Goitrogen-induced thyroid growth in the rat: a quantitative morphometric study

D. Wynford-Thomas, B. M. J. Stringer and E. D. Williams

Department of Pathology, Welsh National School of Medicine, Heath Park, Cardiff, CF4 4XN

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SUMMARY
This study was designed to investigate quantitatively the changes in thyroid morphology which accompany goitrogen-induced thyroid growth in the rat and to relate these changes to the level of serum thyrotrophin (TSH).

Animals treated with the goitrogen aminotriazole were killed, together with controls, at frequent intervals up to 153 days. A carefully controlled perfusion-fixation technique was employed, together with plastic embedding, to ensure optimal preservation of in-vivo morphology, and attention was paid to stereological principles in the methods of tissue sampling. Morphometric analysis was carried out by 'point-counting' to obtain the proportion of thyroid volume occupied by each of four components: epithelial cells, follicular lumen, blood vessels and non-vascular stroma. Total thyroid weight was measured, from which the total volumes of each component were calculated. Serum TSH was measured by radioimmunoassay.

Goitrogen treatment led to a rapid rise in the level of serum TSH, reaching a sustained fivefold maximum by day 82. Total thyroid weight increased to a 12-fold plateau after 116 days. There was a rapid change during the first week in the proportional volumes of component tissues; a rise in the epithelial cell and blood vessel components being accompanied by a corresponding fall in follicular lumen and non-vascular stroma. Total epithelial cell volume rose in parallel with thyroid weight to reach a 16-fold maximum. There was a more marked (34-fold) increase in vascular volume and there were smaller increases in the other two components. No significant growth occurred after 116 days.

The results show that goitrogen-induced changes in thyroid morphology are complete at submaximal levels of TSH stimulation and that growth then proceeds by a proportional increase in all components. The morphological changes persist, but growth eventually ceases, despite a sustained rise in the level of serum TSH, thus pointing to the existence in the thyroid of a growth-limiting control mechanism.

INTRODUCTION
It is well established that blockade of thyroid hormone synthesis by chemical inhibitors (goitrogens) leads to thyroid growth (Kennedy & Purves, 1941) and that this is largely due to the increase in the level of serum thyrotrophin (TSH) which results from release of pituitary thyrotrophs from inhibition by thyroid hormone (Bakke & Lawrence, 1964). Early qualitative studies (Kennedy & Purves, 1941; Thyssen, 1947) showed that this growth is due to an increase in both the size and number of follicular cells, and is accompanied by a marked loss of colloid and an increase in vascularity.

Previous attempts to quantify these changes in morphology, however, have all been unsatisfactory. First, with the exception of recent work by Herveg & Wollman (1977) and
Wollman, Herveg, Zeligs & Ericson (1978), none has taken account of the considerable shrinkage and distortion which are known to occur during conventional fixation and tissue-embedding procedures (Denev, Cordier, Mesquita & Haumont, 1979). Secondly, only a few of the component tissues of the thyroid have been studied in any one experiment (Santler, 1957; Philp, Crooks, Macgregor & McIntosh, 1969). Thirdly, unreliable sampling methods have been employed without reference to stereological principles, often employing a single central section (Santler, 1957; Matovinovic & Vickery, 1959) and finally, no attempt has been made to relate these changes in morphology to the level of the TSH stimulus.

The purpose of this study was to provide a reliable, quantitative assessment of the components of thyroid growth in the rat during prolonged goitrogen administration and to correlate these changes with the level of serum TSH. It was hoped that the results obtained would provide an accurate baseline for further studies now being undertaken into the mechanisms regulating thyroid growth.

**Materials and Methods**

Ninety-six male Wistar rats aged 10–11 weeks and weighing 190 ± 13 g at the start of the experiment were housed in a thermostatically controlled room with a constant lighting cycle and fed a standard laboratory rat and mouse breeding diet (iodine content 600 μg/kg; Pilsbury, Edgbaston, Birmingham). They were randomly segregated into 12 groups of eight animals each. Eight groups were given goitrogen, aminotriazole (ATA; Koch-Light Laboratories Ltd, Colnbrook, Bucks), at a concentration of 0·1% in the drinking water, a dose which has previously been shown to block thyroid hormone synthesis completely (Stringer, Wynford-Thomas, Jasani & Williams, 1981), while the other four acted as controls. Goitrogen-treated groups were killed after 3, 7, 14, 24, 46, 82, 116 and 153 days, control groups after 0, 25, 83 and 154 days.

