THE PROTEOLYTIC ENZYME OF THE THYROID IN THE MACAQUE MONKEY

(*Macaca mulatta*)

by

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CHAPTER I

INTRODUCTION

Diseases of the thyroid gland have been observed for many centuries. The precise mechanisms of thyroid gland function have been determined in the twentieth century in which adequate scientific techniques and equipment for studying precise function have been available. Prior to this period, basic investigations of thyroid were handicapped by the lack of suitable methods. Thyroid function has been investigated from various aspects. One of these has been the studies of the enzymatic steps that regulate hormone production, storage, and release. The present study is concerned with investigating the properties of the enzyme(s) responsible for regulating the release of thyroid hormones into the circulation.

One of the first investigations of thyroid gland function using basic scientific techniques was reported by Oswald in 1899. He reported the isolation of an iodine containing protein fraction from saline extracts of thyroid gland by precipitation in $(NH_4)_2SO_4$.[27,35] He further purified this iodine containing protein, thyroglobulin, by isoelectric precipitation. The protein

was found to be very slightly soluble in water and biologically active, manifesting the functional effects of the crude thyroid substance. A succeeding important step in the investigations of the thyroid gland function occurred in 1914 when Kendall, working at the Mayo Clinic, demonstrated the existence of thyroxine in thyroglobulin.(18,19) Sir Charles Harington confirmed Kendall's work and determined the structure of thyroxine which was subsequently synthesized by Harington and Barger.(12,13,14) These investigations laid the groundwork for an ultimate understanding of the thyroid gland function, although many years passed before thyroxine was finally accepted as the principal circulating thyroid hormone. This acceptance was principally through the investigations of Taurog and Chaikoff who, in 1947, clearly demonstrated that thyroxine was the principal circulating thyroid hormone.(43) Their findings were confirmed by Leblond and Gross and by Laidlaw.(10,22,24,25) Since then thyroxine has been repeatedly shown to be the principal circulating thyroid hormone even though it is accompanied by small amounts of triiodothyronine.

Most workers now agree that thyroxine is the principal active circulating thyroid hormone, that it is formed in the thyroid gland, and that it is secreted into

the circulation. In the blood stream it is transported in a loose association with a protein. The transporting protein is called thyroxine binding protein (TBP) and it has an electrophoretic mobility between α_1 and α_2 globulin at pH 8.6. Thyroglobulin is itself capable of binding thyroxine in a similar fashion.(16,25,46) This would provide a mechanism by which the thyroxine released from the thyroglobulin molecule could be held on its surface prior to passing into the blood stream.

It is not known whether the biosynthesis of thyroid hormone takes place within the thyroglobulin molecule or not. However, there is good evidence that it does.(46,51) That iodination of thyroglobulin bound tyrosine to form monoiodotyrosine (MIT) and diiodotyrosine (DIT) can occur has been demonstrated using microsomal-mitochondrial preparations.(45,46,51) Furthermore, the ease of coupling of diiodotyrosine derivates in vitro suggests that thyroxine is formed from bound diiodotyrosine in the gland.(38,39)

The steps theoretically involved in the biosynthesis of thyroxine are:

1. Concentration of iodide from the circulation by the gland.

2. Oxidation of trapped iodide to iodine and its incorporation into tyrosine to form MIT (organification).

$$2\ I^- \longrightarrow I_2 + 2\ e$$

$$I_2 \longrightarrow I^+ + I^-$$

$$I^+ + \text{tyrosine} \longrightarrow \text{MIT}$$

3. Further iodination of MIT to form DIT.

4. Coupling of two molecules of DIT to form thyroxine.

5. Coupling of MIT and DIT to form triiodothyronine.

Thyroxine and triiodothyronine are considered to be the circulating thyroid hormones, with thyroxine being the principal one. During transport they are loosely bound to plasma proteins, especially TBP for which thyroxine has a greater affinity. They also exist in the free state on the thyroglobulin molecule where they are held in a similar loose association. Free MIT and DIT also exist in the thyroid gland but are normally present in very small amounts in the serum.(10,44,46) This is due to the action of a deiodinase system which deiodinates MIT and DIT when they are not bound to protein and, therefore, are in the process of release from the gland. Since thyroxine and triiodothyronine are held in association with thyroglobulin and later, plasma proteins, they are not deiodinated and can pass into the circulation.(40,42)

The iodide liberated by deiodination in the thyroid gland is re-utilized by the gland in the formation of additional iodo-amino acids. This constitutes an especial system called the intra-thyroidal iodide pump and serves to increase the efficiency of the utilization of thyroid iodide in the formation of hormonal iodo-amino acids. This enzyme will not deiodinate MIT and DIT when they are bound to thyroglobulin.

In the interval between the isolation and characterization of thyroxine by Kendall and Harington and the establishment of acceptance of it as the principal circulating thyroid hormone, investigation of the mechanisms of the storage of thyroid hormone and secretion was conducted. These investigations were limited by the failure to recognize the principal secreted product. One of the arguments against the acceptance of thyroxine as the hormone was the observation that thyroglobulin had a greater effect on oxidation; an effect probably due to the triiodothyronine contained in thyroglobulin. Many of the investigations that were conducted during this period, between the identification of thyroxine and the establishment of the primary role of that hormone, were limited to the mechanism of storage and release of active hormone without identification of its true nature. These

studies generally confused the problem, chiefly because histological and histochemical techniques were the major methods of study. It is well known that these techniques have definite limitations; structures that were later shown to be artefacts of fixation and staining were frequently the basis for formulation of theories.(41,49) The nature of the colloid was not precisely known, and non-uniform staining of colloid in various follicles was thought to be of major importance. It was later shown that non-uniform staining was most often due to non-uniform fixation.(53,54) Histological and histochemical investigations led to two basic hypotheses explaining the mechanism of thyroid hormone storage and release. The idea that colloid material was produced by the follicular epithelial cells and then released into the follicle where it was stored until it was needed was basic to both hypotheses. According to one hypothesis, the hormone then flowed between the cells via inter-epithelial capillaries and lymphatics. Immediate peripheral need for hormone could be met by its direct secretion into the blood stream from the epithelial cells. Thus, a two-way stream for hormone secretion was postulated.(9,49,50) The observation of an inter-epithelial capillary network in submammalian vertebrates was the fundamental basis for this hypothesis.(15)

The alternate hypothesis proposed that colloid escaped through the follicular cells and the intercellular spaces by simple diffusion.(41,53,54,55) This hypothesis was prompted by the observation of cyclical filling and emptying of individual follicles studied by trans-illumination.(53,54) The content of each follicle was thought to be determined by the rate of secretion into the follicle and the rate of diffusion from it. Secretion and diffusion would depend equally on the functional activity of the follicle. The follicle was supposedly polarized, and therefore, no direct secretion of colloid from the cells into the blood stream occurred. Other support for this hypothesis was the experimentally observed passage of colloid through the inter-epithelial spaces during ultra-centrifugation.(31)

The mechanisms of storage and release of thyroid hormone were further developed by investigations which identified thyroglobulin as the primary constituent of the colloid. The large size of the thyroglobulin molecule to which the epithelial and endothelial cells were impermeabile raised doubts as to the validity of the previous theories. These doubts were reinforced by the failure to find circulating thyroglobulin in euthyroid subjects.(26) This suggested that fragments of

thyroglobulin were split off by proteolysis and that they passed into the circulation as small molecular weight peptides.(9) Initial experimental evidence supporting this suggestion was the demonstration of a proteolytic enzyme in the follicular fluid of rat thyroid.(5) Subsequent investigations have confirmed the presence of a thyroid proteolytic enzyme.(1,2,3,6,8,17,23,32,33,52) These investigations, coupled with the proof that thyroxine and triiodothyronine are the circulating thyroid hormones, have led to general acceptance of the theory that a proteolytic enzyme(s) is critical for secretion of stored thyroid hormones.

