Despite the many years of investigation of the thyroid, some of the most fundamental problems concerning this ductless gland are still the subject of speculation and controversy, and the certain progress which follows quantitative knowledge has not yet materialized. Since quantitative values cannot be obtained by clinical observations alone, and since pathologic studies of the glands cannot of themselves solve the function of the thyroid, it becomes apparent that nothing short of the actual isolation in pure crystalline form of the chemical substance or substances within the gland, which are responsible for its activity, can furnish the necessary knowledge with which to gain quantitative relationship. Having accomplished the isolation in pure crystalline form of the active agents within the gland, quantitative results may be obtained and ultimate solution of the thyroid problem is within the power of the investigator.

Investigation of the Chemical Constituents of the Gland, with Isolation of the Iodine-Containing Compound in Pure Crystalline Form.

Starting with fresh and desiccated thyroid the present investigation was at first concerned with the diffusibility of the iodine-containing compound. Iodine is not dialyzable from the thyroid proteins, and will withstand rather severe chemical treatment
126 Iodine Compound in Thyroid

and still be undialyzable through a collodion sack in running water. In order to determine the stability of the iodine compound, various hydrolytic processes were applied to the thyroid proteins. Among others the alcoholic alkaline hydrolysis used by Vaughan was tried and, with some slight modifications, was found to produce a deep-seated hydrolysis without breaking off iodine from its organic combination. Furthermore, the hydrolysis so alters the iodine-containing compounds that about 70 per cent of the total iodine is dialyzable. Another change brought about by the hydrolysis is the solubility of the iodine compound in acid. About 50 per cent of the total iodine contained in the hydrolyzed products is soluble in acids, and 50 per cent is insoluble. The iodine in the acid-insoluble portion is to a large extent not dialyzable. The presence of iodine in organic combination and in non-dialyzable form was encouraging evidence of the stability of the iodine compound and invited further investigation.

The physical and chemical properties of the acid-insoluble group of hydrolyzed constituents will be described somewhat in detail, since this will bring out the chemical problems involved and some of the difficulties encountered.

An alkaline solution of the acid-insoluble constituents is dark brown, almost black, with a green fluorescence, and shows a Tyndall phenomenon with a beam of light. The non-diffusibility, fluorescence, and Tyndall phenomenon show that the solution is one of colloidal nature. Among the acid-insoluble constituents are fatty acids resulting from the original fat in the desiccated thyroid, and sulfur, which results from the decomposition of cystine. The solution has no characteristic odor other than a general fatty smell. The first step in the method of separating the iodine compound is to dry the acid-insoluble constituents, mix with infusorial earth, and extract with petroleum ether to remove fatty acids and sulfur.

Later it was found that fresh thyroid glands could be substituted for desiccated thyroid as a source of material, and that hydrolysis in alcohol was not necessary. Hydrolysis of the proteins may be carried out by use of aqueous sodium hydroxide alone, and the length of the time can be reduced to 24 hours. A quantitative separation of all fats as the sodium soap may be
effected, and a perfectly clear alkaline filtrate of the hydrolyzed thyroid proteins containing practically the entire iodine content of the gland is obtained. On acidification of this solution, a fine flocculent precipitate separates. If this precipitate is filtered off and dried, it is found to contain approximately 0.1 per cent of the total weight of the fresh glands used. It contains, on the average, 26 per cent of the total iodine. The total iodine in the gland, therefore, is divided by aqueous alkaline hydrolysis into acid-soluble and acid-insoluble compounds. Approximately three-fourths of the total iodine-containing compounds are soluble in acid and only one-fourth insoluble. This proportion is remarkably constant during different times of the year, and in different samples of thyroid from different species. The significance of the figure is not entirely clear, but in all probability it represents the equilibrium existing within the glands between the completed iodine compound which possesses physiologic activity and the materials which are used by the gland in the building up of the substance. Physiologically tested, the acid-soluble hydrolyzed constituents are inactive, and the small portion which is precipitated by acid possesses the entire physiologic activity of the gland.

