

STUDIES ON THE ROUTE OF SYNTHESIS
OF THE THYROID HORMONE

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OF THE THYROID HORMONE

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TITLE: STUDIES ON THE ROUTE OF SYNTHESIS OF THE THYROID HORMONE

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SCOPE AND CONTENTS:

Certain aspects of the biochemistry of the thyroid gland have been studied. The techniques of filter paper chromatography and radioautography were used to separate and identify the iodine-containing amino acids of the gland, and were modified somewhat, in order to obtain reliable results.

Although a series of preliminary experiments were performed with the thyroid glands of rats in vivo, the main part of this work made use of the in vitro technique. Surviving tissue slices were incubated in the presence of the radioactive tracer, iodine¹³¹. Evidence was obtained which indicated that at least two of the amino acids found "free" in the thyroid gland were degraded by the gland to inorganic iodide.

The mechanism of action of a number of thyroid gland inhibitors was investigated. It was found that all but two of the materials studied led to the formation in the tissue slices of unidentified iodine-containing materials with the simultaneous disappearance of inorganic iodide. Hence, at least a part of the goitrogenic nature of these inhibitors would appear to be due to

the "removal" of iodide.

It was found that one portion of the inhibition caused by 3 - fluorotyrosine could be "reversed" in vitro with tyrosine. Interesting sex variations in thyroid gland activity were observed during these experiments.

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GENERAL INTRODUCTION

Within the last few years a good deal of interest has been shown in a method of study and treatment of human thyroid disorders utilizing iodine ¹³¹ which emits β and γ -radiations.(1) One such clinic is being held regularly at this university. Therefore, when facilities became available for original research in biochemistry, it was decided to devote some time to an investigation into certain aspects of thyroid gland biochemistry, in particular the route of synthesis of the thyroid hormone in the gland.

The experimental section of this thesis deals with the initial research which has been carried out on the problem in this laboratory.

Consultation of the literature revealed that although a great deal of work had been done on the chemistry of the thyroid gland during the last fifty years there were still many features of the tissue and its synthetic processes about which relatively little was known. For example: the roles taken as precursors of the thyroid hormone by the various iodine-containing amino acids found in the gland; the identity of the enzymes involved in thyroxine synthesis, the actual nature of the thyroid hormone, and the manner in which goitrogenic materials exert their inhibitory action were still uncertain.

However, before any definite line of research could be begun, a selection of suitable techniques for the study of the thyroid gland had to be made. Since the isotope, iodine ¹³¹, was available for use as a biological tracer, the rest of the procedures were chosen so that this radioactive material could be utilized to the best advantage. Paper chromatography seemed to be the most convenient method available for

the rapid separation and identification of the constituents of the thyroid. Nevertheless, the results being obtained with chromatography by other workers were not too satisfactory, and left much to be desired in some respects. Hence, a considerable part of the time devoted to this work was spent on adapting this technique to give reliable results with thyroid gland extracts. This involved selection of a suitable quality of filter paper and at least one solvent mixture which would bring about a complete separation of the compounds being studied. Other modifications which would improve the usefulness of the chromatography were also tested, and if found to be suitable, were adopted as a general procedure.

Radioautography of the paper chromatograms was chosen to detect and identify the presence of iodine-labelled materials.

Surviving thyroid tissue slices, incubated in a physiological medium, provided a convenient method for the controlled study of the gland. In this manner the composition of the environment of the thyroid tissues could be strictly standardized so that definite amounts of various compounds could be administered to the slices to determine their effects. Therefore, after a preliminary series of experiments with living, whole animals, performed in order to familiarize the author with the techniques involved, and to form a normal basis to which the in-vitro results could be compared, the remainder of the research was carried out utilizing the in-vitro approach.

A fairly varied group of topics was briefly investigated during the latter part of this work in order to ascertain in what direction further detailed research would prove most successful with the techniques

available. These include, among others, the following: the effect of nembutal anaesthesia on thyroid metabolism in-vivo, the effect of high intensity of γ -radiation on the synthetic processes of the gland, and the administration of labelled diiodotyrosine and monoiodotyrosine, two proposed precursors of thyroxine, to surviving tissue slices, in-vitro. However, the most concentrated study was concerned with the effect and mechanism of compounds which have been found to be inhibitory to thyroid synthesis. The materials were administered to surviving thyroid slices, in the presence of isotopic iodine, and the results analyzed by chromatography and radioautography. One of these materials, 3-fluorotyrosine, is a known competitive inhibitor of tyrosine in certain metabolic pathways(2). Therefore, the final section of this work deals with the effect of tyrosine on thyroxine synthesis by tissue slices in the presence of 3-fluorotyrosine.

The results obtained are discussed in relation to the present hypotheses of thyroid hormone synthesis, with special emphasis placed on the mechanism of action of the inhibitory materials studied. In addition, certain conclusions were drawn as to the most advisable method of approach to a more detailed study of the biochemistry of the thyroid gland.

HISTORICAL INTRODUCTION

The Thyroid Gland; its Physiology and Function (3,4)

The thyroid gland is an endocrine gland present in all vertebrates, but absent in the invertebrates. It consists of characteristic groups of closed follicles which contain a gelatinous mass, the colloid, in which is stored the hormone secreted by the gland. The thyroid is derived phylogenetically from the endostyle organ, a structure of considerable importance in the tunicates.

The thyroid first assumes a relatively large size in the elasmobranch fishes where it consists of a group of follicles which lie at the anterior end of the aorta. In the teleost fishes, the thyroid consists of widely scattered follicles which lie under the gill arch. In the amphibia, the gland consists of a pair of oval bodies on each side of the lingual bone. In the reptiles the thyroid is usually unpaired and lies over the pericardium. In birds, it is a paired organ which lies in the thorax, partially imbedded in the thymus. In mammals, the thyroid consists of two lobes situated on both sides of the trachea at the level of the larynx.

The chief function of the thyroid hormone seems to be concerned with the regulation of the rate of metabolism in the animal body. This thyroid effect on oxidative metabolism is so exerted as to speed up the utilization of carbohydrate, fat, and protein. All of the many and varied thyroid effects might be attributed to this general action.

In certain vertebrates, metamorphosis occurs under the influence of the thyroid. Thus, the thyroidectomized tadpole does not become transformed into a frog, but just grows into a large tadpole, while

small tadpoles fed with thyroxine soon metamorphize into tiny frogs.

Abnormal and defective thyroid glands have been of considerable interest, especially in human subjects. Some of the more common types of thyroid disturbances are:

Cretinism - caused by a developmental defect or atrophy of the thyroid during infancy so that the gland fails to produce sufficient hormone to maintain metabolism at a normal level. The cretin is stunted in growth, and incompetent, physically and mentally.

Myxedema - occurs during adulthood and is due to a deficiency or absence of the thyroid secretion. It is characterized by a marked decrease in basal metabolism, retardation of all nervous reactions, and mental deterioration. The individual shows the symptoms of dropsy, with puffy hands and face.

Both cretinism and myxedema may be successfully treated by thyroid therapy leading to the restoration of higher metabolic rates, increased general activity and disappearance of other symptoms.

Simple goiter - is a deficiency disease resulting from an insufficient amount of iodine in the diet. The individual is hypothyroid even though the glands may be conspicuously enlarged. This enlargement is brought about in order to compensate for the lack of iodine by increasing the secretory surface of the thyroid follicles.

Exophthalmic goiter or Grave's disease - is characterized by hypertrophy of the thyroid tissue and by the excessive production of thyroid hormone. The principal functional disturbances are increased metabolism, shortness of breath, nervous agitation, insomnia, rapid heart beat, loss of weight, and protrusion of the eyeball.

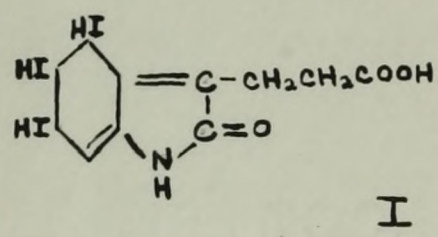
Hyperthyroidism can be controlled permanently only through reduction of the hormone output of the gland. This involves decreasing the secretory surface of the thyroid, either by surgical removal of parts of the gland or by application of X-radiation. Recently, radioactive iodine has been used for the purpose (1). Since this element is concentrated in the gland, internal radiations replace the necessity of externally applied X-rays. Treatment of exophthalmic goiter is also carried out, using antithyroid drugs such as thiouracil and iodide. The action of the latter in reducing hyperactivity of the gland has not yet been satisfactorily explained.

Early Ideas of the Thyroid Gland and Its Hormone

Probably the first discovery of major importance in connection with the biochemistry of the thyroid gland occurred in 1895 when Baumann announced the identification of the element, iodine, in the gland. Until that time apparently no one had suspected the presence of that element in the body. This discovery led to a variety of work being done on the effects of various iodine compounds on the thyroid (5), the amount of iodine present in the gland (6), what other constituents were present (7), variations of iodine content with age, sex and seasons (8,9), and attempts at fractionation of the materials present into active and inactive portions. It was soon realized that the iodine content of the gland had a definite relationship to its action on the metabolic rate of the animal. The active iodine-containing principle of the thyroid is present soon after conception and takes an active part in the development of the animal (10). Blum and Grutzner

announced that most of the thyroid iodine is in the form of a protein, but that some was soluble in acetone, including a portion identified as free inorganic iodide (11).

In 1915, Kendall decomposed the proteins of the gland by alcoholic alkaline hydrolysis and fractionated the hydrolysate into two parts, namely, those constituents which were soluble in acids and those which were insoluble (12). From the latter portion he isolated a pure crystalline compound (60 per cent iodine) which he first claimed to be diiodo-dihydroxyindole (13). It was highly active, and Kendall proposed that it acted as a true catalyst during metabolic reactions (14). In 1918, he announced that the correct nature of this active, crystalline material was hydrotriiodooxyindolepropionic acid. He shortened this to "thyrooxyindole" or "thyroxine"(I)(15).

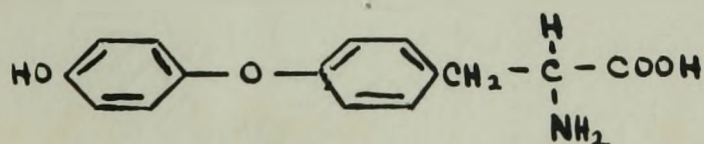


Thyroxine: Its Discovery and Isolation

Kendall's isolation of "thyroxine" required eight years. From two tons of thyroid gland he isolated only 33 g. of the active material. The first reaction to Kendall's announcement seems to have been one of doubt. Swingle (16) fed iodine to thyroidectomized tadpoles and observed their metamorphosis, so concluded that iodine itself was the active principle of the gland. The purpose of the gland, he said, was simply to attract and

store the iodide. Howells (17) claimed the isolation from the thyroid of an iodine-free product which produced the typical thyroid effect in tadpoles. However, in 1919, Kendall (18) published further proof for the action of his thyroxine and claimed that he had synthesized the compound, proving it to be 4, 5, 6, trihydro - 4, 5, 6 - triiodo - 2 keto - 3-indolepropionic acid. (Same compound as above.) This structure was accepted for several years. Due to its similarity to tryptophane, the latter was proposed as the biological precursor for thyroxine. At first the evidence seemed to confirm this. Cramer (19) claimed that a low tryptophane diet caused atrophy of the gland. Hicks (20) examined the spectra of thyroxine, tryptophane and 2 - hydroxyindole - 3 - propionic acid, and reported a definite "family" resemblance. Kendall (21) proposed a theory for the action of thyroxine involving the indole ring of his structure.

In 1926, seven years later, Harington (22) brought forward evidence that the empirical formula for thyroxine should be $C_{15}H_{11}O_4NI_4$ and not $C_{11}H_{10}O_3NI_3$ as announced by Kendall. He criticized many of the methods and interpretations of Kendall and succeeded in synthesizing the p - hydroxyphenylether of tyrosine (thyronine) (II)



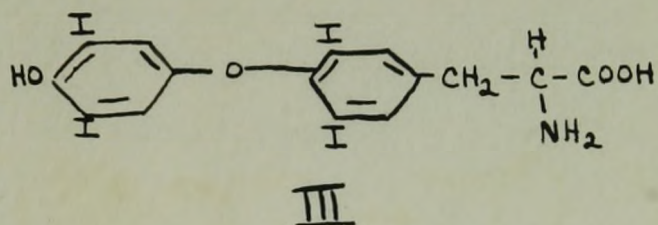
II

This was shown to be completely identical to the material obtained by catalytically deiodinating natural thyroxine. From this fact, he

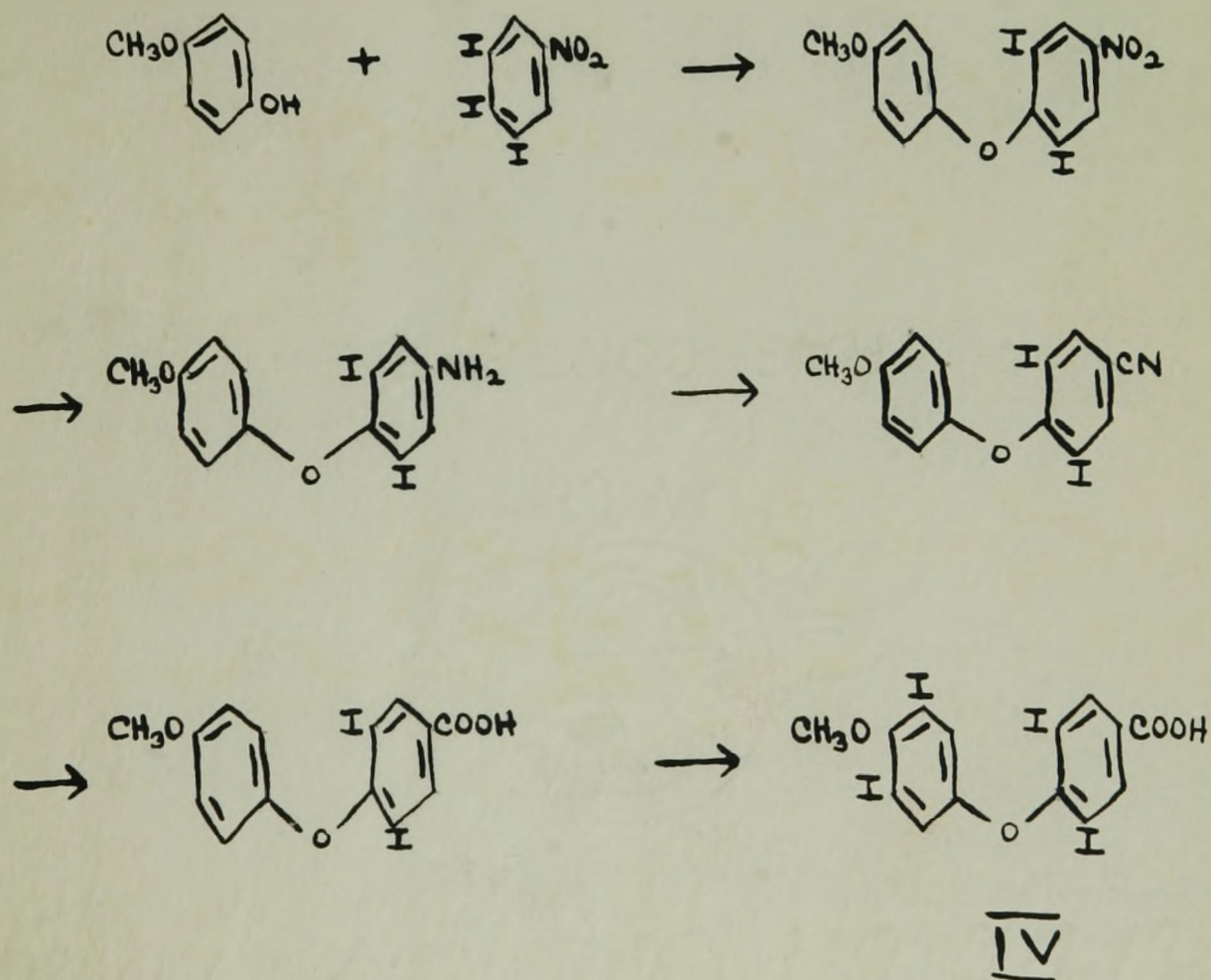
deduced that thyronine formed the "nucleus" of the thyroxine molecule.

The next year, in collaboration with Barger (23), he synthesized thyroxine for the first time, thus proving the positions of the iodine atoms in the molecule. The true structure was

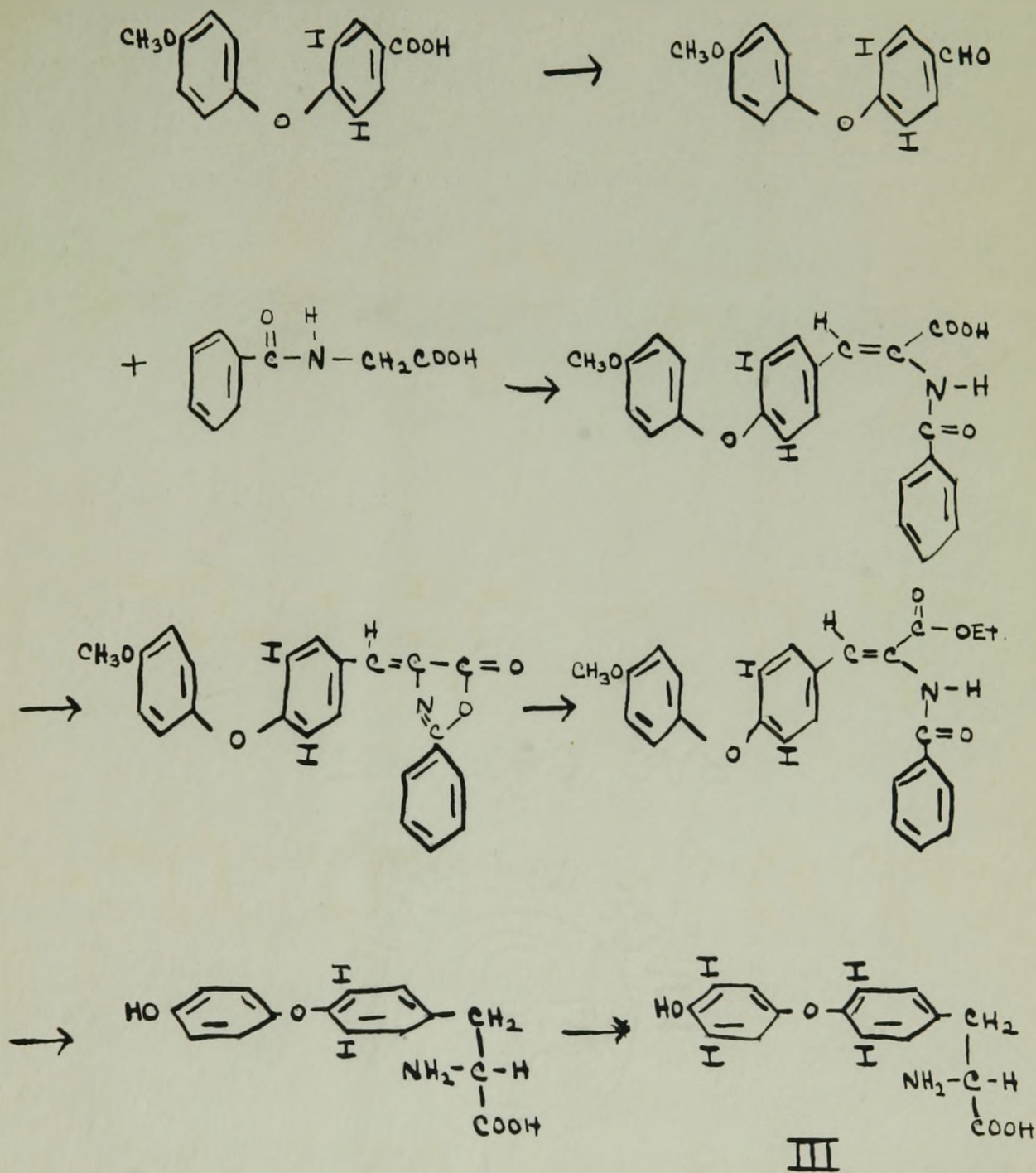
3, 3¹, 5, 5¹ - tetraiodothyronine (III).