**Histological preparation**

Animals were anaesthetized with an i.p. injection of urethane (16·8 mmol/kg). The abdomen was then opened and 2–3 ml blood withdrawn from the inferior vena cava for subsequent hormone assay. Perfusion fixation was then carried out by a modification of the method of Zeligs & Wollman (1976). The thoracic cavity was approached through the diaphragm and, with the heart still beating, a cannula was inserted through the left ventricle and clamped in the aorta. Perfusion was begun at a pressure approximating that of the mean systolic arterial pressure of the rat (120 mmHg) and at the same time the right atrium was incised. For the first 5 s a ‘pre-perfusate’ was introduced consisting of 1·5% dextran-40 and 0·1% procaine in 0·03 m-phosphate buffer (pH 7·35) which was found to facilitate subsequent perfusion of fixative. The animal was then perfused for 6 min with the fixative solution, a mixture of 2·5% glutaraldehyde and 1% formaldehyde in 0·03 m-phosphate buffer containing 1·5% dextran-40. Previous studies had established this to be the optimum schedule for these fixatives, since it produced minimal shrinkage of follicular cells, nuclei or colloid (as shown by absence of intercellular clefts, crenation or edge retraction respectively), while avoiding interstitial oedema or ballooning of blood vessels. Completeness of perfusion was indicated by absence of red cells.

After fixation, the thyroid was dissected from the trachea, weighed and ‘post-fixed’ in 2% osmium tetroxide for 2 h. The gland was then ‘diced’ (Wynford-Thomas, Stringer & Williams, 1981a) into approximately 30 cuboidal fragments which were randomly embedded in Epon (EMscope Laboratories Ltd, London SW8 4TE). We have shown that this procedure effectively overcomes the non-randomness of thyroid architecture and allows a single section to be used as a representative sample of the whole gland (Stringer, Wynford-Thomas & Williams, 1981). Two 1 μm sections (each containing at least eight profiles of diced fragments) were cut from each block and stained with toluidine blue. This enabled
epithelial cells, follicular lumen and stroma to be identified. However, C-cells were not reliably distinguishable from follicular cells by this technique and both were therefore counted together as the 'epithelial' component. Since C-cells form less than 5% of the gland (Thurston, 1980), the results for this epithelial component may be assumed to reflect changes in the follicular cell population alone with negligible error.

Quantification

Quantification was carried out by a systematic ‘point-counting’ method (Weibel, 1979). The section was projected at a magnification of ×860 onto the screen of a Visopan microscope and a square field equivalent to 116 μm × 116 μm was covered by a grid of 100 evenly spaced points. The proportion of points lying on each component tissue was determined for the four components of epithelial cells, follicular lumen, blood vessels (lumen plus wall) and remaining (non-vascular) stroma. This was repeated for each thyroid on five fields from each of two sections. The fields were chosen by a systematic sampling method, one being taken from the top left hand corner of each diced fragment (starting with the top left hand fragment) until the required number of five had been reached. It was found that the mean proportion of points of ten fields gave an estimate for both control and ATA-treated animals with a relative standard error of less than 15% for all components with the exception of follicular lumen in the treated groups. For the latter, this figure rose to 25%, but this seemingly large analytical error was small (<20%) in relation to the very large biological variation for this component (interanimal coefficient of variation averaged 120%).

The proportional volume (Vv) occupied by each component was assumed to equal the mean proportion of points (Weibel, 1979) and was expressed as a percentage. The specific gravity of the thyroid was determined by the fluid-displacement technique of Scherle (1970) and found to be approximately equal to 1 for both control and treated glands. The total volume and mass for each component were hence calculated as the product of Vv and thyroid weight.

Serum TSH

Serum levels of TSH were measured by radioimmunoassay using a homologous rat TSH assay supplied by the NIAMDD Pituitary Hormone Distribution Program (Stringer, Wynford-Thomas, Jasani & Williams, 1981).

RESULTS

Goitrogen treatment led to a cessation of body growth but was otherwise well tolerated. The changes in histological appearance of the thyroid after different durations of goitrogen treatment are illustrated in Pl. 1, figs 1, 2 and Pl. 2, fig. 3.

Serum TSH (Text-fig. 1)

After the introduction of goitrogen, the level of serum TSH rose rapidly from 979 ± 120 (S.E.M.) to 4507 ± 244 ng/ml by day 24. This was followed by a slower rise to reach a sustained maximum of over 5000 ng/ml by day 116. There was a slight but significant \( P < 0.05 \) upward trend in controls from 979 ± 120 at day 0 to 1407 ± 103 ng/ml at day 154.