The solubility of the acid-insoluble iodine compounds in organic solvents varies, depending on the solvent used, the presence of water, whether or not acid is present, and the temperature. About 10 per cent of the total iodine is soluble in ethyl ether, but only a small amount is soluble in petroleum ether. If desiccated thyroid has been used as a source of material the dry acid-insoluble products freed from fatty acids by extraction with petroleum ether are dissolved in sodium hydroxide and again acidified. If the aqueous hydrolysis has been used the precipitate obtained by acidifying the alkaline solution of hydrolyzed products is dissolved in sodium hydroxide and reprecipitated with acid. This precipitate is heavy, flocculent, and amorphous, and, when heated above 40-50°C. in aqueous solution, turns to a black, tarry mass. On cooling and drying it is found to be brittle and may be broken into a fine, dry, almost black powder. The dry powder is soluble in acid ethyl alcohol and aqueous solutions of sodium, potassium, and ammonium hydroxides and carbonates. It is in part precipitated from acid
alcohol by the addition of sodium carbonate, the portion precipitated being a sticky, black, tarry mass. Most of the iodine-containing compounds are soluble in alcohol in the presence of sodium carbonate, so that this is a valuable aid in the gross separation of the black, tarry impurities from the constituents which contain iodine. However, some of the iodine is carried down by the sodium carbonate precipitation, showing that the iodine compound, although probably the same throughout all the different precipitates, is attached to various groups of compounds which possess different solubilities.

If barium hydroxide is added to the alcohol solution after removal of the sodium carbonate precipitate, practically all the rest of the dark-colored compounds are precipitated by the barium. This precipitate carries down varying percentages of iodine, depending on conditions. If the solution is made acid with hydrochloric acid before the addition of barium, a very high percentage of the iodine may be precipitated. If the solution is made alkaline with sodium hydroxide, a very small percentage may be carried down. Between these two limits the amount removed by the barium depends on the acidity and the temperature. Since the alcohol solution remaining after precipitation with barium is light straw-colored, the alcohol-barium-soluble constituents to which the iodine compound is attached are almost colorless.

Similar separations may be produced in aqueous solution. If the dark brown alkaline solution of the hydrolyzed products is acidified, almost all the color and iodine compounds are precipitated. Barium, calcium, and magnesium salts, added to an alkaline aqueous solution of the acid-insoluble hydrolyzed constituents, precipitate practically all the dark-colored compounds, leaving a very light-colored solution. This precipitation divides the iodine present; about one-half is precipitated by the alkaline earths and one-half is soluble.

These precipitation and solubility properties clearly show the nature of the material with which one has to work. It is a mixture of compounds in colloidal form, and the problem is not only one of separating a mechanical mixture. It was necessary to gain a clear insight into the chemical nature of the iodine compound and the other constituents before the separation of the two could be realized.
The most striking property of the acid-insoluble group of hydrolyzed products is their acidic nature. They may be dissolved in alkali and reprecipitated by acid without appreciable loss of iodine.

Many attempts were made to separate the iodine compound by its solubility and precipitation properties with various reagents. No specific precipitant was found. No difference in solubility was found which could be used to effect a separation. After many attempts to separate the iodine compound had failed, it became apparent that the compound was not present in free form, but was still firmly bound to some unknown substances.

Experiments showed that iodine was not broken off when dissolved in moderately strong sodium hydroxide. In the hope that heating in aqueous sodium hydroxide might produce a further hydrolysis, the acid-insoluble constituents of the alkaline-alcoholic hydrolysis were heated several hours in 5 per cent sodium hydroxide. It was then found that treatment with sodium hydroxide, followed by precipitation with an acid, will not effect a separation of the iodine compound from the black colloidal impurities. The solubility of the compounds present which do not contain iodine closely parallels the solubility of the iodine-containing compound, and, as far as alkali and acid are concerned, no appreciable separation can be brought about by alternate treatment with each.

Since the addition of a soluble barium salt to an alkaline solution of the acid-insoluble products of hydrolysis precipitates practically all the brown impurities and does not carry down all the iodine, this separation was investigated to see whether the percentage of iodine in the acid-insoluble constituents could thereby be increased. The acid-insoluble constituents were dissolved in dilute sodium hydroxide, and barium hydroxide was added. The solution was heated in a nickel crucible at 100°C. for 18 hours. This treatment produced a precipitation of the brown compounds, giving a light yellow filtrate of the barium-soluble constituents. Determination of the iodine content of the filtrate showed that about 50 per cent of the iodine present had been precipitated from the alkaline solution by barium hydroxide, and 50 per cent remained soluble. Sodium sulfate was added to the water solution containing the barium-soluble con-
stituents, and the barium was removed as sulfate. The solution was then acidified, and a copious precipitate obtained which still retained iodine. The precipitate was filtered off and dried. Analysis showed that the percentage of iodine had been very materially increased by this treatment. The iodine in the starting material amounted to about 5 per cent; after treatment with barium, as outlined above, and reprecipitation with an acid, the iodine was found to be about 15 per cent.

