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Thyrotropin is not a growth factor for human thyroid cells in culture

(pituitary trophic hormones/goiter/cell differentiation)

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ABSTRACT **Thyroid cells, obtained from both normal human tissue and benign nodular goiter, were cultured and maintained *in vitro* in 4–18 passages. Cultures with confluent cells accumulated cyclic AMP (10–150 times the basal amount) upon addition of bovine thyrotropin (100 milliunits/ml), indicating that the cells in culture maintained a thyrotropin-sensitive adenylate cyclase system. Addition of high doses of thyrotropin also induced a characteristic and reversible change in the morphology of the cells.**

The effect of thyrotropin on cell growth was studied in short- and long-term experiments. Thyrotropin reduced [^{3}H]thymidine incorporation in a dose-dependent fashion in all cultures of thyroid cells. The maximal inhibition over a 24-hr period was about 50%. The thyroid cells were notably sensitive, and the half-maximal effect occurred at about 100 milliunits of thyrotropin per ml. In contrast, the hormone had no effect on [^{3}H]thymidine incorporation into human glial cells. Low doses of thyrotropin also had no effect on human fibroblasts and, at high doses, a stimulation of [^{3}H]thymidine incorporation was seen. Thyroid cell cultures grown in the presence of 10 milliunits of thyrotropin per ml for 7–14 days had a slower growth rate and 24–36% lower cell numbers at saturation density than control dishes, indicating that the hormone also had a long-term effect on cell proliferation. The data agree with *in vitro* studies by others of the effects of corticotropin and lutropin on target cells and suggest that *in vivo* the primary action of pituitary trophic hormones on endocrine tissues is not stimulation of growth.

Isolated thyroid cells from various species, including human beings, can be maintained in culture for a limited period of time (1–10). The addition of thyrotropin (TSH) to the cultures at the time of explantation makes the cells organize into follicular structures (3–7) that maintain the capacity to concentrate iodide and synthesize iodinated thyroglobulin (7, 11). It is generally assumed that an increased cyclic AMP level has an important role in the transmission of the hormonal effects since dibutyryl cyclic AMP and phosphodiesterase inhibitor can mimic certain TSH actions on cells *in vitro* (5–7) and TSH activates a membrane-bound adenylate cyclase in thyroid cells (reviewed in ref. 12).

In several cell systems the development of differentiated functions is accompanied by an inhibition of cell multiplication (reviewed in ref. 13). TSH is both considered important for the expression of differentiated functions of the thyroid and generally believed to be a trophic factor for the thyroid *in vivo*. The object of the present investigation was to analyze the effect of TSH on thyroid cell division *in vitro* by using human thyroid cell lines. The results demonstrate that TSH is not a growth factor for thyroid cells *in vitro* and have implications for the understanding of thyroid growth *in vivo*. The results suggest that in the development of goiter the growth stimulus is not provided by TSH.

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MATERIAL AND METHODS

Materials. Bovine TSH (3 units/mg) was purchased from Ferring AB (Malmö, Sweden). Human TSH (2.5 units/mg) was kindly provided by AB Pharmacia (Uppsala, Sweden). The thyrotropin solutions were prepared by dissolving the hormone in 0.02 M phosphate buffer/0.15 M NaCl at pH 7.40, containing 1 mg of bovine serum albumin per ml, and dialyzing against the same buffer at 4°C overnight.

Collagenase (153 units/mg) was purchased from Worthington; 3-isobutyl-1-methylxanthine from Aldrich (Gillingham, U.K.); [^{3}H]thymidine (2 Ci/mmol, 1 Ci = 3.7×10^{10} Bq) from Radiochemical Centre (Amersham, U.K.); N^6,$O^{2'}$-dibutyryl cyclic AMP from Sigma; and human serum albumin from AB Kabi (Stockholm, Sweden). A radioimmunoassay kit for determinations of cyclic AMP was obtained from Schwarz/Mann.

