



THE SYNTHESIS OF THYROACTIVE IODINATED PROTEINS

by

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A THESIS

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TABLE OF CONTENTS

	<u>Page</u>
I. Introduction	1
A. The Thyroid Gland and Thyroxine	1
B. Artificial Iodinated Proteins	3
1. Early Production of Iodoproteins	3
2. Mode of Combination of Iodine with Proteins	4
3. Thyroidal Activity of Iodoproteins	5
4. Practical Aspects of Iodoproteins	6
5. Recent Production of Iodoproteins	9
C. Mechanism of Thyroxine Formation	12
II. Experimental	15
A. Equipment	15
B. Assay Procedure	17
C. Experiments and Results	20
1. Investigation of Reinske's Procedure	20
2. A Simplified Method of Producing Iodocassia	24
3. Further Investigation of Variables	27
4. Formulation of an Improved Process	29
5. Oxygen- KMnO_4 Inter-relations	31
6. Metal Catalysis	35
7. Enzymatic Conversions	37
8. Time and Temperature Relationships	39
III. Summary	80
IV. Bibliography	82

LIST OF TABLES

	<u>Page</u>
I. Tabulation of Results of Reactions #1-79	21
II. A Summary of Reactions Concerning Catalysis	25
III. A Summary of Reactions Concerning Concentration	32
IV. Oxygen-KMnO ₄ Inter-relations	34
V. Effect of KMnO ₄ Concentration	35
VI. Effect of Presence of Metals	37
VII. Effect of Enzymatic Pre-digestion	39
VIII. Effect of Time and Temperature: Procedure I	43
IX. Effect of time and Temperature: Procedure II	44
X. Comparison of Averages of Table VIII and Table IX	45

LIST OF ILLUSTRATIONS

<u>Figure</u>	<u>Page</u>
1. Effects of Time and Temperature on Thyroxine Yield: Procedure I	46
2. Effects of Time and Temperature on Thyroxine Yield: Procedure II	47
3. Effects of Time and Temperature on Thyroxine Yield: Procedure I and II	48
4. Yield vs. Temperature: Procedure I and II	49

I. INTRODUCTION

A. THE THYROID GLAND AND THYROXINE

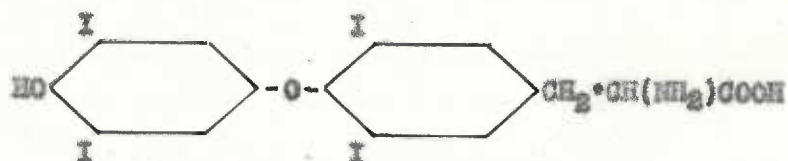
The purpose of this thesis is to study the production of iodinated thyroactive proteins. Such compounds have resulted from a research for an artificial thyroactive material similar to that produced by the thyroid gland of the body.

Clinical observations led to the majority of discoveries concerning the function of the thyroid gland. There are numerous conditions due to misfunction of this gland such as goiters, hypothyroid states (cretinism, myxedema), and hyperthyroid states. According to Harington⁽¹⁾, "The function of the thyroid gland is to maintain the general metabolic rate of the organism at its proper level and to achieve this by the production of the requisite amount of a specific secretion or hormone." This represents a general overall evaluation of the function of the thyroid gland.

It was noted early that iodine exerted favorable effects in the treatment of certain thyroid conditions. In 1895 Baumann⁽²⁾ established that the gland contains a large amount of iodine in organic combination. That it is present as an iodoprotein was discovered by Hutchison⁽³⁾ and Oswald⁽⁴⁾. Naturally, work was directed towards isolation of the active principle of the thyroid gland. Baumann⁽²⁾ succeeded in obtaining a hydrolytic concentrate which he named iodothyria. Hutchison⁽³⁾, in furthering the work of Baumann, obtained an even more potent concentrate. Finally, thyroglobulin, which contains the active iodine constituent, was prepared by Oswald⁽⁴⁾.

The active principle, thyroxine, was finally isolated by Kendall⁽⁵⁾ in 1919. This achievement provided the final proof of the essential connection

of iodine with the activity of the thyroid. He believed the empirical formula to be $C_{11}H_{10}O_3NI_3$. It was not until 1927 that the correct chemical formula, $C_{15}H_{11}O_4NI_4$, and the configuration of thyroxine were established by Harington and Barger⁽⁷⁾. They confirmed their work by synthesizing thyroxine, whose structural formula is shown below.



3,5,3',5' tetra-iodothyronine

Thyroxine has the following physical and chemical characteristics. It occurs as colorless, odorless, tasteless, needle-like crystals. It is unstable to light. The melting point is 231-232°C. with decomposition and evolution of iodine. The d and l forms differ from the racemic compound only in their slightly higher melting points of 235-236°C. The molecular weight of thyroxine is 776.77. The iodine content is 65.36% and the nitrogen content is 1.80%.

Thyroxine is insoluble in water, in alcohol, and in the usual organic solvents, but in the presence of mineral acids or alkalies, it dissolves in alcohol. It is soluble in solutions of the alkali hydroxides and in hot solutions of the alkali carbonates. When alkali hydroxide solutions of thyroxine are saturated with sodium chloride, the sodium salt of thyroxine separates.

The monosodium salt is also prepared by dissolving thyroxine in the minimum quantity of a boiling 0.1 N solution of sodium carbonate. Upon cooling, the very insoluble salt precipitates. This procedure is of use in separating pure thyroxine from other substances.

Commercial thyroid powder which is used therapeutically contains from 0.17 to 0.23% iodine, over half of it as thyroxine. The dosage in thyroid deficiencies is from 30 to 60 mg. When pure thyroxine is used, 0.2 to 2 mg. is the dosage. Thyroxine produces less effect when given orally than when injected. This appears to be due to inefficient absorption because of the limited solubility of the compound. Since thyroid powder is effective orally, is less expensive and is readily obtained, it is generally used therapeutically. (1,8,9)

B. ARTIFICIAL IODINATED PROTEINS

1. Early Production of Iodoproteins

Simultaneous with the work on the natural thyroid proteins and thyroxine, investigations on artificial iodinated proteins were being conducted. Apparently the earliest work on such substances was carried out by Boehm and Berg⁽¹⁰⁾ in 1876. Blum⁽¹¹⁾, in 1897, made claims to the production of iodoprotein, but these were not confirmed. Hutchison⁽⁵⁾ had attempted to increase the potency of thyroid protein by further iodination but was unsuccessful. Liebricht⁽¹²⁾ iodinated casein and after further treatment obtained a series of products with varying iodine contents. He reacted 20 gm. of iodine with 80 gm. of casein at 100°C. Further procedures consisted in extracting the brown powder obtained with ether, in treatment with alkaline bisulfite, and in digestion with sulfuric acid. About the same time, both Hofmeister⁽¹³⁾ and Kuraieff⁽¹⁴⁾ iodinated proteins with iodine liberated from a mixture of potassium iodide, potassium iodate and sulfuric acid. Various time intervals and temperatures were used, but, in general, the temperatures employed were low.

In 1906, Blum and Vaubel⁽¹⁵⁾ found that the hydriodic acid liberated as a side product prevented the reaction from going to completion. This led to the use of a sodium bicarbonate buffer solution which neutralized the acid. The techniques now used for making iodinated proteins are very similar to this process of forty years ago.

Ammoniacal solutions have been used more recently for the iodination of proteins. Blum and Strauss⁽¹⁶⁾ employed a 1.5-2% solution of protein in 5-4% ammonia to which was added 0.1 N iodine solution in small portions. The reaction was carried out at room temperature.

Fitzgerald⁽¹⁷⁾ produced a "protein-iodine" compound by bringing the protein and iodine together in the dry state and grinding in a closed vessel. The grinding and heat thus produced apparently vaporized the iodine and caused it to combine with the protein.

The foregoing investigations were only the beginnings of the problem of preparing iodoproteins. A new era began with the work of Ludwig and von Mutzenbecher^(18,19) in the late 1930's. The fact that their process for making iodoproteins formed a substance similar to the active protein of the thyroid gland was established by the actual isolation of thyroxine from their product. They first accomplished this isolation. To prepare iodoprotein they mixed 100 gm. of casein, 4 l of water and 30 gm. sodium bicarbonate and then added 20 gm. of finely ground iodine in small portions. The reaction temperature was 37°C. and the mixture was stirred for 24 hours. From the iodocasein produced in this reaction, which contained 7% iodine, they were able to isolate thyroxine.

2. Mode of Combination of Iodine with Proteins

These investigations on the iodination of proteins led to speculations as to the nature of the combination of iodine with the proteins. One of the first facts noted about the reaction was that the Millon and Adamkiewicz reactions became negative after iodination, therefore both tyrosine and

tryptophane were involved. In addition, protein sulfur was oxidized indicating some breakdown of the protein. (13,14)

The importance of tyrosine as an iodine-binding group was further indicated by the discovery of diiodotyrosine in gorgonin, a protein derived from the axial skeleton of the coral. Diiodotyrosine was isolated from iodinated proteins by Oswald⁽²⁰⁾ in 1910, and Harington⁽²¹⁾ isolated it from a hydrolyzate of the thyroid gland in 1931.

Iodine-binding groups other than tyrosine were found to be present in proteins. Histidine, for example, takes up iodine, the extent depending upon the alkalinity of the solution and the amount of iodine used. Side reactions with tryptophane and cystine take place in the presence of excessive amounts of iodine.

In summary, the reactions of iodine with proteins are as follows: carbon iodination of tyrosine and histidine, HI formation from substitution, nitrogen iodination of histidine, and oxidation of cystine and tryptophane.

3. Thyroidal Activity of Iodoproteins

The ultimate aim of this work on iodinating proteins was the production of a physiologically active substance similar to the active protein of the thyroid gland. Various claims to thyroidal activity of iodoproteins were made during the early investigations.

Gudernatsch⁽²²⁾, in 1913, observed that thyroid substance stimulates the metamorphosis of frog tadpoles. That iodinated proteins are capable of producing the same effect was indicated by the work of Morse⁽²³⁾, Lenhart⁽²⁴⁾, and Rogoff and Marine⁽²⁵⁾. However, their experiments were not considered conclusive.

Brandt, Mattie, and Holte⁽²⁶⁾ were the first to concentrate a physiologically active substance from iodinated proteins. The acid insoluble

precipitate obtained after alkaline hydrolysis stimulated the metamorphosis of frog tadpoles. Abelin⁽²⁷⁾ designated this substance as homothyroxine. As stated before, Ludwig and von Mutzenbecher^(18,19) were the first to isolate thyroxine from iodinated proteins, and this work established that iodinated proteins contain the same active substance as the thyroid gland.

There has been prolonged controversy in the literature as to whether or not hydrolysis of iodoprotein preliminary to oral administration is necessary for physiological activity. Brandt⁽²⁸⁾, and Abelin⁽²⁷⁾ believed hydrolysis to be necessary. Zaer⁽²⁸⁾, Lerman and Selter⁽²⁹⁾, Harrington and Pitt Rivers⁽³⁰⁾ considered hydrolysis to be unnecessary. Reineke and Turner⁽³¹⁾ recently established that their preparations of iodocasein produce thyroidal effects when given orally without preliminary hydrolysis.

