THE QUANTITATIVE DETERMINATION OF THE IODO-AMINO ACIDS OF THYROID TISSUE

By T. H. KENNEDY*

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Summary

The ability of three different pancreatin preparations to liberate iodo-amino acids from thyroid tissue has been studied. Two of the pancreatin preparations, while freeing substantial amounts of the iodotyrosines, released only a part of the thyroxine.

A method of sodium hydroxide hydrolysis is described which produces less destruction of organic iodine-containing compounds, with reduced formation of iodide and identifiable artefacts.

The third pancreatin, a total extract of pig pancreas, gave thyroxine values in good agreement with those obtained by the modified alkaline method.

Most hydrolysates were analysed both by column and paper chromatographic methods, and the similarity of the values obtained indicates that both methods may be regarded as satisfactory.

I. INTRODUCTION

The classical methods of alkaline hydrolysis of thyroid tissue or thyroglobulin as a preliminary to the determination of the iodinated amino acids have been discarded in recent years in favour of enzymic methods although little direct comparison of the two methods has been made. Braasch, Flock, and Albert (1954) have compared the action of trypsin and sodium hydroxide on human thyroid tissue, and concluded, on the bases of apparent incompleteness of hydrolysis and high iodide formation, that sodium hydroxide was inferior to tryspin, although in only one case was tissue from the same patient hydrolysed by both methods. Furthermore five samples of tissue from the same source gave, after trypsin hydrolysis, thyroxine values of 4–26 per cent. and di-iodotyrosine values of 17–36 per cent. of total iodine (two of the determinations gave thyroxine values of 20 and 26 per cent. and di-iodotyrosine values of 36 and 36 per cent. of total iodine). This wide range of values, for which no explanation is apparent, would not justify the use of tryptic hydrolysis as part of a quantitative procedure.

In the course of another investigation it was found that sodium hydroxide liberated nine times as much thyroxine from a rat thyroid homogenate as did a commercial trypsin preparation. The chromatographic system used was *tert.*-pentanol–ammonia in which the artefacts produced by the action of alkali on the iodinated tyrosines are separated from thyroxine (Stanley 1953; Kennedy 1957). This finding has led to an investigation of the ability of some different pancreatin preparations to release the iodo-amino acids from thyroglobulin. The amounts liberated have been compared with the amounts liberated by a modified sodium hydroxide hydrolysis.

*Endocrinology Research Department, New Zealand Medical Research Council, Medical School, University of Otago, Dunedin, N.Z.

II. Materials and Methods

Rats were killed 8–96 hr after the injection of ^{131}I the dose of which was calculated so that the thyroids contained not more than 7 μc at the time of maximum uptake. Thyroids were removed immediately after death of the rat and placed in a weighing bottle in ice. After weighing, the pooled glands were ground in a small mortar, previously cooled in ice, with 0·1 ml of water, 10–20 mg of glass powder, and 200 μg of methyl thiouracil or thiourea. This addition was made to prevent artefact formation during the process of homogenizing and dividing for analysis (Taurog, Potter, and Chaikoff 1955). The suspension was then diluted with water to contain 50–100 mg tissue per ml.

In some experiments, the iodide and free iodo-amino acids of the gland were removed by five extractions with an equal volume of *n*-butanol after dilution with an equal volume of water and adjustment of the pH to about 2·5 with 0·1N HCl. Another 200 μg of methyl thiouracil or thiourea was added to the aqueous phase and precipitated protein, which were then dried from the frozen state after removal of butanol by vacuum distillation at 30°C. When required, the dry residue was taken up in an appropriate amount of water, adjusted to pH 8–8·3 with 0·1N NaOH, and the suspension divided for enzyme and sodium hydroxide hydrolysis.

(*a*) *Sodium Hydroxide Hydrolysis*

The thyroid homogenate containing up to 100 mg tissue was placed in a tube 8–10 mm in internal diameter, made 2N with respect to sodium hydroxide by the addition of 10N reagent, and then diluted with 2N NaOH to 4–5 ml. A further addition of about 100 μg of methyl thiouracil or thiourea was made, and after thorough degassing at the water pump, the tube was sealed under vacuum. The sealed tubes were heated in an oven at 98–100°C for 16–18 hr.

