Conversion of Thyroxine into Tri-iodothyronine by Rat Liver Homogenate

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By using a highly specific radioimmunoassay the formation of tri-iodothyronine by the deiodination of thyroxine was studied in rat liver homogenate. Several observations suggest that the reaction observed is enzymic in nature. Pre-heating the homogenate for 30 min at 56°C completely abolished conversion of thyroxine into tri-iodothyronine; the component of rat liver homogenate responsible could be saturated with substrate; iodotyrosines displayed competitive activity. Between 0° and 37°C, the tri-iodothyronine-production rate was positively correlated with incubation temperature. The addition of NAD⁺ enhanced conversion into tri-iodothyronine, which suggests that an oxidative mechanism is involved. 5-Propyl-2-thiouracil and 6-propyl-2-thiouracil, both known to prevent deiodination *in vivo*, greatly decreased the deiodination activity of rat liver homogenate.

Since the discovery of 3,3',5-tri-iodothyronine in human plasma (Gross & Pitt-Rivers, 1952) an abundance of evidence has been presented which clearly shows that in the circulation in humans (e.g. Pitt-Rivers *et al.*, 1955; Braverman *et al.*, 1970; Pittman *et al.*, 1971; Sterling *et al.*, 1970) and rats (e.g. Schwartz *et al.*, 1971) it is mainly derived by the peripheral deiodination of thyroxine. These observations have repeatedly been confirmed by studies *in vitro* on perfused rat heart (Rabinowitz & Hercker, 1971), cultured human liver and kidney cells (Sterling *et al.*, 1973), cultured human fibroblasts (Refetoff *et al.*, 1972) and human leucocytes (Klebanoff & Green, 1973; Woeber & Ingbar, 1973). No clear evidence that an enzymic reaction is involved in the mechanism of this conversion is as yet at hand. In rats a role for peroxidase in thyroxine deiodination has been proposed (Galton & Ingbar, 1963; Dawber *et al.*, 1971), as well as the involvement of reduced flavin nucleotide as a cofactor in the deiodination of di-iodotyrosine (Rosenberg & Ahn, 1969). These two supposed enzymic processes, however, seem to be functionally separated (Haibach, 1971). Thyroxine deiodination, in the absence of any apparent tri-iodothyronine generation, has been observed in rat skeletal-muscle homogenate stimulated by flavins (Tata, 1960), and in several other homogenized rat tissues (Galton & Ingbar, 1963). In these and several other studies only the formation of iodide and chromatographically immobile 'origin material' was observed. For instance, this was the case when deiodination was studied in chemically defined milieus, e.g. in the presence of peroxidase and H₂O₂

or H₂O₂ alone (Galton & Ingbar, 1963; Dawber *et al.*, 1971), by the combination of H₂O₂ and chelated metal ions (Reinwein *et al.*, 1968) or by illumination of a solution containing FMN (Reinwein & Rall, 1966). Almost all studies referred to have been performed with radioactive iodine-labelled thyroxine and by chromatographic analysis of the reaction products.

A different approach has been undertaken by Hesch *et al.* (1974), who applied a specific radioimmunoassay to detect tri-iodothyronine generation, when rat liver homogenate was incubated with thyroxine. We have extended these studies and the results are the subject of the present paper.

Experimental

Materials

L-Thyroxine (free acid), 3,3',5-tri-iodo-L-thyronine, 3,5-di-iodo-L-thyronine, 3,3',5-tri-iodothyroacetic acid, 3,3',5-tri-iodothyropropionic acid, 3,5-dinitro-L-tyrosine, NAD⁺, NADH, NADP⁺ and NADPH were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A., L-Tyrosine, 3-iodo-L-tyrosine and 3,5-di-iodo-L-tyrosine were from Calbiochem A.G., Lucerne, Switzerland. Tri-[¹²⁵I]iodo-L-thyronine (specific radioactivity approx. 500 µCi/µg) was from Abbott Laboratories, North Chicago, Ill., U.S.A.; goat anti-(rabbit γ-globulin) antiserum was from Antibodies Inc., Davis, Calif., U.S.A. 5-Propyl-2-thiouracil was synthesized by the method described by Anderson *et al.* (1945) by Dr. P. J. H. Eggels, University Hospital 'Dijkzigt', Rotterdam, The Netherlands.

Preparation of rat liver homogenate

Male Wistar rats weighing approx. 200 g were used. After perfusion *in situ* through the portal vein, rat liver was minced, washed and subsequently homogenized in 3 vol. of 0.25 M-sucrose in 0.05 M-Tris-HCl, pH 7.4 (sucrose-Tris buffer) at 4°C. The homogenate was centrifuged for 5 min at 2000g at 2°C. The resulting pellet was discarded.

