Dissociation of growth and function in the rat thyroid during prolonged goitrogen administration

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Abstract. This study was designed to investigate the changes in growth and function which occur in the rat thyroid during prolonged TSH stimulation. Animals maintained on the goitrogen aminotriazole were sacrificed together with controls at frequent intervals over a period of 5 months. The levels of serum T₃ and T₄ and TSH were measured by radioimmunoassay. Functional activity was assessed by measurement of the thyroid/serum iodide ratio (T/S) and growth by measurement of thyroid weight, follicular cell number and follicular cell mitotic activity. Serum T₃ and T₄ rapidly fell to undetectable levels within 2 weeks. The level of serum TSH rose to a stable 5-fold maximum after 4 weeks. The T/S ratio followed a closely similar pattern rising to a sustained 7-fold maximum. Thyroid weight and follicular cell number increased rapidly for the first few weeks but the growth rate declined progressively, falling almost to zero after 80 days. Mitotic activity rose dramatically to a 30-fold peak after 7 days but then declined almost to normal after 80 days, consistent with the observed change in cell number. The results thus demonstrate a clear dissociation between the functional and proliferative activity of the thyroid follicular cells during prolonged stimulation by a sustained elevation of serum TSH and point to the existence of specific growth regulating mechanisms which limit the mitotic response.

It has been known for many years that inhibition of thyroid hormone synthesis by goitrogen leads to increased pituitary TSH secretion and hence to stimulation of thyroid growth (Kennedy & Purves 1941; Bakke & Lawrence 1964) involving both hypertrophy and hyperplasia of follicular cells (Santler 1957; Philp et al. 1969). The resulting goitre is always self-limiting however, growth greatly diminishing after 1 to 2 months despite continued absence of circulating thyroid hormone.

The mechanisms responsible for this limitation of growth are unknown, but are clearly of great relevance to the understanding of the control of cell division in general. This study is the first of a series of experiments designed to elucidate this phenomenon and specifically considers two possibilities: a) whether the decline in growth is the result of a drop in the level of TSH or in its biological effectiveness; and b) whether the functional activity of the thyroid responds in the same way as thyroid growth.

Materials and Methods

One hundred and ninety-two male Wistar rats aged 10 to 11 weeks (190 ± 13 g) were housed in a thermostatically-controlled room with a constant lighting cycle, and fed a standard laboratory diet — Pilsbury's rat and mouse breeding diet (iodide content 4.7 μmol/kg). They were randomly segregated into groups of 16 animals. Eight groups were given the readily water soluble goitrogen aminotriazole (ATA) in the drinking water at a concentration (0.1 %) known to block thyroid hormone synthesis completely (Stringer et al. 1981). The other four groups acted as controls.

Goitrogen-treated groups were sacrificed after 3, 7, 14, 24, 46, 82, 116 and 153 days, and control groups after 0, 25, 83, and 154 days.

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Serum $T_3$, $T_4$ and TSH levels were measured by radioimmunoassay on all animals, $T_3$ and $T_4$ by a solid-phase antibody kit (Corning Medical Ltd) adapted for rat samples (Stringer et al. 1981), and TSH using the NIAMD/D double antibody rat TSH assay. Eight animals from each group were used for the assessment of function, and 8 for the assessment of growth.

**Assessment of function**

Since organification had been blocked, the activity of the iodide pump was used as the index of functional status, and measured as the ratio of thyroid to serum inorganic iodide concentrations – T/S ratio (Vanderlaan & Vanderlaan 1947). A sc injection of 10 mg ATA (to block iodide binding in controls) was followed 40 min later by an ip injection of 10 µCi of carrier-free Na$^{131}$I tracer. One hour later, the animals were anaesthetised (urethane 16.8 mmol/kg ip), exsanguinated, and the thyroids resected. After careful dissection, the glands were weighed and fixed in 12 ml of formalin for 3 days to allow free $^{131}$I to diffuse out. The radioactivity in the fixative, always more than 95% of the total radioactivity, and in 1 ml of serum was counted in a gamma scintillation counter and the T/S ratio calculated as:

$$\text{CPM in fixative} \\ \text{CPM in serum} \times \text{weight of thyroid in g}$$

**Assessment of growth**

Three indices of growth were used: a) total thyroid weight; b) follicular cell number, and c) follicular cell mitotic activity – estimated as the 4 h metaphase index using a stathmokinetic technique reported previously (Wynford-Thomas et al. 1982a).