His route of synthesis was:



This compound (IV) was found to be identical with the acid obtained by methylation and subsequent oxidation of thyroxine.



The product was racemic thyroxine. This structure for the active compound of the thyroid was also confirmed by synthesis by Baggesgaard Rasmussen (24) in 1928. The naturally occurring form of thyroxine is

L - thyroxine, isolated from the products of proteolytic enzyme digestion of the thyroid protein (25).

The optically active isomers of thyroxine were prepared by resolution of the formyl derivatives of 3, 5 - diiodothyronine, followed by hydrolysis and iodination. At first L - thyroxine was reported to be twice as active physiologically as the racemic product (26,27), but more recent work indicates that D - thyroxine has a biologically active nature in the rat of 0.3 compared to L - thyroxine as 1.0. The ratio of activities was found to be L:DL equal to 1.5:1.0. These last figures are based on the thyroxine requirement needed to prevent pituitary basophil cell changes in rats made thyroxine-deficient with methyl thiouracil, while the former are based on the thyroid-weight changes during thyroxine administration (28, 29).

Thyroglobulin; Other Iodine-containing Materials of the Thyroid Gland

It was recognized during most of the early work on the thyroid that there were other iodine-containing materials in the gland besides thyroxine (30). Interest was shown in the iodinated protein from which thyroxine is obtained. It had become known as thyroglobulin. In 1934, Heidelberger and Svedberg (31) determined the molecular weight of pure thyroglobulin by the sedimentation equilibrium method and by the ultra-centrifuge, announcing the values to be 700,000 and 800,000 respectively. The next year an empirical formula for the protein was given, namely:

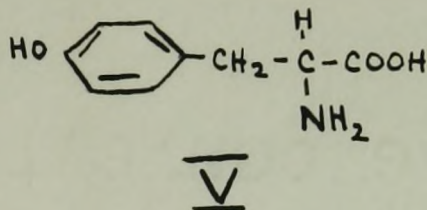
$C_{415}H_{660}O_{134}N_{114}S_2K_2P_3I$ (32). One of the more recent papers on thyroglobulin gives the following values for its iodine content (33).

Iodine 0.52 to 0.73 per cent

Thyroxine iodine 0.155 to 0.242 per cent

Since the announcement of the true structure for thyroxine, opinions changed as to the precursor for this compound. The amino acid tyrosine

(v)



seemed to be the logical starting material. Analysis of the thyroglobulin for non-iodinated amino acids showed approximately 3 to 5 per cent of this material to be present (34,35). Then in 1934, Harington (36), starting with natural L - tyrosine, synthesized optically active thyronine. He compared this to the optically active thyronine prepared by deiodination of natural L - thyroxine and found them to be identical. This stereochemical relationship between natural thyroxine and natural tyrosine supports the hypothesis that tyrosine is the precursor of thyroxine.

Diiodotyrosine

The first indication that this amino acid was present in the gland seems to have been in 1926 when Ingvaldsen and Cameron (37) were devising a color reaction for thyroxine. They found that treatment of this compound with nitrous acid, followed by ammonia, gave a red colour. The colour was also given by diiodotyrosine. Testing extracts of the thyroid for thyroxine they discovered that the red colour persisted, even though

all thyroxine was chemically removed.

DL - diiodotyrosine was isolated from the gland in 1929 by Harington and Randall (38) who stated that this compound constituted all of the acid-soluble iodine present in the gland. In the same year Foster (39) announced that 33 per cent of the total iodine of the gland was diiodotyrosine. After 3,5 - diiodo - DL - tyrosine was isolated from the hydrolysate of proteolytic enzyme action on the thyroid, it was decided that all of the organic iodine in the gland could be accounted for by the two compounds, thyroxine and diiodotyrosine. The losses in recovery were assumed to be due to the procedure used (40).

Over the next few years the reported actions of diiodotyrosine on the thyroid gland itself, and on the basal metabolism of organisms, were many and varied. Some workers reported that the amino acid lowered the basal metabolic rate in hyperthyroid animals, suggesting an antagonism between it and thyroxine (41,42). Others reported that it could maintain life indefinitely in thyroidectomized animals, inhibiting the symptoms of thyroid insufficiency (43,44). The present opinion seems to be that diiodotyrosine exerts very little, if any, action on the basal metabolic rate. It may be very slightly antigoutogenic (45).

That diiodotyrosine and thyroxine made up the total of the organic iodine compounds in the thyroid was well accepted for many years.

3 - Monoidotyrosine

Not until 1948 was it recognized that another iodinated amino acid was present in the thyroid gland. A few years before Ludwig and von Mutzenbecher (46) had isolated monoidotyrosine from artificially iodinated

casein. This same compound was demonstrated to exist in thyroglobulin by Fink and Fink (47) who used paper chromatography and radioactive iodine in their work. They claimed the presence of from one-third to two-thirds as much moniodotyrosine as diiodotyrosine in the gland. The next year Chaikoff et al. (48) confirmed their work by identifying moniodotyrosine on a two-dimensional chromatogram. The specific activity data obtained by him indicated that the new amino acid was the precursor of diiodotyrosine. He also gave good evidence that the moniodotyrosine was not produced by deiodination during the relatively drastic alkaline hydrolysis of the thyroglobulin, for when known quantities of thyroxine or diiodotyrosine were added to rat thyroid before its hydrolysis only 2 to 3 per cent of the added iodine appeared in the moniodotyrosine fraction. Chaikoff (49) gave 15 per cent as the extent to which this new compound was present in the thyroid compared to 30 per cent as diiodotyrosine. No 3, 5 - diiodothyronine could be detected in the gland by his methods.

From this evidence it was proposed that moniodotyrosine is one of the precursors of thyroxine and takes part in the step leading to diiodotyrosine. Roche et al. recently demonstrated that moniodotyrosine is produced as an intermediate in the iodination of free tyrosine to diiodotyrosine in alkaline solutions (50).

The Relationship Between the Thyroid and Pituitary Glands

The activity of the thyroid gland seems to be chiefly under the control of a hormone secreted by the anterior pituitary gland. In 1930 Aron (51) noted that the vesicles of the thyroid became empty and the epithelial cells enlarged when the gland was observed after pituitary extract injection. The next year a group of workers reported that removal of the hypophysis in dogs caused an initial increase in the blood iodine which then gradually fell until, after sixty hours, it was at a subnormal level (52). Soon it was confirmed that extracts from the anterior lobe of the hypophysis did definitely induce hyperactivity of the thyroid (53,54). That the relationship was reciprocal was shown in 1934 by Loeser (55) who found that production of the thyrotropic hormone by the pituitary was decreased by injection of small quantities of iodide, whereas Leblond (56) reported that iodine-deficient rats (with hyperplastic thyroid glands), increased their ability to fix iodide in the thyroid over normal rats due to an excessive release of the thyrotropic factor.

Using radioactive iodide Chaikoff and Taurog (57) showed that after thyrotropic hormone injection there resulted a more rapid removal of circulating iodide by the thyroid gland, a more rapid conversion of this to thyroxine and a more rapid release of active material by the gland into the plasma. Rawson's (58) work also confirmed that there is a delicate balance maintained between the thyroid and anterior pituitary glands whereby excessive production or administration of thyroid hormone may depress production of thyrotropic hormone, while lowered production

of thyroid hormone results in an increased liberation of thyrotropic hormones.

Much of the work done on the isolation of a pure preparation of the hormone of the anterior hypophysis was performed by Collip (59). As yet little of the chemical nature of the material is known except that it is protein in nature.

The action of the thyrotropic hormone on the thyroid gland takes place quickly. De Robertis (60) showed that as early as fifteen minutes after administration of the hormone there is a great increase in the intracellular colloid. This histological change is recognized as one of the most sensitive methods for detecting thyrotropin.

Again, by means of thyrotropin, Borell and Holmgren (61) have shown that the phosphorus balance of the thyroid gland in guinea pigs is markedly affected by thyrotropic hormone injection. The animals were treated for two days with the hormone, followed by injection of phosphorus³² forty minutes before sacrifice. The phosphorus³² atoms of the thyroid increased tenfold above controls, whereas the cell height was doubled on the same dosage. This may indicate that phosphate plays an important role in the synthetic processes leading to thyroid hormone release.

The Nature of Plasma Iodine

For many years little was known about how the hormone of the thyroid was transported, or just what constituted the circulating hormone. The only material associated with the thyroid gland identified in the blood was iodide ion itself; no organic forms of iodine were known. In

1940 Stellar and Olken (62) devised a method for the detection of thyroglobulin down to one part in one hundred and fifty thousand, but none of this protein was found in the blood. However, the first studies performed by Chaikoff with the radioactive tracer, iodine¹³¹, gave the results that an appreciable amount of the labelled plasma iodine was as diiodotyrosine with almost negligible traces as thyroxine (63). Later, when better techniques became available, these results were found to be erroneous.

In 1944, Joliot (64) reported that thyroxine was abundant in the plasma after injection of labelled thyroxine but was present to only a negligible extent in the blood corpuscles. It soon became quite apparent that nearly all of the organic iodine of the plasma was "protein-bound" or at least associated with the plasma proteins (65). Chaikoff (66) observed that when he fed rats on diets which contained 2 μ g. of iodine per day, and gradually increased this to 75 μ g. per day, the protein-associated iodine of the plasma rose also. However, a further increase in the iodine intake failed to produce any significant rise in the level. In the normal animal, this level of protein-bound iodine of plasma appeared to be dependent on the thyroxine content of the gland and to be limited by the gland's capacity to produce thyroxine.

The next year, 1947, Taurog and Chaikoff (67) analyzed the plasma after tracer iodine injection and reported that 90 per cent of the plasma iodine behaved as thyroxine, following the pure carrier through several recrystallizations without loss of specific activity. This protein-bound iodine decreases rapidly after thyroidectomy, whereas thyrotropic hormone injections augmented the plasma levels (68).

In 1948, the same workers presented more evidence that the circulatory hormone of the thyroid is thyroxine loosely bound to protein, α - globulin carrying the highest fraction (69). This iodine of the plasma was almost completely extractable with butyl alcohol, but was not removed by sodium hydroxide from the extract (as diiodotyrosine would be). In addition, crystalline thyroxine added to plasma behaved as though it were bound to protein, being precipitated quantitatively with zinc hydroxide and not being dialyzable. These findings were confirmed by Laidlaw (70) in 1949, who repeated Chaikoff's work using paper chromatography and butanol extractions. He concluded that the circulatory thyroid hormone in the normal animal consists of thyroxine loosely attached to plasma protein. Further work by Chaikoff (71) still further strengthened this conclusion. The only time thyroglobulin is present in the plasma seems to be after an abnormally high administration of radioactive iodine to the gland, when some protein leaks into the blood due to disruption of the secretory mechanism (72).

The Enzyme Systems of the Thyroid Gland

Relatively little is yet known about the enzyme systems of the thyroid gland, which bring about the synthesis of the thyroid hormone from iodide, and which effect the concentration of iodide from that of the plasma. Several postulates have been made, some of which will be discussed later. However, a few of the facts which have been learned about enzymes present in the gland will now be presented.

As early as 1921, Turro (73) reported that it was possible to extract from the thyroid enzymes possessing hydrolytic properties. Milkovitch (74) also found enzymes present that had pronounced hemolytic powers which became apparent only in the presence of free hydroxyl ions. The active substance was thermolabile.

The next year (1924) Herzfeld and Engel (75) isolated extracts of the thyroid which contained lipases stable to quinine. An esterase which was quite active in saponifying acetyl choline was announced in 1930 by Plattner and Hinter (76). It resisted sodium fluoride inhibition.

De Robertis (77), in 1941, described a proteolytic enzyme found in the colloid of the gland which digested gelatin. He suggested that it might be involved in the hydrolysis of colloidal protein and the subsequent reabsorption of products. In 1943, Galli-Mainini noted a direct relationship between the activity of the thyroid and the oxidase content of its tissue (78). This activity, as expected, was increased by thyrotropin and decreased by hypophysectomy. A peroxidase activity was demonstrated in normal rat thyroids by De Robertis and Grasso (1946) (79), further confirmation being given, using color reactions such as Benzidine and hydrogen peroxide and various peroxidase inhibitors (80). This will be discussed more fully in the next section.

Llamas (1947)(81) reported a proteolytic enzyme from the thyroid which acted on gelatin at a pH of 6.5. It was inhibited by cyanides and copper sulfate, but not by p - aminobenzoic acid or those compounds related to urea. However, an enzyme which was strongly inhibited by

thiourea and thiouracil was described by Ambrus in 1949 (82). He named it as a cholinesterase.

Preparations of rabbit thyroid cathepsin contain a nuclease which liberates phosphate from ribose-nucleic acid at pH 5.0 to 5.5 and temperatures of 40 to 50°C. It is inhibited by a 0.008 M. cyanide (83).

As can be seen from these diversified reports, no specific data has been brought forward as to the actual enzyme systems responsible for thyroid hormone synthesis. The present work will supply many techniques required in further studies with tissue homogenates which will have as their aim the elucidation of the pathways of synthesis present in the thyroid gland and the enzyme systems involved. Useful information on the enzyme systems present is also to be found in connection with the thyroid inhibitors, discussed next.

Goitrogenic Compounds

Probably a greater volume of work has been concentrated on this aspect of the thyroid problem than on any other single phase. No doubt this is because of the interest shown in antithyroid drugs for the treatment of patients with abnormal thyroids.

One of the first antithyroid effects to be noted was that of a peculiar property of blood. Blum (84) reported that feeding blood to patients with overactive thyroids neutralized the action of the iodized thyroid proteins and reduced the basal metabolic rate. This preparation was later named "catechin" (85). In 1938, it was

announced by Renki Kin that the antithyroid substance of rabbit and chicken serum was secreted by the pancreas into the venous blood (86). As far as could be found, no further investigation into this material has been done.

Two of the vitamins have effects on the hormone production by the gland. Vitamin A arrests and Vitamin C stimulates the synthesis of thyroxine (87). In fact, Vitamin C has an anti-goitrogenic effect which protects the gland against doses of cyanide. Chickens which synthesize this vitamin are resistant to methyl cyanide (88).

The first antithyroid compounds to be synthesized for treatment of hyperactive patients were α (p - hydroxyphenyl) - α - aminoacetic acids (89). They were not too successful.

It was early recognized that carbon monoxide causes hypertrophy of the thyroid cells (90) (i.e., it is a tissue poison). Schachner, Franklin, and Chaikoff (91) later showed that even in vitro thyroid slices, in the presence of carbon monoxide, cyanide, azide or sulfide, will not synthesize the thyroid hormone. All of these are known to inhibit the heavy metal-containing enzymes (e.g. the cytochrome system), so probably exert their action by inactivating the "oxidative" enzymes of the thyroid gland.

Some of the other compounds of a more general type which have been found to have some inhibitory influence on the basal metabolic rate, or to be goitrogens are:

- (1) 1, 7-Dimethyl xanthine (92)
- (2) 4 - Hydroxy - 3, 5-diodobenzoic acid

- (3) 4 - Benzyloxy - 3, 5 - diiodobenzoic acid
- (4) N - (4¹ - hydroxy - 3¹,5¹ - diiodobenzoyl) -
3, 5 - diiodotyrosine (93)
- (5) Ethers of n - alkyl 3,5 - diiodo - 4 - hydroxybenzoates (94)
- (6) Resorcinol
- (7) 2, 4 - Dihydroxybenzoic acid
- (8) Phoroglucinol
- (9) Hydroxyquinol (95)
- (10) α - Tocopheryl acetate (96)
- (11) Dibromotyrosine (97)
- (12) n - Alkyl 3, 5 - diiodo - 4 - hydroxy benzoates (98)

(This latter group of substances which are rather closely related to thyroxine, structurally, were found to be active inhibitors, especially if esterified with n - butanol.)

However, the majority of the work has been concerned with five main classes of compounds.

Thio Compounds

Kennedy (1952)(99) in an attempt to isolate the goitrogen of rape seed suggested that it was a derivative of urea. Allyl thiourea was found to be active. In 1943, Astwood (100) et al. announced that hyperemia of the thyroid could be caused by thiourea, but could be prevented by hypophysectomy, or by administration of thyroid. Iodide would not counteract its effect. Later, he found that certain allied compounds were

even more effective (101). These included 2 - thiouracil, 2 - thiobarbituric acid and diethyl thiourea (102). At first it was thought, by Lawson (103) that thiouracil blocked the uptake of the iodine by the gland, and hence was goitrogenic. However, this was soon corrected by Chaikoff (104) who showed that when thiouracil was fed daily to rats injected with tracer iodide the uptake of the iodide by the gland was "normal" but synthesis of thyroxine was decreased. He concluded that thiouracil must act by inhibiting one or more of the oxidative enzyme systems concerned with thyroxine synthesis. Glock (105) reported in 1946 that 0.005 M. thiouracil added to slices of thyroid did not inhibit the rate of oxidation of p - phenylene - diamine or ascorbic acid by them and concluded that inhibition was not due to a blocking of cytochrome oxidase activity since that system is required in the above oxidation. Thiouracil also has an indirect action on the thyrotropic activity of the hypophysis. This gland shows increased activity when young male rats were fed 3 mg. of methyl thiouracil daily for ten days (106).

When tracer amounts of iodide¹³¹ were given to rats, it was reported by Albert and Tenney (107) that administration of thiouracil accelerated the thyroid secretion by six times, while injection of thyroxine inhibited it. Sodium iodide had no effect.

Sulfonamides

In 1941, experiments performed by Mackenzie, Mackenzie and McCollum (108) showed that rats which received sulfaguanidine (one to two per cent in their diet) for six to sixteen weeks developed hypertrophied thyroids. In 1943, the same workers (109) showed that many of the sulfonamides were active as goitrogens causing marked thyroid enlargement, hyperemia, colloid reduction and epithelial cell height increase. Affected were rats, mice and dogs, but not chicks or guinea pigs. In order of effectiveness the substances were sulfaguanidine, sulfadiazine, sulfapyridine, sulfamethyldiazine, sulfanilamide and sulfathiazole. Astwood (100) added sulfalilylurea and sulfasuxidine to the list. The same year Franklin and Chalkoff (110) discovered that sulfanilamide at 0.01 to 0.001 M. inhibits the in vitro conversion of tracer iodide to diiodotyrosine and thyroxine.

In 1944, (111,112) they found the same to be true for sulfapyridine, sulfathiazole, and sulfaguanidine at 10^{-3} M. However, in their next report they showed that the sulfonamides and thioureas have no effect on the oxygen consumption of thyroid tissue slices so must interfere with some other system than a cytochrome (113). This is in contrast to the cyanide, azide group which do depress oxygen consumption and are known to be inhibitors of cytochrome-like systems.

p - Aminobenzoic Acid

This compound was first reported to be inactive as an antithyroid material (109), but after further investigation it was found to be goitrogenic, by Astwood (102) in 1943. In in vitro studies by Chaikoff, it was inhibitory at 10^{-3} M, as was p - aminophenylacetic acid (112). Like thiourea and the sulfonamides it had no effect on the oxygen consumption by thyroid slices (113). Also like these materials, it did not inhibit the uptake of iodide by the gland; only the conversion of the tracer to an ~~in~~organic form.

