ENZYLES WHICH CATALYZE THE ORGANIC BINDING OF IODINE

A STUDY OF THE CHARACTERISTICS OF THYROIDAL AND EXTRATHYROIDAL ENZYME SYSTEMS WHICH CATALYZE THE ORGANIC BINDING OF IODINE

By

GEORGE SAMUEL SERIF, M.Sc.

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Author: George Samuel Serif, B.Sc. (McMaster University)
M.Sc. (McMaster University)

SUPERVISOR: Professor S. Kirkwood

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SCOPE AND CONTENTS:

An iodide oxidizing enzyme system has been discovered in extracts of thyroid and certain salivary gland tissues which promotes the formation of elemental iodine. This enzyme system appears to function as a part of the previously reported tyrosine iodinase system which catalyzes the formation of monoiodotyrosine, a precursor of thyroxine, from tyrosine.

A mitochondrial enzyme system, which iodinates tyrosine residues in protein chains and which was previously reported to occur solely in the thyroid gland, has been detected in a number of extrathyroidal centers.

Similarities in behaviour towards substrates and the co-existence of the mitochondrial and tyrosine iodinase systems in tissues may indicate that the systems are basically the same.

The tyrosine iodinase system has been used to distinguish between two mechanisms developed to explain the anti-thyroid action of excess iodide ion.

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GENERAL INTRODUCTION

Although the isolation of monoiodotyrosine (MIT)[1] from the thyroid gland (1) provided indirect evidence that this substance was an intermediate in the *in vivo* elaboration of thyroxine, a more direct proof was supplied by the detection of an enzyme in thyroid tissue extracts which catalyzes the formation of MIT when supplemented with L-tyrosine and cupric ion (2,3). This same enzyme, tyrosine iodinase, was also detected in several extrathyroidal sites where its presence was explained on the basis that it was involved in the degradation of MIT derived from metabolized thyroxine (Tx)(4). The work outlined in this thesis is a continuation of these earlier investigations.

The first division of the thesis deals with the detection of an enzyme system in tyrosine iodinase extracts which catalyzes the oxidation of iodide ion to an oxidation state equivalent to elemental iodine. Evidence for the existence of such an enzyme system is advanced and the system is discussed in terms of its function within the tyrosine

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[1] The following abbreviations are used: MIT, monoiodotyrosine; DIT, diiodotyrosine; Tx, thyroxine; TIT, triiodothyronine; BMR, basal metabolic rate.

iodinase complex. An *in vitro* technique for the detection of the iodide oxidizing enzyme system is developed and is employed to investigate the presence of this enzyme system in those extrathyroidal sites known to possess tyrosine iodinase activity. Possible mechanisms by which the iodide oxidizing system may act are discussed and a tentative mechanism of action is advanced.

The realization that the tyrosine iodinase system is more complex than was originally thought has prompted a more extensive investigation of this system. Thus a further division of this thesis is concerned with a detailed study of tyrosine iodinase substrate specificity and a discussion of the role of metal ions as activators for this enzyme system. The enzyme has also been detected in several new extrathyroidal sites.

During the course of the work described above Taurog *et al.* (5) announced the discovery of a new enzyme system capable of catalyzing the organic binding of iodine. This enzyme system, unlike the soluble tyrosine iodinase system, was reported to be associated with thyroid mitochondria and required no supplementation for maximum activity. In addition, the product of iodination, MIT, appeared to be protein bound. This protein released free MIT only when treated with pancreatin. The existence of this enzyme in thyroid mitochondria was verified employing the techniques for mitochondrial isolation and incubation devised by Taurog *et al.* An exten-

sion of this study revealed the existence of the mitochondrial system in a number of extrathyroidal centers. The discovery of free MIT as an additional product in those mitochondria derived from extrathyroidal sources initiated a study of the nature of the protein component synthesized by mitochondria.

Despite initial conflicts which appeared to exist between the tyrosine iodinase system and the mitochondrial enzyme system it became increasingly apparent, as data accumulated, that certain basic similarities existed. Thus a portion of this thesis has been devoted to a correlation of these systems with respect to their substrate requirements, site of occurrence, and mode of action.

The ready availability of iodide-free preparations of the enzyme system which catalyzes the formation of the first intermediate in Tx biosynthesis presented the opportunity of employing this tyrosine iodinase system as a "model" with which to evaluate the antithyroid action of iodide ion. Thus the final portion of this thesis is devoted to a quantitative study of this anomalous action of iodide ion and its interpretation in terms of current theories advanced to explain its mechanism.

HISTORICAL INTRODUCTION

The Thyroid Gland

Description and Origin

The thyroid gland, first named and accurately described by Wharton in 1659 (6), is an endocrine or ductless gland found only in the vertebrates. This gland varies surprisingly little in basic structure from cyclostomes to mammals. In humans the gland may be described as attached to the upper end of the trachea and possessing a right and left lobe connected by a band of typical thyroid tissue termed an "isthmus". Thyroid tissue is composed of cuboidal epithelial cells arranged in a single layer around spaces roughly ovoid in shape. These spaces or follicles, contain a gelatinous substance, called "colloid", which is believed to be the storehouse for the secretion of the gland.

Two classical methods of approach, embryology and comparative anatomy have been utilized in efforts to establish the origins of the thyroid gland.

A study of the human foetus reveals that the thyroid arises about the end of the fourth week as an out-growth or "bud" developing from the ventral surface of the primitive forgut or pharynx. The gland then divides, caudally, into a right and left lobe, remaining attached to the pharynx by a structure termed the thyroglossal duct. This

duct atrophies during the fifth week of foetal development. The disappearance of the duct is concurrent with a migration of the gland down the front of the trachea where it takes up its ultimate position opposite the first thoracic vertebra.

An examination of the origin and development of the thyroid in lower vertebrates (7) reveals a similar picture of the gland arising through differentiation from gut tissue. In the larval stage of the lamprey eel, the thyroid exists as a "sac" or endostyle connected by a duct to the anterior digestive tract. During metamorphosis the duct becomes an endocrine or ductless gland in the adult animal.

Gorbman (8) has proposed a theory, based on this embryological data, to describe the evolution of the thyroi gland. He suggests that iodoproteins existed, prior to the advent of the thyroid gland, in the upper digestive tract of invertebrates. At some stage during evolutionary development these iodoproteins, after hydrolysis by alimentary proteases, began to provide a valuable control of metabolic activity. By chance this store of iodoproteins became localized in the pharyngeal region and eventually evolved into a "sac"-like organ connected to the gut by a duct which permitted a discharge of iodoprotein into the area of protease activity. Development of a proteolytic enzyme in the "sac" or endostyle itself provided a freedom from dependence on the gut enzymes. As a result the duct was elimin-

ated and the thyroid became an endocrine gland releasing its hormonal secretion directly to the bloodstream.

Functions of the Thyroid

Although a swelling of the thyroid gland had been shown, by Graves in 1835, and by von Basedow in 1840 (9), to be one of the symptoms of a disease characterized by intense nervousness, exophthalmos, and tachycardia, it was not until 1874 that the importance of the gland as a factor in the control of metabolic rate was demonstrated. In that year Gull (10) connected atrophy of the thyroid with the symptoms of a disease now called by his name. These symptoms included apathy, increased body fat, and greater susceptibility to the cold. Murray in 1891 (11) was able to prepare extracts of sheep thyroid which would completely correct the metabolic defects resulting from an hypoactive thyroid. In 1895 Magnus-Levy (12) showed that greatly reduced metabolic rate was a symptom of Gull's disease and that treatment with desiccated thyroid restored the rate to a normal level.

Thus these three workers laid the ground-work which established the thyroid as a gland secreting an active principle or hormone absolutely necessary for normal growth and the maintenance of a metabolic rate correctly adjusted to the environment. This general picture of thyroid function is still considered correct but details of such features as the

interplay between the thyroid and other hormone-secreting glands, the means of elaboration of the hormone, the mode and locus of action of the hormone, and even the exact nature of the active principle are still obscure.

Since a goodly measure of that which is known about thyroid function has been derived from a consideration of abnormal glands, it is of interest to describe briefly these defects, their symptoms, and their causes. Although much individual variation is found in the symptoms of patients suffering thyroid faults this does not prevent the classification of such disorders under certain main headings.

(a) Hypothyroid state

(i) Cretinism- a cretin is one whose normal growth has been retarded through the absence or deficiency of the thyroid hormone through childhood. This state is characterized by undeveloped skeletal growth, arrested sexual development, mental deficiency, and a lowered basal metabolic rate.

(ii) Myxedema (Gull's Disease)- a result of thyroid hormone deficiency in adults or older children, which inevitably follows atrophy or destruction of the thyroid. Symptoms are a low metabolic rate, thick puffy appearance of the skin, apathy, and increased susceptibility to cold.

(b) Hyperthyroid state

The hyperthyroid state is indicated by a number of

syndromes and has been termed variously Graves', Parry's and Basedow's disease. It is undoubtedly due to excessive secretion of the thyroid hormone since symptoms of the hyperthyroid state may be induced in normal persons by administration of the hormone. Common symptoms of the disease are an accelerated pulse, nervous excitability, dissipation of fat stores, disturbance of carbohydrate metabolism and an increased metabolic rate.

(c) Goiters

Goiters, or enlargement of the thyroid gland are observed in hyper- hypo- and normal states of thyroidal activity:

(i) Simple goiter- usually associated with a dietary deficiency of iodide in otherwise normal patients and is apparently a compensatory mechanism.

(ii) Exophthalmic goiter- associated with an overactive secretion of hormone.

(iii) Goiter - associated with an underactive secretion of hormone. There is a low natural incidence of this type of goiter, but it can be produced at will by antithyroid drugs.

Isolation of an Active Principle of the Thyroid and its Identification and Biogenesis

The first step in the separation of the active principle from the thyroid gland was taken by Murray who in 1891 (12) prepared a glycerine extract of sheep's thyroids and successfully employed this extract to dispel symptoms of myxedema. Iodine was demonstrated to be an important constituent of the extract in 1896 by Baumann (13, 14) who hydrolyzed thyroid tissue to obtain a dry preparation containing 10 percent iodine and possessing the physiological activity of the whole gland. Baumann named this preparation iodothyrin and considered it to contain iodine in organic combination. Oswald in 1899 (15) demonstrated that the active constituent of the thyroid was attached to a protein of the follicular colloid. He termed this protein thyroglobulin.

A pure preparation of the active principle of the thyroid was isolated and named thyroxin by Kendall in 1919 (16). An elemental analysis of the compound indicated that it possessed 65 percent iodine. The chemical structure of thyroxine was deduced as 3,5,3',5' -tetraiodothyronine by Harington (17) and the compound was synthesized by Harington and Barger in 1927 (18).

The elucidation of the structure of thyroxine (Tx) aroused much speculation as to the mode of biosynthesis of this unique molecule. The obvious structural similarities between thyroxine and tyrosine and the occurrence in nature of iodogorgoic acid (3,5-di-iodotyrosine) led Harington to propose that the hormone arose through a metabolic pathway involving iodination of tyrosine followed by a unique coupling of the iodination products (19). Support for this concept was obtained by Harington in 1929 when di-iodotyrosine (DIT) was isolated from thyroid protein (20). The inexact techniques of amino acid isolation and determination available at this time led Harington to the erroneous conclusion that the total organically bound iodine could be accounted for by the sum of DIT and Tx concentrations (21).

In 1939 Ludwig and von Mutzenbecker (22) made the remarkable discovery that proteins containing tyrosine residues could be iodinated <u>in vitro</u> to yield small quantities of DIT and Tx. This work, later confirmed by Harington and Pitt-Rivers (23), lent powerful support to the hypothesis of Tx genesis through a coupling reaction involving DIT. Expanding his study of thyroxinogenesis, von Mutzenbecker showed that the tyrosine residues need not be bound to protein since incubation of "free" DIT in alkaline solution for a few days resulted in isolable amounts of Tx (24). Reaction conditions for this coupling were established by

Harington (19) and involved such factors as an obligatory aerobic atmosphere, the presence of excess H_2O_2 and an optimum pH of 10. Johnson and Tewkesbury in 1942 (25) developed a hypothetical mechanism for the *in vivo* coupling of DIT to form Tx as shown in Fig.1 based upon the studies of Pummerer *et al.*(26) on the oxidation of *o* and *p* substituted phenols in alkaline solution and their own investigation of the oxidation of DIT by HOl. An extension of the theoretical consideration involved in the preceding reaction sequence was proposed by Harington in 1946 (19) who proposed a mechanism based on free radical formation through oxidation of the phenoxide ion. (Fig. 2).

Pitt-Rivers studied the *in vitro* oxidative coupling reaction using acetyl-DL-tyrosyl-glutamic acid rather than tyrosine (27) and made the discovery that the reaction proceeded more readily with the peptide. Thus in comparison to the coupling of "free" DIT which provides maximal yields of 1.63 percent Tx at pH 10 and 70° C, the coupling of tyrosyl-peptide provides net Tx yields of approximately 35 percent at the physiological temperature of 37° C and at pH 7.5. A consideration of the high net yields of Tx obtained by Pitt-Rivers has led Harington to postulate that the *in vivo* formation of Tx follows a pathway of non-enzymatic iodination of tyrosine residues of proteins with elemental iodine acting in the double role of tyrosine iodinating

agent and oxidizing agent for the coupling of the iodinated residues (19).

The principle excreted by the thyroid gland has an intense physiological activity. Due to this fact only minute amounts of Tx are formed in the normal functioning of the gland. An estimate of daily hormone production has been made through studies of the effects of L-Tx medication upon the basal metabolic rate (BMR) of patients with thyroid conditions varying from normal to the profound myxedematous state (28). The value obtained by these studies has been set at 0.2 mg. per day. It is impossible, by conventional techniques, to investigate the *in vivo* generation of such small amounts of material. Thus early workers interested in the biosynthesis of the thyroid hormone were forced back to speculations based on observations of *in vitro* model systems. The advent of readily available radioactive iodine as a biological tracer permitted, for the first time, a more intimate view of the thyroid processes.

One of the first experiments employing radioactive iodine, was carried out by Hamilton and Soley in 1939 (29). These authors fed radioiodine to normal patients and those suffering from non-toxic goiter, myxedema, and Graves' disease and established a characteristic iodide ion uptake curve for each thyroidal state.

Using radioactive iodide, Perlman *et al.* (30) provided

suggestive evidence that DIT was a precursor of thyroxine. These authors injected rats with radioiodide ion and detected labelled DIT and Tx in alkaline hydrolysates of the isolated thyroglobulin. Following up these experiments with quantitative measurements of the radioactive iodide-131 and stable iodide-127 incorporated into DIT and Tx with time, Taurog and Chaikoff (31) were able to demonstrate that their specific activity results satisfied the graphical tests of Silversmit et al. (32), designed to establish one compound as the precursor of another. They thus suggested this data as positive proof that DIT is a precursor of Tx in the thyroid gland. However, a criticism may be leveled at this work. It has been observed that, during the treatment of iodo-amino acids with aqueous alkali in the presence of radioiodide, exchange occurs between inorganic and organic iodine as well as extensive decomposition of DIT and MIT (33). If this decomposition and exchange occurred with the iodo-amino acids and iodide-131 in the Taurog-Chaikoff experiments, the specific activity data of these authors may be of little value. An indication that this may be the case is given in the work of McGuillan et al. (34), who obtained specific activity results similar to those of Taurog and Chaikoff (31) with alkaline hydrolysis of thyroglobulin, but differing from those of these authors when hydrolysis was carried out by enzymatic means. Thus it is seen that although DIT would appear to be

a logical precursor of Tx the relationship has not yet been established with complete surety.

Monoiodotyrosine (MIT), like DIT, would appear to be a logical intermediate in Tx biogenesis. The presence of this amino acid in the thyroid gland had not been detected until 1948, although it had been isolated from the hydrolysate of artificially iodinated proteins (22). In 1948, MIT was demonstrated to be present in significant amounts, in the thyroid gland, by Fink and Fink who used a combination of the techniques of radioiodine labelling and filter paper chromatography (1). This work was later confirmed by Taurog et al. (35) and Fishkoff et al. (36).

Employing the same specific activity technique used with DIT, Taurog, et al. (37) demonstrated that MIT fulfilled the graphical tests of Liversmit et al. (32), required in order that it may be a precursor of DIT. The criticism of the early work done with DIT and Tx however, applies here also since isotopic exchange and decomposition may have altered specific activity values.

Thyroidal Enzymes

Despite Harington's suggestion of a non-enzymatic formation of Tx *in vivo*, attempts to identify enzyme systems potentially involved in hormone synthesis have met with some success.

In 1942 Morton and Chaikoff (38) showed that surviving slices of thyroid tissue were capable of labelled Tx formation when incubated in a physiological medium containing I-131. These authors reported no iodinating capacity in thyroid homogenates (39). Eleven years later Weiss (40) discovered that if thyroid homogenates were supplemented with tyrosine and cupric ion organic binding of I-131 occurred. On the basis of a non-specific butanol extraction technique the author concluded that this organically bound iodine was present mainly as Tx.

Fawcett and Kirkwood studied Weiss' system employing paper chromatography to separate the radioactive components (2,3). Instead of Tx and its precursors these authors found only "free" MIT. Since boiled thyroid homogenates lost the ability to bind I-131, it was suggested that cell free thyroid homogenates contained the enzyme concerned with the first step in Tx synthesis *in vivo*. Fawcett and Kirkwood termed this enzyme tyrosine iodinase and postulated that

Tx biogenesis occurred with free amino acids rather than with those bound to protein chains.

In 1955 Chaikoff and co-workers (5) fractionated thyroid homogenates into their component parts by differential centrifugation and investigated the ability of each fraction to bind I-131 organically. They found that the mitochondrial fraction bound I-131 without supplementation. Furthermore, this organically bound iodine was immobile upon chromatography with their solvents. Digestion of this component with a pancreatin preparation released only MIT. Chaikoff and his collaborators contended that the Fawcett-Kirkwood system was an artifact and offered the results of their investigation of mitochondria as strong support for the theory of protein bound conversion of tyrosine to Tx.

The question of whether Tx is or is not formed from tyrosine moieties incorporated into proteins is an interesting one. The theory of "protein bound" formation of the hormone arose quite naturally from early <u>in vitro</u> work concerned with the artificial iodination of proteins (22). It was believed, as a result of these experiments, that the free amino acids, MIT, DIT, and Tx did not exist as such in the thyroid gland but could only be obtained through hydrolysis of thyroglobulin. However, in 1950 Gross <u>et al.</u> (41) showed that small amounts of Tx, DIT, and MIT could be extracted with butanol from unhydrolyzed thyroid protein. This report

was partially confirmed in 1951 by Tong, Taurog and Chaikoff (42), who isolated free Tx and MIT from the thyroid gland in amounts corresponding to 0.5 percent of the total iodine of the gland. An interesting report concerned with the existence of free iodinated tyrosines in the thyroid has recently appeared. Stanbury (43), who studied iodine uptake in a group of hereditary cretins found, surprisingly, that this group rapidly and avidly concentrated I-131 in their thyroids and then just as rapidly released the radioactivity to the blood stream. A chromatographic analysis of the blood of these patients revealed only free MIT and DIT. Presumably these patients possessed a metabolic fault which prevented conversion of the iodinated residues to Tx.

Other enzyme systems which may be directly or indirectly involved in Tx formation have been detected in the thyroid. Reports of a thyroidal peroxidase appeared as early as 1910 (44). In 1943 Schachner and co-workers (45) observed that the formation of organically bound I-131 was inhibited by reagents known to inhibit peroxidases. Dempsey in 1944 (46) claimed to have demonstrated a peroxidase in rat follicular cells by a histochemical technique. In the same year Glock (47) challenged Dempsey's observations and suggested that the activity observed had been due to non-specific haemoglobin. DeRobertis et al. in 1946 (48) supported Dempsey's claim of a peroxidase in thyroid tissue by the

demonstration that perfused thyroid glands could still be demonstrated by histochemical means to possess a peroxidase activity. These authors prepared a glycerol extract of thyroid tissue which was shown, in a qualitative manner, to intensify the oxidation of iodide to elemental iodine in the presence of hydrogen peroxide.

Functions for a thyroidal peroxidase have been suggested. According to Harington (19) a peroxidase might be involved in the generation of an oxidized form of iodide which might be the reactive iodinating species. Westerfeld and Lowe (49) have suggested a further role for a thyroidal peroxidase as the enzyme involved in the DIT oxidative coupling step. Hydrogen peroxide required by the peroxidase would be presumably supplied by the oxidase reported to occur in the thyroid (46).

Keston has devised an ingenious model system to illustrate the possible connection between an oxidase, a peroxidase and the iodination of tyrosine (50). Addition of xanthine and I-131 to unpasteurized milk (known to contain xanthine oxidase and lactoperoxidase) resulted in the formation of labelled casein which upon hydrolysis and butanol extraction yielded a butanol soluble radioactive compound which Keston presumed to be Tx. Later workers, however, have shown that at least 90 percent of the iodinated material in this preparation is MIT (57).

A thyroidal protease has been reported by a number of workers (52,53,54,55,56). It has been suggested that the protease is necessary for the mobilization and secretion of the thyroid hormone through its potential ability to cleave Tx from thyroglobulin. It is interesting to note, however, that the pH optimum of this protease has been established at pH 2.5-3.5 and it has no proteolytic activity near pH 7 (56). Since the colloid pH has been shown to be essentially neutral (57), it is questionable whether the proteolytic function of the enzyme is important in vivo. On the other hand proteases have been observed to function as transpeptidases at pH's other than those of optimum proteolytic activity (58). It might be possible therefore that this protease is functioning to exchange free iodinated tyrosines with other amino acids in the thyroglobulin molecule.

The detection of 3',3,5-triiodothyronine (TIT) 3',5',3-triiodothyronine and 3',3-diiodothyronine in the thyroid gland (59,60) has focused attention on the possibility that these compounds may be derived from Tx in the thyroid by enzymatic dehalogenation. That this process might be a normal pathway for the metabolism of Tx was strengthened by reports of the high biological activity of TIT and 3',3-diiodothyronine (61,62). The presence of a dehalogenase in the thyroid gland has been detected by Roche et al. (63). These workers have demonstrated that thyroid gland slices deiodinate MIT and DIT and that this ability is

destroyed by boiling. This dehalogenase, however, was shown to possess no ability to deiodinate Tx. The presence of this enzyme in the gland has been suggested, by Roche and his co-workers, to provide a means of recycling iodine tied up in MIT and DIT residues which were unavailable for conversion to Tx rather than as a means of converting Tx to a more active hormone. This suggestion implies that the iodination process in the thyroid is spontaneous.

A lipase and an esterase have been reported in the thyroid (44,64), but their function in hormone biogenesis remains obscure.

Extrathyroidal Synthesis and Degradation of the Thyroid Hormone

An intact thyroid gland is apparently not required for Tx formation in the animal body. Invertebrates which do not possess this gland have been shown to elaborate MIT, DIT and Tx. DIT was detected in the protein skeletons of corals prior to its observation in the thyroid gland (65,66, 67,68). Therefore it is not unexpected that experimental data have been obtained suggesting an extrathyroidal synthesis of Tx in vertebrates.

Thus Chapman in 1941 (69) produced data to show that a high level of iodine in the diet of thyroidectomized rats results in significant changes in weight, surface area, water intake, food utilization, and metabolic rate over thyroidectomized controls on low iodine diets. The author suggests that these differences were caused by an extrathyroidal synthesis of a Tx-like substance in the peripheral tissues. Chapman and Higgins (70) obtained further evidence for this theory by their observation that the cytological changes in the pituitary gland characteristic of thyroidectomy are intensified in animals on a low iodide diet as compared to controls receiving adequate supplies of iodide. This work was extended by Purves and Griesbach (71), who showed with

immature rats, that the changes in the pituitary of surgically thyroidectomized rats described above can be largely prevented by the administration of large amounts of iodide. They concluded that the action of iodide in preventing degranulation of acidophils in the thyroidectomized animal was equivalent to daily injections of 0.125 micrograms of Tx. It is questionable whether a surgical thyroidectomy ever completely removes all thyroid tissue. To overcome this objection Hum et al.(72) conducted a similar study using rats thyroidectomized with I-131, a technique which has been shown to destroy completely all thyroid tissue (73). The results of Hum et al. were quantitatively identical with those of Purves and Griesback. A number of attempts have been made to establish potential sites of extrathyroidal Tx formation. Studies of I-131 uptake in the normal animal, however, show little localization of I-131 outside of the thyroid with the exception of the saliva and gastric juices where concentration of I-131 of 30 and 40 times that of plasma I-131 have been observed (74). An interesting I-131 concentration effect was reported by Foster et al. (75). These authors detected a 250 percent increase in thymus uptake of I-131 following thyroidectomy. It has been suggested that the thymus compensated for thyroid tissue loss by development of an ability to synthesize Tx. Earlier Van Dyke (76) observed the presence of typical thyroid cells, including follicles, in the thymus glands of thiourea treated rats.

A second phase of extrathyroidal Tx metabolism

involves the pathways whereby the hormone is degraded. The observation has been made that iodide-131 may be isolated from the tissues of rats injected intravenously with Tx labelled with I-131 (77). This observation, coupled with the fact that more hormonal iodine may be produced than can be accounted for by the dietary iodide (78), makes it quite obvious that a degradative process is present in animals which permits a recycling of the iodine originally bound in organic form. It has been known for some time that DIT injected into the animal body is rapidly deiodinated (79) and early observations have been confirmed and extended through the use of labelled DIT (80). Roche's discovery of a thyroidal dehalogenation enzyme has already been mentioned and similar activities have also been reported to occur in liver, intestine and kidney preparations (81). Roche has found his dehalogenase to be incapable of deiodinating Tx. More recently an active Tx dehalogenase has been detected in kidney slices by Larson et al. (82). This dehalogenase is effective in the formation of TIT from Tx. Recent data have supplied some evidence that TIT may be the active thyroid hormone (83). In view of this Larson et al. have suggested that their dehalogenase may have an important physiological function.

The enzyme tyrosine iodinase detected by Pawcett and Kirkwood in cell free thyroid extracts has been shown by these authors to be present in extrathyroidal sites, notably in the salivary glands (3,4). Pawcett and Kirkwood have suggested

that it may function in a reverse direction when located outside the thyroid as a dehalogenase involved in the degradation of MIT to iodide ion and tyrosine. These authors proposed a cyclic or "dynamic" balance with Tx generation in the thyroid and degradation in the salivary glands. Evidence for this scheme was obtained by their demonstration that salivariectomized rats deiodinated injected DIT at a much slower rate than normal animals (4). More recently this observation has been challenged by Neugamer (84), and Chaikoff et al. (85). The former author showed that deiodination of DIT proceeds at the same rate in salivariectomized and normal dogs and suggested that the decreased DIT deiodination in salivariectomized rats observed by Fawcett and Kirkwood may be an artifact resulting from a depression of blood circulation in the rat tail vein, from which blood samples were taken. That salivary degradation of Tx has a minor role is also suggested by the results of Newcomer (86), who observed no increased BMR in salivariectomized rats. These results taken in conjunction with the observation that completely eviscerated rats deiodinate DIT just as rapidly as normal animals (87), indicate the deiodination phenomenon may be a widely disseminated process in the animal body.

An additional inference as to the nature of the MIT, DIT dehalogenase, may be drawn from the recent report of Stanbury et al. (43). If the deiodination process were a non-

specific reaction involving, for example, a reductive dehalogenation with reduced diphosphopyridine nucleotide, then all persons would be expected to possess this metabolic pathway. Stanbury et al., however, have demonstrated quite conclusively that hereditary cretins exist who do not possess a means of degrading the iodotyrosines. Thus, presumably, a specific enzymatic process is required for effective dehalogenation in the peripheral tissues. This conclusion is supported by the earlier work of Snapper (79), who showed that the DIT deiodination process had the characteristics of an enzyme catalyzed reaction.

