until after the third day. In the following sections the behaviour of the thyroidal iodoproteins during these phases of goitre induction is examined.

iii) Metabolism of thyroidal iodine in the rat during goitre induction

The results are summarised in table 2 and figure 5. Table 2 shows the turnover of thyroidal ^{125}I per unit of thyroid weight. The ^{125}I content of the whole gland, represented by the tissue homogenate, and the soluble gland iodine, represented by the 125,000 g supernatant, decreased as expected after initiation of the MTU regime. The particulate iodine of the gland represented by the 125,000 g pellet, increased over the first five days of treatment then decreased over the next few days when the thyroid gland weight was undergoing a rapid increase. In table 2 and figure 5 the iodine remaining in each of these compartments is also expressed as a per cent of the zero time value. As can be seen from figure 5, total thyroidal iodine and soluble iodine content decreased in an apparent logarithmic manner. This is in/

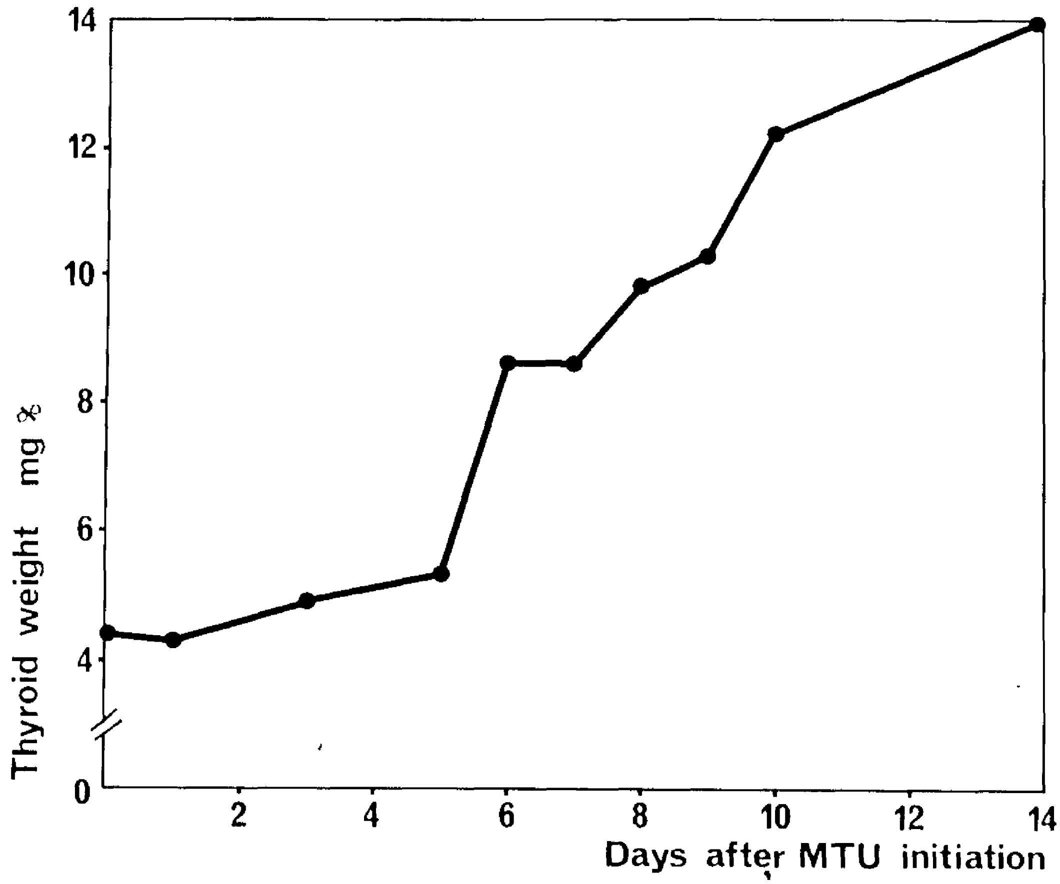

Fig.4. Male rats were given daily, by gavage, 40 mg M.T.U. The response of the thyroidglands to the goitrogen regime is expressed as thyroid gland weight (mg) per 100 g body weight. Details of the experiment are shown on table 1.

Table 1   Response of the rat thyroid gland to MTU

Days after MTU initiation	No. of animals	mgms thyroid wt per 100 g body weight		
		Mean	Range	S.D.
0	14	4.4	2.8- 7.1	± 1.8
1	14	4.3	3.5- 6.2	± 0.8
3	14	4.9	3.6- 6.6	± 0.9
5	10	5.3	5.0- 5.8	± 0.1
6	7	8.6	7.5- 9.6	± 0.8
7	4	8.6	6.5-11.2	± 1.9
8	4	9.8	9.1-10.4	± 0.2
9	5	10.3	9.6-10.8	± 0.15
10	6	12.2	10.4-14.0	± 1.1
14	4	14.0	12.6-15.4	± 1.6

Table 2

Days after MTU initiation	^{125}I cts per 100 secs x 10^{-6} x (mg thyroid weight per cent body weight)			^{125}I % Values					
	Homogenate	125,000g supnt.	Particulate fraction	Homogenate		125,000g supnt.		Particulate fraction	
				a	b	a	b	a	b
0	2.99	2.52	0.35	100	100	88.3	100	11.7	100
1	2.72	2.24	0.48	100	89.2	83.1	82.8	17.9	136.5
3	1.74	1.23	0.50	100	64.7	71.0	52.0	29	160.5
5	1.32	0.75	0.57	100	53	56.5	34.9	43.5	199.5
6	0.77	0.40	0.32	100	50.2	52.2	29.7	47.8	206
8	0.48	0.23	0.25	100	35.4	48.3	19.4	51.7	156.5
10	0.29	0.14	0.16	100	27.4	46.7	14.5	53.3	125.1

Rats, in a state approaching 'isotopic equilibrium' with ^{125}I, were given daily by gavage 40 mg MTU. The ^{125}I remaining in the whole thyroid gland, the 125,000g supernatant and the 125,000g particulate fraction, at intervals throughout goitrogenesis is shown. The results are also expressed as (a) a percent of the ^{125}I in the whole gland at the time of sacrifice
(b) a per cent of the ^{125}I present in each of the fractions at zero time

The results are the mean of two experiments. Animals were used in groups of 3.

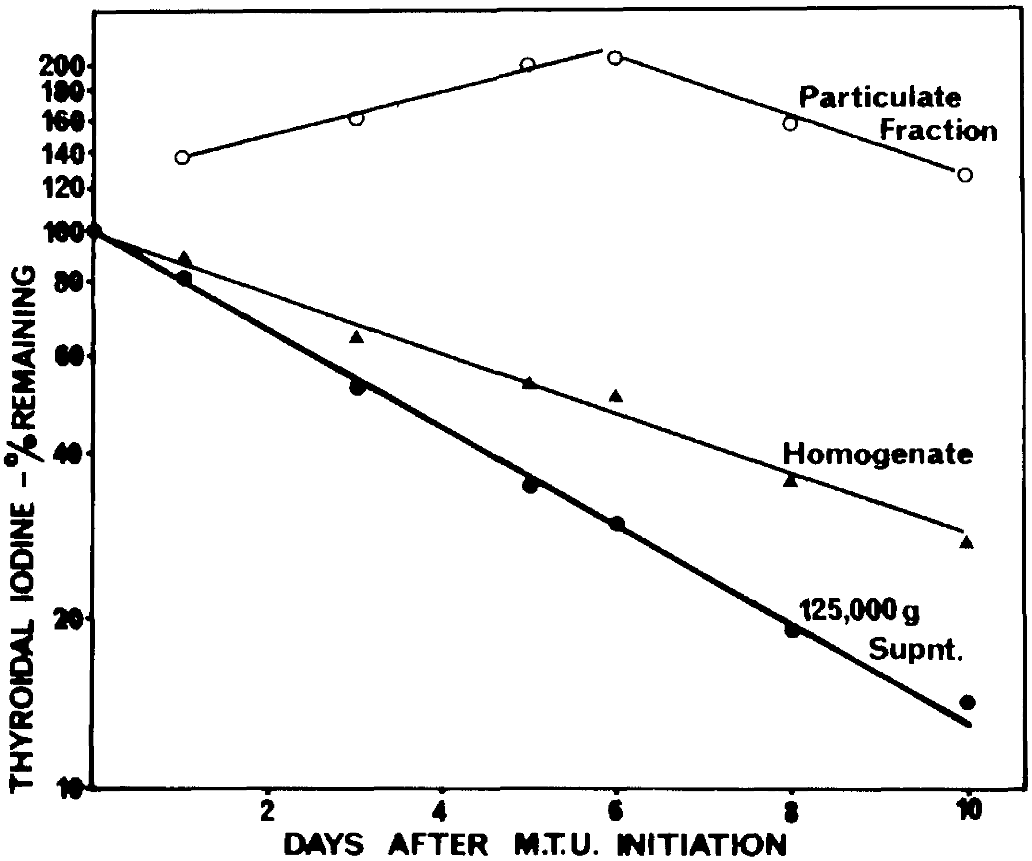

Fig. 5. Metabolism of Thyroidal Iodoprotein in the rat during goitrogenesis.

The ^{125}I remaining in the whole thyroid gland, the 125,000g supernatant and the 125,000g particulate fraction is expressed as a percent of the ^{125}I present in each of the fractions at zero time.

in agreement with the results of Rosenberg, La Roche and Ehlert (1966). However, from the slopes of the lines it can be seen that iodine was lost from the soluble compartment at a faster rate than from the whole gland. This can be related to the particulate iodide compartment. As seen in figure 5 this increased over the first 5-6 days before decreasing over the remaining period of goitrogen treatment. In addition, the slope of the line during the latter phase is similar to that showing loss of iodide from the whole gland. Finally, in table 2 the iodide contents of the soluble and particulate compartments are expressed as a per cent of the total gland iodide at the appropriate time interval. At zero time the soluble iodide represented 88 per cent of the total gland iodide. Over the first 6 days of goitrogen treatment this fell sharply until after 6 days the iodide was more or less equally distributed between the two cellular compartments.

iv) Study of the turnover of thyroidal iodoproteins by DEAE cellulose chromatography

The amount of ^{125}I-labelled iodoprotein eluted at each of the steps in the elution gradient was expressed as a per cent of the total radioactivity eluted from the column at each interval. Step 1 (0.20 M NaCl) represents that fraction of the total radioactivity eluted between 0.15M and 0.20M NaCl. The results are summarised in table 3. It can be seen that during goitrogenesis changes occurred in the relative proportions of the ^{125}I-labelled iodoprotein fractions.

At/

Table 3

Step in elution gradient	Days After Initiation of MTU Regime											
	0		1		3		6		10		12	
	a	b	a	b	a	b	a	b	a	b	a	b
Step 1 0.20M NaCl	65.3	100	60.8	65.9	56.38	33.8	43.2	9.4	33.8	3.5	23.6	0.97
Step 2 0.25M NaCl	25.8	100	28.2	73.2	33.4	51.5	37.1	21.8	29.1	6.7	25.2	2.61
Step 3 0.30M NaCl	3.57	100	4.15	77	4.8	54	5.8	20.0	5.1	8.4	7.8	5.9
Step 4 2.0M NaCl	1.60	100	2.07	91.3	3.3	81.6	6.5	61.0	13.6	50.1	23.3	39

The ^{125}I remaining in each of the iodoprotein fractions isolated by stepwise gradient elution from DEAE cellulose is expressed as:-

(a) a per cent of the total ^{125}I content of the 125,000g supernatant fraction at each interval

(b) a per cent of the ^{125}I eluted in the fractions at the appropriate zero time

The results represent the mean of two experiments. Animals were used in groups of 3

At zero-time the greatest proportion (65.3 per cent) of the iodoprotein was eluted at step 1. This, however, decreased with time until after 12 days on MTU it constituted only 23.6 per cent of the total ^{125}I-labelled iodoprotein eluted. The proportion of the total iodoprotein in step 2 on the other hand increased from 25.8 per cent at zero time to 37 per cent after 6 days before decreasing to 25 per cent after 12 days. The iodoprotein eluted at step 3 and step 4 represented only a small proportion of the total radioactivity at zero time. However, over the period of goitrogenesis this increased, being more pronounced in step 4 than step 3.

In table 3 and figure 6 the results are also shown expressed as a per cent of the radioactivity eluted at the appropriate zero time value. When these results are produced as a semi-logarithmic plot (figure 6) it can be seen that the ^{125}I-labelled iodoprotein eluted at each of the steps in the elution gradient decreased logarithmically. Furthermore, from the slopes of the lines it is apparent that iodoprotein was lost from these fractions at different rates. Thus the iodoprotein eluted at step 1 appears to be metabolised at the fastest rate while that eluted at step 4 was metabolised at a much slower rate. The iodoprotein fractions eluted at steps 2 and 3 were metabolised at intermediate rates. There would not appear to be any significant difference between the rates of metabolism of the iodoprotein eluted at steps 2 and 3.

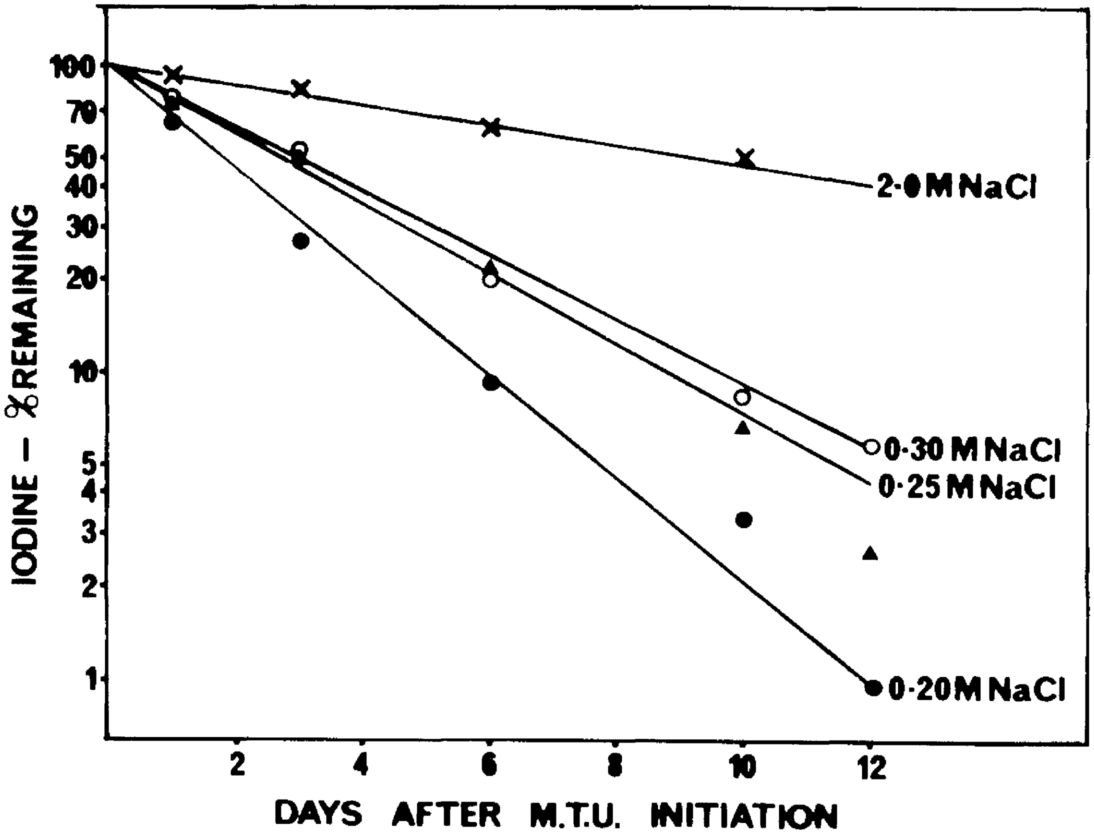

Fig. 6 Study of the turnover of Thyroidal Iodoproteins during goitrogenesis by DEAE Cellulose Chromatography.

The ^{125}I remaining in each of the iodoprotein fractions isolated by stepwise gradient elution from DEAE cellulose is expressed as a per cent of the ^{125}I eluted in the fractions at the appropriate zero time.

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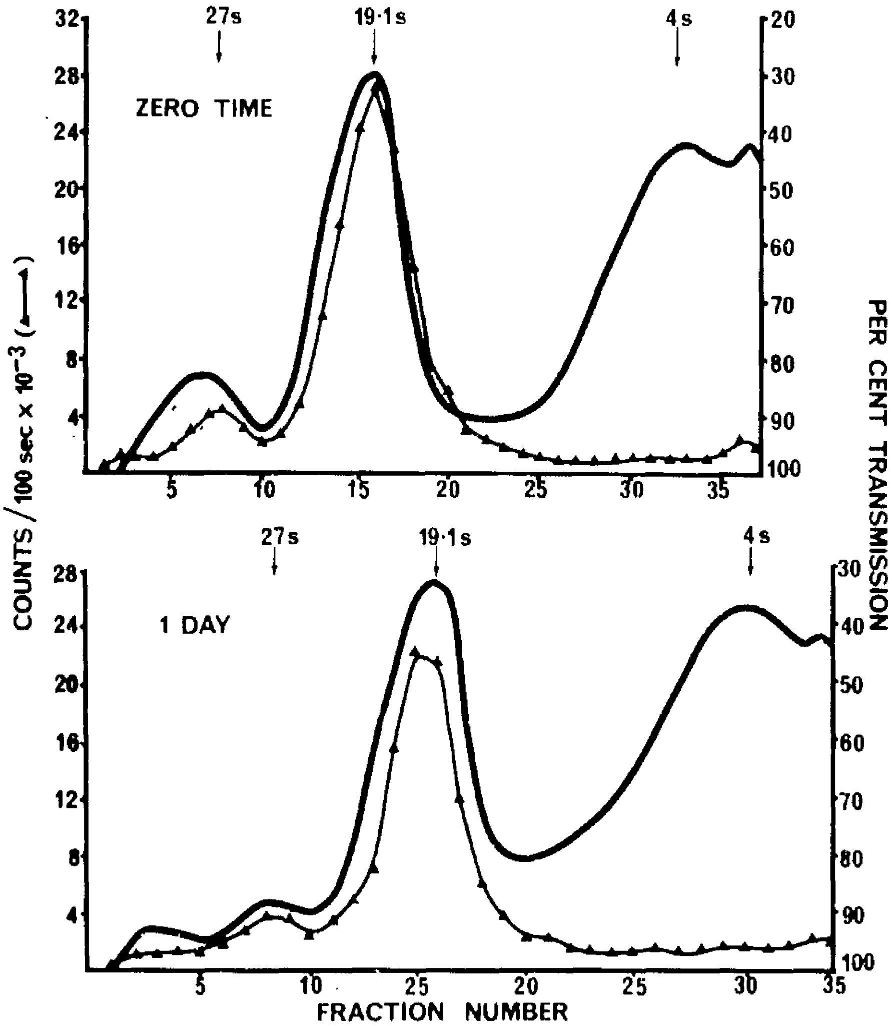
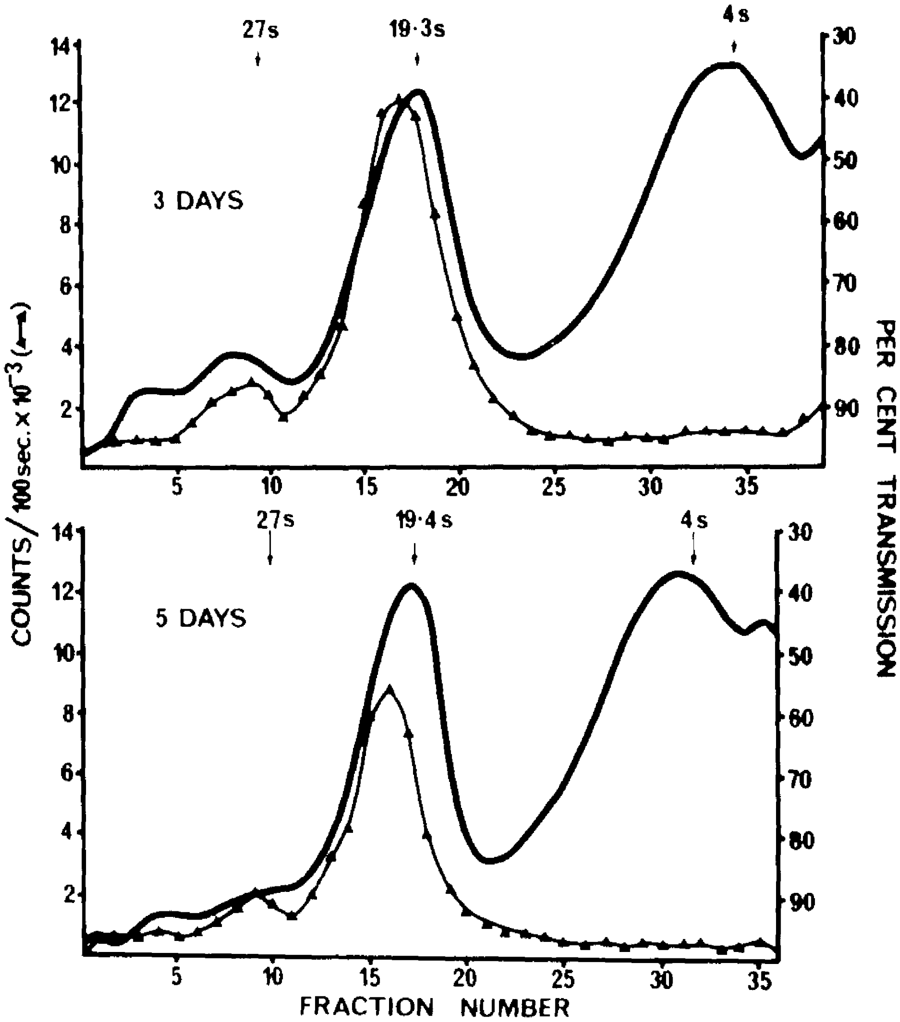
v) A study of the metabolism of thyroidal iodoproteins by sucrose gradient ultracentrifugation

The results of sucrose gradient centrifugation of the soluble iodoproteins in the rat thyroid gland during the goitrogen regime are shown in figures 7-9. The zero time pattern shows the normal distribution of thyroidal proteins of control animals; a 27S component, a major 19S component and a 3-8S component. The large 3-8S peak in the control rats is due to the fact that no initial salting out of the thyroidal iodoproteins was carried out. As can be seen in 'equilibrium labelled' rat thyroids at zero time the labelled peak and the protein peak are coincident at the 19S position. After initiation of the MTU regime at each interval studied there was a small but definite shift in the position of the labelled peak towards the bottom of the gradient. Thus, after 7-9 days the labelled peak had shifted from the 19S position at zero time to a position corresponding to 20.5S at 9 days. Considering the protein pattern, as shown by the absorbance at 280 mμ, there would appear to be a discrepancy. If, as expected in 'equilibrium labelling', the iodoproteins of the thyroid gland are uniformly labelled it would be expected that any shift in the labelled peak would be paralleled by a corresponding shift in the protein peak. As can be seen, however, this is not the situation - the protein peak is always displaced towards the top of the gradient.
A possible explanation is that the inherent error in fraction collection produces/



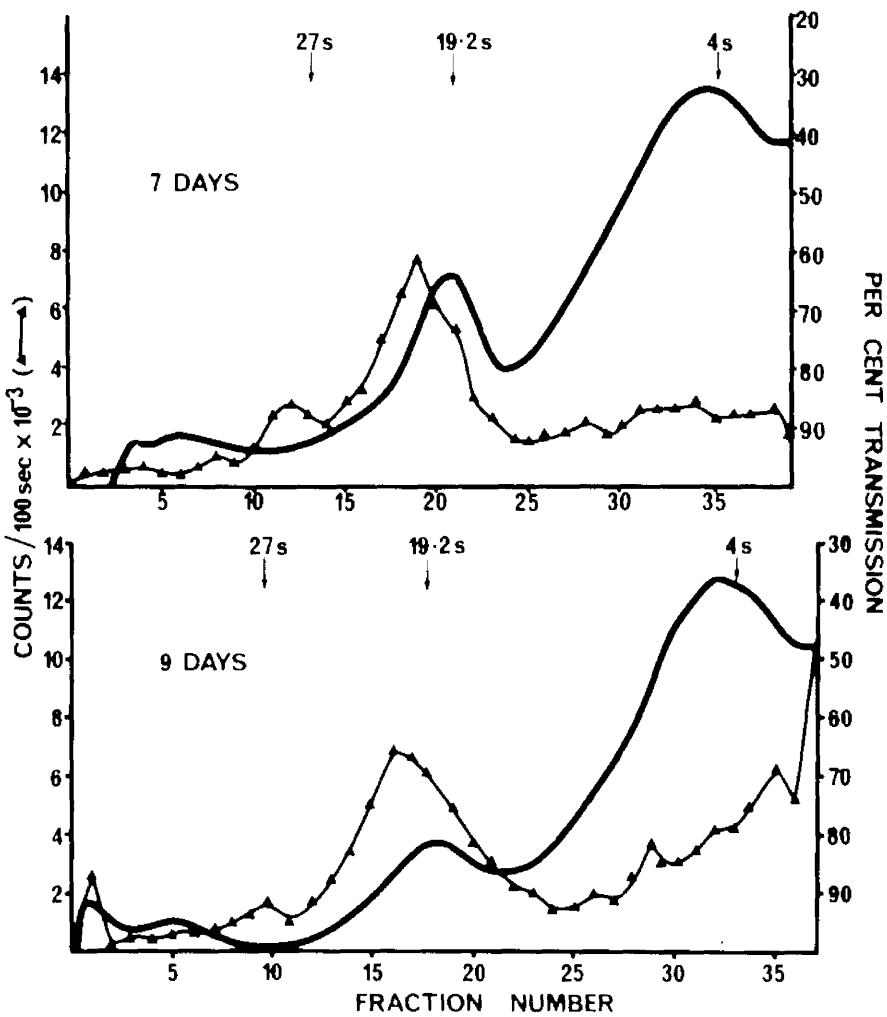
Figs. 7–9.

Sucrose gradient ultracentrifugation patterns of the soluble proteins prepared from rat thyroid glands during goitrogenesis. Goitres were induced in the rats, which were in a state approaching "isotopic equilibrium" with ^{125}I, by the daily administration of 40 mg M.T.U. Animals were used in groups of 3.



27s
19·3s
4s
3 DAYS
27s
19·4s
4s
5 DAYS
COUNTS/100sec. x 10^-3
PER CENT TRANSMISSION
FRACTION NUMBER

Fig. 8



27s
19·2s
4s
7 DAYS
9 DAYS
COUNTS / 100 sec × 10⁻³ (▲—▲)
PER CENT TRANSMISSION
FRACTION NUMBER

Fig. 9

produces an artifactual shift in the labelled peak. This was, however, ruled out by the fact that in three separate experiments a similar small shift in the position of the labelled peak was obtained. Alternatively, the failure of the protein peak and the labelled peak to coincide could be due to the increased protein synthesis which occurs during goitrogenesis. The labelled peak represents only iodoprotein while the OD peak measures protein in general. As there was no initial purification of the iodoproteins, it is possible that the increased protein synthesis causes contamination of the iodoprotein peak with lighter protein molecules. This could conceivably prevent the fractional changes in the position of the iodoprotein peak which are likely to occur. At any rate, the evidence suggests that a small but definite shift in the ^{125}I-labelled iodoprotein peak occurs during goitrogenesis.

