LIPOPROTEIN IN ASSOCIATION WITH THYROGLOBULIN

by

JOHN M BALLANY

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

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CHAPTER 1

INTRODUCTION

HISTORICAL

The thyroid was first recognised as a secretory organ by King (1) in 1836, who studied the gland's anatomy and vascular system and submitted the viscous fluid which could be expressed from it to chemical tests. By the end of the 19th century it was realised that the thyroid gland produced some substance necessary for good health. (2) Baumann in 1896 found that a protein fraction from the thyroid gland contained considerable amounts of iodine. This fraction on hydrolysis was effective in the relief of symptoms of myxoedema. Purification of this iodine containing constituent was not achieved until 1915 when Kendall (3+4) isolated a crystalline product containing 65% iodine, which was named Thyroxine (T_4). Improvements to Kendall's methods (5) led to the synthesis and elucidation of the chemical structure of thyroxine and so the nature of the thyroid hormone was seemingly established.

However, Gross and Pitt-Rivers (6) found a compound with three iodine atoms, Triiodothyronine (T_3), both in the thyroid gland and in plasma. This compound proved to be quicker acting on the metabolism than the four iodine atomed thyroxine and also was effective in the relief of myxoedema. Both thyroxine and triiodothyronine act to regulate the general metabolic state of the body.

Another type of hormone found in the thyroid is the hormone calcitonin, which acts as a calcium lowering agent. (7)

The function of the thyroid gland thus appears to be the synthesis, storage and secretion of its unique hormones, controlled by thyroid stimulating hormone (TSH) from the anterior pituitary. An outline of the biochemistry of the thyroid gland, with particular reference to the subject being investigated, namely the lipoprotein associated with thyroglobulin, will be given in the following chapters.

FUNCTIONAL ANATOMY OF THYROID GLAND

The thyroid gland consists of an aggregation of follicles (Fig 1), each made up of a single layer of epithelial cells surrounding a central lumen which contains an amorphous, viscous fluid known as the colloid. The follicular cells possess a definite polarity, their apices directed toward the lumen of the follicle and their bases toward the basement membrane, the height of these cells being determined by the state of activity of the thyroid. The thyroid cell nucleus is large and usually situated in a basal position. In the normal human adult the size of the follicles varies from 50 to 500μ in diameter. The follicular cells are the site of hormone synthesis, the colloid representing the inert storage form of the hormone.

On electron microscopy, the apical end of the cell has an unusual irregular border which appears to be composed of microvilli which are believed to play a part in the secretion of the thyroid hormones into the colloid. These microvilli could also play a role in the resorption of the colloid from the follicles. The cytoplasm of the thyroid cell contains mitochondria, granules or vescicles thought to represent colloid (thyroglobulin) resorption particles, and a variety of organelles./

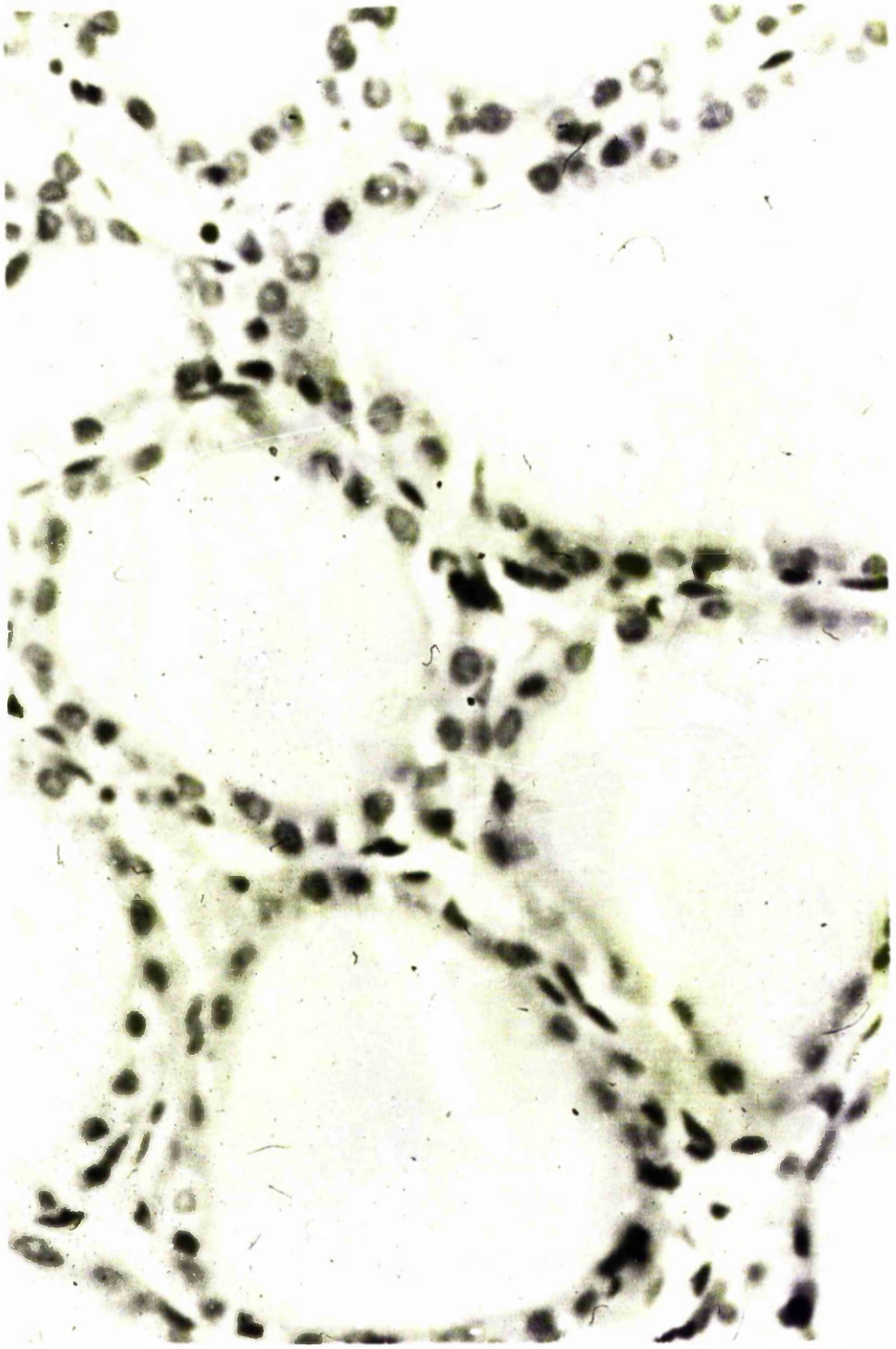


Fig. 1. Section through normal rat thyroid gland showing colloid filled follicles (x 40), stained with Haematoxylin and Eosin.

/organelles. There is an extensive network of endoplastic reticulum and prominent Golgi apparatus involved in the synthesis and secretion functions of the cell. Unlike exocrine cells which secrete into the body cavity, thyroid cells not only secrete into the colloid but resorb from it and release the thyroxine and triiodothyronine into the perifollicular capillaries.

SYNTHESIS OF THE THYROID HORMONES

The process whereby iodine is concentrated can be divided into two stages, iodine transport and iodine organification. The trapping of iodine by the thyroid from the capillary blood into the follicular cell occurs by two processes, namely by diffusion and by transport.(7A)* Diffusion is an almost negligible method of iodine entry into the thyroid gland, active transport is therefore the more important mechanism. Iodine is transported into the thyroid against an electrochemical gradient (8) and this process is dependent on oxidative phosphorylation. This active transport mechanism accumulates 25 to 100 times the concentration of iodine in the thyroid than found in the serum.

The details of iodine organification are still mainly speculative although the conversion of trapped iodine is probably an oxidative process carried out by the thyroid peroxidase enzyme system. This "activated" or "charged" iodine ion is then bound to tyrosine molecules in the primary structure of thyroglobulin. These iodinated tyrosine residues then couple together to form T4 and T3. The latter could be derived from the joining of monoiodotyrosine and diiodotyrosine molecules or by deiodination of T4. As can be deduced from this organification process the synthesis of the thyroglobulin skeleton is one of the most/

*(7A) Textbook of Endocrinology, 5th Edition, Edited by R.H. Williams, Published by W.B. Saunders Company, p.102 (1974)

/most important steps in the formation of the thyroid hormones. It has been suggested that the protein moeity of thyroglobulin is synthesised on the ribosome studded membrane, then moves in the follicular cells through the endoplastic reticulum, the Golgi vesicles, apical vesicles into the colloid.

When T3 and T4 are required for metabolism, stored thyroglobulin is resorbed from the colloid by a pinocytosis-like process and is hydrolysed probably by thyroid proteases, thus releasing the thyroid hormones. The iodotyrosines released are de-iodinated by specific enzymes and their iodine recycled for further use by the gland. A schematic representation of the iodine metabolism pathway in the thyroid is shown in fig. 2. The iodine released from the breakdown of iodotyrosines however appears to be handled differently from newly trapped iodine as it is not easily discharged from the thyroid by substances such as thiocyanate or perchlorate. This iodine has been referred to as the second iodine pool of the thyroid.

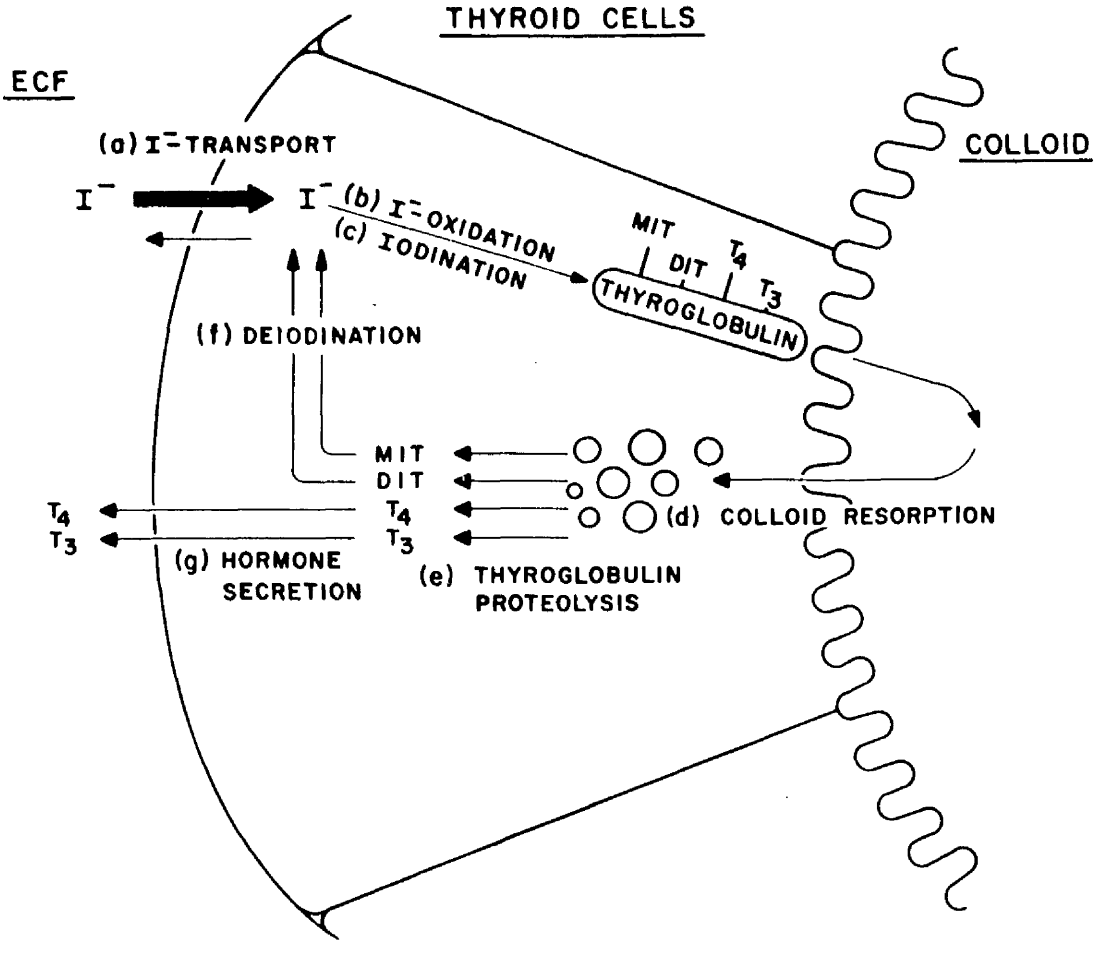


Fig. 2. Iodine Metabolism Pathways in the Thyroid.

a) Active iodide transport through basal membrane.
b) Iodide oxidation within the cell.
c) Iodination of thyroglobulin.
d) Resorption of stored thyroglobulin from the colloid.
e) Hydrolysis of thyroglobulin to release iodotyrosines and iodothyronines.
f) Deiodination of iodotyrosines and recycling of released iodine through the iodide pool of the thyroid.
g) Discharge of iodothyronines into the extra cellular fluid.

After Werner "The Thyroid" 3rd Edition.

CHAPTER 2

THYROGLOBULIN BIOSYNTHESIS & CHEMISTRY

Thyroglobulin biosynthesis can be sub-divided into three major stages: (a) synthesis and assembly of polypeptide chains, (b) carbohydrate attachment; and (c) iodination and maturation of the molecule. Current beliefs are that these three processes are independent and occur sequentially in the thyroid.

Polypeptide synthesis in the thyroid has been studied by incorporating ^{14}C or ^{3}H-labelled amino-acids into proteins *in vitro* and *in vivo*. Sheep thyroid slices incubated with radio-labelled amino-acids and analysis of the subsequently labelled proteins by means of sucrose density gradient ultracentrifugation (9-10) showed that most of the radioactivity was firstly associated with a 12S component corresponding to a structural sub-unit of thyroglobulin. 3-8S proteins, which may possess single polypeptide chains of the thyroglobulin molecule were also labelled at this stage. Further incubation with unlabelled amino-acids showed radioactivity accumulating in the region of 19S thyroglobulin with concomitant decreases in 12S label. (11). It was thus concluded from these kinetic studies that 12S protein and presumably 3-8S proteins are precursors of thyroglobulin, that is to say thyroglobulin is synthesised from structural sub-units. Newly synthesised thyroglobulin, however, possesses some different properties from older, more mature thyroglobulin. These differences are found mainly in slightly, but difinitely, lower sedimentation coefficients. for/

for example, newly synthesised sheep thyroglobulin sediments at 17-18S and rat thyroglobulin at 16-16.8S compared to 19S for mature thyroglobulin from these animals. (15).

Thyroglobulin biosynthesis can be summarised as follows:-

n(3-8S) ⟶ 2(12S) ⟶ non-iodinated thyroglobulin

non-iodinated thyroglobulin + iodine ⟶ matured, iodinated thyroglobulin.

n represents the unknown number of polypeptide chains comprising thyroglobulin.

In vivo incorporation experiments (12) confirm that 12S and presumably 3-8S proteins are precursors of thyroglobulin, but the possibility of the 12S component being a dissociation product of newly formed thyroglobulin cannot be entirely ruled out. The incorporation of amino-acids and conversion of low molecular weight proteins into thyroglobulin appears to be controlled by TSH since injection of this material into rats immediately enhances conversion of 12S protein into 18-19S thyroglobulin.

Carbohydrate Incorporation

Thyroglobulin contains 8-10% carbohydrate comprising two, or possibly three different types of unit. (13).

Calf thyroid slices incubated with ^{14}C-labelled glucose (13) showed that radioactivity was incorporated into five sugar components of both particulate bound protein and soluble thyroglobulin. On solubilisation of the particulate bound protein using deoxycholate, it was proved from its chemical, immunochemical and electrophoretic properties to be a precursor of thyroglobulin. Thyroid particulate fractions could also incorporate radioactivity from ^{14}C-labelled glucose into sugars in the particulate-bound thyroglobulin-like protein, which suggests/

/suggests carbohydrate synthesis of the thyroglobulin molecule occurs at a particulate site. Incubations containing puromycin, using both thyroid slices and particulate fractions, reveal that carbohydrate incorporation occurs independently of and later than the synthesis of the polypeptide portion of the molecule. This is proven by the fact that incorporation of radioactivity into soluble thyroglobulin did not decrease even though amino-acid incorporation was completely inhibited by puromycin. Particulate bound thyroglobulin precursors, however, showed partial impairement of carbohydrate incorporation in the presence of puromycin; the more internally located sugars being affected to a greater degree than those externally located. This variable degree of inhibition suggests that carbohydrate incorporation is accomplished in a stepwise manner by the addition of one sugar molecule at a time to the enlarging carbohydrate chain.

A difference in mechanism between the incorporation of mannose and galactose was reported by Herscovics (14). Mannose appeared to be incorporated in a manner and time course similar to that of leucine in that only the 12S and 3-8S fractions were labelled at early times after incubation. On the other hand galactose was incorporated into 19S thyroglobulin directly. These differences in time of incorporation provide further evidence for stepwise attachment of the monosaccharide molecules. It should also be noted that thyroglobulin newly labelled with ^{14}C carbohydrate had a lower sedimentation coefficient (17-18S) than that of mature thyroglobulin (19S). Carbohydrate incorporation is not impaired by propylthiouracil which totally inhibits iodine uptake and so indicates that carbohydrate attachment is independant of/

/ independant of iodination. Since little if any carbohydrate radioactivity was observed in the polysomes it was concluded that carbohydrate incorporation was independent of polypeptide chain synthesis.

Iodination

After completion of polypeptide and carbohydrate moieties of thyroglobulin, iodination of tyrosyl residues and subsequent formation of T_3 and T_4 takes place within the matrix of the molecule. Iodination proceeds in a stepwise fashion, the degree of iodination being variable depending on the physiological state of the thyroid gland. In vitro cell free systems show that whole homogenates and various subcellular fractions incorporate iodine into thyroid protein at least to the stage of monoiodotyrosine (MIT). This incorporation of iodine into protein may not necessarily be an enzymatic process since other proteins such as albumin and casein can be non-enzymatically iodinated. Seed and Goldberg (15) incorporated ^{125}I into a thyroglobulin-like protein which sedimented at 18S which is slightly lower than native thyroglobulin. No light-weight proteins were found to be iodinated and further work involving the use of propylthiouracil showed that this compound completely abolished iodination of thyroglobulin. Taking this lighter than normal 18S protein and iodinating chemically transforms it into a true 19S thyroglobulin. In addition to the in vitro evidence Thomson and Goldberg (12) showed by in vivo studies in the rat that iodine is incorporated into a protein which sediments at 18S; light-weight proteins were only minimally iodinated if iodinated at all.

Chemistry of Thyroglobulin/

Chemistry of Thyroglobulin

Thyroglobulin is an iodoprotein of molecular weight of the order of 660,000-670,000 (16) and axial ratio 10.

Variation of pH on thyroglobulin has produced interesting data on the ultracentrifugal pattern of the protein. (16) 19S thyroglobulin was the major protein up to pH9.5, a 12S protein being found to a certain extent at this pH. Above pH9.5, 15S and 12S protein components were found, the 12S protein being the major constituent. It was postulated that the 12S protein accounted for half the molecular size of the 19S protein, which was in keeping with the fact that the process was reversible up to pH11. At this pH and above an 8S protein predominated and at pH12 and above all protein was present in a 3-4S form.

The effect of heating on native thyroglobulin was studied by Edelhoch and Metzger (17), who found that thyroglobulin denatured measureably at room temperature and pH11.3. At neutral pH, high temperatures are required for denaturation to commence; the process being aided by neutral salts and alkaline pH. The above mentioned workers also showed that at pH 9.5 and 53°C, 19S thyroglobulin dissociated to produce 12S and 17S proteins, which denatured immediately since their solubility (it was shown later) was lost above pH5. At temperatures below 53°C, these two products were soluble at pH5. The loss of solubility was found by various methods to be due to minor configurational changes.

Protein denaturants such as sodium dodecyl sulphate (SDS), guanidine and urea rupture protein non-covalent bonds, both intra and/

/and inter molecularly. At low concentrations SDS (<0.001M) a 12S component was produced from native 19S thyroglobulin. This process was shown to be reversible on dialysis of the SDS from the protein solution in a manner similar to pH induced changes. Increasing concentrations of SDS leads to progressive unfolding of both 19S and 12S components to forms which behave as random chain polymers. Urea was found to behave in a similar manner, but at no concentration of urea or SDS was transformation from 19S to 12S found to be complete. This would suggest that at neutral pH a fraction of 19S thyroglobulin remains resistant to dissociation.

Reduction of disulphide linkages, which can be inter or intra chain, can result in changes of molecular size or in changes of shape. The extent of reduction (18) is a function of pH, reductant concentration, solvent and reduction time. Beta mercaptoethanol (BME) is a substance which has the property of reducing disulphide bonds. Starting with a 12S thyroglobulin preparation and reducing with BME two equal sized components sedimenting at approximately 6S result. On prolonged dialysis, reconstitution of the reduced products ensues and 12S and even 19S proteins can be restored. This restored protein is found to be more resistant to reduction with BME than native 19S thyroglobulin. From this and other data it has been suggested that 19S thyroglobulin is composed of two non-covalently bound components of 12S size.

Study of 27S iodoprotein (approximately twice the molecular weight of 19S protein) resulted in not only 19S but also 12S and 3-8S components being formed, which suggests that 27S iodoprotein/

/iodoprotein is made up of a complex aggregation of 19S thyroglobulin and sub-units.

Recently there has been some dispute concerning the theory that 19S thyroglobulin is formed by the coupling of two 12S sub-units which in turn is formed from two 6S units. On fractionation of the labelled 3-8S peak from the rat thyroid (19), three components were found, namely 3S, 6S and 7S proteins. The latter two were found to be homogeneous ultra-centrifugally by polyacrylamide gel electrophoresis and precipitable with antithyroglobulin antibodies. More detailed studies (20) show that smaller sub-units than 19S and 12S account for approximately 20% of thyroglobulin. These smaller polypeptides range in size from molecular weight of 20,000 to 250,000. Incorporation of these sub-units into thyroglobulin gives rise to an interesting number of possibilities (20+21).

It has been shown (22) that the carbohydrate component accounts for approximately 10% of the thyroglobulin molecule. This carbohydrate moiety consists of galactose, mannose, fucose, N-acetylglucosamine and sialic acid. Two distinct glycopeptides account for these carbohydrates: glycopeptide A with molecular weight 1,050 consists of five mannose residues to one residue of N acetylglucosamine; glycopeptide B with molecular weight 3,200 contains mannose, N-acetylglucosamine, galactose, fucose and sialic acid in the ratio 3:5:4:1:2. Thyroglobulin has been shown to possess 9 moles A and 14 moles B/mole protein.

From amino-acid analysis it appears that the amino-acid, aspartic acid is most probably the one involved in the glycopeptide linkage./

/linkage. Sialic acid, being the easiest of the sugars to hydrolyze from the molecule, would appear to be the terminal sugar in the glycopeptide. More recent studies have produced results (23+24) which showed that an additional unit (unit C) was possibly present in human thyroglobulin. Unit C was composed of galactosamine residues occuring as an integral part of the protein, and completely distinct from units A and B. These galactosamine units being linked to serine and threonine residues.

The iodine content of thyroglobulin has been shown to vary considerably. Diethylaminoethyl (DEAE) cellulose chromatography fractionates thyroglobulin into three fractions dependent on their iodine content, the earlier eluting fraction containing less iodine than the later eluting fractions and the starting material. All three fractions had identical MIT contents but differed in DIT content and to a lesser extent T_4. Iodination of the starting material (in vitro) resulted in the disappearance of the early eluting fraction and an increased, higher iodinated, later eluting fraction. Further evidence of differences in iodine content can be obtained using the ultracentrifuge, since poorly iodinated thyroglobulin sediments with an "S" value less than 19S, for example, 17-18S. Thus it would appear that thyroglobulin represents a mixture of molecules which differ only in the relative proportions of their constituent iodo-amino acids.

CHAPTER 3

REVIEW OF PUBLISHED WORK ON PURIFICATION OF THYROID PROTEINS AND THYROLIPIDS

Since this work arises mainly from observations made during purification of thyroid proteins and since lipid products are to be described, a review of purification procedures and lipids contained in the thyroid gland would seem appropriate.

PURIFICATION PROCEDURES APPLIED TO THYROID PROTEINS

For many years it was thought that only one kind of iodoprotein, thyroglobulin, was contained in the thyroid, but several iodoproteins (24-A)* both soluble and particulate-bound exist in the thyroid although usually to a much lesser degree. This necessarily leads to the term thyroglobulin having to be defined. Robbins and Rall (25) recommended in 1960 that a soluble iodoprotein of thyroid origin must possess the following properties to be named thyroglobulin:-

(a) sedimentation coefficient (S_{20W}) of approximately 19S;

(b) electrophoretic mobility in veronal buffer, pH8.6 of approximately $-5 \times 10^{-5} cm^2/s.$ V; and (c) a sharp insolubility at about 38% ammonium sulphate. The most important discriminatory property is the sedimentation coefficient. It must also be noted that this definition only denotes a family of related molecules and not a single molecular species.