The Functions of Iodide Ion in the Thyroid

The action of iodide ion presents one of the most intriguing anomalies in the field of thyroid research. Iodide ion has been shown to be absolutely necessary for normal thyroid hormone production but yet it will, when in excess, actually inhibit hormone formation.

The necessity for an adequate supply of iodide has been recognized for many years. Seaweed ash has been used from early times in the treatment of certain thyroid goiters now known to be caused by iodide deficiency (88). In 1821 Coindet (89) linked the active principle of seaweed ash to iodide and advocated use of the latter in the therapy of hyperplastic goiter. Due, however, to indiscriminate use of the drug its true value was lost sight of for over a quarter of a century. Chatin from 1850 to 1860 studied the relationship between iodide and goiter and showed that the iodide content of the soil and water of districts in which goiter was prevalent was low. He suggested, on the basis of his observations, that iodide be used as a goiter preventative (90). The credit for firmly establishing a thyroidal requirement for iodide, however, is generally now accorded Marine who demonstrated the relationship between goiter formation and iodide intake unequivocally by a study of school children in the United States goiter belt

(91,92,93). Kendall's isolation of an organically bound iodine compound, Tx, as the active principle of the thyroid gland (16), and Harington and Barger's elucidation of its structure (18), show beyond any reasonable doubt that adequate iodide intakes are required for the express purpose of providing adequate precursor material for the elaboration of the hormone.

The concept that excess iodide acts as an antithyroid agent dates from its use by Plummer, an American surgeon, as a pre-operative treatment for patients with Graves' disease (94). Plummer discovered that when these patients ingested large quantities of iodide, a marked reduction in hyperthyroid characteristics and an apparent depletion of Tx stores resulted. This depletion allows surgical removal of thyroid tissue without accompanying "thyroid storms" which were frequently a dangerous consequence of the operation.

In the face of present day knowledge of the action of iodide ion as a precursor in Tx formation, its use as an antithyroid agent would appear to be a most unlikely treatment of hyperthyroidism. However, no doubts may now be entertained as to the inhibitory action of iodide ion. Chaikoff and coworkers (95), have demonstrated the direct antithyroid action of iodide ion on surviving thyroid tissues. Wolff et al. (96) have extended this work by an in vivo study in the rat and more DIT is formed in the thyroid of the normal animal after injections of small amounts of iodide (5 to 10 micrograms)

than when 10 to 20 times this amount is injected. These authors have also shown that in rats in which the level of plasma iodide is maintained above 30 micrograms / 100 ml. the uptake of radioiodine by the thyroid is completely prevented. Clinical confirmation of the antithyroid action of iodide ion is afforded by Stanley's (97) observation that increased dietary iodide effected a definite inhibition of organic binding of iodine in both normal and thyrotoxic patients.

A number of theories have been advanced to explain the antithyroid action of iodide ion. A mechanical theory holds that the thyroid gland becomes packed with hormone when supplied with large quantities of building material. The pressure of the accumulated secretion then shuts off the blood supply and prevents diffusion of the hormone to other parts of the body (98).

Another theory suggests that the antithyroid action of iodide ion is mediated through inhibition of thyrotrophic hormone formation or action. Thus Loeser and Thompson (99) found that when iodide was fed to the normal animal the quantity of thyrotrophic hormone in the pituitary was reduced, and Silberberg (100) and Siebert et al. (101) demonstrated that treatment of normal guinea pigs with potassium iodide could effect a partial inhibition of the stimulating action of the thyrotrophic hormone (TSH) on the thyroid gland. These results have since been confirmed by other workers (102,103,104).

Wright and Trikojus (105) have modified this theory slightly by suggesting that it is not iodide which inhibits the effect of TSH but rather elemental iodine which is formed from iodide ion within the thyroid gland. *In vitro* work by Rawson and co-workers (106) on the inactivation of the thyrotrophic hormone by elemental iodine lends some support to this modification of the general theory.

Another hypothesis proposed by De Robertis and Nowinski (107) is based on their observation of the inactivation of the proteolytic enzyme of the thyroid gland by elemental iodine, *in vitro*. These authors suggest that the thyroid protease is essential in conversion of thyroglobulin to the circulating thyroid hormone. Destruction of this proteolytic activity *in vivo* with elemental iodine (generated from excess iodide in the thyroid gland) would then render the thyroid hormone unavailable.

Two final theories advanced to explain the antithyroid action of excess iodide ion attempted to reduce the phenomenon to the molecular level by a consideration of well known chemical reactions of iodide ion. The first of these mechanisms was postulated by Pitt-Rivers (108) and Harington (109), who suggested that hypoiodous acid is the reactive form of iodine which is actually effective in the iodination of the tyrosine in the thyroid gland. This substance is formed by the hydrolysis of elemental iodine as in equation [1] .

$$I_2 + H_2O \longrightarrow HIO + HI \qquad [1]$$

EXPERIMENTAL

A number of avenues of approach have been used in this study of the phenomena concerned with the thyroidal and extrathyroidal metabolism of the thyroid hormone. As a direct result the techniques and procedures used are many and varied. These methods will now be discussed, where possible, in their more general aspects. Where extensions or alterations of the general techniques are necessary in later discussions, these changes will be discussed at pertinent points throughout the thesis.

Chromatographic Procedures for Separation and Identification of Radioactive Components

The chromatographic procedures employed in the separation and identification of radioactive components of an assay medium were essentially those devised by Fawcett and Kirkwood (2,3), and may be described as follows:

The chromatographic support used was invariably Whatman No. 1 filter paper cut to 56 cm. x 5 cm. lengths. These strips were pretreated by spraying with 0.1 M phosphate buffer at a pH of 7.8. This pretreatment allowed sharper definition of separated components and was based on a technique used by Carless and Woodhead for the separation of alkaloids (111). In addition to the buffer treatment 3 micrograms of sodium

From a consideration of equation [1] it is seen that any increase in iodide ion will, by the Mass Law effect, decrease the concentration of hypoiodous acid with consequent depression in the formation of organically bound iodine. A second molecular theory, advanced by Fawcett and Kirkwood (110), contends that I^- acts through its ability to combine with elemental iodine to form a molecular compound as in equation [2].

$$I_2 + I^- \longrightarrow I_3^- \qquad [2]$$

This interaction would effectively remove the reactive iodinating species and inhibit hormone formation. It should be possible to study quantitatively the mechanisms proposed by Harington and Pitt-Rivers and Fawcett and Kirkwood since the mode of action of iodide ion in both these mechanisms is clearly defined.

thiosulfate were dried on each strip at the origin position which was centrally located 3 cm. from the bottom end of the paper strips. This procedure was found to prevent some of the radioactivity from running at the solvent front. This front activity is believed to be attributable to an iodine complex and is undoubtedly an artifact. In practice carrier organic compounds were found to be unnecessary and were not used. Carrier iodide, however, in 3 microgram amounts was spotted to the origin of each paper strip prior to spotting with the sample under investigation. Where a comparison of unknown radioactive components to known amino acids was desired, 20 microgram samples of the reference amino acid were dried at the origin position at this stage. In general 30 microliter aliquots of the material to be chromatographed were delivered at the origin by means of a micro-pipette. Drying of these samples was greatly hastened by exposing the wet spot to a current of warm air. The solvent system most commonly used for separation was the top layer of an n-butanol : acetic acid : water equilibrate, (63:2:27). Other solvents used in particular instances were the top layers of equilibrates prepared from n-butanol : 2N ammonium hydroxide (1:1) and n-butanol : 2N formic acid (1:1) Chromatographic development was carried out for 16 hours in a closed insulated cabinet using the ascending technique. At the end of this period the paper strips were removed from the cabinet, the position of the solvent front marked immediately,

and the strips then dried at room temperature. When thoroughly dry, the chromatograms were used to prepare radioautographs by the simple procedure of placing the radioactive strips against Kodak "No Screen" X-ray film. Exposure times varied with radioactivity used. With a total of 20 microcuries of activity in each individual experiment, 16 hours of exposure were required. With 100 microcuries of I-131 an exposure of 3 hours was sufficient. After development of the X-ray film the position of the radioactive spots could be determined by laying the developed radioautograph against the paper chromatogram. In cases where unlabelled amino acids had been used as reference materials the presence and position of these compounds were determined by spraying the paper strips with a 0.1 percent solution of ninhydrin in n-butanol. The sprayed chromatograms, when compared with the radioautographs, permitted the identification of the labelled materials present in the assay medium.

Assay Chromatography

Where the identity of the radioactive components had been previously determined and where a large number of assay tubes were to be evaluated with respect to the percent composition of each of the various components, the "long strip" chromatographic method just described was too tedious and time consuming. Thus a method of chromatography suited for assay purposes was developed.

Small strips of Whatman No. 1 filter paper 1 cm. x 16.5

cm. were sprayed with 0.1 M phosphate buffer. One microgram of sodium iodide and one microgram of sodium thiosulfate were spotted to the origin located 1 cm. from the bottom end of the strips. Eight microliter samples of the desired assay mediums were spotted on separate strips and dried with a current of warm air. A rectangular glass jar 18 cm. x 24 cm. x 17 cm. was used as the chromatography chamber. This container was immersed in a constant temperature bath at 38° C and the strips were introduced, suspended from a specially prepared cover, in such a manner that their bottom edge was 2-3 mm. below the solvent surface. Chromatographic development of the strips was carried on for precisely 40 minutes, at which time the strips were removed from the chamber and dried at room temperature. It was observed that strict adherence to the details of this procedure resulted in chromatograms in which the R_f values were completely reproducible. Since the position of the components were thus known it is no longer necessary to radioautograph the strip. Instead the radioactive spots could be cut out and their activities directly determined.

Quantitative Evaluation of Radioactivity

The method of estimation of radioactivity below was used for both "long" and "short" chromatographic papers.

The radioactive spots were located and cut from the paper chromatograms and then folded into small squares approximately 5 mm. x 5 mm. These squares were mounted in aluminum counting plates by means of "Scotch Tape" and counted with a

scintillation counter. Since high energy gamma radiation was being measured the effect of absorption by either paper or "Scotch Tape" could be ignored. Activities measured were calculated as a percent of the total activity. Thus the concentration of any one labelled component could be expressed as a percentage with respect to all others. This method automatically corrects for decay of radioiodine and also corrects for any small errors introduced in measuring the aliquot dried on the paper chromatogram.

Incubation Procedures for the Tyrosine Iodinase Assay

Assays for tyrosine iodinase activity were conducted along lines originally suggested by Fawcett and Kirkwood. Some modifications have, however, been introduced.

Enzyme preparations to be assayed were added in graded amounts to 125 mm. x 15 mm. Pyrex test tubes which contained an incubation media consisting of:

(a) 0.5 ml. of 0.1 M phosphate buffer pH 7.4
(b) 0.3 ml. of 1.1×10^{-4} M iodide
(c) 0.2 ml. of 0.04 M L-tyrosine
(d) 0.1 ml. of I-131 (20 microcuries of activity)
(e) 0.2 ml. of 0.04 M cupric ion
(f) sufficient distilled water to provide a constant volume of 3.3 ml.

Immediately after addition of the cupric ion activator the assay medium was incubated at 38° C for a period of 30 minutes

and then spotted to chromatographic papers, which were developed and counted as described. Since essentially all radioactivity was found in two spots:

(a) an iodide spot

(b) an MIT spot

the activity of the enzyme preparation was expressed as a percentage conversion of radioiodine to MIT.

It was originally assumed that the percentage conversion of I-131 to MIT would be a straight line function of the concentration of the tissue preparation. However it has been found that only at low tissue preparation concentrations does the activity versus concentration plot follow a straight line. The assay procedure was limited to the evaluation of low-concentration enzyme preparation (no higher than a 1.0 ml. sample of a 5 percent tissue homogenate supernatant in 3.3 ml. of assay medium). The activities of more concentrated solutions were then determined by extrapolation. The reason for this deviation from a straight line has not been determined as yet, but it is believed that the presence of large quantities of extraneous protein in crude enzyme preparations causes some inhibiting action.

Anaerobic Assay for Tyrosine Iodinase Activity

During the study of the factors concerned with the function of the tyrosine iodinase system the question of this system's dependence on the presence of molecular oxygen became

of interest. An investigation of the enzymatic process of tyrosine iodination under anaerobic conditions was carried out in the following manner.

Two test tubes 2.5 cm. in diameter and 10 cm. long containing 1.0 ml. of rat submaxilliary enzyme preparation and all components of the tyrosine iodinase assay medium with the exception of cupric ion, were fitted with two-holed rubber stoppers. Through one of these openings in each stopper a glass tube with a drawn out tip was inserted until it extended beneath the surface of the assay medium. Nitrogen gas was passed through the lead-in tube in such a manner that it swept through the assay medium and issued from the remaining orfice with sufficient pressure to prevent entry of atmospheric O_2. Nitrogen flow was continued for a five minute period. After 4 minutes had elapsed the cupric ion solution (O_2 free) was added. One of the assay tubes was then immediately sealed under a slight positive pressure of nitrogen. Nitrogen flow to the remaining tube was stopped and the assay medium re-equilibrated with atmospheric oxygen by shaking, in air, for one minute. The two tubes were then incubated for a 3 hour period at 38° C. Following incubation, aliquots from each tube were spotted on chromatograms, developed, radioautographed and counted in the normal manner. Duplicate assays were run concurrently.

Iodide Oxidizing Enzyme Assay

The same assay medium and methods of chromatography used in the tyrosine iodinase assay procedures were employed in the assay for the iodide oxidizing enzyme with two exceptions. Meta-aminophenol, an aromatic compound not accepted by tyrosine iodinase but which reacts avidly with elemental iodine, was substituted for L-tyrosine in the assay medium and incubation time was increased to two hours. Radioautographs of the chromatography strips again revealed only two radioactive spots:

(a) an iodide spot

(b) an iodo-m-aminophenol spot

Thus the activity of the iodide oxidizing enzyme may be measured in terms of the percentage conversion of I-131 to organically bound form.

Homogenate Supernatant Preparation

Homogenates and homogenate supernatants used in preliminary studies concerned with purification of tyrosine iodinase were prepared by means of the "Waring Blendor". In this procedure fresh tissue freed of fat and connective tissue was weighed and added to the appropriate ice cold extraction solution in a chilled glass "Waring Blendor" receptacle. The blendor was then run at "high speed" for one minute. At the end of this period the homogenates were decanted into pre-cooled "Lusteroid" test-tubes and centrifuged at 2,500 g and 0° C for

20 minutes. The supernatant was then decanted through several layers of surgical gauze into chilled containers and maintained at a temperature of approximately 4° C prior to use. Where a second or third extraction was desired, the sediment in the centrifuge tubes was resuspended in the appropriate concentration of ice cold solvent by the aid of a glass stirring rod. Supernatants of these extractions were obtained as previously by centrifugation at 2,500 g and 0° C for 20 minutes, followed by decantation.

Where large quantities of homogenate supernatant were not required the Potter-Elvehjem glass homogenizer was used. Except where otherwise stated, supernatants used were prepared in the following fashion. Fresh, trimmed, tissue was quickly weighed on a chilled watch glass and added to an homogenizer tube immersed in an ice-water bath. The desired volume of ice-cold isotonic potassium chloride solution was added and homogenization carried to completion as quickly as possible. Homogenates so prepared were decanted into chilled "Lusteroid" tubes and centrifuged at 2,500 g and 0° C in a refrigerated centrifuge for 20 minutes. The supernatant was decanted through gauze to remove fat particles and then maintained at approximately 4° C until used.

Preparation and Incubation of Media for Studies of the Antithyroid Action of Iodide Ion

Rat submaxilliary gland tissue was used in this series of experiments as a source of tyrosine iodinase. Supernatants were obtained from 20 percent homogenates prepared by the Potter-Elvehjem homogenizer technique already described. The supernatants were made iodide free by dialysis against iodide free salts (see page 45). Experiments were carried out in 125 mm. x 15 mm. Pyrex test tubes containing the following mixture:

(a) 1.0 ml. of the dialyzed enzyme preparation

(b) 0.5 ml. of 0.1 M phosphate buffer pH 7.4 (prepared from purified salts)

(c) 0.2 ml. of 0.04 M L-tyrosine

(d) 1.4 ml. of the appropriate concentration of iodide ion and I-131

(e) 0.2 ml. of 0.04 M cupric ion

The radioiodine used was carrier-free and was added to the medium in varying amounts governed by the level of stable iodine. In experiments where iodide ion concentrations from 10^{-6} to 10^{-5} were used 20 microcuries of I-131 were added to each assay tube. In those containing from 10^{-5} to 10^{-3} M iodide a constant specific activity of I-131 was used such that the tube containing 10^{-3} M iodide ion contained 0.75 mc. of radioactivity. Parallel experiments with radioiodine concentrations of one-tenth this specific activity demonstrated conclusively that radiation was not affecting the performance of the enzyme system.

The high levels of radioactivity were used in order to enable the small amounts of MIT formed at maximum inhibition to be measured with sufficient accuracy, Incubations were conducted at 38° C for 3 hours and at the end of this period the media from the appropriate tube were chromatographically analyzed and the percentage conversion to MIT determined.

Preparation of Tissue Mitochondria

Mitochondria from the various tissues investigated for organic iodine binding ability were prepared by the differential centrifugation procedure of Taurog et al. (5). Lots of the appropriate tissue weighing 1.5 g. each were ground in a chilled Potter-Elvehjem glass homogenizer with 10 ml. of ice-cold Krebs-Ringer bicarbonate solution prepared from the following stock solutions:

Solutions	Parts
0.9 percent sodium chloride solution	100
1.15 percent potassium chloride solution	4
1.22 percent calcium chloride solution	3
2.11 percent potassium hydrogen phosphate solution	1
3.82 percent hydrated magnesium sulphate solution	1
1.30 percent sodium bicarbonate solution gassed for 1 hour with CO_2	21

Immediately prior to use the Krebs-Ringer bicarbonate solution was gassed with 95 percent O_2: 5 percent CO_2 for 10 minutes.

Several lots of homogenates were pooled and centrifuged at 600 g and 0° C for 10 minutes to remove whole cells and nuclei. The supernatant was decanted through gauze to separate fat particles and the sediment discarded. The supernatant was then recentrifuged at 25,000 g and 0° C for 30 minutes and decanted from the resulting mitochondrial "pill". The mitochondria were resuspended in ice-cold Krebs-Ringer bicarbonate solution by means of light homogenation with the Potter-Elvehjem glass homogenizer. Volumes were then adjusted in order that 3.0 ml. of the final suspension was equivalent to 1.5 g. of original tissue. The mitochondrial preparation was added to Warburg flasks together with 100 microcuries of I-131 in 50 to 100 microliters of solution. In general 3.0 ml. quantities of suspension were used for each flask. Where difficulties were encountered in preparing sufficient quantities of suspension, one-half quantities were used. The flasks were suspended in a Warburg bath maintained at 38° C and were gassed for 10 minutes with 95 percent O_2:5 percent CO_2. Incubation was then carried out with shaking, for two hours. At the end of this period the flasks were removed from the bath, cooled to ice temperature, and treated with 50 microliters of a saturated solution of thiouracil to inhibit further organic binding of I-131. Aliquots of these preparations were spotted to prepared chromatography strips and treated as previously described. In cases where enzymatic hydrolysis of the incubated material was desired

0.5 ml. aliquots were pipetted into 15 ml. tapered centrifuge tubes. Proteolytic enzyme preparations were then added and the tubes allowed to digest at 38° C under toluene. After 16 hours digestion the hydrolyzed preparations were spotted to chromatography strips.

Dialysis Procedures

Homogenate supernatants to be dialyzed were transfered by means of a chilled pipette to "Visking" cellophane casings sealed at one end. Generally 20 ml. of supernatant were placed in each casing. The top end of the container was then closed in such a manner that a small quantity of air was entrapped. The cellophane bags were tied by one end to a magnetic stirring bar and suspended in a 4 liter beaker of 0.1 M phosphate buffer solution (pH 7.4) prepared from iodide-free salts. The air bubble served to keep the bags erect. The beaker was then placed over a magnetic stirrer in such a fashion that the rotating magnetic bar gently moved the cellophane bags through the dialysis medium. The temperature of the buffer was maintained at 4° C throughout the dialysis. In general, dialysis was continued for 40 hours against two changes of buffer solution (4 liters per change). During dialysis protein material was observed to precipitate. This precipitate was removed by centrifuging for 20 minutes at 2,500 g and 0° C. The supernatant solution from this centrifugation was then

made up to the original volume of the homogenate supernatant with 0.1 M phosphate buffer (pH 7.4) and used as such in enzyme assays.

Assay for Proteolytic Activity

Proteolytic activity was determined by a modification of Anson's procedure for measurement of pepsin activity (112).

Pyrex test tubes 15 mm. x 125 mm. containing 5.0 ml. of a 2 percent aqueous solution of dialyzed bovine haemoglobin substrate powder (Worthington Biochemical Co., Freehold N.J.) were brought to 38° C in a constant temperature bath. One ml. quantities of graded concentrations of the enzyme preparations to be assayed were added to the tubes which were then incubated for 30 minutes. At the end of this period 10 ml. of 0.3 M trichloracetic acid were added to stop proteolytic action and to precipitate protein material which was removed by suction filtration. Five ml. of the clear filtrate was then added to 10 ml. of 0.5 N sodium hydroxide solution. Three ml. of Folin-Ciocalteu reagent (diluted to one-half concentration) was added and the intensity of the colour read in a spectrophotometer at 535 mμ. The concentration of the material which gave a colour with the reagent was expressed in terms of the amount of tyrosine required to give a colour of equal intensity.

Folin-Ciocalteu Reagent

The Folin-Ciocalteu reagent was prepared according to the method of its originators (113).

One hundred ml. of sodium tungstate ($Na_2WO_4 \cdot 2H_2O$) 25 g. of sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$), 700 ml. of distilled water, 50 ml. of 85 percent phosphoric acid and 100 ml. of concentrated hydrochloric acid were added to a 1500 ml. flask. This mixture was refluxed gently for 10 hours. The condenser was removed and 150 g. of lithium sulphate, 50 ml. of distilled water and a few drops of bromine added. Boiling was recommenced for 15 minutes to remove excess bromine. The solution was cooled, diluted to one liter and filtered. Before use the solution is diluted with an equivalent volume of distilled water.

Iodine Free Salts Used in Dialysis

In order to reduce the iodide level of certain preparations to as low a value as possible they were dialyzed against a buffer solution prepared from carefully purified salts. These salts ($Na_2HPO_4 \cdot 7H_2O$ and KH_2PO_4) were prepared by disolving the analytical reagent grade chemical in a volume of distilled water approximately twice that required to prepare a hot saturated solution. Hydrogen peroxide (3.0 ml. of a 30 percent solution) was added to the solution and it was then evaporated to one-half its volume. The solution was cooled and the precipitated salt filtered off on a Buchner funnel. This sequence was repeated three times followed by a final recrystallization from distilled water in the absence of hydrogen peroxide.

Radioiodine

The radioactive iodine used throughout this thesis was the "Isotag" brand of I-131 supplied by Charles E. Frosst and Co. as an aqueous solution of carrier free NaI-131.

Catalase

The crystalline beef liver catalase used in peroxidase inhibition studies was prepared by Worthington Biochemical Corp., Freehold, N.J. using a procedure described by Tauber and Petit (114). This preparation consisted of a suspension of enzyme crystals in water. Its activity was 15,000 units per ml. where a unit is defined as that weight of enzyme required to decompose 1 mg. of hydrogen peroxide per minute under the conditions described by Beers and Sizer (115). The weight of dry protein per catalase unit was determined as 0.66 micrograms per unit.

Common Reagents and Solvents

All inorganic reagents used in the experimental work of this thesis were of "analytical" grade quality. The majority of organic compounds used were of this same standard. Where "purified" grade crystalline organic compounds were not obtainable however the "technical" grade chemicals were recrystallized by standard techniques to a constant melting point. All organic solvents, regardless of quality, were redistilled before use.

RESULTS AND DISCUSSION

Introduction-Enzyme Systems Involved in Organic Binding of Iodine

Surviving thyroid slices, like the thyroid gland *in situ*, have been demonstrated to be capable of incorporating iodide-131 into MIT, DIT, and Tx (38). This evidence would appear to indicate that the pathway for Tx formation involves the sequence [3] :

$$\text{Tyrosine} \longrightarrow \text{MIT} \longrightarrow \text{DIT} \longrightarrow \text{Tx.} \qquad [3]$$

Prior to 1953, however, little was known concerning the actual mechanism by which the iodinated compounds were formed. Harington (109) suggested that the iodination reactions might proceed non-enzymatically with the enzymatic formation of elemental iodine as the controlling step.

In 1953 Weiss (40) found that cell free homogenates of thyroid tissue would incorporate iodide-131 into an organically bound form if they were supplemented with cupric ion and L-tyrosine. Furthermore, although anaerobiosis abolishes the synthesis by slices (45), he found it to be without effect on homogenates supplemented with cupric ion. Weiss reported the organically bound iodine of his system to consist of protein bound Tx and DIT since he observed it to be readily soluble in n-butanol during extraction of hydrolyzed homogenates. Fawcett and Kirkwood (2,3) reinvestigated Weiss' system with the object

of identifying the substances formed by the use of paper chromatography rather than by this non-specific solvent extraction procedure. These authors detected only one iodinated organic compound, MIT and produced evidence to show that its formation was dependent on the action of a soluble enzyme that carries out a single step iodination of tyrosine with elemental iodine. Furthermore, this MIT was shown to be present as the free amino acid and required no hydrolysis of the homogenate for its release. Fawcett and Kirkwood detected high concentrations of this enzyme, which they named tyrosine iodinase, in salivary glands of several animals and suggested that its presence might be explained by the hypothesis that these tissues degrade Tx and that the enzyme functions in the deiodination of MIT resulting from Tx degradation.

More recently organic binding of iodide has been described with other thyroid preparations. Taurog et al. (5) have observed that suspensions of sheep thyroid mitochondria incorporate iodide-131 into an organically bound form and Stanbury and Wyngaarden (116) have reported organic binding of iodide-131 with whole rat thyroid homogenates. Both these preparations carry out the synthesis without the addition of either cupric ion or tyrosine. Taurog et al. (5) have separated the radioactive components of their system by means of paper chromatography. Three radioactive spots were observed on their chromatograms: an immobile spot, an iodide spot and an uniden-

tified spot running at the solvent front. Pancreatin treatment of the labelled mitochondria, prior to chromatographic separation, results in greatly reduced origin activity and the appearance of free MIT. Taurog et al. suggest that the origin component is protein bound MIT from which the free amino acid is released on pancreatin hydrolysis and offer these observations as support of the theory that primary organic binding of iodine is through iodination of protein. The iodide binding system of Stanbury and Wyngaarden (116) bears a strong resemblance to that of Taurog et al.(5) in that supplementation is not required for synthesis and the initial product of iodination appears to be protein bound L-tyrosine. The iodination observed with this system, however, is less than that with isolated mitochondria. It is entirely possible that these two systems are essentially the same and that the weaker iodination observed in the Stanbury-Wyngaarden system is due to the lower concentration of mitochondria present.

The foregoing discussion illustrates the state of confusion which now exists in thyroid enzyme work. At least two organic iodine binding systems are presently defined and these have been shown to differ with respect to substrate requirements, need for supplementation, and solubility of the enzyme system. Since it appears probable that only one thyroidal iodine binding system exists, it would be of value to deter-

mine which, if either, of these systems has physiological significance. A major portion of the work carried out in this thesis has been directed toward a clarification of this problem.