In addition to the labelled peak at 19S, as already mentioned there was also a small labelled peak corresponding to 27S iodoprotein. The labelled peak was evident throughout the MTU regime, although at 7-9 days no corresponding OD peak was discernible. Nevertheless, when the amount of radioactivity in the 19S and 27S peaks at the appropriate time intervals were expressed as a per cent of the values at zero time both were found to decrease logarithmically. From the slopes of the lines it is apparent that 19S iodoprotein is metabolised at a faster rate than 27S iodoprotein (figure 10).

vi/

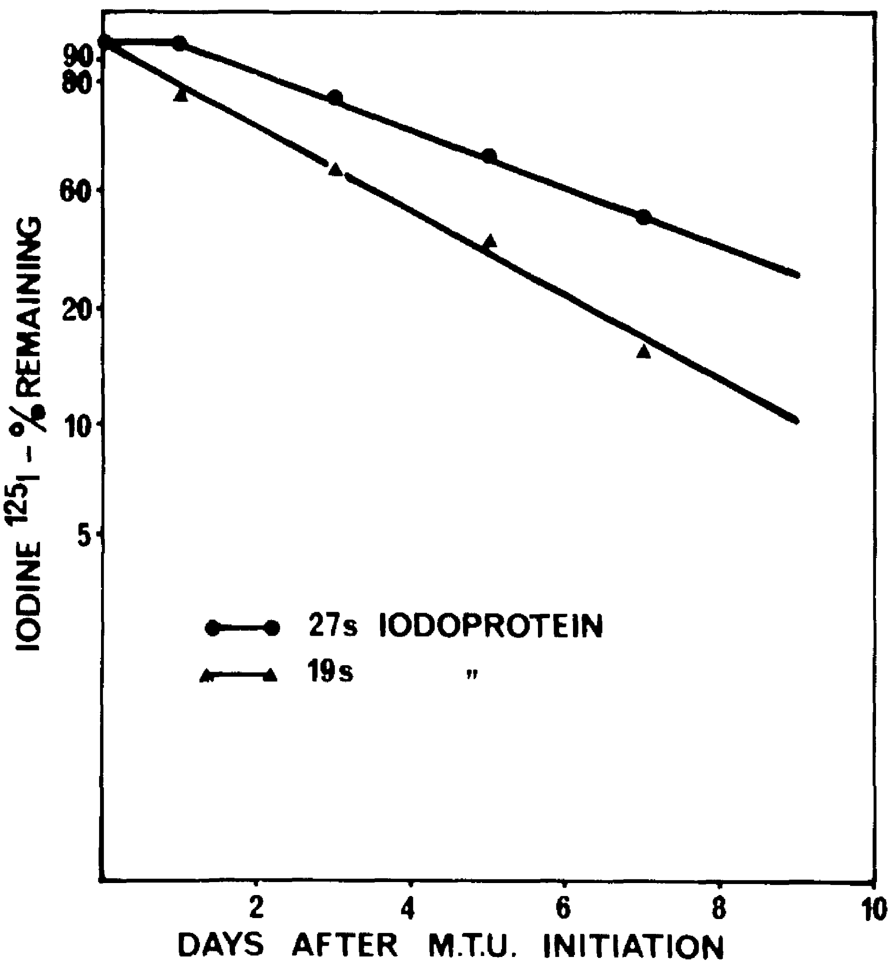
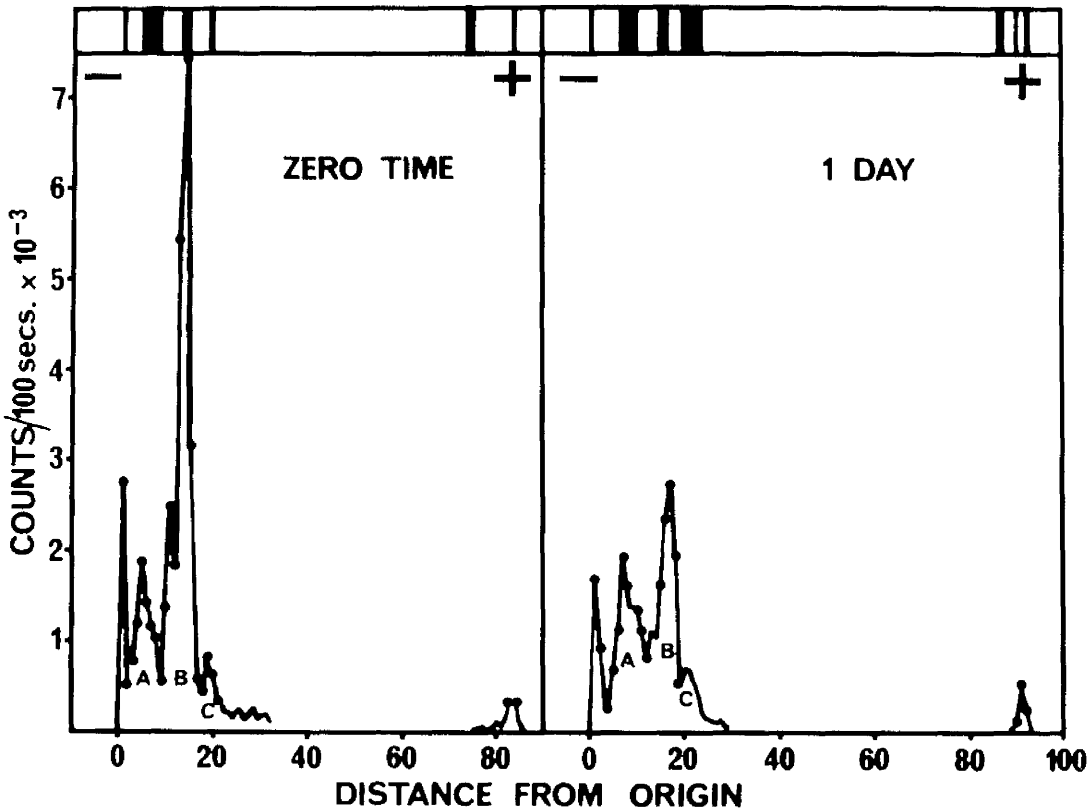


Fig.10. Metabolism of the 19S and 27S iodoproteins in the rat thyroid during goitrogenesis.

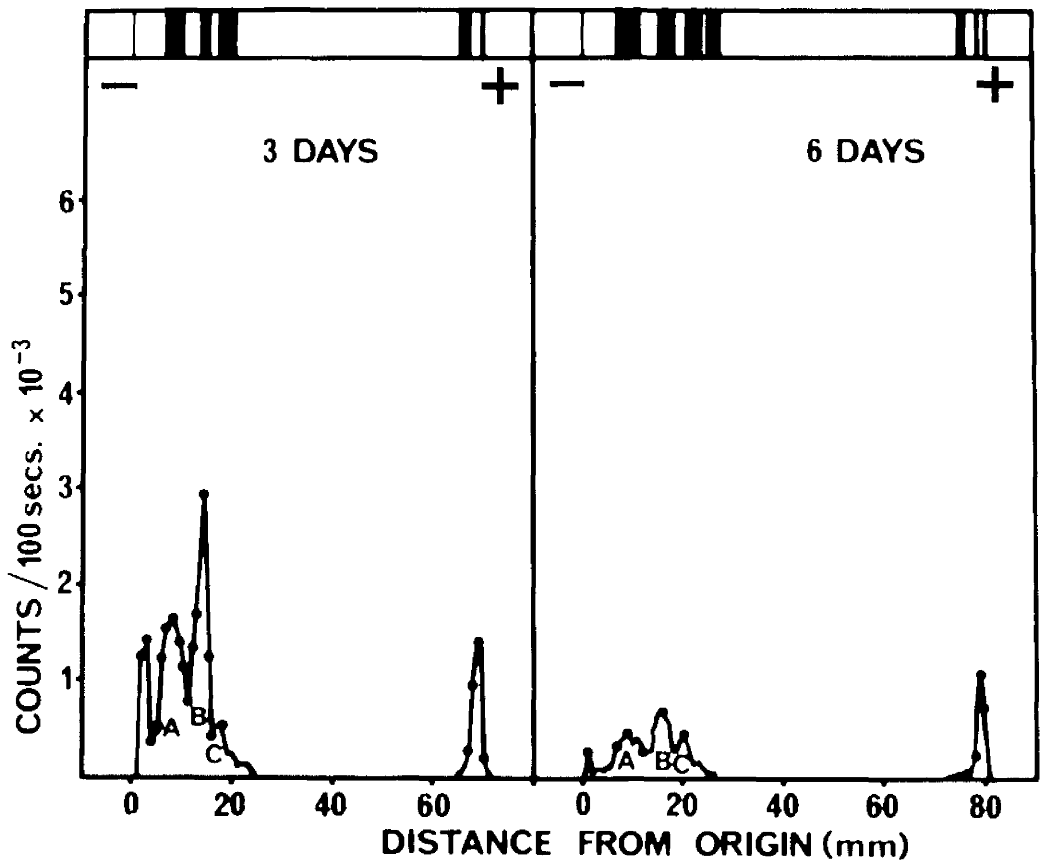
The 19S and 27S iodoproteins were isolated from the soluble proteins of rat thyroid gland homogenates by sucrose density gradient ultracentrifugation. (of Figs.7-9). The ^{125}I remaining in each of the iodoprotein components is expressed as a percent of the ^{125}I content of the component at the appropriate zero time. Each point represents the mean of 2 experiments. Animals were used in groups of 3.

vi) Study of the metabolism of thyroidal iodoproteins by polyacrylamide gel electrophoresis

The position of the stained proteins and the distribution of ^{125}I along the gel at intervals after initiation of MTU are shown in figures 11-12. At zero time the major protein band (comp.B) coincided with the largest labelled peak which contained approximately 59.5 per cent of the total radioactivity. A lightly stained slower migrating component (comp.A) had 19.5 per cent of the total radioactivity and a small labelled peak (comp.C) corresponding to a faster migrating component had 9.7 per cent. The remainder of the radioactivity was distributed between origin material which had barely entered the gel and a small labelled peak which migrated ahead of serum albumin at the front of electrophoresis. Figure 13 and table 4 show the changes occurring in each of these labelled components following initiation of the goitrogen regime. As can be seen, iodoprotein was lost within one day from both comp B and comp C. However, it is obvious from figure 13 that iodoprotein was lost at a faster rate from comp B than comp C. On the other hand, there was little change in the ^{125}I-labelled iodoprotein content of comp A and the origin material prior to the third day of the MTU regime. On the fifth day, however, there was a dramatic fall in the ^{125}I content of both components to 20.5 and 15.3 per cent respectively of the zero time value. The iodinated material at the front (table 4) increased to almost 3 times the zero time value after three days before decreasing over the next few days. However/



Figs. 11-12. Polyacrylamide gel electrophoresis patterns of the soluble proteins prepared from rat thyroid glands during goitrogenesis. Rats were treated as described under Figs. 7-9. The lower part of each fig. shows the distribution of ^{125}I - labelled iodoproteins along the gel. The upper part represents the distribution of protein along the gel after staining with 1% Amido black.



3 DAYS
6 DAYS
−
+
−
+
COUNTS / 100 secs. × 10⁻³
A
B
C
A
B
C
0
20
40
60
0
20
40
60
80
1
2
3
4
5
6
DISTANCE FROM ORIGIN (mm)

Fig. 12

Table 4 A study of the metabolism of thyroidal iodoproteins by polyacrylamide gel electrophoresis

	Per Cent ^{125}I Remaining				
Days after MTU Initiation	"Origin"	Component A	Component B	Component C	"Front"
0	100	100	100	100	100
1	98	100	51.5	82	120
3	96	90.5	34.5	66.5	350
5	15.3	20.5	25.8	42.3	273
6	8.4	20.5	12.1	42.3	227

The table shows the ^{125}I remaining in each of the protein components separated by electrophoresis expressed as a per cent of the zero time value.

"Origin" refers to iodinated material at the origin of electrophoresis which had not entered the gel.

"Front"" refers to iodinated material migrating with the electrophoretic front

Components A, B and C are described in figs. 11 - 12.

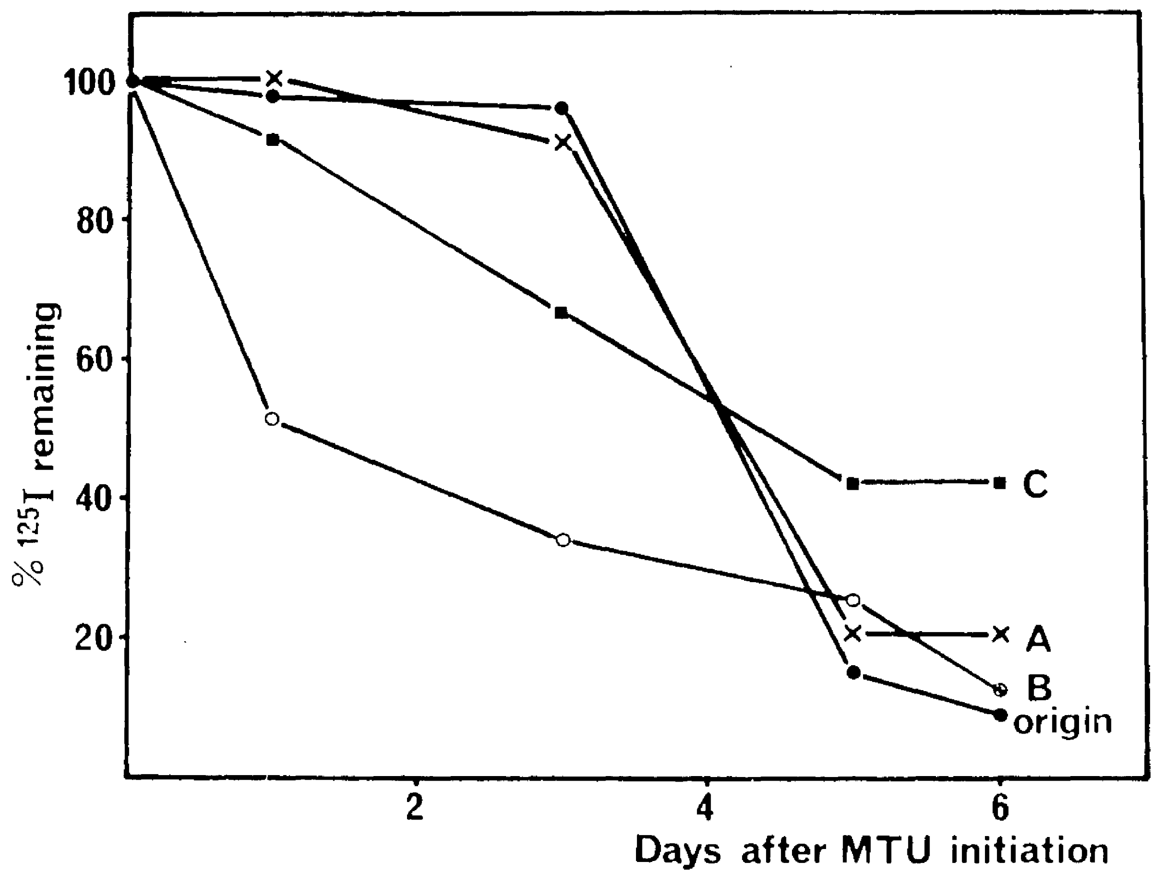


Fig. 13. ^{125}I remaining in each of the iodoprotein components separated by polyacrylamide gel electrophoresis expressed as a percent of the appropriate zero time value.

However at six days it was still double the control value.

In the stained gels (figures 11-12) comp B corresponded to the major iodoprotein component of sheep thyroglobylin, i.e. 19S iodoprotein. Comp A, however, did not coincide with the slower migrating component (27S iodoprotein) in the sheep control. Instead it migrated as a broad diffuse band ahead of the control. The comp C migrated with a similar minor 12S component in the sheep control. After five days Comp C appeared to be composed of two separate bands - a heavily stained band with a lighter band migrating ahead of it.

vii) Iodoamino acid content of the thyroidal iodoproteins during goitre induction

Aliquots of pronase digests of the supernatants of rat thyroid homogenates at different times after initiation of the MTU regime were analysed by TLC and paper chromatography. The results are expressed in table 5. At zero time, by paper chromatography, iodotyrosines constituted 61.4% and the iodothyronine 16.8% of the total radioactivity. Over the next five days the proportion of the total radioactivity released as iodotyrosines decreased slightly while that of the iodothyronines increased. Consequently the ratio of iodothyronines to iodotyrosines increased marginally over the first five days of goitrogenesis. However, an important factor is that at all times there was a large proportion (17-18 per cent) of the total radioactivity which remained at the origin. After seven days there was a sharp fall/

Table 5

Days after MTU initiation	Thyroidal ^{125}I - Per Cent Distribution							
	Origin		MIT+DIT		T_3+T_4		T_3+T_4/MIT+DIT	
	PC	TLC	PC	TLC	PC	TLC	PC	TLC
0	17.4	23.9	61.4	59.8	15.4	16.3	0.251	0.273
1	17.5	23.9	58.8	58.6	16.8	16.9	0.286	0.289
3	18.2	24.1	57.6	58.7	16.5	17.2	0.286	0.293
5	18.1.	25.0	57.0	57.1	18.7	17.9	0.328	0.331
7	39.7	-	44.2	-	9.7	-	0.22	-
9	60.0	-	27.4	-	5.8	-	0.212	-

The iodoamino acid content of thyroidal iodoproteins during goitrogenesis

Rats were treated as described under figs 7 - 9. The ^{125}I contents of the iodoamino acids are expressed as a per cent of the total ^{125}I recovered from the chromatograms. Each result is the mean of two estimations. Rats were used in groups of 3.

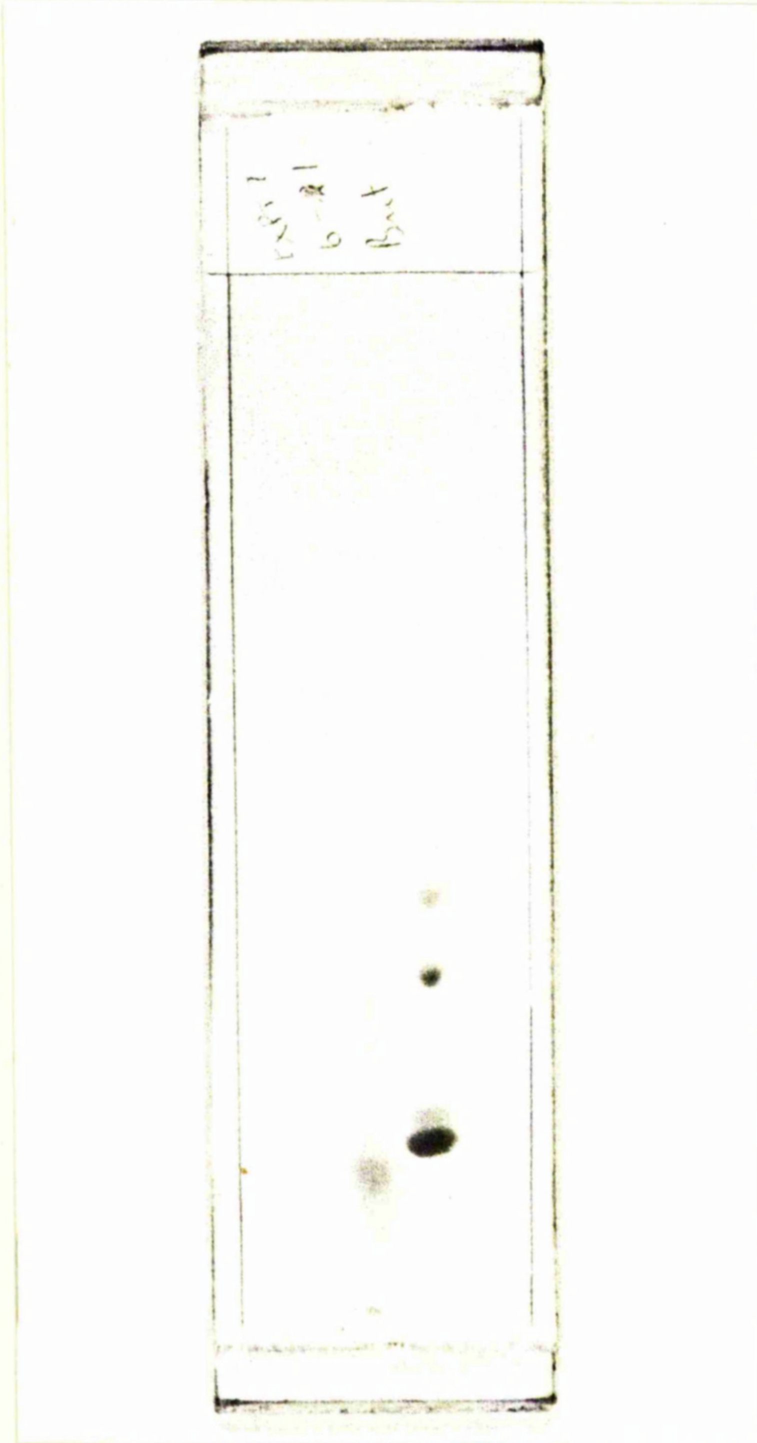


Fig. 14. Thin Layer Chromatography of Thyroidal Iodotyrosines and Iodothyronines.

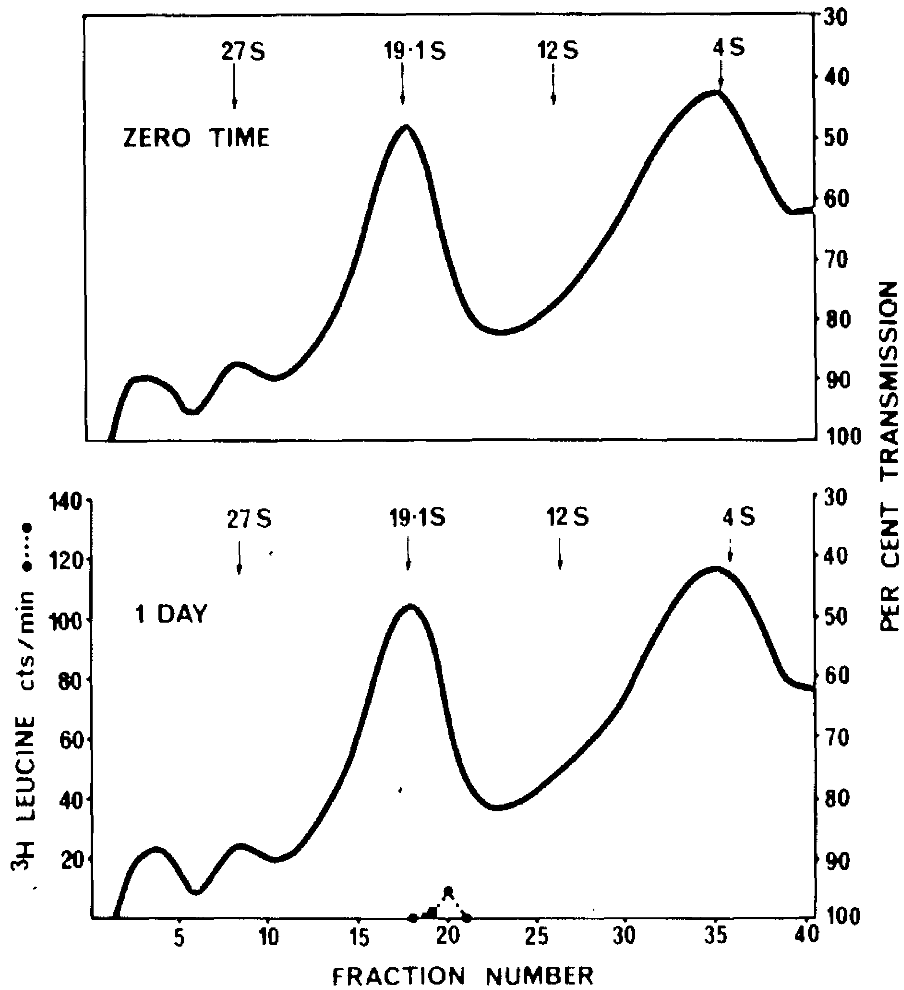
The plates were prepared and developed as described in materials and methods. From origin to solvent front the order of separation is DIT, MIT, T4 and T3.

fall in the per cent of the total ^{125}I released as iodotyrosines and iodothyronines to 44 per cent and 9.4 per cent respectively. This trend continued over the next two days and at nine days on the goitrogen the origin material had increased to 60 per cent of the total radioactivity. Furthermore, the ratio of iodothyronines to iodotyrosines fell below the control value over this latter period.

A typical separation of iodoamino acids as effected by TLC is shown in figure 14. When the pronase digests were analysed by this technique, as shown in table 5, the results for the first five days closely approximated to the results obtained by paper chromatography. No values were obtained for days 7-9 since, due to the smaller amounts of material which can be analysed by this technique, the radioactivity recorded for the iodothyronines was not significant. Nevertheless, the greatest proportion of the radioactivity recorded was found at the origin in agreement with the results obtained by paper chromatography.