The protein content of the homogenate was determined after solubilization in 0.1 M-NaOH by using the biuret method.

Deiodination studies

Thyroxine and other substances were dissolved and diluted in sucrose-Tris buffer immediately before use and added in 10 μ l quantities for incubation with 1 ml samples of homogenate. Control experiments were conducted in which thyroxine was incubated with sucrose-Tris buffer or with homogenate preheated for 30 min at 56°C. Since these controls yielded identical results, the former was subsequently omitted. At increasing time-intervals 200 μ l portions of the reaction mixtures were taken and added to 400 μ l of ice-cold 95% (v/v) ethanol. The precipitates were spun down (1500g, 15 min) and the tri-iodothyronine concentrations were measured directly in 100 μ l portions of the supernatants in duplicate. The recovery of added tri-[¹²⁵I]iodothyronine amounted to 85.8 $\pm$ 4.5% (mean $\pm$ s.d., $n = 22$).

Radioimmunoassay for tri-iodothyronine

The radioimmunoassay utilizes rabbit antiserum raised against a tri-iodothyronine-bovine serum albumin conjugate (Docter *et al.*, 1972). In Table 1 the

composition of the assay mixtures is shown. The mixtures were incubated for 16 h at 4°C. In the assay 100 μ l of previously titred goat anti-(rabbit γ -globulin) antiserum was subsequently added to each tube and the mixtures were kept for an additional 16 h at 4°C. The tubes were centrifuged and the supernatants were subsequently aspirated. The pellets were counted for radioactivity in a Packard gamma spectrometer. Non-specific binding of tri-[¹²⁵I]iodothyronine to the precipitate was evaluated by preparing controls containing normal rabbit serum without antiserum. The amount of tri-[¹²⁵I]iodothyronine specifically bound to the antibody was expressed as a percentage of the radioactivity precipitated in the tubes without added unlabelled tri-iodothyronine, which was usually approx. 45% of the total amount of radioactivity. To calculate the tri-iodothyronine content of unknown samples the standard curve was transformed into a logit-log plot (Feldman & Rodbard, 1972).

Results and Discussion

Radioimmunoassay

Fig. 1 shows a typical radioimmunoassay standard curve. The specificity of the antiserum used was assessed by estimating the relative affinity of various thyroid-hormone analogues for the antibody (Table 2). It is concluded that the antiserum is highly specific for tri-iodothyronine, only 3,3',5-tri-iodothyroacetic acid displaying a significant cross-reactivity. When dilutions of ethanol extracts of the reaction mixtures were assayed, the curve for antibody-bound tri-[¹²⁵I]iodothyronine versus log(μ l of extract) (not detailed here) paralleled the standard curve. These tests establish the reliability of this assay for the

Table 1. Composition of radioimmunoassay mixtures

All reagents were dissolved in 0.05 M-potassium phosphate buffer, pH 7.4, containing 0.4% bovine serum albumin (dilution buffer). Antiserum, final dilution 1:20 000 was dissolved in 2.5% normal rabbit serum in dilution buffer. Extracts of reaction mixtures were made as described in the Experimental section.

	Volume for standard curve (μ l)	Volume for samples (μ l)
Tri-[¹²⁵ I]iodothyronine, 20 pg	100	100
Tri-iodothyronine standard	100	
64% (v/v) Ethanol	100	
Extract of reaction mixture		100
Antiserum	100	100
Dilution buffer	600	700

Fig. 1. Standard curve for the radioimmunoassay of tri-iodothyronine

Conditions are detailed in the Experimental section and in Table 1. Ordinate values show the amount of tri-[¹²⁵I]iodothyronine bound (B) as a percentage of radioactivity precipitated without added unlabelled tri-iodothyronine (B₀).

Table 2. *Cross-reactivity of thyroid-hormone analogues with anti-tri-iodothyronine antiserum*

Cross-reactivity is defined as the ratio of the concentration of tri-iodothyronine to the concentration of analogue both resulting in a 50% decrease of antibody-bound tri-[125 I]iodothyronine.

Compound	Cross-reactivity
3,3',5-Tri-iodo-L-thyronine	1.0
3,3',5,5'-Tetraiodo-L-thyronine	<0.001
3,5-Di-iodo-L-thyronine	0.003
3,3',5-Tri-iodothyroacetic acid	0.77
3,3',5-Tri-iodothyropropionic acid	<0.001
3-Iodo-L-tyrosine	<0.001
3,5-Di-iodo-L-tyrosine	<0.001
3,5-Dinitro-L-tyrosine	<0.001
L-Tyrosine	<0.001

Fig. 2. *Dependence of tri-iodothyronine accumulation on incubation time and temperature*

Portions (1ml) of rat liver homogenate (43mg of protein/ml) were made to react in duplicate with $1\mu\text{g}$ of thyroxine at 0°C (▲), 22°C (■) and 37°C (●). At the times indicated 200 μl samples were added to ice-cold ethanol (400 μl). The resulting mixtures were processed as detailed in the Experimental section.

measurement of the conversion of thyroxine into tri-iodothyronine.