The Iodide Oxidizing Enzyme System

Evidence for an Iodide Oxidizing Enzyme System

The existence of an enzyme in the thyroid involved in iodide oxidation was first postulated by von Mutzenbecker (22), in 1939, as a result of his observation that Tx was formed during the *in vitro* iodination of protein with elemental iodine. In 1944 Dempsey (46) presented histochemical evidence for the existence of a peroxidase in thyroid tissue and suggested that this peroxidase was responsible for the formation of elemental iodine in the gland. De Robertis and Grasso (48) confirmed the existence of a peroxidase in the thyroid gland in 1946. They prepared glycerol extracts of thyroid tissue and obtained qualitative evidence for an enzyme-catalyzed oxidation of iodide ion to elemental iodine in the presence of hydrogen peroxide. Fawcett and Kirkwood, in 1953 (110) produced direct evidence that elemental iodine was formed in intact thyroid tissue through the action of an enzyme system and that this elemental iodine was a key intermediate in the synthesis of organically-bound iodine in the gland. The current wide acceptance of the theory of a peroxidase-iodide oxidizing system may be illustrated by reference to the many hypotheses advanced to explain the inhibiting action of antithyroid compounds as a consequence of their ability to poison peroxidases (117). The question of the

occurrence and nature of an iodide oxidizing system in tyrosine iodinase extracts is thus one of considerable interest.

Weiss (40) suggested that a minimum of two enzymes were involved in his system: an enzyme, presumed to be a peroxidase, involved in the formation of elemental iodine and an enzyme involved in the coupling of two moles of DIT to form Tx. Since iron has been demonstrated to be the prosthetic group in all known peroxidases, Weiss concluded that cupric ion must function as a co-factor for the second enzyme. Because of Weiss' claim that, in copper supplemented thyroid extracts, the formation of organically bound iodine was not affected by anaerobiosis, Fawcett and Kirkwood (2) postulated that cupric ion must supply the oxidizing power that is known to be normally supplied by oxygen (45). They suggested therefore that cupric ion might act as a non-enzymatic generator of elemental iodine through the well-known reaction [4]

$$2Cu^{2+} + 2I^- \longrightarrow 2Cu^{+} + I_2 \qquad [4]$$

and that it replaced the iodide oxidizing enzyme originally present in the thyroid which had been destroyed or damaged by extraction procedures. However, several considerations point to the conclusion that copper is not functioning in the fashion postulated by Fawcett and Kirkwood. Thermo-dynamic calculation of the quantity of elemental iodine which would be expected to be in equilibrium with cupric ion sets the value at 10^{-14} M or approximately 10^{-16} moles of I_2 per assay tube (118). This

extremely small concentration raises reasonable doubt that reaction [4] can replace the natural iodide oxidizing system. Further, extraction experiments with carbon tetrachloride show that the concentration of elemental iodine in the cupric ion supplemented enzyme incubation medium is below 10^{-10} M (119), a value consistent with the thermo-dynamic calculations. In addition, a study of the effect of excess iodide on the kinetics of the tyrosine iodinase system permits an analysis of the function of cupric ion in this preparation. It is possible to show, from Mass-Law considerations, that cupric ion cannot possibly be functioning in the manner originally postulated by Fawcett and Kirkwood. (see Appendix I).

The only reasonable explanation for the inhibition of the tyrosine iodinase system by excess iodide ion involves the assumption that the iodide oxidizing step is enzyme-catalyzed. If this is the case, then as the iodide concentration is increased, the oxidizing enzyme will become saturated and the rate of formation of I_3^- will approach a maximum. However, if the iodide concentration is increased beyond the saturation point inhibition will result since the excess iodide can only serve to remove elemental iodine as triiodide ion,

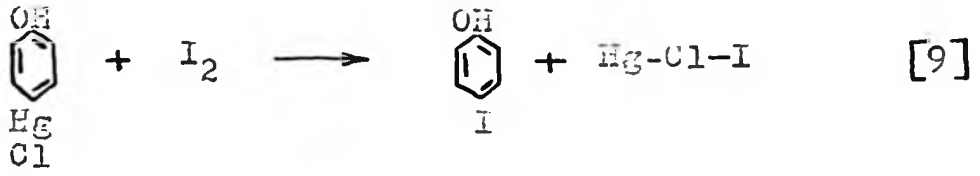
Since there appears to be considerable evidence on hand indicating that an iodide oxidizing enzyme functions in the tyrosine iodinase system, a series of experiments was de-

signed to provide direct evidence on this point. The most likely means of demonstrating such an iodide oxidizing enzyme is through the detection of the formation of its primary product, elemental iodine. A method of collecting and stabilizing elemental iodine was therefore required. Such a method was adapted from a procedure devised by Fawcett and Kirkwood (110) to study the mode of action of certain aromatic antithyroid compounds. These authors observed that certain aromatic compounds functioned in their capacity as inhibitors of organic binding of I-131 in surviving thyroid slices by virtue of their ability to "collect" elemental iodine by undergoing a rapid substitution reaction with it. Applying this information to the problem of the detection of the iodide oxidizing enzyme system, it should be possible to substitute the highly reactive aromatic iodine "collector" resorcinol for L-tyrosine in the tyrosine iodinase reaction medium. Thus, if an iodide oxidizing enzyme exists in tyrosine iodinase preparations, labelled, iodinated resorcinol (formed by a non-enzymatic substitution with enzymatically generated I_2) should be detectable by paper chromatography in unboiled but not in boiled enzyme preparations incubated with I-131. This experiment was conducted with the result that a weak, but definite iodoresorcinol component could be demonstrated chromatographically in the assay medium.

The possibility that tyrosine iodinase is accepting

resorcinol as a substrate in the preceeding experiment must not be overlooked. In this event, the enzymatic phenomenon of the formation of iodinated resorcinol with unboiled but not boiled preparations could well be due to the action of tyrosine iodinase in catalyzing resorcinol iodination and not to enzymatic generation of I_2. If this situation exists, then the iodination of any series of aromatic "collectors" should be a function of their structural resemblance to L-tyrosine. On the other hand, if the substitution reaction with the "collectors" is non-enzymatic, the order of the iodination would be a function of the activating groups on the benzene ring. Since aromatic amines would be expected to be non-enzymatically substituted with greater ease than phenols under the neutral conditions employed in the assay medium (120), the order of iodination expected with the following series of aromatic "collectors" would be phenol < aniline < resorcinol < m-aminophenol < m-phenylene diamine. The results of such a study are given in Table 1. The order of iodination is in the direction predicted by the theory of aromatic substitution rather than in order of resemblance to the L-tyrosine. Metaphenylenediamine, the most rapidly iodinated compound, bears the least structural resemblance to L-tyrosine. Final conclusive evidence was brought to bear on this problem by the use of the iodine "collector" p-chloromercuriphenol. This compound,

which reacts quantitatively with elemental iodine but not iodide (121) according to equation [9] is wholly unlike L-tyrosine because of its bulky metal group. Therefore any



[9]

formation of p-iodophenol with unboiled enzyme preparations in excess of that formed with boiled preparations could only be explained by the non-enzymatic substitution reaction [9]. This thesis was investigated using one ml. of a saturated solution of p-chloromercuriphenol as a "collector" in a tyrosine iodinase assay medium in place of the normal addition of L-tyrosine. A weak but persistent radioactive component was observed which was demonstrated chromatographically to be identical with an authentic sample of p-iodophenol. Boiling the enzyme preparation prior to incubation completely prevented the formation of p-iodophenol.

The Antithyroid Action of Aromatic Inhibitors

Arnott and Doniach (122) have studied the ability of a large number of diphenols to prevent accumulation of I-131 in the thyroids of intact rats. They suggest that this type of antithyroid compound may act through inhibition or "poisoning" of the enzyme involved in I_2 formation (i.e. the iodide oxidizing enzyme). Fawcett and Kirkwood (110) have challenged this picture on the basis of their observations that iodophloroglucinol and iodoresorcinol are formed when phloroglucinol and

resorcinol are incubated with surviving rat thyroid slices. These authors postulate that the primary mechanism of actio. of the aromatic antithyroid agents occurs, rather, through removal of the reactive iodinating species, elemental iodin. A consideration of the bell-shaped curves of Fig. 3 and 4, which represents the results of a study of the effect of "collector" concentration on the I_2 generation rate of the io dide oxidizing enzyme, with several sources of enzyme, may shed some light on this controversy. The first increase in t formation of iodo-compounds with increased amounts of "colle tor" may be readily explained as a simple mass-law effect produced by virtue of the fact that the rate of any reaction is a direct function of the concentration of its reactants. the same basis the subsequent decline in iodination rate must be due to a diminished concentration of one of the re actants. Since the concentration of "collector" has actually increased then, of necessity, we must assume that the concen tration of elemental iodine has in some way been decreased. applied to the understanding of the action of aromatic inhib tors it is seen that the data of Fig. 3 and 4 indicate that the primary action of resorcinol, m-aminophenol, and m-phenyl enediamine is to remove elemental iodine by reacting with it hence competitively inhibiting tyrosine iodination. However the data would also suggest that a secondary inhibition phenomenon (as evidenced by a decrease in iodo-organic produc

formed) may also occur with increased concentration of inhibitor. Since it appears that this inhibition is due to a decrease in the concentration of elemental iodine, it is entirely possible that the suggestion of Arnott and Doniach that aromatic inhibitors function by "poisoning" the iodine generating enzyme is an explanation of this secondary inhibition effect. Thus the mechanism by which aromatic compounds inhibit Tx biogenesis may well be a function of the actual concentration of these inhibitors in the thyroid gland.

Development of an Assay for the Iodide Oxidizing Enzyme System

The knowledge that, with a given tissue and iodine "collector", a maximal peak of iodination occurs at a definite "collector" concentration (see Fig. 3 and 4) is of value in the development of an assay for the iodide oxidizing enzyme system. With beef submaxillary tissue as enzyme source and m-aminophenol as "collector" the maximum iodination peak has been established at 10^{-5} M concentration of "collector". It should be possible to develop an assay for the iodide oxidizing enzyme system by substituting this molar concentration of m-aminophenol for L-tyrosine in the tyrosine iodinase assay medium. If this assay medium is satisfactory then a straight line response should result if the percent iodination is plotted against the concentration of tissue preparation in the medium. Fig. 5 is a typical plot of enzyme concentration against percent iodination of m-aminophenol and the method of assay is seen to be entirely satisfactory.

Nature of the Iodide Oxidizing Enzyme System

The demonstration that an oxidizing enzyme is responsible for the production of I_2 in copper-supplemented thyroid homogenates raised some question as to the final electron acceptor in this system. As a consequence, it was decided to reinvestigate Weiss' claim that his system showed no requirement for oxygen. Five percent extracts of tyrosine iodinase prepared from rat submaxillary gland were used to study the iodination of L-tyrosine under aerobic and anaerobic conditions. The experimental procedure used to establish anaerobic conditions is described in the Experimental section of this thesis. The results of this study are given in Table II. Anaerobiosis is seen to cause a drastic inhibition of the organic binding of iodine. These data conflict with Weiss' observation and demand that the iodide oxidase scheme, involving cupric ion as an acceptor of electrons, be abandoned. The discrepancy which exists between the two sets of data is difficult to explain. The possibility that Weiss' nitrogen supply was contaminated with oxygen would appear unlikely in view of his care in providing O_2 free nitrogen by passing tank nitrogen over hot metallic copper.

Similarly, it is not possible that a toxic factor was present in the nitrogen supply used in our experiments since both control and test media were exposed to identical nitrogen volumes during sweeping procedures, (see p.). Thus there is no explanation of the differences in results at this point.

Since the demand for oxygen must obviously be connected with an oxidizing step, and hence the iodide oxidizing step, it was of interest to examine the effects of anaerobiosis on the iodide oxidizing enzyme system. Thus 5 percent rat submaxillary enzyme preparations were incubated aerobically in the presence of I-131 with m-aminophenol as an elemental iodine "collector". The results of this study are given in Table III. Although not as dramatic as the results obtained with the tyrosine iodinase enzyme, the data of Table III are seen to be entirely consistent with those of Table II. Thus the iodide oxidizing enzyme system detected in tyrosine iodinase extracts contains an oxidase that may be one of three fundamental types. It may involve an oxidase specific for iodide which catalyzes the transfer of two electrons from iodide ion to some acceptor system and hence either directly or indirectly to O_2. The I^+ ion formed may then react with tyrosine immediately through the agency of tyrosine iodinase or combine with I^- ion to form elemental iodine which may be the requisite form demanded by tyrosine iodinase. Schematically this system may be represented by Fig. 6.

An alternate enzyme system for iodide oxidation might involve a hydrogen peroxide generating oxidase coupled with a specific or non-specific peroxidase which would employ the oxidizing power of hydrogen peroxide to generate I_2. This system may be schematically represented as in Fig. 7a.

A third feasible enzyme system is one which is identical

to the last described system with the exception that no peroxidase is present. Thus I_2 is generated non-enzymatically from iodide ion through the agency of hydrogen peroxide released by an oxidase as in Fig. 7b.

An experiment designed to aid in the elucidation of the nature of the iodide oxidizing enzyme system was conducted with the enzyme catalase.

Catalase (123) is an iron porphyrin enzyme which catalyzes the decomposition of hydrogen peroxide according to equation [10].

$$2H_2O_2 \longrightarrow 2H_2O + O_2 \qquad [10]$$

It was decided that if hydrogen peroxide were involved in iodide oxidation then, by virtue of its ability to destroy hydrogen peroxide, catalase should inhibit I_2 formation by the iodide oxidizing enzyme. Since catalase has a peroxidative action common to all iron porphyrin compounds, it was decided to attach no significance to the results of the experiment if inhibition was not detected, since catalase might conceivably not inhibit elemental iodine formation through breakdown of hydrogen peroxide but rather might enhance it through a catalysis of the hydrogen peroxide oxidation of iodide to iodine. If inhibition was observed, however, a definite conclusion might be drawn since, presumably, the only possible inhibiting action of catalase would be through destruction of hydrogen peroxide. Consequently an assay was conducted for the iodide oxidizing enzyme, in the presence of graded amounts of beef

liver catalase, using beef submaxillary homogenate supernatant as a source of enzyme and m-aminophenol as the I_2 "collector". The results of this study are graphically depicted in Fig. 9. Inhibition was observed to occur to the extent of 50 percent at a catalase concentration of 0.05 mg. in the assay media of 3.3 ml. From these results one may conclude that hydrogen peroxide is involved in iodide oxidation and that one of the two mechanisms schematically represented in Fig. 7a and 7b must hold.

A further experiment was performed in an attempt to distinguish between the mechanisms of Fig. 7a and 7b. It has been stated that peroxidases are unable to oxidize halides of lower atomic number than iodine (117). Since hydrogen peroxide, however, has sufficient oxidation potential to oxidize bromide ion to elemental bromine (124), the inability of any hydrogen peroxide system to bring about bromide oxidation in the presence or absence of a peroxidase must be a result of a low hydrogen peroxide concentration. Thus if bromide ion were supplied to the iodide oxidizing enzyme system, this system might or might not oxidize it to bromine. If oxidation was observed, no firm conclusions could be drawn since oxidation might be effected either by hydrogen peroxide alone or by hydrogen peroxide in the presence of a non-specific peroxidase. On the other hand if oxidation does not occur, a definition of the system might be possible. In this latter case two possible situations might

be advanced to explain the inability of the iodide oxidizing system to furnish elemental bromine:

(a) the oxidation of iodide to iodine in vitro and in vivo may be the result of a direct reaction with hydrogen peroxide in the presence of a peroxidase (Fig. 7b) but a low concentration of hydrogen peroxide may lower the potential of the H_2O_2-H_2O couple to a point where it is below the Br_2-Br^- couple

(b) a similar situation to (a) may exist, with respect to a low hydrogen peroxide concentration but a peroxidase specific for iodine, may be involved (Fig. 7a) in the oxidation.

If situation (a) obtained then any member of the halogen group with a lower oxidation potential than iodine, e.g. astatine (125), should be oxidized to the elemental form. Fortunately an in vivo study has been made of the behaviour of astatine in the thyroid gland (126). This study revealed that no organic binding of the halogen occured suggesting that astatine is not oxidized by the iodide oxidizing system of the thyroid. This data would thus appear to dictate against situation (a). If the concentration of hydrogen peroxide were sufficiently low to prevent the oxidation of appreciable amounts of astatine a peroxidase mechanism as in situation (b) would be required to explain the oxidation of iodide to iodine. In view of these conclusions a study was made of the ability of the iodide oxidizing enzyme to oxidize bromide ion. An assay was conducted using: 5 percent beef submaxillary homogenate supernatant as the enzyme source,

m-aminophenol as the halogen "collector", and bromide ion as a substitute for iodide ion with Br-82 as an isotopic tracer. Incubation was carried out for a three hour period. At the end of this interval the assay medium was chromatographed and the chromatograms radioautographed. The X-ray film revealed only one radio-active spot - a bromide ion component. No organically bound bromine was observed and hence no elemental bromine would appear to have been produced by the oxidizing enzyme system. This result is consistent with the report of an in vivo study of Simon (127) which similarly showed no organic incorporation of bromine, and, hence, presumably no oxidation of bromide ion to elemental bromine in the thyroid gland. On the basis of these results it would appear that a peroxidase specific for iodide ion is an integral part of the iodide oxidizing enzyme system. This finding is not without precedent since a highly specific peroxidase has been shown to exist (128). Although this evidence is by no means conclusive, it would appear likely that a hydrogen peroxide-producing oxidase coupled with a peroxidase is involved in iodide oxidation in tyrosine iodinase extracts. An experiment to directly confirm this postulated function of hydrogen peroxide was carried out after a major portion of this thesis had been written. The results were of sufficient importance, however, to warrant inclusion in the thesis at this point. The sole function of an oxidase, as a unit of the iodide oxidizing system, is to provide hydrogen peroxide for the peroxidase through the reduction of elemental oxygen. Thus the depen-

dence of the system on oxygen is essentially an expression of its requirement for hydrogen peroxide. It should be possible therefore to remove this dependence on oxygen by supplementing the iodide oxidizing system with hydrogen peroxide. This hypothesis was examined experimentally by a study of the ability of varying concentrations of hydrogen peroxide to overcome the inhibition of the tyrosine iodinase system brought about by anaerobiosis. It has been demonstrated that the manner in which hydrogen peroxide is supplied to a peroxidase system controls the efficiency with which it is utilized. Thus Laser (130) found that 80 percent of the hydrogen peroxide diffused through a cellophane membrane was used in the peroxidatic oxidation of ethanol to acetaldehyde in the presence of catalase whereas only minor amounts were peroxidatically employed where direct additions of hydrogen peroxide were made to the system. In view of these data it was decided that hydrogen peroxide should be added to the tyrosine iodinase system in a manner which would simulate, as closely as possible, the controlled enzymatic generation of hydrogen peroxide which presumably would be effected by an oxidase. This was accomplished by an adaptation of the technique of Laser which involved the suspension of Visking casing bags containing hydrogen peroxide solution in the assay medium and permitting diffusion of hydrogen peroxide through the casing wall. The result of a typical series of experiments are presented in Fig. 19. It is seen from these data that hydrogen peroxide completely removes all necessity

for elemental oxygen and that the formation of MIT observed with hydrogen peroxide supplemented anaerobic system is actually increased above unsupplemented aerobic systems —— convincing proof for the postulated function of hydrogen peroxide in the system.

The question of the identity of the hydrogen peroxide-producing oxidase is one of very obvious future interest. Dempsey (129) suggests that the cytochrome oxidase-cytochrome c system may be involved. Oxidases which have been looked for but not detected in thyroid tissue or extracts are the xanthine oxidase system (40,47), and the glucose oxidase system (40). It has also been suggested that hydrogen peroxide may arise from the copper catalyzed oxidation of ascorbic acid (117).

Occurence of the Iodide Oxidizing Enzyme System

Since the iodide oxidizing enzyme system is intimately bound up with tyrosine iodinase it must be present in all tissues in which tyrosine iodinase has been observed. As far as this proposition has been examined the expected relationship has been observed. Table IV contains a list of tissues examined for the iodide oxidizing enzyme system and the tyrosine iodinase system.

The Tyrosine Iodinase System

Fawcett and Kirkwood (2,3) originally considered that tyrosine iodinase preparations contained a single enzyme which specifically catalyzed the substitution reaction between elemental iodine and tyrosine. Data presented in the preceeding section of this thesis indicate, however, that an oxidase and a peroxidase are also present in these preparations and that these function with tyrosine iodinase by virtue of their ability to supply its substrate, elemental iodine. Therefore the term "tyrosine iodinase system" will be used henceforth to signify the co-existence of the iodide oxidizing system and tyrosine iodinase. Further modifications of the original concept will now be discussed.

Substrate Specificity

The two basic observations which led to the postulation of a tyrosine iodinase were:

(a) boiled tissue preparations exhibited none of the catalytic qualities observed with unboiled preparations.

(b) tissue preparations exhibited a substrate specificity of a type which is characteristic of enzymes.

The demonstration of an iodide oxidizing enzyme system means that (a) can no longer be considered evidence for an enzyme catalyzing tyrosine iodination since this phenomenon may just

as conveniently be explained on the basis that either the oxidase or peroxidase is inactivated. Hence, were it not for the demonstration of a specificity requirement, the possibility would be open that the iodide oxidizing enzyme system is the only enzymatic process involved in the organic binding of iodine in thyroid tissue extracts. In view of the crucial importance of the specificity data to the concept of a tyrosine iodinase, it was decided to reinvestigate and extend this study. Table V contains the results of iodination studies with the tyrosine iodinase system using a wide range of aromatic substrates and a number of enzyme sources. On the basis of these results some modifications and extensions of the original concept of substrate specificity must be made.

Fawcett and Kirkwood claimed that tyrosine iodinase exhibits a number of specificity requirements. No substrate tested was as effective as L-tyrosine. In short time experiments the extent of iodination of D-tyrosine was approximately 60 percent of that of the L-isomer. On the basis of the observation that p-hydroxyphenylpropionic acid and p-hydroxybenzoic acid were accepted by the enzyme while p-hydroxyphenylacetic acid was not, it was suggested that a p-hydroxyphenyl-ring with a carboxylic acid side chain is required for substrate activity and that the carboxyl group must be capable of orientation into the plane of the aromatic ring when the centers of negative charge (phenolic hydroxyl and carboxylic acid group) are maximally separated.

A point of importance established by the results shown in Table V is that p-hydroxyphenylacetic acid is iodinated, and to a slightly greater extent than either p-hydroxybenzoic acid or p-hydroxyphenylpropionic acid. This is in sharp contrast to the earlier report (3) and is puzzling in view of the fact that the same preparation of p-hydroxyphenylacetic acid was used in both cases. However, an examination of Fawcett's original data has disclosed that he used a frozen preparation of enzyme and ran the experiment without an L-tyrosine control. It is thus entirely possible that his enzyme preparation was denatured. Since the postulated specificity requirement, concerned with the necessity for a carboxyl- side chain in the plane of the aromatic ring, was predicated on the observation that p-hydroxyphenylacetic acid was not accepted as a substrate, a revision of the original theory is necessary. A minor difference between the results of Table V and earlier data (3) is seen in the observation that N-acetyl-DL-tyrosine is iodinated at a rate approaching that of L-tyrosine. An explanation of this discrepancy is not available at present. The data of Table V confirms the fact that, with rat tissue, L-tyrosine is iodinated to a greater extent than D-tyrosine although the differential between the two substrates is less pronounced than that previously reported. The more extensive differences reported by Fawcett and Kirkwood (3) may be due, in part, to the fact that their experiments

were of short time duration. Although this procedure has the advantage of maintaining the time versus percent-iodination curve at its steepest slope, it also reduces the extent of iodination markedly and any errors in the determination of percentage conversion of I-131 to MIT are consequently magnified. Since this source of error was greatly reduced in the experiments reported in Table V it is felt that these results are more reliable.

The relative rates of D- and L-tyrosine iodination observed with the same tissue from different species are of interest. It is seen that, with beef submaxillary tissue, the relative rates of iodination are reversed. If the enzyme system is of physiological significance, then its fundamental nature would not be expected to differ from species to species. Since the enzyme extracts used are relatively crude, it is not improbable that other enzyme systems are making demands upon the tyrosine pool. The extent of these demands may well vary from species to species. Thus an explanation of the reverse order of iodination rate may be that, in beef submaxillary extracts, some enzyme or enzymes are removing L-tyrosine (e.g. through transamination) thus lowering its concentration and hence its rate of iodination as compared to the D-isomer. This explanation would also appear, at first sight, to be applicable to the explanation of the superiority of L-tyrosine over D-tyrosine as a substrate with rat tissue preparations. However, here it would be

necessary to postulate a very active enzyme involved in D-tyrosine metabolism. This situation, though not impossible, is less likely since D-amino acid metabolizing enzymes have a very limited occurence in animal tissue (131). It is evident from the scarcity of data on this point that no firm decision may be made at this time. However, it is obvious that the situation is much more complex than originally suspected.

A revised concept of tyrosine iodinase specificity must be drawn up in view of the data of Table V and the preceeding discussions. For effective substrate activity (arbitrarily set at 25 percent iodination with respect to L-tyrosine) an aromatic compound must possess:

(a) a free phenolic hydroxyl group

(b) a saturated side chain which may be o, m, or p, to the phenolic function

(c) a carboxylic acid group attached to the side chain.

The presence of a free amino group does not appear necessary. When the amino group is effectively covered, as with N-phthaloyl-L-tyrosine or N-acetyl-DL-tyrosine the rate of iodination is unchanged or even enhanced.

Model Systems

One of two possible situations must exist in view of the findings just reported. Either the enzyme tyrosine iodinase does exist but is much less specific than was

originally supposed, or no iodinating enzyme is present in these tissue extracts and iodination is accomplished non-enzymatically by elemental iodine liberated through the action of the iodide oxidizing enzyme. If the iodination is non-enzymatic, it should be possible to obtain relative rates of iodination comparable to those in Table V with a synthetic iodinating system. With this idea in mind a series of aromatic compounds was iodinated with elemental iodine generated by the action of hydrogen peroxide. Iodide concentrations were maintained at the level normally used in enzyme assays. The results of this study are given in Table VI. From a consideration of this Table it is seen that, in general, where iodination occurs, the rates of iodination as compared to L-tyrosine are appreciably higher in the non-enzymatic than in the enzymatic process. Particularly striking is the fact that phenol, p-cresol, and p-hydroxycinnamic acid, which are not iodinated in the presence of enzyme extracts, are extensively iodinated non-enzymatically. These data suggest the existence of an iodinating enzyme, exhibiting definite rules of specificity. Other explanations of the data of Table VI, must not be overlooked. It is conceivable that the failure to observe iodination with p-hydroxycinnamic acid, p-cresol, and phenol might be due to inhibition of the iodide oxidizing enzyme by these substrates rather than to a manifestation of a specificity requirement. However, since inhibition would presumably be a function of the phenolic

hydroxyl, it becomes most difficult to understand why p-hydroxyphenylpropionic acid, for example, does not exhibit a similar inhibitory action.

