The author wishes to thank Miss M. O. Hecht for assistance with the animal work. The work reported in this paper has been carried out on behalf of the Medical Research Council.

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Biochem. J. (1961) **78**, 652

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

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(*Received* 19 *October* 1960)

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).

* This work forms part of a Ph.D. Thesis submitted to the University of London.

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METHODS

Synthesis of radioactive compounds

[3:5-$^{131}I_2$]*Thyroxine and* [3:5-$^{131}I_2$]*tri-iodothyronine.* These compounds were prepared by a modification of the method of Michel, Roche & Tata (1952). A specific radioactivity of about 240 μc/μmole 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 μg. 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. 2N-NH_3 soln. (1:1, v/v) and chromatographed in butan-1-ol–pentan-1-ol–aq. 2N-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. 9N-NH_3 soln. to check against contamination with [^{131}I]di-iodothyronine.

[3′:5′-$^{131}I_2$]*Thyroxine and* [3′:5′-$^{131}I_2$]*tri-iodothyronine.* The procedures employed were those described by Fletcher (1956), being modifications of the original method of Gross & Leblond (1950).

Chromatography. This was carried out by the descending method on Whatman no. 3MM paper. Marker substances were detected by a spray of diazotized sulphanilic acid (Gross & Leblond, 1951) or by ceric sulphate staining (Bowden, Maclagan & Wilkinson, 1955; Fletcher & Stanley, 1955). Four solvent systems were used: butan-1-ol–dioxan–aq. 2N-NH_3 soln. (4:1:5), butan-1-ol–pentan-1-ol–aq. 2N-NH_3 soln. (1:1:2), butan-1-ol–2N-acetic acid (1:1), butan-1-ol–ethanol–water (4:1:5).

Location and measurement of radioactivity. Radioactive chromatograms were scanned with an automatic device which draws the paper at the rate of 1 in./hr. past the end window of a G.M. β-particle counter. Alternatively, radioautography was employed, with Ilford No-screen X-ray film. The radioactivity of liquids was determined by use of a G.M. tube (20th Century Electronics, type M6) of 10 ml. capacity and a sensitivity towards ^{131}I in aqueous solution of 12 400 counts/min./μc.

Preparation of rat-liver extracts. The liver was removed from a male Wistar albino rat and plunged into ice-cold 1·15% (w/v) KCl. It was then removed, a lobe at a time, freed from superficial moisture and quickly cut into small pieces with surgical scissors. A portion (3 cm.^{3}) of the tissue was added to 27 ml. of 1·15% KCl soln., shaken vigorously for 5 min. and squeezed through muslin. The extract was made up to 30 ml. with 1·15% KCl and mixed with 30 ml. of 0·1M-sodium phosphate soln., pH 6·0. This preparation contained about 200 mg. of protein/100 ml. Microscopic examination revealed no whole liver cells. There were fragments of cells and cell membranes and numerous intact erythrocytes. The oxygen consumption of the extract when shaken in air at 37° in a Warburg manometer was 5·6 μl./hr./ml.

Incubation mixture. Samples were made up for incubation by adding labelled thyroxine or tri-iodothyronine (1 μg. unless otherwise stated) in 0·5 ml. of 10% propylene glycol, to 10 ml. of the buffered extract. Incubation was carried out at 37°.

Separation of reaction products. After incubation, 4 ml. of 10% horse serum was added, followed by 5 ml. of 20% trichloroacetic acid. The mixture was filtered and the residue washed with 5% trichloroacetic acid. The filter paper bearing the protein residue was then immersed for 16 hr. in butan-1-ol (35 ml.) to extract the unmetabolized hormone. The butanol-extracted protein was subsequently brought into solution by heating for 7 min. with 0·2N-NaOH at 100° to ensure complete recovery of substrate radioactivity. The sample was thus divided into three fractions: a filtrate (Fraction 1), a butanol extract (Fraction 2) and a NaOH-digest (Fraction 3). Iodide passes into Fraction 1, unchanged thyroxine appears in Fraction 2 and organic degradation products may be expected in any of the three fractions.

In some experiments, when Fractions 1 or 2 were to be studied by chromatography, 20% trichloroacetic acid was replaced by 2% phosphotungstic acid as the protein precipitant.