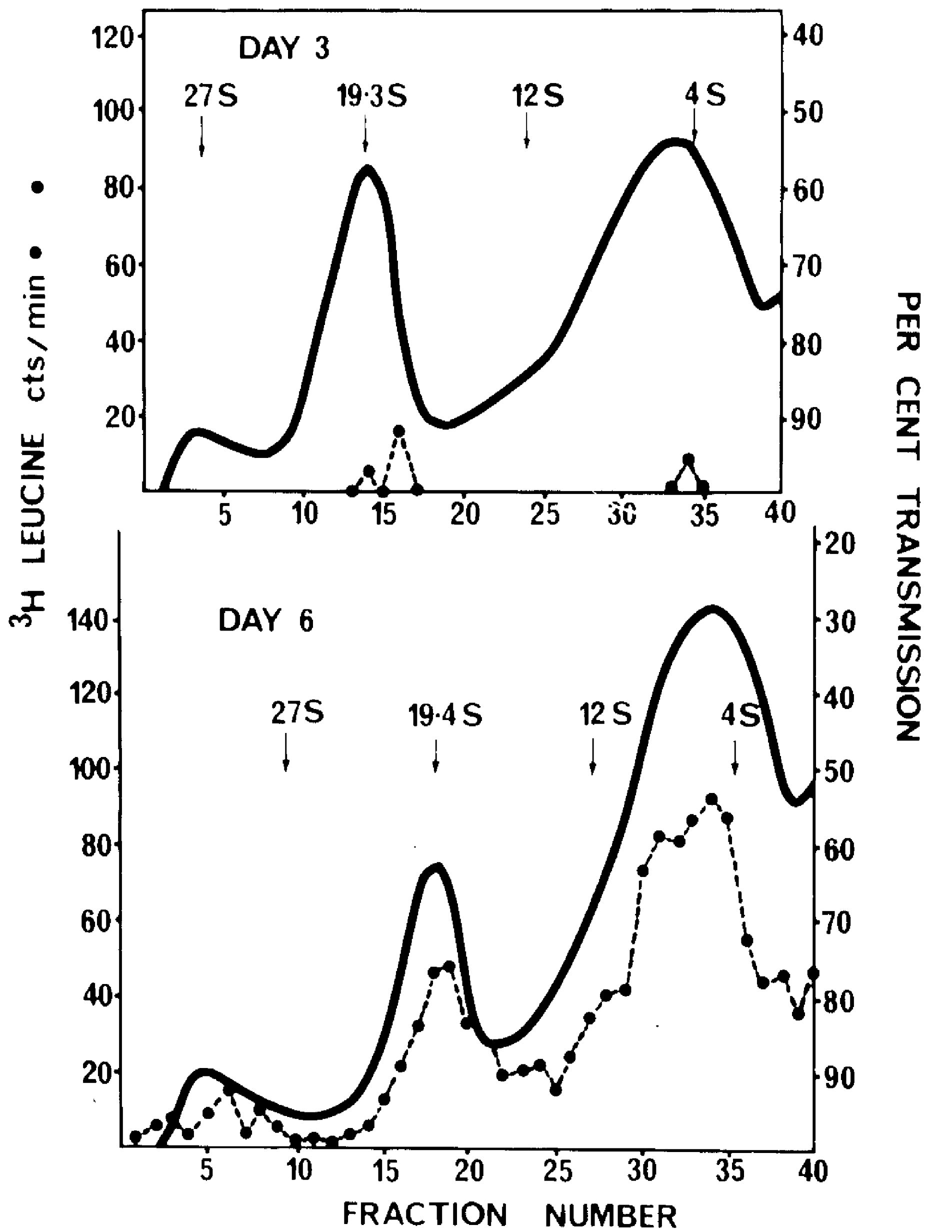
viii) Protein synthesis in the thyroid gland during goitre induction

The results are shown in figures 15-17. During the first three days after initiation of the MTU regime, there was no significant incorporation of ^{3}H-leucine into thyroidal proteins. Between day 3 and day 6 however the labelled amino acid was incorporated into thyroidal protein to a significant extent. At day 6 the largest labelled peak corresponded to the protein in the 3-8S region and there/



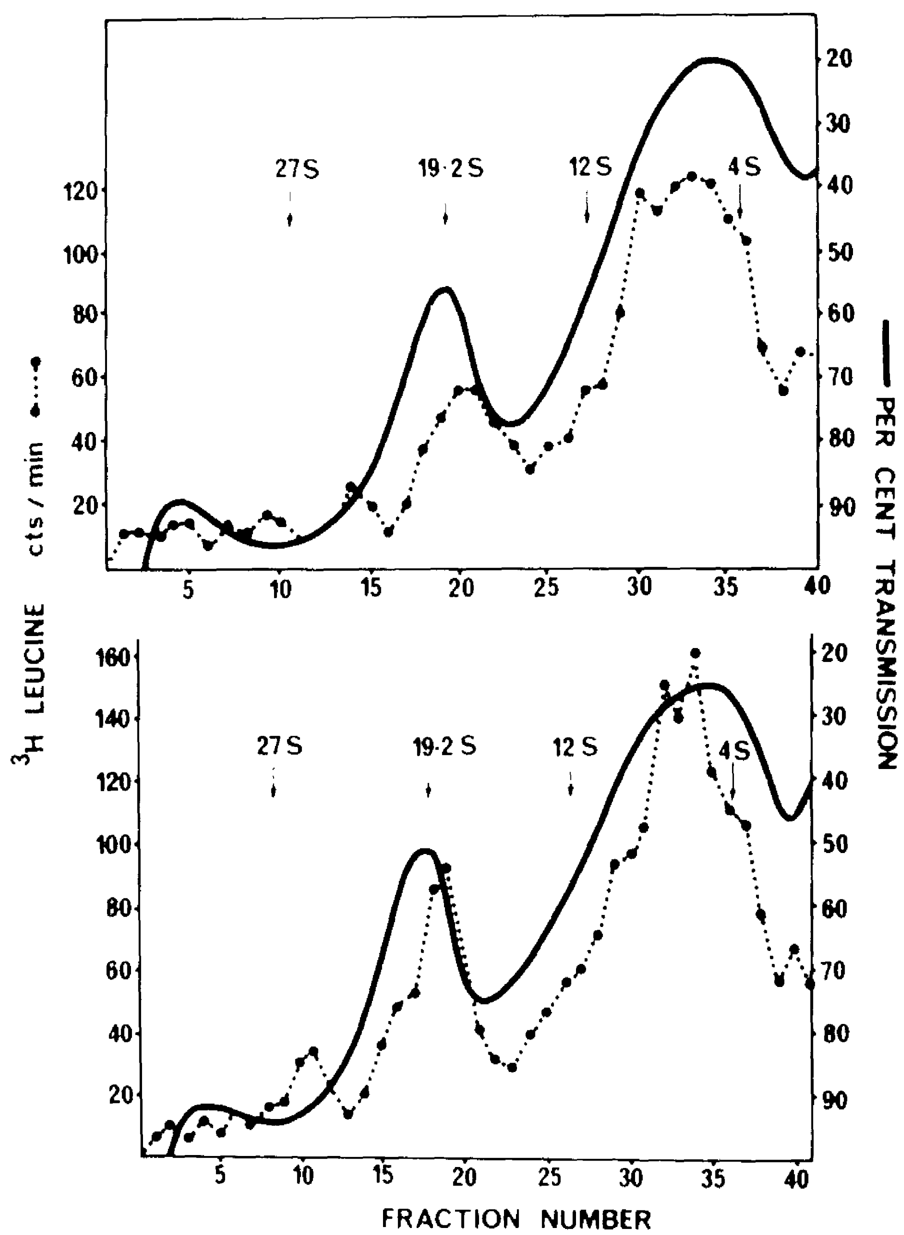
Figs. 15-17. Sucrose gradient ultracentrifugation patterns of the soluble proteins prepared from rat thyroid glands. Pattern of the incorporation of ^{3}H-leucine into the thyroidal proteins during goitrogenesis. Rats, maintained on a low iodine diet, were given daily, by gavage, 40 mg . M.T.U. and 50µC ^{3}H-leucine intraperitoneally. Animals were used in groups of 3.

Note: Fig. 17 shows the pattern of ^{3}H-leucine incorporation after 8 and 10 days.



DAY 3
27S
19·3S
12S
4S
DAY 6
27S
19·4S
12S
4S
^{3}H LEUCINE cts/min •
PER CENT TRANSMISSION
FRACTION NUMBER

Fig. 16



27S
19·2S
12S
4S
120
100
80
60
40
20
5
10
15
20
25
30
35
40
cts / min
^{3}H LEUCINE
PER CENT TRANSMISSION
160
140
FRACTION NUMBER

Fig. 17

there was also a labelled 12S component present. The labelled peak in the thyroglobulin region corresponded to 18.5-19S. After eight days there was an increased incorporation of the label into all components and the labelled peak in the thyroglobulin region now corresponded to 17.5-18S. In addition there was also a small discrete peak corresponding to 24-25S indicating that the labelled amino acid was also incorporated into a heavier protein component. After ten days there was a further increase in the label incorporated. The peak in the thyroglobulin region remained in the 17.5-18S region and the small labelled peak corresponding to 24-25S was more significant.

DISCUSSION

i) Metabolism of thyroidal iodine during goitre induction

As outlined in the general introduction, the metabolism of colloidal iodoprotein which represents most of the soluble gland iodine involves engulfment of the colloid by a process of endocytosis. This is followed by fusion of the engulfed colloid or colloid droplet with lysosomal structures (Seljelid, 1967d) which are presumed to contain the enzymatic machinery necessary for hydrolysis of the iodoprotein. Seljelid (1967c) has also shown that during stimulation of the thyroid gland with TSH the resultant colloid droplets are much larger and more numerous than in normal glands and the size depends on the degree of stimulation. Furthermore, stimulated glands compared to normal glands had a higher proportion of colloid droplets which did not exhibit the signs of possessing the hydrolytic enzyme system (Seljelid, 1967d). This implied that in the stimulated gland, at least at early times after stimulation, the resorption of the colloid occurred at a faster rate than the existing hydrolytic machinery could contend with. This affords an explanation for the difference in rates of disappearance of ^{125}I from the whole gland and the 125,000g supernatant. Administration of MTU would lead to increased TSH stimulation of the thyroid and result presumably in the sequence of events described. Initially the rate of colloid resorption would be greater/

greater than the rate of acquisition of hydrolytic enzymes by the colloid droplets. This in turn would lead to an accumulation of colloid filled particles and produce the increased ^{125}I content of the 125,000g particulate fraction and decreased ^{125}I content of the supernatant fraction encountered (figure 5), since the rate of disappearance of ^{125}I from the 125,000g supernatant and the whole gland would reflect the differences in the rate of colloid resorption and the overall rate of proteolysis. With time, however, it is not inconceivable that continued TSH stimulation, either directly or indirectly could lead to the induction of further lysosomal units or increase the rate of fusion between existing lysosomes and colloid droplets. Sobel (1961) showed increased acid phosphatase activity, which is an index of lysosomal presence, in the rat thyroid after cold exposure - conditions known to produce increased TSH stimulation. If this is indeed the course of events the colloid droplets would eventually be supplied with hydrolytic enzymes and this could account for the later decrease in the ^{125}I content of the particulate fraction. In this connection the decrease in ^{125}I content of the particulate fraction coincides with the time when the gland size is rapidly increasing and, as will be shown later, thyroidal protein synthesis is under stimulation. However, another possible explanation for the results obtained is that due to continuous depletion/

depletion of the colloid the rate of resorption eventually declines and the decrease in ^{125}I content of the particulate fraction is the result of a decreased rate of resorption. It is interesting that at this time the ^{125}I content of the thyroid gland is uniformly distributed between the particulate and soluble fractions.

ii) A study of the metabolism of thyroidal iodoproteins by DEAE cellulose chromatography

It has been shown by several workers (Ingbar, Askonas and Work, 1959; Robbins, 1961; 1963; Ui, Tarutani, Kondo and Tamura, 1961; Shulman and Stanley, 1961; Bouchilloux, Rolland, Torresani, Roques and Lissitsky, 1964) that DEAE cellulose chromatography fractionates thyroglobulin according to the iodine content of the molecules. The higher the di-iodotyrosine content of the molecule the more strongly it is retained by DEAE cellulose and the higher the ionic strength of the buffer required to elute it. Furthermore, it has been shown that newly accumulated iodine is incorporated into the early eluting fractions before the later eluting fractions. Thus fractionation of thyroglobulin on DEAE cellulose reflects the maturity of the constituent iodoprotein molecules - the younger molecules being eluted before the older, more highly iodinated, molecules. The results of the present investigation would therefore appear to indicate that the heterogeneity of thyroglobulin molecules obtained by DEAE cellulose chromatography has/

has indeed a functional significance - the rate of metabolism of the individual iodoprotein fractions eluted being a function of the iodine content and maturity of the molecules. The protein eluted at step 1, which constitutes the greatest per cent of the total iodoprotein in the controls, represents that fraction with the lowest iodine content and contains on average the younger molecules. As shown (figure 6), this fraction has the highest turnover rate. The oldest and most highly iodinated molecules represented by the fraction eluted at step 4 is metabolised at a rate approximately 20 per cent of that eluted in step 1. This also represents the smallest per cent of the total ^{125}I-iodo protein in control rats. The fractions eluted at steps 2 and 3 which are intermediate in iodine content and maturity are correspondingly metabolised at a rate intermediate between those eluted at step 1 and step 4.

Thus the results indicated that the heterogeneity displayed by thyroglobulin on DEAE cellulose is functional and confirms the 'last come, first served' hypothesis (Schneider, 1964). However, it is not possible to demonstrate by this technique whether this heterogeneity is interfollicular or intrafollicular or a combination of both.

iii)/

iii) Study of the metabolism of Thyroidal Iodoproteins by sucrose gradient ultracentrifugation

Although thyroglobulin sediments as a symmetrical peak in the analytical ultracentrifuge, Robbins, Salvatore, Vecchio and Ui (1966), Seed and Goldberg (1963, 1965a) Lissitsky et al (1964, 1966) have all demonstrated a heterogeneity in the thyroglobulin peak by ultracentrifugation in a linear sucrose density gradient. The slower sedimenting fractions have the lower iodine content and are the earliest labelled following a single injection of radio-iodine. Thomson and Goldberg (1968) found that the label did not coincide with 19S thyroglobulin until 48 hours after a pulse dose of ^{125}I. Thus, the newly formed thyroglobulin molecules are found in the slower sedimenting fractions of the 19S protein peak while the more mature molecules are found in the faster sedimenting fractions. In the present investigation, the shift in the labelled peak towards the bottom of the gradient during goitrogenesis indicates that 19S thyroglobulin is not metabolised at a uniform rate. The slower sedimenting molecules are turned over at a faster rate than the faster sedimenting fractions, confirming the results of DEAE cellulose that the younger newly iodinated molecules are metabolised at a faster rate than the older more highly iodinated molecules.

That the 27S iodoprotein is a structural and functional entity which is independent of 19S iodoprotein is becoming apparent.

Robbins/

Robbins et al (1966) showed that although there was little difference in incorporation of iodine into 19S and 27S iodoproteins after two days during 'equilibrium labelling', pulse labelling experiments showed that 19S iodoprotein was labelled at a faster rate than 27S. Vecchio and Claar (1967) demonstrated also, that ^3H-leucine was incorporated into 27S iodoprotein at a much slower rate than 19S iodoprotein and Vecchio, Edelhoch, Robbins and Weathers (1966) provided evidence for the structural individuality of the 27S iodoprotein. The present study has also provided evidence which suggests that the 27S iodoprotein also differs from 19S iodoprotein in its rate of metabolism. The difference in the slopes of the lines in fig. 10 suggests that 27S iodoprotein is turned over at a slower rate. Furthermore, it would appear that while 19S catabolism is initiated within 24 hours of commencement of the goitrogen regime, there is a delay in the turnover of the 27S protein. It is possible that 27S protein is not a product of all the follicles in the thyroid gland and that the delay is related to TSH levels in the blood. Only when a critical level of stimulation is reached are the follicles producing 27S iodoprotein stimulated. This would agree with the hypothesis that 27S iodoprotein represents a thyroxine store which is only metabolised when the metabolism of 19S iodoprotein alone cannot meet the functional requirements.

iv)/

iv) <u>Study of the metabolism of Thyroidal Iodoproteins using polyacrylamide gel electrophoresis.</u>

The data show clearly that differences in metabolism exist between comps B and C and comp A and the origin material, which appear to be metabolised at similar rates. However, it is unlikely that these components per se exist in the thyroid gland. Comp B, since it corresponds in electrophoretic mobility with the major protein component of sheep thyroglobulin, represents a fraction of 19S thyroglobulin. However, the results obtained for the control rats are not consistent with comp A being analogous to 27S iodoproteins. By sucrose gradient ultra-centrifugation of the iodoproteins from 'equilibrium labelled' rat thyroids, the 27S iodoprotein accounted for only 10 - 12% of the total radioactivity whereas by gel electrophoresis, comp A amounted to 20%. It follows, therefore, that other iodoproteins are contributing to comp A. A possible explanation is that, under the conditions of electrophoresis, there is a breakdown of the 27S iodoprotein and a fraction of the 19S iodoprotein. Vecchio, Edelhoch, Robbins and Weathers (1966) showed that rat 27S iodo-protein was unstable under alkaline conditions dissociating to form 19S, 12S and 6S components. The same workers also demonstrated that, under their conditions the older 19S thyro-globulin molecules were more labile than the younger molecules.

Under/

Under the alkaline conditions and slightly elevated temperatures prevailing during electrophoresis, it is highly probable that the 27S iodoprotein molecules and a fraction of the older 19S protein molecules undergo dissociation to their respective subunits. It is not inconceivable that some artifactual aggregation of those subunits could then take place to produce larger molecules which migrate as the broad diffuse band of comp A. By the same reasoning, larger aggregates or unfolded forms of these labile components might account for the labelled material at the origin. Furthermore, this could also account for comp C and since its metabolism occurs at a slower rate than comp B, this would suggest that it was derived from a different fraction of the iodoproteins than comp B. In conclusion, although the conventional terms applied to thyroglobulin species cannot be used in connection with the components fractionated by gel electrophoresis, the results confirm a heterogeneity in the turnover of the thyroidal iodoproteins.

The exact nature of the labelled material migrating with the electrophoretic front is not known. However, since it increases with time after initiation of the MTU regime, it seems not unlikely that it represents large undialysable iodopeptides produced during proteolysis of the iodoproteins. The additional fast migrating component migrating ahead of comp C. which appeared on the 5th day/

day on MTU could be, presumably, a serum protein contaminant since it is not labelled to any significant degree. However, since it is well established that MTU does not inhibit thyroglobulin precursor synthesis, it could represent a non-iodinated subunit.

v) <u>Iodoamino acid content of Thyroidal Iodoproteins during Goitre Induction.</u>

Lissitsky, Simon, Roques and Torresani, (1966) concluded that as maturation of thyroglobulin proceeds, the di-iodotyrosine and iodothyronine content of the molecule increased. The intention of the present investigation was to confirm this by demonstrating that as metabolism of thyroglobulin progressed during goitrogenesis, there was a corresponding increase in the hormonal content of the thyroglobulin remaining. Although the observed increase in the ratio of iodothyronines to iodotyrosines (table 5) is small that similar results were obtained by two different systems would suggest that the increase is significant. However, it is not possible to conclude that this proves that there is an increase in the iodothyronine content of the iodoprotein remaining at each interval since there is always a large proportion of the total radioactivity remaining at the origin after chromatography. There are several interpretations of the origin material. It could be the result of poor pronase preparation since only one preparation, supplied by Koch-Light Labs.,

Labs., was used. It could represent either completely undigested protein, the undigested core of partially hydrolysed iodoprotein or both. Without more detailed knowledge of the exact nature of the origin material, it is difficult to interpret the results on the basis of the above proposition. A further factor is the large increase in the proportion of the total radioactivity which remains at the origin during the latter half of the goitrogenic regime. Sucrose gradient centrifugation and DEAE cellulose chromatography of the iodoproteins have shown that between days 7 and 9 on MTU the predominant iodoprotein fractions represent the older, more highly iodinated proteins. Pitt-Rivers (1963) showed that the higher the degree of iodination of the thyroglobulin molecules the more they resisted hydrolysis. Therefore, a possible explanation of the nature of the origin material is that it represents a highly iodinated iodoprotein species which is very slowly metabolised and resists proteolysis by pronase. However, a more detailed investigation of the origin material is necessary before firm conclusions regarding its nature can be drawn.

vi) <u>Protein synthesis in the Thyroid Gland during Goitre Induction</u>

Cavalieri and Searle (1967) showed that TSH stimulated the synthesis of protein in the intact rat. Ekholm and Strandberg (1967) showed a similar result in guinea pigs. In the present investigation no incorporation of ^{3}H-leucine into thyroidal proteins/

proteins was detected until after the 3rd day on MTU. Since Thomson and Goldberg (1968) and Vecchio, Salvatore and Salvatore (1966) both demonstrated ^{3}H-leucine incorporation into thyroid protein within 30 minutes of administration to control rats, failure to detect significant incorporation of the label for the first three days must be due to the lower dose level of ^{3}H-leucine administered in this experiment. However, the appearance of labelled amino acid in the thyroid proteins on the 6th day must reflect the stimulatory influence of TSH on protein synthesis during the third and sixth days. Presumably, during the early period of MTU treatment, the hormone stores in the gland are sufficient to maintain a low level of circulating TSH. After 3 days, however, as shown previously the iodoprotein content and hence the hormone store of the gland have been severely depleted. This would result in a drop in the levels of circulating hormone and effect a stimulation of the pituitary gland to release TSH. This, in turn, would stimulate the general metabolism of the thyroid gland and result in the increased incorporation of ^{3}H-leucine into protein during the 3rd and 6th days.

Thomson and Goldberg (1968) showed in "in vivo" experiments, that goitrogenic drugs which block iodide organification cause incorporation of labelled amino acids into a 17 - 18S protein rather than true 19S thyroglobulin. In the present investigation a/

a similar incorporation of ^{3}H-leucine into a 17 - 18S protein was found between days 8 and 10 of the goitrogen regime. On the 6th day, however, the label was incorporated into a slightly heavier protein with sedimentation constant in the range 18.5 - 19S. Nunez, Mauchamp, Pommier, Cirkovic and Roche (1966) showed that the higher the iodine content of the thyroglobulin molecule, the higher the sedimentation constant of the protein in the ultracentrifuge. It follows, therefore, that between day 3 and day 6 of goitrogenesis, the labelled amino acid is incorporated into a protein with a higher iodine content than that formed later, Although Tong (1965) could find no evidence that the stimulatory influence of TSH on iodide organification was related to increased enzyme protein synthesis, the conditions of the experiment do not completely rule out this possibility. Furthermore, since under the conditions of the present investigation, the period of stimulation is presumably much longer, the possibility that net synthesis of enzyme protein has occurred, is increased. This could lead to a certain degree of relief from the MTU induced inhibition of iodide organification. Iodide produced by dehalogenase activity within the gland could then be recycled to a limited extent into the newly synthesised thyroglobulin precursors to produce a ^{3}H-leucine labelled protein with the sedimentation/

sedimentation constant found after 6 days on MTU. It is not unlikely that under the prevailing conditions of increased protein catabolism that this protein would be rapidly metabolised. Furthermore, with the continuous loss of glandular iodide there would eventually be insufficient iodide available for this residual reaction. The result would be the incorporation of labelled amino acid into the lighter protein molecules with sedimentation constants 17 - 18S found after 8 days on MTU.

A further feature of ^{3}H-leucine incorporation throughout the MTU regime, is the appearance of the labelled amino acid in a protein with sedimentation constant 24 - 25S between days 8 and 10. Vecchio and Claar (1967) showed that after 10 days, ^{3}H-leucine is incorporated into a small 27S peak in the rat. They found no precursor product relationship between 19S and 27S proteins and concluded that the latter is synthesised at a much slower rate. It is possible that the 24 - 25S component found in the present investigation bears the same relationship to the 27S iodoproteins as 17 - 18S protein to 19S thyroglobulin. It may represent a precursor of 27S iodoproteins which accumulates under the conditions produced by MTU administration. The similarity between the time of labelling of the 27S by Vecchio and Claar (1967) and the 24 - 25S protein would add support to this. Furthermore, the appearance of this component is additional proof that iodination is not a prerequisite for aggregation of the newly synthesised subunits.

Summary

Investigations have been carried out on the metabolism of rat thyroidal iodoproteins during goitre induction. The following results were obtained .

1. The iodoprotein content of the thyroid gland decreased logarithmically and the results showed that the rate of colloid resorption is faster than the rate of release of iodide from the whole gland.

2. The heterogeneity of the thyroglobulin molecules which is obtained by fractionation of the protein by DEAE cellulose chromatography, sucrose gradient ultracentrifugation and polyacrylamide gel electrophoresis was shown to have a functional significance during goitre induction. The former techniques indicated that the newly iodinated iodoprotein molecules are metabolised at a faster rate than the older more highly iodinated molecules. Furthermore, the evidence indicated that the 27S iodoprotein is metabolised at a slower rate than the 19S protein.

3. Attempts to show that, during the metabolism of the thyroidal iodoproteins, the iodothyronine content of the remaining molecules increased were not conclusive. This was primarily due to the presence, at all times during goitrogenesis, of a large per cent of the total radioactivity of the pronase digests remaining at the origin/

origin of the chromatograms. It is suggested that this may represent a highly iodinated iodoprotein which has a slow turnover rate and is particularly resistant to pronase digestion.

4. Incorporation of ^{3}H-leucine into thyroidal proteins of the rat 'in vivo' showed that, during goitrogenesis induced by MTU administration, stimulation of protein synthesis occurred after 3 days on the goitrogen. Initially the labelled amino acid was incorporated into a protein with sedimentation constant 18.5-19S but after 8 days the label was predominantly present in a 17-18S protein. It is suggested that the heavier component is the result of a residual iodination reaction resulting from TSH stimulation of the gland. Finally there is evidence that during MTU treatment, between 8 - 10 days, ^{3}H-leucine is also incorporated into a protein with sedimentation constant 24-25S a probable non-iodinated precursor of the 27S iodoprotein.

SECTION 2

A STUDY OF THE IODOPROTEINS PRESENT IN ESTABLISHED GOITRES IN HUMANS

INTRODUCTION

The present knowledge regarding the structure and biosynthesis of thyroglobulin has been obtained from the study of thyroglobulin from animal sources. For the most part, comparable work with human thyroglobulin has been largely neglected primarily due to the difficulty in obtaining fresh normal human thyroid glands. Most of the studies involving human thyroglobulin have, therefore, been directed towards the comparison of the iodoproteins from normal and pathological thyroid glands. In general this has involved estimation of the solubility of the iodoproteins, their electrophoretic mobilities, sedimentation coefficients and immunologic properties. From this approach certain abnormalities in the iodoproteins from certain pathological glands have been described eg. Easty, Slater and Stanley (1958) described an electrophoretically abnormal thyroglobulin in thyroid cancer and iodoproteins with electrophoretic mobilities identical to serum albumin have been described in adenomas, nodular goitres (Robbins, Wolff and Rall, 1959b); congenital goitre (De Groot and Stanbury, 1959); endemic cretinism (Beckers, De Visscher, 1961; Lobo Da Silva, Hargreaves and Conceiro, 1964) and colloid goitre (De Groot and Carvalho, 1960b). Furthermore, iodoproteins with electrophoretic mobilities similar to pre-albumins have also been described in goitrous/

goitrous subjects by Lissitsky, Codaccioni, Cartouzou and Mante (1964) and Murray, McGirr, Thomson and Hutchison (1965). The exact nature of these abnormal proteins and their role in thyroidal iodoprotein metabolism is still largely conjectural. It has been suggested that they could be iodinated subunits of thyroglobulin or normal constituents of the thyroid gland, independent of thyroglobulin, whose synthesis is accelerated in the conditions prevailing in thyroid disease. In this respect Shulman, Mates and Bronson (1967) have isolated proteins from normal human thyroids which had sedimentation constants of 4S and 7S and they have demonstrated an immunological relationship between these proteins and thyroglobulin.

Recently by virtue of gel filtration techniques, highly purified preparations of human thyroglobulin have been studied. Ramogopal, Spiro and Stanbury (1965) showed that thyroglobulin from goitrous glands was less iodinated and had higher monoiodotyrosine (MIT) to diiodotyrosine (DIT) ratios than that from normal controls. They also showed that the level of iodination had no effect on the electrophoretic mobility or solubility of the thyroglobulin preparation.. Bismuth, Rolland and Lissitsky (1966) could find no significant difference in the amino acid compositions of 19S thyroglobulin from normal and goitrous glands. Similarly Pierce, Rawitz, Brown and Stanley (1965) found no difference in the amino acid compositions, carbohydrate contents, electrophoretic or centrifugal/

centrifugal properties between the thyroglobulin from normal glands and non-toxic goitres.

The following section represents a report of the results obtained from the analysis of the properties of the iodoproteins present in established human goitres which were collected over a period of 2 - 3 years. Data regarding the iodide and carbohydrate contents, electrophoretic mobilities and sedimentation properties of the iodoproteins have been collected. Furthermore, information regarding the degree of heterogeneity of the iodoprotein present in established goitres has been obtained by fractionation of the protein by DEAE cellulose chromatography. Finally, the primary structures of these iodoproteins have been studied by analysing tryptic digests of the peptide fingerprinting technique.

MATERIALS AND METHODS

1. MATERIALS

Normal human thyroid gland was collected on ice following post mortem examination of a patient with no previous history of thyroid disease. Goitrous glands were collected on ice immediately following surgical removal. All glands were stored frozen until used.

Sephadex G200 was obtained from Pharmacia, Uppsala, Sweden. Whatman DEAE cellulose type DE 11 was used in all ion-exchange experiments. ^{131}I and ^{125}I were purchased from the Radiochemical Centre, Amersham, Bucks. Crystallised trypsin was the product of BDH Ltd., Poole.

2. Preparation of Thyroid Extracts

The glands were washed free from excess blood and the extraneous tissue removed. They were then frozen, sliced by hand and the slices extracted for 12 hours at $4^{\circ}C$ with 0.9% saline (2 ml per g tissue). The tissue debris was removed by filtration through cheese-cloth and the filtrate centrifuged at $4^{\circ}C$ for 30 minutes at 38,000 rpm in the Spinco model L. (50 rotor). The extracts were finally dialysed against distilled water for 24 hours at $4^{\circ}C$ and lyophilised. These lyophilised saline extracts of the glands were stored at $-14^{\circ}C$ until required.

3. G200 Sephadex Gel Filtration

The dry gel beads were allowed to swell for 48 hours in deionised/

deionised water to which a bacteriostat had been added. The 'fines' were removed by repeated decantation and the gel was finally equilibrated with 0.1 M KCl - 0.02 M sodium phosphate buffer pH 7.5 (KCl-phosphate buffer). Columns were packed by a continuous flow technique until the required bed volume was attained - at no time during packing was the difference between the level of fluid in the column and the outlet allowed to exceed 10 cms. The column was then stabilised with several column volumes of KCl-phosphate buffer at room temperature and then equilibrated at 4°C. Column packing was checked by applying a small volume of a concentrated solution of Blue Dextran 2000 (Pharmacia, Sweden) and eluting with the KCl-phosphate buffer.

