PURIFICATION AND PROPERTIES OF THYROID PROTEASE

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Summary

Thyroglobulin, purified to electrophoretic homogeneity by fractional precipitation of hog thyroid extracts with ammonium sulphate, has been shown to retain appreciable proteolytic activity.

By solvent-salt fractionation thyroid protease has been obtained substantially free from thyroglobulin.

The enzyme has optimum activity within the range pH 2.5-3.5. It is not activated by sulphydryl reagents, metal ions, trypsin, or thyrotrophic hormone.

I. INTRODUCTION

It seems now well established that the thyroid gland secretes L-thyroxine, as such, into the blood stream where it circulates in loose attachment to protein (Taurog and Chaikoff 1948; Leblond and Gross 1949; Taurog, Chaikoff, and Tong 1950; Rosenberg 1951; Gordon *et al.* 1952). Tri-iodothyronine is also present in normal plasma (Gross and Pitt-Rivers 1952).

There is, however, no certainty as to the structure of the thyroid hormone when it acts peripherally. Moreover, clarification is needed in regard to the mechanisms which effect the liberation of thyroxine from the more complex forms in which it is stored in the thyroid gland—although it might be assumed that simple proteolysis would be the prime motivator in this process. Gersh and Caspersson (1940), as a result of histochemical studies of thyroid glands of guinea pigs under different physiological conditions, formed the opinion that, in the normal animal, the production of colloid is a continuous process and suggested that a protease secreted by the gland cell may digest the colloid in the lumen to products—polypeptides or peptones—capable of rapid transfer across the cell membrane. In extending these observations, de Robertis (1941) was able to show that the colloid extracted from single thyroid follicles possessed appreciable proteolytic activity while Dziemian (1943) claimed that such activity varied with fluctuations in the physiological status of the animal.

In the course of experimental work with purified thyroglobulin we were able to confirm the existence of a close thyroglobulin-enzyme association by showing that such preparations still exhibited appreciable proteolytic activity even though they behaved on electrophoresis as homogeneous proteins. This led to attempts to separate the enzyme component(s) both from such purified thyroglobulin and from cruder material and to devise methods for its preparation and purification. The results of a study of the general pro-

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perties of the protease are also reported here, while its application in an investigation of the enzymic breakdown of thyroglobulin labelled with ^{131}I will be reported in detail later.*

II. Materials and Methods

All chemicals used were of A.R. grade and all solvents were purified by standard methods.

Purified Thyroglobulin.—This was prepared according to the procedure of Derrien, Michel, and Roche (1948) which involves fractional precipitation of a saline extract of thyroid glands with $(NH_4)_2SO_4$, the fraction soluble at 37 per cent. saturation and insoluble at 41 per cent. saturation being retained. Further treatment of the end-product so obtained was omitted in this work although in one instance (batch I) the complete fractionation procedure was followed through a second time. Fresh hog thyroids were used.

Table 1

COMPARISON OF THE PROTEOLYTIC ACTIVITY OF PURIFIED THYROGLOBULIN AND OF THYROID ENZYME PREPARATIONS (METHOD 1)

Preparation	Batch No.	Nitrogen (mg/ml test solution)	Iodine (μg/ml test solution)	$\frac{N}{I}$	Activity (m-equiv. $\times 10^4$ tyrosine liberated per mg N)
Thyroglobulin	H	3·70	139	27	8·9
Thyroglobulin	I*	3·44	—	—	6·3
Thyroglobulin	I†	4·47	134	33	5·7
Thyroglobulin	J	6·15	312	20	7·1
Thyroglobulin	L	3·00	105	29	6·0
Enzyme	2	0·25	2·2	114	57·6
Enzyme	6	0·28	1·5	187	63·6
Enzyme	7	0·26	1·1	236	55·4

* After first fractionation with $(NH_4)_2SO_4$.
† After second fractionation with $(NH_4)_2SO_4$.

Thyrotrophic Hormone.—Two specimens were used—one prepared by Schering A.G. and the other by Parke-Davis (preparation No. 099931) containing 34 and 9-10 Junkmann-Schoeller units/mg respectively.

Proteolytic Activity.—This was tested by the method of Anson (1938) with minor modifications—0.5 ml of enzyme solution was incubated with 2.5 ml of 2 per cent. haemoglobin as substrate for 30 min at 37°C; the supernatant, following precipitation with trichloracetic acid, was assayed with the phenol reagent of Folin-Dennis and results expressed as m-equiv. $\times 10^4$ of tyrosine liberated. The haemoglobin was preserved as a freeze-dried powder.

