BBA 65552

THE DISTRIBUTION OF PROTEOLYTIC ENZYMES IN THE THYROID GLAND

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(Received August 23rd, 1966)

SUMMARY

1. Proteolytic activity of pig thyroid homogenates was determined over the pH range 2.5 to 10, using as substrates denatured haemoglobin and thyroglobulin. There were two pH optima in the acid and neutral range: haemoglobin substrate, pH 3.5 to 3.8 and pH 7.0 to 8.1; thyroglobulin substrate, pH 4 to 5 and pH 7.3 to 8.3. Activity at neutral pH (haemoglobin substrate) was labile and affected by sulphydryl reagents.

2. The distribution of protease and peptidase activity was studied in subcellular fractions using acid phosphatase as a lysosomal marker. The peptidases examined included *N*-acetyl-L-phenylalanyl-L-tyrosine hydrolase (APAT hydrolase), L-cysteinyl-L-tyrosine hydrolase, benzyloxycarbonyl-L-glutamyl-L-tyrosine hydrolase ("Cathepsin A"), benzyloxycarbonyl-glycyl-L-phenylalanine hydrolase ("carboxypeptidase") and L-leucyl-4,methoxy-β-naphthylamide hydrolase ("leucine aminopeptidase").

3. APAT hydrolase, cysteinyltyrosine hydrolase and "cathepsin A" were predominantly particulate. Acid protease (haemoglobin substrate) and acid phosphatase were equally distributed between particulate and soluble fractions whereas neutral protease (haemoglobin substrate), "carboxypeptidase" and "leucine aminopeptidase" were mainly soluble.

4. The particulate enzymes, with the exception of APAT hydrolase which was more resistant to solubilization, were affected to a similar extent by treatment of the lysosomal/mitochondrial fraction by freezing and thawing, dialysis, detergent action, sonication and incubation at acid pH.

5. Similar distribution patterns for the particulate enzymes were obtained on density-gradient centrifugation.

6. The significance of these findings is discussed in relation to the location of these enzymes "*in vivo*".

Abbreviations: Ac–, acetyl; Bz–, benzoyl; Z–, benzyloxycarbonyl; T_4, thyroxine; MIT, monoiodotyrosine; DIT, diiodotyrosine; APAT, *N*-acetyl-L-phenylalanyl-L-tyrosine; PCMB, *p*-chloromercuribenzoate; TSH, thyrotrophic hormone.

INTRODUCTION

Proteolysis of the storage protein, thyroglobulin, is essential for the release of the thyroid hormone. Since proteolysis was first demonstrated in the isolated follicular colloid[1] a number of proteolytic enzymes have been purified: acid protease[2,3], APAT hydrolase[4] and cysteinyltyrosine hydrolase[5]. The acid protease hydrolyses ^{131}I-labelled thyroglobulin to release T_4 together with DIT and MIT and labelled peptides while at least one of the peptidases appears to act synergistically with the protease to release, particularly, additional DIT and MIT[3,6]. Other proteolytic enzymes have also been noted but have not been purified, for example, neutral protease[7,8] and leucine aminopeptidase[9]. The determination of the location of these enzymes in the thyroid is of importance in elucidating their role in proteolysis *in vivo*. It has been shown, in TSH-stimulated rat thyroids, that pinocytosis of colloid occurs[10,11]. Histochemical evidence, with acid phosphatase as marker, indicates the 'fusion' of lysosome-like granules with the colloid droplet[12]. However, little work on the location of thyroid proteases and peptidases has been reported apart from the original work of DE ROBERTIS[1]. WEISS[13] demonstrated catheptic and peptidase activity in the 20 000 $\times$ *g* particulate fraction of the cell, findings which were essentially confirmed by LAVER AND TRIKOJUS[2]. A more precise localization of the proteolytic enzymes was therefore undertaken.

MATERIALS AND METHODS

Experimental animals

Pig thyroids were obtained fresh on ice from the abattoirs.

Male rats, a crossbreed of the Wistar and the *Rattus norvegicus* strains weighing 300–400 g, were used. Thyroids were obtained, usually after the rats were killed with chloroform. Chloroform appeared to have no effect on the distribution of the enzymes since similar results were obtained when animals were killed by exsanguination.

Reagents

Glass-distilled water and A.R. grade reagents were used throughout.

APAT and L-Cys–L-Tyr were synthesized by V. M. TRIKOJUS and T. A. A. DOPHEIDE of this Department. Z–Gly–L-Phe, Bz–L-Arg-amide, Z–L-Glu–L-Phe, Z–L-Glu–L-Tyr, Gly–L-Phe-amide and L-Leu-amide were obtained from Mann Research Labs., L-leucine-4-methoxy-β-naphthylamide from Sigma and *p*-nitrophenyl phosphate from British Drug Houses.

Preparation of homogenates

All media were adjusted to pH 6.8. Pig thyroids (at least 10 glands) were homogenized with 2 to 4 volumes of the appropriate medium in a Servall Omni-mix (metal container) for 2 $\times$ 30 sec at 8000 rev./min at 0°. The homogenate was then strained through two thicknesses of muslin.

Rat thyroids were homogenized in a Potter–Elvehjem type apparatus, consisting of glass tube and Teflon plunger attached to an electric drill. Four thyroids (approx. 100 mg wet weight) were homogenized with 0.5 ml medium for 30–40 sec (15–25 passes).

The most satisfactory medium was found to be 0.25 M sucrose–1% bovine serum albumin (Cohn Fraction V, Commonwealth Serum Laboratories). However, the albumin was omitted in experiments where proteolytic activity was being assessed and in the sucrose gradient runs.

Tissue fractionation

For the study of the distribution of the enzymes in the pig thyroid, the homogenate was fractionated according to the method of DE DUVE *et al.*[14] (with minor modifications) into four particulate and two soluble fractions. The homogenate was centrifuged for 10 min at 650 × *g* to remove nuclei, unbroken cells and debris (N fraction). The supernatant was then centrifuged for 20 min at 14 000 × *g*. This precipitate was resuspended in the medium (M fraction) and the supernatant was centrifuged for 90 min at 78 000 × *g*. The pellet was termed the microsomal fraction (Mi) and the supernatant, the soluble fraction (S). In many experiments the M fraction was recentrifuged for 20 min at 5000 × *g* to give the heavy mitochondrial fraction (M_H) and at 14 000 × *g* for a further 20 min to give a light mitochondrial fraction (M_L) and the mitochondrial washing (M_W).

In the studies on the rat no attempt was made at subfractionation. The homogenate was diluted to 4 ml and centrifuged for 90 min at 110 000 × *g* to give soluble (S) and particulate (P) fractions.

