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INCORPORATION OF LABELLED CARBOHYDRATES INTO THE SOLUBLE THYROID PROTEINS OF RAT AND MAN

by

John A. Thomson

M.D. University of Glasgow

F.R.C.P. (Glasgow)

M.R.C.P. (London)

Senior Lecturer in Medicine,

University Department of Medicine,

Royal Infirmary,

Glasgow.

Thesis submitted for the degree of Doctor of Philosophy,

University of Glasgow

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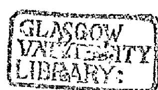

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SECTION 1

INTRODUCTORY

Chapter 1	Introduction
Chapter 2	Chemistry of thyroglobulin
Chapter 3	Biosynthesis of thyroglobulin

CHAPTER 1

INTRODUCTION

In all vertebrates the thyroid gland is one of the important endocrine organs. Like all endocrine organs it has the function of synthesising within itself active substances which it releases as required into the blood stream to act at distant sites. In the case of the thyroid these active hormonal substances formed may be divided into two groups. Firstly, there are the two important hormones, thyroxine (T_4) and triiodothyronine (T_3) which act to regulate the general metabolic state of the body. The second type of hormone formed in the thyroid is the recently discovered calcium-lowering hormone, calcitonin.

On looking at a section of thyroid tissue, for instance the rat thyroid as shown in Fig. 1, it will be seen that the thyroid gland is made up in the resting state of approximately circular rings of cellular material, the spaces within the circle of cells being filled by an amorphous protein material, the colloid. Serial sections reveal that the cells surrounding the colloid form spheres which are referred to as follicles. The amount of colloid present and the height of the epithelial cells of the thyroid follicles is determined by the state of activity of the thyroid gland. The normal vertebrate thyroid gland is rather unusual amongst the endocrine organs in having in its substance a much larger supply of its secretion product, T_3 and T_4, than exists in

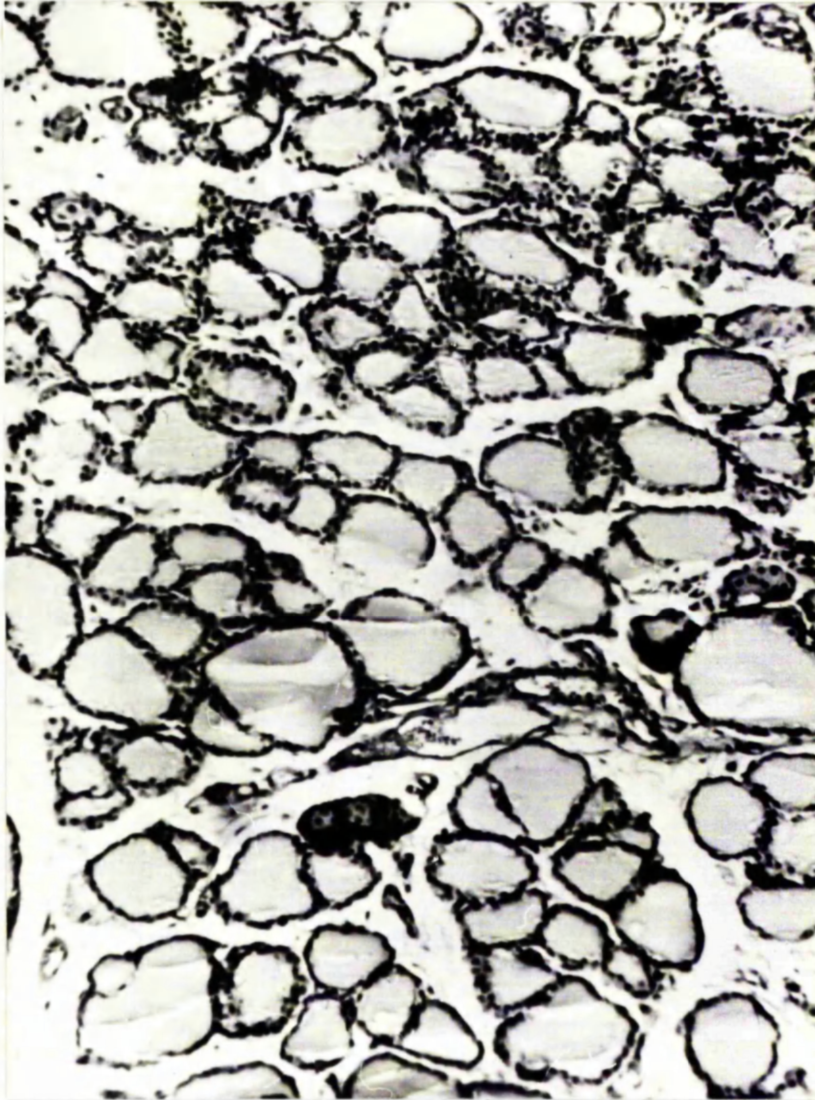


Fig. 1

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

other endocrine organs such as the adrenal which has only a small supply of preformed cortisol present.

The normal thyroid gland is not autonomous but functions physiologically under the control of the pituitary which releases from certain cells of the anterior pituitary a glycoprotein hormone, thyroid stimulating hormone (TSH), which is secreted in response to a falling blood level of T_4 and T_3 to stimulate the thyroid to greater activity (Fig. 2). The pituitary itself is likewise under control of the hypothalamus by means of the recently described tripeptide hormone, thyrotrophin releasing hormone (TRH). One must also bear in mind that the hypothalamus appears to be under a similar negative feedback control from the thyroid gland by means of falling levels of the thyroid hormones. It is also under a regulatory effect from the higher centres in the cortex. The exact nature of this controlling mechanism has not yet been defined clearly.

When stimulation of the thyroid gland by the TRH and TSH mechanisms comes into play the resting thyroid gland responds by an additional release of its preformed hormone from the colloid. The epithelial cells are stimulated to hypertrophy which they do by changing from a flattened cuboidal epithelium to a rather tall columnar epithelium and therefore in the stimulated gland the ratio of colloid to cell as seen on histological section is greatly diminished.

Apart from the follicular epithelial cells, there is another variety of cells to be found in the thyroid especially in certain

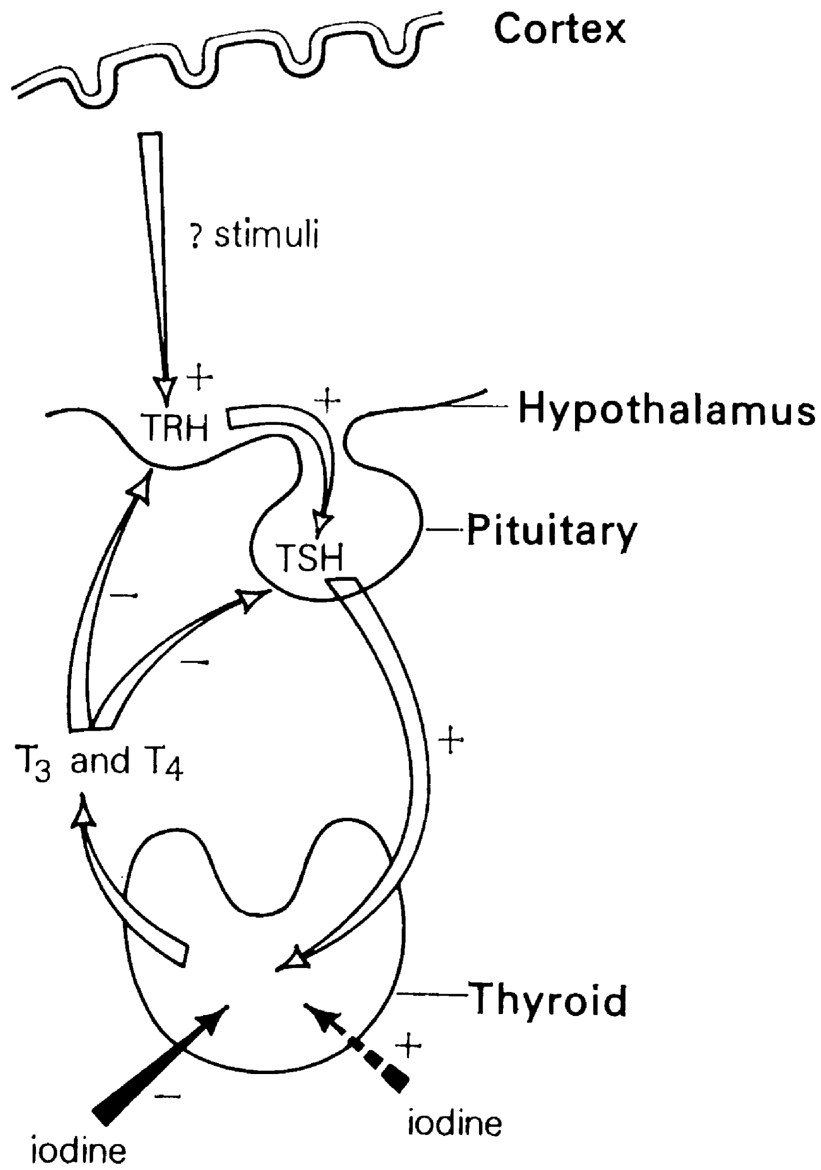


Fig. 2

Mechanisms of regulation of thyroid function.

species such as the bat. These can be shown by special stains to belong to a different class of cell from the thyroid follicular cells and have been called the parafollicular cells, 'C' cells or light cells, and are currently believed to be the source of the calcium-lowering hormone, calcitonin, alluded to above. The effects of this calcium-lowering hormone are not pertinent to the present work and are not discussed further.

More detailed microscopic study of the thyroid epithelial cell has shown it to have a rather unusual structure. It rests on a basement membrane which places it in very close juxta-position to the capillary networks around the follicles. The apical end of the cell, that is the part which points towards the central colloid, has an irregular border which appears on electronmicroscopy to be composed of microvilli. These structures are believed to play an important role both in the secretion of the thyroid hormones into the follicular colloid and also in resorption of the colloid from the follicle. Prominent vesicles can be seen within the cell cytoplasm which are thought to represent resorption particles of thyroglobulin. The thyroid cell has a large nucleus usually situated in a basal position. The cell is well supplied with the subcellular particles associated with an active synthetic and secretory cell, namely a rough endo-plastic reticulum, mitochondria and a prominent Golgi apparatus.

Since the advent of radioactive isotopes of iodine coupled to chromatographic, electrophoretic and ultracentrifugal techniques for examining the thyroid gland we have gained a great deal of insight

into the mechanism of the formation of the thyroid hormones. These studies have been greatly helped by the use of such modern techniques to study families who have congenital defects in biosynthesis of the thyroid hormones and by the advent of various antithyroid drugs which block different steps in the biosynthetic pathway. The broad steps involved in the process of synthesis of T_3 and T_4 are outlined below but it should be remembered that we have by no means a final idea of the intimate details of the processes involved. The accepted main steps consist of:

(a) The trapping of iodine by the thyroid from the capillary blood. This step is blocked by perchlorate and thiocyanate ions.

(b) The conversion of the trapped iodide to some as yet undefined form of iodine probably by some form of oxidative process mediated by the peroxidase enzymes present in the thyroid gland.

(c) The incorporation of this changed iodine into tyrosine residues. Where one iodine atom per tyrosine residue is added the substance monoiodotyrosine (MIT) is formed and where two iodine residues per tyrosine molecule are added the substance diiodotyrosine (DIT) is formed. This step is blocked by many drugs including the thiouracil series and the thiocarbamide drugs such as carbimazole.

(d) The coming together of these iodinated tyrosine residues to form T_4 and T_3. The latter theoretically could be derived either from the joining together of one DIT and MIT molecule

or conceivably from two DIT molecules joining with the loss of one iodine atom.

Step a, the trapping of iodine, would seem clearly to be an aspect of thyroid cellular function as is step b, the oxidisation of iodide probably by some thyroid peroxidase system to give an 'active form' of iodine. The weight of evidence however, which will be discussed later, is that step c and d, the iodination of tyrosine and the formation of T_3 and T_4 take place not within the cell but within the intraluminal colloid probably just at the cell colloid interface. It is not at present considered that free tyrosine molecules are iodinated to give MIT and DIT but it is thought that the tyrosine residues which are iodinated are those already present on preformed, newly synthesised non-iodinated thyroglobulin chains.

(e) Because of this it must be considered that the synthesis of the protein backbone of thyroglobulin is one of the important steps in the formation of T_3 and T_4.

(f) When the hormones T_3 and T_4 are required to exert their metabolic effect they must be first split off from the thyroglobulin chains and released into the plasma. It would seem likely therefore that there is a proteolytic step involved at this point and indeed thyroid proteases have been described.

(g) If there is excess formation of MIT and DIT these iodinated tyrosines are not lost to the thyroid when the thyroglobulin is broken down but it is believed that a dehalogenase or deiodinase enzyme system in the thyroid splits the iodine off

the tyrosine residue and that this iodine which is released is then recycled within the thyroid and is available for further hormone synthesis. There is however a certain amount of information which would seem to indicate that this iodide released from iodotyrosine breakdown is not handled by the thyroid cell in quite the same way as the iodine newly acquired from the plasma. It appears to enter a different compartment of the cell than newly trapped iodide and unlike the latter iodide is not readily dischargeable from the thyroid by substances like perchlorate or thiocyanate.

Thyroglobulin which is the main soluble protein of the thyroid gland accounting for over three quarters of all the soluble proteins in the resting gland is a very complex iodinated glycoprotein. In recent years a great deal of interest has been paid to the synthesis of this important substance. As its name indicates, it is a protein with a significant sugar component which has iodine incorporated into it. The following two chapters will deal respectively with a review of the knowledge of the chemistry of this protein and with its biosynthetic pathways.

CHAPTER 2

CHEMISTRY OF THYROGLOBULIN

The chemical studies on the nature of thyroglobulin have approached the problem in two main ways. Firstly, detailed studies have been made, especially by Edelhoch's group, of various factors affecting the controlled breakdown of this large protein molecule and, secondly, other studies have been done on the non-enzymatic synthesis of thyroglobulin *in vitro*.

Protein structure of thyroglobulin

One of the reasons why a study of the breakdown of thyroglobulin has been rewarding is that it does enable more detailed study of this very large complex protein to be undertaken. There is general agreement that the molecular weight of thyroglobulin as estimated by ultracentrifugation, light scattering or diffusion techniques, is of the order of 660,000 - 670,000 (Edelhoch, 1960). When studied in the ultracentrifuge, thyroglobulin from a normal gland gives a sedimentation constant of 19S. In addition, however, in almost all vertebrate thyroids studied, small amounts of other proteins are present amongst soluble thyroid proteins. These include proteins which are heavier than 19S such as the 27S protein which, as discussed later, is thought to represent an aggregation of 19S and possibly other proteins, and also proteins lighter than 19S, in particular a protein or series of proteins running in the region of 3 - 8S. This group is thought to consist not only of probable thyroglobulin

precursors but also as isolated from thyroid tissue, to include such proteins as haemoglobin, serum albumin and serum globulins. A 12S protein is also found in certain animals especially in guinea pigs, land turtles, dogfish and rabbits (Salvatore et al, 1965a). In these animals however the main protein present still is 19S and the 12S component, which is thought to represent a protein of half the molecular size of 19S protein, is a minor component. This same group of workers however (Aloj et al, 1967) have described finding in the lamprey, which is a primitive vertebrate fish, 12S as the predominant thyroid protein.

Studies by the Edelhoch group on the effect of alterations of pH on thyroglobulin has given interesting data (Edelhoch et al, 1960). They showed that up to a pH of 9.5 that 19S thyroglobulin remained the predominant protein but a 12S protein was also found to a certain extent. Above the level of pH 9.5, a 15S component and a 12S protein were also found but the main protein present at this pH level was 12S. The thesis was that the 12S protein constituted half the molecular size of the 19S protein and this view was in keeping with the fact that the process was reversible up until pH 11 was reached. Until this point it was found that changing the pH back to approaching neutrality resulted in the reconstitution of the 19S protein and the disappearance of the 12S protein. As the pH was changed even more toward the alkaline side an 8S protein became the predominant unit at the region of pH 12 and at higher pH values than this all the

protein was present in a 3 - 4S form.

The effect of heat was also studied by Edelhoch and Metzger (1961). They showed that very mild heating of the 19S protein resulted in the formation of two slower sedimenting components at a point before denaturation was achieved.

Edelhoch and his group also studied the effect of the detergent agent, sodium dodecylsulphate (SDS), on the breakdown of thyroglobulin. They were able to show that at low concentrations of SDS of < 0.001 M that thyroglobulin dissociated into 12S fragments. In a similar fashion to the effects of pH these changes were reversible on dialysing out the SDS from the thyroglobulin solution.

Another substance which has been used in such studies has been betamercaptoethanol (BME). This substance has been widely used in the field of protein chemistry because like many proteins of a complex nature thyroglobulin depends for stability on the presence of disulphide bonds. BME is utilised as a substance which has the property of reducing disulphide bonds and this property has been used in relation to thyroglobulin by De Crombrugghe et al (1965, 1966). These workers were able to show that, starting with a 12S thyroglobulin preparation, exposure to BME resulted in formation of two approximately equal sized parts with sedimentation constants of the order of 6S. Once again, they were able to show that during prolonged dialysis of the BME that they were able to reconstitute the 12S and even the 19S proteins. This re-oxidised protein had

the interesting property of being much more resistant to further breakdown by BME than a thyroglobulin preparation which had not passed through these stages.

Study of the breakdown of the 27S protein of the thyroid has given interesting data. Since this is approximately twice the molecular weight of the 19S protein it seemed possible that it merely was an aggregate of the 19S molecules. Studies by Salvatore et al (1965b) and Vecchio et al (1966) have however shown using mild heat, alkaline pH and low ionic strength buffers, i.e. methods which should not break disulphide bonds, that treatment of a 27S preparation resulted in not only a 19S protein but also a 12S and 3 - 8S component. This work suggests therefore that the 27S protein is made up of a more complex aggregation of 19S thyroglobulin and subunits. The same group also showed that the iodine content of the 27S protein was approximately twice that of the 19S material and suggested that this might act as a slowly turning over iodine store. This view has been reinforced by a recent paper on long term iodine deficiency in rats (Rossi et al, 1973) in which it was found a large percentage of the iodine present was in the 27S protein and not in the 19S thyroglobulin.

Standard enzymatic techniques of protein breakdown using such substances as trypsin had been utilised by Metzger et al (1962). These studies have been relatively disappointing in that breakdown to very small components was achieved without any insight into the intermediate stages.

Since, as mentioned above, the thyroid gland contains a proteolytic enzyme system, it would seem attractive to utilise what one assumes to be the physiological proteolytic system to break down thyroglobulin. In practice although studies of this type have been undertaken by Alpers et al (1956) and Pastan and Almqvist (1965) these have not been very informative. In general the thyroid proteases have not been very effective _in vitro_ against thyroglobulin. They seem to require an 'unphysiological' pH of the order of 3.5 and in the studies mentioned above there was no evidence of formation of any presumed subunits.

Goldberg and Seed (1965) carried out studies on the non-enzymatic synthesis of thyroglobulin from presumed subunits. They were able to show that starting with thyroid proteins of the order of 12S and 3 - 8S that they were able to reconstitute proteins of 19S size. They were able also to show that this process was accelerated by the presence of a small amount of stable thyroglobulin and an _in vitro_ iodination system.

This neat theory that 19S thyroglobulin is formed by the union of two 12S subunits which in turn are formed by two 6S units has been recently challenged. Vecchio et al (1971) found on fractionation of the labelled 3 - 8S peak from the rat thyroid, three components; namely 3S, 6S and 7S. The 6S and 7S appeared homogeneous by ultracentrifugation and polyacrylamide gel electrophoresis and could be precipitated by antithyroglobulin antibodies.

More recently Spiro (1973) has performed detailed studies of

possible thyroglobulin subunits. She found that approximately 20% of the thyroglobulin was present in subunits smaller than 19S and 12S. This series of polypeptides ranged in size from a molecular weight of 215,000 to 20,000. A variety of possibilities for the incorporation of these subunits into 19S thyroglobulin were presented by Spiro (1973). Studies presenting similar data are those by Rolland and Lissitzky (1972).

Iodine content of thyroglobulin

The iodine content of thyroglobulin has been the subject of much study over the last two to three decades. It was noted in 1948 by Derrien et al that the iodine content of apparently pure thyroglobulin varied substantially. This work has been confirmed and extended by more recent studies and in particular by column chromatography with DEAE cellulose. In this particular technique the iodinated proteins of the thyroid are separated mainly on their iodine content. An example of studies of this type is that of Robbins (1963) who showed that beef thyroglobulin could be split into three separate fractions by this technique. In his studies the earlier eluting fraction had a lower iodine content both from initial material and the later eluting fractions. These studies also correlated well with the analysis of the iodotyrosine content of the thyroid tissue. Robbins was able to show that although the MIT content of all fractions was similar, the DIT and T_4 content of the later eluting fractions was increased. Confirmation of the technique was obtained by the demonstration that in vitro iodination greatly diminished the earlier eluting fraction and increased the

later eluting fractions. The different iodine contents of the 19S and 27S proteins have been already discussed.

In the ultracentrifuge, poorly iodinated proteins run with an 'S' value of rather less than 19S, for instance of the order of 17 - 18S (Goldberg and Seed, 1965; Thomson and Bissett, 1969b).

Inoue and Taurog (1968) have claimed that in iodine deficient rats the main protein present was a 12 - 14S protein but this has not been found by other workers. One likely explanation for the discrepancy is the fact that the poorly iodinated protein is much more liable to breakdown _in vitro_ than the normally iodinated 19S protein.

In vitro or _in vivo_ iodination can be shown to increase the 'S' value of iodine deficient thyroglobulin to the more usually found 19S or sometimes even to higher values. Pommier et al (1966) have in addition shown that the artificially iodinated material is more resistant to the effect of SDS than is the starting poorly iodinated material.

Carbohydrate structure of thyroglobulin

Our knowledge of the carbohydrate structure of thyroglobulin has owed much to the work of Spiro (1965) and Spiro and Spiro (1965). These workers showed that thyroglobulin which contains approximately 10% of carbohydrate contains galactose, mannose, N-acetyl glucosamine, sialic acid and fucose. It has been shown that different vertebrates such as sheep, pig and calf have broadly similar sugar components. Minor differences were found between human thyroglobulin and, for example, sheep thyroglobulin in that the human thyroglobulin contains

more carbohydrate due to a larger number of mannose and glucosamine residues. It was estimated that human thyroglobulin contained approximately 350 monosaccharide residues and the other species 290 residues per molecule of glycoprotein.

Following digestion with pronase Spiro (1965) was able to demonstrate the presence of two carbohydrate-containing thyroglobulin subunits. The first, which was labelled unit A, consisted of five residues of mannose to one of N-acetyl glucosamine and had a molecular weight of 1050. The other, unit B, consisted of three mannose residues and five of N-acetyl glucosamine, four of galactose, two of sialic acid and one of fucose. The molecular weight of this latter fragment was of the order of 3200. It was estimated that there were approximately nine units A and fourteen units B in each thyroglobulin molecule. Similar results have been presented by Fukuda and Egami (1971a and b).

Spiro (1965) also used the technique of pronase digestion to obtain the carbohydrate portion of thyroglobulin relatively free from protein except for the few amino acids adjacent to the carbohydrate-peptide linkage. Analysis of these amino acids showed that for both unit A and unit B that aspartic acid was the only one present in sufficient quantity and constancy to be the amino acid likely to be involved in the glycopeptide linkage.

Other studies in this field have been by Murthy et al (1965) who found a single glycopeptide in a pronase digest of sheep thyroglobulin which contained 60% of the total carbohydrate present.

This glycopeptide had a molecular weight of 2,400 and contained 0.5 residues of fucose, two of N-acetyl glucosamine and five of hexose and one of sialic acid. This finding has not been confirmed by other workers.

Robbins (1963) in earlier studies demonstrated that the different thyroglobulins isolated in a DEAE cellulose column had different sugar contents in addition to different iodine contents. He demonstrated that the later eluting fraction contained more sialic acid than the earlier eluting material. This work was not, however, confirmed by Bouchilloux et al (1964).

In more recent papers Spiro and his co-workers have extended their studies (Arima et al 1972; Arima and Spiro, 1972). They showed that the unit A of human and calf thyroglobulin were similar but that there was significant microheterogeneity in the mannose content which varied from five to eleven units per residue, the glucosamine content remaining constant at two residues in each unit. Similarly unit B was shown to have pronounced microheterogeneity when fractionated in a DEAE cellulose column. Once again the variation only affected some of the sugar residues, the mannose remaining constant at three residues but there was considerable variation in the sialic acid, galactose and glucosamine residues. The results found by these workers were also considered consistent with an additional unit (unit C) being present in human thyroglobulin. This unit was composed of galactosamine residues occurring as an integral part of the protein and being distinct from carbohydrate

units A and B. The galactosamine residues appeared to be linked to serine and threonine residues and not to aspartic acid residues as in the case of units A and B.

Detailed studies of the structure of unit A were also undertaken (Arima and Spiro, 1972) using various glycopeptidase enzymes. These workers have put forward a tentative structure of unit A as a mannose core with additional mannose-containing residues added to the glucosamine residues to constitute the normal complement of mannose units.

SUMMARY OF THE CHEMISTRY OF THYROGLOBULIN

The data presented in this section is consistent with the view that thyroglobulin (19S) is composed of subunits of 12S and 6S size. Larger proteins such as the 27S protein are also present and various minor constituents have also been described. At least three carbohydrate subunits would appear to be involved in the structure of normal thyroglobulin and iodine appears to constitute an integral part of the structure. The weight of evidence at present is that iodine, in addition to being required for the formation of T_3 and T_4, is also required for full structural development of the thyroglobulin molecule perhaps by influencing its tertiary structure.

CHAPTER 3

THE BIOSYNTHESIS OF THYROGLOBULIN

In this chapter will be presented the evidence for the present state of knowledge of the biosynthesis of the three aspects of thyroglobulin formation, namely iodine incorporation, amino acid incorporation and carbohydrate incorporation respectively.

Of these three, that of iodine incorporation has been studied to the greatest extent. This was largely because of the availability early on of radioactive isotopes of iodine. Studies have been done using a variety of experimental techniques. At its simplest it can be shown ~~that~~ *in vitro* using a cell free system that whole thyroid homogenates and various subcellular fractions can incorporate iodine into thyroid protein at least to the stage of forming MIT (Taurog et al, 1955). It must also be borne in mind that under certain conditions ~~that~~ the iodination of proteins like casein can be made to proceed non-enzymatically. Such protein iodinating techniques are, of course, now widely used in the preparation of radio-pharmaceuticals and indicate that the incorporation of iodine into protein is not necessarily an enzymatic process.

Using a thyroid slice technique it has readily been shown by many workers that iodine is incorporated into the thyroid proteins. Seed and Goldberg (1963) and Lissitzky et al (1964) showed that ^{125}I is incorporated very rapidly into a thyroglobulin like protein which had a sedimentation constant of the order of 18S, that is

slightly less than the stable 19S thyroglobulin. Seed and Goldberg found that no lighter protein was iodinated as a regular occurrence although Lissitzky et al claimed to have found incorporation into 3 - 8S and 12S proteins at early times of labelling. The results of the author and most other investigators are in keeping with the findings of the former workers. Seed and Goldberg (1965) were further able to show ~~that~~ in the presence of propylthiouracil (PTU) in concentrations of 10^{-3} M that ^{125}I incorporation into thyroglobulin was virtually completely abolished. They were, however, able to show that substances like actinomycin D which interfere with protein synthesis do not affect iodine incorporation into thyroglobulin at least in the early stages.

The fact ~~that~~ it appears in most studies that a protein running just short of 19S thyroglobulin is formed is of interest. Both Goldberg and Seed (1965) and Nunez et al (1965) have shown that chemical iodination will result in the formation of a truly 19S protein from this lighter weight protein.

In addition in vivo studies by the author amongst others (Thomson and Goldberg, 1968) has shown that in the rat, iodine is usually incorporated into a protein of the order of 18S size with lighter proteins only being minimally labelled at very early intervals thus confirming the data obtained from the in vitro studies.

Incorporation of amino acids

This has been studied mainly using tissue slice techniques in vitro. A series of papers by Seed and Goldberg (1963, 1965),

Lissitzky et al (1964) and Nunez et al (1965) are all in agreement that labelled amino acids are incorporated *in vitro* into lighter weight proteins of 3 - 8S and 12S size before the label becomes incorporated into the 19S thyroglobulin. Seed and Goldberg showed further by using puromycin and actinomycin D that protein synthesis can be ~~specifically~~ inhibited without inhibiting iodination. The studies using actinomycin showed that there was delay before this substance had an effect. Since it acts on protein synthesis by interfering with the formation of messenger RNA they were able to estimate that the RNA template for the thyroglobulin synthesis had a half-life of at least fifteen hours.

There have been fewer studies on the cell free incorporation of amino acids into thyroid proteins but the studies of Singh et al (1964, 1965) and Nunez et al (1965) have shown that it was possible to incorporate labelled amino acids into protein using a cell sap preparation. This process was inhibited by puromycin. Nunez et al were able to show after purification of the protein that 3 - 8S and 19S protein had been formed. Similar results were found in a cell free system by Morais and Goldberg (1967).

Vecchio et al (1972) have extended these studies *in vivo* and *in vitro* and have shown that ^{3}H leucine is incorporated into the particulate proteins of the rat and guinea pig thyroid before labelling of the soluble proteins occur. The pattern of labelling *in vivo* suggested that the 12S units are precursors of 19S thyroglobulin but that the 6S or 7S units are probably derived from breakdown of

newly formed thyroglobulin.

Incorporation of carbohydrate

The work of Cartouzou et al (1967) has shown that sheep thyroid polysomes were unable to incorporate ^{14}C glucosamine and ^{14}C mannose into thyroglobulin. Using a calf thyroid slice system, however, Spiro and Spiro (1966) were able to show that labelled glucose could be incorporated into a protein with similar electrophoretic and immunological characteristics to thyroglobulin. They were further able to show that this process was related to the subcellular particles and in studies using puromycin they suggested that the incorporation of carbohydrate occurred after the synthesis of the polypeptide chain. Bouchilloux and Cheftel (1966) have produced very similar results.

Great interest was aroused by papers of Herscovics (1969, 1970) who published in vitro studies using rat thyroid hemilobes in which she showed that there seemed to be a difference in mechanism between the incorporation of galactose and mannose. Mannose appeared to be incorporated in a manner and time course similar to that of leucine; that is that there was incorporation of the labelled sugar into presumed thyroglobulin subunits before incorporation into the 19S protein occurred. On the other hand galactose was almost immediately incorporated into protein of 17 - 18S size. She further showed ~~that~~ during the first hour of incubation that puromycin almost completely inhibited the incorporation of the labelled leucine and mannose into the protein fraction but had little effect on incorporation of

galactose. Fucose was incorporated in the same manner as galactose and likewise was not inhibited by substances such as cycloheximide which block protein synthesis. Herscovics suggested that the results were consistent with the fact that mannose was incorporated earlier into the thyroglobulin protein than galactose and that galactose and fucose were added at the stage of formation of the 17 - 18S protein prior to the iodination of the protein.

The study of these different types of carbohydrate incorporation was the starting point of the present work which was designed to explore the factors which might influence the incorporation of sugars particularly mannose and galactose into the rat thyroid. During the course of this work other papers have been published which are relevant to the present thesis. An attempt is made in the general discussion section to put these other publications into perspective and to incorporate the author's own data into our present scheme of knowledge of thyroglobulin structure.

SECTION 2

MATERIALS AND METHODS

Chapter 4 Materials

Chapter 5 Dietary regimes

Chapter 6 Experimental techniques

CHAPTER 4

MATERIALS

The materials and the suppliers utilised in this work are as follows.

Materials	Suppliers
Male Sprague-Dawley rats approx. 150 g	A. Tuck & Son, Rayleigh, Essex
Potassium iodide (KI)	British Drug Houses Ltd., Poole
Thyroxine (T_4)	British Drug Houses Ltd.
Propylthiouracil (PTU)	L. Light & Co., Colnbrook
Potassium perchlorate ($KClO_4$)	British Drug Houses
Methimazole (2-mercapto-1-methylimidazole)	Aldrich Chemical Co. Inc., Milwaukee, Wisconsin
Carbimazole	Nicholas Research Institute
Thytropar (thyrotropin)	Armour Pharmaceutical Co.
Sodium pyruvate	Boehringer Corporation (London) Ltd.
D(+) galactose	Sigma Chemical Co.
D(+) mannose	British Drug Houses
Sucrose	British Drug Houses
Cycloheximide	Sigma Chemical Co.
2-mercaptoethanol	British Drug Houses
D-galactose-1-C14 (30-40 mCi/m mole)	The Radiochemical Centre, Amersham
D-mannose-1-C14 (20-30 mCi/m mole)	The Radiochemical Centre, Amersham
L-fucose-C14 (U) (> 100 mCi/m mole)	The Radiochemical Centre, Amersham

Materials	Suppliers
Instagel	Packard Instrument Co. Inc.
α-mannosidase (from jack bean)	The Boehringer Corporation (London) Ltd.
β-galactosidase (from Escherichia coli)	The Boehringer Corporation (London) Ltd.

CHAPTER 5

DIETARY REGIMES

Control rats

Primrose, diet 41 (pelletted).

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

PTU treated rats

LID supplemented with 5 mg KI + 0.2 g PTU/kg diet.

Methimazole treated rats

LID supplemented by 5 mg KI + 1,500 mg methimazole/kg diet.

Carbimazole treated rats

LID supplemented by 5 mg KI + 1,500 mg carbimazole/kg diet.

$KClO_4$ treated rats

LID + 20 g $KClO_4$/kg diet.

These goitrogenic diets were fed for a period of three weeks before sacrifice. The $KClO_4$ group received distilled water to drink. The remaining groups of rats received tap water. In some experiments animals received additional iodine supplementation in their drinking water for one week before sacrifice. This was given as KI 0.05% in distilled water unless otherwise stated.

CHAPTER 6

EXPERIMENTAL TECHNIQUES

Methods

Male Sprague-Dawley rats of approximately 150 g were killed under ether anaesthesia. The thyroid glands were rapidly removed and placed in ice-cold phosphate buffered saline (PBS) (0.15 M sodium chloride in 0.01 M potassium phosphate, pH 6.8) contained in a Petri dish resting on a bed of crushed ice. After removal of all fatty tissue visible to the naked eye the thyroid lobes were bisected using a razor blade. The thyroids from three rats, i.e. twelve hemilobes, were incubated in a 25 ml Erlenmeyer flask with 2 ml of a modified Krebs No. 2 buffer (Bisset and Alexander, 1960) which had previously been gassed with oxygen for 10 minutes. The incubations were carried out at 37°C in a Gallenkamp metabolic shaker under an atmosphere of oxygen. Following a 15 minute preincubation, 10 μCi (ie 0.3 μmole) of the appropriate isotope were added to each flask, and incubations carried out for times varying from ½ to 4 hours. At the end of the incubation period, the total flask contents were homogenised using a TRI-R STIR-R fitted with a glass homogenising tube with a teflon pestle. The homogenising tube was kept in crushed ice during the homogenisation process. As few strokes of the pestle were used as was consistent with good disruption of the tissues.

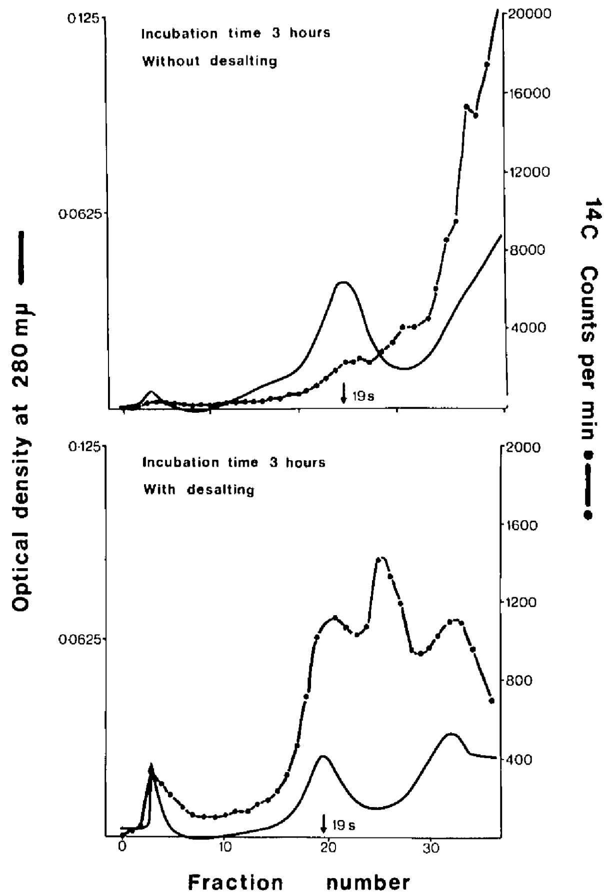
The homogenate was then spun in an MSE refrigerated centrifuge at 15,000 rpm (20,000 g) for 10 minutes to remove cellular debris.

The supernatant was then passed through a Pharmacia K9/30 column containing Sephadex G25 (coarse), bed length 25 cm to remove unincorporated labelled sugar.

The value of this particular step is shown in Fig. 3. In this and all subsequent figures depicting the ultracentrifugal analysis of the thyroid proteins the pattern of the stable proteins recorded at 280 mμ is shown by the solid line and that of the radioactivity present by the broken line. The main stable protein present is thyroglobulin and its position (19S) is indicated by the peak of the stable protein from control rat thyroid tissue. The top of the gradient is to the right and therefore all proteins lighter than 19S appear to the right of the 19S peak. Conversely those of greater molecular weight than 19S appear to the left of the 19S peak.

