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SYNTHESIS *IN VITRO* OF THYROXINE FROM DIIODOTYROSINE BY MYELOPEROXIDASE AND BY A CELL-FREE PREPARATION OF BEEF THYROID GLANDS

I. GLUCOSE–GLUCOSE OXIDASE SYSTEM

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SUMMARY

1. The action of a purified peroxidase, myeloperoxidase, on diiodotyrosine was investigated in the presence of glucose and glucose oxidase as a hydrogen peroxide, generating system. Thyroxine was identified by paper chromatography as one of the reaction products.

2. The incubation of diiodotyrosine with a cell-free thyroid particulate preparation under similar conditions also led to the formation of a substance which was indistinguishable from thyroxine by paper chromatography, ultraviolet and infrared analyses and by its stimulation of tadpole metamorphosis.

3. The synthesis of thyroxine was inhibited by ergothioneine, reduced glutathione, ascorbic acid, semicarbazide, Tapazole, 2-thiouracil and catalase.

4. Significant amounts of ammonia were released on the incubation of diiodotyrosine with either myeloperoxidase or the thyroid preparation in the presence of glucose and glucose oxidase.

5. These results are discussed in relation to the mechanism of thyroxine synthesis by peroxidase. The involvement of a thyroid peroxidase in the synthesis of thyroxine from diiodotyrosine by the thyroid particulate preparation under the conditions employed in this study is suggested.

INTRODUCTION

In 1927, HARINGTON AND BARGER[1] proposed that T_4 is synthesized *in situ* by the condensation of 2 molecules of DIT. VON MUTZENBECHER[2] in 1939 demonstrated the synthesis of T_4 in low yield during the incubation of DIT in an alkaline medium in the presence of oxygen. This finding has been confirmed by many investigators and the yield of T_4 in this non-enzymic reaction can be increased by iodine, H_2O_2 (see ref. 3)

Abbreviations: T_4, thyroxine; DIT, diiodotyrosine; DIHPPA, 4-hydroxy-3,5-diiodophenylpyruvic acid.

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manganese[4] and by substitutions on the amino and carboxyl groups of DIT[5]. HILLMANN[6] in 1958 reported that the incubation of DIT with its deaminated analogue, DIHPPA, for 5 days under anaerobic conditions resulted in the synthesis of T_4 in a yield of 3%. MELTZER AND STANABACK[7] demonstrated an oxygen requirement in this reaction and reported a yield of 10–20% and this has been confirmed by others[8,9]. The results of these studies have led to the demonstration[9,10] that T_4 is synthesized in good yield by the action of L-amino acid oxidase (EC 1.4.3.2) from snake venom on DIT. The initial deamination of DIT and the synthesis of thyroxine by the condensation of DIT and DIHPPA is the proposed mechanism.

The role of peroxidases in the synthesis of T_4 from DIT was studied by KOLLI[11] and LJUNGGREN[12]. KOLLI reported that the formation of T_4 during the incubation of DIT in an alkaline medium was accelerated by either horse-radish peroxidase or lactoperoxidase in the presence of added hydrogen peroxide. On the other hand, LJUNGGREN could not demonstrate the formation of T_4 from DIT when myeloperoxidase was employed under his experimental conditions. An enzyme with the properties of a peroxidase is present in the thyroid gland and has been implicated in the iodination of tyrosine[13–16]. IGO AND MACKLER[17] have stated that thyroxine is synthesized from DIT by cell free preparation of thyroid in the presence of glucose and glucose oxidase.

The present paper is the first of a series dealing with the possible role of the thyroid peroxidase in the synthesis of T_4 from DIT. Thyroxine was identified as a product of the incubation *in vitro* of DIT with a purified myeloperoxidase preparation or with a cell-free preparation of beef thyroid in the presence of glucose and glucose oxidase as the hydrogen peroxide-generating system. The deamination of amino acids by peroxidase has been reported[18]. Therefore the possibility that the synthesis of thyroxine might be a result of the initial deamination of DIT followed by the condensation of DIT and DIHPPA was considered. The release of free ammonia was observed on the incubation of DIT with myeloperoxidase or with a cell-free preparation of beef thyroids in the presence of glucose and glucose oxidase.