4. Practical Aspects of Iodoproteins

When iodoproteins were found to show thyroid activity without preliminary hydrolysis, a new field of research was opened up. This concerned the possible use of thyroactive iodoproteins for the control of certain processes in domestic animals. For example, by 1934 the thyroid gland had been found to exert a great influence on the milk and butterfat secretion in cows.⁽³²⁾ Either desiccated thyroid tissue or thyroxine increases milk and butterfat production.

Different types and breeds of animals exhibit variable basal metabolic rates which indicates normal variations in thyroid hormone secretion. Types of livestock used for fattening purposes have a low thyroid activity while others, such as dairy cows, have a higher activity. Also, there is a normal decline in metabolism with advancing age. Environmental temperatures affect the metabolic rate for cold weather causes it to increase.

These observations show that certain processes in domestic animals can

be controlled with the thyroid hormone. Early experimentation showed that partial thyroidectomy increases fattening in steers and causes a reduction in the milk production of dairy cattle. As stated, Graham⁽³²⁾, in 1934, reported the effects of thyroxine in stimulating milk production. This was confirmed by Jack and Bechdel⁽³³⁾, Folley and White⁽³⁴⁾ and Herman, Graham, and Turner⁽³⁵⁾.

These findings, however interesting, were not practical due to the high cost of the pure thyroxine and the desiccated thyroid gland. The development of thyroxine iodoproteins made such uses of this hormone possible and set off a number of investigations. Not only are the iodoproteins inexpensive and simple to produce, but they also contain a high percentage of thyroxine, approximately 3% compared to the less than 0.2% in desiccated thyroid. Research was directed towards increasing milk production in cows and improving growth and egg production in poultry.

The feeding of iodoprotein develops a hyperthyroid state in the animal. Some of the symptoms of extreme hyperthyroidism are: accelerated pulse, increased circulation rate, cardiac dilatation and hypertrophy, myocardial failure, nervous excitability, muscular weakness, protrusion of the eyeballs, metabolic rate increases, lowered tolerance to high temperatures and dissipation of the fat stores. The degree of these symptoms depends on the degree of hyperthyroidism. Thus the feeding of thyroxine in the form of iodoprotein to normal animals must be carefully controlled so that the hyperthyroidism remains sub-clinical.

When the production of thyroproteins made the hormone available for large scale tests, Reineke and Turner led the field of investigation⁽³⁶⁾. Blaxter⁽³⁷⁾, Reece⁽³⁸⁾, Van Landingham⁽³⁹⁾, Archibald⁽⁴⁰⁾, Seath⁽⁴¹⁾,

Moore (43) and many others did extensive work with iodinated proteins and domestic animals. Although many of the results conflict, and undoubtedly the complete story is unknown, the work to date allows some definite conclusions to be drawn.

As mentioned, most of the research concerned the effects of feeding thyroprotein to dairy cows. It is agreed that milk production is increased, sometimes as much as 20%. The butterfat may show a 50% increase. The usual dosage is now considered to be around 1 gm. per 100 pounds of body weight. It is important to watch the individual cow so that, if necessary, the dosage may either be reduced or raised, whatever the case may be. There are a great many variables governing the effects of thyroprotein on the animal, such as: the phase of lactation, the amount fed, individual differences, breed differences and environmental temperature.

Thyroprotein is particularly beneficial when fed in the declining phases of lactation as it maintains the milk production at a high level. In older animals whose thyroid activity has decreased normally, thyroprotein again maintains milk production. When the feeding of thyroprotein is stopped, the cows go dry. It is necessary to increase the food intake of the animals corresponding to the increase in milk production.

The effect of this treatment on the composition of the milk has been studied. The most important fact found is that no thyroxine appears in the milk.

There is no definite evidence that thyroprotein has harmful effects on the cow when it is fed correctly. As the basal metabolism is increased, the heart beat is increased, but within limits this cannot be considered harmful.

The effects of feeding thyroprotein to poultry have been studied. Experimentation on growth effects shows a stimulation of growth in chicks.

Feathering is also speeded up. Egg production in laying hens is improved, especially in the summer and in the case of older hens. Thyroxine does not appear in the egg.

5. Recent Production of Iodoproteins

The possible large consumption of iodoproteins in the field of animal husbandry would make attractive any chemical manufacturing process that could inexpensively yield iodoproteins of high thyroidal potency. Recently, Reineke and Turner⁽³⁶⁾ investigated the various factors influencing the reaction of iodinating proteins and their relation to the potency of the final product.

a. Procedure

Although Reineke and Turner used the general procedure for iodinating proteins set up by Blum and Vaubal⁽¹⁵⁾, which was also used by Ludwig and von Hutzenbecher^(18,19), they made two major changes. First, they limited the amount of iodine used and second, they incubated the mixture at elevated temperatures. Their general procedure is as follows⁽³⁶⁾:

Twenty gm. of casein is placed in 700 cc. of distilled water containing 5 gm. of sodium bicarbonate, and is dissolved by stirring. The mixture is then placed in a water bath held at 38-40°C., and a total of 3.7 gm. of finely powdered iodine is added in small portions over a period of 3-4 hours, the solution meanwhile being agitated vigorously with a mechanical stirrer. When the requisite amount of iodine has been added, the solution is incubated at 70°C., with vigorous stirring for 18 to 20 hours. After dialysis, the iodinated protein is recovered by isoelectric precipitation, dried and ground to a fine powder.

Their products were tested for thyroïdal potency by both biological and chemical assays.

b. Iodination

Reineke and Turner first studied the effects of the extent of iodination on the reaction. Ludwig and von Mützenbecher⁽¹⁸⁾ suggested the limitation of the amount of iodine used in the process, but the effect of this on the potency of the product was not stated. Maus⁽⁴³⁾, while working with serum albumin in an ammoniacal medium, had shown thyroïdal activity to increase until 3-4 atoms of iodine per mole of tyrosine were combined. However, Reineke and Turner⁽⁴⁴⁾ obtained different results. They found the activity increased with increasing iodination until 4-5 atoms of iodine per mole of tyrosine had been added. Since in the iodination reaction one half of the total iodine added is converted to hydriodic acid, two atoms of iodine per mole of tyrosine are substituted, according to the following equation:



Further iodination caused a decrease in the potency of the iodoprotein. This observation held even at the higher incubation temperatures. The reason for this decrease in activity at higher levels of iodination is probably the further oxidations and side reactions which take place. In addition to this limitation of iodination, Reineke also used low, 35°C., temperatures for iodination and added the iodine in small increments over a 3-4 hour period.

c. pH

The pH of the reaction mixture was also found to be quite important⁽⁴⁵⁾. The pH affects the relative reactivity of the iodine-binding groups in the protein and thus the amount of thyroxine formed. As mentioned before, a good buffer system is necessary to take care of the hydriodic acid which is continually being formed. The optimum pH for the reaction is pH 7 or above.

d. Temperature

The most noticeable deviation from the early methods of forming iodoproteins is the increased temperature of the incubation period. Early workers had formed their iodoproteins at 37°C. and had incubated for 2 to 3 days in some cases. Reineke and Turner⁽⁴⁵⁾ used the low temperatures at first but found that raising the temperature to 60 and 70°C. increased the potency of the product greatly and shortened the incubation time. A temperature of 39°C. for 28 hours gave little increase in potency, but raising the temperature to 65°C. during the last 18 hours of incubation gave large increases in the percentage of thyroxine of the final product. They then incubated at various temperatures from 30 to 97°C. From 30 to 45°C. the potency remained at a uniform level. There was a rise at 60°C. and the maximum temperature was found to be 70°C. Further increases to 97°C. resulted in a decline in activity. The thyroxine content also increased progressively upon increasing the length of the incubation period up to 24 hours. At this point the thyroxine formation appears to continue, but at a constantly diminishing rate.

e. Catalyst

Reineke and Turner⁽⁴⁶⁾ observed that iodoproteins prepared in the presence of a common brass stirrer contained a higher percentage of thyroxine than those made with glass equipment. They believed the metal to be catalyzing the reaction. Salts of the oxides of copper, iron, and cerium were tried, with no effect. However, manganese compounds affected the results markedly. With all other factors constant, Mn_2O_4 and glucose reduced $KMnO_4$ increased markedly the thyroxine content of iodoproteins. Other manganese compounds gave smaller but definite increases.

The rate of stirring is another important factor. From the results of

their experiments on diiodotyrosine⁽⁴⁷⁾, Reineke and Turner believe the effect of stirring to be due to aeration and oxygenation of the solution. The necessity for the presence of air was further established by experiments conducted in an atmosphere of nitrogen. It is postulated that manganese will accelerate the reaction only in the presence of oxygen, acting possibly as an oxygen carrier.

By the addition of the catalyst and with sufficient stirring, the thyroxine content of iodocasein was increased from 2.8 to 3.37% in 24 hours. Since the tyrosine content of casein is 5.82%, the theoretical yield of thyroxine is 10.6%, so that a thyroxine content of 3% represents a 30% yield.

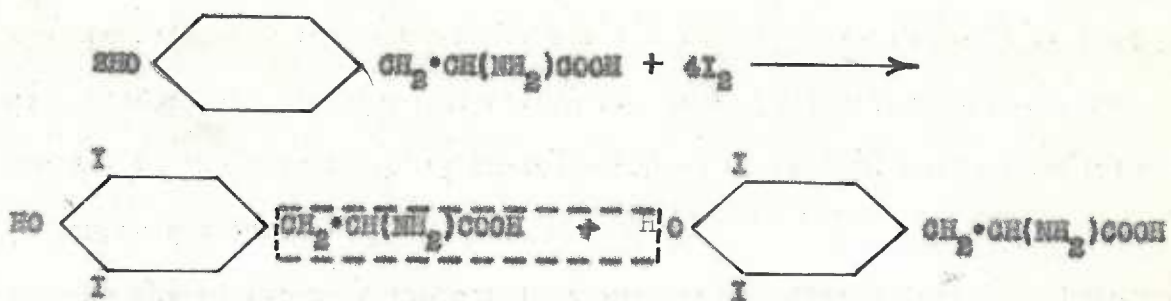
F. Proteins suitable for iodination

Although casein generally was used by Reineke and Turner and previous investigators, other proteins are suitable for iodination and the formation of thyroactive compounds if they contain tyrosine. The relative positions of the tyrosine molecules within the protein, as well as its tyrosine content, undoubtedly govern the thyroidal potency of the iodinated product. As all previous work shows, the percentage of thyroxine depends also on a suitable method of iodination. Some of the proteins used other than casein are serum albumin, serum globulin, silk fibroin, edestin, milk proteins, soybean proteins, and egg albumin.

