XLV. OBSERVATIONS ON THE IODINE-CONTAINING COMPOUNDS OF THE THYROID GLAND. ISOLATION OF *dl*-3:5-DIIODOTYROSINE.

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About three years ago one of the authors [Harington, 1926] described an improved method for the isolation of thyroxine from the thyroid gland, which was based on a graduated hydrolysis of the gland substance with barium hydroxide. When desiccated thyroid was boiled with dilute barium hydroxide the iodine was separated into three fractions, the first being contained in the precipitate of insoluble barium salts, the second being obtainable by acidification of the alkaline filtrate, and the third remaining soluble in presence of acid. Thyroxine was isolated by subjecting the first two fractions to more intensive hydrolysis with barium hydroxide, whilst no evidence could be obtained of the presence of thyroxine in the third, acid-soluble, portion. A similar separation of the iodine compounds of the thyroid into acid-soluble and acid-insoluble had been effected previously by Kendall by hydrolysis with dilute sodium hydroxide, and he was able to show [1919] that the physiological activity was confined to the acid-insoluble fraction, *i.e.* to that fraction from which alone thyroxine could be isolated. This observation has been confirmed by an experiment kindly performed for us by Dr J. H. Gaddum. Desiccated thyroid gland was boiled with 10 % crystalline barium hydroxide under the usual conditions and the hydrolytic products were separated into acid-insoluble and acid-soluble fractions; injection of 2·6 mg. per kg. of the iodine of the acid-insoluble portion produced an increase of 60 % in the oxygen consumption of a rat, whilst a definitely smaller effect was obtained after injection of 26 mg. per kg. of the iodine of the acid-soluble fraction. It is evident, therefore, that this preliminary hydrolysis, although it is of so mild a character that it cannot conceivably involve any destruction of thyroxine, effects a practically complete separation of the latter from the other iodine-containing compounds which may be present.

At the time of the previous investigation the isolation of thyroxine itself was the exclusive object in view and the further examination of the acid-soluble fraction was therefore postponed. The present communication deals

with the results of this examination, leading eventually to the isolation of 3 : 5-diiodotyrosine, and we shall adduce evidence in support of the view that the whole of the acid-soluble iodine is indeed present as diiodotyrosine, whilst the whole of the acid-insoluble iodine belongs in all probability to thyroxine.

If the alkaline filtrate obtained after hydrolysis of desiccated thyroid gland with 10 % barium hydroxide be carefully acidified with hydrochloric acid (to about p_H 5·0) the thyroxine-iodine is, as previously indicated, almost quantitatively precipitated; after removal of this precipitate one is left with a solution containing 50 % or more of the original iodine of the gland, practically the whole of which remains in organic combination. The solution so obtained is, however, of little use for further investigation, owing to its content of barium chloride. Our first step was, therefore, to substitute sulphuric acid for the hydrochloric acid formerly used for the precipitation of the thyroxine fraction. This step resulted in the removal of a certain proportion of the acid-soluble iodine by adsorption on the precipitate of barium sulphate and thyroxine; this amount could be easily recovered, however, by extraction of the precipitate with alkali, removal of the barium sulphate, and acidification, when the thyroxine was again precipitated, and the filtrate containing the acid-soluble iodine which had been removed by adsorption on the barium sulphate could be reunited at a later stage with the main acid-soluble fraction.