MATERIALS AND METHODS

Myeloperoxidase was prepared from dog uterine pus as described by AGNER[19] to the end of Step 6. The stock preparation had absorbancy ratios of 430 mμ/280 mμ = 0.84 and 430 mμ/390 mμ = 1.48 and contained 3.0 mg of myeloperoxidase/ml. The stock solution was diluted 10 times with water before use.

Beef thyroid glands were obtained fresh from the slaughterhouse and were frozen for periods up to 3 months before use. The frozen glands were passed 3 times through a hand-operated meat grinder, and extracted with 1 volume of 0.154 M KCl at 0–5° for 20 min. The extract was passed through cheese-cloth and centrifuged at 105000 × *g* for 1 h in a Spinco Model L preparative ultracentrifuge. Each pellet was resuspended in 3.0 ml of water in a Teflon Potter–Elvehjem homogenizer before use.

The following materials were obtained commercially: glucose oxidase (EC 1.1.3.4), Type IV, 90000 units/g, and ninhydrin spray (NIN-3) from Sigma Chemical Company; L-3,5-diiodotyrosine from Mann Research Lab; [^{131}I]diiodotyrosine from Abbott Laboratories and crystalline catalase (beef-liver catalase (EC 1.11.1.6), 150000 units

per ml) from Worthington Biochemical Corp. Tapazole (1-methyl-2-mercaptoimidazole) was a gift from Eli Lilly and Company.

Synthesis of T_4 from DIT

The reaction mixture which contained the components indicated in the text and in the legends to the figures and tables was incubated at 37° for 3 h in an atmosphere of air. Upon completion of the incubation, 19 N NaOH was added to the reaction mixture to a final concentration of 2 N and the solution was extracted twice with equal volumes of *n*-butanol. Centrifugation was employed to separate the phases. The combined butanol extract was washed once with an equal volume of 2 N NaOH and was evaporated to dryness at room temperature under vacuum. The residue was redissolved in 0.2 ml of 2 N NH_4OH and aliquots were taken for chromatography. Three solvent systems were employed: a, *tert.*-amyl alcohol – ethanol – water (5:1:4, v/v), the upper phase in an atmosphere saturated with ammonia; b, *n*-butanol – ethanol – 2 N NH_4OH (5:1:2, v/v); c, *n*-butanol – acetic acid – water (4:1:1, v/v). Two chromatographic procedures were used:

1. Two-dimensional ascending paper chromatography on Whatman No. 1 filter paper with appropriate standards was employed for the identification of thyroxine. Solvent systems a and b or a and c were used. Radioautograms were prepared and the chromatograms were stained with a ferric chloride–ferricyanide–arsenic acid reagent[20].

2. Descending chromatography on Whatman No. 1 filter paper strips, using solvent system b, was employed to quantitate the yield of T_4. Cold T_4 was added as a standard. The T_4 spot was revealed by staining with ninhydrin. The spot was cut out and counted in a Nuclear-Chicago well scintillation counter and the radioactivity was compared with that of an appropriate standard. The yield was calculated as the amount (mμmoles) of DIT converted to T_4. The spots were pasted back onto the chromatograms with cellophane adhesive tape and radioautograms were prepared to confirm the separation of T_4 from other radioactive components.

A large-scale experiment was conducted as follows. 15 g of L-diiodotyrosine were dissolved in 200 ml of 0.2 N NaOH. To this solution were added 150 ml of water, 0.4 ml of 2 N HCl, 100 ml of 0.2 M phosphate buffer (pH 7.0), 10 ml of 0.01 M glucose, 30 ml of the thyroid particulate suspension and 10 ml of a 1 mg/ml solution of glucose oxidase. The reaction mixture was incubated at 37° with gentle shaking for 3 h. Oxygen was bubbled into the reaction mixture during the incubation period. At the end of the incubation period, the mixture was filtered through glass-wool and the filtrate was acidified to approx. pH 5 by the dropwise addition of 9 N H_2SO_4. The solution was kept at 0–5° overnight. The brownish precipitate was collected by centrifugation and dissolved in 60 ml of 2 N NaOH. The solution was extracted twice with equal volumes of *n*-butanol and the combined extract was washed with 120 ml of 2 N NaOH. The alkaline wash was extracted with 120 ml of *n*-butanol and the combined butanol extracts were evaporated to dryness. The residue was dissolved in 60 ml of water. Acetic acid was added to the solution to a concentration of 5 % by volume and the acidified solution was kept at 0–5° overnight. The acid-insoluble material was collected by centrifugation at 5000 × *g* for 20 min and was washed once with ice-cold 1 % acetic acid. The washed precipitate was dissolved in a minimum volume of boiling 1 % Na_2CO_3. A precipitate was obtained on cooling the solution to 0–5° and acidification with 1 % acetic acid. The precipitate was washed with 1 %