Thyroglobulin is almost exclusively found in the intrafollicular colloid, amounting to 20-30% concentration of this medium, and so may be extracted fairly readily by slicing thyroid glands thinly (or/

*(24A) Textbook of Endocrinology, 5th Edition, Edited by R.H. Williams, Published by W.B. Saunders Company, p.107 (1974)

/(or homogenising) and suspending in cold neutral saline. Early purification procedures were described by Harrington (26) but later work by Heidelberger and Palmer (27) and Cavett et al (28) showed that ammonium sulphate precipitation was an extremely useful method. This salting out method was extensively studied by Derrien et al (29) who recommended fractional precipitation between 37 and 43% saturation with ammonium sulphate at pH6.5 and 22°C. However, an equimolar mixture of 3.5M monopotassium and dipotassium phosphate at pH6.5 and 20°C at 43-48% saturation provided similar results. Although these authors claim homogeneity both by ultracentrifugation and electrophoretically, salting out does not remove all contaminating proteins. Shulman and Witebsky (30) using narrower ammonium sulphate limits (39.3-41.7% saturation) showed that, "fairly concentrated solutions" (0.5%), ultracentrifugally consisted of one principal component (95%), although the yield was low.

Thyroglobulin prepared by salting out is heavily contaminated by proteolytic enzymes and so Ui and Tarutani (31) recommended fractional precipitation in the cold with between 1.50 and 1.80M ammonium sulphate at pH6.8 followed by reprecipitation between 1.55 and 1.75M.

Further purification can be achieved by differential centrifugation (32) which produces highly purified 19S thyroglobulin after multiple runs, however, the yield is fairly low. Sucrose density-gradient centrifugation (33) gives complete separation in good yield but the amount that can be processed is limited, and hence is suitable for small scale analytical purposes only. /

/only.

Gel filtration fractionates protein mixtures according to size and has proved useful in the purification of thyroidal iodoproteins. Perelmutter et al (34) have reported a simple purification procedure for thyroglobulin using Sephadex G200. After two passages an ultracentrifugally homogeneous fraction was obtained. Again limited (34) amounts of pure 19S thyroglobulin are recovered owing to poor separation from 27S iodoprotein. The use of agar or agarose gel, such as Sepharose 4B or 6B, gives better separation and is both suitable for small and large-scale preparations, but combined use of other fractionation procedures is necessary for isolating a highly purified sample of 19S thyroglobulin. DEAE-cellulose chromatography can be used. Ingbar et al (35) showed that sheep thyroglobulin prepared by salting out gave an asymetrical elution curve when gradient elution was used with a DEAE-cellulose column. At constant pH(6.5) thyroglobulin and other related iodoproteins are eluted from DEAE-cellulose at higher ionic strength than that of contaminating proteins, (36+37) hence a preparation of thyroglobulin along with small amounts of 27S and other iodoproteins but free from non iodoproteins, can easily be obtained by stepwise elution. Since 27S and other heavier thyroglobulin related iodoproteins are bound more strongly to DEAE-cellulose than most of 19S thyroglobulin, an ultracentrifugally pure thyroglobulin may be obtained by elution at lower ionic strength than that necessary for complete elution of all related molecules. Karlsson et al (38) combined pretreatment with sephadex G200 and DEAE-cellulose chromatography to obtain pure samples of both pig and human thyroglobulin. DEAE-cellulose other than/

/than providing a vehicle for the production of pure thyroglobulin. samples is also extremely useful in subfractionation of 19S thyroglobulin.

Thyroid lipids

The follicular epithelium and the colloid of the thyroid gland possess an abundance of material with an affinity for lipid stains, and lipids have been extracted in large amounts from thyroid tissue (39). The chemical nature of these lipids has not yet been fully investigated but it has been suggested that they may act as iodide carriers, or serve as substrates and regulators of thyroid metabolism (40+41). Normal thyroid slices actively oxidise free fatty acids even in the presence of glucose, which becomes the preferred substrate only in the presence of TSH.

Iodine uptake by the thyroid gland may be due to a specific thyroidal lecithin fraction which binds iodine reversibly in vitro (42). These active binding lipids were found to be unsaturated lecithins or choline plasmalogens and the inactivity of soy bean and calf brain lecithins shows that anion binding is more than a trivial property of these lipids. This also suggests that these thyroidal lecithins may be involved in anion transport within the gland. Further evidence for this "carrier" or transport role is the fact that phosphatidic acid turnover is increased when transport of secretion were stimulated.

Nervonic acid is found in appreciable amounts in iodide-complexing samples but not in lecithins which do not possess this property. The affinity of inactive thyro-lysolecithin for iodide can be restored/

/restored by reattachment of nervonic acid into the/β position and so it is suggested that α'-acyl-/β-nervonic-L-lecithin is the structure responsible for iodide-complexing (43). The exact nature of the iodide-lecithin linkage is not known, but appears to be a loose one probably depending on the micellar state of lecithin and so no enzymatic activity or energy is needed for the complex to form. The significance of this system is dependent on the fact that the specific lecithin was only found in significant quantities in the thyroid gland and so the first step in iodide uptake, that is, from the blood to the thyroid cell may be explained by this system. Posner and Ordonez (44) showed that lipid-iodide binding is ionic in nature and that iodine is bound co-valently to lipid.

Neutral glycosphingolipids have been identified as minor constituents of the lipid fraction of most mammalian tissues, these compounds being most concentrated in the plasma membrane of the cell and so may play some role in membrane functions. The membrane of the thyroid cell may be the locus of the iodine trapping mechanism. The lipids of the thyroid gland have been shown (45) to consist of neutral glycosphingolipids and sulphatides. The pattern of human thyroid glycolipids differs considerably from that of other non-nervous human tissues. There is, however, some similarity between human thyroid and kidney in that glycosphingolipid content is similar although total glycolipid content in the thyroid is reduced. This may indicate that the role played by these lipids is common for the two tissues, namely active ion transport.

Other lipids such as triglyceride, free fatty acids and/

/and cholesterol, present in the thyroid have been studied by Lipshaw and Foa (46), both under normal conditions and after treatment with TSH for various periods of time. These workers suggest that after treatment with TSH the triglyceride content of the thyroid increased because the TSH-induced lipolysis increased the amount of free fatty acid available for esterification. During this initial phase of TSH stimulation there was a generalized increase in the turnover of all lipid moieties, except cholesterol, and fatty acid synthesis was accelerated. Glucose serves as a primary oxidative substrate and provides glycerol for fatty acid esterification. Prolonged TSH treatment shows fatty acid synthesis is still elevated, but overtaken by fatty acid catabolism. Acetyl-CoA produced in this manner is converted to cholesterol and it is suggested that this cholesterol production is acting as an irreversible trap for acetyl-CoA, which may minimise the inhibitory effect of this substance on glycolysis and fatty acid biosynthesis and so act as a metabolic regulator in the thyroid.

The current interest being shown in the lipids of the thyroid and purification procedures prompted the present work which was designed to elucidate and characterise a lipid fraction found on attempted fractionation and purification of the thyroid proteins.

SECTION 2

MATERIALS AND METHODS

CHAPTER 4 .. .. MATERIALS

CHAPTER 5 .. .. DIETARY REGIMES

CHAPTER 6 .. .. EXPERIMENTAL TECHNIQUES

CHAPTER 4

MATERIALS

The materials and suppliers are as follows:-

MATERIALS	SUPPLIERS
Ampholines	LKB Ltd., South Croyden, Surrey.
Acrylamide	British Drug Houses Ltd., Poole.
N,N,N'N' - tetramethyl-ethylenediamine (TEMED)	L. Light & Co. Ltd., Colnbrook.
Sucrose	British Drug Houses
β mercaptoethanol (BME)	British Drug Houses
Sodium dodecyl sulphate (SDS)	British Drug Houses
Propylthiouracil (PTU)	Koch-Light Ltd., Poole.
Potassium iodide	British Drug Houses
Male Sprague-Dawley rats	A. Tuck & Sons, Rayleigh, Essex.
Sepharose 6B	Pharmacia Ltd., London.
Sephadex G200	Pharmacia Ltd., London
Ammonium persulphate	British Drug H ouses
N',N' - methylenebis - acrylamide	Kodak Ltd., London.
Agarose	British Drug Houses
Ammonium sulphate	British Drug Houses
Iodine-125) Iodine-131)	The Radiochemical Centre, Amersham.
Thyroglobulin (Porcine)	Sigma Ltd., London.

MATERIALS	SUPPLIERS
Oil Red O	George Gurr Ltd., London.
Sudan Black	
Coomassie Blue	
Pyronin	
Toluidine Blue	
Tris (Hydroxymethyl)-aminomethane (Trizma base)	Sigma Ltd.

CHAPTER 5

DIETARY REGIMES

Two basic diets were fed to the rats throughout the course of the study.

Control Rats

Oxoid Diet 41B from Herbert C. Styles (Bewdley) Ltd. Tapwater to drink.

Experimental Rats

The experimental diet fed to the rats throughout the course of this study was the low iodine test diet (LID), powdered form, supplied by the Nutritional Biochemical Corporation, Cleveland, Ohio, U.S.A. Drinking water for all LID fed groups, unless otherwise stated was distilled water.

SUPPLEMENTS TO BASIC DIETS

P.T.U. Supplementation

Hypo-Iodine P.T.U. Group

LID was supplemented by the addition of 0.2g P.T.U./kg diet, distilled water was given to drink.

Hyper-Iodine P.T.U. Group

Oxiod 41B was supplemented by the addition of 0.2g P.T.U./kg diet, the drinking water was 0.05%KI solution.

Normal-Iodine P.T.U. Group

As a control diet for the above two groups oxoid 41B was supplemented by the addition of 0.2g P.T.U./kg diet, tap water was given to drink.

The above diets were fed for 28 days before sacrifice.

CHAPTER 6

EXPERIMENTAL TECHNIQUES

In this section a description of various techniques employed routinely is given. However, various methods used only to clarify certain aspects of the work are not included, but are described, where relevant, in the results section.

Purification of Thyroglobulin

Thyroid glands from freshly slaughtered sheep were brought from the local abattoir in plastic bags on ice contained in a vacuum flask. The glands were carefully dissected from fibro-fatty tissue and placed in ice-cold phosphate buffered saline (PBS) (0.15M sodium chloride in 0.01M potassium phosphate, pH6.8) contained in a boro-silicate glass MSE Homogeniser mortar resting on a bed of ice. The thyroids were then homogenised in PBS (5mls PBS/g wet weight thyroid) using an MSE Tissue Homogeniser until homogenisation was complete (approximately 3 minutes at maximum revolutions). The homogenised glands were placed in the refrigerator overnight to facilitate extraction of the thyroglobulin. At the end of the extraction period the crude thyroglobulin containing homogenate was filtered through glass wool to trap most of the extracted tissue, the filtrate being centifuged at 20,000 g for 10 minutes, in a Beckman Spinco Model L refrigerated ultracentifuge, to spin down any other contaminating thyroid tissue.

Purification of the thyroglobulin containing supernatant was accomplished by 70% ammonium sulphate precipitation and centrifugation/

/centrifugation (5,000rpm for 15 minutes, MSE Super Medium Centrifuge), the resulting precipitate being redissolved in PBS. This procedure was repeated six times to give a reasonably pure iodoprotein sample (mainly 19S thyroglobulin). The purification procedure was carried out at room temperature.

Rat thyroglobulin was prepared by the same procedure, except that the male Sprague-Dawley rats (120-150g) were sacrificed by ether anaesthesia, the thyroid glands being quickly excised and placed in ice-cold PBS before being freed from fibro-fatty tissue.

Purified sheep thyroglobulin was stored at $4^{o}C$ after freeze-drying using an Edwards Speedivac pump and a Quickfit freeze drying apparatus.

Electrophoretic Methods

Agarose Gel Electrophoresis

1% agarose gel was prepared in 200ml batches by heating 2g agarose gel granules in 200mls Barbital buffer pH8.6 and 0.05 ionic strength (I), in a constant boiling water bath. The agarose solution was stirred and measured into test-tubes (15mls/tube) and allowed to cool and set before being stored in the refrigerator at $4^{o}C$. This method ensures that each gel is identical to every other from the same batch. Before electrophoresis was commenced 15mls of agarose gel was re-melted in a water bath and poured evenly (1mm thick) over 6 glass microscope slides contained in a heat resistant plastic holder placed on a levelling table. The six gels were allowed to cool and set, dust and other contaminants being excluded from the gels by a/

/a stainless steel hood.

2.5mm diameter sample wells were cut in the gels (two in each gel at mid-point of microscope slide) and the plastic holder placed in a Shandon electrophoresis system containing a water cooled compartment which prevented the gels overheating on electrophoresis. The samples to be electrophoresed were then added to the wells at a concentration of 1mg/ml, sufficient volume being added to just fill the well. This method allowed six different gels to run simultaneously. Filter paper wicks previously soaked in barbital buffer pH 8.6, 0.05I, completed electrical contact between gels, buffer and electrodes.

The gels were electrophoresed at 150v. for one hour before staining overnight with Amido Black stain (1% amido black in 5% glacial acetic acid). This stain also fixes the proteins in the gel and thus prevents leaching. Excess stain was removed from the gels by frequent washes of 5% acetic acid.

Electrofocussing

The equipment used was as follows:-

LKB	8102	440ml Ampholine column
LKB	3371	Power Supply
LKB	8301A	Flow Analyser, control unit
LKB	8303A	Flow Analyser, detector unit
LKB	10200	Perpex Pump
LKB	8121	Gradient Mixer

The ampholine column was assembled, the cooling water turned on and the procedure for filling the column begun. Dense electrode/

/electrode solution was prepared by dissolving 0.2mls concentrated sulphuric acid and 12.0g sucrose in 14.0mls distilled water. This electrode solution was then pumped carefully into the central tube of the column and so became the anode. The column outlet was opened to remove trapped air and closed when the electrode solution emerged. The meniscus of the anode solution was positioned at least 20mm above the lower end of the central column tube.

The dense solution for the density gradient was prepared by diluting three-quarters (9mls) of the carrier ampholytes (pH 3.5-10 or pH 4-6 dependent on range required) to 150mls with distilled water and dissolving 100g sucrose in this solution. The light solution was prepared by diluting one-quarter (3mls) of the carrier ampholytes to 215mls with distilled water and dissolving the sample to be focussed in this solution. The density gradient was prepared by means of a gradient mixer, the dense solution being poured into the vessel containing an electrically driven stirrer and the light solution into the vessel containing a perspex conical density compensator. 210mls of solution were added to each vessel. The outlet tube from the gradient mixer was attached to a pump and thence into the electrofocussing compartment of the column so that the end of the tubing reached 2-3cm below the compartment inlet. The gradient mixer was started by connecting to the mains and the clamp between the light and dense solution vessels removed as was the clamp on the outlet tube, the liquid being pumped smoothly down the inner wall of the electrofocussing compartment (flow rate not more than 4ml/min). The density/

/density gradient should not reach the upper electrode but if this is the case then some was sucked out.

Light electrode solution was prepared by dissolving 0.4g sodium hydroxide in 40.0mls distilled water and this solution was pumped on to the top of the density gradient to form the cathode. The cooling water and the power supply were then switched on and the voltage set at 500v (maximum power at beginning did not exceed 4-6W). Voltage value at end of run was usually 600-1,000v and the run lasted for 48-72 hours.

The column was emptied by sucking off the electrode solution in the central tube to decrease the risk of mixing. The outlet was opened and the column contents forced out by pumping water onto the top of the column, which subsequently filled the column with water. A flow rate of 1-2ml/min was used and 2ml fractions collected after the effluent had been analysed by a flow analyser linked to a recorder. The pH values of the collected fractions were obtained by using an MIL model 23A pH meter, the samples being read at the same temperature as they were collected. Care was taken that fractions were not exposed to carbon dioxide from the air and so all tubes were stoppered immediately on collection.

Polyacrylamide Gel Electrophoresis

Stock Solutions

(1) Tris buffer solution

36.3g Trizma base, 48.0mls 1N HCl, 0.46ml TEMED, distilled water to 100mls.

(2) Acrylamide solution/

/ (2) Acrylamide solution

30.0g Acrylamide, 0.8g N',N', methylenebisacrylamide, distilled water to 100mls.

(3) Initiator

0.14g Ammonium persulphate, distilled water to 100mls.

Eight running tubes (7.5cms x 0.5cms internal diameter) with one end stoppered were set up vertically and gel solution prepared by mixing - 3mls tris buffer, 6mls acrylamide solution, 12mls initiator and 3mls distilled water. This gel solution was deaerated for approximately one minute using a vacuum pump. The running tubes were then filled with gel solution to within 1.5cm of the top, the gel solution being overlayered with 0.5cms distilled water and polymerisation allowed to proceed. After polymerisation was complete (approximately 45 minutes) the water was removed from the top of the gels, the stoppers from the base and the running tubes were set up in the electrophoresis apparatus (Shandon Disc Electrophoresis system). The gels so produced were at a concentration of 7.5%.

Reservoir buffer was prepared by dissolving 28.8g glycine and 6.0g Trizma base in distilled water, adjusting the pH to pH8.5 and making up to one litre with distilled water. Approximately 50µl of electrophoresis sample in either glycerol or sucrose solution was applied to the top of the gel, one drop 0.001% bromo-phenol blue added as an indicator, and reservoir buffer layered carefully on top of the samples. The buffer compartments were filled with buffer and the power supply connected with the positive electrode to the bottom chamber and the negative to the top chamber. Electrophoresis was/

/was carried out at 5mA/tube, that is, 40mA total current for approximately 90 minutes or until the bromophenol blue band had migrated nearly to the end of the gel. The gels were removed from their running tubes by forcing 5% acetic acid from a syringe with a narrow guage needle between the gel and the tube wall and staining was accomplished with Coomassie brilliant blue (0.5% in7% acetic acid) stain for four hours. Destaining was carried out by frequent washings of 7% acetic acid.

SDS and EME Polyacrylamide Gel Electrophoresis

This method was utilised to help determine molecular weights of proteins and their constituent chains. Similar experiments were set up as described above but a 9% gel was used, which was prepared from the following solutions:-

(1) Acrylamide Gel

20.0g acrylamide and 0.06g N',N', methylenebisacrylamide made up to 100mls with distilled water.

(2) 1.5g Ammonium persulphate made up to 100mls with distilled water.

(3) N,N,N',N', - tetramethylenediamine.

(4) Electrophoresis Buffer:

6.82g $NaH_2PO_4 \cdot 2H_2O$
25.8g $Na_2HPO_4 \cdot 2H_2O$
2.0g Sodium dodecy sulphate (SDS) pH7.5

} per litre

This buffer was diluted 1:1 with distilled water before being/

/being used as the reservoir buffer.

The gel was prepared by mixing 13.5mls solution (1), 15mls solution (4), 0.045mls solution (3) and 1.5mls solution (2). The mixture was deaerated using a vacuum pump and the solution quickly transferred into the electrophoresis running tubes (polymerisation occurs in approximately 20 minutes). The gels were then treated exactly as described previously.

Preparation of Samples for Electrophoresis

Samples of protein at 1.0mg/ml protein concentration were incubated for two hours at 37°C in 0.01M phosphate buffer, pH7.0, with SDS and BME at a final concentration of 1% each. Before applying the 20µl samples to the gels the samples were made more dense by the addition of sucrose or glycerol, and one drop bromophenol blue added as an indicator.

Sucrose Density Gradient Ultracentrifugation

Approximately 0.1-0.4mls of protein solution containing 2-4mg protein was applied to a 12ml, 5-20% linear sucrose density gradient in PBS made using a Beckman gradient former. Gradients were then centrifuged for 16 hours at 28,000 rpm using an SW 41 Ti rotor in a Beckman model L2-65B ultracentrifuge.

After ultracentrifugation the optical density at 280nm of the gradient was recorded with an automatic absorbance recorder (Gilford 2000) by suction from the bottom of the ultracentrifuge tube; 15 drop fractions were collected. The ultracentrifugal pattern of the thyroid iodoproteins was thus obtained. Other thyroid/

/thyroid iodoprotein ultracentrifugation patterns were obtained using 0-5% and 20-40% sucrose density gradients.

Column Chromatography

Sephadex G200 gel, was allowed to swell in excess solvent and was left to stand for three days. During swelling excessive stirring was avoided as rupture of the gel beads may be caused. For all gel types the swelling stage may be accelerated by placing the gel slurry on a boiling water bath. This method of swelling avoids the problem of trapped air bubbles in the gel and was found to be more satisfactory.

The glass column (Pharmacia type K26/100) was mounted vertically and the dead space under the gel supporting net and the tubing were filled with eluant to remove any trapped air bubbles and the outlet closed. The gel, previously swollen and deaerated was allowed to settle and excess eluant decanted to leave a fairly thick slurry, which was carefully poured down the inner wall of the column. Since the column was not completely filled with slurry eluant was added until the column was completely full and an eluant reservoir was connected to the column. Trapped air was removed by bleeding through an air vent in the column top piece. The flow was started immediately after filling the column in order to obtain an even sedimentation of the gel. Two or three column volumes of eluant were passed through the column to stabilize and equilibrate the gel bed, the same flow rate being used as that for experimental work , (2mls/6mins), and the operating pressure was not allowed to vary from the range 4-16cms H_2O.

As/

/As a check on the homogeneity of the gel bed a 2mg/ml solution of Blue Dextran 2000 was run through the column and a sharply defined band giving sharp peaks indicated that the column was suitable for use.

Sample application was achieved by sucking off most of the eluant above the gel surface and then opening the column outlet to drain the remaining eluant away. After closing the column outlet the sample was carefully layered on top of the gel bed; the column outlet was then opened and the sample allowed to drain into the gel bed. The gel surface and column wall in contact with the sample were washed with a small amount of eluant which was then allowed to drain into the gel bed. The column was filled with eluant and connected to the eluant reservoir. Fractions (2ml) were collected and analysed using an LKB Ultrorac. and flow analyser system.

Sephadex G200 has an exclusion limit of 800,000 for peptides and globular proteins and so it was necessary to chromatograph thyroglobulin in Sepharose 6B, which has an exclusion limit of 4×10^6 for proteins, to obtain more detailed separations. The Sepharose 6B columns were packed as described for Sephadex G200, the only difference being that Sepharose gels are supplied as dense suspensions of swollen beads in distilled water, and hence only dilution of the pre-swollen slurry was necessary before column packing.

Estimation of T_3 Resin Uptake and T_4 in Experimental Animal's Serum

Both T_3 Resin Uptake and T_4 levels in experimental animal's sera were determined using the methods of Oxford Laboratories, Foster City, California, U.S.A.

T_3 Resin Uptake Estimation/

/T_3 Resin Uptake Estimation

The vial assemblies containing the reagent system and control sera were removed from the refrigerator and allowed to come to room temperature before testing was begun. The vial caps were removed from the counting vials and 0.2mls of serum sample (or standard) was pipetted into the vial and subsequently diluted with 3.0mls distilled water. The caps containing the test reagents were placed on the counting vials which were shaken until the reagents were uniformly suspended. Incubation was allowed to proceed for a minimum of 10 minutes. Slight variations in room temperature have little or no effect on the test results, and incubation may proceed for up to two hours with no effect on the results. The vial assemblies were shaken and inverted so that the cap was at the bottom before being centrifuged at 5,000 rpm for 10 minutes. When the vials were removed from the centrifuge they were turned over immediately so that the precipitate in the cap was at the top and remixing was avoided. The radioactivity in the liquid portion was counted in a Packard Selektronik Model A5142-C1 gamma counter set for ^{125}I, care being taken that radioactivity in the precipitate was not being counted. A minimum of 10,000 counts/minute should be recorded for each sample, the background being subtracted automatically. Results were calculated as follows:-

$$\text{Sta } T_3 \text{ index} = \frac{\text{reference serum cpm}}{\text{net test cpm}} \times \frac{1}{\text{reference serum value}}$$

T_4 Estimation /

/ T_4 Estimation

0.2mls of serum (or standard) were dispensed into a suitably labelled vial. The Sta T_4 Silica Extractant was vigourously shaken before 3 mls were dispensed into the vials. Each vial was capped and the vial assemblies placed in a centrifuge (cap uppermost), care being taken not to shake the vials end-to-end or cause the suspension to splatter up into the cap. The vials were centrifuged at 1,500rpm for five minutes to lightly pack the silica. After centrifugation the vials were inverted as each was removed from the centrifuge: this procedure prevented resuspending the silica. The vials were removed from the caps which were discarded along with their contents. The magnesium carbonate absorbent was vigourously shaken before 3 mls were dispensed into each vial containing the lightly packed silica. Each vial was capped with a clean cap and shaken to resuspend the silica. The assemblies were allowed to stand for 10-20 minutes at room temperature before being shaken briefly and centrifuged (5,000rpm for 10 minutes) to pack the precipitate into the caps. The vials were inverted and the liquid phase counted in a Packard Selektronik Model A5142-01 gamma counter set for ^{125}I. The counter was programmed to read out the time necessary for 20,000 counts and the results obtained from a standard graph of time (seconds) versus concentration T_4 (nm/l).

SECTION 3

RESULTS

CHAPTER 7 .. .. ASSESSMENT OF HOMOGENEITY OF SAMPLES

CHAPTER 8 .. .. IDENTIFICATION OF PEAK 1

CHAPTER 9 .. .. STUDY OF POSSIBLE ARTEFACTS PRODUCING PEAK 1

CHAPTER 10 .. .. ELECTROPHORESIS AND ELECTROFOCUSSING OF PEAKS 1 AND 2

CHAPTER 7

ASSESSMENT OF HOMOGENEITY OF SAMPLES

Thyroglobulin was prepared by ammonium sulphate precipitation as previously described, and gel filtration carried out with Sephadex G200 and Sepharose 6B, as a check on purity.

