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HIGH MOLECULAR THYROTROPHIN ("BIG"-TSH) FROM HUMAN PITUITARIES: PREPARATION AND PARTIAL CHARACTERIZATION

By

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ABSTRACT

Homogenates of human pituitaries were centrifuged at 30 000 × *g* and the supernatant chromatographed on Sephadex G-100. Approximately 1 % of the radioimmunologically measured total activity of TSH was eluted in the void volume. Rechromatography of this material on Sephadex G-200 usually showed TSH-activity at $K_{av} = 0.5$ (regular TSH), $K_{av} = 0$ (void volume-TSH) and at $K_{av} = 0.3$ ("big"-TSH). "Big"-TSH was extracted from the corresponding fractions by affinity-chromatography with solid phase anti-TSH. It was eluted with 5 mol/l ammonium thiocyanate and further characterized:

1. The molecular weight was approximately 200 000 by comparison with bovine catalase on Sephadex G-200.
2. Immunoidentity as compared with standard-TSH (M. R. C. 68/38) was shown by parallel dilution curves in the radioimmunoassay.
3. Concanavalin-A-Sepharose adsorbed "big"-TSH, which could be eluted with α-methyl-D-mannoside, indicating the glycoprotein nature of "big"-TSH.
4. On polyacrylamide-gel-electrophoresis pH 7.5, "big"-TSH migrated faster ($R_F = 0.32$) than regular TSH ($R_F = 0.1$), indicating a more negatively charged molecule.
5. "Big"-TSH, in contrast to regular TSH, was remarkably stable against 6 mol/l guanidine hydrochloride, suggesting a covalently linked (aggregate) structure.

6. 1 % mercaptoethanol destroyed the immunological activity of both regular and "big"-TSH.
7. "Big"-TSH was digested by trypsin, under mild conditions, to radioimmunologically active products with molecular weights between "big"- and regular TSH, but practically no regular TSH was formed.
8. "Big"-TSH and guanidine-treated "big"-TSH, as well as regular TSH and TSH from the void volume of Sephadex G-200 columns, exhibited biological activity in a cytochemical bioassay in good agreement with the respective immunological activities.

High molecular forms of proteohormones known as "big"-hormones have been described for several years as reviewed by *Yalow* (1974). Recently, "big"-hormones were also observed in the field of pituitary glycoprotein hormones: "big"-LH (*Prentice & Ryan* 1975; *Graesslin et al.* 1976) "big"-TSH (*Erhardt* 1975; *Erhardt & Scriba* 1976) and "big"-β-TSH (*Golstein-Golaire & Vanhaelst* 1975; *Kourides et al.* 1976). There is still little known about the chemical structure and biological significance of the high molecular species of glycoprotein hormones.

MATERIALS AND METHODS

Reagents

Rabbit IgG for coprecipitation in the radioimmunoassays; bovine catalase, equine ferritin for the calibration of Sephadex columns; materials for polyacrylamide-gel-electrophoresis (PAGE): Acrylamide, tetramethylethylenediamine, N,N-methylenebisacrylamide and ammonium persulphate; Triton X 100; α-methyl-D-mannoside; trypsin (less than 0.24 % chymotrypsin) and trypsin inhibitor from lima bean were obtained from Serva GmbH (Heidelberg, Germany).

All Sephadex gels (G-75; G-100; G-200; QAE-A-50; Cl-Sepharose 2 B; Con A-Sepharose) were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden), and sodium phosphate, sodium chloride, sodium EDTA, sodium azide, ammonium thiocyanate, and guanidine (mostly analytical grade), from Merck AG (Darmstadt, Germany). Bovine albumin (Pentex®) was purchased from Roth (Munich, Germany). TSH for labelling and anti-TSH for radioimmunoassay were kindly donated by the NIAMDD (Bethesda, Maryland). Anti-TSH for affinity-chromatography was donated by Farbwerke Hoechst AG (Frankfurt, Germany). Sodium ^{125}iodide for labelling was also obtained from Hoechst AG. Anti-TSH-serum for affinity chromatography was also produced in our laboratories by immunization of two rabbits with TSH (4 U/mg) purchased from Deutsche Kabi GmbH (Munich, Germany). HCG, Primogonyl® for absorption in the radioimmunoassay of TSH and for immunization of rabbits, was obtained from Schering AG (W-Berlin, Germany). Anti-rabbit precipitating serum (donkey) was obtained from Wellcome Ltd. (England). "Eppendorf" plastic tubes (vol. 1.5 ml) for all radioimmunoassays were purchased from Netheler & Hinz (Hamburg, Germany). Human pituitaries obtained at autopsy, usually performed 24–36 h after exitus, were kept frozen at –28°C. Pituitaries kept in acetone were also used.

