A STUDY OF THE ACTION OF PURIFIED THYROID PROTEASE ON ^{131}I-LABELLED THYROGLOBULIN

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Summary

The preparation of pig thyroglobulin labelled with ^{131}I is described and the results of the action of purified thyroid protease on different samples are presented. The major iodine-containing compounds liberated were found to be mono- and di-iodotyrosine, thyroxine, and tri-iodothyronine, together with a "peptide" fraction; of these mono-iodotyrosine, di-iodotyrosine, and the "peptide" predominated. Specific activities of these components were determined and compared with the values obtained following alkaline hydrolysis of the labelled protein.

A radioactive spot of intensity similar to that of thyroxine and of slightly lower R_F value in the solvent system used was also observed. Its identity has not yet been established.

Preliminary experiments with labelled synthetic iodopeptides as substrates indicate that the protease possesses peptidase activity at acid pH which may be preferentially concerned with the fission of bonds joining aromatic amino acid residues; the thyroid enzyme would thus behave, in this respect, similarly to pepsin.

I. INTRODUCTION

The application of the radioiodine technique has contributed much in recent years to our knowledge of the processes underlying thyroid function both from the experimental and clinical angles. Not only does it enable an assessment to be made of the level of thyroid function *in vivo* but, in association with partition chromatography and allied techniques, the unravelling of many of the details of the biochemical reactions involved has been accomplished using comparatively small amounts of material.

The investigations described in this paper have been concerned with the action of a purified protease isolated from the thyroid gland (McQuillan and Trikojus 1953) on thyroglobulin biologically labelled with ^{131}I. These studies are an extension of those forming the subject of a preliminary report to the Oxford Isotope Conference, 1951 (Gordon *et al.* 1953). Roche and colleagues (Roche *et al.* 1950; see also Michel 1952) have also investigated the proteolysis of labelled thyroglobulin, using unpurified glycerol extracts of thyroid tissue as the source of enzyme. In several important aspects, our results have been in line with those of the French workers.

In order to understand further the mechanisms involved, an investigation of the action of the purified enzyme on synthetic ^{131}I-labelled and other peptides has been initiated and some preliminary results are also included in this paper.

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II. Materials and Methods

Thyroid protease.—This was prepared and tested according to McQuillan and Trikojus (1953). The enzyme preparations used had proteolytic activities in the range 25-40 units*/mg.

Radioiodine.—This was supplied in solution as $Na^{131}I$, carrier-free.

Labelled thyroglobulin was prepared from the thyroids of pigs which had been injected with $Na^{131}I$ and was purified as described in the experimental section. Since it was found (McQuillan and Trikojus 1953) that thyroglobulin purified in this manner still retained proteolytic activity, each preparation was tested, using haemoglobin as substrate, as for purified thyroid protease. As the rate of reaction of the protease with haemoglobin follows a straight line over 2 hr, the period of incubation at 37°C was extended to 90 min in order to conserve the labelled thyroglobulin.

Iodoamino acids, which served to identify spots on the chromatograms, were recrystallized samples obtained by standard methods. A specimen of synthetic 3,5,3′-tri-iodothyronine was kindly provided by Glaxo Pty. Ltd. (England); one of the same substance labelled in the 3′-position was prepared from 3,5-di-iodothyronine by the micro-iodination procedure of Lemmon, Tarpey, and Scott (1950). *The peptides* used were synthetic samples as indicated in the text.

Iodine (^{127}I) values were determined by the method of Barker (1950).

pH.—Throughout the work, pH was checked with the glass electrode.

Chromatography.—Solvents for use in chromatography were purified by standard procedures. Solvent systems used were: phenol-water with an atmosphere of ammonia (Partridge 1948); *n*-butanol-*n*-pentanol-ammonia in the volume proportions 1 : 1 : 2; *n*-pentanol-propionic acid-water in the volume proportions 20 : 3 : 15. These systems are subsequently referred to in the text as PWA, BPA, and PPW respectively.

Radioactivity was measured by standard methods. Solutions, after suitable dilution, were assayed in M12, M6, or M6H Veall tubes (20th Century Electronics) by comparison with standard solutions of known radioactivity. In certain instances chromatograms of extracts of enzyme-treated labelled thyroglobulin, prepared on washed No. 1 or No. 3 Whatman paper, were scanned, after removal of the solvent, at 1 cm intervals using 1 min counts; the papers were held in a lead-shielded carrier fitted at the top level of the "Perspex" frame of a standard lead castle under a GM4 tube (Oddie and Mibus 1949); more usually radioautograms were obtained by contact with "No-screen" X-ray film ("Kodirex" or "Ilfex").

* One unit is the amount of enzyme required to liberate 10^{-4} m-equiv. tyrosine in 30 min at 37°C (haemoglobin substrate).

III. Experimental Procedure

(*a*) *Preparation of ^{131}I Thyroglobulin*

Young pigs weighing 17-40 kg were injected subcutaneously in the abdominal region with measured doses of purified carrier-free ^{131}I. Twenty-four hr later the animals were killed by bleeding from the shoulder. The thyroid glands, after clean dissection, weighing, and sampling, were thinly sliced in the frozen state and extracted twice with saline (3 vol. and 1 vol. respectively). The extract was then put through a single fractionation run by the method of Derrien, Michel, and Roche (1948), modified in that the equilibration period at each concentration of ammonium sulphate was shortened from 12 to 5 hr. The 37-41 per cent. fraction was rapidly dialysed and freeze-dried. The distribution of ^{131}I was followed throughout, the various fractions being dissolved, where necessary, in 2N NaOH before counting. Results obtained in seven preparations together with values for ^{127}I content and proteolytic activity of the final products are listed in Table 1. The more reliable uptake figures recorded in this table are considered to be those calculated in each case from the sum of the radioactivity values of the individual fractions; those derived from the small assay samples can only be approximate.

(*b*) *Preparation of Enzyme Digests and Extracts*

Aqueous solutions of thyroglobulin and of protease at pH 3·5 were prepared by adjustment with 2N or 0·5N acetic acid. The requisite volumes of substrate and of enzyme were then mixed, toluene added as preservative, the tubes stoppered and shaken at 37°C (usual period of incubation 24 hr). Experiments were usually controlled in two ways: (i) by a sample containing added enzyme maintained at 0°C during the incubation period; (ii) by one in which the added enzyme was inactivated by heating at pH 3·5 and 100°C for 15 min before mixing with the solution of thyroglobulin. At the conclusion of the incubation period, ethanol was added to 70 per cent. concentration and the precipitate, which was readily flocculated by the addition of a micro-drop of conc. NH_4OH, was removed by centrifuging and washed twice with minimum amounts of 70 per cent. ethanol.