Cell Cultures. Thyroid cell lines were initiated from two nonfunctioning thyroid adenomas (lines designated HTh 1 and HTh 11), one functioning adenoma (HTh 31), and in one case from nonpathological tissue surrounding a functioning adenoma (HtTh 14). The tissue was fragmented with a scalpel, suspended in phosphate-buffered saline containing 1 mg of collagenase per ml, and incubated in a 37°C rocking waterbath for 60 min. Cells were further liberated from the fragments by pipetting. Large fragments were allowed to sediment and the supernatant was decanted and centrifuged ($200 \times g$ for 10 min). The pellets were suspended in Eagle's minimum essential medium (14) supplemented with 10% fetal calf serum, 100 units of penicillin, 1.25 μg of amphotericin B, and 50 μg of streptomycin per ml. The cells were incubated in 50-mm Nunc petri dishes (Nunc, Roskilde, Denmark). About 20 dishes were initiated from 1 g of wet weight tissue. The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Primary confluent monolayers were usually formed within 7–14 days. The cultures were then subcultivated at 1:2 split ratio. Cells were detached after incubation in phosphate-buffered saline with 0.02% EDTA for 5 min followed by a film of phosphate-buffered saline with 0.25% trypsin for about 10 min. Cultures of normal human skin fibroblasts and human glial cells were initiated and maintained as described (15, 16).

Incorporation of [^{3}H]Thymidine. Cells were detached and suspended in minimum essential medium containing 10% fetal calf serum and seeded into 35-mm Nunc dishes. One 50-mm dish with a confluent monolayer was split into eight 35-mm dishes. After 24 hr of incubation, medium was changed to thymidine- and serum-free Ham's nutrient mixture F-10 (17). After 24 hr of serum starvation, medium was changed to F-10 containing 1 mg of human serum albumin per ml; fetal calf serum and TSH were added as specified below. The cultures

Abbreviation: TSH, thyrotropin.

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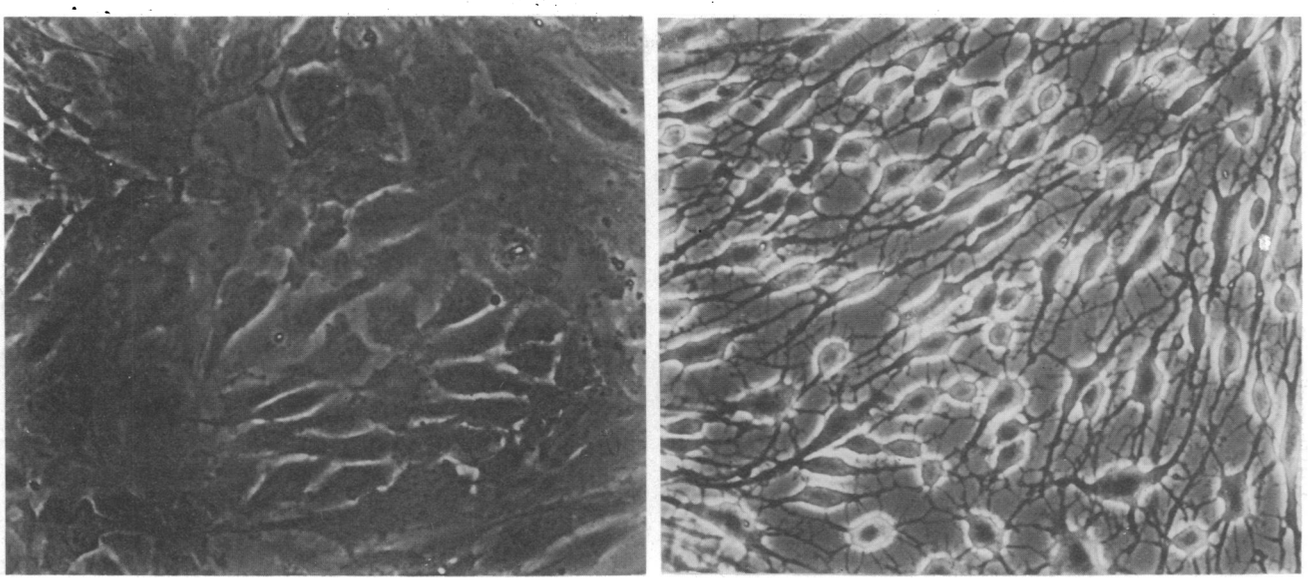

FIG. 1. (*Left*) Appearance of human thyroid cells obtained from benign nodular goiter (HTh 1). (*Right*) Same cells after treatment for 60 min with bovine TSH (100 milliunits/ml).

were preincubated for 18 hr. Tritiated thymidine was then added to a final concentration of 0.02 μCi/ml. After 24 hr of incubation, the cultures were harvested and radioactivity incorporated into trichloroacetic acid-precipitable material was measured as described (18).

