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# Synthesis of a thyroxine derivative for conjugation with malate dehydrogenase

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# Synthesis of a Thyroxine Derivative for Conjugation with Malate Dehydrogenase

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# SYNTHESIS OF A THYROXINE DERIVATIVE

# FOR

# CONJUGATION WITH MALATE DEHYDROGENASE

by

WEN SHI

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## ABSTRACT

Thyroxine is a competitive inhibitor of malate dehydrogenase. For decades, studies have been conducted to improve the inhibitory effect of the thyroxine. The objective of this project is to synthesize a thyroxine derivative for malate dehydrogenase conjugation that is used for a competitive homogeneous enzyme immunoassay. Malate dehydrogenase labelled with the thyroxine derivative, a N-methyl, N-carboxymethylglycyl thyroxine methyl ester (T4-MEMIDA) N-hydroxysuccinimide ester, shows over 90% inhibition of enzyme activity. The conjugated malate dehydrogenase is enzymatically inactive but becomes active after binding with anti-thyroxine antibodies. The thyroxine derivative appears to inhibit the enzyme by hindering its active site, whereas the antibody reactivates the enzyme by pulling the thyroxine away from the active site. The thyroxine conjugate is stable at 4°C for one week without adding any preservative and stabilizer. In competitive homogeneous enzyme immunoassay, the thyroxine from a sample competes with the enzyme conjugate for antibody binding sites and results in reduction of the enzyme reactivation. A set of six samples with different concentrations of thyroxine is used to generate a standard curve. Without separation steps involved, the distinction between free and antibody-bound labeled antigen can be made entirely based on enzymatic activity.

## RATIONALE

The interpretation of variation in T4, T3, TSH, and TBG levels is very important in clinical practice. When the thyroxine concentration falls outside the normal range, hyper- or hypothyroidism is suspected. Measurement of T3 uptake, TSH, or total T3 can confirm the diagnosis. A multiple assay system for simultaneous detection of all these components will reduce costs in diagnosis. However, multiple assay systems involve separate sequential steps for each enzyme reaction. Elimination of separation steps will bring simultaneous assays a significant advance for practical application. With the characteristics of rapid testing and no separation step required, homogeneous enzyme-immunoassay is more favorable for multiple assay systems than others. Therefore, our study is to synthesize the thyroxine derivative and malate dehydrogenase conjugation that can be used for competitive homogeneous enzyme immunoassay. It is expected that advantages of fast testing procedure, small sample volume, and higher precision will help to create a simpler simultaneous detection for thyroid hormone diagnosis.

# INTRODUCTION

## 1. Background Information on Thyroxine

Thyroxine, the iodinated amino acid 3,5,3',5'-tetraiodo-L-thyronine (T4), is secreted by the thyroid gland along with 3,3',5'-triiodo-L-thyronine, (T3) (Fig. 1) [1, 2].

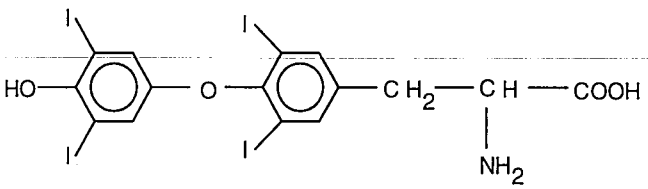



Fig. 1 Molecule of Thyroxine (T4)

The secretion is controlled by the pituitary thyroid-stimulating hormone (TSH) which is affected by hypothalamic thyrotropin-releasing hormone (TRH). Normally, the increase in blood level of T4 and T3 leads to the decrease in the amount of TSH secreted, thereby reducing the production and secretion of T4 and T3. On the other hand, the decrease in blood level of T4 and T3 results in the opposite effect, leading to more production and secretion of T4 and T3. In this manner, a normal balance of circulating thyroid hormone is maintained. Upon entering into blood, T4 is strongly bound by specific plasma proteins, especially by thyroid-binding globulin (TBG). Because of the very high affinity of TBG for T4, more than 99.9% of T4 is bound and less than 0.1% of T4 is free. The free T4 penetrates into cells, induces stimulation of metabolism, and controls feedback via pituitary. Although the free fraction of T4 is influenced not only by

the total circulating T4, but also by the amount of thyroxine-binding proteins in the blood, measurement of total thyroxine concentration is the most common preliminary procedure used to detect thyroid disease. When the thyroxine concentration falls outside the normal range, hyper- or hypothyroidism is suspected. Measurement of T3 uptake, TSH, or total T3 can confirm the diagnosis.

## 2. Classification of Immunoassay

Several methods in determining total T4 levels are available. Immunoassay is one of the common diagnostic methods that is applied using an antibody/antigen reaction. Immunoassay can be classified as radioimmunoassay (RIA), enzyme immunoassay (EIA), and fluoroimmunoassay (FIA). Most of radioimmunoassay utilizes the competition of labeled ligand that may be either a hapten or a macromolecular antigen (Ag*) with the corresponding unlabeled ligand or an analyte (Ag) from a sample for a limited number of antibody binding sites (Ab) [4, 5, 6].

```
Ab + Ag*  ==  Ab.Ag*
+
Ag
||
Ab.Ag
```

As shown above, the concentration of antibody binding sites available to bind the radio-labeled ligand is inversely related to the concentration of analyte present in the sample. Radioimmunoassay is a sensitive and specific technique and has been proved extremely valuable in clinical chemistry. However, this method does have several

limitations. 1) Most useful radioisotopes have relatively short half-lives. 2) Potentially hazardous levels of radioactivity are encountered during labeling procedures. 3) There are problems with disposal and release of radioactivity into the environment. 4) The requirement of a separation step during the assay is a particular disadvantage of RIA. Therefore, a variety of non-isotopic labels has been investigated in an attempt to retain the advantages of RIA without the association with radioisotopes. Two assay methods, enzyme immunoassay and fluorescence immunoassay, have been developed to compete favorably with radioimmunoassay in many areas of performance. Fluorescence immunoassay is based on the principle that certain molecules (fluorophores) can absorb light at one wavelength and emit light at a longer wavelength. However, the sensitivity of fluorescence determination is low in practice because of the background 'noise'. The noise can be due to either light scattering or the presence of fluorophores other than the label itself. Enzyme labels are currently the most widely used non-isotopic labels for the following reasons. First of all, there is no contact with radioactive material required. Secondly, the activities of several enzyme labels can be easily determined with commonly available spectrophotometer. Also, the reagents have a much longer half-life. Furthermore, the separation step is not required in some assays.

### 3. Types of Enzyme-Immunoassay

Enzyme-immunoassay can be categorized as either "heterogenous" or "homogeneous." In heterogeneous enzyme-immunoassay, the separation of free and bound labeled molecules is necessary. However, in homogeneous assays, the separation step is

not required. An essential difference between these two assays is that the enzyme on the labeled antigen or antibody retains its activity in heterogeneous assay [4, 7].

(1) Competitive heterogeneous EIA for Antigen -- Labeled antigen competes with unlabeled antigen for the binding to a limited quantity of antibody. This procedure is

Table 1

Comparison of RIA, EIA, and FIA

| | RIA | Homogeneous EIA | Heterogeneous EIA | FIA |
|---|---|---|---|---|
| Sensitivity | Very High | Low | High | High |
| Precision | Good | Good | Good | Low |
| Complexity | Rather Complex | Simple | Rather Complex | Complex |
| Assay Time | Hours | Minutes | Hours | Hours |
| Equipment | Isotope Counter | Photometer | Photometer | Fluorescence Microscope |
| Automation Potential | High | Very High | High | Low |
| Reagent Stability | Low | High | High | Variable |
| Required Expertise | High | Medium | Medium | High |

analogous to the classical radioimmunoassay and fluorescence-immunoassay. The only major difference is in the use of an enzyme rather than an isotope or fluorescent dye as the label on antigen.

(2) Sandwich EIA for Antigen -- This procedure requires the antigen to have at

least two binding sites. Antigen reacts with excess solid-phase antibody, and after incubation followed by washing, the bound antigen is treated with excess labeled antibody. After further washing the bound label is assayed, and this provides a direct measure of the amount of antigen present.

(3) Homogeneous EIA for Hapten -- This type of EIA does not require the separation of free and bound label because the assay depends on inhibition or activation of the enzyme label.

### 3. Background information about MDH

Malate dehydrogenase (MDH), a common enzyme in the tricarboxylic acid cycle, is composed of two identical subunits that are held together by varying interactions [4]. These two subunits can dissociate without losing catalytic activity and reassemble in the presence of substrate. Each subunit has a molecular weight about 35000 daltons and contains one binding site for a coenzyme. Malate dehydrogenase activity is measured with spectrophotometer that monitors absorbance at 340 nm due to NAD reduction or NADH oxidation. With oxaloacetate and NADH as substrates, initial reaction velocities are about four times greater than that with malate and NAD as substrates (Table 2) [9, 10].