(*c*) *Chromatography of Extracts*

An extract to be chromatographed was evaporated to dryness under reduced pressure and the residue dissolved in the minimum quantity of a solution composed of ethanol:conc. NH_4OH:water in the proportions 25 : 1 : 25. This material was then applied to washed Whatman No. 1 or No. 3 paper either as a spot or, more frequently, as a continuous line, particularly when relatively large amounts of material were to be chromatographed. After comparison with the corresponding radioautogram, the radioactive areas were traced on the paper strips, these sections were then cut into small pieces and eluted overnight at 0°C with 0·1N NH_4OH plus a few drops of toluol. The eluates were then treated as were the initial extracts and rechromatographed either in the

same or in another solvent system. Radioactive components on chromatograms were identified by coincidence in position and shape with ninhydrin-reacting spots which were formed from authentic iodoamino acids added to the extract before chromatography; when specific activities were to be determined, the mixture of unlabelled iodoamino acids was applied in an adjacent position. R_F values are given singly, from the centres of small discrete spots, or as a range to define the limits of larger diffuse areas.

IV. Results

The results of preliminary experiments indicated that there is a well-marked action of the protease on thyroglobulin leading to the liberation of 3-iodotyrosine, 3,5-di-iodotyrosine, thyroxine (in small amount), and peptides containing iodoamino acids, apart from iodine-free fragments. Later experiments, involving larger amounts of labelled thyroglobulin of higher specific activity, were designed to obtain more quantitative data and additional information as to the products of hydrolysis. Details of the experiments using the last three preparations of thyroglobulin (*RA*6, *RA*13, *RA*21; see Table 1) are reported below.

(*a*) *Experiment* 1. *Thyroglobulin Preparation* RA6 *and Enzyme Preparation* E17 (39 *"units"/mg*)

The bulk of the labelled thyroglobulin was submitted to the action of the protease as follows:

	Thyroglobulin	Enzyme	Treatment
E_{37}	253·3 mg; 16·0 ml	32 mg; 3·2 ml	37°C; 24 hr
e_{37}	15·8 mg; 1·0 ml	2 mg; 0·2 ml	37°C; 24 hr
Control (*C*)	15·8 mg; 1·0 ml	2 mg; 0·2 ml (heat-inactivated)	37°C; 24 hr

The digest (e_{37}) was included in order to effect a more exact comparison with the control (*C*). Following incubation, proteins were precipitated at room temperature and the radioactive contents of the 70 per cent. ethanol supernatants and residues determined. Results are set out in Table 2. It is apparent that the ^{131}I contents of the ethanol supernatants of the test samples, containing added active enzyme, were more than twice that of the control, where the radioactivity of the 70 per cent. ethanol-soluble extract was presumably due to the action of enzyme residual in the thyroglobulin substrate (for enzyme activities of the thyroglobulin preparations see Table 1).

(i) *Treatment of Ethanol Extracts.*—Enzyme digest (*E*) was applied to a number of paper sheets and chromatographed in phenol-water-ammonia (PWA); the amount applied was 76·6 μc. Three radioactive zones were eluted (with centres of maximum intensity at R_F 0·63 (*L*), 0·81 (*M*), and 0·94 (*H*)—

the radioactivity recovered being 40·3, 10·9, and 3·18 μc respectively). The eluates were then re-run in PWA. Since further investigation indicated that the zone of radioactivity at R_F 0·94 was an artefact (possibly by phenol-iodine interaction) this material was discarded. Radioactive zones *L* and *M* were again cut out, care being taken to exclude mutual contamination, and eluted. These eluates contained 24·4 and 7·69 μc respectively, representing recoveries of approx. 80 per cent. of material applied. They were evaporated to small volumes and one-third of each was taken for immediate chromatography in *n*-butanol-*n*-pentanol-ammonia (BPA) (see below). To each remaining two-thirds an equal volume of 4N NaOH was added and the alkaline solutions obtained then divided into two equal parts: one-half was maintained at 0°C, the other at 105°C for 16 hr. At the conclusion of this period, the alkaline solutions were extracted three times with an equal volume of butanol, and the butanol extracts, after partitioning three times against water (to reduce the ionic content), were counted and chromatographed in BPA. Water used for washing the butanol extracts was adjusted to pH 10 with NH_4OH.

In order to isolate di-iodotyrosine and related compounds the residual solutions resulting from the above procedure were adjusted to pH 3·5 with 2N HCl, and butanol extraction, washing (with water at pH 3·5 (HCl)), radioactivity estimation, and chromatography repeated as above.

The main chromatographic findings but not the quantitative results are presented since it was found that the water washings of the butanol extracts contained a high proportion of the initial activity of these extracts. The procedure was, therefore, modified in subsequent experiments.

(ii) *Chromatographic Identification of the Components of Zones* L *and* M.—The results obtained by chromatography of the purified zones before and subsequent to alkali treatment (at 0° and 105°C) established the presence of mono-iodotyrosine, di-iodotyrosine, thyroxine, tri-iodothyronine, and two unknown *n*-butanol-soluble components which migrated more rapidly than thyroxine in the solvent system BPA (Plate 1, Figs. 1, 2, and 3). The position occupied by tri-iodothyronine corresponded to that observed in earlier work by Hird and Trikojus (1948), using a similar solvent system. Presence of peptide material was suggested by the increase in intensity of the thyroxine and of the unidentified spots following alkaline hydrolysis. The two unknown radioactive spots in Plate 1, Figure 2, have been shown in later work (Stanley 1953) to be artefacts formed from di-iodotyrosine (and mono-iodotyrosine) when their alkaline solutions are heated.

In Experiments 2 and 3, initial resolution with BPA followed by rechromatography of the individual fractions in either BPA or PPW (pentanol-propionic acid-water) was substituted for the preliminary chromatographic separation in PWA as used in Experiment 1. Specific activities (μc $^{131}I/\mu g$ ^{127}I) of the main ethanol-soluble components of the enzyme digests were also determined and compared with the values obtained for these compounds following alkaline hydrolysis of the same sample of labelled thyroglobulin.

