PREPARATION OF IODINATED CASEIN

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

I. INTRODUCTION

A. The Natural Thyroid Hormone

The discovery of large amounts of organically bound iodine in extracts of the thyroid gland by Baumann[1] stimulated investigations into the nature of the active principle of the gland. Although as far back as the middle ages[2] the beneficial effects of iodine and iodine-containing materials such as sponges and seaweed on goiters were known, the chemical nature of the thyroid hormone was unknown. Through various procedures of extraction, fractions with increasing iodine content and biological activity were obtained from thyroid tissue. In 1899 Oswald[3] obtained a purified protein product from the thyroid follicles and called it thyroglobulin.

Further efforts were made to concentrate the active principle. In 1919 Kendall[4,5] isolated a crystalline compound containing 65% iodine from an alkaline hydrolysate of hog thyroid, which he called thyroxine. Harington[6] determined the correct structure of thyroxine and confirmed it by the synthesis of the compound[7], and by the use of the synthetic product in the successful treatment of a myxedematous patient.

B. Artificial Thyroproteins

While these experiments on natural thyroid products were

going on, other workers were attempting to produce thyroactive compounds synthetically by adding iodine to various proteins. The earlier attempts by Berg, Blum, Hutchinson, Liebricht, Hofmeister and Kurajeff (see bibliography reference 8 for a review of early work) successfully combined iodine into the protein molecule; however, the few claims for biological activity were not confirmed, and these preparations probably did not contain thyroxine.

In 1936 Ludwig and Mutzenbecher[9] produced an iodinated casein with marked biological activity from which they later isolated thyroxine[10]. Their method with some modifications is essentially the one used today.

C. Uses of Thyroactive Proteins

1. Milk Production

The physiological action of thyroactive compounds is complex, and a detailed discussion is beyond the scope of this thesis. Many of the effects are due to the well-known ability of thyroxine to increase the metabolic rate. In 1934 Graham[11] found that thyroxine and desiccated thyroid tissue greatly increased the volume output and butterfat content of the milk of dairy cows. Although the use of thyroid gland preparation materials would not be feasible on a large scale, Reineke and Turner[8] in 1942 found that feeding synthetic thyroprotein had essentially the same effect as natural thyroid substances.

The massive amount of data collected since these early experiments is well reviewed by Reineke[8] and Blaxter[12,13]. With wide variation, depending on factors to be mentioned subsequently, the milk yields of dairy cattle can be increased up to 50 per cent in a period of one to three weeks with thyroprotein feeding of about 15 gms. per day. The increase in yield correlates roughly with dose, up to about 30 gms. per day. At this point some of the undesirable side effects of hyperthyroidism are likely to appear. Along with the increase in yield, the fat content of the milk is increased up to 2.0 percentage units. The lactose content of the milk from iodinated casein fed cows is also generally increased about 10%, but studies on other constituents such as proteins, minerals and vitamins are inconclusive. There is no significant change in these substances except for vitamin C; here, with all types of thyroid stimulation, there is about a 30% decrease. Milk is not an especially good source of ascorbic acid; therefore Blaxter[13] concludes that milk from dairy cows on iodinated casein is safe for human consumption. The high intake of iodine, both organic and inorganic, of cows on thyroprotein, is reflected in an increase in the inorganic iodine content of the milk. There have been no reports of hyperthyroidism in calves of cows stimulated with iodinated casein.

The best results have been obtained by feeding 15 to 20 gms. of iodinated casein per day to productive cows starting

generally about 40 days after freshening. With this procedure the cow's metabolism, heart rate, respiratory rate, and body temperature are increased; and her weight will drop if on a poor or limited food intake. With proper handling the untoward actions of thyroprotein are few. The loss in weight can usually be compensated by an increased food supply. With the above facts in mind, Blaxter[13] estimates that short term thyroprotein treatment will give about a 30% profit in milk supply. Other factors altering the response of cows are: stage of lactation, nutritional intake, age, weight, and breed of cow.

2. Swine Growth

Thyroprotein has also been used in altering the thyroid status of swine. Blaxter et al[13] have reviewed some of the earlier work, and some promising results have been obtained by feeding recently weaned pigs 0.006 to 0.012% iodinated casein in their rations. Weight gains have been reported up to 27 pounds above control values. In this work the known growth stimulating properties of the thyroid hormone are used to good advantage.

3. Chickens

Stimulation of growth in young chicks has been much less conclusive than the work with pigs. Thyroprotein has been shown useful, however, in stimulating egg production,

especially during the summer months and particularly in older hens where there is a normal decline in metabolism.

At present the commercial use of iodinated casein on domestic animals is limited to certain areas that afford the particular geographic and economic circumstances necessary for commercial success. There are many conflicting reports and, although with intelligent use some advantage is gained, more data must be collected before the merit of wide scale use of thyroprotein is definitely established.

4. Human Application

The use of artificially iodinated proteins in humans has been investigated only to a limited extent. Lerman and Salter[14] used hydrolysates of iodinated serum albumin successfully to relieve myxedema. Later the relief of myxedema was used for a biological assay of some iodinated proteins[15]. Further reports of myxedema relief by artificially iodinated proteins prepared essentially by the Ludwig and Mutzenbecher method[16,17] have established their biological effectiveness in humans.

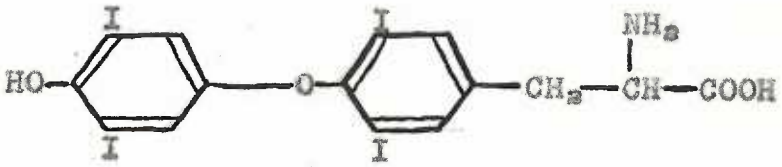
D. Chemical Theory of Thyroxine Formation

1. Incubation of Diiodotyrosine

The isolation of thyroxine from artificially iodinated proteins by Ludwig and Mutzenbecher[10] aroused the

interest of theoretical chemists as to the mechanism of its formation in the intact protein. After the structural formula of thyroxine had been determined (figure 1), Harrington and Barger[6] suggested that its formation in the thyroid gland is through coupling of two molecules of diiodotyrosine with the loss of one side chain.

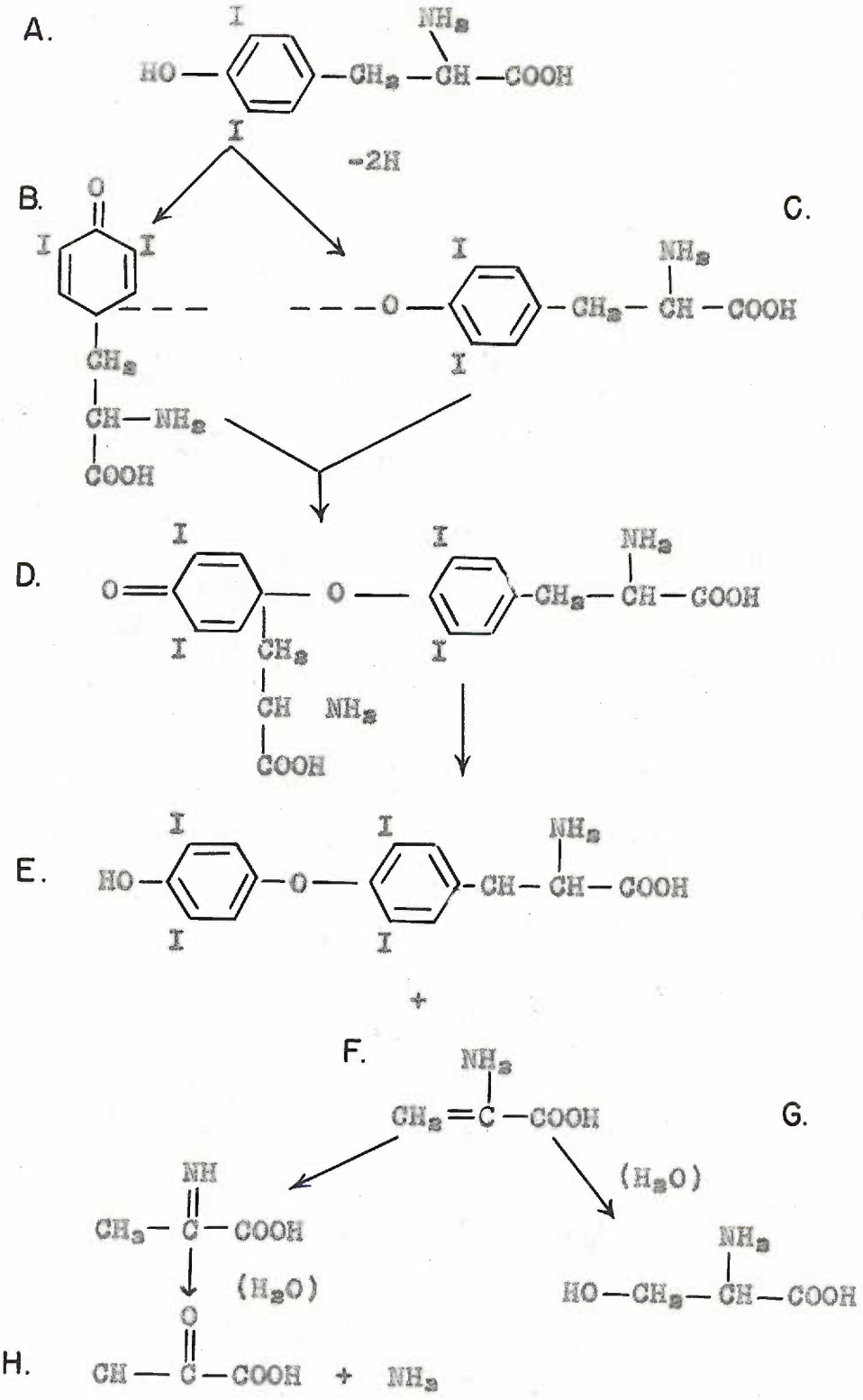
Figure 1



In order to avoid the complications encountered when working with the complex structures of proteins, many investigations into the reaction of thyroxine formation were made on relatively simple systems, that is, those involving only free amino acids. Further evidence that diiodotyrosine is the precursor was given by Mutzenbecher[18] when he obtained a small amount of thyroxine by incubating diiodotyrosine in alkaline solution at 38°C for several days. Johnson and Tewkesbury[19], in confirming Mutzenbecher's experiment, postulated an oxidative mechanism in which hypoiodite, formed from the iodine split off from diiodotyrosine during incubation, is the oxidizing agent. They gave as evidence for their theory a slight