Measurement of Cyclic AMP. The procedure was essentially that described by Rapoport (9) for studies of maximal cyclic AMP production in dog thyroid cell cultures. Duplicate confluent cell cultures were incubated with 100 milliunits of bovine TSH per ml and 0.5 mM 3-isobutyl-1-methylxanthine and control dishes with 0.5 mM isobutylmethylxanthine for 60 min at 37°C. Cyclic AMP was measured by use of a radioimmunoassay kit based on the method of Steiner *et al.* (19).

Determination of Cell Number. Cells were detached in 0.02% EDTA/0.25% trypsin and counted in an electronic particle counter (Celloscope, AB Ljungberg, Stockholm, Sweden).

Protein Determination. Cells were lysed with 0.3 M NaOH and protein content was determined by the method of Lowry *et al.* (20).

RESULTS

Culture and Morphology of Human Thyroid Cells. In the present investigation, we tried to initiate cell lines from 21 thyroid biopsies of nonmalignant tissue. Cell growth occurred in all cases. However, the majority of cell cultures gave rise to mixed populations of epithelial cells and fibroblasts. In four instances cell lines free of visible fibroblast contamination were initiated. The cells grew as monolayer cultures which initially could be passaged one to two times a week (Fig. 1). After several passages, growth rates typically declined until no increase in cell number was detected (see Table 1).

Addition of bovine TSH (10–100 milliunits/ml) to the culture medium had a marked effect on cell morphology. After a few hours' treatment of sparse and confluent dishes with bovine TSH, the cells became arborized (Fig. 1 *right*). A similar morphological response was also obtained by addition of 0.5 mM dibutyryl cyclic AMP. Upon prolonged treatment with TSH, the cell pattern gradually reversed to the original. After 24 hr of incubation, very few arborized cells could be detected. At this state renewed challenge with fresh TSH was without any influence on the appearance of the cells.

TSH-Sensitive Cyclic AMP Production in Human Thyroid Cell Cultures. Basal intracellular levels of cyclic AMP in confluent human thyroid cell cultures were 8–14 pmol of cyclic AMP per mg of cell protein. The challenge of 100 milliunits of bovine TSH per ml for 60 min caused a 10- to 150-fold increase in intracellular content of cyclic AMP in thyroid cell cultures (Table 1). The basal level and the degree of TSH stimulation correspond to those found by Rapoport in studies of dog thyroid cell cultures (9) under similar experimental conditions.

Effect of TSH on [^{3}H]Thymidine Incorporation and Cell Density in Thyroid Cultures. Addition of bovine or human TSH to thyroid cells cultured in the presence or absence of 1% fetal calf serum caused a dose-dependent reduction in [^{3}H]-thymidine incorporation in all thyroid cell lines studied (Fig. 2). The effect was readily detectable in cultures kept in 1% fetal calf serum. The presence of serum was, however, not an absolute requirement since the inhibitory effect of TSH was noticed and showed a similar dose–response relationship in serum-free

Table 1. Effect of TSH on thyroid cell cultures

Cell line	Tissue origin	Life span, passages	Cyclic AMP, pmol/mg cell protein	
			0.5 mM MIX	0.5 mM MIX + TSH
HTh 1	Nonfunctioning nodule	15	14	143
HTh 11	Nonfunctioning nodule	6	12	501
HTh 14	Normal tissue surrounding a functioning nodule	4	ND	ND
HTh 31	Functioning nodule	18	8	1173

MIX, methylisobutylxanthine; ND, not determined.