Animals were first given an ip injection of the metaphase arrest agent vincristine (1 mg/kg). Four hours later, anaesthesia was induced with urethane and perfusion-fixation carried out by a modification of the method of Zeligs & Wollman (1976), fuller details of which are given elsewhere (Stringer 1981; Wynford-Thomas et al. 1982b). Briefly this involved perfusion via the aorta for 5 s with 0.1% procaine/1.5% dextran-40, followed by buffered 1% formaldehyde/2.5% glutaraldehyde for 6 min. These procedures give excellent preservation of in vivo morphology.

Following fixation, the thyroid was blotted, weighed and then 'diced' with a razor blade into approximately 30 cuboidal fragments which were randomly embedded as a single block in the plastic resin hydroxyethylmethacrylate. We have shown that this procedure effectively overcomes the non-random distribution of tissue components in the thyroid (Stringer et al. 1982) and allows a single section to be used as an unbiased sample of the whole gland. To provide the required number of fields, six 3 µm sections were cut from each block at 100 µm intervals and stained with haematoxylin for 20 min at 60°C.

Follicular cell metaphases were counted manually in square fields of side 109 µm with a × 63 objective, 50 fields being viewed from each section giving a minimum of 25 metaphases per animal.

Cell counting was carried out on the same sections at the same magnification but due to the much higher population density of cells compared with metaphases, 30 fields per animal were sufficient to give a precise estimate. Counting was performed by a computer-linked automatic image analyser which was capable of distinguishing accurately between stromal and follicular cells on the basis of nuclear size (Wynford-Thomas et al. 1982a; Wynford-Thomas & Garrahan 1981). This gave follicular cell (nuclear) counts per 5 fields with a precision of better than 5%.

From the mean number of metaphases and nuclei per field, $N_F$, the population densities $N_v$ (number per mm$^3$) were calculated from the formula:

$$N_v = \frac{N_F}{A} \frac{1}{t + D}$$

(Weibel 1979)

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where A is area of field (0.012 mm²), t is section thickness (3 × 10⁻³ mm) and D is mean tangent diameter of nucleus or metaphase.

To obtain D for follicular cell nuclei, the tangent diameter parallel to a horizontal eyepiece scale of 100 nuclear profiles per thyroid was measured and the mean, d, calculated. D is then given (assuming uniform nuclear size) by the formula:

\[ D = \frac{d}{1 - \frac{0.21d}{d + t}} \] (Abercrombie 1946)

This precaution of correcting for nuclear size was taken because it is known that nuclear volume increases significantly following TSH stimulation. Since we had no reason to suspect any change in metaphase size, however, a constant value for D of 10 μm was assumed throughout for metaphase calculations.

The ratio of follicular cell metaphase density to follicular cell nuclear density gave the follicular cell metaphase index, which was expressed as metaphases per 10 000 nuclei. This is proportional to the mean mitotic rate over the 4 h period prior to sacrifice, assuming linear accumulation of metaphases (Wright & Appleton 1980).

Follicular cell number per gland was calculated as the product of nuclear density (per mm³) and gland weight (in mg), the latter having been shown to be numerically equal to the volume in mm³ by the method of Scherle (1970).

![Graph](image_url)

**Fig. 2.** Changes in serum TSH with time. Each point represents the mean of 16 rats ± SE.

**Results**

Goitrogen treatment led to a cessation of normal body growth after 2 weeks but was otherwise well tolerated.

**a) serum T₃ and T₄ (Figs. 1 and 2)**

Following the introduction of goitrogen, serum T₃ and T₄ levels dropped rapidly, falling to below the detection limits of 0.014 and 1.1 ng/ml by days 7 and 14 respectively, while control values remained fairly constant. The level of serum TSH rose rapidly from the control level of 979 ± 120 ng/ml to 3296 ± 394 ng/ml by day 14 (P < 0.001), following which there was a continued but progressively slower rise stabilising at a maximum of around 5000 ng/ml by day 82. Control animals showed a slow but significant rise to 1407 ± 103 ng/ml between day 83 and 154 (P < 0.05).

