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The Free Iodotyrosines of the Rat Thyroid Gland

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Iodide in the thyroid gland is derived from two sources: (*a*) the blood, and (*b*) precursors containing organic iodine, thought to be free iodotyrosines, formed during proteolysis of thyroglobulin. Iodide that enters the gland from the circulation has been called the 'first iodide pool'; iodide derived from breakdown products within the gland, the 'second iodide pool' (Hickey & Brownell, 1954). The former is discharged from the thyroid by perchlorate and thiocyanate; the latter is not.

The iodide of the thyroid has been determined (as $^{131}I^-$ ion) by a number of workers using different techniques (Wollman & Scow, 1953; Ingbar & Freinkel, 1956; Rosenberg, Athans & Behar, 1960; Wollman, 1962); it appears to represent 0·2–0·3% of the total thyroidal ^{131}I.

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The second iodide pool is larger than the first; its size has been estimated in the rat thyroid (Halmi & Pitt-Rivers, 1962) to be approximately 0·26% of the thyroidal iodine, a finding that has been confirmed by Nagataki & Ingbar (1963); it is nearly 100 times as large as the first pool.

Halmi & Pitt-Rivers (1962) estimated the specific activity of iodide in the second pool at different times up to 24 hr. after single injections of [^{131}I]-iodide and compared the values with the specific activity of thyroglobulin-bound mono- and di-iodotyrosine at corresponding times, determined in a previous study (Pitt-Rivers, 1962). During the first 8 hr., the specific activity of second-pool iodide rose at a faster rate and reached a maximum sooner than that of any organic iodinated compound. It was therefore suggested that monoiodotyrosine and possibly di-iodotyrosine in thyroglobulin might be heterogeneous with respect to turnover; the fraction turning over more rapidly

than the average was presumed to be the precursor of the second iodide pool.

The presence of free mono- and di-iodotyrosine in butan-1-ol extracts of unhydrolysed ^{131}I-labelled rat thyroids was first reported by Gross, Leblond, Franklin & Quastel (1950). It is probable that these are the immediate precursors of iodide in the second pool, since Roche, Michel, Michel & Lissitzky (1952) showed that thyroid slices deiodinate these amino acids only when they exist in the free (non-peptide) state.

The present investigation was undertaken to determine whether free iodotyrosines could be the precursors of intrathyroidally formed iodide; the finding of the labelled mono- and di-iodotyrosine in diffusates of intact ^{131}I-labelled thyroid glands has been briefly reported (Pitt-Rivers & Cavalieri, 1962). Details of this work are described below, together with a consideration of possible sources of free thyroidal iodotyrosines.

METHODS

Determination of free iodotyrosines in the thyroid. Male hooded rats weighing about 200 g. were used. They were given diet 41B (Bruce, 1958) containing about 0·6 μg. of iodide/g., and tap water *ad libitum*. Groups of four to six animals were injected intraperitoneally or subcutaneously with carrier-free Na^{131}I (20–200 μc) in aq. 0·9% NaCl. At intervals between 30 min. and 72 hr. after the injection, the animals were anaesthetized with chloroform and killed by exsanguination. The thyroids were removed, weighed and frozen in solid carbon dioxide within 1 min. of death. The frozen glands were transferred to dampened Visking 24/32 dialysis bags and dialysed against 10 ml. of water or 0·01 M-NH_3 with gentle shaking for 16–18 hr. at 2°. The radioactivity in the diffusate was then determined in a well-type scintillation counter; the radioactivity in the contents of the bag was determined in a ring counter described by Campbell, Cuthbertson, Matthews & McFarlane (1956).