It will be noted that before passage through the Sephadex G25 column, a process referred to as desalting, there is a suggestion of a peak of radioactivity associated with the 19S peak after 3 hours incubation of normal rat thyroid with ^{14}C mannose but very little evidence of other protein labelling. However after passage through the column it can be clearly seen that there is a peak of radioactivity associated with the 19S protein, a second peak to the right of the 19S peak lying in the 12S position and a third peak nearer the top of the gradient in the 3 - 8S position. It should be noted that the removal of unincorporated radioactivity has resulted in a marked fall of radioactivity towards the top of the gradient.

The protein eluate was taken to 50% saturation with ammonium



<u>Fig. 3</u>

Ultracentrifugal analysis of the patterns of incorporation of ^{14}C mannose into control rat thyroid without desalting (upper portion) and with desalting (lower portion). Time of incubation 3 hours; SW 41 Rotor at 28,000 rpm for 16 hours.

sulphate, was thoroughly mixed and kept on ice for one hour, after which it was again spun at 15,000 rpm for 10 minutes in a refrigerated centrifuge. The supernatant was discarded, the tube inverted and allowed to drain to remove as much ammonium sulphate as possible. The tube was then blotted with tissues and the protein either processed immediately or else stored in the deep freeze at - 20°C.

The protein precipitate was dissolved in as small a volume of PBS as possible and layered on the top of a 5 - 20% sucrose gradient in PBS made using a Beckman gradient former. The samples were then spun using an SW41 rotor in a Beckman model L2-65B ultracentrifuge at 28,000 rpm for 16 hours.

At the end of the period of ultracentrifugation, the pattern of the thyroid proteins was determined by aspiration from the bottom of the ultracentrifuge tube and passage through a Gilford 2000 absorbance recorder at 280 mμ. Fifteen drop fractions were collected into polyethylene counting vials and, after the addition of 5 ml Instagel, were counted in a Packard Tricarb liquid scintillation counter.

When dealing with human material, this was collected fresh from the operating theatre. It was transported from the theatre to the laboratory in a plastic bag on ice inside a vacuum flask. On arrival in the laboratory the specimen was placed in a Petri dish containing a small amount of ice-cold PBS, the Petri dish resting on crushed ice. Any obvious fatty tissue was dissected away following which thin slices of thyroid tissue were made by hand using a razor blade. Approximately 300 mg of human thyroid tissue per Erlenymeyer flask was incubated and the material dealt with following appropriate time

of incubation in the manner already described for the rat thyroid tissue.

Use of glycosidase enzymes

Rat thyroids were incubated as previously described. Following incubation, the whole flask contents were homogenised, centrifuged at 20,000 g to remove cell debris and the homogenate was then passed through Sephadex G25 columns to remove unincorporated isotope. Each sample was eluted from the column into a 25 ml Erlenmeyer flask, to which was added a few mgs of calcium chloride ($CaCl_2$) and 25 mg pronase, along with a few drops of toluene. This mixture was incubated at 37°C for 3 days, with the addition of a further few mgs pronase after 24 hours.

At the end of 3 days, the action of pronase was stopped by boiling the flask contents for a few minutes. The samples were then transferred to clean Erlenmeyer flasks and the pH adjusted to approximately 4.5. To each flask was added a few mgs of magnesium chloride ($MgCl_2$) and 0.2 ml (1 mg) (α -mannosidase or β -galactosidase). This mixture was incubated at 25°C overnight. The samples were then boiled for a few minutes to stop the action of the glycosidase enzyme and centrifuged to remove debris. The supernatants were then concentrated to 0.5 ml using lyphogel. 60 µl of each sample was then chromatographed overnight on Whatman 3^M_M chromatography paper using N-butanol/pyridine/water (50 : 30 : 20) as solvent, with markers of mannose, galactose and sialic acid. After drying, the chromatograms were cut into strips and scanned using a Packard chromatogram scanner. The position of the stable marker solutions was determined following

spraying with a saturated solution of para-anisidine hydrochloride
in N-butanol.

SECTION 3

INCORPORATION OF RADIOACTIVE SUGARS INTO RAT THYROID PROTEINS IN VITRO

Chapter 7	Incorporation of ^{14}C mannose and ^{14}C galactose in control rats
Chapter 8	Effect of goitrogenic diets on the incorporation of ^{14}C mannose and ^{14}C galactose
Chapter 9	Effect of administration of sodium thyroxine (T_4) to control rats on the incorporation of labelled sugars
Chapter 10	Effect of the administration of potassium iodide (KI) on the incorporation of ^{14}C mannose and ^{14}C galactose
Chapter 11	Effect of TSH *in vitro* and *in vivo* on the incorporation of ^{14}C mannose and ^{14}C galactose
Chapter 12	Effect of cycloheximide on the incorporation of ^{14}C mannose and ^{14}C galactose
Chapter 13	Effect of addition of pyruvate to the incorporation of ^{14}C mannose
Chapter 14	Effect of the addition of stable sugars on the incorporation of ^{14}C mannose and ^{14}C galactose
Chapter 15	Effect of added stable mannose and galactose on labelling with ^{3}H leucine and ^{125}I

Chapter 16 Incorporation of ^{14}C fucose in control rats

Chapter 17 Effect of propylthiouracil (PTU) on the incorporation of ^{14}C fucose

Chapter 18 Effect of sodium thyroxine (T_4) on the incorporation of ^{14}C fucose

Chapter 19 Effect of iodine supplementation on incorporation of ^{14}C fucose

Chapter 20 Effect of cycloheximide on the incorporation of ^{14}C fucose

CHAPTER 7

INCORPORATION OF ^{14}C MANNOSE AND ^{14}C GALACTOSE IN CONTROL RATS

The pattern of incorporation of ^{14}C mannose into the thyroid proteins of control rats is as shown in Fig. 4. It can be seen that at half an hour there is no significant incorporation of ^{14}C mannose into the 19S peak. There is a small amount of incorporation into a radioactive peak running between the 19S and the 3 - 8S stable peaks, that is in the 12S position; associated with the 3 - 8S peak itself there is no discrete peak of incorporation but merely a tail of radioactivity extending to the top of the gradient. At one hour it will be noted that significantly more ^{14}C mannose is incorporated into the labelled 12S peak and now the appearances at the 3 - 8S peak are more that of incorporation of labelled ^{14}C mannose into the protein and not merely a non-specific contamination of the gradient. There is as yet, however, no significant incorporation into the 19S peak although there is the suggestion of such an incorporation. By the time 2 hours has elapsed there is a very definite pattern of three discrete radioactive peaks. There is for the first time significant incorporation of the label into the area of 19S thyroglobulin. This peak is not, however, quite symmetrical, is of a slightly lower 'S' value than thyroglobulin and corresponds to an 'S' value of approximately 18S. The 12S labelled peak has increased in specific activity and in proportion and there is now much more definite incorporation into the 3 - 8S protein. At 3 hours there is

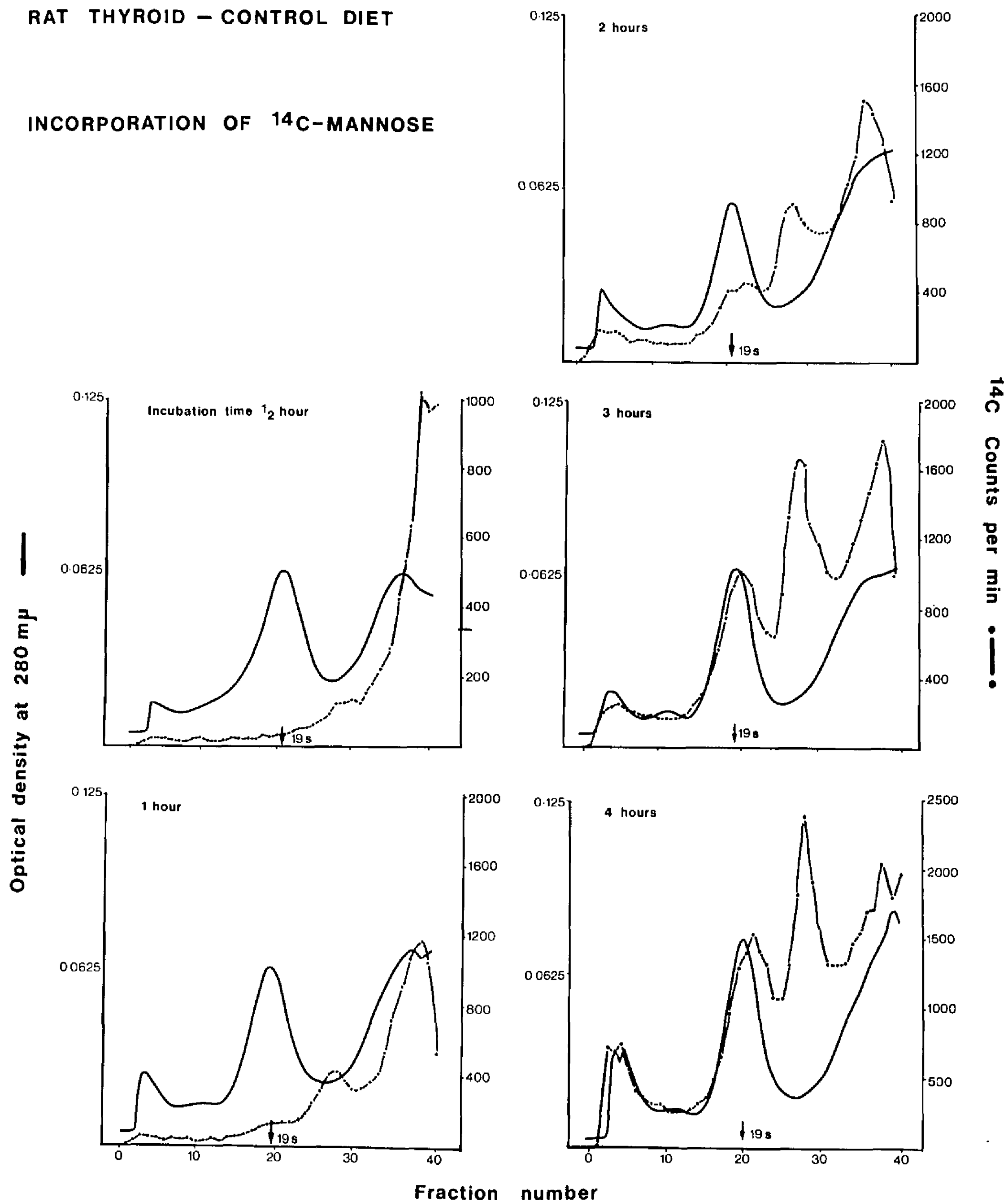


Fig. 4

Ultracentrifugal study of the time course of incorporation of ^{14}C mannose into control rat thyroid. SW 41 Rotor at 28,000 rpm for 16 hours.

increased incorporation of the label into the peak in the 18S position and into the 12S peak although the radioactivity associated with the 3 - 8S peak remains more or less static. Proportionately there is much more incorporation of the label into the 18S peak than in the previous tracing. At the end of this particular experiment, that is 4 hours, the incorporation of the label into the 18S peak had increased and similarly the incorporation into the 12S peak had increased. The radioactivity associated with the 3 - 8S peak was only marginally increased and therefore by proportion appeared to have decreased.

Fig. 5 shows the pattern of incorporation of ^{14}C galactose into the thyroid proteins of the control rat in vitro and here it can be seen that in contrast to the pattern found for ^{14}C mannose incorporation there is at half an hour already some incorporation of the labelled sugar into the thyroglobulin peak. There is a rather ill-defined 12S peak of radioactivity and a broad peak of labelling in the region of the 3 - 8S proteins. At one hour these three peaks are much better defined. That associated with the 19S has increased markedly in specific activity and still exceeds the 12S peak which has, however, also increased in activity. Likewise, the amount of labelled material associated with the lightweight 3 - 8S proteins has also increased. By the time 2 hours has elapsed there is a marked increase in radioactivity associated with the thyroglobulin peak which now more or less corresponds in position to the stable 19S peak. The labelled 12S peak has also increased in specific activity but the amount of label associated with the 3 - 8S peak is static. At 3 hours the pattern of incorporation of radioactivity and the specific activity of

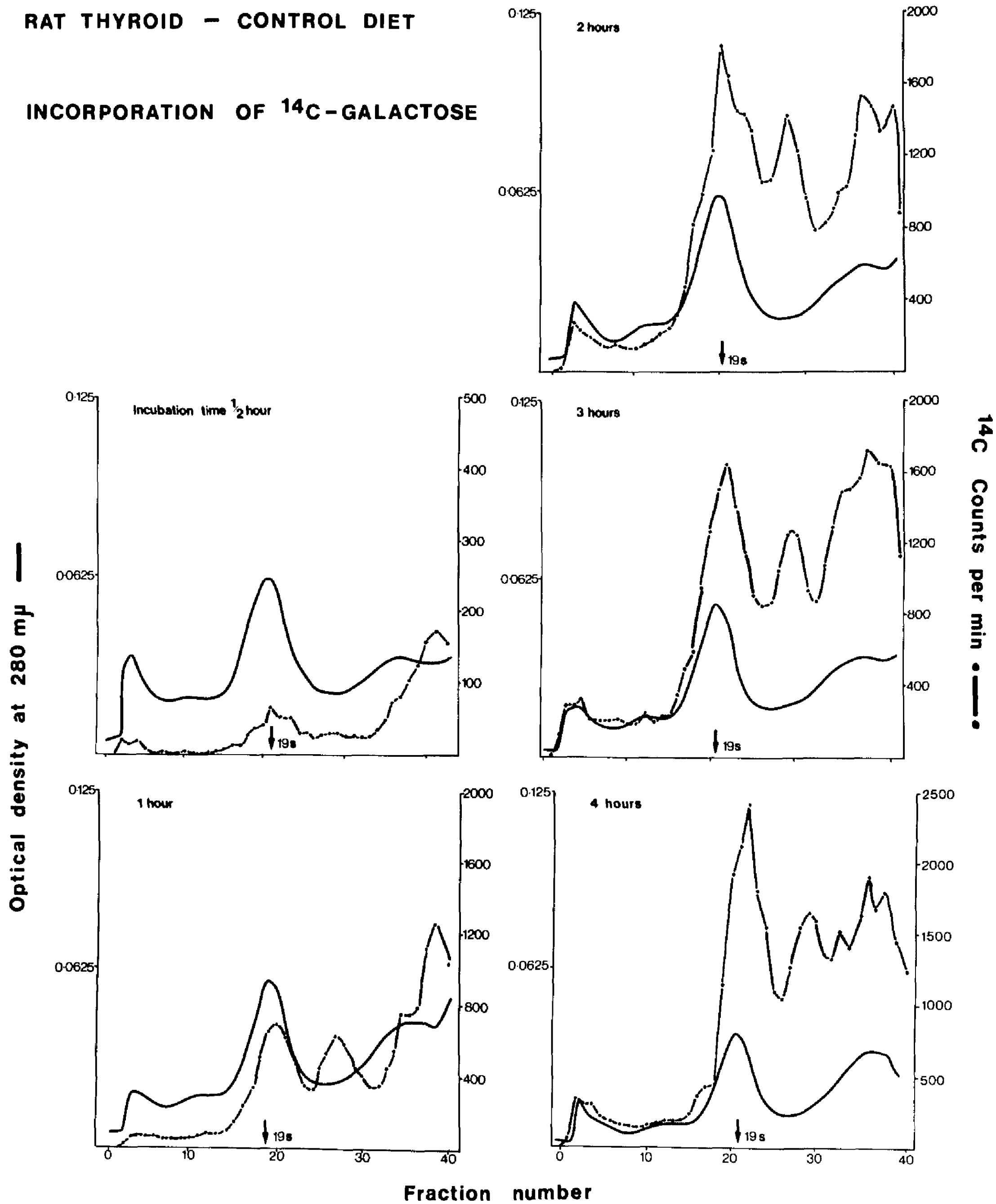


Fig. 5

Ultracentrifugal study of the time course of incorporation of ^{14}C galactose into control rat thyroid. SW 41 Rotor at 28,000 rpm for 16 hours.

incorporation of the labelled galactose is very similar to that found on the 2 hour sample. There still are predominant labelled peaks in the 19S and 3 - 8S regions with a rather smaller labelled 12S peak. In this particular tracing there is a slight suggestion of a labelled peak in the 27S region situated to the left of the 19S peak. At 4 hours there is a change in proportion of the labelled peaks. The predominant peak is now that in the 19S region which has increased in specific activity. The peak in the 12S area has shown a slight increase in specific activity whereas that in the 3 - 8S peak is now static and therefore has proportionately decreased. Once more in this sample there is some suggestion of labelling of proteins higher than the 19S proteins.

In view of the early appearance of a ^{14}C galactose labelled peak associated with the thyroglobulin area, earlier incubation times were studied in addition. After 5 minutes incubation there was no incorporation of the label into either the thyroglobulin or 12S region but by 15 minutes of incubation a tracing like that of the 30 minute sample was obtained, that is there was a small labelled peak in the thyroglobulin region without the appearance of the label in the 12S area.

From these experiments which were repeated on several occasions with reproducible results it can be concluded that the pattern of incorporation of ^{14}C mannose and ^{14}C galactose is consistent with incorporation of the labelled sugar into presumed thyroglobulin subunits, namely 12S and 3 - 8S proteins, before there is incorporation of the label into the 19S proteins. The only tracing which might

raise some doubts is the very small peak of incorporation of ^{14}C galactose into the thyroglobulin region at early time intervals after incubation. The rest of the pattern of galactose incorporation was, however, consistent with the incorporation into presumed thyroglobulin subunits. The main difference between the patterns of incorporation of mannose as opposed to galactose is that the incorporation of labelled galactose into thyroglobulin occurs at earlier time intervals and that the specific activity of labelling of the thyroglobulin-like protein using ^{14}C galactose is always much higher at any given time than was found with ^{14}C mannose.

CHAPTER 8

EFFECT OF GOITROGENIC DIETS ON THE INCORPORATION OF ^{14}C MANNOSE AND ^{14}C GALACTOSE

In Fig. 6 is shown the incorporation of ^{14}C mannose into the thyroid proteins of rats which have been maintained on a goitrogenic diet as the result of the administration of potassium perchlorate ($KClO_4$). As a result of this dietary manipulation, goitres have been induced in these animals. This has resulted as previously described (Thomson and Goldberg, 1968) in an alteration of the stable thyroid proteins. It will be noted that there is not a predominant stable 19S peak present. The predominant peak is now in the lighter 3 - 8S region. Although not specifically run to show the proteins heavier than 19S thyroglobulin there is an abolition of the 27S peak and its replacement seen in some tracings by a peak running in the 32S region (Thomson and Bissett, 1970).

Following 30 minutes incubation with ^{14}C mannose *in vitro* it will be noted that there is a definite labelled peak running in the 12S region with no significant incorporation of radioactivity into either the peak in the thyroglobulin region or the 3 - 8S region. At one hour however there now is a definite although small peak of radioactivity in the 19S region. The 12S peak has increased greatly in size and there is still no definite incorporation of label into the 3 - 8S region. At 2 hours the specific activity of the peak in the 19S region has increased, as has that in the 12S region. There remains no definite incorporation into the 3 - 8S area. At 3 hours

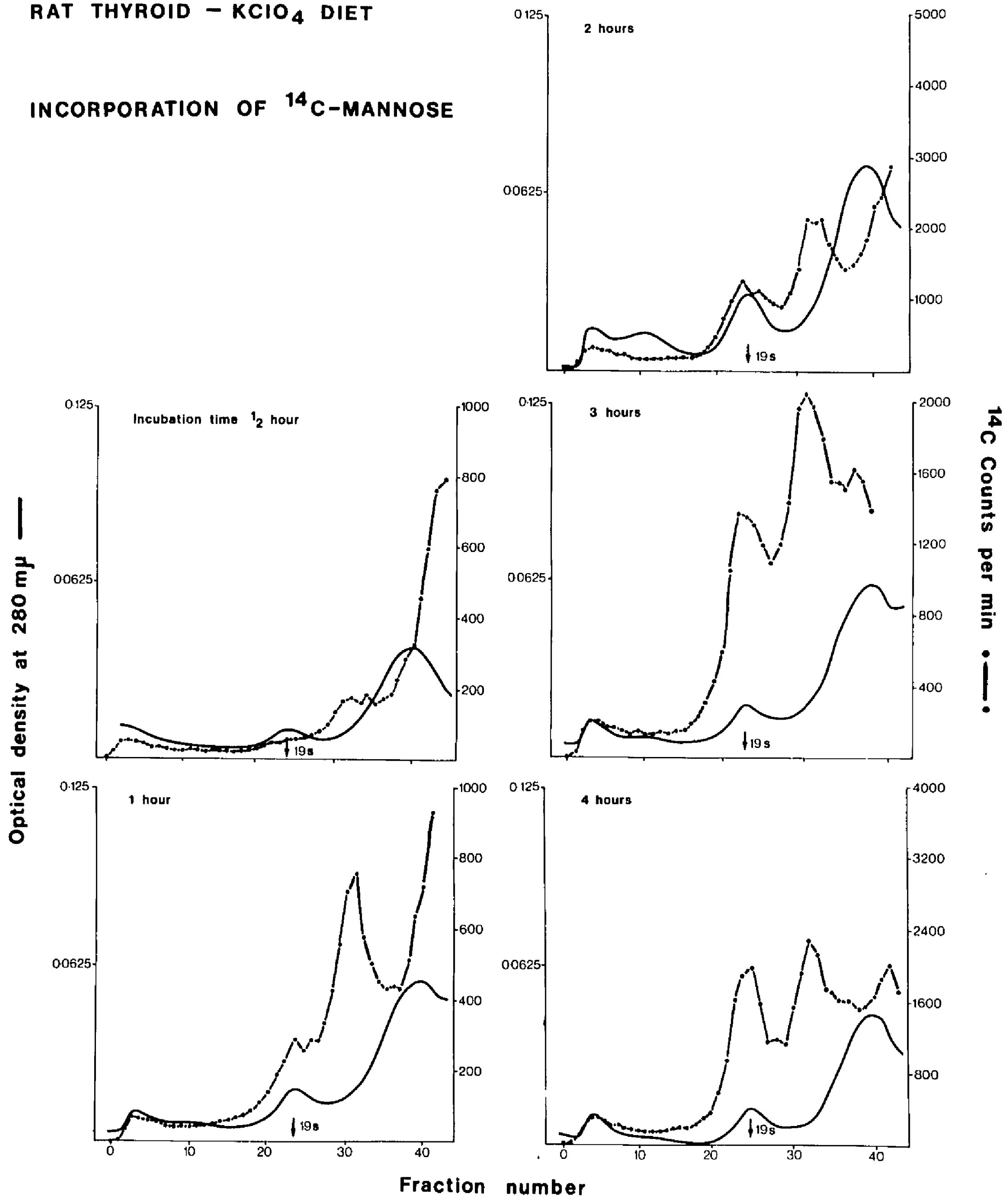


Fig. 6

Ultracentrifugal study of the time course of incorporation of ^{14}C mannose into the thyroid proteins of rats receiving $KClO_4$.
SW 41 Rotor at 28,000 rpm for 16 hours.

the specific activity of the peak in the 19S region has increased only slightly. Likewise the peak in the 12S region has remained at a similar specific activity. There now appears to be a definite peak of labelling in the 3 - 8S region with its activity slightly greater than that in the 19S area. At 4 hours the specific activity of the peak in the 19S region has increased both absolutely and in proportion to the 12S peak which has only increased slightly. The 3 - 8S peak has likewise remained almost stationary. It will be noted by comparing Fig. 6 with Fig. 4, which shows the incorporation of ^{14}C mannose into control rat thyroid, that the radioactivity incorporated in rats on the goitrogenic regime is significantly greater than that of the control animal, that in all tracings there is an earlier appearance of the label in the 12S and in 19S peaks and that the proportion of the label incorporated in each tracing into the 19S peak is greater on the goitrogenic diet.

Fig. 7 shows the pattern of incorporation of ^{14}C galactose *in vitro* into the thyroid proteins of rats on a $KClO_4$ diet. It will be seen that at 30 minutes there is an appreciable incorporation of the labelled sugar in the 19S protein. A smaller, though well-defined 12S peak is present and there is a suggestion of incorporation of the label into the 3 - 8S peak. At one hour a broadly similar pattern is obtained although the specific activity of labelling has increased such that the 19S peak and 12S peak show almost double the radioactivity of the 30 minute specimen. By 2 hours this process had increased markedly so that the specific activity of labelling of the 19S peak had increased by more than twice; the proportion of

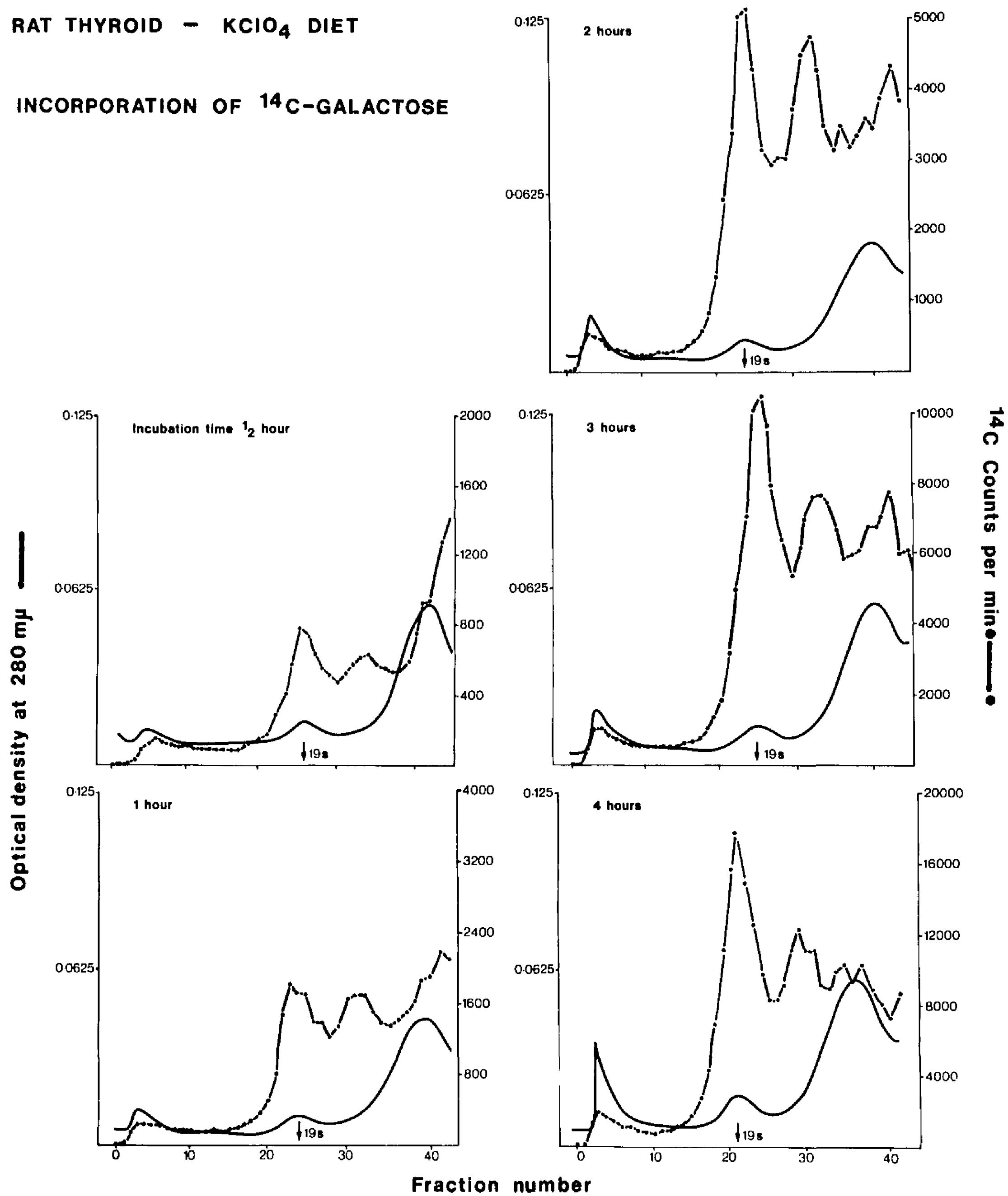


Fig. 7

Ultracentrifugal study of the time course of incorporation of ^{14}C galactose into the thyroid proteins of rats receiving $KClO_4$. SW 41 Rotor at 28,000 rpm for 16 hours.

labelling of the 12S and 3 - 8S peaks remained in step with this increase in specific activity. By 3 hours the specific activity of the 19S peak had increased again markedly and although the labelling of the 12S and 3 - 8S peaks had also increased, proportionally there was much more label associated with the 19S peak. In the 4 hour specimen it will be seen that this general tendency continued, that is that there was increased labelling associated with all peaks but that the proportion associated with the 19S peak greatly increased. There was no significant association of the labelled sugar with proteins higher than 19S.

In a manner similar to that found with incorporation of ^{14}C mannose the pattern obtained with labelled galactose in the goitrogenic as compared to the control diet was of a greatly increased specific activity of labelling at any given time and also a greater tendency for earlier labelling of the 19S protein and proportionally much greater labelling of the 19S protein compared to the other proteins present.

Various other goitrogenic regimes were used, namely propylthiouracil (PTU), methimazole and carbimazole. A similar pattern of labelling to that found on the $KClO_4$ diet was obtained using these other goitrogenic regimes, that is that the specific activity of labelling was increased with the goitrogenic regime and there was a greater tendency for labelling of the 19S protein earlier on the goitrogenic diet. Since these other diets gave a pattern virtually identical with that of the $KClO_4$ diet they have not been shown. The fact that the effect was found no matter what goitrogenic regime

was used, for instance one acting at the site of iodine uptake such as $KClO_4$ or one acting at the site of iodine incorporation into tyrosine such as PTU or the other drugs mentioned would suggest that the effect shown is not specific to the drug itself but that it is the effect of goitre formation perhaps mediated by the increased levels of TSH which are undoubtedly present in these circumstances.

CHAPTER 9

EFFECT OF ADMINISTRATION OF SODIUM THYROXINE (T_4) TO CONTROL RATS ON THE INCORPORATION OF LABELLED SUGARS

In this chapter is shown the effect of adding sodium thyroxine (T_4) to the diet of control rats. Fig. 8 shows the pattern of incorporation of ^{14}C mannose *in vitro* into the thyroid proteins of such rats. At half an hour there is very little incorporation of label but there does appear to be a small peak of radioactivity associated with the 19S protein without significant incorporation of label being associated with the 12S protein although there is a large 3 - 8S peak labelled. At one hour the label associated with the 19S peak has increased in specific activity. A definite 12S labelled peak is seen and the specific activity of the labelled 3 - 8S peak has increased. By 2 hours there has been no significant increase in label associated with the 19S peak; the 3 - 8S peak has increased in specific activity and the 12S peak has also increased in activity but is not well-defined in this particular tracing. At 3 hours the radioactivity associated with the 19S peak has increased both absolutely and proportionately. The labelled 12S peak remains at approximately the same specific activity in the 3 hour as in the 2 hour sample and the specific activity of labelling of the 3 - 8S peak has decreased. There is a suggestion of slight labelling of the 27S protein in this tracing. By 4 hours the specific activity of labelling of 19S peak has doubled; that of

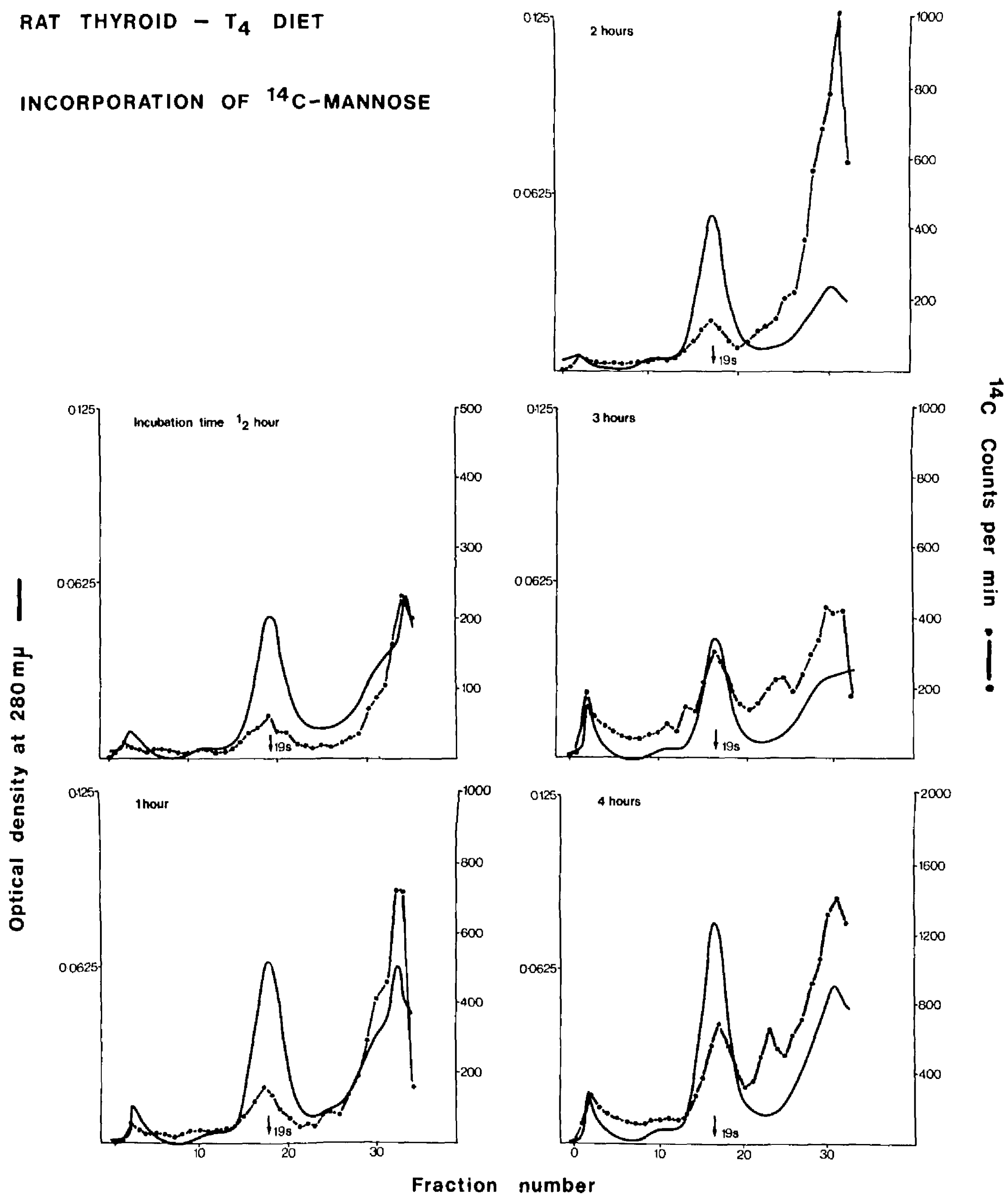


Fig. 8

Ultracentrifugal study of the time course of incorporation of ^{14}C mannose into rats on a diet containing T_4. SW 41 Rotor at 28,000 rpm for 16 hours.

the 12S peak has also increased in specific activity. Likewise the labelled 3 - 8S peak has also increased both absolutely and proportionately.

Compared to the control experiment, incorporation of ^{14}C mannose into the rat on a diet containing sodium thyroxine is characterised by a very much lower incorporation of the labelled sugar than in the control run. There is, however, this interesting finding of earlier incorporation of the labelled sugar into the 19S protein although in the very early sample the absolute count is so low that one should beware of reading too much into the small peak obtained. One feature which is of interest is that the labelled peak in the 19S area in the thyroxine-treated animal does correspond very well with the stable protein peak in this area. It will be noted that compared with Fig. 4 the radioactive peak and the stable peak in the thyroxine-treated animal are virtually symmetrical whereas it will be recalled that in the control animal the ^{14}C mannose peak runs in approximately the 18S position.