The portion of the starting material which had formed a dark, flocculent precipitate with barium hydroxide was dissolved in sodium hydroxide and sodium sulfate. The barium sulfate was removed and the solution was acidified. A precipitate formed on the addition of the acid and when this was filtered off and dried it too was found to contain an increased percentage of iodine. By treatment with barium hydroxide the percentage of iodine contained in the hydrolyzed constituents of the thyroid proteins had been increased.

As this was the only material advance which had been made toward isolation of the iodine-containing compound, it was decided to investigate more fully the reactions involved between barium and the hydrolyzed products of the thyroid proteins. The two precipitates were dissolved in separate solutions of sodium hydroxide, barium hydroxide was added to each, and the solutions were heated in nickel crucibles for 18 hours at 100°C. The insoluble portions were filtered off, the barium was removed as outlined above, the barium-soluble constituents of each crucible were combined and again acidified, the precipitate was filtered off and dried, and analysis showed that the percentage of iodine present had increased in the barium-soluble portion to 26 per cent. With many misgivings the precipitation with barium and heating in a nickel crucible were repeated still another time. The percentage of iodine in the precipitate obtained was 33 per cent. Still another treatment yielded a product containing 42 per cent of iodine. The amount of material now consisted of less than 200 mg. The success of the method, however, called for still further treatment. It was carried out in the same manner, and a precipitate containing 47.3 per cent of iodine was obtained. The character of the precipitate had changed and the color of the solution had almost disappeared. It seemed
probable that some other method of purification could now be applied to the preparation containing 47.3 per cent iodine.

The material was dissolved in 95 per cent alcohol; solution was complete. The alcohol was evaporated on a water bath to a small volume, in the hope that some crystals might separate. By a chance, however, the dish containing the alcohol evaporated to dryness, and the dried precipitate was heated for about 1 hour after the alcohol had been driven off. It was thought that on the addition of more alcohol the white powder on the bottom of the dish would again be dissolved, and, possibly, crystals might still separate. More alcohol was therefore added, but a white incrustation on the bottom of the dish was insoluble in alcohol. I believed that this treatment had effected a further separation, that the iodine-containing compound was redissolved in the alcohol, and that the white incrustation represented impurities. It was filtered off. The weight of this white powder was 18.6 mg. It was dissolved in sodium hydroxide, and a portion of it was used to determine iodine, when it was found that the iodine amounted to 60 per cent. The white incrustation was, therefore, not an impurity, but the iodine compound itself. Its solubility in alcohol had been changed by heating the residue left after evaporation of the alcohol. More of the acid-insoluble constituents resulting from hydrolysis of the thyroid proteins were treated in precisely the same manner, and about 200 mg. of the white residue were obtained. When this was dissolved in aqueous sodium hydroxide, precipitated by adding sulfuric acid and boiling, it was converted into fine white microscopic crystals. For reasons which will be given this iodine compound has been named "thyroxin," and it will be referred to by that name throughout the remainder of this article.

As the yield of thyroxin depended on the amount of desiccated thyroid which could be treated at any one time, it was decided to enlarge our facilities for the hydrolysis of desiccated thyroid, which, up to this time, had been carried out in glass flasks. An 11 gallon galvanized iron tank was constructed, which could take care of 500 gm. of desiccated thyroid at one time. The acid-insoluble constituents were obtained as before. They were dissolved in dilute sodium hydroxide, barium hydroxide was added, and the steps outlined above were carried out. No crystals of thyroxin were obtained.
The work from this point will be presented in a logical rather than a chronological order, as many months were spent in elucidating the factors which prevented the isolation of more crystals.

After working for 14 months in an endeavor to repeat the first isolation of the crystals, it was found that there are five conditions which influence the isolation of thyroxin in pure crystalline form:

1. **Effect of Temperature on Precipitation with an Acid.**—When the partially purified, iodine-containing constituents are precipitated by an acid, the precipitate is flocculent and amorphous. If this is heated to 40–50°C., it assumes a fine, granular form which can be filtered and washed very readily. This procedure was followed because of the facility of handling the precipitate. Warming of the acid solution does not break off any iodine in inorganic form. If, however, this precipitate, which was prepared by warming the solution after the addition of the acid, is dissolved in alkali and heated with barium hydroxide, and the solution is again acidified, some iodine in the inorganic form may be found in the solution. If the precipitation by an acid is carried out in the cold, subsequent heating with barium hydroxide and reprecipitation does not break off iodine in the inorganic form.1