(a) Sephadex G200 Chromatography

A Pharmacia K 26/100 (100 x 2.6cms internal diameter) glass column was set up and Sephadex G200 gel slurry added to give a bed height of 95.0cms. After equilibration of the column with three column volumes of eluant (PBS, pH6.8), 10 mls of thyroglobulin solution, in PBS, concentration 1mg/ml, was applied to the top of the column. The flow rate was adjusted to 2mls/6mins by slowing the rate of escape of eluant from the column with an LKB Perpex pump. By using the pump as a brake, packing of the column bed was prevented and so column life enhanced. Two ml fractions were collected.

The separation produced (Fig 3), gave an almost symmetrical peak at fraction 46, which indicated a fairly high degree of purity. It was, however, noted that this peak was found extremely near to the void volume of the column (Blue Dextran 2,000 eluted at fraction 42) and so the sample may not have penetrated the gel sufficiently to separate it into its constituents. It was thus decided to use another gel type with a higher exclusion limit than Sephadex G200, and so an identical column was prepared using Sepharose 6B, again to check on sample purity.

(b) Sepharose 6B Chromatography /

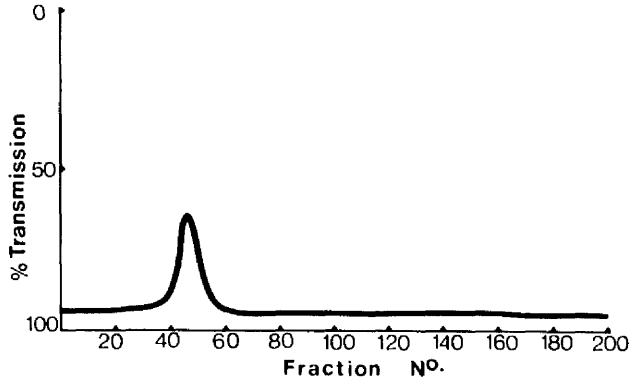


Fig. 3. Elution pattern of sheep thyroglobulin from a sephadex G200 colu..n, (bed height)5.0 cms., 2.6 cms. diameter), eluted with PBS buffer, pH 6.8.

(b) Sepharose 6B Chromatography

The separation produced (Fig 4) gave an assymmetrical elution pattern which contained three main peaks in accordance with the literature (47). This study was limited to the first two main peaks, which were designated Peak 1 and Peak 2. The latter will be subsequently shown to be 19S thyroglobulin.

As assymmetry can be taken to indicate non-heterogeneity, it was assumed that the sample passed through the 6B column was impure, so the fractions containing peak 1 were pooled, dialysed overnight against distilled water to remove buffer salts and freeze dried, before dissolving in PBS and again passing through the Sepharose 6B column. This passage produced a single symmetrical peak at the same position as in the first passage through the column, that is, fraction 60. On repeating this procedure with the pooled fractions containing peak 2 a similar result was produced, in that a single symmetrical peak was found in the position of peak 2 on the first passage, that is, fraction 100. It would appear from these results that this procedure was a reasonable method for producing a fairly pure sample of peak 1 and peak 2, free from contaminants of each other or other lighter weight proteins (the third main peak), and so this method was adopted for the production of stocks of freeze dried samples of peak 1 and peak 2 to be used for further study.

The void volume of the Sepharose 6B column was found to be 116mls (fraction 58) by the use of Blue Dextran 2000, and so peak 1 was just being retarded by the gel in the column since its elution volume was 120mls (fraction 60), which also indicated that peak 1 was a large/

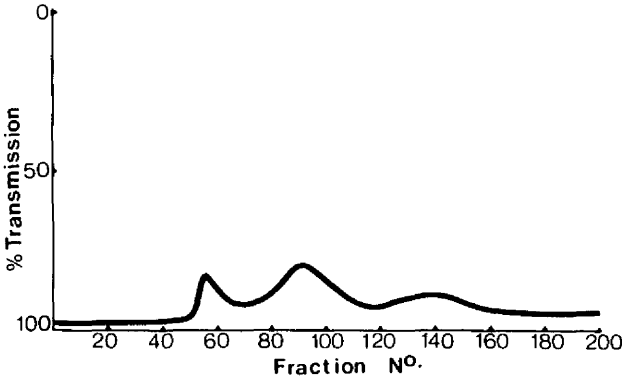


Fig. 4. Elution pattern of sheep thyroglobulin from a sepharose 6B column, (bed height 95.0 cms., 2.6 cms. diameter), eluted with PBS buffer, ph 6.3.

/large molecular weight species.

According to the literature (47) the separation pattern obtained with Sepharose 6B was that of pure thyroglobulin which raised the question, whether peak 1 was an aggregation of 19S thyroglobulin or a completely different molecule, present due to an incomplete purification procedure.

On passing a sample of commercially available thyroglobulin (Porcine thyroglobulin) through the Sepharose 6B column (Fig 5) an identical separation pattern was found, which, although not excluding the possibility of incomplete purification, lent support to the hypothesis that peak 1 was something other than thyroglobulin. Further study into the possibility of peak 1 being produced by an aggregation effect of peak 2 was then carried out.

(c) Absorbance Spectrum Analysis

The spectrum (Fig 6) of a 1mg/ml solution of peak 1 from 230nm to 300nm was measured using a Gilford 2000 absorbance spectrophotometer to determine whether peak 1 CONSISTED of protein, RNA or carbohydrate and the spectrum of peak 2 measured in the same manner was compared with peak 1. The spectrum of peak 2 was that of a protein solution with an absorbance peak at 280nm, whereas that of peak 1 appeared to be of something other than protein, which indicated that this molecule may not be an aggregation of 19S thyroglobulin. This simple test virtually ruled out the possibility of peak 1 being 27S aggregation of thyroglobulin, however, a 32S aggregation still remained a possibility as previous work in the department has suggested that/

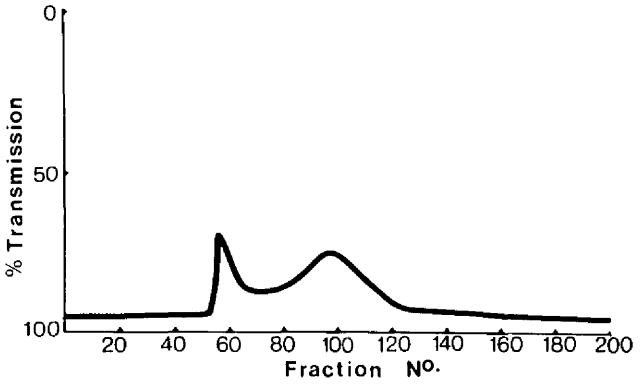


Fig. 5. Elution pattern of commercially available thyroglobulin (Porcine thyroglobulin), from a sepharose 6B column, (bed height 95.0 cms., 2.6 cms. diameter), eluted with PBS buffer, pH 6.8.

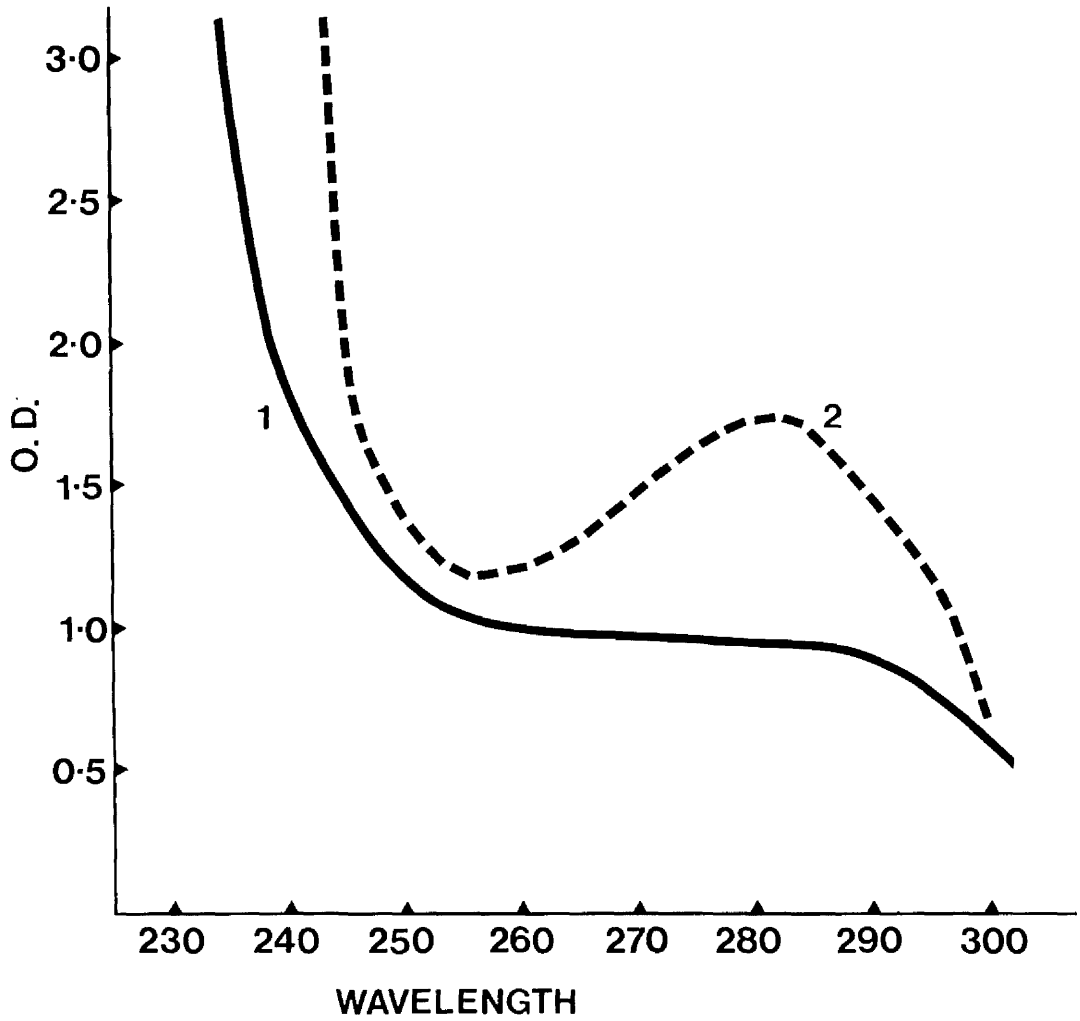


Fig. 6. Absorbance Spectrum between wavelengths 230 nm and 300 nm of a 1 mg/ml solution of each of peaks 1 and 2.

Solid line 1 refers to peak 1.
Dotted line 2 refers to peak 2.

/that 32S thyroglobulin is composed of a mixture of protein and RNA therefore a 1mg/ml solution of peak 1 was treated with absolute ethyl alcohol to precipitate the RNA/protein complex if one did indeed exist. RNA was extracted with 10% sodium chloride solution and the spectrum again measured, with the same result as previously, namely a broad plateau between 260-290nm. This result excluded the possibility of RNA contributing to this pattern: since if any RNA was present a strong absorbance peak at 260nm would be found. The plateau effect must therefore be produced by some other molecule than RNA.

(d) Agarose Gel Electrophoresis

To try to clarify this situation agarose gel electrophoresis was carried out on samples of both peak 1 and peak 2, (both at a concentration of 1mg/ml), using a 1% gel in barbital buffer pH8.6, 0.05I for one hour at 150V. Two staining regimes were used on the gels, namely one set of gels were stained for the detection of protein and another set of gels being stained for the presence of RNA. The protein stains used were amido black (1% in 5% acetic acid) and coomassie brilliant blue (0.5% in 7% acetic acid). Both stains showed that peak 1 migrated slightly further towards the anode than peak 2 (Fig 7) and so must be slightly more electronegatively charged than peak 2. The gels stained for RNA (toluidine blue 1% in 5% acetic acid or pyronin 1% in 5% acetic acid) proved to be negative, and so it appeared that no RNA was present in either peak 1 or peak 2. Appropriate controls were run along with the test samples, namely albumin and an RNA sample from/

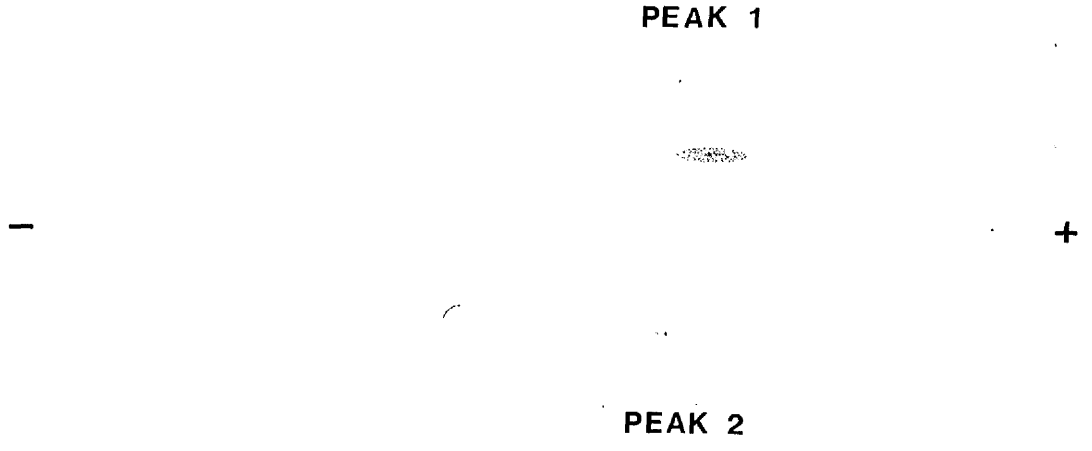


Fig. 7. Agarose gel electrophoresis of peaks 1 and 2 (1 mg/ml solutions of each) using a 1% gel and barbital buffer pH 8.6, 0.05I for one hour at 150 V.

/from rat brain, both of which stained with the appropriate stain. The electrophoresis experiments were repeated using polyacrylamide gels as previously described, the staining regimes used were identical to those in the agarose gel experiments. The results obtained were again negative for RNA but positive for protein which again indicated that peak 1 was not a 32S aggregation molecule of protein and RNA.

(e) Comparison with 32S Peak from PTU Treated Rat Thyroid

As a further check that peak 1 was not a 32S aggregation molecule an experiment to prepare a 32S thyroid protein sample was set up. Rats were fed a diet containing 200mg PTU/Kg diet, for 28 days before their thyroids were excised and the thyroglobulin extracted and purified as previously described. PTU enhances the production of 32S thyroid (47A)* protein and so after passage through the Sepharose 6B column a sample of 32S thyroid protein was obtained. This sample's spectrum (200-350nm) was compared with the spectrum of a sample of 19S thyroglobulin (also from rat). This showed that the spectrum of 32S thyroglobulin was completely different from that of peak 1, since 32S thyroglobulin possessed a peak at approximately 260nm. This is similar to that found by Thomson and Bissett (1970) (Fig 8).

(f) Sucrose Density Gradient Ultracentrifugation

Sucrose density gradients were prepared (5-20% sucrose in PBS) to elucidate the "S" values of peak 1 and peak 2. A few drops of each sample solution were carefully dropped on to the surface of a 5% sucrose solution in PBS contained in a test tube to check that the sample solutions were not denser than the top of the gradient. This procedure eliminated the possibility of the sample sinking in the gradient before ultracentrifugation began. A small volume of sample solution was then/

*(47A) Thomson J.A. Ph.D. Thesis pp.38-41.

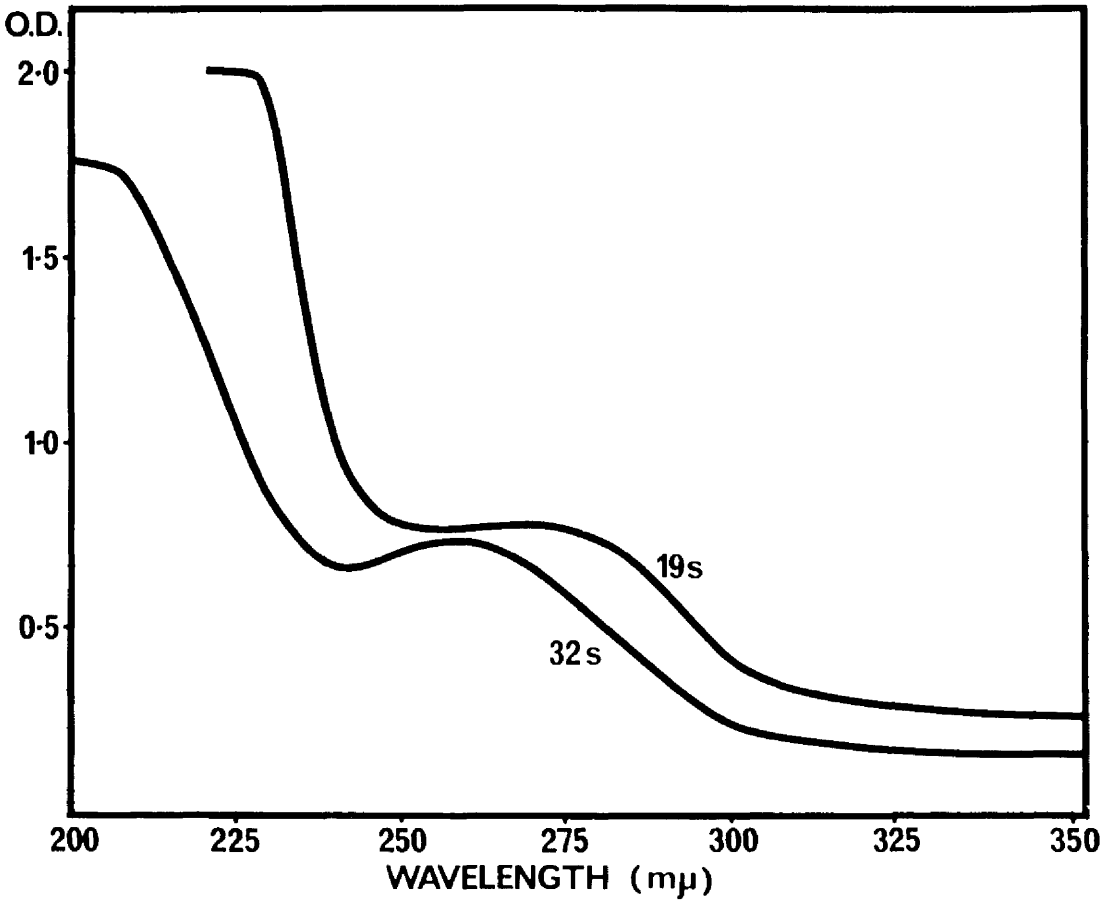
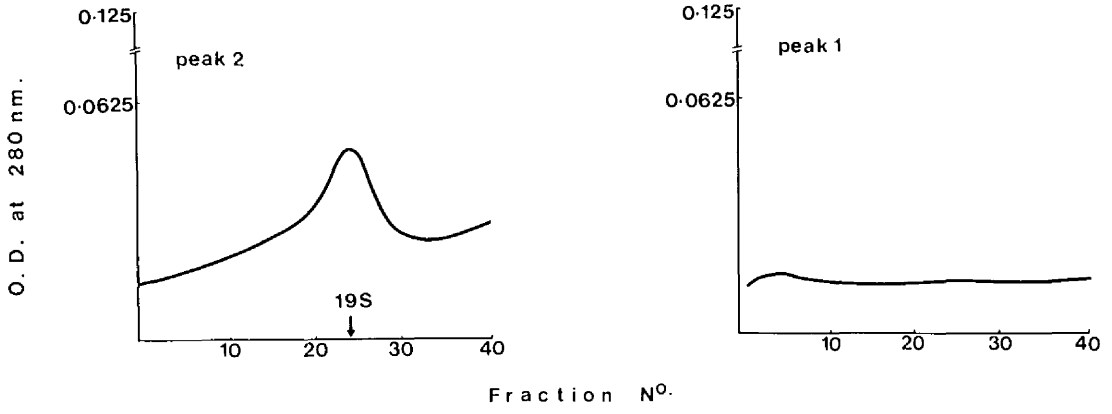


Fig. 8. Absorbance Spectrum of 19S thyroglobulin and 32S thyroid protein between wavelengths 200 mμ and 350 mμ

From Thomson J.A., Bisset J.M. Acta Endocrinologica 65 175 (1970)

/then floated on top of the sucrose density gradients and the samples spun at 28,000rpm for 16 hours using an SW 41 Ti rotor in a Backman Model L2-65B ultracentrifuge. The temperature throughout the ultracentrifugal run was maintained at 23°C (room temperature). At the end of the run the gradients were removed from the centrifuge and carefully placed in a rack to avoid mixing of the gradient. The ultracentrifugal pattern was determined at 280nm with a Gilford 2000 absorbance spectrophotometer which gave an automatic print out on a chart recorder. Peak 1 did not appear at any point on the 5-20% sucrose gradient, but peak 2 sedimented in a position expected of 19S thyroglobulin; no 27S peak was found (Fig 9). These results gave the initial impression that peak 1 most probably sedimented to a greater density than that allowed for in the gradient, so the experiment was repeated (Fig 10) using 20-40% sucrose density gradients, (length of spin, speed and temperature constant). Again peak 1 was not detectable at any point on the gradient, peak 2 was not seen as it would be expected to remain at the top of the gradient. This led to the conclusion that peak 1 was possibly lighter than peak 2 and has been sedimenting at a position near to the top of the 5-20% gradient and so was not being shown on the absorbance trace. If this was correct peak 1 would only float on top of the 20-40% gradient and so would be lost on the chart print out.

Further gradients (Fig 11) were prepared using less dense sucrose solutions so that the final gradient was 2-10%. The samples of peak 1 and peak 2 were then layered on top of these gradients and again spun in the ultracentrifuge. Yet again peak 1 was undetectable on the gradient but peak 2 was readily identified. No other sucrose density/

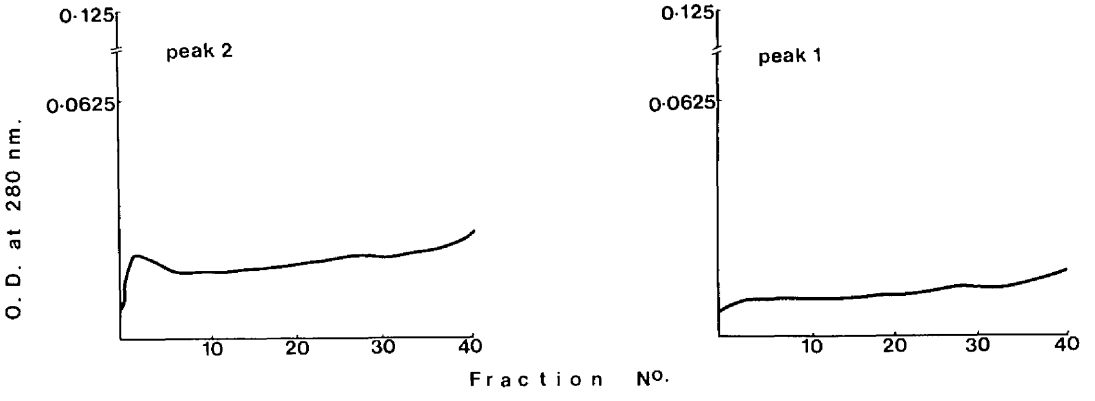


Gradient	5–20% sucrose solution
Speed	28×10^3 rpm
Temp	23°C
Length of run	16 hours

Fig. 9. Ultracentrifugation of peaks 1 and 2 in sucrose density gradients using an SW 41 Ti rotor in a Beckman model L2 - 65B ultracentrifuge.

The peak at the bottom of the gradient for peak 1 (between fractions 1 - 5) is an artefact produced by the entry of an air bubble to show the origin of the gradient.

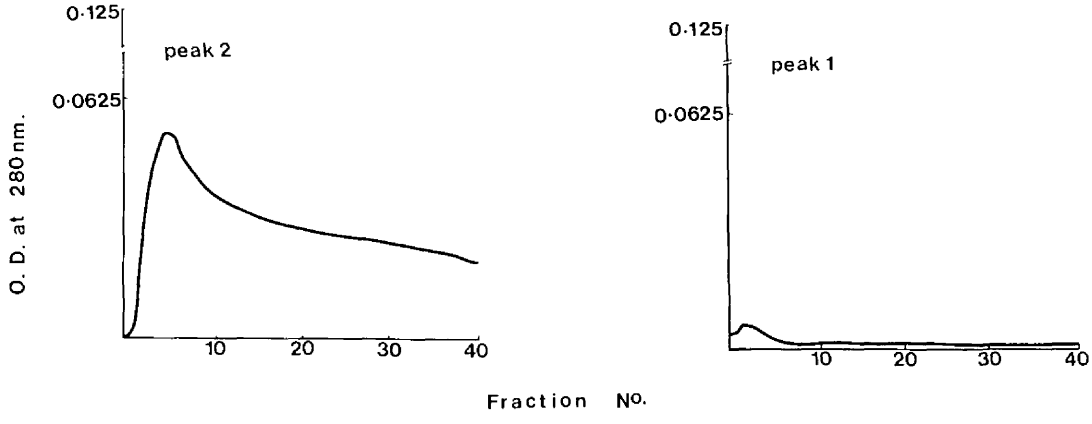
The bottom of the gradient is at the vertical axis.