In preliminary experiments, the nature of the compounds present in the diffusates was investigated by concentrating the latter and analysing by paper chromatography in butan-1-ol saturated with 2 N-acetic acid. At short time-intervals after the administration of [^{131}I]iodide, the iodide and iodotyrosine contents of the diffusates were readily determined; however, at longer time-intervals interfering substances increased. These were probably a mixture of iodinated peptides, which were subsequently detected in material which remained at the origin of chromatograms with butan-1-ol–2 N-acetic acid, butan-1-ol–dioxan–aq. 2 N-NH_3 (4:1:5, by vol.) and collidine–water (20:7, v/v) as solvent systems in an atmosphere of NH_3. To eliminate these substances as far as possible, the following method was used. The entire diffusate was passed through a column (1 cm. × 3 cm.) of Dowex resin A.G. 1 (X2; 200–400 mesh) prepared by the method of Pitt-Rivers & Sacks (1962). The column was then eluted with five 3 ml. portions of aq. 1% (v/v) acetic acid; this removed all the monoiodotyrosine from the column, but only a part of the di-iodotyrosine; quantitative estimates of the latter were abandoned, since prolonged elution of the column even with dilute acetic acid removed more and more of the peptide-like material. The five 3 ml. fractions were pooled, concentrated in a rotary evaporator to a low volume and spotted on Whatman no. 1 paper strips. The chromatograms were developed in butan-1-ol–2 N-acetic acid and carrier iodotyrosines were revealed with ninhydrin [0·15% (w/v) in aq. 50% (v/v) ethanol]. The radioactivity on the strips was measured in a strip counter fitted with an end-window Geiger–Müller tube and an automatic integrating recorder.

At time-intervals up to 24 hr. after the injection of [^{131}I]iodide, analysis was made on single thyroid glands; at 48 and 72 hr. after injection, the amounts of labelled iodotyrosine in the diffusate were so small that it was necessary to pool the glands from two or three animals for analysis.

Effect of propylthiouracil on free and thyroglobulin-bound thyroidal monoiodotyrosine. Groups of four rats were injected with graded doses of propylthiouracil (6, 2, 1 and 0·1 mg.) 0·5 hr. before and 1·5 hr. after the administration of [^{131}I]iodide. They were killed 4 hr. after the [^{131}I]iodide was given. Thyroid diffusates were analysed for free monoiodotyrosine as described above. The thyroglobulin-bound monoiodotyrosine was determined as described by Pitt-Rivers (1962) after hydrolysis of the protein with pancreatin (U.S.P., crystallized three times).

Hydrolysis of ^{131}I-labelled rat thyroid glands with thyroid protease. Rat thyroid glands were labelled with [^{131}I]iodide for 1, 4, 8 and 24 hr., as described above. A crude preparation of thyroid protease was prepared as follows: 25 g. of fresh calf thyroid was homogenized in 250 ml. of aq. 0·9% NaCl and briefly centrifuged to remove cellular debris and stroma. The suspension was then centrifuged for 3½ hr. at 100 000*g* to sediment the thyroglobulin (Robbins, Petermann & Rall, 1954). To prevent dilution of the protease solution by incompletely sedimented thyroglobulin, only the top third of the contents of the centrifuge tubes was used. The protease solution was freeze-dried and the solid redissolved in water at 4 times its original concentration; this solution contained 40 mg. of protein/ml. Sheep-thyroid protease was prepared in the same way from 14 g. of sheep thyroid glands homogenized in 100 ml. of aq. 0·9% NaCl. The enzyme solution was not further concentrated and contained 5·2 mg. of protein/ml.

Three or four labelled rat thyroids were homogenized in an all-glass homogenizer in 0·5 ml. of aq. 0·9% NaCl; to the homogenate was added 1·5 ml. of 0·2 M-acetate buffer, pH 3·6, 0·5 ml. of protease solution, and, in most experiments, 0·1 ml. of a solution containing propylthiouracil, diiodotyrosine and monoiodotyrosine (all at 1 mg./ml.) in tris-HCl buffer, pH 8·4. This was done to diminish any deiodination of labelled iodotyrosines or re-utilization of iodide liberated. Samples of the hydrolysates were removed at approximately hourly intervals and analysed for unhydrolysed material, monoiodotyrosine and di-iodotyrosine by chromatography in butan-1-ol–2 N-acetic acid. After 24 hr., about 60% of the labelled thyroglobulin was hydrolysed, and no further hydrolysis occurred after 30 hr. An estimate of the iodotyrosines present in the different samples of labelled thyroid was made after hydrolysis with pancreatin (U.S.P., crystallized three times); with this preparation, we have found that 90–95% of the labelled thyroglobulin was hydrolysed in 24 hr.