Additional investigations have helped our understanding of iodine metabolism in the thyroid. These investigations have helped to identify the site of action of the proteolytic enzyme(s). Approximately one-third of thyroid gland iodide is present as hormonal iodide. Approximately 2% of this is non-protein iodide and occurs as iodinated amino acids and possibly some peptides, all of which have been or will become part of thyroglobulin. Thought these fractions constitute a small percentage of the total gland iodine, they are critically important.(42,46) Thyroxine iodide comprises three-fourths of the hormonal iodide.(42) The remaining iodide is

present in various other amino acids and is retained within the thyroid by the deiodinase intra-thyroidal pump.

Thyroglobulin is a relatively large molecule with a molecular weight of 670,000.(7,27,42) It is produced in the follicular epithelial cells and is secreted into the follicle where it is stored as colloid. Evidence suggests that the biosynthesis of the thyroid hormones occurs intracellularly within the thyroglobulin molecule.(46,51) This, plus the fact that thyroid hormones but not thyroglobulin are normally present in the circulation, suggests that these hormonal iodo-amino acids are released from thyroglobulin. The best evidence suggests that they are released by a proteolytic enzyme.

Investigations of the Proteolytic Enzyme(s) of the Thyroid

Relatively few studies of this enzyme(s) have been done since De Robertis first demonstrated its existence in 1941. The studies have been chiefly concerned with the demonstration of its presence, the relationship of its activity during various physiological states, and its action against various substrates.

In 1941 De Robertis demonstrated the existence of an intra-follicular proteolytic enzyme in rat thyroids.(5)

He used a micro-dissection apparatus to remove intra-follicular material from the isthmus of the gland. He placed this material on x-ray films and noted proteolytic activity toward the gelatin. Activity was observed at pH 6.6 and 7.0, the normal pH of the follicle. Maximal activity was observed at pH 3.0 and negligible activity at pH 8.2. De Robertis found that the activity of this enzyme(s) could be changed by altering the physiological status of the animal with administration of potassium iodide (KI) or pituitary thyroid stimulating hormone (TSH).

The properties of this enzyme(s) have been further investigated in various physiological states in a more quantitative manner by Dziemian in 1943, who used glycerol-water extracts of rat and guinea pig thyroids.(8) Edestin was used for the substrate and proteolytic activity was measured by micro-acetone titration of protein. The activity of the thyroid proteolytic enzyme(s) was increased for the first 48 hours after injection of TSH, returning to normal at 72 hours. Activity varied with the duration of KI therapy. Cysteine was found to activate the enzyme whereby the suggestion was made that it was a catheptic type enzyme. In general these studies confirmed those of De Robertis.

In 1946 De Robertis and Nowinski reported on investigations of the proteolytic activity in normal and

abnormal human thyroid glands.[6] Glycerol-water extracts were used for the enzyme source, and edestin was used as the substrate. Proteolysis was estimated by the amount of tyrosine and tryptophan liberated. The results confirmed those of Dziemian, suggesting that the enzyme was of the catheptic type and that the optimal pH was near 4.0. Activity varied in patients with normal thyroid glands, severe diffuse toxic goiter, mild toxic goiter, and simple diffuse goiter. Proteolytic activity was highest in those with severe diffuse toxic goiter, lowest in those with simple diffuse non-toxic goiter, and below normal in patients with toxic goiter treated with iodine suggesting correlation with the TSH effect on the gland.

In still other investigations, the quantitative estimation of thyroid proteolytic enzyme activity was performed in glycerol extracts of dog thyroid tissue slices.[17] Beef hemoglobin was used for the substrate. Activity was based upon the amount of tyrosine liberated after 24 hours incubation at 37° C. The effect of cysteine to increase proteolytic activity was again confirmed, further establishing the catheptic nature of the enzyme. The observed pH optimum was 3.55. Heat inactivation of enzymic activity by boiling was demonstrated.

The previous experiments proved the existence of the enzyme and defined some of its properties. However, none had attempted to define the enzyme or its action in comprehensive terms. The first such approach was that of McQuillan and co-workers using a partially purified enzyme preparation made from hog thyroids.(32,33) The substrate was a purified I^{131}-labelled (*in vivo*) thyroglobulin. Chromatogram radioautographs were made to determine the products of hydrolysis. The specific activities of these substances on the chromatogram were compared with those liberated by alkaline hydrolysis of the labelled thyroglobulin. The major iodine containing compounds liberated by hydrolysis were MIT, DIT, thyroxine, triiodothyronine, and a "peptide" fraction. Of these, MIT, DIT, and "peptide" predominated. Proteolysis, using labelled synthetic iodopeptides as substrates, indicated that the enzyme(s) possesses activity at pH 3.5 which preferentially attacked bonds joining aromatic amino acid residues. They concluded that, in this respect, the thyroid enzyme would behave similarly to pepsin.

Another systematic investigation of the thyroid proteolytic enzyme(s) was made by Weiss using a partially purified fraction of thyroid homogenate.(52) Proteinase and also peptidase activity were evaluated. Hemoglobin

was used as the substrate for estimation of proteinase activity and synthetic di- and tri- peptides for estimation of the peptidase activity. Proteinase activity was demonstrated at pH 4.0 and peptidase activity at pH 7.8. Minimal proteinase activity occurred at the higher pH; peptidase activity was not measured at the lower pH. Proteinase activity was augmented in the presence of cysteine and ascorbic acid. It was diminished in the presence of p-chloromercurobenzoate, Cu^{++}, aqueous iodine, iodoacetate and sulfide. These studies again confirmed the cysteine activation previously noted, indicating the probable necessity of sulfhydryl groups for activity, as has been suggested for the cathepsin group of enzymes. No effect on proteinase activity was observed in the presence of thyroxine, Pb^{++}, Zn^{++}, Mg^{++}, Fe^{++}, Co^{++}, and Cd^{++}. Temperature effects were studied by heating the preparation in a water bath for five minutes at the desired temperature and then cooling them prior to use. No appreciable inactivation was noted by heating at 50° C, but beyond this temperature proteolytic activity was decreased and at 65° C no activity remained. Subcellular fractionation studies revealed that proteinase activity did not partition itself into a fraction but was distributed throughout, concentrating primarily in the nuclei and mitochondria.

The conditions necessary for optimal activity of the enzyme were investigated independently by Abelin and Bigelow.[1] This study is important for two reasons. First, it serves as a model for characterization of certain properties of the enzyme against a thyroglobulin substrate. Second, it represents an attempt to correlate general proteolytic effects with those of specific importance, namely, the release of iodinated products. It substantiated the results of previous studies regarding temperature and pH optima and cysteine activation and defined them more specifically.

They used a fraction of thyroglobulin that contained enzyme in high concentration. They investigated the influence of temperature, pH, cysteine concentration, and storage conditions upon enzyme activity. Proteolytic activity was estimated by the analysis of free amino-nitrogen and by the presence of 5% trichloroacetic acid (TCA) soluble iodine after proteolysis. Iodo-proteins are precipitated by 5% TCA whereas the products of hydrolysis are not. At conditions of optimum pH and temperature, 50% of previously 5% TCA insoluble amino-nitrogen was soluble by nine hours. After nine hours, the rate of proteolysis decreased but continued for 72-96 hours. The effect of pH was investigated over the range 3.0 to 7.0 at

0.25 unit increments. Optimal activity was found in the region 4.5 to 4.8. Cysteine activation was studied and found to activate the enzyme maximally at a concentration of 0.08 grams percent per 100 ml. It increased proteolysis by 42%. Double this amount of cysteine caused no additional increase in proteolysis. Temperature effects were studied. Samples were incubated at 40°, 50°, and 60° C. At 50° C, proteolytic activity was decreased 38% from that at 40° C and at 60° C, decreased 70%. Studies of the effects of storage on stability revealed that at pH 7.0, 2% of activity was lost by storage at -10° C by 15 days, and 10% of activity was lost by storage at 0° C by 15 days.