Gradient	20 – 40 % sucrose solution
Speed	28×10^3 rpm
Temp	23^{o}C
Length of run	16 hours

Fig. 10. Ultracentrifugation of peaks 1 and 2 in sucrose density gradients using an Sw 41 Ti rotor in a Beckman model L2 - 65B ultracentrifuge.

The bottom of the gradient is at the vetical axis.



Gradient	2 – 10 % sucrose solution
Speed	28×10^3 rpm
Temp.	$23^{\circ}C$
Length of run	16 hours

Fig. 11. Ultracentrifugation of peak 1 and 2 in sucrose density gradients using an SW 41 Ti rotor in a Beckman model L2 - 65B ultracentrifuge.

The peak at the bottom of the gradient for peak 1 (between fraction 1 - 5) is an artefact produced by the entry of an air bubble to show the origin of the gradient.

The bottom of the gradient is at the vertical axis.

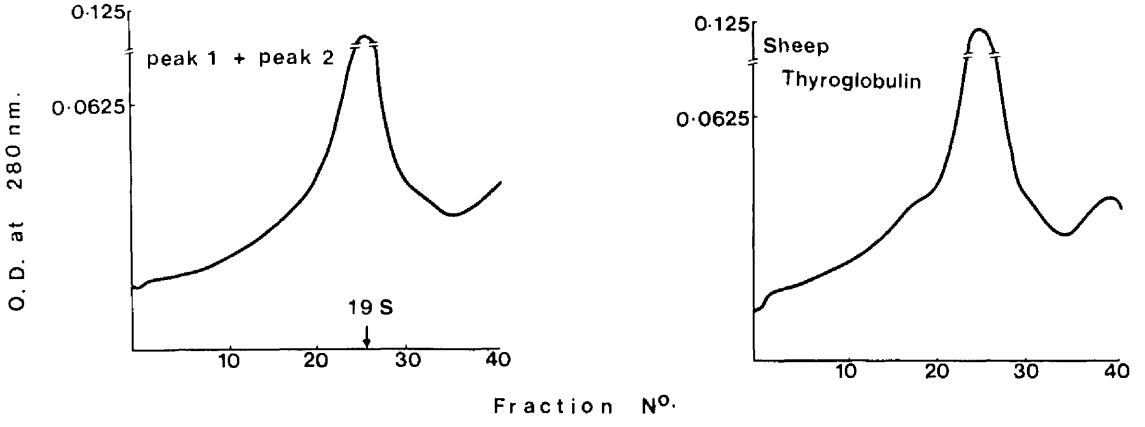
/density gradient ranges were possible since peak 1 would not layer on top of a gradient which was less than 2% sucrose.

5-20% sucrose density gradients were again prepared and samples of a 1:1 mixture of peak 1 and peak 2 layered on top, whole sheep thyroglobulin being run with these gradients as an internal control (Fig 12). All the gradients were spun as before and again no trace of peak 1 was found in either the mixture gradients or sheep thyroglobulin gradients. This showed that peak 2 did not exert any influence on peak 1, such as binding with peak 1 and so causing it to sediment in the gradient. Peak 2 was not affected by this admixture with peak 1 since it sedimented at the 19S position.

The only other factor which might have affected the sedimentation of peak 1 was the temperature of the gradients. To eliminate this factor 5-20% gradients containing peak 1 or peak 2 or peak 1 and peak 2 mixture and sheep thyroglobulin were spun at $4^{o}C$ (Fig 13) and compared with those spun at $23^{o}C$ (Fig 14). The gradients at $4^{o}C$ were found to be more viscous and so it was thought that peak 1 might sediment within the gradient, but on examination these gradients gave identical results to those at $23^{o}C$.

These sucrose density gradient results gave rise to the hypothesis that peak 1 must be a very low density molecule, which was simply floating or "sedimenting" on the top of the gradient, no matter what density range was being used. It has already been established from the results obtained from column chromatography (Sephadex G200, Sepharose 6B) that peak 1 was a large, high molecular weight molecule.

(g) Amino Acid Analysis /

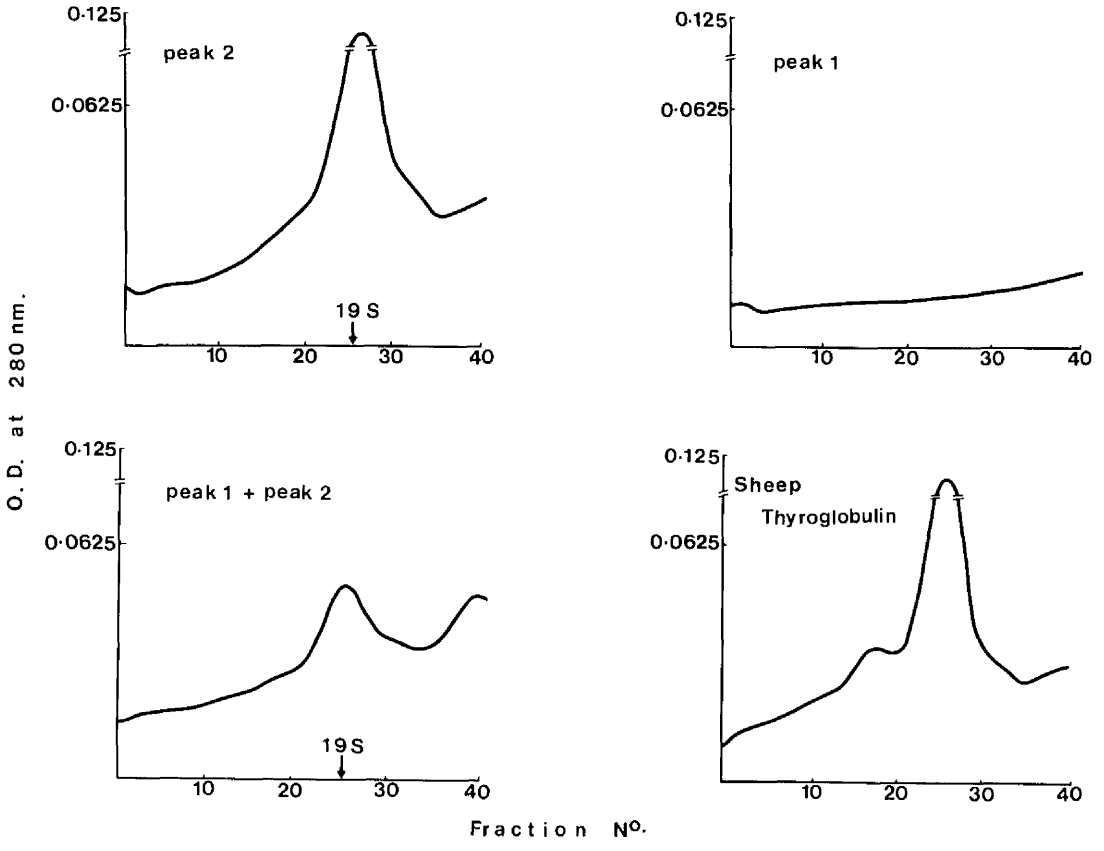


Gradient	5 – 20 % sucrose solution
Speed	28×10^3 rpm
Temp	23°C
Length of run	16 hours

Fig. 12. Ultracentrifugation of a 1:1 mixture of peaks 1 and 2 and whole sheep thyroglobulin in sucrose density gradients using an SW 41 Ti rotor in a Beckman model L2 -65B ultracentrifuge.

The shoulder found on the 19S peak of the whole sheep thyroglobulin gradient is in a position comparable to a 27S sedimenting molecule.

The bottom of the gradient is at the vertical axis.



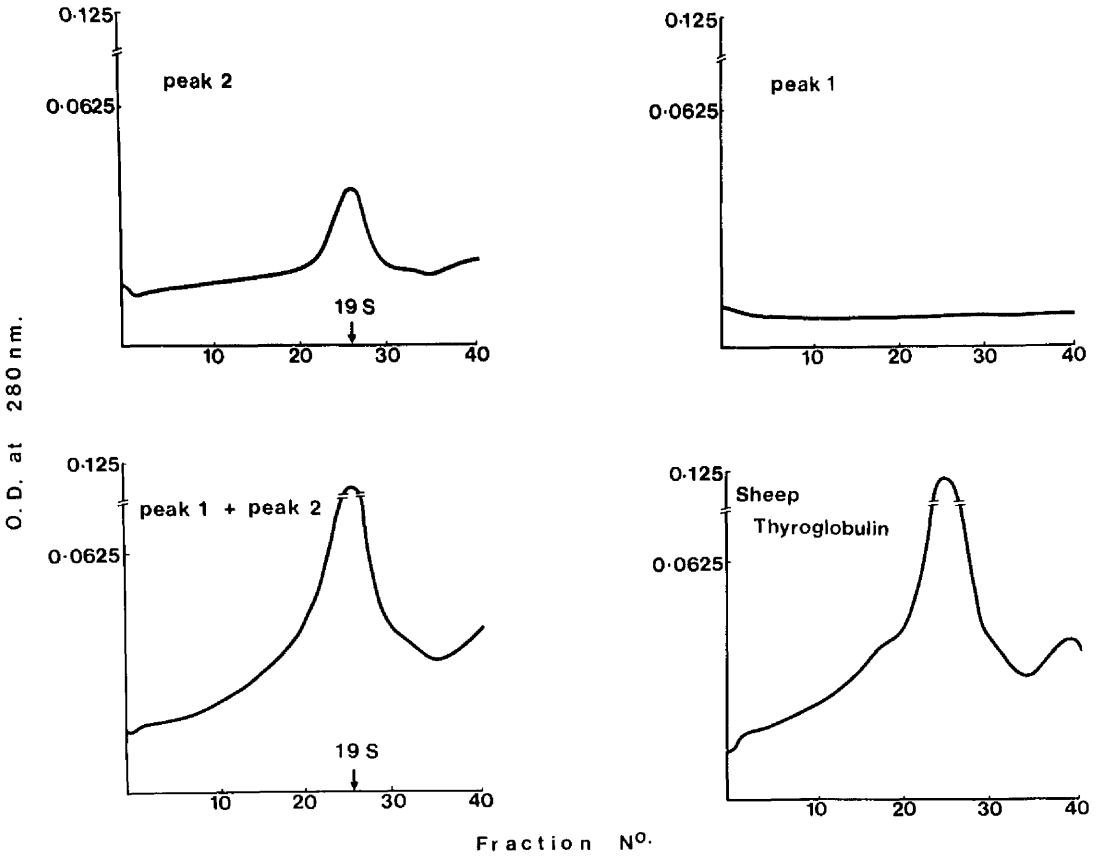
Gradient	5 – 20 % sucrose solution
Speed	28×10^3 rpm
Temp	4° C
Length of run	16 hours

Fig. 13. Ultracentrifugation of peak 1, peak 2, 1:1 mixture of peak 1 and peak 2 and sheep thyroglobulin in sucrose density gradients, to show any temperature effects. SW 41 Ti rotor, Beckman model L2 - 65B ultracentrifuge.

The shoulder found on the 19S peak of the sheep thyroglobulin gradient is in a position comparable to a 27S sedimenting molecule.

The peak at the bottom of the gradient for peak 1 is an artefact produced by the entry of an air bubble to show the origin of the gradient.

The bottom of the gradient is at the vertical axis.



Gradient	5 – 20 % sucrose solution
Speed	28×10^3 rpm
Temp	$23^{\circ}C$
Length of run	16 hours

Fig. 14. Ultracentrifugation of peak 1, peak 2, 1:1 mixture of peak 1 and peak 2 and sheep thyroglobulin in sucrose density gradients, to show any temperature effects. SW 41 Ti rotor, Beckman model L2 - 65B ultracentrifuge.

The shoulder found on the 19S peak of the sheep thyroglobulin gradient is in a position comparable to a 27S sedimenting molecule.

The peak at the bottom of the gradient for peak 1 is an artefact produced by the entry of an air bubble to show the origin of the gradient.

The bottom of the gradient is at the vertical axis.

(6) Amino Acid Analysis

Since peak 1 was identifiable with protein stains, but did not give a true "protein type" spectrum it was decided to carry out an amino acid analysis on both peak 1 and peak 2. By this means it was sought to verify peak 2 as being 19S thyroglobulin and to try to identify which molecular species peak 1 belonged.

Acid hydrolysis of freeze dried samples (5mg) of peak 1 and peak 2 using 3mls 6N hydrochloric acid was carried out in evacuated tubes at 110°C for 48 hours to break down the proteins into their constituent amino acids and so provide the starting material for subsequent analysis by Amino Acid Analyser using the method of Efron (52).

0.2-0.5ml of the acid hydrolysis samples pH2.2 was used for the complete determination of the acidic, neutral and basic amino acids. The sample was applied to a short water-jacketed column filled with ion-exchange resin (UR-40) by means of a micro-syringe which was then washed out with one drop of sodium citrate buffer, pH2.2.

The chromatographic conditions were that the initial column temperature was 30.5°C, but at 76 minutes elution time the temperature was raised to 60°C and maintained at this level until arginine was eluted (time gradient was eight minutes). Flow rates were:- 67ml/hour for the buffer, 43ml/hour for ninhydrin and the back pressure was not allowed to exceed 300psi.

Elution was started with sodium citrate buffer pH3.17, after 99 minutes the buffer pH was changed to pH4.24 and at 182 minutes the pH was again changed to pH4.15. The final buffer pH change was carried out at 323 minutes to pH5.36 (all buffers used being sodium citrate)./

/citrate). 27 minutes were required for the buffer pH changes to appear on the chart, colour development was observed at 570 and 440nm and the total time for complete analysis was 6 hours.

The results (Table 1) showed that peak 1 differs from peak 2 in amino acid content, the most noticible differences being that methionine and ϒ amino butyric acid were absent from peak 1 but present in peak 2. On closer comparison of the amino acid analysis of both proteins it was noted that four amino acids were present in approximately similar amounts in both peak 1 and peak 2 (Table 2), however, ten amino acids were present in peak 1 and peak 2 in an approximate ratio of 1:5 (Table 3). The most significant of these was the amino acid tyrosine which in thyroglobulin gives rise to all the thyroid hormones. The amino acid analysis was repeated using the same conditions for the acid hydrolysis and amount of protein being hydrolysed except that the hydrolysis tubes were filled with oxygen-free nitrogen instead of being evacuated to eliminate the possibility of oxidation producing anomalous results. The subsequent amino acid analysis was identical to that of the previous experiment. Four other amino acids contained in peak 1 and peak 2 were also found to be significantly different from each other, which gave a total of sixteen differences in peak 1 from peak 2, out of a total of twenty-one amino acids. It was thus concluded that peak 1 was a different protein from peak 2, and so different from 19S thyroglobulin, since the amino acid analysis of peak 2 was the same as that established as thyroglobulin. The purification procedure for the production of thyroglobulin was obviously incomplete in both personally prepared and commercially available thyroglobulin. /

	PEAK 1		PEAK 2	
	µM/1	mg%	µM/1	mg%
Nor Leucine (STD)	500	6.55	500	6.55
Aspartic acid	17.8	0.2	14.3	0.2
Threonine	6.8	0.08	8.1	0.1
Serine	20.2	0.2	21.0	0.2
Glutamic acid	13.5	0.2	20.0	0.3
Proline	258.1	3.0	840.2	9.7
Glycine	66.1	0.5	341.3	2.6
Alanine	397.4	3.5	1940.0	17.3
Valine	171.3	2.0	794.4	9.3
Methionine	-	-	5.3	0.08
Iso Leucine	46.4	0.6	208.8	2.7
Leucine	149.5	2.01	619.4	8.1
Tyrosine	21.9	0.4	97.3	1.76
Phenylalanine	51.7	0.9	258.9	4.3
Ornithine	Not separated from other Ninhydrin Positive component.			
Lysine	25.3	0.4	144.0	2.1
Histidine	12.4	0.2	35.5	0.5
Arginine	84.6	1.5	480.1	8.4
α Amino butyric acid	31.4	0.3	190.9	2.0
Allo Iso Leucine	34.7	0.5	91.7	1.2
β Alanine	30.4	0.3	48.1	0.4
γ Amino butyric acid	-	-	34.8	0.4

TABLE 1

Amino Acid Analysis of Peak 1 and Peak 2

Residue	Moles Amino Acid Residue/ Mole Thyroglobulin		
	Hog	Sheep	Peak 2
Lysine	155	164	113
Histidine	77	66	27
Arginine	371	326	453
Aspartic Acid	368	385	386
Threonine	289	270	219
Serine	482	494	567
Glutamic Acid	673	660	540
Proline	412	365	262
Alanine	498	456	467
Valine	327	329	251
Methionine	54	72	72
Isoleucine	131	136	145
Leucine	541	489	437
Tyrosine	101	118	97
Phenylalanine	251	257	232
Glycine	425	417	523

Table 1(a) Amino Acid composition of thyroglobulin peak 2 values calculated from those given in Table 1.

Hog and sheep values from Spiro. M.J. J. Biol. Chem. 245 5820 (1970).

	PEAK 1		PEAK 2	
	µM/l	mg%	µM/l	mg%
Nor Leucine (STD)	500	6.55	500	6.55
Aspartic acid	17.8	0.2	14.3	0.2
Threonine	6.8	0.08	8.1	0.1
Serine	20.2	0.2	21.0	0.2
Glutamic acid	13.5	0.2	20.0	0.3

TABLE 2

Amino acids present in approximately similar quantities in both Peak 1 and Peak 2

	PEAK 1		PEAK 2	
	µM/l	mg%	µM/l	mg%
Nor Leucine (STD)	500	6.55	500	6.55
Proline	258.1	3.0	840.2	9.7
Glycine	66.1	0.5	341.3	2.6
Alanine	397.4	3.5	1940.0	17.3
Valine	171.3	2.0	794.4	9.3
Iso Leucine	46.4	0.6	208.8	2.7
Leucine	149.5	2.01	619.4	8.1
Tyrosine	21.9	0.4	97.3	1.76
Phenylalanine	51.7	0.9	258.9	4.3
Lysine	25.3	0.4	144.0	2.1
Arginine	84.6	1.5	480.1	8.4

TABLE 3

Amino acids present in Peak 1 and Peak 2 in approximately 1:5 ratio

/thyroglobulin.

(h) Carbohydrate Analysis of Peaks 1 and 2

Another difference between peak 1 and peak 2 was found to be the presence of carbohydrate. The carbohydrate content of peak 1 and peak 2 was estimated by the phenol-sulphuric method which utilizes 5% aqueous phenol solution and Analar concentrated sulphuric acid. The carbohydrate containing solution (1ml) was mixed with 2ml of the phenol solution and then 5ml of the concentrated sulphuric acid was added directly on to the surface of the phenol/carbohydrate solution (vigourously from an all-glass syringe). The solution became heated causing the hydrolysis of the carbohydrate material. The solutions were allowed to stand at room temperature for 15 minutes and then cooled in water before the optical density was measured at 490nm.

This method is very sensitive to traces of unwanted carbohydrate material, for example, filter paper, which may adhere to the test-tubes, and so a set of test-tubes are kept solely for this method, having been previously washed out with the reagents.

The phenol/sulphuric test showed that peak 1 did not contain any carbohydrate but peak 2 contained a substantial amount of carbohydrate material. This reinforced the belief that peak 1 was some molecule other than thyroglobulin.

CHAPTER 8

IDENTIFICATION OF PEAK 1

In the previous chapter it was shown that peak 1 was a completely different molecule from peak 2 and so different from 19S thyroglobulin. This chapter will now deal with the identification of peak 1.

A solution of peak 1 was found to be an opalescent cloudy oily type of solution, whereas a solution of peak 2 was transparent. This opalescence was thought to arise from the possibility that peak 1 was not a pure protein molecule and may have been contaminated by fat in some way, or may be a lipoprotein. The separation produced on Sepharose 6B gave a sharp spike-like peak for peak 1 which was typical of that produced by a lipoprotein subjected to column chromatography. Peak 2 on the other hand gave a normal distribution type curve which again indicated that this molecule was different from peak 1, and since peak 1 was eluted from the column just behind the void volume it was taken to be a large molecular weight molecule, but as peak 1 was not detected on sucrose density gradients and was presumably floating on top of these gradients, it was thought to be a low density molecule. Since most lipoproteins are large molecular weight, low density molecules, it was possible that peak 1 was a low dennity lipoprotein.

Lipid Analysis of Peaks 1 and 2

A lipoprotein screen was carried out on both peak 1 and peak 2 to establish whether or not any lipoprotein or related molecule such as triglyceride and cholesterol was present in these peaks.

The/

/The triglyceride estimation utilised a semi-automated procedure based on the method of Kessler and Ledever (48) and used a Technicon Auto Analyzer. The sample was extracted with isopropanol in the presence of a slurry of zeolite, copper lime and Lloyd reagent. The lipid extract was then sampled into an air-segmented alcoholic potassium hydroxide solution and saponification of triglycerides to glycerol occurred, on-stream in a 50°C heating bath. After saponification, periodate reagent was added to the reaction mixture to oxidise the glycerol to formaldehyde. This was followed by condensation with diacetylacetone and ammonia to give a fluorescent product, 3,5 - diacetyl - 1,4 dihydrolutidine. The oxidation and condensation steps were also carried out in a 50°C heating bath. After heating the reaction mixture entered the fluorimeter where the air was removed and the fluorescence activated. Comparison with standards gave the results as mg/100mls.

Total cholesterol was estimated by the modification of the Liebermann-Burchard reaction (49) with the following modifications:-

(1) The amount of sample was reduced from 0.4ml to 0.02ml. The final volume for photometry nevertheless remained at 1.22ml and was sufficient for photometry in semi-micro cuvettes.

(2) The number of reagents was further reduced so that only three pipettings, (sample, cholesterol reagent and sulphuric acid) were required during the manipulations.

The cholesterol standard used was 400mg/100mls acetic acid; the cholesterol was recrystallised several times from methanol and dried well/

/well to free from impurities. The results were again reported as mg/100mls.

Lipoproteins were estimated by the electrophoretic method reported by Frings et al (50), in which lipoproteins were separated in polyacrylamide gel, both on electrophoretic mobility and by molecular size. The sample was prestained with Sudan Black B in a sample or loading gel and then resolved by electrophoresis in a discontinuous pH system consisting of the sample gel, a concentrating gel and a separating gel. A Shandon Disc Electrophoresis system was employed for this separation. The peak 1 sample was compared with human serum lipoprotein samples after electrophoresis for 35 minutes at 5mA per gel.

The results obtained from the lipoprotein screen showed that peak 1 contained triglyceride at a concentration of 20mg/100mls, cholesterol at 10mg/100mls and a very low density lipoprotein (VLDL) present on polyacrylamide electrophoresis in the pre-β region as compared to human serum lipoproteins, (Fig 15). Peak 2 was found to possess triglyceride in a concentration of 10mg/100mls, but both cholesterol and VLDL were absent.

The above procedure verified peak 1 as being a low density molecule, namely a lipoprotein, but as a final check it was decided to prepare a sample of VLDL from sheep serum and compare the elution pattern from the Sepharose 6B column to that of peak 1. The method used was essentially that of Lees & Hatch (51). Sheep's blood from freshly slaughtered sheep was allowed to clot at room temperature for two hours before the serum was spun off in a low speed centrifuge./

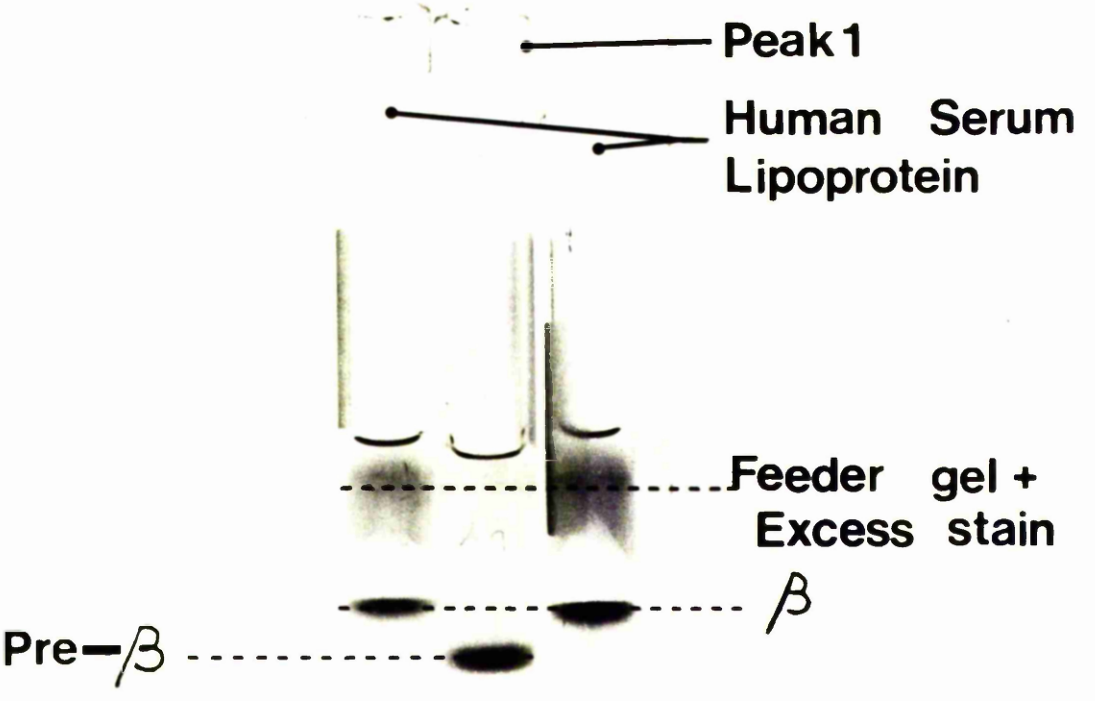


Fig. 15. Polyacrylamide Gel electrophoresis of peak 1 and human serum β lipoprotein. Samples pre-stained with Sudan Black B and electrophoresis carried out in a Shandon Disc Electrophoresis Apparatus for 35 minutes at 5mA/Gel.