Malate dehydrogenase from pig heart mitochondria consists of 318 amino acids on each subunit. The amino acid composition is shown in table 3. This enzyme contains 14 SH groups that are not involved in catalytic reaction but are required for maintaining the three dimensional structure of the enzyme. Although the modification of many amino

acids can result in enzyme inactivation, only histidine has been clearly proved as a catalytic active residue. Malate dehydrogenase is stable for at least a year at 4°C in 50% glycerol solution. Once dialyzed against phosphate buffer, it should be stored at -20°C.

Table 2

Molecular Properties of Malate Dehydrogenase

| | |
|---|---|
| Mass ( daltons ) | 70,000 |
| Subunits | 2 |
| Isoelectric point | 6.2 |
| Dissociation constant<br>NADH ( Tris buffer, pH 8.0 ) | 2.16 uM |
| Apparent Km<br>Oxaloacetate<br>NADH | 4.5x10-5 M<br>3.2x10-5 M |
| pH optimum | 7.4 |
| Activators | Phosphate, $Zn^{2+}$, Malate |
| Inhibitors | Oxaloacetate, Thyroxine, Adenine, Phenols, and Substituted Phenols |

Table 3

Amino Acid Composition of the Mitochondrial Malate Dehydrogenase from Pig Heart

| | | | |
|---|---|---|---|
| Lysine | 26 | Histidine | 5 |
| Glutamic acid | 25 | Aspartic acid | 25 |
| Threonine | 21 | Serine | 18 |
| Arginine | 8 | Proline | 23 |
| Glycine | 29 | Alanine | 33 |
| Cystine | 7 | Valine | 27 |
| Methionine | 6 | Isoleucine | 21 |
| Leucine | 28 | Tyrosine | 5 |
| Phenylalanine | 11 | Tryptophan | 0 |

# THEORY

## 1. Assay Method Design

In a competitive, homogeneous enzyme-immunoassay, three components are usually required [2, 4]. They are antibody, labeled-analyte (antigen or hapten), and an unknown amount of analyte. Upon binding to antibody, the signal arising from the immunochemical label must be modified, directly or indirectly. The first and still the most widely accepted homogeneous enzyme-immunoassay for thyroxine is the enzyme activation immunoassay. The activity of the enzyme is modulated by the thyroxine and anti-thyroxine antibody due to the change in the configuration of the enzyme.

### *1.1 Principle of the Assay*

Among the various enzymes, malate dehydrogenase from pig heart is a particularly important enzyme for thyroxine because of its ability to form an inactive hapten conjugate. Conjugation of thyroxine derivatives to pig heart mitochondrial malate dehydrogenase results in powerful inhibition of enzyme activity [11]. When the thyroxine-specific antibody binds to enzyme-labeled thyroxine, the inhibition of the conjugated enzyme is partially released. Free thyroxine in the patient's sample competes with the enzyme-labeled thyroxine for a limited number of antibody binding sites. As a result of blocking effects of the antibody, activation of the enzyme is reduced [12]. There is an inverse relationship between the conjugated enzyme activity and thyroxine concentration

in the sample (Fig. 2). The relationship between thyroxine concentration and enzyme activity can be developed by testing the conjugated enzyme activity in a series of known thyroxine concentration. The concentration of T4 in a sample is calculated by comparing the enzyme activity with the standard calibration curve [1, 13, 14].

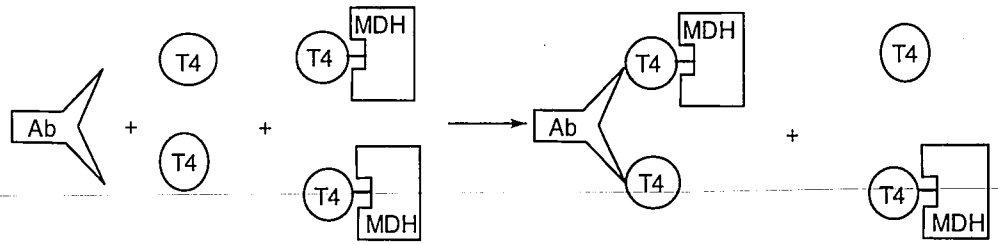



Fig. 2 Principle of Competitive, Homogeneous Enzyme Assay for Thyroxine

### *1.2 Test of Enzyme Activity*

Malate dehydrogenase consists of two identical subunits that have one active site on each. The reactivated enzyme converts oxaloacetate into malate employing NADH as coenzyme. MDH catalyzes the reversible reaction [4]:

$$\text{Oxaloacetate} + \text{NADH} + \text{H}_{+} \text{ ---- Malate} + \text{NAD}$$

Generally, the absorbance of NADH can be measured with spectrophotometer at 340 nm. According to the reaction showing above, MDH activity can be monitored by the change in the optical density at 340 nm per min due to disappearance of NADH. If the final volume of the assay mixture is V ml and the optical pathway (b) 1 cm, the total activity and specific activity of enzyme can be determined as follows: [15]

$$\text{Total Units} = \frac{\Delta A}{\text{min}} \times \frac{\text{umol}}{6.22\ A \times \text{ml}} \times V\ \text{ml} \times \text{Dilution Factor}$$

$$\text{Specific Activity} = \text{units / mg protein}$$

## 2. Conjugation Design

The labeling of malate dehydrogenase with thyroxine is to be carried out by the usage of N-hydroxysuccinimide (NHS) ester of the thyroxine derivatives that leads to coupling at the amino groups of the enzyme (Fig. 3) [2].

Since the thyroxine derivative is not commercially available, an organic synthesis is applied to couple N-methyliminodiacetic acid and thyroxine. The method used for production of the thyroxine derivative is similar to the peptide synthesis.

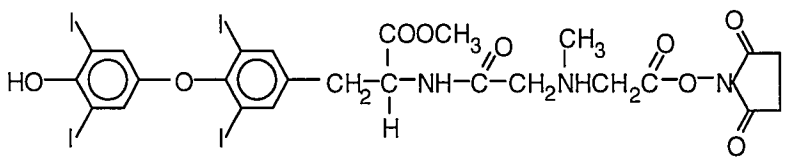



Fig. 3 Molecular Structure of T4-MEMIDA N-hydroxysuccinimide Ester

### *2.1 Methyl thyroxine hydrochloride*

This step includes activation of the amino group and protection of the carboxyl group. Since thyroxine contains one amino and one carboxyl group, a cyclic dipeptide may be formed by thyroxine itself. To avoid formation of undesired compounds, the

carboxyl group of thyroxine must be blocked. Esterification is suitable protection for the carboxyl group. A classical method of esterification requires dry HCl gas that is a major disadvantage in the operation [16]. In this experiment, an improved method is applied for the preparation of the methyl ester thyroxine in which 2,2-dimethoxypropane serves as a source of the methoxyl group, the major solvent in the reaction system, and a reactive reagent for the removal of water (Fig. 4) [17, 18].

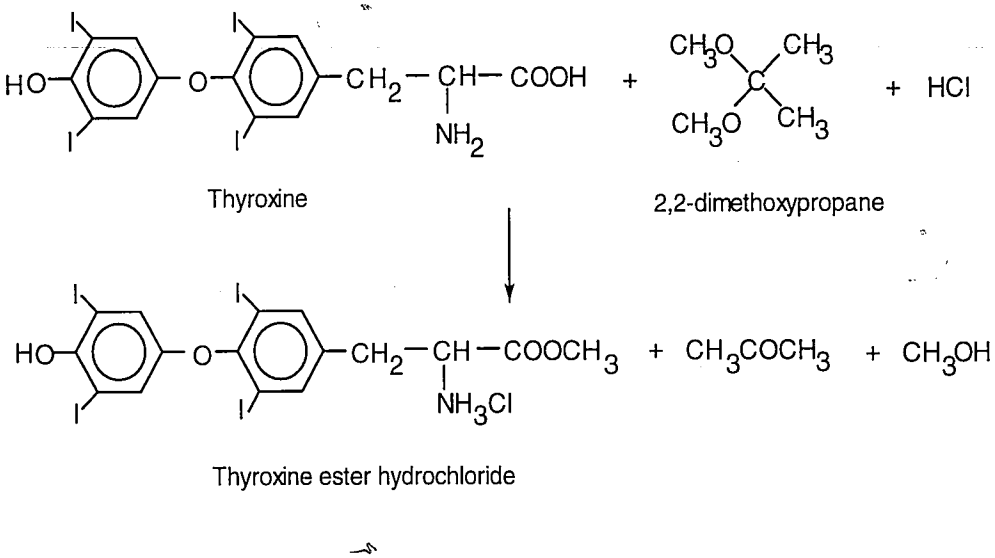



Fig. 4 Formation of Methyl Thyroxine Hydrochloride

### *2.2 N-Methyl, N-carboxymethylglycyl Thyroxine Methyl Ester (Compound I)*

The formation of an amide bond is the crucial step in peptide synthesis. Although many coupling methods have been studied and improved, most of them have undesired side reactions. The method for peptide synthesis in this paper utilizes acid anhydride as the reactive acylating intermediate. N-methyliminodiacetic acid anhydride is a cyclic

compound, in which the anhydride group is sensitive to a nucleophilic attack. Therefore, even traces of moisture can lead to the forming of diacid. The only by-product in this step is hydrochloric acid that can be easily removed. There are several shortcomings for this anhydride method, such as the water sensitivity of reagent. To avoid polymerization, the acid anhydride reagent has to be stored under careful exclusion of moisture [17,19].