TABLE 1

LABELLED PIG THYROGLOBULIN (^{131}I) PREPARATIONS. SUMMARY OF DISTRIBUTION OF ^{131}I BETWEEN FRACTIONS AND OTHER RELEVANT DATA

	*RA*2*		*RA*3		*RA*4		*RA*5		*RA*6*		*RA*13		*RA*21	
	μc	%†	μc	%	μc	%	μc	%	μc	%	μc	%	μc	%
$Na^{131}I$ injected	(*a*) 500; (*b*) 521		488		505		500		(*a*) 743; (*b*) 743		1050		969	
Distribution in Thyroid Fractions:														
Assay samples	1·7		2·1		5·0		0·6		3·1		7·3		6·6	
Saline residues	9·9	8·1	12·7	7·8	10·4	8·0	4·6	5·7	62·0	11·3	15·3	10·0	30·2	13·1
Supernatant (42% $(NH_4)_2SO_4$)	9·7	7·9	15·1	9·2	5·7	4·4	2·1	2·7	20·5	3·7	12·5	8·2	6·5	2·8
Precipitate (37% $(NH_4)_2SO_4$)	32·2	26·3	40·4	24·7	38·8	29·9	18·1	22·5	94·1	17·2	19·0	12·4	49·1	21·5
Supernatant (41% $(NH_4)_2SO_4$)	6·1	5·0	13·5	8·3	10·6	8·2	0·6	0·7	49·0	8·9	25·0	16·4	11·9	5·2
Thyroglobulin (37-41% $(NH_4)_2SO_4$)	64·8	52·7	81·8	50·0	64·1	49·5	55·6	68·5	322·9	58·9	81·3	53·2	132·0	57·4
Total uptake														
(i) By addition	124·4	12·2	165·6	33·9	134·6	26·7	81·6	16·3	551·6	37·1	160·4	15·3	236·3	24·4
(ii) Calc. on assay sample	149		220		184		133		709		233		295	
Wt. pig (kg)	(*a*) 17; (*b*) 26		—		—		22		(*a*) 40; (*b*) 40		35		48	
Wt. of dissected thyroid gland (g)	(*a*) 1·95; (*b*) 2·47		1·70		4·36		2·41		(*a*) 3·49; (*b*) 3·46		2·95		3·07	
Wt. of thyroglobulin (Tg) (mg)	242		193		219		104		414		162		251	
^{127}I content of Tg (%)	0·86		0·34		0·75		0·48		0·40		0·52		0·77	
Proteolytic activity of Tg‡	2·1		2·4		3·3		2·9		2·0		3·8		3·1	

* Two pigs injected. (In *RA*2 the injections were made intraperitoneally, not subcutaneously; that given to the second animal was presumably lost in the bladder as the uptake was only 5·3 μc (calc. on assay sample). In *RA*6 the respective uptakes were 372·4 and 336·3 μc.)

† With the exception of the values for total uptake, which are based on the quantities injected, the figures in the percentage columns relate to the contribution of the particular fraction to the total radioactivity recovered.

‡ Units expressed as m-equiv. × 10^4 of tyrosine liberated per mg thyroglobulin.

(*b*) *Experiment* 2. *Thyroglobulin Preparation* RA13 *and Enzyme Preparation* E22 (37 *"units"/mg*)

(i) *Enzymic Hydrolysis*

Enzymic hydrolysis of thyroglobulin was carried out as in Experiment 1. The following tests were set up:

	Thyroglobulin	Enzyme	Treatment
E_{37}	59 mg; 4·00 ml	12 mg; 1·2 ml	37°C; 24 hr
Control (E_0)	59 mg; 4·00 ml	12 mg; 1·2 ml	0°C; 24 hr
Control (C)	4·9 mg; 0·33 ml	1 mg; 0·1 ml (heat-inactivated)	37°C; 24 hr

At the conclusion of the 24-hr period, proteins were precipitated and the radioactivity of the 70 per cent. ethanol supernatants and residues determined. Results are recorded in Table 2. The fraction of the total radioactivity recovered in the 70 per cent. ethanol-soluble supernatant of the test sample maintained at

TABLE 2

DISTRIBUTION OF RADIOACTIVITY FOLLOWING ETHANOL PRECIPITATION OF ENZYMIC DIGESTS OF LABELLED THYROGLOBULIN (EXPERIMENTS 1, 2, AND 3)

Test	Activity of Thyroglobulin Sample (μc)	Soluble in 70% Ethanol		Insoluble in 70% Ethanol		Total ^{131}I Recovered (μc)
		μc	%	μc	%	
Experiment 1						
Digest at 37°C (major sample)	197·3	79·32	38·1	129·0	61·9	208·3
Digest at 37°C (minor sample)	12·33	4·94	38·0	8·09	62·0	13·03
Control digest at 37°C (added enzyme heat-inactivated) ..	12·33	1·68	15·3	9·33	84·7	11·01
Experiment 2						
Digest at 37°C ..	29·7	12·3	37·5	20·5	62·5	32·8
Digest at 0°C ..	29·7	2·51	8·4	27·5	91·6	30·0
Digest at 37°C (added enzyme heat-inactivated)	2·45	0·48	20·4	1·87	79·6	2·35
Experiment 3						
Digest at 37°C ..	57·6	12·3	19·7	50·4	80·3	62·7

37°C (E_{37}) is seen to be about twice that of the control (C), with heated enzyme, and more than four times that of the control sample (E_0) maintained at 0°C.

Treatment of ethanol extracts and chromatographic identification.—The three extracts were applied to paper and chromatographed in BPA. Five radioactive zones were apparent (at R_F 0·02 (IA), 0·07 (IIA), 0·13 (IIIA), 0·16-0·30 (IVA), 0·38-0·48 (VA)) in the radioautogram of the E_{37} digest (see Plate 2, Fig. 4), whereas in the corresponding radioautograms of the controls (E_0 and C), the first three zones were well marked but no radioactivity was detected in the regions of R_F 0·16-0·30 and 0·38-0·48. However, chromatograms of all three samples were cut in a similar manner at the limits of the radioactive zones in E_{37} and the radioactivity, after elution, estimated. Results are set out in Table 3.

TABLE 3

RADIOACTIVITY OF VARIOUS ZONES ELUTED FROM CHROMATOGRAMS (*n*-BUTANOL-*n*-PENTANOL-AMMONIA) OF 70 PER CENT. ETHANOL EXTRACTS OF ENZYME DIGESTS (EXPERIMENTS 2 AND 3)

	μc	%*	μc	%	μc	%	μc	%	μc	%
Experiment 2	Zone I*A*† R_F 0·02		Zone II*A* R_F 0·07		Zone III*A* R_F 0·13		Zone IV*A* R_F 0·16-0·30		Zone V*A* R_F 0·38-0·48	
Digest at 37°C	4·11	63·1	1·48	22·7	0·22	3·38	0·44	6·75	0·26	3·99
Digest at 0°C	0·80	69·0	0·20	17·3	0·05	4·31	0·06	5·08	0·05	3·97
Digest at 37°C (added enzyme heat-inactivated)	0·16	66·6	0·03	13·8	0·03	10·4	0·01	4·16	0·01	5·00
Experiment 3	Zone I*C*† R_F 0-0·07		Zone II*C* R_F 0·09		Zone III*C* R_F 0·13-0·21		Zone IV*C* R_F 0·23-0·34		Zone V*C* R_F 0·42-0·52	
Digest at 37°C	4·48	58·6	1·72	22·5	0·73	9·6	0·35	4·6	0·36	4·7

* Expressed as percentage of total activity recovered.