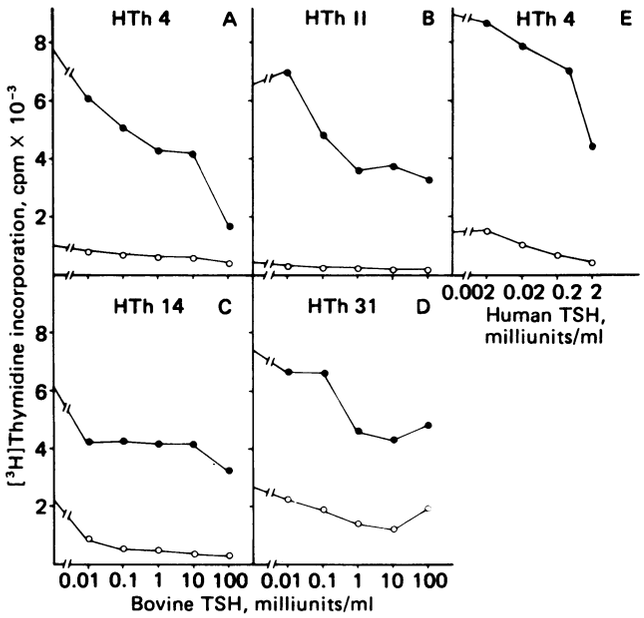

FIG. 2. Effect of bovine TSH (*A–D*) and human TSH (*E*) on [³H]thymidine incorporation (mean of duplicate dishes) into acid-precipitable material of human thyroid cells in culture. TSH was added in doses indicated, alone (○) or along with 1% fetal calf serum (●), to cultures starved for serum for 18 hr. [³H]Thymidine incorporation during the following 24-hr was then measured.

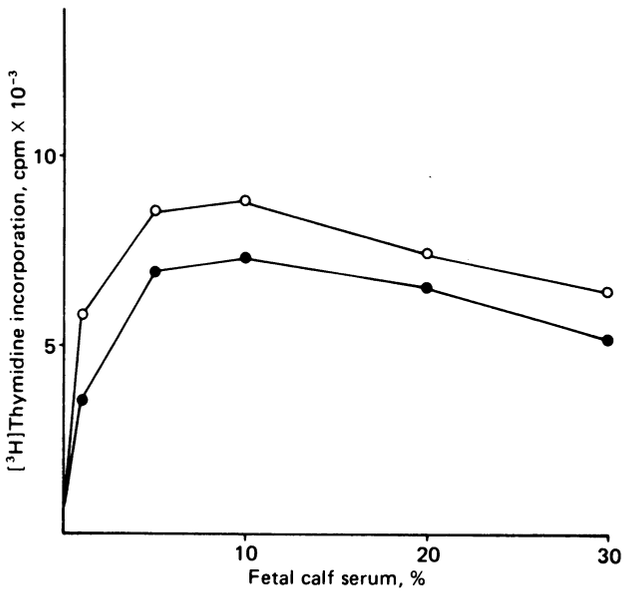

FIG. 4. Influence of increasing concentrations of fetal calf serum on the effect of a fixed amount of bovine TSH on [³H]thymidine incorporation into acid-precipitable material of human thyroid cells in culture (line HTh 1). Fetal calf serum was added in concentrations indicated, alone (○) or along with 10 milliunits of bovine TSH per ml (●). [³H]Thymidine incorporated between 18 and 42 hr after addition of TSH was then measured.

cultures also. The sensitivity of different thyroid cultures to the inhibitory activity of TSH of bovine or human origin was similar, with a half-maximal effect occurring at 100 microunits/ml (Fig. 3). With increasing TSH concentrations the inhibitory effect leveled off. The maximal reduction in [³H]-thymidine incorporation obtained was about 50%.

The following experiment was performed to see whether the TSH-mediated inhibition of [³H]thymidine incorporation could be overcome by an increase in serum concentration. Serum-starved cultures were fed with media containing different concentrations of fetal calf serum (1–30%) with or without 10 milliunits of bovine TSH per ml and the incorporation of [³H]thymidine was measured as above. As shown in Fig. 4, an inhibitory effect of TSH was noticed at all serum concentrations tested. The relative inhibition was most pronounced at 1% fetal calf serum.

