Hydrolysis of ^{125}I -Labelled Thyroglobulin by Pancreatin, Pronase and Pepsin

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1. Pancreatin or Pronase hydrolysis of partially purified heat-denatured ^{125}I -labelled thyroglobulin does not release all the iodoamino acids from peptide linkage. 2. The addition of thyroidal proteases is essential to obtain maximum hydrolysis of thyroglobulin. 3. Pronase plus a fresh non-radioactive thyroid homogenate gives the best hydrolysis results, but peptide 'mapping' shows that about 10% of the radioactivity remains in the form of peptides. 4. These peptides have approximately the same R_F as the iodotyrosines on one-dimensional chromatograms developed in butan-1-ol-2*N*-acetic acid (1:1, v/v). 5. Peptic hydrolysis of thyroglobulin releases virtually all of the iodothyronines but little of the iodotyrosines, in contrast with the action of pancreatin. 6. Hydrolysis of thyroglobulin with pepsin followed by Pronase plus a source of thyroidal proteases is satisfactory, but the results are not superior to those obtained by using Pronase plus a source of thyroidal proteases.

Previous work on the enzymic liberation of iodoamino acids from thyroid protein was carried out on homogenates of whole thyroid by Tong & Chaikoff (1958) and Tong, Raghupathy & Chaikoff (1963). These authors achieved virtually complete release of the iodoamino acids from the protein of their homogenates. However, when their procedures are applied to partially purified thyroglobulin they prove inadequate.

The work described here demonstrates this inadequacy and establishes a satisfactory procedure for use with thyroglobulin preparations.

METHODS AND MATERIALS

Animals. Female Wistar albino rats (200–220g.) were used. They were given a low-iodine diet (Boyd & Oliver, 1960) for 2 weeks before the experiments.

Preparation of ^{125}I -labelled thyroglobulin. Each rat was injected subcutaneously with $125\mu\text{C}$ of carrier-free Na^{125}I (The Radiochemical Centre, Amersham, Bucks.) and the animals were killed 24 hr. later. The thyroid glands were removed immediately and homogenized by hand in 0.15M-NaCl (1.5 ml./thyroid gland) in an all-glass homogenizer at 0°. The homogenate was centrifuged at 2000*g* to remove the cell debris and then at 125 000*g* for 40 min. (Spinco preparative centrifuge, swinging-bucket rotor, SW.39L). The supernatant was dialysed at 2° for 24 hr. against two changes of 2l. of water adjusted to pH 10 with NH_3 and containing 0.04% of thiouracil; the non-diffusible material was freeze-dried. This partially purified thyroglobulin was dissolved in water (3.33%, w/v) and denatured by heating it at $80\pm 1^\circ$ for 5.0 min.

Enzymic hydrolysis of thyroglobulin. (a) Pancreatin.

Hydrolysis conditions were similar to those described by Tong & Chaikoff (1958), except that pancreatin (8 U.S.P. units) obtained from Weddel Pharmaceutical Co., London, E.C. 1, was used instead of Viokase (4 U.S.P. units) from the Viobin Corp., Monticello, Ill., U.S.A. Comparative trials showed that these two preparations result in identical degrees and patterns of hydrolysis. A sample (100 μl .) of ^{125}I -labelled thyroid homogenate (1.33%, w/v) was added to 0.4 ml. of a buffer, pH 8.5, containing 0.07M-tris, 0.11M-NaCl, 4mM-MnSO₄ and 0.04% of thiouracil. Alternatively, 25 μl . of heat-denatured ^{125}I -labelled thyroglobulin (3.33%, w/v) was added to 0.3 ml. of the same tris-NaCl-MnSO₄ buffer. The pH was adjusted to about 8.5; 6 mg. of pancreatin was added and the pH readjusted to 8.5. Incubation was carried out for 48 hr. at 37° under toluene.

(b) Pronase. The volumes of buffer and thyroid material were identical with those described above. The buffer used (Tong *et al.* 1963) contained 0.07M-tris, 0.14M-NaCl, 14mM-CaCl₂ and 0.04% of thiouracil, and was adjusted to pH 8.0. Pronase (2mg.) and 1 drop of toluene were added and the mixture was incubated at 37° for 16 hr. Pronase was purchased from Calbiochem, Los Angeles, Calif., U.S.A.