A further model experiment to evaluate the enzymatic or non-enzymatic nature of iodination in the tyrosine iodinase system was based on a study of casein iodination in milk devised by Keston (50). This author showed that raw milk, which contains xanthine oxidase and lactoperoxidase, yields labelled iodinated casein when supplemented with I-131 and xanthine. Presumably a similar enzyme system could be devised to replace thyroid and submaxillary extracts if iodination were not controlled by the iodinating enzyme tyrosine iodinase. Notatin (glucose oxidase) preparations with and without horseradish peroxidase were substituted for tyrosine iodinase extracts in regular enzyme assays conducted under an atmosphere of 95 percent O_2 : 5 percent CO_2 in the presence of glucose. Under these conditions only slight iodination of L-tyrosine was observed and this was insignificant when compared with the iodination observed with tyrosine iodinase tissue extracts.

Although not conclusive, the evidence produced by the preceeding studies appears to favour the theory of the existence of a tyrosine iodinase. The existence of a tyrosine iodinase will therefore be assumed throughout this thesis. The ultimate answer to this question cannot be

reached with available data. An obvious future approach to a solution of this problem would involve a purification and hence a separation of the components of the tyrosine iodinase system.

Metal Requirements

The mechanism whereby cupric ion functions in the organic binding of iodine is one of the more puzzling facts of the tyrosine iodinase system. It has been variously suggested that cupric ion acts as an artificial acceptor for electrons replacing elemental oxygen (3), as a co-enzyme (40) and as a complexing agent (117). Data already presented (see p. 59) established that cupric ion does not replace elemental oxygen as an electron acceptor since a requirement for oxygen may be demonstrated in tissue extracts even in the presence of added cupric ion. A possible objection to the theory of cupric ion as a coenzyme in the tyrosine iodinase system is that the concentration of the ion demanded by the extracted enzyme system (0.0024 M) is of greater magnitude than that observed in tissues. This point, however, does not exclude the possibility that local concentrations of cupric ion may exist in thyroid tissue since quantitative estimations of cupric ion in the thyroid give the average concentration of cupric ion in the whole gland (132). The suggestion that the function of cupric ion is to bind tyrosine to form a water soluble complex, and hence permit a

higher concentration of tyrosine in solution, would appear to gain some support from the observation that a 1:1 correspondance of cupric ion to tyrosine is required for optimum iodination in the tyrosine iodinase system (118).

Since it is possible to measure the activity of both the iodide oxidizing system and the whole tyrosine iodinase system, it should be possible to determine with which system the cupric ion functions. If the iodide oxidizing enzyme system functions without exhibiting a requirement for copper, the conclusion may be reached that copper acts in some capacity to promote the action of tyrosine iodinase. With this idea in mind the activities of tyrosine iodinase and iodide oxidizing enzyme were determined in the presence and absence of cupric ion. The results of this study are shown in Table VII. In the case of tyrosine iodinase small but detectable quantities of organically bound iodine are formed without cupric ion supplementation, whereas high conversions of iodide to MIT are observed in the presence of the ion. With the iodide oxidizing enzyme system the extent of iodination appears much less dependent upon copper supplementation although a definite increase in elemental iodine "collected" in the presence of cupric ion is apparent. Two possible explanations of these data may be advanced:

(a) Cupric ion may function with both tyrosine iodinase and the iodide oxidizing system. However, the concentrations of the ion required in each instance might be

markedly different. Thus a low level of cupric ion, i.e. that amount already present in the homogenate, may permit an efficient functioning of the iodide oxidizing enzyme system with the result that little increase in the activity of this system will be apparent upon copper supplementation. If, on the other hand, a high level of cupric ion is required for the efficient functioning of tyrosine iodinase, then this system will be markedly stimulated by the addition of copper.

(b) Cupric ion may function to activate only tyrosine iodinase, for which a high level of the ion is necessary. The slight increase in generation of elemental iodine observed when the iodide oxidizing system is supplemented with cupric ion may be the result of a non-enzymatic generation of elemental iodine which supplements that produced by the action of the peroxidase of the iodide oxidizing system. Thus it has been determined that a number of metallic ions will catalyze the non-enzymatic oxidative action of hydrogen peroxide (133). Cuprous ion is among these "activators". Since cuprous ion would undoubtedly be formed to some extent when cupric ion is placed in contact with the organic matter of the enzyme extract, it is possible that cuprous ion may function to generate free iodine non-enzymatically through formation of the extremely active oxidizing radical $\cdot OH$ according to equation [11]

$$Cu^{+} + HOOH \longrightarrow Cu^{2+} + \cdot OH + (:OH)^{-} \qquad [11]$$

A decision between situations (a) and (b) may not be reached with the data available. It is believed, however, that cupric ion is concerned with the step of tyrosine iodination since both situations (a) and (b) suggest this possibility.

A discovery with considerable bearing on the function of cupric ion in the tyrosine iodinase system has come to hand in the observation that ferrous ion will completely replace cupric ion in this system. The results of a series of experiments in which ferrous ion has been used to replace cupric ion with beef submaxillary, rat submaxillary and rat thyroid preparations are graphically depicted in Fig. 10. Optimum rates of iodination observed with both cupric ion and ferrous ion are given in Table VIII. Prior to this series of experiments cupric ion was the only metallic ion which allowed extensive iodination of tyrosine in the tyrosine iodinase system. Ferric ion had been shown to possess some slight activity in this respect (2) but it is now apparent that its action was a function of its ferrous ion content. Concurrent experiments with ferric ion indicated negligible activation with respect to the dramatic values of Fig. 10. Slight variation in maximum iodination with ferrous ion as compared to cupric ion (Table VIII) may be ignored. These undoubtedly are due to the fact that the concentration of ferrous ion used was not optimal but ranged on either side on this optimal value of 7.5×10^{-4} M ferrous ion.

A requirement for an activator other than cupric ion or ferrous ion has also been demonstrated in the tyrosine iodinase system. Preliminary studies on the isolation of the enzymes of this system has revealed that the dialysis of 5 percent beef submaxillary tissue extracts against ice cold isotonic potassium chloride results in substantial drops in activity, as shown in Fig. 11. In order to establish whether denaturation of the enzyme had occured or whether a small molecular weight activator was being removed by dialysis, additions of boiled undialyzed 5 percent beef submaxillary homogenate supernatants were made to the regular tyrosine iodinase medium (see p. 35) which contained 1.0 ml. of dialyzed enzyme preparation as the enzyme source. The response to this treatment is demonstrated in Fig. 11. Approximately 60 percent of the original tyrosine iodinase activity was restored in this manner. Thus, in addition to cupric or ferrous ion, a thermostable factor appears to be involved in activation of MIT formation. In order to determine if the activator is organic or inorganic in nature; the following experiment was done. Undialyzed beef submaxillary preparations were ashed to destroy all organic matter. The residue was taken up in distilled water, such that the final volume of solution was equivalent to the original volume of 5 percent submaxillary extract ashed. Addition of this solution to a dialyzed enzyme preparation

caused the increase in iodination rate depicted in Fig. 11. Although the curve produced is not as regular as that obtained with boiled preparations, the increased iodination is unmistakable. The activator has been shown to be independent of species by the demonstration that ignited beef submaxillary preparations will activate dialyzed rat submaxillary enzyme preparations. Since the activator appears to be inorganic, a series of ions was investigated with respect to their ability to activate iodination produced by dialyzed beef submaxillary extracts. The ions used in this study are recorded in Table X. Although inhibition effects were observed none of these ion activated the system. Although the list of metal ions examined is by no means exhaustive, it includes most of the ions demonstrated to possess activator action with other known enzyme systems. The failure to elicit a response with these ions must not necessarily be construed to mean that the metals represented in Table X are ineffective as activators. It is possible that the presence of more than one of the ions is necessary, or again the valence state of the ions may be the important factor. Much further work needs to be done before the metallic ion activation phenomenon in the tyrosine iodinase system is completely understood.

The Mitochondrial Enzyme System

The classical biochemical approach to the understanding of reactions which occur in tissues generally involves the following sequence:

(a) observation of the phenomenon *in vivo*

(b) detection of the process in whole or sliced tissue preparations

(c) study of the reaction in cell free homogenates

(d) isolation of the enzyme or enzymes controlling the reaction

As far as it has proceeded the study of organic binding in the thyroid has followed this general scheme. Although the importance of organic binding of iodine in the thyroid gland was realized early in the present century (16) it was not until the advent of radioactive iodine as a biological tracer that a competent study of this process could be made. The early studies of the I-131 uptake and binding *in vivo* (30), were rapidly followed by investigations of the phenomenon in whole and sliced thyroid glands (38). Past this point difficulties were encountered since it was observed that thyroid homogenates would not synthesis organically bound iodine (39). Reasons offered for this failure ranged from a suggested dilution of some essential co-factor or substrate to the suggestion that the architectural integrity of the complete cell was demanded by the

process (117). The first possibility seemed favoured when the classical scheme was advanced one further step by Weiss's (40) demonstration of organic iodine formation in cell free homogenates of thyroid tissue supplemented with cupric ion and tyrosine. Thus the subsequent claim by Chaikoff and his group (5) that isolated sheep thyroid mitochondria were capable of the organic binding of I-131, without supplementation, was received with some surprise.

Verification of the Existence of a Mitochondrial Iodinating System

In order to clarify the relationship between the tyrosine iodinase system and the mitochondrial system it was decided to confirm and extend the observations of Taurog et al. (5) on the mitochondrial enzyme system. The first stage in this program was to confirm the observation that mitochondria derived from thyroid tissues would organically bind I-131. Frozen beef thyroid tissue was homogenized and a Krebs-Ringer bicarbonate solution suspension of the mitochondria was prepared employing the differential technique described in the Experimental Section of this thesis. The mitochondria prepared in this fashion were incubated with I-131 and aliquots of reaction mixture, before and after hydrolysis with a pancreatin preparation, were spotted on large chromatography strips and developed. A reproduction of the radioautographs obtained from the chromatograms is

shown in Fig. 12. It is seen that the mitochondrial suspension, untreated with proteolytic enzymes, contains three radioactive components: an immobile origin component, an iodide ion component and a component which travels at the solvent front under the chromatography conditions used (n-butanol : acetic acid : water solvent (68:2:27)). The suspension presents a different picture when hydrolyzed with pancreatin. The origin component has almost completely disappeared and is replaced by a new substance which appears slightly in advance of iodide ion. This radioactive spot has been shown to be free MIT by direct comparison with the authentic material. The loss in radioactivity of the origin component was shown to correspond to the activity of the newly formed MIT component within experimental error. Although the Chaikoff group used a different solvent system in the separation of these radioactive substances and mitochondria from a different species, the results obtained in our laboratory are essentially the same as those reported by Taurog et al. (5). Further studies with sheep thyroid mitochondria revealed qualitatively identical results.

Chaikoff's group has suggested that these results may be explained by the assumption that the origin component consists of protein bound iodinated tyrosine. Thus treatment of this fraction with a protein cleaving enzyme results in its destruction with consequent formation of free MIT.

Extrathyroidal Occurence of the Mitochondrial System

Chaikoff and his co-workers investigated the possibility that mitochondria from extrathyroidal scurces might possess iodinating capacity. They prepared mitochondria from sheep kidney and liver tissues and incubated these with I-131. No formation of organically bound iodine was observed in either case. These authors therefore concluded that the mitochondrial enzyme system was a unique process associated only with thyroid gland tissue and hence not detectable extrathyroidally. However, the demonstration by Fawcett and Kirkwood that a relationship existed between the thyroid and the salivary glands (3) at least with respect to the presence of tyrosine iodinase, raised the possibility that a similar situation might exist with respect to the mitochondrial enzyme system. With this hypothesis in mind, mitochondrial preparations of the rat submaxillary tissue were obtained, using the same techniques employed with thyroid mitochondria. These suspensions were then incubated with I-131. The resulting mitochondrial suspensions, before and after hydrolysis with pancreatin, were separated by chromatography on paper and radioautographs of the chromatograms prepared. The results of these studies are depicted in Fig. 13. Organic binding of I-131 is seen to have occured to an appreciable extent.

The demonstration that the mitochondrial enzyme

system exists in extrathyroidal sites necessitated that a comprehensive study be conducted to determine just how wide-spread this system might be. The rat was chosen as the experimental animal, because of ready availability, and its tissues examined to see if their mitochondria were capable of effecting the organic binding of iodine. The results of this study are shown in Table XV. It is readily seen from this study that the mitochondrial system is not confined to thyroid tissue but is rather wide-spread.

Existence of Free MIT in Extrathyroidal Mitochondrial Systems

An unusual feature of the mitochondria derived from rat submaxillary and extraorbital lacrimal glands is their ability to synthesize free, as well as protein-bound MIT when incubated with I-131. This may be readily seen in Fig. 13 which shows the products produced by the rat submaxillary mitochondrial system.

A number of observations seem to indicate that free MIT is formed within the mitochondria and then diffuses into the medium. Thus supernatant solutions prepared by centrifuging the mitochondrial suspensions at 25,000 g for 20 minutes do not synthesize MIT when supplemented with I-131. Mitochondria incubated with I-131 can be washed free of soluble MIT by repeated centrifugations in Krebs-Ringer bicarbonate solution. When these washed mitochondria are allowed to stand, under toluene, in buffer, for 12 hours

at 38° C, free MIT is easily demonstrated in the supernatant.

The observation that rat submaxillary salivary and extraorbital lacrimal gland mitochondria synthesize appreciable amounts of free MIT raises some doubt as to the correctness of the claim of the Chaikoff group that the product produced in the mitochondria is protein bound MIT. For this reason a series of experiments was designed to cast light on this matter.

Two alternate possibilities might exist other than the interpretation by Chaikoff's group that the cleavage of protein bound MIT occurs when mitochondria are treated with pancreatin. The immobile origin component might consist of:

(a) denatured mitochondria with intact membranes containing free MIT

(b) protein to which free MIT is bonded through physical rather than chemical forces.

An experimental evaluation of each of these possibilities will now be discussed.

Free MIT within a denatured mitochondrial membrane

Palade (136) has carried out a series of electron microscope studies of the external and internal structure of the mitochondrion. His observations lead him to conclude that mitochondria, for the most part, consist of rod-shaped particles whose external feature is a double-layered membrane

completely enclosing a highly compartmentalized inner structure. Applying this picture to the mitochondrial system concerned with organic binding of iodine, it is not inconceivable that free tyrosine is iodinated within the membrane wall. If this wall were impermeable to free MIT then it would be retained within the membrane while the suspension was in the process of being dried on chromatographic strips. Thus free MIT might become immobilized by the denatured mitochondrial walls and be observed as an immobile radioactive component upon chromatography. Treatment of incubated mitochondria with pancreatin prior to chromatographic analysis would rupture the membrane and thus release free MIT which could be demonstrated chromatographically. If this situation exists then it should be possible to rupture the mitochondrial membrane by physical means to release free MIT. Three mechanical procedures were investigated. Claude (137) has demonstrated that exposure of mitochondria to hypotonic solutions may cause their rupture. Thus beef thyroid mitochondria which had been incubated with I-131 were separated from the incubation medium by centrifugation at 25,000 g for 20 minutes and subsequently treated with distilled water. No free MIT could be detected by a chromatographic analysis of this suspension. Incubated beef thyroid mitochondria were then subjected to the action of abrasives in a shaking machine[1] at 60 C.P.S. This was

1 Bacterial Disintegrator, Mickle and Co., England

ineffective in releasing MIT. Finally, incubated beef thyroid mitochondria were exposed to ultrasonic vibrations (0.5 megacycles/sec.) (138). Again no free MIT could be detected. It is extremely unlikely that mitochondria walls could remain intact under these drastic physical conditions. It may therefore be concluded that free MIT does not exist within the mitochondrial membrane.

Free MIT attached to protein by other than chemical bonds

The concept of the strong physical bonding of a small molecular weight organic compound to a protein molecule is not without precedent. Thus avidin, a protein found in egg white, forms an extremely tight complex with biotin whose dissociation constant had been estimated to be in the region of 10^{-21} (139). If this type of complex should exist between free MIT and the protein of the mitochondria then the destruction of the structure of the particular protein involved by chemical procedures, other than the rupture of peptide bonds, should destroy its complexing ability and hence release free MIT. Two experimental approaches to the detection of this phenomenon were tried. Reagents possessing sulfhydryl groups have been shown to profoundly alter the structure of proteins by virtue of their ability to reduce disulfide bonds in the molecule (140). I-131 incubated beef thyroid mitochondria were treated with thioglycollic acid and the resulting mixture analyzed by paper chromatog-

raphy. Radioautographs revealed no free MIT. The second attempt at protein destruction without rupture of peptide bonds centered around the idea that proteins contain nucleic acid, lipid and carbohydrate moieties as well as amino acids in their internal structure (141). Presumably alterations of these groupings could "open up" the protein molecule and release bound MIT. I-131 incubated beef thyroid mitochondria were treated with lipase , and α and β amylase, and crystalline preparations of ribonuclease, and desoxyribonuclease, all obtained from commercial sources[1]. In no case was free MIT liberated.

The inability to free MIT by physical, chemical and enzymatic means, other than through cleavage of peptide bonds, strongly suggests that the MIT formed in the mitochondrial system is indeed bound to protein.

Proteolytic Activity of Mitochondrial Preparations

Since the free MIT that is formed by extraorbital lacrimal mitochondrial preparations cannot be explained on the basis of iodination of free L-tyrosine within the mitochondrial walls, it must be concluded that this free MIT is derived from protein by proteolytic cleavage. If this is the case the mitochondria from rat submaxillary and extraorbital lacrimal tissue must have a proteolytic enzyme associated with them. This proposition was examined by determining

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1 Gen. Biochemicals Inc., Chagrin Falls, Ill.; Worthington Biochemicals Co., Freehold N.J.

the proteolytic activity associated with those mitochondria which do release free MIT and those which do not. (The order of MIT release has been determined as: submaxillary > extra-orbital lacrimal > thyroid).

The results of this investigation are shown in Table XII. It is seen that those mitochondrial preparations which liberate free MIT are found to contain appreciable proteolytic activity whereas none can be detected with beef thyroid preparations. Thus a correlation appears to exist between the observed proteolytic activity and the proportion of free MIT found upon incubation of the respective mitochondria with I-131.

Nature of the Iodinated Protein Produced by Mitochondrial Preparations

Since the work of Chaikoff and his group, (5) and the results just reported, support the theory that MIT formed in the mitochondrial system is associated with proteins by means of peptide bonds, it is highly desirable to obtain some information concerning the characteristics of these proteins.

Roche et al. (142) have studied the effects of pure proteolytic enzymes on hog thyroglobulin, and artificially iodinated proteins. These authors established that crystalline trypsin did a very efficient job of releasing MIT, DIT, and Tx from thyroglobulin. However, trypsin will release only negligible quantities of iodinated residues from

artificially iodinated proteins. Pepsin, on the other hand, liberates a high proportion of MIT containing peptides, rather than MIT. In view of these observations of Roche and his co-workers it was decided worthwhile to undertake a similar study of iodinated proteins derived from thyroid submaxillary and extraorbital lacrimal mitochondrial systems. If any or all of these proteins are produced by a random iodination procedure this fact will become immediately obvious through the action of trypsin in the release of free MIT.

Consequently I-131 labelled iodoproteins derived from rat submaxillary, rat extraorbital lacrimal, and beef thyroid glands, were submitted to the action of preparations of crystalline trypsin and pepsin. The results of this study are seen in Table XIII. It is obvious that all three iodinated proteins bear a strong resemblance to the protein thyroglobulin, at least with respect to their release of free MIT under the action of crystalline trypsin. These proteins, like thyroglobulin, release little free MIT when treated with crystalline pepsin. The results, in the case of salivary and extraorbital lacrimal gland mitochondria, are somewhat obscured by the hydrolysis effected by the associated proteolytic enzymes. It is quite clear, however, that both trypsin and pancreatin markedly accelerate the release of MIT in these preparations while pepsin does not increase the rate of spontaneous hydrolysis.

Crystalline trypsin has been demonstrated to attack only those peptide bonds between the amino acids arginine lysine and an adjacent amino acid. This specificity has been demonstrated both in model systems (143) and in work with native proteins. This specificity of action allows certain conclusions to be drawn concerning the nature of thyroglobulin and the iodoprotein of the mitochondrial system. These protein must contain a large number of tyrosine residues directly attached to either arginine or lysine carboxyls. Since trypsin releases mainly free MIT, and not MIT peptides, it would appear that these tyrosine fragments are located directly at the ends of protein chains with their amino groups attached to either arginine or lysine. The inability of pepsin to release appreciable quantities of free MIT may be more readily understood with this physical picture since pepsin has a markedly different specificity.

Nature of the "Front" Material found in Mitochondrial Preparations

A characteristic of all I-131 incubated mitochondrial preparations is the presence of a radioactive component which, upon chromatography, runs near the solvent front with all solvent systems that have been employed. Chaikoff's group (5) has suggested that this component might be some organic form of "active" iodine which functions as an iodinating agent. It is unlikely that this component can be any

form of oxidized iodide since it has been shown to persist in the presence of reducing reagents such as sodium thiosulfate and thiouracil. Centrifugation of I-131 incubated mitochondrial suspensions at 25,000 g shows that this "front" component is associated with the mitochondria and is not appreciably soluble in the incubation medium (Fig. 14). The component may be extracted from mitochondria using an n-butanol : acetic acid solvent. When this extracted material is hydrolyzed with acids or bases, the only degradation product that can be demonstrated is iodide ion. When chromatography with the n-butanol : acetic acid : water system is carried out at reduced temperatures the front component is resolved into at least three different materials. The identity of these iodinated products has as yet not been established. Presumably one or all of these compounds is unstable since deiodination (as manifested by streaking of the front component) occurs even under the mild conditions employed in chromatography.

Nature of the Enzymes of the Mitochondrial System

The Chaikoff group has suggested (5) that the I-131 containing protein formed in thyroid mitochondria may simply be the result of an exchange reaction between previously iodinated protein and elemental iodine produced by some enzyme system in the mitochondria. This possibility exists in thyroid mitochondria since large stores of iodinated

proteins are known to be present within the gland (15). However, in the case of mitochondria from extrathyroidal sites, exchange is not probable since, in mammals, stores of iodo-proteins in extrathyroidal sites presumably do not occur (146). Further, although it may still be possible that exchange occurs within thyroid mitochondria, the similarity between extra- and intra-thyroidal systems, with respect to both the nature and composition of iodoprotein, appears to rule against this mechanism. Since simple exchange does not appear to explain the formation of iodoproteins in mitochondria a series of experiments were designed to provide a better understanding of the enzyme, or the enzymes involved.

Catalase inhibition studies

In order to determine whether hydrogen peroxide is involved in the mitochondrial enzyme system, the effects of catalase on the iodinating system in beef thyroid mitochondria were investigated (see p.61). The results of this study are graphically depicted in Fig. 15. It is seen that catalase markedly inhibits the organic binding of I-131. This suggests that the mitochondrial system is dependent on the oxidizing power of hydrogen peroxide.

Since mitochondria are enveloped by a membrane, the point of attack of the enzyme catalase is of considerable interest, It is unlikely that a protein of the molecular

weight of catalase (estimated at 248,000 (147)) could diffuse across the membrane, particularly in view of the fact that mitochondria retain approximately 60 percent of their nitrogen as soluble protein within these mitochondrial membranes (148). If iodination occurs only with mitochondria which possess an impaired membrane then the catalase protein might conceivably enter the interior of the mitochondrion and inhibit iodination sites within the particle.

Br-82 studies

The use of Br-82 as a tool in determining the mechanism of action of the iodine generating system in soluble preparations has already been described (see p.62). When Br-82 is substituted for I-131 in the mitochondrial system no organically bound bromine is formed. This result suggests that, as is the case in the soluble system, a specific iodide peroxidase functions in the mitochondria.

Trypsin studies

The conclusion has been reached, from trypsin cleavage studies, that the iodoproteins obtained from the mitochondrial system contain MIT residues at specific sites in the protein chain. This finding suggests that an enzyme is involved in the determination of the point of attack of the active iodinating species. The fact must not be overlooked, however, that these tyrosine sites may be particularly

susceptible to spontaneous iodination.

Taken as a whole the results of the catalase, Br-82, and trypsin studies would appear to indicate that the mitochondrial enzyme system contains a hydrogen peroxide generating system coupled with a peroxidase and this combination is concerned with the formation of elemental iodine whose point of attack on a protein molecule is controlled by an iodinase.

Correlations Between the Tyrosine Iodinase System and the Mitochondrial System

When the report of a mitochondrial system capable of organically binding I-131 first appeared some conflicts appeared to exist between this system and the tyrosine iodinase system of Fawcett and Kirkwood. Since it was unlikely that two iodinating mechanisms would exist in the thyroid gland, it was thought that one of these systems might be an artifact (5). The work previously described in this thesis has already eliminated some of the differences between the two systems. Thus the mitochondrial system has been located in extrathyroidal sites and the tyrosine iodinase system has been demonstrated to provide some iodinated amino acid in the absence of added cupric ion. As a result, the possibility now arises that these two systems might be basically identical but differ only in their associations with cell particles, with a consequent difference in solubility. The observations that the two systems behave identically in the presence of Br-82 and catalase add weight to this suggestion.

Substrate Requirements of the Mitochondrial and Tyrosine Iodinase Systems

The observation that the tyrosine iodinase system iodinates free L-tyrosine while the mitochondrial system

iodinates proteins has proved an obstacle to the conception that the two enzyme systems are of the same nature. The idea of an enzyme system which will handle both free and protein bound amino acids is, however, not out of the question. Thus tyrosinase has been demonstrated to oxidize both free and protein bound tyrosine (149). It was decided, to present the tyrosine iodinase system with a series of tyrosine dipeptides and a poly-tyrosine to determine if they would function as substrates. Consequently L-leucyl-L-tyrosine, L-glycyl-L-tyrosine, L-lysyl-L-tyrosine and poly-L-tyrosine (the latter prepared from the Leuch's anhydride) were substituted for L-tyrosine in the tyrosine iodinase assay system using beef submaxillary and beef thyroid tissue extracts as enzyme sources, The results of this study are given in Table XIV. It is seen that all dipeptides studied served as substrates. In view of the conclusion reached, through trypsin studies, that the tyrosine residues in mitochondrial iodoproteins are located adjacent to lysyl- or arginyl-moieties, it is of particular interest to note that L-lysyl-L-tyrosine is a better substrate for the tyrosine iodinase system than is L-tyrosine itself. The protein-like molecule poly-L-tyrosine is also accepted by the tyrosine iodinase system. That it is not iodinated to the same extent as the dipeptides may well be a pH effect. It was necessary to dissolve the polypeptide in sodium hydroxide solution prior to addition to the assay

medium. Thus the pH of the assay medium was somewhat higher than desirable for optimum iodination. Since the tyrosine iodinase system has been shown to accept protein-like molecules as substrates, a corollary of this observation is that the mitochondrial system should accept free amino acids as substrates. Chaikoff and his co-workers, however, (5) claim that the mitochondrial system will not accept the free amino acid L-tyrosine to any appreciable extent. In order to investigate this matter further beef thryoid mitochondria were supplemented with the amino acids L-p-tyrosine, DL-o-tyrosine, DL-m-tyrosine and the phenols, p-cresol, p-hydroxycinnamic acid, and phenol and incubated with I-131. The radioautographs obtained from this study are shown in Fig. 16. It is seen that all substrates accepted by the tyrosine iodinase system are accepted by the mitochondrial system while the phenols unacceptable to the tyrosine iodinase system are unacceptable to the mitochondrial system, and, indeed, appear to inhibit the protein binding of iodine.