Thiocyanate

This has proved to be the only inhibitor of the thyroid hormone synthesis scheme whose locus of action is known. As with some of the other inhibitors, the first reports about this material were erroneous. Mackenzie (109) stated that it was ineffective as an antithyroid material in 1943, while in 1944 Rawson (103) recognized it as an inhibitor but concluded that it acted upon the hormone-forming mechanism, but not with iodide uptake. Both these ideas were soon shown to be incorrect.

Chaikoff (112), in his in vitro studies, showed that thiocyanate at 10^{-3} M. inhibited conversion by depressing the ability of the gland to concentrate the tracer iodide present in the medium. This was confirmed by Vander Laan and Bissel (114) in 1946, and by Stanley and Astwood (115) in 1948. The latter showed that after practically complete inhibition of

thyroid hormone synthesis by mercaptoimidazole, the human thyroid could still collect appreciable quantities of iodide. However, the iodide was discharged by feeding sodium thiocyanate. The concentration of inorganic iodide in a normal thyroid gland is at least 500 times greater than its concentration in plasma (170).

Iodide Ion

Strangely enough, under certain circumstances iodide itself acts as a depressor on thyroid function. Perhaps the first indication that this was the case occurred in 1934 when Shimasaki found that rats kept on a basic diet with added potassium iodide decreased in weight and showed hypofunction of the thyroid (116). However, it was not until 1944, when Morton, Chaikoff and Rosenfeld (117) performed an in vitro experiment with excess iodide added to the medium that any definite evidence was obtained. They reported that inhibition occurred with an iodide level between 10 and 50 μg . Later, Wolff and Chaikoff (118,119) found that far more diiodotyrosine was synthesized by a normal rat thyroid when 5 to 10 μg . of iodide was injected than when ten or twenty times that amount was used.

We see, then, that the action of antithyroid compounds indicates that there are at least two separate "mechanisms" at work in the thyroid gland. One of them is concerned with the synthesis of the thyroid hormone from inorganic iodide

and is inhibited by a wide range of seemingly unrelated materials including the thioureas, sulfonamides, p - aminobenzoic acid and others.

The second "mechanism" is operating to trap iodide in the thyroid gland so that a "high" concentration of the ion may be available for hormone synthesis. Thiocyanate holds the unique role of being an inhibitor of this mechanism.

Proposed Mechanisms of Inhibition (120)

Of the large group of inhibitors which prevent synthesis of organic iodine compounds, but do not interfere with the concentration of iodide, carbon monoxide, azides, and sulfides almost certainly exert their action on some enzyme system having chemical properties closely related to the cytochrome systems (91). However, very few, if any, of the other inhibitors act upon this system for, in their presence, the uptake of oxygen by the thyroid tissue is not inhibited (113).

In 1945, Taurog, Chaikoff and Franklin (121) attempted to find some correlation between the structure of the remaining inhibitors and their mode of action. On this basis they discovered it to be very difficult to predict accurately whether a substance would be active as an anti-thyroid compound or not. Free amino or hydroxyl groups seemed to favour inhibition; if the amino group were blocked by acetylation, the inhibition was reduced. The structures of the sulfonamides, sulfonic acids and carboxylic acids appeared to be wholly unrelated to their activity. These workers suggested that there is, however, a correlation between

the ease of oxidation and the inhibitory activity.

Substances such as thiourea may keep the oxidation potential of the gland at such a level that the oxidation required to convert inorganic iodide to organically bound iodine is impossible (122). Pitt-Rivers (123) in 1950 agreed with this view and stated that the experimental evidence is strongly in favour of the hypothesis that drugs of the thiocarbamide type exert a direct chemical reducing action which inhibits the formation of diiodotyrosine and possibly thyroxine. Further evidence for this view was presented by Cheymol et al. (124) in 1951. The addition of mobile hydrogen atoms (on nitrogen) augments the antithyroid action of dithiocarbamates, whereas dithioureas are inactive, whether they are linked through sulfur-sulfur, or nitrogen-nitrogen bonds.

Some work on the action of resorcinol compounds appeared in the literature recently (125). The resorcinols interfere with the organic binding of iodide without lowering the concentration of inorganic iodide in the gland. No correlation was found between the reducing power of these compounds and their action, as was postulated by Pitt-Rivers for the thiocarbamides. However, resorcinols are known to inhibit the peroxidase activity of milk so it is quite likely that they act similarly in the thyroid gland. Since the second major step in hormone synthesis involves the oxidation of iodide, it is probable that some peroxidase-like enzyme system is responsible for this conversion.

The coupling of diiodotyrosyl groups to form thyroxyl radicals

also requires an oxidation but just how this is brought about is unknown. Thus far, no specific inhibitor for this conversion has been discovered.

We may conclude, therefore, that much is yet to be learned about antithyroid compounds and the enzyme systems upon which they act.

Compounds with Thyroxine-like Activity; Proposed Mechanisms of Hormone Synthesis

Ever since it was discovered that thyroxine exerted an influence on the metabolic rate of animals, workers have been preparing synthetic thyroxine and related compounds and testing them for similar activity.

As far back as 1908, one worker (126) discovered the product he obtained by the iodination of blood proteins acted as an artificial "iodothylin" but no information as to the structure of this substance was obtained. Several years later, in 1936, Salter and Pearson (127), starting with a diiodotyrosine peptone, obtained from the enzymatic hydrolysis of thyroglobulin, reversed the original digestion (i.e., a peptic synthesis) and isolated an iodine-containing protein whose chemical properties resembled natural thyroid protein and which was active against myxedema. Similar work has been done by Abelin (128,129).

Harrington and Pitt-Rivers (130), in 1939, were successful in isolating a small amount of thyroxine from artificially iodinated casein. In the same year von Mutzenbecker (131) discovered that if he incubated diiodotyrosine at pH 8.8 (0.1 N. sodium hydroxide solution) for fourteen days he obtained a measureable yield of thyroxine. This was successfully repeated by Block (132) who used an incubation

temperature of 37°C. Based on this conversion, Johnson and Tewkesbury (133) suggested a reaction mechanism for the natural in vivo conversion of diiodotyrosine to thyroxine.

Not only was thyroxine isolated from the artificially iodinated proteins, but also moniodotyrosine and diiodotyrosine (134), suggesting that a definite similarity exists between this process and the one occurring in the gland.

Of the structures related to thyroxine, one of the first to be synthesized was an isomer of thyroxine, namely, DL - 3,5, - diiodo - 4 - (2¹,4¹ - diiodo - 3¹ - hydroxyphenoxy) phenyl alanine. The workers (135) found it to be inactive and suggested that the reason was because this isomer is unable to assume a quinoid structure. Another inactive isomer of thyroxine was prepared the same year (1941) by Niemann and Mead (136), namely, DL - 3, 5 - diiodo - 4 - (3¹,5¹ - diiodo - 2 - hydroxyphenoxy) phenyl alanine. Like the former, it cannot form a quinoid structure.

In 1949, Leblond and Grad (137) prepared a number of chloro and bromo-thyronine derivatives. They fed them to experimental animals and measured the per cent increase in oxygen consumption over controls. They found the following order of activity (all less than thyroxine):

3,5-dibromo - 3¹,5¹-diiodo > 3,5-diiodo - 3¹,5¹-dibromo > 3,5 - diiodo - 3¹,5¹-dichloro > tetrabromo > 3,5 - diiodo > 3,5-dichloro - 3¹,5¹ - diiodo > 3,5-dichloro.

Several reaction mechanisms have been proposed and discussed for the synthesis of thyroxine from inorganic iodide in the thyroid gland. Nearly all involve the general scheme: tyrosine → moniodotyrosine → diiodotyrosine → thyroxine.

Harrington (138) in the Pedler Lecture of 1944 outlined some of the more reasonable suggestions which have been given for such a mechanism, and proposed intermediate stages for the last step.

His theoretical reasoning not only demonstrated a possible mechanism for the oxidative coupling of two molecules of diiodotyrosine to thyroxine but suggested that such a reaction is rendered more likely by the presence of iodine atoms in the former compound, which inhibit the possible formation of a diphenyl.

Little conclusive proof has been presented for the presence of the intermediates or side-products of such a scheme (alanine, pyruvic acid or serine) although Pitt-Rivers (139) claimed the identification of alanine as a reaction product in the artificial synthesis of thyroxine from diiodotyrosine (1948).

Techniques Being Used at Present in the Thyroid Field

Up until the last few years workers in the thyroid field, as in other aspects of biochemistry, have been limited by the techniques available for studying the chemistry of biological systems. Often classical methods of analysis were not sensitive enough to detect the small differences in concentrations etc., occurring during the processes. Also, working with whole animals limited the scope of research that could be carried out. Three methods of study now being used for thyroid work will be discussed. They have aided greatly to the knowledge of the biochemistry of this gland.

Tracer Work with Radioactive Iodine

In 1940, Hertz et al. (140) used iodine¹³¹ with a half life of slightly over eight days as an indicator of iodide uptake by the thyroid gland. They found that the percentage collection of tracer by the gland rose to a maximum in ten minutes and that the normal thyroid could collect at least eighty times the amount of iodide expected on the basis of simple diffusion. They suggested the use of this material for the internal radiation of diseased glands.

Other workers quickly adopted the use of this valuable tracer for thyroid studies. In 1941, Perlman, Chaikoff and Morton (141) announced that as much as 65 per cent of the radioactive iodide injected into rats became incorporated into the thyroid within 48 hours. Using isolation with carrier, they determined that up to 16 per cent of this was in thyroxine, and up to 32 per cent in diiodotyrosine, the ratio between the two remaining fairly constant. The same workers began the study of plasma iodine that year with this tracer.

In 1947, Taurog and Chaikoff (143) gave good evidence based on specific activities that diiodotyrosine was the precursor of thyroxine. They noted that very large amounts of isotope activity could prove harmful to the synthetic processes of the gland, but that small tracer amounts would not disturb its normal functioning (144). Radioactive iodide

(47) was also instrumental to the discovery of monoiodotyrosine in the gland. Thyroid inhibitors could be followed much more readily with tracer than without (145). In fact, little specific knowledge of their action was known before this technique became available.

Paper Partition Chromatography

Martin and his co-workers (146) extended the technique of partition chromatography of amino acids to the micro scale by using filter paper instead of silica gel as the "column". This method of detection, separation and analysis has proved to be very useful in carrying out studies on the amino acids present in the thyroid gland. Once the tracer, iodine¹³¹, was available, the two could be used together. The first workers to utilize this combination were Fink, Dent, and Fink (147) in 1947. They injected rats with 250 μ c. of iodide¹³¹ and after twenty-four hours analyzed extracts of basic hydrolysates of their thyroid glands. Two-dimensional chromatography was used and radioautographs prepared. Thyroxine and diiodotyrosine spots were obtained as well as several unknown ones. Later, they showed that one of these corresponded to monoiodotyrosine (47). Soon a large number of workers in the thyroid field were also making use of paper chromatography. They included Fishkoff et al. (148) and Taurog, Tong and Chaikoff (149)

who confirmed the findings of Fink in part, but modified the procedures so that better results were obtained.

Several improvements to paper chromatography in general have been presented by different workers during the last few years. They include new solvents (150,151) and the use of buffered filter paper (152,153). The author has adapted this use of buffers to the thyroid field; this will be discussed in the experimental section of this thesis.

The two types of solvents which have been most frequently used with thyroid extract chromatography have been collidine: water, and butanol: acetic acid: water of various proportions. The latter was utilized by Roche (154) for the quantitative analysis of iodinated amino acids (155). In most cases, however, the results obtained were not completely satisfactory.

Synthesis of the Thyroid Hormone In Vitro by Surviving Tissue Slices

In 1915, Marine and Feiss (156) found that surviving thyroid glands perfused with potassium iodide would absorb and retain the iodide. This was one of the earliest experiments which showed

that thyroid cells exhibit a specific affinity for iodide (157). Similar work was reported by Rabinowitch (158) in 1925.

However, it was not until 1942 that Morton and Chaikoff (159) showed that thyroid slices incubated in a physiological buffer solution containing tracer iodide¹³¹ would actively absorb from 80 to 90 per cent of the tracer and synthesize diiodotyrosine and thyroxine from it.

The next year they repeated these experiments and found that rat, dog and sheep thyroid slices were all capable of this in vitro synthesis (160). Rat thyroid slices were especially active; with them, as much as 12 per cent of the added tracer was isolated as thyroxine, and up to 70 per cent as diiodotyrosine. Tissue slices were more active than minces, which in turn were capable of more synthesis than ground glands. Homogenized thyroids were incapable of synthesis. The authors suggested that this indicates the participation of an intracellular enzyme system. The thyroid inhibitors were found to work equally well in these in vitro studies as with whole animals (161). The conditions could be controlled much more easily in the former case (162).

These three techniques, either alone or combined, form the most

effective, available methods for further thyroid studies; they have all been used in the present work.

The Influence of Age, Sex and Environment
on the Thyroid Activity of Animals

As has been found with other endocrine glands, certain differences occur in thyroid activity from one individual to another. Several papers have been published on the research carried out to determine whether these differences were due to the age, sex or environment of the animal. As nearly "normal" subjects as possible were used as the basis of observation.

In 1912, Morgenstern (7) while analyzing the thyroids from human subjects, found that women have a higher percentage of all inorganic constituents, except chloride, than men.

Seidell and Fenger (8) reported a seasonal variation of iodine in normal glands. From June to November there is approximately three times as much iodine in the gland as from December to May. Fenger (9) stated that female animals contain more thyroid tissue and more iodine in thyroid combination per unit of body weight than do male animals. However, no difference was seen between pregnant and non-pregnant females.

The thyroid gland is also known to undergo hyperplastic changes during menstruation, pregnancy and lactation (163). This suggests some correlation between the sex glands (female) and the thyroid.

That the nature of the gland changes with the age of the animal was shown by Hertzler (164) who reported that in the young adult the

thyroid colloid is acidophilic; in senility, it changes to basophilic. It is also well known that the basal metabolic rate of an animal decreases as it ages, suggesting changes in the character of the gland.

Doy et al. (165) made an analysis of several of the thyroids from slaughtered animals and found that the female thyroids were, on the average, heavier than the male. However, these results were based on relatively large animals such as the sheep and the cow. Since the present work was carried out using albino rats the author was interested in the thyroid glands of these animals. According to Remington, Remington and Welch (166) the weight of the normal rat thyroid, in full-grown rats, was 7.5 mg. per 100 g. of body weight, and for both sexes. They reported no significant variations attributable to age or sex. Nevertheless, we have found definitely detectable differences in activity of glands from the two sexes, even though the weight and size of the thyroids are approximately equal. This is reported in the present thesis.

Recent Advances in Thyroid Biochemistry

A few of the more recent advances made in the thyroid field will now be discussed briefly.

Analysis of Extracts of Unhydrolyzed Thyroid Tissue

All of the early work with the thyroid gland was concerned with an analysis of hydrolysates of thyroglobulin. No one had attempted to determine what, if any, free iodine-containing amino acids were present in the gland.

In 1950, Gross, Leblond, Franklin and Quastel (167) chromatographed aliquots of a butanol extract from unhydrolyzed rat glands to which tracer iodide¹³¹ had been administered. They showed the presence of free moniodotyrosine diiodotyrosine and thyroxine, as well as three radioactive unknown compounds.

Similar studies were done the next year by Chaikoff (168) who homogenized the thyroid glands from rats with 0.9 per cent saline and then chromatographed butanol extracts. He concluded that free diiodotyrosine and thyroxine each constituted about 0.5 per cent of the total iodine of the gland but that free moniodotyrosine was absent or only present in an extremely small amount. The free thyroxine was about one hundred times more concentrated in the gland than in the plasma. The results also indicated that iodination of tyrosine may occur within the protein molecule since the thyroglobulin had a higher specific activity than the free thyroxine.

The same conclusion had been reached by Leblond (169) who reported the order of specific activity of thyroxine to be maximal in thyroglobulin hydrolysate, intermediate in butanol extract of

the thyroid (i.e., free thyroxine) and lowest in plasma thyroxine. This is in agreement with the hypothesis that thyroxine originates in thyroglobulin, is freed in the thyroid and then is released into the circulation as thyroid hormone.

Chaikoff (170) also determined that the free inorganic iodide of the gland comprised 1 per cent of the total iodine present in the thyroid, a concentration at least 500 times that of inorganic iodide in the plasma.

Evidence for the Breakdown of Iodine Compounds in the Thyroid Gland

As early as 1934, it was reported that diiodotyrosine could be easily broken down in the body with the liberation of iodide (171). This was confirmed by Gross and Leblond (172,173) who injected radioactive iodine-containing amino acids into rats and found after two hours that part of the activity was then in the form of inorganic iodide.

However, not until 1951 was there evidence that this had anything to do with the thyroid gland itself. Then Roche et al. (174) demonstrated that slices of dog and sheep thyroids in isotonic media would split off some iodine

from small amounts of added moniodotyrosine and diiodotyrosine. The iodide split off was probably reincorporated into the tyrosine of the protein (175). Just how this mechanism is utilized in the normal gland has not been determined. This work has been confirmed in the present thesis, independently of the above workers.

Triiodothyronine

Gross and Pitt-Rivers (176) in 1951 demonstrated that thyroxine, iodide and two unknown iodine-containing materials were present in the plasma of patients who had received up to 100 *mc.* of isotopic iodine. No moniodotyrosine or diiodotyrosine were present. The next year these same workers (177) reported that one of the unknown compounds was identical to 3, 5, 3¹ - triiodo - L - thyronine. Synthetic triiodothyronine was assayed by determining its effect in preventing goiter in rats treated with thiouracil. Its activity was three to four times that of L - thyroxine (178). This suggests that it may be the true hormone of the gland, instead of thyroxine.

Moniodohistidine and diiodothyronine have also been detected in a butanol extract of

thyroglobulin, but their importance is not yet known (179).

One other reference to some recent research is of interest; thyroxine has been identified in gorgonins by Roche, Yagi, Michel and Lissitzky, (180), indicating that it may be even more universal in its occurrence than expected.

EXPERIMENTAL WORK AND DISCUSSION

Special Reagents and Materials

The technique of paper partition chromatography, used to a large extent in this present work, requires the availability of a number of pure materials to act as reference substances or carriers for the compounds being identified by this method. In addition thyroid inhibiting materials, chromatography solvents and a few other rather special chemicals were needed. Some of these reagents were available commercially or from other sources, but a few were synthesized in this laboratory.

The most important of this latter group was 3 - monoiodotyrosine which is one of the postulated intermediates in thyroid hormone biosynthesis. Certain details in the synthesis have been modified from those presented in the literature. These have been included below. Otherwise the procedure used followed that given in the references noted with each material. If it was felt that further improvement in the methods involved was still possible, some suggestion of this is also presented here.

Reagents Synthesized for Use in this Work

L - 3 - nitrotyrosine (181)

m.p. (in closed tube with fast heating) 222 - 224°C. corr.

(decomposition occurred)

When synthesizing this material it was found necessary to evaporate slightly, under diminished pressure, the neutralized solution of L - 3 - nitrotyrosine nitrate before the product would begin to crystallize.