In early experiments the hydrolysate was acidified to pH 2 with hydrochloric acid and extracted repeatedly with an equal volume of *n*-butanol until less than 5 per cent. of the total ^{131}I was left in the aqueous phase. Concentration of the pooled butanol extracts at 30°C gave a residue which contained too much salt for satisfactory paper chromatograms to be run and it was found necessary to dilute the concentrate with water and repeat the butanol extraction. A simpler method of removing the excess sodium ion was to run the hydrolysate through a column of the cation-exchange resin "IRC 50" in the acid form. The column was then washed with 1·0N NH_4OH until the residual ^{131}I was less than 1 per cent. of the total. The effluent was concentrated *in vacuo* at 30°C nearly to dryness, when the solution usually became semi-solid with colloidal silica. The solid was extracted two or three times with 1 ml 90 per cent. methanol–water (v/v) 0·1N with respect to ammonia, and filtered through a small cotton-wool plug. The methanol solution was taken to dryness *in vacuo*.

(*b*) *Enzyme Hydrolysis*

Three different preparations of pancreatin were used, two of which, A and B, were commercial preparations. The third, C, was prepared by extracting minced fresh pig pancreas with two volumes of water overnight. After centrifuging, the

supernatant was dried from the frozen state. The dry powder was extracted twice with anhydrous ether, dried in air, taken up in a smaller amount of water, centrifuged, and dried again from the frozen state.

About 5 mg of pancreatin was added to 1 ml of homogenate containing 50–100 mg thyroid tissue, the pH adjusted to 8–8·3 with sodium hydroxide, and the digest kept at 37°C. Where aliquots were taken at intervals they were frozen until analysed. Some enzyme digests were dried from the frozen state before analysis.

In some experiments the homogenate was heated for 2–3 min in a boiling water-bath to inactivate thyroid enzymes, particularly the dehalogenase (Roche *et al.* 1953) which it was thought might be contributing to the inorganic iodide formed during hydrolysis.

TABLE 1

APPROXIMATE R_F VALUES IN *tert.*-PENTANOL–AMMONIA AND *tert.*-PENTANOL–ACETIC ACID SYSTEMS

Compound	R_F in:	
	tert.-Pentanol–Ammonia	*tert.*-Pentanol–Acetic Acid
Thyroxine	0·30	0·85
3,5,3′-Tri-iodothyronine	0·50	
3,5-Di-iodotyrosine	<0·1	0·45
Mono-iodotyrosine	<0·1	0·37
Iodide	0·15	0·11
3-Iodo-4-hydroxybenzaldehyde	0·50	0·85
3,5-Di-iodo-4-hydroxybenzaldehyde	0·65	

(c) *Analysis of Hydrolysates*

(i) *Column Chromatograms.*—Dry residues were taken up in 2·5N NaOH, and enzyme digests which were not dried were made 2·5N by addition of 10N NaOH, kieselguhr and a little mobile phase added, and the slurry packed on top of a prepared column. Columns were run as described by Kennedy and Purves (1956) with a modified solvent system, the *tert.*-butanol being followed by *tert.*-butanol–*n*-propanol mixtures instead of the *n*-propanol–*cyclo*hexane mixture previously described. Added carrier compounds were located as previously described and the activity of each fraction measured by γ-counting. Fractions were pooled for final counting on the basis of radioactivity and carrier distribution. The combined fractions were evaporated to 3–4 ml before counting.

(ii) *Paper Chromatograms.*—Whatman No. 1 paper, 10 × 40 cm was used. The paper was cut to give five parallel strips separated by 2-mm spaces.

