Enhanced release of T₃ and rT₃ compared to T₄ from thyroglobulin during autolysis of dog thyroid homogenate

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Abstract. Previous studies on the secretion of thyroxine (T₄), 3,5,3′-triiodothyronine (T₃), and 3,3′,5′-triiodothyronine (rT₃) from perfused dog thyroids have indicated that a differential rate of secretion of various iodothyronines may take place. The aim of the present study was to evaluate whether the proteolysis of thyroglobulin taking place during secretion could be involved in this phenomenon. Homogenate from the same dog thyroid was incubated either at pH 3.6 for 18 h without added protease or with pronase at pH 8.4 for 18 h. Iodothyronines were measured radioimmunologically in ethanol extracts of the hydrolysates. No significant deiodination of T₄ to T₃ and rT₃ took place during incubation. During acid autolysis 17.5 ± 3.5% (mean ± SE, n = 5) of the T₄ found after pronase hydrolysis was liberated, while 31.6 ± 4.8% of the T₃ and 21.2 ± 4.2% of the rT₃ were liberated (both values were significantly higher than that found for T₄). Since iodothyronines in thyroglobulin are released nearly quantitatively during pronase hydrolysis, the results indicate that thyroid proteases acting at acid pH, liberates T₃ and rT₃ more easily than T₄ from thyroglobulin.

This could be the mechanism behind the relatively high secretion of T₃ and rT₃ observed during acceleration of secretion from perfused thyroid lobes, and the relatively high secretion of T₄ observed during deceleration of secretion.

Intracellular hydrolysis of engulfed thyroglobulin with release of iodothyronines is important in the process of thyroid hormone secretion. This proteolysis seems to take place in phagolysosomes at acid pH (Greer & Haibach 1974). It has been shown that various iodinated compounds may be released at different rates during enzymatic hydrolysis of thyroglobulin (Roche et al. 1950; Tong & Chaikoff 1958; Pitt-Rivers & Cavalleri 1963; Kobayashi & Greer 1971). Inoue (1968) and Kobayashi & Greer (1970) have reported in abstract form that during enzymatic proteolysis at acid pH of thyroglobulin the T₃/T₄ ratio of the digest was relatively high compared to that obtained during enzymatic hydrolysis at alkaline pH. Earlier observations from our laboratory on the mixture of iodothyronines secreted from perfused canine thyroid lobes can be explained by a more rapid secretion of T₃ and rT₃ than of T₄ (Laurberg 1980a,b). The mechanism could be a more easy liberation of T₃ and rT₃ than of T₄ from thyroglobulin during intracellular hydrolysis at acid pH.

In the present study we have studied the degree to which T₃, rT₃, and T₄ were liberated from thyroglobulin during partial autolysis of dog thyroid homogenate at acid pH. As a reference we used pronase hydrolysis of the same homogenate as this is considered to be a nearly quantitative hydrolysis (Inoue & Taurog 1967). The liberated iodothyronines were measured radioimmunologically.

Material and Methods

Five mongrel dogs weighing 17–27 kg were anaesthetized with pentobarbital, intubated and ventilated with O₂/N₂O in a ratio of 1/1. Exsanguination was performed through catheters in the femoral arteries. Meanwhile the thyroid was exposed and immediately after exsanguination each lobe was perfused with 10 ml 37°C NaCl, 150 mmol/l, through the superior thyroid artery. The thyroid...
lobes were quickly removed, weighed, minced with scissors and homogenized in 10 ml of NaCl, 150 mmol/l, using an Ultraturrax homogenizer (3 times 5 s) followed by 3 strokes in all-glass Potter Elvehjem homogenizer.

For autolysis incubations consisted of 100 µl of homogenate, 500 µl of acetate buffer, 150 mmol/l, pH 3.6 and 50 µl of toluol. For pronase hydrolysis 100 µl homogenate, 500 µl Tris buffer 40 mmol/l, pH 8.4 with 50 mmol/l methimazol and 10 g/l pronase (Calbiochem) and 50 µl toluol were incubated. All processing and incubation was performed at 37°C. Incubation took place in a N₂ atmosphere in 12 × 65 mm glass vessels stoppered with a rubber bung, in a Dubnoff type metabolic shaker (60 strokes/min, stroke length 5 cm). Two vol of ice-cold 99% ethanol was added immediately (time 0) or after 18 h of incubation. The sample was mixed on a Vortex mixer, and centrifuged at 1500 g for 30 min. The supernatant was removed for measurements of iodothyronines.