L - 3 - aminotyrosine (181)

m.p. (heated rapidly in a sealed tube) 286 - 289°C. corr.

(with decomposition)

An improvement in the final yield of 3 - monoiodotyrosine would probably result if, instead of using "crude" 3 - nitrotyrosine as the starting material for this synthesis (as directed in the literature), a purified material were utilized. Unless this is done, a considerable amount of an unidentified sideproduct, probably 3, 5 - diaminotyrosine, is also produced. However, it may be separated from the desired material by making use of its small solubility in water. The combined precipitates brought down by neutralizing the acidic filtrates are extracted with several volumes of boiling water. The monoaminotyrosine dissolves and may be removed by filtration from the impurity. The final crop of L - 3 aminotyrosine is then obtained by evaporating the filtrate under diminished pressure until crystallization commences.

L - 3 - monoiodotyrosine (182)

m.p. 207 - 210^o C. corr.

(with decomposition)

Since copper bronze powder was not available, copper powder was substituted as the catalyst during the replacement reaction. It gave satisfactory results.

If a solution of the product, 3 - monoiodotyrosine, is kept at an elevated temperature for even a few minutes, some decomposition occurs, marked by the darkening of the solution due to liberation of elemental iodine. Therefore, any recrystallizations must be carried out rapidly in order to obtain a good yield of pure product. The final product was shown to be a single material by chromatography.

4 - amino - 3 - iodobenzene sulfonamide

(3 - iodosulfanilamide) (183)

m.p. 182 - 183^o C. corr.

The product was recrystallized from 30 per cent alcohol. It ran as a single spot when chromatographed on Whatman number one paper in butanol: dioxane (4:1) saturated with 2 N. ammonium hydroxide.

Reagents Obtained from Other Sources

- (1) 3,5 - diiodo - L - tyrosine. $2H_2O$ -
Eastman Kodak Co.
- (2) DL - thyroxine - Hoffman La Roche Inc.
- (3) 3,3¹,5 - triiodo - L - thyronine -
J. Gross and R. Pitt - Rivers (178)
- (4) 3 - fluoro - DL - tyrosine -
C. Niemann (2)
- (5) 3 - fluoro - 5 - iodo - DL - tyrosine -
C. Niemann (2)

Note: These five amino acids were chromatographed on filter paper strips and all found to be single compounds

- (6) n - butanol
b.p. 117° - $118^{\circ}C$.
- British Drug Houses
- (7) Pyridine
b.p. 115° - $116^{\circ}C$.
- Matheson Co. Inc.
- (8) Ethylene glycol monomethyl ether
(methyl cellosolve)
b.p. 124° - $125^{\circ}C$.
- Fisher Scientific Co.

(9) 2,4,6 - Collidine

b.p. 171° - 172° C.

- Matheson Co. Inc.

(10) 1, 4 - dioxane

b.p. 101° - 102° C.

- Eastman Kodak Co.

Note: These five solvents used in chromatography were all redistilled shortly before they were to be utilized.

(11) Sulfanilamide

m.p. 163 - 165° C. corr.

(recrystallized from water)

(12) p - Aminobenzoic acid

- General Biochemicals Incorporated

(13) Ninhydrin

- Brickman and Co.

(14) Soluble starch reagent

- Baker and Adamson Co.

Partition Chromatography on Filter Paper Strips

Introduction

When Consden, Gordon and Martin (146) introduced the technique of partition chromatography of amino acids on filter paper strips in 1944, several workers in the field of thyroid biochemistry (147,148,149) began to make use of it as a method for separating and identifying amino acids of the thyroid gland. When this present work was begun it soon became clear that a number of improvements in the technique would be necessary before it could be utilized with the assurance that the results obtained were reliable and reproducible. A survey of the more recent literature revealed that other workers were being limited by this method as it then was being used, so it was considered profitable to devote some time to the development of an improved and dependable chromatographic technique suited to the particular compounds which would be studied by this method.

This involved a selection of the most suitable grade of filter paper, the choice of a solvent mixture which would bring about the most complete separation of the iodine-containing amino acids of the thyroid gland, and the introduction of several auxiliary procedures, such as buffering the filter paper strips devised to eliminate some of the difficulties connected with partition chromatography. These are discussed in the following sections.

General Experimental Procedure

Preparation and Development of Chromatograms

A solution of the materials to be separated by chromatography was applied from a syringe-controlled micropipette in 50 μ l aliquots to filter paper strips, 8 cm. wide by 56 cm. long. The application was made as a 5 cm. line between two faint pencil marks which had been placed on the strips, 2.5 cm. from the bottom edge. In this manner, a total of 100 μ l. to 250 μ l. of the solution was added to the paper, each application being allowed to dry before the next one was made.

To hasten the process of solvent evaporation, a stream of hot air was used. Excess or prolonged heating was avoided to prevent decomposition of the materials (184).

An alternative method of applying the solution was used in later experiments: the material was added dropwise to a small circular area, approximately 1 cm. in diameter, marked near the bottom edge of the filter paper strip. Less than one-half the above amount of solution was required for this method. It was found that the smaller the area involved in spotting, the more distinct would be the resulting chromatogram.

The two types of container used for developing the chromatograms in these experiments were:

(1) A large insulated cabinet ("chromatocab") so arranged that up to thirty separate filter paper strips could be run at one time, the solvent being placed in glass trays on the bottom. The ascending technique of chromatography was always used. The strips were hung

in the cabinet from glass rods so that their bottom edges dipped evenly below the surface of the solvent. In this cabinet a constant temperature was assured. However, since the volume of the cabinet was relatively large, the time required to attain an atmosphere saturated with solvent was long. The equipment was, therefore, most useful for routine experiments in which the same solvent mixture would be in use for some time.

(2) Tall cylindrical pyrex vessels (46 cm. high by 15 cm. in diameter.) In this case, a filter paper sheet formed into a cylinder and clipped together, top and bottom, was used instead of the strips described above (185). This paper cylinder was lowered into the glass apparatus, in the bottom of which there was approximately one inch of solvent. With this type of vessel, the "spot" technique of solvent application to the filter paper, described above, was always used. These glass containers being small and hence easily prepared with an atmosphere saturated with solvent vapor were found to be of most use when a variety of solvent mixtures were being tested, necessitating frequent changes in the contents of the apparatus.

The time required to develop the chromatograms in the solvents described below varied slightly but was usually in the vicinity of sixteen hours. At the end of this time the strips or sheets were removed from the containers and the solvent fronts immediately marked. The chromatograms were dried at room temperature in a fume hood, heating being avoided since it has been found to cause considerable decomposition of the amino acids (188).

The color produced by the second method takes longer to become visible, but is reasonably permanent. In addition the chromatogram is less discolored as a whole. In either case, the spots were outlined with a pencil on the first day so that in future one could easily see where the compounds were situated.

The ninhydrin colors produced by this treatment are:

Moniodotyrosine - mauve with a pink cast

Diiodotyrosine - deep purple

Thyroxine - brownish yellow, changing to light purple on standing

In many experiments inorganic iodide was separated from the amino acids by chromatography. Its position on the filter paper was located by spraying the chromatogram with a dilute freshly prepared aqueous starch solution. When this dried a 10 per cent acetic acid solution which was approximately 0.1 per cent with respect to nitrite was applied. The nitrous acid oxidized the iodide to molecular iodine on contact so that the characteristic blue-purple color was produced instantly between iodine and starch. This color fades to a pale brown in a few hours so the spot was outlined as soon as the paper dried.

Control Solutions

All preliminary investigation into the techniques of chromatography, as well as the work with radioactive iodine, demanded the use of certain solutions of the pure, non-radioactive compounds being studied. These materials were utilized to determine the location on chromatograms of the iodine-containing amino acids of the thyroid, under the varying

conditions imposed by employing different filter papers, and chromatography solvents. Therefore, solutions of the amino acids, moniodotyrosine, diiodotyrosine and thyroxine were prepared in butanol to give a concentration of 50 μg . per 100 μl . of solution. All solutions were acidified to pH 3 with 6 N. hydrochloric acid. 50 μl . of such a solution when dried as a single spot on filter paper or 150 μl . when dried as a 5 cm. line (see above) gave a good color reaction when treated with ninhydrin (150).

A control solution of inorganic iodide was prepared by dissolving potassium iodide in water to give a final concentration of 10 μg . per 10 μl . 10 μl . of this solution was sufficient to give a distinct color reaction with nitrous acid and starch.

Experimental Results

(1) Selection of Filter Paper

Six different grades and qualities of Whatman filter paper were available for trial, so these were tested under a variety of conditions.

The papers used were:

Whatman No.1	-	medium ash, coarse porosity
" " 4	-	coarse porosity, loose texture
" " 40	-	ash free, double acid washed
" " 41	-	ash free, coarse porosity, soft and double acid washed
" " 42	-	ash free, double acid washed
" " 3 M K	-	fine porosity and close texture medium ash, very thick and strong

In analyzing the results it was decided that in order to accept a paper as being entirely suitable for the chromatographic separation of the amino acids being studied, it should have all of the following desirable properties:

- (1) It should tend not to curl up during the time it was hanging in the chromatograph cabinet, allowing the end of the strip to slip out of the solvent tray.
- (2) The paper fibers should be uniformly distributed throughout the strip so that the ascending amino acids pass up a column of constant composition.
- (3) The developed chromatogram should have an even, straight solvent front.
- (4) The amino-acid bands or spots on the developed chromatogram should be narrow and distinct.
- (5) The R_f values of the amino-acids should be reasonably reproducible.
- (6) The rate of solvent movement up the chromatogram should be fast enough so that complete separation of the amino acids is obtained by an over-night development.

The results for the various filter papers are presented in the following table.

TABLE I
Chromatographic Properties of Whatman Filter Papers

Paper (Whatman)	Property					
	No tendency to curl	Uniform composition	Straight solvent front	Narrow bands	Reproduc- ible R_f	Satisfactory solvent rate
1	++	+	+	+	+	++
4	++	+	+	--	-	-
40	--	-	+	+	+	++
41	--	-	+	+	+	--
42	-	-	++	+	++	++
3 MM	++	+	-	--	-	+

Legend: Very good results ++

Good results +

Poor results -

Very poor results --

Thus the papers listed in order of their usefulness are:

1 42 40 4 and 3 MM 41

Since No.1 paper was the only one which had all six of the suitable properties, it was used in most of the subsequent experiments, although occasionally No.42 paper was utilized for a special purpose.

(2) Selection of Solvent Mixture

A relatively large selection of different solvent mixtures have been tested in the attempt to find one which would give good clear separation of the four substances, moniodotyrosine, diiodotyrosine, thyroxine and inorganic iodide. Very little information regarding the separation of

these particular materials could be located in the general literature on chromatography since very rarely were any of the compounds in question discussed.

Hence it was decided first to determine the usefulness of the solvents being utilized by other workers in the thyroid field, and then modify these, if necessary, or supplement them with a few original mixtures until one with the properties desired was located.

In the table below are listed the solvent mixtures which have been compared in order to select the one having the most desirable properties. The " R_f values" refer to the ratio of the distance which the amino acid has moved from the origin to the distance the solvent front has moved during the development of the chromatograms. All the values included are the average obtained for the total number of experiments performed in that particular solvent mixture. It should be noted that all the chromatograms were prepared by the one-dimensional, ascending technique.

TABLE II
Chromatographic Solvent Mixtures

Solvent Constituents	Propor- tions	Ref- erence	M	R _F Values			Comments
				D	T	I	
(1)Collidine: water (ammonia atmosphere)	(a) 125:44	49	0.40	0.17	0.60	0.75	Collidine was difficult to obtain and keep in a pure condition. It decomposed readily to give a mixture which discolored the paper and produced streaking and diffuse bands. It had an unpleasant clinging odor
(2)Methyl cellosolve: water	(a) 90:10	151	0.68	0.63	0.75	0.83	This solvent did not discolor the filter paper, as did collidine. However, the R _F values were too close together for reliable separation and identification of the compounds. An ammonia atmosphere did not prevent the streaking of one band into the one above it.
(3)Collidine: Methyl cellosolve: water (ammonia atmosphere)	(a) 137:126: 62		0.49	0.29	0.67	0.80	This solvent was devised in an attempt to combine the desirable properties of solvents (1) and (2). In some respects this result was achieved, i.e., there was a good separation of the materials. However, the mixture decomposed even more rapidly than collidine: water alone giving discolored chromatograms and bad streaking after being used only a few times.

TABLE II (continued)

Solvent Constituents	Propor- tions	Ref- erence	R _F Values				Comments
			M	D	T	I	
(4) Ethanol: water	(a) 77:23	187	0.60	0.45	0.75	0.55	This solvent gave colorless chromatograms but overlapping was a drawback to its use, especially the streaking between iodide and monoiodotyrosine
(5) Butanol: acetic acid: water	(a) 74:19:51	188	0.70	0.80	0.97	0.40	All the R _F values, except iodide's, were near the top of the chromatogram. Hence there was considerable overlapping between them, as well as interference between the solvent front band and thyroxine.
	(b) 68:5:27	154	0.40	0.56	0.90	0.30	This was one of the most satisfactory solvents tested, since it did not discolor the paper, and also gave good separation of the compounds in question. However, thyroxine often ran as a fairly wide band close to the solvent front, and was occasionally indistinguishable from it. Iodide and monoiodotyrosine also ran close enough together to give trouble in some cases, especially on the radioautographs.

TABLE II (continued)

Solvent Constituents	Proportions	Ref- erence	R_F Values				Comments
			M	D	T	I	
	(c) 68:2:27 (upper phase)		0.41	0.54	0.85	0.30	By cutting down the acid content of the solvent mixture, the R_F of thyroxine was decreased so that it no longer was interfering with the solvent front band. There was good separation of the amino acids and iodide.
(6) Pyridine: ethanol: water	(a) 5:3:2		0.48	0.60	0.70	0.75	Overlapping between thyroxine and iodide cut down the usefulness of this solvent. However, pyridine, although it still has an objectionable odor, gives a much cleaner chromatogram than collidine.
	(b) 3:1:1		0.60	0.70	0.80	0.78	There was almost complete overlapping between thyroxine and iodide while the R_F values for the other two amino acids were not as good as above either.
	(c) 2:2:1		0.55	0.65	0.75	0.77	Again bad overlapping between iodide and thyroxine prevents this solvent from being useful.
	(d) 3:5:2		0.50	0.60	0.80	0.75	This mixture gave the best results of the pyridine solvents. The overlapping between thyroxine and iodide was not serious.

Legend: Monoiodotyrosine - M
Diodotyrosine - D
Thyroxine - T
Inorganic Iodide - I

Further discussion of some of these solvent mixtures is given at the end of the next section when the results obtained here are compared to those which resulted from the technique of buffering the filter paper strips.

Procedures developed to improve chromatography results

At this point a suitable filter paper had been chosen and at least two solvent mixtures were available which gave good separation of the thyroid amino acids. However, two further improvements were desired. Occasionally the radioautographs prepared from these chromatograms were marred by spots or streaks; also, even in the better solvent mixtures there were still times when overlapping of spots occurred.

Therefore several methods were tried to see which, if any, would give more suitable and reliable results during the process of chromatography.

Adding carrier to radioactive runs:- It was found that, especially with inorganic iodide, the very small amounts of material which are present on a chromatogram in tracer experiments often resulted in bad streaking or indistinct lines or spots on the subsequent radioautograph.

To prevent this, the addition of small amounts of non-radioactive "carrier" materials was usually found to clarify the bands considerably. In fact in the case of iodide, where tracer amounts alone were giving severe streaking, the addition of as little as 1 μ g. of iodide¹²⁷ to the spot improved the results vastly. This addition of carrier to the paper not only clarified the chromatograms but allowed the paper to be sprayed for a direct comparison of radioactive bands to known compounds.

Pretreatment of the filter paper with the chromatography solvent mixture:-

To see whether some impurity in the paper was causing the streaks running up to the solvent front on the autographs, some of the strips of filter paper were processed in the chromatography solvent first, and dried. The amino acids were spotted on as usual and the paper re-run in the same solvent. Any soluble compounds present in the paper should have run to the solvent front during the first treatment so should not be present to interfere with the procedure the second time. However, on comparing these with untreated controls little difference could be seen. It was concluded that whatever the difficulties were, they were not due to impurities on the paper which were moving with the solvent front during chromatography.

Versene (189):- Some paper was treated with a solution of this metal-complexing material, but was found to be weakened and easily torn after the process. In fact it was difficult to finish the treatment without making the paper unusable, so this procedure was discontinued.

Calcium sulfate (190):- Since most of the materials placed on the paper during actual experimental runs were butanol solutions from protein extracts, it was thought that perhaps some of the more soluble proteins were interfering with the chromatography, causing streaks. Therefore, the butanol extracts were treated with anhydrous calcium sulfate for thirty minutes before application to the paper in an attempt to remove any proteins from solution. This was found to improve many of the runs in which high protein concentrations were present. It was especially effective when coupled with Whatman No.42 paper, the paper producing narrower bands, and the "drierite" less spotting. Hence, this combination was used in all subsequent runs where high protein concentration was involved.

8 - Hydroxyquinoline (191):- 50 μ g of this metal-complexing material was spotted on filter paper strips and run overnight in the solvent to be used. It was thought that if the material causing the spots on the autographs was some metal in the paper this treatment would improve the situation. However, after using these treated papers and comparing them to untreated controls, no difference could be detected so it was concluded that the metals, if any, in the paper were not interfering.

buffered filter paper strips (152,153):- A range of aqueous buffer solutions were prepared at pH units of approximately 2, 4, 6, 8, and 10 (192). These 0.066 M. solutions were sprayed evenly on filter paper strips or sheets which were allowed to dry at room temperature.

Making use of these with the control solutions described above, and with the two most successful solvents of the preceding section, the following R_F values were obtained:

TABLE III
Variation of R_F with the pH of Filter Paper Strips

Solvent Mixture	pH				
	of Buffer	Moniodo-tyrosine	Diiodo-tyrosine	Thyroxine	Inorganic Iodide
Butanol:acetic acid: water (68:2:27) (upper phase)	2.0	0.21	0.28	0.64	0.09
	4.0	0.17	0.23	0.48	0.06
	6.0	0.28	0.44	0.80	0.14
	8.0	0.20	0.28	(0.45)*	0.11
	10.0	0.18	0.28	(0.37)*	0.12
Pyridine:ethanol: water (3:5:2)	2.0	0.73	0.76	0.85	0.70
	4.0	0.71	0.80	0.89	0.69
	6.0	0.65	0.63	0.81	0.74
	8.0	0.62	0.40	0.85	0.72
	10.0	0.49	0.20	0.51	0.73

* Very diffuse band

It will be noted that a considerable variation in the R_F values may result by simply changing the pH of the paper. The buffering had the further advantage of preventing a large part of the streaking which occasionally occurred without it.

For the best results the solvent mixture being used was equilibrated with the buffer solution in place of the water. This helped to maintain a constant pH throughout the chromatography development (153).

The most satisfactory pH values for the buffered filter paper strips to be used in conjunction with the two solvent mixtures, butanol-acetic acid-water (68:2:27), and pyridine-ethanol-water (3:5:2) were 6.0 and 8.0, respectively.

For most experiments in this work the former combination was chosen, but the second solvent mixture proved valuable for confirmation experiments where the results obtained by the first method required duplication.

Conclusions and Comments

The development of a successful chromatography procedure proved to be one of the major portions of this work; without a good method it would be impossible to obtain results of any value.

The conclusions which may be drawn after these experiments are that paper chromatography, while being very useful, is not as reproducible and exact as one might expect from casual references to it in the literature. This is confirmed by the lack of agreement among different literature sources.