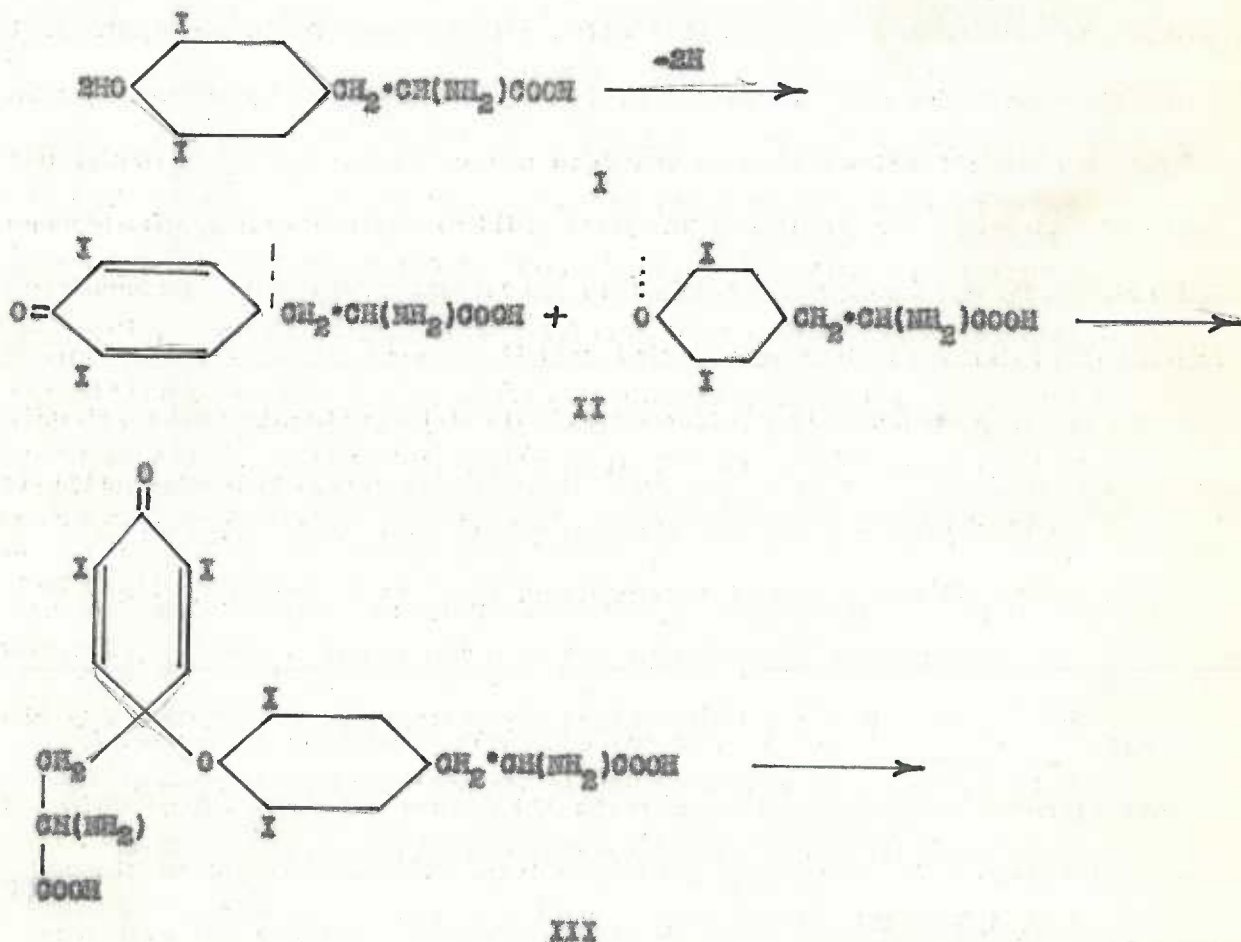
G. MECHANISM OF THYROXINE FORMATION

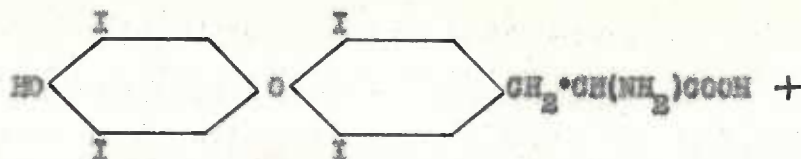
The equation mentioned above showing the combination of iodine with tyrosine to form diiodotyrosine illustrates merely the first stage in the synthesis of thyroxine. The previously discussed incubation period is concerned with the coupling or condensation of diiodotyrosine molecules to form

thyroxine. Harington and Barger⁽⁷⁾ suggested the following scheme to account for this reaction.

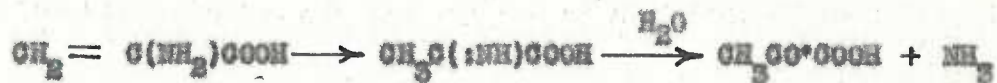


At that time this oxidative mechanism seemed the most feasible explanation. A more recent detailed scheme was proposed by Johnson and Tewkesbury⁽⁴⁸⁾ who used diiodotyrosine rather than the complex protein for their experimentation.





IV



Compound III would be formed from the oxidative coupling of two molecules of diiodotyrosine. It could then follow one of two courses, (a) molecular dissociation with the loss of one alanine side chain and the formation of thyroxine, IV, and imino pyruvic acid, or (b) hydrolysis, with the production of serine. Since pyruvic acid and ammonia have been identified in the reaction mixture, it is likely that scheme (a) is the predominant one.

Many of the early reports (19,30,48,49,50) believed the coupling reaction to be brought about by the mild oxidative reaction of hypiodite. However, the necessity for air and/or oxygen in the system was shown by Barkdoll and Ross (51) and Reineke and Turner (47). Also manganese oxide is effective as a catalyst in the presence, but not in the absence of added air. It appears, then, that atmospheric oxygen rather than the hypiodite is responsible for the oxidative coupling reaction.

II. EXPERIMENTAL

A. EQUIPMENT

With the realization that many phases of the production of thyroactive iodoproteins are as yet not completely understood, I undertook an investigation of the various factors influencing the reaction. In addition to attempting to simplify and modify existing procedures, the time, temperature and catalyst were studied with the aim of improving the thyroxine content of iodocasein.

To begin with, Reineke's procedure was referred to. However, the first problem to solve was that of obtaining the right type of reaction flask and accessory equipment.

ASSEMBLY A

A Fisher Unitized constant temperature water bath was used to maintain incubation temperatures. For stirring the reaction mixture, the electric stirrer of the Warburg bath, rpm 1550, was utilized. This was fitted with a 1/4 inch stainless steel shaft and a 1-1/2 inch paddle with four blades.

With open reaction vessels, the problem of excessive evaporation at the high temperatures used during the long incubation period was encountered. If the preparations became too concentrated or completely dry, the reaction had to be discarded. Therefore, a closed system was devised with the use of a two liter three-necked flask fitted with stirrer, water condenser, and air inlet tube. This flask was arranged in the constant temperature, constant level water bath. Washed air was supplied to the system through a tube which extended below the surface of the mixture, and the stirrer shaft was sealed by a bearing mounted in a tight rubber stopper. The condenser was made

especially long to take care of the excessive evaporation encountered due not only to the temperature, but also to the stream of air blowing through the system. With this apparatus the volume of the reaction mixture remained constant. This assembly served to study reaction mixtures of one liter water volume.

ASSEMBLY B

As Assembly A was quite cumbersome and not too practical, a new arrangement of equipment was devised. It was found that a two liter round bottom flask with a 1-5/8 inch neck opening supported in a 20 gallon steam jacketed kettle for the constant temperature water bath gave a satisfactory assembly. The temperature control was a Terrice steam regulator valve which could be adjusted for temperatures within the range of 140-160°F. A 1/4 horsepower Lightnin' mixer with a 1/2 inch stainless steel shaft fitted with a small 1-1/2 inch paddle was mounted over the kettle on a 2x8 inch plank which was securely bolted to the masonry wall. Stirring was adjusted to achieve vigorous agitation and to give a good vortex, assuring maximum aeration. Due perhaps to the size of the shaft and/or some other unknown conditions, serious evaporation was not encountered to the extent it had been in the early experiments with open flasks.

ASSEMBLY C

When it was found necessary to run two batches simultaneously, another apparatus was set up. A small Lightnin' mixer with a 1/4 inch stainless steel shaft and a 1-1/2 inch paddle with four blades was used. The Fisher Unitized constant temperature, constant level water bath was used again. Some evaporation from the two liter round bottom reaction flask occurred, but was at a minimum.

ASSEMBLY D

The above assemblies served to study reactions of 1 liter water volume. In an attempt to simulate pilot plant conditions, a twenty gallon, stainless steel, steam jacketed kettle was utilized for large preparations of a 10 gallon water volume. Mounted over the kettle was the 1/4 horsepower Lightnin' mixer equipped with a 1/2 inch stainless steel shaft 30 inches long and two 3 inch, three bladed paddles. The rpm of the stirrer was adjusted to achieve vigorous agitation and to give a good vortex, assuring maximum aeration. The steam jacketed stainless steel kettle was equipped with a Terrier temperature control which could be adjusted for temperatures within the range of 140 to 190°F.

B. ASSAY PROCEDURE

Much of the early work on iodinated proteins was difficult to evaluate due to the absence of suitable standard methods of analysis. Biological assays were used for the most part. To assay my preparations for thyroxine, the chemical procedure which Reineke⁽⁵²⁾ modified from Blau's method⁽⁵³⁾ was used. He established that this chemical assay agreed with the biological assays which were in use, such as the stimulation of metamorphosis in frog tadpoles and the elevation of the metabolic rate and decrease in body weight of guinea pigs. The chemical procedure with modifications is as follows:

HYDROLYSIS

Exactly 1 gm. of iodinated casein, 3.2 gm. of $\text{Ba}(\text{OH})_2 \cdot 8\text{H}_2\text{O}$ and 6.4 cc. of distilled water are placed in a 14x3 cm. test tube. An air cooled reflux condenser is attached and the test tube is placed in a vigorously boiling

water bath. When the barium has dissolved, the contents of the tube are stirred well, care being taken to work any material adhering to the side walls down into the solution. The preparation is then replaced in the boiling water bath and the heating is continued for a total of 20 hours. The hydrolysate is mixed with 25 cc. of distilled water and set aside for a few minutes to allow the coarse precipitate of barium salts to settle. The supernatant fluid is then decanted into a 100 cc. volumetric flask. The precipitate of barium salts remaining in the test tube is then decomposed by adding 2 cc. of n-butanol (re-distilled) and exactly 5.0 cc. of 3.5 N HCl, shaking and warming slightly in a water bath to effect complete solution of the precipitate. The dissolved substance is transferred quantitatively to the volumetric flask, and distilled water is added to bring the volume up to 100 cc.

EXTRACTION

A 20 cc. aliquot of the well-mixed hydrolysate is delivered into a 250 cc. separatory funnel, and one drop of a 0.5% bromocresol green indicator solution, made up in 0.1 N sodium carbonate, is added. The solution is then acidified with the 3.5 N HCl to a definitely yellow color (about 3 drops).