Since only one stage of this treatment could be carried out in 1 day, 24 hours intervened between the first acidification and warming of the solution and the second precipitation, after which iodine was demonstrated in the inorganic form in the filtrate. This time interval formed a convenient screen behind which the deleterious action of heat remained hidden for many months. This influence of temperature on acidification was one of the most important causes for the failure to separate more crystals. Fearing that a concentrated sodium hydroxide solution would destroy the iodine compound, we had been neutralizing the alkali which was used to effect the primary hydrolysis with sulfuric acid before evaporating off the alcohol. As the amount of free alkali remaining in the alcohol was variable, depending on the amount of ammonia which had been evolved and the amount of

1 Inorganic iodine has been tested for during this investigation by acidifying the solution with hydrochloric acid, adding starch and a few drops of sodium nitrite, producing a blue ring if inorganic iodine is present.
water and carbonate in the alkali, it frequently happened that slight excess of acid was added, so that the alcohol was evaporated off in the presence of a small amount of acid. This we finally showed has a very destructive action on partially purified thyroxin. In this connection it seems probable that the lack of physiologic activity of iodothyrin is explained by this action of acid on the iodine-containing compound. The iodine may not be broken off by treatment with acid, but the chemical properties, and undoubtedly the nucleus to which the iodine is attached, are altered so that the compound loses its physiologic activity.

2. Effect of Heating the Alkaline Hydrolysis Solution in the Presence of Metal.—At first the hydrolysis was carried out in glass flasks. When an 11 gallon galvanized iron tank was substituted, the hydrolysis was carried out apparently just the same, but it was found that with the metal the hydrolysis in an alkaline solution resulted in the breaking off of the iodine in the inorganic form. Investigation showed that in alkaline solution, iron, zinc, copper, tin, lead, German silver, and, in fact, all metals except nickel and the heavy metals, gold, silver, and platinum, break off iodine from its organic combination. A heavily enameled, cast iron kettle was found to give a satisfactory surface for a container in which to carry out the primary hydrolysis with sodium hydroxide in alcohol. Later this was replaced by a nickel kettle.

3. Effect of Carbon Dioxide.—During the first separation of crystals carbon dioxide was neither employed directly nor was its effect excluded from the various steps in the process. After many months of failure to separate more of the iodine compound in crystalline form it was found that carbon dioxide plays an important rôle in the separation of thyroxin from the impurities. By chance an alkaline solution containing a partially purified preparation of thyroxin mixed with a large amount of impurities was precipitated with carbon dioxide instead of sulfuric acid. The precipitate was filtered on a Buchner funnel and washed with distilled water. Instead of the distilled water running through lighter colored than the first filtrate, it was almost black (Fig. 1). Investigation showed that the black impurities were insoluble in water saturated with carbon dioxide but were soluble in distilled water. Even after the effect of the carbon dioxide on the separation of thyroxin was discovered, its explanation was, for many months, obscure.
4. Effect of Temperature during Treatment of a Solution with Carbon Dioxide.—After it was found that carbon dioxide had a very important action which allowed thyroxin to be isolated in crystalline form, carbon dioxide was added to the various solutions, first in the cold, and then it was added to the solutions warmed to various temperatures up to 100°C. It was found that with the preparations which were contaminated with a large amount of impurities, the passing of carbon dioxide into a solution above 50-60°C. resulted in breaking off iodine. This was confirmed many times, and although for a long time unexplained, the solutions were always cooled before treatment with carbon dioxide.

5. Effect of Different Samples of Desiccated Thyroid.—The fifth point is one beyond control, and was found to rest on the condition of the desiccated thyroid employed. The thyroxin content of different samples of desiccated thyroid varies as much as 400
per cent. It appears probable that bacterial or other decomposition so alters the proteins in some samples that it is impossible to separate any of the iodine compound.

These five factors finally became apparent after a consideration of all the results obtained over a course of 2 years investigation. It is remarkable that all these factors were unconsciously controlled during the first purification, especially as it took many months to find out that there were so many separate and distinct influences at work causing the destruction of the compound.

The investigation of the chemical constituents of the thyroid was begun by the writer in September, 1910. The use of barium salts to effect a separation was tried in November, 1914, and thyroxin was first isolated in December, 1914. Although over 100 mg. were isolated at that time, it was not until February, 1916, that the effect of carbon dioxide was established and more of the substance was obtained. During the summer of 1916 several grams of thyroxin were separated, and by May, 1917, over 7 gm. were available for its chemical identification. The empirical and structural formulas were determined during the summer of 1917. In December, 1917, Mr. Osterberg succeeded in synthesizing a small amount of thyroxin. The synthesis was repeated and the structural formula confirmed in April, 1919.

