Basal and Thyroliberin-Stimulated Prolactin Synthesis in Single-Cell Cultures and in Populations of Rat Pituitary Cells

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1. Newly synthesized prolactin was obtained from cultures of rat pituitary tumour cells (GH₄C₁ cells) after incubation with [³⁵S]methionine. 2. Radioactive synthesized and secreted prolactin was quantified by an immunoprecipitation method by using disc-gel electrophoresis of the dissolved immunoprecipitate in the presence of sodium dodecyl sulphate. By using a microanalytical modification, hormone synthesis and secretion could also be studied in single-cell cultures. This technique was combined with a cytoimmunofluorescence method in which rhodamine-conjugated antibodies were used for studying intracellular prolactin. 3. The presence of radioactive synthesized and secreted prolactin was demonstrated in nine out of 13 single-cell cultures. Cell cultures containing 10 cells or more and clonal populations originating from one cell always secreted radioactive prolactin. 4. Thyroliberin treatment (2 μM) for 24 h increased the extracellular accumulation of radioactive prolactin in five out of seven single-cell cultures and always in populations of cells. 5. The number of cells showing prolactin specific fluorescence increased from 20 to 50% and the intensity of this fluorescence became greater after thyroliberin treatment. 6. Studies of [³⁵S]prolactin secretion from single cells and immunochemical detection of intracellular prolactin showed that some cells in an unsynchronized population did not secrete radioactive prolactin or show prolactin specific fluorescence. 7. The quantitative effect of thyroliberin as studied in single-cell cultures suggested that the main if not the only effect was to increase prolactin synthesis in cells already producing hormone.

The prolactin-producing cells (GH₄C₁ cells) used were clonal strains derived from a rat pituitary tumour (Tashjian *et al.*, 1968; Tashjian & Hoyt, 1972). The cells were adapted to culture conditions *in vitro* and retained the ability to synthesize and secrete prolactin spontaneously into the culture medium (Tashjian *et al.*, 1968). Unsynchronized populations of GH₄C₁ cells grew exponentially for about 7 days, with a mean doubling time of about 50–60 h. Then they reached a plateau phase where cell growth and [³H]thymidine incorporation into DNA were greatly decreased (E. Haug, H. Tjernshaugen & K. M. Gautvik, unpublished work). Hormone synthesis and secretion increased temporarily during experimental growth. However, the cells responded to treatment with the hypothalamic tripeptide, thyroliberin, by increasing prolactin synthesis and secretion during both the exponential and the plateau phases of cell growth (E. Haug, H. Tjernshaugen & K. M. Gautvik, unpublished work). In the present work, we have investigated whether all individual cells in an unsynchronized population synthesize and secrete prolactin. Further, we have studied whether thyroliberin acts on cells already producing hormone or if the action of the tripeptide is mediated through the recruitment of cells previously not producing prolactin. An indirect

fluorescent-antibody technique was used to study the proportion of the cell population that contains detectable amounts of prolactin during basal conditions and after treatment with thyroliberin. In addition we have used a sensitive micro-technique for measuring prolactin synthesis and secretion from individual GH₄C₁ cells cultured without or in the presence of thyroliberin. Since we found that only a fraction of the cells produced and secreted prolactin, we examined newly cloned populations of cells for the ability to synthesize and secrete the hormone.

Materials and Methods

Hormones and chemicals

Rat prolactin (rat prolactin-RP-1) was supplied by the National Institute of Arthritis and Metabolic Diseases, Rat Pituitary Hormone Distribution Program, Bethesda, MD 20014, U.S.A. The following chemicals were purchased: L-[4,5-³H]leucine (51 Ci/mmol) and L-[³⁵S]methionine (145 Ci/mmol) from The Radiochemical Centre, Amersham, Bucks., U.K.; sodium dodecyl sulphate from Sigma, St. Louis, MO, U.S.A.; 2-mercaptoethanol from Bio-Rad Laboratories, Richmond, CA, U.S.A.;

Soluene^(R) 350 (sample solubilizer) from Packard, Zürich, Switzerland; toluene, PPO (2,5-diphenyloxazole) and dimethyl-POPOP [1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene] from Koch-Light Laboratories, Colnbrook, Bucks., U.K.; anti-(immunoglobulin G) from Microbiological Associates, Bethesda, MD, U.S.A. Chemicals used for polyacrylamide-gel electrophoresis were all purchased from Bio-Rad Laboratories.