† The approximate activity ratios of di-iodotyrosine to "peptide", the two components of this zone, in both Experiments 2 and 3 were, respectively, 51 : 49 and 45 : 55 (E_{37} digests).

Since the radioactivity was too low, the eluates of control (C) were not further investigated. Those of E_{37} and of the control (E_0) were rechromatographed and the identity of the major radioactive components established as follows:

Di-iodotyrosine (DIT) and "Peptide."—(1) E_{37}. The eluate of zone IA (R_F 0·02) of the original chromatograms was re-run in PPW. The radioautogram indicated activity at the origin, a second centre at R_F 0·31 (identified as DIT) with a trail between the main radioactive spots. DIT, the "trail," and the material at the origin were eluted. The specific activity of DIT was esti-

mated from the corresponding eluate; the other two eluates were re-run in PPW. From the resultant chromatogram it was established that the trail consisted almost entirely of DIT; this amino acid was calculated to contain approx. 51 per cent. of the activity of the original zone I eluate. The material from the origin, presumably a peptide, was not resolved on re-running and was eluted for the estimation of its specific activity.

(2) E_0. From the first run in PPW of the eluate of zone IA, DIT, and "peptide" were eluted. The activity ratio of "peptide" to DIT (approx. 3 : 1) was appreciably greater than in E_{37} (approx. 1 : 1).

Mono-iodotyrosine (MIT).—E_{37} and E_0. Eluates of zone IIA (R_F 0·07) of the BPA chromatograms were run in PPW. Apart from MIT (R_F 0·25), only traces of radioactivity were detected. MIT was eluted and the specific activity of the E_{37} eluate determined.

Iodide.—E_{37} and E_0. Eluates of zone IIIA (R_F 0·13) were re-run in BPA. Iodide in small amount was identified as the principal constituent in each case.

Thyroxine (Tx).—Eluates of zone IV*A* (R_F 0·16-0·30) were re-run in BPA.

(1) E_{37}. Thyroxine was identified as the major constituent and its specific activity determined; traces of unidentified compounds were also present.

(2) E_0. A faint trace of Tx was detected and eluted.

Tri-iodothyronine (TIT).—Eluates of zone V*A* (R_F 0·38-0·48) were re-run in BPA.

(1) E_{37}. Radioactivity coincided with the R_F value given by a specimen of synthetic radioactive TIT. The spot was eluted and the specific activity estimated.

(2) E_0. A faint streak of radioactivity was detected in the same position as in E_{37}, but was not eluted.

The specific activities of identified compounds are listed in Table 5.

(ii) *Alkaline Hydrolysis of Thyroglobulin*

The labelled thyroglobulin sample *RA*13 (19·6 mg) was hydrolysed with 2N NaOH (6 ml) for 16 hr at 105°C. After adjustment to pH 3·5 with 2N HCl, the hydrolysate was extracted twice with an equal volume of *n*-butanol (which had been saturated with water at pH 3·5 (HCl)) and the butanol extract washed once with one-third its volume of water at pH 3·5. The butanol extract was found to contain 5·43 μc (84·3 per cent.), the aqueous residue 0·74 μc (11·5 per cent.), and the aqueous washings 0·27 μc (4·2 per cent.). The butanol extract was then chromatographed in BPA. Four major zones of radioactivity were detected in the radioautograms (R_F 0·01 (I*B*), 0·07 (II*B*), 0·12 (III*B*), 0·19-0·29 (IV*B*)); these were eluted and the ^{131}I contents determined. The results are given in Table 4.

Further treatment of the eluates of the four zones was carried out as with the E_{37} material in Experiment 2. DIT was recovered from zone I*B* (which also contained traces of MIT), MIT from zone II*B*, and iodide from zone III*B*.

Tx and TIT were tentatively identified in the first re-run in BPA of the eluate of zone IV*B*; they were eluted together and re-run in BPA. Tx only was eluted from this chromatogram.

TABLE 4

RADIOACTIVITY OF VARIOUS ZONES ELUTED FROM CHROMATOGRAMS (*n*-BUTANOL-*n*-PENTANOL-AMMONIA) OF *n*-BUTANOL EXTRACTS OF ALKALINE HYDROLYSATES OF LABELLED THYROGLOBULIN (EXPERIMENTS 2 AND 3)

	μc	%*	μc	%	μc	%	μc	%	μc	%
Experiment 2	Zone I*B* R_F 0·01		Zone II*B* R_F 0·07		Zone III*B* R_F 0·12		Zone IV*B* R_F 0·19-0·29		Zone V*B* —	
	1·56	50·8	0·52	16·8	0·51	16·8	0·48	15·6	—	
Experiment 3	Zone I*D* R_F 0-0·07		Zone II*D* R_F 0·09		Zone III*D*† R_F 0·12-0·18		Zone IV*D* R_F 0·20-0·28		Zone V*D* R_F 0·36-0·46	
	10·6	49·6	5·21	24·4	1·68 1·79	7·9 8·4	1·66	7·8	0·42	2·0

* Expressed as percentage of total activity recovered.

† Zone III*D* comprised two separately eluted bands of radioactivity, (*a*) and (*b*) respectively (cf. Plate 2, Fig. 6).

(*c*) *Experiment* 3. *Thyroglobulin Preparation* RA21 *and Enzyme Preparation* E35 (28·3 *"units"/mg*)

In this experiment the entire quantity of thyroglobulin was used for proteolysis at 37°C and for alkaline hydrolysis.

(i) *Enzyme Hydrolysis*

Thyroglobulin	Enzyme	Treatment
119·5 mg; 6·5 ml	26·9 mg; 3·05 ml	37°C; 24 hr

The subsequent treatment followed that used for E_{37} in Experiment 2.

(ii) *Alkaline Hydrolysis.*—Two separate hydrolyses were carried out with 30·1 mg and 30·8 mg thyroglobulin at 105°C for 15 hr; 6 ml 2N NaOH was used in each case. After hydrolysis the further treatment followed that used in Experiment 2 except that the pH was lowered to 2·7 prior to extraction with *n*-butanol, as this was found to give higher recoveries of labelled compounds.

After the first application to paper the eluates of the corresponding zones for each hydrolysate were combined.

The radioactivity distribution after ethanol precipitation of the enzyme digest is given in Table 2 and for the eluates of the primary chromatographic zones following enzymic and alkaline hydrolysis in Tables 3 and 4 (see also Plate 2, Figs. 5 and 6). The subsequent chromatographic resolution clearly distinguished the following as the major components of the primary zones indicated (specific activities are given in Table 5).