Buffers

A: 0.015 mol/l sodium phosphate; 0.15 mol/l sodium chloride; 0.2 % bovine-albumin; 0.002 % sodium azide; 0.02 mol/l EDTA, pH 7.4.
B: As buffer A, with 4.2 % bovine albumin and no EDTA.
C: As buffer A, but without albumin and EDTA.
D: 0.1 mol/l sodium phosphate, pH 6.5.
E: 0.5 ml/l sodium carbonate, pH 8.6.
F: As buffer E, but with 2 mol/l sodium chloride, pH 8.6.
G: 0.1 mol/l sodium carbonate; 2 mol/l sodium chloride, pH 8.6.
H: 0.1 mol/l sodium acetate; 2 mol/l sodium chloride, pH 4.5.
I: 0.01 mol/l Tris, pH 7.5.

Radioimmunoassays of hTSH, hLH and hGH

Radioimmunoassays were carried out using the double antibody technique and a modular analyzer system (*Marschner et al.* 1975), RIA-E-6000 laboratorium Prof. Berthold (Karlsruhe, Germany) and Ismatec (Zürich, Switzerland). All hormones were labelled with ^{125}I by slight modification of the chloramine-T-method (*Greenwood et al.* 1963). HTSH was measured with minor modifications as described previously (*Erhardt et al.* 1973) using the international standard M. R. C. 68/38 (NIMR, Mill Hill, London). HLH was measured using anti-HCG from rabbits from our laboratory, LH for labelling was purchased from Calbiochem (California, USA), and standard L. E. R. 907 obtained from NIAMDD. HGH was measured using rabbit anti-HGH from our laboratory, HGH "reinst" for labelling and "creschormone" for standards from Deutsche Kabi GmbH (Munich, Germany).

Preparation of "big"-TSH

4–50 pituitaries were thawed at 4°C and homogenized in 5–30 ml cold buffer C for 1 min in a Vortex homogenizer at low speed and for 1 min at high speed. The temperature was allowed to rise to 27°C. The homogenate was centrifuged for 30 min at 30 000 × *g* at 4°C in a Beckmann ultracentrifuge type L-40. The red-brown and still turbid supernatant was chromatographed on a Sephex G-100 glass column (3 × 130 cm) with buffer C under hydrostatic pressure (flow rate 10–15 ml/h). Fractions from 3–6 ml were collected in glass tubes using fraction collector Linear II, Serva GmbH (Heidelberg, Germany). The hormone activities, TSH, LH and HGH were measured in each fraction in adequate dilutions (10^0–10^5) in buffer B. Crude "big"-TSH was eluted constantly in the turbid fractions of the void volume ($K_{av} = 0$) and was usually measured in 1 to 100 µl of the eluates (Fig. 1).

For further purification the "big"-TSH containing fractions were concentrated to a volume of 2–5 ml by ultrafiltration at 4°C, using collodion bags, obtained by Sartorius AG (Göttingen, Germany).

After centrifugation at 30 000 × *g* for 30 min, the turbid concentrate was re-chromatographed on Sephadex G-200 under hydrostatic pressure. The columns were previously coated with 20 ml buffer B and were equilibrated over-night at 4°C with elution buffer C; 3–6 ml fractions were collected (Fig. 2).

Immunization of rabbits

TSH-antiserum was raised in 2 rabbits, which were each immunized by 4 injections, beginning with 100 µg subcutaneously followed by 3 injections of 50 µg TSH, all in incomplete Freund's adjuvant. After eight weeks one rabbit showed 50 % binding of [^{125}I]TSH in a final dilution of 1:100 000.

Preparation of the anti-TSH-affinity gel

Activation of Cl-Sepharose-2 B by cyanogen bromide purchased from Merck AG (Darmstadt, Germany) was performed according to the instructions of Pharmacia Fine Chemicals. Ten g of the wet gel cake was suspended in 30 ml buffer E and reacted with 4 g cyanogen bromide, dissolved in 20 ml of distilled water. Under continuous control by a glass-electrode and under magnetic stirring, the pH of the mixture was kept between pH 10 and 11 by dropwise addition of 20 % sodium hydroxide. The temperature was kept below 30°C by addition of ice. After 20 min the reaction mixture was filtered through a sintered glass funnel and washed with 500 ml of 0.1 mol/l sodium carbonate, pH 8.6.