The following factors have all to be watched, controlled and standardized:

- (1) Paper quality
- (2) Solvent quality and composition
- (3) Saturation of cabinet with vapors

- (4) Temperature
- (5) pH and salt effects
- (6) Spotting and drying techniques

The most useful solvent of those tried for one-dimensional work with extracts from the thyroid gland is butanol:acetic acid:water (68:2:27) with filter paper strips buffered to pH 6.0. Since butanol is used as the extraction solvent during treatment of the thyroid gland, (see the following sections), the use of it as chromatography solvent introduces no new, or possibly harmful, variable. It is easily obtainable in a pure condition. If necessary, for confirmation purposes, an auxiliary solvent mixture such as pyridine:ethanol:water (3:5:2) may be used.

Radioautography

A. Introduction

Very shortly after the introduction of paper partition chromatography as a means of study in the field of thyroid biochemistry, it was recognized that its value as a technique for separating and identifying traces of materials from the thyroid gland would be greatly increased if iodine¹³¹ were used to "label" the iodine-containing compounds being separated by the process (47).

Once the tracer had been incorporated into the compounds involved (by methods described in later sections) the identification procedure consisted of placing a strip of X-ray film over and in close contact with the filter paper for a predetermined period of time. When the

film was developed, all radioactive areas on the paper strip would have an exactly corresponding exposed area on the film, the density of which would correspond "qualitatively" to the amount of the labelled substance present. If the dark band on the film corresponded exactly to the ninhydrin spot of a known compound on the original chromatogram, it was good evidence that the radioactive material was identical to the known (147).

Once the process of chromatography had been developed so that it was giving reliable and reproducible separations, the technique of radioautography could be used with little change from the original procedures given by other workers. In this section will be given a general statement of how the process has been used in connection with the present work.

B. General Experimental Procedure

The X-ray films used in our work were:

(1) Kodak No-Screen film (147,49)

This film is the one chosen by nearly all those who use the technique of radioautography, since it is especially suited to the process, showing "graded" response to the varying radioactivity present. It was used in order to make the present work comparable to that in the recent literature.

(2) Ilford X-ray film

This film gave satisfactory results, but was used only if Kodak "No-Screen" film were unobtainable.

The general procedure followed consisted of placing the chromatograms with their film strips into the special Kodak X-ray

holders. These allowed convenient handling of the strips, individually, or as a group. For some experiments a large metal cassette was used so that a whole uncut sheet of filter paper, as from a two-dimensional paper chromatogram, could be autographed.

At first a strip of cellophane paper was placed between the chromatogram and the film during exposure (147). X-rays can easily penetrate this thin barrier. The cellophane prevented any extraneous contaminants on the paper from contacting the film. However, it was found, by comparing duplicate autographs from a relatively large number of experiments, that no improvement was being obtained by the use of the cellophane. In fact, if the same piece were used more than three or four times it became contaminated itself and had to be discarded. Therefore, the use of cellophane was discontinued.

The development of the exposed autographs was done in large photographic trays. The films were developed for three and one-half minutes in Kodak X-ray Developer, rinsed for thirty seconds in a dilute acetic acid stop bath and "fixed" for from ten to twenty minutes in a Kodak X-ray Fixing Bath. The films were then washed well with cold tap water for at least forty-five minutes and hung to dry after treatment with Kodak Photo-Flo to prevent streaking. When dry the autographs were compared to the ninhydrin (or starch) treated chromatograms from the same experiment by placing the film directly over the paper strips.

C. Autograph Exposure Time as Related to Isotope Activity

The majority of the iodine-¹³¹ activities involved in these experiments varied from approximately 10 μc . to 300 μc . However, the actual activity applied to one filter paper strip was considerably less than this (approximately 1 μc). For this order of X-ray emission the exposure time was at least twenty-four hours and preferably forty-eight hours. In cases near the lower end of the range quoted, exposure times up to one week were required.

The use of a "probe" Geiger counter was very useful in this respect, for one could estimate the exposure time required by monitoring the paper beforehand. The time of exposure was not critical. Several hours, or even a day over the usual exposure, would not change the appearance of the films very much unless the activity being utilized was abnormally high. However, all autographs which were to be compared to one another were given exactly the same exposure times.

D. Estimating the Results

In some cases, when only a rough indication of where the active areas on a chromatogram were situated was required, the paper was marked into narrow strips and each strip "counted" with the Geiger "probe" instrument (154). The area in question was shielded from the rest of the chromatogram by lead bricks. In this way the positions and variations of the activity over the whole strip was learned. The positions of the active areas could be compared to the R_F values of known materials when chromatographed under identical conditions of

solvent, paper and pH.

This was a good technique for a quick indication of what results could be expected without having to wait the day or more required for the autograph exposure.

Thyroid Gland Studies Performed *In Vivo* Using Isotopic Iodine and Albino Rats

Introduction

Although it was contemplated that the main part of this work, concerned with the route of synthesis of the thyroid hormone, would be performed under in vitro conditions, (see next section), it was felt that some time spent using living, whole animals would be valuable to familiarize the author with the techniques involved. In this way the results obtained in vitro could be compared to those observed when the gland was in its natural environment, and an idea of the reliability of the in vitro method determined.

In this section the data collected are compared to those reported by other workers; a few variations of procedure were introduced and their effects are discussed.

General Experimental Procedure

Several experiments were performed by administering a carrier free tracer quantity of iodine ¹³¹* to white rats. After a

* In order to be acceptable as a tracer in biological systems, a radioactive material should have the following properties:
(1) The chemical concentration of the tracer must be so small that it may be assumed that it does not alter the normal biological processes occurring in the system.
(2) The level of the activity of the isotope must be below the point where abnormalities in metabolism could result from the action of radiation on the system.

predetermined period, a sample of the blood was withdrawn and the thyroid glands removed. These were analyzed by the processes of chromatography and autography in order to determine what radioactive compounds were present.

The activities of isotope injected varied from 10 μc . to 2000 μc ., the most common being 100 μc . in approximately 2.5 ml. of aqueous solution.

The time between injection of the tracer and removal of the specimens varied from twenty minutes to twelve days with most experiments requiring an hour or less.

Albino rats varying in age from three months to one and one-half years, in weight from 150 to 400 g., and of both sexes, were obtained from the Zoology Department animal room of McMaster University. They were of the Connaught Laboratory strain. Their diet, consisting of oatmeal, dog biscuits, and water, supplied an adequate iodine level for "normal" thyroid health.

Before the thyroid glands were removed, the rats were anaesthetized with Nembutal, which was injected intraperitoneally as a 10 per cent alcoholic solution containing 64.8 mg. nembutal per ml. 0.1 ml. of this solution per 100 g. bodyweight rendered the animal completely unconscious in a few minutes; it was not sufficient to be fatal.

The blood sample was obtained by heart puncture, using the conventional technique (193). To obtain the plasma, the whole blood was centrifuged at 2000 r.p.m. for ten minutes, and the clear supernatant withdrawn. The radioactive iodine-containing

compounds of the plasma were extracted by one double and two equal volumes of butanol (69). The extract was evaporated under reduced pressure to approximately 2 ml. and 250 μ l. aliquots applied to filter paper strips, as indicated in the section concerned with chromatography. The completed chromatograms were autographed and the darkened areas compared to the positions taken by the carriers, monoiodotyrosine, diiodotyrosine, thyroxine, and inorganic iodide, which had been separated on the same filter paper strips.

The thyroid glands were excised from still surviving animals (194) following removal of the blood sample (when this was necessary). A lateral incision was made over the trachea in the region of the larynx, the lymph nodes and the muscles covering the trachea gently separated with a probe and the desired region of the trachea quickly cut out. The two thyroid lobes were cautiously pulled away from the larynx with blunt tweezers, and placed immediately into a cold glass mortar to be ground with 1 ml. of chilled 0.9 per cent saline solution (167). The glands from two rats were combined. The saline extracts contain 90 per cent of the isotope activity of the gland in a homogeneous solution (195).

Two ml. of butanol were used to extract the free iodine-containing amino acids from the saline. The mixture was resolved into two layers by centrifuging the alcoholic layer withdrawn. 250 μ l. aliquots were analyzed chromatographically.

By this procedure only the free iodinated amino acids of the gland were extracted, and not those combined in protein form. Any thyroglobulin which dissolved in the butanol would not be separated by the

chromatography but remained at the origin (168). Only the free amino acids were identified.

Where the protein, thyroglobulin, was to be analyzed following its hydrolysis, two methods were used:

(1) If an analysis of the protein alone without any identification of the free amino acids was required, the whole gland was ground with 1 ml. of 10 per cent trichloroacetic acid in a cold glass mortar and centrifuged to obtain the precipitated proteins. The precipitate was washed with 2 ml. of 2.5 per cent trichloroacetic acid and finally with 5 ml. of water (49).

(2) If the gland had been extracted with saline followed by butanol, as above, the protein was precipitated during the process by the alcohol, and could be separated by centrifuging the mixture.

In either case, the precipitated protein was hydrolyzed on the steam bath, under reflux, in 2 ml. 10 per cent sodium hydroxide, for at least sixteen hours. Nearly all the material went into solution. The basic hydrolysate was acidified to pH 3 with 6.0 N. sulfuric acid or 6.0 N. hydrochloric acid, the latter being preferred. The acidic solution was then extracted with an equal volume of butanol (49). The butanol extracts were chromatographed and autographed in order to identify the materials present.

General Experimental Results

The autographs of the plasma experiments, carried out at an activity level of 100 μc ., indicated that inorganic iodide¹³¹ and labelled thyroxine were present in the plasma after forty-eight hours,

the latter being present to a very small extent after even one hour. The intensity of the iodide spot decreased with time and was lighter after forty-eight hours than at one hour. No traces of the two amino acids, monoiodotyrosine and diiodotyrosine, were found. However, in the forty-eight hour experiment autographs, a second fainter band, below thyroxine, (collidine:water solvent), was noted, corresponding to some unidentified material.

Towards the end of the experimental work done in connection with this thesis, a sample of 3, 3¹, 5 - triiodothyronine was obtained (170). The forty-eight hour plasma experiments were repeated at two levels of iodine¹³¹ activity - 100 μ c. and 500 μ c.) adding triiodothyronine as a reference material to each of the chromatograms prepared. Use was made of a new chromatography solvent, butanol:dioxane (4:1) saturated with 2 N. ammonium hydroxide, to assure complete separation of the unknown and thyroxines. In both cases, it was found that the unidentified spot corresponded exactly to the added material. This result shows that labelled triiodo-thyronine is a constituent of the plasma of rats forty-eight hours after the animals have been injected with a tracer amount of iodide¹³¹. It confirms the recent work reported by Gross and Pitt-Rivers (178) who noted that the same material is present in human plasma and may be the true hormone of the thyroid gland.

It is of interest that in the autographs from the plasma of rats treated with 500 μ c. iodide¹³¹ there were two further unidentified spots. These run at R_f values of 0.33 and 0.90 in butanol:dioxane (4:1), saturated with 2 N. ammonium hydroxide (195).

The earlier results of one hour experiments agreed with those of all previous workers who had reported thyroxine as the only "thyroid amino acid" to be present in the blood (196). However, the forty-eight hour experiments show other iodide¹³¹-containing materials to be present at later stages in the plasma, and one, at least, may be of considerable importance.

Comparison of the autographs from the thyroid gland experiments with the corresponding chromatograms revealed that when 100 μ c. of isotopic iodide had been administered to the animals, labelled moniodotyrosine, diiodotyrosine and thyroxine, as well as inorganic iodide, were present in the extracts of both the unhydrolyzed and hydrolyzed thyroid protein. In the former, inorganic iodide was by far the darkest band, while diiodotyrosine predominated among the iodinated amino acids present. Free moniodotyrosine was present to a lesser extent than diiodotyrosine or thyroxine. Very little inorganic iodide was associated with the hydrolyzed protein, and practically no thyroglobulin (labelled) remained at the origin of the chromatograms. This indicated a complete hydrolysis. The diiodotyrosine and moniodotyrosine bands were usually of approximately equal intensities and darker than the thyroxine band. This confirmed the results published during recent years (49). Occasionally unidentified bands appeared on the autographs.

The hydrolysis procedure was tested to make sure it was not altering the R_f values of the amino acids. Two mg. each of moniodotyrosine, diiodotyrosine and thyroxine were dissolved in 2 ml. of

10 per cent sodium hydroxide and heated under reflux on the steam bath for several hours. The solution was neutralized, extracted and chromatographed exactly as described above. The results showed that the amino acids were not harmed by the process unless sulfuric acid were used for acidification. If it were, then bad streaking occurred. This could be due to the salts formed. Examination of the chromatographs revealed that no new bands were present after the hydrolysis procedure, nor any of the expected ones missing, so no change serious enough to alter the qualitative results being obtained was produced by the treatment. Hydrochloric acid was used for all subsequent neutralizations.

Some Specific Experiments Done by the In Vivo Method

Effect of Nembutal as Anaesthetic

To determine what effect, if any, the use of nembutal as anaesthetic was having on the experimental results, two groups of two rats each were injected intraperitoneally with 100 μ c. iodine¹³¹. After one hour had elapsed the first two rats were anaesthetized with nembutal as usual, their thyroids removed, hydrolyzed (after precipitation with trichloroacetic acid) and analyzed chromatographically. Likewise, the other two rats were thyroidectomized, but no anaesthetic was used. Instead, the rats were sacrificed by a blow on the head. These thyroids were similarly hydrolyzed, chromatographed and autographed.

The results indicated that, as far as could be determined by these qualitative means, no difference whatever had been made by the use of the nembutal. The usual bands for monoiodotyrosine, diiodotyrosine and thyroxine could all be identified in both cases. In other words, the strips were completely interchangeable.

The Time of Appearance of Isotopic Iodine in the Compounds Present in the Thyroid

Most of the in vivo experiments carried out in this work involved a time of one hour between the introduction of tracer, iodine¹³¹, into the animal, and removal of its thyroid. The results in these cases showed that thyroxine labelled with the isotope was present to a relatively small amount in the thyroglobulin compared to monoiodotyrosine and diiodotyrosine.

However, in the experiments involving longer periods of time, (up to forty-eight hours), the thyroxine spot was more distinct while little difference could be seen in the intensities of the monoiodotyrosine and diiodotyrosine bands. This would indicate that thyroxine builds up in the gland rather slowly, while the "maximum" amounts of diiodotyrosine and monoiodotyrosine are formed comparatively rapidly from inorganic iodide.

Effect of Solvent

The chromatographs of the first in vivo thyroid gland experiments were developed, using collidine:water (125:44) as solvent, but some later experiments were done with methyl cellosolve:water (90:10), and butanol:acetic acid:water (68:2:27). The same bands were identified

in all cases, so this was evidence that no band on the one-dimensional chromatograms was due to an overlapping of two other spots.

At least one case was obtained in each solvent where an unknown band was observed. These were, however, always distinct spots so do not invalidate the preceding statement. Although these bands may be artifacts, they warrant further investigation.

Effect of Iodine¹³¹ Activity on Synthesis in the Thyroid Glands

It is reported in the literature that high activities of iodine¹³¹ cause destruction of the thyroid gland's ability to synthesize thyroxine (72). It was decided to investigate this phenomenon to find out what level of activity would be suitable for further work.

(a) Two sets of two rats each were injected intraperitoneally with 10 μc . and 200 μc . iodine¹³¹ respectively. After two and one-half hours the thyroid glands were removed and analyzed by the process described above. Both the "free" and the organically-bound radioactive materials were identified.

No great difference was observed on the autographs for the two activity levels, other than the obvious one of intensity of the bands, but there was one variation which may have some significance. In the autographs of the 10 μc . experiment, thyroxine was present to a "normal" extent. In other words, the same results were observed as had been obtained many times. Likewise, after the injection of 200 μc . of iodine¹³¹, the extracts of the unhydrolyzed protein again led to a distinct thyroxine spot. However, only a very faint thyroxine spot could

be detected with the dark monoiodotyrosine and diiodotyrosine bands on the autographs from the hydrolysates.

Perhaps the mechanism which, presumably, involves some oxidative enzyme system, whereby diiodotyrosine is converted into thyroxine, was slowly inactivated by the higher radioactivity. If no thyroxine were being synthesized by the gland, the level of this material would gradually be depleted as demand for the compound continued. Eventually, as indicated by the results obtained here, the situation might be that nearly all of the newly synthesized thyroxine, if any were being produced, was immediately liberated for removal in the plasma. Therefore, 200 μ c. of iodine ¹³¹ activity affects the mechanism whereby thyroxine is synthesized, but does not prevent the formation of the intermediates, monoiodotyrosine and diiodotyrosine.

(b) Two groups of rats were injected intraperitoneally with 1 mc. doses of iodine ¹³¹. Four hours later the glands from the first group of two rats were removed and analyzed as usual.

The extracts of the unhydrolyzed protein led to autographs containing four distinct bands of approximately equal intensity, corresponding to thyroxine, diiodotyrosine, monoiodotyrosine and inorganic iodide. The extracts of the hydrolyzed protein contained relatively large amounts of monoiodotyrosine and diiodotyrosine, but no evidence of thyroxine. This confirms the above results.

The other group of four rats was kept under normal conditions for twelve days. They were observed to be increasingly inactive in their habits. At the end of this time, two of the rats were injected

with another 1 mc. of iodine¹³¹, and two hours later the thyroid glands were removed from both groups of rats and analyzed.

The glands were noted to be abnormal, having a shrunken and unhealthy appearance as far as color and size were concerned. Those from the twice-injected rats were monitored by a Geiger counter and were found to be much less active than those from normal animals receiving one-tenth of the dose of activity.

The glands from the rats which had received no second administration of iodine¹³¹ led to autographs which were almost completely free from any organic iodine bands. An extremely faint thyroxine spot was produced from the extract of the unhydrolyzed protein. There were always definite bands for inorganic iodide¹³¹, however, so if any of the normal synthetic mechanisms remained in the gland there should have been at least some of the organic materials present in the labelled condition. Apparently the high level of radiation had inactivated all of the gland's ability to synthesize its hormone.

The autographs from the experiments with the twice-injected rats were exposed for an extremely long time so that any active compounds present would be detected, even if they were there in only very small amounts. The extracts of the unhydrolyzed protein were shown to contain traces of iodide and occasionally monoiodotyrosine, while the hydrolysates led to faint bands for monoiodotyrosine and diiodotyrosine (but not for thyroxine). Considering the amount of activity injected, and the exposure time allowed, these compounds must have been present in almost negligible amounts. The glands had lost a great deal of their ability

to concentrate iodide as well as all that required to synthesize the thyroid hormone. Therefore, the enzyme systems involved must all be sensitive to extreme γ -radiation, but those which iodinate tyrosine and moniodotyrosine are less so than the enzyme(s) which produce thyroxine. In all future experiments low activities of iodine¹³¹ (approximately 70 μc .) were used to prevent any serious inactivation of these synthetic pathways.

All results in this section were confirmed by repetition.

Conclusions

The results obtained in this section indicate that these data agree well with those of previous workers. It will now be interesting to compare the results with those obtained under the in vitro conditions described in the next section.

Thyroid Gland Studies Performed In Vitro Without the Presence of Inhibiting Materials

Introduction

In the historical introduction it was stated that several workers, notably Chaikoff et al. (160), have investigated, using iodine¹³¹ the problem of whether thyroid gland tissue slices will synthesize the thyroid hormone when they are incubated in a physiological medium containing small amounts of inorganic iodide.