In the next figure (Fig. 9) can be seen the pattern of incorporation of ^{14}C galactose into the thyroid proteins of rats on a diet containing thyroxine. At half hour of incubation there appears to be a small labelled 19S peak present. There is a broad band of labelling in the 12S position without a well-defined peak being seen but there is a definite peak of labelling in the 3 - 8S position. At one hour the specific activity of labelling of the 19S peak had increased, the pattern of labelling of the 12S peak was now much more defined and it too had increased in specific activity;

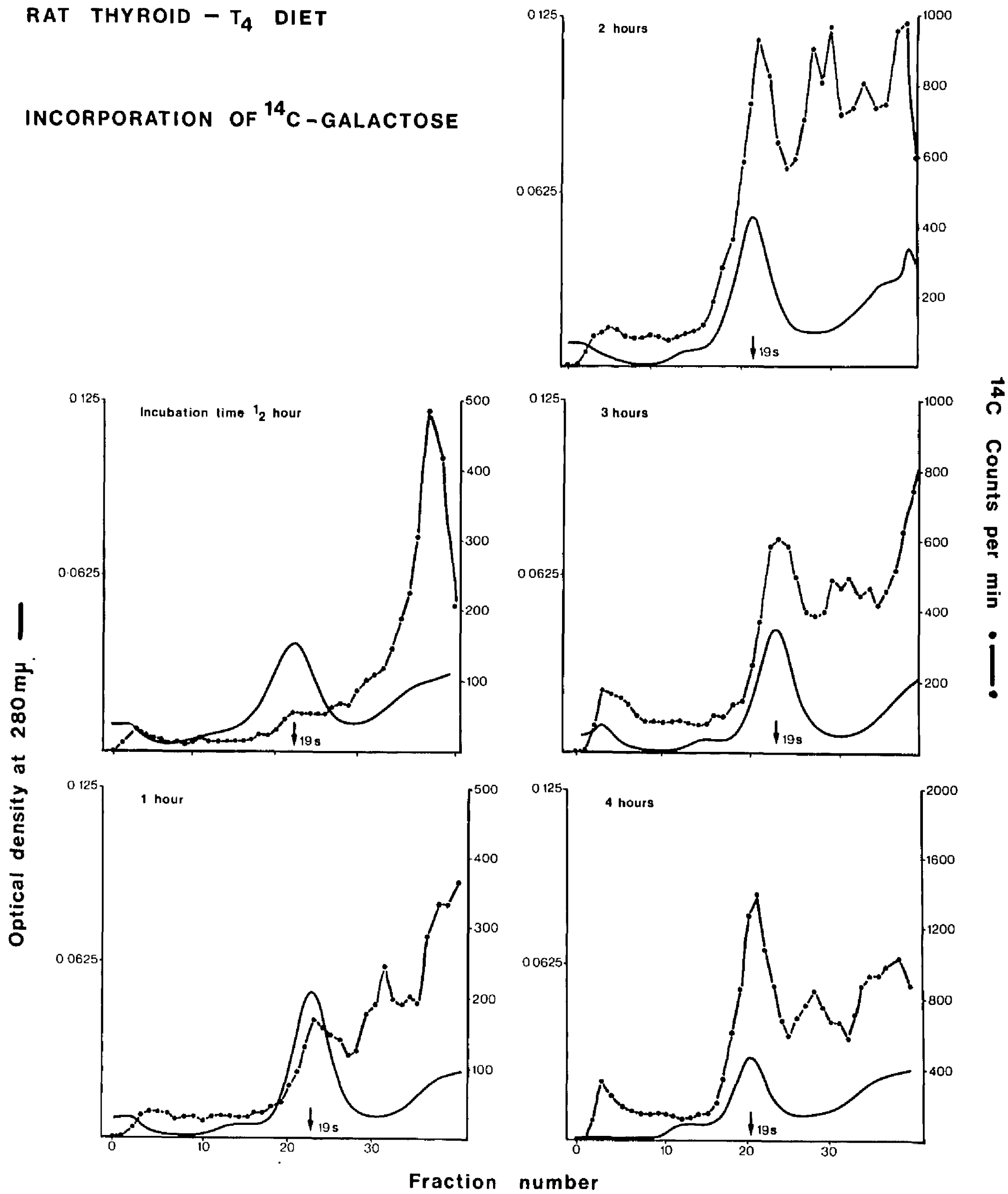


Fig. 9

Ultracentrifugal study of the pattern of incorporation of ^{14}C galactose into the thyroid proteins of rats on a diet containing T_4. SW 41 Rotor at 28,000 rpm for 16 hours.

the labelling of the 3 - 8S peak was not so well-defined but did appear to have decreased. By 2 hours there was marked increase in specific activity of labelling of the 19S peak which now corresponded more or less with the stable protein peak. Likewise the specific activity of labelling of the 12S and 3 - 8S peaks had also increased, all three peaks being approximately similar in size. At 3 hours there was a diminution in labelling of the 19S and 12S peaks; the 19S peak was the predominant labelled peak now present and by 4 hours the pattern of labelling had again changed further in this direction with the 19S peak increasing in specific activity and the 12S and 3 - 8S peaks relatively decreasing.

Compared to the ^{14}C galactose incorporation into the control rat, there was a constant decrease in all tracings of the specific activity of labelling of the thyroid proteins of rats on the thyroxine diet and a relative decrease in labelling of the proteins in the 19S region.

CHAPTER 10

EFFECT OF THE ADMINISTRATION OF POTASSIUM IODIDE (KI) ON THE INCORPORATION OF ^{14}C MANNOSE AND ^{14}C GALACTOSE

In Fig. 10 is shown the effect of the addition of 0.05% potassium iodide (KI) to the drinking water of rats on a normal diet on the incorporation of ^{14}C mannose into the rat thyroid proteins *in vitro*. It will be seen that at 30 minutes there is no significant incorporation of the isotope into the region of 19S thyroglobulin. A small peak of radioactivity in the 12S region is visible and there is a large peak of radioactivity in the lightweight 3 - 8S region. At one hour there is a small amount of the labelled sugar incorporated into the 19S area. The 12S peak has increased in size but the peak in the 3 - 8S area has decreased. After an incubation of 2 hours the radioactive peak in the 19S region has increased in size and has increased proportionately compared to the 12S peak, the 3 - 8S peak shows a further increase in activity. At 3 hours of incubation the 19S peak and 12S peak have increased further with the 3 - 8S region remaining almost static in activity. This process continued further until the 4 hour time of incubation where the 19S and the 12S peak had increased in size but the 3 - 8S peak had remained fairly static.

Compared with labelled mannose incorporation into the control rat thyroid proteins, it will be seen that the pattern of incorporation is basically very similar, the only difference being that at any given time the specific activity of labelling of the thyroid proteins of

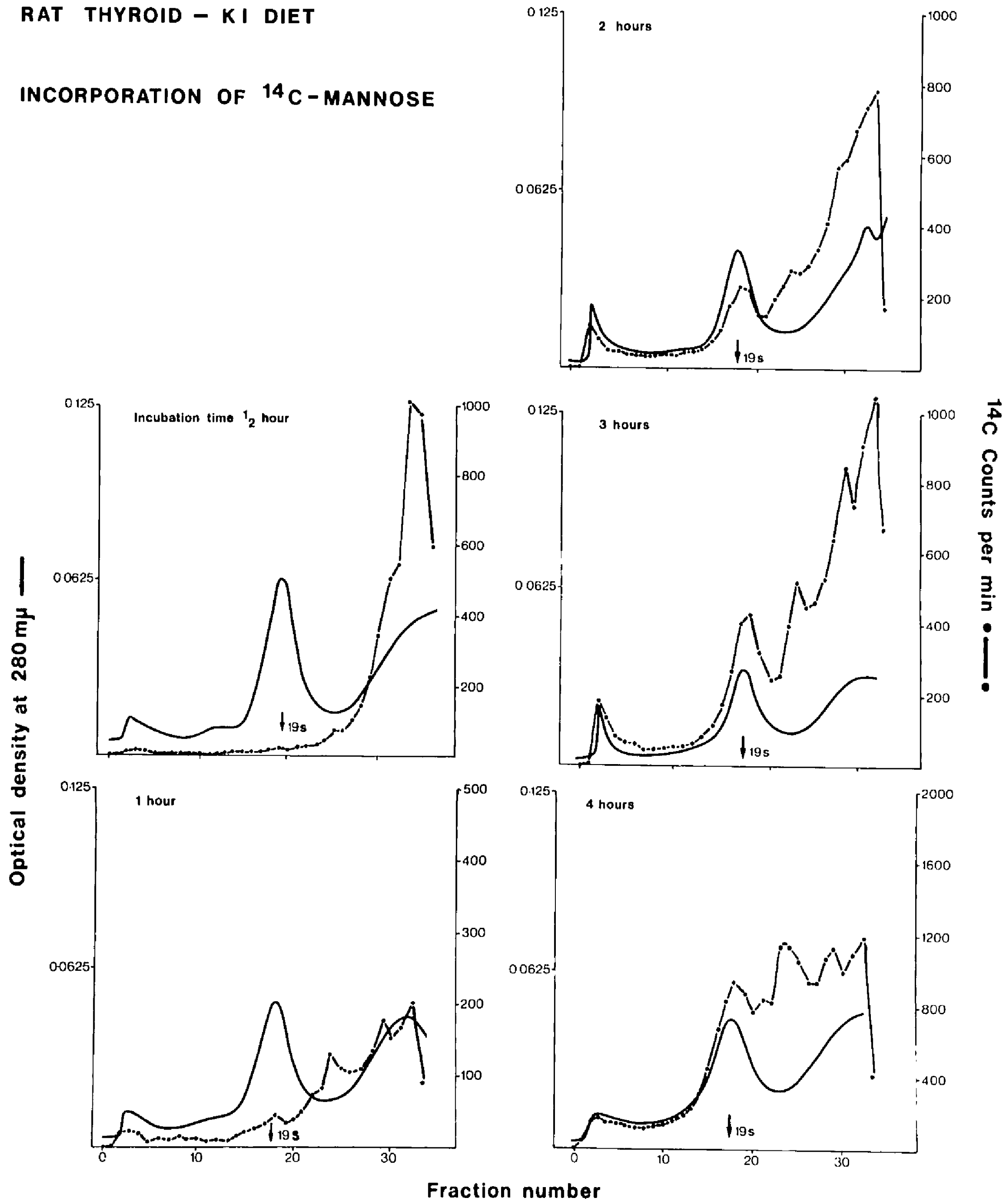


Fig. 10

Ultracentrifugal study of the time course of incorporation of ^{14}C mannose into the thyroid proteins of rats on a KI supplemented diet. SW 41 Rotor at 28,000 rpm for 16 hours.

rats on an iodine supplemented diet is lower than in the control animals. The radioactivity on average is decreased by a factor of 2.

In Fig. 11 is shown the pattern of incorporation of ^{14}C galactose into the thyroid proteins where the rats are maintained on the KI supplemented diet. It will be seen that at half an hour the labelled galactose appears in the 19S, 12S and 3 - 8S peaks. At one hour there is increased incorporation of the label into all three peaks occurring in approximately the same proportions as in the half hour sample. By the time 2 hours has elapsed the pattern has changed somewhat. The labelled peak in the 19S region is now the predominant labelled peak present and has increased markedly in specific activity compared to that of the one hour sample. The 12S peak has also increased in specific activity, as has the 3 - 8S peak, but it now is the smallest of the three peaks which are labelled. At 3 hours the radioactivity incorporated into the 19S region has again increased further as has the radioactivity incorporated into the 12S and 3 - 8S peaks; again at this time the labelled peak in the 19S region is by far the largest. This pattern of incorporation continues at the 4 hour sample in which there is increased incorporation into all three peaks with once more the labelled peak in the thyroglobulin region being the predominant one.

Compared to the galactose incorporation into the control animals, it will be seen that there is no striking difference in degree of specific activity achieved with the exception of the one hour sample in which the activity in the KI treated animals is significantly less than in the control rats.

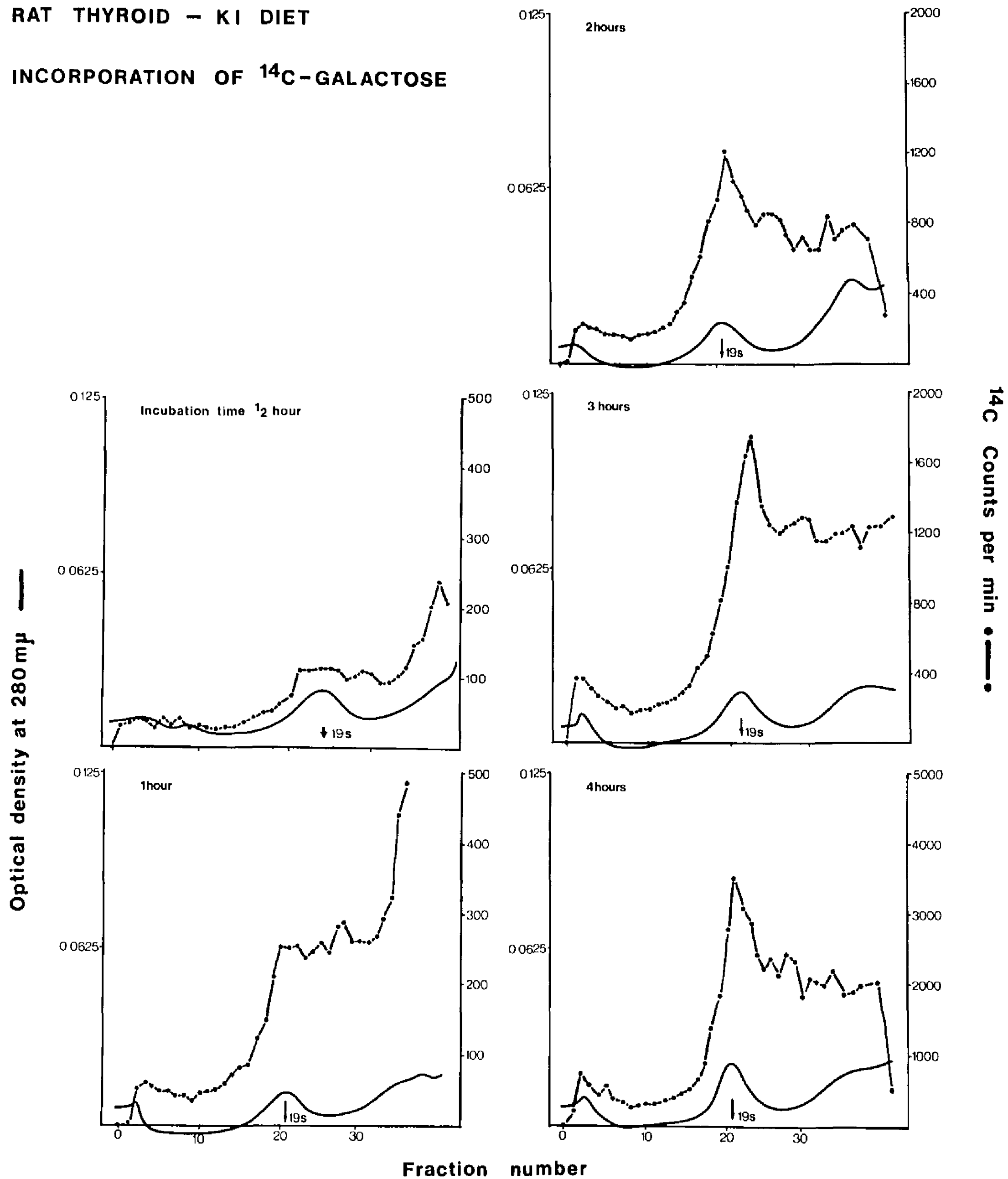


Fig. 11

Ultracentrifugal study of the pattern of incorporation of ^{14}C galactose into the thyroid proteins of rats on a KI supplemented diet. SW 41 Rotor at 28,000 rpm for 16 hours.

CHAPTER 11

EFFECT OF TSH IN VITRO AND IN VIVO ON THE INCORPORATION OF ^{14}C MANNOSE AND ^{14}C GALACTOSE

Effect of TSH in vitro

In Fig. 12 can be seen the effect of adding 2 units of bovine TSH to the incubation medium on the incorporation of ^{14}C mannose into control rat thyroid proteins. At half an hour it will be seen there is already some incorporation of the labelled mannose into the 19S region with very definite incorporation of the labelled sugar into the 12S and 3 - 8S regions. At one hour this incorporation has increased strikingly with a very well-defined peak in the 19S region and the activity in the 12S region has also increased markedly as has that associated with the 3 - 8S peak. After 2 hours of incubation there is a further striking increase in the incorporation of ^{14}C mannose into the 19S peak which has increased relatively in size compared to the other peaks although the labelled 12S peak is still the predominant one; the activity incorporated into the 3 - 8S peak has remained virtually static from the one hour sample. At 3 hours the total incorporation of the labelled sugar into the thyroid proteins has increased and the pattern has changed so that the labelled peak in the thyroglobulin region is now the predominant one although the radioactivity in the 12S peak has also increased; the radioactivity in the 3 - 8S peak has increased somewhat but is now much less than the other two peaks. This pattern of incorporation is continued in the 4 hour incubation sample in which again the

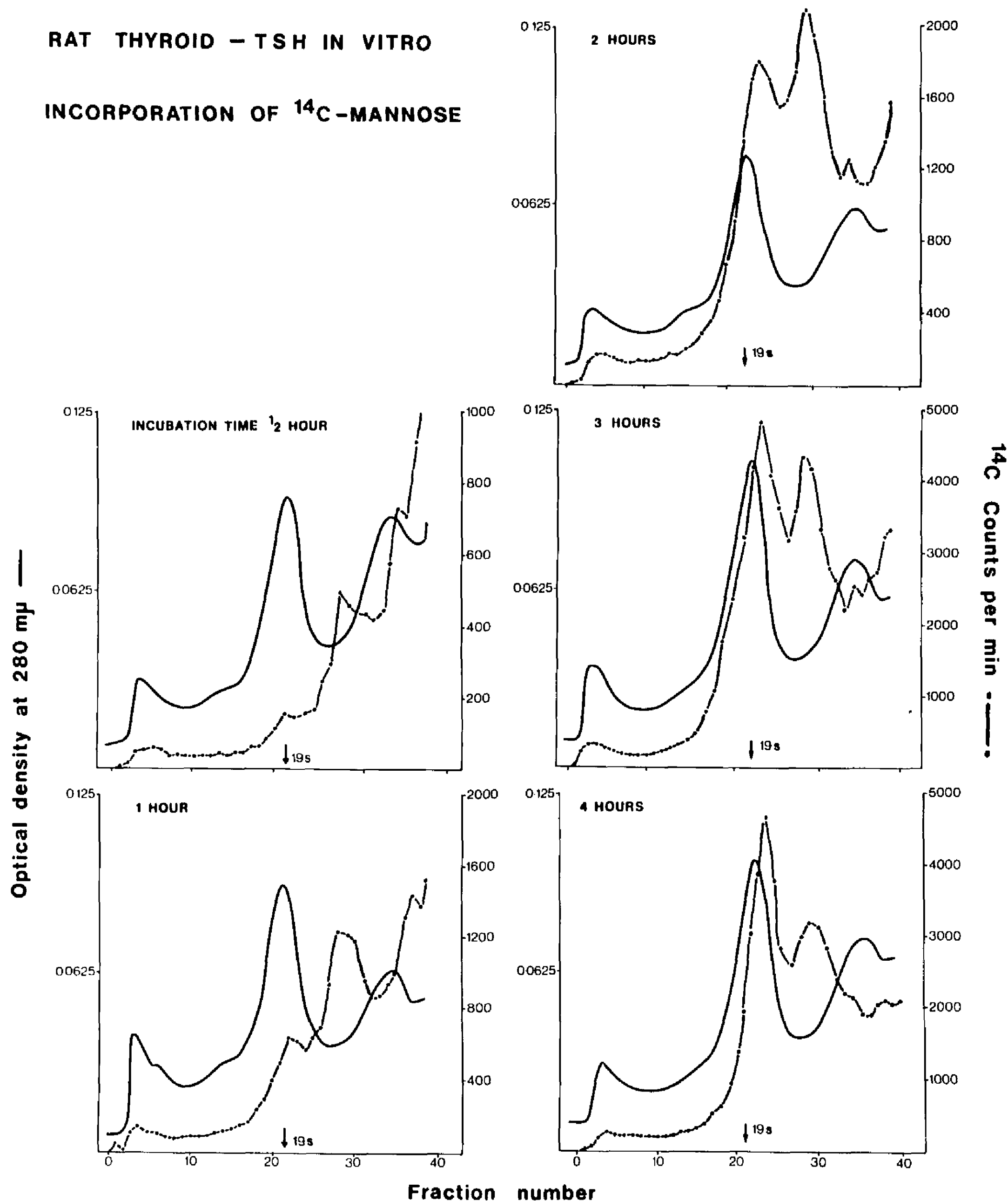


Fig. 12

Ultracentrifugal study of the patterns of incorporation of ^{14}C mannose into the thyroid proteins of control rat thyroids incubated in a medium containing 2 units of bovine TSH per flask. SW 41 Rotor at 28,000 rpm for 16 hours.

labelled sugar in the 19S peak is the predominant one with the radioactivity in the 12S peak now much smaller. There is not much evidence of any discrete incorporation into the 3 - 8S proteins at this time.

It will be seen comparing the effect of TSH in vitro with the incorporation of ^{14}C mannose in the control rats that the addition of TSH to the medium has resulted in a much greater incorporation of the labelled sugar into the thyroid proteins and has also resulted in the much earlier appearance of a labelled peak in the thyroglobulin region. This now appears as early as the half hour sample and at this time the 12S peak is much greater than in the control sample. Similarly at one hour there is much greater incorporation of the labelled sugar into the thyroglobulin peak and into the 12S peak. This pattern of increased total incorporation and more marked incorporation of the labelled sugar into the thyroglobulin region is continued in the 2 hour sample and it will be noted in the 3 hour sample where in the TSH experiment the label is predominantly in the thyroglobulin peak that this is not the case in the control experiment. This difference is also marked in the 4 hour sample.

Fig. 13 shows the pattern of incorporation of ^{14}C galactose into the rat thyroid proteins where 2 units of TSH is added to each incubation flask. Using this technique, at half an hour there is very good incorporation of ^{14}C galactose into the thyroglobulin region which indeed exceeds the activity of the 12S peak significantly. There also is evidence of lightweight protein labelling present. At one hour the specific activity of the labelling has increased markedly

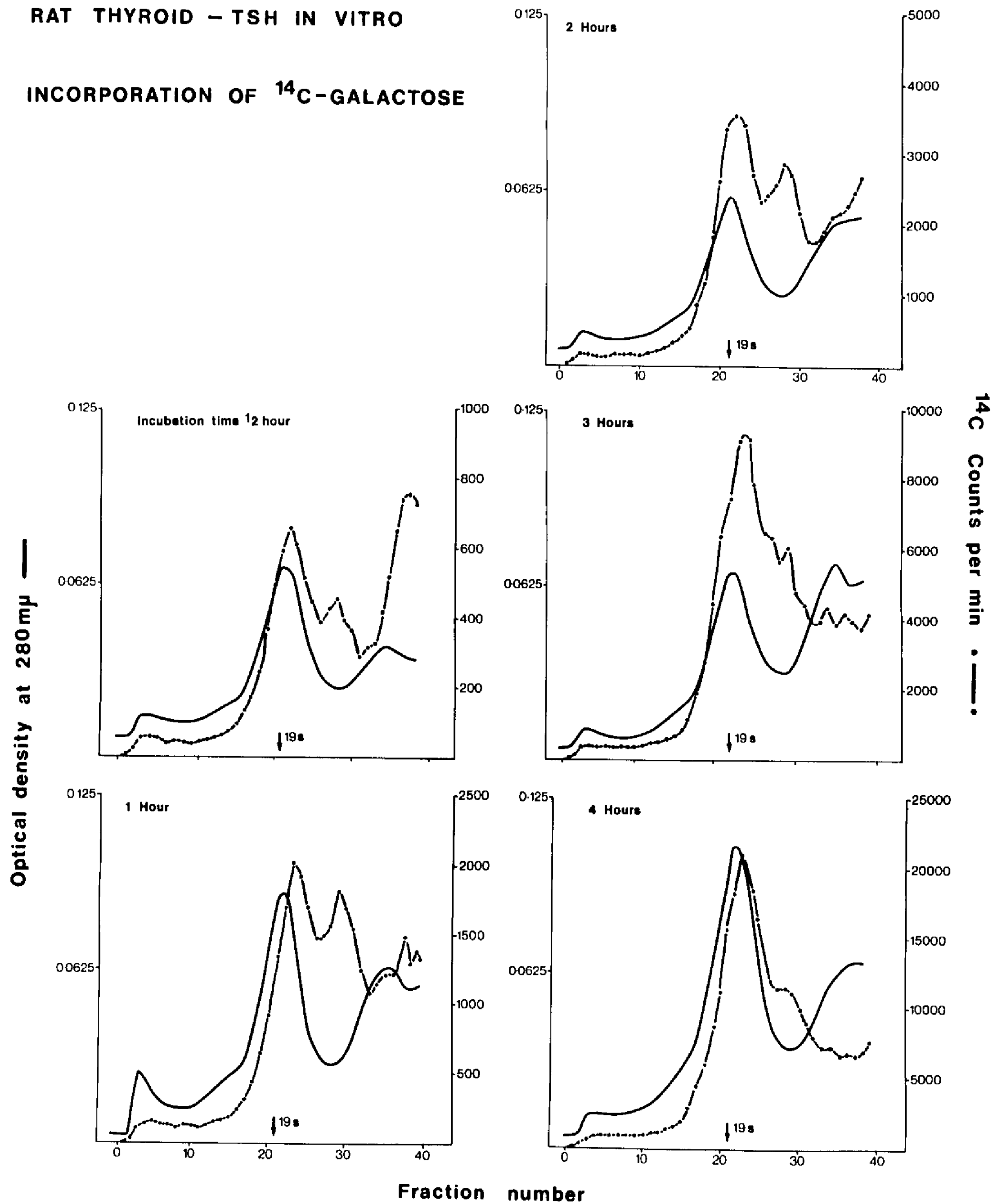


Fig. 13

Ultracentrifugal study of the patterns of incorporation of ^{14}C galactose into control rat thyroids incubated in a medium containing 2 units of bovine TSH per flask. SW 41 Rotor at 28,000 rpm for 16 hours.

and the 12S label has increased relative to the 19S label, the labelling in the 3 - 8S region has also increased. At 2 hours the total incorporation of the ^{14}C galactose has increased further. The label in the thyroglobulin region has relatively increased as compared to the 12S region but there still is a well-defined labelled 12S peak present. In the 3 hour sample there has been further striking increase in the incorporation of the ^{14}C galactose into the thyroglobulin region. The 12S peak in this particular tracing is not well-defined but also it has increased compared to the 2 hour sample. The label in the lightweight region is proportionately much less than it was in the earlier samples. At 4 hours there has been a further increase in incorporation of the labelled sugar into the 19S protein and a further incorporation of label into the 12S protein. There is no definite labelling of the lightweight proteins at this time.

It will be seen that compared to the incorporation of ^{14}C galactose into the control animals (Fig. 5) that when TSH is added to the medium there is a striking increase in the total incorporation of the label and also in the early labelling to a large extent of protein in the thyroglobulin and in 12S regions. It will be seen that as early as half an hour in the TSH sample that the predominant labelled peak present is the one in the thyroglobulin region whereas this is not achieved in the control experiment until the 2 hour sample. It will be noted in the TSH experiment at later times of incubation such as 3 and 4 hours that there is very little incorporation into the 12S and 3 - 8S regions compared to the control sample.

Effect of TSH in vivo

Fig. 14 shows the pattern of labelling of the thyroid proteins with ^{14}C mannose in rats which were injected with 2 units of bovine TSH by intraperitoneal injection 24 hours before sacrifice. At half an hour there is a suggestion of a small labelled peak in the thyroglobulin region; there is a broad band of radioactivity in the 12S area and a rather non-specific increase in activity over the lightweight protein region. At one hour there is a more definite peak in the thyroglobulin region although the predominant labelled peak is in the 12S area; labelling in the lightweight region is not particularly marked. By 2 hours there is well-defined incorporation of the labelled sugar of higher specific activity into the thyroglobulin region, the 12S region which is the predominant label present, and into the 3 - 8S proteins. At 3 hours this process is continued but the labelled 12S protein is still the predominant peak, the specific activity of labelling of the thyroglobulin and 12S peaks has increased but that of the 3 - 8S peak has remained relatively static. At 4 hours of incubation there is a further increased incorporation of the labelled sugar into the 19S peak but the labelled 12S peak is still the predominant one.

Compared to the incorporation of ^{14}C mannose into the control animal it will be seen that at half an hour the pattern of incorporation of the labelled sugar is very similar in the two samples. It is of interest that in the 1, 2 and 3 hour incubations that although the pattern of incorporation of the ^{14}C mannose is broadly similar in the TSH and control samples, in fact the specific activity of labelling of the sugar into the TSH samples is rather

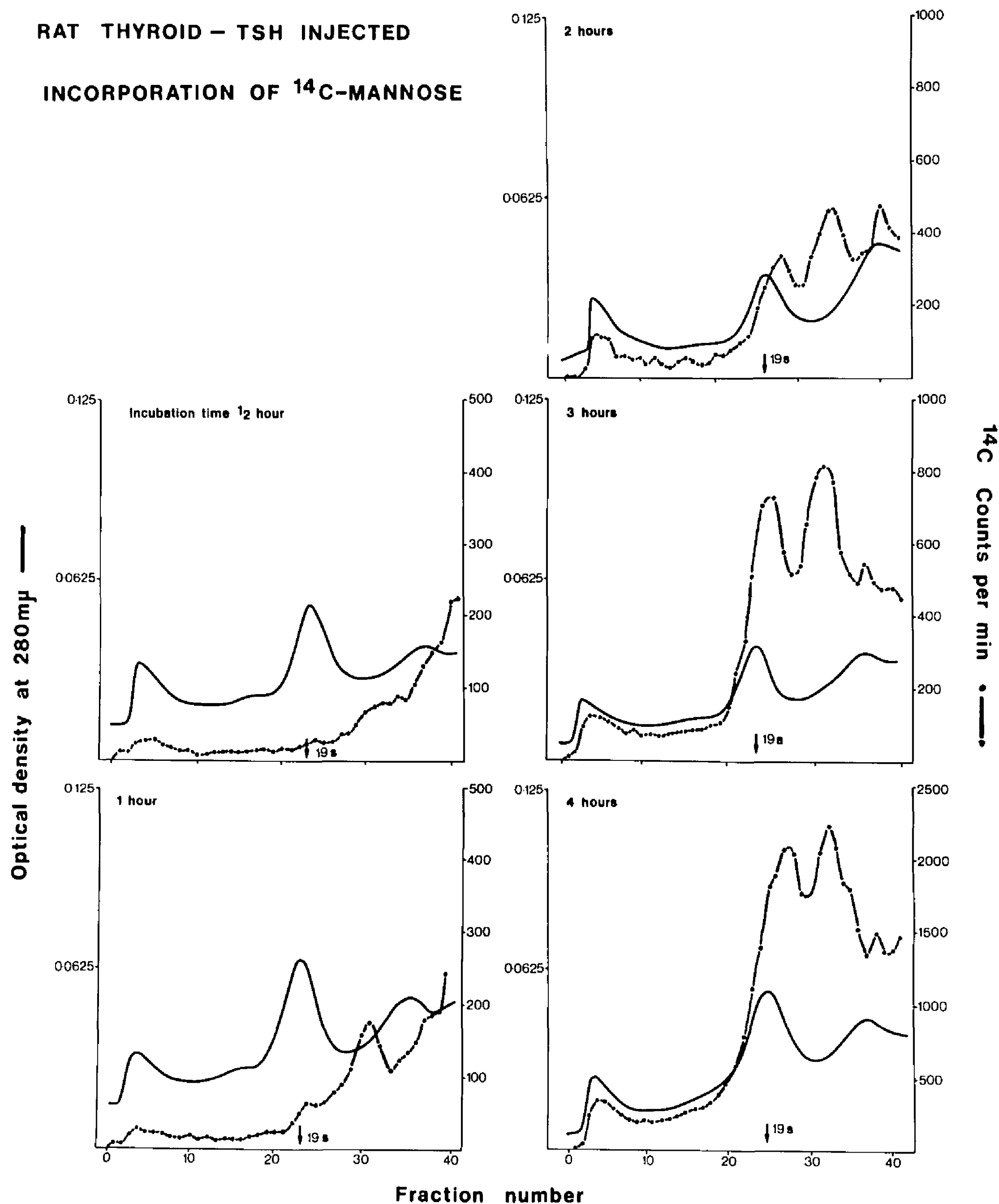


Fig. 14

Ultracentrifugal study of the patterns of incorporation of ^{14}C mannose into the thyroid proteins of rats who were injected 24 hours before sacrifice with 2 units bovine TSH by intraperitoneal injection. SW 41 Rotor at 28,000 rpm for 16 hours.

less than in the control experiments. It is only at the 4 hour sample of incubation that there is the suggestion of an increased incorporation of the ^{14}C mannose into the thyroglobulin region in the TSH treated animals. Part of the explanation of these discrepancies may be due to the fact that inspection of the stable protein patterns in the two tracings would suggest that less protein is present in the gradients from the TSH treated animals.

Fig. 15 shows the pattern of incorporation of ^{14}C galactose into the thyroids of rats injected into the peritoneum with 2 units of TSH 24 hours before sacrifice. It will be seen that at half an hour there is significant incorporation of the label into an 18S, 12S and 3 - 8S peak, all three peaks being well labelled but with the 12S peak being the smallest of the three. At one hour the specific activity of labelling has increased and the 12S peak is now proportionately larger than the 3 - 8S peak and approximately equal to that of the thyroglobulin peak. At 2 hours the label in the thyroglobulin and 12S peak has increased markedly with the labelled peak in the thyroglobulin region being now the predominant one; the incorporation of the label into the 3 - 8S peak has also increased. This pattern of labelling continues in the 3 hour sample in which the specific activity of labelling of the thyroglobulin peak becomes more marked as does the labelling of the 12S peak; that of the 3 - 8S peak increases slightly. At 4 hours there is further marked increase in specific activity of the thyroglobulin and of the 12S peak; the labelling of the 3 - 8S peak is rather ill-defined but continues at approximately the same level as in the 3 hour sample.

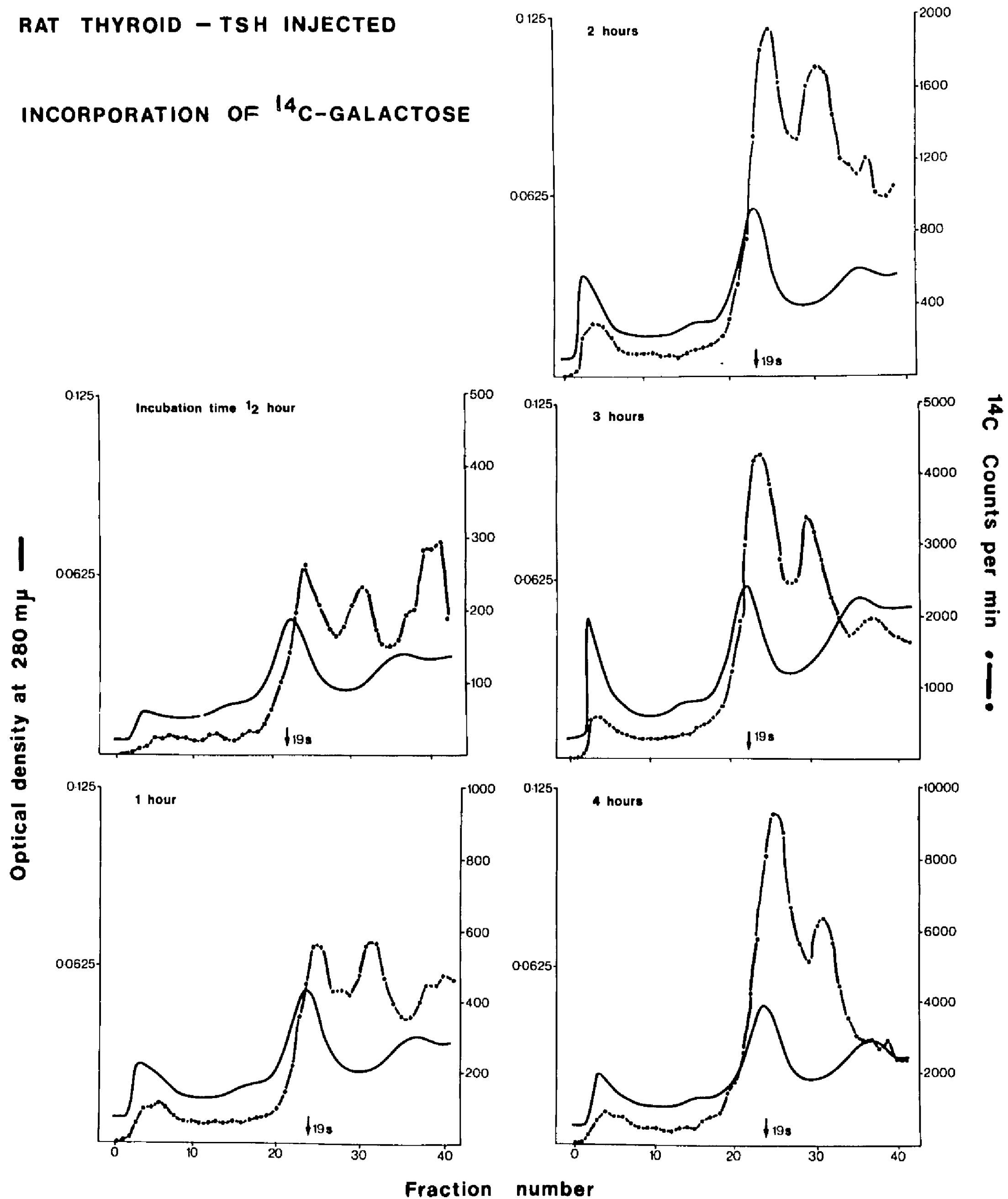


Fig. 15

Ultracentrifugal study of the patterns of incorporation of ^{14}C galactose into the thyroid proteins of rats who were injected 24 hours before sacrifice with 2 units of bovine TSH by intraperitoneal injection. SW 41 Rotor at 28,000 rpm for 16 hours.

Compared to the pattern of labelling of ^{14}C galactose into the control animals it will be noted that in the TSH treated animals there is an increased incorporation of the label into the thyroid proteins and also that this incorporation of label appears much more strikingly at early time intervals into the protein running just short of the thyroglobulin region, that is in approximately the 18S position. The labelling of the 12S protein fraction is more marked in the TSH treated animals at early (30 minutes) and late (3 and 4 hours) times of incubation but this does not apply at 1 or 2 hours incubation times.