Parallel Existence of the Two Enzyme Systems

One of the first tests devised to evaluate the hypothesis of the essential identity of the mitochondrial and tyrosine iodinase systems was that of their occurence in the same extrathyroidal sites. Because of the prior discovery of the tyrosine iodinase system, extensive investi-

gation of its sites has already been carried out. The mitochondria of all tissues with tyrosine iodinase activity were examined for iodination capacity when incubated with I-131. Table IV demonstrates that wherever the tyrosine iodinase system is found the mitochondrial enzyme system is also detected. Further studies of the extrathyroidal mitochondria have since shown, however, that this system occurs in four sites where tyrosine iodinase activity is not observed, (see Table IV). This finding does not militate against the concept of identity of the two systems, however, since these additional sites possess low mitochondrial activity which may be an indication that the tyrosine iodinase system is present but in concentrations which are too low to be detectable.

The Mechanism of the Antithyroid Action of Iodide Ion

A number of theories have been advanced to explain the fact that excess iodide ion inhibits normal thyroid function. Two of these hypotheses, which attempt to explain the phenomenon on a molecular basis, have been singled out for experimental attention.

Pitt-Rivers (108) and Harington (109) have postulated that hypoiodous acid is the reactive form of iodine which reacts with tyrosine in the thyroid gland. This substance is formed by the hydrolysis of elemental iodine as shown in equation [1] .

$$H_2O + I_2 \rightleftharpoons HIO + H^+ + I^- \qquad [1]$$

It is evident that addition of excess iodide ion to the above system will decrease the concentration of hypoiodous acid. Any decrease in the effective concentration of the iodinating species will be reflected in a decreased level of organic binding of iodine.

The second hypothesis, advanced by Fawcett and Kirkwood (110) suggests that the effect of excess iodide ion is exerted through the ability of the ion to combine with elemental iodine to form the triiodide ion [2] ,

$$I^- + I_2 \rightleftharpoons I_3^- \qquad [2]$$

hence reducing the effective concentration of elemental

iodine. This mechanism can be expected to result in inhibition of the iodination process regardless of the nature of reactive species involved. Before iodide can enter an aromatic substitution reaction it has to be oxidized to the level of elemental iodine and it is either elemental iodine, or some positive species in equilibrium with it, which is involved in all aromatic substitution reactions involving this element. These two theories receive some support through the demonstration that elemental iodine is formed in the thyroid gland (110). They have the additional merit that not only will they explain the *in vivo* action of iodide but will also account for the observation that iodine inhibits the general reaction between iodine and aromatic substances (150,151). On theoretical grounds both mechanisms of inhibition are equally probable. However, since the equilibrium constants for both equations [1] and [2] are known, it is possible to predict, with considerable accuracy, how they would affect an iodine utilizing system. Thus if quantitative data were available on the extent of inhibition produced by various concentrations of iodide ion, a decision between the two could be made. Because of a host of independent variables, it is most difficult to obtain such data from a study of the action of iodide ion on the thyroid *in vivo*. However, such an attempt has recently been made (152).

Since iodide ion in sufficient concentration will

totally prevent the formation of organically bound iodine in the thyroid, it must necessarily inhibit the first step in the organic binding process, i.e. the reaction of oxidized iodide with tyrosine to form MIT through the agency of tyrosine iodinase. Since an iodide free tyrosine iodinase system may be prepared by dialysis, it forms a suitable "model" with which to make a quantitative study of iodide inhibition. With this idea in mind a study of the effect of iodide ion on the rate of synthesis of MIT by dialyzed preparations of rat submaxillary gland was carried out using the procedures in the Experimental Section of this thesis (see p. 40). The data derived from this study are graphically depicted in Fig. 17. A typical substrate saturation curve would be predicted for this plot since the rate of formation of MIT should be limited either by the saturation of the iodide oxidizing enzyme system with iodide ion or by the saturation of tyrosine iodinase with elemental iodine produced by the iodide oxidizing enzyme system. Either of these mechanisms would produce a curve that increases with iodide concentration and then approaches a limiting velocity. It is seen from Fig.17 that the velocity increases to a maximum value and then falls as the iodide concentration is further increased. Thus this enzyme system is inhibited by concentrations of iodide above the optimum value just as is the process of organic binding in the thyroid gland. The curve could not be extended much beyond iodide concentrations

of 10^{-3} M. The amount of MIT synthesized in a given time was determined by multiplying the fraction of radioiodide converted to MIT by the molar concentration of iodide present in the medium. Since the fraction of radioiodide converted to MIT became increasingly smaller as the iodide concentration was raised, both owing to inhibition and to dilution of the specific activity of the iodide, the accuracy of its measurement steadily decreased. Although this was partially compensated for by increasing the amounts of radioiodide used, 10^{-3} M iodide represents the highest iodide concentration at which reliable values could be obtained.

The Mechanism of Inhibition

The possibility that the enzyme system is being inhibited by either reaction [1] or [2] can be investigated by calculating the extent of inhibition to be expected from each reaction and comparing it to the observed inhibition.

The equilibrium constant of reaction [2] is known (153) and has the value:

$$K = \frac{(I_3^-)}{(I^-)(I_2)} = 590 \text{ at } 38^\circ \text{ C} \qquad [12]$$

This can be rearranged to:

$$\frac{(I_2)}{(I_3^-)} = \frac{0.00170}{(I^-)} \qquad [13]$$

Expression [13] allows calculation of the extent of inhibition produced by any concentration of iodide ion, provided that the mechanisms of inhibition is through reaction [2] . This follows since (I_2) / (I_3^-) is a measure of the iodinating species in bound form, and the steady state concentration of elemental iodine is constant at iodide concentrations above the optimum. A calculation of this ratio at the optimum iodide concentration (5×10^{-5} M) shows it to be in accord with the experimental fact.

$$\frac{(I_2)}{(I_3^-)} = \frac{0.00170}{5 \times 10^{-5}} = 34 \qquad [14]$$

Under these conditions only about 3 percent of the iodine is bound in the form of the complex. The value of this ratio at the level of iodine producing 55 percent inhibition (10^{-3} M) is 1.7, that is, 35 percent of the available iodine is bound in the inactive form. It is obvious from this that the major part of the inhibition can be accounted for on the basis that it is mediated through reaction [2] .

The inhibition to be expected from reaction [1] can be calculated in the following fashion. The equilibrium constant for this reaction is:

$$K = \frac{(HIO)(H^+)(I^-)}{(I_2)(H_2O)} \qquad [15]$$

This can be rearranged to:

$$(HIO) = \frac{K(I_2)(H_2O)}{(H^+)(I^-)} \qquad [16]$$

It is possible to calculate the ratio of HIO concentration at the optimum iodide concentration to the HIO concentration at 55 percent inhibition by the use of this expression thus:

$$\frac{(HIO)\ \text{max.form.}}{(HIO)\ 55\%\ \text{inhib.}} = \frac{(I_2)\ \text{max.form.}\ (I^-)\ 55\%\ \text{inhib.}}{(I_2)\ 55\%\ \text{inhib.}\ (I^-)\ \text{max.form.}} \qquad [17]$$

It is possible to calculate (I_2) 55% inhib. as a fraction of (I_2) max. form. from equation [13] which yields a value (I_2) max. inhib. = 0.65 (I_2) max. form. When this and the appropriate iodide values substituted in equation [17] the value for the ratio is:

$$\frac{(HIO)\ \text{max.form.}}{(HIO)\ \text{max.inhib.}} = 31 \qquad [18]$$

Since the velocity of the reaction will be directly proportional to the HIO concentration, if it is the iodinating species, an inhibition of 97 percent would be expected. It is obvious that this is considerably more than the observed inhibition and, in the author's opinion, rules out reaction [1] as a mechanism for the observed inhibition.

Correlation of the Inhibition Observed In Vivo with that Observed with the Enzyme Preparation

Recently Wollman and Scow (152) have attempted to decide between the Fawcett and Kirkwood mechanism and the Pitt-Rivers and Harington mechanism on the basis of the inhibition of organic binding of radioiodine observed in

intact mouse thyroid, after the injection of graded doses of iodide. They come to the conclusion that the Fawcett-Kirkwood mechanism will account reasonably well for the inhibition produced by higher levels of iodide, but will not account for the inhibition first observed as the level of iodide in the gland is increased. They make the erroneous assumption that a decrease in the amount of radioiodide bound by the gland is a direct measure of inhibition of the binding process. It should be pointed out that this is not the case and that the addition of stable iodide to any iodinating system will lower the amount of radioiodide bound merely by decreasing its specific activity while at the same time it may not affect, in any way, the amount of organic-iodine formed. This is seen if both the percent radioiodide bound in MIT, and the actual number of moles of MIT synthesized, are plotted against added iodide, using the dialyzed enzyme system previously described (Fig.18). It is evident that the percent incorporation of radioiodide falls off at iodide levels considerably below those that produce the maximum rate of synthesis of MIT. An examination of Wollman and Scow's data on the actual amount of iodine bound in micrograms / hour shows that they observe no inhibition in organic binding at iodide concentrations where Fawcett-Kirkwood mechanism predicts there should be none and that the amount observed at higher iodide concentrations agrees quite well with that predicted

by this theory.

The Active Iodinating Species in the Gland

The identification of the active species in the iodination of aromatic substances in aqueous solution has been the subject of considerable investigation (154). There can be little doubt that the reactive species is an electrophilic agent and probably is either positive iodide ion (I^+, iodinium ion) or the hydrated form of this ion, IOH_2^+. The former seems to be favoured by the recent evidence (151,154). Indeed there is no evidence as yet that any iodine carrier, other than the above cationic species, is effective for aromatic iodination in aqueous solution. It would not be expected then, that HIO would be the active iodinating species in the enzyme-catalyzed iodination of tyrosine, and the present work confirms this expectation. Neither would it be expected that fluctuations in the concentration of HIO would affect the concentration of the above mentioned cations. Elemental iodine is a more effective source of I^+ than is HIO (151) and the equilibrium of reaction [1] is very far to the left which prevents this reaction from appreciably influencing the concentration of elemental iodine.

The position with regard to elemental iodine is quite different. It is in equilibrium with both I^+ and the hydrated form of this ion, and any agent that decreases the concentration of elemental iodine will also decrease the

concentration of both of these cations. As has been pointed out, iodide ion causes marked decrease in the concentration of elemental iodine because the equilibrium point of reaction [2] is to the right (153). It has been conclusively demonstrated that the inhibiting action of iodide on the iodination of aniline is mediated through its ability to form a complex with molecular iodine (151). There is no obvious reason why the enzyme catalyzed iodination of tyrosine should behave in a different fashion.

The method of conversion of elemental iodine into the active iodinating species in the gland cannot, as yet, be settled with any degree of certainty. Two pathways for utilization of elemental iodine appear possible. Thus elemental iodine may be released to its environment where it will enter into equilibrium with I^+. This positive form of oxidized iodide may then be accepted by tyrosine iodinase to be employed in L-tyrosine iodination. Alternately elemental iodine may be directly taken up by tyrosine iodinase which could catalyze the iodination of L-tyrosine by causing the elemental iodine to dissociate to I^+ on its surface. A method of distinguishing between these two mechanisms is not readily available.

Correlation with Clinical Observations on the Action of Iodide on the Thyroid

If iodide exerts its action, in whole or in part, through the mechanism of triiodide binding of elemental

iodine, then it would be predicted that the prolonged administration of iodide in sufficient quantities should cause the appearance of hypothyroidism and goiter in normal subjects. Prior to 1953, little evidence was available on this response of euthyroid patients to large excess of iodide ion. In this year, however, Raben (155) reported symptoms of myxedema in a patient 9 months after treatment with an iodinated oil used as an X-ray contrast medium. In the discussion of this report it was suggested by Astwood that this condition might be caused by iodide inhibition of Tx formation due to exposure of the thyroid to high iodide ion concentrations from degradation of the iodinated oil in the system. The suggestion was offered that the extreme rarity of observation of such cases might be due to necessity for prolonged administration of iodide ion. In the same year Morgans and Trotter (156), reported myxedema in two patients who were found to have been taking, for other than thyroid conditions, preparations which contained high concentrations of iodide ion. When the preparations without iodide were administered the symptoms of myxedema disappeared indicating that iodide alone was responsible for the symptoms. That this phenomenon is not so rare as originally proposed by Astwood is emphasized in a report by Turner and Howard in 1955, (157), of twelve cases of myxedema and simple goiter resulting from the prolonged

ingestion of iodide-containing mixtures by normal subjects. Although the reported incidence of iodide precipitated myxedema is sufficiently large to be of indisputable significance, it is obviously not near the value that would be expected if all euthyroid persons exposed to iodide treatment succumbed to its antithyroid action. The Fawcett-Kirkwood mechanism would predict, however, a thyroid inhibition in all cases. The answer to this seeming contradiction may lie in an evaluation of the results of a recent study of the antithyroid action of iodide ion conducted by Scharf (158). This author administered large doses of iodide ion to rats and followed the progress of its effect on the thyroid through measurements of the basal metabolic rate of the animals. Scharf found that in all cases a thyroidal response could be detected, for a transitory depression in BMR, indicative of a depression in hormone release was observed with all animals. This depression was followed by an increase in BMR to a point above the normal value. After a period of 15 to 20 days however the animals regained their noraml BMR level. Scharf also observed a transitory formation of "thyroidectomy" cells" in the pituitary glands of animals which received high concentrations of iodide. These cells are generally observed, as the name implies, after thyroidectomy and are indicative of a drastic curtailment in thyroid hormone production. As applied to the Fawcett-Kirkwood theory,

these data would suggest that all patients fed large quantities of iodide ion may suffer a temporary disruption in Tx synthesis with the majority recovering after a short period of time. Since presumably the iodide level in the thyroid may reach only a certain maximum value then recovery could be explained through the action of the gland in processing a sufficiently increased concentration of elemental iodine to compensate for that lost through equation [2]. The failure of this compensatory mechanism, which might occur in normal patients with a borderline tendency to hypothyroidism, would result eventually in the display of typical symptoms of an under-active thyroid such as the myxedema and goiter which have been observed.

The beneficial effects of iodide ion in Graves' disease may possibly be explained by the Fawcett-Kirkwood mechanism as well. It is significant that the effect of iodide ion in Graves' disease is not permanent for the patient eventually "escapes" iodine control, (159). This clinical picture is consistent with the work of Scharf and may be explained on the basis of an increased formation of elemental iodine to compensate for that lost through triiodide ion formation.

Other Theories of Iodide Inhibition

While it would appear that the data of this thesis favours the triiodide mechanism of inhibition, it is not inconceivable that additional mechanisms may exist whereby

excess iodide ion affects the gland. Thus it is of value to consider other theories, at least with respect to the evidence for, and deficiencies of these systems.

One of the earliest theories of the antithyroid action of iodide ion was proposed by Marine in 1927 (98). Observing the beneficial effects of iodide ion in Graves' disease, Marine suggested that iodide functioned to permit an increased production of thyroid hormone. The resulting swelling of hormone stores caused a pressure retention of hormone and thus a lowered B.M.R. This theory was originally supported by the claim of Gutman et al. (160), that Graves' diseased patients treated with excess iodide possessed greater concentrations of organically bound iodine in their thyroids than those who were untreated. This study, however, was carried out employing a non-precise extraction technique for removal of inorganic iodide from gland tissues. Thus the observed differential between organic iodine content of iodide treated and untreated thyrotoxic glands must be viewed with some skepticism. That this skepticism is justified was shown by a more recent study of the effects of excess iodide ion in the thyroid gland in vivo using I-131 as a tracer. Iodide ion was demonstrated to cause a definite inhibition of organic binding in both normal and thyrotoxic patients (97). Thus, although a build-up of colloid has been demonstrated in iodide treated Graves' diseased tissue (161), the theory that this colloid is more heavily

iodinated as a result of iodide treatment must be abandoned.

Early observation that the response of the thyroid to the thyroid stimulating hormone (TSH) was conditioned by the dietary iodide level (99,100,101,102), has aroused considerable interest in the possibility that this phenomenon might be concerned with the inhibiting action of excess iodide. Thus the discovery by Rawson et al. that high iodide diets fed concurrently with administered TSH prevented the increase in thyroid cell height, which is a normal response to TSH, inspired the suggestion that excess iodide ion functions through control of the reaction which is presumed to occur between TSH and the thyroid gland. Further evidence for this hypothesis has been found in the additional observation that TSH inactivation in vitro by thyroid tissue is inhibited by adding iodide to the medium (162). Trikojus and co-workers suggested an alteration in this theory and proposed that TSH inactivation or control is a function of elemental iodine, as derived form iodide ion in the gland, and not iodide ion itself. This proposal is based on the in vitro demonstrations by Trikojus et al. of the denaturation of TSH with elemental iodine (105). Rawson, however, questions the physiological significance of this work and suggests the phenomenon to be a simple in vitro oxidation, (162), presumably involving sulfhydryl groups

since iodine inactivated TSH may be reactivated by exposure to reducing reagents. Recently some question has been raised as to the validity of the observation of Rawson et al. that high iodide concentrations inhibit the morphological response of the thyroid to TSH (163). If this criticism is found to be justified, then the earlier observation of the effect of iodide in partially inhibiting the stimulating action of the thyrotrophic hormone, as measured for example by O_2 uptake of guinea pigs (103), may just as reasonably be explained as a consequence of the triiodide inhibition mechanism of inhibition of the organic binding process in the thyroid gland.

A final theory of iodide inhibition of thyroid function was prompted by an observation of de Robertis et al. (107), that elemental iodine destroyed the activity of a thyroid protease in vitro. Since these authors have assumed this protease to be essential in release of Tx from thyroglobulin in the thyroid (a questionable assumption since this protease does not function at a neutral pH) they suggested destruction of the enzyme in vivo by elemental iodine, generated from excess iodide, as an explanation of the antithyroid action of iodide ion. An immediate criticism of this proposal can be based on reports that other enzymes may be inactivated and denatured by elemental iodine (164). This inactivation phenomenon is

thus not a specific function of the thyroid and, in view of the large quantities of elemental iodine which were required for the *in vitro* denaturation of the protease, is most likely of little physiological significance.

Thus, on the basis of the data discussed in this thesis, it would appear that, of all theories advanced to explain the antithyroid action of iodide ion, the triiodide inhibition mechanism merits the fewest objections. It would appear to fit all of the presently available data.

SUMMARY AND CONCLUSIONS

The thyroid gland has two unique functions which other vertebrate tissues apparently do not possess. These are the ability to concentrate iodide ion within its boundaries and the ability to incorporate the ion into an organically bound form. Efforts have been made to isolate the enzymes responsible for these actions of the thyroid. However, only in the latter case have they met with some success. Within the last three years two cell free preparations derived from the thyroid tissue have been demonstrated to incorporate iodine into MIT, the first iodinated intermediate in the metabolic sequence leading to Tx. This thesis is a study of the nature and characteristics of these two enzyme systems

The tyrosine iodinase system, the first enzyme extracted from thyroid tissue which possessed the ability to organically bind iodine, had been shown to require supplementation with cupric ion and L-tyrosine before it would act. It had also been established that the tyrosine iodinase system may be detected in several extrathyroidal sites, in particular in salivary gland tissue, where it exists in high concentration. Prior to the work of this thesis, this preparation was believed to contain one enzyme, tyrosine

iodinase, which was assumed to employ elemental iodine, generated by cupric ion oxidation, to iodinate L-tyrosine. Calculations of the minute quantity of I_2 which would be expected to be formed through cupric ion oxidation of iodide ion, taken in conjunction with a study of the effect of excess iodide ion on the kinetics of the tyrosine iodinase system, now lead to the conclusion that, the function of cupric ion cannot possibly be to generate elemental iodine. The results of the kinetic study described in the thesis are more consistent with the idea that a further enzyme system is responsible for the oxidation of iodide ion to elemental iodine. Direct evidence for an iodide oxidizing enzyme system is presented through the observation that reactive aromatic iodine acceptors such as resorcinol, m-aminophenol, m-phenylenediamine, and p-chloromercuriphenol will "collect" iodine from unboiled tyrosine iodinase preparations but not from those in which the enzymatic activity has been destroyed by boiling. A study of the rate of iodination of these aromatic "collectors' as a function of their concentration is employed to shed light on the mechanism of action of certain aromatic antithyroid agents. Two mechanisms have been advanced to explain the behaviour of this group of compounds. The first of these suggests that inhibition of thyroid activity is effected by a competition of the aromatic compounds with L-tyrosine for elemental iodine. The second mechanism suggests that inhibition is

effected by a "poisoning" of the enzyme system of the thyroid which is responsible for elemental iodine formation. The data of this thesis support the concept that the primary action of the aromatic inhibitors is to remove elemental iodine by reacting with it. However, with increasing concentrations of aromatic inhibitor a secondary inhibition effect becomes prominent which appears to be explainable only on the basis of decreased formation of elemental iodine. This decrease in I_2 generation may well be a result of a "poisoning" of the iodide oxidizing enzyme system. Hence the suggestion is advanced that the mechanism by which certain aromatic compounds inhibit Tx formation may be a function of the actual concentration of these inhibitors in the thyroid gland.

A suitable assay procedure which should prove of value in the future isolation of the iodide oxidizing enzyme is described.

The nature of the enzymes of the iodide oxidizing enzyme system is elucidated. In contradiction to earlier work the tyrosine iodinase system is shown to be dependent on elemental oxygen even when supplemented with cupric ion. This demand for oxygen must obviously be connected with an oxidation step and hence with the iodide oxidizing step. Thus the suggestion is advanced that an oxidase is a component enzyme of the iodide oxidizing enzyme system. Three possible

roles for an oxidase in this system are discussed. On the basis of the observation that catalase inhibits the system while bromide ion is not accepted by it, the conclusion is reached that the complete iodide oxidizing enzyme system consists of: an oxidase which generates hydrogen peroxide; this hydrogen peroxide is then accepted by a peroxidase that is specific for iodide ion. Conclusive proof for the position of hydrogen peroxide in this system is offered through the observation that it is possible to remove the dependence of the tyrosine iodinase system on elemental oxygen by supplementing it with hydrogen peroxide. Under these conditions the synthesis of MIT proceeds with unboiled but not with boiled enzyme preparations. Since the iodide oxidizing enzyme system is intimately connected with tyrosine iodinase, it should be detectable in all tissues known to possess the tyrosine iodinase system. This expected relationship is shown to exist.

The basic observations which led to the postulate of a tyrosine iodinase were that boiled tissue preparations exhibited none of the catalytic properties of the unboiled extracts, with respect to MIT formation and that these tissue preparations exhibited a substrate specificity of a type characteristic of enzymes. Since the first of these phenomena may now be explained just as conveniently on the basis that the enzymatic activity destroyed is that of the iodide oxidizing enzyme system; the specificity data become

of crucial importance to the existence of a tyrosine iodinase. Previous data is re-examined and extended in this thesis by a more comprehensive investigation of potential substrates and by a study of "model" systems. On the basis of specificity, the conclusion is reached that a tyrosine iodinase enzyme exists. However, the specificity requirements are of a more general nature than was originally believed. Thus for effective substrate activity an aromatic compound must possess; a free phenolic hydroxyl, a saturated side chain o,m, or p to the phenolic group and a carboxylic acid group attached to this side chain. The presence of a free amino group does not appear necessary for when this group is effectively covered, as in N-phthaloyl or N-acetyl tyrosine, the rate of iodination is unchanged or even enhanced.

The role of cupric ion in the tyrosine iodinase system is investigated. A study of the activities of the iodide oxidizing enzyme system and the tyrosine iodinase system as a whole, in the presence and absence of cupric ion, revealed a slight copper activation of the iodide oxidizing system and an intense copper activation of the tyrosine iodinase system. Two possible explanations of this data are advanced. Both suggestions imply a requirement by the tyrosine iodinase enzyme for the metal activator. The mechanism by which this activator is effective is unknown. The possibility that cupric ion may function as a coenzyme for tyrosine iodinase, however has not been eliminated. A discovery with considerable

bearing on the function of cupric ion in the tyrosine iodinase system is that ferrous ion will completely replace cupric ion as an activator. Prior to this discovery, cupric ion was the only metallic ion which permitted extensive iodination of tyrosine by tyrosine iodinase extracts. Ferric ion had been shown to possess some slight activity but it is now apparent that its action was a function of its ferrous ion content. The optimal value of ferrous ion required in the assay medium is established as 7.5×10^{-4} M. A requirement for an activator other than cupric ion or ferrous ion is also demonstrated. This activator which is present in crude tissue extracts, is dialyzable and is inorganic in nature.

Further extrathyroidal sites of tyrosine iodinase activity have been established. Histological similarities between salivary gland and lacrimal gland tissue prompted an investigation of this latter site for an ability to organically bind iodine. Tyrosine iodinase activity is found to be detectable, in high concentration, in both extra- and intra-orbital lacrimal gland tissue. The tyrosine iodinase system in extrathyroidal sites has been suggested to function in the deiodination of MIT and DIT. Thus the detection of this system in lacrimal gland tissue is consistent with current views that the deiodination system is widely disseminated in vertebrate tissues.

The second enzyme system which is capable of organically binding iodine had been shown to be associated with thyroid mitochondria. This mitochondrial system, in contrast to the tyrosine iodinase system, appeared to iodinate protein bound tyrosine since free MIT was reported to be released only when the mitochondria were treated with pancreatin, a proteolytic enzyme preparation. The existence of the mitochondrial system in thyroid tissue is verified in this thesis. The discovery is made, however, that this system is not unique to the thyroid, for its presence may be demonstrated in, salivary gland, lacrimal gland, stomach, intestine, spleen, and lung. This widespread occurence of a system capable of organically binding iodine would appear to signify that this process of the thyroid is not as unique as was originally believed. Thus the thyroid's iodide concentration mechanism may be the major factor differentiating it from these other tissues, at least with respect to organic binding of iodine. Presumably they too might incorporate iodine into Tx provided that they were supplied with a sufficiently high concentration of iodide. This concept is consistent with a general body of evidence which now exists supporting the idea that extrathyroidal formation of Tx occurs under certain conditions.

An unusual feature observed with mitochondria from extrathyroidal sites is that appreciable quantities of free

MIT are formed in addition to that which appears to be bound to protein. This observation raises a reasonable doubt as to whether the original substrate iodinated in the mitochondrial system is free or protein bound L-tyrosine. The possibility exists that the MIT observed associated with protein in the mitochondrial iodinating system might be free MIT held to the protein by physical rather than by chemical forces. Exposure of mitochondria to ultrasonic vibrations, mechanical abrasion, hypotonic solutions, thioglycollic acid, ribonuclease, desoxyribonuclease, lipase, and α- and β-amylase liberates no free MIT. This inability to release free MIT by physical, chemical, or enzymatic means, other than by cleavage of peptide bonds, strongly suggests that the L-tyrosine iodinated within the mitochondrial system is protein bound. Since free MIT formed in extrathyroidal mitochondrial systems cannot be explained as the result of the iodination of free L-tyrosine, then it must be concluded that a protease is associated with these mitochondria. This conclusion is verified and a correlation is established between proteolytic activity of the mitochondria and the quantity of free MIT formed by them.

A study is made of the iodoproteins derived from thyroid, submaxillary, and extraorbital lacrimal mitochondria with respect to their behaviour in the release of free MIT under the action of crystalline pepsin and trypsin. A marked similarity is observed between these iodoproteins and thyro-

globulin. All release a major portion of their MIT as the free amino acid when treated with trypsin but relatively little when treated with pepsin. Since it has been reported that artificially iodinated proteins release little free MIT upon treatment with trypsin it is concluded that the iodination of thyroglobulin and of the proteins of submaxillary and extraorbital lacrimal mitochondria, is a controlled enzymatic process. In view of the specificity requirements of trypsin, it is felt that these iodoproteins contain a large number of tyrosine residues attached to the carboxyl groups of arginine or lysine. Since trypsin is shown to release free MIT and not MIT peptides, it may also be concluded that these tyrosine moieties are located at the ends of protein chains. The striking similarity between these proteins from different tissue sources strongly supports earlier theories which suggested that a relationship between these tissues exists.

