CXV. THE ISOLATION OF *d*-3:5-DIIODOTYROSINE FROM THE THYROID GLAND BY THE ACTION OF PROTEOLYTIC ENZYMES.

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(Received June 30th, 1931.)

ABOUT two years ago the present authors [1929] described the isolation of *dl*-3:5-diiodotyrosine by means of graduated hydrolysis of the thyroid gland with barium hydroxide. The isolation was repeated shortly afterwards by Foster [1929] who used a simpler method and obtained a better yield. Although in the previous work every precaution was taken to exclude the possibility of artificial production of diiodotyrosine during the course of the isolation, and although such artificial production was in any case very unlikely to occur, it remained nevertheless desirable to complete the demonstration of the natural occurrence of diiodotyrosine in the thyroid by its isolation by gentler means and in the optically active condition.

Promising material for this investigation offered itself in the various mother-liquors containing acid-soluble organically combined iodine which were obtained as by-products in the course of the work of Harington and Salter [1930] on the isolation of *l*-thyroxine by the enzymic digestion of thyroid gland.

As a first step the experience gained in previous work on diiodotyrosine was utilised to effect a preliminary concentration of the iodine-containing compounds present in the above-mentioned mother-liquors. Thus the mother-liquor obtained after removal of the material insoluble at p_H 5.0, following on the peptic digestion of crude iodothyreoglobulin, contained much iodine, a considerable proportion being present as iodide but a definite amount being still in organic combination. After preliminary clearing of this solution with lead acetate, practically the whole of the iodine was precipitated with silver nitrate in neutral or faintly alkaline solution. The organic iodine compounds were separated from iodide by extraction of the precipitated silver salts with dilute nitric acid and re-precipitated by addition of ammonia; decomposition of the re-precipitated silver salts in the usual manner yielded a solution of the organic iodine compounds in high concentration which was subjected to further digestion with trypsin.

After the tryptic digestion was complete the solution was subjected to a

process of fractionation closely similar to that employed in the previous work [Harington and Randall, 1929] on the isolation of diiodotyrosine, yielding finally a soluble gummy material containing much iodine and giving an intense reaction for diiodotyrosine with nitrous acid and ammonia, but at the same time still giving a biuret reaction.

A product with similar characteristics was obtained by analogous concentration of the organic iodine-containing compounds which remained in solution after removal of the thyroxine-containing fraction of the tryptic digestion products of iodothyreoglobulin [*cf.* Harington and Salter, 1930].

The final successful isolation of diiodotyrosine was achieved by subjecting the combined solution of a number of such end-products of tryptic digestion to the action of an active preparation of erepsin. After this treatment it was possible by a simple fractionation to separate diiodotyrosine in the pure condition.

The losses involved at the various stages of the procedure outlined above were very heavy and the amount of diiodotyrosine finally obtained has no quantitative significance. Evidence was offered previously [Harington and Randall, 1929] bearing on the probable amount of diiodotyrosine actually present in the thyroid, and the suggestions made at that time have since been largely confirmed by the improved yield obtained by Foster [1929]. The present work is to be regarded as the final evidence that *d*-diiodotyrosine does actually occur as a constituent amino-acid of the characteristic protein of the thyroid.

EXPERIMENTAL.

Isolation of d-3 : 5-diiodotyrosine from the acid-soluble fraction of peptic digestion products from iodothyreoglobulin.

The filtrate from a peptic digestion of crude iodothyreoglobulin [*cf.* Harington and Salter, 1930], amounting to 7200 cc. and containing in all 1.986 g. of iodine, was treated with a concentrated aqueous solution of 350 g. of lead acetate and then with sodium hydroxide until just neutral to litmus. The solution was filtered, and the filtrate, containing 1.4 g. of total iodine, was made faintly alkaline to litmus with ammonia and treated with silver nitrate to complete precipitation. The insoluble silver salts were filtered off, suspended in about 800 cc. of water, and treated with freshly boiled out nitric acid until the suspension was permanently acid to Congo red. The silver halides were removed as rapidly as possible by filtration, and the organic silver salts were re-precipitated from the filtrate by the cautious addition of ammonia until present in slight excess. The precipitate was collected, thoroughly washed with water, suspended in 1000 cc. of water and decomposed with hydrogen sulphide. The filtrate from the silver sulphide, containing 423 mg. of iodine was concentrated under diminished pressure to 360 cc.

This solution, which was slightly acid, was brought to p_H 8.0 by addition of sodium hydroxide and was treated with 0.8 g. of trypsin (Merck) dissolved in

10 cc. of water. Toluene was added as a preservative and the solution was placed in an incubator at 38°. At the commencement of the digestion the total N amounted to 20.0 mg./cc. and the amino-N to 1.0 mg./cc. After 48 hours the amino-N had increased to 2.65 mg./cc.; a further addition of 0.8 g. of trypsin was made at this point, which, in the course of another 24 hours, increased the amino-N to 3.01 mg./cc. At this stage the digestion was interrupted; the solution was diluted to 2 litres, neutralised and brought to the boil. A saturated solution of uranium acetate was then added until no further precipitation occurred. The precipitate was removed and the excess of uranium was thrown out with ammonia. The filtered solution was concentrated to 200 cc. and contained 271 mg. of iodine. Silver nitrate was added to complete precipitation, and the insoluble silver salts were fractionated by extraction with nitric acid as described above.