The thyroxine is extracted from the hydrolysate by shaking with 20 cc. of n-butanol, then with 10 cc. of n-butanol. The combined butanol extracts are purified in turn by shaking with 25 cc., then with 15 cc. of 4 N sodium hydroxide solution containing 5% sodium bicarbonate, prepared as needed a liter at a time. The combined sodium hydroxide washes are then extracted with 15 cc. n-butanol. After each extraction the solutions are left long enough to insure good separation before the aqueous layer is drawn off.

The purified n-butanol extract is filtered directly into a 250 cc. nickel crucible through a softly packed plug of glass wool that is placed

in the stem of the separatory funnel. Finally, the separatory funnel is rinsed with 5 cc. of n-butanol, which is drained through the glass wool into the crucible. The n-butanol is most conveniently removed by evaporation on a steam bath under a stream of air in a hood.

IODINE DETERMINATION⁽¹⁾

After evaporation, 5 gm. of sodium hydroxide pellets and 5 cc. of water are added to the crucible. When total iodine is being determined on the sample of iodocasein, 0.3 gm. of iodocasein is used. The mixture is evaporated by cautious heating with a small Bunsen burner and when the water has been driven off, the fusion is continued by strong heating with a Meker burner in a deep sand bath. Towards the end of the reaction, periodical additions of a few milligrams of potassium nitrate are made until the organic matter is entirely destroyed, as indicated by the absence of effervescence after addition of nitrate. After cooling, the melt is transferred quantitatively to a 500 cc. wide-mouthed Erlenmeyer flask with the aid of 200 cc. of water; a little methyl orange and 1 cc. of 10% sodium bisulfite are added to the solution, followed by syrupy phosphoric acid until the indicator is faintly pink. Sufficient bromine is introduced to color the solution strongly; the mixture is stirred with an electric mixer, and then boiled briskly for 10 minutes with the addition of a few Hanger granules to promote smooth ebullition. After addition of 10 drops of 5% aqueous sodium salicylate, the solution is cooled, treated with 5 cc. of 10% potassium iodide and 5 cc. of syrupy phosphoric acid. The liberated iodine is titrated with 0.005 N sodium thiosulfate prepared and standardized as directed in the Student Biochemistry Laboratory Manual⁽⁵⁴⁾, using freshly prepared 0.5% soluble starch solution, made up according to Hawk⁽⁵⁵⁾, as indicator. One cc. of 0.005N thiosulfate is equivalent to 0.106 mg. of iodine. The percent thyroxine in the sample is then estimated by multiplying the percent iodine by 1.529.

The modifications consisted of extracting the 20 cc. aliquot of the hydrolysate a second time with 10 cc. of n-butanol and extracting the combined sodium hydroxide washes with 15 cc. of n-butanol. In an investigation of the assay procedure, using crystalline thyroxine, it was found that a significant amount of thyroxine was recovered in these extra washes.

From the results of assays on mixtures of 1 gm. of casein and 4-6 mg. of pure thyroxine, it was determined that around 97% of the thyroxine could be recovered by this assay procedure.

For convenience, a factor for computing thyroxine and total iodine percentages was formulated each time new thiosulfate was prepared. Example of a calculation follows:

% Thyroxine:

$$\frac{(\text{N of Thiosulfate}) (0.106) (5) (1.529) (100)}{(0.1) (1000)} = \text{Factor}$$

Factor x cc. of 1 to 20 dil. of approx. 0.1 N Thio = % Thyroxine

% Total Iodine:

$$\frac{(\text{N of Thiosulfate}) (0.106) (100)}{(0.005) (0.3) (1000)} = \text{Factor}$$

Factor x cc. of approx. 0.1 N Thio = % Total Iodine

M.005N
0.081

G. EXPERIMENTS AND RESULTS

1. INVESTIGATION OF REINEKE'S PROCEDURE

The first objective was to follow Reineke and Turner's work in an attempt to duplicate their results. The results of the various reactions which were run are found on Table I. Using Harris' purified casein, Reineke's procedure was followed, and after a number of failures due to difficulties with apparatus a 24 hour product containing approximately 3% thyroxine and 7% total

TABLE I

TABULATION OF RESULTS OF REACTIONS #1-78

#	Assembly	Procedure	Treatment	% Thyroxine	
				8 hours	24 hours
1-4	A	Reineke	KMnO ₄ oxides		----
5	A	Reineke	KMnO ₄ oxides		2.49
6	A	Reineke	KMnO ₄ oxides		3.24
7	A	Reineke	Avocet casein		3.34
8	A	Reineke	Dialysis		3.24
			No dialysis		3.51
9	A	Reineke	Raney nickel		3.20
10	A	Reineke	60 gm. casein/l; I ₂ at 70°C.; glucose reduced		
			KMnO ₄		3.33
11	A	Reineke	No catalyst		1.70
13	A	Reineke	Same as # 10		2.75
15	A	Reineke	Casein reduced KMnO ₄		2.45
17	A	Reineke	Casein reduced KMnO ₄		3.22
19	A	Reineke	KMnO ₄ in solution		2.78
20	A	Reineke	Heated KMnO ₄		2.17
23	A	Reineke	KMnO ₄ in solution; I ₂ added all at once; 70°C.		3.03
24	D	I	Time; yield; temp.; pH	1.92	2.90
25	D	I		2.40	3.50
26	D	I	I ₂ at 33°C.	1.36	1.70
27	D	I		1.80	2.40
28	D	I	HgO ₂	2.60	3.32
29	D	I	5 gm. KMnO ₄	1.55	2.29
30	D	I			2.81
31	D	I	Misc. salts; KMnO ₄		3.50
32	D	I			3.20
33	D	I			3.23
34	A	I			2.30
35	A	I	Tenkage		2.40
36	A	I	Butyl peroxide		1.80
37	A	I	Butyl peroxide; KMnO ₄		2.06
38	A	I	Mg. ions		----
39	A	I	Tenkage; butyl peroxide		2.50
40	A	I	Ni. ions		2.51
41	A	I	Tenkage; HgO ₂		2.34
42	A	I	Tenkage; HgO ₂		2.51
43	A	I	75 gm. casein/l		2.65
44	A	I	20 gm. NaNO ₃		3.03

TABLE I (Continued)

#	Assembly	Procedure	Treatment	% Thyroxine	
				8 hours	24 hours
45	A	I	15 gm. I ₂		2.45
46	A	I	Solubilizing agent		2.17
47	A	I	1 gm. K ₂ MnO ₄		1.90
48	B	I			2.00
49	B	I			3.27
50	B	I			3.42
51	B	I	Hg. ions		1.49
52	B	I	75 gm. casein/l		2.95
53	B	I	Tyrosine; 11.6 gm. I ₂		2.91
56	B	I	75 gm. casein/l; solu- bilizing agent		2.77
57	B	I	Rennin		3.28
58	B	I	Pre-treatment, NaOH		1.40
59	D	I	75 gm. casein/l		3.29
60		I	Pressure; 4 hours-1.40%		
61	B	I	Charcoal; 11 hours-2.51%		
62		I	Pressure; 7 hours-1.86%		
63	B	I	Early K ₂ MnO ₄	2.77	3.45
64	B	II		3.27	3.97
65	B	II		3.15	4.10
66	B	II	H ₂ O ₂	3.07	3.45
67	B	II	75 gm. casein/l 70°C.	2.23	3.41
68	B	II	No K ₂ MnO ₄ ; 70°C.	2.17	2.47
69	B	II	75 gm. casein/l 70°C.	2.65	3.52
70	B	II	75 gm. casein/l 70°C.	2.86	3.63
71	B	II	70°C.	2.89	3.17
72	B	II		3.19	4.15
73	B	I		2.25	3.10
74	B	II	No K ₂ MnO ₄		3.23
75	B	II	Caroid	4.13	4.74
76	B	II	75 gm. casein/l	2.85	3.49
78	B	II	Caroid; 75 gm. casein/l	2.72	3.24 - 8
79	C	II			2.64

iodine was obtained. (#6) In this reaction, 100 cc. of colloidal manganese oxides prepared according to Peters and Van Slyke⁽⁵⁶⁾ were used as the catalyst. The mixture was well aerated and rapidly stirred. During reaction of the casein with bicarbonate to form soluble sodium caseinate, much carbon dioxide is evolved with attendant foaming. Foaming is encountered also during the iodination step due to the bicarbonate reacting with the hydroiodic acid which is formed. To overcome this foaming, caprylic alcohol was used.

Crude feed casein was used in place of Harris casein and was found to react in the same manner. (#7) This casein was used in subsequent preparations

Reinske dialyzed his preparations against water as a purification procedure. In order to determine whether or not this is necessary, half a preparation obtained after precipitation and re-solution was dialyzed. (#8) Both total iodine and the percentage of thyroxine in the dialyzed portion were the same as found in the undialyzed fraction. Dialysis, accordingly, was omitted in succeeding preparations.

In one preparation (#10) the catalyst, concentration and method of iodination were varied relative to Reinske's procedure. The catalyst was prepared by reducing 1 gm. of $KMnO_4$ with 0.2 gm. glucose dissolved in 30 cc. of water containing 0.5 gm. of $NaHCO_3$. One half of this preparation was used. The iodination was carried out at $70^{\circ}C$. over a period of an hour. The concentration of casein in the reaction mixture was increased to 60 gm. per 1000 cc. of water and the other ingredients were increased proportionately. The thyroxine content of the resulting preparation after 24 hours reaction time was satisfactory.

A control without catalyst was tried (#11) giving a product containing less than 2% thyroxine in 24 hours.

Various reactions were run under the conditions of increased concentration and increased temperature of iodination with the aim of finding a

suitable method of catalysis. Table II summarizes these results. Reineke recommended Mn_2O_3 which was unavailable. In an attempt to make this compound, $KMnO_4$ was heated in a furnace at 600 to 800°C. and the resulting material used to catalyze a reaction. (#20) In addition to using the colloidal oxides of manganese prepared according to Peters and Van Slyke⁽⁵⁶⁾ (#1-8) and those formed by the reduction with glucose as described above (#10-13), a solution of $KMnO_4$ was reduced with casein and the mixture used as catalyst. (#14,16) As the latter procedure gave good results, permanganate was dissolved in water and added directly to the reaction mixture. (#19) All of these products prepared with the above catalysts gave high yields of thyroxine with the exception of that prepared with the catalyst obtained by heating $KMnO_4$ in the furnace. As the use of potassium permanganate, $KMnO_4$, directly is the most convenient, this method of catalyzing the reaction was utilized.