CHAPTER 12

EFFECT OF CYCLOHEXIMIDE ON THE INCORPORATION OF ^{14}C MANNOSE AND ^{14}C GALACTOSE

Fig. 16 shows the effect of the addition of cycloheximide *in vitro* on the incorporation of ^{14}C mannose into the rat thyroid. The pattern of incorporation of the ^{14}C mannose in the control experiment at half an hour is shown in the top part of the figure where it will be seen, as previously noted, that the main incorporation at this time is into the 12S and 3 - 8S protein with very little incorporation into the thyroglobulin region. The middle portion of the figure shows the anticipated result at 4 hours of incubation in the control animal, that is that the predominant incorporation of the ^{14}C mannose is now into the thyroglobulin region with a well-defined 12S peak still present but with little incorporation into the 3 - 8S area. When the cycloheximide is added in the concentration of 2.0 mM after half an hour of incubation and the incubation is continued until 4 hours (lower portion of figure) it will be noted that the predominant incorporation is into the 19S protein with a small 12S peak still present and a rather broad band in the 3 - 8S region. Compared to the control 4 hour figure the total incorporation of ^{14}C mannose into the thyroglobulin region is diminished by a factor of some sevenfold as is the incorporation into the 12S region. Compared however to the half hour sample, that is the time at which the cycloheximide was added, there obviously has been incorporation of mannose from peaks lighter than the 19S peak

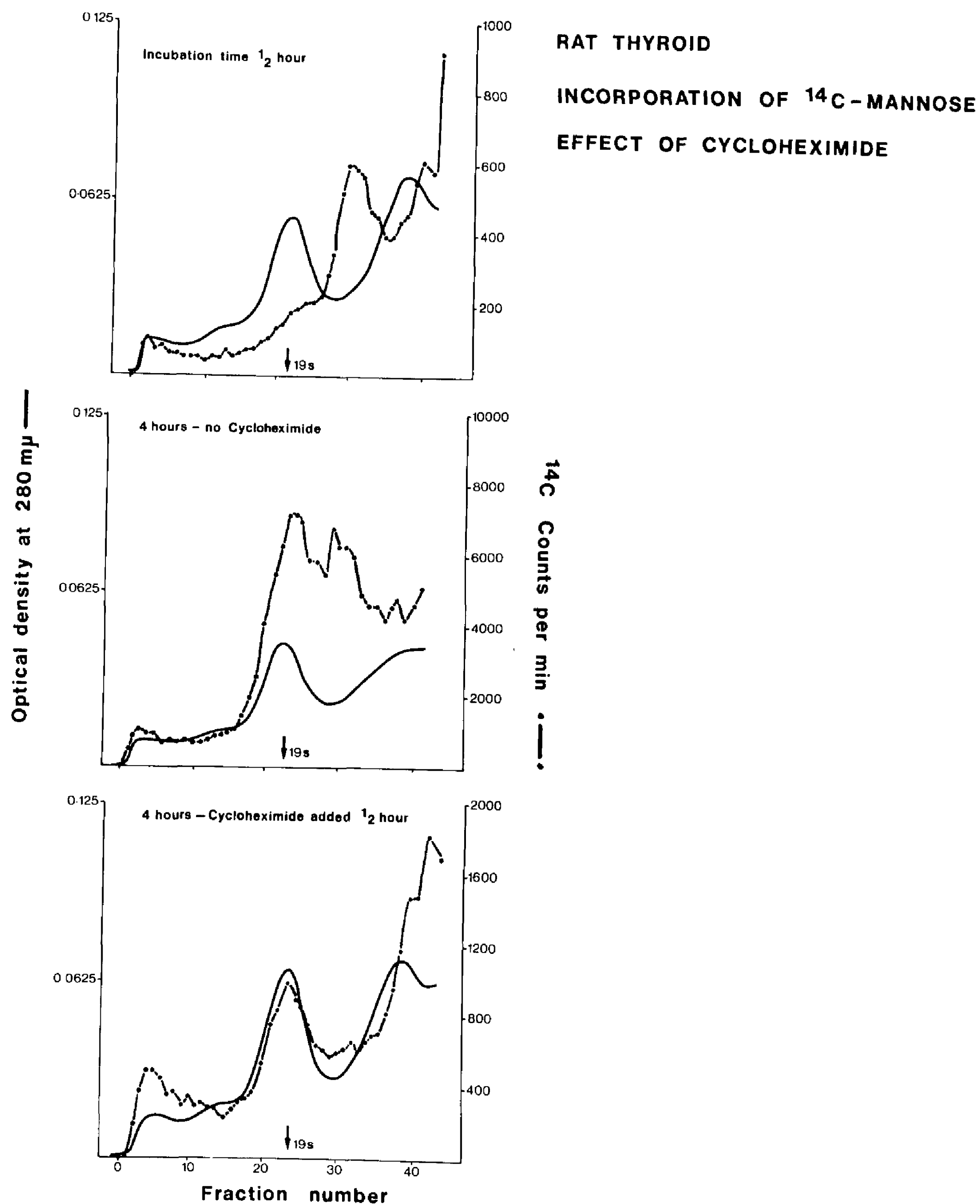


Fig. 16

Ultracentrifugal study of the effect of cycloheximide on the incorporation of ^{14}C mannose into control rat thyroid protein. Upper portion: control half hour incubation. Middle portion: control four hour incubation. Lower portion: 4 hour incubation in which cycloheximide was added to the medium at 30 minutes from the beginning of the incubation. SW 41 Rotor at 28,000 rpm for 16 hours.

into the thyroglobulin region. This process has occurred despite the use of a concentration of cycloheximide which is sufficient to effectively block further uptake of sugars or amino acids into the rat thyroid _in vitro_. This would therefore suggest that once the 12S protein has been formed that cycloheximide is ineffective in stopping the formation of the 19S protein.

Fig. 17 shows a similar experiment on the effect of the addition of cycloheximide _in vitro_ on the incorporation of ^{14}C galactose into the thyroid proteins of the rat. The control half hour incubation is shown again in the top part of the figure and this shows that at this time there is, as previously noted, a small peak running approximately in the thyroglobulin region but not quite in 19S position. There is a rather broad band of radioactivity in the 12S region and in the 3 - 8S region. By 4 hours of the control incubation (middle part of the figure) the expected pattern is obtained with the predominant peak being in the thyroglobulin region and with a well marked, although smaller 3 - 8S and 12S peak present. The lower part of the figure again shows the pattern when 2.0 mM cycloheximide is added after half an hour of incubation but with the incubation continued for 4 hours. It will be noted that compared to the 4 hour control incubation the specific activity of labelling of the protein in the thyroglobulin region is down by approximately threefold. The labelling of the 12S protein is also strikingly lower than the 4 hour control incubation. Compared to the control half hour incubation however there obviously has been increasing accumulation of the labelled material in the 19S region and to a lesser extent in the 12S

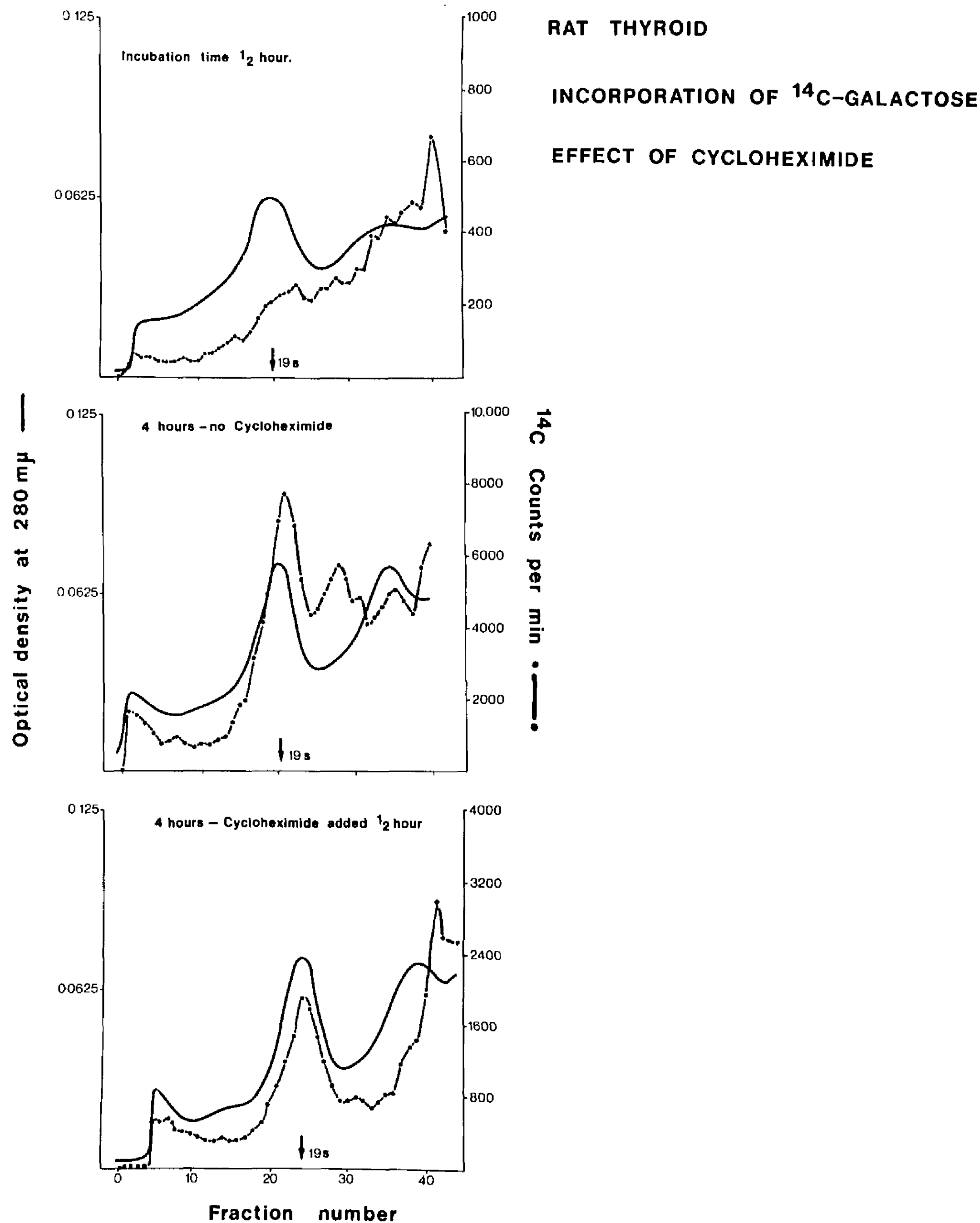


Fig. 17

Ultracentrifugal study of the effect of cycloheximide on the incorporation of ^{14}C galactose into control rat thyroid protein. Upper portion: control half hour incubation. Middle portion: control 4 hour incubation. Lower portion: 4 hour incubation, cycloheximide added at 30 minutes from the commencement of incubation. SW 41 Rotor at 28,000 rpm for 16 hours.

region. This would again suggest that the addition of cycloheximide does not prevent the formation of labelled 19S protein provided that smaller units such as 12S and 3 - 8S proteins are allowed to be labelled before the addition of cycloheximide.

CHAPTER 13

EFFECT OF ADDITION OF PYRUVATE TO THE INCORPORATION OF ^{14}C MANNOSE

Since it has been claimed (Herscovics, 1969) that the incorporation of ^{14}C mannose into the thyroid proteins is decreased by the addition of pyruvate, an experiment was performed to look at this effect in our particular system. Figs. 18 and 19 show a simultaneously performed comparison between the incorporation of ^{14}C mannose into the rat thyroid proteins *in vitro* without and with the addition of 10 mM sodium pyruvate. Fig. 18 shows the expected pattern of incorporation of ^{14}C mannose as previously outlined, that is at half an hour with the most obvious peak being in the 12S region with labelling of the 3 - 8S proteins in addition. A peak running just short of a 19S region appears at one hour but still the predominant labelling is in the 12S peak and some labelling is also seen in the 3 - 8S area. The incorporation of the radioactivity increases by 2 hours of incubation but still with a predominant 12S peak labelled. This pattern was maintained at 3 hours with increasing incorporation of the label. By 4 hours the predominant labelled peak was running just short of the thyroglobulin region with a well-defined labelled 12S peak and 3 - 8S peak still present.

In Fig. 19 the effect of pyruvate on this accumulation of ^{14}C labelled mannose into the thyroid proteins of the rat is shown. It will be seen that a very similar pattern of incorporation of radioactivity is shown with the possible exception of the 4 hour incubation

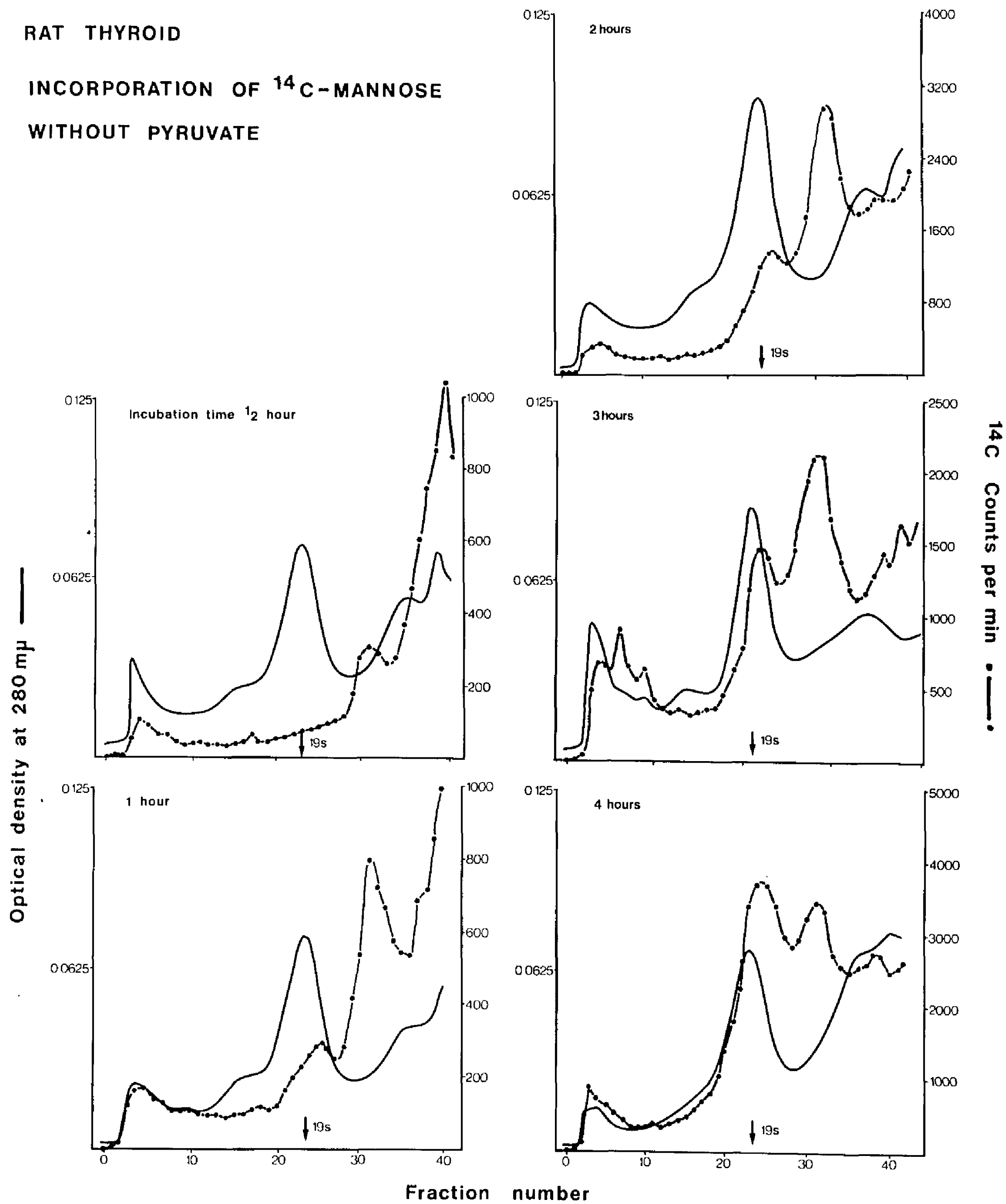


Fig. 18

Ultracentrifugal study of the time course of incorporation of ^{14}C mannose into control rat thyroid protein. No pyruvate added to the incubation medium. SW 41 Rotor at 28,000 rpm for 16 hours.

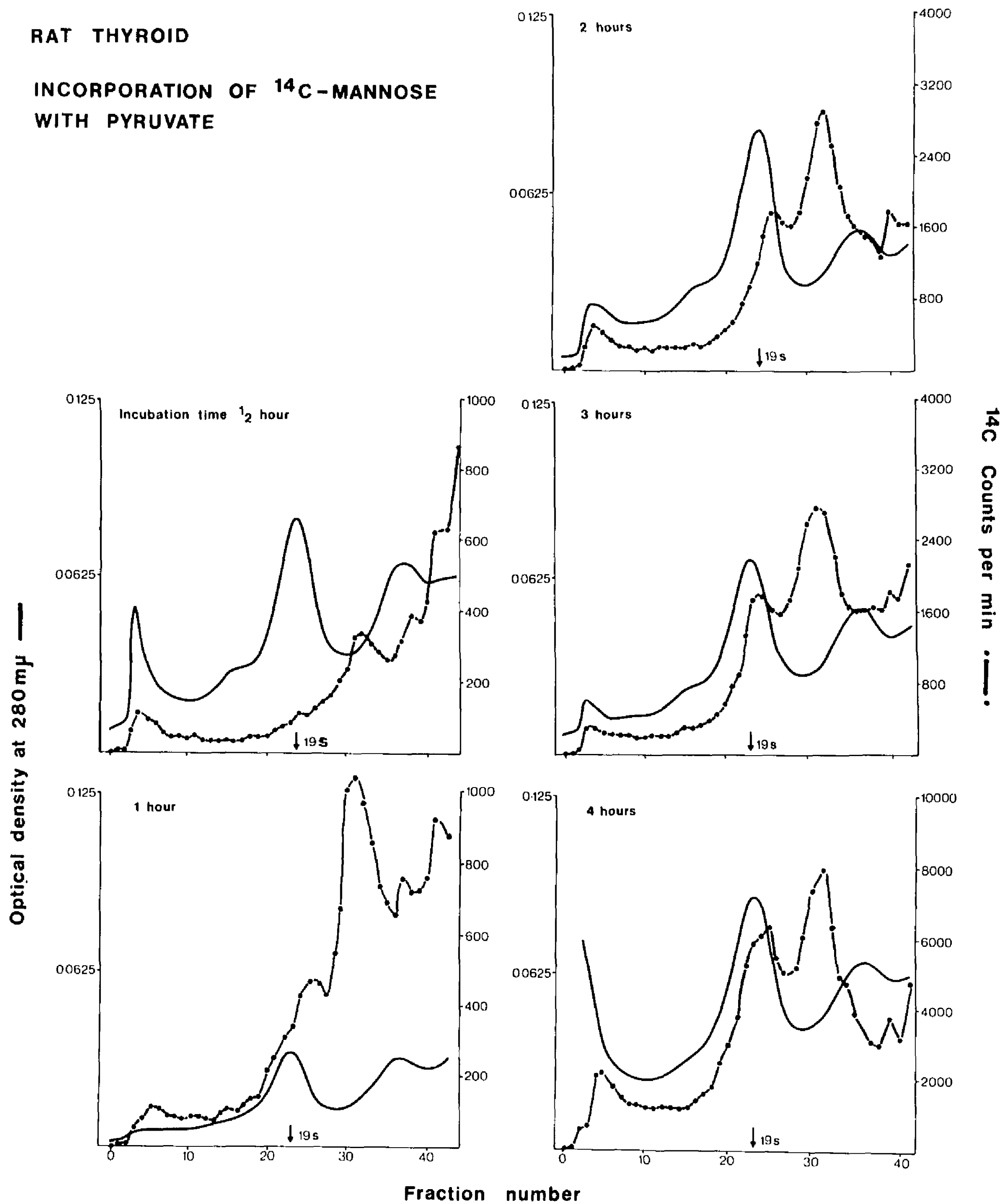


__Fig. 19__

Ultracentrifugal study of the time course of incorporation of ^{14}C mannose into control rat thyroid protein when 10 mM sodium pyruvate was added to the medium. SW 41 Rotor at 28,000 rpm for 16 hours.

where there is still a predominant 12S peak which contrasts with the pattern in the control experiment where the 19S peak is predominant at this time. The specific activity of labelling of the thyroglobulin peak was not, however, less than the control experiment. With this exception, however, there certainly was no evidence of any inhibition of incorporation of the ^{14}C mannose into the thyroid proteins as a result of adding pyruvate and if anything the total count rate achieved in the experiment with the addition of pyruvate exceeded that in the control run.

CHAPTER 14

EFFECT OF THE ADDITION OF STABLE SUGARS ON THE INCORPORATION OF ^{14}C MANNOSE AND ^{14}C GALACTOSE

The effect of the addition of stable mannose on the incorporation of ^{14}C mannose into the rat thyroid *in vitro* is shown in Fig. 20. In this figure all the incubation times are 4 hours. The control incubation without any added mannose gives the expected pattern at this time of incubation of a predominant peak just short of the thyroglobulin region with a large 12S peak still present. When 10 mM mannose is added it will be seen that the specific activity of labelling of the protein in the thyroglobulin region drops by a factor of about eight-fold, as does the incorporation of the label into the 12S protein. As the concentration of added stable mannose is increased to 50 mM the incorporation of labelled mannose decreases further by a factor of fourfold, as does the incorporation into the 12S protein. At 100 mM stable mannose added *in vitro* there is now only a small peak of incorporation of the labelled material in the thyroglobulin and in the 12S region with a rather broad peak of activity associated with the 3 - 8S proteins. At the concentration of 500 mM mannose added *in vitro* there is only the faintest suggestion of incorporation into the thyroglobulin region and no obvious incorporation into other proteins.

Similarly, in Fig. 21 is shown the effect of the addition of graded doses of stable galactose on the pattern of incorporation of

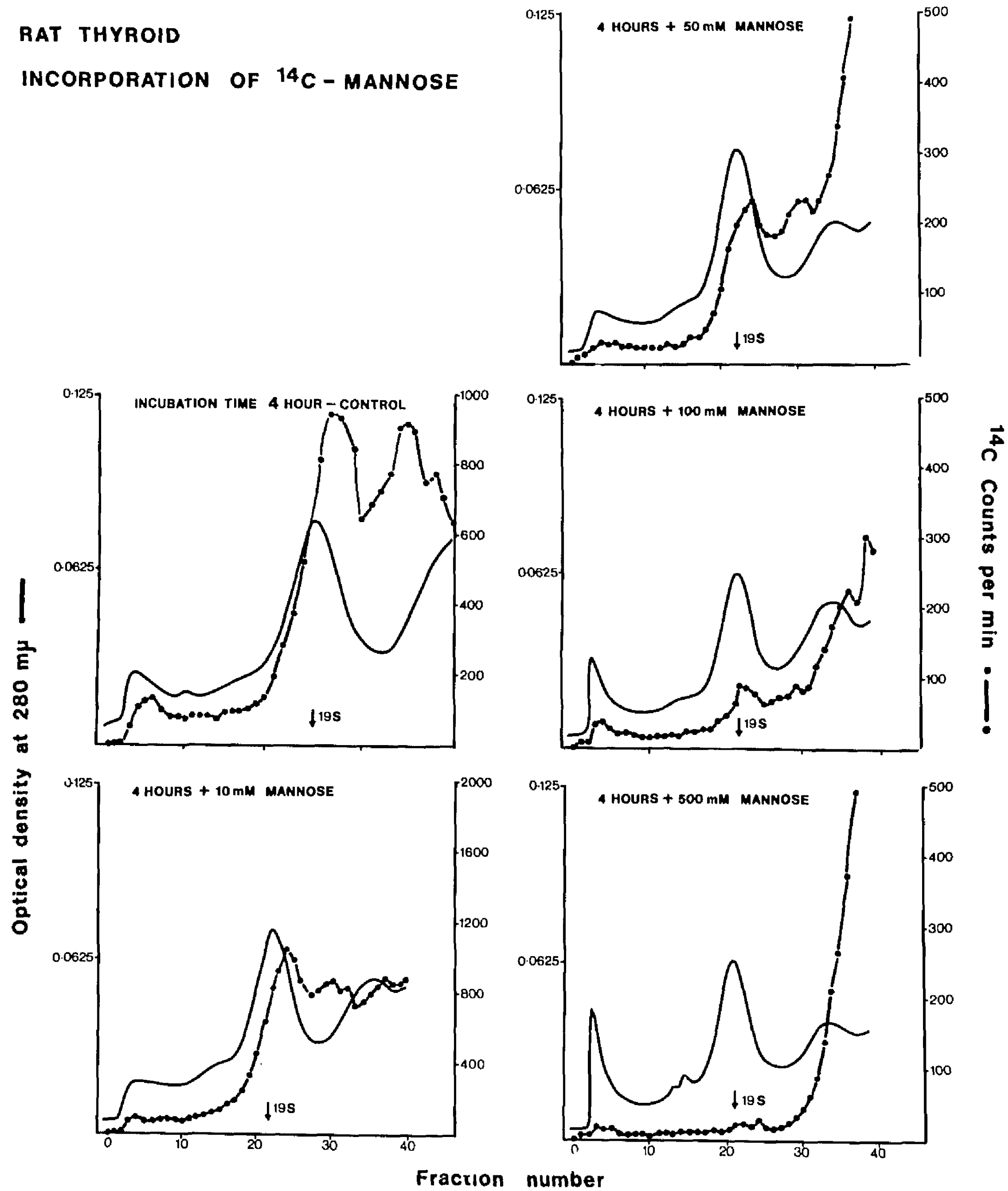
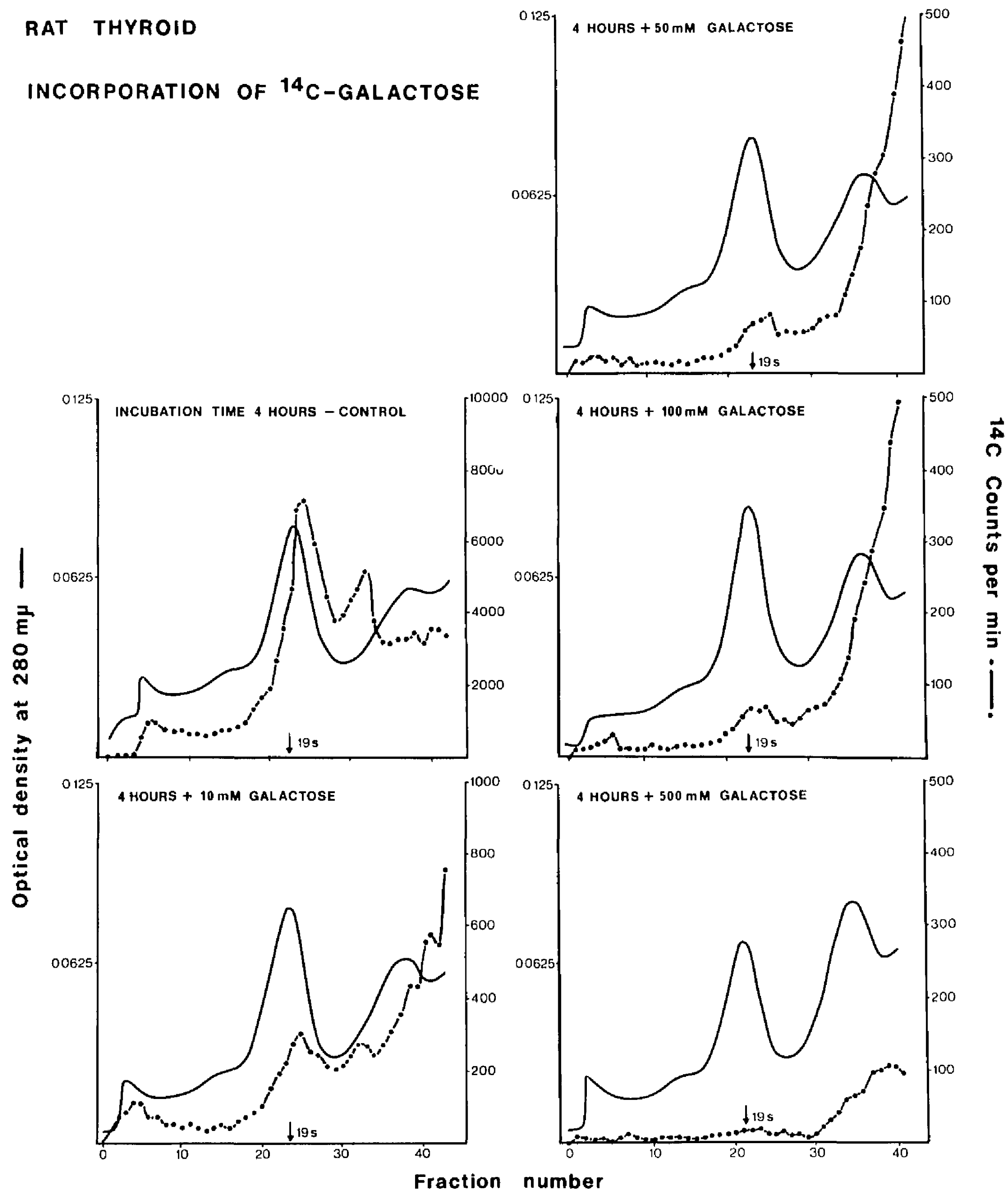


Fig. 20

Ultracentrifugal study on the effect of adding graded doses of stable mannose on the time course of incorporation of labelled mannose into control rat thyroid protein. SW 41 Rotor at 28,000 rpm for 16 hours.



<u>Fig. 21</u>

Ultracentrifugal study of the effect of the addition of graded doses of stable galactose on the incorporation of labelled galactose into control rat thyroid protein. SW 41 Rotor at 28,000 rpm for 16 hours.

^{14}C galactose into the rat thyroid proteins after 4 hours incubation. At 4 hours in the control experiment the predominant labelled protein is, as expected, in the thyroglobulin region although just short of 19S. There is a well-defined labelled peak in the 12S region with no very obvious peak in the 3 - 8S region. The addition of 10 mM stable galactose added in vitro causes a fall in the incorporation of radioactivity into the thyroglobulin region by a factor of some twentyfold. A slightly lesser effect is shown on 12S protein relative to the 19S but this still falls dramatically in activity. The addition of 50 mM galactose brings about a further marked fall in the incorporation of radioactivity into the thyroglobulin region and into the 12S region where only a faintly discernable peak is now present. This situation is similar to that found when 100 mM galactose is added in vitro and when 500 mM galactose is added there is virtually no incorporation of the labelled galactose into the thyroglobulin region but there is rather a broad non-specific peak of radioactivity over the area of the 3 - 8S proteins.

Studies were also undertaken to see what effect the addition of mannose would have on galactose incorporation. The effect of galactose on mannose incorporation was also studied. A concentration of 200 mM added stable mannose and galactose were used in these experiments because this concentration, based on the immediately preceding work, fell just short of total inhibition of incorporation. Fig. 22 shows the effect of added stable mannose and galactose on the incorporation of ^{14}C mannose into the rat thyroid proteins in vitro. The incubation times are all at 4 hours and the control experiment

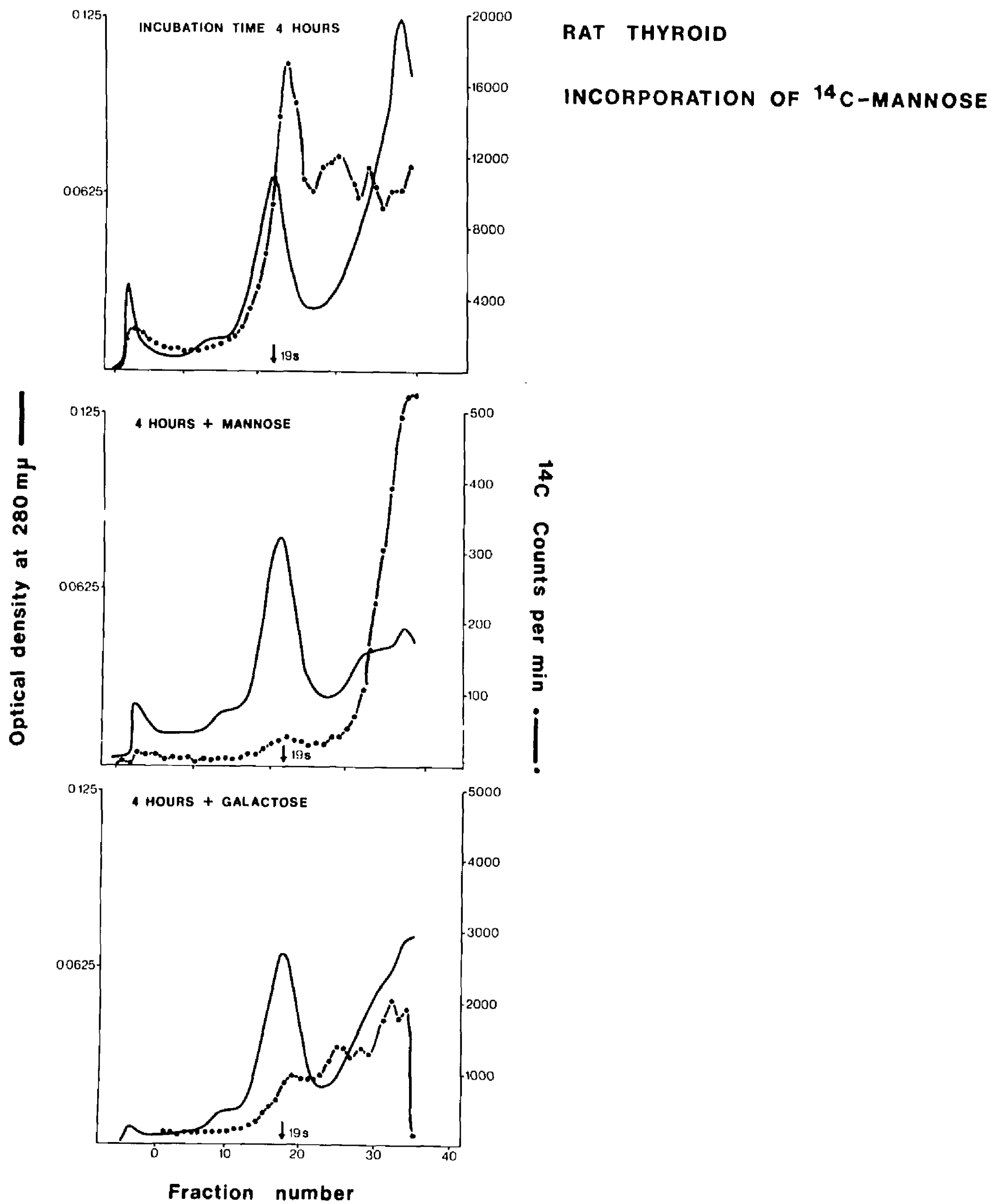


Fig. 22

Ultracentrifugal study of the effect of added stable sugars on the incorporation of ^{14}C mannose into control rat thyroid. Upper portion: control 4 hour incubation. Middle portion: 4 hour incubation plus 200 mM mannose. Lower portion: 4 hour incubation plus 200 mM galactose. SW 41 Rotor at 28,000 rpm for 16 hours.

(top of figure) shows the expected pattern of the predominant peak running just short of the thyroglobulin region and a well marked 12S peak and a smaller 3 - 8S labelled peak present. As anticipated at the concentration of 200 mM mannose, there was only a small amount of incorporation of ^{14}C mannose into the proteins in the thyroglobulin region with a rather non-specific band at the top of the gradient (middle part of figure). The addition of 200 mM stable galactose, however, resulted in much less inhibition of incorporation of the ^{14}C mannose and it will be seen that there are small but well-defined peaks occurring in the thyroglobulin region, the 12S region and in the 3 - 8S region (lower part of the figure).

Conversely, Fig. 23 shows the effect of 200 mM stable galactose and mannose on the incorporation of ^{14}C galactose into the rat thyroid proteins <u>in vitro</u>. The expected pattern found in the control incubation at 4 hours is shown (top of figure) and it will be seen that there is a predominant labelled peak in the thyroglobulin region running just short of a 19S peak, a well marked 12S peak and less well marked 3 - 8S peak are also seen. The addition of 200 mM galactose <u>in vitro</u> (middle of figure) results in virtual inhibition of labelling of the proteins with the exception of a minimal peak in the thyroglobulin region. The effect of the addition of 200 mM mannose is also shown and it will be seen that there is now a better defined peak of labelling in the thyroglobulin region, a small 12S peak and a peak in the 3 - 8S region.