Density-gradient centrifugation

Discontinuous gradients were prepared by layering 1 h before use. Linear gradients were prepared by a modification of the methods of PARR[15] and BOCK AND LING[16]. The apparatus consisted of two identical cylindrical vessels placed side by side and connected by a narrow tube. Both vessels were open to the atmosphere. The vessel containing the heavier sucrose was stirred by a magnetic stirrer and had an outlet of fine Teflon tubing which was attached to the top of the centrifuge tube so that the sucrose flowed slowly down the wall of the tube. The linearity of the gradient so produced was tested by addition of 2,6-dichlorophenolindophenol to the heavier sucrose. The centrifugation was carried out at 10 400 × *g* for 80 min, using the Spinco Model-L centrifuge (SW 39 head).

Enzyme assays

The enzyme activities were determined after dialysis overnight, except in the case of the "neutral protease" (due to its instability), or unless otherwise stated. Appropriate controls were always included. The results given throughout were obtained after subtraction of control from test values.

Acid phosphatase

This was assayed by the method of GIANETTO AND DE DUVE[17] with sodium β-glycerophosphate as the substrate. Released phosphate was estimated by the ALLEN[18] method. One enzyme unit is defined as that amount which releases 1 μmole phosphate in 1 min at 37°. In later experiments, the method of BESSEY, LOWRY AND BROCK[19] was used with *p*-nitrophenyl phosphate as substrate. One unit is that activity which hydrolyses 1 μmole of the substrate in 1 min at 37°.

Proteolytic enzymes

The substrate, haemoglobin, was prepared from defibrinated ox blood and freeze-dried. Prior to use, the haemoglobin was dissolved in water, denatured with either 2 M HCl at pH 1.5 or 6 M urea, and then adjusted to either pH 3.5 with 2 M NaOH or to the desired pH with suitable buffers.

A mock incubation of substrate *plus* enzyme fraction was always set up to check the actual pH level, which is the value given throughout. The assay method was a modification of that of ANSON[20]. Incubation periods of 10 min were generally used since the activity in the alkaline pH range was unstable. The "tyrosine equivalents" released were estimated from the absorbance of the filtrate at 280 mμ (using Unicam SP 500 spectrophotometer) after precipitation of the unreacted substrate with 10% trichloroacetic acid.

Thyroglobulin was prepared from pig thyroids according to the method of DERRIEN, MICHEL AND ROCHE[21] and freeze-dried. Fresh solutions were made by dissolving the protein in water and adjusting the solution to the required pH by addition of the appropriate buffers. pH levels were checked as with the haemoglobin substrate. 0.5 ml of the buffered 1% thyroglobulin solution was incubated with 0.5 ml of the enzyme solution for 3–4 h at 37°. Control tubes containing only 0.5 ml of the thyroglobulin solutions were also incubated. 2 ml 10% trichloroacetic acid was then added to all tubes and 0.5 ml of the enzyme preparation to the controls. The tubes were then centrifuged and the absorbance of the supernatant at 280 mμ determined. The reaction was found to be linear for at least 4 h.

In both assays one unit of proteolytic activity is defined as that amount of enzyme which releases the equivalent of 0.1 μmole of tyrosine in 1 min at 37°.

Peptidases

Quantitative. Activity against a number of characteristic substrates was measured as follows:

APAT (substrate for APAT hydrolase) by the method of MENZIES AND MCQUILLAN[4], the time of incubation being increased to 2 or 3 h. When assaying rat preparations the assay was carried out at pH 5.6 instead of 4.1 since the rat enzyme was found to have a higher optimum pH*. One unit of APAT hydrolase activity is expressed as that amount of enzyme which releases 1 μmole of tyrosine from the substrate in 1 min at 37°.

L-Cys–L-Tyr (substrate for cysteinyltyrosine hydrolase) by a modification of the method of LOUGHLIN AND TRIKOJUS[5]. 50 μl of the enzyme was preincubated with 50 μl 0.1 M sodium acetate buffer (pH 5.3) containing 0.6 M Zn^{2+} (the acetate salt) for 30 min at 37°. 100 μl 32 mM L-Cys–L-Tyr was then added and the incubation continued for 30–120 min. Samples of 50 μl were taken at zero time and at the end of the incubation. One unit is defined as that amount of enzyme which releases 1 μmole of tyrosine and cysteine (as measured by ninhydrin colour increase) in 1 min at 37°.

Z–L-Glu–L-Tyr (substrate for cathepsin A) as for APAT hydrolase, substrate, 10 mM.

Z-Gly–L-Phe (substrate for carboxypeptidase) as for APAT hydrolase, substrate, 10 mM.

* N. W. DUNN AND M. T. MCQUILLAN, unpublished results.

L-Leucine-4-methoxy-β-naphthylamide (substrate for leucine aminopeptidase) by the method of BERNT AND BERGMEYER[22], with certain modifications: L-leucine-4-methoxy-β-naphthylamide was used as the substrate in place of L-leucine-β-naphthylamide and preincubation with $MnCl_2$ was omitted since there was no apparent activation of the enzyme. One unit of activity is defined as that amount of enzyme which releases 1 μmole of 4-methoxy-β-naphthylamine in 1 min at 37°.

Qualitative. When a quantitative estimation was not practicable 50 μl of the fraction was incubated with 50 μl of the buffered substrate for 4 h at 37°. 10 μl of each incubation was then applied to chromatography paper. Control tubes, containing buffer *plus* either enzyme or substrate, were similarly treated. 10 μl samples of "marker" solutions of the appropriate amino acids in concentrations calculated to be equivalent to 100% hydrolysis of the peptide were also applied. Chromatograms were developed with *n*-butanol–acetic acid–water (4:1:1, by vol.), dried in air and sprayed with ninhydrin. The degree of hydrolysis was estimated visually by comparison of incubation and "marker" spots.

Protein determinations

Protein determinations were carried out according to the method of ELLMAN[23].

RESULTS

Preliminary investigations, using the technique of DE DUVE *et al.*[14], indicated that the distribution of acid protease (haemoglobin substrate, pH optimum 3.5) closely paralleled that of acid phosphatase, suggesting that the proteolytic enzyme was also of lysosomal origin. The distribution, however, was quite different from that found by other workers for similar enzymes in the liver. This could be due, as DE DUVE[24] had noted, to the fact that the thyroid is a much more difficult tissue to homogenize. The homogenizing technique and medium were subsequently modified (to that given in MATERIALS AND METHODS) and yields of up to 65% "lysosomes" (determined from

TABLE I

DISTRIBUTION OF ACID PROTEASE AND ACID PHOSPHATASE IN PIG-THYROID AND RAT-LIVER TISSUE

Preparation: Pig thyroids homogenized in 4 vol. 0.25 M sucrose. Homogenate fractionated as in MATERIALS AND METHODS and precipitates resuspended in one-half the original volume. All samples dialysed overnight before assay. M_H diluted, other fractions assayed undiluted. Assay procedures: Protease, incubation with 2% haemoglobin for 30 min at pH 3.5. Phosphatase, incubation with 0.05 M β-glycerophosphate in the presence of 0.1% Tween 20 for 60 min at pH 5.0.