Another fundamental study was done by Alpers, Robbins, and Rall, who investigated the activity of the enzyme(s) on a purified thyroglobulin extract.(2) They used phosphate purified I^{131}-labelled rat thyroglobulin for the substrate, and an isotonic saline extract of rat thyroid tissue for the enzyme source. Their aim was to define the properties of the substrate as well as those of the enzyme. Proteolysis was estimated by salting-out, dialysis, electrophoresis, and chromatography. Activity was measured by the I^{131} present in the soluble fraction after salting-out and in the dialysate. Chromatography was used

to separate the products of hydrolysis, and the I^{131} in each fraction was measured to quantitate them. Electrophoretic studies were done to observe the effects on the substrate. Results from the salting-out experiments revealed 55% of the radioactivity was present in the soluble fraction. No additional hydrolysis occurred after 48 hours. The pH optimum was 4.9, and no hydrolysis took place at pH 6.6-6.7. Dialysis experiments concurred with the above. Thirty-five to 50% of the I^{131} was removed by dialysis after 24 hours incubation at 37° C and in the pH range 3.5-5.0. No radioactivity was present in the dialysate in non-incubated specimens. Dialysis prior to incubation did not affect proteolysis. Chromatography showed all of the I^{131} at the origin in untreated samples. Peaks of radioactivity characteristic of MIT, DIT, thyroxine, and inorganic iodide were seen. The fraction of radioactivity present in each compound was comparable in all of the experiments. The values were MIT 9%, DIT 15%, thyroxine 6%, and inorganic iodide 4%. Electrophoretic studies revealed denaturation of the substrate was a primary step in the enzymatic process.

The above studies were followed by ultracentrifugal studies on thyroglobulin during hydrolysis.(3) The aim was to observe the substrate during proteolysis.

Proteolysis was measured by the same means as in the previous experiment, salting-out, dialysis, chromatography, and electrophoresis. Samples were incubated at 37° C for 35 hours at pH 5.2. Control specimens were not incubated. Thirty-three per cent of the original radioactivity was present in the soluble fraction in salting-out studies. Electrophoresis of the precipitate revealed the major radioactive peak between α_1 and α_2 globulin, characteristic of denatured thyroglobulin. Dialysis removed 46% of the initial radioactivity. No iodinated amino-acids or iodide were present in the non-dialysable fraction. Electrophoresis showed a loss of radioactivity in the albumin fraction (diiodotyrosine). These findings confirmed those of the previous study.

These ultracentrifugal analyses by Alpers, Petermann and Rall revealed that only 19% of the thyroglobulin was unhydrolyzed, one-third in the form of native thyroglobulin and two-thirds as denatured thyroglobulin. Partition cell studies revealed that 36% of the original radioactivity passed the partition. Six per cent was thyroglobulin; 15% was large fragments of substrate in various states of aggregation, and the rest was a slowly sedimenting material. Salting-out data agreed with this. Thirty-four per cent of the initial radioactivity had the insolubility in phosphate

characteristic of thyroglobulin. Assuming the partition cell studies to be accurate, 6% of this was unchanged thyroglobulin and 28% was material which resembled thyroglobulin in solubility but not in sedimentation behavior. This was thought to be the slow sedimenting material.

The above observations led to the postulation that the hydrolysis of thyroglobulin by this enzyme liberated intermediate substances of many sizes. This would be compatible with the proposal by Linderstrom-Lang that a proteolytic enzyme works in a zipper fashion, attacking one peptide bond in every molecule before attacking other bonds.(28) This would result in a mixture of fragments of assorted sizes as was observed. This type of reaction would depend upon an initial denaturation or unfolding of the molecule which would open it up and expose bonds for further proteolysis.

Studies pertaining to the nature of thyroglobulin have further contributed to the above hypothesis.(29,30,34) These studies revealed a reversibly, partially denatured form of thyroglobulin, the " α " form to be present under certain conditions.

Native form $\rightleftharpoons$ " α " - Form $\rightarrow$ Denatured form

Papain hydrolysis of thyroglobulin in preparations which favored the " α " form was studied. The sedimentation pattern of the hydrolysate showed that the " α " form was broken down most rapidly and that it was broken down by the interrupted type of proteolysis. Papain was also active against native thyroglobulin, and broke it down in a one-by-one manner.(34) These studies are of additional interest since papain behaves similar to the cathepsin type enzymes.

Characteristics of the Proteolytic Enzyme(s) Resolved by the Previous Studies

The previously cited experiments permit certain conclusions to be made relative to the proteolytic enzyme(s). The existence of a thyroid gland proteolytic enzyme(s) has been demonstrated. It behaves as if it were a cathepsin. It is active in the pH range 2.5-5.2 and has optimal activity between pH 4.0 and 4.9. It is activated by cysteine. It is thermolabile, being greatly inactivated at temperatures over 50° C and almost totally inactivated above 65° C. Dialysable components are not necessary for its activation or activity.

Its proteolytic effects have been evaluated against various substrates, including its most logical natural

substrate, thyroglobulin. It is capable of hydrolyzing the iodo-amino acids from thyroglobulin. Its activity has been compared to and is less than that of pepsin in its ability to hydrolyze thyroglobulin. Variation in activity has been demonstrated by *in vivo* alteration of the physiologic state of animal and human thyroid metabolism. *In vitro* studies with some of the same drugs that were used to alter *in vivo* thyroid function have not produced similar effects.(4)

Discussion of the Previous Experiments

The thyroid proteolytic enzyme(s) is of physiologic importance in the release of hormonal iodo-amino acids from thyroglobulin. The previously cited studies are limited by the fact that no quantitative chemical measurements of these compounds were made. The studies based upon the I^{131} radioactivity present in the hydrolytic products, which were separated by chromatography, cannot be accepted as a quantitative measure of those compounds because the total quantities of the fractions present were not measured. Solubility differentiation of I^{127} and I^{131} during dialysis, salting-out, and acid precipitation indicates iodide containing compounds have become separable from thyroglobulin after incubation with an enzyme containing preparation of the thyroid. However, this does not

indicate the precise nature of these substances nor their relative amounts. The limitations imposed by using non-specific parameters for estimating and defining proteolytic activity was commented upon by one group.(3)

The estimation of proteolytic activity by indirect chemical means, that is, the measuring of tyrosine or terminal amino-nitrogen, has merit when substrates other than thyroglobulin are used; however, thyroglobulin is the natural and most biologically important substrate. In most of the cited studies a truly purified preparation of the enzyme was not used and thyroglobulin was present in the enzyme preparation. Consequently, some of the tyrosine that was measured may have been that released from the thyroglobulin in the enzyme aliquot rather than all of it coming from the substrate. In the studies in which substrates other than thyroglobulin were used, a preferential release of tyrosine from thyroglobulin rather than the artificial substrate may have taken place.

