SUMMARY

1. [3:5-¹³¹I₂]Thyroxine has been incubated with extracts of rat liver and it was found that deiodination from the thyroxine α ring occurs very slightly or not at all.

2. Experiments with [3':5'-¹³¹I₂]thyroxine confirmed previous findings that the iodine atoms on the β ring are released as inorganic iodide.

3. Organic metabolites were produced which adhered to the protein of the liver extract. These contained the iodine from the α ring, since on treatment with alkali they yielded 3:5-di-iodotyrosine and 4-hydroxy-3:5-di-iodophenyl-lactic acid.

4. The possible identity of these metabolites is discussed.

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Determination of the Distribution of ¹³¹I in Biosynthetically Labelled [¹³¹I]Tri-iodothyronine Isolated from the Thyroid Glands of Rabbits

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The reaction between thyroxine and *m*-nitro-benzenediazonium chloride has been utilized for the localization of ¹³¹I in biosynthesized thyroxine (Plaskett, 1959). Coupling occurs in the position *ortho* to the phenolic group and some of the iodine from the thyroxine β ring is displaced from organic combination. The inorganic iodide is separated from the organic constituents of the mixture, and its radioactivity determined separately.

This paper reports the application of this tech-

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nique to a study of 3:5:3'-tri-iodothyronine (referred to as tri-iodothyronine). The work was undertaken on account of its importance in elucidating the pathway of tri-iodothyronine biosynthesis. The formation of tri-iodothyronine from thyroxine in the thyroid gland was long thought unlikely because the thyroid deiodinases (Roche, Michel, Michel & Lissitzky, 1952; Roche, Michel, Michel, Gorbman & Lissitzky, 1953) were reported to be inactive towards thyroxine. However, Pitt-Rivers & Tata (1959) have found that pig-thyroid deiodinase deiodinates thyroxine if freed from blood proteins.

An alternative route of tri-iodothyronine formation is the coupling of 3-monoiodotyrosine and 3:5-di-iodotyrosine with the elimination of one alanine side-chain by a reaction analogous to the one which generates thyroxine from di-iodotyrosine (Harington, 1944).

It has already been demonstrated that thyroxine formed in the thyroid gland of the rat or the rabbit after a dose of [^{131}I]iodide is equally labelled with radioactivity on the two rings (Plaskett, 1961). If tri-iodothyronine were formed from thyroxine by the loss of one iodine atom, it would be expected that the labelling of the tri-iodothyronine would be such that the iodine on the two rings is of equal specific radioactivity. If, alternatively, tri-iodothyronine is formed by the coupling of monoiodotyrosine and di-iodotyrosine, the two rings are being contributed by two dissimilar precursors, which may differ in specific radioactivity.

The localization of ^{131}I in tri-iodothyronine was undertaken in an attempt to distinguish between these two possibilities.

EXPERIMENTAL AND RESULTS

Adaptation of the diazo-coupling technique for use with tri-iodothyronine. It had been hoped originally that the ^{131}I in tri-iodothyronine could be studied by a preliminary iodination of the 5'-position with ^{127}I , followed by diazo-coupling of the thyroxine product. In practice, however, it was not possible to effect the iodination without also causing loss of ^{131}I from the 3'-position by an exchange reaction. Coupling was therefore carried out by using unmodified tri-iodothyronine. In this reaction it was expected that the yield of inorganic iodide from the β ring would be very small, since the reacting diazonium salt may couple in the untenanted 5'-position. It was expected that this position would have to be blocked by some substituent other than iodine before the coupling reaction could be usefully employed. Surprisingly, when the reaction was carried out with [$3'\text{-}^{131}\text{I}$]tri-iodothyronine, 47.8% of the total radioactivity appeared in the supernatant (iodide) fraction. This can be attributed to a preferential coupling in the position occupied by the iodine atom, since in the work on thyroxine it was established that no important quantity of any 3':5'-bisazo derivative was formed.

In adapting the coupling reaction for use with tri-iodothyronine some modifications of procedure were inevitable. The technique as originally described for thyroxine (Plaskett, 1959) is divisible into four stages of manipulation: (1) purification of radioactive thyroxine by precipitation; (2) preparation of the thyroxine solution for coupling; (3) coupling; (4) separation of the fractions by precipitating the azo-compounds and assay of ^{131}I

in the supernatant and precipitate. Stage (3) was in no way altered. Stage (4) was rendered faster and more accurate by the use of a different solvent for bringing the precipitate into solution: the original solvent [0.2N-NaOH-aq. 40% (v/v) ethanol-aq. 5.4% Teepol 530 (2:1:1, by vol.)] was modified by mixing it with pyridine in the ratio 1:1 (v/v). Stage (2) was altered only by improved accuracy in measuring the components of the mixture; this was achieved by handling larger volumes. Small inaccuracies in the concentration of $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ significantly affected percentage precipitation. Each batch of purified tri-iodothyronine was dissolved therefore in 5.3 ml. of a mixture of 2.00N-NaOH-water- $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ soln. (5.4 mg./ml.) (12:31:10, by vol.). The weight of tri-iodothyronine used throughout was 16.75 mg.

