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Metabolism of 3-iodotyrosine and 3,5-diiodotyrosine by thyroid extracts

Shih-ying Sun

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METABOLISM OF 3-IODOTYROSINE AND 3,5-DIODOTYROSINE

BY THYROID EXTRACTS

BY

SHIH-YING SUN, 1943-

A

THESIS

submitted to the faculty of
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ABSTRACT

A porcine thyroid enzyme was extracted from thyroid tissue by homogenization and differential centrifugation. The enzyme was capable of metabolizing both 3-iodotyrosine and 3,5-diiodotyrosine. All of the thyroid subcellular particles produced a fluorescent compound when incubated with these substrates. When the 1,000 x g sediment was supplemented with the 48,000 x g supernatant a second compound was formed. The compound was UV absorbing and has been identified as either 3-iodo-4-hydroxybenzaldehyde or 3,5-diiodo-4-hydroxybenzaldehyde depending upon which substrate was used.

The 1,000 x g sediment was solubilized with 1% Conoco 1012-6 detergent. The activating factor in the 48,000 x g supernatant was shown to be a macromolecule, probably a protein, by dialysis and ammonium sulfate precipitation. The enzymes in both fractions are required to form 3-iodo-4-hydroxybenzaldehyde.

Conversion of the reaction products to their dinitrophenylhydrazones followed by paper chromatography indicated that the benzaldehydes were formed by decomposition of the corresponding p-hydroxyphenylpyruvic acid. This rules out the enzymatic formation of the benzaldehydes. This study lends support to model system studies which have suggested that 3,5 diiodo-4-hydroxyphenylpyruvic acid should be an intermediate in the formation of thyroxine.
ACKNOWLEDGEMENTS

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The loving help and encouragement of her parents made her graduate study possible and it is to them that this thesis is affectionately dedicated.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iii</td>
</tr>
<tr>
<td>LIST OF ILLUSTRATIONS</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>ix</td>
</tr>
<tr>
<td>I. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>II. REVIEW OF LITERATURE</td>
<td>2</td>
</tr>
<tr>
<td>A. Morphology and Phylogeny of the Thyroid</td>
<td>2</td>
</tr>
<tr>
<td>B. Iodine Containing Compounds of the Thyroid</td>
<td>3</td>
</tr>
<tr>
<td>1. Thyroxine</td>
<td>3</td>
</tr>
<tr>
<td>2. 3, 5, 3'-Triiodothyronine</td>
<td>4</td>
</tr>
<tr>
<td>3. Other Iodinated Compounds</td>
<td>5</td>
</tr>
<tr>
<td>C. Hormonal Biochemistry of the Thyroid Gland</td>
<td>6</td>
</tr>
<tr>
<td>1. Iodoproteins of the Thyroid</td>
<td>6</td>
</tr>
<tr>
<td>2. Thyroxine Biosynthesis</td>
<td>7</td>
</tr>
<tr>
<td>a. Iodination of Tyrosine</td>
<td>7</td>
</tr>
<tr>
<td>i. Thyroid Peroxidase</td>
<td>7</td>
</tr>
<tr>
<td>ii. Tyrosine Iodinase</td>
<td>9</td>
</tr>
<tr>
<td>b. Thyroxine Synthesis</td>
<td>10</td>
</tr>
<tr>
<td>i. Studies with Model Systems</td>
<td>10</td>
</tr>
<tr>
<td>ii. In Vivo Studies</td>
<td>15</td>
</tr>
<tr>
<td>3. Chemistry of the Iodoamino Acids</td>
<td>16</td>
</tr>
<tr>
<td>III. EXPERIMENTAL PROCEDURE</td>
<td>19</td>
</tr>
<tr>
<td>A. Materials</td>
<td>Page</td>
</tr>
<tr>
<td>---------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>1. Reagents</td>
<td>19</td>
</tr>
<tr>
<td>2. Thyroid Tissue</td>
<td>19</td>
</tr>
<tr>
<td>3. Homogenization Medium</td>
<td>20</td>
</tr>
<tr>
<td>B. Methods</td>
<td>20</td>
</tr>
<tr>
<td>1. Tissue Preparations</td>
<td>20</td>
</tr>
<tr>
<td>2. Enzyme Assay</td>
<td>20</td>
</tr>
<tr>
<td>3. Chromatographic Procedures</td>
<td>22</td>
</tr>
<tr>
<td>4. Analysis of Chromatograms</td>
<td>22</td>
</tr>
<tr>
<td>a. UV Absorption</td>
<td>22</td>
</tr>
<tr>
<td>b. Amino Acid Localization</td>
<td>23</td>
</tr>
<tr>
<td>c. Localization of Iodide</td>
<td>23</td>
</tr>
<tr>
<td>5. Spectral Measurement</td>
<td>23</td>
</tr>
<tr>
<td>6. Preparation of Chelate Complexes</td>
<td>24</td>
</tr>
<tr>
<td>a. Pyridoxal-Diiodotyrosine-Aluminum Chelate</td>
<td>24</td>
</tr>
<tr>
<td>b. Pyridoxal-Monoiodotyrosine-Aluminum Chelate</td>
<td>24</td>
</tr>
<tr>
<td>7. Preparation of Keto Acid 2,4-dinitrophenyl hydrazones</td>
<td>24</td>
</tr>
<tr>
<td>8. Preparation of 3,5-diiodo-4-hydroxybenzaldehyde</td>
<td>25</td>
</tr>
<tr>
<td>9. Enzyme Extraction and Isolation</td>
<td>25</td>
</tr>
<tr>
<td>a. Solubilization of Fraction 1</td>
<td>25</td>
</tr>
<tr>
<td>b. Treatment of Fraction 6</td>
<td>26</td>
</tr>
<tr>
<td>C. Results</td>
<td>26</td>
</tr>
</tbody>
</table>
## LIST OF ILLUSTRATIONS

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Fractionation Procedure for Thyroid Tissue</td>
<td>21</td>
</tr>
<tr>
<td>2. Chromatography of Products Formed During the Incubation of 3,5-diiodotyrosine with Thyroid Fractions</td>
<td>29</td>
</tr>
<tr>
<td>3. Chromatography of Products Formed During the Incubation of 3,5-diiodotyrosine with Thyroid Fractions</td>
<td>30</td>
</tr>
<tr>
<td>4. Chromatography of Products Formed During the Incubation of 3,5-diiodotyrosine with Thyroid Fractions</td>
<td>31</td>
</tr>
<tr>
<td>5. Chromatography of Products Formed During the Incubation of 3-iodotyrosine with Thyroid Fractions</td>
<td>32</td>
</tr>
<tr>
<td>6. Chromatography of Products Formed During the Incubation of 3,5-diiodotyrosine with Thyroid Fractions</td>
<td>34</td>
</tr>
<tr>
<td>7. Chromatography of Products Formed During the Incubation of 3-iodotyrosine with Thyroid Fractions</td>
<td>35</td>
</tr>
<tr>
<td>8. UV Absorption Spectra of the Compounds DIT-F-1, and DIT-F-2 in 30% Alcoholic NH₄OH</td>
<td>37</td>
</tr>
<tr>
<td>10. UV Absorption Spectrum of the Compound MIT-A-3 in 30% Alcoholic NH₄OH</td>
<td>39</td>
</tr>
<tr>
<td>11. UV Absorption Spectrum of the Compound MIT-F-2 in 30% Alcoholic NH₄OH</td>
<td>40</td>
</tr>
<tr>
<td>12. UV Absorption Spectra of the Standard Thyroxine, 3,5-Diiodotyrosine, and 3-Iodotyrosine in 30% Alcoholic NH₄OH</td>
<td>41</td>
</tr>
<tr>
<td>13. Chromatography of DIT-Chelate, 4-Hydroxyphenylpyruvic Acid, and Thyroxine</td>
<td>42</td>
</tr>
<tr>
<td>14. UV Absorption Spectra of MIT-Pyridoxal-Aluminum Chelate and DIT-Pyridoxal-Aluminum Chelate in 0.1M Acetate Buffer</td>
<td>43</td>
</tr>
<tr>
<td>15. UV Absorption Spectrum of 4-Hydroxyphenylpyruvic Acid in</td>
<td></td>
</tr>
<tr>
<td>Figure</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
</tr>
<tr>
<td>30% Alcoholic NH₄OH</td>
<td>45</td>
</tr>
<tr>
<td>16. Chromatography of Recrystallized 3,5-diido-4-hydroxybenzaldehyde, Unrecrystallized 3,5-diido-4-hydroxybenzaldehyde, and p-hydroxybenzaldehyde in Tertiary Amyl Alcohol Saturated with 2N NH₄OH</td>
<td>46</td>
</tr>
<tr>
<td>17. UV Absorption Spectra of p-hydroxybenzaldehyde, 3,5-diido-4-hydroxybenzaldehyde, and 3-iodo-4-hydroxybenzaldehyde in 30% Alcoholic NH₄OH</td>
<td>47</td>
</tr>
<tr>
<td>18. Chromatography of the Products of the Assay Mixture Stopped by Addition of 2,4-dinitrophenylhydrazine in Different Solvents</td>
<td>48</td>
</tr>
<tr>
<td>19. Quantitative Relationship Between Solubilized Enzyme and Production of MIT-A-3</td>
<td>51</td>
</tr>
</tbody>
</table>
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. The ( R_f ) Values of 3-iodotyrosine, 3, 5-diiodotyrosine, and Thyroxine in Various Solvents</td>
<td>27</td>
</tr>
<tr>
<td>II. Ammonium Sulfate Fractionation of Fraction Six</td>
<td>50</td>
</tr>
</tbody>
</table>
I. INTRODUCTION