To return to the latter, in the first experiment, the solution, which gave a strong biuret reaction and evidently contained for the most part peptones and other higher protein degradation products, was concentrated and subjected to more intensive hydrolysis either with acid or alkali; hydrolysis with acid was soon dismissed as useless, since it resulted in the entire destruction of the organic iodine compounds, the iodine appearing in the solution of hydrolytic products as iodide. Hydrolysis with barium hydroxide was evidently much more favourable since a large proportion of the iodine remained in organic combination; no success, however, attended attempts to isolate the iodine compound from the hydrolytic products. Nevertheless these early experiments were useful in that they gave an indication of the probable nature of the compound for which we were searching; for it was observed that, after partial separation of the hydrolytic products, those fractions which were rich in iodine gave the colour reaction with nitrous acid and ammonia which is given also by thyroxine, and which was previously shown [Harington and Barger, 1927] to be characteristic of the *o*-diiodophenolic grouping, and was put on a roughly quantitative basis by Ingvaldsen and Cameron [1926]. At this stage we returned to the original solution to see whether a preliminary partial separation of the iodine compound might not be effected at this early stage, and the mixture of products obtained after the second hydrolysis be thereby simplified. We found that precipitation with basic lead acetate served our purpose in this respect. Addition of basic lead acetate to the neutralised solution until no further immediate precipitate was formed removed about 80 % of the iodine; the precipitation of the iodine could indeed be made

quantitative by making the solution alkaline with ammonia, but the last 20 % of the iodine was accompanied by so large a preponderance of other material that we found ourselves finally in no better position than before the lead treatment; we therefore contented ourselves with the 80 % precipitation obtained as indicated above. The lead salts were filtered off and decomposed with sulphuric acid, and the filtrate, after removal of sulphuric acid, was united with the portion recovered, as described above, from the first acid precipitate, and was concentrated and further hydrolysed with 40 % barium hydroxide. After removal of barium with carbon dioxide the solution was treated with silver nitrate which precipitated the whole of the iodine; the silver salts were extracted with dilute nitric acid, the iodide formed during the hydrolysis being left undissolved, and were reprecipitated with ammonia; they were then decomposed with hydrogen sulphide in the usual way. We thus obtained a solution which gave a strong nitrous acid reaction, and it became more and more evident that the properties of the substance under investigation were closely similar to those of diiodotyrosine.

Earlier workers who have isolated this compound from natural sources [cf. *e.g.* Wheeler and Mendel, 1910] have employed hydrolysis with barium hydroxide followed by silver precipitation, and have then utilised phosphotungstic acid in order to separate the diiodotyrosine from the dicarboxylic amino-acids which are the chief accompanying impurity. In our hands this reagent did not prove satisfactory either from the point of view of completeness of precipitation of the iodine, or from that of cleanness of separation. We therefore had recourse to extraction of the neutralised solution with butyl alcohol for the purpose in view. Continuous extraction with this solvent is undesirable, since, on prolonged boiling with butyl alcohol, diiodotyrosine appears to undergo some decomposition; by shaking out the warm solution with successive quantities of butyl alcohol [cf. Onslow, 1921] we were, however, successful in extracting the greater part of the iodine with no disadvantageous effects. The aqueous solution of the material extracted by butyl alcohol still contained some colloidal substances which inhibited the crystallisation of the diiodotyrosine; these substances could be removed by treatment, under the appropriate conditions, with uranium acetate, and then, after one more precipitation as the lead salt, the diiodotyrosine could be obtained crystalline without difficulty.

Reference to the experimental part below, and to the diagrammatic representation of a typical experiment, will show that, although the actual quantity of diiodotyrosine isolated amounts only to about 11 % of the total iodine of the thyroid, the losses during the process of isolation are essentially the continuous small losses of material which are inevitably associated with a somewhat complex manipulation of this type. An exception to this statement may at first sight appear to be indicated by the large loss of iodine as iodide during the intensive hydrolysis with barium hydroxide; we have satisfied ourselves, however, that even pure diiodotyrosine admixed with a protein

and subjected to similar treatment, loses a considerable proportion of its iodine as iodide, and such loss may well be greater when the compound is at the same time being split off from peptide combination; we do not, therefore, regard this phenomenon as invalidating our general argument that at no stage is there any sharp break in the recovery of the iodine, such as would indicate the presence of an iodine-containing compound of a different character. In order further to confirm this conclusion we carried out, in one experiment, colorimetric determinations by the nitrous acid reaction coincidently with our organic iodine determinations, and, although no pretence is made that the colorimetric results were more than approximations, a definite parallelism between the two sets of observations was apparent[1].