From this series of experiments it can be seen that the incorporation of labelled sugar is inhibited at increasing concentrations

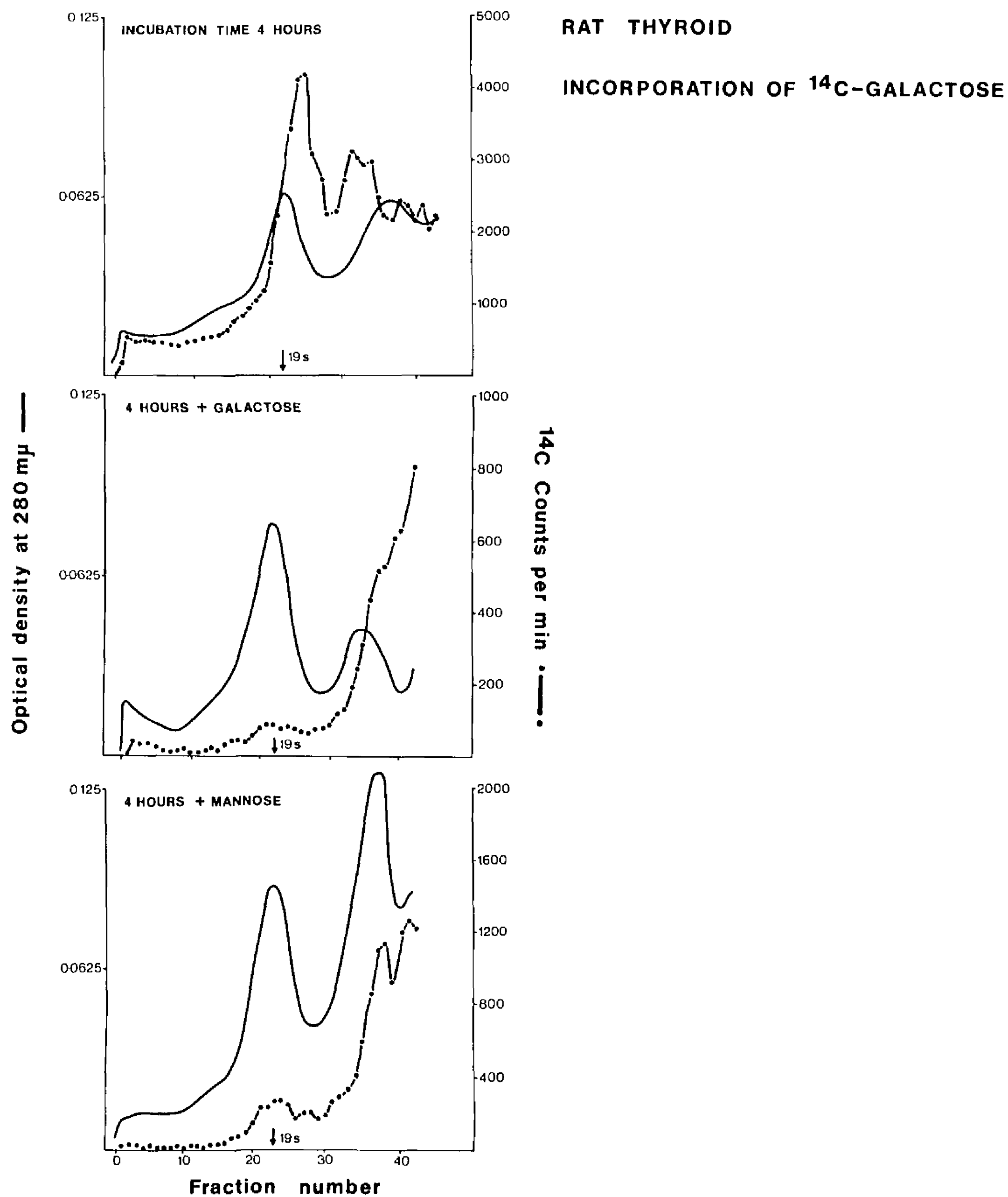


Fig. 23

Ultracentrifugal study of the effect of added stable sugars on the incorporation of ^{14}C galactose into control rat thyroid proteins. Upper portion: control 4 hour incubation. Middle portion: 4 hour incubation plus 200 mM galactose. Lower portion: 4 hour incubation plus 200 mM mannose. SW 41 Rotor at 28,000 rpm for 16 hours.

of the stable sugar. Furthermore, the results suggest that there is not merely a non-specific inhibition of labelling due to such things as osmotic pressure but that each stable sugar is more effective in inhibiting the incorporation of its own labelled compound than is the other sugar tested.

The effect of the added stable sugars on the osmolality of the buffers is shown in Table 1. These were measured using an Advanced Osmometer. It will be seen that at equal quantities of added mannose and galactose an equivalent increase in osmolality was obtained.

Table 1

EFFECT OF ADDED STABLE MANNOSE AND GALACTOSE ON THE OSMOLALITY OF KREBS NO. 2 BUFFER

Buffer	Osmolality (mOsm/Kg)
Krebs no. 2 Buffer	273
" " " " + 10 mM Mannose	286
" " " " + 50 mM Mannose	324
" " " " + 100 mM Mannose	375
" " " " + 200 mM Mannose	470
" " " " + 500 mM Mannose	759
" " " " + 10 mM Galactose	284
" " " " + 50 mM Galactose	323
" " " " + 100 mM Galactose	372
" " " " + 200 mM Galactose	465
" " " " + 500 mM Galactose	746

CHAPTER 15

EFFECT OF ADDED STABLE MANNOSE AND GALACTOSE ON LABELLING WITH ^{3}H LEUCINE AND ^{125}I

In this section some experiments are illustrated to show the effect the addition of stable mannose and galactose on the incorporation of labelled leucine and iodine into the rat thyroid proteins *in vitro*. In Fig. 24 is shown the effect of added stable mannose and galactose in the concentration of 200 mM on incorporation of ^{3}H leucine into the rat thyroid proteins. The control incubation is shown and it will be noted that at 4 hours, which is the time of incubation in all experiments in this section, that there is a well-defined peak of labelling running just short of the thyroglobulin region, a rather broad 12S peak of labelling is present and there also is a labelled peak in the 3 - 8S region. This pattern of labelling with ^{3}H leucine is what is regularly found in the experience of the author (Thomson and Goldberg, 1968) and of other workers. When 200 mM stable mannose is added to the incubation medium it will be seen that this pattern of labelling is broadly preserved although there is a definite fall in radioactivity by a factor of some threefold. When 200 mM stable galactose is added to the incubation medium it will be seen that there is a change in pattern of radioactivity with now the labelled peak in the thyroglobulin region being decidedly smaller than the other two peaks. There is an apparent fall in the radioactivity in addition but it must be borne in mind that there is less stable protein present in this run as judged by the size of the thyroglobulin peak,

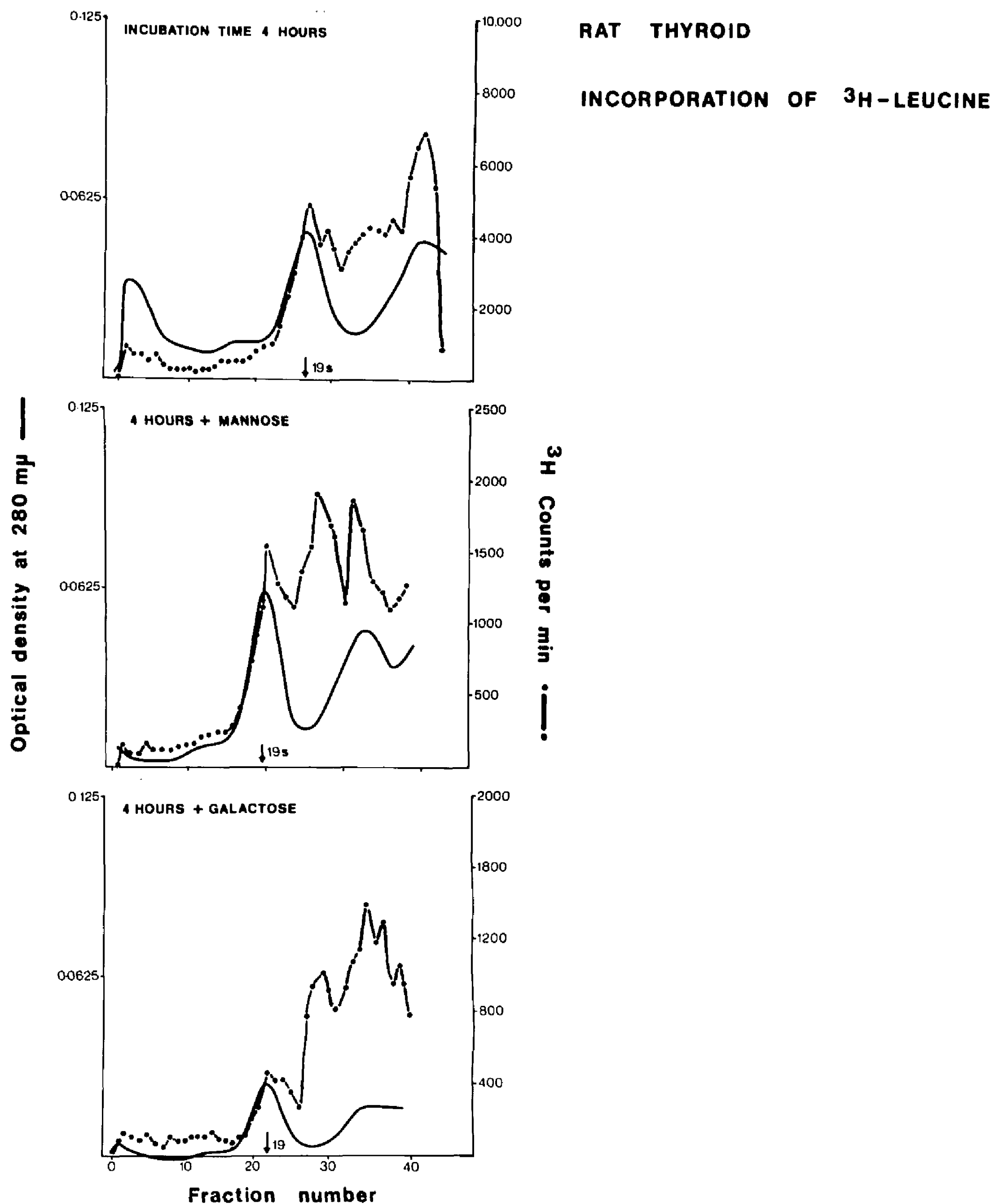


Fig. 24

Ultracentrifugal study of the effect of added stable sugars on the incorporation of ^{3}H leucine into control rat thyroid proteins. Upper portion: control 4 hour incubation. Middle portion: 4 hour incubation plus 200 mM mannose. Lower portion: 4 hour incubation plus 200 mM galactose. SW 41 Rotor at 28,000 rpm for 16 hours.

therefore one could not be too dogmatic about changes in total counts incorporated. It would therefore seem that the addition of stable mannose and galactose in the concentration used has some slight effect in inhibiting the incorporation of ^{3}H leucine into the rat thyroid in vitro with a suggestion that galactose may be more effective in inhibiting the incorporation into proteins in the thyroglobulin region than into other areas.

In Fig. 25 is shown the effect of the same concentrations of stable mannose and galactose on incorporation of ^{125}I into the rat thyroid proteins in vitro. The incubation times are all 4 hours and at this time in the rat thyroid, as shown by previous work, the pattern of radioactivity is virtually that of a single peak in the region of thyroglobulin with perhaps a little labelling of 27S protein present in this particular specimen. The addition of 200 mM stable mannose results in approximately 50% reduction in the radioactivity incorporated but the same pattern of incorporation is maintained. With the added stable galactose a very similar pattern to the control experiment was found and certainly there was no evidence of inhibition of ^{125}I incorporation. If anything there was an augmentation of the ^{125}I incorporation under this circumstance.

It can therefore be concluded that in the rat the addition of stable sugars in vitro at the concentrations used do not have a fundamental effect on the pattern of incorporation of iodine into the rat thyroid proteins. There is some suggestion that mannose might inhibit the total incorporation of the radioactivity but certainly galactose does not seem to have this effect.

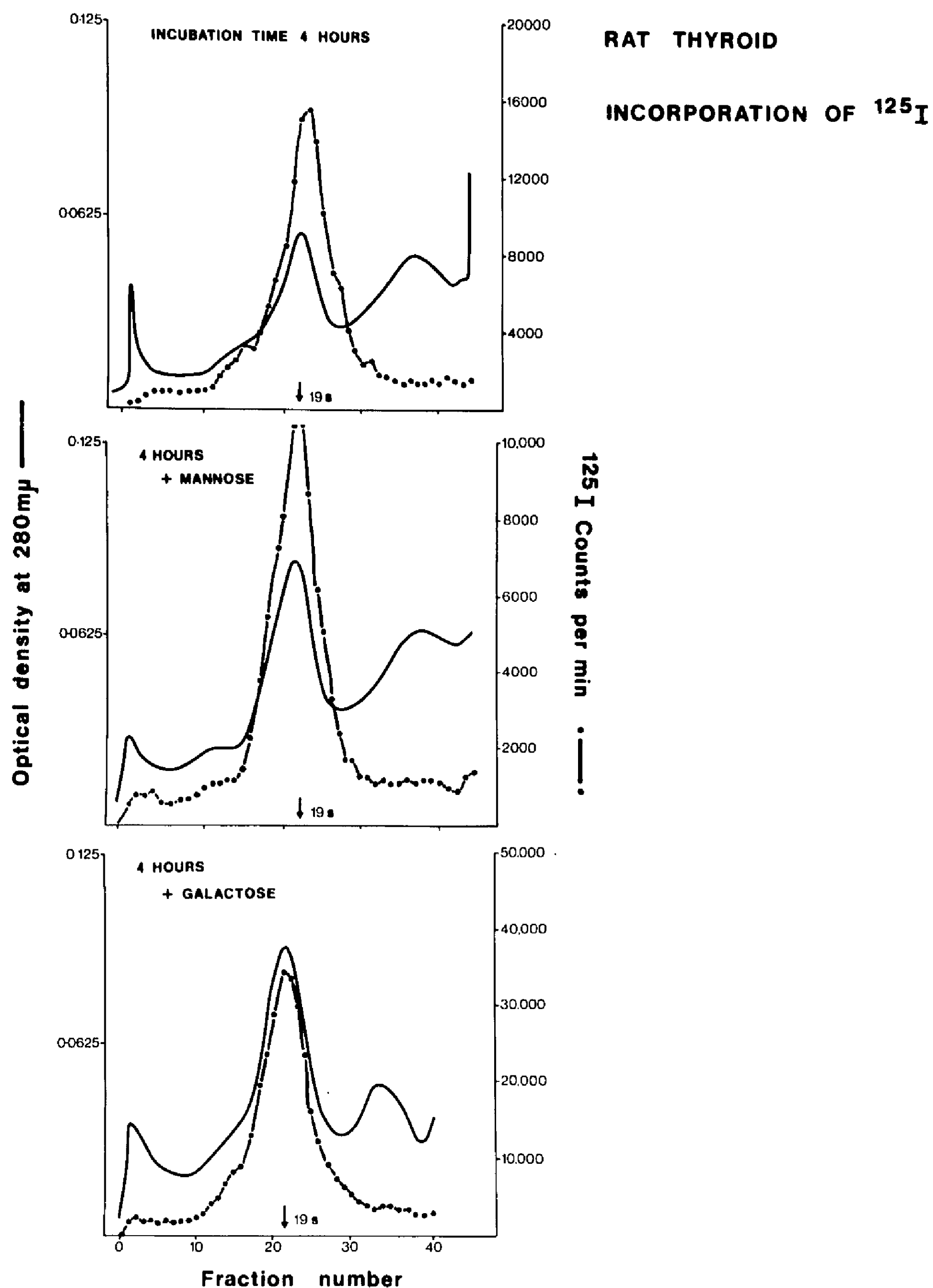


Fig. 25

Ultracentrifugal study of the effect of added stable sugar on the incorporation of ^{125}I into control rat thyroid proteins. Upper portion: control 4 hour incubation. Middle portion: 4 hour incubation plus 200 mM mannose. Lower portion: 4 hour incubation plus 200 mM galactose. SW 41 Rotor at 28,000 rpm for 16 hours.

CHAPTER 16

INCORPORATION OF ^{14}C FUCOSE IN CONTROL RATS

Fig. 26 shows the pattern of incorporation *in vitro* of ^{14}C fucose into the thyroid proteins of control rats. At half an hour it will be noted that there is a small amount of radioactivity associated with the thyroglobulin peak with a broad 3 - 8S pattern of labelling being present. At one hour the predominant labelled peak present was in the thyroglobulin region. This had increased in specific activity compared to the 30 minute tracing and a small 12S peak could also be discerned; a labelled peak in the 3 - 8S region was also seen. At 2 hours the labelled peak in the thyroglobulin region had increased markedly and was now much the largest labelled peak present; there was a rather ill defined 12S peak labelled and the 3 - 8S proteins were also labelled to some extent. At 3 hours a similar pattern was obtained of predominant incorporation of the label into the thyroglobulin region although running short of the 19S protein; some shouldering of this radioactivity peak between the 12S and 18S area was seen; the 3 - 8S peak was also labelled. This pattern of labelling was maintained to the 4 hour period of incubation when the specific activity of labelling of the proteins in the thyroglobulin region had increased; a similar shoulder of radioactivity going across from the 18S to 12S region is seen and a small amount of label was associated with the 3 - 8S proteins.

Compared to the patterns of mannose and galactose incorporation it will be seen that the pattern of fucose incorporation corresponded

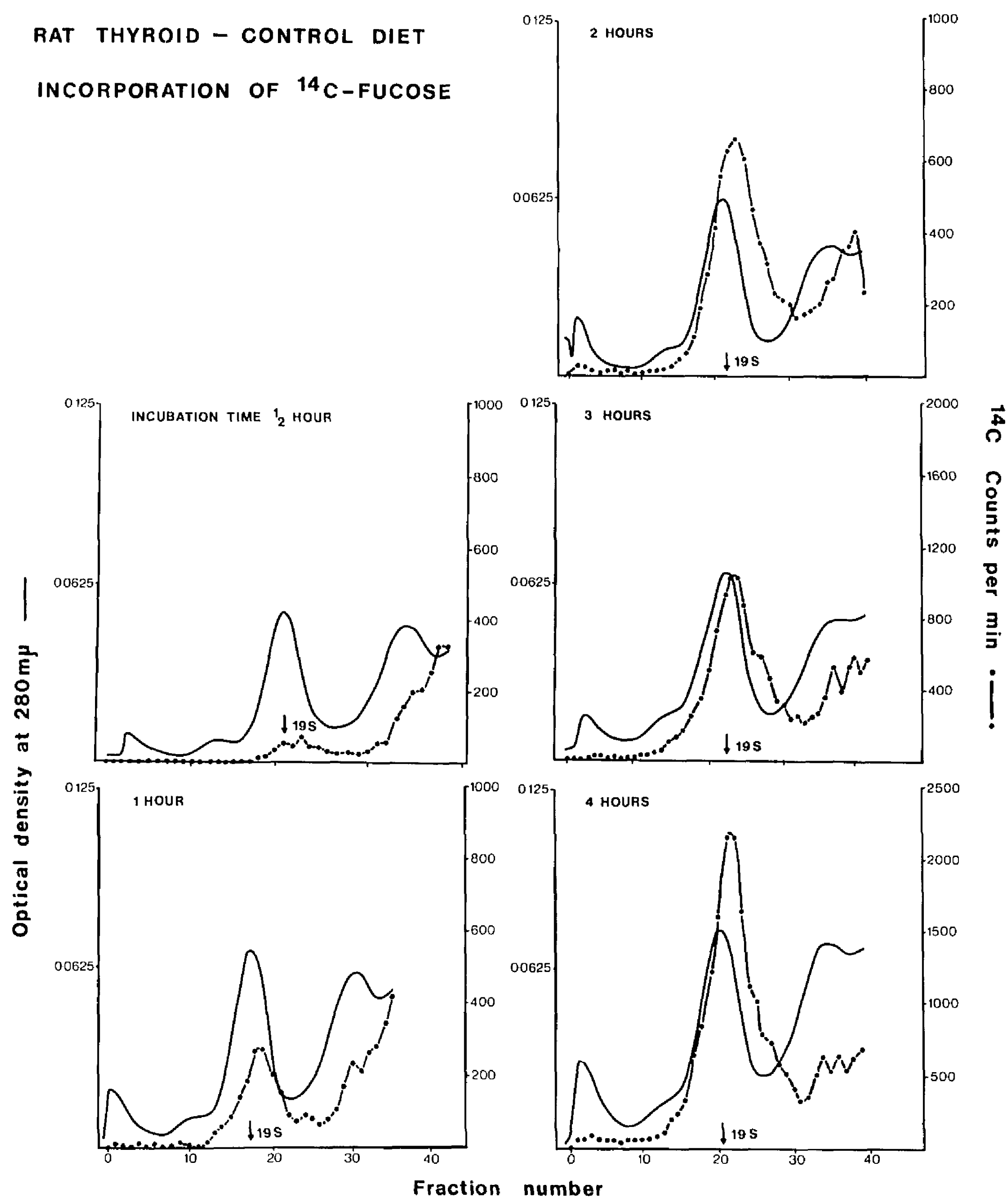


Fig. 26

Ultracentrifugal study of the time course of incorporation of ^{14}C fucose into control rat thyroid proteins. SW 41 Rotor at 28,000 rpm for 16 hours.

rather more to that of galactose than of mannose but at no time was the 12S label as clearly defined as in the pattern of labelling with galactose. There was also the appearance of radioactivity running between the 12S and 18S region and distorting the shape of the thyroglobulin peak which was not seen in the patterns of galactose and mannose incorporation.

CHAPTER 17

EFFECT OF PROPYLTHIOURACIL (PTU) ON THE INCORPORATION OF ^{14}C FUCOSE

The effect of propylthiouracil (PTU) on the pattern of incorporation of ^{14}C fucose into the rat thyroid proteins *in vitro* is shown in Fig. 27. The alteration in the stable protein pattern with diminished protein in the thyroglobulin region and increased protein in the 3 - 8S region previously noted on the $KClO_4$ diet is again seen with PTU. It will be seen that at half an hour there is predominant incorporation of the label into the thyroglobulin region with some shouldering occurring between the 12S and 19S region. At one hour the specific activity of labelling of the protein in the thyroglobulin region had increased and there was only a small labelled peak possibly present in the 12S area. This pattern of labelling persisted on the 2 hour sample with still the predominant labelling being associated with the thyroglobulin area. Further increases in the incorporation of radioactivity in this region were seen at 3 hours and at 4 hours.

Compared to the incorporation of ^{14}C fucose in the control rat the administration of a goitrogenic drug such as PTU resulted in a modest increase in incorporation of radioactivity. The difference in incorporation of labelled fucose between control and goitrogen treated animals was not nearly as marked as with the incorporation of ^{14}C mannose or ^{14}C galactose in the presence of a goitrogenic drug. At no time during the incubation was there any striking incorporation of the fucose into the 12S protein and at all times the bulk of radioactivity appeared to be associated with the proteins in the thyroglobulin region.

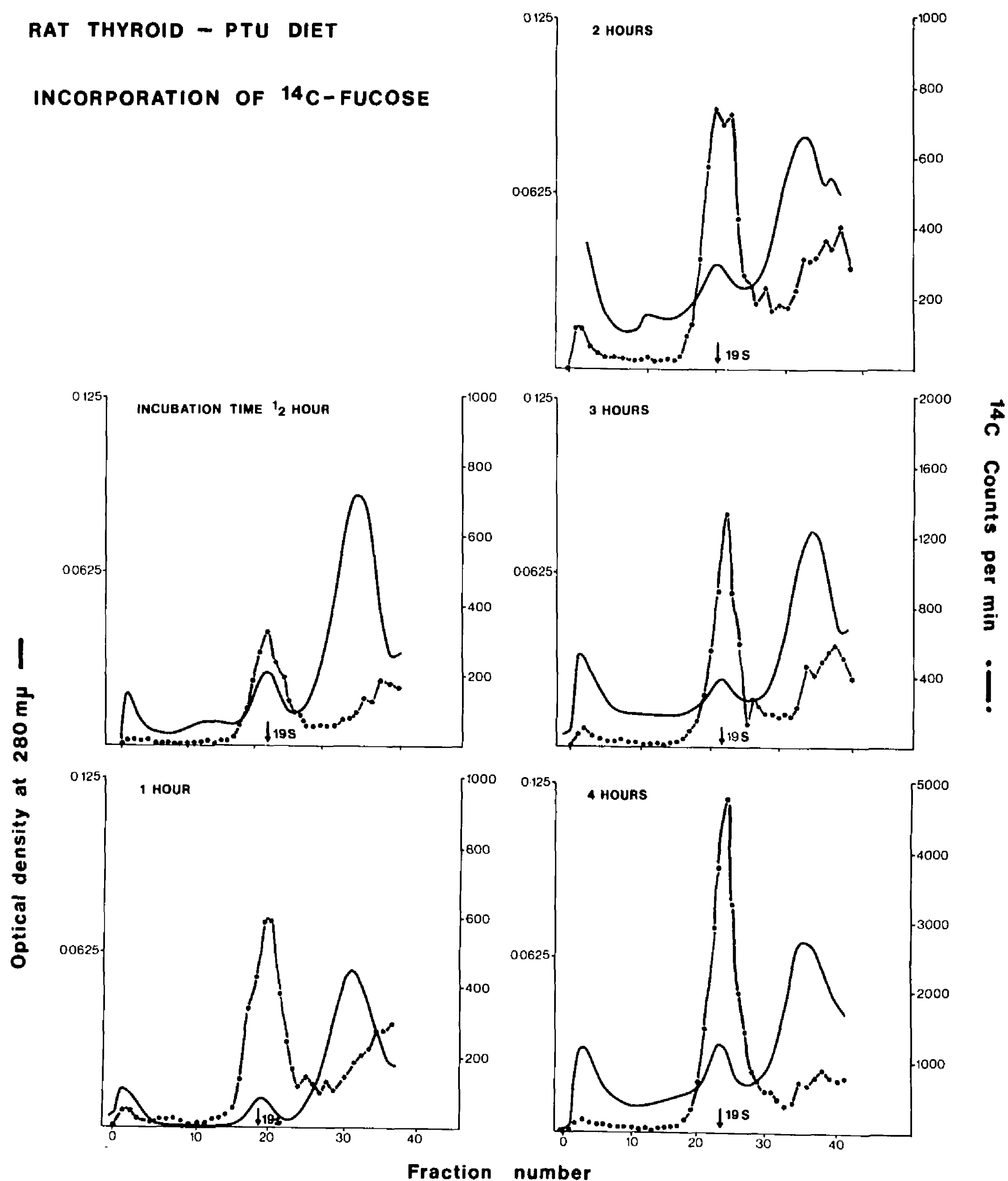


Fig. 27

Ultracentrifugal study of the time course of incorporation of ^{14}C fucose into the thyroid proteins of rats maintained on a PTU diet. SW 41 Rotor at 28,000 rpm for 16 hours.

CHAPTER 18

EFFECT OF SODIUM THYROXINE (T_4) ON THE INCORPORATION OF ^{14}C FUCOSE

Fig. 28 shows the effect of adding T_4 to the diet of control rats on the incorporation of ^{14}C fucose *in vitro*. At 30 minutes there is no significant labelling of proteins other than the 3 - 8S proteins. After 1 hour has elapsed however there is definite labelling of the thyroglobulin peak and of the 3 - 8S peak but no labelling has occurred in the 12S region. At 2 hours the specific activity of labelling of the thyroglobulin peak has increased and now there is a small labelled peak in the 12S area; labelling of the 3 - 8S proteins is still present. A similar pattern of labelling is found at 3 and 4 hours without any significant increase in labelling of the thyroglobulin region with the passage of time. As the incubation proceeds labelling of the 12S peak diminishes.

Compared to the control incubation the addition of T_4 to the diet has resulted in diminution of labelling of thyroglobulin and relative increase of labelling of the 3 - 8S proteins.

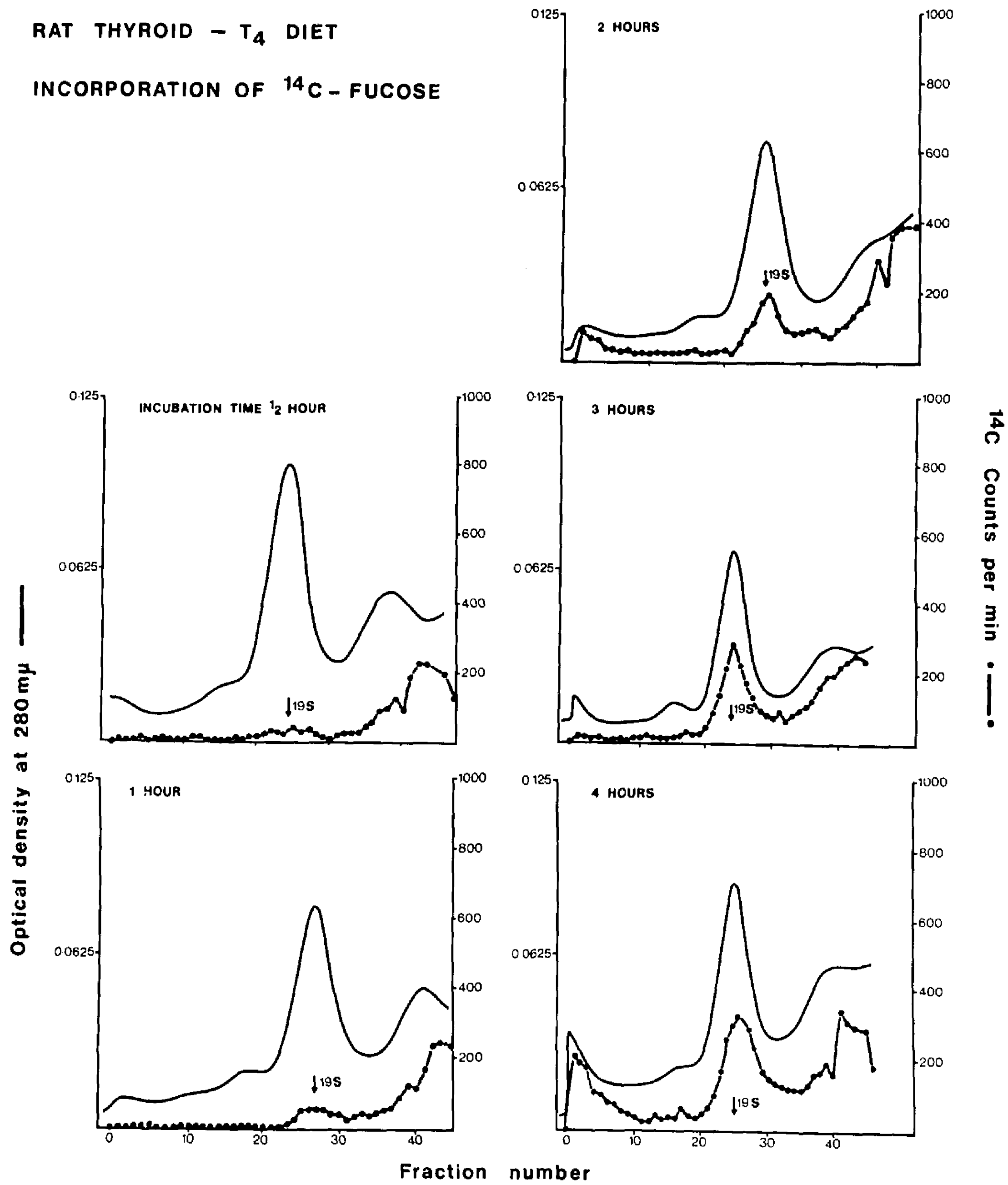


Fig. 28

Ultracentrifugal study of the time course of incorporation of ^{14}C fucose into the thyroid proteins of rats maintained on a diet containing T_4. SW 41 Rotor at 28,000 rpm for 16 hours.

CHAPTER 19

EFFECT OF IODINE SUPPLEMENTATION ON INCORPORATION OF ^{14}C FUCOSE

Fig. 29 shows the pattern of incorporation of ^{14}C fucose into the rat thyroid proteins when the rat's diet had been supplemented with potassium iodide (0.05% KI) in the drinking water for one week. It will be seen that at half an hour of incubation there is a small peak of labelling associated with the thyroglobulin region. At one hour the specific activity of this labelled peak has increased but there is no very obvious peak associated with any other protein present. At 2 hours of incubation the specific activity of labelling of the peak in the thyroglobulin region has increased slightly and there is now the appearance of a small labelled peak in the 12S area. This pattern of labelling is continued for the 3 and 4 hour specimens in which there is increasing specific activity of labelling of the proteins in the thyroglobulin region with no discrete 12S peak present but some shouldering of radioactivity between the 12S and 18S area.

Compared to the control incorporation of fucose the pattern found with the administration of 0.05% KI in the drinking water for one week is very similar although at any given time the specific activity of labelling is rather less. On average the iodine supplemented animals show approximately 50% of the specific activity of labelling of the control animals.

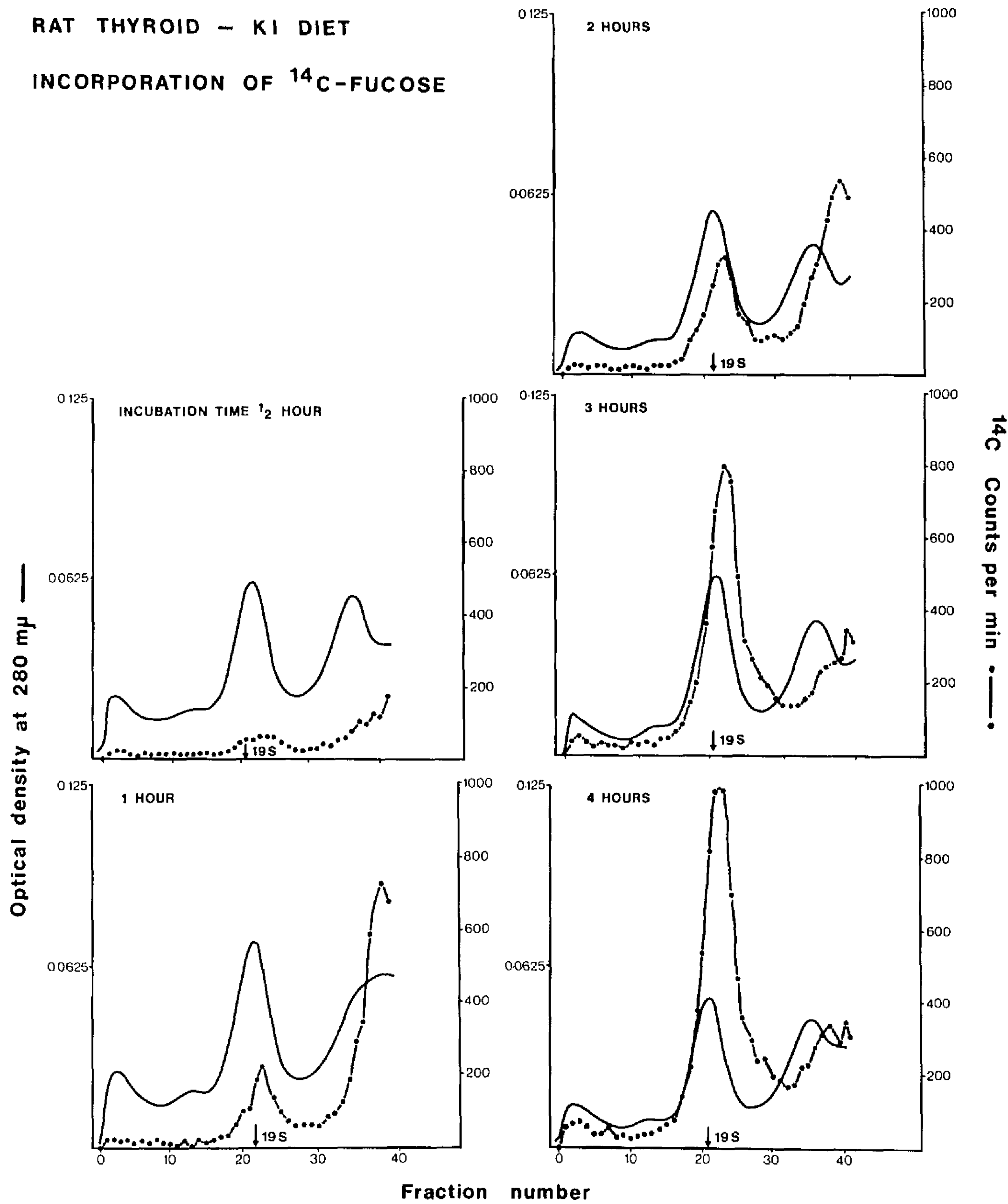


Fig. 29

Ultracentrifugal study of the time course of incorporation of ^{14}C fucose into the thyroid proteins of rats maintained on a KI supplemented diet. SW 41 Rotor at 28,000 rpm for 16 hours.

CHAPTER 20

EFFECT OF CYCLOHEXIMIDE ON THE INCORPORATION OF ^{14}C FUCOSE

In fig. 30 is shown the effect of cycloheximide on the incorporation of ^{14}C fucose into the thyroid proteins of control rats in vitro. The upper part of the figure shows the pattern obtained after 30 minutes incubation without cycloheximide. There is a small peak of labelling in the thyroglobulin area with slight labelling of the 3 - 8S proteins. In the middle part of the figure is shown the pattern of labelling after 4 hours incubation without cycloheximide where, as already shown, there is a predominant peak of labelling in the thyroglobulin region with only a slight shoulder of lighter labelled proteins present. In the lower portion of the figure is shown the pattern obtained when cycloheximide is added after 30 minutes of incubation and the incubation is continued until 4 hours have elapsed. In this circumstance a definite labelled 19S peak is obtained; there is also some radioactivity present at the top of the gradient but not exactly coinciding with the stable 3 - 8S peak.

It can be seen from this figure that the addition of cycloheximide does not prevent the formation of labelled 19S protein although the specific activity of labelling is approximately half that of the control 4 hour incubation.