* A preliminary account of the results of this work was given at the 1951 Oxford Isotope Conference (Gordon *et al.* 1953).

pH.—Throughout the work pH was checked with the glass electrode. In testing the pH of alcoholic solutions the procedure of Cohn *et al.* (1950) was adopted and these were buffered by the addition of 2.5 ml/l of a stock solution of the required pH; the buffers used were acetate (4.3M) for pH 4.0 and mixtures of K_2HPO_4 (2M) and NaH_2PO_4 (4M) in the appropriate proportions to give pH 6.0 and pH 8.0 on dilution.

Iodine and Nitrogen Assays.—Iodine values were determined either by the semi-micro method of Astwood and Bissell (1944) or by the micro method of Barker (1950) and nitrogen by the micro-Kjeldahl procedure with selenium as catalyst.

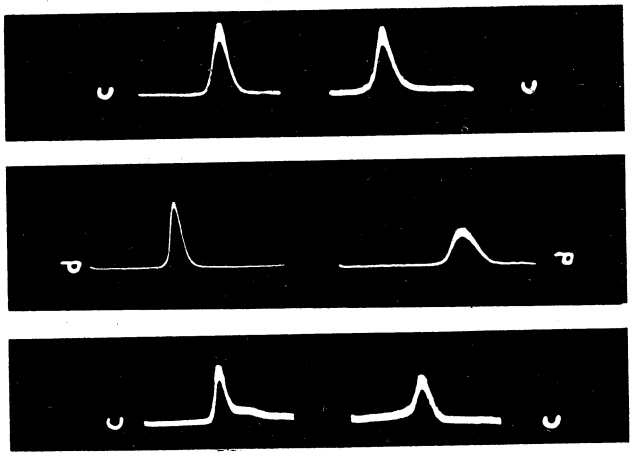


Fig. 1.—Electrophoretic tracings of thyroglobulin preparation I. Top, pH 5.70; middle, pH 7.65; bottom, pH 9.94. L.H.S.: ascending boundary. R.H.S.: descending boundary.

III. Experimental and Results

(*a*) *Proteolytic Activity of Thyroglobulin Preparations*

Results of determinations of the proteolytic activity of thyroglobulin preparations are listed in Table 1.

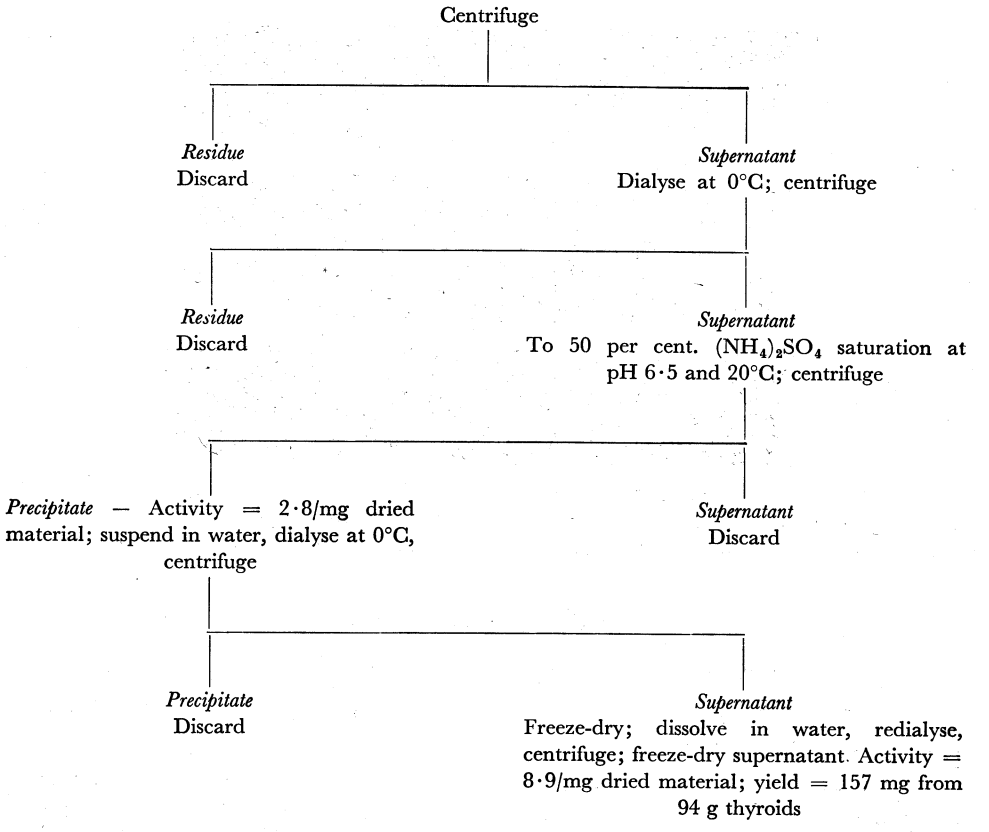
The proteolytic activities, expressed in arbitrary units,* are small but regular in value. Moreover, there was no significant change in content when one of the samples, batch I, was refractionated with $(NH_4)_2SO_4$. In accord with the observations of Derrien *et al.* (1949) on specimens of thyroglobulin similarly prepared, this material was found to be electrophoretically homogeneous. The sample was run at pH levels of 5.70, 7.65, and 9.94 (Fig. 1). No evidence of more than one component was obtained; an apparent second component in tracing *c*, pH 9.94, is a boundary anomaly usual with this buffer (Michaelis universal veronal buffer) and which migrates slowly at this pH.