Binding of anti-TSH-IgG

The preparation of IgG was performed using the ion exchange gel QAE-A-50-Sepharose according to the instructions of Pharmacia Fine Chemicals, elution was with buffer D, and concentration by ultrafiltration. Five mg of the IgG dissolved in 10 ml buffer F was added to 1 g of the wet, freshly prepared activated Cl-Sepharose-2 B-cake in a polystyrene-beaker and slowly rotated at room temperature for 3 h continued over-night at 4°C. The mixture was again filtered over a sintered glass funnel. The optical density of the filtrate at 280 nm was measured and compared with the light absorption before extraction with the activated gel to calculate roughly the proportion of protein linked to the gel. This varied between 40–90 % (2–4.5 mg protein/g wet Sepharose).

To inactivate the anti-TSH gel it was suspended in 20 ml of 1 mol/l glycine and rotated for 3 h at room temperature. After filtration, the affinity gel was repeatedly and alternately washed 10 times with buffer G and buffer H (50 ml each).

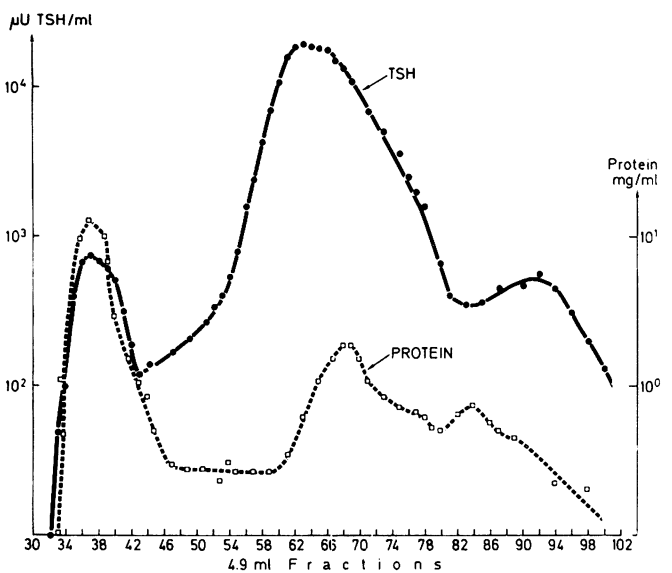

Fig. 1.

Chromatogram of the 30 000 *g* supernatant of 4 human pituitaries on Sephadex G-100 (134 × 3 cm; buffer C; 4°C).

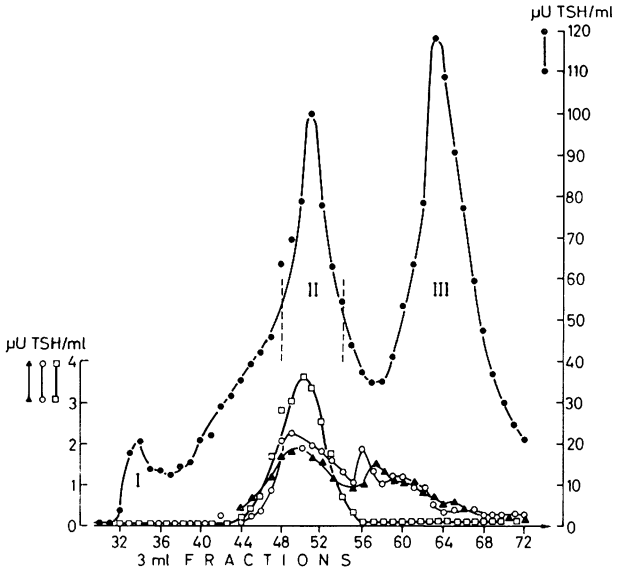

Fig. 2.
Chromatograms of "big"-TSH on Sephadex G-200 before and after mild tryptic digestion. (130 × 1.7 cm; buffer C; 4°C).

—●— Crude product from Sephadex G-100, showing void volume TSH (I); "big"-TSH (II) and regular TSH (III).
—□— Untreated "big"-TSH, re-chromatographed.
—○— 5 min treated with trypsin.
—▲— 10 min treated with trypsin.

Specific extraction of "big"-TSH from Sephadex eluates

One g of wet, freshly prepared affinity-gel was added to the pooled "big"-TSH fractions from the Sephadex chromatography (20–80 ml). After slow rotation at room temperature for 2 h the gel was filtered (Schott AG, Fritte 1 G 2) and again repeatedly washed (at least 4 times), alternating between buffer G and buffer H in order to remove physically adsorbed proteins.