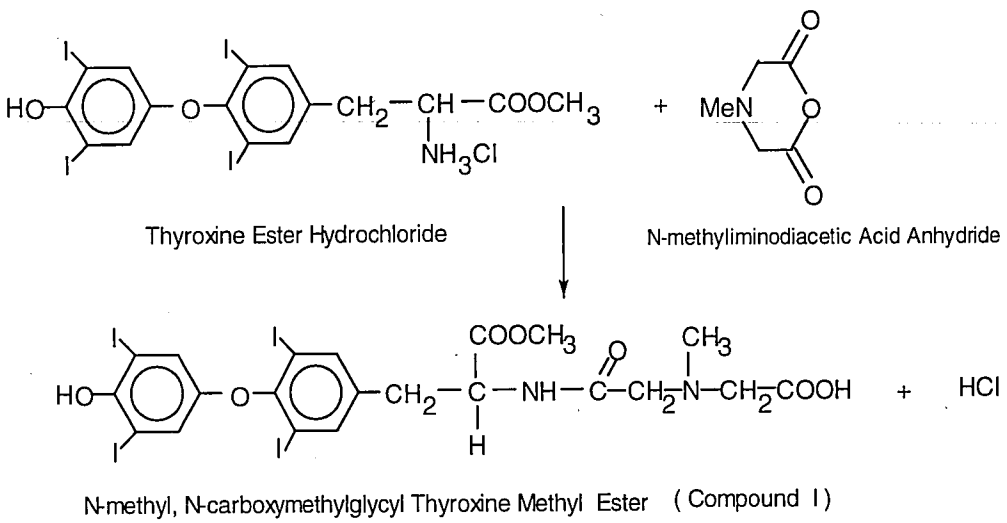



Fig. 5 Formation of N-methyl, N-carboxymethylglycyl Thyroxine Methyl Ester (Compound I)

### *2.3 T4-MEMIDA N-hydroxysuccinimide Ester (Compound II)*

This is an activation step for the T4-MEMIDA conjugation to amino groups of the enzyme. Activation of the carboxyl group of the thyroxine derivative can be achieved by the conversion of the carboxyl group to the reactive ester. N-hydroxysuccinimide ester is widely used in peptide synthesis because of its excellent stability. T4-MEMIDA N-

hydroxysuccinimide ester is formed in presence of "coupling reagent". Water-soluble 1-ethyl-3 (3'-dimethylaminopropyl) carbodiimide hydrochloride is the most successful coupling reagent for the extremely rapid synthesis of pure ester. Since the by-product is water soluble, it can be easily washed away with water [19, 20, 21, 22].

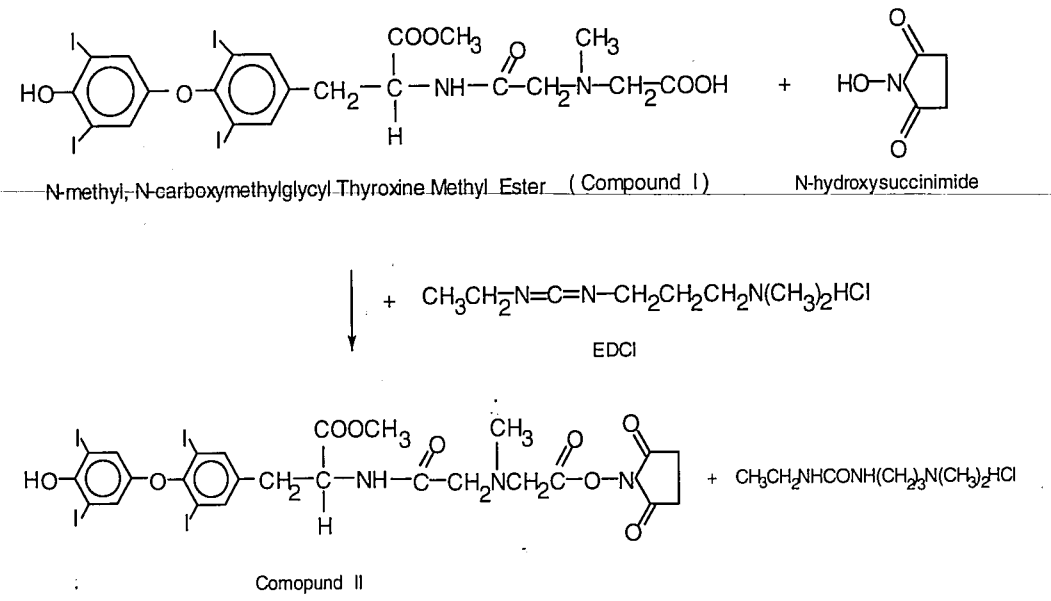



Fig. 6 Formation of T4-MEMIDA N-hydroxysuccinimide Ester (Compound II)

### *2.4 T4-derivative and MDH Conjugation*

The T4-derivative contains a reactive NHS ester that can react under mild conditions. Acylation of the free amino groups of MDH occurs via the active NHS ester [2, 4, 12, 23]. The leaving N-hydroxysuccinimide is readily soluble in water and thus can be easily separated from the insoluble products. Inactivation of enzyme is induced by T4-derivative through blocking of the histidine that has been clearly proved as a catalytic

active residue [24, 25]. Although other research indicates that inactivation of enzyme can be induced by the modification of the thiol groups with iodine-containing compounds, direct oxidation of thiol groups leads to irreversible inhibition of the enzyme [26].

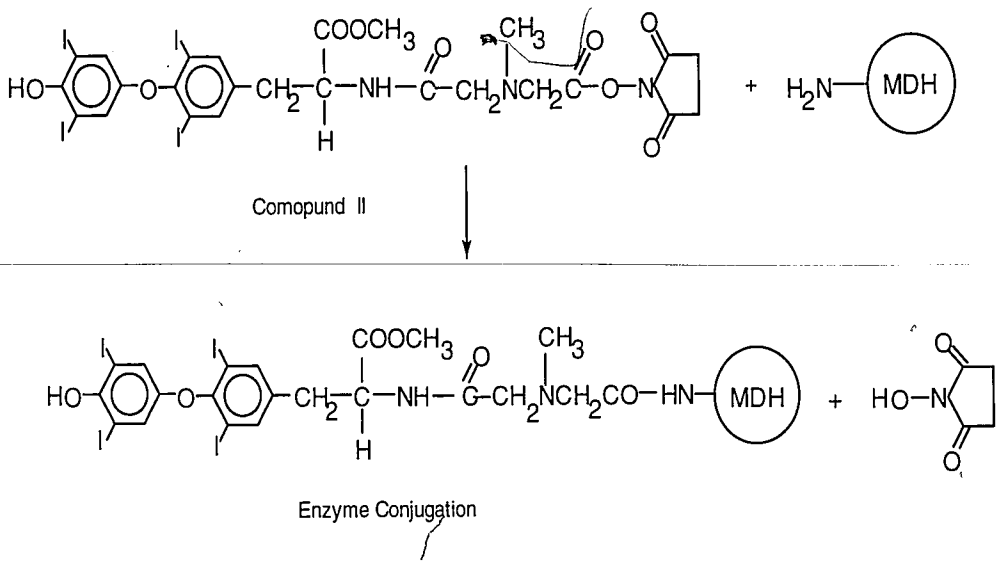



Fig. 7 Formation of T4-derivative and MDH Conjugate

# EXPERIMENTAL

## 1. Materials

Materials used in this experiment were obtained from the following sources:

(1) Pig heart mitochondrial malate dehydrogenase - Calzyme Laboratories, Inc.

(2) Thyroxine - Sigma Chemical Co.

(3) N-methyliminodiacetic acid - Aldrich Chem. Co.

(4) 2,2-dimethoxypropane - Sigma Chemical Co.

(5) Dimethyl formamide (DMF) - Fisher Scientific

(6) Dry tetrahydrofuran (THF) - Sigma Chemical Co.