Fraction	*Thyroid (% distribution)*		*Liver* (% distribution)*	
	Acid protease	*Acid phosphatase*	*Cathepsin*	*Acid phosphatase*
N	4	3	4	4
M_H	31	31	35	24
M_L	21	21	43	41
M_W	8	9	—	—
Mi	3	3	8	20
S	33	32	12	13

* Results obtained by DE DUVE *et al.*[14].

particulate acid phosphatase) could be obtained. Results from a typical experiment are given, together with those of DE DUVE *et al.*[14] for rat liver, in Table I.

pH curves

Total particulate (N + M + Mi) and soluble fractions were assayed over the pH range 2.5 to 10 using both haemoglobin and thyroglobulin as substrates. As indicated in Fig. 1, there are two distinct optima—in the pH range 3.5–3.8 (acid protease) and in the pH range 7.0–8.1 (neutral protease)—when haemoglobin is the substrate.

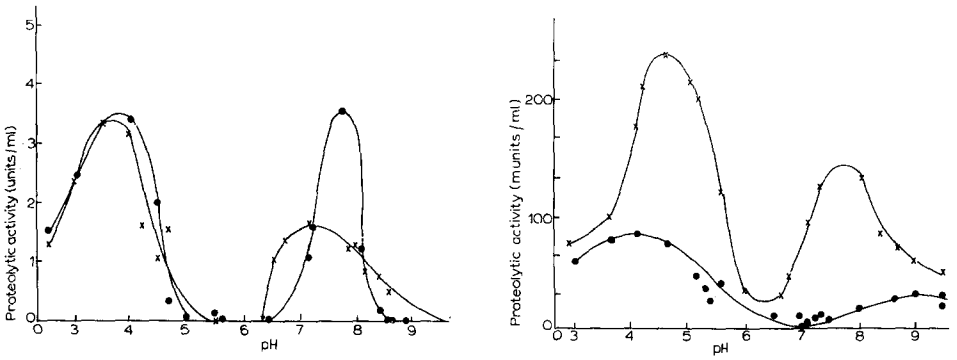


Fig. 1. pH–activity curve: 2% haemoglobin substrate, incubated 10 min, 37°. pH range 2.5–4.5: acid-denatured haemoglobin; pH range 4–9.7: urea-denatured haemoglobin. Buffers used: pH 2.5–3.2: glycine–HCl; pH 3.3–5.5: sodium acetate–acetic acid; pH 5.5–8.5: unbuffered; pH 8.5–9.7: glycine–NaOH. Preparation: Pig thyroids homogenized in 4 vol. 0.25 M sucrose. Homogenate centrifuged 20 min, 14 000 × *g*. Precipitate suspended in one-half the original volume. Assay carried out immediately after preparation of fractions. ●—●, soluble; ×—×, particulate.

Fig. 2. pH–activity curve: 1% thyroglobulin incubated 3 h, 37°. Buffers used: pH 3–5.5: sodium acetate–acetic acid; pH 5.5–7: unbuffered; pH 7–8.1: Tris–HCl; pH 8.5–10: glycine–NaOH. Preparation as given in Fig. 1. ●—●, soluble; ×—×, particulate.

The distribution of acid protease closely follows that of acid phosphatase while neutral protease is mainly in the soluble fraction. When thyroglobulin is the substrate there are again two pH optima (Fig. 2) but acid protease appears to have an optimum in the pH range 4–5 and the percentage in the particulate fraction is higher compared with the activity against haemoglobin. Neutral protease has an optimum in the range 7.3–8.3 and 90% of this activity is particle-bound, whereas it is mainly soluble when haemoglobin is the substrate. It should be noted that total activity with thyroglobulin as substrate was only 5% of that with haemoglobin at the acid pH optimum and 3% in the neutral pH range.

Effect of cysteine, mercaptoethanol and –SH reagents on the activity of the proteases

pH-activity curves were also determined in the presence of 0.01 M mercaptoethanol. Mercaptoethanol had no effect in the acid pH optimum range with either substrate. With haemoglobin as the substrate the mercaptoethanol caused a small shift in the neutral pH optimum (*plus* 0.8 pH unit) and an inconsistent elevation of enzymic activity. The effect of 0.06 M cysteine on the activity of the neutral protease also varied from preparation to preparation. It was considered possible that this

TABLE II

STABILITY OF NEUTRAL PROTEASE (SOLUBLE FRACTION). HAEMOGLOBIN SUBSTRATE

Mercaptoethanol concentration: storage, 0.02 M; assay, 0.01 M. Cysteine concentration: storage, none; assay, 0.06 M. Storage temperature: 4°. Preparation: 35 g pig thyroids homogenized in 140 ml 0.25 M sucrose. Centrifuged 90 min, 78 000 × *g* supernatant used for study.

Atmosphere	*Stabilizer*	*Age of preparation (h)*	*Activity*	
			Units	*% Initial sample*
Air	None	6	923	100
Air	Mercaptoethanol	6	1200	130
Air	None	26	382	42
Air	Frozen	26	234	26
Air	Mercaptoethanol	26	647	71
N_2	None	26	428	47
N_2	Mercaptoethanol	26	623	69
Air	Cysteine	26	877	95

might be due to variation in the glands. Previous results had indicated that the neutral protease was unstable. After ageing of the preparation overnight, which caused a marked reduction in activity, a definite activation with either cysteine or mercaptoethanol could be detected consistently. Similarly, prior addition of mercaptoethanol to the preparation reduced the loss of activity on ageing, whereas N_2 had little effect

TABLE III

EFFECT OF ACTIVATORS AND INHIBITORS ON NEUTRAL PROTEASE (SOLUBLE FRACTION). HAEMOGLOBIN SUBSTRATE

Preparation: 26 g pig thyroids homogenized in 80 ml 0.25 M sucrose. Centrifuged 90 min, 78 000 × *g*; supernatant used for study. Preincubation: All samples preincubated for 30 min with the added reagent. The concentrations of the reagents on preincubation were 6 times those of the actual assays (given in the Table). Dialysis: Overnight against glass-distilled water at 4°.