Evidence for deiodination. Maclagan & Reid (1957), who used only [3′:5′-^{131}I]thyroxine and were concerned primarily with measurements of deiodination, expressed the radioactivity of Fraction 1 as a percentage of that of the total substrate. These authors established that the radioactivity of Fraction 1 was composed wholly of inorganic iodide by a procedure in which the ^{131}I was first oxidized to iodine by treatment with cold $K_2Cr_2O_7$, extracted and studied chromatographically. To eliminate the objection that $K_2Cr_2O_7$ exerts a destructive effect on some iodine compounds, the radioactivity of Fraction 1 was now studied by an alternative method.

Fletcher (1957) found that inorganic iodide could be separated from organic iodine compounds during passage through a column of AgCl at pH 8·5 in the presence of 50% ethanol and 1% NaCl. Fraction 1 from a liver extract incubated with [3′:5′-$^{131}I_2$]thyroxine was subjected to this test, and 100% of the radioactivity remained adhering to the column. This finding was in agreement with the view expressed by Maclagan & Reid (1957) that Fraction 1 contained no radioactive organic substances.

Digestion with trypsin. In experiments which required the rat-liver protein to be digested with trypsin, the protein was first washed free from butanol with ether, dried and powdered. A portion (100 mg.) of the powder was suspended in 5 ml. of 0·1M-sodium phosphate soln. and incubated with 15 mg. of trypsin (Armour 1–75) at pH 8·4. Incubation was continued for 48 hr. at 40° in the presence of toluene.

RESULTS

Properties of the deiodinating system. Some preliminary experiments with [3′:5′-$^{131}I_2$]thyroxine served to confirm the essential findings of Maclagan & Reid (1957). The extracts caused 65–80% deiodination of a sample (1 μg.) of thyroxine on incubation for 15 min. Table 1 records the progress of deiodination with time and Table 2 indicates initial reaction velocities which were observed at different concentrations of substrate. The extracts were more active in deiodinating thyroxine when incubated at 37° than when maintained at 20°, and their activity was almost completely destroyed by heating for 10 min. at 100°.

The extract was next incubated with [3:5-$^{131}I_2$]-thyroxine; very little [^{131}I]iodide was produced. A detailed study of the organic reaction products was undertaken.

Radioactivity measurements and chromatographic studies on the three fractions of the extract. Table 3 gives figures for the distribution of ^{131}I between the fractions and shows the components of each fraction as found by chromatography. Fraction 2 was prepared for chromatography by neutralization and evaporation under reduced pressure. Fractions 1 and 3 were prepared by butanol extraction at pH 3 followed by neutralization and evaporation of the extract.

(i) Fraction 1. In zero-time controls the radioactivity in this fraction consisted only of the iodide impurity of the substrate. In samples incubated with [3′:5′-$^{131}I_2$]thyroxine this fraction contained 73% of total substrate radioactivity as inorganic

iodide. In samples incubated with [3:5-$^{131}I_2$]-thyroxine the radioactivity of Fraction 1 was much lower, only 21 % of the total substrate, and was not entirley inorganic. Up to 60 % of it was present as a compound with the chromatographic properties of 3:5-di-iodotyrosine. These results indicate that whereas deiodination occurs readily from the 3′:5′ positions of thyroxine, the same is not true of the 3:5 positions.

(ii) Fraction 2. The main radioactive component of Fraction 2 was in all cases unmetabolized thyroxine. In samples incubated with [3:5-$^{131}I_2$]-thyroxine this fraction also contained small quantities of some unidentified metabolites. No tri-iodothyronine was present.

Table 1. *Deiodination of* [3′:5′-$^{131}I_2$]*thyroxine* (1 μg.) *by a potassium chloride extract of rat liver at* pH 6·0 *and* 37°

Progress of the reaction is shown.

Duration of incubation (min.)	Percentage of substrate radioactivity appearing in Fraction 1
0	7·9
2	25·0
5	49·3
10	67·2
15	70·7

Table 2. *Metabolism of thyroid hormones by a potassium chloride extract of rat liver at* pH 6·0 *and at* 37°

Initial reaction velocities are given at different concentrations of substrate.