CHAPTER II

PRESENT STUDY

Proposal of the Present Study

The aim of the present study is to affirm some of the characteristics of the thyroid proteolytic enzyme(s) using hormonal iodide released as the parameter for measuring activity. This represents the biologically active end-products of hydrolysis from the natural substrate, thyroglobulin. They are the products that are released *in vivo* if the proteolytic enzyme(s) acts in its proposed role.

The measurement of hormonal iodide was possible by using a micro-method for measuring butyl-alcohol extractable iodide, the hormonal iodide, which was developed in this laboratory. Use of this method has made the present study possible with the small amounts of tissue that were available.

The present study was divided into two parts: (1) the demonstration of the existence of the proteolytic enzyme(s) in the Macaque monkey (*Macaca mulatta*) during the neonatal period of life, and (2) the investigation of some of the properties of the enzyme(s).

The neonatal period was chosen because it is a critical period of thyroid function. Disruption or cessation of activity during this period results in many well known primary and secondary clinical changes. The onset and development of function and the importance of the thyroid in fetal life is not well known; however, the chronological appearance of important enzymes can be inferred by the appearance of histologically recognizable structures at different stages of development. An abrupt need for endogenous thyroid hormone occurs during the perinatal period.(36) Increased proteolysis would be logically thought to take place as part of the mechanism of increased secretion of hormone during this period. If the enzyme(s) functioned at a critical level during this period, differences in activity might be expected to appear at different stages. With this in mind, the activity at various ages in the neonatal life of the Macaque monkey (Macaca mulatta) was studied. The previously described properties of the enzyme were tested to affirm their presence and to ascertain the properties of the enzyme in primate thyroid glands for the present and future investigations.

CHAPTER III

METHODS EMPLOYED IN THE PRESENT STUDY

Preparation of Tissue

Fresh, I^{131}-labelled (*in vivo*), thyroid glands were obtained from sacrificed newborn Macaque monkeys (*Macaca mulatta*). The connective tissue was cleaned from them with a Bard-Parker #11 blade and by blotting on gauze. The cleaned gland was then weighed on a Roller-Smith balance and was homogenized in approximately 3 ml of 0.11 M NaCl-0.04 Tris (hydroxymethyl) aminomethane buffer (Tris buffer) in a motor driven, all glass, tube and pestle tissue homogenizer which was placed in an ice-water bath. Homogenization was judged to be complete when an even suspension (devoid of floating sedimenting particles) was obtained. The homogenate was transferred to a 10 ml volumetric flask and brought to volume with Tris buffer that had been used to rinse the homogenizer tube and pestle. The pH of the homogenate in Tris buffer was 8.1.

An aliquot of the homogenate was transferred to a volumetric flask and brought to volume using a 0.025 M sodium acetate-acetic acid buffer (pH 4.49) as the

diluent. The volume of the aliquot and the final volume depended upon the weight of the thyroid gland. A final concentration of approximately 1.0 mg thyroid tissue per ml was sought. The range of concentrations obtained was approximately 0.60-1.1 mg thyroid per ml. This amount of tissue contained the most satisfactory amount of thyroxine that could be determined by the micro-butyl-alcohol extractable iodine method. Cysteine as cysteine hydrochloride was added to the homogenate with dilution with the acetate buffer to give a cysteine concentration of 0.08 grams per cent. Toluene was added as a preservative. In some of the specimens the remainder of the homogenate in the Tris buffer was digested with pancreatic enzymes and the iodo-amino acids and iodide were determined chemically and radiochemically after separation by paper chromatography.

Preparation of Samples for Chemical Analysis

One ml aliquots of the homogenate in acetate buffer were transferred to glass stoppered tubes. Five drops of 10% H_2SO_4 (v/v) from a 5 ml blow-out serological pipet were added to those tubes which were to serve as controls. The pH after that amount of acid had been added was 0.90-1.0, as determined on the Beckman model G portable pH meter.

The enzyme(s) being studied has been reported to be inactive at this pH.(1) The reaction samples were incubated at 37° C for varying periods of time in an uncovered water bath. Five drops of 10% H_2SO_4 (v/v) delivered from a 5 ml blow-out serological pipet were added to these tubes at the end of their period of incubation. In a separate group of experiments to be described subsequently, the homogenate was placed in the glass stoppered tubes and the tubes placed in a boiling water bath for five minutes. Acid was then added to those that were to serve as controls and the others were incubated at 37° C along with samples handled in the manner described above.

The homogenate was incubated as soon as it was prepared; at the same time acid was added to the control specimens which were not incubated. In most of the experiments the hormonal iodide (thyroxine iodide) was determined immediately after the addition of the H_2SO_4 which inhibited enzyme(s) activity; however, in some of the later experiments immediate determination of hormonal iodide was not feasible and the samples were refrigerated for periods of one to three days before analysis. Previous studies had shown that no enzyme activity exists under these conditions of refrigeration (6° C) and acidification (pH 1.0).

Preliminary Study to Determine the Products of Hydrolysis

A paper chromatographic analysis of incubated, pooled, homogenized I^{131}-labelled (*in vivo*) rat thyroid glands was done to determine the products liberated during the 24-hour incubation period. Radioautograms were made from the chromatograms. Pooled, I^{131}-labelled, rat thyroid homogenate was used for this study because a concentrated thyroid homogenate of pH 4.2-4.5 was needed. This could not be supplied with the monkey thyroid glands since their disposition was fixed and none could be spared for such investigations. The rat thyroids were homogenized in 3 ml of normal saline in the manner described under the section describing preparation of tissue.

The homogenate was diluted with 0.025 M sodium acetate-acetic acid buffer (pH 4.49) and cysteine and toluene were added. One aliquot was placed in a glass stoppered tube and incubated for 24 hours in an uncovered water bath. Aliquots of the non-incubated aliquots were placed directly on paper for chromatographic analysis. Aliquots of the incubated homogenate were placed on the paper at the completion of incubation. The chromatograms were immediately developed. It was assumed that no proteolysis would occur after the sample had been placed

on the chromatography paper and dried. The chromatograms were developed with collidine-water (100:35) in an ammonia atmosphere and butanol-dioxane-ammonia (4:1:5). Descending chromatography was used in both instances.

Chromatography

Aliquots of the I^{131}-labelled, pooled, rat thyroid gland homogenate were applied in streaks to Whatman number 3 filter paper. This paper was found by our laboratory to be the most satisfactory for separation of iodide and iodo-amino acids liberated during hydrolysis and for the chemical analysis of them when those areas were cut from the paper and analyzed for I^{127}. Such findings are in agreement with those reported by Kowkabany.(21) The paper was found to be free of contaminants which could interfere with the determination of I^{127}.

The paper was laid on a dry, suitably cleaned glass plate. A light pencil line was drawn across the width of the paper 5 cm from an end. Eight cm from the same end an interrupted parallel light pencil line was drawn. This line extended 2.5 cm in from each edge and continued after leaving 3.5 cm unlined intervals. This left two unlined 3.5 cm areas in which the sample could be applied in streaks which could be aligned with the straight pencil line. A 10 ml serological pipet was placed under the paper

near the area where the specimen was applied, elevating it from the glass surface and serving to prevent the sample from blotting and being lost on the glass. The line 5 cm from the end was used as a guide for folding the paper to place over the glass rod during development.

The sample was applied in 3.5 cm streaks on the designated areas with 25 ul capillary pipets. After each application the streak was dried with a hair dryer before additional sample was added. Cold air was used since it was felt that warm air might alter the protein in the homogenate causing false products to be released.