Cell culture conditions and source of carrier rat prolactin

In the present experiments, the GH₄C₁ cells were cultured as a monolayer in 60mm×60mm dishes (Falcon, Oxnard, CA 93030, U.S.A.) on glass or in cylindrical micro-wells (Falcon), 7mm in diameter and 12mm deep. The cells were grown in complete Ham's F-10 medium (Ham, 1963) supplemented with 18% horse and 2.5% foetal calf serum in a humidified atmosphere of CO₂+air (5:95) at 37°C. The experiments were carried out with cells in the early exponential phase of cell growth.

Carrier rat prolactin was obtained from GH₄C₁ cell cultures stimulated with thyroliberin (2 μM) for 7 days. The medium, which contained 30 μg of prolactin/ml as determined by radioimmunoassay (Gautvik & Kriz, 1976a), was centrifuged and used as described in the immunoprecipitation reaction.

Synthesis by GH₄C₁ cells of [³⁵S]prolactin and [³H]prolactin

Monolayer cultures growing in plastic dishes or single-cell cultures growing in micro-wells were used. When a one-cell culture was needed an average of 0.2 cell per well was plated. Poisson analysis indicated that about 90% of the colonies originated from one cell only, and this was affirmed by examination in a phase contrast microscope 24h after plating. The radioactive amino acids (L-[³⁵S]methionine or L-[4,5-³H]leucine) were then added for 24h as described for each experiment. The medium containing extracellular radioactive prolactin was used for measurements of synthesized and secreted prolactin as described below. The GH₄C₁ cells do not make other rat pituitary hormones (Haug & Gautvik, 1976).

Immunoprecipitation of radioactive rat prolactin and disc-gel electrophoresis in sodium dodecyl sulphate

Culture medium containing synthesized and secreted [³H]- or [³⁵S]-prolactin was obtained as described above. Prolactin carrier (unlabelled hor-

mone) and rabbit anti-prolactin antiserum were added to give a slight antibody excess, as described for each type of experiment. The antiserum showed no cross-reactivity with other pituitary hormones when tested by radioimmunological methods (Haug & Gautvik, 1976). The mixture was incubated overnight at 4°C and the immunoprecipitate isolated by centrifugation for 30 min (International centrifuge, 3000g, 4°C). The supernatant was removed, the precipitate washed five times in 1 ml of ice-cold 0.15 M-phosphate-buffered saline (0.10 M-NaCl and 0.05 M-NaH₂PO₄), pH 7.5, and dissolved in 5–100 μl of 0.1 M-NaH₂PO₄, pH 7.1, containing 2% (v/v) sodium dodecyl sulphate, 1% 2-mercaptoethanol and 10% (w/v) sucrose. Portions (4–10 μl) were submitted to polyacrylamide-disc-gel electrophoresis in the presence of sodium dodecyl sulphate as described by Gautvik *et al.* (1976). In brief, for measurement of prolactin synthesis in populations (10⁵) of cells, conventional glass tubes were used (length 75 mm, internal diam. 4 mm). For measurement of prolactin synthesis in single-cell cultures, the glass tubes had an internal diameter of 1.7 mm and total length of 60 mm.

Twelve tubes could be electrophoresed together on a modified Canalco model 1200 apparatus. Electrophoresis was for 70–90 min, and a constant current of 2 mA/gel or 0.25 mA/gel was used for the conventional and micro-disc-gel electrophoresis respectively. The tracking dye, Bromophenol Blue, was dissolved in the upper-bath buffer.

Analysis of radioactivity in gel slices

After electrophoresis the gel tubes were immediately removed and frozen at –20°C. Gel pieces (2 mm thick) were then cut and dissolved overnight in a toluene-based scintillation fluid containing PPO (5 g/litre), dimethyl-POPOP (50 mg/litre) and 5% (v/v) Soluene 350. Radioactivity was determined by liquid-scintillation counting, with an efficiency of 23% for ³H and 43% for ³⁵S. The Packard scintillation counter recorded a background radioactivity of 23 c.p.m. for ³H and 44 c.p.m. for ³⁵S. The specific radioactivity of prolactin was obtained by subtracting the background radioactivity in the gel from that in the slices where prolactin was localized (see Figs. 1 and 2). The background radioactivity was the mean c.p.m. in the slices before and after the prolactin peak. The calculated data shown (Figs. 2 and 3, and Tables 1 and 2) are corrected for this gel background.