Elution of "big"-TSH from the affinity-gel

The wet affinity-gel cake loaded with "big"-TSH was suspended in buffer C and poured into a small column (10 ml plastic syringe with filter). "Big"-TSH was eluted with 20 ml of freshly prepared 5 mol/l ammonium thiocyanate in distilled water, pH 7, within 10–20 min and collected in dialysis tubing. Dialysis against 6 l distilled water was carried out over 24 h at 4°C with a single change of the outer fluid. The dialyzed "big"-TSH was subsequently concentrated by ultrafiltration through collodion bags to give a final volume of 0.5–1 ml. HTSH, HGH and HLH activity were determined by radioimmunoassay and the protein concentration by lightabsorption at 280 nm (0.7 O. D.$_{280}$ ≅ 1 mg protein/ml) to monitor the purification. The product was re-chromatographed on Sephadex G-200 and the fractions collected either in siliconized glass

tubes, or with 0.2 ml of buffer B in the bottom of the tubes, in order to reduce adsorption onto the glass. Concentration was again performed by ultrafiltration in collodion bags.

Polyacrylamide-gel-electrophoresis (PAGE)

PAGE was performed in 0.5 mol/l Tris-HCl at p H7.5 (*Smith* 1968). Bromphenolblue was used as a marker. Two hundred μU of "big"-TSH or TSH, respectively, was applied to the columns (separation gel: 7 x 0.5 cm, concentration gel 2 x 0.5 cm). At the end of the electrophoresis, the gels were frozen at –80°C for 30 min and subsequently cut into 45 slices of 1.5 mm using razor-blades. The slices were each placed in 0.6 ml of buffer B and allowed to elute (for 20 h) at room temperature. The TSH-activity in the eluate from each slice was measured by radioimmunoassay (Fig. 5).

Treatment of "big"-TSH and regular TSH with guanidine and Triton X 100

Triton X 100 (0.25 %) and guanidine-HCl (6 mol/l final concentration) were dissolved in 2 ml of "big"-TSH-solution in buffer A (pH 5.1), and the mixture incubated for 1 h at 37°C. For comparison, incubations were also performed without guanidine, but with Triton X 100, and without both. The reaction mixtures were chromatographed on Sephadex G-100 or G-200, respectively (Fig. 6).

Mild tryptic digestion of "big"-TSH

Twenty μg of trypsin in 20 μl buffer C was added to 160 μU of "big"-TSH from Sephadex G-200 in 200 μl of buffer C in a small siliconized glass tube. No calcium was added. The solution was incubated at 37°C for 5 or 10 min, respectively, after which 10 μg of lima bean inhibitor in 20 μl buffer C was added in order to stop the reaction. Re-chromatography was performed on 130 x 1.7 cm Sephadex G-200, as for the undigested "big"-TSH (Fig. 2).

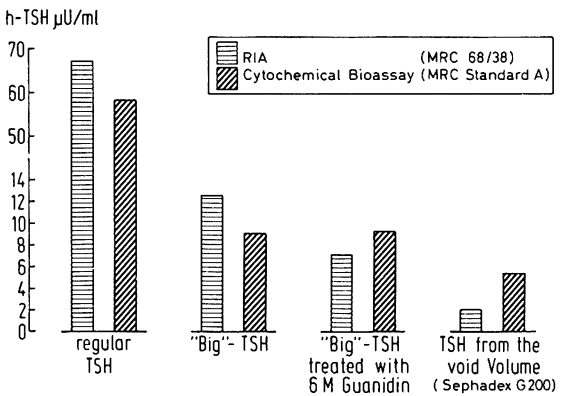

Fig. 3.
Comparison of the radioimmunologically and cytochemically measured TSH-activity of human pituitary TSH-fractions from Sephadex G-200 and of "big"-TSH after treatment with 6 mol/l guanidine-HCl and desalting by re-chromatography.

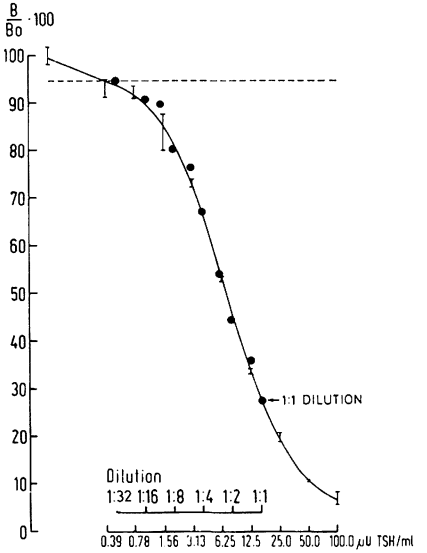

Fig. 4.

Dilution curve of "big"-TSH from affinity chromatography after re-chromatography on Sephadex G-200, compared with TSH-standard M. R. C. 68/38.

—●— Dilutions of "big"-TSH.
——— Standard curve.