State of sample	*Reagent added (M)*	*Activity*	
		Units	*% Initial sample*
Undialysed	None	295	100
Undialysed	Cysteine (10^{-4})	257	88
Undialysed	Cysteine (10^{-2})	576	195
Undialysed	PCMB (10^{-6})	292	99
Undialysed	PCMB ($5 \cdot 10^{-5}$)	118	40
Undialysed	PCMB (10^{-6}) + cysteine (10^{-4})	222	75
Undialysed	PCMB (10^{-6}) + cysteine (10^{-2})	287	97
Undialysed	PCMB ($5 \cdot 10^{-5}$) + cysteine (10^{-4})	92	31
Undialysed	PCMB ($5 \cdot 10^{-5}$) + cysteine (10^{-2})	287	97
Undialysed	Iodoacetate (10^{-4})	136	46
Undialysed	Iodoacetate (10^{-2})	74	25
Undialysed	Iodoacetate (10^{-4}) + cysteine (10^{-4})	225	67
Undialysed	Iodoacetate (10^{-4}) + cysteine (10^{-2})	160	54
Dialysed	None	177	60
Dialysed	Cysteine (10^{-2})	183	62

and freezing (16 h) and thawing caused an even greater loss of activity. Inactivation by dialysis overnight at 4° could not be reversed by cysteine.

Preincubation of the soluble fraction with PCMB resulted in some loss of neutral protease activity. The effect of 10^{-6} M PCMB was variable (20% to zero inhibition); higher concentrations, *e.g.* $5 \cdot 10^{-5}$ M were more effective. This effect could be reversed by 10^{-2} M cysteine. Iodoacetate was a more potent inhibitor and its effect could be reversed only partially by the addition of cysteine.

These results are summarised in Tables II and III. Inhibition (approx. 50%) was also observed following incubation for 30 min at pH 5 or in the presence of either 0.1% Tween 20 or 0.06 M citrate.

Distribution of APAT hydrolase and cysteinyltyrosine hydrolase

Table IV shows the results obtained in a typical experiment using pig thyroids; all assays were carried out on the same preparation. Both peptidases appear to be more firmly particle-bound than the acid phosphatase.

Distribution of leucine aminopeptidase activity

10 mM L-leucine-amide was completely hydrolysed by a thyroid homogenate under the conditions of the qualitative assay at pH 4.1, 5.3 and 7.5. Quantitative estimations of enzymic activity at pH 4.5, 5.3 and 7.5, with L-leucine-4-methoxy-β-naphthylamide as substrate, were carried out to locate the peptidase precisely (Table V). At pH 7.5, 60% of the enzyme was recovered in the soluble fraction and a high percentage of the particulate activity in the N fraction which consists of unbroken cells and cell debris as well as nuclei. At pH 5.3, the total activity was smaller (15% that at pH 7.5) and the distribution essentially the same. At pH 4.5 no activity could be detected.

Distribution of cathepsin A activity

Preliminary qualitative experiments which were carried out at pH 4.1, 5.3 and 7.5 indicated that Z–L-Glu–L-Phe and Z–L-Glu–L-Tyr were readily hydrolysed at pH 4.1 and 5.3. Distribution of activity was determined subsequently at pH 5.3 (Table VI) and was found to be similar to that of APAT hydrolase. However it has not been established that the activity against the characteristic substrates for cathepsin A is due to an enzyme distinct from that hydrolysing APAT. Purified APAT hydrolase has been found to show very low activity against these substrates at pH 4.1 but not at pH 5.3 (ref. 4).

Distribution of carboxypeptidase activity

The activity of the particulate (N + M + Mi) and soluble fractions as well as that of the homogenate against Z–Gly–L-Phe was assessed qualitatively at pH 4.1, 5.3 and 7.5. There was only a slight hydrolysis of the peptide at pH 4.1 and 5.3 and, in contrast to the pancreatic enzyme[25], no hydrolysis at pH 7.5. On quantitative estimation at pH 5.3 and 6.8, no hydrolysis was detected at the higher pH and, at the lower pH, most of the activity was in the soluble fraction (Table VI).

Cathepsins B and C

The activity of the thyroid homogenate, particulate and soluble fractions against

TABLE IV

DISTRIBUTION OF APAT HYDROLASE AND CYSTEINYLTYROSINE HYDROLASE IN PIG THYROID

Preparation: 50 g pig thyroids homogenized in 180 ml 0.25 M sucrose–1% bovine serum albumin. Fractionated as in MATERIALS AND METHODS. All samples dialysed overnight prior to assay. M_H diluted before assay, other fractions assayed undiluted.

Fraction	*APAT hydrolase*		*Cysteinyltyrosine hydrolase*		*Acid phosphatase**	
	Units	*% total*	*Units*	*% total*	*Units*	*% total*
N	1.30	15	0.61	8	2.63	10
M_H	4.15	49	2.30	29	6.03	23
M_L	0.45	5	0.20	3	1.00	4
M_W	0.35	4	1.37	17	0.07	1
Mi	0.10	1	0.08	1	0.83	3
S	2.13	25	3.27	42	16.00	60

* *p*-Nitrophenyl phosphate as substrate.

TABLE V

DISTRIBUTION OF LEUCINE AMINOPEPTIDASE ACTIVITY IN PIG THYROID

Preparation: 40 g pig thyroids homogenized in 80 ml 0.25 M sucrose. Fractionation as in MATERIALS AND METHODS. Assay: Incubation with L-leucine-4-methoxy-β-naphthylamide for 60 min at pH 7.5, 37°. Results given are those obtained with dialysed samples; the assay was also carried out on the undialysed fractions with identical results.

Fraction	*Leucine aminopeptidase activity*		*Acid phosphatase activity*	
	Milliunits	*% total*	*Units*	*% total*
N	183	20	4.87	14
M_H	50	6	9.58	27
M_L	15	2	0.78	2
M_W	47	5	1.07	3
Mi	60	7	1.47	4
S	538	60	17.78	50

TABLE VI

DISTRIBUTION OF CATHEPSIN A AND CARBOXYPEPTIDASE ACTIVITY IN PIG THYROID

Preparation: 20 g pig thyroids homogenized in 50 ml sucrose–1% bovine serum albumin. Centrifuged 90 min, 78 000 × *g*. Dialysed overnight before assay.

Peptide (pH)	*Activity particulate*		*Activity soluble*	
	Milli-units	*% total*	*Milli-units*	*% total*
Z-L-Glu-L-Tyr* (5.3)	43	72	17	28
Z-L-Glu-L-Phe** (5.3)	70	88	10	12
Z-Gly-L-Phe (5.3)	23	29	55	71
Z-Gly-L-Phe (6.8)	0	0	0	0
Acid phosphatase		50		50

* Not completely soluble.
** Also assayed at pH 4.5, with similar results.

the characteristic substrates for cathepsins B and C, Bz–L-Arg-amide and Gly–L-Phe-amide, respectively, was assessed qualitatively at pH 4.1, 5.3 and 7.5. The homogenate showed high activity against Gly–L-Phe-amide at pH 7.5, the activity being greater in the particulate fraction, but there was only slight hydrolysis at the two lower pH levels. Bz–L-Arg-amide was not hydrolysed at any of these pH levels even in the presence of 0.05 M cysteine.