The chromatograms were developed by descending chromatography using collidine-water in an ammonia atmosphere and n butanol-dioxane-ammonia as solvents. The ammonia atmosphere was achieved by placing a beaker of concentrated NH_4OH in the tank. The chromatograms were developed in an all glass tank covered with a fitted plate glass top and sealed with vacuum grease. The glass top was weighted to maintain an airtight seal during the period of development. Development proceeded for 16 hours at room temperature which ranged from 20-22° C. The papers were dried in a chromatography oven at a temperature ranging from 80-90° C for approximately two hours. Separation of the iodo-amino acids and iodide had been satisfactory using these methods

in our laboratory.(37) They incorporate modifications of procedures reported elsewhere.(10,44)

Radioautography

The solvent front and origin were marked with drops of I^{131} and the paper was exposed for four hours to non-screen x-ray film. The developed radioautogram was used to identify the iodo-amino acids and iodide in the samples. These were judged by comparison of their location with the location of them on chromatograms which had been marked with I^{131}-labelled MIT, DIT, triiodothyronine, thyroxine, and iodide. The location was also ascertained by comparison of location with published R_f values of these compounds separated by paper chromatography and developed with these solvents and of pictures of similar radioautographs of chromatograms developed with these solvents.(11)

Chemical Analysis of Thyroid Hormonal Iodide

Thyroid gland hormonal iodide was determined by the micro-method for the estimation of butyl-alcohol extractable hormonal iodide described by Kontaxis and Pickering.(20) This method affords a specific means for evaluating the hormonally active iodo-amino acids.

Chemical Analysis of Thyroid Gland Iodide

Thyroid gland total iodide content was determined by a modification of the butyl-alcohol extractable thyroid hormonal iodide method. The modification consisted of foregoing the n butyl-alcohol extractions and Blau's Solution (4N NaOH - 5% Na_2CO_3) washing of the extract. Thyroid gland homogenate was evaporated with KNO_3 and Na_2CO_3 and ashed in the muffle furnace and the iodide analyzed by the ceric-arsenious oxidation-reduction reaction just as in the determination of hormonal iodide.

CHAPTER IV

RESULTS

Special Preliminary Study

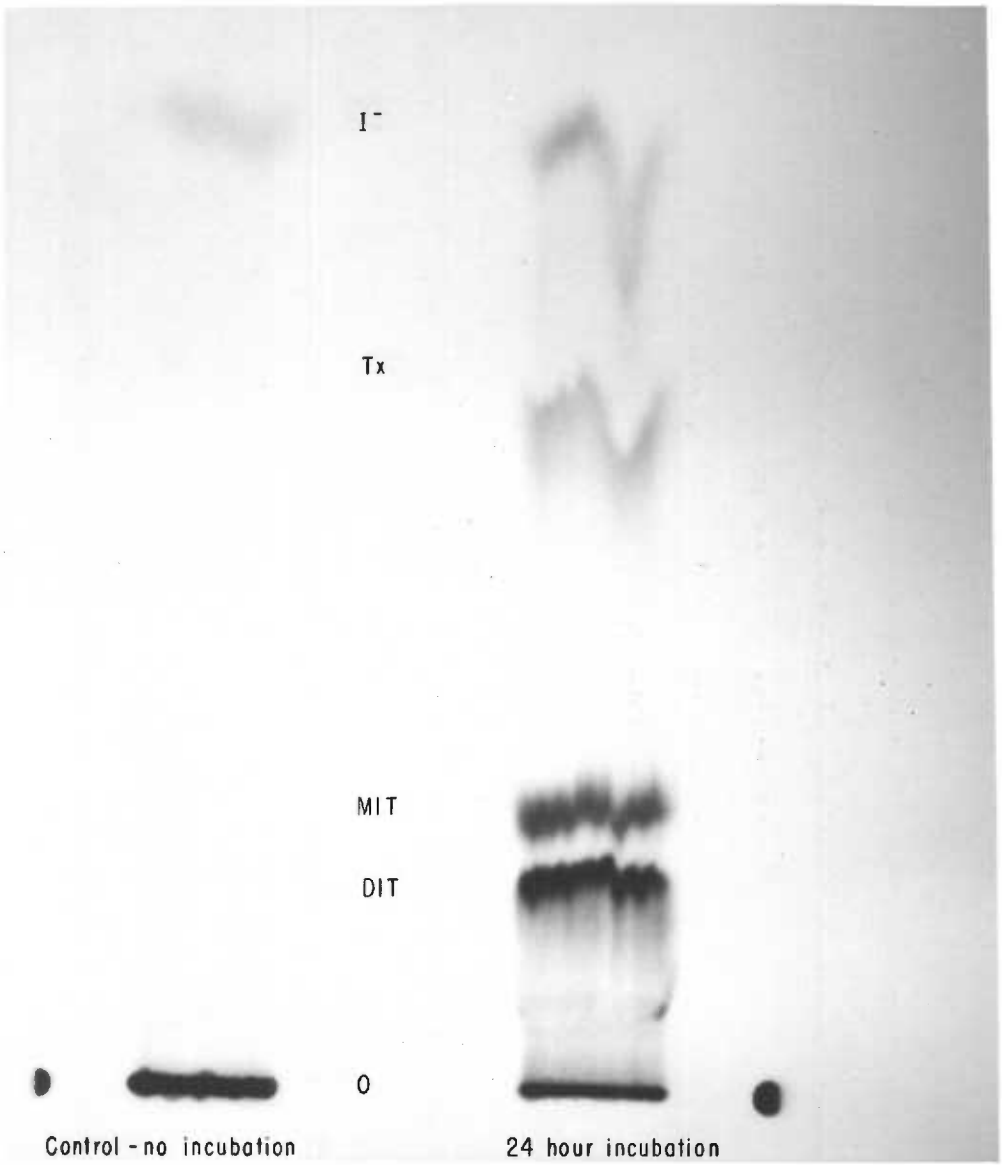
A preliminary study was performed to determine the products of enzymic hydrolysis in thyroid glands. Rat thyroid glands were used to conserve monkey glands. Pooled, I^{131}-labelled (*in vivo*), rat thyroid glands were homogenized and processed, and the products of hydrolysis were determined by paper chromatography and radioautography. Collidine-water-ammonia and butanol-dioxane-ammonia were the solvents used for the chromatographic separation of products. Non-incubated homogenates were also chromatographed and radioautographed to serve as controls. The radioautograms in Figures 1 and 2 validate the method and clearly show the separation of iodo-amino acids released during incubation.