Thyroxine is a hormone secreted by the thyroid gland. During the past twenty-five years, there has been a vast amount of research work on this hormone, but many aspects of its mechanism of action and synthesis are still unexplained. It is generally accepted that the biosynthesis of thyroxine depends first upon the oxidation of iodide ion probably to iodinium ion. The oxidation of iodide is catalyzed by peroxidase enzymes. Iodinium ion is utilized to form 3-iodotyrosine which is further iodinated to form 3,5-diiodotyrosine. The final step in formation of thyroxine is postulated as the coupling of two 3,5-diiodotyrosine molecules with the loss of one alanine side chain.

This study was undertaken to isolate the thyroid coupling enzyme and to study the enzymatic formation of thyroxine from 3,5-diiodotyrosine.
II. REVIEW OF LITERATURE

A. MORPHOLOGY AND PHYLOGENY OF THE THYROID

The thyroid is an endocrine gland which consists of two lobes, one on each side of the trachea with a connecting portion, a thin isthmus, making the entire gland more or less H-shaped in appearance (Harper, 1965). The thyroid consists of many follicles or spheres of single layered epithelial cells surrounding a protein portion called the colloid. Numerous follicles interspersed with blood vessels and connective tissue and covered by an outer membrane forms the thyroid gland. The size of the thyroid follicles varies with the species and within each species there is variation depending on the functional state of the gland. The follicles are smaller when the gland is more active.

The precise location and shape of the thyroid gland varies considerably from species to species. All higher animals have an encapsulated gland. Reptiles, birds, and mammals generally have a single bilobed gland, while amphibians have two widely separated glands (Gorbman, 1955). A few primitive forms of animals have only follicular structures with thyroid-like activity. Such structures first occur in cyclostomes where the thyroid follicles are embedded in the dense fibrous tissue in the pharyngeal floor (Gorbman, 1955).

The sea lamprey is an exception. It lacks a thyroid in the larval stage and follicular structures appear in the adult after metamorphosis. The endostyle is an exocrine gland, located in the larval lamprey's pharynx, in which iodine is concentrated. Iodoproteins are synthesized in the endostyle and secreted through a duct into the digestive tract. During metamorphosis
a small portion of the endostyle becomes true follicular cells (Goldsmith, 1949).

The existence of thyroid hormones as thyroxine or triiodothyronine is not limited to vertebrates even though lower animal forms do not have true thyroid glands. In at least three of the phyla (Annelida, Mollusca, Arthropoda) there are iodoproteins in or very near the mouth or pharyngeal cavity. If this first available thyroxine became involved in the metabolic activities of a prochordate group of animals, it would appear that the evolution of thyroxine preceded the evolution of the thyroid gland (Gorbman, 1955).

B. IODINE CONTAINING COMPOUNDS OF THE THYROID

1. Thyroxine

In 1915, Kendall isolated thyroxine crystals from dessicated thyroid glands (1915). The initial hydrolysis was performed using alcoholic sodium hydroxide. After a number of years, he succeeded in crystallizing a small amount of an active compound, which was named "thyroxin". Four years later, Kendall improved the method by substituting fresh thyroid glands for dessicated thyroids and by hydrolyzing the proteins in aqueous sodium hydroxide instead of alcoholic sodium hydroxide (1919).

The crystalline thyroxine contained approximately 65% iodine and had an apparent empirical formula of $C_{11}H_{10}O_3Ni_3$. Kendall and Osterbery (1919) examined acidic and basic properties of thyroxine and erroneously identified it as an indole. They proposed that thyroxine was trihydrotriiodoxindole propionic acid, and had the structural formula:

\[
\begin{array}{c}
\text{I} \\
\text{I} \\
\text{I} \\
\text{CH}_2\text{CH}_2\text{COOH}
\end{array}
\]
Harrington (1926a) used 40% barium hydroxide for hydrolysis followed by acid precipitation and obtained a white crystalline material. It had the empirical formula $C_{15}H_{11}O_{4}NI_{4}$. Later, Harrington (1926b) showed that desiodothyroxine was a diphenyl ether with the structure:

$$\text{HO--O--CH}_{2}\text{CHNH}_{2}\text{COOH}$$

Harrington (1926b) named this compound thyronine.

Harrington and Barger (1927) suggested that coupling of two molecules of diiodotyroxine occurred to form thyroxine. The following structural formula for thyroxine was proposed.

$$\text{HO--O--CH}_{2}\text{CHNH}_{2}\text{COOH}$$

This structural formula was proven to be correct by degradation and by synthesis (Harrington & Barger, 1927).

2. 3,5,3'-triiodothyronine

Leblond, Gross, and their coworkers (Gross et al., 1950; Gross & Leblond, 1951), using extracts of thyroid glands which had been injected with radioactive iodide, found three radioactive spots on two dimensional chromatograms which did not correspond to known standards. One of these very closely resembled thyroxine and was particularly interesting because its presence was observed in blood along with thyroxine and iodide.

From further work, including measuring the $R_f$ value (Roche et al., 1952),
crystallographic examination (Kennard, 1953), and melting points, the un­
known compound was proven to be 3, 5, 3'-triiodothyronine (Gross and Pitt­
Rivers, 1953).

3, 5, 3'-triiodothyronine has physiological activity equal to or greater
than thyroxine. It is considered the minor hormone of the thyroid because it
amounts to approximately 3% of the total hormone produced in most animals.

3. Other Iodinated Compounds

3-iodotyrosine and 3, 5-diiodotyrosine are formed by iodination of
tyrosine. These two were the first thyroid metabolites recognized by paper
chromatography. 3-iodotyrosine and 3, 5-diiodotyrosine are thought to be the
building blocks of thyroxine. They occur in significant amounts in the gland,
although only traces are found before hydrolysis of thyroid extracts. This
implies that the great majority of organic iodine is protein bound as mono­
iodotyrosine, diiodotyrosine, or thyroxine residues within the peptide chain
of thyroglobulin.

A number of other iodinated compounds have been found in thyroid tissue.
3,3'-diiodothyronine, and 3,3',5'-triiodothyronine were detected by chroma­
tography of rat and pig thyroid extracts (Roche et al, 1956). A number of
iodinated peptide derivatives have also been recognized (Gmelin and Virtanen,
1959). The amounts of these compounds and other minor iodocompounds were
found to be very small. Their physiological activity is not known.

The minor iodinated compounds may be found in the amino acid chain
of the protein, thyroglobulin. In any event, the hormones, thyroxine and
triiodothyronine, as well as diiodotyrosine and monoiodotyrosine are stored
within the colloid of the thyroid. The hormones are released and made available to the body only after a protease breaks down thyroglobulin.

C. HORMONAL BIOCHEMISTRY OF THE THYROID GLAND

1. Iodoproteins of the Thyroid

Iodination of tyrosine residues in thyroglobulin is one of the essential steps in the formation of thyroxine in the thyroid gland.

Thyroglobulin, the major protein constituent of the thyroid gland, has a molecular weight of 670,000 and a sedimentation coefficient of 19S. Proteins with sedimentation coefficients of 27S, 17S, and 12S have also been extracted from the thyroid. It is now agreed that these entities are derived from the 19S by various means. DeCrombrugghe et al (1965) have shown that the 12S protein can be further disassociated into 2.8S and 6S subunits by limited reduction with mercaptoethanol. The 6S subunit is composed of two 2.8S components and the 12S subunit of thyroglobulin is made up of two 6S subunits (Seed and Goldberg, 1963). These two components appear to be in weak association and probably two disulfide bonds stabilize the 12S protein.

The 17S component of thyroid extracts contains two 12S subunits and it is not iodinated. The 19S thyroglobulin is iodinated. Because of this difference, it suggests that the 17S component is probably the immediate precursor of the 19S thyroglobulin (Mauchamp et al, 1965). The 19S thyroglobulin is probably produced by conformational modifications during iodination of the 17S protein (Nunez et al, 1966).