acetic acid and with water and was dissolved in a minimum volume of alkaline ethanol (3 ml of 0.1 N NaOH in 100 ml of 70% ethanol). The solution was centrifuged at 5000 × *g* for 10 min to remove some insoluble material. The clear yellow supernatant solution was acidified with 1% acetic acid to yield the final product as a light yellow precipitate. The product was dried over P_2O_5 *in vacuo*.

A parallel experiment was carried out in the absence of the thyroid particulate suspension. No acid-insoluble material was obtained from the butanol extract under the conditions described above.

Estimation of ammonia

Ammonia was estimated by the colorimetric method of RUSSELL[21] following isothermal distillation in Conway vessels. The hypochlorite solution employed in the colorimetric method was prepared by the dilution of 4.0 ml of a commercial bleaching liquid (Clorox) to 100 ml with boiled deionized water.

Spectrophotometric measurements

Ultraviolet and visible spectral analyses were performed in a Cary M-11 recording spectrophotometer using cylindrical fused quartz cells 1 cm in length. Infrared analyses of KBr pellets of the samples were performed in a Perkin-Elmer Model M-221 infrared spectrophotometer.

RESULTS

The incubation of DIT with either myeloperoxidase or the thyroid particulate suspension in the presence of glucose and glucose oxidase resulted in the formation of a compound which was chromatographically indistinguishable from T_4 in 3 solvent systems. Fig. 1 demonstrates the two-dimensional chromatogram of the butanol extractable products of the reaction mixture. An average of 20 mμmoles, equivalent to 0.2% of the DIT in the medium was converted to T_4 under these conditions. Table I demonstrates the requirement for each component of the reaction mixture (glucose, glucose oxidase, thyroid particulate preparation) for the maximum yield of thyroxine under the conditions employed. Heating the myeloperoxidase or the thyroid particulate suspension at 100° for 3 min resulted in a complete loss of activity (Table I). The pH optimum was between 6.5 and 7.0 in phosphate buffer when myeloperoxidase was employed and was 7.0 when the thyroid particulate suspension was used.

The formation of T_4 was inhibited by ergothioneine, GSH, ascorbic acid, Tapazole, thiouracil, catalase and semicarbazide under the conditions employed in Table II. Bisulfite appeared to have a slight stimulatory effect with myeloperoxidase (+29%) but was inhibitory when the thyroid particulate suspension was used. The synthesis was stimulated by Mn^{2+} when the thyroid preparation was employed (Table I).

A large scale experiment using the thyroid particulate suspension yielded a light-yellow precipitate which was compared to an authentic sample of T_4 as follows: (a) The precipitate was chromatographed on Whatman No. 1 paper in 2 solvent systems (solvent b and c) and stained with ninhydrin or the ferric chloride–ferricyanide–arsenic acid reagent. The chromatographic and staining properties of the precipitate were identical to those of thyroxine. (b) The ultraviolet spectrum of the

product dissolved in 0.005 N NaOH was identical to that of thyroxine. It showed a large band with a maximum absorption at 227 mμ (see ref. 22) and a small band with a maximum at 325 mμ. (c) The product was biologically active. The metamorphosis of tadpoles (*Xenopus laevis*) kept in spring water containing 40 mg %