**b) T/S ratio (Fig. 3)**

The T/S ratio initially rose rapidly from the control value of 24.1 ± 1.6 to 95.7 ± 4.9 by day 3.
Changes in thyroid weight with time (perfused-fixed thyroids). Each point represents the mean of 8 rats ± SE.

$P < 0.001$), but then followed an irregular course with an inflection at day 7 and a significant fall at day 24 to 76.3 ($P < 0.01$). An overall upward trend continued, however, to reach a sustained maximum of 150 to 170 between days 82 and 153. Control values rose significantly to 43.9 ± 3.9 at day 83 ($P < 0.001$).

c) Thyroid weight (Fig. 4)

Thyroid growth showed three phases – an initial lag of a few days, a period of rapid growth for 80 days, and a final period of declining growth rate, the weight reaching a maximum 12-fold increase (266.6 ± 56.1 mg) by the end of the experiment. Control weights rose from 22.1 ± 1.7 to 28.4 ± 1.2 by day 25, following which no further growth occurred. The weights of the immersion-fixed glands used for T/S measurement (not shown) followed the same pattern of growth to a plateau but the absolute values were lower throughout.

d) Follicular cell number (Fig. 5)

Total follicular cell numbers followed a similar pattern to gland weight showing a 9-fold rise from 9.7 ± 0.4 x 10$^6$ to reach a maximum of 89.5 ± 2.1 x 10$^6$ by day 82. Control numbers rose by approximately 30% during the first 25 days but then stabilised.

e) Metaphase index (Fig. 6)

Follicular cell metaphase index (measured at mid-day) rose rapidly from the control level of 5.3 ± 1.5 per 10 000 to reach a peak of 149.7 ± 22.9 by day 7. There was then a rapid decline returning to 3.2 ± 0.6 by day 82, followed by a slight but significant ($P < 0.01$) second rise to 10.0 ± 1.6 by day 153. The control level fell steadily during the experiment to reach 1.4 ± 0.3 by day 83, ie only slightly below that in the equivalent goitrogen-treated group ($0.02 < P < 0.05$).

Discussion

Goitrogen treatment led as expected to reduction of serum thyroid hormone to below detectable levels, and it can be safely assumed that full blockade of hormone synthesis was maintained throughout the experiment (Stringer et al. 1981). The initial rise in serum TSH is adequately ex-
Changes in midday follicular cell metaphase index with time. Each point represents the mean of 8 rats ± SE.

plained by release of feedback inhibition of thyro-troph function, but the continued increase long after undetectable levels of thyroid hormone had been reached requires further explanation, and was probably due to a progressive increase in the thyrotroph mass (Ching et al. 1974). Our results clearly show that there was no fall-off in the levels of immunoreactive TSH even with prolonged (5 month) goitrogen treatment, and in this respect agree with the work of Bakke & Lawrence (1964) and Ching et al. (1974). The possibility of a decline in bioreactive TSH (Klug & Adelman 1978) can largely be excluded by the finding of a sustained level of thyroid function (T/S ratio).

There was a marked functional response to goitrogen-induced TSH stimulation as reflected by the increase in the T/S ratio. This closely paralleled the rise in serum TSH except for unexpected irregularities at around days 7 and 24. (It could be speculated that these dips occurred as the result of a cyclic passage of a synchronised cohort of cells into a part of the cell cycle in which iodide is not concentrated efficiently – for example during and for some time after mitosis). Otherwise these re-

sults are in general agreement with previous studies (Vanderlaan & Vanderlaan 1947; Taurog et al. 1947), although surprisingly, there seem to have been no previous investigations of the sequential changes in T/S ratio during long-term goitrogen treatment.

It is known that TSH stimulation leads to a marked alteration in thyroid morphology, so that the observed change in T/S ratio may have been influenced by a change in the proportion of total thyroid volume occupied by the inorganic iodide compartment. Autoradiographic evidence (Doniach & Logothetopoulos 1955; Andros & Wollman 1967) strongly suggests that this consists of follicular cells plus colloid. We have shown in a companion experiment (Wynford-Thomas et al. 1982b) that the increase in the proportional volume occupied by follicular cells during goitrogen administration is balanced by a corresponding fall in colloid volume, so that it can be assumed that the change in the iodide compartment was negligible in relation to the magnitude of the rise in T/S ratio. It is highly improbable therefore that the pattern of change in the T/S ratio was significantly affected by changes in morphology.