Having then convinced ourselves that we could account for all the acid-soluble iodine as diiodotyrosine, we returned to the acid-insoluble fraction, to see whether we could find here any indication of the presence of an organic iodine-containing compound other than thyroxine. In the method previously described [Harington, 1926] the thyroxine was recovered by extraction with alkaline sodium sulphate; this manipulation is troublesome, and it has been found better to decompose the salts by suspension in warm dilute hydrochloric acid; lipoidal material is then removed with ether, and the remaining insoluble precipitate is united with the main acid precipitate for the second hydrolysis. It was formerly stated that, at the end of the second hydrolysis, the whole of the thyroxine was to be found in the precipitate of barium salts; this remark requires modification. The distribution of thyroxine at this stage depends on the concentration of iodine in the solution; if this concentration (at the commencement of the hydrolysis) is, as in the earlier experiments, 1 mg. per cc. or more, it is true that most of the thyroxine appears in the precipitate, although even here traces may be obtained by acidification of the mother liquor; on the other hand, with more dilute solutions, a large part of the thyroxine may remain in solution; in any case, in carrying out this isolation, the solution should at this stage be acidified and any precipitate which is obtained should be combined with the material obtained on recovery from the insoluble barium salts; the combined acid-insoluble material is then converted into crystalline thyroxine by the method previously described [Harington, 1926].

Reference to the experimental part will show that here again we meet, in the process of isolation of thyroxine, with a series of losses such as can

[1] The quantitative nitrous acid reaction in coloured solutions is conveniently carried out as follows. Into each of two test tubes A and B is introduced a 5 cc. sample of the solution to be tested containing approximately 0·5 mg. of iodine per cc. To A is added 1 cc. of a 0·1 % solution of diiodotyrosine, to B 1 cc. of water. To both tubes are added two drops of 30 % sodium nitrite and 3 drops of concentrated hydrochloric acid; the tubes are shaken and allowed to stand 2 minutes at the ordinary temperature; to each is added 9 cc. of butyl alcohol, the contents being shaken for 1 minute and allowed to separate; 5 cc. of each butyl alcohol layer are transferred to the cups of a colorimeter and 0·5 cc. alcoholic ammonia (0·5 *N*) added; the resulting pink colours are compared immediately.

reasonably be ascribed to unavoidable imperfections of technique. In the particular experiment described, the thyroxine ultimately isolated represented 16 % of the total iodine of the gland substance; this is an average result with the improved technique now employed; we have had even better yields. As in the case of diiodotyrosine, so with thyroxine, the greatest loss occurs at the stage of the intensive alkaline hydrolysis. This loss consists in part of iodine split off as iodide, and for the rest of iodine which is still in organic combination but is soluble in acid. The proportion which appears in the inorganic condition is considerably less than is the case at the corresponding stage in the isolation of diiodotyrosine; this difference, we think, is due to the fact that a great part of the thyroxine, as it is set free by hydrolysis, separates as the sparingly soluble barium salt and is thus removed from the action of the alkali, whilst the soluble diiodotyrosine remains exposed to this action throughout the experiment. As to the further small amount of iodine which appeared at this stage in the acid-soluble organic form (an amount so small as to preclude its detailed examination) two obvious possibilities suggest themselves; in the first place it is naturally not claimed that the division of the products of the preliminary hydrolysis of the thyroid into those containing thyroxine on the one hand and those containing diiodotyrosine on the other is absolutely quantitative; indeed the experiment of Dr Gaddum mentioned above shows that this is not the case, since a trace of residual physiological activity was found in the acid-soluble fraction; if, then, traces of thyroxine may appear among the acid-soluble products, it is equally possible that traces of diiodotyrosine may appear among the acid-insoluble substance; especially does this seem likely to us since one of our principal difficulties in the isolation of diiodotyrosine has been the extreme ease with which, from neutral or slightly acid solutions, this substance is adsorbed on precipitates; it is not unreasonable, therefore, to suppose that part of the fraction of the iodine under consideration belongs in fact to diiodotyrosine. A second possibility which must not be ignored is that, under the somewhat severe conditions of the hydrolysis, a part of the thyroxine may yield a product still containing organically-combined iodine but soluble in acid. Thus it is only those two iodine atoms which are situated *ortho* to the phenolic group of thyroxine which we should expect to be labile towards alkali of the concentration employed during the hydrolysis; were hydrolysis of these two iodine atoms to occur we should have a pyrogallol derivative still containing iodine, which would almost certainly be fairly soluble in water, and such part of which as was not further oxidised would go to make up the fraction which we are discussing. In any case we find it more reasonable to suppose that this small fraction of acid-soluble organic iodine (which is, after all, only 5 % of the total iodine) appears at this stage for some such reason as we have suggested, rather than that it indicates the presence of an independent iodine-containing compound in the original gland.