(c) Pepsin. (i) Initially, to examine the effect of pH on peptic hydrolysis, 100 μl . of the ^{125}I -labelled thyroid homogenate (1.33%, w/v) was added to 0.4 ml. of 0.04M-sodium citrate buffer, pH 1.9, 3.7 or 5.2, containing 0.04% of thiouracil. Pepsin (0.5mg.) was added and incubation was carried out at 37° for 24 hr. under toluene.

(ii) In further experiments 25 μl . of heat-denatured thyroglobulin (3.33%, w/v) was added to 200 μl . of 0.01N-HCl, and pepsin (1mg.) was added. The pH was adjusted to 1.9 and incubation was carried out for 24 hr. as described in (c)(i) above. Pepsin (twice crystallized) was obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A.

(d) Pepsin followed by Pronase. Peptic hydrolysis of heat-denatured thyroglobulin was carried out, as described in (c)(ii) above, with 1 mg. of pepsin at pH 1.9 for 24 hr. The pH was adjusted to about 8.0 with 1 N-NaOH after incubation. Then 25 μ l. of a tenfold concentrated solution of the tris-NaCl-CaCl₂ buffer described in (b) above was added to the peptic digest to give the correct final concentration of buffer components, pH 8.0. Hydrolysis was carried out with Pronase (2 mg.) as described in (b) above.

Thyroidal proteolytic enzymes. A thyroid homogenate was used as a source of thyroidal proteases. A rat thyroid gland was homogenized in 1.0 ml. of either 0.07 M-tris-0.11 M-NaCl-4 mM-MnSO₄ buffer, pH 8.5, or 0.07 M-tris-0.14 M-NaCl-14 mM-CaCl₂ buffer, pH 8.0 (concn. of homogenate approx. 2%, w/v). In some experiments, 100 μ l. of an unlabelled fresh rat thyroid homogenate, in the appropriate buffer, was added to each incubation mixture containing ¹²⁵I-labelled thyroglobulin and either pancreatin or Pronase.

Paper chromatography. The procedure of Plaskett, Barnaby & Lloyd (1963a) for the extraction of free iodoamino acids from the enzymic hydrolysates was followed. An equal volume of methanol-aq. NH₃ (sp.gr. 0.88) (1:1, v/v) was added and the resulting suspension was centrifuged. The supernatant was removed and the precipitate was re-extracted with half the original volume of methanol-aq. NH₃ (sp.gr. 0.88) (1:1, v/v). Samples (0.2 ml.) of the combined supernatants from the centrifuged hydrolysates were each applied as a 3 cm. band on Whatman no. 3MM paper. Two chromatograms of each hydrolysate were developed separately in butan-1-ol-2 N-acetic acid (1:1, v/v) and butan-1-ol-dioxan-2 N-NH₃ (4:1:5, by vol.) (Gross, Leblond, Franklin & Quastel, 1950). These solvents give good resolution of the iodotyrosines and iodothyronines respectively (Plaskett, Barnaby & Lloyd, 1963a,b). The chromatograms were dried at 40° and were counted in 1 cm.-wide segments on a Baird and Tatlock Ltd. radioactive chromatogram counter. Radioactive spots were identified by reference to the position of carrier mono- and di-iodotyrosine (obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks.), thyroxine and 3,3',5-tri-iodothyronine (obtained from Glaxo Laboratories Ltd., Greenford, Middlesex), and potassium iodide (British Drug Houses Ltd., Poole, Dorset).