For preparative purposes the dimensions of the gel bed were adjusted to 2.5 x 35 cms. 2 ml of a 7% solution of the lyophilised saline extracts of the glands were carefully layered on top of the gel under the KCl-phosphate buffer. The density of the protein solution was increased by the addition of 2-3 drops of a 20% sucrose solution. The top of the gel bed was protected during sample application and subsequent elution by a nylon net sample applicator. The protein was eluted with KCl-phosphate buffer at a flow rate of 1-2 ml/cm^2/hr and 2 ml fractions were collected in an automatic fraction collector. The fractions were assayed for protein by measuring the optical density of each fraction at 280 mμ in/

in a Unicam SP500 spectrophotometer and for haemoglobin by measuring the optical density (OD) at 414 mμ. When the patient had been given ^{131}I or ^{125}I pre-operatively, the presence of radioactivity in the eluate was measured by the Ekco sample changer type N695. Further purification of the iodoprotein was accomplished by pooling the necessary fractions from the first cycle through the column and concentrating the protein to the required volume with Carbowax. The above procedure was then repeated. The purified iodoprotein was finally dialysed against distilled water at 4°C for 24 hours, lyophilised and stored dessicated at -14°C.

4. Estimation of Protein Concentration, Iodide and Carbohydrate content of the iodoproteins

The protein concentration of all solutions was determined by the method of Lowry, Rosebrough, Farr and Randall (1951) using a bovine serum albumin standard. Hexose content of the iodoproteins was determined by the orcinol-sulphuric acid method described by Winzler (1955) using a standard of mannose, galactose in the ratio 1:1. Sialic acid estimations were performed by the thiobarbituric acid method of Warren (1959) and total iodide was determined by the method of Farell and Richmond (1961). In the latter case, the estimations were performed on dilutions of iodoprotein solutions in 1% bovine serum albumin to minimise the loss of protein on glassware at high dilutions.

5. Sucrose Gradient Ultracentrifugation

The method was the same as that described in materials and methods section 1./

section 1. The protein applied to the gradient was obtained by making solutions of the lyophilised saline extracts in PBS 50% with respect to ammonium sulphate. After 1 hour at $4^{\circ}C$, the solution was centrifuged at 16,000 rpm in an MSE refrigerated centrifuge with a high speed attachment. The supernatant was discarded and the precipitate dissolved in PBS. In some cases (cf results, 3), the purified and lyophilised iodoprotein dissolved in PBS was used.

6. Polyacrylamide Gel Electrophoresis

The technique was identical to that described in the materials and methods, section 1.

7. DEAE Cellulose Chromatography

The DEAE cellulose was prepared and the columns packed as described in materials and methods, section 1. The dimensions of the columns used were 2.5 x 32 cms. 100 mg to 200 mg of protein were applied to the column and washed on with 1 column volume of 0.025 M potassium phosphate buffer pH 6.5. All operations were performed at $4^{\circ}C$. The protein was eluted by applying a stepwise gradient of NaCl in 0.025 M potassium phosphate buffer pH 6.5 The protein eluted at 0.05 M, 0.10 M etc. to 0.30 M and 2.0 M NaCl was collected. In cases 1 - 10, the iodoprotein purified by gel filtration was applied to the column while in cases 11 - 12, the lyophilised saline extracts of the glands were applied. The flow rates were adjusted to 30 ml per hour and 5 ml fractions were colleted. The presence of protein in the fractions was determined as/

as described previously. Aliquots of each protein fraction eluted were dialysed extensively against 0.9% saline at 4°C and concentrated by Carbowax. The protein in these samples was then analysed by polyacrylamide gel electrophoresis. The remainder of the protein in each fraction was dialysed against distilled water at 4°C until free from chloride, lyophilised and stored desiccated at -14°C.

8. Preparation of S. Carboxymethylated Iodoproteins

The method of reduction and alkylation described by Edelhoch and de Crombrugghe (1966) was used. 5 mg iodoprotein was dissolved in 2 ml 8 M urea - 0.063 M mercaptoethanol - 0.015 M bicarbonate solution pH 10.2. This solution was left at room temperature for 30 minutes and then 1 ml of an 0.15 M iodoacetic acid solution in 8 M urea pH 10.2 was added. After a further 30 minutes, the solution was dialysed against 3 changes of distilled water. Dialysis was then continued for a period of 24 hours at 4°C against 0.2 M ammonium bicarbonate buffer pH 8.5. 0.05 mg trypsin was added and the solution incubated at 37°C for 24 hours. A small quantity of sodium azide was added as a bacteriostat.

9. Peptide Fingerprints of Native and S.Carboxymethylated Iodoproteins

Limited digests of native and S. carboxymethylated (SCM) iodoproteins with trypsin were obtained as described above for SCM iodoproteins. Concentration of the digests was carried out by lyophilisation and redissolving the residue in the minimum of 0.2 M/

0.2 M ammonium bicarbonate buffer pH 8.5. Aliquots of the digests containing equivalent to 1 - 2 mg iodoproteins were then spotted on Whatman 3 MM paper (46 x 57 cms.). Electrophoresis was carried out at 10 mA for 16 hours at pH 3.5 (pyridine, acetic acid, water ; 1:10:295). The voltage varied from 250 V at the start to 200 V at completion of the electrophoresis. Descending chromatography for 17 hours with n butanol, acetic acid, water (4:1:5) was performed in the second dimension. The position of the peptides was detected by spraying the paper with a solution of 0.01% ninhydrin in collidine, acetic acid, ethanol (1:25:7.5 V/V/V) and heating in an oven. The spots were marked by viewing the chromatograms over a fluorescent light and tracings of the chromatograms taken as records. Autioradiographs of the chromatograms which were labelled with radioiodine were obtained with Kodak-Kodirex x-ray film with an exposure period of 1 - 2 weeks.

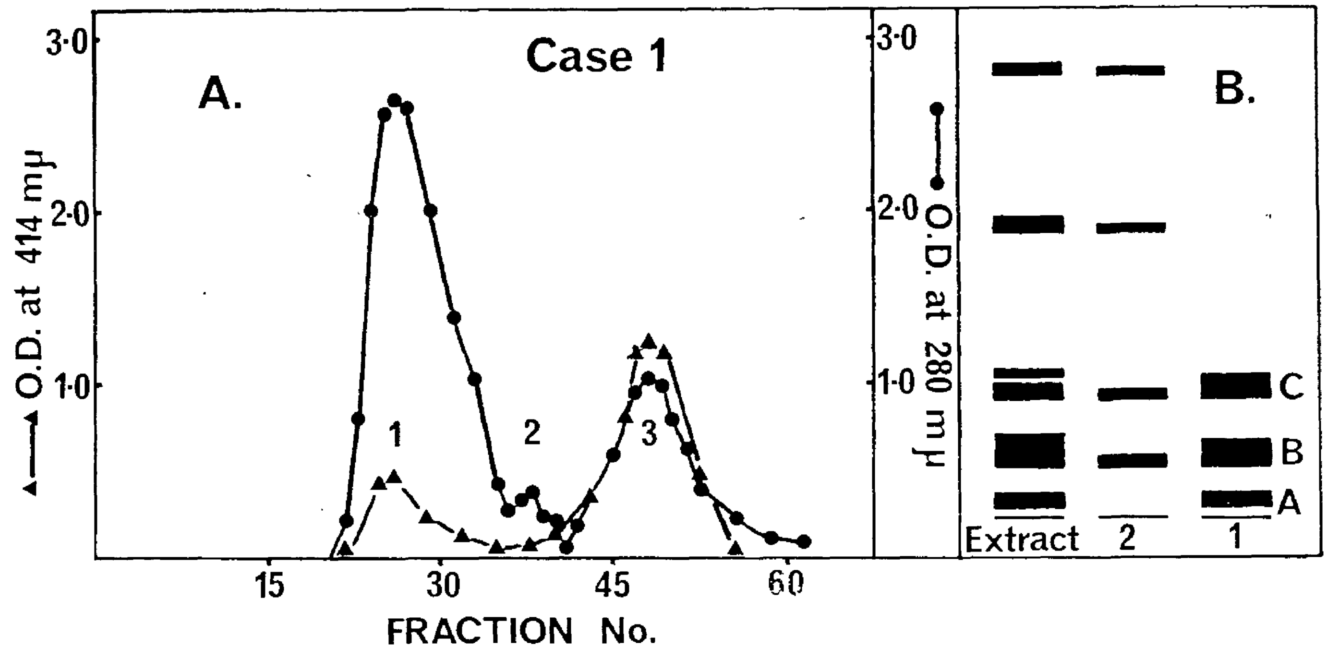
RESULTS

1. G200 Sephadex Gel Filtration

Elution profiles of the proteins from extracts of normal and goitrous human thyroid glands are shown by the distribution curves in figs. 18 - 23. The polyacrylamide gel electrophoretic patterns of the gland extracts and the protein eluted in peak 1 (cf. fig. 18) are also shown. In almost all cases, three protein peaks were eluted. Differences in the relative proportions of these peaks were related to the degree of contamination of the gland extracts with serum proteins. As can be seen from the elution profiles, one cycle through G200 Sephadex was not sufficient to remove all traces of haemoglobin, as measured by OD at 414 mμ from peak 1. However, in most cases this could be accomplished by recycling the protein in peak 1 although in some cases several cycles were required to remove all detectable traces of the haemoglobin.

i) Normal Thyroid Gland (Case 1, fig. 18)

Peak 3 contained predominantly haemoglobin and serum albumin. Peak 2 contained traces of proteins from peak 1 and peak 3 and was not investigated further. Peak 1 contained protein with electrophoretic properties identical to purified sheep thyroglobulin. The protein migrated as three distinct bands (A, B and C) as described by Spiro (1964). Component C can be attributed to dialysis/



Figs. 18 - 23 A. Patterns of elution from G200 Sephadex gel columns of proteins from saline extracts of normal and abnormal thyroid glands. In cases 8, 10 and 12 column dimensions were 1 x 16 cm; fraction volume 1 ml and flow rate 1-2ml/cm^2/hr. In all other cases, the conditions were as described in materials and methods.

B. Polyacrylamide gel electrophoretic patterns of the proteins from normal and abnormal thyroid glands.

Extract - original saline extracts of the glands
1,2 - earliest peak etc. eluted from the G200 Sephadex columns.

dialysis and lyophilisation of the protein prior to electrophoresis. While it has been established that these procedures result in dissociation of the thyroidal iodoproteins, they were adopted as the most convenient method of storage of both the gland extracts and the purified iodoprotein. Since all proteins were treated identically, it was concluded that, bearing in mind the effect on the proteins, this would not seriously affect the results obtained.

ii) Non-toxic Goitre (cases 2 - 4, fig. 19)

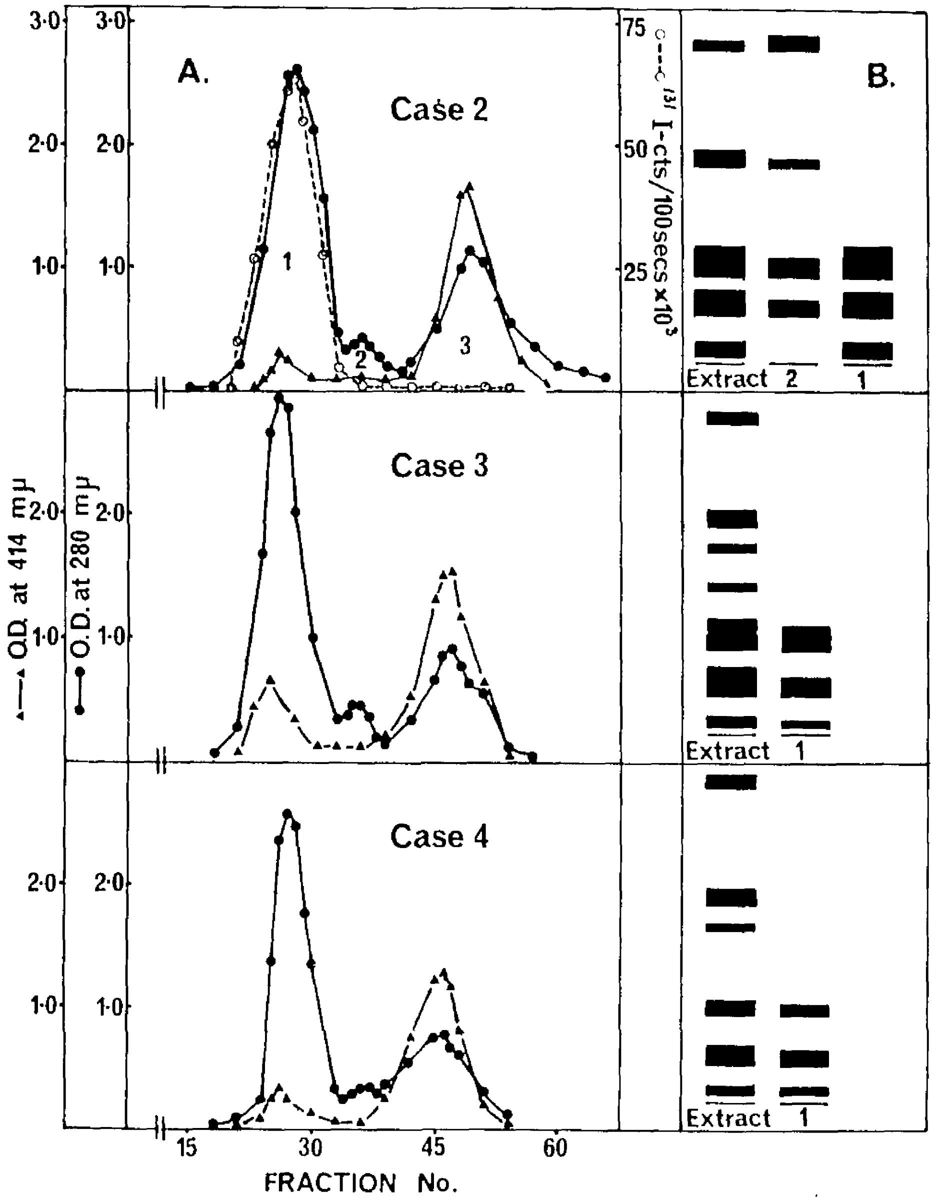
In case 2, the patient was given ^{131}I preoperatively and, as shown, the label was eluted exclusively in peak 1. In all cases the components of peak 1 corresponded to the normal thyroidal iodoproteins.

iii) Congenital Goitre (cases 5 - 6, fig. 20)

Both patients received ^{131}I and, as above, this was confined to the protein fraction eluted in peak 1. Electrophoretically, the labelled protein corresponded to the normal components B and C. Component A was missing.

iv) Thyroid Carcinoma (cases 7 - 8, fig. 21)

Both patients received ^{131}I and, as before, the label was eluted only in peak 1. Protein bands corresponding to the normal iodoprotein components were present in both cases although in case 7 component C appeared as a faint narrow band. In addition, there was minor component migrating between components A and B. The relationship/



A.
B.
Case 2
Case 3
Case 4
O.D. at 414 mμ
O.D. at 280 mμ
131 I-cts/100secs x10^3
FRACTION No.
Extract

Fig. 19

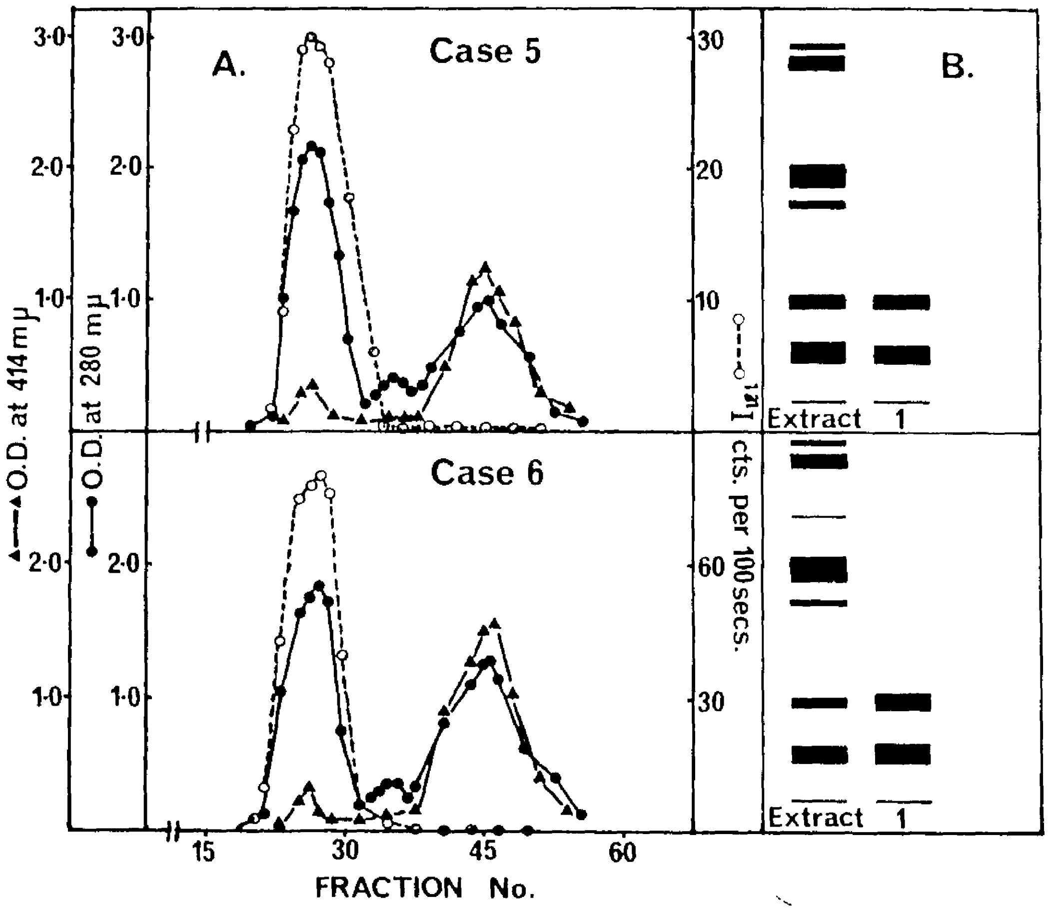
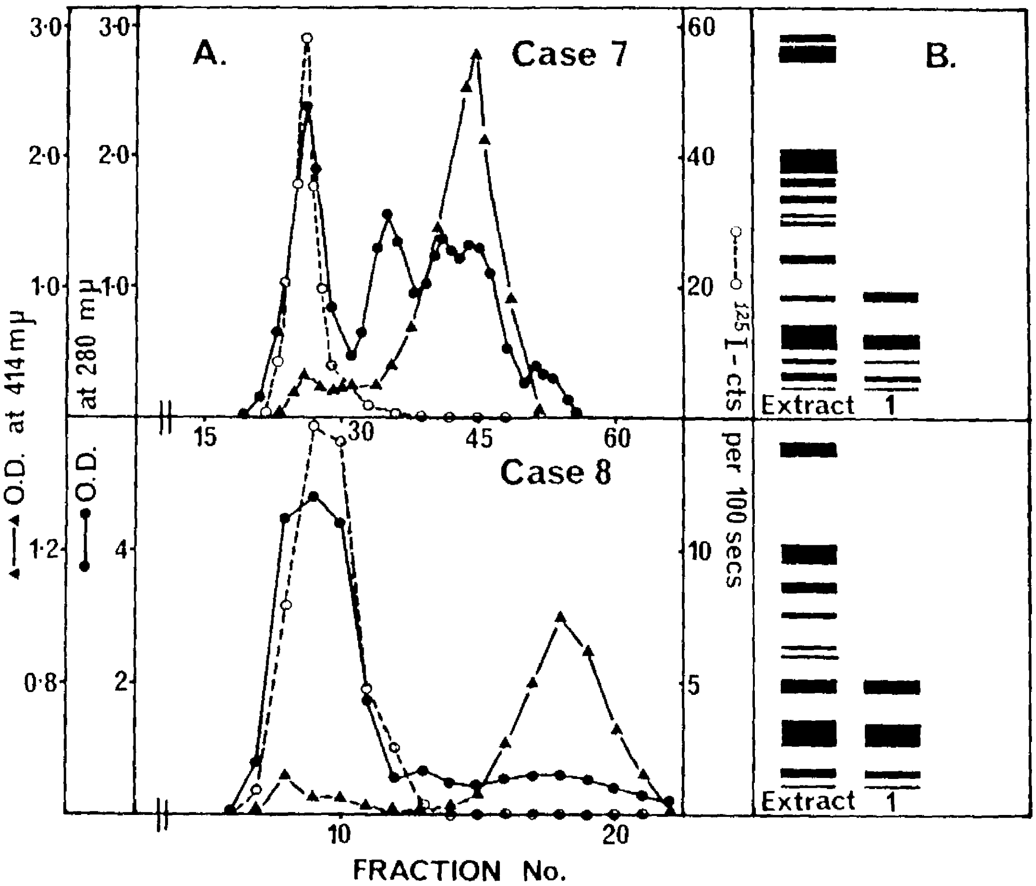

A.
Case 5
B.
Case 6
O.D. at 414 mμ
O.D. at 280 mμ
^{131}I cts. per 100 secs.
FRACTION No.
Extract
1
3·0
2·0
1·0
30
20
10
60
15
45

Fig. 20



A.
Case 7
B.
Case 8
3·0
2·0
1·0
1·2
0·8
4
2
60
40
20
10
5
15
30
45
60
10
20
FRACTION No.
O.D. at 414 mμ
O.D. at 280 mμ
^{125}I - cts per 100 secs
Extract
1
Extract
1

Fig. 21

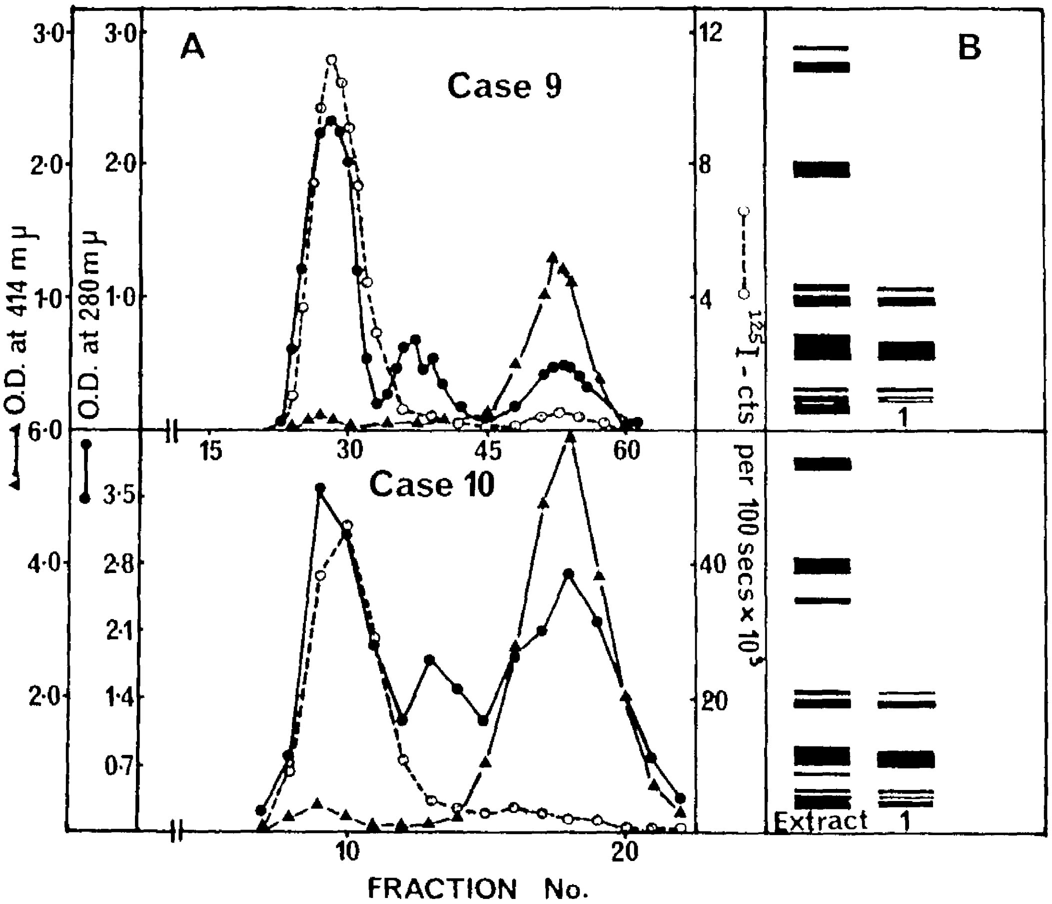
relationship of this protein, if any, to carcinoma of the thyroid gland is not known although it is possible that it represents a serum protein contaminant.

v) Hashimoto's Thyroiditis (cases 9 - 10, fig. 22)

Both patients received ^{131}I preoperatively. In both cases, over 95% of the label was eluted in peak 1; the remainder of the radioiodine was eluted with peak 3. By slicing the stained protein bands from the gel after electrophoresis of the gland extract, the minor labelled component was found to migrate with serum albumin. Electrophoretically, the purified protein from peak 1 corresponded to the normal components band C and there was a faint narrow band with a mobility similar to component A. Component C appeared to have split into two discrete bands both of which were labelled. It was not possible to determine whether this was artifactual or an abnormality of the condition. A similar situation was encountered in section 1 in rats maintained on MTU for over 5 days, although in this case the faster migrating band was not labelled. Finally, both cases showed faintly stained bands in the region of the serum γ globulins.

vi) Dyshormonogenetic Goitres (cases 11 - 12, fig. 23)

Both patients received ^{131}I preoperatively. In addition to the normal labelled peak 1, 40 - 50% of the total ^{131}I was eluted fractionally ahead of the haemoglobin peak. In case 11, electrophoresis/



A
B
Case 9
Case 10
O.D. at 414 mμ
O.D. at 280 mμ
^{125}I - cts per 100 secs × 10^{3}
3·0
2·0
1·0
6·0
4·0
2·0
3·5
2·8
2·1
1·4
0·7
12
8
4
40
20
15
30
45
60
10
20
FRACTION No.
Extract
1

Fig. 22

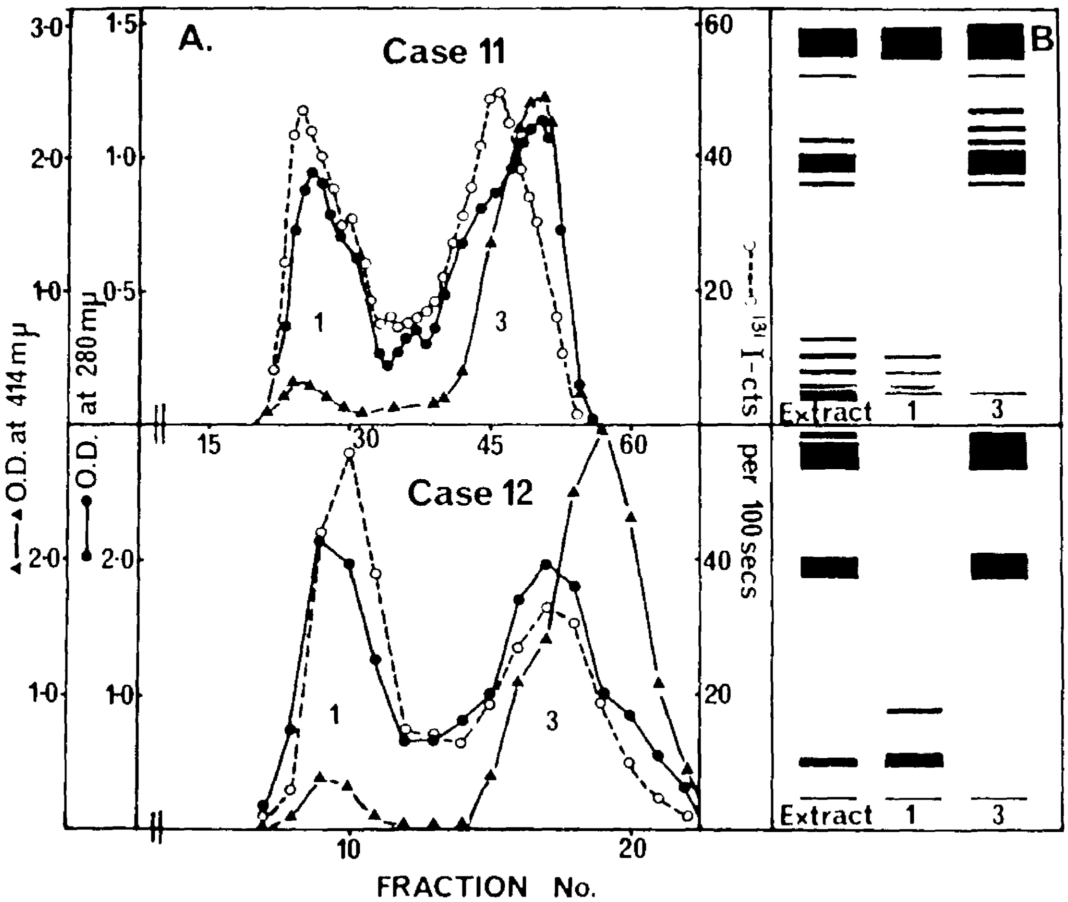

A.
Case 11
Case 12
B
O.D. at 414 mμ
O.D. at 280 mμ
^{131}I-cts per 100 secs
FRACTION No.
Extract
1
3

Fig. 23

electrophoresis of peak 1 showed the presence of a narrow band corresponding to component B and a heavily stained band which migrated with serum albumin. Both bands were labelled. There were also two faintly stained unlabelled bands between the origin and component B. Due to the high degree of contamination of this extract with serum protein these bands are probably derived from the latter. In case 12, peak 1 comprised only one labelled component corresponding to the normal component B. The second peak contained a labelled albumin-like iodoprotein.