increase in the yield of thyroxine on addition of hypoiodous acid to the incubating mixture. Their proposed mechanism for this reaction is shown in figure 2 (page 8). Thus dehydrogenation of diiodotyrosine, compound A, leads to compounds B and C, which couple to form the quinol ether, compound D. This spontaneously decomposes to form thyroxine, compound E, and an unstable amino compound F, which is hydrated to form serine, compound G, or pyruvic acid, compound H. Their identification of pyruvic acid in the reaction mixture supports portions of their theories. Harrington[20] gives further support to this proposed mechanism when by application of the resonance theory he finds the structures given by Johnson and Tewkesbury[18] to be the most probable. Harrington obtained an increase in yields of thyroxine by the use of hydrogen peroxide as an oxidizing agent; however, when potassium ferricyanide was used, only tarry products were obtained. In later experiments Harrington and Pitt-Rivers[21] found the optimum pH for thyroxine formation to be 10 and thus concluded that iodine and not hypoiodite is the oxidizing agent, since the yield of thyroxine falls off sharply in the more alkaline solutions which favor the existence of hypoiodite. Reineke and Turner[22] used manganese dioxide as a catalyst in their experiments and found an increase in yield of thyroxine as long as the incubating solution of diiodotyrosine was well aerated. They concluded that atmospheric oxygen was the oxidizing agent, for no thyroxine was formed if air was completely

Figure 2



excluded from the incubating mixture. The nature of the immediate oxidizing agent is still unproven; however, the fact that oxygen is necessary is well established.

The exact nature of the side chain split off during the coupling reaction is also still undetermined. Pitt-Rivers[23], using paper chromatography and other methods, has identified oxalic acid; 3,5,diiodo-4-hydroxy-benzaldehyde; triiodophenol; and alanine in various mixtures of incubated diiodotyrosine. The aldehyde and triiodophenol are oxidation products which are formed when diiodotyrosine is incubated at a high pH. Oxalic acid could be formed in a number of ways but is probably formed by beta oxidation of the diiodotyrosine side chain. The author suggests that the alanine found is not necessarily split off as such but may be formed from other by-products of the reaction.

2. Thyroxine Formation in Intact Proteins

a. Iodination

When iodine is added to a protein, the first reaction to take place is substitution on the benzene ring of the tyrosine molecules meta to the hydroxyl group. Iodine also combines with histidine and reacts with tryptophane[8]. In addition to chemical combination, iodine is adsorbed by proteins. Under various conditions proteins can be prepared containing large amounts (up to 18%[8]) of iodine; however, there is little or no correlation between total iodine content and biological (thyroxine) activity.

b. pH

The coupling of diiodotyrosine molecules in intact proteins is influenced by the same factors affecting the simple amino acid reactions described above, but is also influenced by additional factors. When iodine is substituted in the benzene ring of tyrosine, hydrogen iodide is split off; therefore a buffer is needed to maintain the pH of the mixture at optimum levels. Ludwig and Mutzenbecher[9] used sodium bicarbonate and were able to maintain their incubating solution at around pH 8. These workers also suggested that a limited amount of iodine is desirable for high thyroxine formation. Muus et al[15] also found that their iodinated serum albumin decreased in activity when the iodine added was greater than four atoms per molecule of tyrosine in the protein. Muus used ammonia in an alcoholic solution as buffering medium. Reineke and Turner[24] found that maximum potency was obtained with casein in a bicarbonate solution when the iodine concentration was four to five atoms per molecule of tyrosine. However, they also found that by increasing the amount of bicarbonate along with the iodine they obtained their best yields when six atoms of iodine were added per molecule of tyrosine.

c. Incubation time and temperature

Early preparations were incubated at 38°C to minimize the denaturation of the protein. Reineke and Turner[22]

obtained a large increase in the thyroxine content by incubating the iodinated casein at elevated temperatures for varying periods up to 24 hours. Osborn[35] studied the time temperature relationships and found the thyroxine content increased proportionately up to 90°C. She also found that the thyroxine content increased as the incubation time was increased, but at a diminishing rate after 24 hours.

d. Catalysts

During the course of their investigations, Reineke and Turner found higher potencies in the preparations made in an apparatus containing a brass stirrer. This led to the investigation of a series of metals as catalysts[36]. Other metals, including iron and copper, were used unsuccessfully; however, they obtained a consistent increase in thyroxine content with colloidal manganese dioxide formed by the reduction of potassium permanganate with glucose. They also found increases with other manganese oxides and salts. Osborn[35] confirmed the catalytic action of the manganese compounds but was unable to obtain any beneficial effect from a brass stirrer or any related metals. Pitt-Rivers and Randall[37] also failed to obtain any effect with a brass stirrer. In further investigations of manganese compounds, Osborn[36] found that when $KMnO_4$ is added to a casein solution, the permanganate is reduced and becomes an effective catalyst.

Osborn also tried various other known oxygen carriers such as butyl peroxide, hydrogen peroxide, and hemoglobin,

but these did not alter the reaction. Pitt-Rivers and Randall[37] also found horse radish peroxidase ineffective.

e. Aeration

Reineke and Turner[36] found that the increased potency of their products obtained with a manganese catalyst was not realized if the stirring motor was slowed down. Osborn[35] also found that rapid stirring increased the amount of thyroxine in her proteins. She also found that aeration of the solution with air or oxygen would catalyze the reaction and increase the amount of thyroxine which could be obtained. Therefore she states that $KMnO_4$ has a catalytic action when oxygen is introduced.

f. Physical factors

(1) Concentration of ingredients

Reineke and Turner[36] used about 3 per cent protein solutions in 0.7 per cent bicarbonate for their preparations. Osborn[35] found a 6 per cent protein solution in 1.5 per cent bicarbonate more convenient to use and found that even 7.5 per cent solutions did not appreciably decrease her yield of thyroxine if the other ingredients were increased proportionately. If marked evaporation from her incubating solutions occurred, the yield of thyroxine was decreased.

(2) Addition of Iodine

Originally it was thought that the iodine must be added to the protein solutions in finely powdered form, slowly, over a period of 4 hours, at 38°C, the method of Mutzenbecher. Pitt-Rivers and Randall[27] iodinated with iodine solutions in KI also at 38°, and Reineke and Turner[28] iodinated at 38° even though they incubated their solutions at much higher temperatures. Osborn[25] found that her yields of thyroxine were increased when she added the iodine all at once at 75-80°C.

(3) Precipitation

After incubation Reineke and Turner[28] dialyzed their solution over night in a cellophane bag. The solution was then isoelectrically precipitated with HCl at pH4. Osborn[25] showed that the thyroxine content was not altered by dialysis; she also found that isoelectric precipitation at 60°C gave a precipitate that settled rapidly and was easily dried.

g. Proteins suitable for Iodination

Tyrosine has been shown to be the important constituent of proteins necessary for thyroxine formation. As one would expect, all proteins containing tyrosine which have been iodinated under the proper conditions have shown thyroidal activity. However, contrary to what might be expected, the amount of thyroxine which can be formed under

standard conditions is not proportional to the amount of tyrosine in the original protein. Roche et al[85] have iodinated a series of proteins by Reineke's method and found silk fibroin which contains 12 per cent tyrosine to form only one fourth as much thyroxine as zein which contains 5.8 per cent tyrosine. They also tested the thyroxine content of iodinated peptones formed from the peptic hydrolysis of casein and found only trace amounts. Osborn[85] at the same time investigated the thyroxine content of the products of papain hydrolysis of casein. With one preparation she obtained the highest thyroxine yield of any of her experiments, but was not able to reproduce this yield.

E. Thyroprotein Assay Methods

One of the most difficult problems in thyroprotein research is the determination of the thyroidal potency of the various products. This subject is well reviewed by Reineke and Turner[80] and Pitt-Rivers[80], so only the basic problems will be presented here.

1. Bioassay

Reineke and Turner have classified present day bioassay methods into four main groups. These are: (1) assay based on the effect of thyroid in altering the energy metabolism or body weight; (2) replacement therapy in thyroidectimized or thiouracil treated animals; (3) main-

tenance of the thyroid-pituitary balance in thiouracil treated animals; (4) stimulation of the processes of maturation and metamorphosis in amphibian tadpoles. All of these methods have been used by various workers and all have some serious drawbacks. The obvious standard for comparison of thyroidal substances is crystalline thyroxine. When the above mentioned responses to thyroidal substances are compared to responses obtained with thyroxine, not only is there great individual variability but obvious discrepancies occur if careful environmental standards are not maintained. Many of these discrepancies are due to the fact that thyroxine is a relatively insoluble material and not quantitatively adsorbed from the gastro-intestinal tract. This makes oral administration less satisfactory than parenteral administration. Parenteral administration presents another problem as described below. Thyroxine is released slowly into the blood stream in small quantities by the thyroid gland. Thus single injections of larger doses are unphysiological and a large part of the injected dose is destroyed, and the test animal thus does not make a quantitative response. This phenomenon probably explains why the response of animals to some thyroproteins is greater than one would expect from their thyroxine content, for the protein is broken down more slowly and the release of thyroxine more closely simulates the action of the thyroid gland.