Properties of the mitochondrial/lysosomal fraction

The M fraction was resuspended in either 0.25 M sucrose (pH 6.8) or in the test medium for various physicochemical tests. Solubilization of the enzymes under the test conditions was determined by recentrifuging at 14 000 × *g* for 20 min and assay of both supernatant (S) and precipitate (P), giving an estimate of total recovery as well as extent of solubilization. This was found to be important in indicating loss or enhancement of activity through treatment. Untreated M fraction was centrifuged and assayed at the same time.

Freezing and thawing. Results are given in Table VII. In contrast to the results obtained by other workers with liver and kidney particles, the maximum release of

TABLE VII

SOLUBILIZATION OF THYROID ENZYMES BY FREEZING AND THAWING

Preparation: 30 g pig thyroids homogenized in 60 ml 0.25 M sucrose. Centrifuged 20 min, 14 000 × *g*. Precipitate washed with 60 ml sucrose and recentrifuged. Precipitate suspended in 60 ml sucrose and subjected to freezing and thawing. All samples centrifuged together 20 min, 14 000 × *g*. Precipitates suspended in the original volume and all samples dialysed prior to assay.

*Treatment**	*% total activity released*			
	Acid protease	*APAT hydrolase*	*Cysteinyl-tyrosine hydrolase*	*Acid phosphatase*
0	10	6	28	9
1	45	42	44	24
2	52	44	52	25
3	50	52	58	30
6	55	54	67	40

* Figures given under Treatment refer to the number of times frozen and thawed.

acid phosphatase was only 40% while that of the proteolytic enzymes was usually higher, particularly cysteinyltyrosine hydrolase, 67% of which could be recovered in the soluble fraction.

Dialysis. The M fraction was dialysed overnight at 4° against glass-distilled water. 38% acid protease, 27% APAT hydrolase and 41% acid phosphatase was solubilized.

Sonication. Sonication of the M fraction suspended in 0.25 M sucrose was carried out in a glass vessel in an ice-bath with the probe just under the surface of the suspension. Results are given in Table VIII. The four enzymes studied can be solubilized in this way, but APAT hydrolase required longer treatment. 1 min sonication con-

TABLE VIII

SOLUBILIZATION OF THYROID PARTICULATE ENZYMES BY SONICATION

Preparation: 70 g pig thyroids homogenized in 140 ml 0.25 M sucrose. Centrifuged 20 min, 14 000 × *g*. Precipitate resuspended in 70 ml sucrose and 10-ml aliquots subjected to sonication for times as stated. Recentrifuged at 14 000 × *g* for 20 min. Dialysed overnight. Sonication: MSE Ultrasonicator, 500-W model, used at stop 3 on maximum output. 1 min = 2 × 30 sec; 6 min = 3 × 2 min; 12 min = 4 × 3 min. Figures in parentheses refer to % of total activity recovered.

Sonication time (min)	*Acid protease (units/ml)*		*APAT hydrolase (milli-units/ml)*		*Cysteinyltyrosine hydrolase (milli-units/ml)*		*Acid phosphatase (milli-units/ml)*	
	Precipitate	*Supernatant*	*Precipitate*	*Supernatant*	*Precipitate*	*Supernatant*	*Precipitate*	*Supernatant*
0	26.9 (87)	4.2 (13)	700 (91)	67 (9)	467 (85)	83 (15)	1800 (89)	233 (11)
1	23.2 (76)	7.2 (24)	700 (91)	67 (9)	383 (72)	150 (28)	1267 (70)	550 (30)
6	15.4 (57)	11.8 (43)	483 (66)	250 (34)	267 (64)	150 (36)	967 (45)	1167 (55)
12*	0.7 (15)	4.1 (85)	33 (7)	417 (93)	67 (17)	317 (83)	67 (12)	467 (88)

* Temperature rose to approx. 30° during last 3 min sonication.

TABLE IX

SOLUBILIZATION OF THYROID PARTICULATE ENZYMES BY TWEEN 20

Preparation: 65 g pig thyroids homogenized in 140 ml 0.25 M sucrose. Centrifuged 20 min, 14 000 × *g*. Precipitate suspended in 70 ml sucrose and aliquots treated as above. All samples recentrifuged 20 min, 14 000 × *g*. Precipitates suspended in sucrose. Tween concentrations during assay: precipitate (P): none; supernatant (S): protease, 0, 0.016 and 0.16%; APAT hydrolase, 0, 0.025 and 0.25%; acid phosphatase, 0, 0.005 and 0.05%.

Conditions of preincubation	*Acid protease*			*APAT hydrolase*			*Acid phosphatase*		
	Units		*% release*	*Milli-units*		*% release*	*Milli-units*		*% release*
	P	*S*		*P*	*S*		*P*	*S*	
Control	21.5	6.8	24	400	50	11	1833	200	10
0.1% Tween, 0 min, 4°	20.0	7.4	27	417	50	11	1667	83	5
30 min, 4°	20.0	7.9	28	400	100	20	1333	267	17
0.1% Tween, 30 min, 4°	20.8	7.6	27	400	100	20	1167	867	43
1.0% Tween, 30 min, 4°	18.2	8.2	31	317	133	30	1200	950	44
30 min, 37°	19.2	7.5	28	400	100	20	1783	217	11
0.1% Tween, 30 min, 37°	19.0	7.8	29	450	217	33	1033	950	48
1.0% Tween, 30 min, 37°	6.6*	15.5	70	217*	367	63	400*	1633	80

* Also assayed in presence of Tween, concentrations during assay being: protease, 0.16%; APAT hydrolase, 0.25%; acid phosphatase, 0.05%. Results were similar: Protease = 5.3 U.; APAT hydrolase = 250 mU.; acid phosphatase = 433 mU.

TABLE X

EFFECT OF TWEEN 20 ON THYROID PARTICULATE ENZYMES

Preparation: As for Table VIII. No preincubation. Tween concentration on assay: As given in above table.

Concentration of Tween 20 (%)	*Acid protease (milli-units/ml)*	*APAT hydrolase (milli-units/ml)*	*Acid phosphatase (milli-units/ml)*
0	1883	40	133
0.1	1650	46	136
1.0	667	46	140

sistently released little, if any, APAT hydrolase in contrast to a release of up to 30% of bound acid protease. 12 min sonication caused some inactivation of the enzymes studied, in particular of acid protease and phosphatase.

Detergent action. (a) Tween 20 was added to the M fraction and the suspension was centrifuged after standing for 30 min at either 4° or 37°. As the results in Table IX indicate, Tween 20 does not readily solubilize the three enzymes studied, a marked effect being obtained only at 37° and 1% Tween. The total recovery of acid protease in the precipitate and supernatant was only 77% that of the original, suggesting inactivation of the enzyme, whereas acid phosphatase was unaffected and APAT hydrolase was consistently activated by both Tween concentrations. (b) Tween 20 was added to the M fraction together with the substrate. Consistent with the results obtained above, a slight activation of APAT hydrolase resulted whereas acid protease was inactivated (Table X).