Assessment of the identity of the immunoprecipitate, the specificity and the reproducibility of the micro-analytical procedure

When rabbit antiserum (Gautvik & Kriz, 1976b) against rat prolactin was added to two separate cul-

ture media containing [^3H]prolactin and [^{35}S]prolactin as described above, an immunoprecipitate was formed. The dissolved immunoprecipitates were electrophoresed in the conventional disc-gel-electrophoretic system as described above. The two radioactive peaks co-migrated and also showed the same electrophoretic mobility as the reference prolactin preparation (30 μg), which was detected after staining with Coomassie Blue (Fig. 1). However, when anti-serum to rat immunoglobulin was added to medium containing [^3H]prolactin, an immunoprecipitate was formed, but no radioactive prolactin peak was recovered after electrophoresis (Fig. 1).

Fig. 2 shows the quantitative recovery and reproducibility of the micro-disc-electrophoretic procedure when the dissolved immunoprecipitate containing [^{35}S]prolactin was diluted before samples were applied to the gels. Figs. 2(a) and 2(b) show the radioactivity profiles of the gels when duplicate samples (5 μl and 2 μl) of the same solution were electrophoresed. The same solution of dissolved precipitate was then diluted 1:10 before duplicate samples (5 μl and 2 μl) were applied to the capillary tubes (Figs. 2c and 2d). The quantitative measurement of prolactin was carried out as described above and given on the graphs.

Cytochemical procedures

The cells were cultured as a monolayer on a microscope objective glass for 1–2 days. Compartments were then made by use of Depex dividing lines across the glass, and the different compartments were processed simultaneously, ensuring identical conditions for control and test groups. A double-antibody technique was used for the demonstration of prolactin specific cytoimmunofluorescence. Hormone-specific rabbit anti-(rat prolactin) antiserum (Gautvik & Kriz, 1976b; Haug & Gautvik, 1976) was absorbed with freeze-dried rat liver powder and used in final dilutions of 1:100 to 1:10000 in phosphate-buffered saline, pH 7.4, containing 0.1% bovine serum albumin. Purified immunoglobulin fractions of sheep anti-(rabbit immunoglobulin G) antiserum were coupled to tetramethylrhodamine isothiocyanate and used at a final dilution of 1:100 (Brandtzaeg, 1973a,b). The reaction procedure was as follows: incubation with primary antiserum for 2h, repeated washings in the phosphate-buffered saline for 18h, incubation with secondary antibody for 2h and finally repeated washings in the same buffer for 3h. Controls included: (1) direct application of the secondary antibody with omission of the primary antiserum; (2) preincubation of primary antiserum with excess of prolactin (reference preparation); (3) application of the antisera to a closely related cell line from the original tumour, which does not produce prolactin. Microscopy was performed on a Leitz Orthoplan instrument equipped with a Ploem objective revolver.

Results

Cytochemical demonstration of intracellular prolactin before and after treatment with thyroliberin

Plate 1 shows that the intensity of the rhodamine fluorescence varied considerably, from brilliant to faint, just discernible, luminescence. Of 800 cells counted per group, 20.1% were registered as prolactin-positive in the control group (Plate 1a)

Fig. 1. Identification of synthesized radioactive prolactin and the specificity of the immunoprecipitation

To two different cultures of GH $_4\text{C}_1$ cells were added [^{35}S]methionine (10 $\mu\text{Ci/ml}$) and [^3H]leucine (10 $\mu\text{Ci/ml}$) respectively. After 24h of incubation, portions of each medium (100 μl) containing [^{35}S]prolactin or [^3H]prolactin received carrier prolactin (0.15 μg in 50 μl) and rabbit anti-prolactin antiserum (25 μl of 1:10 dilution) to slight antibody excess. The immunoprecipitates were isolated by centrifugation, washed, and after dissolution were submitted to conventional disc-gel electrophoresis in the presence of sodium dodecyl sulphate (2mA/gel for 80min). The radioactivity was determined in gel slices by liquid-scintillation counting as described in the Materials and Methods section. The two radioactive forms of prolactin showed the same electrophoretic mobility and co-migrated with the prolactin reference preparation, detected by staining with Coomassie Blue ($\downarrow$). As a control for specificity of the immunoprecipitation, medium was used that contained [^3H]prolactin and unlabelled prolactin carrier to which was added antibody against rat immunoglobulin G, and the immunoprecipitate was isolated as described above. No radioactive prolactin peak was observed after electrophoresis when antibody against rat immunoglobulin G was used (Δ). Bromophenol Blue was used as tracking dye ($\downarrow$). $\circ$, [^{35}S]Prolactin; $\bullet$, [^3H]prolactin.