The 27S iodoprotein of the thyroid gland has a molecular weight of about 1.2x10^6. It appears to be a polymer of thyroglobulin subunits. It has
the same amino acid composition as 19S thyroglobulin. The only significant difference was that the 27S component has a higher iodine content than thyroglobulin (Salvatore et al., 1965). This component has been shown to disassociate into 19S subunits. Vecchio has postulated that 27S is a dimer of 19S or it is made up of equimolar amounts of 19S, 12S, and 6S (1965).

2. Thyroxine Biodynamics

a. Iodination of Tyrosine

i. Thyroid Peroxidase

The iodination of tyrosine to form monoiodotyrosine, diiodotyrosine, and ultimately thyroxine occurs only when iodide is oxidized by the thyroid gland. It is now generally accepted that thyroidal iodide oxidation is catalyzed by a peroxidase.

In 1943, Schachner and his coworkers made the first suggestion that a peroxidase might be involved in the iodination reaction (1943). Modern attempts to identify the various reactions involved in iodination in the thyroid began in 1953 with the work of Weiss, who showed that cell-free homogenates of thyroid tissue fortified with tyrosine and cupric ion synthesized iodinated tyrosine (1953). Subsequently, Fawcett and Kirkwood (1953) reinvestigated Weiss's claim and found that under these circumstances free monoiodotyrosine was the major or only compound formed. They suggested that the role of copper was to oxidize iodide to iodine which was then enzymatically used to iodinate tyrosine. Furthermore, the required enzyme "tyrosine iodinase" was found in the soluble fraction of thyroids (Fawcett and Kirkwood, 1953). Taurog et al. found microsomes and mitochondria isolated from thyroid gland were active in converting
iodide into iodotyrosine, but in this system the monoiodotyrosine was in protein linkage and no free monoiodotyrosine could be detected (1955).

In 1961, Cunningham and Kirkwood (1961) found that hydrogen peroxide could replace the requirement for cupric ion. Serif and Kirkwood (1959), and Alexander (1959) proved the requirement for hydrogen peroxide in the formation of monoiodotyrosine.

There have been numerous attempts to isolate and purify the peroxidase-iodinase enzyme system. In one study (Alexander, 1962) it was observed that iodide peroxidase could be cleaved into an apoenzyme and a prosthetic group. The prosthetic group was identified as ferriprotoporphyrin IX. Another study (DeGroot and Davis, 1960) showed that after extensive purification of thyroid iodide peroxidase, no porphyrin component could be demonstrated, nor was any cytochrome oxidase activity found. Solubilization of the iodide peroxidase was accomplished using deoxycholate and trypsin (Igo and Mackler, 1961). The enzyme purified by DeGroot and Davis (1962) would iodinate tyrosine, monoiodotyrosine or proteins.

Earlier ideas that in vitro iodination resulted only in monoiodotyrosine formation (Tong et al, 1957) now seem incorrect since the solubilized mitochondrial system with $\text{H}_2\text{O}_2$ forms diiodotyrosine quite readily (DeGroot and Davis, 1962). DeGroot and his coworkers (1965) also found that there were no iron or flavin cofactors involved in the iodination reaction.

Igo et al (1964) reported a purification procedure and properties of a highly active particulate enzyme system isolated from beef thyroid tissue which catalyzed the peroxidation of iodide to iodine. The same year, Maloof
and Soodak (1964) reported the purification of a thyroid peroxidase which performs the oxidation of thiocyanate as well as the iodination of tyrosyl groups.

Recently, the characteristics of a hemoprotein peroxidase were determined by Coval and Taurog (1967). Their report describes the purification of a peroxidase from pig thyroid particles after trypsin digestion, ammonium sulfate fractionation, column chromatography on DEAE-cellulose and gel filtration on Bio-gel P-100. The purified preparation contained a hemoprotein with a molecular weight of 63,000. Hosoya and Morrison (1967) supported the hemoprotein nature of thyroid peroxidase. A highly active peroxidase was isolated from thyroid microsomes and purified by gel filtration and diethyl-aminoethyl-cellulose chromatography. Its spectral properties showed that the enzyme was a typical hemoprotein peroxidase.

ii. Tyrosine Iodinase

Fawcett and Kirkwood (1954) gave the first indication that iodination might be enzymatically controlled. They found that if a variety of substrates with similar chemical reactivities were subjected to enzymatic iodination some which were structurally and electronically very similar to tyrosine were not iodinated. In 1955, Wyngarden and Stanbury (1955) reported that the oxidation of iodide was enzymatically controlled, but the iodination reaction was non-enzymatic. Alexander and DeGroot (Alexander, 1961; DeGroot and Davis, 1962) later reported that the thyroid peroxidase did not iodinate thyronine. This suggests that iodination of tyrosine is enzymatically controlled. Klebanoff et al (1962) postulated that thyroid peroxidase was involved
in the iodination reaction and that tyrosine was also oxidized by the peroxidase.

Based on the assumption that separation of the peroxidatic activity and iodinase activity is possible, a number of workers have attempted to separate the two enzymes. DeGroot and Davis (1962) found that isolation of peroxidase from sheep thyroid tissue did not parallel iodinase activity. Yip (1964; 1965; 1966) reported separating a peroxidase and an iodinase by using column chromatography.

Other groups have shown that it is not necessary to postulate two enzymes. Ljunggren (1966) and Taurog and Howells (1966) reported that monoiodotyrosine and diiodotyrosine can be formed by highly purified non-thyroid peroxidases without any other enzymes. Nicholson (1967) showed that there were at least two different peroxidases in the thyroid gland, one of which has an iodinase function. This thyroid peroxidase-iodinase catalyzed the oxidation of iodide and utilized the oxidized iodide in the iodination of tyrosine.

b. **Thyroxine Synthesis**

   i. **Studies with Model Systems**

      Harrington and Barger (1927) suggested, in 1927, that the synthesis of thyroxine involved coupling two molecules of 3,5-diiodotyrosine. In 1939, Ludwig and von Mutzenbecher (1939) discovered that iodinated casein yielded, after hydrolysis, small amounts of thyroxine. The same year, von Mutzenbecher (1939) found that the incubation of 3,5-diiodotyrosine at pH 8.4-8.8 also produced thyroxine in about 0.1% yield. A few years later, Johnson and Tewkesbury (1942) improved the method of von Mutzenbecher by bubbling oxygen through the reaction mixture. They postulated that the
coupling reaction in the presence of oxygen might involve free radicals. Such a mechanism is shown below.

\[
\begin{align*}
\text{I} & \quad \text{e}^- \\
\text{O} & \quad \text{I} \\
\text{I} & \quad \text{R}_1
\end{align*}
\]

Matsuura and coworkers have made extensive studies of model reactions which might be analogous to the \textit{in vivo} formation of thyroxine. Matsuura and Cahnmann (1960) reported that the synthesis of various hindered quinol ethers from desamino analogs of tyrosine and of halogenated tyrosines proceed by a free radical mechanism. Some of these quinol ethers were converted by pyrolysis or acid catalysis to the corresponding analogs of thyroxine. Subsequently, Matsuura and Nishinaga (1962) reported that derivative of tyrosine and of dibromotyrosine reacted with the free radical 2,4,6-tributyl-phenoxyl to form quinol ethers that could be converted to the corresponding di-t-butyl analogs of thyroxine. This sequence of free radical reactions represents a nonenzymatic model for the conversion of diiodotyrosine to thyroxine.
Meltzer and Stanaback (1961) showed that diiodotyrosine reacted with 3,5-diido-4-hydroxyphenylpyruvic acid (DIHPPA) at neutral or slightly alkaline pH in the presence of oxygen to give thyroxine in over 20% yield. Hillmann (1956) had previously suggested that oxidation of diiodotyrosine to DIHPPA might be the first step in the conversion of diiodotyrosine to thyroxine. His work also implied that the conversion of diiodotyrosine to DIHPPA occurred by oxidation, but that the subsequent coupling did not entail oxidation. The formation of thyroxine in good yield by the coupling of DIHPPA with diiodotyrosine has been confirmed by Shiba and Cahnmann. They synthesized various forms of radioactive L-thyroxine, carrying the label $^{131}$I or $^{14}$C either in the phenolic ring or in the non-phenolic ring and in the side chain. They proved that the phenolic ring of the thyroxine formed is derived from DIHPPA, and the non-phenolic ring and its alanine side chain from 3,5-diiodotyrosine (Shiba and Cahnmann, 1962b). Later they found that rattlesnake venom in the presence of oxygen and catalase can convert diiodotyrosine to thyroxine with the intermediate formation of DIHPPA (Shiba and Cahnmann, 1962a).

\[
\begin{align*}
\text{HO-CH}_2\text{C(O)}\text{COOH} & \quad + \quad \text{HO-CH}_2\text{CNH}_2\text{COOH} \\
\text{(DIHPPA)} & \quad \rightarrow \quad \text{HO-CH}_2\text{CNH}_2\text{COOH}
\end{align*}
\]

(DIT)

\[
\begin{align*}
\text{(T}_4\text{)}
\end{align*}
\]
These findings suggest that DlHPPA may be an obligate intermediate in the formation of thyroxine in vivo (Shiba and Cahnmann, 1962a).