Another problem in the use of a standard in thyroxine bioassays is the relative potencies of L and D-L thyroxine. The reported values in the literature vary greatly, and since crystalline thyroxine is most conveniently isolated in the racemic form it is used this way as a standard. Probably the activity of the L form is enough greater than that of the D to enable one to assign all the activity of a racemic mixture to the L form, at least within the limits of accuracy of the bioassays now available.

The final problem to be mentioned is concerned with the specificity of response. One of the most widely used bioassay procedures is that of Deansley and Parkes[31]. They found that the stimulation of metamorphosis in Xenopus tadpoles correlates well with the increase in milk production of dairy cows. They have also shown that the tadpoles respond to diiodotyrosine as well as thyroxine.

2. Chemical assays

Most of the chemical assays for thyroxine in thyroproteins are based on the determination of iodine in various solvent fractions after hydrolysis of the protein. The early methods of evaluating the potency of thyroproteins by a total iodine analysis as mentioned previously are of very little value since only a small fraction of the total iodine is thyroxine iodine. Early attempts to isolate thyroxine from hydrolysates of thyroproteins showed that almost all of the

activity was in the acid insoluble portion. Pitt-Rivers and Randall[27] attempted to estimate the activity of their preparation by measuring the acid insoluble iodine. However, Deansley and Parkes[31] showed that there was very little correlation between acid insoluble iodine and the biological activity of thyroproteins as assayed in tadpoles or cattle. A new method of thyroxine assay was made available in 1932 by Leland and Foster[32]. They found that the thyroxine of hydrolyzed desiccated thyroid could be separated from diiodotyrosine by extraction from an alkaline solution with butanol. Blau[33] modified this procedure by extracting the hydrolysate with butanol at pH 3.5 and purifying the butanol fraction with 4N NaOH containing 5% $NaHCO_3$. This technique has been modified by Reineke et al[34] and Osborn[35], and is the one used in the experimental work to be described. Reineke states that there is good agreement between the values obtained by his chemical assay and those obtained from bioassays based on the increased output of CO_2 by guinea pigs.

There are several other assay procedures using colorimetric or polarographic procedures. These offer no real advantage over the procedure described by Reineke[34].

II. EXPERIMENTAL

A. The Chemical Assay of Thyroproteins

1. Hydrolysis

1.0 gm. of thyroprotein is weighed into a 150 x 25 mm. pyrex test tube containing 3.2 gm. of $BaOH \cdot 8H_2O$. The dry materials are mixed by gently tapping the bottom of the tube. 5 ml. of water are added, rinsing down any material which adheres to the side of the tube. The tube is then placed in a steam or boiling water bath. After 2 to 4 hours the tube is removed and the mixture stirred. 1.2 ml. of water is added, rinsing the stirring rod and sides of the tube. Hydrolysis is then continued for a total of 18 to 22 hours. The tube is then removed from the bath and 25 ml. of cold water are added. The hydrolysate is allowed to settle, or is spun down, and the clear supernatant poured off into a 100 ml. volumetric flask. The undissolved material remaining in the tube is brought into solution by adding 5.0 ml. of 3.5 N HCl and 2 ml. of n-butanol. This mixture is stirred, warmed on the steam bath and then added to the 100 ml. flask. The tube is rinsed with water and the walls cleaned with the aid of a stirring rod, the rinse being added to the 100 ml. flask. This process is repeated with small amounts of water and the volume brought to 100 ml.

2. Separation of the thyroxine fraction

After thorough mixing of the contents of the 100 ml. flask, a 20 ml. aliquot of the hydrolysate is transferred to a 250 ml. separatory funnel. One drop of bromo cresol green indicator is added, and the hydrolysate is acidified with 3.5 N HCl to the first yellow color. 20 ml. of butanol are added and the mixture is thoroughly shaken. The two layers are next allowed to separate, and the aqueous layer is drained off into another 250 ml. separatory funnel. The aqueous layer is then re-extracted with 10 ml. of butanol, and the butanol layers are combined. The butanol solution, which now contains thyroxine and diiodotyrosine, is extracted with 25 ml. of 4 N NaOH containing 5% $NaHCO_3$. The layers are allowed to separate, and the alkaline layer is transferred to a second separatory funnel. The butanol is then re-extracted with 15 ml. of alkali and the alkali portions combined. The combined alkali portions are then extracted with 15 ml. of fresh butanol.

The alkaline washes are discarded and the butanol portions combined. 10 ml. of butanol are used to rinse the second separatory funnel. The butanol solution is then filtered through a small glass wool plug fixed in the stem of the separatory funnel, into a 250 ml. nickel crucible. The crucible is placed on a steam bath and the butanol is evaporated with the aid of a stream of air.

3. Iodine determination

To the dried residue from the butanol evaporation 5 gm. of NaOH and 5 ml. of water are added, and the crucible is heated gently over a burner until no more steam comes off. The crucible is then placed in an open "oven" and heated strongly with a Fisher burner. Toward the end of the fusion a small amount of KNO_3 is added and the mixture is heated until clear. The crucible is allowed to cool, and the melt is then dissolved in about 100 ml. of water and transferred to a 500 ml. wide mouth Erlenmeyer flask with the aid of an additional 100 ml. of water. 1 ml. of 10% $NaHSO_3$ is then added to convert all of the iodine compounds to iodide. Then 3 to 4 drops of Methyl orange are added and the solution is carefully acidified to the first pink color with syrupy H_3PO_4 (85%). 10 drops of liquid bromine are added and the solution is mixed with a glass stirring paddle. 2 Henger granules are added and the solution is boiled under the hood until clear. 10 drops of 5% sodium salicylate are added and the solution is allowed to cool in a water bath. 5 ml. of 10% KI and 5 ml. of syrupy H_3PO_4 are added, and the solution is titrated with fresh 0.005 N $Na_2S_2O_3$. 1 ml. of 1% starch solution is added near the end point.

4. Calculations

After hydrolysis and separation, the iodine of the butanol fraction is assumed to be bound as thyroxine iodine. By fusion this is converted into inorganic iodides. The

iodides are converted into iodate by bromine and determined by the following reaction:

$$KIO_3 + 5\,KI + 6H_3PO_4 \longrightarrow 3I_2 + 3H_2O + 6KH_2PO_4$$

The iodine is then titrated with sodium thiosulfate:

$$I_2 + 2Na_2S_2O_3 \longrightarrow 2NaI + 2Na_2S_4O_6$$

In the above reaction each iodine atom from the separated thyroxine is represented by 6 iodine atoms in the final titration. Therefore each ml. of 0.005N sodium thiosulfate represents

$$\frac{0.005}{1000} \times \frac{127}{6} = 106 \times 10^{-6} \text{ gm., or } 0.106 \text{ mg. iodine,}$$

127 being the atomic weight of iodine. Thyroxine has a molecular weight of 777 and contains 4 iodine atoms; thus we multiply the weight of iodine by $\frac{777}{508}$, or 1.529, to get the weight of thyroxine*. The percentage thyroxine in the 1 gm. of thyroprotein sample is thus calculated from the following formula:

$$\% \text{ thyroxine} = \frac{\text{ml. thiosulfate} \times .106 \times 1.529 \times 5 \times 100}{1000 \text{ (sample wt. in mg.)}}$$

Osborn[35] has shown that it is more convenient to use a factor for each new batch of standardized 0.1 N thiosulfate:

$$\text{Factor} = \frac{\text{Normality of thiosulfate} \times 0.106 \times 1.529 \times 5 \times 100}{0.1 \times 1000}$$

Then, the ml.'s of .005 N thiosulfate x factor = % thyroxine.

A check on the accuracy of the assay procedure is through thyroxine recovery experiments. A small but accurately

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*Since only 20 ml. of the 100 ml. hydrolysate is used, we must multiply the answer by 5.

weighed amount of crystaline thyroxine is transferred to a test tube and carried through the hydrolysis, separation and iodine determination procedure. In our hands, 97% of the thyroxine was recovered, with a range from 94 to 99%. When diiodotyrosine was added, occasionally very slightly higher values were obtained.

5. Sources of Error

Since the assay procedure is long and involves many steps, it is often difficult to trace the source of an error seen in the final result. Values which are too high are often obtained when non-thyroxine iodine is included in the thyroxine fraction. For example, incomplete hydrolysis, due either to shortened hydrolysis times or to incomplete mixing, may leave peptides of diiodotyrosine which follow thyroxine in the separation procedure. Non-thyroxine iodine can contaminate the final fraction if there is incomplete separation of the aqueous and butanol layers. Care must also be used in the final titration, as too much phosphoric acid will promote auto-oxidation of the iodide solution and thus give values which are too high, due to the fading end point.

The most common causes of low values are spattering, or errors occurring during the evaporation or the fusion procedures. Another source of trouble is the formation of persistent emulsions in the first step of the separation procedure. These can usually be avoided by having the volume

of the aqueous layer greater than that of the butanol layer.

In spite of the difficulties pointed out above, reasonably consistent results were obtained in the repeated analysis of a "standard" thyroprotein preparation. This "standard" was run as a control sample in each series of experimental assays.