Mercaptoethanol treatment of "big"-TSH

Ten µl mercaptoethanol was added to 160 µU "big"-TSH in 1 ml buffer B. For comparison, M. R. C. 68/38-TSH was treated identically. After 50 h at 4°C and after 1 h at 37°C, aliquots of the reaction mixtures were diluted 1:10 with buffer B to eliminate unspecific effects of mercaptoethanol. TSH-activity was then measured by radioimmunoassay and compared with the activity before treatment. The "big"-TSH was re-chromatographed on 1.1 × 70 cm Sephadex G-100 (Fig. 7).

Adsorption of "big"-TSH to Con A-Sepharose

Two ml of Con A-Sepharose was applied to a 5 ml plastic syringe fitted with a glassfilter, and 80 µU of "big"-TSH was added in 0.1 ml of buffer C. The column was washed with 8 ml buffer C and the elution subsequently performed with 10 ml 0.05 mol/l α-methyl-D-mannoside in buffer C. One ml fractions were collected into Eppendorf vials containing 50 µl of buffer B.

Determination of the DNA-content of "big"-TSH

Ten mU of "big"-TSH (enriched by affinity chromatography and equivalent to approximately 3 µg of regular TSH), dissolved in 0.1 ml of buffer A was treated with 10 µl (1 mg/ml) proteinase K (Merck AG, Darmstadt, Germany), after dilution with 0.4 ml buffer I, for 17 h at 37°C, to liberate RNA and DNA from the protein moiety

of "big"-TSH. The measurement of DNA and RNA was performed using the sensitive fluorescence method with ethidium bromide in the presence of 0.07 mol/l magnesium chloride (*Paoletti & Le Pecq* 1971), and a Perkin-Elmer fluorescence spectrometer (MPF-2A). The fluorescence emission was monitored at 586 nm after calibration with calf thymus DNA and with unfractionated yeast t-RNA (Boehringer, Mannheim, Germany). The limit of detection for DNA was approximately 0.1 μg/ml and for t-RNA 1 μg/ml.

Measurement of the biological activity

The measurement of the biological activity was performed in the laboratory of Dr. von zur Mühlen, Hannover, using the cytochemical bioassay (*Bitensky et al.* 1974). The biological activity was measured in the three main fractions ($K_{av} = 0$, 0.3 and 0.5) of pituitary-TSH chromatographed on Sephadex G-200 and likewise in a guanidine-treated and re-chromatographed "big"-TSH fraction. M. R. C.-TSH-standard A was used for calibration. TSH activities of the same fractions were determined in our laboratory in Munich by radioimmunoassay using M. R. C. 68/38 as standard. Comparison of standard A and 68/38 in the radioimmunoassay showed standard A to have 85 ± 7 % of the activity of 68/38-TSH (Fig. 3).

RESULTS

Chromatography of pituitary supernatants

Chromatograms of the 30 000 × *g* supernatants from human pituitary homogenates on Sephadex G-100 columns always showed turbid fractions eluting in the void volume with TSH-activities of about 1 % of the total TSH-activity (Fig. 1). However, re-chromatography on Sephadex G-200 showed that these fractions from G-100 were not homogeneous, but were separated into TSH-fractions eluting in the void volume of G-200 ($K_{av} = 0$), "big"-TSH-fractions of K_{av} 0.3 and regular TSH ($K_{av} = 0.5$) (Figs. 2 and 3). The amount of TSH found in the void volume was dependent on the gravity force during centrifugation, indicating that in these fractions TSH was still linked to pituitary cell fragments. The further experiments were mainly performed with "big"-TSH ($K_{av} = 0.3$).

Characterization of "big"-TSH

"Big"-TSH, purified only by re-chromatography on Sephadex G-200 or further purified by affinity chromatography, displayed parallelism of its dilution curves to TSH-standards in the radioimmunoassay (Fig. 4). It was eluted on a calibrated Sephadex G-200 column shortly behind bovine catalase (M. W. 240 000), pointing to a molecular weight of approximately 200 000. The same product chromatographed in the polyacrylamide-gel-electrophoresis in Tris-buffer pH 7.5 migrated with a R_F = 0.32 significantly faster than regular TSH (R_F = 0.1), indicating "big"-TSH to be a more negatively charged mole-

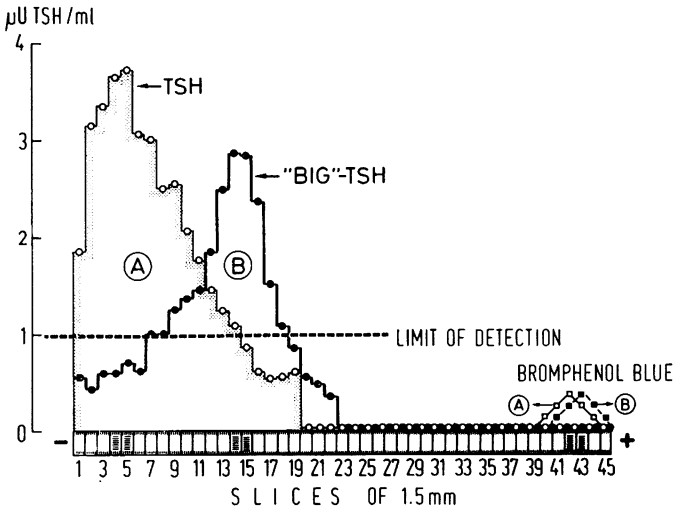

Fig. 5.
Polyacrylamid-gel-electrophoresis of TSH and "big"-TSH.