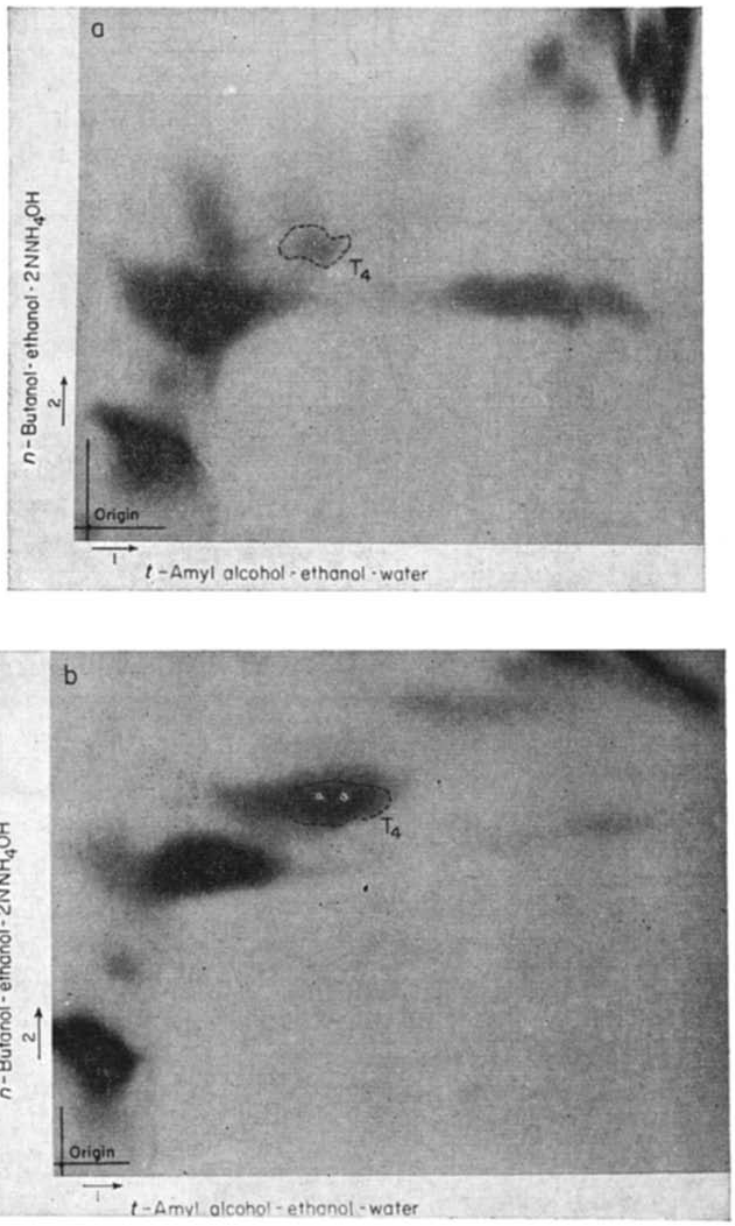


Fig. 1. The synthesis of thyroxine from diiodotyrosine. The reaction mixture contained 200 μmoles of Sørensen's phosphate buffer (pH 7.0), 10 μmoles of DIT, 10–15 μC of [^{131}I]DIT, 1 μmole of glucose, 100 μg of glucose oxidase, and water to a final volume of 5.0 ml. Myeloperoxidase (0.2 ml) was added to (a) and the thyroid particulate suspension (0.5 ml) was added to (b). Two-dimensional paper chromatograms were prepared.

of thiourea was stimulated by the addition of the product to the medium. The rate of metamorphosis was comparable to that produced by T_4 at the same concentration. (d) The infrared spectrum of the precipitate in a solid KBr pellet was identical to that of T_4 (free acid form) at the same concentration (Fig. 2).

Significant amounts of free ammonia were released on the incubation of DIT

TABLE I

REQUIREMENTS FOR THE SYNTHESIS OF THYROXINE FROM DIIODOTYROSINE

The complete system was as described in Fig. 1. Descending chromatography in the butanol–ethanol–NH_4OH solvent was employed. The amount of thyroxine formed was compared to that formed in the complete system which was arbitrarily designated as 100.

Supplements	*Synthesis of thyroxine*	
	Myeloperoxidase	*Thyroid particulate*
Complete system	100	100
Complete system without glucose	0	0
Complete system without glucose oxidase	5	23
Complete system without glucose and glucose oxidase	0	0
Complete system without enzyme	0	0
Complete system with enzyme heated to 100° for 3 min	0	0
Complete system plus Mn^{2+} (0.5 mμmole)	92	178

TABLE II

EFFECTS OF SOME INHIBITORS ON THE SYNTHESIS OF T_4 FROM DIT

The reaction mixture contained all the components described in Fig. 1. The inhibitor was added to the reaction mixture as indicated.