Matsuura et al (1964) reported the investigation of the participation of free radicals in the synthesis of thyroxine from DlHPPA and 3, 5-diiodotyrosine. Free radicals generated in the presence of oxygen from aqueous solution of DlHPPA have been studied by electron spin resonance spectrometry. One of the observed radicals has been identified as the phenoxy radical of DlHPPA which was suggested to be an intermediate in the synthesis of thyroxine from DlHPPA and diiodotyrosine in the presence of oxygen. One of the phenoxy radical forms of DlHPPA is shown:

Later Matsuura and Nishinaga (1964) reported the structural requirements for analogs of diiodotyrosine in the reaction with DlHPPA to form analogs of thyroxine. It was concluded that the formation of the corresponding analogs of thyroxine is favored by the following structural features of the analogs of diiodotyrosine: (1) substitution of both ortho positions to the phenolic hydroxyl with halogen, (2) an aliphatic side chain of the type C-C-COOH in the para or meta position to the phenolic hydroxyl, (3) the presence of an amino group, a hydroxy group or a double bond in the α-position to the carboxyl group of the side chain. 3, 5-diido-4-hydroxybenzaldehyde (DIHB) was always contained in products in addition to the analogs of thyroxine formed.

Toi et al (1965) demonstrated that coupling with DlHPPA was feasible...
when the 3,5-diiodotyrosine molecules were bound to thyroglogulin. Thyroxine
was formed in 9% under these conditions, but it was also found that 3,3',5'-
triiodothyronine was formed in approximately the same yield when DIHPPA was
coupled to protein bound 3-iodotyrosine. 3,3',5'-triiodothyronine is not a
naturally occurring iodocompound.

Matsuura et al (1968a) reported that in the pH range of 7.2-7.6 the
oxidative coupling of DIHPPA and diiodotyrosine proceeds with great ease,
even near 0°C. The reaction takes place in two distinct phases, an aerobic
and an anaerobic one. In the first phase the keto acid, in its enol form, is
oxidized to a thyroxine precursor which then reacts with diiodotyrosine to
form thyroxine. For this second phase oxygen is not required. In the solid
state, the thyroxine precursor is extremely unstable. It is a hydroperoxide
which differs from DIHPPA by having a hydroperoxy group, instead of a hy-
drogen attached to the carbon atom which is adjacent to the aromatic ring. A
mechanism for the formation of the hydroperoxide is shown in the following
chain reactions (1968a).

1. \[ \text{O} \quad \text{I} \quad \text{I} \quad \text{CH}_2\text{CCOO}^- + \text{O}_2 \rightarrow \text{O} \quad \text{I} \quad \text{I} \quad \text{C} \equiv \text{C}^- \text{COO}^- + \text{O}_2^- \]

2. \[ \text{I} \quad \text{I} \quad \text{H} \quad \text{C} \equiv \text{C}^- \text{COO}^- + \text{O}_2^- + \text{H}^+ \rightarrow \text{O} \quad \text{I} \quad \text{I} \quad \text{C} \equiv \text{C}^- \text{COO}^- \]
Recently, Matsurra et al. (1968a) reported that the radical formed in the autoxidation of DIHPPA, which had been suspected to be an intermediate in the synthesis of thyroxine from DIHPPA and 3,5-diiodotyrosine, was identified as 2,6-diiodobenzosemiquinone. This semiquinone did not react with 3,5-diiodotyrosine to form thyroxine.

In 1966, Taurog and Howells (1966) reported the formation of thyroxine during the chloroperoxidase-catalyzed iodination of thyroglobulin. This suggests that a peroxidase may also be involved in the coupling reaction in the thyroid.

**ii. In vivo Studies**

A great deal of work has been done in vivo on the relative rates of labeling (by $^{131}$I) of 3-iodotyrosine, 3,5-diiodotyrosine, thyroxine, and 3,5,3'-triiodothyronine. Pitt-Rivers and Tata (1959) found a precursor-
product relationship between 3,5-diiodotyrosine and thyroxine in rats and rabbits. Feuer (1959) suggested that 3,5,3'-triiodothyronine was the precursor of thyroxine from the relative radiochemical specific activities of 3,5,3'-triiodothyronine and thyroxine. Later Leloup and Lechiver (1955) found that the amount of 3,5,3'-triiodothyronine synthesized depends upon the ratio of 3-iodotyrosine to 3,5-diiodotyrosine. They also showed 3,5,3'-triiodothyronine is not a precursor of thyroxine by tests of iodide deficient animals.

Fischer _et al_ (1965) suggested that the coupling of two 3,5-diiodotyrosine molecules may be enzymatically controlled. They found that the bovine thyroid microsomal fraction contains an enzyme system which catalyzes the synthesis of thyroxine from 3,5-diiodotyrosine. Manganese is required and pyridoxal or pyridoxal phosphate functions as a stimulating material. Pyridoxal phosphate might facilitate the coupling of iodothyrosine molecules and/or the removal of the side chain from the iodothyrosine molecule. Another possibility is that pyridoxal exerts its influence by modifying the substrate.

A rapid spectrophotometric method for the simultaneous determination of tyrosine, monoiodotyrosine, 3,5-diiodotyrosine, and thyroxine in native or iodinated thyroglobulin has been reported by Edelhoch (1962). Edelhoch and Perlman (1969) reported that the increase in the thyroxine to 3,5,3'-triiodothyronine ratio in thyroglobulin with increasing iodine content can be explained by the rapid increase in the 3,5-diiodotyrosine to 3-iodotyrosine ratio.

A composite scheme for thyroxine synthesis is shown on the next page, (page 17).

3. **Chemistry of the Iodoamino Acids**
active serum transport thyroecithin

\[
\text{iodide thyrolecithin} \quad \text{"oxidized iodide"}
\]

\[
\text{thyroid peroxidase + } H_2O_2 \rightarrow \text{thyroidal iodide}
\]

\[
\text{thyroid peroxidase + } H_2O_2 \rightarrow \text{"oxidized iodide"}
\]

\[
\text{"oxidized iodide"} \rightarrow \text{tyrosine iodinase + } H_2O_2
\]

\[
\text{coupling enzyme } H_2O_2 \text{ pyridoxal Mn}
\]

\[
\text{tyrosine iodinase + } H_2O_2
\]

\[
\text{nonenzymatic iodination}
\]

\[
\text{"oxidized iodide"}
\]

\[
\text{HO} \quad \text{CH}_2\text{CHCOOH}
\]

\[
\text{HO} \quad \text{CH}_2\text{CHCOOH}
\]
Analytical techniques for separation and identification of iodoamino acids are extensive.

For separation, paper chromatography is a common and useful method. A variety of solvents can be used. Thin layer chromatography on cellulose powder, silica gel, or mixtures of these supports is also an effective method for separation. Alexander and Scheiz (1968) recently reported that by using the silylating agent N, O-bis (trimethylsilyl) acetamide volatile derivative of the iodoamino acid can be formed. The silylated amino acids yield single peaks when analyzed by gas chromatography. This method is fast and sensitive.

The methods for localization of iodocompounds on chromatograms depends on the localization of the iodine portion of the molecule or the aromatic ring. The iodide catalyzed reaction between arsenious acid and ceric sulfate in sulfuric acid solution is one available method which can be used on paper chromatograms (Bowden et al, 1955). Such a technique yields a white spot on a yellow background due to the unreduced ceric sulfate. This method is sensitive, but lacks permanence. Ultraviolet absorption is a useful method for localization and quantitation of iodoamino acids.
III. EXPERIMENTAL PROCEDURE

A. MATERIALS

1. Reagents

D-glucose (anhydrous), glucose oxidase, pyridoxal-5-phosphate, pyridoxal HCl, and p-hydroxyphenylpyruvic acid were obtained from Sigma Chemical Company, St. Louis, Missouri.

Sucrose was obtained from Mallinckrodt Chemical Works, St. Louis, Missouri.

Triketohydrindene hydrate (ninhydrin) was obtained from Fisher Scientific Company, Fair Lawn, New Jersey.

Tyrosine, thyroxine, 3-iodotyrosine, and 3,5-diiodotyrosine were obtained from Mann Research Laboratories, Division of Becton, Dickinson & Co., N.Y., N.Y.

Whatman No. 1 and No. 3mm paper for chromatography was obtained from Arthur H. Thomas Company, Philadelphia, Pa.

All chemicals were used without further purification.

All inorganic salts and solvents were reagent grade and were used without further purification.

All reagents were prepared in deionized water which was obtained by filtering tap water through a Barnstead Model BD-5 standard resin demineralizer.