Table I lists these "standard assays" accumulated over a two year period, the values being arranged in order of increasing size.

TABLE I

Values obtained by repeated assays of a single, homogenous thyroprotein product

3.60, 3.66, 3.67, 3.68, 3.73, 3.74, 3.75,
3.76, 3.76, 3.77, 3.77, 3.78, 3.80, 3.80,
3.82, 3.83, 3.83, 3.84, 3.85, 3.87, 3.88,
3.90, 3.97

Average	=	3.785
Range	=	3.60 - 3.97
Standard deviation	=	0.095

B. The Preparation of Thyroproteins

The earliest batches of iodinated casein were prepared in this laboratory by the technique described by Osborn[28].

The temperature of a 12 liter Fisher water bath apparatus is controlled at 80°C with an auxiliary heater and a Fenwall thermostat. A 2 liter round bottom flask is immersed in the bath and held by a clamp. Provision is made for rapid stirring by the use of a "Lightnin" stirrer. 1 liter of distilled water and 15 gm. of $NaHCO_3$ are added to the flask, and then 60 gm. of commercial casein are added slowly with a few drops of Antifoam. As soon as the casein has dissolved, 200 mg. of $KMnO_4$, dissolved in a small amount of warm water, are added; and as the temperature approaches 80°C 10.4 gm. of iodine are added all at once. A glass tube is then adjusted well below the surface and oxygen is bubbled through through the rapidly stirred mixture at a rate of one liter per minute. More Antifoam is added as needed, and the mixture is allowed to incubate at 80°C. Incubation times were varied from 4 to 24 hours. At the end of the incubation period, timed from the addition of iodine, the flask is removed from the bath, partially cooled, and the solution filtered through glass wool into a precipitation pan. At 60°C dilute HCl is added slowly to the mixture with constant stirring until there is complete precipitation, the optimum pH being around 3.9. The precipitate is allowed to settle and the supernatant liquid poured off. The residue is then partially dried on a suction filter and the drying completed in an oven at 70°C. The dried material is then ground to granular powder and stored for analysis.

1. Variations of Ingredients and Procedures

Using the procedure essentially as described above, a study was made of the effects of varying one or more of the reaction components. When time course studies were done, serial samples were obtained by the removal of 50 ml. aliquots from the reaction mixture. This was done by pipet without disturbing the temperature, rate of stirring, or oxygen flow. One of the first difficulties encountered was evaporation and concentration of reactants in preparations incubated 8 hours or more; also, the Antifoam became ineffective and allowed solid material to collect on the sides of the flask above the reactant volume. This problem was overcome by constructing a side arm on the 2 liter flask and attaching a constant level water bottle. This apparatus was used in all preparations incubated over 8 hours. Table II contains a summary of a series of investigations. In this and throughout the remainder of this thesis, reactions or studies will be listed by sample number; these refer to the number assigned the experiment at the time of performance.

In several experiments the standard ingredients were varied. In reactions 5, 6, 7, and 10 a commercial, crude, wet casein product was substituted for the usual dry material with allowances for the excess moisture. Also in numbers 6, 7, and 10 a commercial, crude filter cake iodine was used in place of the standard resublimed product. The thyroxine values of these products were well within the usual range.

Table II

A Summary of the Results of Preliminary Experiments

Sample Number	Temperature	Treatment, remarks	% Thyroxine Incubation Time (hours) 4	8	12	24
1	80-85°	Compressed air, evaporation		2.83		
2	80-85°	(Same)		2.38		2.98
3	78-85°	(Same)		2.47	2.63	
4	80°±2	(Same)		2.32	2.37	2.82
5	80°	Wet casein, evaporation			2.12	
6	80°	Wet casein, wet iodine, constant level flask			2.41	
7	85°	(Same as 6)		2.41	2.83	3.75
8	85°	Pre-iodination incubation, compressed air	1.44			
9	85°	Pre-iodination incubation, oxygen	2.73			
10	85°	(Same as 6) + oxygen		3.24 (7 hours)		
11	80°	Standard products, O_2		2.95	3.25	
12	80°	(Same as 11)	2.14	2.60	3.06	
13	85-90°	Pre-iodination incubation	1.91 (3 hours)			
14	90°	(Same as 13)	2.41			
15	90°	(Same as 13)	2.09			
16	80°	Pre-iodination incubation 90°	1.99			
21	80°	I_2 added at 80°	2.22	2.70	2.79	
25	80°	Repeated iodination	3.05 (4.5 hours)			
31	80°	Na_2CO_3		3.20		
36	80°	Stirrer off between 4 and 8 hours	2.75	2.72		

In another short series of experiments (numbers 8, 9, 13, 14, 15, 16) the casein solution, containing all the usual ingredients but not the iodine, was incubated 4 hours prior to the addition of iodine. This procedure had no beneficial effect on the thyroxine content, and in most cases the values were below average. In preparation 32 an equivalent amount of Na_2CO_3 was substituted for the $NaHCO_3$ and there was no deleterious effect on the thyroxine yield. 3 gm. additional iodine were added to preparation 25 at 1.5 and 2.5 hours, and the product contained 3.05% thyroxine after 4.5 hours of incubation. In preparation 36 the stirrer was left off after removing the 4 hour sample and there was no increase in thyroxine formation in spite of 4 hours additional incubation. It is apparent that the changes in procedure and/or ingredients recounted above did not materially affect reaction yields.

2. Investigation of Precipitation, Washing, and Drying Techniques

The color, texture, and granularity of the early preparations varied greatly. Some products which were dried in a vacuum desiccator over concentrated H_2SO_4 were much lighter in color and were easily ground into a fine powder, while the products allowed to dry at room temperature or in the oven at 70°C were darker, coarser, and more difficult to powder. Tables III-A, III-B, and III-C give a summary of

Table III

A Summary of Precipitation, Washing, and Drying Techniques

Table III-A Precipitation

Sample	Technique	% Thyroxine	% Iodine
28 (8 hr)	A. Redissolved and dialized	2.98	6.04
	B. Control (isoelectric precipitation)	3.07	6.34
29 (8 hr)	A. Redissolved and dialized		6.41
	B. Redissolved		6.11
	C. Control		6.94
Stock soln.	A. Precipitated at room temp.		6.38
	B. Control (ppt. at 60°C)		5.99
	C. Ppt. in acid alcohol, room temperature		6.20
	D. Ppt. in acid alcohol, 60°C		6.17
	E. Ppt. in acid acetone, room temperature		6.18
	F. Ppt. in acid acetone, 60°C		5.85
39 (4 hr)	A. Ppt. at room temperature	3.06	8.26
	B. Ppt. at 60°C	2.78	8.16
	C. Ppt. acid alcohol, room temp.	2.81	7.40
	D. Ppt. acid alcohol, 60°C	2.73	7.61
	E. Ppt. acid acetone, room temp.	2.79	
	F. Ppt. acid acetone, 60°C	2.88	
92 (4 hr)	A. Ppt. at 80°C	3.82	
	B. Ppt. at 60°C	4.13	
95 (4 hr)	A. Ppt. at 80°C	2.83	
	B. Ppt. at 60°C	2.71	

Table III-B Washing

Sample	Treatment	% Thyroxine	% Iodine
Stock	A. Acetone wash		8.11
	B. Ethanol wash		7.68
	C. Control		8.19
38 (9 hr)	A. Acetone wash		6.82
	B. Control		8.04
38 (8 hr)	A. Ethanol wash		7.40
	B. Control		8.04
43 (4 hr)	A. Ethanol wash	2.59	7.32
	B. Control	2.63	9.12
44 (8 hr)	A. Ethanol wash	4.12	
	B. Control	3.65	
47 (4 hr)	A. Ethanol wash	3.15	
	B. Control	3.42	
48 (4 hr)	A. Ethanol wash	2.79	7.45
	B. Control	2.49	8.60
49 (4 hr)	A. Ethanol wash	2.38	
	B. Control	2.29	
50 (4 hr)	A. Ethanol wash	2.52	
	B. Control	2.38	
54 (4 hr)	A. Ethanol wash	2.76	
	B. Control	2.75	

Table III-C Drying

Sample	Method	% Thyroxine	% Iodine
24 (4 hr)	A. Oven at 70°C	2.30	
	B. Desiccator	2.29	
51 (4 hr)	A. Desiccator	2.71	
	B. Room Air	2.82	
55 (4 hr)	A. Desiccator	2.75	8.78
	B. Oven at 70°C	2.52	9.08
59 (4 hr)	A. Desiccator		8.51
	B. Oven at 70°C		8.59
87 (4 hr)	A. Desiccator	3.78	
	B. Oven at 70°C	4.03	

thyroxine and total iodine values of products precipitated, washed, and dried in various ways. Normally, the iodocasein was isoelectrically precipitated with HCl at 60°C, collected on a filter funnel, and air- or oven-dried. To reprecipitate, the sample was resuspended in dilute alkali, warmed and stirred until redissolved, and then reprecipitated at 60°C. The samples precipitated in acid alcohol and acid acetone were handled as follows: 50 to 100 ml. of the iodocasein solution were filtered into a 250 ml. round bottom centrifuge cup fixed with a stirrer, and precipitated by the slow addition of an acid alcohol solution prepared by adding 20 ml. of 1N HCl to 80 ml. of 95% ethanol. When no additional precipitate formed, the solid material was collected by centri-

fugation and dried in the usual manner. Precipitation at elevated temperatures was accomplished by first placing the centrifuge cup in a water bath at a temperature of 60°C and proceeding as before. The acid acetone precipitations were done in the same way, acetone being substituted for the 95% ethanol. The samples listed as being washed only were first isoelectrically precipitated in the 250 ml. centrifuge cups. The solid material was spun down and the supernatant fluid decanted, 50 to 100 ml. of solvent added, and the suspension stirred for 5 to 10 minutes; the solid material was recollected and the process repeated until the supernatant solvent layer was clear. Although reprecipitation or washing with a solvent often reduced the total iodine content, no consistent or marked effect was noted on the thyroxine values; likewise the thyroxine values of products dried in the desiccator did not differ significantly from the thyroxine values of oven dried products, in spite of marked differences in the physical appearance of the two products.