On the basis of this investigation, therefore, we feel justified in advancing

the definite view that there are only two iodine-containing compounds in the thyroid gland, namely thyroxine and 3 : 5-diiodotyrosine; in the gland which we have used for our experiments, the iodine would appear to be about equally distributed between these two compounds. This gland material, which we have employed throughout, is all obtained from one geographical source, and it has, moreover, shown a remarkable constancy of iodine content at whatever season of the year it has been purchased. There is, however, little doubt that variations in the distribution of iodine in the thyroid between diiodotyrosine and thyroxine may occur. Such variations would account for the lack of parallelism between iodine content and physiological activity which has been observed in the past for different samples of thyroid, and for the observations of Kendall and Simonsen [1928] and others on the varying proportion of the iodine which can be obtained in the acid-insoluble condition; it has indeed been stated that in some glands the iodine is entirely in the acid-soluble condition. The suggestion has already been advanced [Harington and Barger, 1927] that, biologically, thyroxine is derived from tyrosine through the stage of 3 : 5-diiodotyrosine, two molecules of which may be supposed to undergo oxidative coupling with the loss of one side-chain to give thyroxine; the actual isolation of diiodotyrosine from the thyroid lends strong support to this theory. If we are to regard diiodotyrosine as the precursor of thyroxine, it is evident, that with varying states of activity of the gland we must expect to find varying relationships between the amount of the precursor and of the complete hormone. Taking the possibility of such variations into account, an obvious corollary is that the only reasonable chemical assay of the therapeutic value of a thyroid preparation must be based on the acid-insoluble iodine and not on the total iodine content of the material.

Brief reference must here be made to a publication by Kendall and Simonsen [1928] which has recently appeared. These authors cite their own observations and those of other workers to the effect that the whole of the physiological activity of the thyroid gland cannot be accounted for by its thyroxine. The discrepancy to which Kendall draws attention is, however, not so much between the activity of a gland and its content of thyroxine, as between the activity and the amount of thyroxine which can be isolated. We think that most workers who have had experience of this type of manipulation will agree that a very grave difference exists between the amount of a compound which is present in a tissue and the amount of that compound which can be isolated in the pure condition. We have attempted above to bring forward evidence that the whole of the acid-insoluble iodine of the thyroid may be regarded as thyroxine; if this conclusion can be accepted and if we bear in mind that the acid-insoluble fraction usually represents 40 to 60 % of the total iodine, and further, that the true physiological activity of thyroxine as it occurs in the thyroid may well be different from that of the free compound, the discrepancy no longer appears so serious. It may be recalled that the physiological activity of laevorotatory thyroxine was found to be

definitely greater than that of the dextrorotatory isomeride, whence it was assumed that the former was probably the naturally occurring compound [Harington, 1928]. Recent experiments in this laboratory have cast some doubt on the correctness of this deduction, and, until the point is finally cleared up, we do not wish to base any arguments on the optical activity of the naturally occurring hormone. There remains, however, the possibility that the physiological activity of thyroxine in the natural state, *i.e.* in peptide or other form of combination, may be enhanced with respect to its activity in the free condition. We suggest that, in the present state of our knowledge, any remaining discrepancy between the physiological activity and the acid-insoluble iodine content of the thyroid may be explained on these lines more acceptably than by the gratuitous assumption of the existence in the gland of an "active" form of thyroxine differing in chemical structure from the compound as we know it.