(7) N-hydroxysuccinimide (NHS) - Sigma Chemical Co.

(8) 1-ethyl-3-(3'-dimethylaminopropyl) carbodiimide hydrochloride (ECDI) - Sigma Chemical Co.

(9) Hydrochloric acid - Fisher Scientific

(10) Diethylene glycol monoethyl ether (Carbitol) - Sigma Chemical Co.

(11) Ethyl ether - Sigma Chemical Co.

(12) Ethyl acetate - Sigma Chemical Co.

(13) n-Heptane - Mallinckridt Chemical Works

(14) Triethylamine - Eastman Organic Chemicals

(15) Acetic anhydride - Fisher Scientific

(16) Methanol - Fisher Scientific

(17) Incstar 125I total T4 radioimmunoassay kit

(18) Anti-T4 antibody - Fitzgerald

(19) Oxalacetic acid - Sigma Chemical Co.

(20) NADH - Sigma Chemical Co.

(21) Methylene Chloride - Fisher Scientific

(22) 8-Anilino-1-naphthalene sulfonic acid (ANS) - Sigma Chemical Co.

**2. Reagents (Buffers)** [27]

(1) Dialysis Buffer (1 M $K_2HPO_4$, 1 mM $NaN_3$, and 1 mM EDTA):

Dissolve 17.418 g of $K_2HPO_4$, 0.065 g of $NaN_3$, and 0.03722 g of EDTA into 100 ml of distilled water. Store at 0°-4°C.

(2) Carbonate Buffer (50 mM $NaHCO_3/Na_2CO_3$, 25% Carbitol, and pH 9.2):

Dissolve 0.11 g of $NaHCO_3$, 0.265 g of $Na_2CO_3$, and 25 ml of carbitol into 50 ml of distilled water, adjust the pH to 9.2, and dilute to 100 ml.

(3) Potassium Phosphate Buffer (0.1 M $K_2HPO_4/KH_2PO_4$, pH 7.5):

Dissolve 14.11 g of anhydrous $K_2HPO_4$, 2.18 g of anhydrous $KH_2PO_4$ in 800 ml of distilled water, adjust pH to 7.5, dilute to 1 liter, and store cold.

(4) Substrate Solution (0.5 mM oxaloacetate and 0.2 mM NADH):

Dissolve 0.0066 g of oxaloacetic acid and 0.0156 g of NADH into 100 ml of 0.1 M potassium phosphate buffer. This reagent must be fresh made right before used.

(5) 0.1 % of BSA Solution

Dissolve 0.1 g of BSA into 100 ml of PBS buffer. Store no longer than one week.

(6) Stock Solution

a. Enzyme stock solution: 8 mg/ml

b. Conjugate stock solution: 0.8 mg/ml

c. Anti-T4 antibody stock solution: neat serum, no preservative

(7) Other Solution (Dilution)

a. enzyme dilution:

Pipet 10 ul of 8 mg/ml enzyme stock solution into 990 ul of glycerol to make 80 ug/ml of enzyme solution.

Pipet 10 ul of 100 times diluted enzyme solution into 990 ul of potassium phosphate buffer (pH 7.5) to make 0.8 ug/ml enzyme solution.

b. conjugate dilution:

Dilute enzyme conjugate 1000 times to make 0.8 ug/ml solution.

c. Anti-T4 antibody dilution:

Dilute antibody 100 times with 0.1 % BSA solution.

## 3. Equipment

- Rotavapor R110
- Perkin-Elmer Lambda 3 UV/Vis Spectrophotometer
- Gamma Counter

## 4. Procedure of Conjugation [28]

### *4.1 Preparation of N-methyliminodiacetic Acid Anhydride*

1. Reflux 1 g of N-methyliminodiacetic acid in 5 ml of acetic anhydride until all have been essentially dissolved (about 5 to 30 minute).
2. Remove the excess acetic anhydride and acetic acid under reduced pressure at 100°C until a nearly constant weight of residue is obtained.

### *4.2 Preparation of Methyl Thyroxine Hydrochloride*

1. 1 mmol of thyroxine is suspended in 10-15 ml of 2,2-dimethoxypropane, and 1 ml of concentrated hydrochloric acid is added.
2. The mixture is allowed to stand at room temperature overnight.
3. The volatile reactants are removed in rotary evaporator at a bath temperature not exceeding 60°C.
4. The remaining product is dissolved in a minimum amount of dry methanol, and the solution is diluted with 25 ml of dry ether.
5. Suction filter the precipitation, wash the precipition with ether until white solid forms, and weight the product.

### *4.3 Preparation of N-Methyl, N-carboxymethylglycyl Thyroxine Methyl Ester*

1. Dissolve 0.413 g of the methyl ester thyroxine hydrochloride under nitrogen blanket environment into a solution that contains 4 ml of dimethyl formamide (DMF), 5 ml of dry tetrahydrofuran (THF), and 130 ul of dry triethylamine.
2. Stir the mixture for 15 minutes, then add 1.5 ml of N-methyliminodiacetic acid anhydride in 2.5 ml of dry THF in one addition.

3. Volatilize the solution in a rotary evaporator to make a foamy solid.
4. Dissolve the foamy solid in 12.5 ml of THF, and extract the solution with a combination of 15 ml of distilled water and 25 ml of ethyl acetate.
5. Separate the solution, and extract the aqueous layer three times with 12.5 ml of ethyl acetate.
6. Combine the organic layers, and extract them once with 25 ml of saturated NaCl.
7. Dry the organic layer with anhydrous magnesium sulfate overnight.
8. Suction filter of the organic layer, and evaporate the solvent to produce a white solid.
9. Dissolve the white solid in 15 ml of THF with 10 ml of chloroform, and heat the solution to reflux, then add n-heptane slowly.
10. Reduce the volume of the solution until a definite cloud persisted, and allow the solution to cool down at room temperature, followed by the cooling in a freezer.

### *4.4 Preparation of T4-MEMIDA N-hydroxysuccinimide Ester (Compound II)*

1. Dissolve 10 mg of T4-MEMIDA and 1.3 mg of N-hydroxysuccinimide (NHS) into 250 ul of dry DMF.
2. Keep the reaction mixture at 0°C under a nitrogen blanket while stirring.
3. Add 2.3 mg of 1-ethyl-3(3'-dimethylaminopropyl) carbodiimide hydrochloride (ECDI) into the mixture, and maintain the mixture at 0°C until the ECDI has

been dissolved.

4. Allow the solution to stand at 4°C overnight.

### *4.5 Preparation of Conjugate*

1. Pipet 100 ul of MDH stock (8.0 mg/ml) into 900 ul of carbonate buffer.
2. Add 250 ul of DMF to 1 ml of MDH-buffer, and stir the mixture.
3. Add 12 ul total of compound II in 3 ul increments (15 minutes apart) to ensure complete reaction.
4. Purify the conjugates with dialysis at 2-4°C against 1 M $K_2HPO_4$, 1 mM $NaN_3$ and 1 mM EDTA.

## 5. Procedure of Immunoassay

### *5.1 Test the Concentration of T4-derivative in the Conjugate Solution*

1. Pipet 10 ul of Incstar T4 standard samples and 1000 times dilution of conjugate into antibody coated tubes.
2. Add 1 ml of tracer into the tubes, and incubate for 45 minutes at room temperature.
3. Decant the solution, and count the tubes with gamma counter.

### *5.2 Original Enzyme and Conjugated Enzyme Activity Assay*

1. Pipet 10 ul of 0.8 ug/ml of initial enzyme solution and enzyme conjugate solution into individual test tubes.

2. Add 2 ml of substrate into each tube, and measure the absorbance for 4 minutes.
3. Plot the figure of absorbance changes vs time, and calculate the specific activity.

### *5.3 Reactivation Test of Conjugated Enzyme*

1. Pipet 10 ul of 0.8 ug/ml of conjugated enzyme into series of test tubes.
2. Add different volume of anti-T4 antibodies (Fitzgerald serum fraction, diluted 100 fold) into the test tubes (Table 3).
3. Add 2 ml of substrate into these tubes, and measure the absorbance for 8 minutes.

Table 4

Volume of Each Reagent in Enzyme Activity Test

| Tube # | Antibody ( ul ) | PBS ( ul ) | Substrate (ml) |
|---|---|---|---|
| 1 | 0 | 200 | 2 |
| 2 | 30 | 170 | 2 |
| 3 | 50 | 150 | 2 |
| 4 | 100 | 100 | 2 |
| 5 | 200 | 0 | 2 |

### *5.4 Enzyme Immunoassay*

1. Pipet 10 ul of different concentration of standard T4 sera into test tubes.
2. Add 100 ul of ANS into the tubes, and incubate the mixture for 10 minutes.
3. Add 10 ul of T4 enzyme conjugate and 100 ul of anti-T4 antibody into these test tubes.
4. Add 2 ml of substrate into these tubes, and measure the absorbance for 8 minutes (Table 4).
5. Calculate the enzyme activity change in different T4 concentration.