2. Thyroid Tissue

Porcine thyroid glands were obtained from St. Louis Independent Packing Co., and stored at -20°C. Enzymatic activity was stable for many months in thyroid glands frozen at this temperature.
3. **Homogenization Medium**

The homogenization medium for isolation of subcellular particles was 0.25M sucrose containing 1.0mM ethylenediaminetetraacetic acid, disodium salt.

B. **METHODS**

1. **Tissue Preparations**

Thyroid glands thawed at room temperature were ground in an electric meat grinder with 3/16 inch holes. The minced tissue (100g) was homogenized in 400mL of the homogenization medium for one minute on high speed in a Waring Blender. The homogenate was subjected to differential centrifugation at the following forces and times: 1,000 x g for 10 minutes, 5,000 x g for 10 minutes, 15,000 x g for 60 minutes, 27,000 x g for 60 minutes, and 48,000 x g for 60 minutes. The sediment from each centrifugation and the 48,000 x g supernatant were retained for this study. Each fraction was stored at -20°C until used. The fractionation is summarized in Figure 1, page 21.

In the following discussion, the arabic numerals indicated in Figure 1 will be used to represent the fractional residues and the supernatant.

2. **Enzyme Assay**

The enzyme assay used in this work was a modification of the procedure developed by Fischer et al (1965). The incubation media consisted of 10 millimoles 3-iodotyrosine or 3,5-diiodotyrosine, 3.33 millimoles MnCl₂, 10 millimoles D-glucose, 83 millimoles sucrose, 16.7 millimoles Tris buffer, pH 7.4, 0.67 millimoles ethylenediaminetetraacetic acid, disodium salt, 200μg of glucose oxidase, and from 50 to 75mg of thyroid proteins. The total volume
Ground Thyroid Tissue

1. Homogenize with 0.25M sucrose, 1mM EDTA.
   (all steps were carried out at 0°−5° C)

2. Centrifuge at 1,000 x g for 10 min.

Residue
(1)    Supernatant

1. Centrifuge at 5,000 x g for 10 min.

Residue
(2)    Supernatant

1. Centrifuge at 15,000 x g for 60 min.

Residue
(3)    Supernatant

1. Centrifuge at 27,000 x g for 60 min.

Residue
(4)    Supernatant

1. Centrifuge at 48,000 x g for 60 min.

Residue
(5)    Supernatant
(6)

Figure 1. Fractionation Procedure for Thyroid Tissue.
was 3 ml. The incubation time was two hours at 30°C.

The reaction was stopped by adding 2.5 M hydroxylamine hydrochloride. The mixture was heated to 70°C in a water bath for 10 minutes. Then the mixture was extracted twice with 3.5 ml of 1-butanol, and once with 3 ml of 1-butanol. The butanol was removed under reduced pressure. The residue was dissolved in 0.2 ml of 30% alcoholic NH₄OH. Aliquots of these extracts were used for chromatography.

3. Chromatographic Procedures

20 μl of each extract was analyzed by ascending chromatography on Whatman No. 1 paper. Paper chromatography was carried out in Solvent A, 1-butanol-acetic acid-H₂O (68:2:27); Solvent B, 1-butanol-dioxane-2N NH₄OH (4:1:5); or Solvent C, tertiary amyl alcohol saturated with 2N NH₄OH.

Thin layer plates made by mixing 2 g of silica gel and 8 g of cellulose with 50 ml of deionized water were tried. Solvent D, t-butanol-3% NH₄OH (3:1) was used as the solvent system for thin layer chromatography.

Solvent E, 1-butanol-ethanol-NH₄OH 0.5N (70:10:20), and Solvent F, 1-butanol-ethanol-H₂O (70:10:20), were used as the solvent systems for paper chromatography of the 2,4-dinitrophenylhydrazones. The spots on the paper were detected using an ultraviolet lamp.

3,5-diiodo-4-hydroxybenzaldehyde recrystallized and unrecrystallized dissolved in methanol were chromatographed on Whatman No. 3 mm paper in tertiary amyl alcohol saturated with 2N NH₄OH as the solvent system.

4. Analysis of Chromatograms

a. UV absorption
The spots on paper chromatography were examined under Short Wave
Ultraviolet Filter Model SL 2537. It was used in a dark room.

b. Amino Acid Localization

The presence of $\alpha$-amino acids on the paper chromatograms was de­
tected by ninhydrin. The chromatograms were sprayed with 0.5% ninhydrin
in n-butanol and the color developed at room temperature. The appearance of
a purple color was considered positive for $\alpha$-amino acids.

c. Localization of Iodide

The presence of iodocompounds was confirmed by the use of the
$\text{Ce(NH}_4\text{)}_4(\text{SO}_4)_4-\text{NaAsO}_2$ reagent. Equal volumes of 10% $\text{Ce(NH}_4\text{)}_4(\text{SO}_4)_4$ and
5% $\text{NaAsO}_2$ in 1N sulfuric acid were mixed immediately before use. A piece
of Whatman No. 3mm chromatography paper was saturated with the mixture
and placed on a glass plate. The chromatogram to be analyzed was placed on
top of the saturated paper and pressed firmly in place by a second glass plate.
Various forms of iodide yield a colorless spot on a yellow background.

Iodate and periodate were detected by spraying the chromatograms with
a solution of 1g potassium iodide dissolved in 100ml of 0.2% starch containing
a trace of HCl.

Iodide was localized by spraying the chromatograms with a solution in
which the potassium iodide was replaced by potassium iodate.

5. Spectral Measurement

The spots cut from the paper chromatograms were eluted with 30%
alcoholic $\text{NH}_4\text{OH}$. The UV absorption spectra of the eluted spots and appropriate standards were determined on a Cary Model 14 Recording Spectrophotometer.
In a few cases, a Hitachi Perkin-Elmer 139 Spectrophotometer was used. Agreement between the two instruments was very good.

6. **Preparation of Chelate Complexes**

a. **Pyridoxal-Diiodotyrosine-Aluminum Chelate**

Pyridoxal hydrochloride (0.01 mole), sodium hydroxide (0.01 mole), and 3,5-diiodotyrosine (0.02 mole) were dissolved in 20ml of aqueous 0.1M acetate buffer, pH 5.0. After addition of potassium aluminum sulfate (0.005 mole), the solution was filtered and heated at 90°-100°C for 30 minutes. After standing overnight at room temperature the solution was filtered and the filtrate used for paper chromatography (Metzler et al., 1954). The spot was eluted from the chromatogram with 30% alcoholic NH₄OH and the UV spectrum determined.

b. **Pyridoxal-Monoiodotyrosine-Aluminum Chelate**

The method described above was used to prepare a pyridoxal-monoiodotyrosine-aluminum chelate. The concentrations were the same. The only difference was that 3-iodotyrosine was used instead of 3,5-diiodotyrosine.

7. **Preparation of Keto Acid 2,4-dinitrophenylhydrazones**

Since p-hydroxyphenylpyruvic acids are known to decompose during chromatography to yield the corresponding p-hydroxybenzaldehyde (Shiba and Cahnmann, 1962b), an attempt was made to stabilize the first product formed.

Approximately 0.3g of the concentrated assay mixture was dissolved in 6ml of water containing 0.3g of sodium bicarbonate. A slight excess of a saturated solution of dinitrophenylhydrazone in 2N HCl (approximately 0.5% solution) was added. The mixture was allowed to stand overnight at 5°C
(Smith, 1960). The crystals were filtered, dissolved in ethylacetate and analyzed by paper chromatography.

The dinitrophenylhydrazones of DIHB and p-hydroxybenzaldehyde were also prepared. A solution of DIHB in ethanol was prepared by dissolving 0.04g of the compound in 1.6ml of 95% ethanol. The freshly prepared 2,4-dinitrophenylhydrazine solution was added and the resulting mixture was allowed to stand overnight at 5°C. The crystals were filtered off. The 2,4-dinitrophenylhydrazone of p-hydroxybenzaldehyde was prepared by the same method (Shriner et al, 1962).

8. Preparation of 3,5-diido-4-hydroxybenzaldehyde

3,5-diido-4-hydroxybenzaldehyde was synthesized according to the procedure by Matsuura and Cahnmann (1959). A solution of 3.25g (0.02 mole) of iodine monochloride in 5ml of 20% HCl was added within 5 minutes to a stirred solution of 1.66g (0.01 mole) of p-hydroxybenzaldehyde in 14 ml of 20% HCl and 120ml of water. Stirring was continued for another 2 hours. The precipitate was filtered, washed with water, and recrystallized from an ethanol-water mixture.