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

(*b*) *Preparation of Protease*

Preliminary studies indicated that preferential denaturation of the thyroglobulin would facilitate the separation of the protease component. Consequently it was decided to use cruder material, prepared by precipitation of thyroid extracts with $(NH_4)_2SO_4$, rather than purified thyroglobulin, as the starting point for fractionation studies.

Method 1.—Hog thyroids; extract with 4 vol. 60 per cent. glycerol in Waring Blendor. Add toluol. Overnight at 0°C. Activity of extract = 0.112/mg thyroid.



The activities of three batches of enzymes prepared by this method are compared with those of four batches of thyroglobulin in Table 1. However, this method proved unreliable as it was partly dependent on separation of insoluble material—chiefly denatured thyroglobulin—during dialysis and this precipitation was not always complete.

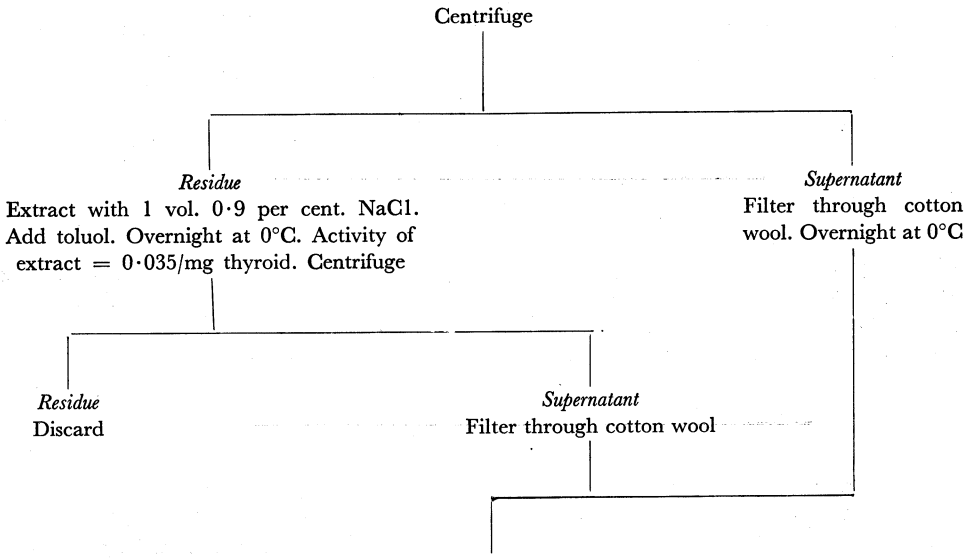
As extracts prepared with glycerol, phosphate buffer at pH 7, and isotonic sodium chloride were found to be of equal activity, saline was used in all future extractions. The crude material for fractionation was then obtained by preci-