2. A Simplified Method of Producing Iodo-casein

With the knowledge gained from the above experiments, Reineke's basic procedure was altered in order to obtain a simplified and improved method of preparing iodo-casein.

a. Concentration

The concentration of casein was increased from 20 gm. per 700 cc. of water to 50 gm. per 1000 cc. of water. The other ingredients were increased in concentration proportionally. Although the concentration was more than double that used by Reineke, the resulting preparations had thyroxine contents similar to his preparations. This increased concentration is of considerable importance in any contemplated large scale operation.

b. Iodination

The iodination step was modified. In Reineke's procedure, finely ground iodine is added over a 3-4 hour period at 33-40°C. It was found that unground

TABLE II

A SUMMARY OF REACTIONS CONCERNING CATALYSIS

#	Assembly	Procedure	Catalyst	% Thyroxine 24 hours
1-3	A	Reineke	$KMnO_4$ oxides	3.00
9	A	Reineke	Raney nickel	3.00
10	A	I	Glucose reduced $KMnO_4$	3.38
13				2.75
15	A	I	Casein reduced $KMnO_4$	2.45
17	A	I		3.22
19	A	I	$KMnO_4$ in solution	2.78
23				3.00
31	D	I	Cu, Co, Fe, CrO_4 red.; $KMnO_4$	3.50
53	B	I	Tyrosine; $KMnO_4$	2.91
57	B	I	Rennin; $KMnO_4$	3.28
64	B	II	Oxygen; $KMnO_4$	4.00
28	D	I	H_2O_2 ; $KMnO_4$	3.32
11	A	Reineke	No catalyst	1.70
20	A	I	Heated $KMnO_4$	2.17
47	A	I	1 gm. $KMnO_4$	1.90
29	D	I	5 gm. $KMnO_4$	2.29
35	A	I	Tankage	2.40
36	A	I	Butyl peroxide	1.60
37	A	I	Butyl peroxide; $KMnO_4$	2.06
39	A	I	Butyl peroxide; tankage	2.50
41	A	I	Tankage; H_2O_2	2.34
42				2.51
51	B	I	Mg. ions	1.48
40	A	I	Nickel ions	2.31

Iodine added at 70°C. all at once gave somewhat better yields of thyroxine. In the large preparations (10 gallon) this method of iodination was found to be necessary for high yields of thyroxine. The higher temperature of 70°C. facilitates solution of the casein and also the iodination reaction.

c. Antifoam

Caprylic alcohol was first used to decrease the foaming encountered. (#1-14) This agent has an objectionable odor, was required in relatively large quantities, and was lost by evaporation. A Dow Corning Silicone Antifoam preparation was obtained and found to be very satisfactory. This agent was developed for suppressing foaming in aqueous solutions. D. C. Antifoam A is a colorless, tasteless, translucent material with a honey-like consistency. It is inert physiologically and only minute quantities are required.

d. Precipitation

The dialysis step was eliminated. A helpful fact concerning the precipitation was noted. It was found that when the reaction solution was precipitated while still warm, the precipitate settled better and was easier to filter than when precipitated in the cold. The hot precipitation produced a partially coagulated product.

The relation of pH to the precipitation was investigated and found to be not too critical. This was done by taking aliquots, precipitating them at various pH values as determined with the Beckman pH meter, filtering the precipitates on Gooch crucibles, and determining the weights and percentages of thyroxine of the precipitates. (#24) The precipitations were carried out both at room temperature and at 60°C. Variations in pH values between 3 and 4.5 were found to have no effect on the yield of iodocasein or the percent thyroxine, despite the fact that the isoelectric pH of iodocasein is 3.9. Accordingly, it was established that indicator paper would suffice for the

pH determination in the isoelectric precipitation. Furthermore, this experiment established that precipitation at the high temperatures had no adverse effects upon the yield of product or its thyroxine content.

e. Procedure I

With these new techniques, a method was formulated for the preparation of thyroactive iodocasein which gave consistently high yields of thyroxine and proved less complicated than previous methods. The improved procedure is as follows:

To one liter of water, which is stirred rapidly to allow maximum aeration, is added 15 gm. of A.R. NaHCO_3 . Sufficient (0.5 to 1 cc.) D.C. Antifoam A is introduced, then 60 gm. of Avocet feed casein is added slowly. The casein is allowed to dissolve for 15 minutes while the temperature is going up to 70°C . At this time, 10.4 gm. of Resublimed iodine is added all at once. This is allowed to go into solution and react for 15 minutes. The solution becomes quite yellow. Then the catalyst, 200 mg. of KMnO_4 dissolved in water, is added, and the solution turns a deep brown. The reaction is allowed to proceed for 24 hours after the addition of the catalyst. The iodocasein is precipitated at 60°C . by the addition of dil. HCl to pH 3.9 as determined with Acutint indicator paper. The precipitate is collected on a Buchner funnel and dried in a vacuum desiccator over conc. sulfuric acid.

After this procedure was found to give consistent results of 3-3.5% thyroxine in 24 hours, research was begun to improve the process so that a higher yield of thyroxine might be obtained.

3. Further Investigation of Variables

a. Catalysis

The most obvious variable to begin with was the catalyst. The results of these investigations are tabulated in Table II. As stated, manganese in the form of $KMnO_4$ or manganese oxides was quite efficient as a catalyst. Reineke believed these substances would not work unless oxygen was present. He postulated that the manganese was an oxygen carrier, but no conclusive proof for this was advanced.

The possible catalysis of the reaction by other metal ions was investigated. Some of the ions which were studied briefly were magnesium, nickel, copper, cobalt, chromate, and iron. These ions were used alone, in combination and with $KMnO_4$. (#31,38,40,51) They were used in the form of salts (100 mg.) or salts pre-reduced with glucose. There was no noticeable catalysis by any of the compounds tried.

Due to the apparent necessity for oxidative conditions to facilitate the coupling reaction, various peroxides were used for catalysis. Butyl peroxide and hydrogen peroxide were used alone and with $KMnO_4$. (#35,36,37,39,41,42) Again the reaction was not altered. Hemoglobin also failed as a catalyst. (#35,39,41,42)

b. Proportions

The proportions of the ingredients had previously been kept the same as in Reineke's procedure. In his procedure, sufficient $NaHSO_3$ to neutralize the HI formed and keep the mixture buffered is present. However, in one reaction (#44) 20 gm., rather than 15 gm., of $NaHSO_3$ was added, with no beneficial effects on the yield of thyroxine.

Earlier investigations showed the adverse effects of excess iodine. So, in one reaction (#45) the amount of iodine was increased from 10.4 to 15 gm. As expected, this treatment caused a decrease in the percent thyroxine of the final product.

c. Solubility

Since a highly concentrated colloidal protein system was being studied, not only the solubility, but also the degree of dispersion of the casein had to be considered. Solubilizing agents manufactured by Atlas (G 2800, ARL/6209 and Tween 20) were added to reactions (#46,56) to investigate these points. The agents had no effect upon the results.

d. Pressure

Having noted the effects of increased temperature on the reaction, it was attempted to study the effects of increased pressure. In many cases chemical reactivity is influenced by increasing the pressure on a system. A rough experiment (#60) investigating this was carried out by incubating the solution after iodination in a pressure cooker with no stirring or added air. Another reaction (#62) was run with oxygenation of the solution and atmosphere thoroughly, previous to applying the pressure. The reaction was not influenced by pressure under these conditions.

e. Concentration

The concentration of the casein had previously been increased to 60 gm. per liter of water. The possibility of using 75 gm. of casein per liter was investigated. In a series of preparations (#45,52,56,59,129) it was found that a high potency product could be produced even with the more concentrated solution. However, as shown in Table III, the percentages of thyroxine were generally slightly lower than those obtained using 60 gm. casein per liter.

4. Formulation of an Improved Process

In all the above-mentioned research, no factor had been found which affected the reaction markedly, either in speeding it up or in increasing the percentage of thyroxine in the final product.

a. Effect of oxygen

As aeration of the mixture is necessary for maximum yields of thy-

roxine, and as this is undoubtedly due to the oxygen of the air, pure oxygen was bubbled through the solution during a reaction. (#64) In this reaction the catalyst, KMnO_4 , was added after the casein had dissolved and was allowed to react for 15 minutes before the iodine was added. One preparation (#65) had indicated that this early addition of the permanganate might be advantageous. The reaction was further varied in that it was carried out at 80°C . Such treatment had a marked effect on the process for the velocity of the reaction was increased so that a product containing 3% thyroxine was obtained at 8 hours. Previously, many 24 hour preparations contained only 3% thyroxine. Furthermore, the 24 hour product of this reaction contained about 4% thyroxine, rather than the usual 3%. This high yield of thyroxine had not been attained previously. Thus, the effect of oxygen appears to be two-fold: (1) speeding the rate of reaction and (2) increasing the possible percentage of thyroxine. This same treatment was tried a number of times and was found to give consistent results. The yield of iodocasein was not affected by this more drastic process. Thus, from these findings, a new process for making iodocasein was formulated.

b. Procedure II

To one liter of water, which is stirred rapidly to allow for maximum aeration, is added 15 gm. A.R. NaHCO_3 . Then sufficient D.C. Antifoam A to prevent foaming is added. Sixty gm. of Avocat casein is added slowly. The casein is allowed to dissolve for 15 minutes while the temperature is going up to 80°C . Then the catalyst, 800 mg. of KMnO_4 , dissolved in water, is added. The solution turns brown. This is allowed to react for 15 minutes. At this time 10.4 gm. of iodine is added all at once. Oxygen is then bubbled through a tube into the so-

lution in a steady stream to allow maximum saturation of the solution with oxygen. The reaction is allowed to proceed for 24 hours from this time. The precipitation is as described in Procedure I.

With this new procedure established, various experiments were conducted concerning the different factors which surround such a process. It was found that by increasing the concentration of casein to 75 gm. per liter good yields of thyroxine were obtained, but they generally were not as high as when 60 gm. per liter were used. (#67,69,70,76,78,115) A summary of the effects of concentration of the solution is on Table III.

5. Oxygen-KMnO₄ Inter-relations

Table IV summarizes these studies. The effect of oxygen alone, without the catalyst, permanganate, was investigated. (#68,74,109) In the case of the 70°C. reaction, the results suggested that oxygen could largely replace permanganate as a catalyst, and at 80°C. this point was even more evident. Used alone, oxygen or permanganate can thus be considered equivalent in their power to affect the percentage of thyroxine of the final product. Pure oxygen and permanganate used together have a definite additive effect in catalyzing the reaction. These statements are based on experiments carried out with KMnO₄ in the presence of atmospheric oxygen.

The action of oxygen is obviously that of providing maximum oxidative conditions for the coupling reaction. The action of permanganate in catalyzing the reaction is not so clear. KMnO₄ is a very efficient oxidizing agent, but since it is reduced immediately upon addition to the casein solution, its oxidative effects are of short duration. The oxygen, on the other hand, provides oxidative conditions throughout the entire incubation period, while the coupling reaction goes on. The coupling does not occur

TABLE III

A SUMMARY OF REACTIONS CONCERNING CONCENTRATION

#	Assembly	Procedure	Concentration of casein	% Thyroxine 24 hours
1-9, 11	A	Reineke	20 gm. / 700 cc. H ₂ O	3.00
10, etc.	A	I	60 gm. / 1000 cc. H ₂ O	3-3.5%
43	B	I	75 gm. / 1000 cc. H ₂ O	2.65
52	B	I	75 gm. / 1000 cc. H ₂ O	2.95
56	B	I	75 gm. / 1000 cc. H ₂ O	2.77
59	B	I	75 gm. / 1000 cc. H ₂ O	3.29
129	B	I	75 gm. / 1000 cc. H ₂ O	2.62
67	B	II, 70°C.	75 gm. / 1000 cc. H ₂ O	3.41
69	B	II, 70°C.	75 gm. / 1000 cc. H ₂ O	3.32
70	B	II	75 gm. / 1000 cc. H ₂ O	3.66
76	B	II	75 gm. / 1000 cc. H ₂ O	3.49
115	B	II	75 gm. / 1000 cc. H ₂ O	3.45
78	B	II, caroid	75 gm. / 1000 cc. H ₂ O	3.24
44	A	I	20 gm. NaHCO ₃	3.03
45	A	I	15 gm. Iodine	2.43
47	A	I	1 gm. KMnO ₄	1.90

until diiodotyrosine is formed and is not an instantaneous process. Under these conditions, it is easier to understand the relation of oxygen to the coupling reaction than the relation of the short oxidative effect of the KMnO_4 . Since KMnO_4 is effective when added either before or after iodination, and since the oxides of KMnO_4 are effective, its role may be that of a simple catalyst and not related to its oxidative powers. Reineke's findings that manganese compounds are not catalytic when the reaction is carried out in an atmosphere of nitrogen indicates that the permanganate somehow makes the oxygen of the reaction atmosphere available to the mixture.