Peptide 'mapping' of enzymic hydrolysates. A sheet of Whatman no. 3MM paper was wetted with pyridine-acetic acid-water (25:1:475, by vol.) buffer, pH 6.5, and covered with a sheet of polythene to restrict evaporation of solvent. The methanol-aq. NH₃ (sp.gr. 0.88) (1:1, v/v) extract of the hydrolysate and carrier iodoamino acids, in a volume of 200 μ l., were immediately applied to the origin of the paper. High-voltage electrophoresis was carried out in pyridine-acetic acid-water buffer, pH 6.5, for 3 hr. at 1.85 kv and 40 ma in a vertical tank with white spirit as coolant (Michl, 1951). The paper was dried at 40° for 2 hr. and then developed at a right angle to the direction of electrophoresis by chromatography in butan-1-ol-2 N-acetic acid (1:1, v/v) for 12 hr. The dried peptide 'map' was radioautographed for 5 days with Ilford No-Screen X-ray film (14 in. $\times$ 17 in.) (Plaskett, 1964). Outlines of radioactive spots on the film were transferred to the chromatography paper. These spots were cut out and counted in an annular-cup scintillation counter with a pulse-height analyser (Ekco, type N610B) set to count the ¹²⁵I radiation (background 50 counts/min.). Radioactivity on the remainder of the

chromatogram was found by cutting it into $\frac{1}{2}$ in. $\times$ 4 in. strips, which were also assayed. Positions of the iodoamino acids on the peptide 'map' were found by eluting the spots and chromatographing them again, and then locating the carrier amino acids by their u.v. absorption on the chromatograms and by spraying with Pauly's reagent (Plaskett, 1964). The position of carrier iodide was found by spraying with aq. PdCl₂ (1%, w/v).

RESULTS

Action of pancreatin and Pronase on denatured thyroglobulin. The results of one-dimensional paper chromatography of the products of hydrolysis of heat-denatured thyroglobulin by pancreatin and Pronase respectively are shown in Table 1. In interpreting this and subsequent Tables, several points should be borne in mind.

(i) The amount of radioactivity left at the origin decreases as hydrolysis is prolonged, and the amount of this radioactivity is always less when the solvent is butan-1-ol-2 N-acetic acid (BA) than when the solvent is butan-1-ol-dioxan-2 N-ammonia (BDA). The best available estimate of high-molecular-weight unhydrolysed material is therefore the radioactivity left at the origin by the solvent BDA.

(ii) In practically every instance, the amount of radioactivity found in the iodide spot is less with solvent BA than with solvent BDA. The estimate of iodide from the BA chromatogram is therefore likely to be closer to the true value.

(iii) Plaskett *et al.* (1963a,b) pointed out that the iodotyrosines are better estimated on BA chromatograms and that the iodothyronines are better estimated on BDA chromatograms.

On the basis of these criteria, Table 1 shows that the Pronase hydrolysate contains significantly less origin material than does the pancreatin hydrolysate, that it may contain less iodide, and that it contains significantly more thyroxine. In both hydrolysates the radioactivity accounted for as free iodotyrosines and iodide (determined from BA chromatograms) and free iodothyronines (determined from BDA chromatograms) amounts to only about 80% of the total (79.7 and 83.9% for the pancreatin and Pronase hydrolysates respectively).

Action of pancreatin and Pronase on thyroid homogenate. The action of the two enzymes on unheated homogenates of thyroid glands that have been labelled *in vivo* with ¹²⁵I is shown in Table 2. Each hydrolysate contains less origin material than does the corresponding heat-denatured thyroglobulin hydrolysate and a higher proportion of the total radioactivity is accounted for (86.6 and 85.5% for the pancreatin and Pronase hydrolysates respectively).

Effect of unlabelled thyroid homogenate on the hydrolysis of denatured thyroglobulin. Table 3 shows that the added thyroid homogenate decreases the

Table 1. *Composition of pancreatin and Pronase hydrolysates of heat-denatured thyroglobulin*

Partially purified ^{125}I -labelled rat thyroglobulin was denatured by heating it at 80° for 5 min. The results of three separate experiments are given as means $\pm$ s.e.m. Experimental details are given in the text. BA and BDA refer to butan-1-ol-2*N*-acetic acid (1:1, v/v) and butan-1-ol-dioxan-aq. 2*N*-ammonia (4:1:5, by vol.) respectively. Other abbreviations: MIT, 3-iodotyrosine; DIT, 3,5-di-iodotyrosine; T4, thyroxine; T3, 3,3',5-tri-iodothyronine.