RESULTS

Autolysis of thyroid protein during dialysis

Analysis of thyroid glands labelled *in vivo* for 4 hr. showed pronounced differences in the amounts of free iodotyrosines, depending on whether dialysis was carried out against water or 0·01 M-ammonia (Fig. 1). Bradley & Taylor (1916) have shown that autolysis of certain tissues such as liver is enhanced by an acid pH and inhibited by an alkaline pH. In the present experiments, it appears that, even at 2°, hydrolysis of ^{131}I-labelled protein may occur if the pH is not prevented from falling. The possibility cannot be excluded that some of the diffusible iodotyrosines found in these experiments were produced by autolysis of thyroglobulin during dialysis against dilute ammonia. However, dialysis against 0·01 M-ammonia failed to reveal any increase in diffusible iodotyrosines between 12 and 24 hr., and this procedure is unlikely to lead to the formation of labelled products by hydrolysis.

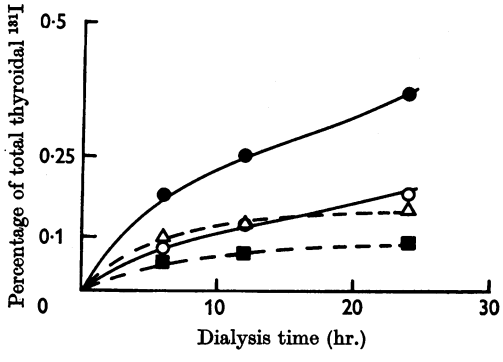

Fig. 1. Rates of appearance of ^{131}I-labelled monoiodotyrosine (○, ■) and di-iodotyrosine (●, △) in diffusates of whole rat thyroid glands when dialysed against water (○, ●; continuous lines) and 0·01 M-NH_3 (■, △; broken lines) at 2°.

Changes in the specific activity of free monoiodotyrosine after the injection of [^{131}I]iodide

Two attempts were made to estimate the concentrations of free iodotyrosines in the thyroid by Van Middlesworth's (1956) method of isotope equilibrium. In the first, seven rats were injected daily for 14 days with a constant amount of a solution containing 1 μc of [^{131}I]iodide on the first day; in the second, six rats were injected daily for 7 days with a solution containing 2 μc of [^{131}I]iodide on the first day. In both experiments, the amounts of radioactivity in diffusible iodotyrosines were too low to permit quantitative assessment; it was thought that the use of higher doses of [^{131}I]iodide would lead to radiation damage to the thyroids.

The relative amounts of free labelled monoiodotyrosine, as percentages of total thyroidal radioactivity, did not change materially between 48 and 72 hr. after a single injection of [^{131}I]iodide (see Table 1). The value for free labelled monoiodotyrosine at 72 hr. must therefore be close to the isotopic equilibrium value; this was used as a basis for the calculation of the specific activity of monoiodotyrosine at all the short time-intervals.

The specific activities of thyroglobulin-bound monoiodotyrosine, second-pool iodide and free monoiodotyrosine 0·5–24 hr. after the injection of [^{131}I]iodide are shown in Fig. 2. The specific activity of free monoiodotyrosine rose more steeply than that of either of the other compounds, reaching a maximum in the gland labelled for 3 hr. Thereafter it fell rapidly and flattened out after 8 hr.

Table 1. *Time-course of changes in ^{131}I-labelled free monoiodotyrosine and di-iodotyrosine*

Experimental details are given in the text. All results are expressed as means ± S.D. MIT, monoiodotyrosine; DIT, di-iodotyrosine (Harington, Pitt-Rivers, Querido, Roche & Taurog, 1957).