Deiodination studies

Fig. 2 shows the accumulation of tri-iodothyronine, when rat liver homogenate is incubated with $1\mu\text{g}$ of thyroxine/ml ($1.3\mu\text{M}$) at different temperatures. During the first 15 min of the reaction the rate of tri-iodothyronine production was 2.5nm/min at 37°C , 0.47nm/min at 22°C and only 0.16nm/min at 0°C . No tri-iodothyronine generation was observed when the homogenate was pre-heated for 30min at 56°C , and when no thyroxine was added to the homogenate the amount of tri-iodothyronine formed was negligible.

Fig. 3. *Dependence of tri-iodothyronine-production rate on thyroxine concentration*

Portions (1ml) of rat liver homogenate (51mg of protein/ml) were made to react with amounts of thyroxine as indicated in duplicate. After incubation for 15min at 37°C the reaction was stopped and the tri-iodothyronine generated was extracted by addition of ethanol as described in the Experimental section.

The relationship between the rate of tri-iodothyronine formation and the thyroxine concentration is shown in Fig. 3. Since an abundance of thyroxine- and tri-iodothyronine-binding sites exists in rat liver homogenate (e.g. Tata, 1962; Visser *et al.*, 1975), the free thyroxine concentration in the reaction system is not known and the kinetics of the reaction studied cannot be characterized simply by the standard procedure for the estimation of the Michaelis constant. At concentrations of thyroxine above $5\mu\text{g/ml}$ ($6.4\mu\text{M}$) no further increase in reaction rate is observed, however, and this suggests that either the catalysing system is saturated in these circumstances and/or the possible cofactors involved are only present in limited concentrations.

Experiments were conducted in which the effect of the addition of millimolar concentrations of nicotinamide-adenine dinucleotides was studied (Table 3). The presence of NAD^+ resulted in a significant enhancement of the deiodination, whereas the addition of NADP^+ was without effect. An insignificant decrease in tri-iodothyronine production was observed when the incubations were performed in the presence of 1mM-NADH or 1mM-NADPH. These results could indicate that an oxidative reaction is involved.

In view of the role of flavins in deiodination reactions (Rosenberg & Ahn, 1969; Tata, 1960; Reinwein & Rall, 1966) 1mM-FAD was added to the reaction mixtures. This resulted in an approximately twofold increase in tri-iodothyronine generation. However, a considerable amount was also produced in the control tubes with pre-heated homogenate in the presence of FAD. When this experiment and the determination of

Table 3. *Effect of possible cofactors on the conversion of thyroxine into tri-iodothyronine*

Incubations were performed at 37°C as outlined in the Experimental section, by using 1 µg of thyroxine/ml. Mean values $\pm$ s.e.m. (number of experiments) are given for the amount of tri-iodothyronine formed in paired experiments conducted on separate occasions. Statistical analysis was with Student's paired *t* test. NS, Not significant ($P > 0.05$).

Incubation conditions	Tri-iodothyronine production (ng)	
	15 min	30 min
Control	16.2 $\pm$ 2.3 (4)	26.5 $\pm$ 5.0 (4)
+1 mM-NAD ⁺	21.8 $\pm$ 2.2 (4) $P < 0.05$	36.7 $\pm$ 6.2 (4) $P < 0.025$
Control	18.7 $\pm$ 0.9 (5)	29.3 $\pm$ 1.9 (5)
+1 mM-NADH	16.5 $\pm$ 2.4 (5) NS	26.5 $\pm$ 3.6 (5) NS
Control	17.9 $\pm$ 1.3 (5)	29.2 $\pm$ 0.9 (5)
+1 mM-NADP ⁺	18.7 $\pm$ 1.9 (5) NS	31.6 $\pm$ 2.0 (5) NS
Control	14.7 $\pm$ 2.3 (4)	22.0 $\pm$ 4.0 (4)
+1 mM-NADPH	9.2 $\pm$ 2.3 (4) NS	12.3 $\pm$ 3.0 (4) NS

Table 4. *Effect of addition of thyroid-hormone analogues on the conversion of thyroxine into tri-iodothyronine*

For details see legend of Table 3; incubation time 15 min.