Enzyme	Chromatography solvent	Radioactivity (% of total on chromatogram)						MIT/DIT ratio
		Origin	Iodide	MIT	DIT	T4	T3	
Pancreatin	BA	2.86 $\pm$ 0.09	6.05 $\pm$ 1.20	33.66 $\pm$ 1.14	27.36 $\pm$ 1.38	14.07 $\pm$ 0.60		1.24
	BDA	13.61 $\pm$ 2.88	8.43 $\pm$ 0.45	55.78 $\pm$ 0.75		10.30 $\pm$ 0.83	2.33 $\pm$ 0.34	—
Pronase	BA	1.10 $\pm$ 0.11	4.19 $\pm$ 0.07	28.31 $\pm$ 0.75	31.56 $\pm$ 0.14	22.24 $\pm$ 0.32		0.90
	BDA	6.62 $\pm$ 0.22	4.98 $\pm$ 0.06	59.27 $\pm$ 1.08		16.97 $\pm$ 0.24	2.85 $\pm$ 0.24	—

Table 2. *Composition of pancreatin and Pronase hydrolysates of ^{125}I -labelled rat thyroid homogenate*

The results of three separate experiments are given as means $\pm$ s.e.m. Experimental details are given in the text. For definition of abbreviations see Table 1.

Enzyme	Chromatography solvent	Radioactivity (% of total on chromatogram)						MIT/DIT ratio
		Origin	Iodide	MIT	DIT	T4	T3	
Pancreatin	BA	1.99 $\pm$ 0.13	3.70 $\pm$ 0.13	29.72 $\pm$ 1.36	33.92 $\pm$ 0.72	17.48 $\pm$ 1.02		0.88
	BDA	4.27 $\pm$ 0.12	6.11 $\pm$ 0.55	58.76 $\pm$ 1.17		16.30 $\pm$ 0.68	3.00 $\pm$ 0.06	—
Pronase	BA	0.73 $\pm$ 0.05	5.74 $\pm$ 0.53	29.30 $\pm$ 0.44	31.22 $\pm$ 1.14	23.17 $\pm$ 0.55		0.94
	BDA	2.50 $\pm$ 1.15	5.82 $\pm$ 0.55	64.01 $\pm$ 1.18		15.87 $\pm$ 0.38	3.41 $\pm$ 0.14	—

Table 3. *Effect of thyroidal proteases on the composition of enzymic hydrolysates of heat-denatured thyroglobulin*

Partially purified ^{125}I -labelled rat thyroglobulin was denatured by heating it at 80° for 5 min. and hydrolysed with pancreatin or Pronase plus non-radioactive thyroid homogenate (as a source of thyroidal proteases). The results of three separate experiments are given as means $\pm$ s.e.m. Experimental details are given in the text. For definition of abbreviations see Table 1.

Enzyme	Chromatography solvent	Radioactivity (% of total on the chromatogram)						MIT/DIT ratio
		Origin	Iodide	MIT	DIT	T4	T3	
Pancreatin	BA	1.58 $\pm$ 0.24	5.42 $\pm$ 0.37	30.21 $\pm$ 1.21	33.56 $\pm$ 1.01	17.59 $\pm$ 1.37		0.90
	BDA	3.28 $\pm$ 0.07	6.29 $\pm$ 0.18	59.04 $\pm$ 1.54		14.39 $\pm$ 1.67	2.57 $\pm$ 0.27	—
Pronase	BA	1.16 $\pm$ 0.10	5.67 $\pm$ 0.19	32.22 $\pm$ 0.52	33.54 $\pm$ 0.12	18.77 $\pm$ 0.51		0.96
	BDA	1.99 $\pm$ 0.43	5.88 $\pm$ 0.31	60.77 $\pm$ 0.04		17.83 $\pm$ 0.29	2.88 $\pm$ 0.08	—

amount of radioactivity left at the origin of BDA chromatograms (compare Table 1). The effect on the amount of iodide is equivocal, but there is certainly no significant increase. The total amount of radioactivity accounted for, however, rises from about 80% (Table 1) to nearer 90% (86.2 and 92.1% respectively).