The data of this thesis indicate that the tyrosine iodinase system and the mitochondrial enzyme system possess many common features. These systems behave similarly toward oxygen, bromide ion, and catalase. This suggests that both systems contain a hydrogen peroxide generating oxidase coupled with a peroxidase and tyrosine iodinating enzyme. Further similarities are established in that both systems will accept the same substrates and are found in the same tissues. In view of these data the suggestion is advanced

that the two systems are basically identical, differing only in their association with cellular particles. This results in a consequent difference in solubility.

A final section of this thesis is concerned with a quantitative evaluation of two molecular mechanisms which have been advanced to explain the antithyroid action of iodide ion. Both mechanisms suggest that iodide ion acts to interfere with Tx formation by removal of the active iodinating species. The suggested species, however, differs in the two mechanisms. The first hypothesis proposed suggested that the reactive species, HIO, is removed by a reversal of the hydrolysis of elemental iodine. The second hypothesis suggested that elemental iodine, or some positive species in equilibrium with it, is the iodinating agent. Removal of this species is effected by triiodide complex formation. Employing the tyrosine iodinase system as a "model", a quantitative study of iodide inhibition of the organic binding of iodine is undertaken over a wide range of iodide concentration. The inhibition observed agrees closely with that calculated on the basis that inhibition is effected through I^-_3 formation. On the other hand, the inhibition calculated on the basis that HIO is the active iodinating species is found to be prohibitively large. Thus this study establishes that I^-_3 complex formation may account for the inhibitory effect of iodide ion in the biogenesis

of Tx and also offers a reasonable basis for the rejection of HIO as an iodinating species.

It is possible to predict that if iodide exhibits its antithyroid action through triiodide complex formation then prolonged administration of excess quantities of iodide ion should cause the appearance of hypothyroidism and goiter in normal subjects. This prediction is verified by reference to the recent literature in which reports of goiter and hypothyroidism, attributable to the effects of excess iodide ion, have become more prevalent. The beneficial effects of iodide ion in Graves' disease are suggested to be a direct result of the inhibition of organic binding of iodine through I_3^- complex formation. A review of other hypotheses advanced to explain the inhibitory effects of iodide ion leads to the conclusion that most of these theories possess flaws which warrant their rejection.

TABLES AND FIGURES

Table I

The Relative Rates of Iodination of Organic Acceptors

I_2 Acceptor	Relative Iodination Rate[a]
Phenol	0.5
Aniline	5.3
Resorcinol	10.5
m-Aminophenol	37.4
m-Phenylenediamine	46.7

(a) the relative rate of iodination is a measure of the percent conversion of radioactive iodide to iodo-organic compound which occurs when the following medium is incubated at 38° C for a period of two hours;

(i) 1.0 ml. of a five percent extract of beef submaxillary tissue
(ii) 1.0 ml. of 3.3×10^{-5} M acceptor
(iii) 0.5 ml. of 0.1 M phosphate buffer pH 7.4
(iv) 0.3 ml. of 1.1×10^{-4} M iodide ion
(v) 0.1 ml. of I-131 (20 μc.)
(vi) 0.2 ml. of 0.04 M cupric ion

Table II

The Effect of Anaerobiosis on the Tyrosine Iodinase System

Experimental Conditions	Sample No.	Percent Conversion of I-131 to DIT
Anaerobic (N_2)	1	4.3
	2	5.6
Aerobic	3	70.9
	4	73.8

The assay conditions used in this series of experiments are described on page 36.

Table III

The Effect of Anaerobiosis on the Iodide Oxidizing Enzyme System

Experimental Conditions	Sample No.	Percent Conversion of I-131 to Iodo-m-Aminophenol
Anaerobic (N_2)	1	1.3
	2	0.6
Aerobic	3	6.0
	4	5.9

The assay conditions used in this series of experiments were essentially those described on page 36 with the exception that 1.0 ml. of 3.3×10^{-5} M m-aminophenol was substituted for 0.2 ml. of 0.04 M tyrosine in the assay medium.

Table IV

Tissues Tested for the Iodide Oxidizing Enzyme System

Tissue	Iodide Oxidizing Activity	Tyrosine Iodinase Activity
Beef Thyroid	+	+
Beef Submaxillary	+	+
Rat Submaxillary	+	+
Rat Thymus	-	-
Rat Liver	-	-
Rat Muscle	-	-
Rat Lymph Node	-	-
Rat Kidney	-	-

One ml. samples of a 5 percent homogenate supernatant of each tissue were used as the enzyme source. Incubation time for the iodide oxidizing enzyme assay was two hours (see page 35) while that for the tyrosine iodinase assay was one-half hour (see page 38).

Table V

Alternate Substrates for Tyrosine Iodinase

Enzyme Source	Substrate	Conversion to Iodo-compound (L-Tyrosine = 100)
Beef submaxillary	N-Phthalyl-L-tyrosine	173
" "	DL-o-Tyrosine	159
" "	DL-m-Tyrosine	118[a]
" "	D-Tyrosine	114
" "	N-Acetyl-DL-tyrosine	95
" "	p-Hydroxyphenylacetic acid	55
" "	p-Hydroxybenzoic acid	42
" "	3-Fluoro-DL-tyrosine	33
" "	p-Hydroxyphenylpropionic acid	27
" "	Tyramine	24
" "	N-Acetyl-L-tyrosine anilide	13[b]
" "	O-Methyl-L-tyrosine	9
" "	p-Aminobenzoic acid	0
" "	3(3,4-Dihydroxyphenyl)-propionic acid	0[c]
" "	3(3-Methoxy-4-hydroxy-phenyl)propionic acid	0[c]
" "	Sulfanilamide	0
Rat submaxillary	D-Tyrosine	94
" "	p-Hydroxyphenylpropionic acid	35
" "	p-Hydroxyphenylacetic acid	44
" "	p-Hydroxybenzoic acid	40
" "	O-Methyl-L-tyrosine	0
" "	p-Hydroxycinnamic acid	0
" "	Phenol	0
" "	m-Cresol	0
" "	p-Cresol	0
Rat thyroid	D-Tyrosine	81
" "	p-Hydroxyphenylacetic acid	16
" "	p-Hydroxybenzoic acid	15
" "	p-Hydroxyphenylpropionic acid	14
" "	O-Methyl-L-tyrosine	0
" "	p-Hydroxycinnamic acid	0
" "	Phenol	0
" "	m-Cresol	0
" "	p-Cresol	0

Table V (continued)

(a) three distinct iodo-organic compounds were detected with this substrate
(b) the substrate appeared to precipitate during the assay
(c) oxidation of the substrate occured during the assay

These experiments were conducted using 1.0 ml. of a 5 percent extract of the respective tissues in each assay tube as a source of tyrosine iodinase and employing an assay medium identical to that described on page 35 with the exception that 0.2 ml of the required substrate (0.04 M) was substituted for 0.2 ml. of 0.04 M L-tyrosine. Incubation was conducted at 38° C for a one hour period. Analysis of the assay tubes was carried out by standard procedures described in the Experimental Section of this thesis.

Table VI

A Comparison of Reaction Rates in Enzymatic and Non-Enzymatic Iodinations

Substrate	Enzymatic Iodination[a] (L-Tyrosine = 100)	Non-Enzymatic Iodination[b] (L-Tyrosine = 100)
N-Phthaloyl-L-tyrosine	173	157
N-Acetyl-DL-tyrosine	95	141
p-Hydroxyphenylacetic acid	55	120
p-Hydroxybenzoic acid	42	103
D-Tyrosine	118	98
p-Hydroxycinnamic acid	0	93
p-Cresol	0	78
Tyramine	24	75
Phenol	0	46
p-Aminobenzoic acid	0	0[c]
Sulfanilamide	0	0[c]

(a) enzymatic iodinations were carried out as reported in Table V using 1.0 ml. of a 5 percent extract of beef submaxillary tissue as the enzyme source.

(b) the medium for non-enzymatic iodination studies contained the following materials:
- (i) 1.5 ml. of 0.1 M borate-HCl-NaOH buffer pH 9.0
- (ii) 0.1 ml. of 3 percent hydrogen peroxide solution
- (iii) 0.3 ml. of 1.1 x 10^{-4} M iodide ion
- (iv) 20 microcuries of I-131 in 0.1 ml. of solution
- (v) 0.2 ml. of 0.04 M substrate
- (vi) sufficient distilled water to bring the volume to 3.0 ml.

Incubation for non-enzymatic iodinations was carried out in Warburg flasks for three hours, with shaking, at 38° C.

(c) it is possible that the lack of iodination observed with these compounds may be a result of their tendency to be easily oxidized.

During these runs the interesting observation was made that no oxidation of iodide ion to elemental iodine occured with hydrogen peroxide (as measured by organic binding of I-131) unless the assay flasks were shaken in the presence of oxygen. Anaerobiosis completely inhibited this non-enzymatic iodination.

Table VII

The Activator Function of Cupric Ion with Enzymes of The Tyrosine Iodinase System

Experimental Conditions	Percent Conversion of I-131 to an Organically Bound Form
Tyrosine iodinating enzyme[a]	
tyrosine - copper	27.5
copper	0.0
tyrosine	4.3
Iodide oxidizing enzyme[a]	
copper	32.0
no copper	25.4

(a) 1.0 ml. of a 5 percent beef submaxillary homogenate supernatant was used as an enzyme source with each assay tube. Assay conditions as described on pages 35 and 38 were used with the presence and absence of copper and tyrosine as indicated above.

Table VIII

A Comparison of the Activating Effect of Cupric Ion and Ferrous Ion

Enzyme Source	Metal Ion	Maximum Iodination[a]
Rat thyroid	Fe^{2+}	12.5
" "	Cu^{2+}	13.5
Rat submaxillary	Fe^{2+}	47.5
" "	Cu^{2+}	56.8
Beef thyroid	Fe^{2+}	34.0
" "	Cu^{2+}	24.4

(a) the term maximum iodination refers to the maximum conversion of I-131 to MIT under the assay conditions outlined in the description to Fig. 10 using the ion activator indicated above.

Table IX

A Study of Tyrosine Iodination With Elemental Iodine Generated by Hydrogen Peroxide

Tube No.	Enzyme Preparation[a] (ml.)	3 Percent H_2O_2 (ml.)	1.65×10^{-2} M Fe^{2+} (ml.)	0.04 M Cu^{2+} (ml.)	3.3×10^{-5} M m-Amino-phenol (ml.)	0.04 M L-tyrosine (ml.)	Percent Iodination
1	1.0	-	0.2	-	1.0	-	65[b]
2	-	0.1	0.2	-	1.0	-	None
3	-	0.1	-	0.2	1.0	-	"
4	-	0.1	-	-	1.0	-	"
5	1.0	-	0.2	-	-	0.2	30[b]
6	-	0.1	0.2	-	-	0.2	None
7	-	0.1	-	0.2	-	0.2	"
8	-	0.1	-	-	-	0.2	"

In addition to the above components each assay tube contained:
(i) 0.5 ml. of 0.1 M phosphate buffer pH 7.4
(ii) 0.3 ml. of 1.1×10^{-4} M iodide ion
(iii) 0.1 ml. of I-131 (50 microcuries)
(iv) sufficient distilled water to bring the volume to 3.3 ml.

Incubation was carried out aerobically for one hour at 38° C without shaking,-thus the incubation procedure is entirely analogous to that used for a tyrosine iodinase or iodide oxidizing enzyme assay but eliminates the H_2O_2-O_2 catalyzed oxidation of iodide ion observed in Table VI.

(a) a 5 percent extract of beef submaxillary tissue was used as the enzyme source
(b) visual determination from X-ray film radioautographs

Table I

Ions Tested for Activator Action with the Tyrosine Iodinase Enzyme System

Ion Studied	Source	Effect
Sb^{+5}	$SbCl_5$	None
As^{+5}	As_2O_5	"
Bi^{+2}	$BiCl_2$	"
Cd^{+2}	$CdSO_4 \cdot 8H_2O$	"
Ce^{+4}	$Ce(NH_4)_2(NO_3)_6$	"
$Cr_2O_7^{-2}$	$K_2Cr_2O_7$	"
Co^{+2}	$CoCl_2$	"
Fe^{+3}	$FeCl_3 \cdot 6H_2O$	"
Fe^{+2}	$FeSO_4 \cdot (NH_4)_2SO_4$	"
Hg^{+2}	$HgCl_2$	Inhibits
Pb^{+2}	$PbCl_2$	None
Li^{+}	$LiCl$	"
Ni^{+3}	$NiCl_3 \cdot 6H_2O$	"
Ag^{+}	Ag_2SO_4	Inhibits
Sr^{+2}	$SrCl_2$	None
Sn^{+4}	$SnCl_4 \cdot 5H_2O$	"
WO_4^{-2}	$Na_2WO_4 \cdot 2H_2O$	"
Ca^{+2}	$CaCl_2$	"
ZrO^{+2}	$ZrO(NO_3)_2 \cdot H_2O$	"
Ba^{+2}	$BaCl_2 \cdot 2H_2O$	"
Pt^{+4}	$H_2PtCl_6 \cdot H_2O$	Inhibits
La^{+3}	$La(NO_3)_3 \cdot 6H_2O$	None

Table X (continued)

Ion Studied	Source	Effect
BO_3^{-3}	H_3BO_3	None
Al^{+3}	$AlCl_3$	"
Co^{+2}	$CoCl_2 \cdot 6H_2O$	"
Cr^{+3}	$(NH_4)_2SO_4Cr_2(SO_4)_3 \cdot 24H_2O$	"
Mg^{+2}	$MgSO_4 \cdot 7H_2O$	"
Mn^{+2}	$MnCl_2 \cdot 4H_2O$	"
MoO_4^{-2}	$Na_2MoO_4 \cdot 2H_2O$	"
Zn^{+2}	$ZnCl_2$	"

The basic tyrosine iodinase assay medium described on page 25 was used to evaluate the action of the above ions. Additions of ions were made such that their molarity in the final assay medium was 1.0×10^{-4} M. One ml. of beef submaxillary extract was used as the enzyme source in each assay tube.

Table XI

Rat Tissue Examined for Tyrosine Iodinase Activity

Tissue Source	Presence of Tyrosine Iodinase
Extraorbital lacrimal	+
Intraorbital lacrimal	+
Thyroid	+
Submaxillary salivary	+
Stomach	+
Thymus	-
Spleen	-
Small intestine	-
Lung	-
Liver	-
Kidney	-
Brain	-
Skeletal muscle	-
Heart muscle	-
Testis	-
Pancreas	-

Tyrosine iodinase activity was tested by the addition of 1.0 ml. of a 5 percent extract of each tissue to the basic tyrosine iodinase assay medium described on page 35 . Incubation was carried out at 38° C for a period of one-half hour and the assay tube contents analyzed for radioactive components by the standard techniques described in the Experimental Section of this thesis.

Table III

Proteolytic Activities of Mitochondrial Suspensions

Source of Mitochondria	Relative Proteolytic Activity[a] Moles x 10^7
Rat submaxillary	9.1
Rat extraorbital lacrimal	3.0
Beef thyroid	n.d.[b]

(a) the relative proteolytic activity is measured in terms of moles of tyrosine x 10^7 which produce a color, equivalent to that which is produced through the reaction of the protease-formed degradation products of bovine haemoglobin with the Folin-Ciocalteu reagent (see p. 44).
(b) not detectable.

Table XIII

The Action of Proteolytic Preparations on Mitochondrial Iodoproteins

Source of Mitochondria	Proteolytic Enzyme	Liberation of Protein Bound MIT (Percent)[a]
Beef thyroid gland	No enzyme	11.3
	Pepsin	11.5
	Trypsin	70.0
	Pancreatin	92.6
Rat salivary gland	No enzyme	49.1
	Pepsin	40.9
	Trypsin	92.0
	Pancreatin	88.3
Rat extraorbital lacrimal gland	No enzyme	37.2
	Pepsin	31.1
	Trypsin	76.3
	Pancreatin	77.4

(a) Mitochondria from the above sources were first incubated with I-131 as described in the procedures outlined in the Experimental Section of this thesis. All free MIT was then extracted from the mitochondria by washing with Krebs-Ringer bicarbonate solution. These mitochondria were then exposed to the action of the above proteolytic enzymes over a 10 hour period and the resulting preparations analyzed to determine the percent conversion of organically bound iodine to free MIT.

Table XIV

Peptides as Substrates for the Tyrosine Iodinase System

Enzyme Source	Substrate	Relative Rate of Iodination (L-Tyrosine = 100)
Beef submaxillary	L-Leucyl-L-tyrosine	108
	L-Glycyl-L-tyrosine	104
	L-Lysyl-L-tyrosine	157
	Poly-L-tyrosine[a]	29
Beef thyroid	L-Lysyl-L-tyrosine	123
	L-Glycyl-L-tyrosine	88

The capacity of the above peptides to replace L-tyrosine as a substrate for tyrosine iodinase was evaluated by determining the extent to which these peptides were iodinated when substituted for L-tyrosine in the basic tyrosine iodinase assay medium described on page 35 . Molarities of all substrates were maintained at 0.0024 M in the assay medium. L-tyrosine controls were run concurrently.

(a) The molarity of poly-L-tyrosine was adjusted to 0.0024 M with respect to the repeating unit:

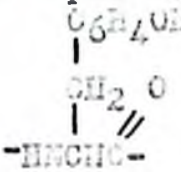


Table XV

Tissues Possessing Iodinating Capacity

Tissue	Enzyme Actvity Present	
	Soluble System	Mitochondrial System
Thyroid		
Rat	+++	NI
Beef	++	+++
Sheep	NI	+++
Human	+++	NI
Submaxillary Salivary		
Rat	+++	++
Beef	+++	+
Guinea-pig	++++	NI
Intraorbital Lacrimal		
Rat	++	NI
Guinea-pig	+	NI
Human	++	NI
Extraorbital (Rat)	+++	+++
Stomach (Rat)	+	++
Thymus (Rat)	-	+
Spleen (Rat)	-	++
Lymph Node (Rat)	NI	+
Small Intestine (Rat)	-	+
Lung (Rat)	-	+

The following rat tissues were found completely devoid of activity for both system; liver, kidney, brain, skeletal muscle, heart muscle, testis, pancreas.

The scale of enzyme activity was based on the percent incorporation of radioiodine into MIT, in excess of boiled controls. For the soluble system a 5 percent homogenate of the appropriate tissue was incubated for 0.5 hour under the conditions described on page 35 . For the mitochondrial system the conditions described on page 42 were followed.

Table XV (continued)

The scale of activities is ;

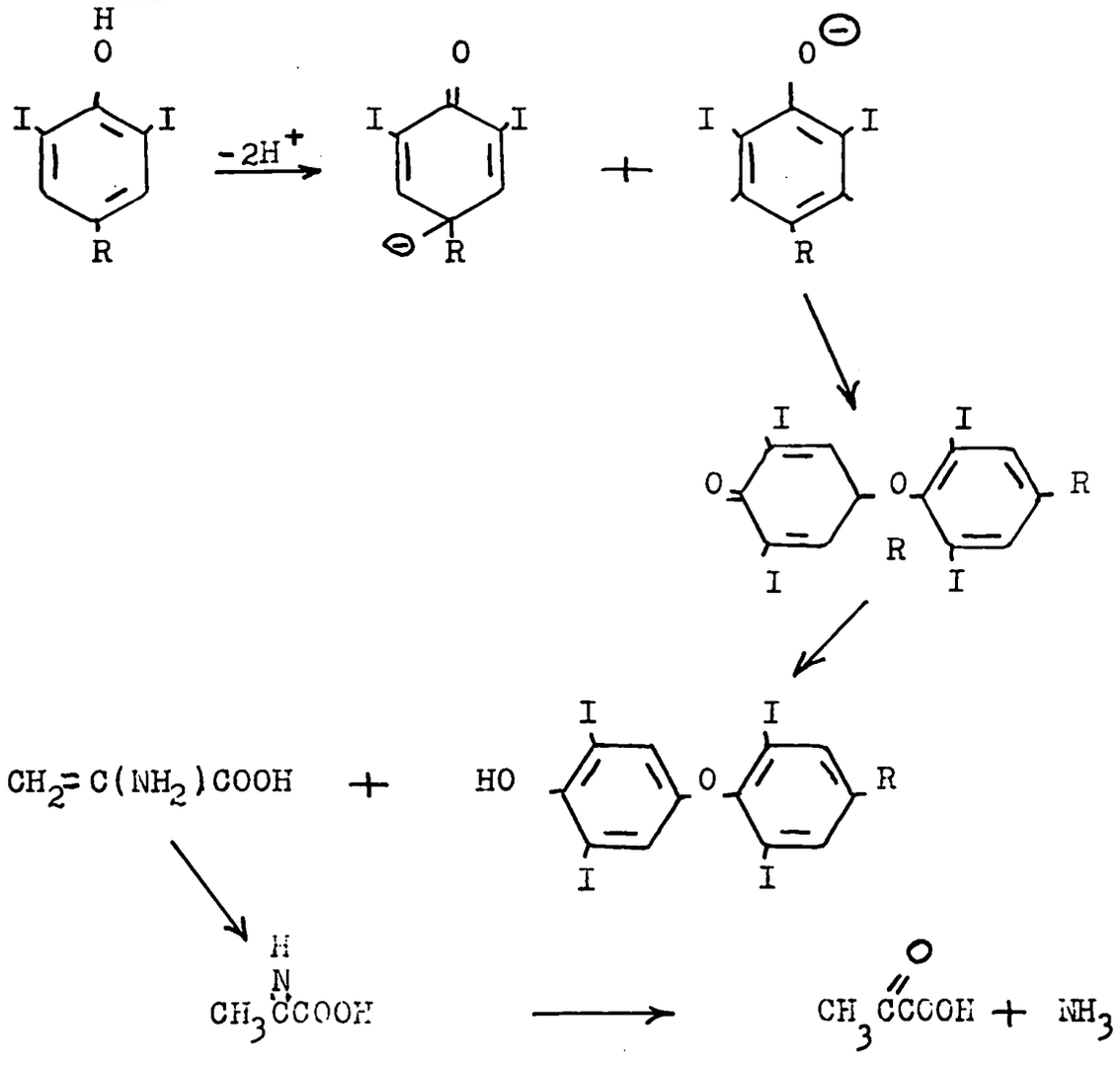
0 - 2 percent incorporation	-
2 - 10 " "	+
10 - 25 " "	++
25 - 50 " "	+++
50 percent + "	++++
Tissue not investigated	NI

Description of Figure 1

A Hypothetical Conversion of Diiodotyrosine to Thyroxine

The reaction sequence depicted in Figure 1 has been suggested by Johnson and Tewkesbury (25) as a mechanism whereby two diiodotyrosine moieties might react to form thyroxine.

Figure 1

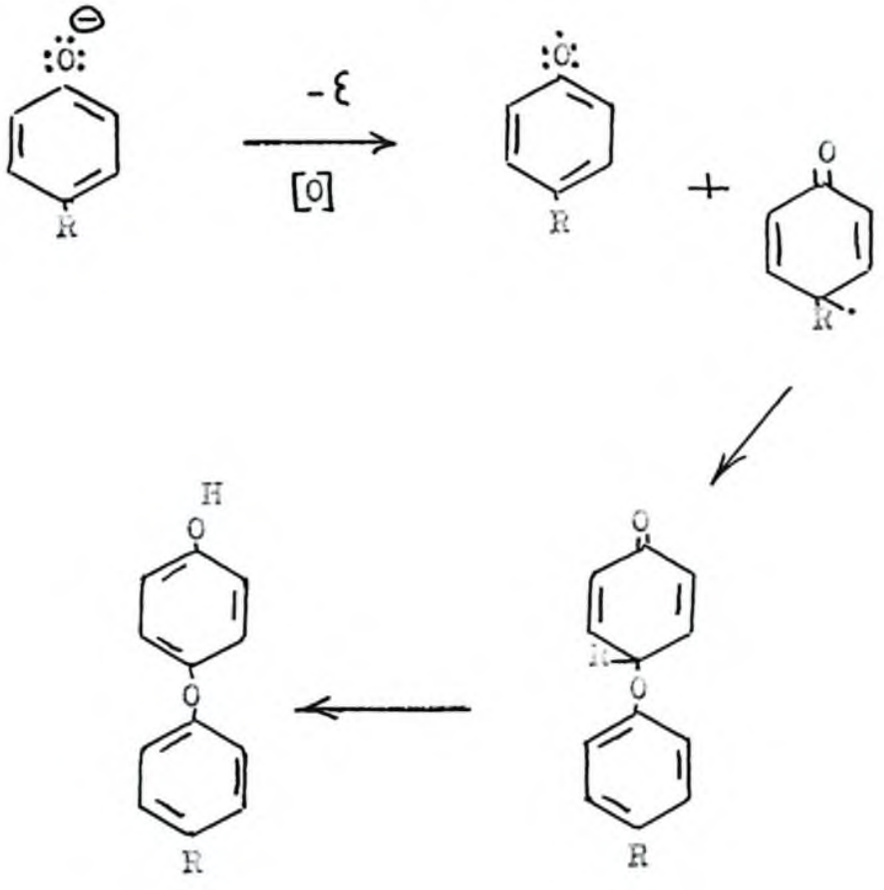

H
O
I
I
R
-2H+
O
I
I
R
O
I
I
R
I
O
O
R
I
I
I
I
R
I
I
CH2=C(NH2)COOH
+
HO
O
R
H
N
CH3CCOOH
O
CH3CCOOH + NH3

Description of Figure 2

A Free Radical Mechanism for Thyroxine Biogenesis

The reaction sequence of Figure 2 involving free radical intermediates has been suggested by Harington (19) as a model of thyroxine biogenesis.

Figure 2



Description of Figure 3

Variation in Iodination Rate with Molarity of Iodine Acceptor

The measurment of the percent conversion of I-131 to iodo-organic compound was carried out as described on page 38 .

The incubation medium used has been described in the addendum to Table I. Molarity of acceptor, however, was varied over the range 10^{-7} to 10^{-3}.

