Determination of the Distribution of ^{131}I in Biosynthetically Labelled [^{131}I]Thyroxine Isolated from the Thyroid Glands of Rabbits and Rats*

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It has often been supposed, on the basis of the current theory of thyroxine biosynthesis, that radioactive thyroxine isolated from the plasma or thyroids of animals is equally labelled on the two rings. This supposition is necessarily true for thyroxine samples taken from an animal several weeks after administration of [^{131}I]iodide, when the radioactive label has equilibrated with the stable iodine of the body. For thyroxine samples taken soon after administration of the radioactive dose, however, experimental verification is necessary.

The current theory of biosynthesis of thyroxine involves, as the final stage, the coupling of two di-iodotyrosine molecules with the elimination of one alanine side chain (Harington, 1944; Pitt-Rivers, 1948). If this coupling reaction occurs between di-iodotyrosine molecules free in solution, there is no possibility that either ring of the resultant thyroxine can become preferentially labelled, since under these conditions the reaction takes place between identical components. The specific radioactivity measurements of Tong, Taurog & Chaikoff (1951), however, indicate that thyroxine is probably generated within the peptide chains of thyroglobulin, and in this case there is a possibility that the protein imparts specificity to the reaction in such a manner that the reacting di-iodotyrosine moieties are of different specific radioactivity.

Plaskett (1959) described a chemical technique for determining the distribution of ^{131}I in the thyroxine molecule by displacing some of the 3':5'-thyroxine iodine by reaction with a diazonium salt. This paper describes the isolation of thyroxine from the thyroid glands of rabbits and rats in a sufficiently pure condition to permit study by this technique. The results of diazo coupling are presented as evidence that during biosynthesis of thyroxine from [^{131}I]iodide the radioactive label becomes equally distributed between the rings of the thyronine nucleus.

METHODS

Chromatography. Chromatograms were developed by the descending method on Whatman no. 3MM paper. Only unidimensional techniques were employed. When spots of the separated substances were eluted for running again in another solvent, the eluent used was ethanol-aq. 2N-NH₃ soln. (1:1, v/v). Marker compounds were stained with diazotized sulphanilic acid (Gross & Leblond, 1951a).

Location of radioactivity. The distribution of radioactivity on the developed and dried chromatograms was determined by means of an automatic scanning counter. The paper moved at the rate of 1 in./hr. under a shielded mica end-window β -particle counter. The number of pulses from the counter were recorded on a continuously moving recording milliammeter.

Preparation and treatment of the thyroxine samples

From rats. Male Wistar albino rats (170–230 g.) were each injected intravenously with 30 μC of [^{131}I]iodide in sterile aq. 0.9% NaCl. They were killed after a suitable period of time by an intraperitoneal injection of Nembutal followed by heart puncture. The thyroid gland was dissected out and hydrolysed by refluxing for 20 hr. in 3 ml. of 2N-NaOH containing 50 μg . of sodium L-thyroxine. The hydrolysate was extracted once with an equal volume of butan-1-ol; the upper layer was separated and evaporated to a small volume under reduced pressure. The concentrated extract was applied quantitatively to the origin of a paper chromatogram and developed in butan-1-ol-2N-acetic acid (1:1, v/v). After drying the chromatogram, the positions of the radioactive peaks were determined. A small iodide peak was present (R_f 0.22) but the bulk of the radioactive material ran as a single peak close to the solvent front. This peak, which contained the iodothyronines, was eluted. The eluate was evaporated to a small volume under reduced pressure before being applied quantitatively to the origin of another paper chromatogram. This was developed in butan-1-ol-pentan-1-ol-aq. 2N-NH₃ soln. (1:1:2, by vol.) and the positions of the peaks were again determined. The largest peak on this chromatogram had R_f identical with that of authentic thyroxine. This peak was eluted and some small samples of it were tested by running again in several different solvents. With the exception of 3–5% of inorganic iodide the material was found to be radiochemically homogeneous, and it was inseparable from authentic thyroxine markers. The major part of the eluted [^{131}I]thyroxine was mixed with 10 ml. of ethanol-aq. 2N-NH₃ soln. (1:1, v/v) containing 20 mg. of sodium L-thyroxine.

