XXXIX. CHEMISTRY OF THYROXINE.

I. ISOLATION OF THYROXINE FROM THE THYROID GLAND.

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(*Received February 16th, 1926.*)

In 1919 Kendall [1919, 1] announced, as the result of a long and painstaking investigation, the successful isolation from the thyroid gland of a crystalline substance containing 65 % of iodine, which he named "thyroxin," and which has been proved by numerous clinical investigations [Boothby and Sandiford, 1924] to be the substance, or at least one of the most important substances, through which the thyroid gland exerts its control of the metabolic rate. Subsequently Kendall [1919, 2] published a second paper in which the empirical formula of "thyroxin" was stated to be $C_{11}H_{10}O_3NI_3$; a number of salts and other simple derivatives of "thyroxin" were described, and finally a structural formula was proposed indicating that the substance was a derivative of α-oxindolepropionic acid in which the benzene ring was reduced and had three iodine atoms attached to the 3-, 4- and 5-carbon atoms respectively. Contained in this formula are several inherent chemical improbabilities, if not impossibilities, but it is perhaps not necessary to enter into this question further here, since a careful study of Kendall's paper reveals the very slender nature of the evidence from which the formula is deduced. Indeed, it is justifiable to say that, from a chemical point of view, there is no evidence. Two points may be mentioned in this connection. In the first place, the presence of an indole nucleus is deduced from the fact that the substance gives the pine-splinter reaction for pyrrole under conditions which practically amount to an alkaline fusion; now it is a well-known fact that many substances other than pyrrole or indole derivatives will give this reaction provided that they contain an open chain grouping of atoms which, under the influence of dehydration or oxidation, can undergo internal condensation with pyrrole ring formation; this piece of evidence, therefore, unsupported by any other in the same direction, is of no value. In the second place, the presence of a reduced benzene ring in the molecule is deduced from the percentage of hydrogen as determined by combustion; the special difficulties of accurate determination of hydrogen by combustion in a substance containing 65 % of iodine are sufficiently obvious, but, even assuming these difficulties to be overcome, a difference of two hydrogen atoms, in a substance of the formula proposed by Kendall, makes a difference to the analytical result which is scarcely outside the ordinary experimental error.

It is fully apparent, therefore, that without considerable further chemical evidence it is impossible to accept the formula proposed by Kendall, and that the constitution of thyroxine must be regarded as not proven.

Considerations of the nature outlined above led the present writer [Harington, 1925] to make a synthetic "shot in the dark" at the constitution of thyroxine by the preparation of a substance agreeing closely in empirical formula with that deduced from Kendall's analyses, and, further, a substance which might be expected to give chemical reactions of the type which was described as characteristic of thyroxine. The compound synthesised was 3:4:5-triiodophenylpyrrolidonecarboxylic acid, and it showed no physiological action resembling that of thyroxine. The failure of this time-consuming attempt indicated the futility of such a method of attack and forced upon the writer the conclusion that further progress was not likely to be made with the elucidation of the constitution of thyroxine until sufficient supplies of the substance were available to permit of its systematic chemical degradation. The first part of the problem resolved itself, therefore, into the question of the isolation of thyroxine from the thyroid gland.

The method employed by Kendall [1919, 1] for the isolation is laborious and time-consuming in the extreme; he himself states that he worked up three tons of fresh thyroid gland with a total yield of 33 g. of thyroxine; such a method is obviously impracticable from an ordinary laboratory point of view. Thyroxine is prepared according to Kendall's method by Messrs Squibb, of New York, but, as might be expected from the technical difficulties of the preparation, the price is prohibitive even for the small amounts that are used for therapeutic purposes. It was essential, therefore, that a simpler and cheaper method of extraction should be devised.