Concn. of substrate (μg./ml.)	Reaction rate (μg./ml. of extract/min.)	
	Thyroxine	Tri-iodothyronine
0·1	0·0097	0·0014
0·2	0·0192	—
0·5	0·0375	—
1·0	0·0592	—

(iii) Fraction 3. In zero-time controls this fraction contained a small amount of thyroxine which had not been extracted by the butanol into Fraction 2. In samples incubated with [3′:5′-$^{131}I_2$]-thyroxine the radioactivity increased slightly above control amounts; when extracted and studied by chromatography this radioactivity was found to be in the form of inorganic iodide. In samples incubated with [3:5-$^{131}I_2$]thyroxine, 53 % of the total substrate radioactivity was found in Fraction 3. The radioactivity was readily extractable into acid butanol and was isolated chromatographically as a compound indistinguishable from 3:5-di-iodotyrosine. It was run in all four solvent systems with markers of 3-monoiodotyrosine, 3:5-di-iodotyrosine, 3:5-di-iodo-4-hydroxyphenylpyruvic acid, 4-hydroxy-3:5-di-iodophenyl-lactic acid, 4-hydroxy-3:5-di-iodophenylacetic acid and 4-hydroxy-3:5-di-iodobenzaldehyde. It corresponded exactly with the di-iodotyrosine marker in all four solvents and did not correspond consistently with any of the other markers. The radioactive compound crystallized with authentic 3:5-di-iodotyrosine from 50 % acetic acid without change of specific radioactivity.

The discovery of radioactive di-iodotyrosine in Fraction 3 does not necessarily indicate that this substance is produced from [3:5-$^{131}I_2$]thyroxine by the liver enzymes, since the preparation of Fraction 3 involves the use of boiling 0·2 N-NaOH soln. Moreover, any free di-iodotyrosine formed by the liver enzymes would pass into Fractions 1 and 2, not into Fraction 3. The result is interpreted to mean that thyroxine is metabolized by the liver preparation to a compound (X) which adheres very firmly to the liver protein and which contains the iodine from the thyroxine α ring. This compound is apparently labile in hot alkali, yielding di-iodotyrosine.

Attachment of compound X to the liver protein. The appearance of 53 % of the substrate radio-

Table 3. *Metabolism of labelled thyroxine* (1 μg.) *by potassium chloride extracts of rat liver during incubation for* 1·5 *hr. at* 37°

Distribution of the substrate radioactivity among the fractions of the extract is shown. Each figure is the mean result of 10 experiments, quoted to the nearest whole number. The compounds found in the fractions by chromatographic analysis are indicated.

	Percentage of total substrate radioactivity		
	Fraction 1	Fraction 2	Fraction 3
Zero-time control ([3:5-$^{131}I_2$]thyroxine or [3′:5′-$^{131}I_2$]thyroxine)	6 (iodide)	90 (thyroxine)	4 (thyroxine)
Incubated sample, with [3′:5′-$^{131}I_2$]-thyroxine	73* (iodide)	18 (thyroxine)	9 (iodide + trace of thyroxine)
Incubated sample, with [3:5-$^{131}I_2$]-thyroxine	21 (iodide + di-iodotyrosine)	26 (thyroxine + trace of di-iodotyrosine + unknowns)	53 (di-iodotyrosine + trace of thyroxine)

* Finding confirmed by the use of an AgCl column.

activity in Fraction 3 indicates that the major thyroxine metabolite is more firmly bound to protein than is thyroxine itself, since the binding resists butanol washing. Attempts were made to break this binding by washing in many different organic solvents. In these experiments, Fractions 1 and 2 were prepared as already described, but NaOH-digestion was omitted. Instead, the butanol-washed liver protein was washed again in ether, benzene, ethanol, $CHCl_3$, acetone or carbon tetrachloride. No radioactivity could be eluted from the protein by any of these solvents. Boiling, exposure to strong mineral acids and treatment with detergents were tried, but were equally ineffective. Treatment for 6 hr. with cold 0·1 M-trisodium phosphate produced the same result as NaOH-digestion, di-iodotyrosine being released almost quantitatively from the radioactive protein. Digestion of the liver protein by trypsin also resulted in the disappearance of the butanol-insoluble radioactivity and the appearance of radioactive di-iodotyrosine.