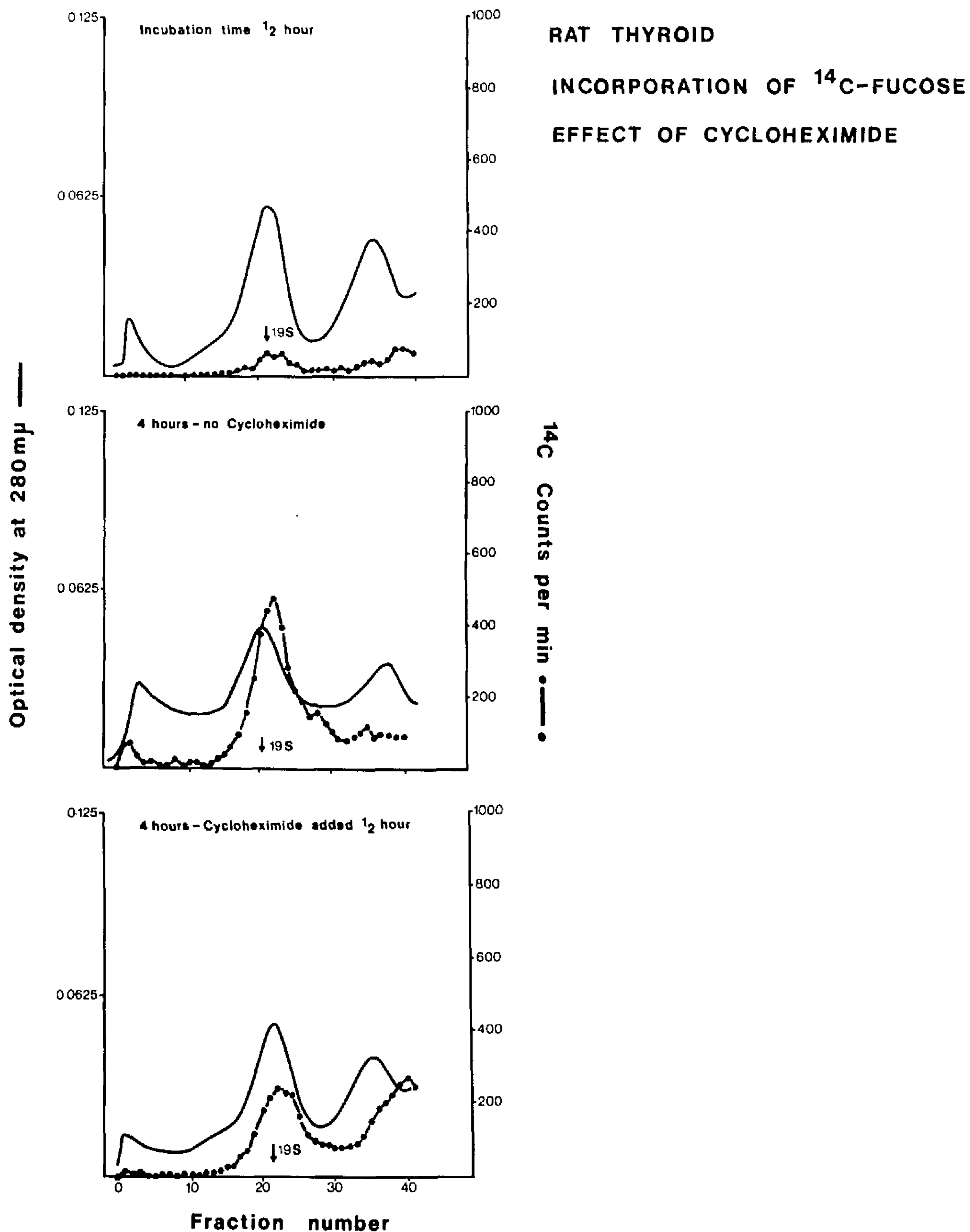


Fig. 30

Ultracentrifugal study of the effect of cycloheximide on the incorporation of ^{14}C fucose into control rat thyroid proteins. Upper portion: control half hour incubation. Middle portion: control 4 hour incubation. Lower portion: 4 hour incubation in which cycloheximide was added at 30 minutes from the commencement of incubation. SW 41 Rotor at 28,000 rpm for 16 hours.

SECTION 4

INCORPORATION OF ^{14}C MANNOSE AND ^{14}C GALACTOSE INTO HUMAN THYROID GLANDS IN VITRO

Chapter 21 'Normal' thyroid gland

Chapter 22 Non-toxic goitre

Chapter 23 Thyroid adenoma

Chapter 24 Thyrotoxic glands

Chapter 25 Hashimoto's thyroiditis

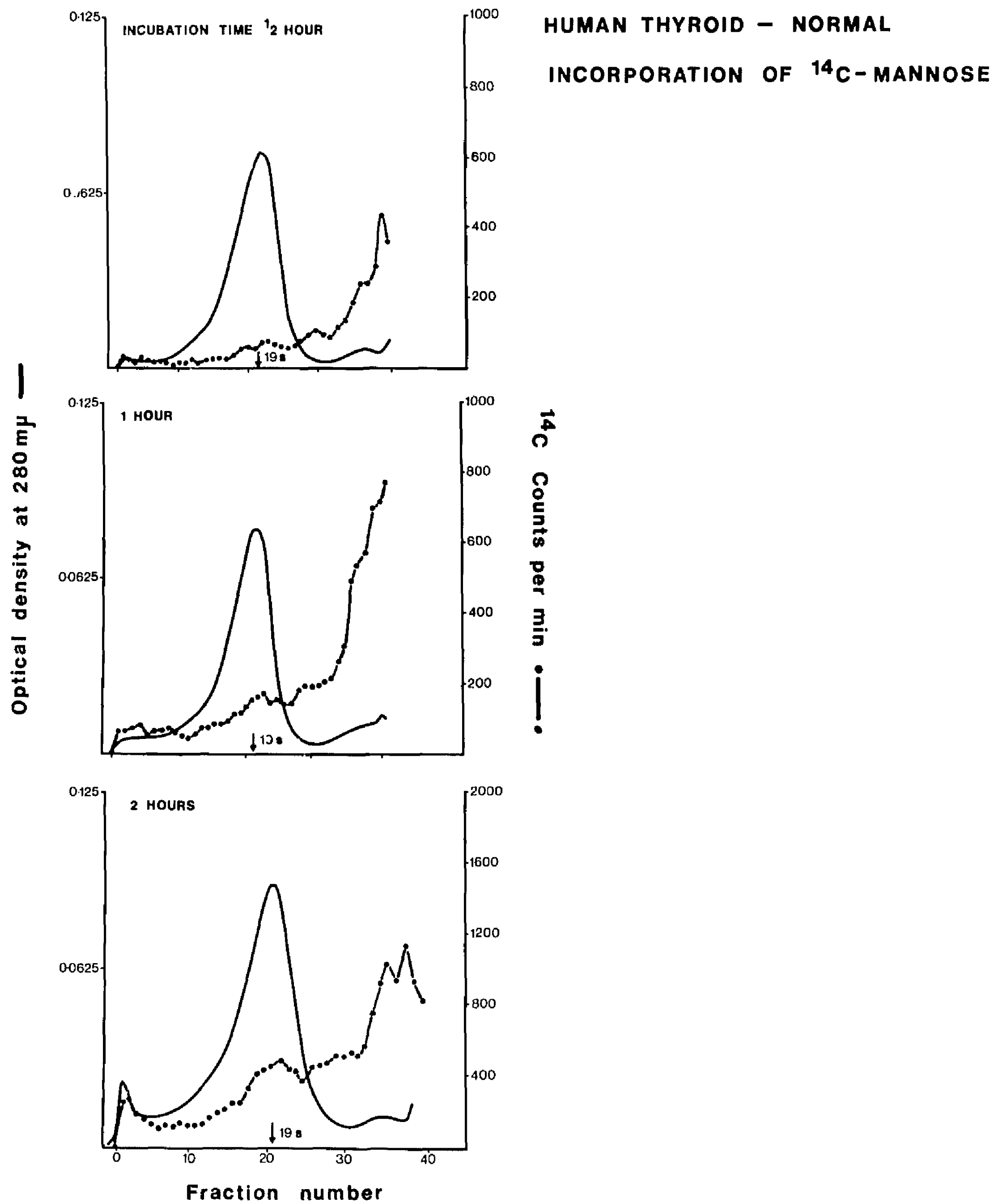
Chapter 26 Carcinoma of thyroid

CHAPTER 21

'NORMAL' THYROID GLAND

Fig. 31 shows the pattern of incorporation of ^{14}C mannose into slices of thyroid gland obtained by biopsy from a patient who was undergoing a neck exploration for possible hyperparathyroidism. There was no thyroid abnormality at operation or on histological examination, and this has been designated as 'normal' thyroid tissue. It may be argued that this gland is not perhaps entirely normal but for ethical reasons it is as close an approximation as one is likely to achieve. It will be seen that with labelled mannose at half an hour there is slight labelling of the proteins in the thyroglobulin region, a definite 12S peak is present and there is label associated in larger quantity with the 3 - 8S peak. At one hour the label in the thyroglobulin region has become more definite and has approximately doubled in specific activity. The label associated with the 12S peak has kept in pace with this change and there is also more radioactivity associated with the top of the gradient. At 2 hours this pattern has continued in approximately the same proportion with the specific activity in all three fractions increasing.

Fig. 32 shows the pattern obtained with labelling of the same gland with ^{14}C galactose <u>in vitro</u>. It will be seen that at half an hour there is a slight amount of label associated with the proteins in the thyroglobulin region; a small rather ill-defined labelled 12S peak is present and there is a large amount of label associated with the 3 - 8S fraction. After one hour incubation the thyroglobulin



<u>Fig. 31</u>

Ultracentrifugal study of the time course of incorporation of ^{14}C mannose into 'normal' human thyroid proteins <u>in vitro</u>.

SW 41 Rotor at 28,000 rpm for 16 hours.

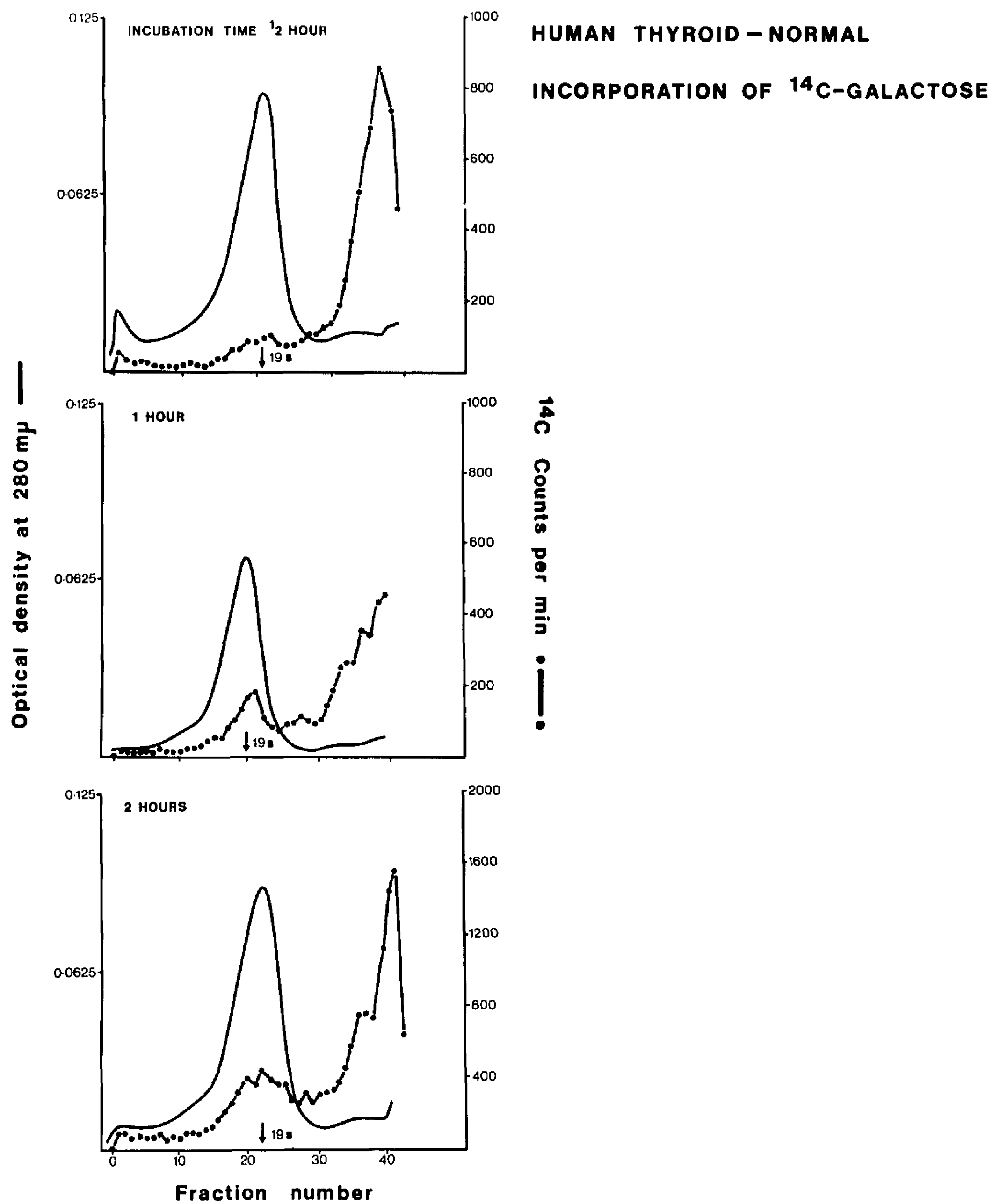


Fig. 32

Ultracentrifugal study of the time course of incorporation of ^{14}C galactose into 'normal' human thyroid proteins *in vitro*. SW 41 Rotor at 28,000 rpm for 16 hours.

peak has increased, that associated with the 12S peak has only increased marginally and that associated with the 3 - 8S peak has declined markedly. This pattern of labelling is continued on the 2 hour sample which shows approximately doubling of the label associated with the thyroglobulin region. The label associated with the 12S region has also increased slightly and the label associated with the 3 - 8S region has also increased.

It will be seen therefore that the pattern of labelling with ^{14}C mannose and ^{14}C galactose in this 'normal' human thyroid gland is remarkably similar both in pattern of incorporation of the label and in the amount of the label incorporated. This similarity was also present amongst the other 2 'normal' human thyroid glands studied.

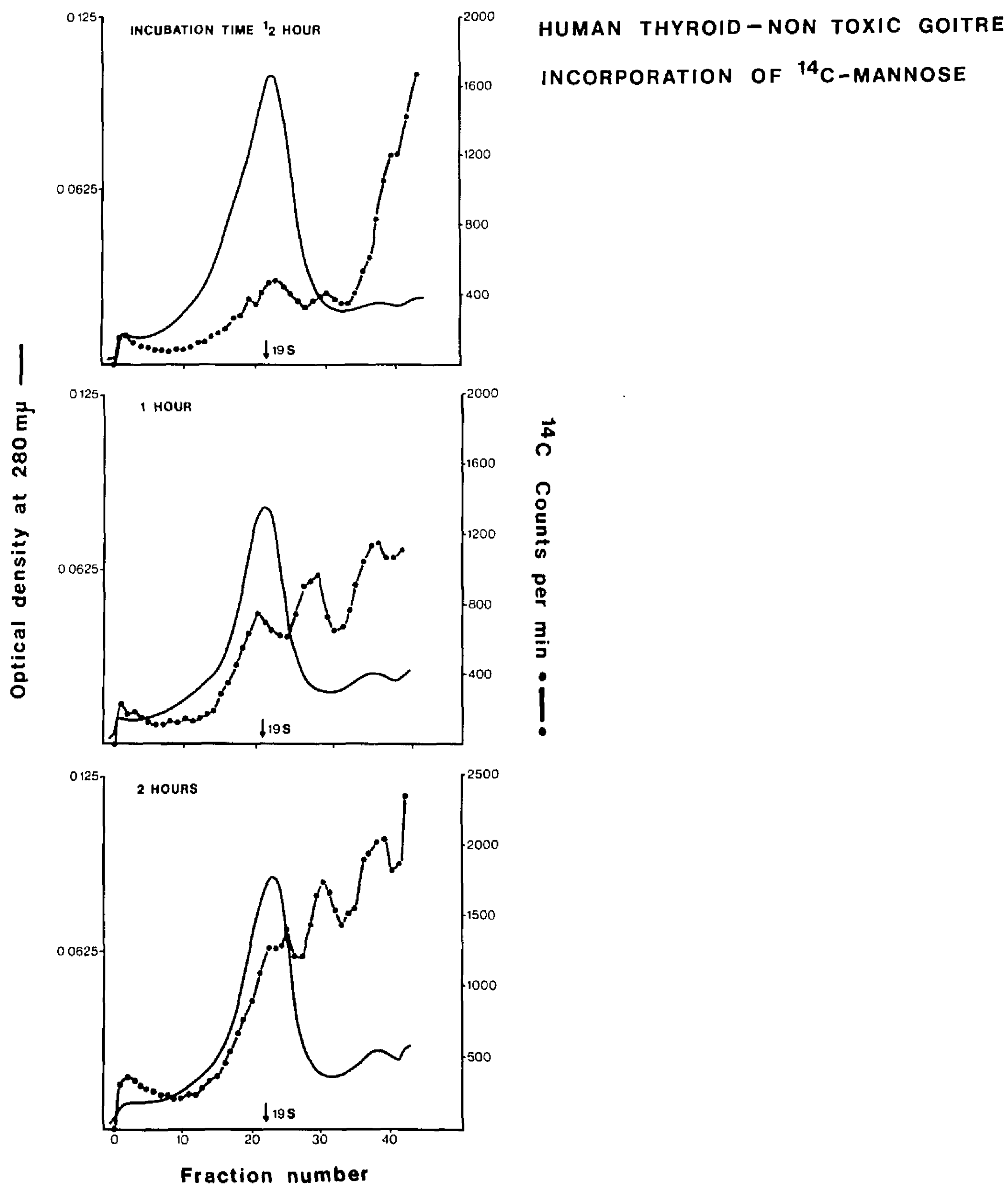
Compared to the patterns of incorporation seen in the rat, the normal human glands show a similar pattern of mannose and galactose incorporation but the specific activity of labelling was much greater in the rat thyroid tissue.

CHAPTER 22

NON-TOXIC GOITRE

Fig. 33 shows the pattern of incorporation of ^{14}C mannose into thyroid tissue *in vitro* from a human non-toxic goitre. It will be seen that at half an hour there is significant labelled protein present in the thyroglobulin region, a definite labelled 12S peak is also present and there is rather non-specific labelling associated with the top of the gradient. At one hour greatly increased radioactivity is associated with the proteins in the thyroglobulin region, the 12S region and the 3 - 8S region. All three peaks have increased in specific activity but that associated with the 12S region to the largest extent. At 2 hours this pattern of labelling continues with increased specific activity being associated with all three peaks with the main labelling being associated with the 3 - 8S peak, then with the 12S peak and lastly but only slightly less in extent with the peak in the thyroglobulin region.

Fig. 34 shows the pattern of incorporation of ^{14}C galactose into the same thyroid gland. At half an hour there is a very definite peak of labelling of the proteins in the thyroglobulin region, a smaller 12S labelled peak is present and there is some labelling associated with the proteins in the 3 - 8S region. At one hour this pattern of labelling is maintained with increased label associated with all three peaks, the 12S peak remaining proportionately the smallest of the three peaks. At 2 hours the same pattern of labelling was found with the specific activities of each peak increasing in proportion.



<u>**Fig. 33**</u>

Ultracentrifugal study of the time course of incorporation of ^{14}C mannose into the thyroid proteins of human non-toxic goitre *in vitro*. SW 41 Rotor at 28,000 rpm for 16 hours.

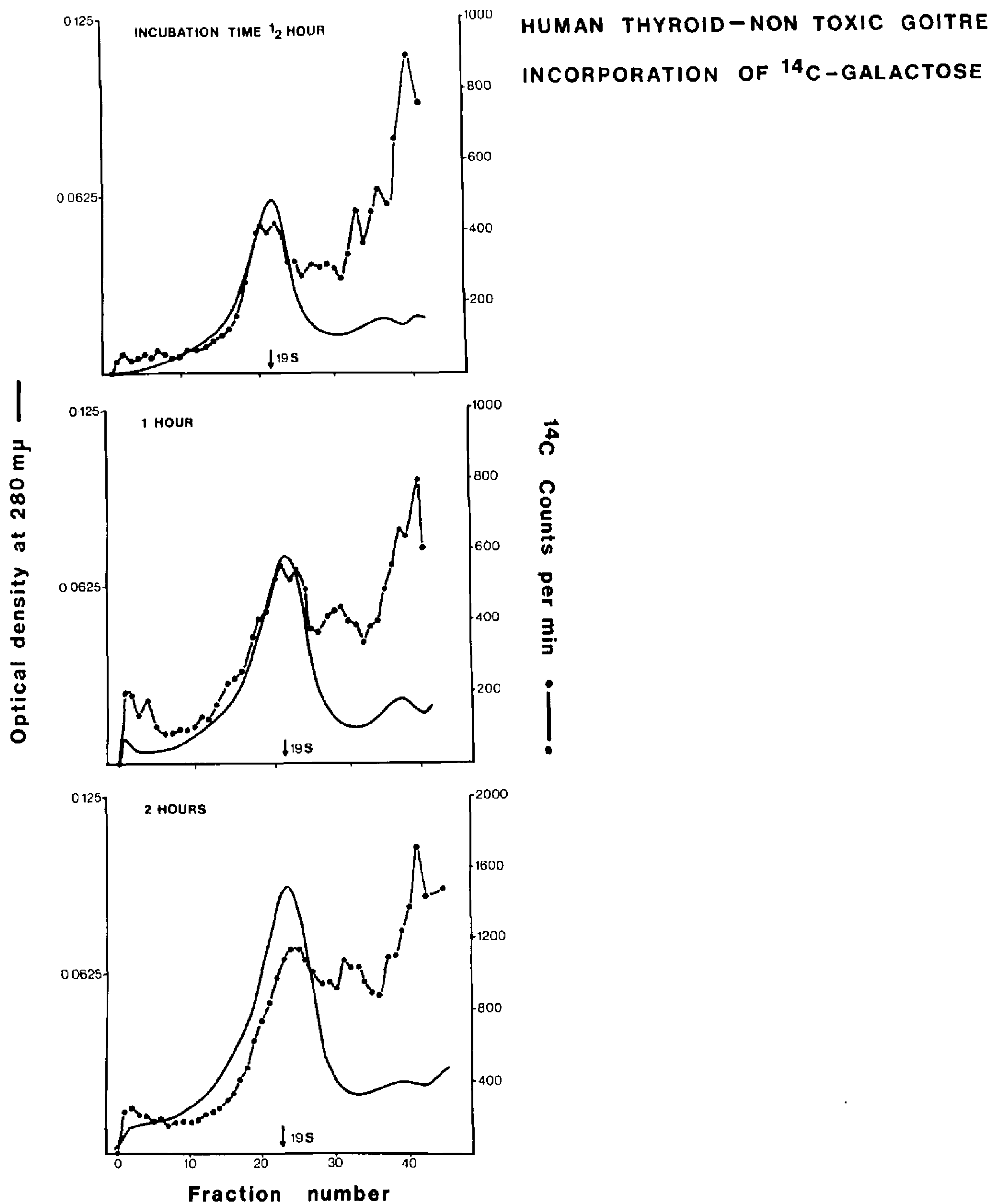


Fig. 34

Ultracentrifugal study of the time course of incorporation of ^{14}C galactose into the thyroid proteins from human non-toxic goitre, in vitro. SW 41 Rotor at 28,000 rpm for 16 hours.

The pattern of labelling shown in fig. 33 and 34 was obtained in 13 of 18 non-toxic goitres examined. In the remaining 5 specimens the patterns obtained were those described for the 'normal' human thyroid.

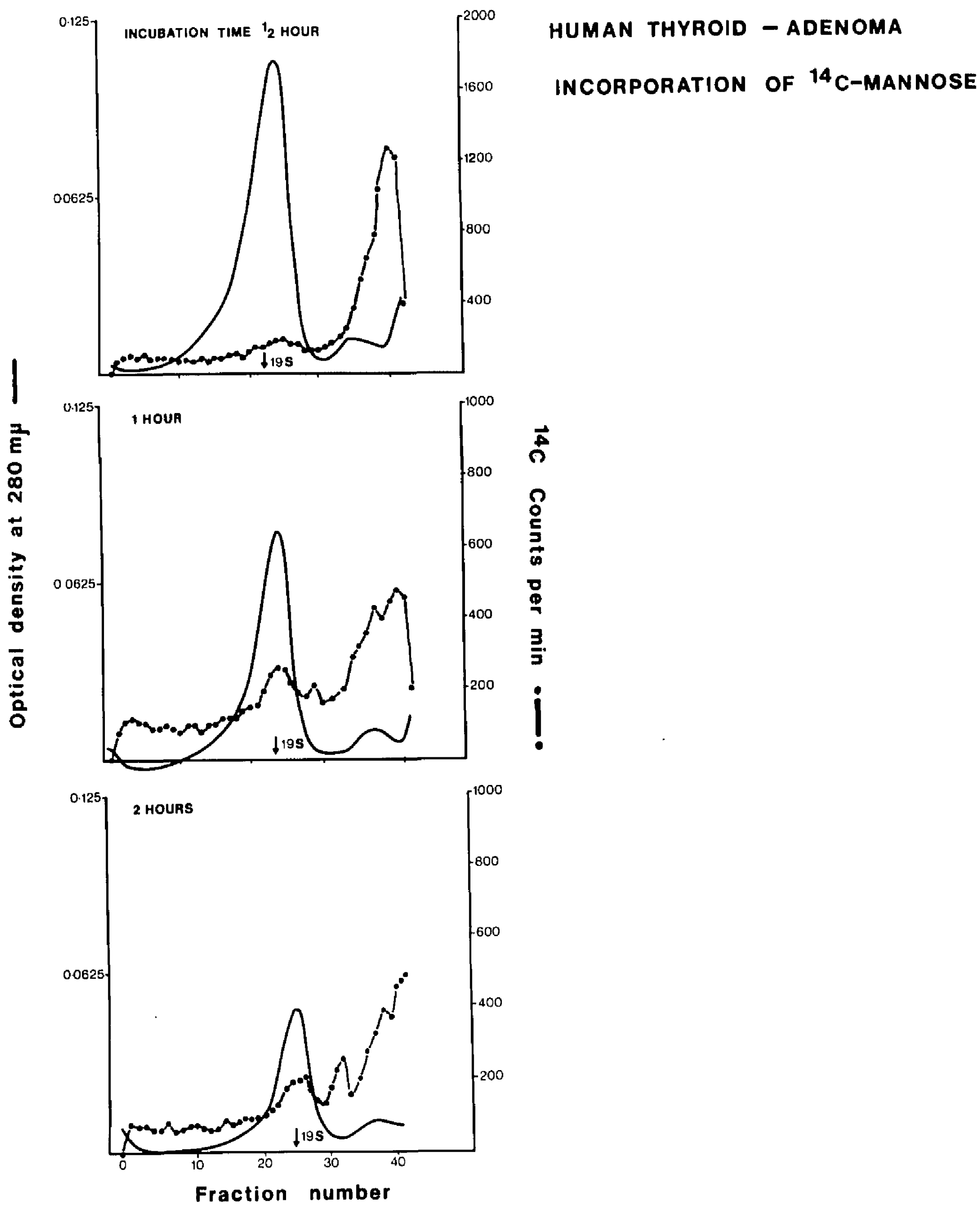
It will be seen that in contrast to that found in the 'normal' human gland the typical pattern of labelling of the non-toxic goitre showed some differences between ^{14}C mannose and ^{14}C galactose incorporation into the soluble thyroid proteins. There is labelling of the proteins in the thyroglobulin region to a greater extent at earlier time intervals with galactose and this larger amount of radioactivity associated with the proteins in the thyroglobulin region is maintained throughout the period of incubation. It will be seen that compared to 'normal' human thyroid that there is typically a greatly increased specific activity of labelling at all time intervals and compared to the rat thyroid tissue studies there is much earlier labelling of proteins in the thyroglobulin region especially when mannose is utilised.

CHAPTER 23

THYROID ADENOMA

Fig. 35 shows the pattern of incorporation of ^{14}C mannose into slices of a human thyroid adenoma. At half an hour there is a slight amount of labelling associated with the peak in the thyroglobulin region, no discrete 12S peak is seen but there is a peak of labelling associated with the 3 - 8S proteins. At one hour there is definite incorporation of label into the thyroglobulin region at higher specific activity, a small labelled 12S peak is now seen and there is again labelling associated with the 3 - 8S proteins. At 2 hours the label associated with the 12S peak had increased proportionately compared to the other peaks. The specific activities in the other peaks were more or less unchanged. It should be noticed, however, that there is rather less protein on this gradient and therefore proportionally to the amount of stable protein present the radioactivity in the three peaks has increased in specific activity.

Fig. 36 shows the pattern of incorporation of ^{14}C galactose into the same human thyroid adenoma. At half an hour there is a small peak of labelling associated with the proteins in the thyroglobulin region with perhaps a small labelled 12S peak present but it is difficult to be dogmatic about this. There is rather non-specific labelling associated with the top of the gradient. At one hour the labelling of the proteins in the thyroglobulin region has increased by about twofold. There is a small peak of 12S labelling now seen and the labelling associated with the 3 - 8S proteins is likewise



<u>Fig. 32</u>

Ultracentrifugal study of the time course of incorporation of ^{14}C mannose into the thyroid proteins from human thyroid adenoma *in vitro*. SW 41 Rotor at 28,000 rpm for 16 hours.

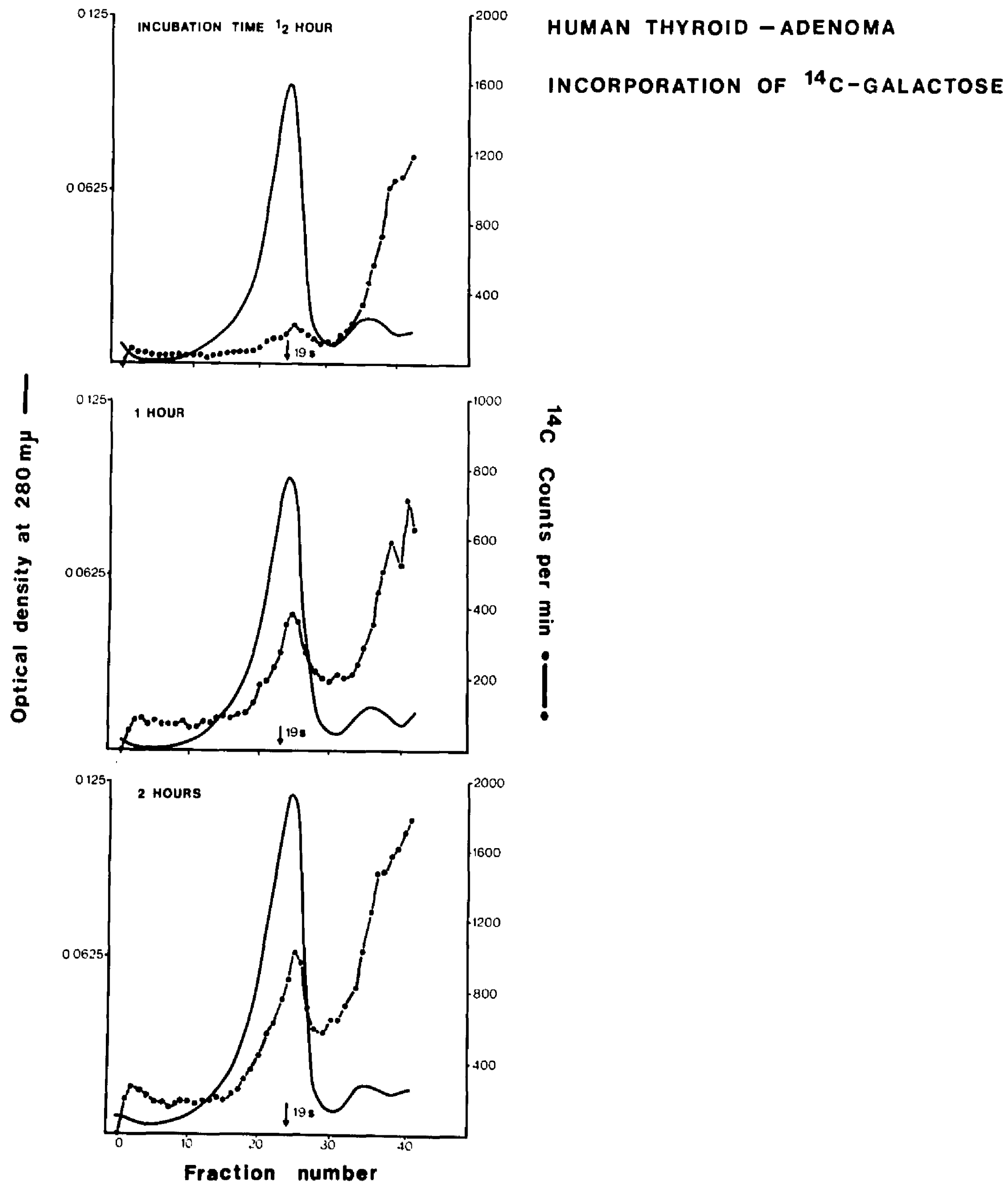


Fig. 36

Ultracentrifugal study of the time course of incorporation of ^{14}C galactose into the thyroid proteins from human thyroid adenoma *in vitro*. SW 41 Rotor at 28,000 rpm for 16 hours.

better defined. At 2 hours the labelling of the proteins in the thyroglobulin region has markedly increased, the 12S peak is again seen but is not well marked and there is rather non-specific labelling of the proteins on top of the gradient.

It will be seen that compared to the 'normal' human tissue the amount of labelling achieved in the thyroid adenoma had increased both with mannose and galactose. It is interesting that in this instance the label associated with the 12S peak using galactose is not at all well-defined as compared to either the labelling in the 'normal' thyroid or in the non-toxic goitre.

In view of the fact that the functional activity of thyroid adenomas vary when judged by the results of *in vivo* scanning with radioiodine it was thought worthwhile looking at the results of mannose and galactose incorporation taking the scanning results into account. In all, 12 nodules were found to show increased incorporation of mannose and galactose compared to 'normal' human thyroid; of these 7 had been scanned before operation and all were found to be 'hot' with respect to their iodine scan. In contrast 7 adenomas showed a pattern of mannose and galactose incorporation similar to that found in 'normal' human thyroid tissue. Of these 4 had had radioiodine scans and all showed less accumulation of radioiodine in the adenoma than in the surrounding thyroid tissue, that is all were 'cold' adenomas.

CHAPTER 24

THYROTOXIC GLANDS

Fig. 37 shows the pattern of incorporation of ^{14}C mannose into slices from the thyroid of a patient with thyrotoxicosis. The patients studied in this section had all been treated pre-operatively by conventional means with a course of carbimazole therapy until rendered euthyroid and then given potassium iodide 30 mg three times a day for the ten days before the operation. It will be seen that at half an hour there is no evidence of any labelling associated with the proteins in the thyroglobulin region or with the proteins in the 12S region. There is some labelling at the top of the gradient, however, in the region of the 3 - 8S proteins. At one hour there is a tiny peak of labelling associated with the thyroglobulin region, a rather broad peak of labelled 12S protein present and the label associated with the 3 - 8S region has decreased in specific activity. At 2 hours the peak of labelling associated with the proteins in the thyroglobulin area has increased in specific activity, that of the proteins in the 12S region rather more than trebled in specific activity and the proteins associated with the 3 - 8S area also show increased labelling.