9. Enzyme Extraction and Isolation

a. Solubilization of Fraction 1

Two hundred and fifty grams of hog thyroid tissue was treated as described on page 20. The first fraction was suspended in 90ml of 0.1M phosphate buffer, pH 8.0. 10ml of 10% Conoco 1012-6 detergent was added to the suspended fraction 1 to make a final concentration of 1%. The mixture was stirred at 0°C for 40 minutes and centrifuged at 48,000 x g for one hour.
b. **Treatment of Fraction 6**

The 6th fraction was dialyzed against 0.2M phosphate buffer, pH 7.0 for 24 hours at 0°C – 4°C. After dialysis the 6th fraction was fractionated with ammonium sulfate. The ammonium sulfate concentration was increased by increments of 10% (Dixon and Webb, 1964). After each addition of ammonium sulfate, the solution was allowed to stand for 10 minutes and centrifuged at 10,000 x g for 10 minutes. Following centrifugation, a minimum volume of phosphate buffer pH 8.0 was added to dissolve the precipitates. The final solutions were assayed for enzyme activity.

C. **RESULTS**

The \( R_f \) values for various solvent systems and support media are summarized in Table I. For paper chromatography, 1-butanol-acetic acid-\( \text{H}_2\text{O} \) (68:2:27) (Solvent A) was chosen as the solvent system because the \( R_f \) values of the standards: thyroxine, 3,5-diiodotyrosine, and 3-iodotyrosine are quite different. Two other solvent systems, 1-butanol-dioxane-2N \( \text{NH}_4\text{OH} \) (4:1:5) (Solvent B) and tertiary amyl alcohol saturated with 2N \( \text{NH}_4\text{OH} \) (Solvent C), were also tried as solvent systems, but the \( R_f \) values of the standards were very close to one another.

Thin layer chromatography in a solvent of t-butanol-3% \( \text{NH}_4\text{OH} \) (3:1) (Solvent D) gave good separation, but the recovery by elution was inferior. The percentage recovery of 3,5-diiodotyrosine and thyroxine on the thin layer chromatograms was not consistent. Therefore, only paper chromatography was used in this study.

An isolation procedure for subcellular particles was carried out as
## TABLE I

THE $R_f$ VALUES OF 3-IODOTYROSINE, 3,5-DIIODOTYROSINE, AND THYROXINE IN VARIOUS SOLVENTS

<table>
<thead>
<tr>
<th>Solvent</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-iodotyrosine</td>
<td>0.24–0.28</td>
<td>0.10–0.13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3,5-diiodotyrosine</td>
<td>0.31–0.34</td>
<td>0.83–0.85</td>
<td>0.22–0.25</td>
<td></td>
</tr>
<tr>
<td>thyroxine</td>
<td>0.64–0.68</td>
<td>0.72–0.74</td>
<td>0.35–0.38</td>
<td>0.50–0.54</td>
</tr>
</tbody>
</table>
described on page 20. All of the fractions were assayed as described on page 20. For each fraction a control, which had been boiled for 20 minutes, was run. After development of the chromatograms, they were analyzed in a variety of ways: UV absorption, ninhydrin for $\alpha$-amino acids, ceric ammonium sulfate-sodium arsenite for the presence of iodocompounds, starch plus iodate for iodide, and starch plus iodide for iodate. The results of these experiments using 3,5-diiodotyrosine or 3-iodotyrosine as substrate are shown in Figures 2, 3, 4, and 5. The standards thyroxine, 3,5-diiodotyrosine, and 3-iodotyrosine were spotted for comparison.

Chromatograms which were stained with ceric ammonium sulfate-sodium arsenite showed that every spot was positive for iodine. However, they were negative for inorganic iodide as was shown by spraying with starch plus iodide or iodate. From this information, it is apparent that each compound contains organically bound iodide.

Henceforth, the spots which were observed shall be referred to by the numbers indicated in Figures 2, 3, 4, and 5. From Figures 2, 3, 4, and 5 it is readily apparent that compounds MIT- and DIT-F-2 are formed by an enzymatic process which is inactivated by heat. Compounds, MIT- and DIT-F-2 have $R_f$ values which are similar to that of thyroxine. These are, however, fluorescent spots while thyroxine is UV absorbing. DIT- and MIT-A-1 have the same $R_f$ values as 3,5-diiodotyrosine and 3-iodotyrosine, respectively. These spots presumably represent unreacted substrate. DIT-F-1 occurs in both the boiled and unboiled fractions. When no enzyme is added (Figures 6 and 7) this spot is not present. The corresponding spot, MIT-F-1, occurs
Figure 2. Chromatography of Products Formed During the Incubation of 3,5-diiodotyrosine with Thyroid Fractions. Numbers with primes indicate that particular fraction was placed in a boiling water bath for 20 min. ○ - fluorescent spots under UV light. $\|$ - absorbing spots under UV light. $\%$ - spots which gave a positive ninhydrin reaction.
Figure 3. Chromatography of Products Formed During the Incubation of 3, 5-diiodotyrosine with Thyroid Fractions. Numbers with primes indicate that particular fraction was placed in a boiling water bath for 20 min. ○ -fluorescent spots under UV light. † -absorbing spots under UV light. ≡ -spots which gave a positive ninhydrin reaction.
Figure 4. Chromatography of Products Formed During the Incubation of 3,5-diiodotyrosine with Thyroid Fractions. Numbers with primes indicate that particular fraction was placed in a boiling water bath for 20 min. ○ -fluorescent spots under UV light. ● -absorbing spots under UV light. ⬿ -spots which gave a positive ninhydrin reaction.
Figure 5. Chromatography of Products Formed During the Incubation of 3-iodotyrosine with Thyroid Fractions. Numbers with primes indicate that particular fraction was placed in a boiling water bath for 20 min. ○ -fluorescent spots under UV light. ¶-absorbing spots under UV light. ‡-spots which gave a positive ninhydrin reaction.
only in assays using unboiled enzyme. The other significant difference between the chromatograms derived using MIT or DIT as substrate was the appearance of MIT-A-2. This compound has the same $R_f$ value as DIT.

Since the thyroid coupling reaction is an extremely complex reaction the possibility of two enzymes or one enzyme and a soluble cofactor was investigated. In such experiments, the sixth fraction was added to each of the other fractions and assayed. The results are shown in Figures 6 and 7.

The significant difference is that 1 plus 6 fraction showed a UV absorbing spot, A-3, which moves very near the solvent front with either 3,5-diiodothyrosine or 3-iodothyrosine as substrate. If either fraction is boiled prior to assaying compounds MIT- and DIT-A-3 are not formed. Another interesting aspect of these chromatograms was that compounds DIT- and MIT-F-2 were formed as long as both fractions had not been boiled.

Since the assay procedure as described on page 20 contains a large number of coenzymes and cofactors, it was possible that some of these were not required. In a series of experiments each component of the reaction mixture was systematically omitted. It was shown that all of the components were necessary for the formation of MIT-A-3.

To determine the nature of the compounds which were being formed during the enzymatic reaction, the spots DIT-F-1, DIT-F-2, DIT-A-1, DIT-A-3, MIT-A-3, and MIT-F-2 were eluted from the chromatograms with 30% alcoholic NH$_4$OH. The eluant was concentrated by reduced pressure and the UV spectra determined for each compound. The spectra are shown in Figures 8, 9, 10, and 11. The spectra of the standard thyroxine, DIT, and MIT are shown
Figure 6. Chromatography of Products Formed During the Incubation of 3,5-diiodotyrosine with Thyroid Fractions. Numbers with primes indicate that particular fraction was placed in a boiling water bath for 20 min. ○ -fluorescent spots under UV light. □ -absorbing spots under UV light. ≡ -spots which gave a positive ninhydrin reaction.
Figure 7. Chromatography of Products Formed During the Incubation of 3-iodotyrosine with Thyroid Fractions. Numbers with primes indicate that particular fraction was placed in a boiling water bath for 20 min. • -fluorescent spots under UV light. • -absorbing spots under UV light. • -spots which gave a positive ninhydrin reaction.
in Figure 12. The spectra determined for the compounds derived from DIT were not conclusive. However, the spectra of MIT-A-3, and MIT-F-2 showed maxima at 335μm and at 305μm, respectively. The maxima for the standard thyroxine, DIT, and MIT were at 326μm, 310μm, and 305μm, respectively. These are in agreement with maxima found previously (Edelhoch, 1962).

Since pyridoxal-5-phosphate is required for the formation of MIT and DIT-A-3, and the compound did not react with ninhydrin, one of the unknown compounds could be a Schiff's base of pyridoxal and the substrate. Therefore, the chelate compounds of a pyridoxal-3,5-diiodotyrosine-aluminum, and a pyridoxal-3-iodotyrosine-aluminum were prepared as described on page 24. The results of paper chromatography are shown in Figure 13. The UV absorption spectra of both chelates were determined, and are shown in Figure 14. The maxima for the major peaks in the MIT- and DIT-chelates are 327μm and 316μm, respectively.