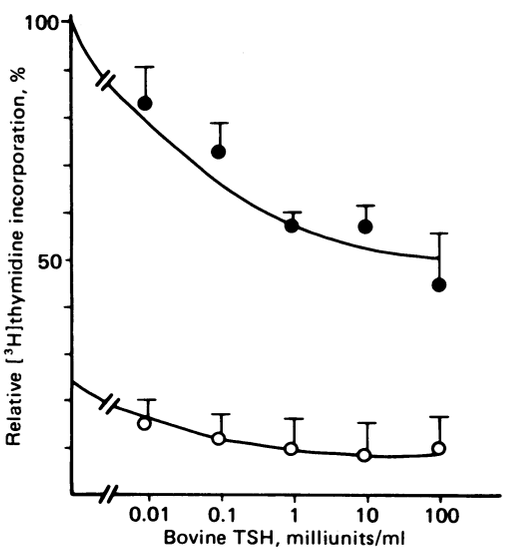

FIG. 3. Dose–response curve for TSH inhibition of [³H]thymidine incorporation into human thyroid cells. Data from experiments shown in Fig. 2 *A–D* were expressed as % [³H]thymidine incorporation (mean and SEM) as a function of TSH concentration, taking the incorporation in cultures fed with 1% fetal calf serum alone as 100%.

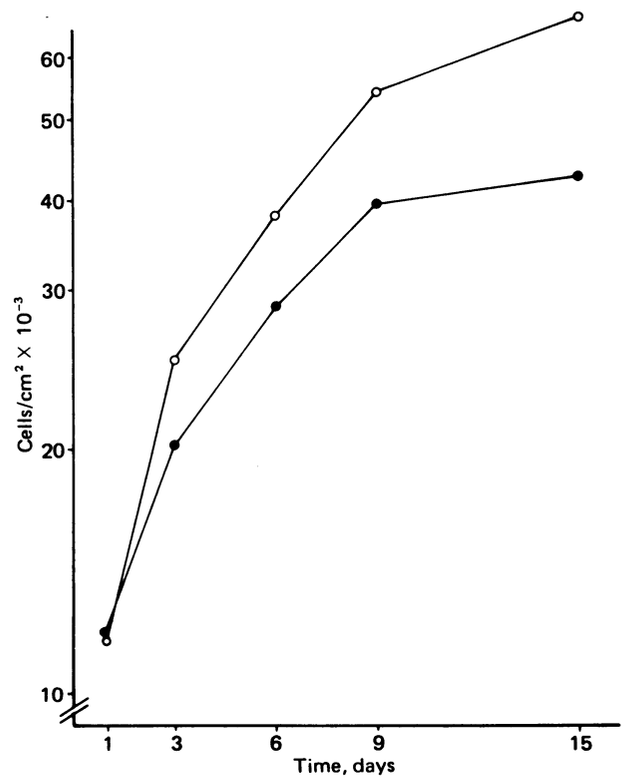

FIG. 5. Long-term effect of TSH on thyroid cell growth. Sparse cultures (HTh 31) were plated and cultured in F-10 medium containing 10% fetal calf serum with or without human TSH (10 milliunits/ml) and medium renewed three times a week. Cell numbers were determined for duplicate dishes.