Some preliminary experiments were carried out in an atmosphere of nitrogen. Reactions with KMnO_4 , without KMnO_4 and with the oxides of KMnO_4 were run. The results obtained with reactions run in nitrogen without KMnO_4 show that a certain amount of thyroxine is formed even under the most adverse conditions. The results from this investigation substantiate Reineke's work, as no noticeable catalysis of the reaction was obtained when KMnO_4 was used in the absence of oxygen.

TABLE IV
OXYGEN-KMnO₄ INTER-RELATIONS

#	Assembly	Procedure	Treatment	% Thyroxine	
				8 hours	24 hours
		I	KMnO ₄	2.0	3.0
62	B	I	No KMnO ₄		2.0
63	B	I	No KMnO ₄ ; O ₂	2.0	2.5
			KMnO ₄ oxides	2.0	3.0
64	B	II	KMnO ₄	3.0	4.0
74	B	II	No KMnO ₄		3.2
89	B	II	No KMnO ₄	2.5	
109	B	II	No KMnO ₄	2.6	3.0
122	B	II	Oxides of KMnO ₄	2.9	3.0
120	C	N ₂	KMnO ₄ ; 80°C.	1.6	2.3
135	C	N ₂	KMnO ₄ ; 70°C.		1.9
123	C	N ₂	No KMnO ₄ ; 80°C.	1.8	2.3
134	C	N ₂	No KMnO ₄ ; 70°C.		1.5
125	C	N ₂	Oxides of KMnO ₄ ; 80°C.	1.2	1.6

In connection with the study on the action of permanganate, an attempt was made to determine the minimum amount of the KMnO₄ necessary for high yields of thyroxine. (#64, 85, 107, 119) As seen in Table V, a quantity as small as 10 mg. per the liter reaction is sufficient to produce a high yield of thyroxine. Assuming a molecular weight of 75,000 for casein, the 10 mg. of KMnO₄ represents a 10 to 1 molar ratio to the casein. But due to the many reducing groups in one molecule of casein, this amount of KMnO₄ must still be considered catalytic.

TABLE V
EFFECT OF THE $KMnO_4$ CONCENTRATION

#	Assembly	Procedure	Amount of $KMnO_4$	% Thyroxine 24 hours
94	C	I	10 mg.	3.0
94	D	I	25 mg.	2.9
93	A	I	200 mg.	3.0
96	B	I	1 gm.	2.8
107	B	II	10 mg.	3.9
94	B	II	200 mg.	4.0
119	B	II	1 gm.	2.8

6. Metal Catalysis

Previously it was stated that Reineke first noticed the effect of metal ions catalyzing the reaction from the results obtained when a brass stirrer was used. The type of brass was not mentioned, but in most brass, copper is present to 60-70% and zinc to 30-40%. Lead and iron are in trace amounts only.

To investigate this metal catalysis, paddles for the stainless steel shaft were made out of brass, copper, zinc, and tin. Reactions were run using these paddles alone or in combination, without $KMnO_4$. Table VI shows the results of these reactions.

The tin paddle had the effect of raising the yield of thyroxine only slightly.

The copper, brass and zinc paddles were used together in one reaction. The results from this were quite strange. Various stages of the assay procedure looked different from the normal and the result was the lowest ever obtained, the figure for thyroxine being about 0.3%. The same

results were obtained in reactions run with the copper and brass paddles used together and with each used alone. Since copper is the metal common to these reactions, it must be the agent responsible for these results.

Just how the metal stirrer acted in catalyzing Reineke's reaction is unknown. Perhaps a type of contact catalysis was operating. The other possibility is that at the surface of the paddle there was solution of the metal and the ions thus produced catalyzed the reaction. It is difficult to explain the results obtained here (Table VI), for the action may have been negative catalysis or actual destruction. The concentration of metal may have been different than Reineke's in these experiments. Perhaps a small amount of such metals is catalytic and a larger amount is not.

A reaction using the copper paddle was run (#132), and at 8 hours, the iodocasein contained 2% thyroxine. This indicates that during the early stages of the reaction copper may be catalytic. The results obtained above with reactions run for 24 hours with copper present indicate that destruction of thyroxine occurs. Again, this might be a concentration effect. At 8 hours, if there is solution of the metal, the concentration would not be as great as at 24 hours. When the copper concentration reaches a certain point, some sort of destruction of the thyroxine may begin.

TABLE VI
EFFECT OF THE PRESENCE OF METALS

#	Assembly	Procedure	Paddle	% Thyroxine 24 hours
82	B	I	Stainless	2.0
80	C	I	Cu, Zn, Brass	0.4
81	C	Reineke	Cu, Zn, Brass	0.5
106	C	I	Cu, Brass	0.5
116	C	Reineke	Cu, Brass	0.6
118	C	Reineke	Cu	0.4
130	C	I	Brass	0.5
133	C	I	Zn	2.1
96	C	I	Tin	2.3
93	C	Reineke	Tin	2.5
132	C	I	Cu; 8 hour--2.0	

7. Enzymatic Conversions

Although the theoretical yield of thyroxine from casein is around 10%, the highest ever obtained was 4%. When the size and configuration of a protein molecule is considered, it seems logical that the position of the tyrosine molecules within the protein may govern the yield of thyroxine. Since the tyrosine molecules occur at certain intervals in the protein structure, and as these intervals and the space arrangement of the protein are unknown, it is impossible to predict the amount of coupling of diiodotyrosine molecules that will occur. It is possible that the 3-4% yields of thyroxine represent the maximum yield in the unaltered protein molecule. Altering the molecule in some way so that more diiodotyrosine units could come in contact might allow more thyroxine to be formed.

To test this theory, the enzyme papain was utilized. This is a relatively stable, proteolytic enzyme which is active at an alkaline pH, and at 60-70°C. It is inactivated by oxidizing agents. A small amount of "Garoid" (an activated commercial papain) suspended in glycerin was added to the casein solution and allowed to react for 30 minutes at 60°C. From this point on Procedure II was used. This first preparation (#76) contained the highest percentage of thyroxine ever obtained. At eight hours, it contained 4% thyroxine and at 24 hours, it contained 4.6%. The yield of iodocasein was very low, indicating breakdown of the protein molecule. This breakdown of the molecule and alteration of the configuration is undoubtedly the factor which brings about the increased formation of thyroxine. More of the diiodotyrosine molecules may come in contact and couple. The problem remains yet to find the amount of enzyme which will allow for maximum yields of thyroxine with a minimum amount of breakdown, so that a reasonable yield of iodocasein may be obtained.

Further experiments were attempted using various brands of papain, but none of the results were as striking as the first. The yields of thyroxine and the iodocasein, however, were satisfactory. Table VII shows the results of this investigation. This preliminary work has opened up a new field of study and many interesting experiments remain to be done.

TABLE VII

EFFECT OF ENZYMATIC PRE-DIGESTION

#	Assembly	Procedure	Treatment	% Thyroxine		Yield gms.
				8 hours	24 hours	
75	B	II	Caroid	4.1	4.7	56
78	C	II	Caroid; 75 gm. casein/l	3.3		62
84	B	II	Papain N F	3.0		55
124	B	II	Optimo papain	3.6		53

8. Time and Temperature Relationships

Since results obtained with Procedure II indicated that temperature had a great effect on the reaction, a series of reactions were run in order to extensively study the time and temperature relations. Both Procedure I and II were used, and as two reactions were run daily, both Assembly B and C were utilized. Four temperatures were investigated; 60°, 70°, 80°, 90°C. Time samples were taken at 4, 8, 12, 16, and 24 hours.

Before these results are presented, a discussion of the variables affecting the reaction and its results is pertinent. First, there is a wide range of normal variation in the percent thyroxine obtained. As can be seen from results with Procedure I (Table I), the thyroxine content may vary from 2.75% to 3.5% under supposedly standard conditions. As a minimum, there is a normal variation of 0.5% thyroxine under the conditions used.

The long assay procedure is a variable, as there are a number of steps where both positive and negative errors may occur. With practice, duplicates show differences of 0.2% thyroxine which represents only 2 or

3 cc. of 0.005 N thiosulfate with titration values of 30-40 cc. This is approximately a 10% error.

There are a number of factors which may vary during the running of a reaction itself as it is very difficult to standardize conditions. The fact that four different assemblies were used adds to the problem of interpreting results. In a 24 hour process, there are many chances for variations. One important factor is the stirring. It was always attempted to have the stirring rapid and vigorous so the maximum amount of air or oxygen would be mixed in. However, the location of the shaft in respect to the center of the flask and also the depth of the paddle may greatly change the amount of air drawn into the reaction.

Another variable encountered was the evaporation from the reaction flask. The effects on the reaction of concentrating the solution are unknown. Either an increase or decrease of the thyroxine content of the final product may be brought about by this concentration of the solution. Although it was always attempted to maintain the level of the solution in the reaction flask, a significant amount of evaporation did occur and was extremely variable.

The other factor which might account for differences and variations in results is the temperature. Constant temperature water baths were used. Normally, they held the temperatures quite well with only 2-3 degree variations on either side of the required temperatures. However, even this variation is significant when there is only a 10 degree difference between the temperatures being tested. During the latter part of the experimentation, the steam kettle temperature control was not functioning well. Variations of 5 degrees on either side of the required temperature were sometimes encountered. Due to the critical effect of temperature

on the results, these excursions of temperature must be responsible for many of the variations in thyroxine yields which were found.

In spite of these uncontrollable variables, the results from the time and temperature reactions do allow for some interesting conclusions. Tables VIII, IX, X and Figures 1-4 summarize the results of this investigation. Some reactions were run 3 or 4 times, but the assays were not done in duplicate. From the general trend of results, it is possible to draw some conclusions. The results of the reactions were average, but certain values which were obtained and known to be grossly in error were not used. Curves were drawn from these averages to illustrate the effects of time and temperature on the yield of thyroxine. Due to the previously mentioned variations to which the results were subject, the values are not absolute, but only representative. Treating the results in this way allows the general effects of time, temperature and the procedure used to be illustrated.