In general, G200 Sephadex gel filtration is a convenient method for the purification of large amounts of thyroglobulin from human gland extracts and has the additional capacity to detect abnormalities in the thyroidal iodoproteins. In those cases where there is a high degree of contamination of the extracts with serum proteins e.g. thyroid carcinoma, Hashimoto's thyroiditis, it is not possible to remove all the contaminating proteins particularly the serum γ globulins. However, it was always possible, by careful selection of the fractions from peak 1, to obtain a preparation of thyroglobulin which was essentially free from these contaminants.

2. Sucrose Gradient Ultracentrifugation

The sedimentation properties of the iodoproteins from human goitrous glands were studied in 5 - 20% linear sucrose density/

density gradients. The OD patterns at 280 mμ of the gradients are shown in figs. 24 - 29. In cases 1, 2, 6, 10 and 12 the protein applied to the gradient was obtained from peak 1 in gel filtration profiles after dialysis and lyophilisation. In all others, the protein was obtained from the redissolved precipitate after ammonium sulphate precipitation of the gland extract.

i) Normal Thyroid Gland (case 1, fig. 24)

Three OD peaks were present corresponding to sedimentation constants 27S, 19S and 12S. The latter, in this case and cases 2, 6 and 12, was the result of dialysis and lyophilisation of the protein.

ii) Non-toxic Goitres (cases 2 - 4, figs. 24 - 25)

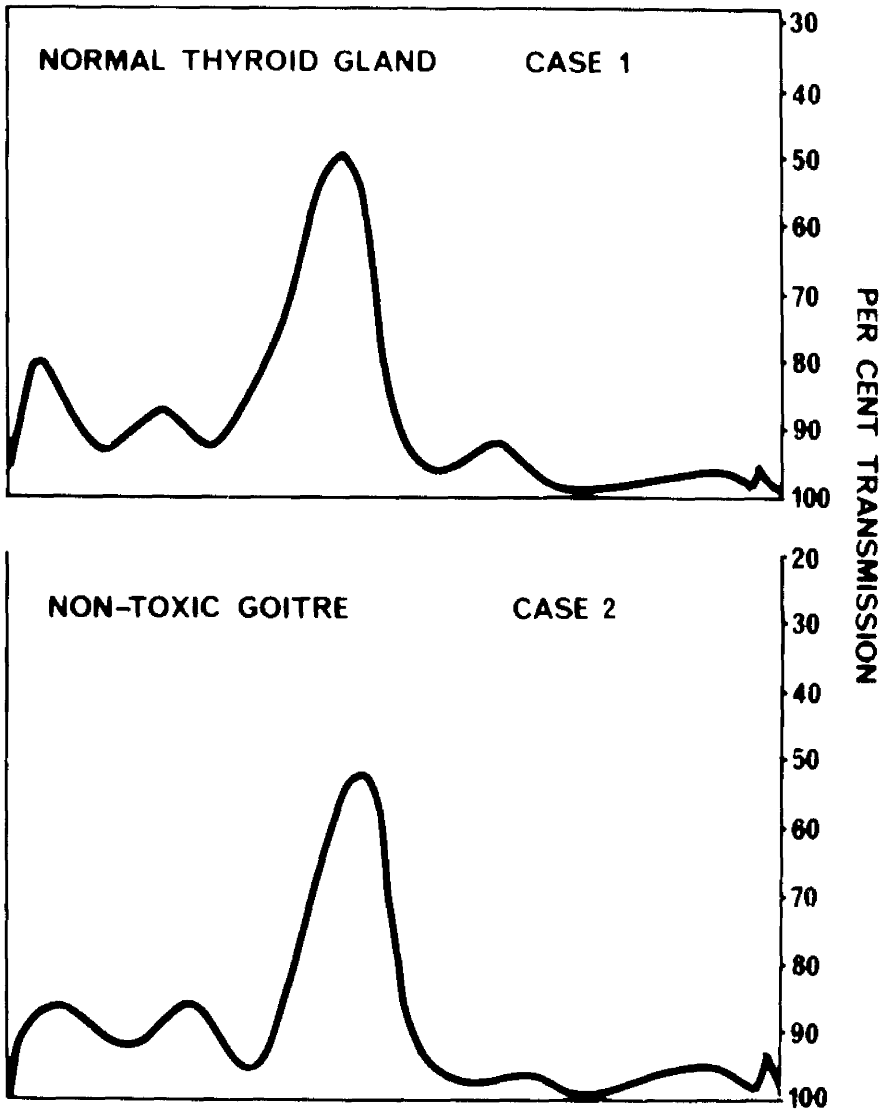
All three cases had components corresponding to 19S and 27S iodoproteins. The 3 - 8S proteins in cases 3, 4 and also cases 5, 7 - 10 are mainly serum proteins not completely removed by the ammonium sulphate precipitation step.

iii) Congenital Goitre (cases 5 - 6, fig. 26)

Both cases contained a 19S iodoprotein peak only - no heavier components were apparent. This agrees with the failure to detect a slower migrating component (comp A) on electrophoresis of the purified proteins.

iv) Thyroid Carcinoma (cases 7 - 8 , fig. 27)

Both cases had the major 19S iodoprotein and a heavier iodoprotein corresponding to 26 - 27S.



Figs. 24 - 29 Sucrose gradient ultracentrifugation patterns of proteins from normal and abnormal thyroid glands. In each case the bottom of the gradient is to the left of the figure.

Fig. 24 From top to bottom of gradient, the peaks correspond to the following sedimentation coefficients: Case 1; 12.5S, 19.5S, 27.5S. Case 2: 12.1S; 19.2S; 27.4S.

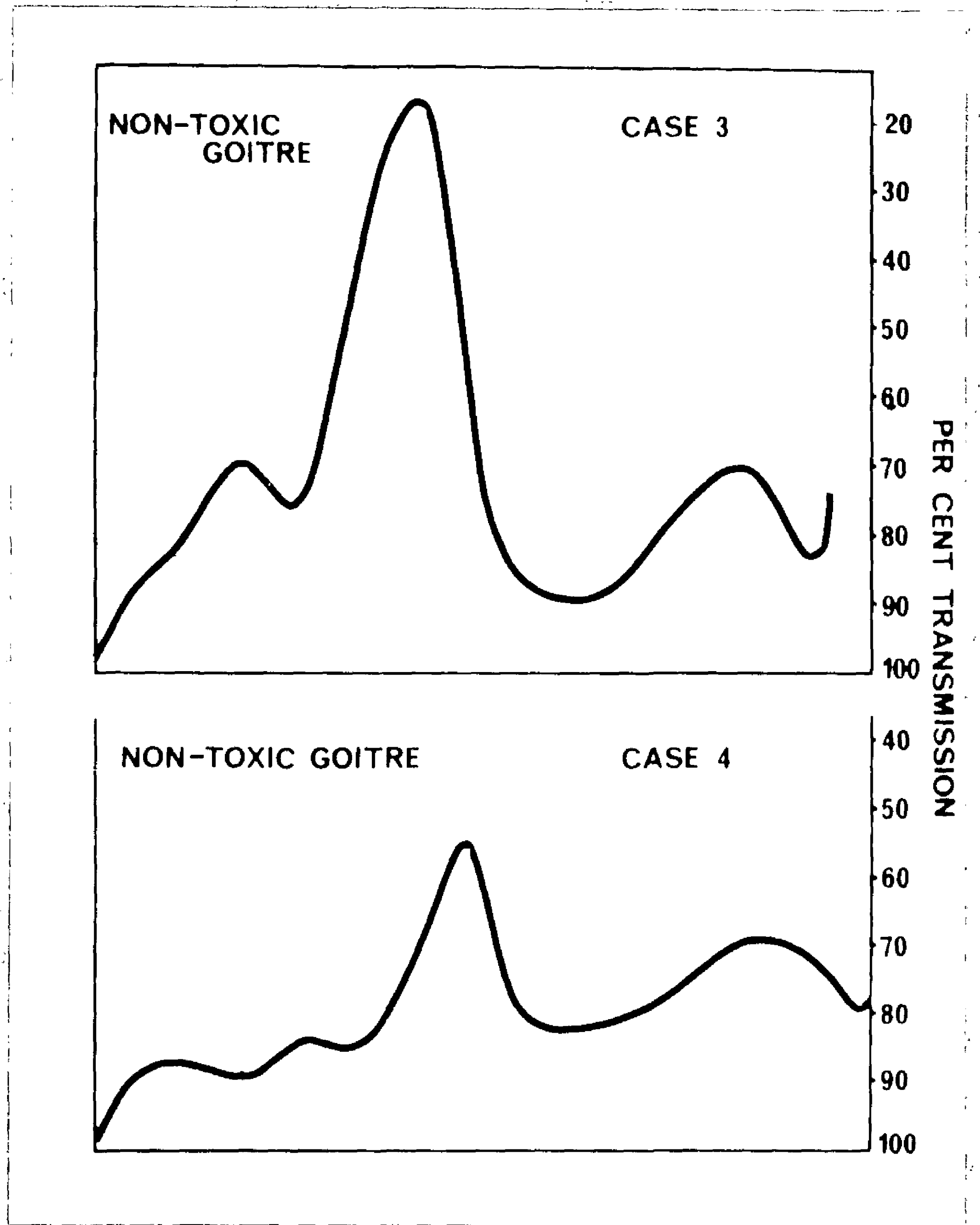


Fig. 25 From top to bottom of the gradient, the peaks correspond to the following sedimentation coefficients: Case 3, 3 - 8S; 19.3S; 27.4S. Case 4, 3 - 8S; 19.4S; 27S.

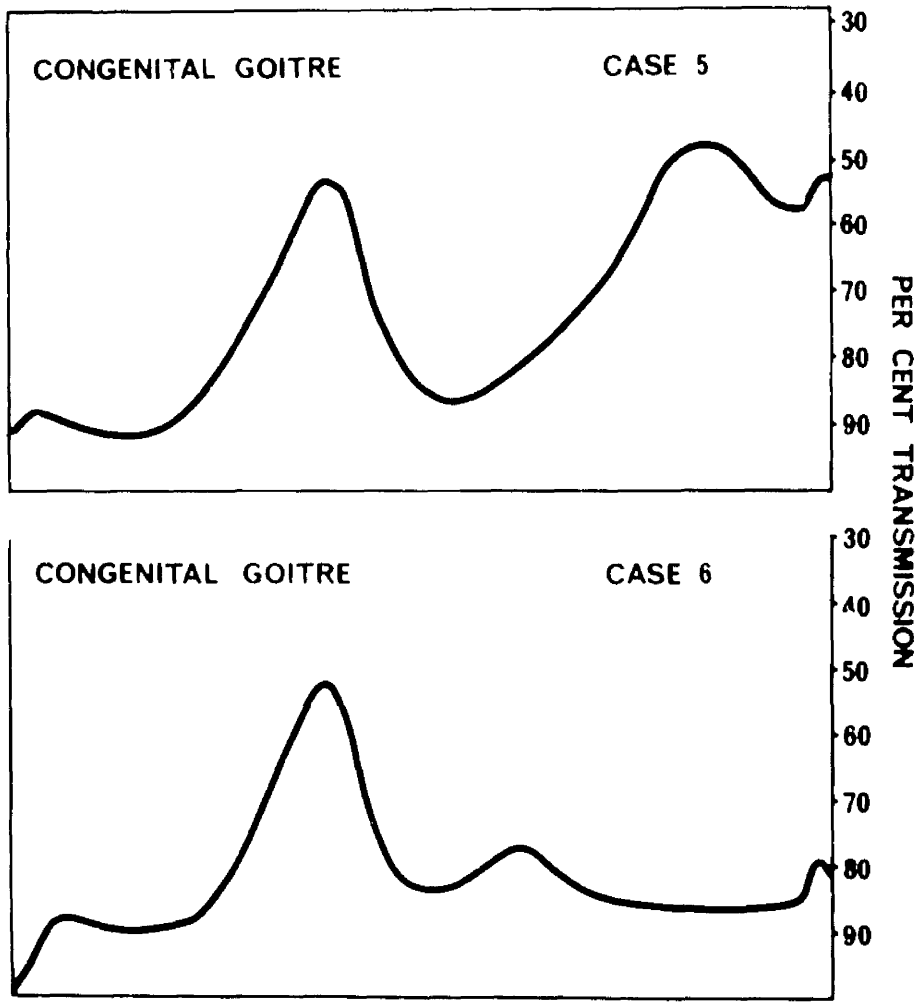


Fig. 26 From top to bottom of gradient, the peaks correspond to the following sedimentation coefficients: Case5, 3 - 8S; 19.2S. Case 6, 12.1S; 19.4S.

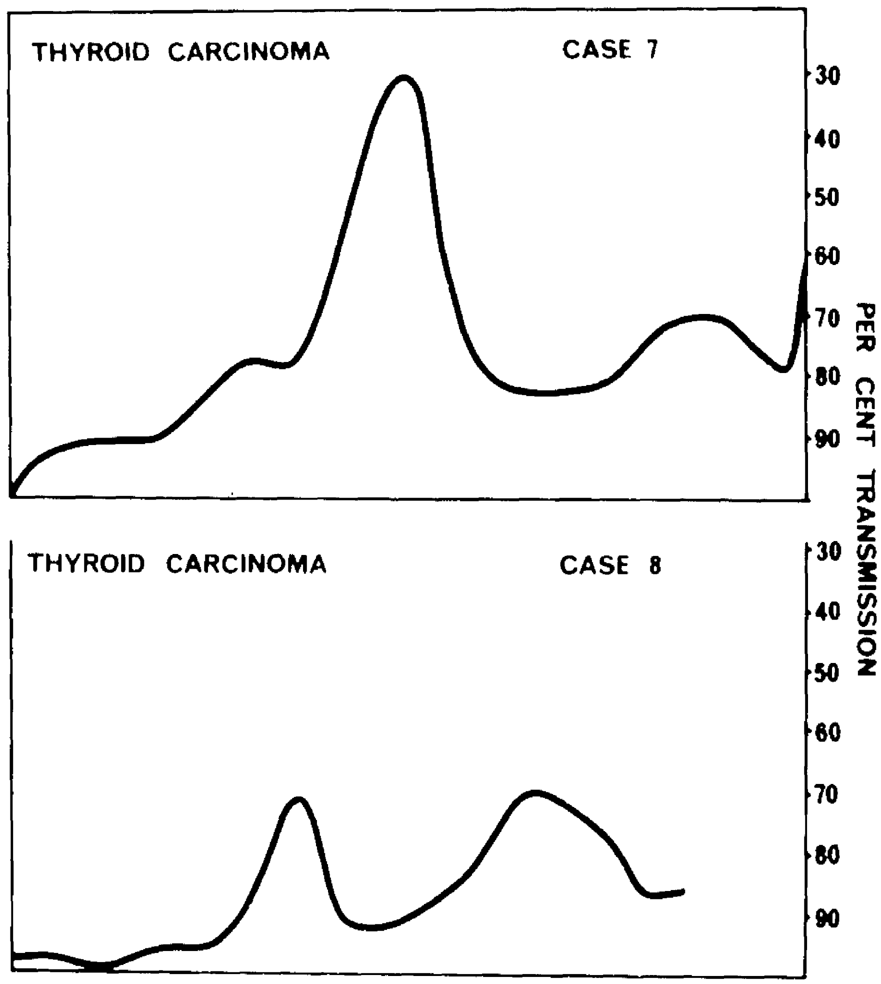


Fig. 27 From top to bottom of gradient, the peaks correspond to the following sedimentation coefficients: Case 7, 3-8S; 18.9S; 26.3S; Case 8, 3-8S; 19.1S; 26S.

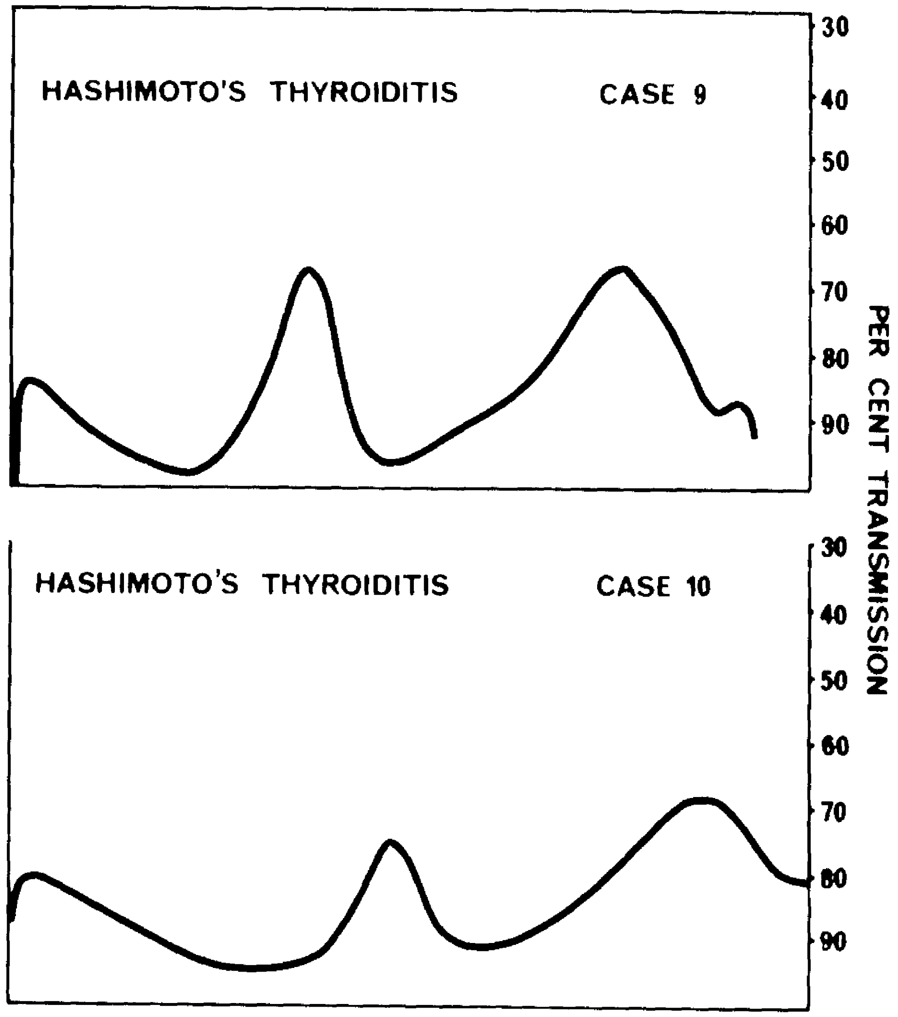


Fig. 28 From top tp bottom of gradient, the peaks correspond to the following sedimentation coefficients: Case 9, 3 - 8S; 20.8S Case 10, 3 - 8S: 20.6S.

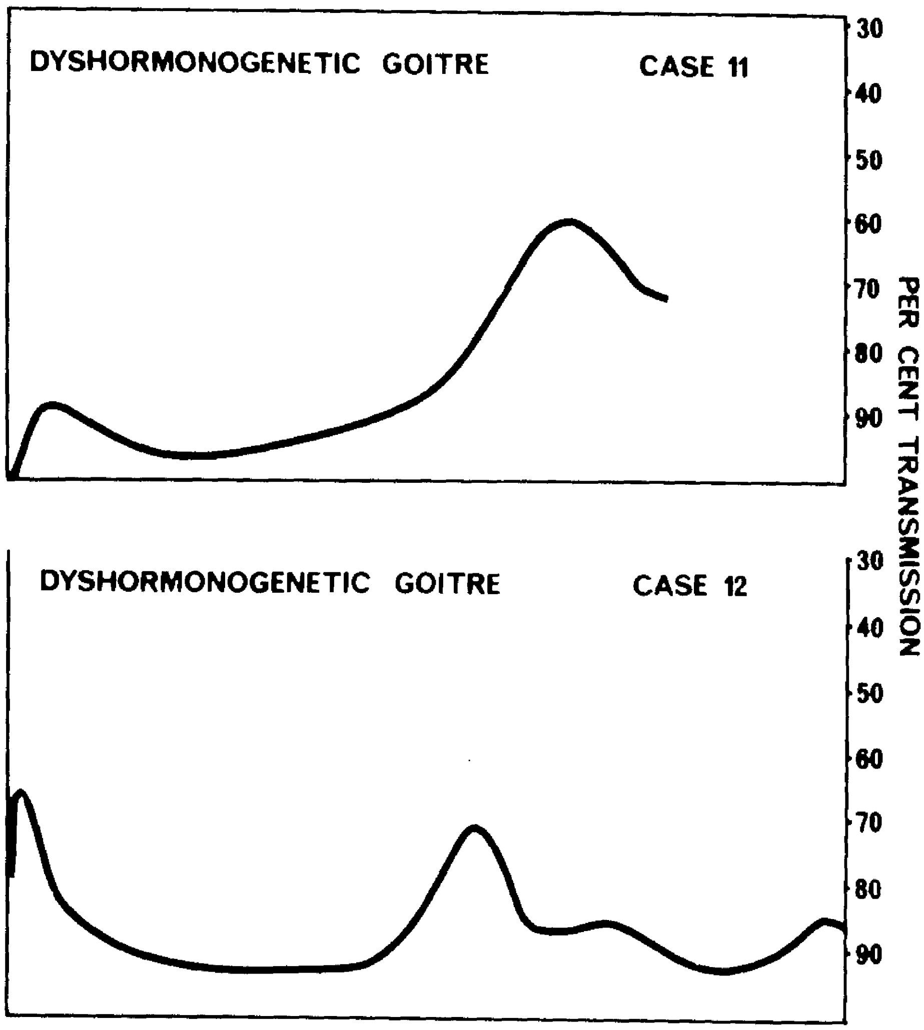


Fig. 29 From top to bottom of the gradient the peaks correspond to the following sedimentation coefficients: Case 11, 5.8S; Case 12, 12S; 18.5S.

v) Hashimoto's Thyroiditis (cases 9 - 10, fig. 28)

In these cases the only OD peak in the region of thyroglobulin had a sedimentation constant corresponding to 20 - 20.5S. There were no heavier components present.

vi) Dyshormonogenetic Goitre (Cases 11, 12, Fig. 29)

Case 11, the iodoprotein purified by gel filtration had no OD peaks in either the 19S or 27S regions of the gradient in spite of the fact that this protein had been eluted in the exclusion volume from G200 Sephadex. The only protein peak present corresponded to a sedimentation constant of 5 - 6S. Case 12, on the other hand, had 2 OD peaks; the major peak corresponded to 18.5S and the minor peak to 12S.

3. Iodide and Carbohydrate content of the Purified Iodoprotein

Samples of the iodoproteins purified by gel filtration were analysed for hexose, sialic acid and iodide. The results are shown in table 6. As expected, the iodide content of the iodoproteins varied widely. However, in general the values obtained for the iodoprotein from goitrous glands were lower than those for normal human thyroglobulin. Normal values for hexose and sialic acid contents were obtained in all cases except Hashimoto's thyroditis and case 11. In the former, higher hexose levels were consistently obtained while in the latter, both the hexose and sialic acid contents were 50% lower than the values recorded for normal thyroglobulin.

4. DEAE Cellulose Chromatography of Purified Iodoproteins

The degree of heterogeneity of the iodoproteins from normal and goitrous/

Table 6 Iodide and carbohydrate contents of purified iodoproteins from human thyroid glands

Case No.	Clinical Condition	PBI µg ^{127}I per mg protein	Hexose mg %	Sialic Acid mg %
1	Euthyroid	3.2	4.0	1.4
2	Non Toxic Goitre	0.56	4.0	1.6
3		3.5	3.8	1.7
4		0.76	4.2	1.7
5	Congenital Goitre	0.18	3.7	1.5
6		0.24	3.8	1.3
7	Carcinoma	0.72	3.9	1.97
8		0.89	4.2	1.8
9	Hashimoto's Thyroiditis	0.23	6.2	1.8
10		0.28	5.9	1.9
11	Dyshormonogenetic Goitre	0.30	1.9	0.52
12		0.32	3.8	1.72
Sheep	-	7.7	4.6	1.7

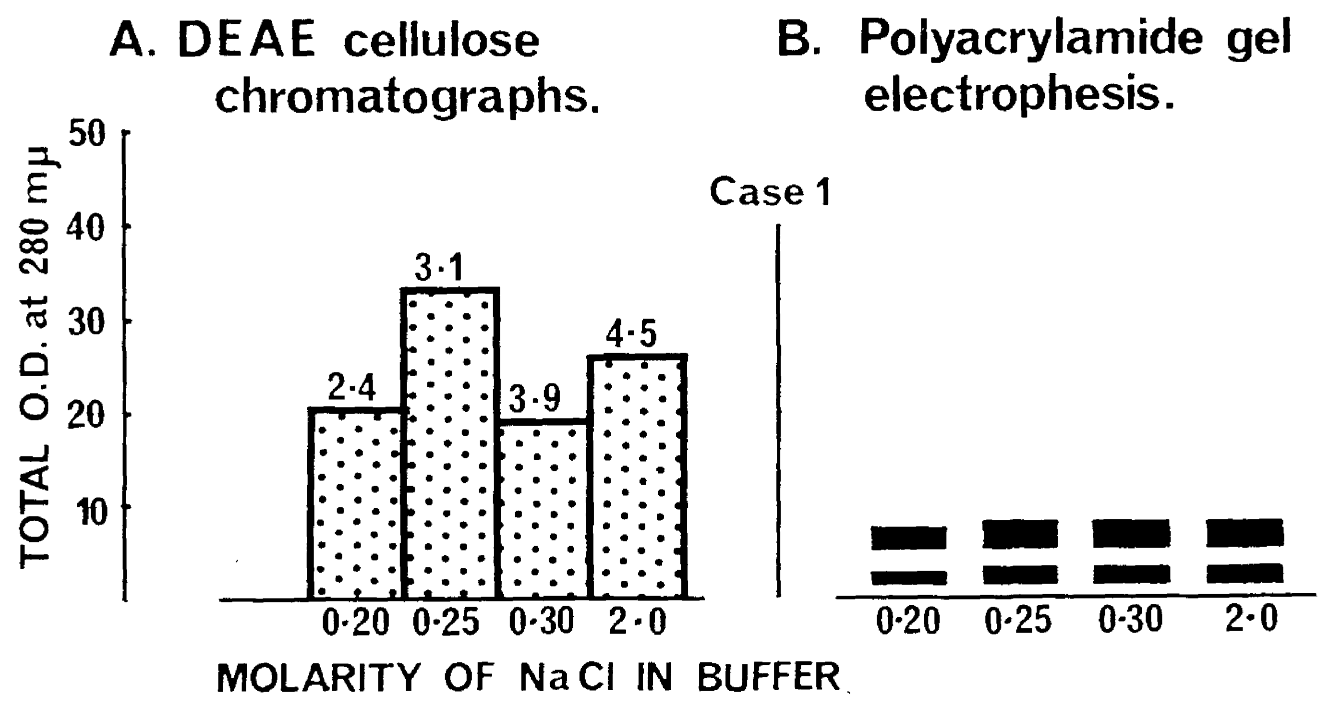
goitrous glands was studied by DEAE cellulose chromatography. In figs. 30 - 34 the proteins, as measured by the OD at 280 mμ, eluted at each step in the elution gradient is expressed as a per cent of the total OD at 280 mμ eluted from the column. In cases 1 - 9, the iodoprotein purified by gel filtration was fractionated.

i) Normal Thyroid Gland (case 1, fig. 30)

Iodoprotein was eluted at each step in the gradient from 0.20 - 2.0 M NaCl. While the highest per cent of the total protein was eluted at 0.25 M NaCl the protein was fairly uniformly distributed between the early eluting steps (o.20 - 0.25 M NaCl) and the later eluting steps (0.3 - 2.0 M NaCl). Electrophoretically the iodoproteins eluted at each step were identical.

ii) Non-toxic Goitre (cases 2 - 4, fig. 31)

In each case, iodoprotein was eluted at the same steps in the gradient as the normal although in case 2 iodoprotein was also eluted at 0.15 M NaCl. In cases 2 - 3, the highest per cent of the total protein was eluted at 0.20 M compared to 2.0 M NaCl in case 4. Furthermore, in cases 3 and 4 the protein was fairly evenly distributed between the early and later eluting steps while, in case 2, over 80% of the total protein was eluted in the early steps. Electrophoretically the iodoproteins fractions in each case were normal although in cases 2 and 4 the slower migrating component was absent/



Figs. 30 - 33 DEAE cellulose chromatography of the purified iodoproteins from normal and abnormal thyroid glands. For conditions of chromatography see materials and methods.