Thyroid weight (Text-fig. 1)

The thyroid growth curve showed three phases: an initial lag of a few days, a period of rapid growth for 46 days, and a final period of declining growth rate, reaching a maximum 12-fold plateau (266.6 ± 44.3 mg) by the end of the experiment. Control weights rose from 22.1 ± 1.7 to 28.4 ± 1.2 mg by day 25, following which no further growth occurred. There was a close correlation between thyroid weight and the level of serum TSH \( (r = 0.93) \).
Text-fig. 1. Changes in serum thyrotrophin (TSH; ●) and thyroid weight (▲) during goitrogen treatment. Each point represents the mean of 8 rats together with its standard error (vertical bar).

Text-fig. 2. Changes in (a) proportional volumes (Vv) and (b) total volumes of thyroid component tissues during goitrogen treatment. (a) Shows changes in the percentage of thyroid volume occupied by each component tissue and (b) the corresponding changes in the absolute volumes of each component tissue: epithelial cells (●), follicular lumen (▲), blood vessels (〇) and non-vascular stroma (△). Each point represents the mean of 8 rats together with its standard error (vertical bar).
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Proportional volume of component tissues (Text-fig. 2a)

Epithelial cells
Epithelial cell Vv rose rapidly from the control value of 52.6 ± 2.2 to 68.9 ± 1.1% by day 7 (P < 0.001) and remained around this level for the remainder of the experiment. Controls showed no significant change apart from a transient rise at day 83 to 64.3 ± 3.3%.

Follicular lumen
Follicular lumen Vv fell from 26.7 ± 3.2% at day 0 to 4.3 ± 0.3% by day 7 and remained at around this level. At all times during goitrogen treatment there was a strong inverse correlation between follicular lumen and epithelial cell Vv (r = -0.97). Controls showed little change apart from a fall to 15.3 ± 3.4% at day 83, corresponding to the rise in epithelial cell Vv at this point.

Blood vessels
Blood vessel Vv increased rapidly from 5.7 ± 0.4 to 14.8 ± 1.7% by day 7 and then more slowly to a sustained maximum of 16–17% from 24 days onwards. There was a significant but transient rise to 22.5 ± 1.9% at day 116 (P < 0.05). Control values remained fairly constant at between 6 and 7%.

Non-vascular stroma
The proportion of non-vascular stroma fell in inverse correlation with blood vessel Vv from 15.3 ± 1.2% at day 0 to 8.0 ± 0.2% by day 82. A transient fall occurred to 5.2 ± 0.3% (P < 0.001) at day 116, corresponding to the rise in blood vessel Vv mentioned above. In controls there was a significant (P < 0.01) downward trend from 15.3 ± 1.2% at day 0 to 9.4 ± 1.0% at day 154.

Correlation of Vv with serum TSH during goitrogen treatment
This was calculated on all goitrogen-treated groups plus day 0 control. Epithelial cell Vv and blood vessel Vv were positively correlated with the level of serum TSH (r = 0.66 and 0.88 respectively), while follicular lumen Vv was negatively correlated (r = -0.73).

Total weights and volumes of component tissues (Text-fig. 2b)

Epithelial cells
Epithelial cell mass rose rapidly as a result of goitrogen treatment and closely paralleled the growth of the whole gland, to reach a 16-fold maximum of 185.3 ± 39.4 mg at day 153. Controls showed a slow but significant (P < 0.01) increase from 11.6 ± 1.0 to 18.4 ± 1.6 mg by day 83.

Follicular lumen
The volume of follicular lumen in goitrogen-treated animals initially fell from 5.7 ± 0.8 to a minimum of 2.0 ± 0.1 μl at day 7. From then on a rapid increase in thyroid volume with no further decline in Vv accounted for a progressive increase in total volume to 11.7 ± 3.2 μl by day 153. There was no significant change in controls from the initial value of 5.7 ± 0.8 μl.

Blood vessels
The volume occupied by blood vessels rose dramatically from the control level of 1.3 ± 0.1 to 44.3 ± 11.5 μl at day 153. There was a slight but significant (P < 0.05) upward trend in controls from 1.3 ± 0.1 to 1.7 ± 0.2 μl by day 154.
Non-vascular stroma

The volume of non-vascular stroma increased from $3.2 \pm 0.3 \mu l$ to an irregular maximum of around 18 $\mu l$ from day 46 onwards. There was no significant change in controls from the initial value of $3.2 \pm 0.3 \mu l$.

Correlation with serum TSH

There was a strong positive correlation between serum TSH and the volume occupied by epithelial cells and blood vessels at all durations of goitrogen treatment ($r = 0.93$ and 0.90 respectively) and a slightly weaker correlation with non-vascular stroma ($r = 0.87$). The volume of follicular lumen was negatively correlated ($r = -0.99$) over the first 7 days but positively correlated over the remaining duration of goitrogen treatment ($r = 0.88$).