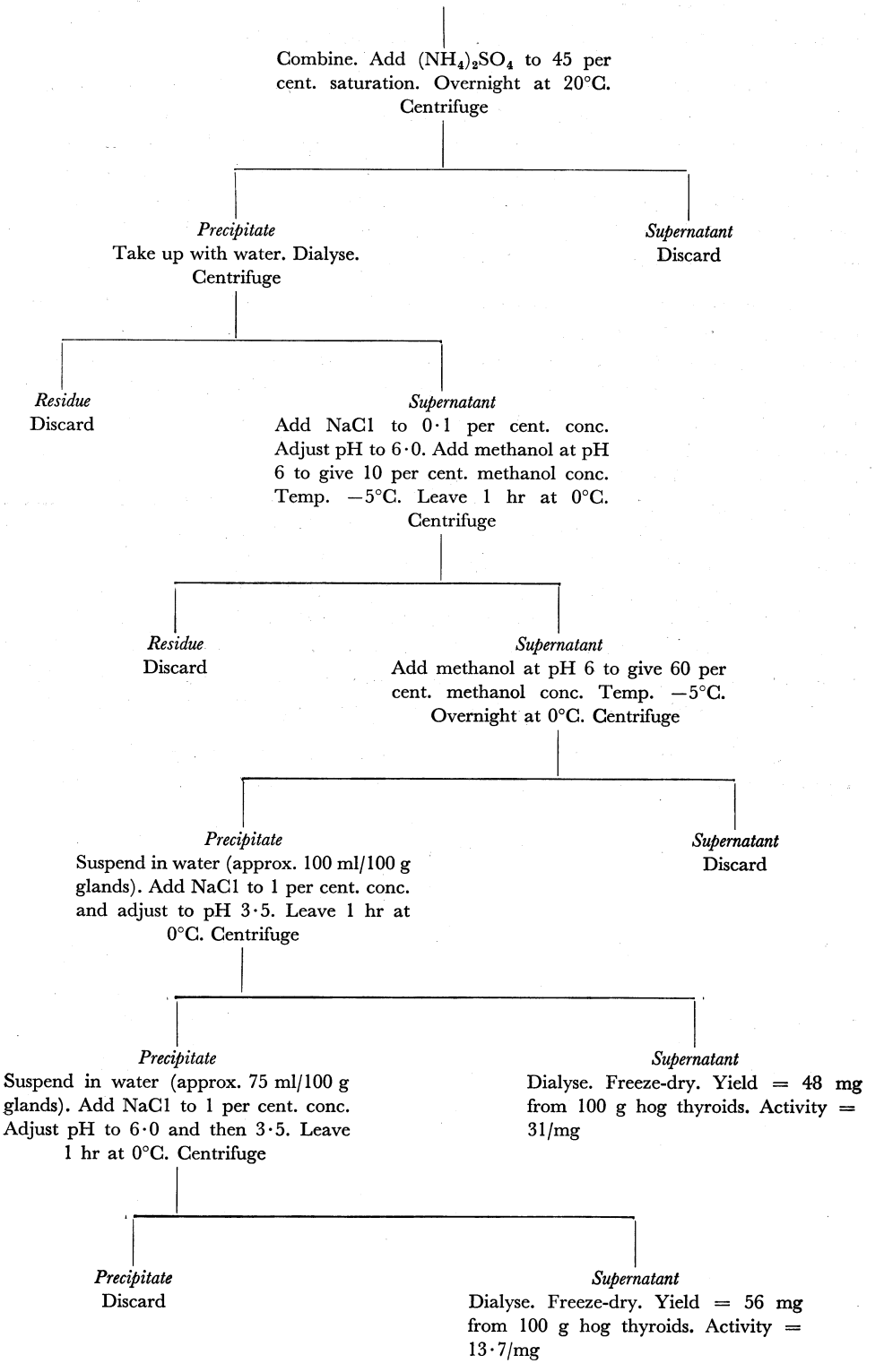
pitation at pH 6.5 with $(NH_4)_2SO_4$ at 45 per cent. saturation at which concentration removal of the enzyme from solution was complete.

Method 2 (*Solvent-Salt Fractionation*).—In a preliminary experiment in which the early stages of the plasma-fractionation procedure of Cohn *et al.* (1950) were carried out, the proteolytic activity was found in the precipitate from a 0.9 per cent. NaCl solution at 20 per cent. ethanol concentration which contained the bulk of the protein. More extensive studies along these lines with methanol also failed to effect a separation when pH levels of 4, 6, 7, or 8 and saline concentrations of 0.01 or 0.1 per cent. were used.

However, when a comparison was made of the solubility of the crude protein in distilled water and in isotonic saline, it was found that, on the addition of dilute acid to a solution in distilled water, precipitation occurred at the isoelectric point of thyroglobulin—pH 4.5—and, as the pH was lowered, the precipitate redissolved. On repeating this procedure using a solution of the crude protein in isotonic saline, precipitation occurred at pH 4.5 but the precipitate appeared to be completely insoluble even at pH 3.2. However, the clear supernatants obtained by centrifuging such solutions below pH 4.5 were found to possess appreciable proteolytic activity. When methanol was added to a solution of the crude protein in 0.1 per cent. NaCl to a concentration of 60 per cent., the precipitate suspended in 1 per cent. NaCl, and the pH adjusted to 3.5, about 60 per cent. of the total activity of the resulting suspension was found to be contained in the water-clear supernatant. The methanol appeared to effect a more rapid and complete denaturation of the bulk protein. These findings were utilized in the method of preparation finally adopted.