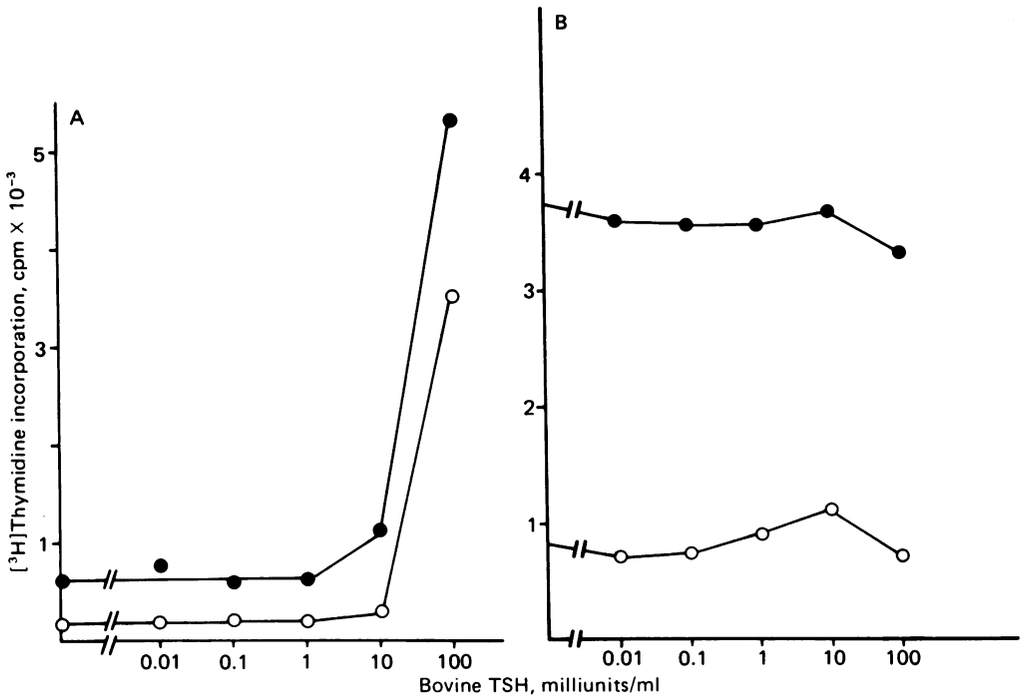

FIG. 6. Effect of bovine TSH on [^{3}H]thymidine incorporation into acid-precipitable material of (*A*) human fibroblasts and (*B*) human glial cells in culture. Bovine TSH was added in doses indicated, alone (○) or along with fetal calf serum (●) to cultures starved for serum for 18 hr. [^{3}H]Thymidine incorporation during the following 24 hr was then measured.

The long-term effect of TSH on thyroid cell growth *in vitro* was also investigated. These experiments were carried out in medium with 10% fetal calf serum. Cultures kept in the presence of bovine TSH (10 milliunits/ml) had a slower growth rate throughout the period of growth to saturation than cultures kept in the absence of TSH. Consequently, the final saturation density was lower in TSH-treated cultures—35% lower as shown in Fig. 5 and 24% lower in a parallel set of experiments (data not shown). Note that these figures are close to the degree of lowered [^{3}H]thymidine incorporation found in short-term cultures cultured in 10% fetal calf serum (Fig. 4).

Effect of TSH on [^{3}H]Thymidine Incorporation in Human Fibroblasts and Glia Cell Cultures. [^{3}H]Thymidine incorporation in human fibroblasts and human glia cells showed an opposite response to TSH compared to thyroid cells. At low levels of TSH no effect on human fibroblasts could be detected, but at higher concentration (10–100 milliunits/ml), stimulation of [^{3}H]thymidine incorporation was found (Fig. 6). Under serum-free conditions the fibroblast cultures showed an ≈20-fold increase. When bovine TSH was added along with 1% fetal calf serum, a 9-fold increase with the highest hormone concentration, 100 milliunits/ml, was determined.

Human glia cell cultures were not affected by bovine TSH when [^{3}H]thymidine incorporation was studied under identical experimental conditions (Fig. 5). Thus, the markedly different results with fibroblasts and glial cells indicate that thyroid cell cultures have a unique sensitivity to an inhibitory activity of TSH on [^{3}H]thymidine incorporation.

DISCUSSION

In the present study human thyroid cell cultures, apparently free of fibroblast-like cells, responded with a marked elevation in cyclic AMP after addition of high doses of bovine TSH. This indicates the presence of a TSH-responsive adenylate cyclase system in the cell cultures. The amounts of cyclic AMP formed and the magnitude of the increase were similar to those reported for dog thyroid cells in tissue culture (9). In addition, the human cells underwent the same morphological alteration after TSH addition as was described for dog thyroid cells (9).