In another experiment the hydroscopicity of thyroprotein samples precipitated in various ways from a stock solution was tested. The results as seen in Table IV show that there was a slight effect of the precipitation technique upon the 2 hour exposure sample but that there was very little change in moisture content of any of the samples on exposure to air for 24 hours after thorough desiccation.

Table IV

An Investigation of Hydroscopicity

Sample	Precipitation Method	% Moisture after 2 hrs exposure	% Moisture after 24 hrs exposure
A	Dilute HCl at 20°C	3.05	3.85
B	Dilute HCl at 60°C	2.88	3.17
C	Acid Ethanol at 20°C	2.69	2.45
D	Acid Ethanol at 60°C	2.44	2.18
E	Acid Acetone at 20°C	2.61	2.70
F	Acid Acetone at 60°C	1.92	1.77

From the results of the above mentioned experiments it was concluded that there was very little change in the thyroxine content of thyroproteins made by any of the simpler purifying techniques. Therefore the procedure described in the opening remarks of this section was used for subsequent preparations.

3. Standard preparations

In order to determine the effect of any variation in technique on the preparation of thyroproteins, it was felt that a standard value must be established for comparison. The technique used was that described previously, using a two liter round bottom flask with a side arm. Every attempt was made to eliminate any possible variables which might influence the per cent of thyroxine in the final product. In spite of these efforts, there was large variation in the

thyroxine values of the "standard preparations". Also, as seen in Table V-A, most of the preparations followed the thyroxine isotherms reported by Osborn[88]; that is, the preparations which had low thyroxine values at 4 hours also had lower values at 8 and 12 hours than the preparations with high thyroxine values at 4 hours. We therefore concentrated on establishing a 4 hour standard preparation value to which future variations could be compared. The values used in establishing this value are listed in Table V-B. In addition to these values, Table V-B lists the weight in grams of the final product. Products of low potency but high yield and those of high potency but low yield might each yield the same amount of thyroxine. The last column of figures of Table V-B lists this calculation, but it can be seen that the relationship does not hold.

4. Papain preparations

In the quest for new methods by which the amount of thyroxine formed in iodinated casein could be increased, Osborn[88] theorized that some alteration of the protein molecule might facilitate the coupling of diiodotyrosine molecules which would otherwise be held apart. To test this theory she used the proteolytic enzyme papain to partially digest the casein. As stated previously, her first preparation contained the highest yield of thyroxine of any thyroprotein products yet made. However, further attempts, using the same method,

Table V

A. Standard Preparations

Sample Number	% Thyroxine 4 hours	8 hours	12 hours
17	2.94	3.17	3.27
18	2.86	2.91	3.20
19	2.86	--	3.48
20	2.99	--	3.19
21	2.22	--	--
27		3.28	
28	2.80	3.07	
29	2.76	3.34	
30	2.50	3.32	
32	2.62	3.00	
33	2.69	2.86	
34	2.79	3.14	
35	2.47	3.02	
36	2.75	--	
37	2.42	3.06	
38	2.36	2.80	
39	2.78	3.15	
72	2.00	--	
77	2.75		
78	2.78		
80	2.19		
81	2.51		
82	2.73		
83	2.55		
Average	2.62	3.09	3.26
Range	2.00-2.99	2.80-3.34	3.19-3.48

Table V

B. List of Standard 4 Hour Preparations

Sample Number	% Thyroxine	Weight in Grams	(Weight) x (Thyroxine)
72	2.00	58.5	117
80	2.19	59.5	130
21	2.22		
38	2.36		
37	2.42		
35	2.47		
30	2.50		
81	2.51	58.5	147
83	2.55	59.3	151
32	2.62		
33	2.69		
82	2.73	59.5	162
36	2.75		
77	2.75	58.5	161
29	2.76		
39	2.78		
78	2.78	59.5	165
34	2.79		
28	2.80		
18	2.86		
19	2.86		
17	2.94		
20	2.99		
Average	2.62	59.0	155

gave only average yields. Table VI shows the results of our preliminary papain experiments.

Papain is a proteolytic enzyme which is active in both acid and alkaline states. It has an optimum temperature of about 70°C and is inactivated by oxidizing agents. For the first experiments, numbers 22, 23, and 26, the same sample of Caroid (a commercial form of papain which is dried in vacuo) was used that yielded Osborn[28] her best results.

The following describes a sample Caroid experiment. 500 mg. of Caroid were weighed out into a crucible and mixed into a homogenous paste with a small amount of glycerol. The paste was then diluted with a small amount of water and washed into the flask containing 60 gm. of casein dissolved in 1 liter of 1.5% $NaHCO_3$. The enzyme was allowed to act for 20 minutes at 65°C. Then the $KMnO_4$ and iodine were added in the usual amounts and oxygen bubbled through as the temperature was raised to 80°C. The rest of the incubation and precipitation were carried out as usual. Approximately 3 minutes after the addition of the papain suspension, the casein solution became cloudy, and at the end of the digestion the casein solution had a definite milky appearance. During the digestion period the stirrer was slowed down in order to minimize the oxidation of papain by atmospheric oxygen. The weight yield of iodinated casein was decreased, but no increase in thyroxine yield was noted.

Table VI

Pre-Digestion with Caroid

Sample	Caroid used (gms)	Digestion Time (min)	Temp. (C)	% Thyroxine 4 hrs	8 hrs	Remarks
22	0.5	20	65°	2.30	(3.18)	12 hrs. incubation
23	0.5	35	65°	2.47	--	Used mother liquor
26	0.5	45	60-65°	2.60	3.05	
40	0.5	15	60-62°	3.30	3.51	Fresh sample of Caroid
41	0.5	15	58-61°	3.32	3.39	
42	0.5	15	58-59°	3.96	4.14	
43	0.5	20	55°	2.63		
44	0.5	20	55-60°	3.25	3.65	
45	0.5	20	57-60°	3.11	3.65	
46	1.0	10	60-62°	3.15	3.42	
47	1.0	15	53-55°	3.42		45 gm. yield
48	0.250	15	59-60°	2.49		58 gm. yield
49	0.250	20	56-58°	2.29		

The mother liquor of preparation 22 was used, with additional $NaHCO_3$, to dissolve the casein for experiment 23. This time the 500 mg. of papain were allowed to act for 35 minutes at 65°C before the regular procedure was carried out. In a third preparation, number 26, 500 mg. of papain were allowed to act 45 minutes at 65°C. In none of these early preparations was any increase in thyroxine formation noted. In later experiments, numbers 40 through 49, a fresh sample

of Caroid was used, and a marked increase in the yield of thyroxine was obtained. Preparation 42 contained 4% thyroxine at the end of 4 hours incubation, and many of the others were well above the range of non-digested preparations. In this brief series of experiments the highest yield was obtained by digesting the casein with 500 mg. of Caroid for 15 minutes. Further variations in the amount of Caroid used and time allowed for digestion yielded products with a smaller increase in thyroxine percentage. A cloudy supernatant fluid was left in many of these papain preparations after isoelectric precipitation. Precipitation of these suspensions with ethanol led to a product which weighed approximately 2 gm. and contained less than 1.0% thyroxine.

In another series of preparations, numbers 50 through 71, a purified sample of papain (Mefford) was used to digest the protein in a procedure similar to that described above using Caroid. The results of these experiments are listed in Table VII. As was noted in the previous experiments with papain, the total weight of thyroprotein precipitated at the end of incubation was less in digested preparations than in standard non-digested preparations. Also there is some correlation between the decrease in weight yield and degree of digestion. For instance, in experiment 71 1.00 gm. of papain was allowed to act for 30 minutes and the final yield was 37.2 gm., a decrease of almost 40% from the average 59 gm. of non-digested preparations. When only 200 mg. of papain were used

Table VII

Predigestion with Purified (Mefford) Papain

Sample	Weight of Papain	Digestion Time (min)	% Thyroxine	Weight
50	0.200	30	2.38	
51	0.200	45	2.71	
52	0.500	45	2.78	
53	0.250	45	3.02	50
54	0.200	60	2.74	52
55	0.300	45	2.75	45
56	0.200	30	2.43	--
57	0.300	30	2.23	46
58	0.250	30	2.81	52
59	0.200	30	2.13	52.5
60	0.200	30	2.02	55
61	0.200	60	2.03	51
62	0.200	45	2.18	53.5
63	0.200	45	2.20	53
64	0.500	30	2.66	43
65	0.500	25	2.22	42
66	1.00	15	2.66	43.5
67	1.00	10	2.41	41.5
68	0.500	15	2.70	50.5
69	0.500	45	2.58	44.0
70	0.500	60	2.37	42.2
71	1.00	30	2.92	37.2

for the same period, the weight loss was approximately 10%. None of the preparations digested with Mefford papain showed any significant increase in thyroxine formation above control values, in spite of evidence of partial digestion such as the milky appearance and weight decrease. A further variation in procedure was tried in this series. Starting with preparation 59, the 60 gm. of casein were dissolved in 1 liter of water containing only 4 gm. of $NaHCO_3$, and the digestion with papain was carried out in this solution at pH 7.2. At the end of digestion, 11 gm. of $NaHCO_3$ were added with the usual amounts of $KMnO_4$ and oxygen to bring the total amount of $NaHCO_3$ to 15 gm. for the iodination and incubation phase. Although there was no increase in thyroxine content in the thyroproteins digested at this lower pH, this procedure (digestion at pH 7.2) was used in subsequent experiments.