ATA administration led to thyroid growth of a pattern similar to that seen with other goitrogens (Thyssen 1947; Greig et al. 1969; Christov 1975), although reaching a higher maximum than most. This is partly explained by the use of perfusion-fixation which preserves vascular volume and prevents follicular cell shrinkage, leading to a 40% weight increase compared to immersion-fixed glands (Stringer 1981). The growth curve for cell number followed a similar pattern to total thyroid weight.

Following the introduction of goitrogen, there was a dramatic rise in mitotic activity followed by an equally marked decline to return almost to normal by day 82. This fall-off is in broad agreement with all previous studies (Santler 1957; Christov 1975; Redmond & Tuffery 1978), but none of these had established the long-term changes with sufficient reliability, due either to the use of too short a follow-up period (Christov 1975) or to insufficient sample size (Redmond & Tuffery 1978). A further source of uncertainty in these studies resulted from their failure to take into account circadian rhythmicity. We have recently demonstrated a marked circadian rhythm in thyroid mitotic activity in control animals with a midday peak and an amplitude of 69%, and have further shown that this is abolis-
hed by 80 days of goitrogen treatment (Wynford-Thomas et al. 1982a). Using this data we corrected the day 82/83 metaphase index results by calculating 24 h means from the observed (midday) values. This produced a significant increase in the difference between treated and control groups, the 24 h mean values being 3.2 ± 0.62 for treated and 0.83 ± 0.18 for control animals (significance of difference 0.002 < P < 0.01). This illustrates the importance of considering changes in the amplitude of circadian rhythms where small differences are being investigated.

The late rise in metaphase index from day 113 was unexpected since it occurred while the control level was declining. Although the clustering of mitoses was not quantified, several foci of intense mitotic activity were observed at day 153, and the overall increase in mitotic rate may have been due to the emergence of pre-neoplastic clones of cells.

Our investigations have therefore confirmed that long-term goitrogen treatment leads to an initial rapid increase in thyroid weight which is not sustained, the rate of increase becoming very small after about 16 weeks of treatment. The curve for follicular cell number matches that for thyroid weight, while the follicular cell mitotic rate matches the rate of change in follicular cell number, showing that the decline in the rate of increase in the latter is due to a decline in the rate of cell production and not to an increase in the cell death rate. The continued absence of T₃ and T₄ shows that no significant escape from goitrogen blockade has occurred, and the measurement of TSH shows that high levels are reached early, and sustained even at a time when the thyroid growth rate has almost ceased.

The major finding in this study is the clear dissociation between the function and the growth response to the prolonged TSH stimulus. The function, as assessed by measurements of the iodide trap, rises as TSH rises, and remains high throughout the sustained TSH rise. This provides evidence that the immunoreactive TSH measured retains its biological activity. The decline in mitotic activity during long-term goitrogen blockade therefore is not due to a failing TSH stimulus, and leads us to suggest the existence of a control mechanism which can modulate the follicular cell growth response to TSH separately from its functional response.

It would seem likely that this control mechanism lies within the follicular cell although whether it exists at a receptor or post-receptor level is uncertain. While the general view is that only one class of TSH receptor exists, recent work has suggested that receptor auto-antibodies can influence growth and function separately (Dreghage et al. 1980), so that the possibility remains that the follicular cell can modulate its responses by controlling the number of growth and function receptors separately. With regard to post-receptor mechanisms, several possibilities exist. Follicular cells may show a progressive loss of the ability to divide in response to a TSH stimulus with each successive division, thus setting a finite limit to the number of divisions which can occur. Such a mechanism has been well demonstrated for tissue cultured fibroblasts (Hayflick 1965). Alternatively, selective growth inhibition may be brought about by a negative feedback control sensitive to cell number, mediated by a chalone-like substance, as has been shown in epidermis and several other tissues (Bullough 1962; Rytöma 1976).

Whatever the control mechanism may be, it would seem reasonable to assume that its failure represents the first step in the pathogenesis of neoplasia. It is clearly of fundamental importance therefore to elucidate its nature, and we are currently undertaking a series of experiments with this aim.

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References


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