Up to the present time about 33 gm. of the compound have been separated from 6,550 pounds of fresh thyroid material which has been made up almost entirely of hog thyroid (Fig. 2). The method may be briefly stated as follows.

The fresh thyroid glands are hydrolyzed in 5 per cent sodium hydroxide. The fats are removed by rendering the sodium soap insoluble, and the clear alkaline filtrate is cooled and acidified. The acid-insoluble constituents containing practically 100 per cent of the thyroxin present are filtered off. This material is redissolved in sodium hydroxide and reprecipitated, using hydrochloric acid. The precipitate is now air-dried and is dissolved in 95 per cent alcohol. The excess hydrochloric acid which remains in the air-dried precipitate is neutralized with sodium hydroxide until it is almost neutral to moistened blue litmus paper. A heavy, black, tarry precipitate forms, which may be removed by filtration. The alcoholic filtrate is treated with barium hydroxide by adding a hot, very concentrated aqueous solution of the
FIG. 2. The three tanks in which 6,550 pounds of hog thyroid glands were treated for the isolation of thyroxin.

hydroxide to the alcohol, and refluxing. The treatment with barium removes some heavy dark impurities. A small amount of sodium hydroxide is added to the filtrate, and carbon dioxide is passed through the solution. The barium and sodium carbonate are removed by filtration, and the alcohol is distilled.
The last traces of alcohol are removed by heating in an evaporating dish. The aqueous residue is now acidified with hydrochloric acid. The precipitate is dissolved in alkaline alcohol, carbon dioxide is passed through the solution, the precipitated sodium carbonate is removed, and the alcohol is evaporated. The last traces of alcohol are removed by heating on a water bath and the solution is allowed to stand. The monosodium salt of thyroxin will separate at this point. The yield is not quantitative, and it must be further purified by dissolving in alkaline alcohol, passing in carbon dioxide, distilling the alcohol, and allowing the monosodium salt to crystallize a second time. This may then be precipitated from an alkaline alcoholic solution by the addition of acetic acid. Resolution in alkaline alcohol and precipitation with acetic acid for five or six times removes the impurities and will yield thyroxin containing the theoretical percentage of iodine. A considerable percentage of the total iodine present is carried down in the neutral alcohol solution by the barium, and another portion is held in solution when the monosodium salt separates, but it is not practicable to try to separate the thyroxin from these precipitates as the yield is very small. Physiologically they possess the same activity as thyroxin when administered according to the iodine content.

After isolating about 7 gm. of thyroxin in the manner described, its empirical and structural formulas were determined and the substance was shown to be 4, 5, 6 tri-hydro-4, 5, 6 tri-iodo,-2 oxy,-beta indolepropionic acid. Thyroxin exists in three forms: (1) the keto form with the carbonyl group adjacent to the imino,

![Diagram of thyroxin]

(2) a tautomeric enol form of this with an alpha hydroxy group and double-bonded nitrogen with no hydrogen attached to the imino,
and (3) a form in which there is an open-ring structure, the elements of water entering between the imino and the carbonyl with the formation of an amino and a carboxyl group. A consideration of the isolation of thyroxin after its structural formula had been determined explains the chemical reactions involved in the purification and isolation of the substance.

A Consideration of the Reactions Involved in the Isolation of Thyroxin.

Thyroxin was first separated by following a method of treatment which was found to increase progressively its iodine content, and for the isolation of much of the material so far prepared this same method was followed without any light being thrown on the exact nature of the chemical reactions involved. The use of barium salts for the separation of thyroxin was first tried because of their ability to precipitate the dark-colored impurities from an alkaline solution of the hydrolyzed thyroid proteins. With the solutions of hydrolyzed proteins at first used, about 50 per cent of the total iodine could be precipitated by barium hydroxide. When the barium-soluble constituents were precipitated by an acid and redissolved in sodium hydroxide or carbonate, barium salts were found to precipitate some of the compounds which had previously been soluble in the presence of barium. This in time led to the discovery that the solubility of thyroxin in the presence of barium hydroxide was a test of its purity.