A —○— Regular TSH (NIAMDD HTSH for labelling).
B —●— "big"-TSH.
Gel: 7 × 0.5 cm; 0.5 M Tris, pH 7.5; 5–3 mA/column.

The TSH-activity was measured by radioimmunoassay in 1.5 mm slices after elution with buffer B (0.6 ml/slice).

cule (Fig. 5). Affinity chromatography of "big"-TSH on Con A-Sepharose showed that it behaves like a glycoprotein, since it was bound to Con A-Sepharose like regular TSH. It could also be displaced and eluted by α-methyl-D-mannoside.

Measurements of the DNA and RNA content of "big"-TSH, after enrichment by affinity chromatography, using the very sensitive fluorescence method with ethidium bromide displayed DNA and RNA content below the limit of detection: less than 1.5 % DNA and less than 15 % RNA.

All TSH-fractions (Fig. 3), including those treated with guanidine, displayed biological activity. The biological activity of the trypsin-treated fractions have not been measured, because no well defined products were formed.

Purification and yield of "big"-TSH after preparation by affinity chromatography

Affinity chromatography with solid phase anti-TSH after prior separation of a well defined "big"-TSH fraction by Sephadex chromatography, resulted in a 15–40-fold purification as compared with protein or HGH, respectively,

Table 1.

Purification and yield of "big"-TSH from 50 pituitaries by affinity chromatography with anti-TSH-Sepharose and Con A-Sepharose after prior separation from regular TSH and void volume TSH by one chromatography run on Sephadex G 100 and two subsequent runs on Sephadex G 200 (results of one experiment).

	Absolute amounts				Purification			Yield
	"Big"-TSH (mU)	HLH (U)	HGH (μg)	Protein (mg)	TSH/HLH (mU/U)	TSH-HGH (mU/μg)	TSH/protein (mU/mg)	"Big"-TSH (%)
Before affinity chromatography	18	47	380	19	0.38	0.047	0.95	100
In the ammonium thiocyanate eluate after extraction by anti-TSH-Sepharose	3.1	2.8	4.1	0.18	1.1	0.76	17.2	16.7
In the α-methyl-D-mannoside eluate after extraction by 1 ml Con A-Sepharose	0.5	0.8	0.12	not measurable (< 0.05)	0.6	4.2	>10	2.8

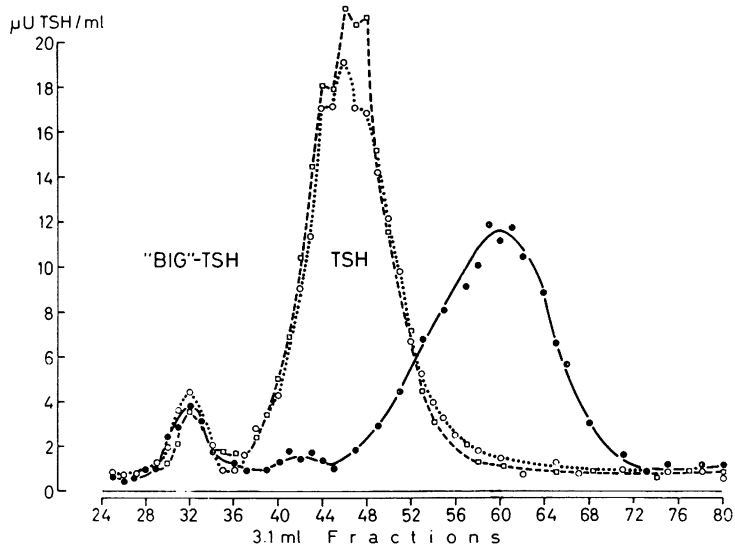

Fig. 6.

Chromatograms of crude "big"-TSH on Sephadex G-100 after various treatments. (127 × 1.7 cm; buffer C; 4°C).

1) ···○··· Untreated.
2) —□— 1 h; 37°C; 0.25 % Triton X 100; pH 7.4.
3) —●— 1 h; 37°C; 0.25 % Triton X 100; 6 mol/l guanidine-HCl; pH 5.1.