/centrifuge. 0.01ml of a 5% EDTA-solution (w/v) per ml of serum was added and the serum stored at 4°C, and 4mls of this treated serum pipetted into 2½in x ⅜in diameter cellulose nitrate centrifuge tubes. The tubes were then filled to the brim by carefully layering on a 1.006 density solution (11.40g sodium chloride and 0.1g of $EDTANa_2$, 1ml 1N NaOH, made up to one litre and 3mls of additional distilled water added) on top of the serum. The tubes were slowly capped, allowing excess fluid to run down the outside of the tube, before being placed in a 40.3 rotor of a Beckman Model L ultracentrifuge, and centrifuged for 16 hours at 40,000rpm at a temperature of 16-18°C. After centrifugation the tubes were placed in a tube slicer and cut to give a top fraction VLDL of approximately 2ml. These 2ml fractions were transferred quantitatively to a volumetric flask using an all glass syringe, small quantities of the 1.006 density solution were used to rinse the slicer and the tube tops. A 10ml sample of this sheep serum VLDL was then passed through a Sepharose 6B column (identical to that used for separating peak 1 and peak 2) and the elution pattern obtained (Fig 16). Sheep serum VLDL was eluted from the column between fractions 46 and 56 (using PBS pH6.8) with a peak at fraction 50, again a sharp spike-like peak being observed. Peak 1 which was eluted at fraction 60 was marginally but significantly different from the serum VLDL peak. This indicated that sheep serum VLDL was a larger molecule than peak 1. Since this sample was eluted in approximately the same elution volume as peak 1, and gave a similarly shaped peak to peak 1, it suggests that peak 1 was indeed a very low/

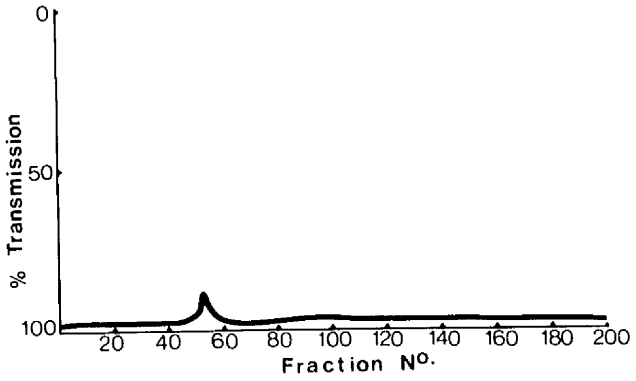


Fig. 16. Elution pattern of sheep serum VLDL from a sepharose 6B column (bed height 95.0 cms., 2.6 cms. diameter) eluted with PBS buffer, pH 6.8.

/low density lipoprotein.

Attempted Extraction of Lipid from Protein of Peak 1

Since peak 1 has been found to be a lipoprotein and since the lipid portion of this molecule contains only triglyceride and cholesterol, that is, no phosphate present, it was decided to strip off the lipid to free the protein part of the molecule for further study such as ultracentrifugation, electrophoresis and electrofocussing.

The lipid portion of peak 1 was separated from the protein by treating a freeze-dried, desalted, sample with an ether:ethanol mixture in the ratio 1:3, with constant shaking for 16 hours at $4^{\circ}C$. The precipitated protein was spun down and the procedure repeated twice, shaking for four hours each time. The protein moiety so produced was ether dried before solubilisation of the sample was attempted. The protein so produced was unfortunately insoluble in aqueous buffer systems and so no further investigation into this part of the molecule could be carried out. This procedure showed that the lipid could be separated from the protein relatively easily which verified the absence of phosphate. However, the method did not clarify the position regarding the properties possessed by the protein part of the molecule.

CHAPTER 9

STUDY OF POSSIBLE ARTEFACTS PRODUCING PEAK 1

STUDY OF POSSIBLE ARTEFACTS PRODUCING PEAK 1

Peak 1 has been identified as being a lipoprotein of the pre-β variety when compared to human serum lipoprotein, and so it was considered necessary to investigate the possibility of this molecule arising from some other source than the colloid of the thyroid cells. In other words, is peak 1 an artefact produced by the purification procedure employed for production of thyroglobulin?

An investigation into possible sources of lipoprotein contamination was carried out with the first possibility being the cell membrane which is known to possess lipoprotein (or lipids and protein) in considerable amounts. Peak 1 could possibly be due to small fragments of cell membrane, produced during tissue homogenisation, which were of small enough size to pass through the sepharose 6B gel column, and so give rise to peak 1 situated just behind the column void volume. This possibility would be ruled out by ultracentrifugation at sufficient speed to spin down any such fragments of cell debris. 400mg of freeze dried sheep thyroglobulin dissolved in 6mls PBS were spun at 100,000g for 16 hours (50 rotor) in a Spinco Model L2-65B refrigerated Ultracentrifuge and after centrifugation the sample was passed through a sepharose 6B column and the elution pattern recorded. The elution pattern did not differ in any respect from those previously obtained, that is, uncentrifuged, and so it was concluded that peak 1 did not consist of fragments of cell membrane./

/membrane.

Another possible source of lipoprotein contamination was incomplete removal of blood serum during the purification procedure. This possibility, however, was discounted since a sheep serum VLDL fraction was eluted from the sepharose 6B column in a significantly different region to that of peak 1.

Since all experiments carried out so far used freeze dried samples of sheep thyroglobulin (commercially available thyroglobulin was also freeze dried), it may be that this drying process was in some way responsible for the appearance of peak 1. To investigate this possibility a freshly prepared sample of sheep thyroglobulin was passed through the sepharose 6B column, since a similar elution pattern of peak 1 and peak 2 were obtained this theory was discounted.

Thyroids obtained from the local abattoir were embedded in fibro-fatty tissue which was cleaned before thyroglobulin was extracted. Cleaning means "cleaned" to the naked eye. Histological sections were prepared from "cleaned" thyroids and stained for lipid with Sudan Black and Oil Red "O" stains (Figs 17+18). As can be seen from these sections, thyroids previously considered to be free from fibro-fatty tissue were still extensively contaminated. On examination of the Oil Red "O" photomicrograph it can also be seen that there are a few pockets of fat within the thyroid gland, and so it was thought this may be the source of the peak 1 lipoprotein. On further investigation, by staining with Sudan IV and Nile Blue stains, of this fibro-fatty capsule surrounding the thyroid and of the fat pockets within the gland it was found to be Neutral Fat and so was not the source of the lipo- /

Untreated

Sudan Black

Fig. 17. Sections through "cleaned" thyroid gland.
Upper portion - Unstained
Lower portion - Stained with Sudan Black stain to show the presence of fat.

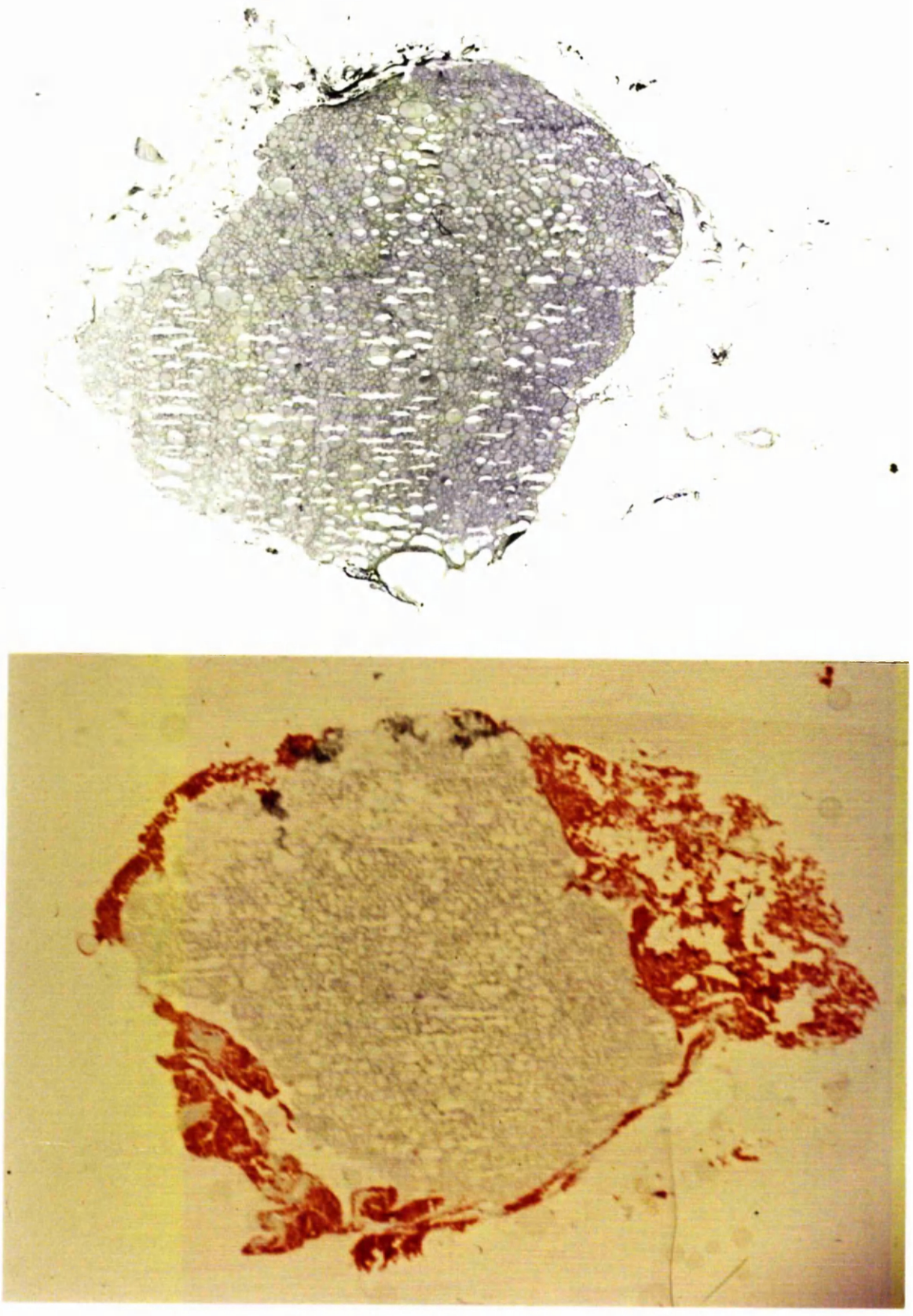


Fig. 18. Sections through "cleaned" thyroid gland.
Upper portion - Unstained.
Lower portion - Stained with Oil Red "O" stain to show the presence of fat.

/lipoprotein peak 1.

From the above experiments it was concluded that peak 1 was most probably thyroidal in origin, and part of the colloid found within thyroid cells, since various other possibilities such as cell membrane, blood serum and fibro-fatty tissue surrounding the thyroid gland have been discounted. Peak 1 has also been shown to be present in freshly prepared thyroglobulin, that is thyroglobulin which has not been subjected to freeze-drying, and so this molecule most probably plays some role in thyroid function.

CHAPTER 10

ELECTROPHORESIS & ELECTROFOCUSSING OF PEAKS 1 & 2

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was carried out on both peak 1 and peak 2 using desalted, freeze-dried samples of both of these molecules. The electrophoresis conditions were as previously described.

Electrophoresis on polyacrylamide gel depends on molecular size and the charge possessed by the molecule. The results obtained from the experiment (Fig 19) show that peak 2 has travelled in the gel to a slightly greater distance than peak 1 (peak 2 has moved into the gel for 2mm whereas peak 1 has moved only 1.5mm). These results indicate that peak 1 is a larger molecule than peak 2.

Another experiment, using SDS-containing gels and SDS treated samples of peak 1 and peak 2, was set up to elucidate the molecular weights of these two molecules. Suitable standards, namely Fibrinogen (MW 340,000), Immunoglobulins, IgA (dissociated MW 200,000), IgG (MW 160,000) and Bovine Serum Albumin (MW 66,500) were treated identically to the peak 1 and peak 2 samples. Electrophoresis conditions were as previously described. The use of SDS in treatment of the electrophoresis samples, in the gels and electrophoresis buffer is to nullify the charge possessed by each molecule to be electrophoresed and so mobility within the gels will be directly proportional to the molecules molecular weight.

The/

Fig. 19. Polyacrylamide gel electrophoresis of peak 1 and peak 2. Electrophoresis carried out in tris-glycine buffer, pH 8.5, for 90 minutes at 5mA/gel.
Sample size - 50µl of a 1mg/ml solution of each molecule.
Denatured material at origin of gel.
Peak 2 - left gel.
Peak 1 - right gel.

/The separation obtained (Fig 20) shows that both peak 1 and peak 2 are large molecular weight molecules, since both have remained near to the top of the gel, whereas the standards have moved in the gel to varying degrees. The smaller less dense bands which can be seen in the standards gel are impurities from the fibrinogen and immunoglobulin samples, (Kabi Fibrinogen is only 94% clottable and was subsequently shown (53) to contain small quantities of Factor VIII, fibrin stabilising factor and Plasminogen), and so these additional bands are to be ignored. On measuring the distances migrated by each standard, (Fig 20A). (Table 4) and plotting these results on semi-log paper against the molecular weights of the standards a straight line graph is obtained. When the distances travelled by peak 1 and peak 2 are measured and read off on this graph the molecular weight of these molecules can be ascertained. Peak 1 moved in the gel to a distance of 1mm from the origin and so the molecular weight of this molecule was found to be 700,000. Peak 2 migrated in the gel to a distance of 3mm which gave a molecular weight of 670,000 for this molecule. The published molecular weight for thyroglobulin is 660,000 and so this method can be taken to be fairly accurate in the determination of molecular weights. These results, therefore, confirm that both peak 1 and peak 2 are high molecular weight molecules.

Further investigation of both peak 1 and peak 2 was carried out using polyacrylamide gel electrophoresis in conjunction with sample pre-treatment with SDS and β mercaptoethanol (BME). This pre-treatment should split the moleculesinto their constituent polypeptide chains/

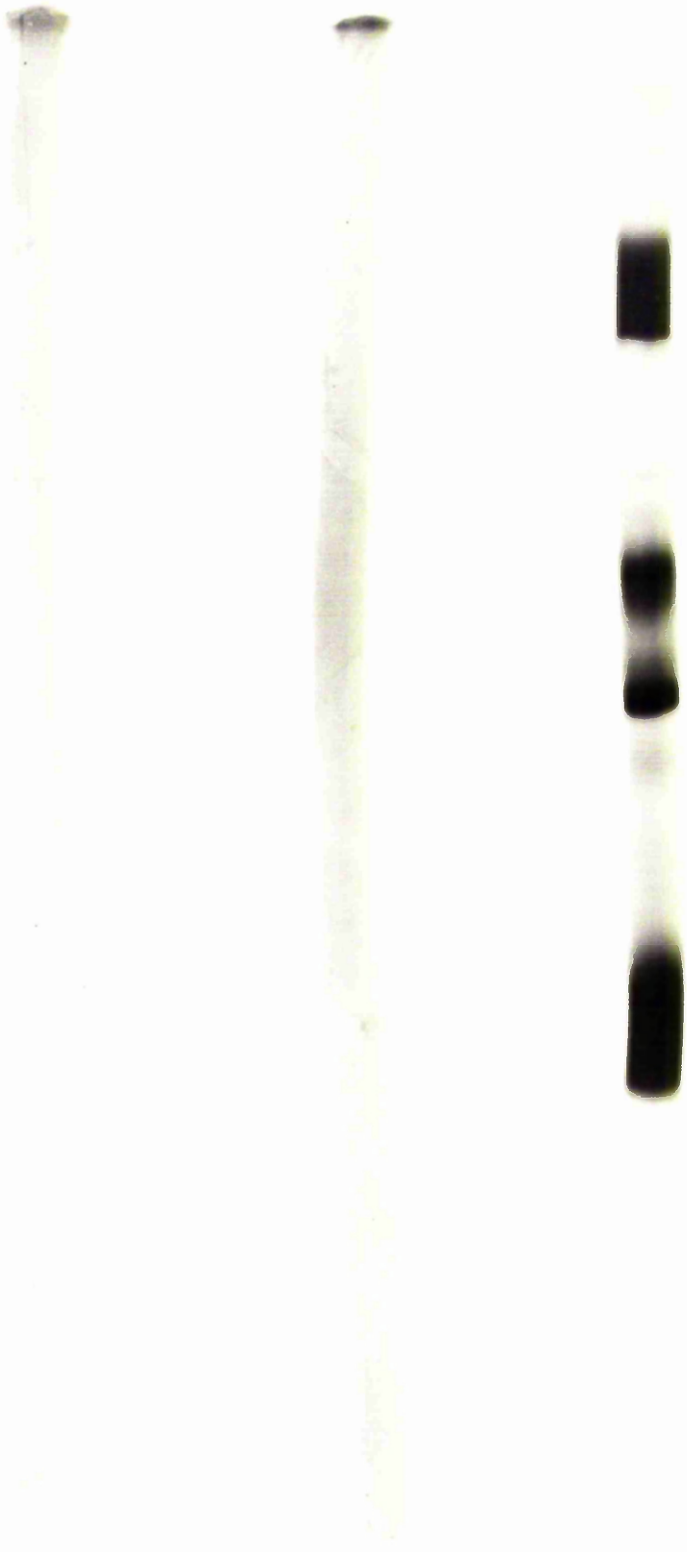


Fig. 20. Polyacrylamide gel electrophoresis of peak 1, peak 2 and standards in sodium phosphate/SDS buffer, pH 7.5, for 90 minutes at 5mA/gel.

Sample size - 20μl of a 1mg/ml solution of each molecule, pre-treated with 1% SDS.

Standards - Fibrinogen, IgA, IgG, Bovine Serum Albumin.

Peak 2 - left gel.
Peak 1 - middle gel.
Standards - right gel.

	Distance Migrated (mm)	Molecular Weight
Fibrinogen	33.0	340,000
IgA (Dissociated)	56.0	200,000
IgG	70.0	160,000
BSA	106.0	66,500
Peak 1	1.0	700,000
Peak 2	3.0	670,000

TABLE 4

Comparison of distance migrated in polyacrylamide gel electrophoresis to molecular weight.

Values given for both peak 1 and peak 2 are estimated from linear graph obtained from standards values.

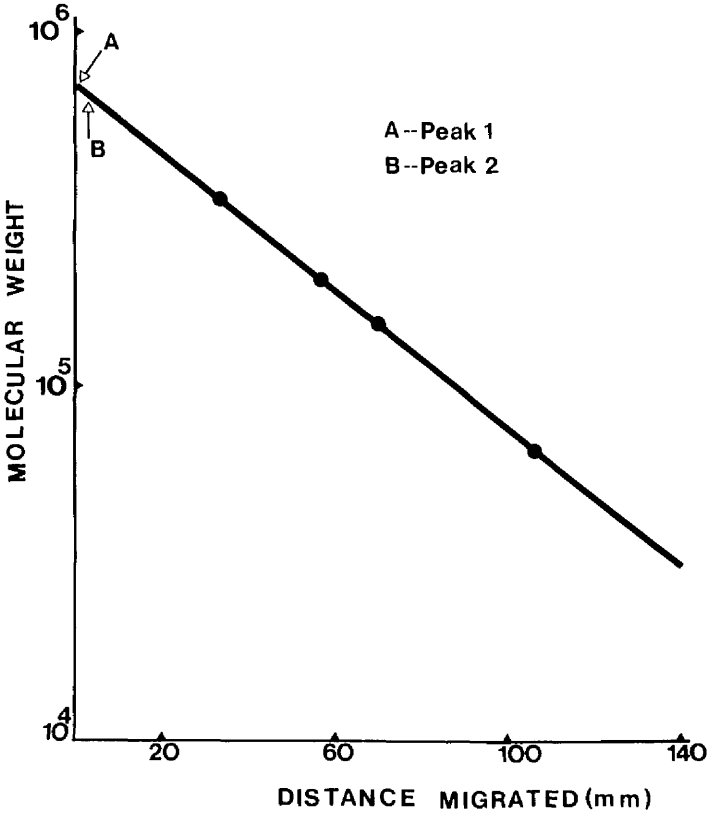


Fig. 20(a) Graph of distance migrated (m.m.) against molecular weight of known staniards.

/chains by the cleaving of disulphide bridges. The results obtained (Fig 21) show six bands in the gel on which peak 1 was electrophoresed and nine bands in that for peak 2. In each case one of the bands is denser than the others which suggests that the polypeptide present in each of these bands is at a higher concentration than the others. The results suggest that peak 1 is composed of at least six different polypeptide chains, however, one polypeptide may be present in more than one part of the molecule, that is, may be present in two or three different sites, (this is suggested by one band being denser than the others). Peak 2 on the other hand possesses at least nine different polypeptide chains, one band again being denser than the others which suggests that this polypeptide is present possibly in more than one position in the molecule. The two denser bands, however, do not appear to be the same polypeptides in both molecules. When measurements of distance migrated (Table 5) from the origin (top of the gel), are taken for each polypeptide band for both peak 1 and peak 2, exact replication of bands are found in five out of the six possible bands in peak 1. The only dissimilarity occurs in band two of peak 1 which migrates 6mm whereas the second band of peak 2 migrates 6.5mm. This small difference may be significant when the total structure of the molecule is considered. The first similar bands (1mm from top of the gel) should probably be discounted as denatured original material trapped in the gel, which still gives four similarities out of five possible in peak 1.

Since/

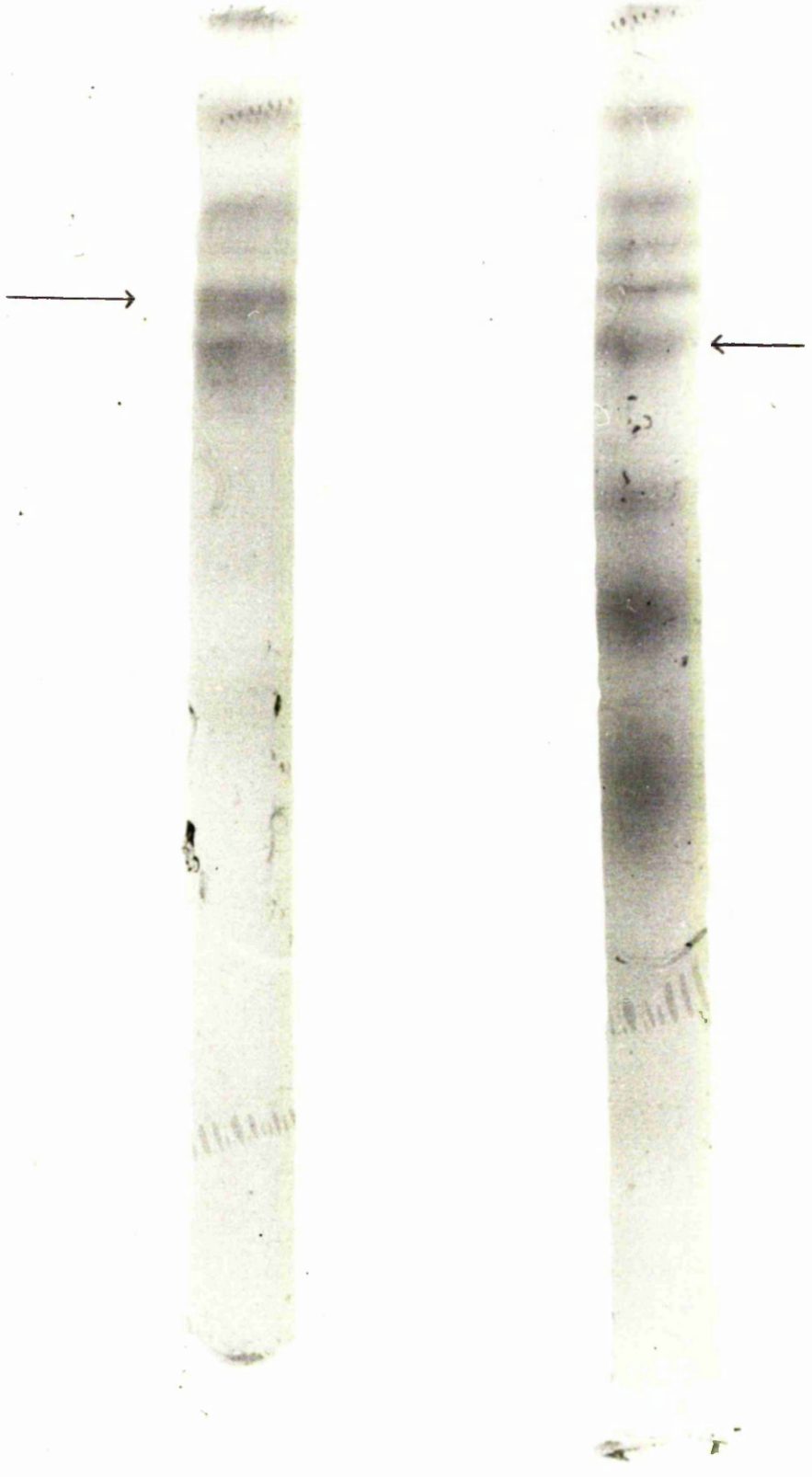


Fig. 21. Polyacrylamide gel electrophoresis of peak 1 and peak 2 in sodium phosphate/SDS buffer, pH 7.5, after pre-treatment with 1% SDS and BME. Electrophoresis for 90 minutes at 5mA/gel.
Sample size - 20μl of a 1 mg/ml solution of each molecule.
Arrows show densest band, gels origionally same length.
Peak 1 - left gel.
Peak 2 - right gel.