Partially purified thyroxin which is soluble in barium hydroxide is also soluble in sodium carbonate and in alcohol. Pure thyroxin is insoluble in these reagents. The process of purification rested essentially on the repeated treatments of the impure preparations of thyroxin with barium hydroxide and the recovery
of the barium-soluble compounds by precipitation with an acid. The barium-insoluble constituents were recovered as sodium salts after decomposition with sodium sulfate and then precipitated with an acid.

After six or eight such barium treatments it was found that an increasing percentage of the total iodine was insoluble in barium hydroxide and finally it was shown that thyroxin could not be separated in crystal form from alcohol so long as it was soluble in barium hydroxide. Treatment with barium hydroxide influenced the separation of thyroxin in four different ways: (1) it effected a separation between the two forms of thyroxin, one barium-soluble, the other barium-insoluble; (2) it caused the destruction of certain of the impurities which were present; (3) it precipitated certain impurities from the solution thereby effecting a separation; and (4) it carried down mechanically, when it was precipitated as barium sulfate, the dark-colored colloidal impurities which had almost identical solubilities as thyroxin itself and which were not removed by any other means.

The chain of events which led to the explanation of the reactions involved in the separation of thyroxin from the other compounds contained in the acid-insoluble products of the alkaline hydrolysis and the conversion of barium-soluble thyroxin into barium-insoluble thyroxin was as follows.

It was found that derivatives attached to the imino group render thyroxin soluble in alcohol. This was found true of the acetyl, the formyl, the ureide, the sulfate, and the hydrochloride. It was also found that derivatives attached to the imino group do not form crystalline disilver salts.

When thyroxin is partially purified, so that it contains from 30 to 50 per cent of iodine, it is soluble in alcohol and it does not form a crystalline silver salt. These reactions suggested that the difficulty in purification arose from the fact that some derivative was attached to the imino group, which rendered the compound soluble in alcohol and prevented its precipitation with silver. Since it had already been found that the sodium salts of the acetyl, the formyl, and other derivatives on the imino were readily thrown out of solution in crystalline form by increasing the amount of sodium hydroxide present, attempts were made to determine the group attached to the imino in partially purified
Iodine Compound in Thyroid

thyroxin by precipitating its sodium salt with a high concentration of sodium hydroxide. Partially purified barium-soluble thyroxin containing about 40 per cent of iodine was therefore dissolved in sodium hydroxide, and a solution of 30 per cent sodium hydroxide was added to this. As had been hoped, the addition of the stronger alkali soon produced a cloudy precipitate which did not settle but remained suspended, due to the high specific gravity of the solution. This was centrifuged, the supernatant liquid contained most of the yellow impurities, and the precipitate remained as a firm felt on the bottom of the tube. It was dissolved in distilled water, sodium hydroxide was again added, the precipitate again formed, but this time it separated in more distinct particles and the solution was less turbid. It was centrifuged, the supernatant liquid was still yellow, but showed much less color than the first solution. The residue in the bottom of the tube was again dissolved in distilled water, sodium hydroxide was added, and this time the precipitate assumed a still different form, coming down in distinct separate particles, practically white, and the solution was almost colorless. These particles were examined under the microscope. They were the typical disodium salt of pure thyroxin itself, and not of a derivative. They were centrifuged from the alkali, dissolved in alcohol, and precipitated by the addition of acetic acid, when they were recovered as pure crystalline thyroxin.

The substitution of sodium chloride for sodium hydroxide permitted the solutions to be filtered instead of centrifuged, and it was then found that sodium chloride precipitated the disodium salt of thyroxin from an alkaline solution even better than sodium hydroxide. Although this sample of thyroxin had been completely soluble in barium hydroxide, it was now insoluble in such a solution. As this change in solubility could not have been brought about by the hydrolysis of a derivative attached to the imino group, it was apparent that solubility in barium hydroxide depends on the presence of certain impurities.

Investigation of the impurities which were separated from thyroxin by salting out the crystals from the alkaline solution showed that they are soluble in ether, that they are acidic in nature, and contain indole derivatives which give the pine-splinter reaction after fusion in caustic alkali. If pure crystalline
thyroxin is dissolved in dilute sodium hydroxide and some of these indole derivatives are added to the solution, there appears to be an immediate reaction between thyroxin and these impurities which completely alters the chemical properties of thyroxin. The presence of these impurities renders thyroxin soluble in barium hydroxide, and instead of separating with sodium chloride as the crystalline disodium salt, it is thrown out of solution as an oily tar.