It appeared probable, from a study of Kendall's work, that a considerable proportion of the total loss of active material occurred during the preliminary process of hydrolysis of the gland for 24 hours in 5 % sodium hydroxide, so that the first attempts were directed towards finding a substitute for this. In particular, many attempts were made to employ tryptic digestion of the gland, in the hope that the gentle but deep-seated hydrolysis so effected might bring about a more favourable separation. The first experiment in this direction did indeed appear promising, in so far that as much as 73 % of the total iodine of the gland was obtained in a soluble condition after tryptic digestion for seven days; but the proportion of the soluble iodine which could be precipitated with acid was no larger than after hydrolysis with sodium hydroxide; larger amounts of iodine could be precipitated with metallic precipitants (*e.g.* mercuric sulphate in almost neutral solution), but the recovery of the iodine-containing compounds from such precipitates was not satisfactory. Attempts were then made to effect a further separation by prolonged tryptic digestion of the acid-insoluble products of the preliminary digestion and, finally, to carry the process still further by the use of erepsin. In no case, however, was it found possible to separate thyroxine from the products of digestion. It was early found that continuous extraction with butyl alcohol, under reduced pressure, of the almost neutral solution of the acid-insoluble products of the first digestion, removed therefrom a small proportion of iodine-containing material which gave the reaction with nitrous acid described by Kendall as characteristic for thyroxine, and it was hoped that the application of this method of extraction to the products of further digestion might yield a substance which could be crystallised. It was, however, found that neither the relative amount nor the purity of the substances extracted by butyl alcohol was much increased by the more prolonged digestion. Further, it was constantly found in these digestion experiments that more or less of the iodine was split off in inorganic form; although as yet no experiments have been made with regard to the action of the enzymes on pure thyroxine, it would appear probable that we have here to deal with a phenomenon similar to that encountered by Oswald [1909] in his work on 3:5-diiodotyrosine. Reflection upon the latter possible similarity led to the idea that the hydrolytic agent found by Oswald to be most suitable for the isolation of diiodotyrosine, namely barium hydroxide, might find a favourable application in this case also. The direct hydrolysis of thyroid gland with high concentrations of barium hydroxide, similar to those employed by Oswald, led apparently to much destruction of thyroxine, since, although a large proportion of the iodine was found in the filtrate after such a hydrolysis, practically none of this iodine could be precipitated with acid. Hydrolysis with dilute barium hydroxide, however, appeared at once to be more favourable since, after such treatment, the filtrate contained 60–70 % of the total iodine, of which about 40 % was precipitated, on acidification, in the form of a cream-coloured flocculent precipitate, which contrasted pleasantly with the dark, almost black precipitate obtained similarly after hydrolysis with sodium hydroxide. Both this precipitate and the insoluble material resulting from the first hydrolysis gave the nitrous acid reaction for thyroxine, whilst the reaction was absent from the filtrate after acidification. Attempts were first made to purify this precipitate further by the method described by Kendall, but iodine determinations indicated such heavy losses at the various stages that this was soon abandoned. Extraction with butyl alcohol of the ammoniacal solution of the precipitate removed about 70 % of the iodine, but even from the material so extracted it was not possible to separate thyroxine. It therefore seemed probable that further hydrolysis was required and the acid precipitate was therefore subjected to more intensive treatment with barium hydroxide; this led to a further separation of the iodine, approximately 50 % of which remained in solution, the remainder being carried down with the insoluble barium salts. The portion which remained in solution was not precipitated with acid. On recovery from the barium salts by boiling with alkaline sodium sulphate solution and reprecipitation with acid under certain conditions, a precipitate was obtained, which, on solution in alkaline alcohol and precipitation with acetic acid, gave a partially crystalline product containing over 63 % of iodine. On further purification this yielded a substance, containing 65·3 % of iodine, which, from its physical properties and physiological action, was evidently thyroxine. Subsequently it was found that, by extracting the insoluble precipitate, obtained after the preliminary hydrolysis, with alkaline sodium sulphate, precipitating with acid, and subjecting this precipitate to the treatment above outlined, a further crop of thyroxine could be obtained. The total amount of crude crystalline thyroxine obtained by this method amounted to 1·2–1·3 g. per kg. of desiccated thyroid gland, or a little over 1 g. of completely purified thyroxine. The desiccated gland employed contained about 0·55 % of iodine; taking 0·12 % of thyroxine as an average yield, this is equivalent to 0·078 % of iodine. A little over 14 % of the total iodine of the gland was, therefore, obtained in the form of thyroxine. This sample of gland, in the fresh condition, weighed approximately 4·4 times as much as in the dry state; the yield of thyroxine calculated on the fresh gland is, therefore, 0·027 %; Kendall's yield of 33 g. from 3 tons (3000 kg.) is about 0·0011 %.