Experimental.

In this part a detailed description is given of one complete experiment which is typical of several; for the sake of clarity a diagrammatic representation of the process is given.

Desiccated thyroid gland (250 g. containing 1·220 g. of iodine) was boiled under a reflux condenser for 6 hours with 2500 cc. of a 10 % solution of crystalline barium hydroxide; after cooling, the solution was filtered, and the filtrate brought to p_H 5·0 by addition of 50 % sulphuric acid; the precipitate of thyroxine and barium sulphate, which carried down also some of the acid-soluble iodine by adsorption, was filtered off, and the filtrate and washings (A) containing 515 mg. of iodine were set aside.

(1) *Isolation of thyroxine.* The precipitate of insoluble barium salts resulting from the preliminary hydrolysis was ground up and suspended in dilute hydrochloric acid; the solution was brought to the boil, adjusted to p_H 5·0 and filtered; the precipitate was ground up with ether, again filtered, and dissolved in dilute sodium hydroxide; the resulting solution was combined with that resulting from extraction of the barium sulphate-thyroxine precipitate with warm dilute sodium hydroxide followed by removal of the barium sulphate; the combined sodium hydroxide solutions contained 549 mg. of iodine. The total loss up to this stage was therefore 156 mg. of iodine; this was accounted for in part by a small unhydrolysed residue of the original material, and, for the rest, was spread over the various operations above described; in numerous experiments the distribution of these losses, as between the different operations, was uniform, and each individual loss was small. The sodium hydroxide solutions were acidified with sulphuric acid to p_H 5·0 and the precipitate was filtered off; the solution was treated with slight excess of barium hydroxide and the filtrate (B) containing 118 mg. of iodine was reserved (see below). The acid-insoluble precipitate (431 mg. of iodine) was dissolved in 300 cc. of water with the aid of a little ammonia,