Since it was shown that this great alteration in the chemical properties of thyroxin could be brought about by the presence of certain indole derivatives of acidic nature, and since the change was not due to the hydrolysis of a derivative attached to the imino group, the first explanation was that the imino group was rendered more reactive by the presence of the impurities and that a salt formation occurred similar to the sulfate or hydrochloride, the acidic group being attached to the imino nitrogen. However, it was difficult to explain all the reactions by such a change.

The first proof of the chemical reactions involved was obtained after preparation and analysis of the acetyl derivative. In the acetyl derivative of thyroxin, only one acid group is left which can react with a metal, therefore, a mono-metal derivative should be obtained. Analysis of the silver salt of the acetyl showed that apparently two atoms of silver had added to the acetyl. The simplest explanation for this would be the opening of the pyrrole ring between the imino and carbonyl groups with the formation of the second free carboxyl group.
Further investigations showed that this is most probably true and that a similar opening of the ring occurs in thyroxin. The relation between the two forms is precisely the same as the relation between creatinine and creatine. The opening of the ring increases the acidic properties of thyroxin and renders it soluble in sodium carbonate, barium hydroxide, and alcohol. It prevents the formation of a crystalline insoluble disilver salt and, in fact, all the changed properties of thyroxin are adequately explained by this reaction.

Analysis shows that the iodine content of barium-soluble thyroxin may be as high as 58 or even 60 per cent. This iodine content excludes the possibility of any group being attached to the imino, having a higher molecular weight than formic acid. Furthermore, barium-soluble thyroxin can be changed and separated in pure crystalline form, insoluble in barium hydroxide, merely by allowing an aqueous sodium carbonate solution to stand several weeks. Under these conditions there is a slow closing of the ring with the formation of the mono-salt which is only slightly soluble. It seems most probable that the impurities present do not react in a stoichiometric relation with all the thyroxin, but that the presence of even a small percentage of the impurities introduces the factor of time and greatly delays the rate at which the ring closes.

Sufficient proof that a group is not attached to the imino group of partially purified thyroxin which is soluble in barium hydroxide, alcohol, and sodium carbonate is furnished by the fact that it is impossible to hydrolyze the acetyl from the imino group without disruption of the molecule. This also applies to all derivatives so far studied in which a group is attached to the nitrogen. Hydrolysis does not remove the group but destroys
the integrity of the molecule. Therefore, the reactions occurring during purification can not be the removal of a group attached to the imino. In the body it appears probable that thyroxin exists in the open-ring form with an amino and two carboxyl groups. The problem then, in isolation is to establish conditions favorable for the closing of the pyrrole ring. The open-ring form of thyroxin will not crystallize and although it is but slightly soluble in acid, its solubility in carbonates, barium and calcium hydroxide, alcohol, and pyridine are in striking contrast to the closed-ring form which is insoluble in all these reagents. In the presence of certain impurities it appears very difficult, sometimes impossible, to close the ring and thereby separate thyroxin. The mere presence of these impurities is sufficient to open the ring if pure crystalline thyroxin is added to a solution of such impurities.

The exact nature of these impurities is still unknown, but they are among the hydrolyzed products of the thyroid proteins and contain the indole nucleus. Animo-acids from gelatin do not cause thyroxin to exist in the open-ring form. The difficulties encountered in separating the closed-ring form of thyroxin from any solution depends on the ratio between the amount of thyroxin present and the amount of impurities present. Thyroxin appears to carry down either chemically or mechanically these impurities so that solution in alkali and precipitation with acid do not effect a separation. The best conditions so far found for the closing of the ring and the separation of thyroxin are obtained by solution of thyroxin in sodium carbonate. Under these conditions the ring slowly closes and the monosodium salt, which is only slightly soluble, deposits in crystalline form on the bottom of the flask.

SUMMARY.

The chemical reactions involved in the five conditions, which influence the isolation of thyroxin, may be summarized as follows.

1. Effect of Temperature on Precipitation with an Acid.—The chemical reactions involved in the acidification with an acid were very obscure until the acetyl derivative was prepared. It was found that when an acid suspension of the acetyl is neutralized with sodium hydroxide, at the neutral point, iodine breaks off
from the acetyl derivative, and the liberation of iodine may be shown to be a matter of oxidation and reduction. Thyroxin is far less susceptible, and an acid suspension of thyroxin may be neutralized with an alkali without any such liberation of iodine occurring. When, however, an impure preparation of thyroxin is neutralized, the impurities appear to influence thyroxin in a manner similar to the addition of the acetyl group to the molecule, and at the proper hydrogen ion concentration, which is very nearly the neutral point, there is a reaction between the impurities and thyroxin resulting in a breaking off of iodine. Temperature greatly influences this reaction. If carried out in the cold solution, no destruction of thyroxin occurs.