Incubation at pH 5.0 and 6.8, 37°. Results are given in Fig. 3. The enzymes under investigation were released at approximately the same rate. At pH 6.8, the effect was relatively slight whereas at pH 5.0 up to 80% of the enzymic activity was soluble after 4 h incubation.

Release was also studied at the pH of assay of APAT hydrolase and acid protease. At these pH levels the effect was more marked. At pH 4.1, 37% of APAT

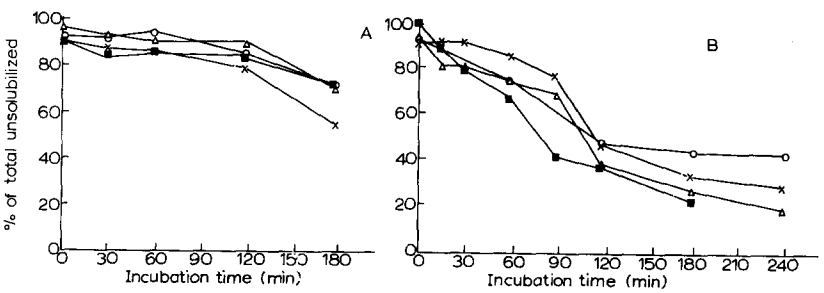
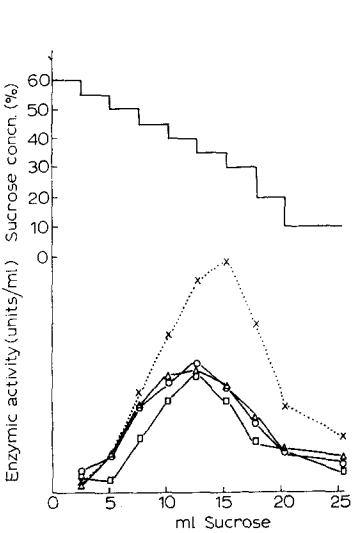


Fig. 3. Release of particulate enzymes at (A) pH 6.8 and (B) pH 5.0. Preparation: M fraction prepared as in MATERIALS AND METHODS from an homogenate of 30 g pig thyroids in 60 ml 0.25 M sucrose and resuspended in 60 ml of (A) 0.25 M sucrose (pH 6.8) or (B) 0.25 M sucrose–1 M sodium acetate–acetic acid (pH 5.0) and incubated at 37° as indicated in the figure. Suspension then centrifuged 20 min, 14 000 × *g* and precipitate suspended in original volume of 0.25 M sucrose (pH 6.8). Supernatant and precipitate dialysed before assay. ○—○, acid protease; △—△, APAT hydrolase; ×—×, acid phosphatase; ■—■, cysteinyltyrosine hydrolase.



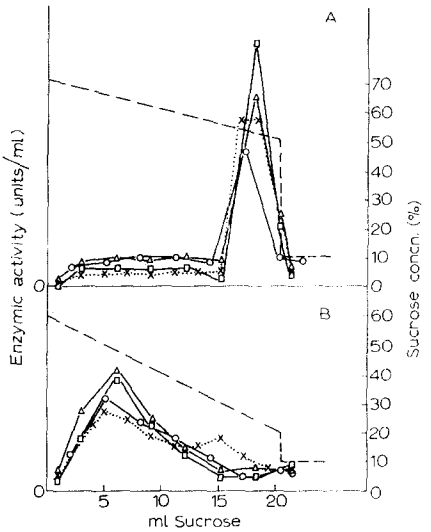


Fig. 4. Discontinuous sucrose gradient centrifugation. Preparation; 30 g pig thyroids homogenized in 60 ml 0.25 M sucrose. Fractionation as given in MATERIALS AND METHODS. M fraction resuspended in 30 ml 10% sucrose. Gradient discontinuous: 20% to 60% sucrose. Sampling, 2.5-ml aliquots were collected for assay. All assayed without dialysis. Acid phosphatase assayed after freezing and thawing once. ○—○, acid protease; △—△, APAT hydrolase; ×.....×, acid phosphatase; □—□, cysteinyltyrosine hydrolase.

Fig. 5. Continuous sucrose gradient centrifugation. Preparation as given in Fig. 4. Gradients: (A) 50% to 70% sucrose; (B) 20% to 60% sucrose. Sampling: 1.0-ml aliquots were collected for assay. Assay as in Fig. 4. ○—○, acid protease; △—△, APAT hydrolase; ×.....×, acid phosphatase; □—□, cysteinyltyrosine hydrolase; — — — —, sucrose concentration.

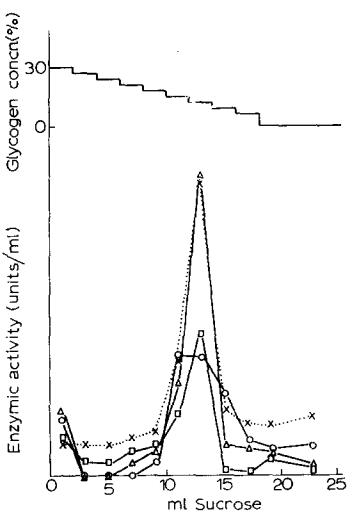


Fig. 6. Discontinuous glycogen gradient centrifugation. Preparation: 30 g pig thyroids homogenized in 60 ml 0.25 M sucrose. M fraction prepared as in MATERIALS AND METHODS and resuspended in 30 ml 0.5 M sucrose. Gradient discontinuous: 0.5 M sucrose to 30% glycogen–0.5 M sucrose. Sampling: 2.5-ml aliquots were collected for assay. Assay as in Fig. 4. ○—○, acid protease; △—△, APAT hydrolase; ×.....×, acid phosphatase; □—□, cysteinyltyrosine hydrolase.

hydrolase was released on standing for 30 min at 4° and 69% after incubating for 16 min at 37°. At pH 3.5, the extent of solubulization of acid protease on standing at 4° for 30 min and after incubation for 16 min at 37° was similar (approx. 50%).