Time after injection of [^{131}I]iodide (hr.)	No. of rats	Thyroid wt. (mg.)	Uptake of ^{131}I (% of dose)	Activity of free MIT (% of thyroidal ^{131}I)	Sp. activity of free MIT*	Activity of free DIT (% of thyroidal ^{131}I)
0·5	5	16·8±3·0	1·0±0·2	0·12±0·03	8·3±1·7	0·068±0·020
1	6	13·4±0·3	2·3±0·2	0·092±0·010	17·6±0·7	0·073±0·010
2	5	15·5±2·1	5·4±1·4	0·072±0·018	29·0±13·5	0·081±0·020
3	8†	12·0±1·5	3·6±0·7	0·105±0·011	35·3±3·3	—
4	4	11·9±1·7	6·5±0·36	0·050±0·010	30·6±5·0	0·092±0·040
8	6	15·1±1·1	14·1±1·5	0·018±0·006	18·4±6·5	—
24	12	12·8±1·2	12·3±1·6	0·015±0·005	15·9±5·5	—
48	6	12·3±1·4	9·7±1·4	0·009±0·001	7·7±0·8	—
72	6	13·9±1·2	6·8±1·0	0·009 (6 pooled glands)	4·9	—

* Sp. activity: % of dose/g. of thyroid = $\frac{\%\text{ of free MIT in gland} \times \text{uptake (\% of dose)} \times 10}{0{\cdot}009 \times \text{thyroid wt. (mg.)}}$.

† This group of animals was studied 5 months after the other groups.

Effect of propylthiouracil on the formation of free and thyroglobulin-bound monoiodotyrosine

The results of this experiment are shown in Fig. 3. With the highest dose of propylthiouracil (6 mg. administered before and after the injection of [^{131}I]iodide) there was complete inhibition of the formation of free monoiodotyrosine and almost complete inhibition of the synthesis of thyroglobulin-bound monoiodotyrosine, 4 hr. after the administration of [^{131}I]iodide; as the dose of propylthiouracil was lowered, the formation of thyroglobulin-bound monoiodotyrosine was partly re-established, and at the lowest dose its inhibition was only moderate (23 %). However, at all concentrations of propylthiouracil used, the inhibition of free monoiodotyrosine was marked and with the lowest dose of propylthiouracil remained as high as 82·5 %.

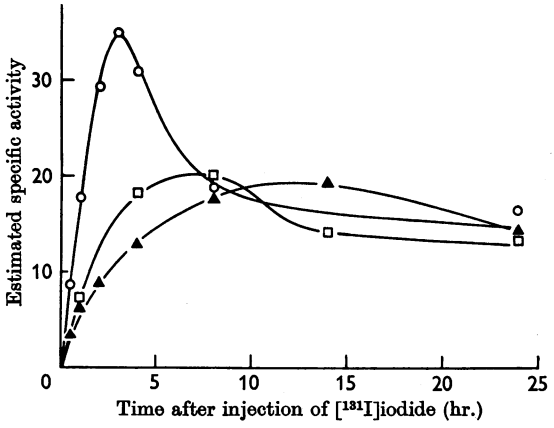

Fig. 2. Changes in the specific activities of thyroglobulin-bound monoiodotyrosine, free monoiodotyrosine and second-pool I^- ion with time after a single injection of [^{131}I]iodide. ▲, Bound monoiodotyrosine; ○, free monoiodotyrosine; □, non-dischargeable I^- ion.