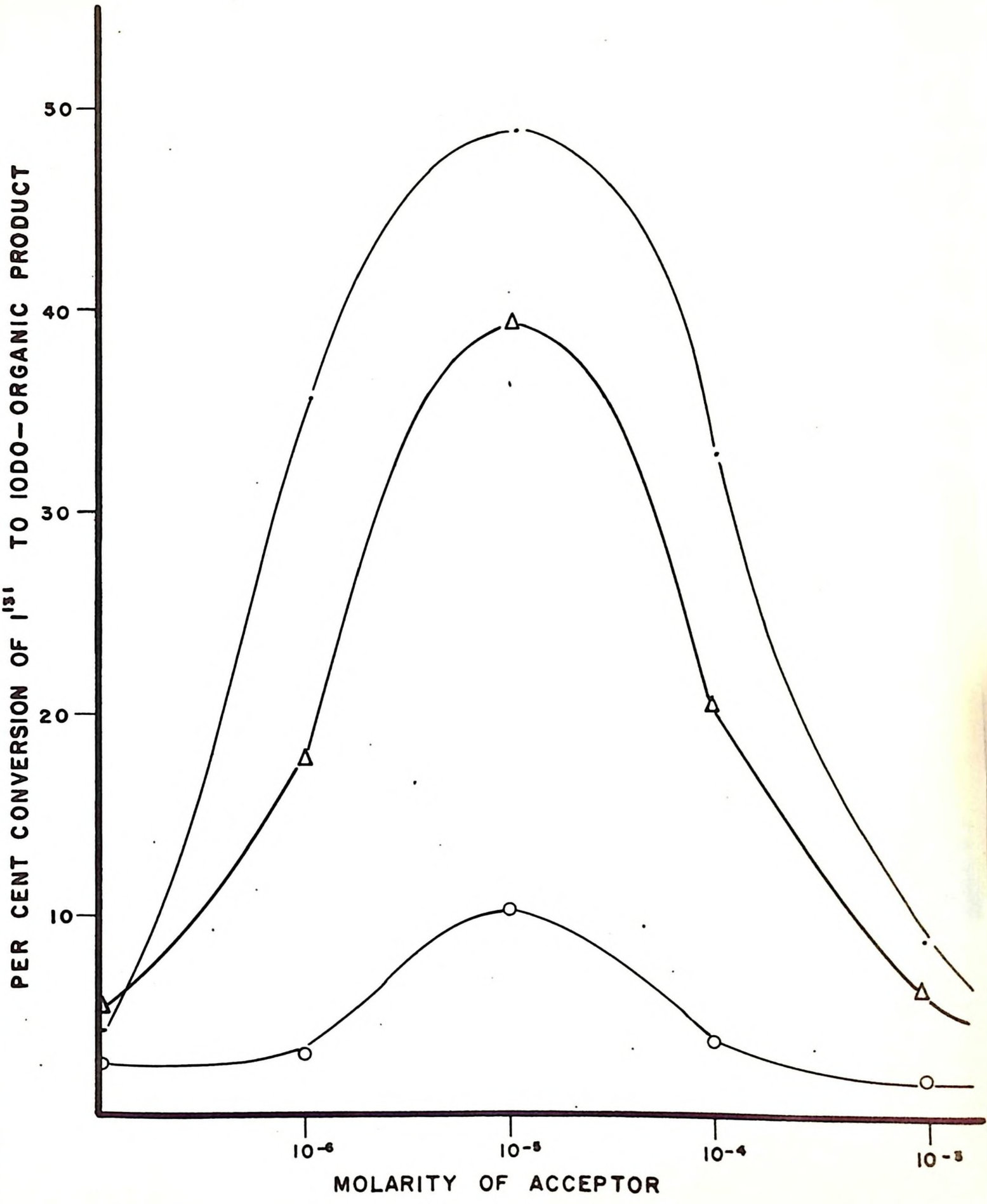
The curves of Figure 3 represent;

m-phenylenediamine .--------.

m-aminophenol Δ--------Δ

resorcinol O--------O

Figure 3

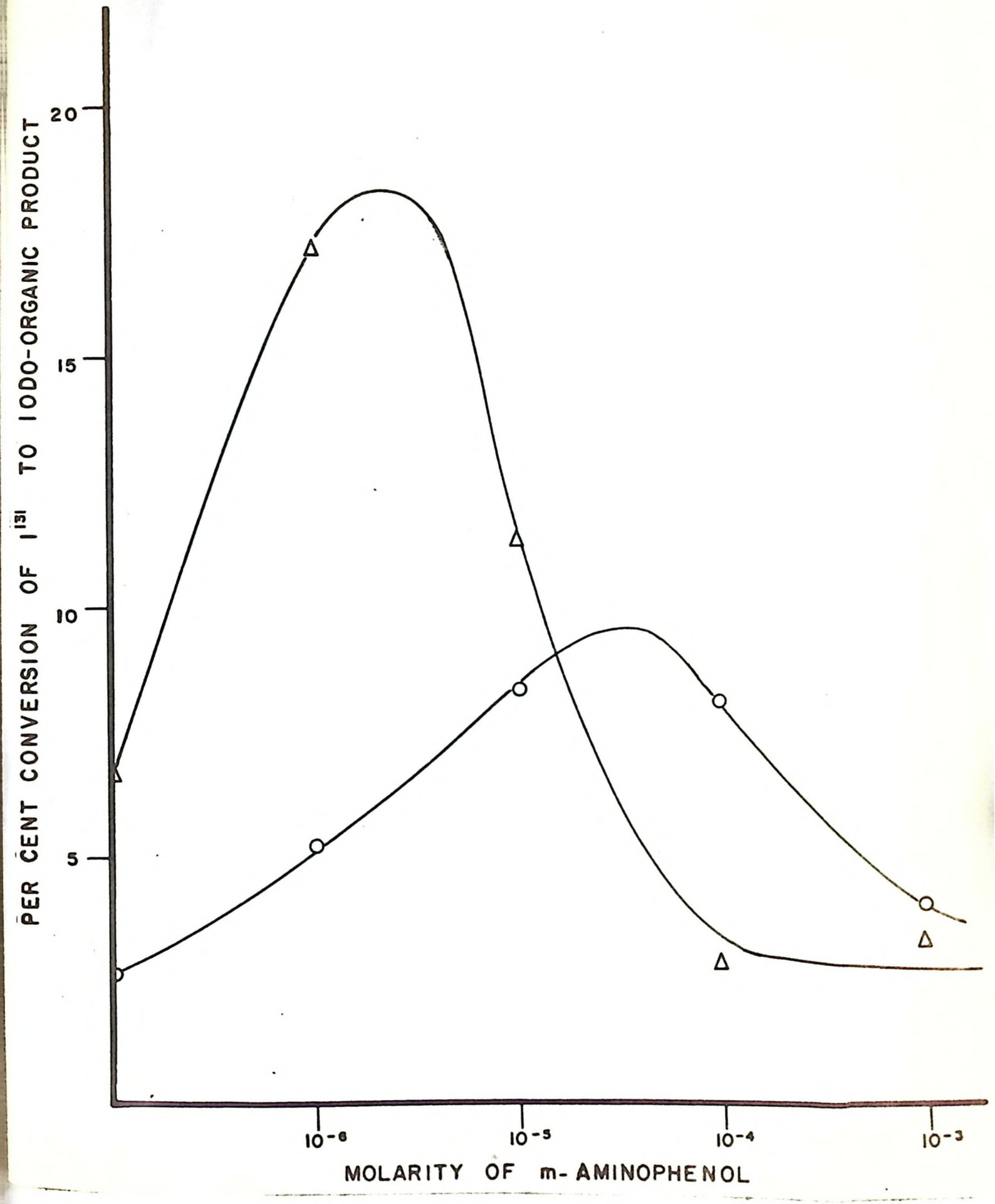

PER CENT CONVERSION OF I^{131} TO IODO-ORGANIC PRODUCT
50
40
30
20
10
10^{-6}
10^{-5}
10^{-4}
10^{-3}
MOLARITY OF ACCEPTOR

Description of Figure 4

The Effect of Enzyme Source upon the Variation in Iodination Rate with Molarity of m-Aminophenol

The variation in the iodination rate of m-aminophenol was studied over the acceptor concentration range of 10^{-7} to 10^{-3} M as in Figure 3. Five percent extracts of rat submaxillary (Δ-------Δ) and beef thyroid (o-------o) tissues were used, as the enzyme sources.

Figure 4

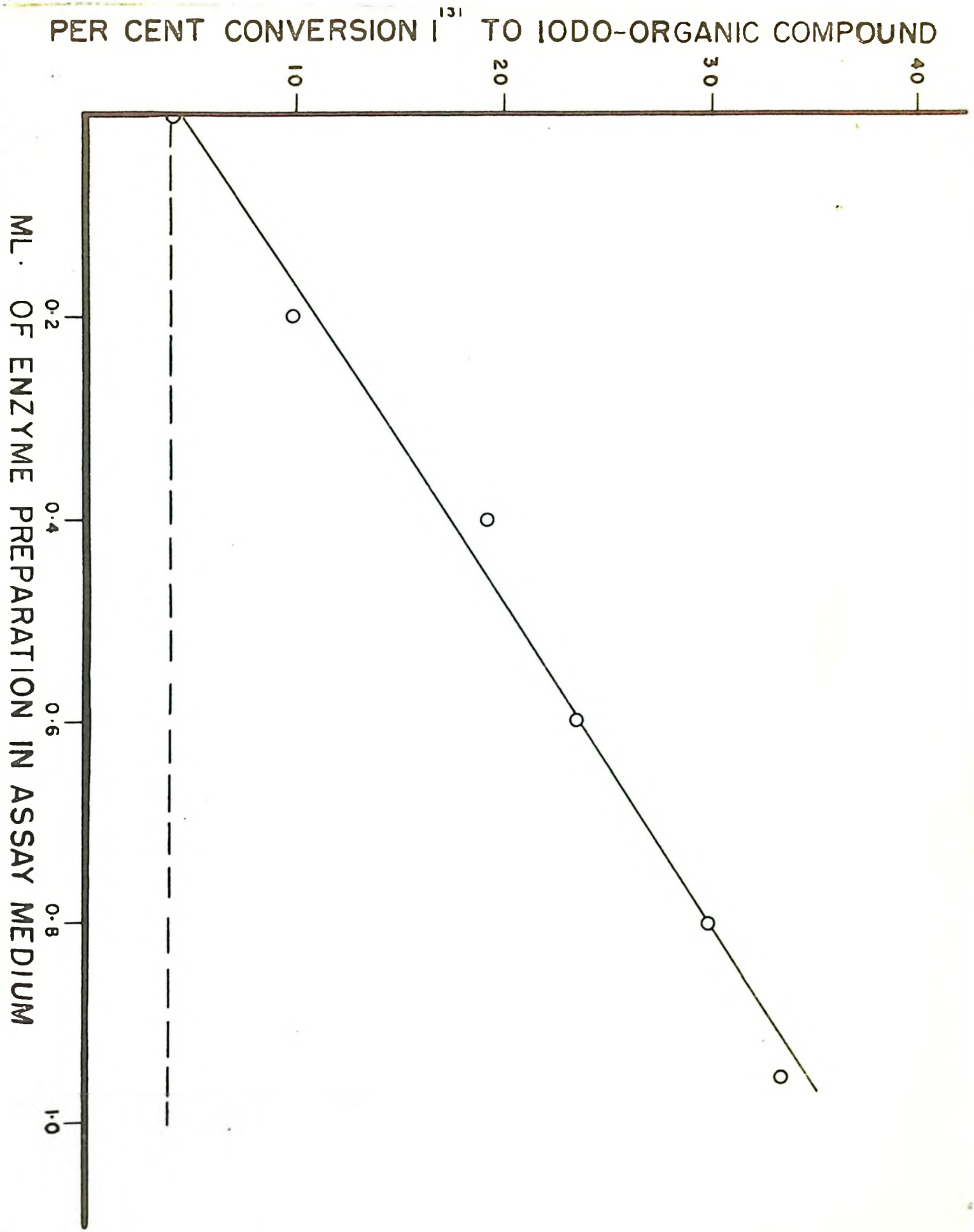

PER CENT CONVERSION OF I^{131} TO IODO-ORGANIC PRODUCT
20
15
10
5
10^{-6}
10^{-5}
10^{-4}
10^{-3}
MOLARITY OF m-AMINOPHENOL

Description of Figure 5

Typical Assay Curve for the Iodide Oxidizing Enzyme System

Graded concentrations of 5 percent beef submaxillary extracts were added to the basic assay medium described in Table I to provide the curve of Figure 5 (O-------O). Incubation was conducted over a two hour period at a temperature of 38° C employing m-aminophenol as the I_2 "collector". A boiled control run concurrently established the base-line activity (-------).

Figure 5


PER CENT CONVERSION I131 TO IODO-ORGANIC COMPOUND
10
20
30
40
ML. OF ENZYME PREPARATION IN ASSAY MEDIUM
0·2
0·4
0·6
0·8
1·0

Description of Figure 6

Iodide Oxidation Via a Specific Oxidase

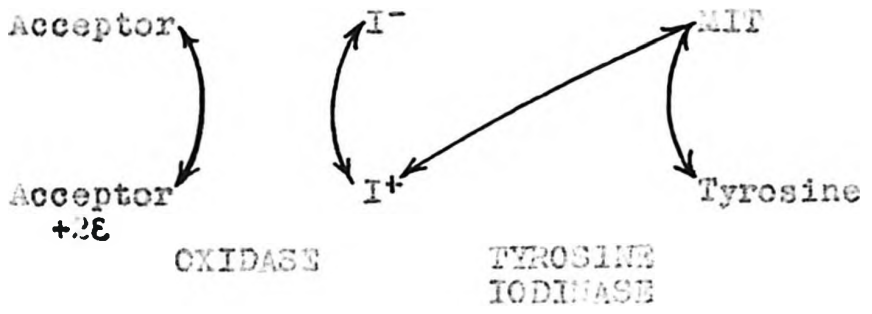
Figure 6 depicts schematically the possible interrelationship which might exist between an oxidase specific for iodide ion and tyrosine iodinase. Two variations of the general scheme might occur:

(a) a specific oxidase functions through the removal of two electrons from iodide ion to provide I^+ which then directly reacts with tyrosine to generate MIT through the agency of tyrosine iodinase.

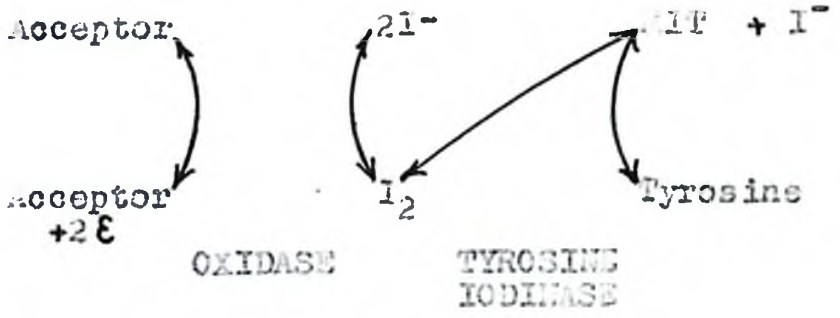
(b) oxidation of iodide occurs as in (a) with the exception that the product of the oxidase is elemental iodine which is accepted in this form by tyrosine iodinase.

Figure 6

(a)


Acceptor
Acceptor
+2ε
I⁻
I+
MIT
Tyrosine
OXIDASE
TYROSINE
IODINASE

(b)


Acceptor
Acceptor
+2 ε
2I⁻
I2
MIT + I⁻
Tyrosine
OXIDASE
TYROSINE
IODINASE

Description of Figure 7

Potential Roles of Hydrogen Peroxide in the Generation of Elemental Iodine

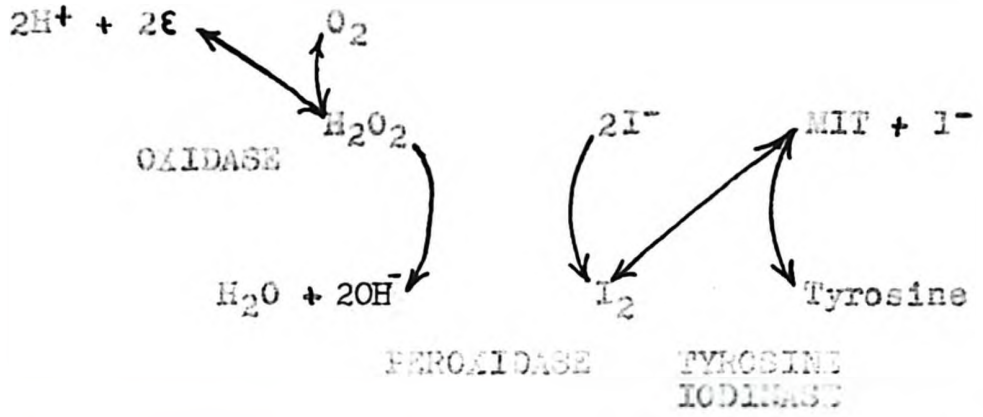
Figure 7 outlines the possible pathways whereby hydrogen peroxide generated by an oxidase might be employed to provide elemental iodine for tyrosine iodinase:

(a) an oxidase reduces molecular oxygen to hydrogen peroxide to provide a substrate for a peroxidase which employs this oxidizing power to convert iodide ion to elemental iodine which is in turn accepted by tyrosine iodinase.

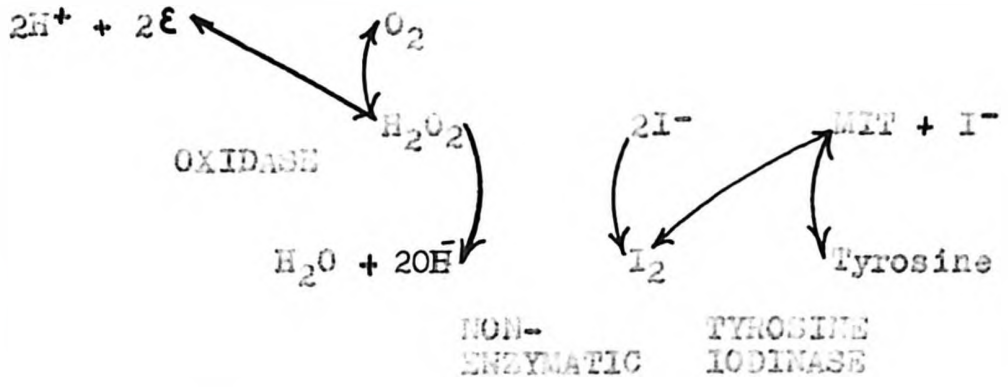
(b) an identical situation to (a) exists with the exception that no peroxidase is involved in the utilization of hydrogen peroxide.

Figure 7

(a)


2H+ + 2ε
O2
H2O2
OXIDASE
H2O + 2OH-
2I-
MIT + I-
I2
Tyrosine
PEROXIDASE
TYROSINE
IODINASE

(b)

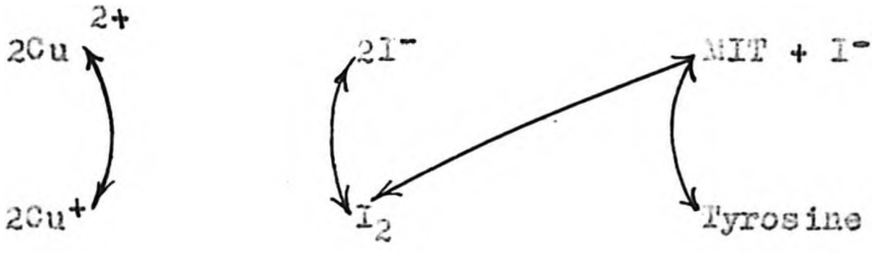

2H+ + 2ε
O2
H2O2
OXIDASE
H2O + 2OH-
2I-
MIT + I-
I2
Tyrosine
NON-
ENZYMATIC
TYROSINE
IODINASE

Description of Figure 8

The Role of Cupric Ion as an Acceptor of Electrons in the Tyrosine Iodinase System

The outline of a possible association of enzymes in the tyrosine iodinase system depicted in Figure 8 is identical to that of Figure 6b with the exception that cupric ion is specified as the electron acceptor.

Figure 8



IODIDE OKIDASE

TYROSINE IODINASE

Description of Figure 9

The Effect of Catalase on the Tyrosine Iodinase System

A series of tyrosine iodinase assay tubes were prepared for this inhibition study as described on page 35 using 1.0 ml. of a 5 percent beef submaxillary homogenate supernatant in each tube as a source of enzyme. Crystalline beef liver catalase was added to these tubes in graded concentrations from zero to 700 units. Incubation was conducted at 38° C for 0.5 hours. The percent conversion of I-131 to MIT was evaluated by standard procedures already described (see p. 34). A boiled control was run concurrently to establish the baseline activity (-------).

Figure 9

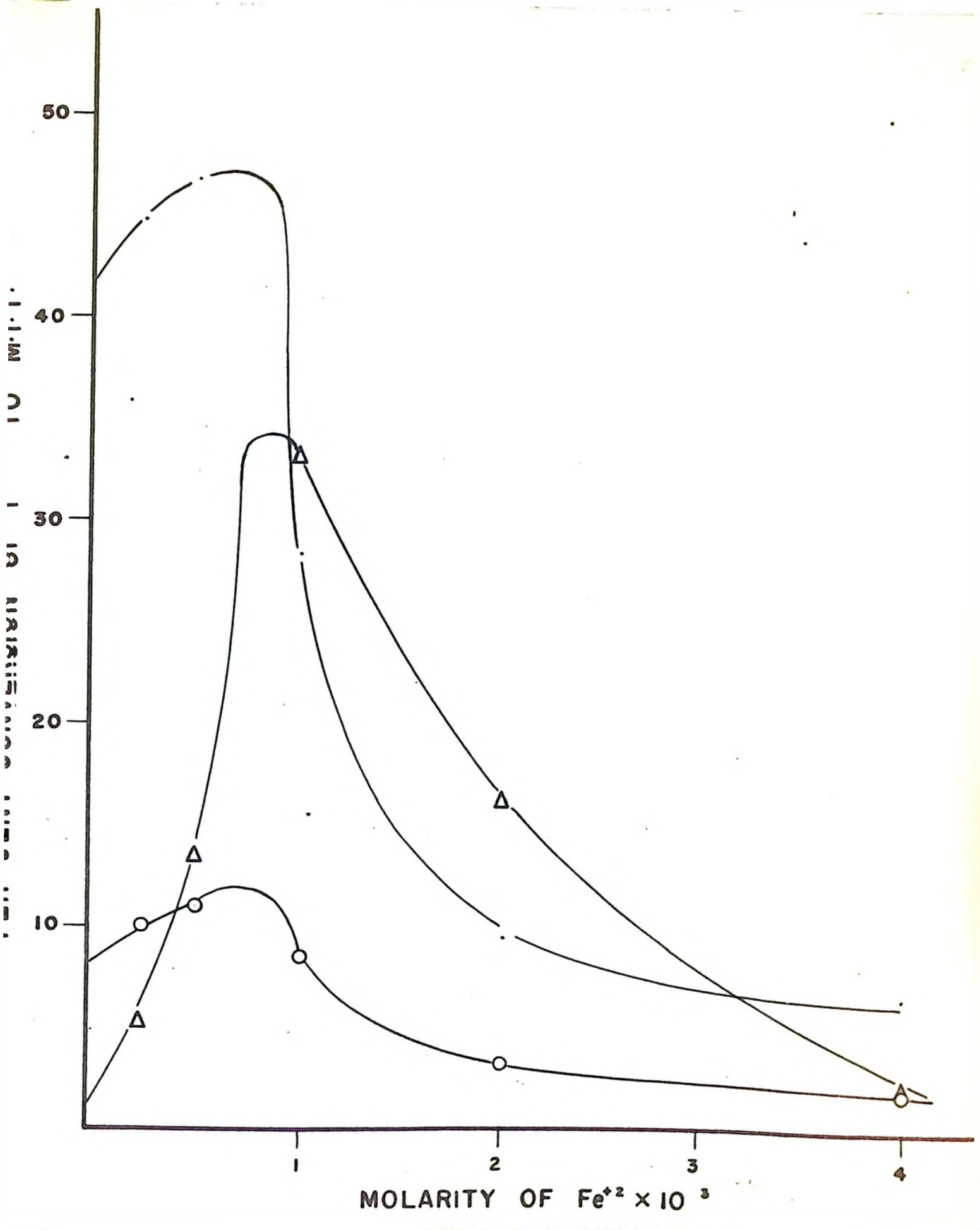
PER CENT CONVERSION OF I^{131} TO M.I.T.
40
30
20
10
UNITS OF CATALASE X 10^{-1}
70
60
45
30
15

Description of Figure 10

The Effect of Varying Concentrations of Ferrous Ion on the Activity of the Tyrosine Iodinase System

The experiments of Figure 10 were conducted using rat submaxillary (•-------•), beef submaxillary (Δ------Δ), and rat thyroid (O------O) tissue extracts as sources of the tyrosine iodinase system. The assay tubes were prepared using 1.0 ml. of the respective tissue extracts (5 percent homogenate supernatants) and the basic assay medium described on page 35 with the exception that no cupric ion was employed. Freshly prepared ferrous ion solutions were added such that the molarity of the ion in the assay medium varied from 2.5 x 10^{-4} to 4.0 x 10^{-3} M. Incubation time for rat enzyme preparations was three hours whereas the beef enzyme preparations were incubated for one-half hour. The incubation temperature was maintained at 38° C in both cases. The assay tubes were analyzed by standard procedures described in the Experimental Section of this thesis.

Figure 10



Description of Figure 11

The Response of Dialyzed Tyrosine Iodinase Preparations to a Factor Present in Undialyzed Preparations

Figure 11a represents a typical assay curve for a given 5 percent beef submaxillary enzyme preparation prior to dialysis (.--------.). A straight line response to increased concentrations of enzyme preparation is demonstrated.

The curves of Figure 11b are based on the same enzyme preparation used with Figure 11a with the exception that the preparation has been dialyzed against 12 liters of 0.1 M phosphate buffer at a pH of 7.4 (three volume changes of four liters each) for a period of forty hours. The baseline activity of 1.0 ml. of this preparation with the regular tyrosine iodinase assay medium is shown (- - - -). The elevation of this baseline activity by the further addition of graded concentrations of boiled undialyzed beef submaxillary homogenate supernatant (5 percent) to the dialysis preparation is also demonstrated (o ------ o). That the factor involved appears to be inorganic in nature was demonstrated by the addition of graded concentrations of ignited undialyzed beef submaxillary preparation solution (prepared by igniting a given volume of undialyzed beef submaxillary extract and dissolving the residue in a volume of distilled water equivalent to the original volume of extract) to the basic 1.0 ml. of dialyzed enzyme preparation in the regular tyrosine iodinase assay medium (Δ------Δ).

PER CENT CONVERSION OF I131 TO M.I.T.
10
20
30
40
ML. OF ENZYME PREPARATION IN MEDIUM
0·4
0·8
1·0
1·2
1·6
2·0
ML. OF ACTIVATOR IN MEDIUM
0·4
0·6
1·0
1·2
1·6
2·0

Figure 11

Description of Figure 12

The Organic Binding of I-131 by Beef Thyroid Mitochondria

The radioautographs presented in Figure 12 are of chromatograms used to analyze the results obtained from the incubation of beef thyroid mitochondria with radioiodine. The procedures and techniques employed in this incubation and analysis are described in the Experimental Section of this thesis.

Chromatogram A represents a beef thyroid mitochondrial suspension directly after incubation with I-131. Proceeding up the chromatogram two radioactive components are obvious. The first of these has not travelled with the solvent, the second component has been identified as iodide ion.

Chromatogram B represents a preparation identical to that of A with the exception that this suspension has been further treated with pancreatin. The iodide component remains but the origin component has disappeared and is replaced by a new component identified as MIT.

Chromatogram C represents mitochondria which have been boiled prior to incubation with I-131. Only an iodide component is apparent and no organic binding of iodine has occured.

The transverse line at the top of each chromatogram represents the solvent front.