Investigation of structure-linked latency. The M fraction was prepared as in

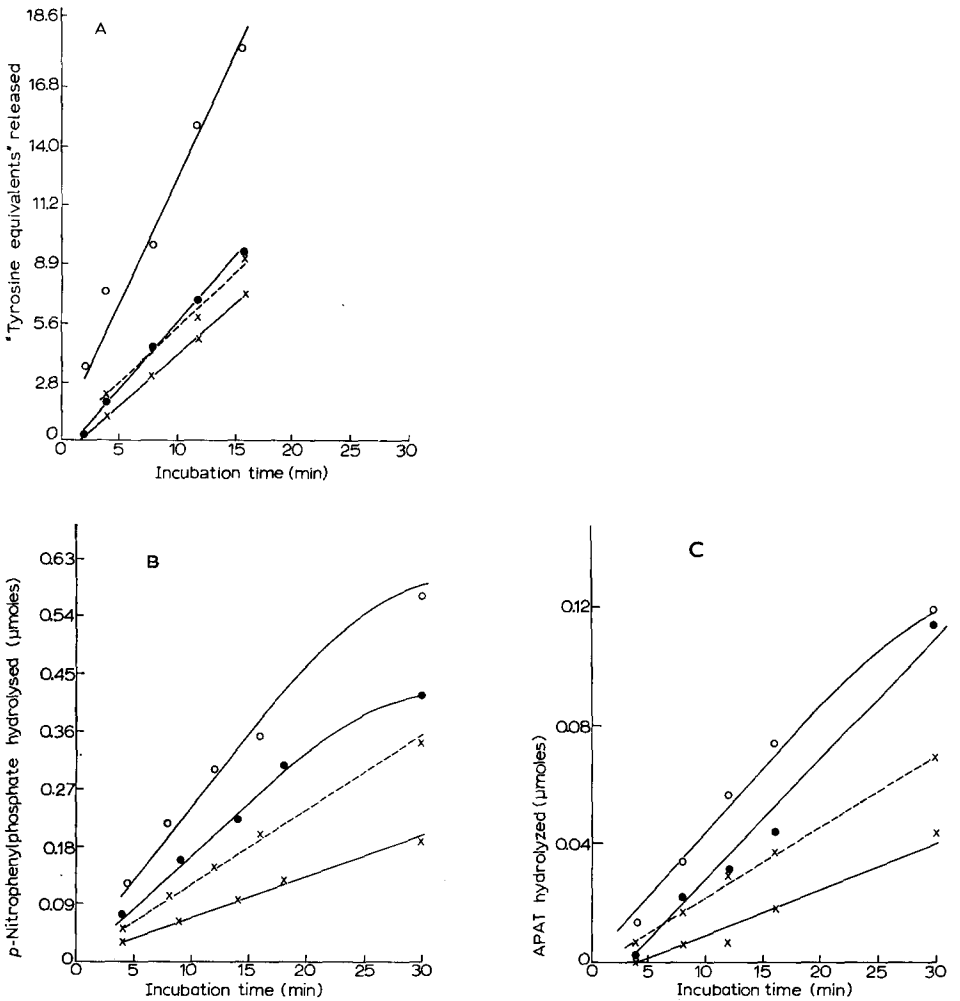


Fig. 7. Structure-linked latency. Preparation: M fraction prepared as in MATERIALS AND METHODS from a homogenate of 100 g pig thyroids in 2 vol. 0.25 M sucrose–1% bovine serum albumin. M fraction suspended in 14 ml 0.25 M sucrose–1% bovine serum albumin. Assays carried out within 2 h of preparation. Assay procedure: Acid protease (A), 1 ml haemoglobin (pH 3.5) incubated at 37° with (i) 0.2 ml M fraction (ii) 0.2 ml M fraction, diluted 1:3. Tyrosine equivalents released determined as in general assay procedure. Acid phosphatase (B), 1 ml *p*-nitrophenyl phosphate–citrate buffer (pH 5.1) incubated at 37° with 0.2 ml M fraction or diluted M fraction; 200-μl samples of the incubation delivered into 4 ml 0.1 M NaOH at different time intervals for estimation of nitrophenol. APAT hydrolase (C), 0.06 ml APAT in 0.1 M NaOH, 0.54 ml acetate buffer (pH 4.1) and 0.2 ml M or diluted M fraction incubated at 37°; 100-μl samples taken at various time intervals and reacted with ninhydrin. ●—●, M fraction; ○—○, M fraction (9:10 diluted), 0.1% Tween; ×—×, M fraction, diluted 1:3; ×- - - -×, M fraction (3:10 diluted), 0.1% Tween.

MATERIALS AND METHODS, but was suspended in a very much smaller volume than normally used (0.14 vol. compared with 0.8 vol.). Incubations were carried out for different time intervals as shown in Fig. 7. Comparison was made with results obtained with a more dilute preparation (0.52 vol.) and in the presence of 0.1% Tween. The time *vs.* activity curves for acid protease and phosphatase showed little, if any, lag period but addition of Tween enhanced activity, thus indicating some latency, especially at the higher particle concentration. The time *vs.* activity curves for APAT hydrolase indicated a lag period of 4 min or less. In addition, activity was increased by Tween. Demonstration of latency of particulate cysteinyltyrosine hydrolase activity was not attempted because of (a) the necessity for preincubation with Zn^{2+} at pH 5 and (b) the high zero time values due to the high extinction of the substrate and protein present.

Sucrose gradient centrifugation. The M fraction was washed with 0.25 M sucrose, centrifuged and resuspended in a volume of 10% sucrose equivalent to the original wet weight of glands used. 5 ml of this preparation was layered on to the gradient. Initially, discontinuous gradients were prepared as shown in Fig. 4. From these results it appeared that the proteolytic enzymes under investigation were concentrated in identical layers whereas acid phosphatase was concentrated at lower sucrose levels. However, using continuous sucrose gradient centrifugation (Fig. 5) this discrepancy was not observed and it seems probable that the enzymes are all located in particles of similar size and osmotic properties. This was further confirmed using a discontinuous glycogen gradient (Fig. 6).

Distribution of enzymes in the rat thyroid

Results of one experiment are shown in Table XI together with maximum variation obtained in a number of others. In contrast to results obtained with pig thyroids, the distribution of acid protease (haemoglobin substrate) differs from that of acid phosphatase, the protease being located mainly in the soluble fraction while neutral protease occurs entirely in the particulate fraction. The pH curve obtained

TABLE XI

DISTRIBUTION OF ENZYMES IN THE RAT THYROID

Preparation: 63 mg rat thyroids homogenized in 4 ml 0.25 M sucrose. Centrifuged 80 min, 110 000 × *g*. Pellet suspended in 4 ml sucrose. Number of experiments: Acid protease, 20; neutral protease, 16; APAT hydrolase, 20; cysteinyltyrosine hydrolase, 20; cathepsin A, 18; acid phosphatase, 20.