A. The O.D. at 280 mμ eluted at each step in the elution gradient is expressed as a per cent of the total O.D. at 280 mμ eluted from the column. The P.B.I. values of each fraction expressed as μg ^{127}I per mg protein are shown.

B. Polyacrylamide gel electrophoresis of the proteins eluted at each step in the elution gradient.

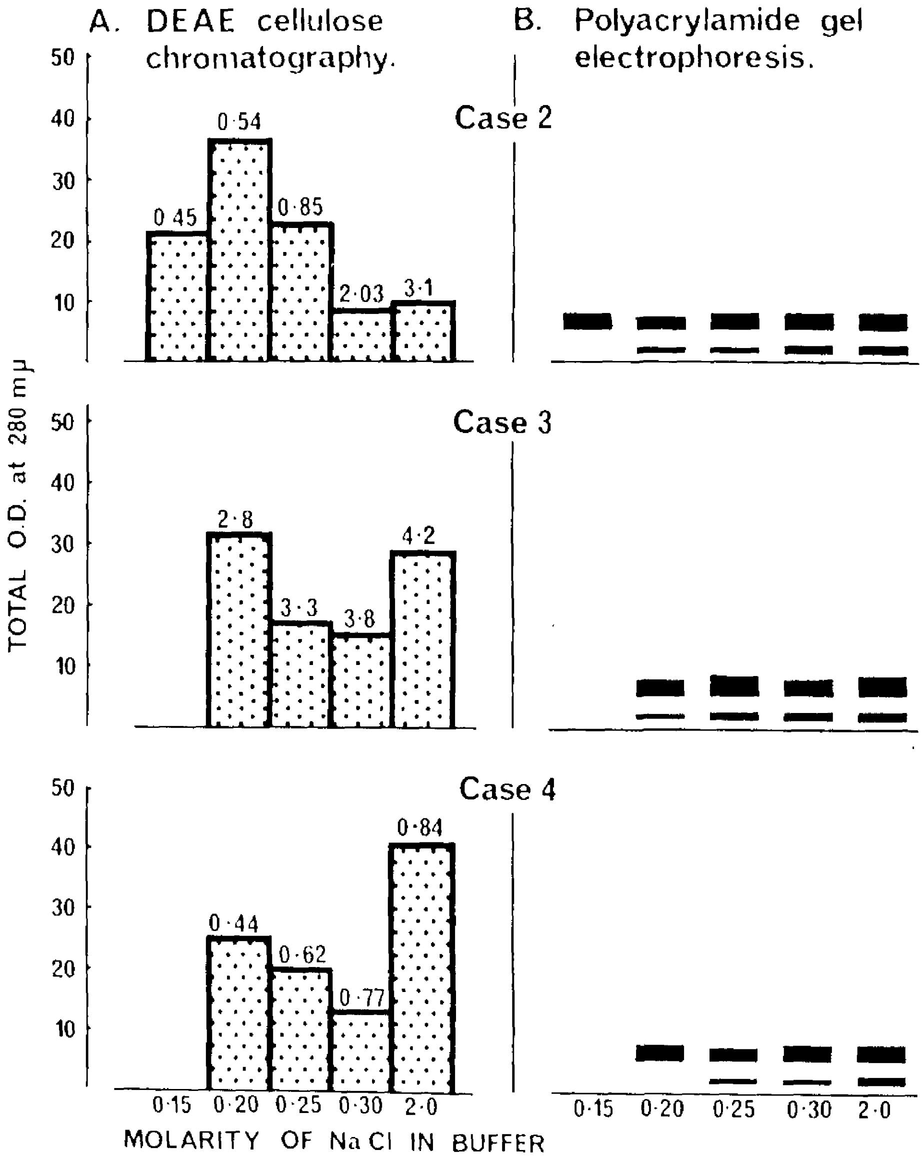

A. DEAE cellulose chromatography.
B. Polyacrylamide gel electrophoresis.
Case 2
Case 3
Case 4
TOTAL O.D. at 280 mμ
50
40
30
20
10
0·54
0 45
0·85
2·03
3·1
2·8
3·3
3·8
4·2
0·44
0·62
0·77
0·84
0·15
0·20
0·25
0·30
2·0
MOLARITY OF Na Cl IN BUFFER

Fig. 31.

absent in the iodoprotein eluted at 0.15 M NaCl and 0.20 M NaCl respectively.

iii) Thyroid Carcinoma (cases 7 - 8, fig. 32)

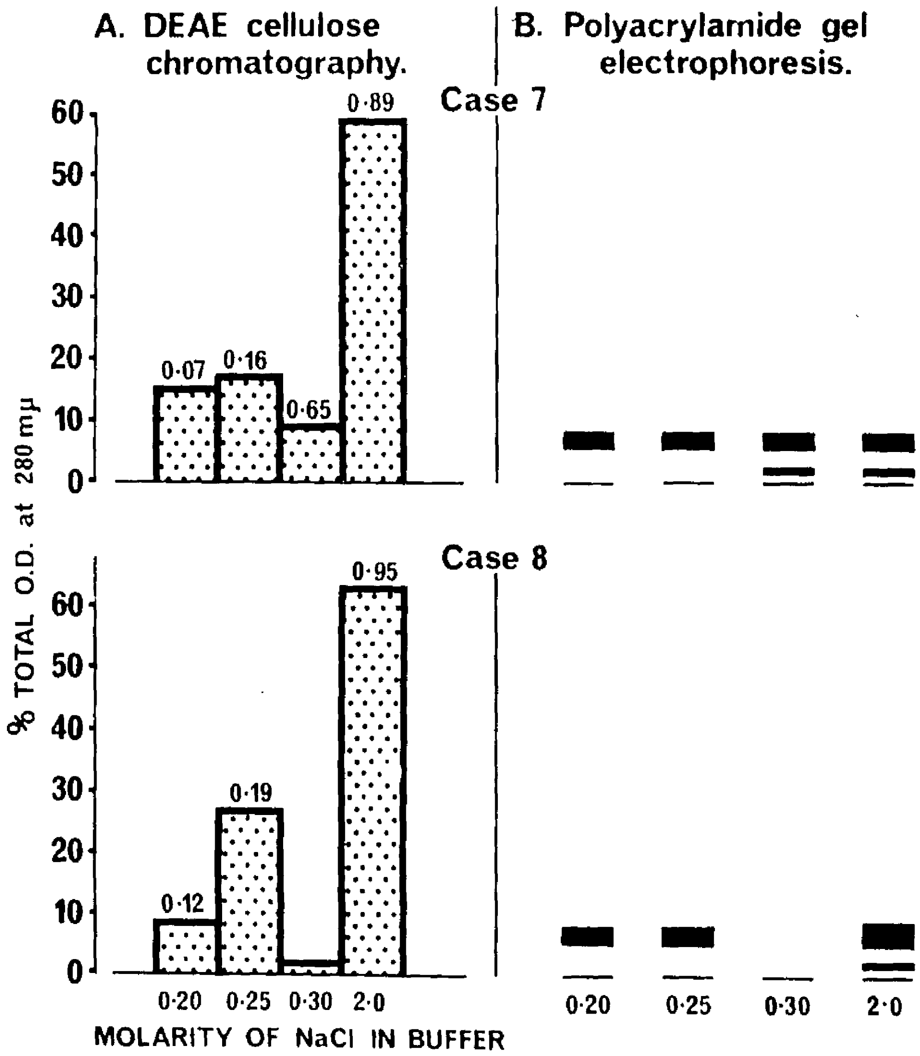
In both cases, iodoprotein was also confined to the steps in the gradient between 0.20 and 2.0 M NaCl. However, the highest per cent of the total protein was eluted at 2.0 M NaCl with the result that 65 - 70% of the protein was present in the later eluting fractions. Electrophoretically the iodoprotein eluted at each of the steps was normal although in case 7, the slower migrating component was absent from the early eluting steps while in case 8 this component was only present in the fraction eluted at 2.0 M NaCl.

iv) Hashimoto's Thyroiditis (cases 9 - 10, fig. 33)

In case 9, iodoprotein was confined to the same fractions as the normal although in case 10 iodoprotein was eluted only at 0.30 and 2.0 M NaCl. In both cases the highest per cent of the protein was eluted at 2.0 M NaCl and, as a result, 76% of the total protein was eluted at the later steps in case 9 and 100% in case 10. Electrophoretically, all iodoprotein fractions contained a protein component indistinguishable from normal thyroglobulin but the slower migrating component was confined to the later eluting steps in case 9 and to the fraction eluted at 2.0 M NaCl in case 10.

v) Dyshormonogenetic Goitre (cases 11 - 12, fig. 34)

Since the gland extracts contained albumin - like iodoproteins when/



A. DEAE cellulose chromatography.
B. Polyacrylamide gel electrophoresis.
Case 7
Case 8
% TOTAL O.D. at 280 mμ
60
50
40
30
20
10
0
0·07
0·16
0·65
0·89
0·12
0·19
0·95
0·20
0·25
0·30
2·0
MOLARITY OF NaCl IN BUFFER

Fig. 32

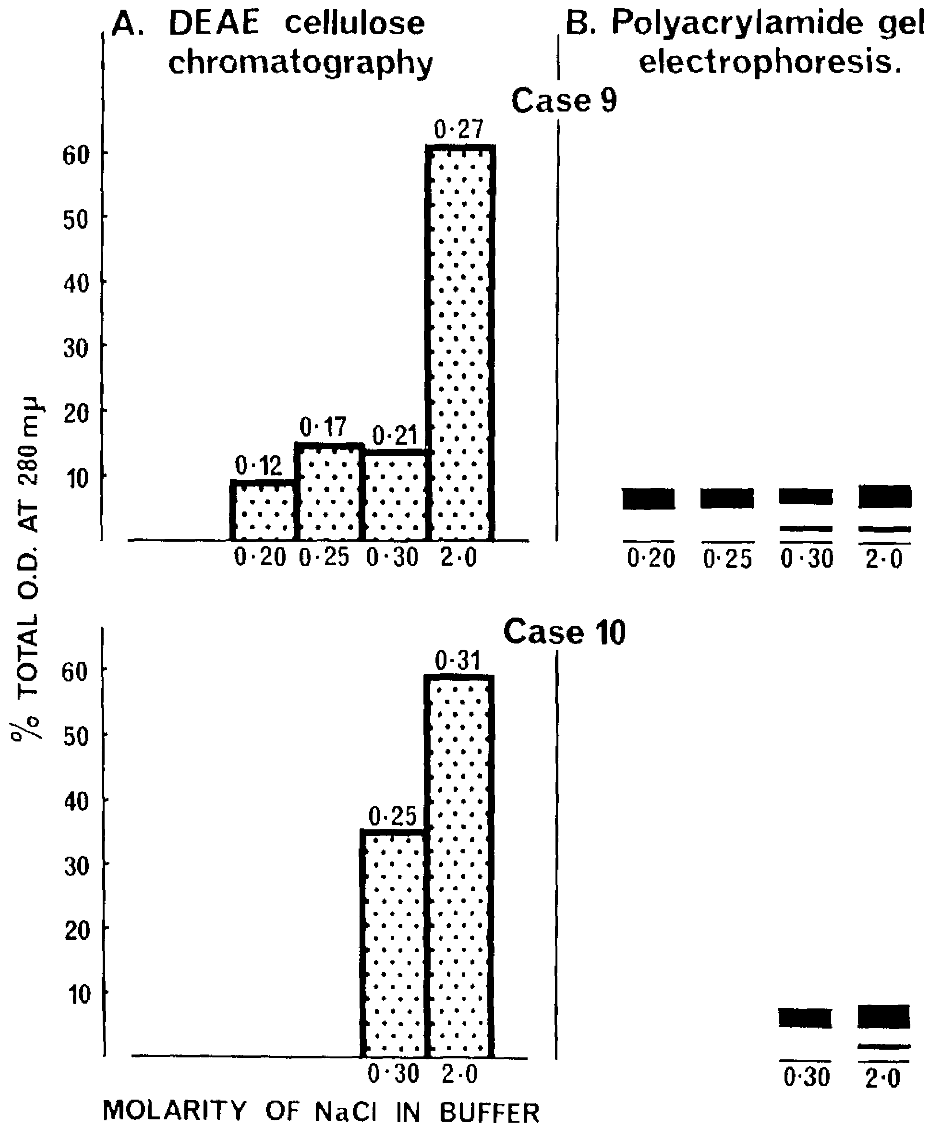

A. DEAE cellulose chromatography
B. Polyacrylamide gel electrophoresis.
Case 9
0·27
60
50
40
30
20
10
0·12
0·17
0·21
0·20 0·25 0·30 2·0
0·20 0·25 0·30 2·0
% TOTAL O.D. AT 280 mμ
Case 10
0·31
60
50
40
30
20
10
0·25
0·30 2·0
0·30 2·0
MOLARITY OF NaCl IN BUFFER

Fig. 33

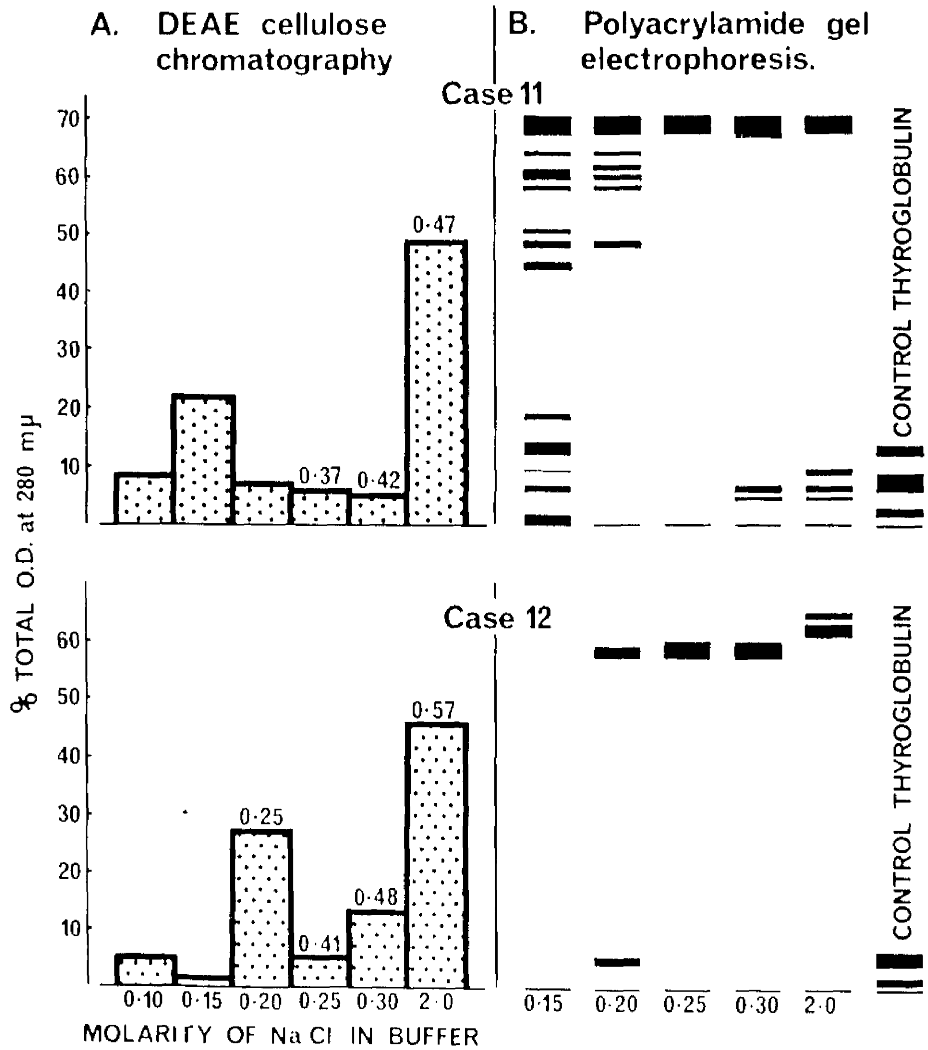


Fig. 34 DEAE cellulose chromatography of the saline extracts of the thyroid glands from cases 11 and 12 (cf. legend figs. 30 - 33)

when fractionated by gel filtration, the gland extracts were also chromatographed on DEAE cellulose in an attempt to isolate these abnormal iodoproteins.

In both cases, serum proteins and haemoglobin were eluted at steps 0.05 - 0.15 M NaCl. In case 11 at 0.20 M, where previously thyroglobulin had been eluted, electrophoretically the proteins eluted corresponded to serum albumin and other unidentified proteins which were possibly other serum proteins. Similarly at 0.25 - 2.0 M NaCl the major protein eluted had an electrophoretic mobility similar to serum albumin. Furthermore, at 0.30 - 2.0 M NaCl other minor protein components were eluted one of which migrated electrophoretically like normal human thyroglobulin. Since serum albumin was elüted, in this system, at 0.15 - 0.20 M NaCl, it seems unlikely that the albumin proteins eluted between 0.25 - 2.0 M NaCl represent true serum albumin. Furthermore, the P.B.I. value recorded for the protein eluted at 0.25 M NaCl is indicative of the iodinated structure of the protein.

In case 12, the fraction eluted at 0.20 M NaCl contained two protein components; one migrated as normal human thyroglobulin, the other as serum albumin. At o.25 - 0.30 M NaCl, there was only one protein component eluted which behaved electrophoretically like serum albumin. However, at 2.0 M NaCl the protein eluted migrated as two distinct/

distinct bands ahead of serum albumin. The P.B.I. values recorded for the protein eluted between 0.25 - 2.0 M NaCl indicates the presence of iodoproteins in these fractions.

5. Peptide Fingerprints of Purified Iodoproteins

In figs. 35 - 40, tracings of the original peptide maps for both native (fig. A) and SCM iodoprotein (fig. B) are shown.

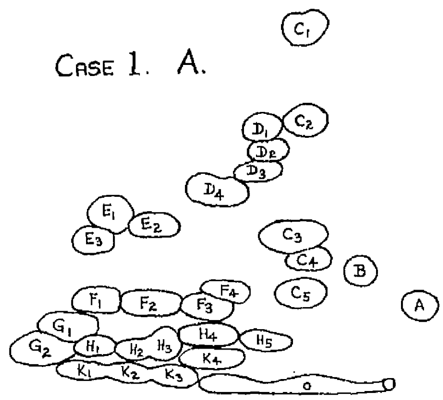
i) Normal Thyroid Gland (case 1, fig. 35)

Fig. 38A represents the peptide map obtained from the tryptic digest of native thyroglobulin. There were 29 distinct ninhydrin positive spots together with a large streak of heavily stained material along the origin line of electrophoretic separation. The latter is presumably derived from large undigestible polypeptides. The peptides have been numbered as shown in fig. 38A for reference purposes since no attempt has yet been made to identify them further. Fig. 38B represents the peptide map obtained for the SCM iodoprotein. The number of peptides which were clearly discernible had increased to 60. Peptides clearly common to both patterns are marked. From the large increase in the number of peptides it is clear that reduction has exposed many additional sites to the trypsin which were previously masked.

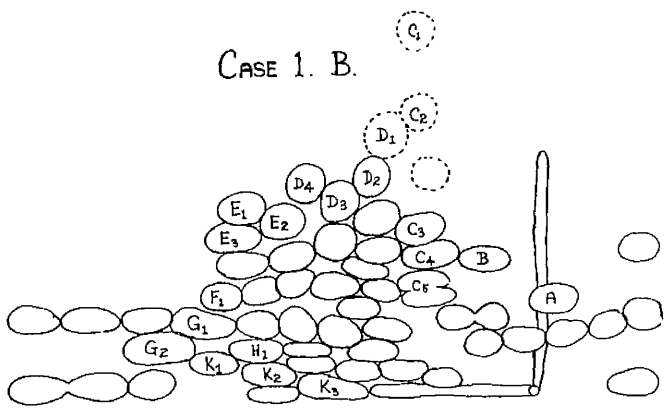
ii) Non-toxic Goitre (cases 2 - 4, fig. 36)

In all cases, the peptide maps for the native and SCM iodoproteins were/

Case 1. A.



Case 1. B.



Figs. 35 - 40 Peptide fingerprints of the tryptic digests of the purified iodoproteins from normal and abnormal thyroid glands.

A. peptide fingerprints of the tryptic digests of the native iodoproteins.

B. peptide fingerprints of the tryptic digests of the SCM iodoproteins.

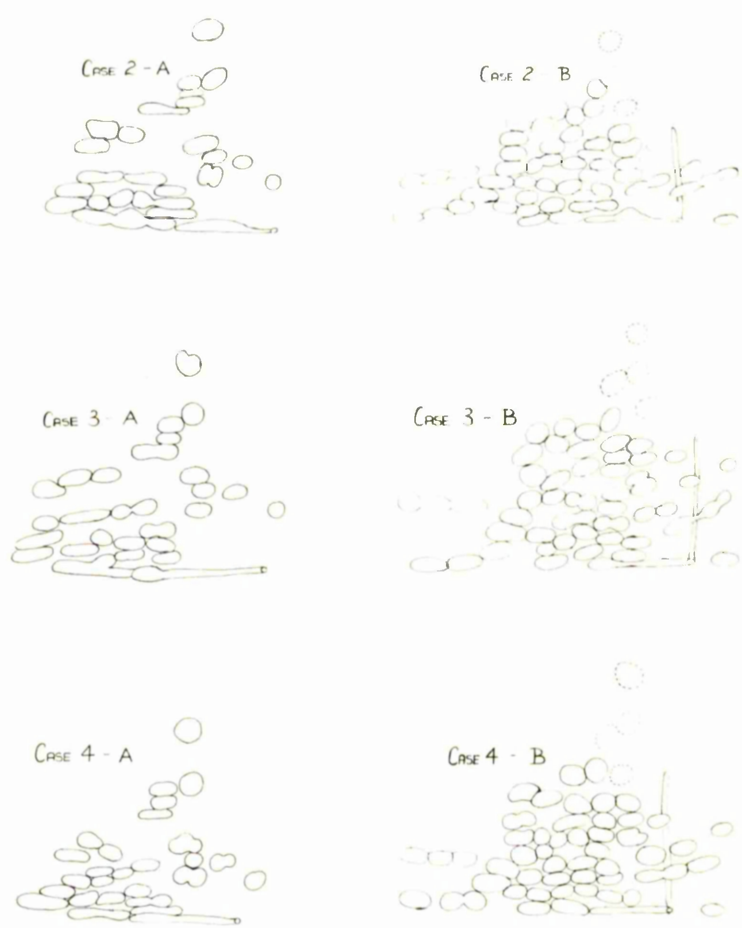


Fig. 36

were undistinguishable from the normal. The position of the peptide spots in these and the other patterns did not always correspond exactly to the position of similar spots in the normal pattern. However, this is an error inherent in the method since, even in duplicate chromatograms, differences in the relative position of the same spots occurred. Nevertheless, groups of peptides clearly similar to those obtained in figs. 38A and B were found in each case.

iii) <u>Congenital Goitre (cases 5 - 6, fig. 37)</u>

Both peptide maps of the native iodoproteins were similar although they differed in many respects from the normal pattern. There were only 11 clearly defined peptides present; the normal peptides A, B and groups C and G together with 2 additional peptides C6 and C7 in group C. Peptides corresponding to groups F, H4 - 5 and K4 were also discernible although it was difficult to identify them due to the indistinctiveness of the ninhydrin colour. The difficulty in distinguishing these latter peptides suggested that possibly the other missing peptides were also present in too small quantities to be clearly identified. This was substantiated by the results obtained for the SCM iodoproteins (fig. B). The number of peptides had increased to 38 - 40 and those corresponding to the 9 groups present in the pattern for normal thyroglobulin were clearly visible. Furthermore, the additional peptides corresponded closely to peptides present in the/