In this work many of the general type-experiments performed by these workers have been repeated. That is, some "normal" syntheses under in vitro conditions were carried out, the products produced

being identified by chromatography and compared to those which have been reported in the literature, and also to those compounds identified under in vivo conditions. This was followed by observing the effects obtained by variation of some of the conditions under which synthesis normally occurred.

In addition, two amino acids which have been postulated as intermediates in the synthesis of thyroxine by the thyroid gland were labelled with isotopic iodine, and incubated with surviving tissue slices. Only recently (175) has any work of this nature been reported, but it was carried out under different conditions and appeared in the literature after the work performed here was completed. The results obtained serve to confirm, independently, those observed by these workers.

It will be noted in this section, and in the subsequent sections, that equal emphasis has been given to identification of both the "free" amino acids and the "protein-bound" amino acids of the thyroid gland. All the reported work in the literature, with the exception of two papers (167,168), has dealt exclusively with the latter. It was felt that the free amino acids may play an important role in the gland, so their behaviour has been especially noted.

General Experimental Procedure

All of the in vitro work reported in this thesis was done, using thyroid tissue slices obtained from albino rats. To obtain the slices the rats were anaesthetized with nembutal and the thyroid

glands were quickly removed to a petri dish containing a physiological Ringer-bicarbonate saline solution. (The composition of this solution is described below.)

With a clean, sharp razor blade moistened with the saline solution, each lobe was cut into two equal pieces, making four slices of tissue from each rat. The slices were then transferred as quickly as possible, and with the least handling necessary, to the reaction vessel, a Warburg flask (197).

The Warburg Respirometer made an ideal constant temperature bath for this work, and the small flasks fitted with two side arms were well suited to in vitro studies of this type.

The physiological medium in which all in vitro work was performed consisted of an aqueous solution of the following composition (198).

<u>Constituent</u>	<u>Parts</u>
0.90 per cent sodium chloride	100
1.15 per cent potassium chloride	4
1.22 per cent calcium chloride	3
2.11 per cent potassium dihydrogen phosphate	1
3.82 per cent hydrated magnesium sulfate	1
1.30 per cent sodium bicarbonate	21

(gassed for one hour with carbon dioxide)

Any other constituents added to the flask were contained in solutions of such a concentration that the final medium was always isotonic to mammalian blood.

The typical in vitro experiment carried out in the absence of inhibitors took the following form (150).

Thyroid slices from two rats were placed into a Warburg flask containing 2 ml. of the Ringer-bicarbonate medium in the main part of the flask. In one of the side arms was placed 0.5 ml. of a solution containing approximately 70 μ c. of iodine¹³¹. It was prepared from a concentrated Ringer mixture diluted by the aqueous isotope solution to the isotonic concentration.

All ground glass joints were greased, the flask was fitted to its manometer and the apparatus was incubated in the Warburg Respirometer bath, which was thermostatically controlled at 38.0°C. (rat body temperature). Throughout the experiment constant mixing of the contents of each flask was maintained by a mechanical shaker.

About fifteen minutes was allowed for temperature equilibration and then the tracer was administered to the slices by tipping the flask.

At the end of the specified time, usually three hours, the flasks were removed from the bath and the thyroid slices quickly transferred to an ice-cold glass mortar, where they were homogenized with 1.0 ml. of chilled 0.9 per cent saline. Extraction with butanol, hydrolysis with 10 per cent sodium hydroxide, and analysis by chromatography and autography was then carried out exactly as reported in the in vivo section. The chromatograms were always prepared with added, non-labelled reference materials in order that any radioactive bands in the autographs could be compared to known substances on the chromatograms.

General Experimental Results

The results obtained in this work following the procedures described above were very similar to those reported by other workers in the thyroid field. They show that in vitro synthesis of all the iodine-containing compounds identified in the thyroid glands of normal rats (described in the preceding section) will take place (160).

Thus, in the extracts of the unhydrolyzed protein were present detectable amounts of monoiodotyrosine, diiodotyrosine, thyroxine and inorganic iodide (167). The latter was present to a much greater extent than any of the amino acids, while thyroxine usually predominated over diiodotyrosine and monoiodotyrosine. Occasionally, unidentified bands were observed in the autographs similar to those noted under in vivo conditions.

The hydrolysate extracts contained monoiodotyrosine and diiodotyrosine with smaller amounts of thyroxine. Unknown bands on the autographs were much less common among these thyroglobulin constituents.

Further discussion of the results obtained here will be found in the concluding paragraphs of this section, following a description of how variation of the experimental conditions influenced the degree of synthesis by surviving tissue slices.

Some Specific Experiments Done Using the In Vitro Technique

Length of Time of Incubation

Three series of in vitro experiments were performed varying only

in length of time of incubation after addition of iodine¹³¹. The times involved were respectively one hour, two hours, and three hours. The results refer to the free amino acids identified from extracts of the unhydrolyzed protein.

After one-hour there was no indication of any iodide¹³¹-containing free amino acids in the thyroid gland; only inorganic iodide was present. After two hours' incubation, the autographs revealed sharp, definite monoiodotyrosine and diiodotyrosine bands but rarely any indication of thyroxine. At the end of three hours thyroxine was always present and usually both the iodotyrosines.

Occasionally, either or both monoiodotyrosine and diiodotyrosine could not be identified on the autographs. As yet no definite explanation for this phenomenon can be presented. However, since the conditions under which synthesis occurred were standardized as much as possible, there remains little doubt that the difficulty must be caused by the use of tissue slices. It is impossible to assure that surviving slices are identical. The success of the in vitro technique depends upon the condition of the tissue surface exposed to the incubation medium containing the iodine¹³¹. This will be discussed further in the concluding part of this section.

Since best results were obtained with three hours' incubation, this time was used in all subsequent experiments.

Pretreatment of Rats with Potassium Thiocyanate

Potassium thiocyanate affects the thyroid gland by making it unable

to concentrate inorganic iodide (112). In fact, after its injection the iodide concentration of the gland falls sharply, approaching the level in the blood (120). It was thought that injection of rats with this substance previous to the removal of their thyroid glands might deplete the glands of iodide to such an extent that in vitro uptake of tracer by surviving thyroid slices from these animals would be much faster than before, and hence lead to more satisfactory results.

Therefore, three adult rats were injected with 1.0 mg. of potassium thiocyanate in 0.2 ml. aqueous solution thirty minutes before they were to be sacrificed. After the regular procedures, the autographs from the extracts of the unhydrolyzed protein revealed that none of the usual materials, not even inorganic iodide, was present. The tissue slices must have retained enough thiocyanate within them to prevent uptake of iodide¹³¹.

The presence of a faint thyroglobulin spot at the origin of the autograph indicated that the protein would show the presence of small amounts of the expected labelled amino acids, but only because of simple diffusion of the tracer into the gland. Potassium thiocyanate does not inhibit thyroxine synthesis but only the concentration of iodide ion (112). Perhaps a small quantity of iodide¹²⁷ added to the incubation medium would reverse the effect of the retained thiocyanate, since the two materials are antagonistic in their action. However, potassium thiocyanate could possibly lead to artifacts in these experiments so its use in this connection was discontinued.

Radioactive Diiodotyrosine

Diiodotyrosine labelled with iodine¹³¹ may be prepared by an exchange between diiodotyrosine and iodide¹³¹ in a buffered solution (199). Conditions which gave maximum exchange were found to be:

Temperature 90°C.
pH 4.0 to 5.0
Time of incubation 10 minutes

The concentration of diiodotyrosine used was 2 mg. per ml., while the activity of iodide¹³¹ was 10 μ c. to 300 μ c. per ml. The exchange was carried out in a 15 ml. centrifuge tube, which was incubated in a water bath heated to the correct temperature by steam. It was discovered that the desired pH could be obtained by adding about 10 mg. of ammonium chloride to the reaction solution.

To determine the completeness of the reaction the solution was chromatographed. The only radioactive band on the strip corresponded exactly to diiodotyrosine added as a reference material to the chromatogram. This showed the exchange was complete.

A sample of labelled diiodotyrosine dissolved in an isotonic solution was incubated with surviving tissue slices in a Warburg flask. The concentration of diiodotyrosine in each flask was such that it represented several hundred times the amount of free diiodotyrosine normally present in a rat thyroid gland (usually about 0.07 μ g.) (168). This maintained a high concentration gradient to facilitate adsorption of the amino acid by the tissue slices.

The activity of the diiodotyrosine was approximately 100 μc . Analysis for the compounds present followed the usual procedure. The results fell into two categories.

In some cases the only radioactive material extracted from the glands was diiodotyrosine. Apparently these tissue slices were impermeable to this amino acid and were unable to utilize it for synthesis of the hormone.

In other cases, in addition to a spot for the added diiodotyrosine, bands for monoiodotyrosine and thyroxine were obtained and under these circumstances inorganic iodide was also invariably present. This meant that either the exchange reaction had not gone to completion, which was very unlikely since control autographs revealed no inorganic iodide associated with the labelled diiodotyrosine, or else some mechanism in the gland itself was present which was degrading the diiodotyrosine to iodide ion. The gland could then use the iodide to synthesize new diiodotyrosine and thyroxine. More evidence for this is reported in the next paragraph so further discussion will be reserved until it is presented.

Radioactive monoiodotyrosine

(a) Labelled monoiodotyrosine was prepared in exactly the same manner described for diiodotyrosine. When it was administered to the thyroid slices, the preparations were incubated for three hours at 38.0°C., extracted, hydrolyzed, chromatographed and autographed as described before.

The autographs of the extracts of the unhydrolyzed thyroid were compared to control autographs of the exchanged material and showed definitely that during incubation with the glands some breakdown of monoiodotyrosine into inorganic iodide had occurred with the subsequent synthesis and liberation of labelled thyroxine and diiodotyrosine. This breakdown of monoiodotyrosine, or diiodotyrosine, described above, could have occurred after the gland had incorporated the labelled amino acid into the thyroglobulin or else while it was still in a free state. Later evidence points to the latter as being the more likely.

The autographs from the protein hydrolysates showed monoiodotyrosine, diiodotyrosine and thyroxine to be present.

Soon after the experimental work reported here was completed, there appeared in the literature results very similar to those just discussed (175). Hence this work serves to confirm independently the presence of an enzyme system which degrades the free iodine-containing amino acids of the thyroid gland to inorganic iodide; this iodide may then be resynthesized into the thyroid hormone.

(b) It was desired to obtain a sample of labelled monoiodotyrosine which was known to be completely free from any inorganic iodide, in order to verify the results obtained above. A cation exchange resin would bring about such a separation.

Therefore, a sample of the amino acid was prepared by the exchange reaction, and dissolved in 1.0 N. hydrochloric acid. A small amount of iodine¹³¹, (10 μ c.), was added to the solution to act as indicator

for any inorganic iodide present. The solution was passed through a 42 cm. by 26 mm. column packed with 20-50 mesh Dowex-50 resin. The majority of the active material remained near the top of the column. The resin was washed with 200 ml. of 1.0 N. hydrochloric acid, followed by 475 ml. of 6 N. hydrochloric acid. No activity could be detected in the last 300 ml. of washings so it was assumed that all inorganic iodide had been removed.

The monoiodotyrosine was then eluted from the column by passing 300 ml. of 5 N. ammonium hydroxide through the resin. The activity which had been located on the column was now all in the basic solution. The solution was evaporated under reduced pressure to dryness and an aliquot of the residue incubated with surviving tissue slices. The results showed that very little uptake of monoiodotyrosine by the thyroid slices had occurred. As a result very little synthesis of thyroxine had taken place. Examination of the remaining portion of the monoiodotyrosine showed that it was discolored and contained an impurity which must have been removed from the Dowex resin by the ammonium hydroxide. It is very likely that this contaminant poisoned the enzyme systems of the thyroid slices and prevented synthesis of thyroxine. Therefore, if this method is to be utilized to separate monoiodotyrosine and inorganic iodide, a different cation exchange resin must be chosen which will not lead to impurities.

Effect of Iodide¹³¹ Concentration

In these in vitro experiments a range of iodide¹³¹ activities

was used during the different runs. It was concluded that the most suitable concentration to use was between 50 and 100 μc . Therefore, 70 μc was chosen as the standard activity. If the activity was much higher than this there was evidence that the excessive - radiation was damaging the enzyme systems of the gland and giving erroneous results, (cf. in vivo work at high iodine¹³¹ activities).

The Addition of D - glucose to the Incubation Medium

In a few in vitro experiments the incubation medium was made 0.1 M. with respect to D - glucose. It was thought that if the synthetic processes occurring in the gland required glucose for their source of energy this material might lead to a greater extent of hormone synthesis. The autographs prepared from these experiments were compared to controls. The results revealed that no more synthesis of thyroxine had occurred when glucose was added to the medium than when it was not. Also, the autographs from the former case were marred by streaks, so the use of glucose was discontinued.

The Effect of Adding Tyrosine to the Incubation Medium (cf. Section VII)

Approximately 0.4 mg. per ml. of L - tyrosine was added to the incubation medium of an in vitro experiment. The results showed that it definitely caused no inhibition of hormone synthesis, by the surviving thyroid slices, and aided it to a slight extent. The latter difference was too slight to draw any definite conclusions as to the value of adding tyrosine to the medium as a general practice. However, these data proved of value when compared to the experiments discussed

later involving reversal of 3 - fluorotyrosine inhibition with tyrosine.

The Relation Between the Size of Thyroid Slice and the degree of Thyroid Hormone Synthesis

Two experiments were performed in order to determine whether the amount of synthesis being obtained under in vitro conditions would be diminished by the utilization of several small slices from each lobe of surviving thyroid tissue instead of the usual two. Chaikoff (160) reported that less synthesis did take place in tissue minces than in tissue slices but did not specify the effect of increasing the number of slices prepared from one thyroid lobe. The thyroid glands were removed from two rats and one lobe from each rat treated in the same manner. One pair was sliced into the usual two pieces per lobe, while the other was cut into six pieces per lobe.

The slices were incubated with isotopic iodine, extracted, and analyzed. The results showed that no detectable decrease in the amount of synthesis in the flask containing the smaller slices had occurred. Apparently this degree of disruption had not been serious enough to prevent the normal synthetic processes from occurring. This information was used in the section dealing with thyroid inhibitors where occasionally the greater number of slices per lobe was used so as to expose a larger surface area of the gland to the incubation medium.

The Use of Carbon Dioxide in the Incubation Medium

One of the substituents of the incubation medium (sodium bicarbonate) was always gassed with carbon dioxide for several minutes before use. Taser (200) in 1942 had shown that in the absence of carbon dioxide the

maximum activity of tissue slices is maintained for only a short time.

It was decided to find out whether this applied to the synthetic process in question and, if so, whether a short time gassing was required each time an in vitro experiment was performed, or just when the stock solution of bicarbonate was prepared.

By comparing autographs, the results showed that without any carbon-dioxide gassing at all synthesis of thyroxine by tissue slices incubated with isotopic iodide was definitely less, but not prevented entirely. On the other hand, one gassing of the sodium bicarbonate solution (200 ml.) on its preparation was sufficient to assure maximum synthesis, if the storage bottle were kept tightly closed and cold between experiments. Further gassings with carbon dioxide just before the next experiment did not improve the synthesis.

Conclusions

The in vitro experiments performed in this section show that synthesis of the thyroid hormone very definitely can, and does, take place in surviving tissue slices incubated with tracer amounts of radioactive sodium iodide.

The conditions of extraction, hydrolysis, and identification of the iodine-containing amino acids produced by the thyroid slices were such that reasonably reliable results were obtained by this method. However, there was always present a certain lack of reproducibility in the results and the only way in which conclusions could be drawn was to carry out a large group of experiments to determine what

constituted an "average" or most common set of results.

In summary, the usual results obtained in a non-inhibited in vitro experiment confirmed that in the thyroid gland were present small amounts of "free" amino acids, namely, monoiodotyrosine, diiodotyrosine and thyroxine. Although several cases were noted where all three amino acids were detectable at the same time, it was often observed that either or both monoiodotyrosine and diiodotyrosine were missing. This would indicate that they are not involved in the active synthesis of the thyroid hormone, but perhaps are "side-paths" in the main reaction scheme which takes place in the protein, thyroglobulin. As examples, there follow the results of eight experiments performed under identical conditions as far as time of incubation, incubation medium, iodine¹³¹ activity, and methods of extraction and identification are concerned. The free amino acids identified are listed in the order of the intensity of their bands on the autographs.

- (1) D and M > T
- (2) D and T > M
- (3) D and T and M
- (4) T
- (5) D > T
- (6) M and D > T
- (7) M > T > D
- (8) D > T

where the symbols are:

M - monoiodotyrosine

D - diiodotyrosine

T - thyroxine

Detectable sex variations do exist in the thyroid gland; this became increasingly evident in the further experiments of this work. In Section VII some definite evidence will be presented for this. A consultation of the literature reveals that the two papers (167,168) which discuss the "free" amino acids present in the gland do not agree with each other on all points so apparently a lack of reproducibility is characteristic of this phase of thyroid studies.

Another point which must be considered is that usually in this work only two rats were used at one time. Probably this number is not sufficient to give a clear idea of the average situation. Individualities in the glands would be conspicuous in each experiment. If the free amino acids of the thyroid gland are involved in a degradation process to give inorganic iodide, instead of a synthetic process, the gland, when excised, could be at one of several stages, depending on the particular demand for the thyroid hormone at the time of removal.

Since the thyroid is under the control of at least one other gland (58) which is not present in vitro, the tissue slices could be "frozen" in the stage in which they were removed from the body. Thus certain enzymes of the gland might be present for one part of the pathway of degradation but not for another, since some enzymes could be stored in an inactive state to be activated as required by the needs of the gland and the body. If this is the case, to obtain a system which indicates the actual synthetic processes occurring in a "normal" thyroid gland, one would have to make use of tissue slices

from a relatively large number of experimental animals.

The results for the hydrolysates were much more consistent. In fact, all but a very few experiments showed that in thyroglobulin, moniodotyrosine and diiodotyrosine were present in considerable amounts, and thyroxine was nearly always detectable in smaller quantities. The results were not only consistent within themselves but agreed with the findings of other workers on these points.

One observation which might be brought forward is that, whereas Chaikoff (160) reports diiodotyrosine to be present consistently in amounts greater than moniodotyrosine, a few examples of the opposite situation have been observed in this work. However, the most common results obtained agree with the findings of Chaikoff on this point.

Thyroid Gland Studies Performed in Vitro in the Presence of Inhibitors

Introduction

One of the main purposes of this work was to study the action of the general types of thyroid inhibitors. In both in vivo and in vitro experiments, compounds related to thiouracil (104), p - aminobenzoic acid and sulfanilamide (110) have been found to inhibit only the actual synthetic mechanisms of the thyroid gland and not its ability to concentrate iodide from the blood. On the other hand, potassium thiocyanate (112) specifically inhibits the concentration mechanism and not the rest of the process.

This section deals with the technique of in vitro incubation of the inhibitor with surviving tissue slices followed by chromatographic analysis.

No other work in the thyroid field had combined these methods or had analyzed thyroid gland extracts after inhibition to see at what point inhibition had occurred, and to what extent. In addition to the established types of inhibitors two compounds, 3 - fluorotyrosine and 3 - fluoro - 5 - iodotyrosine were available (2). The former had been shown to behave as competitive inhibitor in the metabolism of tyrosine by the organism, Neurospora crassa. Likewise, 3 - fluoro - 5 - iodotyrosine is very similar to moniodotyrosine in its structure.