Presence of deaminated metabolites. The liver extracts from some animals differed from the majority in that 4-hydroxy-3:5-di-iodophenyllactic acid was found in Fraction 3 as well as di-iodotyrosine. This compound was identified by the same chromatographic tests as those described for di-iodotyrosine, and its presence indicated that compound X may exist in a deaminated form. The relative amounts of the amino and desamino forms could not be altered either by varying the substrate concentration within the range 0·5–10·0 μg./10 ml. of extract or by varying the pH within the range 5·0–7·0. Nor did the transamination inhibitors *p*-phenylenediamine and *p*-chloromercuribenzoate in the concentration used (see below) alter the relative amounts of these metabolites.

Metabolism of tri-iodothyronine. The liver extracts metabolized tri-iodothyronine less rapidly than thyroxine (Table 2), but the changes which occurred were qualitatively similar. When the extract was incubated with [3:5-$^{131}I_2$]tri-iodothyronine there was a progressive increase in the radioactivity of Fractions 1 and 3 at the expense of Fraction 2. The radioactive component of Fraction 3 was chromatographically indistinguishable from the corresponding thyroxine-breakdown product and from 3:5-di-iodotyrosine.

Inhibition of thyroxine metabolism. It was found that *p*-chloromercuribenzoate at a concentration of 2 mM caused 17% inhibition of deiodination. Thioglycollic acid at a concentration of 10 mM caused total inhibition. *o*-Phenylenediamine and *o*-iodosobenzoate were only effective as inhibitors at concentrations greater than 10 mM and sodium azide was ineffective at 1 mM.

The remaining inhibition data are given in detail in Table 4. Experiments on [3′:5′-$^{131}I_2$]thyroxine give information about deiodination from the β ring. Experiments on [3′:5′-$^{131}I_2$]thyroxine give information about the amount of compound X which becomes bound to the tissue protein. Phenylhydrazine was a very active inhibitor of the reaction and gave the same percentage inhibition when studied by the two methods. Iodoacetate and cystine preferentially inhibited the protein-binding of compound X. In these experiments

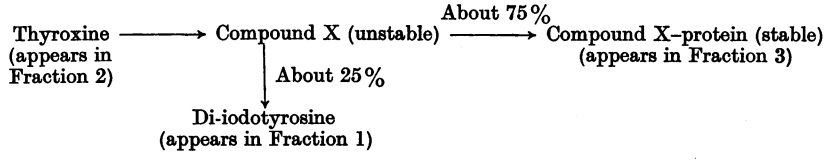
Table 4. *Inhibition of the metabolism of thyroxine in potassium chloride extracts of rat liver*

Inhibitor	Percentage of total substrate radioactivity			Inhibition (%) (measured by loss of yield in Fraction 3)
	Fraction 1	Fraction 2	Fraction 3	
	Experiments with [3:5-$^{131}I_2$]thyroxine			
No addition	21	26	53	—
Iodoacetate (10 mM)	11·0	76·6	12·4	82·9
Phenylhydrazine (0·5 mM)	6·3	86·5	7·2	94·5
Cystine (0·5 mM)	26·7	25·6	47·7	10·8
(2 mM)	24·1	37·2	38·7	29·2
(4 mM)	20·3	53·7	26·0	55·1
				Inhibition (%) (measured by loss of yield in Fraction 1)
	Experiments with [3′:5′-$^{131}I_2$]thyroxine			
No addition	73	18	9	—
Iodoacetate (10 mM)	39·7	52·7	7·6	49·7
Phenylhydrazine (0·5 mM)	9·6	81·0	9·4	94·6
Cystine (4 mM)	66·0	26·8	7·2	12·5

cystine could be replaced by cysteine. Two moles of cysteine produced the same degree of inhibition as a mole of cystine; the substances are presumably interconvertible in the liver extract, and the sulphydryl compound may well be the active inhibitor.