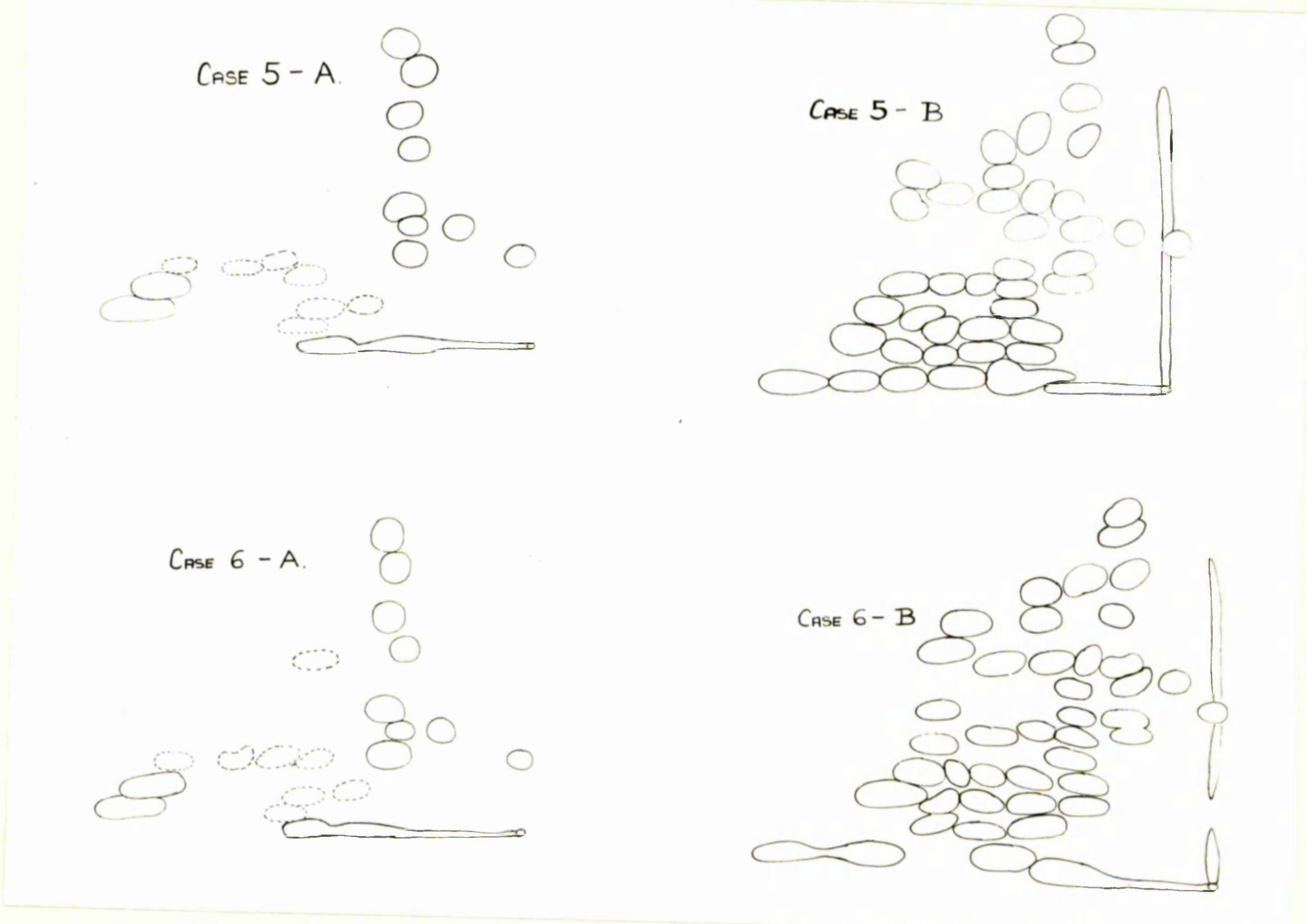


Fig. 37

the normal SCM iodoprotein fingerprint. Nevertheless, there were several peptides which were clearly missing; 5 peptides between groups E and peptides C3 - 5, 9 peptides between peptide A and the origin and the group which migrated ahead of peptide G-1. Therefore, the peptides which were missing from the pattern for native iodoproteins were released by trypsin only after reduction of the protein and a large number of peptides were missing from the pattern for the SCM proteins.

iv) Thyroid Carcinoma (cases 7 - 8, fig. 38)

The peptide maps for both the native and SCM iodoproteins resembled closely these obtained for normal thyroglobulin. However, as in cases 5 - 6, there were two additional faint peptides corresponding to C6 - 7.

v) Hashimoto's Thyroiditis (cases 9 - 10, fig. 39)

v) Hashimoto's Thyroiditis (cases 9 - 10, fig. 39)

The patterns for the native iodoproteins were closely resembled those obtained in cases 5 - 6 than the normal pattern although in case 10 there was an additional peptide C8 in group C. No peptides corresponding to groups F, H or K were visible. In case 9, the iodoproteins had been labelled 'in vivo' with ^{131}I and the dotted broken lines in fig. 39A represents the positions of the ^{131}I-labelled peptides. There were 9 labelled peptides together with streaked areas of dense radioactivity around the origin. 8 of the labelled peptides were confined to group C and it is interesting that none of them/

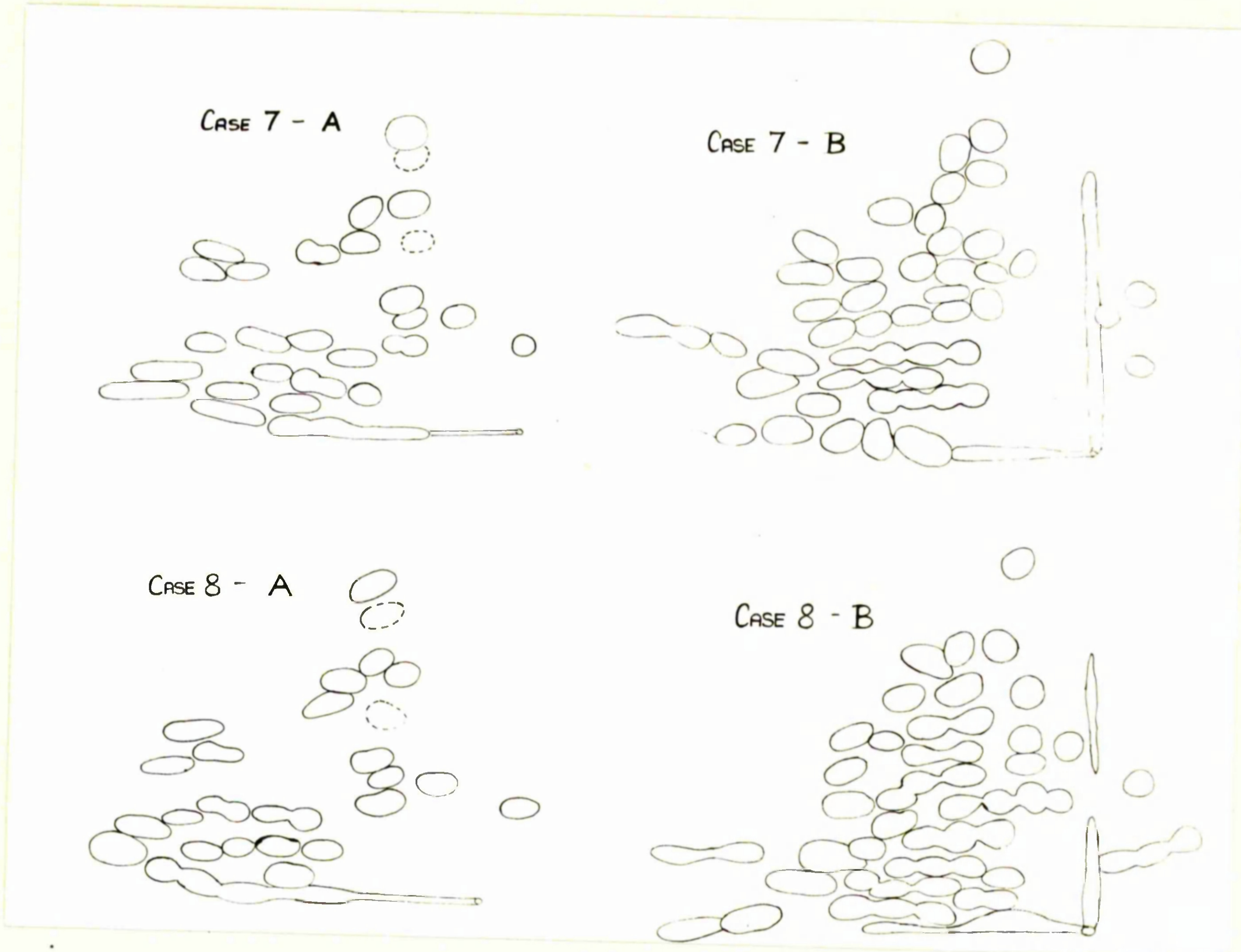


Fig. 38

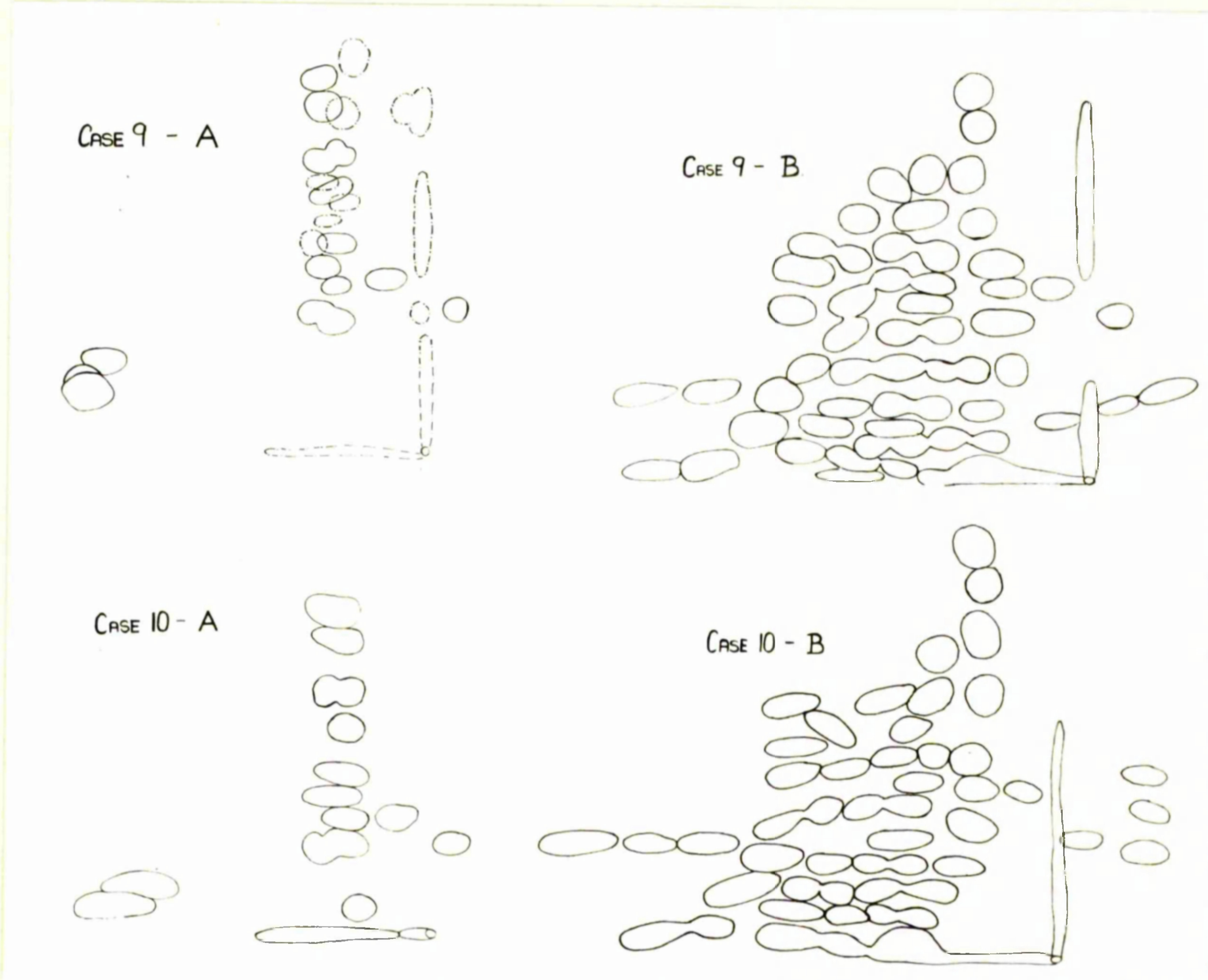


Fig. 39

them coincided exactly with the ninhydrin stained areas. Movement of the x-ray film relative to the chromatogram was ruled out as a reason for this lack of coincidence since the paper and negative were rigidly held by staples placed at each corner.

After reduction of the iodoproteins, the peptide maps for the SCM proteins were indistinguishable from the pattern for normal SCM thyroglobulin.

vi) Dyshormonogenetic Goitre (cases 11 - 12, fig. 40)

The native iodoprotein from case 11 produced a peptide pattern indistinguishable from that of case 10. Reduction of the protein, however, did not produce the normal pattern for SCM thyroglobulin. The pattern more closely resembled that of the normal pattern for native thyroglobulin with 27 distinct peptides.

In case 12, the peptide pattern for the native protein was identical to that described for cases 5 - 6 although there were no detectable peptides corresponding to groups F, H or K. Reduction of the protein, however, produced a pattern which closely resembled that for normal SCM thyroglobulin.

6. Peptide Fingerprints of Iodoprotein fractions separated by DEAE cellulose chromatography

The iodoprotein fractions separated by stepwise gradient elution from DEAE cellulose columns were incubated with trypsin and the resulting/

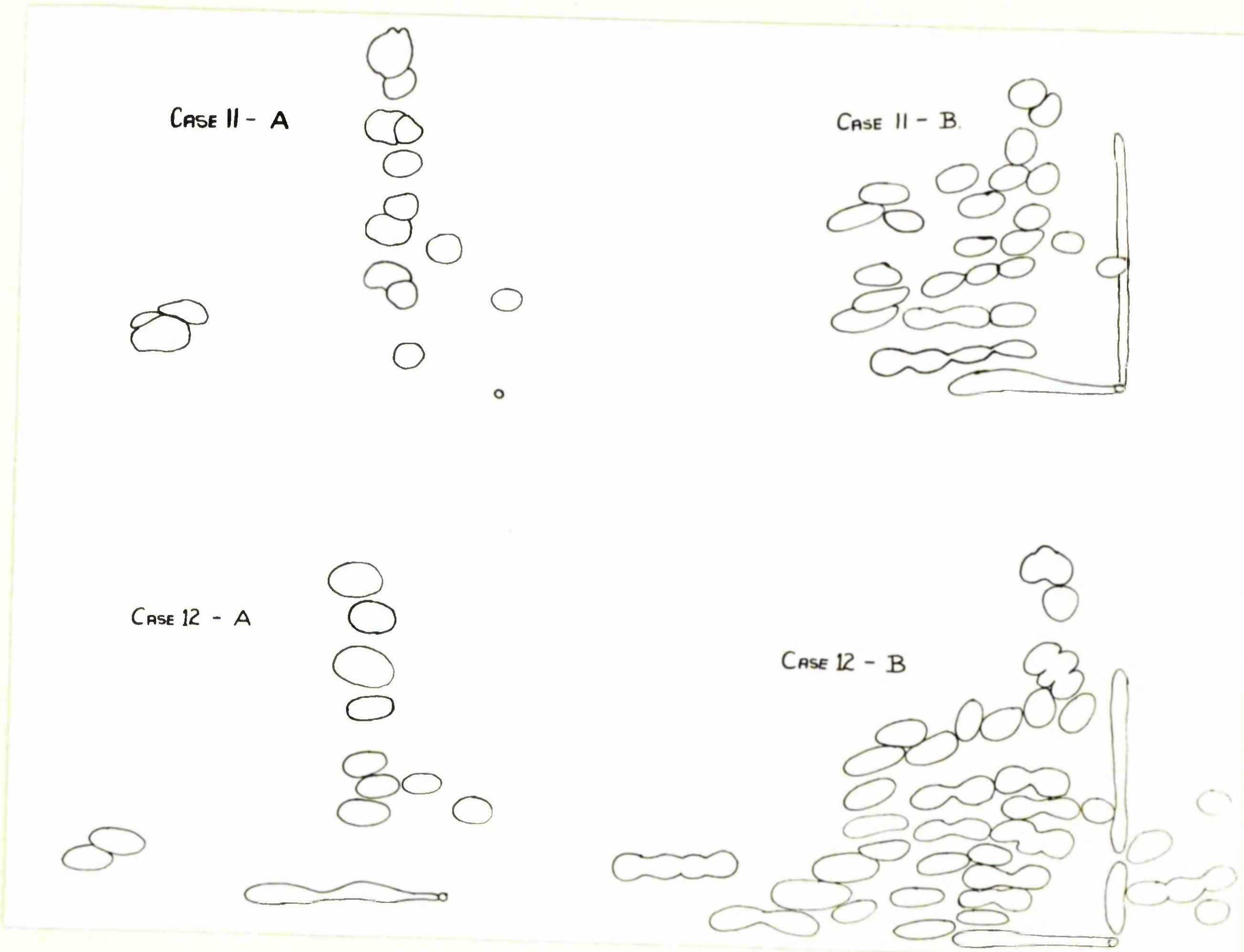


Fig. 40

resulting limit digests analysed by the peptide mapping technique. The tracings of the original peptide maps are shown in figs. 41 - 45.

i) Normal Thyroid Gland (case 1, fig. 41)

The peptide maps for all iodoprotein fractions eluted between 0.20 M - 2.0 M NaCl were essentially similar. Furthermore, there were no detectable differences between these patterns and the pattern for the unfractionated thyroglobulin shown in fig. 41A.

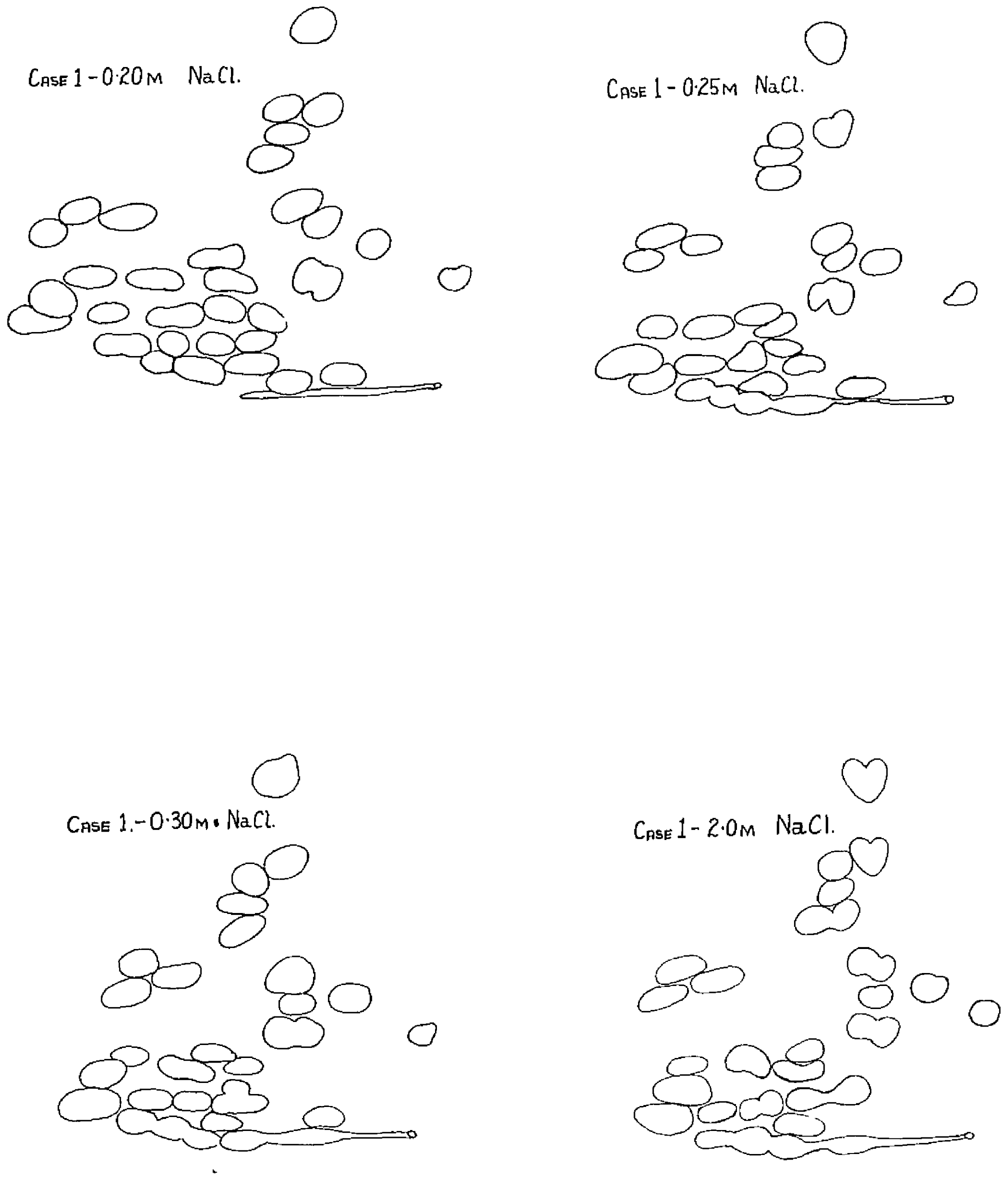
ii) Non-toxic Goitre (case 3, fig. 42)

The results were similar to those described in case 1.

iii) Thyroid Carcinoma (case 7, fig. 43)

In the fraction eluted at 0.20 M NaCl, there were only 11 clearly defined peptides in the peptide pattern obtained. These peptides corresponded to the normal peptides A, B and groups C and G. Faintly stained peptides corresponding to peptides C6 and C7 were also present. A similar pattern was also obtained for the fraction eluted at 0.25 M NaCl although faintly stained peptides corresponding to the peptides in groups F, H and K were also present. The inability to detect peptides in groups D and E and the indistinctiveness of these present in groups F, H and K was unexpected since the pattern for the unfractionated protein was normal. However, the peptide patterns for the remaining iodoprotein fractions eluted at 0.30 M and 2.0 M NaCl were identical

to/



Figs 41 - 45 Peptide fingerprints of tryptic digests of the iodoprotein fractions separated by DEAE cellulose chromatography.

* 0.20 - 2.0 M NaCl refers to the molarity of NaCl at which the iodoproteins were eluted from the columns.

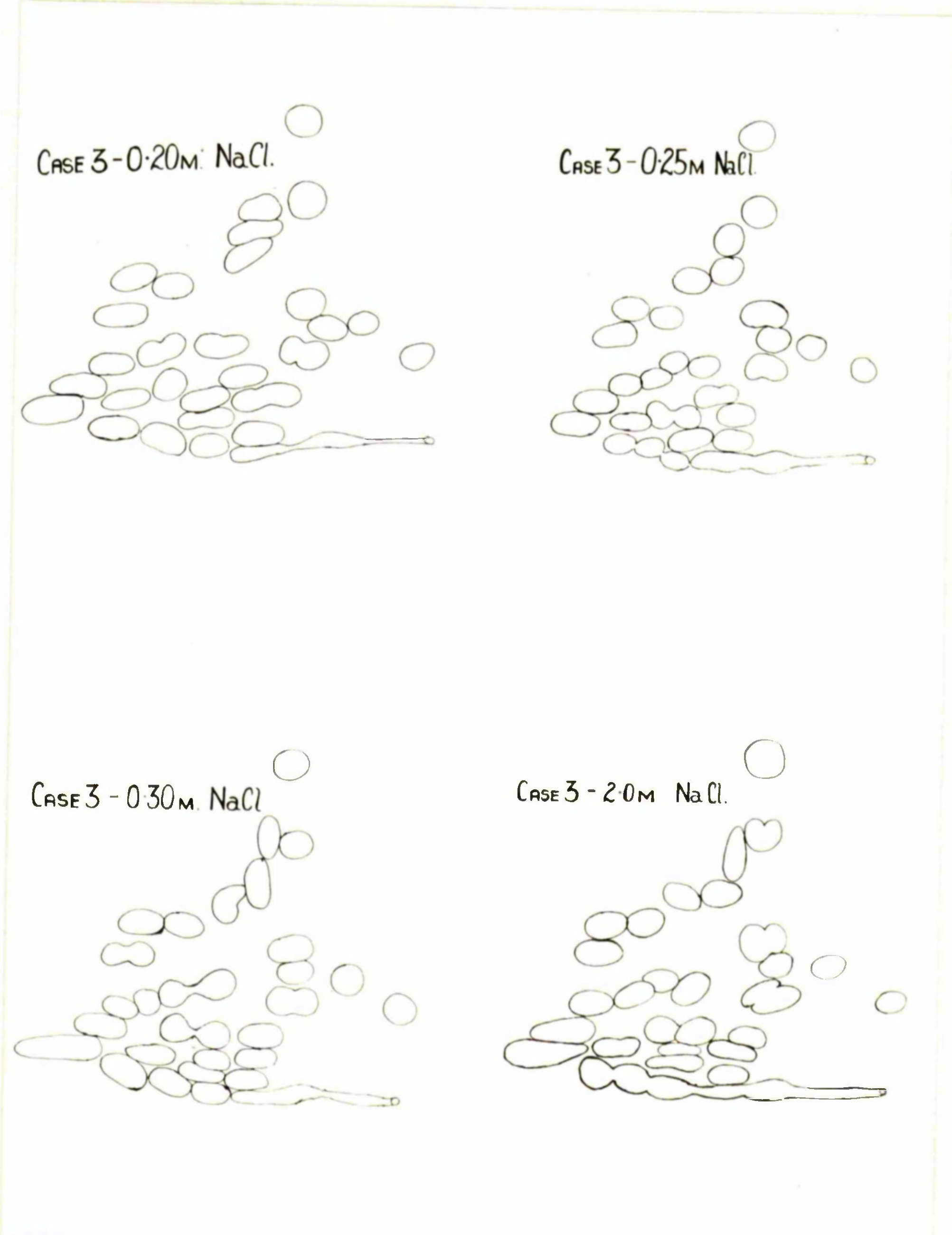

CASE 3 - 0·20M NaCl.
CASE 3 - 0·25M NaCl.
CASE 3 - 0·30M NaCl
CASE 3 - 2·0M NaCl.

Fig. 42

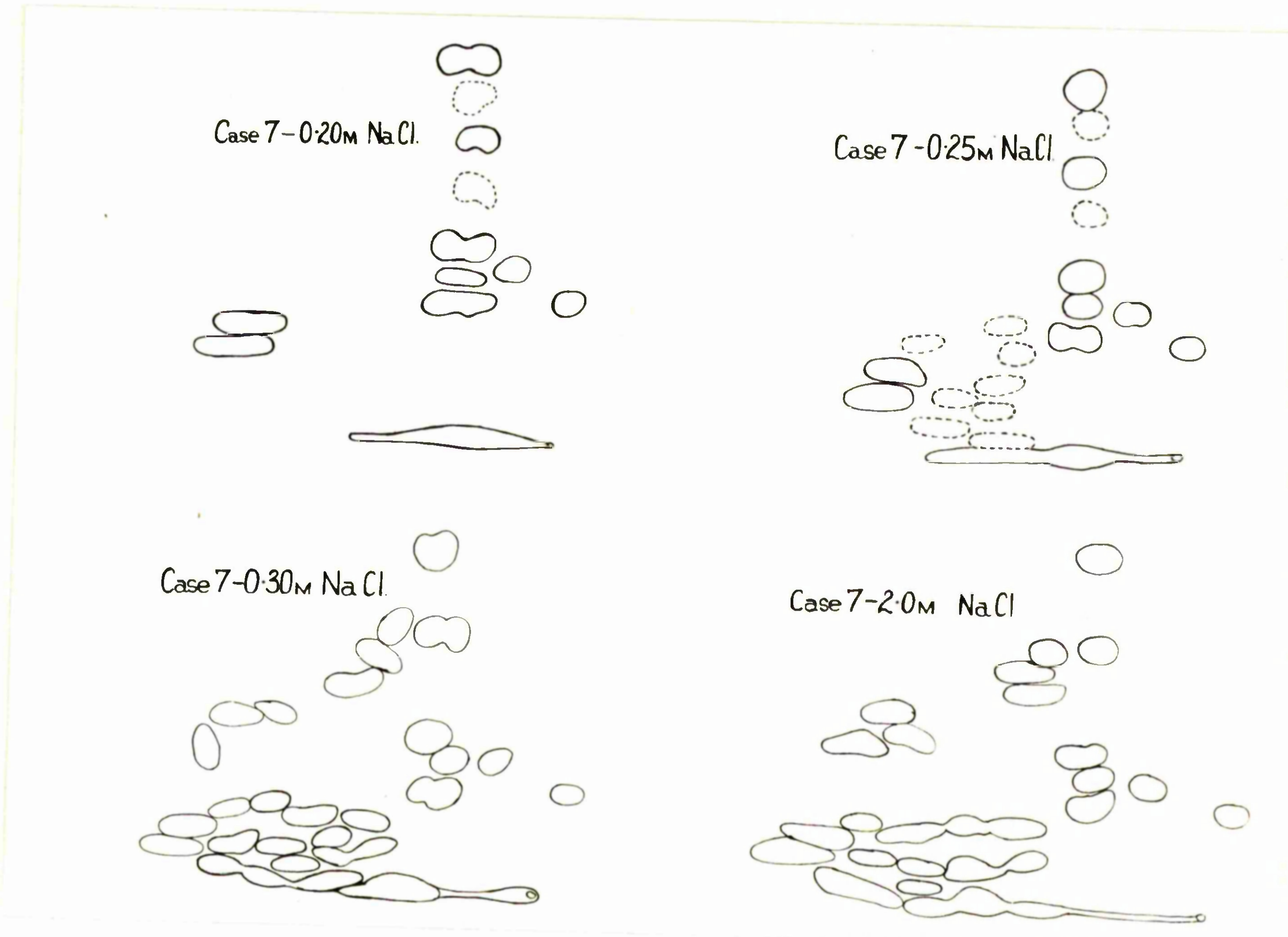

Case 7 – 0·20M NaCl.
Case 7 - 0·25M NaCl.
Case 7 – 0·30M Na Cl.
Case 7 – 2·0M Na Cl

Fig. 43

to that for normal native thyroglobulin - peptides C6 and 7 were not discernible.

v) Hashimoto's Thyroiditis (case 9, fig. 44)

The iodoprotein fractions eluted at each of the steps between 0.20 M and 2.0 M NaCl produced indistinguishable peptide patterns which were identical to that obtained for the unfractionated iodoprotein.

vi) Dyshormonogenetic Goitre (cases 11 - 12, fig. 45)

In case 11, those iodoprotein fractions eluted between 0.25 - 2.0 M NaCl were analysed. The iodoprotein eluted at 0.25 M, which behaved electrophoretically like serum albumin, produced a peptide map with 17 distinct peptides. These included the peptides A, B and group C together with the additional peptides C6 - 8.