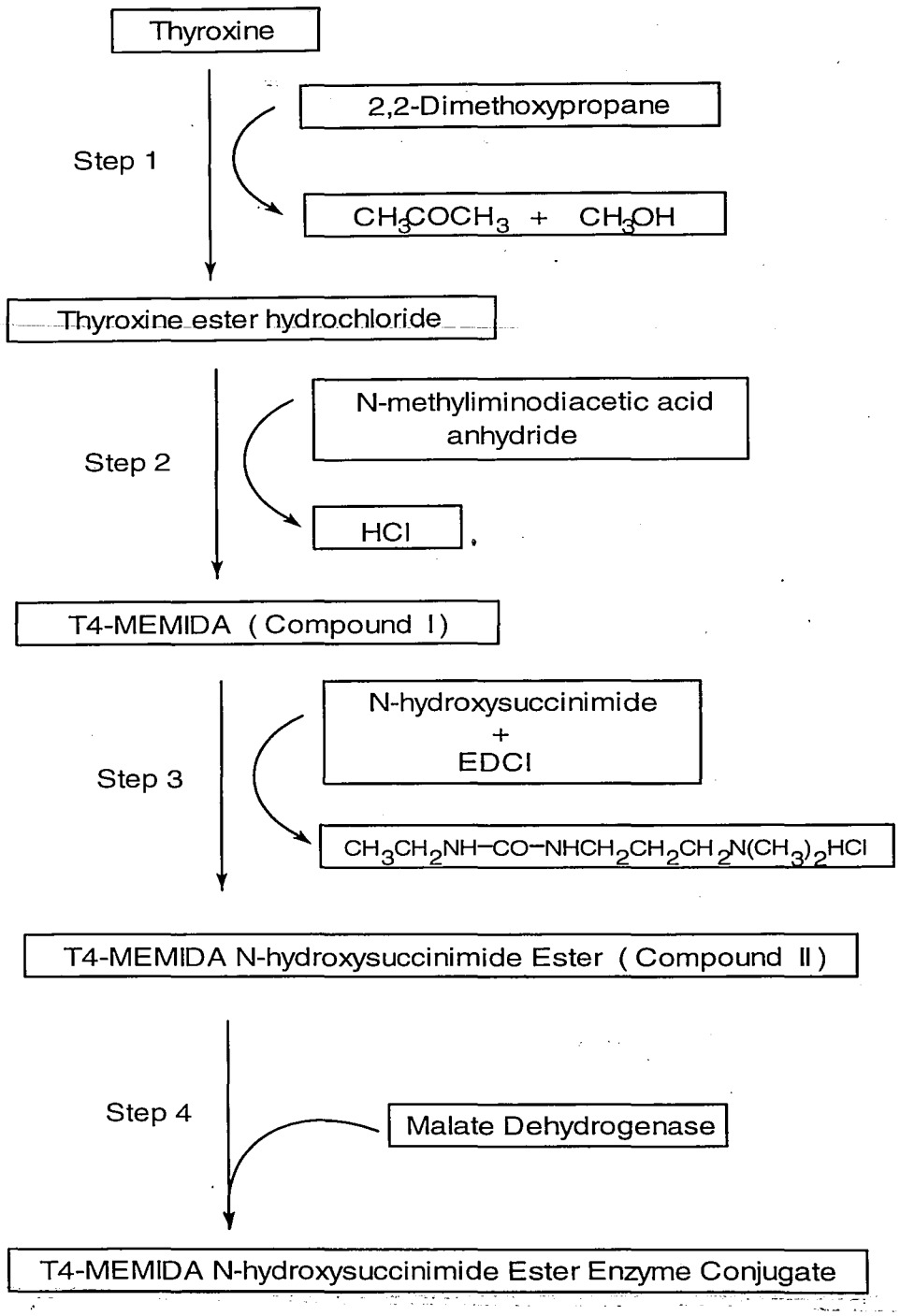
Table 5

Volume of Each Reagent in EIA Test

| Tube # | STD (ul) | Conc. (ug/dl) | Conj. (ul) | ANS (ul) | Ab (ul) | Subs. (ul) |
|---|---|---|---|---|---|---|
| 1 | 10 | 0 | 10 | 100 | 100 | 2 |
| 2 | 10 | 1 | 10 | 100 | 100 | 2 |
| 3 | 10 | 4 | 10 | 100 | 100 | 2 |
| 4 | 10 | 8 | 10 | 100 | 100 | 2 |
| 5 | 10 | 12 | 10 | 100 | 100 | 2 |
| 6 | 10 | 20 | 10 | 100 | 100 | 2 |

## RESULTS

### 1. Reaction Schemes



**2. Enzyme Conjugation**

The thyroxine ester hydrochloride was made according to step 1. The volatile reactants and by-products were evaporated in the rotary evaporator at a bath temperature not exceeding 60°C. Recrystallization from methanol-ether generated a white crystalline ester hydrochloride. This was a single compound by TLC analysis as shown in Fig. 8. Yields of the thyroxine ester hydrochloride was 97% in this procedure.

Theoretically, only compound I is generated in step 2. In practice, however, a couple of side reactions might occur. Because N-methyliminodiacetic acid anhydride was prepared through refluxing the N-methyliminodiacetic acid in excess acetic anhydride, the product was slightly more than the expected amount. Distillation under vacuum can remove most acetic anhydride at 110°C and acetic acid at 90°C. In this step, a dark brown oil was obtained, which might be anhydride mixture instead of pure N-methyliminodiacetic acid anhydride. After the coupling of methyl ester thyroxine hydrochloride with anhydride mixture, a thick brown oil was obtained. When the thick brown oil was extracted by the combination of distilled water and ethyl acetate, and by the subsequent aqueous saturated sodium chloride, a yellow oil was obtained. Most undesired by-products were removed within aqueous phase [29]. Since the product is an oily material, the percentage of yield can not be calculated at this step. Thin-layer chromatography (TLC) was performed in triethylamine/methanol/methylene chloride (0.2:1:10, v/v) solvent to test the product purity. As indicated in Fig. 8, some un-reacted N-methyliminodiacetic acid anhydride and methyl thyroxine hydrochloride remained in

the oil.

In step 3, both N-hydroxysuccinimide and ECDI are water soluble and can be removed by the washing with water. Therefore, the product of compound II is quite pure. Thin-layer chromatography was also performed on the solution with n-hexane/methylene chloride (1:5, v/v) to test the purity of compound II (Fig. 9).

In step 4, 12 ul of compound II was added to malate dehydrogenase solution to inactivate the enzyme completely. In order to remove the un-reacted T4-derivatives from the conjugated solution, dialysis was performed overnight against 1 M $K_2HPO_4$, 1 mM $NaN_3$, and 1 mM EDTA at 2-4°C. When the concentration of T4-derivative on both sides of the membrane reached an equilibrium, the purification stopped.

**3. Enzyme Activity Test**

Reaction of malate dehydrogenase with compound II caused strong inhibition of enzyme activity. Before the dialysis, the conjugated enzyme was 97.4% inhibited. After dialysis, the enzyme activity was 81.3% inhibited (Figs. 10, 11). In order to test the concentration of the T4-derivative in solution, a regular radioimmunoassay was conducted by the using of Incstar total T4 kit. The concentration of T4-derivative in solution was determined by interpolation from the T4 standard curve in the form of count per minute (CPM) vs concentration (ug/dL) (Fig. 12) [30]. The concentration of T4 derivatives was 32,000 ug/dl and 18,300 ug/dl before and after the dialysis respectively. Apparently,

excess thyroxine derivatives were removed through the dialysis, and the binding of thyroxine with enzyme reached a new equilibrium. In this experiment, we assumed that the protein concentrations remain unchanged before and after conjugation. Based on the calculation result, an average of 1.73 thyroxine derivative had bound to each enzyme.

The thyroxine conjugate was stable at 4°C for one week and degraded rapidly on second week. On day eight, there was about 47.2% of inhibition left. And, on day forty, there was only 17.5% of inhibition left (Fig. 13). It seemed that about 17.5% of enzyme inactivation was irreversible.

Addition of the anti-thyroxine antibody to the conjugate led to the restoring of enzyme activity (Fig. 14). The optimal amount of anti-thyroxine antibodies added was 100 ul of 100 fold dilution. Without addition of anti-thyroxine antibody, the conjugated enzyme had 18.7% of initial enzyme activity. With addition of 100 ul of 100 fold dilution antibody, the conjugated enzyme had 59.3% of initial enzyme activity. With addition of 200 ul of 100 dilution antibody, the conjugated enzyme had 69.1% of initial enzyme activity. Further addition of the antibody slightly increased conjugated enzyme activity.