Enzyme	*% Distribution*	
	Particulate	*Soluble*
Acid protease*	28 (± 7)	72 (± 7)
Neutral protease*	100 (± 3)	0 (± 3)
APAT hydrolase	80 (+ 13)	20 (− 13)
Cysteinyltyrosine hydrolase	30 (± 10)	70 (± 10)
Cathepsin A**	67 (− 20)	33 (+ 20)
Acid phosphatase	47 (± 15)	53 (± 15)

* Assayed without prior dialysis.
** Assayed with Z-L-Glu-L-Tyr as substrate.

with rat thyroid homogenate (haemoglobin substrate) was very similar to that with pig with optima at pH 3.9–4.5 and 6.4–7.4.

DISCUSSION

The proteolytic "pH spectra" of homogenates obtained from pig and rat thyroid glands appear to be restricted to approximately one pH unit in both the acid and neutral range. The activity demonstrated with thyroglobulin as substrate at both pH optima is much lower than that with haemoglobin. However, this may merely indicate that the criterion of proteolytic activity utilized, namely, release of "tyrosine equivalents", although deliberately chosen for direct comparison, is less suitable when thyroglobulin is the substrate than that of release of the physiologically important iodoamino acids.

The activity in the acid pH range is probably due to the well-characterized acid protease supplemented by peptidase action. As reported by MENZIES *et al.*[3], the peptidase APAT hydrolase will hydrolyse peptide fragments obtained by proteolysis of ^{131}I-labelled thyroglobulin by acid protease. This supplementary action could be a factor in the elevation of the pH optimum and in the higher percentage of particle-located activity when thyroglobulin is the substrate. While this paper was in preparation, BALASUBRAMANIAM AND DEISS[26] published their results on the purification and properties of a lysosomal cathepsin D from beef thyroids. Their data also indicate no effect of –SH reagents on this enzyme from thyroid lysosomes. They imply that this lysosomal enzyme is latent and disruption in a high-speed homogenizer is essential for assay of total enzyme. However, even though the enzyme was particle-bound, we could detect no latency at the pH of assay and at the particle concentration normally used.

The activity of pig thyroid homogenates determined using haemoglobin as substrate at pH 7.5, in contrast to that at pH 3.5, is located mainly in the soluble fraction whereas with thyroglobulin as substrate the activity at pH 7.5 is almost entirely in the particulate fraction, suggesting the presence of at least two enzymes. BALASUBRAMANIAM AND DEISS[26] are of the opinion that activity of beef thyroid homogenates with urea-denatured haemoglobin as substrate at pH 7.5 is artifactual and is due to release of nucleic acids presumably from the homogenate. However, the marked difference in amount of tyrosine equivalents liberated from haemoglobin and thyroglobulin in our work does not support this assumption with respect to pig thyroid homogenates. It would appear that this activity at pH 7.5 (thyroglobulin substrate) is distinct from that described by PASTAN AND ALMQVIST[8,27] in the particulate fraction of rat thyroid homogenates. The latter enzyme which hydrolysed ^{125}I-labelled thyroglobulin (approx. 50% reduced) showed an absolute dependence on high KCl concentration. In our work no KCl was added. In contrast to our results with pig thyroid homogenates, the activity of rat thyroid homogenates (haemoglobin substrate) at pH 7.5 is confined to the particulate fraction. In a preliminary experiment, hydrolysis of ^{131}I-labelled thyroglobulin (as measured by release of ^{131}I soluble in 10% trichloroacetic acid) was detected only at pH 3.5 with no hydrolysis at pH 7.5. The latter result is consistent with that obtained by PASTAN AND ALMQVIST[8,27] since neither KCl nor mercaptoethanol was added.

The distribution of peptidase and protease activity as determined in our experiments may reflect a true picture of the distribution in the intact thyroid follicle. How-

ever, it is most probable that considerable damage has been done to subcellular organelles during the preparation, particularly since thyroid tissue is difficult to homogenize. The enzymic activity detected in the particulate fraction must therefore be regarded as a minimum. It should also be remembered that the soluble fraction comprises follicular colloid as well as cytoplasmic soluble fraction. However, certain differences in the distribution of the enzymes studied have been established. The peptidases APAT hydrolase, cathepsin A and, to a slightly smaller extent, cysteinyltyrosine hydrolase are located predominantly in the particulate fraction whereas acid protease and phosphatase are distributed equally between the particulate and soluble fractions. There are several possible explanations for this difference in behaviour. The former group of enzymes may be located either in a particle which is more stable to homogenization or in the membrane which co-precipitates with the intact particle on centrifugation after disruption. On the other hand there may be actually a higher percentage of the latter enzymes in the cytoplasm and/or the colloid. DE ROBERTIS[1], using a micromanipulative procedure, claimed that acid protease is present in the colloid of the thyroid follicle. However, WETZEL, SPICER AND WOLLMAN[12], as well as other workers[10,11], were unable, by histochemical techniques, to detect acid phosphatase in luminal colloid but only in intracellular colloid droplets. Leucine aminopeptidase is located essentially in the soluble fraction although at least 20% is particulate—mainly in the nuclear fraction which would also contain unbroken cells. Carboxypeptidase also appears to be mainly in the soluble fraction. The distribution of proteolytic activity at neutral pH has already been discussed.

Attempts to demonstrate differences in the stability of the particles containing the enzymes were, in general, unsuccessful. Although the various procedures, *e.g.* freezing and thawing, dialysis, detergent action and incubation at acid pH, differed in the efficiency of solubilization, all of the enzymes studied, with the exception of APAT hydrolase, were affected to a similar extent. This peptidase appears to be more resistant, especially to sonication. It should be noted, however, that these studies were, of necessity, carried out on the most stable particles, namely, those which had withstood homogenization.

Sucrose and glycogen gradient centrifugation yielded very similar distribution patterns for the enzymes. However, electron micrographs indicated that enzyme-rich fractions from sucrose gradient centrifugation were not homogeneous but included mitochondria, endoplasmic reticulum and debris as well as lysosomes.

Structure-linked latency, which is a general property of lysosomal enzymes, could not be demonstrated at the particle concentration normally used although at higher concentrations some latency was apparent. A complicating factor is that the assay conditions for these enzymes favour autolysis of the particles.

We cannot conclude that the enzymes studied are definitely of lysosomal origin, although the very similar distribution of acid phosphatase and the properties of the particulate fraction would suggest this to be the case. The differential release of proteolytic enzymes from the lysosomes may be an important factor in the control of thyroglobulin breakdown. The initial proteolysis may occur within the thyroid lumen, possibly with the release of large peptides, but further breakdown most probably takes place within the cell. On the other hand, the entire process may occur within the cell. Work in progress is directed towards the solution of this problem. The influence of thyroid status on the process of proteolysis is also under investigation.

ACKNOWLEDGEMENTS

We are indebted to the National Health and Medical Research Council of Australia for a personal grant to one of us (P.J.) and for a contribution towards the costs of the investigation. We wish to express our thanks to Professor V. M. TRIKOJUS for helpful discussions and to Miss E. PARKHILL for skilled technical assistance.

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