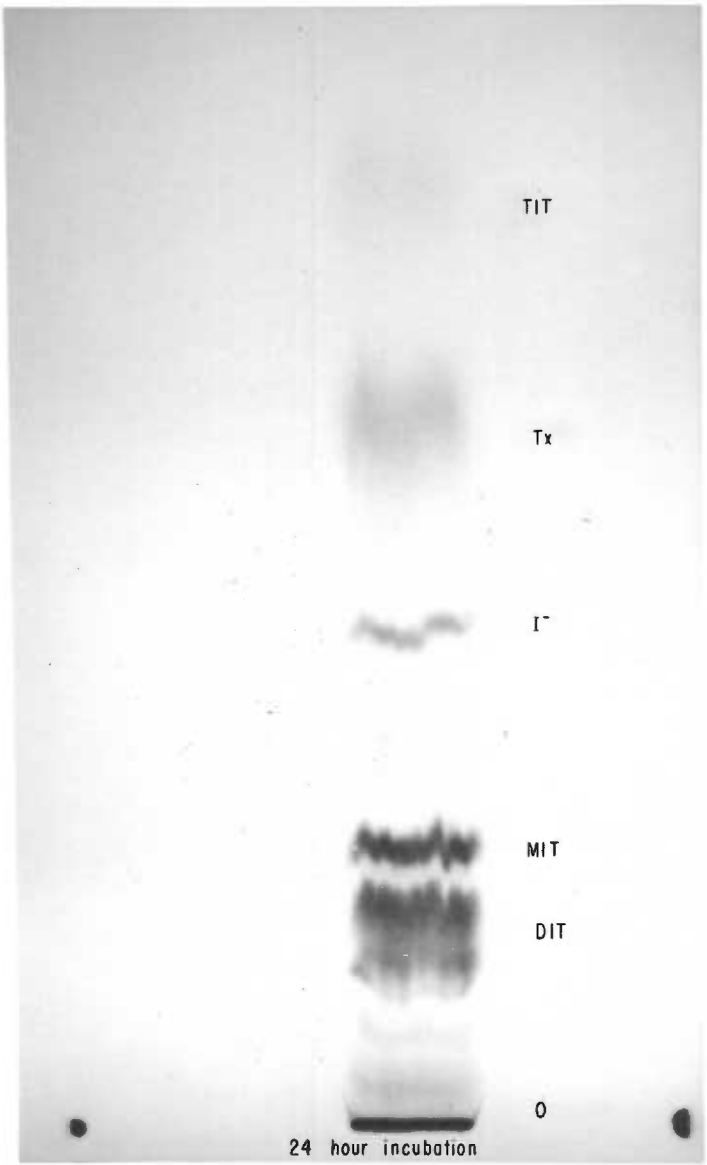
Figure 1

Products separated using butanol-dioxane, ammonia. TIT = Triiodothyronine; other symbols are the same as those used in Figure 2.

Figure 2

Products of hydrolysis separated using collidine-water in an ammonia atmosphere. O = origin; MIT = monoiodotyrosine; DIT = diiodotyrosine; T_X = thyroxine; I^- = inorganic iodide.


I⁻
Tx
MIT
DIT
O
Control - no incubation
24 hour incubation


TIT
Tx
I⁻
MIT
DIT
O
24 hour incubation

Part I

The Presence of the Proteolytic Enzyme(s) in Thyroid Tissue During the Neonatal Period

Thyroid homogenates of freshly sacrificed neonatal Macaque monkeys (Macaca mulatta) of different conceptional ages[1] were prepared in the manner described under the section on methods. The homogenates contained 0.6-1.5 mg thyroid tissue per ml (the majority contained 0.7-0.85 mg thyroid tissue per ml). The homogenates were incubated for 24 hours in a 37° C water bath. The proteolytic enzyme was inactivated at the conclusion of incubation by the addition of five drops of 10% H_2SO_4 which lowered the pH of the homogenate to approximately 1.0, at which the enzyme(s) is inactive. Activity was expressed as the fraction of hormonal iodide expressed as micromoles of thyroxine which was released per mg of thyroid tissue during incubation. Such activity was measured in glands from various neonatal age groups. The results are listed in Table I.

---

[1]Conceptional age is the age in days of the animal dated from the exposure time of the mating partners.

TABLE I

THYROID PROTEOLYTIC ENZYME ACTIVITY IN NEONATAL PRIMATES

Conceptional Age in Days	Animal Number	Gland Weight (mg)	mg Thyroid Hmg Incubated	Total T_g, T_x per mg Thyroid (1x10^3 u Moles)	Free T_x per mg Thyroid (1x10^3 u Moles)	T_x Released During 24-hrs. Incubation (1x10^3 u Moles)	% Hydrolysis	Total Gland T_x (1x10^3 u Moles)[1]
200	PP 59-5	160	1.06	0.257	0.011	0.159	61	43.5
	PP 59-11	183	0.73	0.320	0.005	0.120	37	58.5
	PP 59-16	214	0.86	0.238	0.010	.039	16	50.8
225	CP 59-4	175	0.70	0.564	0.021	0.073	13	103
	CP 59-5	147	0.59	0.180	0.032	0.076	42	31
	PP 59-6	119	0.94	0.200	0.010	0.045	22	24.7
	PP 59-12	204	0.81	0.31	.022	0.025	8	63.8
250	PP 59-4	191	1.55	0.429	0.011	0.075	17	84.8
	CP 59-7	296	1.04	0.385	.008	0.032	8.5	114
	PP 59-7	130	1.11	0.198	.007	0.038	19	23.8
	PP 59-8	107	0.86	0.174	.004	0.086	50	18
	PP 59-9	187	0.75	0.306	.056	0.063	21	68.5
	PP 59-10	156	0.78	0.221	.034	0.034	15	40

[1]Determinations of total gland thyroxine iodide were kindly performed by Miss K. Settergren.

Part II

Properties of the Enzyme

Incubation Time and Enzyme Activity. The degree of hydrolysis at different incubation times was studied in tissue preparations from animals of different age. Thyroid gland samples were hydrolyzed for periods of time ranging from six to 72 hours. The results are presented in Table II and data for animals of conceptional age 250 days are plotted in Figure 3. Each individual point on the graph represents three or more analyses.

TABLE II

INFLUENCE OF INCUBATION TIME

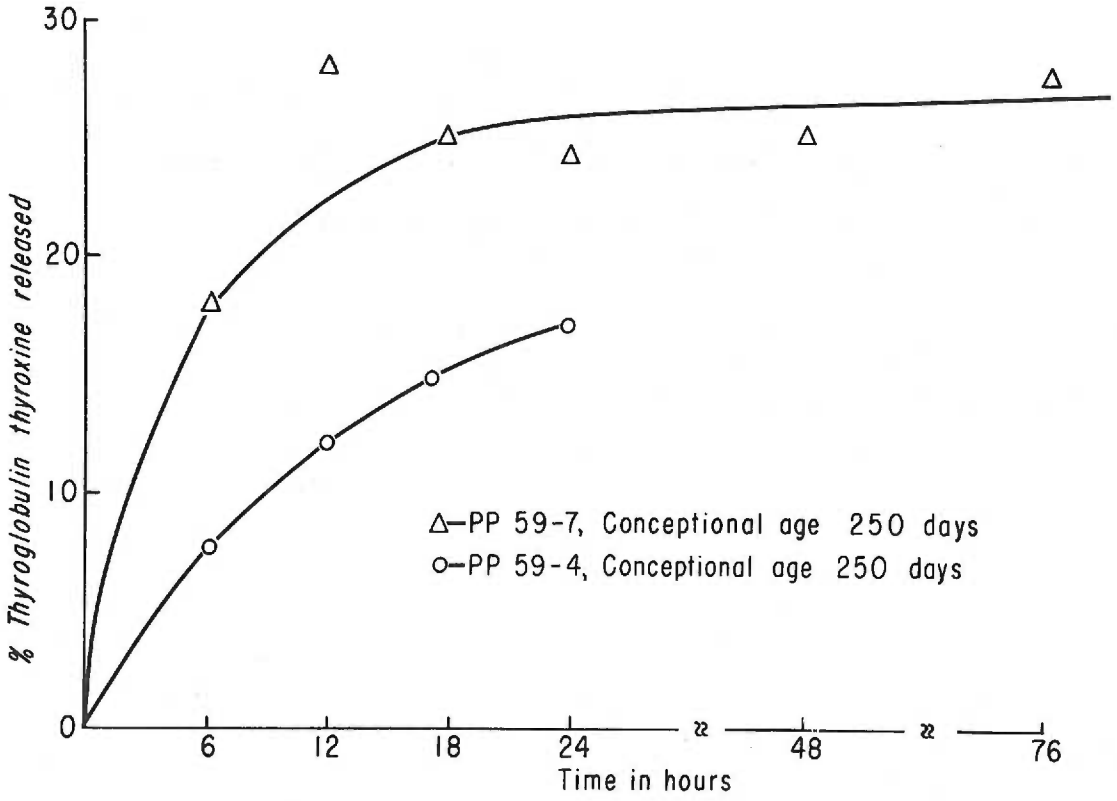
ON ENZYME ACTIVITY

Hours Incubation	% Hormonal I^- Released PP 59-7	% Hormonal I^- Released PP 59-4
6	18	7.6
12	28	12
18	25	15
24	22	17
48	26	----
76	28	----

Figure 3

Progress curve to show the influence of incubation time on enzyme activity.