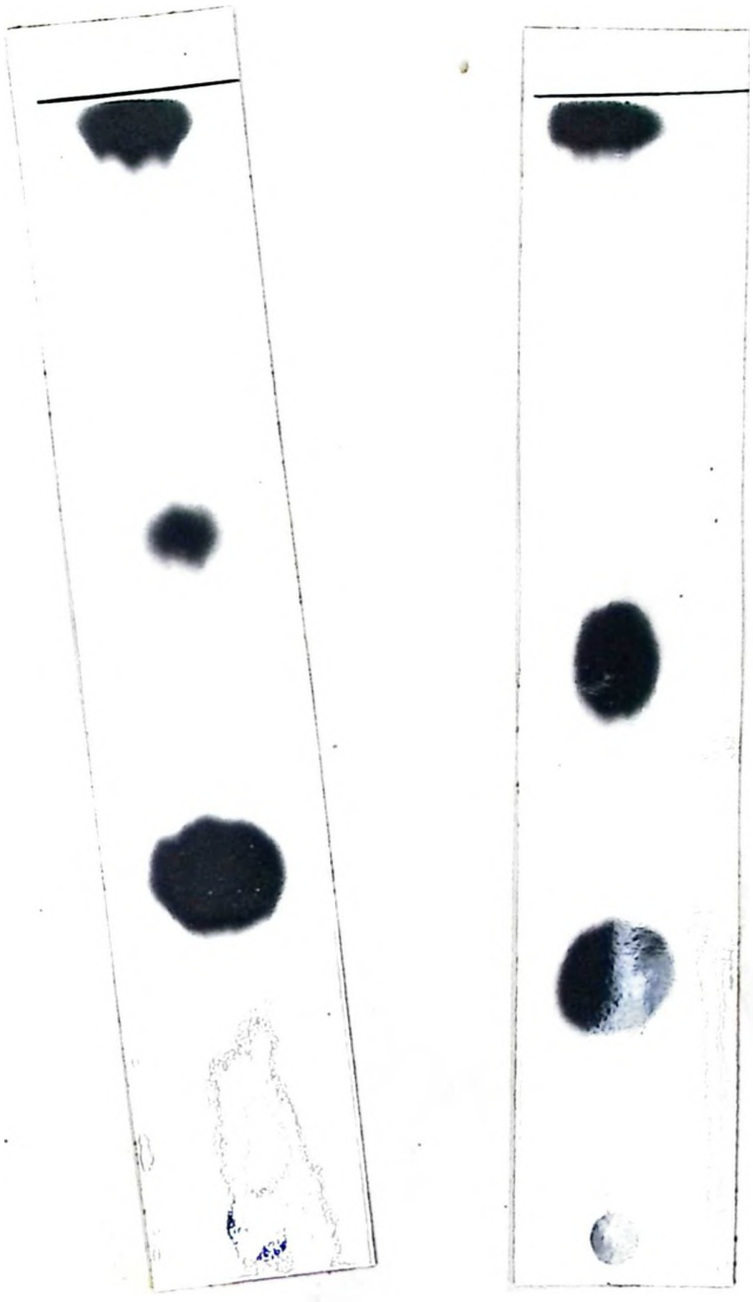
Description of Figure 12 (continued)

Faint "unknown" radioactive components directly beneath the solvent fronts in chromatograms A and B are not visible in these reproductions.

Figure 12

A B C

Figure 13



A B

Description of Figure 13

The Organic Binding of I-131 by Rat Submaxillary Mitochondria

The radioautographs depicted in Figure 13 were obtained from chromatograms of rat submaxillary mitochondria suspensions incubated with I-131.

Radioautograph A represents mitochondria chromatographed directly after incubation with I-131. Four radioactive components are visible. Proceeding up the chromatogram these are:

(i) an immobile component
(ii) an iodide component
(iii) a faint MIT component
(iv) an unknown solvent front

Radioautograph B represents this same mitochondrial preparation after incubation with pancreatin. The origin component has been greatly reduced while the MIT component has been greatly intensified.

The transverse lines at the top of each radioautograph represents the solvent front.

Description of Figure 14

The Association of the "Front" and "Origin" Components of the Mitochondrial Enzyme System with Particulate Material

The radioautographs of Figure 14 demonstrate the close association of the "front" and "origin" components, present in I-131 incubated mitochondria, with the mitochondria or some other water insoluble material.

Radioautograph A is derived from a chromatogram of beef thyroid mitochondria after a two hour incubation with I-131 at 38° C. The "front", iodide, and "origin" components are immediately obvious.

Radioautograph B is derived from a chromatogram of the supernatant of the mitochondrial suspension represented in A obtained through centrifugation of the suspension at 25,000 g for a period of 20 minutes. Both "front" and "origin" components are absent.

Figuro 14

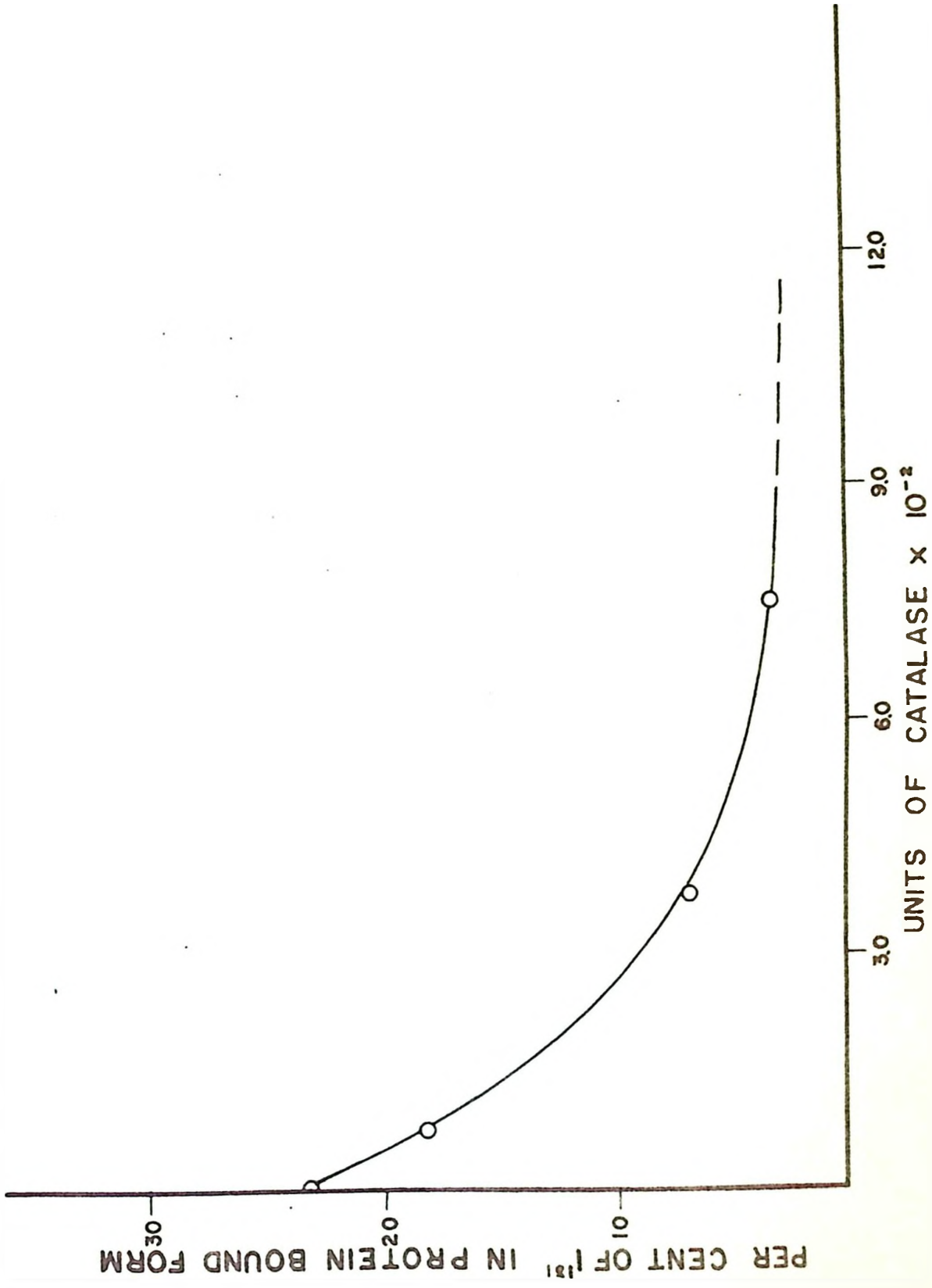
A B

Description of Figure 15

The Effect of Catalase on the Mitochondrial Enzyme System

The experimental curve of Figure 15 was derived from a study of the inhibiting effects of varying concentrations of catalase on the incorporation of I-131 into protein bound form by thyroid mitochondria. For this purpose 3.0 ml. volumes of beef thyroid mitochondrial suspension were incubated with I-131 for two hours at 38° C in the presence of graded concentrations of a crystalline preparation of beef liver catalase. The analyses of the percent of I-131 converted to a protein bound form was carried out by procedures described in the Experimental Section of this thesis.

Figure 15


PER CENT OF I^{131} IN PROTEIN BOUND FORM
10
20
30
UNITS OF CATALASE × 10^{-2}
3.0
6.0
9.0
12.0

Description of Figure 16

Alternate Substrates for the Mitochondrial Enzyme System

The radioautographs reproduced in Figure 16 are derived from the chromatograms of beef thyroid mitochondria incubated with I-131 and a series of substrates (0.0024 M in the suspension) for a period of two hours at 38° C. Each set of two radioautographs depicts the effect of a given substrate on the mitochondrial enzyme system as compared to a boiled control.

Set A: the free amino acid L-tyrosine has been added to the mitochondrial suspension prior to incubation. The unboiled mitochondria are seen to contain four radioactive components, an "origin" or protein bound component, an iodide component, a free MIT component, and a "front" component. The boiled mitochondria contain only radioactive ion.

Set B: the free amino acid m-DL-tyrosine has been added to the mitochondrial suspension prior to incubation. The unboiled mitochondria are seen to contain, an "origin" component, an iodide component, three iodo-m-DL-tyrosine derivatives, and a "front" component. The boiled mitochondria contain only radioiodide ion.

Set C: the free amino acid o-DL-tyrosine has been added to the mitochondrial suspension prior to incubation. The unboiled mitochondria are seen to contain, an "origin" component, an iodide component, an iodo-o-DL-tyrosine derivative,

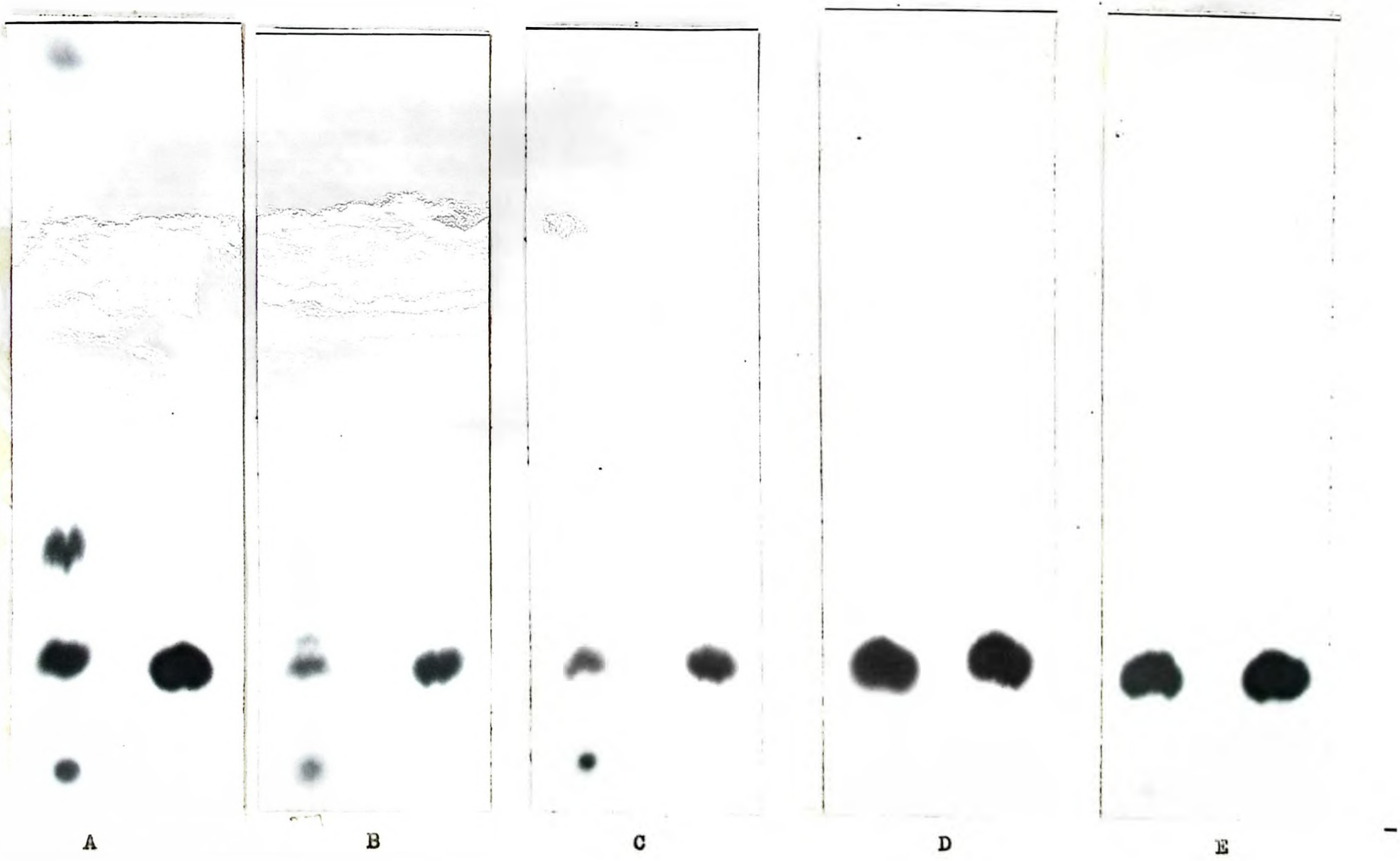
Description of Figure 16 (continued)

and a "front" component. The boiled mitochondria contain only radioiodide ion.

Set D: phenol has been added to the mitochondrial suspension prior to incubation. No organic binding of I-131 has occured in either unboiled or boiled mitochondrial preparations. Phenol appears to almost completely inhibit the iodination of protein.

Set E: p-hydroxycinnamic acid has been added to the miochondrial suspension prior to incubation. As with phenol the ability to form organic iodine compounds has been almost completely inhibited.

Figure 16

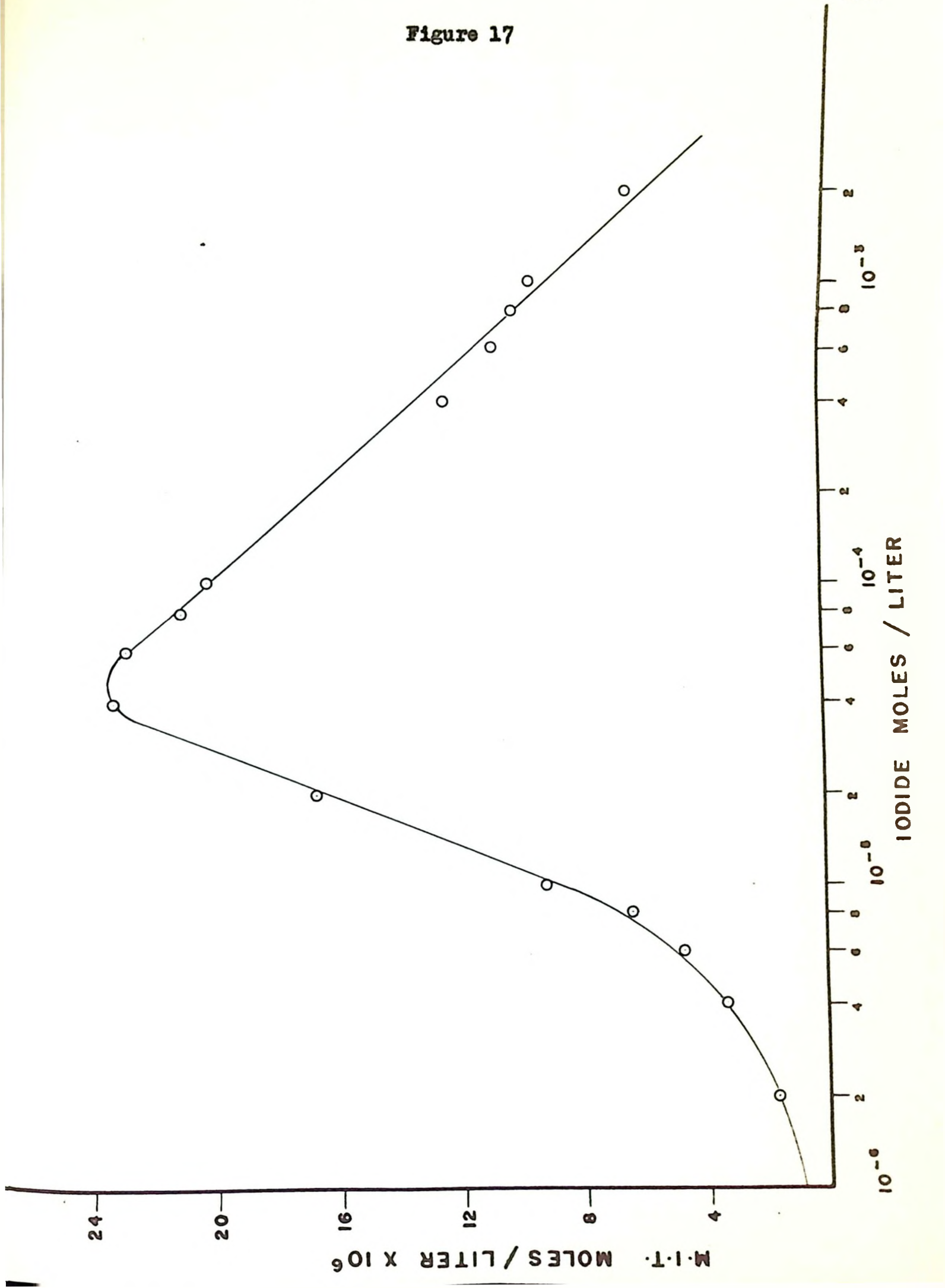


Description of Figure 17

The Effect of Iodide Concentration Upon the Organic Binding of I-131 by the Tyrosine Iodinase System

The experimental techniques employed to derive the data of Figure 17 are discussed in detail on page 40 .

Figure 17

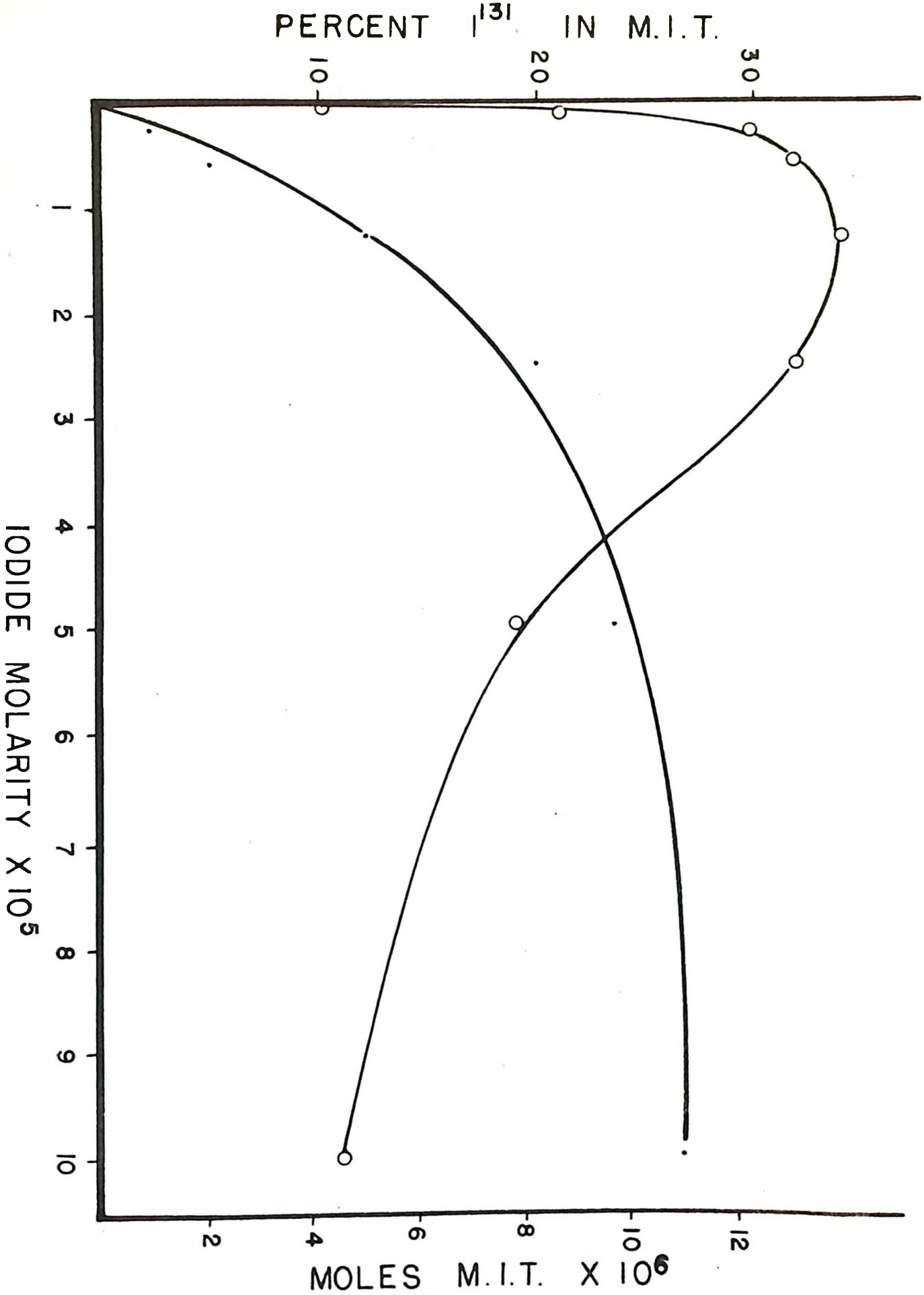

M·I·T· MOLES / LITER X 10⁶
24
20
16
12
8
4
IODIDE MOLES / LITER
10⁻⁶
2
4
6
8
10⁻⁵
2
4
6
8
10⁻⁴
2
4
6
8
10⁻³
2

Description of Figure 18

Percent Incorporation of I-131 into MIT as Contrasted with the Amount of MIT Actually Synthesized

Figure 18 demonstrates a common pitfall encountered in any consideration of organic binding of iodine when I-131 is employed as a biological tracer. It is seen that the percent binding of I-131 (left ordinate, circled points) decreases, with increasing concentrations of iodide, at a time when the actual number of moles of MIT synthesized (right ordinate, uncircled points) is increasing.

Figure 18


PERCENT I^{131} IN M.I.T.
10
20
30
IODIDE MOLARITY X 10^5
1
2
3
4
5
6
7
8
9
10
2
4
6
8
10
12
MOLES M.I.T. X 10^6

Description of Figure 19

The Reversal of Anaerobic Inhibition of the Tyrosine Iodinase System with Hydrogen Peroxide

The experiments represented by the radioautographs in Figure 19 were conducted in Warburg flasks which contained all of the components required for a tyrosine iodinase assay (see p. 35) with the exception that no stable iodide other than that present as a contaminant in the reagents used was supplied. One ml. aliquots of a 5 percent extract of rat submaxillary tissue were used as a source of enzyme. Visking bags containing approximately 1.0 ml. of a hydrogen peroxide solution or 1.0 ml. of distilled water were suspended in the assay medium. Tracer iodide held in a side-arm of the flask was not added until all oxygen had been flushed from the flasks and replaced with nitrogen gas. After the addition of I-131 the flasks were incubated with shaking for a three hour period at 38° C. The reaction mixtures were then treated with approximately 20 mg. of $Na_2S_2O_3 \cdot 5H_2O$ to destroy excess peroxide, chromatographed with n-butanol : acetic acid : water (68:2:27) solvent, radioautographed, and the extent of iodination determined. Individual variations of this treatment and the results obtained are described below:

A In this experiment a 0.01 M hydrogen peroxide solution was used in the Visking bag. The percent conversion of I-131 to MIT was 12.5 percent.

Description of Figure 19 (continued)

B. Reaction conditions in this experiment were identical to those of experiment A with the exception that the enzyme preparation was boiled. The percent conversion of I-131 to MIT was 10.6 percent. The small differential in iodination between experiments A and B would indicate that the major portion of MIT formed was the result of non-enzymatic oxidation of iodide ion to iodine. This non-enzymatic iodination is expected in view of the high concentration of hydrogen peroxide used.

C. In this experiment a 0.001 M hydrogen peroxide solution was used. The percent conversion of I-131 to MIT was 34.2 percent.

D. The reaction conditions of this experiment were identical to those of experiment C with the exception that a boiled enzyme preparation was used. The percent conversion of I-131 to MIT was 0.8 percent. The large differential in iodination observed with experiments C and D would indicate that an enzymatic generation of I_2 has resulted. Since the differential observed with experiments A and B is much less than that of experiments C and D it may be concluded that large excesses of hydrogen peroxide inhibit some component enzyme of the tyrosine iodinase system.

E. In this experiment distilled water was used in the Visking bag. The percent conversion of I-131 to MIT was 1.2

Description of Figure 19 (continued)

This conversion is therefore a measure of the iodination observed under inhibition through anaerobiosis. Thus the difference in extent of iodination observed in experiments C and D is a measure of the reversal of inhibition effected by supplementation with hydrogen peroxide.

F. The conditions of this experiment were identical to those of experiment E with the exception that aerobic conditions were re-established after the addition of tracer iodide. The percent conversion of I-131 to MIT was 31.2 percent and is a measure of the ability of the tyrosine iodinase system to function employing elemental oxygen. Thus a comparison of experiments C and F would indicate that the system is more efficient when supplemented with hydrogen peroxide.

Figure 19

APPENDIX I

It is possible to predict what effect changes in iodide concentration will have on any iodinating system, provided that it derives its iodine from the reaction:

$$2I^- + 2Cu^{2+} \rightleftharpoons I_2 + 2Cu^+ \qquad [1]$$

The equilibrium constant for [1] may be written:

$$\frac{(Cu^+)^2\,(I_2)}{(Cu^{2+})^2\,(I^-)^2} = K \qquad [2]$$

From equation [1] it is seen that $(Cu^+) = 2(I_2)$. Further, a consideration of the numerical value of the equilibrium constant of [1] (3.5×10^{-13} (165)) and the fact that, in the systems discussed here, the (Cu^{2+}) : (I^-) ratio is never less than unity, permits the conclusion that (Cu^{2+}) is essentially constant. These considerations allow the modification of [2] to:

$$(I_2) = (I^-)^2_5\, K' \qquad [3]$$

Equation [3] permits the calculation of the ratio of the iodine concentrations that would be produced by reaction [1] at iodide concentrations corresponding to the optimum rate of formation of MIT by the tyrosine iodinase system (5×10^{-5} M, see p.181) and to 55 percent inhibition of the system (1×10^{-3} M, see p.181). This may be done as follows:

$$\frac{(I_2)\ 55\%\ \text{inhib.}}{(I_2)\ \text{opt.form.}} = \left(\frac{(I^-)\ 55\%\ \text{inhib.}}{(I^-)\ \text{opt.form.}} \right)^{\frac{2}{3}} = 7.4 \quad [4]$$

However, the concentration of free iodine existing in any solution containing both iodine and iodide is markedly influenced by the reaction:

$$I^- + I_2 \rightleftharpoons I^-_3 \quad [5]$$

It is possible to calculate, from the equilibrium constant of [5] the proportion of iodine that will be in the free state at any given concentration of iodide (166). When this is done for the above two iodide concentrations it is found that 97 percent of the iodine will be in the free state at 5×10^{-5} M iodide and 65 percent will be free at 1×10^{-3} M iodide. The ratio given by equation [4] may then be corrected:

$$\frac{(I_2)\ 55\%\ \text{inhib.}}{(I_2)\ \text{opt.form.}} = \frac{65}{97} \times 7.4 = 5.0 \quad [6]$$

It is obvious that, since the free iodine concentration produced by reaction [1] is increased by iodide concentrations that inhibit MIT synthesis, this synthesis cannot be dependent on reaction [1] as a source of iodine.

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