Fig. 2. Quantitative determination of [^{35}S]prolactin by using immunoprecipitation and micro-disc-gel electrophoresis in sodium dodecyl sulphate

Radioactive hormone was obtained when the cells were cultured in the presence of [^{35}S]methionine ($10\mu\text{Ci/ml}$) for 4 h. The culture medium ($500\mu\text{l}$) received carrier prolactin and rabbit anti-prolactin antiserum to slight antibody excess as described in the legend to Fig. 1. Duplicate portions ($5\mu\text{l}$ and $2\mu\text{l}$ respectively) of the dissolved [^{35}S]prolactin immunoprecipitates were submitted to micro-disc-gel electrophoresis and radioactivity was determined in gel slices as described in the Materials and Methods section (a and b). One portion of the dissolved immunoprecipitate was diluted 1:10 and duplicate samples (5 and $2\mu\text{l}$ respectively) were electrophoresed as above (c and d). Specific radioactivity of prolactin was calculated after subtraction of the background radioactivity. The calculated values for each gel are shown on the graph. Duplicate determinations of four different amounts of applied [^{35}S]prolactin reflect the reproducibility of the method. The quantitative recovery of [^{35}S]prolactin corresponded closely to the amounts of dissolved immunoprecipitate applied to the gels. O, ●, individual gels for duplicate determination.

compared with 51.3% in the thyroliberin-treated group ($2\mu\text{M}$ for 24 h) (Plate 1b). Inserting a red filter decreased the light intensity to a point where many of

the weakest fluorescing cells were no longer discernible, thereby raising the 'threshold' for counting a cell as positive. In this situation, 11.2% of prolactin-

EXPLANATION OF PLATE I

Prolactin specific intracellular fluorescence before (a) and after (b) stimulation with thyroliberin (2 μM for 24 h)

Prolactin-negative non-fluorescent cells are indicated by arrows. After stimulation with thyroliberin (see the text) a higher fraction of the cell population contained detectable amounts of prolactin, and the intensity of the fluorescence was greater. In the stimulated cells, the fluorescence was predominantly confined to the cytoplasm on one side of the nucleus, whereas the unstimulated cells usually showed patchy fluorescence all around the nucleus. (Originally photographed on 27 DIN colour film, 60s exposure time.)

positive cells were found in the control group compared with 23.2% in the treated group. In both instances, thyroliberin treatment resulted in a 2.5- and 2.1-fold increase in prolactin-positive cells. Also, in the treated group there were more cells showing a brilliant fluorescence (Plates 1a and 1b). Specific qualitative differences were observed: in untreated cells the fluorescence was distributed evenly in the cytoplasm, whereas in thyroliberin-stimulated cells fluorescence was confined to only one side of the nucleus, presumably the Golgi region.

Prolactin-negative cells are marked with arrows on Plate 1. They have an appearance identical with the controls which were included to ensure the cytochemical specificity for detection of prolactin as outlined in the Materials and Methods section.

Prolactin synthesis and secretion in individually cultured GH₄C₁ cells

The microanalytical procedure, which involves immunoprecipitation and capillary disc-gel-electrophoresis, has the necessary sensitivity to detect prolactin synthesis and secretion from one cell (Gautvik *et al.*, 1976). In addition, the procedure is specific for prolactin and shows a satisfactory reproducibility (Figs. 1 and 2). This method was used for quantitative measurements of prolactin synthesis and secretion from one to several thousand cells. The results obtained from measurements of basal synthesis of [³⁵S]prolactin are shown by filled circles in Fig. 3, and the experimental details are given in the legend. No prolactin was detected in wells that contained no cells. In wells containing one and two cells, prolactin was demonstrated in four out of six wells and six out of eight wells respectively.