Therefore, their action as inhibitors of thyroxine synthesis was determined by chromatographic analysis with special attention paid to the possibility that they might block the synthetic processes at definite points, and cause a building-up of the thyroxine precursor with which they competed, namely, tyrosine and 3 - moniodotyrosine, respectively. Their mode of action and effectiveness as inhibitors was compared to known goitrogenic substances.

It was thought possible that the action of one or more of the inhibitors resulted because the material was able to enter the enzymatic iodination scheme, in the gland, become iodinated itself, and then block further synthesis of the hormone. This final blocking might be due to the inhibitory nature of the new iodine compound itself, or simply because iodide was no longer available for the regular synthesis. One other possibility would be that the inhibitor could become coupled to one of the reaction intermediates, thus removing the material as an effective precursor of thyroxine.

If any such iodinated compounds were being formed, paper chromatography and radioautography would separate them as new, unidentified

radioactive bands. If no new band appeared then it would be evidence that the inhibition did not act by becoming iodinated itself.

Evidence has been presented by other workers for and against the idea that actual hormone synthesis occurs in the thyroglobulin rather than through a pathway involving the free amino acids (168). The results obtained in this work are consistent with the supposition that synthesis does occur in the combined protein form.

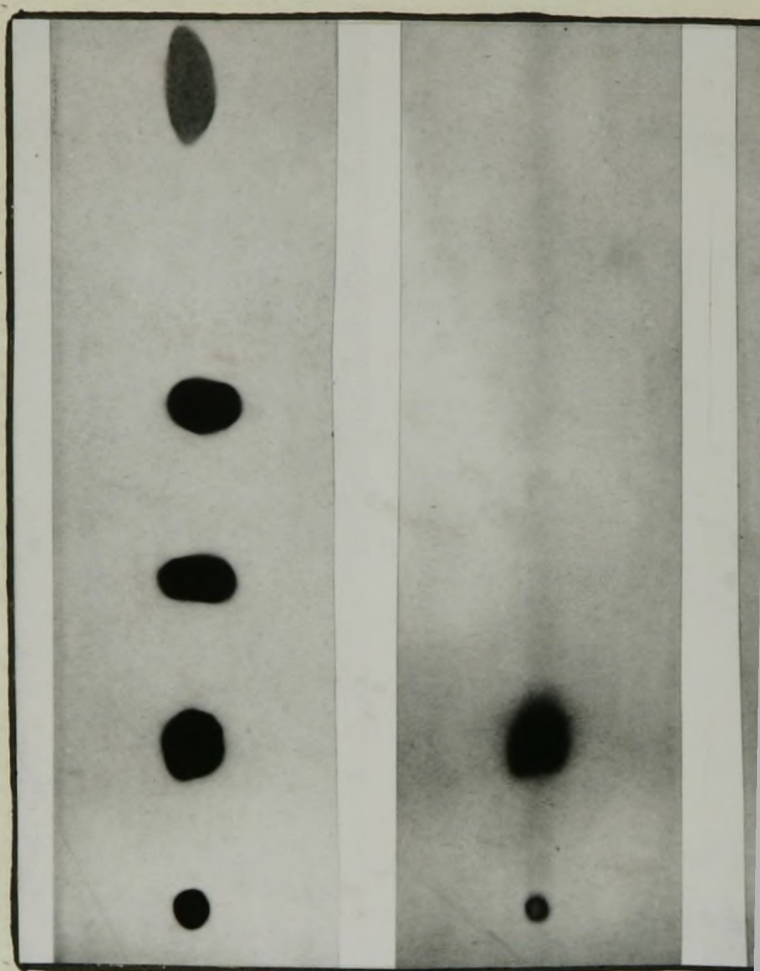
General Experimental Procedure

The in vitro experiments in this section were carried out by means of the same general procedure as outlined before. (See page 75). The second sidearm of the Warburg flask was utilized for the inhibitor. This material was always added to the incubation medium as a Ringer-bicarbonate solution, generally in a volume of 0.5 ml.

The final concentration of the inhibitors in the flasks was of the order of 10^{-3} M. These concentrations had been found to be successfully inhibitory in experiments by other workers (110-112).

Below are listed the materials studied in this section with the concentrations at which they were utilized.

- (1) 3 - fluorotyrosine 1.2 to 2.3×10^{-3} M.
- (2) 3 - fluoro - 5 - iodotyrosine 0.37 to 0.71×10^{-3} M.
- (3) p - aminobenzoic acid 3.0×10^{-3} M.
- (4) Sulfanilamide 1.0 to 3.0×10^{-3} M.
- (5) Thiouracil 1.0×10^{-3} M.
- (6) Potassium thiocyanate 1.0×10^{-3} M.



A

B

F

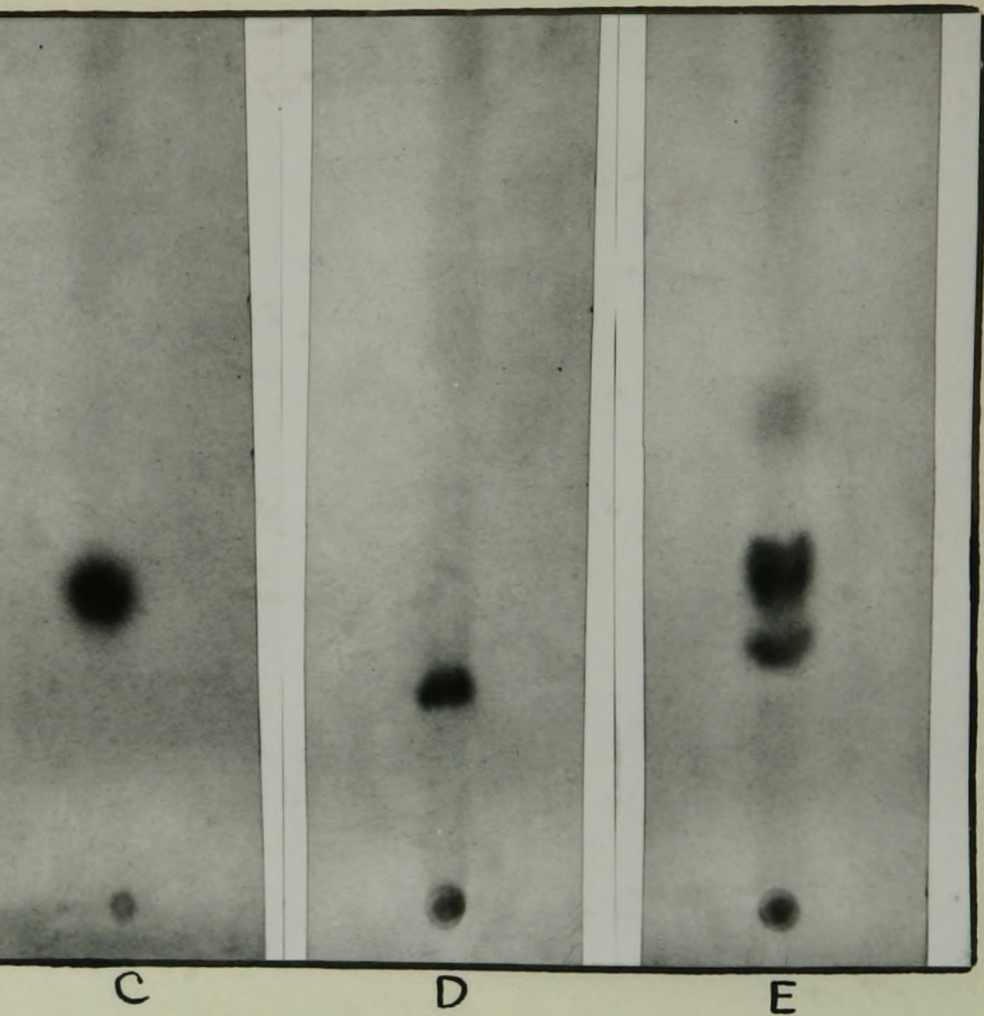


FIGURE 1.

At this same concentration of 3 - fluorotyrosine the autographs from the extracts of the hydrolyzed thyroglobulin revealed that only a slight inhibition of thyroxine synthesis had occurred as compared to an experiment carried out under the same conditions, but utilizing no inhibitor. Since synthesis of thyroxine was not completely prevented by the inhibitor, some of the tracer, iodide ¹³¹, must have entered the gland and been incorporated into thyroxine before the 3 - fluorotyrosine could exhibit its inhibitory powers. However, the fact that no inorganic iodide, as such was allowed to remain in the

* had been incubated in the presence of iodide ¹³¹ with one of the inhibitory materials discussed in this section. The chromatograms corresponding to these autographs (not shown) were all developed in butanol:acetic acid:water, (68:2:27), and were prepared on filter paper strips buffered to pH 6.0.

Figure 1(A) was included in order to illustrate the average positions taken under these conditions of chromatography by the materials which are discussed most frequently in this section. Naming them from the bottom to the top of the strip, the spots represent the origin, inorganic iodide, moniodotyrosine, diiodotyrosine, and thyroxine.

It must be stressed that these are average positions. The R_f values corresponding to these positions were not constant enough to allow the definite identification of a particular band by its R_f alone. In all cases, the band must be compared to the position and shape of spot taken by an added reference material on the same chromatogram.

The four autographs (B-E) are discussed in the sections dealing with the results to which they correspond.

gland, once it had been collected, is shown by the remarkable observation that there is no spot for iodide on any of the autographs. Almost always, however, the monoiodotyrosine band was darker on the hydrolysate autographs than that for diiodotyrosine. This occasionally was observed under "normal" conditions. (See page 90). However, more frequently diiodotyrosine was present to the greater extent in the latter cases.

At a 3 - fluorotyrosine concentration of 2.3×10^{-3} M. the autographs for the unhydrolyzed tissue were very similar to those above, i.e., an unknown band was present just above the reference R_f for inorganic iodide. The hydrolysate autographs indicated considerably more inhibition than before. No thyroxine band was present and there were only extremely faint ones for diiodotyrosine and monoiodotyrosine, the former being negligible. The appearance of an unknown spot on these autographs suggested that the inhibitor, 3 - fluorotyrosine, was being iodinated in some manner by the tissue slices. One possible product of such an iodination would be 3 - fluoro - 5 - iodotyrosine. Therefore, the unknown band was compared, chromatographically, to this material. However, the R_f obtained for 3 - fluoro - 5 - iodotyrosine under the same conditions of chromatography showed the compounds were not identical.

3 - Fluoro - 5 - iodotyrosine

Again it had been hoped that this compound would act specifically in its inhibition and block the over-all pathway of synthesis of thyroxine between monoiodotyrosine and diiodotyrosine because of its

structural similarity to the former. Examination of the autographs showed the following:

(a) At a 3 - fluoro - 5 - iodotyrosine concentration of 0.37×10^{-3} M. the autographs from the extracts of the unhydrolyzed tissue contained but one dark band, located between the R_f value for carrier iodide and moniodotyrosine on the chromatograms. Therefore, the observed compound was identical to neither material. As observed with 3 - fluorotyrosine, there was again no band for inorganic iodide. By similar analysis, the thyroglobulin was shown to contain an easily detectable amount of labelled moniodotyrosine, but a much lower amount of diiodotyrosine and scarcely any thyroxine. These results could be explained by a specificity such as expected for 3 - fluoro - 5 - iodotyrosine, but it was decided that more evidence was needed before any decisions could be drawn. Since no moniodotyrosine was detected in the unhydrolyzed tissue extracts from the gland, it was felt that the action was not as predicted by the structural similarities of 3 - fluoro - 5 - iodotyrosine and moniodotyrosine.

(b) At a concentration of 0.71×10^{-3} M. no change could be observed in the autographs of the free amino acids from those at the 0.37×10^{-3} M. inhibition level. Again a dark unidentified band was present. The autographs prepared from the hydrolysate extracts were almost completely blank except for an extremely faint moniodotyrosine band.

It is concluded that 3 - fluoro - 5 - iodotyrosine probably

behaves in an analogous manner to 3 - fluorotyrosine as a thyroid inhibitor. In butanol: acetic acid: water (68:2:27), using filter paper strips buffered to pH 6.0, the R_f values for the two unidentified iodine-containing materials were very similar, and the unknowns may be identical. 3 - fluoro - 5 - iodotyrosine is a more efficient inhibitor than 3 - fluorotyrosine at the same molar concentrations. For example, it produces just as much inhibition at 0.71×10^{-3} M. as 3 - fluoro - tyrosine does at its solubility limit of 2.3×10^{-3} M.

Moniodotyrosine must be the first step in the pathway of synthesis to the thyroid hormone since it is the one and only spot present on any autographs which are not quite completely inhibited.

The mechanism of inhibition of these two materials is not of the specific nature expected from their structures, but is probably due to a type of competition of the inhibitor with protein-bound tyrosine for inorganic iodide, the unidentified band being the product found between the inhibitor and the iodide.

It is stressed that in none of these cases was inorganic iodide, as such, identified; any of this material entering the tissue must be converted rapidly to the unidentified material. or to di- and moniodo-tyrosine at lower levels of inhibition.

The results which have been observed with 3 - fluorotyrosine and 3 - fluoro - 5 - iodotyrosine are consistent with the idea that synthesis of thyroxine occurs in the thyroglobulin, i.e., with the intermediate amino acids as part of a protein chain. This hypothesis is the one most commonly favored in the recent literature (168,169).

If the synthesis of the thyroid hormone proceeded through a series of "free" amino acids, beginning with tyrosine, 3 - fluorotyrosine would be expected to block this pathway at the initial iodination step, since it is a known antagonist of tyrosine in metabolic pathways (2). However, it has been shown that this inhibitor does not exert its principal action in this manner, but owes at least part of its inhibitory power to the fact that it removes inorganic iodide from the reaction. When inhibition was not complete, labelled monoiodotyrosine and diiodotyrosine were identified, but only in the thyroglobulin. The corresponding "free" amino acids were not detected. This would be expected if synthesis does occur in the protein, the "free" amino acids being normally formed later during a degradation process designed to obtain the iodide from the compounds (see page 82). If synthesis of thyroxine does involve "free" amino acids, then it must be assumed that when inhibition with 3 - fluorotyrosine (or 3 - fluoro - 5 - iodotyrosine) was not complete the amount of the "free" amino acids labelled with iodine¹³¹ had dropped below that which could be detected by the methods used here.

Sulfanilamide

This representative of the sulfanamides was reported to be inhibitory at $10^{-3}M$. (110) so a medium of this concentration was used to incubate surviving thyroid slices with radioactive iodide. The results were obtained by chromatography and radioautography as discussed before.

Using unbuffered filter paper strips :- At 10^{-3} M. sulfanilamide proved to be very similar to 3 - fluorotyrosine in its action. That is, the "free" amino acid autographs were blank except for a band identified as inorganic iodide. The autographs of the hydrolysate extracts were not indicative of much inhibition. All three of the "normal" amino acids were present, although their bands were fainter than usual, especially thyroxine which was just detectable, indicating that the enzyme system which couples diiodotyrosine to thyroxine is more sensitive to the inhibitor than the other enzyme systems.

At 2.0×10^{-3} M. the inhibition was complete. Both types of autographs were free from iodine-containing amino acids. In a few cases monoiodotyrosine was just detectable in the thyroglobulin.

On filter paper buffered to pH 6.0:- The band thought to be inorganic iodide in the above cases on closer examination proved to be different than the starch control spots in some small respects, i.e., they were not of quite identical shapes. The experiments were repeated with buffered filter paper for chromatography.

At 2.0×10^{-3} M. the "free" amino acid autographs showed the same dark spot as before, but now it was noted that its R_f did not correspond to inorganic iodide or to any known amino acid (Figure 1 (c)). The hydrolysates contained no thyroxine or diiodotyrosine but a trace of monoiodotyrosine. This is evidence that sulfanilamide acts by tying up inorganic iodide so it is unavailable for use. However, it appears to compete with protein-bound tyrosine for the iodide because the latter was still iodinated slightly in the protein.

At $1.0 \times 10^{-3}M$. this competition was still in favor of tyrosine. Scarcely any second iodination to diiodotyrosine had occurred, indicating that moniodotyrosine cannot compete as successfully as tyrosine for iodide.

A comparison of the R_F of the unknown spot obtained with sulfanilamide to that obtained with 3 - fluorotyrosine and 3 - fluoro - 5 - iodotyrosine revealed that they were not identical. The new band had an R_F value between those for moniodotyrosine and diiodotyrosine bands developed in butanol:acetic acid:water (68:2:27), while the substituted tyrosines led to an unknown band with an R_F value between those for moniodotyrosine and iodide. This is evidence that the unknown materials do involve the inhibitors themselves, since the R_F of the unknown has been altered by introducing a different inhibitory compound.

This work illustrates the errors which may result in the identification of materials during paper chromatography if more than one solvent mixture is not used to develop the chromatograms, or if the pH of the buffered filter paper strips is not varied. Here, what appeared at first to be a material identical with inorganic iodide was separated from iodide by changing the conditions of chromatography.

3 - Iodosulfanilamide:- 3 - Iodosulfanilamide was synthesized as described on page 40(183). It was chosen as one possibility for the unidentified compound being produced during sulfanilamide inhibition. 50 μg . of the substance was added as a reference material to the

filter paper strip on which had been spotted an aliquot of the butanol extract from thyroid tissue inhibited by sulfanilamide. The developed chromatogram was autographed. The film was compared to the original chromatogram on which the position of 3 - iodosulfanilamide had been made visible (197) by spraying with diazotized sulfanilic acid. It was observed that the R_f values for the two compounds were very different, (0.35 and 0.85). Therefore, the unknown produced with sulfanilamide inhibition is not 3 - iodosulfanilamide.

p - Aminobenzoic Acid

It is reported in the literature that p - aminobenzoic acid is inhibitory between 10^{-2} and 10^{-3} M. (112). Therefore, all experiments with this material were performed at a concentration of 3×10^{-3} M.

Examination of the hydrolysate chromatograms by autography revealed that almost complete inhibition of the synthetic processes had occurred in the thyroid slices, although monoiodotyrosine was occasionally detectable. It is felt that at a higher concentration of the inhibitor no monoiodotyrosine would be formed.

The autographs from the extracts of the unhydrolyzed protein once more indicated the presence of an unknown iodinated material, not corresponding to either inorganic iodide or monoiodotyrosine. The band was at an R_f slightly below that for the unknown observed with sulfanilamide but above that for the material obtained with the substituted tyrosines.

It is therefore proposed that p - aminobenzoic acid exerts its inhibitory action by competition with tyrosine (either protein-bound

or free, depending upon which is involved in the synthetic processes of the gland) for inorganic iodide with which it combines to form the as yet unidentified material observed on the chromatograms.

Thiouracil

Thiouracil belongs to a relatively large group of thyroid inhibitors which contain sulfur and are related to urea or uracil.

The glucogenic substance was incubated with thyroid slices at a concentration of 1.0×10^{-3} M. The results confirmed previous work with this material (104). Complete inhibition of synthesis had resulted. The only spot observed on the autographs from the extracts of unhydrolyzed protein was a very dark one for inorganic iodide comparable to that obtained in a normal uninhibited experiment (Figure 1 (B)).

This observation contrasts sharply with that obtained with 3 - fluorotyrosine, 3 - fluoro - 5 - iodotyrosine, and sulfanilamide. With these three, no inorganic iodide bands were present on any of the autographs. With thioracil, however, there was little doubt that the material was actually iodide, and not some other unidentified material which had an R_F value equivalent to iodide. In two solvents, butanol:acetic acid:water (68:2:27), and pyridine:ethanol:water (3:5:2), using buffered filter paper in both cases, the spot corresponded exactly with inorganic iodide.