The solvent systems used were *tert.*-pentanol–2N NH_4OH (1 : 1 v/v) (Gleason 1955) for measurement of thyroxine, 3,5,3′-tri-iodothyronine, and iodide, and *tert.*-pentanol–acetic acid-water (9 : 1 : 10 v/v) for measurement of iodide and the two iodinated tyrosines. The aqueous phase of the acid system was saturated before each run with hydrogen sulphide. Approximate R_F values are given in Table 1.

Iodide determinations obtained from the acetic acid system were in close agreement with those from the ammonia system, and only the latter are given.

TABLE 2
DETERIORATION OF PANCREATIN A WITH TIME

Hydrolysis	Chromatographic Analysis	Iodo-amino Acids as Percentage of Total Thyroid ^{131}I				
		T_4*	T_3*	DIT*	MIT*	Iodide
Early experiments:						
Pancreatin A, 48 hr	Paper	6·6	1·2	19·0	36·8	3·4
	Column	6·7	1·4	18·9	32·8	3·6
Experiments 6 months later						
Pancreatin A, 96 hr	Paper	0·8	0·0	21·2	33·8	4·6
2N NaOH, 16 hr†	Paper	7·3	1·4	26·2	25·0	24·7

*In this and subsequent tables, T_4, T_3, DIT, and MIT refer to thyroxine, 3,5,3′-tri-iodothyronine, 3,5-di-iodotyrosine, and mono-iodotyrosine respectively.

†Old method of NaOH hydrolysis.

Dry residues from the hydrolysates were taken up in 0·1N NH_4OH and, after thorough grinding with a glass rod, 2–10 μl of the suspension applied in a band across

TABLE 3
COMPARISON OF AMOUNTS OF IODO-AMINO ACIDS LIBERATED FROM A RAT THYROID HOMOGENATE BY PANCREATINS B AND C AND BY SODIUM HYDROXIDE

Hydrolysis	Time (hr)	Chromatographic Analysis	Iodo-amino Acids as Percentage of Total Thyroid ^{131}I				
			T_4	T_3	DIT	MIT	Iodide
Pancreatin B	24	Paper	4·4	0·7	27·5	29·4	4·2
	48	Paper	5·7	0·7	29·1	26·3	4·2
Pancreatin C	24	Paper	8·0	1·2	43·0	25·0	3·9
	48	Paper	8·6	1·4	41·8	25·5	4·6
	48	Paper	7·9	1·5	39·8	24·0	4·4
	48	Column	8·2	1·2	42·6	20·4	3·9
2N NaOH	16	Paper	12·4	1·6	37·4	16·1	8·1
	16	Column	10·1	2·3	40·0	18·6	8·3

the 2-cm strip. Enzyme digests were applied directly to the paper in the same quantity. After drying, the papers were left in the tank for 1–2 hr before application of the

TABLE 4

COMPARISON OF AMOUNTS OF IODO-AMINO ACIDS LIBERATED FROM DIFFERENT THYROID HOMOGENATES BY PANCREATIN C AND SODIUM HYDROXIDE

Homogenate	Hydrolysis	Time (hr)	Chromatographic Analysis	Iodo-amino Acids as Percentage of Total Thyroid ^{131}I				
				T_4	T_3	DIT	MIT	Iodide
1	Pancreatin C	24	Paper	14·2	2·1	33·6	30·8	3·1
		48	Paper	15·6	2·4	35·9	29·3	3·5
		96	Paper	14·6	3·1	38·6	27·6	8·5
		96	Paper	14·1	2·9	37·8	28·6	8·1
		96	Column	13·2	3·6	37·5	27·0	7·3
	2N NaOH	17	Paper	14·7	1·9	27·2	18·6	8·2
	2N NaOH	17	Paper	13·2	1·9	26·3	19·3	7·2
2	Pancreatin C	96	Paper	11·9	6·6	16·3	32·8	4·9
		96	Paper	12·3	6·5	15·3	32·7	5·5
		96	Column	13·5	8·7	13·6	34·3	4·7
	2N NaOH	16	Paper	8·2	7·3	18·8	34·0	12·3
3	Pancreatin C	48	Paper	12·3	2·0	39·4	18·0	9·6
		48	Paper	10·8	2·5	41·1	18·2	10·9
	2N NaOH	16	Paper	11·7	2·1	28·4	12·3	15·5

mobile phase. The chromatograms were run for 16–20 hr after which time the solvent front was about 25 cm from the origin. Mixtures of known substances were run on parallel strips, and all films were autographed on Ilford X-ray paper to check the separation. The papers were cut up for counting on the basis of the autograph and the separate pieces counted under a thin end-window tube (General Electric Co. 2B7).