Fig. 38 shows the pattern of labelling of the same thyrotoxic gland with ^{14}C galactose <u>in vitro</u>. It will be seen that at half an hour there is a well marked labelled peak associated with the 19S region, there is a well marked 12S peak also present and there is a considerable amount of label in the 3 - 8S proteins. At one

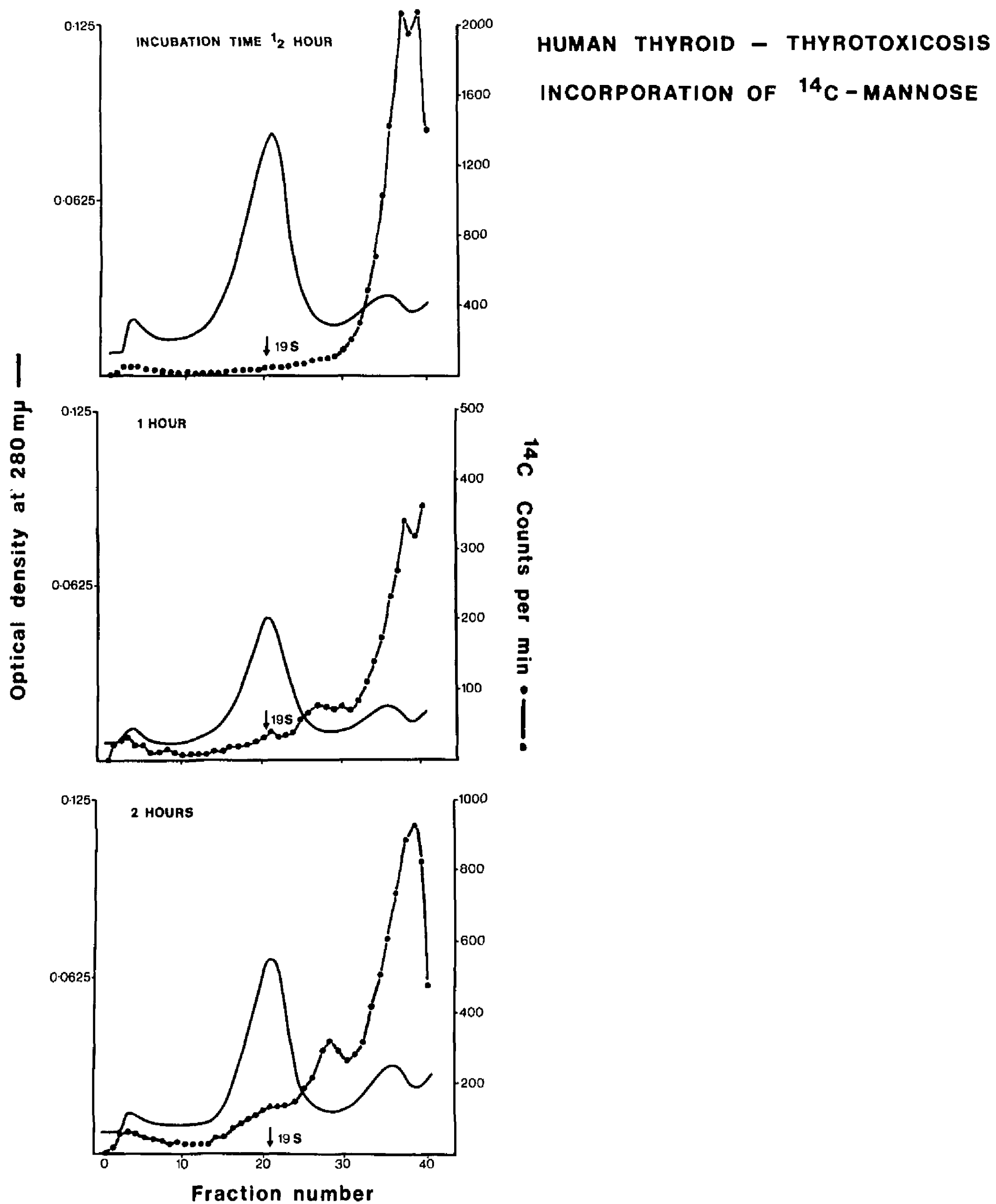


Fig. 37

Ultracentrifugal study of the time course of incorporation of ^{14}C mannose into the thyroid proteins of human thyrotoxic glands pre-treated with carbimazole and potassium iodide. SW 41 Rotor at 28,000 rpm for 16 hours.

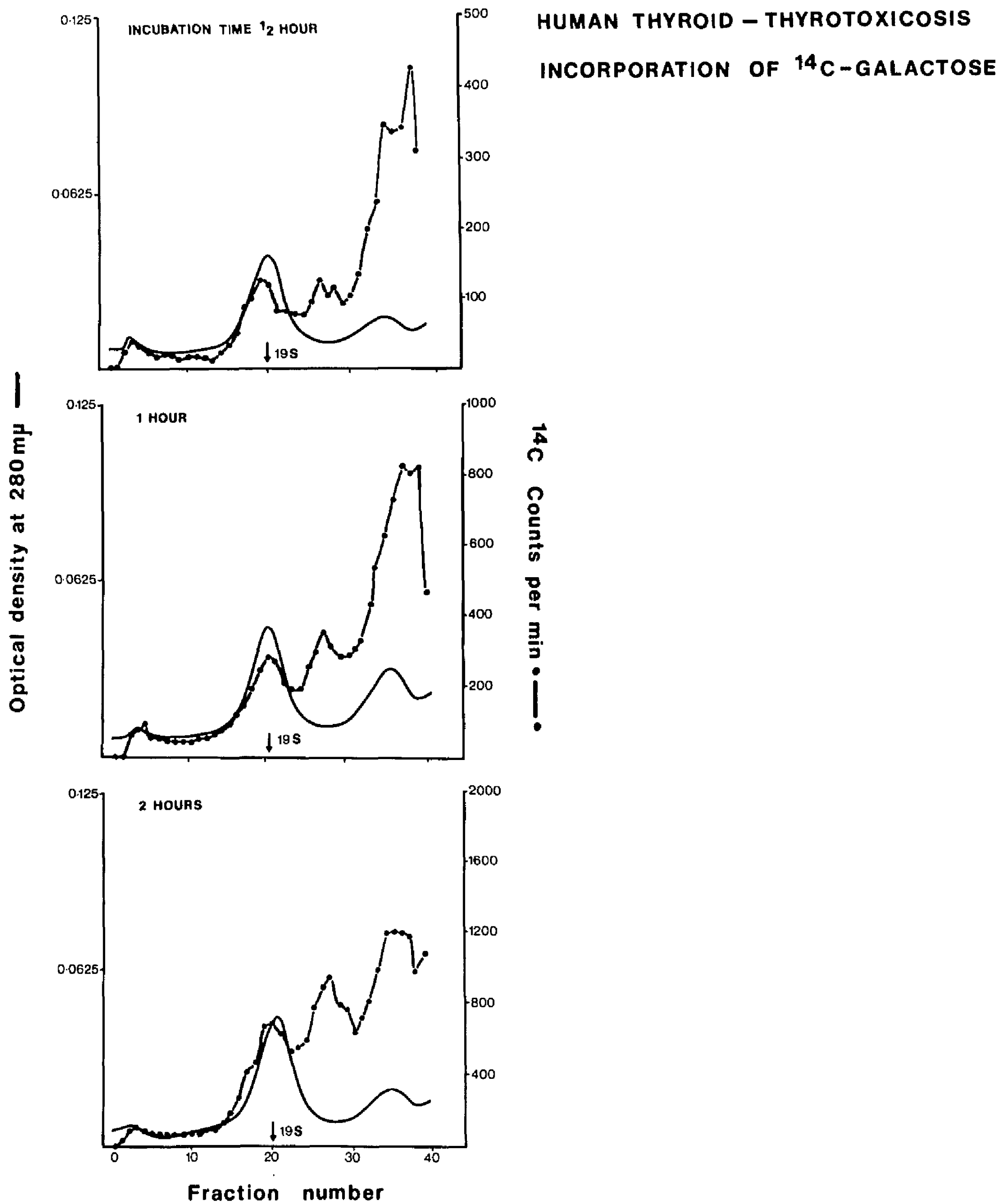


Fig. 38

Ultracentrifugal study of the time course of incorporation of ^{14}C galactose into the thyroid proteins from human thyrotoxic glands pre-treated with carbimazole and potassium iodide.

SW 41 Rotor at 28,000 rpm for 16 hours.

hour the labelling of all three peaks has increased, approximately the same proportion of labelling being maintained as found in the half hour specimen and by 2 hours the same pattern of labelling of the three peaks was obtained with in this instance proportionately much more label being associated with the thyroglobulin and 12S peaks than with the 3 - 8S peaks.

In all, 10 thyrotoxic patients treated pre-operatively with carbimazole and potassium iodide were studied. The pattern of incorporation of labelled mannose and galactose was remarkably uniform. No significant discrepancies from that shown in fig. 37 and 38 were found.

It is of interest that in thyrotoxic glands there is a discrepancy in pattern between labelling with mannose and with galactose. There is much earlier labelling of proteins with galactose than with mannose and the degree of labelling achieved is much greater. It is of interest that the label associated with the 12S peak using galactose is much more marked in the thyrotoxic gland than in the thyroid adenoma.

CHAPTER 25

HASHIMOTO'S THYROIDITIS

In this condition there is alteration of the thyroid epithelium to an abnormal type (Askanazy cells) and there is replacement of a large part of the gland with a lymphocytic infiltrate which causes a diminution in the amount of thyroglobulin present in the gland. This is reflected in the different stable protein patterns seen here from the other human thyroid glands. It will be noted that there is proportionately much less stable protein in the thyroglobulin region and a much greater amount of protein associated with the 3 - 8S area. This alteration of the thyroid protein pattern in Hashimoto's thyroiditis has been described by Thomson and Bissett (1969a) and others.

Fig. 39 shows the pattern of incorporation of ^{14}C mannose into such a gland *in vitro*. It will be seen that at half an hour there is virtually no labelling of proteins above the 3 - 8S area. There is a large peak of incorporation of the label into the lightweight proteins but there is no trace of a labelled 12S or 19S protein. At one hour the labelling of the 3 - 8S peak had increased but again there was no labelling associated with the 12S or 19S proteins. This pattern of labelling was also found in the 2 hour sample in which although the labelled peak of the 3 - 8S protein had increased markedly in specific activity there was no definite labelling of the other proteins.

Fig. 40 shows the pattern of labelling found using ^{14}C galactose. At half an hour it will be seen that there is substantial labelling

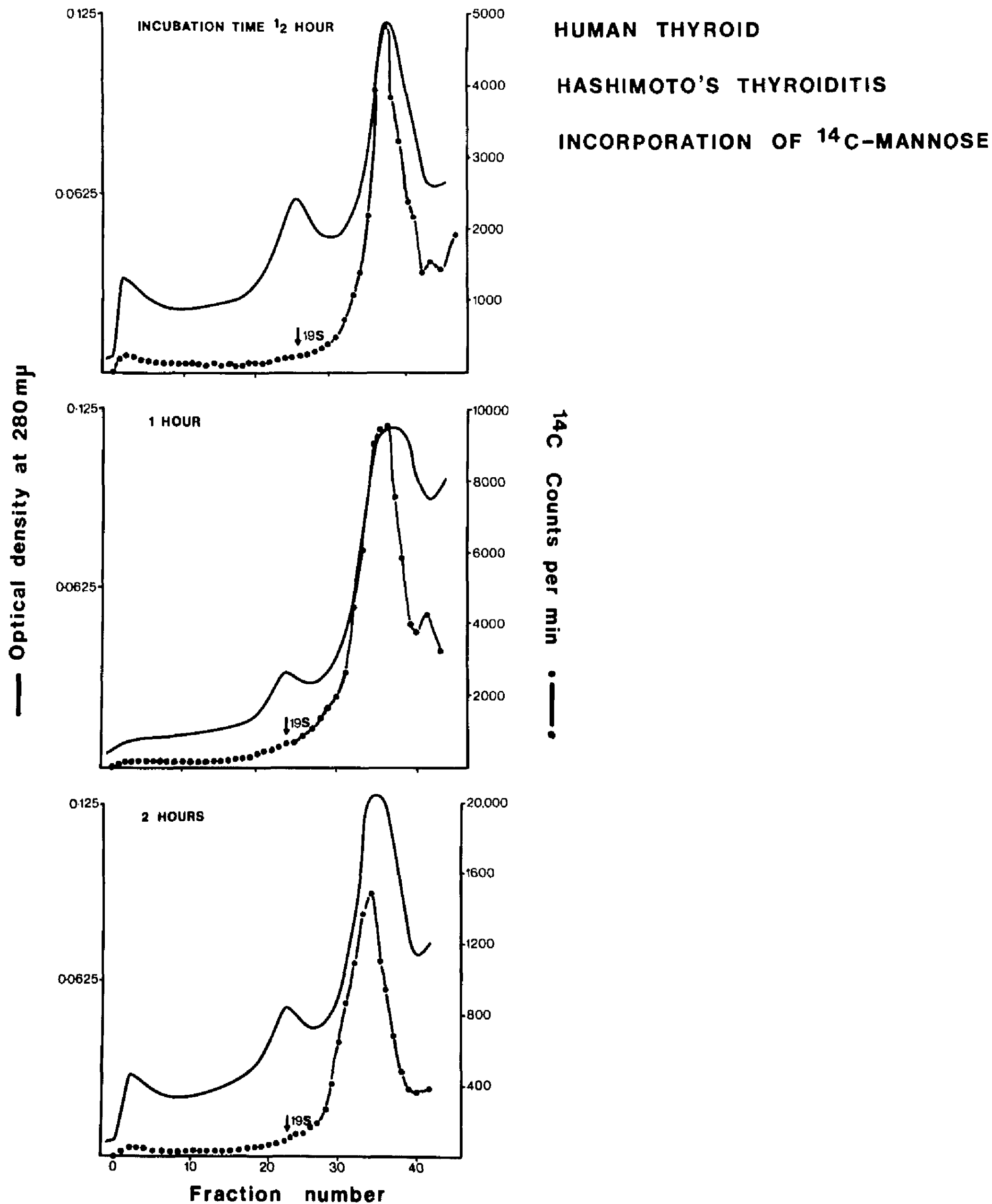
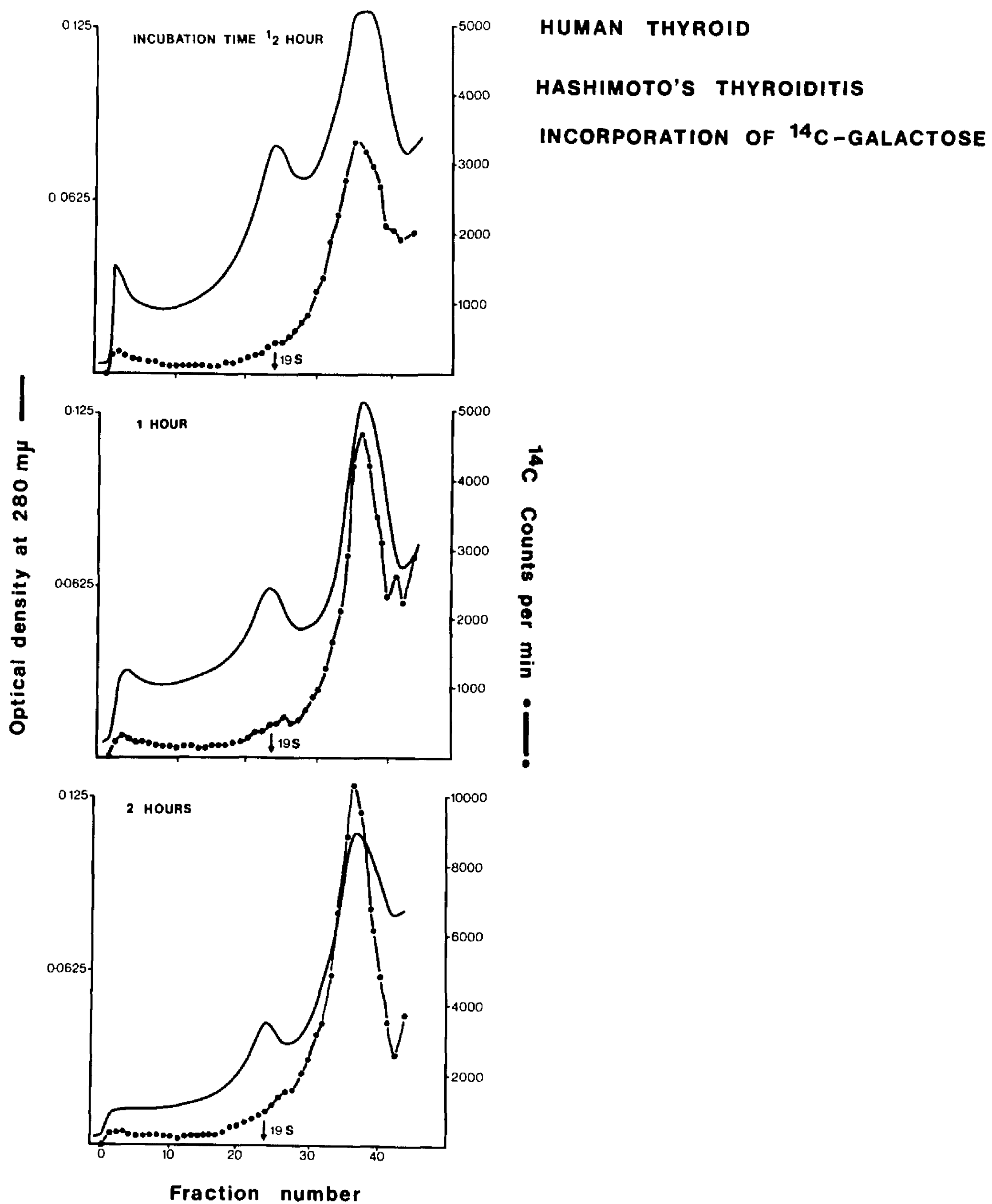


Fig. 39

Ultracentrifugal study of the time course of incorporation of ^{14}C mannose into the thyroid proteins from Hashimoto's thyroiditis *in vitro*. SW 41 Rotor at 28,000 rpm for 16 hours.



<u>Fig. 40</u>

Ultracentrifugal study of the time course of incorporation of ^{14}C galactose into the thyroid proteins from Hashimoto's thyroiditis *in vitro*. SW 41 Rotor at 28,000 rpm for 16 hours.

of the 3 - 8S proteins but at the most only a slight suggestion of labelling of proteins in the thyroglobulin region. At 1 hour there is an increase in specific activity of labelling of the proteins in the 3 - 8S area, there is a small peak running short of the 19S area present but no definite evidence of 12S labelling. At 2 hours the peak of labelling of the 3 - 8S proteins has further increased. There is some increase in labelling in the thyroglobulin area but there is no discrete peak of incorporation found.

Nine patients with Hashimoto's thyroiditis were studied, in 6 the pattern of mannose and galactose incorporation was as shown in figs. 39 and 40. In 3 others however there was a small amount of labelling of the proteins in the thyroglobulin region. This only constituted a small proportion of the total labelled sugar incorporated and the amount of the label associated with the thyroglobulin region did not increase with longer times of incubation.

Fig. 41 shows the 30 minute incubation studies from such a patient illustrating the small proportion of labelling of thyroglobulin found even in such patients. The figure also shows the results of labelling _in vitro_ with ^{3}H leucine and ^{125}I. It will be seen that ^{125}I is incorporated into thyroglobulin but ^{3}H leucine is not.

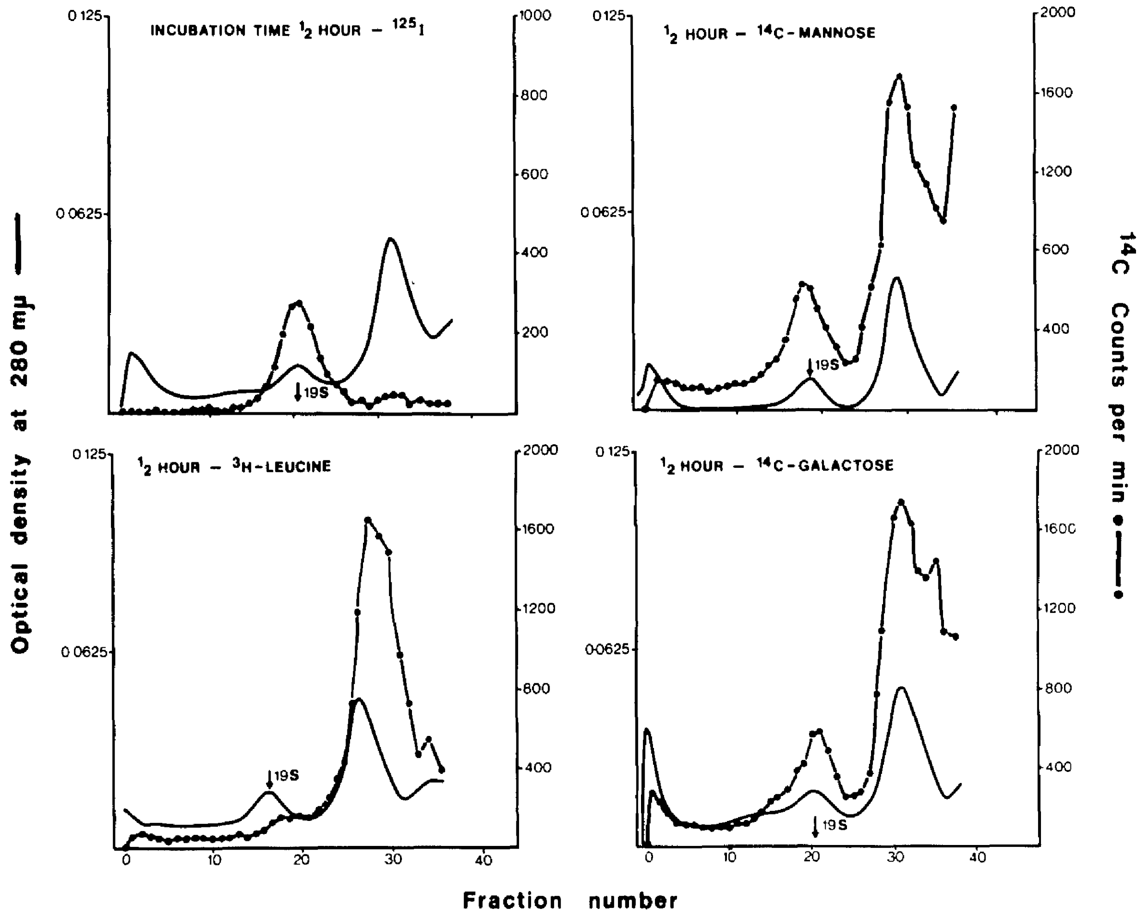


Fig. 41

Ultracentrifugal study of the early incorporation of ^{14}C mannose, ^{14}C galactose, ^{3}H leucine and ^{125}I into the thyroid proteins from Hashimoto's thyroiditis in vitro. Time of incubation – 30 minutes; SW 41 Rotor at 28,000 rpm for 16 hours.

CHAPTER 26

CARCINOMA OF THYROID

Three patients with thyroid carcinoma were studied. One patient had an anaplastic carcinoma, one had a well differentiated papillary carcinoma and the third patient had a medullary carcinoma, that is one arising from the parafollicular or 'C' cells of the thyroid. In this last type of tumour there is replacement in the normal thyroid tissue by masses of the abnormal cells which is reflected in the lack of proteins in the thyroglobulin region in most of the gradients seen and the presence of a large amount of stable protein in the 3 - 8S area. Fig. 42 shows the pattern of labelling with ^{14}C mannose in the medullary carcinoma and it can be seen that in this particular gradient a small amount of protein in the thyroglobulin region is present. There is a rather broad peak of labelling in the 12S area and label associated with the top of the gradient but no discrete peak of labelling in the thyroglobulin region. At 2 hours it can be seen that there is no discrete stable or radioactive protein in the thyroglobulin region or in the 12S region and there is no definite label associated with the 3 - 8S peak. At 4 hours a rather similar picture is obtained with a broad pattern of labelling being associated with the top of the gradient but not specifically with the 3 - 8S peak. There is no stable or labelled protein associated in the other areas.

Fig. 43 shows the pattern of labelling obtained using ^{14}C galactose. At half an hour there is no labelling of the proteins in the thyroglobulin and 12S area but a broad peak of labelling

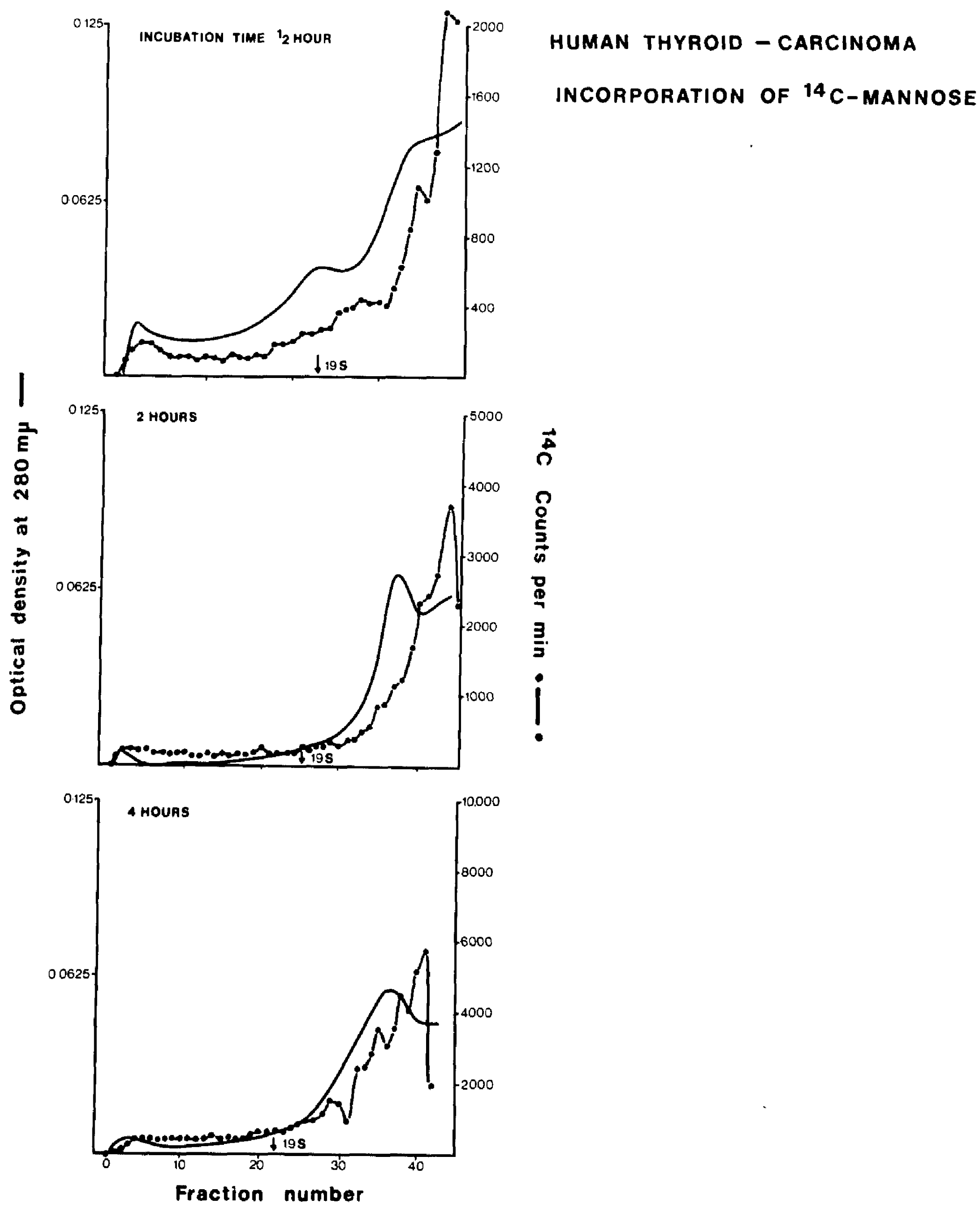


Fig. 42

Ultracentrifugal study of the time course of incorporation of ^{14}C mannose into the thyroid proteins from medullary carcinoma of thyroid *in vitro*. SW 41 Rotor at 28,000 rpm for 16 hours.

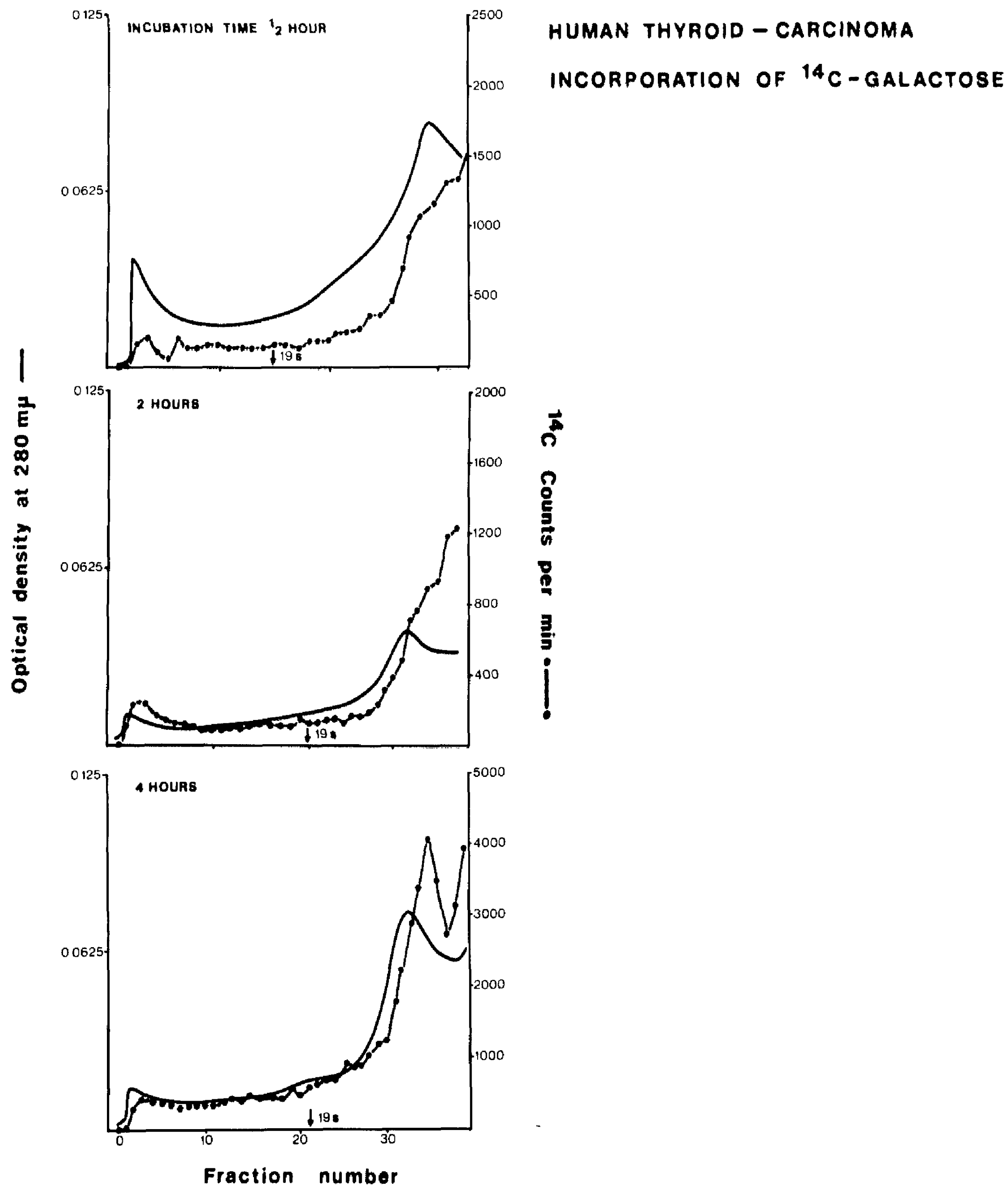


Fig. 43

Ultracentrifugal study of the time course of incorporation of ^{14}C galactose into the thyroid proteins from medullary carcinoma of thyroid *in vitro*. SW 41 Rotor at 28,000 rpm for 16 hours.

associated with the top of the gradient. This pattern is also found at 2 hours and at 4 hours. In this latter specimen there is a small peak which could be associated with the 12S region but it is difficult to be sure of the significance of this one area of raised count associated with this gradient. There is again a broad peak of labelling over the area of the 3 - 8S proteins.

It will be seen that neither using ^{14}C mannose or ^{14}C galactose is there any definite pattern of incorporation of the material into the thyroglobulin-like proteins or into their possible precursors with the exception of the 30 minute mannose sample. In this sample there obviously is some thyroglobulin-like protein present and it is possible that fortuitously some relatively 'normal' thyroid tissue at the edge of the tumour was included in this incubation. Certainly in none of the other gradients except for the 4 hour mannose specimen is there any suggestion of a stable protein in the thyroglobulin region.

The thyroid gland from the patient with anaplastic carcinoma gave a similar lack of incorporation of labelled sugar other than into the 3 - 8S peak. The results from the patient with a well differentiated papillary tumour showed a considerable amount of thyroglobulin to be present and patterns of incorporation similar to non-toxic goitre tissue were obtained.

SECTION 5

STUDIES OF CERTAIN ASPECTS OF THE BREAKDOWN OF THYROGLOBULIN

Chapter 27 Effects of dialysis and betamercaptoethanol (BME)

Chapter 28 Chromatographic studies of thyroid proteins following incubation with ^{14}C mannose and ^{14}C galactose and exposure to specific glycosidase enzymes

Chapter 29 Effect of ultracentrifugation at 23°C on the patterns of incorporation of ^{14}C mannose and ^{14}C galactose into rat thyroid protein

CHAPTER 27

EFFECTS OF DIALYSIS AND BETAMERCAPTOETHANOL (BME)

In fig. 44 is shown the effect of dialysis and BME on the breakdown of thyroglobulin which has been labelled with ^{14}C mannose. At the top of the figure is shown the pattern expected after incubation of rat thyroid hemilobes for 4 hours in the presence of ^{14}C mannose. There is a well marked labelled peak running just short of the thyroglobulin region, a well marked 12S peak is also seen and there is label associated with the 3 - 8S proteins. It should be noted in the figures in this chapter that the speed of ultracentrifugation has been increased so that the thyroglobulin peak now runs much nearer the bottom of the gradient and enables more space for changes in the proteins in the intermediate regions to be seen. The middle part of the figure shows the effect of overnight dialysis at 4°C of the thyroid proteins obtained from the 4 hour incubation against NH_4OH (0.01 M) solution adjusted to a pH of 10. It will be seen that this has the effect of greatly diminishing the concentration of labelled mannose into the proteins running in the thyroglobulin region but greatly increasing the labelling of the 12S proteins. There is some diminution in labelling of the proteins in the 3 - 8S area. This would imply therefore that the effect of dialysis, as anticipated from the work of Edelhoch has resulted in the breakdown of protein in the thyroglobulin region to protein in the 12S region. It should be noted that in this particular instance there also is now the

RAT THYROID

INCORPORATION OF ^{14}C-MANNOSE

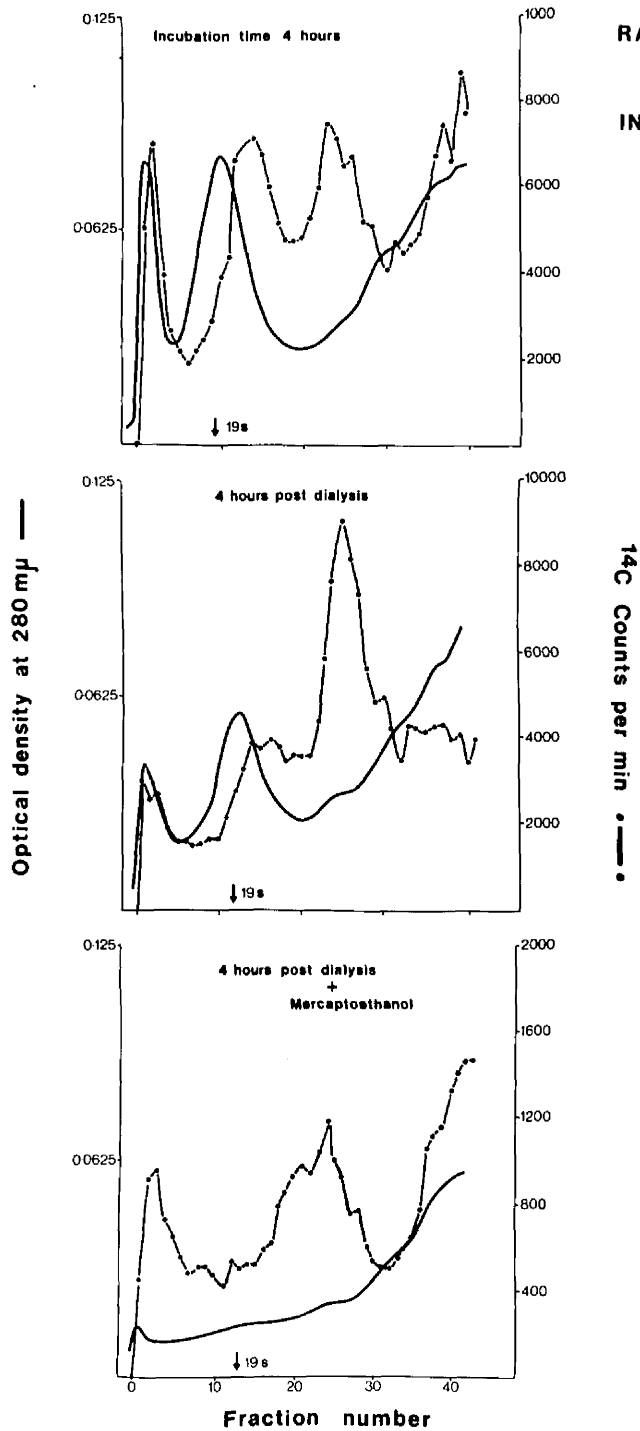


Fig. 44

Ultracentrifugal study of the effect of dialysis at pH 10 and betamercaptoethanol on the thyroid proteins of control rats labelled with ^{14}C mannose *in vitro*. Upper portion: control 4 hour incubation. Middle portion: effect of dialysis at pH 10. Lower portion: effect of added betamercaptoethanol. SW 41 Rotor at 35,000 rpm for 16 hours.

appearance of a stable peak of protein in the 12S area. In the lower part of the figure can be seen what happens when the thyroid proteins, previously dialysed at pH 10, are exposed to the addition of BME and left overnight at $4^{o}C$. In this instance there is almost complete disappearance of the stable protein peak in the thyroglobulin region. There is a small stable protein peak in the 12S area and there is a broad stable protein peak extending over the 3 - 8S area. As regards labelling, there now is only a minimal peak of labelling associated with the 19S area, there is a large broad peak of labelling in the 12S region and proportionately there is much more label associated with the 3 - 8S area. This would therefore fit in with the concept that the labelled material from both the thyroglobulin and from the 12S area which have fallen markedly in specific activity has been transferred to proteins in the 3 - 8S region.