**4. Thyroxine Enzyme Immunoassay**

Sera from clinically diagnosed hypothyroid (n=57) and hyperthyroid patients (n=34) have been evaluated by Becton Dickinson (Table 5) [31]. Therefore, a concentration range between 0 ug/dL and 20 ug/dL was chosen to build up a standard

curve (Fig. 15). A concentration of 0 ug/dL, 1 ug/dL, 4 ug/dL, 8 ug/dL, 12ug/dL, and 20 ug/dL of thyroxine in standard solution competes with enzyme labeled thyroxine derivative for limited antibody binding sites (100 ul of 100 fold diluted serum). If we defined that the conjugated enzyme activity is 100% when the thyroxine concentration is 0 ug/dL in solution, the calculated result indicated that the conjugated enzyme activity is 71.8% when the thyroxine concentration is 20 ug/dL in solution. Each thyroxine concentration between 0 ug/dL - 20 ug/dL corresponded to one activity data.

Table 6

Clinical Values of Total T4

| Population | Mean | Range |
|---|---|---|
| Normal | 8.33 ug/dL | 5 - 12 ug/dL |
| Hypothyroid | 2.21 ug/dL | 0 - 4.8 ug/dL |
| Hyperthyroid | 15.6 ug/dL | 11.7 - 19.5 ug/dL |