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The mixture was then evaporated to dryness under reduced pressure. The dried thyroxine was dissolved in 10 ml. of 0.5 *N*-NaOH soln. and transferred quantitatively to a centrifuge tube. The thyroxine was precipitated by addition of 0.7 ml. of conc. HCl and centrifuged. The supernatant liquid containing the iodide impurity was separated off and the precipitate redissolved in 5.2 ml. of 0.463 *N*-NaOH soln.; 0.1 ml. of 0.218 *M*-Na₂S₂O₃ was added and the diazo-coupling reaction carried out as described by Plaskett (1959).

The radioactivities of the precipitated azo compounds and of the supernatant fraction were determined as previously described, but it was necessary, because of the low counting rates of the specimens under study, to obtain a very accurate estimate of the background before each determination. A background count was therefore made overnight (about 55 000 sec.) with the counting tube filled with water. About 30 000 sec. were then devoted to counting the radioactive specimen.

From rabbits. Male rabbits (2.0–2.5 kg.) were each injected intravenously with 300 μ C of [¹³¹I]iodide in sterile aq. 0.9% NaCl. They were killed after either 6 or 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 2 *N*-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 and the organic layer evaporated to a small volume for chromatography in butan-1-ol–2 *N*-acetic acid (1:1, v/v). In this solvent most of the radioactivity ran as a single peak (*R_F* 0.93) which corresponded in position to the iodothyronines. This peak was eluted and thyroxine was isolated from it by running again in the manner already described for the work on the rat glands. Once again, the thyroxine contained a trace of inorganic iodide as its only radioactive impurity.

Effectiveness of reprecipitation

In order to determine the extent to which reprecipitation removed [¹³¹I]iodide which contaminated the thyroxine, 20 mg. of sodium L-thyroxine was dissolved in 10 ml. of 0.5 *N*-NaOH containing [¹³¹I]iodide and reprecipitated by addition of 0.7 ml. of conc. HCl. Only 3% of the radioactivity was reprecipitated with the thyroxine. Thus if the [¹³¹I]thyroxine was found to be contaminated with 5% of [¹³¹I]iodide immediately after elution from the chromatogram this contamination would be decreased to 0.15% by reprecipitation.

Freedom of thyroxine samples from non-radioactive impurities

It has been pointed out (Plaskett, 1959) that one possible danger in applying the diazo-coupling technique to thyroxine isolated from biological sources is that the biosynthetic hormone, although radiochemically pure, may contain non-radioactive phenols and amines which would interfere with the stoichiometry of the diazo-coupling reaction by consuming some of the diazonium salt.

It was decided to test for such impurities by performing the diazo-coupling reaction several times with different quantities of the biosynthetic [¹³¹I]thyroxine. The radioactive hormone functions only as a tracer in the reaction and makes no significant contribution to the concentration of thyroxine in the reaction vessel. Its quantity can therefore be varied without upsetting the balance of the reactants, 20 mg. of carrier thyroxine being used throughout. If it contained impurities which do upset this balance, their effect should become less apparent as their quantity is decreased. Thus in some experiments the entire yield of thyroxine from a rabbit gland was used in making a single measurement, whereas in others the smallest quantity was used which would permit sufficiently accurate counting of the supernatant and precipitate.

In practice it was found that these variations in the quantity of biosynthetic [¹³¹I]thyroxine did not alter the result obtained from diazo coupling. Hence it was inferred that substances which interfere with diazo coupling had been either eliminated during the purification of the thyroxine or decreased to insignificantly low levels.

RESULTS

Alkaline butan-1-ol extracts of the thyroid hydrolysates from rabbits killed 24 hr. after the dose of ¹³¹I contained 20% of the total radioactivity of the gland. In rabbits killed 6 hr. after the dose, only 13% was extractable into butanol. In each case about 50% of the extractable radioactivity was present as thyroxine. In rats killed 3 hr. after the dose, 12% of the radioactivity of the thyroid hydrolysate was extractable into butanol, but often much less than 50% of it was present as thyroxine.