In stage (1) 16.75 mg. of tri-iodothyronine was mixed with biosynthetic [^{131}I]tri-iodothyronine in solution in ethanol-aq. 2N- NH_3 soln. (1:1, v/v) and then evaporated to dryness under reduced pressure. The residue was dissolved in 10 ml. of 0.5N-NaOH and reprecipitated by addition of conc. HCl. Some experiments were made in order to determine the optimum volume of conc. HCl to use, since when 0.7 ml. was used a rather poor yield of precipitated tri-iodothyronine was obtained from the acid medium. Table 1 shows that tri-iodothyronine is very soluble in this medium and that better recovery is obtained by precipitating close to neutrality. It was extremely important to have a constant recovery at this stage, since loss of tri-iodothyronine upset the ratios of reactants during coupling and gave false values. In the work reported here 0.52 ml. of conc. HCl was used throughout.

Standard slope. The diazo-coupling reaction was carried out several times with [$3'\text{-}^{131}\text{I}$] and [$3:5\text{-}^{131}\text{I}_2$]tri-iodothyronine. Table 2 gives the results of these determinations, which fix the

Table 1. *Precipitation of tri-iodothyronine by acidification of the medium*

In each experiment, acid was added to 16.75 mg. of radioactive tri-iodothyronine in 10 ml. of 0.5N-NaOH solution. The tri-iodothyronine used was iodide-free, having been previously reprecipitated.

Vol. of conc. HCl added (ml.)	Excess of HCl (m-equiv.)	Recovery of tri- iodothyronine (%)
0.80	3.0	89.9
0.76	2.6	90.3
0.72	2.2	91.5
0.70	2.0	91.9
0.66	1.6	92.7
0.64	1.4	93.6
0.62	1.2	93.8
0.56	0.6	95.7
0.50	Nil	97.6

Table 2. *Precipitation results obtained by performing the diazo-coupling test with [¹³¹I]tri-iodothyronine*

Position of label and source of tri-iodothyronine	No. of measurements made	Percentage precipitation	
		Range	Mean
3' (synthetic)	10	51.3-54.2	52.2
3:5 (synthetic)	7	96.3-96.6	96.5
All positions (biosynthesized)	16	80.82-83.05	81.6

extreme points of the standard slope at 52.2 and 96.5 %. Tri-iodothyronine containing equal quantities of ¹³¹I on each ring would therefore give 74.35 % precipitation. Tri-iodothyronine having the same specific radioactivity on the two rings would give 81.74 % precipitation.

Isolation of tri-iodothyronine from the thyroid glands of rabbits. Male rabbits (2-2.5 kg.) were each injected intravenously with 300 μ C of [¹³¹I]-iodide in sterile 0.9 % NaCl soln. They were killed after 24 hr. by an intravenous injection of Nembutal. The thyroid gland was dissected out and hydrolysed by refluxing for 20 hr. in 5 ml. of 2N-NaOH. The hydrolysate was extracted once with an equal volume of ether and once with an equal volume of CHCl₃. These extracts, which together contained less than 2 % of the total radioactivity, were discarded. The hydrolysate was next extracted with an equal volume of butan-1-ol. This extract, which contained 20 % of the total radioactivity, was evaporated to a small volume under reduced pressure, applied quantitatively on the origin of a paper chromatogram, and developed in butan-1-ol-2N-acetic acid (1:1, v/v). The positions of the radioactive peaks were determined by means of an automatic-recording β -particle counter. The iodothyronine peak (R_f 0.93) was eluted and re-run in butan-1-ol-pentan-1-ol-aq. 2N-NH₃ soln. (1:1:2, by vol.). On this chromatogram a small radioactive peak running ahead of thyroxine (R_f 0.53) corresponded with authentic tri-iodothyronine markers. This peak was eluted and re-run in butan-1-ol-aq. 9N-NH₃ soln. (1:1, v/v). Its radioactivity was separated by this procedure into three components, one of which corresponded with authentic tri-iodothyronine markers. This peak was eluted, and on running again in several different solvents was found to contain only tri-iodothyronine. The yield of tri-iodothyronine represented 0.7-0.9 % of the total radioactivity of the gland.

The chromatograms were developed by the descending method on Whatman no. 3MM paper.

Results of diazo-coupling. These are given in Table 2. The mean value for percentage precipitation (81.6) indicates, by reference to the standard slope, that the rabbit tri-iodothyronine contains 33.6 % of its total ¹³¹I on its β ring. This result is very close to the finding which would be expected if the specific radioactivities of the iodine on the

two rings were equal (i.e. random labelling), in which case 33 $\frac{1}{3}$ % of the ¹³¹I would appear on the β ring.

