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Heterogeneity of Rat Thyroglobulin Labelled with ^{131}I *in vivo*

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Halmi & Pitt-Rivers (1962) showed that, at short times after the injection of ^{131}I in rats, the specific-activity curve of the second iodide pool, derived by intrathyroidal deiodination of iodo compounds, rose more steeply than that of any iodo amino acid in rat thyroglobulin (Pitt-Rivers, 1962); they postulated a heterogeneity of thyroglobulin with respect to labelled iodotyrosines, the fraction with a more rapid turnover being the probable precursor of the second iodide pool at short times after the injection of ^{131}I . This did not preclude the possibility that all the iodotyrosine residues, whether labile or not, could be the precursors of the second iodide pool.

Pitt-Rivers & Cavalieri (1963) made quantitative studies of ^{131}I -labelled free iodotyrosines in diffusates of rat thyroids at different times after the

injection of ^{131}I . They found that, up to 4 hr. after the injection, the specific activity of monoiodotyrosine rose more steeply than that of second pool iodide; that of di-iodotyrosine was lower. However, the mean of the sum of the specific activities of the iodotyrosines exceeded that of second pool iodide; it was therefore concluded that both were likely to be a source of this iodide.

In preliminary experiments on the hydrolysis of labelled rat thyroids with crude calf-thyroid protease, Pitt-Rivers & Cavalieri (1963) found that, in glands that had been labelled for 1 or 4 hr., the rate of release of mono[^{131}I]iodotyrosine was rapid for 3–4 hr. and then slowed down to a constant rate up to 10 hr. In the glands labelled for 24 hr., the rate of release of mono[^{131}I]iodotyrosine was constant throughout the experiment. This lent

Table 1. *Thyroidal ^{131}I uptakes, thyroid weights and concentrations of thyroid homogenates in the autolysates*

Experimental details are given in the text.

Group	Time of labelling (hr.)	Thyroidal ^{131}I uptake (% of dose)	Thyroid weight (mg./100 g. body wt.)	Concn. of thyroid homogenates (mg./ml. of autolysate)
I	0.5	2.8	8.0	86.0
II	1	5.6	8.1	83.5
III	2	6.6	8.9	86.7
IV	4	11.0	9.2	90.5
V	18	14.0	8.5	88.8
VI	48	12.4	9.3	87.5
VII	0.5	2.9	8.7	85.4
VIII	0.5	2.5	8.2	84.9

support to the idea that thyroglobulin labelled during the shorter periods contained moniodotyrosine residues that were more labile to thyroid protease than the average iodotyrosine residue of the gland. The fact that the glands labelled for 4 hr. contained more labile mono[^{131}I]iodotyrosine than those labelled for 1 hr. suggested that there might be some other source of free iodotyrosines; labile di-[^{131}I]iodotyrosine was not demonstrable in these experiments. In the work described below, the rate of release of [^{131}I]iodotyrosines during the autolysis of rat-thyroid tissue labelled with ^{131}I for different times has been investigated.

METHODS

Male hooded rats weighing 200–250 g. were given diet 41B (Bruce, 1958) containing about 0.4 μg . of iodine/g. and tap water *ad lib*. (Iodine analyses, by Dr B. I. Sacks at University College Hospital Medical School, on the two batches of the diet gave 0.37 and 0.42 μg . of I/g.) Groups of four animals were injected intraperitoneally with carrier-free Na^{131}I (100–200 μC in aq. 0.9% NaCl) and killed by exsanguination under chloroform anaesthesia after 0.5, 1, 2, 4, 18 and 48 hr. Thyroids were quickly removed, weighed, pooled, chilled in an ice bath and counted in a ring counter. The glands were homogenized in an all-glass homogenizer in 0.3 ml. of aq. 0.9% NaCl containing 100 μg . of thiouracil/ml. and kept overnight at 2°; 0.5 ml. of 0.2M-acetate buffer, pH 3.6, was then added. In one experiment, the rate of release of [^{131}I]iodotyrosines from native and denatured [^{131}I]thyroglobulin labelled for 0.5 hr. at pH 5.6 was examined. For native thyroglobulin, four thyroid glands were homogenized in 0.3 ml. of aq. 0.9% NaCl and immediately treated with 0.5 ml. of 0.2M-acetate buffer, pH 5.6. For denatured thyroglobulin, four thyroid glands were homogenized in 0.3 ml. of aq. 0.9% NaCl and then treated with 0.05 ml. of 0.2N-acetic acid; the mixture was kept at 2° overnight and 0.45 ml. of 0.2M-sodium acetate was then added, bringing the pH to 5.6. The homogenates were incubated at 35° in stoppered test tubes with gentle shaking.