DISCUSSION

Although thyronine was identified as a product of thyroxine breakdown *in vitro* only recently (Lissitzky, Bénévent, Roques & Roche, 1958), it has long been realized that total deiodination was the eventual fate of thyroxine *in vivo* (Myant & Pochin, 1950). Studies on intermediate thyroid-hormone metabolism have therefore centred upon the identification of a metabolite with a modified side chain (Roche, Michel & Tata, 1954; Lachaze & Thibault, 1951; Roche, Michel, Jouan & Wolf, 1956), or upon isolation of partially deiodinated products, especially 3:5:3′-tri-iodothyronine (Albright, Larson & Tust, 1954; Sprott & Maclagan, 1955). The work reported in this paper indicates that tri-iodothyronine is not produced from thyroxine by liver extracts. It establishes the formation of a partially deiodinated metabolite (compound X) which can be recovered from the extract with a yield of over 50% and which contains two iodine atoms corresponding to the 3:5 iodine atoms of the thyroxine substrate. The fact that this metabolite becomes bound to the protein of the extract is presumably responsible for its accumulation in high yield, for in the bound condition it is not subject to further breakdown by the liver enzymes. The great stability of this protein-binding over a wide range of conditions and its ready destruction by cold weak alkali suggests that compound X may be held to the protein by chemical linkage. The disintegration of compound X on tryptic hydrolysis of its binding protein suggests that it is very unstable when in free solution. This fact may account for the appearance of some di-iodotyrosine in Fraction 1.



It is to be regretted that the lability of the free compound X has so far rendered its isolation and identification impossible. The appearance of di-iodotyrosine as its breakdown product indicates that the effect of alkali is to hydrolyse off the deiodinated β ring or some part of it. This lability in alkali is perhaps the best clue to the structure of compound X. 3:5-Di-iodothyronine, for example, which is a theoretically possible metabolite of thyroxine, would not have been alkali-labile. The best parallel to the behaviour of compound X is to be found in the work of Lissitzky & Bouchilloux (1957) on hydroxylated thyronine derivatives. 3′-Hydroxythyronine is auto-oxidizable, and the corresponding *o*-quinone undergoes spontaneous hydrolysis of the diphenylether link, yielding tyrosine. If, as suggested by Wilkinson (1956), deiodination is a hydrolytic reaction rather than a reductive one, the formation of hydroxylated derivatives of 3:5-di-iodothyronine is the likely result of deiodination of the thyroxine β ring.

3′-Hydroxy-3:5-di-iodothyronine has been synthesized by Doskotch & Lardy (1958). A sample of this material was incubated with rat-liver extract to compare its behaviour with that of compound X. Its fate was followed by chemical estimations of ^{127}I. At the concentrations used (0·44 and 4·4 μM) this compound adhered to the protein of the extract in a form which resisted butanol extraction (i.e. its iodine appeared in Fraction 3 in a yield of 35–50%) and the main iodine-containing component of Fraction 3 had the chromatographic properties of 3:5-di-iodotyrosine.

If compound X is indeed structurally related to 3′-hydroxy-3:5-di-iodothyronine, its ready combination with protein may be explained in terms of the known chemical properties of *o*-quinones. Mason (1955) reports that these substances combine with proteins by a reaction involving the free amino or sulphydryl groups. Addition of a high concentration of sulphydryl compounds would inhibit this type of protein-binding, producing the effect recorded in Table 4.

The only work known to the author in which a quinone was claimed to be a product of thyroxine breakdown is that of Allegretti (1954). This was 2:6-di-iodo-*p*-quinone, which was formed, according to that author, without deiodination from either ring. The findings have been questioned by Pitt-Rivers & Tata (1959).

The difference between the findings of the present paper and those of Lissitzky (1958) *et al.* may be due to the use by those workers of surviving tissue slices in place of an extract or homogenate. Whole cells presumably contain a complete enzyme system for the destruction of thyroxine, whereas in less complete tissue preparations the later stages of the metabolic pathway are not active.

SUMMARY

1. [3:5-$^{131}I_2$]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′-$^{131}I_2$]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.

The author wishes to thank Dr H. A. Lardy for his gift of 3′-hydroxy-3:5-di-iodothyronine and Dr E. Shalom for carrying out the iodine estimations. This work was undertaken during tenure of a grant from the Rockefeller Funds of the Medical School and was completed on behalf of the Medical Research Council.

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Biochem. J. (1961) **78**, 657

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

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(*Received* 26 *October* 1960)

The reaction between thyroxine and *m*-nitrobenzenediazonium chloride has been utilized for the localization of ^{131}I 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 technique 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.

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