The remaining peptides were in a group which migrated electrophoretically ahead of group C. They have been labelled N1 - 8 for reference purposes. By comparing the positions of those peptides in the pattern for normal thyroglobulin it is tentatively suggested that peptides N4, 5 and 7 correspond to H1, 2 and 4: N8 to K2 and N1 and 6 to F2 and 3. This comparison is, however, purely superficial since identities cannot be equivocably established without more detailed chemical analysis. An interesting feature, however, is the absence of peptides G1 and 2 since this is the/

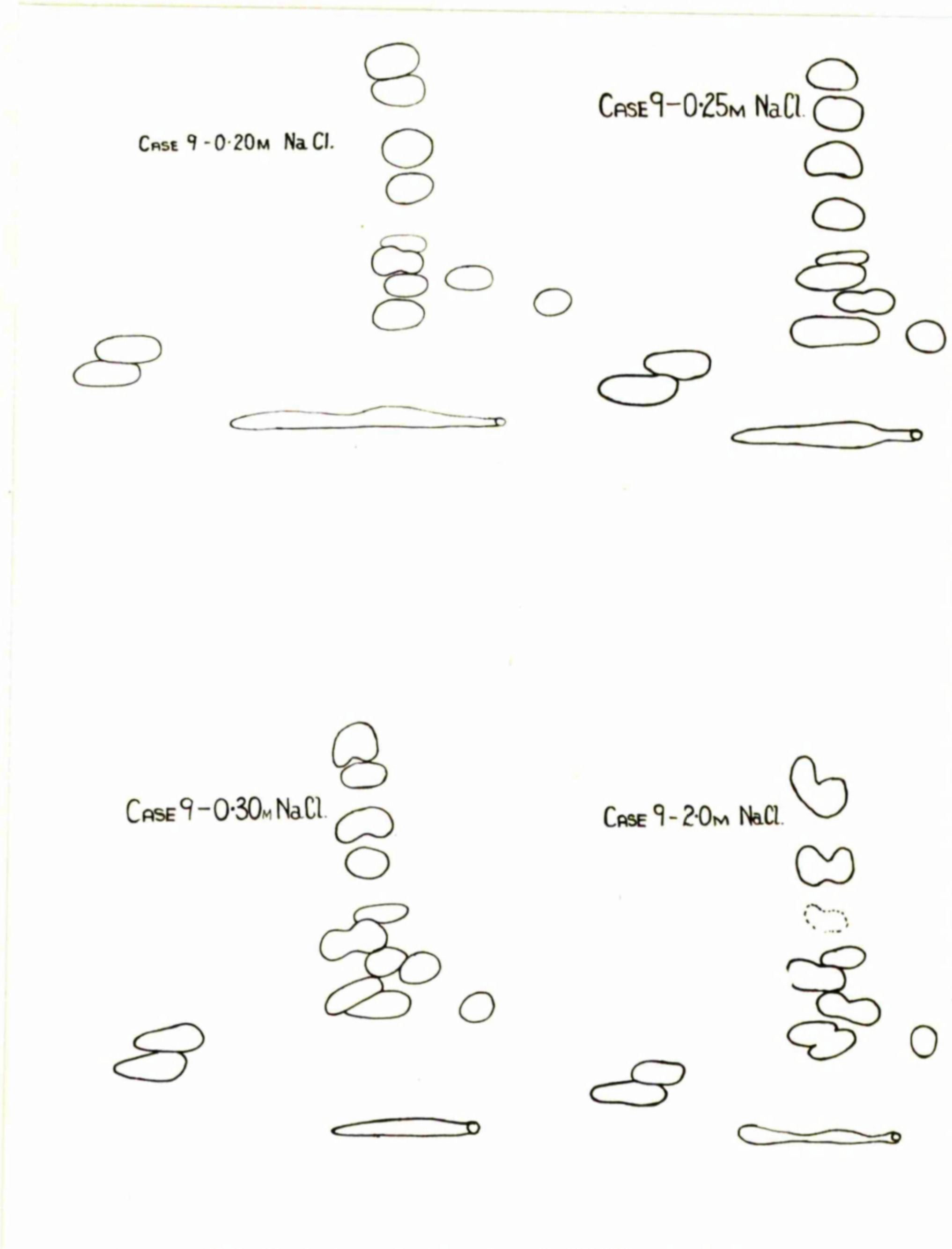

Case 9 - 0·20M Na Cl.
Case 9–0·25M NaCl.
Case 9–0·30M NaCl.
Case 9 - 2·0M NaCl.

Fig. 44

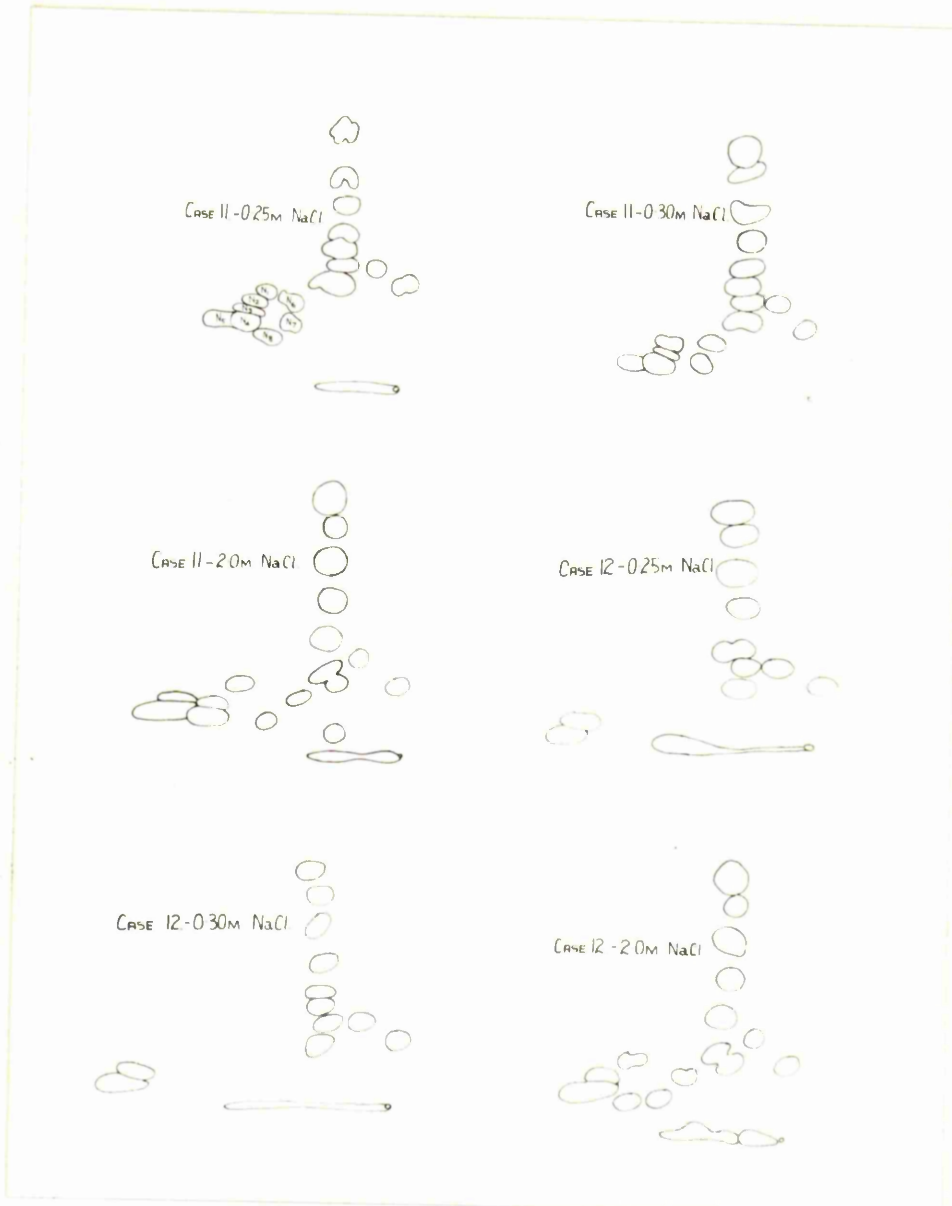

Case 11 - 0.25M NaCl
Case 11 - 0.30M NaCl
Case 11 - 2.0M NaCl
Case 12 - 0.25M NaCl
Case 12 - 0.30M NaCl
Case 12 - 2.0M NaCl

Fig. 45

the only instance where these peptides were missing from the fingerprint. A similar pattern was obtained for the iodoprotein fraction eluted at 0.30 M NaCl although peptides N1 and 8 were missing. The fraction eluted at 2.0 M NaCl produced a pattern which contained peptides corresponding to those found in the fraction eluted at 0.30 M NaCl. However, in addition, the peptides G1 and 2 were now present.

In case 12, the protein fractions eluted at 0.25 M - 2.0 M NaCl were also analysed. The peptide maps for all three fractions were similar and indistinguishable from that described in the case of the native unfractionated protein.

Discussion

1. General Properties of the Iodoproteins in established Human Goitres

The purified iodoproteins from non-toxic goitres and the thyroid carcinomas studied were, in general, indistinguishable from normal human thyroglobulin in electrophoretic properties, sedimentation constants and carbohydrate contents. Since the only significant difference was the total iodine content of the proteins, this clearly indicates that reduction of the iodination levels of iodoproteins to those recorded for these iodoproteins has no effect on these properties of the iodoproteins. Furthermore, in cases 5, 6 and 12 where the iodine content of the iodoproteins were even lower, the major iodoprotein had the same physico-chemical properties as normal human 19S thyroglobulin. However, in these latter cases and cases 9 - 10, no component corresponding to 27S iodoprotein was present. It has been suggested that, in view of the higher level of iodination of the 27S iodoprotein, this component provides a large proportion of thyroxine content of the gland and represents a hormone store (Robbins, Salvatore, Vecchio and Ui, 1966). In section 1 ^{3}H-leucine incorporation studies into rat thyroidal proteins during goitrogenesis suggested that, in the absence of iodination, a precursor of the 27S iodoprotein with sedimentation constant of

24/

24 - 25S was synthesised. It is possible that in those glands where the iodine content of the existing protein is low (0.18 - 0.32 μg ^{127}I per mg), synthesis of a 27S protein precursor continues but due to a lesion in the iodine metabolism of the gland, e.g. iodide organification, iodide conservation or iodide transport, iodination of this precursor to a level which affords a degree of stability to the molecule does not occur. Furthermore, since the turnover of the 27S iodoprotein is considerably slower than the 19S protein, under the above conditions, the available iodine would be mainly channelled into the 19S component.

In cases 9 - 10, Hashimoto's thyroiditis, the major iodoprotein component resembled normal thyroglobulin electrophoretically but sedimented in sucrose gradient centrifugation as a 20 - 21S component. While a similar iodoprotein was encountered in the rat thyroid after 8 - 10 days (cf section 1) on MTU, it is unlikely, in view of the low iodine contents of these iodoproteins (0.23 - 0.28 μg ^{127}I per mg), that these are the older more highly iodinated molecules. Since a higher hexose value than normal was recorded for these proteins it is quite possible that the higher sedimentation constant is related to this. An abnormal carbohydrate moiety in the protein could conceivably affect the configuration of the molecule sufficiently to alter the sedimentation properties of/

of the molecule. However, the lack of sufficient material from these glands prevented a more detailed analysis of the carbohydrate moieties of the iodoproteins.

Cases11 and 12 were similar in that both gland extracts contained iodo albumins in addition to a larger iodoprotein eluted in the exclusion volume from G200 Sephadex. However, while this latter protein, in case 12, resembled the normal 19S iodoprotein in its properties the same protein from case 11 differed in many respects. It was highly unstable to the isolation techniques used, being completely broken down to smaller units, and contained only 50% of the normal hexose and sialic acid content. Edelhoch (1965) and Lissitsky, Rolland and Bergot (1965) showed that 19S thyroglobulin is an aggregate of 2 dissimilar 6S subunits and it is interesting that the sedimentation constant of the dissociated protein in case 11 was approximately 6S. However, since reduction of disulphide linkages in the 19S iodoprotein was necessary to produce these subunits, the ease of dissociation of this iodoprotein suggests that its subunits are held together by non-covalent linkages only. Therefore, at this level of investigation, there would appear to be no real identity between this iodoprotein and normal human thyroglobulin.

2. DEAE Cellulose Chromatography of the Purified Iodoproteins

Thyroglobulin exists as a whole spectrum of molecules differing only in their level of iodination. This confers on the molecules a heterogeneity which can be assessed by ion-exchange chromatography on DEAE cellulose. This capacity can be related to the diiodotyrosine and, to a lesser extent, the thyroxine contents of the molecules: the higher the diiodotyrosine level the more strongly the molecule is retained by the DEAE cellulose. In this study, the degrees of heterogeneity of the iodoproteins from established human goitres were studied.

In general, the iodoproteins from normal goitrous glands were eluted between the same limits of the applied gradient.

In case 2, however, iodoprotein was also eluted at a lower ionic strength and over 80% of the total protein was confined to the early eluting steps. In cases 3 - 4, on the other hand, the iodoprotein was more evenly distributed between the early and late eluting steps and in this respect resembled the pattern for the iodoprotein from the normal gland. Assuming that the diiodotyrosine content of the molecules is the major contributing factor in this reaction, this would imply that in cases 3 - 4, on average, the diiodotyrosine distribution among the molecules closely resembles that of the normal gland, whereas in case 2 there is, on average, a higher/

higher proportion of monoiodotyrosine than diiodotyrosine among the molecules compared to the normal. In cases of Hashimoto's thyroiditis and thyroid carcinoma, on the other hand, the general feature was that over 70% of the total protein was eluted in the later eluting steps. If the above reasoning was applied in this case, it would imply on average that there was a higher proportion of diiodotyrosine residues in these iodoproteins than in the iodoproteins from the normal gland. However, comparing the iodine contents of the proteins in the later eluting fractions with those obtained for the same fractions from the normal gland (cf Figs. 30, 32 and 33), there is a wide divergence of values. A possible explanation is that steric factors are involved. Since only groups on the surface of the protein will be available for interaction with the charged groups on the cellulose, it is possible that even at low levels of iodine similar diiodotyrosine concentrations can occur at the surface of the molecule to that present in the more highly iodinated molecules - the differences in iodine content between the proteins being accounted for by iodotyrosines buried in the molecule and unavailable for interaction with the cellulose. In general, however, the results indicate that in cases of thyroid carcinoma and Hashimoto's thyroiditis the iodoprotein is more homogenous than normal while in non-toxic goitre the degree of heterogeneity/

heterogeneity resembles that found in the normal gland.

3. Peptide Fingerprints of Iodoproteins in Established Human Goitres and Normal Thyroid Gland

In recent years some workers have applied the technique of peptide fingerprinting to the study of the primary structure of thyroglobulin. Spiro (1964) produced peptide maps of salt fractionated sheep thyroglobulin in which she detected 29 - 30 different peptides. This is very similar to the results obtained in this study for the fingerprint of normal human thyroglobulin. Furthermore, allowing for differences in the techniques, there is a definite similarity in the grouping of the peptides between the fingerprints of native sheep and human thyroglobulin. Lissitsky, Rolland and Bergot (1965) performed similar experiments with SCM sheep thyroglobulin. However, there is a large discrepancy in the number of peptides reported for the sheep thyroglobulin and that found in the present study for SCM human thyroglobulin. Since Lissitsky et al did not reproduce their peptide fingerprints, the discrepancy cannot be further elucidated. However, it could be related to the better resolution afforded by high voltage electrophoresis techniques as opposed to the low voltage method employed in this study.

In the present investigation, the peptide fingerprinting technique/

technique was applied to compare the primary structures of the iodoproteins from normal and goitrous thyroid glands. The similarity in the pattern of the peptides for both the native and SCM iodoproteins from non-toxic goitres, thyroid carcinomas and the normal gland suggest that these proteins have identical primary structures. However, the fingerprints of the native iodoproteins from the other cases studied deviated to a significant degree from the normal pattern. This was due to the absence of peptides in the groups D, E, F, H, and K and the presence of additional peptides in group C. It is interesting that the presence of the former groups of peptides coincide with those iodoprotein preparations containing significant amounts of 27S iodoprotein. Further evidence of this was obtained from the fingerprints of the iodoprotein fractions eluted from DEAE cellulose in case 7. The early eluting fractions had no detectable 27S iodoprotein and the corresponding peptide fingerprints lacked these particular peptides. Any explanation, however, must also take into account that, on reduction and alkylation of the iodoprotein preparations lacking the 27S component, the resulting peptide fingerprints were similar to that for the normal SCM iodoprotein which contained the 27S component. Since trypsin can release the same peptides from reduced 19S iodoprotein as a reduced iodoprotein preparation containing/

containing both 19S and 27S components, this would suggest that both these iodoproteins are composed of subunits with identical primary structures. However, since fewer peptides are released from native 19S iodoprotein than from the native protein preparation containing both components this would suggest that in 27S iodoprotein certain peptide bonds are available to the trypsin which are protected in the 19S protein due, presumably, to steric factors. Vecchio, Edelhoch, Robbins and Weathers (1966) showed that, while reduction of disulphide bonds in the 19S iodoprotein was essential to the release of a subunit with sedimentation constant 6S, a similar subunit could be released from 27S iodoprotein under conditions which ruptured non-covalent bonds only. It follows, therefore, that during enzymic digestion of iodoprotein preparations containing 27S iodoprotein, certain sites in the 6S subunit could be exposed to trypsin which would not be available in the 19S protein without reduction and alkylation. Furthermore, in the peptide maps of the native iodoproteins which contained the peptide groups D, E, F, H and K, the intensity of the ninhydrin stained spots in these groups was much less than that in peptides A, B groups C and G. Subsequent reduction, however, greatly increased the intensity of the colour in those regions. Such a situation would occur if these peptides were/

were derived from exposure to the trypsin of additional sites of action by dissociation of the 27S iodoprotein as described above and if similar sites of action were exposed in the 19S iodoprotein components on reduction and alkylation.

The appearance of the additional peptides in group C in cases 5, 6 and 9 - 12 are difficult to explain since no attempt has been made to identify the chemical nature of the peptides released by trypsin. However, the possibility that they are the result of endogenous exopeptidase activity is unlikely since they are also present in the peptide maps of the SCM iodoproteins and the conditions of reduction and alkylation would have inactivated any proteolytic enzymes in the preparations. A possible explanation is that proposed by Alexander (1968) to explain a similar situation encountered in the peptide maps of rat thyroglobulin. In thyroglobulin preparations there could exist a heterogeneity with regard to the iodination of different tyrosyl sites or to the extent of iodination of the same tyrosyl site. In case 9, the autoradiograph of the fingerprint indicated the presence of iodopeptide spots overlapping the ninhydrin colour of peptides C6 and C7. If one assumes that a particular tyrosine residue in the iodoprotein can be either non-iodinated, mono-iodinated or diiodinated/

diiodinated, then in case 9 the peptides C6 - 7 could represent the non-iodinated form of the peptide and the spot on the autoradiograph could represent the monoiodinated derivative. This could explain the lack of coincidence of the ninhydrin and radioactive spots. If under normal conditions these particular tyrosyl sites are generally readily iodinated to the diiodotyrosyl level then it is possible that the omission of these peptides from the normal pattern could be due to this alone or the fact that these iodotyrosyl residues could be involved in the iodotyrosyl coupling reactions leading to thyroxine formation. In either instance it is not inconceivable that the position of the particular peptide in the fingerprint would be altered. Further proof that a heterogeneity of tyrosyl residues with respect to iodination could be the reason for the variability in the presence of these peptides is that, although they were not detected in the fingerprint of the native iodoprotein in case 7, they were present in the fingerprints of the fractions of this iodoprotein which were eluted from DEAE cellulose at the lower ionic strengths.

In cases 5 and 6, although the fingerprint of the SCM iodoprotein closely resembled that of the normal SCM protein, at least 20 peptides were missing. The overall similarity of the physical and/

and chemical properties of this iodoprotein and the normal iodoprotein does not suggest the existence of an abnormality in the primary structure of the protein expected by the omission of 20 peptides. Since the iodine contents of these proteins are very low compared to the other iodoprotein studies, it seems reasonable that these differences might also be related to the heterogeneity of the tyrosyl residues with regard to iodination.

Cases 11 and 12 are discussed separately because, as mentioned earlier, both gland extracts contained iodoproteins which migrated electrophoretically like serum albumins. In case 11, the results of the peptide fingerprints of the iodoproteins are best discussed in relation to the other properties of the iodoproteins; 1. The ease of dissociation of the iodoprotein eluted in the exclusion volume from Sephadex to subunits which behaved electrophoretically like serum albumin and had a sedimentation constant of 6S. 2. The low hexose and sialic acid content of this iodoprotein. 3. The similarity between the fingerprints and the SCM iodoprotein and that of native normal thyroglobulin. 4. The elution of iodoproteins, which resembled serum albumin electrophoretically, from DEAE cellulose between 0.25 M - 2.0 M NaCl. 5. The general similarity between the peptide fingerprints of these iodoproteins and the native iodoprotein/

iodoprotein and the omission of the groupG peptides from the fingerprints of the fractions eluted between 0.25 M - 0.30 M NaCl. It is difficult to find a single explanation which covers all the abnormalities encountered in the iodoproteins from this gland. However, the results of peptide fingerprinting does suggest that there is a structural relationship between these iodoproteins and normal thyroglobulin. The studies of Edelhoch (1965) which provided evidence that thyroglobulin consists of 2 dissimilar 6S subunits and that reduction of the protein is essential to release these components could provide a possible explanation. If it is postulated that in this gland one of the 6S subunits is structurally abnormal then the iodoprotein isolated from gel filtration could represent an artifactual aggregation of the normal subunits. If disulphide linkages are normally only formed between dissimilar 6S subunits, an abnormality in one of the subunits could prevent this occurring and result in the case of dissociation of the iodoprotein purified by gel filtration. The absence of the other subunit in the aggregate could explain the low carbohydrate content and the absence of over 30 peptides from the peptide pattern of the SCM iodoprotein. The fact that the group G peptides were present in all peptide fingerprints except those of the iodo albumins eluted between 0.25 M - 0.30 M NaCl from DEAE cellulose suggest that these fractions might be the abnormal subunit. The iodination of subunits is not unknown/

unknown since Salvatore, Salvatore and Roche (1967) demonstrated the iodination of a 5S subunit in the lamprey. They postulated that under conditions where there is a slow rate of polymerisation of the subunits iodination of the subunit could occur.

In case 12, while two iodoproteins were also eluted from G200 Sephadex, the iodoprotein eluted in the void volume was stable and resembled normal 19S thyroglobulin in electrophoretic properties, sedimentation constant and carbohydrate content. Furthermore, the peptide fingerprint of the SCM iodoprotein suggested that this iodoprotein had the same primary structure as normal 19S thyroglobulin. Therefore it would appear that, in this case, a normal thyroglobulin molecule is synthesised. However, by DEAE cellulose chromatography of the gland extract, in addition to the normal thyroglobulin, iodoproteins with electrophoretic mobilities similar to serum albumin and pre-albumin were eluted. By sucrose density gradient centrifugation, the iodo albumin and the iodo pre-albumin had sedimentation constants in the rage 5 - 6S and 3 - 4S respectively. Since the peptide fingerprints of these iodoproteins closely resembled those of the native thyroglobulin of the gland, a structural relationship would seem to exist between these iodoproteins. A possible explanation is that they are iodinated subunits of a normal thyroglobulin which was particularly/

particularly labile under the conditions of isolation. However, it has been established that subunit aggregation occurs immediately after protein synthesis during thyroglobulin formation and that reduction of disulphide linkages is essential to release the 6S subunits. Furthermore, the existence of what appears to be a normal thyroglobulin species in the gland and the iodine contents of this protein and the iodo albumin suggests that the normal mechanisms which are considered to confer stability to the iodo-proteins are potentially available in the gland. The alternative is that the subunits per se are iodinated. This then raises the question why some subunits should aggregate to form normal thyroglobulin while others do not. If, however, as explained for case 11, there are 2 dissimilar 6S subunits involved in the thyroglobulin formation, it is possible that normally there exists a balanced synthesis of both subunits and that this balance is maintained by some mechanism of protein synthesis control. It is not inconceivable that, if this were the case, a lesion in the control mechanism could result in either an increased or a decreased synthesis of one of the subunits compared to the normal. This would result in the excessive synthesis of one of the subunits and lead to its accumulation since the aggregation of the subunits to form thyroglobulin would then be limited by the rate of synthesis of/

of the other subunit. This subunit could then be iodinated and result in the formation of the iodo albumins. The iodo pre-albumin could then be formed by the dissociation of the more highly iodinated 6S subunits under the conditions of isolation.

SUMMARY

1. Iodoproteins were purified from the thyroid glands of 12 patients presented with established goitres.

2. In 8 of the cases, the iodoproteins were indistinguishable from normal thyroglobulin in electrophoretic properties, sedimentation constants and carbohydrate contents although the iodine content of the proteins was, in general, lower than that recorded for normal thyroglobulin. In two cases of Hashimoto's thyroiditis, the hexose content of the iodoprotein was elevated and the sedimentation constant was in the range 20 - 21S.

3. The heterogeneity of the purified iodoproteins in 10 of the cases was examined by DEAE cellulose chromaography. In non-toxic goitre the heterogeneity of the iodoproteins resembled that of normal thyroglobulin whereas in thyroid carcinoma and Hashimoto's thyroiditis the iodoproteins were considerably more homogeneous.

4. Peptide fingerprints of the native and SCM iodoproteins showed that in all cases the iodoproteins had similar primary structures as the normal thyroglobulin preparation. The differences which were encountered in the fingerprints of the iodoproteins in 7 cases could be attributed to the absence of the 27S iodoprotein in these glands and to the existence of a heterogeneity of the tyrosyl residues with regard to the extent of iodination.

5./

5. In two cases abnormalities were detected in the iodoproteins isolated. In one case, in addition to a normal thyroglobulin, an iodinated albumin and pre-albumin were isolated. However, a structural similarity between those iodoproteins and normal thyroglobulin was revealed by peptide fingerprinting and it is suggested that they represent iodinated subunits of normal thyroglobulin. In the other case, a high molecular weight iodoprotein was isolated which, although related to normal thyroglobulin, had only half the normal carbohydrate content and readily dissociated under conditions of isolation to smaller subunits. An iodo albumin was also isolated from this gland which produced a peptide fingerprint significantly different from normal thyroglobulin. It is suggested that in this gland an abnormal subunit is synthesised which prevents normal aggregation and stabilisation mechanisms occurring.

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