DISCUSSION

Our results show that inhibition of thyroid hormone synthesis by aminotriazole led as expected to a rapid rise in the level of serum TSH to a fivefold maximum which was maintained throughout the 5-5 months of the experiment. This in turn led to goitre formation, total thyroid weight increasing to reach a 12-fold maximum after approximately 4 months.

During the first week of goitrogen administration there was a rapid change in the morphology of the gland; an increase in the proportional volume of the epithelial cell compartment being accompanied by a corresponding decrease in follicular lumen (colloid) and a marked increase in vascularity. Growth then proceeded proportionally in all tissues resulting in a final 16-fold increase in epithelial cell mass and a 34-fold increase in vascular volume.

Qualitatively, these observations agree with earlier work (Kennedy & Purves, 1941; Thyssen, 1947; Strum & Karnovsky, 1971); quantitative comparisons, however, are difficult. Most previous morphometric studies (Tala, 1952; Lever & Vlijim, 1955) were carried out as bioassays for TSH and used guinea-pig or chick thyroid, the morphology of which differs quantitatively from that of rat. Studies on the latter have been surprisingly few and fragmentary. The closest comparison is provided by the work of Santler (1957) in which the proportional volumes occupied by follicles (follicular cells plus colloid) and blood vessels were measured during 56 days of treatment with methylthiouracil (MTU). It was found that the former component remained constant at around 50% while the latter increased from 3.3 to 11.4%. Phlip et al. (1969) in a similar study found that follicular cells formed approximately 40% of total thyroid volume in control rats, rising to 55–60% after 22 days of MTU administration. In a later study of normal rats, Saadeh & Babikian (1978) reported that follicular cells formed 25% of the volume of follicles and hence an even lower percentage of total thyroid volume. All these results, however, were obtained using immersion-fixed wax-embedded tissue and must therefore be regarded as subject to considerable shrinkage and distortion artifacts. Indeed the work of Santler (1957) demonstrated that a change of fixative (from Zenker-acetic acid to Carnoy’s) resulted in a significant alteration in morphology. Follicular percentage volume was increased by 25% and furthermore the density of the follicular cell population was decreased by nearly 50% in goitrogen-treated animals but not in controls. This indicates that not only may fixation affect the relative volumes of tissues but that, more importantly, the effect may differ between treated and control animals and hence may distort the true effect of treatment.

In comparison with these studies, our results show proportional volumes which were greater for follicular cells and blood vessels and lower for colloid. There is no reason to suppose that this was due to any biological differences in our animals. In particular, dietary iodide was within the accepted range for the rat and serum TSH was close to previously reported levels (Bakke & Lawrence, 1964; D’Angelo, Paul, Wall & Lombardi, 1976). Direct
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comparison with immersion-fixed tissue has confirmed that the differences were due to the fixation and processing techniques employed. By careful control of fixative osmolarity and use of post-osmication we have prevented the follicular cell shrinkage seen with most immersion-fixation wax-embedding schedules and the use of perfusion has prevented vascular collapse by fixing vessels in a dilated state. The histological appearance of our thyroids closely resembles that described recently by Wollman et al. (1978) using similar histological methods. Unfortunately, full morphometric analysis of this work has not been published but the data for the blood vessel component show an even greater increase in volume with goitrogen treatment than that reported here: up to 70-fold after 65 days of thiouracil administration.

Although some uncertainty must remain, since there is no reference method which allows direct observation of the in-vivo state, we feel that the results obtained using our techniques more accurately represent the in-vivo morphology of both normal and TSH-stimulated thyroids than have most earlier studies.

Correlation of the morphological changes with the level of the TSH stimulus reveals several interesting relationships. There is initially a strong positive correlation between serum TSH and both epithelial cell and blood vessel Vv, and a similar negative correlation with follicular lumen Vv, but only up to day 7. It seems that the initial response to a rise in serum TSH consisting of follicular cell hypertrophy, loss of colloid and vascular dilatation is essentially complete well before maximum TSH levels are reached. This early response has been noted previously (Wollman, Andros, Cannon & Eagleton, 1968) and is fairly well understood. Loss of colloid is the result of an increased rate of endocytosis, which is one of the earliest responses to TSH stimulation (Ketelbant-Balasse, Roermans, Frederic, Golstein & Neve, 1976). Follicular cell hypertrophy has been shown by electron microscopic studies (Strum & Karnovsky, 1971) to be largely due to a massive dilatation of the endoplasmic reticulum, which is undoubtedly related to the increased rate of thyroglobulin synthesis brought about by TSH stimulation. The vascular response, however, is more difficult to explain. Wollman et al. (1978) have shown that it occurs by an increase in vessel circumference (achieved by endothelial cell proliferation) and by a process of capillary fusion; sprouting of new vessels is not an important mechanism. The nature of the stimulus, however, remains uncertain and may be either direct, by TSH itself, or indirect, by substances released from TSH-stimulated follicular cells.