The pure substance, on analysis, yielded figures similar to those obtained by Kendall except for the nitrogen, which was found to be lower than he states, and indeed the whole analysis leads to the empirical formula $C_{15}H_{11}O_4NI_4$ instead of the formula $C_{11}H_{10}O_3NI_3$. The nitrogen analysis was confirmed on a sample of Squibb's preparation of thyroxine, and the final confirmation will be found in the succeeding paper on the iodine-free derivatives of thyroxine. In view of the self-evident identity of the substance prepared by the above method with Kendall's thyroxine, and the proof just mentioned that his proposed empirical formula is incorrect, no further reference will be made to his subsequent publications, and the work here reported is to be regarded as proceeding, from this point onwards, upon first principles.

EXPERIMENTAL.

Isolation of thyroxine from the thyroid gland. 500 g. of desiccated thyroid gland are boiled for 5–6 hours, under a reflux condenser, with 5000 cc. of a 10 % solution of crystalline barium hydroxide. The length of time of the preliminary hydrolysis does not appear to make much difference either to the yield of acid-precipitable iodine or to the purity of the precipitate. It is, however, of importance that the concentration of barium hydroxide should not exceed that named above. At the end of the hydrolysis the solution is allowed to stand overnight and is then filtered from the heavy dark precipitate (B) through large Buchner funnels; the clear yellow filtrate is acidified with hydrochloric acid until barely acid to Congo-red, and the light-coloured flocculent precipitate (A) allowed to settle; the insoluble material (B) is set aside to be worked up subsequently. After standing for some hours, precipitate

(A) is separated by decantation and filtration, and is washed on the filter with water, the filtrate and washings being rejected; it is then dissolved in 250 cc. of water with the aid of ammonia, treated with crystalline barium hydroxide to make a concentration of 40 % of the latter, boiled to drive off the excess of ammonia and heated for 18 hours on a steam-bath under a reflux condenser; the temperature during this period must not exceed 100°; attempts to quicken the process by boiling for shorter periods met with no success. At the end of this period the solution is filtered hot through a Buchner funnel and the precipitate (C) washed with a little hot water. The filtrate is of no further value as regards thyroxine; it apparently contains some tryptophan (positive glyoxylic and bromine reactions). The precipitate (C) is ground up and suspended in 1 % sodium hydroxide (250 cc.), the solution is boiled, and, while boiling, treated with a strong solution of sodium sulphate in slight excess; the boiling is continued for a few minutes and the solution then filtered from barium sulphate; the filtrate is brought to the boil and, while boiling, is treated with 50 % sulphuric acid until just acid to Congo red; on continuing the boiling for a minute or two the character of the precipitate changes; at first light and flocculent, it becomes heavy and granular; as soon as this change has occurred the boiling is discontinued and the solution cooled. The heavy precipitate is filtered off and dissolved in about 20 cc. of *N* sodium hydroxide, alcohol is added to about 80 % concentration, the solution is filtered from a small amount of tar, brought to the boil and treated with 10 cc. of 33 % acetic acid; thyroxine thereupon separates in a partially crystalline condition. Yield 0·4 g. (0·08 %). The material (B) is worked up as follows: the precipitates from the preliminary hydrolysis of 2000 g. of thyroid are ground up and boiled with 2000 cc. of 2 % sodium hydroxide, a slight excess of sodium sulphate being added to the boiling solution; the filtered solution is acidified with hydrochloric acid, and the precipitate is subjected to subsequent treatment precisely similar to that described for (A) above. Yield 0·9 g. (0·045 %). The total yield is, therefore, 0·125 % of the desiccated gland. The crude thyroxine so obtained may be further purified by repeated solution in alkaline alcohol and reprecipitation with acetic acid; it tends, however, obstinately to retain coloured impurities; the final purification is best effected (though with slight loss) by making use of the insolubility of the sodium salt, a phenomenon already observed by Kendall. The crude material, after one recrystallisation by the above method, is dissolved in boiling 0·5 % sodium carbonate, about 75 cc. being required for 1 g. On cooling, a heavy white micro-crystalline precipitate of sodium salt separates out. After standing overnight in the ice-chest this is separated on the centrifuge, dissolved in 80–90 % alcohol with the aid of a little sodium hydroxide and the boiling solution treated with 33 % acetic acid in excess. The thyroxine so obtained is perfectly white and crystallises in rosettes and sheaves of fine needles, as indicated by Kendall for the "keto form" of thyroxine. On heating, it darkens at 220° and melts with decomposition and evolution of iodine at 231–233°;