Another possibility for the unknown compound is 3-iodo-4-hydroxyphenylpyruvic acid (MIHPPA). Therefore, the desiodo analog of this compound, 4-hydroxyphenylpyruvic acid (HPPA) was dissolved in 30% alcoholic NH₄OH and chromatographed on Whatman No. 1 paper. An UV absorbing spot was found which had an Rf value similar to the spot MIT-A-3. This is shown in Figure 13. The spectrum of HPPA in 30% alcoholic NH₄OH is shown in Figure 15. The maximum wavelength was 332μm. Phenylpyruvic acids are known to be unstable in alkaline solution; under such conditions they break down to form the corresponding benzaldehyde. Therefore, the spectrum of p-hydroxybenzaldehyde was determined. This is shown in Figure 17.
Figure 8. UV Absorption Spectra of the Compounds DIT-F-1, and DIT-F-2 in 30% Alcoholic NH₄OH.
Figure 9. UV Absorption Spectra of the Compounds DIT-A-1 and DIT-A-3 in 30% Alcoholic NH$_4$OH.
Figure 10. UV Absorption Spectrum of the Compound MIT-A-3 in 30% Alcoholic NH₄OH.
Figure 11. UV Absorption Spectrum of the Compound MIT-F-2 in 30% Alcoholic NH$_4$OH.
Figure 12. UV Absorption Spectra of the Standard Thyroxine, 3,5-diiodotyrosine, and 3-iodotyrosine in 30% Alcoholic NH₄OH.
Figure 13. Chromatography of DIT-Chelate, 4-hydroxyphenylpyruvic acid, and Thyroxine. ⨯ - absorbing spots under UV light. ⚫-spots which gave a positive ninhydrin reaction.
Figure 14. UV Absorption Spectra of MIT-Pyridoxal-Aluminum Chelate and DIT-Pyridoxal-Aluminum Chelate in 0.1M Acetate Buffer.
3, 5-diiodo-4-hydroxybenzaldehyde (DIHB) was synthesized as described on page 25. The recrystallized and unrecrystallized DIHB were chromatographed on Whatman No. 3mm paper in tertiary amyl alcohol saturated with 2N NH₄OH as the solvent. Two absorbing spots were found in the unrecrystallized sample. Both spots gave positive tests for iodine with ceric ammonium sulfate-sodium arsenite reagent. Such a chromatogram is shown in Figure 16. Since the second spot had a slightly different Rᵣ value from the starting material, it was assumed to be 3-iodo-4-hydroxybenzaldehyde (MIHB). This spot was eluted with 30% alcoholic NH₄OH and the UV spectrum determined. The spectra of 4-hydroxybenzaldehyde, MIHB, and DIHB are shown in Figure 17.

In order to determine if MIT-A-3 was a product of the reaction or if it was formed during the extraction procedure, the reaction of the first plus the sixth fraction was stopped by adding 2, 4-dinitrophenylhydrazine instead of hydroxylamine hydrochloride. The mixture was extracted with ethylacetate and paper chromatograms spotted. The chromatograms were run in a solvent of 1-butanol-ethanol-H₂O (70:10:20) or 1-butanol-ethanol-NH₄OH 0.5N (70:10:20). The Rᵣ values were compared to p-hydroxyphenylpyruvic acid. These are shown in Figure 18.

The 2, 4-dinitrophenylhydrazones of DIHB and p-hydroxybenzaldehyde were prepared as described on page 24. They were chromatographed on Whatman No. 1 paper in butanol-ethanol-NH₄OH 0.5N (70:10:20). The compounds did not yield distinct spot but gave long streaks from the origin to near the solvent front.

To determine the nature of the species in the sixth fraction which
Figure 15. UV Absorption Spectrum of 4-hydroxyphenylpyruvic acid in 30% Alcoholic NH₄OH.
Figure 16. Chromatography of Recrystallized 3,5-diodo-4-hydroxybenzaldehyde, Unrecrystallized 3,5-diodo-4-hydroxybenzaldehyde, and p-hydroxybenzaldehyde in Tertiary Amyl Alcohol Saturated with 2N NH₄OH. ☼ - absorbing spots under UV light.
Figure 17. UV Absorption Spectra of p-hydroxybenzaldehyde, 3,5-diiodo-4-hydroxybenzaldehyde, and 3-iodo-4-hydroxybenzaldehyde in 30% Alcohol NH₄OH.
Figure 18. Chromatography of the Products of the Assay Mixture Stopped by Addition of 2,4-dinitrophenylhydrazine in Different Solvents. A is in 1-butanol-ethanol-H$_2$O (70:10:20): B is in 1-butanol-ethanol-0.5N NH$_4$OH (70:10:20). —UV absorbing spots.
activates the formation of MIT-A-3, the sixth fraction was dialyzed against
0.2M phosphate buffer, pH 7.0. The material inside the dialysis bag and the
material outside the bag plus the first fraction were assayed. MIT-A-3 was
found only when the material inside the bag was used along with fraction 1.

Fractional precipitation of the sixth fraction by ammonium sulfate was
studied after dialysis. The formation of MIT-A-3 is summarized in Table II.
Unfortunately, the samples were lost before the protein concentrations could
be determined. However, qualitatively the 30-40% and 40-50% fractions con­
tained the most precipitate, while the 50-60% fraction contained the least.
Therefore, there must have been an enrichment of the activating factor in
the 50-60% fraction.

The first fraction treated with Conoco 1012-6 detergent plus the sixth
fraction was assayed. The results showed that after treatment with detergent
and centrifugation at 48,000 x g the activity was retained in the supernatant.

The quantitative relationship of compound MIT-A-3 to the amount of
the thyroid protein added to the assay was determined. Different amounts of
Conoco 1012-6 solubilized enzyme and 0.5ml of the sixth fraction were added as
the thyroid protein. The results are shown in Figure 19. The plot of com­
 pound MIT-A-3 formed vs. the concentrations of solubilized enzyme showed
a nearly linear relationship.
### TABLE II

**AMMONIUM SULFATE FRACTIONATION OF FRACTION SIX**

<table>
<thead>
<tr>
<th>% of (NH$_4$)$_2$SO$_4$ Saturation</th>
<th>Absorbance at 335μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 — 30</td>
<td>0.440</td>
</tr>
<tr>
<td>30 — 40</td>
<td>0.475</td>
</tr>
<tr>
<td>40 — 50</td>
<td>0.485</td>
</tr>
<tr>
<td>50 — 60</td>
<td>0.510</td>
</tr>
</tbody>
</table>
Figure 19. Quantitative Relationship Between Solubilized Enzyme and Production of MIT-A-3.
IV. DISCUSSION

In this study, it was found that the formation of MIT-A-3 (MIT-A-3 is an ultraviolet absorbing compound with a slightly greater $R_f$ than thyroxine) is catalyzed by two of the subcellular fractions. Both fraction one and fraction six are required for synthesis of this compound. This is in contrast to the work of Fischer et al. (1965) with beef thyroids who found that only the microsome fraction is required for synthesis of thyroxine. One possible explanation for the difference is that this study was conducted using porcine thyroid tissue. A similar phenomenon has been observed in studies on the thyroid peroxidase. In beef thyroid tissue the peroxidase was associated with lighter particles than the same enzyme from porcine glands (Hosoya and Morrison, 1967; Igo et al., 1964). It has been suggested that this is due to the difference in the rigidity of the two tissues (Alexander and Corcoran, 1962).

Crude enzyme extracts contain numerous other substances of both large and small molecular weight. Many of the low molecular weight substances can serve as coenzymes and cofactors. The small molecules can be removed by dialysis, leaving the large molecules, which are predominantly proteins. Dialysis of fraction six against phosphate buffer showed that the material remaining inside the dialysis bag plus the first fraction could form MIT-A-3. The material which passed through the dialysis membrane plus the first fraction did not form MIT-A-3 even after concentration of the material outside the bag. This indicates that a macromolecule, most likely a protein, in fraction six is involved in the reaction.
The activating factor in the sixth fraction can be fractionated by ammonium sulfate precipitation. The results of the fractional precipitation of the sixth fraction after dialysis showed that most of the activating factor precipitated between 50 and 60% of saturation with ammonium sulfate. Qualitatively this fraction contained the least protein and therefore there was an increase in the specific activity of this factor.

The first fraction was solubilized by treatment with 1% Conoco 1012-6 detergent. The compound MIT-A-3 could be synthesized by detergent solubilized enzyme plus the sixth fraction. After solubilization the amount of enzyme added to the assay was proportional to the amount of compound MIT-A-3 formed. An increase in specific activity for the solubilization could not be determined because of the difficulty in accurately measuring the extremely viscous first fraction.