Peptide 'mapping' of hydrolysate constituents. In an attempt to account for the radioactivity not accounted for in the identifiable regions of the one-

dimensional chromatograms, the constituents of various thyroglobulin hydrolysates were separated by electrophoresis on paper in one direction and by chromatography in the other by the technique described in the Methods and Materials section. The results are presented in Table 4; in interpreting them, it has to be borne in mind that the iodide (which constitutes about 5% of the radioactivity found on the one-dimensional chromatograms) migrates off the peptide 'maps'. If one makes

Table 4. *Radioactivity on peptide 'maps' of enzymic digests of heat-denatured thyroglobulin*

The Table shows the percentages of the total radioactivity in recognizable spots on peptide 'maps' of enzymic digests of ^{125}I -labelled thyroglobulin that was denatured by heating it at 80° for 5 min. Experimental details are given in the text. For definition of abbreviations see Table 1.

Enzyme	Radioactivity in recognizable spots (% of total on paper)	Radioactivity in MIT + DIT + T4 + T3 (% of total on paper)
Pancreatin	90	80
Pancreatin + non-radioactive homogenate	87	75
Pronase + non-radioactive homogenate	94	90

Table 5. *Effect of pH on composition of peptic hydrolysates of ^{125}I -labelled rat thyroid homogenate*

Composite results are shown from chromatograms developed in solvents BA and BDA. Experimental details are given in the text. For definition of abbreviations see Table 1.

pH	Radioactivity (% of total on chromatogram)				(T4 + T3)/(MIT + DIT) ratio
	MIT	DIT	T4	T3	
1.9	9.3	24.3	16.3	2.1	0.55
3.7	14.3	25.8	14.7	2.0	0.42
5.2	9.6	14.9	17.7	1.1	0.78

Table 6. *Composition of peptic hydrolysates of heat-denatured thyroglobulin*

Partially purified ^{125}I -labelled rat thyroglobulin was denatured by heating it at 80° for 5 min. and it was hydrolysed with pepsin at pH 1.9 for 24 hr. The results of three separate experiments are given as means $\pm$ s.e.m. Experimental details are given in the text. For definition of abbreviations see Table 1.

Chromatography solvent	Radioactivity (% of total on chromatogram)						MIT/DIT ratio
	Origin	Iodide	MIT	DIT	T4	T3	
BA	13.91 $\pm$ 0.26	12.34 $\pm$ 1.92	15.39 $\pm$ 0.67	18.53 $\pm$ 0.51	26.67 $\pm$ 0.17		0.83
BDA	22.15 $\pm$ 0.69	8.88 $\pm$ 0.94	36.25 $\pm$ 0.50	16.24 $\pm$ 0.40	4.49 $\pm$ 0.10		—

allowance for this, the estimate of the percentage of total hydrolysate radioactivity that is recovered as free iodoamino acids is very much the same in the two-dimensional separation as in the one-dimensional.

An appreciable part of the radioactivity is identified in spots that show the characteristics of small peptides, the major portion of which have R_F values in the region of the iodotyrosines. The proportion of the hydrolysate found in such forms is smallest for the Pronase hydrolysis in the presence of thyroid homogenate.

Peptic hydrolysis of thyroid iodoprotein. The yields of iodoamino acids after peptic hydrolysis of ^{125}I -labelled thyroid homogenate at three different pH values are shown in Table 5. Iodoamino acid release by pepsin is maximal after about 6 hr. incubation.

Though not really suitable for the total hydrolysis of thyroglobulin, pepsin has the property of

selectively releasing the iodothyronines. Hydrolysis by pepsin at pH 5.2 produces the higher iodothyronine/iodotyrosine ratio, but this is mainly a reflection of the low iodotyrosine yield. It was found convenient to hydrolyse thyroglobulin preparations at pH 1.9 by acidifying the protein dissolved in water, rather than by dissolving it in the citrate buffers used in the preliminary trials.

Table 6 shows that iodothyronine release from heat-denatured thyroglobulin is virtually complete with pepsin (compare with hydrolysis by Pronase plus thyroid homogenate shown in Table 3), whereas only a low proportion of the iodotyrosines is released.

The large amount of radioactivity appearing in the position normally occupied by iodide on the chromatograms is probably peptide material, since further hydrolysis of the peptic digest by Pronase yields about 6% of the radioactivity in this position (Table 7). The total amount of radioactivity

Table 7. *Effect of peptic and subsequent Pronase hydrolysis on heat-denatured thyroglobulin*

Partially purified ^{125}I -labelled rat thyroglobulin was denatured by heating it at 80° for 5 min. It was hydrolysed with pepsin at pH 1.9 for 24 hr., and then, after readjustment of the pH to 8.0, it was incubated with tris-NaCl- CaCl_2 buffer, pH 8.0, and Pronase for 16 hr. The results of three separate experiments are given as means $\pm$ S.E.M. Experimental details are given in the text. For definition of abbreviations see Table 1.