INFLUENCE OF INCUBATION TIME ON ENZYME ACTIVITY



Influence of pH on Enzyme Activity. Homogenates were adjusted to pH 4.1 and 8.2 and incubated for 24 hours at 37° C. This was done to determine if the peptidase reported to be active at the higher pH value would be able to hydrolyze the thyroglobulin present in the homogenate. The results are presented in Figure 4.

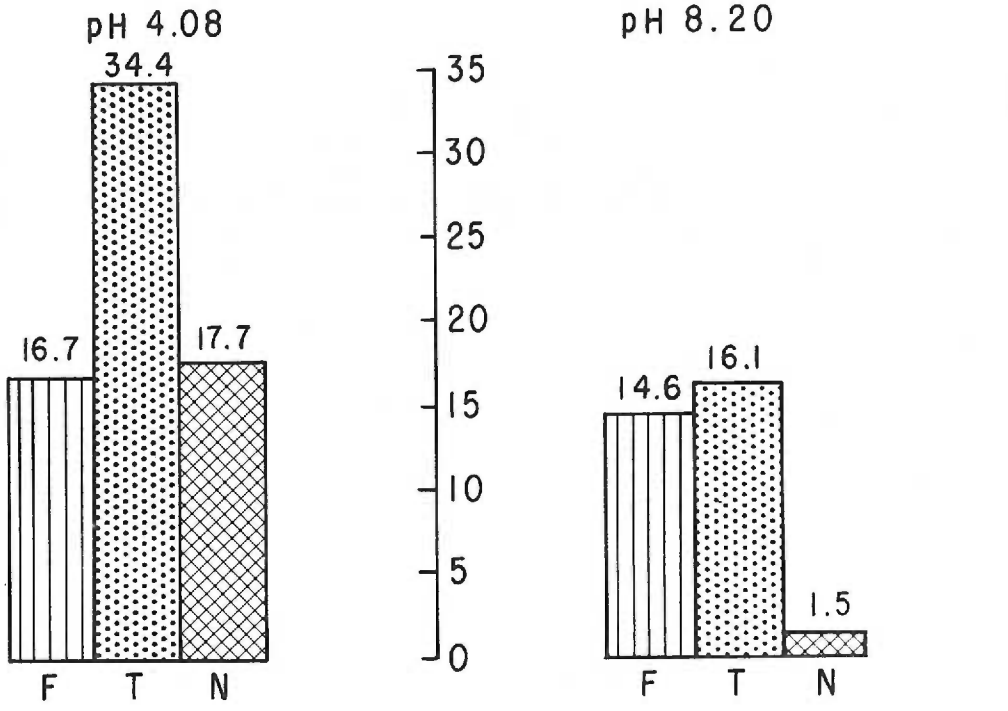
Figure 4

Influence of pH on proteolytic activity.

INFLUENCE OF pH ON PROTEOLYTIC ACTIVITY

Animal PP59-10, Conceptional age 250 days

Micrograms of Hormonal Iodide x 10^3 per mgm Thyroid Gland



F = Free hormonal iodide

T = Total hormonal iodide released during 24 hrs. incubation

N = Net hormonal iodide released during 24 hrs. incubation

Influence of Boiling the Homogenate Prior to Incubation. Homogenates were placed in a boiling water bath for five minutes prior to incubation at 37° C for 24 hours. This was done to determine (1) whether this inactivated the enzyme(s), (2) whether hormonal iodide is liberated by non-enzymic hydrolysis during the 24 hours of incubation at 37° C at a pH of 4.1, and (3) whether hormonal iodide is lost during incubation and by boiling at a pH of 4.1. The results are presented in Table III.

TABLE III

INFLUENCE OF BOILING ON PROTEOLYTIC ACTIVITY

Data Expressed as mg Hormonal I^- per mg/Thyroid Tissue

Animal	Control Tubes (H_2SO_4)		24-Hour Hydrolysis	
	Not Boiled	Boiled	Not Boiled	Boiled
CP 59-5 Age 225 days	2.45	2.34	8.05	1.80
CP 59-4 Age 225 days	1.87	1.66	10.62	1.58

Influence of Refrigeration at pH 1.0. The effect of storage at refrigerator temperatures (6° C) and at pH 1.0 was studied to determine whether under these conditions (1) proteolysis occurred, or (2) hormonal iodide was lost. The thyroid homogenates were acidified and placed in a refrigerator for 24 hours prior to extraction with butyl-alcohol. These data were compared with those of samples extracted immediately after enzymatic activity was stopped by acidification. The results are presented in Table IV.

TABLE IV

INFLUENCE OF REFRIGERATION (6° C) ON PROTEOLYTIC ACTIVITY

	Immediate Extraction	24-Hour Refrigeration Before Extraction
ug Hormonal I⁻ / mg Thyroid	0.001	0.000

Influence of Substrate Concentration on Enzymic Activity. A homogenate in which proteolytic activity had been destroyed by heat and which contained six mg thyroid tissue per ml was used for the substrate. The homogenate in which enzyme activity was not destroyed was prepared in the usual manner. It contained 0.80 mg wet thyroid tissue per ml. The thyroid tissue in this study was from animal PP 60-5, conceptional age 183 days. Inactivation of the enzyme in the substrate homogenate was accomplished by placing the thyroid tissue homogenate in a boiling water bath and bringing it to a temperature of 80-85° C. for 1.5-2.0 minutes and then cooling it in an ice bath to room temperature. This degree of heating inactivated the proteolytic enzyme. Substrate homogenate in amounts of 0.2 and 0.4 ml, containing 1.2 and 2.4 mg thyroid, were added to the reaction vessels which contained 1.0 ml of the enzyme homogenate. The reaction volume was 1.40 ml which was adjusted to volume with 0.025 M acetate buffer (pH 4.49). The pH of the reaction mixture was 4.35. Incubation temperature was 37° C and reaction times were 6, 12, 18, and 24 hours. The results are presented in Table V.

TABLE V

INFLUENCE OF SUBSTRATE ON PROTEOLYTIC ACTIVITY

	ugx10^3 Hormonal Iodide Released per Reaction Vessel			
Incubation Period	6 hrs.	12 hrs.	18 hrs.	24 hrs.
Enz. Hmg. Only	9.3	15.	18.1	20.0
Enz. Hmg. plus 0.2 ml Substrate Hmg.	10.9	20.0	28.7	27.7
Enz. Hmg. plus 0.4 ml Substrate Hmg.	9.4	24.1	33.2	35.6

CHAPTER V

DISCUSSION OF DATA

Preliminary Study to Show the Products of Hydrolysis

The iodinated products of hydrolysis using pooled, I^{131}-labelled (*in vivo*), rat thyroid gland homogenates were determined by paper chromatography. Radioautograms of the chromatogram are shown in Figure 1. The products of hydrolysis are MIT, DIT, thyroxine, triiodothyronine, and inorganic iodide.