Table 1 shows the precipitation results obtained from the diazo-coupling reaction. The quantity of the radioactive thyroxine in the rat thyroids was

Table 1. Results obtained by carrying out the diazo-coupling test with biosynthesized thyroxine

Average of all thirty-four results on biosynthetic thyroxine = 72.8%.				
Source of thyroxine	Duration of expt. (hr.)	Number of times repeated	Precipitation (%)	
			Range	Mean
Rat	1.0	1	—	74.0
	3.0	11	70.9–74.8	72.7
	6.0	1	—	73.1
	28.0	1	—	72.4
	55.0	1	—	72.4
Rabbit	6.0	4	72.0–74.6	73.5
	24.0	14	71.1–75.0	72.4
Human	24.0	1	—	74.6

small, and hence each gland yielded only sufficient to perform a single diazo-coupling test. The 15 results obtained with rat thyroxine therefore represent experiments carried out on 15 different animals. With rabbits, however, the thyroxine fraction contained a larger quantity of radioactivity, more than proportional to the greater size of the administered dose. Eighteen results were therefore obtained from only nine animals, three of which were killed 6 hr. after administration of the dose, and the remainder after 24 hr. The single experiment which was performed on human thyroxine utilized normal thyroid tissue surgically removed from a female patient with thyroid carcinoma. The patient had received 1.2 mc of [¹³¹I]-iodide 24 hr. before operation. The isolation of the thyroxine fraction was carried out by the method already described for the rabbit glands.

Standard slope

The diazo-coupling test was performed several times on 20 mg. samples of [3:5-¹³¹I₂]thyroxine and [3':5'-¹³¹I₂]thyroxine. The results are given in Table 2. The mean precipitation figures were taken as the ends of the standard slope. Thus since this slope runs from 96.3 to 48.9, thyroxine labelled uniformly throughout the molecule would be expected to give a precipitation figure of 72.6%. The experimental values actually obtained for biosynthetic thyroxine (Table 1) were not considered to differ significantly from this theoretical value.

The accuracy of the measurements is such that all except one of the thirty-four experimental results fall within the limits of error which had been indicated by the methodological studies (Plaskett, 1959), i.e. $72.8 \pm 2.0\%$. This range corresponds to thyroxine containing 46.2–54.3% of the ¹³¹I on the 3:5 positions, and the mean result, 72.8, corresponds to thyroxine containing 50.4% of the ¹³¹I on the 3:5 positions.

DISCUSSION

As early as 1948, Frieden, Lipsett & Winzler had considered that the distribution of ¹³¹I in the labelled thyroxine then being used for experimental purposes was an important problem. The labelled

thyroxine prepared by biosynthesis was thought to differ from that prepared by isotope exchange and other labelling techniques, but the difference had not been established and measured. The known precursor/product relationship between 3:5-diiodotyrosine and thyroxine (Harington, 1944; Taugog & Chaikoff, 1947) indicated that biosynthesized thyroxine may be equally labelled on the two rings. For this reason biosynthesized thyroxine has frequently been employed in tracing the metabolic fate of the hormone: such experiments were considered to give a more complete account of the mechanism of thyroxine breakdown than experiments with thyroxine labelled on one ring only (Gross & Leblond, 1951*b*; Lissitzky, Roques, Bénévent & Pinchera, 1958).

The results of the present work confirm the view that the thyroid glands of the animals which were studied exhibit no preferential labelling of either ring of the thyroxine molecule. This finding, obtained with thyroxine isolated very quickly after the administration of a radioactive dose, indicates that the participation of a protein in hormone synthesis exerts no specificity of the kind envisaged above.

In addition to justifying the assumption, frequently made by research workers in the past, that the thyroid glands of animals previously injected with [¹³¹I]iodide constitute a source of randomly labelled thyroxine, the results of this work make possible some further investigations of the biosynthesis of thyroid hormone. Since biosynthesized [¹³¹I]thyroxine is randomly labelled and since chemically synthesized radioactive hormones are normally labelled specifically in one ring or the other, the diazo-coupling technique renders it possible to distinguish between endogenously labelled hormones and radioactive hormones which have been injected. Work is now in progress in an attempt to apply the diazo-coupling technique to the blood and tissue tri-iodothyronine and thus to determine whether this material originates in the thyroid gland, or whether it arises by the peripheral deiodination of thyroxine.

SUMMARY

1. The diazo-coupling technique for determining the distribution of ¹³¹I between the two rings of the thyroxine molecule has been applied to radioactive thyroxine purified from animal thyroids, and in one instance from a human thyroid.