5. Activated papain preparations

The chemistry of the enzymatic action of papain is complicated and its complete discussion is beyond the scope of this thesis. However, it is well established that the proteolytic activity of papain is increased by activation with a variety of substances, all of which are reducing agents. Also, there is some indication[35] that the proteolytic activity is altered qualitatively by some of these activating agents. The next series of thyroprotein preparations were digested with activated papain suspensions. Samples of 5.00 gm. of

Mefford papain were ground to a smooth paste in a mortar with the aid of a small amount of glycerin. The paste was then transferred to a graduated cylinder and distilled water added to make 100 ml., and H_2S was bubbled through the suspension for 60 minutes. During this time a white flocculent precipitate formed on the upper part of the cylinder. This was worked down into the suspension as well as possible. An aliquot of this suspension was then pipetted into the reaction solution, which contained 60 gm. of casein, 4 gm. of $NaHCO_3$, and 1 liter of water at 65°C. The digestion was allowed to proceed for varying lengths of time before the addition of $KMnO_4$, $NaHCO_3$, oxygen, and iodine. Within one minute after the addition of the activated papain suspension the mixture appeared milky.

Table VIII lists the thyroxine values of a series of experiments in which the papain used for digestion was activated with H_2S. Although the effects of the activated papain were clearly seen in these early experiments, a more convenient technique of activation was developed. Samples 73, 75, 76, and 86 were prepared with the papain activation above. All other activations were carried out as below. The papain was weighed out and made into a paste as before. However, after transferring the paste to the graduated cylinder an equal amount of water previously saturated with H_2S was added, and the half saturated solution was incubated in the oven at 45°C with frequent agitation for 1 hour. The concentrations

Table VIII

Four Hour Preparations Predigested with H_2S Activated Papain

Sample	Weight of Papain	Digestion Time	% Thyroxine	Avg.	Yield of Product (grams)
117	100	5	2.56		51.9
116	100	30	3.13		43.9
122	100	60	3.39		38.8
95	250	5	2.83	2.80	48.6
102	250	5	2.76		51.1
75	250	15	3.19		43.0
90	250	15	2.97	3.16	44.2
91	250	15	3.32		42.1
76	250	30	3.37		38.5
92	250	30	4.00		29.0
121	250	30	3.61	3.71	32.0
132	250	30	3.64		32.3
134	250	30	3.53		35.2
93	250	60	3.87		31.4
101	250	60	3.18	3.60	30.5
120	250	60	3.76		29.0
94	500	5	3.38		39.8
127	500	5	3.06	3.29	45.8
136	500	5	3.42		42.6
73	500	15	3.22	3.36	35.5
118	500	15	3.50		34.1

Table VIII (continued)

Sample	Weight of Papain	Digestion Time	% Thyroxine	Avg.	Yield of Product (grams)
86	500	20	3.70		33.5
87	500	30	4.03		27.3
88	500	30	4.11	4.00	30.8
128	500	30	3.85		27.2
97	500	60	3.87	3.83	23.5
124	500	60	3.79		23.2
98	1000	5	3.43		37.0
100	1000	15	3.89		29.0
89	1000	30	3.79		20.3
99	1000	60	3.51		18.0

were adjusted so that the suspensions contained 100 mg. of papain per ml. of H_2S treated water. In an effort to find the optimum conditions for digestion, the amount of activated papain used for digestion was varied from 100 to 1000 mg. and the time of proteolysis varied from 5 to 60 minutes. Again we see the variation in thyroxine yield in products prepared with supposedly identical conditions and ingredients that was seen in our efforts to establish a standard value. However, there is an overall very significant elevation in thyroxine values, and three values are over 4%, an increase of over 50% above the average of the standard preparations. Figure 3 shows graphically per cent thyroxine plotted against time of

Figure 3

PER CENT THYROXINE VS. DIGESTION TIME

(Data from Table VIII, pages 42-43)

These samples were all predigested with H_2S activated papain. 100, 250, 500 and 1000 mg. portions of papain were allowed to digest the casein for periods varying from 5 to 60 min. The per cent thyroxine of the resultant products is plotted against the time allowed for digestion.

FIGURE 3

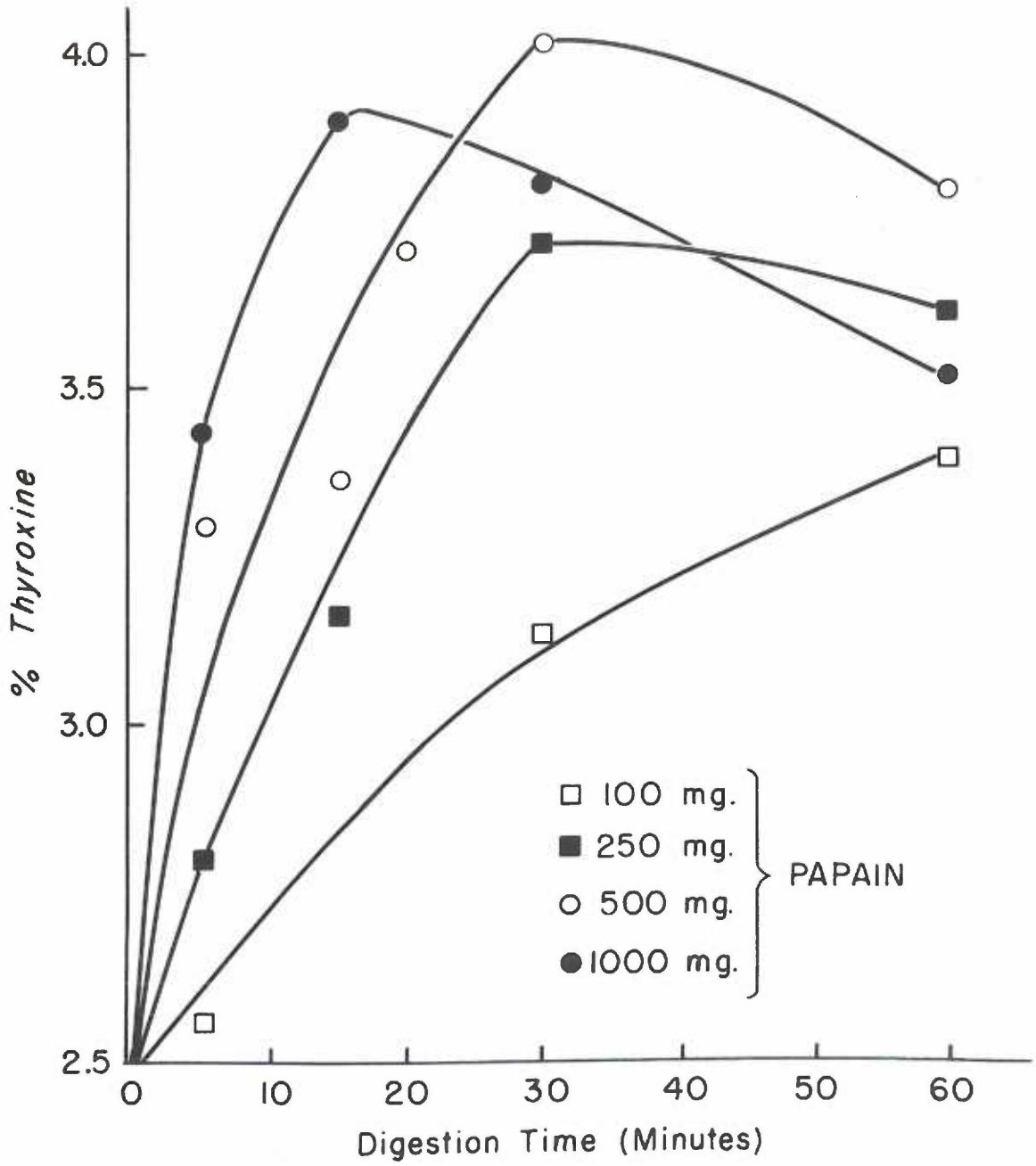

4.0
3.5
3.0
2.5
% Thyroxine
0
10
20
30
40
50
60
Digestion Time (Minutes)
100 mg.
250 mg.
500 mg.
1000 mg.
PAPAIN

Table IX

Incubation Time and Temperature Variations on H_2S Activated Papain Preparations

A. Predigestion with 250 mgs. of H_2S Activated Papain for 30 Minutes

Sample	Incubation Time	% Thyroxine	Grams Yield
129	24 hours	4.38	30.5
130	12 "	3.93	34.2
Average Table VIII	4 "	3.71	35.2

B. 4 Hour Preparations Digested 250 mgs. Activated Papain for 30 minutes

Sample	Incubation Temp.	% Thyroxine	Grams Yield
131	95° C	4.09	30.4
Average Table VIII	80° C	3.71	35.2
133	65° C	2.69	35.1
	500 mgs. for 5 minutes		
125	95° C	3.50	39.7
Average Table VIII	80° C	3.06	45.8

activated papain digested products was made by digesting the casein in the mother liquor of a previous experiment. However, the yield, after digestion with 250 mg. for 30 minutes and with the usual incubation procedures, was only 21 gm., which compares unfavorably with the usual amount of 30 gm.