Table I. *Separation of iodine into acid-soluble and acid-insoluble fractions.*

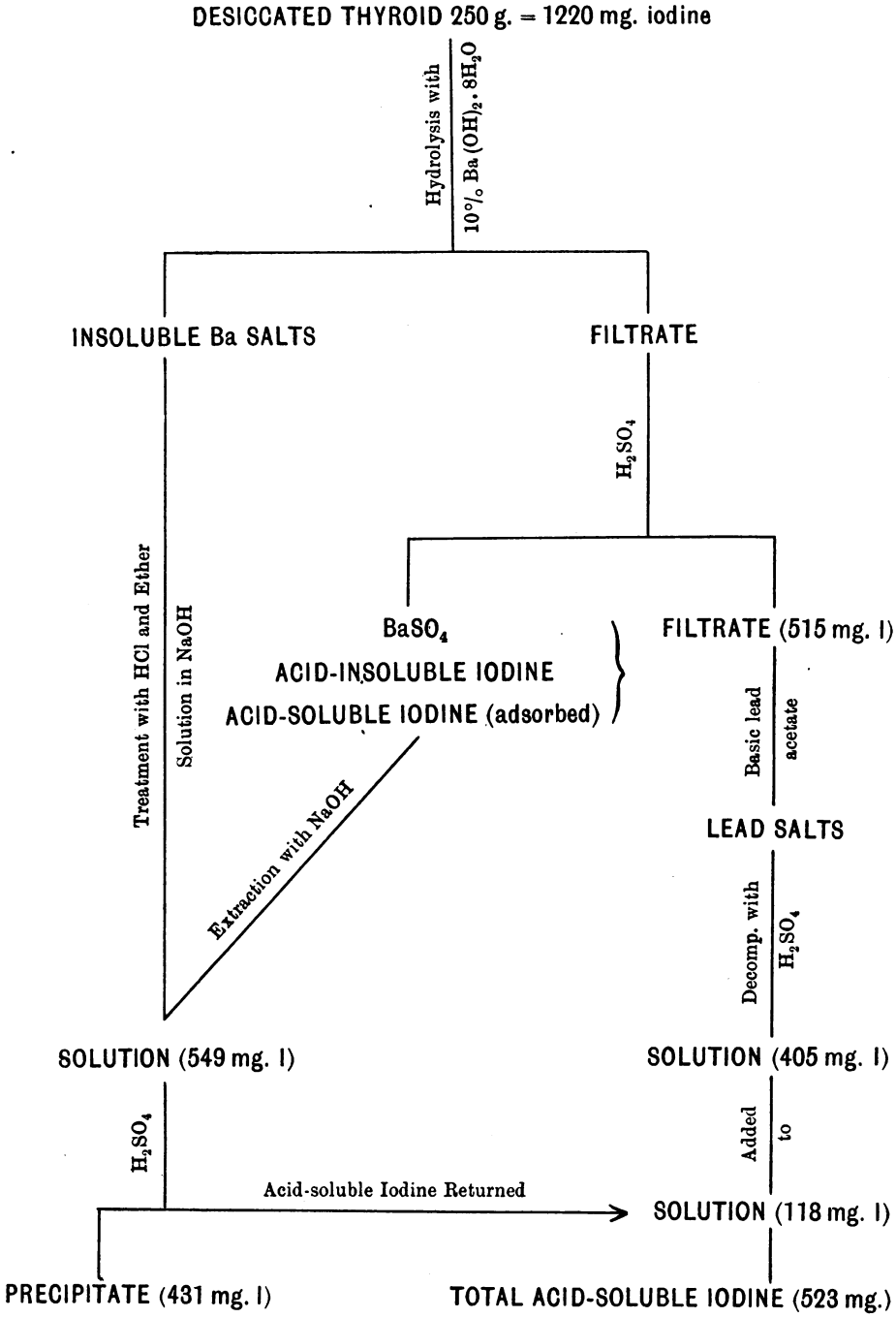
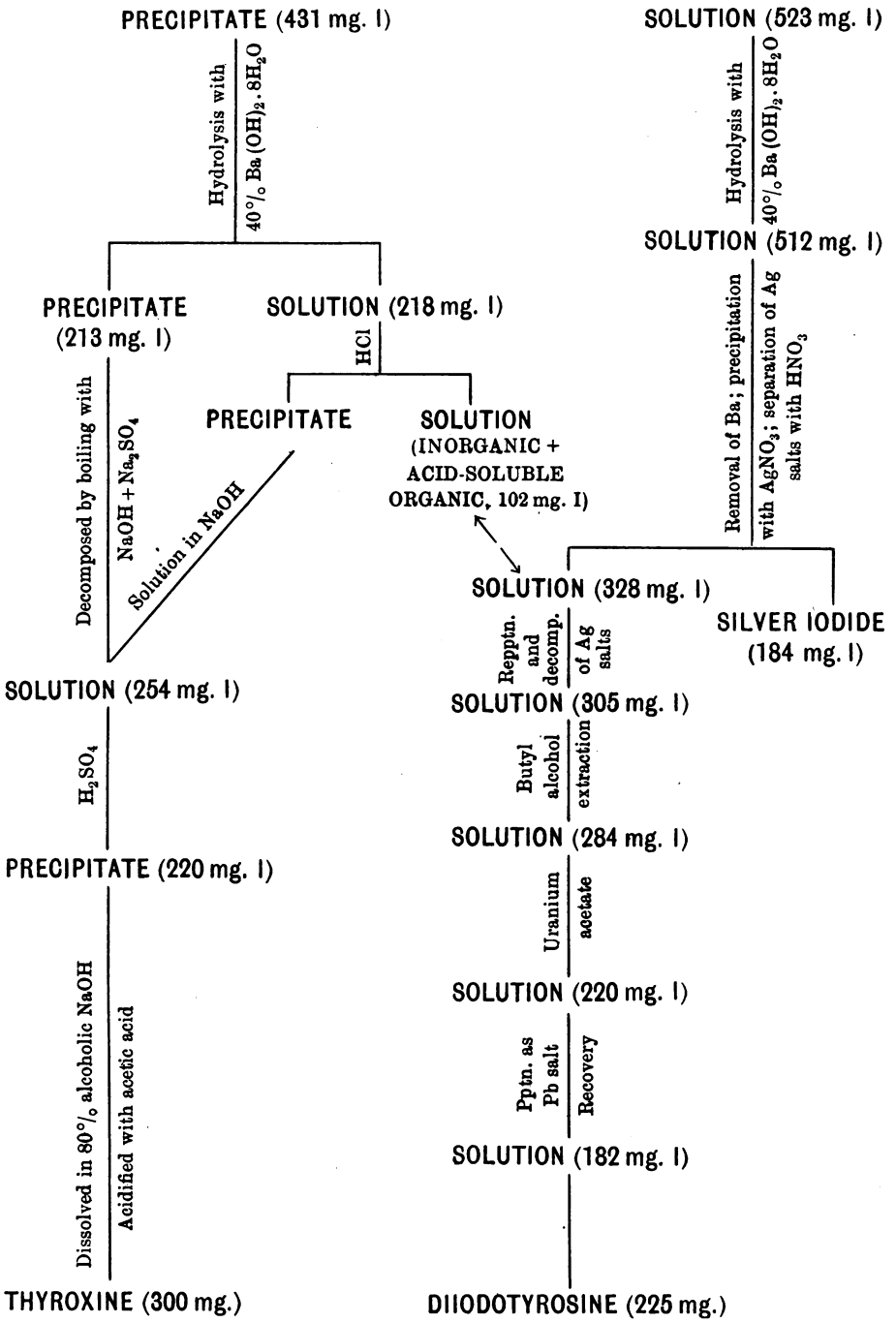