When 3,5-diiodotyrosine was used as the substrate, four compounds appeared on the chromatograms. The UV absorbing compound DIT-A-3 was formed only when both the first and the sixth fractions were added. The \( R_f \) value of DIT-A-3 was slightly greater than the \( R_f \) for thyroxine. This compound did not give a positive ninhydrin test, was positive for iodide, and did not give an interpretable spectrum. This information shows that the compound DIT-A-3 is not thyroxine. The compound DIT-A-3 has an \( R_f \) value and an absorption spectrum which is identical to the substrate 3,5-diiodotyrosine. In addition DIT-A-1 gave positive ninhydrin and iodide tests. On this basis DIT-A-1 is thought to be unreacted substrate. The compound DIT-F-2 gave a negative ninhydrin test, a positive iodide test, was fluorescent under UV
light, and had the same $R_f$ value as thyroxine. Even though this compound has the same $R_f$ value as thyroxine the other data indicates that DIT-F-2 is not thyroxine. The fourth compound DIT-F-1 was present on all chromatograms whether the thyroid protein had been heat denatured or not. It, therefore, must be a non-enzymatically formed, ninhydrin negative, iodine containing compound.

When 3-iodotyrosine was used as the substrate, a similar chromatographic pattern resulted. The major exception was that MIT-A-2 appeared. Also using 3-iodotyrosine as the substrate sufficient material could be eluted to determine the UV spectra of these compounds. Therefore the remainder of the study was conducted using 3-iodotyrosine as the substrate.

Of the five compounds formed when 3-iodotyrosine was the substrate three appear to be unrelated to thyroxine synthesis. The compounds, MIT-A-1 and MIT-A-2, were UV absorbing, gave positive ninhydrin and iodide tests, and had the same $R_f$ values as the standards MIT and DIT, respectively. In addition the compounds had the same absorption maxima as standard MIT and DIT. From this it was assumed that MIT-A-1 was unreacted substrate. MIT-A-2 appears to be DIT. DIT could either have been synthesized from MIT by thyroid enzymes or have been a trace contaminate in the MIT. The compounds MIT-F-1 was also formed nonenzymatically so it was not studied further.

Two of the compounds which appear on the chromatograms are more significant with respect to thyroxine synthesis. MIT-F-2 is formed in the presence of any of the native subcellular particles. Although MIT-F-2 and thyroxine have similar $R_f$ values MIT-F-2 did not contain an $\alpha$-amino group.
It showed maximum absorption at 305\(\mu\). MIT-A-3 was synthesized only in the presence of both the first and the sixth fractions. It was UV absorbing compound, and has the same \(R_f\) value as the compound DIT-A-3, but MIT-A-3 showed an absorption maximum at 335\(\mu\). In Fischer's report, 3,3'-diiodothyronine was synthesized from 3-iodotyrosine by the thyroid enzyme system. It showed maxima at 311\(\mu\) and 225\(\mu\) (1965). Neither of the compounds MIT-A-3 and MIT-F-2 showed maxima in this region. Neither compound contained an \(\alpha\)-amino group. Therefore, 3,3'-diiodothyronine was not synthesized in this study.

Since pyridoxal phosphate is required in this reaction, the most likely origin for iodocompounds without \(\alpha\)-amino groups is shown in the following sequence:

\[
\begin{align*}
\text{HO-} & \begin{array}{c}
\text{I} \\
\text{C-CH}_2 \text{COOH}
\end{array} & \text{H} & \begin{array}{c}
\text{NH}_2
\end{array} & \xrightarrow{\text{pyridoxal phosphate}} & \text{enzyme (E)} & \begin{array}{c}
\text{I}
\end{array} \\
\text{MIT}
\end{align*}
\]

\[
\begin{align*}
\text{HO-} & \begin{array}{c}
\text{I} \\
\text{C-CH}_2 \text{COO}^\Theta
\end{array} & \text{H} & \begin{array}{c}
\text{N}
\end{array} & \text{E} & \begin{array}{c}
\text{O}^\Theta \text{PO}_2 \text{H}_2 \text{C}
\end{array} & \text{CH}_3 \\
\text{pyridoxal phosphate - iodotyrosine - enzyme complex (I)}
\end{align*}
\]
In this sequence there are three compounds that should not give positive ninhydrin reactions: the Schiff's base of pyridoxal phosphate and MIT, MIHPPA, and MIHB. These compounds or similar model systems were compared to MIT-A-3 and MIT-F-2.

The Schiff's base of pyridoxal phosphate and MIT would most likely not be stable under the conditions of the assay unless it is stabilized by an enzyme or a metal ion. For comparison the pyridoxal-MIT-aluminum complex was prepared. The spectra of MIT-pyridoxal-aluminum was measured and the maxima were at 327μ and 288μ. This is quite different from the compounds MIT-A-3 and MIT-F-2. The difference was also demonstrated by paper chromatography. It is not possible, however, to rule out the presence of such a Schiff's base since this work was limited to studying the metal complex analog.

A second possibility is that MIT-A-3 or MIT-F-2 might be MIHPPA. This would appear to be an unlikely situation since the p-hydroxyphenylpyruvic acids are known to be very unstable particularly under basic conditions (Shiba
and Cahnmann, 1962b). To determine the extent of this instability the spectrum of the desiodo analog of MIHPA, p-hydroxyphenylpyruvic acid (HPPA) was measured in 30% alcoholic NH₄OH. Under this condition HPPA gave a spectrum which was identical to that of p-hydroxybenzaldehyde (HB).

The third possibility is that MIT-A-3 or MIT-F-2 could be MIHB. For this work 3,5-diiodo-4-hydroxybenzaldehyde (DIHB) was prepared. Upon chromatography this standard was shown to contain two compounds. The major one was assumed to be the desired product and the minor one which also contained iodine was assumed to be MIHB. DIHB showed maxima at 347μm and 263μm. These are very close to the values 345μm, 263μm and 212μm reported by Shiba and Cahnmann (1962b). HB showed a maximum at 332μm, and MIHB showed a maximum at 337μm.

The maximum which was observed for MIHB is almost identical to MIT-A-3. It was also confirmed by chromatography in a solvent of 1-butanol-aceticacid-H₂O (68:2:27) that DIHB and HB had the same Rf values as MIT-A-3 and DIT-A-3. Therefore, it was concluded that MIT-A-3 was most likely MIHB. This finding is supported by the work of Nishinaga and Matsuura (1964). They reported that 3,5-diiodo-4-hydroxybenzaldehyde was always produced in the chemical synthesis of thyroxine from 3,5-diiodotyrosine. In this case using 3-iodotyrosine as the substrate it is very possible to produce the compound MIHB.

The finding that MIHB is produced during this reaction leaves two possible interpretations. One is that MIHB is formed enzymatically from 3-iodotyrosine. The second possibility is that MIHPPA is formed enzymatically
from 3-iodotyrosine and this compound decomposes under the basic conditions used for extraction. To ascertain which is occurring the reaction was stopped by addition of 2,4-dinitrophenylhydrazine. After extraction with ethylacetate the material was chromatographed. If MIHPPA is formed the 2,4-dinitrophenylhydrazine should react with it to produce the dinitrophenylhydrazone. The results of the paper chromatograms showed the compound which formed had an $R_f$ value similar to the dinitrophenylhydrazone of p-hydroxyphenylpyruvic acid. It definitely does not appear to be MIHB since these aldehydes do not separate but yield long streaks in this solvent. From these results it would appear that 3-iodotyrosine is converted to MIHPPA which in turn decomposes to MIHB.
V. CONCLUSIONS

Both MIT and DIT are metabolized to form a fluorescent compound, MIT- or DIT-F-2, when any thyroid protein is incubated with the appropriate cofactor. If fraction 1 and fraction 6 are used simultaneously either MIT-A-3 or DIT-A-3 is formed in addition to the fluorescent compound. Fraction 1 was solubilized by treatment with 1% Conoco 1012-6 detergent. After detergent treatment the enzyme remained in the supernatant when centrifuged at 48,000 x g. The activating factor in the sixth fraction was shown to be a macromolecule, probably a protein, by dialysis and ammonium sulfate precipitation.

Due to difficulties in working with DIT as a substrate, MIT-A-3 was investigated by paper chromatography and spectral measurements. It showed an absorption maximum at 335μμ, gave a negative ninhydrin test, and gave a positive iodide test. By comparison to standards this compound was identified as 3-iodo-4-hydroxybenzaldehyde.

The source of 3-iodo-4-hydroxybenzaldehyde was investigated by addition of 2,4-dinitrophenylhydrazine to stop the reaction. The dinitrophenylhydrazone which was formed appeared to be that of 3-iodo-4-hydroxyphenylpyruvic acid rather than that of 3-iodo-4-hydroxybenzaldehyde. This would indicate that the first product is 3-iodo-4-hydroxyphenylpyruvic acid which decomposes to 3-iodo-4-hydroxybenzaldehyde in the alkaline solution used for extraction. The results of this work show that 3,5-diiodo-4-hydroxyphenylpyruvic acid can be found by thyroid enzymes. This lends support to suggestions that DIHPPA is an intermediate in the thyroxine synthesis.
VI. BIBLIOGRAPHY


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VII. VITA

Shih-ying Sun was born on January 14, 1943, in Kirin, China. She received her high school education at the First Girls Middle School in Taipei, Taiwan and graduated in June 1961.

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