After the experiments, about 70–80% of the isolated cells formed colonies, and no difference in viability was observed between prolactin-producing and non-producing cells. There were marked variations in the prolactin specific radioactivity from the medium of secreting cells, suggesting different rates of prolactin synthesis.

Effect of thyroliberin on prolactin synthesis and secretion in single-cell cultures

To some wells containing one cell, thyroliberin (2 μM) was added 24h before incubation with [³⁵S]methionine. Two out of seven wells did not contain detectable amounts of [³⁵S]prolactin (Fig. 3). The remaining wells, however, contained more prolactin specific radioactivity, suggesting that thyroliberin treatment increased synthesis and secretion in individually cultured cells (Fig. 3, open circles).

Prolactin synthesis in clones of GH₄C₁ cells

Fig. 3 also shows that in wells containing 10–2000 plated cells, [³⁵S]prolactin synthesis was always detected. However, the finding of cells producing no

Fig. 3. Measurements of prolactin synthesis in GH₄C₁ cells as a function of number of cells plated per well

The cells were cultured in 100 μl of medium without (●) or with (○) thyroliberin (2 μM) for 48 h in the presence of 30 μCi of L-[³⁵S]methionine for the last 24 h. The medium was harvested and extracellular prolactin determined as described in the Materials and Methods section. Note that in some wells containing zero, one or two cells, no prolactin was detected.

prolactin (Fig. 3), raised the question whether such cells show a temporary inability to make the hormone or whether this is the permanent characteristic feature of a certain fraction of a cell population. We therefore examined the hormone-producing ability of cells in clones originating from single-cell cultures. Table 1 shows that prolactin was synthesized by all clones examined, although the amount of radioactive hormone varied.

Effects of thyroliberin on prolactin synthesis in populations of GH₄C₁ cells

Thyroliberin increases the rate of prolactin synthesis in large populations of cells (Tashjian *et al.*, 1971; Dannies & Tashjian, 1973). We have compared the relative quantitative effects of thyroliberin in single-cell cultures and in large populations of GH₄C₁ cells. Table 2 shows the effect of thyroliberin treatment (2 μM) for 48 h on synthesized intra- and extra-cellular prolactin analysed as described in the Materials and Methods section, by using conventional

Table 1. *Synthesis and secretion of [35 S]prolactin in GH $_4$ C $_1$ -cell clones originating from single-cell cultures*

The cells were plated from a single-cell suspension to give an average of 0.2 cell per well. The presence of single-cell cultures was thereafter determined by microscopic examination (see the text). After 17 days of culture (about seven cell divisions), 5 μ Ci of [35 S]methionine was added to the colonies for 24 h. Synthesized and secreted [35 S]prolactin was measured as described in the Materials and Methods section. Approx. 64–128 cells were present in the wells.

Clone no.	Radioactivity in [35 S]prolactin (c.p.m.)
1	51
2	41
3	96
4	68
5	48
6	57
7	73
8	31
9	33
10	57
11	90

Table 2. *Synthesis and secretion of [35 S]prolactin in a GH $_4$ C $_1$ -cell population*

Monolayer cultures received new medium with and without thyroliberin (2 μ M) for 48 h. Intracellular and extracellular radioactive prolactin was determined after incubation with [35 S]methionine (25 μ Ci/ml) for the last 1 h of the experimental period (see the Materials and Methods section). Each dish contained on average 300 μ g of cell protein. The results are expressed as means of duplicate dishes that differed by less than 15%.

	[35 S]Prolactin (c.p.m./dish)		
	Medium	Cells	Total
Controls	210	2010	2220
Thyroliberin	340	3150	3490
Control	1.62	1.56	
Thyroliberin			

sodium dodecyl sulphate/polyacrylamide-disc-gel electrophoresis. After a labelling period of 60 min, most of the newly synthesized prolactin is found intracellularly. The stimulatory effect of thyroliberin was about 160%, whereas the stimulation in single-cell cultures was calculated to be 230% (Table 2 and Fig. 3).

