

## CLXXVIII. RESOLUTION OF *dl*-THYROXINE.

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OWING to the alkaline hydrolysis which, so far as we know at present, is an essential step in any method for the isolation of thyroxine from the thyroid gland, the fact that the compound is always obtained in the racemic condition is not surprising. It is, however, to be assumed that, in the natural state, thyroxine occurs as one or other optically active modification, and, further, that the physiological activity of the naturally occurring isomeride may be considerably greater than that of its enantiomorph.

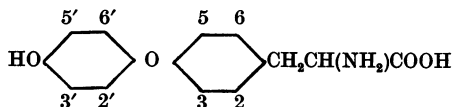
The fact that so-called natural thyroxine, as we know it, is the *dl*-compound rendered the complete chemical and physiological identification of the natural and synthetic substance an easy matter. Should the naturally occurring variety of thyroxine be considerably more active physiologically than its isomeride, an explanation of the discrepancy observed by certain workers [cf. Reid Hunt, 1922] between the physiological activity of thyroxine and of amounts of thyroid gland containing equivalent quantities of iodine, would be afforded by the fact that the thyroxine had been artificially racemised, for in the process of racemisation the thyroxine might lose anything up to one-half of its physiological activity depending on the relationship between the activities of the two isomerides.

The best solution of the problem would obviously be the isolation of optically active thyroxine from the thyroid gland. A large number of experiments in this direction have been made, but so far all have proved fruitless. As is indicated by the experiments of earlier workers on the subject and as fully borne out by the experience of the present writer, acid hydrolysis is entirely useless. From the constitution of thyroxine indeed this is to be expected; since 3 : 5-diiodotyrosine, in which the iodine atoms are present in a configuration similar to that of the 3' : 5'-iodine atoms of thyroxine, is also readily destroyed by boiling with acids. Assuming the necessity of a drastic hydrolysis then, a necessity which is indicated by all the available evidence, the only possible line of attack seems to be through the action of enzymes. Many attempts in this direction have been made, but success has not as yet attended any of them. One of the main difficulties in this connection is the fact observed many years ago by Oswald [1909], and again by the

present writer [Harington, 1926], that certain preparations of trypsin have the property of splitting off more or less of the iodine of the thyroid gland as iodide. Certain experiments have indeed indicated that this is not a universal property of all trypsin preparations, and the hope has not been abandoned that a preparation may be obtained which will effect the desired hydrolysis; it is evident, however, that many further experiments will be required in order to determine the necessary conditions.

Since no further immediate progress could be made in this direction, it was decided to tackle the question from the other end, and to attempt the resolution of synthetic *dl*-thyroxine. It will be evident from the description which has already been given of the properties of thyroxine that the chief difficulty to be anticipated in this work is insolubility, which makes the preparation and purification of salts a troublesome task. In order to minimise this difficulty it was decided, in the first instance, to resolve *dl*-3 : 5-diiodothyronine<sup>1</sup> into its optically active components, and then to iodinate these to the corresponding optically active thyroxine. *dl*-3 : 5-Diiodothyronine, on warming with anhydrous formic acid, readily yielded a formyl derivative, a substance readily soluble in alcohol but quite insoluble in water. It was first attempted to prepare salts of this compound with alkaloids such as brucine, strychnine, or cinchonine which have been used with success in the resolution of the formyl derivatives of other *dl*-amino-acids. The alkaloid salts of *dl*-formyldiiodothyronine, however, were compounds with unattractive properties. They were almost entirely insoluble in water, but little soluble in absolute alcohol, and fairly readily soluble in mixtures of the two solvents. They invariably separated from solution as oils which could in no case be induced to crystallise; in short, it became evident that they were useless for the purpose in view. It appeared from this experience that one had to seek not only for greater solubility but for greater tendency to crystallise; that is to say, that the employment of a base with lower molecular weight was more likely to give favourable results. With this in view attention was directed to the employment of  $\alpha$ -phenylethylamine, and the use of this base has led to at least a partial success. On warming equivalent amounts of the acid and base in a large volume of water, solution took place, and on cooling about 70 % of the acid separated in the form of the phenylethylamine salt. This insoluble fraction could not be obtained optically pure by recrystallisation; the mother liquor on the other hand yielded a soluble fraction which after one or two recrystallisations appeared pure; the acid was recovered from this salt, the