TSH inhibited the incorporation of [^{3}H]thymidine into all thyroid cell lines, which were derived from nonpathological and functional or nonfunctional nodular tissues. In contrast, TSH had no effect on [^{3}H]thymidine incorporation in glial cell cultures; when tested in fibroblast cultures, TSH was without effect at low concentrations, whereas at high concentrations a stimulation of the incorporation of [^{3}H]thymidine was found. It is likely that the stimulation seen with high doses of TSH in fibroblast cultures was caused by growth factors previously found to contaminate certain TSH preparations (see ref. 21). These findings and the marked sensitivity of thyroid cell cultures to preparations of both bovine and human TSH, with noticeable inhibition at doses within the physiological range (0.01 milliunit/ml) and half-maximal inhibition at one order of magnitude higher concentration, strongly suggest that this phenomenon reflects a specific interaction between TSH and the TSH receptor of the thyroid cell.

Culture of human thyroid cells has been described in two previous reports. In one study human thyroid cells were maintained in culture for up to 6 days and thyroid hormone production was measured (10). No data on TSH effects on cell growth were given. In another study (8), the effect of TSH on cultured human, dog, and bovine thyroid cells was reported. Nonconfluent thyroid cells (species origin not given) grew faster to saturation density in the presence of 100 milliunits of TSH per ml than the control cells. However, after 6 days in culture the same terminal cell density was reached in both instances. Further, bovine TSH (100 milliunits/ml) was reported to stimulate the incorporation of [^{3}H]thymidine in a linear fashion during a 6-hr incubation. This study thus reported observations that differ from those found by us. The reason for this discrepancy is not clear. However, differences in the homogeneity of the cell populations studied and possible impurities in the TSH preparations may be of importance.

The lack of stimulation of TSH on thyroid cell multiplication *in vitro* found in the present study prompts a discussion of data concerning the effects of TSH on thyroid growth *in vivo*. TSH injections give rise to a wide range of morphological responses (see review, ref. 12), the earliest reflecting an increased thyroid secretion. After 2–4 hr an increased cell height, suggestive of cell hypertrophy, can be observed. Prolonged administration of TSH for up to 6 days leads to an increase in thyroid weight with proliferation of capillaries preceding an increase of the parenchyma (22, 23). In rat or mouse thyroids chronically stimulated by TSH a 3-fold increase of the total cell number, estimated by DNA determinations, has been reported (24–26). The labeling index was higher in endothelial cells and fibroblasts than in follicle cells (27). Thus, it is clear that the increased growth of the thyroid gland caused by prolonged TSH administration can be attributed only to a minor degree to an actual development of new follicle cells. Moreover, in the latter studies (24–27) animals were used that had been pretreated with iodine-blocking agents to depress thyroid hormone production and increase endogenous TSH secretion. Such treatment makes an interpretation of the pure TSH effects difficult. Finally, TSH has no effect on thyroid cell division, but causes marked cellular hypertrophy when infused into dwarf mice over a 3-week period (28). Thus, in our opinion data supporting a view that TSH itself is a growth factor for thyroid epithelial cells *in vivo* are not convincing. It is conceivable that in the development of goiter the primary stimulus for thyroid growth is iodine deficiency or disturbed thyroid metabolism and not TSH.

The present results should be compared to observations on the interaction between other pituitary trophic hormones and their respective target cells in culture. Thus, lutropin has been reported to suppress the mitotic rate in cultured luteal cells (29), whereas differentiated luteal cell functions are induced and maintained. An antimitotic effect of corticotropin on rat adrenocortical (30) or rat adrenal tumor cells (31–33) in culture has recently been documented. All three hormones (corticotropin, lutropin, and TSH) activate membrane-bound adenylate cyclase and thereby stimulate the hormone production of their respective target cells. Taken together, the different studies support the concept that cell multiplication and expression of differentiated functions may be antagonistic.

While it is generally believed that TSH has a trophic action *in vivo*, conclusive experimental evidence supporting this concept is lacking. The present study shows that TSH does not stimulate growth of human thyroid cells in culture. It is suggested that TSH might mediate a proliferation response *in vivo* via the induction of specific growth factors.

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