The time course of thyroxine formation in activated papain preparations was compared to that of standard preparations by taking frequent samples from a large scale operation. 300 pounds of casein were digested for one half hour at 65°C with one pound of papain which had been suspended and activated in five gallons of one-half saturated H_2S water. Iodine, $KMnO_4$ and oxygen were then added and the incubation carried on in the usual manner. The results of two such experiments are listed in Table X, as are those of two non-digested runs; this data is presented graphically in Figure 4. The digested preparations have reached, in five hours, values seen in standard preparations only after 20 hours or more incubation. Also, after 8 hours, the digested preparations reached over 4%, a value rarely achieved in standard preparations.

Another series of experiments was performed in which the papain solution was activated with cyanide. The papain was weighed, made into a paste with glycerine and water, and transferred to a graduated cylinder as before. 0.2M KCN was added and titrated to pH 7 with 1.0 N HCl so that the final concentration contained 10 mg. of papain per ml. of neutral-

Table X

Time Course of Thyroxine Formation from Large Scale Preparations

Activated Papain Preparations

A. 1 lb. papain/300 lbs. casein

Time (hours)	% Thyroxine
0*	1.64
1	1.93
2	2.61
4	3.21
6	3.95
7	4.00
8	4.32

B. 3/4 lb. activated papain/ 300 lbs. casein

Time (hours)	% Thyroxine
0*	1.37
1	1.95
3	2.81
5	3.69
8	3.88

Standard Large Scale Preparations

A.

Time (hours)	% Thyroxine
1	1.37
5	2.38
11	3.02
15	3.40
20	3.65

B.

Time (hours)	% Thyroxine
0*	1.12
1	1.46
2	1.88
4	2.21
8	3.02
12	3.20
16	3.27
18	3.58

*0 time sample was taken just after all the iodine had been added.

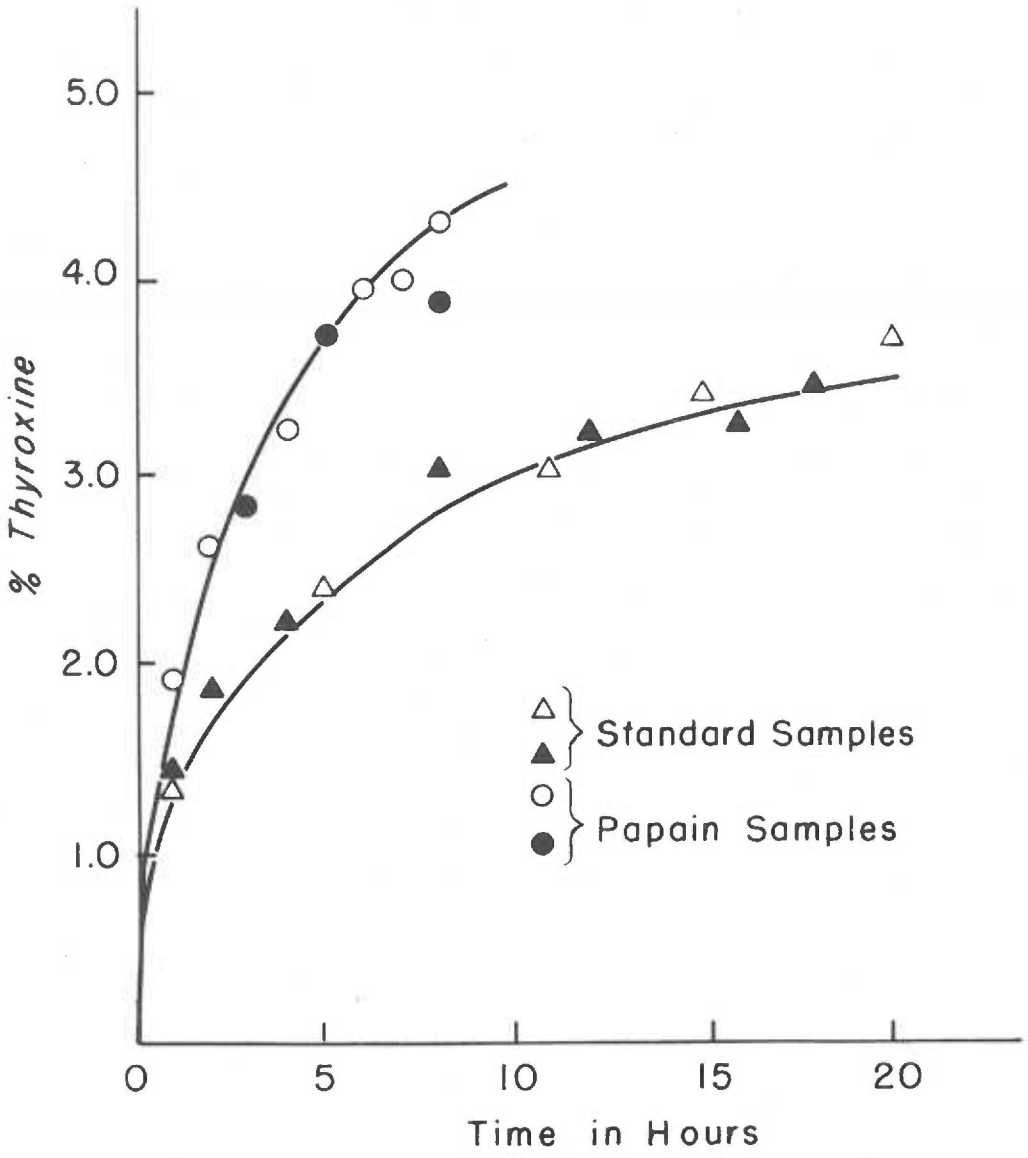
Figure 4

TIME COURSE RELATIONSHIPS

(Data from Table X,
page 48)

Numerous samples were taken from large scale productions. Two preparations were made using predigestion with H_2S activated papain and two were standard preparations. The per cent thyroxine is plotted against incubation time.

FIGURE 4


5.0
4.0
3.0
2.0
1.0
% Thyroxine
0
5
10
15
20
Time in Hours
Standard Samples
Papain Samples

ized 0.1M KCN. For example: 1.00 gm. of papain was dissolved in 50 ml. of 0.2M KCN, titrated with 8.5 ml. of 1.0 N HCl (under the hood), and the solution made up to 100 ml. The mixture was then incubated in the oven at 45°C for 45 minutes. The KCN activated papain was then used to digest the casein in the same manner as with H_2S activated papain. Table XI shows the results of this series.

Table XI

Four Hour Preparations Digested with KCN Activated Papain

Sample	Weight of Papain	Digestion time	% Thyroxine	Weight Yield
109	100	5	2.52	56.0
114	100	30	2.70	41.7
113	100	60	3.10	37.5
106	250	5	2.51	49.1
107	250	5	2.56	51.5
105	250	30	3.93	29.3
112	250	60	3.85	23.3
104	500	5	2.90	38.8
103	500	30	3.18	20.6
108	500	60	2.84	17.5
115	1000	5	2.76	33.7
110	1000	30	2.57	12.4

Although the number of experiments does not warrant any final conclusions, many of the preparations were well above

those prepared without digestion. The highest values were obtained by digestion with 250 mg. of papain. Higher amounts of papain did not give as great an increase in thyroxine yield although an increase in the amount of digestion was seen by the decrease in iodocasein yield which was as much as that found with H_2S activated papain. Due to the toxic nature of cyanide, further investigations in this field were not undertaken.

6. The Effects of Prolonged Storage

Another interesting effect was discovered more by accident than by intent. A 125 ml. Erlenmeyer flask containing the unprecipitated solution of preparation 39 was inadvertently left in storage for 15 months. On precipitation, the thyroxine content was found to be 6.44%. In order to substantiate this effect of prolonged storage, another sample, no. 137, was prepared by the standard method, and aliquots were precipitated at various intervals. These results are listed in Table XII.

Table XII

Effect of Storage at Room Temperature on Thyroxine Formation

Sample	Incubation Procedure	Storage Time	% Thyroxine
39	8 hrs. incubation 80°	0	3.15
		15 months	6.44
137	4 hrs. incubation 80°	0	2.60
		3 days	2.48
		3 weeks	2.74
		3 months	3.19
		20 months	4.17

III. DISCUSSION

The reaction of coupling of two diiodotyrosine molecules to form thyroxine has been previously discussed in the introduction to this thesis and the better known conditions necessary for its occurrence have been reviewed. Some new light has been shed on this subject by our experimental findings. Since the amount of thyroxine formed in intact proteins is not directly proportional to the content of tyrosine (see reference 36 for a review of the various proteins and their thyroxine content), one can assume that the spacial arrangement of tyrosine is an important factor. Our efforts to increase the thyroxine yield by partial digestion of casein with purified papain preparations were to no avail. Roche et al[88] also found that the peptones of casein, formed by partial digestion with pepsin, contained far less thyroxine than the intact casein when iodinated by Reineke and Turner's method. In this laboratory an attempt was made to iodinate a pepsin hydrolysate of casein by the usual technique; the amount of thyroxine formed was negligible.

When the casein is partially digested with papain which has been activated with H_2S or KCN, thyroxine formation is greatly enhanced. Therefore the activity of this enzyme has been altered in such a way that the products formed from its action on casein have tyrosine molecules in an advantageous position for coupling and thyroxine formation. Our highest

yields of thyroxine were obtained after the casein had been digested by 500 mg. of H_2S activated papain for 30 minutes. Further digestion, either by prolonging the digestion time or increasing the amount of papain, led to products which contained less than maximum amounts of thyroxine. Therefore we may assume that the size of the peptide chain was optimum for thyroxine formation at this time.