this melting point is lower than that recorded by Kendall, but a sample of Squibb's "thyroxin crystals" behaved similarly, darkening at 220° and melting with decomposition and evolution of iodine at 229–231°; a mixture of the two preparations melted at 231–232°. The pure thyroxine is insoluble in water and in all the ordinary organic solvents. It is soluble in the cold in solutions of the alkali hydroxides, provided that the concentration of alkali be not too high; it is precipitated in an amorphous condition from its solution in alkali by the addition of acid, in excess of which it is not soluble. It dissolves in sodium carbonate solution only on boiling. It is soluble in 90 % alcohol in the presence of either alkali hydroxide or of mineral acid, but not in presence of acetic acid.

A solution of 0·75 g. thyroxine in 5 cc. *N* sodium hydroxide and 15 cc. of alcohol was examined in a 1 dm. tube in the polarimeter; no signs of optical activity could be observed[1]. This is in agreement with the observation of Kendall [1919, 2]. In view, however, of the process of isolation involving, as it does, prolonged alkaline hydrolysis, this cannot be taken as evidence of the absence of an asymmetric carbon atom, since, if the asymmetric carbon atom were the α-carbon of an α-amino-acid, the compound would probably have been more or less completely racemised. In view of the constitution of thyroxine, as described in the succeeding paper, racemisation during the alkaline hydrolysis is the probable explanation of the absence of optical activity in the product.

Analyses.

0·2504 g. gave 0·2125 g. CO_2; 0·0392 g. H_2O.
24·6 mg. gave 0·46 mg. N (micro-Kjeldahl).
(Squibb's "thyroxin crystals": 20·8 mg. gave 0·39 mg. N.)
0·1570 g. gave 0·1898 g. AgI (Carius).

	C	H	N	I
Found	23·14	1·74	1·87	65·3
Calculated for $C_{15}H_{11}O_4NI_4$	23·18	1·42	1·80	65·3

For final identification a physiological test was made as follows: 10 mg. of the substance were administered intravenously to a normal individual with a basal metabolic rate of – 5 %; at the end of 48 hours the basal metabolic rate had begun to rise; on the fifth day it reached its maximum of + 40 %, the pulse rate having risen from 72 to 120; the effects continued to be noticeable for a fortnight after the injection; the whole reaction was, therefore, typical of those which have been described by the American workers with thyroxine.

For assistance in making this physiological test I am indebted to Dr K. S. Hetzel.

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[1] I am indebted to Mr H. J. Channon for kind assistance in making this determination.

SUMMARY.

A method is described by which thyroxine may be isolated from the thyroid gland in a yield of about 0·12 % of the dried gland.

The probable empirical formula of thyroxine is $C_{15}H_{11}O_4NI_4$.

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