Figures 1-4 indicate that the maximum temperature for the reaction is not 70°C ., as was stated by Reineke. The differences in the procedures used may account for this discrepancy, but this is not likely. In the range of temperatures investigated, $60-90^{\circ}\text{C}$., a direct relationship exists between the temperature of the reaction and the percentage of thyroxine obtained. (Figure 4) This is true with both Procedures I and II. These effects of temperature can probably be explained by the fact that increasing the temperature in a system usually speeds up the rate of a chemical reaction.

The ability of oxygen to increase the yield of thyroxine over that obtained with Procedure I is illustrated in Figures 2, 3, and 4. This effect is most noticeable in the lower temperature range. At 90°C ., the

great effect of this high temperature, though not over-riding the effect of oxygen, decreases it.

The work of previous investigators indicated that a 3-3.5% thyroxine yield was maximum. Even when the reaction was allowed to continue for more than 24 hours, this figure was not surpassed. Figures 1-4 show that products containing over 4% thyroxine are obtained when the reaction is run at 80 and 90°C. The results obtained with enzymatic conversions suggest that these high reaction temperatures may be functioning similar to a proteolytic enzyme. In addition to the effect of increasing the rate of the chemical reaction, 80 and 90°C. may possibly bring about some breakdown or change in the configuration of the protein molecule. While at 70°C. the unaltered protein molecule may be reacting, at 80°C. the molecule may be altered so that more diiodotyrosine residues are in contact, thus bringing about increased thyroxine formation.

The results as seen in Figures 1, 2, 3, seem to indicate the importance of the zero to 4 hour period in thyroxine formation. The greatest amount of thyroxine per unit time is formed during this period. The treatment employed in the early part of the reaction probably influences the rest of the process and the thyroxine content of the final product. The four hour reaction time should, therefore, be investigated more thoroughly with the aim of improving the yield of thyroxine and shortening the reaction time necessary for high yields of thyroxine.

TABLE VIII
EFFECTS OF TIME AND TEMPERATURE: PROCEDURE I

Hours #-Assembly	4	8 (% Thyroxine)	12	16	24
<u>60°C.</u>					
88-C	1.56	1.65	1.95		
92-C	1.49	1.65	1.76		
114-C			2.68		2.84
127-C	1.80	2.00			2.68
Average	1.6	1.8	2.1		2.7
<u>70°C.</u>					
24-D	1.70	1.90	2.00	2.76	3.10
25-D		2.40			3.50
28-D		2.49			3.32
73-B		2.25			3.10
85-C	2.25	2.45	2.76	2.82	3.06
90-C		2.15	2.33	2.54	2.94
103-B	1.65	1.91	2.33	2.57	3.04
140-B		2.39	2.52	2.71	3.13
Average	1.9	2.2	2.3	2.7	3.2
<u>80°C.</u>					
87-B	2.20	2.58	2.96		3.54
100-C	2.12	2.67	3.07	3.00	3.55
113-B	2.65	3.11	3.20	3.57	3.81
Average	2.3	2.8	3.1	3.3	3.6
<u>90°C.</u>					
88-C	2.94	3.43	3.49		3.84
93-C	3.01				4.19
112-C	2.84	3.39	3.69	3.76	4.03
Average	2.9	3.4	3.6	3.8	4.0

TABLE IX
EFFECTS OF TIME AND TEMPERATURE: PROCEDURE II

Hours #-Assembly	4	8 (% Thyroxine)	12	16	24
<u>60°C.</u>					
104-C	1.87				
108-C	2.44	2.57			2.93
128-C	1.67	1.71			
Average	1.8	2.1			2.9
<u>70°C.</u>					
71-B		2.69			3.17
105-B	2.10	2.46	2.61		3.14
126-B	2.13	2.59	2.62		
Average	2.1	2.6	2.6		3.2
<u>80°C.</u>					
64-B		3.27			3.97
65-B		3.15			4.10
72-B		3.19			4.15
111-B	2.73	3.02	3.40		
Average	2.7	3.1	3.4		4.1
<u>90°C.</u>					
102-C	3.12	3.32		4.00	4.26
110-C	3.15	3.40	3.62		
Average	3.1	3.4	3.8	4.0	4.3

TABLE X
COMPARISON OF AVERAGES OF TABLE VIII AND TABLE IX

Hours	Procedure I	Procedure II
<u>60°C.</u>	(% Thyroxins)	
4	1.8	1.8
8	1.8	2.1
12	2.1	
16		
24	2.7	2.9
<u>70°C.</u>		
4	1.9	2.1
8	2.2	2.6
12	2.5	2.8
16	2.7	
24	3.2	3.2
<u>80°C.</u>		
4	2.3	2.7
8	2.8	3.1
12	3.1	3.4
16	3.3	
24	3.6	4.1
<u>90°C.</u>		
4	2.9	3.1
8	3.4	3.4
12	3.6	3.8
16	3.8	4.0
24	4.0	4.3