2. The results indicate that from the earliest time after administration of [¹³¹I]iodide when thyroxine samples could be obtained, the radioactive atoms were equally distributed between the rings of the thyronine nucleus.

Table 2. *Precipitation results obtained by performing the diazo-coupling test with chemically synthesized [3:5-¹³¹I₂]thyroxine and [3':5'-¹³¹I₂]thyroxine*

Position of label	Number of measurements made	Precipitation (%)	
		Range	Mean
3:5'	11	47.3–50.2	48.9
3:5	6	96.1–96.5	96.3

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Studies on the Degradation of Thyroid Hormones *in vitro* with Compounds Labelled in Either Ring*

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Several independent groups of workers have established that preparations of mammalian tissue catalyse the deiodination of thyroxine and 3:5:3'-tri-iodothyronine (Sprott & MacLagan, 1955; Lissitzky, Michel, Roche & Roques, 1956; Tata, 1958; Yamasaki & Slingerland, 1959; Etling & Barker, 1959). Throughout this work, where labelled hormones were employed, the radioactive tracer was confined to the β ring of the iodothyronine molecule. Deiodination involved loss of the labelled atoms from organic combination, and the fate of the thyronine carbon skeleton could not be traced.

Recently, biosynthetic [^{131}I]thyroxine was employed for studies *in vitro* (Lissitzky, Roques, Bénévent & Pinchera, 1958; Lissitzky, Bénévent, Roques & Roche, 1958) and from the results of this work it was claimed that deiodination was the simultaneous fate of all four of the thyroxine iodine atoms.

This paper describes work on the metabolism *in vitro* of chemically-synthesized radioactive thyroxine and 3:5:3'-tri-iodothyronine with the tracer confined to the 3:5 positions. It reports the finding of an intermediate metabolite, in breakdown of thyroid hormone, which has undergone deiodination only in the β ring.

The enzyme system employed was present in a rat-liver extract almost identical with that used by MacLagan & Reid (1957).

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METHODS

Synthesis of radioactive compounds

[3:5- $^{131}\text{I}_2$]Thyroxine and [3:5- $^{131}\text{I}_2$]tri-iodothyronine. These compounds were prepared by a modification of the method of Michel, Roche & Tata (1952). A specific radioactivity of about 240 $\mu\text{C}/\mu\text{mole}$ was obtained in the following manner. 3:5-Diamino-4-(4'-methoxyphenoxy)-*N*-acetylphenylalanine ethyl ester (50 mg.) was employed in the tetrazotization reaction. Part of the resulting solution was diluted tenfold with a conc. H_2SO_4 -acetic acid mixture (1:2, v/v) at 0°. A portion (0.1 ml.) of this diluted solution was used for the radioiodination reaction. The quantity of the tetrazotized compound which was employed corresponded to 333 $\mu\text{g.}$ of the original diamine. It was added to 0.66 mg. of NaI, 0.33 mg. of I_2 , 0.08 mg. of urea and 1 mc of [^{131}I]iodide in 0.2 ml. of water. Approximately 0.05 ml. of CHCl_3 was present and the mixture was maintained at 40°.

Chromatographic purification of the radioactive intermediate 3:5-di-iodo-4-(4'-methoxyphenoxy)-*N*-acetylphenylalanine ethyl ester was found to be unnecessary. By omission of this step a day was conserved during the preparation. Removal of the protective methyl, acetyl and ester groups was carried out by refluxing for 2½ hr. with 0.4 ml. of acetic acid and 0.4 ml. of redistilled HI.

The radioactive 3:5-di-iodothyronine was iodinated in 1 ml. of conc. aq. NH_3 soln. by addition of 2 ml. of ether containing non-radioactive iodine. For the preparation of thyroxine 3.0 mg. of iodine was used, and for the preparation of tri-iodothyronine 1.0 mg. The mixture was evaporated almost to dryness, redissolved in a few drops of ethanol-aq. 2*N*- NH_3 soln. (1:1, v/v) and chromatographed in butan-1-ol-pentan-1-ol-aq. 2*N*- NH_3 soln. (1:1:2). The band of thyroxine or tri-iodothyronine was eluted from the paper for use. A sample of the tri-iodothyronine was always run again in butan-1-ol-aq. 9*N*- NH_3 soln. to check against contamination with [^{131}I]di-iodothyronine.