sively by Ryhage and von Sydow.[1] This phenomenon was overlooked by Thomas and Willhalm [4] in their study of γ-terpinene, where the high intensity of $m/e = 119$ indicates the occurrence of a thermal breakdown in the mass spectrometer.

Similar decompositions of monoterpene alcohols were discussed by von Sydow,[2] who is very well aware of the instability of sabinol, although this was inadvertently overlooked when Ref.[2] was prepared. Fig. 14 in that paper shows the mass spectrum of sabinol when the combining system was kept at 200°C. It contains a large amount of thujone, as pointed out by Willhalm *et al.*[5] When the combining system was kept at 100°C the spectrum shown in Fig. 1 of this paper was obtained (March 25, 1963). This should substitute Fig. 14 in Ref.[2] and agrees nicely with the one published by Willhalm *et al.*[5] In the case of thujone there is also good agreement.[3,5]

As to fenchol,[2] it seems likely that the specimen contained 10 % isofenchol.[5] A better gas chromatographic separation has recently been obtained.

The data presented in Refs.[1-3], although in need of future improvements — like all experimental data — have been applied successfully in two investigations on black currant material.[8,9]

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Proteolytic Activity in Extracts from Human Thyroid Tissue

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The thyroid gland is an aggregate of follicle cells, acini, which are filled with a gelatinous substance called colloid. It is known that the thyroid hormones thyroxine and triiodothyronine are stored within the colloid as thyroglobulin. Thyroglobulin, however, has a molecular weight of about 650 000 to 670 000 and cannot pass the follicle membranes. Gersh and Caspersson [1] postulated that in the normal animal the production of colloid is a continuous process and they suggested that proteases in the gland bring about hydrolysis of thyroglobulin into polypeptides and peptones which can pass the cell membranes. In extending these observations De Robertis [2] was able to show that thyroid follicle extract possessed proteolytic activity and Dziemian [3] stated that the activity of the protease depended upon the state of the thyroid. The hydrolysis of thyroglobulin and the proteases and peptidases in thyroid extracts, in most cases of animal origin, have been studied by several authors.[4-12] The proteolytic activity is considered to be of catheptic nature but has not yet been identified. Attempts to purify the proteases in thyroid extracts from different animals have been carried out by some of the above authors, but only in extracts of sheep thyroids have two proteases with pH optima at 3.8 and 5.7 been isolated.[13] In human thyroid only one protease has been found. In the present communication the proteolytic activity of thyroid extracts on gelatin and haemoglobin, and the nature of a thyroid cathepsin are examined and the separation of two of these enzymes by means of gel filtration is reported.

Material and methods. Thyroid tissue from non-toxic goitre patients was frozen at −20°C, freed from fat and connective tissue, cut in pieces and extracted (toluene added) for 20 h at + 4°C in a volume of 3.0 ml per g tissue of a mixture of 4 parts 0.9 % NaCl and 1 part 0.067 M Na-phosphate buffer, pH 7.4.

The mixture was then filtered through nylon-cloth 140 mesh and centrifuged at 6000 × *g* for 20 min at + 2°C. The supernatant was frozen and stored at −20°C and thawed just before investigations without concentration. For gel filtration [14] a part of the extract was concentrated 5 times by ultrafiltration and samples were applied to Sephadex G-200 (AB Pharmacia, Uppsala, Sweden) in a 2.1 × 145 cm column. Elution was made at + 5° with 0.1 M Na-phosphate, pH 7.4, in 1 M NaCl and 1 % butanol. The optical density of the fractions collected was measured at 280 and 260 mμ.

For the proteolytic assay three substrates were used. Gelatinase activity was determined viscosimetrically according to a method of Hultin.[15,16] Haemoglobin splitting activity was measured by the Anson-Kunitz method [17] using urea-denatured bovine haemoglobin, interruption of the digestion with TCA and measurement of liberated tyrosine in the clear filtrate at 280 mμ. The catheptic activity was tested on N-benzoyl-L-arginine amide and measured by the Conway diffusion method as reported by Greenbaum and Fruton.[18]

Results. The first substrate used to test the protease activity in thyroid extracts was gelatin. The results are shown in Table 1. Activity was found at three different pH ranges, *i.e.* around 4, 6 and

Table 1. Gelatinase activity of a thyroid extract expressed in Hultin units (H.U.) was measured at various pH values with and without cysteine in 0.01 M final concentration. 1.00 ml extract and 0.50 ml H_2O or cysteine (pH corr.) was mixed with 3.00 ml of a 4.00 % gelatin solution and the viscosity was measured at 35.5°C.