The first peak represents "big"-TSH, the second peak regular TSH, the third peak, only seen on the guadinine-treated material, has not fully been investigated, but possibly contains β-TSH, which shows almost the same binding characteristics with the antibody as the TSH-standard used in the assay.

and 2–10-fold when compared with HLH. In this step the yield of "big"-TSH dropped from 100 to between 15 and 30 %.

Further purification was achieved on Con A-Sepharose, the protein concentration then being below the limit of detection (< 50 µg protein/ml). As compared with HGH this procedure resulted in a further 5–10-fold purification. However, compared to HLH no further purification was achieved. The yield of "big"-TSH dropped by an additional factor of about 5.

Table 1 shows the results of one experiment, where the purification of "big"-TSH was approximately 100-fold as compared to somatotrophin.

Stability of "big"-TSH

In an attempt to convert "big"-TSH to regular TSH and to find some indications concerning the structure of "big"-TSH, the product was treated with 6 mol/l guanidine, 1 % mercaptoethanol and trypsin, respectively.

Treatment with 6 mol/l guanidine for 1 h at 37°C in the presence of 0.25 %

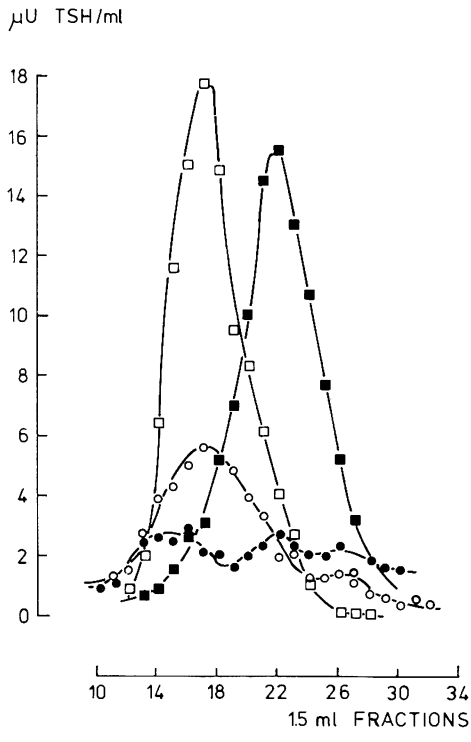

Fig. 7.
Chromatograms of TSH and "big"-TSH on Sephadex G-100 after treatment with mercaptoethanol. (70 × 1.1 cm; buffer C; 4°C).

—■— HTSH (M. R. C. 68/38) untreated.
—□— "big"-TSH untreated.
—●— "big"-TSH in buffer B; 1 h; 37°C; 1 % mercaptoethanol, pH 7.4.
—○— "big"-TSH 66 h; 4°C; 1 % mercaptoethanol, pH 7.4.

of the detergent Triton X 100 at pH 5.1 resulted in only a minor degradation of about 10–15 % in contrast to regular TSH, which was almost completely destroyed under the same conditions (Fig. 6). Triton X 100 alone had no significant effect on "big"-TSH. Moreover, the guanidine-treated "big"-TSH did not lose its biological activity, as shown in the cytochemical bioassay (Fig. 3).

Treatment with 1 % mercaptoethanol at 4°C and at 37°C led to loss of the immunological activity of "big"-TSH and regular TSH at similar rates, so that an intermediate formation of regular TSH from "big"-TSH could not conclusively be shown by this experiment. At 37°C, both "big"-TSH and regular TSH lost 90 % of their immunological activities in the radioimmunoassay. At 4°C, approximately 50 % was lost within 48 h. Re-chromatography of the mercaptoethanol-treated "big"-TSH showed only a minor peak in the region where regular TSH would be expected (Fig. 7).

The digestion experiments with trypsin were performed in a mild procedure at pH 7.4 in the absence of calcium. Re-chromatography of the digestion mixture on Sephadex G-200 demonstrated the formation of still immunologically active cleavage products, which were eluted between "big"-TSH and regular TSH, indicating molecular weights between "big"- and "regular" TSH. There was practically no products formed which could be eluted in the volume where TSH should appear (Fig. 2).

So far none of the cleavage experiments have succeeded in the formation of regular TSH from "big"-TSH.