In three of the experiments the residue after 18 h autolysis was hydrolysed with pronase: after ethanol extraction of the autolysate the pellet was washed twice with 2 ml 99% ethanol, each wash followed by mixing and re-centrifugation. The washed pellet was dried overnight in an exsiccator at room temperature, and re-suspended in 100 µl NaCl, 150 mmol/l, 500 µl Tris buffer 40 mmol/l, with 50 mmol/l methimazol and 10 g/l pronase, and 50 µl toluol. After 18 h incubation at 37°C, ethanol extraction was performed as usual.

In one experiment an ethanol extract of 18 h autolysate was chromatographed on a 1 × 7 cm Sephadex G25 fine column at 4°C. To 1 ml of ethanol extract was added 1 ml of phosphate buffer 50 mmol/l pH 7.5 and the mixture applied on the column. Elution was performed with phosphate buffer 50 mmol/l pH 7.5 overnight. The volume of the first 20 samples was 0.4 ml, the remainder samples were 2 ml.

T₄, T₃, and rT₃ were measured radioimmunologically (Weeke & Ørskov 1975, 1978; Lauberg 1978a). Standards were always prepared in the buffer/ethanol medium of the samples to be measured. All samples were measured in triplicate. In previous studies the suitability of these assays for measurements of iodothyronines in pronase hydrolysate of dog thyroid homogenate had been tested (Lauberg 1976, 1978a). When T₄, T₃, and rT₃ were measured in four consecutive 2-fold dilutions of ethanol extract of 18 h autolysate the measured concentrations were T₄: 3.65, 1.95, 0.93, and 0.42 mmol/l, T₃: 1.56, 0.78, 0.39, and 0.19 mmol/l, rT₃: 0.039, 0.019, 0.007, and 0.003 mmol/l.

Librated iodothyronines were calculated as the difference between the amount measured in ethanol extract at 0 h and after 18 h autolysis or pronase hydrolysis. The 0 h values were less than 10% of the amount liberated during autolysis.

Each value given is the mean of three incubation vessels.

Results

The amounts of T₄, T₃, and rT₃ liberated from thyroid homogenate during 18 h autolysis at acid pH or during 18 h pronase hydrolysis are depicted in Table 1. The absolute amounts of iodothyronines varied considerably from dog to dog, but the relative composition was more uniform, the molar ratios being T₄/T₃: 10.2 ± 1.1 and T₄/rT₃: 49.0 ± 10.6, mean ± SE.

During acid autolysis the liberated amounts of iodothyronines were considerably less than during pronase hydrolysis. This was always most pronounced for T₄ (17.5 ± 3.5%) while the relative yield compared to pronase hydrolysis was higher for rT₃ (21.2 ± 4.2%) and especially for T₃ (31.6 ± 4.8%, mean ± SE, n = 5).

The possibility that this pattern of enhanced release of T₃ and rT₃ compared to T₄ was due to methodological faults, was tested in the following control experiments.

Evaluation of a possible deiodination of some of the liberated T₄ to T₃ and rT₃ during autolysis

T₄ standard leading to a 3–4-fold increase in the final amount of free T₄ was added before incubation. The measured amounts of free T₃ and rT₃ after incubation were 18.1 ± 1.2 and 1.07 ± 0.11 compared to 19.5 ± 0.4 and 1.11 ± 0.04 pmol/mg thyroid in autolysates with no T₄ added (mean ± SE of 5 autolysates). The mean T₄/T₃ and T₄/rT₃ in vessels with 1 mmol/l propylthiouracil, which inhibits intrathyroidal deiodinases (Lauberg 1978b, 1979; Green 1978) were 5.8 and 35.4 while it was 6.2 and 39.3 in control vessels. Thus we found no signs of significant deiodination T₄ to T₃ or rT₃ during autolysis.

Recovery of iodothyronines added to incubations

When T₄, T₃, and rT₃ standards leading to a 3–4-fold increase in free iodothyronines after 18 h autolysis were added to incubation vessels at 0 h, the recovery in ethanol extract was at 0 h: T₄: 106.8%; T₃: 99.6%; rT₃: 87.2%, and after 18 h autolysis: T₄: 80.0%; T₃: 89.4%; rT₃: 80.2%. Thus the pattern of a relatively high yield of T₃ and rT₃ compared to T₄ during acid autolysis was not due to a major loss of one or more iodothyronines during incubation or extraction. No attempts were made to correct the final values for loss.
Interference of iodothyronine containing peptides in the various assays

Since proteolysis of thyroglobulin was only partial during autolysis the possibility existed that the ethanol extract contained considerable amounts of iodothyronine containing peptides which by interfering differently in the various assays could lead to a false estimate of the composition of iodothyronines liberated during autolysis. To test this possibility, an ethanol extract of autolysate was chromatographed on Sephadex G25 fine, and the fractions examined for immunoreactive T4, T3, and rT3. In all three assays an early peak appeared corresponding to approximately 15% of the measured T4, and 10% of the measured T3 and rT3. The remaining immunoreactivity was eluted late and corresponding to the respective iodothyronine standards, as previously described (Laurberg 1978a). Thus only small parts of other immunoreactive substances than free iodothyronines were found and only negligible differences between their contribution to the measured T4, T3, and rT3 were observed.

