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### Thyroxine Augmentation of Growth Hormone-Induced Endochondral Osteogenesis.\* (31460)

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The actions of thyroxine in enhancing body growth, skeletal and somatic, are among the most sensitive of this hormone's effects(1), but remain incompletely studied and understood. Thyroxine participates both in (A) incremental growth, and in (B) differentiation.

A, 1. It has long been known that thyroxine augments the action of pituitary growth hormone, in the absence of the pituitary and/or the thyroid glands, as well as in intact animals (2). Whether this augmentation is true synergism is unresolved, but the demonstration by Geschwind and Li(3) that a dose of thyroxine so minute as to have no discernible effect alone could yet increase the sensitivity of the "tibia

line" assay for growth hormone, appears strong evidence for synergism.

A, 2. Alone, thyroxine induces a discernible, but slight and unsustained, elongation of bones in hypophysectomized rats, a transient effect which is thus independent of pituitary growth hormone(4).

B. In differentiation, thyroxine can induce the appearance of new epiphyseal ossification centers and subsequently their fusion to the diaphysis through resorption of the epiphyseal cartilage plate. This action occurs in the absence of the pituitary(4) or the thyroid(5,6), or both(7). It is apparently effected through continued erosion of cartilage in the absence of an equivalent rate of chondrogenesis; "chondroclasts" have appeared at the marrow-cartilage junction during thyroxine administration(8).

In most circumstances, the principles underlying these actions are not known; in one circumstance (growth in thyroxine-treated

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thyroidectomized rats) it has been well-documented that repair of the pituitary and restoration of endogenous growth hormone secretion is a substantial part of the action(9,10, 11). To complicate the situation, distinctions have not always been well drawn between the immediate effects of hormone administration (*i.e.*, observation after a few days or even weeks) and the effects seen following nearly a year of sustained treatment. Nor does it follow that the widening of an epiphyseal cartilage plate (such as is used as an indicator of growth in the assay of growth-promoting substances) is always associated with active bony elongation.†

The present inquiries arose, in part, from the observation that it proved impossible to stimulate skeletal growth to the point of gigantism in thyroidectomized rats by long-continued administration of high doses of growth hormone, although it was possible to achieve this effect in intact or hypophysectomized rats(12). The growth hormone stimulated osteogenesis, as such, as judged by widening and thickening of bones, but not the elongation of bones which depends on chondrogenesis at epiphyseal cartilage plates. Only when traces of thyroxine were added to the treatment was full endochondral osteogenesis resumed. As a possible explanation, it appeared that growth hormone alone might support osteogenesis, but that thyroxine might be necessary for growth hormone to stimulate sustained chondrogenesis; conceivably, the needs for thyroid hormone were so low that they might be met by endogenous activity even in hypophysectomized rats. (Taurog *et al*(13) have shown continued, albeit low-level thyroxine formation in rats 8-9 weeks after hypophysectomy.)

The present study is intended to search for the lower limit of this potential thyroxine requirement for endochondral ossification. It was decided to use, as the basic test situation, the standard tibia line assay procedure, for this test depends upon evocation of chondro-

genesis in the epiphyseal cartilage plate. The conditions for performing the test have been defined carefully by Geschwind and Li(3), who also describe the earlier contribution of Marx, Simpson, and Greenspan in the test's development. In brief, female rats are hypophysectomized when 26-28 days of age, and after a 12-day post-operative interval are screened for good condition and presumptive evidence of completeness of the operation. The hormonal material under test is injected daily for a 4-day period; 24 hours after the last injection the animals are autopsied and the tibias dissected free, split, and "stained" with silver nitrate solution to mark calcified regions (von Kossa reaction). The width of the uncalcified portion of the proximal epiphyseal cartilage plate is measured in a compound microscope with a micrometer eyepiece. Typically the width in untreated controls is of the order of 150  $\mu$ . If, following treatment, the width does not achieve at least 200  $\mu$ , the effect is considered to be in the "non-specific" range(3). With a potent growth hormone a linear dose-response increase is found over a wide range of dosages. Dose is always expressed as total administered during the 4-day test.