Distance Migrated (mm) by Constituent Polypeptides

PEAK 1	PEAK 2
1.0	1.0
6.0	6.5
9.0	9.0
11.5	11.5
15.5	15.5
18.0	18.0
	30.5
	38.0
	44.0

TABLE 5

Comparison of distance migrated by constituent polypeptides, of peak 1 and peak 2, on polyacrylamide gel electrophoresis.

/ Since peak 1 appears to be almost identical to peak 2 in its higher molecular weight polypeptide chains, the only marked differences being the absence of three small molecular weight components, it would seem that the protein moiety of peak 1 is in fact a shortened type of peak 2 molecule. This relationship was further investigated by repeating the above experiment using greatly increased concentrations (5mg/ml) of peak 1 and peak 2, to show whether any low molecular weight polypeptide chains were present and had not been stained in the previous experiment due to low concentration. The results obtained were identical to those described above and so it was assumed that the hypothesis that peak 1 and peak 2 had a proportion of similar peptides was correct.

The polyacrylamide gel electrophoresis results show the differences between peak 1 and peak 2 due to molecular size, and so experiments were designed to show any differences between these molecules simply due to the overall net charge possessed by them.

Electrofocussing

The technique employed for this was iso-electric focussing, which concentrates molecules of differing overall net charge at their iso-electric points (IEP). The experimental details have been described previously.

The results obtained (Fig 22) show that peak 1 has a slightly lower IEP than peak 2, namely peak 1 IEP is pH4.48, whereas peak 2 IEP is pH4.68. These results were obtained by iso-electric focussing of a desalted mixture of peak 1 and peak 2. The experiment was repeated using desalted sample of peak 1, the results obtained were/

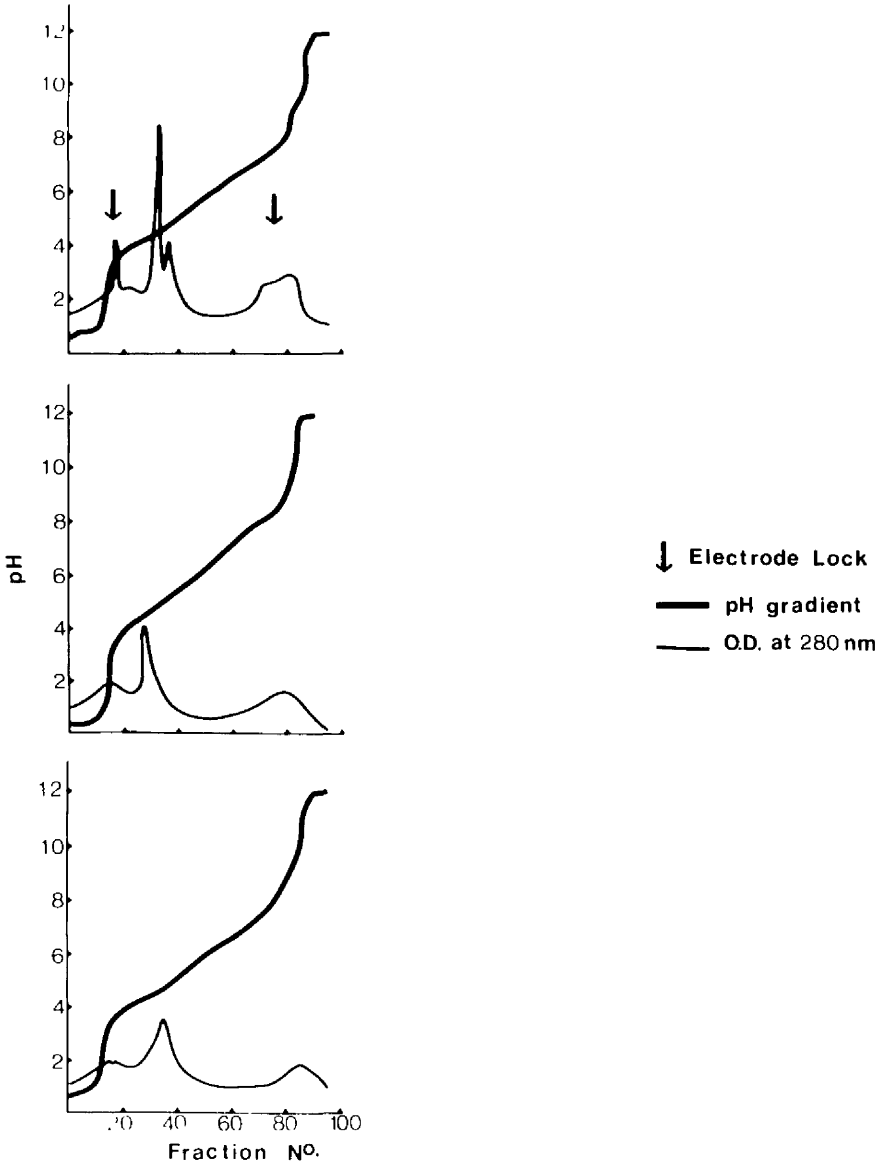


Fig. 22. Electrofocussing of peak 1 and peak 2. Procedure carried out for 48 - 72 hours, voltage at end of run 600 - 1,000 V. Effective pH gradient pH3 - pH 10.

Upper portion - peak 1, peak 2 mixture.
Middle portion - peak 1.
Lower portion - peak 2.

/were identical to the previous experiment. When a desalted sample of peak 2 was focussed, the result obtained was an IEP of pH4.70, which is 0.02 pH units higher than that obtained from the previous experiment and represents an error of 0.43% from the previous result. This result can therefore be taken to be within the experimental error of the method employed.

These results again show that the molecules are of a similar type since both iso-electric points are found within the same pH region, and also show that there is not a marked difference in overall net charge possessed by the molecules although peak 1 is a lipoprotein and peak 2 is a protein. The results also show that the method gives a good reproduction of the iso-electric point since the published value for peak 2 is pH4.60.

SECTION 4

IN VIVO AND IN VITRO IODINATION

CHAPTER 11 .. .. IODINATION OF PEAK 1 AND PEAK 2

CHAPTER 11

IODINATION OF PEAK 1 AND PEAK 2

It has been established in the previous section that peak 1 is a lipoprotein, thyroidal in origin, and contains an appreciable amount of the amino-acid tyrosine. Since tyrosine gives rise to all of the thyroid hormones, and so is iodinated in thyroglobulin, it was decided to establish whether or not the tyrosine in peak 1 was iodinated, and if so to try to elucidate the function of this other iodine-containing molecule in the thyroid.

Chemical Demonstration of Iodination of Peaks 1 and 2

Protein bound-iodine (PBI) tests were carried out on samples of both peak 1 and peak 2. Inorganic iodine was removed from the sample by shaking with a moistened anion exchange resin (Amberlite IRA 400), the supernatent poured into an Autoanalyzer cup and transferred to the Autoanalyzer. After dilution with water the sample was mixed with concentrated sulphuric acid and then with a perchloric/nitric acid mixture, and heated at 280°C to digest the proteins and so liberate the iodine. After digestion the sample was mixed with arsenious acid followed by ceric ammonium sulphate.

The reduction of the yellow coloured ceric to the colourless cerous ion was measured at 410nm, to measure the rate of the reaction which is dependent on the iodine ion concentration. A standard curve was prepared from potassium iodate solutions and results reported as µg/100ml. /

µg/100ml.

Peak 1 was found to contain iodine, the PBI being 1,100µg/100ml. Peak 2 as expected was also iodinated, the PBI being 5,760µg/100ml. The amount of freeze-dried material from each peak was identical. Again it was noted that the PBI results showed an approximate 1:5 ratio between peak 1 and peak 2, which was in keeping with the results of tyrosine content of the two peaks obtained from the amino acid analysis.

In vivo Iodination Studies

(a) Control Rats

Peak 1 has been shown to possess stable iodine, in fairly large quantity, and so the mechanism by which this lipoprotein was iodinated was sought. In vivo iodination experiments were carried out using 120-150g male Sprague-Dawley rats, in which 30 rats were fed a diet containing normal amounts of iodine (oxoid 41B) and tap water to drink for a period of 28 days. On day 26, 1 ml of radio-labelled KI solution (^{125}I specific activity <10µg/ml) containing 4µCi ^{125}I was injected intraperitoneally into each rat. This procedure was repeated on day 27 so that the total ^{125}I dosage was 8µCi ^{125}I. This ensured that both peak 1 and peak 2 were background labelled with this particular isotope. On the 28th day of the experiment a further intraperitoneal injection of radio-labelled KI solution was administered, but ^{131}I was used on this occasion and the dosage was 8µCi in 1ml (^{131}I specific activity <20µg/ml). After a time lapse of five minutes from the time of the injection, the rats were sacrificed under ether anaesthesia and their thyroids quickly excised and the procedure, as previously described/

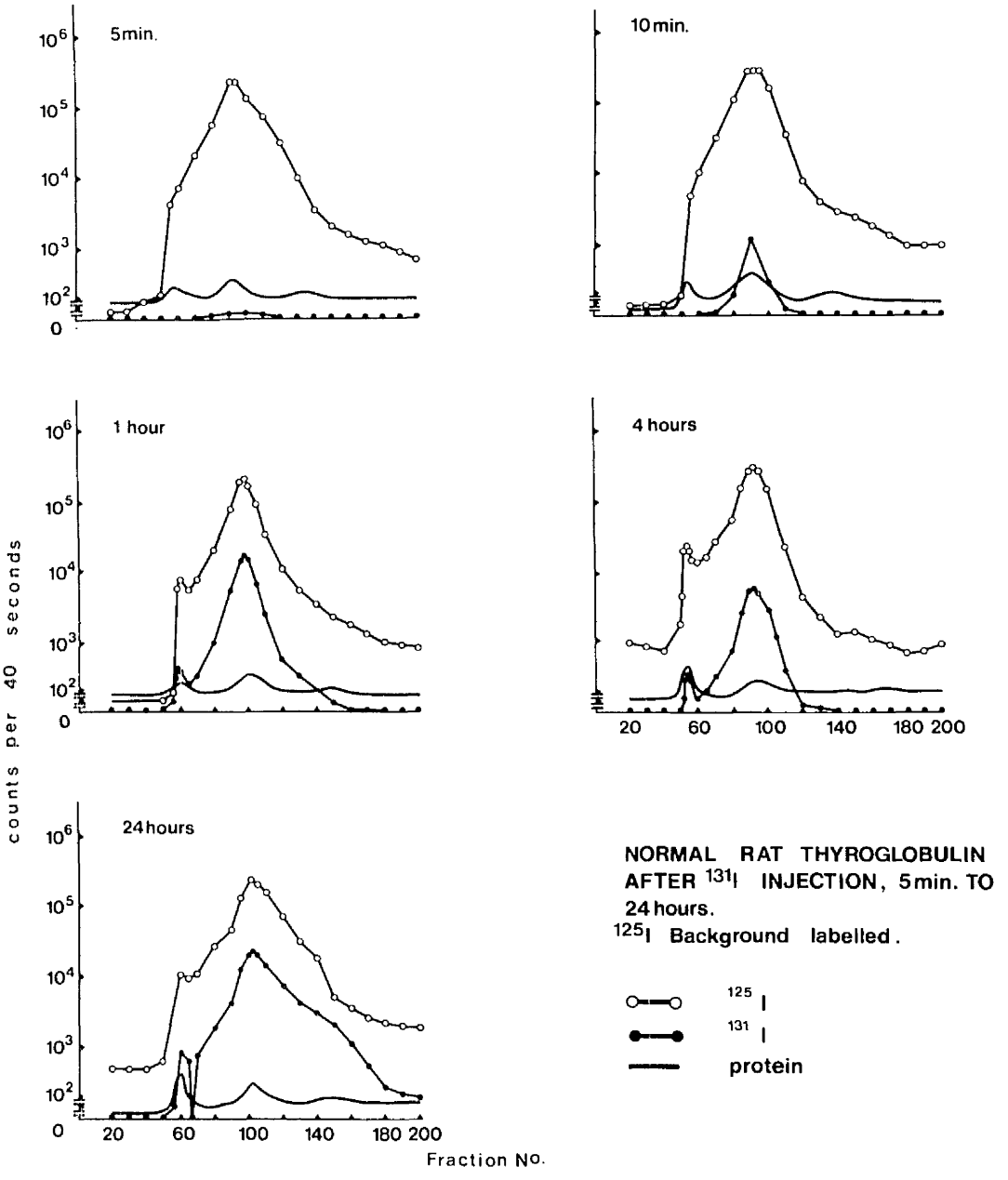
/described, for the harvesting and purification of the thyroglobulin within the glands begun.

The freshly obtained rat thyroglobulin was then passed through a sepharose 6B column, identical to those used previously, and the thyroglobulin elution pattern recorded. The 2ml fractions which were collected simultaneously, were then placed in a Packard Selektronik Model A5142-01 gamma counter, set for ^{131}I, and the radioactivity of the samples was counted for forty seconds. The gamma counter was then re-set to monitor ^{125}I and the samples were again counted for radioactivity (40sec count). The counter was able to differentiate between the ^{125}I and ^{131}I, isotopes of iodine and so could count them independently of each other, so no allowance for interferance of ^{125}I in the ^{131}I channel and vice versa was necessary.

The results thus obtained were plotted on semi-log graph paper and the column elution protein tracing superimposed. By this means it was possible to give a pictorial representation of iodine incorporation at a specific time after radio-iodine administration.

The above experiment was repeated at 10 minutes, 15 minutes, 30 minutes, 1 hour, 4 hours, 6 hours, 12 hours and 24 hours after injection of ^{131}I, and so a time course pattern of incorporation of radio-labelled iodine into both peak 1 and peak 2 was obtained (Fig 23).

As can be seen from this figure the 8μCi of ^{125}I have successfully background labelled both peak 1 and peak 2 in all of the time course experiments. The ^{131}I (8μCi) is immediately taken up and incorporated (5 minutes) by peak 2 and becomes more pronounced as time of sacrifice after iodine administration increases. Peak 1, however, only shows signs of ^{131}I incorporation at 15 minutes after injection/



NORMAL RAT THYROGLOBULIN AFTER ^{131}I INJECTION, 5min. TO 24 hours.
^{125}I Background labelled.

^{125}I
^{131}I
protein

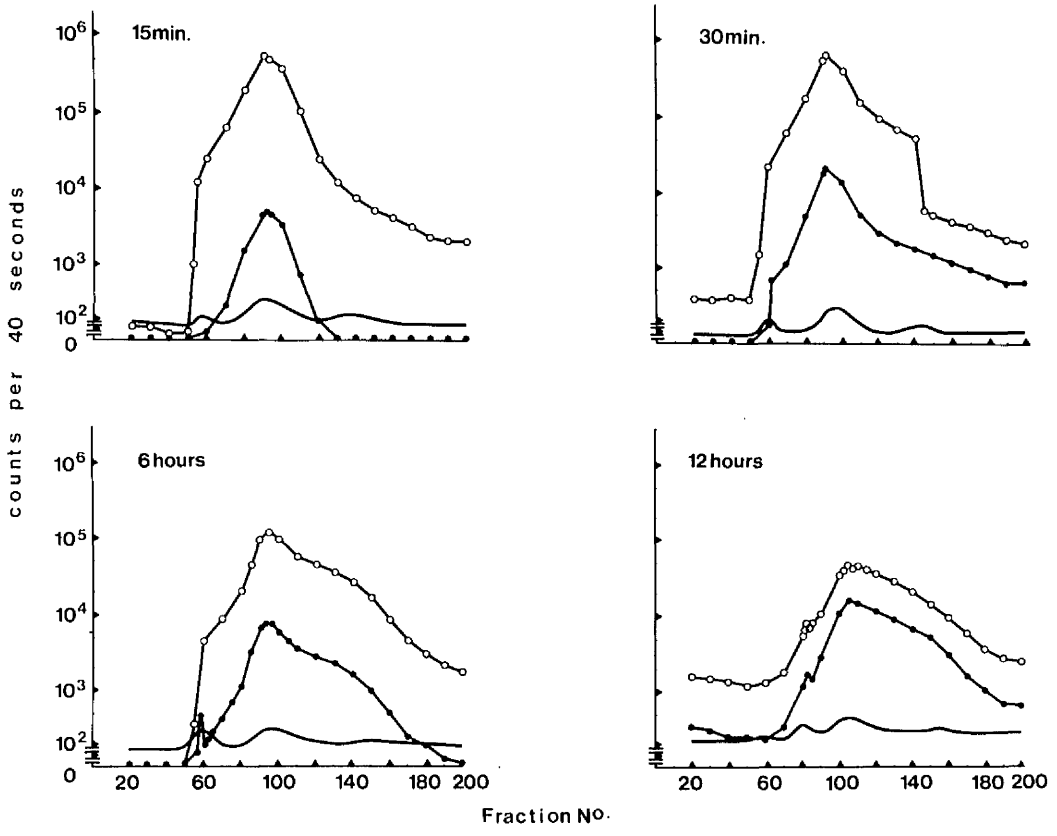


Fig. 23. Time course of radioactive iodine incorporation into peak 1 and peak 2 of normal-iodine rat thyroglobulin in vivo.

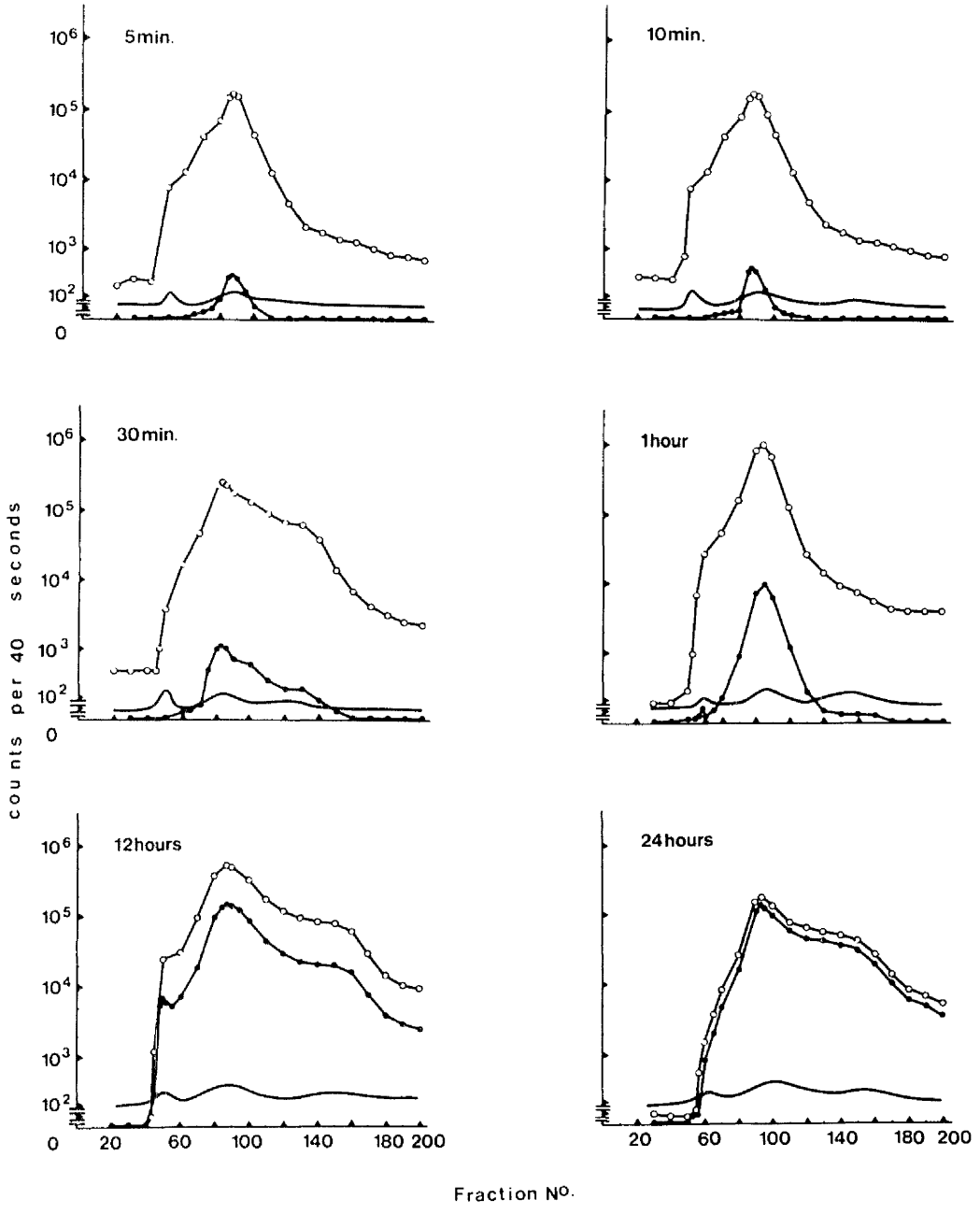
/injection and this incorporation is only of a very limited degree. At 30 minutes, incorporation of ^{131}I can be said to be proceeding as radioactivity counts are accumulating and giving a pronounced shoulder (at the position of peak 1) on the larger peak 2 radio-iodine incorporation graph. This shoulder has differentiated itself into a true radio-iodine incorporation peak in the position of peak 1 by 1 hour after injection, and remains so until 12 hours after injection, where it once more becomes a shoulder on the overall "blanket" of ^{131}I incorporation. This suggests that peak 1 has incorporated as much ^{131}I as is possible. After a further 12 hours (that is, 24 hours after injection) peak 1 has again become differentiated in the overall radio-iodine incorporation graph. Indeed differentiation is very marked in that at fraction 67 no radioactivity has been incorporated into the lipoprotein at all. This differentiation suggests that peak 1 has incorporated and stabilized the ^{131}I administered and has also overcome the possible "overload" situation found at 12 hours after injection. The time lag of incorporation of radio-iodine between peak 2 and peak 1 suggests that peak 1 is being only passively labelled, that is, is not actively trapping iodine as is the case in peak 2. The first radio-labelling experiment was taken as the base line (control) for further experiments with rats fed on varying diets.

(b) Iodine Deficient Rats

Since it was postulated that peak 1 was only being passively iodinated in the control experiment, an experiment was designed to show whether or not any difference in iodine uptake into peak 1 would/

/would be found if the experimental rats were made iodine deficient. Thirty male Sprague-Dawley rats (120-150g) were fed a low iodine diet (LID) for 28 days with only glass re-distilled water to drink before injection of 4μCi ^{125}I contained in 1ml KI solution on each of days 26 and 27. On day 28, 8μCi ^{131}I contained in 1ml KI solution was injected intraperitoneally and the rats sacrificed, under ether anaesthesia, 5 minutes after injection of the ^{131}I. The procedure as previously described gave a sample of hypo-iodine rat thyroglobulin which was passed through a sepharose 6B column and the protein elution pattern obtained. The same procedure as before was carried out to obtain the radioactivity graph and the protein trace was superimposed on this graph. This experiment was repeated at 10 minutes, 15 minutes, 20 minutes, 30 minutes, 1 hour, 2 hours, 6 hours, 12 hours and 24 hours after ^{131}I administration and so the time-course pattern of radio-labelled iodine into peak 1 and peak 2 from iodine deficient rats was obtained (Fig 24).

As can be seen from this figure the ^{125}I has again successfully background labelled both peak 1 and peak 2 in all of the time-course experiments. The ^{131}I is immediately taken up into peak 2 (5 minutes after injection) and since this molecule has been deprived of iodine the uptake is quite naturally greater than that of the control series. The incorporation of ^{131}I into peak 2 continues up to 24 hours, by which time the amount of label (^{131}I) in peak 2 is almost the same as the ^{125}I background, which is again different from the control series. Since the hypo-iodine rat thyroid was found to be taking /


5min.
10min.
30min.
1hour
12hours
24hours
10^6
10^5
10^4
10^3
10^2
0
counts per 40 seconds
20
60
100
140
180
200
Fraction No.