Incubation conditions	Tri-iodothyronine formed (ng)
Control	21.0 $\pm$ 1.3 (3)
+1 µg of 3-iodotyrosine	18.9 $\pm$ 1.3 (3) NS
Control	20.2 $\pm$ 1.2 (4)
+10 µg of 3-iodotyrosine	9.4 $\pm$ 2.6 (4) $P < 0.05$
Control	18.3 $\pm$ 1.5 (5)
+1 µg of 3,5-di-iodotyrosine	21.3 $\pm$ 0.9 (5) NS
Control	18.7 $\pm$ 2.0 (4)
+10 µg of 3,5-di-iodotyrosine	10.2 $\pm$ 2.8 (4) $P < 0.05$
Control	18.9 $\pm$ 1.3 (4)
+1 µg of 3,5-dinitrotyrosine	18.1 $\pm$ 1.2 (4) NS
+10 µg of 3,5-dinitrotyrosine	17.7 $\pm$ 1.5 (4) NS
Control	19.2 $\pm$ 1.6 (5)
+1 µg of tyrosine	19.3 $\pm$ 0.9 (5) NS
+10 µg of tyrosine	19.3 $\pm$ 1.0 (5) NS

tri-iodothyronine by radioimmunoassay were repeated in the dark, this enhancement with FAD totally disappeared. The results of experiments without added FAD, performed in the dark, were as under normal illumination. These findings are in accordance with the work of Reinwein & Rall (1966), who detected thyroxine deiodination by illuminating a solution containing thyroxine, FMN and tracer amounts of certain cations.

The reaction was investigated in the presence of different quantities of thyroid-hormone analogues known to be unreactive in the radioimmunoassay (Table 4). Of the compounds used only 3,5-di-iodotyrosine and 3-iodotyrosine are potent inhibitors, affecting the reaction to a comparable extent. 3,5-Dinitrotyrosine was tested since it is reported to be a specific di-iodotyrosine dehalogenase inhibitor (Haibach, 1971), and this enzyme is a constituent of rat liver homogenate (Rosenberg & Ahn, 1969). The

Table 5. *Effect of addition of propylthiouracil on the conversion of thyroxine into tri-iodothyronine*

For details see legend to Table 3; incubation time 15 min.

Incubation conditions	Tri-iodothyronine formed (ng)
Control	16.6 $\pm$ 2.1 (4)
+10 µM-5-Propyl-2-thiouracil	5.5 $\pm$ 1.1 (4) $P < 0.005$
+100 µM-5-Propyl-2-thiouracil	4.3 $\pm$ 0.9 (4) $P < 0.005$
Control	17.0 $\pm$ 2.6 (4)
+10 µM-6-Propyl-2-thiouracil	5.1 $\pm$ 2.5 (4) $P < 0.005$
+100 µM-6-Propyl-2-thiouracil	3.2 $\pm$ 1.2 (4) $P < 0.001$

presence of this compound, however, did not affect the reaction, from which it is concluded that the reaction proceeds via a deiodinase different from di-iodotyrosine dehalogenase. Haibach (1971) also reported on the functional separation between di-iodotyrosine deiodinase and thyroxine deiodinase in rat thyroid tissue. The addition of tyrosine to the incubation mixtures was without effect.

6-Propyl-2-thiouracil and 5-propyl-2-thiouracil, when administered to rats (Oppenheimer *et al.*, 1972; Bernal & Escobar del Rey, 1974) or humans (Hershman, 1964; Saber *et al.*, 1975; Geffner *et al.*, 1975), prevent peripheral deiodination of thyroxine and tri-iodothyronine besides displaying goitrogenic activity. Another goitrogen, methimazole, leaves the conversion of thyroxine into tri-iodothyronine unaffected. These goitrogens exert their anti-thyroid activity mainly by inhibiting oxidative iodination of tyrosine by thyroidal peroxidase (Astwood, 1949). 5-Propyl-2-thiouracil and 6-propyl-2-thiouracil were tested for their potential inhibitory effect on the deiodination. Table 5 shows that propylthiouracil strongly impairs the conversion of thyroxine into tri-iodothyronine. Addition of methimazole in equimolar concentrations was without effect (T. J. Visser & I. van der Does-Tobé, unpublished work). From the

experiments involving NAD^+ , 5-propyl-2-thiouracil and 6-propyl-2-thiouracil we suggest that thyroxine is deiodinated by rat liver homogenate, resulting in a production of tri-iodothyronine, by an oxidative mechanism.

Preliminary experiments showed that the catalysing activity is predominantly associated with the microsomal fraction (T. J. Visser & I. van der Does-Tobé, unpublished work). Whether the deiodination occurs at random, resulting in a concomitant production also of 3,3',5'-tri-iodothyronine, and whether 3,3',5'-tri-iodothyronine itself is also deiodinated by the same enzyme or exhibits some kind of product inhibition remains to be established.

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