Another effect of temperature during acidification is probably that of polymerization. Indole compounds polymerize very readily in the presence of acid. Thyroxin in pure state is slowly changed by boiling in strong acid, but when only partially purified the action of strong acid and heat brings about such a deep seated alteration in the molecule that its physiologic activity is destroyed.

2. Effect of Heating the Alkaline Hydrolysis Solution in the Presence of Metal.—Thyroxin is very susceptible to reduction. An alkaline solution of thyroxin may be completely decomposed by heating with metallic zinc; the iodine is broken off from the molecule. Without doubt, the other metals, iron, copper, tin, lead, German silver, and, in fact, all metals except nickel, gold, silver, and platinum, react in the same manner, causing the destruction of thyroxin by reduction.

3. Effect of Carbon Dioxide.—There are two distinct actions produced by carbon dioxide. One is partial purification of thyroxin by precipitation from an alkaline solution. If an alkaline solution of partially purified thyroxin is treated with carbon dioxide until no further precipitation is produced, and the precipitate is removed by filtration, a large amount of material may be precipitated in the filtrate by the addition of a stronger acid. Thyroxin is precipitated by carbon dioxide more completely than, and at a point far in advance of, certain of the impurities. The effect of carbon dioxide as a precipitant depends on the ratio between the amount of thyroxin and the amount of impurities present. In the first solution of the acid-insoluble constituents
of the hydrolyzed thyroid proteins, carbon dioxide will not precipitate any thyroxin. As the impurities are removed, the precipitation of thyroxin by carbon dioxide becomes more and more complete until in a solution of pure thyroxin the precipitation with carbon dioxide is quantitative. This influence of the impurities on the precipitation of thyroxin by carbon dioxide is one of the most striking examples of the effect of impurities on the chemical properties of thyroxin.

Another influence of carbon dioxide on the separation of thyroxin is more deep seated and was, at first, very difficult to explain. Thyroxin is soluble in sodium hydroxide with the formation of the disodium salt. If carbon dioxide is passed into such a solution, the hydroxy group gives up its sodium and exists in the free form. The carboxyl group, however, retains the sodium in the form of a mono-metal derivative. This is far less soluble than the di-metal derivative and readily crystallizes from the solution. When partially purified thyroxin, which exists in the open-ring form, is treated in this manner with sodium hydroxide and carbon dioxide, the optimum conditions are produced for the closing of the ring, and after standing for a considerable length of time the ring closes with the crystallization of the mono-metal salt of the closed-ring form of thyroxin.

4. Effect of Temperature during the Treatment of a Solution with Carbon Dioxide.—This is closely related to, and is probably identical with, the effect of temperature on acidification of a solution with a mineral acid. The effect is the breaking off of iodine from the thyroxin molecule, and the mechanism is undoubtedly a reduction due to the presence of impurities and accelerated by the increase in temperature, the carbon dioxide functioning merely in producing the proper hydrogen ion concentration for this reaction to take place.

5. Effect of Different Samples of Desiccated Thyroid.—One of the most important reasons for the failure to separate thyroxin consistently was the variability of the samples of desiccated thyroid used. Analysis of the samples obtained at different times of the year shows that in the winter months, January, February, and March, the iodine content of the glands may be so low as to make the isolation impracticable. During the summer months the thyroxin content of the gland increases from 400 to
500 per cent and allows a much more liberal and much simpler purification of thyroxin. The amount of impurities present appears to be more nearly constant, so that in the winter months the problem is greatly complicated by having to deal with approximately the same amount of impurities and with a greatly diminished amount of thyroxin. It is significant that the real progress was made with the problem only when a satisfactory sample of desiccated thyroid was obtained, but this was demonstrated only after the completion of the work. Another influence, which undoubtedly is very important, not only in the isolation of the substance but in the consideration of the therapeutic value of any sample of desiccated thyroid is the state of preservation of the thyroid proteins. Thyroxin has been shown to contain two carboxyl groups and one amino group, when existing in openring form, in which state it undoubtedly does exist in the thyroid proteins. Deamination and decarboxylation by bacteria are well known, and it seems highly probable that some samples of desiccated thyroid are without therapeutic value because of bacterial decomposition. Bacterial action could very readily result in deamination and decarboxylation of thyroxin, which would render the substance without physiologic activity although the iodine content of the material would not be altered.

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