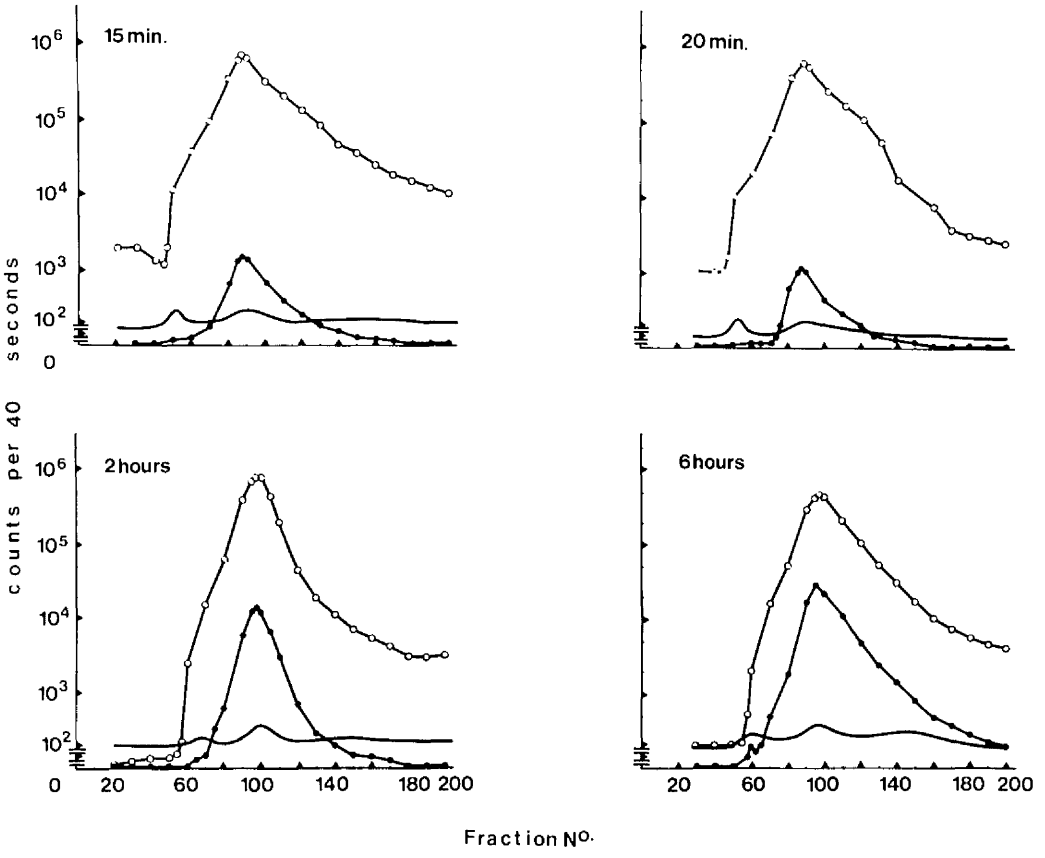


Fig. 24. Time course of radioactive iodine incorporation into peak 1 and peak 2 of hypo-iodine rat thyroglobulin in vivo.

/taking up iodine avidly, it was expected that peak 1 (if active trapping of iodine was possible for this molecule) would become labelled with ^{131}I more quickly than in the control experiments. This is not the case as peak 1 only becomes labelled one hour after the time of injection, (control experiments label appeared in peak 1 at 15 minutes), which again indicates that this molecule does not play an active role in the trapping of iodine. In fact the level of ^{131}I incorporation remains fairly static from 1 hour to 12 hours after injection, when, by this time, a dramatic increase in peak 1 labelling occurs. At 24 hours after injection the ^{131}I label approximately mirrors the ^{125}I background label graph both in shape, and in the degree of labelling present, which again differs dramatically from the results, for this time, of the control experiments, as there is no differentiation in label between peak 1 and peak 2.

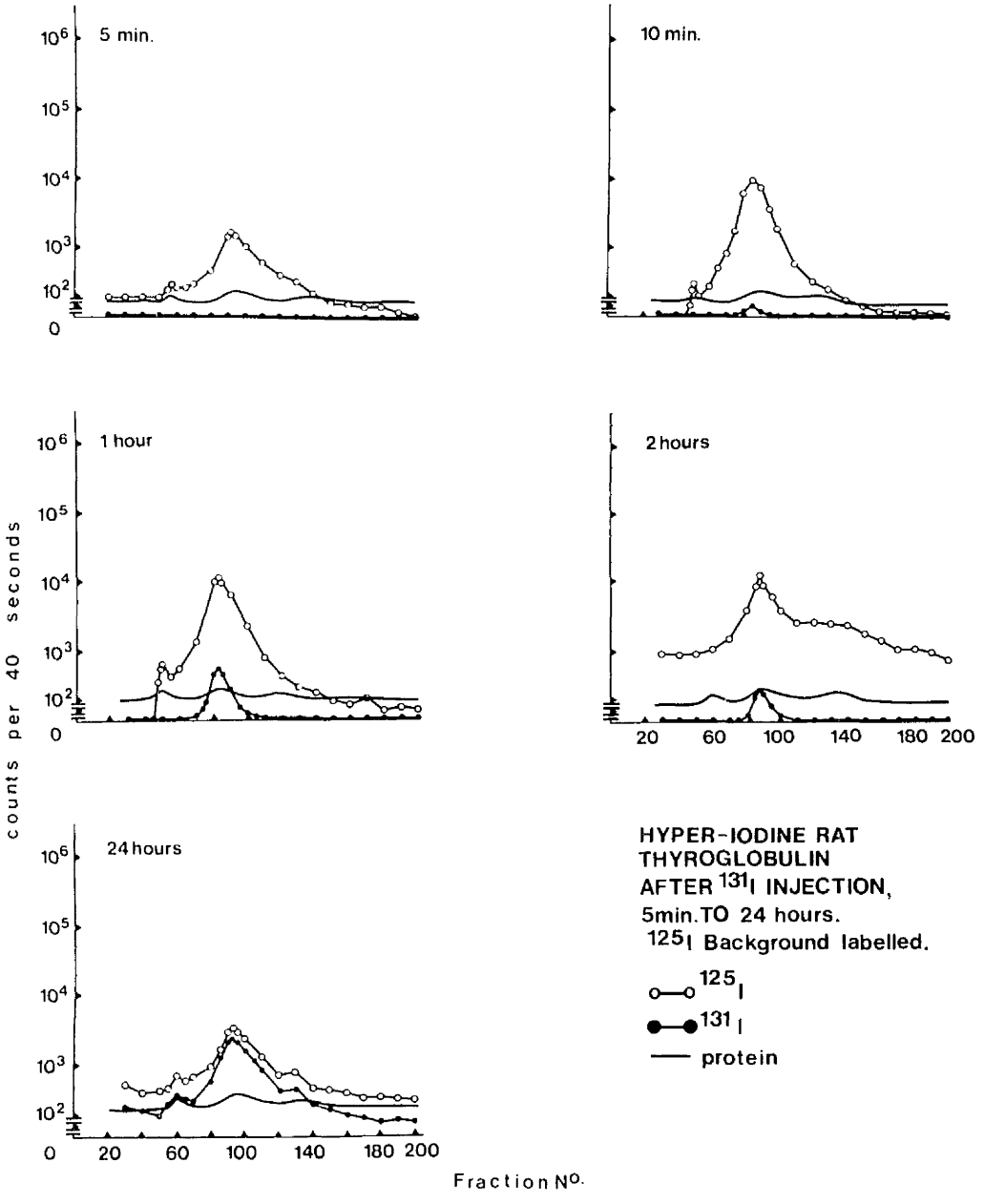
The greater time-lag between incorporation of ^{131}I into peak 2 and peak 1 indicates that peak 1 may be labelled due to breakdown of labelled components of peak 2, and so only passively stores iodine. If this was the case, the time-lag should be extended in thyroids which possessed an abundance of iodine.

(c) Iodine Supplemented Rats

To explore this possibility further, another series of time-course labelling experiments were designed, again using thirty rats for each experiment. In this series of experiments the rats were fed on normal iodine diet (oxiod 41B) for 28 days, but instead of tap water to drink, these rats were given a 0.05% KI solution (KI dissolved/

/dissolved in tap water). This drinking solution represented a massive supplement of iodine when compared to the normal requirements. The rats were again injected, the thyroglobulin extracted and passed through the sepharose 6B column as before. The protein trace was recorded and the fractions counted both for ^{125}I and ^{131}I and the results plotted on semi-log paper as previously described. The time-course for this experiment was sacrifice at 5 minutes, 10 minutes, 15 minutes, 1 hour, 2 hours, 6 hours, 12 hours and 24 hours, after ^{131}I injection.

The administered ^{125}I (Fig 25) again gave a "blanket" background label of both peak 1 and peak 2, but to a much lesser degree than previously. Peak 1, however, was differentiated from peak 2 by this label in the 5 minute to 1 hour experiments, whereas no differentiation of the two peaks was observed from 2 hours to 12 hours, the 24 hour experiment results again showed peak 1 being differentiated from peak 2. These results (that is, ^{125}I graph) do not represent an acute uptake of iodine into the peaks at the times quoted since iodine incorporation had been progressing for up to 48 hours prior to the times of ^{131}I injection. The acute iodine uptake results are those obtained from the ^{131}I counts, (pulse labelling), which show no iodine incorporation into peak 2 until 10 minutes after ^{131}I injection. This incorporation builds up over the whole time-course study but again total incorporation was much less than that previously obtained in both the control experiments and hypo-iodine experiments. This is to be expected since the thyroid of these rats has been subjected to virtual "overload" conditions. Peak 1 only becomes radioactively labelled/



HYPER-IODINE RAT THYROGLOBULIN AFTER ^{131}I INJECTION, 5min. TO 24 hours.
^{125}I Background labelled.

o—o ^{125}I

●—● ^{131}I

— protein

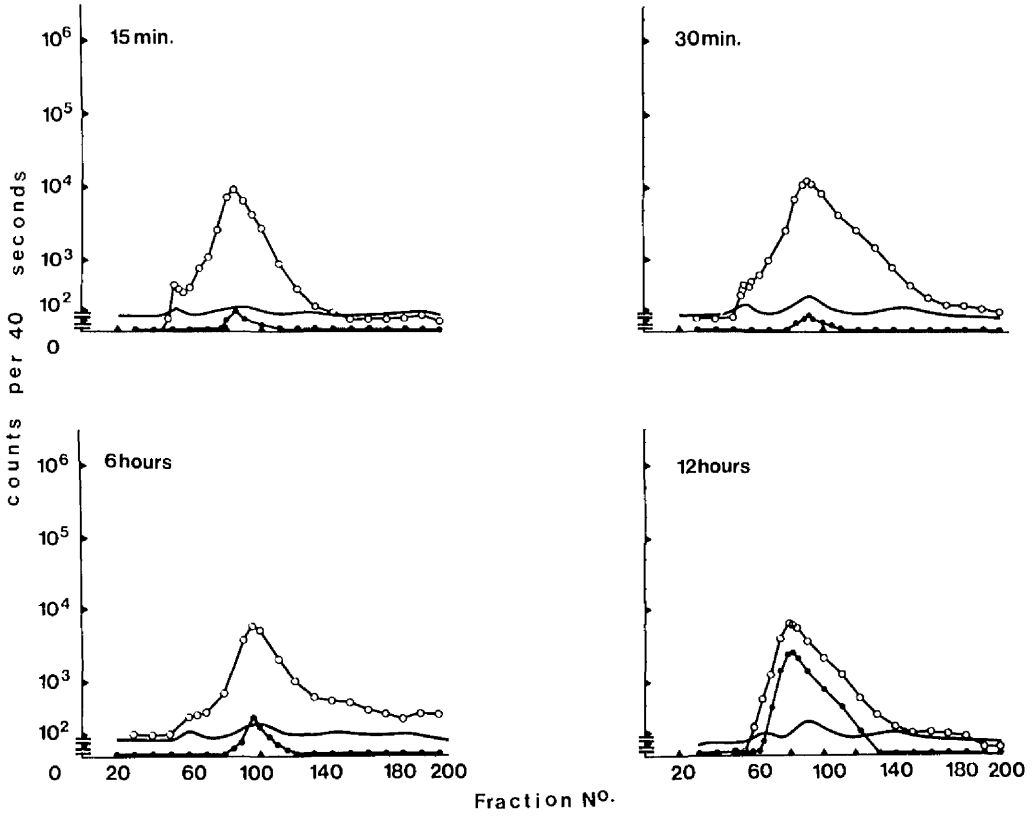


Fig. 25. Time course of radioactive iodine incorporation into peak 1 and peak 2 of hyper-iodine rat thyroglobulin in vivo.

/labelled 12 hours after ^{131}I injection and even then does not show differentiation from the label found in peak 2. This is due to peak 1 only beginning to be labelled at this particular time. The 24 hour results show peak 1 has been labelled with ^{131}I and is also differentiated from the label found in peak 2 (that is, peak 1 possesses a separate labelled peak and not just a shoulder on peak 2). This slowing down of iodine uptake by both peak 1 and peak 2 was due to the hyper-iodine diet which was fed to the experimental animals. The fact that peak 1 was not labelled until 12 hours had elapsed from the time of injection whereas peak 2 was iodinated after 10 minutes indicates that peak 1 might be iodinated due to the breakdown, or recycling of iodinated molecules found in peak 2.

The mechanism involved could therefore consist of the breakdown of iodinated tyrosine residues within the thyroid (represented previously in Fig 2), with release of iodine within the thyroid cell which is then able to be taken up by the lipoprotein which forms Peak 1. This experiment shows that by the intake of large quantities of non-radioactive iodine the processes carried on in the thyroid gland may be monitored at a relatively "slower speed" due to the dilution factors involved when radioactive iodine pulse is administered.

It would, therefore, seem that the iodinated lipoprotein which makes up peak 1 is some form of storage molecule for recycled iodine in the thyroid.

Further dietary experiments were set up to investigate the iodine storage role of peak 1. These experiments included, as supplements /

/supplements to the diets used in the preceeding labelling experiments, the addition of an iodine organification inhibitor PTU.

(d) PTU Treated Rats

To study the effect of an iodine organification inhibitor, three experiments were carried out, using a group of experimental animals in each of the three previously described dietary regimes, that is, control diet, hypo-iodine and hyper-iodine diets. Each of these diets was supplemented with 200mg PTU/Kg diet to cause partial inhibition of iodine organification. These diets were fed for the same period as before but only one time of sacrifice after ^{131}I injection, namely 6 hours, was used. This procedure was adopted to give sufficient time for the ^{131}I pulse (8μCi) to be incorporated into the various iodoproteins in each group so that each experiment could be directly compared to the others within the group. The injections and representation of results were as previously described.

As can be seen (Fig 26) in all three cases the protein trace is significantly different from all others previously obtained.

This is due to the effect of PTU on the thyroid, in that there are now presumably present 32S iodoprotein and 27 S iodoprotein (as shown by previous work in this department) as well as peak 1 and peak 2 (19S thyroglobulin). This different elution pattern from the sepharose 6B column shows that peak 1 still elutes first from the column followed by 32S, 27S iodoproteins and then 19S thyroglobulin. The production of both 32S and 27S iodoproteins, however, does not interfere with the labelling experiments to any great degree.

In/

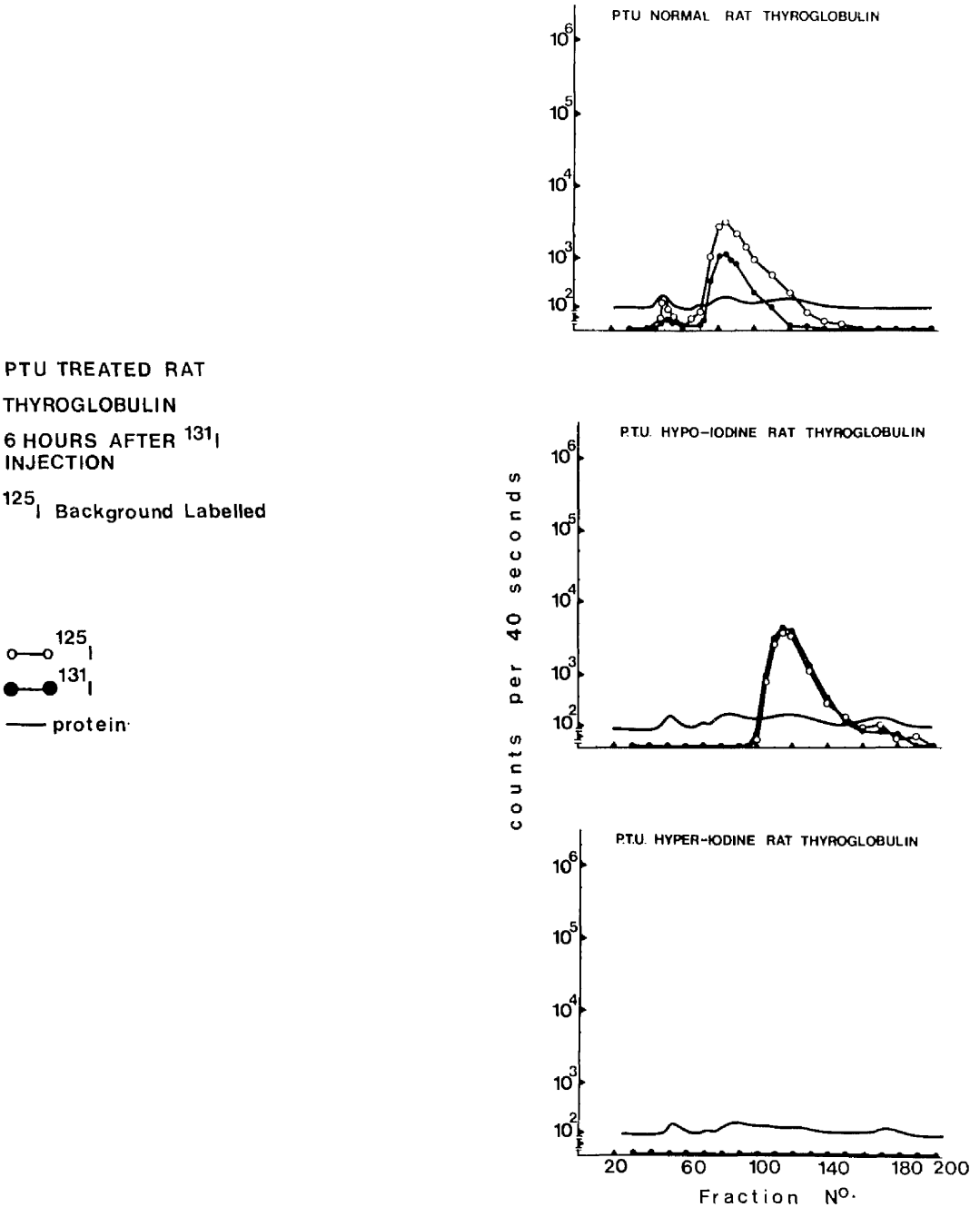


Fig. 26. In vivo radioactive iodine incorporation into peak 1 and peak 2 of normal-iodine, hypo-iodine and hyper-iodine rat thyroglobulin, from animals fed a diet containing an inhibitor of iodine organification (PTU) in conjunction with varying iodine concentrations in the diet.

/ In the control diet, PTU treated group the incorporation of ^{125}I has proceeded to a greatly reduced degree than in the corresponding control group which shows that the PTU addition to the diet is having a marked effect. Both peak 1, 27S iodoprotein peak, and peak 2 have been labelled by this isotope, indicating that peak 2 has still possessed sufficient iodine for the iodination of peak 1 and 27S iodoprotein to be carried out. (19S thyroglobulin has a greater affinity for iodine than 27S iodoprotein). The ^{131}I pulse has been taken up by these three molecules in a similar manner to the ^{125}I but again to a much lesser degree than normally would have occurred. This set of results, thus sets the base-line for comparison of the other two experiments in the series.

In the case of the hyper-iodine, PTU treated group, the administration of PTU has again produced the 32S and 27S iodoprotein peaks, but incorporation of both ^{125}I and ^{131}I has not occurred. This is due to two factors, namely the inhibitory effect of PTU and the dilution effect on the isotopes of the massive amounts of non-radioactive iodine present in the animals diet. These results are helpful in clarifying the position, in that they show the effects of diet and also provide corroboration of the lessening uptake of radioactivity produced in the previous series of experiments by the presence of large quantities of non-radioactive iodine.

The final group in this series of experiments were those animals fed a hypo-iodine PTU supplemented diet. The PTU treatment again produced the other peaks in the column elution pattern but in this/

/this case the only peak which had incorporated either ^{125}I or ^{131}I was found to be peak 2, that is, 19S thyroglobulin. Both of these isotopes had been taken up in similar quantities by this peak, which indicates that the dietary regime had been effective in making the thyroid deficient in iodine. Since no other iodoprotein or other iodine containing molecule was labelled it would appear that 19S thyroglobulin had avidly trapped any available iodine and incorporated it for the production of thyroid hormones, and so no overlap of labelling was permitted.

This series of results again indicates that peak 1 does not actively trap iodine but only becomes iodinated passively, probably due to an iodine re-cycling process from peak 2.

(e) Effect of Diets on Circulating Thyroid Hormone Levels

To obtain further indication of the effect of all the diets used and to provide a comparison between experimental groups both T_3 resin uptake and T_4 tests by the Oxford method were carried out. The experimental animals were fed the same diets, for the same period, as those previously fed to each of the experimental groups before the thyroid function tests were done. The results (Table 6) give an indication of thyroid hormone levels both in normal untreated (control) animals and in the experimental groups. The results show some degree of variation in hormone levels, the most striking being those of the groups fed a PTU supplemented diet, which again shows that the dosage of this compound used was effective.

(f) In vitro Studies of Iodine Incorporation /

Experimental Group	T_3 Index	T_4 nm/l
Control (Normal Iodine)	1.29	59.5
Hyper-iodine	1.32	89.0
Hypo-iodine	1.26	53.6
PTU Normal Iodine	1.05	3.0
PTU Hyper-iodine	1.21	10.0
PTU Hypo-iodine	1.17	0.0

TABLE 6

Results of Oxford T_3 Resin uptake and T_4 tests carried out on each of the experimental groups.

/ (f) In vitro Studies of Iodine Incorporation

An in vitro labelling experiment was carried out to discover if the incorporation of iodine into the thyroid differed from the incorporation in vivo. This experiment was set up for the maximum time possible, which was four hours, since after this time the thyroglobulin in the glands would begin to be leached out into the buffer system. Freshly prepared Krebs No 2 buffer pH7.4 ± 0.05 was gassed in a stream of oxygen to ensure that the thyroids would not be starved of oxygen. Thirty rats fed on normal-iodine diet were sacrificed and their thyroids excised and bisected. The bisected glands were placed into 5mls of the buffer in a flask and allowed to pre-incubate at 37°C (water bath) under a constant stream of oxygen for 15 minutes. At this point 16μCi of ^{125}I and 8μCi ^{131}I were added to the flask (0.2mls of each isotope) and the glands allowed to incubate for four hours. After this time the thyroglobulin was harvested and purified as before and the incorporation results obtained as previously described (Fig 27). The only significant difference obtained in the incorporation was a decrease in the background label of ^{125}I. This is to be expected since background labelling usually proceeds for some 48 hours. A decrease in ^{131}I incorporation into both peak 1 and peak 2 was also noted, again this is to be expected since iodine uptake will necessarily be impaired under in vitro conditions. The overall incorporation pattern, however, is similar to those found in the control group. These results also show that peak 1 can be labelled in vitro which would rule out any interference possibilities such as peak 1 becoming iodinated due to proximity/

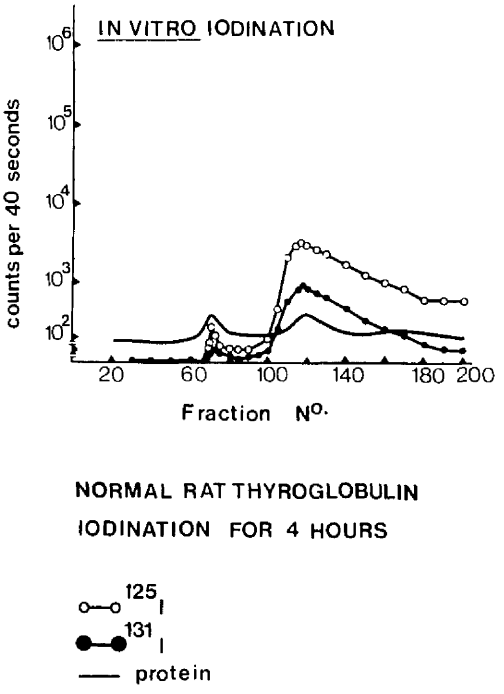


Fig. 27. In vitro radioactive iodine incorporation into peak 1 and peak 2 of normal-iodine rat thyroglobulin.

/proximity with iodinated molecules in the bloodstream of the experimental animals.

The in vitro experiment shows that both ^{125}I and ^{131}I were incorporated into peak 1 and peak 2 in proportion to their concentrations. The pattern of labelling in vitro is similar to that in vivo, in that the labelled iodine is preferentially incorporated into peak 2 with a smaller amount of label incorporated into peak 1.