III. Results

One sample of commercial pancreatin, A, liberated considerable amounts of 3, 5-di-iodotyrosine and mono-iodotyrosine from thyroid tissue but only 1 per cent. of the total iodine was present as thyroxine at 48 hr. Further addition of enzyme and readjustment of the pH did not produce any substantial alteration in the

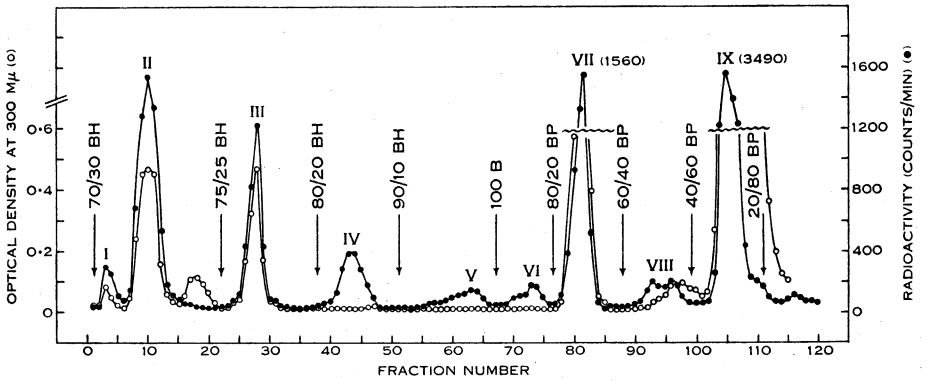

Fig. 1.—Column chromatogram of pancreatin digest of rat thyroid homogenate 2. Solvent changes were made at the points indicated. BH, *tert.*-butanol–*cyclo*hexane (v/v) mixture; BP, *tert.*-butanol–*n*-propanol (v/v) mixture. The radioactivity of the fractions is shown by the solid circles, and the optical density at 300 mμ, giving the distribution of the carriers, is shown by open circles. Identified peaks are: II, thyroxine; III, 3,5,3′-tri-iodothyronine; IV, iodide; VII, 3,5-di-iodotyrosine; and IX, mono-iodotyrosine. The small absorption peak between II and III is the methyl thiouracil added to the digest. Values in parenthesis give the maximum radioactivity for the respective peaks. The companion ultraviolet absorption peaks were off the scale.

proportion of these amino acids. Subsequent alkaline hydrolysis of this digest liberated 7·3 per cent. of the total iodine as thyroxine. In experiments carried out some months earlier this pancreatin had given much greater amounts of thyroxine from similar rat thyroids, although the results had not been compared with those of alkaline hydrolysis. The results are given in Table 2.

The amounts of the different iodo-amino acids liberated by the enzyme preparations B and C from the same thyroid homogenate are shown in Table 3, together with the amounts liberated by modified sodium hydroxide hydrolysis. Further comparison of the values obtained by the action of enzyme preparation C and sodium hydroxide on different thyroid homogenates is given in Table 4.

The method of sodium hydroxide hydrolysis used here differs in three respects from that used previously (i) in the presence of small quantities of thiourea or methyl thiouracil, (ii) in the reduction of the amount of oxygen present, and (iii) in the increased ratio of sodium hydroxide solution to protein. No attempt has been

made to assess the influence of these factors individually but the system described did not produce identifiable artefacts, and the amount of iodide formed was less than that frequently encountered with the old method where the volume of sodium hydroxide was less and air was not excluded.