This information confirms the report that "thio" inhibitors of the thyroid gland exert their action by preventing the oxidation

of inorganic iodide to the level of molecular iodine (123). Such an oxidation is necessary before tyrosine can be converted into monoiodotyrosine.

Thiocyanate

It was of interest to learn whether thiocyanate behaved in vitro in the same manner it did in vivo, i.e., by preventing hormone synthesis due to a blocking of the iodide concentration mechanism of the gland (115).

Potassium thiocyanate was utilized at 1.0×10^{-3} M., the results being compared to those from an experiment in which no inhibitor was present. The autographs of the "free" iodine-containing amino acids present in the uninhibited tissue slices revealed the usual very dark iodide spot plus lighter bands for the amino acids, monoiodotyrosine, diiodotyrosine and thyroxine. Thiocyanate, on the other hand, produced a light iodide band alone, but none of the amino acids. Apparently, uptake of iodide by the surviving thyroid tissue was very slight. That which did occur was probably due to straight diffusion and not to any active mechanism (120).

The autographs prepared from the two hydrolysate extracts were also compared. The control autographs showed the normal bands for the three amino acids (see page 70). The thiocyanate autograph, however, was much fainter in all respects, revealing only a light monoiodotyrosine, a very faint diiodotyrosine and no thyroxine. The slight synthesis which did occur may have been due to the small amount of iodide which had diffused passively into the gland. It indicates that the synthetic

mechanisms of the gland were still in operation but had not sufficient iodide to produce a detectable amount of thyroxine. No new or unidentified bands were present on any autographs.

Inorganic Iodide

The inhibitory power of relatively large quantities of inorganic iodide on the synthesis of thyroxine has been reported (118). Confirmation of this was obtained by the addition of 25 μ g. sodium iodide to Warburg flasks for in vitro incubation with thyroid slices.

In all cases, the material brought about almost complete inhibition of the synthetic processes in the slices. However, it did not influence uptake of iodide by the slices, as indicated by the dark iodide band present on the autographs prepared from the unhydrolyzed protein extracts.

It should be kept in mind, therefore, that the salts used in preparing the Hinger-bicarbonate medium, especially sodium chloride, may be contaminated with small amounts of iodides, so special care must be taken to obtain pure materials for this purpose.

The Effect of Varying the Order of Addition of Inhibitor and Isotopic Iodine to the Reaction Flask

The inhibitor experiments discussed previously were performed with duplicate systems, the only variable being the order in which the inhibitor and the radioactive tracer were added to the incubation medium containing the thyroid slices. In some cases, the iodide was added twenty minutes after the inhibitor and in some cases at exactly

the same time. A comparison of the results showed that usually no difference was detectable. However, in two cases, consistent differences were obtained.

p - Aminobenzoic acid at $3.0 \times 10^{-3}M$.:- If this inhibitor were added to the tissue slices before the tracer, the inhibition observed was more complete than when the iodide and p - aminobenzoic acid were added together. In the latter case, the autographs from the extracts of the hydrolyzed thyroglobulin always contained a very faint moniodotyrosine band. The amino acid was consistently absent under the former conditions. This is further evidence that moniodotyrosine is the first material produced in the synthetic pathway from tyrosine to thyroxine.

The effect observed could be explained by assuming that p - aminobenzoic acid permeates the cell walls more slowly than inorganic iodide, so that the first step in the synthesis had begun before the inhibitor became effective. When the inhibitor was added first, no synthesis would occur.

3 - Fluorotyrosine at $2.3 \times 10^{-3}M$.:- Again a difference was present in the autographs from the hydrolysates, but in the opposite direction to that observed with p - aminobenzoic acid. If the inhibitor was added before the tracer, faint bands for two of the three usual amino acids were detectable. (Moniodotyrosine and diiodotyrosine.) If iodide and 3 - fluorotyrosine were added to the tissue slices together, the resulting autograph was almost completely blank, indicating complete inhibition.

Such a result would be predicted if 3 - fluorotyrosine, when converted to the observed unknown compound with iodide was a less effective antagonist of tyrosine than 3 - fluorotyrosine is itself (2). Thus, when the material was incubated with the thyroid slices, a portion of it would be inactivated by the inorganic iodide associated with the tissue. Then when the tracer iodide was added, the isotope would be incorporated into a system which, although definitely inhibited below the normal extent of hormone synthesis, was still capable of some synthesis which would continue until the remaining 3 - fluoro - tyrosine had tied up all the tracer as the unidentified compound.

On the other hand, when both tracer and inhibitor were added together, the isotope would immediately begin to be removed at a time when the gland was being most extensively inhibited. Very little labelled amino acids could be produced until the 3 - fluoro - tyrosine level had dropped to a considerable extent. But by that time there would be very little radioactive tracer left.

Conclusions

The results with inhibitors confirm the present knowledge of the mechanisms at work in the thyroid gland. One of these is concerned with the concentration of iodide, and seems to be specifically inhibited only by thiocyanate. The remainder of the inhibitors studied do not prevent concentration of iodide by the gland, but seem to act either by tying up the inorganic iodide in some unuseable form, probably by competing with protein-bound

tyrosine or by preventing the oxidation of inorganic iodide to the level of molecular iodine. None of these inhibitors were "specific," i.e., they did not appear to block the path of synthesis at any definite point.

One pair of the inhibitors studied, 3 - fluorotyrosine and 3 - fluoro - 5 - iodotyrosine had such similar structures to two of the "free" amino acids of the thyroid, (tyrosine and monoiodotyrosine), it is felt on the basis of other work (2) that if normal synthesis of thyroxine did occur through "free" amino acids, these materials would likely have inhibited the pathway at specific points. However, when the inhibitors were incubated at concentrations lower than that required for complete inhibition, synthesis, detectable only in the protein, went past the point where the block was expected, while with higher concentrations synthesis did not occur at all.

These results are so similar to those from the other inhibitors utilized that it is suggested that all the aromatic-type goitrogenic compounds act by a similar mechanism, probably involving a tying-up of inorganic iodide.

It was noted in this section that monoiodotyrosine, bound in thyroglobulin, was the first iodinated material to be detectable in the gland. It was also the last one observed before complete inhibition as the concentration of an inhibitor was gradually increased. Again this is evidence consistent with the hypothesis that synthesis does occur in the protein.

The second material observed was always diiodotyrosine, again protein-bound, which under normal uninhibited conditions builds up to a greater concentration than moniodotyrosine. Finally, thyroxine became observable.

The reason no free amino acids were present on the autographs from the inhibited experiments, even when inhibition was not complete, could be caused by one of two things:

(1) If there exists an "equilibrium" between the iodinated amino acids bound in the protein and the "free" amino acids, it is very much in favor of protein-binding. Hence, when inhibition begins it wouldn't take much lowering of the concentration of the bound amino acids for the level of "free" amino acids to drop below the point of detection.

(2) If the "free" amino acids are liberated so that their iodide may be recovered and re-used, perhaps these same inhibitors also act upon the systems by which liberation occurs.

Reversal of 3 - Fluorotyrosine Inhibition with Tyrosine

Introduction

The results obtained with the thyroid inhibitor, 3 - fluorotyrosine, indicated that part, or all, of the action of this material resulted from an interaction of it with inorganic iodide. It was noted that except with the highest concentrations of this compound utilized - 2.3×10^{-3} M. - (when inhibition was virtually complete) there was always a small amount of labelled moniodotyrosine detectable in the thyroglobulin. One possibility was that tyrosine and 3 - fluorotyrosine were competing for the iodide. In this section several experiments were performed in which L - tyrosine was added to the incubation medium, as well as 3 - fluoro - tyrosine, to see whether the tyrosine would effect any reversal of the inhibition. The ratio of the concentrations involved for these two compounds was of the order of those reported by Kiemann (2) in his work where he had shown 3 - fluorotyrosine to be a competitive inhibitor of tyrosine in the organism, Neurospora crassa.

Experimental Procedure

To study the possible reversal with tyrosine of 3 - fluorotyrosine inhibition, the concentrations of 3 - fluorotyrosine used ranged from 1.2×10^{-3} M. to 2.3×10^{-3} M., combined with tyrosine concentrations ranging from 1.1×10^{-3} M. to 4.4×10^{-3} M.

Thus, in eight experiments, the corresponding values were:

<u>3 - Fluorotyrosine</u>	<u>Tyrosine</u>
2.3×10^{-3} M.	1.1×10^{-3} M.
1.2	2.2
2.3	2.2
1.8	4.4
1.3	2.8
1.2	2.2
1.2	2.2

Rats of both sexes were used as experimental animals. However, the thyroid tissue placed in any one reaction flask was always from rats of the same sex. The results could then be compared to see whether there were any variations attributable to sex. The overall procedure followed exactly the same form as outlined in previous sections dealing with the in vitro technique of study.

Experimental Results

Indications that reversal of inhibition was occurring in these experiments were, at first, detectable only when female rats served as the source of thyroid tissue, and when the concentrations of 3 - fluorotyrosine and tyrosine were 1.2×10^{-3} M. and 2.2×10^{-3} M., respectively.

In such cases, a "reversal" was noted to have occurred but it was observed only on the autographs prepared from the extracts of the unhydrolyzed thyroid gland. The autographs from the tissues which had been exposed to tyrosine as well as 3 - fluorotyrosine contained detectable amounts of the "free" amino acids, monoiodotyrosine,

difluorotyrosine and thyrosine, labelled with iodide ¹³¹ (Figure 1(E)), while those which had been incubated with inhibitor demonstrated the absence of any labelled amino acids (Figure 1(D)).

A comparison of the autographs with those from a normal uninhibited run revealed that the intensities of the labelled amino acid bands were darker in the reversal cases, i.e., apparently more of these amino acids were present in the "free" state than under normal conditions in the gland.

The unknown band, discussed before, (see page 92), was present both when tyrosine was present or when the inhibitor had been utilized by itself. Therefore, the "reversal" process had not prevented 3 - fluorotyrosine from tying up all the inorganic iodide which was being collected by the slices, but which was not being incorporated into tyrosine. The results expected from the hydrolysate extracts of these same experiments were not observed. In fact, the results were exactly the opposite to what would be predicted. Where 3 - fluorotyrosine and tyroxine had both been present, the labelled amino acid bands on the radioautographs were fainter and indicative of more inhibition than in the cases where just 3 - fluorotyrosine had been present. In other words, the effect of these two materials seems to be additive towards causing inhibition of the synthesis of thyroxine in the protein, instead of competitive. It was noted before that tyrosine alone does not act as a thyroid inhibitor, so the effect observed here must be due to something which occurs only when both 3 - fluorotyrosine and tyrosine are present together (see page 85). As yet, no explanation can be

presented for the phenomenon.

When glands from male, instead of female, rats were used, the results obtained indicated that very little "reversal" had occurred. However, towards the end of this work longer exposure times were allowed for the radioautographs. They then revealed that the same phenomenon of "reversal" was occurring with tissue from male rats, although to a noticeably lesser extent than with female rat glands. A comparison was made of the autographs from normal uninhibited experiments and they, too, showed almost consistently that slightly more synthesis (i.e., darker bands on the autographs) occurred with tissue from female rats than from male rats.

The experiments leading to "reversal" were repeated three times and the results were always the same. There can be little doubt that there does exist some difference in the synthesizing abilities of glands from rats of different sexes. In all cases these rats were of the same age and of nearly the same weight.

A consideration of the results obtained in this section, especially the unusual ones for the protein-bound (thyroglobulin) amino acids, indicates that the "reversal" observed in these experiments is probably not a true reversal resulting in more of the thyroid hormone being produced due to a direct competition of tyrosine with the inhibitor, 3 - fluorotyrosine. An increase in the thyroid-bound thyroxine was not noted to occur. Some other mechanism, as yet unexplained, must be involved.

The Relation Between Age of Animal and Activity of Its Thyroid Gland

The above results indicate that a difference exists in the activity of thyroid slices from male and female rats.

Another interesting phenomenon which has been observed on occasion has been a difference in the synthetic abilities of thyroid slices from different-aged rats. The small thyroid slices from very young rats (e.g. two months) were found to be just as active, and usually more so, than the much larger slices obtained from older rats. This is not unexpected. Since the thyroid controls the metabolic rate of the animal body, it would be most active when the animal is at the age of maximum growth. Young animals are known to have a higher basal metabolic rate than those which have reached maturity (201).

CONCLUSIONS

The majority of the work done in connection with this thesis has been of a developmental nature, to obtain procedures which may be used in future research work on the biochemistry of the thyroid gland. Most, but not all, of the difficulties have been eliminated in these techniques. One of the greatest problems has been the lack of reproducibility in some of the experiments, even though conditions, as nearly identical as possible, were supplied. Usually, the differences have been ones of degree rather than kind, so have not affected the qualitative conclusions which have been drawn throughout the experimental sections. However, they would become important if research on the thyroid gland were continued. It is felt that almost certainly this difficulty which remains is associated with the use of thyroid slices. Working with a tissue which is so small means that it is almost impossible to obtain slices which are always identical. But more important, when tissue slices are used, permeability problems which may not be present within the living animal arise. For example, it has been demonstrated that when a compound postulated as an intermediate in a biological reaction scheme is administered to an organism in its incubation medium, and is not utilized as expected, it does not necessarily mean that the material is not an intermediate (202). The cell walls of the organism may be impermeable to the material so that it is not getting into the actual location where the reaction is occurring. Later, in the

work referred to here, experiments with broken cell preparations proved that the compound in question was actually an intermediate, as first postulated.

With tissue slices, depending upon just where the cut occurred, it is also conceivable that quite different results could be obtained. It is believed, therefore, that if parts of this work were repeated with tissue homogenates, that is, broken cell preparations, in which these permeability problems would not exist, consistent results would be obtained. To obtain synthesis of the thyroid hormone by homogenates would first require the availability of a "complete" incubation medium containing all the necessary materials (i.e., buffer, inorganic ions, cofactors, a source of high energy phosphate, etc.). This has not yet been done, but with the present available supply of these naturally occurring, required materials, synthesis of the thyroid hormone by homogenates could be expected without too much difficulty. Once this mode of synthesis is available, it will be a gateway to many inaccessible facts about thyroid synthesis, the enzyme systems present in the gland, and the roles of cell constituents postulated as intermediates.

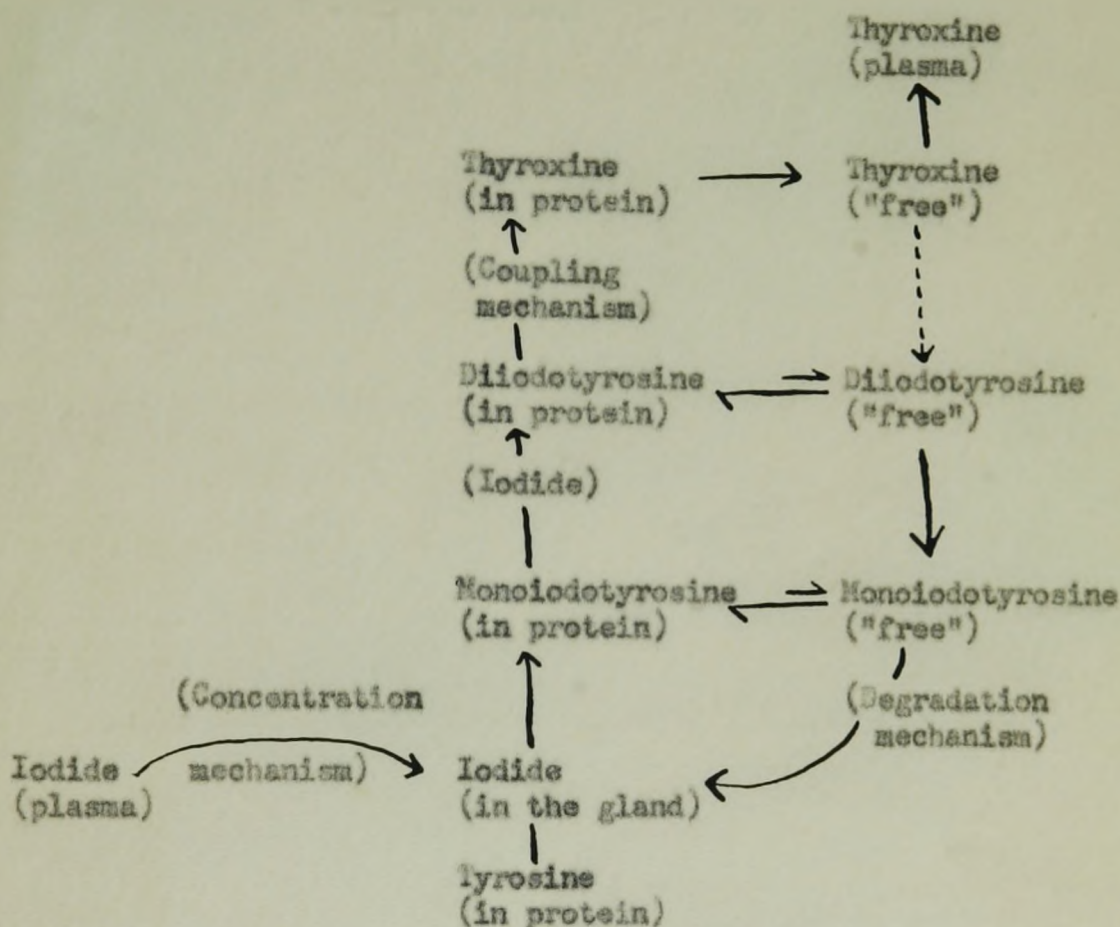
Other work which should prove of interest is the characterization of the unidentified radioactive bands which appear in the autographs from certain inhibitor experiments. Similarly, it would be of value to know the identity of the other unknown bands which occasionally appeared on "normal" autographs, and which did not correspond to any of the known compounds. Two of these may be identical with the newly-discovered iodine-containing materials of the thyroid gland or plasma, namely, triiodothyronine (177) an amino acid which has recently come

to the forefront of thyroid chemistry, and monoiodohistidine (179).

When a pure sample of an iodine-containing material which is suspected to be one of the unidentified compounds is available, the process simply involves the chromatography of the unknown material and the known compound together on a filter paper strip, followed by radioautography. If the unknown spot corresponds exactly to that of the known, good evidence would be available that the two are identical. Conclusive proof would be obtained by developing the samples in several different chromatographic solvents, buffered to various pH values. If there were always exact correspondence between the two, then one could safely say the two materials were identical. The more intermediates present in thyroid tissue, which are identified, the sooner will a complete knowledge of how the thyroid synthesizes its hormone be available.

It has been noted that in the majority of inhibitor experiments the bands for monoiodotyrosine were usually darker than any of the other active amino acid bands. When the inhibition was almost but not quite complete, monoiodotyrosine was always the only labelled material detectable in the hydrolysates. This would suggest that it is the first step in the route of synthesis of thyroxine or thyroid hormone. It would, therefore, be of interest to know whether monoiodotyrosine (or more specifically, the initial iodination, tyrosine to monoiodotyrosine) had any direct connection with the mechanism whereby iodide is concentrated in the gland. Scarcely anything is known about this mechanism except that it is inhibited by thiocyanate (112).

In conclusion, the following is a brief diagram indicating one possible route of synthesis and liberation of the thyroid hormone (assuming it to be, at this time, thyroxine).



This picture is consistent with the majority of the results discussed previously, and they may be fitted into such a scheme. However, some cannot (see page 112). Therefore, there must yet be many additions to our knowledge of thyroid biochemistry before a complete reaction system can be stated.

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