Samples were applied to Whatman no. 1 paper strips for chromatographic analysis at zero time and at intervals of about 2 hr. for 8 or 10 hr. A final sample was taken at 24 or 30 hr. The pH of the mixture was adjusted to about 8.6 with 0.5N- NH_3 ; 0.4 ml. of 0.2M-tris buffer, pH 8.6, was

Fig. 1. Rates of release of mono[^{131}I]iodotyrosine, during autolysis at pH 3.6, in glands labelled for: I, 0.5 hr.; II, 1 hr.; III, 2 hr.; IV, 4 hr.; V, 18 hr.; VI, 48 hr.

added and the amounts of [^{131}I]iodotyrosines were determined chromatographically after hydrolysis for 16 hr. with pancreatin (U.S.P.; three-times crystallized). The chromatograms were developed in butan-1-ol saturated with 2N-acetic acid and in butan-1-ol-dioxan-2N- NH_3 (4:1:5, by vol.). The radioactivity on the strips was measured in a strip counter.

RESULTS

Thyroid weights and ^{131}I uptakes. Thyroidal ^{131}I uptakes, thyroid weights and concentrations of thyroid homogenates are shown in Table 1. The ^{131}I uptakes are somewhat higher than found by Halmi & Pitt-Rivers (1962); this is probably due to the rather low iodine content of the diet.

Autolysis of the glands. The results of autolysis at pH 3.6 of thyroid glands labelled with ^{131}I for between 0.5 and 48 hr. are shown in Figs. 1 and 2.

The rates of appearance of free [^{131}I]iodotyrosines are expressed as percentages of the total (pancreatin value). The rates of release of both iodotyrosines are greater the shorter the time of labelling. As found by Pitt-Rivers & Cavalieri (1963) the glands labelled for 18 hr. (or more) contained no labile moniodotyrosine residues. In the present experiments, however, thyroglobulin-

bound di-iodotyrosine residues were as labile to thyroid protease in the same tissue as was moniodotyrosine.

The amounts of the labelled iodotyrosines present in the autolysates after 24 or 30 hr. varied between 65 and 90 % of the pancreatin values, except in group VI where it was only 40 %. Almost no further autolysis was observed after 48 hr.

Thyroids labelled for 0.5 hr. were also autolysed at pH 5.6, with and without preliminary denaturation of thyroglobulin. The results are compared in Fig. 3 with curve I of Fig. 1. Curve VII shows that, during autolysis at pH 5.6 without denaturation of thyroglobulin, the rate of release of mono[^{131}I]iodotyrosine is only about one-tenth of that during autolysis at pH 3.6. When the thyroglobulin is denatured (curve VIII) the rate of release of mono[^{131}I]iodotyrosine is about one-third of that occurring at pH 3.6.

DISCUSSION

The proteolytic activity of thyroid extracts is sensitive to pH and is maximal below neutrality. De Robertis (1941) first showed by histochemical methods that proteolysis of gelatin by colloid from rat-thyroid follicles was stimulated when the pH was lowered to 2-3. Partially purified pig-thyroid protease was found by McQuillan, Mathews & Trikojus (1961) to be five times as active at pH 3.5 as at pH 5.2 when bovine haemoglobin was the substrate; however, the rate of release of [^{131}I]labelled compounds from rat thyroglobulin was more rapid at the higher pH. Haddad & Rall (1960) fractionated sheep-thyroid protease on diethylaminoethylcellulose and carboxymethylcellulose columns and separated two proteases with maximal activities at pH 3.8 and 5.7. Pitt-Rivers & Cavalieri (1963) obtained a fourfold diminution of activity of a crude calf-thyroid protease towards [^{131}I]labelled rat thyroglobulin when the pH was raised from 3.6 to 5.6.

Similar findings have been obtained in the present work; the activity of rat-thyroid protease towards rat thyroglobulin is about ten times as great at pH 3.6 as at pH 5.6, and preliminary acid denaturation of the substrate enhances the rate of proteolysis at the higher pH. Alpers, Petermann & Rall (1956) demonstrated by electrophoretic methods that denaturation of rat thyroglobulin occurred during autolysis at a pH (5.2) that would not of itself produce acid denaturation (Heidelberger & Pedersen, 1935).