TABLE 5

SPECIFIC ACTIVITIES OF THE MAJOR ^{131}I-CONTAINING COMPOUNDS LIBERATED FROM LABELLED THYROGLOBULIN* BY THYROID PROTEASE AND BY ALKALINE HYDROLYSIS (EXPERIMENTS 2 AND 3)†

	Mono-iodotyrosine		Di-iodotyrosine		Thyroxine		Tri-iodothyronine		"Peptide"	
	Expt. 2	Expt. 3	Expt. 2	Expt. 3	Expt. 2	Expt. 3	Expt. 2	Expt. 3	Expt. 2	Expt. 3
Digest at 37°C (24 hr)										
^{131}I content (μc)	0·69	1·06	0·66	1·25	0·101	0·206	0·075	0·118	0·56	1·51
^{127}I content (μg)	5·3	12·4	5·9	7·9	0·84	4·2	1·7	3·2	6·4	23·0
Specific activity (μc/μg)	0·13	0·086	0·11	0·16	0·12	0·049	0·044	0·037	0·088	0·066
Alkaline hydrolysate										
^{131}I content (μc)	—	3·06	—	6·03	—	0·625	—	0·143	—	—
^{127}I content (μg)	—	26·4	—	70·0	—	12·0	—	2·9	—	—
Specific activity (μc/μg)	—	0·12	—	0·086	—	0·052	—	0·049	—	—

*Specific activities of thyroglobulin 0·096 μc/μg (Experiment 2) and 0·076 μc/μg (Experiment 3).

† It should be noted that the figures quoted do not necessarily represent total recoveries since the care taken to elute pure samples necessitated more than one chromatographic resolution and cutting of the radioactive areas within narrow limits.

(i) *Enzyme Hydrolysis.*—Zone IC: DIT, "peptide"; zone IIC: MIT; zone IIIC: unknown, Tx; zone IVC: Tx; zone VC: TIT.

(ii) *Alkaline Hydrolysis.*—Zone I*D*: DIT, MIT (ratio of DIT : MIT approx. 4·6 : 1); zone II*D*: MIT; zone III*D* (*a* and *b*): I^- and unknown (same R_F as unknown in IIIC), see also Plate 3, Figures 7 and 8; zone IV*D*: Tx; zone V*D*: TIT.

Zone II*C* contained in addition to MIT a small amount of radioactivity in a position subsequently shown to be occupied by an authentic sample of labelled mono-iodohistidine. This also appeared in zone III*D* of the alkaline hydrolysate between MIT and iodide. The unknown mentioned as occurring in the eluates of III*C* and III*D* was approximately of the same radioactive intensity as thyroxine. It could not have arisen as an artefact during alkaline hydrolysis as it was present in both types of hydrolysate; moreover the main alkali-produced artefacts observed by Stanley (1953), being of higher R_F, were deliberately excluded when the positions of the primary zones were defined. Identity with 3,5-di-iodothyronine was excluded on the basis of R_F comparison.

The small amounts of iodide found in the enzyme hydrolysates of Experiments 2 and 3 could have arisen in the original 70 per cent. ethanol extracts by elution of free iodide which had become adsorbed by the thyroglobulins during their preparation; alternatively the evaporation and chromatographic procedures associated with the resolution of the primary zones could have caused iodide fission from the iodine-containing components. Roche and colleagues (Roche *et al.* 1951) have demonstrated the presence of a deiodase in thyroid tissue acting specifically on MIT and DIT and have shown (Roche *et al.* 1952) that the enzyme is still active though unstable in a cell-free medium (pH optimum about 8·0). Our protease preparations were tested on labelled DIT at pH 3·5 and 7·5 and shown to be without deiodase activity.

(*d*) *Action of Thyroid Protease on Labelled Di-iodotyrosyl Peptides*

Three peptides were iodinated with ^{131}I plus carrier by the micro-procedure of Lemmon, Tarpey, and Scott (1950): L-leucyl-L-tyrosine (LT); glycyl-L-tyrosine (GT); *N*-acetyl-L-phenylalanyl-L-tyrosine (APAT). The first two were authentic samples from Roche Products Ltd. while the third was synthesized according to Baker (1951). The three iodinated reaction products were purified by paper chromatography in the solvent systems BPA (APAT) and PPW (LT and GT). From these chromatograms radioactive zones corresponding to the labelled di-iodotyrosyl peptides were eluted and purified by re-application to paper, using the same solvents except with iodinated APAT where phenol-water was found to achieve a better separation from contaminating iodide. The final eluates of ^{131}I peptides were evaporated to dryness *in vacuo* and each residue was dissolved in 0·5 ml acetate buffer, pH 3·2. Thyroid protease (activity 33·6 "units"/mg) and crystallized pepsin (Armour) were dissolved in the buffer to give concentrations of 5 mg/ml. The test solutions were prepared as shown in Table 6.

The tubes were incubated for 1 hr at 38°C and the reactions then retarded by rapid cooling to 0°C. To each tube unlabelled di-iodotyrosine was added as marker on the chromatograms, which were prepared with aliquots of the solutions and developed in a variety of solvent systems. The best resolutions were obtained with BPA on Whatman No. 3 paper run for 24 hr (using a pad of filter paper to absorb solvent at the bottom of the filter paper sheet). Radioautograms of the various digests are presented in Plate 3, Figure 9. Hydrolysis