*Experiment 1.* A standard dose of growth hormone was sought to form the base of comparison for all further tests. The preparation of growth hormone employed was highly purified. To give still further assurance of minimal contamination with thyrotropic hormone—TSH—(which might stimulate the thyroid, if present, or even aberrant thyroid follicles such as have been seen in the thymus(15)), the hormone was treated with periodate(16) to inactivate any residual glycoprotein moieties.‡ Table I shows the epiphyseal plate widths encountered in the various experimental groups. At 40  $\mu$ g total dose this preparation gave a response in the "non-specific" range; 50  $\mu$ g gave a well-defined response, almost 50% above the control level. The dose chosen for further testing was 60  $\mu$ g, since this gave an increase which was invariably significantly above the non-specific range.

† Of several situations which support this remark, one may mention an experiment in which bone length was greater in a thyroxine-growth hormone-treated group than with growth hormone alone, but the epiphyseal cartilage plates were narrower in the doubly-treated group(14).

‡ We acknowledge gratefully a generous supply of such growth hormone from Dr. Lawson L. Rosenberg.

TABLE I. Widening of Uncalcified Portion of Tibial Epiphyseal Cartilage Plate in Response to Growth Hormone.

Treatment	No. of rats	Width of plate, $\mu$ †
Hypophysectomized* controls	25	142 $\pm$ 1.9
Hypophysectomized* & growth hormone ( $\mu$ g/4 days)		
40	18	182 $\pm$ 9.3
50	16	208 $\pm$ 8.3
60	20	225 $\pm$ 4.0
Normal controls	6	256 $\pm$ 8.6

\* Completeness of hypophysectomy verified at autopsy by inspection of sella under dissecting microscope.

† Means and standard errors. Value for hypophysectomized controls is significantly below normal ( $p < 0.001$ ); all values for treated animals are significantly above hypophysectomized controls ( $p < 0.001$ ); highest treatment value is significantly below normal ( $p < 0.01$ ).

*Experiment 2.* This experiment was intended to disclose what level of thyroxine would influence the tibia response of *thyroidectomized* rats. The age at operation and the post-operative interval were the same as used in the studies on hypophysectomized rats. A broad dosage range of l-thyroxine was tested, from 0.001  $\mu$ g/day to 10.0  $\mu$ g/day, or a 10,000-fold spread. (*N.B.: hereafter, in accord with the convention of expressing total dose in this test, these doses are multiplied by 4, reflecting the 4 daily injections.*) The evidence for completeness of thyroidectomy was obtained following the procedure of Evans *et al*(1) using a tracer dose of  $I^{131}$ . Any rat in which the iodine uptake of the perilyngeal and peritracheal tissues (corrected by comparison with an equal mass of muscle) exceeded 0.05% of the tracer dose was presumed to be incompletely thyroidectomized and was rejected from the study.

Fig. 1 illustrates the results. Each point represents the mean value for 6 to 10 animals, with the exception of 20 thyroidectomized controls and 4 animals in the 1.0  $\mu$ g dose group. The brackets indicate 1 standard error. Thyroidectomized controls showed a plate width of 229  $\mu$ . Widening of the plate above the control value was found with all thyroxine doses tested. At the lowest dose, 0.004  $\mu$ g, the widening was of questionable statistical significance ( $p < 0.05$ ), but thereafter "t"-

tests for difference from controls gave p-values of 0.02 to 0.001. In the dosage range of 0.01 to 1.0  $\mu$ g there was no indication of response proportional to dosage, and the various widths did not differ significantly among themselves. The width characteristic of normal controls was encountered between 1 and 2  $\mu$ g of thyroxine dosage. It should be noted that this was achieved in 4 days, after a 12-day period of hormonal deficiency. *Maintenance* treatment (*i.e.*, treatment started immediately after thyroidectomy) might have allowed a similar level to be attained with a lower dose.