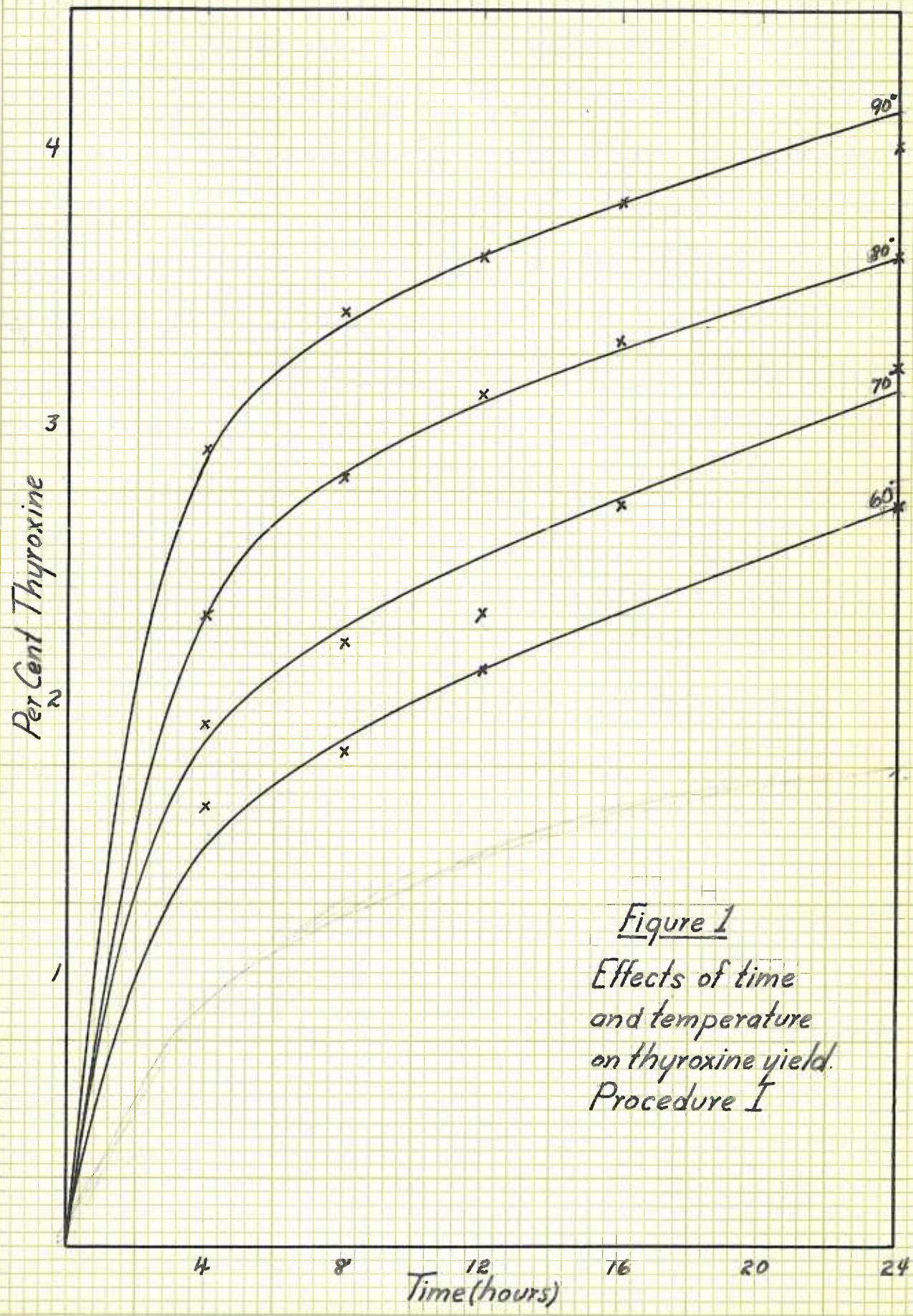


Figure 1
Effects of time
and temperature
on thyroxine yield.
Procedure I

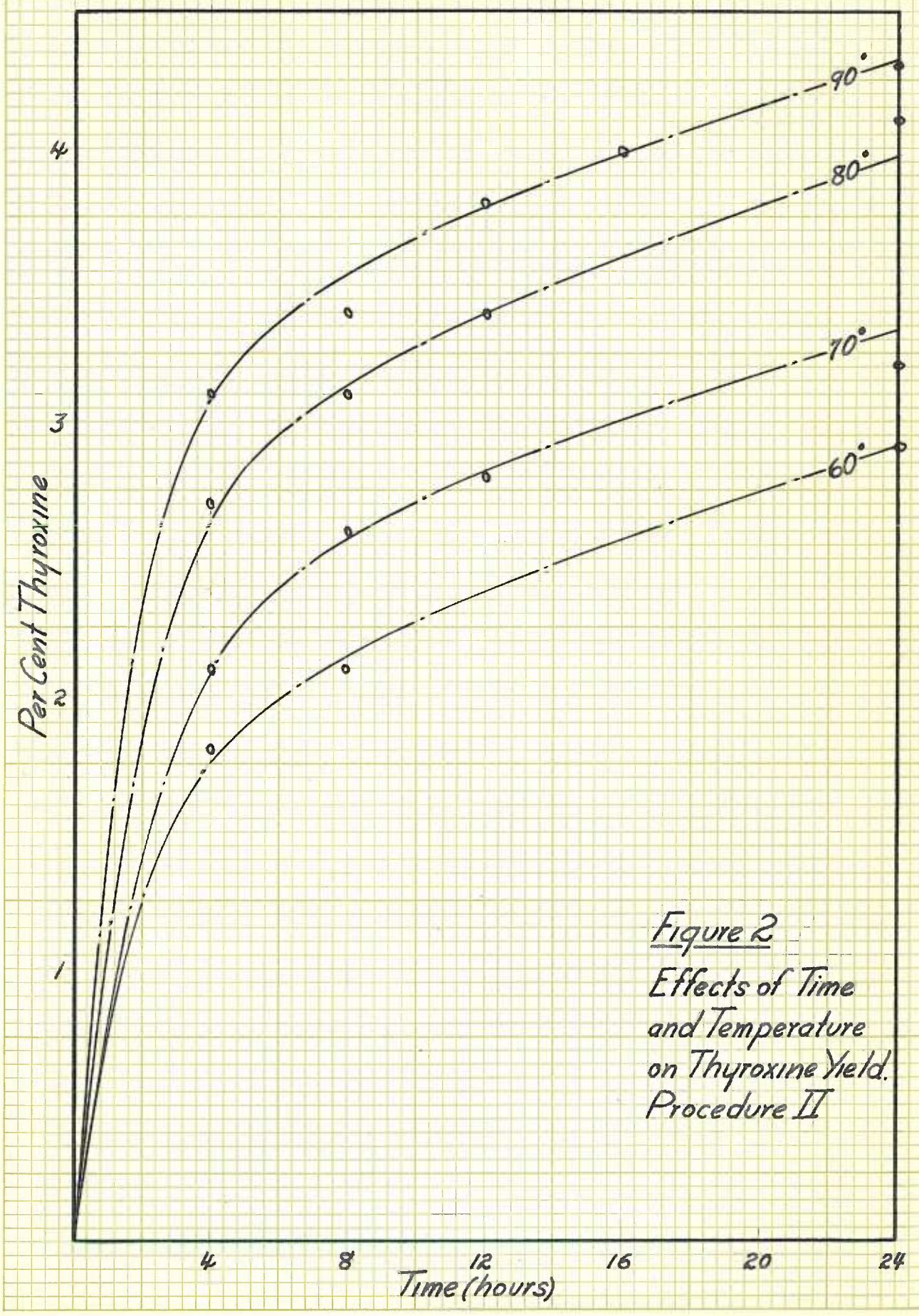


Figure 2
Effects of Time
and Temperature
on Thyroxine Yield.
Procedure II

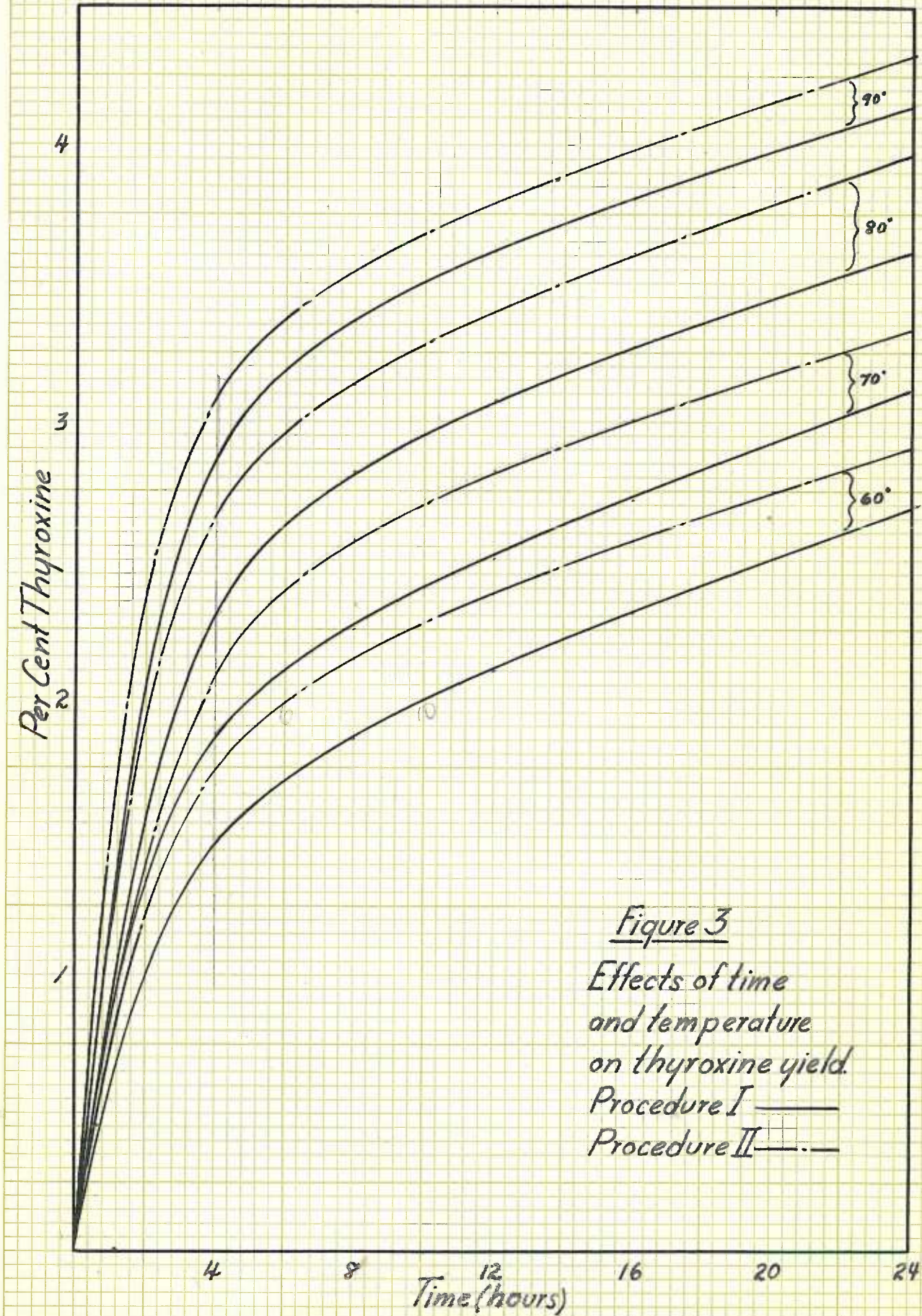


Figure 3
Effects of time
and temperature
on thyroxine yield.
Procedure I ———
Procedure II - - -

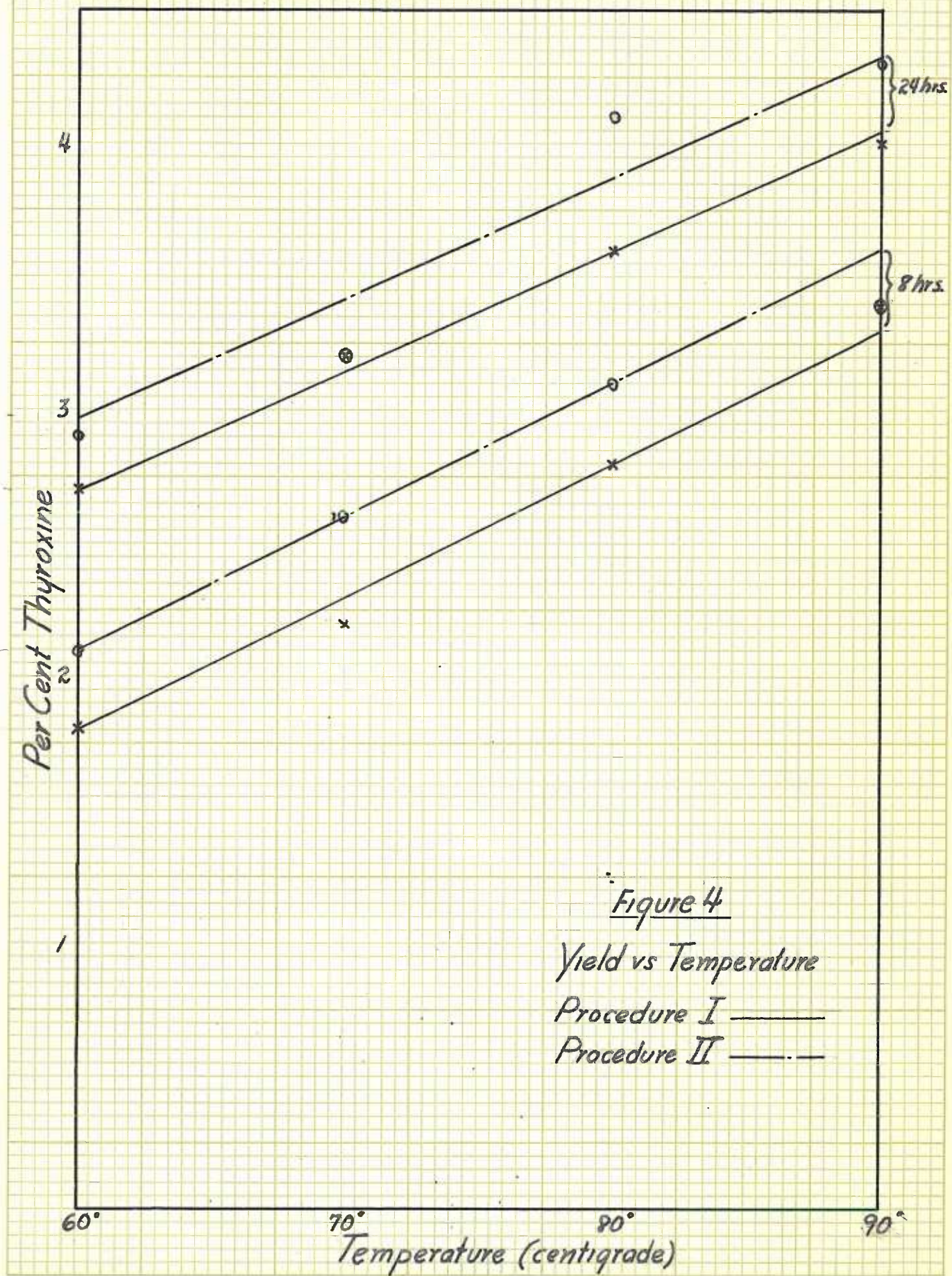


Figure 4
Yield vs Temperature
Procedure I —●—
Procedure II —×—

III. SUMMARY

This thesis presents the results of an investigation of the production of iodocasein. New procedures for the preparation of iodinated casein containing high percentages of thyroxine have been devised. Procedure I represents a simplification of previous methods.

During an investigation of the many variables influencing the reaction, the use of pure oxygen at 80°C. was found to give greatly improved yields of thyroxine in shorter reaction times. From this finding Procedure II was formulated.

The role of oxygen and $KMnO_4$ in the reaction was investigated. It was concluded that the oxygen provides maximum oxidative conditions for the coupling reaction. The $KMnO_4$ apparently acts only as a catalyst, possibly as an oxygen carrier.

The metal, copper, was found to affect the reaction adversely. This was manifested by abnormally low 24 hour thyroxine values. The reason for this is unknown.

Preliminary work on enzymatic pre-digestion of the casein shows that much higher yields of thyroxine than those obtained previously are possible. The enzyme papain probably acts by breaking down the large protein molecule into smaller fractions, so that more diiodotyrosine residues are in close contact and thus able to couple.

The time and temperature relations of Procedure I and II were investigated. The rate of the reaction increased with increased temperature between 60 and 90°C. High yields of thyroxine may be obtained by employing high reaction temperatures. In the temperature range studied

the use of pure oxygen in the reaction mixture increases the yield of thyroxine over that obtained with Procedure I at any one time or temperature.

The results presented in this thesis have clarified certain phases of the reaction and in addition have indicated new lines of investigation to be followed in research on thyroactive iodinated proteins.

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