ACTION OF THYROID PROTEASE ON THYROGLOBULIN

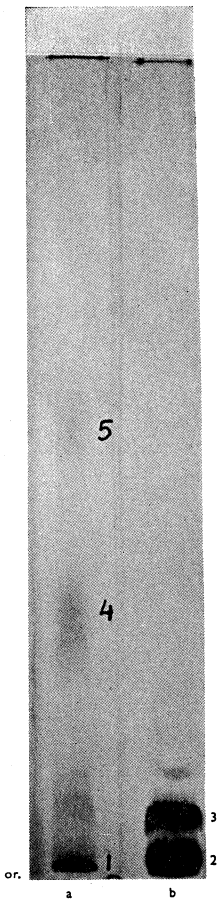


Fig. 1

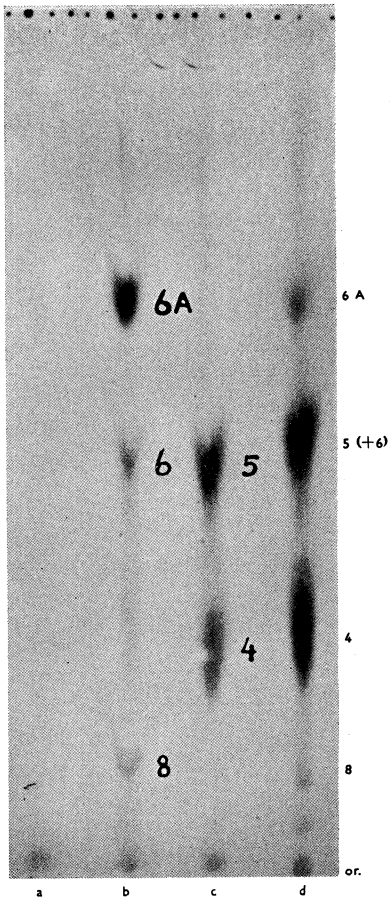


Fig. 2

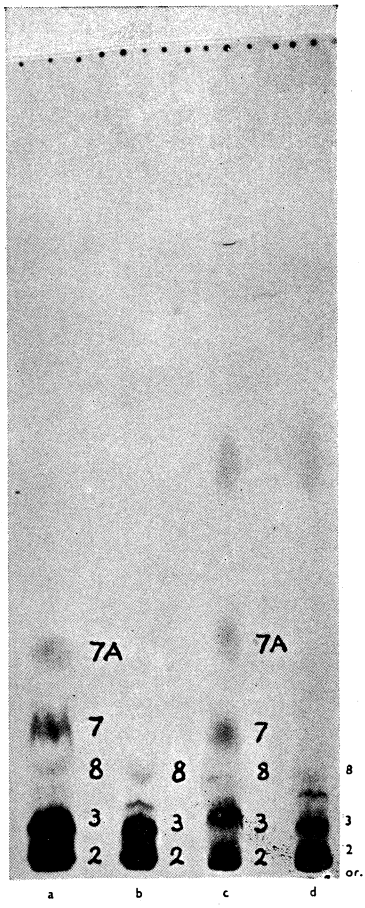


Fig. 3

ACTION OF THYROID PROTEASE ON THYROGLOBULIN

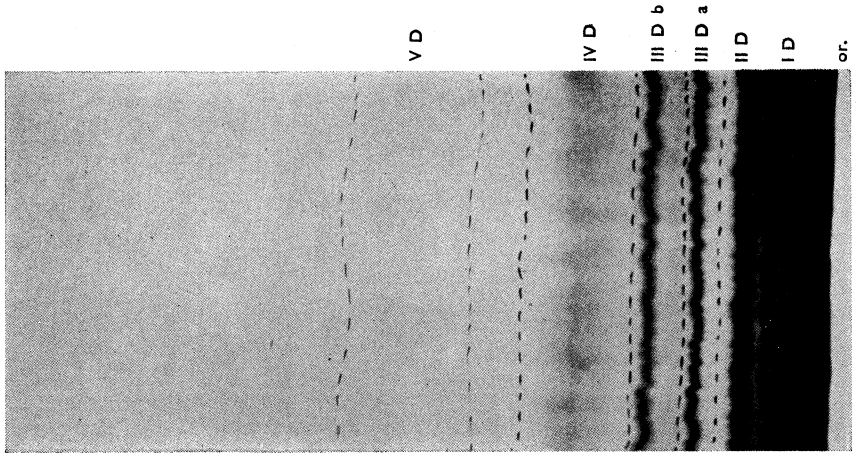


Fig. 6

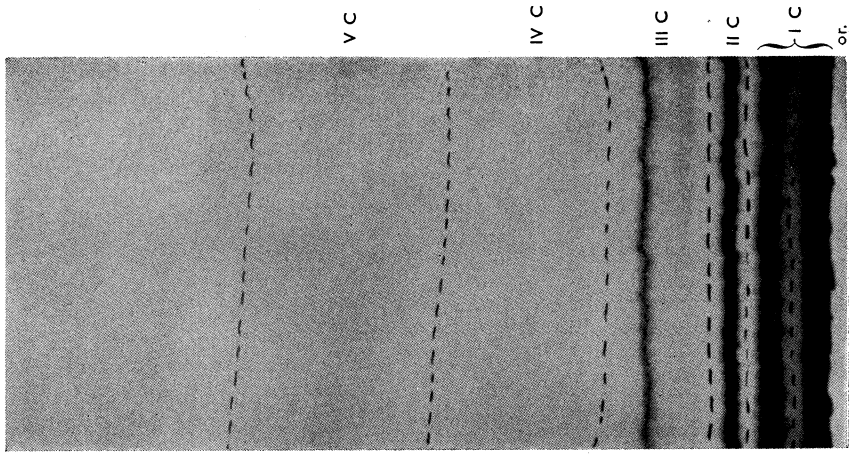


Fig. 5

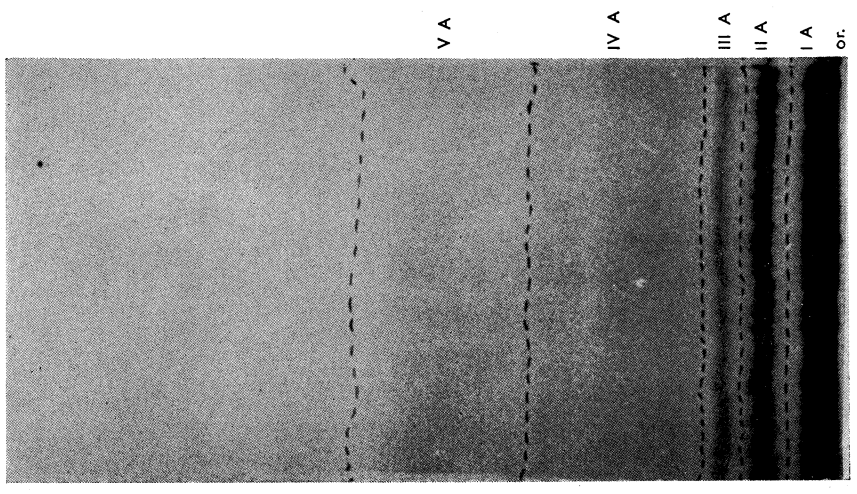
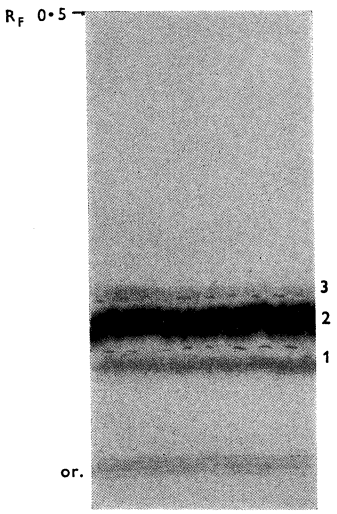
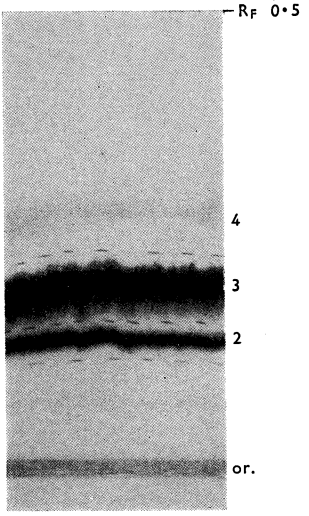