Inhibitor ($4 \cdot 10^{-3}$ M)	*Percent inhibition of T_4 synthesis*	
	Myeloperoxidase	*Thyroid particulate*
Ergothioneine	100	100
Reduced glutathione	100	100
Ascorbic acid	100	100
Semicarbazide	87	72
Bisulfite	—	60
Tapazole	100	100
2-Thiouracil	100	100
Catalase (15000 units)	81	89

TABLE III

RELEASE OF AMMONIA

The reaction mixture contained 5 μmoles of DIT, 1 μmole of glucose, 0.1 mg of glucose oxidase, 0.2 ml of myeloperoxidase or 0.5 ml of the thyroid particulate suspension, and 200 μmoles of either phosphate buffer (pH 6.5) (when myeloperoxidase was employed) or Tris buffer (pH 9.0) (when the thyroid preparation was used) and water in a total volume of 2.5 ml. The flasks were incubated at 37° for 3 h in an atmosphere of air. The myeloperoxidase and the thyroid preparation were found to contain some ammonia. These amounts of ammonia were subtracted from the total except for "Complete system without enzyme".

Supplements	*Amounts of ammonia released (mμmoles)*	
	Myeloperoxidase	*Thyroid particulate*
Complete system	100	164
Complete system without glucose	—	36
Complete system without glucose oxidase	0	14
Complete system without diiodotyrosine	16	0
Complete system without enzyme	31	31

with either myeloperoxidase or the thyroid particulate suspension, glucose and glucose oxidase under the conditions employed in Table III. The amounts of ammonia released in the presence of DIT was 50–200 mμmoles greater than the amounts released in the absence of DIT. Considerable variation was observed from day to day. All the components of the reaction mixture were required for maximum yields (Table III). Heating the myeloperoxidase or the thyroid preparation at 100° for 5 min completely inhibited ammonia formation. The formation of ammonia also was completely inhibited by the addition of catalase (15000 units).

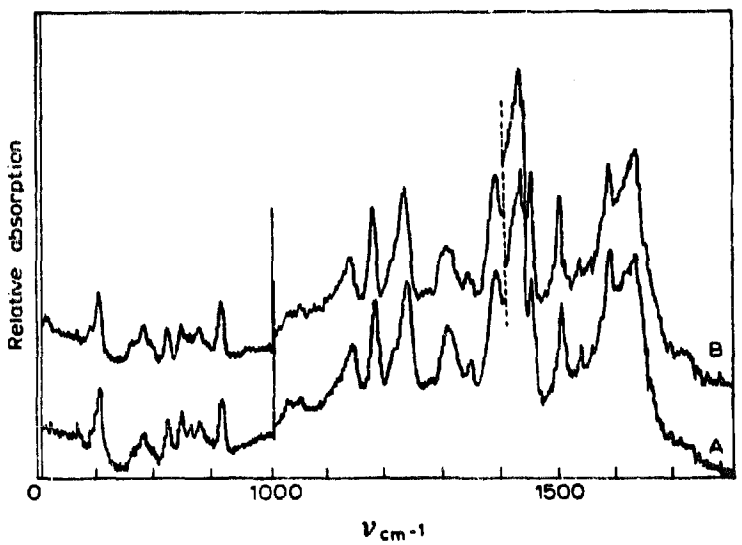


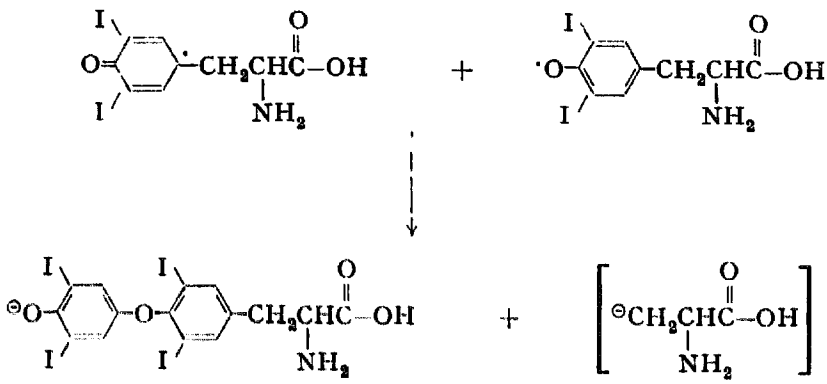
Fig. 2. Infrared spectra of thyroxine (free acid) and the reaction product (free acid) in KBr pellets. A, 0.138 g % of thyroxine (free acid) in KBr pellets; B, 0.138 g % of the reaction product (free acid); see text.