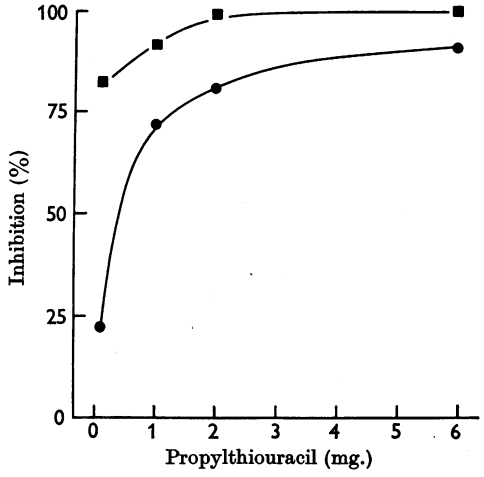

Fig. 3. Effect of graded doses of propylthiouracil administered *in vivo* on the formation of thyroglobulin-bound and free monoiodotyrosine. ■, Free monoiodotyrosine; ●, bound monoiodotyrosine. Rats were killed 4 hr. after the administration of [^{131}I]iodide, and propylthiouracil was given 0·5 hr. before and 1·5 hr. after this injection of [^{131}I]iodine.

Hydrolysis of labelled thyroid by crude thyroid protease

Calf-thyroid protease. The results of this experiment are shown in Fig. 4. With glands labelled for 1 and 4 hr. *in vivo*, hydrolysis by thyroid protease proceeded at first at a rapid rate which slowed down after 3–4 hr. The effect was most marked in the gland labelled for 4 hr. In the glands labelled for 24 hr., the appearance of labelled monoiodotyrosine in the hydrolysate occurred at a constant rate up to 9½ hr. These findings have been interpreted to mean that at the short time-intervals after the injection of [^{131}I]iodide the thyroid gland possesses a fraction of monoiodotyrosine which is more labile to thyroid protease than is the average. At no time of labelling the gland was any significant amount of labile di-iodotyrosine demonstrable.

In one experiment, the substrate was heated in a boiling-water bath for 2 min. before the protease was added. After 9 hr., only about 5 % of the mono- and di-iodotyrosine in the substrate had been liberated.

The activity of our protease solution towards labelled rat thyroid was reduced fourfold when the incubation was carried out at pH 5·6. This may have been due to loss during preparation of a

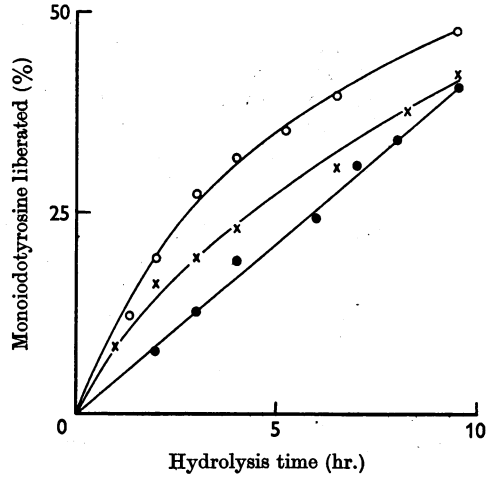

Fig. 4. Rate of liberation of ^{131}I-labelled monoiodotyrosine by crude calf-thyroid protease from rat thyroglobulin labelled with ^{131}I for: ×, 1 hr.; ○, 4 hr.; ●, 24 hr.

protease fraction demonstrated by Haddad & Rall (1960) and by McQuillan, Mathews & Trikojus (1961) to be active at this pH.

No attempt was made to inactivate the rat-thyroid protease in the glands themselves, or to separate it from rat thyroglobulin. Subsequent experiments showed that the contribution of rat-thyroid protease amounted to about 10% of the total hydrolytic effect.

Sheep-thyroid protease. Rat thyroids labelled with [^{131}I]iodide for 1, 2, 4 and 24 hr. were hydrolysed with sheep-thyroid protease. The rates of hydrolysis of corresponding substrates were similar to those found with the calf-enzyme preparation. In both experiments the maximum amount of labile monoiodotyrosine appeared in the glands labelled for 4 hr.