Fig. 4

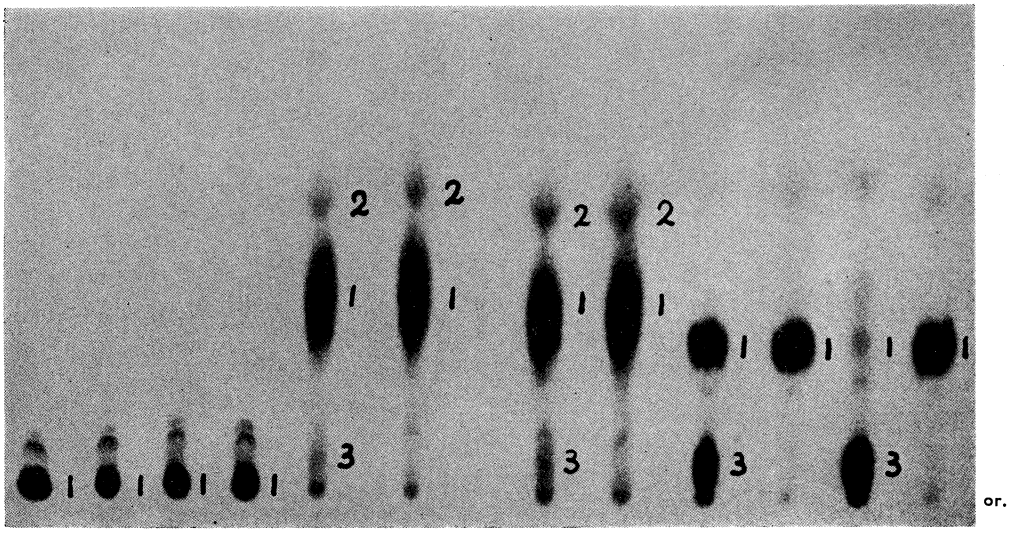
ACTION OF THYROID PROTEASE ON THYROGLOBULIN



7



8



9

of di-iodo-APAT by pepsin was almost complete compared with about 50 per cent. by the thyroid protease. By contrast only trace amounts of di-iodotyrosine were liberated by either enzyme from the leucyl peptide. The iodinated glycyl peptide was found to have an R_F value very close to di-iodotyrosine in all the solvent systems tried. However, inspection of Plate 3, Figure 9, will show a very close similarity between the four radioautograms corresponding to the digests of the peptide with both heated and unheated enzymes. Any appreciable release of di-iodotyrosine would have led to an elongation of the peptide spots. The significance of these findings will be discussed later.

TABLE 6

DESIGN OF EXPERIMENT TO COMPARE THE ACTION OF THYROID PROTEASE AND PEPSIN ON LABELLED IODOPEPTIDES (pH = 3·2)

Tube No.	Peptide*	Thyroid Protease (ml)	Pepsin (ml)
1	Glycyl-	0·05	—
2	Glycyl-	0·05 (HI)†	—
3	Glycyl-	—	0·05 (HI)
4	Glycyl-	—	0·05
5	Leucyl-	0·05	—
6	Leucyl-	0·05 (HI)	—
7	Leucyl-	—	0·05
8	Leucyl-	—	0·05 (HI)
9	Acetyl PA-	0·05	—
10	Acetyl PA-	0·05 (HI)	—
11	Acetyl PA-	—	0·05
12	Acetyl PA-	—	0·05 (HI)

*Glycyl-, leucyl- and acetyl PA- = the iodinated derivatives of glycyl-L-tyrosine, L-leucyl-L-tyrosine and *N*-acetyl-L-phenylalanyl-L-tyrosine respectively. In all cases volume of peptide solution = 0·1 ml.

† HI = Heat-inactivated.

V. DISCUSSION

The results of seven preparative experiments recorded in this paper (Table 1) show that it is possible to obtain rapidly by the fractional precipitation method of Derrien, Michel, and Roche (1948) purified labelled thyroglobulin in which 50 per cent. or more of the ^{131}I taken up by the animals' thyroids is retained by this fraction. The dry weight of the protein recovered amounted to approximately 5 per cent. of that of the dissected thyroid tissue in five out of seven of the experiments. With such preparations as substrates it has been demonstrated that digestion with purified thyroid protease can lead to the release, after 24 hr at 37°C and pH 3·5, of nearly 40 per cent. of the radioactivity in the form of compounds now soluble in 70 per cent. ethanol. This was considerably greater than the degradation which followed incubation at 0°C or that obtained when the endogenous protease in the thyroglobulin was

allowed to act at 37°C in the presence of heat-inactivated added enzyme. From Table 3 (Experiment 2) it is seen that the percentage distribution of radioactivity in the several chromatographic zones is substantially similar for the digest at 37°C and for the two controls. It therefore seems likely that the added protease is closely related to, if not identical with, the enzyme retained by the thyroglobulin during its preparation.

Following resolution of the alcoholic extracts of the enzymic digests by paper chromatographic procedures, 3-mono-iodotyrosine (MIT), 3,5-di-iodotyrosine (DIT), thyroxine (Tx), 3,5,3′-tri-iodothyronine (TIT), and a "peptide" fraction have been demonstrated as the major radioactive iodine-containing components present. Mono-iodohistidine (characterized by Roche, Lissitzky, and Michel (1952) as a component of thyroglobulin) was also identified in trace amounts, while the nature of another radioactive spot of moderate intensity, in a position on the chromatogram between iodide and Tx (*n*-butanol-*n*-pentanol-2N NH_4OH system), could not be determined. Both these substances were also present in the alkaline hydrolysates of Experiment 3 (Plate 3, Fig. 7). After allowing for the lower content of Tx and TIT in thyroglobulin compared with DIT and MIT the former two iodoamino acids do not appear to be released preferentially by the enzyme (cf. Michel 1952).

The nature of the "peptide" fraction was not ascertained. The increase, greater than threefold (2·02 μc compared to 0·59 μc) in the amount of "peptide," corresponding to the increase in incubation temperature from 0° to 37°C in Experiment 2, suggests, as more likely, that this fraction is a product of enzyme action rather than a pre-existing loosely attached component of the thyroglobulin dissociable in 70 per cent. ethanol. Furthermore, since the activity ratios of DIT to "peptide" observed in zone I of both Experiments 2 and 3 were found to be approximately equivalent (Table 3), this could be used as an argument against contamination by thyroglobulin itself; it is, however, true that the specific activity values of the "peptide" found in both experiments were close to those of the corresponding thyroglobulins (Table 5).