Fig. 45 shows a similar experiment conducted with ^{14}C galactose using rat thyroid hemilobes. Once again the control 4 hour incubation is shown in the top part of the figure and here will be seen the typical appearance of a predominant labelled peak running just short of the 19S peak, a labelled 12S peak present and some labelling over the 3 - 8S area. As a result of overnight dialysis (middle portion of the figure) at pH 10 there is a marked diminution of labelling of the peak in the thyroglobulin area; a marked increase in labelling of the peak in the 12S area and this is again associated with the appearance of a small stable 12S peak; the labelling associated with the 3 - 8S region has also fallen. The

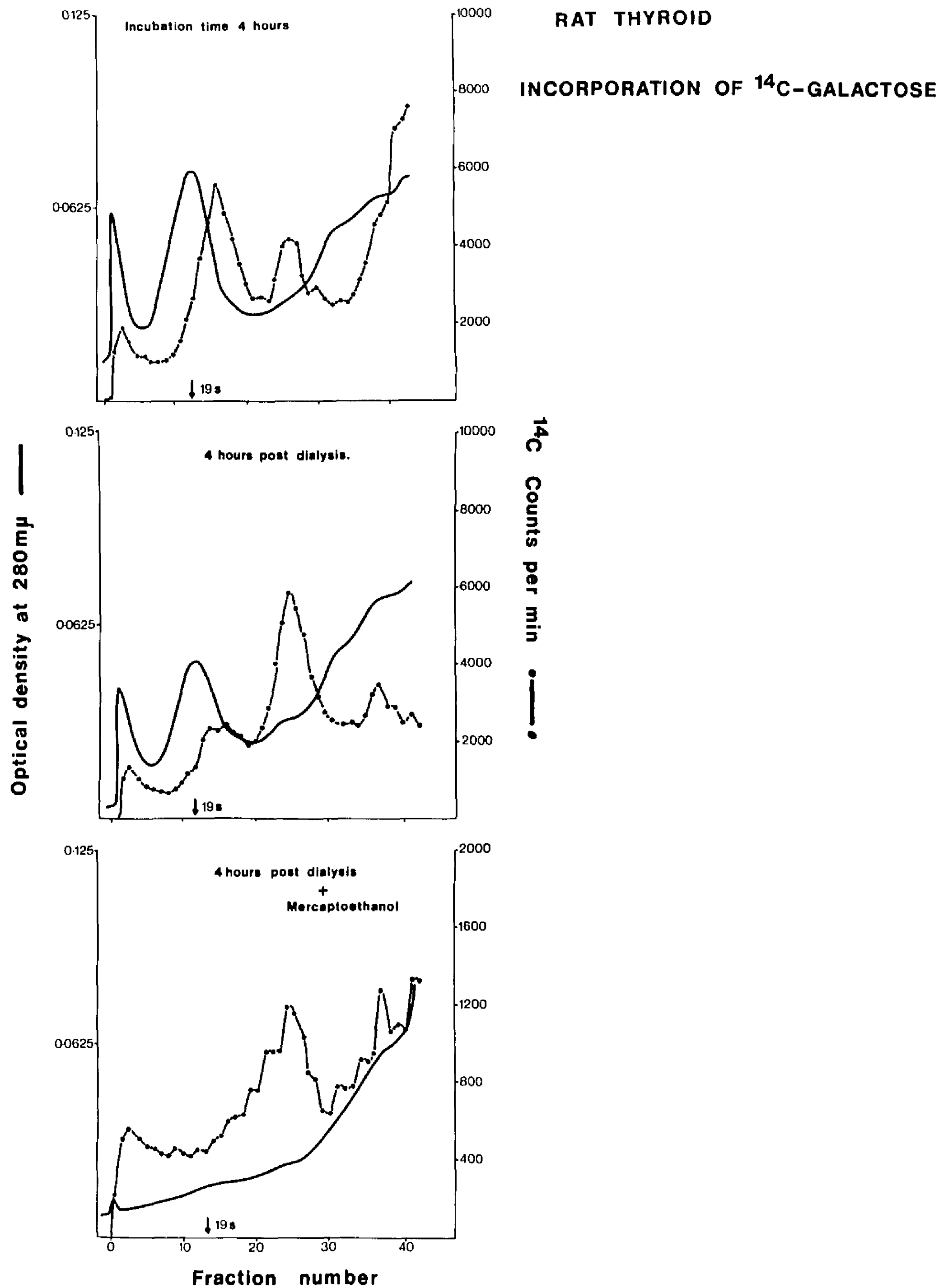


Fig. 45

Ultracentrifugal study of the effect of dialysis at pH 10 and betamercaptoethanol on the thyroid proteins of control rats labelled with ^{14}C galactose *in vitro*. Upper portion: control 4 hour incubation. Middle portion: effect of dialysis at pH 10. Lower portion: effect of additional betamercaptoethanol. SW 41 Rotor at 35,000 rpm for 16 hours.

effect of the addition of BME is shown in the lower part of the figure. It will be seen that once more the stable proteins are altered so that there is now abolition of the protein peak in the 19S region, there is no discrete 12S stable peak present but there is merely a broad area of protein lying over the top of the gradient. There is no significant labelling of the 19S proteins present, there is a large 12S labelled peak although diminished in specific activity from the post-dialysis specimen and proportionately there is much more labelling of the peak in the 3 - 8S region. Once again this would imply the effect of the dialysis has been to break the protein in the thyroglobulin region down into the 12S region and the effect of BME has been to break the protein in the 12S region down to proteins in the 3 - 8S region.

These studies would therefore suggest that mannose and galactose are associated not only with the proteins in the thyroglobulin region but also are associated with the 12S and 3 - 8S proteins during breakdown of the thyroglobulin molecule. It is therefore conceivable that incorporation of the sugars could occur into the 3 - 8S and 12S proteins initially and the proteins in the thyroglobulin region be built up from these presumed submits.

CHAPTER 28

CHROMATOGRAPHIC STUDIES OF THYROID PROTEINS FOLLOWING INCUBATION WITH ^{14}C MANNOSE AND ^{14}C GALACTOSE AND EXPOSURE TO SPECIFIC GLYCOSIDASE ENZYMES

Herscovics (1969) has suggested that during incubation with of ^{14}C mannose there is some conversion of the ^{14}C mannose to galactose and that this could result in apparent early incorporation of ^{14}C mannose into proteins in the thyroglobulin region.

In view of this, studies were performed, as described in the methods section, to see whether this applied under the conditions used in this study.

Using ^{14}C mannose and ^{14}C galactose individually studies were performed using rat thyroid tissue at incubation times of $\frac{1}{2}$, 1, 2, 3, 4 hours. Following the incubations the flask contents were submitted to digestion with either α -mannosidase or β -galactosidase under the conditions outlined. Following this chromatography using butanol/pyridine/water solvent showed only a single peak corresponding to the stable mannose or galactose respectively.

No evidence was therefore obtained which would suggest that significant conversion of labelled sugars occurred in the present studies.

CHAPTER 29

EFFECT OF ULTRACENTRIFUGATION AT 23°C ON THE PATTERNS OF INCORPORATION OF ^{14}C MANNOSE AND ^{14}C GALACTOSE INTO RAT THYROID PROTEIN

Schneider et al (1971) have suggested from their studies in the guinea pig that the appearance of 12S labelling using ^{3}H leucine is a technical artefact. They suggest that storage at 2-4°C and subsequent ultracentrifugation at 4°C results in a reversible alteration of the thyroglobulin-like proteins. Their studies were consistent with the breakdown of 19S protein to initially 15S protein and later to 12S protein. On raising the temperature of the experimental conditions to room temperature (23°C) 12S protein was not seen and 19S protein was reconstituted.

In view of this, studies were undertaken where following the usual incubation at 37°C, the flask contents were kept at room temperature thereafter and ultracentrifuged at 23°C.

The results are shown in figs. 46 and 47. It will be seen, both using ^{14}C mannose and ^{14}C galactose, that ultracentrifugation at 23°C still gives the pattern of incorporation into 3 - 8S, 12S and thyroglobulin-like proteins. The results obtained using either sugar are very similar to the control rat experiments shown in figs. 4 and 5 with the exception of slightly earlier incorporation of mannose into thyroglobulin.

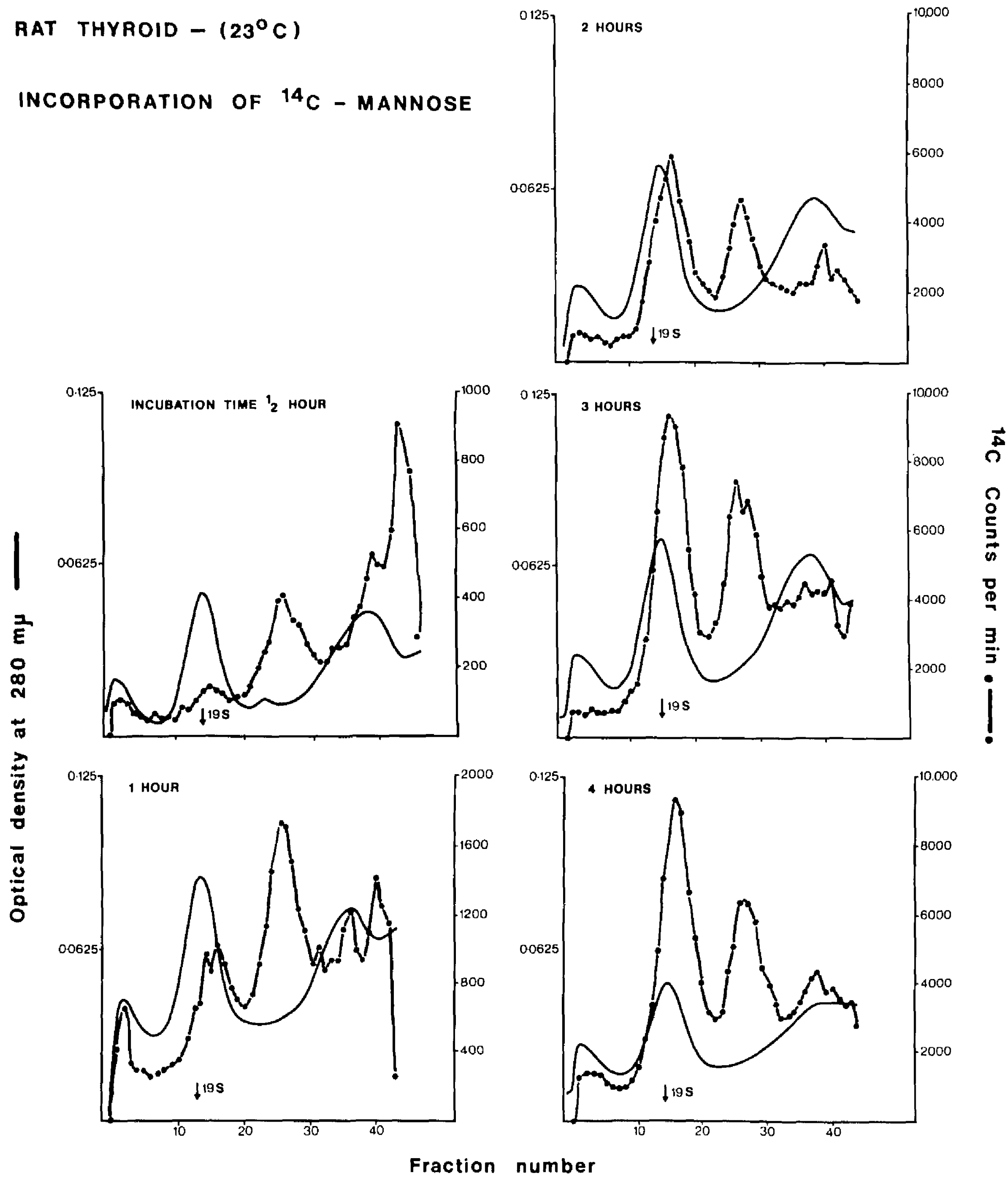
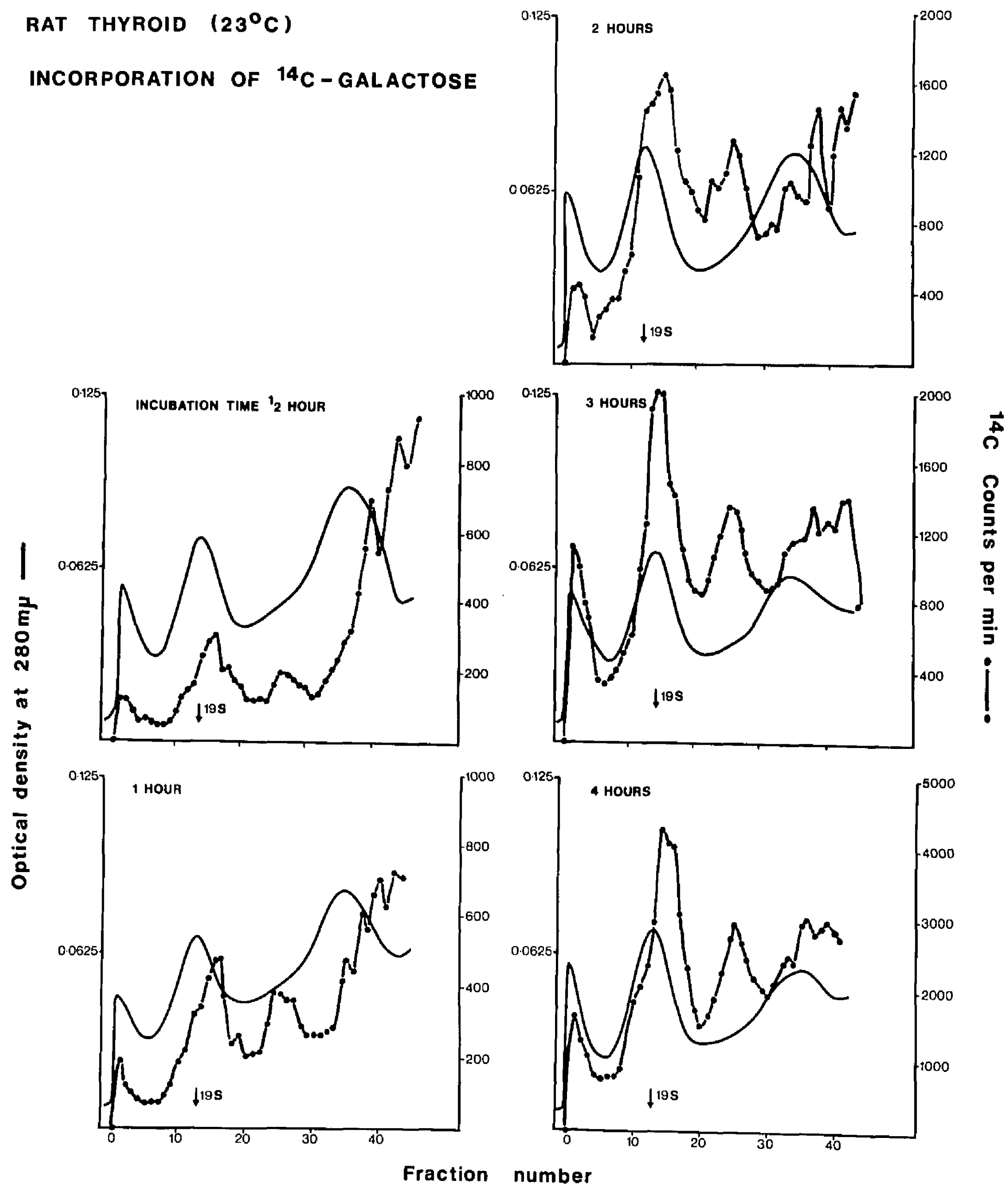


Fig. 46

Ultracentrifugal study of the time course of incorporation of ^{14}C mannose into control rat thyroid proteins *in vitro*.

SW 41 Rotor at 28,000 rpm for 16 hours: temperature of ultracentrifugation: 23°C.



<u>Fig. 47</u>

Ultracentrifugal study of the time course of incorporation of ^{14}C galactose into the thyroid proteins of control rat thyroids. SW 41 Rotor at 28,000 rpm for 16 hours; temperature of ultracentrifugation: 23°C.

GENERAL DISCUSSION

The author's previous work (Thomson 1969) was devoted to looking at the mechanisms of incorporation of iodine and leucine into rat thyroglobulin *in vivo* and into human pathological glands *in vitro*. As already presented in the introductory section the evidence is that ^{3}H leucine is incorporated into lightweight protein fractions (3 - 8S and 12S) before incorporation into thyroglobulin occurs. In contrast ^{125}I is incorporated directly into the thyroglobulin region although usually just short of the true 19S position.

The work reported in the present thesis is therefore an extension of these studies looking at the patterns of incorporation of labelled sugars into the soluble thyroid proteins of the rat thyroid *in vitro* and in the human pathological glands *in vitro*. The extension of these studies was prompted by the work of Herscovics (1969) who reported differences in the incorporation of certain labelled sugars into the soluble thyroid proteins of the rat. In particular, she found that labelled mannose was incorporated into the thyroid proteins in a manner similar to that for ^{3}H leucine, that is the pattern was consistent with incorporation into presumed thyroglobulin subunits, 3 - 8S and 12S, before there was incorporation of the label into 19S thyroglobulin. In contrast she found that labelled galactose was incorporated in a manner similar to that of iodine, that is there was no incorporation of galactose into proteins lighter than the immediate thyroglobulin precursors, that is proteins of approximately 18S size. She, therefore, postulated that galactose in contrast to mannose was

incorporated only into the completed non iodinated polypeptide chains of thyroglobulin. Consistent with these studies were her results using substances which inhibit protein synthesis. She was, for instance, able to show using puromycin that there was almost complete inhibition of incorporation of labelled leucine and mannose but no effect on the incorporation of galactose. She later extended her studies to look at another sugar, fucose (Herscovics 1970). This sugar theoretically should appear on the outer aspect of the carbohydrate chains of the thyroglobulin molecule. Herscovics found that labelled fucose was incorporated in a manner similar to that of labelled galactose, that is into a 17 - 18S protein without incorporation into the presumed thyroglobulin subunits. She also found using fucose that the incorporation was not immediately affected by the use of cycloheximide, another drug which inhibits protein synthesis. From the same laboratory autoradiographic studies supporting these biosynthetic differences in incorporation of labelled sugars have been reported (Whur et al 1969). Labelled galactose was quickly concentrated over the Golgi apparatus whereas mannose was seen diffusely over the thyroid follicular cell cytoplasm; galactose was incorporated into colloid as early as 30 minutes but mannose took 2 hours to reach the colloid.

Because of the above results the initial study conducted by the author was to look at the pattern of incorporation of ^{14}C mannose and ^{14}C galactose _in vitro_ into rat thyroid hemilobes using the incubation techniques which the author has used extensively in studies of leucine and iodine incorporation.

Before considering the similarities and differences between the present results and those found by Herscovics various technical points should perhaps be considered. 1) The incubation system used in the two studies were broadly similar but one or two minor differences did exist. The buffer used in the present study was a modified Krebs No. 2 buffer which has been used very successfully in the author's laboratory for some years. Herscovics used a Krebs Ringer bicarbonate buffer. 2) Following incubation Herscovics used only the thyroid hemilobes and did not use the total flask contents as was used in the present study. The importance of this is that at long times of incubation there is significant leaching of thyroid proteins from the thyroid slices or hemilobes and these then appear in the medium. This was well documented some years ago by Seed and Goldberg (1965) who showed that after six hours of incubation there was very little thyroglobulin left in thyroid slices *in vitro*. 3) In the present study the use of Sephadex G25 columns to remove unincorporated labelled sugars was particularly useful as documented in fig. 3. This revealed easily seen labelled peaks in proteins lighter than 19S which were obscured before the desalting step. This step in our studies preceded the ammonium sulphate precipitation step which was done in both studies. 4) In the present studies it was found that pyruvate made no difference to the pattern of incorporation of ^{14}C mannose and therefore this was not added in the present study as a routine (see Chapter 13). 5) One further point was the present author's inability to find any metabolism of ^{14}C mannose or ^{14}C galactose into any other labelled sugars

during the course of the incubation. This is in contrast to the finding of Herscovics that there was a significant conversion of ^{14}C mannose into ^{14}C galactose and this she postulated could alter the pattern of the results obtained. The author's data in regard to this point in particular is reassuring since there is another paper (Mitranic and Moscarello 1972) which purports to show that following the injection of ^{14}C mannose into the bloodstream of a rat there is a rapid conversion of most of the carbohydrate to sialic acid.

As regards the results of the present studies of the incorporation of labelled sugars into rat soluble thyroid proteins _in vitro_ it will be seen that the results for mannose follow closely those established for leucine, that is the pattern of incorporation is consistent with incorporation initially into 3 - 8S, and 12S proteins and then progressively into 19S protein. This is in keeping with the results of Herscovics (1969). In contrast to her findings, however, the pattern of galactose incorporation followed a broadly very similar pattern in the present studies. There was progressive labelling of all three peaks, the 3 - 8S, the 12S and the 19S peaks with the passage of time. The only differences between galactose and mannose incorporation in the control rat in our studies was the fact that using galactose there was persistently earlier incorporation of the label into the thyroglobulin-like proteins at any given time and that the specific activity of labelling of thyroglobulin-like proteins was higher at any given time of incubation compared to the same time interval using labelled mannose.

Recently Schneider et al (1971) have suggested that the appearance of a 12S peak following the administration of a pulse of labelled amino acid in vitro is an artefact.* They proposed that the appearance of this 12S peak and gradual accumulation as time passes of increasing amounts of label in the thyroglobulin proteins is due not to a precursor/product relationship between the 12S and 19S peaks but rather that the opposite is the case. They proposed that newly formed thyroglobulin which is known to be more liable to breakdown than older thyroglobulin is rapidly labelled but easily breaks down to form a labelled 12S peak. The studies of the effect of dialysis of the soluble thyroid proteins at pH 10 show that certainly the labelled sugar remains as an integral part of the protein molecule when broken down from 19S to 12S under these conditions. The reason for the gradual lessening of the 12S peak with the passage of time, according to Schneider et al, is because of the increasing stability of the 19S thyroglobulin. They were able to show using the guinea pig as the experimental animal that these changes in their studies were temperature dependent and that the usual techniques of maintaining the proteins, either frozen or at low temperatures, would result in the preferential formation of a labelled 12S peak whereas maintaining it at room temperature (23°) resulted in the formation of a labelled 19S peak. It should be noted that their studies were performed in the guinea pig which is rather an unusual laboratory animal in that most workers have found regular stable 12S thyroid protein present in this animal (Roche et al 1968).

* See pages 9-12 for discussion of relationship of 19S thyroglobulin and subunits

Roche and co-workers have shown that the guinea pig alone out of sixteen different mammals studied was the only one found to have appreciable 12S protein present constituting approximately 14 per cent of the total soluble thyroid proteins. In the author's previous work (Thomson 1969) it has been noted that the guinea pig is unusual in having thyroid proteins which are much less iodinated than other vertebrate thyroid proteins. This presumably is due to its largely unsupplemented vegetarian habits. The fact that poorly iodinated thyroglobulin is much more liable to show breakdown than normally iodinated thyroglobulin therefore makes the guinea pig a rather unsatisfactory animal with which to study the relationship of possible thyroglobulin precursor subunits to 19S thyroglobulin. Certainly as outlined in Section 5, Chapter 29, the author was unable to show in the rat that ultracentrifugation at 23° gave a significantly different pattern of incorporation of ^{14}C mannose and ^{14}C galactose into the soluble thyroid proteins compared to the similar studies at 4°C. Further support for the author's view that the presence of a labelled 12S protein in the present studies is not just a technical artefact is the previous work from this laboratory using an identical incubation technique to study the labelling of extremely iodine deficient rat thyroid proteins with ^{125}I, no protein $<$ 18S size was iodinated (Thomson and Bissett 1969b).

Having therefore validated the studies on technical grounds and examined the various factors which could possibly cause different results to be obtained from those found by Herscovics, the various factors possibly involved in the incorporation of

^{14}C mannose and ^{14}C galactose into rat soluble thyroid proteins were examined. The effect of administering a goitrogenic drug certainly did not result in any blockage of the incorporation of ^{14}C mannose or ^{14}C galactose. In fact, the incorporation of the labelled sugars increased; this has also been found previously using leucine. The effect of the drug apart from increasing the amount of incorporation of the label was to cause increased labelling of the thyroglobulin-like proteins at earlier time intervals and proportionately to greater extent than in the control animals. As previously pointed out, this effect was not specific for any single goitrogenic drug and suggested that perhaps the effect of the thyroid blocking drugs was to cause increasing thyroid stimulation via the TSH mechanism by means of the well known negative feed back mechanism activated by falling peripheral blood hormone levels.

In keeping with this hypothesis were the results obtained using thyroxine from which it can be seen that there is significant inhibition of incorporation of both mannose and galactose with mannose being rather more affected than galactose. More importantly one was able to reproduce the increased incorporation of ^{14}C mannose and ^{14}C galactose and also the early incorporation into the thyroglobulin-like proteins with the use of TSH added to the incubation medium <u>in vitro</u>. It should be noted that although TSH injected <u>in vivo</u> did produce some stimulation of incorporation this was by no means marked and indeed occurred only at later time intervals such as 3 - 4 hours and not early time intervals when if anything there was relative inhibition of incorporation.

This discrepancy between the results of the *in vivo* and *in vitro* administration of TSH might at first sight be considered bizarre. It is possible that the differences found were merely technical and that if a different dose or different time interval of administration had been used then different results might be obtained. Various studies were performed using different dosages up to 3 units per rat at different time intervals but essentially the same results were obtained. Assuming a broadly similar sensitivity of human and rat thyroid tissue to the effects of bovine TSH a dose of 1 - 3 units of TSH to a 150 gm rat is not a small dose compared to a dose of 10 units usually used in a 70 Kilo man. There is however supporting evidence in the literature for this unusual effect of TSH. Wägar et al (1973) showed discrepancies between the action of TSH on thyroid protein synthesis *in vivo* and *in vitro* using labelled leucine. They found that when the concentration of stable leucine in the medium was low that there was initial inhibition of *in vitro* incorporation of labelled leucine into the thyroid slices. This could be abolished by adding stable leucine to the medium. *In vivo* they found that there was no stimulation of incorporation of labelled leucine into the thyroid proteins till after a lag phase of 4 hours after TSH administration. The finding of similar discrepancies with regard to labelled sugars is of interest but this topic has not been further looked at in the present study.

It can be seen from Chapter 10 that iodine in the form of potassium iodide in the drinking water does result in a similar

pattern of incorporation of ^{14}C mannose and ^{14}C galactose to that found using control rats. There is, however, some diminution in labelling under these circumstances; it is known that potassium iodide has rather ill understood effects on the thyroid gland, one of which almost certainly is to diminish the activity of TSH and therefore the mild inhibition of incorporation could be explained by postulating this mechanism.

In contrast to the studies of Herscovics (1969) it can be seen from figs. 16 and 17 that the results of adding cycloheximide were broadly similar for mannose and galactose incorporation, that is that the addition of the drug, although completely inhibiting protein synthesis, did not inhibit the steady accumulation of labelled 19S protein. This pattern is consistent with mannose and galactose being incorporated into thyroglobulin subunits such as 3 - 8S and 12S rather than galactose being incorporated directly into thyroglobulin. It should be noted that the amount of cycloheximide used in the present experiments was much greater than that used by Herscovics in her study on ^{14}C fucose incorporation. The amounts used in her study would have been quite ineffective in inhibiting polypeptide synthesis using the author's techniques.

The results of the use of added stable sugars to study the effect on incorporation of the specific labelled sugar are interesting. It was found, as anticipated, that adding increasing quantities of the stable sugar resulted in progressive inhibition of incorporation of the specific labelled sugar. This presumably happens by a mass action effect although as outlined in Table 1

it is possible that the osmotic effect shown also played a part. It will be seen that mannose was more effective in inhibiting the incorporation of labelled mannose than labelled galactose and the converse was found for the addition of stable galactose. It has been suggested that specific glycosyltransferase enzymes exist in the thyroid (Spiro and Spiro 1968a and b). Certainly the studies reported in the present work would be in keeping with specificity of incorporation of the labelled sugars. It was interesting, however, as seen in fig. 24 that the addition of mannose and galactose had some inhibitory effect on the incorporation of labelled leucine but not that of iodine. It is difficult on the present evidence to be sure that this effect is not merely that of alteration of osmotic pressure with resulting impairment of thyroid cell function. One might postulate if this were the case that the incorporation of leucine into polypeptide chains might be more affected than the addition of iodine to the completed polypeptide chain.

The experience with the incorporation of ^{14}C fucose reported in the present work is consistent with the studies of Herscovics (1970). Certainly there seemed no good evidence of incorporation of fucose into presumed thyroglobulin subunits. Labelling of the 19S proteins occurred very early and was progressive. One might postulate that some of the slight shouldering effect of the radioactive peak of ^{14}C fucose incorporation was due to slight breakdown of the thyroglobulin proteins. It is interesting that the administration of a goitrogenic drug did not alter the pattern of

this incorporation nor did it increase the amount of incorporation to nearly the same extent as was found using ^{14}C mannose, ^{14}C galactose, ^{3}H leucine or ^{125}I. The effect of added thyroxine on the incorporation of ^{14}C fucose did suggest, however, that the incorporation was also mediated through the TSH mechanism in view of the fact that under these circumstances the incorporation of ^{14}C fucose was much diminished. This was supported by the modest reduction in incorporation as a result of the administration of potassium iodide. Cycloheximide could be shown to be ineffective in inhibiting the formation of labelled thyroglobulin. This is in agreement with Herscovics (1970).

The overall conclusion from these studies in the rat is that the patterns of incorporation of ^{14}C mannose and ^{14}C galactose are consistent with the incorporation of both sugars into presumed subunit proteins of 3 - 8S and 12S size before there is striking incorporation of the labelled protein into 19S, that is the pattern in both sugars more resembled that of the incorporation of amino acids such as ^{3}H leucine rather than that of ^{125}I. It should be noted that labelling of a 12S protein occurred routinely using galactose. Even the studies with added T_4 to the diet which theoretically should inhibit thyroglobulin breakdown still showed labelling of the 12S protein (fig. 9). In this instance the labelling of the 12S protein was in keeping with it being a pre-cursor of the labelled 19S peak. It is of interest that the studies of the breakdown of thyroglobulin (Chapter 27) have shown during the degradation of the thyroglobulin molecule that the

carbohydrate which had been incorporated into a 19S protein remained attached as far as the 3 - 8S stage and did not seem to be lost into the medium as unincorporated sugar.

In contrast, however, fucose appeared to be incorporated by a different mechanism and as stated by Herscovics (1970) appeared to be incorporated directly into thyroglobulin-like proteins without passing through stages of subunit incorporation.

The next main section of the thesis concerns the patterns of incorporation of ^{14}C mannose and ^{14}C galactose found in human thyroid glands studied <u>in vitro</u>. The pattern found in the 'normal' thyroid gland of the human was similar to that of the rat although the specific activity of labelling was not so great. This might be, of course, due to the fact that these patients were not strictly normal; they all had a neck exploration for a possible parathyroid tumour and there is no evidence at present of what effect an elevated serum calcium might have on the incorporation of these sugars. In keeping with this possibility is the fact that the non-toxic goitres showed by and large an increased amount of incorporation of labelled mannose and galactose than the 'normal' human gland. One might postulate that this effect could be mediated through the goitres being stimulated by increased levels of serum TSH. Unfortunately, however, it has been regularly shown using current techniques of radioimmunoassay that there is no consistent picture of increased serum TSH levels in patients with simple goitre (Hall et al 1971). It is possible in these circumstances that the results found in patients with non-toxic

goitre are basically more 'normal' than those found in so-called 'normal' thyroid glands.

It should be noted in the human also that the pattern of incorporation of ^{14}C mannose and ^{14}C galactose was consistent with significant amounts of labelled 3 - 8S and 12S protein being formed in addition to labelling of the thyroglobulin proteins.

As regards the studies of the thyroid adenomas, it is of interest that their behaviour in vitro could be predicted from the results of the in vivo scanning with radioactive iodine or technetium. Where the adenoma showed increased accumulation of either of these two isotopes in vivo it also always showed increased incorporation of the labelled sugars in vitro. In contrast where the adenoma was 'cold', there was a decreased incorporation of the sugars. The fact that an adenoma was 'hot' or 'cold' did not reflect the activity of the gland as a whole. In particular, thyroid adenomas are not usually under increased TSH stimulation and the effect of increased incorporation of the various substances mentioned would seem to be due to the autonomous increase in activity of the adenomatous tissue. It is of interest that in the thyroid adenomas there is much less evidence of 12S labelling than in any other condition studied. The reason for this is not at all clear.

The results of studies in patients with thyrotoxicosis are of interest in that this was the only situation in the present studies where there was a major discrepancy between the results of mannose and galactose incorporation. Galactose was incorporated to a much

greater extent and much earlier than mannose. It is possible that this discrepancy was the result either of the underlying thyrotoxic process or of the treatment administered. With regard to the latter possibility the results of the studies in the rat would be against the therapy having this effect. An antithyroid drug like carbimazole would be expected to increase labelling both with mannose and galactose, and iodide would be expected to cause some slight diminution in the incorporation of both but not a major degree of discrepancy between the incorporation of both sugars. It may therefore be that in thyrotoxicosis there is something in the disease process itself that gives this effect. Perhaps for instance there might be a specific lack of the transferase enzymes responsible for the incorporation of mannose and this could well cause the results found. This possibility obviously will require further study but has not been examined further at present.

In contrast, the results found in Hashimoto's thyroiditis were quite the opposite. In general, there was very poor incorporation of both mannose and galactose into the proteins in the thyroglobulin region. Indeed this was virtually absent in the majority of patients studied. Even in those in whom there was slight incorporation this did not increase with time and there was no suggestion of labelling of a 12S protein. This pattern of labelling virtually only 3 - 8S protein is that previously reported by the author for ^{3}H leucine (Thomson and Bissett 1969a) whereas iodine is usually well incorporated into

the thyroglobulin-like proteins. The explanation for this difference is not immediately apparent especially since obviously some stable thyroglobulin is formed as judged by the ultra-centrifugal pattern of the stable thyroid proteins and also the fact that most patients studied were indeed euthyroid. Hashimoto's thyroiditis is the one thyroid condition in which increased levels of TSH are universally found (Hall et al 1971). Because of the high endogenous level of TSH one might have anticipated increased incorporation of labelled sugars and amino acids into the thyro-globulin-like proteins as this is the expected effect of TSH. Perhaps again this is a condition in which there are specific deficiencies of glycosyltransferase enzymes and this area would obviously repay further study.

The pattern of results found in a small number of carcinomas of thyroid were as anticipated from histology of the tumour. Where the tumour was well differentiated as, for instance, in papillary carcinoma there was good incorporation of the labelled sugar into thyroglobulin. Where the carcinoma was undifferentiated as in an anaplastic or a medullary carcinoma there was no significant incorporation of labelled sugars into the proteins of the tumour further than the 3 - 8S stage. This pattern of incorporation is consistent with that previously found using leucine (Thomson 1969).

The overall results of the incorporation of ^{14}C mannose and ^{14}C galactose into the human thyroid glands were therefore of interest, in that certain conditions, notably thyroid adenoma, thyrotoxicosis, Hashimoto's thyroiditis and thyroid carcinoma

provide evidence of situations in which there are changes in the pattern of incorporation of the labelled sugars from that anticipated on theoretical grounds. As already mentioned, it is possible that in these abnormal glands there are deficiencies of particular transferase enzymes and indeed such a defect has been found for a rat thyroid tumour studied by Monaco and Robbins (1973). Study of such defects as well as their intrinsic scientific interest might possibly throw some light on the pathogenesis of conditions such as thyrotoxicosis, thyroid adenoma and Hashimoto's thyroiditis which are at present still poorly understood.

SUMMARY

This thesis examines the patterns of incorporation of labelled carbohydrates into the soluble proteins of the rat thyroid and of human pathological glands *in vitro*.

It has been previously postulated by Herscovics (1969) that ^{14}C mannose is incorporated like amino acids, that is into presumed thyroglobulin subunits of 3 - 8S and 12S size before there is labelling of the 19S protein. In contrast to this it was claimed that ^{14}C galactose and ^{14}C fucose are both incorporated directly into a protein just short of thyroglobulin running at approximately the 17 - 18S position on ultracentrifugation.

The present studies using the rat thyroid are in agreement with Herscovics with respect to the incorporation of ^{14}C mannose. However in the present studies ^{14}C galactose was also incorporated in a pattern consistent with subunit incorporation and not direct incorporation into a 17 - 18S protein. ^{14}C fucose, however, did seem to be incorporated directly into thyroglobulin-like protein and not to be incorporated into presumed thyroglobulin subunits.

Various factors affecting the incorporation of these labelled sugars into the rat thyroid protein were examined. The process of incorporation was stimulated by the administration of an antithyroid drug or by TSH. All goitrogenic drugs gave the same effect suggesting that they might act through a TSH controlled mechanism. Further support for this possibility came from the study of the effect of sodium thyroxine and potassium iodide on the incorporation of

labelled sugars when it could be shown that both substances interfered with the incorporation of all sugars studied. These effects were probably mediated via a TSH inhibiting mechanism.

In vitro studies of human thyroid glands showed results in 'normal' human thyroids and non-toxic goitres similar to those anticipated from the rat study, that is that the pattern of incorporation of ^{14}C mannose and ^{14}C galactose was consistent with that of subunit incorporation before labelling of thyroglobulin-like proteins. In certain human pathological conditions, however, discrepancies from this were found. In thyrotoxicosis there was very poor incorporation of mannose as compared to galactose; in thyroid adenomas there was very little labelling of 12S protein compared to labelling of proteins in the thyroglobulin region using either mannose or galactose and in Hashimoto's thyroiditis there was in general very poor labelling of proteins in the thyroglobulin region with either sugar in contrast to the good labelling obtained with iodine. These discrepancies suggest that perhaps the differences in these pathological conditions might be due to deficiencies of specific glycosyltransferase enzymes.

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