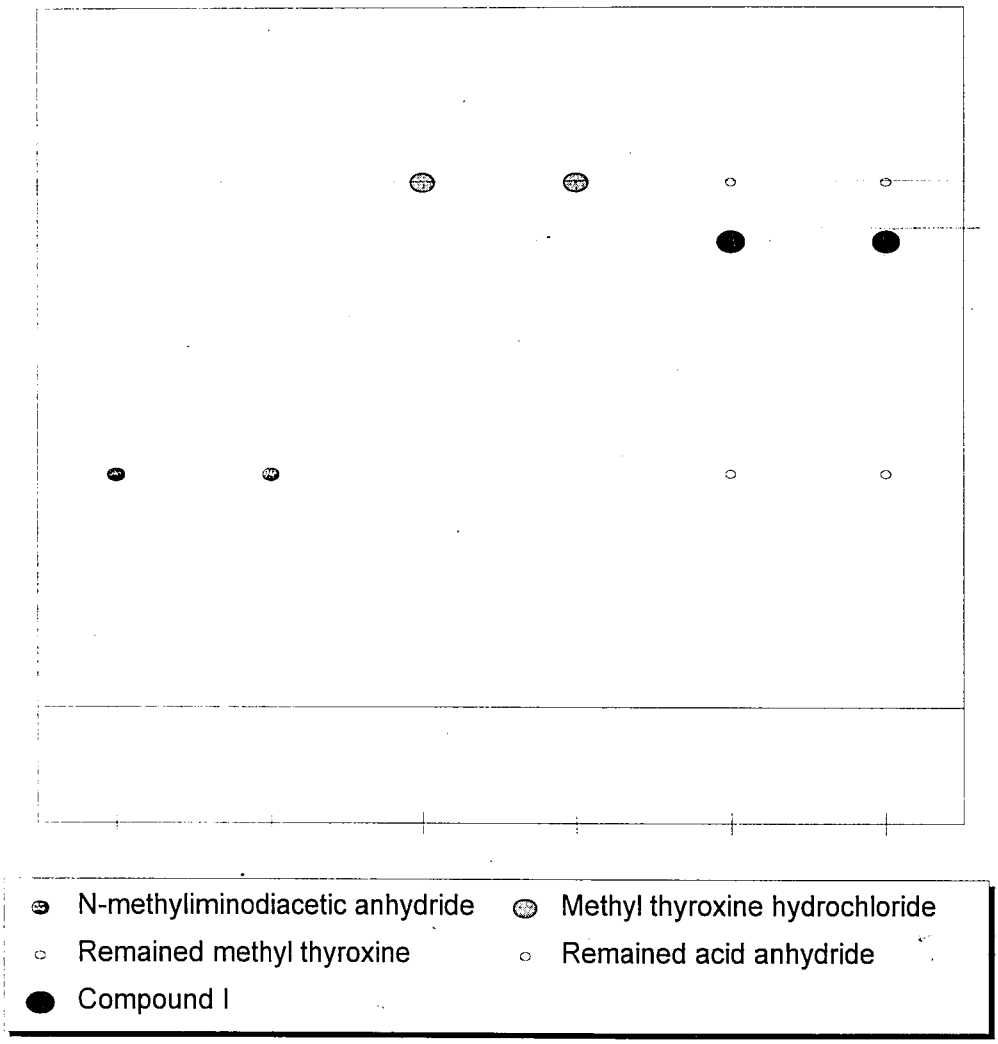



Fig. 8 Thin Layer Chromatography of Compound I

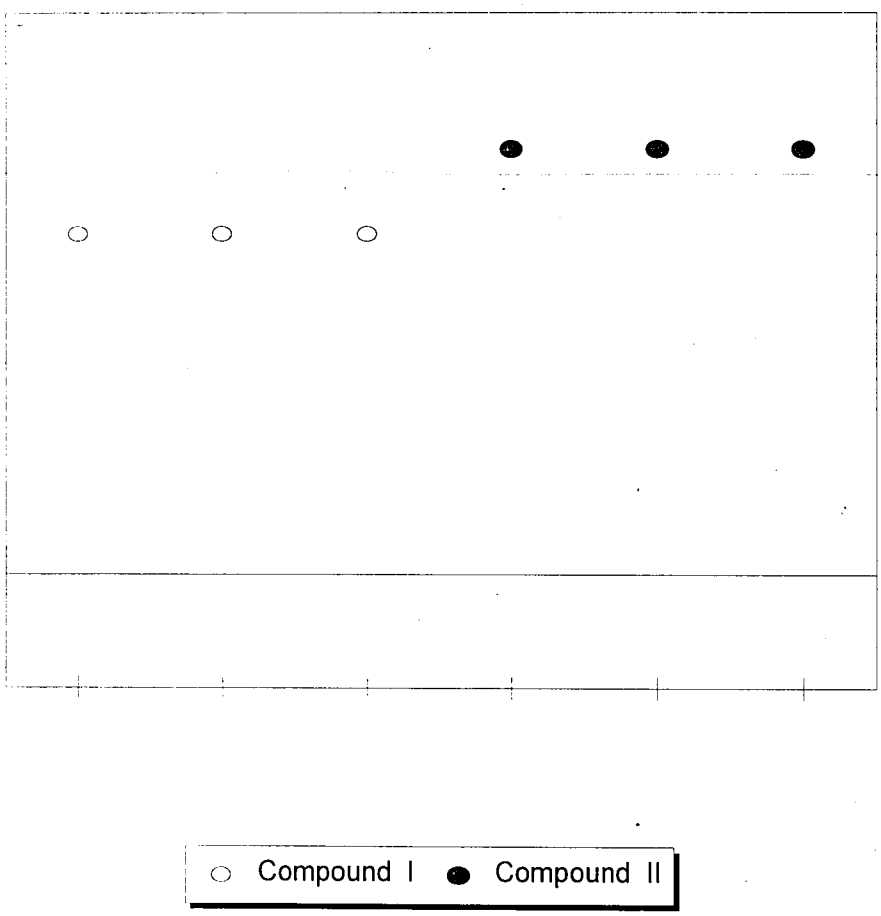



Fig. 9 Thin Layer Chromatography of Compound II

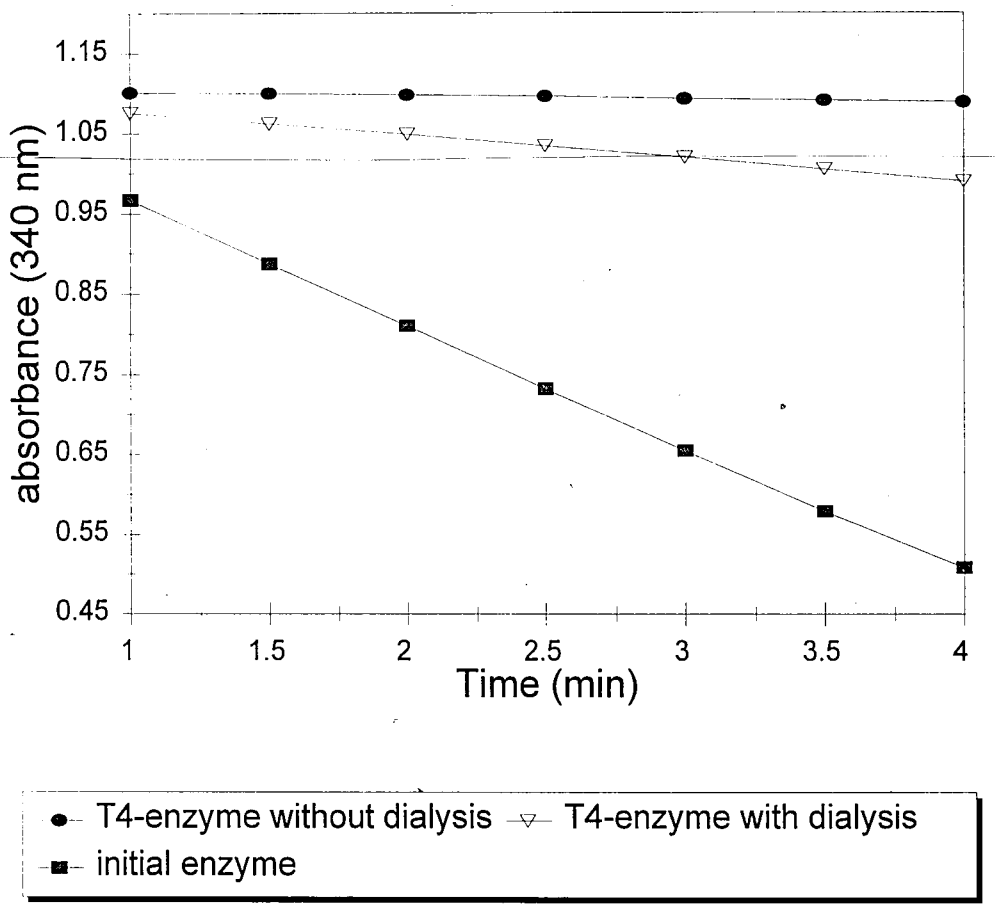



Fig. 10 Comparison of Reaction Rate

between Conjugate Enzyme and Original Enzyme

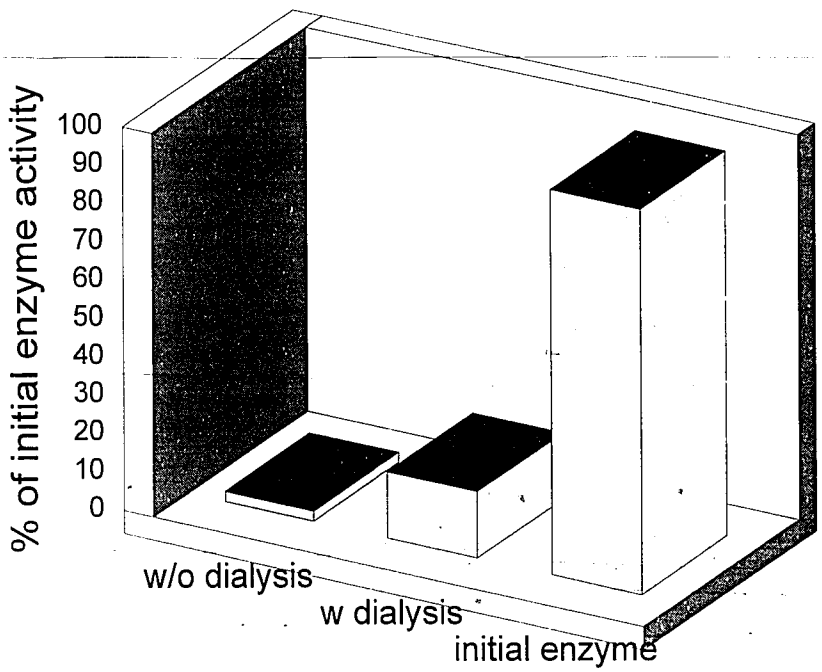



Fig. 11 Inhibition of Conjugated Enzyme before and after Dialysis

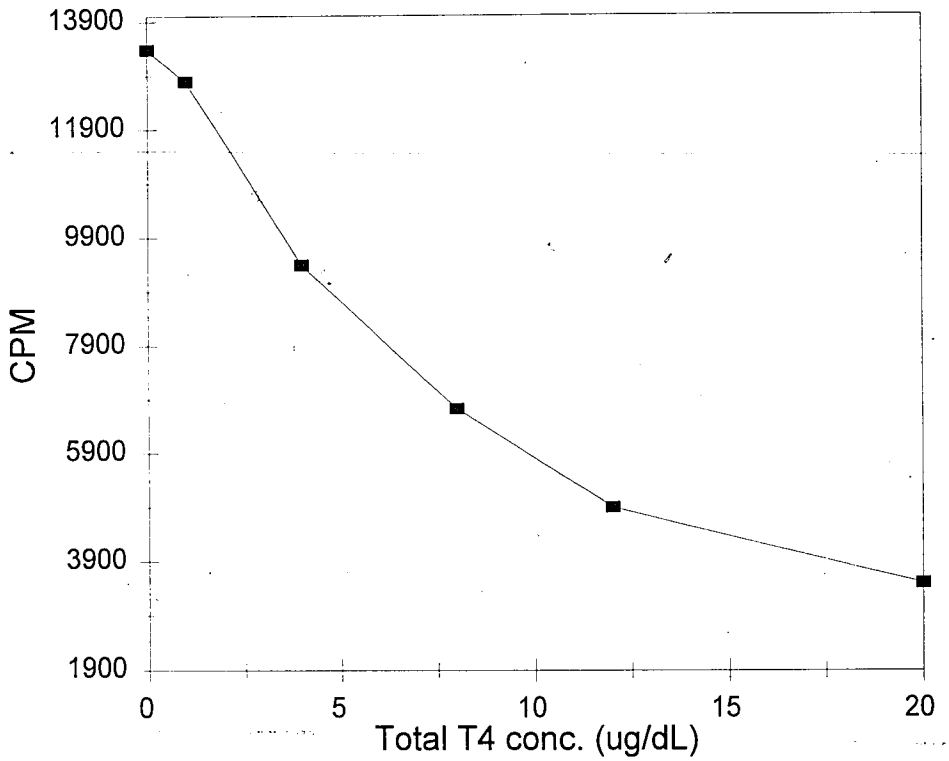



Fig. 12 RIA Response Curve for Thyroxine Derivative

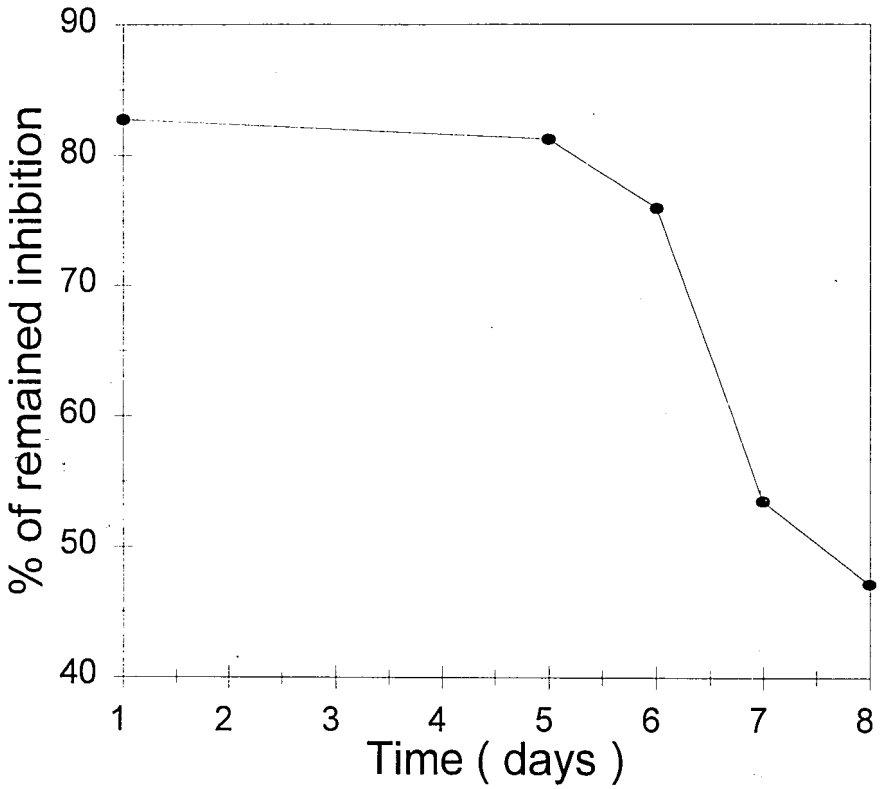



Fig. 13 Conjugate Dissociation vs Time

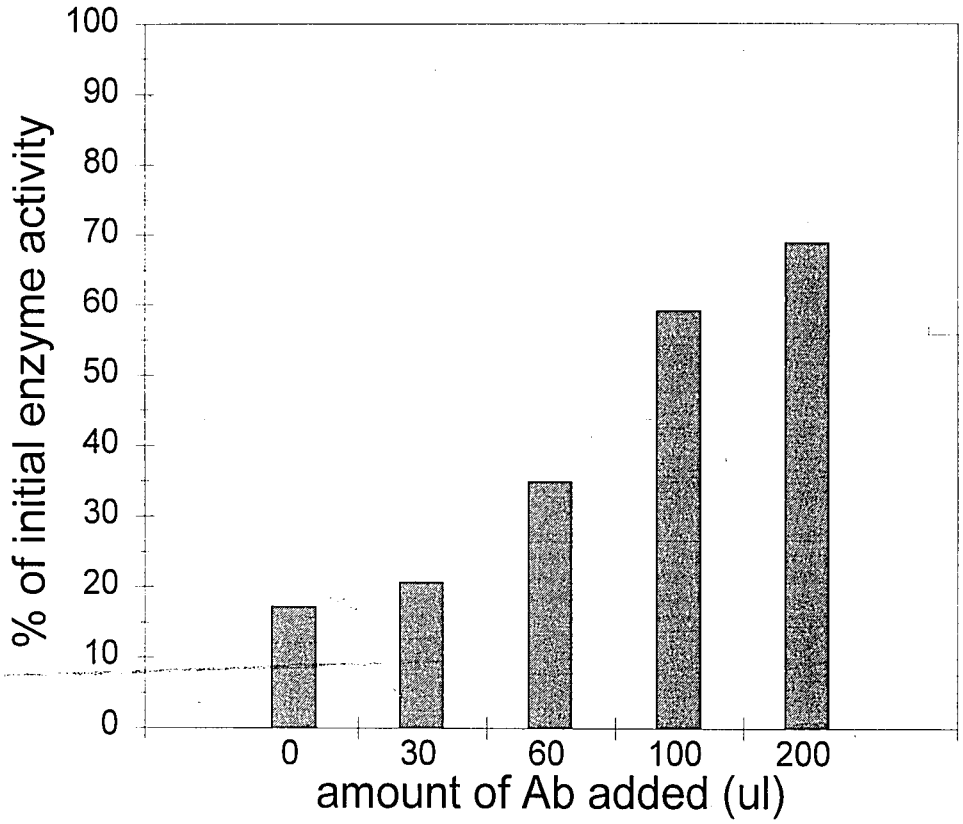



Fig. 14 Reactivation of T4-MDH Conjugate by Adding Anti-T4 Antibody

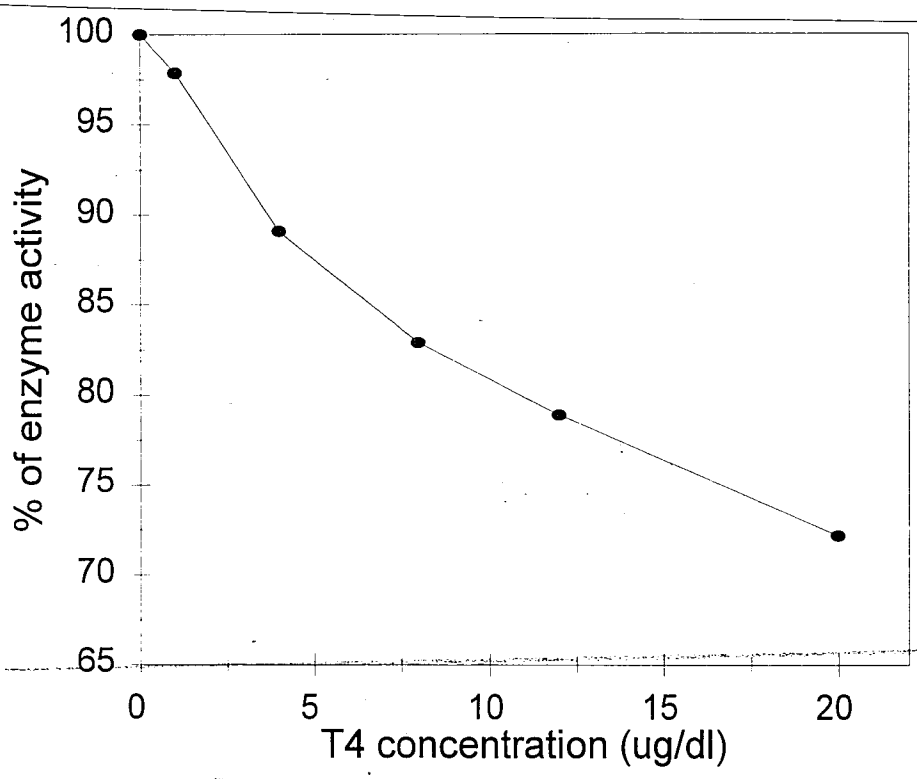



Fig. 15 Homogeneous Enzyme-immunoassay Response Curve for Thyroxine

# DISCUSSION

## 1. Enzyme Immunoassay of Thyroxine

For over ten years, the major significance of a homogeneous enzyme immunoassay for serum thyroxine has been the fact that it can be adapted to an automated biochemical profiling instrument. At present, the major advantage for us to study thyroxine homogeneous enzyme immunoassay is that it can be applied in the simultaneous enzyme immunoassay for thyroid function tests. In RIA, the simultaneous assay can not be approached to test more than two analytes because of the requirement of the separation steps and the limited source of the radioisotope. On the other hand, simultaneous testing three or four analytes is possible in EIA by labeling these analytes with different enzymes and testing enzyme activities in different wavelengths. Since the enzyme activity of thyroxine-MDH conjugate can be detected in ultraviolet wavelength, there is no color interfere with other visible wavelength absorbance. Therefore, it is significant to study the thyroxine-MDH conjugate for developing simultaneous assay.

## 2. Stability of the Conjugate

One of the advantages of EIA is the high stability of reagents. In our experiment, the enzyme conjugate is less stable than expected. Since some of stabilizers may interfere with the malate dehydrogenase activity, the synthesized enzyme conjugate remains unstable. In order to store the conjugate for longer time, an effective approach is to

lyophilize the conjugate, store it in a solid form, and dissolve the required amount right before use.

### 3. Purification of Antibody

In this experiment, anti-thyroxine antibody sera were used to reactivate enzyme. Many proteins in the serum might interfere with enzyme activity detection. Besides, the unpurified antibody might respond to the conjugate slowly. Therefore, for better assay performance, purification of antibodies is suggested through precipitation with 50% saturated ammonium sulfate and DEAE-cellulose chromatography.