The main factor limiting the accuracy of the measurements in this work was statistical error in the assay of ¹³¹I. It can be shown that the standard deviation (δ) of the percentage precipitation is given by

$$\delta = 100 \sqrt{\frac{xy}{(x+y)^3}},$$

where x and y are the total counts recorded on the supernatant and precipitate respectively in a given time, t . The minimum value of t used was such that $x > 3000$ and $y > 12\,000$. This gives $\delta = 0.33$ as a maximum. Ten of the sixteen measurements gave results within the range 81.61 ± 0.33 . The results are therefore no less accurate than the best accuracy permitted by the counting data, and it may be supposed therefore that errors in the chemistry of the reaction do not contribute significantly to the variation observed. This situation is attributed to the improvements in manipulation which are described in this paper, and it is in contrast with the work on thyroxine where manipulative error was considered to contribute significantly to the variation of the results.

DISCUSSION

It is clear that if the thyroid gland were capable of incorporating ¹³¹I more rapidly into one ring of a hormone than into the other, the effect would only be apparent for a short period after giving the radioactive dose before the various iodine pools of the body have become fully equilibrated with the injected isotope. It might be argued therefore that although the tri-iodothyronine of the rabbit thyroid was randomly labelled after 24 hr., this does not necessarily indicate that it was also randomly labelled during the first few hours after the dose was given. It is valid to assume, however, that much of the [¹³¹I]tri-iodothyronine synthesized during the first few hours will remain in the gland until 24 hr. Thus if the hormone is synthesized with preferential labelling of one ring at any time during the 24 hr., this preference would be detected in spite of dilution with randomly labelled material during the later stages of the experiment.

The finding that the tri-iodothyronine of the rabbit thyroid is randomly labelled after 24 hr. can be interpreted in two ways. Either tri-iodothyronine is formed in the thyroid gland by deiodination of thyroxine, or it is formed from monoiodotyrosine and di-iodotyrosine pools, which are of equal specific radioactivity for most of the 24 hr. period. It becomes important therefore to know how the radioactivity of the thyroidal monoiodotyrosine and di-iodotyrosine varies after a single dose of ^{131}I , since, if their specific activities are markedly different, they are precluded from a role as immediate precursors of tri-iodothyronine.

Few records of direct measurements of specific radioactivity are available in the literature, but information about specific radioactivity may be deduced from measurements of di-iodotyrosine/monoiodotyrosine ratios. Thus in a chapter on thyroid-hormone biosynthesis, Pitt-Rivers & Tata (1959) give a diagram showing the 'distribution of ^{131}I in the principal iodinated amino acids of hydrolysed rat thyroid gland in function of time after the administration of ^{131}I '. The ^{131}I of monoiodotyrosine is shown rising rapidly to exceed the ^{131}I of di-iodotyrosine by a factor of six to seven times at about 6 hr. after the dose. It then declines, and di-iodotyrosine/monoiodotyrosine is unity at 24 hr. Dr J. R. Tata (personal communication) has stated that this diagram is being modified in the light of new experiments. The nature and sequence of the events are confirmed by the new experiments, but the time relations are altered so that all the changes occur about twice as rapidly. If these events occur similarly in the rabbit, the formation of tri-iodothyronine from monoiodotyrosine and di-iodotyrosine is precluded.

In a review on thyroid hormones Michel (1956) is in agreement with the findings of Pitt-Rivers & Tata (1959) when he states that, after ^{131}I is injected into animals, monoiodotyrosine is high in relation to di-iodotyrosine in the first hours after administration of the dose. However, Bois & Larsson (1958), in contradiction to this, state that, after giving ^{131}I to rats, the di-iodotyrosine/monoiodotyrosine ratio was constant during the

period 30 min.–7 days after the dose. This disagreement in the literature must be resolved before a confident statement can be made about the pathway of tri-iodothyronine biosynthesis.

In the present work, the discovery of preferential labelling in tri-iodothyronine would have been decisive. The discovery, instead, of random labelling supports the hypothesis that tri-iodothyronine is generated by deiodination of thyroxine; the drawing of a definite conclusion awaits specific-radioactivity measurements on the iodotyrosines.

SUMMARY

1. Radioactive tri-iodothyronine has been isolated from the thyroid glands of rabbits 24 hr. after administration of [^{131}I]iodide.

2. It has been established that the labelling of this tri-iodothyronine was such that the specific radioactivity of the iodine on the two rings was equal.

3. This finding is discussed in relation to the possible pathways of tri-iodothyronine biosynthesis; it is consistent with the view that tri-iodothyronine is formed by the deiodination of thyroxine.

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