DISCUSSION

High-molecular forms of HTSH have not been observed earlier probably because of the minute amounts found in the supernatant of pituitary homogenates, and because of the necessity of using long columns to separate "big" from regular TSH. Additionally, the "big"-TSH-fractions obtained after chromatography on Sephadex G-100 columns are not homogeneous. On Sephadex G-200, turbid fractions can again be obtained, in which TSH is probably still linked to cell-fragments ("void volume-TSH"). The second fraction, which was eluted on G-200 between the void volume TSH and regular TSH, differs mainly in three aspects from regular TSH:

Firstly, it showed a molecular weight of about 7 times that of regular TSH by chromatographic comparison with bovine catalase, secondly, in contrast to regular TSH, "big"-TSH was not cleaved into subunits by 6 mol/l guanidine, and finally, it migrated faster on PAGE pH 7.5 when compared with highly purified TSH in spite of its much bigger molecular weight.

This indicates more negatively charged groups, which could for instance be sialic acid groups. As tested by fluorescence with the ethidium bromide method, "big"-TSH contains no DNA or RNA in significant amounts.

However, "big"-TSH also displays a series of features very similar to regular TSH which do not exclude a relationship to regular TSH. Firstly it showed parallelism of its dilutions when compared with standard TSH in the radioimmunoassay, indicating identity of the antigenic sites and good accessibility for the specific antibody, so that the antigenic site does not seem to be folded into the big protein. Moreover, "big"-TSH was bound to Con A-Sepharose like glycoproteins just as regular TSH and could be displaced readily by α-methyl-D-mannoside. These features make it very likely that "big"-TSH contains, in some still unknown manner, regular TSH. The high stability against 6 mol/l guanidine is surprising, but is in accordance with the high stability of "big"-LH as described by *Prentice & Ryan* (1975). This suggests, that "big"-TSH is not a mere aggregate but a covalently linked product, and indicates in addition, that the subunits in this "big"-TSH may also

be covalently linked, in contrast to those of regular TSH, which are easily cleaved by 6 mol/l guanidine.

We still do not know, if "big"-TSH is a product, in which regular TSH is linked to a second "big"-protein or whether "big"-TSH is a covalently linked polymer of regular TSH. The latter possibility could be clarified, when sufficient amount of purified "big"-TSH has been prepared for the determination of its specific activity. Our attempts to cleave "big"-TSH with mercaptoethanol showed only, that the immunological activity was destroyed in the same way as that of regular TSH under these conditions. From these experiments, we are therefore not able to elucidate on the combining groups in the "big"-TSH. But at the same time we cannot exclude, that disulphide groups play a role in binding.

Our attempts to cleave "big"-TSH by tryptic digestion did not result in the production of regular TSH. This is in contrast to "big"-ACTH, which is transformed to regular ACTH by trypsin as described by *Yalow* (1974). But at least tryptic digestion products were formed, which still showed immunological activity in the radioimmunoassay and which on re-chromatography on Sephadex G-200 displayed molecular weights between "big"-TSH and regular TSH.

Interestingly, regular TSH, as well as "big"-TSH, guanidine-treated "big"-TSH and void volume TSH, showed biological activity in the cytochemical bioassay in good agreement with the radioimmunological findings. This allows the suggestion that in "big"-TSH as in void volume TSH the immunologically/biologically active sites are accessible to the TSH-receptors of thyroid cells.

The question of the amount of alpha and beta subunits in the "big"-TSH molecule can only be answered when firstly, "big"-TSH has been further purified, mainly using affinity chromatographic techniques with Con A-Sepharose and solid-phase-anti-TSH, and secondly, when a mild cleavage procedure has been found. Attempts to determine the amount of alpha and beta subunits included in the "big"-TSH, based on measurements in poorly purified Sephadex eluates, are not valid because of the cross-reaction of the accompanying glycoproteohormones and their subunits which complicate the quantitation.

In a few experiments, where serum of hypothyroid patients was chromatographed on Sephadex G-100 or 200 no TSH-activity was found at the position, where "big"-TSH should appear, even with a very sensitive radioimmunoassay (limit of detection 0.1 μU/ml). Interestingly *Golstein-Golaire & Vanhaelst* (1975) and *Kourides et al.* (1976) reported beta-TSH-subunit-activity in some hypothyroid sera, corresponding to a "big"-beta-TSH with a molecular weight of approximately 160 000. It is still not known whether there is any relationship between the "big"-beta-TSH found occasionally in serum and "big"-TSH regularly present in pituitaries.

The physiological significance of "big"-TSH is still obscure and open to speculation. Up to date, it cannot be excluded that "big"-TSH is merely a side-product or an oxidation product of no physiological importance. If "big"-TSH has any physiological significance, then we rather believe, because of the minute amounts present, that it is an intermediate between an even larger precursor and regular TSH, rather than a storage form, as has been suggested by *Golstein-Golaire & Vanhaelst* (1976). The "intermediate" hypothesis can, however, only be proven when we succeed in showing the formation and the disappearance of "big"-TSH in pituitary cells.

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