DISCUSSION

Source of second iodide pool

The curve describing the specific activity of free monoiodotyrosine as a function of time after the injection of [^{131}I]iodide rises at a more rapid rate and reaches a peak sooner than the specific activity curve of I^- ion in the second pool (Fig. 2). This finding supports but does not prove the hypothesis that free monoiodotyrosine may be a precursor of the second iodide pool.

The equilibrium concentration of free di-iodotyrosine has not been determined; it has not therefore been possible to estimate its specific activity. However, in our initial experiments, in which dialysis of thyroids was carried out against water, the ratio of free di-iodotyrosine to monoiodotyrosine was about 3:1 after labelling for 72 hr.; that is, they were present in the free state in the same proportion as in thyroglobulin-bound linkage. Unless the free iodotyrosines are deiodinated at a different rate, a suggestion for which there is no evidence, it seems reasonable to use the above proportion of free iodotyrosines in assessing their contributions to the second iodide pool. As has been shown, free monoiodotyrosine represents about 0·01% of the total thyroidal iodine; free di-iodotyrosine on this basis will represent 0·03% of the total thyroidal iodine. Using this value to calculate the specific activity of free di-iodotyrosine at 2 and 4 hr. after the injection of [^{131}I]iodide, we get values of 9·4 and 17% of dose/g. of thyroid; if both iodotyrosines contribute equally to second iodide pool, then the average value for the specific activities of both compounds at these times must be made. These averages are 16 at 2 hr. and 21 at 4 hr. Both averages exceed the specific activity of second iodide pool at this time, which agrees with the suggestion that both free mono- and di-iodotyrosine are precursors of the second iodide pool.

Whether compounds other than free iodotyrosines contribute to intrathyroidally formed iodide remains an open question. Roche *et al.* (1952) found that only free iodotyrosines were deiodinated by sheep-thyroid slices. J. Wolff (unpublished work) showed that incubation of sheep thyroid homogenates with ^{131}I-labelled acetyl-DL-di-iodo-tyrosyl peptides led to the formation of free di-iodotyrosine and iodide. R. R. Cavalieri (unpublished work) found that incubation of sheep thyroid homogenates with [^{131}I]iodide and glycyl-L-tyrosine gave rise to labelled monoiodotyrosine whereas similar incubations with glycyl-D-tyrosine gave rise to glycyl-D-monoiodotyrosine.

From these results it seems reasonable to suggest that the peptide fragments of thyroglobulin, which contain the natural isomers of the iodotyrosines, are rapidly hydrolysed *in vivo* by the thyroid peptidases before deiodination.

Formation of free iodotyrosines

On theoretical grounds, free monoiodotyrosine may arise either from the iodination of free tyrosine or from the hydrolysis of iodinated peptides or protein. The first reaction has not been directly demonstrated *in vivo*. However, experiments with thyroid homogenates or cells (Fawcett & Kirkwood, 1953; Serif & Kirkwood, 1956; Pastan, 1961), as well as partially purified peroxidase systems (De Groot & Davis, 1962; Alexander, 1959; Klebanoff, Yip & Kessler, 1962), demonstrate the ease with which free tyrosine, added as substrate, is iodinated. It seems reasonable to assume that in the intact gland tyrosine residues may be iodinated whether they exist within the thyroglobulin molecule, in smaller peptides, or as the free amino acid. It appears likely that free tyrosine is present within the thyroid gland although its concentration has not been measured as far as we are aware. If indeed free tyrosine or tyrosine peptides other than thyroglobulin are iodinated directly in the thyroid, their rates of iodination may be quite different. Further, free di-iodotyrosine may be formed by the iodination of free monoiodotyrosine. De Groot & Davis (1962) have shown that, during the iodination of free tyrosine by their purified thyroidal iodide–peroxidase system, the ratio of di-iodotyrosine to monoiodotyrosine formed varies from 0·1 to 0·7, depending on the relative concentrations of iodide to tyrosine.