pH	cysteine	Activity H.U./ml	pH	cysteine	Activity H.U./ml
2.0	+	0	6.0	+	50
»	−	0	»	−	50
3.25	+	270	7.0	+	0
»	−	140	»	−	0
3.75	+	245	8.0	+	25
»	−	50	»	−	140
4.5	+	150	9.0	+	80
»	−	95	»	−	250
5.5	+	0	10.0	+	0
»	−	0	»	−	0

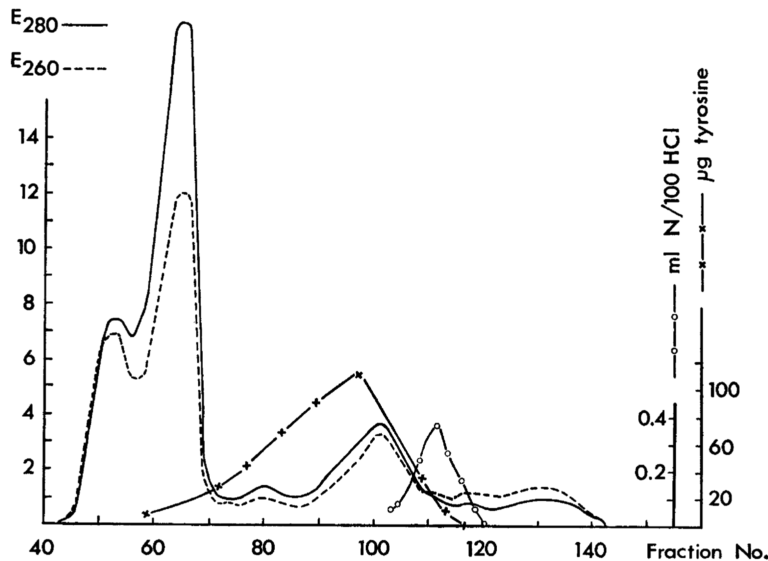

Fig. 1. Chromatographic fractionation of a buffered saline extract of human thyroid tissue on Sephadex G-200. A 5 ml sample (1.9 % protein) was applied to a 2.1 × 145 cm column. × = Proteolytic activity with 2 % haemoglobin as substrate, incubation at pH 3.75, 4 h at 37°C; O = cathepsin B activity in presence of 0.01 M cysteine, incubation at pH 5.0, 16 h at 37°C. One ml fraction samples were used throughout.

8.5. Cysteine was found to have an activating effect at acid pH, no effect at pH 6, and an inhibiting effect at alkaline pH.

One of the proteases which brings about hydrolysis of the thyroglobulin is considered to be a cathepsin but has not been classified. However, a weak benzoyl-arginine amide splitting effect, activated by cysteine, was now found in the thyroid extracts; according to the definition of Fruton *et al.*,[19,20] this means that this enzyme is of cathepsin B type. The maximum activity was at pH 5 and the pH-activity curve had a symmetrical shape approaching zero at pH 4 and 6. Table 2 shows the effect of some agents on the cathepsin B activity at pH 5. The strong inhibiting effect of iodine, iodoacetamide and alloxan is normal for an SH dependent enzyme such as cathepsin B. It is of interest to note that serum has no influence on the activity as cathepsin B is not found in serum.

The thyroid extracts were investigated by means of gel filtration and one of the chromatograms is shown in Fig. 1. The highest peak contained the thyroglobulin and the fractions were tested for proteolytic activity on haemoglobin at pH 3.75 which was found to be optimal, and for cathepsin B activity on BAA at pH 5.0 with cysteine as activator. As can be seen the cathepsin B is partly separated from the haemoglobin splitting protease.

Since this work was completed two papers have been published on the purification of thyroglobulin by means of gel filtration.[21,22] All results seem to be in good agreement. The long column bed used by the present authors seems to be well fitted for separations of this kind. Further work is in progress.

Table 2. Influence of some agents on thyrocathepsin B. Incubation mixture: 1.00 ml extract, 0.50 ml agent (pH corr.), 0.20 ml 0.1 M BAA* and 0.50 ml 0.2 M citrate buffer pH 5.20 h at 35.5°.

Agent	Final conc.	Rel.act.
H_2O	—	100**
Cysteine	10^{-2} M	115
I_2	2.5×10^{-3} M	18
Iodoacetamide	»	0
Alloxan	»	0
EDTA	»	99
Heparin	1:2000	101
Normal human serum	1:4	100

* BAA = Benzoyl-arginine amide.
** 100 = 560 μl 0.01 M HCl consumed in 20 h.

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