The curve shown could be explained on the hypothesis that (1) there is a slight degree of restoration of cartilage structure which can occur with extraordinarily low thyroxine supplies, although it does not represent active and sustained chondrogenesis, and (2) there is a higher thyroxine dose level which restores endogenous pituitary growth hormone secretion to a significant degree and at which (together with the synergic effect of the exogenous thyroxine) active chondrogenesis is evoked. The second part of this hypothesis, at least, is susceptible to testing by pituitary bioassay. The relationship of thyroxine dosage to pituitary growth hormone elaboration has been studied repeatedly by others but not with animals of

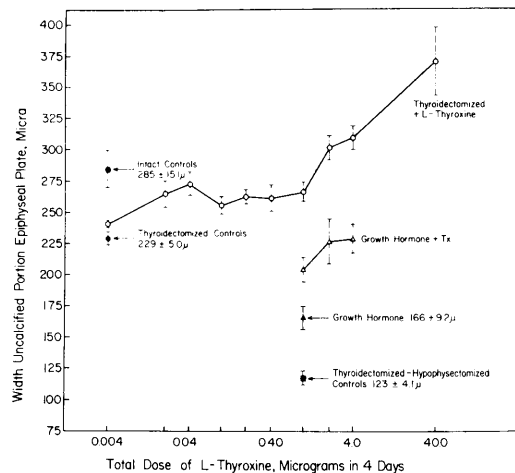


FIG. 1. Showing (above) response of tibial epiphyseal cartilage plate of thyroidectomized rats to graded doses of thyroxine, and (lower right) response of hypophysectomized-thyroidectomized rats to growth hormone, with and without "concurrent" thyroxine.

TABLE II. Bioassay of Growth- and Thyroid-Stimulating Factors in Anterior Pituitary Glands of Thyroidectomized Rats.

Donors		Recipients (hypophysectomized)		
Operation	Thyroxine dose ( $\mu\text{g}/4$ days)	No.	Epiphyseal plate width ( $\mu$ )	Thyroidal $I^{131}$ uptake (% dose*)
Dose: $\frac{1}{2}$ pituitary gland in 4 days†				
Thyroidect.	none	8	$138 \pm 4.6$	$2.2 \pm .12$
"	.4	6	$208 \pm 8.0$	$2.9 \pm .04$
"	1.0	6	$190 \pm 6.0$	$2.8 \pm .20$
"	2.0	7	$229 \pm 9.0$	$3.3 \pm .10$
Normal	none	6	$223 \pm 9.0$	$3.4 \pm .04$
Dose: $\frac{1}{8}$ pituitary gland in 4 days†				
Thyroidect.	none	6	$147 \pm 10.0$	$1.2 \pm .05$
"	.4	6	$151 \pm 10.4$	$1.3 \pm .04$
"	1.0	6	$171 \pm 14.0$	$1.9 \pm .05$
"	2.0	8	$194 \pm 12.5$	$2.1 \pm .50$
Normal	none	5	$218 \pm 12.1$	$2.3 \pm .12$

\* % of dose administered 20 hr before autopsy, corrected by comparison with equivalent weight of gastrocnemius muscle.

† Pooled from adenohypophyses of 4 rats in each group, except for 8 thyroidectomized controls and 8 normal controls.

this young age nor with dosages as low and injection periods as short as used here.

The pituitary glands of the thyroidectomized animals represented in Fig. 1 had been frozen at autopsy. Subsequently, glands from selected groups of animals were dissected free from their posterior lobes, homogenized and suspended in saline, and tested for growth hormone content by injection into hypophysectomized rats under the standard assay conditions. The glands were those from animals whose thyroxine dosage had bracketed the abrupt change in direction of the response curve in Fig. 1, namely 0.4, 1.0, and 2.0  $\mu\text{g}$  total dose of thyroxine. Of these, 2 pituitary dose levels were assayed, one-half gland and one-eighth gland total dose, respectively. TSH content was also estimated, by measuring the effect on uptake of radioactive iodine tracer ( $I^{131}$ ) in the thyroids of the hypophysectomized test animals. Other pituitary hormones (e.g., gonadotrophins, adrenocorticotrophin) were not assayed, as it has been clearly demonstrated (11) that they require substantially higher doses of thyroxine for adequate measurement by bioassay than were tested here.

