HYDROLYSIS OF THYROGLOBULIN CATALYZED BY RAT THYROID PARTICLES.

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The thyroid gland contains the protein thyroglobulin in which are bound in peptide linkage the iodotyrosines and iodothyronines. Thyroglobulin must be hydrolyzed for the iodothyronines, the active hormones of the thyroid, to be released into the blood stream. Previous investigators have identified proteases in the thyroid which have acid pH optima and are inactive near neutrality (Weiss, 1953; Alpers et al., 1955; Laver & Trikojus, 1955).

This report describes the identification of a particulate enzymatic activity in the thyroid which hydrolyzes thyroglobulin. This degradation of thyroglobulin occurs at a physiological pH and is dependent on the presence of a salt and a mercaptan for maximal activity.

Methods: Thyroids were removed from 125-350 g. Sprague-Dawley rats and homogenized in a ground glass homogenizer in 3-10 volumes of 0.01 M tris-HCl (pH 7.6). The particulate fraction was sedimented at 100,000 G for 1 hour in a Spinco Model L ultracentrifuge, and the unwashed pellet was resuspended in 0.01 M tris (pH 7.6), dialyzed for 16 hours against the same buffer and stored frozen in liquid nitrogen. Thyroglobulin labeled with I^{125} was prepared by sucrose gradient centrifugation from the thyroids of rats injected 20 or 48 hours previously with 100-200 μc of I^{125} and diluted with unlabeled material (Salvatore et al., 1964). Routine incubations were carried out in 6 x 50 mm glass tubes which were shaken at 37°C.

Reactions were stopped by the addition of 3 volumes of cold 10% trichloracetic acid (TCA). The TCA soluble radioactivity was used as a measure of hydrolysis. Determinations of pH were made on larger reaction mixtures with a glass electrode. The ninhydrin reaction was done by the method of Moore and Stein (1948).

Results: Incubation of the complete system containing thyroid particles, thyroglobulin-I^{125}, and optimal concentrations of KCl and β-mercaptoethanol (BME) results in degradation of thyroglobulin to TCA soluble material (Table 1). The reactions are linear for 1 hour and proportional to enzyme concentration. In the absence of both KCl and BME there is no measurable enzymatic activity. Omission of BME decreases the rate 80% while omission of KCl decreases it 93%. There is no proteolytic activity detectable in the 100,000 G supernatant.

TABLE 1

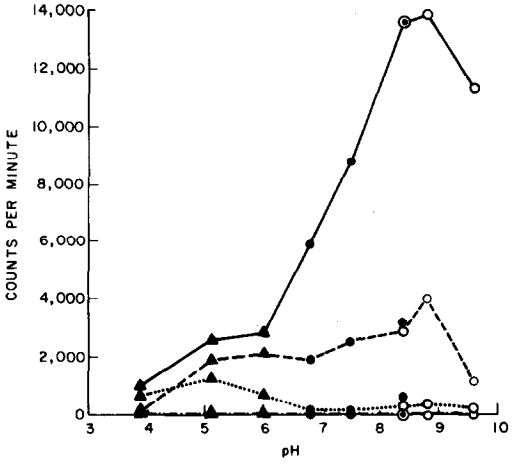
Reaction Components	c.p.m. soluble in TCA by particulate enzyme	soluble enzyme	Equivalent mμmoles leucine released by particulate enzyme
Complete	3,627	3,010	16
KCl omitted	181	258	-
BME omitted	833	704	-
BME; KCl omitted	1	0	0
Boiled Enzyme	56	-	0

Hydrolysis of thyroglobulin by particulate and soluble enzymes. Reaction mixtures contain tris-HCl, 0.1 M; KCl, 0.5 M; BME, 0.05 M; thyroglobulin I^{125} 110 μg (28,600 cpm); and where indicated thyroid particles isolated from 0.62 mg. wet weight of gland or soluble enzyme, 38 μg protein; Total volume 0.05 ml, pH 7.5, incubation time, 1 hour.

To demonstrate that peptide bond cleavage is occurring, the ninhydrin reaction was performed on 0.1 ml samples incubated in a similar manner.

The data of Table 1 show that there is an increase in ninhydrin positive material.

The optimum pH of the reaction is about 8.6 with about 50% activity at pH 7.0 (Fig. 1). In the absence of both BME and KCl there is no detectable activity at any pH.