T4, T3, and rT3 in the residue after autolysis

As a final test T4, T3, and rT3 were measured in pronase hydrolysate of the residue after autolysis (Table 1). It should be expected that the sum of iodothyronines liberated during autolysis and during pronase hydrolysis of the residue would be similar to the amount liberated during pronase hydrolysis. In the three experiments T4 in autolysate + pronase hydrolysate of residue was 103.9 ± 9.9% of that in pronase hydrolysate, while T3 was 94.1 ± 4.3% and rT3 88.5 ± 2.9% (mean ± SE). Thus there was no indication that T3 or rT3 were overestimated or T4 underestimated in autolysates.

Discussion

The present study has shown that iodothyronine liberation from thyroglobulin by thyroidal acid proteases is not a random process: T3 and rT3 are released more easily than T4. In previous studies on secretion from perfused canine thyroids, we have regularly observed that a sudden stimulation of thyroid secretion induces a more steep increase in the secretion of T3 than of T4, leading to a fall in the T4/T3 ratio in thyroid secretion. When the secretion reaches a new stable level, the T4/T3 ratio returns to near pre-stimulation values (Laurberg 1976, 1977, 1978a,c, 1979). Furthermore, a sudden cessation of thyroid secretion induces the opposite variation in the T4/T3 ratio in secretion: a transient increase (Laurberg 1980a). Similar, but less pronounced variations are observed in the T4/rT3 ratios (Laurberg 1978a, 1980a). We have suggested
that this pattern of secretion is due to a more rapid secretion of triiodothyronines than of T4 from the thyroglobulin taken into the follicular cells (Laurberg 1980a,b). This might be caused by differences in the rate of release of various iodothyronines during the intracellular proteolysis of thyroglobulin or in the rate of transport or diffusion of the liberated iodothyronines from the phagolysosomes into the capillary. The results of the present study suggest that the mechanism behind such a rapid secretion of triiodothyronines compared to that of T4 is a disparity in the release rate during intracellular proteolysis of thyroglobulin.

The more easy release of T3 and rT3 from thyroglobulin during autoysis at acid pH (thus involving acid denaturation of thyroglobulin (Ui et al. 1974)) could be a result of thyroglobulin heterogeneity. Iodine poor thyroglobulin is more easily hydrolysed than iodine rich thyroglobulin (Lamas & Ingar 1978) and iodine poor thyroglobulin has a relatively high T3 and low T4 content compared to iodine rich thyroglobulin (Studer & Greer 1965). The results of the present study could thus be due to a preferential hydrolysis of a population of iodine poor, relatively T3 rich thyroglobulin molecules. However, it has been shown by Belshaw et al. (1975) in dogs kept on different iodine containing diets, that the iodine content of thyroglobulin has to be very low to induce an appreciable shift in the T4/T3 ratio. The normal dog thyroid probably does not contain major populations of thyroglobulin with such great differences in iodine content. In a previous study we have used DEAE chromatography to separate dog thyroglobulin fractions with different iodine content (Laurberg 1978d). Even if a more than 3-fold difference in iodine content was observed between different fractions, only small differences in the T4/T3 and no differences in the T4/rT3 ratios were observed. Using RbCl density gradient centrifugation for separation of thyroglobulin with different iodine content obtained from human thyroids, Izumi & Larsen (1977) also found identical T4/T3 ratios in various fractions of thyroglobulin from the same thyroid. Thus it seems not likely that differences in iodine content of thyroglobulin molecules is the cause for the relative ease of T3 and rT3 release observed in the present study. Still it could be that irrespective of iodine content, thyroglobulin molecules containing T3 or rT3 have a secondary or tertiary structure which make them more susceptible to hydrolysis, or that T3 and rT3 tend to be located in a part of the molecule which is easily hydrolysed by thyroid proteases.

Another possibility is that peptide bonds involving T3 or rT3 are more readily attacked during proteolysis than bonds involving T4. Such a mechanism was suggested by Kobayashi et al. (1973) to explain the difference in release rate of MIT and DIT during in vitro proteolysis of rat thyroglobulin (Kobayashi & Greer 1971).

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