<sup>1</sup> In order to lessen the clumsiness of the systematic nomenclature of thyroxine derivatives it is proposed to call the amino-acid, desiodothyroxine, "thyronine," the positions being numbered as shown



so that thyroxine would be "3 : 5 : 3' : 5'-tetraiodothyronine."

formyl group removed by hydrolysis with hydrobromic acid, and the resulting optically active diiodothyronine iodinated in the ordinary way. Since the insoluble fraction could not be purified it was necessary to employ in turn the two isomerides of the base; in this way, using *l*- $\alpha$ -phenylethylamine there was obtained *l*-thyroxine having  $[\alpha]_{5461}^{21^\circ} - 3.2^\circ$  and, using *d*- $\alpha$ -phenylethylamine, *d*-thyroxine having  $[\alpha]_{5461}^{21^\circ} + 2.97^\circ$ . I am indebted to Dr J. H. Gaddum for the physiological investigation of these samples of optically active thyroxine. A brief account of his results is included in this paper, and the work will be more fully described elsewhere. Briefly, according to his results, *l*-thyroxine is about three times as active physiologically as the *d*-compound. If we assume that the pure *d*-compound is inactive, this result would indicate that each isomeride was contaminated with about 25 % of the other; the chemical work seems to indicate that the separation was probably better than this, but the point must remain in doubt until natural optically active thyroxine has been obtained. At present the most that can be said is that *l*-thyroxine is very definitely the more active physiologically, and is therefore presumably the naturally occurring isomeride.

The numerical value of the rotation of optically active thyroxine according to these experiments is indeed surprisingly low, and it might be that partial racemisation had been induced during the final iodination of the diiodothyronine. In order to control this point, an experiment was performed in which *l*-tyrosine was iodinated and the resulting 3 : 5-diiodotyrosine reduced to tyrosine again by shaking in alkaline solution with hydrogen and palladium-calcium carbonate. The tyrosine finally obtained in this way showed the same optical rotation as the starting material. If the analogy holds, therefore, as seems not unreasonable to expect, it should be possible to assume that no racemisation has accompanied the introduction of the last two iodine atoms into thyroxine.

#### EXPERIMENTAL.

*Formyl-3 : 5-diiodothyronine.* 15 g. *dl-3 : 5-diiodothyronine*<sup>1</sup> were warmed on the water-bath for 3 hours with 100 cc. of 99 % formic acid; the solution was evaporated under diminished pressure and the residual syrup warmed for a further 3 hours with 50 cc. of formic acid; the process was once more repeated, distillation of the formic acid this time leaving a crystalline residue; the latter was extracted with warm absolute alcohol; a small amount of unchanged amino-acid remained undissolved and was removed by filtration; the filtered solution was boiled with charcoal, filtered and poured into a large volume of hot water; on slow cooling the formyl derivative crystallised out in colourless plates, M.P. 207°; the yield was 12 g. The compound was practically insoluble in water; it was readily soluble in alcohol, but sparingly so in other organic solvents.

<sup>1</sup> For the 3 : 5-diiodothyronine used in this work and in that described in the following paper I am indebted to Messrs Hoffmann la Roche of Basel to whom, and in particular to Dr M. Guggenheim, I wish to express my best thanks.

*Analysis.* 1.13 mg. required 4.9 cc. *N*/200 thiosulphate [Kendall, 1914].  
22.8 mg. gave 0.537 mg. N (micro-Kjeldahl).

	I	N
Calculated for $C_{16}H_{13}O_5NI_2$	45.9	2.5
Found ... ..	46.0	2.4

*dl*- $\alpha$ -Phenylethylamine, prepared by the reduction of acetophenoneoxime with sodium and alcohol, was resolved by the method of Aeschlimann [1925]: the *l*-base had  $[\alpha]_{5461}^{20^\circ} - 41.0^\circ$  and the *d*-base  $[\alpha]_{5461}^{10^\circ} + 40.5^\circ$  in benzene solution.