Table II shows the results. In  $\frac{1}{2}$  of a gland, the 2 lower dose levels of thyroxine were at the border-line between non-specific and specific growth hormone response, and were not significantly different in their hormone content,<sup>§</sup> whereas the highest level

showed growth hormone content equivalent to that in  $\frac{1}{2}$  of the gland of a normal rat. In contrast, at the  $\frac{1}{8}$ th gland assay level, none of the thyroxine-treated rats showed a growth hormone content above the conventional non-specific range, although, in truth, the responses obtained rose with thyroxine dosage.

While it is true that the assay reflects pituitary hormone *content*, rather than *release*, the results support the interpretation that Fig. 2 distinguishes between two thyroxine effects on cartilage—a low-level non-dose-specific effect and a higher level effect through the pituitary gland, and that the point of critical distinction between these effects lies approximately between 1.0 and 2.0  $\mu\text{g}$  total dose (or 0.25 and 0.5  $\mu\text{g}$  daily dose) of l-thyroxine in a *repair* test (as distinguished from a *maintenance* test).

Although thyrotropic hormone content of the pituitary gland is not at issue in this experiment, Table II shows that the thyroid gland was stimulated by pituitary homogenates at all doses of thyroxine used, and the highest dose of thyroxine brought this level of stimulation virtually to normal, both in

§ The reversal of the expected order of response at the 2 lower thyroxine dose levels, both in epiphyseal plate width and  $I^{131}$  uptake, suggests differences in reactivity to these marginal doses among the individual rats whose pituitaries were pooled for the test material.

$\frac{1}{8}$ th of a gland as well as in  $\frac{1}{2}$  of a gland. Without additional information on pituitary release, these data cannot be discussed further in terms of the reciprocal relationships between TSH and thyroid hormone.

*Experiment 3.* With a standard dose of the specified preparation of growth hormone and a limited but meaningful dose range of l-thyroxine to be tested for synergistic effects, the test animals in the 3rd experiment were both hypophysectomized and thyroidectomized to provide a complete deficiency of these 2 hormones. The 60  $\mu\text{g}$  total dose of growth hormone was administered alone to 9 rats, and was also given with thyroxine, 1  $\mu\text{g}$  (10 rats), 2  $\mu\text{g}$  (10 rats), and 4  $\mu\text{g}$  (13 rats). Six doubly operated rats served as controls. The results are graphed on the lower right of Fig. 1. Alone, the growth hormone gave some cartilage plate widening, although in the "non-specific" range. One  $\mu\text{g}$  of thyroxine augmented this effect markedly ( $204 \pm 9.8 \mu$ ); 2  $\mu\text{g}$  produced a plate width ( $227 \pm 18.0 \mu$ ) corresponding closely to the width achieved by growth hormone alone in hypophysectomized rats (225  $\mu$ , Table I). Administration of 4  $\mu\text{g}$  did not significantly increase this augmentation ( $229 \pm 11.9 \mu$ ), and it may thus be suggested that the optimal synergistic effect was attained at the intermediate thyroxine dose.