Procedure.—Extract glands with 3 vol. 0.9 per cent. NaCl in Waring Blendor. Add toluol. Overnight at 0°C. Activity of extract = 0.200/mg wet thyroid tissue.




Combine. Add $(NH_4)_2SO_4$ to 45 per cent. saturation. Overnight at 20°C. Centrifuge
Precipitate
Take up with water. Dialyse. Centrifuge
Supernatant
Discard
Residue
Discard
Supernatant
Add NaCl to 0·1 per cent. conc. Adjust pH to 6·0. Add methanol at pH 6 to give 10 per cent. methanol conc. Temp. −5°C. Leave 1 hr at 0°C. Centrifuge
Residue
Discard
Supernatant
Add methanol at pH 6 to give 60 per cent. methanol conc. Temp. −5°C. Overnight at 0°C. Centrifuge
Precipitate
Suspend in water (approx. 100 ml/100 g glands). Add NaCl to 1 per cent. conc. and adjust to pH 3·5. Leave 1 hr at 0°C. Centrifuge
Supernatant
Discard
Precipitate
Suspend in water (approx. 75 ml/100 g glands). Add NaCl to 1 per cent. conc. Adjust pH to 6·0 and then 3·5. Leave 1 hr at 0°C. Centrifuge
Supernatant
Dialyse. Freeze-dry. Yield = 48 mg from 100 g hog thyroids. Activity = 31/mg
Precipitate
Discard
Supernatant
Dialyse. Freeze-dry. Yield = 56 mg from 100 g hog thyroids. Activity = 13·7/mg

Certain preparations gave lower recoveries of purified enzyme. This is probably linked with the low protein concentrations during dialysis at the later stage of the preparation. In two of these preparations in which only small amounts of protease were recovered, the enzyme was of relatively high activity —90/mg—perhaps owing to removal of a contaminant by adsorption on the Cellophane.

The proteolytic activities and iodine contents of four specimens of protease prepared according to the above method are listed in Table 2. The fact that iodine is still present is presumably due to contamination and not to inherent association of iodine and the enzyme protein, while the variation in iodine content of the specimens probably reflects the nature of the thyroglobulin contaminant; thus in a parallel investigation the iodine values of a series of purified thyroglobulin samples were found to range between 0.15 and 0.86 per cent.

TABLE 2

PROTEOLYTIC ACTIVITY OF THYROID ENZYME PREPARATIONS (METHOD 2)

Preparation No.	Iodine (g/100 g)	Activity (m-equiv. $\times$ 10^4 tyrosine liberated per mg enzyme)
14	0·016	33·6
16	0·034	23·2
17	0·018	39·5
18	0·042	37·0

(*c*) *Properties of Protease*

The enzyme preparations made according to either method were white powders, readily soluble in water. Solutions were found to be without activity following heating at 100°C for 5 min at pH 3.5.

pH activity curves were determined for a number of specimens of protease, mostly within the pH range 2-4. Where tests were carried out at pH values above 4 it was found that the addition of urea to denature the haemoglobin substrate (as recommended by Anson 1938) inactivated the enzyme. Consequently, the following general procedure was used for the preparation of denatured substrate: haemoglobin powder was dissolved in water, the pH lowered to 1.15, and the solution left 1 hr at room temperature. Aliquots were then taken, adjusted to the required pH with 1N NaOH, and diluted to give a 2 per cent. solution. Owing to the strong buffering action of haemoglobin, individual adjustment to the correct pH was preferred to the addition of buffer solutions.

The results obtained with two specimens of enzyme prepared according to method·2 are shown in Figure 2. Although our earlier observations with cruder preparations had indicated a pH maximum at 3.5 in accord with Dziemian

(1943), present findings show a practically constant value between pH 2.5 and 3.5. In other tests it was found that the activity continued to drop from pH 4 to 6.