Conditions are identical to those of Table 1 except that the specific activity of the thyroglobulin is 8 x 10^5 cpm/mg. The buffers are: 0.1 M citrate, A; 0.1 M tris, · ; and 0.1 M triethylamine, O. Complete system,—— ; BME omitted, --- ; KCl omitted, ··· ; BME and KCl omitted, ·-·.

To demonstrate release of iodoamino acids from thyroglobulin, incubations were extended to 4 hours. At the end of the incubation the particulate material which contained about 10% of the radioactivity was sedimented at 40,000 G for 10 minutes. Measured aliquots of the supernatant were applied to Whatman 3 mm paper for chromatography. Internal standards of thyroxine (T_4), triiodothyronine (T_3), monoiodotyrosine (MIT), diiodotyrosine (DIT) and KI were also applied and the chromatograms developed in four solvent systems: n-butanol-dioxane-2N NH_4OH, 4:1:5 upper phase; n-butanol saturated with 2N NH_4OH, n-butanol-ethanol-0.5N NH_4OH 5:1:2; n-butanol saturated with 2N acetic acid. The strips were scanned in a strip counter, autoradiographed and stained. The

correspondence between the stained carriers and the radioactive spots showed that MIT, DIT, and iodide were present (Table 2). In addition there were several compounds with chromatographic behavior nearly identical to T_4. These compounds are probably T_4 and related thyronines formed from T_4 by deiodination since in control experiments using I^{131} labelled MIT, DIT and T_4, only T_4 was extensively deiodinated. The low yield of T_4 was not due to selective adsorption of T_4 to the centrifuged pellet.

TABLE 2

Reaction Components	% of I^{125} at Origin	Peptide+	I^-	MIT	DIT	T_4 Region
Complete*	6	66	6	13	6	3
KCl omitted	97	0	1	1	0	0
BME omitted	99	0	0	0	0	0
Boiled Enzyme	99	0	1	0	0	0

*Release of iodoamino acids from thyroglobulin. Each reaction mixture contains tris-HCl, 0.1 M; KCl, 0.5 M; BME, 0.05 M; thyroglobulin I^{125} 89 µg (49,500 cpm); thyroid particles from 8.0 mg wet weight of gland; Total volume 0.10 ml, pH 7.5, incubation time 4 hours. To each chromatographic strip 40,000 cpm were applied. Solvent, n-butanol sat. with 2N acetic acid.

+This radioactivity is in 4-6 bands between the origin and I^- regions. It is believed to represent iodoamino acids in peptide linkage.

The data of Table 2 also show that both KCl and BME are necessary for significant release of iodoamino acids. The ultimate release of iodoamino acids is probably not due to a single proteolytic activity in the particles but rather to the combined activity of several enzymes.

During the reaction proteolytic activity becomes soluble. A soluble enzyme preparation is made from 0.6 ml reaction mixtures incubated for 30 minutes under standard conditions (Table 1) but without thyro-

globulin. The particles are sedimented and the soluble enzyme passed over a Sephadex G-25 column equilibrated in 0.1 M tris-HCl pH 7.5 to separate the protein from the KCl and BME. With this soluble enzyme there is still dependence on KCl and BME (Table 1). This demonstrates that the function of these substances is not simply solubilization of the enzyme.

The role of the mercaptan appears to be to reduce the thyroglobulin, since chemically reduced thyroglobulin freed of BME by passage through Sephadex G-25 is attacked by the enzyme at the same rate whether or not BME is present. This suggests that in the thyroid gland thyroglobulin may first require reduction or denaturation before it can be hydrolyzed. There are high levels of glutathione present in the thyroid to accomplish this (Klebanoff et al. 1962). A precedent is seen in keratin where reduction of the disulfide bonds facilitates its hydrolysis by enzymes in the intestine of the clothes moth (Linderstrom-Lang and Duspina, 1935). The KCl requirement for thyroglobulin hydrolysis is not just that of ionic strength since it is replaced by NaCl, but not by LiCl or urea. Recent microscopic studies have suggested that thyroglobulin is reabsorbed from the lumen into the thyroid cell in droplets in which thyroglobulin may be degraded (Wollman et al., 1964). The proteolytic activity may be attached to one of the subcellular structures involved in the resorbtion and degradation of thyroglobulin.

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