In this connexion, the effect of small doses of propylthiouracil on the formation of free and bound monoiodotyrosine *in vivo* merits some comment. It is obvious from Fig. 3 that the formation of free monoiodotyrosine is inhibited to a much greater degree than is that of thyroglobulin-bound monoiodotyrosine. This could be interpreted to

mean that iodination of free tyrosine is more affected by propylthiouracil than is that of bound tyrosine. However, other explanations can be found. It is possible that propylthiouracil accelerates the rate of deiodination of free monoiodotyrosine or retards the rate of proteolysis of thyroglobulin. The latter is unlikely, since we did not detect any difference in the rates of hydrolysis of labelled thyroglobulin by crude thyroid protease in the presence or absence of propylthiouracil.

Iodination of di- and tri-peptides of tyrosine by thyroid-tissue preparations *in vitro* is difficult to demonstrate because of the presence of peptidase activity (Weiss, 1953). However, studies by R. R. Cavalieri (unpublished work) have indicated that simple peptides containing D-tyrosine are iodinated as rapidly as free tyrosine.

During chromatographic analysis of diffusates of ^{131}I-labelled thyroid glands, it was found that these contained radioactive material that did not migrate from the origin of the chromatograms in the solvent systems used. The amount of 'origin material' increased with the time of labelling and reached a maximum of about 0·6% of total thyroidal ^{131}I after 72 hr. This material was not fractionated further, but it almost certainly represents some, if not all, of the peptides studied by Lissitzky, Grégoire, Grégoire & Limozin (1961) using different techniques. Whether these peptides resulted from direct iodination or from partial breakdown of thyroglobulin is not known.

From the experiments on the hydrolysis of ^{131}I-labelled rat thyroid by crude preparations of thyroid protease, it appears that at short time-intervals of labelling the thyroid contains a portion of monoiodotyrosine that is more labile than the average. The proportion of labile monoiodotyrosine was higher in the glands labelled for 4 hr. than in those labelled for 1 hr. If a labile fraction of thyroglobulin were the only source of free monoiodotyrosine, this should be present in the greatest amount at times of labelling shorter than that at which the iodotyrosines reach their maximum specific activity, i.e. at about 3 hr. Since this has not been found, it is concluded that the other sources of free iodotyrosines in the thyroid discussed above also contribute to the pool of free iodotyrosines.

The following sequence of events appears to explain the results most simply: iodide transported into the thyroid from the blood is largely incorporated into the tyrosine residues of thyroglobulin, giving first bound iodotyrosines and later iodothyronines; to a much smaller extent, free tyrosine and tyrosine peptides are also iodinated to their mono- and di-iodo derivatives. Some of the thyroglobulin and the iodotyrosine peptides is rapidly hydrolysed by thyroid proteases and peptidases to give the free iodoamino acids. The latter comprise a final common pathway in the formation of the second iodide pool of the gland.

SUMMARY

1. The free monoiodotyrosine of ^{131}I-labelled rat thyroid glands has been determined by column and paper chromatography of thyroid diffusates at different times after the administration of [^{131}I]-iodide.

2. An estimate of the specific activity of free monoiodotyrosine at 0·5 to 72 hr. after the injection of [^{131}I]iodide was made. It rose more sharply than the specific activities of thyroglobulin-bound labelled monoiodotyrosine and of intrathyroidally formed I^- ion. It is suggested that both the iodotyrosines are likely precursors of this I^- ion.

3. The effect of graded doses of propylthiouracil administered *in vivo* was examined. This goitrogen inhibited the appearance of free monoiodotyrosine much more markedly than that of thyroglobulin-bound monoiodotyrosine.

4. The rate of hydrolysis of ^{131}I-labelled rat thyroglobulin with crude preparations of calf- and sheep-thyroid protease was studied at different times of labelling. For short time-intervals, up to 4 hr., the thyroglobulin contained a fraction of monoiodotyrosine that was more labile to the protease than the average. Labile di-iodotyrosine was not demonstrated.