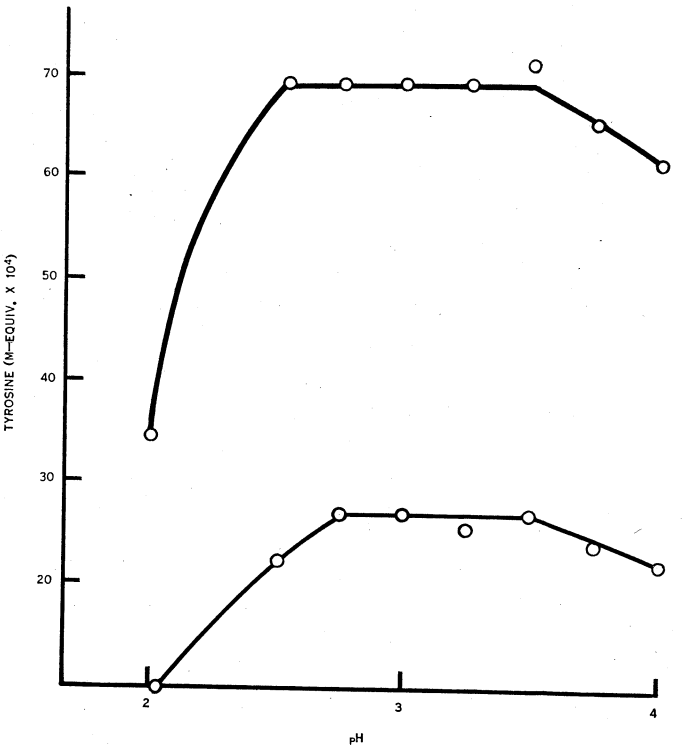


Fig. 2.—pH activity curves of purified protease, preparations 34 and 35 (method 2). Activity at pH 3.5 = 71.5 and 27.0 respectively.

The action of the protease was tested only on the substrates haemoglobin and thyroglobulin. On the latter protein, under the conditions of assay as applied to haemoglobin, the enzyme did not appear to exert any effect. However, that there is a well-marked action on this substrate was evident in the more intensive studies with longer periods of incubation in which thyroglobulin labelled with ^{131}I was utilized (unpublished data).

(*d*) *Experiments with Activators*

(i) Using crude thyroid extracts, Dziemian (1943) and Kamner, Peranio, and Bruger (1950) reported increased proteolytic activity in the presence of cysteine. The effect of incubation of the purified enzyme with cysteine and thioglycollic acid was therefore investigated. Results are listed in Table 3. In this table are also recorded the results of attempts to activate the protease by incubation with Mn^{++} (as $MnSO_4$). This metal was chosen in view of its recognized role as a cathepsin activator (Lehninger 1950) and particularly in

consequence of the report by Deysach and Ray (1949) that Mn is preferentially absorbed by the thyroid gland. Enzyme solution plus activator was usually incubated for 2 hr and the activity subsequently compared with that of a control solution incubated at the same pH for a similar period.

TABLE 3

ACTIVITY OF ENZYME FOLLOWING INCUBATION FOR 2 HR WITH THIOGLYCOLLIC ACID, CYSTEINE, Mn^{++}, AND Ca^{++}

Preparations 8, 10, 12*a*, and 12*b* were obtained according to method 1, preparation 18*a* according to method 2

Preparation No.	Agent Under Test	Concentration of Agent	pH of Incubation	M-equiv. × 10^4 Tyrosine Liberated per mg Enzyme*
Crude NaCl extract	Thioglycollic acid	0·003M	3·2	7·8†
Crude NaCl extract	Control	—	3·2	9·3†
8	Thioglycollic acid	0·015M	3·85	7·1
8	Control	—	3·85	7·1
10	Cysteine	0·004M	3·5	15·5
10	Cysteine	0·008M	3·5	15·8
10	Control	—	3·5	15·4
12*a*	Cysteine	0·017M	3·7	19·5
12*a*	Control	—	3·7	17·9
8	Mn^{++}	0·02M	3·5	6·2
8	Control	—	3·5	7·2
8	Mn^{++}	0·02M	7·0	7·5
8	Control	—	7·0	7·2
8	Mn^{++}	0·02M	7·5	6·2
8	Control	—	7·5	6·1
12*b*	Mn^{++}	0·02M	6·95	11·2
12*b*	Control	—	6·95	11·2
12*b*	Mn^{++}	0·02M	3·5	8·3
12*b*	Control	—	3·5	11·2
18*a*	Ca^{++}	0·02M	‡	50·2
18*a*	Control	—	‡	50·2

* pH of test, 3·5. † Figures represent activity per ml extract. ‡ Not incubated.

In order to reduce to a minimum the high value for the blank determination usually obtained in experiments with cysteine, due to the reaction of this substance with the phenol reagent, the enzyme powder was either dissolved directly in one drop of cysteine hydrochloride solution of appropriate concentration (experiments with enzyme preparation No. 10) or in one drop of water and an equal volume of cysteine hydrochloride added (experiments with enzyme

preparation No. 12*a*). At the conclusion of the incubation period, the solution of enzyme was diluted to the requisite volume before assaying with haemoglobin substrate.