The decreasing trend in the order of specific activities, namely MIT > DIT > Tx, in the alkaline hydrolysate of Experiment 3 (Table 5) demonstrates the generally accepted sequence of these amino acids during their biosynthesis as MIT → DIT → Tx (cf. Taurog and Chaikoff 1947). However, this order does not apply to the specific activities of the labelled components of the corresponding enzyme digest (Table 5). Furthermore, the individual values for MIT and DIT liberated by enzyme action are appreciably different from those obtained following alkaline hydrolysis. These discrepancies could have risen from errors in technique although all procedures were standardized and estimations of ^{127}I made in duplicate in most instances. For example, even though our laboratory has had considerable experience in the micro-estimation of iodine, such procedures as elution and evaporation, associated with the preparation of the purified zonal components, may have introduced contamination with ^{127}I. All chromatographic paper was, however, subjected to prolonged washing with distilled water before use. It should also be noted that the specific activities

of the DIT and "peptide" fractions in E_0 (Experiment 2) were determined although the values are not included in Table 5; these were 0·11 and 0·082 μc ^{131}I/μg ^{127}I respectively, which are close to the values obtained for the same two components in E_{37}. Furthermore, replicate determinations of the specific activities of the thyroglobulin used in Experiment 3 gave the following values: 0·072, 0·080, 0·077, 0·074 μc ^{131}I/μg ^{127}I.

If the observed variation in the specific activity values between the corresponding components of the enzymic and alkaline hydrolysates is accepted as significant, this might indicate a non-homogeneous biosynthesis of thyroglobulin. This suggestion lacks, however, the evidence which could have been provided by determinations of the specific activities of the components of the fraction insoluble in 70 per cent. ethanol; these were, unfortunately, not carried out.

The action of thyroid protease on the labelled peptides (within the optimal pH range of the enzyme for protein (haemoglobin)) suggests that this enzyme has properties similar to those found for pepsin by Baker (1951), namely that it hydrolyses preferentially links between two aryl-amino acids. Although we have no evidence for a preferential release by the enzyme of thyroxine from thyroglobulin, compared with the other iodoamino acids, it might prove to be the case that the release of *all* iodoamino acids is favoured during proteolysis. This could be brought about by the presence, adjacent to the iodoamino acids (all of which contain aromatic residues), of other arylamino acids. Weiss (1953) has recently effected a partial separation of the peptidase and protease activities of thyroid tissue (tested respectively at pH 7·8 and pH 4·0). However, he does not appear to have examined his protease fraction for peptidase activity at acid pH.

VI. Acknowledgments

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EXPLANATION OF PLATES 1-3

PLATE 1

Fig. 1.—Radioautogram of chromatogram of samples of zones *M* and *L* eluted from the PWA (phenol-water-ammonia) chromatograms of the products of thyroid protease action. Experiment 1. Compare with Figures 2 and 3 but note that more material applied in preparing chromatograms in latter cases. (*a*) Zone *M* eluate. Solvent system, BPA (*n*-butanol-*n*-pentanol-ammonia); (*b*) zone *L* eluate. Solvent system, BPA. 1, "Peptide"; 2, di-iodotyrosine; 3, mono-iodotyrosine; 4, thyroxine; 5, tri-iodothyronine; *or.*, origin.

Fig. 2.—Radioautogram of chromatogram of *n*-butanol extracts of eluates of zones *L* and *M* after treatment with alkali at 0° and 105°C, respectively, for 16 hr. Butanol extract at pH 14 (2N NaOH). (*a*) Zone *L* eluate, 0°C; (*b*) zone *L* eluate, 105°C; (*c*) zone *M* eluate, 0°C; (*d*) zone *M* eluate, 105°C. Solvent system, BPA. 2-5 As for Figure 1; 6, alkali-produced artefact not identical with but close to 5; 6*A*, alkali-produced artefact; 8, iodide; *or.*, origin.

Fig. 3.—Radioautogram of chromatogram of *n*-butanol extracts of eluates of zones *L* and *M* after treatment with alkali at 0° and 105°C, respectively, for 16 hr. Butanol extract prepared after adjustment to pH 3·5 of residue from extraction at pH 14 (Fig. 2). (*a*) Zone *L* eluate, 0°C; (*b*) zone *L* eluate, 105°C; (*c*) zone *M* eluate, 0°C; (*d*) zone *M* eluate, 105°C. Solvent system, BPA. 1-6 As for Figures 1 and 2; 7, unknown; 7*A*, unknown, R_F as 4 but identity with thyroxine disproved; 8, iodide; *or.*, origin.

PLATE 2

Fig. 4.—Radioautogram of chromatogram of the 70 per cent. ethanol-soluble fraction of E_{37} enzyme digest. Experiment 2. Solvent system, BPA. The zones indicated refer to those in Table 3. *or.*, origin.

Fig. 5.—Radioautogram of chromatogram of the 70 per cent. ethanol-soluble fraction of E_{37} enzyme digest. Experiment 3. Solvent system, BPA. The zones indicated refer to those in Table 3. *or.*, origin.

Fig. 6.—Radioautogram of chromatogram of the *n*-butanol extract prepared at pH 3·5 from the alkaline hydrolysate of labelled thyroglobulin. Experiment 3. Solvent system, BPA. The zones indicated refer to those in Table 4. *or.*, origin.

PLATE 3

Fig. 7.—Radioautogram of chromatogram of zone III*Da* eluate after re-running in the solvent system BPA. 1, Mono-iodohistidine; 2, iodide; 3, unknown; 4, thyroxine; *or.*, origin.

Fig. 8.—Radioautogram of chromatogram of zone III*Db* eluate after re-running in the solvent system BPA. 2, Iodide; 3, unknown; 4, thyroxine; *or.*, origin.

Fig. 9.—Radioautogram of chromatograms of three labelled dipeptides containing di-iodotyrosine after incubation with active and heat-inactivated (HI) enzymes. Solvent system, BPA. (*a*) Glycyl— + thyroid protease; (*b*) glycyl— + thyroid protease (HI); (*c*) glycyl— + pepsin (HI); (*d*) glycyl— + pepsin; (*e*) L-leucyl— + thyroid protease; (*f*) L-leucyl— + thyroid protease (HI); (*g*) L-leucyl— + pepsin; (*h*) L-leucyl— + pepsin (HI); (*i*) *N*-acetyl-L-phenylalanyl— + thyroid protease; (*j*) *N*-acetyl-L-phenylalanyl— + thyroid protease (HI); (*k*) *N*-acetyl-L-phenylalanyl— + pepsin; (*l*) *N*-acetyl-L-phenylalanyl— + pepsin (HI). 1, Unchanged peptide; 2, iodide; 3, di-iodotyrosine; *or.*, origin.