5. It was concluded that both the free iodotyrosines in the thyroid are the precursors of intrathyroidally formed I^- ion (second iodide pool). The free monoiodotyrosine may come in part from a moiety of thyroglobulin that is more labile than the average. Other sources of free iodotyrosines are discussed.

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Neutral Proteinases in the Lens

2. PARTIAL PURIFICATION AND PROPERTIES*

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The term 'neutral proteinase' denotes a widely occurring class of proteolytic enzymes that are most active at about pH 7·5. This property distinguishes neutral proteinases from cathepsins, as cathepsins are defined as having pH optima of about 4 (Fruton, 1960). Waley & van Heyningen (1962*a*) used as source of enzyme the fraction of the soluble proteins of ox lens that is precipitated at pH 5 (α_1-crystallin). This fraction contains both the proteinase and proteins that serve as substrates. The enzyme has now been purified; the 'endogenous' substrate has been shed, and this permits a comparison of substrates. Moreover, the proteinase has been largely separated from the leucine aminopeptidase in lens. Part of these results have been published in a symposium (van Heyningen & Waley, 1962; Waley & van Heyningen, 1962*b*).

METHODS

Veronal buffer. This contained 400 ml. of 0·025 M-diethylbarbituric acid, 14·5 ml. of 0·5 M-sodium diethylbarbiturate and 8·6 ml. of M-$MgCl_2$ in 1725 ml. of solution; CO_2-free water was used; pH was 7·5 at room temperature.

Assays. The proteinase was assayed with α_2-crystallin (1%, w/v) as substrate in veronal–Mg^{2+} buffer at 55° (Waley & van Heyningen, 1962*a*). Four samples were usually taken, after incubation for 0, 1, 2 and 3 hr. The extent of reaction (followed by the ninhydrin reaction) was proportional to the time for at least 3 hr. under these conditions. The unit of proteinase is that amount which produces 1 μmole of amino acid (leucine equivalent) in 1 ml. of incubation mixture/hr. Leucine aminopeptidase was assayed with L-leucylglycine as substrate, the enzymic hydrolysis being followed by paper chromatography. The unit of peptidase is that amount which produces 1 μmole of glycine/ml. of assay mixture/hr. (Waley & van Heyningen, 1962*a*).

Gel-filtration. This was carried out on 2·7 cm. × 15 cm. long columns of Sephadex G 25 (medium grade; Pharmacia, Uppsala, Sweden) in the cold room; the columns were equilibrated and eluted with the veronal–Mg^{2+} buffer. The concentration of protein in the fractions was estimated by E at 280 mμ, read against the veronal buffer, which scarcely absorbs at this wavelength; $E^{1\%}_{1\,cm.}$ 10 was assumed for all protein fractions. For α_1-crystallin, calibration by the biuret reaction (Gornall, Bardawill & David, 1949) with bovine serum albumin as standard gives $E^{1\%}_{1\,cm.}$ 9·3.

Diethylaminoethylcellulose. Diethylaminoethyl (DEAE)-cellulose (Bio-Rad Laboratories, Richmond, Calif., U.S.A.) was graded by repeatedly allowing it to settle in water for about 20 min. and removing the supernatant. The DEAE-cellulose was washed on a sintered-disk funnel with 0·5 N-NaOH, and then with water; it was converted into the chloride form by washing with N-HCl and then washed with water. Finally, the DEAE-cellulose was equilibrated with the veronal–Mg^{2+} buffer, pH 7·5, and then packed into a column, 2·25 cm. × 12 cm. long. Chromatography was carried out in the cold room (at about 6°); the rate of elution was 1–2 ml./min. with a pressure of about 80 cm. water.

Hydroxyapatite. This was prepared by the method of Tiselius, Hjerten & Levin (1956) and a column about 1·7 cm. × 7 cm. long used. Before use it was washed with 50 ml. of M-phosphate, pH 7·4, prepared from NaH_2PO_4,-

* Part 1: Waley & van Heyningen (1962*a*).