Chromatography solvent	Radioactivity (% of total on chromatogram)						MIT/DIT ratio
	Origin	Iodide	MIT	DIT	T4	T3	
BA	1.17 $\pm$ 0.11	5.56 $\pm$ 0.22	31.63 $\pm$ 0.92	30.34 $\pm$ 0.59	17.63 $\pm$ 0.38		1.05
BDA	4.86 $\pm$ 0.14	7.28 $\pm$ 0.18	62.44 $\pm$ 0.57		13.52 $\pm$ 0.41	3.18 $\pm$ 0.19	—

accounted for is 84.2%, which compares favourably with the results for Pronase hydrolysis in Table 2, though it does not represent an improvement in hydrolysis. Pronase followed by peptic hydrolysis is found to be unsatisfactory, however.

DISCUSSION

Rosenberg & LaRoche (1964) concluded that iodothyronines were preferentially adsorbed on particulate matter in enzymic digests of thyroid material. Plaskett *et al.* (1963a) minimized iodothyronine loss by chromatographing methanol-aq. ammonia (sp.gr. 0.88) extracts of thyroid homogenate digests; this procedure was followed in the present work. Any origin material on the chromatograms represents unhydrolysed material and not free iodoamino acids non-specifically adsorbed on protein.

In view of the virtually complete hydrolysis of ^{125}I -labelled rat thyroid homogenates by Pronase and also by pancreatin, it is surprising to find that hydrolysis of partially purified heat-denatured thyroglobulin is not satisfactory. The results apparently show that enzymic hydrolysis of thyroid iodoprotein relies on the quite important but previously unrecognized role of thyroid enzymes present in the thyroid homogenate.

It is likely that thyroglobulin is hydrolysed mainly by the thyroid mast-cell enzyme at pH 8.0 or 8.5 *in vitro* (Pastan & Almquist, 1966a,b), rather than by the thyroidal proteases and peptidases that McQuillan, Mathews & Trikojus (1961) showed to have acid pH optima.

McQuillan *et al.* (1961) showed that heat denaturation of thyroglobulin at 80° for 4 min. destroys the thyroidal proteases still present in partially purified thyroglobulin. I have confirmed that this is true for my thyroglobulin preparation. Addition of an unlabelled fresh thyroid homogenate to heat-denatured thyroglobulin thus provides thyroidal proteases that augment hydrolysis by pancreatin or Pronase.

One-dimensional chromatography shows that enzymic hydrolysis of heat-denatured thyroglobulin with pancreatin or Pronase and fresh thyroid homogenate yields about 90% of the radioactivity as free iodoamino acids or iodide, and peptide 'mapping' shows that about 10% of the radioactivity is still peptide-bound. After peptide 'mapping' and development in BA solvent, most of the peptide material has approximately the same R_F value as the iodotyrosines. Some peptide material has the same R_F value as the iodothyronines, however, which accounts for the higher percentage of iodothyronines estimated from one-dimensional BA chromatograms than that estimated from BDA chromatograms (e.g. see Table 6). One-dimensional BA chromatograms thus yield too high estimates of both iodotyrosines and iodothyronines.

The specificity of Pronase (Hiramatsu, 1967) suggests that incomplete hydrolysis of thyroglobulin may be due to resistance of prolyl, cystinyl or aspartyl bonds to enzymic attack. Alternatively, the carbohydrate units of the glycoprotein may prevent access of the enzyme to peptide bonds (Murthy, Raghupathy & Chaikoff, 1965).