DISCUSSION

It may be concluded from the chromatographic, ultraviolet and infrared analyses that thyroxine is synthesized from diiodotyrosine by a cell-free thyroid particulate preparation in the presence of glucose and glucose oxidase as a hydrogen peroxide-generating system. FMN and Mn^{2+} can replace glucose and glucose oxidase as an hydrogen peroxide-generating system[23]. These findings would support the report by IGO AND MACKLER[17]. The supplements required for the synthesis of thyroxine by the thyroid preparation under the conditions employed here were the same as those required for the synthesis of thyroxine by myeloperoxidase. Catalase was inhibitory in both systems as were a number of other inhibitors. These similarities, plus the earlier observations that a peroxidase is present in the thyroid gland[13,16,24] make it not unreasonable to suggest the involvement of a peroxidase in the synthesis of thyroxine from diiodotyrosine by the thyroid preparation under the experimental conditions employed in this study. It is not possible to conclude from the data presented here that the enzyme involved in the conversion of diiodotyrosine to thyroxine by the thyroid particulate preparation is the same one that catalyzes the iodination of tyrosine. However, if they are not identical they are closely similar enzymes.

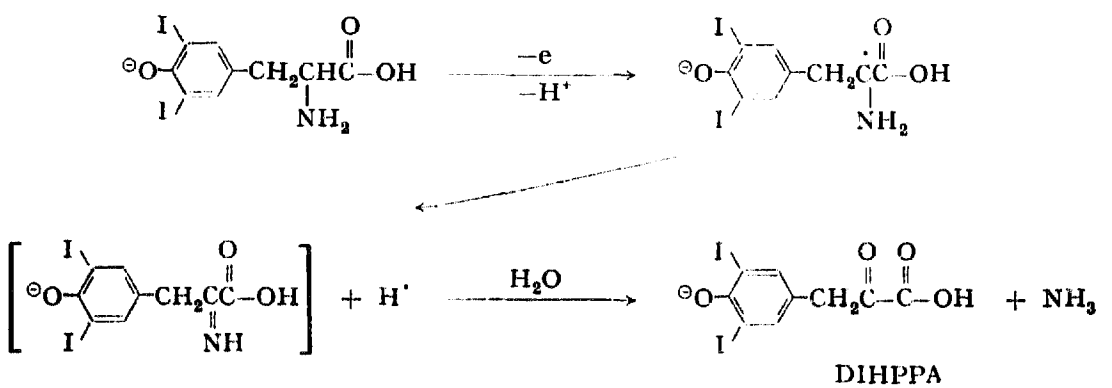
The mechanism of thyroxine synthesis by either myeloperoxidase or the thyroid particulate preparation is unknown. Two possibilities exist. Free radicals have been

identified by electron paramagnetic resonance spectroscopy as products of the oxidation of a number of substances by peroxidase and H_2O_2 (see ref. 25). The loss of an electron from the phenolic hydroxyl group of diiodotyrosine would result in the formation of the phenoxy radical, and the synthesis of thyroxine by the reaction of the phenoxy radical with a second radical in the appropriate resonance state might result in the formation of thyroxine as follows:



If the side chain is eliminated in such a way that ammonia is one of the products, then it might be expected that the amount of ammonia released would be equal to the amount of thyroxine formed.

A second possible mechanism for the formation of thyroxine from diiodotyrosine by peroxidase would involve the loss of an electron from the α-carbon of diiodotyrosine. The radical so formed might undergo further transformation to an imino acid which is deaminated in an aqueous medium to form DIHPPA as follows:



DIHPPA

The mechanism of thyroxine synthesis under these conditions would involve the coupling reaction between DIHPPA and diiodotyrosine. Under these conditions, a stoichiometric relationship between the formation of thyroxine and the release of ammonia would not be expected since the DIHPPA formed can either react with diiodotyrosine to form thyroxine or be decomposed in the aqueous medium[9]. The amount of ammonia released under the conditions reported here was about 10 times the amount of thyroxine formed. This would support the latter mechanism for thyroxine synthesis. However, it should be emphasized that the chemical changes underway in the reaction mixture under the conditions employed here are certainly not limited to either one or the other of the two outlined above, but rather consist of a number of different simultaneous reactions. For example, thyroxine itself is

oxidized by peroxidase and its decomposition includes deiodination and splitting of the ether linkage[26]. The yield of thyroxine reported here therefore may not represent the actual amount of thyroxine formed but rather the steady state concentration resulting from its synthesis and degradation.

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