Discussion

The synthesis and secretion of prolactin from individual GH $_4$ C $_1$ cells were studied by using immunocytochemical and biochemical microanalytical methods. The results of the two methods are compatible in that they examine hormone production

in single cells, in contrast with previous studies which measured the product of large populations of cultured cells. However, the results obtained by the two methods are different in the following respects. The immunofluorescence method gives information about the presence of prolactin in cells at the moment of fixation, whereas the other method measures hormone synthesis and secretion during 24 h. However, earlier studies have shown that these cells have a high rate of prolactin synthesis (Gautvik & Kriz, 1976b), but contain only very small amounts of the hormone (Gautvik & Kriz, 1976b; Haug & Gautvik, 1976), which is not degraded intracellularly (Dannies & Tashjian, 1973). Thus we assume that cells synthesizing prolactin also release the hormone. The combination of relatively low sensitivity of the immunofluorescence method and small intracellular stores of prolactin probably leads to an underestimation of the fraction of hormone-producing cells. Thus Hoyt & Tashjian (1973), using a prolactin-specific peroxidase-staining method, concluded that two-thirds of the cells were prolactin-positive and that thyroliberin treatment did not increase the fraction of cells staining positively for prolactin. The microanalytical method for quantitative measurement of prolactin synthesis and secretion by single cells was able to detect as little as 10 pg of prolactin (Gautvik *et al.*, 1976). Using this method we also found that about two-thirds of the individually cultured cells secreted prolactin. Provided that the culture conditions prevailing for single cells did not alter the cell's ability to produce and secrete hormone, we interpret the results as follows.

The measurements of prolactin synthesis and secretion in single cells strongly suggest that a certain fraction of a GH $_4$ C $_1$ -cell population does not make the hormone. This conclusion is supported by the present and a previous (Hoyt & Tashjian, 1973) histochemical study. Since all the cell clones originating from single-cell cultures did synthesize and secrete prolactin (Table 1), the cells cannot have permanently lost their hormone-producing ability. Rather, the presence of prolactin non-producers must represent a temporal inability, which probably reflects the existence of cells in different phases of growth. It is noteworthy that about two-thirds of the cells were found to be hormone producers by the two independent methods, and that about 70% of the exponentially growing cells in an unsynchronized population exist in the G $_1$ phase (P. O. Clausen, K. M. Gautvik & T. Lindmo, unpublished work). Obviously most of the hormone-producing cells must be in the G $_1$ phase, although we cannot rule out that cells in other phases of growth are also able to synthesize and secrete prolactin.

Calculation of the mean specific radioactivity in prolactin gave (c.p.m./cell): 18, 13, 6, 6, 9 and 7 for 1, 2, 10, 20, 100 and 2000 cells/well respectively. Therefore colonies of 10 and 20 cells apparently behave like

populations of cells with respect to synthesis and secretion of prolactin. Whether the number of cell divisions or the size of colonies (cell density) influences the amount of hormone synthesized and secreted needs to be determined.

Prolactin synthesis and secretion were stimulated by thyroliberin in single-cell cultures, as in populations of cells. Moreover, a greater relative increase in prolactin synthesis was observed after thyroliberin treatment in hormone-producing single-cell cultures than in populations of cells (Fig. 3 and Table 2). This result agrees with the suggestion of Hoyt & Tashjian (1973) that treatment with thyroliberin does not change the percentage of prolactin-producing cells in a given population. The finding in the present study that thyroliberin increased the number of prolactin-fluorescing cells may be explained by the lower sensitivity of this method. Our immunocytochemical data also showed that thyroliberin treatment made the prolactin specific fluorescence stronger, as well as increasing the number of hormone-positive cells. Taken together, the available cytochemical and biochemical data strongly suggest that thyroliberin induces prolactin synthesis by affecting GH₄C₁ cells already producing hormone. There is thus no need to postulate a recruitment of previously non-producing cells to account for the effect of this tripeptide.

It is not known whether the GH₄C₁ cells are functionally coupled, in the sense that the effect of thyroliberin is transmitted through one cell to its adjacent neighbour, and whether this influence is sufficient to induce prolactin synthesis. If functional coupling between two neighbouring cells exists, it would not be necessary to postulate the existence of hormone receptors on every cell to explain the action of thyroliberin. Since this tripeptide induces prolactin

synthesis and secretion in single cells in culture, intimate cell-to-cell interaction is not a prerequisite for the action of thyroliberin.

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