After the initial phase of redistribution of tissue volumes, thyroid growth proceeded by a proportional increase in all components. The 16-fold increase in total follicular cell mass is largely achieved by hyperplasia and we have shown in a companion study (Wynford-Thomas, Stringer & Williams, 1981b) that there is a nine-fold increase in the number of follicular cells per gland by the time that maximum growth has been achieved. We have also shown that mitotic activity increases dramatically to a 30 fold peak after 7 days and then declines to little more than control levels after 82 days, which accounts for the observed limitation of total growth of the goitrogen-treated thyroid. However, the underlying mechanisms responsible for this fall in mitotic activity despite a sustained TSH stimulus remain unknown. The possibility of a decline in the bioactivity of circulating TSH has been largely refuted by the finding that the functional response of the gland (activity of iodide pump) remains maximal throughout (Wynford-Thomas et al. 1981b). It seems likely, therefore, that there is a specific growth-regulating control mechanism based either on an inherent limitation of the number of mitoses which follicular cells can undergo, or on the production of a mitotic inhibitor by these cells. We are actively investigating these possibilities at present. It may be noted here, however, that a close correlation was observed throughout between total follicular cell mass and serum TSH, which would be consistent with a feedback control system based on a balance between the TSH stimulus and an endogenously synthesized inhibitor, the concentration of which was proportional to cell mass.
While it is clear that follicular cell multiplication accounts for the greater part of the increase in follicular cell mass, it is not certain how this increase is achieved in terms of the structural organization of the gland, i.e. whether there is an expansion of pre-existing and/or formation of new follicles. It can be seen from Pl. 1, figs 1, 2 and Pl. 2, fig. 3 that as follicular cell hyperplasia proceeds, follicles are converted into large convoluted masses of cells possessing one or a few tiny lumina. Follicular margins become obscured and this, together with loss of colloid, renders accurate quantification of follicle number difficult. If however, goitrogen is withdrawn after growth has ceased, colloid re-accumulates and we have shown (Pl. 2, fig. 4) that after a few weeks, distinct, clearly demarcated follicles are again visible. We are currently carrying out quantification of thyroids treated in this way and preliminary data indicate that there is a large increase (approximately eightfold) in the number of follicles compared with glands not exposed to goitrogen. Hence we may tentatively conclude that expansion of the follicular cell population is accompanied by a corresponding proliferation of follicles. The thyroid may therefore resemble the salivary glands in its capacity for reproducing its functional units, in contrast for example with the kidney, in which this does not occur in the adult.

The results therefore provide, within the limits of current histological techniques, an accurate quantitative analysis of the components of thyroid growth during long-term TSH stimulation. We have shown that after a marked initial change in the relative volumes of component tissues, growth proceeds by a co-ordinated expansion of all components, reaching a maximum after 4 to 5 months.

Two questions remain unanswered: first, the mechanism by which co-ordination is achieved between the growth of parenchymal and stromal elements, and secondly, the nature of the control system which limits total growth in the face of a sustained stimulus. Further work is currently being undertaken to investigate these problems and will form the subject of further publications.

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REFERENCES


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DESCRIPTION OF PLATES
(1 \mu m \text{Epon section; toluidine blue; } \times 370)

\textbf{Plates 1}

Fig. 1. Photomicrograph of thyroid from control rat. Gland consists of colloid-filled follicles of varying diameter, lined by cuboidal epithelial cells and surrounded by a vascular stroma.

Fig. 2. Photomicrograph of thyroid from a rat treated with goitrogen for 3 days. Note increased epithelial cell height (E), loss of colloid from many follicles (F) and capillary dilatation (C).

\textbf{Plates 2}

Fig. 3. Photomicrograph of thyroid from a rat treated with goitrogen for 46 days. Note almost complete absence of colloid, and massive dilatation of capillaries.

Fig. 4. Photomicrograph of thyroid from a rat, initially treated with goitrogen for 82 days and then returned to a normal diet for 2 weeks. Note large number of small follicles lined by low epithelium, and complete regression of vascular dilatation.