Table II. *Isolation of thyroxine and of diiodotyrosine.*



crystalline barium hydroxide was added to 40 % concentration, and the whole was heated for 18 hours at 100°; the solution was filtered hot, the filtrate cooled and the barium hydroxide which separated was removed; hydrochloric acid was then added to p_H 5·0 and the precipitate (C) collected; the hydrochloric acid filtrate contained 102 mg. of iodine (in a parallel experiment this was shown to consist to an almost equal extent of inorganic iodide and acid-soluble organic iodine). The insoluble barium salts were decomposed by boiling with alkaline sodium sulphate, and to the alkaline filtrate was added the hydrochloric acid precipitate (C); the alkaline solution was heated to boiling and brought to p_H 5·0 with sulphuric acid; the precipitate was collected and dissolved in 80 % alcohol with the aid of sodium hydroxide; the solution was filtered, heated to boiling, and acidified with acetic acid, when there separated 300 mg. of thyroxine. The serious loss in this process occurs therefore during the drastic hydrolysis; the remaining loss is accounted for by the additive effect of incomplete recovery from the insoluble barium salts, incomplete re-precipitation, and finally incomplete separation on crystallisation from acetic acid-alcohol, each individual loss again being small in amount.

(2) *Isolation of dl-3 : 5-diiodotyrosine.* The filtrate A (see above) was treated with basic lead acetate (the B.P. solution) until no further immediate precipitation occurred; after standing overnight the lead salts were filtered off and suspended in water (2000 cc.); the mixture was heated to boiling and 50 % sulphuric acid was added until the reaction remained acid to Congo red; lead sulphate was removed by filtration and the filtrate was freed from sulphuric acid by addition of a slight excess of barium hydroxide. The alkaline filtrate from the barium sulphate contained 405 mg. of iodine, 20 % of the iodine thus having been sacrificed at the stage of lead precipitation for reasons already given. The alkaline filtrate was combined with solution B (see above), and the whole, containing in all 523 mg. of iodine, was boiled down to 500 cc.; 200 g. of crystalline barium hydroxide were added, and the solution was heated for 18 hours at 100°. A small greenish precipitate was filtered off hot, but contained no significant amount of iodine; the filtrate (512 mg. of iodine) was cooled, the barium hydroxide was filtered off and recrystallised; the combined mother liquors were treated with carbon dioxide and the barium carbonate removed by filtration and well washed; the filtrate and washings contained 512 mg. of iodine. Silver nitrate (20 % solution) was now added until precipitation was complete, the mother liquor being free from iodine; the silver salts were filtered off and ground up with dilute nitric acid (free from nitrous acid); the filtered solution contained 328 mg. of iodine, indicating a loss at this stage of 184 mg. as iodide. The organic silver salts were re-precipitated by the careful addition of ammonia, filtered off, washed, suspended in water and decomposed with hydrogen sulphide; the solution was brought to the boil, the silver sulphide removed by filtration, and the filtrate, containing 305 mg. of iodine, was concentrated to 200 cc. under diminished pressure. The solution was now extracted by shaking out nine times with

butyl alchohol (previously purified by agitation with saturated sodium bisulphite followed by distillation) at a temperature of about 70°; the combined butyl alcohol extracts were evaporated to dryness under diminished pressure and the residue dissolved in water; the solution contained 284 mg. of iodine; the volume was made up to 500 cc., the solution was brought to the boil and treated with uranium acetate solution in slight excess; the precipitate was filtered off and the filtrate freed from uranium with ammonia and concentrated to 320 cc. under diminished pressure; it now contained 220 mg. of iodine; basic lead acetate solution was added to complete precipitation, and after standing overnight the precipitate was collected, well washed, and decomposed by saturating its suspension in hot water with hydrogen sulphide; the lead sulphide was boiled out with much hot water, and the filtrate and washings were concentrated to a small volume under diminished pressure; the solution, which was faintly acid to Congo red, was exactly neutralised to litmus with ammonia; on further concentration in a vacuum desiccator over sulphuric acid 225 mg. of a crystalline compound separated out. On recrystallisation from 50 % acetic acid it formed pale straw-coloured prismatic needles having M.P. 198·4° (decomp.); a sample of *dl*-3 : 5-diiodotyrosine, prepared by iodination of *dl*-tyrosine, had M.P. 197·5°, whilst a mixture of the natural and synthetic products melted at 198·0°[1]. The product from the thyroid gave the colour reaction with nitrous acid and ammonia with intensity; on evaporation of a small amount on the water-bath with concentrated hydriodic acid, a residue was left which gave a strong Millon's reaction.

Analysis. 2·168 mg. required 5·00 cc. *N*/200 thiosulphate [Kendall, 1914]
20·3 mg. gave 0·577 mg. N (Micro-Kjeldahl)
20·3 mg. gave 1·035 cc. moist N_2 at 21·5° and 764 mm.

	I	N
Found:	58·2	2·8, 2·9
Calc. for $C_9H_9O_3NI_2$:	58·6	3·2

There remained, therefore, no doubt as to the identity of the compound isolated from the thyroid gland with *dl*-3 : 5-diiodotyrosine.

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[1] The melting point of *dl*-3 : 5-diiodotyrosine is variously reported in the literature, values from 190° to 213° being given; using synthetic samples of indubitable purity, and heating fairly rapidly, we have never found it to lie above 199°.