In an attempt to exploit the acid pH optima of the thyroidal proteases and peptidases, peptic hydrolysis of a ^{125}I -labelled thyroid homogenate was examined. There is no indication that the thyroidal enzymes augment hydrolysis by pepsin. It is unlikely that either (a) the buffer, (b) the omission of possible activating metal ions or (c) the conditions of incubation could decrease the activity of the thyroidal enzymes (Ekholm, Smeds & Strandberg, 1966; Kress, Peanasky & Klitgaard, 1966). The purified thyroidal protease with acid pH optimum attacks various synthetic substrates containing aromatic amino acids or aromatic and aliphatic amino acids (McQuillan, Stanley & Trikojus, 1954; Kress *et al.* 1966), which indicates that the protease has a specificity similar to that of pepsin. However, neither McQuillan *et al.* (1954) nor Kress *et al.* (1966) found preferential release of

the iodothyronines over the iodotyrosines by this thyroidal enzyme.

Pepsin is shown to release the iodothyronines completely from heat-denatured thyroglobulin, but a large amount of unhydrolysed material remains. Virtually complete hydrolysis of heat-denatured thyroglobulin is achieved by using pepsin followed by Pronase, though these results are not superior to those obtained with Pronase plus a source of thyroidal proteases.

Studies on peptic hydrolysis of synthetic substrates (Jackson, Schlamowitz & Shaw, 1965; Inouye, Voynick, Delpierre & Fruton, 1966) indicate that the active site of pepsin requires a non-polar group on either side of the bond to be split: charged groups near the pepsin-sensitive bond influence hydrolysis by inhibiting either binding or catalysis. Thus the results of peptic hydrolysis suggest that the iodothyronine residues are bound to other hydrophobic residues in the thyroglobulin molecule (see also Tang, 1963).

The conclusion is that the best procedure for the release of labelled iodoamino acids from labelled thyroglobulin is to use Pronase plus a non-radioactive thyroid homogenate. About 10% of the radioactivity then remains in a peptide form that migrates from the origin on one-dimensional chromatography. Peptic hydrolysis releases iodothyronine residues preferentially from peptide linkage, but hydrolysis of thyroglobulin is incomplete.

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REFERENCES

- Boyd, G. S. & Oliver, M. F. (1960). *J. Endocrin.* **21**, 25.
 Ekholm, R., Smeds, S. & Strandberg, U. (1966). *Exp. Cell Res.* **43**, 506.
 Gross, J., Leblond, C. P., Franklin, A. E. & Quastel, J. H. (1950). *Science*, **111**, 605.
 Hiramatsu, A. (1967). *J. Biochem., Tokyo*, **61**, 168.
 Inouye, K., Voynick, I. M., Delpierre, G. R. & Fruton, J. S. (1966). *Biochemistry*, **5**, 2473.
 Jackson, W. T., Schlamowitz, M. & Shaw, A. (1965). *Biochemistry*, **4**, 1537.
 Kress, L. F., Peanasky, R. J. & Klitgaard, H. M. (1966). *Biochim. biophys. Acta*, **113**, 375.
 McQuillan, M. T., Mathews, J. D. & Trikojus, V. M. (1961). *Nature, Lond.*, **192**, 333.
 McQuillan, M. T., Stanley, P. G. & Trikojus, V. M. (1954). *Aust. J. biol. Sci.* **7**, 319.
 Michl, H. (1951). *Mh. Chem.* **82**, 489.
 Murthy, P. V. N., Raghupathy, E. & Chaikoff, I. L. (1965). *Biochemistry*, **4**, 611.
 Pastan, I. & Almqvist, S. (1966a). *Endocrinology*, **78**, 350.
 Pastan, I. & Almqvist, S. (1966b). *J. biol. Chem.* **241**, 5090.
 Plaskett, L. G. (1964). *Chromat. Rev.* **6**, 91.
 Plaskett, L. G., Barnaby, C. F. & Lloyd, G. I. (1963a). *Biochem. J.* **87**, 473.
 Plaskett, L. G., Barnaby, C. F. & Lloyd, G. I. (1963b). *Biochem. J.* **89**, 479.
 Rosenberg, L. L. & LaRoche, G. (1964). *Endocrinology*, **75**, 776.
 Tang, J. (1963). *Nature, Lond.*, **199**, 1094.
 Tong, W. & Chaikoff, I. L. (1958). *J. biol. Chem.* **232**, 939.
 Tong, W., Raghupathy, E. & Chaikoff, I. L. (1963). *Endocrinology*, **72**, 931.