SECTION 5

DISCUSSION

CHAPTER 12

DISCUSSION OF RESULTS

Purification Procedures

This work was prompted in the first instance by the seemingly anomolous elution pattern obtained from passage of a sample of "purified" thyroglobulin through a sepharose 6B column. This elution pattern gave three main peaks which indicated that more than one molecular species was present in the thyroglobulin sample.

Column chromatography or gel filtration can be described in the following way: molecules larger than the largest pores of the swollen gel, that is, above the exclusion limit, cannot penetrate the gel particles and therefore pass through the bed in the liquid phase outside the particles, and so are eluted first. Smaller molecules, however, penetrate the gel particles to a varying extent depending on their size and shape and are therefore eluted from the gel in order of decreasing molecule size.

Since the exclusion limit of sepharose 6B is molecular weights in excess of 4×10^6 and the first of the peaks (peak 1) of the so-called purified thyroglobulin sample elutes just behind the void volume of the column it can be assumed that this molecule is of high molecular weight. This assumption is verified by the fact that the separation obtained on sephadex G200 gave one single peak of the normal distribution type, again just behind the void volume, and so would comprise of molecules both larger and smaller than thyroglobulin./

/thyroglobulin. For these reasons it was assumed that the "purified" sample of thyroglobulin was in fact still in an impure condition, and, since the sample used was personally prepared, it was decided to chromatograph a commercially available sample of thyroglobulin to give an indication of its purity. The results obtained showed that commercially available material possessed the same elution pattern and so was also impure. These results indicated that the purification procedure carried out in the laboratory and by chemical suppliers was incomplete.

On further examination of the purification procedure employed in the laboratory, namely fractional precipitation of proteins with 70% ammonium sulphate solution, it was found that after up to 20 precipitations the same elution pattern was obtained, and, therefore, the fault in the purification seemingly lay in this method. Various other concentrations of ammonium sulphate solution, both more concentrated and less concentrated were employed to obtain a more highly purified sample or at least to furnish a sample free from high molecular weight contaminants. These methods were found to give identical results to those obtained previously and so it was assumed that the purification procedure using ammonium sulphate was not entirely satisfactory. Since sepharose 6B has the ability to separate a sample of thyroglobulin into three main peaks, this method could be employed as a final step in the purification procedure. When fractions collected from a sepharose 6B column were pooled, (only the three fractions making up the summit of the peak, to avoid any possibility of contamination from other peaks), desalted, freeze-dried and separately/

/separately applied to the column a single peak was obtained in the same position as the pooled fractions eluted previously. This method therefore, produced pure samples of all the peaks which comprised the impure thyroglobulin sample. The method also produced fairly large quantities of purified material, for example, peak 2 can be obtained in yields of up to 400mg, and so with the use of larger sepharose 6B columns gram quantities of purified thyroglobulin could be produced.

This method of purification was adopted for the production of samples for this present study, since adequate quantities of material could be obtained, simply and easily, without the need to design and test a completely new method of purification, and also a higher degree of purity could be obtained than in commercially available products.

Analysis of Absorbance Characteristics and Ultracentrifugal Patterns

The unusual absorbance spectrum obtained for peak 1 (Fig 6) showed that this molecule was something other than pure protein, and since, by various methods the possibility of a protein-RNA complex was ruled out, the conclusion reached was that this molecule contained protein but also some other molecule. The value of the spectrum (Fig 6) results is that the field of possibilities could be quickly narrowed to certain molecules which are composed of protein complexed with, for example, carbohydrate or lipid.

Considering the possibility of a protein-carbohydrate complex; it would be unlikely that the spectrum results obtained would be produced by only a small portion of the complex being carbohydrate since peak 2 (that is, thyroglobulin) has been shown to contain/

/contain 8-10% carbohydrate material, which still gives a spectrum which is similar to that normally found with protein. This leads to the assumption that for any marked effect by carbohydrate, in the spectrum of a protein-containing molecule the amount of carbohydrate present must be in a concentration in an excess of 10% of the protein present. This would suggest that the biosynthesis of peak 1 was totally different from that of thyroglobulin. The purification procedure employed was for protein and so a large excess of carbohydrate present within the molecule would tend to keep the molecule in solution under the conditions required for the precipitation of protein. This suggestion is strengthened by the results obtained from sucrose density gradients. For a molecule to be comprised of an excess of carbohydrate and seemingly disappear or float on top of any given sucrose density gradient range is highly unlikely, since carbohydrate polymers would be expected to behave like any other high molecular weight species and so sediment within the gradient. It was not expected, therefore, that a protein/carbohydrate complex would be of such low density as to be untraceable under these conditions. Further investigation of peak 1 by methods designed specifically for the detection of carbohydrate showed that peak 1 was lacking in this material, whereas peak 2 possessed substantial amounts of carbohydrate. This result finally excluded the possibility that the unusual spectrum was due to the presence of a protein/carbohydrate complex, which, by process of elimination led to the investigation of the possibility of peak 1 being a protein/lipid complex.

It /

/ It would be reasonable to assume that for lipid material to have any marked effect on the spectrum of a protein-containing molecule it would have to be present in excess. However, it would be possible to precipitate a protein/lipid complex under the conditions necessary for the precipitation of protein since the lipid fraction of the molecule would probably be composed of long chain fatty acids or triglycerides which would have no effect in keeping the molecule soluble in aqueous systems. The gradient results, that is, the disappearance of peak 1, can also be explained by the presence of lipid in the molecule. Lipids tend to be high molecular weight but low density molecules and so peak 1 was thought to possibly be a lipid-containing protein such as a lipoprotein. In due course by various means this peak 1 molecule was eventually identified as a pre-/3 lipoprotein when compared to human serum lipoproteins. This result, however, does not mean that peak 1 is a serum lipoprotein, since various factors, which will be discussed later show it to be thyroidal in origin. Another factor which indicates that the results obtained, both for spectrum and gradients, are likely to be valid if the molecule is a lipoprotein is the fact that lipoproteins usually only consist of up to 20% protein, and therefore up to 80% lipid would obviously interfere with the spectrum produced and also give the molecule high molecular weight but low density characteristics.

Amino Acid Analysis

The amino acid analysis shows the overall amino acid composition of both peak 1 and peak 2. On examination of the results obtained, it was found that six amino acids in peak 1 were significantly/

/significantly different from peak 2. These amino acids were methionine, histidine, α amino-n-butyric acid, allo-isoleucine, β alanine and γ amino butyric acid. The most significant differences were the absence of methionine and γ amino butyric acid from peak 1. Since methionine is a sulphur-containing amino acid, the absence of this particular molecule from the lipoprotein might have been expected to produce fewer sites for disulphide bridge formation. This is not the case since the sulphur of methionine is contained within the carbon skeleton of the amino acid and not in a terminal position as in the case of cysteine. Therefore the possibility of disulphide bridge formation may be ruled out. The absence of methionine from the peak 1 molecule, although being a significant difference, would not be expected to produce any major structural differences between peak 1 and peak 2.

A further ten amino acids (Table 3) were found to be present in peak 1 and peak 2 in an approximate ratio of 1:5, the most significant of these being tyrosine which would impart the facility of iodination to peak 1, which has been proven by testing both peaks 1 and 2 for iodine content by the estimation of the protein bound iodine (PBI).

The ratio of 1:5 found for approximately half of the amino acid composition of the peak 1 molecule can be explained in terms of lipoprotein structure. As previously stated lipoproteins are composed of up to 20% protein and so taking peak 2 as fairly normal protein, (it is actually a glycoprotein, but consists of 90-92% protein), it would be reasonable to expect the amino acid analysis of peak 1 should contain a lower concentration of amino acids than that for peak 2./

/peak 2. Since the concentration difference is approximately 1:5 it would appear that peak 1 is only composed of one-fifth of the protein of peak 2. Taking this result into consideration and also that thyroglobulin is 8-10% carbohydrate the composition of peak 1 can be estimated as being 18-18.4% protein and the remainder lipid.

Another hypothesis which can be made when considering this ratio between peak 1 and peak 2 is that peak 1 may be a shortened, precursor or storage form of the protein which makes up peak 2, the lipid being attached to render the molecule inert until such times as it is required by the thyroid for the production of thyroglobulin. This hypothesis does not appear to be correct since other molecules such as 12S, 27S and 3-8S iodoproteins take part in the biosynthesis of the thyroglobulin molecule, and it seems more logical to produce sub-units or aggregations of the completed molecule than to produce a shortened molecule and then render it inert with long chain fatty acids or triglycerides which would then require to be broken down by specific enzymes before resynthesis of the thyroglobulin molecule could take place. It would, therefore, appear that the peak 1 molecule played some other role, perhaps a storage function within the thyroid.

Carbohydrate

The absence of a carbohydrate moiety from the peak 1 molecule indicates that the biosynthesis of this molecule is different from that of thyroglobulin, since after polypeptide chain formation, carbohydrate material is added in a stepwise manner to the growing thyroglobulin molecule. It, therefore, would not seem logical for the thyroid/

/thyroid gland to assemble thyroglobulin-like polypeptide chains and then instead of attaching carbohydrate material add in lipid, so producing a molecule which was not utilizable in the production of thyroid hormones. This would again indicate a different role for this molecule.

Differences in the structure of the peak 1 molecule due to the absence of carbohydrate and certain amino acids could produce differences in the secondary and tertiary structures, and so would cause major differences in the properties possessed by the molecule such as different electrophoretic mobility, solubility, and other physical properties.

Origin of Peak 1

Various methods, including ultra-centrifugation, staining and column chromatography have excluded the possibility of the peak 1 lipoprotein being an artefact of the purification procedure for thyroglobulin, contamination from cell membrane particles, or from serum lipoproteins, which suggests that this particular molecule is thyroidal in origin.

Most lipoproteins are manufactured either by the liver or small intestine and as a result eventually appear in the serum. Since the peak 1 lipoprotein does not seem to be similar to serum lipoproteins, its site of manufacture may be other than those mentioned previously. It would also be a strange coincidence, for example, for the liver to produce a lipoprotein with a protein moiety very similar to that possessed by thyroglobulin, because if this were the case the liver could probably manufacture thyroglobulin and there would be no necessity for/

/for the thyroid gland.

The small intestine processes dietary lipoproteins and also produces chylomicrons which are passed to the lymphatic system. Although the thyroid has lymphatic drainage it is very unlikely that any dietary lipoprotein would possess a similar peptide structure to thyroglobulin. Therefore, it seems reasonable to assume that the peak 1 lipoprotein is thyroidal in origin.

Electrophoresis and Electrofocussing

The results obtained from agarose gel electrophoresis show that peak 1 is more electronegatively charged than peak 2 and so migrates slightly further towards the anode. These results are seemingly contradicted by the results obtained from electrophoresis on polyacrylamide gel, since peak 2 was found to have migrated slightly further than peak 1. These results cannot be explained on the grounds of the molecules possessing different overall net charges, since, the buffer pH in both types of electrophoresis was pH8.5. The difference in migration can, however, be explained by the fact that in agarose gel electrophoresis, sample mobility is dependent solely on net charge characteristics possessed by the molecule, whereas migration in polyacrylamide gel electrophoresis depends on both net charge and molecular size of the sample molecule. The effect of peak 1 being more electronegatively charged than peak 2 is obviously more than cancelled out by the fact that peak 1 is a larger molecule and so is retarded by the gel. These/

/These results, therefore, show that peak 1 is a larger molecule than peak 2 which tends to lend support to the results obtained from column chromatography.

SDS acts as a form of detergent and so breaks hydrogen bonding within the molecule, and being a highly charged molecule thus nullifies charge characteristics. Results obtained from SDS-containing gels again show that the peak 1 molecule is larger than the peak 2 molecule. When compared with suitable standards the sample molecular weights can be determined since under these conditions molecular weight is directly proportional to distance migrated. The molecular weight thus obtained for peak 2 (thyroglobulin) is 670,000 which shows an error of 1.52% from the published value of 660,000 and so the determined molecular weight of 700,000 for peak 1 would presumably be judged to have an error of the same magnitude, which still shows that the peak 1 molecule is larger than peak 2.

Further investigation of samples of both peak 1 and peak 2 on polyacrylamide gel electrophoresis, but in the presence of SDS and BME which disrupts disulphide bonds show the sub-unit or at least the constituent polypeptide chains, which have been separated due to the rupture of disulphide bridges in both molecules. The main differences between peak 1 and peak 2 under these conditions are the total absence of three low molecular weight polypeptides from peak 1. Further evidence that this lack of three low molecular weight polypeptides may be the main structural difference between both molecules is the fact that five out of the other six polypeptide bands present on the/

/the gels migrate to exactly the same distance, which indicates that they are of similar molecular weights if not identical in structure. It would seem reasonable to assume, therefore, that the absence of the three low molecular weight polypeptides from the peak 1 molecule was the reason for the peak 1 protein moiety appearing to be a shortened peak 2 molecule.

One polypeptide in each of the peak 1 and peak 2 gels appears darker than all the others in their respective gels (Fig. 21). This indicates that these polypeptides are present in larger quantities than the others which would presumably mean that they are present in more than one position in the protein molecule. This repetition of certain polypeptides is fairly common within protein molecules. However, the repeated polypeptide in peak 1 does not correspond (on molecular weight/distance-migrated evidence) to that of the peak 2 molecule. This evidence again shows that peak 1 and peak 2 are similar but significantly different molecules with perhaps different functions within the thyroid gland.

So far comparisons between peak 1 and peak 2 have been made mainly on molecular size differences except for electrophoresis carried out on agarose gel, which showed a charge difference between the two molecules. Further investigation by the technique of iso-electric focussing, the two main applications of which, in biochemistry, are the analytical separation of high molecular weight ampholytes, specifically proteins, according to their iso-electric points and characteristion/

/characterisation of proteins by their iso-electric points, gave differing results. This method needs only a difference of 0.02 pH units between two proteins to enable a separation and characterisation to be successful. The IEP found for peak 1 was pH4.48 and that for peak 2 was 4.68 which substantiated the difference in mobility between these two molecules on agarose gel electrophoresis. The results were also found to be reproducible to within 0.02 pH units which would appear to be the limiting error of the method. The IEP results for the two molecules are sufficiently different from each other, but, within the same pH region, for the molecules to be entirely different but to be related in some manner, either structurally or functionally.

Iodination

The protein bound iodine content of peak 1 was found to be 20% of that of peak 2 which helps to substantiate the fact that peak 1 is a lipoprotein molecule, since lipoproteins are composed of up to 20% protein the remainder being lipid. The results also show that the iodine is probably protein bound, presumably, by the amino acid tyrosine and not bound to the lipid fraction of the molecule as the PBI results for peak 1 and peak 2 are found to be in a 1:5 ratio which directly relates to data obtained for amino acid content of both molecules. Since lipid possesses an affinity for iodine, if the iodine was bound to the lipid part of the molecule it would be expected that the iodine so bound would not bear any relationship to the data obtained for amino acid content of both peaks since the greater part (up to 80%) of the peak 1 molecule is composed of lipid, and so far greater binding of iodine would occur. (54). The PBI results do not show what mechanism,/

/mechanism, if any, is utilized for the trapping or retention of iodine by the peak 1 molecule. Further insight into the peak 1 iodination mechanism is given by the results obtained from the in vivo incorporation of radio-iodine into both peak 1 and peak 2 molecules under different dietary regimes with subsequent different thyroid status. The results obtained in the control group indicate that peak 1 does not actively trap iodine but is passively labelled with radio-iodine. Only after a time-lag of some 15 minutes does peak 1 begin to accumulate iodine which suggests that an iodine recycling process within the thyroid was taking place. This could be for instance iodine being passed from thyroglobulin to the peak 1 lipoprotein. Further evidence of this recycling might be found and the above results enhanced if the thyroid was made iodine deficient. Incorporation of iodine into thyroglobulin was increased dramatically in iodine deficient rats, from that observed for the control experiment but the time-lag of incorporation of iodine into peak 1 was increased from 15 minutes to one hour which indicates that iodine incorporation is passive in that the peak 1 molecule does not seem to actively trap iodine like peak 2, that is thyroglobulin. The increased time-lag of incorporation of ^{131}I into peak 1 between the control and iodine deficient experiments, suggests that the labelling of peak 1 is dependent on the prior labelling of peak 2. This supports the suggestion that peak 1 is a recycling or storage depot for excess iodine within the thyroid. The time-lag increase is most probably due to the thyroglobulin molecule being so avid for iodine that no recycling of iodine is permitted until a satisfactory level of iodination can be maintained in the peak 2/

/peak 2 molecule.

Results obtained from hyper-iodine experiments show that radio-iodine incorporation proceeds at a much slower rate than either of the control or hypo-iodine experiments. This is probably due to both isotope dilution effects and iodine saturation effects on the molecules concerned. The time-lag of incorporation of ^{131}I is extended to 12 hours after the time of injection, which substantiates the theory that peak 1 is a passively labelled storage compound since under the extreme conditions of overload with iodine, virtual steady state conditions would be produced and so any recycling of radio-iodine from peak 2 to peak 1 would require a longer period of time. The 12 hour lag also provides additional evidence that peak 1 becomes labelled with iodine from peak 2 which is recycled, since peak 2 becomes labelled only 10 minutes after isotope administration. It is possible that in iodine supplemented rats, peak 2 possesses so much unlabelled iodine which can be either used in thyroid hormone production or recycled, that the recycling effects of the pulse of ^{131}I do not show for such a long period of time.

The results of the above experiments suggest that the lipoprotein molecule, peak 1, does not avidly trap iodine but merely becomes passively labelled as some type of storage molecule for either recycled iodine from thyroglobulin or from breakdown products of thyroid hormones. The postulated mechanism of iodination of peak 1 would therefore involve the breakdown of thyroid hormones or the breakdown of the thyroglobulin molecule itself to release tyrosine in the form of monoiodotyrosine and diiodotyrosine which would be deiodinated. The/

/The iodine subsequently released from these two hormones would then be recycled through the iodine pool of the thyroid. Peak 1 lipoprotein may well be part of the thyroid iodide pool. It has been known for some time that iodine derived from the breakdown of MIT and DIT is treated by the thyroid in a different manner from newly trapped iodine. The former iodine fraction has been referred to as the second iodine pool of the thyroid gland.

The results from further dietary experiments involving the use of an inhibitor of iodine organification (PTU) clarify the role played by peak 1 in the thyroid.

The results obtained from the PTU experiment show that although the protein pattern from the sepharose 6B column now contains 32S and 27S iodoproteins as well as peak 1 and peak 2, the iodine incorporation into peaks 1 and 2 is not interfered with by the presence of these other iodoproteins. This is due to the affinity of 19S thyroglobulin (peak 2) for iodine superceding that of any other thyroidal iodoprotein. The iodine incorporation pattern although similar to that of the control experiment proceeds at a vastly reduced rate and since both peak 1 and 27S iodoprotein are labelled in addition to peak 2, it can be assumed that the PTU does not have any effect on the deiodination of MIT and DIT and the subsequent re-iodination of the tyrosine residues of the peak 1 molecule.

This set of results can be taken as the base-line for the PTU experiments in which the iodine content of the diet was varied. The hyper-iodine PTU experiment shows no incorporation of radio-iodine into either peak 1 or peak 2, but the effectiveness of the diet in/

/in altering the thyroid protein pattern can still be seen by studying the protein elution pattern which includes 32S and 27S iodoproteins. The lack of incorporation of radio-iodine can be explained by the effects of the PTU and the presence of an excess of unlabelled iodine. This result is, however, helpful in that when taken into consideration with the other results in the series a definite pattern emerges, namely that the incorporation of labelled iodine into peak 1 is hindered by both an excess of stable iodine coupled with an organification inhibitor and also by a low iodine diet coupled with PTU. The latter result was shown by an experiment where a hypo-iodine PTU diet was fed before administration of radio-iodine. The likely explanation for the lack of radio-iodine in peak 1 of the hypo-iodine diet experiment is that the affinity of 19S thyroglobulin for iodine is so great that no breakdown of thyroid hormones is permitted until every possible site within the thyroglobulin molecule has been iodinated. Comparison of this hypo-iodine result with that of the control group shows that incorporation of radio-labelled iodine into peak 2 is reduced (due to the presence of PTU) but no incorporation of background label has occurred in peak 1, which suggests that the thyroid hormones are not being broken down to release iodine to be recycled. This would seem to verify in a negative manner, that peak 1 is iodinated by an iodine recycling process.

So far, all the results considered have been for <u>in vivo</u> studies but the incorporation of iodine into peak 1 may differ significantly from the control group if carried out as an <u>in vitro</u> experiment. This was not found to be the case since radio-iodine incorporation/

/incorporation was found to proceed in a similar manner to that of the *in vivo* control group the only difference being in the amount of both isotopes incorporated into the two peaks. Quite naturally this must be lower than *in vivo* since thyroid function will necessarily be diminished under *in vitro* conditions and also labelling is carried out for a fraction of the time involved for the *in vivo* experiments. The result obtained, did, however, substantiate the labelling hypothesis which had been proposed as a result of the *in vivo* experiments and adds weight to the suggestion that the lipoprotein molecule which comprises peak 1 can be said to play a storage role for iodine in the thyroid.

CHAPTER 13

GENERAL DISCUSSION

The results presented in this work show that an iodinated lipoprotein of the pre/3 type exists in the thyroid in addition to thyroglobulin. This lipoprotein appears to incorporate iodine, but using radio-iodine is labelled much more slowly than thyroglobulin. Therefore, a brief discussion of how this molecule fits into current knowledge of thyrolipids would seem appropriate.

Controversy exists as to whether thyroidal lipids bind iodine specifically and whether lipid-bound iodine in the thyroid can be reversibly released in the cell. Work has shown that in the rat thyroidal lipids are not preferentially iodinated as compared to those of other organs (55) Shah et al (56+57) have found an unidentified lipid fraction (fraction II), which is Ninhydrin negative, phosphorus negative, alkali labile, acid stable, phospholipase A stable, and orcinol positive, indicating the absence of a free amino group, phosphorus group, vicinal phosphorus ester linkage, vinyl ether linkage and the presence of a sugar moiety. This fraction II seems to be iodinated specifically by the thyroid since other iodide concentrating tissues such as the submaxillary salivary gland and the stomach localise radio-iodine with phosphatidyl choline. This fraction II may be the peak 1 lipoprotein discovered during this work. However, the presence of a sugar moiety is against this explanation.

/The

/The function of fraction II appears to be related to thyroxine formation, the degree of iodination of fraction II being modulated by the amount of thyroxine formed within the gland. This function is similar to that found for the peak 1 lipoprotein since this molecule only becomes iodinated as iodine is recycled from the breakdown of thyroid hormones, and iodination of peak 1 could be said to be dependent on thyroid hormone levels.

The fraction II of Shah was prepared using methods for the extraction and purification of lipids, whereas the peak 1 lipoprotein was prepared using techniques for the extraction and purification of proteins. Fraction II has been studied as a lipid whereas peak 1 lipoprotein has been investigated mainly as a protein. Both of these molecules could therefore be synonymous, as a carbohydrate moiety of the peak 1 lipoprotein could presumably be lost during the aqueous extraction/purification procedure adopted for the production of this molecule, whereas the organic procedure adopted by Shah for the production of fraction II would preserve this carbohydrate moiety.

Other forms of lipid found in the thyroid gland can be classified as phospholipids, glycolipids, sulphatides, fatty acids, or triglycerides, do not fall within the same category as the peak 1 lipoprotein and so can be discounted as being relevant to this study. It would therefore appear that the fraction II of Shah is the most similar of the thyrolipids, (so far described in the literature) to the peak 1 lipoprotein. This latter molecule can most readily be placed in a comparable position to that of fraction II, described by Shah, in the scheme of current knowledge of thyrolipids.

SUMMARY

Purified thyroglobulin, both commercially available and personally prepared by ammonium sulphate precipitation, when fractionated on sephadex G200 shows only one peak whereas on sepharose 6B three peaks are found, two of which were examined further and designated peak 1 and peak 2. Both peaks are large molecular weight molecules.

Ultracentrifugation, on sucrose gradients, of purified peak 1 and peak 2 showed peak 2 to behave similarly to 19S thyroglobulin. Peak 1, however, was not found on these gradients, which suggests that it is a low density molecule.

Polyacrylamide gel and agarose gel electrophoresis show peak 1 to have different electrophoretic mobility and to be composed of different components from peak 2. Iso-electric focussing showed peak 1 to have a lower iso-electric point that peak 2.

Amino acid analysis showed significant differences between the two peaks, which is further verified by values for protein bound iodine, peak 1 having 20% of the iodine content of peak 2. The absorbance spectrum between 230nm and 300nm proves that both peaks are composed of different substances. Peak 2 was shown to be 19S thyroglobulin. Peak 1 contained triglyceride and cholesterol in addition to amino acids and was therefore considered to be a larger molecular weight very low density lipoprotein of the pre-beta type.

Time course of radioactive iodine labelling (^{125}I, ^{131}I) of both peaks by intraperitoneal injection using iodine deficient, normal iodine, and iodine enhanced rats showed that peak 2 actively trapped/

/trapped iodine but peak 1 appeared to be passively labelled. Further time course labelling experiments using an inhibitor of iodine organification verified that peak 1 was passively labelled but appeared to be iodinated from the recycling of iodine from deiodinated thyroid hormones.

Since the lipoprotein which constitutes peak 1 contains significant stable iodine, this may be the second iodine pool of the thyroid gland.

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