A preliminary experiment was carried out as follows: 11.06 g. *dl*-formyl-3 : 5-diiodothyronine were suspended in 1100 cc. of boiling water and treated with 2.42 g. *l*- $\alpha$ -phenylethylamine dissolved in 200 cc. of warm water; after boiling for a minute the acid had passed into solution; a trace of impurity was removed by filtration and the solution allowed to cool slowly and stand for 24 hours at the ordinary temperature; the salt which separated was crystalline but evidently not homogeneous, appearing under the microscope as clumps of stout needles mixed with a felt of fine ones. It was filtered off, washed with water and dried; it amounted to 8.35 g. (theoretical 6.74 g.). On concentrating the mother liquor under diminished pressure to about 100 cc. the more soluble salt separated at first as an oil, which, however, soon crystallised to an apparently homogeneous felted mass of fine colourless needles (3.7 g.). The latter salt was fairly soluble in alcohol, whilst the first fraction seemed to be much less so; the 8.35 g. were therefore ground up with cold alcohol, filtered off and washed with alcohol; it was then recrystallised by dissolving in dilute alcohol and boiling until the alcohol was removed; it still, however, did not appear homogeneous. The two fractions were then decomposed and the optical rotations of the acids observed; that from the soluble salt had  $[\alpha]_{5461}^{23^\circ} + 21.3^\circ$ ; that from the insoluble salt  $[\alpha]_{5461}^{23^\circ} - 16.9^\circ$ .

In the light of the above results the following series of experiments were carried through; repetition of the resolution has given essentially similar results.

#### A. *l*-Thyroxine.

6.1 g. formyl-*dl*-3 : 5-diiodothyronine were converted into the salt with *l*-phenylethylamine under the conditions described above; the insoluble salt amounted to 4.6 g. and the soluble salt to 2.5 g.; the latter had  $[\alpha]_{5461}^{22^\circ} + 22.0^\circ$  ( $c = 5$  in 50 % alcohol); after two recrystallisations from water it had  $[\alpha]_{5461}^{22^\circ} + 23.8^\circ$  under the same conditions; it crystallised in masses of fine colourless needles which were anhydrous and melted at 188–189°.

*Analysis.* 1.26 mg. required 4.45 cc. *N*/200 thiosulphate [Kendall, 1914].  
15.3 mg. gave 0.637 mg. N (micro-Kjeldahl).

	I	N
Calculated for $C_{24}H_{24}O_5N_2I_2$	37.7	4.2
Found ... ..	37.4	4.2

*Formyl-l-3 : 5-diiodothyronine*. The above salt was dissolved in dilute alcohol and decomposed by the addition of slightly more than the theoretical amount of dilute hydrochloric acid. Precipitation of the acid was completed by cautious dilution with water. The formyl-*l-3 : 5-diiodothyronine* formed colourless plates, darkening at 195° and melting at 214° (decomp.). In 5 % solution in alcohol in a 2 dm. tube  $\alpha = 2.78^\circ$ , whence  $[\alpha]_{5461}^{21^\circ} = + 27.8^\circ$ .

*l-3 : 5-Diiodothyronine*. The formyl derivative was boiled for 1 hour under a reflux condenser with 15 % hydrobromic acid; the solution was evaporated to dryness under diminished pressure and the residual hydrobromide dissolved in warm aqueous alcohol; the solution was then cautiously neutralised with ammonia whereupon the amino-acid separated in glistening colourless plates, m.p. 256° (decomp.).

In 5.4 % solution in 0.880 ammonia it had  $[\alpha]_{5461}^{20^\circ} = - 1.3^\circ$ .

*l-Thyroxine*. The above amino-acid was iodinated in ammoniacal solution as previously described [Harington and Barger, 1927], and the thyroxine purified through the sodium salt. Mention may here be made of the observation that in this final step in the synthesis of thyroxine it is better to employ the strongest iodine solution (*i.e.* above 2.5 *N*); by adopting this measure the greater part of the thyroxine is obtained as the ammonium salt, and the yield is raised to 75–80 % of the theoretical. The *l*-thyroxine obtained in this experiment melted at 235–236° with decomposition; 0.66 g. was dissolved in 6.07 g. of 0.5 *N* sodium hydroxide and 13.03 g. of alcohol; under these conditions it had  $[\alpha]_{5461}^{21^\circ} = - 3.2^\circ$ .

#### B. *d*-Thyroxine.

The steps in the preparation of *d*-thyroxine were precisely similar, so that detailed description is not necessary.

11.06 g. formyl-*dl-3 : 5-diiodothyronine* were converted into the salts with *d*- $\alpha$ -phenylethylamine. The soluble fraction amounted to 3.6 g. and, on recrystallisation, formed colourless needles, melting at 187–188°, and having  $[\alpha]_{5461}^{19.5^\circ} = 21.9$  in 5 % solution in 50 % alcohol.