### 4. Purification of Conjugate

In order to remove excess compound II, dialysis has been conducted. During a 12 hour dialysis against 1 M phosphate buffer, the enzyme inhibition losses 16.1%. Therefore, a better purification method for conjugate needs to be investigated. Moreover, because the impurity of the N-methyliminodiacetic acid anhydride interferes with the purity of the followed products, the preparation of the N-methyliminodiacetic acid anhydride must be further improved in such experiments.

## CONCLUSION

Conjugated malate dehydrogenase with the thyroxine derivative, a T4-MEMIDA N-hydroxysuccinimide ester, leads to over 90% inhibition of initial enzyme activity. Addition of anti-thyroxine antibody reverses the inhibition in different degrees. Before the addition of anti-thyroxine antibody, the conjugated enzyme has 18.7% of initial enzyme activity. After the addition of 100 ul of 100 fold dilution antibody, the conjugated enzyme has 59.3% of initial enzyme activity. The conjugated enzyme activity has been restored 40.7%. This volume of anti-thyroxine antibody is used for the competitive, homogeneous enzyme immunoassay in which separation steps are no longer required. A conjugated enzyme activity in the assay corresponds to a concentration between 0 ug/dL and 20 ug/dL. The whole procedure can be done in about 20 minutes which is three times shorter than other procedures. The possibility of environmental hazards is eliminated by the replacement of radioisotope with enzyme label.

The project has completed the most essential step in the multiple enzyme assay system. Further studies are needed to identify the effective combination of different enzymes in the simultaneous T4, T3, T3U and TSH testing system.

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## BRIEF BIOGRAPHY

The author, Wen Shi, was born in Fuzhou, China on March 18, 1967. Her father is Xiangquan Shi, and mother is Yuzhi Lin. On September 1985, she attended the South China University of Technology, and graduated with a B.S. degree in Chemical Engineering on July 1989. From 1989 to 1991, the author was an Assistant Engineer at Commercial Service Co., Shenzhen, China. In 1992, she attended Lehigh University to pursue her M.S. degree in Chemistry. During her studying in Lehigh University, she also served Laboratory Services, Inc. as a Research Assistant.

# END OF TITLE