The solution obtained by recovery from the organic silver salts contained 174 mg. of iodine. It was concentrated to 200 cc. and extracted by shaking out 9 times at 70° with purified (aldehyde-free) butyl alcohol. The butyl alcohol extracts were evaporated to dryness under diminished pressure, the evaporation being several times repeated after addition of water. The residue contained 128 mg. of iodine; it was dissolved in water (600 cc.) and the clearing process with uranium acetate was repeated. After removal of the uranium the solution, containing 109 mg. of iodine, was concentrated under diminished pressure to 140 cc. and precipitated with basic lead acetate. After standing overnight the precipitate was filtered off, suspended in water and decomposed with hydrogen sulphide. The filtrate from the lead sulphide, when concentrated, yielded a yellow gum which contained 67 mg. of iodine all in organic combination. The material gave a strong colour reaction for diiodotyrosine with nitrous acid and ammonia, but gave also a biuret reaction and could not be induced to crystallise.

The residues from two such experiments, containing in all 142 mg. of iodine, were therefore united in solution in 30 cc. of water; 60 cc. of an active erepsin extract were added and the mixture was brought to p_H 8.0, saturated with toluene and placed in an incubator at 38°. At the commencement of the digestion the mixture contained total N 2.18 mg./cc. and amino-N 0.51 mg./cc.; the digestion was interrupted after 67 hours when the amino-N had reached 0.96 mg./cc. and the rate of increase had become very low.

The solution was diluted with 50 cc. of water and treated with excess of silver nitrate. The insoluble silver salts were filtered off, washed and decomposed with hydrogen sulphide. The filtrate from the silver sulphide (containing 124 mg. of iodine) was freed from hydrogen sulphide and cleared with uranium acetate; after removal of excess of uranium the solution was concentrated to 80 cc. and contained 100 mg. of iodine. Basic lead acetate was added in slight excess, and after standing overnight the precipitate was collected, washed, suspended in water and decomposed with hydrogen sulphide. The lead sulphide was thoroughly boiled out several times with water to extract adsorbed diiodotyrosine. The filtrate and extracts from the lead sulphide, which con-

tained 88 mg. of iodine, were concentrated to a low bulk under diminished pressure.

On keeping overnight an amorphous precipitate, admixed with nodules of colourless needles, had separated. The latter were collected and the mother-liquor was cleared on the centrifuge and concentrated further, when a second crop of crystals was obtained. A still further amount of crystals was obtained by dissolving the amorphous precipitate in boiling 50 % acetic acid and allowing the solution to cool. The whole of the crystalline material was collected and recrystallised from 50 % acetic acid, yielding 77 mg. of prismatic needles. (Found: N, 3.18 %; calc.: N, 3.2 %.) The compound gave the characteristic colour reaction with nitrous acid and ammonia with intensity; it had M.P. 194° (decomp., uncorr.) and this M.P. was unchanged by admixture with a sample of *d*-3 : 5-diiodotyrosine prepared by the iodination of *l*-tyrosine. In 3.64 % solution in *N* hydrochloric acid, using a 0.5 dm. tube, the observed rotations of the material from the thyroid were $\alpha_{5461} + 0.07^\circ$ and $\alpha_D + 0.05^\circ$; whence $[\alpha]_{5461} + 3.85^\circ$ and $[\alpha]_D + 2.75^\circ$. Abderhalden and Guggenheim [1908] record $[\alpha]_D + 3.0^\circ$ for the acid dissolved in 4 % hydrochloric acid.

Isolation of d-3 : 5-diiodotyrosine from the acid-soluble fraction of tryptic digestion products of iodothyreoglobulin.

The filtrates obtained by removal of all material insoluble at p_H 5.0 after the tryptic digestion of iodothyreoglobulin [*cf.* Harington and Salter, 1930] were subjected to a process of fractionation similar to that just described for the tryptic digest of the acid-soluble peptic products. The procedure in one such instance is summarised below.

	Iodine (mg.)
Tryptic filtrate (3600 cc.)	481
After clearing with uranium acetate	348
After precipitating with silver nitrate, fractionation of silver salts and recovery	168
After butyl alcohol extraction	153
After second uranium treatment	119

The gummy residues from four such experiments, containing in all 318 mg. of iodine, were united; one-half was subjected to digestion with erepsin at p_H 8.0 for 93 hours. The digest was worked up as before and yielded finally 150 mg. of crude crystalline *d*-3 : 5-diiodotyrosine.

Note on preparation of active erepsin extract.

The following method has been found convenient for the preparation of active erepsin extract.

The mucous membrane of the small intestine of a recently killed dog was scraped off with a spatula and ground up with sand in a mortar under 5 times its weight of 66 % glycerol. After standing overnight the mixture was diluted with 4 vols. of water and rapidly centrifuged. The extract was submitted to dialysis under diminished pressure in an apparatus of the type described by Sørensen [1918] until free from glycerol and was finally concentrated by

filtration through the collodion bag until the volume was one-half that of the original extract. A slight precipitate was removed by filtration through ordinary filter-paper. The resulting solution was kept at 0° in presence of toluene, but was used as soon as possible. It contained a very active erepsin, being capable of hydrolysing glycylglycine quantitatively in a few hours.

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