Bergman et al[35] have shown that activation of papain with H_2S, KCN, and other agents alters the proteolytic activity in such a way that peptides and even amino acids are found in the digestant. He also has investigated the specificity of activated papain on synthetic peptides, and although certain preferences as to site of hydrolysis are demonstrated, no specific pattern has yet been evolved.

Pitt-Rivers[33] has studied this coupling reaction by using synthetic peptides containing diiodotyrosine. She has incubated various peptide solutions under oxidative conditions and found wide variations in the amount of thyroxine formed by various peptides. For instance N-benzoyl-3,5,-diiodotyrosine formed almost no thyroxine, while N-acetyl-3,5,-diiodotyrosylglutamic acid formed up to 36% after incubation at 38°C for 11 days.

With continued studies it is possible that enough can be learned of the kinetics of this coupling reaction to enable us to find precisely the peptide structures which are necessary for maximum thyroxine formation. At the present time

this thesis presents a method by which high potency iodinated casein products can be prepared within a short period of time.

IV. PRELIMINARY INVESTIGATIONS OF THE ASSAY PROCEDURE

One of the main criticisms of the chemical assay used to determine thyroxine in the experimental work just presented is the possibility of high values being obtained by contamination of the butanol fraction with non-thyroxine iodine-containing compounds. Pitt-Rivers[23] has shown that 3,5-diiodo-4-hydroxybenzaldehyde is an oxidation product of diiodotyrosine and is found in the hydrolysates of some thyroproteins. It is also carried along with thyroxine in the butanol extraction and is not removed by reextraction of the butanol with alkali. In order to test the effects of this compound on our assay procedure and also to see if the increase in value we obtained in papain preparations was due to this contaminant, the following experiments were undertaken.

3,5-diiodo-4-hydroxybenzaldehyde was prepared according to Paal[27]. 12 gm. of parahydroxybenzaldehyde (Eastman) were dissolved in 50 ml. of ethanol mixed with 25 gm. of iodine dissolved in 90 ml. of ethanol. 2.1 gm. of I_2O_5 dissolved in 300 ml. of water were added and the mixture was refluxed for 30 minutes, cooled, and the residue collected on a filter funnel. The precipitate was washed twice with 100 ml. of 10% $NaHSO_3$, acidified to congo red with HCl, washed with water and dried. The crude material was then purified by recrystallizing from hot ethanol with the aid of a small amount

of activated charcoal. After repeated recrystallizations a white compound was obtained which melted at 194-197°C with decomposition. Paal's compound decomposed at 198-199°C. The yield was 8.5 gm., 30% of theoretical. The oxime of this aldehyde was prepared according to Schriner and Fuson[38]. 0.5 gm. of hydroxylaminehydrochloride was dissolved in 3 ml. of water. 3 ml. of 2.5 N NaOH and 0.2 gm. of the aldehyde were added with 1 ml. of ethanol. The mixture was warmed in boiling water for 15 minutes and then placed in the refrigerator over night. The crystals of oxime were collected by centrifugation and recrystallized from 50% ethanol. The melting point was 201-202°C with decomposition. This agrees well with the 203°C found by Paal[39]. The phenylhydrazone was more difficult to prepare, and the orange crystals formed very slowly and in poor yield. The melting point of a product from a single recrystallization was 168°C with decomposition, considerably higher than the 160°C reported by Paal.

The first experimental assays were carried out to follow the aldehyde through the normal assay procedure. When 1 gm. of casein and 32.8 mg. of 3,5-diiodo-4-hydroxybenzaldehyde were hydrolyzed together, 24 mg. of iodine or 43.2% of the added aldehyde showed up in the butanol layer. When thyroxine and the aldehyde were hydrolyzed with casein, the butanol layer contained an additional 33% of the added aldehyde assuming 100% recovery of thyroxine. In the next series of experiments we tested the ability of various solvents to extract

this aldehyde from an acid aqueous solution. 20 ml. aliquots of an aqueous solution of 3,5-diiodo-4-hydroxybenzaldehyde were pipetted into a series of separatory funnels. The solutions were acidified with dilute HCl to a yellow color with bromocresol green and then extracted with 20 and 10 ml. portions of the following solvents: butanol, ether, chloroform, carbon disulfide, carbon tetrachloride, benzene, toluene, and xylene. The solvents were then evaporated under the hood and the iodine determined as in the normal thyroxine method. All the solvents extracted a major portion of the aldehyde. The highest, butanol, extracted 99%. Chloroform and carbon tetrachloride extracted 84 and 90%, respectively. This series of solvents was then tested on a solution containing only thyroxine. Butanol extracted 99% of the thyroxine. All the rest of the solvents extracted 2% or less. Re-extraction of the thyroxine solutions, which had been extracted previously with chloroform and carbon tetrachloride, with butanol yielded 97% thyroxine recovery. When an iodocasein hydrolysate was extracted, all the solvents except chloroform and carbon tetrachloride formed emulsions which resisted all efforts to separate them. For convenience chloroform was used in subsequent experiments. Various iodocasein preparations were assayed by the standard method and the results compared to those obtained by extracting the acidified hydrolysate with two 20 ml. portions of chloroform prior to extraction with

butanol. The results are calculated in terms of thyroxine and are listed in Table XIII.

Repeated efforts to isolate 3,5-diiodo-4-hydroxybenzaldehyde from hydrolysates of iodocasein were to no avail. 50 gm. of iodocasein were carried through the regular assay extraction on a corresponding large scale and the residue from evaporation of butanol extracted with several portions of ether. Isolation of the ether soluble residue yielded only a fatty material, M.P. 70°C, and a tar which we were not able to crystallize.

The experiments reported above are just preliminary investigations and no final conclusions can be drawn. We have, however, confirmed the findings of Pitt-Rivers[88] that 3,5-diiodo-4-hydroxybenzaldehyde will follow thyroxine in the regular assay procedure and, if present in the iodocasein hydrolysate, will be determined as thyroxine. We have also found that chloroform and other solvents will extract a major portion of this aldehyde from the hydrolysate without appreciably lowering the thyroxine concentration. If the major portion of chloroform extracted iodine is in the form of this aldehyde, a rough estimation shows that iodocasein preparations contain from 8 to 33%. (Since thyroxine is 65% and 3,5-diiodo-4-hydroxybenzaldehyde is 68% iodine, no correction was made for the thyroxine factor used in these calculations.) Both standard and papain preparations were tested

Table XIII

Comparison of Assay Results
with and without
Chloroform Extraction

Sample and Description	Procedure	% Thyroxine	Difference	% Loss (as Thyroxine)
1 gm. assay control + 13.2 mg. 3,5-diiodo-4-hydroxybenzaldehyde	Routine	4.99		
	$CHCl_3$	3.52	1.47	--
Assay control product (see page 23)	Routine	3.70		
	$CHCl_3$	3.39	.31	8%
Large scale papain preparation	Routine	4.06		
	$CHCl_3$	3.38	.68	17%
No. 34 4 hr. routine prep.	Routine	2.54		
	$CHCl_3$	2.23	.31	12%
No. 72 Low value 4 hr. routine preparation	Routine	1.88		
	$CHCl_3$	1.25	.63	33%
No. 83 4 hr. routine prep.	Routine	2.51		
	$CHCl_3$	2.08	.43	17%
No. 92 Activated papain prep.	Routine	3.96		
	$CHCl_3$	3.13	.83	21%
No. 134 Activated papain prep.	Routine	3.51		
	$CHCl_3$	2.69	.82	23%
No. 39 Prolonged storage prep.	Routine	6.17		
	$CHCl_3$	5.18	.99	16%

and each group showed an average 20% decrease in "thyroxine" with chloroform extraction. Therefore we can assume that the increase in thyroxine value obtained by predigestion with activated papain is not due to an increase in this aldehyde.

SUMMARY

The effects of many factors on the formation of thyroxine in iodinated casein have been studied, using a method previously developed in this laboratory by Osborn[35]. The yield of thyroxine was not appreciably altered by variations in the quality of a number of ingredients including casein, iodine and $NaHCO_3$. Changes in the physical appearance of the final iodocasein product caused by variations in the precipitation, washing and drying techniques also did not alter the thyroxine content.

Increases in the thyroxine content by predigestion of the casein prior to iodination with Caroid (commercial papain), were found previously by Osborn[35] and duplicated by us when fresh Caroid was used. However, we obtained no increase in thyroxine with older samples of Caroid and also found purified papain to be ineffective in spite of evidence of good proteolytic activity.

When the papain used for digestion was first activated with H_2S or KCN, a marked increase in thyroxine yield was obtained. This increase reached a maximum when 500 mg. of H_2S activated papain were allowed to digest the casein for 30 minutes at 65°C. Using this procedure iodocasein products containing over 4% thyroxine were prepared with only 4 hours of incubation. The weight yield of these digested products

was considerably below that of non-digested products. We also found a sample to contain large amounts of thyroxine after storage of the unprecipitated solution at room temperature for long periods.

Some preliminary experiments on the assay procedure were carried out. 3,5-diiodo-4-hydroxybenzaldehyde, a by-product of diiodotyrosine oxidation, was found to follow thyroxine in the usual course of extractions. Preliminary extraction of the aqueous hydrolysate with chloroform prevented the contamination of the thyroxine fraction with this aldehyde. The increase in thyroxine formation found in our activated papain preparations is not due to this aldehyde.

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