It is apparent that, under these test conditions, no activation of the protease by any of these agents was observed. Results obtained with purified enzyme, therefore, do not confirm the claims of previous workers cited above using crude thyroid extracts. It should be noted, however, that Dziemian (1943) used a different protein substrate, edestin, in his assays. Incubation with Mn^{++} at pH 3.5 decreased somewhat the activity of the enzyme, although this effect was not seen when prior incubation was carried out at pH levels between 6.95 and 7.5. It may also be noted that we were unable to confirm the observation of Kamner, Peranio, and Bruger (1950) that proteolytic activity was increased in the presence of acetate (Watson and Trikojus, unpublished data).

TABLE 4

ACTIVITY OF ENZYME (PREPARATIONS 6*a*, 6*b*; METHOD I) FOLLOWING INCUBATION FOR 2 HR WITH THYROTROPHIC HORMONE (34 J.S. UNITS/MG)

	TSH Concentration (J.S. units/mg enzyme)	pH of Incubation	pH of Test	M-equiv. × 10^4 Tyrosine Liberated per mg Enzyme
Enzyme (6*a*)+TSH	4	3·6	3·5	13·0
Enzyme (6*a*)+TSH	4	3·6*	3·5	14·0
Enzyme (6*a*) control	—	3·6	3·5	14·1
TSH control	4	3·6	3·5	—
Enzyme (6*b*)+TSH	4	7·0	3·5	9·5
Enzyme (6*b*)+TSH	4	7·0*	3·5	10·9
Enzyme (6*b*) control	—	7·0	3·5	10·2
TSH control	4	7·0	3·5	—
Enzyme (6*b*)+TSH	4	7·0	7·0	—
Enzyme (6*b*)+TSH	4	7·0*	7·0	—
Enzyme (6*b*) control	—	7·0	7·0	—
TSH control	4	7·0	7·0	—

* Enzyme and TSH were incubated separately and combined for testing.

(ii) In view of the report (Dziemian 1943) that the proteolytic enzyme content of the thyroid gland is increased after administration of thyrotrophic hormone (TSH) to rats and guinea pigs (Watson and Trikojus, unpublished data 1951), it was decided to investigate the effect of TSH on the protease *in vitro* since TSH is known to exert its action within the thyroid gland. Enzyme + TSH were incubated together for 2 hr at two different pH levels and the activity of the solution subsequently compared with controls consisting of enzyme incubated alone and of enzyme and TSH incubated separately and combined for testing. TSH was also tested for proteolytic activity. Results are given in

Table 4. It can be seen that TSH neither possesses proteolytic activity nor does it influence the activity of the enzyme.

In order to determine whether there might be an accessory factor in thyroid tissue essential for activation of the protease by TSH, a cat thyroid was finely sliced and the slices held in Ringer solution. After removal of excess moisture, one aliquot of 50 mg was added to 4 ml of enzyme solution (preparation No. 7; 2 mg/ml) and an equal amount to 4 ml enzyme solution + 8 mg TSH (preparation No. 099931) in Warburg flasks which were then shaken for 4 hr at 37°C, the pH being 7.4. At the conclusion of the incubation period, the contents were centrifuged and 0.5 ml supernatant tested. The values obtained, 9.3 and 9.7 units/mg enzyme for presence and absence of TSH respectively, indicate again no effect of the hormone.

(iii) The influence of incubation together with another proteolytic enzyme, trypsin, on the activity of the thyroid protease was determined. Thyroid protease, dissolved in a solution of trypsin in 0.02M $CaCl_2$, was incubated at 37°C at pH 6.5 or 8.15. Aliquots were withdrawn at intervals and proteolytic activity tested at pH 3.5. Results are set out in Table 5.

TABLE 5

ACTIVITY OF THYROID PROTEASE (PREPARATION NO. 19; METHOD 2) FOLLOWING INCUBATION WITH TRYPSIN

Concentration of Trypsin (mg/ml)	pH of Incubation	Period of Incubation (min)	M-equiv. × 10^4 Tyrosine Liberated per mg Enzyme*
0·01	6·5	0	53·0
0·01	6·5	15	48·0
0·01	6·5	30	49·5
0·01	6·5	120	51·0
0·05	6·5	0	57·0
0·05	6·5	15	50·0
0·05	6·5	30	50·7
0·05	6·5	60	55·8
0·05	6·5	120	52·4
0·06	8·15	0	56·4
0·06	8·15	30	58·7
0·06	8·15	120	58·7

* pH of test, 3·5.

It is apparent that incubation with trypsin did not significantly influence the activity of the thyroid enzyme.