*Discussion.* Caution must be exercised in deducing the physiological equivalents of the thyroxine dosage effects herein demonstrated, due (as already indicated) to the *repair* rather than the *maintenance* nature of the tests, and also to the fact that the hormones were administered once daily rather than in a constant supply. Nevertheless, it appears that even in hypophysectomized rats the thyroid gland provides some degree of assistance to the chondrotropic effects of pituitary growth hormone. Furthermore, this assistance may be simulated by administering a dose of thyroxine lying between the minimally significant amount of 0.25  $\mu\text{g}$  *per day* (1  $\mu\text{g}$  total dose) and an optimum no more than twice that amount. It is worth noting that Geschwind and Li found the lower figure significant in a *maintenance* test on hypophysectomized rats (3), although not testing as broad a range of dosages as employed here. This dose is much

less than the thyroxine secretion rate of young normal rats(17). Finally, this proposal is compatible with biochemical demonstrations of the output of hormone by the thyroid gland of hypophysectomized rats(13,18).

Although supporting the suggestion that thyroxine is necessary for the regulation of endochondral osteogenesis, these experiments do not disclose its role. Evidence is required relating the chondrogenetic process to some known function of thyroxine at the cellular level. It may, perhaps, be sought in the association of thyroid hormone with tissue and cell differentiation. Rigal(19,20) has emphasized the importance of the progenitor cells lying in the narrow germinal zone of the epiphyseal cartilage plate. These cells must undergo proliferation and transformation to provide what are virtually "clones" of chondrocytes. Subsequent proliferation of the latter, as long columns of flattened cells, is responsible for widening of the plate. Possibly thyroxine is required in the transformation of the progenitors. Experiments are in progress employing critical conditions derived from the present study, extended by tritiated thymidine administration and autoradiographic study of the cells in the various regions of the epiphyseal cartilage plate.

*Summary.* The possibility has been investigated that thyroxine is required to support the chondrogenetic effects of growth hormone in endochondral osteogenesis. Effects of these hormones have been determined in hypophysectomized, thyroidectomized, and hypophysectomized-thyroidectomized rats, being measured by the tibial epiphyseal plate widening response commonly employed in growth hormone assay. A dose of l-thyroxine lying between 0.25 and 0.5  $\mu\text{g}/\text{day}$  (a) restored vigorous endochondral osteogenesis in thyroidectomized rats, (b) increased their pituitary growth hormone content to normal, and (c) in hypophysectomized-thyroidectomized rats augmented the effect of a dose of growth hormone to equal that when the growth hormone dose was given alone to hypophysectomized rats.

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### Trypsin Inhibitor in Guar Meal. (31461)

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*Cyamopsis tetragonoloba* (Guar) is a drought-resistant annual legume which is adapted to a wide range of soil types. The seed has long been used as a source of gum. The residue remaining after extraction of the gum contains 36-45% protein. Attempts to feed this protein concentrate to animals have met with varying degrees of success. Guar meal has been reported to contain toxic factors which have caused diarrhea, a decrease in growth rate, anorexia, and increased mortality(1,2). Guar gum has been reported to decrease growth and increase mortality in studies with chicks(1,2). The meal contains a trypsin inhibitor which could be destroyed by heat, according to preliminary reports from this laboratory(3,4). It was reported earlier (5) that the nutritive value of Guar meal was not improved by autoclaving. The oil or fat of the Guar meal has been reported not to contain a toxic factor(6).

The present report is concerned with the trypsin inhibitor found in Guar meal.

*Experimental.* An extract of Guar meal was made by placing 20 g of Guar meal in a 250 ml Erlenmeyer flask containing 100 ml of phosphate buffer (pH 7.6, 0.02 M). The flask was shaken gently and allowed to stand in a cold room at 5°C for 6 to 8 hours with intermittent shaking. The mixture was then filtered through Whatman No. 1 filter paper, and the filtrate was used for assay of the toxic factor.

Worthington lyophilized, salt free hemoglobin was prepared according to the method of Anson(7). The procedure of Northrop *et al*(8) was used for the trypsin assay. Tryptic activity was determined at 37°C with the urea-denatured hemoglobin substrate. The extent of proteolysis was determined by reading the absorbance of the 5% trichloroacetic acid soluble degradation products at 280 m $\mu$  with a Beckman model DB spectrophotometer.

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