These results indicate that the proteolytic enzyme(s) is not specific for the release of thyroid hormone, but that all of iodo-amino acids are released. Radioautography using I^{131} as the source of radioactivity is useful for depicting the iodinated amino acids. This study confirms the findings of McQuillan and Trikojus that all of the iodo-amino acids and inorganic iodide are released, but it fails to support their claim that a fast moving iodine containing "peptide" fraction is liberated.(33) No doubt peptides are liberated as has been stated and is shown by the tail at the origin in the radioautogram shown in

Figures 1 and 2, but it is certainly not fast moving. The other authors used different solvents and had to extract and re-chromatograph spots to get their complete separation of products. In doing this they had to concentrate the extracted material *in vacuo* prior to re-development by chromatography. The extra handling could have caused alteration of some of the compounds and thus been responsible for the unknown products that were designated "peptides." Concentration of *in vacuo* butanol extracts of labelled thyroid homogenate has been observed to interfere with chromatographic separation of products.(46)

Studies in Macaque Monkeys (Macaca mulatta)

The properties of the thyroid gland proteolytic enzyme(s) were studied using a semi-quantitative means for measuring enzyme activity. The method developed depended on quantitative measurement of the hormonal iodide released with incubation of homogenized tissue under controlled conditions. This method, specific in that it measures the hormonal end-products of proteolysis in thyroid tissue, has not been designed for studies of comparative, quantitative assay of enzyme activity. Later studies, in which proteolytic activity was shown to vary with the amount of substrate present, made apparent the fact that the substrate concentration must be controlled to provide

a basis for such comparative quantitative assessment of activity. As employed in the studies reported in this thesis, the method used has been applicable in investigations of the properties of the enzyme(s). Other investigators reported studies using similar tissue preparations, but have used less direct means for evaluating the products of hydrolysis.(2,3)

Proteolytic Activity of Thyroid Gland Tissue from Animals at Different Ages

Thyroid gland proteolytic activity was demonstrated in thyroid tissues of all of the animals studied at the various ages noted. No quantitative comparative estimation of proteolytic activity was made between the different age groups for the reasons stated above.

Influence of Incubation Time on Proteolytic Activity

Enzymic proteolytic activity varied with time in all cases studied. These relationships are shown in Figure 3.

Maximum proteolytic activity occurred between 18-24 hours after which a plateau was reached. These results corroborate the reported observations of others.(1,2)

Effect of pH, High Temperature, and Refrigeration After Acidification

Proteolytic activity was observed in the pH range 4.0-4.5. No activity was observed at pH values of 8.2 or 1.0. These findings are consistent with the reports of others.(1) The absence of activity at pH 1.0 provided a convenient basis for stopping proteolysis. The lack of activity at pH 8.2, a pH at which peptidase activity had been reported, may indicate that the peptidase does not play a role in the initial hydrolysis of thyroglobulin.(52)

Proteolytic activity was destroyed by boiling the homogenate for five minutes prior to incubation. No hydrolysis occurred when previously boiled homogenate was incubated at 37° C for 24 hours. The hormonal iodide released after incubation at 37° C and for 24 hours was due to the activity of the thyroid proteolytic enzyme(s) and was not due to the conditions of the experiment. The effect of temperature was further defined in other studies in which a markedly greater concentration of thyroid homogenate was used (30 mg thyroid/ml vs 0.89 mg/ml), in which more enzyme(s) might have been expected to be present. The enzyme(s) present was destroyed by heating the homogenate to a temperature of 80-85° C for 1.5-2 minutes. This was consistent with the values of heat inactivation

of the enzyme(s) reported by others.(1,52) Denaturation of thyroglobulin takes place during incubation for 24 hours at 37° C at pH 3.6 which is near the iso-electric pH of thyroglobulin and is below the pH at which thyroglobulin becomes irreversibly denatured.(2,27) Other studies have shown that denaturation of thyroglobulin is an initial step in enzyme hydrolysis.(3) Therefore, heat denaturation of the homogenate provided a suitable useful means for preparing a thyroglobulin containing preparation in which proteolytic activity has been destroyed, but in which the susceptibility of the substrate to proteolytic enzyme action has not been hindered.

No hormonal iodide was lost nor did additional proteolysis occur during 24 hours of refrigeration (13° C) at a pH of 1.0. Thyroxine iodide is easily oxidized at room temperature and at acid conditions, and it had been important to establish that storage did not affect assay when immediate extraction with butyl-alcohol was not done.

The studies reported in this thesis have defined certain properties of the thyroid proteolytic enzyme(s). The information derived from these studies is useful as a frame of reference for future work with the enzyme(s). The method presented has the advantage of being based upon determinations of enzyme activity by a more specific

parameter of thyroglobulin proteolysis than had been used by previous investigators. It is a very suitable basis for future studies with purified enzyme and substrate preparations. Detailed or quantitative definition of the enzyme(s) and its properties must await such investigations.

Influence of Substrate Concentration on Enzymic Activity

The influence of substrate concentration on enzymic activity was investigated by analysis of hydrolytic activity of constant amounts of enzyme incubated with varying amounts of substrate. The results indicated that activity varied directly with substrate concentration, as would be expected in an enzyme catalysed process. These studies indicated a severe limitation in investigations attempting to quantitatively measure enzyme activity in preparations of unknown substrate concentration.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Summary

A brief historical account of the development of knowledge of the thyroid gland has been presented. Especial attention has been given to the theories of the mechanism of storage and secretion of thyroid hormone based on studies reported prior to the suggestion that a proteolytic enzyme(s) may be responsible for the release of thyroid hormone from thyroglobulin. The investigative data leading to the discovery of this enzyme(s) have been described along with subsequent studies describing what is known about the properties of this enzyme(s).

This study has presented a new approach to the detection of the products of hydrolysis and was concerned with establishing the existence of the thyroid proteolytic enzyme(s) in neonatal primates, and determining some of its properties. The reasons for using a method based on the determination of the amount of hormonal iodide released from thyroglobulin as the parameter for measuring activity have been discussed along with details of the

method which may be performed on small amounts of tissue.

The results of the experiment have been presented and discussed. The enzyme(s) has been shown not to be specific for hydrolysis of hormonal iodo-amino acids but hydrolyzes all of the iodo-amino acids from thyroglobulin. It has been shown to be present in Macaque monkeys (Macaca mulatta) throughout early extrauterine life. Certain properties of the enzyme(s) have been described and discussed.

Conclusions

Certain conclusions may be made from the results obtained in the present investigations. These are:

1. The enzyme(s) was not specific for hydrolysis of the hormonal iodo-amino acids. All of the iodo-amino acids were released during proteolysis.

2. A proteolytic enzyme(s) capable of splitting iodo-amino acids from thyroglobulin was present in thyroid gland homogenates in neonatal Macaque monkeys (Macaca mulatta). No difference in activity at different ages in the neonatal period was demonstrated in this study.

3. The enzyme(s) showed proteolytic activity in the pH range 4.15 to 4.5. It was not active at pH 8.1 or at pH 1.0.

4. Proteolytic activity was destroyed by heating the enzyme(s) to a temperature of 80° to 85° C for 1.5 minutes.

5. No non-enzymic proteolysis occurs under the condition of incubation at 37° C for 24 hours at a pH of 4.1.

6. No proteolysis occurred and no hormonal iodide was lost when thyroid homogenate was stored at a pH of 1.0 at 6° C temperature for 24 hours.

7. Proteolysis increased with increased substrate concentration as would be expected in an enzymatic process. This amount of substrate makes it necessary to use specific quantities of substrate for assay of enzyme activity. The determination of hydrolysis from homogenates alone did not give a satisfactory measure of proteolytic activity.

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