IV. DISCUSSION

The studies reported here provide indirect evidence that there is a close association of thyroglobulin with protease in the thyroid gland; they also indicate

a procedure whereby the components can be separated. In unpublished experiments during which thyroid extracts were fractionated with ammonium sulphate, the proteolytic activity of each fraction was determined using haemoglobin at pH 3.5; whereas about half the total activity was found in the fraction insoluble at 42 per cent. and soluble at 37 per cent. ammonium sulphate saturation (i.e. purified thyroglobulin), the remainder was distributed between the fraction soluble at 42 per cent. and that soluble at 37 per cent. saturation. It is therefore possible that the proteolytic activity of thyroid tissue is not confined to a single protease although no attempt was made to characterize the other fractions.

Compared with the proteolytic enzymes of the digestive tract (Northrop, Kunitz, and Herriott 1948) the activities of even the purest thyroid protease preparations are relatively low; crystalline pepsin and trypsin would be respectively roughly 100 and 50 times as potent. However, our usual preparations appear to be about 40 times as active as a specimen of cathepsin isolated from thymus tissue by Maver and Greco (1949). A close comparison with the cathepsins of spleen as studied by Fruton and colleagues (e.g. Tallan, Jones, and Fruton 1952) was not possible owing to the different assay techniques used by these workers. It is of interest that Adams and Smith (1951) found that their most active preparation of proteinase I (pH optimum 3.8) isolated from pituitary extracts exhibited a proteolytic activity one-sixtieth that of crystalline trypsin when assayed against haemoglobin.

Activity studies with purified enzyme failed to demonstrate a pH optimum at 3.5 as reported by previous workers (e.g. Dziemian 1943) using crude extracts of the gland; moreover earlier claims of activation by sulphydryl reagents could not be confirmed. Negative results were also obtained with Mn^{++} and Ca^{++}. However, these findings are similar to those reported for the pituitary proteinases by Adams and Smith (1951) since cysteine, ascorbic acid, and various metal ions, including Mn^{++}, were found to be without effect on the activity of these enzymes. Of further interest is the failure to influence the activity of the thyroid enzyme by incubation with trypsin or with purified thyrotrophic hormone even in the presence of surviving slices of thyroid tissue.

V. Acknowledgments

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VI. References

Adams, E., and Smith, E. (1951).—*J. Biol. Chem.* **191**: 651.
Anson, M. L. (1938).—*J. Gen. Physiol.* **22**: 79.
Astwood, E. B., and Bissell, A. (1944).—*Endocrinology* **34**: 282.
Barker, S. B. (1950).—*J. Biol. Chem.* **173**: 715.
Cohn, E. J., et al. (1950).—*J. Amer. Chem. Soc.* **72**: 465.
Derrien, Y., Michel, R., and Roche, J. (1948).—*Biochim. Biophys. Acta* **2**: 454.

DERRIEN, Y., MICHEL, R., PEDERSEN, K. O., and ROCHE, J. (1949).—*Biochim. Biophys. Acta* **3**: 436.
DEYSACH, L. D., and RAY, T. W. (1949).—*Proc. Soc. Exp. Biol. Med.* **71**: 353.
DZIEMIAN, A. J. (1943).—*J. Cell. Comp. Physiol.* **21**: 339.
GERSH, I., and CASPERSSON, T. (1940).—*Anat. Rec.* **78**: 303.
GORDON, A. H., GROSS, J., O'CONNOR, D., and PITT-RIVERS, R. (1952).—*Nature* **169**: 19.
GORDON, A. W., MCQUILLAN, MARY T., STANLEY, P. G., and TRIKOJUS, V. M. (1953).—Proc. Isotope Techniques Conference, Oxford, July 1951 **1**: 248.
GROSS, J., and PITT-RIVERS, R. (1952).—*Lancet* **262**: 439.
KAMNER, M. E., PERANIO, A., and BRUGER, M. (1950).—*Endocrinology* **46**: 353.
LEBLOND, C. P., and GROSS, J. (1949).—*J. Clin. Endocrinol.* **9**: 149.
LEHNINGER, A. L. (1950).—*Physiol. Rev.* **30**: 393.
MAVER, M. E., and GRECO, A. E. (1949).—*J. Biol. Chem.* **181**: 853.
NORTHROP, J. H., KUNITZ, M., and HERRIOTT, R. M. (1948).—"Crystalline Enzymes." (Columbia University Press: New York.)
DE ROBERTIS, E. (1941).—*Anat. Rec.* **80**: 219.
ROSENBERG, I. N. (1951).—*J. Clin. Invest.* **30**: 1.
TALLAN, H. H., JONES, M. E., and FRUTON, J. S. (1952).—*J. Biol. Chem.* **194**: 793.
TAUROG, A., and CHAIKOFF, I. L. (1948).—*J. Biol. Chem.* **176**: 639.
TAUROG, A., CHAIKOFF, I. L., and TONG, W. (1950).—*J. Biol. Chem.* **184**: 99.