Pooled concentrated fractions from the column analysis of homogenate 2, the effluent curve of which is shown in Figure 1, were run through an "IRC 50" column which was washed with dilute ammonia as described earlier, and the effluents dried from the frozen state. The residues were taken up in 0·1 ml of 0·1N NH_4OH and aliquots run on paper with the two solvent systems described. The papers were autographed and then sprayed to locate the carriers. The thyroxine, tri-iodothyronine, iodide, di-iodotyrosine, and mono-iodotyrosine peaks all gave a strong radioactive band corresponding in position with the carrier, in both solvent systems. In all the iodo-amino acid fractions faint iodide bands were also present. The first column peak, fractions 1–6, when run on paper showed faint bands on the origin, and in the iodide position in both solvents. There were no bands corresponding to the iodohydroxybenzaldehydes (Kennedy 1957). No identifiable bands, other than traces of iodide, were found in the paper runs of the other column peaks.

IV. Discussion

The chief objections to the conventional method of alkaline hydrolysis have been the production of artefacts and the liberation of considerable amounts of iodide. The artefacts in some solvent systems run in the same position as thyroxine and lead to high values. Thus Roche, Michel, and Volpert (1954) concluded that all of the apparent thyroxine found in an alkaline hydrolysate of casein iodinated enzymically by the procedure of Keston (1944) was an artefact as it was not produced during enzyme hydrolysis. Taurog *et al.* (1955) found that trypsin hydrolysis of sheep thyroid slices gave thyroxine values lower than those obtained in earlier work where sodium hydroxide was used. This error may be avoided by the use of a chromatographic system which separates the artefacts from thyroxine, but the very presence of these substances, together with inorganic iodide, which is frequently 20–30 per cent. of the total iodine, is evidence of destruction during the hydrolytic procedure. Using such systems very low thyroxine values, amounting to about 1 per cent. of the total iodine, have been frequently obtained, particularly in hyperplastic thyroids where the total iodine content is low (Kennedy and Purves 1956).

The modified sodium hydroxide hydrolysis described here reduces the magnitude of some of these errors. The iodide found is considerably less than that produced by the conventional method although in general it is higher than that found in enzymic hydrolysates. The thyroxine values, in most cases, are in reasonable agreement with those found after hydrolysis by a total pancreas extract preparation. In addition, in no instance has any detectable amount of either of the iodohydroxybenzaldehydes been encountered (Kennedy 1957).

Two of the enzyme preparations used liberated mono- and di-iodotyrosine and thyroxine, and in the acetic acid–pentanol system only traces of radioactivity were left on the origin in the thyroglobulin position. The amount of thyroxine, however, was less than that obtained after alkaline hydrolysis. The third enzyme

preparation gave thyroxine values in good agreement with those obtained by the modified sodium hydroxide method and there was also a reasonable agreement between the figures obtained from either hydrolysate when analysed by two different chromatographic systems. The values for the iodinated tyrosines after chemical hydrolysis were sometimes much lower than those after enzymic hydrolysis. In this case the latter values are to be preferred since, for a given enzymic hydrolysate, the different analytical systems gave substantially the same result.

Trypsin, like sodium hydroxide, liberated variable but significant amounts of iodide from thyroid tissue. This was found in enzymic hydrolysates of thyroglobulin which had been previously extracted with butanol, as well as in those which had been heated to destroy the thyroid dehalogenase system. Most of this iodide must be formed during the hydrolysis since different methods of analysis gave closely similar results. This would be unlikely if it were formed during the separation procedures. This iodide probably arises by spontaneous breakdown in solution of the major constituents, as these fractions from column analyses always contain some iodide when they are run on another column or on paper.

The failure of some trypsin preparations to liberate all of the thyroxine, although substantial amounts of the iodo-tyrosines are produced, may be responsible for some of the very low thyroxine values reported in the literature, e.g. the value of 0·45 per cent. total iodine reported by Stanley (1956) for normal human thyroglobulin.

It is suggested that enzyme preparations should be checked from time to time for their ability to release thyroxine, by comparison either with the alkaline method described here or with another enzyme preparation.

V. References

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