*Formyl-d-diiodothyronine*. On decomposition the above salt gave formyl-*d-3 : 5-diiodothyronine*, which formed colourless plates melting at 210°, and having, in 5 % solution in alcohol  $[\alpha]_{5461}^{21^\circ} = 26.9^\circ$ .

*d-3 : 5-Diiodothyronine*. The formyl derivative, on hydrolysis, yielded *d-3 : 5-diiodothyronine*, m.p. 256° with decomposition. In 4.35 % solution in 0.880 ammonia this had  $[\alpha]_{5461}^{18^\circ} = + 1.15^\circ$ .

*d-Thyroxine*. The thyroxine prepared by iodination of the above amino-acid melted at 237° with decomposition, and had  $[\alpha]_{5461}^{21^\circ} = + 2.97^\circ$  (0.74 g. dissolved in 6 g. of 0.5 *N* sodium hydroxide and 14 g. of alcohol).

*C. Effect of iodination on optical activity of tyrosine.*

The *l*-tyrosine used in these experiments had  $[\alpha]_{5461}^{23^\circ} - 12.0^\circ$  (4.8 % solution in *N* hydrochloric acid). For the preparation of 3 : 5-diiodotyrosine, the tyrosine was dissolved in 20 parts of concentrated ammonia (Sp. Gr. 0.880); the solution was cooled in ice and treated drop by drop with a concentrated (2.5 *N*) solution of iodine in potassium iodide until the theoretical 2 mols. had been added. On removal of the ammonia by distillation *in vacuo*, or by spontaneous evaporation, the diiodotyrosine crystallised out; it was filtered off, washed with cold water, and recrystallised from 50 % acetic acid with the addition of charcoal. The yield was 60 % of the theoretical. The product, in 4.8 % solution in *N* hydrochloric acid, had  $[\alpha]_{5461}^{23^\circ} + 2.6^\circ$ .

The diiodotyrosine so obtained was dissolved in 100 parts of *N* potassium hydroxide and the solution shaken in an atmosphere of hydrogen in presence of the palladium-calcium carbonate catalyst, exactly as described for the reduction of thyroxine [Harrington, 1926]; the theoretical uptake of hydrogen was complete within 45 minutes. The catalyst was removed by filtration and the solution neutralised with acetic acid and concentrated on the water-bath; the yield of tyrosine was about 80 % of the theoretical, and the product had  $[\alpha]_{5461}^{23^\circ} - 11.8^\circ$  (4.8 % solution in *N* hydrochloric acid). No racemisation had therefore been induced by iodination followed by reduction.

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Samples of the optical isomers obtained from Dr Harrington have been tested for their effects on the growth of tadpoles, and on the oxygen consumption and weight of rats.

*Effect on tadpoles.*

Evidence has been presented elsewhere [Gaddum, 1927] in support of the belief that, under suitable conditions, the effect of thyroxine on tadpoles may be used as a specific quantitative test for this substance. The optical isomers were compared by the method described in the above-mentioned paper. The tadpoles are immersed in batches of 12 for 24 hours in dilute solutions of the substance to be tested. They are then kept in tap water and the average length of each batch is determined at intervals. Several such tests were performed and it was consistently found that both the *d*-thyroxine and the *l*-thyroxine produced, in small concentrations, definite effects on the length and development and also that the *l*-thyroxine produced these effects in slightly smaller concentrations than the *d*-thyroxine. No definite value has yet been obtained by this method for the proportion between the potencies of the preparations.

*Effect on rats.*

The oxygen consumption was determined by a method devised by Richards and Collison [1928]. This method enables a continuous record both of the oxygen consumption and of the movements of the animal to be kept, and, for the determination of the basal oxygen consumption, periods have been chosen during which the record showed no movement. The rats were kept without food each night, and each morning they were weighed and their basal oxygen consumptions were measured. When these had reached a fairly steady level, the thyroxine was injected subcutaneously (2 mg. of thyroxine being dissolved in 1 cc. of *N*/100 NaOH). It was found that this injection produced a rise in oxygen consumption lasting 3-14 days. The experiments are not yet complete, but it is already clear that both preparations produce definite effects in doses of 4 mg. per kg. both on the weight and on the oxygen consumption. The *l*-thyroxine appears to be about three times as potent as the *d*-thyroxine.

*Conclusion.*

The two tests applied both point to the conclusion that the *l*-thyroxine is definitely more active physiologically than the *d*-thyroxine, but that the latter possesses considerable activity.

There are two possible explanations of this fact.

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