Heterogeneity of thyroid tissue with regard to iodine metabolism has been postulated by a number of workers. Dobyns & Lennon (1948) showed, in radioautographic studies of human-thyroid adenomas, that fixation of [^{131}I] in the follicles paralleled

Fig. 2. Rates of release of di-[^{131}I]iodotyrosine, during autolysis at pH 3.6, in glands labelled for: I, 0.5 hr.; II, 1 hr.; III, 2 hr.; IV, 4 hr.; V, 18 hr.; VI, 48 hr.

Fig. 3. Rates of release of mono[^{131}I]iodotyrosine in thyroid labelled for 0.5 hr. during autolysis: I, at pH 3.6; VII, at pH 5.6; VIII, at pH 5.6 with preliminary acid denaturation of thyroglobulin.

to some extent the degree of cell differentiation. Nadler, Leblond & Bogoroch (1954) made quantitative radioautographic studies of ^{131}I metabolism in rat thyroid glands and found that the turnover of ^{131}I was greater in small follicles than in large ones. Triantaphyllidis (1958) showed that in rats the specific activity of ^{131}I -labelled thyroid hormone secreted into the blood was 3–4 times that of the intrathyroidal hormone during the first few days after injection of ^{131}I . Ingbar, Askonas & Work (1959) described heterogeneity of electrophoretically and ultracentrifugally homogeneous sheep thyroglobulin with regard to ^{131}I turnover and amino acid content. From studies on the distribution of ^{131}I and stable iodine in serum (Werner & Block, 1959) and thyroid tissue (Štolc, 1962), heterogeneity of the thyroid with regard to biosynthesis and secretion of hormonal and non-hormonal iodine has been postulated.

The present work supports the hypothesis that the thyroid gland is heterogeneous with respect to the biosynthesis of iodotyrosines. At short times of labelling with ^{131}I , both mono- and di-iodotyrosine residues are present in thyroglobulin or other thyroid protein in a form that is readily hydrolysable by the thyroid proteases present in the same glands. The shorter the time of labelling, the more labile mono- and di-iodotyrosine can be demonstrated. No evidence has been obtained of a differential rate of release of mono- ^{131}I -iodotyrosine, contrary to the findings of Litonjua (1960) and Pitt-Rivers & Cavalieri (1963).

The marked diminution in the rate of autolysis at pH 3.6 of thyroid homogenates from glands labelled for 48 hr. merits comment. At this time, ^{131}I and stable iodine in the gland are approaching equilibrium, and the rate of release of the ^{131}I -iodotyrosines reflects the rate of release of the unlabelled amino acids. The continuous decrease in the rates of autolysis found in these experiments suggests that some change may be occurring in the structure of the thyroglobulin molecule that makes the iodotyrosine residues less accessible to the thyroid protease with time. Nevertheless, even at a time approaching isotopic equilibrium, thyroglobulin contains iodotyrosyl residues that can be hydrolysed by the thyroid protease. These results are compatible with the hypothesis that both the free iodotyrosines represent the principal precursors of the second iodide pool of the thyroid.

More recent experiments have shown much lower rates of thyroid autolysis than those described above. Thyroidal ^{131}I uptakes are lower, although

dietary iodine is only $0.2\text{ }\mu\text{g./g.}$ However, thyrotrophin injections *in vivo* stimulate thyroid autolysis *in vitro*.

SUMMARY

1. The rates of release of mono- and di- ^{131}I -iodotyrosine during autolysis of homogenates of rat thyroid labelled for 0.5, 1, 2, 18 and 48 hr. *in vivo* have been studied at pH 3.6.

2. In all the experiments both the ^{131}I -iodotyrosines are released from thyroglobulin at equal rates at the same times of labelling.

3. The rates of release of the ^{131}I -iodotyrosines are greatest at the shortest time of labelling, and decrease as the time of labelling is increased.

4. In glands labelled for 0.5 to 4 hr., but not for 18 hr. or longer, there is evidence of labile mono- and di- ^{131}I -iodotyrosine, as shown by an initial rapid appearance of the free labelled amino acids in the autolysates.

5. The rate of autolysis of rat-thyroid homogenates is about 10 times as great at pH 3.6 as at pH 5.6. Preliminary acid denaturation of the substrate enhances the rate of autolysis at pH 5.6.

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